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TITLE: A Targeted Alpha-Particle Radiopharmaceutical for Bone Metastatic Breast Cancer

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CONTRACTING ORGANIZATIONS: Wake Forest University Health Sciences

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14. ABSTRACT The proposed studies will develop and evaluate a new therapeutic strategy for treating breast cancer bone metastasis by disrupting the interactions between breast cancer cells and sensory neurons within bone using alpha particle-emitting radiopharmaceuticals that target the CGRP-CRLR axis. The successful completion of this project will establish a new therapeutic target for cancer-induced bone pain and bone metastatic breast cancer; while providing the foundation required to develop new therapies that will decrease suffering and improve survival of breast cancer patients with bone metastases. During the entire grant period, (i) we further validated that CRLR is a suitable target to treat bone metastatic breast cancer and successfully manipulated CRLR levels in breast cancer cells using lenti-virus technique; (ii) we validated that CRLC-overexpressing breast cancer cells can grow in vivo without affecting growth rate; (iii) we prepared the PTCA-CGRP ₂₇₋₃₇ peptides and completed the radiochemistry experiments with the PCTA-CGRP ₂₇₋₃₇ peptides and developed strategies to overcome the severe lipophilicity of the agents under investigation; and (iv) we completed the preparation and stability analysis of several new ligands using the positron emitting radioisotope zirconium-89 (89Zr: t _{1/2} = 78.4 h, β ⁺ : 22.8 %, E _β +max = 901 keV; EC: 77%, E _γ = 909 keV; 99%).					
15. SUBJECT TERMS Breast Cancer; Bone metastasis; Cancer-induced bone pain; Radiopharmaceuticals; Calcitonin receptor-like receptor; Actinium-225					
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1. INTRODUCTION:

When breast cancer (BCa) patients develop bone metastases, their 5-year survival rate declines by more than 70%. External beam radiation is used primarily for palliative treatment for patients with bone metastases. Bisphosphonate and anti-receptor activator of nuclear factor κ B ligand (RANKL) antibody to decrease bone resorption can reduce the onset of pain, but they ultimately fail to improve overall survival. Radium-223 ($^{223}\text{RaCl}_2$) can extend overall survival of prostate cancer patients with bone metastases, but only to a limited extent. Since these treatments mainly target bone remodeling, **new approaches that target factors other than bone remodeling are needed.**

Cancer-induced bone pain is the most common and intractable symptom of bone metastases, and it substantially impairs quality of life. Eighty percent of patients with bone metastasis have cancer-induced bone pain, and its management is a tremendous challenge for patients and caregivers. It has also been suggested that cancer-induced bone pain may be a negative indicator of survival. The ALSYMPCA trial, which investigated the role of $^{223}\text{RaCl}_2$ in prostate cancer patients with bone metastases, revealed that decreased pain levels correlated with increased overall survival. Thus, treating bone metastases by targeting pain signals are a promising approach to improve mortality.

Calcitonin gene-related peptide (CGRP), a 37-amino acid neuropeptide widely distributed in the peripheral and central nervous systems, is closely associated with pain behavior. Sensory nerves that express CGRP were enriched in the periosteum and bone marrow of a murine model of BCa bone metastasis. Patients with bone metastatic prostate cancer have elevated serum levels of CGRP. Sensory nerve-derived CGRP stimulated lung cancer growth. We have also found that: (i) bone metastatic prostate cancer directly affects sensory nerves to induce bone pain; (ii) sensory nerves that express CGRP are enriched in the marrow of those with bone metastatic prostate cancer; (iii) prostate cancer patients with bone metastatic disease have elevated serum levels of CGRP; (iv) samples from patients with bone metastatic BCa expressed higher levels of a CGRP receptor, calcitonin receptor-like receptor (CRLR); and (v) CGRP induces proliferation of BCa cells through CRLR. These data suggest that the CGRP/CRLR axis influences bone metastatic progression and could be a valuable therapeutic target.

The recent therapeutic efficacy of $^{223}\text{RaCl}_2$ ($t_{1/2} = 11.4$ d; $E_{\alpha_{\max}} = 6-7$ MeV) for treatment of prostate cancer patients with bone metastases has renewed interest in the development of alpha particle-based therapies. The effectiveness of $^{223}\text{RaCl}_2$ results from its ability to form complexes with hydroxylapatite in remodeling bone. $^{223}\text{RaCl}_2$ emits alpha particles and deposits substantial ionizing energy via high linear energy transfer (LET) over < 100 μm within bone tissue. When DNA is exposed to high LET, irreparable double-stranded breaks occur, resulting in cell death. $^{223}\text{RaCl}_2$ is more potent than low LET β -emitting bone-seeking agents, and its cytotoxicity is not affected by hypoxia or mechanisms of radio- or chemoresistance. However, its chemical properties make it difficult to conjugate to molecular delivery systems, such as antibodies that target solid tumors.

There is growing consensus that actinium-225 (^{225}Ac) ($t_{1/2} = 10$ d; $E_{\alpha_{\max}} = 6-8$ MeV) could be a superior alternative for targeted alpha particle therapy of bone metastases. Unlike other alpha particle-emitting radionuclides, ^{225}Ac of high specific activity and radionuclide purity is available. It has a long half-life, which synergizes well with the biological half-life of a circulating antibody. At the target site, it releases 30 million electron volts of total kinetic energy per nuclear decay, making it highly cytotoxic when properly targeted. **Unlike $^{223}\text{RaCl}_2$, ^{225}Ac 's chemistry is amenable to chelation and conjugation to targeting molecules such as antibodies and peptides.** Recent *in vivo* preclinical evaluation of ^{225}Ac radiotherapeutics demonstrates that ^{225}Ac may provide new strategies for targeted alpha particle therapy development. Although the recent clinical success of ^{225}Ac radiotherapeutics that target prostate-specific membrane antigen (PSMA) demonstrate that targeted alpha

particle therapy approaches could have a significant and positive impact on the management of prostate cancer, targeting PSMA is not applicable for other cancer types including breast cancer.

Hypothesis:

The delivery of alpha particle radiation to bone metastatic foci using CRLR-targeting ligands will reduce BCa tumor burden and cancer-induced bone pain.

Specific Aims:

Aim 1: Determine the best alpha particle radiopharmaceuticals targeted to the CRLR.

We will develop CRLR-targeted peptide (CGRP₂₇₋₃₇) conjugated with actinium-225 (²²⁵Ac, ²²⁵Ac-CGRP₂₇₋₃₇). Then, the binding affinity of ²²⁵Ac-CGRP₂₇₋₃₇ to CRLR will be determined *in vitro*. The biodistribution of ²²⁵Ac-CGRP₂₇₋₃₇ and organ dosimetry will be determined *in vivo*.

Aim 2: Determine the impact of CRLR-targeted alpha particle therapy on BCa bone metastases and cancer-induced bone pain.

Using an *in vivo* bone metastatic model that allows us to measure tumor growth, bone remodeling, skeletal innervation, and pain behaviors within each mouse, we will determine the safety and efficacy of ²²⁵Ac-CGRP₂₇₋₃₇ in the treatment of bone metastatic progression and its resultant pain.

Our proposal would be the first to rigorously investigate the use of ²²⁵Ac-based targeted alpha particle therapy to target the CRLR, a receptor for the pain-related neuropeptide CGRP, in breast cancer bone metastases. If successful, the proposed studies will yield a paradigm-shifting treatment strategy for BCa patients with bone metastases.

The **immediate benefits** of this research will provide essential preliminary data for the design of subsequent clinical translational studies. Our results could be quickly translated into novel treatment strategies to reduce breast cancer-associated morbidity and mortality.

The potential for **long-term and positive impact** of our work is substantial. This work will inform new treatment strategies to address both bone metastatic progression and cancer-induced bone pain of BCa.

2. KEYWORDS:

Breast Cancer; Bone metastasis; Cancer-induced bone pain; Radiopharmaceuticals; Calcitonin receptor-like receptor; Actinium-225

3. ACCOMPLISHMENTS:

What were the major goals and objectives of the project?

This application will test a new and innovative approach for ^{225}Ac -based targeted alpha particle therapy of breast cancer bone metastases by exploiting CRLR as a potential therapeutic target for breast cancer bone metastases, with the goal of improving both patients' quality of life and overall survival.

Task 1: *In vitro* selection of CRLR-targeted alpha particle radiopharmaceuticals.

Months 1-12.

- Synthesize the CGRP₂₇₋₃₇ (**Months 1-2; Dr. Shiozawa**).
- Conjugate CGRP₂₇₋₃₇ to ^{225}Ac -PCTA (**Months 3-5; Dr. Wadas**).
- Test the serum stability of ^{225}Ac -CGRP₂₇₋₃₇ (**Months 6-8; Dr. Wadas**).
- Establish CRLR-down-regulated, CRLR-overexpressed breast cancer cells (**Months 6-8; Dr. Shiozawa**).
- Perform receptor binding assays and internalization assays (**Months 9-12; Dr. Wadas**).

Task 2: *In vivo* selection of CRLR-targeted alpha particle radiopharmaceuticals.

Months 1-18.

- Submit documents for local IACUC review. (**Months 1-2; Dr. Shiozawa**).
- Submit IACUC approval and necessary documents for review by the Animal Care and Use Review Office (ACURO). (**Months 3-4; Dr. Shiozawa**).
- Perform biodistribution and dosimetry assays (**Months 13-18; Drs. Shiozawa and Wadas**).

Task 3: Determine the maximum tolerated dose of CRLR-based targeted alpha particle therapy.

Months 18-24.

- Perform maximum tolerated dose studies (**Months 18-24; Drs. Shiozawa and Wadas**).
- Co-author comprehensive review of radiopharmaceutical treatment and bone metastasis. (**Months 20-14; Drs. Shiozawa and Wadas**).

Task 4: Determine treatment efficacy of CRLR-based targeted alpha particle therapy.

Months 25-34.

- Test the effects of ^{225}Ac -CGRP₂₇₋₃₇ on tumor growth in the marrow, bone remodeling, and pain-related behavior within the same animal (**Months 25-34; Drs. Shiozawa and Wadas**).
- Co-authored manuscript summarizing all the results. (**Months 33-36; Drs. Shiozawa and Wadas**).

What was accomplished under these goals?

(2019-2020)

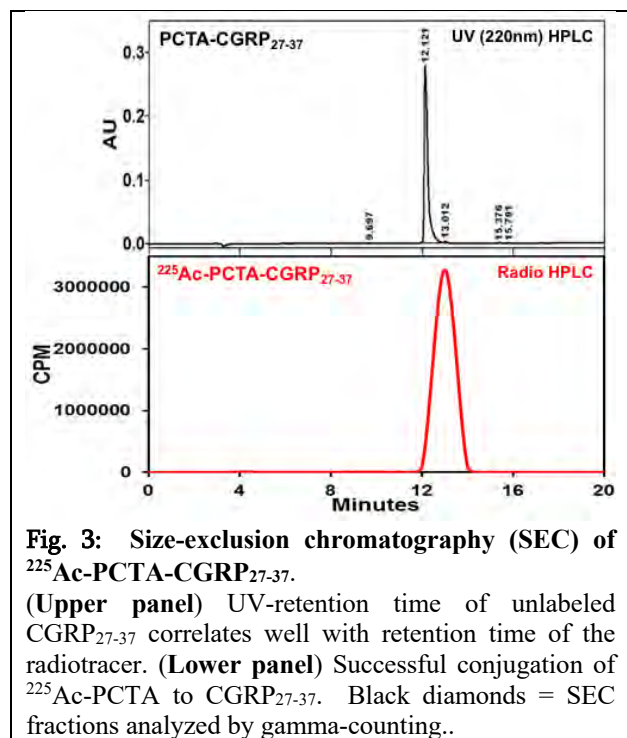
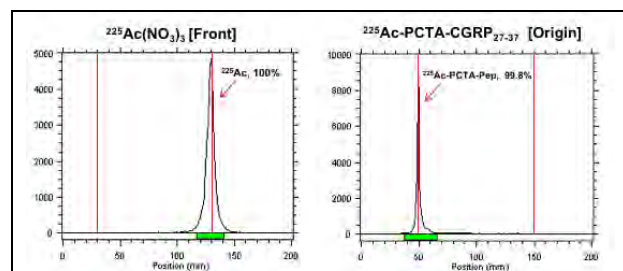
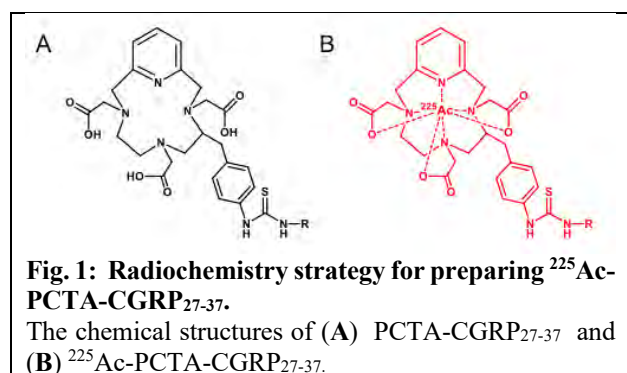
The Award Transfer (Drs. Wadas and Shiozawa).

As of 12/01/19, the Partnering PI, Dr. Wadas accepted a position as an Associate Professor at the University of Iowa. Upon his arrival at the University of Iowa, Dr. Wadas has been working on obtaining the necessary institutional approvals (Institutional animal care and use committee, Institutional biosafety committee, etc) to submit the grant transfer request to the Department of Defense from the Wake Forest University Health Sciences (WFUHS) to the University of Iowa. Currently, the award has been transferred and all necessary institutional approvals have been obtained for studies to commence at the University of Iowa.

Additionally, to accomplish the remaining goals of the award at Wake Forest, Dr. Shiozawa was required to obtain approval for his own radioactive materials protocol. Currently, he has received the necessary didactic training provided by WFUHS Radiation Safety Committee. However, before his protocol is approved, the WFUHS Radiation Safety Committee has required him to gain “hands-on” training at the University of Iowa from Dr. Wadas, who is an internationally recognized expert in the safe use of alpha-particle-emitting radionuclides. Unfortunately, The University of Iowa shut-down and non-essential air travel restrictions put in place due to the COVID-19 outbreak have delayed this training.

^{225}Ac -PCTA-CGRP₂₇₋₃₇ was stable in human serum (Dr. Wadas): Task 1.

To assess the stability of ^{225}Ac -PCTA-CGRP₂₇₋₃₇, we first synthesized the CGRP₂₇₋₃₇ by conjugating with a PCTA chelator (**Fig. 1A**) and radiolabeling it with ^{225}Ac using standard protocols (Tafreshi NK et al., J. Nucl. Med. 2019; 60:1124-33) (**Fig. 1B**). Formation of ^{225}Ac -PCTA-CGRP₂₇₋₃₇ was verified by Radio-TLC (**Fig. 2**) as well as Radio-HPLC (**Fig. 3**). Then, *in vitro* stability of ^{225}Ac -PCTA-CGRP₂₇₋₃₇ was determined by incubating with 500 μL of human serum at 37°C. The radiopharmaceutical was stable after 10 days in human serum at 37°C (**Fig. 4**).



Day	% Intact	
	TLC scanner	Gamma-counter
0	100	100
1	98.5 ± 0.5	98.1 ± 0.4
3	96.6 ± 0.7	96.1 ± 0.8
5	93.9 ± 0.4	93.2 ± 1.1
7	92.4 ± 0.8	91.7 ± 0.9
10	90.1 ± 0.6	89.8 ± 0.5

Fig. 4: *In vitro* serum stability of ^{225}Ac -PCTA-CGRP₂₇₋₃₇.

In vitro serum stability was carried out by adding 50 μL of ^{225}Ac -PCTA-CGRP₂₇₋₃₇ (45 μCi) to 1 mL of human serum. The solutions were incubated at 37°C for 10 days and were analysed at suitable time points by TLC scanner and quantified by gamma-counter.

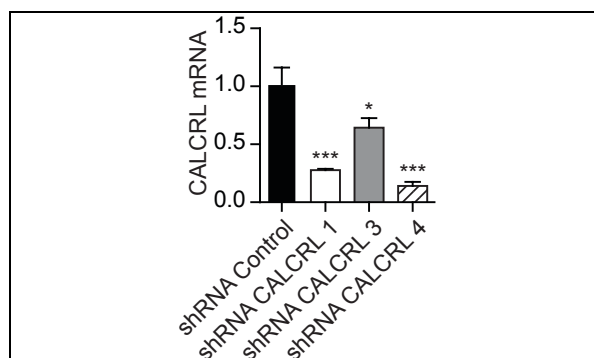
The CALCRL in breast cancer cells were down-regulated using shRNA (Dr. Shiozawa): Task 1.

Fig. 5: The down-regulation of CALCRL in breast cancer cells.

CALCRL knockdown in MDA-MB-231 cells. $p \leq 0.05$, *** $p \leq 0.001$ vs. shRNA control (One-way ANOVA, Dunnett's multiple comparisons). Presented as mean fold change $\pm$ SEM.

To test the binding affinity of ^{225}Ac -PCTA-CGRP₂₇₋₃₇ to CRLR on breast cancer cells, we first needed to manipulate the levels of CRLR in breast cancer cells. To do so, we tested 4 different shRNAs targeting CALCRL (gene name of CRLR). Three out of four shRNAs successfully down-regulated CALCRL in MDA-MB-231 cells (**Fig. 5**), while one of them (shRNA CALCRL 2) killed cells (data not shown). Based on the knock-down efficiency, we chose CALCRL-down-regulated MDA-MB-231 cells by shRNA CALCRL 1 and 4 to be used in future binding affinity analyses.

(2020-2021)

The plan for training Dr. Shiozawa in the safe use of radioactive materials (Drs. Wadas and Shiozawa).

To accomplish the remaining goals of the award at Wake Forest, Dr. Shiozawa was required to obtain approval for his own radioactive materials protocol due to Dr. Wadas' institution change. Currently, he has received the necessary didactic training provided by WFUHS Radiation Safety Committee. However, before his protocol is approved, the WFUHS Radiation Safety Committee has required him to gain "hands-on" training at the University of Iowa from Dr. Wadas, who is an internationally recognized expert in the safe use of alpha-particle-emitting radionuclides. Unfortunately, the University of Iowa shut-down and non-essential air travel restrictions put in place due to the COVID-19 outbreak have delayed this training. Therefore, Drs. Shiozawa and Wadas are currently trying to conduct the online training (we are currently setting this up and we may require 2-3 times) between Wake Forest and University of Iowa. This will be followed by in-person training at the University of Iowa so that Dr. Shiozawa may gain experience with the safe handling of alpha particle emitting radionuclides.

The joint publication (Drs. Shiozawa and Wadas): Task 3.

Drs. Shiozawa and Wadas published together a review manuscript relevant to this study. This paper is selected by the editors as the cover of the 2021; Volume 26 (Issue 8) (<https://www.mdpi.com/1420-3049/26/8>).

Patel CM, **Wadas TJ**, **Shiozawa Y**. Progress in Targeted Alpha-Particle-Emitting Radiopharmaceuticals as Treatments for Prostate Cancer Patients with Bone Metastases. *Molecules*. 2021; In Press. PMID: In Progress. PMCID: In Progress.

The synthesis of CGRP₂₇₋₃₇ analogs (Dr. Wadas): Task 1.

During the current funding period, we focused on the largescale synthesis of the CGRP peptides described in the application. This was completed to ensure that sufficient material would be available once the resumption of research is allowed at the University of Iowa and at Wake Forest University Health Sciences. Briefly, the CGRP peptides were prepared on an AAPTEC APEX-396 automated peptides synthesizer using standard Fmoc chemistry, HBTU and 9-hydroxybenzotriazole. Deprotection using a standard trifluoroacetic acid, water and triisopropylsilane solution afforded all of the peptides in good yields. To conjugate the PCTA chelator to each peptide, standard NCS coupling chemistry was utilized. In these reactions, 1.2 mole equivalents of the PCTA-NCS bifunctional chelator (Macrocylics, Inc.) was reacted with 1 mole equivalent of the unconjugated peptide in HEPES buffer (pH 9). The crude conjugates were then purified by semi-preparative reverse phase HPLC. The purity and identity of each product was assessed using an Agilent 1200 series HPLC interfaced with

an AB Sciex 4000QT LC-MS system. After the confirmation of purity and identity, each conjugate underwent lyophilization to yield a white fluffy powder. The structures of each complex to be studied are shown in **Fig. 6**. The conjugates are highly lipophilic and insoluble on the macro-scale in aqueous media. Considering this observation, we have expended additional efforts to test different organic-aqueous solvent systems that will 1) keep the peptides in solution once the lyophilized powder is rehydrated, 2) not cause conjugate degradation after solubilization, and 3) be robust enough to withstand the degradative effects of ionizing radiation. These studies are ongoing.

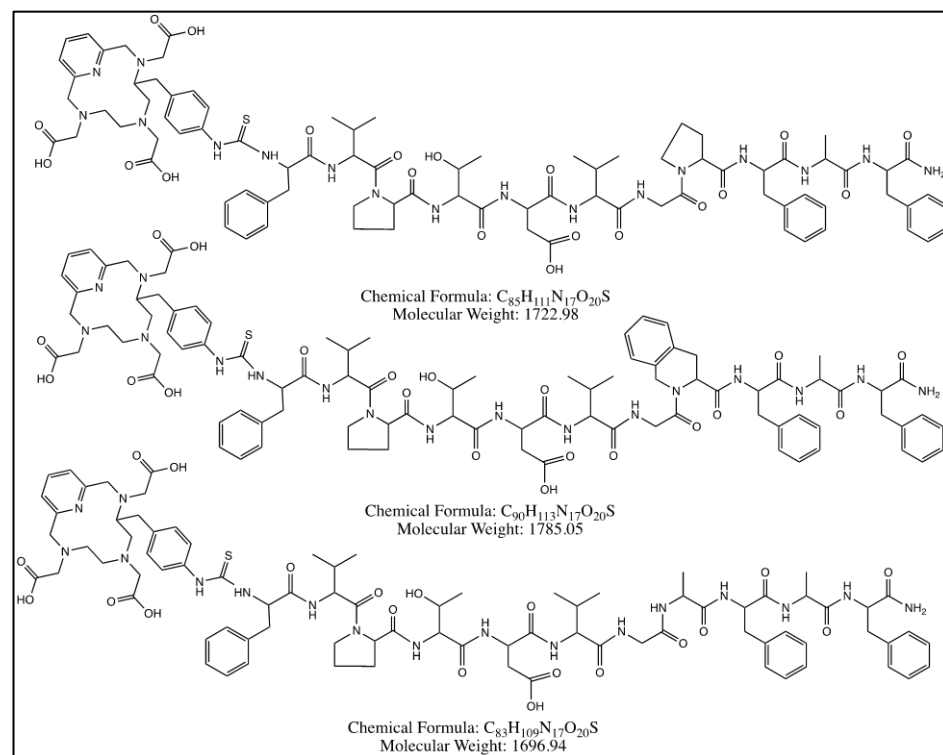


Fig. 6. The chemical structure of the PCTA-CGRP peptides.

The development of the over-expression virus vector for CRLR (Dr. Shiozawa): Task 1.

To first create CRLR-over-expressing breast cancer cells, MDA-MB-231 and ZR-75-1 cells were transfected with pCMV3-CALCRL (gene name of CRLR) expression vector [pCMV3-CALCRL vector (SinoBiological, cat: #HG11518-UT)] using lipofectamine reagents (Lipofectamine 2000) following a company's recommended protocol. However, the survival rate of resulting cells after antibiotic selection was extremely low, suggesting that the transfection efficiency was low. Since MDA-MD-231 cells are known to be a hard-to-transfect cell line, we upgraded lipofectamine reagents to Lipofectamine 3000, which is a more optimized reagent for hard-to-transfect cells and then repeated transfection using pCMV3-CALCRL expression vector. However, the survival rate of resulting cells after antibiotic selection was still extremely low. After this attempt, we also modified the ratio between the number of cells and the amount of transfection reagents several times. However, these protocol modifications also failed to improve the transfection efficiency. To overcome this problem, we decided to

generate a lentiviral vector containing a full length human CALCRL ORF. We are currently subcloning CALCRL ORF into a lentiviral vector (Biobasic company). This subcloning includes (i) the synthesis of the full length CALCRL ORF sequences with addition of the 5' cloning site (SpeI) and the 3' cloning site (EcoRI), (ii) the deletion of Oct4 ORF in the receiving plasmid [pSin-EF2-Oct4-pur (Addgene, cat: #16579) (**Fig. 7**)] between 5' SpeI and 3' EcoRI cloning sites, (iii) the ligation of the synthesized CALCRL ORF into the receiving plasmid between 5' SpeI and 3' EcoRI cloning sites, and (iv) the verification of the successful ligation by sequencing the final plasmid, pSin-EF2-CALCRL-puro plasmid. Thereafter, the lentiviral particles will be generated using these final plasmids. CALCRL ORF be transduced into the MDA-MD-231 and ZR-75-1 cells using the resulting lentiviral vector.

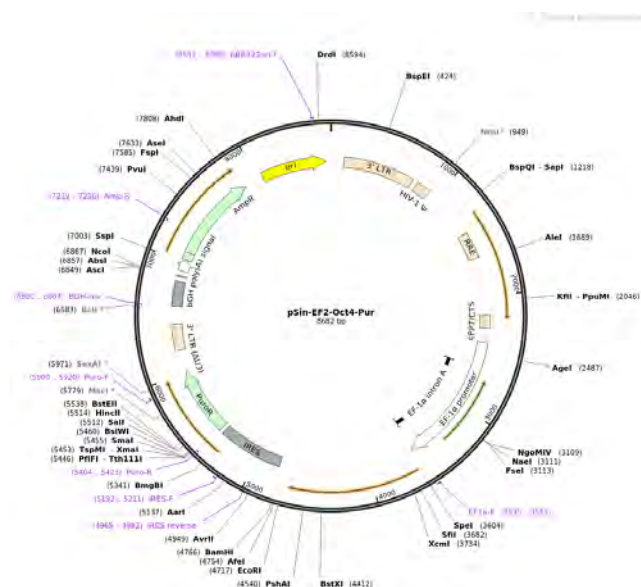


Fig. 7. The map of pSin-EF2-Oct4-pur plasmid.

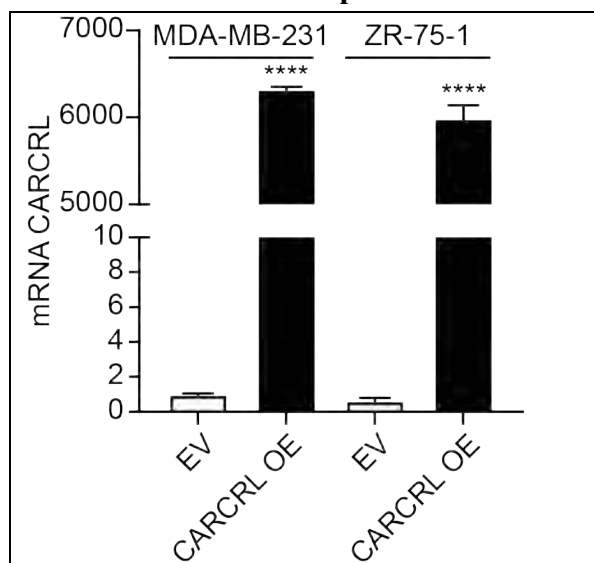
(2021-2022)

No cost extension on this award (Drs. Shiozawa and Wadas).

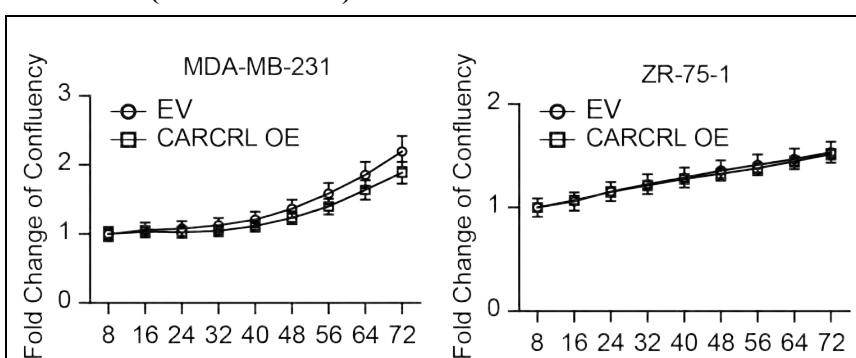
One-year no cost extension on this award was approved due to the COVID-19 pandemic.

Institutional radiation safety approval by Wake Forest School of Medicine (Dr. Shiozawa).

Due to the relocation of Dr. Wadas from Wake Forest School of Medicine to University of Iowa, Dr. Shiozawa must have obtained institutional approval for the radioactive work to perform the proposed experiments and receive the appropriate training. However, we experienced delays in obtaining institutional approval to perform the proposed radio isotope experiments due to the COVID-19 pandemic as well as the relocation and lab renovations of Dr. Wadas. After conducting online training with Dr. Wadas, Dr. Shiozawa obtained institutional radiation safety approval to perform proposed work in November 2021.

The CALCRL over-expression in breast cancer cells (Dr. Shiozawa): Task 1.**Fig. 8: The over-expression of CALCRL in breast cancer cells.**

CALCRL over-expression in MDA-MB-231 and ZR-75-1 cells. **** $p \leq 0.0001$ vs. empty vector control (Student's *t*-test). Presented as mean $\pm$ SEM.

**Fig. 9: CALCRL over-expression did not affect proliferation in breast cancer cells.**

CALCRL-over-expressed and empty vector-transfected control breast cancer cells (MDA-MB-231 and ZR-75-1) were seeded in a 96 well plate (1000 cells/well) and proliferation was monitored by Incucyte Live-Cell Analysis Systems for 72 hours. Presented as mean fold change $\pm$ SEM.

To test the binding affinity of ^{225}Ac -PCTA-CGRP₂₇₋₃₇ to CRLR on breast cancer cells, we needed to manipulate the levels of CRLR in breast cancer cells. To do so, during the previous period, we developed (i) the CALCRL (gene name of CRLR)-downregulated breast cancer cells and CALCRL-over-expression lentiviral vector. During the current funding period, we successfully over-expressed CALCRL in both MDA-MB-231 and ZR-75-1 cells with CALCRL-over-expression lentiviral vector (**Fig. 8**). Upon successful cell line establishment, CALCRL-over-expressed and control breast cancer cells lines were evaluated for proliferation, and CALCRL-over-expression did not affect cell proliferation (**Fig. 9**).

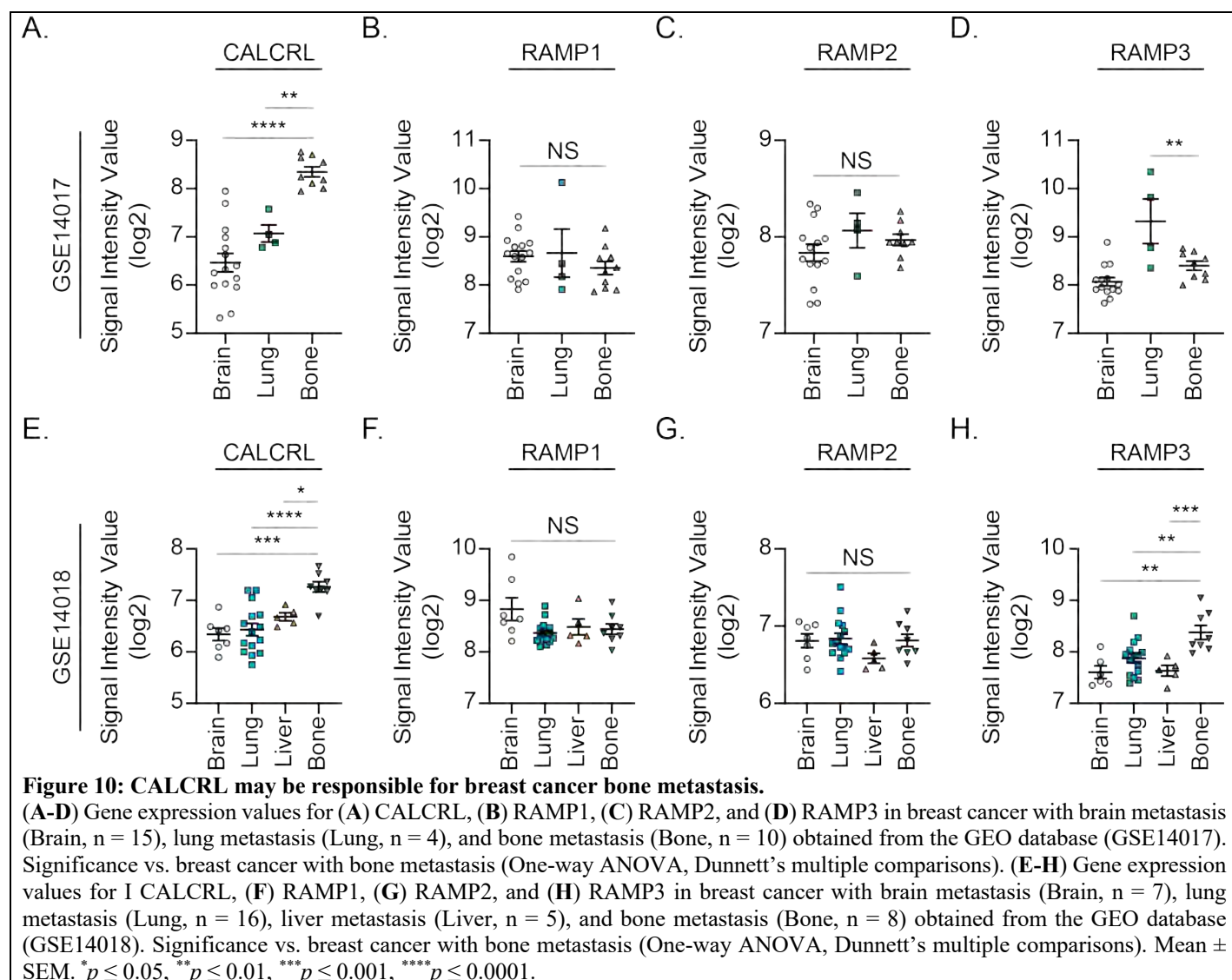
(2022-2023)

Setting up study using radiopharmaceuticals in Wake Forest School of Medicine (Dr. Shiozawa): Task 4.

Due to the extremely limited amounts of ^{225}Ac produced (please see CHANGES/PROBLEMS section), the generation and testing of the CRLR-targeted ^{225}Ac is now beyond the scope of our goal. Since we have planned to use ^{223}Ra as a control for the CRLR-targeted ^{225}Ac but never performed any study using radiopharmaceuticals, and ^{223}Ra is currently available as a product from Oak Ridge National Labs reactors bi-monthly, we decided to test if we could safely operate a radiopharmaceutical study in our institution using ^{223}Ra , while waiting for ^{225}Ac availability. We started working with Atrium Wake Forest IACUC and we acquired the designated mouse barrier room and biosafety cabinet for this study in December, 2022. The initial studies were originally scheduled to start in January, 2023 and would have completed the initial ^{223}Ra study by March, 2023. However, due to difficulties filling out paperwork through the Department of Energy (DOE) and internally for Atrium Wake Forest Radiation Safety/IACUC, we were unable to receive the compound for this date. The next available window for Radium delivery was March, and we were scheduled to receive 1 millicurie (mCi) of ^{223}Ra from Oak Ridge National Labs the week of March 20th. We ordered 36 mice and performed all basic preparations including the examination of baseline pain behavior and BLI/X-Ray imaging for these mice the week before. Our lab team performed the surgery of these mice on March 17th, injecting MDA-MB-231 cells through interfemoral injection. We were contacted by Oak Ridge that afternoon that the ^{223}Ra would not be able to be delivered on the set date due to a precursor reactor product not yielding enough decayed material to complete our order on time, and that the order would be filled on the next available delivery window (May). Although we were not able to perform ^{223}Ra study within the grant period, we will plan to follow up this study using other funding sources.

The CRLR may be responsible for development of breast cancer bone metastasis, but not brain, lung, or liver metastases (Dr. Shiozawa): Task 4.

Our preliminary data have indicated that bone metastatic breast cancer expresses higher levels of CRLR (gene name: CALCRL). Since breast cancer patients develop not only bone metastasis at a later stage, but also other types of metastases, we investigated the roles of CRLR and RAMPs in breast cancer metastases using two breast cancer patient cohorts in the Gene Expression Omnibus (GEO) Datasets. Intriguingly, samples from breast cancer patients with bone metastases expressed higher CALCRL levels, compared to those with brain, lung, or liver metastases (**Fig. 10A & E**). However, none of RAMPs were consistently expressed higher in the samples from breast cancer patients with bone metastases (**Fig. 10B-D & F-H**). These data further suggest that the CRLR expressed by bone metastatic breast cancer could be a valuable therapeutic target.



The CALCRL over-expression didn't affect the growth of breast cancer cells *in vivo* (Drs. Shiozawa and Wadas): Task 4.

During the previous period, we developed CALCRL-overexpressing human breast cancer MDA-MB-231 cells (Fig. 11A) and we confirmed the expression of CALCRL on the cell surface of these cells (Fig. 11B). During this award period, we tested if CALCRL overexpression influenced the growth of breast cancer cells *in vivo*. To do so, CALCRL-overexpressing cells (CALCRL OE) and empty vector-transfected cells (Vector) were subcutaneously inoculated into nude mice. As expected, both CALCRL OE and Vector cells developed palpable tumor in flank (Fig. 11C). However, CALCRL overexpression didn't affect breast cancer growth *in vivo*. This data suggests that the CALCRL-overexpressing MDA-MB-231 cells can be used for further analyses to determine if CRLR-targeting radiopharmaceuticals can bind to CRLR on breast cancer *in vivo*, and the impact of radiopharmaceuticals on breast cancer bone metastases and cancer-induced bone pain *in vivo*.

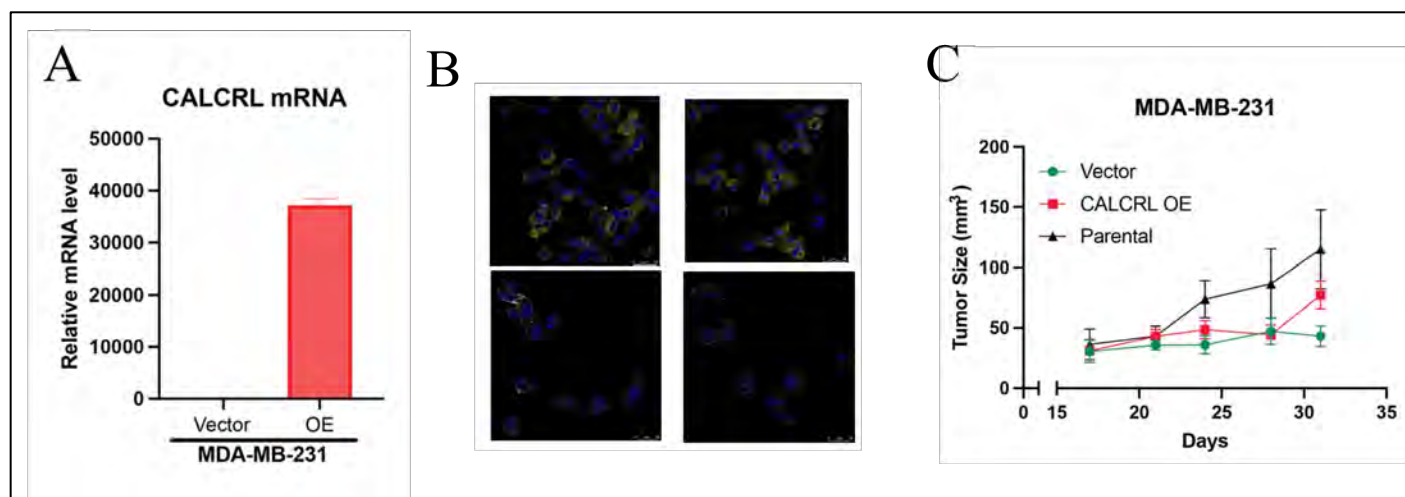


Figure 11: CALCRL may be responsible for breast cancer bone metastasis. (A) CALCRL overexpression in MDA-MB-231 cells. (B) Immunohistochemistry of engineered MDA-MB-231 cells confirming cell surface expression of CALCRL. **Top:** MDA-MB-231 cells overexpressing CALCRL on the surface of viable cells. **Bottom:** MDA-MB-231 cells without CALCRL expression on cell surface (negative control). DAPI (blue; nucleus); WGA (white; cell membrane); CALCRL expression (green; cell surface). (C) Female 6-8-week-old mice were inoculated with either 1.0×10^6 CALCRL-overexpressing or empty vector-transfected MDA-MB-231 cells through subcutaneous injection into the right flank. Tumor volume was tracked using a caliper, starting 17 days after injection of the tumor cells.

Synthesis of anti CRLR radiopharmaceuticals using ^{89}Zr -chloride (Dr. Wadas): Task 4.

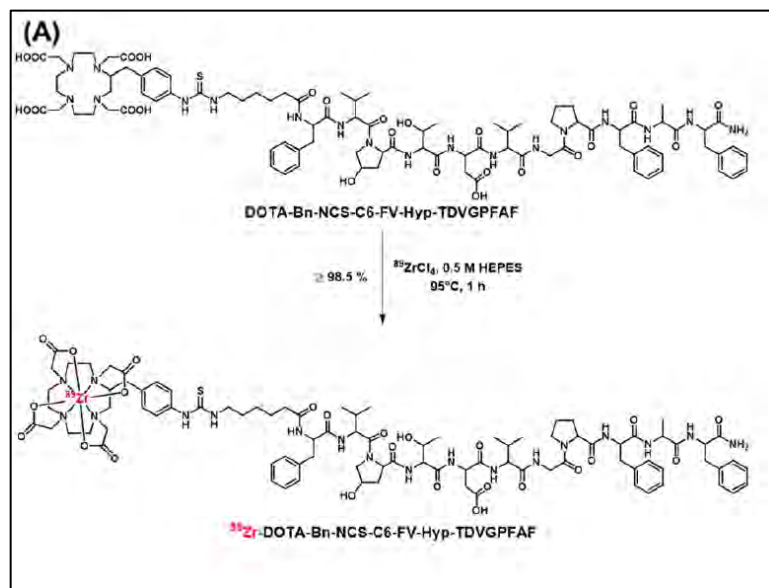


Figure 12. Representative synthesis of novel anti-CALCRL radiopharmaceuticals. Using ^{89}Zr -chloride, the unlabeled conjugate could be radiolabeled with ^{89}Zr in high yield, high purity, and specific activity.

During the previous reporting period we synthesized several new analogs for testing. Unlike the structures originally proposed, these novel systems are only 10 amino acids in length and have K_i values in the picomolar range. We synthesized these peptides in bulk and added the macrocyclic chelator DOTA to the peptides so that these conjugates could be radiolabeled with the PET isotope ^{89}Zr (Fig.12). Radiolabeling of the conjugates proceeded smoothly to afford the radiopharmaceuticals in excellent radiochemical purity, radiochemical yield, and high specific activity (Fig. 13). To obtain a preliminary assessment of radiopharmaceutical stability, all radiolabeled agents were incubated in human serum for 72 h. During the time course of this experiment no degradation was observed. This suggests that the peptide ligands and the ^{89}Zr -DOTA complex are both resistant to the proteins contained within human serum. It also suggests that these radiopharmaceuticals will

remain stable once injected *in vivo*. Current work is focused on characterizing the affinity and selectivity of

each radiopharmaceutical for CRLR using the cell lines recently provided by the Shiozawa lab.

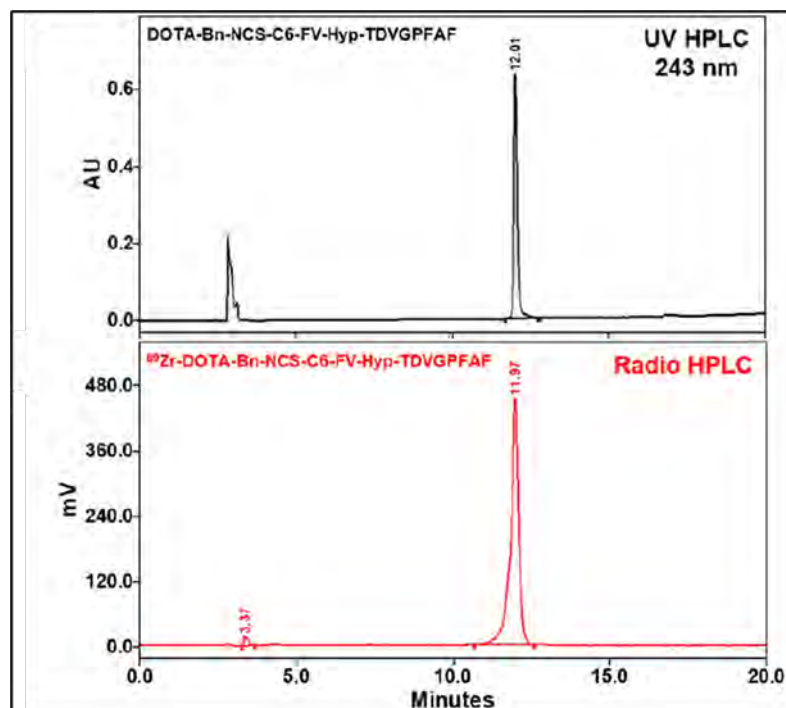


Figure 13. Quality control of the newly developed radiopharmaceuticals. Upper Panel: Representative UV-HPLC of the cold conjugate depicted in Figure 12. Lower Panel: Representative Radio-HPLC of the radiopharmaceutical depicted in Figure 12. There is good agreement in the retention time between the unlabeled conjugate and the radiopharmaceutical.

What opportunities for training and professional development did the project provide?

(2019-2020)

Dr. Shiozawa was invited to give a talk at The U.S. Bone and Joint Initiative (USBJI) and Bone and Joint Canada (BJC) Young Investigator Initiative Workshop Spring 2019 (Rosemont, IL) on April 27, 2019.

Dr. Shiozawa was invited to give a seminar at University of North Carolina at Chapel hill, Adams School of Dentistry (Chapel hill, NC) on December 03, 2019.

Dr. Wadas gave a lecture at the University of Iowa's Free Radical and Radiation Biology Program. This lecture dealt with the use of alpha particle therapy in cancer care. University of Iowa, Iowa City, IA (February 25, 2020).

AWARD: BC180720, BC180720P1

TITLE: A targeted alpha particle radiopharmaceutical for bone metastatic breast cancer

PI: Yusuke Shiozawa, M.D., Ph.D., Partnering PI: Thaddeus Wadas, Ph.D.

Dr. Wadas accepted the role as Guest Editor of a Special Issue of *Molecules* that focuses on the use of radiotherapy of disease. The Issue description and call for papers can be found at https://www.mdpi.com/journal/molecules/special_issues/radiopharmaceutical_chemistry_radiotherapy.

(2020-2021)

Dr. Wadas gave a lecture at the University of Iowa's Cancer Biology Program. This lecture dealt with the development of targeted alpha particle-based radiotherapy and its use in oncology. University of Iowa, Iowa City, IA (November 26, 2020).

Dr. Wadas gave a lecture at the University of Iowa's Human Toxicology Program. This lecture dealt with the use of PET imaging in the precision medicine era. University of Iowa, Iowa City, IA (December 20, 2020).

(2021-2022)

As of July 1st, 2021, Dr. Shiozawa was promoted to an Associate Professor with Tenure.

Dr. Shiozawa was chosen to participate in the 2022 cohort of the Wake Forest Clinical and Translational Science Institute's Mentor Academy (February 2022-June 2022).

Dr. Shiozawa was chosen to participate in the 2022 cohort of the Wake Forest Clinical and Translational Science Institute's Leadership Academy (February 2022-June 2022).

Dr. Shiozawa became the Cancer Biology representative to the Molecular and Cellular Biosciences (MCB) (PhD) track admission committee (2021-Present).

Dr. Shiozawa became a member of Biomedical Science (BMSC)-Research Pathway (Master of Science) program recruitment committee (2022-Present).

Dr. Wadas received a secondary appointment as an Associate Professor of Radiation Oncology. Department of Radiation Oncology, University of Iowa, Iowa City, IA.

Dr. Wadas served on NIH Special Emphasis Panel/Scientific Review Group ZCA1 RPRB-H (M1) S. February 2022.

(2022-2023)

Dr. Shiozawa was chosen to become a member of Membership Engagement Committee of American Society for Bone and Mineral Research (2022-Present).

Dr. Shiozawa served on NIH grant review panel (i) 2022/10 ZCA1 TCRB-5 O1 S, SEP-3: NCI Clinical and Translational Cancer Research, May 27, 2022; (ii) 2022/10 ZNS1 SRB-W (03): EPPIC-NET: Review of asset applications for the Early Phase Pain Investigation Clinical Trials Network, June 22, 2022; and (iii) 2023/05 Oncology 1-Basic Translational Integrated Review Group, Tumor Host Interactions Study Section, February 22-23, 2023.

Dr. Shiozawa became a member of Cancer Biology Promotion and Tenure Committee (2022-Present).

Dr. Shiozawa became a member of Cancer Biology Strategic Plan Committee (2022-Present).

Dr. Wadas served on NIH Special Emphasis Panel/Scientific Review Group ZCA1 RPRB-H (M1) S. February 2023.

Dr. Wadas served on NIH Special Emphasis Panel/Scientific Review Group ZCA1 RPRB-H (M1) S. October 2023.

How were the results disseminated to communities of interest?

There is nothing to report.

What do you plan to do during the next reporting period to accomplish the goals and objectives?

- 1) Determine if CRLR-targeting radiopharmaceuticals can bind to CRLR on BCa *in vivo* (Aim 1&2).
- 2) Determine the impact of radiopharmaceuticals on BCa bone metastases and cancer-induced bone pain *in vivo* (Aim 2).
- 3) Assess the affinity and selectivity of the radiopharmaceuticals for the CRLR in vitro using the cell lines provided by Shiozawa lab (Aims 1& 2).
- 4) Assess the biodistribution and tumor retention of the radiopharmaceuticals in vivo (Aims 1& 2).

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

(2019-2020)

1. We found that ^{225}Ac -PTCA-CGRP₂₇₋₃₇ were stable in human serum.
2. We were able to down-regulate CRLC expression levels in BCa using shRNA.

(2020-2021)

1. Drs. Shiozawa and Wadas published together a review manuscript relevant to this study.
2. We were able to synthesize several different analogs of CGRP₂₇₋₃₇.
3. We are currently developing the over-expression virus vector for CRLR.

(2021-2022)

1. Drs. Shiozawa and Wadas obtained the one-year no cost extension on this award due to the COVID-19 pandemic.
2. Dr. Shiozawa obtained the institutional radiation safety approval from Wake Forest School of Medicine to perform the proposed experiments.
3. We were able to over-express CRLR in breast cancer cells.
4. The Wadas research group developed methods to overcome the solubility issues with the bioconjugates under investigation.
5. The Wadas group synthesized several new analogs for evaluation that are in addition to the molecules proposed in the original application.

(2022-2023)

1. Dr. Shiozawa acquired the designated mouse barrier room and biosafety cabinet in December 2022 and filled out the paperwork through the Department of Energy and Atrium Wake Forest Radiation Safety/IACUC to perform the proposed experiments.
2. We further validated that CRLR is a suitable target to treat bone metastatic breast cancer.
3. We validated that CRLC-overexpressing breast cancer cells can grow in vivo without affecting growth rate.
4. The Wadas group radiolabeled several new analogs for evaluation that are in addition to the molecules proposed in the original application.

AWARD: BC180720, BC180720P1

TITLE: A targeted alpha particle radiopharmaceutical for bone metastatic breast cancer

PI: Yusuke Shiozawa, M.D., Ph.D., Partnering PI: Thaddeus Wadas, Ph.D.

What was the impact on other disciplines?

We were able to develop new methods of radiopharmaceutical production. While specific to this project, they may have broad applicability to radiopharmaceutical development in general.

What was the impact on technology transfer?

There is nothing to report at this time.

What was the impact on society beyond science and technology?

There is nothing to report at this time.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

(2019-2020)

Nothing to report.

(2020-2021)

There has been some non-key personnel turnover (Technician) and maternity leave (Postdoc) in Dr. Shiozawa's lab as well as the recent COVID-19 pandemic which have impacted the timeliness of conducting the proposed studies. Due to COVID-19, Wake Forest University Health Sciences (WFUHS) issued an institutional stop work order from the middle of March to the end of May 2020. The personnel have been replaced and the lab has resumed normal operations.

In the original application, all of the experimental work involving radioactivity was to be conducted by Dr. Wadas' laboratory and under his radiation safety protocol. Shortly after this application was funded however, Dr. Wadas accepted a position in the Department of Radiology at the University of Iowa and moved his laboratory there. Unfortunately, his move to the University of Iowa coincided with the COVID-19 pandemic. At that time the University of Iowa enacted an institutional stop work order from the middle of March to the end of July 2020. This stop work order hindered Dr. Wadas' productivity on two levels. Foremost, he was unable to work on the aims as currently outlined in the SOW after the award was successfully transferred to the University of Iowa. Secondly, this stop work order prevented the renovation of Dr. Wadas' laboratories to accommodate radioactive materials. The expected completion date of renovations is July 1, 2021. The renovation schedule also affected the timeline of the hands-on radiation safety training, which we are planning to conduct so that Dr. Shiozawa may receive approval to use radioactive materials at WFUHS. Although Dr. Shiozawa has completed the appropriate radiation safety training required by WFUHS and additional didactic training with Dr. Wadas via videoconference, the WFUHS Radiation Committee is still requiring that Dr. Shiozawa receive hands-on training in the proper handling, use, storage and disposal of alpha particle-emitting radioisotopes from Dr. Wadas at the University of Iowa. Once the WFUHS' COVID-19 travel restriction order, which prevents work-related travel, is rescinded on June 30, 2021 Drs. Wadas and Shiozawa will schedule a week-long radiation safety training session at the University of Iowa.

In the next year term, we will try to catch up the speed of project productivity and continue to (i) determine the binding affinity of radiopharmaceuticals to breast cancer cells *in vitro* (Aim 1) and (ii) determine the biodistribution and dosimetry of radiopharmaceuticals *in vivo* (Aim 1).

(2021-2022)

Nothing to report.

(2022-2023)

In the original application, we proposed to conjugate CRLR-targeting CGRP₂₇₋₃₇ analogs with Actinium-225 (²²⁵Ac) to target bone metastatic breast cancer cells. However, ²²⁵Ac compounds are in extreme demand for patient treatment/research purposes and compound availability for researchers continues to be problematic. The United State Department of Energy recently had a large group session in March 2023 detailing current challenges in generation and highlighting a new method which has the potential to greatly increase availability for future studies (<https://www.isotopes.gov/information/actinium-225>). While these developments do show much promise for researchers and feasibility of these type of treatments; unfortunately, these availability issues will not be resolved in time for this grant's scheduled completion date. After much consideration and contact with Oak Ridge National Labs (a major source of ²²⁵Ac) and other National Isotope Development Center (NIDC) sources for availability, we decided to change our approach. The current approach has focused on developing the imaging radiopharmaceuticals with the PET radioisotope zirconium-89 (⁸⁹Zr: $t_{1/2} = 78.4$ h, β^+ : 22.8 %, $E_{\beta+\max} = 901$ keV; EC: 77%, $E_{\gamma} = 909$ keV; 99%) instead of the therapy-based radiopharmaceuticals using ²²⁵Ac. We chose this route for several practical and scientific reasons. First, unlike ²²⁵Ac, ⁸⁹Zr is produced and shipped from academic and industrial production centers on a regular schedule. Secondly, the cost per millicurie of ⁸⁹Zr is 10-fold less than that of ²²⁵Ac. Scientifically, developing the anti-CRLR imaging radiopharmaceuticals first would allow the research team to compare the tumor targeting and biodistribution properties of the anti-CRLR ligands more efficiently. Once the best anti-CRLR ligand was identified, then the research team would be able to synthesize the best ²²⁵Ac analog for therapy studies.

Changes that have a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations

Journal Publications

Peer reviewed journal

(2019-2020)

1. Lycan TW, Hsu F, Ahn CS, Thomas A, Walker FO, Sanguenza OP, **Shiozawa Y**, Park SH, Peters CM, Romero-Sandoval EA, Melin SA, Sorscher S, Ansley K, Lesser GJ, Cartwright MS, Strowd RE. Neuromuscular ultrasound for taxane peripheral neuropathy in breast cancer. **Muscle and Nerve**. 2020; 61(5):587-594. PMID: 32052458. PMCID: In Progress.

Status of Publication: Published

Acknowledgement of federal support: Yes

2. Tafreshi NK, Tichacek CJ, Pandya DN, Doligalski ML, Budzevich M, Kil H-J, Bhatt NB, Kock ND, Messina J, Ruiz EE, Delva NC, Weaver A, Gibbons WR, Boulware DC, Khushalani NL, El-Haddad G, Triozzi PL, Moros EG, McLaughlin ML, **Wadas TJ**, Morse DL. Melanocortin 1 Receptor-Targeted α -Particle Therapy for Metastatic Uveal Melanoma. **J. Nucl. Med.** 2019; 60(8): 1124-1133. PMID: 30733316. PMCID: PMC6681690.

Status of Publication: Published

Acknowledgement of federal support: No

3. Tichacek CJ, Budzevich MM, **Wadas TJ**, Morse DL, Moros EG. A Monte Carlo Method for Determining the Response Relationship between Two Commonly Used Detectors to Indirectly Measure Alpha Particle Radiation Activity. **Molecules**. 2019; 24(18): 3397-3402. PMID: 31546752. PMCID: PMC6767018.

Status of Publication: Published

Acknowledgement of federal support: No

4. Tafreshi NK, Doligalski ML, Tichacek CJ, Pandya DN, Budzevich MM, El-Haddad G, Khushalani NI, Moros EG, McLaughlin ML, **Wadas TJ**, Morse DL. Development of Targeted Alpha Particle Therapy for Solid Tumors. **Molecules**. 2019; 24(23): 4314-4362. PMID: 31779154. PMCID: PMC6930656.

Status of Publication: Published

Acknowledgement of federal support: No

(2020-2021)

1. Wu S-Y, Xing F, Sharma S, Wu K, Tyagi A, Liu Y, Zhao D, Deshpande RP, **Shiozawa Y**, Ahmed T, Zhang W, Chan M, Ruiz J, Lycan TW, Dothard A, Watabe K. Nicotine promotes brain metastasis by polarizing microglia and suppressing innate immune function. *JEM*. 2020;217:e20191131. PMID: 32496556. PMCID: PMC7398164.

Status of Publication: Published

Acknowledgement of federal support: No

2. Eber MR, Park SH, Contino KF, Patel CM, Hsu F-C, **Shiozawa Y**. Osteoblasts derived from mouse mandible enhance tumor growth of prostate cancer more than osteoblasts derived from long bone. *J Bone Oncology*. 2021;26:100346. PMID: 33425674. PMCID: PMC7779864.

Status of Publication: Published

Acknowledgement of federal support: Yes

3. Park SH, Eber MR, M. Fonseca M, Patel CM, Cunnane KA, Ding H, Hsu F-C, Peters CM, Ko M-C, Strowd RE, Wilson JA, Hsu W, Romero-Sandoval EA, **Shiozawa Y**. Usefulness of the measurement of neurite outgrowth of primary sensory neurons to study cancer-related painful complications. *Biochem Pharmacol*. 2021;188:114520. PMID: **33741328**. PMCID: In Progress.

Status of Publication: Published

Acknowledgement of federal support: Yes

4. Widner DB, Liu C, Qingxia Z, Sharp S, Eber MR, Park SH, Files DC, **Shiozawa Y**. Activated Mast Cells in Skeletal Muscle Can be a Potential Mediator for Cancer-Associated Cachexia. *J. Cachexia Sarcopenia Muscle*. 2021; In Press. PMID: In Progress. PMCID: In Progress.

Status of Publication: In Press

Acknowledgement of federal support: Yes

(2021-2022)

1. Wu K, Feng J, Lyu F, Xing F, Sharma S, Liu Y, Wu S-Y, Zhao D, Tyagi A, Deshpande RP, Pei X, Ruiz MG, Takahashi H, Tsuzuki S, Kimura T, Mo Y-Y, **Shiozawa Y**, Singh R, Watabe K. Exosomal miR-19a and IBSP cooperate to induce osteolytic bone metastasis of estrogen receptor-positive breast cancer. *Nat Commun*. 2021;12:5196 PMID: 34465793. PMCID: PMC8408156.

Status of Publication: Published

Acknowledgement of federal support: No

2. Kiguchi N, Ding H, Park SH, Mabry KM, Kishioka S, **Shiozawa Y**, Romero-Sandoval EA, Peters CM, Ko M-C. Functional roles of neuromedin B and gastrin-releasing peptide in regulating itch and

pain in the spinal cord of non-human primates. *Biochem Pharmacol.* 2022;198:1144972. PMID: 35189108. PMCID: In Progress.

Status of Publication: Published

Acknowledgement of federal support: No

3. Tafreshi NK, Pandya DN, Tichacek CJ, Budzevich MM, Wang Z, Reff JN, Engelman RW, Boulware DC, Chiappori AA, Strosberg JR, Ji H, **Wadas TJ**, El-Haddad G, Morse DL. Preclinical evaluation of [²²⁵Ac]Ac-DOTA-TATE for treatment of lung neuroendocrine neoplasms. *Eur J Nucl Med Mol Imaging.* 2021 Oct;48(11):3408-3421. doi: 10.1007/s00259-021-05315-1. PMID: 33772332.

Status of Publication: Published

Acknowledgement of federal support: No

4. Tafreshi NK, Kil H, Pandya DN, Tichacek CJ, Doligalski ML, Budzevich MM, Delva NC, Langsen ML, Vallas JA, Boulware DC, Engelman RW, Gage KL, Moros EG, **Wadas TJ**, McLaughlin ML, Morse DL. Lipophilicity Determines Routes of Uptake and Clearance, and Toxicity of an Alpha-Particle-Emitting Peptide Receptor Radiotherapy. *ACS Pharmacol Transl Sci.* 2021;4(2):953-965. doi: 10.1021/acspsci.1c00035. PMID: 33860213; PMCID: PMC8033749.

Status of Publication: Published

Acknowledgement of federal support: No

(2022-2023)

1. Zaher, A., Stephens, L.M., Miller, A.M., Hartwig, S.M., Stolwijk, J.M., Petronek, M.S., Zacharias, Z.R., **Wadas, T.J.**, Monga, V., Cullen, J.J., Furqan, M., Houtman, J.C.D., Varga, S.M., Spitz, D.R., Allen, B.G. Pharmacological ascorbate as a novel therapeutic strategy to enhance cancer immunotherapy. *Front. Immunol.* **2022**;13:989000. doi: 10.3389/fimmu.2022.989000. eCollection 2022. PMID: 36072595.

Status of Publication: Published.

Acknowledgement of federal support: No

2. Abou, D.S., Zerkel, P., Robben, J., McLaughlin, M., Hazlehurst, T., Morse, D., **Wadas, T.J.**, Pandya, D.N., Oyama, R., Gaehle, G., Nickels, M.L., Thorek, D.L. J. Radiopharmaceutical Quality Control Considerations for Accelerator-Produced Actinium Therapies. *Cancer Biother. Radiopharm.* **2022**;37(5):355-363. doi: 10.1089/cbr.2022.0010. PMID: 35695807.

Status of Publication: Published.

Acknowledgement of federal support: No

3. Tran, C.G., Borbon, L.C., Mudd, J.L., Abusada, E., AghaAmiri, S., Ghosh, S.C., Vargas, S.H., Li, G., Beyer, G.V., McDonough, M., Li, R., Chan, C.H.F., Walsh, S.A., **Wadas, T.J.**, O'Dorisio, T.,

O'Dorisio, M.S., Govindan, R., Cliften, P.F., Azhdarinia, A., Bellizzi, A.M., Fields, R.C., Howe, J.R., Ear, P.H. Establishment of Novel Neuroendocrine Carcinoma Patient-Derived Xenograft Models for Receptor Peptide-Targeted Therapy. *Cancers (Basel)*. **2022**;14(8):1910-1920. doi: 10.3390/cancers14081910.PMID: 35454817.

Status of Publication: Published.

Acknowledgement of federal support: No

Invited reviews

(2019-2020)

Nothing to report.

(2020-2021)

1. Patel CM, **Wadas TJ**, **Shiozawa Y**. Progress in Targeted Alpha-Particle-Emitting Radiopharmaceuticals as Treatments for Prostate Cancer Patients with Bone Metastases. *Molecules*. 2021; In Press. PMID: In Progress. PMCID: In Progress.

Status of Publication: In Press

Acknowledgement of federal support: Yes

(2021-2022)

1. Contino KF, Yadav H, **Shiozawa Y**. The gut microbiota can be a potential regulator and treatment target of bone metastasis. *Biochem Pharmacol*. 2022;197:114916. PMID: 35041811. PMCID: PMC8858876.

Status of Publication: Published

Acknowledgement of federal support: Yes

(2022-2023)

1. Ollodart J, Contino KF, Deep G, **Shiozawa Y**. The Impacts of exosomes on bone metastatic progression and their potential clinical utility. *Bone Rep*. 2022;17:101606. PMID: 35910404. PMCID: PMC9335387.

Status of Publication: Published

Acknowledgement of federal support: Yes

2. Yu Y, Ollodart J, Contino KF, **Shiozawa Y**. Immunotherapy as a potential treatment approach for currently incurable bone metastasis. *J Bone Miner Metab*. 2023; In Press. PMID: 36752903. PMCID: In Progress.
Status of Publication: Published
Acknowledgement of federal support: Yes

Book

(2019-2020)

1. Park SH, **Shiozawa Y**. (2019) Genomic mutation as a potential driver of the development of bone-related cancers. In Zaidi M (Ed.), *Encyclopedia of Bone Biology*. In Press. Elsevier.
Status of Publication: In Press
Acknowledgement of federal support: Yes
2. **Shiozawa Y**. (2019) The roles of bone marrow-resident cells as a microenvironment for bone metastasis. In Birbrair A (Ed.), *Tumor Microenvironments in Different Organs – Part A*. In Press. Switzerland: Springer Nature.
Status of Publication: Published
Acknowledgement of federal support: Yes

(2020-2021)

1. Eber MR, Jimenez-Andrade JM, Peters CM, **Shiozawa Y**. (2020) A method of bone-metastatic tumor progression assessment in mice using longitudinal radiography. In Deep G (Ed.), *Cancer Biomarkers: Methods and Protocols*, Methods in Molecular Biology, In Press. New York: Humana Press. Methods Mol Biol. In Press. PMID: In Progress. PMCID: N/A.
Status of Publication: In Press
Acknowledgement of federal support: Yes

(2021-2022)

Nothing to report.

(2022-2023)

Nothing to report.

Presentation

(2019-2020)

1. Strowd RE, Lycan T, Thomas A, Hsu FC, Ahn C, Sanguenza O, **Shiozawa Y**, Park SH, **Peters CM**, EA Romero-Sandoval, Melin SA, Sorscher S, Lesser GJ, Walker FO, Cartwright MS. Neuromuscular ultrasound for the non-invasive assessment of breast cancer patients with peripheral neuropathy from taxanes. The American Academy of Neurology 71st Annual Meeting, Philadelphia, PA, USA, May 4-10, 2019. Poster.
2. Tsuzuki S, Park S, Eber M, Widner B, Kamata Y, Kimura T, Bianchi-Frias D, Coleman I, Nelson P, Hsu F-C, **Peters C**, **Shiozawa Y**. A pain-related neuropeptide calcitonin gene-related peptide promotes bone metastatic progression of prostate cancer through p38. American Society for Bone and Mineral Research (ASBMR) 2019 Annual Meeting, Orland, FL, USA, September 20-23, 2019. Poster.
3. Park S, Eber M, Tsuzuki S, Cain R, Widner B, Kamata Y, Kimura T, Bianchi-Frias D, Coleman I, Nelson P, Hsu F-C, **Peters C**, **Shiozawa Y**. The role of the SCF/c-kit pathway in cancer-induced bone pain. American Society for Bone and Mineral Research (ASBMR) 2019 Annual Meeting, Orland, FL, USA, September 20-23, 2019. Poster.
 - **Selected as a Plenary Poster**
4. Cain R, Park SH, Eber M, Martin TJ, Parker R, Jimenez-Andrade JM, **Shiozawa Y**, **Peters C**. Characterization of a syngeneic mouse model of prostate cancer induced bone pain. Neuroscience 2019, Chicago, IL, USA, October 19-23, 2019. Poster.

(2020-2021)

1. Ding H, Kiguchi N, Park SH, Kishioka S, **Shiozawa Y**, Peters C, Ko M-C. Functional roles of neuromedin B and gastrin-releasing peptide in regulating itch and pain in the spinal cord of primates. **FASEB J.** 2030;34 (S1):03569. Experimental Biology 2020 (Cancelled, but abstract was published).
2. Eber M, Park S, Hsu F-C, **Shiozawa Y**. Osteoblasts Derived from Mouse Mandible Enhance Tumor Growth of Prostate Cancer More Than Osteoblasts Derived from Long Bone. The Cancer and Bone Society (CABS) / American Society for Bone and Mineral Research (ASBMR) Pre- Meeting, The Seed and Soil: Therapeutic Targets for Cancer in Bone, Virtual, September 9-10, 2020. e-Poster.
3. Eber M, Park S, Hsu F-C, **Shiozawa Y**. Osteoblasts Derived from Mouse Mandible Enhance Tumor Growth of Prostate Cancer More Than Osteoblasts Derived from Long Bone. American Society for

Bone and Mineral Research (ASBMR) 2020 Annual Meeting, Virtual, September 11-14, 2020. e-Poster.

4. Widner DB, Eber M, Park S, **Shiozawa Y**. Skeletal Muscle Resident Mast Cells as a Potential Novel Biomarker for Cancer-Associated Cachexia. Cancer Cachexia 2020, Virtual, September 10-11, 2020. e-Poster.

(2021-2022)

1. Li J, Hilimire T, Cheng C, Broude EV, Liu Y, Lilly M, **Shiozawa Y**, Wilding G, Roninson IB, Chen M. Inhibition of CDK8/19 Mediator kinase suppresses primary and metastatic growth of castration-resistant prostate cancer. 28th Annual PCF Scientific Retreat, Virtual, October 28-29, November 4-5, 2021. e-Poster.
2. Park S, Eber M, Widner B, Tsuzuki S, Kimura T, Cain RM, Parker R, Jimenez-Andrade JM, Ramirez-Rosas M, Husu F-C, Peters C, **Shiozawa Y**. The role of the SCF/c-Kit pathway in cancer-induced bone pain. The Cancer and Bone Society (CABS) Young Investigator Symposium, Virtual, February 15-16, 2022. Oral.
3. **Wadas, TJ**. Improving the kidney toxicity profile of actinium-225 radiopharmaceuticals American Chemical Society Meeting, San Diego, CA. Virtual. March 22, 2022
4. **Wadas, TJ**. Using polyazamacrocyclic ligands to improve the zirconium-89 immuno-PET paradigm. Department of Chemistry Colloquium Series. University of Iowa, Iowa City, IA. October 15, 2021.
5. **Wadas, TJ**. The therapeutic potential of actinium-225 radiopharmaceuticals. College of Pharmacy Seminar Series. University of Iowa, Iowa City, IA. November 2, 2021.

(2022-2023)

1. Li J, Hilimire T, Cheng C, Broude EV, Liu Y, Lilly M, **Shiozawa Y**, Wilding G, Roninson IB, Chen M. Inhibition of CDK8/19 Mediator kinase suppresses primary and metastatic growth of castration-resistant prostate cancer. American Association for Cancer Research (AACR) Annual Meeting 2022, New Orleans, LA, USA, April 13-18, 2022. Poster.
2. Oladeji A, Contino K, Ollodart J, Yu Y, Steele L, **Shiozawa Y**. Stem Cell Factor Derived From Cancer Cells Induces the Sprouting of Sensory Neurons Through Its Receptor c-Kit. The Annual Biomedical Research Conference for Minoritized Scientists (ABRCMS) 2022, Anaheim, CA, USA, November 9-12, 2022. Poster.

Dr. Wadas' Invited Lectures

1. Improving the Kidney Toxicity Profile of Actinium-225 Radiopharmaceuticals. 3/22/2022. 258th

ACS National Meeting, San Diego, CA.

2. Using Cerium-134 in Immuno-PET. 9/19/2022. Ce-134 User Group Meeting. DOE Isotope Program. Oak Ridge National Laboratory, Oak Ridge, TN.
3. Interrogating the Immune System with Molecular Imaging. 9/20/2022. Microbiology and Immunology Seminar Series. Carver College of Medicine, University of Iowa, Iowa City, IA.
4. Developing Anti-Fibroblast Activation Protein Nanobodies as a Platform for Pancreatic Cancer Theranostics. 10/8/2022. GI Cancer Symposium. Carver College of Medicine, University of Iowa, Iowa City, IA.
5. Targeting Fibroblast Activation Protein as a Biomarker for Glioblastoma. 10/22/2022. Center for Biocatalysis and Bioprocessing Conference. Carver College of Medicine, University of Iowa, Iowa City, IA.
6. Polyazamacrocyclic Ligands as Zirconium-89 Chelators: Teaching Old Dogs New Tricks. 11/2/2022. ARIA Workshop. Oak Ridge National Laboratory, Oak Ridge, TN.
7. **Plenary Lecture:** The Therapeutic Potential of Actinium-225 Radiopharmaceuticals. 12/15/2022. International Conference on Nuclear Analytical Techniques. Seoul, Republic of Korea.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Yusuke Shiozawa

Project Role: PI, W81XWH-19-1-0045

Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0001-9814-9230

Nearest person month worked: 1.2

Contribution to Project: Dr. Shiozawa is an Assistant Professor in the Department of Cancer Biology. He has extensive experience in the study of prostate cancer bone metastasis, and will provide oversight of the entire program including development and implementation of all policies, procedures, and processes. In this role, Dr. Shiozawa will be responsible for the completion of the project and for ensuring that systems are in place to guarantee institutional compliance with US laws, including biosafety and animal research guidelines, data collection and analyses, and facilities. Dr. Shiozawa will supervise other personnel on the project to ensure timely and effective studies.

Funding Support: Department of Defense, National Cancer Institute, Matavivor, TEVA Pharmaceuticals

Name: Thaddeus Wadas

Project Role: Partnering PI, W81XWH-19-1-0046

Researcher Identifier (e.g. ORCID ID): [0000-0002-9913-0336](https://orcid.org/0000-0002-9913-0336)

Nearest person month worked: 1.2

Contribution to Project: Dr. Wadas is an Associate Professor in the Department of Radiology at University of Iowa. Dr. Wadas will share oversight of the entire program with Dr. Shiozawa, including development and implementation of all policies, procedures, and processes. Dr. Wadas has a Ph.D. in Chemistry and has extensive experience in peptide chemistry, radiochemistry and developing *in vivo* studies in various tumor models as well as microPET imaging to evaluate radiopharmaceuticals. Dr. Wadas will assist Dr. Shiozawa's laboratory in radiochemistry, radiopharmaceutical-related *in vitro* experiments and all imaging data analysis. Dr. Wadas has access to all the resources and facilities needed to execute this research successfully, and his lab has successfully collaborated with the Dr. Shiozawa. Dr. Wadas will meet with Dr. Shiozawa remotely on a monthly basis to discuss progress on projects related to this Award.

Funding Support: Department of Defense, National Cancer Institute, Matavivor

Name: Christopher Peters

Project Role: Co-Investigator, W81XWH-19-1-0045

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.6

Contribution to Project: Dr. Peters is an Assistant Professor in the Department of Anesthesiology. Dr. Peters' research interests are in central and peripheral mechanisms of pain chronicity including postsurgical, bone cancer and chemotherapy induced pain and neuropathy. Dr. Peters will be responsible for supervising and

AWARD: BC180720, BC180720P1

TITLE: A targeted alpha particle radiopharmaceutical for bone metastatic breast cancer

PI: Yusuke Shiozawa, M.D., Ph.D., Partnering PI: Thaddeus Wadas, Ph.D.

training personnel in evoked and non-evoked bone cancer pain behavioral assays. Dr. Peters will also assist with histological and immunohistochemical analysis of skeletal tissue as part of Aims 2. He has extensive experience with the behavioral analysis, immunohistochemistry, biochemical analysis and fluorescent, brightfield, and confocal microscopic imaging required for this project.

Funding Support: National Institutes of Health, Department of Defense

Name: Fang-Chi Hsu

Project Role: Co-Investigator, W81XWH-19-1-0045

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.36

Contribution to Project: Dr. Hsu is a Professor in the Department of Biostatistical Sciences. She will work closely with Dr. Shiozawa and the team to analyze the results of the project.

Funding Support: National Institutes of Health, Department of Defense

Has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations have been involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS:

QUAD CHARTS.

9. APPENDICES:

An Award Expiration Transition plan and the original copies of manuscript are attached.

A targeted alpha particle radiopharmaceutical for bone metastatic breast cancer

BC180720, BC180720P1

W81XWH-19-1-0045, W81XWH-19-1-0046

PI: Yusuke Shiozawa
Thaddeus Wadas

Org: Wake Forest University Health Sciences
University of Iowa

Award Amount: \$299,999 (Shiozawa)
\$299,999 (Wadas)

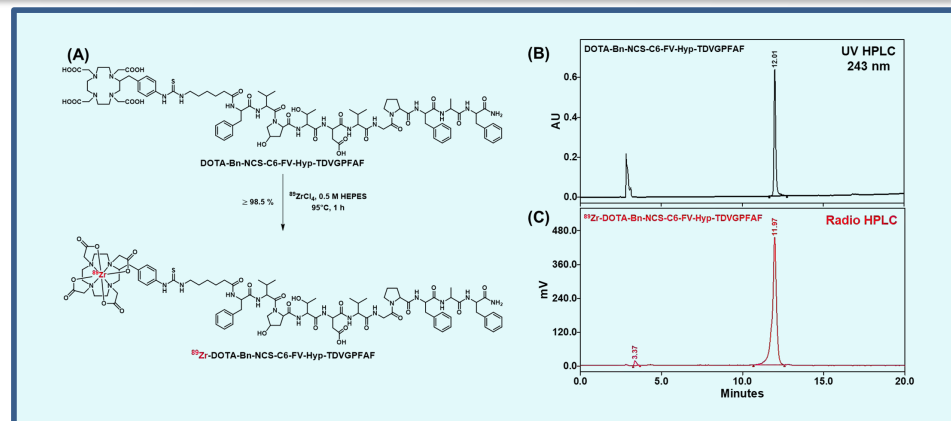


Study/Product Aim(s)

- **Aim 1: Determine the best alpha particle radiopharmaceuticals targeted to the CRLR.**
- **Aim 2: Determine the impact of CRLR-targeted alpha particle therapy on breast cancer bone metastases and cancer-induced bone pain.**

Approach

The proposed studies will develop a new therapeutic strategy for treating bone metastatic breast cancer using an alpha-particle radiopharmaceutical. This work will lay the needed foundation to develop new therapeutic targets for both cancer-induced bone pain and bone metastatic breast cancer, to decrease suffering and improve survival of breast cancer patients with bone metastases.



Representative synthesis and quality control of anti-CRLR radiopharmaceuticals.

(A) Using ^{89}Zr -chloride, the unlabeled conjugate could be radiolabeled with ^{89}Zr in high yield, high purity, and specific activity. (B) Representative UV-HPLC of the cold conjugate. (C) Representative Radio-HPLC of the radiopharmaceutical. There is good agreement in the retention time between the unlabeled conjugate and the radiopharmaceutical.

Timeline and Cost

Activities	CY	19	20	21	22
Aim 1 (In vitro studies)					
Aim 1 (Animal studies)					
Aim 2 (Animal studies)					
Estimated Budget (\$K)		\$100	\$100	\$200	\$200

Updated: 03/31/2022

Goals/Milestones (Example)

CY19 Goal – Team development

✓ Set up and organize the monthly meeting

CY20 Goals – In vitro studies

☐ *In vitro* selection of ^{225}Ac -CGRP₂₇₋₃₇

☐ *In vivo* selection of ^{225}Ac -CGRP₂₇₋₃₇

CY21 Goal – Animal studies

☐ *In vivo* selection of ^{225}Ac -CGRP₂₇₋₃₇

☐ Determine the maximum tolerated dose of ^{225}Ac -CGRP₂₇₋₃₇

CY22 Goal – Animal studies

☐ Determine the maximum tolerated dose of ^{225}Ac -CGRP₂₇₋₃₇

☐ Determine treatment efficacy of ^{225}Ac -CGRP₂₇₋₃₇

Comments/Challenges/Issues/Concerns

- Limited amounts of ^{225}Ac produced and COVID-19-related issues have negatively affected our productivity.

Budget Expenditure to Date

Projected Expenditure: \$600K

Actual Expenditure: \$300K (Shiozawa) + \$300K (Wadas)

Transition Plan Questionnaire

Directions: Please answer all questions that apply for each product under development. Please fill out one document per product. *This is not an application for funding; however, answers will help us understand the outcomes and products from your award.*

1. After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? Yes ☒ or No ☐

These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.

2. What **conclusion(s)** does your final data support?

We further validated that CRLR is a suitable target to treat bone metastatic breast cancer and successfully manipulated CRLR levels in breast cancer cells using lenti-virus technique.

We validated that CRLC-overexpressing breast cancer cells can grow in vivo without affecting growth rate.

We prepared the PTCA-CGRP27-37 peptides and completed the radiochemistry experiments with the PCTA-CGRP27-37 peptides and developed strategies to overcome the severe lipophilicity of the agents under investigation.

We completed the preparation and stability analysis of several new ligands using the positron emitting radioisotope zirconium-89 (^{89}Zr : $t_{1/2}$ = 78.4 h, β^+ : 22.8 %, E_{β^+max} = 901 keV; EC: 77%, E_{γ} = 909 keV; 99%).

3. Will you/have you applied for/obtained follow-on-funding for this project? **If yes**, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. *This information will be recorded as an outcome to this award.*

We have not applied for follow-on-funding yet.

4. What will be the **next step(s)** for this project?

- 1) Determine if CRLR-targeting radiopharmaceuticals can bind to CRLR on breast cancer in vivo (Aim 1&2).
- 2) Determine the impact of radiopharmaceuticals on breast cancer bone metastases and cancer-induced bone pain in vivo (Aim 2).
- 3) Assess the affinity and selectivity of the radiopharmaceuticals for the CRLR in vitro using the cell lines provided by Shiozawa lab (Aims 1& 2).
- 4) Assess the biodistribution and tumor retention of the radiopharmaceuticals in vivo (Aims 1& 2).

5. How would you classify your **lead candidate product**? f

(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy): Please choose, if applicable

(b) Diagnostic

(c) Device

(d) Research Tool to Address a Research Bottleneck

(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)

(f) Other - Please Specify: theranostic radiopharmaceuticals

6. How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

Due to high levels of chemical and radiation exposures, the incidence rate of both cancer among military service members and veterans is high and continues to grow. Especially, the 5-year survival rate of breast cancer patients with bone metastasis is less than 30%. Moreover, as bone metastasis progresses, patients experience symptoms such as significant pain. This progressive pain greatly hinders performance of normal daily activities, resulting in (i) reduced quality of life for military service members, veterans, and their families, and (ii) preclusion from returning to active duty for military service members. The conventional standards of care for prevention or delay of bone metastatic disease are not curative, rather they only provide palliative or supportive care. Therefore, our strategy to mitigate bone metastatic disease and its associated pain using CRLR-targeting radiopharmaceuticals will help alleviate many of the challenges and hurdles that bone metastatic breast cancer patients and their families face every day. Moreover, considering the prevalence of bone metastasis, the successful completion of our work may have broad positive implications for all bone metastasis. Improving care for patients with bone metastasis will (i) improve their physical and emotional health, and reduce the financial burden (from treatment costs and lost work) of military service members, veterans, and their families, and (ii) enable military service members to return to active duty.

7. Therapy / Product Development, Transition Strategies, and Intellectual Property

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.

At this moment, we do not plan to pursue intellectual property on our product.

Neuromuscular ultrasound for taxane peripheral neuropathy in breast cancer

Thomas W. Lycan DO, MHS¹  | Fang-Chi Hsu PhD² | Christine S. Ahn MD³ | Alexandra Thomas MD¹ | Francis O. Walker MD⁴ | Omar P. Sanguenza MD⁵ | Yusuke Shiozawa MD, PhD⁶ | Sun Hee Park PhD⁶ | Christopher M. Peters PhD⁶ | Edgar Alfonso Romero-Sandoval MD⁷ | Susan A. Melin MD¹ | Steven Sorscher MD¹ | Katherine Ansley MD¹ | Glenn J. Lesser MD¹ | Michael S. Cartwright MD⁴ | Roy E. Strowd MD⁴

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Funding information

Department of Defense, Grant/Award Numbers: W81XWH-19-1-0045, W81XWH-17-1-0541, W81XWH-14-1-0403

Abstract

Background: Our study aim was to evaluate neuromuscular ultrasound (NMUS) for the assessment of taxane chemotherapy-induced peripheral neuropathy (CIPN), the dose-limiting toxicity of this agent.

Methods: This cross-sectional study of breast cancer patients with taxane CIPN measured nerve cross-sectional area (CSA) by NMUS and compared with healthy historical controls. Correlations were determined between CSA and symptom scale, nerve conduction studies, and intraepidermal nerve fiber density (IENFD).

Results: A total of 20 participants reported moderate CIPN symptoms at a median of 3.8 months following the last taxane dose. Sural nerve CSA was 1.2 mm² smaller than healthy controls ($P \leq .01$). Older age and time since taxane were associated with smaller sural nerve CSA. For each 1 mm² decrease in sural nerve CSA, distal IENFD decreased by 2.1 nerve/mm (R^2 0.30; $P = .04$).

Conclusions: These data support a sensory predominant taxane neuropathy or neuronopathy and warrant future research on longitudinal NMUS assessment of CIPN.

KEYWORDS

breast cancer, chemotherapy, neuropathy, taxane, ultrasound

1 | INTRODUCTION

Chemotherapy-induced peripheral neuropathy (CIPN) is a common toxicity of many classes of chemotherapy, including taxanes, platinum, vinca alkaloids, and others. Taxanes, such as paclitaxel and docetaxel,

have had an increasing role in the treatment of breast cancer and are now used in the majority of patients receiving perioperative chemotherapy.¹ When chemotherapy cannot be given as intended due to dose reductions, it is associated with worse progression-free and overall survival.² Although spontaneous recovery is possible, in roughly half

Abbreviations: AANEM, American Association of Neuromuscular & Electrodagnostic Medicine; ALS, amyotrophic lateral sclerosis; BMI, body mass index; CIPN, chemotherapy-induced peripheral neuropathy; CSA, cross-sectional area; DRG, dorsal root ganglion; DWL, distal wrist crease; H&E, hematoxylin and eosin; IENFD, intraepidermal nerve fiber density; LMN, lower motor neuron; NCS, nerve conduction study; NMUS, neuromuscular ultrasound; PGP, protein gene product; QLQ, Quality of Life Questionnaire; SNAP, sural sensory nerve action potential.

of cases functional disability and risk of falls from CIPN persists for years.^{3,4} Currently, there is no predictive method to identify patients who are at high risk for developing permanent, debilitating CIPN.

There are few clinically available point-of-care tests to objectively predict, diagnose, or monitor CIPN. Self-reported symptom scales such as the European Organization for Research and Treatment of Cancer CIPN 20 Quality of Life Questionnaire (QLQ)⁵ are potentially predictive of CIPN but have inter-scale and inter-observer variability.^{6,7} Quantitative sensory testing fails to predict the severity of CIPN.^{8,9} Nerve conduction studies (NCSs) are insensitive to small fiber changes; typical findings include mild conduction velocity slowing at sites of entrapment or reduction of sural sensory responses.^{10–12} Skin biopsy for measuring intraepidermal nerve fiber density (IENFD) is the gold standard for the diagnosis of sensory-predominant small fiber neuropathy but is not an ideal test due to its invasive nature.^{3,6}

Studies have found that neuromuscular ultrasound (NMUS) can detect peripheral nerve lesions generally with enlargement of CSA in patients with demyelinating hereditary,^{13,14} inflammatory,^{15,16} entrapment,¹⁷ and diabetic neuropathies¹⁸ though reduction of CSA has also been reported (eg, amyotrophic lateral sclerosis [ALS],¹⁹ spinocerebellar ataxia syndrome^{20–23}). CSA is generally larger in demyelinating than axonal polyneuropathies²⁴ and more commonly reported in large fiber neuropathies, though one study of patients with small fiber neuropathies demonstrated enlargement of CSA compared with matched-healthy controls.²⁵

Less is known about the usefulness of NMUS for assessing the effect of chemotherapy upon peripheral nerves. Four studies have used NMUS for the detection of CIPN among gastrointestinal cancer patients who received oxaliplatin. Compared with historical reference values, three studies found no consistent change in CSA,^{24,26} although one did note an increase in peripheral nerve CSA at compression sites (median nerve at wrist, ulnar nerve at elbow).²⁷ The fourth study compared neurological measures before and after chemotherapy and found increased CSA of the tibial nerve and at one compression site of the fibular nerve (fibular head), but did not find changes in CSA in other nerves (sural, median, ulnar, radial).²⁸ Importantly, the mechanism and symptoms of neurotoxicity are markedly different between taxanes (eg, microtubular stabilization, hypoesthesia) and oxaliplatin (eg, inhibition of sodium channels; cold-induced paresthesias). Therefore, while prior studies of oxaliplatin CIPN are certainly informative, taxane-specific studies are needed to further characterize this syndrome.

In this study, our aim was to test NMUS as a noninvasive, point-of-care test to measure nerve CSA in patients with active taxane CIPN symptoms with the hypothesis that nerve CSA would be altered.

2 | METHODS

2.1 | Eligibility characteristics

Our single-arm, prospective pilot study enrolled breast cancer patients with self-reported symptomatic CIPN for comprehensive neurological assessment, including NMUS. The trial was conducted in accordance

with the International Conference on Good Clinical Practice Standards and the Declaration of Helsinki. The protocol was approved by institutional review board and ethics committees of our institution and the trial was registered on ClinicalTrials.gov (NCT03139435). All patients provided informed consent. Eligible patients were ≥ 18 -year-old women with breast cancer (any stage) and clinical symptoms of peripheral neuropathy due to either previous or current taxane exposure, as clinically attributed by the patient and their medical oncologist. We noted the presence of comorbidities (such as diabetes), which can predispose toward peripheral neuropathy with nerve enlargement.¹⁸ We excluded patients with a self-reported or documented history of pre-existing peripheral neuropathy before initiation of the taxane. Patients were followed, either by telephone or in person, for 30 days to ensure the biopsy site healed.

2.2 | Assessments

Participants underwent a cross-sectional assessment that included focused history, neurological exam, and diagnostic testing by a neurologist at a single visit. NMUS with a GE S8 system and a routine 15-MHz linear transducer (GE Healthcare, Chicago, IL) was used to measure CSA at nerve sites as per previously described methodology and institutional practice.²⁹ These sites were at the posterior distal leg (sural nerve), the ankle (tibial nerve), and the forearm and distal wrist crease (DWC; median nerve). The median nerve was assessed at two places to control for enlargement that can occur just proximal to entrapment at the carpal tunnel (at the DWC) due to subclinical carpal tunnel syndrome. Reference values for NMUS were from a published historical dataset.²⁹ The 20-item self-reported neuropathy scale (QLQ-CIPN20) was administered as previously described with the exception of the hearing loss item; this item was excluded from data collection because taxanes are not associated with ototoxicity.³⁰

NCS assessed the tibial motor nerve, median motor nerve, and the sural sensory nerve (antidromic, taken at two sites on the ankle) as per AANEM standards³¹ and compared with reference values from healthy adults.³² IENFD was measured with two skin biopsies (4–5 mm), one at the upper thigh and the second on the ipsilateral distal leg in sural nerve territory (10 cm above lateral malleolus). As previously described, biopsied tissues were cut into 50- μ m sections for H&E staining and immunostaining with an antibody to protein gene product (PGP) 9.5, a neuron- and neuroendocrine cell-specific ubiquitin carboxy-terminal hydrolase expressed throughout the peripheral nervous system.³³

Slides were processed and read by two collaborators in dermatopathology with experience in reading skin biopsy samples for IENFD. The same two dermatopathologists reviewed all cases. IENFD was measured as the linear density (per millimeter) of PGP 9.5-positive fibers that crossed the dermal-epidermal junction. Serum and additional unstained biopsy slides were stored and transferred for exploratory analyses. Although not blinded to the diagnosis of CIPN, the examiner performing NMUS did not review the patient's symptoms before performing the study and was not aware of the cumulative dose,

timing since taxane therapy, severity of clinical symptoms, or IENFD results at the time of testing. Dermatopathologists were not blinded to the patient but did not have access and were blinded to the NCS and NMUS data at the time of their analysis.

2.3 | Study endpoints

The primary outcome was tibial nerve CSA by NMUS compared with historical controls. Secondary outcomes included sural nerve CSA by NMUS and determination of whether tibial or sural nerve CSA correlated with QLQ-CIPN20, NCS, or IENFD. Exploratory outcomes included measures of median nerve CSA.

2.4 | Statistical analysis

The tibial nerve CSA, sural nerve CSA, and sural nerve amplitude were compared with historical data^{29,34} from healthy patients using two-sample t-test; primary historical data were used for the analysis of CSA measures. Phase of treatment was defined as active (≤ 30 days since last taxane administration), recent (>30 and ≤ 180 days), and remote (>180 days). Comparison of CSA, QLQ scales, and IENFD

measures between different phases of taxane exposure were performed using the Kruskal-Wallis tests. Spearman's rank correlation coefficients were used to study the associations between two measures (eg, distal and proximal IENFD). Linear regression models were used to examine the associations between the dependent variable (nerve CSA) and covariates of interest (NCS measures, self-reported neuropathy scale, and IENFD). Clinical factors (eg, age) that were thought to be potentially associated with nerve CSA were fitted in the model one at a time.

3 | RESULTS

Between May 4, 2017, and November 13, 2018, 25 patients were screened for eligibility; 3 did not meet inclusion criteria, and 2 declined to participate as summarized in CONSORT flow diagram (Figure 1). Recruitment was by provider referral and by paper fliers posted in both patient and provider areas of the oncology clinic. All patients completed their planned evaluation and follow-up. One patient was excluded from median nerve analysis due to symptoms and NCS findings that were diagnostic of carpal tunnel syndrome.

A total of 20 patients underwent evaluation with their baseline clinical characteristics summarized in Table 1. Average cumulative

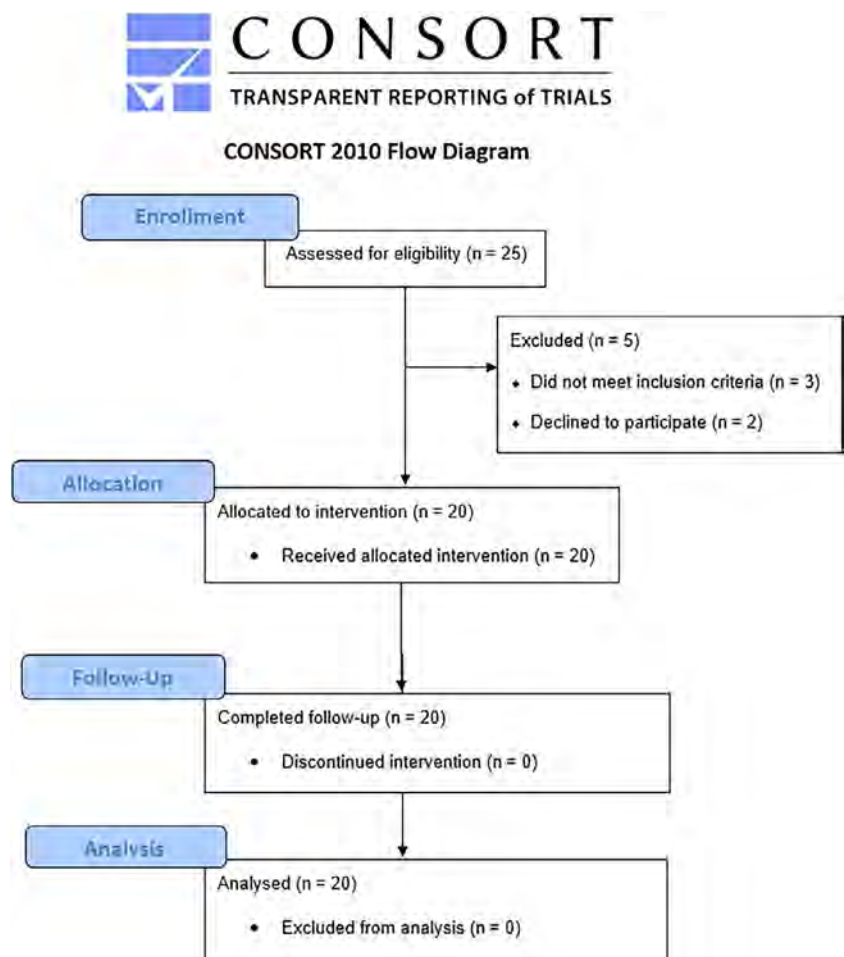


FIGURE 1 CONSORT flow diagram [Color figure can be viewed at wileyonlinelibrary.com]

taxane dose was 817 mg/m²; for reference, the typical dosing regimen for adjuvant paclitaxel in breast cancer is 80 mg/m²/week for 12 weeks (total cumulative dose of 960 mg/m²). CIPN patients reported moderate-to-severe CIPN symptoms by QLQ-CIPN20, which were predominantly sensory (19.1 ± 4.9; max 32) as opposed to motor (15.6 ± 5.8; max 32) or autonomic (3.3 ± 1.6; max 8). All patients had normal strength (5/5) in foot dorsiflexion; deep tendon reflexes at the ankles were absent in 13/20 (65%), reduced in 4/20 (20%).

Sural nerve amplitude was significantly lower (mean 10.9 ± 5.8 μV) than healthy historical controls (mean 17.2 ± 10.1 μV; $P < .01$) with 8 patients below the normal range (<7.1 μV).³⁴ There were no associations between tibial nerve CSA and tibial compound muscle action potential amplitude (Spearman's $r = 0.12$), distal latency (−0.24), or conduction velocity (0.21). There were no associations between sural nerve CSA and sural sensory nerve action potential (SNAP) amplitude (Spearman's $r = 0.20$), latency (−0.26), or conduction velocity (0.27). Sural SNAP amplitude was not associated with symptom severity by QLQ-CIPN20 (Spearman $rs [18] = -0.23$).

Distal IENFD had mean 6.6 ± 3.8 nerves/mm (range, 2.4–15 nerves/mm) with 30% of participants below the normal range, defined as the 5th

percentile among historical healthy controls (3.8 nerves/mm). Proximal IENFD had mean 11.0 ± 6.0 nerves/mm (range, 9–24 nerves/mm) with 15% of participants below the normal range, defined as the 5th percentile among historical healthy controls (5.2 nerves/mm).³⁵ Distal and proximal IENFD were associated with each other (Spearman $rs [19] = 0.49$; $P = .03$). Neither distal nor proximal IENFD was associated with symptom severity by QLQ-CIPN20 (Spearman $rs [19] = -0.16$; 0.12, respectively) or with cumulative dose (Spearman $rs [20] = 0.42$; 0.19, respectively). Proximal IENFD was negatively associated with tibial amplitude (Spearman $r = -0.46$; $P = .04$); otherwise, there was no association between IENFD (either proximal or distal) and any other NCS measures.

CSA was evaluated by NMUS at 74 nerve sites among the 20 patients with no repeated measurements. The average sural nerve CSA was significantly smaller than healthy historical controls (Table 2). Older age was associated with significantly smaller sural nerve CSA (Spearman $rs [20] = -0.65$, $P \leq .01$). More days since taxane chemotherapy was also associated with smaller sural nerve CSA ($rs [20] = -0.46$; $P = .04$). Sural nerve CSA was not significantly associated with either body mass index (BMI) (Spearman $rs [20] = 0.25$) or cumulative taxane dose (Spearman $rs [20] = 0.40$); neither were the CSA of other nerves. Unadjusted sural nerve CSA did not have an association with distal IENFD, but when controlled for age and days from last taxane administration, distal IENFD reduced by 2.1 nerve/mm for each 1 mm² decrease in sural nerve CSA (Table 3).

Descriptive data of nerve findings by phase of treatment (active, recent, remote) are summarized in Table 4. When compared by phase of treatment and independently from each other, tibial nerve CSA ($P = .44$), sural nerve CSA ($P = .17$), proximal IENFD ($P = 0.43$), distal IENFD ($P = .32$), QLQ sensory scale ($P = .46$), and sural amplitude ($P = .45$) did not meet statistical significance by Kruskal-Wallis one-way analysis of variance.

Median nerve CSA at the compressive site (DWC) was larger than among healthy historical controls, whereas at the noncompressive site (forearm), there was no significant difference. These results were the same even when including the participant with carpal tunnel syndrome ($n = 20$), with median nerve CSA at the DWC larger than healthy historical controls ($P = .02$) and no significant difference at the

TABLE 1 Demographic and disease characteristics of the participants

Parameter		Mean (SD) or n (%)
Age, mean (SD)		55.4 (10.5)
BMI, mean (SD)		29.8 (6.9)
Stage of breast cancer, n (%)	Stage I	8 (40%)
	Stage II	5 (25%)
	Stage III	2 (10%)
	Stage IV	5 (25%)
Prior radiation therapy, n (%)		10 (50%)
Prior surgery for breast cancer, n (%)		19 (95%)
Prior hormone therapy for breast cancer, n (%)		8 (40%)
Prior lines of systemic cancer therapy, median (IQR)		1 (1–3)
Most recent taxane, n (%)	Paclitaxel	10 (50%)
	Docetaxel	8 (40%)
	Nab-paclitaxel	2 (10%)
Cumulative taxane dose, mg/m ² , mean (SD)		817.3 (414.9)
Months since last taxane dose, median (IQR)		3.8 (1.0–6.1)
Relevant comorbidities, n (%)	Diabetes, no end-organ damage	4 (20%)
	Hypothyroidism	3 (15%)
	Depression	3 (15%)
	Alcoholism	1 (5%)
	Cerebrovascular disease	1 (5%)
	Vitamin B12 deficiency	1 (5%)

IQR, interquartile range; Nab, nanoparticle albumin-bound.

TABLE 2 Comparison of nerve CSA by NMUS between CIPN patients and healthy historical controls

	CIPN patients, mm ² , mean (SD)	Healthy controls, mm ² , mean (SD)	t value	P value
Tibial nerve CSA	13.0 (3.6)	14.0 (4.3)	0.94	.35
Sural nerve CSA	4.1 (1.3)	5.3 (1.8)	2.85	<.01*
Median nerve CSA (forearm)	7.3 (1.4)	7.7 (1.7)	0.89	.37
Median nerve CSA (DWC)	12.5 (3.9)	10.1 (2.7)	−2.39	.03**

*Statistically significant at $\alpha \leq .05$. Df = 138.

**Statistically significant at $\alpha \leq .05$. Satterthwaite unequal variances; Df = 16.

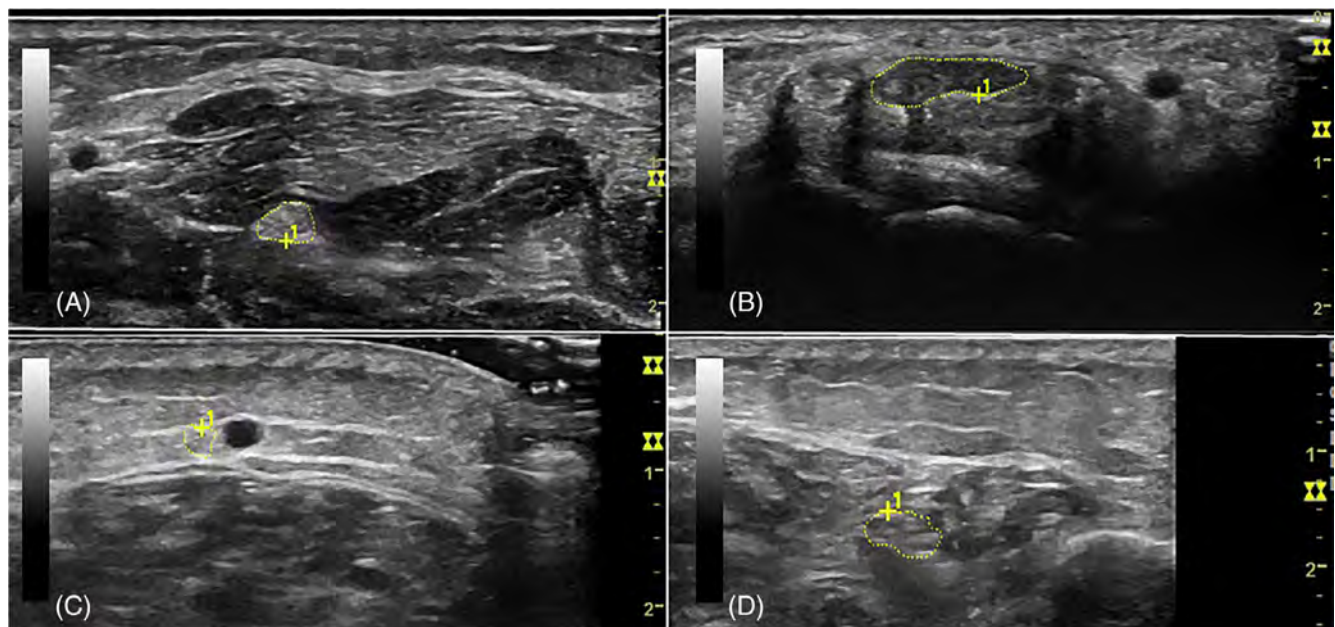
TABLE 3 Multiple regression analysis of factors to predict distal IENFD

Factors	Univariate		Multivariate model 1*		Multivariate model 2†	
	Beta	P value	Beta	P value	Beta	P value
Sural CSA	0.35	.13	0.99	.15	2.10	.04‡
Days since last taxane	−0.29	.21	−0.01	.25	0.19	.13
Age	−0.12	.62			−0.01	.09

*Model 1: R² = 0.19; F(2,19) = 2.03; P = .16.†Model 2: R² = 0.30; F(3,19) = 2.32; P = .11.‡Statistically significant at $\alpha \leq .05$.**TABLE 4** Comparison of findings between different phases of taxane exposure

	Active taxane, mean (SD)	Recent taxane, mean (SD)	Remote taxane, mean (SD)
No. of participants	6	9	5
Cumulative dose (taxane mg/m ²)	995.8 (95.8)	713.9 (440.7)	789.0 (587.1)
Tibial nerve CSA (mm ²)	14.2 (4.8)	13.2 (3.2)	11.2 (2.3)
Sural nerve CSA (mm ²)	4.8 (1.6)	3.9 (0.6)	3.6 (1.5)
Forearm median nerve CSA (mm ²)	7.3 (1.9)	8 (1.0)	6.2 (0.4)
DWC median nerve CSA* (mm ²)	13.8 (6.5)	13.6 (3.0)	11.0 (2.4)
QLQ sensory scale	17.2 (4.3)	20.4 (5.2)	19.2 (5.4)
QLQ motor scale	12.3 (3.4)	18.6 (6.6)	14.2 (4.4)
QLQ autonomic scale	2.5 (0.5)	3.9 (1.8)	3.2 (1.6)
Proximal IENFD (nerves/mm)	13.1 (6.7)	8.9 (3.6)	12.2 (8.5)
Distal IENFD (nerves/mm)	8.9 (4.9)	5.5 (2.9)	5.9 (3.0)

*P value significant to <.05 (.0219) by Kruskal–Wallis one-way analysis of variance.

**FIGURE 2** Representative imaging by NMUS. A, Median nerve at forearm in participant 3 (CSA, 9 mm²). B, Median nerve at wrist in participant 3 (CSA, 25 mm²). C, Sural nerve in participant 6 (CSA, 4 mm²). D, tibial nerve in participant 6 (CSA, 19 mm²) [Color figure can be viewed at wileyonlinelibrary.com]

forearm ($P = .41$). Representative NMUS imaging is shown in Figure 2. Descriptive data from all participants is shown in Supporting Information Table S1, which is available online.

The study was well-tolerated without any adverse effects and with a high uptake among participants with just 2 of 25 participants declining to complete the study protocol after registration. Skin

biopsy led to one adverse event in a patient who had redness and irritation attributed to an inflammatory local reaction and not an infection.

4 | DISCUSSION

The key findings of this study are that, compared with healthy historical controls, patients with a small fiber neuropathy caused by taxane exposure had (1) decreased sural nerve size, (2) increased median nerve size but only at a compression site, and (3) NMUS findings that correlated well with IENFD loss on skin biopsies. Reduced CSA was specific to the sural sensory nerve and was not seen in the tibial nerve. This is likely reflective of the predominantly sensory manifestation of taxane CIPN, which was confirmed in our sample using a validated symptom scale.

NMUS assessment of the median nerve at its compression site (DWC) found that CIPN patients had increased CSA compared with healthy controls. When moved proximally to assess the median nerve at a noncompressive site (forearm), there was no difference in CSA between CIPN patients and healthy controls. This discrepancy is often seen with NMUS evaluation of symptomatic carpal tunnel syndrome and other polyneuropathies.³⁶ These findings suggest that, at compression sites, CIPN may aggravate nerve entrapment and cause an increase in CSA of peripheral nerves. This discrepancy in the effects of CIPN between compression and noncompression anatomic sites has been described in NCS¹¹ and was suggested in a prior study of oxaliplatin CIPN.²⁷

The finding of decreased peripheral nerve CSA is uncommon and has been reported in ALS¹⁹ and spinocerebellar ataxia syndrome.²⁰⁻²³ In vitro³⁷ and animal studies³⁸ have shown that the neurotoxicity of microtubule-targeting agents (eg, taxanes) is predominantly due damage to the dorsal root ganglion (DRG).^{39,40} This leads to axonal loss in the longest sensory nerves and contributes to the clinical symptoms consistent with a ganglionopathy. In ALS, the reduction in nerve CSA is thought to reflect nerve atrophy from lower motor neuron (LMN) dropout⁴¹ and may have utility as a marker of LMN involvement.⁴² We hypothesize that the decreased CSA observed in our study may reflect the sensory ganglionopathy of taxane CIPN with sensory nerve atrophy. Because this study was limited to taxane CIPN, these results are not broadly applicable to other classes of chemotherapies whose neurotoxic mechanisms vary.

Multivariable analysis was notable for two confounding factors between CIPN and CSA, that is, age and time since taxane exposure. Age has previously been reported as having a very small negative correlation with peripheral nerve CSA.²⁹ The association with time is a novel finding; we hypothesize that this may be due to the gradual atrophy of damaged peripheral nerves following the initial insult. This is supported by the finding of decreased distal IENFD among patients with more time since taxane. Future studies will need to further account for these confounding factors.

There were several limitations of this pilot study, most of which were inherent to its design and small sample size. This study was designed to provide a broader evaluation of the pathophysiology of CIPN by including participants at all timepoints during and after

taxane (active, recent, remote). However, this heterogeneity decreased the statistical power to detect changes in CSA, which may only occur in a time-specific manner. Because there is no intervention available for CIPN once the patient has completed taxane chemotherapy, there is little clinical utility for improved evaluation of chronic CIPN. Therefore, future studies of the clinical use of NMUS, including one that is currently enrolling patients,⁴³ will focus on patients with CIPN who are being considered for additional taxane chemotherapy so that dosing may be adjusted accordingly. This study was limited to female participants; reassuringly, in the prior study of healthy controls, there was no significant difference in nerve CSA by sex.²⁹ Prior studies⁴⁴ have reported variability among healthy controls with sural nerve CSA approximately 2–6 mm², our healthy control data had a mean of around 5 mm². Our study was not funded to support collection of another set of matched control values, which is why we used our previously published set.²⁹ A newer high-resolution probe could also be considered for future studies, as ultrahigh-frequency NMUS has been found to be useful for assessing changes at the fascicular level.⁴⁵

CIPN is a common complication of breast cancer treatment with a critical need for a better diagnostic imaging modality. NMUS was found to be a rapid, noninvasive assessment of CIPN, which was well-tolerated and available at the point-of-care using a routine 15-Hz linear probe. This study's findings warrant future research on the longitudinal assessment of sural nerve CSA in CIPN.

CONFLICT OF INTEREST

Michael Cartwright has intellectual property with Elsevier for a textbook on Neuromuscular Ultrasound. Francis Walker has received honoraria and support for expenses from Grifols and AskBio; research funding from Vaccinex, TEVA Pharmaceuticals, and Pfizer; intellectual property with Elsevier and UpToDate; and support with interest-free equipment loans from Natus, Monarch Medical, and Terason. Yusuke Shiozawa has received research funding from TEVA Pharmaceuticals. The remaining authors have no conflicts of interest.

ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Melanocortin 1 Receptor–Targeted α -Particle Therapy for Metastatic Uveal Melanoma

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New effective therapies are greatly needed for metastatic uveal melanoma, which has a very poor prognosis with a median survival of less than 1 y. The melanocortin 1 receptor (MC1R) is expressed in 94% of uveal melanoma metastases, and a MC1R-specific ligand (MC1RL) with high affinity and selectivity for MC1R was previously developed. **Methods:** The ²²⁵Ac-DOTA-MC1RL conjugate was synthesized in high radiochemical yield and purity and was tested in vitro for biostability and for MC1R-specific cytotoxicity in uveal melanoma cells, and the lanthanum-DOTA-MC1RL analog was tested for binding affinity. Non-tumor-bearing BALB/c mice were tested for maximum tolerated dose and biodistribution. Severe combined immunodeficient mice bearing uveal melanoma tumors or engineered MC1R-positive and -negative tumors were studied for biodistribution and efficacy. Radiation dosimetry was calculated using mouse biodistribution data and blood clearance kinetics from Sprague–Dawley rat data. **Results:** High biostability, MC1R-specific cytotoxicity, and high binding affinity were observed. Limiting toxicities were not observed at even the highest administered activities. Pharmacokinetics and biodistribution studies revealed rapid blood clearance (<15 min), renal and hepatobiliary excretion, MC1R-specific tumor uptake, and minimal retention in other normal tissues. Radiation dosimetry calculations determined pharmacokinetics parameters and absorbed α -emission dosages from ²²⁵Ac and its daughters. Efficacy studies demonstrated significantly prolonged survival and decreased metastasis burden after a single administration of ²²⁵Ac-DOTA-MC1RL in treated mice relative to controls. **Conclusion:** These

results suggest significant potential for the clinical translation of ²²⁵Ac-DOTA-MC1RL as a novel therapy for metastatic uveal melanoma.

Key Words: melanocortin 1 receptor; ²²⁵Ac alpha therapy; uveal melanoma; mouse model

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Uveal melanoma is the most common primary intraocular malignancy and differs from the more common cutaneous melanoma in terms of risk factors, primary treatment, anatomic spread, molecular changes, and response to systemic therapy (1,2). Patients who develop uveal melanoma metastases, primarily in the liver, have a very poor prognosis with a median survival of about 1 y. Because uveal melanomas have different characteristic mutations from cutaneous melanomas, targeted therapies that have been effective for the latter, such as BRAF, are not indicated for the former (3). Immune checkpoint inhibition therapies that are successful in cutaneous melanoma have had poor efficacy in ocular melanoma, with fewer than 10% of patients responding and with rapid recurrence (3).

The melanocortin 1 receptor (MC1R) is highly expressed in uveal melanoma metastases (4). MC1R is a member of a family of 5 G-protein–coupled melanocortin receptors, 4 of which bind melanocyte-stimulating hormone (MSH) and related ligands (MC1R, 3R, 4R, and 5R) (5). Unlike the other members of this G-protein family, MC1R is not expressed in most normal human tissues (6), lessening concern for therapy-related toxicity. Although expression is found in the brain (7) and normal melanocytes (8), this is not a major concern because conjugates can be designed to not cross

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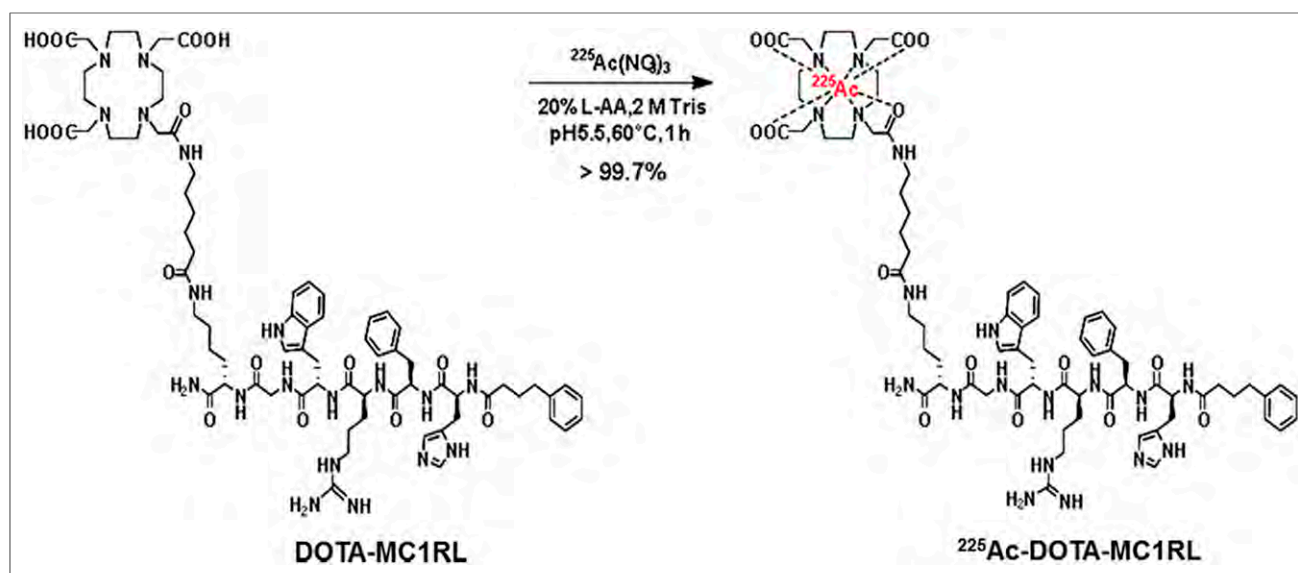


FIGURE 1. Radiochemical synthesis of ^{225}Ac -DOTA-MC1RL.

the blood–brain barrier and, in the most severe cases of melanocyte loss, the most serious symptom is vitiligo (9). MC1R expression has been reported on activated monocytes, macrophages, and dendritic cells derived from monocytes (10). This is also not a significant concern since the population of activated monocytes and macrophages can be replenished within days and lymphoid dendritic cells, which do not express MC1R, will not be depleted. MC1R is highly polymorphic (11), but the wild-type frequency is about 50% (8) and the most common mutations occur with a frequency of 21.5% in cytoplasmic domains, 19.7% in transmembrane domains, and 0% in the extracellular domain (11). Hence, most patients will have an MC1R isoform that is suitable for ligand binding. An MC1R-specific ligand (MC1RL) and conjugates were previously developed with high specificity (>200 fold) and affinity (0.2–0.4 nM inhibition constant) for MC1R (12,13). A fluorescent-dye conjugate was rapidly internalized by MC1R-expressing tumor cells, does not cross the blood–brain barrier, and is rapidly cleared from circulation (7).

Herein is reported the preclinical development and testing of a novel MC1R-targeted radiopharmaceutical, ^{225}Ac -DOTA-MC1RL, for targeted α -particle therapy (TAT) (14,15) of uveal melanoma. α -particle emissions consist of dicationic helium nuclei (He^{2+}) that have high linear-energy transfer and a short mean free path of only a few cell diameters (<100 μm) in tissue (16). ^{225}Ac is an α -particle-emitting radionuclide that has a 10-d half-life (17), 4 α -emissions in its decay chain, and high (28 MeV) total energy release (18).

MATERIALS AND METHODS

Compound Synthesis and Loading with Lanthanide

MC1RL (13) was synthesized according to a conventional N^α -fluorenylmethyloxycarbonyl (Fmoc) peptide synthesis strategy, except the Fmoc-Lys(Alloc)-OH was coupled to allow orthogonal alloc deprotection of the linker on the ϵ -amino group of the lysine after the linear peptide synthesis. The alloc group is removed, and Fmoc-aminohexanoic acid linker and tri-*t*-butyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (DOTA; TCI Chemicals) were coupled sequentially using *O*-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate activation. The DOTA-MC1RL peptide was cleaved

from the resin with a cocktail of trifluoroacetic acid (Chem-Impex International), water, and triisopropylsilane (Sigma-Aldrich) (95:2.5:2.5, v/v), precipitated in cold diethyl ether, pelleted/decanted, and lyophilized. The crude white powder was purified by reverse-phase high-performance liquid chromatography (Agilent) and characterized by both matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (JEOL USA Inc.) and analytic high-performance liquid chromatography. A scrambled peptide ligand (DOTA–substance P [SP]) was synthesized by changing the order of amino acids (sequence: 4-phenylbutyric acid-Trp-Gly-His-Arg-(D)-Phe-Lys(aminohexanoic acid-DOTA)-CONH₂). The europium–diethylenetriaminepentaacetic acid (DTPA)–MC1RL was synthesized as described before (19) except that MC1RL was used as the binding ligand (Supplemental Figs. 1A–1C; supplemental materials are available at <http://jnm.snmjournals.org>). Competition binding assays were performed as previously described using the europium-DTPA-NDP α -MSH ligand. The europium-DTPA-MC1RL binding affinity was determined using saturation binding assays. To determine MC1RL binding affinity for murine MC1RL, saturation binding assays were performed using the europium-DTPA-MC1RL and B16-F10 murine melanoma cells with high expression of murine MC1R (12).

Cell Culture and Characterization

Uveal melanoma cell lines were acquired (OCM1, OCM3, and OCM8 from June Kan-Mitchel, University of Southern California;

TABLE 1
In Vitro Serum Stability of ^{225}Ac -DOTA-MC1RL

Day	% intact	
	Thin-layer chromatography scanner	γ -counter
0	100	100
2	97.3 $\pm$ 0.5	96.9 $\pm$ 0.4
4	95.6 $\pm$ 1.1	95.1 $\pm$ 0.8
6	93.5 $\pm$ 0.8	93.2 $\pm$ 1.3
8	91.4 $\pm$ 1.2	91.0 $\pm$ 0.9
10	90.2 $\pm$ 0.7	89.9 $\pm$ 1.3

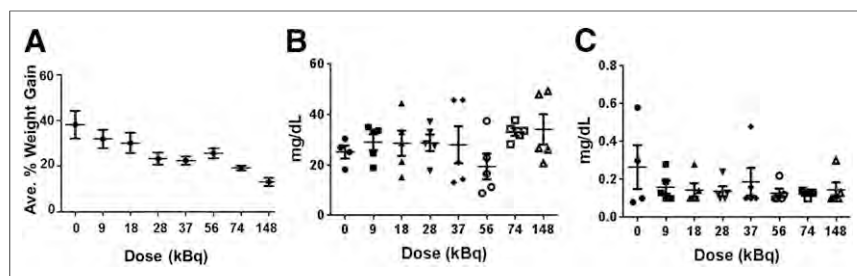


FIGURE 2. MTD study for non-tumor-bearing mice: percentage weight gain (A), blood urea nitrogen (B), and blood creatinine (C).

OMM1 from Gregorius P. Luyten, University Hospital at Rotterdam; and MEL270, MEL290, and OMM2.3 from Timothy Murray, Bascom Palmer Eye Institute) and grown in RPMI medium, 10% fetal bovine serum, a 100 units/mL concentration of penicillin, a 100 mg/mL concentration of streptomycin, 1% 200 mM L-glutamine, 1% 100 mM sodium pyruvate, 1% minimal essential medium essential vitamin mixture ($\times 100$), 1% nonessential amino acid mixture ($\times 100$), and 1% 1 M (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in 5% CO_2 at 37°C. A375, A375/MC1R human cutaneous melanoma cells, and Hek293/MC1R cells were obtained and grown as before (12,20). Cells were authenticated per American Type Culture Collection guidelines (21), monitored for original morphology, and tested for *Mycoplasma* (MycoAlert kit; Lonza), and only passage numbers of less than 25 cells were used. MC1R expression and receptor number were determined as previously described (12,13) except that europium-DTPA-MC1RL was used for saturation binding. Cytotoxicity was determined as described in Supplemental Figure 2.

Radiochemical Synthesis and Characterization

DOTA-MC1RL or DOTA-SP (10 $\mu\text{g}/10\ \mu\text{L}$ of water), $^{225}\text{Ac}(\text{NO}_3)_3$ (3.4 MBq), 90 μL of water, and 10 μL of 20% L-ascorbic acid were added to a 1.5-mL tube followed by pH adjustment to 5.5–6 (1 M Tris buffer; 10–12 μL) and incubation at 60°C for 1 h (Fig. 1). Specific activity was calculated using a standard method (22). Radiochemical purity was assessed 24 h after collection by a γ -counter, and in vitro serum stability was determined by adding 50 μL of ^{225}Ac -DOTA-MC1RL (2,072 kBq) to 1 mL of human serum ($n = 4$), incubated at 37°C for 10 d, and quantified at multiple time points by thin-layer chromatography and γ -counting using established methods (23).

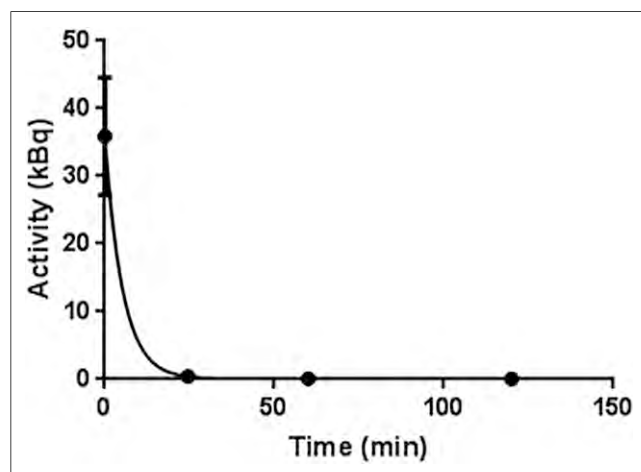


FIGURE 3. Plot of rat blood clearance: exponential decay nonlinear regression line fit of ^{225}Ac α -activity in rat blood over time, after intravenous administration of ^{225}Ac -DOTA-MC1RL ($n = 4$ rats).

Animal Studies

All protocols were approved (University of South Florida Institutional Animal Care and Use Committee protocol IS00000805 and Wake Forest University Health Sciences Institutional Animal Care and Use Committee protocol A11-144). Male and female animals were used. Sprague-Dawley rats, 10–12 wk old and weighing 200–250 g, were purchased with jugular vein catheters installed (Charles River). Nontumor studies used BALB/c mice (10–12 wk old, 18–22 g; Charles River). Severe combined immunodeficient (SCID) mice (6–8 wk old, 15–20 g; Charles River) were used for xenografting cell lines. Tail vein catheters were used for agent administration to mice.

For xenografting, 10×10^6 cells in 80 μL of phosphate-buffered saline and 20 μL of Matrigel (phenol red-free; Corning) were injected subcutaneously into the flank. Tumor volumes were determined by caliper using the following formulas: volume = (length $\times$ width²)/2 for A375 and A375/MC1R, and volume = (length $\times$ width $\times$ height)/2 for MEL270 tumors, which were initially flat with a gradual shift to a rounded shape.

Histology and Immunohistochemistry

Excised tissues were prepared for histology, hematoxylin and eosin staining, MC1R immunohistochemistry staining, and slide scanning as previously described (12). Metastasis burden was determined using images of 3 sections (25%, 50%, and 75%) through each liver and lung. Metastasis area was determined by segmentation using intensity and size threshold classifiers on the triple-red channel (Visiopharm software, version 6.7.0.2590). Total tissue area was determined with an intensity threshold classifier on the immunohistochemistry intensity channel, and the percentage metastasis was calculated.

To quantify MC1R expression in tumors, images from serial hematoxylin and eosin and immunohistochemistry sections were analyzed using Visiopharm, version 2017.7. Each serial section pair (hematoxylin and eosin and immunohistochemistry) was aligned using the tissue align module, and viable tumor was segmented by thresholding the hematoxylin channel. A multithreshold-marker-area analysis was then performed within the viable tumor region on each immunohistochemistry image. Each pixel was categorized as negative, weak, moderate, or strong on the basis of thresholds set by a pathologist, and the percentages of each category were normalized by total area of interest.

Maximum Tolerated Dose (MTD)

The MTD study was performed as previously described (23).

Measurement of Activity

Because α -particles from ^{225}Ac cannot be directly measured in tissue because of the short mean free path (18), ^{225}Ac α -activities were estimated using measurements of related γ -emissions. For the initial MTD study, syringes were prepared with a range of activities as determined by the γ -counter (Wallac 1470 Wizard; Perkin-Elmer). For subsequent studies, a dose calibrator (Atomlab 500; Biodex) was used to prefll syringes with $148\ \text{kBq} \pm 10\%$ (per Appendix E of the Biodex manual) of ^{225}Ac -conjugate activity. Activities were measured for 2 min using dial number 38.2 as recommended by Biodex. Activities of ^{225}Ac , and the ^{221}Fr and ^{213}Bi daughter products (18), were measured by acquiring isomeric γ -spectra (Supplemental Fig. 3) before administration using a 4π well-type wipe-test γ -counter (Atomlab 500). Activities (^{225}Ac) were calculated using factors for γ -ray abundance per α -decay using calibration parameters and correction coefficients from Appendices A and E of the instrument manual. A full energy window

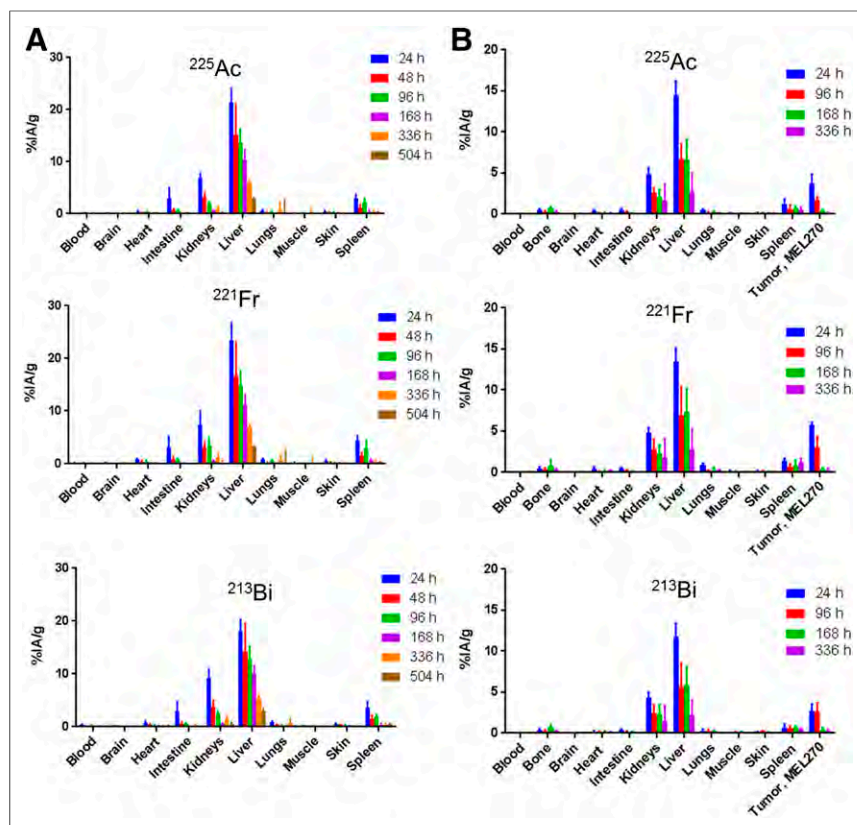


FIGURE 4. Biodistribution of ^{225}Ac -DOTA-MC1RL: ^{225}Ac , ^{221}Fr , and ^{213}Bi activities in tissues from non-tumor-bearing BALB/c mice ($n = 6$ per time point) (A) and SCID mice bearing MEL270 human uveal melanoma tumors ($n = 5$ per time point) (B).

(0–800 keV) was used for spectra acquisition that included γ -counts from ^{225}Ac (99.8-keV peak, 1% abundance) and 2 γ -emitting daughters, ^{221}Fr (218.1-keV peak, 11.4% abundance) and ^{213}Bi (440.5-keV peak, 25.9% abundance) (24). The α -activities were determined by fitting each peak with a multigaussian fit and integrating to determine the net

nonlinear regression, and dosimetry calculations were performed for ^{225}Ac , ^{221}Fr , ^{217}At , ^{213}Bi , and ^{213}Po using the generalized internal dosimetry schema of the MIRD Committee for α -particle emitters (26,27). The β^- decay branching ratio for ^{217}At to ^{217}Rn is only 0.01%; therefore, it was assumed that all decays of ^{217}At were by α -emission to ^{213}Bi . The

number of counts while incorporating the acquisition time. Spectra were acquired at least 24 h after radiosynthesis or tissue rendering, ensuring that ^{225}Ac and daughters were in secular equilibrium (25). Activity remaining in the syringe and catheter after injection was calculated and subtracted to determine net administered activity.

Blood Pharmacokinetics

Sprague-Dawley rats were weighed before injection with radioactivity and injected with 148 kBq ($\pm 10\%$) of ^{225}Ac -DOTA-MC1RL in the syringe. Serial blood draws (45 μL) were taken from 5 min to 24 h after injection. ^{225}Ac α -activity was calculated as described above. Data were fitted using an exponential decay nonlinear regression.

Biodistribution

Non-tumor-bearing BALB/c mice, or SCID mice bearing MEL270 xenografts (160–650 mm^3) or A375 and A375/MC1R bilateral xenografts (189–1,680 mm^3), were intravenously administered 148 kBq ($\pm 10\%$) of ^{225}Ac α -activity in the syringe. Tissues were rendered and weighed at multiple time-points between 24 h and 3 wk after injection. For each tissue, ^{225}Ac , ^{221}Fr , and ^{213}Bi α -activities were calculated as described above and reported as percentage injected activity per gram (%IA/g).

Radiation Dosimetry

Biodistribution data for the different tissues were fitted using an exponential decay nonlinear regression, and dosimetry calculations were performed for ^{225}Ac , ^{221}Fr , ^{217}At , ^{213}Bi , and ^{213}Po using the generalized internal dosimetry schema of the MIRD Committee for α -particle emitters (26,27). The β^- decay branching ratio for ^{217}At to ^{217}Rn is only 0.01%; therefore, it was assumed that all decays of ^{217}At were by α -emission to ^{213}Bi . The branching ratios for decay of ^{213}Bi to ^{213}Po (98%) or ^{209}Tl (2%) were included in the calculation. Because of the relatively low linear-energy transfer and the small dimensions of the target tissues, the β^- emissions from ^{217}At , ^{213}Bi , ^{209}Tl , and ^{209}Pb were assumed negligible and were not included in the calculations (28). The following assumptions were made: uniform distribution of activity in the tissue volume; no α -particles escaping from the source tissue due to the short range; and electron and photon contributions that were negligible compared with α -particle energy deposition (28). It was also assumed that α -particles from ^{221}Fr (4.9-min half-life), ^{217}At (32.2-ms half-life), ^{213}Bi (46-min half-life), and ^{213}Po (4.2- μs half-life) were deposited in the same location as ^{225}Ac (10-d half-life) because of the relatively shorter half-lives of these daughter isotopes. Although ^{217}At and ^{213}Po do not have detectable γ -emissions, under the assumption that the decay chain had reached secular equilibrium, the accumulated activity of these 2 daughters would equal that of ^{221}Fr

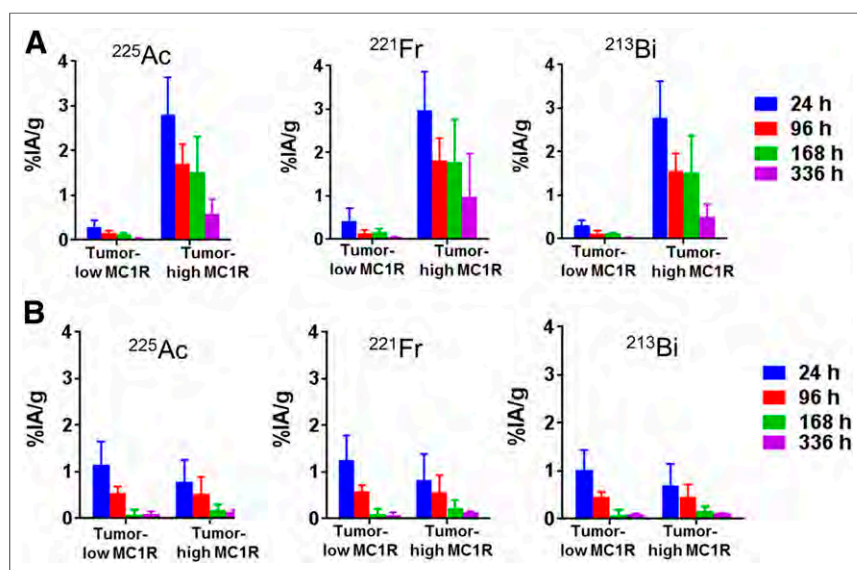


FIGURE 5. Biodistribution of ^{225}Ac -DOTA-MC1RL (A) and ^{225}Ac -DOTA-SP (B) in bilateral A375 and A375/MC1R tumors ($n = 5$ per time point).

TABLE 2
Radiation Dosimetry and Clearance Kinetics Parameters for ^{225}Ac -DOTA-MC1RL in Non-Tumor-Bearing BALB/c Mice

Parameter	Blood	Brain	Heart	Intestine	Kidney	Liver	Lung	Muscle	Skin	Spleen
^{225}Ac										
Initial activity/organ (kBq)	ND	0.0065	0.0161	1.9113	0.7647	7.6597	0.0512	0.0044	0.0483	0.0426
Effective decay rate constant (h^{-1})	ND	0.0070	0.0030	0.0060	0.0060	0.0040	0.0030	0.0030	0.0050	0.0030
Effective decay half-life (d)	ND	4.1259	9.6270	4.8135	4.8135	7.2203	9.6270	9.6270	5.7762	9.6270
Accumulated activity/organ ($\text{kBq} \times \text{h}$)	ND	0.7621	3.8089	260.3419	104.1599	1484.6038	12.1242	1.0529	7.7927	10.0784
Absorbed dose/injected activity (Gy/kBq)	ND	0.0002	0.0023	0.0102	0.0300	0.1485	0.0042	0.0004	0.0024	0.0092
^{221}Fr										
Initial activity/organ (kBq)	0.0153	0.0222	0.0349	1.9927	1.3795	8.4464	0.0723	0.0211	0.0705	0.0647
Effective decay rate constant (h^{-1})	0.0010	0.0030	0.0050	0.0070	0.0080	0.0040	0.0020	0.0030	0.0040	0.0040
Effective decay half-life (d)	28.8811	9.6270	5.7762	4.1259	3.6101	7.2203	14.4406	9.6270	7.2203	7.2203
Accumulated activity/organ ($\text{kBq} \times \text{h}$)	5.6907	5.2622	5.6292	232.2869	139.2517	1637.0734	21.2642	4.9899	13.6614	12.5317
Absorbed dose/injected activity (Gy/kBq)	0.0022	0.0013	0.0037	0.0098	0.0434	0.1770	0.0080	0.0018	0.0045	0.0124
^{217}At										
Initial activity/organ (kBq)	0.0153	0.0222	0.0349	1.9927	1.3795	8.4464	0.0723	0.0211	0.0705	0.0647
Effective decay rate constant (h^{-1})	0.0010	0.0030	0.0050	0.0070	0.0080	0.0040	0.0020	0.0030	0.0040	0.0040
Effective decay half-life (d)	28.8811	9.6270	5.7762	4.1259	3.6101	7.2203	14.4406	9.6270	7.2203	7.2203
Accumulated activity/organ ($\text{kBq} \times \text{h}$)	5.6907	5.2622	5.6292	232.2869	139.2517	1637.0734	21.2642	4.9899	13.6614	12.5317
Absorbed dose/injected activity (Gy/kBq)	0.0025	0.0014	0.0042	0.0110	0.0486	0.1983	0.0090	0.0021	0.0050	0.0139
^{213}Bi										
Initial activity/organ (kBq)	0.0236	0.0195	0.0309	1.8886	1.0318	6.5122	0.0717	0.0152	0.0627	0.0511
Effective decay rate constant (h^{-1})	0.0010	0.0010	0.0020	0.0050	0.0040	0.0040	0.0020	0.0020	0.0030	0.0030
Effective decay half-life (d)	28.8811	28.881	14.4406	5.7762	7.2203	7.2203	14.4406	14.4406	9.6270	9.6270
Accumulated activity/organ ($\text{kBq} \times \text{h}$)	8.7917	7.2573	9.0914	304.6111	199.9839	1262.1858	21.0948	4.4603	14.8376	12.1064
Absorbed dose/injected activity (Gy/kBq)	0.0001	0.0000	0.0001	0.0002	0.0012	0.0025	0.0001	0.0000	0.0001	0.0002
^{213}Po										
Initial activity/organ (kBq)	0.0236	0.0195	0.0309	1.8886	1.0318	6.5122	0.0717	0.0152	0.0627	0.0511
Effective decay rate constant (h^{-1})	0.0010	0.0010	0.0020	0.0050	0.0040	0.0040	0.0020	0.0020	0.0030	0.0030
Effective decay half-life (d)	28.8811	28.881	14.4406	5.7762	7.2203	7.2203	14.4406	14.4406	9.6270	9.6270
Accumulated activity/organ ($\text{kBq} \times \text{h}$)	8.7917	7.2573	9.0914	304.6111	199.9839	1262.1858	21.0948	4.4603	14.8376	12.1064
Absorbed dose/injected activity (Gy/kBq)	0.0044	0.0023	0.0079	0.0168	0.0811	0.1778	0.0103	0.0021	0.0063	0.0156
Total absorbed dose/injected activity (Gy/kBq)	0.0092	0.0053	0.0183	0.0481	0.2042	0.7042	0.0317	0.0064	0.0182	0.0512

ND = not detected.

and ^{213}Bi , respectively. The total absorbed α -particle dose was calculated from the summation of doses from ^{225}Ac , ^{221}Fr , ^{217}At , ^{213}Bi , and ^{213}Po .

Antitumor Efficacy

Tumor-bearing mice ($n = 11/\text{group}$) were injected with activities of ^{225}Ac -DOTA-MC1RL or ^{225}Ac -DOTA-SP, cold lanthanum-DOTA-MC1RL, or saline solution (0.9%, Cardinal Pharmaceuticals). Surpassing a 2,000 mm^3 tumor volume was the experimental endpoint unless clinical endpoints, such as 20% weight loss, tumor ulceration, hunched back, lack of grooming, or lethargy, were observed. Metastasis formation was identified by necropsy.

Statistical Analysis

The t test was used for the MTD study. The following analyses were used for comparison of the efficacy study groups: Kaplan-Meier for time to endpoint, a mixed-model analysis for tumor growth change, a paired Wilcoxon signed-rank test for initial decrease in tumor volume, a Fisher exact test with corrections for multiple testing using the Holm stepdown method for metastasis

burden, and a nonparametric Kruskal-Wallis test for immunohistochemistry staining.

RESULTS

Synthesis and Characterization of Parent Compound and Lanthanide Chelates

The unmetallated DOTA-MC1RL was synthesized and, since there are no nonradioactive isotopes of actinium, the analogous lanthanum-DOTA-MC1RL chelate was prepared for use as a non-radioactive control (Supplemental Figs. 4–8) (23,29). Both DOTA-MC1RL and lanthanum-DOTA-MC1RL had high binding affinity for human MC1R, 0.24 ± 0.20 and 0.23 ± 0.18 nM inhibition constants, respectively (Supplemental Fig. 9A). The binding affinity of europium-DTPA-MC1RL to human MC1R was determined to be a 4.4 ± 2.3 nM dissociation constant (Supplemental Fig. 9B). Lower, 1.3 μM , dissociation constant affinity was observed for europium-DTPA-MC1RL binding to murine MC1R (Supplemental Fig. 9C). The scrambled peptide controls, lanthanum-DOTA-SP and europium-DOTA-SP, did not bind (Supplemental Figs. 9D and 9E).

TABLE 3
Radiation Dosimetry and Clearance Kinetics for ^{225}Ac -DOTA-MC1RL in SCID Mice Bearing MEL270 Tumors

Parameter	Blood	Bone	Brain	Heart	Intestine	Kidney	Liver	Lung	Muscle	Skin	Spleen	Tumor
^{225}Ac												
Initial activity/organ (kBq)	0.0010	0.0172	0.0031	0.0151	0.3026	0.6417	4.8193	0.0265	0.0042	0.0242	0.0228	0.0518
Effective decay rate constant (h^{-1})	0.0010	0.0030	0.0020	0.0050	0.0130	0.0050	0.0060	0.0050	0.0020	0.0020	0.0040	0.0080
Effective decay half-life (d)	28.8811	9.6270	14.4406	5.7762	2.2216	5.7762	4.8135	5.7762	14.4406	14.4406	7.2203	3.6101
Accumulated activity/organ ($\text{kBq} \times \text{h}$)	0.2726	3.2443	0.6873	2.1154	16.7417	89.9040	588.513	3.7089	0.9236	5.3441	3.6979	4.9055
Absorbed dose/injected activity (Gy/kBq)	0.0001	0.0041	0.0003	0.0020	0.0012	0.0273	0.0662	0.0025	0.0007	0.0010	0.0107	0.0045
^{221}Fr												
Initial activity/organ (kBq)	0.0036	0.0185	0.0059	0.0192	0.2655	0.6167	4.4828	0.0495	0.0071	0.0362	0.0257	0.0806
Effective decay rate constant (h^{-1})	0.0017	0.0030	0.0010	0.0030	0.0090	0.0040	0.0060	0.0040	0.0020	0.0020	0.0010	0.0070
Effective decay half-life (d)	16.9889	9.6270	28.8811	9.6270	3.2090	7.2203	4.8135	7.2203	14.4406	14.4406	28.881	4.1259
Accumulated activity/organ ($\text{kBq} \times \text{h}$)	0.8370	3.4960	1.5396	3.6259	22.3311	99.8475	547.425	8.0162	1.5699	8.0166	6.7303	8.6407
Absorbed dose/injected activity (Gy/kBq)	0.0002	0.0047	0.0007	0.0037	0.0017	0.0328	0.0666	0.0058	0.0014	0.0017	0.0211	0.0086
^{217}At												
Initial activity/organ (kBq)	0.0036	0.0185	0.0059	0.0192	0.2655	0.6167	4.4828	0.0495	0.0071	0.0362	0.0257	0.0806
Effective decay rate constant (h^{-1})	0.0017	0.0030	0.0010	0.0030	0.0090	0.0040	0.0060	0.0040	0.0020	0.0020	0.0010	0.0070
Effective decay half-life (d)	16.9889	9.6270	28.8811	9.6270	3.2090	7.2203	4.8135	7.2203	14.4406	14.4406	28.881	4.1259
Accumulated activity/organ ($\text{kBq} \times \text{h}$)	0.8370	3.4960	1.5396	3.6259	22.3311	99.8475	547.425	8.0162	1.5699	8.0166	6.7303	8.6407
Absorbed dose/injected activity (Gy/kBq)	0.0003	0.0053	0.0007	0.0041	0.0019	0.0367	0.0746	0.0065	0.0015	0.0019	0.0236	0.0097
^{213}Bi												
Initial activity/organ (kBq)	0.0016	0.0133	0.0027	0.0082	0.2160	0.5664	3.9089	0.0175	0.0017	0.0240	0.0131	0.0381
Effective decay rate constant (h^{-1})	0.0013	0.0020	0.0007	0.0040	0.0100	0.0040	0.0060	0.0060	0.0020	0.0050	0.0030	0.0060
Effective decay half-life (d)	22.2163	14.4406	41.2588	7.2203	2.8881	7.2203	4.8135	4.8135	14.4406	5.7762	9.6270	4.8135
Accumulated activity/organ ($\text{kBq} \times \text{h}$)	0.4096	2.9358	0.7531	1.3342	16.2384	91.7024	477.346	2.1354	0.3859	3.3617	2.4736	4.6565
Absorbed dose/injected activity (Gy/kBq)	2.20E-06	7.39E-05	5.96E-06	2.51E-05	2.32E-05	5.61E-04	1.08E-03	2.88E-05	6.19E-06	1.32E-05	1.44E-04	8.68E-05
^{213}Po												
Initial activity/organ (kBq)	0.0016	0.0133	0.0027	0.0082	0.2160	0.5664	3.9089	0.0175	0.0017	0.0240	0.0131	0.0381
Effective decay rate constant (h^{-1})	0.0013	0.0020	0.0007	0.0040	0.0100	0.0040	0.0060	0.0060	0.0020	0.0050	0.0030	0.0060
Effective decay half-life (d)	22.2163	14.4406	41.2588	7.2203	2.8881	7.2203	4.8135	4.8135	14.4406	5.7762	9.6270	4.8135
Accumulated activity/organ ($\text{kBq} \times \text{h}$)	0.4096	2.9358	0.7531	1.3342	16.2384	91.7024	477.346	2.1354	0.3859	3.3617	2.4736	4.6565
Absorbed dose/injected activity (Gy/kBq)	0.0002	0.0052	0.0004	0.0018	0.0016	0.0392	0.0756	0.0020	0.0004	0.0009	0.0101	0.0061
Total absorbed dose/injected activity (Gy/kBq)	0.0007	0.0193	0.0021	0.0115	0.0065	0.1366	0.2842	0.0168	0.0040	0.0056	0.0656	0.0290

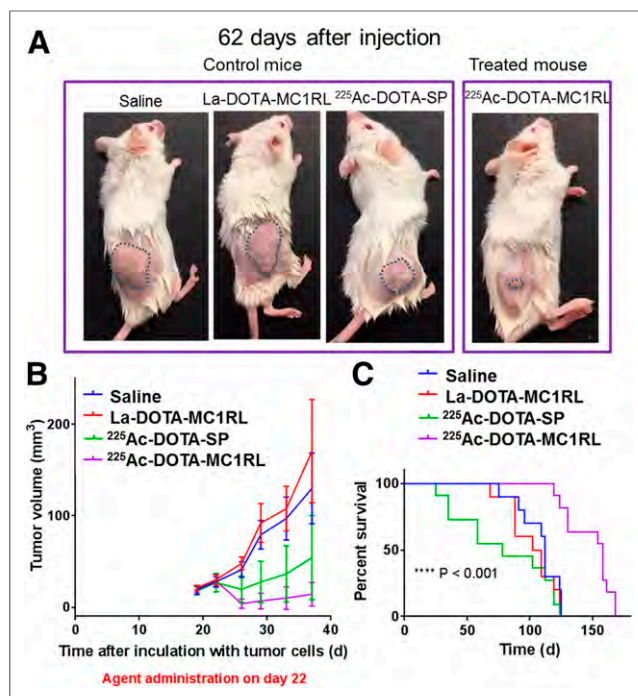


FIGURE 6. Efficacy study in mice bearing MEL270 tumors: representative images of tumors (outlined) (A); initial tumor growth volumes (B); and Kaplan–Meier plots (C).

Radiosynthesis and Characterization of ²²⁵Ac Radiopharmaceutical

Radiochemical purity of 99.8% and specific activity of 181.3 ± 92.5 kBq/ μ g and 140.6 ± 55.5 kBq/ μ g for ²²⁵Ac-DOTA-MC1RL and ²²⁵Ac-DOTA-SP, respectively, were observed (Supplemental Fig. 10). In vitro serum stability was high, with 90% intact after 10 d (Table 1).

MC1R Expression on Uveal Melanoma Cell Lines and Xenograft Tumors

MC1R messenger RNA and protein expression were confirmed in a set of uveal melanoma cell lines (Supplemental Fig. 11). Only MEL270, OMM2.3, and OMM1 cells carry the GNAQ or GNA11 mutations found in nearly all uveal melanomas (30). The MEL270 and OMM1 cells formed tumors in immunocompromised mice, and all xenografts had high and uniform MC1R protein expression.

Receptor Number for Tumor Cell Lines

MEL270 cells were selected for the in vivo studies, and it was determined that MEL270 cells have 410,000 receptors per cell (Supplemental Fig. 12), which is a higher level of endogenous expression than that of the engineered A375/MC1R cells, which have 75,000 receptors per cell (12). The parental A375 melanoma cell line has extremely low expression, at 400 ± 93 MC1Rs per cell (31).

In Vitro MC1R-Specific Cytotoxicity

Cytotoxicity assays were performed with the goal of demonstrating target-specific cytotoxicity. Assay conditions were not optimized to demonstrate maximal toxicity. Significantly reduced proliferation ($P < 0.0001$) was observed in uveal melanoma cells and the engineered A375/MC1R cells treated with ²²⁵Ac-DOTA-MC1RL relative to the untargeted ²²⁵Ac-DOTA-SP or phosphate-

buffered saline controls (Supplemental Fig. 2). All cell lines also had a significant ($P < 0.001$) response to incubation with ²²⁵Ac-DOTA-SP relative to phosphate-buffered saline. However, there was no significant difference in A375 cell proliferation (extremely low MC1R) when treated with either the targeted or the untargeted radiopharmaceutical. These results demonstrate MC1R-specific cytotoxicity. Assay replicates yielded comparable results.

MTD

The MTD was evaluated in immune-competent non-tumor-bearing BALB/c mice ($n = 5$ /cohort). Cohorts received a single intravenous injection of ²²⁵Ac-DOTA-MC1RL over the range of 0–148 kBq in the syringe. At completion of the study (>11 ²²⁵Ac half-lives, 118 d after injection), serum and tissues (adipose, bone, cecum, colon, duodenum, esophageal, heart, ileum, kidney, liver, lung, lymph nodes, muscle, pancreas, small intestine, spleen, and stomach) were collected for histology and then examined in a masked manner by a veterinary pathologist to assess radiation-induced tissue damage. No remarkable damage was observed in any of the tissues (Supplemental Figs. 13–16). For example, the control kidneys had minimal multifocal interstitial fibrosis and minimal medullary protein in tubules, which were both considered to be incidental findings. The incidental minimal medullary protein was also found in some kidneys from the groups that received treatment activities, but each treatment group also included kidneys that were within normal limits for all types of damage. The cortex of one kidney from the group with the highest administered activity had a focal extracellular cortical hyaline substance that was healing and was considered to be an incidental finding (Supplemental Fig. 14D). Blood urea nitrogen and creatinine, which are important indicators of renal function, were also determined and were not significantly elevated among the groups (Figs. 2B and 2C). All animals had gained weight by the end of the study, albeit less weight was gained by animals at the highest dose level than in the lowest (Fig. 2A).

Pharmacokinetics and Biodistribution

In rats, ²²⁵Ac-DOTA-MC1RL rapidly cleared (<15 min after injection) from blood circulation (Fig. 3). After administration to non-tumor-bearing BALB/c mice, ²²⁵Ac activities were observed primarily in clearance tissues. At 24 h after injection of ²²⁵Ac-DOTA-MC1RL, the liver, kidneys, spleen, and intestine had 21.2 ± 2.8 , 6.9 ± 0.9 , 2.9 ± 0.8 , and 2.9 ± 2.0 %IA/g, whereas negligible activity was observed in the other tissues measured. Activity had largely cleared from the tissues at 1–3 wk (Fig. 4A). For tumor-bearing animals, activity was retained in MC1R-positive tumors, that is, MEL270 (Fig. 4B) and A375/MC1R tumors (Fig. 5A), which had 3.6 ± 1.2 and 2.8 ± 0.8 %IA/g, respectively, compared with the nominal 0.30 ± 0.1 %IA/g in the MC1R-negative A375 tumors at 24 h after injection. The clearance tissues in tumor-bearing animals had lower activities than in non-tumor-bearing mice: for example, 14.4 ± 1.7 %IA/g in the livers of MEL270 tumor-bearing mice at 24 h (Fig. 4B), compared with the 21.2 ± 2.8 %IA/g in the non-tumor-bearing mice (Fig. 4A). The ²²⁵Ac-DOTA-SP tumor distribution in the bilateral A375 and A375/MC1R model was also determined, and as expected, uptake was minimal and did not differ between the positive and negative A375 tumors (Fig. 5B). The distribution of ²²¹Fr and ²¹³Bi was also determined (Figs. 4 and 5). However, since ²²⁵Ac and daughters are at secular equilibrium by 24 h after injection and the ²²¹Fr and ²¹³Bi atoms

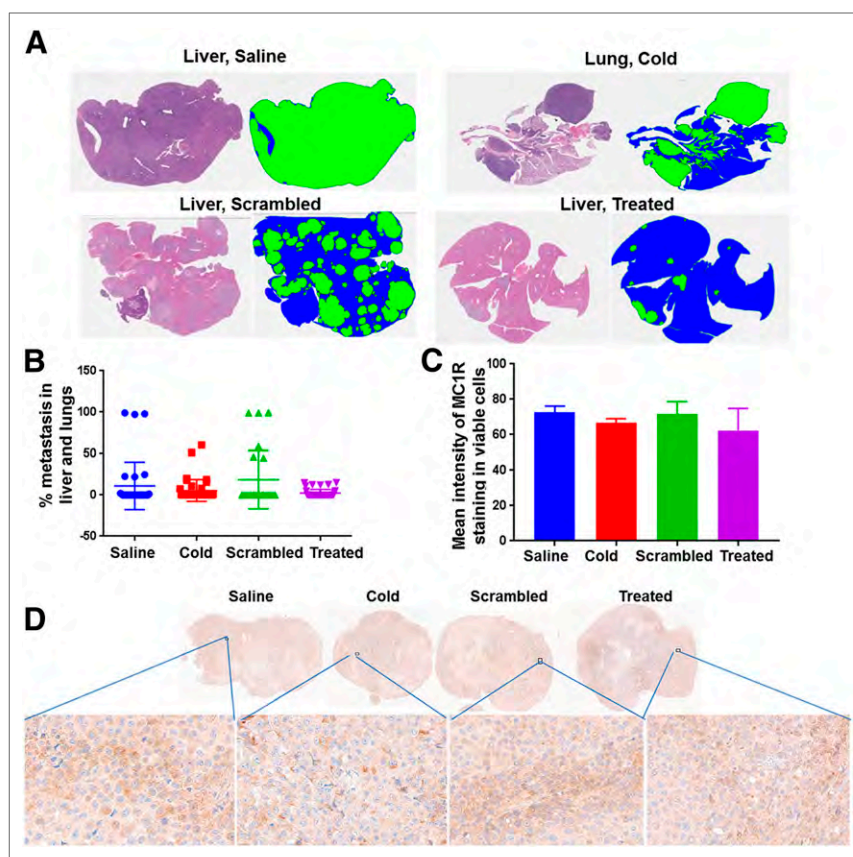


FIGURE 7. Metastasis study in MEL270 uveal melanoma mouse model and MC1R expression in tumors reaching endpoints from each treatment group: representative hematoxylin and eosin staining and corresponding threshold segmentations of sections containing liver and lung metastases (cold = lanthanum-DOTA-MC1RL; scrambled = untargeted; treated = ^{225}Ac -DOTA-MC1RL; blue = normal tissue; green = metastasis) (A); quantified metastasis burden (B); graph (C) and sections (D) for MC1R immunohistochemistry staining of MEL270 tumors after reaching endpoints.

present during injection will be mostly decayed, the ^{221}Fr and ^{213}Bi detected in the samples are from decay of the ^{225}Ac taken into the tissues.

Radiation Dosimetry

Biodistribution data were fitted (Supplemental Fig. 17), and clearance kinetics, tissue biologic half-life, accumulated activity, and absorbed dose/injected activity (Gy/kBq) were estimated for each radionuclide in each tissue for non-tumor-bearing and MEL270 tumor-bearing mice (Tables 2 and 3). The effective decay half-lives calculated for ^{225}Ac in tissues—for example, 7.2 d in liver—were shorter than the radiodecay half-life of ^{225}Ac (10 d), indicating biologic clearance. The calculated total absorbed dose per injected activity (Gy/kBq) for ^{225}Ac -DOTA-MC1RL was minimal in all tissues except clearance organs and positive tumor. Since the positive tumors shrank rapidly in response to the treatment and the total absorbed doses were extrapolated from data collected over a 2-wk period, the dose values for the tumors are likely subdued relative to the clearance organs, which did not have appreciable cellular toxicity at the administered activities. The total absorbed dose in the liver was generally lower in mice with tumors than in nontumor mice: for example, 0.284 and 0.704 Gy/kBq, respectively.

Antitumor Efficacy

SCID mice bearing MEL270 tumors ($124 \pm 36 \text{ mm}^3$ pretreatment tumor volumes) were injected with a single administration of ^{225}Ac -DOTA-MC1RL ($92.5 \pm 9.3 \text{ kBq}$), ^{225}Ac -DOTA-SP ($99.9 \pm 9.9 \text{ kBq}$), lanthanum-DOTA-MC1RL (1 pmol/mouse), or saline. Representative images show much smaller tumors in treated mice than in controls (Fig. 6A), and tumor volumes decreased immediately after treatment relative to controls ($P = 0.001$) before eventual regrowth (Fig. 6B). Treated mice had a significantly delayed time to experimental or clinical endpoint ($P < 0.001$), with a median survival of 148 d, compared with the median survival of control groups (79–108 d), and differences among the controls were not significant (Fig. 6C). In this study, some animals were euthanized because of reaching clinical endpoints instead of the experimental endpoint (Supplemental Table 1). Some of the animals that reached clinical endpoints had metastases in the liver or lungs, and metastasis burden was significantly lower in the ^{225}Ac -DOTA-MC1RL treated group than in the controls ($P = 0.024$) (Figs. 7A and 7B). Mice bearing A375/MC1R tumors ($240 \pm 110 \text{ mm}^3$ pretreatment volume) were also injected with either sterile saline, lanthanum-DOTA-MC1RL, $107.3 \pm 11.1 \text{ kBq}$ of ^{225}Ac -DOTA-SP, or $59.2 \pm 5.9 \text{ kBq}$ of ^{225}Ac -DOTA-MC1RL, and significant decreases in tumor volume ($P = 0.005$) and tumor growth delay ($P < 0.0001$) were observed. Some tumors that disappeared did not recur, and those mice lived their natural

life span (Supplemental Figs. 18A–18C). After tumors reached an endpoint, MC1R staining was quantified, and the level of MC1R expression was not significantly different in treated tumors that responded by shrinking before regrowth relative to control tumors ($P = 0.60$ for MEL270 and $P = 0.82$ for A375/MC1R) (Figs. 7C and 7D; Supplemental Fig. 18D).

DISCUSSION

We have developed and evaluated a novel MC1R-targeted radiopharmaceutical, ^{225}Ac -DOTA-MC1RL, for TAT of metastatic uveal melanoma. The choice of using a peptide-targeting ligand is reinforced by the recent preclinical and clinical successes of TAT radiopeptides (14,15,32–34). Another group has also reported the development of a peptide-based TAT, ^{212}Pb -CCMSH, that is targeted to melanocortin receptors for treatment of melanoma (35). However, ^{212}Pb -CCMSH was associated with renal toxicity. This is likely due to the use of an α -MSH derivative–targeting ligand, as α -MSH has specificity for multiple melanocortin receptor isoforms, including MC5R, which is expressed in the human kidney and lungs (6). The MC1RL-targeting moiety used in the current work has specificity for the MC1R isoform (13), greatly reducing the potential for renal toxicity. Another advantage of ^{225}Ac -DOTA-MC1RL over the ^{212}Pb -TAT agent is that ^{225}Ac has greater cell-killing potential through

generation of 4 α -particle emissions per radionuclide, compared with the single α -emission of ^{212}Pb , in their relative decay chains (35,36).

^{225}Ac -DOTA-MC1RL has high affinity for MC1R, high radiochemistry yield and purity, high biostability, and MC1R-specific cytotoxicity in vitro. In vivo studies demonstrated low toxicity, rapid blood clearance, and uptake into MC1R-positive tumors and clearance organs. Biodistribution studies demonstrated that ^{225}Ac remains in the compartments where ^{225}Ac -DOTA-MC1RL was initially distributed, that is, tumors and clearance organs, and the corresponding clearance kinetics parameters and radiation dose delivered by all α -particle-emitting radioisotopes in the decay chain were calculated. Considering the 10-d half-life of ^{225}Ac , most of the administered ^{225}Ac -DOTA-MC1RL will have either been taken into tumor cells (7) or cleared from the blood before decay. Hence, ^{225}Ac -DOTA-MC1RL likely functions as an in vivo α -particle generator, concentrating α -emissions in the target tumor tissues, with limited translocation of daughter isotopes (32). This is consistent with the recent observations of efficacy with low toxicity observed for an ^{225}Ac -PSMA-targeting small-molecule conjugate (37).

In vivo efficacy studies demonstrated significant tumor and metastasis growth delay and prolonged survival in human uveal and cutaneous melanoma xenograft models in mice after a single treatment of ^{225}Ac -DOTA-MC1RL, including some cures. Tumors that shrank and regrew after treatment had the same MC1R expression levels as controls, suggesting that multiple treatment regimens would increase efficacy.

CONCLUSION

We have developed and evaluated a novel MC1R-targeted radiopharmaceutical for TAT of metastatic uveal melanoma. In vivo studies demonstrated low toxicity, rapid blood clearance, uptake into MC1R-positive tumors and clearance organs, significant tumor and metastasis growth delay, and prolonged survival in human uveal melanoma xenograft models in mice after a single treatment of ^{225}Ac -DOTA-MC1RL. This novel radiopharmaceutical has strong potential to benefit patients with metastatic uveal melanoma, which has had no significant improvement in treatment in the last 20 y.

DISCLOSURE

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Melanocortin 1 Receptor–Targeted α -Particle Therapy for Metastatic Uveal Melanoma

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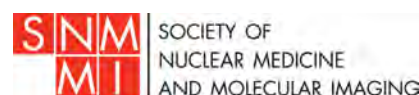
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Review

Development of Targeted Alpha Particle Therapy for Solid Tumors

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Abstract: Targeted alpha-particle therapy (TAT) aims to selectively deliver radionuclides emitting α -particles (cytotoxic payload) to tumors by chelation to monoclonal antibodies, peptides or small molecules that recognize tumor-associated antigens or cell-surface receptors. Because of the high linear energy transfer (LET) and short range of alpha (α) particles in tissue, cancer cells can be significantly damaged while causing minimal toxicity to surrounding healthy cells. Recent clinical studies have demonstrated the remarkable efficacy of TAT in the treatment of metastatic, castration-resistant prostate cancer. In this comprehensive review, we discuss the current consensus regarding the properties of the α -particle-emitting radionuclides that are potentially relevant for use in the clinic; the TAT-mediated mechanisms responsible for cell death; the different classes of targeting moieties and radiometal chelators available for TAT development; current approaches to calculating radiation dosimetry for TATs; and lead optimization via medicinal chemistry to improve the TAT radiopharmaceutical properties. We have also summarized the use of TATs in pre-clinical and clinical studies to date.

Keywords: targeted alpha-particle therapy; solid tumors; mechanism of cell death; targeting moieties; chelation; radiation dosimetry; medicinal chemistry; clinical studies

1. Introduction

Over the past two decades, radioimmunotherapy (RIT) has proven to be an effective treatment for non-solid tumors (reviewed in [1,2]); e.g., radiolabeled anti-CD20 monoclonal antibodies for the

treatment of lymphoma. These antibody-radionuclide conjugates have typically used beta (β)-particle emitting radionuclides; e.g., ^{131}I , ^{67}Cu , ^{177}Lu or ^{90}Y . However, due to the relatively long range of the associated β -emissions and the poor tumor penetration of antibodies, there has been (compared) regarding the use of RIT for the treatment of solid tumors, where much of the energy is deposited in the surrounding normal tissues relative to the tumor, particularly in the case of small tumor cell foci or metastases [1]. Alpha (α)-particle emissions have a much shorter range and greater linear energy transfer (LET) relative to β -emissions, depositing more energy into smaller volumes [3]. Hence, there has been significant interest in the development of targeted alpha-particle therapy (TAT) for the treatment of solid tumors. Recently, the FDA approved the use of $^{223}\text{RaCl}_2$ (Xofigo[®]) for the palliative care of prostate bone metastases, and the efficacy of $^{223}\text{RaCl}_2$ and $^{225}\text{Ac-PSMA-617}$ have been demonstrated in the treatment of prostate bone metastases (Figure 1) [4–6]. These developments have further elevated interest in the development of novel α -emission cancer treatments [7,8]. Typically, TAT for solid tumors involves attaching the α -particle emitting radionuclide to a tumor-targeting scaffold, followed by the intravenous administration and systemic targeting of tumors and metastases. The α -particle range is only a few cell diameters, ensuring that the greatest effect of tumor TAT remains within the tumor volume [3]. Herein we discuss the current progress, challenges and approaches toward the development of novel TATs for the treatment of solid tumors.



Figure 1. $^{68}\text{Ga-PSMA-11}$ PET/CT images of a treatment-naïve patient with extensive bone metastasis at primary diagnosis. A complete remission was observed after three cycles of $^{225}\text{Ac-PSMA-617}$ with de-escalating activities of 8/7/6 MBq. The patient remained symptom-free with undetectable serum PSA and a negative $^{68}\text{Ga-PSMA-11}$ PET/CT at 11-month follow-up evaluation. This figure and legend were reproduced from Sathekge, et al. [6].

There are differences in the mechanism of tumor cell killing when comparing β -emission to α -emission therapies. β -particles scale to the size of electrons travel over a relatively longer range (0.3–12 mm) in tissues in comparison to α -particles, have relatively lower LET and generate hydroxyl free radicals by indirect action compared to α -particles, have relatively lower LET and generate hydroxyl free radicals by direct breaking covalent bonds of water molecules in the tissue [9]. The α -particles result in oxidative damage to the size cellular DNA macromolecules, causing single (10–99) and double (100–1000) strand breaks [10]. In contrast, α -particles have higher LET (size 100 keV/ μm) versus β (size 0.2 keV/ μm) [11–14]. Hence, α -emissions deposit large amounts of energy in a smaller volume relative to β -emissions and result in the direct breaking of covalent bonds; e.g., DNA double-strand breaks. In either case (α or β), the DNA damage can activate DNA damage checkpoints and double-strand break repair [15]. Where the damage is significant or if there are defects in the checkpoint or repair pathways such that repairs cannot be made, i.e., irreparable damage, programmed cell death (apoptosis) is initiated [16]. In apoptosis-deficient tumor cells, the resulting damage to the cellular machinery eventually results in necrotic cell death [16]. Hence, α -emission therapy potentially has several distinct advantages relative to β therapy. First, the shorter range of α -emissions result in lower off-target damage but will

still allow for killing of adjacent tumor cells. Since tumors are known to be heterogeneous in marker expression [17], nearby cells that do not express the target can also be damaged and killed, potentially eliminating a mechanism of resistance; i.e., killing of marker expressing cells with the clonal expansion of non-marker expressing cells. Second, the greater energy deposition will result in greater DNA damage and a correspondingly greater level of cell killing. Third, α -emission-mediated DNA damage does not rely on the generation of free radicals, potentially eliminating the development of a major mechanism of resistance to radiation therapy; e.g., upregulation of superoxide dismutase, etc. [3].

There are many α -emitting radionuclides available for use in the development of TAT, and factor, including nuclear characteristics, availability, chemistry, specific activity, synthesis yield, chemical and biological stability of conjugates, and costs, need to be considered. Choice of targeting scaffold is also a major consideration in TAT development. Antibodies, antibody fragments, peptides and passive targeting strategies have been employed and the type of targeting moiety chosen will have a bearing on the type of radionuclide attachment used, the route of clearance and blood pharmacokinetics (PK), tissue biodistribution (BD) and radiation dosimetry. The relative merits of available radionuclides, attachment chemistries and targeting moieties are discussed herein. Radiation dosimetry (RD) is an area of key importance in development of TATs. The discrete decay chains of some α -particle-emitting radionuclides involve the generation of daughter products with individual radioactive emission properties. Hence, the biological fate of the daughter products and resulting tissue exposure to ionizing radiation is a major concern that must be evaluated and the current status of RD studies in the context of TAT development are also discussed. Strategies for lead optimization via medicinal chemistry to improve the TAT radiopharmaceutical properties and methods of current good manufacturing practice (cGMP) production are discussed. Also included are brief reviews of preclinical and clinical TAT studies conducted to date.

2. Mechanism of Action/Tumor Cell Killing

Since the discovery of radioactive materials, the effect of radiation on the properties of matter has been of significant interest in the disciplines of material science, geology and astrophysics. For example, the first large-scale effect of radiation on solid material was observed by E.P. Wigner via exposure to a nuclear fission reactor, and that was thus named the Wigner effect, or “Wigner’s disease” [1]. Since then, the large spread of medical technology involving sources of ionization radiation [2] and the development of nuclear weapons has caused a spike in studies on the effect of radiation on living tissue. That work increased our understanding of the concept of radiation risk and created new fields of scientific study; for example, radiation health safety, radiation dosimetry and radiation oncology.

Because of the high energy of α -particles and stochastic nature of ionization radiation, their effects may be observed on all levels of a biological system. Any molecule, cell, tissue or organ can display α -decay radiation damage, and such damage can be localized, or occur throughout the entire body of any multicellular organism [3].

The first step in producing radiation effects is the generation of a primary recoil atom and α -particles by a radioactive decay. Such events take place very rapidly in much less than 1 fs [4]. In the case of α -decay of radiopharmaceutical isotopes of interest, the average kinetic energy per recoil atom is ~100 keV, and the average kinetic energy deposited within the range of a single α -particle is 5 MeV. It is clear that the relatively high levels of energy deposited by the combination of the fast-moving heavy ions and high energy α -particles can cause large amounts of damage to solid matter. There are many methods to estimate the effect of radiation in solid materials; e.g., the stopping theory based on coulombic interactions, molecular dynamics and transport theory. However, unlike solid materials, biological tissues do not consist of solid crystalline structures, and this significantly increases the complexity of estimating the effects of α -decay (as well as other ionization radiation), making the application of the aforementioned methods impossible or extremely difficult numerically. To overcome such limitations, a semi-quantitative approach has been applied. First, the biological effects observed in irradiated subjects were separated into one of two categories [3,6]: deterministic effects, which have

a practical threshold absorption dose below which effects are negligible or not evident; and stochastic effects, where the relationship between dosage and severity of effect is either less evident or absent.

Maintaining the integrity of many different types of macromolecular structures is important to cell viability and all cellular organic molecules are subject to damage by ionizing radiation. However, the genomic DNA molecules are considered to be the most critical targets for the biological effects of ionizing radiation because intact DNA is required for cellular replication and damaged but repaired DNA can result in the fixation of genetic mutations that can affect normal cellular function and viability [18]. Ionizing radiation interacts with DNA either by directly transferring energy to the biological material or indirectly by creating reactive free radicals from the radiolysis of water. These interactions result in damage to the DNA's structure via broken covalent bonds. Linear energy transfer (LET) is an approach to describe the spatial distribution of ionization and excitation produced by direct or indirect effects of different types of radiation along a linear path [15]. Alpha (α) particles have high LET radiation because they create dense ionizations and excitations in matter due to coulombic interactions with atoms. Being a heavy charged particle, an α -particle will continuously slow down along its track with minimal deflection. Through the process of slowing down, the interaction cross-section towards the end of travel increases, resulting in increased LET, which is known as the Bragg Peak (Figure 2) [19,20].

Relative biological effectiveness (RBE) is the ratio of the dose of a reference radiation and the dose of a test radiation that produces the same biological effect. Some of the most common biological effect measurements are necrotic and apoptotic (programmed) cell death, DNA damage, chromosomal aberrations and genetic mutations. The RBE of α -particles can range from 3.5 to 4 for cell killing or 6 to 12 for mutation, and be up to 10 for cell transformation [15]. As a comparison, the RBE for low LET electrons and photons is 1.

An important biological endpoint is cell killing. Damage to cells can be classified as either sub-lethal events or lethal events. Sub-lethal events are due to the accumulation of damage that has the potential to be repaired, typically as a result of exposure to lower doses, and lethal events typically result from irreparable damage due to exposure to higher doses [3]. The ability to repair these events is seen as a shoulder on the cell survival curve and is characteristic of low LET radiation (Figure 3) [21]. A single event of high LET radiation can be lethal. The cell survival curve for a lethal event does not have a shoulder, indicating the inability to repair [22].

The DNA double-strand break is the most biologically significant type of damage, which occurs as a result of two single strand breaks in close proximity or a rupture of the double-strand at the site of interaction [23]. Cell survival is highly dependent on the spatial distribution of double-strand breaks [15]. Given the same dose, high LET radiation can create up to four times more double-strand breaks of low LET radiation. Additionally, the formation of high LET double-strand breaks are more complex compared to low LET in that they are less randomly distributed and form clustered DNA damage to multiple base-pairs [19]. It has been widely accepted that high oxygen levels play a large role in a cell's sensitivity to ionizing radiation, and hence, tumor hypoxia is an established factor in resistance to radiation therapy [24]. This is due to enhancement of free radical production by the presence of oxygen. Free radical production occurs as a result of indirect action, or low LET interactions. Since α -particles interact directly with the DNA, the level of oxygen becomes irrelevant; hence, eliminating a major mechanism of resistance to therapy [3].

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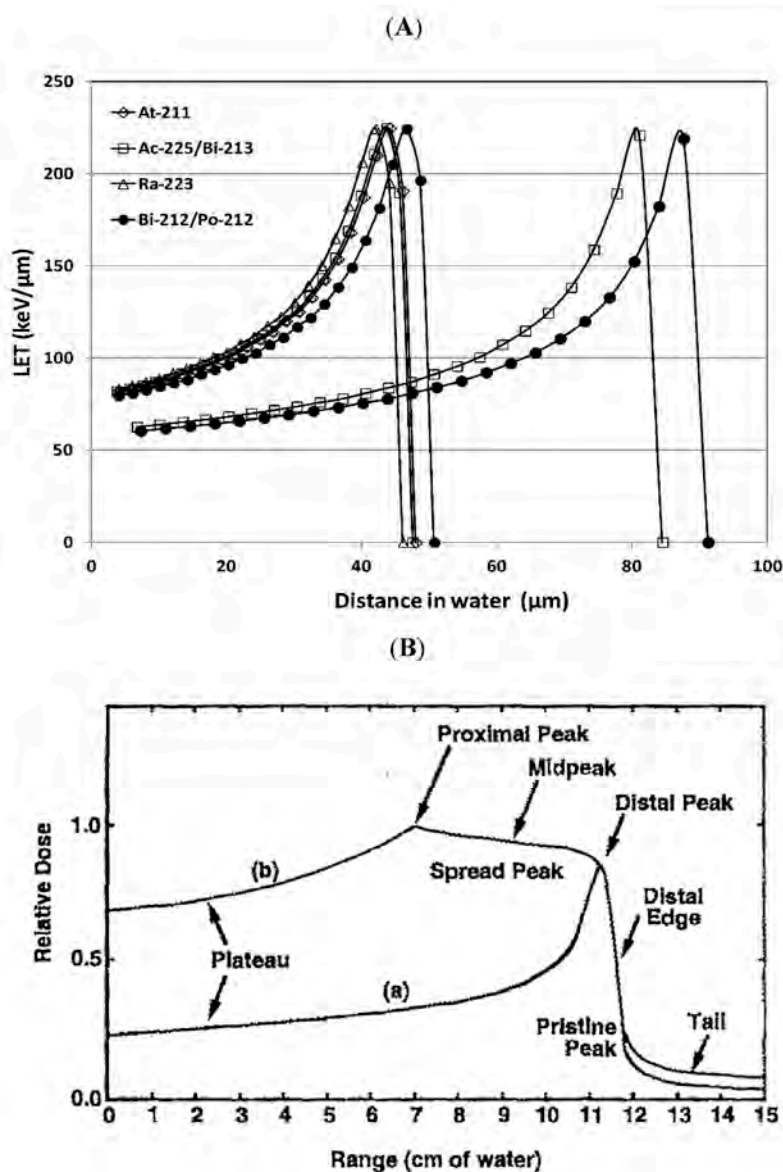


Figure 2. (A) Linear energy transfer (LET) versus distance in water traveled by typical α -particles emitted by radionuclides in development for α -particle radioimmunotherapy: ^{225}Ac (5.829 MeV)/ ^{213}Bi (8.375 MeV), ^{211}At (5.867 MeV), ^{212}Bi (6.08 MeV)/ ^{212}Po (8.78 MeV), ^{223}Ra (5.716 MeV). The range of the α -particle and the position of the Bragg peaks are correlated with the initial energy of the α -particles. LET of α -particles in water was calculated using stopping-power and range tables (continuous slowing down approximation range) for electrons, protons and helium ions from the National Institute of Standards and Technology (NIST). (B) The deposition of heavy ion energy as a function of penetrating depth of (a) a pristine beam and (b) a modulated beam with widened stopping region (spread out Bragg peaks). This figure and legend were reproduced from [19].

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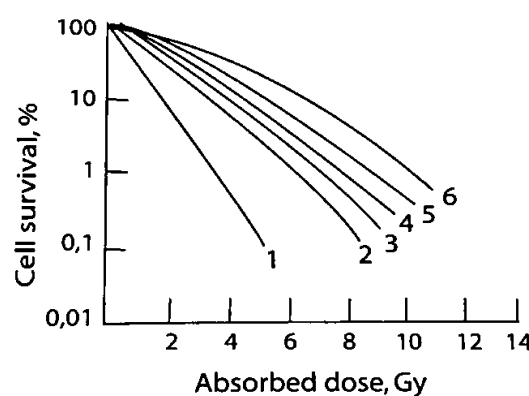


Figure 3. Survival of a human kidney T-cell culture irradiated with ionizing particles of different kinds: (1) particles with $E = 2.5$ MeV, $LET = 165$ keV/m; (2) particles with $E = 27$ MeV, $LET = 25$ keV/m; (3) deuterons with $E = 3.0$ MeV, $LET = 20$ keV/m; (4) X-rays with $E = 20$ keV and $LET = 6$ keV/m; (5) X-rays with $E = 250$ keV and $LET = 2.5$ keV/m; and (6) particles with $E = 2.2$ MeV, $LET = 0.3$ keV/m. This figure and legend were reproduced from [21].

After exposure to radiation that results in DNA damage, the cell cycle can be stopped at cell-cycle checkpoints which allow the cell to repair the damage via multiple repair mechanisms in order to preserve genomic integrity [23]. The DNA double-strand break is the most biologically significant type of damage, which occurs as a result of two single strand breaks in close proximity or a rupture of the double-strand at the site of interaction [23]. Cell survival is highly dependent on the spatial distribution of double-strand breaks [15]. Given the same dose, high LET radiation can create up to four times more double-strand breaks of low LET radiation. Additionally, the formation of high LET double-strand breaks are more in the late S and G2 phases of DNA synthesis where an intact DNA template is available, resulting in more efficient and higher-fidelity repair. Non-homologous end joining occurs throughout the cell cycle but is the only means of repair in G1 and early S phases. In this error prone repair method, DNA ends are rejoined with no sister templates [22]. In this case, chromosomal aberrations can occur as a result of recombining incorrect DNA ends; i.e., combining a loose end to some other molecule, presence of oxygen. Free radical production occurs as a result of indirect action, or low LET and the truncation of ends. If incorrect repair occurs prior to DNA replication, these errors can be replicated in daughter DNA which can lead to mitotic cell death or can lead to the generation of genomic mutations without cell death [22]. There is also the situation where double-strand breaks are not repaired and the dividing cell enters mitosis, leading to mitotic catastrophe and eventual cell death. A higher proportion of double-strand breaks remain un-rejoined after exposure to high LET radiation [15]. When it comes to damage from high LET α -particles in close proximity to the cells being irradiated, the main radiobiological effect is complex and irreparable DNA damage resulting in cell death by either apoptosis or necrosis.

In the last few years, successful attempts have been made to explain the bystander effect [24,25]. The bystander effect is defined as a group of effects that are observed in cells that have not been directly irradiated following the irradiation of other nearby cells. Two mechanisms were proposed. One is the transfer of genomic instability through p53-mediated pathways, and the other suggests that irradiated cells secrete cytokines or other factors that transit to other cells that are not irradiated and signal for increased levels of intracellular reactive oxygen species [26]. One of the defined sub-classes of the bystander effect is the “abscopal effect,” in which radiation treatment of a tumor propagates to tumors outside the irradiated volume [26]. A more recent publication demonstrated an effect that might explain the abscopal effect [27]. It was demonstrated that α -particle treatment of prostate cancer cells generated an adaptive antitumoral immune response, as has been previously reported for other forms of radiation. Combinations of bystander effects and the abscopal (likely immune) response in vivo are potential mechanisms of the efficacy for tumors that are not vulnerable to the targeted α -emitter radiotherapy in a patient with heterogeneous target expression.

3. Alpha-Particle Emitting Radionuclides

Using radiation as a method of cancer therapy requires delivering the maximum dose to the tumor while minimizing the dose to healthy tissues. Targeted radionuclide therapy is advantageous in that it seeks molecular and functional targets within patient tumor sites [28]. Beta (β)-emitting radionuclides (^{90}Y , ^{131}I , ^{177}Lu and ^{186}Re) are used for cancer-targeted therapy but have problems with cross-fire irradiation of normal tissues due to their relatively long range in tissue, which is 0.5–12 mm. In contrast, α -particles deposit higher energy over a much shorter range (40–90 μm), potentially causing higher cytotoxicity to tumor cells while delivering a lower dose to normal, adjacent tissues [29]. Alpha (α)-emission is the process by which an unstable nucleus ejects a highly-energetic, heavy, charged particle consisting of two protons and two neutrons. Alpha (α)-particles have a higher LET (100 keV/ μm) compared to β -particles (0.2 keV/ μm) which results in a dense ionization track in matter. The short range of α -emission provides specificity to the target cell population with a minimal effect on surrounding normal cells, and the high LET leads to a high frequency of irreparable DNA double-strand breaks [18]. This limits cytotoxic effects to within a small distance from the location of decay. It has been estimated that only one cell traversal by an α -particle track is necessary to kill a cell while, thousands of β -particle traversals are required for the same effect [30]. Because of the long range of β -particles and the need of a high number of hits for cell killing, a large portion of the dose deposited is outside of the intended target. In addition to treatment of solid tumors, the use of α -particles for targeted treatment of circulating disease could be an improvement due to the potentially reduced damage to normal tissues. Use of TAT has been considered for killing isolated cancer cells in transit in the vascular and lymphatic systems, in regressing tumors by disruption of tumor capillary networks and in treatment of micrometastatic foci [31]. In particular, TAT may be ideal for treatment of solid metastases, as the short range will primarily kill tumor cells, with little deleterious impact on surrounding normal tissue. Additionally, heterogeneity of target expression has been observed within a given tumor or metastasis [32] and this is thought to be a mechanism of developing resistance to targeted therapies; non-target expressing cells survive treatment and clonally expand into a resistant population. In this case, α or β -emissions from a targeted cell will serve to kill surrounding untargeted cells within the effective range, potentially reducing the development of resistance.

A number of factors need to be considered when choosing an α -emitting radionuclide for therapy. These include proper nuclear characteristics, ease of radiochemical incorporation, specific activity, synthesis yields, chemical and biological stability, availability and cost [29].

The physical half-life of the radionuclide should be long enough to allow for radiosynthesis preparation and be compatible with the pharmacokinetics of tumor localization [33]. The decay pathway of the α -emitter should be carefully analyzed. Due to the conservation of energy and linear momentum, a daughter nuclide which subsequently decays by α -emission could detach from the radioimmunoconjugate; see for example [34]. These free products could travel away and deposit doses to healthy tissues. A decay chain that is long and complicated, having many different decay types, could also present an issue dosimetrically, especially if the daughter products are metabolized differently than the parent. A possible way to overcome this issue is to use ^{225}Ac as an *in vivo* generator in which the delivery system is designed to be internalized into the target cell where the toxic daughter elements may detach from the targeting vector but remain trapped in the cell [35].

Another important nuclear characteristic is having a large number of α -emissions per decay. The radiotoxicity associated with having multiple emissions could be high enough to kill a tumor cell in a single decay. An accompanying gamma (γ) photon emission with energy suitable for *in vivo* imaging is also beneficial for assisting with pharmacokinetic and dosimetric evaluations [33].

Another important element for radionuclide selection is availability. Alpha (α)-emitters are produced either by cyclotron bombardment or by reactor irradiations, are incorporated into a generator and are eluted from a parental source. Obtaining radionuclides in pure form with high specific activity and large quantities is essential for adequate therapeutic evaluation. High specific activity is important to avoid receptor saturation by the unlabeled targeting agent [36]. If membrane antigenic density is

low, insufficient binding to tumor cells will occur [36]. Transportation of these radionuclides safely and economically is also a key issue in selection.

While there are over 100 α -emitting radionuclides, only several have been investigated in preclinical and clinical studies. This is mostly due to radionuclides lacking nuclear properties, the absence of viable chemistry, complicated decay chains and production and economic issues [37]. Therefore, radionuclides meeting the criteria for therapeutic use have been limited to ^{225}Ac , ^{211}At , ^{212}Bi , ^{213}Bi , ^{212}Pb , ^{223}Ra , ^{224}Ra , ^{149}Tb and ^{227}Th . The physical characteristics of these isotopes can be seen in Table 1.

Table 1. Physical characteristics of α -emitting radioisotopes investigated for clinical use.

Isotope	Half-Life	Max Energy	Emissions Per Decay
^{225}Ac	10.1 d	5.83	4 α , 2 β -
^{211}At	7.2 h	5.87	1 α , 1 EC
^{212}Bi	1.01 h	6.09	1 α , 1 β -
^{213}Bi	45.6 min	5.87	1 α , 2 β -
^{212}Pb	10.6 h	6.09	1 α , 2 β -
^{223}Ra	11.4 d	5.87	4 α , 2 β -
^{224}Ra	3.6 d	8.8	5 α , 2 β -
^{149}Tb	4.1 h	3.96	1 α , 1 β +
^{227}Th	18.7 d	6.04	5 α , 2 β -

The first α -emitter to be used in human clinical trials for therapy was ^{213}Bi in 1997, when it was labeled to the anti-leukemia antibody HuM195 [38]. ^{213}Bi is available through generator based ^{225}Ac and decays with a 45.6 min half-life by emission of one α (8.37 MeV) and two β -particles. The generator is produced at Oak Ridge National Laboratory in the US and at the Institute for Transuranium Elements in Karlsruhe Europe. In the decay of ^{213}Bi , there is an emission of a 440 keV isomeric γ , which is beneficial for imaging studies.

^{211}At decays with a half-life of 7.2 h and emits two α -particles through a split decay pathway with energies of 5.87 and 7.45 MeV. One path is to ^{207}Bi by α -emission followed by electron capture to ^{207}Pb and the other is by electron capture to ^{211}Po followed by α -emission to ^{207}Pb . An advantage of this decay path is that ^{211}Po emits 77–92 keV characteristic X-rays which can be used for imaging [37]. The main disadvantages are availability and purity. Conventionally, the production of ^{211}At requires α -particle cyclotrons, which are only available in a few places worldwide, to produce the $^{209}\text{Bi}(\alpha, 2n)^{211}\text{At}$ reaction with minimal ^{210}At contamination [39]. Astatine has significant metallic characteristics that lead to complications in standard antibody labeling and results in a rapid release of free ^{211}At [37,40,41]. To resolve this problem, approaches have been developed by several research groups based on small linker molecules that create an aryl carbon–astatine bond involving an astatodemetalation reaction using tin, silicon or mercury precursors [42]. Other alternative methods for astatination, e.g., boron–astatine, rhodium–astatine or nanoparticle encapsulation, are also being pursued [43].

^{225}Ac is a radiometal with a half-life of 10.1 days and produces six radionuclide daughters in the decay path to stable ^{209}Bi . For each decay event of ^{225}Ac , there are successively, four α and two β emissions with high energy (α 8.38 MeV, β 1.42 MeV). In the decay of ^{225}Ac and its daughters there are several isomeric γ emissions with energy suitable for imaging studies. The relatively long half-life allows for a centralized production site that can ship ^{225}Ac to users [44]. The main method for generating ^{225}Ac for clinical studies is through the decay of ^{229}Th which originates from ^{233}U . In the world there are three main sources of ^{229}Th : Oak Ridge National Laboratory (USA), The Institute of Physics and Power Engineering (Russia) and The Institute for Transuranium Elements (Germany). The quantities produced are not enough for a global application of ^{225}Ac . To keep up with the increasing demand for ^{225}Ac for clinical applications, it has been found that large scale quantities can be produced through high-energy proton irradiation of ^{232}Th [45,46]. To address the shortage, the US Department of Energy formed a Tri-lab collaboration of Los Alamos (LANL), Brookhaven (BNL) and Oak Ridge (ORNL) National Laboratories with the goal of developing an alternative route for production of Ac [47].

Another limitation for this radionuclide has been with the radiochemical stability of the attachment to immunoconjugate. McDevitt et al. [34], state that the instability of these attachments is due to the high classical recoil energy (100–200 keV) of the daughter product which breaks the molecular bonds of the chelator. Significant advances have been made in developing chelators that form thermodynamically stable and kinetically inert complexes with ^{225}Ac . Khabibullin et al., recently calculated the chelation stability of ^{225}Ac and daughters in the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelator [48].

As one potentially abundant starting material, ^{212}Bi (1.01 h half-life) can be obtained from ^{228}Th and decays via a branched pathway to ^{208}Tl (36% α) and ^{212}Po (64% β); then, both decay to stable ^{208}Pb [33]. However, ^{212}Bi has several disadvantages that potentially limit its use. First, its short half-life can be problematic if the production and shipping processes are lengthy. This issue can be solved by using ^{224}Ra as a generator to locally produce ^{212}Bi . Another complication is the high energy γ emission (2.6 MeV) which requires a considerable amount of shielding to minimize exposure. This, along with the short half-life, makes shipping difficult, resulting in an availability problem.

^{223}Ra is found naturally in trace amounts following the decay of ^{235}U , but it is mainly produced artificially by the decay of ^{227}Th ($T_{1/2} = 18.7$ days), which is produced from ^{227}Ac ($T_{1/2} = 21.77$ years). Since ^{227}Ac is found only in traces in uranium and thorium ores, it is mainly synthesized by ^{226}Ra ($T_{1/2} = 1600$ years) irradiation in a nuclear reactor [49]. ^{223}Ra has a half-life of 11.4 days and emits four α -particles, two β -particles and γ rays on the path to stable ^{207}Pb [34]. While the emission of four α -particles is advantageous for tumor toxicity, ^{219}Rn in gaseous form is also emitted, which can redistribute in the body and dose non-targeted cells. See Figure 3 for comparable energies of α -particles.

The major challenge using ^{223}Ra is finding a suitable ligand for in vivo sequestration. However, similarly to cations of the alkaline earth elements, radium has natural bone-seeking properties without the need for a carrier agent. As radium mimics calcium, when ^{223}Ra dichloride (Xofigo®) is injected intravenously, it forms complexes with the bone mineral hydroxyapatite at areas of increased metabolic bone activity, such as bone metastases, thereby exerting a highly localized antitumoral effect [7]. In addition, the ^{223}Ra daughter isotopes are also retained in the bone matrix [50].

Thanks to this characteristic, ^{223}Ra dichloride ($^{223}\text{RaCl}_2$) has demonstrated improvement in overall survival for the treatment of bone metastases in castration-resistant prostate cancer and has received FDA approval for this application [34], making it the first FDA approved TAT.

^{227}Th can be produced continuously from ^{227}Ac and decays with a half-life of 18.7 days to ^{223}Ra [51]. The long half-life is beneficial for radiolabeling and targeting. ^{227}Th also decays with accompanying γ emissions of 236 keV and 50 keV, which can be used for imaging but are low enough to avoid the need for patient shielding.

^{212}Pb has a 10.6 h half-life and is produced either from the decay chain of ^{228}Th or by the ^{224}Ra generator [39]. Because of its long half-life and the fact that ^{212}Pb is a β -emitter that decays to ^{212}Bi , one approach has been to use this radionuclide as an in vivo generator to compensate for the short half-life of ^{212}Bi [52]. ^{212}Pb can deliver over ten times the dose per unit of administered activity compared to ^{212}Bi or ^{213}Bi [53]. The major issue with ^{212}Pb is the electron capture and auger electron emissions which can cause significant recoil of the ^{212}Bi daughter [54]. The free ^{212}Pb has been shown to cause severe bone marrow toxicity [55], while the free Bi has shown to cause kidney toxicity. By co-injecting DTPA or EDTA chelating agents, rapid release of free ^{212}Bi can be achieved [56].

4. Targeting Moieties

In the burgeoning field of targeted radiopharmaceuticals, there is no universal answer to the question: what is the best targeting molecule? Rather, the abundance of target molecule classifications allow for a more customized approach to developing TATs. Globally, the requirements of a targeting ligand include the ability to concentrate at and bind to extracellular targets and the availability of chemical functional groups amenable to the attachment of linkers and chelators. A balance of many other factors like off-target binding, biodistribution and pharmacokinetics (particularly with

respect to the decaying half-life of the chosen radionuclide) are all critical factors for the selection of a proper targeting ligand. Recently, small molecules, peptides, antibodies, antibody fragments and even some passive targeting strategies have been investigated to deliver radioisotope payloads. See Tables 2 and 3 for examples of TAT conjugates that have been studied in the pre-clinical and clinical environments. This section will explore the advantages and challenges of the aforementioned targeting ligand categories.

4.1. Small Molecules

An advantage of using low molecular weight ligands as targeting moieties for α -therapy include rapid penetration into the tumor and rapid clearance of unbound conjugates from circulation; thus, reducing toxicity. Internalizing ligands are particularly beneficial for ^{225}Ac 's application in order to harness the multiple α -particles emitted in its decay chain. A successful example of using small molecules in TAT is ^{225}Ac -PSMA-617 for the therapy of metastatic, castration-resistant prostate cancer [57,58]. The favorable pharmacokinetics properties of the PSMA-617 small molecule include its fast tumor uptake, high internalization rate, extended tumor retention and rapid clearance of unbound ligand [58]. These features make the molecule highly desirable for labeling with an α -emitter with a half-life of several days and multiple α -emissions in its decay chain, like ^{225}Ac . The first report of the significant therapeutic efficacy of ^{225}Ac -PSMA-617 involved two patients with late-stage, metastatic, castration-resistant prostate cancer (mCRPC) and complete remissions [59]. Additional clinical studies have further revealed the remarkable anti-tumor activity and the promising duration of tumor-control of treatment with ^{225}Ac -PSMA-617 [6,59,60]. These studies have further demonstrated the significant potential of TAT (Table 3, Figure 1). Xerostomia (dry mouth syndrome) was the main side effect, indicating that further modifications in clinical trial design might be necessary to further enhance the therapeutic range.

4.2. Peptides

Peptides are oligomers of amino acids that may sometimes exhibit secondary structure; they may have branched or linear frameworks, and may be composed of varying amounts of non-canonical monomers. The polypeptide chains of peptides can have anywhere from 2 to 70 amino acids but more typical examples of targeting peptides are made of less than 10–15 amino acids (1000–1500 MW). Owing to this molecular weight and their capacity to be synthesized and modified with conventional organic synthesis techniques, peptides have long been utilized as targeting agents for radionuclide therapies and diagnostic applications.

Since the 1980s, analogs of the endogenous peptide hormone somatostatin have been developed as therapeutics for neuroendocrine disorders [61]. The FDA approved octreotide (OC, Figure 4), the cyclic octapeptide, upon which much of the early peptide receptor radionuclide therapy (PRRT) was based. Chelating molecules attached and various radionuclides for β and α -emission therapies have been reported. Figure 4 details the structure of OC and two of its commonly-used PRRT ligand analogs, DOTATOC and DOTATATE.

Nephrotoxicity is often one of the highest concerns in PRRT due to tubular reabsorption of the labeled peptide after glomerular filtration [62]. Seventy-four human patients were followed (>1 year) after PRRT with the β -emitter ^{177}Lu -Octreotate. Renal function was monitored by accessing glomerular filtration rate (GFR). Somewhat surprisingly, renal impairment was minimal, with only 43% of patients experiencing a decrease in GFR (<2 mL/min/m² per year) and 11 patients actually saw increased GFR (>10 mL/min/m² per year) [63]. The first published account of the α -emitter ^{213}Bi in an OC analog (DOTATOC, Figure 4) was reported in 2006. The radio-peptide ligand was shown to retain its affinity for the somatostatin receptor and inhibited tumor growth in a somatostatin receptor-positive rat pancreatic tumor model (CA20948) [64]. Importantly, the study followed major organ toxicities in rodents. Little to no nephrotoxicity was observed at the various administered activities (13–22.2 Mbq).

More recently, even tumors that had become radioresistant to β -therapy (^{90}Y and ^{177}Lu -DOTATOC) responded to ^{213}Bi -DOTATOC in a clinical model of eight human patients [65]. The treatment for each patient was individualized according to each particular disease state and each patient had substantially positive outcomes in terms of tumor regression and survival. Critically, the acute hematological toxicity normally associated with the analogous β -therapies was only moderate. This limited report suggested only a mild reduction in acute renal function. No other major acute toxicities were reported. This first-in-human report of this peptide-targeted alpha therapeutic may lay the ground work for future human TAT using peptide-targeted systems.

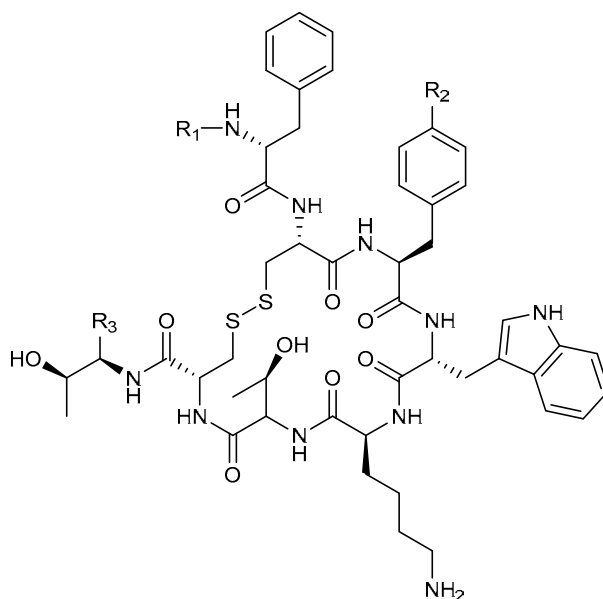


Figure 4. The somatostatin mimetic octreotide and its DOTMA-containing analogs. Octreotide: $R_1 = \text{H}$, $R_2 = \text{H}$ and $R_3 = \text{CH}_2\text{OH}$; DOTATOC (endotreotide): $R_1 = \text{DOTMA}$, $R_2 = \text{OH}$ and $R_3 = \text{CO}_2\text{H}$; DOTATATE (Octreotate): $R_1 = \text{DOTMA}$, $R_2 = \text{OH}$ and $R_3 = \text{CH}_2\text{OH}$.

It has been the common supposition that TAT would work well for small, disseminated tumors but not as well for large, solid tumors. Recently, DOTATATE (Figure 4) was labeled with ^{213}Bi and used to investigate efficacy differences in tumor size among two tumor models, rat pancreatic (CA20948) and human small cell carcinoma (H69) with each containing a small (50 mm^3) and large (200 mm^3) tumor group [66]. Both tumor lines maintained high expression of SSTR₂ and all tumor groups saw delayed tumor growth and higher survival over controls. Three mice from the smaller tumor groups were effectively put into remission through the end of the study. This report has implications for TAT in regard to tumor receptor heterogeneity, tumor size and perfusion. More recently, α -emitting radionuclides have been conjugated to melanocortin-1 receptor targeting peptides and efficacy determined in the pre-clinical treatment of mouse xenograft models of cutaneous and uveal melanomas [67–69].

4.3. Antibodies
Full length immunoglobulins (IgGs) are typically in the 150 kDa molecular weight range, and have been proven to have high binding affinity and specificity to a broad range of extracellular receptors. Developments in hybridoma cell line technology have opened the door to the production of monoclonal antibodies (mAbs) which can be labeled with chelating molecules, to which radionuclides can be added. This approach to specifically deliver ionizing radiation payloads is termed radioimmunotherapy (RIT). While many of the examples here [67–69] involve radioactive payloads directly conjugated to the protein, antibodies have also been employed to target macromolecular payloads, such as nanoparticles and liposomes to cellular targets [70–72].

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Molecular research group based in the Memorial Sloan-Kettering Cancer Center (MSKCC) has reported several accounts of their work labeling trastuzumab with actinium 225. This mAb is the well-known anti-HER2/ERBB2 targeting agent. Initially, they showed through a three-cell line sphere-in-vitro model that the RITx scheme could prevent the growth and growth dependent of growth factors and dose dependant [70]. While a [70] While a paper, this work the importance of important expression and also suggested challenges of RITx of targeting agent of targeting agents in normal tissues lead to toxicity of related free decaying daughter product [M3K76]. Scheinberg group's work eventually led to the first clinical trial of an actinium 225 chelated targeting antibody (IgG) primed by eventually led to the first clinical trial of an actinium 225 chelated targeting antibody (ClinicalTrials.gov Identifier: NCT00672165). This RITx scheme utilized the subsequent explant of humanized anti-CD33 antibody of Alemtuzumab to target acute myeloid leukemia cells. Subsequently, a preliminary study in both preclinical and clinical pipeline pharmaceuticals, Inc. And in portfolio of targeted alpha conjugated isotopes. One in both preclinical and clinical pipelines cage stracture (B10, Figure 5) been utilized as CP-BT antibody in 2013 in Gracemore coupled a leucocyte antigen structure (B10, Figure 5) with a AAs reported CD45 epitope using anti-CP20 target conjugated with leukemia with a similar probably Green and [99]. Others reported an example using anti-residual disease conjugated with a AAs with a similar animal labeling scheme [71]. This study sought to 70% eradication of the MGD in animals bearing disseminated melanoma even animal model. Their lymph node results showed 70% eradication of the MGD in animals bearing a disseminated model. However, the subcutaneous lymphoma xenograft group treated with the same agent perfusion saw prolonged survival (two to three-fold) without any cured animals. This study is clear evidence of the

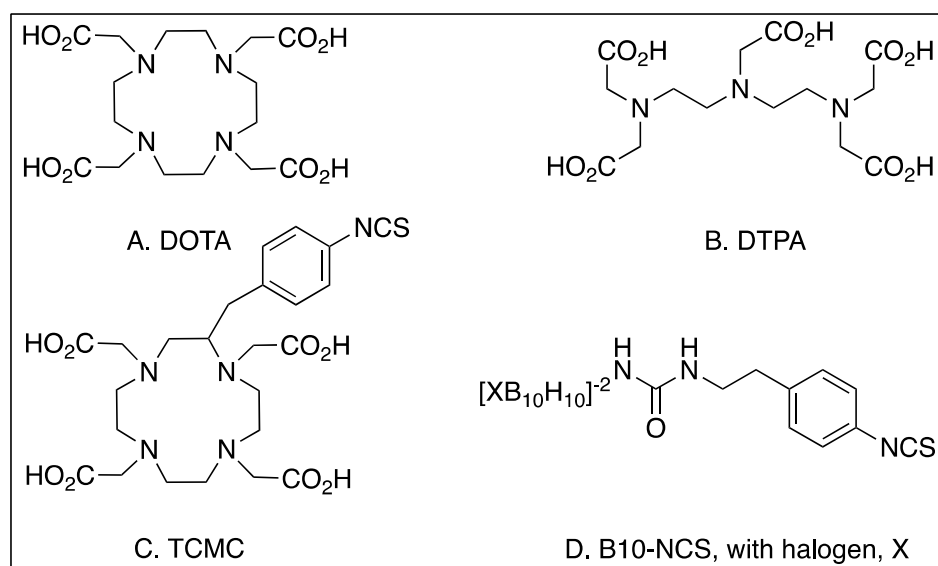


Figure 5. Common metal chelators and binders used to attach radionuclides to targeting ligands.

4.4. Antibody Fragments

4.4. Antibody Fragments
A limitation of full-size IgGs is their typical 1–3 week serum stability. While this kind of circulation may be advantageous for certain therapeutic applications, for radioimmunotherapy (RIT) it presents a serious liability: Excess antibody can continue to circulate, lowering the tumor-to-nontumor (T/N) ratio, particularly with respect to the tumor-to-blood (T/B) ratio. Finally, high levels of continuously-circulating α -emitters result in hematological toxicities, as well as extravasation in normal tissues. To shorten the long plasma half-life of full-length antibodies, many groups have sought

In attempts to shorten the long plasma half-life of full-length antibodies, many groups have sought to reduce the sizes of the mAbs while retaining their remarkable binding characteristics. To those ends, antibodies have been cleaved into smaller sections through enzymatic digestion and also engineered to those ends, antibodies have been cleaved into smaller sections through enzymatic digestion and also engineered ab initio [78,79]. For comparison, the engineered antibody fragments of diabodies (Db) and minibodies (Mb) have typical circulating half-lives of 2-5 and 5-12 h, respectively, while the circulating half-life of full-length IgGs is more typically 1-3 weeks. Monovalent and bivalent

ab initio [78,79]. For comparison, the engineered antibody fragments of diabodies (Db) and minibodies (Mb) have typical circulating half-lives of 2–5 and 5–12 h, respectively, while the circulating half-life of full-length IgGs is more typically 1–3 weeks. Monovalent and bivalent affibodies (7 and 15 kDa respectively) have been developed with targeting specificity to *HER-2* and they have plasma half-lives of roughly 45 min [80]. The science of antibodies is ever progressing and new classes are still being observed in nature; nanobodies (13–14 kDa) are heavy chain-only antibodies, that have been isolated from *Camelidae* and have lately been conjugated with theranostic radionuclides [81]. These new categories of targeting constructs are adding to the growing body of evidence that there may be a so-called ‘Goldilocks’ zone in terms of the size of targeting constructs.

4.5. Passive Targeting

Another strategy often employed for TAT is based on the natural selective uptake and accumulation of certain elements. For instance, the actively restructuring environment of osteoblastic bone metastases is conducive to calcium accumulation in the form of hydroxyapatite. Due to the resemblance of radium-223 to calcium, it can act as a surrogate and be incorporated into the rapidly-growing matrix [7,29]. Indeed, the recent approval of ^{223}Ra dichloride therapies for metastatic castrate-resistant prostate cancer has been followed in patients for over twelve months with moderately successful outcomes [82]. Other studies have looked to ^{223}Ra dichloride for the treatment of the bone metastases from hormone-refractory breast cancer [83,84].

The enhanced permeability and retention (EPR) effect is often regarded as a rationale for the passive targeting of large macromolecules like nanoparticles and liposomes. This accumulation of macromolecules through the leaky vasculature of quickly-dividing tumor cells also provides the opportunity to incorporate multiple radionuclides into the framework of a targeting molecule. Additionally, it has been proposed that nanoparticles or liposomes could be used to sequester decaying daughter products that would otherwise be released to freely circulate in the blood because of damage to molecular bonds due to α -decay or metabolism of the radiopharmaceutical [85,86].

5. Chelation/Attachment

5.1. Radiosynthesis

The recent approval of $^{223}\text{RaCl}_2$ has reinvigorated research into TAT development [87]. Several α -particle-emitting radionuclides have been described in the literature, and these include ^{211}At , $^{212/213}\text{Bi}$, ^{212}Pb and ^{225}Ac [44,88,89]. With the exception of ^{211}At [41,42,90], all are radiometals that require a bifunctional chelator for the attachment of the therapeutic radioisotope to the targeting moiety. The chemistries of $^{212/213}\text{Bi}$ and ^{212}Pb have been covered extensively elsewhere [91–94]. The radiochemistry of radium has recently been reviewed [95]. This section will cover recent advances in ^{225}Ac chelation chemistry that have occurred since the publication of the excellent review by Scheinberg and McDevitt in 2011 [96].

^{225}Ac , whose decay chain was not finally resolved until the middle of the 20th century is one of 29 radioactive isotopes of actinium, which is the first element of the actinide series [97]. Although the lack of non-radioactive actinium isotopes has hindered the exploration of ^{225}Ac chemistry, the pioneering work of Seaborg and others has provided an important understanding of the basic reaction chemistry of this element [97–99]. Actinium chemistry has been observed to closely follow that of lanthanum chemistry, since both exist as trivalent ions in solution. These observations have led to the belief that La^{3+} can be an important non-radioactive surrogate for Ac^{3+} , with the caveat that the differences in ionic radii must be considered carefully [100].

Despite the relatively limited knowledge of actinium chemistry, the medical community has seized upon its therapeutic potential in TAT. Using lanthanide chemistry as a guide, many attempts to attach this radioisotope to targeting molecules have been reported with a variety of acyclic and cyclic ligands that would effectively chelate this radioisotope [101–106]. To date, however,

only 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) has found widespread acceptance as an ^{225}Ac chelator in both preclinical and clinical applications [96], despite the low radiochemical yields and specific activities that have been reported [73,104,107]. Maguire et al. have described an improved, one-step ^{225}Ac radiolabeling procedure of monoclonal antibodies that resulted in a 10-fold increase in radiolabeling yield and a 30-fold increase in specific activity [108]. The authors concluded that the improvements in yield and specific activity resulted from greater control over the DOTA-mAb conjugation reaction and the improved chelation of ^{225}Ac during radiosynthesis. Additionally, the new procedure did not alter antibody activity or therapeutic efficacy in vivo, which further suggests the usefulness of this strategy. More recently, Poty et al. reported a single-step click chemistry approach to ^{225}Ac -radioimmunoconjugate synthesis [109]. The click approach generated superior radiochemical yields compared to the standard two-step strategy, suitable specific activities, good in vitro stability and excellent biodistribution profiles. The authors proposed that said novel click strategy could be applied when the targeting vector is unstable under the conditions used for the standard method.

Despite improving the radiochemical yield and specific activity of ^{225}Ac radiopharmaceuticals, important challenges, which include minimizing the detrimental effects of recoil energy and effectively sequestering the cytotoxic daughter products ^{221}Fr and ^{213}Bi after ^{225}Ac decay, remain to be overcome. While several reports have described cellular internalization as an effective method of sequestering ^{225}Ac and its daughter products [35], scientists are increasingly turning to nanotechnology to resolve this issue, and a variety of nanoparticles, including liposomes and carbon-based nanoparticles, have been explored [96,110–115]. Recent reports have demonstrated that LaPO_4 nanoparticles hold promise as safe and effective nanocarriers for ^{225}Ac [71]. In these studies, the authors synthesized LaPO_4 (monazite) nanoparticles doped with ^{225}Ac . These particles were prepared with functionalized surfaces that facilitated the attachment of 201B mAb, which targets murine thrombomodulin on lung epithelium. Upon in vivo injection, targeted particles accumulated rapidly in the lung through specific interactions between the 201B mAb and its antigen. Moreover, retention of ^{225}Ac and half of the ^{221}Fr and ^{213}Bi daughter nuclei within the particles demonstrated the potential for trapping radionuclides after high energy emissions. McLaughlin et al. improved upon this work by examining ^{225}Ac -doped LaPO_4 nanoparticles that were encapsulated by GdPO_4 and Au shells [86]. These particles had an average diameter of 27 nm, and were easily conjugated to the 201b mAb using standard bioconjugation techniques. Biodistribution and small animal single-photon emission computed tomography (SPECT) studies revealed the specific targeting of murine thrombomodulin within the lung epithelium that was reduced upon the administration of non-radioactive 201b mAb. Interestingly, the multi-layered nanoparticle design seemed to increase the retention of ^{225}Ac , ^{221}Fr and ^{213}Bi within the particle, which reduced the radiation dose experienced by the kidney. Approximately four and five-fold reductions were observed at 1 and 24 h post-injection, respectively. These data demonstrate that multi-functional, layered nanoparticles have the potential to deliver and retain ^{225}Ac and its daughter radionuclides at the target site, while minimizing off-target toxicities that can occur from errant daughter products in the ^{225}Ac decay chain.

5.2. Linkers/Rational Design

The most utilized chelating molecules for targeted radionuclide therapies using biological molecules are 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and diethylene triamine pentaacetic acid (DTPA), and their related analogs (Figure 5). Depending on how the compound is linked to the targeting ligand, these organic structures feature 3 or 4 carboxylates that are negatively charged at physiological pH, and along with the lone pair electrons from each of the 3 or 4 nitrogens, they can coordinate metal ions like α and β -emitting radionuclides. Often, these chelating molecules are linked to the targeting molecule by forming a new amide bond between an amine on the targeting molecule and a carboxylate on the DOTA/DTPA. This strategy is particularly advantageous in the case of peptide synthesis since the reaction, characterization and purification of the linker addition can be part of the overall synthesis of the targeting ligand.

When DOTA or DTPA are linked to proteins like antibodies, amide bonds are typically created between a primary amine of surface-exposed lysine residue and an activated carboxylate on the DOTA or DTPA (Figure 6). Another widespread chemistry available to link protein lysines with DOTA or DTPA is through an isothiocyanate which yields a stable thiourea (Figure 6) [53,116]. While these newly formed amide bonds create the same covalent structure as the peptide-synthesized version, there are important distinctions. In the stochastic reaction of any accessible lysine, many different combinations are possible, and there is little to no control over the regioselectivity of the conjugation. It is unlikely, but possible, that targeting ligands could block or hinder the conjugation. It is unlikely, but possible, that targeting ligands could block or hinder the conjugation.

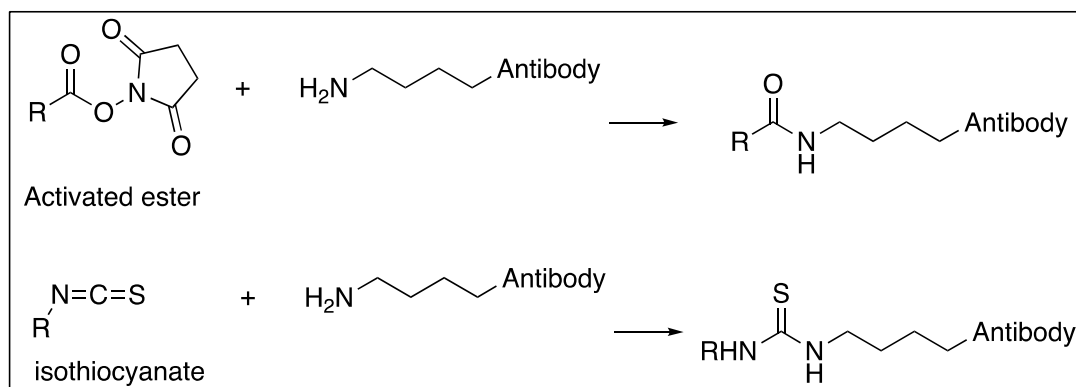


Figure 6. Common conjugation chemistries used to functionalize biomolecules. Countless other schemes have been well characterized and the reader is directed to Bioconjugates Techniques for a review and protocols [117].

The importance of site-specific modifications to biologic targeting motifs is only recently becoming clear. In a 2014 paper from UCLA [118], diabodies (Dbs) were conjugated both site-specifically through reduced cysteines and non-specifically through accessible lysine ϵ -amines to DOTA chelators. While the tumor-to-blood ratio of the specifically labeled protein was moderately higher than the more heterogeneous product, the more striking result was the renal and hepatic distribution. Kidney uptake levels were almost doubled for the cysteine-labeled Db, and liver uptake levels were reduced for the non-specific amine-labeled Dbs.

Other important research regarding linkers has uncovered different methods to reduce kidney toxicity, via the renal reabsorption of radiolabeled peptides and antibody fragments, as they are filtered by the glomerulus. By taking advantage of renal brush border enzymes, the radionuclide can be cleaved from targeting ligand and excreted. By engineering an antibody fragment with a C-terminal lysine, and subsequently modifying the ϵ -amine with DOTA, Indium-111, Li and coworkers demonstrated a 50%–60% reduction in the kidney uptake of the radionuclide [119]. More recently, a Japanese group demonstrated a 50%–60% reduction in the kidney uptake of the radionuclide [119]. More recently, a Japanese group probed the brush border enzymes to understand more about their specificity and they were able to pinpoint a glycine-tyrosine linkage that specifically cleaved a radio-labeled benzoyl derivative from an antibody Fab fragment [120]. Presumably, a similar strategy could be used for the α -emitting halogen, ^{211}At .

Poly(ethylene glycol) (PEG) linkers are made of repeating $-\text{CH}_2\text{CH}_2\text{O}-$ monomers and are commonly employed by chemists to alter biodistribution and pharmacokinetics. PEGs can be prepared in two categories, polymeric and discrete oligomers. Bifunctionalized versions of these polymers can link targeting molecules with the chelating agent or just add bulk/solubility to lower molecular weight entities. Researchers wishing to reduce immunologic response to nanoparticles have been known to decorate their macromolecules with PEG [80], as was the case with McLaughlin and coworkers with PEG12 linker used to link ^{225}Ac containing nanoparticles to mAb [86].

6. Radiation Dosimetry

Dosimetry provides means for evaluating the efficacy of a radiation therapy modality [3]. Because the goal is to deliver high dose to tumor cells with a minimal dose to normal tissues, it is of great importance to quantify accurately where, when and how a dose is being deposited. The Medical Internal Radiation Dose Committee (MIRD) developed an approach to determine the

6. Radiation Dosimetry

Dosimetry provides means for evaluating the efficacy of a radiation therapy modality [3]. Because the goal is to deliver high dose to tumor cells with a minimal dose to normal tissues, it is of great importance to quantify accurately where, when and how a dose is being deposited. The Medical Internal Radiation Dose Committee (MIRD) developed an approach to determine the average absorbed dose from internal radionuclides [121]. The absorbed dose method accounts for low LET radiation where thousands of statistically independent deposition events across a cell are required to induce a biological effect [3]. With respect to an α -particle's interactions, the averaged absorbed dose will fail to characterize biological outcome. Because the α -particle range is only a few cell lengths, some cell nuclei will receive multiple traversals while others will receive zero traversals. Additionally, the location of the α -particle track will determine the energy absorption by the cell. These factors result in large statistical variability of energy deposition and stochastic effects become important. To account for those effects, Roeske developed a microdosimetric methodology based on the mean dose to target cells, probability distribution of specific energy absorbed by target cells, and the fraction of cells receiving zero hits [122]. Kellerer and Chmelevsky described the requirement that microdosimetry must be used when the relative deviation of local dose is greater than 20% [122]. Following this criterion, microdosimetric techniques are more important in analyzing non targeted cells where the local average dose is small, resulting in larger variations than targeted cells.

In most cases, the activity level and mean absorbed doses to the target are large, resulting in a low expected stochastic deviation, and therefore, there is no need to use microdosimetric techniques. The MIRD Pamphlet No. 22: Radiobiology and Dosimetry of Alpha-Particle Emitters for Targeted Radionuclide Therapy recommends using the conventional MIRD formalism [3]. The mean absorbed dose to the target region, r_T , from a source region, r_S , due to a particular emission type, x , over a dose integration period, T_D , is given by:

$$D_x(r_T, T_D) = \tilde{A}(r_S, T_D) \frac{\sum_i \Delta_i^x \varphi(r_T \leftarrow r_S; E_i^x)}{M(r_T)} \quad (1)$$

where $\tilde{A}(r_S, T_D)$ is the total number of nuclear transitions in the target region; Δ_i^x is the mean energy emitted per disintegration for the i -th emission of type x ; $\varphi(r_T \leftarrow r_S; E_i^x)$ is the fraction of energy emitted per nuclear transition in the source region that is absorbed in the target region by the i -th emission of type x that is emitted with initial energy E ; and $M(r_T)$ is the mass of the target region. The total number of nuclear transitions is determined by measuring activity levels in tissue samples at several points in time post administration. The activity levels plotted against time gives a time-activity curve which is integrated to obtain the total number of nuclear transitions. The mean energy emitted per emission Δ_i is a physical property of the radionuclide and can be obtained from nuclear decay tables. The absorbed fraction for each decay type is dependent on the reference phantom geometry and obtained through Monte Carlo calculation. The dose contribution from each emission type is then weighted by its relative biological effectiveness, and then summed:

$$D_{RBE}(r_T, T_D) = RBE_\alpha D_\alpha(r_T, T_D) + RBE_e D_e(r_T, T_D) + RBE_{ph} D_{ph}(r_T, T_D) \quad (2)$$

Several assumptions are made when using this formalism of dose calculation. It is assumed that the activity is uniformly distributed in the organ and that α -energy deposition is also uniformly distributed in the organ. It is also assumed that dose from both α and β -emissions are locally deposited. The calculation of the absorbed fractions is based on idealized phantoms that have standard geometries which cannot account for the individual anatomy of a patient. It is also important to note that radioactive daughters are not taken into account. Therefore, daughter decays must also be calculated, as well as accounting for daughters' biodistribution. The formalism was developed as an adequate

method for the dosimetry of internal radionuclides used for diagnostic purposes and lacks the accuracy needed for therapeutic applications.

As a first method to improve on the original MIRD formalism, phantoms were developed to better match the standard human anatomy. For example, Christy and Eckerman developed phantoms that represented a male, a female and children [123]. Later, as computational power increased, voxelized phantoms like VoxelMan were created based off 3D imaging [124]. MIRD Pamphlet No. 17 describes a method to extend the S value formalism to the voxel level to account for nonuniform distributions of activity [125]. The most extensive software that has adapted the MIRD S factors is OLINDA/EXM, which has calculated an internal dose for over 200 radionuclides, including α -emitters, in 10 different phantoms [126].

The voxel S method is still a model-based approach to dosimetry. There is much less tolerance for inaccuracies in therapeutic applications, which calls upon the need for patient-specific dosimetry. In order to accomplish this, the patient's own anatomy, obtained from CT, is used in combination with SPECT imaging to obtain 3D spatial distribution of activity. It is advantageous for the α -emitting radionuclide to also have γ photon emission as it decays so that SPECT imaging can provide information about regional uptake. The method to calculate a 3D, imaged-based dose is as follows [127]. Serial SPECT studies taken over time post administration of the radiopharmaceutical can determine the pharmacokinetic variations within an organ on the voxel level. The SPECT/CT images are registered together and integrated voxel by voxel over time to obtain the 3D, time-integrated activity. The CT provides a map of tissue electron density for each voxel, which is necessary for dosage calculations. A Monte Carlo package is then employed using the activity distribution as the source definition and energy deposition is tallied in each voxel. Several software packages are available for 3D, patient-specific dosimetry, including MCID [128], OEDIPE [129] and SCMS [130] which are based on MCNP, 3D-RD [131] and DOSIMG [132] which are based on EGS [133], RAYDOSE [134] and RAPID [135] which are based on GEANT4 [136], and DPM [137], which is not a public domain code. Both MCNP and GEANT4 can simulate α -particles, while EGS can only transport photons and electrons.

While imaging-based dosimetry can account for non-uniformity and calculate dose at the voxel level, it is still a macroscopic quantity. The spatial resolution of current SPECT imaging ranges from 5 to 25 mm [31]. These dimensions are much larger than the 40–90 μm range of an α -particle. This results in the stochastic variations due to α -particle energy depositions not being taken into account and the voxel dose could be misleading. For α -particles, the ideal dosimetric targets are isolated cancer cells in transit in the vascular and lymphatic systems, micro-metastases and tumor capillary networks [31]. The only way to study the dosimetry of α -particles on a small scale is pre-clinically. These have been limited to in vitro measurements and microdosimetric Monte Carlo models. However, recently, several groups have developed alpha cameras, such as the Ionizing-Radiation Quantum Imaging Detector, which provides images of the ex vivo activity distribution with a spatial resolution on the order of tens of microns μm [138,139]. Monte Carlo methods can then be employed to calculate the dose absorbed on that scale. This is viewed as an important advancement in alpha dosimetry because no further modeling was needed to obtain the spatial and temporal activity distribution.

Table 2. Preclinical targeted alpha-particle therapy (TAT) studies.

Isotope	Study	Molecular Target	Targeting Moiety	Drug(s) & Route	Cancer Type & Animal Model	Key Results	Ref
^{225}Ac	Efficacy, toxicity	PSMA, CD19	J591 & B4 mAbs	^{225}Ac -DOTA-J591, ^{225}Ac -B4, i.v.	Human LNCaP prostate s.c. xenografts & disseminated Daudi lymphoma in male nude mice.	Both effective without toxicity.	[35]
^{225}Ac	Efficacy, toxicity	HER-2/neu	Trastuzumab	^{225}Ac -DOTA-trastuzumab, i.p.	SKOV3 human ovarian cancer s.c. xenografts in female nude mice.	Effective with no toxicity.	[148]
^{225}Ac	PK, RD, toxicity	CD33	HuM195 Ab	^{225}Ac -DOTA-HuM195, i.v.	Cynomolgus monkey leukemia (does not express the human CD33 target).	12 d blood $T_{1/2}$, dosimetry kinetics estimated, efficacy without renal toxicity.	[149]
^{225}Ac	BD, efficacy, toxicity	Ganglioside GD2	3F8 Ab	^{225}Ac -DOTA-3F8, i.v.	NMB-7 human neuroblastoma xenografts in nude mice (BD), meningeal carcinomatosis xenografts in nude rats (efficacy) & cynomolgus monkeys (toxicity).	Tumor specificity, increased survival, no toxicity.	[150]
^{225}Ac , ^{177}Lu	BD, efficacy, toxicity	Somatostatin receptors	DOTATOC peptide	^{225}Ac and ^{177}Lu -DOTATOC, i.v.	AR42J rat pancreatic exocrine s.c. xenografts in nude mice.	^{225}Ac -TAT had greater efficacy relative to ^{177}Lu -TBT with low toxicity.	[151]
^{225}Ac , ^{213}Bi , ^{90}Y	BD, dosimetry, efficacy, toxicity	HER-2/neu	7.16.4 mAb	^{225}Ac , ^{213}Bi and ^{90}Y -7.16.4, i.v.	neu-N transgenic mouse model with rat HER-2/neu expression and spontaneous lung metastases & NT2.5 mouse mammary fat pad xenografts with rat HER-2/neu.	^{225}Ac -TAT had greater efficacy but with renal toxicity relative to ^{213}Bi -TAT & ^{90}Y -TBT.	[152]
^{225}Ac , ^{213}Bi	BD, efficacy, toxicity	nucleolin	F3 peptide	^{225}Ac -DOTA-F3, ^{213}Bi -DTPA-F3, i.p.	MDA-MB-435 human peritoneal carcinomatosis in SCID mice.	^{225}Ac -TAT had greater efficacy relative to ^{213}Bi -TAT with specific tumor uptake and minor renal toxicity.	[153,154]
^{225}Ac	Safety and efficacy	IL13RA2	Pep-1L peptide	^{225}Ac]Pep-1L, stereotactic intracranial injection	U8251 human glioblastoma orthotopic xenografts in male nude mice.	Efficacy with no significant toxicity.	[155]
^{225}Ac	Vascular normalization & efficacy	Vascular endothelial (VE)-cadherin	E4G10 Ab	^{225}Ac -DOTA-E4G10, i.v.	LS174T human colon s.c. xenografts in female nude mice.	Improved tumor vascular architecture & increased efficacy when combined with chemotherapy.	[107]

Table 2. Cont.

Isotope	Study	Molecular Target	Targeting Moiety	Drug(s) & Route	Cancer Type & Animal Model	Key Results	Ref
^{225}Ac	BBB and BTB permeabilization	Integrin $\alpha_v\beta_3$	small-molecule antagonist	^{225}Ac -labeled targeted liposomes (^{225}Ac -TL), intracranial injection	U87 MG human glioblastoma orthotopic xenografts in male nude mice.	Enhanced blood-brain barrier (BBB) and blood-tumor barrier (BTB) permeability.	[156]
^{225}Ac	BD, Efficacy	Thrombo-modulin	201b mAb	LnPO_4 nanoparticles (NPs) doped with ^{225}Ac -201b, i.v.	Syngeneic EMT6 mouse breast epithelial cell metastases in BALB/c mouse lung following i.v. injection of cells	Retention of ^{225}Ac and daughters in lung tissue, metastasis burden reduced.	[157]
^{225}Ac	Micro BD, RD	PD-L1	anti-PD-L1-BC Ab	^{225}Ac -DOTA-anti-PD-L1-BC, i.v.	NT2.5 mouse mammary xenografts in female nude mice.	Uniform distribution in liver, non-uniform in kidney and tumor, liver RD was limiting.	[158]
^{225}Ac	BD and toxicity	Bone metastasis	Zoledronic acid (ZOL)	^{225}Ac -DOTAZOL, i.v.	Wistar rats.	High bone:blood ratio. Kidney toxicity.	[159]
^{225}Ac	BD, RD and dose response	PSMA	PSMA ligands with albumin-binding moiety	^{225}Ac -RPS-074, i.v.	LNCaP human prostate cancer s.c. xenografts in BALB/c mice.	Decreased clearance rate, single administration had complete response in 86% of tumors.	[160]
^{225}Ac	PK, BD, specificity, RD, toxicity, efficacy	MC1R	MC1RL peptide	^{225}Ac -DOTA-MC1RL, i.v.	PK (Sprague-Dawley rats), BALB/c mice (toxicity and BD) and MEL270 human uveal melanoma s.c. xenografts in SCID mice (BD and efficacy).	Renal and hepatobiliary excretion, rapid blood clearance, low toxicity, prolonged survival and decreased metastasis after single injection.	[67]
^{225}Ac	Efficacy, toxicity	CA19.9	5B1 human mAb	^{225}Ac -labeled tetrazine radioligand and a transcyclooctene5B1 for pretargeting, i.v.	Bilateral MIA PaCa-2 (CA19.9-negative) and BxPC3 (CA19.9-positive) pancreatic cancer s.c. xenografts, and BxPC3 orthotopic xenografts in nude mice.	Pretargeting has similar efficacy compared to conventional TAT with reduced hematotoxicity.	[144]
^{225}Ac	Vascular normalization & efficacy	Vascular endothelial (VE)-cadherin	E4G10 Ab	^{225}Ac -DOTA-E4G10, i.v.	LS174T human colon s.c. xenografts in female nude mice.	Improved tumor vascular architecture & increased efficacy when combined with chemotherapy.	[107]

Table 2. Cont.

Isotope	Study	Molecular Target	Targeting Moiety	Drug(s) & Route	Cancer Type & Animal Model	Key Results	Ref
^{211}At	BD, RD, specificity, efficacy, toxicity	Tenascin glycoprotein	81C6 mAb	^{211}At -81C6, subarachnoid catheter or i.v.	Female athymic rat model of neoplastic meningitis by inoculation of human rhabdomyosarcoma cells via subarachnoid catheter.	Efficacy without significant toxicity. RD estimates.	[161,162]
^{211}At	PK, BD, efficacy, toxicity	gp38	MOv18 mAb	^{211}At - & ^{131}I -MOv18, i.p. or i.v.	Peritoneal OVCAR-3 human ovarian xenografts in BALB/c v/v or nude mice following IP injection of cells.	^{211}At -TAT had greater efficacy relative to ^{131}I -TBT.	[163–166]
^{211}At	Tumor neo-vasculature targeting	Fibronectin ED-B domain	Human scFv(L19)	^{211}At -scFv(L19), i.v.	Murine F9 teratocarcinoma & rat FE8 sarcoma in female nude mice.	Retained at tumor blood vessels resulting in increased tumor to blood ratios.	[140]
^{211}At	BD, tumor dosimetry, efficacy, toxicity	95-kDa glycoprotein	MX35 mAb	^{211}At -MX35, i.p. or i.v.	OVCAR-3 human ovarian cancer micrometastases in nude mice.	Fractionated treatment increased efficacy without significant toxicity.	[23,167,168]
^{211}At , ^{90}Y	Efficacy	CD30	HeFi-1 mAb	^{211}At -, ^{90}Y HeFi-1, i.v.	Human anaplastic large cell lymphoma cells in SCID/NOD mice. Karpas 299 cell i.v. injection for leukemia & SUDHL-1 xenografts for lymphoma.	^{211}At -HeFi-1 increased survival in the leukemia model & combination with unlabeled HeFi-1 further improved efficacy. ^{90}Y -HeFi-1 TBT increased survival in the lymphoma model.	[169]
^{211}At	BD, efficacy, toxicity	CD44v6	U36 chimeric mAb	^{211}At -U36, i.v.	UT-SCC7 human head and neck squamous cell carcinoma s.c. xenografts in nude mice.	Reduced tumor growth with no significant toxicity. BD consistent with targeting.	[170]
^{211}At	BD, efficacy, toxicity	HER2/neu	C6.5 diabody	^{211}At -SAPS-C6.5, i.v.	HER2/neu-positive MDA-MB-361/DYT2 breast xenografts in nude mice.	Tumor growth delay with low renal toxicity.	[142]
^{211}At	Efficacy	NIS-transduced tumor cells	Astatide (HAt) peptide	^{211}At -astatide, i.p.	NIS transduced LNCaP human prostate (NP-1) and parental (P-1) s.c. xenografts in male nude mice.	Accumulation similar to iodine with efficacy against NP-1 tumors relative to control P-1 tumors.	[147]

Table 2. Cont.

Isotope	Study	Molecular Target	Targeting Moiety	Drug(s) & Route	Cancer Type & Animal Model	Key Results	Ref
²¹¹ At, ²¹³ Bi	BD, myelo suppression, toxicity	CD45	30F11 Ab	²¹¹ At-30F11-ADTM, ²¹³ Bi-30F11-CHX-A", i.v.	Female BALB/c mice.	²¹¹ At-TAT induced myeloablation in haematopoietic tissues with greater efficacy and less toxicity relative to the ²¹³ Bi conjugate.	[171]
²¹¹ At	Efficacy	HER-2/neu	Trastuzumab	²¹¹ At-trastuzumab, i.p.	SKOV3 human ovarian i.p. xenografts in nude mice.	Combination of trastuzumab and ²¹¹ At-trastuzumab resulted in complete tumor eradication.	[172]
²¹¹ At	Dosimetry, toxicity, efficacy	Lewis Y epitope	BR96, chimeric IgG1 mAb	²¹¹ At-BR96, i.v.	BN7005-H ₁ D ₂ rat syngeneic sub-peritoneal colon engraftments.	Resulted in undetectable tumors with tolerable toxicity.	[173]
²¹¹ At	Efficacy	CD20	1F5 mAb	²¹¹ At-1F5, i.v.	Human Ramos (Burkitt lymphoma) s.c. xenografts in nude mice and i.v. injection of Ramos cells in SCID mice for disseminated lymphoma.	Highly effective in minimal residual disease mouse model.	[77]
²¹¹ At	BD, dosimetry	Sigma-2 receptor	MM3 ligand	²¹¹ At-MM3, i.v.	EMT6 murine breast syngeneic tumor in female BALB/c mice.	Tumor specific targeting.	[146]
²¹¹ At	Efficacy	Norepineph-erine transporter	Benzyl-guanidine	meta-[²¹¹ At]-astatobenzyl-guanidine, i.v.	PC12 rat pheochromocytoma s.c. xenograft in nude mice.	Reduced tumor size without weight loss.	[174]
²¹¹ At	BD, efficacy	MICA/B	anti MICA/B Ab	²¹¹ At-anti MICA/B Ab, i.v.	HCT116 (p53 ^{-/-} & MICA/B positive) human colon cancer s.c. xenograft in nude mice.	Significant reduction in tumor growth, no weight loss, erythrocytopenia with recovery in 3–4 wks.	[175]
²¹³ Bi, ⁹⁰ Y	Toxicity and efficacy	CO17-1A	CO17-1A Fab'	²¹³ Bi-Fab' and ⁹⁰ Y-Fab', i.v.	GW-39 human colon cancer s.c. xenograft in nude mice.	TAT had greater efficacy and lower toxicity than TBT.	[176]
²¹² Bi	Specificity, efficacy, toxicity	gp70	103A mAb	²¹² Bi-CHX-A-DTPA-103A, i.v.	RLV induced erythroleukemia in BALB/c mice.	Clinical and histological remission of erythroleukemia and prolonged survival with low toxicity.	[177]

Table 2. Cont.

Isotope	Study	Molecular Target	Targeting Moiety	Drug(s) & Route	Cancer Type & Animal Model	Key Results	Ref
^{212}Bi	BD, efficacy, toxicity	CD25	Anti-Tac, humanized mAb	^{212}Bi -CHX-A-anti-Tac, i.v.	SP2 and SP2/Tac syngeneic murine lymphoma in nude mice.	Effective in treatment of bulky solid tumors.	[178]
^{213}Bi	Stability, PK, toxicity	CD33	HuM195 mAb	^{213}Bi -CHX-A-DTPA-HuM195, i.p. or i.v.	Normal BALB/c mice without leukemia.	Favorable stability, PK and toxicity.	[179]
^{213}Bi , ^{90}Y	Pretargeting efficacy	CD25 (Tac)	Humanized anti-Tac mAb (HAT)	^{213}Bi - & ^{90}Y -DOTA-HAT; & HAT-streptavidin & ^{213}Bi -DOTA-biotin or ^{90}Y -DOTA-biotin, i.v.	Intraperitoneal MET-1 human adult T-cell leukemia in SCID/NOD mice.	Pre-targeted ^{213}Bi TAT increased survival relative to ^{213}Bi -DOTA-HAT, ^{90}Y TBT & pre-targeted TBT.	[143]
^{213}Bi , ^{131}I	Efficacy	TAG-72	Humanized, domain-deleted CC49 mAb (HuCC49ΔCH2)	^{213}Bi - or ^{131}I -HuCC49ΔCH2, i.p.	TAG-72+ LS-174T & TAG-72 negative MIP human colon i.p. xenografts in nude mice.	^{213}Bi -TAT had greater growth inhibition or regression relative to ^{131}I -TBT.	[180]
^{213}Bi	Efficacy, toxicity	d9-E-cad	d9-E-cad mAb	^{213}Bi -d9-E-cad mAb, i.p.	HSC45-M2 human gastric i.p. xenografts with d9-E-cad mutation in female nude mice.	Double administration had greater efficacy relative to single administration, with no toxicity.	[181]
^{213}Bi	BD, efficacy, toxicity	Somatostatin receptors	DOTATOC peptide	^{213}Bi -DOTATOC, i.v.	CA20948 rat pancreatic adenocarcinoma tumors in Lewis rats.	Antitumor efficacy with low toxicity.	[64]
^{213}Bi	Specificity, BD	CD87	P-P4D peptide	^{213}Bi -P-P4D, i.p.	OV-MZ-6 human ovarian i.p. xenografts in female nude mice.	Specific tumor uptake, kidney uptake reduced by co-injection of gelofusine.	[182]
^{213}Bi	Efficacy, toxicity	MUC1, uPAR and BLCA-38	C595 & BLCA-38 mAbs, & PAI2 protein	^{213}Bi -C595, -BLCA-38 & -PAI2, i.p.	PC-3 human prostate orthotopic, intratibial and s.c. xenograft tumors in NOD SCID mice.	Multiple TAT can overcome heterogeneous antigen expression with efficacy against micrometastases.	[145]
^{213}Bi	Efficacy, toxicity	EGFR	Matuzumab	^{213}Bi -matuzumab, intravesical	EJ28 human orthotopic bladder xenografts in nude mice.	Increased survival without toxicity. Combination with mitomycin C increased efficacy with nephrotoxicity.	[183]
^{213}Bi	Efficacy	TAG-72	Humanized CC49 mAb (HuCC49DCH2)	^{213}Bi - HuCC49DCH2, i.p.	LS-174T human colon i.p. xenografts in female nude mice.	Combination trastuzumab and i.p. TAT increased efficacy and was well tolerated.	[184]
^{213}Bi	Pretargeting efficacy	CD20	scFv-1F5-SA (streptavidin fusion protein)	1F5-SA & ^{213}Bi -DOTA-biotin, i.v.	Ramos human lymphoma xenografts in nude mice.	Tumor regression and increased survival in mice with small tumors via pretargeting.	[141]

Table 2. Cont.

Isotope	Study	Molecular Target	Targeting Moiety	Drug(s) & Route	Cancer Type & Animal Model	Key Results	Ref
^{213}Bi , ^{177}Lu	BD, dosimetry, efficacy, toxicity	GRP	PESIN and AMBA peptides	^{177}Lu -DOTA-PESIN, ^{213}Bi -DOTA-PESIN, or ^{213}Bi -AMBA, i.v.	PC-3 human prostate s.c. xenografts in female nude mice.	^{213}Bi -TAT had greater efficacy compared to ^{177}Lu -TBT. ^{213}Bi -DOTA-PESIN had lower renal toxicity relative to ^{213}Bi -AMBA.	[185]
^{213}Bi , ^{177}Lu	Efficacy	CD138	9E7.4 mAb	^{213}Bi -9E7.4 and ^{177}Lu -9E7.4, i.v.	5T33 murine multiple myeloma cell syngeneic i.v. injection into C57/BL6 mice.	^{213}Bi -9E7.4 increased survival and cured 45%, ^{177}Lu -9E7.4 increased survival, no cures.	[186]
^{213}Bi , ^{177}Lu	Efficacy, toxicity	Mutant d9-E-cadherin	d9MAb	^{213}Bi -d9MAb & ^{177}Lu -d9MAb, i.p.	HSC45-M2 human gastric cancer cell i.p. injection in nude mice.	^{213}Bi had comparable efficacy with lower toxicity.	[187]
^{213}Bi	BD, efficacy, toxicity	CD138	Anti-mouse CD138 Ab	^{213}Bi -CD138, i.v.	5T33 mouse multiple myeloma cell engraftment into syngeneic C57BL/KaLwRij mice.	Increased survival with only moderate and transient toxicity.	[188]
^{213}Bi	Efficacy, toxicity	EGFR	Matuzumab	^{213}Bi -matuzumab, intravesical.	EJ28 human orthotopic bladder xenografts in nude mice.	Increased survival with low toxicity.	[189]
^{213}Bi	PK, efficacy, dosimetry, toxicity	SSTR2	DOTATATE peptide	^{213}Bi -DOTATATE, i.v.	Neuroendocrine H69 human small cell lung carcinoma and CA20948 rat pancreatic s.c. xenografts in nude mice.	Effective in small and large tumors (both types), with dose limiting renal toxicity.	[66]
^{212}Pb	Efficacy	HER-1	Cetuximab	^{212}Pb -cetuximab, i.p.	ILS174T human colon i.p. xenografts in nude mice.	Extended survival and combined with gemcitabine & carboplatin increased efficacy.	[190]
^{212}Pb	Efficacy	MC1R	DOTA-Re (Arg ¹¹)CCMSH peptide	^{212}Pb [DOTA]-Re (Arg ¹¹)CCMSH, i.v.	B16/F1 murine melanoma syngeneic s.c. engraftments in C57BL/6 mice.	Tumor eradication at higher activities.	[191]
^{212}Pb	Efficacy	HER-2 and CEA	Trastuzumab & 35A7	^{212}Pb -trastuzumab & ^{212}Pb -35A7, i.p.	A-431 HER-2 positive and CEA transfected vulvar squamous carcinoma cells i.p. in nude mice.	Internalizing anti-HER2 labeled Ab had greater efficacy than non-internalizing anti-CEA labeled Ab.	[192]
^{212}Pb	Efficacy, toxicity	HER-2/neu	Trastuzumab	^{212}Pb -trastuzumab, i.p.	LS174T human colon & Shaw human pancreatic i.p. xenografts in nude mice.	Increased survival with low toxicity.	[193]

Table 2. Cont.

Isotope	Study	Molecular Target	Targeting Moiety	Drug(s) & Route	Cancer Type & Animal Model	Key Results	Ref
^{212}Pb	Efficacy	HER-2/neu	Trastuzumab	^{212}Pb -trastuzumab, i.p.	LS-174T human colon i.p. xenografts in nude mice.	Combination with gemcitabine increased survival.	[194]
^{212}Pb	BD, efficacy	B7-H3	376.96 mAb	^{212}Pb -376.96, i.p.	ES-2 or A2780cp20 human ovarian cancer cells i.p. into nude mice.	High peritoneal retention, tumor tissue accumulation & increased survival.	[195]
^{212}Pb	BD, efficacy	B7-H3	376.96 mAb	^{212}Pb -376.96, i.v.	Panc039 pancreatic cancer orthotopic xenografts in nude mice.	High tumor uptake & tumor growth inhibition.	[196]
^{212}Pb	BD, efficacy	CSPG4	225.28 mAb	^{212}Pb -225.28, i.v.	SUM159 & 2LMP human triple negative breast cancer (TNBC) orthotopic mammary fat pad xenografts in nude mice.	Dose-dependent growth inhibition.	[197]
^{212}Pb	Administration route, toxicity, efficacy	EGFR	Panitumumab F(ab') ₂ fragment	^{212}Pb -panitumumab F(ab') ₂ , i.p. & i.v.	ILS-174T human colon i.p. xenografts in nude mice.	Increased survival with tolerated toxicity via i.p. or i.v. injection.	[198]
^{212}Pb	Efficacy, combination therapy	MC1R	ee-cyclized α -MSH peptide	^{212}Pb -DOTA-MC1L, BRAFi & HDACi	A2058 & MEWO human melanoma xenografts in nude mice.	Improved tumor response by combination therapy.	[69]
^{227}Th	BD, efficacy, toxicity	CD20	Rituximab	^{227}Th -DOTA-p-benzyl-rituximab, i.v.	BALB/c mice & Raji human B-cell lymphoma s.c. xenografts in nude mice.	Increased efficacy with manageable toxicity.	[199]
^{227}Th	BD, efficacy, toxicity	HER-2/neu	Trastuzumab	^{227}Th -DOTA-trastuzumab, i.v.	SKBR-3 human breast cancer xenografts in nude mice.	Tumor growth inhibition with no toxicity.	[200]
^{227}Th	BD, efficacy, toxicity	CD70	Anti-human CD70 mAb	CD70-TTC, i.v.	786-O human renal cancer s.c. xenografts in nude mice	Well tolerated with inhibition of tumor growth.	[201]
^{224}Ra	Efficacy, toxicity	peritoneal metastases	Injection into peritoneum	^{224}Ra -labeled calcium carbonate microparticles, i.p.	ES-2 and SKOV3 human ovarian cancer i.p. xenografts in nude mice.	Well tolerated with antitumor effect.	[202]

7. Pre-Clinical Studies

7.1. Preclinical Therapeutics Studies

In recent years, a number of pre-clinical studies have been reported that provide strong evidence of the potential for use of TAT in the treatment of cancers. See Table 2 for a summary of the published in vivo preclinical TAT studies ($n = 48$) that have used animal models of cancer. Most of these studies (81%) have involved the use of immunoconjugates where monoclonal antibodies serve both as the targeting moiety and as the attachment scaffold for the α -emitting radionuclide. Two of these immunoconjugates involved the use of antibody fragments (scFv) [140,141] and one used a diabody [142]. Three studies involved pretargeting, where a cancer targeting antibody–streptavidin construct, or antibody–TCO (*trans*-cyclooctene) construct for click chemistry, was injected prior to delivery of an α -emitting radionuclide attached to biotin [141,143,144]. In this study, pre-targeting demonstrated increased efficacy over TAT using the targeting antibody alone. Peptide-based targeting moieties were used in 15% of these studies. In other individual studies, a protein [145], a small molecule ligand [146] and an inorganic compound [147] were used as targeting groups.

As is the case for most radioimmunotherapies in current use, that typically deliver β -emitting radionuclides, a number of the reported TAT studies (23%) targeted non-solid tumors; i.e., leukemias, lymphomas and multiple myeloma. A majority of these non-solid tumor studies reported significant efficacy and/or decreased systemic toxicity relative to controls. Since α -emissions have higher LET over a shorter range, there is significant interest in the use of TAT for treatment of solid tumors. This is evident in the preclinical studies reported, as 79% involved targeting of solid tumors and nearly all of these studies reported significant efficacy against solid tumors relative to controls, with nearly half also reporting low or manageable systemic toxicity. Four of these studies demonstrated increased efficacy using TAT relative to targeted beta (β)-emission therapy (TBT) using the same targeting moiety and tumor model [143,151,152,163–166,180,185].

7.2. Preclinical Imaging

Whether developing a small molecule or antibody-based therapeutic, pharmaceutical companies traditionally rely upon molecular imaging to assist them in identifying the most promising molecules in the research pipeline to be carried forward into clinical trials [203–206]. Furthermore, this strategy has been particularly effective in developing targeted, systemic β -based radiotherapies when the β -emitting radioisotope can be paired with a diagnostic surrogate for SPECT or PET imaging. Several of these pairs include $^{64/67}\text{Cu}$, $^{86/90}\text{Y}$ and $^{124/131}\text{I}$ [207]. However, with the exception of the $^{203/212}\text{Pb}$ system [68,191,208], researchers developing TATs with other α -emitters such as ^{213}Bi , ^{211}At and ^{225}Ac , have not been able to capitalize on this strategy. This limitation has hindered TAT development, but also stimulated attempts by the scientific community to observe the pharmacokinetics of TAT therapies using the decay of TAT radioisotopes in preclinical or clinical settings. For example, Sgouros et al. attempted to use the 440 keV γ emission of ^{213}Bi to monitor the biodistribution of ^{213}Bi -HuM195 in patients with leukemia [209]. Due to the short half-life of the radioisotope, data acquisition was limited to sixty minutes after patient injection, but this was sufficient to detect elevated levels of radioactivity in the red marrow, liver and spleen. While the data suggested that it would be possible to derive important pharmacokinetic and dosimetric data from the in vivo imaging of the ^{213}Bi emissions, the large amount of activity necessary for imaging has precluded its routine use in a clinical setting.

The decay scheme and extreme cytotoxic potency of ^{225}Ac further complicates the TAT community's ability to develop preclinical and clinical imaging methodologies to monitor pharmacokinetics and estimate dosimetry. Since the 440 keV emission derived from ^{213}Bi decay is considered too energetic for preclinical imaging applications, attention has focused on the less energetic 218 keV γ ray emission of ^{221}Fr , which has been used to monitor the biodistribution of ^{225}Ac -doped nanoparticles in mice [71,86], and this approach has shown promise. However, in this imaging paradigm, animals are typically euthanized 1 h after receiving the radiopharmaceutical and imaged 24 h after euthanasia to ensure

isotopic equilibrium. Hence, these experiments do not address the potential of longitudinal SPECT studies to inform development of ^{225}Ac -radiopharmaceuticals. Clearly, new approaches are needed, since the ability to image and visualize ^{225}Ac -based radiopharmaceutical biodistribution, metabolism, and clearance in animal models through longitudinal imaging studies would be advantageous [210,211].

Cerenkov luminescence imaging has emerged as an alternative to traditional nuclear medicine techniques for visualizing the delivery and biodistribution of TATs. Cerenkov luminescence is derived from the emission of ultraviolet light when certain charged particles exceed the phase velocity of light in a given medium [212,213], and this effect can be observed using standard optical imaging systems originally designed to detect bioluminescence and fluorescence. Recently, Cerenkov emissions were observed with the decay of several medically relevant isotopes, including ^{225}Ac , which was reported to yield the largest optical signal among all isotopes examined [211,214]. Alpha (α) particles however, do not travel with sufficient velocity to generate Cerenkov emissions, which led researchers to postulate that the emissions observed resulted from the β -decay of the ^{213}Bi , ^{209}Tl and ^{209}Pb daughter products. Subsequent publications, using theoretical and experimental means, described the association between Cerenkov radiation and ^{225}Ac decay [214,215]. While both research groups determined that Cerenkov luminescence imaging (CLI) could be accomplished with ^{225}Ac and other α -particle emitting radionuclides, it was determined that radionuclides' decays solely by α -particle emission do not produce sufficient Cerenkov radiation to be useful for imaging. Secondly, a time delay would be needed for equilibrium to be established between the parent radionuclide and its daughter products. For ^{225}Ac , this delay was determined to be ten hours. This precludes the imaging of small molecules and peptides at early time points, but allows one to monitor the biodistribution of ^{225}Ac -labeled antibodies and nanoparticles, which require extended circulation time for effective tumor targeting and blood clearance. Finally, the high recoil energy associated with ^{225}Ac and daughter product release from the original conjugate must be considered. The emission of Cerenkov radiation may not reflect the actual biodistribution of the radiopharmaceutical in question, but of the daughter products producing the Cerenkov emissions [216]. Sequestering the daughter products with the original conjugate is important if this technique is to provide any benefit to the TAT community. In proof of principle studies, Pandya and coworkers seized upon these recommendations and further tested the association between ^{225}Ac decay and Cerenkov radiation in living animals [217]. The authors synthesized ^{225}Ac -DOTA-c(RGDyK) and evaluated its stability, biodistribution and potential use as an imaging agent in CLI in a murine model expressing human glioblastoma U87mg tumors, which overexpress the $\alpha_v\beta_3$ integrin. Additionally, they exploited the well-documented ability of RGD-containing ligands to be internalized on integrin binding to sequester the ^{225}Ac and the daughter products within the tumor. This technique, often referred to as the nanogenerator approach, has been used successfully to sequester ^{225}Ac -based radiotherapeutics and daughter decay products within a cell to increase their therapeutic effectiveness [35,149,150]. Surprisingly, all animals demonstrated no signs of distress during these experiments. When compared to $\alpha_v\beta_3^-$ tissues, *in vivo* CLI revealed five-fold more luminescence from the $\alpha_v\beta_3^+$ tumors. Luminescence was also observed in the liver and kidney tissues, which have been shown in related biodistribution studies to be involved in the clearance and excretion of this radiopharmaceutical [218–220]. *Ex vivo* image analysis also revealed a similar trend as the *in vivo* results, and the addition of a blockade reduced the luminescence emitted from the tumors by 80%, which suggested to the authors that activity delivered by the radiopharmaceutical and internalized through specific receptor-interactions was being retained in the tumor. To further validate the theranostic potential of their approach, they also performed preliminary therapy studies in U87mg tumor-bearing mice, which illustrated the cytotoxic potency of their α -emitting radiotherapeutic [221]. While more studies are needed, these promising results suggest that this area of research may have broader implications for molecular imaging and may help to facilitate TAT development.

8. Medicinal Chemistry

8.1. Lead Optimization

As previously touched upon, the characteristics of various targeting ligands, like lipophilicity, molecular weight and ionization potential are all critical variables that help modulate radiopharmaceutical properties. For example, small, polar compounds may undergo rapid renal excretion. The addition of a PEG linker will add water solubility, bulk, and may help avoid kidney toxicities. Conversely, the removal of an ionizable group (e.g., sulfonate or protonated amines at physiologic pH) during medicinal chemistry design may make the targeting ligand more hydrophobic and shift clearance toward initial liver processing. Therefore, when working to reduce targeting ligand complexity or increase the ease of synthesis, these physical attributes of the compound must be taken into consideration.

Antibodies and engineered fragments can also benefit from medicinal chemistry optimization. Many chelators and linkers covalently attached to antibodies and their fragments produce heterogeneous products. In the most typical fashion of linker attachment, surface accessible lysine side chains are utilized to form covalent bonds with radionuclide chelating molecules (Figure 6). Site-specific antibody modifications can yield more homogeneous products. The Rader group at Scripps Florida has made such progress by engineering a selenocysteine residue into targeting antibodies, allowing for complete control of regiochemistry during functionalization [222].

Another route to increasing T/NT ratios is through the notion of pretargeting. These strategies operate under the premise that nonradiolabeled, bifunctional targeting agents can be administered to the patient and allowed to converge to their target. Once the unbound portion has cleared, a fraction of radiolabeled molecule with a binding affinity for the bifunctional targeting agent is administered. Typically, the radiolabeled agent is designed to clear quickly if unavailable for binding; thus, lowering off-target radiation. Typical binding partners amenable to pretargeting schemes include avidin/streptavidin–biotin, DNA–DNA or antibody–hapten [223]. For a more in-depth analysis of pretargeting strategies please see the recent review article from Frampas and coworkers [224].

Novel combination therapies are also now being investigated. Combination therapies can be particularly useful in heterogeneous diseases like multiple myeloma. Anti CD138 mAb conjugated with ^{213}Bi has been tested synergistically with the chemotherapeutic melphalan to help eliminate residual disease in a multiple myeloma model [116]. Interestingly, this study saw no benefit to the combination therapy when compared with RIT alone. The speculation was that the follow-up treatment with CD138-targeted mAb was less successful due a swift reduction in CD138 antigen in cells progressing through apoptosis [225], illustrating the importance of the orthogonality in study design as it relates to combination treatments and target expression.

8.2. cGMP Production

Current good manufacturing practice (cGMP) standards for the production of the targeting ligand precursors before the incorporation of the α -emitting radioisotopes are similar to those used for the manufacturing of peptide, antibody or antibody fragment drug and drug candidates. There are numerous cGMP-compliant peptide synthesis companies, and often the preclinical grade peptides will be prepared by standard solid-phase synthesis and purified to $\geq 95\%$ purity, but cGMP-grade peptides will often be prepared via routes that allow greater control over the purity and reliability of the synthesis. For instance, cGMP peptides are more likely to be prepared using a greater reliance on solution phase-synthesis procedures than preclinical grade peptides because the costs can be much lower on scaled-up batches of peptide, and more importantly, impurities can be removed as they build up during a solution-phase synthesis, whereas impurities just build up during standard solid-phase synthesis procedures. The recent development of a fully solid-phase synthesis of cGMP-quality Fuzeon[®] is an exception to this trend that may lead to a greater reliance on fully solid-phase synthesis procedures for the production of cGMP peptides. Frequently, solid phase methods are used in

combination with solution phase synthesis methods. As an example, preclinical grade linear peptides can be prepared and cyclized to the final product on resin, whereas cGMP grade peptides will often be prepared on resin and purified and then cyclized in solution, due to the easier analysis of reactions in solution.

The cGMP production of antibody and antibody fragments will be focused on for the development of robust methods of production often using hybridoma technologies and purification by at least two orthogonal separation methods, usually involving an affinity column purification step followed by a size exclusion type purification system. Often these biotechnology-based manufacturing methods produce antibody or antibody fragments that are chemically programmed to provide specific sites for metal chelate attachment. For a recent review of this topic please, see the two-part article from Adumeau et al. [226,227].

The results of these manufacturing processes are lyophilized powders or relatively-concentrated, sterile, aqueous buffer solutions of the targeting moieties that are ready to chelate the α -emitters. The main difference between cGMP radiosynthesis for clinical trials and research grade radiosynthesis in the United States is the implementation of an FDA-compliant audit trail. Since the development of α -emitting radiopharmaceuticals is happening globally, another major issue concerning production and distribution of these novel therapies is the consideration of the local regulatory environment. A discussion of the potential for harmonization of the various international regulations has recently been published [228].

Table 3. Clinical TAT studies.

Isotope	Molecular Target	Targeting Moiety	Drug	Cancer Type	Trial/# of Patients	Administration Route	Key Results	Ref
²²⁵ Ac	CD33	HuM195	²²⁵ Ac-DOTA-HuM195	AML	Phase I/20, Ongoing multicentric phase I, II	Intravenous	Safe at doses $\leq 3 \mu\text{Ci/kg}$, anti-leukemic activity across all dose levels studied, no acute toxicities, myelosuppression	[235]
²²⁵ Ac	PSMA	PSMA-617 ligand	²²⁵ Ac-PSMA-617	Prostate cancer	NA/40	Intravenous	Remarkable anti-tumor response was observed in the patients. Xerostomia in salivary gland was the main side effect.	[5,6,60,233,236,237]
²²⁵ Ac	Somatostatin receptors	DOTATOC peptide	²²⁵ Ac-DOTATOC	Neuroendocrine tumors	NA/34	Not mentioned	Well-tolerated with promising treatment efficacy	[234]
²¹¹ At	Tenascin-C	chimeric 816 antibody	²¹¹ At-ch81C6	Glioblastoma	Phase I/18	surgically created resection cavity	Increased Median survival (54 weeks), No dose-limiting toxicity, No-grade 3 toxicity	[238]
²¹¹ At	NaPi2b	MX35 F(ab') ₂	²¹¹ At-MX35 F(ab') ₂	Ovarian carcinoma	Phase I/9	Intraperitoneal	No adverse effects, grade I toxicity, no bone marrow toxicity	[239]
²¹³ Bi	CD33	HuM195	²¹³ Bi-CHX-A-DTPA-HuM1 95	AML	Phase I/18	Intravenous	14 patients had reductions in marrow blasts	[240]
²¹³ Bi	CD33	HuM195	²¹³ Bi-CHX-A-DTPA-HuM1 95	AML	Phase I, II/31	Intravenous	dose-response relationship with remission at the highest doses	[241]
²¹³ Bi	CD20	Rituximab	²¹³ Bi-CHX-A''- Rituximab	Non-Hodgkin lymphoma	Phase I/9	Intravenous	Myelosuppression and no other toxic side, two patients responded	[242]
²¹³ Bi	Neurokinin type-1 receptor	Substance P	²¹³ Bi-DOTA-[Thi8, Met (O2) 11]-substance P	Glioblastoma	NA/2, 9, 20	Intrathecal	Well-tolerated, favorable response	[229,230,243]
²¹³ Bi	NG2 proteoglycan	9.2.27 antibody	²¹³ Bi-cDTPA-9.2.27	Melanoma	Phase I/38	Intralesional	TAT was safe up to 450 mCi and effective at 200 mCi	[244,245]
²¹³ Bi	Somatostatin receptors	DOTATOC peptide	²¹³ Bi-DOTATOC	Neuroendocrine tumors	NA/7	Intraarterial infusion	responses were observed in all patients	[65]
²¹³ Bi	EGFR	Cetuximab	²¹³ Bi-CHX-A-DTPA-anti-EGFR	carcinoma in situ (CIS) of the bladder	Pilot studies/9 and 12	Intravesical	TAT well tolerated and showed therapeutic efficacy	[231,232]
²¹² Pb	HER2	Trastuzumab	²¹² Pb-TCMC-trastuzumab	Ovarian Cancer	Phase I/3	Intraperitoneal	Dose escalation showed a little agent-related toxicity, consistent with the dosimetry data	[246,247]
²²³ Ra	Hydroxy-apatite	NA	²²³ Ra-chloride	Prostate cancer mets	Phase I-III/921	Intravenous	radium-223 improved overall survival	[7,8,82,248–251]

9. Clinical Studies

9.1. Recent TAT Clinical Trials

Despite the potential therapeutic benefits of the α -particle emitting radioisotopes over β -particle emitters, the restricted availability of relevant radionuclides and limited number of phase III clinical trials has so far limited the clinical use of TAT. Table 3 summarizes the TAT clinical studies conducted to date. Clinical trials prior to 2014 were reviewed by Elgqvist et al. [34] and we will focus on the more recent trials herein. The use of short-lived α -emitters like ^{211}At ($T_{1/2} = 7.2\text{ h}$), ^{212}Bi ($T_{1/2} = 60\text{ min}$) and ^{212}Pb ($T_{1/2} = 10.6\text{ h}$) has been subdued so far, due to production and distribution limitations. However, this is not the case for ^{213}Bi ($T_{1/2} = 46\text{ min}$), which, going back over the past two decades, has been used in the largest number of TAT clinical trials to date. Recent ^{213}Bi studies have included the targeting of peptides for the treatment of glioblastoma [229,230] and neuroendocrine tumors [65], and the targeting of monoclonal antibodies for the treatment of bladder cancer [231,232]. Since the approval of ^{223}Ra -dichloride for the treatment of CRPC in 2013, ^{225}Ac has also emerged as a targeted therapeutic radionuclide of interest that has been used for targeting a small molecule [5,6,233], peptide [234] and monoclonal antibody [235] for the targeting of prostate cancer, neuroendocrine tumors, and acute myeloid leukemia (AML), respectively. Clinical studies of TATs using ^{225}Ac or ^{213}Bi as the therapeutic radionuclide have recently been reviewed [58]. Although the responses have been impressive for some, ^{225}Ac -PSMA-617 in particular, the completion of more randomized clinical trials comparing TAT to standard of care will be essential to determine the clinical utility of these new therapeutic approaches.

9.2. Radium-223 Dichloride ($^{223}\text{RaCl}_2$) Trials

In the following section, we will discuss the first and only clinically-approved TAT in the United States so far. After more than a 100 years since the discovery of the radium element in 1898 by Marie and Pierre Curie, on May 15, 2013, one of its radioisotopes, radium-223 (Ra-223) became the first α -emitting radionuclide to be approved by the Food and Drug Administration (FDA) in the form of radium-223 dichloride ($^{223}\text{RaCl}_2$, Xofigo[®] formerly Alpharadin, made by Bayer HealthCare Pharmaceuticals Inc.) for the treatment of patients with castration-resistant prostate cancer (CRPC), with symptomatic bone metastases, and no known visceral metastatic disease [252]. This was based on the improvement in overall survival following intravenous administration of $^{223}\text{RaCl}_2$ in patients with advanced prostate cancer with metastases to the bone, demonstrated in a double-blind randomized placebo-controlled trial called the ALSYMPCA (alpharadin in symptomatic prostate cancer) trial [7].

The role of systemic radionuclide therapy to the bone is to deliver therapeutically-effective radiation doses to multiple foci, including microscopic disease, as opposed to external beam radiotherapy, that gives more focal treatment and affects both normal bone marrow and metastases [253]. The two most commonly used radionuclide bone seeking agents for bone pain palliation are ^{89}Sr (Metastron) and ^{153}Sm -ethylene diamine N,N' -tetra(methylene) phosphonic acid (^{153}Sm -EDTMP; Quadramet) [254], but these β -particle therapies, although effective for pain palliation, frequently cause myelosuppression and have not been shown to affect patient survival [255].

Cations of the alkaline earth elements (strontium, barium, and radium) have natural bone-seeking properties without the need for a carrier agent. As radium mimics calcium, when Ra-223 dichloride is injected intravenously, it forms complexes with the bone mineral hydroxyapatite at areas of increased metabolic bone activity, such as bone metastases, thereby exerting a highly localized antitumoral effect [7,256]. In addition, the Ra-223 daughter isotopes are also retained in the bone matrix [50]. The shorter penetration range of Ra-223 causes less bone marrow toxicity than bone-seeking β -particles [256]. The peak skeletal uptake of Ra-223 occurs within 1 h of injection, and blood radioactivity levels are <1% after 24 h with almost no redistribution of daughter nuclides from bone [256]. The total skeletal uptake ranges between 40% and 60% of the administered activity [257], and the excretion is predominantly via the gastrointestinal tract, with much less renal clearance [258]. Due to their significantly higher potency, the activities administered of α -emitters are much lower

than β emitters, with significantly less radiation exposure to hospital staff and family members [259]. Merely basic hygiene measures and routine contamination protection barriers, such as latex gloves, are enough to protect both health care personnel involved in the patient's radionuclide administration and the patient's family.

In the first-in-human single dosage phase I trial for patients with breast and prostate cancer skeletal metastases, single dose Ra-223 was well tolerated at all therapeutically relevant doses (46–250 kBq/kg intravenously [IV]) [257]. There was no dose-limiting hematological toxicity, only mild, transient myelosuppression (nadir 2 to 4 weeks after the injection) and mild transient diarrhea, with nausea and vomiting more frequently observed in the highest dosage group [257]. Subsequently, the phase I dose-escalation study in advanced prostate cancer with bone metastases, showed that repeated Ra-223 dosing was well tolerated to a total dose of 250 kBq/kg [50].

In the following phase II study of CRPC patients with symptomatic bone metastases, Ra-223 given at different doses (5, 25, 50 and 100 kBq/kg) was well tolerated with a positive effect on pain assessments (visual analogue scale [VAS] and analgesic use). At week 8, 56% and 71% of patients had reduced pain and stable analgesic consumption in the 50 and 100 kBq/kg groups, respectively. A statistically significant decrease in bone turnover markers, including alkaline phosphatase, was noted in the highest dose-group ($p = 0.0067$) [260].

The phase III, double-blind, randomized, placebo-controlled, multicenter, multinational ALSYMPCA trial enrolled patients from June 2008 through February 2011 [7]. Eligible patients had progressive metastatic symptomatic disease and regular use of analgesic medication or treatment with external beam radiation therapy (EBRT) for cancer-related bone pain within the previous 12 weeks. Patients were randomized 2:1 to receive six injections of radium-223 (50 kBq/kg IV) or a saline placebo infusion; a cycle was comprised of one injection every 4 weeks. The primary endpoint was overall survival (OS). Various secondary endpoints included time to first symptomatic skeletal events (SSE), time to alkaline phosphatase increase and time to PSA progression. A planned interim analysis was conducted, and on June 3, 2011, the Independent Data Monitoring Committee recommended stopping the trial early due to evidence of a significant treatment benefit. Median OS was 14.0 months for Ra-223 (95% CI (confidence interval): 12.1–15.8) versus 11.2 months for placebo (95% CI: 9.0–13.2) ($p = 0.00185$; HR (hazard ratio) = 0.695; 95% CI: 0.552–0.875).

An updated analysis included 528 events from 921 randomized patients prior to placebo patients crossing over to Ra-223 [7,261]. For this analysis, median OS was 14.9 months for Ra-223 arm (95% CI: 13.9–16.1) and 11.3 months for patients receiving placebo (95% CI: 10.4–12.8) (HR = 0.695; 95% CI: 0.581–0.832). OS favored Ra-223, with a 30% reduction in the risk of death compared with placebo. The OS benefit with Ra-223 was supported by a delay in time to first SSE compared to the placebo (15.6 versus 9.8 months; HR = 0.66; 95% CI: 0.52–0.83; $p < 0.001$). Improvement in survival was independent of both prior exposure to docetaxel [250] and the use of bisphosphonates [261]. Ra-223 significantly prolonged time to total ALP progression (HR = 0.167; 95% CI: 0.129–0.217; $p < 0.00001$); and time to PSA progression (HR = 0.643; 95% CI: 0.539–0.768; $p < 0.00001$) was associated with a beneficial effect on pain and quality of life (QOL) [262], and was associated with a highly favorable safety profile with a low rate of adverse hematological events. The most common (at least two percent) of the adverse reactions in patients receiving Ra-223 were nausea, diarrhea, vomiting, renal impairment and peripheral edema.

Based on these data, the National Comprehensive Cancer Network (NCCN) supports the use of $^{223}\text{RaCl}_2$ (Xofigo[®]) as a first-line therapy for patients with symptomatic metastatic CRPC and no visceral metastases with a Category 1 recommendation [227]. The recommended dose for $^{223}\text{RaCl}_2$ is 50 kBq/kg (1.35 microcuries/kg) administered by slow intravenous injection, over 1 min, every 4 weeks, for a total of six cycles.

Since completion of the ALSYMPCA trial, Ra-223 has been studied in combination with other, newly-approved CRPC therapies, such as abiraterone, enzalutamide, docetaxel, cabazitaxel, sipuleucel-T, olaparib and pembrolizumab [263–265]. A phase 3, randomized, double-blind study of

radium-223 or placebo, each in combination with abiraterone plus prednisone in chemotherapy-naïve patients with asymptomatic or mildly symptomatic mCRPC with bone metastases (ERA 223; NCT02043678) [266], was recently prematurely unblinded. The independent data monitoring committee (IDMC) recommended unblinding the trial due to the observation of more fractures and deaths in the combination treatment arm. The European Medicines Agency (EMA)'s Pharmacovigilance Risk Assessment Committee (PRAC) has reviewed the preliminary data from the ongoing clinical study. In the study 34.7%, of patients treated with Xofigo, Zytiga and prednisone/prednisolone have died so far, compared with 28.2% of patients given placebo, Zytiga and prednisone/prednisolone. Fractures have also occurred more frequently with the Xofigo combination than the placebo combination (26% versus 8.1%). Unblinded data from the study are currently being analyzed to confirm the preliminary findings of the IDMC. Given these results from ERA 223 trial, the current recommendation is not to combine radium-223 with concomitant abiraterone acetate and prednisone (<https://www.ema.europa.eu/news/prostate-cancer-medicine-xofigo-must-not-be-used-zytiga-prednisoneprednisolone>). Hence, the administration of concomitant chemotherapy with Xofigo is not recommended due to an increased risk of adverse reactions [267].

Immune modulation has been examined *in vitro* in human prostate, breast and lung carcinoma cells following exposure to sub-lethal doses of ^{223}Ra . Malamas and his colleagues showed that ^{223}Ra significantly enhanced T cell-mediated lysis of each tumor type by CD8+ cytotoxic T lymphocytes (CTLs) specific for MUC-1, brachyury, and CEA tumor antigens. Therefore, ^{223}Ra may be more effective in combination with immunotherapies [268].

In view of the increased long term risk of secondary malignancies, such as sarcomas and leukemias, that were seen in patients who were treated with high doses of another radium isotope, radium-224 ($T_{1/2} = 3.7$ days) (primarily used in Europe) for bone pain associated with ankylosing spondylitis, there is concern for development secondary malignancy with the use of Ra-223. So far there has been no evidence of secondary malignancies following Ra-223 therapy [7,269]. However, it is important to note that patients with metastatic CRPC have a much shorter overall survival than patients with ankylosing spondylitis. Care should be taken when expanding the use of Ra-223 to other patient groups with longer expected overall survival times, since the rate of secondary tumor development may increase.

Ra-223 trials have been performed for patients with diseases other than CRPC. Ra-223 was found to be safe and well tolerated when studied in a phase II, open-label trial investigating the safety and short-term efficacy in patients with breast cancer who had bone-dominant disease [270], but the data are still limited. In contrast to prostate cancer, multiple myeloma bone lesions are predominantly lytic. It is not yet known if Ra-223 will have any role in this category of patients, but based on previous clinical trials using ^{153}Sm -EDTMP plus chemotherapy, the combination showed efficacy in some patients with far-advanced myeloma [271], so theoretically, these results could be replicated with Ra-223, with potentially fewer side effects. In view of the limited but positive experience in ^{153}Sm -EDTMP for osteosarcoma due to its osteoblastic nature [272], there is currently a phase I dose trial for the use of Ra-223 in patients with osteosarcoma at the M.D. Anderson Cancer Center (www.clinicaltrials.gov; NCT01833520).

10. Conclusions

There has recently been significant interest and progression toward the development of targeted alpha-particle therapy (TAT) for the treatment of solid tumors. Many pre-clinical and clinical studies have demonstrated promising anti-tumor efficacy results (Tables 2 and 3). The success of $^{223}\text{RaCl}_2$ in the care of patients with bone metastases and the apparent efficacy of additional TAT agents in recent clinical studies, e.g., ^{213}Bi -DOTATOC and ^{225}Ac -PSMA, has brought attention to this area of radiopharmaceutical development and increased the potential for successful translation of TAT for treatment of solid tumors and metastases [65,233]. Three radioisotopes have seen the most use in preclinical and clinical studies, ^{213}Bi , ^{225}Ac and ^{211}At . Each of these radionuclides has distinct advantages and disadvantages regarding availability, cost, decay chain attributes, tissue distribution

and stability of attachment. Of these, ^{225}Ac is readily available and has the distinct advantage of having a long, 10 d half-life, which allows for centralized production and distribution compared to the requirement of local production for the other radionuclides with shorter half-lives. Since TAT does not rely on the generation of free-radicals for the generation of DNA damage, as is the case for β -emission therapies, a major mechanism of resistance to radiation treatment can be bypassed; i.e., upregulation of superoxide dismutase. Some evidence of this was seen in a report stating that patients with tumors that have developed resistance to $^{90}\text{Y}/^{177}\text{Lu}$ -DOTATOC therapy were sensitive to subsequent treatment with ^{213}Bi -DOTATOC [65]. Additionally, the use of α -emitters enables solid-tumor targeted therapy with decreased peripheral tissue damage and the potential for decreased development of resistance due to the ability to kill surrounding tumor cells that do not express the target marker in a heterogeneous tumor microenvironment. Small molecules, peptides and monoclonal antibodies have been used for tumor targeting (Tables 2 and 3). However, due to the shorter clearance time (minutes to hours), small molecules and peptides may have an advantage over antibodies that can circulate for days in that they can accumulate in the target tumor and clear systemically prior to decay and deposition of energy, maximizing the beneficial effects of the ionizing radiation while minimizing the off-target exposure. This is especially true when using radionuclides with a long half-life; e.g., ^{225}Ac . Peptide-targeted agents may have a greater risk of generating renal toxicity [66], but there is evidence that lead optimization via medicinal chemistry could potentially improve the renal toxicity of a given peptide agent. For example, a recent preclinical study of two different gastrin-releasing peptide receptor (GRP-R)-targeting agents using two different ^{213}Bi -labeled GRP-R targeting peptides demonstrated differential renal toxicities [185]. TAT is currently emerging as a potentially new and effective approach toward the treatment of solid tumors, and the clinical translation of novel TAT radiopharmaceuticals appears to be on the near horizon. Since the abscopal effect has been observed in external beam therapy in animals, the ability to target internal radiation to tumors via TAT may have additional added value as a companion to the recently-successful immune checkpoint targeted therapies.

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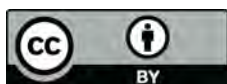
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Article

A Monte Carlo Method for Determining the Response Relationship between Two Commonly Used Detectors to Indirectly Measure Alpha Particle Radiation Activity

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Abstract: Using targeted ligands to deliver alpha-emitting radionuclides directly to tumor cells has become a promising therapeutic strategy. To calculate the radiation dose to patients, activities of parent and daughter radionuclides must be measured. Scintillation detectors can be used to quantify these activities; however, activities found in pre-clinical and clinical studies can exceed their optimal performance range. Therefore, a method of correcting scintillation detector measurements at higher activities was developed using Monte Carlo modeling. Because there are currently no National Institute of Standards and Technology traceable Actinium-225 (²²⁵Ac) standards available, a well-type ionization chamber was used to measure 70.3 ± 7.0 , 144.3 ± 14.4 , 222.0 ± 22.2 , 299.7 ± 30.0 , 370.0 ± 37.0 , and 447.7 ± 44.7 kBq samples of ²²⁵Ac obtained from Oak Ridge National Lab. Samples were then placed in a well-type NaI(Tl) scintillation detector and spectra were obtained. Alpha particle activity for each species was calculated using gamma abundance per alpha decay. MCNP6 Monte Carlo software was used to simulate the 4 π -geometry of the NaI(Tl) detector. Using the ionization chamber reading as activity input to the Monte Carlo model, spectra were obtained and compared to NaI(Tl) spectra. Successive simulations of different activities were run until a spectrum minimizing the mean percent difference between the two was identified. This was repeated for each sample activity. Ionization chamber calibration measurements showed increase in error from 3% to 10% as activities decreased, resulting from decreasing detection efficiency. Measurements of ²²⁵Ac using both detector types agreed within 7% of Oak Ridge stated activities. Simulated Monte Carlo spectra of ²²⁵Ac were successfully generated. Activities obtained from these spectra differed with ionization chamber readings up to 156% at 147.7 kBq. Simulated spectra were then adjusted to correct NaI(Tl) measurements to be within 1%. These were compared to ionization chamber readings and a response relationship was determined between the two instruments. Measurements of ²²⁵Ac and daughter activity were conducted using a NaI(Tl) scintillation detector calibrated for energy and efficiency and an ionization chamber calibrated for efficiency using a surrogate calibration reference. Corrections provided by Monte Carlo modeling improve the accuracy of activity quantification for alpha-particle emitting radiopharmaceuticals in pre-clinical and clinical studies.

activity quantification for alpha-particle emitting radiopharmaceuticals in pre-clinical and clinical studies

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1. Introduction

1. Introduction

The use of targeted ligands to deliver alpha particles directly to cancer cells has become a promising strategy to treat tumors [1–4]. This is because of their short path length of 40–80 μm and high linear energy transfer (LET~100 keV/ μm) [5]. These two properties make alpha particles highly cytotoxic to targeted cells, with little damage to surrounding normal cells.

Owing to the large amount of energy deposited by alpha particles, it is important to quantify the radioactivity of the targeted radiopharmaceutical initially injected and its subsequent biodistribution, pharmacokinetics, and radiation dose deposited in various tissues. As a result of its short path length, the direct measurement of alpha particles' activity in tissues is not possible in most, if not all, preclinical scenarios. If a parent metastable radioisotope also emits gamma rays, indirect methods of detection such as gamma spectroscopy can be used to estimate activity using scintillation detectors and well-type ion chambers that are commonly available in research laboratories and nuclear medicine clinics.

Actinium-225 (^{225}Ac) was chosen as the radionuclide for alpha-particle therapy (TAT) targeted to the melanocortin 1 receptor for treatment of metastatic uveal melanoma [6], the prostate-specific membrane antigen (PSMA) for metastatic prostate cancer [1], and CD33 for acute myeloid leukemia [7]. Clinical studies have been carried out for the PSMA and CD33 TATs and others in pre-clinical development will soon go to clinical trials [6].

^{225}Ac has a 10-day half-life and decays via six daughter radionuclides resulting in a net of four alpha particles per Actinium disintegration (Figure 1.) [8,9]. ^{225}Ac and two of its daughters, ^{221}Fr and ^{213}Bi , also decay with accompanying isomeric gamma photons. Using gamma detection systems, such as ion chambers or scintillation detectors, it is possible to indirectly determine the alpha activity by gamma ray abundance per decay conversions.

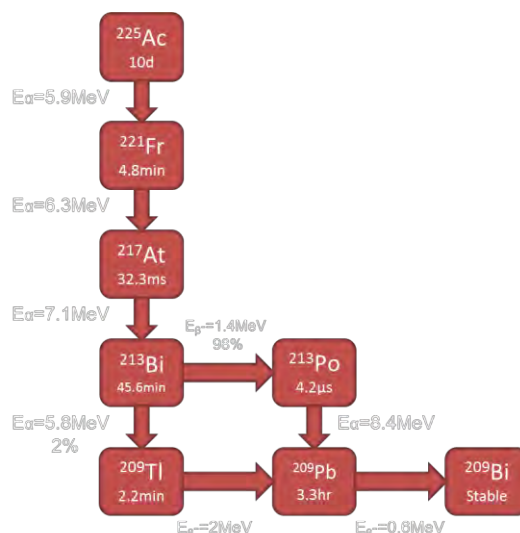


Figure 1. Decay chain of Actinium-225 (^{225}Ac).

Figure 1. Decay chain of Actinium-225 (^{225}Ac).

In the clinic, ion chambers are readily available and their use is the standard of practice for checking activities for diagnostic and therapeutic agents. An ion chamber reading does not provide information on the parent and daughter radionuclides because it does not discriminate the different changes between the parent and daughter radionuclides. A detector providing energy identification, such as a sodium iodide or a high-purity germanium (HPGe) detector, can be used. With this approach, it is possible to determine the activity of each gamma emitting daughter, which gives more information on how the decay species behave.

While HPGe seems to be the attractive option owing to its superior energy resolution, its performance suffers as a result of its low detection efficiency, cost, and requirement of sophisticated cooling systems. Despite the advantages of the NaI(Tl), dead time losses at higher activities and poor energy resolution may also provide incorrect activity measurements, leading to underestimation or overestimation of radiation dose [10].

In pre-clinical and clinical biodistribution studies, activity measurements of collected blood and tissues are used to calculate pharmacokinetics and radiation dosimetry. Therefore, accurate measurements for ^{225}Ac and daughters are needed. Hence, scintillation detector measurements are needed to generate these spectra. Alpha radiation dosimetry is performed using the methods recommended by the Committee on Medical Internal Radiation Dose [11]. It is essential that these measurements are accurate because the dosimetry estimates are then extrapolated to human estimations and, therefore, serve as the fundamental basis for all patient safety measures.

Scintillation detectors are calibrated by analyzing three aspects of the detector: energy, resolution, and efficiency [12]. Once the detector is fully calibrated, it can be used to perform gamma spectroscopic measurements in order to determine the activity of radioactive samples [13–18]. Ion chambers are calibrated by measuring a sample of the radiopharmaceutical in question with known activity, correcting for decay, and applying a calibration factor. The American National Standards Institute (ANSI) recommends that the applied calibration factor adjusts the measurement to within $\pm 10\%$ of the known activity [19]. Although reference standards have been developed for isotopes used in internal radiotherapy, for example, ^{177}Lu [20] ^{225}Ac standards are currently under development by NIST and are not yet available. For this study, ^{225}Ac provided by Oak Ridge National Lab was used as a cross-reference source.

While there are evident limitations in making clinical predictions when translating a radiopharmaceutical from mice to humans, it is important to minimize these limitations that are the result of instrumentation. Herein, a threshold is identified above which the scintillation detector cannot accurately measure the activities of ^{225}Ac and daughters. An activity response relationship between the ion chamber and scintillation detector measurements is reported and used in a method to improve activity determination above the threshold by correcting scintillation detector measurements via Monte Carlo simulations. These corrections improve the dosimetry of pre-clinical work and ultimately facilitate the translation of the new radiopharmaceutical to the human clinic.

2. Results

The system of ordinary differential equations describing the ^{225}Ac decay chain was solved to determine the daughters' activities as a function of time; these results are plotted in Figure 2 for ^{225}Ac , ^{221}Fr , and ^{213}Bi . For an initial activity of 3.7×10^4 kBq of ^{225}Ac , ^{221}Fr reaches secular equilibrium with ^{225}Ac in 55 minutes, while ^{213}Bi takes 380 minutes. Therefore, after 380 minutes (6.37 h), the parent and all daughter nuclides are in secular equilibrium.

To account for the statistical variance of physical spectra, a Gaussian energy broadening function was integrated with the ideal Monte Carlo simulation model. The parameters of this function were determined by nonlinear fitting of the measured FWHM versus energy. These parameters were determined to be $a = 0.005616$, $b = 0.0521$, and $c = 2.027$ with an R-squared value of 0.9984. This allowed the benchmark validation of the Monte Carlo model using the ^{137}Cs standard source (Figure 3). Simulating 155.4 kBq, the known source activity, showed a 1.02 mean percent difference at analogous bins between measured and simulated spectra.

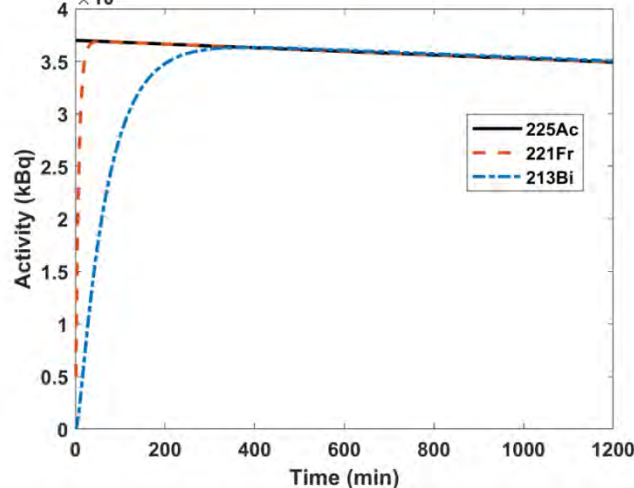


Figure 2. Solutions to system of decay ordinary differential equations of for an initial activity of 3.7×10^4 kBq of ^{225}Ac .

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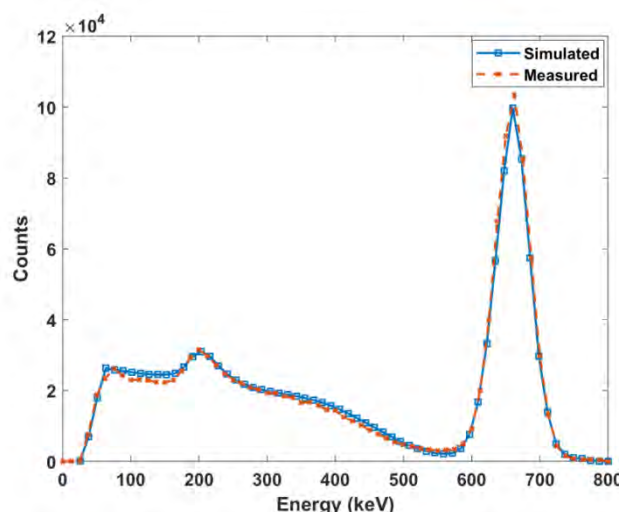


Figure 3. Comparison of measured and simulated spectra of ^{137}Cs .

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The measured NaI(Tl) gamma spectrum and Gaussian peak fitting of ^{225}Ac and its daughters can be seen in Figure 4. The two source method was used to determine the dead time of the scintillation detector to characterize the saturation of the signal with increasing activity. The value was determined to be $4.6 \times 10^{-5} \text{ s}$. The dead time value was incorporated into the gamma spectrum fitting method that was used to determine the scintillation detector activities of ^{225}Ac , ^{221}Fr , and ^{213}Bi , and these values were compared to the ion chamber measurements (Table 1). All samples were in secular equilibrium during measurement, as shown in Figure 2. The ion chamber manufacturer error is reported as 3%. Measurements using NIST-traceable sources within the activity range of 5.705 to 39.257 MBq had 3% uncertainties. However, when measuring NIST-traceable sources at the lower activities of 158.73 and 391.09 kBq, the uncertainties were 9.7% and 9.6%, respectively.

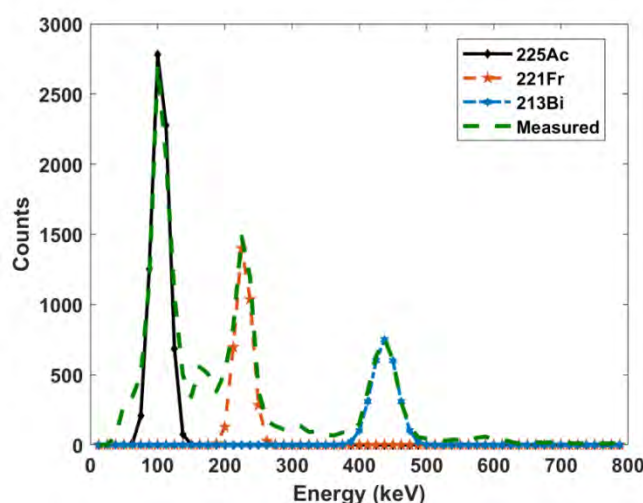


Figure 4. NaI(Tl) measured gamma spectrum with Gaussian fitting.

Table 1. Ion chamber and NaI(Tl) measured activities. Single measurements were taken in each detector. Notice that as the ion chamber readings increase, the discrepancies with the NaI(Tl) increase. All values are in kBq. Note that the uncertainties in the ion chamber readings are expressed as 10% in accordance with the calibration measurements in the activity range (above). The NaI(Tl) uncertainties are expressed as the propagation of uncertainties in all steps of the calculation. The difference between the ion chamber readings and the NaI(Tl) determined activity for ^{225}Ac are indicated in the last column to the right.

Ion Chamber Reading	NaI(Tl) Determined Activities				^{225}Ac Percent Difference
	^{225}Ac	^{221}Fr	^{213}Bi		
57.35 $\pm$ 7.57	66.97 $\pm$ 8.18	63.64 $\pm$ 7.98			
144.3 $\pm$ 14.4	96.20 $\pm$ 9.81	115.07 $\pm$ 10.73	111.74 $\pm$ 10.57		
222.0 $\pm$ 22.2	124.69 $\pm$ 11.17	152.81 $\pm$ 13.36	143.93 $\pm$ 12.00		
299.7 $\pm$ 30.0	140.23 $\pm$ 11.84	177.97 $\pm$ 13.34	165.39 $\pm$ 12.86		
370.0 $\pm$ 37.0	152.07 $\pm$ 12.33	198.32 $\pm$ 14.08	182.41 $\pm$ 13.51		
447.7 $\pm$ 44.7	140.23 $\pm$ 11.84	211.64 $\pm$ 14.55	187.59 $\pm$ 13.97		
299.7 $\pm$ 30.0	140.23 $\pm$ 11.84	177.97 $\pm$ 13.34	165.39 $\pm$ 12.86		
370.0 $\pm$ 37.0	152.07 $\pm$ 12.33	198.32 $\pm$ 14.08	182.41 $\pm$ 13.51		
447.7 $\pm$ 44.7	140.23 $\pm$ 11.84	211.64 $\pm$ 14.55	187.59 $\pm$ 13.97		

The spectra acquired by the scintillation detector (Figure 4) have overlapping multiple peaks that are further masked by the poor resolution of the detector. Monte Carlo simulations with 1 keV resolution, the spectra acquired by the scintillation detector (Figure 4) have overlapping multiple peaks that are further masked by the poor resolution of the detector. Monte Carlo simulations with 1 keV resolution allowed for the counting of contributions from each individual isotope as opposed to the overlapping ^{225}Ac and ^{213}Bi all exhibit many peaks in the 90 to 200 keV range. Monte Carlo simulations allowed for the counting of contributions from each individual isotope as opposed to the overlapping ^{225}Ac and ^{213}Bi all exhibit many peaks in the 90 to 200 keV range. Monte Carlo simulations allowed for the counting of contributions from each individual isotope as opposed to the overlapping ^{225}Ac and ^{213}Bi all exhibit many peaks in the 90 to 200 keV range.

Monte Carlo simulations were run for the NaI(Tl) detector radioisotope to the readings. The resulting spectra were compared to the corresponding scintillation detector spectra and it was observed that as the readings increased, the percent difference between the measured and simulated spectra increased. The percent differences ranged from 19% at 70.3 kBq up to 156% at 447.7 kBq. This is illustrated in Figure 6. The readings that minimized the mean percent differences were used as the equilibrium input activities in the Monte Carlo simulations. Figure 7 shows the matching of simulated spectra to measured spectra for all six of the samples of different quantities. This procedure was repeated for all ion chamber readings. Figure 8 shows the ion chamber reading and the simulated activity that matches to within 1% of the NaI(Tl) spectra for all of the samples. At low readings, the measured and simulated quantities agree reasonably well: 70.3 kBq measured in the ion chamber matched with 62.9 kBq equilibrium Monte Carlo activity and 144.3 kBq measured in the ion chamber matched with 107.3 kBq Monte Carlo activity. For larger ion chamber readings, the matched response began to plateau.

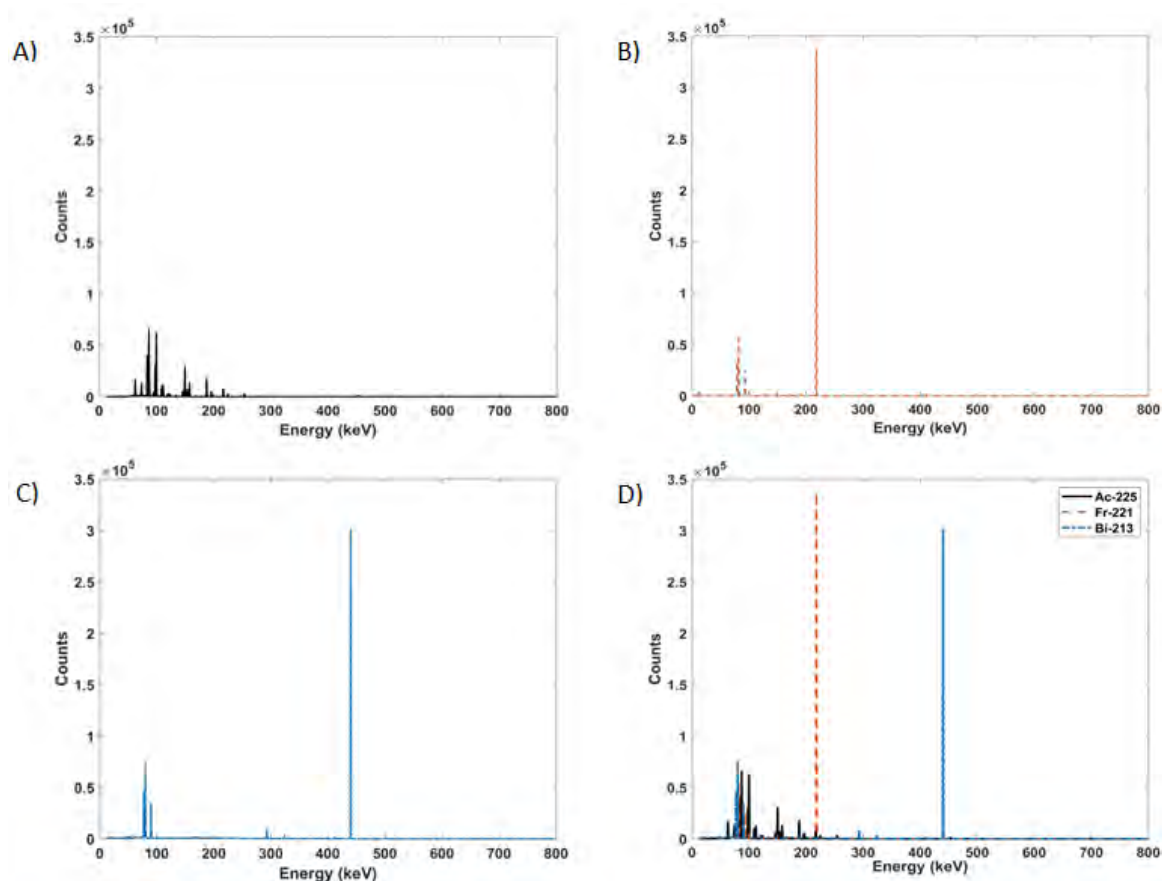


Figure 5. High-definition Monte Carlo simulated gamma spectra of (A) Ac-225, (B) Fr-221, (C) Bi-213, and (D) all three superimposed.

Monte Carlo simulated gamma spectra were compared to ion chamber readings. The resulting spectra were compared to the measured spectra. It was observed that as the simulated activity increased, the simulated spectra increasingly matched the measured spectra. This is illustrated in Figure 6. The simulated spectra were used as the equilibrium of simulated spectra. The procedure was repeated for the simulated activity of 100, 200, 300, 400, and 500 kBq. As the simulated activity increased, the measured chamber matched with the simulated chamber. At low activity, the measured chamber matched with the simulated chamber. As the activity increased, the response began to plateau.

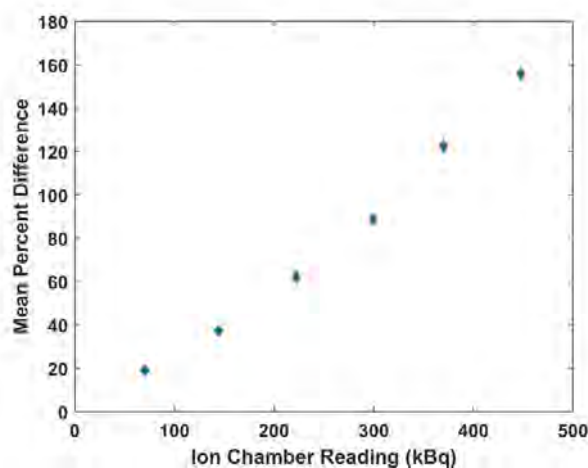
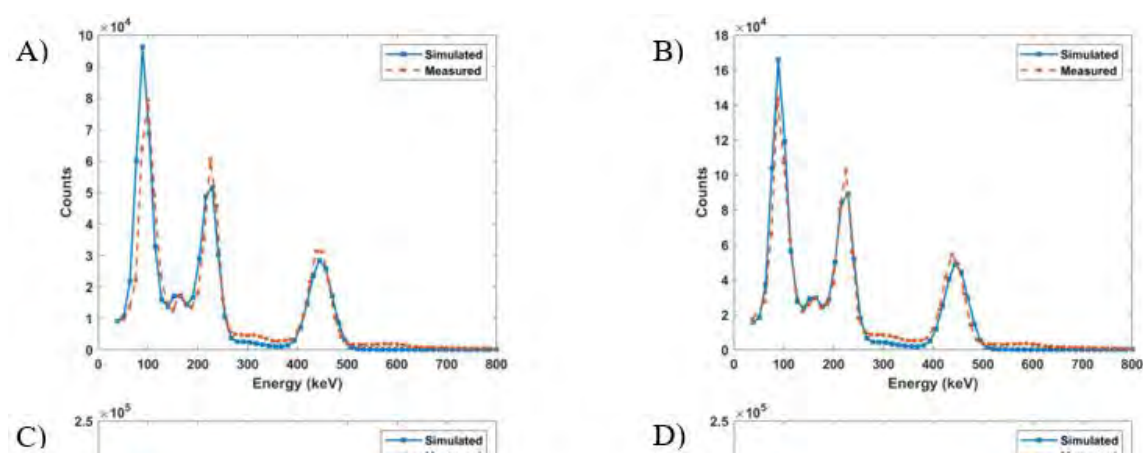


Figure 6. The mean percent difference between measured spectra and simulated spectra using the ion chamber reading measurement as input for the Monte Carlo model.



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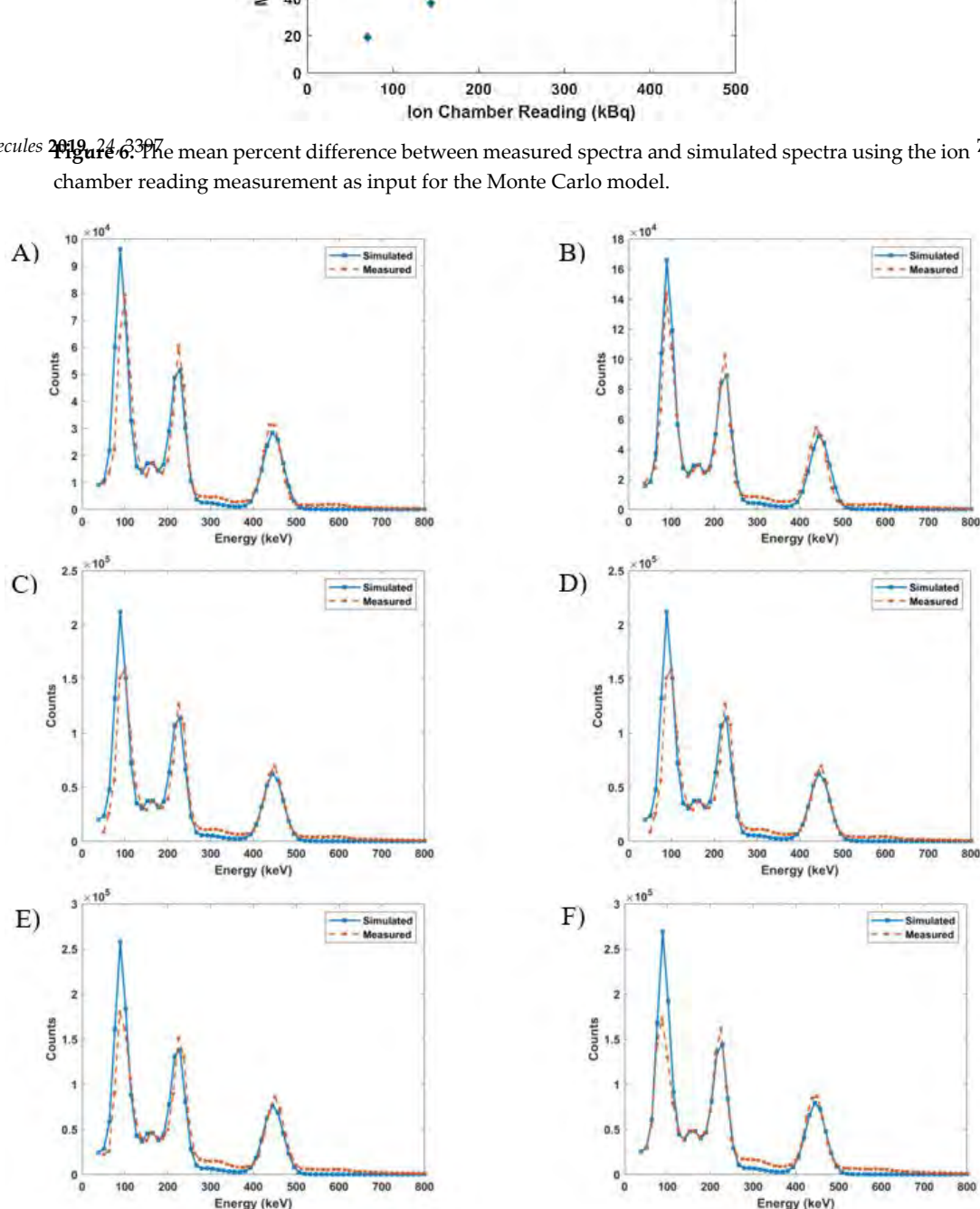


Figure 7. Measured vs. simulated ^{225}Ac spectra for (A) 70.3 kBq (0.17%), (B) 144.3 kBq (0.5%), (C) 222.0 kBq (0.18%), (D) 292.4 kBq (0.99%), (E) 379.0 kBq (0.11%), and (F) 447.7 kBq (0.02%). The values in parentheses are the mean percent differences between the two spectra.

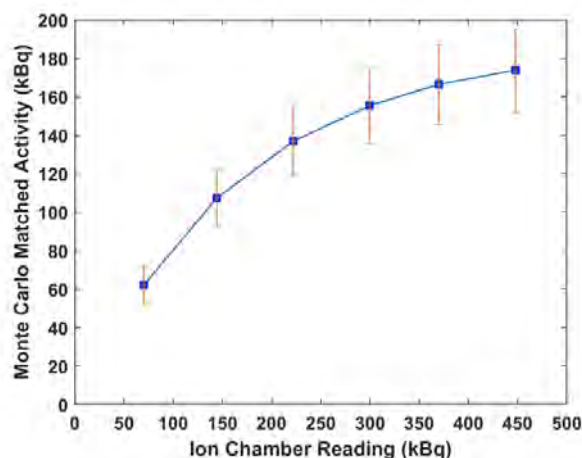


Figure 8. Activity response relationship between ion chamber reading and Monte Carlo-NaI(Tl) corrected spectra activity.

3. Discussion

The purpose of this work is to develop a method to determine the activities of ^{225}Ac and daughters in samples using standard clinical instruments. This is important for the determination of blood clearance pharmacokinetics and tissue biodistribution, both of which are used for radiation dosimetry calculations. In this work, an activity response relationship is determined between two

3. Discussion

The purpose of this work is to develop a method to determine the activities of ^{225}Ac and daughters in samples using standard clinical instruments. This is important for the determination of blood clearance pharmacokinetics and tissue biodistribution, both of which are used for radiation dosimetry calculations. In this work, an activity response relationship is determined between two radiation detectors commonly used for pre-clinical and clinical applications, a well-type NaI(Tl) scintillation detector and an ion chamber, respectively, where ^{225}Ac activities between the range of 70 to 450 kBq are not accurately measured by the scintillation detector. This relationship was used to inform Monte Carlo simulations that corrected scintillation detector measurements for accurate readings within this range. Although the error of the ion chamber measurements increased to ~10% within this activity range, compared with errors of 3% observed when measuring activities as low as 6 MBq, the error within this range for the scintillation detector as compared with the benchmarked Monte Carlo simulations ranged from 20% to 160% (Figure 6).

Several preclinical studies have reported using a NaI(Tl) gamma counter to measure ^{225}Ac injection and biodistribution activities [21–25]. Ion chambers are commonly used in nuclear medicine departments to prepare radiopharmaceuticals for patient injection. Measuring the activity of radioisotopes with complicated decay pathways, such as in the case of ^{225}Ac , the readings are not only the result of the parent's activity, but also from the contributions from the daughters' activities. From the solution of the system of decay equations, it was determined that ^{225}Ac and its daughters reached secular equilibrium in less than seven hours. While the daughters emit alpha particles and/or beta particles, the ranges of these particles are not penetrative enough to enter the ion chamber. Therefore, the ion chamber reading is a result of the gamma rays emitted from ^{225}Ac , ^{221}Fr , and ^{213}Bi . For example, a recent human ^{225}Ac therapy study reported injection activities for ^{225}Ac , but it was not clear if the reported values were from bulk measurements or if spectra were acquired and only ^{225}Ac emissions were considered [1].

Several groups have built Monte Carlo models of NaI(Tl) detectors to simulate response functions based on standard benchmark simulations and measurements of ^{137}Cs without description of further extensions of their models [26–31]. Herein, this procedure was extended to include the detector response for ^{225}Ac and its daughters. Implementation of the adjustment coefficients obtained by the nonlinear fitting into the Monte Carlo model adequately described the Gaussian spread of the detector pulses and was evident in benchmark testing. For all simulations, it was assumed that ^{225}Ac , ^{221}Fr , and ^{213}Bi were in secular equilibrium and thus had equal activities. The high-definition Monte Carlo simulations showed that both ^{221}Fr and ^{213}Bi exhibit peaks in the energy range of ^{225}Ac gamma pulse height distribution. Because of the 12.5 keV energy resolution of the NaI(Tl) detector, these peaks are summed to form a larger composite photopeak resulting in greater net counts, and thus over-calculated activities.

Gamma spectroscopic measurements were performed using the NaI(Tl) scintillation detector. All samples were measured after the calculated time to reach secular equilibrium, but the resulting activities were not equal. Further, the sum of activities for all three species greatly overestimated the ion chamber readings. Although the activities determined for the three isotopes should theoretically be identical, discrepancies are the result of the low energy bin resolution of the detector and the dead time count saturation. Using the measured ion chamber readings as input to the Monte Carlo simulations (Figure 6) largely overestimated the measured NaI(Tl) spectrum.

After minimizing the difference between each sample spectra and the Monte Carlo output, an activity response function relationship was determined for the two detectors (Figure 8). Both the Monte Carlo activity and the ion chamber reading represent the sum of the activities of ^{225}Ac , ^{221}Fr , and ^{213}Bi . The plot shows a curve with a decreasing positive slope as the ion chamber measurements increased beyond 148 kBq. This is a result of the NaI(Tl) dead time losses. As the activity of a sample increases, more pulses are lost, leading to errors in counting. The discrepancy between the low energy measured and simulated peaks in Figure 7 is also a result of dead time effects. The ratio of simulated

to measured peaks increases with increasing activity because the Monte Carlo model does not account for dead time. Further, the disagreement in the simulated and measured spectra in the low count region arises from the fact that the model represents an idealized detector and does not account for the metallic impurities of a real detector.

Standard NIST-traceable samples of ^{225}Ac are currently unavailable, so ^{225}Ac obtained from Oak Ridge was used as a surrogate calibration reference. These samples did not come with uncertainty estimations. As measurements using the recommended dial number agreed with the Oak Ridge surrogate reference samples to less than 10%, the readings of the ionization chamber were considered reliable for the study carried out in this work. The scintillation detector's measured energy resolution and detection efficiency over the energy range of ^{225}Ac gamma emissions (40–662 keV) agreed with other published studies and reports [28,32–34]. Consequently, this detector was also considered to be calibrated in efficiency and energy for ^{225}Ac analysis. The calibration sources used were all less than 185 kBq. When calibrating the ion chamber with sources with activity above 5.71 MBq, the error was less than 3%. When testing with sources in the lower activity range, error in the measurements increased to 10%, which is still within ANSI recommended tolerance. The measurement results showed that the responses from these two detectors do not correlate well in the activity range of 70 to 450 kBq, even though both detectors were calibrated for efficiency and energy resolution according to their respective recommended methods. The Monte Carlo model developed herein corrects NaI(Tl) measurements, allowing for a more accurate estimate of ^{225}Ac and daughter activities over a range of activities useful for both pre-clinical and clinical use.

4. Materials and Methods

4.1. Mathematical Model

The ^{225}Ac decay chain can be characterized by a system of ordinary differential equations that describe the activities and abundances of each species as a function of time (Figure 9) [35].

$$\begin{aligned}\frac{dN_{Ac^{225}}}{dt} &= -\lambda_{Ac^{225}} \times N_{Ac^{225}} \\ \frac{dN_{Fr^{221}}}{dt} &= -\lambda_{Fr^{221}} \times N_{Fr^{221}} + \lambda_{Ac^{225}} \times N_{Ac^{225}} \\ \frac{dN_{At^{217}}}{dt} &= -\lambda_{At^{217}} \times N_{At^{217}} + \lambda_{Fr^{221}} \times N_{Fr^{221}} \\ \frac{dN_{Bi^{213}}}{dt} &= -\lambda_{Bi^{213}} \times N_{Bi^{213}} + 0.9999 \times \lambda_{At^{217}} \times N_{At^{217}} \\ \frac{dN_{Rn^{217}}}{dt} &= -\lambda_{Rn^{217}} \times N_{Rn^{217}} + 0.0001 \times \lambda_{At^{217}} \times N_{At^{217}} \\ \frac{dN_{Po^{213}}}{dt} &= -\lambda_{Po^{213}} \times N_{Po^{213}} + 0.98 \times \lambda_{Bi^{213}} \times N_{Bi^{213}} + \lambda_{Rn^{217}} \times N_{Rn^{217}} \\ \frac{dN_{Tl^{209}}}{dt} &= -\lambda_{Tl^{209}} \times N_{Tl^{209}} + 0.02 \times \lambda_{Bi^{213}} \times N_{Bi^{213}} \\ \frac{dN_{Pb^{209}}}{dt} &= -\lambda_{Pb^{209}} \times N_{Pb^{209}} + \lambda_{Po^{213}} \times N_{Po^{213}} + \lambda_{Tl^{209}} \times N_{Tl^{209}} \\ \frac{dN_{Bi^{209}}}{dt} &= \lambda_{Pb^{209}} \times N_{Pb^{209}}\end{aligned}$$

Figure 9. System of equations describing the decay of ^{225}Ac to the very long-lived ^{209}Bi .

In Figure 9, N_x and λ_x denote the number of atoms of radionuclide and the decay constant of radionuclide x , respectively. These equations are not easily solvable in Mathematica with the initial (time zero), respectively, the steady state of 405 kBq and all daughters' activities of 100 kBq (the determinant (time zero) of ^{225}Ac activity is better 370 d104 kBq and all daughters' activities are set to 0 kBq to determine the times at which daughter products reach equilibrium with the parent.

4.2. Monte Carlo Model

The Monte Carlo N-Particle version 6.1 (MCNP6.1) package was used to simulate the pulse height gamma distribution from ^{225}Ac [36]. A computational model of the 2" × 2" NaI(Tl) 4π detector was built using the material compositions and dimensions given in the manufacturer's manual, as illustrated in Figure 10 [32].



radionuclide X , respectively. These equations were simultaneously solved in Mathematica with the initial (time zero) ^{225}Ac activity set to 3.7×10^4 kBq and all daughters' activities set to 0 kBq to determine the times at which daughter products reach equilibrium with the parent.

The Monte Carlo N-Particle version 6.1 (MCNP6.1) package was used to simulate the pulse height gamma distribution from ^{225}Ac [36]. A computational model of the $2'' \times 2''$ NaI(Tl) 4π detector was built using the material compositions and dimensions given in the manufacturer's manual, as illustrated in Figure 10 [32].

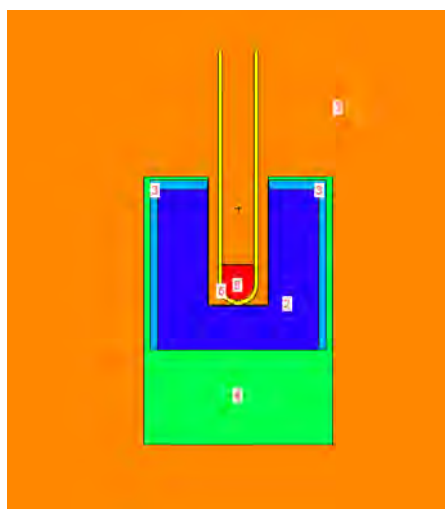


Figure 10. Geometry of the Monte Carlo model wipe test scintillator detector. Image generated using the Visual Editor software for MCNP. 22: NaI; 33: MgO; 4: Al; 6: polyethylene; 7: air; 8: H₂O with ^{225}Ac , ^{223}Fr , ^{213}Bi source distributions.

A standard 5 mL polyethylene test tube filled with 1 mL of water was placed in the well of the detector. The source was defined to be uniformly distributed in the 1 mL water volume. All 75 photon emissions from ^{225}Ac , ^{223}Fr , and ^{213}Bi were defined by energy and abundance [9]. The photomultiplier tube was modeled as a 30 mm diameter aluminum cylinder to account for backscatter [26].

Photon transport was conducted using the mcplib84 photoatomic data library and detailed photon physics interactions [37]. These include Thomson scattering, Compton scattering, and photoelectric absorption with the creation of fluorescence photons or Auger electrons. Full photon and electron simulations were performed and spectra were compared to those obtained from only photon transport. Photon and electron cell importances were set to 1 for all cells in the geometry. A mean percent difference of less than 1% between photon/electron transport and photon only transport was observed in benchmark generated spectra of ^{137}Cs . There were also less than 1% differences in the Compton edge and Compton regions. Therefore, the computationally expensive tracking of electrons was omitted in subsequent simulations and electron cell importances were set to 0. In this case, the electrons generated are assumed to deposit their energy locally. Any electron induced bremsstrahlung photons produced are accounted for in the thick-target bremsstrahlung model [38]. This model skips the electron tracking step and transports the generated photon in the direction of the parent electron.

The pulse height distribution tally (F8) was used to calculate the deposited energy in the NaI(Tl) crystal. This tally accumulates the kinetic energy lost by local photon-induced secondary electrons, per history, in a specified volume. The tally was divided into 64 bins, each with a 12.5 keV width to match the energy resolution of the detector. To relate the simulated response to activity, values for the number of gamma emissions per disintegration for each isotope, scaled by the activity, were placed on the source probability card in the source definition. The resulting tallies were then multiplied by the sum of these values, the live acquisition time, and an activity conversion factor containing photon abundance per decay. For each simulation, 2×10^8 histories were run to achieve less than 1% error under each photopeak.

MCNP6.1 models an ideal detector and thus does not take into account the Gaussian shape of experimental NaI(Tl) energy resolution resulting from intrinsic light collection inefficiency, statistical fluctuation in charge multiplication in the photomultiplier tube, and electronic noise [39]. To account for the statistical variance of physical spectra, a Gaussian energy broadening function (MCNP6.1

FT8 GEB) was applied to the simulated pulse height distribution. The GEB function was obtained experimentally from measurements of the detector's specific full width at half maximum (FWHM) from a set of isotopes of differing energies. Spectra from five isotopes were measured and the FWHM of each peak was calculated. The isotopes used and their physical characteristics are listed in Table 2.

Table 2. Physical characteristics of the radioactive sources used for NaI(Tl) detector energy response measurements [9].

Source	Half-Life	Energy (keV)
^{129}I	1.6 ⁷ yr	39.5
^{241}Am	432.6 yr	59.5
^{57}Co	271.7 d	122.0
^{68}Ga	67.7 m	511.0
^{137}Cs	30.1 yr	661.6

A curve of FWHM as a function of energy was fit with the following nonlinear function shown in Equation (1) [36]:

$$FWHM = a + b\sqrt{E + cE^2}, \quad (1)$$

where E is the incident energy of the incident photon in keV; and a, b, and c are parameters that were determined by least squares fitting and input in the FT8 command.

4.3. Monte Carlo Simulations

There are no standard NIST-traceable calibration sources for ^{225}Ac to validate the detector model. Instead, the spectrum of a NIST-traceable 155.4 kBq ^{137}Cs (662 keV photopeak) source was measured in the NaI(Tl) detector and compared to a simulated spectrum of the same activity for validation.

After the model was validated, a high definition version of the F8 pulse height tally (1 keV bin width) was simulated with only ^{225}Ac defined as the source. This was followed by separate high definition simulations of ^{221}Fr and ^{213}Bi . These simulations were done to see what radiations contribute to each peak, which are masked by the poor energy resolution of the NaI(Tl) detector.

4.4. Experimental Measurements

The ^{225}Ac used in this work was obtained from Oak Ridge National Laboratory (ORNL). The Atomlab™ Wipe Test Counter (Biodex Medical Systems, Inc., Shirley, New York, USA) was used to perform gamma spectroscopy measurements with ^{225}Ac . The wipe test counter is a 2" × 2" NaI(Tl) well-type scintillation detector with 1.2 cm of lead shielding. The multichannel analyzer associated with the wipe test NaI(Tl) detector has 64 12.5 keV channels ranging from 0 to 800 keV. Spectra obtained with the NaI(Tl) detector for 30 s acquisition time and full energy window to include gamma counts from ^{225}Ac , with composite peak at 99.8 keV, and from its two gamma-emitting daughters, ^{221}Fr ($T_{1/2} = 4.9$ min) with a peak at 218.1 keV and ^{213}Bi ($T_{1/2} = 46$ min) with a peak at 440.5 keV. Background spectrum measurements were performed and subtracted from all obtained ^{225}Ac spectra. The net background readings for the NaI(Tl) detector were in the range of 100 to 200 net counts per minute. The net counts for an ^{225}Ac sample of 45 kBq activity was 444,000 per minute.

Dead time measurements and calculations were performed according to the two-source method [10]. Two different sources of ^{22}Na with different activities were each measured individually and then in combination.

In-house software was developed using MATLAB to calculate the net number of gamma counts, N, by fitting each peak with a Gaussian fit and integrating. Alpha particle activity for each species was calculated using factors for gamma abundance (Y) per alpha decay: ^{225}Ac (1%), ^{221}Fr (11.4%), ^{213}Bi (25.9%) [9].

The detector efficiency, ϵ , and energy calibration for the NaI(Tl) detector were determined by performing measurements of NIST-traceable ^{137}Cs , ^{133}Ba , and ^{57}Co sources. These radionuclides have numerous photopeaks covering the gamma energy spectrum of ^{225}Ac . The activity is then given by the following Equation (2):

$$A = \frac{N}{t * \epsilon * Y'} \quad (2)$$

where t is live time. The activity of ^{225}Ac determined using this approach was compared to a decay corrected sample of known activity from ORNL. A factor of 0.082 was applied to Equation (2) to obtain ^{225}Ac activity values to within 5% of the ORNL specified ^{225}Ac activity. The uncertainty in the NaI(Tl)-determined activity was calculated by propagating individual uncertainties in the net number of counts and the efficiency measurements, as is typically done in nuclear counting measurements.

The Atomlab™ 500 Dose Calibrator is a well-type pressured ion chamber filled with argon gas that is commonly used in the clinic. This instrument was calibrated for energy and efficiency with the following NIST-traceable sources: ^{137}Cs (6.63 MBq), ^{57}Co (39.26 MBq), and ^{133}Ba (5.71 MBq) by the vendor. Lower activity NIST-traceable sources provided by Eckert & Ziegler were also used: ^{137}Cs with 158.73 kBq and ^{57}Co with 391.09 kBq activities. No significant variation in the calibration coefficient as a function of photon energy was observed. An ion chamber linearity test was performed with ^{18}F in the activity range of 1.2 GBq to 266.0 kBq and a variance of 1.4% was observed. The dial number was set at 38.2 for ^{225}Ac , as indicated by the vendor and confirmed by our comparison with two decay corrected samples of different activities obtained from Oak Ridge. Measurements of these samples in the ion chamber agreed with the Oak Ridge measurements to within 7%. In order to determine the relationship between this ion chamber and the NaI(Tl) detector, six different activities of ^{225}Ac samples were measured using the ion chamber: 70.3 ± 7.0 , 144.3 ± 14.4 , 222.0 ± 22.2 , 299.7 ± 30.0 , 370.0 ± 37.0 , and 447.7 ± 44.7 kBq. Each sample was measured and placed in the NaI(Tl) detector, and a spectrum was obtained and compared. Using the activity reading from the ion chamber as the initial parent–daughter equilibrium activity input for the Monte Carlo model, simulated spectra were calculated and compared to the measured spectra from the NaI(Tl) detector. Mean absolute percent differences at analogous bins between the experimental and simulated spectra were calculated. Using the ion chamber measured activity as input to the Monte Carlo simulations underestimated the readings from the NaI(Tl), so several successive runs were completed using different activities as input in symmetrical range around the initial point in order to match the spectra. The percent differences were plotted against input activity and fitted with a linear model. The x-intercept was found in order to determine the minimum mean percent difference between the measured and simulated spectra. This process was repeated for each sample and an activity response function relationship was determined between the ion chamber and the NaI(Tl) detector. The uncertainty in the activity relationship determined using this methodology was analyzed by adding the uncertainties in the NaI(Tl) measurements, σ_{NaI}^2 ; the ion chamber measurements, σ_{IC}^2 ; and the Monte Carlo calculations, σ_{MC}^2 , in quadrature (Equation (3)):

$$\sigma_A = \sqrt{\sigma_{\text{NaI}}^2 + \sigma_{\text{IC}}^2 + \sigma_{\text{MC}}^2} \quad (3)$$

5. Conclusions

In this work, the ^{225}Ac activity response function between two commonly used instruments was investigated, a range of activities (70 to 450 kBq) was identified, and a method was developed using Monte Carlo simulations to correct scintillation detector measurements within this range. The scintillation detector was calibrated in energy and efficiency using NIST-traceable sources covering a broad energy range, while the ion chamber was calibrated in efficiency using a surrogate calibration reference. The error of ion chamber measurements increased with decreasing activity, but remained within the ANSI recommended value of 10%. The NaI(Tl) performed poorly from 70 to 450 kBq. Therefore, a Monte Carlo model was built to correct the NaI(Tl) measurements, thereby providing

improved accuracy. The corrections provided by the Monte Carlo model will provide a useful tool to make corrections to the activity values measured by the well-type NaI(Tl) detector. This allows for the better quantification of alpha-particle emitting radiopharmaceuticals and daughter byproducts in pre-clinical and clinical studies. In this case, use of scintillation gamma spectroscopy will be the preferred method.

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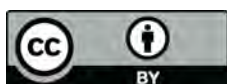
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Genomic Mutation as a Potential Driver of the Development of Bone-Related Cancers

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Nomenclature

AID	Activation-induced cytidine deaminase
CML	Chronic myeloid leukemia
CSR	Class switch recombination
DSBs	Double strand breaks
Ig	Immunoglobulin
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
NSG	The next-generation sequencing
Ph	Philadelphia chromosome mutation or Philadelphia translocation
PI3K	Phosphoinositide 3-kinases
PTHrP	Parathyroid hormone-related protein
RANKL	Receptor activator of nuclear factor κ B ligand
RB1	Retinoblastoma 1
SOH	Somatic hypermutation
TGF-β	Transforming growth factor-beta

Introduction

“What causes cancer?” This is still an unanswered, and likely unanswerable (for now), question, despite recent advances in understanding the molecular and cellular basis of cancer development and progression. One might conclude that cancer is an aging-related disease. Seventy-eight percent of people in US who are diagnosed with cancer are older than 55 years ([American Cancer Society, 2015](#)). Indeed, the average ages when patients are diagnosed with breast, lung, and prostate cancer, are around 61, 70, and 66 years, respectively ([Zullig et al., 2012](#)). These epidemiological data clearly support the notion that aging impacts cancer development. However, this doesn’t explain why cancer also often occurs in younger age groups, including pediatric populations. Moreover, a recent trend has shown that the incidence of cancer is rising among young adults ([Sung et al., 2019](#)). Mechanisms of carcinogenesis between young and old age patients may not be the same.

Although it is still controversial, the development of some of the pediatric cancers are thought to be associated with inherited gene mutations or alterations. In Alfred Knudson’s observation of patients of retinoblastoma ([Knudson, 1971](#)), a pediatric cancer of retinal cells, he found that (1) there are inherited and non-inherited (sporadic) types of retinoblastoma; and (2) the inherited retinoblastoma occurs more often bilaterally at early age, whereas the sporadic retinoblastoma usually presents unilaterally at later age. Based on these findings, Knudson hypothesized that the inherited retinoblastoma already acquires one mutation or a “hit” (e.g. the tumor suppressor retinoblastoma 1 (RB1) gene mutation) on one allele of all genes and therefore another “hit” can happen easily and on the multiple genes. On the contrary, it is very difficult to develop the second “hit” on a single gene, when there is no inherited mutation. Thus, it usually takes a long period of time to develop the sporadic retinoblastoma and it happens unilaterally. This is now known as the two-hit theory. If this theory is plausible, the notion that the risk of developing cancer increases as we grow older certainly makes sense, since most genes do not show phenotypic changes unless two or multiple “hits” occur in the specific locations ([Knudson, 1971](#)). Although we all have some inherited gene mutations, the prevalence of cancer risk-associated inherited gene mutations is not very high. Therefore, it takes time to develop multiple mutations on one single gene. Moreover, genes are

more frequently mutated in older individuals. Older people have been exposed to more carcinogens, such as UV light, tobacco, and alcohol, than younger people. Carcinogens are known to cause mutations, such as in the TP53 gene, which is associated with P53 protein production (Ouhit et al., 1998; Rivlin et al., 2011; Ronchetti et al., 2004). P53 serves as a tumor suppressor protein by inducing cell cycle arrest or cell death of fast dividing cells, which may eventually become cancerous. Because gene mutations or alterations can contribute to cancer at any age, the determination of oncologic gene signatures is a useful first step toward understanding cancer development and progression.

Cancer genomic research has been intensively conducted in the last 30–40 years (Stratton et al., 2009), in parallel with the improvement of high-throughput DNA sequencing technology. The recent development of the next-generation sequencing (NGS) technologies have allowed for the production of large volumes of sequencing data rapidly (4 h/run vs. 24 h/run with the first-generation sequencing technologies) and cost effectively (4 cents/kilobyte vs. 20 cents/kilobyte with the first-generation sequencing technologies) (Kchouk et al., 2017). Using NGS, thousands of innate and acquired mutations associated with cancer development (e.g. BRCA1 and BRCA2, known to be associated with development of breast and ovarian cancers Mersch et al., 2015) have been discovered (Kamps et al., 2017). Although these findings result in deeper understanding of cancer genomics, and improvement of diagnoses and treatments of cancer, we are still losing the battle against particularly “metastatic disease.” Bone is one of the major metastatic sites (Coleman, 2001). When cancer patients with cancer develop bone metastases, their 5-year survival rate declines by more than 70% (Svensson et al., 2017) and the median survival time is approximately 2–3 years (Bienz and Saad, 2015). Although bisphosphonate and denosumab, a human monoclonal anti-receptor activator of nuclear factor κ B ligand (RANKL) antibody, which both decrease bone resorption, are established treatments for bone metastases, they ultimately fail to improve overall survival (Stopeck et al., 2010; Fizazi et al., 2011). Radium-223, which forms complexes with hydroxyapatite in bone (Badrising et al., 2016; Vignani et al., 2016; Abou et al., 2016), can extend overall survival of prostate cancer patients with bone metastases (Parker et al., 2013), but only by a few months (average = 3 months). Since these treatments mainly target the bone microenvironment, targeting mechanisms other than the bone microenvironment are needed if eradicating bone metastasis is to be an achievable clinical goal.

Targeted therapies against BCR-ABL gene mutation with small molecular kinase inhibitors (e.g. imatinib) have successfully improved overall survival of patients with chronic myeloid leukemia (CML) (Iqbal and Iqbal, 2014; Massoud et al., 2017; Hochhaus et al., 2017). Since specific mutations associated with development and progression of cancers found in bone (or bone-related cancers), not only bone metastatic cancers, but also cancers with mesenchymal origin (e.g. osteosarcoma) and cancers with hematopoietic origin (e.g. multiple myeloma, leukemia) have recently been discovered, targeting the mutations can lead to new therapeutic approaches for these cancers. In this review, we will discuss (i) the recent discovery of gene mutations in bone-related cancers; (ii) progress and shortcomings of the mutation-targeted therapies for bone-related cancers; and (iii) future directions for genomic studies in bone-related cancers.

Gene Mutations in Bone Metastasis

Each cancer with metastatic ability has a particular preference for organs where it disseminates. For example, prostate cancer preferably metastasizes to bone, but rarely spreads to brain (Taddei et al., 2012; Sutton et al., 1996). Surprisingly, the anatomical location of cancers and blood flow patterns do not explain preference of metastatic sites of cancers. The metastatic process requires the interaction between cancer cells and the secondary sites, or metastatic microenvironment. Stephen Paget first hypothesized (Ribatti et al., 2006; Paget, 1989; Fidler and Poste, 2008), and recent evidence now strongly supports, that the metastatic microenvironment (soil) controls the metastatic fate of cancer cells (seed) (Liu et al., 2017). Moreover, it has been demonstrated that subpopulations of tumor cells can metastasize to certain organs (Langley and Fidler, 2007). This is believed to be due in part to heterogeneous nature of cancer. In fact, there is a distinct pattern between gene expression among cancer cells that possess different organ-tropisms (Chiang and Massague, 2008; Weigelt et al., 2003), suggesting that there could be gene expression patterns associated with bone metastatic potential.

Parathyroid hormone-related protein (PTHrP) is one of the well-studied molecules that are relevant for bone metastatic progression. PTHrP derived from bone metastatic cancer cells stimulates osteoblasts to release RANKL, which enhances osteoclastogenesis, resulting in osteolytic bone lesions (Soki et al., 2012). This bone resorption process enables bone matrix to release transforming growth factor- β (TGF- β) which further induces PTHrP release from bone metastatic cancer cells (Clines and Guise, 2005). Along with this notion, 90% of breast cancer tissues found in bone metastatic sites express high levels of PTHrP, whereas only 17% of those found in metastatic sites other than bone highly express PTHrP (Powell et al., 1991). Interestingly, the oncogenic KRAS gene mutation correlates with increased PTHrP levels in cancer (Kamai et al., 2001; Rudduck et al., 1993). The KRAS mutation is specifically associated with poor prognosis of lung adenocarcinoma patients with bone metastases, while KRAS mutation fails to affect prognosis of those patients with other metastases (Lohinai et al., 2017). Although further studies are clearly needed, these findings suggest that KRAS mutation plays a crucial role in metastatic progression in bone by increasing PTHrP levels in bone metastatic cancer. Furthermore, a recent NGS study identifying gene mutations associated with bone metastasis in 8 bone metastatic samples obtained from non-small cell lung cancer patients revealed that among 3620 mutated genes discovered, mutations of hepatocyte nuclear factor 1 alpha, adenomatous polyposis coli, and CD22 are found in all 8 samples and have higher consistent mutations (>50%) (75%, 62.5%, and 50%, respectively) (Zhang et al., 2016). These findings suggest that gene mutations also play a crucial role in the development of bone metastasis, and that targeting gene mutations can be a potential alternative therapeutic approach for bone metastasis.

Gene Mutations in Osteosarcoma

Osteosarcoma is one of the most common malignant cancers of mesenchymal origin. Osteosarcoma cells are potentially derived from cells along the differentiation spectrum from mesenchymal stem cells to mature osteoblasts (Le Nail et al., 2018; Marina et al., 2004). Osteoblast-lineage cells are naturally slow-growing. During growth spurts and puberty, these cells undergo rapid cell division, resulting in an increased error rate in DNA synthesis (Cotterill et al., 2004). The accumulation of DNA errors and/or genomic instability is thought to cause transformation of osteoblasts to osteosarcoma (Yang et al., 2017; Martin et al., 2012). Osteosarcoma therefore occurs more often in adolescence. Although fairly uncommon, osteosarcoma can also be found in older patients. Unlike other types of cancers, adult osteosarcoma is not considered a lifestyle-related cancer. Instead, adult osteosarcoma is believed to be associated with genetic disorders caused by both somatic and germline gene mutations, such as multiple exostoses syndrome and tuberous sclerosis (Bukara et al., 2018; Farid and Ngeow, 2016). Therefore, genomic mutation is a key driver of the development of osteosarcoma.

Osteosarcoma is often associated with complex karyotypes due to a high frequency of chromosomal translocations, amplifications, and deletions (Biegel et al., 1989). The deletion mutation of a tumor suppressor TP53 is a hallmark of osteosarcoma (Guo et al., 1996). It has been revealed that more than 50% of osteosarcoma tissue samples have allelic deletion on chromosome 17p13 (where the TP53 gene is located) (Guo et al., 1996). Additionally, about 25% of samples without 17p13 deletion showed rearrangements (addition of exon fragments) and point mutations (nonsense) in the TP53 gene, resulting in loss-of-function mutation (Toguchida et al., 1992). Consistently, a study using a whole genome sequencing analysis of 34 osteosarcoma tumor samples found that 80% of the tumors have mutations in both alleles of the TP53 gene and 90% have mutations in at least one allele (Chen et al., 2014). It has been suggested that loss of TP53 functions, such as cell cycle arrest, DNA repair, and apoptosis, are responsible for the development of many cancers including osteosarcoma (Rivlin et al., 2011). TP53 is also known to inhibit DNA damage (e.g. gene amplification and deletion) mediated by genotoxic treatments like radiation, further suggesting that loss of TP53 functions leads to genetic alterations in other oncogenes and tumor suppressor genes (Overholtzer et al., 2003). Consistently, studies have shown that treatments of PRIMA-1 [2,2-bis (hydroxymethyl)-3-quinuclidinone], which is a compound that can restore mutant TP53 activities by converting its protein conformation to a wild type TP53 (Rangel et al., 2019), inhibits proliferation of a mutant TP53-expressing human osteosarcoma cell line SaOS-2 by restoring a pro-apoptotic pathway induced by TP53 (Bykov et al., 2002; Bykov et al., 2005; Rokaew et al., 2007; Lambert et al., 2009). In addition, the PRIMA-1 analog PRIMA-1MEt (APR-246) inhibited growth of a chemotherapeutic agent camptothecin-resistant osteosarcoma, lung cancer, prostate cancer, and ovarian cancer cells due to their TP53 mutation (Bykov et al., 2005). Although PRIMA-1 and APR-26 have not yet been tested in a clinical trial in osteosarcoma patients, the safety and clinical activity of APR-246 in combination with chemotherapies are currently tested in prostate cancer, ovarian cancer, and melanoma patients (Parrales and Iwakuma, 2015). An oncogenic E3 ubiquitin-protein ligase MDM2 promotes degradation of TP53 through indirect binding (Moll and Petrenko, 2003; Kussie et al., 1996; Haupt et al., 1997). A potent MDM2 antagonist RG7388 (Idasanutlin) has been entered phase III clinical trials in acute myeloid leukemia patients (Tisato et al., 2017). However, RG7388 failed to induce apoptosis in TP53 mutation-positive osteosarcoma cells, resulting in development of resistance to RG7388 (Skalniak et al., 2018). Although RG7388 might be a potential therapeutic option for osteosarcoma since it can inhibit degradation of TP53 protein, further investigations are warranted.

Besides TP53, the mutation of a tumor suppressor Rb1 gene, a key regulator of cell cycle, is also frequently found in osteosarcoma (39–64%) (Chen et al., 2014) and often presents as a null or dysfunctional mutation (Harbour and Dean, 2000; Scholz et al., 1992). Similar to TP53 loss, the loss of Rb1 increases genomic instability (van Harn et al., 2010). Interestingly, in 25–30% of hereditary retinoblastoma, which is caused by a germline Rb1 mutation, patients develop osteosarcoma (Hansen et al., 1985). Rb1 protein induces cell cycle progression by binding to the E2F transcription factor in G1 phase (Giacinti and Giordano, 2006). This binding can be disrupted when Rb1 is phosphorylated by CDK4 or CDK6 kinases, and consequently Rb1 becomes inactive (Knudsen and Wang, 1997). Therefore, activation of Rb1 function can be achieved by the inhibition of CDK4/6 kinases (Spring et al., 2016). Although not in osteosarcoma, preclinical studies in Rb1 mutation-positive breast and pancreas cancer cells have shown anti-tumor effect of CDK4/6 inhibitors in combination with other cytotoxic agents, such as chemotherapies, targeted signaling inhibitors, and endocrine therapies (Spring et al., 2016).

Amplifications and gain-of-function mutations in an oncogene phosphoinositide 3-kinases (PI3K) are also seen in osteosarcoma (Zhang et al., 2015). Indeed, missense mutations in the PIK3CA and PIK3R1 genes and deletion and/or nonsense mutations in the PIK3R5, PIK3R6, and PIK3C3 genes were observed in osteosarcoma patient samples (Perry et al., 2014; Chen et al., 2014). A PI3K inhibitor BKM120 (buparlisib) inhibited proliferation and induced apoptosis of osteosarcoma cells by disrupting Akt/mammalian target of rapamycin (mTOR) pathway, a major downstream targets of PI3K (Bavelloni et al., 2019). Phase II clinical trials of a potent mTOR inhibitor everolimus in advanced osteosarcoma patients with progressive disease after standard targeted therapies, including sorafenib, anthracycline and ifosfamide, revealed that 29%–45% of patients are progression free at 4–6 months (Grignani et al., 2015; Yoo et al., 2013; Mita et al., 2013; Chawla et al., 2012).

Gene Mutations in Hematopoietic Malignancies

Within the bone marrow, cells of hematopoietic origin can also be cancerous. These types of cancer are called “liquid cancers” or “blood cancers” and can be mainly classified as leukemias, lymphomas, and myeloma. When plasma cells, which produce antibodies, become mutated and grow in an uncontrolled manner in the marrow, they progress into multiple myeloma. Although the mechanisms responsible for the development of multiple myeloma remain yet unclear, studies have suggested that genetic alterations that occur during the differentiation and maturation process of plasma cells cause multiple myeloma (Boyle et al., 2014). During the antibody production process, immature B cells, which migrate from bone marrow to the germinal center of lymph nodes, undergo affinity maturation that involves two processes; somatic hypermutation (SOH) and class switch recombination (CSR) (Gonzalez et al., 2007). SOH occurs in the variable antigen-binding coding sequence of immunoglobulin (Ig) genes that regulates specificity and binding affinity of antibodies (Klein et al., 1998; Neuberger and Milstein, 1995). CSR occurs in the constant region of the heavy chain producing genes to generate antibodies of different immunoglobulin isotypes, such as IgG, IgA, and IgM (Stavnezer, 1996; Honjo et al., 2002). Both SOH and CSR processes are regulated by activation-induced cytidine deaminase (AID, an enzyme that creates point mutations in DNA converting cytosine bases to thymidine bases) (Stavnezer et al., 2008; Chaudhuri and Alt, 2004). AID is also known to create DNA damage, such as double strand breaks (DSBs), in Ig loci during the affinity maturation process (Peled et al., 2008; Stavnezer, 2011). These DSBs are usually repaired within the region where Ig genes locate, but also results in the abnormal chromosomal translocations (~1000 cells per day) (Adams et al., 1982; Ramiro et al., 2004). Mutations in oncogenes or tumor suppressor genes can be induced by the accumulation of the abnormal chromosomal translocations overtime, and these mutations cause malfunction in matured plasma cells, eventually becoming myeloma (Bandelow et al., 2007). Additionally, although it is rare, AID can also induce DSBs in ATM genes, associated with the DNA repair system and responsible for activation of TP53-dependent apoptosis (Cheng and Chen, 2010). Indeed, 7181C/T mutation in the ATM gene is observed in a subpopulation of multiple myeloma patient tumor samples where ATM expression significantly decreases (Austen et al., 2008). This study also suggested that the loss of ATM function is one of the underlying mechanisms of myeloma development (Austen et al., 2008). Moreover, despite low frequency (4%), the association between ATM mutations and aggressiveness of multiple myeloma has recently been demonstrated. Whole-exome sequencing in 463 myeloma patient samples revealed that ATM mutations are associated with impaired progression-free survival (median, 15.4 vs. 26.6 months, $P = 0.05$) and 2-year overall survival (95% CI, 50% vs. 80%, $P = 0.01$) (Walker et al., 2015).

Whole genome sequencing studies have identified that recurrent mutations in NRAS, KRAS, and BRAF oncogenes are frequently detected in myeloma patient samples (Lionetti et al., 2015). NRAS, KRAS, and BRAF genes are associated with RAS protein kinases, which are the most common proto-oncogenes in cancers (Prior et al., 2012; Davies et al., 2002; Pratilas et al., 2012). These RAS protein kinases are downstream of mitogen-activated protein kinase (MAPK) pathway, known as a critical signal transduction pathway that regulates cell proliferation and survival (Burotto et al., 2014). Among them, BRAF mutation is the most common mutation (~90%) in melanoma and several BRAF inhibitors have been used as targeted therapies for melanoma patients (Cheng et al., 2018). Besides MAPK pathway associated genes, mutations in PI3K are also detected in a subpopulation of multiple myeloma. The phosphorylated form of Akt, a direct downstream target of PI3K (Franke et al., 1995), and the mutation of DEP domain-containing mTOR-interacting protein, a positive regulator of PI3K (Boyd et al., 2010), have been found in myeloma patient samples.

Leukemia is another type of blood cancer that is derived from white blood cells. Several gene mutations have also been identified in leukemia. Among them, Philadelphia chromosome mutation or Philadelphia translocation (Ph) is a well-known genomic alteration associated with acute and chronic leukemia (Kang et al., 2016). Ph was originally found in the patients with CML (Nowell, 1962). Ph is a resultant product of the chromosomal translocation between chromosome 22 which contains breakpoint cluster region (BCR) gene and chromosome 9 which contains proto-oncogene tyrosine protein kinase (ABL1) (Collins and Groudine, 1983). The BCR-ABL1 fusion protein is closely associated with development of CML; more than 95% CML patients are Ph positive (Faderl et al., 1999). ABL1 is a tyrosine kinase which is involved in cell differentiation and regulates normal cell cycle (Wang, 2014). The fusion of ABL1 with BCR results in abnormal cell proliferation by generating the constitutively active form of a tyrosine kinase (Pendergast et al., 1991). A kinase inhibitor against BCR-ABL1, imatinib mesylate, has been widely used and very successful for treatment of Ph positive CML (Shah et al., 2002). However, some patients develop resistance to imatinib. A study examining the mutations of CML cells obtained from 32 CML patients who develop resistance to imatinib revealed that 29 samples out of 32 imatinib-resistant CML cells possess BCR-ABL1-dependent mutations, which prevent the binding of imatinib to the kinase domain of BCR-ABL1 protein (Shah et al., 2002). Although several BCR-ABL1-independent resistant mechanisms have been identified, BCR-ABL1-dependent mutations are thought to be major drivers of resistance to imatinib (Gorre et al., 2001). Thereafter, the second- and third-generation of BCR-ABL1 inhibitors, such as dasatinib, nilotinib, bosutinib, and ponatinib, have been developed and clinically tested (Rossari et al., 2018). These inhibitors can target almost all resistance-related mutations, except for the T315I mutation (Rossari et al., 2018). To overcome T315I mutation-dependent BCR-ABL1 inhibitor resistance, newer generation BCR-ABL1 inhibitors (e.g. bafetinib, rebastinib, tozasertib, danusertib, HG-7-85-01, GNF-2, and 1,3,4-thiadiazole derivatives) are currently under preclinical evaluation (Rossari et al., 2018). Although the results seem to be promising, further validation studies are required prior to clinical use.

Future Direction and Conclusions

The recent advancement in DNA sequencing technologies has enabled us to discover many important oncogenes and tumor suppressor genes responsible for the development of cancer, including bone-related cancers. Based on these findings, small molecule inhibitors that can target specific mutations have been made clinically available (Fig. 1). Like other cytotoxic agents, such as chemotherapies and radiation, most of these mutation-targeted inhibitors initially show anti-tumor effects, but eventually cause treatment resistance. It could be due in part to the activation of compensatory pathways mediated by the blockade of mutation-specific downstream pathways. If this is the case, the mutations targeted with these inhibitors may not be the key drivers leading to cancer development. Therefore, further studies identifying the initial mutations, which regulate hundreds or thousands of the mutations in oncogenes and tumor suppressor genes, are necessary in order to develop more effective targeted therapies.

Although treatments targeting mutations in cancer cells sounds promising, bone-related cancers are still difficult to eradicate since their progression is also influenced by the bone marrow microenvironment, or the “niche” (Decker et al., 2016; Zhang et al., 2019; Ren et al., 2015; Shiozawa et al., 2011; Park et al., 2018). When the *dicer1* gene is deleted in osteoblastic hematopoietic stem cell niche, the abnormal osteoblastic niche induces bone marrow dysfunction with myelodysplasia (Raaijmakers et al., 2010), suggesting the involvement of bone marrow microenvironment in the oncogenesis of bone-related cancers. In this regard, attention needs to be paid not only to genomic mutations in bone-related cancers, but also to those in the bone marrow microenvironment.

Particularly in bone metastasis, current treatments often target the bone marrow microenvironment. Although these bone-targeted therapies can reduce the onset of skeletal-related events (e.g. bone pain, pathological fractures, spinal cord compression) (Tsuzuki et al., 2016), they are primarily palliative. Targeting only the bone marrow microenvironment may not be sufficient to eradicate bone metastatic disease. Since bone metastasis-specific gene mutations have been discovered (Lohinai et al., 2017; Zhang et al., 2016), bone-targeted therapies can be combined with small molecules which target these mutations, if available. Alternatively, these bone metastasis-specific gene mutations can be used to guide precision treatment decisions.

Although cancer genome research has made available novel gene-targeted treatment options for bone-related cancers, there is still a gap to be filled if we want to further lower mortality of these patients. Therefore, we must continue to promote scientific and technological progress in the both fields of the cancer genome and bone-related cancer. Further multidisciplinary and multi-institutional research efforts are clearly warranted.

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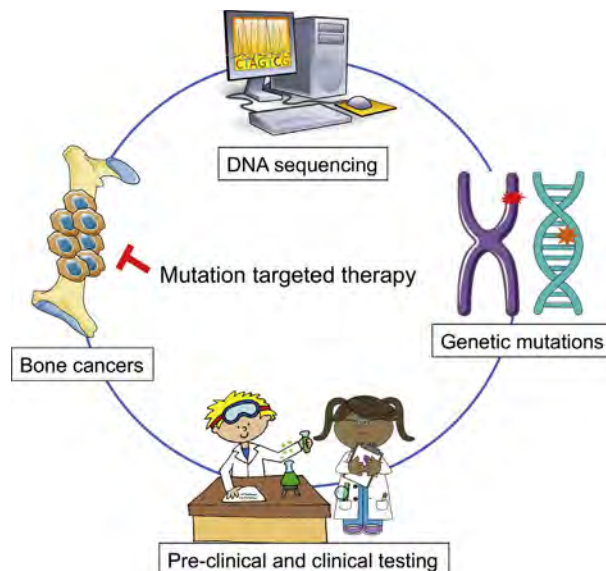


Fig. 1 Development of mutation-targeted therapies for bone-related cancers. Development of small molecule inhibitors specifically targeting gene mutations involves multiple steps. Once bone-related cancer is detected, a bone biopsy is performed to determine the precise diagnosis of cancer type. In some cases, biopsy samples are sent to the clinical laboratory to run DNA sequencing. The mutational status of oncogenes and tumor suppressor genes in these samples is analyzed, and the functions of these mutations and their therapeutic potential are assessed in preclinical settings. Successful preclinical studies eventually lead to the development of small molecule inhibitors. After preclinical validation, these potential inhibitors are moved into clinical trials and finally become available in the clinic when the results from the trials turn out to be positive. Graphics are adapted from Smart Servier Medical Art (<https://smart.servier.com/>) and Fly clipart (<https://flyclipart.com/>).

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The Roles of Bone Marrow-Resident Cells as a Microenvironment for Bone Metastasis

5

Yusuke Shiozawa

Abstract

It has been appreciated that the cross talk between bone metastatic cancer cells and bone marrow microenvironment influence one another to worsen bone metastatic disease progression. Bone marrow contains various cell types, including (1) cells of mesenchymal origin (e.g., osteoblasts, osteocytes, and adipocytes), (2) cells of hematopoietic origin (e.g., osteoclast and immune cells), and (3) others (e.g., endothelial cells and nerves). The recent studies have enabled us to discover many important cancer-derived factors responsible for the development of bone metastasis. However, many critical questions regarding the roles of bone microenvironment in bone metastatic progression remain elusive. To answer these questions, a deeper understanding of the cross talk between bone metastatic cancer and bone marrow microenvironment is clearly warranted.

Keywords

Bone marrow microenvironment · Tumor microenvironment · Bone metastasis · Vicious cycle · Cells of mesenchymal origin · Cells of hematopoietic origin · Blood

formation · Bone formation · Osteoblasts · Osteocytes · Adipocytes · Osteoclasts · Immune cells · Endothelial cells · Nerves

5.1 Introduction

Bone metastasis is one of the major causes of cancer death. Full understanding of the underlying mechanisms of bone metastasis, however, remains unrevealed. The presence of micrometastatic cells in the marrow at early stage of cancer has been associated with a poor prognosis [1] since micrometastatic cells can result in full-blown bone metastases at later stage [2] and metastatic progression of these cells may be environmentally directed by influences from the bone marrow-resident cells (Fig. 5.1). Indeed, it has been suggested that the cross talk between bone metastatic cancer cells and bone marrow microenvironment plays a crucial role in regulating the dissemination cascade of cancer cells to the bone and the progression of micrometastatic cells within the bone [3–5].

Bone marrow contains many cell types, including cells of hematopoietic and mesenchymal origin. Interactions among these cells are crucial for regulating blood and bone formation. Osteoblasts [6–8], adipocytes [9, 10], endothelial cells [11, 12], and nerves [13, 14] are known to serve as a specific microenvironment for hematopoietic stem cells. Osteoblasts, osteoclasts, osteocytes, and endothelial cells collectively

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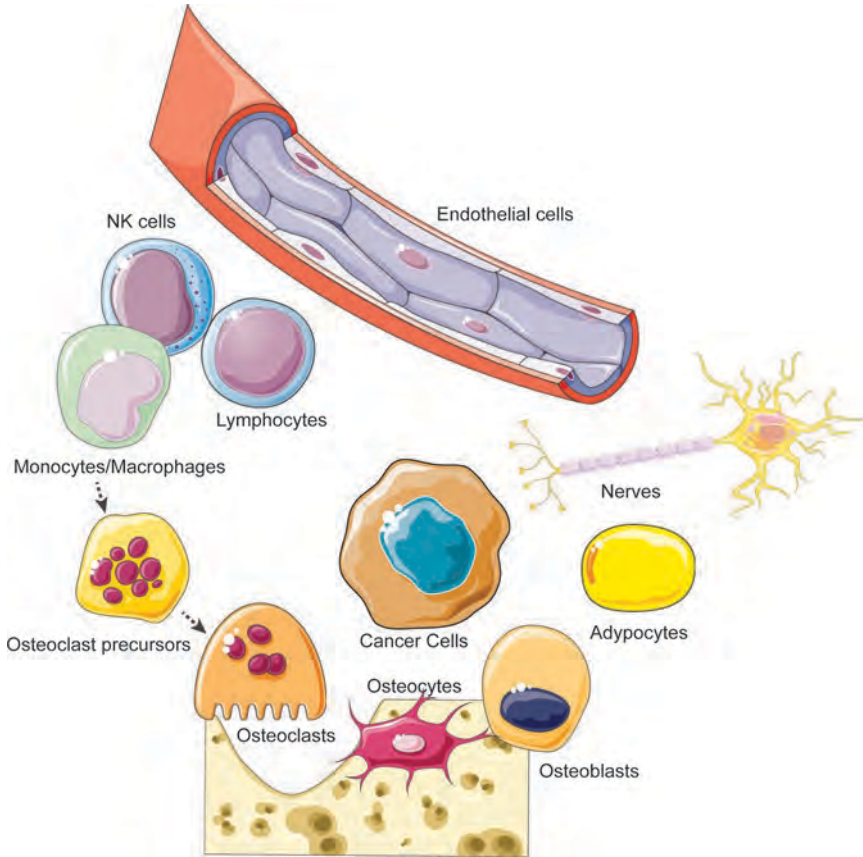


Fig. 5.1 The model of the bone marrow tumor microenvironment Bone marrow contains many cell types that directly influence blood formation (e.g., osteoblasts, adipocytes, endothelial cells, nerves) and bone formation (e.g., osteoblasts, osteocytes, osteoclasts, endothelial cells). Recent studies have revealed that bone metastatic cancer cells adopt mechanisms whereby these bone-resident cells maintain hematopoiesis and bone remodeling in

order to gain access to and grow within the bone. Bone marrow-resident immune cells are also involved in the process of bone metastasis in both a direct and an indirect manner. By interacting one another, all these cells play crucial roles in controlling bone metastatic progression as a bone marrow tumor microenvironment. Graphics adapted from Smart Servier Medical Art (<https://smart.servier.com/>)

organize bone remodeling [15]. We have demonstrated that bone metastatic cancer cells target and commandeer the microenvironment for hematopoietic stem cells, using similar mechanisms whereby hematopoietic stem cells home to the marrow to gain access to the bone [16]. Furthermore, bone metastatic cancer cells cause “vicious cycle” of bone metastasis by disrupting the balance between normal osteoclastogenesis and osteoclastogenesis [17, 18]. These evidences suggest that a microenvironment in the marrow also conversely affects the development and progression of bone metastasis.

Stephen Paget famously hypothesized in his “seed and soil” theory that metastatic cancer cells (seed) seek a specific metastatic site (soil) in order to survive outside of the primary tumor site [19]. Although the molecular mechanisms whereby bone metastatic cancer cells (seed) disseminate to bone have been extensively studied, tumor-supportive roles of bone marrow microenvironment (soil) are still largely unknown. In this review, we will explore what is currently known about bone marrow microenvironment-mediated bone metastatic progression and also suggest future directions for the microenvironment-targeted therapies for bone metastatic disease.

5.2 Bone Marrow Tumor Microenvironment

5.2.1 Cells of Mesenchymal Origin

5.2.1.1 Osteoblasts

Osteoblasts are the cells of mesenchymal origin, responsible for new bone formation, and known to be involved in regulating the homing and maintenance of hematopoietic stem cells [20]. Several lines of evidence suggest that osteoblasts also play significant roles in the development of bone metastasis. When human prostate cancer cell line PC-3 cells (intracardiac injection) and hematopoietic stem cells (intravenous injection) were simultaneously injected into irradiated severe combined immunodeficient (SCID) mice, both PC-3 cells and hematopoietic stem cells homed to osteoblasts and localized very closely each other [16]. Moreover, PC-3 cells and human prostate cancer cell line C4-2B cells prevented the binding of hematopoietic stem cells to murine primary calvarial osteoblasts [16]. Likewise, more numbers of intracardially inoculated human prostate cancer cell line PC-3NW1 cells homed to lateral endocortical surfaces of tibia of athymic nude mice where more osteoblasts reside compared to medial endocortical surfaces [21]. However, there were no differences in between the minimum distance from disseminated PC-3NW1 cells to lateral endocortical surfaces and medial endocortical surfaces [21]. When animals were treated with a C-X-C motif chemokine receptor 4 (CXCR4) antagonist AMD3100, differential dissemination patterns of PC-3NW1 cells to lateral and medial endocortical surfaces were no longer detected, and the minimum distance from disseminated PC-3NW1 cells to both endocortical surfaces was extended [21]. When human breast cancer cell line MCF-7 cells were inoculated into athymic nude mice by an intra-iliac artery injection, they initially colonized to alkaline phosphatase (ALP)-positive/collagen I-positive osteoblasts through E-cadherin (MCF-7 cells) and N-cadherin (osteoblasts) interaction [22]. This cancer-to-osteoblast interaction activated mammalian target of rapamycin (mTOR) pathway, and mTOR inhibitors torin 1

and rapamycin delayed the early colonization of MCF-7 cells to osteoblasts [22]. Similarly, human prostate cancer cell line C4-2B4 cells [23] and human breast cancer cell line MDA-MB-231 cells [24] bound to murine pre-osteoblast cell line MC3T3-E1 cells through homophilic cadherin-11–cadherin-11 interaction.

The interaction between bone metastatic cancer cells and osteoblasts might be involved in the bone metastatic progression within the marrow. More PC-3 cells bound to murine primary calvarial osteoblasts obtained from Annexin II (Anxa2) wild-type mice than those from Anxa2 knockout mice, and PC-3 cells grew greater on Anxa2 wild-type osteoblasts [25]. When PC-3 cells were implanted into athymic nude mice with vertebral bodies obtained from either Anxa2 wild-type or Anxa2 knockout mice, more growth of PC-3 within Anxa2 wild-type vertebral bodies was observed compared to that within Anxa2 knockout vertebral bodies [25]. When receptor for Anxa2 were downregulated in PC-3 cells, metastatic burdens within athymic nude mice were inhibited [25]. Anxa2 treatments induced AXL expression, a receptor for growth arrest-specific 6 (GAS6), in PC-3 cells [26]. Similarly, when PC-3 cells were cocultured with MC3T3-E1 cells, PC-3 cells become dormant by upregulating AXL expression [27]. Additionally, when primary human osteoblasts were cocultured with PC-3 cells, the secretion of GAS6 from osteoblasts was increased [26]. The GAS6 (osteoblasts)/AXL (PC-3 cells) interaction induced the expression of transforming growth factor (TGF)- β 1, TGF- β 2, TGF- β receptor 2 (TGFR2), and TGFR3 in PC-3 cells [27]. TGF- β 2 was responsible for osteoblast-mediated PC-3 dormancy [27]. Moreover, PC-3 cells became stemlike phenotype (CD133 positive/CD44 positive) on wild-type murine primary calvarial osteoblasts, while the conversion of PC-3 cells to stemlike phenotype was prevented when they were cocultured with osteoblasts obtained from GAS6 knockout mice [28]. The GAS6 derived from osteoblasts induced the conversion of PC-3 cells into stemlike phenotype by activating mTOR pathway through another GAS6 receptor, Mer [28]. When PC-3 cells were

implanted into SCID mice with vertebral bodies obtained from either GAS6 wild-type or GAS6 knockout mice, more stemlike PC-3 cells were observed near osteoblasts within GAS6 wild-type vertebral bodies [28]. When PC-3 cells pretreated with rapamycin were intracardially inoculated into SCID mice, the stemlike conversion of PC-3 cells within the bone marrow were diminished [28].

When Jagged1-overexpressing bone-tropic MDA-MB-231 subline SCP28 cells were cocultured with MC3T3-E1 cells, SCP28-derived Jagged1 induced interleukin 6 (IL-6) secretion from MC3T3-E1 by upregulating Hey1 (a downstream of notch pathway), resulting in the increased proliferation of SCP28 cells on MC3T3-E1 cells [29]. The γ -secretase inhibitor MRK-003, which abolishes notch activity [29], and human monoclonal anti-Jagged1 antibody 15D11 [30] attenuated (1) the increased proliferation of Jagged1-overexpressing SCP28 cells mediated by MC3T3-E1 cells and (2) bone metastatic burden of intracardially inoculated Jagged1-overexpressing SCP28 cells in athymic nude mice. Moreover, the combination of 15D11 and paclitaxel synergistically prevented bone metastatic progression of Jagged1-overexpressing SCP28 cells compared to 15D11 or paclitaxel alone [30]. Interestingly, 15D11 and paclitaxel combination also inhibited bone metastatic progression of low Jagged1-expressing parental SCP28 cells since paclitaxel induced Jagged1 expression in osteoblasts, leading to chemoresistance [30]. When osteoblasts were induced into senescence using a conditional transgenic mouse model [fibroblasts accelerate stromal-supported tumorigenesis (FASST) mouse: Col-Cre-ER^{T2} mouse x ROSAlox-stop-lox-p27^{Kip1} mouse], greater numbers of intracardially injected murine breast cancer cell line NT2.5 cells disseminated to bone [31]. In FASST mice, senescent osteoblasts indirectly enhanced the growth of disseminated NT2.5 cells by stimulating osteoclastogenesis via IL-6 production [31]. In addition, bone disseminated murine multiple myeloma cell line 5TGM1 cells (intravenous injection into C57BL/KaLwRijHsd mice) became dormant and consequently resistant to

melfhalan when they localized near osteoblasts [32]. However, when animals were treated with receptor activator of nuclear factor κ -B ligand (RANKL) to stimulate osteoclast activities, the dormancy of 5TGM1 cells was reversed [32]. These data suggest that osteoblasts are important for the early dissemination and colonization of bone metastatic cancer cells as well as the regulation of dormancy, stem cell-like conversion, and later disease outgrowth of bone metastatic cancer cells.

5.2.1.2 Osteocytes

Bone is a dynamic organ continuously remodeled throughout life. Bone remodeling is controlled by proper balance between osteoblastogenesis and osteoclastogenesis [15]. Osteocytes are also known to be involved in regulating bone remodeling. Osteocytes negatively regulate osteoblastic activities by releasing dickkopf-1 (DKK1) and sclerostin (inhibitors of Wnt pathway which are important for osteoblastic differentiation) and enhance osteoclastic resorption by releasing RANKL when they undergo programmed cell death [15, 33–35]. Osteocytes are the terminally differentiated cells of osteoblastic lineage and reside within the bone matrix [33–35]. Approximately 90–95% of adult skeletal tissues consist of osteocytes [33–35]. However, it has been only recently appreciated the roles of osteocytes as a tumor microenvironment. Osteocyte death and enhanced osteoclast activity were observed in osteolytic bone lesions of multiple myeloma patients [36]. Higher levels of pro-osteoclastogenesis cytokine IL-11 were found in bones of multiple myeloma patients with osteolytic lesions than those without osteolytic lesions [36]. Conditioned medium (CM) obtained from the coculture between human multiple myeloma cell line JJN3 cells and human pre-osteocyte cell line HOB-01 cells stimulated osteoclastic formation of human primary peripheral blood mononuclear cells by enhancing the secretion of IL-11 and C-C motif chemokine ligand 3 (CCL3) [36]. These data suggest that osteocytes create favorable environment for bone metastatic cancer cells by promoting osteolytic bone damage.

Osteocytes not only provide the environment for cancer but also directly influence cancer progression. CM obtained from murine osteocyte cell line MLO-Y4 cells stimulated *in vitro* cell proliferation of human prostate cancer cell lines (PC-3 and DU145) and human breast cancer cell lines (MDA-MB-231 and MCF-7) [37]. CM obtained from murine osteocyte cell line MLO-A5 cells also enhanced the proliferation of murine breast cancer cell line 4 T1.2 cells [38, 39]. However, when 4T1.2 cells were three-dimensional (3D) cocultured with MLO-A5 cells, the size of MLO-A5/4T1.2 spheroid was shrank [38, 39]. Additionally, *in vitro* 3D coculture system between human osteocytes and prostate cancer organoids revealed that prostate cancer organoids significantly impair bone structure through the induction of osteocyte apoptosis while promoting bone mineralization [40]. In this model, prostate cancer organoids significantly enhanced the protein expression of fibroblast growth factor 23 (FGF23) and DKK1 in osteocytes but reduced sclerostin [40]. However, these changes failed to be observed in 2D coculture conditions [40], suggesting that the 3D culture system provides a unique opportunity to study the interaction between osteocytes and cancer cells.

MLO-Y4 CM also enhanced the anchorage-independent growth, migration, and invasion of MDA-MB-231 cells and murine breast cancer cell line Py8119 cells [41]. These phenomenon were inhibited with bisphosphonate [alendronate and zoledronic acid (ZOL)] treatments and fluid flow-induced shear stress by opening connexin 43 hemichannels in osteocytes [41]. The growth of Py8119 cells in the bone mediated by intratibial injections was enhanced when cells were injected into osteocyte-specific connexin 43 knockout mice or mice with osteocyte-specific connexin 43 junction impairment [41]. Alendronate upregulated ATP release from MLO-Y4 cells, and this ATP inhibited the MLO-Y4 CM-mediated anchorage-independent growth, migration, and invasion and *in vivo* primary tumor growth of MDA-MB-231 cells and Py8119 cells through P2X7 receptors [42]. In addition, these were reversed by adenosine treat-

ment [42]. ATP treatments prevented the growth of intratibially injected Py8119 cells [42]. Likewise, CM from MLO-Y4 cells primed with CM of human prostate cancer cell lines (PC-3 and C4-2B) induced the growth, migration, and invasion of PC-3 and C4-2B cells by upregulating growth-derived factor 15 (GDF15) in MLO-Y4 cells and enhancing early growth response 1 (EGR1) expression in prostate cancer cells [43]. When C4-2B cells were co-inoculated with murine primary osteocytes into the flank of mice, these osteocyte/C4-2B mixtures grow greater than C4-2B cells alone, due in part to the upregulation of EGR1 in C4-2B cells [43]. The growth of osteocyte/C4-2B mixture was diminished when GDF15 was downregulated in osteocytes [43]. When PC-3 or C4-2B cells were injected into the tibia of mice in which GDF15 expression in tibia was downregulated using siRNA, prostate cancer cell growth in bones and EGR1 expression in prostate cancer cells were decreased compared to those in bones infected with control siRNA [43]. However, MLO-A5-CM conversely reduced the migratory ability of 4T1.2 cells through type I collagen by downregulating SNAIL [an epithelial to mesenchymal transition (EMT) inducer] and upregulating phosphorylation of Akt [38, 39]. Further study is warranted to conclude the effects of osteocytes on migratory ability of bone metastatic cancer cells.

Osteocytes are mechanosensitive cells [33–35], and the impact of mechanical stress on tumor microenvironmental abilities of osteocytes has been elucidated. Fluid flow-induced shear stress stimulated the differentiation of MLO-Y4 cells regardless of the existence of CM derived from MDA-MB-231 in the culture [44]. Fluid flow-induced shear stress on the 3D coculture between MLO-Y4 cells and human umbilical vein endothelial cells (HUVECs) inhibited the extravasation of MDA-MB-231 cells [45–47]. This was in part due to the reduction of IL-6 in MLO-Y4 cells (chemoattractant for cancer cells; inhibition of osteoclast apoptosis), the reduction of intercellular adhesion molecule 1 (ICAM-1) in HUVECs (cancer cell adhesion to endothelial cells; inhibition of osteoclast apoptosis), and the reduction of matrix metalloproteinase 9 (MMP9) in

MDA-MB-231 cells (cancer cell migration; reduction of endothelial junctional integrity) [45–47]. When hydrostatic pressure was applied to MLO-Y4 cells, MLO-Y4-CM promoted the growth, migration, and invasion of human prostate cancer cell lines (PC-3, DU145, and LNCaP) in part due to the upregulation of CCL5 and MMP2 and MMP9 [48].

When human multiple myeloma cell line RPMI 8226 cells were cocultured with HOB-01 cells or MLO-Y4 cells, RPMI 8226 cells induced death of HOB-01 and MLO-Y4 cells by activating autophagy [49]. This multiple myeloma-induced osteocyte death and autophagy were inhibited by proteasome inhibitors, bortezomib, and MG 262 [49]. Consistently, multiple myeloma patients treated with regimens including bortezomib showed significantly higher osteocyte viability in the bone lesions compared to those treated with regimens without bortezomib ($p = 0.017$) [49]. Moreover, a combination agent of nanoparticle loaded with a natural compound, plumbagin and ZOL (PUCZP), prevented the MLO-Y4-mediated osteoclastogenesis of murine bone marrow monocytes mediated by downregulating of RANKL and sclerostin expression in MLO-Y4 cells [50]. PUCZP also attenuated bone metastatic progression and cancer-induced bone resorption in athymic nude mice intracardially inoculated with MDA-MB-231 cells by downregulating of RANKL and sclerostin expression in osteocytes, reducing osteoclastogenesis, and inducing apoptosis of tumor cells [50]. These findings suggest that targeting osteocytes is a potential new therapeutic strategy for bone metastatic disease.

5.2.1.3 Bone Marrow Adipocytes

Bone marrow is known to contain significant numbers of adipocytes. Bone marrow adipocytes take up about 70% of marrow space in adults [51] and serve as one of the major components of the bone marrow microenvironment for hematopoietic stem cells [9, 10]. However, the effects of bone marrow adipocyte on bone metastatic progression have been only recently uncovered [52–59]. Direct contact between human primary bone marrow mesenchymal stem cell (MSC)-derived

adipocytes (BMAs) and promyelocytic leukemia protein-retinoic acid receptor α (PML-RAR α)-expressing human acute promyelocytic leukemia cell line U937/PR9 cells protected U937/PR9 cells from apoptosis through the leptin (adipocytes)/leptin receptor (leukemia) axis [60]. Additionally, BMAs activated the phosphorylation of signal transducer and activator of transcription 3 (STAT3) and ERK mitogen-activated protein kinase (MAPK) in U937/PR9 cells [60]. Likewise, coculture with human primary BMAs protected human acute monocytic leukemia cell line U937 cells from spontaneous apoptosis by increasing leukemia cells' free fatty acid intake and consequent fatty acid β -oxidation [61]. This is in part due to the upregulation of peroxisome proliferator-activated receptor γ (PPAR γ), fatty acid binding protein 4 (FABP4), CD36, and B-cell lymphoma 2 (BCL2) genes; the phosphorylation of AMP-activated protein kinase (AMPK) and p38 MAPK; and the upregulation of antiapoptotic chaperone 70-kDa heat-shock protein (HSP70) [61]. Human primary BMAs also supported the proliferation of human primary acute myeloid leukemia (AML) blasts by upregulating FABP4 in adipocytes and enhancing lipids intake and fatty acid β -oxidation in AML cells [62]. When connective tissue growth factor (CTGF) was downregulated in human bone marrow MSCs, these MSCs differentiated into adipocyte lineage [63]. When CTGF-downregulated MSCs were implanted with endothelial cells into the flank of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) IL-2r^{null} (NSG) mice, they contained more adipocytes than the implants of CTGF-normal MSCs with endothelial cells [63]. Intravenously inoculated human AML cell line Molm13 cells and acute lymphoblastic leukemia cell line Nalm6 cells homed better to CTGF-downregulated MSC implants compared to those with CTGF-normal MSCs since adipocytic CTGF-downregulated MSC implants expressed higher levels of C-X-C motif chemokine ligand 12 (CXCL12) and leptin [63]. Moreover, bone marrow of multiple myeloma patients contained more adipocytes than that of healthy counterparts [64]. The 5TGM1 cells migrated toward CM

from murine pre-adipocyte cell line 3T3-L1 cells through CCL2 and CXCL12 [64]. When 5TGM1 cells were cocultured with 3T3-L1 cells, 3T3-L1 cells enhances the viability of 5TGM1 cells by activating β -catenin and inhibiting caspase3 in 5TGM1 cells [64]. When 5TGM1 cells primed with 3T3-L1 cells were intravenously injected into C57BL/KaLwRij mice, these 3T3-L1-primed 5TGM1 cells grew greater in the bone than those without 3T3-L1 priming [64]. These findings suggest that bone marrow adipocytes are also crucial components of microenvironment for hematopoietic malignancies, similar to their roles for hematopoietic stem cell [9, 10].

Recent studies have also revealed the impact of bone marrow adipocytes on bone metastatic progression on solid tumors. When MDA-MB-231 cells were cocultured with bone fragments obtained from patients who undergo hip replacement, MDA-MB-231 cells migrated toward bone fragments and extensively colonized within the bone marrow adipose tissue compartment [65]. When human bone marrow stromal cells were transformed into adipocytic lineage with omega-6 polyunsaturated fatty acid (PUFA) treatments, more PC-3 cells migrated toward these omega-6 PUFA-treated human bone marrow stromal cells [66, 67]. Additionally, more lipid intake of PC-3 cells from bone marrow stromal cells was observed [66, 67]. Murine primary BMMA also induced glycolytic shift in human prostate cancer cell lines PC-3 and ARCaP(M) cells by upregulating glycolytic markers [enolase 2 (ENO2), lactate dehydrogenase (LDH), hexokinase 2 (HK2), pyruvate dehydrogenase kinase 1 (PDK1), and phosphorylated pyruvate dehydrogenase (p-PDH)], increasing lactate release, reducing oxygen consumption rate, and decreasing ATP levels [68]. This was due to the upregulation of HIF-1 α target genes [carbonic anhydrase 9 (CA9), vascular endothelial growth factor (VEGF), and glucose transporter 1 (GLUT1)] [68]. Conversely, CM from PC-3 and ARCaP(M) cells promoted lipolysis in murine primary BMMA by increasing free glycerol release, which was uptaken by prostate cancer cells [68]. Bone metastatic samples obtained from prostate cancer patients with bone metastases showed

higher levels of heme oxygenase 1 (HO-1; a protector from oxidative cell damage) compared to primary prostate tumors or samples from other metastatic sites [69]. When PC-3 and ARCaP(M) cells were cocultured with murine primary BMMA, the expression of HO-1 and endoplasmic reticulum (ER) stress chaperone glucose-regulated protein 78 (GRP78; another protector from oxidative cell damage) was enhanced [69]. Adipocyte-induced oxidative stress and overexpression of HO-1 in PC-3 and ARCaP(M) cells promoted the invasion of these cells [69]. Similarly, CM of murine primary BMMA induced the invasion of PC-3 cells by upregulating HO-1, IL-1 β , and FABP4 but downregulating PPAR γ in PC-3 cells [70]. Overexpression of HO-1 in PC-3 and ARCaP(M) cells also induced the enhanced levels of GRP78 and pro-survival factors Bcl-xl and survivin in prostate cancer cells. HO-1 overexpression significantly increased growth in the bone of intratibially injected PC-3 and ARCaP(M) cells compared to those with control vector infection [69].

Intratibial injections of PC-3 and ARCaP(M) cells into mice with high-fat diet (60 kcal% fat) showed the enhanced numbers of bone marrow adipocytes osteolytic lesions [71]. CM from PC-3 and ARCaP(M) cells stimulated the secretion of CXCL1 and CXCL2 from murine primary BMMA, and BMMA-derived CXCL1 and CXCL2 induced osteoclastogenesis of murine bone marrow macrophages [71]. Along with this notion, when murine melanoma cell line B16-F10 cells were directly injected into the tibia of high-fat diet-fed C57BL/6 N mice, more growth of B16-F10 cells in the bone was observed along with the increased numbers of bone marrow adipocytes and osteoclasts, and enhanced osteolytic lesions, compared to those in the bone of C57BL/6 N mice with normal diet (10 kcal% fat) [72]. Additionally, serums of tumor-bearing mice with high-fat diet, and macrophages and osteoclasts found in their bone expressed higher levels of osteopontin than those from animals with normal diet [72]. Murine primary BMMA induced IL-6 mRNA expression in B16-F10 cells, and higher serum IL-6 concentration was observed in of tumor-bearing mice with high-fat diet [72].

Neutralizing anti-osteopontin antibody and neutralizing anti-IL-6 antibody or Janus kinase 2 (JAK2; a downstream of IL-6) inhibitor AG490 reduced the enhanced B16-F10 growth in the bone of tumor-bearing mice with high-fat diet [72]. When B16-F10 cells were injected into C57BL/6 mice by intracardiac injection, the numbers of bone marrow adipocytes and the serum levels of leptin increased at day 7 of injection, and these increased adipocytes and leptin levels decreased by day 10 [73]. Similar trend was observed in osteoclast activities [73]. Interestingly, B16-F10 cells localized around bone marrow adipocytes [73]. Consistent with the observations in *in vivo*, CM from B16-F10 cells induced differentiation of murine primary BMMA while mature BMMA were dedifferentiated into immature BMMA when they were cocultured with B16-F10 cells [73]. On the other hands, CM from murine primary BMMA induced proliferation and accelerated cell cycle of B16-F10 cells [73]. Similarly, CM from murine primary BMMA enhanced DNA synthesis of murine multiple myeloma cell line 5T33MM cells, stimulated their migration toward adipocyte CM, and protected from apoptosis [74]. These data suggest that bone marrow adipocytes also play an important role in the regulation of bone metastatic progression of solid tumors.

5.2.2 Cells of Hematopoietic Origin

5.2.2.1 Osteoclasts

Osteoclasts are the multinucleated cells derived from a monocyte/macrophage lineage and are associated with the development of osteolytic bone metastatic lesions. Osteoclasts are known to indirectly serve as a bone marrow tumor microenvironment. Osteoclasts create a sufficient space for bone metastatic cancer cells to grow by resorbing bone matrix [75]. Indeed, osteolytic bone metastatic lesions in athymic nude mice inoculated with SCP28 cells (intracardiac injection) were prevented by treatment of microRNA (miR)-141 and miR-219 which downregulated in activated osteoclasts, while miR-141 and miR-219 did not directly affect SCP28 cells prolifera-

tion [76]. Osteoclasts promote extracellular calcium release and create extracellular acidic environments during bone resorption. Higher levels of calcium-sensing receptor were observed in bone metastatic tumor samples of prostate cancer [77] and renal cell carcinoma patients [78] compared to primary tumor samples. Extracellular calcium stimulated the proliferation of human breast cancer cell lines BT474 and MDA-MB-231-1833 cells [79] and PC-3 cells [80] through calcium-sensing receptor. Extracellular pH promoted the spheroid and colony formation of PC-3 cells [81]. Although there is no direct evidence yet, these findings suggest that osteoclasts may also play a crucial role as a bone marrow tumor microenvironment in an indirect manner by developing extracellular high-calcium and extracellular acidic environments.

5.2.2.2 Immune Cells

It has been extensively studied antitumor or protumor effects of immune system at primary tumor site. Although bone marrow contains various immune cells, the cross talk between bone metastatic cancer cells and bone marrow immune cells remains largely unexplored. Higher numbers of CD4-positive/Foxp3-positive regulatory T (Treg) cells were found in the bone marrow of prostate cancer patients with bone metastases than that of patients without bone metastases [82]. Treg cells obtained from bone metastatic prostate cancer patients had greater migratory and proliferative capacities and were responsible for the inhibition of osteoclasts, resulting in osteoblastic bone lesions [82]. Contrary, RANKL levels in CD3-positive T cells within the marrow of BALB/c mice in which 4 T1 cells were inoculated into mammary fat pad were enhanced compared to those of mice inoculated with nonmetastatic 4T1subline, 67NR cells [83]. However, there were no changes in the numbers of CD3-, CD4-, and CD8-positive T cells between 4T1- and 67NR-bearing mice [83]. Consequently, the enhancement of RANKL in T cells increased osteoclastic activities in the marrow, leading to bone metastatic colonization of 4 T1 cells [83]. Intracardiac injection of 4 T1 cells into BALB/c mice increased the numbers of B220-positive/

CD11c-positive plasmacytoid dendritic cells (pDCs) and the increased CD4-positive T cells in their marrow [84]. pDCs and CD4-positive T cells produced osteolytic bone lesions and subsequently increased tumor burden and bone damage [84]. Depletion of pDCs with PDCA-1 antibody led to decreased tumor burden and bone damage by reducing the numbers of CD4-positive T cells and CD11b/Gr-1 double-positive myeloid-derived suppressor cells (MDSCs) and activating cytotoxic CD8-positive T cells in the marrow [84].

More number of CD11b/Gr-1 double-positive MDSCs were observed in the marrow of C57BL/6/KaLwRij mice intravenously injected with 5TGM1 cells compared to non-tumor-bearing mice [85]. The MDSCs from 5TGM1-bearing mice showed greater potential to differentiate into mature and functional osteoclasts than those from control mice [85]. When 5TGM1-bearing MDSCs were delivered to 5TGM1-bearing mice, osteolytic bone lesions were further enhanced compared to 5TGM1-bearing mice without MDSC treatments [85]. Additionally, the enhancement of osteoclastic activities mediated by 5TGM1-bearing MDSCs both in vitro and in vivo were inhibited by ZOL treatments [85]. Likewise, CD11b/Gr-1 double-positive MDSCs obtained from bone marrow cells of BALB/c mice which were intracardially inoculated with 4T1 cells and developed bone metastases differentiated into osteoclasts by increasing NO levels, but not MDSC from lung of 4T1-bearing mice with bone metastases or from bone marrow of 4 T1-bearing mice without bone metastases [86].

In bone samples obtained from prostate cancer patients with bone metastases, more CD68-positive macrophages were observed near tumor mass and pathological woven bone [87]. When F4/80-positive macrophages were depleted in the macrophage Fas-induced apoptosis (MAFIA) mice with AP20187 treatments, the growth of murine prostate cancer cell line RM-1 cells (intratibial inoculation) in their bone and resultant osteolytic lesions were significantly reduced [88]. Similarly, F4/80-positive macrophage depletion by clodronate liposome treatments resulted in the reduction of the growth of intrati-

bially injected RM-1 cells (into C57BL6 mice) and PC-3 cells (into athymic nude mice) [88]. However, contrarily to MAFIA mice, depletion of macrophages by clodronate liposome treatments increased bone volume [88] but reduced pathological woven bone development [87]. When CD169-positive macrophages were depleted in the CD169-diphtheria toxin receptor (DTR) mice with DT treatments, the woven bone development in RM-1-bearing bone (intratibial injection) were attenuated, while CD169 macrophage depletion did not affect the tumor growth in the bone [87]. The treatments of anti-mouse CD115 monoclonal antibody which only target murine myeloid cells also inhibited osteolytic bone metastatic progression in athymic nude mice intracardially inoculated with MDA-MB-231 cells [89]. The beneficial effects of anti-mouse CD115 monoclonal antibody were comparable to ZOL [89]. When marrow-resident F4/80-positive/CD206-positive pro-tumorigenic M2-like macrophages, which enhance prostate cancer proliferation by efferocytosis, were reduced by trabectedin treatments, the growth of PC-3 cells (intratibial or intracardiac injection) in the bone of athymic nude mice was attenuated [90]. However, trabectedin treatments failed to change osteoclastic activities in the tumor-bearing bones [90]. These findings suggest that bone marrow immune cells also support bone metastatic progression as a bone marrow tumor microenvironment directly in an immunosuppressive manner and indirectly by influencing bone remodeling.

5.2.3 Other Components of Bone Marrow Microenvironment

5.2.3.1 Endothelial Cells

Bone is a highly vascularized organ and subsequently supplies hematopoietic cells and cytokines produced in the marrow. Although the vasculature is known to be crucial for tumor growth and metastasis, little is known as to the roles of bone marrow endothelial cells as a tumor microenvironment. Human breast cancer cell lines MDA-MB-231 cells (orthotopic inocula-

tion) and HMT-3522-T4-2 cells (intracardiac inoculation) disseminated into the bones of NOD/SCID mice, and those disseminated tumor cells became dormant when they localized microvascular endothelium [3]. Consistently, HMT-3522-T4-2 cells stayed dormant on the organotypic bone microvascular mimic (the mixture of HUVECs and murine primary bone marrow stromal cells) [3]. More dormant HMT-3522-T4-2 cells were observed when they reside on the thrombospondin 1 (TSP-1)-expressing mature endothelium, while TGF- β 1 and periostin-expressing immature neovascular tips accelerated the growth of HMT-3522-T4-2 cells [3]. Murine breast cancer cell line 4T07 cells (orthotopic inoculation) disseminated closer to vasculature compared to megakaryocytes or osteoblasts, within the marrow of BALB/c mice, and vasculature-resident 4T07 cells had more resistance to chemotherapy (Adriamycin plus cyclophosphamide, AC) [91]. Coculture between the organotypic bone microvascular mimic and HMT-3522-T4-2 cells treated with doxorubicin revealed that the induction of chemoresistance mediated by vasculature was not due to the cell cycle arrest but the binding with vasculature through the integrin β 1/von Willebrand factor axis and the integrin α v β 3/vascular cell adhesion molecule (VCAM)-1 axis [91]. Indeed, the resistance of 4T07 cells to AC regimen in the marrow of BALB/c mice were reversed by (1) the downregulation of integrin β 1 and integrin α v in 4T07 cells using shRNA and (2) the treatments of the integrin β 1 inhibitory antibody AIIB2 and integrin α v β 3 inhibitory antibody LM609 [91]. More branching and sprouting of bone marrow vessels along with tumor burden and osteolytic bone lesions were observed by volumetric computed tomography (VCT) in the femur of nude rat injected with MDA-MB-231 cells through femoral artery [92]. When MDA-MB-231-bearing rat were treated with anti-VEGF antibody bevacizumab, branching and sprouting of bone marrow vessels as well as tumor burden and osteolytic bone lesions were inhibited [92]. These findings suggest that bone marrow endothelial cells are involved in the regulation of dormancy, chemoresistance, and outgrowth of bone metastatic cancer cells.

5.2.3.2 Nerves

The importance of nerves for cancer progression has been appreciated [93]. The sympathetic nervous system regulates the metastatic process of prostate cancer to bone [94], and denervation can suppress tumorigenesis and metastasis [94–97]. Moreover, the roles of sympathetic nervous system in bone metastatic progression have recently begun to be revealed. Chronic immobilization stress and a nonselective β 1/ β 2 adrenergic receptor agonist isoproterenol treatments enhanced the colonization of intracardially injected MDA-MB-231 cells to the bone of athymic nude mice through the RANK (cancer)/RANKL (bone) axis by inducing the RANKL expression in bones [98]. However, chronic immobilization stress and isoproterenol treatments did not affect the growth of MDA-MB-231 cells in the bone [98]. The enhancement of tumor colonization mediated by isoproterenol treatments was also thought to be due to the increased neovascularization of tumor-bearing bones [99]. Consistently, isoproterenol stimulated the VEGF secretion of MC3T3-E1 cells and murine primary bone marrow stromal cells [99]. The tube formation of HUVECs was increased when they were treated with CM from isoproterenol-treated murine primary bone marrow stromal cells [99]. The tube formation was prevented when cultures were treated with an antibody targeting VEGF-A:VEGF receptor 2 signaling mcr84 [99]. Isoproterenol-induced increased neovascularization and enhanced tumor colonization of MDA-MB-231 cells were reversed in (i) osteoblastic-specific β 2 adrenergic receptor knockout mice (Rag2 background) and (ii) Rag2 mice treated with mcr84 [99]. Isoproterenol-treated promoted the adhesion between murine primary bone marrow endothelial cells or murine endothelial cell line C166 cells and MDA-MB-231 cells in vitro [100]. The expression levels of adhesion molecules E-selectin and P-selectin in murine primary bone marrow endothelial cells and C166 cells were increased via β 2 adrenergic receptor when they were treated with CM from isoproterenol-treated murine primary bone marrow stromal cells [100]. High levels of IL-1 β were observed in the CM of isoproterenol-treated

murine primary bone marrow stromal cells, and IL-1 β treatments enhanced E-selectin and P-selectin expression in C166 cells and promoted the adhesion between C166 cells and MDA-MB-231 cells [100]. Additionally, when PC-3 cells were cocultured with murine primary calvarial osteoblasts, their proliferation was slowed and most of them were in the G1 cell cycle phase [101]. Norepinephrine induced the proliferation and G2-M cell cycle of PC-3 cells cocultured with murine primary wild-type calvarial osteoblasts, while norepinephrine did not affect the proliferation and cell cycle of PC-3 cells cocultured with murine primary GAS6 knockout calvarial osteoblasts [101]. Since bone is a richly innervated organ, further investigations on the impact of nerves on bone metastatic progression are clearly warranted.

5.3 Conclusions and Future Directions

When cancer patients develop bone metastases, their prognosis is very poor. Current treatments for bone metastases mainly target bone remodeling. Denosumab (a human monoclonal antibody against RANKL) and bisphosphonates (which suppress osteoclast activity) are established treatments for bone metastases and are thought to work by decreasing bone resorption. However, they ultimately fail to improve overall survival [102, 103]. Only a new α -emitting radiopharmaceutical radium-223, which binds to hydroxyapatite in the marrow, can extend overall survival and then only by three months [104]. Thus, approaches that target factors other than bone remodeling are needed to lower mortality of cancer patients with bone metastases. In recent years, the supportive roles of immune cells, adipocytes, and nerves as a tumor microenvironment have been appreciated, and in the bone marrow, as described above, the interactions between these cells and bone metastatic cancer cells also seem to influence disease processes. Although further studies are clearly warranted, treatments targeting these bone marrow-resident cells, or in combination with bone-targeted therapies, may be new thera-

peutic strategies for bone metastatic disease. A better understanding of how bone metastatic cancer and bone marrow microenvironment influence one another to worsen bone metastatic disease progression will aid in discovering new therapeutic targets for bone metastatic cancer—area in which current therapies are wanting—to decrease suffering and improve the survival of cancer patients with bone metastases.

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Review

Progress in Targeted Alpha-Particle-Emitting Radiopharmaceuticals as Treatments for Prostate Cancer Patients with Bone Metastases

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Abstract: Bone metastasis remains a major cause of death in cancer patients, and current therapies for bone metastatic disease are mainly palliative. Bone metastases arise after cancer cells have colonized the bone and co-opted the normal bone remodeling process. In addition to bone-targeted therapies (e.g., bisphosphonate and denosumab), hormone therapy, chemotherapy, external beam radiation therapy, and surgical intervention, attempts have been made to use systemic radiotherapy as a means of delivering cytotoxic radiation to every bone metastatic lesion. Initially, several bone-seeking beta-minus-particle-emitting radiopharmaceuticals were incorporated into the treatment for bone metastases, but they failed to extend the overall survival in patients. However, recent clinical trials indicate that radium-223 dichloride (²²³RaCl₂), an alpha-particle-emitting radiopharmaceutical, improves the overall survival of prostate cancer patients with bone metastases. This success has renewed interest in targeted alpha-particle therapy development for visceral and bone metastasis. This review will discuss (i) the biology of bone metastasis, especially focusing on the vicious cycle of bone metastasis, (ii) how bone remodeling has been exploited to administer systemic radiotherapies, and (iii) targeted radiotherapy development and progress in the development of targeted alpha-particle therapy for the treatment of prostate cancer bone metastasis.

Keywords: bone metastases; radiopharmaceuticals; radium-223; actinium-225; targeted alpha-particle-emitting radiopharmaceuticals



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1. Introduction

As a result of improvements in cancer research, prevention, early diagnosis, and treatment, the survival time of cancer patients with localized disease has increased. However, the prognosis for cancer patients with disseminated disease has decreased dramatically; distant metastases are responsible for 90% of all cancer-related deaths [1]. Although cancer cells may spread to any part of the body, different cancers have been observed to colonize different organs of the body at different rates and bone is a major metastatic site for several cancers. The relative incidence of bone metastasis is 65–75% in breast cancer, 65–75% in prostate cancer, 60% in thyroid cancer, 30–40% in lung cancer, 40% in bladder cancer, 20–25% in renal cell carcinoma, and 14–45% in melanoma [2]. Furthermore, the median survival time of patients with bone metastases is 19–25 months for breast cancer, 12–53 months for prostate cancer, 28 months for thyroid cancer, 6 months for lung cancer, 6 months for bladder cancer, 12 months for renal carcinoma, and 6 months for melanoma [3]. Importantly, the presence of metastatic bone disease alters the course of clinical care of patients, increases the immense physical and emotional burdens faced by patients, and augments the economic burden faced by patients and society [4–13]. For example, prostate cancer patients experiencing bone metastases incurred health care costs that were approximately \$8000 more than those incurred by men without bone metastasis [14]. Interestingly, it has

been demonstrated that the total medical care costs for breast cancer patients with bone metastases who experienced skeletal-related events (SREs) are nearly \$50,000 greater than for those without SREs [4,7].

Since bone metastasis is one of the major causes of death of cancer patients, eradicating cancer-induced bone diseases represents one of the greatest challenges of modern health care. Thus, there is a critical need to integrate our current understanding of cancer metastasis with emerging concepts in bone biology to advance our understanding of cancer-induced bone diseases, with the goal of improving treatment strategies and clinical outcomes, while reducing the financial difficulties experienced by patients. The treatment strategies for bone metastases are somewhat unique when compared to those for other metastases. Normally, the treatment strategies for both primary and metastatic tumors are similar—targeting the tumors themselves or inducing the immune system surrounding the tumors. However, for bone metastases, the treatments target the function of the metastatic organ, which is bone (an organ that continuously remodels throughout life by coupling osteoclast and osteoblast activity, which is called bone remodeling [15]). It has been suggested that the cells involved in bone remodeling (e.g., osteoclasts, osteoblasts, and osteocytes) and bone metastatic cancer cells interact with each other, and this crosstalk between bone-related cells and bone metastatic cancer cells stimulates further bone metastatic progression, known as “the vicious cycle of bone metastases” [16]. It is therefore natural to target bone remodeling to interfere with this cycle. Indeed, bisphosphonate and denosumab, a human monoclonal anti-receptor activator of nuclear factor κ B ligand (RANKL) antibody, which decreases osteoclastic activity, have been used as treatments for bone metastases [17,18]. These treatments have been effective in reducing the painful complications of bone metastases but ultimately fail to improve the overall survival of cancer patients with bone metastases [17,18]. However, recent clinical trials indicate that an alpha-particle-emitting radiopharmaceutical radium-223 dichloride ($^{223}\text{RaCl}_2$), which targets hydroxyapatite or osteoblastic bone metastatic lesions, improves the overall survival of prostate cancer patients with bone metastases. Importantly, to date, this is the only bone-targeted treatment modality that can prolong the survival time of cancer patients with bone metastases, although several combinations of systemic treatments (e.g., hormone therapies and chemotherapies) are known to enhance the overall survival of metastatic castration-resistant prostate cancer patients, including patients with bone metastases [19]. Although $^{223}\text{RaCl}_2$'s success holds promise for alpha-particle-emitting radiopharmaceuticals for bone metastatic disease and has renewed interest in the development of these therapies [20–32], little is known as to the targeted treatment strategies for bone metastatic disease using alpha-particle-emitting radiopharmaceuticals.

While many excellent reviews relating to targeted radiotherapy have been published [22,33–35], this review will highlight (i) the biology of bone metastases by emphasizing the vicious cycle of bone metastasis, (ii) the treatment strategies for bone metastasis by mainly focusing on radiopharmaceuticals, and (iii) the future directions for targeted alpha-particle-emitting radiopharmaceutical treatment strategies in bone metastasis.

2. The Biology of the Vicious Cycle of Bone Metastases

Although personalized medicine strategies continue to be adopted in the clinic, the tumor phenotype at the primary site typically dictates the treatment regimen. However, these conventional treatment strategies usually fail to eradicate metastases. This leads to a more aggressive combination treatment, including chemotherapies, radiotherapies, immunotherapies, and/or targeted therapies, for cancer patients with metastases. Importantly, when considering the treatment of bone metastasis, the dynamics of bone turnover also needs to be considered during treatment planning.

Unlike other organs, bone is continuously renewed throughout life to maintain its structural integrity. Bone is composed of three parts: compact bone, trabecular bone, and bone marrow. Compact bone is a hard, solid bone tissue and forms the outside layer of bone. Trabecular bone (or spongy bone) and bone marrow are found in the inside of

bones. A part of trabecular bone eventually converts into compact bone. The bone marrow is composed of two distinct stem cell lineages, cells of hematopoietic origin and those of mesenchymal origin. Hematopoietic stem cells (HSCs) give rise to all blood cell types, including macrophages that differentiate into osteoclasts, while mesenchymal stem cells (MSCs) are responsible for the generation of stromal cells, osteoblasts, and osteocytes [36]. Additionally, these cells of mesenchymal origin are crucial for trabecular bone development. Interestingly, these two cell lineages interact with each other to maintain each other's functions. For example, osteoblasts serve as the microenvironment for HSCs, or the HSC niche [37–42], while HSCs support osteoblastic differentiation to establish the HSC niche [43]. Another example is that osteoblasts are responsible for the activation of osteoclasts [44], while these activated osteoclasts resorb the bone matrix to create the space for osteoblasts to form new bone [45]. As a result, compact bone, trabecular bone, and bone marrow crosstalk to maintain healthy bone development. This process, called bone remodeling, is a delicate balance that is often exploited by cancer cells that successfully colonize the bone [46].

Since Paget's seed and soil theory was proposed over a century ago [47], efforts have been made to understand why particular cancers prefer specific organs over others. Although blood flow and anatomical structure are considered as among the major contributing factors to the development of bone metastasis [48,49], proper physiological mechanisms of why particular types of cancers disseminate to the bone have not been fully uncovered. Additionally, recent evidence suggests that the establishment of organ-specific metastases is the result of not only the passive reception of circulating tumor cells through blood flow and anatomical structure but also the fact that the bone microenvironment selectively and actively recruits these circulating tumor cells [50]. This indicates that the interactions between the microenvironment of the bone marrow and bone metastatic cancer cells are crucial for the bone metastatic progression process.

Once these tumor cells have effectively seeded on the bone marrow, they begin to proliferate and interact with the cells involved in bone remodeling (e.g., osteoclasts, osteoblasts, and osteocytes) through paracrine and juxtacrine signaling events. This interaction creates an imbalance in the normal bone remodeling process in what has been termed the vicious cycle of bone metastases (Figure 1) [51]. Recent studies have revealed that bone metastatic prostate cancer cells hijack the interaction between the cells of hematopoietic and mesenchymal lineages, which is important for maintaining healthy bone remodeling, to establish metastatic growth within the marrow. For example, bone metastatic prostate cancer cells target the osteoblastic HSC niche during their dissemination to the bone and compete for occupancy of the HSC niche [52,53]. These observations are consistent with previous research that has demonstrated that metastatic colonization is frequently observed in bones that contain red marrow, where blood cell formation and bone formation are active (e.g., the axial skeleton, vertebrae, ribs, and the pelvis) [54].

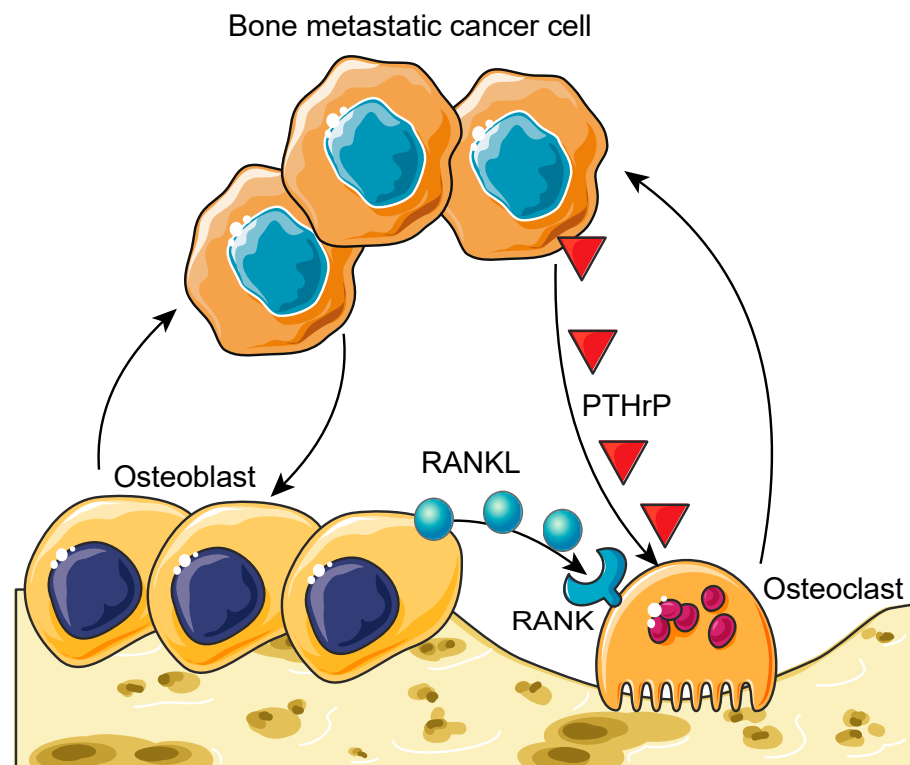


Figure 1. The vicious cycle of bone metastases. Bone metastatic cancer cells break the healthy bone remodeling processes to create a suitable microenvironment for the growing cancer cells and induce hyperosteoclastogenesis by activating osteoclasts through the secretion of parathyroid hormone-related peptide (PTHrP). This process leads to osteolytic bone lesions and provides bone metastatic cancer cells more space to grow. On the other hand, cancer cells can over-activate osteoblasts, resulting in osteoblastic bone lesions. These hyper-activated osteoblasts also stimulate osteoclastogenesis through the receptor activator of the nuclear factor κ B ligand (RANKL) (secreted from osteoblasts)/RANK (expressed on osteoclasts) axis. Furthermore, these hyper-activated osteoblasts and osteoclasts enhance the growth and survival of bone metastatic cancer cells. This process is called the vicious cycle of bone metastases. Graphics adapted from Smart Servier Medical Art (<https://smart.servier.com/bone-metastasis-March2021>) adapted from Smart Servier Medical Art (<https://smart.servier.com/>, accessed on 18 March 2021).

Additionally, once the vicious cycle has been initiated, bone metastases may present as osteolytic (bone destructive), osteoblastic (bone forming), or mixed metastases, depending on how the infiltrating cancer cells have exploited the normal bone remodeling mechanisms. Although there are osteolytic and osteoblastic-only bone metastases, most metastases arising from solid tumors have a heterogeneous phenotype [55]. Osteolytic bone metastatic lesions are characterized by the destruction of normal bone formation, primarily mediated by the hyper-activation of osteoclasts (multinucleated cells that resorb the bone matrix and develop from a monocyte-macrophage lineage) [56]. In this destructive mechanism, bone metastatic cells induce the release of parathyroid-hormone-related peptide (PTHrP), which is known to activate osteoclasts and induce bone resorption [57,58]. Conversely, osteoblastic bone metastatic lesions are characterized by the deposition of new bone. Although the mechanisms of osteoblastic bone metastases are still poorly understood, they are believed to occur through the hyper-activation of osteoblasts [59]. However, the newly formed bone matrix is poorly organized, weak, and fragile [60,61]. This leads to a lack of mechanical strength and frequent fracture [60,61]. Moreover, as osteoblasts continue to proliferate, they can inadvertently cause increased bone resorption, since they release cytokines, such as RANKL, which stimulates osteoclast differentiation and activation [59,60,62].

Taken together, the bone marrow microenvironment, especially where active hematopoiesis and bone remodeling take place, may play a crucial role in the establishment

Taken together, the bone marrow microenvironment, especially where active hematopoiesis and bone remodeling take place, may play a crucial role in the establishment and development of bone metastases, and therefore bone-marrow-microenvironment-targeting strategies have been used to treat bone metastatic disease.

3. Treatment of Bone Metastases

Current treatment options for bone metastasis include agents targeting bone remodeling (e.g., bisphosphonate and denosumab), hormone therapy, chemotherapy, external beam radiation therapy (EBRT), systemic radiotherapy, and surgical intervention [3,63,64]. Often, more than one intervention is used to suppress bone metastatic growth and maintain a patient's quality of life [65]. Although EBRT, bisphosphonate, and denosumab can reduce the onset of the painful complications of bone metastasis [17,18,66–71], delivering EBRT to every bone metastatic lesion is not practical and bisphosphonate and denosumab fail to improve the overall survival of bone metastatic patients (since these therapies mainly target bone remodeling but not cancer cells within the bone). Because of these limitations, bone metastasis is currently considered a hard-to-treat disease. Therefore, significant effort has been expended to develop ways to use the systemic delivery of therapeutic radionuclides to every bone metastatic lesion so that bone metastatic cancer cells can be exposed to the cytotoxic effects of radiation.

3.1. Radiopharmaceuticals for Bone Metastases

Radionuclides derive their cytotoxicity from the particles they release during radioactive decay, which has been considered a systemic radiotherapy for bone metastasis. These particles include auger electrons (e^-), beta minus (β^-) particles, and alpha (α^{++}) particles (Table 1) [72].

Table 1. General characteristics of therapeutic radionuclides.

Decay	Particle	Maximum Particle Range (mm)	Maximum Particle Energy (MeV)	Linear Energy Transfer (keV/ μ m)
Electron capture internal conversion	Non-energetic electrons	0.0005	0.001	26
Beta minus particle	Energetic electrons	12	2.3	0.2
Alpha particle	Helium nuclei	0.1	9	80

Auger electrons are released from an atom that undergoes electron capture or internal conversion. These particles have low energy, travel a maximum distance of a micron in tissue and have moderately high linear energy transfer (LET), which allows for many destructive ionization events along the path traveled by the particles [73]. These properties require that the emitting atom be localized in close proximity to the cancer cell's DNA to have a therapeutic effect [74,75].

Beta-minus-particle-emitting radionuclides decay by β^- emission. These negatively charged electrons have widely varying energies and path lengths, which may range from 0.05 keV to 2.3 MeV and from 0.05 mm to 12 mm, respectively. As these particles travel, they exhibit a lower LET than auger electrons. This results in few ionization events along the particles' path. Their cytotoxic effect is believed to occur through the particles' ability to generate reactive oxygen species (ROS). In turn, these ROS generate single-stranded DNA breaks within the cancer cell. High concentrations of these β^- -particles are required to create enough single-stranded DNA breaks to overwhelm DNA damage repair mechanisms and yield a therapeutic benefit.

Alpha-particle-emitting radionuclides are, by far, the most cytotoxic radionuclides that have been used as systemic radiotherapy [76,77]. These particles have mass and charge equivalent to those of a helium nucleus. Alpha-particles are emitted with an energy range of 5–9 MeV, travel short distances (equivalent to no more than 10 cell diameters), and have an

LET that surpasses the LET of auger electrons to ensure a significant number of ionization events along the alpha-particle's path through tissue. These properties make the therapeutic efficacy of these radionuclides less dependent on chemo- and radio-resistance mechanisms or hypoxia. Although their cytotoxic properties have been recognized for decades, they are only now achieving clinical translation due to successful research initiatives that have explored how to efficiently attach these α -emitting radionuclides to targeting ligands such as antibodies and peptides for effective delivery to cancer cells.

Historically, the Food and Drug Administration (FDA) has approved beta-minus-particle-emitting radiopharmaceuticals as adjunct treatments for bone metastases because of their chemical properties that give them an affinity for the bone matrix [78–81]. These radiotherapies are either administered as an ionic salt solution or chelated to bone-seeking ligands, such as hydroxyethylidene diphosphonic acid (HEDP) or tetramethylene phosphonic acid (EDTMP) (Figure 2A); a brief review of each radionuclide is outlined below.

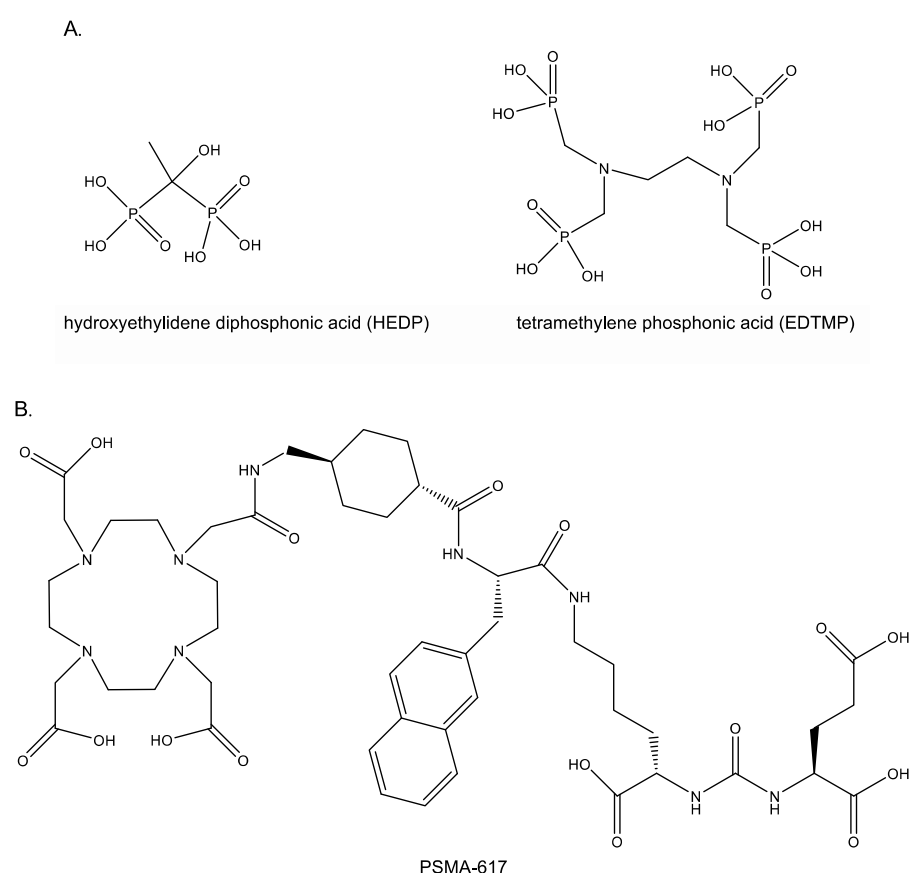


Figure 2. Several ligands used to deliver alpha-particle-emitting and beta-minus-particle-emitting radionuclides to bone metastasis. While ligands such as (A) hydroxyethylidene diphosphonic acid (HEDP) and tetramethylene phosphonic acid (EDTMP) target bone remodeling to deliver therapeutic radiation to bone metastases, ligands like (B) prostate-specific membrane antigen (PSMA)-617 target prostate cancer cell biomarkers.

Phosphorus-32 and strontium-89: Phosphorus-32 (^{32}P ; $t_{1/2} = 14.3$ d; $E_{\beta\text{max}} = 1.71$ MeV; maximum penetration in tissue (average) = 8 mm (3 mm)) is produced by the irradiation of sulfur-32 (^{32}S) through neutron capture and is distributed as ^{32}P orthophosphate. It has been investigated as a bone pain palliating radiopharmaceutical for more than 50 years [82]. After intravenous injection, approximately 90% of the injected dose is known to be incorporated into sites of bone remodeling, due to the radiopharmaceutical's affinity for the hydroxyapatite within the bone matrix. In dose-palliative studies, response rates approached 80% of treated bone [83–85] and were observed to last more than a year in patients with multiple sites of the disease. Unfortunately, due to the long particle range and relatively high relatively high energy of the β -particle, pancytopenia was observed in most patients, resulting in disfavor of its clinical use [86,87]. Strontium-89 (^{89}Sr ; $t_{1/2} = 50.5$ d; $E_{\beta\text{max}} = 1.46$

energy of the β^- -particle, pancytopenia was observed in most patients, resulting in disfavor of its clinical use [86,87]. Strontium-89 (^{89}Sr : $t_{1/2} = 50.5$ d; $E_{\beta\text{max}} = 1.46$ MeV; maximum penetration in tissue (average) = 6 mm (2.4 mm)) is produced by nuclear fission or through neutron capture and is distributed as $^{89}\text{SrCl}_2$. Since $^{89}\text{Sr}^{2+}$ ions exhibit similar chemical properties to Ca^{2+} ions, they are readily incorporated into the hydroxyapatite where active bone remodeling is occurring due to metastatic disease [78]. Typically, overall response rates, as defined by a reduction in bone pain, for patients receiving ^{89}Sr therapy approached 80%, with the palliative effects lasting approximately 15 months [87,88]. Although significant changes in red blood cell counts are not observed with this therapy, a decrease in white blood cell counts and platelets has been observed in 80% of patients [89–92], but these toxicities are resolved within 4 months of treatment cessation.

Rhenium-186 and Rhenium-188: Rhenium-186 (^{186}Re : $t_{1/2} = 3.8$ d; $E_{\beta\text{max}} = 1.07$ MeV; maximum penetration in tissue (average) = 4.5 mm (1.1 mm)) is routinely produced in nuclear reactors by direct neutron activation of metallic-enriched ^{185}Re . Rhenium-186 decays with a maximum beta energy of 1.07 MeV and a low abundance 137 keV gamma emission. It has a physical half-life of 89.3 h. Because of its maximum beta energy and particle path length, efforts have been made to determine its ability to treat bone metastasis, but since its chemical properties are not similar to those of calcium, it must be chelated to a bone-seeking ligand before injection. In the comparative study between the skeletal and soft-tissue uptake of ^{186}Re -HEDP (0.13 GBq) and ^{153}Sm -EDTMP (37 MBq/kg-body weight) in patients with confirmed bone metastasis, significantly less bone uptake was observed in patients receiving ^{186}Re -HEDP therapy, while soft-tissue retention was comparable for both radiopharmaceuticals [78]. Despite this fact, several studies have demonstrated that more than 80% of patients receiving ^{186}Re therapy reported an improvement in their bone pain [93–97]. Similar to other agents, thrombocytopenia was observed to be a major but reversible side effect of treatment [98,99]. Rhenium-188 (^{188}Re : $t_{1/2} = 0.7$ d; $E_{\beta\text{max}} = 2.12$ MeV; maximum penetration in tissue (average) = 10.4 mm (3.1 mm)), although not approved for clinical use, has also been investigated as an agent for bone pain management, since it can be produced and shipped to clinical sites as a $^{188}\text{W}/^{188}\text{Re}$ generator [100]. When chelated to HEDP, it demonstrated therapeutic efficacy, and repeated administrations improved progression-free and overall survival [101]. Additionally, it was observed that only 63% of the patients receiving ^{188}Re -HEDP therapy reported grade I thrombocytopenia, 3% reported Grade II thrombocytopenia, and 3% of the patients reported grade I leukopenia, which were comparable for patients treated with ^{186}Re -HEDP or ^{153}Sm -EDTMP [101]. Furthermore, platelet and leukocyte counts returned to pretreatment levels within 3 months, suggesting that these toxicities are clinically manageable.

Samarium-153: Samarium-153 (^{153}Sm : $t_{1/2} = 1.9$ d; $E_{\beta\text{max}} = 0.81$ MeV; maximum penetration in tissue (average) = 2.5 mm (0.6 mm)), which is produced by the neutron irradiation of a samarium-152 target, has a half-life of 46.3 h and a range in bone of 1.7 mm [102]. To be effective, however, ^{153}Sm needs to be chelated to a ligand such as EDTMP, which like other phosphonic acid complexes has a high affinity for skeletal tissue while being rapidly cleared from the blood and local soft tissue. This radiopharmaceutical has shown clinical benefit in several clinical trials, with the majority of patients experiencing pain relief [103]. In one study involving 100 patients with confirmed metastatic disease, subjects received 18–37 MBq/kg of ^{153}Sm -EDTMP and were followed for several weeks after treatment [102]. Nearly 70% of the patients experienced symptomatic relief of their bone pain. Myelotoxicity was the main side effect of treatment, with nearly 90% of the subjects experiencing grade II thrombocytopenia or leukopenia, which resolved approximately 2 months after therapy. While several patients experienced grade III/IV myelotoxicity, these patients were observed to have a depleted hematopoietic reserve because of previous therapeutic interventions.

Lutetium-177: Lutetium-177 (^{177}Lu : $t_{1/2} = 6.7$ d; $E_{\beta\text{max}} = 0.50$ MeV; maximum penetration in tissue (average) = 2.2 mm (0.67 mm)) is produced by neutron irradiation of ^{176}Lu or ^{176}Yb targets. Although several cancer-targeting agents, such as Lutathera® and ^{117}Lu -PSMA-617, have been used to deliver targeted radiation therapy to cancer cells expressing

specific cancer cell antigens [104], additional studies have sought to use the radioactive decay properties of ^{177}Lu for treating bone metastasis. Several groups have investigated the utility of ^{177}Lu -EDTMP as a bone-seeking radiopharmaceutical since the energy of the emitted β^- is low enough to reduce bone marrow suppression, which has consistently been a major disadvantage of other β^- -emitting bone-seeking agents [105,106]. Recently, the use of low- and high-dose ^{177}Lu -EDTMP was examined in a phase II study in patients with metastatic breast or prostate cancer [107]. In this study, subjects received either a low (0.13 GBq) or a high (0.26 GBq) dose of ^{177}Lu -EDTMP and were followed for 16 weeks post-treatment. Based upon pain assessment scores, the overall response rate was 86%. Additionally, treatment-related toxicity was evaluated in all patients. Grade I/II hematological toxicities were observed in 34% of the subjects, while grade III/IV toxicities were observed in 23% of the study participants. However, the observed toxicities were not significantly different in either the low- or the high-dose cohort, and this finding prompted the authors to conclude that ^{177}Lu -EDTMP is safe and effective as a treatment for bone pain palliation. More recently, several groups investigated whether using a bisphosphonate to deliver the cytotoxic radiation released by ^{177}Lu to the metastatic deposit in bone would provide greater relief. While many bisphosphonates were studied, zoledronic acid was chosen for further study since it had significantly higher incorporation into hydroxyapatite [108,109]. Consequently, several groups studied ^{177}Lu -DOTA-zoledronic acid (^{177}Lu -DOTA-ZOL) and demonstrated that it has pharmacokinetics comparable to those of ^{177}Lu -EDTMP [110,111]. These studies also revealed higher absorption of the dose by the trabecular bone in patients who received ^{177}Lu -DOTA-ZOL vs. ^{177}Lu -EDTMP, but the dose absorbed by critical organs was much lower for the former radiopharmaceutical. Recently, the safety and efficacy of ^{177}Lu -DOTA-ZOL in 40 patients with metastatic bone disease was evaluated [112]. Eligible subjects received 0.13 GBq of ^{177}Lu -DOTA-ZOL at monthly intervals and then were monitored for a 12-week period after therapy. Based upon criteria such as pain palliation, an overall response rate of 90% was observed. However, unlike patients receiving ^{177}Lu -EDTMP, grade III/IV hematological toxicities were not observed, although several patients did experience grade II anemia. Moreover, renal toxicity and hypercalcemia, which can be complications of bisphosphonate administration, were not observed in study participants. Interestingly, pain palliation was observed within 7 days of treatment, with relief lasting 10 months, which is more than twice that experienced by patients receiving ^{177}Lu -EDTMP.

Not only beta-minus-particle-emitting radiopharmaceuticals but also alpha-particle-emitting radiopharmaceuticals have been used as a treatment modality for bone metastatic disease. Radium-223 dichloride ($^{223}\text{RaCl}_2$; $T_{1/2} = 11.4$ d; $E_{\alpha_{\text{max}}} = 6\text{--}7$ MeV) is a water-soluble salt that was approved nearly a decade ago by the FDA for the treatment of bone metastasis associated with metastatic prostate cancer. Similar to calcium ions, this alkaline earth ion accumulates in bone, where it decays through seven daughter radionuclides, while releasing four α^{++} -particles and two β^- -particles, generating approximately 30 MeV of total kinetic energy, which is deposited in the surrounding bone cancer microenvironment [67–69,78,113]. This large energy deposition is believed to generate irreparable double-stranded DNA breaks within the DNA of tumor cells that have localized in the bone, causing cancer cell death [114–117]. This strategy has yielded clinical success. For example, in the ALSYMPCA trial (NCT00699751), which was a randomized, double-blind, placebo-controlled phase III trial that investigated the role of $^{223}\text{RaCl}_2$ in metastatic castration-resistant prostate cancer patients ($n = 921$), the median overall survival of patients treated with $^{223}\text{RaCl}_2$ ($n = 614$, 14.9 months) significantly improved compared with those treated with a placebo ($n = 307$, 11.3 months) (hazard ratio, 0.70; 95% confidence interval, 0.58 to 0.83; $p < 0.001$) [66]. Moreover, patients treated with $^{223}\text{RaCl}_2$ experienced decreased pain levels that correlated with increased overall survival [118]. Diarrhea, nausea, and vomiting were the most common side effects reported by patients. Additionally, pancytopenia was observed in many patients with grade III/IV thrombocytopenia, and neutropenia was observed in patients with compromised bone marrow function [119]. Since those initial trials, the use of $^{223}\text{RaCl}_2$ therapy in combination with other agents using a strategy that has

proven successful with non-radioactive therapeutics has been sought [120]. For example, a post hoc exploratory analysis of an international, early access, open-label, single-arm phase IIIb trial of the testing efficacy of $^{223}\text{RaCl}_2$ in metastatic castration-resistant prostate cancer patients following the ALSYMPCA trial revealed that a combination of $^{223}\text{RaCl}_2$ and androgen deprivation therapies (ADTs; abiraterone or enzalutamide) extended the patients' overall survival compared to therapy with $^{223}\text{RaCl}_2$ alone [118]. However, a recent multinational, multicenter, randomized, double-blind, placebo-controlled phase III trial of the combination of abiraterone and $^{223}\text{RaCl}_2$ in patients with metastatic castration-resistant prostate cancer (NCT02043678) not only failed to improve skeletal event-free survival but also increased the frequency of bone fractures compared to the placebo [121]. These contradictory results suggest that additional prospective studies are needed and caution is warranted when choosing an effective combination strategy that involves the use of ADTs and $^{223}\text{RaCl}_2$ therapy for treating prostate cancer patients with bone metastasis.

3.2. Targeted Radiopharmaceuticals for Bone Metastases

To date, the strategy for integrating systemic radiotherapies into the treatment plans of cancer patients with bone metastases has relied on bone metabolism to target bone metastatic disease. While successful, the results of this strategy have been primarily palliative, since the bone metastatic cancer cells are not specifically targeted by the radiopharmaceutical. As a result, efforts are being made to expand this strategy by developing agents that can selectively target biomarkers that are over-expressed on the cancer cells within the bone metastatic microenvironment, with the goal of improving therapeutic efficacy and patient outcomes. For example, one such strategy that has been successful in the treatment of metastatic prostate cancer has been the development of radiotherapies that specifically target prostate-specific membrane antigen (PSMA) [122]. PSMA is known to be highly expressed on prostate cancer cells at the primary tumor and within visceral and bone metastases [123]. PSMA-617, a small molecule that binds to PSMA with high affinity, was designed to target PSMA-expressing prostate cancer cells (Figure 2B) [124]. In a recent single-arm, single-center, phase II trial, ^{177}Lu -PSMA-617 was administered to men with metastatic castration-resistant prostate cancer ($n = 30$) [125]. After four cycles of radiotherapy, 57% of these patients experienced minimal toxicity and achieved a greater-than-50% reduction in prostate-specific antigen (PSA) levels, which is one of the clinical surrogate markers for prostate cancer treatment response [125]. Based on these promising results, an international prospective open-label randomized phase III trial comparing the treatment efficacy between ^{177}Lu -PSMA-617 and the best standard of care in men with metastatic castration-resistant prostate cancer (VISION trial, NCT03511664) is currently underway [126], and as of 23 March 2021, the initial result that ^{177}Lu -PSMA-617 significantly improves the overall survival and radiographic progression-free survival of PSMA-positive metastatic castration-resistant prostate cancer patients was announced (<https://www.novartis.com/news/media-releases/novartis-announces-positive-result-phase-iii-study-radioligand-therapy-177lu-psma-617-patients-advanced-prostate-cancer>, (accessed on 1 April 2021)).

Despite the promise of ^{177}Lu -PSMA-617 therapy, approximately 30% of patients do not respond to this treatment [127]. These observations have led to the investigation of the use of ^{225}Ac -PSMA-617 as an alternative alpha-particle-emitting radiotherapy for refractory patients [127–129]. Actinium-225 (^{225}Ac ; $t_{1/2} = 10$ d; $E_{\alpha_{\text{max}}} = 6\text{--}8$ MeV) is a radioactive metal of the actinide series. Similar to $^{223}\text{RaCl}_2$, ^{225}Ac has a relatively long half-life and emits four α^{++} - and two β^{-} -particles per nuclear decay. However, unlike $^{223}\text{RaCl}_2$, ^{225}Ac can be linked to a variety of targeting ligands [130–137]. In the early stage of the development of ^{225}Ac -PSMA-617, two patients received this therapy [127]. The first patient exhausted conventional chemotherapies, bone remodeling therapy, ADTs, and six cycles of $^{223}\text{RaCl}_2$ therapy. The patient received three cycles of approximately 10 MBq (100 kBq/kg body weight) of ^{225}Ac -PSMA-617. After 8 weeks, all visible visceral and bone metastases had decreased to a size that was below the limit of detection of clinical imaging scanners, while PSA levels had decreased from 3000 to 0.26 ng/mL. Similar to the first

in both preclinical and clinical settings (Table 2). Since PSMA is expressed by not only bone metastatic prostate cancer cells but also those at the primary site and soft-tissue metastatic sites, this strategy is not specific for bone metastases. However, a majority of the patients with metastatic castration-resistant prostate cancer experience bone metastases during the course of disease progression. Therefore, the development of PSMA-targeted alpha-particle-emitting radiopharmaceuticals is crucial if eradicating bone metastatic prostate cancer is to be an achievable clinical goal. Further studies, including the assessments of their clinical efficacy and safety, are clearly warranted.

Table 2. The recent status of PSMA-targeted alpha-particle-emitting radiopharmaceuticals.

Agent	Conjugator	Development Phase	Refs.
^{213}Bi -J591	PSMA-targeting murine monoclonal antibody, J591	Preclinical study	[143–145]
^{213}Bi -PSMA I&T	PSMA-targeting (3S,7S)-29,32-dibenzyl-5,13,20,28,31,34-hexaoxo-37-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)-4,6,12,21,27,30,33-heptaazaheptatriacontane-1,3,7,26,37-pentacarboxylic acid (DOTAGA-FFK(Sub-KuE)), PSMA I&T	Preclinical study	[146]
^{213}Bi -JVZ-008	PSMA-targeting nanobody, JVZ-008	Preclinical study	[146]
^{211}At -6	PSMA-targeting (2S)-2-(3-(1-carboxy-5-(4- ^{211}At -astatobenzamido)pentyl)ureido)-pentanedioic acid, compound 6	Preclinical study	[147]
^{225}Ac -PSMA-617	PSMA-targeting small molecule, PSMA-617	Clinical study	[127,138,141,148]
^{227}Th -PSMA-TTC	PSMA-targeting fully human antibody, BAY 2315158	Clinical trial (phase I: NCT03724747)	[149]
^{225}Ac -J591	PSMA-targeting murine monoclonal antibody, J591	Clinical trial (phase I: NCT03276572)	

4. Conclusions

Bone metastasis creates enormous physical, emotional, and financial burdens for cancer patients and society; it is a significant contributor to cancer mortality rates. Systemically delivered radionuclides have been explored as potential therapies for bone metastasis. Initially, β^- -emitting radionuclides were used because they could be delivered easily to sites of active bone remodeling. However, their efficacy has been limited to pain mitigation. Recently, systemically delivered $^{223}\text{RaCl}_2$, which decays through α^{++} -particle emission, has been observed to extend the overall survival of men with metastatic prostate cancer. These results have renewed interest in alpha-particle-emitting radiotherapies, and new molecularly targeted strategies to deliver this highly cytotoxic radiation directly to bone metastatic cancer cells within the bone marrow microenvironment are being investigated. Using this strategy, ^{225}Ac -PSMA-617, which has been an effective treatment for men with metastatic prostate cancer, which was previously believed to be refractory to conventional chemotherapies and standard of care radiotherapy, has been developed. While further clinical evaluation is warranted, current data suggest that targeted alpha-particle-emitting radiopharmaceuticals used alone or in concert with current standard treatments may lead to much-needed improvements in the clinical management of cancer bone metastasis.

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Research Paper

Osteoblasts derived from mouse mandible enhance tumor growth of prostate cancer more than osteoblasts derived from long bone

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ABSTRACT

Prostate cancer (PCa) metastasizes to bone, where the bone marrow microenvironment controls disease progression. However, the cellular interactions that result in active bone marrow metastases are poorly understood. A better understanding of these interactions is critical to success in the pursuit of effective treatments for this life ending disease. Anecdotally, we observe that after intracardiac injection of PCa cells, one of the greatest tools to investigate the mechanisms of bone-metastatic disease, animals frequently present with mandible metastasis before hind limb metastasis. Therefore, in this study, we investigated whether the bone cells derived from the mouse mandible influence PCa progression differently than those from the hind limb. Interestingly, we found that osteoblasts harvested from mouse mandibles grew faster, expressed more vascular endothelial growth factor (VEGF), increased vascularity and formed more bone, and stimulated faster growth of PCa cells when cultured together than osteoblasts harvested from mouse hind limbs. Additionally, these findings were confirmed *in vivo* when mouse mandible osteoblasts were co-implanted into mice with PCa cells. Importantly, the enhancement of PCa growth mediated by mandible osteoblasts was not shown to be due to their differentiation or proliferation activities, but may be partly due to increased vascularization and expression of VEGF.

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1. Introduction

Prostate cancer (PCa) is deadly when it metastasizes to bone, often years after a patient is cured of the primary disease. These metastases are believed to originate from disseminated tumor cells (DTCs) shed from the primary tumor that made their way to the bone marrow and became dormant. Once in the bone marrow, progression of dormant DTCs in the bone is directed by influences from the bone marrow microenvironment. Still, a full understanding of the more complex interactions that result in active bone marrow metastases remains elusive. Our group recently discovered that early in the metastatic process, PCa cells target and commandeer the hematopoietic stem cell (HSC) specific marrow microenvironment, or HSC niche, using mechanisms similar to those involved in HSC homing to bone [1]. Osteoblasts are a major component of the HSC niche and have been shown to participate in the regulation of HSC homing and quiescence (or dormancy) [2–5]. Interestingly, DTCs also utilize osteoblasts to establish early colonization within the marrow [1,6]. Thereafter, the DTCs lie dormant

within the osteoblastic niche until they begin to proliferate again, controlled by mechanisms still poorly understood, and eventually develop into full-blown metastases [7].

A tool frequently used to investigate the mechanisms of metastasis is the intracardiac injection mouse model. After a small volume of cancer cells is inoculated into the left ventricle of the mouse heart, the circulatory system becomes flooded with cancer cells that eventually colonize organs of preference, such as bone. As expected, this model consistently results in long bone metastases, including lesions in the femur and tibia. However, mandible metastases are also frequently observed in this model [8]. This appears to be a phenomenon unique to this model, as metastasis to the human mandible is uncommon for solid tumors known to metastasize to the bone, such as PCa [9]. Anecdotally, using this model we have found that sometimes the only site of metastasis is the mandible, and that in animals with multiple bone metastatic lesions, many demonstrate faster growth in the mandible than those in the hind limbs of the same animal (unpublished observations). These findings raise the question whether the bone marrow microenvironment of the mandible stimulates the growth of DTCs more than that of the hind limbs. One difference between bone cells derived from mandible and long bones that has been documented is their proliferative and differentiation potential. Indeed,

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osteoblasts derived from rat mandible bone marrow grew faster and formed more bone *in vivo* than those differentiated from long bone marrow [10]. The same effects were seen in human tissue cultures of mandible and iliac marrow derived osteoblasts [11]. Interestingly, it has recently been revealed that different subpopulations of osteoblasts contribute differently to bone metastatic progression [12]. Therefore, we hypothesized that osteoblasts harvested from mouse mandible bones would enhance the growth of PCa cells when they interact with each other, more than those harvested from hind limb bones.

In this study, we found that osteoblasts harvested from mouse mandible bones grew faster than those harvested from hind limb bones, similar to the reports of osteoblasts derived from rat and human tissues. Additionally, osteoblasts harvested from mouse mandibles expressed higher levels of vascular endothelial growth factor (VEGF). Moreover, when these osteoblasts were co-cultured with PCa cells, osteoblasts harvested from mouse mandibles significantly increased the growth of PCa cells compared to osteoblasts harvested from hind limbs. Most importantly, these findings were confirmed *in vivo*, and immunofluorescent staining of pathological specimens revealed that vascularity of mandible osteoblast implants was greater than hind limb osteoblast implants.

2. Materials and methods

2.1. Cell culture

Human PCa cell line PC-3 [American Type Culture Collection (ATCC), Manassas, VA, Cat #: CRL-1435] and DU145 cells (ATCC, Cat #: HTB-81) were transformed to stably express green fluorescent protein (GFP) and firefly luciferase (PC3-GFP-luc, and DU145-GFP-luc) by transduction with a lentivirus (Lenti-GF1-CMV-VSVG) generated by the University of Michigan Vector Core. The transduced cells were sorted for GFP positive cells at the Wake Forest Baptist Comprehensive Cancer Center Flow Cytometry Shared Resource using an Astrios EQ (Beckman Coulter, Pasadena, CA), expanded and frozen at low passage (<10). The growth media for PC-3 and DU145 was RPMI 1640 (Gibco, Gaithersburg, MD, Cat #: 11875093) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, Cat #: F2442), 1% Penicillin-Streptomycin (Gibco, Cat #: 15140122), and 1% L-Glutamine (Gibco, Cat #: 25030081). Murine calvarial pre-osteoblast cell line MC3T3-E1 Subclone 4 (ATCC, Cat #: CRL-2593) were cultured with MEM α without nucleosides (Gibco, Cat #: 12561056) supplemented with 10% FBS, 1% Penicillin-Streptomycin, and 1% L-Glutamine. Before supplementation, this formulation of MEM α already contains 50 mg/L Ascorbic Acid, and therefore no additional Ascorbic Acid was required. Cells were incubated at 37°C, 5% CO₂, and 100% humidity and were routinely passaged when no more than 80% confluent.

2.2. Animal care and use certification

All animal studies were approved by the Institutional Animal Care and Use Committee (Protocol A18-036) at Wake Forest University Health Sciences.

2.3. Intracardiac PCa inoculation and imaging of tumor growth

DU145-GFP-Luc (1×10^6 cells/100 μ L of PBS) cells were injected into immunocompromised Athymic Nude mice (Charles River Laboratories, Wilmington, MA, Cat #: 490) by left ventricular intracardiac injection, as previously described [13]. To monitor cancer growth, luciferase signal was followed at least once a week for

10 weeks using the IVIS Lumina Series III system and Living Image software (Xenogen, Alameda, CA).

2.4. Primary murine osteoblast culture

Hind limbs and mandibles were dissected from 5-week old male C57BL6/J mice (The Jackson Laboratory, Bar Harbor, ME, Cat #: 000664). Epiphyses from the femurs and tibias were cut and the marrow was removed by centrifugation, as previously described [14]. Briefly, an 18g hole was bored into the bottom of a 0.6 mL microcentrifuge tube with a needle, and then the tube was placed in a 1.7 mL microcentrifuge tube. The diaphyses of the bones were placed in the smaller tube, and the apparatus was then centrifuged at max speed for 30 sec. Afterward, the bone fragments remained in the upper 0.6 mL tube, and the marrow was pelleted in the bottom 1.7 mL tube. The empty diaphyses were placed into a 10 cm dish in HBSS, no calcium, no magnesium, no phenol red (Gibco, Cat #: 14175095) supplemented with 1% Penicillin-Streptomycin and minced into smaller pieces using a scalpel. The molars, incisors and dental pulp of the mandibles were removed and the remaining pieces (ascending ramus containing the coronoid, condylar and angular processes) were placed into a 10 cm dish in HBSS supplemented with 1% Penicillin-Streptomycin and minced into smaller pieces using a scalpel. After forceful washing to remove all marrow, a 10 min Trypsin-EDTA digestion was performed on the bone and mandible pieces at 37°C. Complete MEM α [MEM α without nucleosides supplemented with 10% FBS, 1% Penicillin-Streptomycin, 1% L-Glutamine, and 10nM dexamethasone (Sigma-Aldrich, Cat #: D4902) [15]] was then added to the explant cultures and the mature bone cells were allowed to migrate out of the bone and mandible pieces and expand for two weeks. In some cases, the cells were grown in bone mineralization medium (BMM) to induce further osteoblastic differentiation and mineral deposition: this media is complete MEM α supplemented with 0.5mM L-Ascorbic acid (Sigma, Cat #: A5960), 2mM β -Glycerophosphate disodium salt hydrate (Sigma-Aldrich, Cat #: G9422), and 10mM HEPES (Thermo Fisher Scientific, Hampton, NH, Cat #: BP299). The control media for BMM is complete MEM α supplemented with 10mM HEPES (vehicle).

2.5. MTT assay

Cells were seeded at a density of 4×10^3 cells/100 μ L complete MEM α into each well of a 96-well plate and incubated for 1, 3, and 5 days. To measure relative cell numbers, 500 μ g/mL MTT (Tocris Bioscience, Minneapolis, MN, Cat #: 5224) was added to each well and incubated for 4 h. The reaction was halted, and the formazan precipitate was dissolved by the addition of an equal volume of 10% SDS in 0.01 M HCl (10 g/100 mL). The plate was incubated at 37°C, 5% CO₂, and 100% humidity overnight and read on a plate reader at 560 nm with a background measurement at 650 nm.

2.6. In vitro mineralization assay

Cells were seeded at a density of 1×10^5 cells/250 μ L complete MEM α into each well of a 24-well plate and incubated for 24 h. The complete MEM α was then replaced with 500 μ L fresh complete MEM α and the plate was incubated for 48 h. The media was replaced with vehicle or BMM and incubated for 72 h. This process was continued every other day for a total of 14 days from first treatment. Half of the wells were harvested for mRNA. The remaining wells were stained with Alizarin Red S (ARS) to quantify total mineralization. Briefly, cells were fixed with 10% neutral buffered formalin for 5 min at room temperature and then stained with 2% ARS (Sigma-Aldrich, Cat #: A5533-25G) at pH 4.1 in water (2 g/100 mL) for 30 min at room temperature. Excess stain was

washed out in running tap water until the water ran clear. Representative images were captured on an EVOS inverted microscope (Thermo Fisher Scientific). Stain from each wells was quantified after lysing entire contents as described elsewhere using an acetic acid extraction method and plate reader absorbance at 405 nm [16].

2.7. Real Time qPCR

Confluent and multilayered osteoblastic cells were lysed, and RNA was harvested using the RNeasy Plus Mini Kit (Qiagen, Germantown, MD, Cat#: 74134). The RNA concentrations were determined and subsequently normalized between samples prior to cDNA generation using Invitrogen SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, Cat#: 18064022). Real time qPCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA Cat#: 4369016) and Taqman Gene Expression Assays [Applied Biosystems, Cat#: 4331182, Assay IDs: Mm03413826_mH (*Bglap*: Osteocalcin, OCN), Mm00436767_m1 (*Spp1*: Osteopontin, OPN), Mm00437306_m1 (*Vegfa*: vascular endothelial growth factor, VEGF), Mm00441906_m1 (*Tnfrsf11*: RANKL), Mm00435454_m1 (*Tnfrsf11b*: Osteoprotegerin, OPG), Hs00921372_m1 (*TNFRSF1*: RANK), Hs02786624_g1 (*GAPDH*), and Mm99999915_g1 (*Gapdh*)] on the Bio-Rad CFX Connect instrument (Hercules, CA). Data is presented as relative gene expression using the delta-delta Ct method, with *Gapdh* used as the reference gene.

2.8. Co-culture assay

Primary cells, or MC3T3-E1 cells, were seeded at a density of 2×10^4 cells/100 μ L complete MEM α into each well of a 96-well plate and incubated for 24 h. In some cases, cells were growth arrested for 2.5 h with 10 μ g/mL mitomycin-C (Sigma-Aldrich, Cat #: 10107409001). Thereafter, cancer cells were seeded on top of the bone cells or by themselves in new 96-well plates, at a density of 2×10^3 cells/100 μ L complete MEM α . Plates were incubated for 48 h and the media replaced with vehicle or BMM, and incubated for 1, 3, and 5 days, with media replacement at each time point. To monitor cancer growth in real time, luciferase signal was checked before each media replacement using the IVIS system, after addition of 200 μ g/mL D-Luciferin (PerkinElmer, Waltham, MA, Cat #:122799) and 10 min incubation at 37°C.

2.9. Ossicle implantation

1×10^5 DU145-GFP-luc and/or 1×10^6 primary cells [mandible osteoblasts (MaOBs) or hind limb osteoblasts (HLOBs)] were suspended in 10 μ L BMM in individual microcentrifuge tubes (inoculum tube) and stored on ice until implantation. Under sterile conditions, absorbable gelatin sponges (Ethicon, Somerville, NJ, Cat #: 1973) were cut into small cubes (~ 5 mm³) and placed in a petri dish containing BMM on ice until implantation. Mice were anesthetized, backs shaved if necessary, and the surgical site was prepared using betadine. Blunt tipped scissors were used to create a small incision (~ 1 cm) in the dorsal skin parallel to the spine, and the scissors were used to create subcutaneous pouches on both sides of the incision. Using fine tipped forceps, a sponge piece was removed from the petri dish, excess liquid was blotted using sterile gauze, and then seeded by placing it in the bottom of an inoculum tube and allowing it to absorb the entire inoculum. The seeded sponge was then implanted into one of the subcutaneous pouches. Each animal received an implant seeded with HLOBs in one pouch and an implant with MaOBs in the opposite pouch. The incision was closed with surgical staples, which were removed after 7 days. Animals implanted with tumor seeded cells were

athymic nude mice and animals implanted with only primary cells were C57BL6/J (male, 5 weeks old). In order to monitor cancer growth in co-culture seeded ossicles *in vivo*, luciferase signal was followed at least once a week for 20 weeks using the IVIS system, whereas primary cell only seeded ossicles were grown for 7 weeks *in vivo*.

2.10. Tissue processing

After euthanasia, the implants were dissected and placed in 10% neutral buffered formalin for 24 h at 4°C under agitation. They were then radiographed using the MultiFocus 10x15 Digital Radiography System (Faxitron Bioptics, Tucson, AZ). Radiographs were analyzed using ImageJ software (NIH, Bethesda, MD) for densitometry. Briefly, tissue was outlined using the freehand tool and Max Gray values were determined for each implant. Next, the implants were placed in cassettes and decalcified for 14 days in 10% ethylenediaminetetraacetic acid (EDTA), with fresh solutions replaced on day 7. The tissues were then cryopreserved in 30% sucrose for 48 h. They were halved with a razor blade and one half of each tissue was arranged in plastic molds containing optimal cutting temperature (OCT) media and frozen on dry ice. Using a cryostat, 10 and 20 μ m sections were made and stored at -80°C. The other tissue halves were processed for paraffin embedding and sectioned on a microtome at 5 μ m sections. Hematoxylin and Eosin (H&E) staining was performed on sections, as well as immunohistochemistry (IHC) for Ki67 (Abcam, Cambridge, MA, Cat#: ab16667), and immunofluorescence (IF) for Osteocalcin (Takara Bio USA, Mountain View, CA, Cat#: M173), Pancytokeratin (Novus Biologicals, Centennial, CO, Cat #: NB600-579SS), Endomucin (Santa Cruz Biotechnology, Dallas, TX, Cat#: sc-65496), VEGF (Santa Cruz Biotechnology, Cat#: sc-7269), and CD31 (R&D Systems, Minneapolis, MN, Cat #: AF3628) as previously described [17]. Antigen retrieval was performed using Biogenex DeCal Retrieval Solution (Biogenex, San Ramon, CA, Cat#: HK089-5K) before incubating with the primary antibody at a concentration of 1:500 overnight at 4°C. Sections were then labeled with secondary rabbit antibody (Biogenex, Cat#: HK336-5R), Vectastain Elite ABC HRP kit (Vector, Burlingame, CA, Cat#: PK-6100) and DAB Peroxidase (HRP) Substrate Kit (Vector, Cat#: SK-4100). Slides were mounted in permanent mounting media and scanned on a NanoZoomer slide scanner (Hamamatsu, Japan) at 40x magnification. Quantitative analysis was performed using Visiopharm software (Westminster, CO) and an APP designed to automatically count DAB and hematoxylin alone positive nuclei, as well as determine tissue section area on the entire slide.

2.11. Statistical analysis

Metastatic tumor growth was measured by bioluminescent imaging (BLI), and radiance values were log transformed in order to satisfy the conditional normality assumption. The mean and standard error of the mean (SEM) of log transformed radiance values were calculated by time and location of metastatic lesions (mandible vs. hind limb). For exploratory purposes, comparisons of log transformed radiance values between locations at each time point were performed using the paired Student's t-tests. Mean differences in log transformed radiance between locations were also estimated using the mixed effects model with time, location, and interaction between time and location included in the model. The advantage of using this model was that all data were analyzed simultaneously, resulting in more efficient estimates. The hypothesis test for location effect at each time point was performed using a contrast. The nested (location within each mouse) random intercepts were used to take into account the correlated structure (e.g., repeated measures over time; measures at different locations in

the same mouse). Sub-analysis of the same data was performed to compare metastasis-free survivals, as measured with a 75% signal cut off. Event time was defined as the time from the beginning of the study to the initial endpoint ($\geq$ the 75th percentile of log transformed radiance) and censoring time as the time from the beginning of the study to the last assessment or death. The Kaplan-Meier survival curves for the initial endpoint by location were plotted. The comparison between the two survival curves were performed using the Cox proportional hazards model with robust sandwich estimates to take into account the correlated structure (e.g., different locations in the same mouse). The same statistical approaches were applied to analyses of tumors co-cultured with primary cells (MaOBs or HLOBs).

The distributions for the other outcome measures such as gene expression and OD were confirmed to be normal. The means and SEM of the outcome measures were calculated by tissue origin (mandible vs. hind limb) and by treatment (vehicle vs. BMM), and comparisons of the outcome measures were performed using the unpaired Student's t-test. The means and standard errors of the tissue section areas and of MaOB or HLOB ossicles were calculated and compared using the paired Student's t-test.

The comparison of log transformed average radiance between the combination of primary cells (MaOBs vs. HLOBs) and treatment (vehicle vs. BMM) at each time point was performed using the analysis of variance (ANOVA) for exploratory purpose. Mean differences in log transformed radiance between primary cells and treatments at each time point were also estimated using the mixed effects model with time, location, treatment, and two-way and three-way interactions included in the model. If higher order interactions were not statistically significant, they were removed from the model. For PC3 co-culture analyses, the interaction between primary cells and time as well as the interaction between treatment and time were included in the model. For DU145 co-culture analyses, only the interaction between primary cells and time was included in the model. The random intercept was used to handle repeated measurements over time. The hypothesis tests for primary cell effect and BMM effect at each time point were performed using contrasts. All the analyses were performed using the GraphPad Prism statistical program (GraphPad Software, San Diego, CA) and the SAS software (SAS Inc, Cary, NC) with significance at $P \leq 0.05$.

3. Results

3.1. Intracardiac injection results in lesions in the mandibles before the hind limbs.

Since we have anecdotally found that mandible metastases occur faster than hind limb metastases in mice following intracardiac injection of PCa cells, we sought to examine more detailed metastatic patterns and tumor growth capacities of PCa cells in the intracardiac model. To do so, human PCa cell line DU145-GFP-luc cells were inoculated intracardially into immunocompromised athymic nude mice. When the bioluminescent imaging (BLI) signals of nude mice inoculated with DU145-GFP-luc were followed, detectable tumors in the mandibles were significantly brighter, and therefore larger in size than those in the hindlimbs at day 29, but over time signals at both sites became similar (Fig. 1A&B). Curiously, the BLI signals of the mandibles actually begin to decrease after day 29, which might be explained by tumor necrosis or hypoxic environments associated with fast-growing tumors [18]. Sub-analysis of the same data was performed to compare metastasis-free survivals, as measured with a 75% signal cut off, and the onset of metastasis in the mandible appeared to occur

sooner than the hindlimb, although not statistically significant (Fig. 1C).

3.2. Cells derived from mandible and hind limb bone explants have osteoblastic characteristics.

To dissect out the differences between cells in the mandibles and hind limbs, we decided to compare the functions of osteoblasts from these two areas, since proliferation of PCa is known to be regulated in part by osteoblasts [19–21]. After cleared of soft tissue and bone marrow, bone pieces obtained from the mandibles and hind limbs (femurs and tibias) of adult C57BL6/J mice were minced (Fig. 2A) and cultured in media containing dexamethasone (10nM) to differentiate any remaining cells into osteoblasts [15]. After two weeks of culture, cells derived from the bone explants were assessed of their osteoblastic characteristics. Both cultures expressed relatively high levels of the osteoblastic markers Osteocalcin and Osteopontin (Fig. 2B&C). Basal expression of Osteocalcin and Osteopontin were both lower in the mandible cell cultures than the hind limb cultures, but the presence of both markers in vehicle-treated cells revealed that the cultured bone explant cells are mostly differentiated even without the use of differentiation supplements, unlike traditional bone marrow stromal cell culture preps (Fig. 2B&C). After treatment with known osteoblastic differentiation supplements L-Ascorbic acid and β -Glycerophosphate, the levels of these markers were significantly increased in both explant cultures, however cultures of mandible cells responded to L-Ascorbic acid and β -Glycerophosphate to a greater degree than cultures of hind limb cells (Fig. 2B&C). As vascular endothelial growth factor (VEGF) derived from osteoblasts, encoded by the mouse gene *Vegfa*, has been shown to be involved in osteoblast differentiation and mineralization [22], and VEGF is implicated in PCa migration to bone and growth through induction of angiogenesis [23], we tested whether *Vegfa* expression was increased in mandible cultures. Basal expression of *Vegfa* was higher in cultures of mandible cells than those of hind limb cells and was not significantly changed in response to L-Ascorbic acid and β -Glycerophosphate supplementation in either mandible or hind limb cultures (Fig. 2D). To further test osteoblastic functional capabilities, the mineralization potentials of these cells were evaluated using Alizarin Red S (ARS) staining. Both cells derived from mandible and hind limb explants were able to mineralize, as evidenced by ARS staining (Fig. 3A&B). Interestingly, cells from mandible explants showed more mineralization than cells from hind limb explants, regardless of L-Ascorbic acid and β -Glycerophosphate supplementation (Fig. 3B). We attribute this observation to the previous finding that differentiation supplements were not required for osteoblast marker expression in our bone explant cultures (Fig. 2B&C), and therefore, not required for mineralization either. Altogether, this suggests that the cells derived from mandible and hind limb bone explants have osteoblastic characteristics. Therefore, from here on, the cells derived from mandible and hind limb bone explants will be referred to as mandible osteoblasts (MaOBs) and hind limb osteoblasts (HLOBs), respectively.

3.3. Mandible osteoblasts form more bone than hind limb osteoblasts *in vivo*

To further confirm the osteoblastic characteristics of MaOBs and HLOBs, these cells were implanted together with gelatin scaffolds (ossicles) subcutaneously into C57BL6/J mice, and the ossicles were allowed to grow *in vivo* for 7 weeks. Radiographs of the resulting ossicles revealed that the MaOB seeded ossicles had more mineralization than HLOB seeded ossicles (Fig. 4A&B). The ossicles were decalcified and cryosectioned in order to perform immunofluores-

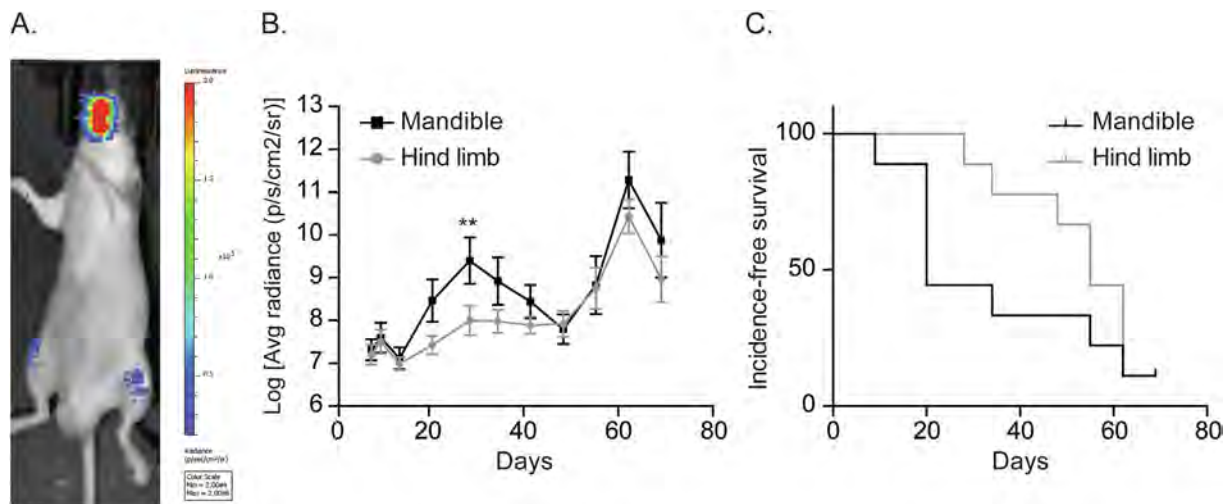


Fig. 1. Metastasis to mandible occurs first after intracardiac injection of PCa cells. Five-week old male nude mice were injected intracardially with 1×10^6 DU145-GFP-luc cells ($n=8$) and observed with bioluminescent imaging (BLI) for 10 weeks. (A) Representative image of a mouse at day 29 demonstrating brighter signal in the mandible than the hindlimbs. (B) All collected longitudinal BLI data after log transformation. Results displayed as Mean $\pm$ SEM. Mixed effects model. $p \leq 0.01$. (C) A 75% cut-off was employed to examine metastasis-free survival (mandible vs. hind limb). Cox proportional hazards model with robust sandwich estimates.

cence for Osteocalcin, hematoxylin and eosin (H&E) staining, and immunohistochemistry for Ki67 (Fig. 4C-E). In order to test whether the cells contained in the ossicles were still osteoblasts, immunofluorescence was performed on the cryosections for Osteocalcin. It is clear that the cells located near and on bone nodules in the ossicles remained Osteocalcin positive, and substantial Osteocalcin appeared to be deposited within the bone itself (Fig. 4C). H&E staining confirmed what was seen by radiograph, as evidenced by increased frequency of woven bone nodules in the MaOB seeded ossicles (Fig. 4D). Visiopharm software was used to automatically quantify tissue section area, and Ki67 positive and negative nuclei. MaOB seeded ossicles were no larger in area than HLOB ossicles (Fig. 4F), but contained a significantly greater number of total and proliferating cells, as evidenced by Ki67 nuclear positivity (Fig. 4G&H).

A possible explanation for the increased proliferation of MaOB cells *in vivo* could be increased vascularity of the MaOB ossicles due to higher levels of VEGF. Interestingly, we did observe greater immunostaining for the endothelial cell markers Endomucin and CD31 in the tissue surrounding and within the implants seeded with MaOBs, when compared to those seeded with HLOBs (Fig. 5A). Still, vascularity of all the implants appeared somewhat scarce and could explain why relative levels of bone formation in the implants appeared lower than other models which use bone marrow stromal cells as the inoculum for ossicle models of bone formation [15]. We also performed immunofluorescence for VEGF using ossicle cryosections as we found differential expression of *Vegfa* in our cell cultures *in vitro* (Fig. 2D). Strangely, we did not see VEGF staining in Osteocalcin positive MaOBs within the implants. However, we did detect VEGF near Osteocalcin positive MaOBs (Fig. 5B), but not HLOBs (data not shown). These results suggest that MaOBs seeded in ossicles may secrete VEGF to the tissues surrounding the implant.

3.4. Mandible osteoblasts support prostate cancer growth more than hind limb osteoblasts *in vitro* and *in vivo*

Our next attempt was then to determine whether there are differential effects between MaOBs and HLOBs on PCa growth. To address this question, human PCa cell lines PC3-GFP-luc and DU145-GFP-luc cells were seeded on top of MaOB or HLOB cells. After 3–5 days of co-culture, more PCa cells were observed on

MaOBs than HLOBs using BLI (Fig. 6A&B). However, L-Ascorbic acid and β -Glycerophosphate supplement failed to induce further tumor proliferation (Fig. 6A&B), suggesting that osteoblastic mineralization was not the major cause of greater PCa proliferation mediated by MaOBs. Then, the changes in the relative amounts of viable cells during *in vitro* culture between MaOBs and HLOBs were compared. As previously reported in rat and human bones [10,11], the cultures containing MaOBs had more viable cells compared to those containing HLOBs (Fig. 6C), suggesting that MaOBs grew significantly faster than HLOBs. However, when mitomycin-C growth arrested MC3T3-E1 cells were cocultured using the same methods, PCa cells actually grew faster on top of growth arrested osteoblasts (Fig. 6D&E). These data suggest that the differences between PCa growth in culture with MaOBs vs. HLOBs are not likely due to baseline differences in mineralization or proliferation.

Next, DU145-GFP-luc cells were co-implanted with either MaOB or HLOB ossicles subcutaneously into athymic nude mice. Similar to what was seen *in vitro*, the growth of DU145-GFP-luc cells co-implanted with MaOB ossicles, as evidenced by significantly brighter BLI signals on the last five measurements, appeared to be greater than those co-implanted with HLOB ossicles (Fig. 7A&B). These results were significantly different despite the fact that one mouse had a much smaller MaOB co-implant tumor than the other four mice (Fig. 8A). With such a limited group size, this tumor cannot be considered an outlier, but it is the reason why the differences observed in the longitudinal data also seem limited (Fig. 7A&B). Sub-analysis of the same data was performed to compare incidence-free survivals, as measured with a 75% signal cut off, and tumors in the MaOB ossicles occurred significantly sooner than in the HLOB ossicles (Fig. 7C). These differences were consistent with *ex vivo* BLI analyses performed on the dissected tumors which again showed significantly brighter signal in the MaOB ossicles (Fig. 7D). As before, radiographs revealed that the MaOB seeded ossicles had more calcification than HLOB seeded ossicles (Fig. 8A&B). In order to test the composition of the cells contained in the co-implanted ossicles, immunofluorescence for pancytokeratin was performed on the cryosections of decalcified ossicles. Clearly, the implants with both MaOBs and HLOBs at 20 weeks contained PCa tumor cells (Fig. 8C). H&E staining and immunohistochemistry for Ki67 were also performed on the co-implanted ossicles to further determine their morphology and cell proliferation status (Fig. 8D&E). Visiopharm software was used to auto-

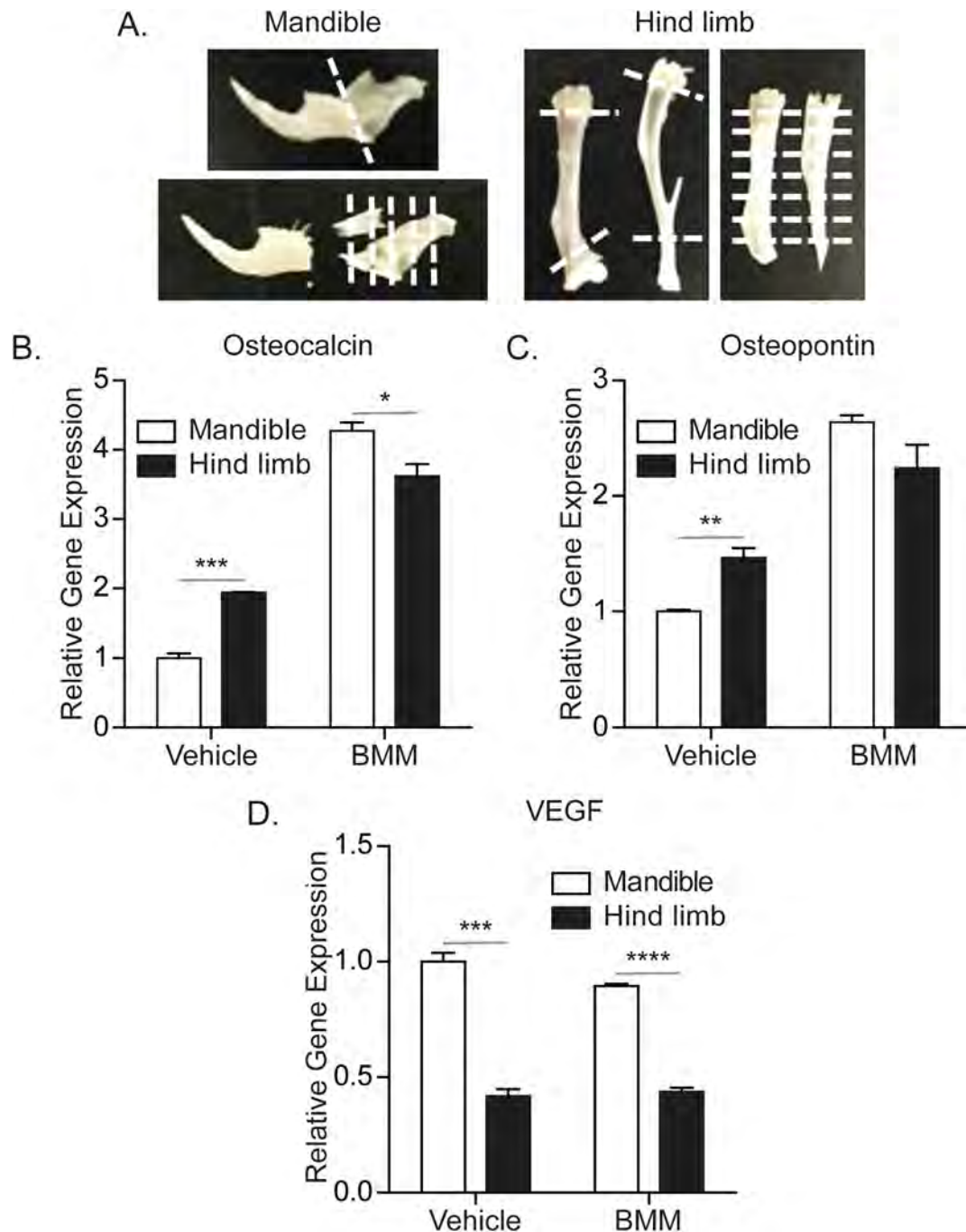


Fig. 2. Cells harvested from mandible and hind limb bones express osteoblast markers. Five-week old male C57BL/6J mice were euthanized, and mandibles and hind limbs were harvested. Mandible and hind limb cells were cultured with bone mineralization medium (BMM) (0.5mM L-Ascorbic acid, 2mM β -Glycerophosphate disodium salt hydrate, and 10mM HEPES) or vehicle (10mM HEPES) for 10 days. (A) Schematic of bone culture preparation: molars, incisors and dental pulp of the mandibles were removed and discarded; epiphyses from the femurs and tibias were cut and the marrow was removed by centrifugation; remaining pieces of the mandibles and femurs/tibias were minced into smaller pieces, digested with trypsin, and cultured and expanded for two weeks before experiments. (B) Relative gene expression of Osteocalcin (Bglap), normalized to vehicle treated mandible cell cultures. (C) Relative gene expression of Osteopontin (Spp1), normalized to vehicle treated mandible cell cultures. (D) Relative gene expression of vascular endothelial growth factor (VEGF), normalized to vehicle treated mandible cell cultures. Gapdh was used as reference gene. Delta-delta Ct method. Results displayed as Mean $\pm$ SEM. Unpaired Student's *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

matically quantify tissue section area, and Ki67 positive and negative nuclei. MaOB seeded ossicles appeared larger in area than HLOB ossicles, although not quite significant (Fig. 8F), but contained a significantly greater number of total and proliferating cells, as evidenced by Ki67 nuclear positivity (Fig. 8G&H).

4. Discussion

In this study, we first demonstrated that in the intracardiac model of metastatic PCa, mandible bone lesions often occur before hind limb bone lesions, which is anecdotally well-recognized. We

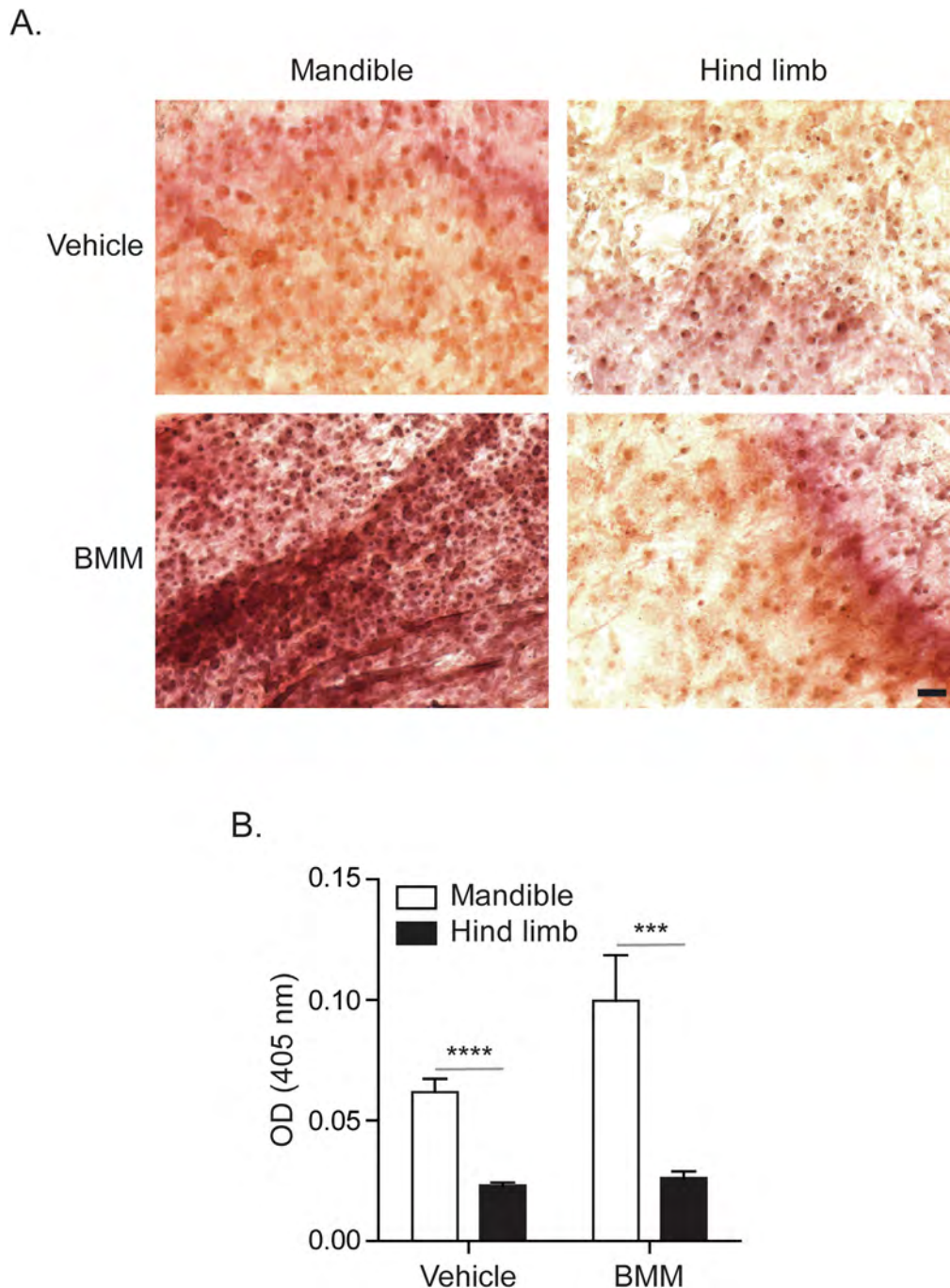


Fig. 3. Cells harvested from mandibles mineralize more than those from hind limbs. Mandible and hind limb cells were cultured with bone mineralization medium (BMM) (0.5mM L-Ascorbic acid, 2mM β -Glycerophosphate disodium salt hydrate, and 10mM HEPES) or vehicle (10mM HEPES) for 14 days. Cells were stained with 2% Alizarin Red S. (A) Representative Alizarin Red S image. Magnification 10x. Bar = 50 μ m. (B) Residual stain from whole plates were extracted with acetic acid and read at 405 nm on a plate reader. Results displayed as Mean $\pm$ SEM. Unpaired Student's *t*-test. ****p* $\leq$ 0.001, *****p* $\leq$ 0.0001.

showed that cells derived from both the mandible and hind limb bones of mice have osteoblastic properties, and that MaOBs have a higher potential for osteoblastic differentiation than HLOBs both *in vitro* and *in vivo*. Interestingly, when PCa cells were co-cultured with these osteoblasts, PCa cells grew significantly faster in co-culture with MaOBs compared to HLOBs. Consistently, PCa cells co-implanted with MaOBs into animals grew faster and larger tumors than those co-implanted with HLOBs. Altogether, the data suggest that these differences may not be due to differences in mineralization status, as L-Ascorbic acid and β -Glycerophosphate supplementation failed to increase PCa growth *in vitro*, nor differ-

ences in osteoblast proliferation, as mitomycin-C growth arrest of osteoblasts actually increased PCa growth *in vitro*. However, the observations that MaOBs vascularize more *in vivo* and express significantly higher levels of *Vegfa* *in vitro* may be clues to a possible mechanism, although careful mechanistic studies must be performed in order to investigate this further.

The idea that there are different subpopulations of osteoblasts that exhibit different functions in the bone microenvironment has been discussed. For example, while mature osteoblasts form mineral deposits by differentiating osteocytes in healthy bone during homeostasis [24], immature osteoblasts are involved in the

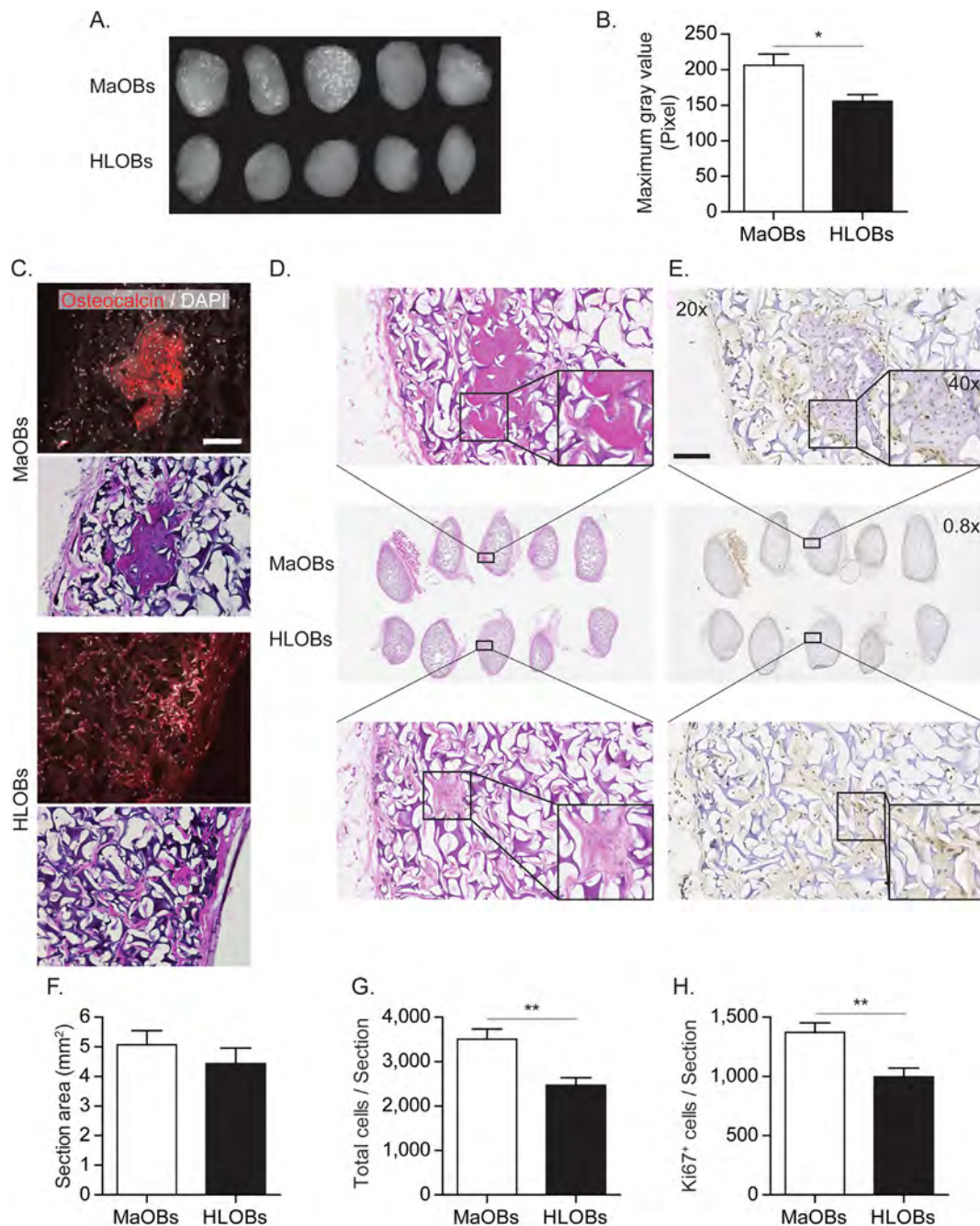


Fig. 4. Cells harvested from mandibles are more proliferative and form more bone than from hind limbs. 1×10^6 primary cells (MaOBs or HLOBs) were seeded onto absorbable gelatin sponges and implanted into subcutaneous pouches of 5-week old male C57BL6/J mice ($n=5$) and grown for 7 weeks *in vivo*. (A) After euthanasia, implants were dissected, fixed, and radiographed. (B) Densitometry quantification using ImageJ of (A). (C) Representative osteocalcin immunofluorescence and H&E image of (A). DAPI was used for nuclear staining. Magnification 20x. Bar = 100 μ m. (D) Representative H&E image of (A). Bar = 100 μ m. (E) Representative Ki67 immunohistochemical image of (A). Bar = 100 μ m. (F-H) Automated quantification using Visiopharm software of (F) tissue section area, (G) total cells per tissue section, and (H) Ki67 nuclear positive cells per tissue section. Results displayed as Mean $\pm$ SEM. Paired Student's *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$.

process of bone resorption by activating osteoclasts through the production of receptor activator of NF- κ B ligand (RANKL) [25,26]. In fact, when we tested relative gene expression of RANKL, we found that MaOBs express higher levels than HLOBs (1.00 ± 0.023 vs 0.097 ± 0.021), although they also expressed higher levels of osteoprotegerin (1.00 ± 0.12 vs 0.29 ± 0.014). Osteoprotegerin is a molecule that binds RANKL, inhibiting its ability to activate its receptor RANK on osteoclasts [27]. Similarly, intercellular adhesion molecule (ICAM)-1 positive osteoblasts, which are in G0/G1 phase of the cell cycle arrest, contributed more to osteoclastogenesis than

bone formation, by interacting with monocytes [28]. This phenomenon is also known in the process of hematopoiesis. It has been demonstrated that the activated leukocyte cell adhesion molecule (ALCAM) positive/Sca-1 negative immature osteoblasts are responsible for maintenance of long-term reconstitution activity of HSCs [29]. On the other hand, it has been suggested that the major function of mature osteoblasts is to control the maintenance of lymphoid progenitors, but not hematopoietic stem cells or myeloid progenitors [30]. Moreover, it has been recently revealed that specific subpopulations of osteoblasts influence bone

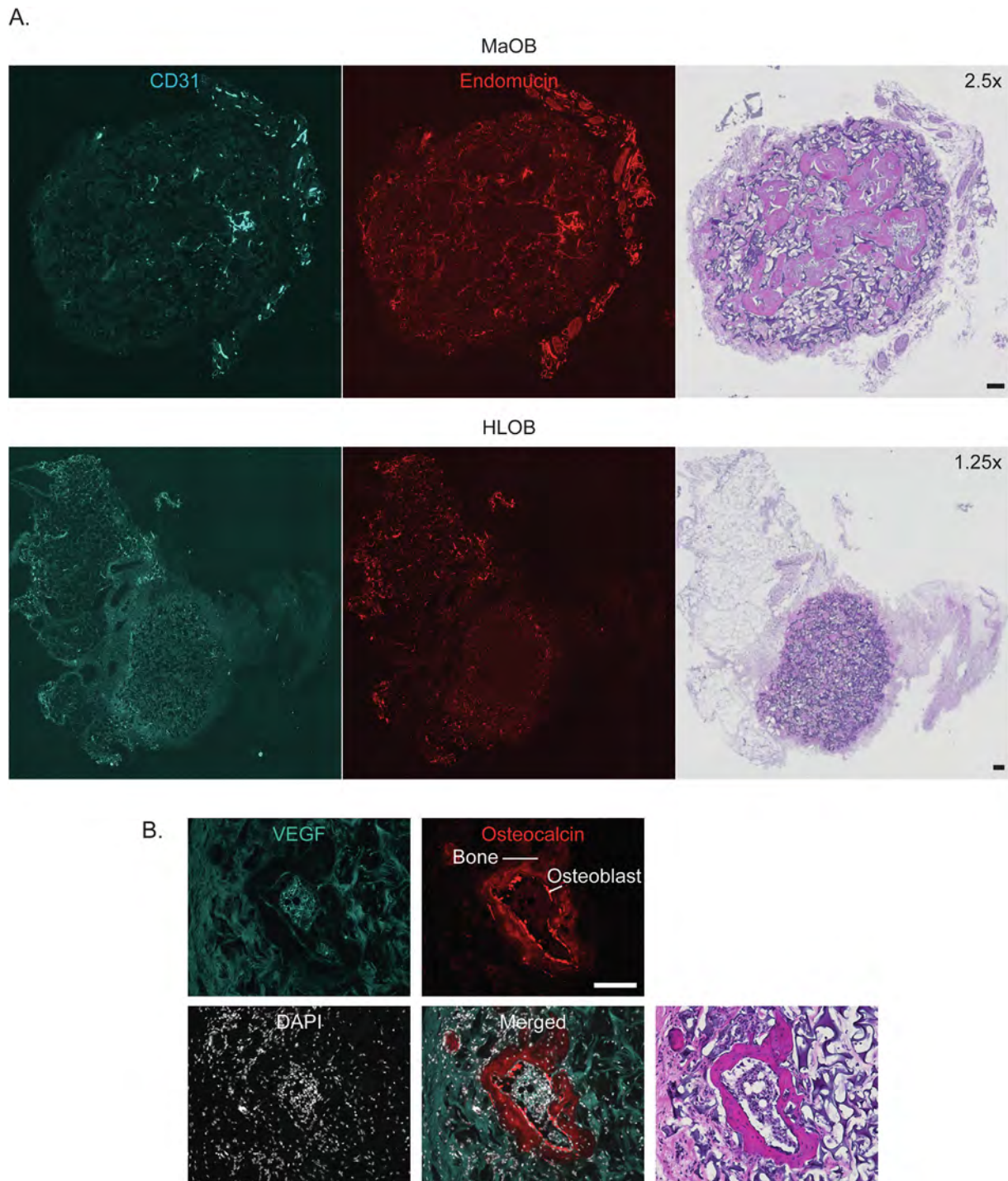


Fig. 5. Ossicle vascularity is mainly localized to tissue surrounding implant. Immunofluorescence was performed on cryosections from Fig. 4 to assess ossicle vascularity. (A) Representative CD31 and Endomucin staining, and H&E image. DAPI was used for nuclear staining. Magnification 10 $\times$. Bar = 100 μ m. (B) Representative vascular endothelial growth factor (VEGF) and Osteocalcin immunofluorescence, and H&E image. DAPI was used for nuclear staining. Magnification 20 $\times$. Bar = 100 μ m.

metastatic progression in the tumor microenvironment. Osteoblast progenitors, but not differentiated or mature osteoblasts, were shown to promote breast cancer cell migration through the release of hepatocyte growth factor (HGF), suggesting that the immature osteoblasts are involved in the early steps of the bone seeding process [31]. When osteoblasts interact with breast cancer cells, they are transformed into OPN positive/interleukin (IL)-6 negative/ α -smooth muscle actin (SMA) negative osteoblasts [12]. When these cancer-associated osteoblasts were co-cultured with breast cancer

cells, the growth of breast cancer cells was significantly suppressed and the expression of the cell cycle arrest marker p21 was activated [12].

To investigate the specific effect of osteoblasts on bone metastatic growth of PCa, we took a simplified approach, by focusing solely on the osteoblast component of the bone-metastatic tumor microenvironment. This is an obvious limitation of this study, as there are many cell types other than osteoblasts that exist in the marrow, and these cells are also known to contribute to tumor

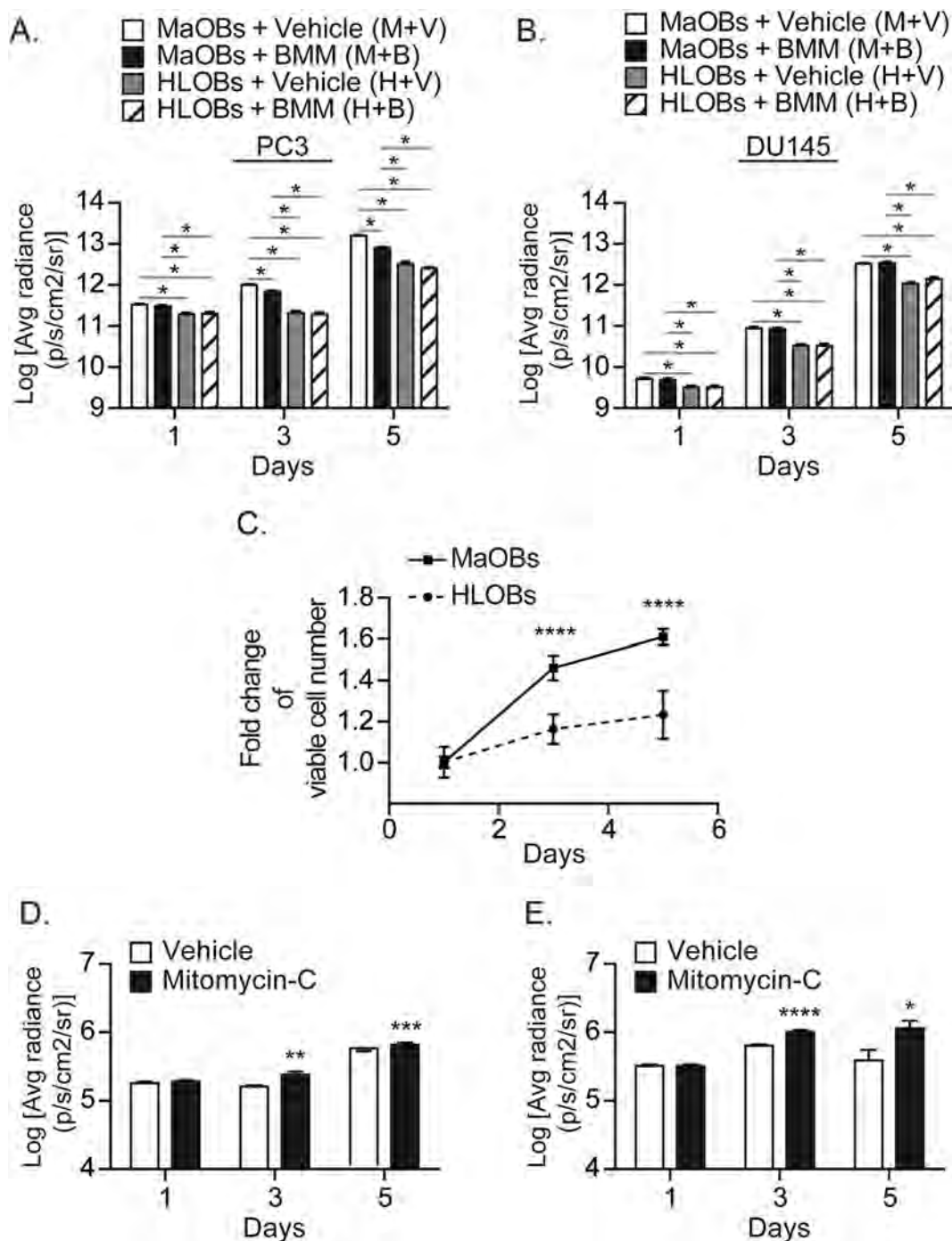


Fig. 6. PCa cells grow faster on osteoblasts harvested from mandibles than osteoblasts from hind limbs. (A) PC3-GFP-luc and (B) DU145-GFP-luc human prostate cancer cells were seeded on top of the primary cells (MaOBs or HLOBs) at a 1:10 ratio. Co-cultures were treated with bone mineralization medium (BMM) (0.5mM L-Ascorbic acid, 2mM β -Glycerophosphate disodium salt hydrate, and 10mM HEPES) or vehicle (10mM HEPES) for 5 days. Bioluminescent imaging (BLI) was measured at day 1, 3, and 5. Two-way ANOVA with Tukey's Studentized Range (HSD) Test for multiple comparisons. (C) MaOBs and HLOBs were seeded in multi-well plates and analyzed for cell proliferation after 1, 3, and 5 days of culture by MTT assay. Proliferation displayed as fold change normalized to day 1. Mixed effects models. (D) PC3-GFP-luc and (E) DU145-GFP-luc human prostate cancer cells were seeded on top of the MC3T3-E1 cells at a 1:10 ratio. To induce cell growth arrest, the MC3T3-E1 cells were pre-treated with vehicle or 10 μ g/mL mitomycin-C for 2.5 h prior to co-culture. BLI was measured at day 1, 3, and 5. Paired Student's *t*-test. Results displayed as Mean $\pm$ SEM. **p* $\leq$ 0.05, ***p* $\leq$ 0.01, ****p* $\leq$ 0.001, *****p* $\leq$ 0.0001.

growth, homeostasis, or dormancy [32]. Although further study is clearly needed, this may be part of the reason why osteoblasts failed to express VEGF during differentiation within the ossicles. Additionally, osteoblasts are not the only cells responsible for general bone health. We believe this is a major reason that we did not observe as robust bone formation as others have reported in their ossicle models of bone marrow stromal cell or mesenchymal stem

cell implantation [10,11]. We believe that in the absence of other stromal cells, the VEGF and other growth factors such as RANKL released by osteoblasts are less effective at promoting angiogenesis and general bone health in an implant model. For instance, osteoblast-derived VEGF has been shown to promote bone formation in a paracrine fashion (which might explain why MaOB ossicles formed more bone than HLOB ossicles in this study), but was

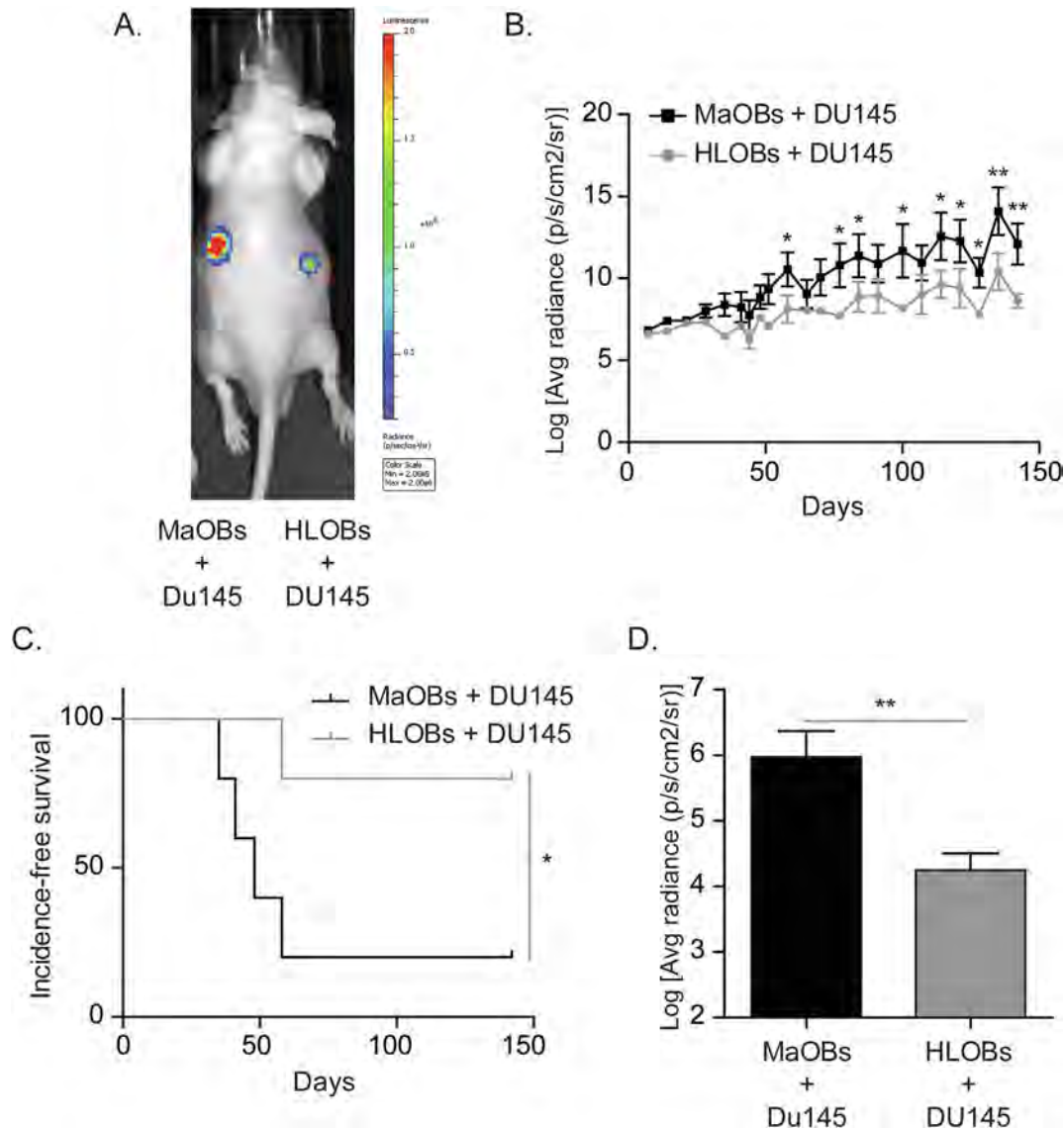


Fig. 7. PCA cells grow faster *in vivo* co-implanted with mandible bone cells. 1×10^6 primary cells (MaOBs or HLOBs) were seeded onto absorbable gelatin sponges and co-implanted into subcutaneous pouches of 5-week old male athymic nude mice ($n=5$) with 1×10^5 DU145-GFP-luc cells and grown for 20 weeks *in vivo*. (A) Representative bioluminescent imaging (BLI) at 20 weeks. (B) All collected longitudinal BLI data after log transformation. Results displayed as Mean $\pm$ SEM. Mixed effects model. (C) 75% cut-off was employed to examine incidence-free survival (MaOBs vs. HLOBs). Cox proportional hazards model with robust sandwich estimates. (D) After euthanasia, tumors were dissected and *ex vivo* BLI performed. Results displayed as Mean $\pm$ SEM. Paired Student's *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$.

also shown to recruit endothelial cells, macrophages, and promote osteoclastogenesis [22]. Further studies to reveal the effects of not only osteoblasts, but also the stroma, endothelium, nerves, osteocytes, osteoclasts, immune cells, and hematopoietic and mesenchymal stem cells on bone and tumor physiology are clearly warranted. The strength of our study is in the *in vivo* co-implant model, which demonstrated that MaOBs contributed to faster tumor cell proliferation than HLOBs. Although the mechanisms behind these differences remain uncertain, the observation that MaOBs promoted greater vascularization *in vivo* could serve as a foundation for further studies. Another finding deserving of future studies was the increased gene expression of RANKL and OPG in MaOBs, as we were also able to detect RANK gene expression in DU145 cells (32.2 ± 1.2 mean cycle threshold; and GAPDH: 26.3 ± 1.1 mean cycle threshold). This finding may help to inform future investigations into mouse mandible tumor frequency, as the question regarding possible differences in tissue seeding and tumor cell anchorage following intracardiac injection remains, and RANKL has

previously been implicated in the bone-metastatic process [33]. Altogether, we believe that these culture methods will be valuable tools in further investigating the mechanisms of tumor cell proliferation in the bone and discovering new interventions to manipulate osteoblast proliferation, activity, or growth factor signaling, which may improve the susceptibility of PCa cells to existing cytotoxic treatments, including chemotherapies.

The findings of this study can be interpreted through the lens of existing treatments used to treat bone metastatic PCa. Current established treatments for bone metastases mainly target bone remodeling, specifically resorption, but these have only had modest success so far, namely denosumab (a human monoclonal antibody against RANKL) and bisphosphonates, which suppress osteoclast activity. Osteoclasts promote bone resorption, creating sufficient space for osteolytic bone tumors to expand and grow [34]. Denosumab and bisphosphonates antagonize bone resorption and as such were hypothesized to slow tumor growth. However, both treatments ultimately fail to improve overall survival

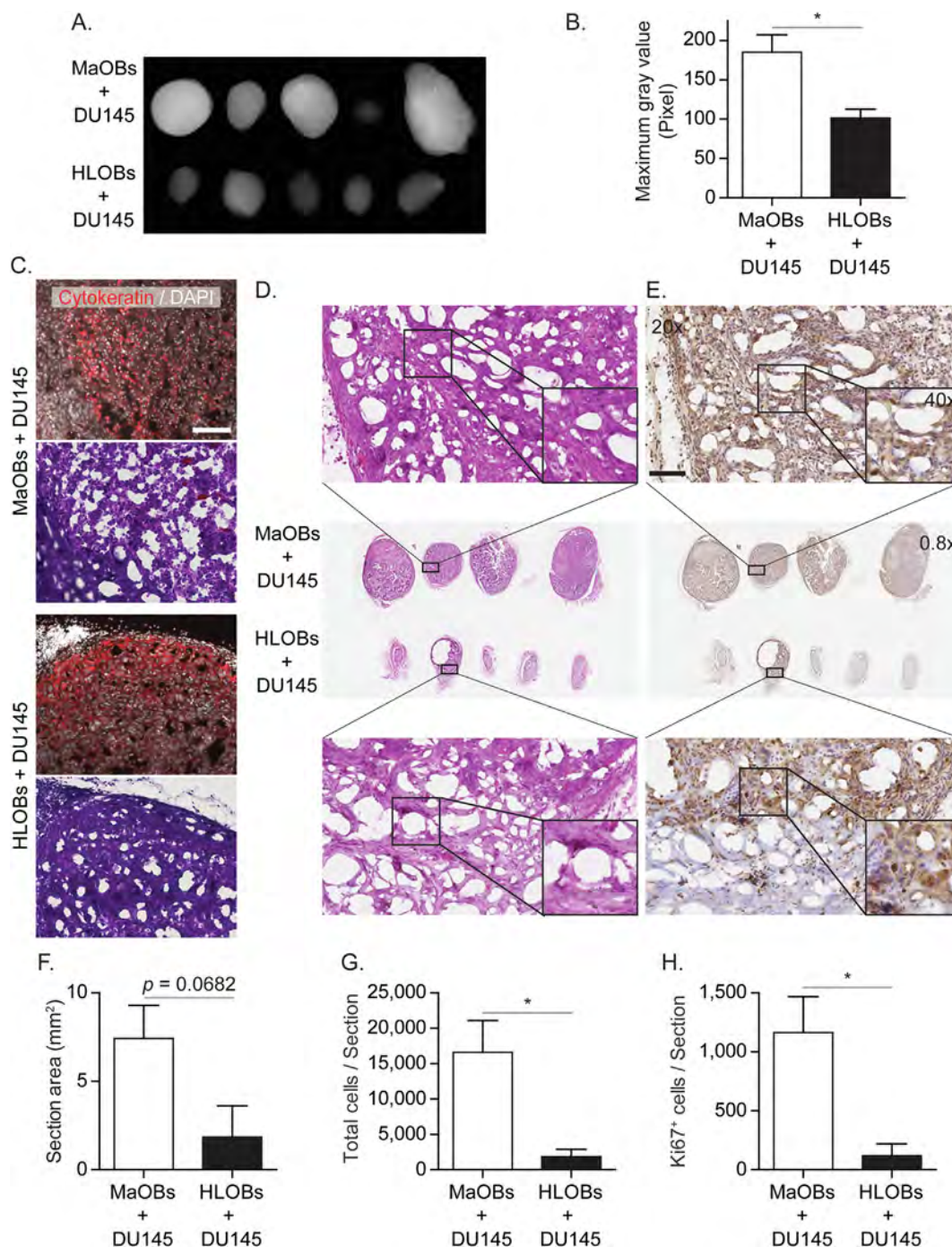


Fig. 8. Tumors from PCa/mandible bone cell co-cultures are more proliferative. (A) After euthanasia, tumors from Fig. 6 were dissected, fixed, and radiographed. (B) Densitometry quantification using ImageJ of (A). (C) Representative Pan-cytokeratin immunofluorescence and H&E image of (A). Magnification 20x. Bar = 100 μ m. (D) Representative H&E image of (A). Bar = 100 μ m. (E) Representative Ki67 immunohistochemical image of (A). Bar = 100 μ m. (F–H) Automated quantification using Visiopharm software of (F) tissue section area, (G) total cells per tissue section, and (H) Ki67 nuclear positive cells per tissue section. Results displayed as Mean $\pm$ SE. Paired Student's *t*-test. **p* $\leq$ 0.05.

[35,36]. On the other hand, radium-223 (Ra^{223}), which forms complexes with hydroxyapatite in bone [37–39], can extend overall survival in PCa patients with bone metastases [40], but only by a few months (mean = 3 months). A variety of combinations of therapies [e.g. docetaxel, second generation androgen deprivation therapies (ADTs) such as abiraterone, enzalutamide, or others] have been shown to extend overall survival of metastatic PCa patients [41]. However, when choosing an effective combination strategy for PCa bone metastases, it may be best to avoid ADTs

since they are known to negatively affect bone health [42,43]. Indeed, a recent trial of the combination of abiraterone and Ra^{223} in patients with bone-metastatic PCa not only failed to improve skeletal event-free survival, but it also increased the frequency of bone fractures compared with placebo [44]. Alternatively, the idea of inducing bone formation by enhancing osteoblastic activity as a treatment for bone metastatic disease has recently been appreciated [45,46], as inactive/immature osteoblasts can reduce PCa susceptibility to chemotherapy [47,48]. However, the results of this

study suggest that inducing osteoblastic differentiation may not be the best strategy for treating progressive bone metastatic tumors, as BMM treatments failed to increase PCa growth in co-culture models. A popular strategy to treat many cancers has been to use drugs that inhibit angiogenesis, but this strategy has been somewhat unsuccessful in the treatment of bone-metastatic PCa [49,50]. This may be in part because these inhibitors mainly target the receptor for VEGF, but not VEGF itself. Here, we see that high *Vegfa* expressing, fast growing, and bone forming MaOBs induced more tumor growth *in vivo* than HLOBs, which expressed less *Vegfa* *in vitro*, are slower growing, and form less bone *in vivo*. More studies are clearly necessary to elucidate whether osteoblast-derived VEGF, or other angiogenic factors, is required for the faster tumor growth observed in the mandibles of intracardially-injected mice, whether osteoblast-derived VEGF can be targeted to treat bone metastatic disease, and ultimately whether these findings correspond with bone metastatic PCa in humans. We believe that the models described here are valuable tools to aid in this research.

Author contributions

Matthew R. Eber and Yusuke Shiozawa wrote the manuscript with input from all authors. Matthew R. Eber and Yusuke Shiozawa designed study and contributed to data interpretation. Matthew R. Eber, Sun H. Park, Kelly F. Contino, and Chirayu M. Patel performed *in vitro* and *in vivo* experiments. Fang-Chi Hsu performed statistical analyses. Yusuke Shiozawa supervised the study. All authors reviewed and approved the final manuscript.

CRedit authorship contribution statement

Matthew R. Eber: Conceptualization, Data curation, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Sun H. Park:** Data curation, Investigation, Methodology, Writing - review & editing. **Kelly F. Contino:** Data curation, Investigation, Writing - review & editing. **Chirayu M. Patel:** Data curation, Investigation, Writing - review & editing. **Fang-Chi Hsu:** Formal analysis, Methodology, Software, Writing - review & editing. **Yusuke Shiozawa:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

Yusuke Shiozawa has received research funding from TEVA Pharmaceuticals, but not relevant to this study. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute.

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Usefulness of the measurement of neurite outgrowth of primary sensory neurons to study cancer-related painful complications

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ABSTRACT

Abnormal outgrowth of sensory nerves is one of the important contributors to pain associated with cancer and its treatments. Primary neuronal cultures derived from dorsal root ganglia (DRG) have been widely used to study pain-associated signal transduction and electrical activity of sensory nerves. However, there are only a few studies using primary DRG neuronal culture to investigate neurite outgrowth alterations due to underlying cancer-related factors and chemotherapeutic agents. In this study, primary DRG sensory neurons derived from mouse, non-human primate, and human were established in serum and growth factor-free conditions. A bovine serum albumin gradient centrifugation method improved the separation of sensory neurons from satellite cells. The purified DRG neurons were able to maintain their heterogeneous subpopulations, and displayed an increase in neurite growth when exposed to cancer-derived conditioned medium, while they showed a reduction in neurite length when treated with a neurotoxic chemotherapeutic agent. Additionally, a semi-automated quantification method was developed to measure neurite length in an accurate and time-efficient manner. Finally, these exogenous factors altered the gene expression patterns of murine primary sensory neurons, which are related to nerve growth, and neuro-inflammatory pain and nociceptor development. Together, the primary DRG neuronal culture in combination with a semi-automated quantification method can be a useful tool for further understanding the impact of exogenous factors on the growth of sensory nerve fibers and gene expression changes in sensory neurons.

1. Introduction

Recent evidence suggests that crosstalk between the peripheral nervous system and cancer plays an important role in both cancer development and cancer-associated pain [1–3]. In head and neck cancer, tumors innervated by peripheral nerves are known to be more aggressive than those with less innervation [4]. Recent studies using mouse models have revealed that the sympathetic nervous system regulates prostate cancer tumorigenesis and bone metastasis [5], and that

denervation can even attenuate tumorigenesis and metastasis in several types of cancer [5–8]. Moreover, it has been indicated that cancer-associated pain may be a negative indicator of survival in cancer patients [9], suggesting that sensory nerves influence disease progression in patients with poor prognoses. This accumulating evidence indicates that the peripheral nervous system within the tumor microenvironment may enhance disease progression.

Conversely, cancer cells are also known to affect neuronal activities. Cancer often causes pain by interacting with sensory nerves. Especially,

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when cancer cells metastasize to the bone, patients experience bone pain, referred to as cancer-induced bone pain (CIBP). Bone is richly innervated by sensory nerves [10], and it has been suggested that the acidic environment surrounding cancer, or factors, such as growth factors, cytokines, or chemokines, secreted by cancer cells can all stimulate receptors on sensory nerves to induce CIBP [11–14]. Additionally, recent studies have also demonstrated that sensory nerve outgrowth (or sprouting) is one of the potential mechanisms of CIBP [15–19]. A neurotrophic factor nerve growth factor (NGF) is known to be involved in the regulation of survival and growth of neurons [20]. NGF derived from osteosarcoma cells stimulated neuroma-like formation in the periosteum of cancer-inoculated mouse bones, resulting in CIBP [19]. When an NGF antagonist was administered to cancer-bearing mice, CIBP as well as the accompanying nerve sprouting were attenuated [19]. Consistently, abnormal sensory nerve sprouting also causes pain behaviors in other chronic pain conditions [10,21–23].

Not only nerve growth, but also nerve damage is associated with the induction of chronic pain conditions. Chemotherapy-induced peripheral neuropathy (CIPN) is a consequence of the neurotoxic effects of chemotherapy agents [24–26]. Indeed, paclitaxel reduced the density of intraepidermal nerve fibers in hind paw skin and produced pain-related behaviors in rodents [26,27].

These findings suggest (i) that nerves and cancer interact very closely with one another and (ii) abnormal plasticity or remodeling of sensory neurons may contribute to cancer-induced pain. Therefore, it is crucial to understand how nerve growth is regulated in the context of cancer and its treatments. In pursuit of this goal, animal studies are often used to investigate the systemic effects of disease states and therapies on peripheral nerves, but they are not always suitable to study local interactions. For that reason, various *in vitro* neuronal culture systems have been developed to understand the mechanisms of neurite outgrowth. The mouse pheochromocytoma cell line PC12, which is known to form neurite-like structures in response to NGF [28], has been previously used to assess the effects of cancer-derived factors on neurite outgrowth. Conditioned medium (CM) derived from pancreatic cancer cells [29] or head and neck cancer-derived exosomes [30] were shown to enhance neurite sprouting of PC12 cells. Cell lines are easy to maintain and handle but may not fully recapitulate the phenotype of sensory neurons. Moreover, only few neuronal cell lines which extend neurites are currently available. The use of primary culture of dorsal root ganglia (DRG) neurons may overcome this hurdle. To date primary DRG culture has mainly been used to investigate physiological alterations in neuronal activity [31] and to a lesser degree neurite outgrowth.

In the current study, we established methods for reliable *in vitro* primary cultures of sensory neurons derived from mouse, non-human primate, and human DRGs. Additionally, we found that these primary DRG neuronal cultures were suitable to study the neurite length alterations and changes in gene expression associated with nerve growth, and neuro-inflammatory pain and nociceptor development, mediated by CM obtained from cancer cells or paclitaxel-induced neurotoxicity. We also developed a semi-automated method for the measurement of neurite outgrowth, resulting in the reduction of overall analysis time.

2. Materials and methods

All methods were carried out in accordance with relevant guidelines and regulations. All human studies and all animal studies followed the Declaration of Helsinki and the Institutional Animal Care and Use Committee Guidelines, respectively.

2.1. Human subjects research certification

All human studies are approved by the Institutional Review Board (IRB #00056846) at Wake Forest University Health Sciences. Informed consent was obtained from all subjects involved in the current study.

2.2. Animal care and use certification

All animal studies are approved by the Institutional Animal Care and Use Committee (Protocol A18-026 for mouse and Protocol A18-161 for non-human primate) at Wake Forest University Health Sciences.

2.3. Murine DRG isolation and primary neuronal culture establishment

Primary DRG neuronal culture was prepared, according to previously published protocols [32,33] with modifications. Nine-twelve lumbar DRGs (L2–L4) of male C57BL/6 mice (8–12 weeks of age, Jackson Laboratory, Bar Harbor, ME) were dissected and directly placed into a 15 mL conical tube, containing 14 mL of ice-cold $1 \times$ Hanks' balanced salt solution (HBSS) without Mg^{++}/Ca^{++} (Thermo Fisher Scientific, Gibco, Waltham, MA). Isolated DRGs were enzymatically digested in 3 mL of papain solution [30 U/mL papain (Worthington Biochemical Corp., Lakewood, NJ), 0.1% saturated $NaHCO_3$ solution (Sigma-Aldrich, St. Louis, MO), 0.3 mg/mL L-Cys (Sigma-Aldrich) in HBSS without Mg^{++}/Ca^{++}] for 30 min at 37°C with 5% CO_2 , and then incubated in 3 mL of collagenase type II (CLS2)/dispase type II (Dispase II) solution [4 mg/mL CLS2 (Worthington Biochemical Corp.) and 4.7 mg/mL dispase (Sigma-Aldrich) in HBSS without Mg^{++}/Ca^{++}] for 30 min at 37°C with 5% CO_2 . The DRGs were mixed gently every 10 mins. The resulting DRGs were centrifuged at $200 \times g$ for 2 min and washed with HBSS without Mg^{++}/Ca^{++} . The pellet was transferred to a 15 mL conical tube, containing 500 μ L of neuronal growth (NG) medium [Neurobasal-A (Thermo Fisher Scientific, Gibco), 1% N2 (Thermo Fisher Scientific), 2% B-27 (Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), 1% penicillin–streptomycin (Thermo Fisher Scientific), and 0.4% glucose (Sigma-Aldrich)]. The DRGs were triturated 15–20 times using p1000 and then p200 pipette tips and filtered through a stainless mesh sieve (40 μ m, Thermo Fisher Scientific) to obtain single-cell suspensions and remove undigested tissue debris. After bovine serum albumin (BSA) purification [3.5% (W/V) BSA solution] (see below), 500–1,000 cells of DRGs in 30 μ L of warm NG medium were seeded onto the center of 12 mm round coverslips (MatTek Corp., Ashland, MA), pre-coated with Poly-D-lysine (50 μ g/mL, overnight at 4°C, Thermo Fisher Scientific, Corning) and laminin (20 μ g/mL, 1 h at 37°C, Thermo Fisher Scientific, Corning), in 24-well plate. After 1–2 h, 1 mL of warm NG medium was gently added to the sides of wells and the cells were maintained at 37°C with 5% CO_2 .

2.4. BSA purification

After trituration and filtration, single-cell suspensions from DRGs were centrifuged through 3.5% (W/V) BSA solution (Thermo Fisher Scientific) (5 mL of BSA solution: 1 mL of HBSS without Mg^{++}/Ca^{++} ; 1 mL of cell suspension in a 15 mL conical tube) at $14 \times g$ for 20 min at room temperature (RT) to separate sensory neurons (in pellet) from satellite cells and debris (in BSA layer).

2.5. Non-human primate DRG isolation and primary neuronal culture establishment

Non-human primate primary neuronal culture was prepared similarly as described in the murine primary neuronal culture with minor modifications. DRGs (T12 and L1) of a healthy 8-year old female Rhesus macaque (Worldwide Primates, Inc., Miami, FL) were dissected and directly placed in a 15 mL conical tube containing 14 mL of ice-cold HBSS without Mg^{++}/Ca^{++} and transferred to the laboratory for further procedures. Isolated DRGs were cleaned up by removing non-nervous tissues and nerve root under the laminar flow hood using sterile blade, scissors, and forceps, and cut into small pieces achieved at approximately 1–3 mm side length of a cubical volume in ice-cold HBSS without Mg^{++}/Ca^{++} and transferred to a 15 mL conical tube containing papain solution. DRGs were enzymatically digested in 6 mL of papain

solution for 1 h at 37°C with 5% CO₂ and then incubated in 6 mL of CLS2/Dispase II solution for 1 h at 37°C with 5% CO₂. During the digestion, DRGs were triturated 10–15 times using p1000 tips every 20 min and filtered through a stainless mesh sieve (100 µm, Thermo Fisher Scientific) to obtain single-cell suspensions and remove undigested tissue debris. After BSA purification [3.5% (W/V) BSA solution], 600 cells of DRGs in 30 µL of warm NG medium were seeded onto the center of 12 mm round coverslips, pre-coated with Poly-D-lysine and laminin, in 24-well plate. After 1–2 h, 1 mL of warm NG medium was gently added to the sides of wells and the cells were maintained at 37°C with 5% CO₂.

2.6. Human DRG isolation and primary neuronal culture establishment

Human primary neuronal culture was prepared similarly as the non-human primate neuronal culture with minor modifications, according to previously published studies [34–38]. DRG (T10) of a male patient (41 years old age) were dissected during a T10 corpectomy for correction of kyphotic deformity secondary to discitis/osteomyelitis and directly placed in a 15 mL conical tube containing 14 mL of ice-cold HBSS without Mg⁺⁺/Ca⁺⁺ and transferred to the laboratory for the further procedures. Isolated DRGs were cleaned up by removing non-nervous tissues and nerve root under the laminar flow hood using sterile blade, scissors, and forceps, and at approximately 1–3 mm side length of a cubical volume in ice-cold HBSS without Mg⁺⁺/Ca⁺⁺ and transferred to a 15 mL conical tube containing papain solution. DRGs were enzymatically digested in 9 mL of papain solution for 1 h at 37°C with 5% CO₂ and then incubated in 9 mL of CLS2/Dispase II solution for 1.5 h at 37°C with 5% CO₂. During the digestion, DRGs were triturated 10–15 times using p1000 tips every 20 min and filtered through a stainless mesh sieve (100 µm) to obtain single-cell suspensions and remove undigested tissue debris. After BSA purification [3.5% (W/V) BSA solution], DRGs in 30 µL of warm NG medium were seeded onto the center of 12 mm round coverslips, pre-coated with Poly-D-lysine and laminin, in 24-well plate. After 1–2 h, 1 mL of warm NG medium was gently added to the sides of wells and the cells were maintained at 37°C with 5% CO₂.

2.7. Cancer cell or normal prostate epithelial cell derived conditioned medium generation

To collect the conditioned medium (CM) from cancer cells, 5×10^5 cells of a human breast cancer cell line established from a pleural effusion, MDA-MB-231 [American Type Culture Collection (ATCC), Manassas, VA], a human prostate cancer cell line established from bone metastasis, PC-3 (ATCC), a human lung adenocarcinoma cell line established from lung carcinomatous tissue, A549 (ATCC), or a murine lung carcinoma cell line established from a primary tumor nodule from the Lewis lung carcinoma model, LL/2 (ATCC) were seeded onto a 10 cm dish with 10 mL of growth medium. These cancer cell lines were chosen since they have been shown to grow in the bone and/or can induce CIBP [39–42]. The passage number for all cell lines used was less than 25 passages. MDA-MB-231 and LL/2 cells were cultured in Dulbecco's modified eagle medium (DMEM) (Thermo Fisher Scientific, Gibco) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Gibco), 1% penicillin–streptomycin and glutamine (PSG) (Thermo Fisher Scientific, Gibco), while PC-3 and A549 cells were cultured in Roswell park memorial institute (RPMI) 1640 medium (Thermo Fisher Scientific, Gibco) containing 10% FBS and 1% PSG. At 24 h, the growth medium was removed and replaced with 10 mL of serum free DMEM or RPMI 1640 medium (Thermo Fisher Scientific, Gibco) containing 1% PSG. For the control medium, 10 mL of serum free DMEM or RPMI medium were added to a 10 cm dish without adding any cancer cells. To collect the CM from non-cancer cells, 5×10^5 cells of a human normal epithelial prostate cell line, PWR-1E (ATCC) were seeded onto a 10 cm dish with 10 mL of Keratinocyte-serum free medium (K-SFM) containing human recombinant EGF (5 ng/mL) and bovine pituitary extract (0.05 mg/mL) provided with the K-SFM kit (Thermo Fisher Scientific, Gibco).

The passage number for all cell lines used was less than 25 passages. At 24 h, the growth medium was removed and replaced with 10 mL of K-SFM without any supplements. For the control medium, 10 mL of K-SFM were added to a 10 cm dish without adding any cells. After 24 h of incubation at 37°C with 5% CO₂, the CM were collected and filtered through a 0.2 µm syringe filter (Thermo Fisher Scientific) to remove any cell debris. Additionally, cancer-derived CM were concentrated to $4 \times$ using an Amicon Ultra-15 centrifugal filter tube (MilliporeSigma, Burlington, MA, NMWL10K) performed at $4,000 \times g$ for 20 min at RT. The CM was stored at 4°C until use.

2.8. Neurite outgrowth assay

After 48 h (murine and non-human primate) or 72 h (human) of primary DRG neuronal culture establishment (the time required to achieve 20–30% nerve fiber occupancy on the slide based on Fig. 3A), half (500 µL) of medium was replaced with 500 µL of either control, normal epithelial cell-derived CM, or cancer-derived CM, and then the cells were incubated another 48–72 h (the time required to achieve 70–80% nerve fiber occupancy on the slide based on Fig. 3A). In some cases, the cells were treated with 500 µL of NG medium containing either dimethyl sulfoxide (DMSO) control or paclitaxel (Sigma-Aldrich) for 24 h. At the termination of the experiments, the cells were fixed in 500 µL of 4% paraformaldehyde (PFA) for 10 min at RT, and immediately subjected to immunofluorescence or stored in $1 \times$ D-PBS at 4°C until use.

2.9. Immunofluorescence assay

Fixed cells were blocked with immunofluorescence (IF) buffer [$1 \times$ D-PBS supplemented with 5% Normal Donkey serum (Jackson ImmunoResearch, West Grove, PA) and 0.03% Triton X-100 (Sigma-Aldrich)] for 1 h at RT and incubated with primary antibodies overnight at 4 °C in IF buffer. Primary antibodies used: mouse anti-β-III tubulin antibody (1:1,000, Biolegend, San Diego, CA, cat #: 801201); chicken anti-200kD neurofilament (NF200) antibody (1:3,000, Neuromics, Cambridge, MA, cat #: ab134459); rabbit anti-protein gene product 9.5 (PGP9.5) antibody (1:1,000; Cederlane, Rosemont, IL, cat #: 14730–1-AP); rabbit anti-calcitonin gene-related peptide (CGRP) antibody (1: 5,000, Sigma-Aldrich, cat #: C8198); biotinylated isolectin B4 (IB4) antibody (1: 2,500, Sigma-Aldrich, cat #: L2140); rabbit anti-S100 antibody (1: 1,000, Abcam, Cambridge, UK, cat #: ab868); or goat anti-glial fibrillary acidic protein (GFAP) antibody (1: 200, Santa Cruz, Dallas, TX, cat #: sc-6170). Thereafter, the cells were incubated with secondary antibodies for 2 h at RT in IF buffer. The specific choices of secondary antibodies were made based on primary antibodies used. Secondary antibodies used: anti-rabbit cyanine 3 (CY3) (1:700, Jackson ImmunoResearch, cat #: 711–165-152); anti-chicken CY2 (1:600, Jackson ImmunoResearch, cat #: 703–225-155); anti-streptavidin CY5 (1:500, Jackson ImmunoResearch, cat #: 016–170-084); anti-goat CY5 (1:500, Jackson ImmunoResearch, cat #: 705–175-147); or anti-mouse CY2 (1:600, Jackson ImmunoResearch, cat #: 715–225-150). After washing 5 times with $1 \times$ D-PBS, the cells were mounted with ProLong Gold antifade mountant with DAPI (Thermo Fisher Scientific).

2.10. Imaging and quantification of immunofluorescence

For each group, 2–3 coverslips were quantified, and 6–10 images were taken from each coverslip using a Nikon Eclipse Ni fluorescent microscope system (Nikon, Tokyo, Japan). Images were saved in nd2 or tiff files for further analysis using Visiopharm (Hørsholm, Denmark) or Image J (NIH, Bethesda, Maryland) software, respectively.

2.11. Image J analysis

Neuron J, a plugin for Image J software was used to manually analyze nerve density, as previously described [43,44]. Briefly, images

were opened in Image J (<https://imagej.net/Fiji/Downloads>) and converted to 8-bit, which were then individually opened in Neuron J plugin. Thereafter, total neurite lengths of neuronal markers NF200 or β -III tubulin positive neurites per each image were traced and normalized with the numbers of neurons, which were counted using a multi-point tool of Image J.

2.12. Visiopharm image analysis

An algorithm (called an “APP”) was created in the Visiopharm software, which uses digital masks and associated labels to automatically detect and count the somas of NF200 positive neurons, as well as detect and measure total NF200 positive neurite length. In some cases, PGP9.5, β -III tubulin, or activating transcription factor 3 (ATF3) were used as markers instead of NF200 for neurite length or soma quantification. The APP was designed to exploit the observations that somas are usually brighter in fluorescence intensity than neurites, and neurites are long, thin structures whereas somas are round and larger in diameter. This APP uses thresholds, which were set manually according to the set of images to be analyzed, as sets of images can vary in fluorescence intensity between experiments due to staining and microscope setting differences. The APP includes post-processing for cleaning up the classification (i.e. small object removal, skeletonization, erosion, dilation, etc.) and the calculation of the variable included in our measurements.

2.13. Real time qPCR

After murine primary DRG neuron cultures were treated with (i) either control or cancer-derived CM (48 h), or (ii) either DMSO or paclitaxel (10 μ M) (24 h), cells were lysed in 350 μ L RLT- β -ME buffer. RNA was extracted using the RNeasy plus micro kit (Qiagen, Germantown, MD) and cDNA was generated using Invitrogen SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real time PCR (qPCR) was performed using Taqman gene expression master mix (Applied Biosystems, Foster City, CA) and Taqman gene expression assays on the Bio-Rad CFX Connect instrument (Hercules, CA). Taqman gene expression assays used: growth associated protein 43 (GAP43) (Mm00500404_m1), NF200 (Mm01191456_m1), CGRP (Mm00801463_g1), Substance P (Mm01166996_m1), Bradykinin (Mm04207315_s1), tumor necrosis factor- α (TNF- α) (Mm00443258_m1), suppressor of cytokine signaling 3 (SOCS3) (Mm00545913_s1), immunoglobulin superfamily containing leucine rich repeat 2 (ISLR2) (Mm00623260_s1), and GAPDH (Mm99999915_g1). For interleukin-6 (IL-6), cDNAs were amplified by qPCR using SsoAdvanced Universal SYBR green supermix (Bio-rad laboratories, Hercules, CA). Primers used: mouse IL-6 Forward: 5'-TTCCTACCCCAATTCCAAT-3' and Reverse: 5'-CCTTCTGTGACTC-CAGCTTATC-3' (Integrated DNA Technologies, Newark, NJ). Data is presented using the delta-delta Ct method, with GAPDH used as the reference gene.

2.14. Statistical analysis

Numerical data are expressed as mean $\pm$ the standard error of the mean (SEM). Statistical analysis was performed by unpaired two-tailed Student's *t* test or one-way ANOVA with Tukey's multiple comparisons, using the GraphPad Prism statistical program (GraphPad Software, San Diego, CA) with significance at $p \leq 0.05$. The chi-square goodness-of-fit test was used to test whether the distribution of the three soma size groups (0–599 μ m², 600–1,199 μ m², and 1,200–1,300 μ m²) of one species was the same as that of the other species.

3. Results

3.1. Murine DRGs can survive, and sensory neurons can grow neurites even in serum-free condition

To establish a primary 2D DRG neuron culture, L2-4 DRGs were collected from C57BL/6 mice and dissociated into single cell suspension by enzymatic digestion. The resulting DRGs were plated onto Poly-D-lysine/laminin-coated coverslips and cultured in neuronal growth medium. Unlike other studies [45–48], DRGs were maintained in the condition that serums and nerve-related growth factors (e.g. NGFs, GDNFs) were withdrawn to avoid their effects on the survival of neurons and neurite outgrowth. As shown in Fig. 1A, cells obtained from DRGs, including sensory neurons and satellite cells, could maintain viability for at least 15 days in serum and growth factor-free conditions. Although approximately 90% of sensory neurons extended neurites, it was very difficult to observe under the light microscope since two cell types overlapped with each other. To visualize sensory neurons, DRGs were stained with DAPI and three different neuronal markers: 200kD neurofilament (NF200) (all myelinated A-fibers); calcitonin gene-related peptide (CGRP) (peptidergic myelinated thin A δ -fibers and unmyelinated C-fibers) and Substance P (peptidergic unmyelinated C-fibers). Sensory neurons expressed these markers, whereas satellite cells failed to do so (Fig. 1B).

3.2. Murine DRG sensory neurons were purified using a BSA gradient centrifugation

It has been demonstrated that, in the peripheral nervous system, satellite cells are involved in the regulation of survival and axonal growth of neurons [49,50]. Since one of our main purposes of establishment of primary DRG neuronal culture is to investigate the direct effects of exogenous factors on the neurite outgrowth of sensory neurons or neurite toxicity, our next attempt was to separate sensory neurons from satellite cells. To do so, density gradient centrifugation was performed using 3.5% BSA solution. After purification, sensory neurons were enriched in the pellet fraction, while satellite cells were concentrated in the BSA layer. When cells in the pellet fraction were plated, the numbers of satellite cells (S100 positive) were reduced, compared to those before purification (Fig. 2A). To further confirm the quality of purification, both cells in the pellet fraction and the BSA layer were plated. As expected, cells in the pellet fraction could extend neurites, while cells in the BSA layer failed to do so (Fig. 2B).

Afterwards, the neurite outgrowth of neuronal cells in the pellet fraction were followed over time. The nerve fiber occupancy on the slides increased in a time dependent manner (10% nerve fiber occupancy at 24 h, 20–30% nerve fiber occupancy at 48 h, 50–60% nerve fiber occupancy at 72 h, and 70–80% nerve fiber occupancy at 96 h) (Fig. 3A). Neuronal cells in the pellet fraction were also segregated into three groups based on soma size (Fig. 3B). The size of soma was divided into small ($\leq 599 \mu$ m²), medium (600–1,199 μ m²) and large (1,200–1,300 μ m²), which has been used for *in vivo* characterization of rodent DRGs [51–55]. The majority of murine sensory neuron somas were smaller than 599 μ m² (Fig. 3B). Additionally, cells were stained with NF200, CGRP, and isolectin B4 (IB4) (non-peptidergic unmyelinated C-fibers) to further characterize the subpopulation of the DRG neurons. Interestingly, NF200 positive neurons always co-localized with CGRP positive neurons, but not IB4 positive neurons (Fig. 3C). Additionally, some CGRP positive neurons overlapped with IB4 positive neurons (Fig. 3C). Moreover, small size neurons consisted of NF200 negative/CGRP negative/IB4 positive neurons; medium size neurons consisted of NF200 negative/CGRP positive/IB4 positive, NF200 negative/CGRP positive/IB4 negative, or NF200 positive/CGRP positive/IB4 negative neurons; and large size neurons consisted of NF200 positive/CGRP positive/IB4 negative neurons (Fig. 3C).

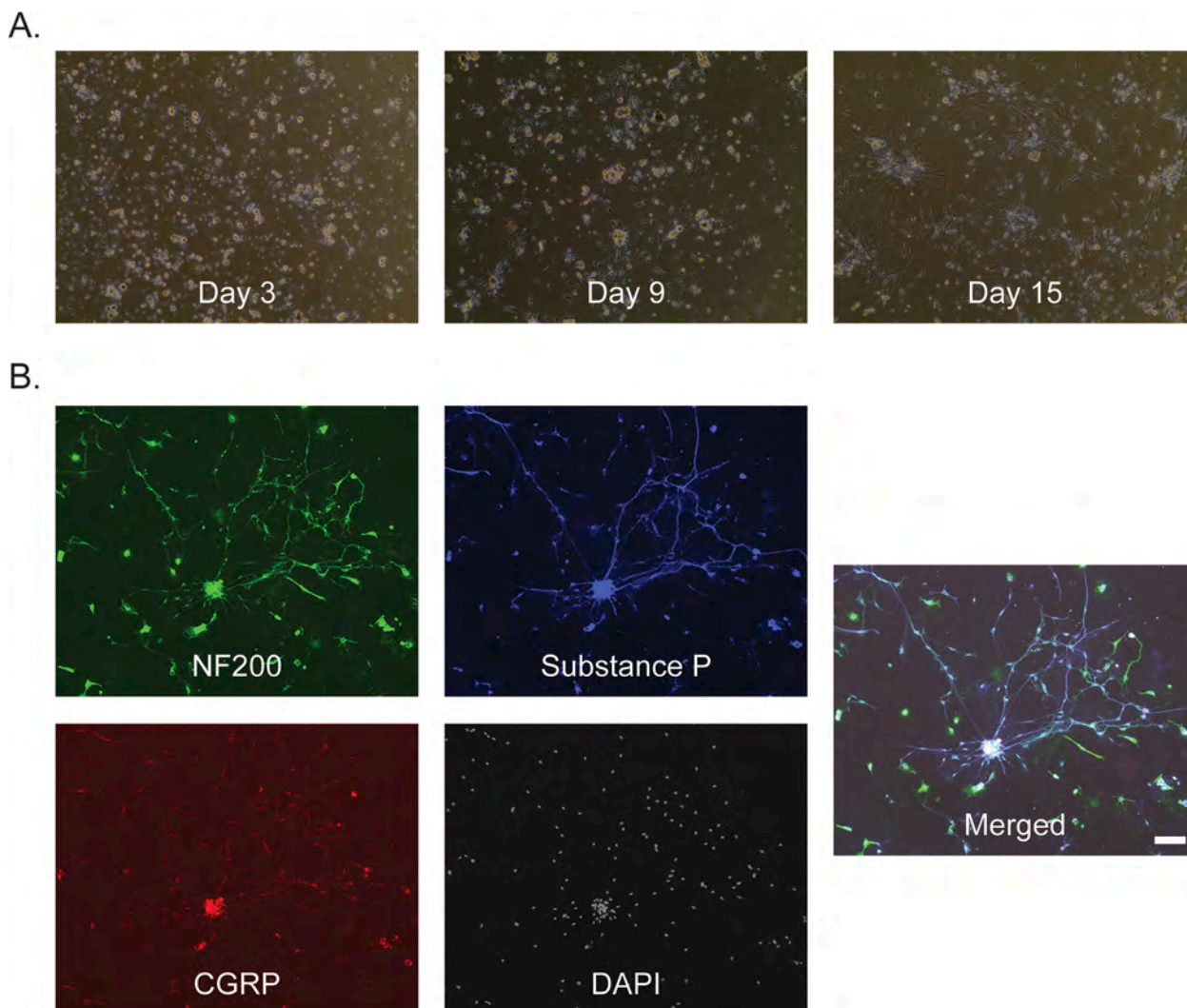


Fig. 1. Establishment of a murine primary 2D DRG neuron culture. (A) Representative bright field images of murine primary DRG culture (9–12x lumbar DRGs (L2–L4) from 8 to 12-week-old male C57BL/6 mice) seeded onto Poly-D-lysine/laminin coated coverslips at Day 3, 9, and 15. Magnification 4 $\times$. (B) Representative immunofluorescence images of (A) at Day 15. Sensory neurons were stained with antibodies against 200 kD neurofilament (NF200) (green) (all myelinated A-fibers), calcitonin gene-related peptide (CGRP) (red) (peptidergic myelinated thin A δ -fibers and unmyelinated C-fibers), and Substance P (blue) (peptidergic unmyelinated C-fibers). DAPI (gray) is used for nuclear staining. Magnification 10 $\times$. Bar = 100 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Cancer-derived factors stimulated the neurite outgrowth of murine sensory neurons, whereas a chemotherapeutic agent reduced.

Next, to test whether the growth of sensory neurons can be manipulated *in vitro* by exogenous factors, which again is our main purpose of the establishment of primary sensory neuron cultures, murine primary sensory neurons were treated with either cancer-derived CM or paclitaxel. We chose cancer-derived CM treatments since it is known to (i) enhance neurite sprouting [29,30] and (ii) induce pain behaviors in rodents when delivered with an intraplantar injection [56]. When sensory neurons were exposed to CM derived from human breast cancer cell line MDA-MB-231 cells, human prostate cancer cell line PC3 cells, human lung cancer cell line A549 cells, or murine lung cancer cell line LL/2 cells, the sprouting of sensory neurons significantly increased, compared to those exposed to control CM (Fig. 4A&B). However, CM derived from normal prostate epithelial cells did not affect the growth of sensory nerves (Fig. 4C). Interestingly, we observed more nerve sprouting in sensory neurons treated with CM derived from lung cancer cell lines (A549 and LL/2). This finding might be consistent with orthopedic surgeons' anecdotal evidence that bone metastasis from lung

cancer are more painful than those from other cancer types (e.g. prostate cancer and breast cancer) (personal communications), although further studies to confirm this observation are clearly warranted. On the other hand, when sensory neurons were treated with paclitaxel, the growth of sensory nerve fibers was reduced in a dose dependent manner (Fig. 4D&E). Mouse sensory neurons were treated with 10 μ M paclitaxel, since this dose is close to a physiological-dose [57].

For quantification of neurite outgrowth, we used a commercially available image analysis software, Visiopharm to automatically measure a total neurite length and Image J to count the numbers of the soma in each coverslip. Then, a total neurite length was normalized with soma count. To validate whether an automated method can accurately be used to measure neurite outgrowth, we first created a novel algorithm (called an "APP") using Visiopharm that enabled us to detect the somas and measure the length of their neurites based on structural differences and fluorescence intensity, respectively (Fig. 5A). Then, values obtained automatically (Visiopharm) were compared to those obtained manually (Image J). As shown in Fig. 5B, total neurite lengths obtained using these two different methods were highly correlated ($r^2 = 0.8975$). However, soma counts were not as highly correlated as total neurite lengths ($r^2 =$

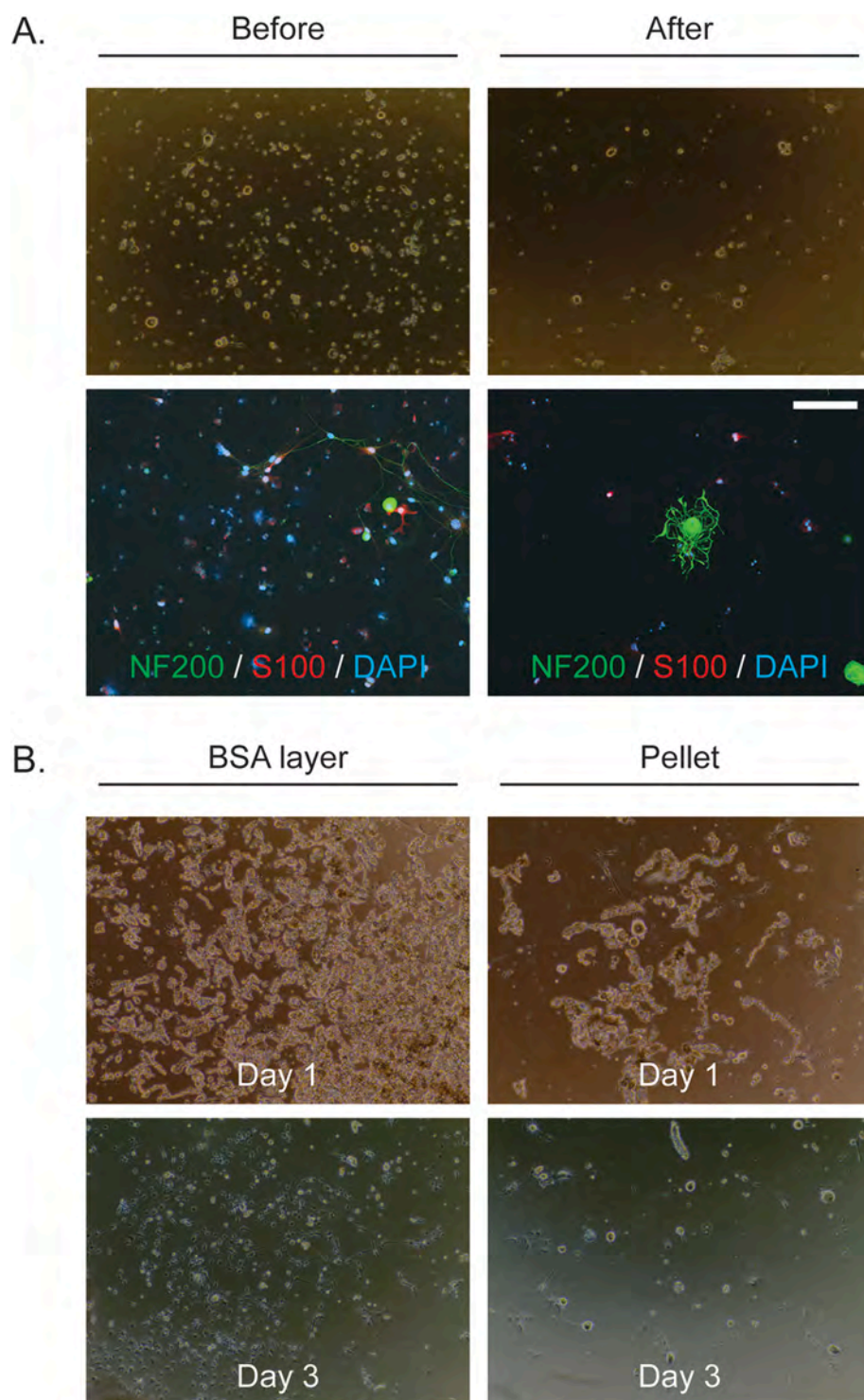


Fig. 2. Purification of murine primary DRG sensory neurons using a BSA gradient centrifugation. (A) DRG cells were seeded onto Poly-D-lysine/laminin coated coverslips before or after a BSA gradient centrifugation [3.5% (W/V) BSA solution]. Representative bright field images of murine DRGs before and after a BSA gradient centrifugation (top panels). Magnification 4 $\times$. Representative immunofluorescence images murine DRGs before and after BSA centrifugation (bottom panels). Sensory neurons were stained with antibodies against 200kD neurofilament (NF200) (green), and satellite cells were stained with antibodies against S100b (red), DAPI (blue) is used for nuclear staining. Magnification 20 $\times$. Bar = 100 μ m. (B) After a BSA gradient centrifugation, cells obtained from the pellet fraction (pellet) and BSA layer were seeded onto Poly-D-lysine/laminin coated coverslips. Representative bright field images of cells from the pellet fraction and BSA layer at Day 1 (top panels) and 3 (bottom panels). Magnification 4 $\times$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.1561) (Fig. 5C). This might in part be due to the difficulty to segregate the cluster of the somas into a single soma. These studies demonstrated the following: (i) the total neurite length obtained using an automated method were similar to that obtained manually; (ii) a manual counting method provided more accurate information regarding the numbers of soma than an automated method; and (iii) counting somas manually was not as time consuming as measuring neurite length. Therefore, we chose the quantification strategy described above.

3.4. The impact of cancer-derived factors and a chemotherapeutic agent on the expression of genes, associated with nerve growth and neuro-inflammatory pain, in murine sensory neurons

Next, to determine whether the alterations of sensory neuron length mediated by exogenous factors are associated with pain, gene expression analyses were performed by qPCR on murine primary sensory neurons treated with either cancer-derived CM or paclitaxel. In this case, we chose CM derived from A549 cells (A549 CM), since the greatest enhancement of murine primary sensory neuron sprouting was observed

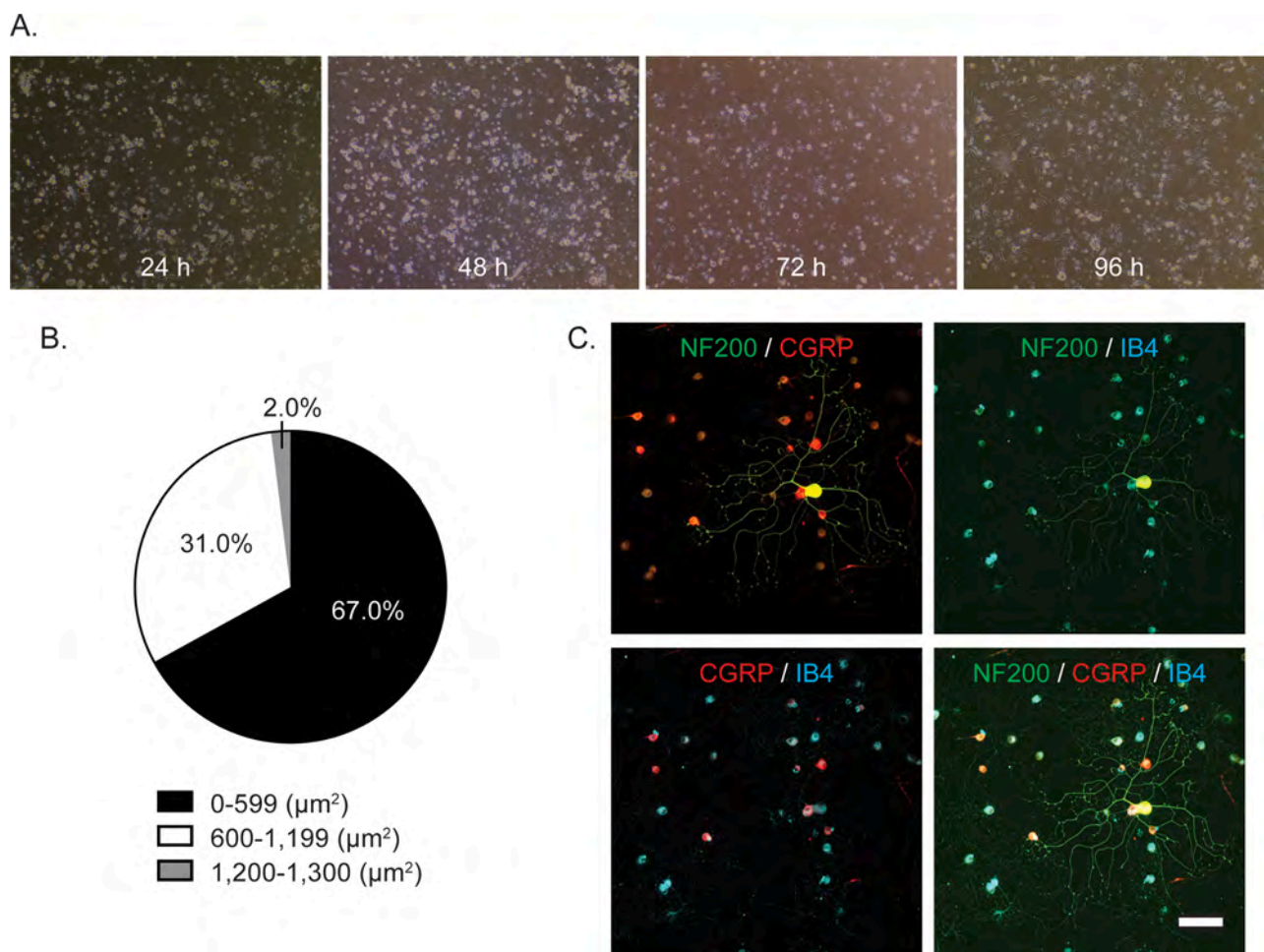


Fig. 3. Characterization of murine primary DRG sensory neurons. (A) Representative bright field images of murine primary DRG culture (9-12x lumbar DRGs (L2-L4) from 8 to 12-week-old male C57BL/6 mice) after a BSA gradient centrifugation [3.5% (W/V) BSA solution] seeded onto Poly-D-lysine/laminin coated coverslips at 24, 48, 72, and 96 h. Magnification 4x. (B) Quantification analysis of the cell size distribution of murine primary DRG sensory neurons (n = 467). Areas of soma (μm^2) of cells from (A) were measured using image J software. (C) Representative immunofluorescence images of murine primary DRG sensory neurons after a BSA gradient centrifugation. Sensory neurons were stained with antibodies against 200kD neurofilament (NF200) (Green) (all myelinated A-fibers), calcitonin gene-related peptide (CGRP) (red) (peptidergic myelinated thin A δ -fibers and unmyelinated C-fibers), and isolectin B4 (IB4) (light blue) (non-peptidergic unmyelinated C-fibers). Magnification 10x. Bar = 100 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

when treated with A549 CM (Fig. 4A). To determine whether our observations in the neurite length changes mediated by exogenous factors correlate to gene expression patterns, the levels of growth associated protein 43 (GAP43), a nerve growth marker [58], and NF200 gene expression in A549 CM or paclitaxel-treated murine sensory neurons were measured. Consistent with our findings in the neurite length changes, A549 CM significantly increased GAP43 expression, while paclitaxel exerted opposite effects (Fig. 6A). A similar trend was observed in changes in NF200 gene expression patterns as those of GAP43, although it did not reach statistical significance (Fig. 6B). Then, gene expression levels of pain-related molecules, including neuropeptides (CGRP, Substance P, Bradykinin) and cytokines involved in pathological pain conditions [tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6)] [59,60] were measured to determine the association between nerve growth and pain. Interestingly, patterns of gene expression changes were different depending on the treatments used. Neither A549 CM nor paclitaxel affected CGRP expression levels (Fig. 6C), while A549 CM significantly decreased Substance P expression levels (Fig. 6D), and both A549 CM and paclitaxel significantly increased Bradykinin expression levels in murine primary sensory neuron (Fig. 6E). Paclitaxel significantly increased TNF- α expression levels (Fig. 6D), whereas A549 CM significantly decreased IL-6 expression levels (Fig. 6G). Additionally, the gene expression levels of suppressor of cytokine signaling 3

(SOCS3) and immunoglobulin superfamily containing leucine rich repeat 2 (ISLR2), which were significantly increased in DRGs derived from patients with neuropathic pain, compared to those derived from patients without pain [36], were measured. SOCS3 and ISLR2 are known to be involved in development of cancer-related pain [61] and nociceptive sensory neuron development [62], respectively. In these cases, A549 CM significantly impacted on the gene expression of SOCS3 (increased, Fig. 6H) and ISLR2 (decreased, Fig. 6I), while paclitaxel failed to do so.

3.5. Sensory neurons obtained from non-human primate and human DRGs act similar to those of mice.

To enhance translational potential, non-human primate primary DRG sensory neuron culture was established using similar methods to establish murine primary sensory neuron culture. Consistent with murine culture, the BSA purification method could largely reduce satellite cell population, and about 90% of the viable neurons grew neurite in serum and growth factor-free conditions (Fig. 7A). Additionally, the majority of non-human primate sensory neuron somas were between 600 μm^2 to 1,199 μm^2 (Fig. 7B). Furthermore, non-human primate sensory neurons responded to cancer-derived CM (Fig. 7C&D) and paclitaxel treatment (Fig. 7E&F), similar to murine sensory neurons.

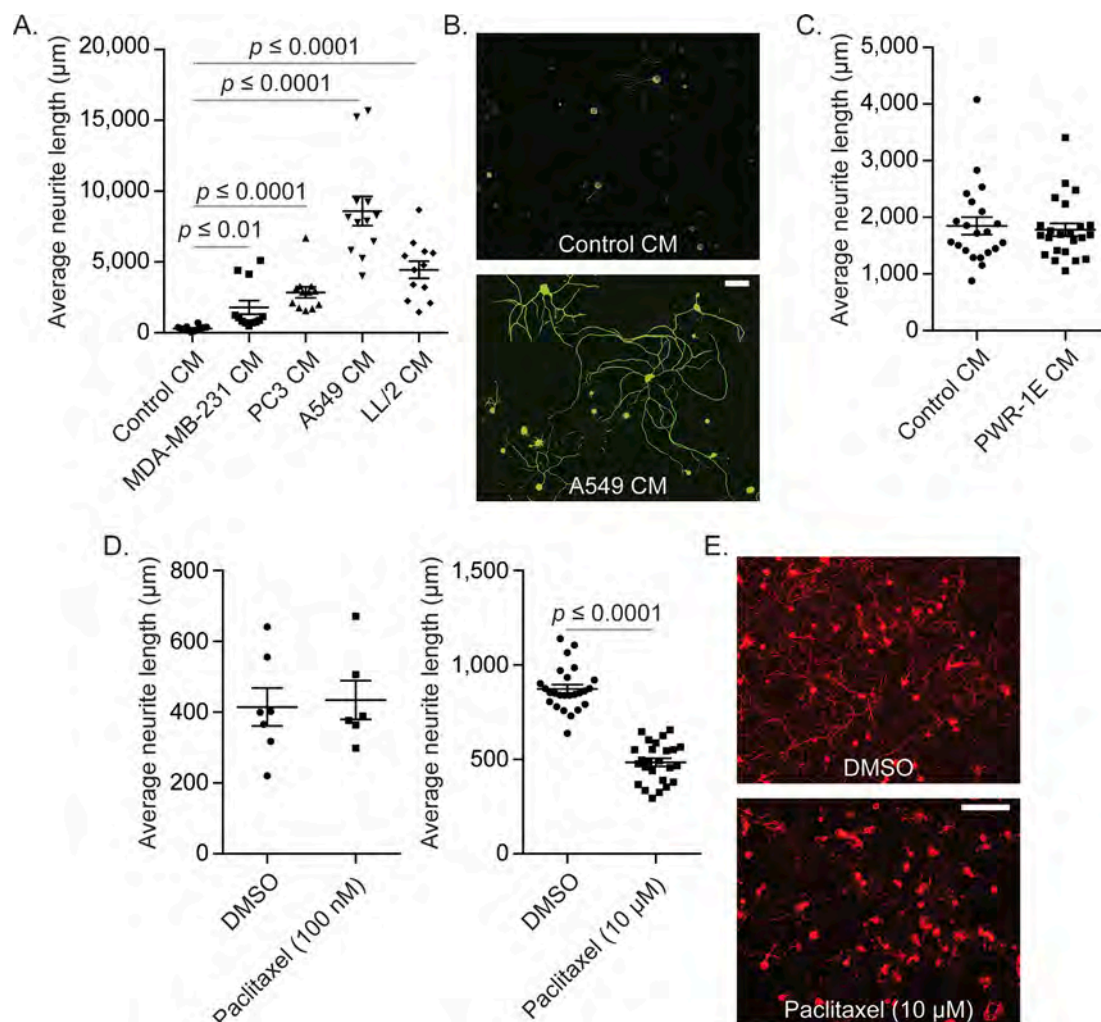


Fig. 4. Manipulation of the sprouting of murine primary DRG sensory neurons by exogenous factors. (A) Murine primary DRG sensory neurons were treated with either control medium (Control CM) or conditioned medium (CM, 0% serum) derived from human breast cancer cell line MDA-MB-231 cells (MDA-MB-231 CM), human prostate cancer cell line PC3 cells (PC3 CM), human lung cancer cell line A549 cells (A549 CM), or murine lung cancer cell line LL/2 cells (LL/2 CM) for 48 h. Sensory neurons were stained with antibodies against 200kD neurofilament (NF200). Quantification of the average of the total neurite length. Mean $\pm$ SEM. Student's *t*-test, significance at $p \leq 0.05$. The figure is a representative of two independent experiments, and 6 random images from each coverslip ($n = 2$) were analyzed in a blind manner. (B) Representative immunofluorescence images of murine primary DRG sensory neurons treated with either control or human lung cancer cell line A549 CM. Magnification 10 $\times$. Bar = 100 μ m. (C) Murine primary DRG sensory neurons were treated with either control medium (Control CM) or CM derived from human normal prostate epithelial cell line PWR-1E cells (PWR-1E CM) for 48 h. Sensory neurons were stained with antibodies against NF200. Quantification of the average of the total neurite length. Mean $\pm$ SEM. Student's *t*-test. The figure is a representative of one independent experiment, and 7–8 random images from each coverslip ($n = 3$) were analyzed in a blind manner. (D) Murine primary DRG sensory neurons treated with either DMSO or paclitaxel (100 nM, 10 μ M) for 24 h. Sensory neurons were stained with antibodies against PGP9.5. Quantification of the average of the total neurite length. Mean $\pm$ SEM. Student's *t*-test, significance at $p \leq 0.05$. Experiment was performed once, and 8–10 random images from each coverslip ($n = 3$) were analyzed in a blind manner. (E) Representative immunofluorescence images of murine primary DRG sensory neurons treated with either DMSO or paclitaxel (10 μ M) for 24 h. Magnification 10 $\times$. Bar = 100 μ m.

Non-human primate and human sensory neurons were treated with 1 μ M paclitaxel, since a concentration of 10 μ M used for mouse sensory neurons was too toxic to them (Data not shown).

Primary sensory neuron culture of human DRGs was also established. Consistent with murine and non-human primate culture, the BSA purification method could reduce satellite cell population, and about 93% of the viable neurons grew neurite in serum and growth factor-free conditions (Fig. 8A), and the majority of the soma size was smaller than 599 μ m² (Fig. 8B). Since sensory neurons from three different species showed different size distributions of cell population, we sought to determine whether there are any similarities in the soma size distribution among murine, non-human primate, and human sensory neurons. Interestingly, the chi-square goodness-of-fit test showed that the distributions of the soma size were different among the three species (all *p*-values from pairwise comparisons less than 0.0001). Additionally, when

neurons were treated with cancer-derived CM, the lengths of neurite were significantly longer than those treated with control CM (Fig. 8C&D). On the other hand, when neurons were treated with paclitaxel, the growth of neurite was significantly reduced compare to the neurons treated with vehicle (Fig. 8E&F).

4. Discussion

In this study, we demonstrated four important findings: (1) primary DRG sensory neuron cultures can be derived from mouse, non-human primate, and human tissues and cultured in serum and growth factor-free conditions, (2) a BSA gradient centrifugation method is useful to separate sensory neurons from satellite cells, (3) DRG sensory neurons maintain their heterogeneous subpopulations, and (4) the length of nerve fibers and gene expression pattern, associated with nerve growth,

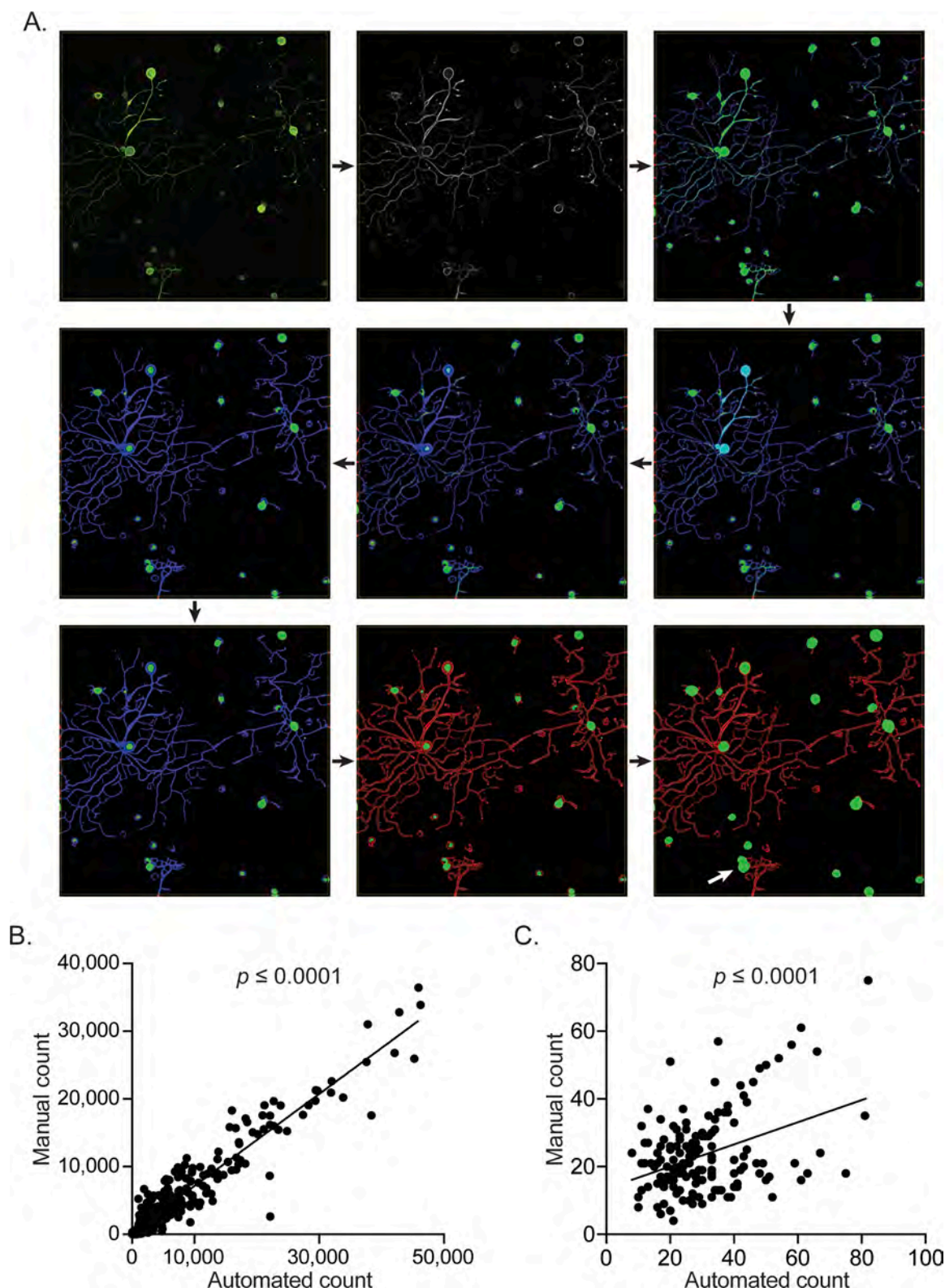


Fig. 5. Quantification of total neurite length by automated imaging analysis. (A) Representative images of the image-processing steps performed in the Visiopharm APP used for automated neurite length quantification and soma count, starting from the original fluorescent image of 200kD neurofilament (NF200) stained mouse DRG neurons (top left) to the final quantified image (bottom right) with relevant masks for neurite length (red) and soma count (green). Black arrow: direction of processing steps. White arrow: two neurons inaccurately counted as one. (B) 316 images of murine primary DRG sensory neurons stained with antibodies against NF200 were analyzed using the Visiopharm APP and by manual tracing using ImageJ software and values for total neurite length were plotted (μm): Linear regression analyses, significance at $p \leq 0.05$. (C) 164 images of murine primary DRG sensory neurons stained with antibodies against NF200 were analyzed using the Visiopharm APP and by manual counting using ImageJ software and values for total soma numbers were plotted (counts): Linear regression analyses, significance at $p \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

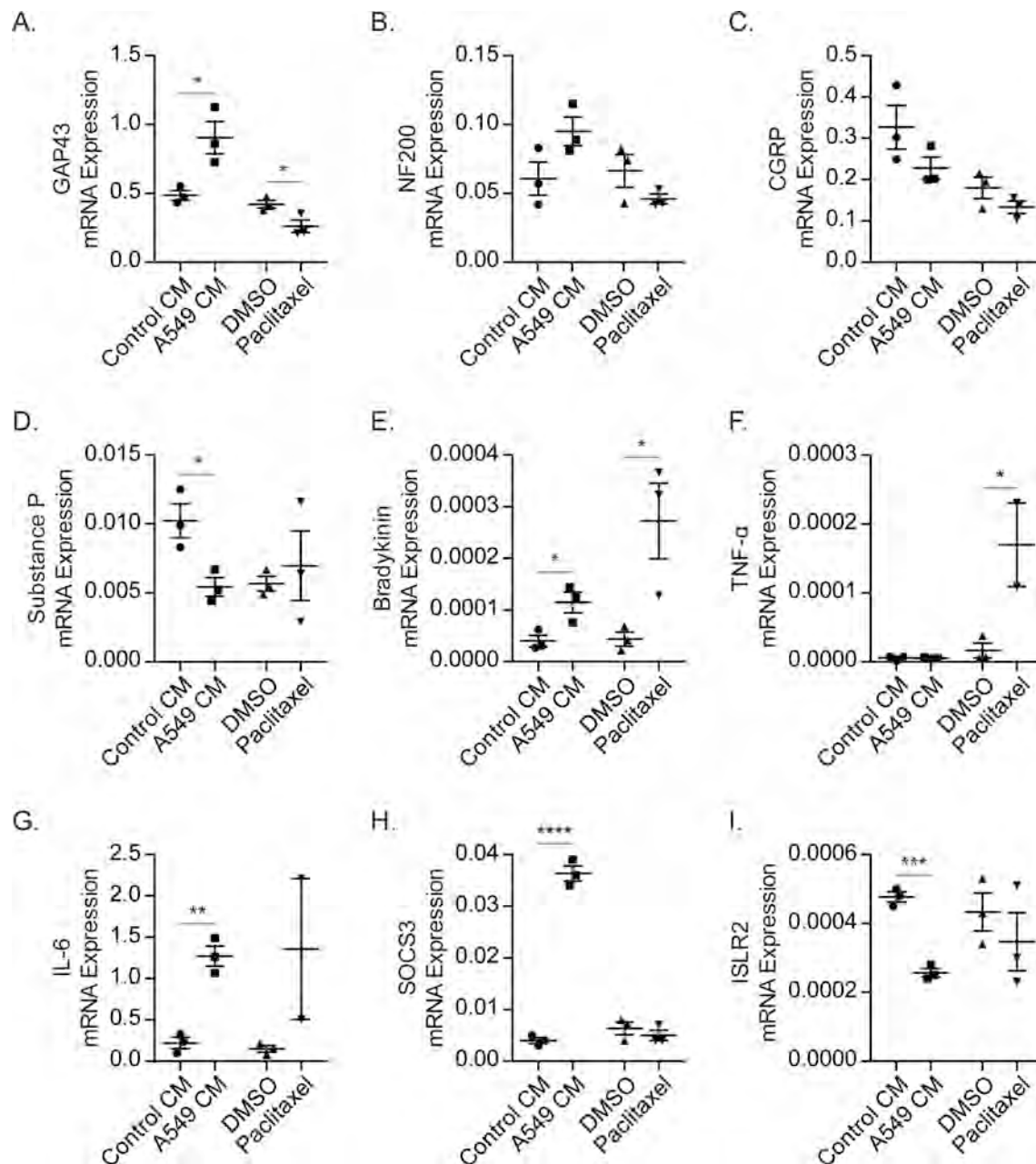


Fig. 6. Changes in expression of genes associated with nerve growth and pain in murine primary DRG sensory neurons by exogenous factors. Murine primary DRG sensory neuron cells were treated with control medium (Control CM) or human lung cancer cell line A549 cell-derived conditioned medium (A549 CM) for 48 h, and DMSO or paclitaxel (10 μ M) for 24 h. Expression of (A) GAP43 and (B) NF200, which are genes associated with nerve growth. Expression of (C) CGRP, (D) Substance P, (E) Bradykinin, (F) TNF- α , (G) IL-6, (H) SOCS3, and (I) ISLR2, which are genes associated with neuro-inflammatory pain and nociceptor development. GAPDH was used reference gene. Delta-delta Ct method. Results were displayed as Mean $\pm$ SEM. Student's *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. GAP43: growth associated protein 43; NF200: 200kD neurofilament; CGRP: calcitonin gene-related peptide; TNF- α : tumor necrosis factor- α ; IL-6: interleukin-6; SOCS3: suppressor of cytokine signaling 3; ISLR2: immunoglobulin superfamily containing leucine rich repeat 2.

and neuro-inflammatory pain and nociceptor development, of sensory neurons were altered by responding to exogenous factors, such as cancer-derived CM and reduced by responding to a chemotherapeutic agent. We developed a semi-automated quantification method which allowed us to analyze the neurite growth in an accurate and time-efficient manner. Together, these findings suggest that a semi-automated measurement of the neurite growth, along with gene expression analyses, of primary DRG sensory neurons can be a useful *in vitro* tool to study the impact of exogenous factors on the growth of sensory neurons. The schematic workflow of the primary DRG sensory neuron culture preparation and analyses, is described in Fig. 9.

Animal models have been widely used to study pathological

mechanisms underlying human diseases and to test therapeutic strategies. Although *in vivo* studies are useful for addressing large-scale biological questions, *in vitro* methods are better suited for exploring micro-level mechanisms. In this study, using primary neuronal cultures, we demonstrated that (i) cancer-derived CM induced the sprouting of nerve fibers and (ii) the chemotherapeutic agent, paclitaxel inhibited the neurite outgrowth. One of the most important aspects of our studies is that the structural changes observed in our *in vitro* setting recapitulate pathological conditions observed in *in vivo* rodent models. For example, the inoculation of cancer cells into the bone of rodents resulted in increased neurite density around the bone, which is associated with CIBP behaviors [17,18]. Similarly, the systemic administration of

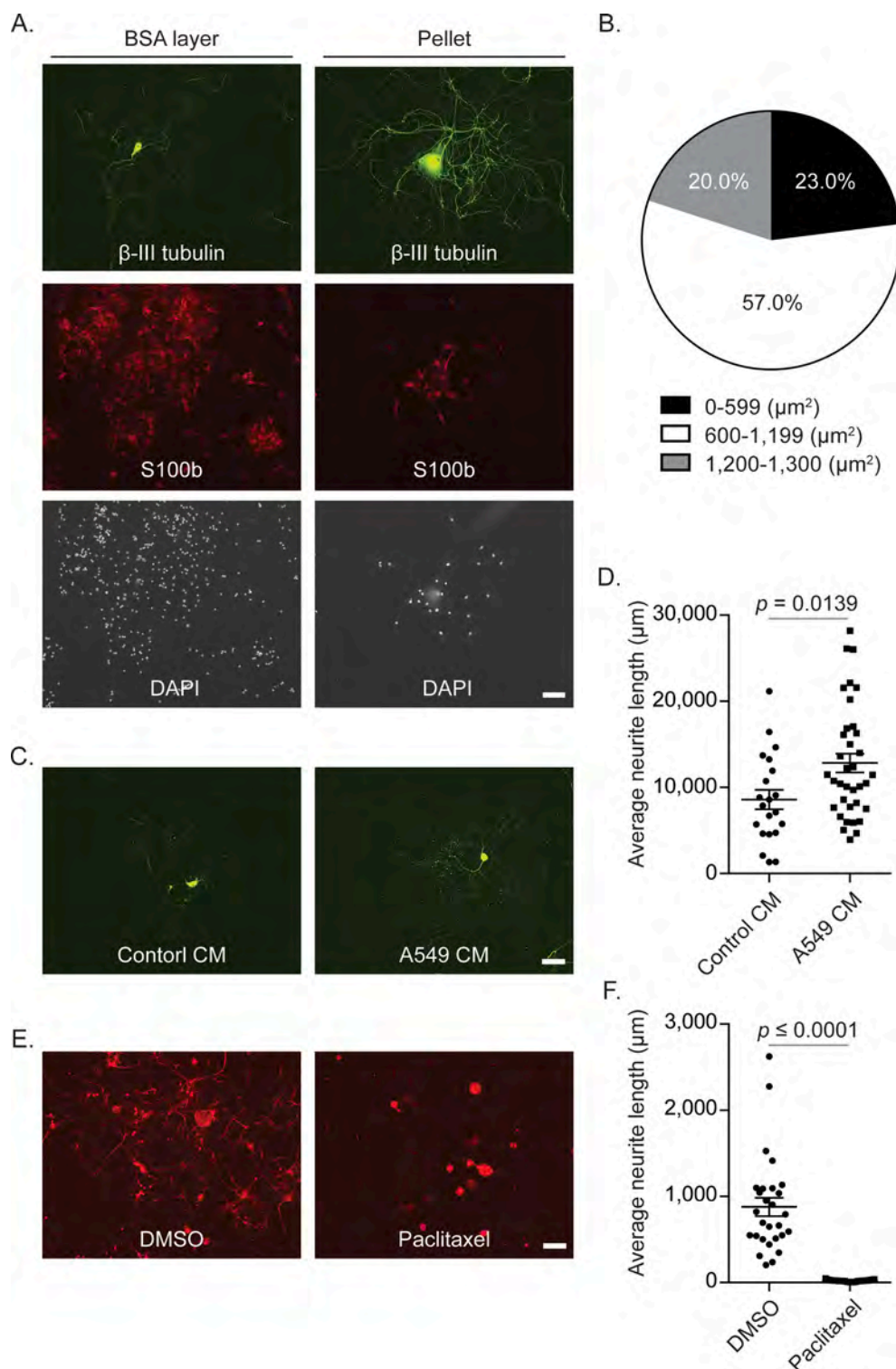
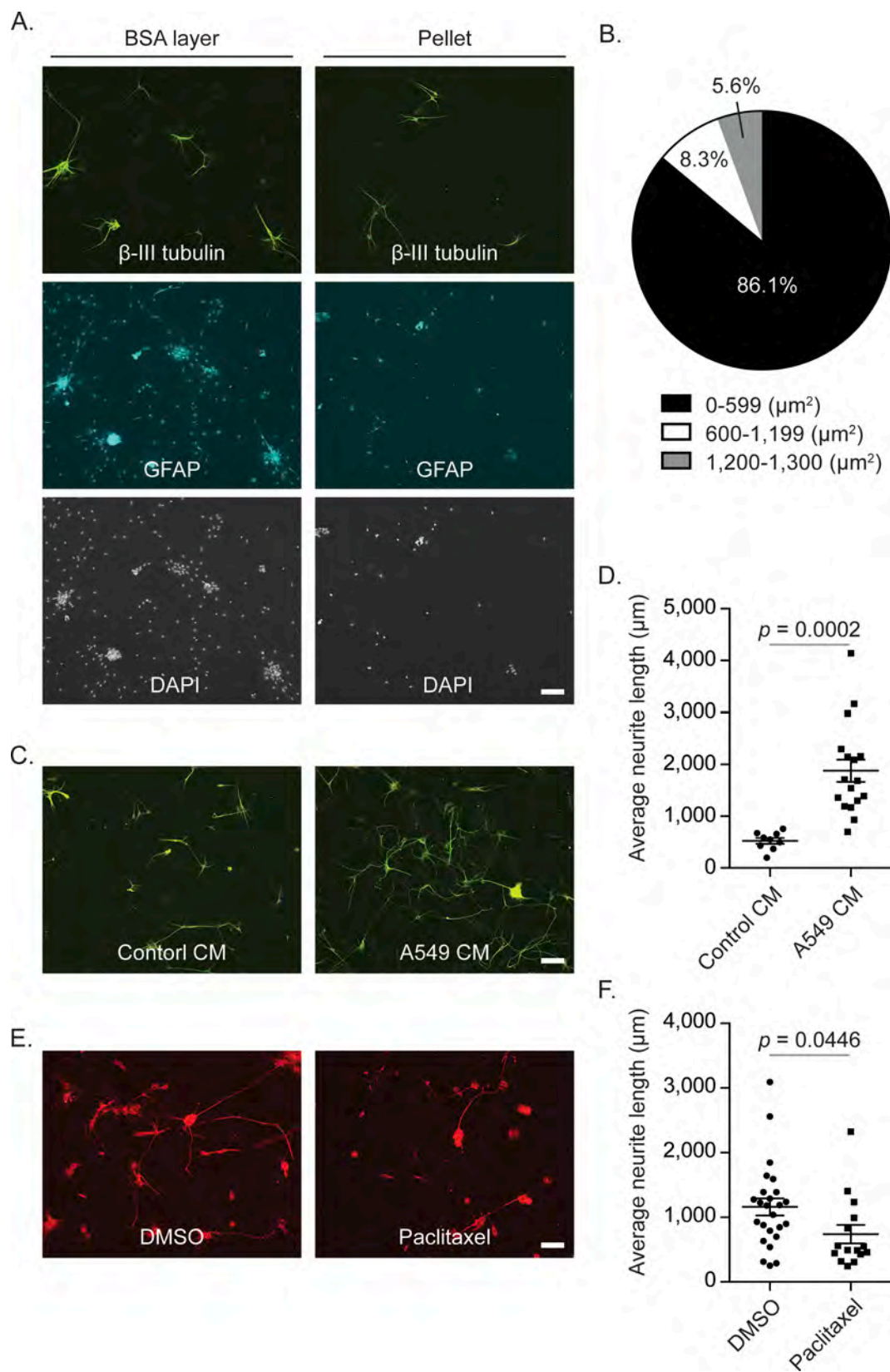


Fig. 7. Establishment of a non-human primate primary DRG sensory neuron culture. (A) Representative immunofluorescence images non-human primate DRGs (T12 and L1 of a healthy 8-year old female Rhesus macaque) before and after BSA centrifugation [3.5% (W/V) BSA solution]. Sensory neurons were stained with antibodies against β -III tubulin (green), and satellite cells were stained with antibodies against S100b (red), DAPI (gray) is used for nuclear staining. Magnification 10 $\times$. Bar = 100 μm . (B) Quantification analysis of the cell size distribution of non-human primate primary DRG sensory neurons ($n = 309$). Areas of soma (μm^2) of cells from (A) were measured using image J software. (C) Representative immunofluorescence images of non-human primate primary DRG sensory neurons treated with either control medium (Control CM) or human lung cancer cell line A549 cell-derived conditioned medium (A549 CM) (0% serum) for 72 h. Sensory neurons were stained with antibodies against β -III tubulin. Magnification 10 $\times$. Bar = 100 μm . (D) Quantification of the average of the total neurite length per image of (C): Mean $\pm$ SEM. Student's t -test, significance at $p \leq 0.05$. The figure is a representative of two independent experiments, and 8–12 random images from each coverslip ($n = 3$) were analyzed in a blind manner. (E) Representative immunofluorescence images of non-human primate primary DRG sensory neurons treated with either DMSO or paclitaxel (1 μM) for 24 h. Sensory neurons were stained with antibodies against PGP9.5. Magnification 10 $\times$. Bar = 100 μm . (F) Quantification of the average of the total neurite length per image of (E): Mean $\pm$ SEM. Student's t -test, significance at $p \leq 0.05$. Experiment was performed once, and 8–12 random images from each coverslip ($n = 3$) were analyzed in a blind manner. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

paclitaxel to rodents led to a reduction of intraepidermal neuronal fiber density in the skin, which is a hall mark of CIPN [26,27]. Our *in vitro* primary DRG sensory culture manipulations were in line with these *in vivo* structural changes in peripheral sensory neurons. Thus, *in vitro* primary DRG sensory neuron culture system established in this study is an alternative tool to study the impact of soluble factors and/or direct cell–cell interactions on the architectural remodeling of sensory neurons.

Even though the use of rodent *in vivo* and *in vitro* models is essential

for the advancement of science, the results of pre-clinical studies do not always translate to the human condition. In fact, a recent meta-analysis of 2,000 scientific studies using animal models revealed that more than half of these studies (~60%) either could not be replicated or failed to move forward to clinical trials [63]. To overcome this hurdle, studies using human tissues are receiving growing attention. Therefore, an additional salient finding of our studies is the reproducibility of pathologic neurite outgrowth following cancer-derived CM and paclitaxel-induced neurotoxicity in non-human primate and human primary



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Fig. 8. Establishment of a human primary DRG sensory neuron culture. (A) Representative immunofluorescence images human primary DRGs (T10 of a 41-year-old male patient with discitis/osteomyelitis) before and after BSA centrifugation [3.5% (W/V) BSA solution]. Sensory neurons were stained with antibodies against β -III tubulin (green), and satellite cells were stained with antibodies against glial fibrillary acidic protein (GFAP) (blue), DAPI (gray) is used for nuclear staining. Magnification 10x. Bar = 100 μ m. (B) Quantification analysis of the cell size distribution of human primary DRG sensory neurons ($n = 72$). Areas of soma (μ m²) of cells from (A) were measured using image J software. (C) Representative immunofluorescence images of human primary DRG sensory neurons treated with either control medium (Control CM) or human lung cancer cell line A549 cell-derived conditioned medium (A549 CM) (0% serum) for 72 h. Sensory neurons were stained with antibodies against β -III tubulin. Magnification 10x. Bar = 100 μ m. (D) Quantification of the average of the total neurite length per image of (C): Mean $\pm$ SEM. Student's *t*-test, significance at $p \leq 0.05$. The figure is a representative of two independent experiments, and 8–13 random images from each coverslip ($n = 2$) were analyzed in a blind manner. (E) Representative immunofluorescence images of human primary DRG sensory neurons treated with either DMSO or paclitaxel (1 μ M) for 24 h. Sensory neurons were stained with antibodies against PGP9.5. Magnification 10x. Bar = 100 μ m. (F) Quantification of the average of the total neurite length per image of (E): Mean $\pm$ SEM. Student's *t*-test, significance at $p \leq 0.05$. Experiment was performed once, and 8–10 random images from each coverslip ($n = 2$) were analyzed in a blind manner. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DRG dissection



DRG dissociation to obtain single cell suspensions

Enzymatic digestion

Papain (Mouse: 30 min, Non-human primate: 1 h, Human: 1 h @ 37°C)
 Collagenase/Dispase (Mouse: 30 min, Non-human primate: 1 h, Human: 1.5 h @ 37°C)

Mechanical dissociation (Gentle trituration 10–15 times using pipette tips)



BSA centrifugation to separate sensory neurons (pellet) from satellite cells (BSA layer)

3.5% (W/V) BSA solution, 14 x g, 20 min @ room temperature



Monolayer sensory neuron culture on Poly-D-lysine and laminin-coated coverslips

Treatment

Cancer-derived CM (48–72 h @ 37°C)
 Chemotherapy (48–72 h @ 37°C)



Analyses

Immunohistochemistry and imaging
 Neurite length analyses by Visiopharm app
 Gene expression analyses by qPCR
 Statistical analyses

Fig. 9. The schematic workflow of the primary DRG sensory neuron culture preparation and analyses. Dorsal root ganglia of mice, non-human primate, or human, were dissociated by enzymatic digestion and mechanical dissociation to obtain single cell suspensions. Then, the resulting cells were segregated into sensory neurons and satellite cells using a BSA gradient centrifugation. Thereafter, sensory neurons were plated onto Poly-D-lysine and laminin-coated coverslips to establish monolayer culture. In some cases, sensory neurons were exposed to exogenous factors, such as cancer-derived conditioned medium (CM) or chemotherapeutic agents, and then changes in nerve fiber length and gene expression were analyzed by immunohistochemical analyses and qPCR, respectively.

neuronal cultures, similar to murine neuronal cultures. Our findings also indicate that our approach with rodent sensory neurons provides an alternative to the use of non-human primate or human primary sensory neuron samples so that these precious resources can be used specifically for translational assessments after more thorough characterization with mouse neuron cells. We also recognized that the use of non-human primate or human primary sensory neurons is not trivial and requires a great deal of resources to obtain fresh DRGs. In fact, one of the major challenges that we faced in the course of our studies was the coordination of multiple departments and laboratories. However, these constraints become minor when there is solid coordination and commitment among different departments and laboratories, and a frequent communication and engagement between physicians and basic scientists.

Our study also uncovered that our approach has some limitations. For example, although successful, the numbers of sensory neurons recovered from human DRG were relatively lower than those from mice and non-human primate, which limited the number of conditions

performed in each experiment. This may be in part due to the difficulty in the digestion step of human DRGs. Human DRGs contains more connective tissues, including fibroblasts, blood vessels, and base membrane, than murine and non-human primate DRGs [64]. Consistent with this notion, during preparation of culture, the large number of fibroblasts were observed in human DRG, while few or no fibroblasts were seen in murine and non-human primate DRGs. Since thick connective tissues surrounding neurons may interfere with enzymatic reaction, it is crucial to cut the human DRGs into small pieces prior to the digestion step. Another potential limitation is the fact that the most abundant population of neurons in human DRG was small neurons in contrast to murine and non-human primate DRGs. Although the result is in line with previous findings [65,66], this suggests that our approach might not be ideal for studies targeting medium or large size of human sensory neurons. However, it is important to note that the cell size characterization method used in this study might not translate into non-human primate and/or human sensory neurons. Not only cell size differences, but also

there may be differences in responses to exogenous factors among DRGs derived from these species at a molecular and functional levels, although the effects of cancer-derived CM and paclitaxel treatments on nerve growth were consistent in all species. Indeed, nicotine evoked currents as well as nicotinic receptor expression patterns are significantly different between human and rodent DRGs [34]. Therefore, further subpopulation, molecular, and functional characterizations of murine, non-human primate, and human DRGs are clearly warranted.

Once logistic and technical optimization issues were overcome, we found that the image analysis of neurite length represents a challenge due to the lack of reliable methods to accurately measure the length of nerve fiber, and perhaps this is the reason why very few studies include this outcome. We found that manual measurement is accurate when performed by the same experimenter using a blinded method, but at the same time it is extremely time consuming and experimenter biases might reduce its reproducibility. These challenges prompted us to develop a method of image analysis aimed to reduce potential experimenter biases and increase efficiency in analysis time. In this study, we successfully developed a semi-automated method using the Visiopharm platform for the measurement of neurite length. The highly correlated values between this method and the manual approach using Image J demonstrate that our unbiased approach using the Visiopharm interface is accurate enough to consistently detect neurite length. One of the limitations of our approach however is the lack of tight correlation between automatically and manually generated soma counts. This might be attributed to the use of only one marker for two different cellular structures; i.e. NF200 staining was used to detect both soma count and neurite length. We uncovered that this approach does not allow the algorithm (APP) to differentiate a cluster of multiple neurons from individual neurons, something that the human eye could discern or infer. This limitation could be overcome by using different markers for neuronal soma (or nucleus) from neurites. Thus, the APP in this study was designed to maximize the accuracy of its neurite length measurements at the expense of accurate soma counts, since manually counting somas is significantly easier than manually tracing neurites. We are currently working to develop soma quantification APPs using nuclear stains for future studies capable of generating counts that are highly correlated with manual methods.

Although the importance of nerves for cancer progression has been appreciated [1], there are no treatments yet available which target the nerve-cancer interaction (e.g. cancer-induced nerve sprouting). In addition, chemotherapy is still the gold standard for cancer treatments and results in CIPN in many patients due to drug neurotoxicity. It is, therefore, critical to understand the interactions between cancer and/or chemotherapeutic agents, and sensory nerves, as well as how these interactions affect the progression of these painful complications, so that safer and more effective treatments can be developed. Although further electrophysiological analyses, phenotypical analyses, and chemical neural stimulation (e.g. capsaicin or KCL) on neuronal cell bodies might strengthen our findings, the measurement of neurite length and gene expression changes mediated by exogenous factors (e.g. cancer-derived factors, chemotherapeutic agents) can be a useful tool to elucidate the causes and mechanisms of these painful symptoms. For example, it has been suggested that nerve-related growth factors [29] or exosomes [30,56] derived from cancer cells induce cancer-related pain by stimulating neurite outgrowth [15–19], and that axonal damage mediated by chemotherapeutic agents through mitochondrial dysfunction in nerve cells and oxidative stress on nerve cells are responsible for CIPN symptoms [67]. We believe that our developed approaches could be used to reveal these underlying mechanisms. Moreover, the use of higher species' neuronal tissues in tandem with the development of an accurate and time-efficient methodology to measure neurite length under different pathological conditions will enhance clinical translational of our discovery. For success of this approach, a well-coordinated multi-disciplinary team between physicians and basic scientists, which we began to establish, is clearly essential.

Author contributions

S.H.P., M.R.E., and Y.S. wrote the manuscript with input from all authors. S.H.P., M.R.E., C.M.P., M.C.K., R.S., W.H., E.A.R-S., and Y.S. designed study and contributed to data interpretation. S.H.P., M.M.F., H. D., and C.M.P. performed animal surgeries. M.C.K. provided monkey DRG. J.A.W. and W.H. provided human DRG. S.H.P. and M.M.F. established DRG culture. S.H.P., M.R.E., M.M.F., K.A.C., and C.M.P. performed immunohistochemistry and quantification analyses. F.H. performed statistical analyses. C.M.P., M.C.K., R.S., W.H., E.A.R-S., and Y.S. supervised the study. All authors reviewed and approved the final manuscript.

Declaration of Competing Interest

Y.S. has received research funding from TEVA Pharmaceuticals, but not relevant to this study. No conflict of interest exists for remaining authors.

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ARTICLE

Nicotine promotes brain metastasis by polarizing microglia and suppressing innate immune function

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Up to 40% of lung cancer patients develop brain metastasis, and the median survival of these patients remains less than 6 months. Smoking is associated with lung cancer. However, how smoking impacts the development of brain metastasis remains elusive. We examined 281 lung cancer patients with distant metastasis and found that smokers exhibited a significantly high incidence of brain metastasis. We found that nicotine enhanced brain metastasis, while a depletion of microglia suppressed this effect in vivo. Nicotine skewed the polarity of microglia to the M2 phenotype, thereby increasing the secretion of IGF-1 and CCL20, which promoted tumor progression and stemness. Importantly, nicotine enhanced the expression of SIRPα in microglia and restricted their phagocytic ability. We also identified a compound, parthenolide, that suppressed brain metastasis by blocking M2 polarization. Our results indicate that nicotine promotes brain metastasis by skewing the polarity of M2 microglia, which enhances metastatic tumor growth. Our results also highlight a potential risk of using nicotine for tobacco cessation.

Introduction

Approximately 170,000 patients in the United States are diagnosed with brain metastasis every year (Ellis et al., 2012). Lung, breast, and skin cancers are the most common tumor types that metastasize to the brain (Bacha et al., 2018; Huang et al., 2018; Redmer, 2018; Xing et al., 2018). Patients with metastatic lung cancer have a very poor prognosis (Mujoomdar et al., 2007), and those with brain metastasis have a 1-yr survival rate of 4–20% (Gaspar et al., 1997; Sperduto et al., 2008; Verger et al., 2005). Despite significant improvements in the treatment of metastatic cancer, the therapeutic options for brain metastasis are still limited mainly due to inefficient drug delivery to brain as well as limited surgical approaches to multiple metastases. Radiation therapies, such as stereotactic radio surgery and fractionated whole brain radiation, are standard therapeutic techniques employed for brain metastasis; however, they provide only limited local tumor control and, more often than not, intracranial failure leading to neurological death. The development of lung cancer brain metastasis is a complex process, requiring the invasion of primary tumor cells into surrounding tissue and vessels, trafficking through the circulatory system, and colonizing into the brain parenchyma (Fidler, 2015). Therefore, understanding the underlying mechanisms that drive brain

metastasis and developing more effective therapies are urgently needed.

Cigarette smoking is one of the major risk factors for lung cancer (Lee et al., 2012). Our previous study have shown that smoking is correlated to rapid progression of lung cancer brain metastasis (Shenker et al., 2017). Tobacco smoke contains a toxic mix of >7,000 chemicals. Among them, nicotine is a major cigarette component associated with human tobacco consumption and addiction (Braverman, 1999; Heishman, 1999). Nicotine is not a carcinogen; however, several studies have reported the tumor-promoting effects of nicotine via activation of nicotinic acetylcholine (nACh) receptor in the tumor cells. The nACh receptor is comprised of combination of multiple subunits, and they are expressed in a variety of brain cells including neuron, astrocytes, endothelial cells, and microglia (Mazzaferro et al., 2017). The α4β2 is the most abundant class of nACh receptor in the brain and is the principal mediator of nicotine dependence (Hawkins et al., 2004; Mazzaferro et al., 2017). This suggests that nicotine may be capable of reprogramming the brain tumor microenvironment (TME) via activation of its receptor to promote tumor progression.

Several studies have shown that the interaction of neoplastic cells with microglial cells, brain-residing macrophages, profoundly

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affects tumor progression in glioblastoma (da Fonseca and Badie, 2013; Roesch et al., 2018; Wei et al., 2013). In brain metastatic lesions of lung cancer patients, abundant activated microglia were found to be infiltrated (He et al., 2006). Importantly, nACh receptor has been shown to be expressed on microglia (Morioka et al., 2015; Sadigh-Eteghad et al., 2016), raising the possibility that nicotine may stimulate microglia's immunomodulatory pathways, which affects their immune function. Microglia are known to polarize into classical (M1) or alternative (M2) phenotypes by various microenvironment stimuli (Wei et al., 2013; Wu and Watabe, 2017). M1 cells suppress tumors by releasing cytotoxic factors and also by phagocytosis. In contrast, M2 cells have a pro-tumor response by activating immunosuppressive factors. It is virtually unknown how metastatic cells escape immune attack of microglia and then colonize in the brain. In this study, we showed that nicotine promoted lung cancer brain metastasis by enhancing both tumor progression and stemness by skewing microglial polarization and inhibiting its anti-tumor phagocytic ability. We also found that a natural compound, parthenolide (PTL), effectively suppressed tumor progression and stemness by blocking nicotine-induced polarization of the microglia in the brain.

Results

Smoking increases brain metastasis of lung cancer

To examine the effect of tobacco smoking on brain metastasis, we analyzed the incidence and mortality rate of 281 patients with lung cancer brain metastasis (Fig. S1) with or without a history of smoking. All patients were diagnosed with stage IV disease. Among them, 79 patients were diagnosed with brain metastasis. 59 patients were current smokers at the time of diagnosis and treatment, while 222 of them had either a previous or no smoking history. We found that the incidence of brain metastasis was significantly higher in current smokers compared with the never or the former smokers group (Fig. 1 A). The results of Kaplan–Meier analyses indicate that the current smokers were associated with worse brain metastasis progression free (Fig. 1 B) and overall survival (Fig. 1 C) compared with those who were not currently smoking. Since activation of microglia was previously reported to be associated with brain tumor progression (da Fonseca and Badie, 2013; He et al., 2006; Roesch et al., 2018; Wei et al., 2013), we examined the status of microglia activation in brain metastatic lesions and found that current smokers exhibited a significantly increased number of Iba1⁺ (pan-microglial marker) cells in the brain metastatic lesions (Fig. 1 D) compared with noncurrent smokers. Importantly, the majority of infiltrated microglia in current smokers was identified to be pro-tumor M2 microglia (CD206⁺ cells; Fig. 1 D). These results suggest a role of smoking-mediated M2 microglial polarization in brain metastatic tumor growth.

Nicotine promotes brain metastasis through activation of microglia

Because M2 microglia are abundantly infiltrated in brain metastatic lesions of current smokers, we examined the possibility that nicotine promotes brain metastasis by polarizing microglia to M2 microglia. We first examined the expression of nicotine receptors in microglia cells and in tumor cells and found that

microglia expressed high levels of $\alpha 4\beta 2$, which is the most sensitive receptor of nicotine in the brain (Fig. 2 A). We also found that microglia expressed a significantly higher level of $\alpha 4\beta 2$ receptor than macrophage (Fig. S2 A). Moreover, the expression of $\alpha 4\beta 2$ receptor in microglia dramatically increased upon nicotine treatment (Fig. S2 A). We then examined the effect of nicotine on microglia polarization and tumor progression using the LL/2 mouse Lewis lung carcinoma model. The LL/2 cell is originated from the C57BL/6 mouse; however, this cell line can grow tumors in both C57BL/6 and BALB/c mice as reported previously (Janker et al., 2018; Kamei et al., 2009; Scholar et al., 1989). Therefore, we tested the effect of nicotine in both models. We intracardially inoculated luciferase-labeled LL/2 cells into both wild-type mice (C57BL/6 or BALB/c) followed by injecting nicotine (1 mg/kg) every 3 d until the end point. We found that nicotine significantly increased the incidence and growth of brain metastasis in both models (Fig. S2, B–D, for C57BL/6 model; Fig. 2 B and Fig. S2, E and F, for BALB/c model). On the other hand, nicotine did not promote bone metastasis (Fig. 2 B). The result of our immunohistochemical analysis for brain metastatic lesions revealed that high levels of activated microglial cells accumulated in the tumor mass in the nicotine-treated group (Fig. 2 C). Importantly, the majority of these microglia in the nicotine-treated mice were found to be M2 microglia (CD206⁺/Iba1⁺), and fewer M1 microglia (Iba1⁺/F4/80⁺) were identified in the brain lesions (Fig. 2 D and Fig. S2, G and H), suggesting that nicotine promoted microglia polarization to the M2 phenotype in the brain metastatic lesions. Infiltrated peripheral macrophages/microglia are known to modulate brain tumor progression (Bacha et al., 2018; Ellis et al., 2012). To define the exact role of macrophage and microglia on brain metastasis, we stained brain metastatic tumors with antibody against the macrophage marker CD45. We also examined the ratio of the number of microglia (CD45⁺/Iba1⁺) and peripheral macrophages (CD45⁺/Iba1⁺) in the brain metastatic lesions and found that macrophages comprised only 12–14% of CD45⁺/Iba1⁺ cells, and the number of macrophages was not altered between nicotine-treated and -untreated groups (Fig. S2 I). These results strongly suggest that nicotine promotes brain metastasis by skewing microglia to the M2 phenotype. To further validate this hypothesis, we examined the effect of depletion of “original” microglia by treating animals with the inhibitor for CSF1 receptor (CSF1R), PLX3397. CSF1 signaling is necessary for microglia viability, and treating mice with PLX3397 has been previously shown to eliminate ~99% of all microglia in the brain (Elmore et al., 2014). We therefore treated immune-competent wild-type mice with PLX3397 and transplanted them with LL/2 mouse lung cancer cells followed by nicotine treatment. To avoid a whole-body effect of PLX3397, the PLX3397 was locally injected into brain through intracranial injection 1 wk after the transplantation of LL/2 cells followed by nicotine treatment (Fig. S2 J). Strikingly, blocking microglia by PLX3397 significantly suppressed nicotine-related brain metastasis of lung cancer (Fig. 2, E–G), prolonged brain metastasis-free survival (Fig. 2 H), and repressed the nicotine-related M2 microglial polarization (Fig. 2, I and J) in the BALB/c mouse model. Similarly, PLX3397 suppressed nicotine-related brain metastasis and inhibited M2

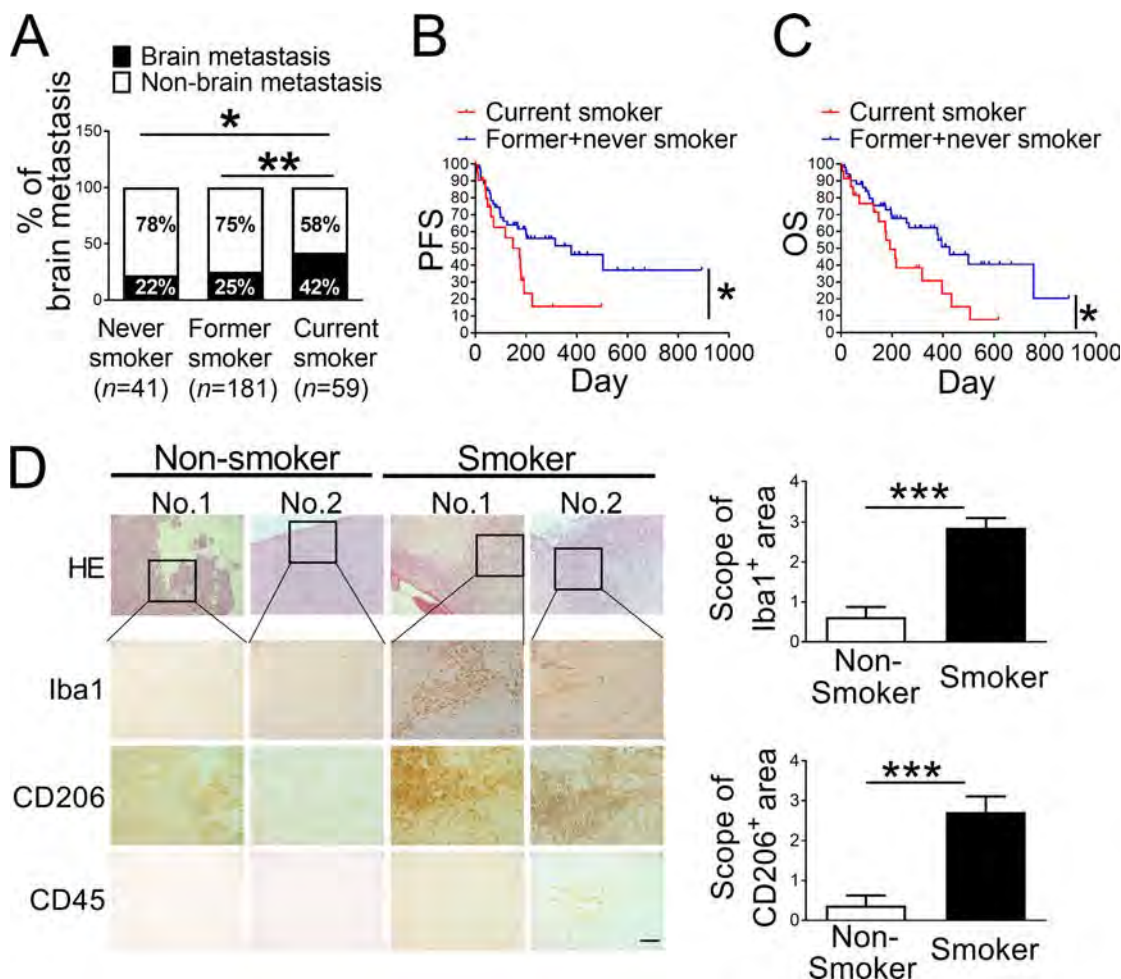


Figure 1. Cigarette smoking increases incidence and mortality rate of lung cancer brain metastasis. (A) Incidence of brain metastasis among 281 stage IV lung cancer patients composed of both current and noncurrent smokers at the time of diagnosis. All patients were admitted to Wake Forest Baptist Hospital. (B and C) Progression-free survival (PFS; B) and overall survival (OS; C) of patients with brain metastasis (n = 79) and with or without smoking history were examined by Kaplan–Meier analysis. (D) Representative images of immunohistochemical analysis for Iba1⁺, CD206⁺, and CD45⁺ cells in the H&E-stained brain metastatic lesions of lung cancer patients who were current smokers (n = 7) and noncurrent smokers (n = 4). Scale bar, 100 μ m. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

microglial polarization in the C57BL/6 mouse model (Fig. S2, K–N). We also treated nude mice with PLX3397 and transplanted them with H2030BrM cells via an intracardiac injection followed by nicotine treatment (Fig. S2, O–R). Nicotine-induced brain metastasis was significantly suppressed after PLX3397 treatment (Fig. S2, O–Q). The brain metastasis-free survival was also significantly prolonged by PLX3397 treatment (Fig. S2 R). PLX3397 treatment showed no detectable liver toxicity (Fig. S2 S). These results suggest that the nicotine-induced brain metastasis is indeed mediated by the effect of nicotine on microglia.

Nicotine skewed M2 microglial polarization

As shown in Fig. 2 D, the number of M2 microglia was increased after nicotine treatment, suggesting that nicotine promotes microglial polarization. To further investigate the effect of nicotine on microglia polarization, the microglial cells were treated with nicotine followed by measurement of expression of M1 and M2 markers. Our results showed that nicotine did not affect tumor cell proliferation (Fig. S2 T) at 1 μ M concentration, but the

same dose of nicotine significantly increased the mRNA expression of M2-related genes, *arginase-1* (*Arg1*), *arginase-2* (*Arg2*), and *CD204*, in both human and mouse microglial cells lines (Fig. 3, A and B) and mouse primary microglial cells (Fig. 3 C). Since STAT3 pathway is involved in M2 microglia (Qin et al., 2017), we examined the status of phospho-STAT3 and JAK expression in microglia with or without nicotine treatment, and found that nicotine treatment indeed enhanced the expressions of JAK/STAT3 (Fig. 3 D). We then treated the microglia with nicotine in the presence of STAT3 inhibitor (STATITC) and found that inhibiting the STAT3 by STATITC significantly suppressed the nicotine-induced STAT3 activation (Fig. 3 E). The *Arg1* gene is an M2 marker and is known to be regulated by STAT3 (Yi and Kim, 2017). We found that nicotine-induced up-regulation of the *Arg1* gene promoter was significantly suppressed by STATITC treatment (Fig. 3 F). Moreover, inhibition of STAT3 reversed the nicotine-mediated suppression of Iba1⁺/F4/80⁺ (M1) and promotion of Iba1⁺/CD206⁺ cells (M2; Fig. 3, G and H). These results suggest that the nicotine promotes M2 microglial polarization via activation of the STAT3 pathway.

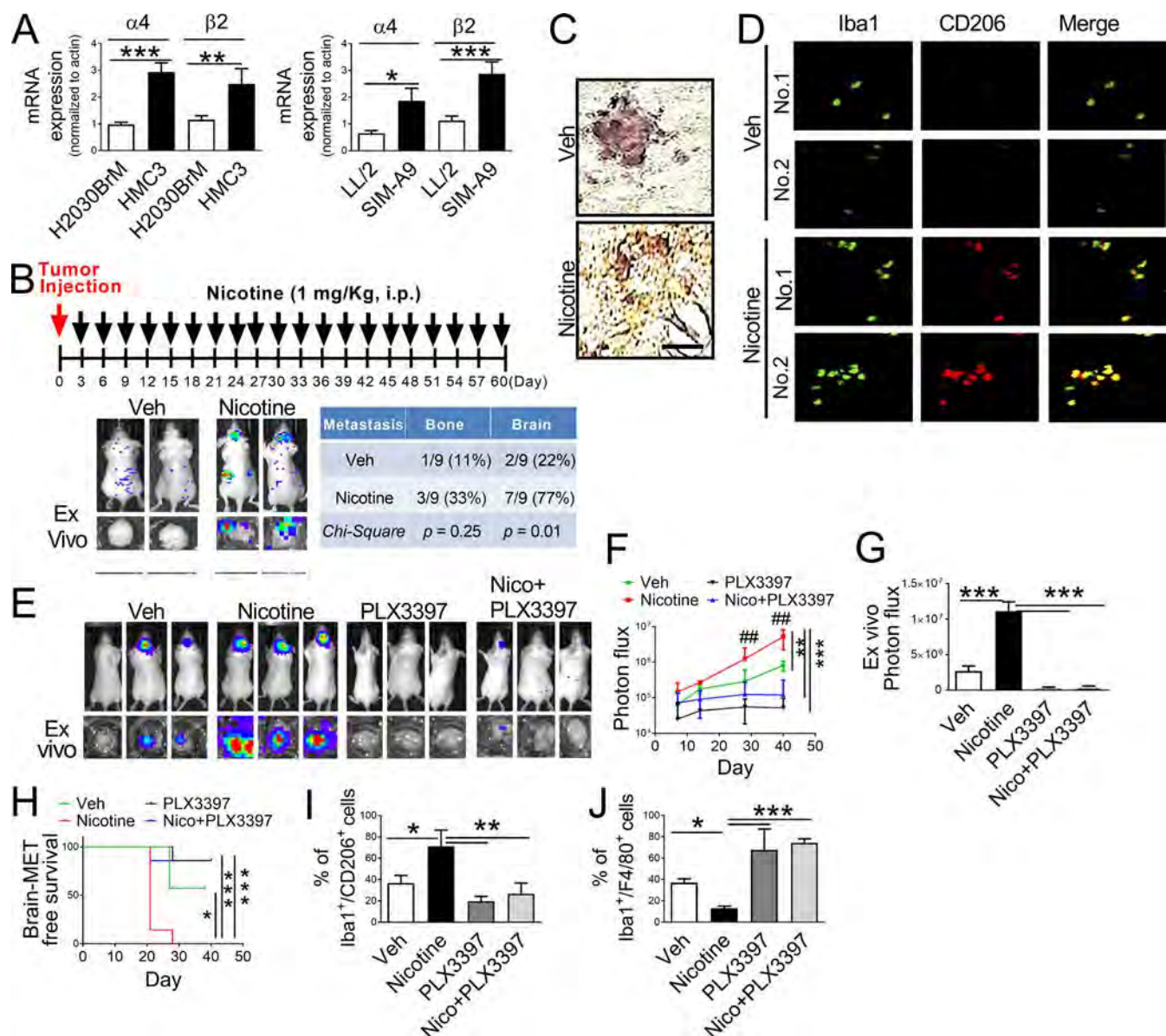


Figure 2. Nicotine promotes brain metastasis by activating microglia in vivo. (A) Expression of $\alpha 4 \beta 2$ nicotine receptor on human microglia (HMC3), mouse microglia (SIM-A9), human lung cancer cells (H2030BrM), and mouse lung cancer cells (LL/2) were evaluated by qRT-PCR ($n = 3$ /group). (B) LL/2 (5×10^4 cells) were intracardially injected into immune-competent BALB/c mice ($n = 9$). After 3 d of intracardiac transplantation of LL/2 cells, mice received nicotine treatment (1 mg/kg) by intraperitoneal injection every 3 d for 60 d. Upper panel: BLI images of brain metastatic lesions of representative mice from each experimental group (vehicle alone or nicotine treatment). Lower panel: total photon flux of ex vivo image of brain metastatic lesions was measured by BLI at the end point (day 60). Quantitative data of bone and brain metastasis of lung cancer are shown in the right panel. (C) At the end point, the brain sections from mice with or without treatment with nicotine were examined for Iba1⁺ signal (brown) on microglia. Scale bar, 20 μ m. (D) Representative images of immunohistochemical analysis for CD206⁺ and Iba1⁺ microglia in the metastatic brain lesions of mice that were treated with or without nicotine ($n = 9$ /group). Scale bar, 100 μ m. (E) Mouse lung cancer LL/2 cells were intracranially injected into wild-type BALB/c mice ($n = 9$ /group) followed by administering PLX3397 by intracranial injection. Upper panels are BLI images of brain metastatic lesions of representative mice from each experimental group at day 40. Lower panels represent the total photon flux of ex vivo brain metastatic lesions as measured by BLI at the end point (day 40). (F) Quantitative data of in vivo brain metastasis of lung cancer ($n = 9$ /group). (G) Ex vivo BLI signals in the brain at the end point ($n = 9$ /group). (H) Kaplan–Meier analysis of brain metastasis-free survival was performed ($n = 9$ /group). (I and J) Metastatic brain tumors in E were isolated from the brain and were examined by FACS for M2 (I) and M1 (J) microglial polarization ($n = 4$ or 5/group). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus respective nicotine group. ##, $P < 0.01$ versus respective PLX3397 or Nico+PLX3397 group.

Nicotine-treated microglial cells promote tumor growth and stemness

To examine the effect of nicotine-induced M2 microglia on tumor progression, we treated lung cancer brain metastatic cells, H2030BrM and PC9BrM, with conditioned medium (CM) generated from human microglia that were treated with or without

nicotine. We found that CM derived from nicotine-treated microglia significantly increased sphere formation (Fig. 4 A) and population of ESA⁺/CD44⁺ or aldehyde dehydrogenase (ALDH⁺) cancer stem cells (CSCs; Fig. 4 B). The expression of stemness-inducing genes, SOX2 and NANOG, was also significantly up-regulated when the brain metastatic cells were treated with

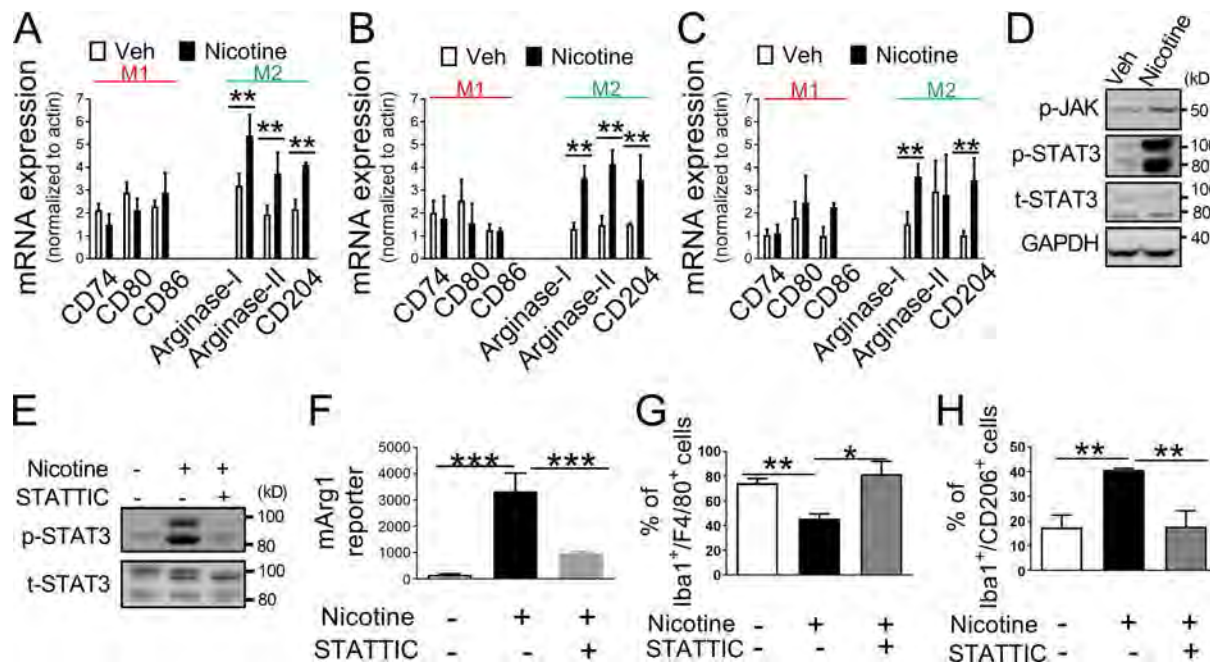


Figure 3. Nicotine promotes M2 microglial polarization via STAT3 activation. (A–C) Expressions of M1/M2 microglia markers were examined after the treatment of human microglia (HMC3) (A), mouse microglia (SIM-A9; B), and primary mouse microglia (C) with either vehicle or nicotine (1 μ M). The value of qRT-PCR in each figure was normalized using actin as a control. The y axis indicates arbitrary unit ($n = 3$ /group). (D) Human microglia (HMC3) were treated with or without nicotine (1 μ M), and the expressions of JNK and STAT3 were examined by Western blot. (E) Human microglia (HMC3) were treated with or without nicotine and/or STATTIC (0.5 μ M) for 24 h, and the expression of STAT3 was examined by Western blot. (F) Mouse microglia cells (SIM-A9) were treated with or without nicotine and/or STATTIC (0.5 μ M) for 24 h followed by examining the luciferase-promoter activity of *Arg1* (M2; $n = 4$ /group). (G and H) Mouse microglia (SIM-A9) were treated with or without nicotine (1 μ M) and/or the STAT3 inhibitor (0.5 μ M) for 24 h. Cells were then subjected to FACS analysis for analysis of Iba1⁺/F4/80⁺ (M1 cell; G) and Iba1⁺/CD206⁺ (M2 cell; H; $n = 9$ /group). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

the CM of nicotine-treated microglia (Fig. 4, C and D). Furthermore, nicotine-treated microglial CM also promoted the colony-forming ability of both H2030BrM and PC9BrM cells (Fig. 4 E). We then sought to identify the factor(s) in CM that are responsible for the increase in stemness. We performed the cytokine array analysis (consisting of 99 factors) and found that nicotine significantly augmented the secretion of CCL20 and IGF-1 in the microglial CM (Fig. S3 A). The results of screening were verified using human microglial cells (Fig. S3 B). Furthermore, blocking STAT3 by STATTIC significantly suppressed nicotine-mediated up-regulation of CCL20 and IGF-1 (Fig. S3 B). We observed that microglia produced higher levels of IGF-1 and CCL20 than macrophage (Fig. S3 C). The expression of receptors of IGF-1 and CCL20 were significantly increased in H2030BrM cells after the treatment with CM of microglia that were exposed to nicotine (Fig. 4 F). We then treated lung cancer cells with recombinant CCL20 and IGF-1 and examined their effect on CSC properties. We found that both cytokines significantly enhanced the sphere formation (Fig. 4 G), ESA⁺/CD44⁺ CSC population (Fig. 4 H), and mRNA expression of CSC markers SOX2 and NANOG (Fig. 4, I and J). In addition, IGF-1 and CCL20 significantly increased the colony-forming ability of brain metastatic lung cancer cells (Fig. 4 K). To understand the clinical relevance of these results, we used The Cancer Genome Atlas (TCGA) dataset and found that high expression of CCL20 was associated with poor overall survival in lung cancer patients (Fig. S3 D);

however, no such correlation was observed for the IGF-1 gene (Fig. S3 E). Furthermore, expressions of both CCL20 and its receptor, CCR6, were up-regulated in the primary tumor site of lung cancer patients compared with normal lung tissue in the same dataset (Fig. S3, F and G). Moreover, microglia expressed a higher level of CCL20 mRNA than lung cancer cells, indicating that microglia are potentially major sources of CCL20 in brain TME (Fig. S3, H and I). To examine whether CCL20 is involved in the growth-promoting effect of CM derived from nicotine-treated microglia, CCL20 was depleted in the CM using CCL20 neutralizing antibody. Our result indicates that depletion of CCL20 significantly suppressed the generation of CSC by nicotine-treated CM (Fig. 4 L). To further validate our results, we examined the levels of CCL20 in brain and serum of the nicotine-treated mice represented in Fig. 2 E and found that CCL20 indeed increased in the brain and serum of nicotine-treated animals (Fig. 4, M and N). In contrast, depletion of microglia by PLX3397 significantly suppressed the levels of nicotine-induced CCL20 in brain and serum of nicotine-treated animals (Fig. 4, M and N). Furthermore, consistent with our in vivo results, we found that the serum level of CCL20 was significantly higher in lung cancer patients who were current smokers compared with noncurrent smokers (Fig. 4 O).

We also examined the direct effect of nicotine on cell growth by treating the human lung cancer brain metastatic cells, H2030BrM, with nicotine alone or with nicotine-treated

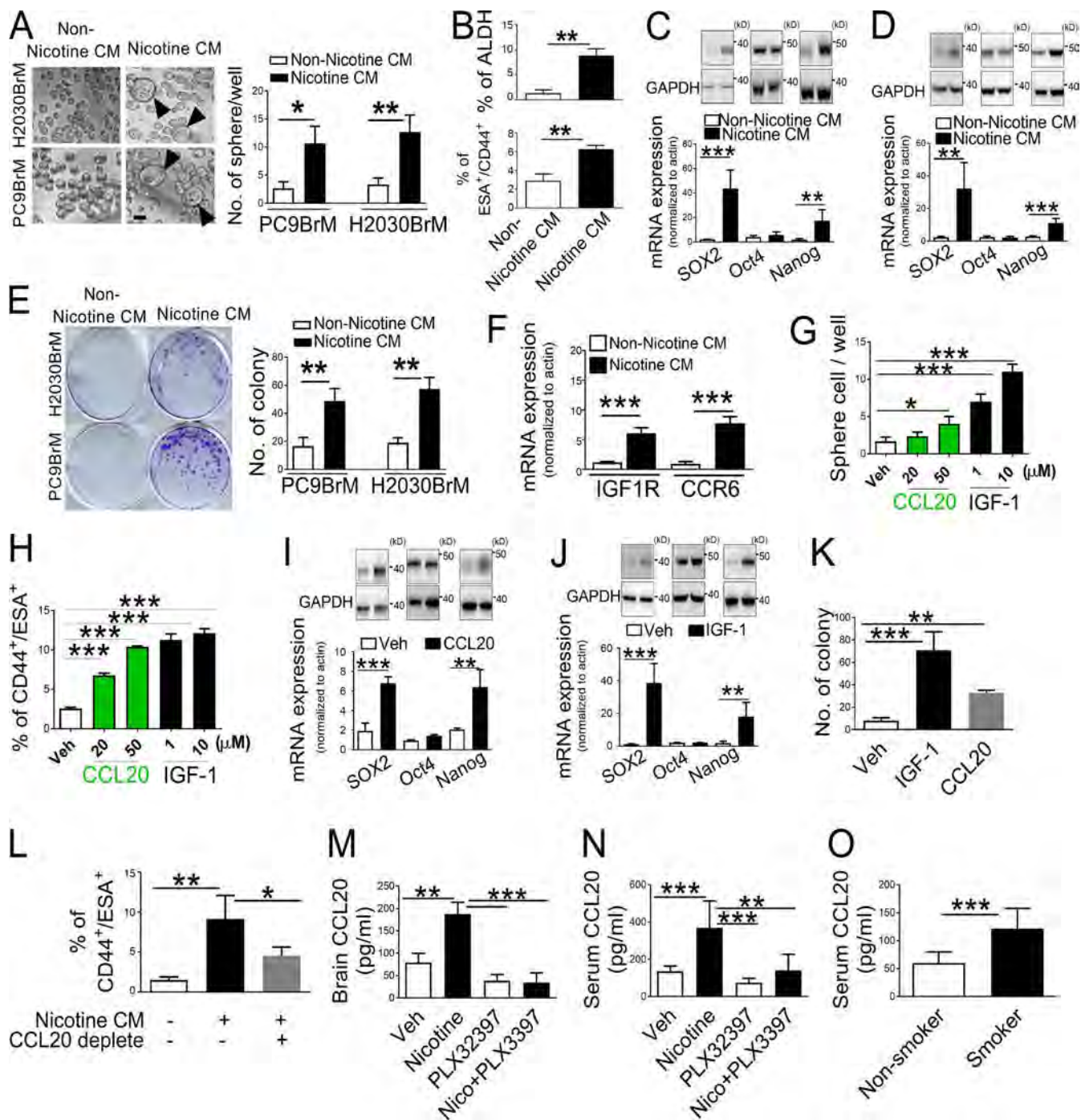


Figure 4. Nicotine-mediated microglial polarization promotes tumor stemness. (A) Effect of human microglial cells (HMC3) treated with or without nicotine (1 μM), and collection of CM. Brain metastatic lung cancer cells (H2030BrM and PC9BrM) were treated with the non-nicotine and nicotine CM for 24 h, and their sphere-forming ability was measured. Left panel: representative images. Arrowheads indicate sphere formation. Right panel: quantification of number of spheres. Non-nicotine or nicotine CM: microglia cells were treated with PBS or nicotine (1 μM) for 24 h, and they were washed with PBS and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for 24 h (*n* = 4/group). Scale bar, 10 μm. (B) Effect of the human microglial CM on CSC markers, ESA⁺/CD44⁺, and ALDH activity were examined using FACS (*n* = 4/group). (C and D) Expressions of stemness genes in H2030BrM (C) and in PC9BrM cells (D) were examined after the treatment with or without nicotine-derived human microglial CM by qRT-PCR (*n* = 6/group). (E) Colony-forming abilities of H2030BrM and PC9BrM cells were measured in the presence or absence of the CM derived from nicotine-treated human microglia. Left panel: representative images. Right panel: quantification of number of colonies (*n* = 4/group). (F) Expressions of IGF-1 and CCR6 receptors in H2030BrM cells were examined after the treatment of cells with or without nicotine-derived human microglial CM by qRT-PCR (*n* = 4/group). (G) Effect of recombinant proteins, CCL20 and IGF-1, on sphere formation ability of H2030BrM was measured (*n* = 3/group). (H) H2030BrM cells were treated with CCL20 or IGF-1 for 24 h followed by measuring the ESA⁺/CD44⁺ stem cell population by FACS (*n* = 4/group). (I and J) H2030BrM cells were treated with or without 20 μM of CCL20 (I) or 1 μM of IGF-1 (J) for 24 h, and the expressions of stemness genes were examined by qRT-PCR and Western blot (*n* = 4/group). (K) Colony-forming assay was performed for the same set of samples as in G and H (*n* = 4/group). (L) Human microglia were treated with or without nicotine (1 μM) for 24 h, and their CM was prepared. The CM was then

added with anti-CCL20 antibody to deplete CCL20. H2030BrM cells were then treated with the CCL20-depleted CM followed by assaying the ESA⁺/CD44⁺ stem cell population by FACS ($n = 4/\text{group}$). **(M and N)** Metastatic lesions in the brain and serum were prepared from the mice used in Fig. 2 E. The protein expression of CCL20 in the brain (M) and in the serum (N) in each group was measured by ELISA ($n = 6/\text{group}$). **(O)** The protein expression of CCL20 in the serum of lung cancer patients with or without smoking history were measured by ELISA ($n = 10/\text{group}$). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

microglial CM. Although colony-forming ability increased in both groups (Fig. S3 J), the CSC population and expression of stem cell markers were significantly elevated only in cells treated with the nicotine-treated microglial CM (Fig. S3, K and L). Consistent with these results, treating the mouse lung cancer cell, LL/2, with CM derived from nicotine-treated mouse microglia significantly increased the levels of CSCs (Fig. S3 M) and promoted the colony-forming ability (Fig. S3 N) and tumor cell viability (Fig. S3 O). We also examined whether nicotine-induced brain metastasis is mediated via M2 microglial polarization by STAT3 activation. We blocked the M2 polarization of microglia by STAT3IC and examined the expression of *IGF-1* and *CCL20* by quantitative real-time PCR (qRT-PCR). We found that the nicotine treatment significantly up-regulated the *IGF-1* and *CCL20*, while STAT3IC reversed the effect (Fig. S3, P and Q). The CM was then added to the culture of LL/2 cells. We found that CM derived from microglia that were treated with both nicotine and STAT3IC significantly suppressed CSC population (Fig. S3 R) and tumor cell viability (Fig. S3 S).

Nicotine suppresses innate immune function of microglia

Microglia are known to exhibit phagocytic activity against brain tumor cells, like macrophages (Hutter et al., 2019). However, to escape from microglia-mediated phagocytosis, tumor cells express CD47 on their cell surface, which activates so-called “do not eat me” signaling. To investigate if nicotine affects this immune function of microglia, we measured the expression of signal-regulatory protein α (SIRP α) on microglia and CD47 on tumor cells. We found that the mRNA and protein expression of SIRP α were up-regulated in human microglia after nicotine treatment (Fig. 5 A). We also found that nicotine significantly increased the mRNA and protein expression of CD47 in both H2030BrM and PC9BrM cells (Fig. 5 B). Importantly, the expression of CD47 is up-regulated in brain metastasis of lung cancer patients with smoking history (Fig. 5 C). We then examined the effect of nicotine on phagocytic ability of microglia. We prelabeled H2030BrM cells with the fluorochrome-PKH26, a red-fluorescent vital dye that binds tightly to cell membranes (Munn and Cheung, 1990). The labeled cells were incubated with microglia that were treated with or without nicotine. We found that the size of PKH26⁺ tumor cells and microglia cells was similar at 0 h in the co-culture (Fig. 5 D). However, after 24 h of incubation, the small and irregular shape of PKH26⁺ cells was accumulated in the microglia. This result suggests that tumor cells were rapidly degraded by nicotine-treated microglia through phagocytosis. As shown in Fig. 5, D and F, both human and mouse microglial cells showed strong phagocytic activities toward the H2030BrM and LL/2 cells. Importantly, this phagocytic ability was greatly compromised in the presence of nicotine (Fig. 5, E and G). Blocking STAT3 signaling by STAT3IC

treatment suppressed nicotine-mediated down-regulation of phagocytic ability of microglia (Fig. 5 E). We also examined the expression of SIRP α in microglia of the nicotine-treated mouse brain represented in Fig. 2 E and found that the CD47 and SIRP α expression were strongly elevated in the brain of mice treated with nicotine (Fig. 5, H–J). These results suggest that nicotine promotes M1/M2 conversion and suppresses the innate immune function of microglia, which promotes brain metastasis progression.

PTL suppresses nicotine-induced M2 microglia polarization and enhances their phagocytic ability

Our results strongly suggest that blocking M2 polarization of microglia may be an effective therapeutic approach for brain metastasis. Therefore, we screened a natural compound library to identify compounds that block M2 macrophage polarization using an *Arg1* promoter reporter system. We screened a total of 103 compounds that are known to be blood–brain barrier (BBB) permeable. As shown in Fig. S4 and Fig. 6 A, we identified three compounds (>15-fold change, $P < 0.001$) that effectively suppressed the nicotine-induced activation of *Arg1* promoter at a concentration of 1 μM . We selected the top compound, PTL, which suppressed the *Arg1* promoter activity by >20-fold, for further study (Fig. 6 A). PTL is an abundant sesquiterpene lactone found in the medicinal herb Feverfew (*Tanacetum parthenium*). It has been used to treat inflammation and headache for centuries in Europe (Liu et al., 2018). To confirm the suppressive effect of PTL on nicotine-induced M2 microglial polarization, microglia were treated with nicotine in the presence or absence of PTL (Fig. S5 A), and expressions of M1 and M2 markers were examined. We found that PTL significantly decreased the nicotine-mediated M2 polarization of microglia (Fig. 6 B). Furthermore, the nicotine-mediated reduction of Iba1⁺/CD11b⁺ cells (M1) and elevation of Iba1⁺/CD206⁺ cells (M2) were significantly reversed by PTL (Fig. 6 C). Previous studies have shown that PTL modulated the JAK2/STAT3 signaling in response to cytokine stimulations (Liu et al., 2018; Sobota et al., 2000). We indeed found that PTL suppressed the nicotine-induced JAK2/STAT3 activation (Fig. 6 D), suggesting that PTL blocks the nicotine-induced M2 microglial polarization by targeting STAT3. Furthermore, the nicotine-induced suppression of phagocytic ability of microglia was restored when cells were treated with PTL (Fig. 6 E). The H2030BrM cells were treated with CM of microglia that were exposed to nicotine only or nicotine plus PTL. We found that the ESA⁺/CD44⁺ CSCs (Fig. 6 F) and colony-forming ability (Fig. 6 G) were significantly suppressed by CM of microglia treated with nicotine plus PTL. We also examined the mRNA expression of *CCL20* and found that the nicotine-mediated secretion of *CCL20* was down-regulated by the PTL treatment (Fig. 6 H). Next, to investigate the effect of PTL on

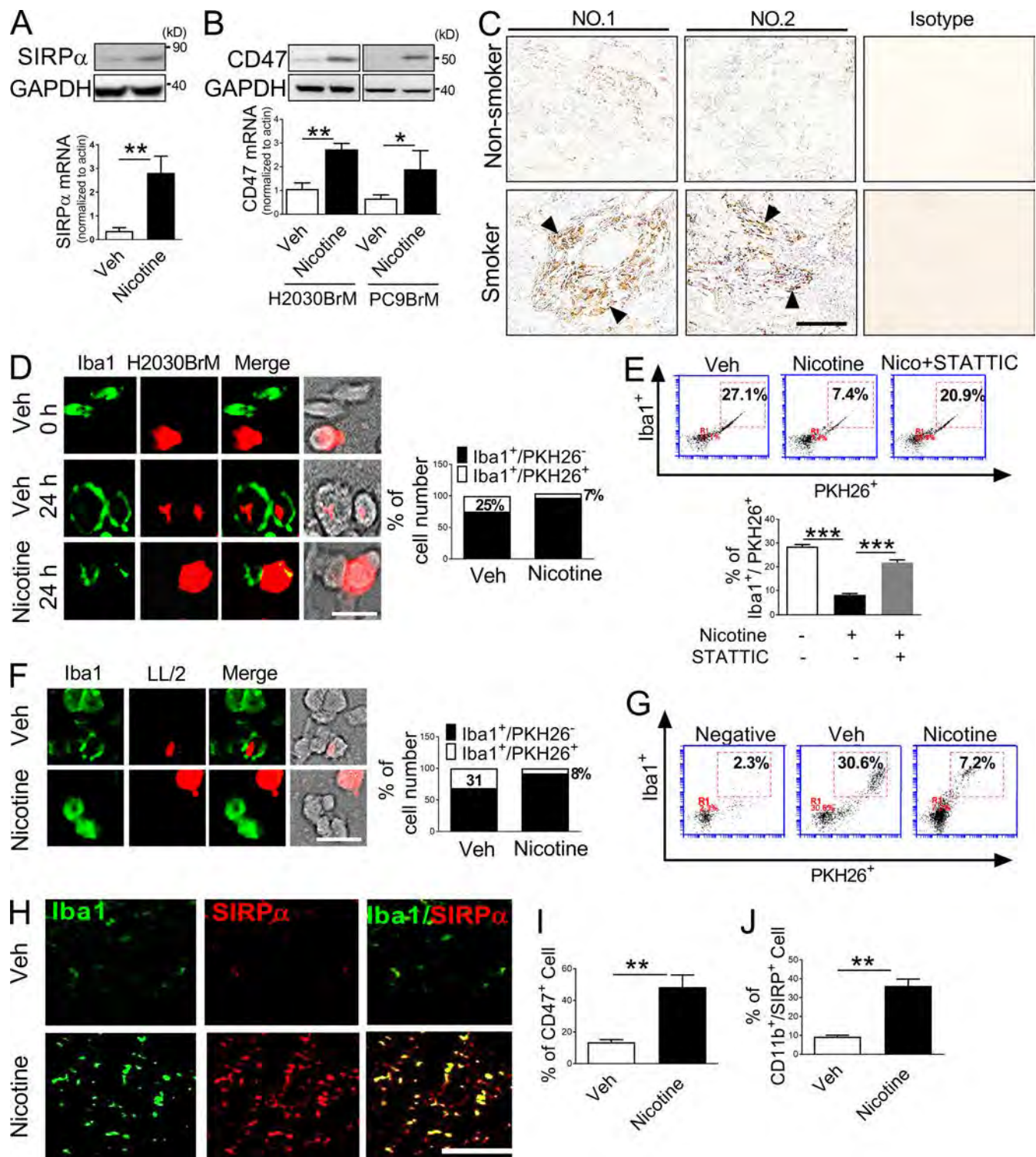


Figure 5. Nicotine suppresses innate anti-tumor immune function of microglia. (A) Human microglia cells (HMC3) were treated with or without nicotine (1 μ M) for 24 h, and the expression of SIRP α was examined by qRT-PCR and Western blot ($n = 4$ /group). (B) H2030BrM and PC9BrM cells were treated with or without nicotine (1 μ M) for 24 h, and the expression of CD47 was examined by qRT-PCR and Western blot ($n = 4$ /group). (C) Representative images of immunohistochemical analysis for CD47 $^{+}$ cells in the metastatic brain lesions of lung cancer patients with or without smoking history. Arrows indicate CD47 $^{+}$ cells. Non-smoker ($n = 4$) and smoker ($n = 7$). Scale bar, 50 μ m. (D) Phagocytic activities of HMC3 human microglial cells (green) with or without nicotine treatment were examined using PKH26-labeled H2030BrM cells (red). Both cells were incubated together for 0 or 24 h and photographed (left panels), and the number of microglia that phagocytosed tumor cells was counted to quantify microglial phagocytic activity as shown in the right panel ($n = 4$ /group). Scale bar, 10 μ m. (E) A parallel set of cells in D was analyzed by FACS to quantify phagocytic abilities of microglia after nicotine or STATTC treatment. Upper panels: representative FACS data. Lower panel: statistical analysis of the FACS data ($n = 4$ /group). (F) Mouse microglia (SIM-A9; green) were incubated with PKH26-labeled LL/2 mouse lung cancer cells (red) for 24 h. They were then photographed under a fluorescent microscope ($n = 4$ /group). Scale bar, 100 μ m.

(G) Phagocytic abilities of microglia with or without nicotine treatment were examined by incubating mouse microglia with PKH26-labeled LL/2 mouse lung cancer cells for 24 h, followed by analysis of the cells by FACS ($n = 4$). (H) The metastatic lesions in the brain of mice in Fig. 2 E were sectioned, and they were subjected to immunohistochemical analysis for SIRP α and Iba1. Representative immunohistochemistry images of the microglia are shown ($n = 9$ /group). Scale bar, 100 μ m. (I and J) The metastatic lesions in the brain of mice in Fig. 2 E were subjected to FACS analysis for CD47 $^{+}$ tumor cell (I) and SIRP α /CD11b $^{+}$ (J) microglia cells ($n = 9$ /group). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

nicotine-related brain metastasis in vivo, wild-type mice (C57BL/6 and BALB/c) were transplanted with LL/2 mouse lung cancer cells followed by administering nicotine (1 mg/kg) only or nicotine plus PTL (1 mg/kg) via an intraperitoneal injection. As shown in Fig. 6, I–K (BALB/c) and Fig. S5, B and C (C57BL/6), PTL treatment significantly suppressed nicotine-mediated brain metastasis and prolonged the brain metastasis-free survival (Fig. 6 L). To examine whether the PTL treatment also reverses microglia polarization in the brains of these mice, we performed flow cytometry (FACS) analysis of mouse brain and stained brain sections for M1 and M2 markers. A significant increase in Iba1 $^{+}$ /F4/80 $^{+}$ (M1 marker) and decrease in Iba1 $^{+}$ /CD206 $^{+}$ (M2 marker) were observed in brain microglia of PTL-treated mice (Fig. 6, M and N; and Fig. S5, D and E). To validate our results in human cell line, we used the xenograft model of H2030BrM cell and tested the efficacy of PTL. As shown in Fig. S5, F–K, PTL significantly suppressed the nicotine-induced brain metastasis and M2 microglia polarization. PTL treatment did not affect body weight of mice and showed no detectable liver toxicity (Fig. S5 L). We also examined the direct effect of PTL on cell viability and CSC population in vitro (Fig. S5 M). Our results showed that PTL suppressed the abundance of CD44 $^{+}$ /ESA $^{+}$ CSC and cell viability at the concentration of 10–20 μ M, but not at 1 μ M, which is the dose used throughout our in vitro experiments (Fig. S5, N and O), indicating that the anti-tumor effect of PTL is not mediated by direct cytotoxic effect, and it is rather mediated by the effect on microglia. Furthermore, PTL significantly suppressed stemness promoting effect of nicotine on microglia (Fig. S5 P) and the expression of stemness-related genes (Fig. S5, Q and R). These results suggest that PTL suppresses nicotine-related brain metastasis of lung cancer by skewing polarity of M1 microglia and up-regulating their phagocytic ability.

Discussion

The incidence of brain metastasis among lung cancer patients is 10–36% (Bacha et al., 2018; Barnholtz-Sloan et al., 2004; Schouten et al., 2002), although these data are considered to be underestimated (Bacha et al., 2018; Schouten et al., 2002). The majority of the lung cancer patients are diagnosed with advanced stage disease, most with stage IV cancer. More than 90% of lung cancer deaths are attributed directly to the metastatic disease and include those brain metastases (Ali et al., 2013; Kakusa et al., 2018). Cigarette smoking is a major risk factor for lung cancer (Lee et al., 2012), and our previous study showed a negative impact of smoking on lung cancer brain metastasis (Shenker et al., 2017). In the present study, we have found a significantly higher incidence of brain metastasis among current smokers with stage IV lung cancer.

Nicotine is not a carcinogen, but it causes addiction by activating nACh receptor, which is abundantly expressed by various

brain cells including microglia. Direct tumor promoting effects of nicotine have been previously reported (Schaal and Chellappan, 2014; Sun et al., 2017; Wang et al., 2017; Yoneyama et al., 2016); however, how nicotine promotes tumor by activating TME is still poorly understood. The current report is the first to show the critical impact of nicotine on brain TME to promote brain metastasis by skewing microglia to M2 phenotype and suppressing their role in innate immunity. Our results indicate that the phenotypic conversion of microglia by nicotine is mediated through the nACh receptor–STAT3 pathway, and that inhibition of STAT3 blocked the M2 conversion, suppressed the secretion of pro-tumor M2 cytokines, and reactivated their phagocytic activity toward tumor cells (Fig. 6 O).

Previous studies showed that microglial cells and macrophages in the brain are derived from two different sources, parenchymal resident microglia and monocytes/macrophages that enter the brain from the bone marrow (Wu and Watabe, 2017). Although microglia and macrophage cells show similarity in their surface markers and physiological functions, recent studies have demonstrated that microglia and macrophages are two distinct myeloid populations with different developmental origins (Gomez Perdiguero et al., 2015; Prinz and Priller, 2014; Schulz et al., 2012). Microglial cells originate from erythromyeloid progenitors that begin on embryonic day (E) 7.5–E8.0 in the blood islands of the yolk sac. Until E9.5, erythromyeloid progenitors migrate to the developing central nervous system and mature into microglia (Gomez Perdiguero et al., 2015; Prinz and Priller, 2014; Schulz et al., 2012). These early microglial cells reside in the brain throughout life and are thought to sustain the local microglial population. In contrast, macrophages originate from the hematopoietic stem cells that start in the aorta–gonad–mesonephros region at E10.5, and then in the fetal liver at E12.5. After the postnatal stage, macrophages are produced from monocytes in the bone marrow (Gomez Perdiguero et al., 2015; Prinz and Priller, 2014; Schulz et al., 2012). Furthermore, recent studies demonstrated that microglial cells and macrophage have distinct and specific surface antigens (Feng et al., 2015). Studies on CX3CR1 $^{+}$ /GFP/CCR2 $^{+}$ /RFP knock-in fluorescent protein reporter mice revealed that microglial cells are CX3CR1 $^{+}$ /CCR2 $^{-}$ /CD45 $^{-}$, while the macrophages are CX3CR1 $^{-}$ /CCR2 $^{+}$ /CD45 $^{+}$ (Feng et al., 2015), strongly supporting the notion that microglial cells and macrophages are from different populations and they can be distinguished. Badie and Scharfner performed flow cytometric analysis and characterized the distribution of microglial cells and macrophages in experimental gliomas and found that microglial cells (CD11b/c ^{high} , CD45 ^{low}), mainly present at the site of tumor or tumor periphery, accounted for 13–34% of the tumor mass. In contrast, macrophages (CD11b/c ^{high} , CD45 ^{high}) were less prominent within the tumors or the tumor periphery and accounted for just

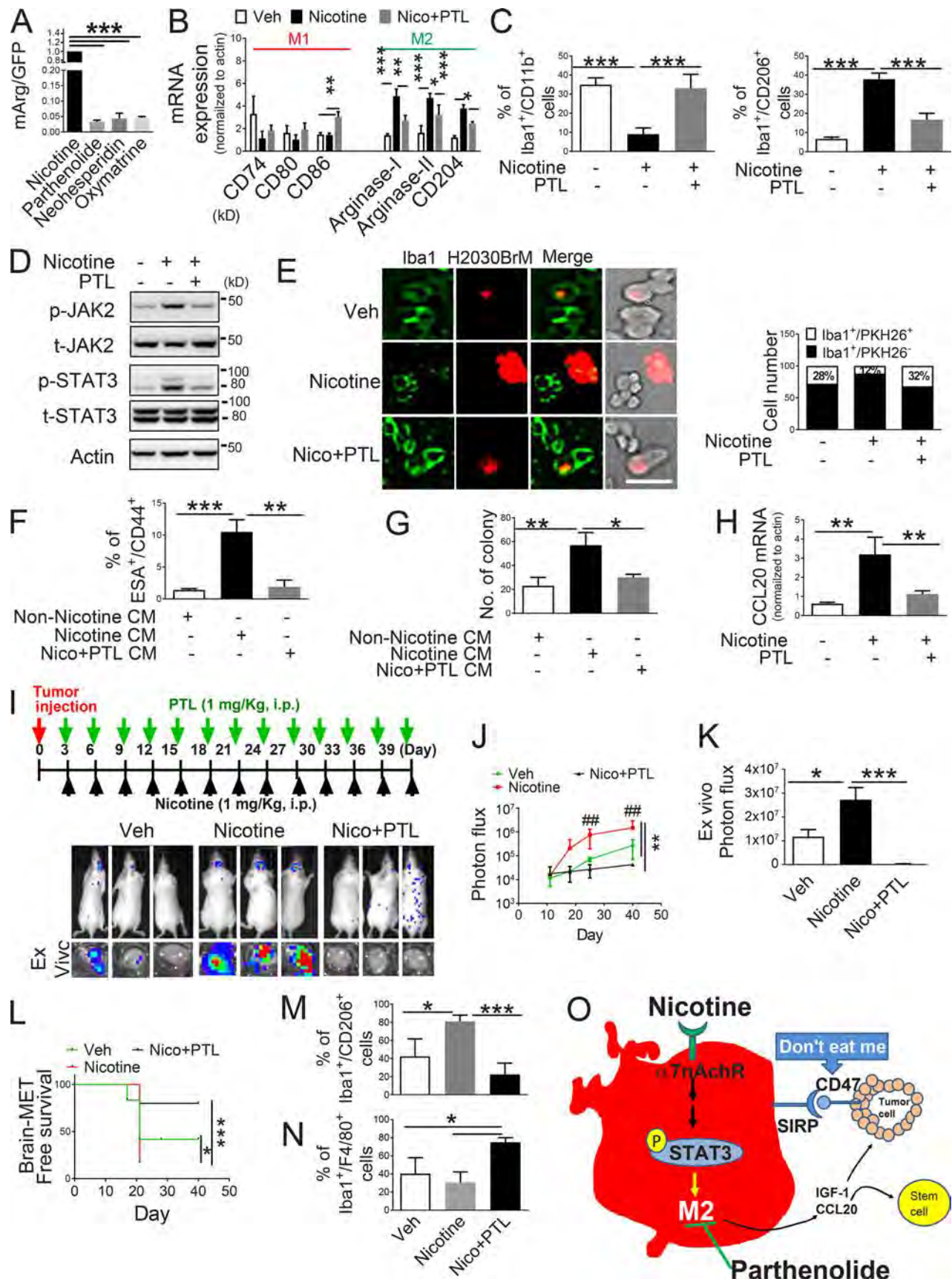


Figure 6. PTL suppresses brain tumor progression by blocking nicotine-induced M2 microglia polarization. (A) Human microglia cells (HMC3) with the Arg1 reporter plasmid were cultured in the presence or absence of compounds that were identified as the top three most effective inhibitors for Arg1 during our

initial screening (see Materials and methods). After 48 h of incubation, luciferase reporter activity was measured ($n = 4/\text{group}$). **(B)** Expression of surface markers of M1/M2 microglia was examined by qRT-PCR after microglial cells were treated with or without nicotine plus PTL ($n = 4/\text{group}$). **(C)** The same set of samples in B was evaluated for quantification of Iba1⁺/CD11b⁺ (M1) cells and Iba1⁺/CD206⁺ (M2) cells by FACS ($n = 4/\text{group}$). **(D)** The same set of samples in C was examined for quantification of the protein expression of JAK2 and STAT3 by Western blot. **(E)** Human microglial (HMC3) cells (green) with or without nicotine treatment (1 μM) in the presence or absence of PTL (1 μM) were incubated with PKH26-labeled H2030BrM cells (red) for 24 h and photographed (left panels), followed by measurement of the microglial phagocytic activity (right panel; $n = 4/\text{group}$). Scale bar, 10 μm . **(F)** CM was prepared from human microglia (HMC3) treated with or without nicotine and PTL. The CM was added to the culture of H2030BrM, and cells were incubated for 48 h followed by evaluation of CSC population by FACS. Non-nicotine, nicotine, or Nico+PTL CM: microglia were treated with PBS, nicotine, or nicotine plus PTL for 24 h. They were then washed twice with PBS and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for a further 24 h ($n = 4/\text{group}$). **(G)** For the same set of samples as F, colony-forming ability was also measured ($n = 4/\text{group}$). **(H)** Human microglia were treated with or without nicotine (1 μM) in the presence or absence of PTL for 24 h, followed by assessment of the expression of CCL20 by qRT-PCR ($n = 4/\text{group}$). **(I)** The mouse lung cancer cells, LL/2, were intracardially injected into wild-type BALB/c mice. After 3 d of intracranial transplantation of LL/2 cells, mice received nicotine (1 mg/kg) plus PTL (1 mg/kg) by an intraperitoneal injection every 3 d for 40 d. Upper panel: BLI images of representative mice from each experimental group at day 40. Lower panel: total photon flux of ex vivo brain metastatic lesions was measured by BLI at the endpoint (day 40; $n = 9/\text{group}$). **(J)** Quantitative data of BLI in the brain regions are shown ($n = 9/\text{group}$). **(K)** Ex vivo signals in the whole brains at the end point were quantified. **(L)** The Kaplan–Meier analysis of brain metastasis-free survival was performed ($n = 9/\text{group}$). **(M and N)** Metastatic brain tumors in I were isolated from the brain and were examined by FACS for M2 (M) and M1 (N) microglial polarization ($n = 9/\text{group}$). **(O)** A proposed model illustrating a nicotine-induced brain metastasis ($n = 9/\text{group}$). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

4.2–12% of the tumor mass (Badie and Schartner, 2000). These results also suggest that microglial cells play a key role in mediating brain tumor progression.

We have also found that nicotine dramatically increased the mRNA expression of $\alpha\beta 2$ receptor on microglia cell compared with macrophage. These results suggest that microglia is more responsive to nicotine compared with macrophage, resulting in increase in the incidence of brain metastasis. Furthermore, it was reported that the numbers of macrophage/microglia were significantly higher in the central nervous system than in the peripheral system after injury (Leskovar et al., 2000). In addition, the peak concentrations of three cytokines, TNF α , IL-1, and IL-6, appeared earlier and were significantly higher in the injured central nervous system than peripheral system (Leskovar et al., 2000), suggesting differences in the reparative responses of macrophage/microglia in the PNS and central nervous system to injury.

In this study, we showed that blocking microglia CSF1R by PLX3397 significantly suppressed nicotine-mediated brain metastasis, suggesting that cancer cells lose growth support function from M2 microglia. Previous studies showed that PLX3397 reprogrammed tumor-infiltrating macrophages, enhanced antigen presentation (Zhu et al., 2014), and suppressed TAM infiltration in the tumor (Butowski et al., 2016; Cannarile et al., 2017; Cuccarese et al., 2017). We also found that PLX3397 skewed the population of microglia/macrophage to M1 phenotype. Therefore, while blocking the proliferation of microglia/macrophage, PLX3397 also promotes the residual microglia's innate immune function to tumor cells. Our results indicate that nicotine increased the secretion of IGF-1 and CCL20 in the CM of microglial cells. IGF-1 is known to enhance tumor cell survival and proliferation via activation of the PI3K-Akt signaling (Park et al., 2016; Zhu et al., 2011). However, the role of CCL20 on tumor progression has not yet been well defined. CCL20 is a critical chemoattractant responsible for the recruitment of inflammatory immune cells (Nandi et al., 2014). The expression of CCL20 has been shown to be overexpressed in a variety of tumors including lung cancer (Liu et al., 2016; Wang et al., 2015, 2016). In pancreatic cancer, CCL20 has been found to be

overexpressed in metastatic tumor tissues (Liu et al., 2016). It was reported to promote epithelial-mesenchymal transition and invasive ability of tumor cells by activating the MAPK and PI3K pathways and matrix metalloproteinase signaling (Marsigliante et al., 2016; Wang et al., 2016). Previous studies showed that CCL20 promotes cell proliferation through activation of MAPK pathway (Lu et al., 2017) and up-regulating transcription of c-Fos and c-Myc. Others have shown that CCL20 enhanced the chemotherapy resistance of triple-negative breast cancer by promoting ALDH⁺ population of breast cancer cells through PKC ζ - and p38 MAPK-mediated NF- κ B activation (Chen et al., 2018). We found that stem cell genes NANOG and SOX2 were up-regulated in CCL20-treated cells, suggesting that CCL20 promoted the stemness of lung cancer brain metastatic cells via NANOG/SOX2. One of the major sources of CCL20 is a tumor-associated macrophage, especially M2 macrophage (Liu et al., 2016). In this study, we found that nicotine strongly promoted phenotypic conversion of microglia to M2 and enhanced secretion of CCL20 to stimulate tumor cell growth and cancer cell stemness. The depletion of microglia in our animal model significantly reversed the effect of nicotine on brain metastasis. Furthermore, we showed that the effect of CCL20 on tumor stem cell was mediated by up-regulation of SOX2 and NANOG genes. These results suggest that CCL20 may serve as a novel therapeutic target and biomarker for brain metastasis of lung cancer.

SIRP α is expressed predominantly in myeloid and neuronal cells (Adams et al., 1998; Kharitononkov et al., 1997). Normal cells express CD47 that interacts with SIRP α on macrophage/microglia to suppress their phagocytic activity. Several studies have shown that the expression of SIRP α is also associated with polarization of microglia/macrophage (Pan et al., 2013). Furthermore, knockdown of SIRP α in macrophages increased the production of M1 cytokines in the tumor environment (Pan et al., 2013). Blocking SIRP α also triggered the Akt survival signal in macrophages and enhanced macrophage migratory activities toward tumors (Pan et al., 2013), suggesting that SIRP α also affects microglia's function. In this study, we found that the nicotine increased the expression of SIRP α on microglia, while nicotine also promoted the expression of CD47 on the tumor

cells, resulting in suppression of phagocytic ability of microglia. There are two possible mechanisms that are involved in the anti-tumor effect of microglia. One is direct internalization of tumor cells into the lysosome of microglia by phagocytosis. The other possibility is that tumor cells are first killed by cytotoxic factors released by microglia followed by phagocytosis of the apoptotic debris by microglia. In addition, it is noteworthy that the CD47-SIRP α signal was previously found to modulate T cell differentiation and regulate T cell priming to the tumor site (Liu et al., 2015b; Zhang and Petro, 1996). Therefore, the CD47-SIRP α signal that was activated by nicotine plays a pivotal role in modulating anti-tumor innate as well as adaptive immune responses in TME.

By screening a BBB permeable natural compound library, we identified PTL as a potent inhibitor for nicotine-induced M2 conversion of microglia. PTL is an abundant sesquiterpene lactone found in the medicinal herb Feverfew (*T. parthenium*). It has been used for centuries as a traditional medicine to treat arthritis, fever, and headache (Liu et al., 2015a; Rummel et al., 2011). PTL was also previously shown to exhibit direct tumor suppressive activity (Talib and Al Kury, 2018) by modulating multiple pathways, including p53, vascular endothelial growth factor (Talib and Al Kury, 2018), B-Raf/MAPK/Erk (Lin et al., 2017), and STAT3 pathways (Carlisi et al., 2011; Skoumal et al., 2011; Song et al., 2014). However, these direct tumor cytotoxic effects mediated by PTL require a relatively high dose, typically >10 μ M. In this study, we found that sub-micromolar dose (<1 μ M) of PTL effectively suppressed nicotine-induced polarization of microglia to M2 phenotype and enhanced its M1 phenotype. On the other hand, the low concentration (1 μ M) of PTL did not affect the population of CSC or tumor cell growth, which strongly suggests that the anti-tumor effect of PTL on nicotine-promoted brain metastasis shown in our study is mediated via modulation of microglial function and not by direct effect of PTL on tumor cells. Importantly, PTL was shown to be able to cross the BBB due to high membrane permeability (Könczöl et al., 2013). Considering the inhibitory effect of PTL on nicotine-induced microglial activation and their minimum toxicity at the effective dose, PTL could be used for the prevention and treatment of brain metastasis, particularly for patients with past and current smoking history.

Nicotine is highly addictive and considered as the major obstacle for smoking cessation (Henningfield et al., 1991). Unfortunately, many cancer patients have difficulty in quitting smoking even after their diagnosis due to nicotine addiction. E-cigarette, nicotine patch, and nicotine gum are nicotine replacement therapies for smoking cessation used by these patients (Hartmann-Boyce et al., 2018; Stead et al., 2012). Although nicotine, per se, is not a carcinogen, many reports document its tumor-promoting effects (Li et al., 2017; Schaal and Chellappan, 2014; Schaal et al., 2018) as well as its pro-inflammatory effect, which suppresses the phagocytosis ability of the alveolar macrophages (Scott et al., 2018). Considering our results that clearly demonstrated profound and long-term effects of nicotine on brain metastasis progression, extra caution should be paid in the use of nicotine for smoking cessation.

Materials and methods

Human subjects

We used a cohort of patients with a diagnosis of nonsmall cell lung cancer with or without brain metastases treated at Wake Forest Baptist Comprehensive Cancer Center between May 2015 and April 2018. We collected a total of 281 stage IV nonsmall cell lung cancer patients with or without a history of smoking. Among them, 79 patients were diagnosed with brain metastasis. 59 patients were current smokers at the time of diagnosis and treatment. We defined the “never smokers” ($n = 44$) as those who have no smoking history and those who had smoked <10 pack/year but stopped smoking. The “former smokers” ($n = 181$) are defined as those who smoked >10 pack/year but quit smoking in the past. Patient characteristics included histology status, smoking history, pack/years, progression status, and date of death. Patient information about age, gender, cancer type, and smoking status are described in Fig. S1. All samples used in this study were obtained under the approval of the institutional review boards of Wake Forest School of Medicine Institutional and Wake Forest Baptist Comprehensive Cancer Center. Written informed consent was obtained from all participants.

Cell culture and reagents

Human lung cancer cell lines, H2030BrM and PC9BrM, were kind gifts from J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY). H2030BrM and PC9BrM cell lines were authenticated by using GenePrint 10 STR System (Promega, B9510). SIM-A9 was obtained from Kumi Nagamoto-Combs, through Kerafast.com. HMC3 and LL/2 cells were purchased from American Type Culture Collection (ATCC) and were authenticated by ATCC. The H2030BrM and PC9BrM were cultured in RPMI supplemented with 10% FBS, streptomycin (100 mg/ml), and penicillin (100 U/ml). LL/2 cells were cultured in DMEM medium supplemented with 10% FBS. SIM-A9 and HMC3 were cultured in DMEM/F12 medium supplemented with 5% FBS. All cells were grown at 37°C under 5% CO₂. All cell lines were obtained between 2010 and 2016, and they were ensured to be negative of mycoplasma. The HMC3 and SIM-A9 cells were seeded in a 10-cm dish. After reaching 70% confluence, cells were incubated with or without 1 μ M nicotine in DMEM/F12 medium supplemented with 2% FBS or in medium containing nicotine plus the STAT3 inhibitor STATTIC (0.5 μ M). After 24 h, cells were washed twice with PBS and then incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for 24 h. The CM harvested from the cell culture were centrifuged at 300 g for 10 min to remove the cells and stored at -80°C. To investigate the effect of nicotine-derived CM on tumor progression, we harvested CM from microglial cells that were treated with or without nicotine, followed by treatment of tumor cells with the CM. All cell lines were tested by using universal mycoplasma detection kit (ATCC, 30-1012k, lot 70008746).

Animal models

Animals were treated in accordance with the U.S. National Institutes of Health Animal Protection Guidelines, and the protocol was approved by the Wake Forest Baptist Health Institutional

Animal Care and Use Committee. Female nude, BALB/c, and C57BL/6 mice of 5–6 wk of age were used. The mice were housed (four to five per cage) at a stable temperature ($24 \pm 1^\circ\text{C}$), in a 12-h light/dark cycle, and with unrestricted access to food and water. Several studies showed that human lung adenocarcinomas cell H2030BrM and mouse Lewis lung carcinoma cell LL/2 exhibited their highly metastatic abilities in the nude, C57BL/6, and BALB/c mice, respectively (Chen et al., 2016; Janker et al., 2018; Kamei et al., 2009; Malladi et al., 2016; Nguyen et al., 2009; Scholar et al., 1989). Actively growing luciferase-labeled H2030BrM and LL/2 cells were intracardially injected at a concentration of 2×10^5 H2030BrM cells or 5×10^4 LL/2 cells in 100 μl PBS into the left cardiac ventricle. To confirm a successful injection, the photon flux from whole body of the mice was immediately measured using IVIS Xenogen bioimager (Caliper). To examine the effect of nicotine, the mice received nicotine (1 mg/kg) by intraperitoneal injection every 3 d. To examine the role of microglia on nicotine-related brain metastasis, the mice were administered PLX3397 (20 mg in 7 μl , Selleck Chemicals USA), a selective inhibitor of CSF1R (also known as macrophage colony-stimulating factor receptor), which reduces viability of microglial cells. To avoid whole-body effect, the PLX3397 was directly injected into the right mouse brain after 1 wk of transplantation of mouse lung cancer cell LL/2. Mice were divided into four groups: (1) tumor transplant only, (2) tumor transplant plus nicotine, (3) tumor transplant plus PLX3397, and (4) tumor transplant plus PLX3397 and nicotine. To examine the effect of natural compound (PTL) on modulation of microglial cell polarization, the mice were administered PTL (1 mg/kg) plus nicotine by intraperitoneal injection. Mice were divided into three groups: (1) tumor transplant only, (2) tumor transplant plus nicotine, and (3) tumor transplant plus PTL and nicotine. Tumor growth was monitored by bioluminescence until day 60. For bioluminescent imaging, the mice were injected with D-luciferin intraperitoneally (100 mg/kg), followed by capturing images every week using IVIS Xenogen bioimager. The brain metastasis was monitored, and the luminescence was quantified once per week. Finally, whole brain was removed and incubated in PBS with 0.6 mg/ml luciferin for 5 min, and photon flux was measured by an in vivo image system (IVIS).

Intracranial implantation

Mice were anesthetized with a ketamine/xylazine (90–120/7–10 mg/kg) mixture and fixed into a stereotactic head frame (Kopf stereotactic frame). A 1.5-mm bur hole was drilled 1 mm anterior to the coronal suture on the right hemisphere and 2 mm lateral from the midline. A Hamilton syringe fixed onto the head frame was used to inject LL/2 (2×10^5 cells/5 μl) into the right frontal lobe of the brain. The skin incision was then closed with 4–0 silk thread. The ketoprofen (2 mg/kg) was given to the mice to reduce pain. After 1 wk of tumor transplantation, 36 mice were randomly divided into four groups: (1) tumor transplant only (control group), (2) tumor transplant plus nicotine, (3) tumor transplant plus PLX3397, and (4) tumor transplant plus PLX3397 and nicotine. The PLX3397 and PLX3397 plus nicotine groups received PLX3397 (20 mg in 7 μl) treatment by intracranial injection every 2 wk at the same area of tumor transplantation. The control group received the same volume of PBS.

Arg1 gene promoter reporter assay and natural compound screening

To examine the nicotine-induced M2 microglial polarization and examine the effect of natural compounds on microglial polarization, SIM-A9 cells were first infected with lentivirus containing GFP gene, and GFP⁺ cells were sorted by flow cytometry (FACS). The GFP⁺ SIM-A9 cells were seeded in 96-well plates for 1 d and were transfected with the Arg1 promoter reporter plasmid (Addgene) using Lipofectamine 2000 (Invitrogen). After 24 h, the cells were treated with only nicotine or nicotine plus natural compound in presence of 2% FBS and cultured for another 24 h. Cells were washed twice and were treated with D-luciferin for 5 min. The expression of luciferase was detected by using an IVIS Xenogen bioimager. For Arg1 promoter luciferase normalization, the photon flux was divided by GFP signal, which was measured by a Multi-Mode Reader (Biocompare). Each experiment was conducted a minimum of three times.

qRT-PCR

For qRT-PCR, the treated cells were washed with PBS and lysed using the TRIzol reagent. The isolated total RNA was quantified by a NanoDrop 2000 (Thermo Fisher Scientific). Then, RNA was reverse transcribed to cDNA using a Reverse Transcription Superscript kit (Bio-Rad). The cDNA was then amplified with a pair of forward and reverse primers for the following genes: hCD74-F 5'-GCGACCTTATCTCCAACAATG-3', hCD74-R 5'-GTCCTCCAGTTCCAGTGACT-3', hCD80-F 5'-CTCTTGCTGGCTGGCTGCTTT-3', hCD80-R 5'-GCCAGTAGATGCGAGTTTGTGC-3', hCD86-F 5'-CCATCAGCTTGTCTGTTTCATTCC-3', hCD86-R 5'-GCTGTAATCCAAGGAATGTGGTC-3', hArginase-1-F 5'-GCGGGCGGACGC TGGCG-3', hArginase-1-R 5'-GCAGCGGGACCATGTCCACTC-3', hArginase-2-F 5'-AGACGCGAGTGCATTCCATC-3', hArginase-2-R 5'-CACTAATGGTACCGATTGCCA-3', hCD204-F 5'-GGTTTCAATTGTAAGAGAGAGAAG-3', hCD204-R 5'-CTGAGCAATTCTTCGTTTCCC-3', mCD74-F 5'-AGAGCCAGAAAGGTGCAGC-3', mCD74-R 5'-GATGCATCACATGGTCTCTGG-3', mCD80-F 5'-ACGTATTGGAAGGAGATTACAGCT-3', mCD80-R 5'-TCTGTACAGCTTACTATCCCGC-3', mCD86-F 5'-TTACGGAAGCACCCACGATG-3', mCD86-R 5'-CCTGTTACATTCTGAGCCAGT-3', hCHR NA7-F 5'-CGTGGATGAGAAGAACCAAG-3', hCHR NA7-R 5'-CCATTGGGGATATAGCCACT-3', hSOX2-F 5'-GGGAAATGGGAGGGGTGCAAAAGAGG-3', hSOX2-R 5'-TTGCGTGAGTGTGGATGGATTGGTG-3', hOCT4-F 5'-GCTGGAGAAGGAGAAGCTGGAGC-3', hOCT4-R 5'-GCTAAGCTGCAGAGCCTCAAAGC-3', hNanog-F 5'-CTTGGAAGCTGCTGGGGAAG-3', hNanog-R 5'-GATGGGAGGAGGGGAGAGGA-3', hCD47-F 5'-GCTAAAATATCGTGTGTTTTCATGGTTTTC-3', hCD47-R 5'-GCTGTACTAAACACATAGTAGTGAAGT-3', hSIRP-F 5'-GCCTTAGTCGTTCCGCCGCG-3', hSIRP-R 5'-GCCGCTTGGCCCTACTCCT-3', hIGF-1-F 5'-GCTGTGCCTGCTCACCTTCACCAG-3', hIGF-1-R 5'-GCCTCCTTAGATCACAGCTCCGGAAG-3', hCCL20-F 5'-TCTCGCCTCCAGCATGAAAG-3', and hCCL20-R 5'-CAGATCTCCTTGGCCACAATG-3'.

PCR reactions were performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and CFX Connect (Bio-Rad). The thermal cycling conditions composed of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of PCR using the following profile: 94°C , 30 s; 60°C , 30 s; and 72°C , 30 s.

Immunocytochemistry and immunofluorescence

The human brain metastasis tissues of lung cancer were obtained from Wake Forest Baptist Comprehensive Cancer Center Tumor Tissue and Pathology Shared Resource. Tissue sections were stained using anti-CD206 (1:200, R&D Systems) for M2 microglia and anti-ionized calcium-binding adapter molecule-1 (Iba1; 1:1,000, Abcam) for microglia and CD45 (1:500, Abcam) for macrophage. Brain sections were then incubated with appropriate biotin-conjugated secondary antibodies and diaminobenzidine as the substrate. As a control, isotype antibodies were used. To examine the expression of SIRP α on microglia, 10- μ m-thick frozen mouse brain sections were stained using rabbit anti-Iba1 (1:1,000; Abcam) for microglia and anti-SIRP α (1:200) (Cell Signaling). Brain sections were incubated for 1 h in a PBS solution containing 0.1% Triton X-100 and 3% bovine serum albumin at room temperature, and then transferred to a solution containing anti-SIRP α antibody overnight at 4°C. The secondary antibody conjugated with fluorescent dye Alexa Fluor 594 (1:1,000, Invitrogen) was used to detect the expression of SIRP α . Sections were then incubated overnight at 4°C in a solution containing anti-Iba1 antibodies. Antibodies against rabbit IgG conjugated with fluorescent dye Alexa Fluor 488 (1:1,000, Invitrogen) were used to detect the microglial cells. Fluorescence images were acquired using a microscope (BZ-X710, Keyence) connected to a computer equipped with imaging software (BZ-X Viewer). Appropriate isotype antibodies were used as controls. To determine the area of Iba1⁺ and CD206⁺ cells in brain metastasis, we chose three randomly selected fields in each tumor and measured staining intensity after taking images followed by using the Image-Pro software. We also chose three nontumor areas in the corresponding slide and measured intensity as background, and this background was subtracted from the intensity in the tumor area. The average intensity of three fields was used as the score of that patient. Therefore, each patient has a single score. We then divided the score range by setting the lowest score as 0 and the highest to be +3.

Western blot

Cells were homogenized in the radioimmunoprecipitation assay buffer and then centrifuged at 17,000 *g* for 30 min at 4°C. The protein concentrations of the supernatants were determined by NanoDrop 2000. Supernatants (30 μ g of total protein) were mixed with sample buffer containing 0.5 M dithiothreitol, heated to 80°C for 10 min, loaded into each well of 10% polyacrylamide gel, and resolved at 120 V for 2 h. The separated proteins bands were transferred to a polyvinylidene fluoride membrane, blocked with 5% milk, and probed with respective primary antibodies: total-STAT3 (1:1,000, Cell Signaling), phospho-STAT3 (1:1,000, Cell Signaling), GAPDH (1:10,000, Cell Signaling), SOX2 (1:500, Cell Signaling), OCT4 (1:1,000, Cell Signaling), NANOG (1:500, Cell Signaling), and SIRP α (1:1,000, Cell Signaling). The bound antibodies were detected using an enhanced chemiluminescence detection kit (GE Healthcare Lifescience). The band intensities were measured using an imaging system (Amersham Imager 600; UVP) and analyzed using ImageJ (1.43u; <http://rsb.info.nih.gov/ij/>). For gel loading control, membranes were incubated with monoclonal GAPDH antibody.

CCL20 ELISA

Mouse brain specimens were homogenized in radioimmuno-precipitation assay lysis buffer and centrifuged at 13,000 *g* for 30 min at 4°C. The protein concentrations of the supernatant were measured and adjusted to 1 mg/ml. The human serum of lung cancer patients was obtained from FOX CHASE Cancer Center. The serum was further diluted fourfold in assay buffer. The levels of CCL20 in the mouse brain supernatants and mouse and human serum were determined using mouse and human CCL20 ELISA kits (Sigma-Aldrich). Briefly, 96-well plates were first coated with anti-CCL20 monoclonal antibodies followed by addition of 100 μ l of the supernatants and serum under investigation or the same volume of the CCL20 standards. The plate was incubated at 4°C overnight on a rocking platform. After washing the plates, the detection antibody and streptavidin solution were added to each well. After incubation, the 3,3',5,5'-tetramethylbenzidine (TMB) and stop solution were added to each well. Finally, the plates were examined at an absorbance wavelength of 450 nm. Standard curves were obtained from values generated from known concentrations of mouse and human CCL20 provided by the kits.

IGF-1 ELISA

Microglial cells (HMC3) were treated with or without nicotine (1 μ M) plus STATTIC (0.5 μ M) for 24 h. Cells were washed with PBS twice and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for an additional 24 h. The human monocyte/macrophage cells were treated with or without nicotine for 24 h. CM was collected and analyzed by the human IGF-1 ELISA kit (Sigma-Aldrich). Briefly, 96-well plates were first coated with anti-IGF-1 monoclonal antibodies followed by addition of 100 μ l of the microglia conditional medium or the same volume of the IGF-1 standards. The plate was incubated at 4°C overnight on a rocking platform. After washing the plates, the detection antibody and streptavidin solution were added to each well. After incubation, the TMB and stop solution were added to each well. Finally, the plates were examined at an absorbance wavelength of 450 nm. Standard curves were obtained from values generated from known concentrations of mouse and human IGF-1 provided by the kit.

Phagocytosis assay

H2030BrM and LL/2 cells were labeled with PKH26 dye (Sigma-Aldrich). The labeled cells were then washed three times and cultured overnight to reduce nonspecific leaking of dye during the assay. Tumor cells were 95% viable after the incubation. Labeled tumor cells were mixed with microglia that were pretreated with or without nicotine. Co-cultured tumor and microglia cells in the culture slides were harvested after 24 h and fixed with 4% paraformaldehyde. Microglial cells were counterstained with anti-Iba1 antibody overnight. Secondary antibody conjugated with the fluorescent dye Alexa Fluor 488 (1:1,000, Invitrogen) was used to detect the microglia. The phagocytic activities were measured by immunofluorescence microscope and FACS. For quantification of phagocytosis, the phagocytic percentage was calculated as $100 \times (\text{percent Iba1}^+ /$

PKH26⁺ cells/[percent Iba1⁺/PKH26⁺ cells + percent Iba1⁺/PKH26⁺ cells]).

Statistical analysis

Data are represented as mean $\pm$ SD. Significance was set at $P < 0.05$. The nicotine effect on M1/M2 gene expression, effect of nicotine-derived microglial CM on tumor growth, and effect of nicotine on microglia phagocytosis ability were analyzed using the unpaired Student's *t* test. One-way ANOVA was used to analyze the effect of nicotine, PTL3397, or STAT1C treatment on M1/M2 gene polarization and tumor progression, the effect of different concentrations of IGF-1 or CCL20 on cancer progression, the effect of different natural compounds on M2 gene polarization, and the effect of nicotine or PTL treatment on M1/M2 gene polarization and cancer cell progression and nicotine effect in animal studies. Significance between groups was represented as *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Online supplemental material

Fig. S1 shows the effect of smoking and nicotine on brain metastasis. Fig. S2 demonstrates that the nicotine promotes brain metastasis in a mouse model. Fig. S3 shows that nicotine treatment of microglia increases secretion of CCL20 and IGF-1 followed by promotion of tumor cell stemness. Fig. S4 shows the effect of natural compounds on nicotine-mediated activity of Arg1 reporter. Fig. S5 shows PTL suppressed nicotine-promoted brain metastasis in syngeneic mouse model.

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Supplemental material

	Adenocarcinoma	Non-adenocarcinoma
Age	62.78	65.21
Male gender (%)	94 (33%)	60 (21%)
Smoking status		
-Never/Former	149	73
-Current	43	16
Pack years		
<20	81	23
20-60	88	38
>60	23	28

Figure S1. **Effect of smoking and nicotine on brain metastasis.** Patient profile and their smoking history.

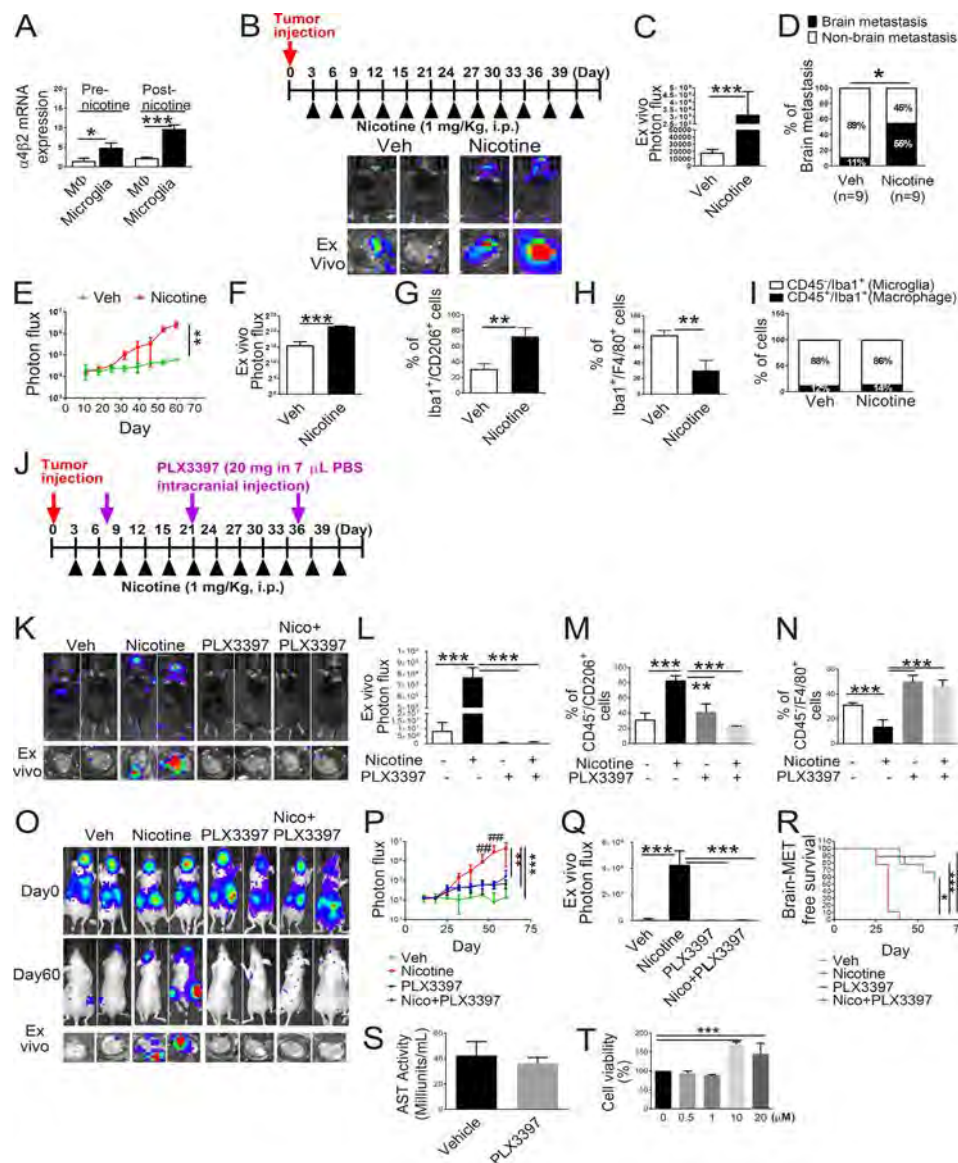


Figure S2. Nicotine promotes brain metastasis in mouse model. (A) Expression of the $\alpha 4 \beta 2$ nicotine receptor on human microglia (HMC3) and human monocyte/macrophage (THP-1) were evaluated before and after nicotine treatment by qRT-PCR ($n = 4$ /group). (B) Mouse lung cancer LL/2 cells were intracardially injected into wild-type C57BL/6 mice ($n = 9$ /group). After 3 d of intracardiac transplantation of LL/2 cells, mice received nicotine (1 mg/kg) treatment by intraperitoneal injection every 3 d. Upper panels are BLI images of brain metastatic lesions of representative mice from each experimental group at day 40. The lower panels represent the total photon flux of ex vivo brain metastatic lesions as measured by BLI at the endpoint (day 40). (C) Quantitative data of in vivo brain metastasis of lung cancer ($n = 9$ /group). (D) Percentage of brain metastasis of lung cancer in C57BL/6 mice with or without nicotine treatment ($n = 9$ /group). (E and F) Quantitative data of in vivo brain metastasis of lung cancer (left panel) and ex vivo signals in the brain at the end point (right panel) of the experiment conducted in Fig. 2 B ($n = 9$ /group). (G and H) Metastatic brain tumor tissues were dissociated, and the nicotine was examined on Iba1⁺/CD206⁺ (G) or Iba1⁺/F4/80⁺ cells (H) by FACS ($n = 9$ /group). (I) Metastatic brain tumor tissues in Fig. 2 B were dissociated, and the Iba1⁺/CD45⁻ (microglia) and Iba1⁺/CD45⁺ (macrophage) were measured and plotted in relation to the nicotine treatment ($n = 9$ /group). (J) Scheme of the experimental design. After 3 d of intracranial transplantation of LL/2 cells, the mice received nicotine (1 mg/kg) by intraperitoneal injection every 3 d. After 1 wk of tumor transplantation, PLX3397 (20 mg/kg in 7 μ L PBS) was directly injected into the right brain of the mice every 2 wk. (K) Upper panels are BLI images of brain metastatic lesions of representative mice from each experimental group at day 40. The lower panels represent the total photon flux of ex vivo brain metastatic lesions as measured by BLI at the endpoint (day 40; $n = 9$ /group). (L) Quantitative data of ex vivo brain metastasis of lung cancer at the end point. (M and N) Metastatic brain tumors in K were isolated from the brain and examined by FACS for M2 (M) and M1 (N) microglial polarization ($n = 9$ /group). (O) H2030BrM (2×10^5 cells) were intracardially injected into nude mice ($n = 9$ /group) followed by administering them with nicotine plus PLX3397 (1 mg/kg) by intraperitoneal injection every 3 d. Upper and middle panels are BLI images of brain metastatic lesions of representative mice from each experimental group at day 0 and day 60, respectively. Lower panels represent the total photon flux of ex vivo brain metastatic lesions as measured by BLI at the endpoint (day 60; $n = 9$ /group). (P and Q) Quantitative data of in vivo brain metastasis of lung cancer and (Q) ex vivo signals in the brain at the end point (day 60; $n = 9$ /group). (R) Kaplan–Meier analysis of brain metastasis-free survival was performed. (S) Serum of mice in Fig. 2 E was collected, and an aspartate aminotransferase test (AST) was measured using the AST activity assay kit (Sigma-Aldrich) ($n = 9$ /group). (T) H2030BrM cells were incubated with the indicated concentration of nicotine for 24 h. They were then examined for cell viability ($n = 9$ /group). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus respective nicotine group. ##, $P < 0.01$ versus respective PLX3397 and Nico+PLX3397 groups.

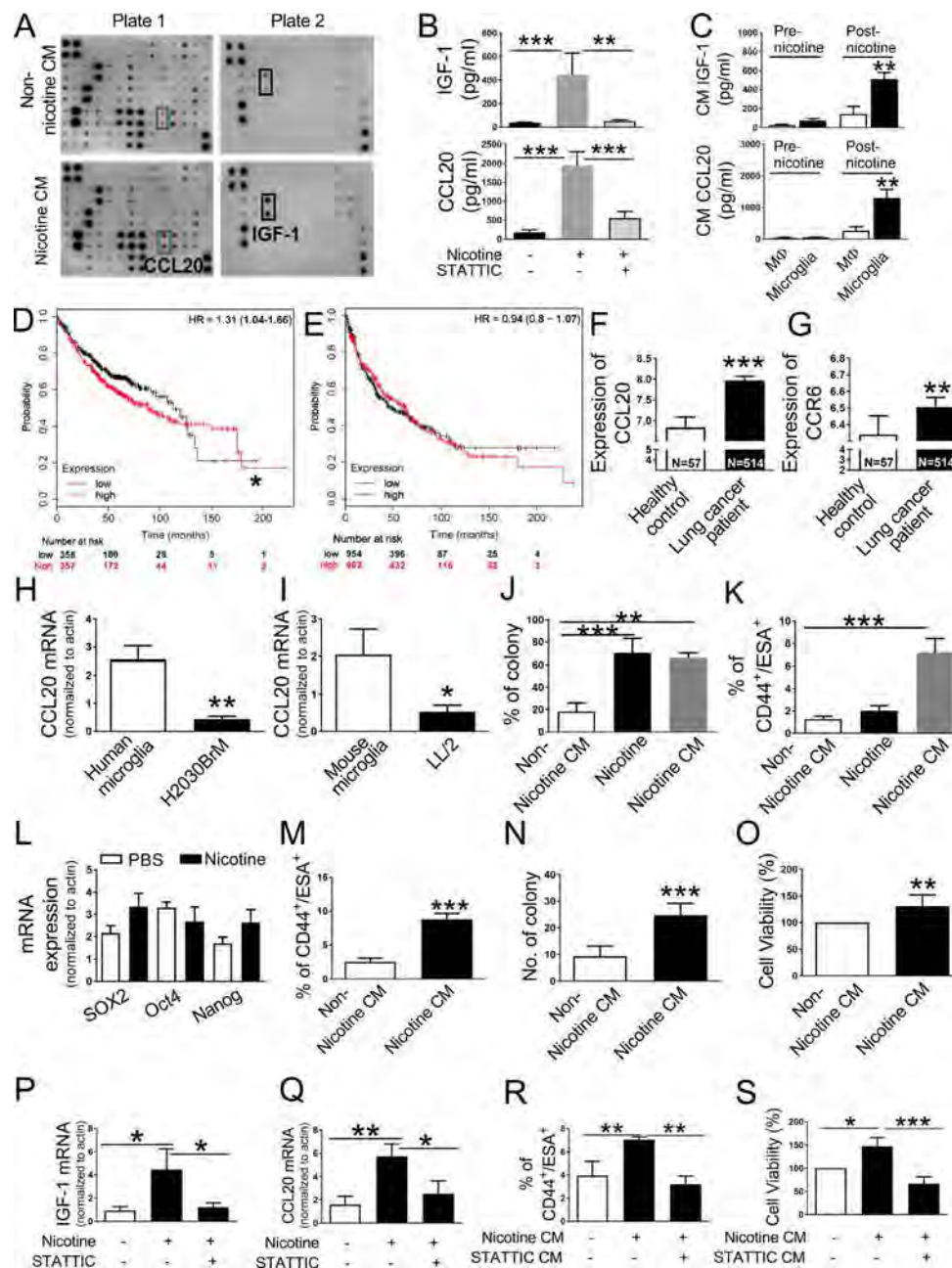


Figure S3. Nicotine treatment of microglia increases secretion of CCL20 and IGF-1 followed by promotion of tumor cell stemness. (A) Human microglial (HMC3) cells were treated with PBS or nicotine (1 μ M) for 24 h. CM was collected and analyzed by using the cytokine/growth factor array (Raybio). (B) Human microglial (HMC3) cells were treated with or without nicotine (1 μ M) plus STAT3C (0.5 μ M) for 24 h. Cells were washed with PBS twice and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for additional 24 h. CM was collected and analyzed by IGF-1 and CCL20 ELISA ($n = 4$ /group). (C) Human microglial (HMC3) cells and macrophage (THP-1) were treated with or without nicotine (1 μ M) for 24 h. Cells were washed with PBS twice and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for an additional 24 h. CM was collected and analyzed by IGF-1 and CCL20 ELISA ($n = 4$ /group). (D and E) Kaplan-Meier analysis was performed for examining relapse-free survival of lung cancer patients in relation to CCL20 ($P = 0.02$; D) and IGF-1 ($P = 0.36$; E) expression using the TCGA dataset. (F and G) Expressions of CCL20 (F) and CCR6 (G) were examined in lung tumor tissues from healthy individual and lung cancer patients using the GEO dataset (GSE22220). (H and I) Expression of CCL20 in human microglia and tumor cells (H) or mouse microglia and mouse tumor cells (I) were examined by qRT-PCR ($n = 4$ /group). (J-L) H2030BrM cells were treated with or without nicotine or CM extracted from human microglia that were treated with nicotine for 24 h. These cells were examined for their colony-forming ability (J), CD44⁺/ESA⁺ CSC population (K), and mRNA expression of stemness-related genes (L; $n = 4$ /group). (M) Effect of CM extracted from nicotine-treated mouse microglial cells on LL/2 mouse lung CSC population was examined by FACS. (N) Colony-forming ability of LL/2 was measured in the presence or absence of the CM derived from nicotine-treated microglia by qRT-PCR ($n = 4$ /group). (O) LL/2 cells were incubated with the CM derived from nicotine-treated microglia for 24 h. They were then examined for cell viability ($n = 4$ /group). (P and Q) Mouse microglia cells were treated with or without nicotine in the presence or absence of STAT3 inhibitor for 24 h. Cells were then examined for the expression of IGF-1 (P) or CCL20 (Q) by qRT-PCR. LL/2 cells were treated with CM derived from microglia that were treated with or without nicotine and/or STAT3C for 24 h ($n = 4$ /group). (R and S) The cells were then examined for CD44⁺/ESA⁺ CSC population (R) and for cell viability (S; $n = 4$ /group). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

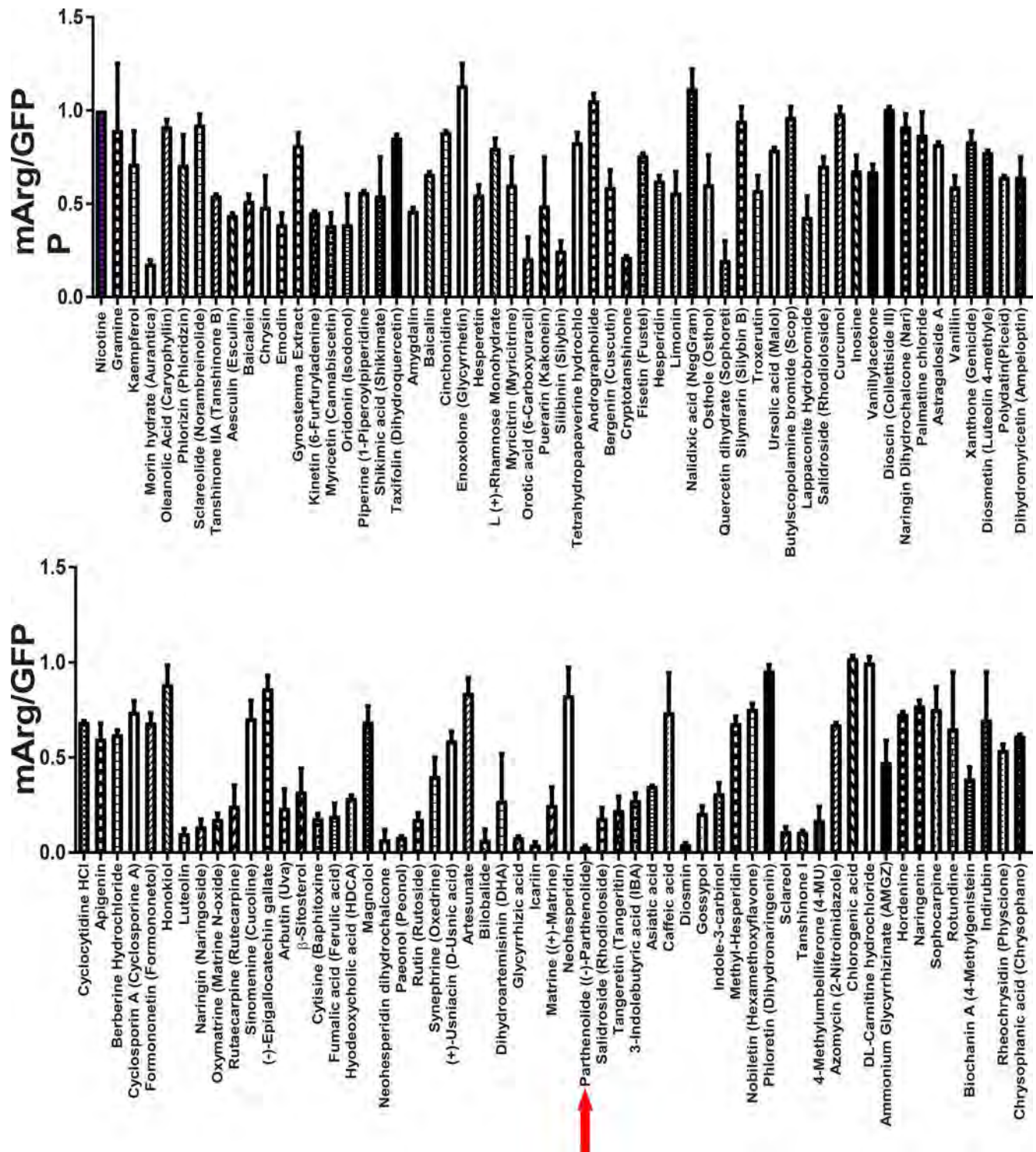


Figure S4. **Effect of natural compounds on nicotine-mediated activity of Arg1.** HMC3 cells containing Arg1 reporter plasmid were cultured in the presence or absence of nicotine (1 μ M) and/or each natural compound for 24 h. These cells were then examined for direct effect of compound on nicotine-mediated activity of Arg1 reporter ($n = 4/\text{group}$).

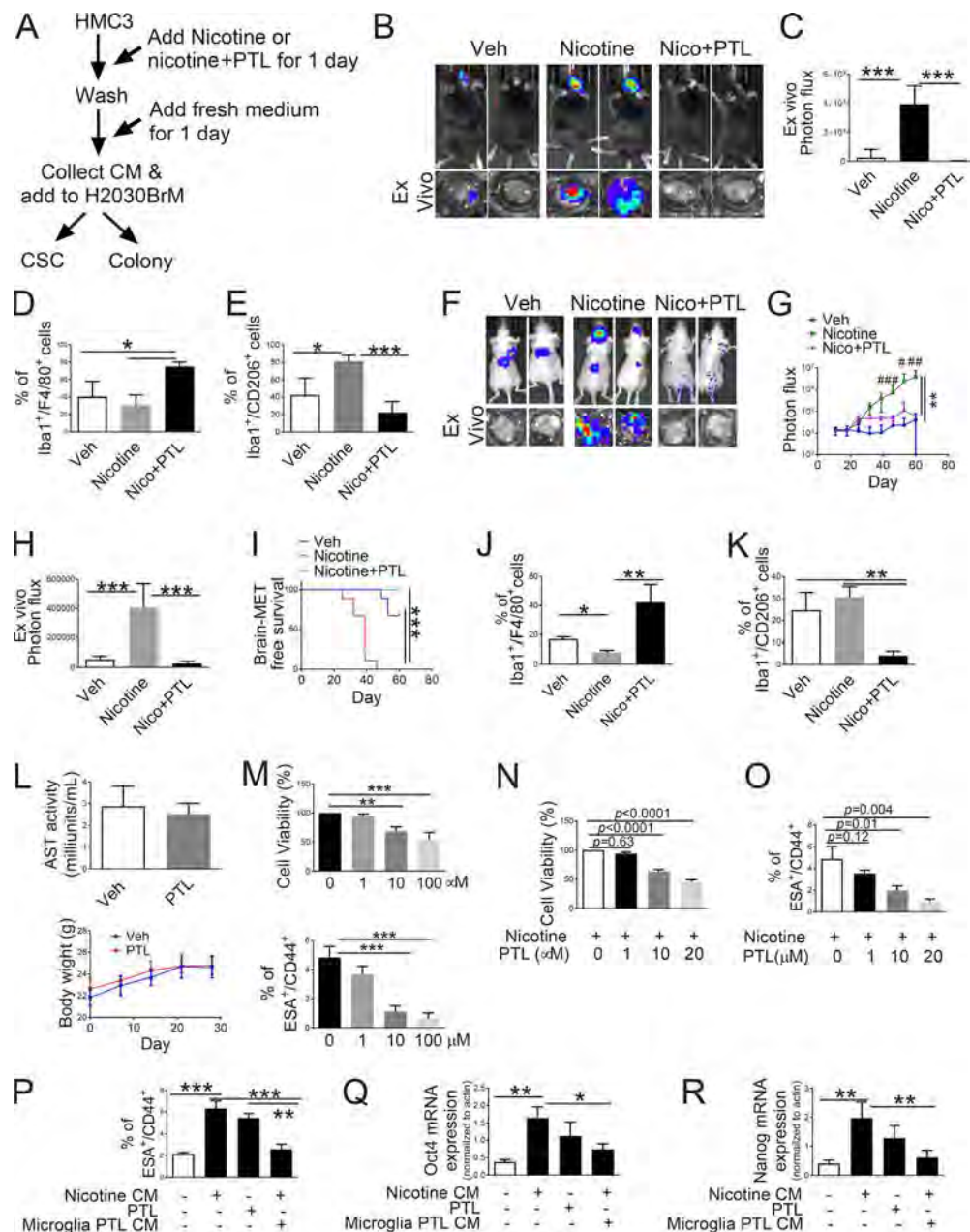


Figure S5. PTL suppressed nicotine-promoted brain metastasis in syngeneic mouse model. (A) Scheme of the experimental design. (B) Mouse lung cancer LL/2 cells were intracardially injected into wild-type C57BL/6 mice ($n = 9/\text{group}$) followed by administering nicotine (1 mg/kg) or PTL (1 mg/kg) by intraperitoneal injection every 3 d after tumor injection. Upper panels are BLI images of brain metastatic lesions of representative mice from each experimental group at day 40. Lower panels represent the total photon flux of ex vivo brain metastatic lesions as measured by BLI at the end point (day 40). (C) Quantitative data of ex vivo brain metastasis of lung cancer in the brain at the end point of the experiment conducted in B ($n = 9/\text{group}$). (D and E) Metastatic brain tumor tissues in B were dissociated and the effect of nicotine was examined for Iba1⁺/F4/80⁺ (D) and Iba1⁺/CD206⁺ (E) by FACS ($n = 9/\text{group}$). (F) H2030BrM (2×10^5 cells) were intracardially injected into nude mice followed by administering them with nicotine (1 mg/kg) plus PTL (1 mg/kg) by an intraperitoneal injection every 3 d for 60 d. Upper panel: BLI images of brain metastatic lesions of representative mice from each experimental group at day 60. Lower panel: total photon flux of ex vivo brain metastatic lesions was measured by BLI at the endpoint (day 60; $n = 9/\text{group}$). (G) Quantitative data of brain metastasis of lung cancer in vivo are shown ($n = 9/\text{group}$). (H) Ex vivo signals in the brain at the end point were examined ($n = 9/\text{group}$). (I) The Kaplan–Meier analysis of brain metastasis-free survival was performed. ($n = 9/\text{group}$). (J and K) Metastatic brain tumors were isolated from the brain and were examined by FACS for M1 (J) and M2 (K) microglial polarization using FACS ($n = 5/\text{group}$). (L) Serum of mice in Fig. 6 I was collected, and AST was measured by AST activity assay kit (Sigma-Aldrich). Body weight of the mice was also measured at the indicated time points ($n = 9/\text{group}$). (M) Direct effect of PTL on tumor cell viability by MTS assay and ESA⁺/CD44⁺ CSC population by FACS ($n = 4/\text{group}$). (N and O) H2030 cells were cultured in the presence or absence of nicotine (1 μ M) and PTL at various concentrations for 24 h. These cells were then examined for direct effect of PTL on cell viability by MTS assay (N) and on ESA⁺/CD44⁺ CSC population by FACS (O; $n = 4/\text{group}$). (P) H2030BrM cells were incubated with CM derived from microglia that were treated with or without nicotine and/or PTL (1 μ M) for 24 h, followed by examination of ESA⁺/CD44⁺ CSC population by FACS ($n = 4/\text{group}$). (Q and R) The same set of samples in P was examined for the expression of stemness-related genes, OCT4 (Q) and NANOG (R), by qRT-PCR ($n = 4/\text{group}$). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$ versus respective Nico+PTL group.

ARTICLE



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OPEN

Exosomal miR-19a and IBSP cooperate to induce osteolytic bone metastasis of estrogen receptor-positive breast cancer

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Bone metastasis is an incurable complication of breast cancer. In advanced stages, patients with estrogen-positive tumors experience a significantly higher incidence of bone metastasis (>87%) compared to estrogen-negative patients (<56%). To understand the mechanism of this bone-tropism of ER⁺ tumor, and to identify liquid biopsy biomarkers for patients with high risk of bone metastasis, the secreted extracellular vesicles and cytokines from bone-tropic breast cancer cells are examined in this study. Both exosomal miR-19a and Integrin-Binding Sialoprotein (IBSP) are found to be significantly upregulated and secreted from bone-tropic ER⁺ breast cancer cells, increasing their levels in the circulation of patients. IBSP is found to attract osteoclast cells and create an osteoclast-enriched environment in the bone, assisting the delivery of exosomal miR-19a to osteoclast to induce osteoclastogenesis. Our findings reveal a mechanism by which ER⁺ breast cancer cells create a microenvironment favorable for colonization in the bone. These two secreted factors can also serve as effective biomarkers for ER⁺ breast cancer to predict their risks of bone metastasis. Furthermore, our screening of a natural compound library identifies chlorogenic acid as a potent inhibitor for IBSP-receptor binding to suppress bone metastasis of ER⁺ tumor, suggesting its preventive use for bone recurrence in ER⁺ patients.

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Bone metastasis is the most predominant complication of breast cancer. More than 50% of patients have bone as the first site of distant metastases, followed by lung (17%), brain (16%), and liver (6%)¹. Ultimately, 70% of metastatic breast cancer patients experience a distant bone relapse during the course of disease^{2,3}. Bone metastasis leads to a worse prognosis in breast cancer patients, with a 16-month median survival⁴, and it causes many other complications. Patients develop skeletal-related events (SREs) such as severe bone pain, pathological fracture, hypercalcemia, and infiltration of the bone marrow⁵. Crosstalk between cancer cells and bone stroma cells, including mesenchymal stem cells, osteoblast cells, and osteoclast (OC) cells, play critical roles in bone metastasis⁶. Most breast cancer patients develop osteolytic lesions, which are mainly resulted from OC-mediated bone resorption rather than physical destruction by the cancer cells^{7,8}. The interaction with breast cancer cells activates OC cells, induces the bone resorption^{9,10}, and liberates the growth factors embedded in the bone matrix, which in turn stimulate the growth of cancer cells¹¹. This process is termed “the vicious cycle of bone metastases”¹². To impede this pathway, bisphosphonate (Zoledronic Acid) and RANKL inhibitor (Denosumab) were developed as bone-targeted therapies^{13,14}. However, the use of these drugs in breast cancer patients has several limitations. As to the preventive use of bone-targeted therapies for bone metastasis, while bisphosphonate showed a moderate preventive effect in postmenopausal breast cancer patients, it did not decrease the rate of bone recurrence in premenopausal patients¹⁵. The significant side effects, including osteonecrosis of the jaws¹⁶, make it an unfavorable preventive regimen. Moreover, RANKL inhibitor (Denosumab) failed to prevent bone recurrence among both premenopausal and postmenopausal patients¹⁷. For therapeutic use, these drugs have been proven to reduce the symptoms, but not to extend the patients’ survival^{18,19}. Thus, there is an urgent need for effective treatments for preventive and curative management of breast cancer bone metastasis (BCBM).

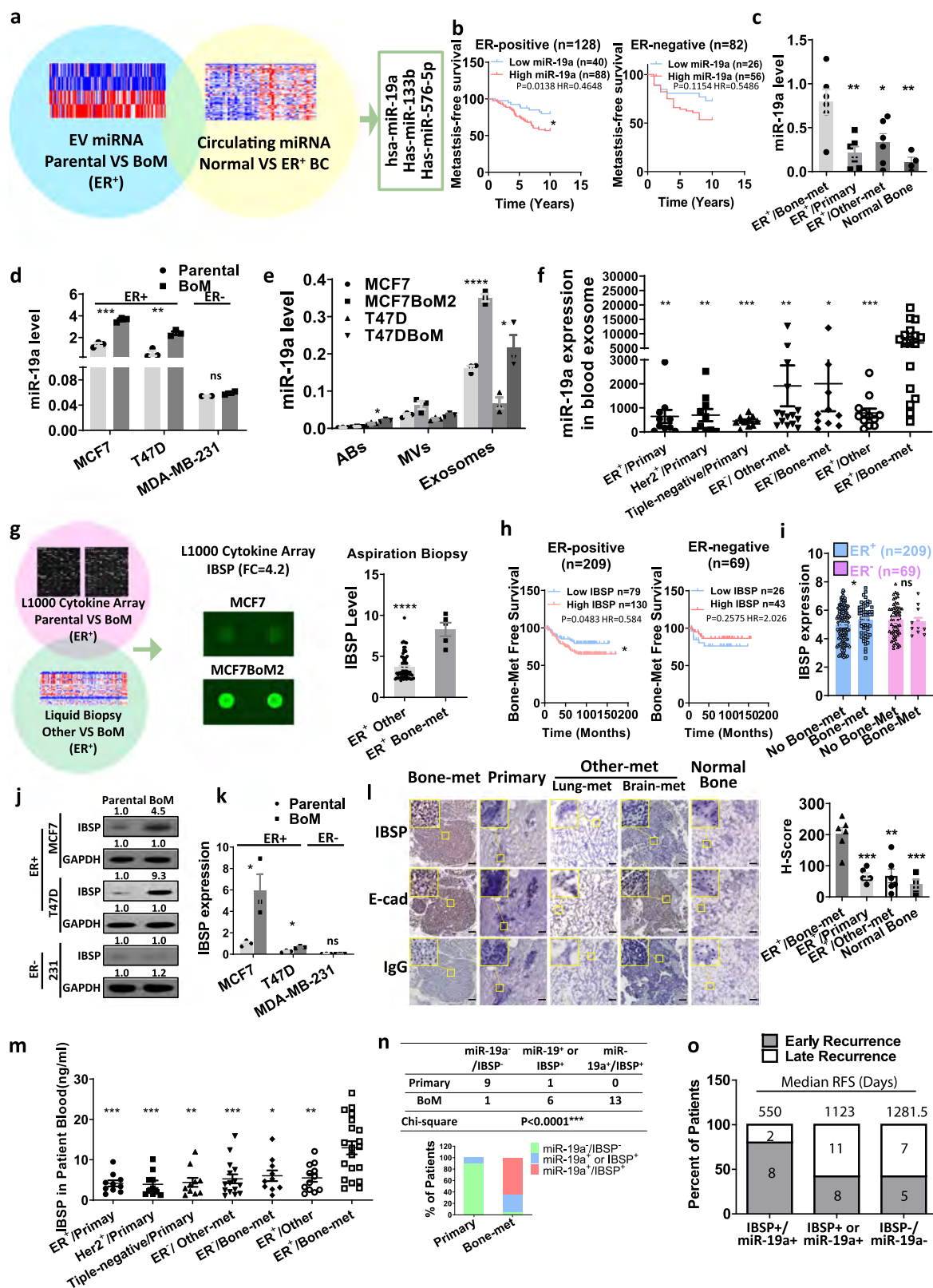
Among different subtypes of breast cancer, patients with estrogen receptors⁺ (ER⁺) tumors experience the most frequent bone metastases. Bone metastases account for as high as 87% of metastatic diseases among ER⁺ breast cancer patients, while the incidence of bone metastasis is <56% among ER⁻ breast cancer patients²⁰ with metastatic diseases. ER positivity is also associated with a higher rate of bone recurrence^{21,22}, and ER⁺ breast cancer relapses more frequently in the bone than in other organs²³. Although ER⁺ breast cancer patients are regularly treated with adjuvant endocrine therapy for a long-term after surgery, cancer cells frequently develop late-onset recurrence in bone²². This suggests drug resistance to endocrine therapy developed in the bone-metastatic cells. The long-dormant period also deprioritizes current preventive bone-targeted therapy due to their significant side effects. Thus, it is important to identify biomarkers that can be used for predicting the risk of bone recurrence among ER⁺ breast cancer patients. Despite the strong association between ER positivity and bone metastasis, how ER⁺ cancer cells acquire the bone tropism remains unclear. Understanding the mechanism of such organotropism warrants major opportunities to identify specific biomarkers and preventive/therapeutic targets for ER⁺ breast cancer bone metastasis. To survive in the bone microenvironment, breast cancer cells need to interact with bone microenvironment cells. Cell-secreted free proteins, including PTHrP, IL-1, and IL-6 were known to facilitate the communication between cancer cells and bone cells²⁴. In addition to the cytokines, cancer cells are also able to interact with microenvironment cells through extracellular vesicles (EVs)²⁵. These vesicles, including apoptotic bodies (ABs), microvesicles (MVs), and exosomes, encapsulate tumor-specific content, and transmit

them into environmental cells and circulation. They promote tumor growth by directly transducing tumor cells²⁶, or affecting surrounding cells to generate metastatic niche^{27–29}. As yet, no secretory factors specific to ER⁺ breast cancer have been identified to elucidate the association between ER positivity and bone metastasis.

In the current study, to decipher the molecular mechanism by which ER⁺ cancer preferentially metastasizes to the bone, we examined both the EVs and the free proteins secreted by ER⁺ bone-tropic breast cancer. We found that the expression of exosomal miR-19a and IBSP is significantly upregulated in the secretion of ER⁺ bone-tropic breast cancer cell lines, as well as in ER⁺ breast cancer patients with bone metastases. While the miR-19a was found to be upregulated in breast cancer, its exact role in tumor progression has been poorly understood^{30,31}. IBSP is a major structural protein of the bone matrix and is mainly expressed by bone cells³². We found that ectopic expression of both factors promoted bone metastasis in our in vivo models, suggesting both factors cooperate for ER⁺ breast cancer bone metastasis. We also screened a natural compound library and identified chlorogenic acid (CGA) that is capable of blocking this pathway. These findings illustrate a unique pathway of how ER⁺ breast cancer interacts with the bone microenvironment and establishes bone colonization. Furthermore, these two secretory factors would serve as prognostic markers for predicting the risk of bone metastasis in ER⁺ breast cancer patients.

Results

Bone-metastatic ER⁺ breast cancer cells secrete miR-19a and IBSP. Considering the importance of cell–cell communication in bone metastasis, ER⁺ cells may shed secretory factors in the bone microenvironment to exert strong bone tropism. To test this hypothesis, we examined both EVs and proteins secreted from bone-tropic breast cancer cells. Small non-coding RNA is known to be selectively transported and enriched in EVs^{33,34}, suggesting its importance as a disease regulator and feasible biomarker. Thus, we compared the transcriptome profiles of miRNA in EVs isolated from MCF7BoM2, a previously reported organotrophic ER⁺ breast cancer cell line that prefers bone colonization³⁵, with the EVs from its parental cell line MCF7 (Fig. 1a). We made sure that the increased bone metastasis rate of the MCF7BoM2 line was not due to an alteration in the cell growth ability, as implantation of MCF7 and MCF7BoM2 into the mammary fat pads generated primary tumors with similar sizes and weights (Supplementary Fig. 1a–d). We found 157 miRNAs that were significantly upregulated in the EVs from MCF7BoM2. To validate the clinical relevance of these exosomal miRNAs, we examined whether they are secreted into the blood of ER⁺ patients using the cohort data set of the circulating miRNA profiles from 23 ER⁺ breast cancer patients and 22 healthy donors (Fig. 1a). Among the 157 miRNAs, three of them (miR-19a, miR-133b, and miR-576-5p) were found to be significantly upregulated in the blood of ER⁺ breast cancer patients. Kaplan–Meier survival analyses were performed to investigate the association between these three miRNAs with metastatic events, and only miR-19a was found to be positively correlated with metastatic disease in ER⁺ breast cancer patients (Fig. 1b). There is no difference in metastatic incidence between ER⁻ breast cancer patients with or without elevated miR-19a expression (Fig. 1b), and both miR-133b and miR-576-5p are not significantly associated with metastasis in either ER⁺ or ER⁻ patients (Supplementary Fig. 2a). We also compared the ER⁺ breast cancer tissue from bone-metastatic lesions with ER⁺ primary breast cancer tissue from breast cancer patients without recurrence for 10 years, and with brain and lung metastatic lesions from ER⁺ breast cancer patients with visceral metastases



only, as well as with normal bone tissue. It was found that miR-19a is indeed only increased in bone-metastatic tissues (Fig. 1c). To validate the specificity of miR-19a associated with ER⁺ tumor, we established another ER⁺ bone-tropic cell line, T47DBoM. Similar to MCF7BoM2, T47DBoM generated comparable size and weight of primary tumors when implanted in the mammary fat pads. On the other hand, T47DBoM caused more aggressive

bone metastases when it was implanted intravenously (Supplementary Fig. 1e–h). We examined the expression of miR-19a in ER⁺ and ER⁻ cancer cell lines and found that the ER⁺ bone-tropic breast cancer cells, MCF7BoM2 and T47DBoM, have a higher endogenous expression of miR-19a compared to the parental cell lines, MCF7 and T47D (Fig. 1d). On the other hand, the expression of miR-19a did not show any difference in the bone-

Fig. 1 Bone-metastatic ER⁺ breast cancer cells secrete miR-19a and IBSP. **a** EVs were prepared from the conditioned medium of bone-metastatic cell line MCF7BoM2 and the parental cell line MCF7, followed by extraction of RNA. They were then subjected to miRNA profiling by GeneAtlas miRNA Array. Using the criteria of FDR < 0.05 and Fold change > 1.3, 156 miRNAs were selected for further study. These miRNAs were then examined whether they are secreted in the blood of ER⁺ patients using the cohort data set (GSE41922) of the circulating microRNA profiles between 23 ER⁺ breast cancer (BC) patients and 22 healthy donors (Normal). Three miRNAs (miR-19a, miR-133b, miR-576-5p), were found to be significantly upregulated (FDR < 0.05 and logFC > 0.5) in the blood of breast cancer patients. BoM, bone metastatic. **b** Kaplan-Meier analysis of miR-19a was performed between breast cancer patients with low miR-19a and high miR-19a expression using GSE22220. **c** RNA from bone-met (metastatic) lesions ($n = 6$), primary tumors ($n = 6$), lung and brain lesions ($n = 6$) of ER⁺ breast cancer patients, and normal bones ($n = 4$) was extracted and miR-19a expression was examined by Taqman-PCR. A two-sided student's *t*-test was performed (Primary vs Bone-met, $p = 0.0047$; Other-met vs Bone-met, $p = 0.0249$; Normal Bone vs Bone-met, $p = 0.0059$). Data are presented as mean values $\pm$ SEM. **d** The expression of miR-19a in parental cell lines (MCF7, T47D, and MDA-MB-231) and bone-tropic cell lines (MCF7BoM2, T47DBoM, and 231BoM-1833) were assayed by Taqman PCR. Two-sided student's *t*-tests were performed between parental groups and bone-metastatic (BoM) groups. $p = 0.0003$ (MCF7 vs MCF7BoM2, $n = 3$), $p = 0.0028$ (T47D vs T47DBoM, $n = 3$), $p = 0.1996$ (MDA-MB-231 vs 231BoM-1833, $n = 2$). Data are presented as mean values $\pm$ SEM. **e** The expressions of miR-19a in ABs, MVs, and exosomes from the parental cell lines (MCF7 and T47D) as well as their bone-tropic variants (MCF7BoM2 and T47DBoM) were examined by Taqman PCR. Two-sided student's *t*-tests were performed between parental groups and bone-metastatic (BoM) groups, $n = 3$ in each group. $p = 0.0408$ (ABs, T47D vs T47DBoM), $p < 0.0001$ (Exosomes, MCF7 vs MCF7BoM2), $p = 0.0167$ (Exosomes, T47D vs T47DBoM). Data are presented as mean values $\pm$ SEM. **f** Exosomes were isolated from the serum of breast cancer patients and the expression of miR-19a was compared among seven groups: (i) ER⁺ ($n = 10$), (ii) Her2⁺ ($n = 10$), and (iii) triple-negative ($n = 10$) breast cancer patients who were recurrence-free for 10 years, and (iv) ER⁻ breast cancer patients with visceral metastases ($n = 15$), (v) ER⁻ breast cancer patients with bone metastasis ($n = 10$), (vi) ER⁺ breast cancer patients with visceral metastases ($n = 12$) and ER⁺ breast cancer patients with bone metastasis ($n = 20$). Two-sided student's *t*-tests were performed to compare the miR-19a expression between the ER⁺/Bone-met group with each of the other groups. $p = 0.0012$, 0.003, 0.0008, 0.0036, 0.016, and 0.0005 from left to right. Data are presented as mean values $\pm$ SEM. **g** Cytokine/growth factor profiling was performed on conditioned media from MCF7 and MCF7BoM2 using L1000 array (Raybiotech), and 49 upregulated cytokines (fold change > 3) were selected. This list was cross-examined with gene profiles of the ER⁺ breast cancer biopsy database (GSE56493). Genes upregulated (FDR < 0.05 and fold change > 1.2) in biopsy of bone metastasis ($n = 5$) compared to other sites ($n = 75$) were selected. IBSP in conditioned media, as well as in ER⁺ breast cancer biopsy from the bone lesion and other sites (GSE56493, $p < 0.0001$, presented as mean values $\pm$ SEM) were presented. **h** Kaplan-Meier analyses were performed for the association between IBSP expression and bone metastasis-free survival among 209 ER⁺ breast cancer patients and 69 ER⁻ breast cancer patients using GSE2034. **i** The IBSP expression was compared between ER⁺ breast cancer patients with ($n = 57$) or without ($n = 152$) bone metastasis ($p = 0.04625$, two-sided student's *t*-test), as well as between ER⁻ breast cancer patients with ($n = 11$) or without ($n = 58$) bone metastases ($p = 0.6944$, two-sided student's *t*-test). Data are presented as mean values $\pm$ SEM. **j** Western blot analysis for IBSP expression in MCF7 and MCF7BoM2, T47D and T47DBoM, MDA-MB-231 and 231BoM-1833. Quantification of the bands was performed using the ImageJ program and the value was normalized to that of control (left panel). **k** PCR analysis for IBSP expression was performed in MCF7 and MCF7BoM2 ($p = 0.0341$), T47D and T47DBoM ($p = 0.0120$), MDA-MB-231 and 231BoM-1833 ($p = 0.3494$), $n = 3$ in all groups. Two-sided student's *t*-tests were used in each pair of comparisons. Data are presented as mean values $\pm$ SEM. **l** IBSP was examined by IHC on bone lesions ($n = 6$), primary breast cancer tissue without recurrence for 10 years ($n = 6$), brain and lung lesions ($n = 6$) and normal bones ($n = 4$). The staining was quantified by *H*-score assessment and compared by the two-sided student's *t*-test. $p = 0.0002$ (Primary vs Bone-met); $p = 0.0014$ (Other-met vs Bone-met); $p = 0.0006$ (Normal Bone vs Bone-met). Data are presented as mean values $\pm$ SEM. E-cad (E-cadherin) was stained as the marker of breast cancer cells. Rabbit IgG was used as isotype control. Scale bar, 100 μ m. **m** ELISA was performed to quantify the IBSP level in the serum of ER⁺ ($n = 10$), Her2⁺ ($n = 10$) and triple-negative ($n = 10$) breast cancer patients without recurrence for 10 years, and ER⁻ breast cancer patients with visceral metastases ($n = 15$), ER⁻ breast cancer patients with bone metastasis ($n = 10$), ER⁺ breast cancer patients with visceral metastases ($n = 12$) and ER⁺ breast cancer patients with bone metastasis ($n = 20$). Two-sided student's *t*-tests were performed to compare the miR-19a expression between the ER⁺/Bone-met group with each of the other groups. $p = 0.0009$, 0.0009, 0.0016, 0.0010, 0.0101, and 0.0024 from left to right. Data are presented as mean values $\pm$ SEM. **n** ER⁺ breast cancer patients with or without bone metastases were separated into three groups according to the serum levels of IBSP and exosomal miR-19a, including miR-19a low/IBSP low group (miR-19a⁻/IBSP⁻), miR-19a low/IBSP high or miR-19a high/IBSP low group (miR-19a⁺ or IBSP⁺) and miR-19a high/IBSP high group (miR-19a⁺/IBSP⁺). The percentage in each group was calculated and plotted as the bar graph. The chi-square test was performed. **o** ER⁺ breast cancer patients with recurrent disease were selected from TCGA. Patients with early recurrence (<1000 days) and late recurrence (≥ 1000 days) were separated into three groups: miR-19a low/IBSP low group (miR-19a⁻/IBSP⁻), miR-19a low/IBSP high or miR-19a high/IBSP low group (miR-19a⁺ or IBSP⁺) and miR-19a high/IBSP high group (miR-19a⁺/IBSP⁺). The percentage of patients and the median RFS (recurrence-free survival) days in each group were calculated.

tropic triple-negative cell line derived from MDA-MB-231³⁶ (Fig. 1d). To examine whether free or vesicle-encapsulated miRNAs are the major source of secreted miR-19a, we treated the conditioned medium from MCF7BoM2 with RNase. The RNase treatment did not degrade the secreted miR-19a from breast cancer cells, suggesting that they are protected in the membrane vesicles (Supplementary Fig. 2b). This finding is consistent with the previous report that the majority of miRNA in serum is encapsulated by EVs^{37,38}. EVs include ABs, MVs and exosomes. To further clarify the source of EV-derived miR-19a, we examined the miR-19a expression in these three components of EVs from MCF7 and T47D as well as their bone-tropic variants. After confirming the purity of the isolated vesicles by nanoparticle tracking analysis (NTA), electron microscope (EM), as well as a western blot (Supplementary Fig. 2c, d), the expression of miR-

19a in these vesicles, were examined by Taqman PCR. We found that miR-19a is most highly expressed in exosomes compared to other EVs (Fig. 1e). Importantly, there is a strong upregulation of miR-19a in the exosomes from ER⁺ bone-metastatic variants, MCF7BoM2 and T47DBoM (Fig. 1e), compared to their parental cell lines. However, ER⁻ 231BoM showed only a negligible increase of miR-19a in the exosomes (Supplementary Fig. 2e). Next, we examined whether the increase of exosomal miR-19a can be detected in the serum of ER⁺ breast cancer patients with bone metastases. Strikingly, ER⁺ breast cancer patients with bone metastasis are significantly associated with higher expression of exosomal miR-19a in serum (Fig. 1f). It should be noted that the expression of exosomal miR-19a remained low in the patients with primary breast cancer with no recurrence within 10 years. We also did not observe any increase in exosomal miR-19a in the

serum of ER⁺ breast cancer patients at advanced stages nor in ER⁺ patients with visceral metastases but with no bone involvement (Fig. 1f). These data suggest that miR-19a is specifically upregulated in both endogenous and exosomal levels of ER⁺ bone-tropic breast cancer cells.

We also examined secreted proteins and cytokines from ER⁺ bone-tropic breast cancer cells. By performing L1000 cytokine array analysis, 1000 secreted proteins were examined in the conditioned medium from MCF7 and MCF7BoM2, and 49 proteins were found to be upregulated in the cell culture media from MCF7BoM2. Among these 49 proteins, IBSP was the only significantly upregulated gene in the aspiration biopsy from bone-metastatic lesions of ER⁺ breast cancer patients, compared to the biopsy samples from other sites (Fig. 1g). IBSP is a major structural protein of the bone matrix. It is synthesized by skeletal-associated cell types as a secreted protein, and is able to bind to calcium and hydroxyapatite via its acidic amino acid clusters³⁹. In breast cancer, we observed cancer cell-specific upregulation of IBSP, while the paired normal breast epithelial cells did not express IBSP (Supplementary Fig. 2f). This finding suggests a possible role of IBSP in breast cancer progression. Since many cytokines were previously reported to bind to the surface of EVs, we also looked at the membrane fractions of EVs by western blot (Supplementary Fig. 2g), but IBSP was not detected. We also performed immunostaining of IBSP and CD63 for the exosomes isolated from MCF7BoM2, and found that only CD63 but not IBSP was detected on the surface of exosomes (Supplementary Fig. 2h). Thus, we concluded that IBSP in the extracellular region is mainly in the form of free secreted protein. To further clarify the connection between IBSP and bone metastasis of ER⁺ breast cancer, we performed Kaplan–Meier analyses among 278 breast cancer patients. IBSP was found to be positively correlated with bone metastasis only in ER⁺ breast cancer patients, but not in ER[−] breast cancer patients (Fig. 1h). There is an increase in IBSP expression in the bone-metastatic group compared to the non-bone-metastatic group among ER⁺ breast cancer patients, but not among ER[−] breast cancer patients (Fig. 1i). We also verified that ER⁺ bone-metastatic cell lines have higher expression of IBSP compared to the parental cell lines. The PCR and western blot analyses confirmed the upregulation of IBSP in MCF7BoM2 and T47DBoM, but not in ER[−] bone-metastatic cell line 231BoM (Fig. 1j, k). When ER⁺ breast cancer tissue from bone lesions was compared with ER⁺ primary breast cancer tissue from patients with no recurrence within 10 years, and with brain and lung metastatic lesions from ER⁺ breast cancer patients with visceral metastases only, as well as with normal bone tissue, IBSP was found to be significantly increased in bone-metastatic tissues (Fig. 1l). Next, we examined whether the increase of IBSP can be detected in the serum of ER⁺ breast cancer patients with bone metastases. A significant upregulation of IBSP protein in the serum of ER⁺ breast cancer patients with bone metastasis was detected (Fig. 1m), but not in the serum of patients with only primary breast cancer, advanced ER[−] breast cancer patients, or ER⁺ breast cancer patients with visceral metastases other than bone.

Our data (Fig. 1a–m) suggest both miR-19a and IBSP are positively related to bone metastasis of ER⁺ cancer cells. However, how they are associated with bone metastasis remains unknown. We further investigated whether miR-19a and IBSP are mutually related to bone metastasis of ER⁺ breast cancer cells. We combined the results from Fig. 1f with Fig. 1m, and stratified the patients into three groups: both miR-19a and IBSP low, miR-19a or IBSP high, both miR-19a and IBSP high. Strikingly, ER⁺ breast cancer patients with bone metastasis are significantly associated with higher expression of both IBSP and miR-19a. Upregulation of sole factor led to a negligible, but not significant

increase of the bone-metastatic incidence, suggesting a possible cooperative role of these two factors in bone metastasis of ER⁺ breast cancer cells (Fig. 1n). Next, we examined the expression of miR-19a and IBSP in breast cancer patients using the TCGA database. Among 41 ER⁺ breast cancer patients with recurrence, IBSP and miR-19a could predict the recurrence risk as the majority of patients with high levels of both factors experienced earlier recurrence within 1000 days (Fig. 1o). The median recurrence-free survival (RFS) time of patients with high levels of both miR-19a and IBSP is only 550 days, while the median RFS time of patients with high expression of either miR-19a or IBSP and patients with low levels of both miR-19a/IBSP are 1123 days and 1281.5 days, respectively. These results indicate that miR-19a and IBSP are positively associated with bone metastasis, and they serve as biomarkers for a higher risk of bone metastasis when they are both upregulated in ER⁺ breast cancers.

Both IBSP and miR-19a are essential for bone metastasis of ER⁺ breast cancer.

To test whether both miR-19a and IBSP are required for bone metastasis of ER⁺ breast cancer cells, we ectopically expressed IBSP and miR-19a in MCF7, and established stable overexpression cell lines (Supplementary Fig. 3a–d). Ectopic expression of miR-19a did not alter the level of IBSP, and vice versa, indicating there is no regulatory network involved between IBSP and miR-19a. Neither miR-19a nor IBSP could induce any change of exosome production (Supplementary Fig. 3e), proliferation, or migration of the cells in vitro (Supplementary Fig. 3f, g). Intra-mammary fat pad injection of MCF7 with miR-19a and/or IBSP forced-expression did not induce any difference in tumor growth in vivo (Fig. 2a, b). Strikingly, ectopic expression of both miR-19a and IBSP together in MCF7 led to a significant increase in bone metastasis in the xenograft model with the intracardiac injection of cancer cells (Fig. 2c). Nonetheless, miR-19a or IBSP alone did not increase the incidence of bone metastasis. These data suggest miR-19a and IBSP promote bone metastasis through the tumor microenvironment instead of affect cancer cells directly. Since IBSP is known to regulate bone turnover by binding to integrin α V β 3 receptor on the surface of OC cells⁴⁰, we examined the bone densities of mice implanted with the breast cancer cells. We found that ectopic expression of both IBSP/miR-19a resulted in a significant decrease in bone density in the tumor lesions (Fig. 2d). We also quantified the total area of TRAP⁺ OC surface and its percentage of total bone surface area (OC.S/BS%) in the tumor-bearing bones and found that synchronous overexpression of miR-19a and IBSP significantly increased the osteoclastogenesis (Fig. 2e). We then repeated this experiment in another ER⁺ cell line, T47D. Both IBSP and miR-19a were ectopically introduced into T47D (Supplementary Fig. 3h, i), and stable overexpression cell lines had been established (Supplementary Fig. 3j, k) before the cells were inoculated into mouse tibia. Similar to MCF7, ectopic expression of both miR-19a and IBSP resulted in increased tumor burden in the bones (Fig. 2f), while discrete expression of miR-19a or IBSP alone did not affect the tumor growth. Bone lesions of T47D/IBSP/miR-19a presented decreased bone densities (Fig. 2g) and increased OC activities (Fig. 2h). These results indicate that miR-19a and IBSP work together to induce osteolytic bone metastasis in ER⁺ breast cancer patients, though not directly affecting cancer cell growth or motility.

miR-19a promotes osteolytic bone metastasis. Because both miR-19a and IBSP are necessary to promote the bone metastasis of ER⁺ breast cancer cells, we investigated how each of them contributes to the bone-tropic metastasis of cancer cells. MiR-19a,

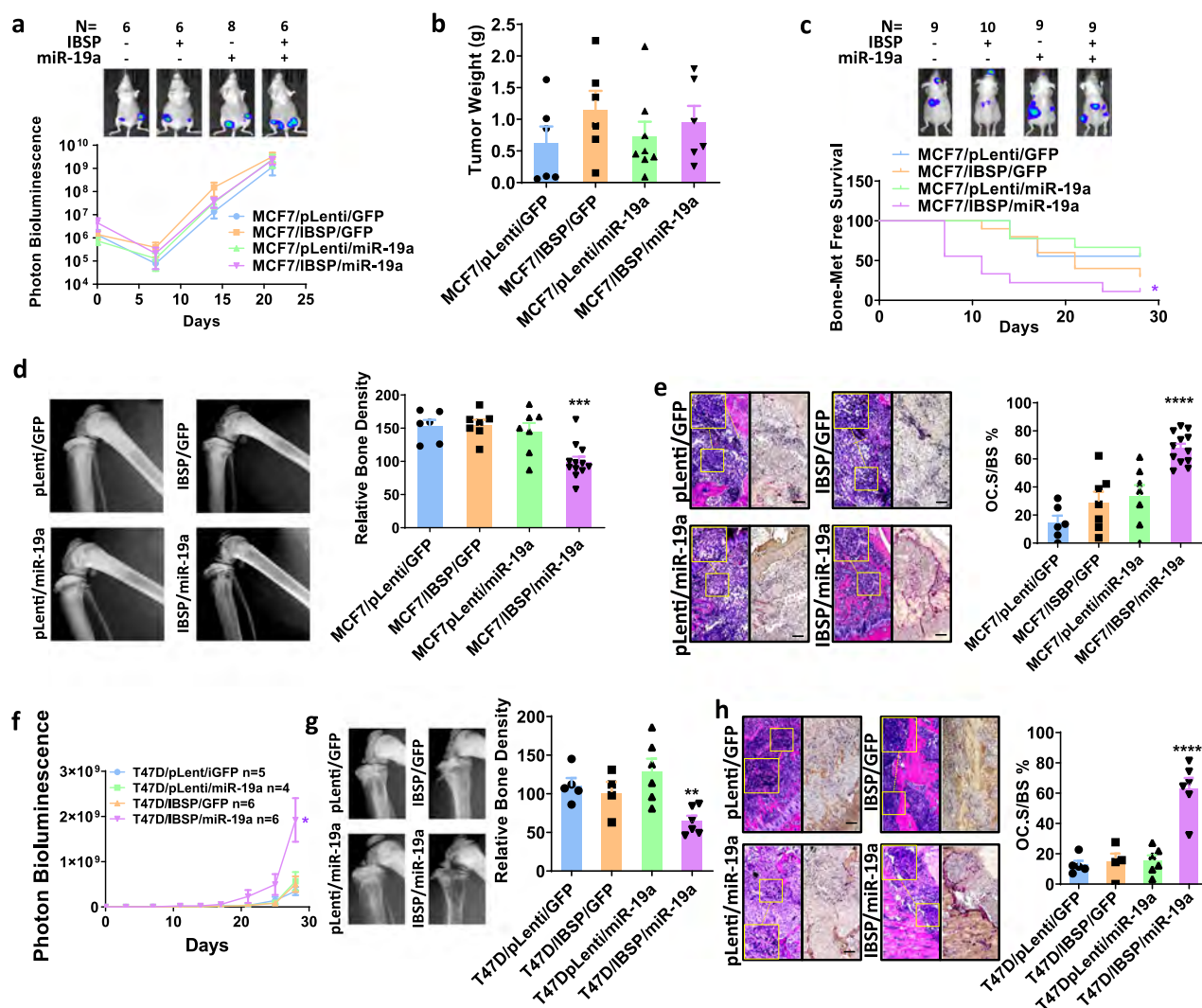


Fig. 2 Both IBSP and miR-19a are essential for bone metastasis of ER⁺ breast cancer. **a** miR-19a and/or IBSP were ectopically expressed in MCF7, and they were transplanted into mammary fat pads of female nude mice. The growth of the tumor was monitored by measuring the luciferase activity by IVIS Bioluminescence. $p = 0.3110$ (MCF7/pLenti/GFP vs MCF7/IBSP/miR-19a; $n = 6$ vs 6), $p = 0.2649$ (MCF7/pLenti/GFP vs MCF7/IBSP/GFP; $n = 6$ vs 6), $p = 0.4739$ (MCF7/pLenti/GFP vs MCF7/pLenti/miR-19a; $n = 6$ vs 8). Data are presented as mean values $\pm$ SEM. **b** At day 21, tumors were removed from the mammary fat pad and their weights were measured. $p = 0.3971$ (MCF7/pLenti/GFP vs MCF7/IBSP/miR-19a; $n = 6$ vs 6), $p = 0.2178$ (MCF7/pLenti/GFP vs MCF7/IBSP/GFP; $n = 6$ vs 6), $p = 0.7632$ (MCF7/pLenti/GFP vs MCF7/pLenti/miR-19a; $n = 6$ vs 8). Data are presented as mean values $\pm$ SEM. **c** miR-19a and/or IBSP were ectopically expressed, and they were transplanted into female nude mice via intracardiac injection. The incidence of bone metastasis was monitored by measuring the luciferase activity by IVIS Bioluminescence. Log-rank (Mantel-Cox) test was performed to calculate the p -value. $p = 0.0105$ (MCF7/pLenti/GFP vs MCF7/IBSP/miR-19a), $p = 0.3982$ (MCF7/pLenti/GFP vs MCF7/IBSP/GFP), $p = 0.9230$ (MCF7/pLenti/GFP vs MCF7/pLenti/miR-19a). **d** The legs of mice were imaged by X-ray and the bone density was measured by ImageJ. $p = 0.0007$ (MCF7/pLenti/GFP vs MCF7/IBSP/miR-19a; $n = 6$ vs 12), $p = 0.8621$ (MCF7/pLenti/GFP vs MCF7/IBSP/GFP, $n = 6$ vs 7), $p = 0.6397$ (MCF7/pLenti/GFP vs MCF7/pLenti/miR-19a, $n = 6$ vs 7). Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM. **e** TRAP staining was performed in tumor-bearing bones from mice. The total OC surface area relative to the bone surface area was measured by ImageJ, then calculated and compared among different groups. H&E staining of the same field was shown together with the TRAP staining. Scale bar, 100 μ m. $p < 0.0001$ (MCF7/pLenti/GFP vs MCF7/IBSP/miR-19a, $n = 6$ vs 12), $p = 0.1598$ (MCF7/pLenti/GFP vs MCF7/IBSP/GFP, $n = 6$ vs 7), $p = 0.0867$ (MCF7/pLenti/GFP vs MCF7/pLenti/miR-19a, $n = 6$ vs 7). Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM. **f** miR-19a and/or IBSP were ectopically expressed, and they were transplanted into female nude mice via intra-tibia injection. The growth of tumor in bone was monitored by measuring the luciferase activity by IVIS Bioluminescence. $p = 0.0175$ (T47D/pLenti/GFP vs T47D/IBSP/miR-19a), $p = 0.4877$ (T47D/pLenti/GFP vs T47D/IBSP/GFP), $p = 0.3305$ (T47D/pLenti/GFP vs T47D/pLenti/miR-19a). Data are presented as mean values $\pm$ SEM. **g** The legs of mice were imaged by X-ray and the bone density was measured by ImageJ. $p = 0.0034$ (T47D/pLenti/GFP vs T47D/IBSP/miR-19a, $n = 5$ vs 6), $p = 0.5750$ (T47D/pLenti/GFP vs T47D/IBSP/GFP, $n = 5$ vs 4), $p = 0.3809$ (T47D/pLenti/GFP vs T47D/pLenti/miR-19a, $n = 5$ vs 6). Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM. **h** TRAP staining was performed in tumor-bearing bones from the mice. The OC surface relative to the bone surface was calculated and compared among different groups. H&E staining of the same field was shown together with the TRAP staining. Scale bar, 100 μ m. $p = 0.0001$ (T47D/pLenti/GFP vs T47D/IBSP/miR-19a, $n = 5$ vs 6), $p = 0.7711$ (T47D/pLenti/GFP vs T47D/IBSP/GFP, $n = 5$ vs 4), $p = 0.5701$ (T47D/pLenti/GFP vs T47D/pLenti/miR-19a, $n = 5$ vs 6). Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM.

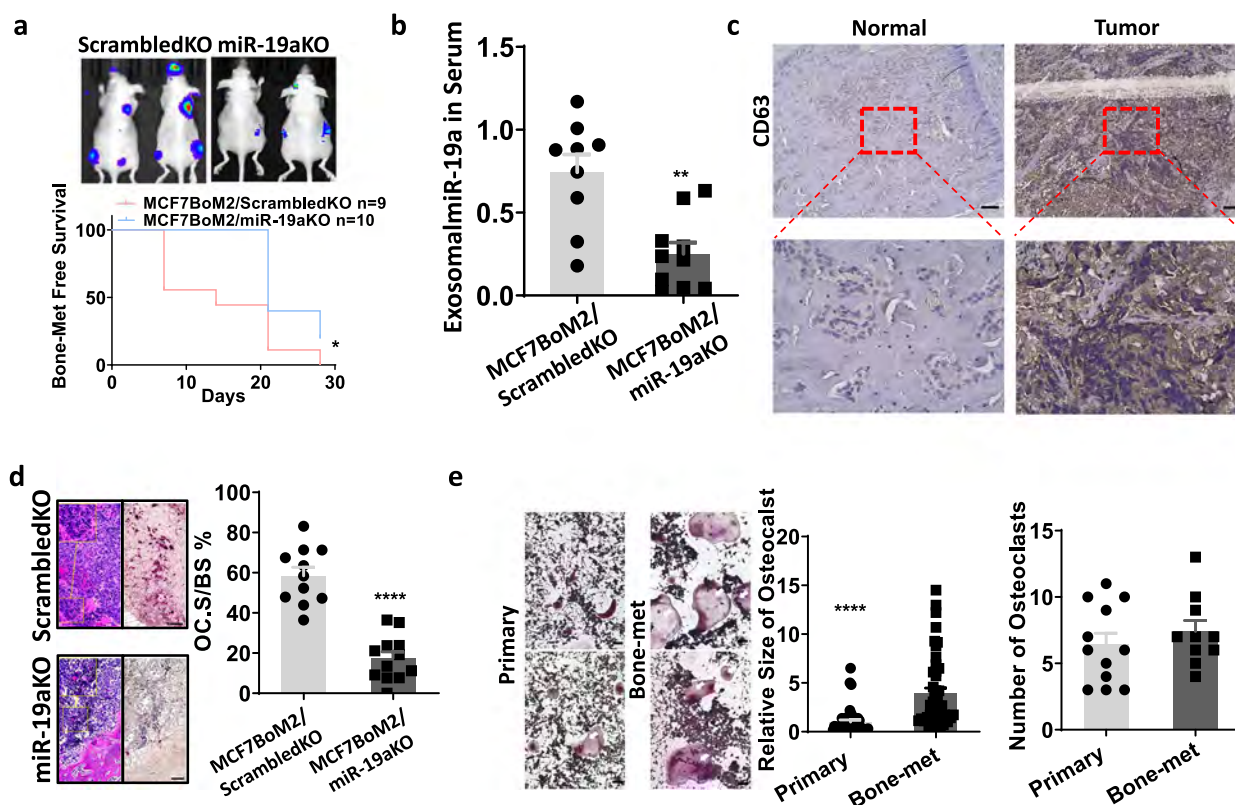


Fig. 3 miR-19a promotes osteolytic bone metastasis. **a** MCF7BoM2/ScrambledKO and MCF7BoM2/miR-19aKO were transplanted into female nude mice via intracardiac injection. The growth of bone metastasis was monitored by measuring the luciferase activity by IVIS Bioimager. Log-rank (Mantel-Cox) test was performed to calculate the p -value ($p = 0.0109$). **b** At the endpoint (day 28), mice were sacrificed and the expression of exosomal miR-19a from serum was examined by Taqman. A two-sided student's t -test was performed ($p = 0.0011$, $n = 9$ vs 10). Data are presented as mean values $\pm$ SEM. **c** Representative figures of the expression of exosomal marker CD63 in normal bone and tumor-bearing bone from the mice. Scale bar, 100 μ m. **d** TRAP staining for osteoclast cells in tumor-bearing bones from the mice transplanted with MCF7BoM2/ScrambledKO or MCF7BoM2/miR-19aKO. OC surface relative to the bone surface was calculated and compared. H&E staining of the same field was shown together with the TRAP staining. Scale bar, 100 μ m. A two-sided student's t -test was performed ($p = 0.00000014$, $n = 11$ vs 12). Data are presented as mean values $\pm$ SEM. **e** Exosomes from the serum of ER⁺ breast cancer patients with ($n = 10$) or without ($n = 12$) bone metastasis were isolated. The harvested exosomes were incubated with mouse bone marrow-derived monocytes. After 1 week, the cells were fixed and TRAP staining was performed. The size ($p = 0.0000039$) and number ($p = 0.4254$) of differentiated OC were quantified and compared. Scale bar, 100 μ m. Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM.

as a member of the OncomiR-1 family, was previously found to be upregulated in multiple malignancies^{41–43}. It was also found to be expressed in breast cancer cells, although its role remains unclear^{30,44}. We found both endogenous and secreted levels of miR-19a were higher in the bone-metastatic cell lines compared to the parental cell lines. However, endogenous expression of miR-19a did not affect breast cancer cells per se because the ectopic expression of miR-19a did not alter the growth or motility of cells in vitro (Supplementary Fig. 3f, g). To further clarify the role of miR-19a in bone metastasis of ER⁺ breast cancer cells, we applied the CRISPR/Cas9 technology to knock out the miR-19a in MCF7BoM cells. A specific gRNA (guide RNA) was designed to target the stem-loop of miR-19a precursor so that the biogenesis of mature miR-19a was hindered. The genomic DNA cleaving ability of our gRNA targeting miR-19a was verified by the T7E1 enzyme (Supplementary Fig. 4a). Four independent clones with minimal miR-19a expression and unaltered proliferation were selected and combined to establish the stable knockout cell line (Supplementary Fig. 4b–d). When tested in vitro, we found that knockout of miR-19a did not alter the cell migration or invasion abilities (Supplementary Fig. 4e–f). However, when miR-19a knockout cells were implanted into nude mice by intracardiac

injection, loss of miR-19a significantly decreased bone metastasis (Fig. 3a). These data are consistent with our previous finding that miR-19a is needed for bone-tropic metastasis of MCF7BoM2. MCF7BoM2 has a high expression of both miR-19a and IBSP. Knockout of miR-19a, but leaving IBSP alone, compromised the bone-metastatic ability of MCF7BoM2. Interestingly, when we examined the serum exosomes in the mice, we found that the knockout of miR-19a significantly decreased exosomal miR-19a in the serum (Fig. 3b). This finding suggests that serum exosome could represent the dynamic changes of breast cancer cells. These results are also in agreement with the previous finding that miR-19a could not induce the intrinsic change of cancer motility. Accordingly, we investigated whether exosomal miR-19a could promote bone metastasis by remodeling environmental cells in a paracrine manner instead of directly affecting cancer cells. The tumor lesion in the bone exhibited abundant expression of exosomal marker CD63 (Fig. 3c), implying exosome activity in the microenvironment. Since we found an aberrant osteolytic change in our previous data, we examined the OC in the bone lesions. There was a decrease of osteoclastogenesis in the tumor-bearing bones when miR-19a was knocked out from the cancer cells (Fig. 3d), indicating a possible role of exosomal miR-19a in

promoting OC cells. To verify this hypothesis, exosomes from serum of ER⁺ breast cancer patients with or without bone metastases were isolated and incubated with primary OC precursors. Exosomes prepared from the patients with bone metastases significantly promoted OC maturation and differentiation (Fig. 3e). These results indicate that miR-19a is involved in the osteolytic bone metastasis and exosomal miR-19a could directly promote OC activity in the bone microenvironment.

Exosomal miR-19a targets PTEN/AKT pathway to promote osteoclastogenesis. To validate our hypothesis that exosomal miR-19a promotes the activity of OC cells, we first examined whether exosomes can be directly up-taken by OC cells. OC precursor RAW264.7 cells were treated with PalmGFP-labeled exosomes derived from bone-metastatic breast cancer cells. The result indicates that exosomes from MCF7BoM2 were indeed incorporated into RAW264.7 cells 24 h after the treatment (Fig. 4a). It was reported that miR-19a targeted PTEN 3'UTR⁴⁵ and that OC differentiation and their activity were regulated by NF- κ B and AKT pathways^{46,47}, both of which were known to be inhibited by PTEN^{48,49}. Thus, it is plausible that cancer cells secrete miR-19a and suppress PTEN expression in OC cells when exosomes are internalized. The decrease of PTEN leads to activation of NF- κ B and AKT pathways that promote OC cells. To test this notion, we examined whether exosomal miR-19a modulates OC activity by suppressing PTEN expression, using the 3' UTR reporter assay. We confirmed that the miR-19a-targeted sequence on PTEN is conserved between human and mouse (Supplementary Fig. 5a, b), and found that ectopic expression of miR-19a indeed targeted PTEN, while mutation of the miR-19a binding site on PTEN 3'UTR rescued the reporter activity (Fig. 4b). Furthermore, ectopic expression of miR-19a in OC precursor cells, RAW264.7, suppressed PTEN expression and induced NF- κ B and AKT pathway with increased phosphorylated P65 and AKT (Fig. 4c, d). We also examined whether increased miR-19a could promote OC differentiation of RAW264.7 by performing TRAP staining. The ectopic expression of miR-19a significantly increased the number of mature OC cells and their size (Fig. 4e). Next, we tested whether direct treatment of OC precursor cells with exosomal miR-19a, instead of lentiviral overexpression, could also induce the activation of P65 and AKT pathways. Although there was no difference in the efficiency of exosome uptake (Supplementary Fig. 5c), compared to exosomes from the parental cell line, exosomes from bone-metastatic MCF7BoM2 increased miR-19a, decreased PTEN and induced AKT, and P65 activation in mouse bone marrow monocyte (mBMM), which is the primary precursor of OC (Fig. 4f, g). In addition, exosomes from MCF7BoM2 promoted the differentiation of mBMM into OC (Fig. 4h). In a bone resorption assay, mBMMs were seeded on the bone chips and incubated with exosomes from MCF7 and MCF7BoM2. Exosomes from MCF7BoM2 exhibited significantly higher competence to induce the osteolytic activities of OC cells as shown by the increased area of resorption pit (Fig. 4i). Consistent with our finding of enriched miR-19a in exosomes, only exosomes, but not MV or AB, could induce the differentiation of OC cells (Supplementary Fig. 5d). To further investigate whether miR-19a is responsible for the OC-promoting effect of the exosomes, OC cells were incubated with exosomes isolated from MCF7BoM2/ScrabmbleKO cells or MCF7BoM2/miR-19aKO cells (Fig. 4j–m and Supplementary Fig. 6a–c). When miR-19a was knockout, exosomes were unable to deliver miR-19a into OC cells and suppress PTEN expression (Fig. 4j and Supplementary Fig. 6b). We also found that exosomes prepared from bone-metastatic breast cells with miR-19a knockout could not induce P65 and AKT activation (Fig. 4k

and Supplementary Fig. 6c) and reversed the OC-promoting effect (Fig. 4l, m and Supplementary Fig. 6a). Collectively, we demonstrated that bone-metastatic cells delivered miR-19a-containing exosomes to OC precursor cells and suppressed PTEN expression. Decreased PTEN induced the activation of NF- κ B and AKT signaling pathways, promoting the differentiation and osteolytic activity of OC cells. This notion was also verified by examining the effect of exosomes from MCF7 and MCF7/miR-19a cell lines on OC cells. Compared to exosomes from parental MCF7 cells, exosomes from MCF7 with ectopic expression of miR-19a significantly increased the miR-19a expression and decreased PTEN expression in both recipient RAW264.7 and mBMM cells, increased OC number, and promoted the differentiation of OC in vitro (Supplementary Fig. 6d–i).

ER⁺ breast cancer cells secrete IBSP to recruit osteoclast precursors. Next, we examined the role of IBSP in osteolytic bone metastasis induced by miR-19a. IBSP is known to act as a ligand of integrin α V β 3 receptor that is highly expressed on the surface of OC⁴⁰. A previous study found that IBSP knockout in mice resulted in decreased OC number in bones⁴⁰. Integrin α V β 3 is known to be associated with OC migration and adhesion^{50,51}, and IBSP was previously found to be a cell-secreted protein with bone matrix binding ability⁵². Therefore, we hypothesize that IBSP secreted from the bone lodging cancer cells can recruit the OC precursors to the micro-metastatic site followed by creating an OC precursor-enriched niche. Such an environment enhances the uptake of miR-19a-containing exosomes by the OC precursors in a concentration-dependent manner. In a trans-well migration assay, we found that both MCF7BoM2 and MCF7/IBSP recruited more OC precursors to abluminal chambers compared to MCF7 (Fig. 5a), which supports our hypothesis that IBSP attracts OC precursors. We then performed the knockout of IBSP using the CRISPR-Cas9 system in MCF7BoM2. To rule out the clonal effect, three clones that showed no IBSP expression and no alteration of growth ability were selected and mixed into one heterogeneous line, MCF7BoM2/IBSPKO (Fig. 5b, c and Supplementary Fig. 7a). The knockout of IBSP expression did not alter the cell migration ability of MCF7BoM2/IBSPKO (Fig. 5d). However, the bone-metastatic ability was significantly compromised by IBSP knockout in MCF7BoM2/IBSPKO cells in the xenograft mouse model (Fig. 5e). Loss of IBSP also preserved the density of tumor-bearing bones (Fig. 5f). Results from TRAP staining of the tumor-bearing tibias also suggested that the IBSP knockout led to decreased osteoclastogenesis (Fig. 5g). These results strongly support our hypothesis that IBSP recruits OC cells to the tumor lesions in the bone, which is essential for the uptake of exosomes and establishment of bone metastasis of ER⁺ breast cancer cells.

CGA blocks cooperative function of miR-19a and IBSP in ER⁺ BCBM. We hypothesized that cooperation between exosomal miR-19a and IBSP generates the osteolytic bone microenvironment, enabling the ER⁺ tumor growth in the bone. Therefore, we examined the changes in bone microenvironment induced by directly injecting IBSP and exosomal miR-19a prepared from MCF7BoM2 into the tibial bone of mice every two days without tumor cell injection. This treatment resulted in a significant increase in the number of OC at the treatment site (Supplementary Fig. 7b). We also found that the DiD-labeled exosomes from MCF7BoM2 were incorporated into OC cells when they were injected into the tibial bone (Supplementary Fig. 7c). These results suggest that exosomal miR-19a and IBSP cooperatively create an osteolytic bone microenvironment. We then tested whether such change could enhance the growth of primary MCF7

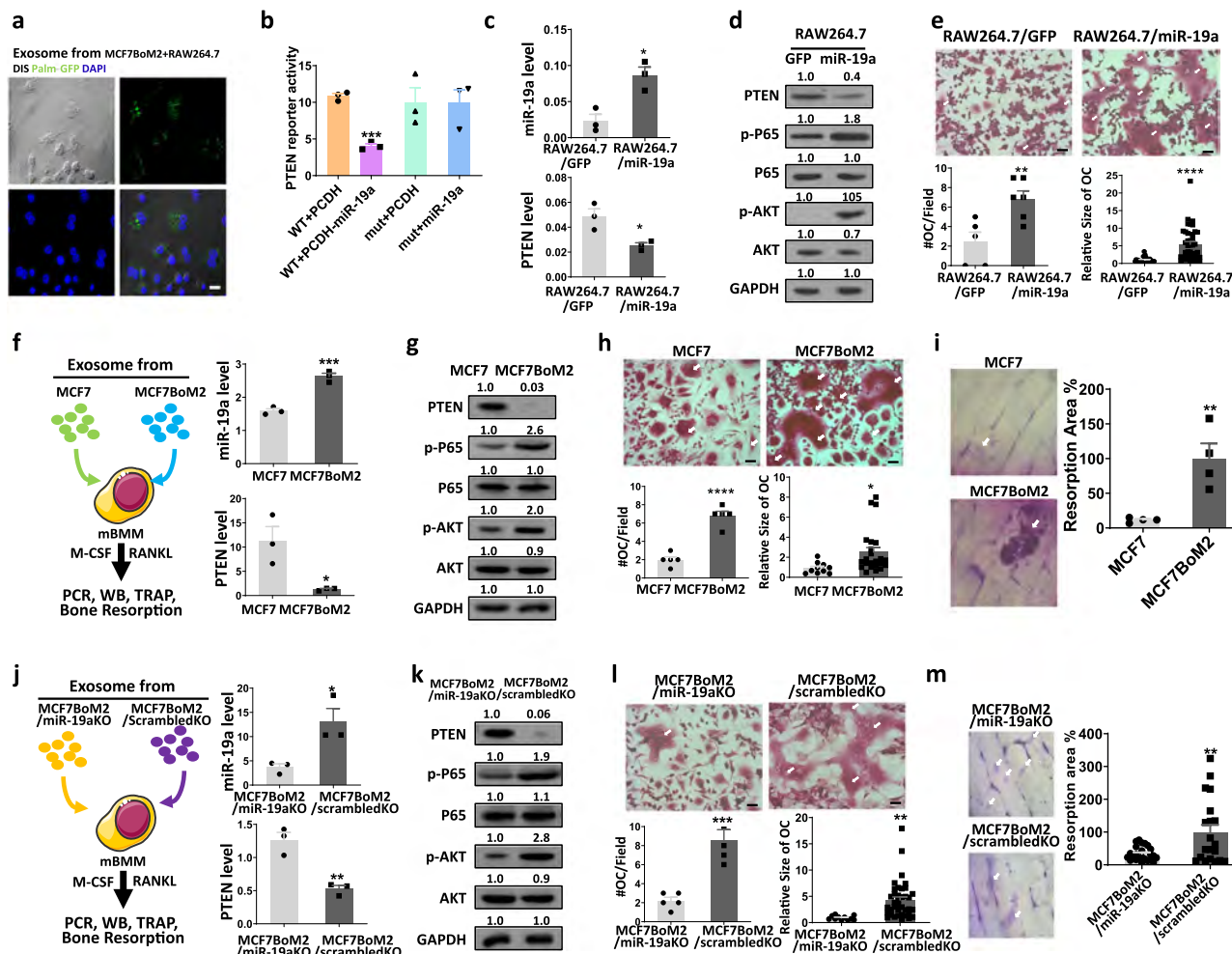


Fig. 4 Exosomal miR-19a targets PTEN/AKT pathway to promote osteoclastogenesis. **a** Exosomes were labeled with GFP by ectopic expression of PalmGFP in MCF7BoM2 cells. OC cells were then treated with the labeled exosomes. After 24 h, uptake of exosome by osteoclast cells was monitored by confocal microscope. Scale bar, 10 μ m. **b** Luciferase reporter assay for the constructs containing wild-type PTEN 3'UTR or mutant PTEN 3'UTR was performed after transfecting the cells with the miR-19a expression vector. $p = 0.0001$ (WT + PCDH vs WT + PCDH-miR-19a, two-sided student's t -test). Data are presented as mean values $\pm$ SEM. **c** Taqman PCR for miR-19a expression ($p = 0.0131$, $n = 3$), and SYBR Green qPCR for PTEN expression ($p = 0.0232$, $n = 3$) in RAW264.7/GFP and RAW264.7/miR-19a were performed. Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM. **d** Western blot analysis for PTEN, phospho-NF- κ B P65, total NF- κ B P65, phospho-AKT and total AKT expression in RAW264.7/GFP and RAW264.7/miR-19a cell lines. The band intensity was quantified and normalized to that of control (left panels). **e** TRAP staining of RAW264.7/GFP and RAW264.7/miR-19a cells. On day 4, the number of differentiated osteoclasts ($p = 0.0080$, $n = 5$ vs 6) and the size of these cells ($p < 0.0001$, $n = 30$ vs 40) were measured. Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM. Scale bar, 100 μ m. **f** Mouse BMMs were induced into OC cells with the treatment of exosomes and cytokines. Taqman PCR for miR-19a expression ($p = 0.0008$, $n = 3$) and SYBR Green qPCR for PTEN ($p = 0.0271$, $n = 3$) expression were performed for OC cells treated with exosomes from MCF7 and MCF7BoM2. Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM. **g** Western blot analysis for PTEN, phospho-NF- κ B P65, total NF- κ B P65, phospho-AKT and total AKT expression in mouse BMMs treated with exosomes from MCF7 and MCF7BoM2. The band intensity was quantified and normalized to that of control (left panels). **h** TRAP staining was performed for mouse BMMs treated with exosomes from MCF7 and MCF7BoM2. On day 4, the number of differentiated osteoclast ($p = 0.000036$, $n = 5$ vs 5) and their sizes ($p = 0.0323$, $n = 10$ vs 23) were measured. Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM. Scale bar, 100 μ m. **i** Bone-resorbing activities of OC treated with exosomes from MCF7 and MCF7BoM2 were measured by the bone-resorption assay. BMMs were seeded on a bone chip and treated with exosomes and cytokines. Resorption pits were measured and compared ($p = 0.0070$, $n = 4$). Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM. **j** Taqman PCR for miR-19a expression ($p = 0.0291$, $n = 3$) and SYBR Green qPCR for PTEN expression ($p = 0.0047$, $n = 3$) were performed for mouse BMMs treated with exosomes from MCF7BoM2/miR-19aKO and MCF7BoM2/ScrambledKO. Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM. **k** Western blot analysis was performed for PTEN, phospho-NF- κ B P65, total NF- κ B P65, phospho-AKT, and total AKT expression in mouse BMMs treated with exosomes from MCF7BoM2/miR-19aKO and MCF7BoM2/ScrambledKO. The band intensity was quantified and normalized to that of control (left panels). **l** TRAP staining was performed for mouse BMMs that were treated with exosomes from MCF7BoM2/miR-19aKO and MCF7BoM2/ScrambledKO. On day 4, the number of differentiated osteoclast cells ($p = 0.0005$, $n = 5$ vs 5) and their sizes were measured ($p = 0.0029$, $n = 11$ vs 38). Scale bar, 100 μ m. Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM. **m** The bone-resorbing activity of OC was measured by the bone-resorption assay using BMMs that were treated with exosomes from MCF7BoM2/miR-19aKO and MCF7BoM2/ScrambledKO ($p = 0.0025$, $n = 24$ vs 20). Two-sided student's t -tests were performed. Data are presented as mean values $\pm$ SEM.

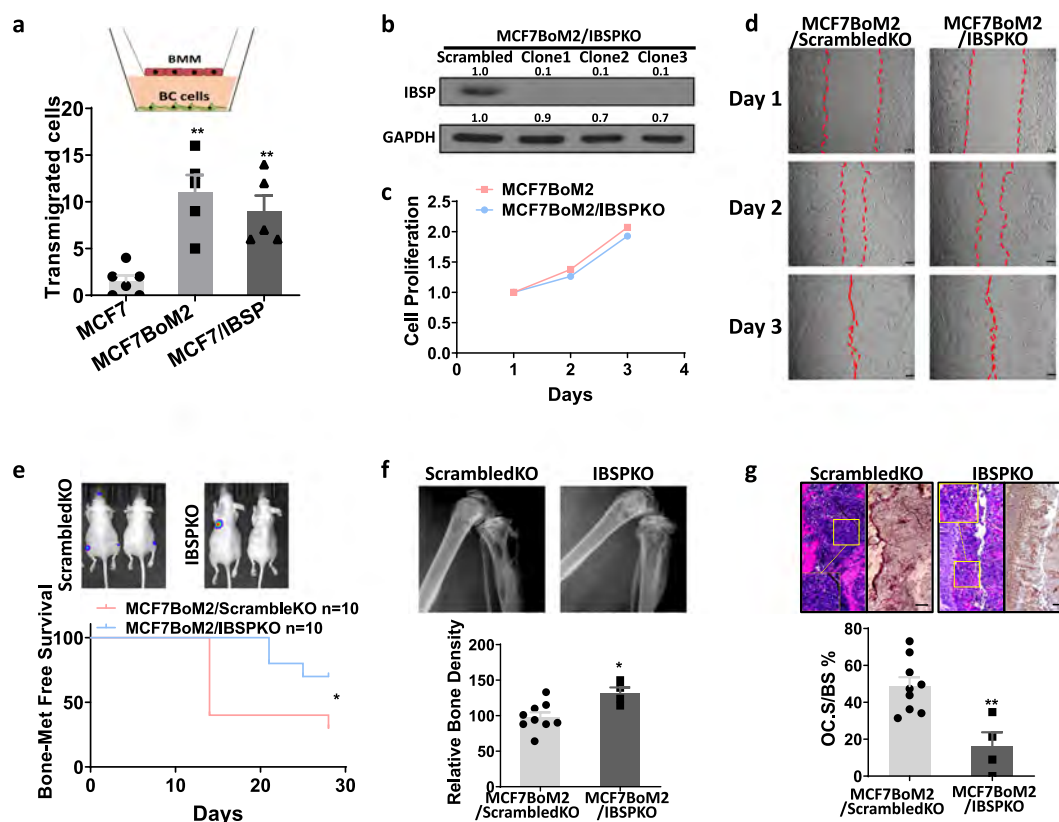


Fig. 5 ER⁺ breast cancer cells secrete IBSP to recruit osteoclast precursors. **a** Trans-well migration assays were performed to measure the migration ability of BMMs towards breast cancer cells. BMMs were seeded on the luminal side and breast cancer cells (MCF7, MCF7BoM2, and MCF7/IBSP) were seeded on the abluminal chamber. Data are presented as mean values $\pm$ SEM. **b** CRISPR-Cas9 mediated knockout of IBSP in MCF7BoM2. Three knockout clones were confirmed by western blot. The band intensity was quantified and normalized to that of control (left panels). **c** The selected clones were mixed and designated as MCF7BoM2/IBSPKO. Cell proliferation was measured for MCF7BoM2 and MCF7BoM2/IBSPKO by MTS assay. A two-sided student's *t*-test was performed on day 3 and $p = 0.0496$ ($n = 3$ vs 3). Data are presented as mean values $\pm$ SEM. **d** Cell migration ability of MCF7BoM2 and MCF7BoM2/IBSPKO was measured by wound-healing assay. Scale bar, 100 μ m. **e** MCF7BoM2/ScrambledKO and MCF7BoM2/IBSPKO (500,000/0.1 ml PBS) were transplanted into female nude mice via intracardiac injection. The growth of bone metastasis was monitored by measuring the luciferase activity by IVIS Bioimager, and the Kaplan-Meier analysis was done for bone metastasis-free survival ($p = 0.0496$, HR = 4.101). **f** The tumor-bearing bones were imaged by X-ray, and the relative bone densities were quantified by ImageJ ($p = 0.0124$, $n = 9$ vs 4). Two-sided student's *t*-tests were performed. Data are presented as mean values $\pm$ SEM. **g** OC surface relative to the bone surface was calculated and compared after TRAP staining. H&E staining of the same field was shown together with the TRAP staining ($p = 0.0036$, $n = 9$ vs 4). Two-sided student's *t*-tests were performed. Data are presented as mean values $\pm$ SEM. Scale bar, 100 μ m.

cells in the mice's tibias (Fig. 6a). MCF7 cells were transplanted into the tibial bone. At the same time, both IBSP and exosomes from MCF7BoM2 were given every two days into the tumor site. To evaluate the roles of exosomal miR-19a, exosomes from MCF7BoM2/miR-19aKO were used as a control. We found that the injection of miR-19a-containing exosomes and IBSP significantly promoted tumor growth (Fig. 6a) in the bones, which resulted in a decrease in bone density (Fig. 6b) and an increase in OC activity (Fig. 6c). These results suggest the indispensable roles of exosomal miR-19a and IBSP in bone metastasis of ER⁺ breast cancer cells. Consistent with these results, knockout of miR-19a or IBSP from MCF7BoM2 significantly decreased the bone-metastatic ability of cancer cells, while ectopic expression of each factor alone did not rescue the effect. (Supplementary Fig. 7d, e).

Because IBSP plays a critical role in recruiting OC precursor cells and enabling the delivery of exosomal miR-19a to promote bone metastasis, targeting IBSP could be an effective preventive strategy to block the IBSP/exosome pathway, and decrease the risks of bone metastasis/recurrence. Therefore, we used a competitive binding assay to screen a natural compounds library aiming at identifying compound(s) that block the binding of IBSP with its receptor integrin α V β 3. In this assay, we layered integrin

α V β 3 on the bottom of wells, followed by adding IBSP in the presence or absence of 176 natural compounds. The binding of IBSP protein to integrin α V β 3 was quantified using an enzyme-tagged antibody to measure the inhibitory effect of natural compounds. We found that Chlorogenic acid (CGA) most significantly inhibited the binding of IBSP to integrin α V β 3 (Fig. 6d). To ensure that the inhibitory ability is not a result of direct interaction between the natural compound and the enzyme tag, we repeated our screening with a different enzyme-tagged antibody (Supplementary Fig. 8a). In a chemotaxis assay, we found that CGA significantly blocked the recruitment of OC precursors (mBMM) by IBSP (Fig. 6e). On the other hand, CGA did not directly suppress the growth or migration of cancer cells (Supplementary Fig. 8b, c). These data indicate CGA is a competent inhibitor of the recruitment of OC by IBSP. Importantly, in the xenograft model, both CGA and exosome inhibitor GW4869 effectively suppressed ER⁺ breast cancer bone metastasis (Fig. 7a), while no notable side effects were observed with the mice received CGA treatment (Supplementary Fig. 8d, e). It should be noted that the inhibitory effect of CGA on bone metastasis is not a result of its effect on tumor cell growth, because CGA did not affect the primary tumor growth

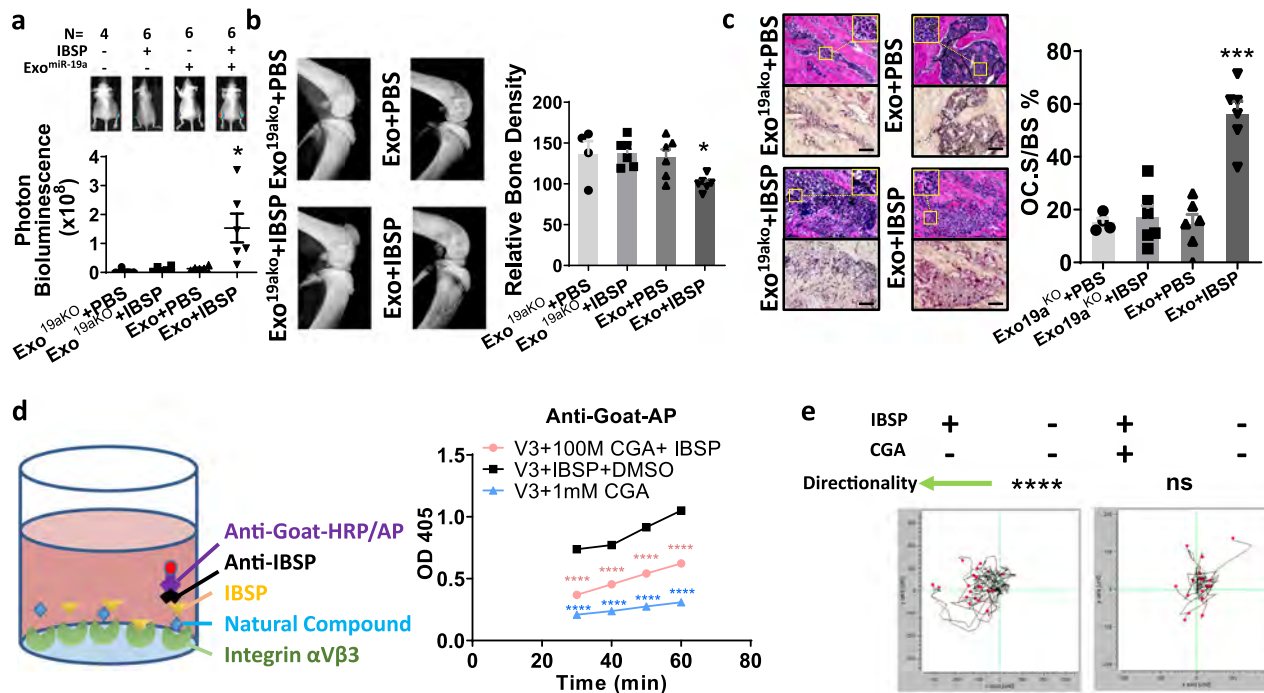


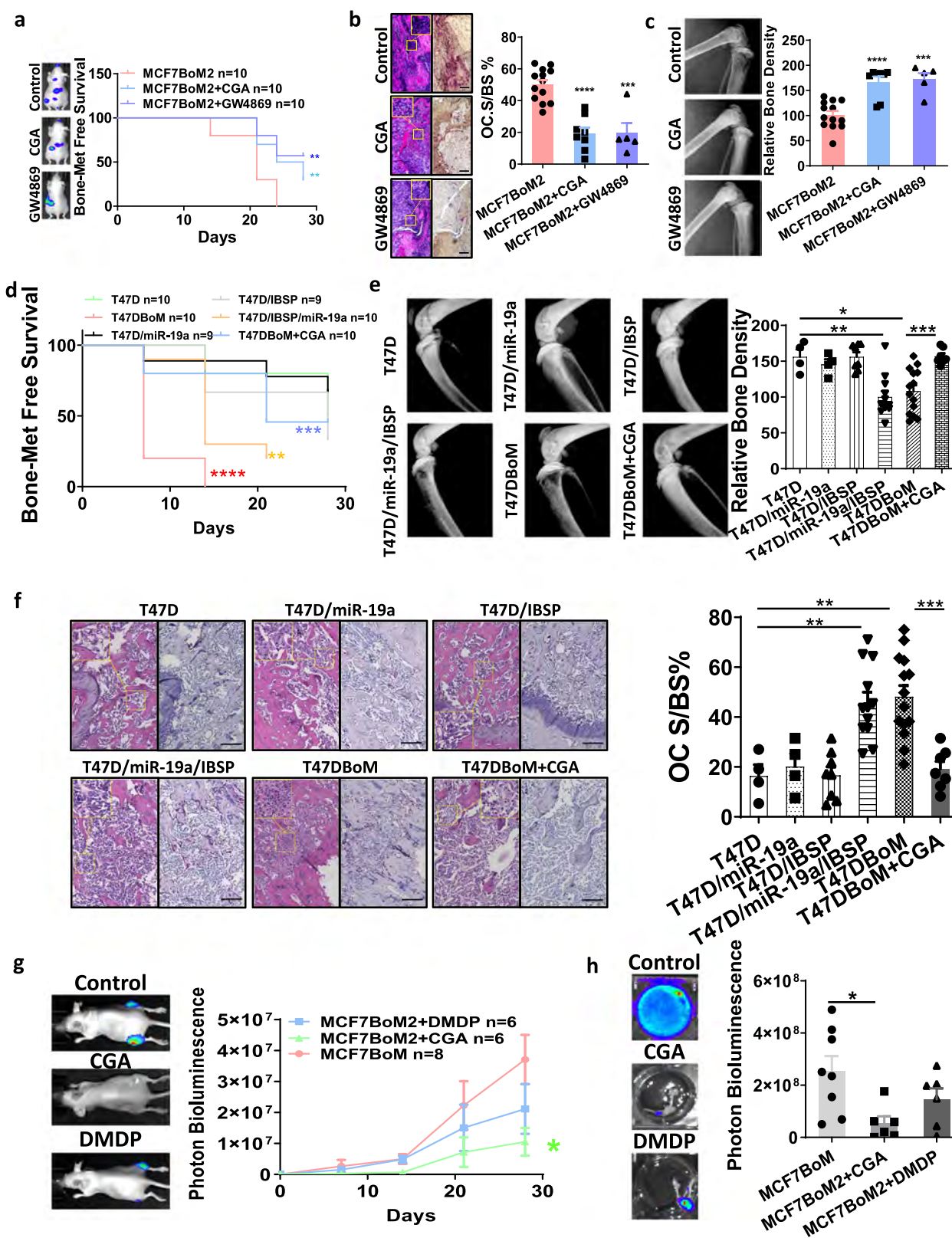
Fig. 6 IBSP and miR-19a cooperatively induce osteoclastogenesis and promote cancer cell growth in the bone. **a, b** MCF7 cells were injected into the tibias of mice. These mice also received 2 μ g exosomes prepared from MCF7BoM2 and 0.5 μ g recombinant IBSP injected into the tibias every 2 days. The exosomes prepared from MCF7BoM2/miR-19aKO and PBS were used as controls. After three weeks, the bioluminescence signals of tibial bones were measured. Two-sided student's *t*-tests were performed. $p = 0.4670$ (Exo^{19aKO} + PBS vs Exo^{19aKO} + IBSP, $n = 4$ vs 6), $p = 0.1949$ (Exo^{19aKO} + PBS vs Exo + PBS, $n = 4$ vs 6), $p = 0.0446$ (Exo^{19aKO} + PBS vs Exo + IBSP, $n = 4$ vs 6). Data are presented as mean values $\pm$ SEM (**a**). The bones were then removed and bone densities were measured by X-ray. Two-sided student's *t*-tests were performed; $p = 0.9280$ (Exo^{19aKO} + PBS vs Exo^{19aKO} + IBSP, $n = 4$ vs 6), $p = 0.9143$ (Exo^{19aKO} + PBS vs Exo + PBS, $n = 4$ vs 6), $p = 0.0257$ (Exo^{19aKO} + PBS vs Exo + IBSP, $n = 4$ vs 6). Data are presented as mean values $\pm$ SEM (**b**). **c** The removed bones were decalcified, sectioned, and examined for the amount of OC surface by TRAP staining followed by quantification using the ImageJ. Two-sided student's *t*-tests were performed; $p = 0.7224$ (Exo^{19aKO} + PBS vs Exo^{19aKO} + IBSP, $n = 4$ vs 6), $p = 0.8773$ (Exo^{19aKO} + PBS vs Exo + PBS, $n = 4$ vs 6), $p = 0.0002$ (Exo^{19aKO} + PBS vs Exo + IBSP, $n = 4$ vs 6). Data are presented as mean values $\pm$ SEM. **d** Integrin α V β 3 was seeded on the bottoms of 96-well plates. IBSP was then added to the well in the presence or absence of the library of natural compounds. The bound IBSP was measured by colorimetric substrates of alkaline phosphatase (AP). The OD 405 was compared between CGA-treated groups and the DMSO-treated group. Two-sided student's *t*-tests were performed; $p < 0.0001$ ($n = 3$) in all groups. CGA was found to most significantly inhibit the binding of IBSP to integrin α V β 3. Data are presented as mean values $\pm$ SEM. **e** The role of IBSP as a chemoattractant of osteoclast cells, and the inhibitory effect of CGA on this chemoattraction were examined by the μ -Slide Chemotaxis assay. The movement of 20 OC cells with the highest mobility was tracked and the directionality was calculated by Chemotaxis and Migration Tool. *p*-value was calculated with the Rayleigh test (left: $p = 0.0000002$, right: $p = 0.194763$).

(Supplementary Fig. 8f, h). On the other hand, CGA did not suppress the bone metastasis of 231BoM cells in our ER⁻ breast cancer xenograft model (Supplementary Fig. 8i). These results demonstrate that CGA is a promising preventive reagent for bone metastasis of ER⁺ breast cancer. With either CGA treatment or exosome blockade, the bone-metastatic lesions showed significantly decreased osteoclastogenesis (Fig. 7b) and increased bone density (Fig. 7c). We also confirm our findings in the T47D xenograft mouse model. When the T47D cells were implanted into mice by intracardiac injection, ectopic expression of miR-19a and IBSP significantly increased the rate of bone metastasis (Fig. 7d), decreased the bone density of the metastatic lesions (Fig. 7e), and increased the OC activities (Fig. 7f). The CGA treatment significantly decreased the rate of bone metastasis, and rescue the osteolytic changes in the bone lesions (Fig. 7d–f). Interestingly, when we generated organ culture of tumor tissues from various distant metastatic lesions in the T47D inoculated mice, we found significantly more exosomal miR-19a in the culture media of bone-metastatic lesions compared to other metastatic sites (Supplementary Fig. 8j). We also examined whether CGA was able to suppress the established bone metastasis. We first transplanted MCF7BoM2 into the tibial bone of mice. After confirming the tumor growth by measuring

the bioluminescent signals, CGA or bisphosphonate-DMDP (Dichloromethylenediphosphonic acid disodium salt) was given to the mice. We found that CGA was able to suppress the cancer cell growth of established tumors in the bone more effectively compared to the control group or the bisphosphonate treatment group (Fig. 7g, h).

Discussion

ER⁺ breast cancer patients have a considerably higher risk of bone metastasis^{20,22,53–55} compared to other subtypes. In this study, we identified exosomal miR-19a and IBSP as key secreted factors from ER⁺ bone-metastatic cells. Ectopic expression of one factor alone did not increase bone lesions in our animal models. However, upregulation of both factors together significantly promoted the tumor growth in the bone and osteoclastogenesis, indicating the cooperative roles of exosomal miR-19a and IBSP in promoting osteolytic bone metastasis. We found that ER⁺ cancer cells secrete IBSP as a chemoattractant for precursors of OC cells, while exosomal miR-19a enhances the osteoclastogenesis in the early metastatic site, which promotes the bone colonization of ER⁺ breast cancer cells (Fig. 8). Importantly, in both serum and transcriptome profiles of breast cancer patients, we verified that miR-19a and IBSP are correlated with bone metastasis and recurrence



status. While the serum of advanced breast cancer patients in this study was collected during the course of treatment with tumor burden, we did not see a significant difference in the expression of exosomal miR-19a and IBSP between groups with different treatment histories (Supplementary Data 5). These data suggest the potential use of exosomal miR-19a and IBSP of serum as liquid

biomarkers to stratify ER⁺ breast cancer patients with a high risk of bone recurrence.

MiR-19a is located in the miR-17-92 cluster (oncomiR-1), which is characterized as a set of mammalian microRNA oncogenes⁵⁶. The tumor-promoting role of miR-19a has been reported in multiple types of cancers^{57–61}, and it is known to

Fig. 7 CGA blocks cooperative function of miR-19a and IBSP in ER⁺ BCBM. **a** The mice were treated with 2 μ g exosomes prepared from MCF7BoM2 and 0.5 μ g recombinant IBSP by injecting them into the tibias every two days. The exosomes from MCF7BoM2/miR-19aKO and PBS were used as controls. After two weeks, the bones were removed, and the TRAP staining of the bones was performed. The OC surface of the bone surface was quantified by ImageJ and compared by the two-sided student's *t*-test. $p = 0.0181$ (PBS + Exo^{19aKO} vs IBSP + Exo), $p = 0.2436$ (PBS + Exo^{19aKO} vs PBS + Exo), $p = 0.4594$ (PBS + Exo^{19aKO} vs IBSP + Exo^{19aKO}). $n = 5$ in all groups. **b** The ratio of OC surface relative to the bone surface was calculated after TRAP staining. $p < 0.0001$ (MCF7BoM2 vs MCF7BoM2 + CGA, $n = 13$ vs 8), $p = 0.0001$ (MCF7BoM2 vs MCF7BoM2 + GW4869, $n = 13$ vs 5). Representative H&E staining of the same field was shown (left panel) together with the TRAP staining (right panel). Scale bar, 100 μ m. Two-sided student's *t*-tests were performed. Data are presented as mean values $\pm$ SEM. **c** The legs of tumor-bearing mice were imaged by X-ray, and the bone density was measured by ImageJ. Two-sided student's *t*-tests were performed. $p < 0.0001$ (MCF7BoM2 vs MCF7BoM2 + CGA, $n = 13$ vs 8), $p = 0.0002$ (MCF7BoM2 vs MCF7BoM2 + GW4869, $n = 13$ vs 5). Data are presented as mean values $\pm$ SEM. **d** T47D, T47DBoM, and T47D with ectopic expression of miR-19a or/and IBSP were intra-cardially injected into mice. One group of mice injected with T47DBoM also received CGA treatment. The growth of bone metastasis was monitored by IVIS Bioimager, and the Kaplan-Meier analysis and log-rank tests were performed for bone metastasis-free survival. $p = 0.000036$ (T47D vs T47DBoM); $p = 0.0078$ (T47D vs T47D/IBSP/miR-19a); $p = 0.0003$ (T47DBoM vs T47DBoM + CGA). **e** The legs of tumor-bearing mice were imaged by X-ray, and the bone density was measured by ImageJ. $p = 0.0027$ (T47D vs T47D/IBSP/miR-19a, $n = 4$ vs 12); $p = 0.0111$ (T47D vs T47DBoM, $n = 4$ vs 14); $p = 0.0009$ (T47DBoM vs T47DBoM + CGA, $n = 14$ vs 7). Two-sided student's *t*-tests were performed. Data are presented as mean values $\pm$ SEM. **f** The ratio of OC surface relative to the bone surface was calculated after TRAP staining. H&E staining of the same field was shown (left panels) together with the TRAP staining (right panels). Scale bar, 100 μ m. $p = 0.0028$ (T47D vs T47D/IBSP/miR-19a, $n = 4$ vs 12); $p = 0.0023$ (T47D vs T47DBoM, $n = 4$ vs 14); $p = 0.0003$ (T47DBoM vs T47DBoM + CGA, $n = 14$ vs 7). Two-sided student's *t*-tests were performed. Data are presented as mean values $\pm$ SEM. **g** One week after intra-tibia injection of MCF7BoM2, mice were treated with CGA or DMDP. The growth of bone tumors was monitored by measuring the luciferase activity by IVIS Bioimager. Two-sided student's *t*-tests were performed. $p = 0.0215$ (MCF7BoM2 vs MCF7BoM2 + CGA, $n = 8$ vs 6), $p = 0.1920$ (MCF7BoM2 vs MCF7BoM2 + DMDP, $n = 8$ vs 6). Data are presented as mean values $\pm$ SEM. **h** After 24 days, the legs were removed and the bioluminescence of each leg was quantified. Two-sided student's *t*-tests were performed. $p = 0.0144$ (MCF7BoM2 vs MCF7BoM2 + CGA, $n = 8$ vs 6), $p = 0.1747$ (MCF7BoM2 vs MCF7BoM2 + DMDP, $n = 8$ vs 6). Data are presented as mean values $\pm$ SEM.

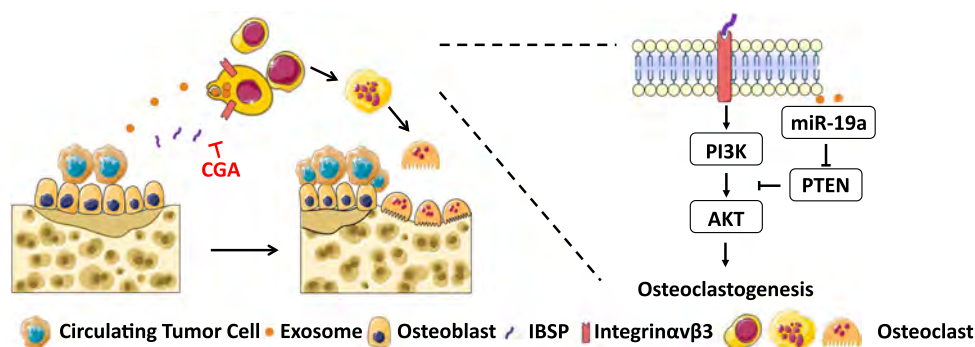


Fig. 8 Proposed mechanism of exosomal miR-19a and IBSP mediated bone metastasis in ER⁺ breast cancer. In the early metastatic niche, ER⁺ breast cancer cells secrete IBSP as a chemoattractant to recruit precursors of OC cells, creating an OC precursor-enriched microenvironment. OC precursors incorporate exosomes from breast cancer cells, and miR-19a is transported into OC precursors. Inside the OC precursors, miR-19a suppresses PTEN expression, activates NF- κ B and AKT pathways, and promotes osteoclastogenesis. Mature OC cells induce bone resorption, liberating growth factors from the bone matrix, which further promote the growth of cancer cells.

directly target tumor suppressor PTEN^{42,62,63}. It is also predicted to target ER. However, overexpression of miR-19a did not alter the level of ER in breast cancer cells⁶⁴, and the exact role of miR-19a in breast cancer is yet poorly understood. The previous study has characterized miR-19a targets in breast cancer by quantitative proteomic approach³⁰. No growth-suppressive effect was found when those targets were ectopically expressed, suggesting that endogenous miR-19a does not directly alter the growth of cancer cells per se. Consistent with their finding, the results of our study revealed that modulation of miR-19a expression in breast cancer cells did not alter the growth or migration abilities in vitro. Interestingly, ectopic expression of miR-19a did not affect the expression of PTEN in the cancer cells (data not shown). A possible explanation is that miR-19a is actively sorted out of cancer cells by exosomes. Accordingly, miR-19a expression is positively correlated with osteolytic bone metastasis in vivo, strongly suggesting a role of miR-19a as a key modulator of tumor microenvironment in the process of bone metastasis of breast cancer. In the “vicious cycle of bone metastasis”⁶⁵, breast cancer cells are known to interact with host cells to generate a favorable microenvironment for cancer cell growth in the bone

through direct cell-cell contact or via secreted proteins^{66–68}. We found that the oncogenic role of miR-19a in ER⁺ breast cancer is dependent on its secretory nature as exosomes. Exosomal miR-19a secreted from tumor cells are internalized into the OC precursor and induces the differentiation of OC into mature OC. OC osteoclastogenesis is one of the key steps related to the establishment of bone metastasis, and miR-19a augments this process by activating the p-AKT pathway. Therefore, this study revealed a specific role of miR-19a in ER⁺ breast cancer bone metastasis. The nature of miR-19a encapsulated inside exosomes also makes it a promising candidate as a circulating biomarker for diagnostic and prognostic purposes^{69–72}.

IBSP constitutes around 12% of the non-collagenous proteins in human bone and it is synthesized by bone resident cells. Several types of cancers, including colon, breast, prostate, and lung cancers were reported to have upregulated IBSP expression⁷³. In breast cancer, IBSP was also detected in cancer cells⁷⁴, and we found that IBSP is only expressed in breast cancer cells but not in normal breast tissue, making it a good candidate as a tumor-specific biomarker. Indeed, IBSP was previously reported to be associated with poor survival of breast cancer

patients⁷⁵. Interestingly, it was also found to be positively associated with bone metastasis, but negatively associated with lung metastasis in breast cancer⁷⁵. These observations can be well explained by our results, as we found IBSP regulates bone metastasis in ER⁺ breast cancer. ER⁺ breast cancer is known to have a higher incidence of bone metastasis compared to other subtypes, while they have a lower incidence of lung metastasis⁵⁵. By classifying subtypes of breast cancer patients, we have shown the feasibility of using IBSP, together with exosomal miR-19a to predict the risk of bone metastasis in ER⁺ patients. More importantly, we clarified a cooperative pathway between IBSP and miR-19a, that mobilizes the bone microenvironment to guide the ER⁺ breast cancer cells to the bone and support their cell growth. However, in this study, we did not observe a regulatory effect in gene expression between miR-19a and IBSP (Supplementary Fig. 2a, b). Therefore, the increased bone metastasis is likely to be a result of the colonial selection of high miR-19a/IBSP cells that adapt well to the early metastatic niche due to the interaction with OC cells.

Currently, there is no effective and safe regimen to prevent bone recurrence in breast cancer. The present bone-targeted therapy does not improve the survival of breast cancer patients, and patients with bone metastases still rely on systemic treatment. However, patients with bone recurrence develop resistance to systemic treatment as the cancer cells acquired resistance to these drugs at an early stage. In this study, we found that CGA blocks the interaction between IBSP and OCs, inhibiting the pro-osteoclastogenesis effect of cancer-derived exosomes. CGA is abundant in the extract from plants, and it has minimum side effects for long-term preventive treatment. By using exosomal miR-19a and IBSP to predict the risk of bone metastasis, ER⁺ breast cancer patients could benefit from this agent in preventing late-onset recurrence in the bone. CGA could also be used as a combination therapy to treat ER⁺ breast cancer patients with bone metastasis.

Collectively, our study identified exosomal miR-19a and IBSP as mediators of cell–cell communication between breast cancer cells and OC cells, which promotes the vicious cycle of bone metastasis in ER⁺ breast cancer. Importantly, the natural compound CGA can block this process. This study sheds light on the molecular mechanism of bone metastasis in ER⁺ breast cancer, which warrants further investigation on the miR19a-IBSP axis for the development of an effective therapeutic strategy.

Methods

Cells and cell culture. Human breast carcinoma cell lines, MDA-MB-231, MCF7, T47D, and mouse monocyte/macrophage cell line RAW 264.7 were purchased from American Type Tissue Culture Collection (ATCC, Manassas, VA, USA). MCF7BoM2 and 231BoM-1833, the bone-metastatic derivative of breast cancer cell lines were kindly provided by Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center)^{76,35}. To select bone-tropic T47DBoM, 100,000 T47D cells were injected into the left cardiac ventricle of athymic-nu/nu mice. Subsequently, bone-metastatic tumor cells were isolated from the tibias. Three rounds of selection were performed to establish the T47DBoM cell line. Luciferase-labeled cells were generated by infecting the lentivirus carrying the firefly luciferase gene. MDA-MB-231, MCF7 cells and their variants, as well as RAW264.7, were cultured in DMEM medium supplemented with 10% FBS and antibiotics. Murine bone marrow-derived monocytes and macrophages (BMMs) were generated as previously described⁷⁷. Briefly, bone marrow cells were harvested by flushing the femurs and tibias of six to eight-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA) with minimum essential medium- α (α -MEM; Invitrogen, Carlsbad, CA, USA). The cells were then washed, centrifuged, and incubated in complete α -MEM containing 10% heat-inactivated fetal bovine serum and penicillin/streptomycin for 18 h to separate the floating and adherent cells. The floating cells were collected and cultured in complete α -MEM supplemented with 50 ng/ml murine recombinant Macrophage Colony Stimulating Factor (M-CSF; Peprotech, Rocky Hill, NJ, USA). After 3 days, non-adherent cells were washed out and the adherent cells were used as BMMs.

Animal experiments. For experimental metastasis assay, cancer cells in 100 μ l of PBS were injected into the left cardiac ventricle of the athymic-nu/nu mice (7–8 weeks). For intratibial implantation of cancer cells, cells with or without ectopic expression of miR-19a and IBSP were injected into the tibia at a final concentration of 100,000 cells/10 μ l. To investigate the effect of miR-19a and IBSP on the primary tumor, cancer cells (1×10^6) in 50 μ l PBS were mixed with Matrigel matrix (Corning, Corning, NY, USA) at 1:1 ratio, and implanted into mammary fat pads of athymic-nu/nu mice. All mice received implantation of 1.7 mg estradiol continuous release pellets (Innovative Research of America). To confirm a successful injection, we immediately monitored photon flux from the whole body of the mice after injection. The growth of tumors in the bones was monitored and quantified by measuring luminescence using IVIS Xenogen bioimager (Caliper Life Science), and the images were analyzed using Living Image (Caliper Life Science) version 4.7.3 software and Aura software (Spectral Instruments Imaging, LLC) version 3.2. For CGA (Sigma-Aldrich, St. Louis, MO, USA) treatment, the compound dissolved in deionized water was administered orally at doses of 200 mg/kg body weight per day. For GW4869 (Cayman Chemical, Ann Arbor, MI, USA) treatment, the drug was intraperitoneally (i.p.) injected at the dose of 2.5 μ g/g every three days. For DMDP (Dichloromethylenediphosphonic acid disodium salt) treatment, the drug was intraperitoneally (i.p.) injected at the dose of 10 mg/kg twice a week. For *in vivo* exosome uptake assay, the mice were treated with 2 μ g Vybrant DiD-labeled exosomes with or without 0.5 μ g recombinant IBSP by injecting them into the tibias every two days. After two weeks, the bones were extracted and sectioned with a diamond knife. The sectioned bone was then stained with FITC-CTSK (Cathepsin K) and DAPI. The co-localization of OC and exosome was examined by Keyence All-in-one Fluorescence Microscope (BZ-X700). For measurement of the percentage of OC surface per bone surface (OC.S/BS%), bioluminescence and H&E staining were performed to identify the metastatic sites. For each of the mice, all the bone-metastatic sites were TRAP stained and cumulative osteoclast surface and bone surface were measured by ImageJ (Fiji, 1.52n)⁷⁸.

X-ray imaging. On days 28 before euthanization, X-ray images of the mice were captured with Faxitron Multifocus 10 $\times$ 15 Digital Radiography System. Mice were anesthetized and placed dorsal side up in the X-ray chamber with legs in the capture region. X-ray images of the tibias were analyzed using ImageJ (ImageJ 1.8.0_112; 64-bit; National Institute of Health) according to previous publications^{79,80} and relative bone density was quantified. The image was calibrated to a standard aluminum wedge. The average mean grayscale value of the area of interest was recorded after the value of background soft-tissue was subtracted.

Human L1000 array. The cell culture supernatants were dialyzed and biotinylated according to the manual of L-Series Human Antibody Array 1000 (RayBiotech, Peachtree Corners, GA, USA). The glass slide arrays were then locked and incubated with biotin-labeled samples to allow the binding of target proteins to the pre-printed capture antibodies. Streptavidin-conjugated fluorescent dye is then used to interact with the bound proteins. Finally, when the slide is dried, laser fluorescence scanning was performed with GenePix[®] 4000B Microarray Scanner to visualize signals. To compare the expression of each protein target, Protein Array Analyzer was used to quantify the signals.

Gene knockout and overexpression. IBSP and miR-19a knockout was carried out with CRISPR/Cas9 system. lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961; <http://n2t.net/addgene:52961>; RRID:Addgene_52961) Guide RNAs for IBSP (AGAACTTACTGAGAAAGCAC) and miR-19a (AATCTATG-CAAAACTGATGG) were cloned into vectors according to the provided protocol. Lentivirus was prepared by transfection of plasmids into HEK 293T cells. MCF7BoM2 was infected with lentivirus and screened with puromycin. The success of knockout was confirmed by the T7E1 assay (NEB). Single-cell clones were picked by limit dilution. Four miR-19a knockout clones and three IBSP knockout clones were selected. The knockout clones of each gene were mixed to generate the heterogeneous knockout cell lines and they were used for functional assays. The stabilities of the knockout were verified every five passages by PCR and western blot analysis. The plasmid for overexpression of IBSP (LV185867) was purchased from ABM (Applied Biological Materials, Richmond, BC, Canada) and the plasmid for overexpression of miR-19a (PCDH-miR-19a) was a kind gift from Dr. Yin-yuan Mo.

Osteoclastogenesis assay and tartrate-resistant acid phosphatase (TRAP) staining. Murine BMMs at a density of 1×10^6 cells/ml or RAW264.7 cells at a density of 1×10^5 cells/ml were plated in a chamber slide (ThermoFisher, Waltham, MA, USA) and cultured in complete α -MEM supplemented with 50 ng/ml murine recombinant M-CSF, 25 ng/ml murine recombinant RANKL (Peprotech, Rocky Hill, NJ, USA) in the presence or absence of 20 μ g/ml exosomes, a concentration used previously in multiple studies^{27,66,81,82}. On day 4, TRAP staining was performed using a leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The multinucleated TRAP-

positive cells from five biological replicates were considered mature OC cells and monitored by Zeiss Axioplan2 microscope. The size of OC cells was measured by ImageJ as reported⁸³.

Bone-resorption assay. To determine the role of exosomal miR-19a in the bone resorption activity of OC cells, pit assay was performed on bone slices (Immunodiagnostic Systems). 50,000 bone marrow-derived OC precursor BMMs were seeded on sterilized bone slices placed in a 96-well plate. The BMMs were cultured with complete α -MEM supplemented with 50 ng/ml murine recombinant M-CSF, 25 ng/ml murine recombinant RANKL (Peprotech, Rocky Hill, NJ, USA) in the presence or absence of 20 μ g/ml exosomes. The media was changed every 2–3 days. After two weeks of cell culture, cells were washed, and resorption pits were stained with 1% toluidine blue. The resorption area was photographed under microscope and the image was analyzed by ImageJ.

Extracellular vesicles purification and tracking analysis. Extracellular vesicles (EVs) from cells were isolated by ultracentrifugation. Cells were grown in EVs-depleted media for 48 h before CM was collected for EVs purification. To isolate total EVs, CM was centrifuged at $300 \times g$ for 10 min to remove cells. EVs were collected by ultracentrifugation at $120,000 \times g$ for 70 min. Apoptotic bodies (ABs), microvesicles (MVs), and exosomes were isolated by differential centrifugation as published before^{84,85}. Briefly, the harvested CM was centrifuged at $300 \times g$ for 10 min to remove cells. ABs were collected by centrifugation at $2000 \times g$ for 20 min. The supernatant was centrifuged at $16,500 \times g$ for 20 min to collect MVs. After that, the Supernatant was passed through a 0.2 μ m filter (Sarstedt, Nümbrecht, GERMANY) to remove the particles larger than 200 nm. Exosomes were then pelleted by ultracentrifugation at $120,000 \times g$ for 70 min. The isolated EVs were analyzed and quantified by nanoparticle tracking analysis (NTA) and electron microscope with negative staining.

Imaging of exosomes by transmission electron microscopy. For negative staining, Purified exosomes were incubated onto discharged 200 mesh copper EM grids and fixed with 2% Paraformaldehyde (PFA). 1% uranyl acetate (UA) solution was used to stain the exosomes on the surface of the EM grids. The exosomes were imaged by FEI Tecnai BioTwin Transmission Electron Microscope. For immunostaining of IBSP and CD63 on the surface of exosomes, Anti-CD63 (1:100) (Santa Cruz Biotechnology, Dallas, Texas, USA) and anti-IBSP (1:100) (ThermoFisher, Waltham, MA, USA) were used to incubate the fixed exosomes on the grids. The 10 nm colloidal gold-labeled protein A (1:50)(BOSTER BIOLOGICAL TECHNOLOGY, Pleasanton, CA, USA) was then used to label the primary antibodies. After that, negative staining was performed with 2% uranyl acetate and the grid was imaged by transmission electron microscopy.

Exosome uptake assay. Breast cancer cell lines were labeled with lentivirus expressing PalmGFP (a kind gift from Dr. Xandra O. Breakefield). Cells with intense fluorescence were selected by BD FACSAria Cell Sorter. Exosomes labeled with PalmGFP were isolated by differential ultracentrifuge. To monitor the uptake of exosomes in OC cells, RAW264.7 cells were seeded in chamber slides overnight and 20 μ g/ml exosomes were added into the media. After 24 h of incubation, the cells were washed and fixed. Cell nuclei were stained with DAPI followed by sealing the slides with coverslips. The uptake of exosomes was confirmed by examining the GFP signal under Olympus FV1200 SPECTRAL laser scanning confocal microscope.

ExoELISA. To compare the exosome production from cells after ectopic expression of miR-19a and IBSP, exosome concentration in the cell culture media was determined using ExoELISA from System Biosciences (SBI, Mountain View, CA, USA). According to the manufacturer's instruction, exosomes from a comparable amount of different cell lines were purified by ExoQuick-TC precipitation solution (SBI, Mountain View, CA, USA). Diluted exosomes, together with protein standards, were incubated with a provided microtiter plate. The detection antibody was added to the wells, followed by a horseradish peroxidase enzyme-linked secondary antibody. Finally, TMB ELISA substrate was added to develop the signal, and a stop buffer was used to stop the reaction. OD value was recorded with a spectrophotometric plate reader at 450 nm. The data were normalized to the cell count.

Immunohistochemistry. Immunohistochemical analysis was carried out for paraffin-embedded, surgically resected specimens of breast cancer bone metastases. Briefly, the sections were de-paraffinized, rehydrated, and heated at 100 °C for 20 min in Tris-buffered saline (pH 9.0) for antigen exposure. They were treated with 3% H₂O₂ to block endogenous peroxidase activity, followed by treatment with 5% BSA solution for 30 min. The slides were then incubated with primary antibodies targeting IBSP (Invitrogen, PA5-50633, 1:100) and E-cadherin (Cell Signaling Technology, clone 24E10, #3195, 1:200) for 16 h at 4 °C. After washing in PBS/0.1% Tween-20, the sections were treated with the secondary antibody anti-rabbit (Bio-Rad, #1706515, 1:500). The sections were washed extensively, and DAB substrate chromogen solution was applied followed by counterstaining with hematoxylin. For negative control, we added the rabbit IgG isotype control

(Invitrogen, 02-6102, 1:250) instead of primary antibodies during the primary antibody incubation. The expression of the protein of interest was evaluated by H scoring as the sum of the frequency and the intensity (0: none; 1: weak; 2: moderate; 3: strong). The final score = $1 \times (\% \text{ of } 1 + \text{cells}) + 2 \times (\% \text{ of } 2 + \text{cells}) + 3 \times (\% \text{ of } 3 + \text{cells})$. IHC scores were determined by concordance among the scores of two independent reviewers.

MTS assay. Viability of cell lines was quantified by using the CellTiter 96 Aqueous One solution cell proliferation assay kit (Promega, Madison, WI, USA). Cells were plated in 96-well tissue culture plates at a density of 5000 cells per well. At the time of measurement, 20 μ l of CellTiter 96 Aqueous One solution reagent was added into each well of the 96-well assay plate containing the samples in 100 μ l of culture medium. The plate was incubated at 37 °C for 1 h in a humidified, 5% CO₂ atmosphere. The absorbance at 490 nm was measured and recorded.

Wound-healing migration assay. The cell migration was estimated by a wound-healing migration assay and monitored by the Olympus IX-70 microscope. Cells were seeded in 12 well cell culture dish and a scratch was made on the monolayer using a sterile tip when cells reach 100% confluence. The distance of migration by cancer cells was measured every 24 h.

Real-time PCR and western blotting. Total RNA was isolated by using Direct-zol RNA MiniPrep Plus (Zymo Research Corp, Irvine, CA, USA) and miRNeasy Mini Kit (Qiagen, Germantown, MD, USA) for mRNA and microRNA, respectively. We used iScript Reverse Transcription Supermix (Life Science Research, Hercules, CA, USA) and TaqMan MicroRNA Reverse Transcription kit (ThermoFisher Scientific, Waltham, MA, USA) for mRNA and miRNA reverse transcription, respectively. Quantitative PCR (qPCR) for miRNA was conducted by using TaqMan Universal Master Mix II and TaqMan microRNA assays (ThermoFisher Scientific, Waltham, MA, USA). All miRNA assays were purchased from ThermoFisher Scientific. U6 snRNA and RNU48 TaqMan probes were used as endogenous controls for human and mouse cells, respectively. For coding genes, qPCR was conducted by using iTaq Universal SYBR Green Supermix (Life Science Research, Hercules, CA, USA). Primers used in this study were listed in Supplementary Data 1. Western blotting was carried out using general methods. Briefly, the protein was extracted from the cells using RIPA buffer, quantified by Protein Assay (Bio-Rad, Hercules, California, USA), resolved by 10% SDS-polyacrylamide gel, and then electro-transferred to nitrocellulose membrane. Primary antibodies against IBSP (Invitrogen, PA5-50633, 1:500), Phospho-NF- κ B p65 (Ser536) (Cell Signaling Technology, clone 93H1, #3033, 1:1000), NF- κ B p65 (Cell Signaling Technology, #8242, 1:1000), Phospho-Akt (Ser473) (Cell Signaling Technology, clone D9E, #4060, 1:3000), Akt (Cell Signaling Technology, #9272, 1:2000), HSP70 (Invitrogen, Clone 5A5, #MA3-007, 1:1000), PTEN (Santa Cruz Biotechnology, clone A2B1, sc-7974, 1:1000), CD63 (Santa Cruz Biotechnology, clone MX-49.129.5, sc-5275, 1:500), TSG101 (Santa Cruz Biotechnology, clone C-2, sc-7964, 1:1000), GRP94 (Santa Cruz Biotechnology, clone H-10, sc-393402, 1:500), α -actinin-4 (Santa Cruz Biotechnology, clone G-4, sc-390205, 1:500), Calregulin (Santa Cruz Biotechnology, clone A-9, sc-166837, 1:500), GAPDH (Cell Signaling Technology, clone D16H11, #5174, 1:15,000), α -tubulin (Cell Signaling Technology, #2144, 1:2000) were used. HRP-conjugated anti-mouse IgG (Cell Signaling Technology, #7076) or anti-rabbit IgG (Bio-Rad, #1706515) and mouse IgG kappa binding protein m-IgG κ BP-HRP (Santa Cruz Biotechnology, sc-516102) were used as secondary antibodies. Immunoblotting images were captured by Amersham Imager 600 or the X-ray film processor. Images were quantified with ImageJ software.

Receptor-ligand in vitro binding assay. The receptor–ligand in vitro binding assay was developed according to previous publications^{86,87}. Briefly, a high-binding 96-well plate was coated with integrin α V β 3 in the carbonate-bicarbonate buffer. The plate was sealed and incubated at 4 °C overnight. The plate was then washed and blocked with PBS (0.1% Tween and 1% BSA). IBSP with or without the natural compound library was incubated with coated integrin α V β 3 at room temperature for 2 h. The plate was then washed and further incubated with primary antibody Goat anti-IBSP (R&D SYSTEMS, AF4014, 1:100) or Goat IgG Control (R&D SYSTEMS, AB-108-C, 1:100). Anti-goat IgG tagged with either HRP (horseradish peroxidase) (Invitrogen, A15999, 1:5000) or AP (alkaline phosphatase) (Invitrogen, A16002, 1:2500) was used as the secondary antibody. After washing the plates, the substrate was added, and the plates were incubated for 30 min followed by adding the stop solution. The binding of integrin α V β 3 and IBSP was quantified by measuring the absorbance value with a plate reader.

μ -Slide chemotaxis assay. The μ -Slide chemotaxis assay was carried out according to the manufacturer's instructions. Briefly, OC precursors BMMs at 3×10^6 cells/ml were mixed with collagen type I gels and other ingredients before being seeded into the middle channel. Left and right chambers were filled with FBS media with or without IBSP. The movement of cells was tracked according to a previous publication⁸⁸. Time-lapse video microscopy was performed using an inverted Nikon microscope. Cell tracking was performed using the ImageJ software (National Institutes of Health, Bethesda, USA) plugin “Manual Tracking” (Fabrice

Cordelières, Institut Curie, Orsay, France), then analyzed with Chemotaxis and Migration Tool (Version 2.0, IBIDI).

Clinical samples. Clinical samples from breast cancer patients and healthy individuals were obtained from Tumor Tissue and Pathology Shared Resource of Wake Forest Baptist Comprehensive Cancer Center and Cooperative Human Tissue Network. The serum samples from early-stage breast cancer patients were collected before surgery and the serum samples from advanced breast cancer patients were collected during the course of treatment. Population characteristics including patient age, race, diagnosis, metastasis, receptor status, and treatment history were retrieved from medical records, and summarized in Supplementary Data 2 and 3. The patient information of tumor tissue and healthy bone tissue was summarized in Supplementary Data 4.

MiRNA array, bioinformatics, statistical tests, and reproducibility. EVs from MCF7 and MCF7BoM2 were collected and miRNA was extracted using miRNeasy Micro Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). Expression profiling of miRNA was performed by using the Affymetrix GeneChip miRNA Array. For calculation of FDR and fold change in differential expression analyses, the ComparativeMarkerSelection module from GenePattern⁸⁹ (Broad Institute, Cambridge, MA, USA) was used. For clustering and visualization, the HierarchicalClustering and HierarchicalClusteringViewer modules from GenePattern were used. For metastasis-free survivals, Kaplan–Meier curves were plotted using the time to event as the outcome. Statistical differences in survival across groups were assessed using the log-rank test. For other analyses, student's *t*-tests (two-sided) were used unless otherwise noted. All other statistical analyses were performed using the GraphPad Prism 8 Program (GraphPad Software, San Diego, CA, USA). Asterisk: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.001, ^{ns}*p* ≥ 0.05. Representative experiments were repeated independently three times with similar results.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

CEL and expression files associated with the GeneChip miRNA array in this study are deposited at GEO (<https://www.ncbi.nlm.nih.gov/geo/>) with the accession number GSE176105. The GEO data set (GSE41922, GSE22220, GSE56493, GSE2034), TCGA referenced during the study are available in a public repository from <https://www.ncbi.nlm.nih.gov/geo/> and <https://portal.gdc.cancer.gov/> websites. The source data underlying Figs. 1–7 and Supplementary Figs. 1–8 are provided as a Source Data file. All the other data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file. Source data are provided with this paper.

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The authors declare no competing interests.

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Functional roles of neuromedin B and gastrin-releasing peptide in regulating itch and pain in the spinal cord of non-human primates

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ABSTRACT

Despite accumulating evidence in rodents, the functional role of neuromedin B (NMB) in regulating somato-sensory systems in primate spinal cord is unknown. We aimed to compare the expression patterns of NMB and its receptor (NMBR) and the behavioral effects of intrathecal (i.t.) NMB with gastrin-releasing peptide (GRP) on itch or pain in non-human primates (NHPs). We used six adult rhesus monkeys. The mRNA or protein expressions of NMB, GRP, and their receptors were evaluated by quantitative reverse transcription polymerase chain reaction, immunohistochemistry, or *in situ* hybridization. We determined the behavioral effects of NMB or GRP via acute thermal nociception, capsaicin-induced thermal allodynia, and itch scratching response assays. NMB expression levels were greater than those of GRP in the dorsal root ganglia and spinal dorsal horn. Conversely, NMBR expression was significantly lower than GRP receptor (GRPR). I.t. NMB elicited only mild scratching responses, whereas GRP caused robust scratching responses. GRP- and NMB-elicited scratching responses were attenuated by GRPR (RC-3095) and NMBR (PD168368) antagonists, respectively. Moreover, i.t. NMB and GRP did not induce thermal hypersensitivity and GRPR and NMBR antagonists did not affect peripherally elicited thermal allodynia. Consistently, NMBR expression was low in both itch- and pain-responsive neurons in the spinal dorsal horn. Spinal NMB-NMBR system plays a minimal functional role in the neurotransmission of itch and pain in primates. Unlike the functional significance of the GRP-GRPR system in itch, drugs targeting the spinal NMB-NMBR system may not effectively alleviate non-NMBR-mediated itch.

1. Introduction

Of all nociceptive somatosensory systems, acute itch/pruritus is an essential alert system that eliminates noxious components by scratching responses. However, intractable itch associated with several skin or systemic diseases (e.g., atopic dermatitis, dry skin, diabetes mellitus, and cholestatic liver disease) significantly affects the quality of life and causes huge economic losses globally [1,2]. Several lines of evidence reveal critical molecules that contribute to the transmission of itch from the periphery to the central nervous system [3–5]. The intrathecal (i.t.) administration of gastrin-releasing peptide (GRP), a mammalian bombesin-related peptide, elicits robust itch-related scratching behavior

in rodents and primates [6,7]. Moreover, the ablation of GRP receptor (GRPR)⁺ neurons in the spinal dorsal horn (SDH) markedly reduces scratching behavior induced by different types of pruritic stimuli, without affecting the pain threshold in rodents [8,9]. The enhancement of GRP-GRPR activity based on the upregulation of each molecule and the sensitization of GRPR⁺ neurons may not only underlie acute but also chronic itch [10,11].

Neuromedin B (NMB) is a decapeptide that bears close homology with bombesin-related peptide, and it mediates several physiological functions such as smooth muscle contraction, hormone secretion, and cell growth [12,13]. In contrast to GRP, NMB may be involved in both itch and pain signaling. Initially, intracerebroventricular administration

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of NMB elicits itch-related scratching behavior [14], supported by other rodent studies using i.t. or an intracerebroventricular delivery route [15–17]. However, the degree of NMB-induced scratching is considerably less than that of GRP. Conversely, the intraplantar administration of NMB elicits nociceptive hypersensitivity and the ablation of NMB receptor (NMBR)⁺ neurons results in thermal hypoalgesia [18]. NMBR and GRPR, which have close structural homology, are predominantly expressed in separate subpopulations of spinal neurons, and the NMB-NMBR system may function upstream of the GRP-GRPR system in rodents [15,19]. Despite accumulating evidence in rodents, there is no evidence regarding the functional role of spinal NMB-NMBR system for regulating itch and pain in primates.

A substantial neurochemical gap between rodents and primates [20–22] warrants confirming the functional significance of key molecules contributing to sensory processing in primates. The pharmacological effects of opioid peptides (i.e., μ -opioid peptides and nociceptin/orphanin FQ) on pain and itch-related behaviors in non-human primates (NHPs) differ from those in rodents [23–25]. The i.t. administration of μ -opioid receptor agonists, such as morphine, produces itch sensation in patients and elicits robust scratching behavior in NHPs [6,26]; however, the degree of scratching in rodents is much less than that in NHPs [16,27,28]. Unlike opioids, the behavioral effects of i.t. GRP in rodents are similar to those in NHPs [6,29], thereby indicating the functional significance of GRP-GRPR system in rodents can be translated to NHPs. NMBR is distributed in the central nervous system of primates [30], thus functional evidence for the NMB-NMBR system in NHPs is needed to understand the mechanisms in the spinal transmission of itch. We aimed to compare the expression patterns of NMB-NMBR and GRP-GRPR systems and the behavioral effects of i.t. NMB with GRP on itch or pain sensation in NHPs.

2. Materials and methods

2.1. Animals

We used six adult rhesus monkeys (*Macaca mulatta*), comprising four males and two females, with a body weight of 6–11 kg and aged 10–16 years. The animals were housed at an indoor facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Frederick, MD, USA). They were individually placed in cages with a floor space of 6–12 square feet, 2.7–5.4-foot high ceiling, and an environmentally controlled room (21–25 °C, 40–60% relative humidity) with a 12-h light/dark cycle (lights on: 6:30–18:30). They were provided with water, monkey chow (LabDiet St. Louis, MO, USA), and fresh fruit *ad libitum*. We provided them with primate enrichment devices and treats daily. They were not subjected to any experiment 1 month before initiating the study. The animals were assigned to each experiment based on the tasks they were trained to perform. All experiments followed a within-subject design (i.e., each group of animals served as its own control and all dosing conditions were randomized by a counterbalanced design). All experiments were conducted during late mornings of weekdays until the completion of time courses or testing sessions. All animal care and experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (Bethesda, MD, USA), and were approved by the Institutional Animal Care and Use Committee of Wake Forest University (Winston-Salem, NC, USA). The present study was reported in accordance with the Animal Research: Reporting of In Vivo Experiments [31].

2.2. Tissue collection

Researchers have previously described detailed procedures for tissue collection [32]. Briefly, the animals were deeply anesthetized with sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA) and the vascular system was flushed with saline (Baxter, Deerfield, IL, USA). The

spinal column was dissected to expose the spinal cord and the attached dorsal root ganglia (DRG). For reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR), the spinal dorsal horn (SDH) and DRG tissues from the lumbar segment 4 (L4) were flash frozen in dry ice. For *in situ* hybridization and immunohistochemistry, one block of L4 spinal cord tissue was fixed in 4% paraformaldehyde (Sigma-Aldrich), cryopreserved in 30% sucrose (Sigma-Aldrich) prepared in phosphate buffered saline (PBS) (Sigma-Aldrich), and embedded in an optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA). All tissues were stored at –80 °C until use.

2.3. RT-qPCR

We performed SDH and DRG tissue processing and the RT-qPCR protocol according to a previous study [32]. The mRNA expression levels of *NMB*, *GRP*, *NMBR*, and *GRPR* from five animals were quantified based on the comparative threshold cycle (C_T) method [33]. PCR amplification efficiency for each primer was determined by the slope of standard curve generated from tenfold serial dilutions (0.02–20 ng) of the cDNA mixture from subjects. The average C_T values were obtained from three replicates and normalized against that of β -actin (*ACTB*) using the following formula: $2^{-(C_{T \text{ target gene}} - C_{T \text{ ACTB}})} (2^{-\Delta C_T})$.

2.4. Immunohistochemistry

Fixed frozen spinal cord tissues were sectioned at a thickness of 30 μ m using a cryostat (Leica CM3050S; Leica Biosystems, Wetzlar, Germany) for free-floating immunostaining. The tissue sections were washed in PBS containing 0.3% Triton X-100 (Sigma-Aldrich) and treated with TrueBlack Lipofusin Autofluorescence Quencher (Biotium, Fremont, CA, USA) for 2 min. After washing with PBS, we blocked the tissues with PBS containing 3% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 1 h. The sections were incubated with a primary antibody against GRPR (rabbit polyclonal, 1:500 dilution; MC-831, MBL International, Woburn, MA, USA), NMBR (rabbit polyclonal, 1:500 dilution; ABR-004, Alomone Labs, Jerusalem, Israel), and NeuN (mouse monoclonal, 1:500 dilution; MAB377, MilliporeSigma, Burlington, MA, USA; markers for neuronal nuclei) at 4 °C overnight. The following day, we rinsed the sections in PBS before incubation with donkey anti-rabbit or anti-mouse IgG secondary antibody conjugated to AlexaFluor 594 or 488, respectively (1:500 dilution; Jackson ImmunoResearch Laboratories), at room temperature for 3 h. All antibodies were prepared in PBS containing 1% normal donkey serum. The tissue sections were rinsed in PBS, mounted on glass slides to air dry, and cover-slipped with Prolong Gold AntiFade mounting media (ThermoFisher Scientific, Waltham, MA, USA).

2.5. *In situ* hybridization

Fixed frozen spinal cord tissues were sectioned at a thickness of 10 μ m using a cryostat (Leica) for detecting mRNA expression. We performed *in situ* hybridization according to the protocol of the RNAscope Multiplex Fluorescent v2 Assay (Advanced Cell Diagnostics, Newark, CA, USA). Briefly, the spinal cord sections were fixed in 4% paraformaldehyde, dehydrated in ethanol, and treated with H₂O₂, target retrieval reagent, and protease III. The tissues were hybridized with commercial probes for NMBR (# 821681, GRPR (# 821671), protein kinase C γ (PKCG; # 837971; marker for pain processing neurons), vesicular glutamate transporter 2 (VGLUT2; # 540961; marker for excitatory neurons), and vesicular GABA transporter (VGAT; # 428881; marker for inhibitory neurons). Moreover, we visualized the nuclei with the DAPI. The tissue sections were cover-slipped with Prolong Gold AntiFade mounting media, followed by the capture of confocal images using Olympus FV1200 confocal microscope (Olympus America, Center Valley, PA, USA). For quantifying the percentage of co-expression, we captured mosaic images with Z series by setting a fixed matrix that

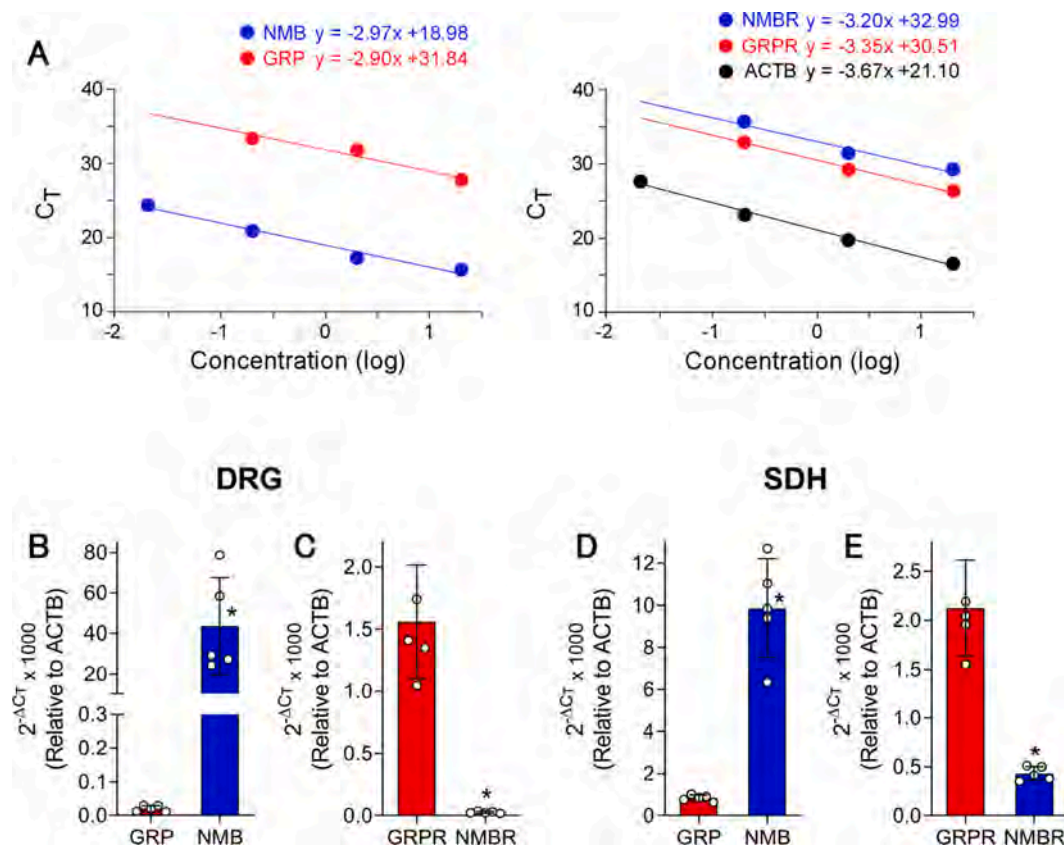


Fig. 1. A comparison of mRNA expression of *GRP* versus *NMB*, and *GRPR* versus *NMBR*, in the dorsal root ganglia and spinal dorsal horn of monkeys. mRNA levels were quantified using RT-qPCR and analyzed by the comparative C_T method. (A) PCR amplification efficiency for each primer (*NMB*, *GRP*, *NMBR*, *GRPR*, and *ACTB*) was determined by the slope of standard curve generated from tenfold serial dilutions (0.02–20 ng) of the cDNA mixture from subjects. (B–E) Relative mRNA expression levels in the dorsal root ganglia (B, C) and spinal dorsal horn (D, E). Data represent the mean $\pm$ SD ($n = 5$), with dots representing individual NHP. Data were analyzed by the paired t -test. * $p < 0.05$, significantly different between *GRP* and *NMB* (B, D) or *GRPR* and *NMBR* (C, E).

contained the entire spinal dorsal horn. To quantify the distribution of *NMBR*⁺ and *GRPR*⁺ cells, we identified laminae I–III versus IV–V using standard lamination criteria [34]. Cells containing at least three dots of punctate staining were counted with the software Image J, developed by the National Institute of Health (NIH, Bethesda, MD, USA).

2.6. Itch scratching responses

We recorded the scratching responses of six animals in their home cages to assess the itching sensation caused by test compounds [35]. Each 15-min recording session followed the i.t. administration of *NMB*, *GRP*, or a mixture of *NMB* or *GRP* with different antagonists. The number of scratches was the primary outcome. A scratch was defined as one brief (<1 s) scraping on the skin surface of other body parts using the forepaw or hind paw. The total number of scratches for each 15-min period was calculated by experimenters blinded to the dosing conditions.

2.7. Acute thermal nociception

We performed the warm water tail-withdrawal assay to measure nociceptive responses of five animals to thermal stimuli and the effects of test compounds [36,37]. The animals were seated in primate restraint chairs, followed by the immersion of lower parts of their shaved tails (~15 cm) in water maintained at 42 °C, 46 °C, or 50 °C. We used water at 42 °C or 46 °C as a normally non-noxious stimulus (i.e., no tail-withdrawal expected), whereas that at 50 °C was used as an acute noxious stimulus (i.e., 2–3 s tail-withdrawal latency); however, it did not cause thermal injury. The tail-withdrawal latency was the primary

outcome. The animals were randomly assigned to the dosing condition. Experimenters unaware of the dosing conditions measured the tail-withdrawal latencies at each temperature, randomly using a computerized timer. They recorded a maximum duration of 20 s (the cut-off) if the animal did not withdraw its tail within 20 s. Test sessions commenced with the baseline measurements at each temperature. Subsequently, the tail-withdrawal latencies were measured at 15 min, 30 min, 45 min, and 60 min following the i.t. administration of the test compound.

2.8. Capsaicin-induced thermal allodynia

We evaluated the antiallodynic effects of the test compound using a 1-h pretreatment regimen (i.e. 1 h before capsaicin administration) [38,39] in five animals. Capsaicin (1.2 mg/mL $\times$ 0.3 mL) was topically administered via a bandage attached to the terminal 3–5 cm of the tail for 15 min. The allodynic response was manifested as reduced tail-withdrawal latency from a maximum value of 20 s to approximately 2–3 s in 46 °C water. The aforementioned allodynic effect peaked at 15 min following the removal of the capsaicin bandage, during which we measured the tail withdrawal latency in 46 °C water (i.e., to determine the antiallodynic effects of the test compound).

2.9. Drugs

NMB (Sigma-Aldrich), *RC-3095* (Sigma-Aldrich), *GRP* (Tocris Bioscience, Minneapolis, MN, USA), and *DAMGO* ([D-Ala²,N-MePhe⁴,Gly-oI⁵]-enkephalin) (Cayman Chemical, Ann Arbor, MI, USA) were dissolved in sterile water. *PD168368* (Tocris) stock solution was

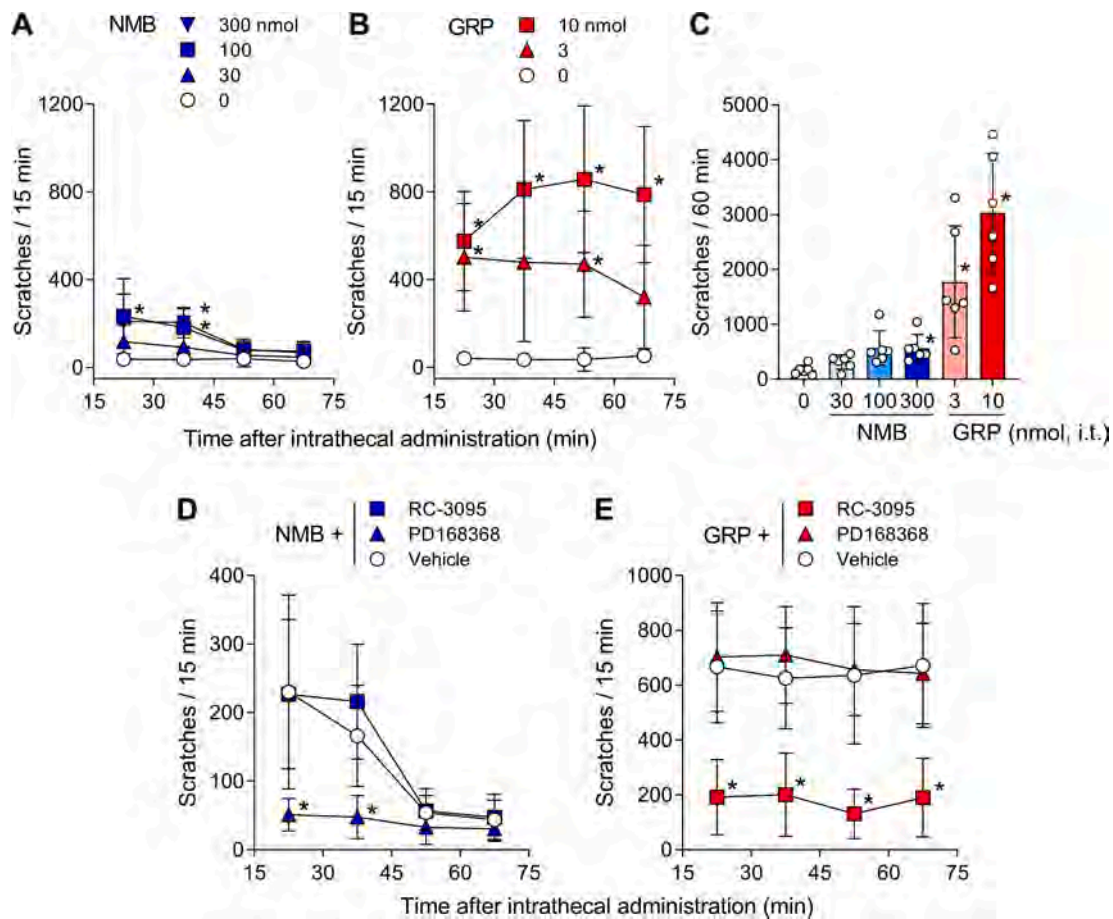


Fig. 2. Effects of intrathecal NMB and GRP on itch scratching responses in monkeys. (A, B) Time courses of itch scratching responses elicited by varying doses of NMB and GRP. (C) Total number of scratches summed from the four time points displayed in A and B. (D, E) Time courses of itch scratching responses elicited by NMB (100 nmol) or GRP (10 nmol), in combination with the GRPR antagonist RC-3095 (100 nmol) or NMBR antagonist PD168368 (300 nmol). Data represent the mean $\pm$ SD ($n = 6$), with dots representing individual NHP. Data were analyzed by the two- (A, B, D, and E) or one-way (C) repeated measures ANOVA, followed by Dunnett's multiple comparison test. * $p < 0.05$, significantly different from the vehicle condition.

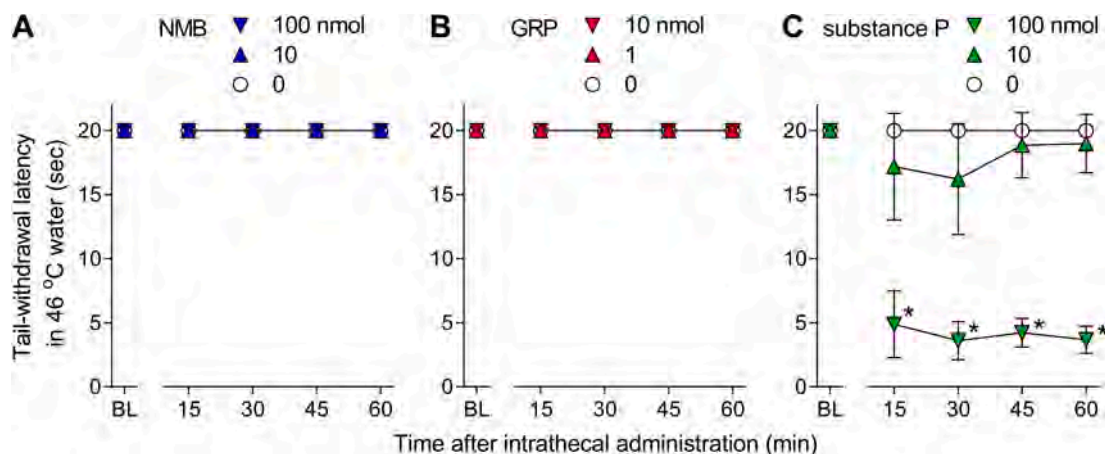


Fig. 3. Effects of intrathecal NMB and GRP on the thermal nociceptive threshold, compared with substance P in monkeys. (A-C) Time courses of tail-withdrawal latencies measured in 46 °C water following the intrathecal administration of varying doses of NMB (A), GRP (B), or substance P (C). Data represent the mean $\pm$ SD ($n = 5$), and were analyzed by the two-way repeated measures ANOVA, followed by Dunnett's multiple comparison test * $p < 0.05$, significantly different from the vehicle condition.

prepared in a 1:1:8 ratio of dimethyl sulfoxide (ThermoFisher Scientific), Tween 80 (ThermoFisher Scientific) and sterile water, and diluted with sterile water to obtain the target working solution. The doses of these test ligands were based on previous NHP studies [6,35,40]. We

administered the drugs intrathecally following a previously described drug delivery procedure [35]. Briefly, the animals were positioned in primate restraint chairs and anesthetized by intravenous propofol (2.5–4.0 mg/kg for bolus infusion and 0.3–0.4 mg/kg/min for

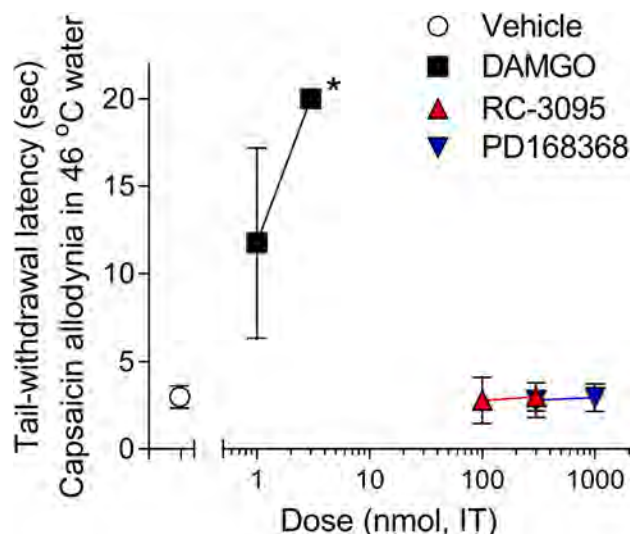


Fig. 4. Effects of intrathecal NMBR and GRPR antagonists on peripherally elicited pain, compared with DAMGO ([D-Ala²,N-MePhe⁴,Gly-ol⁵]-enkephalin) in monkeys. Topical capsaicin-induced allodynia was evaluated by tail-withdrawal assays in 46 °C water following the intrathecal administration of μ -opioid receptor agonist DAMGO (1 and 3 nmol), GRPR antagonist RC-3095 (100 and 300 nmol), or NMBR antagonist PD168368 (300 and 1000 nmol). Data represent the mean $\pm$ SD ($n = 5$), and were analyzed via a one-way repeated measures ANOVA, followed by Dunnett's multiple comparison test. * $p < 0.05$, significantly different from the vehicle condition.

continuous infusion; AstraZeneca Pharmaceuticals LP, Wilmington, DE, USA). A spinal needle (22-gauge $\times$ 1.5; BD Biosciences, Franklin Lakes, NJ, USA) was inserted into the subarachnoid space between the L4/L5 lumbar vertebra. A 1-mL solution of compound was slowly infused through the spinal needle within 30 s. Animals recovered from anesthesia within 10 min after the termination of propofol infusion, and were returned to their home cages.

2.10. Statistical analyses

Data are presented as mean values $\pm$ SD. We performed comparisons for similar monkeys across all test sessions for one experiment. We conducted the paired t -test (Fig. 1) and two- or one-way repeated measures analysis of variance (ANOVA) (Figs. 2, 3, and 4) to compare the outcome measures (i.e., tail-withdrawal latency and the number of scratches). The criterion for significance was set at p -value < 0.05 . We used GraphPad Prism version 9 software for statistical analyses. Behavioral data collection was performed under blinded conditions. No statistical power calculations were performed prior to the study. The sample size was based on our previous experience with this design [38,40].

3. Results

3.1. Expression of NMB, GRP, and their receptors in the DRG and SDH

First, we investigated the gene expression levels of *NMB*, *GRP*, and their receptors in the DRG and SDH of NHPs by RT-qPCR. The slopes of the standard curves for each gene were almost identical, which indicated that the PCR amplification efficiencies were similar among same categories of molecules (Fig. 1A). In both the DRG and SDH, mRNA expression levels of *NMB* (relative to *ACTB*) were substantially greater than those of *GRP* (Fig. 1B, D), whereas the levels of *NMBR* were significantly lower than those of *GRPR* (Fig. 1C, E).

3.2. Effects of i.t. NMB and GRP on itch sensation

We conducted behavioral analyses to evaluate the effects of spinally-delivered NMB and GRP on itch sensation in NHPs. The i.t. administration of NMB transiently elicited scratching behavior in a dose-dependent manner (Fig. 2A, C). In contrast, the i.t. administration of GRP caused robust and prolonged scratching behavior (Fig. 2B, C). NMB-induced scratching behavior was completely blocked by the NMBR antagonist (PD168368), but not the GRPR antagonist (RC-3095) (Fig. 2D). Conversely, GRP-induced scratching behavior was antagonized by RC-3095, but not PD168368 (Fig. 2E).

3.3. Effects of i.t. NMBR or GRPR ligands on pain sensation

Subsequently, we determined the impact of i.t. NMB and GRP on pain sensitivity in NHPs. Neither NMB nor GRP affected withdrawal latency to innocuous stimuli, whereas the i.t. administration of substance P reduced the latency to stimuli, thus displaying thermal allodynia (Fig. 3). Moreover, we assessed the anti-allodynic effects of PD168368 or RC-3095 on capsaicin-induced thermal allodynia. Unlike DAMGO, a μ -opioid receptor agonist with anti-allodynic effects, PD168368 and RC-3095 did not exert an effect on thermal allodynia over the behaviorally active dose range (Fig. 4).

3.4. Localization of NMBR in different subsets of neurons in the SDH

We characterized the distributions of NMBR and GRPR in the SDH. By immunohistochemistry with NeuN antibody that labels neuronal nuclei, protein expressions of NMBR and GRPR were located on the NeuN⁺ neurons mainly in the superficial laminae and much less in the deeper area (Fig. 5A, B). By *in situ* hybridization using RNAscope, close to 90% of the NMBR⁺ neurons and GRPR⁺ neurons were distributed in laminae I-III and only about 10% were in laminae IV-V (Fig. 6A, D). Less than 20% of the NMBR was colocalized with GRPR (Fig. 6A, E), and the majority of NMBR and GRPR did not colocalize with PKC γ , a marker of pain-responsive neurons (Fig. 6B, C, F). Approximately 60% and 70% of the NMBR⁺ neurons and GRPR⁺ neurons, respectively, were VGLUT2⁺ excitatory neurons (Fig. 7A, B, E), whereas the minority of NMBR⁺ and GRPR⁺ neurons were VGAT⁺ inhibitory neurons in the SDH (Fig. 7C, D, F).

4. Discussion

We provided three key findings to define the functional roles of the NMB-NMBR system in the SDH of NHPs. First, the i.t. administration of NMB elicited a milder scratching behavior with lower potency than GRP. Moreover, the activation of spinal NMB-NMBR or GRP-GRPR systems resulted in scratching responses via independent receptor mechanisms. Second, i.t. NMB and GRP did not induce thermal hypersensitivity. Neither did i.t. NMBR and GRPR antagonists modulate peripherally elicited thermal allodynia induced by capsaicin. Third, NMBR displayed lower expression in both itch- and pain-responsive neurons in the SDH, namely GRPR⁺ and PKC γ ⁺, respectively. In other words, the spinal NMB-NMBR system plays a limited role in the neurotransmission of itch and pain in NHPs.

Exploring the functional roles of spinal NMB-NMBR and GRP-GRPR systems in regulating itch and pain in primates necessitates determining their expression patterns in the sensory neurons and SDH. Despite the functional significance of GRP in itch transmission [8,19,41,42], sources of GRP contributing to itch sensation are still controversial in rodents. GRP is produced in the interneurons located on the SDH [43–45], whereas a recent report demonstrated that functionally-important GRP is located on the sensory neurons, despite lower expression levels of GRP in the DRG than that in the SDH [46]. Nevertheless, expression patterns of GRP in NHPs are consistent with findings from rodent studies that GRP-expressing cells are principally located on the SDH, but not the

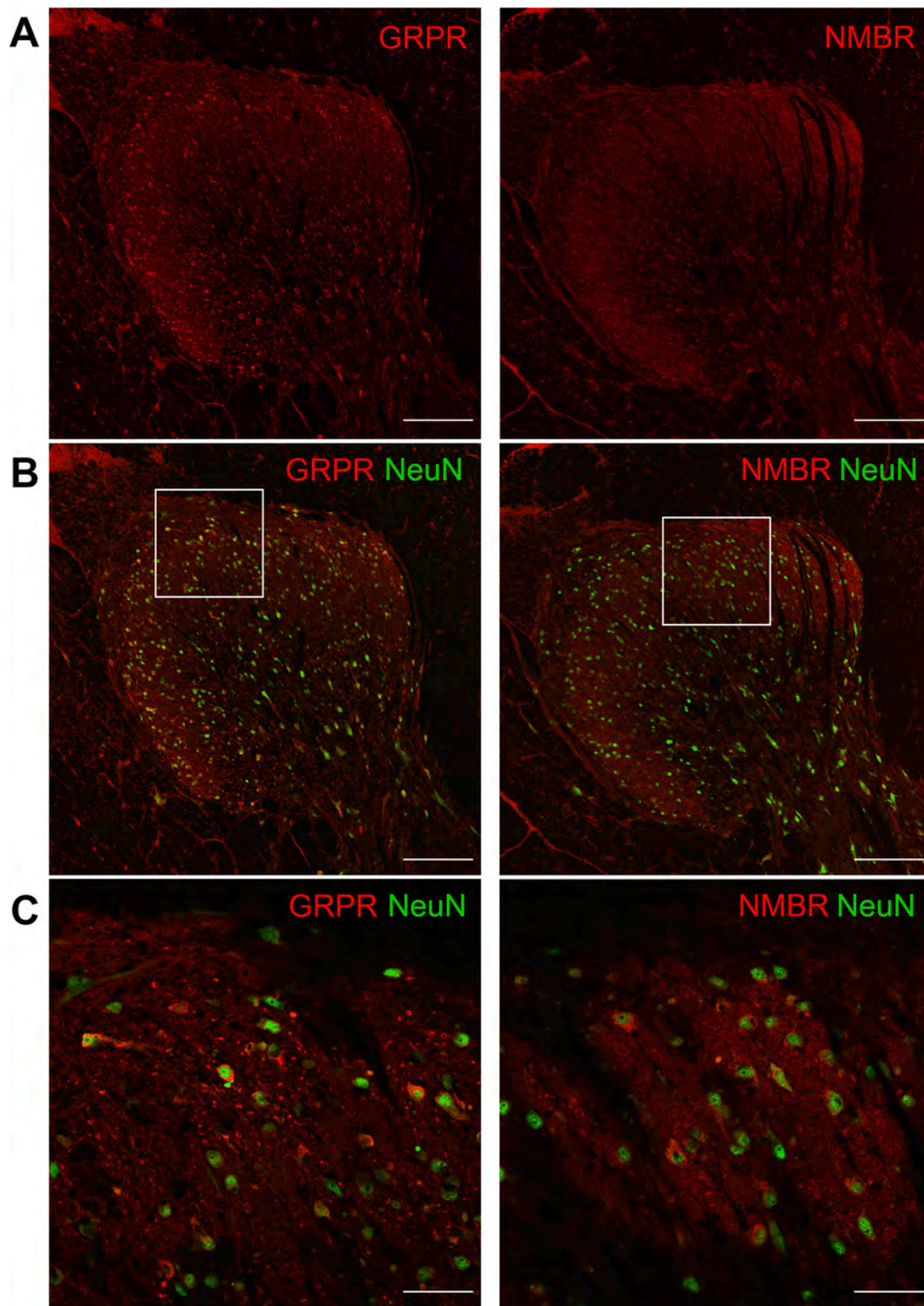


Fig. 5. Expression of NMBR and GRPR protein in the spinal dorsal horn of monkeys. (A, B) Representative images of immunohistochemistry depict the expression of GRPR (left column) and NMBR (right column) in NeuN⁺ cells. (C) Higher magnification of the boxed areas in (B). Scale bar = 200 μ m (A, B), and 50 μ m (C).

DRG. However, NMB is produced by DRG neurons and contributes to nociceptive transmission in rodents [15,18,47]. The expression levels of NMB are greater than that of GRP in both the DRG and SDH of NHPs, thus suggesting NMB may modulate sensory processing, such as itch, in NHPs. Regardless of the exact neuroanatomical site releasing endogenous ligands, it is important to understand that NMB and GRP are

released within the SDH, and subsequently mediate itch sensation. I.t. GRP elicited robust scratching behavior, whereas NMB caused mild scratching behavior. The expression patterns of NMB and NMBR in comparison with GRP and GRPR in NHPs are similar to a previous study performed in dogs [48]. Possibly, lower expression levels of NMBR relative to GRPR and shorter half-life of neuromedin peptides may

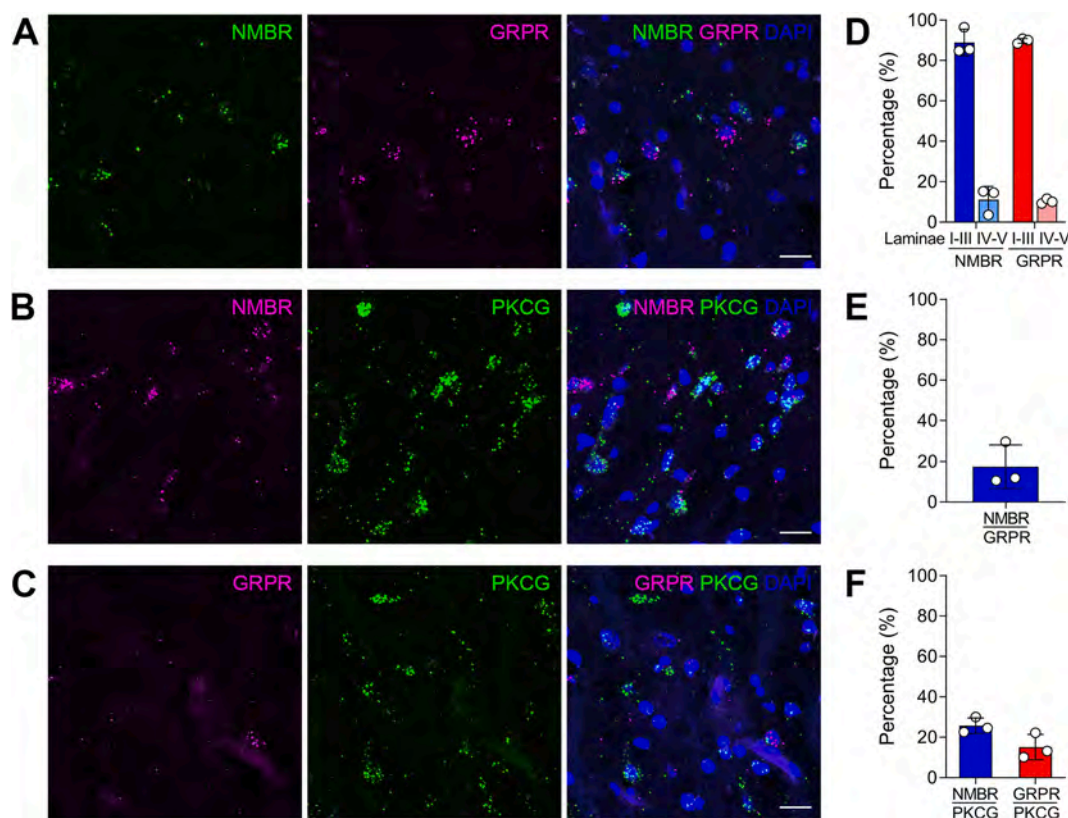


Fig. 6. Expression of NMBR and GRPR mRNA in the spinal dorsal horn of monkeys (A–C). Representative confocal images of *in situ* hybridization (RNAscope) depict the mRNA expression of NMBR in GRPR⁺ (A) or PKCG⁺ neurons (B) and the mRNA expression of GRPR in PKCG⁺ neurons (C). (D) Quantification of the distribution of NMBR⁺ and GRPR⁺ neurons in laminae I–III and IV–V of the spinal dorsal horn. (E, F) Bar graphs depict the percentage of co-expression. Data represent the mean $\pm$ SD (n = 3), with dots representing individual NHP. Scale bar = 20 μ m.

explain these behavioral differences in scratching [49]. Higher expression level of NMB in the peripheral tissues may indicate that this peptide is involved in other peripheral functions such as muscle contraction and endocrine secretion [12,13]. Moreover, the majority of NMBR and GRPR are distributed on different subpopulations, and rarely observed in PKC γ ⁺ pain-associated neurons. Therefore, NMB-NMBR and GRP-GRPR pairs in spinal cord may not significantly contribute to pain in primates.

Itch-associated functions of NMB have been determined in rodents, as the i.t. or intracerebroventricular administration of NMB increases scratching behavior. Although i.t.-administered agents could be distributed in the DRG [50], the likelihood of i.t. NMB acting on NMBR in the DRG seems low as NMBR is expressed in the DRG of NHPs at a minimal level compared to the SDH. This may indicate a species difference considering that i.t. NMB affected the NMBR-expressing DRG neurons in rodents [51]. Scratching responses by centrally administered NMB were considerably lower than those of GRP [14,16,17]. Nevertheless, another group demonstrated that NMB-induced scratching responses were significantly higher than those of GRP, based on a single dose in mice [15,19]. Different mouse strains or species may contribute to the aforementioned discrepancy. Herein, the full dose-response curve confirmed the mild scratching effects of NMB, compared with GRP in the same group of NHPs, consistent with previous rodent studies [14,16,17,52]. NMB and GRP bind to NMBR and GRPR, respectively; however, the cross-reactivity of NMB and GRP against other receptors (i.e., GRP to NMBR) constitute the cross-binding theory for itch regulation in rodents [15]. In primates, NMB-induced scratching responses were blocked by a NMBR antagonist, but not a GRPR antagonist, and *vice versa*, consistent with other rodent studies [16]. Therefore, each ligand (NMB and GRP) acts on its cognate receptor to elicit scratching. Unlike substance P, i.t. NMB and GRP did not affect pain sensitivity in NHPs, despite NMB-induced thermal hypersensitivity in rodents [18]. I.t. GRP

did not exert anti-hypersensitive effects on carrageenan-induced thermal hyperalgesia in NHPs [6]. Furthermore, we tested if NMBR and GRPR antagonists affected thermal hypersensitivity. Normally, μ -opioid receptor agonists, such as DAMGO and morphine, induce significant antinociceptive and antiallodynic effects in rodents and primates [53]. Unlike DAMGO, neither NMBR nor GRPR antagonists attenuated capsaicin-induced allodynia. Therefore, the spinal NMB-NMBR system partially contributed to itch; however, it did not affect pain regulation in NHPs.

Behaviorally, NMB and GRP only acted on their cognate receptors for regulating itch in NHPs. Anatomically, NMB-saporin only ablated NMBR⁺ neurons, but not GRPR⁺ neurons, and *vice versa* in rodents [15,18]. In NHPs, the localization of NMBR and GRPR were restricted to the NeuN⁺ neurons. Moreover, the aforementioned receptors were principally expressed in VGLUT2⁺ excitatory interneurons, but not in VGAT⁺ inhibitory neurons. Therefore, both NMB-NMBR and GRP-GRPR systems directly facilitated itch transmission in the SDH. The majority of NMBR did not colocalize with GRPR, a marker of itch-responsive neurons, in the SDH of both rodents and NHPs. Thus, NMB-NMBR and GRP-GRPR systems may activate the spinal transmission of itch via independent mechanisms. Particularly, the spinal NMB-NMBR system plays a functional role in regulating mild itch.

The pharmacological effects of ligand-receptor systems vary between rodents and primates [23,24]. Particularly, opioid ligands (i.e., μ -opioid receptor and nociceptin receptor agonists) often exert diverse effects in sensory processing between rodents and primates [54,55]. μ -opioid receptor agonists, such as morphine, produces itch sensation in humans and exerts robust scratching behavior in NHPs [36,56]. However, the degree of scratching behavior caused by μ -opioid receptor agonists in rodents is considerably less than that in NHPs [16,27], thereby indicating itch sensation elicited by μ -opioid receptor agonists in humans is

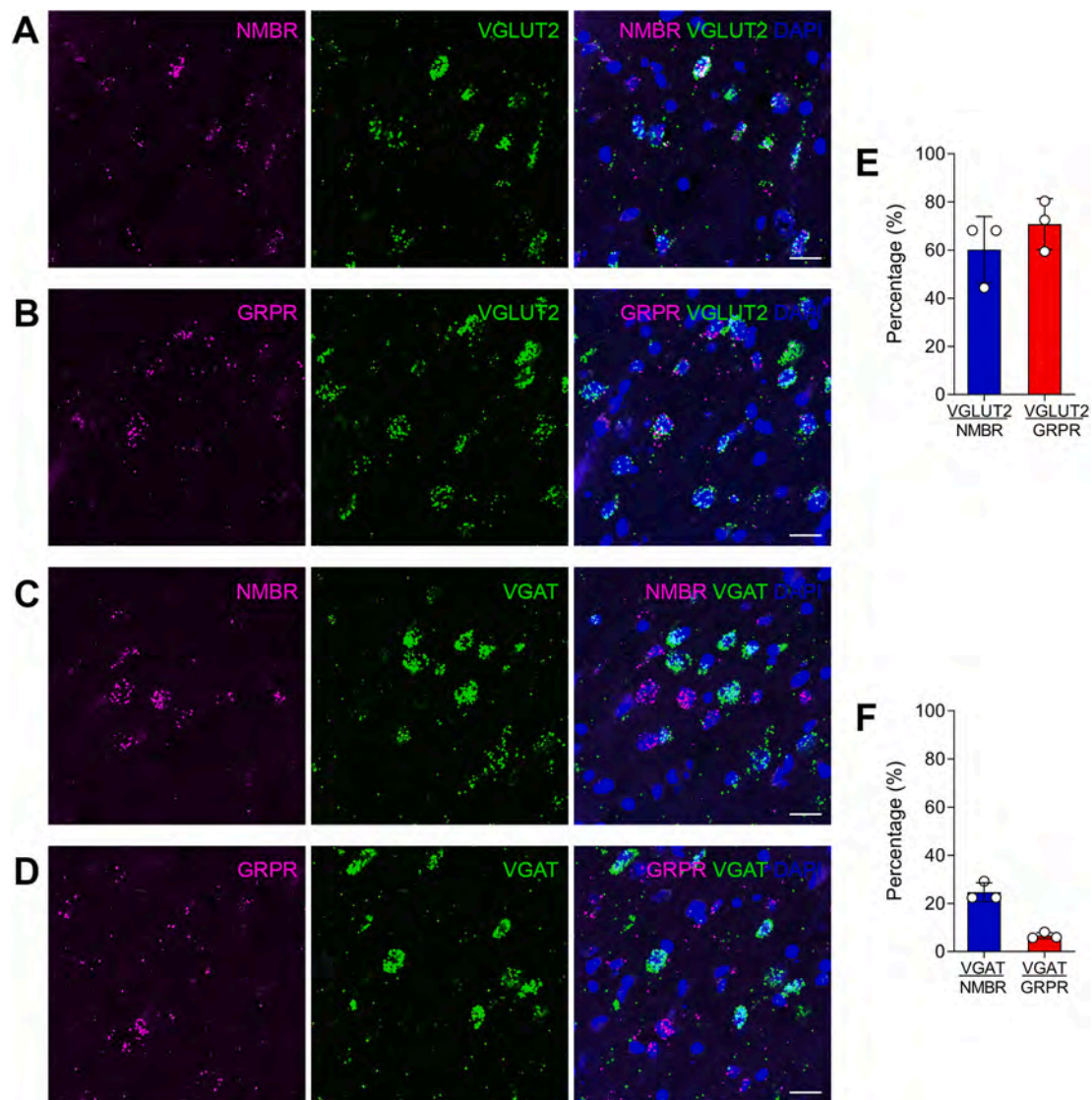


Fig. 7. Expression of VGLUT2 and VGAT mRNA in NMBR⁺ and GRPR⁺ neurons in the spinal dorsal horn of monkeys. (A–D) Representative confocal images of *in situ* hybridization (RNAscope) show the mRNA expression of VGLUT2 in NMBR⁺ (A) or GRPR⁺ (B) neurons and the mRNA expression of VGAT in NMBR⁺ (C) or GRPR⁺ (D) neurons. (E, F) Bar graphs show the percentage of co-expression. Data represent the mean $\pm$ SD ($n = 3$) with dots representing individual NHP. Scale bar = 20 μ m.

modeled in NHPs, but not in rodents [23]. Moreover, nociceptin receptor agonists demonstrate differential effects between rodents and NHPs. The effects of an endogenous nociceptin receptor agonist, nociceptin/orphanin FQ, on pain regulation in rodents depends on the administration route or pain modality [24,55], whereas nociceptin/orphanin FQ exerts distinct antinociceptive effects in NHPs under varied experimental conditions [25,54]. Therefore, pharmacological and behavioral studies in NHPs are pivotal for the translational relevance in humans [23]. GRP-induced scratching behavior is robust in both rodents and primates; thus, it may be reasonable to emphasize the functional and translational significance of GRP and explore the neurobiological mechanisms of itch mediated by the GRP-GRPR system in rodents. In contrast, i.t. NMB caused mild scratching behavior in NHPs; however, the potency was $< 1/100$, compared with that of GRP. Moreover, intradermal NMB resulted in weak scratching behavior in NHPs [51]. Taken together, NMB is highly expressed in the DRG and SDH, but the NMB-NMBR pair plays a minimal “functional” role in regulating itch than the GRP-GRPR pair in primates. Both NMBR⁺ and GRPR⁺ neurons are mainly expressed in laminae I–III of SDH, which may not explain the observed differences between GRP and NMB in NHP behavioral tests. Further studies are warranted to elucidate the other functions of NMB in

the peripheral tissues and central nervous system [12].

By focusing the translational relevance of the spinal NMB-NMBR system previously evaluated only in rodents, our study revealed the neuroanatomical and functional evidence for the NMB-NMBR system in primates. Spinal NMB-NMBR and GRP-GRPR systems can independently elicit scratching; nonetheless, both systems are insignificantly involved in pain regulation. In terms of the expression patterns of NMB and NMBR in the DRG and SDH, our findings for NHPs were consistent with previous studies in rodents. Despite the high expression of NMB in DRG and SDH, low expression of NMBR in the GRPR⁺ neurons of the SDH may limit its function in regulating itch. Unlike the functional significance of spinal GRP-GRPR system in itch, drugs targeting the spinal NMB-NMBR system may not effectively alleviate non-NMBR-mediated itch. This warrants future neuropharmacological studies assessing translational properties from rodents to primates to validate the functional significance of these target molecules.

CRediT authorship contribution statement

Norikazu Kiguchi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review

& editing. **Huiping Ding:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Sun H. Park:** Validation, Investigation, Writing – review & editing. **Kelsey M. Mabry:** Validation, Investigation, Writing – review & editing. **Shiroh Kishioka:** Validation, Writing – review & editing. **Yusuke Shiozawa:** Validation, Writing – review & editing. **E. Alfonso Romero-Sandoval:** Validation, Writing – review & editing. **Christopher M. Peters:** Validation, Investigation, Writing – review & editing. **Mei-Chuan Ko:** Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Preclinical evaluation of [^{225}Ac]Ac-DOTA-TATE for treatment of lung neuroendocrine neoplasms

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Abstract

Purpose There is significant interest in the development of targeted alpha-particle therapies (TATs) for treatment of solid tumors. The metal chelator-peptide conjugate, DOTA-TATE, loaded with the β -particle emitting radionuclide ^{177}Lu (^{177}Lu]Lu-DOTA-TATE) is now standard care for neuroendocrine tumors that express the somatostatin receptor 2 (SSTR2) target. A recent clinical study demonstrated efficacy of the corresponding [^{225}Ac]Ac-DOTA-TATE in patients that were refractory to [^{177}Lu]Lu-DOTA-TATE. Herein, we report the radiosynthesis, toxicity, biodistribution (BD), radiation dosimetry (RD), and efficacy of [^{225}Ac]Ac-DOTA-TATE in small animal models of lung neuroendocrine neoplasms (NENs).

Methods [^{225}Ac]Ac-DOTA-TATE was synthesized and characterized for radiochemical yield, purity and stability. Non-tumor-bearing BALB/c mice were tested for toxicity and BD. Efficacy was determined by single intravenous injection of [^{225}Ac]Ac-DOTA-TATE into SCID mice-bearing human SSTR2 positive H727 and H69 lung NENs. RD was calculated using the BD data.

Results [^{225}Ac]Ac-DOTA-TATE was synthesized with 98% yield, 99.8% purity, and displayed 97% stability after 2 days incubation in human serum at 37 °C. All animals in the toxicity study appeared healthy 5 months post injection with no indications of toxicity, except that animals that received ≥ 111 kBq of [^{225}Ac]Ac-DOTA-TATE had chronic progressive nephropathy. BD studies revealed that the primary route of elimination is by the renal route. RD calculations determined pharmacokinetics parameters and absorbed α -emission dosages from ^{225}Ac and its daughters. For both tumor models, a significant tumor growth delay and time to experimental endpoint were observed following a single administration of [^{225}Ac]Ac-DOTA-TATE relative to controls.

Conclusions These results suggest significant potential for the clinical translation of [^{225}Ac]Ac-DOTA-TATE for lung NENs.

Keywords ^{225}Ac targeted alpha therapy · [^{225}Ac]Ac-DOTA-TATE · Lung neuroendocrine neoplasms

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Introduction

Neuroendocrine tumors (NETs) arise from the neuroendocrine system in the lung, gastrointestinal tract, and pancreas, among other organs [1]. A large fraction (25%) of well-differentiated NETs are of bronchopulmonary origin. Well-differentiated and moderately differentiated lung NETs are referred to as lung carcinoids and are divided into typical and atypical classifications. Poorly differentiated lung neuroendocrine carcinomas are divided into large cell neuroendocrine carcinoma and small cell lung cancer (SCLC) [2, 3]. Few trials have investigated systemic treatments for patients with lung NETs [4–8]. The only medication approved by the US Food and Drug Administration (FDA) for progressive lung NETs is everolimus, an oral inhibitor of the mechanistic target of rapamycin (mTOR) [9].

Overexpression of somatostatin receptors in patients with neuroendocrine neoplasms (NEN) is utilized for both diagnosis and treatment. Somatostatin receptor 2 (SSTR2) is widely expressed in pulmonary NENs, including typical carcinoids, atypical carcinoids, large cell neuroendocrine carcinoma, and SCLC [10]. Therefore, somatostatin analogues (SSAs) with high affinity to SSTR2 can be used for targeting these types of tumors [10]. Peptide receptor radionuclide therapy (PRRT) with the [^{177}Lu]Lu-DOTA chelate conjugated to the octreotate SSA ([^{177}Lu]Lu-DOTA-TATE) has proved to be an effective therapy option for NETs [6, 7, 11].

The limitations of [^{177}Lu]Lu-DOTA-TATE therapy are that only 26–55% of patients achieve stable disease, 18–32% are treatment refractory, and half progress within 2 to 3 years after starting treatment [12–14].

^{177}Lu is a β -particle emitting radionuclide and there is evidence that a superior therapeutic index could be achieved using targeted α -particle therapy (TAT) as an alternative [15–20]. Compared to β particles, α particles have a 200–400 $\times$ greater linear energy transfer and a shorter range in solid tissue, <100 μm for α -emissions relative to a few mm for β -emissions. Hence, α -particle emissions are more likely to deposit energy within the boundaries of a tumor or metastasis relative to β -emissions, which can cause toxicity in surrounding normal tissues, and greater energy deposition leads to greater cell killing. Unlike β -emissions, it is hypothesized that α -emissions do not rely solely on generation of free radicals to generate DNA damage. Instead, the energy deposited is sufficient to directly cause DNA double-strand breaks [21]. This enables TATs to potentially evade a common mechanism of radiation resistance, i.e., free radical scavenging. To demonstrate these advantages, [^{225}Ac]Ac-DOTA-TOC was compared to [^{177}Lu]Lu-DOTA-TOC in the treatment of rat pancreatic exocrine tumor xenografts in immunocompromised mice and TAT demonstrated greater efficacy and lower toxicity relative to the TBT [16]. In recently published case studies, significant efficacy of [^{225}Ac]Ac-DOTA-TATE was

demonstrated in patients' refractory to [^{177}Lu]Lu-DOTA-TATE [22]. Furthermore, [^{177}Lu]Lu-DOTA-TATE has been compared with [^{177}Lu]Lu-DOTATOC in patients and a greater tumor residence time was observed for [^{177}Lu]Lu-DOTA-TATE, indicating that DOTA-TATE may be a superior targeting ligand for delivery of radiotherapy [23].

^{225}Ac is an α -particle emitting isotope that has demonstrated significant utility in TAT and decays with a 10-day half-life via a complex decay scheme, emitting four α particles and depositing significantly higher energy levels in local tissue, 28 MeV, compared to single α -particle emitters, e.g., 7.8 MeV for ^{212}Bi (or ^{212}Pb), or 384 keV for the β -emitter ^{177}Lu [24, 25]. Hence, ^{225}Ac has been described as an in vivo α -particle generator [26]. The main method for generating ^{225}Ac for clinical studies is through the decay of ^{229}Th which originates from ^{233}U . There are three main sources of ^{229}Th in the world: Oak Ridge National Laboratory (USA), The Institute of Physics and Power Engineering (Russia), and The Institute for Transuranium Elements (Germany). In addition, it has been shown that large-scale quantities can be produced through the decay of ^{232}Th [27, 28]. To address the shortage of the clinical need of ^{225}Ac , the US Department of Energy formed a Tri-lab collaboration of Los Alamos (LANL), Brookhaven (BNL), and Oak Ridge (ORNL) National Laboratories with the goal of developing an alternative route for production of ^{225}Ac by use of a linear accelerator [29]. Due to the long half-life, ^{225}Ac -based TATs can be produced at regional radiopharmaceutical facilities and distributed for use by Nuclear Medicine Departments within 1 to 3 days post-production.

Herein, we investigate the toxicity, biodistribution (BD), radiation dosimetry (RD) in mice, and efficacy of [^{225}Ac]Ac-DOTA-TATE in xenograft tumor models of pulmonary NENs. These preclinical studies are needed to support the clinical translation of [^{225}Ac]Ac-DOTA-TATE.

Materials and methods

Cell culture

NCI-H69 human SCLC, NCI-H727 human lung carcinoid, and HEK293 cells were purchased from American Type Culture Collection, expanded for 2 passages and cryopreserved. NCI-H69 and NCI-H727 cells were grown in RPMI-1640 Medium, (Life Technologies), 10% FBS (Life Technologies), 100 units/mL penicillin, 100 mg/mL streptomycin in 5% CO_2 at 37 $^\circ\text{C}$. HEK293 cells were grown in DMEM/F12 medium (Life Technology). Cells were authenticated using short tandem repeat (STR) DNA typing according to ATCC's guidelines [30], monitored by microscopy to assure maintenance of their original morphology, tested for

mycoplasma contamination using the MycoAlert kit (Lonza) and experiments used cells of passage numbers <25.

SSTR2 transfection of HEK293 cells

HEK293 cells were transfected with 2 µg of SSTR2 (NM_001050) Human Tagged ORF Clone in pCMV6 vector with Neomycin selection marker (Origene) using the FuGENE HD Transfection Reagent (Promega) as described before [31]. SSTR2 expression was determined by quantitative real-time RT-PCR (qRT-PCR) using the primers described below. The HEK293/SSTR2 clone with the highest expression was maintained in medium containing 400 µg/mL of G418.

Chelation of lanthanum and europium

DOTA-(Tyr3)-octreotate (DOTA-TATE), ≥98% purity, was purchased (Bachem). DOTA-TATE was loaded with lanthanum (La^{3+}) by addition of $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ (3.9 mg, 10.5 µmol) to DOTA-TATE (5.0 mg, 3.5 µmol) in 1 mL of 0.1 M AcONa buffer (pH 6.0) and europium (Eu^{3+}), by addition of $\text{EuCl}_3 \cdot 6\text{H}_2\text{O}$ (1.6 mg, 4.2 µmol) to DOTA-TATE (2 mg, 1.4 µmol) in 5 mL H_2O and 0.1 mL DMSO, and stirring 12 h at room temperature (RT). For both chelates, reaction completion was determined by HPLC (Agilent 1260 Infinity II HPLC system with a quaternary pump, a vial sampler, and a DAD detector) and a C18 column yielded the final product (~3.3 mg and 1.9 mg, respectively). A Phenomenex C18 column (Luna 5 µL C18(2) 100 Å, 4.6 × 250 mm) was used. The DAD detector was set to 214 nm. Phase A: 0.1% TEA/AcOH (TEAA) in water (pH 6.0) and Phase B: 90% acetonitrile in Phase A (pH adjusted to 6.0 with AcOH) using the following: 10–45% Phase B in Phase A in 50 min. Mass and purity were determined by HRMS (ESI) and HPLC, respectively.

Binding assay

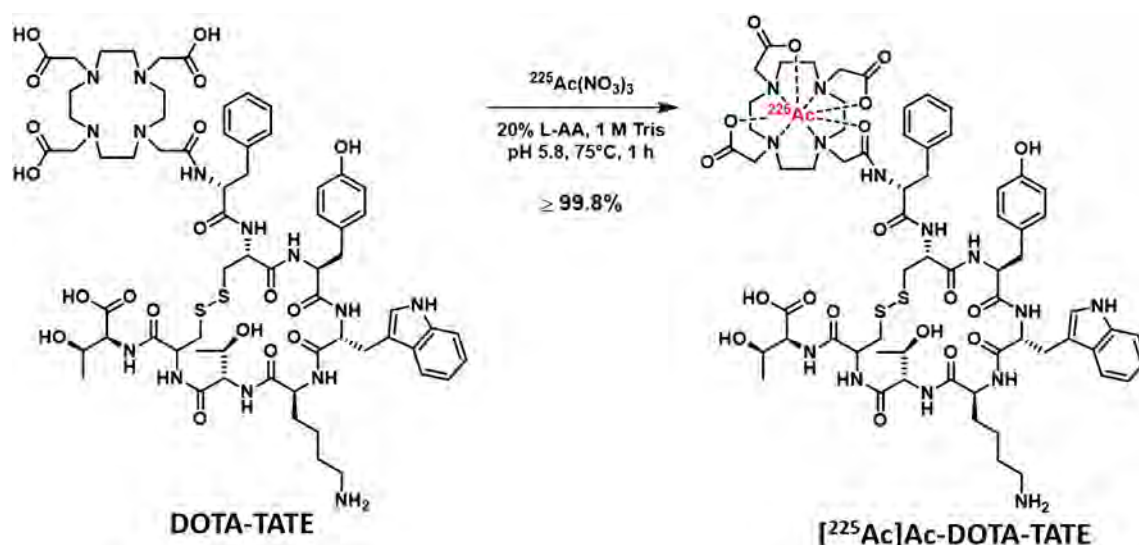
The receptor number of HEK293/SSTR2 cells was determined using time-resolved fluorescence (TRF) saturation binding assay. Different concentrations of Eu^{3+} -DOTA-TATE (0–2500 nM) were used as the test ligand and 5 µM of La^{3+} -DOTA-TATE was used as the blocking ligand. Briefly, black 96-well plates (clear bottom, Corning, #3603) were coated with 0.25 mg/ml of PDL (Poly-D Lysine, Sigma). HEK293/SSTR2 cells were plated in the coated plates, at a density of 30,000 cells/well. On the day of the experiment (1 day after seeding the cells), the medium was aspirated and for the top half of the plate (total binding) 50 µL of the Eu^{3+} -DOTA-TATE (2.5–2500 nM) test ligand was added to each well in a series of decreasing concentrations, followed by 50 µL of binding medium (DMEM, 1 mM 1,10-phenanthroline, 200 mg/L bacitracin, 0.5 mg/L leupeptin,

0.3% BSA). The bottom half of the plate (non-specific binding), was prepared as the top half, except that 5 µM of blocking La^{3+} -DOTA-TATE ligand was added instead of binding medium. Cells were incubated with the ligands for 1 h at 37 °C in a cell culture incubator, followed by two washes with PBS. After washing, the cells were incubated with 50 µL of 2.0 M HCl for 2 h at 37 °C followed by neutralization with 55 µL of 2.0 M NaOH. Then, 115 µL of enhancement solution (PerkinElmer) was added to each well and cells were incubated for an additional 30 min at 37 °C prior to reading. The plates were read on a PerkinElmer VICTORx4 2030 multilabel reader using the standard Eu^{3+} time-resolved fluorescence (TRF) measurement (340-nm excitation, 400-µs delay, and emission collection for 400 µs at 615 nm). The standard curve was used to determine the amount of ligand present at the B_{max} obtained in the saturation binding assay. The average number of cells per well at the end of the assay was calculated. To determine the receptor number, the following equation was used: (Eu^{3+} amount for B_{max} (moles)/average cell number per well) × 6.023×10^{23} = receptor number per cell. This B_{max} value was then used to calculate ligand association and binding kinetics for each cell using the following equation: $B = B_0 + (B_{\text{max}} - B_0)/(1 - e^{-kt})$, where B corresponds to a receptor saturation parameter, an analog of ligand-receptor complex formation [RL], with values between initial saturation B_0 and the maximum saturation B_{max} ; k is the reaction rate constant, and t is time. NCI-H69 receptor number was determined as above except that ligand incubation step was reduced to 30 min with only × 1 PBS wash.

La^{3+} -DOTA-TATE binding affinity was determined using a TRF competitive binding assay as previously [32], using HEK293/SSTR2 cells and 50 nM Eu^{3+} -DOTA-TATE used as the competing ligand. Data points were acquired in quadruplicate and each assay was repeated 3 times.

Radiochemical synthesis and characterization

The $^{225}\text{Ac}(\text{NO}_3)_3$ (5.80×10^4 Ci/g; carrier free; >10 mCi/ml concentration) was purchased from Oak Ridge National Laboratory (TN, USA). Complexation was achieved by reacting DOTA-TATE (5–10 µg in 5–10 µL water from 1.0 mg/mL solution) with $^{225}\text{Ac}(\text{NO}_3)_3$ (3.4 MBq) that was diluted in 100 µL of water containing 10 µL of 20% L-ascorbic acid. The pH was adjusted to 5.8 using 1 M Tris buffer (10–12 µL), and then incubated at 75 °C for 1 h (Scheme 1). Reaction progress and radiochemical purity of [^{225}Ac]Ac-DOTA-TATE were measured without further purification 24 h after collection (to ensure secular equilibrium among ^{225}Ac and its daughters) using ITLC with γ counting, radio-TLC, and γ counting of radio-HPLC fractions. Stability was determined by adding 50 µL of [^{225}Ac]Ac-DOTA-TATE (1702 kBq) to 1 mL of human serum ($n = 4$), 37 °C incubation



Scheme 1 Radiochemical synthesis of $[\text{}^{225}\text{Ac}]\text{Ac-DOTA-TATE}$

for 10 days, and TLC scanner (Bioscan) and γ -counter (PerkinElmer) quantification at time points [33].

Animal studies

Protocols were approved: University of South Florida IACUC protocol IS00006466. Animals were purchased from Charles River (Wilmington, MA). An equal number of males and females were used for each cohort.

Injected activity measurement

Syringes were prefilled with $\pm 10\%$ of $[\text{}^{225}\text{Ac}]\text{Ac-DOTA-TATE}$ activity. Activities were measured using the Atomlab 500 Dose Calibrator (Biodex) as described before [34].

Toxicity

Cohorts ($n = 6$) of non-tumor-bearing BALB/c mice (6–8 weeks old) were given a single intravenous injection by tail-vein catheter of 200 μl of 55.5, 111 or 185 kBq of $[\text{}^{225}\text{Ac}]\text{Ac-DOTA-TATE}$ activity in 0.9% sterile saline (Cardinal Pharmaceuticals) in the syringe (45.8 ± 14.8 , 94.7 ± 4.8 , and 163.9 ± 9.6 mean injected activity), or 0.9% sterile saline alone as a control. These administration activities were selected based on results from a previous preclinical study using an ^{225}Ac -based peptidic TAT [34]. Injected activity is determined for each animal by subtracting the amount of activity remaining in the syringe and catheter from the pre-injection amount of activity in the syringe. Mice were monitored for distressed behavior and weighed twice per week for 5 months, followed by euthanasia and tissue collection. Blood urea nitrogen (BUN), creatinine, alkaline phosphatase (ALKP), alanine aminotransferase (ALT), and aspartate

aminotransferase (AST) in serum were analyzed using an automated biochemical analyzer (CLIP, Catalyst DX, IDEXX). Bone, bone marrow, brain, cecum, duodenum, kidney, liver, lymph nodes, muscle, pancreas, salivary gland, small intestine, and spleen were harvested and fixed in 10% formalin. Bone was decalcified in 14% ethylene diaminetetraacetic acid (EDTA) solution after fixation. Tissues were embedded in paraffin, sectioned (4–6 μm thickness), stained with hematoxylin and eosin (H&E), and examined blind by the veterinary pathologist (R.E.).

Biodistribution

Non-tumor-bearing BALB/c mice (6–8 weeks old) were administered by tail-vein catheter 200 μl of 74 kBq of $[\text{}^{225}\text{Ac}]\text{Ac-DOTA-TATE}$ activity in 0.9% sterile saline in the syringe (66.6 ± 2.9 kBq mean injected activity), euthanized at 24, 48, and 96 h p.i., $n = 4$ mice per cohort. This administration activity was selected based on results from a previous preclinical BD study using an ^{225}Ac -based peptidic TAT [34]. Tissues were removed, weighed, and the percent injected activity per gram (%IA/g) calculated as before [34].

Radiation dosimetry

Dosimetry calculations were performed using BD data as previously described [34].

qRT-PCR and immunocytochemistry

SSTR2 expression was verified by qRT-PCR and ICC performed to determine protein. NCI-H69 and NCI-H727 RNA was extracted (RNeasy, Qiagen). Two sets of human SSTR2 primers were designed (Gene Runner for Windows 3.05): set

1: forward, 5'-AACCAGACAGAGCCGTACTA-3' and reverse, 5'-GCATAGCGGAGGATGACA-3'; and set 2: forward, 5'-GCTGGCTTCCCTTCTACATATT-3' and reverse, 5'-GAGGACCACCACAAAGTCAA-3'. B-actin was used for normalization as previously [35]. NCI-H69 and NCI-H727 ICC was performed using 30 µg/mL anti-SSTR2 antibody (Sigma, HPA007264) as before [34], except that H69 were grown on coverslips coated with 0.25 mg/ml poly-D-lysine (Sigma).

Anti-tumor efficacy

NCI-H69 and NCI-H727 subcutaneous xenograft tumor-bearing SCID mice (6–8 weeks old, $n = 10/\text{cohort}$) were injected by tail-vein catheter with 200 µl of 185 kBq [^{225}Ac]Ac-DOTA-TATE activity in 0.9% sterile saline in the syringe (148.0 ± 17.8 and 144.3 ± 18.8 kBq mean injected activity), or 0.9% sterile saline alone as a control. This administration activity was selected based on preliminary results from the toxicity study herein. The efficacy study was initiated 4–1/2 months after initiation of the toxicity study and no toxicities were detected at this activity at that time. Animals underwent euthanasia after reaching 2000 mm³ tumor volume or if clinical endpoints were observed, e.g., 20% weight loss, tumor ulceration, hunched back, lack of grooming, and lethargy.

Histology and immunohistochemistry

Following euthanasia, organs (toxicity) and tumors (efficacy) were excised, formalin fixed, paraffin embedded, sectioned (5 µm), and stained with H&E (tumors and organs) and IHC (tumors) using rabbit SSTR2 polyclonal antibody (1:200 dilution, GTX70735, GeneTex) and a Discovery XT automated system (Ventana Medical Systems). Slides were scanned using a ScanScope XT digital slide scanner (Aperio, CA). To quantify SSTR2 expression in tumors, images from serial H&E and IHC central sections were analyzed using Visiopharm 2017.7 as before [34].

SSTR2 expression was evaluated in using gray-level co-occurrence texture analysis in three main steps: (1) tumor segmentation, (2) target expression extraction, and (3) texture analysis. The tumor region was segmented to limit the analysis to cells within the tumor. For target expression, a binary mask was created from the positive and negative stained cells with each pixel representing a cell with the value of 1 if unstained or 2 if stained. A co-occurrence texture analysis was applied to the binary mask to calculate the heterogeneity score for target expression. Specifically, the diagonal values in the gray-level co-occurrence matrix measure the closeness of the distribution of all gray levels. Therefore, heterogeneity is calculated from diagonal entries that are related, i.e., with target expression. The

heterogeneity score ranges from 0 to 1, where 0 is completely homogeneous and 1 is completely heterogeneous.

Statistics

Cohort size was determined by power analyses. Analysis of variance (ANOVA) was used to assess differences in toxicity by activity and Dunnett's test to determine differences among activities and saline control. Prior to ANOVA, the Anderson Darling test assessed whether a transformation was required. The unpaired *t*-test compared the ICC or IHC expression level of SSTR2 in NCI-H69 and NCI-H727 cells and tumors. Two-way ANOVA was used to compare tumor volume among treatment and saline groups and Kaplan-Meier analyses (GraphPad Prism 7 software) determined significance among the cohorts.

Results

Binding affinity of La³⁺-DOTA-TATE

There are no non-radioactive isotopes of actinium and lanthanum is a useful surrogate for Ac since both are trivalent ions in solution [36]. The HRMS (ESI) calculated mass for the prepared La³⁺-DOTA-TATE, C₆₅H₈₇LaN₁₄O₁₉S₂ (M + H)⁺, 1571.4855 MW, was 1571.4867 with 100% purity, $t_R = 22.15$ min (Supplemental Fig. 1). La³⁺-DOTA-TATE bound SSTR2 expressing HEK293/SSTR2 cells with high, 19.00 ± 9.2 nM K_i (Fig. 1).

The HRMS (ESI) calculated mass for the Eu³⁺-DOTA-TATE chelate, C₆₅H₈₇EuN₁₄O₁₉S₂ (M + H)⁺, 1585.5004 MW, was 1585.5031 with 100% purity, $t_R = 22.14$ min (Supplemental Fig. 2). Eu³⁺-DOTA-TATE binding affinity for SSTR2 was 22.12 nM K_d , and HEK293/

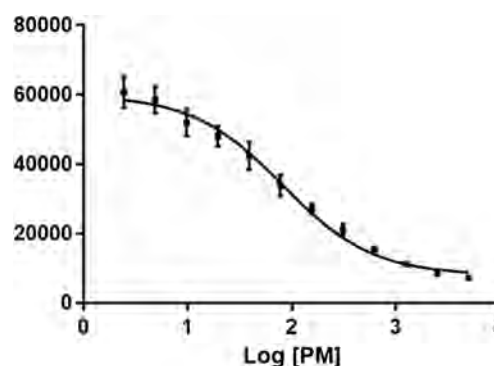


Fig. 1 Whole-cell competitive binding assay of La³⁺-DOTA-TATE. The binding affinity of La³⁺-DOTA-TATE was calculated to be 19.00 ± 9.2 nM K_i ($n = 3$ repeats). In this whole-cell lanthanide time-resolved fluorescence assay, HEK293/SSTR2 cells were used and 50 nM of Eu³⁺-DOTA-TATE was used as the competing ligand

Table 1 In vitro serum stability of [^{225}Ac]Ac-DOTA-TATE

Day	% Intact TLC scanner
0	100
1	100.44±0.88
3	99.27±0.29
5	96.74±2.06
7	93.19±1.50
10	90.29±4.00

SSTR2 cells were shown to express 1.87×10^6 SSTR2 receptors on the cell-surface per cell (Supplemental Fig. 3A).

Radiosynthesis and characterization of [^{225}Ac]Ac-DOTA-TATE

Radiosynthesis (Scheme 1) provided >98% yield with high radiochemical purity ($\geq 99.8\%$) (Supplemental Fig. 4). Also, [^{225}Ac]Ac-DOTA-TATE had an excellent in vitro stability, with 97% integrity remaining after 2 days in human serum at 37 °C (Table 1).

Toxicity

Weight gain was observed in all animals at 5 months post injection (p.i.), albeit less weight was gained by animals at the highest dose level relative to the lowest (Fig. 2a). Significant differences in weight change were observed among saline and 111 kBq and 185 kBq treatments ($p < 0.01$ and $p < 0.001$, respectively), but not 55.5 kBq ($p = 0.50$) (Supplemental Table 1). Blood BUN and creatinine were all in the normal range (Fig. 2b and c), p values = 0.12 and 0.33, respectively (Supplemental Table 2 and 3). No significant differences in ALT, AST, or ALKP liver enzymes were observed among the cohorts (Fig. 2d–f and Supplemental Table 4–6).

No pathologic findings were observed for 55.5 and 111 kBq cohorts. In contrast, animals in cohorts receiving ≥ 111 kBq in the syringe (≥ 94.8 kBq actual injected activity) began to lose weight at ~100 days p.i. (Supplemental Fig. 5), and at necropsy chronic progressive nephropathy was observed (Fig. 3). No other organ (bone, bone marrow, brain, cecum, duodenum, liver, lymph nodes, muscle, pancreas, salivary gland, small intestine, and spleen) showed pathologic changes related to the treatment. Glycogen accumulation was observed in the livers of some animals (including the saline control group), but this was considered an incidental finding.

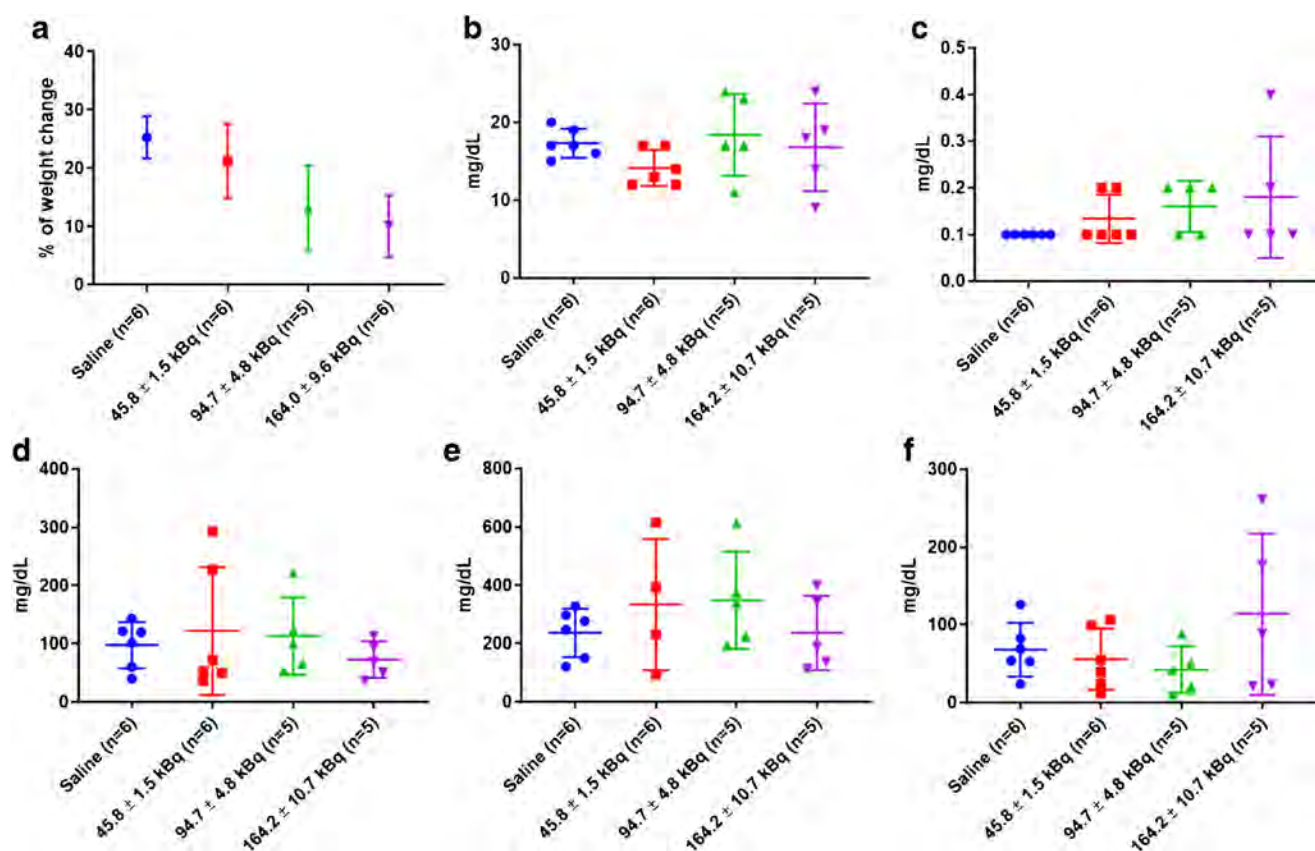


Fig. 2 Toxicity of [^{225}Ac]Ac-DOTA-TATE in BALB/c mice. **a** Percent weight gain, **b** BUN (reference range: 18–29 mg/dl), **c** blood creatinine (reference range: 0.2–0.8 mg/dL), **d** ALT (reference range: 28–132 U/L),

e AST (reference range: 59–247 U/L), and **f** ALKP (reference range: 62–209 U/L)

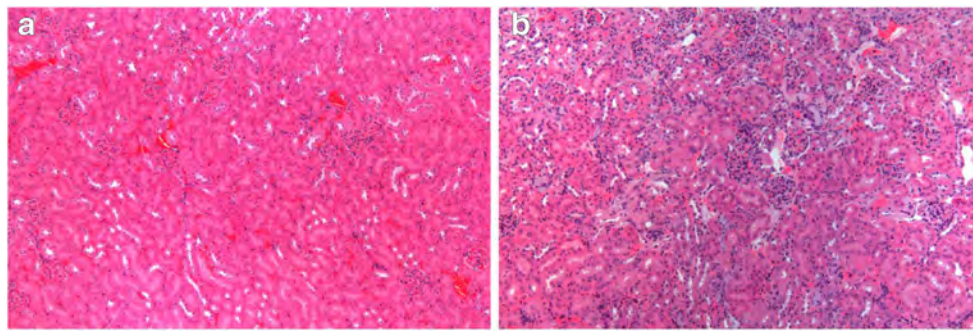


Fig. 3 Histological appearance of kidney from a mouse administered **a** saline or **b** 179.0 kBq. (a) Kidney histology of a saline administered mouse, without significant abnormalities, where glomeruli are normocellular, with open capillary loops, and tubular epithelium is uniformly cuboidal, with round nuclei, and a prominent brush border. (b)

Kidney histology of a 179.0-kBq administered mouse, with chronic progressive nephropathy. Extensive tubular cell regeneration, diffuse fibrosis, mild mononuclear inflammatory cell infiltrates, and thickened, hypercellular glomerular tufts are evident

Biodistribution

BALB/c mice ($n = 4$ per cohort) were injected intravenously with 74 kBq of [^{225}Ac]Ac-DOTA-TATE α activity in the syringe. The activities from ^{225}Ac and daughters ^{221}Fr and

^{213}Bi in equilibrium were determined for each organ and time point using γ spectroscopy (Fig. 4a–c and Supplementary Fig. 6). [34, 37] At 24 h p.i., the kidneys, liver, and stomach had 1.63 ± 0.72 , 0.147 ± 0.07 , and 0.31 ± 0.08 %IA/g, respectively, while only negligible activity was observed in the other

Fig. 4 Biodistribution of **a** ^{225}Ac , **b** ^{221}Fr , and **c** ^{213}Bi after intravenous injection of [^{225}Ac]Ac-DOTA-TATE in BALB/c mice

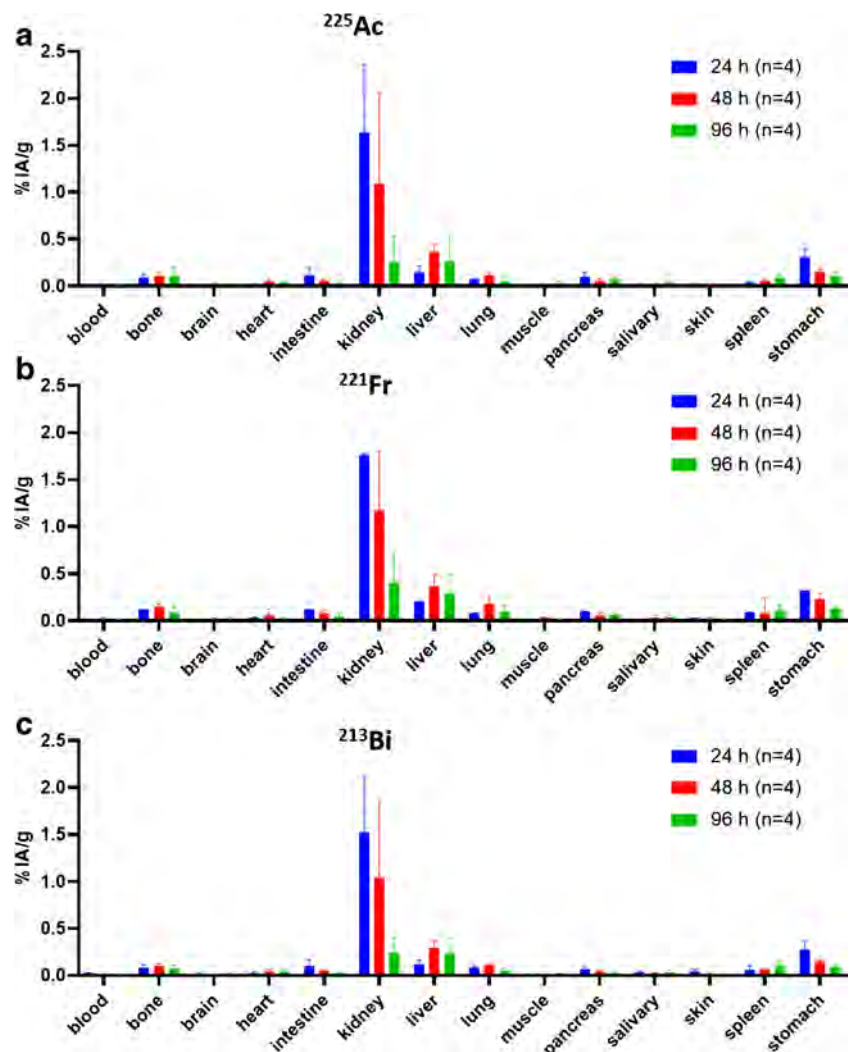
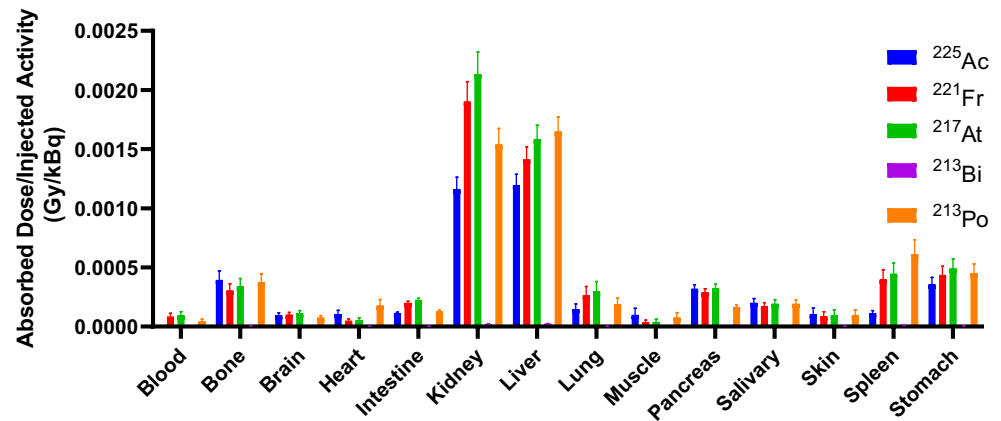


Table 2 Radiation dosimetry and clearance kinetics parameters for [^{225}Ac]/Ac-DOTA-TATE in BALB/c mice

Parameter	Blood	Bone	Brain	Heart	Intestine	Kidney	Liver	Lung	Muscle	Pancreas	Salivary	Skin	Spleen	Stomach
^{225}Ac														
Initial activity/organ, A_0 (kBq)	0.0003	0.0131	0.0019	0.0033	0.1527	0.3862	0.1200	0.0227	0.0024	0.0171	0.0028	0.0023	0.0039	0.0859
Decay rate constant, λ_{eff} (h^{-1})	0.0206	0.0030	-0.0103	-0.0004	0.0200	0.0250	-0.0053	0.0130	-0.0049	0.0060	-0.0077	-0.0092	0.0062	0.0190
Decay half-life, T_{eff} (days)	1.4020	9.6270	2.8040	72.2028	1.4441	1.1552	5.4493	2.2216	5.8941	4.8135	3.7508	3.1393	4.6582	1.5201
Accumulated activity/organ, $\tilde{A}$ (kBq $\times$ h)	0.0080	1.4274	0.8190	0.4871	4.4589	8.2462	29.4458	1.0829	0.5613	1.4271	0.8906	0.8656	0.3228	2.6801
Absorbed dose/injected activity (Gy/kBq)	0.0000	0.0004	0.0001	0.0001	0.0001	0.0012	0.0012	0.0001	0.0001	0.0003	0.0002	0.0001	0.0001	0.0004
^{221}Fr														
Initial activity/organ, A_0 (kBq)	0.0019	0.0170	0.0036	0.0067	0.01532	0.4011	0.1612	0.0251	0.0052	0.0169	0.0037	0.0039	0.0097	0.0852
Decay rate constant, λ_{eff} (h^{-1})	-0.0071	0.0100	-0.0044	0.0190	0.0130	0.0190	-0.0033	0.0080	0.0170	0.0080	-0.0028	-0.0015	0.0030	0.0170
Decay half-life, T_{eff} (days)	4.0678	2.8881	6.5639	1.5201	2.2216	1.5201	8.7519	3.6101	1.6989	3.6101	10.3147	19.2541	9.6270	1.6989
Accumulated activity/organ, $\tilde{A}$ (kBq $\times$ h)	0.5594	1.0195	0.7934	0.2102	7.2991	12.5136	32.1637	1.7734	0.1848	1.1933	0.7036	0.6489	1.0604	3.0452
Absorbed dose/injected activity (Gy/kBq)	0.0001	0.0003	0.0001	0.0000	0.0002	0.0019	0.0014	0.0003	0.0000	0.0003	0.0002	0.0001	0.0004	0.0004
^{217}At														
Initial activity/organ, A_0 (kBq)	0.0019	0.0170	0.0036	0.0067	0.1532	0.4011	0.1612	0.251	0.0052	0.0169	0.0037	0.0039	0.0097	0.852
Decay rate constant, λ_{eff} (h^{-1})	-0.071	0.0100	-0.0044	0.0190	0.0130	0.0190	-0.0033	0.0080	0.0170	0.0080	-0.0028	-0.0015	0.0030	0.0170
Decay half-life, T_{eff} (days)	4.0678	2.8881	6.5639	1.5201	2.2216	1.5201	8.7519	3.6101	1.6989	3.6101	10.3147	19.2541	9.6270	1.6989
Accumulated activity/organ, $\tilde{A}$ (kBq $\times$ h)	0.5594	1.0195	0.7934	0.2102	7.2991	12.5136	32.1637	1.7734	0.1848	1.1933	0.7036	0.6489	1.0604	3.0452
Absorbed dose/injected activity (Gy/kBq)	0.001	0.0003	0.0001	0.0000	0.0002	0.0021	0.0016	0.00003	0.0000	0.0003	0.0002	0.0001	0.0004	0.0005
^{213}Bi														
Initial activity/organ, A_0 (kBq)	0.0042	0.0125	0.0066	0.0053	0.1340	0.3639	0.0948	0.0270	0.0034	0.0119	0.0056	0.0056	0.0068	0.0777
Decay rate constant, λ_{eff} (h^{-1})	0.01100	0.0070	0.0080	0.0030	0.0210	0.0250	-0.0073	0.0170	0.0050	0.0140	0.0030	0.0040	-0.0024	0.0190
Decay half-life, T_{eff} (days)	2.6256	4.1259	3.6101	9.6270	1.3753	1.1552	3.9563	1.6989	5.7762	2.0629	9.6270	7.2203	12.0338	1.5201
Accumulated activity/organ, $\tilde{A}$ (kBq $\times$ h)	0.2345	0.9591	0.4653	0.5789	3.6681	7.7700	28.7854	0.9636	0.3058	0.5253	0.6047	0.5575	1.2470	2.4230
Absorbed dose/injected activity (Gy/kBq)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.000	0.000	0.0000	0.0000
^{213}Po														
Initial activity/organ, A_0 (kBq)	0.0042	0.0125	0.0066	0.0053	0.1340	0.3639	0.0948	0.0270	0.0034	0.0119	0.0056	0.0056	0.0068	0.0777
Decay rate constant, λ_{eff} (h^{-1})	0.0110	0.0070	0.0080	0.0030	0.0210	0.0250	-0.0073	0.0170	0.0050	0.0140	0.0030	0.0040	-0.0024	0.0190
Decay half-life, T_{eff} (days)	2.6256	4.1259	3.6101	9.6270	1.3753	1.1152	3.9563	1.6989	5.7762	2.0629	9.6270	7.2203	12.0338	1.5201
Accumulated activity/organ, $\tilde{A}$ (kBq $\times$ h)	0.2345	0.9591	0.4653	0.5789	3.6681	7.7700	28.7854	0.9636	0.3058	0.5253	0.6047	0.5575	1.2470	2.4230
Absorbed dose/injected activity (Gy/kBq)	0.0000	0.0004	0.0001	0.0002	0.0001	0.0015	0.0016	0.0002	0.0001	0.0002	0.0002	0.0001	0.0006	0.0005
Total absorbed dose/injected activity (Gy/kBq)	0.0002	0.0014	0.0004	0.0004	0.0007	0.0068	0.0059	0.0009	0.0003	0.0011	0.0008	0.0004	0.0016	0.0017

Fig. 5 Radiation dosimetry of ^{225}Ac and daughters following administration of [^{225}Ac]Ac-DOTA-TATE in BALB/c mice



tissues measured. Activity had largely cleared from the tissues by 96 h p.i. Since at the time of the BD measurement, ^{225}Ac was in equilibrium with its daughters, whereas the actual distributed daughters had been decayed, ^{221}Fr and ^{213}Bi only represent the biodistribution of ^{225}Ac .

Radiation Dosimetry

Radiation dosimetry (RD) calculations were based on the data obtained from the BD studies at 24, 48 and 96 h p.i. The α -particle dose from ^{225}Ac and each of its α -emitting daughters was calculated (Supplementary Fig. 6). The decay of ^{221}Fr to ^{217}At was assumed to take place in the same location as ^{221}Fr . Similarly, the decay of ^{213}Po was assumed to take place at the same location as ^{213}Bi , while accounting for its 98% branching ratio.

BD data for the different tissues were fitted using an exponential decay nonlinear regression, allowing the estimation of the initial activity due to uptake (A_0), the decay rate constant (λ_{eff}), decay half-life (T_{eff}), accumulated activity ($\tilde{A}$), and absorbed dose/injected activity (Gy/kBq) for each radionuclide in each tissue (Table 2). The total absorbed dose is the summation of the values for the five α -emitting radionuclides.

The calculated total absorbed dose for [^{225}Ac]Ac-DOTA-TATE was minimal in all tissues except kidney and liver, with 0.0068 and 0.0059 Gy/kBq, respectively. Figure 5 represents graphs of the absorbed dose from each radionuclide per tissue.

The effective decay half-lives (T_{eff}) calculated for ^{225}Ac in tissues that had significant uptake were shorter than the radioactive decay half-life of ^{225}Ac (10 days), indicating biological clearance. For example, the calculated T_{eff} in kidney and liver were 1.16 and 5.45 days, respectively (Table 2). Hence, T_{eff} is a composite of radioactive decay and active biological clearance. The T_{eff} was only calculated to be longer in tissues with minimal uptake where instrument background likely interfered with the accuracy of measurement. It is notable that the kidneys had the highest initial activity, 0.39 kBq, relative to liver, 0.12 kBq, but the kidneys had a nearly 5-fold lower effective half-life compared to liver, leading to faster kidney clearance relative to liver and a higher but comparable total absorbed dose.

SSTR2 expression in NEN cells

NCI-H69 cells have significantly higher SSTR2 mRNA ($p < 0.001$) and protein expression ($p < 0.05$) relative to NCI-

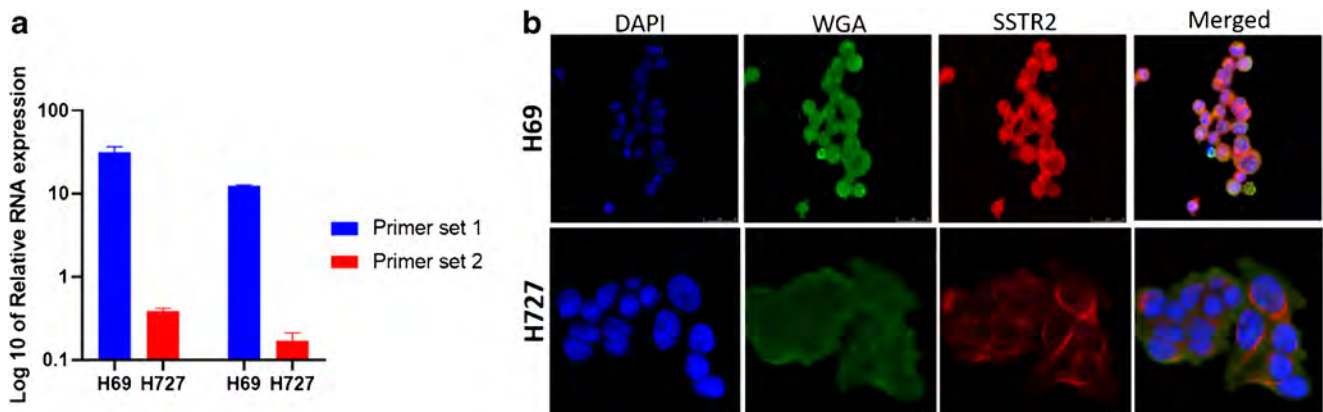


Fig. 6 SSTR2 expression on NCI-H69 and NCI-H727 cells. **a** qRT-PCR. Note the Log10 scale for the expression level. **b** ICC using DAPI (blue), WGA (green), and anti-SSTR2 antibody (red)

H727 (Fig. 6). The SSTR2 cell-surface receptor number per NCI-H69 cell was 1.60×10^6 (Supplemental Fig. 3b). SSTR2 expression in NCI-H727 cells was not high enough to accurately calculate the receptor number.

Anti-tumor efficacy

Eight days after tumor cell xenoengraftment, mice ($n = 10$ per cohort) were intravenously injected with a single bolus of 148.7 kBq or 144.3 kBq mean ^{225}Ac activity for H69 and H727 cohorts, respectively, or saline solution. Figure 7a and b show representative images at 25 days p.i. Tumor volumes decreased significantly after treatment relative to saline controls ($p < 0.001$) prior to eventual regrowth (Fig. 7c and d). Saline control mice had significantly greater tumor volumes at the 25 d p.i. time point ($p < 0.0001$) compared to mice treated with ^{225}Ac Ac-DOTA-TATE. Fig. 7e and f show Kaplan-Meier analyses. Animals treated with ^{225}Ac Ac-DOTA-TATE had a significantly delayed time to experimental endpoint ($p < 0.0001$ for H69 and $p = 0.0009$ for H727) relative to the saline controls. The median time to endpoint was 93.6 ± 10.1 and 62.7 ± 6.7 days for the H69-treated and saline cohorts, respectively, and the median time to experimental endpoint was 62.7 ± 7.9 days and 51.4 ± 4.4 for the H727-treated and saline cohorts, respectively.

IHC staining determined SSTR2 expression in tumors with and without treatment after reaching the experimental endpoint of 2000 mm³ tumor volume (Fig. 7 g–j). As seen in the in vitro ICC results, saline treated H69 tumors had significantly greater SSTR2 expression relative to saline treated H727, $p < 0.0001$ (Fig. 7i). Heterogeneity of SSTR2 expression was quantified and saline treated H727 tumors had significantly greater heterogeneity scores relative to saline treated H69 tumors, $p < 0.0001$ (Fig. 7j). Following a single administration of ^{225}Ac Ac-DOTA-TATE, SSTR2 expression significantly decreased by 10%, $p < 0.01$, in H69-treated tumors relative to saline treated tumors, with no significant difference observed in H727 tumors (Fig. 7i). Similarly, H69 tumors demonstrated increased heterogeneity by 22%, $p < 0.05$, following treatment, with no significant difference observed for H727 tumors (Fig. 7j). Hence, the decrease in expression in H69 tumors likely represents a population of cells with decreased SSTR2 expression rather than a general decrease in every cell within the tumor.

Discussion

Ballal, et al. recently presented the first clinical experience and early results on the efficacy and safety of ^{225}Ac Ac-DOTA-TATE in 32 patients with SSTR expressing metastatic GEP-NETs who were ^{177}Lu Lu-DOTA-TATE refractory [22]. For these case studies, systemic TAT using ^{225}Ac Ac-DOTA-

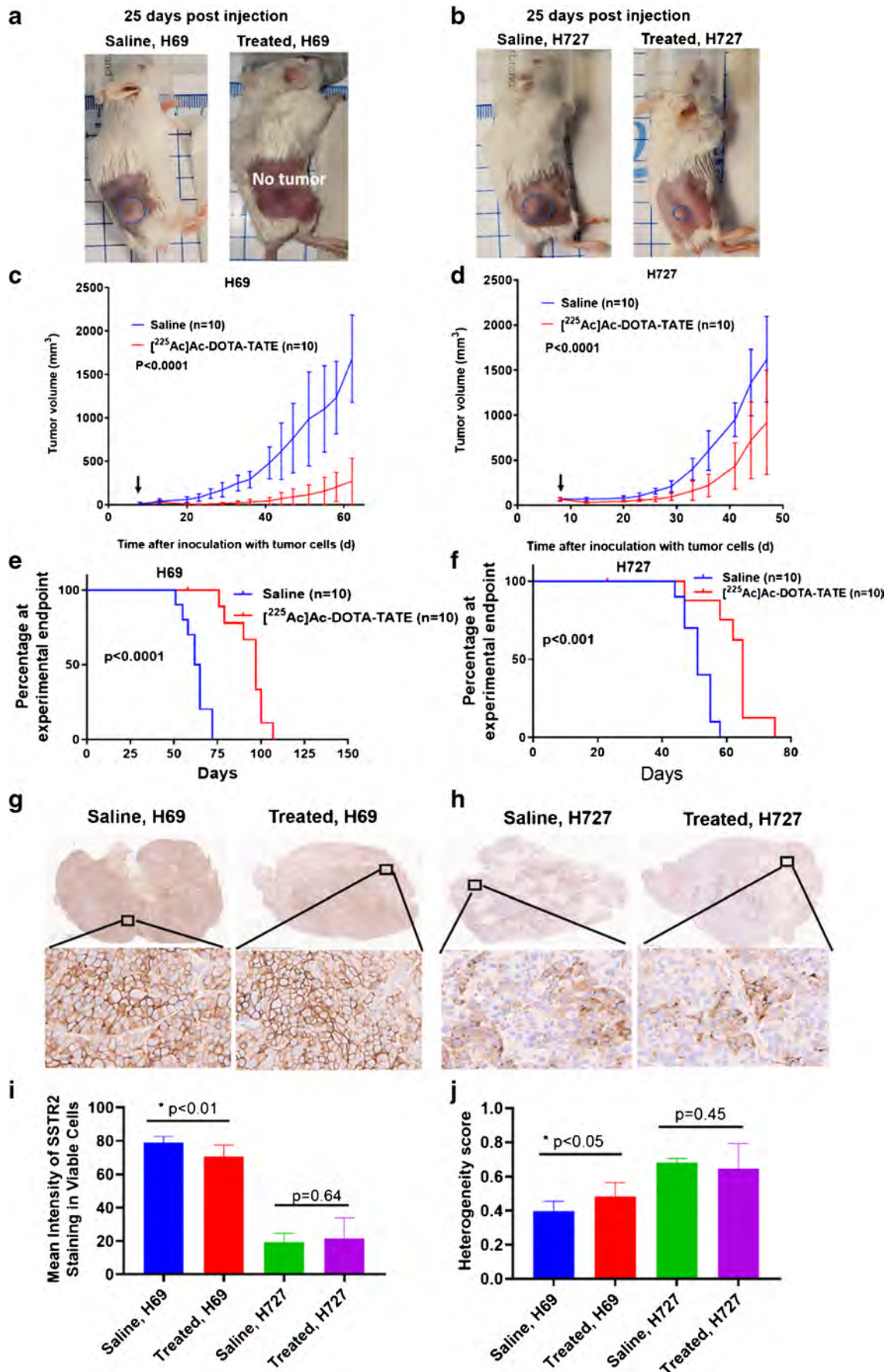
Fig. 7 Efficacy of ^{225}Ac Ac-DOTA-TATE in SCID mice-bearing H69 and H727 tumors. The treated animals were intravenously injected with a single bolus of 148.7 kBq and 144.3 kBq mean ^{225}Ac activity for H69 and H727 cohorts, respectively, or saline solution at 8 days after tumor cells inoculation. Representative images of mice-bearing a H69 and b H727 tumors (outlined), 25 days p.i. Tumor growth volumes of c H69 and d H727 tumors relative to saline controls (arrow indicates the day of injection). Kaplan-Meier plots of e % H69 and f H727 tumor-bearing mice that have reached the experimental endpoint. (g) Representative SSTR2 IHC images of treated and control g H69 tumors and h H727 tumors at experimental endpoint. i Quantified SSTR2 IHC expression j and quantified heterogeneity in treated and control H69 tumors and H727 tumors

TATE was performed in all the patients with ^{225}Ac Ac-DOTA-TATE (100 kBq/kg body weight) at an interval of 8 weeks, to a cumulative dose of 55,500 kBq [22]. This pilot study was reported at an early stage, too soon to calculate overall and event-free survival. However, the short-term clinical results with a median follow-up duration of 8 months suggests that treatment with ^{225}Ac Ac-DOTA-TATE can overcome resistance to ^{177}Lu Lu-DOTA-TATE and that ^{225}Ac Ac-DOTA-TATE can be used as a promising treatment option.

Despite this early clinical experience, preclinical animal studies are needed to better understand the biodistribution and radiation dosimetry of ^{225}Ac Ac-DOTA-TATE, and to support the development of novel methods of determining radiation dosimetry in patients. Furthermore, preclinical studies are required to support an investigational new drug (IND) application to the United States Food and Drug Administration (US FDA) to enable clinical trials in the USA and to enable development of advanced radiation dosimetry methods for clinical use. In addition, the method for synthesis and quality assurance of ^{225}Ac Ac-DOTA-TATE were not presented in the Ballal study.

Herein, we report the first preclinical study for ^{225}Ac Ac-DOTA-TATE. We have synthesized ^{225}Ac Ac-DOTA-TATE with high radiochemical yield and purity, and performed stability, BD, RD, toxicity, and efficacy studies in preclinical models of somatostatin receptor 2 (SSTR2) positive lung tumors. DOTA-TATE was chosen because ^{177}Lu Lu-DOTA-TATE has been compared with ^{177}Lu Lu-DOTA-TOC in patients and a greater tumor residence time was observed for ^{177}Lu Lu-DOTA-TATE, indicating that DOTA-TATE may be a superior targeting ligand for delivery of radiotherapy [23].

Previous somatostatin receptor-targeted PRRT studies have shown that nephrotoxicity is a dose-limiting factor since reabsorption of radio-labeled SSAs by cells in the proximal tubule of the nephron occurs [38, 39]. A preclinical study on PRRT with ^{225}Ac Ac-DOTA-TOC conducted in nude mice bearing AR42J rat pancreatic NET xenograft tumors demonstrated significant kidney tubular necrosis at activities higher



than 30 kBq [16]. Our BD results demonstrated that [^{225}Ac]Ac-DOTA-TATE is primarily cleared by the renal route, with some hepatic clearance. Clearance kinetics parameters and RD were calculated, and the highest accumulated doses were observed in the kidneys and liver, with the highest uptake (initial accumulation, A_o) and fastest clearance (T_{eff}) in the kidney relative to the liver.

In the toxicity study, all animals survived to 5 months p.i., and there was no acute kidney damage observed by pathology at any dose level. BUN and creatinine levels were in the normal range for all animals in the study. However, chronic progressive nephropathy was observed in animals injected with $\geq 2.56 \mu\text{Ci}$ (94.72 kBq) and, despite overall weight gain, animals that were injected with the highest activities, averaging $4.43 \mu\text{Ci}$ (164 kBq) began losing weight at ~ 100 days p.i. It is notable that for clinical administrations, SSA PRRTs are co-infused with amino acids for renal protection by blocking peptide reabsorption in the proximal tubules, which significantly diminishes the radiation dose to the kidneys [6, 40]. Liver enzymes were normal and there was no treatment related damage observed in the liver or other tissues by pathology.

We confirmed a high and moderate endogenous expression of SSTR2 in H69 and H727 human lung cancer cells, respectively. Following a *single* treatment of [^{225}Ac]Ac-DOTA-TATE, the *in vivo* efficacy study demonstrated significantly decreased tumor volume, increased tumor growth delay, and a prolonged time to experimental endpoint for animals bearing both tumor types. The responses were greater in H69 tumor-bearing animals compared to H727, which is likely due to the relatively higher and more homogeneous SSTR2 expression in H69 tumors. It is notable that H69 tumors that regrew following treatment had 10% lower SSTR2 expression and 22% increased heterogeneity of SSTR2 expression relative to controls, and these differences were significant, but the H727 tumors regrew with unaltered expression. Tumor regrowth with retained target expression suggests the potential for multiple dosing regimens to prolong survival. However, the loss of some expression in regrowth suggests the potential for development of treatment resistance over time.

In conclusion, our overall results suggest significant potential for the clinical translation of [^{225}Ac]Ac-DOTA-TATE as a novel therapy for lung neuroendocrine neoplasms. Nephrotoxicity results suggest that renal protective measures will be needed, such as the co-injection of amino acid that is currently done during clinical administration of [^{177}Lu]Lu-DOTA-TATE [6, 40]. Furthermore, since it is not practical to directly image ^{225}Ac in patients, personalized methods for determining radiation dosimetry need to be developed using companion imaging agents, e.g., [^{68}Ga]Ga-DOTA-TATE positron emission tomography and image voxel-based Monte Carlo simulations.

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Authors' contributions Drs. Morse and El-Haddad are the corresponding authors and principal investigators that initiated and led the coordination, planning, and execution of the multi-institutional and multi-disciplinary studies reported herein. Dr. Wadas planned, led, and conducted the radiochemistry at University of Iowa. Dr. Tafreshi contributed to the planning and coordination of the biological studies conducted at Moffitt. Dr. Tafreshi also prepared the first draft of the manuscript. Dr. Pandya performed the radiochemistry at University of Iowa. Drs. Tichacek and Budzevich participated in activity measurements, biodistribution data analyses, and radiation dosimetry calculations. Jordan Reff contributed to the animal studies at Moffitt. Dr. Engelman performed the pathology analyses for the toxicity studies at Moffitt. Drs. Ji and Wang performed the lanthanum and europium chelations. David Boulware performed statistical analyses. Drs. Chiappori and Dr. Strosberg advised regarding the clinical perspective and clinical aspects of this work.

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Declarations

Conflict of interest Drs. Morse and Wadas are co-inventors on a provisional patent application. No other potential conflicts of interest relevant to this article exist.

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Lipophilicity Determines Routes of Uptake and Clearance, and Toxicity of an Alpha-Particle-Emitting Peptide Receptor Radiotherapy

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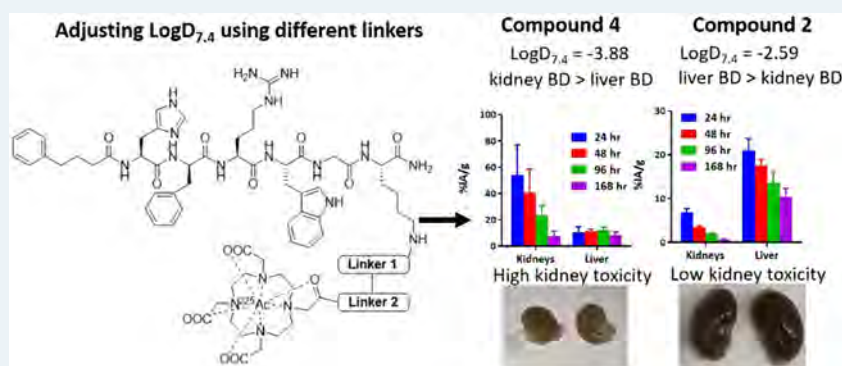
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Supporting Information



ABSTRACT: Lipophilicity is explored in the biodistribution (BD), pharmacokinetics (PK), radiation dosimetry (RD), and toxicity of an internally administered targeted alpha-particle therapy (TAT) under development for the treatment of metastatic melanoma. The TAT conjugate is comprised of the chelator DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate), conjugated to melanocortin receptor 1 specific peptidic ligand (MC1RL) using a linker moiety and chelation of the ²²⁵Ac radiometal. A set of conjugates were prepared with a range of lipophilicities (log *D*_{7.4} values) by varying the chemical properties of the linker. Reported are the observations that higher log *D*_{7.4} values are associated with decreased kidney uptake, decreased absorbed radiation dose, and decreased kidney toxicity of the TAT, and the inverse is observed for lower log *D*_{7.4} values. Animals administered TATs with lower lipophilicities exhibited acute nephropathy and death, whereas animals administered the highest activity TATs with higher lipophilicities lived for the duration of the 7 month study and exhibited chronic progressive nephropathy. Changes in TAT lipophilicity were not associated with changes in liver uptake, dose, or toxicity. Significant observations include that lipophilicity correlates with kidney BD, the kidney-to-liver BD ratio, and weight loss and that blood urea nitrogen (BUN) levels correlated with kidney uptake. Furthermore, BUN was identified as having higher sensitivity and specificity of detection of kidney pathology, and the liver enzyme alkaline phosphatase (ALKP) had high sensitivity and specificity for detection of liver damage associated with the TAT. These findings suggest that tuning radiopharmaceutical lipophilicity can effectively modulate the level of kidney uptake to reduce morbidity and improve both safety and efficacy.

KEYWORDS: Biodistribution, Melanocortin receptor 1, Actinium-225, Lipophilicity, Radiotherapy, α -Particle emitter

Drug discovery and development is a complex, labor-intensive, and lengthy process. This long road includes determining a clinical need, identification and validation of clinically relevant target(s), discovery of targeting moieties, preclinical testing of the drug candidates, and documentation of the efficacy and safety of the drug candidate in the clinical trial phase.^{1–3} On average, this process can take 12–15 years with a cost of hundreds of millions of dollars.^{4,5} Furthermore, roughly 10% of compounds that are selected for further clinical

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study make it to the marketplace as safe and effective drugs, demonstrating that the overall drug development process is formidable.⁶

The success of a new drug candidate is determined not only by its efficacy but also by proper pharmacokinetic behavior. Many promising drug candidates with appreciable activity *in vitro* fail to become marketable products, most often due to inappropriate pharmacokinetic behavior. Physicochemical properties can be used in the optimization of pharmacokinetics and the early prediction of absorption, distribution, metabolism, and excretion (ADME) can save time and money.^{7,8}

Lipophilicity, which is the affinity of a molecule or a moiety for a lipophilic environment, is a key physicochemical property that plays a crucial role in ADME characteristics of drugs.^{9,10} It is one of the factors included in the rule of five formulated by Lipinski in 1997, a tool for medicinal chemists that is used for quick assessment of compounds during drug discovery.¹¹ It also has considerable impact on solubility and membrane permeability,^{12,13} potency,¹⁴ selectivity,¹⁵ and promiscuity¹⁵ of drugs, therefore affecting their biodistribution (e.g., hepatic elimination vs renal excretion), pharmacodynamics, and toxicological profile.^{9,10,16} Lipophilicity is commonly described by the processes of partition between two phases, a nonpolar (organic phase) and a polar (mostly aqueous phase), and expressed as the logarithm of the *n*-octanol partition coefficient ($\log P$ or $\log D$).¹⁷

Peptide–ligand drug conjugates can have high binding affinity and specificity for targets that are highly expressed on cancer cells but have low or nonexpression on normal cells of concern for toxicity. These properties have created significant interest in the use of peptide ligands in pharmaceutical research and development (R&D),¹⁸ as there are currently ~140 therapeutics with peptide targeting ligands in clinical trials.² The systemic clearance route of peptide–drug conjugates can be a significant factor in terms of dose-limiting toxicity (i.e., renal vs hepatic). Although several studies have correlated the route of clearance with the physicochemical properties of small molecules,^{9,19–21} to the best of our knowledge, these correlations are largely unexplored for peptide conjugates.

Because of the high linear energy transfer (LET) and short-range of α particles in tissue, there is significant interest in the development of targeted alpha-particle therapy (TAT) for the treatment of solid tumors (reviewed recently by our group).²² We recently published studies demonstrating the conjugation of a peptide based ligand (MC1RL) with high affinity and selectivity for the melanocortin 1 receptor (MC1R)²³ to a metal chelator (dodecane tetraacetic acid, DOTA) using an aminohexanoic acid (Ahx) linker (DOTA-Ahx-MC1RL). This conjugate was radiolabeled with the α -particle-emitting radionuclide, ²²⁵Ac, for use as TAT for treatment of metastatic uveal melanoma.²² The melanocortin 1 receptor (MC1R) is expressed in 94% of uveal melanoma metastases.

Our preclinical studies demonstrated low toxicity, significantly prolonged survival, and decreased metastasis burden following treatment with ²²⁵Ac-DOTA-Ahx-MC1RL.²² We also observed that ²²⁵Ac-DOTA-Ahx-MC1RL was cleared more via the hepatic route, which differed from our previously reported near-infrared fluorescent dye conjugate (MC1RL-800) that has predominant renal clearance.^{24,25} We observed that the $\log D_{7.4}$ value of ²²⁵Ac-DOTA-Ahx-MC1RL was significantly higher than the value of MC1RL-800 and hypothesized that lipophilicity may have a role in determining

the clearance route of these two conjugates. To test this hypothesis, we designed and synthesized a library of DOTA-linker-MC1RL compounds with diverse linkers and a predicted range of $\log D_{7.4}$ values. Herein, we report the synthesis and characterization of lipophilicity, biodistribution, clearance route, radiation dosimetry, and toxicity profile of this new library of compounds.

MATERIALS AND METHODS

Synthesis of DOTA-linker-MC1RL Compound Library.

MC1RL compounds²³ with different linkers were synthesized on Rink Amide resin (initial loading: 0.568 mmol/g) using N $^{\alpha}$ -Fmoc-protecting amino acids and a HCTU/DIEA strategy. After the resin was swollen in DCM for 30 min, the Fmoc protecting group was removed with 2% DBU in DMF (2 $\times$ 5 min). The resin was washed with NMP and DCM three times each, and the first amino acid was coupled using HCTU and DIEA in NMP (4 equiv of N $^{\alpha}$ -Fmoc amino acid, 4 equiv of HCTU, and 8 equiv of DIEA). The double coupling was performed at all steps under the same coupling conditions due to the sequence deletion and the slower coupling rate in longer sequences. After coupling, the resin was washed with NMP ($\times$ 3) and DCM ($\times$ 3), and any unreacted free amine groups on the resin were treated using 50% acetic anhydride in pyridine for 5 min. After the resin was washed with NMP ($\times$ 3) and DCM ($\times$ 3), the same procedure was repeated for the next amino acid coupling until every residue in the sequence was coupled.

The Alloc protecting group of the C-terminal Lys was deprotected with piperidine (5–10 drops) and Pd(PPh₃)₄ (0.2 equiv) in anhydrous chloroform. Under nitrogen gas, the reaction mixture was stirred for 15 min and then repeated. After the resin was washed with chloroform, NMP, and DCM; DOTA (no-linker), aminohexanoic acid–DOTA, D-Lys-D-Lys–DOTA, or D-Lys-D-Glu–DOTA, were coupled to the free amine via HCTU coupling as described above.

The peptide and every protecting group was cleaved using cleavage cocktail (88% TFA, 5% water, 5% phenol, and 2% triethylsilane) for 4 h. The crude peptide was isolated from the resin by filtration, and the filtrate was concentrated. The peptide was precipitated in ice cold diethyl ether, dissolved in water, and lyophilized. The off-white crude powder was purified by reverse-phase chromatography.

Complexation of the peptides with lanthanum (La³⁺) was accomplished by incubation (25 °C) of the purified peptide in 0.1 M of sodium acetate, pH 6 buffer solution with 3 equiv of lanthanum chloride heptahydrate. The complexation reaction was assayed for completion by monitoring the retention time shift of the ligands on a linear gradient of analytical RP-HPLC. After 16 h of incubation, the reaction was complete, and peptide ligand solutions were separated from the excess metal and buffer by semiprep RP-HPLC. Eu-DTPA-MC1RL was synthesized as described before.²²

Cell Culture. HEK293/MC1R (HEK293 cells engineered to express MC1R)²⁶ were grown in DMEM/F12 (Life Technologies) containing 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin in 5% CO₂ at 37 °C. All experiments were performed with cells of passage number less than 25. Cells were authenticated using short tandem repeat (STR) DNA typing according to ATCC's guidelines by the Molecular Genomics Core Facility at Moffitt²⁷ and monitored by microscopy to ensure maintenance of their original

morphology. Cells were tested for mycoplasma contamination using the MycoAlert kit (Lonza).

Binding Assay. HEK293/MC1R cells²⁸ were used to assess ligand binding in a lanthanide-based time-resolved fluorescence competitive binding assay as described previously.²³ Briefly, the cells were plated on SigmaScreen poly-D-lysine coated plates (Sigma-Aldrich) at a density of 30 000 cells/well 1 day before the experiment. On the day of the experiment, the medium was aspirated, and 50 μ L of nonlabeled competing ligand was added to each well in a series of decreasing concentrations (ranging from $\sim$ 1 μ M to 0.1 nM), followed by 50 μ L of Eu-DTPA-MC1RL at 10 nM. All ligands were diluted in binding medium (DMEM, 1 mM 1,10-phenanthroline, 200 mg/L bacitracin, 0.5 mg/L leupeptin, and 0.3% BSA). Cells were incubated with ligands for 30 min at 37 °C. Following incubation, cells were washed two times with PBS, and 100 μ L of enhancement solution (PerkinElmer) was added to each well. Cells were incubated for an additional 30 min at 37 °C prior to reading. The plates were read on a PerkinElmer VICTORx4 2030 multilabel reader using the standard Eu time-resolved fluorescence (TRF) measurement (340 nm excitation, 400 μ s delay, and emission collection for 400 μ s at 615 nm). Competitive binding curves were analyzed with GraphPad Prism software using the sigmoidal dose-response (variable slope) classical equation for nonlinear regression analysis.

Lipophilicity. The lipophilicities of the unmetallated and La³⁺-chelated DOTA-linker-MC1RL compounds were determined by a miniaturized shake-flask method similar to a previous report.²⁴ Stock solutions of the compounds (200 μ M) in 10 mM, pH 7.4, phosphate buffer were prepared. Aliquots of the stock solutions were vortexed with three different ratios of *n*-octanol. The resulting emulsions were centrifuged and the PBS and *n*-octanol layers separated. Unknown compound concentrations in the aqueous and organic layers were measured by HPLC-triple quadrupole MRM mass spectroscopy. Triplicate measurements of compound concentration were used to calculate the log of partition between the lipid and aqueous phases at pH 7.4 (log $D_{7.4}$).

Radiochemical Synthesis and Quality Control for ²²⁵Ac-DOTA-linker-MC1RL Compounds. Actinium-225 (²²⁵Ac; $t_{1/2}$ = 10 days; $E_{\alpha\max}$ = 6–8 MeV) was purchased from Oak Ridge National Laboratories. The complexation of ²²⁵Ac was achieved for DOTA-Ahx-MC1RL as previously described²⁹ and by reacting DOTA-MC1RL (no linker), DOTA-DLGL-MC1RL (D-Lys-D-Lys linker), or DOTA-DLDG-MC1RL (D-Lys-D-Glu linker) (5–10 μ g in 5–10 μ L water from 1.0 mg/mL solution) with ²²⁵Ac(NO₃)₃ (3.4 MBq) that was diluted in 100 μ L of water containing 10 μ L of 20% L-ascorbic acid. The pH of the resulting solution was adjusted to 5.5–6 using 1 M Tris buffer (10–12 μ L) and then incubated at 60 °C for 1 h. Reaction progress and radiochemical purity were measured without further purification using ITLC with gamma counting, radio-TLC, and gamma counting of radio-HPLC fractions. Specific activity was calculated using a standard method.³⁰ Analysis was performed 24 h after sample collection to ensure secular equilibrium was achieved between ²²⁵Ac and its daughter products. The proportion of chelated ²²⁵Ac in this preparation relative to the proportion of chelated La³⁺ is described in Supplemental Experimental Methods.

Animal Studies. All protocols were approved as University of South Florida IACUC protocol IS00004454.

Injected Activity Measurement. Syringes were prefilled with ²²⁵Ac radiopharmaceutical activity, and since α -particles from ²²⁵Ac cannot be directly measured in tissue due to the range,³¹ the activity was measured using the isomeric gamma spectra by the 4 π well-type Atomlab 500 Wipe Test Gamma Counter (BioDex) and converted to ²²⁵Ac alpha activity using factors for γ ray abundance per alpha decay as described previously.²² The spectra were acquired using a full energy window to include gamma counts from ²²⁵Ac, with peak at 99.8 keV (abundance 1%), and its two γ -emitting daughters, ²²¹Fr (4.9 min $T_{1/2}$) with peak at 218.1 keV (abundance 11.4%) and ²¹³Bi (46 min $T_{1/2}$) with peak at 440.5 keV (abundance 25.9%).³² The alpha activity in 1 μ Ci of each radionuclide was determined by fitting each peak with a multi-Gaussian fit and integrating to determine the net number of counts while incorporating the acquisition time. The activity was injected into each mouse via tail-vein catheter. Activity remaining in the syringe and catheter postinjection were calculated and subtracted to determine net administered activity. Spectra were acquired at $\geq$ 24 h post-radiosynthesis or tissue rendering ensuring that ²²⁵Ac and daughters were in secular equilibrium.³³

Biodistribution (BD). Male and female BALB/c mice (10–12 weeks, 18–22 g, Charles River) were used for the BD studies. Tail vein catheters were used for agent administration to mice. All mice were intravenously administered 148 kBq $\pm$ 10% of ²²⁵Ac alpha activity in the syringe. The animals were euthanized at multiple time-points between 24 h and 1 week post-injection (p.i.) (n = 5 mice per time point), and tissues were removed and weighed. The isomeric gamma spectra were measured for each tissue sample as described above. Using the net administered activity for ²²⁵Ac, ²²¹Fr, and ²¹³Bi, the percent injected activity per gram (%IA/g) were then calculated and compared to a weighed, counted standard for all groups.

In-house-designed Matlab (MathWorks) code was used to calculate the pharmacokinetics (PK) rates of uptake and clearance for each compound. Data were fit using the nonlinear least-squares approach and the logarithmic equation for uptake and two-exponential decay equation for clearance to find relevant parameters (e.g., rates of uptake and clearance).³⁴ The following equation was used for uptake:

$$y = A \times \log(x) + \lambda$$

where y [%IA/g] is the percentage of measured radioactivity per gram of tissue, A [%IA/g] is the peak percentage of radioactivity per gram of tissue, and λ [%IA/g/h] is the rate of uptake.

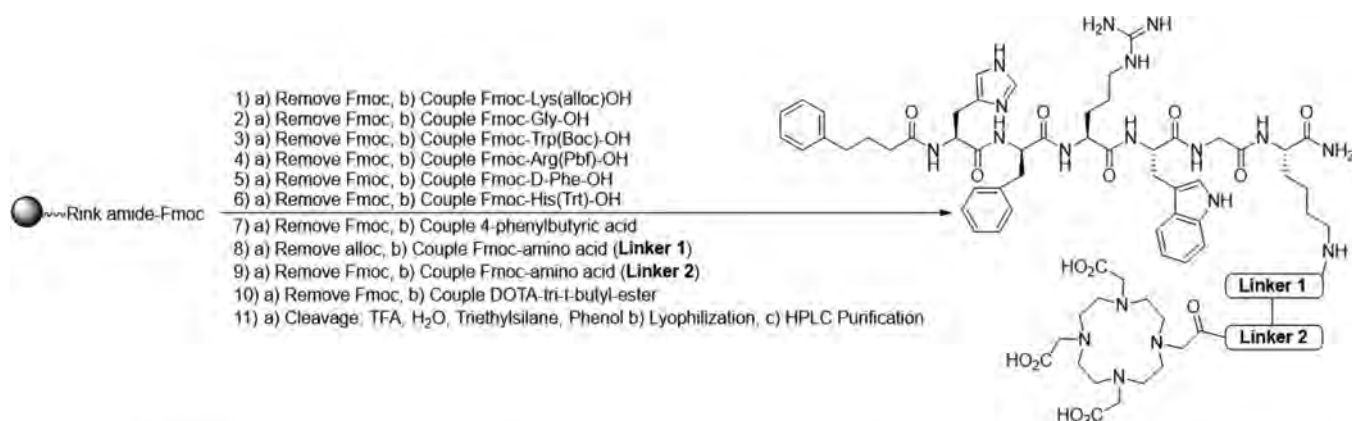
A two-compartment model was used where rapid clearance of radiopharmaceutical circulating in the blood and a slower clearance of radiopharmaceutical from tissues are observed. Hence, the following two-exponential equation was used for clearance:

$$y = A_1 \times e^{(-\lambda_1 x)} + A_2 \times e^{(-\lambda_2 x)}$$

where y [%IA/g] is the percentage of measured radioactivity per gram of tissue, A_1 [%IA/g] and A_2 [%IA/g] are the initial percentages of radioactivity per gram of tissue at two different phases of clearance, and λ_1 [%IA/g/h] and λ_2 [%IA/g/h] are the rates of clearance for two different phases.

Radiation Dosimetry (RD). Dosimetry calculations were performed as previously described.²⁹ As described above for BD studies, isomeric γ -emission spectra of ²²⁵Ac and the two

Scheme 1. Synthetic Route of DOTA-Linker-MC1RL Compounds



Compounds	MW	Linker Residue 1	Linker Residue 2
1	1361	-	-
2	1474	Ahx	-
3	1586	D-Lys	D-Lys
4	1618	D-Lys	D-Glu

radionuclides in the decay chain with detectable emissions, ^{221}Fr and ^{213}Bi , were acquired in tissues rendered at different p.i. time points and used for these calculations. Because of their short half-lives, ^{217}At (32.2 ms) and ^{213}Po (4.2 μs) were assumed to decay at the site of their parent radionuclides, ^{221}Fr and ^{213}Bi , respectively. The β decay branching ratio for ^{217}At to ^{217}Rn is only 0.01%; therefore, it was assumed that all decays of ^{217}At progressed by α emission to ^{213}Bi . The branching ratios for decay of ^{213}Bi are 98% to ^{213}Po and 2% to ^{209}Tl and were accounted for in the calculation. Due to the relatively low LET and the small dimensions of the target tissues, the β emissions from ^{217}At , ^{213}Bi , ^{209}Tl , and ^{209}Pb were assumed to be negligible and are not included in the calculations.³⁵

Organ and tumor absorbed doses from ^{225}Ac , ^{221}Fr , ^{217}At , ^{213}Bi , and ^{213}Po were determined using acquired BD data. Dosimetry calculations were performed using the generalized internal dosimetry schema of the MIRD Committee for α -particle emitters.^{35,36} According to MIRD #21, the absorbed radiation dose due to particle type x , $D_x(r_T, T_D)$, is the mean energy imparted to target tissue r_T per unit tissue mass, defined by eq 1:

$$D_x(r_T, T_D) = k \tilde{A}(r_S, T_D) \frac{\sum_i \Delta_i^x \phi(r_T \leftarrow r_S; E_i^x)}{M(r_T)} \quad (1)$$

where $\tilde{A}(r_S, T_D)$ is the total number of nuclear transitions in the target region (accumulated activity), Δ_i^x is the mean energy emitted per nuclear transition, $\phi(r_T \leftarrow r_S; E_i^x)$ is the fraction of energy emitted per nuclear transition in the source region that is absorbed in the target region by the i th emission that is emitted with initial energy E_i , k is a conversion factor, and $M(r_T)$ is the mass of the target tissue. Time-activity curves were generated for each organ and fit with an exponential decay nonlinear regression. Accumulated activity in each organ/tumor was determined by analytically integrating the resulting equation of fit. Several assumptions were made in the

calculation of absorbed dose. It was assumed that the α -particle activity was uniformly distributed in each organ/tumor volume, and as a result of its short-range in tissue, no α particles escaped from its source organ/tumor. Electron and photon contributions were assumed to be negligible compared to α -particle energy deposition.³⁷ After these assumptions, eq 1 simplifies to

$$D_\alpha(r, T_D) = \frac{k \tilde{A}(r, T_D) \Delta^\alpha}{M(r)} \quad (2)$$

It was assumed that α particles from ^{221}Fr (4.9 min $T_{1/2}$), ^{217}At (32.2 ms $T_{1/2}$), ^{213}Bi (46 min $T_{1/2}$), and ^{213}Po (4.2 μs $T_{1/2}$) were deposited in the same location as ^{225}Ac (10 day $T_{1/2}$) due to the relatively shorter half-lives of these daughter isotopes. Although ^{217}At and ^{213}Po do not have detectable γ emissions, under the assumption that the decay chain had reached secular equilibrium, the accumulated activity of these two daughters would equal that of ^{221}Fr and ^{213}Bi , respectively. The total absorbed α -particle dose was calculated from the summation of doses from ^{225}Ac , ^{221}Fr , ^{217}At , ^{213}Bi , and ^{213}Po .

Toxicity. Toxicity studies were conducted using cohorts of normal BALB/c mice. After a single intravenous injection of each ^{225}Ac conjugate ranging from 3 to 314 kBq ^{225}Ac activity or saline, cohorts ($n = 6$ mice/cohort) were weighed 2 times per week and monitored for 7 months for signs of distressed behavior. At the end of that period, animals were euthanized, and serum was collected by centrifugation at $1500 \times g$. Blood urea nitrogen (BUN), creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) content in serum were analyzed using the Catalyst DX automated chemistry analyzer and CLIP (IDEXX, ME). Liver, kidney, spleen, pancreas, cecum, small intestine, lymph nodes, bone marrow, muscle, salivary gland, and bone were harvested and fixed in 10% formalin. Bone was decalcified in 14% ethylene diaminetetraacetic acid (EDTA) solution after fixation in formalin. All tissues were embedded in

paraffin, sectioned (4–6 μm thickness), and stained with hematoxylin and eosin. The slides were scanned in the Moffitt Analytical Microscopy Core Facility using a ScanScope XT digital slide scanner (Aperio, CA) and examined by the veterinary pathologist (R.E.), in a blind manner.

Statistical Analysis. Analysis of variance (ANOVA) was used to assess the relationship of BD (%IA/g) with the log D value, with log D and time as the independent variables. These analyses were modeled on liver BD, kidney BD, and the kidney/liver BD ratio (i.e., three separate models). An interaction effect with time and BD (either kidney or liver or the ratio) was included too.

Logistic regression analyses were used to assess the toxicities for each compound and injected activity group using standard reference ranges (adjusted for control levels if necessary) based on the injected activity. Weight change for each compound and activity was evaluated relative to the control group's weight change. A predicted probability of toxicity was then generated across the range of injected activity levels to generate curves for each of compound group.

Using the Spearman's correlation coefficient, the correlation of log D values with uptake values for both kidney and liver were estimated for each radiopharmaceutical. Also, for each derived toxicity measure for either kidney or liver, we calculated the sensitivity and specificity of toxicity using pathology as the standard.

RESULTS

Synthesis and Characterization of DOTA-linker-MC1RL Compounds. The library of DOTA-linker-MC1RL compounds were synthesized using the synthetic route shown in Scheme 1. Synthesis of the DOTA-Ahx-MC1RL version was previously described.²⁹ Each compound was chelated with La^{3+} to facilitate *in vitro* characterizations. La^{3+} has been reported as a useful surrogate for compounds being developed with Ac, since both exist as trivalent ions in solution.²⁸ See Figures S1–S8 for HPLC chromatograms and MALDI-TOF spectra of each compound, with and without La^{3+} chelation.

Via competitive binding assay, we determined that all of the La^{3+} -DOTA-linker-MC1RL compounds have high binding affinity for MC1R (i.e., 0.08, 0.20, 0.06, and 0.23 nM K_i) for versions with the following linkers: no linker, Ahx, DLDL, and DLDG, respectively (Figure 1).

Log $D_{7.4}$, which can influence the tissue distribution of a compound and is an indicator of lipophilicity or solubility, was determined for the unmetallated and La^{3+} -chelated compounds (Table S1). Complexation with La^{3+} reduced the log $D_{7.4}$ for

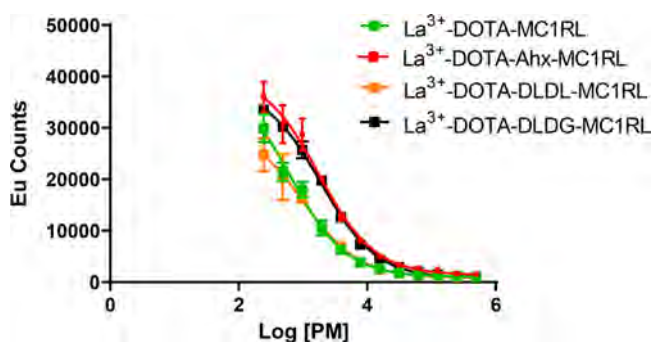


Figure 1. Competitive binding assay for La^{3+} -DOTA-linker-MC1RL compounds.

each compound. These values suggest that the compounds are soluble in aqueous buffer, but with variable lipophilicities (ranging from -1.79 to -2.75 for the unmetallated compounds and from -2.14 to -3.88 for the compounds chelated with La^{3+}).

Radiosynthesis and Characterization of ^{225}Ac -DOTA-linker-MC1RL Radiopharmaceuticals. Radiosynthesis (Scheme 2) for all ^{225}Ac -DOTA-linker-MC1RL radiopharmaceuticals provided a greater than 95% yield with high radiochemical purity ($\geq 99.0\%$) as determined by radio-TLC and radio-HPLC (Figures S9–S12).

Biodistribution and Pharmacokinetics. Tissue BD of the ^{225}Ac -DOTA-linker-MC1RL radiopharmaceuticals were determined in BALB/c mice. Following intravenous injection, ^{225}Ac activity was observed primarily in clearance tissues (i.e., liver, kidneys, and intestine), and clearance occurred over a period of weeks (Figure 2). Using the BD data, the pharmacokinetics of kidney and liver uptake and clearance rates, and blood clearance rates were calculated for each radiopharmaceutical (Table 1). The uptake data were best fit using the logarithmic uptake equation. A two-compartment model for clearance was assumed, and clearance data were fitted using the two-exponential clearance equation.

Radiation Dosimetry. Radiation dosimetry (RD) calculations were based on the data obtained from the BD studies. RD for targeted radiotherapy is the determination of the absorbed energy deposited per unit mass by ionizing radiation in the different tissue compartments within the body. As described in the Materials and Methods, the α -particle dose from ^{225}Ac and each of its α -emitting daughters was calculated using γ spectroscopy of ^{225}Ac , ^{221}Fr , and ^{213}Bi (Figure S13).

BD data for the different tissues were fitted using an exponential decay nonlinear regression, allowing the estimation of clearance kinetics, tissue biological half-life, accumulated activity, and absorbed dose/injected activity (Gy/kBq) for each radionuclide in each tissue. See Table S2A–C for ^{225}Ac -DOTA-MC1RL, ^{225}Ac -DOTA-DLDL-MC1RL, and ^{225}Ac -DOTA-DLDG-MC1RL. RD for ^{225}Ac -DOTA-Ahx-MC1RL was previously reported.²⁹ The total absorbed dose is the summation of the values for the five α -emitting radionuclides. The calculated total absorbed dose for each of the ^{225}Ac -labeled compounds was minimal in all tissues except clearance organs (kidneys and liver). The total absorbed dose of ^{225}Ac and daughters was greater in the liver relative to the kidneys for ^{225}Ac -DOTA-MC1RL (no linker) and ^{225}Ac -DOTA-Ahx-MC1RL with 0.56 and 0.24 Gy/kBq for kidneys and 0.79 and 0.70 Gy/kBq for liver, respectively. The total absorbed dose of ^{225}Ac and daughters was greater in the kidneys (1.59 and 0.92 Gy/kBq) relative to the liver (1.36 and 0.45 Gy/kBq) for ^{225}Ac -DOTA-DLDL-MC1RL and ^{225}Ac -DOTA-DLDG-MC1RL, respectively. Figure 3 presents graphs of the absorbed dose from each radionuclide per tissue for all four radiopharmaceuticals.

It is noted that in tissues with significant uptake, the effective decay half-lives (T_{eff}) calculated for ^{225}Ac were shorter than the radiodecay half-life of ^{225}Ac (10 days) for all the compounds, indicating biological clearance. For example, the calculated T_{eff} in kidneys and liver for ^{225}Ac —no linker was 4.8 and 5.7 days, respectively (Table S2A). Hence, T_{eff} is a composite of radiodecay and active biological clearance. The T_{eff} was only calculated to be longer in some tissues with minimal uptake where instrument background likely interfered with the accuracy of measurement.

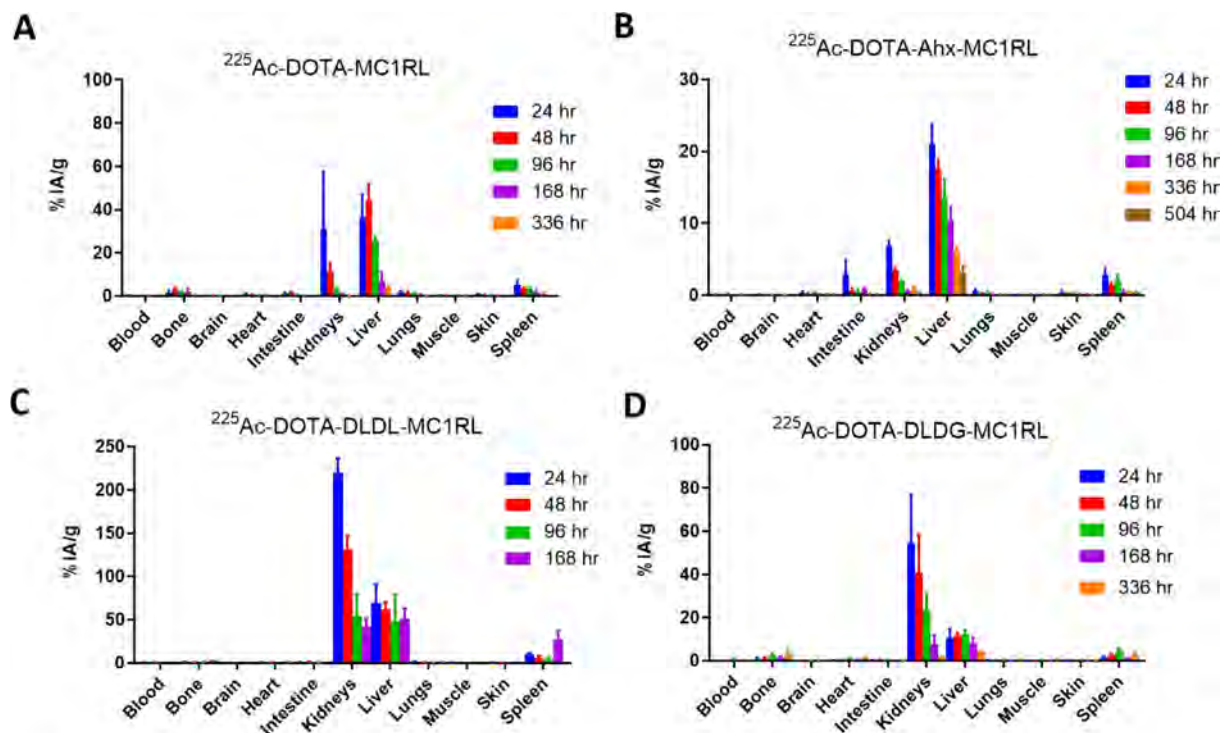
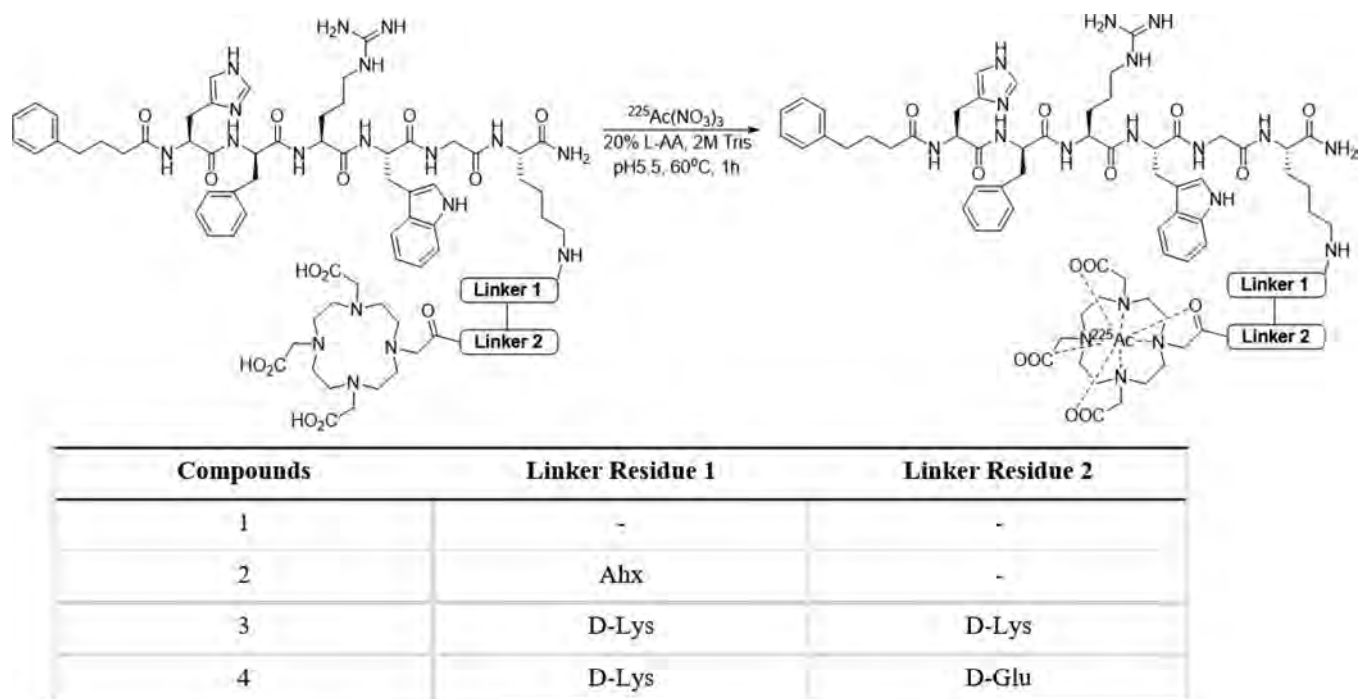
Scheme 2. Radiochemical Synthesis of ^{225}Ac -DOTA-linker-MC1RL Compounds

Figure 2. BD results for (A) ^{225}Ac -DOTA-MC1RL, (B) ^{225}Ac -DOTA-Ahx-MC1RL, (C) ^{225}Ac -DOTA-DLGL-MC1RL, and (D) ^{225}Ac -DOTA-DLDG-MC1RL. Activities were calculated for tissues rendered from BALB/c mice ($n = 6$ per time point). The time courses vary among the different compounds due to differences in animal survival and liver clearance.

Toxicity. Cohorts of mice received a single intravenous injection of a range of ^{225}Ac activities from 3 to 314 kBq of each radiopharmaceutical. Animals were weighed twice per week and monitored for 7 months for signs of discomfort or declining condition. At the completion of the study, serum was collected for renal and hepatic toxicity assays, and tissues were collected for histological staining and pathology examination.

See Table S3 for the histopathology scoring of all tissues in the study.

Death due to acute renal failure was observed in some cohorts. There were no deaths observed in mice administered ^{225}Ac -DOTA-MC1RL (no linker) or ^{225}Ac -DOTA-Ahx-MC1RL at any injected activity. All mice treated with ≥ 77.6 kBq of ^{225}Ac -DOTA-DLGL-MC1RL and a single mouse

Table 1. Pharmacokinetics of Uptake and Clearance Rate Constants for Each Compound

compound	organ	uptake rate constant, λ (%IA/g/h)	fast clearance rate constant, λ_1 (%IA/g/h)	slow clearance rate constant, λ_2 (%IA/g/h)
^{225}Ac -DOTA-MC1RL	blood		0.00756	1.89
	kidney	3.98	0.0361	0.0275
	liver	30.1	63.8	0.00737
^{225}Ac -DOTA-Ahx-MC1RL	blood		0.507	0.0125
	kidney	5.63	0.0605	0.00514
	liver	17.3	0.0662	0.00319
^{225}Ac -DOTA-DLDDL-MC1RL	blood		1.87	0.00577
	kidney	179	0.0229	−0.0109
	liver	55.9	0.00511	−0.0323
^{225}Ac -DOTA-DGDG-MC1RL	blood		0.0198	2.00
	kidney	44.1	0.0117	−0.0118
	liver	8.97	0.0412	0.00360

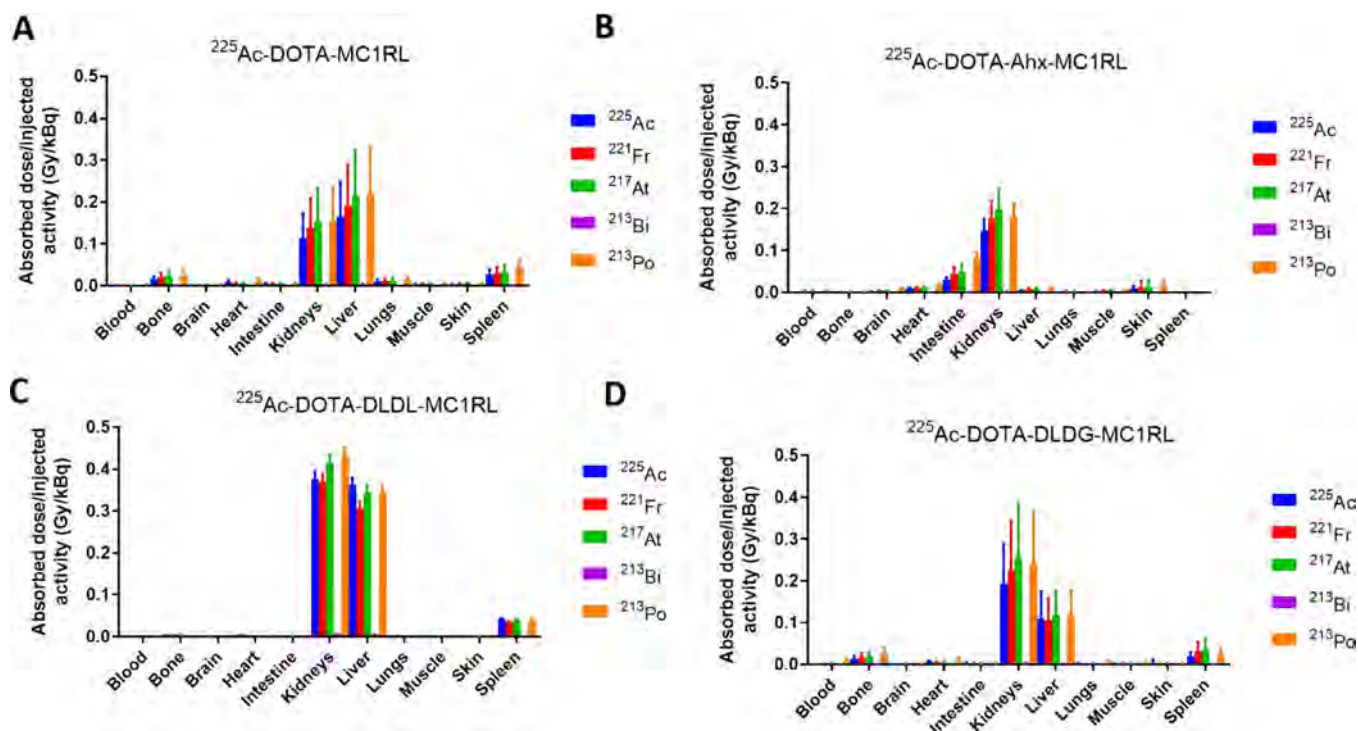


Figure 3. Radiation dosimetry of ^{225}Ac and daughters following administration of (A) ^{225}Ac -DOTA-MC1RL (no linker), (B) ^{225}Ac -DOTA-Ahx-MC1RL, (C) ^{225}Ac -DOTA-DLDDL-MC1RL, and (D) ^{225}Ac -DOTA-DLDG-MC1RL in BALB/c mice.

injected with 44.0 kBq of ^{225}Ac -DOTA-DLDG-MC1RL died within 1–2 weeks p.i., and at necropsy, kidneys appeared grossly pale, pitted, small, and irregular in shape (Figure 4A). Histologically, tubular cell necrosis was characterized by intense cytoplasmic eosinophilia with pyknotic nuclei, while other tubules appeared regenerative with cytoplasmic basophilia and nuclear crowding (Figure 4C).

By the end of the study, mice injected with ^{225}Ac -DOTA-MC1RL, ^{225}Ac -DOTA-Ahx-MC1RL, ^{225}Ac -DOTA-DLDDL-MC1RL, and ^{225}Ac -DOTA-DLDG-MC1RL at ≥ 268.9 , 181.5, 11.5, and 50.2 kBq of ^{225}Ac activity, respectively, had chronic progressive nephropathy. Histologically, this pathophysiological entity was characterized by little normal renal parenchyma remains due to extensive tubular cell necrosis, epithelial sloughing and cast formation, extensive tubular cell regeneration, diffuse interstitial edema and fibrosis with mild mononuclear inflammatory cell infiltrates, and hypercellular glomerular tufts (Figure 4D,E). Animals injected with less than

the above activities survived without abnormal kidney pathology.

None of the animals injected with ^{225}Ac -DOTA-MC1RL or ^{225}Ac -DOTA-DGDG-MC1RL had histopathological liver damage. By the end of the study, mice injected with greater than >317.8 kBq of ^{225}Ac -DOTA-Ahx-MC1RL had treatment-related liver damage such as bile duct hyperplasia and hepatocyte apoptosis (Figure 4G), and mice injected with >27.0 kBq of ^{225}Ac -DOTA-DLDDL-MC1RL had treatment related hepatocyte granular degeneration, apoptosis, and necrosis (Figure 4H,I). Incidental hepatocyte glycogen accumulation and mild, focal, hepatocellular fatty changes were observed in the livers of some treated and untreated control mice.

The spleen of some animals injected with >125.8 kBq of ^{225}Ac -DOTA-DLDDL-MC1RL had moderate lymphoid depletion. A mild multifocal lymphoplasmacytic infiltration of salivary glands was observed in some treated mice.

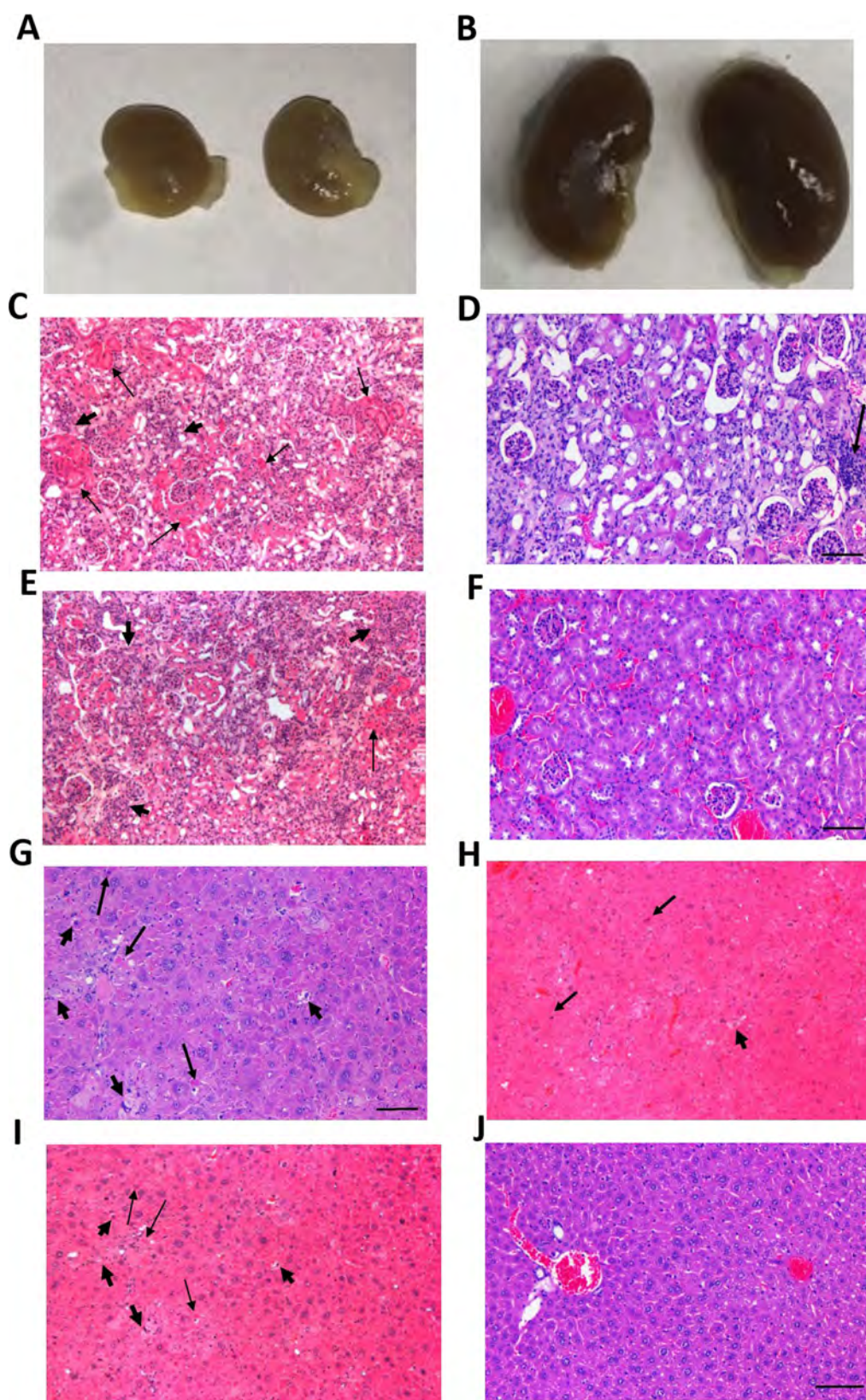


Figure 4. Gross appearance of kidneys (A, B) and histological appearance of kidney (C–F) and liver (G–J) from mice treated with different peptides in the toxicity study. (A, B) Gross appearance of kidneys from mice injected with 79.4 kBq of ^{225}Ac -DOTA-DL DL-MC1RL and 305.4 kBq ^{225}Ac -DOTA-MC1RL. (C) Kidney histology of a 135.4 kBq of ^{225}Ac -DOTA-DL DL-MC1RL administered mouse, with acute tubular necrosis resulting in death at 11 days after administration. At necropsy, kidneys appeared grossly pale, pitted, small, and irregular in shape. Histologically, tubular cell necrosis was characterized by intense cytoplasmic eosinophilia with pyknotic nuclei (arrows), while other tubules appeared regenerative with cytoplasmic basophilia and nuclear crowding (arrowheads). (D) Kidney of a 293.4 kBq of the ^{225}Ac -No linker administered mouse with nephropathy comprised of tubular epithelial cell degeneration with cytoplasmic vacuolization, necrosis, and regeneration with cytoplasmic

Figure 4. continued

basophilia and nuclear crowding, hypercellularity of glomerular tufts, and focal infiltration of mononuclear cells (arrow). (E) Kidney histology of a 84.2 kBq of ^{225}Ac -DOTA-DLDG-MC1RL administered mouse euthanized 7 months after administration, with chronic progressive nephropathy. Little normal renal parenchyma remains due to extensive tubular cell necrosis (arrow), epithelial sloughing and cast formation, extensive tubular cell regeneration (arrowheads), diffuse interstitial edema and fibrosis with mild mononuclear inflammatory cell infiltrates, and hypercellular glomerular tufts. (F) Kidney of saline-treated mouse with normal eosinophilic cuboidal tubular epithelium, and normal glomeruli. (G) Liver of 292.2 kBq of the ^{225}Ac -DOTA-Ahx-MC1RL administered mouse with hepatocellular eosinophilic cytoplasmic swelling, single hepatocellular apoptosis (arrows) with hypereosinophilic cytoplasm and pyknotic nuclei, and single hepatocellular necrosis (arrowheads) with pale eosinophilic cytoplasm and karyolysis. (H) Liver histology of a 135.4 kBq of ^{225}Ac -DOTA-DLDL-MC1RL administered mouse, with focal hepatocellular eosinophilic cytoplasmic swelling, single hepatocellular apoptosis (arrows) with hypereosinophilic cytoplasm and pyknotic nuclei, and single hepatocellular necrosis (arrowhead) with pale eosinophilic cytoplasm and karyolysis. (I) Liver histology of a 36.0 kBq of ^{225}Ac -DOTA-DLDL-MC1RL administered mouse, with hepatocellular eosinophilic cytoplasmic swelling, hepatocellular apoptosis (arrows), and hepatocellular necrosis (arrowhead). (J) Liver of saline administered mouse with normal, well-delineated hepatic cords and sinusoids, a portal triad (left), and a central vein (right).

The animal weights for each radiopharmaceutical and administered activity are plotted in Figures S14–S17. By the end of the study, mice injected with ^{225}Ac -DOTA-MC1RL, ^{225}Ac -DOTA-DLDL-MC1RL, and ^{225}Ac -DOTA-DGDL-MC1RL at ≥ 268.9 , 25.5, and 44.4 kBq of ^{225}Ac activity, respectively, decreased in weight over the course of the study. Mice injected with ≥ 192.9 kBq of ^{225}Ac -DOTA-Ahx-MC1RL lost weight, but three mice injected with 200, 212, and 315 kBq of activity gained weight. Mice administered lower activities gained weight over the course of the study.

Serum specimens were tested for BUN and creatinine levels as indicators of renal toxicity, and ALKP, ALT, and AST levels as indicators of hepatic toxicity (Table S4).³⁸ Elevated BUN levels were observed in some mice injected with ^{225}Ac -DOTA-MC1RL, ^{225}Ac -DOTA-Ahx-MC1RL, ^{225}Ac -DOTA-DLDL-MC1RL, and ^{225}Ac -DOTA-DLDG-MC1RL at ≥ 193.6 , 200.1, 11.5, and 19.36 kBq of ^{225}Ac activity, respectively.

Since histopathology is considered the gold standard of determining toxicity of exposure to ionizing radiation and since weight loss and serum biomarkers are indirect indicators of damage due to toxicity, the sensitivity and specificity of these indirect measures were evaluated relative to pathology (Table 2). Weight loss was shown to have high sensitivity and

kidney-to-liver BD ratio, $p < 0.001$ (Table S5). It is noted that no relationship was observed between $\log D_{7.4}$ and liver BD. Since BD is a composite of agent uptake and clearance, the correlations among $\log D_{7.4}$ and kidney and liver uptake, and clearance rates and toxicity indicators (weight loss, pathology, BUN and ALKP levels) were examined (Table S6). The statistical analysis showed that $\log D_{7.4}$ is significantly associated with weight loss, $p < 0.0001$, and that both $\log D_{7.4}$ and weight loss are significantly associated with kidney clearance rates, $p < 0.0001$. Furthermore, BUN levels are significantly associated with kidney uptake rates, $p < 0.0001$, and liver clearance rates, $p < 0.0001$.

DISCUSSION

Lipophilicity is a major determinant of route of clearance of small molecule drugs due to the dependence of renal clearance on membrane permeability.³⁹ However, this observation has not been fully substantiated for peptide conjugates.⁴⁰ Mass can influence the route of clearance (i.e., molecules < 50 kDa are removed from circulation via renal glomeruli, while larger macromolecules clear via the splenic and hepatic routes).⁴⁰ With the goal of characterizing the relationship between lipophilicity and clearance route, we designed and synthesized a set of peptide-conjugate based radiopharmaceuticals for TAT with a range of lipophilicities ($\log D_{7.4}$ values) and comparable mass values ranging from 1361 to 1618 MW without metal chelation. Also, unnatural amino acids are incorporated into the peptidic component of these compounds, and high radiochemical stability has been previously demonstrated for the ^{225}Ac -DOTA-Ahx-MC1RL radiopharmaceutical.²⁹ Hence, peptide metabolism is likely a minor factor in determining the route of clearance. Herein, we report characterization of the BD, PK, RD, and toxicity of these compounds in mice and the relationships of $\log D_{7.4}$ values with the route and PK of uptake and clearance of the administered TAT.

Since the lipophilicities of the compounds were adjusted by inserting linkers that have different structures and chemical properties between the peptide targeting moiety and the metal chelator, quality control characterizations were also performed. As a nonradioactive surrogate of ^{225}Ac , La^{3+} chelates were used for these assays. The high binding affinity (low nM K_d) of the MC1RL peptide for the MC1R receptor and $\log D_{7.4}$ values were confirmed for each compound.

For BD, PK, and RD determinations, each compound was labeled with ^{225}Ac with high radiochemical yield and purity. When evaluating the BD, PK, and RD data, general observations can be made that the metalated compounds

Table 2. Determination of Sensitivity and Specificity of the Indirect Indicators of Toxicity Incomparison to Pathology

parameter	% sensitivity	% specificity
Kidney Pathology		
weight loss	87	95
BUN	74	97
Liver Pathology		
weight loss	85	74
AST	14	90
ALKP	85	86
ALT	28	95

specificity for tissue damage to either kidney or liver. BUN levels had high sensitivity and specificity for kidney damage, and ALKP levels had high sensitivity and specificity for liver damage. The probabilities of kidney or liver damage by pathology, elevated BUN or ALKP levels, or weight loss per injected activity for each radiopharmaceutical are shown in Figure 5A–E.

Relationship of BD, PK, RD, and Toxicity with Compound Lipophilicity. Using the BD data for all four compounds, a significant relationship was determined between $\log D_{7.4}$ and kidney BD, $p < 0.05$, and between $\log D_{7.4}$ and the

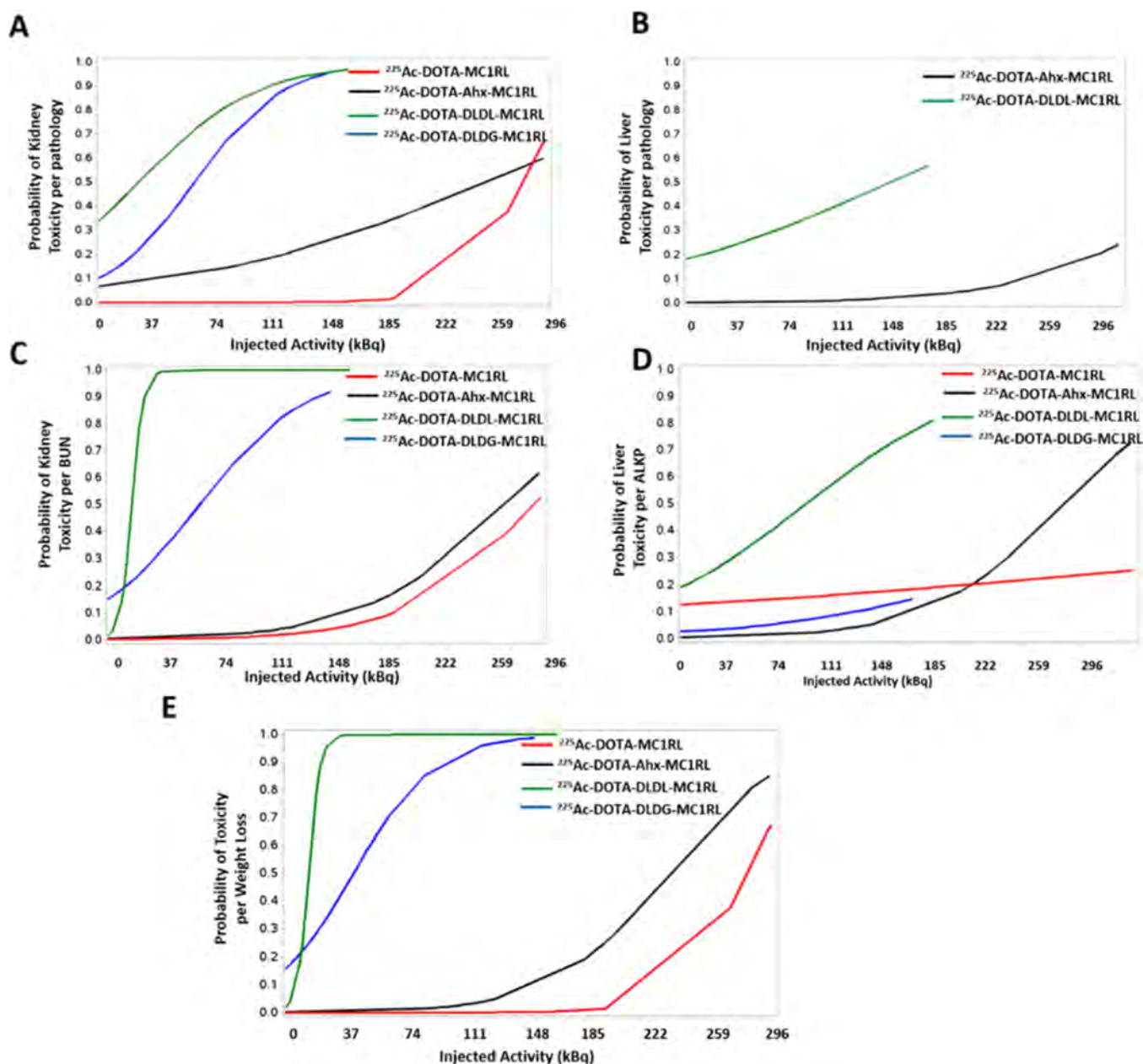


Figure 5. Toxicity probability curves per injected activity for each radiopharmaceutical per (A) kidney pathology, (B) liver pathology [no toxicities were observed for the other two radiopharmaceuticals], (C) BUN, (D) ALKP, and (E) weight loss.

with log $D_{7.4}$ values above -2.6 have lower kidney uptake rates, are cleared more by the hepatic route than renal, and have a lower radiation dose to the kidney when compared to the compounds with log $D_{7.4}$ values below -3.4 , which are cleared more by the renal route, have higher kidney uptake rates, and have a higher radiation dose to the kidney. This negative correlation of decreased kidney uptake and decreased radiation dose with increasing lipophilicity can be explained as a result of an increase in passive reabsorption in the kidney tubule, leading to less renal uptake.^{21,39}

Radiation-induced renal toxicity is a major concern in the therapeutic application of peptides labeled with radiometals.⁴¹ In this study, we observed two distinct pathological features: acute kidney damage and chronic kidney damage. Acute kidney failure was observed in animals that were administered compounds with log $D_{7.4}$ values below -3.4 and was grossly

characterized by pale, pitted, small, and irregular shaped kidneys at necropsy and by acute nephropathy via pathology examination. Acute kidney damage resulted in a rapid loss of kidney function and death within the week following treatment. Animals administered compounds with log $D_{7.4}$ values above -2.6 lived for the duration of the 7 month study and exhibited chronic pathologies of the kidney and liver at high administered activities. The relative probabilities of pathology in the kidney and liver by administered activity were calculated for all cohorts. Weight loss and serum biomarkers of kidney and liver toxicity were also quantified, and weight loss was shown to have high sensitivity and specificity for detection of both kidney and liver pathology. While creatinine and BUN levels are typically used as biomarkers of kidney damage,⁴² BUN was identified in our study as having higher sensitivity and specificity of detection of

Table 3. Relationship of log $D_{7.4}$ with Kidney Uptake, Radiation Doses, and Toxicity

linker	log $D_{7.4}$ ^a	uptake rate constant, λ (%IA/g/h)	total absorbed dose (Gy/kBq)	probability of toxicity at 148 kBq injected activity		
				pathology	BUN	weight loss
no linker	-2.41 ± 0.26	3.985	0.5628	0.00	0.04	0.01
Ahx	-2.59 ± 0.20	5.625	0.1366	0.23	0.10	0.10
DLDL	-3.49 ± 0.27	178.5	1.5980	0.95	1.00	1.00
DGDL	-3.88 ± 0.17	44.08	0.9200	0.95	0.94	0.99

^aDetermined using the La³⁺ chelate. ^bDetermined using the ²²⁵Ac chelate.

kidney pathology. Of all liver enzymes tested, ALKP levels had both high sensitivity and high specificity for detection of liver damage. Statistical analyses identified significant relationships between log $D_{7.4}$ and kidney BD, the kidney-to-liver BD ratio, weight loss, and kidney clearance rates. Weight loss also significantly correlated with kidney clearance rates. BUN levels were significantly correlated with kidney uptake and liver clearance rates.

Table 3 was prepared to highlight the relevant relationships among the log $D_{7.4}$ values of the La³⁺-chelated compounds and the kidney uptake, kidney total absorbed dose, and kidney toxicity values of the corresponding ²²⁵Ac-chelated compounds. It is notable that the compounds with log $D_{7.4}$ values above -2.6 have kidney uptake rates, total absorbed doses, and probabilities of toxicity that range from 3- to 100-fold lower than the compounds with log $D_{7.4}$ values below -3.4 .

The arginine side chain in the MC1R targeting ligand is likely to be positively charged in all of the peptide conjugates at pH 7.4. This likely gives an overall plus one charge to the metalated no-linker, Ahx linker, and DLDG analogs and no overall charge for those same unmetalated analogs. The +3 metals, La and ²²⁵Ac, are balanced by the three carboxylates giving no overall charge for the DOTA-metal-chelated portion of the conjugates. The free DOTA-MC1RL species have three negative charges from the DOTA carboxylates and two positive charges from the protonation of two of the four tertiary amines in the 1,4,7,10-tetrazadodecane ring system. The metalated and unmetalated DLDL MC1RL analogs are expected to have overall +3 or +4 charge, respectively, but they are less polar than the relevant DLDG MC1RL analogs, which suggests significantly reduced basicity for the Lys side chains at physiological pH due to the overall charge density of DLDL analogs.

All of the linkers used for conjugation are expected to be stable to metabolic changes and all of the conjugates have epsilon lysine-amide linkages which are unlikely to be substrates of proteolytic enzymes. D-Amino acids were used in the polar linkers to inhibit possible proteolytic cleavage between the first and second amino acids in the linkers themselves.

In conclusion, by using different linker chemistry to increase the lipophilicity of a TAT radiopharmaceutical, we demonstrated that the kidney uptake rate can be significantly decreased, which also decreases the total absorbed dose and probability of toxicity to the kidney. Liver uptake, dose, and toxicity were not significantly influenced by the increase in lipophilicity. Hence, there is significant potential to decrease the kidney toxicity of radiopharmaceuticals via medicinal chemistry approaches that adjust lipophilicity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acspsci.1c00035>.

Lipophilicity (log $D_{7.4}$) for unmetalated and La³⁺-chelated compounds, radiation dosimetry and clearance kinetics parameters in BALB/c mice; relationship between log D and kidney, liver and kidney/liver biodistribution (BD): statistical analysis, correlation of log $D_{7.4}$ and kidney and liver uptake and clearance with indicators of toxicity (weight change, pathology, BUN and ALKP); analytical HPLC chromatograms and MALDI-TOF spectra; radiochemical purities of ²²⁵Ac-DOTA-MC1RL, ²²⁵Ac-DOTA-Ahx-MC1RL, ²²⁵Ac-DOTA-DLDL-MC1RL, and ²²⁵Ac-MC1RL-DLDG-DOTA; isomeric gamma spectrum of mouse kidneys for ²²⁵Ac-DOTA-MC1RL (no linker) at 24 h post-injection; weights over time of the animals in this study injected with different activities of radiopharmaceutical or saline (PDF)

Histopathology scoring for all tissues in the study (XLSX)

Toxicity indicators: weight change, pathology and serum markers (XLSX)

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Notes

The authors declare the following competing financial interest(s): D.L.M. and N.K.T. are co-inventors of an awarded patent. D.L.M., T.J.W., M.L.M., H.K., and N.K.T. are co-inventors on a pending patent application. Modulation Therapeutics, Inc., has licensed related intellectual property, and M.L.M. is a co-founder of that company.

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ABBREVIATIONS

MC1RL, Melanocortin receptor 1 ligand; Ahx, 6-Amino-hexanoic acid linker; BD, Biodistribution; BUN, Blood urea nitrogen; Fmoc, Fluorenylmethyloxycarbonyl; HCTU, O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIEA, Diisopropylethylamine; DCM, Dichloromethane; DBU, 1,8-Diazabicyclo(5.4.0)undec-7-ene; DMF, Dimethylformamide; NMP, N-methyl-2-pyrrolidone; Alloc, allyloxycarbonyl; Pd(PPh₃)₄, Tetrakis-(triphenylphosphine)palladium(0); DOTA, 1,4,7,10-Tetraazacyclododecane-1,4,7-tris-*tert*-butyl acetate-10-acetic acid; D-Glu, Fmoc-D-glutamic acid; D-Lys, Fmoc-D-lysine; TFA, Trifluoroacetic acid; RT-HPLC, Reverse-phase high-performance liquid chromatography; Ahx, Amino-hexanoic acid; DLDL, D-Lys-D-Lys; DLDG, D-Lys-D-Glu

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Review

The gut microbiota can be a potential regulator and treatment target of bone metastasis

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ABSTRACT

The gut microbiota, an often forgotten organ, have a tremendous impact on human health. It has long been known that the gut microbiota are implicated in cancer development, and more recently, the gut microbiota have been shown to influence cancer metastasis to distant organs. Although one of the most common sites of distant metastasis is the bone, and the skeletal system has been shown to be a subject of interactions with the gut microbiota to regulate bone homeostasis, little research has been done regarding how the gut microbiota control the development of bone metastasis. This review will discuss the mechanisms through which the gut microbiota and derived microbial compounds (i) regulate gastrointestinal cancer disease progression and metastasis, (ii) influence skeletal remodeling and potentially modulate bone metastasis, and (iii) affect and potentially enhance immunotherapeutic treatments for bone metastasis.

1. Introduction

In the human body, it is estimated that there are ten times as many microorganisms as nucleated host cells [1]. The genome of these microorganisms, known as the human microbiome, is found to contain approximately 100 times the number of genes present in the human genome [2]. Microorganisms, established in the human body *in utero*, begin to multiply and diversify immediately after birth until stabilization occurs at three years of age [3,4]. One of the major sites where microorganisms exist in the human body is the gut [5]. The collection of microorganisms that exists in the gut is labeled as the gut microbiota, as microorganisms that live in a specific environment are coined the microbiota. The gut microbiota consists of a vast number of bacteria, viruses, fungi, archaea, and protists, and can have a tremendous impact

on human health [6,7]. Especially, with all components working in conjunction, the gut microbiota plays a fundamental role in human digestion, as it is capable of breaking down nutrients that the host would be incapable of digesting on its own [8]. For example, a single gene locus in *Bacteroides ovatus* is responsible for the digestion of xyloglucans, a sugar from plants that would otherwise be indigestible for the human host [9]. Similarly, there are specific microbiota that regulate the uptake of vitamins and minerals [10]. Additionally, *Prevotella corpi* and *Lactobacillus plantarum* are just two of the gut microorganisms believed to be necessary for vitamin B2 synthesis [11]. Through these functions, the gut microbiota contribute to maintaining host health.

Another important role of the gut microbiota in the host environment is to equilibrate and maintain the interaction between the gut and host microorganisms. In a healthy individual, this equilibrated state is

Abbreviations: GI, gastrointestinal; CRC, colorectal cancer; *H. pylori*, *Helicobacter pylori*; *B. fragilis*, *Bacteroides fragilis*; *E. coli*, *Escherichia coli*; CagA, cytotoxin-associated gene A; AKT, protein kinase B; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; GRB2, growth factor receptor bound protein 2; MEK, mitogen-activated protein kinase; WNT, wingless/integrated; IL, interleukin; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; ROS, reactive oxygen species; BM-MSC, bone marrow-derived mesenchymal stem cell; SCFA, short chain fatty acid; GPR, G protein-coupled receptor; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; LPS, lipopolysaccharide; TLR4, toll-like receptor 4; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor β; DTC, disseminated tumor cell; PTHrP, parathyroid hormone-related protein; RANKL, receptor activator of NF-κB ligand; TNFR-1, tumor necrosis factor receptor 1; NFATc1, nuclear factor of activated T cells c1; EP4, prostaglandin E receptor 4; LCA, lithocholic acid; HSC, hematopoietic stem cell; IGF-1, insulin-like growth factor 1; ADT, androgen-deprivation therapy; FMT, fecal microbiota transplantation; ²²³RaCl₂, radium-223 dichloride; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; CTLA-4, cytotoxic T lymphocyte antigen 4; ICI, immune checkpoint inhibitor; T_H17, T helper 17; Tregs, regulatory T cells; T_H1, T helper 1; CEA, carcinoembryonic antigen; VEGFR, VEGF Receptor.

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achieved by crosstalk and cross-regulation between the gut microbiota and their human host, which serves to create a stable balance in the gastrointestinal (GI) tract [12]. The primary functions of the healthy GI tract are to regulate nutrient and water absorption in addition to preventing toxins from entering circulation, and the design of the gut barrier is indicative of these roles [13]. The gut barrier is comprised of three layers (i) a mucus layer that serves as protection for epithelial cells from gut microbiota, (ii) a single layer of epithelial cells that comprise the majority of the physical intestinal wall, and (iii) a series of junctional complexes that regulate the transport of molecules and maintain the intestinal barrier [14–16]. Typically, gut microbiota can reside within the gut without problem due to this barrier system; however, problems arise when the gut microenvironment is thrown into a state of dysbiosis, the state in which equilibrium between the gut microbiota and their human host is disrupted [17,18]. Dysbiosis is brought on by a variety of causes primarily related to environmental contaminants, excess alcohol consumption, and unhealthy eating [19]. The physical, biochemical, and immune barriers that serve to protect the host become compromised during dysbiosis resulting in systemic inflammation [20–23]. When dysbiosis is induced, this can lead to permeability within the gut barrier, essentially impairing the protective responses and normal function within the gut, creating what is known as a “leaky gut”. When the gut barrier becomes compromised, the bacteria that in some cases initially created the leaky gut, along with toxins and food particles, can leak out into the blood stream stimulating a proinflammatory response and secreting cytokines [24–27]. A chronic state of gut dysbiosis is known to be linked to irritable bowel syndrome, osteoarthritis, obesity, diabetes, cancer, and many other diseases [28–30].

Chronic inflammation has long been known to be associated with cancer development [31]. Innate immune cells are capable of detecting infection or injury in a tissue through pattern recognition receptors in the cytoplasm [32]. When damage is detected, innate immune cells move into the damaged, precancerous tissue and induce an inflammatory response; this leads to angiogenesis, tissue remodeling, and cytokine and chemokine presence, creating an ideal environment for tumorigenesis [33]. Recent evidence has come to suggest that environmental factors that induce inflammation can cause the composition of the gut microbiota to fluctuate, which can then influence different types of cancer, namely colorectal cancer (CRC), through gut microbiota-related mechanisms [34]. As previously stated, one of these environmental agents is infection, and several types of bacteria are known to be linked to cancer. *Helicobacter pylori* (*H. pylori*) infection, for example, has a known association with GI cancer development [35]. *Streptococcus bovis* was the first bacterium to be associated with CRC, and since then several other bacteria including *Bacteroides fragilis* (*B. fragilis*), *Enterococcus faecalis*, *Fusobacterium nucleatum*, and *Escherichia coli* (*E. coli*) have all been implicated in colorectal carcinogenesis [36]. These findings corroborate the idea that the gut microbiota play a role in tumorigenesis.

Although there are clear examples of the gut microbiota's involvement primarily in GI cancer, research regarding gut dysbiosis and cancer outside of the gut remains elusive. In this review, we will discuss the effects of the gut microbiota and gut dysbiosis on cancer development and metastasis, specifically as it pertains to bone metastasis, and potential therapeutic strategy for bone metastatic disease using probiotics.

2. The mechanisms whereby gut microbiota induce GI cancer tumorigenesis

H. pylori was linked to stomach inflammation, and ultimately gastric cancer, in what some would consider a reckless, albeit dedicated demonstration of scientific commitment that later won two researchers the Nobel Prize. Subject to much scrutiny, Dr. Barry Marshall intentionally infected himself with *H. pylori* and underwent a stomach biopsy in order to prove that the bacterium was linked to gastric disease [37,38]. While this was the first known class I oncogenic bacterium with

a carcinogenic effect, this discovery was a gateway for further exploration into bacteria that induce cancer and the mechanisms through which this induction occurs [39]. When exploration of bacteria as carcinogenic agents first began, it was learned that bacteria induce cancer by both creating a chronic state of inflammation and generating metabolites that are known to be carcinogenic [40]. Since this discovery in the late 1900s, the knowledge of these mechanisms has expanded.

It is currently known that bacteria are capable of promoting carcinogenesis through several different mechanisms, and one of the primary courses of action for which the gut microbiota induce GI carcinogenesis is through chronic inflammation caused by bacterial infection. When bacteria such as *H. pylori* breach the gut epithelium, this bacterium induces chronic inflammation by invading the gastric cells where it can reside, potentially asymptomatic, for years, all while releasing the oncoprotein, cytotoxin-associated gene A (CagA) [41,42]. CagA ultimately effects gastric epithelial cells in multiple ways. Inducing cell proliferation of gastric epithelial cells is one of the known functions of CagA, and this occurs through (i) the activation of the protein kinase B (AKT) pathway through phosphoinositide 3-kinase (PI3K), (ii) the activation of the extracellular signal-regulated kinase (ERK) pathway through growth factor receptor bound protein 2 (GRB2) and mitogen-activated protein kinase (MEK), and (iii) β -catenin activation of the wingless/integrated (WNT) pathway [43–47]. CagA has also been found to induce an inflammatory response through its contribution to interleukin (IL)-8 expression in gastric epithelial cells; by acting on the Ras pathway, CagA helps stimulate IL-8 induction which triggers an inflammatory response through nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) signaling [48]. Additionally, CagA stimulates reactive oxygen species (ROS) in infected gastric epithelial cells. When ROS production occurs in excess, DNA damage and carcinogenesis can occur in gastric epithelial cells [49]. These findings suggest that *H. pylori* infection contributes to carcinogenesis in the gut through the induction of inflammation and DNA damage.

In recent years, there has been some success in treating *H. pylori* infection, which has led to the finding that this bacterium is primarily responsible for non-cardia gastric cancer (cardia gastric cancer is located near the esophageal-gastric junction, while non-cardia gastric cancer is located in the distal section of the stomach [50]), whose rates have been declining with lower *H. pylori* infection rates [51]. Subsequently, cardia gastric cancer rates have been seen to increase seven-fold [52]. While *H. pylori* infection increases the risk of gastric cancer, it is also thought to play a protective role in esophageal cancer by reducing acid secretion in this area [50]. There is some evidence to suggest that by eradicating *H. pylori* in the developing world, the incidence of the cardia subtype has increased, because the quantity of acid in this area has decreased [52,53]. Regardless of gastric subtype, it is through mechanisms that induce inflammation and DNA damage that *H. pylori* infection, or lack thereof, contributes to carcinogenesis in the gut.

Other bacteria also utilize similar mechanisms to induce DNA damage and ultimately cancer. When *B. fragilis* breach the gut epithelium and release *B. fragilis* toxin, an inflammatory immune response, one of the hallmarks of cancer, occurs in the body [54]. This bacterium induces an inflammatory response through (i) the activation of β -catenin and the WNT pathway and (ii) the induction of IL-8 secretion [55–58]. *Helicobacter hepaticus* and *E. coli* are examples of gut bacteria that act to promote carcinogenesis through mechanisms that induce an inflammatory response. These two bacteria produce DNA double-strand breaks in epithelial cells by inducing cytolethal distending toxin, which causes cancer proliferation in conjunction with inflammation [59,60].

An alternative mechanism through which gut bacterial carcinogenesis occurs is the mediation of bile acid metabolism by the gut microbiota [61]. In a healthy microenvironment, bile acids are expelled into bile where they are then digested by the gut microbiota for reuptake into the blood stream [62]. Problems can occur however, when bile acids are present at high levels, because bile acids are cholesterol derivatives whose detergent properties act to disrupt bacterial and host cell

membranes, denature proteins, and cause oxidative DNA damage [63,64]. When these acids are highly concentrated, a process that can be facilitated by bacteria such as *Salmonella typhi*, they quickly induce inflammatory reactions in the body's attempt to repair the damaged tissue [41,65,66]. In the process of tissue repair, an accumulation of bone marrow-derived mesenchymal stem cells (BM-MSCs) is seen in the mucosal layer of the gut barrier, contributing to the now precancerous state of the gut [67,68]. In addition to generating this precancerous state, bile acids are also known to stimulate ROS, which disrupts the base excision repair pathway, contributing to DNA damage which leads to carcinogenesis through gene mutation [66,69]. When exposure to excess bile acids occurs chronically, this causes degradation of p53, a tumor suppressor, leading to a decrease in the apoptosis of cancer cells [65]. This degradation further impairs the process of DNA repair as the cell cycle will not arrest in response to DNA damage and apoptosis will not be induced [65,70]. This evidence implicates bile acids in the induction and progression of the cancer disease state, primarily in gastric cancer and CRC.

While contributing heavily to gut carcinogenesis, the gut microbiota can also play anti-tumorigenic roles in the gut. Gut bacteria, in the process of digesting carbohydrates and fibers that the body would otherwise be unable to digest, generate short chain fatty acids (SCFAs) which interact with cancer cells [71]. It was recently discovered that the concentration of SCFAs – and SCFA producing bacteria – is reduced in patients with CRC, as compared to a control group [72]. One specific SCFA, butyrate, can act as a ligand to G protein-coupled receptor (GPR) 109A, a cell surface receptor of macrophages and adipocytes that was shown to correlate with CRC development when expression of butyrate is decreased [73]. Butyrate, in conjunction with GPR109A, can down-regulate the activity of NF- κ B and decrease the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, known to be involved in tumorigenesis [74–76].

3. The mechanisms whereby gut microbiota control GI cancer metastasis

While primary tumorigenesis remains a problem, metastasis is considered to be the predominant cause of poor prognosis in patients with GI cancer [77]. In patients diagnosed with CRC, approximately 60% develop metastases to secondary sites within five years, and when CRC metastasizes, patients are faced with poor prognosis [78]. The five-year survival rates for patients with distant metastasis from CRC are just 14% (colon cancer) and 16% (rectal cancer), while those for patients without metastasis are 91% and 89%, respectively [79]; this accounts for 9.4% of all the patient deaths attributed to cancer in 2020, second only to lung cancer [80,81]. One of the GI cancer counterparts, stomach cancer has an average five year survival rate of just 31% across all disease states, largely because this cancer has already metastasized upon diagnosis [52], thus making gastric cancer the third leading cause of cancer related death globally [82]. When cancer metastasizes from the GI system, the most common secondary sites include liver, peritoneum, lung, and bone [83]. Although not as thoroughly studied as its role in primary GI carcinogenesis, the gut microbiota has also been found to heavily influence metastasis of GI cancers.

Bacteria are known carcinogens, functioning to promote carcinogenesis and metastasis, in part, through the release of toxins, primarily lipopolysaccharide (LPS), which interfere with immune and inflammatory cell signaling pathways in a healthy individual [84,85]. LPS is a glycolipid on the outer membrane of gram-negative bacteria [86], many of which safely reside in the gut microenvironment [87]. LPS can be released from the bacterial cell wall through bacterial lysis or shedding [88–90], and is known to interact with immune cells through ligand/receptor binding [91,92]. In a healthy gut microenvironment, LPS will not breach through the gut wall; however, the leaky gut status caused by dysbiosis enables toxins from bacteria, including LPS, to leak through the intestinal epithelium via a defective tight junction barrier [93–95].

Thereafter, lipid A on LPS interacts with macrophages through the activation of toll-like receptor 4 (TLR4) [96]. This activation of TLR4 activates NF- κ B to induce the release of inflammatory cytokines, associated with metastatic progression, such as tumor necrosis factor (TNF)- α , IL-1, and IL-6 [97,98], as well as vascular endothelial growth factor (VEGF) [99] and transforming growth factor β (TGF- β) [100]. This LPS-induced VEGF promotes CRC metastasis by (i) enhancing angiogenesis [101,102] and lymphangiogenesis [103–105]; (ii) inducing epithelial-mesenchymal transition of CRC cells; and (iii) stimulating the mobile ability of CRC cells by upregulating the transcription of protein coding gene, CNTN-1 [106] and the protein moesin through the RhoA/ROCK-2 pathway [107].

While it is evident that the gut microbiota directly influence GI cancer metastasis, recent evidence exists to implicate the gut microbiota as a contributor to the development of the pre-metastatic niche. In order for a primary tumor to metastasize, the local microenvironment in the secondary site must be favorable for metastatic tumor cells, or disseminated tumor cells (DTCs), to grow within this new environment [108]. This is the premise of Paget's 1889 "seed and soil" hypothesis; just like a seed needs to land on nutrient rich soil to grow, DTCs (seeds) need a fertile environment in the metastatic site (soil) in order to take hold [109]. Indeed, a recent study of CRC showed that *E. coli* from the primary tumor site in the colon penetrated through the gut barrier and migrated to the liver, where they enhanced pre-metastatic niche formation, leading to DTC recruitment and eventually full-blown liver metastasis [110].

In addition to gut bacteria being found in secondary tumor sites, evidence supports the idea that the gut microbiota communicate and interact with other cell types to facilitate cancer metastasis [111,112]. Gut microbiota also provide another pathway for GI cancers to metastasize by communicating with BM-MSCs. It is known that BM-MSCs have an affinity for tumor or cancer cells, and that once recruited, these cancer-associated BM-MSCs can differentiate into fibroblasts and macrophages to influence progression of tumors [113]. However, recent evidence has begun to suggest that BM-MSCs are also recruited to metastatic sites, and then serve as the metastatic niche to promote metastatic progression [114–118]. For example, a study of lymph node metastasis from gastric cancer demonstrated the ability of these gastric cancer cells to educate BM-MSCs via secretory exosomes to create the metastatic niche in the lymph nodes, resulting in enhancement of the dissemination of gastric cancer into lymph node, as well as enhancement of lymphangiogenesis which promotes further dissemination [119]. These findings suggest that the gut microbiota also facilitate GI cancer metastasis by influencing the microenvironment in the metastatic site in either a direct or indirect manner.

4. The potential mechanisms whereby gut microbiota regulate bone metastatic process of solid tumors

While little evidence exists to support the idea that the gut microbiota influence GI cancer metastasis, there are a number of parallels between the mechanisms regulating metastasis of solid tumors out of gut and the mechanisms through which the gut microbiota act on the metastatic microenvironment "soil". Bone is one of the most common metastatic sites of solid tumors. While how gut microbiota control the bone dissemination process of solid tumors outside of the gut has not been thoroughly explored, there is a growing body of evidence that exists to support the gut microbiota as a regulator of the bone microenvironment and skeletal health. For example, the gut microbiota has been implicated as a modulator of other bone diseases, such as osteoarthritis and osteoporosis, through the brain-gut-bone axis [120,121]. The brain-gut-bone axis - sometimes referred to as the gut-bone axis - is a term used to reference the communicative interactions between the brain/nervous system, the gut microbiota, and the skeletal system through which bone health is affected [122,123]. Enhanced osteoclast activity found in osteoarthritis and osteoporosis likely contributes to

bone metastasis, creating space for tumor cells to infiltrate the bone microenvironment. It was shown that arthritic mice have higher incidence of bone metastasis from breast cancer cells than their non-arthritic counterparts [124]. Further, while osteoporosis was not deemed to be a risk factor for bone metastasis, it was found that bone metastasis is accelerated in breast cancer patients with untreated osteoporosis [125]. Together, these findings suggest that the gut microbiota may play a crucial role in both the development of bone metastasis and an environment suitable for bone metastatic diseases.

Bone homeostasis is a result of the balance of bone formation mediated by osteoblasts and bone resorption induced by osteoclasts [126]. When cancer cells disseminate to the bone, they disrupt this homeostatic process [127]. Bone metastases are derived into three primary categories: osteolytic, osteoblastic, and mixed; which types of bone metastasis develop appears to be associated with how DTCs interfere with the balance between osteoclastogenesis and osteoblastogenesis [128]. As is the case in osteolytic bone metastases, the tumor cells present in the bone upregulate the cytokines IL-1, IL-6, and TNF- α , all of which promote osteoclast formation from macrophages [129] and induce osteoblast apoptosis [130]. In addition to these proinflammatory cytokines, the parathyroid hormone-related protein (PTHrP), derived from bone metastatic cancer cells, also contributes to osteoclastogenesis. The cancer-derived PTHrP is known to induce bone resorption by activating a G-protein coupled receptor PTHrP receptor that is highly expressed on the osteoblasts and osteocytes [131,132]. Upon binding, PTHrP enhances the production of receptor activator of NF- κ B ligand (RANKL) by osteoblasts and osteocytes, which binds to its receptor RANK on osteoclasts and their precursors acting to stimulate bone resorption and osteoclast differentiation, respectively [133,134]. This induced osteoclastogenesis contributes to significant bone destruction, creating space for tumor cells to proliferate in the bone [135].

Osteoclastogenesis can also be influenced by the gut microbiota. The relationship between the gut microbiota and bone homeostasis, especially osteoclastogenesis, was exemplified in a study comparing skeletal development of germ-free mice with that of conventionally raised mice. In this study, significantly higher trabecular bone volume and reduced osteoclast cell counts were observed in the bones of germ-free mice, compared to those of the conventionally raised mice. When germ free mice were treated with gut microbiota from conventionally raised donor mice, trabecular bone volume and osteoclast numbers became comparable with those of the conventionally raised mice [136]. As stated earlier, LPS derived from the gut microbiota enters circulation when dysbiosis occurs. LPS in circulation stimulates the osteoclast differentiation of monocytes and macrophages and promotes the maturation and survival of the resulting osteoclasts [137,138]. High levels of systemically circulating LPS also induce the release of the proinflammatory cytokine TNF- α from macrophages, known to control osteoclast activities by binding with the TNF- α tumor necrosis factor receptor 1 (TNFR-1) [137,139–143]. Indeed, LPS-induced TNF- α can induce bone resorption by driving osteoclastogenesis of a murine macrophage cell line RAW 264.7 cells without going through the RANK/RANKL pathway [143]. One proposed mechanism of LPS-associated osteoclastogenesis is that LPS induces the upregulation of a transcription factor nuclear factor of activated T cells c1 (NFATc1), essential for the differentiation of osteoclast cells, in the nucleus of osteoclast precursors [144,145]. Along with these notions, LPS treatments stimulate the reduction of bone density in two rodent models of chronic inflammation and one rodent model of prostaglandin E receptor 4 (EP4) deficient mice, which lack a receptor on osteoblasts (EP4) that regulates osteoclast formation [146–148]. Further, the LPS-TLR4 axis has also been implicated in enhancing the severity of osteoporosis and osteoarthritis [146,149–151]. In addition to circulating LPS, the gut microbiota can also regulate osteoclastogenesis through bile acid metabolism. Bile acid mediated osteoclastogenesis is facilitated primarily by lithocholic acid (LCA), a secondary bile acid converted through the 7 α -dehydroxylation of chenodeoxycholic acid, a process heavily influenced by bacterial

hydrolases located on the cell walls of gut bacteria [152,153]. Once converted, LCA serves as a ligand for the vitamin D receptor. Traditionally, the vitamin D receptor, which binds with vitamin D, is known for its role in calcium absorption, and high levels of vitamin D are positively correlated with bone mineral density [154,155]. However, when LCA binds to the vitamin D receptor on osteoblasts, it inhibits bone formation [156]. Furthermore, LCA directly damages osteoblasts, resulting in bone loss [157,158]. These studies suggest that osteoclastogenesis is regulated in part by gut microbiota-derived systemic LPS and LCA.

While osteoclastogenesis proves to be essential for creating space for bone metastatic tumor cells to grow, the osteoblasts also play a vital role in the bone metastatic progression. Osteoblasts, located at the endosteum of bone [159], support self-renewal, multi-potency, homing and migration of hematopoietic stem cells (HSCs) [160,161]. Therefore, osteoblasts are known as the specific microenvironment for HSCs, or the osteoblastic HSC “niche”. Importantly, the osteoblastic HSC niche is not only involved in the HSC maintenance, but also implicated in early colonization of bone DTCs. Prostate cancer cells compete directly with HSCs to gain space in the osteoblastic HSC niche when they disseminate to the bone [162]. Furthermore, bone metastatic breast cancer cells first colonized the osteoblastic niche through cell–cell adherens junctions [163]. Thereafter, these DTCs within the osteoblastic niche can remain in a dormant state for years before being reactivated to form full blown bone metastases [164,165]. These findings suggest that osteoblasts are crucial for the early onset of cancer colonization and its survival within the bone.

The gut microbiota can also be involved in the establishment of early colonization of bone metastatic cancer cells, as they also induce bone formation by activating osteoblasts. As discussed above, the gut microbiota are capable of breaking down nutrients that the host would be incapable of digesting on its own through anaerobic fermentation [8] and often, SCFAs are the product of this biochemical reaction [166]. These gut microbiota-inducing SCFAs are known to up-regulate skeletal formation. Indeed, treatments with probiotics, which are fermented into SCFAs by the gut microbiota, improved skeletal health in various animal models [167,168]. Moreover, SCFAs enhanced bone formation by stimulating the production of insulin-like growth factor 1 (IGF-1), a growth factor known to enhance skeletal formation, from the intestinal epithelial cells [169–172]. An additional mechanism by which the gut microbiota influence bone formation is through the regulation of the sex hormone androgen. Androgens are known to promote the growth of normal and cancerous prostate cells, and often androgen-deprivation therapy (ADT) is the first-line treatment for prostate cancer. The gut microbiota *Clostridium scindens* is known to convert glucocorticoids into androgens through enzymatic processes [173]. Further, a transplantation of gut microbiota from male mice into the female intestines resulted in an increase in systemic testosterone, from which androgen is derived. It is proposed that gut microbiota-derived sex steroids moderate bone remodeling [174] through (i) androgens stimulating longitudinal bone growth due, in part, to the upregulation of IGF-1 [175] and (ii) androgens activating androgen receptors on the osteoblasts which in turn inhibit bone resorption and osteolytic activity; this androgen activity has been shown to preserve cortical thickness, predominately in aging [176]. The importance of androgens in bone regulation is also supported by a meta-analysis evaluating studies of the efficacy of androgen-deprivation therapies (ADT) and/or androgen receptor inhibitors on prostate cancer patients, which revealed that non-metastatic castration-resistant prostate cancer patients presented with increased bone fragility and decreased bone mineral density following these anti-androgen treatments [177]. Moreover, a recent work demonstrated that ADT enhanced the commensal microbiota that contribute to castration resistance, and when resistance to ADT developed, the gut microbiota that enhance androgen formation were increased [178]. These suggest that gut derived androgens contribute to ADT resistance in prostate cancer patients [179], a disease state which is often accompanied by

bone metastasis, since androgen signaling likely contributes to and accelerates this metastatic process [180,181]. Furthermore, antibiotic treatments and fecal microbiota transplantations (FMT) were both found to prolong the time to castration resistance and control tumor growth [178]. Together, these findings suggest that the gut microbiota serve as an extraneous source of androgen in the presence of ADT and may contribute to bone metastasis.

Although evidence does not exist specifically implicating the gut microbiota as a causative factor in bone metastasis, the gut microbiota have been seen to affect the metastatic process of breast cancer that commonly disseminates to bone. In a mouse model of hormone receptor positive breast cancer, gut dysbiosis can promote breast cancer metastasis through increased tumor cell dissemination into the blood and secondary sites, as well as the activation of macrophages and the release of proinflammatory cytokines [182]. Additionally, treatments of an antioxidant and -inflammatory agent muscadine grape extract reduced metastases of triple negative breast cancer by inducing microbial richness *in vivo* [31,183].

Taken together, although further studies are clearly warranted, these findings on the roles of the gut microbiota in both bone remodeling and metastases of solid tumors that commonly metastasizes to bone suggest that the gut microbiota may affect bone metastatic development (Fig. 1).

5. The potential roles of gut microbiota in enhancing the efficacy of treatments for bone metastasis

When compared with treatment strategies for other metastatic sites, approaches for treating bone metastasis are fairly unique. In typical cancer disease treatment, primary and metastatic tumors are subject to similar therapeutic approaches focused on either targeting the tumors themselves or stimulating the immune system around the area encompassing the tumor. However, treatment strategies for bone metastasis

engage a different target. Bone metastasis therapies usually aim to target the function of the bone, an organ that couples osteoclast and osteoblast activity to continuously remodel [184]. It has been suggested that bone metastatic cancer cells are capable of interacting with the osteoclasts, osteoblasts, osteocytes, and other cells involved in the bone remodeling process. This crosstalk between bone metastatic cancer cells and cells involved in bone remodeling induces what is known as “the vicious cycle of bone metastasis” in which bone metastatic progression hastens [185]. The involvement of the bone organ in metastatic progression makes it logical to target bone remodeling in order to treat bone metastasis. Two current bone metastasis treatment options are bisphosphonates and the human monoclonal anti-RANKL antibody denosumab, both of which function to decrease osteolytic activity [186,187]. While these treatments provide necessary palliative relief for patients with bone metastasis, they ultimately fail to improve survival outcomes [186,187]. Recent clinical trials, however, show some promise for a new therapeutic agent that has been shown to improve survival outcomes for patients with bone metastasis, albeit only slightly. Radium-223 dichloride ($^{223}\text{RaCl}_2$), an alpha particle-emitting radiopharmaceutical that targets hydroxyapatite or osteoblastic bone metastatic lesions has been shown to improve survival for a few months (mean = 3 months) in patients with bone metastases from prostate cancer [188]. While the overall survival of patients with bone metastasis can also be enhanced for a few months through treatment with complementary systemic therapies (eg. hormone therapies, chemotherapies), $^{223}\text{RaCl}_2$ is the only bone-targeted therapy that is capable of improving survival outcomes for patients with bone metastases [189]. Since at current moment treatment options that target bone remodeling have had only moderate success, elucidating treatment options that function through mechanisms other than targeting bone remodeling is essential in order to eradicate bone metastasis in patients.

In recent years, immune checkpoint inhibition, a type of

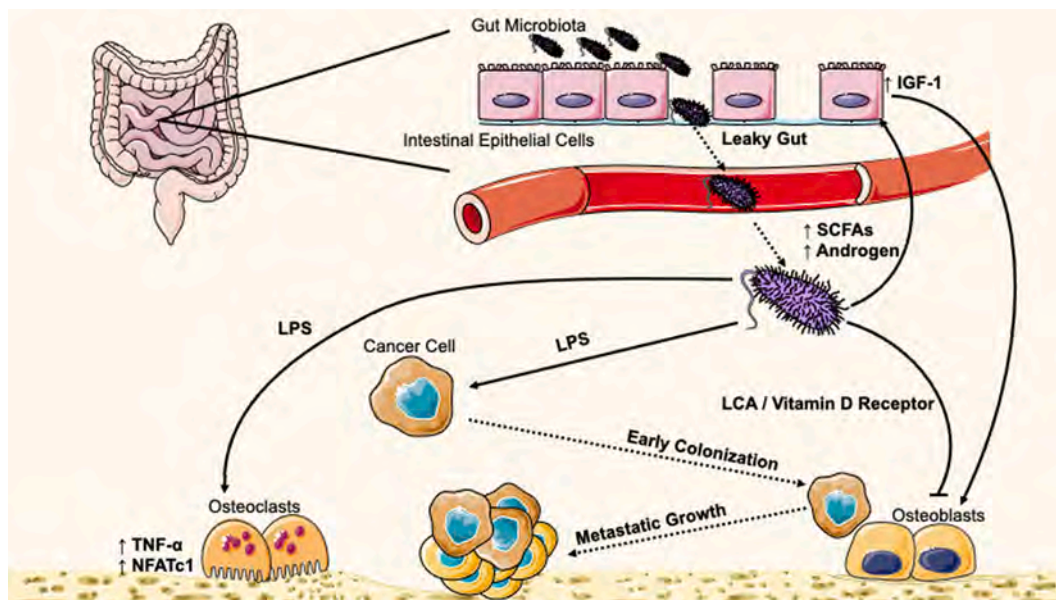


Fig. 1. Potential mechanisms whereby gut microbiota induce bone metastatic progression. The intestines are comprised of a single layer of epithelial cells that allow for absorption of nutrients into circulation. However, when dysbiosis occurs, the tight junctions in the gut become loose, creating what is known as a leaky gut epithelium. This status allows the gut microbiota to escape from the intestines and enter circulation. Once in circulation, the gut microbiota stimulate cancer cells through lipopolysaccharide (LPS) to acquire mobile ability. These cancer cells have disseminated from the primary tumor site to the bone through systemic circulation. The gut microbiota also directly interact with osteoclasts and osteoblasts. To induce bone resorption, gut microbiota-derived LPS enhances osteoclast activities by upregulating tumor necrosis factor (TNF)-α and the transcription factor nuclear factor of activated T cells c1 (NFATc1) in osteoclasts. Further, lithocholic acid (LCA), which is converted from bile acid by the gut microbiota, inhibits osteoblast activities by binding with the vitamin D receptor on osteoblasts. To induce bone formation, gut microbiota-mediated increased short chain fatty acids (SCFAs) and upregulated androgen stimulate osteoblastogenesis by inducing insulin-like growth factor (IGF)-1 secretion from intestinal epithelial cells. Since osteoclasts are also creating space for bone metastatic cancer cells and osteoblasts are a key microenvironment for the early colonization of bone metastatic cancer cells, this suggests that the gut microbiota modulate both cancer cells and the bone microenvironment to create an environment favorable for bone metastasis. Graphics adapted from Smart Servier Medical Art (<https://smart.servier.com/>).

immunotherapy, has emerged as a novel cancer treatment. The immune checkpoint programmed cell death-1 (PD-1) is expressed on several immune cells, including T cells, B cells, monocytes, natural killer T cells, and dendritic cells, with high quantities on cancer antigen-specific T cells [190]. Its ligand programmed death-ligand 1 (PD-L1) is generally expressed by macrophages, however, in the case of cancer, cancer cells express PD-L1 as a pro-tumor response to repress T cell activity [191,192]. Similarly, the immune checkpoint cytotoxic T lymphocyte antigen 4 (CTLA-4), expressed by activated effector T cells, functions as a pro-tumorigenic agent through T cell suppression [193]. When the immune checkpoints PD-1 or CTLA-4, located on T cells, are activated, they function to restrain T cell functions; however, when PD-1 and/or CTLA-4 are inhibited, as is the case in immune checkpoint inhibitor (ICI) therapy, previously restrained T cells are unrestricted and are capable of targeting cancer cells [194]. PD-1 has become a primary immunotherapeutic target as blocking its activation by PD-L1 has shown high efficacy and low toxicity clinically in its aim to normalize the immune system, rather than boost the immune cells effect on cancer specifically [195]. A meta-analysis performed to evaluate the efficacy and toxicity of anti-PD-1/PD-L1 inhibitors in patients with advanced stage and metastatic cancers (lung cancer, head-and-neck cancer, gastric cancer, renal-cell carcinoma, multiple myeloma, urothelial carcinoma, breast cancer, and CRC) revealed that enhanced survival outcomes and overall patient safety are the result of ICI treatment [196].

While ICIs have shown to be somewhat effective in controlling metastasis in general, the treatment efficacy of ICIs on bone metastasis has not been superior to conventional therapies for bone metastatic disease, listed above. In a recent phase III clinical trial investigating the efficacy of an anti-CTLA-4 antibody ipilimumab in advanced stage metastatic castration-resistant prostate cancer patients, including bone metastatic patients, ipilimumab failed to enhance patients' overall survival, although it positively influenced progression free survival (4.0 months, 95% confidence interval (CI): 3.6–4.3 vs. 3.1 months, CI 95%: 2.9–3.4) [197]. A phase II trial of an anti-PD-1 antibody pembrolizumab was conducted in three cohorts of metastatic castration-resistant prostate cancer patients (tumor with measurable PD-L1 positive, tumor with measurable PD-L1 unknown, and bone predominant disease). It revealed that in patients treated with docetaxel and at least one targeted endocrine therapy, pembrolizumab treatment did slightly increase progression free survival (2.1 months, 2.1 months, and 3.7 months) though it did not reach statistical significance; however, interestingly, it appeared to be most effective in patients with bone-predominant metastases (estimated 12 month survival: 41%, 35%, and 62%) [198]. Additionally, non-small cell lung cancer patients with bone metastases treated with an anti-PD-1 monoclonal antibody nivolumab had significantly shorter survival outcomes than those treated with docetaxel alone (patient deaths: 26/86 nivolumab treated, 11/75 docetaxel treated, $p = 0.019$) [199]. Moreover, in bone metastatic animal models using PD-1 knockout mice or wild type mice treated with nivolumab, anti-PD-1 effects could only prevent fractures of tumor-bearing bones and diminish cancer-induced bone pain, but not improve their survival [200]. Together, these findings suggest that ICI therapies themselves generally cannot reverse the negative survival outcomes of cancer patients with bone metastases.

It has been proposed that ICI therapy is not successful in patients with bone metastasis due to the immune regulatory nature of the bone metastatic environment. Once in the bone, bone metastatic cancer cells interact with osteocytes to induce their release of TGF- β , which in turn activates cancer cells to produce osteolytic factors, including PTHrP, IL-6, and IL-11 [201]. Importantly, the abundant TGF- β within the marrow is known to act as an immunosuppressant. When TGF- β is rampant in the bone microenvironment, TGF- β drives polarization of immune suppressor T cells, T helper 17 (T_H17) cells and regulatory T cells (Tregs) from naïve CD4⁺ T cells, whereas it prevents the differentiation into immune effector T cell, T helper 1 (T_H1) cell [202,203]. Indeed, in a bone metastatic mouse model, the high levels of TGF- β created during the

bone metastatic process prevented T_H1 cell development in the marrow, resulting in reducing the optimal function of ICI therapy and eventually the development of resistance to it [204]. In addition, in a syngeneic breast cancer model, when the levels of TGF- β were reduced using either an oncolytic adenovirus expressing a soluble form of the TGF- β receptor 2, sTGF- β RII-Fc, that can directly target TGF- β , or pan-TGF- β neutralizing antibody 1D11, CD8⁺ T cell tumor-infiltration and the cytotoxic factors they release (e.g. perforin and granzymes) were increased in the breast tumors [205,206].

Interestingly, recent studies demonstrated that alteration of the gut microbiota can enhance the efficacy of ICI therapy for several cancer types, including GI cancer, melanoma, non-small cell lung cancer, and renal cell carcinoma through two mechanisms: (i) antigen-specific mechanisms in which the gut microbiota share epitopes with tumor antigens to reduce anti-tumor responses and (ii) antigen-independent mechanisms involving innate immune cells such as effector T cells and myeloid cells, both of which can be found in the gut mucosa [207–211]. For example, a study conducted an analysis on renal cell carcinoma and non-small cell lung cancer patient stool samples treated with ICIs, and identified that *Akkermansia muciniphila* (*A. muciniphila*) was positively correlated with progression free survival and patients' response to PD-1 inhibition [212]. Based on this finding, mouse intestines were colonized with *A. muciniphila* alone or in combination with *E. hirae*, and then these mice were injected orthotopically with either murine Renal melanoma cells or Lewis lung carcinoma cells. As expected, colonization with these two gut microbiota in the gut of the tumor-bearing mice enhanced PD-1 blockade efficacy. Furthermore, treatment using anti-PD-1 antibody with *A. muciniphila* and *E. hirae* induced accumulation of memory CD4⁺ T cells in the mouse tumors and increased the levels of IL-12, a cytokine that induces T_H1 differentiation, in the circulation [212]. More interestingly, recent case reports demonstrated that cytotoxic T cell-based immunotherapy completely attenuated bone metastases in a chemotherapy-resistant gastric cancer patient [213], and that pembrolizumab induced complete response in lung cancer patients with bone metastases [214,215]. In the case of the patient with bone metastases from chemotherapy-resistant gastric cancer, complete attenuation was obtained through treatment with dendritic cell-cytotoxic T lymphocytes specific to carcinoembryonic antigen (CEA) and VEGF receptor (VEGFR)-2 (the two most commonly expressed antigens in gastric cancer) with adjunct low dose cyclophosphamide (an immune suppressor which in low doses depletes Treg cells, increasing T cell activity) [213]. In the three patients with lung cancer, systemic treatment with pembrolizumab, an anti-PD-1 antibody, in addition to palliative treatment of the bone metastases showed success in driving patients into remission [214,215]. These findings suggest that ICI therapy can be effective in treating bone metastasis when immune effector T cell activities are enhanced by manipulating the gut microbiota. To do so, probiotics, which enhance immune effector T cell activities, as well as reduce immune suppressor T cell activities, can be combined with ICI therapy for bone metastasis. Indeed, increased T_H1 cell number and decrease in T_H17 cell number were observed in peripheral blood mononuclear cells of atopic dermatitis mice treated with the probiotic Duolac ATP (which contains *Lactobacillus casei*, *L. plantarum*, *Lactobacillus rhamnosus*, and *B. lactis*) [216]. Additionally, *Lactobacillus casei* and Bio-Three (a combination of *Bacillus mesentericus*, *Clostridium butyricum*, and *Enterococcus faecalis*) are known to promote T_H1 cell differentiation [217,218]. Since the modulation of immune compartment mediated by the gut microbiota depends on the bacteria contained in each respective probiotic cocktail, selecting the right probiotics specific to enhancing T_H1 cell lineage may be a key to improve the efficacy of ICI therapy in patients with bone metastasis. Moreover, FMT - often thought of as a super probiotic - may also prove useful in the enhancement of ICI therapy for patients with bone metastasis. FMT was first used in patients with recurrent *Clostridium difficile* infection [219,220], and involves taking stool from a healthy donor and delivering it via colonoscopy, nasogastric tube, or ingestible capsules to the dysbiotic GI tract of a recipient

with the goal of repopulating the microbiota to restore normal gut function [221,222]. FMT has already been used to successfully treat patients with complications from cancer or cancer treatments, such as ICI colitis and *Clostridium difficile* infection [223,224]. A recent clinical trial demonstrated that FMT, using stool samples from long-term responders to PD-1 treatment, can be used in conjunction with anti-PD-1 ICI therapy in advanced melanoma patients with PD-1 resistance in order to re-colonize the gut and re-educate the tumor microenvironment to respond to anti-PD1 treatment [225]. Further studies in this area are clearly needed to strengthen ICI efficacy for bone metastatic disease.

While FMT has not been studied in prostate cancer patients, FMT from patients with castration-resistant prostate cancer exasperated castration-resistant prostate cancer in mice, whereas FMT from ADT-sensitive prostate cancer patients suppressed tumor growth in mice with castration-resistant prostate cancer [178]. The significance of this data warrants studies into FMT as a therapeutic agent for advanced prostate cancer patients with bone metastases, which do not successfully respond to most current treatments, including ADT and ICI. ADT is known to induce osteolytic activity in prostate cancer patients, resulting in a decrease in bone mineral density and an increase in associated risks, including fracture [226]. Currently, bisphosphonates and denosumab have both shown to enhance bone mineral density in prostate cancer patients without bone metastases receiving ADT [227,228]; however, these treatment strategies are not widely implemented in patients without bone metastasis, since they do not improve survival outcomes and have associated side effects (e.g. osteonecrosis of the jaw). Recent studies conducted in osteoarthritis patients suggest that FMT or probiotic treatment may prove useful in improving bone health in prostate cancer patients receiving ADT. For example, treatment with *Lactobacillus casei* Shirota was found to reduce both pain and levels of the inflammatory marker high-sensitivity C-reactive protein - associated with enhanced risk of fractures - in osteoarthritis patients [229,230]. Therefore, if FMT or probiotic treatment can be geared towards repopulating the gut with bacteria that re-educate the bone microenvironment to enhance osteoblastic activity, this may prolong the length of time for which patients can endure ADT treatments and mitigate the risk of skeletal related events. Further research is needed, however, these findings, combined with the evidence supporting FMT enhancing ICI, suggests that the manipulation of gut microbiota by probiotics and/or FMT may be a useful tool to treat advanced cancer patients with bone metastasis. The concept of re-colonizing the gut microbiota and re-educating the tumor microenvironment using probiotics and/or FMT to respond to treatment and suppress tumor growth in untreatable patients is clinically novel, and further research into this field - exploring the ability of both probiotics and/or FMT to mitigate the effects of the dysbiotic gut on the bone microenvironment - may yield key insight into successful treatments of bone metastasis (Fig. 2).

6. Discussion/Conclusion

In this article, we have detailed the apparent involvement of the gut microbiota in tumorigenesis, cancer metastasis, and skeletal health. In the human body, the gut microbiota interact with the host to maintain homeostasis. However, when microbial dysbiosis is induced, changes in these gut microbiota/host interactions enhance pro-tumor and metastatic activities [231] and alter the bone remodeling process [174]. Although little is known regarding the mechanisms through which the gut microbiota influence bone metastasis, evidence we discussed here suggest that the gut microbiota, by stimulating both bone metastatic cancer cells and the bone microenvironment, contributes to the development of bone metastasis. However, the following outstanding questions remain to be answered:

1. Which specific gut microbiota are implicated in bone metastatic progression?

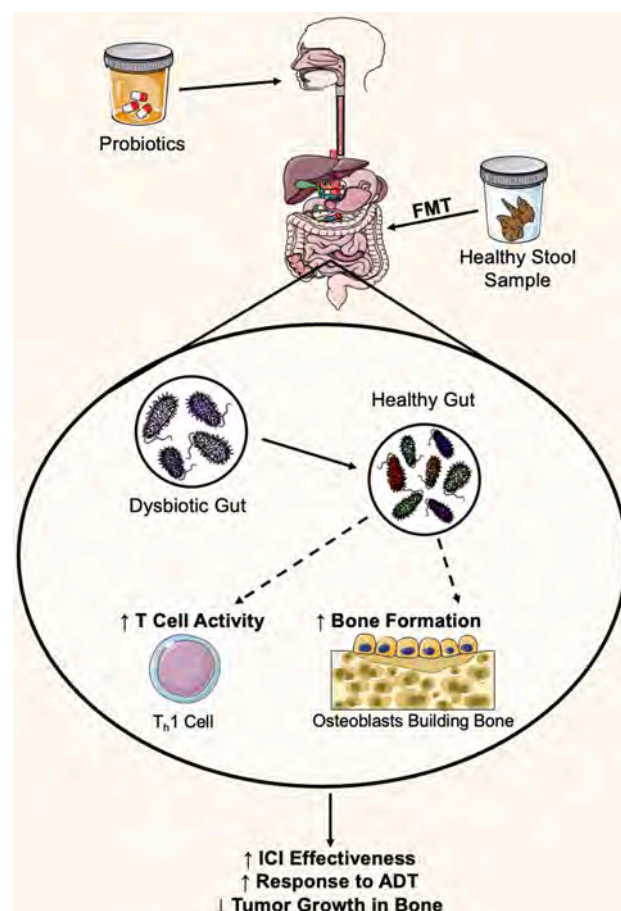


Fig. 2. Proposed treatment strategies for bone metastasis by manipulation of gut microbiota. Probiotics, ingested orally, provide the gastrointestinal system with a new supply of beneficial bacteria and yeasts that help maintain or restore gut health. Similarly, fecal microbiota transplantations (FMT), which involves inserting stool from a healthy donor into a recipient with a dysbiotic gut, can be used to repopulate the gut with healthy bacteria. When the dysbiotic gut microbiota are repopulated with healthy bacteria, both T cell activity and bone formation are increased. If this recolonization is performed specifically with bacteria that enhance T_H1 cell activity, this helps increase the effectiveness of immune checkpoint inhibitor (ICI) treatment. This technique may be useful to enhance ICI efficacy in patients with bone metastasis, where ICI treatment is generally ineffective. Further, repopulating the gut with healthy bacteria, specifically from prostate cancer patients who are responsive to androgen-deprivation therapy (ADT), has been shown to re-educate the tumor microenvironment to respond to ADT in patients who have resistance to this treatment. This concept of re-educating the tumor microenvironment, using bacteria that favor bone formation and a balanced gut, has the potential to decrease tumor growth in the bone by making the bone microenvironment unfavorable for tumor growth and enhancing the effectiveness of common cancer therapies. Graphics adapted from Smart Servier Medical Art (<https://smart.servier.com/>).

- Does gut microbiota composition differ among different bone metastatic phenotypes (e.g. osteolytic, osteoblastic, mixed)?
- Do changes in gut microbiota directly influence the process of cancer dissemination to the bone?
- Can FMT be used to enhance the effectiveness of treatments for bone metastasis, by repopulating the gut?
- Can probiotics really enhance ICI efficacy for bone metastasis? If so, what types of probiotics?
- Can prebiotics, which induce the growth of beneficial bacteria in the gut, further enhance the effect of probiotics on ICI efficacy?
- Does preventing and/or blocking gut dysbiosis prevent bone metastasis or increase progression free survival?

Clinically, when cancer cells disseminate to the bone, patient survival outcomes diminish significantly. Unfortunately, treatments for bone metastasis at this point are generally palliative and have been shown to provide minimal curative effect [232]. Therefore, there is an apparent need for further studies elucidating the mechanisms through which bone metastasis occurs in order to identify novel treatment options for patients. These mechanisms are elusive; however, recently the gut microbiota have emerged as a likely contributor to bone metastasis, and their manipulation may enhance the efficacy of immunotherapies which are currently known to be ineffective on bone metastasis. Part of the mystery that needs to be uncovered is how bacteria from “the outside world (e.g. gut mucosal tissues)” can influence and educate the non-mucosal organ system that is the bone, to create an environment favorable for metastatic disease. While further clinical evaluation is warranted, current data suggest that ICI therapy plus probiotics, including FMT, alone or in concert with current standard treatments for bone metastasis may lead to much-needed improvements in the clinical management of cancer bone metastasis.

Author Contributions

K.F.C., H.Y., and Y.S. wrote the manuscript. All authors reviewed and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Pharmacological ascorbate as a novel therapeutic strategy to enhance cancer immunotherapy

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Pharmacological ascorbate (i.e., intravenous infusions of vitamin C reaching ~20 mM in plasma) is under active investigation as an adjuvant to standard of care anti-cancer treatments due to its dual redox roles as an antioxidant in normal tissues and as a prooxidant in malignant tissues. Immune checkpoint inhibitors (ICIs) are highly promising therapies for many cancer patients but face several challenges including low response rates, primary or acquired resistance, and toxicity. Ascorbate modulates both innate and adaptive immune functions and plays a key role in maintaining the balance between pro and anti-inflammatory states. Furthermore, the success of pharmacological ascorbate as a radiosensitizer and a chemosensitizer in pre-clinical studies and early phase clinical trials suggests that it may also enhance the efficacy and expand the benefits of ICIs.

KEYWORDS

pharmacological ascorbate, immune regulation, anti-PD-1, cancer immunotherapy, immune checkpoint inhibitors, antioxidant therapy, prooxidant therapy

1 Introduction

Immune evasion is one of the cancer hallmarks, defined as a mechanism by which cancer cells either suppress the immune response or mask themselves from immune detection and destruction (1, 2). Anti-tumor immunity is crucial during cancer initiation, progression, and metastasis. Tumors develop multiple mechanisms to evade anti-tumor immunity depending on the level of immune infiltration in the tumor microenvironment that defines it canonically as “cold” or “hot” (3). Cold tumors are considered “immune deserts” characterized by low immune cell infiltration (3). The decreased immunogenicity of cold tumors enables them to evade immune destruction by decreasing levels of tumor antigens, inhibiting maturation of dendritic cells, and suppressing T cell activation, infiltration, and migration (3). Hot tumors are highly infiltrated by immune cells; however, they evade anti-tumor immunity through impaired T cell tumor recognition, increased immune suppressor cell activation, and increased expression of inhibitory immune checkpoint molecules such as programmed death-ligand 1 (PD-L1) (3).

The work of James P. Allison and Tasuku Honjo in the 1990s and early 2000s led to the discovery of immune checkpoint inhibitors (ICIs). ICIs are drugs or antibodies that bind immune checkpoint proteins and prevent the activation of their immune-suppressive function, subsequently leading to increased immune activity. In 2011, Ipilimumab (anti-cytotoxic T-lymphocyte antigen 4 or anti CTLA-4) was the first ICI to receive FDA approval after demonstrating positive clinical outcomes in melanoma patients (4). In 2014, the programmed death protein 1 (anti PD-1) Nivolumab and Pembrolizumab received FDA approval following successful clinical trials in metastatic melanoma (5).

ICIs have since expanded from melanoma to multiple malignancies such as lung, bladder, breast, kidney, and head and neck cancers, etc. (6). However, their application in clinical settings faces numerous challenges such as primary and acquired resistance, toxicity, and limited response in some patient populations. Therefore, it is essential to develop new approaches that increase the sensitivity of tumors to ICIs to meet the clinical need. One approach to overcome these ICI limitations is pharmacological ascorbate (intravenous infusions of vitamin C resulting in ~ 20 mM concentrations in plasma (7)).

Ascorbate (L-ascorbic acid, $C_6H_8O_6$) is a six-carbon small molecule antioxidant with two ionizable hydroxyl groups and a molecular mass of 176.12 grams discovered by Szent-Györgyi and colleagues in the 1930s (8, 9). Commonly known as vitamin C, ascorbate is an essential micronutrient for humans who need to meet a daily intake of 100 – 200 mg *via* consuming fruits and

vegetables (10). Humans are not capable of ascorbate biosynthesis due to a mutation that prevents the conversion of L-gulonolactone to L-ascorbic acid (11, 12). Without adequate ascorbate intake, the homeostasis of multiple physiological functions is disturbed. Ascorbate is a donor antioxidant capable of undergoing two one-electron oxidations; this function plays a central role in collagen biosynthesis and elasticity, redox regulation, enzyme function (as a cofactor), hormone regulation and biosynthesis, and amino acid metabolism (13). As a cofactor, ascorbate functions as an epigenetic regulator for enzymes such as Ten-eleven translocation (TET) proteins and Jumonji C domain-containing histone demethylases (JHDMs) (14–16). By reducing Fe^{3+} to Fe^{2+} ascorbate promotes DNA demethylation, thus epigenetically modifying gene expression in multiple cell populations including cancer cells (as an anticancer effector) and immune cells (14–22).

At a physiological state with adequate dietary vitamin C intake, ascorbate concentrations in the plasma are ≤ 0.08 mM (23). With intravenous administration, ascorbate has unique pharmacokinetics that affects its final plasma concentration and urinary excretion. Oral ascorbate administration with doses up to 6 g/day yields plasma concentrations ≤ 0.22 mM due to the presence of tight physiologic control that leads to urinary excretion of excess ascorbate (23–25). Interestingly, administering ascorbate intravenously can yield plasma concentrations of 15–20 mM temporarily bypassing the strict ascorbate transporter regulation plasma concentrations (7, 23, 25). A remarkable outcome of these unique ascorbate pharmacokinetics is the selective cancer cell killing millimolar plasma concentrations, whilst sparing non-malignant cells, making it an emerging potential anticancer therapy with a plethora of clinical applications (23, 25, 26).

This review discusses the potential of pharmacological ascorbate as a sensitizer for cancer immunotherapy. We discuss the role of ascorbate in the context of immune function and immune regulation, review the most recent findings combining pharmacological ascorbate with ICIs, and highlight potential future directions for this promising approach.

2 Ascorbate and immune regulation

Because of ascorbate’s potential to enhance ICIs in cancer therapy, it is imperative to evaluate the effects of ascorbate on the immune function. However, experimental evidence regarding the effects of pharmacological dosing of ascorbate on immune function remains limited but a great deal of information can be learned from previous studies of the effects of ascorbic acid on cells of innate and the adaptive immune systems and how it

orchestrates immune function by acting on their proliferation, differentiation, and cytokine secretion.

2.1 Ascorbate and the innate immune system

2.1.1 Macrophages and monocytes

Macrophages are phagocytic tissue-differentiated monocytes essential for innate immune defense (27). The main functions of macrophages include phagocytosis, cytokine secretion, and professional antigen presentation to the adaptive immune system (28). In the tumor microenvironment, the role of macrophages can be profoundly influenced by their polarization status which determines their effector functions. Based on their polarization, tumor-associated macrophages (TAMs) can be broadly categorized into M1 and M2 macrophages. M1 macrophages are pro-inflammatory and exert anti-tumor effects by secreting IL-1 β , TNF, IL-12, and IL-18 while the several subtypes of M2 macrophages are anti-inflammatory and promote tumor progression by secreting IL-4, IL-10, IL-13, and TGF- β (28–30). Monocytes and macrophages accumulate high ascorbate concentrations (4–6 mM) by upregulating the ascorbate transporter, SVCT2 (31–35). In these cells, ascorbate is a cofactor for the hydroxylase enzyme responsible for regulating the hypoxia-inducible factors (HIF) family (22) whose activation impacts the polarization of TAMs towards the M2 phenotype that suppresses anti-tumor immunity and the associated T cell cytotoxicity (36–39). Additionally, some evidence in macrophages pointed at TET proteins and Jumonji C domain-containing histone demethylases that use ascorbate (21, 22) suggesting that ascorbate may play a role in macrophage function and polarization through epigenetic regulation.

Generally, ascorbate plays a pro-phagocytic role that promotes monocyte survival, protects against reactive oxygen species (ROS), and enhances phagocytosis (32, 40). A study by Oberritter and colleagues showed that ascorbate levels in macrophages decrease from baseline after phagocytosis, indicating that it is required to protect macrophages from ROS generated during phagocytosis (41). Monocytes treated with ascorbate (4–20 mM) were protected from FAS-induced cell death, thus promoting survival and differentiation (42). Ascorbate can also exert an anti-inflammatory effect on macrophages. In an *in vitro* study, ascorbate reduced the secretion of the pro-inflammatory cytokines, TNF, and IL-6 (43). Thus, ascorbate may selectively drive macrophage activity in either a pro or anti-inflammatory direction. A potential explanation for this duality is maintaining the balance between pro and anti-inflammatory responses and protecting normal tissues; however, more research is needed to elucidate the complex relationship between ascorbate and macrophage differentiation.

2.1.2 Neutrophils

Neutrophils make up to 70% of total leukocytes, and they are the most abundant immune cells in circulation produced at approximately 10^{11} cells per day (44). Neutrophils are the first responders of the innate immune system. They can carry out phagocytosis or degradation of pathogens by ROS and reactive nitrogen species (RNS) production (superoxide, hydrogen peroxide, and nitric oxide) or release their cytotoxic granules. The function of neutrophils has been well-established in innate immune function and antimicrobial activity, and recently, interest in neutrophils as regulators of the tumor microenvironment has developed. Neutrophils have been linked to tumor suppression -via hydrogen peroxide and nitric oxide production- and metastasis promotion (by inducing ROS-mediated DNA damage, IL-1 secretion, and other mechanisms) (45).

Neutrophils maintain high concentrations of ascorbate that can reach up to 14 mM (46). Motility is vital to neutrophils as they must travel to the primary site of infection to eliminate the pathogen or damaged tissue, and exposure to ROS can significantly hinder their motility. *In vitro* and *in vivo* work by Anderson demonstrated that the antioxidant action of ascorbate can rescue neutrophils from ROS exposure and stimulate migration by inhibiting the peroxidase-H₂O₂-halide system (47). These high levels of ascorbate promote chemotaxis and stimulate neutrophil superoxide and hydrogen peroxide production, thus allowing them to carry out their phagocytic and killing function once they are at the site of infection (32). *In vitro*, neutrophils treated with 0.1–0.3 mM ascorbate showed a significant increase in ROS production (48). Similarly, human studies with ascorbate supplementation demonstrated improved phagocytic activity and increased ROS production in neutrophils (49, 50).

Neutrophil extracellular traps (NETs) are large web-like extensions of decondensed chromatin with cytosolic and granule proteins that neutrophils form upon stimulation by ROS and the subsequent downstream activation of neutrophil elastase (NE) and protein-arginine deiminase type 4 (PAD4) in a process known as NETosis (51). NETs can also lead to neutrophil death by causing a loss of the nuclear envelope and plasma membrane degradation (51). NETs can promote tumor proliferation, cancer metastasis, and increase the infiltration of immunosuppressive T regulatory lymphocytes into the tumor microenvironment (45, 51, 52). Treating human neutrophils with ascorbate resulted in a decrease in phorbol ester-induced NETosis *in vitro*. In ascorbate deficient mice, NETosis was increased (53). Recently, an *ex vivo* study showed that ascorbate reduced sepsis-induced NETosis in a dose-dependent manner. At 1 mM, it induced a reduction in NETosis, while concentrations > 10 mM increased NETosis (54). These findings suggest that ascorbate is a potential regulator of NETosis which may have beneficial implications for cancer therapy.

2.1.3 Natural killer cells

Natural killer (NK) cells are innate immune granular lymphocytes that kill pathogen infected cells and tumor cells *via* cytotoxic action and produce cytokines such as IFN- γ (55). NK cell depletion is linked to cancer development and a poor prognosis (55). NKs have a unique ability to recognize cancer cells that escape T lymphocytes. 40-90% of human tumors downregulate major histocompatibility complex 1 (MHC-I) molecules used for antigen recognition by CD8⁺ T lymphocytes needed for an immune response (56). However, NK cells can recognize cancer cells regardless of their MHC-I status as they can recognize other surface markers on tumor cells (55, 57), highlighting their importance in anti-tumor immunity and as targets for immunotherapy.

Treating NKs with ascorbate (0.28 mM) increased cell proliferation and promoted the expansion and differentiation of cytokine-stimulated cultures containing NK progenitors without hindering the cytotoxic capacity of the differentiated NKs against leukemia cells under normoxic and hypoxic conditions [46]. In a human toxicology study where NK function was dramatically compromised by toxic chemical exposure (e.g., formaldehyde), 78% of individuals who received ascorbate (0.06 g/Kg body weight dose) showed over a ten-fold enhancement in NK function compared to untreated subjects (58). Similarly, treating functionally-impaired NKs derived from patients with β -thalassemia with 1 mM ascorbate resulted in a significant rescue of NK function compared to untreated controls (59). These data suggest that ascorbate supports NK survival and function. The susceptibility of NKs to oxidative stress (60) – a classic feature of the tumor microenvironment – suggests that ascorbate functions as an antioxidant that can protect NKs in the tumor microenvironment and enable them to carry out their anti-tumor function.

2.2 Ascorbate and the adaptive immune system

2.2.1 B lymphocytes

B lymphocytes carry out the humoral immune response. They differentiate into antibody-secreting plasma cells and memory B-cells that play a role in a prolonged immune response. Ascorbate accumulation has been described in B lymphocytes suggesting its functional relevance in humoral immunity (61). Anti- μ -primed spleen-derived B lymphocytes showed enhanced differentiation into plasma cells represented by increased IgM production and increased viability upon treatment with ≥ 0.5 mM 2-O- α -D-glucopyranosyl-L-ascorbic acid (a stable derivative of ascorbate) compared to untreated controls (62). A more recent study demonstrated that treating B-lymphocytes with 0.002-0.01 mM ascorbate promotes plasma cell differentiation by enhancing TET2/3-Mediated DNA demethylation; a similar response was reproduced in animals

receiving 4 mg/g body weight ascorbate (18). In a study by Woo et. al, activated B-cells treated with 0.0625 mM – 1 mM ascorbate demonstrated no changes in proliferation but apoptosis was induced in a dose-dependent manner *in vitro* (63).

In human studies, evidence of ascorbate's role in B cell function is conflicting. Vallance, Prinz, and colleagues demonstrated that vitamin C supplementation (1 g daily) increased IgG and IgM plasma levels suggesting an association between ascorbate and B cell differentiation and function (64, 65). In contrast, Kennes and colleagues found that ascorbate (0.5 g daily) did not affect immunoglobulin levels (66). However, Kennes used a lower dose of ascorbate compared to Prinz and tested an older patient population, which could justify the contradictory findings between the two studies. Hence, more investigation is needed to elucidate the complex role of ascorbate in mediating humoral immunity.

2.2.2 T lymphocytes

T-lymphocytes are responsible for cellular immune response through the action of CD4⁺ T helper cells (Th) and CD8⁺ cytotoxic T cells (Tc) (67). CD4⁺ T cells differentiate into different subsets such as Th1, Th2, Th17, and T Regulatory cells (Treg) (67). Th1 cells drive a pro-inflammatory cell mediated immune response by secreting IFN- γ , IL-2, TNF, and Th2 cells carry out an inflammatory immune response mediated by the cytokines IL4, IL-5, IL-6, IL-10, and IL-13 (67). Th17 cells are pro-inflammatory cells that produce IL-17 and IL-22 and some studies indicate that they may play a role in tumorigenesis (67, 68). Treg cells regulate immune cells by a number of mechanisms including secreting suppressive cytokines such as IL-10 and TGF- β , inducing apoptosis, or using cytokine deprivation to directly suppress other CD4⁺ and CD8⁺ T cells to downregulate cell-mediated immune response (67, 69). Tc cells secrete pro-inflammatory cytokines TNF and IFN- γ that have cytotoxic effects on tumor cells, further, Tc cells can directly kill cells through secretion of perforin and granzyme B that generate pores in the plasma membrane and activate cellular caspases, respectively (67, 70). All T cell subsets and their respective cytokines play essential roles in tumor immunity (67, 70), and major cancer-killing role of T lymphocytes has made them the target of the majority of immunotherapies such as CAR-T (chimeric antigen receptor) cell therapy and ICIs that target PD-1 and CTLA-4 on T cells (71).

Like macrophages, neutrophils, and B cells, T cells accumulate high millimolar levels of ascorbate (72, 73). Ascorbate is vital for numerous cellular functions in the life of a T cell, from development and maturation, proliferation and differentiation, to activation and regulation. When it comes to thymic T cell maturation and development, Manning and colleagues treated T cells with 0.03 mM ascorbate and observed increased T cell maturation, enhanced T cell receptor selection, and CD8 coreceptor upregulation. Similarly,

ascorbate-deficient mice showed impaired T cell maturation compared to mice that accumulate ascorbate (73). The Huijskens group treated T cell progenitors with 0.095 mM ascorbate and found it induced T cell maturation and promoted differentiation of double-positive ($CD4^+CD8^+$) thymocytes in the absence of stromal cells, and that ascorbate is required for T cell development when stromal cells are present (74).

As for peripheral T cells, *in vitro*, ascorbate acted as an inhibitor of multiple forms of T cell apoptosis, such as growth factor withdrawal, spontaneous apoptosis, and steroid-induced death (75). Some evidence suggests a potential dose-dependent effect of ascorbate on T cell viability and proliferation. Low ascorbate concentrations (0–0.125 mM) had no effect on T cell viability and proliferation while higher concentrations (0.25–0.5 mM) decreased both (76, 77). Ascorbate polarizes Th cells toward the Th1 phenotype (78, 79). Similarly, naïve T cells had a preference towards differentiating into Th1 cells in the presence of IL-12 produced by dendritic cells treated with 0.08 – 2 mM ascorbate (80). Moreover, dendritic cells treated with 0 – 2 mM ascorbate had elevated IL-12 production that led to increased effector $CD8^+$ differentiation and activation causing improved tumor killing *in vivo* (81). With regard to Treg cells, studies show that ascorbate may promote the conversion of effector $\gamma\delta$ T cells into Treg cells through the TET-mediated epigenetic regulation of Foxp3 expression through DNA demethylation, suggesting increased immune-suppressive functions (82, 83). In contrast, Oyarce and colleagues reported that although ascorbate promotes the differentiation of Tregs, their ability to suppress immune response against skin allograft was diminished (84). The impaired Treg function observed by Oyarce is consistent with suppressed Treg function and increased effector T cell activation with ascorbate supplementation in ascorbate-deficient mice as reported by Maeng and colleagues (85).

The first evidence on pharmacological ascorbate as a modulator of anti-tumor immunity in humans was reported in a clinical trial where NSCLC patients received pharmacological ascorbate with carboplatin and paclitaxel, and those with progression-free survival ≥ 6 months showed enhanced $CD8^+$ activation by 4.2 fold compared to 1.6 fold in patients with progression-free survival < 6 months (86). Through its role in regulating T cell maturation, differentiation, and activation, ascorbate is not only an immune modulator, but also a very promising regulator of T cell mediated antitumor immunity. More investigation is needed in this area in order to elucidate the multifaceted function of ascorbate in immune response and clearly define the role of dose and timing on immune cell proliferation and activation.

3 Ascorbate and cancer therapy

Cameron and Pauling lead clinical trials on ascorbate and cancer in the 1970s. Terminal patients were treated with high dose intravenous and oral ascorbate and it was found that ascorbate supplementation prolonged survival by 210–300 days and 22% of ascorbate-treated patients had greatly increased survival time (over 2.4 years) compared to matched controls (87, 88). Subsequent clinical trials with oral ascorbate in patients with advanced colorectal, lung, stomach, liver, and pancreatic cancer failed to reproduce Cameron and Pauling's findings (89, 90). Unfortunately, this decreased the interest in ascorbate as an anti-cancer therapy. A pivotal criticism of these trials is that they did not use intravenous ascorbate which was later shown to have a pharmacokinetic ability to increase plasma ascorbate concentrations to the tens of milli-molar range (91). Multiple studies by Levine and colleagues then confirmed that for cancer cell killing to be accomplished, pharmacological doses of ascorbate are required as they lead to the generation of toxic concentrations of hydrogen peroxide in the extracellular space in the tumor but not in the blood leading to selective killing (92–94). These findings initiated a new wave of ascorbate research in cancer therapy in the early 2000s (7, 95–100).

A growing body of evidence shows that ascorbate can selectively kill cancer cells by acting as a prooxidant by generating high fluxes of hydrogen peroxide that can react with intracellular labile iron *via* Fenton chemistry while it is believed to simultaneously protect normal tissue by acting as a donor antioxidant (26, 93). Chen and colleagues showed that ten cancer cell lines had an EC50 of < 4 mM with ascorbate while the non-malignant cell lines could survive ascorbate concentrations up to 20 mM (93).

These findings were confirmed *in vivo* in xenograft models of ovarian, pancreatic, and glioblastoma (GBM) cancers where pharmacological ascorbate significantly reduced tumor growth (93). These findings inspired the investigation of the potential of ascorbate as a sensitizing agent in combination with chemotherapy and radiation. In pancreatic cancer, pharmacological ascorbate was a radiosensitizer *in vitro* and in a xenograft mouse model (95). Concordantly, clinical trial data suggests that pharmacological ascorbate increases overall survival in patients with pancreatic cancer receiving concurrent chemotherapy (97). As a chemosensitizer, pharmacological ascorbate exhibited a synergistic effect when combined with gemcitabine *in vitro* and pre-clinical pancreatic cancer models (101). Consistent with these results, subsequent early phase clinical trials showed that pharmacological ascorbate is safe with promising clinical outcomes when combined with gemcitabine (102, 103). Additional early phase clinical trials in

GBM, non-small cell lung carcinoma (NSCLC), and ovarian cancer also found pharmacological ascorbate to be safe with promising clinical outcomes (96, 98, 104, 105). In addition to enhancing radiation and chemotherapy effectiveness, pharmacological ascorbate has also significantly reduced chemotherapy induced normal tissue toxicity as reported by Ma and colleagues in ovarian cancer, and radiation-induced normal tissue injury as reported by Alexander and colleagues in pancreatic cancer (97, 104). Taken together, data from all the aforementioned *in vitro*, pre-clinical, and clinical studies demonstrate its excellent capacity to kill cancer cells, improve response to conventional therapy -even in cancers known for poor response and poor prognosis such as pancreatic cancer-, and minimize the normal tissue damage associated with chemotherapy and radiation. With our current understanding of the importance of tumor immunology, ascorbate may increase the efficacy of ICIs.

4 Ascorbate as a sensitizer for cancer immunotherapy

While multiple basic and clinical studies focused on ascorbate and its clinical application in the context of chemotherapy and radiotherapy, very few studies have explored its potential as a sensitizer for cancer immunotherapy. The roles that ascorbate plays in immune regulation previously discussed in this review, along with its prooxidant action against cancer and antioxidant protective action in normal tissues, suggest pharmacological ascorbate may enhance the response of cancer cells to ICIs and protect against ICI-induced normal tissue injury such as pneumonitis.

Luchtel and colleagues evaluated pharmacological ascorbate (1.5 M) in a mouse lymphoma model in combination with anti-PD-1. This study showed an increase in lymphoma immunogenicity represented by a 15%- 21% increase in Tc-mediated cell killing (106). Another finding from this study was that combining high doses of ascorbate with anti PD-1 significantly increased Tc and macrophage infiltration in the tumor microenvironment and increased IL-12 production, increased Tc and NK activation, and increased granzyme B production (106). Lastly, this group showed that combining pharmacological ascorbate (4g/kg) with anti-PD-1 has a synergistic effect that significantly decreased tumor growth compared to untreated controls or treatment with single agents (106). Magri, A., et al. evaluated mouse models of melanoma, breast, pancreatic, and colorectal cancers to test the effect of ascorbate in combination with anti-PD-1 and anti-CTLA-4. First, they examined the effect of ascorbate on anti-tumor immune response and found it is primarily a Tc mediated

process emphasizing the potential of ascorbate sensitizing against anti-PD-1 and anti-CTLA-4 (107). Consistent with Luchtel's group, they reported significantly increased Tc infiltration in the tumor microenvironment and reduced tumor growth when combining ascorbate with anti-PD-1 and anti-CTLA-4 and (107). Tumor volume was significantly reduced in breast, colorectal, and pancreatic cancers by the ascorbate anti-PD-1 and anti-CTLA-4 combinations (107). Mice that showed complete response exhibited progression-free survival for up to a year (107). Most recently, a study reported a synergistic effect in a mouse model of renal cell carcinoma treated with high dose (0.5 g/kg) ascorbate combined with anti PD-L1 (17). Compared to untreated controls, high dose ascorbate alone, or anti PD-L1 alone, the combination showed the highest levels of CD4⁺ and CD8⁺ infiltration in the tumors, in addition to elevated IFN- γ and IFN- γ -induced CXCL9, CXCL10 and CXCL11 (17). Interestingly, the expression levels of PD-L1 were significantly increased by high dose ascorbate, suggesting that it can potentially make tumors more responsive to checkpoint blockade (17). These initial findings describing pharmacological ascorbate and cancer immunotherapy, ICIs in particular, are promising and warrant further investigation.

5 Discussion and future directions

Through its antioxidant activity and epigenetic regulation, ascorbate is a crucial modulator of immune function and the anti-tumor immune response (Figure 1). Moreover, as a small molecule antioxidant, it protects normal tissue against ROS. Metabolic changes in cancer cells, such as their reduced ability to metabolize hydrogen peroxide and their increased iron accumulation, make them susceptible to the prooxidant action of ascorbate. As a result, pharmacological ascorbate has been recently exploited in multiple clinical trials as a chemo- and radiosensitizer, but the field of ascorbate and cancer immunotherapy has yet to be explored significantly. Three studies showed pharmacological ascorbate combinations with ICIs: a) increase Tc infiltration in the tumor microenvironment and b) exert synergistic effects on tumor suppression. These findings emphasize the importance of this novel approach to cancer therapy. Future studies should include other malignancies and clinically relevant combinations such as using ICIs with chemotherapy and radiation. Additionally, future studies should address how pharmacological ascorbate affects primary and acquired resistance to ICIs, and whether or not it protects normal tissue from adverse effects associated with ICIs such as pneumonitis and colitis. Mechanistic studies that elucidate the route of action of ascorbate as a sensitizer to ICIs

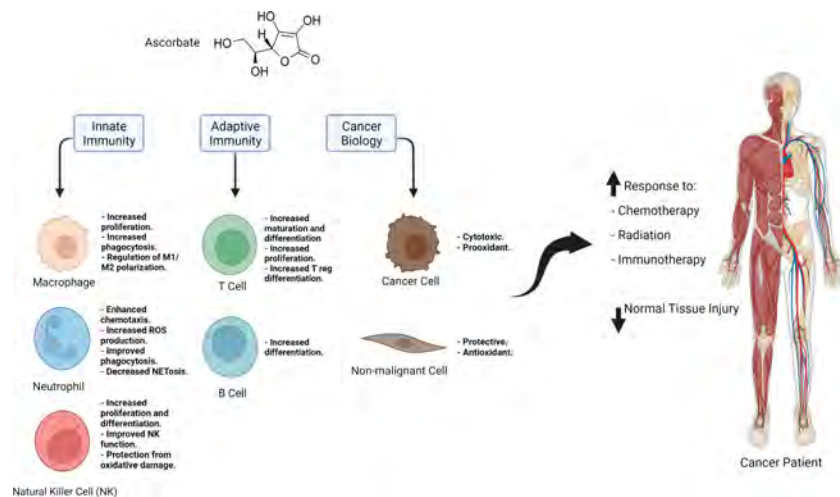


FIGURE 1

Summary of the immune regulatory effects of ascorbate, the role it plays in cancer and non-malignant cells, and its potential applications in cancer therapy.

are also critical. A noteworthy potential mechanism that can be studied in this context is the epigenetic regulation of TET2; prior research suggests that ascorbate promotes TET2 activation and leads to the expression of genes associated with anti-tumor immunity (19). Peng and colleagues explored this mechanism in their study and showed that with the loss of TET, high dose ascorbate lost the synergistic effect it had with anti PD-L1 in a mouse model of renal cell carcinoma, thus emphasizing the importance of epigenetic regulation as a focus for mechanistic studies on ascorbate and immunotherapy (17). There are many questions that need to be answered about ascorbate and cancer immunotherapy. Answering these questions will help expedite the establishment of clinical trials that will create a new and potentially improved standard of care regimens for cancer patients.

Author contributions

AZ and BA wrote the manuscript. LS, AM, SH, JS, MP, ZZ, TW, VM, JC, MF, JH, SV, and DS provided expertise and feedback. BA, DS, MF, SV, and JH provided funding. All authors contributed to the article and approved the submitted version.

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Radiopharmaceutical Quality Control Considerations for Accelerator-Produced Actinium Therapies

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Abstract

Background: Alpha-particle-emitting radiotherapies are of great interest for the treatment of disseminated cancer. Actinium-225 (²²⁵Ac) produces four α -particles through its decay and is among the most attractive radionuclides for use in targeted radiotherapy applications. However, supply issues for this isotope have limited availability and increased cost for research and translation. Efforts have focused on accelerator-based methods that produce ²²⁵Ac in addition to long-lived ²²⁷Ac.

Objective: The authors investigated the impact of ²²⁵Ac/²²⁷Ac material in the radiolabeling and radiopharmaceutical quality control evaluation of a DOTA chelate-conjugated peptide under good manufacturing practices. The authors use an automated module under identical conditions with either generator or accelerator-produced actinium radiolabeling.

Methods: The authors have performed characterization of the radiolabeled products, including thin-layer chromatography, high-pressure liquid chromatography, gamma counting, and high-energy resolution gamma spectroscopy.

Results: Peptide was radiolabeled and assessed at >95% radiochemical purity with high yields for generator produced ²²⁵Ac. The radiolabeling results produced material with subtle but detectable differences when using ²²⁵Ac/²²⁷Ac. Gamma spectroscopy was able to identify peptide initially labeled with ²²⁷Th, and at 100 d for quantification of ²²⁵Ac-bearing peptide.

Conclusion: Peptides produced using ²²⁵Ac/²²⁷Ac material may be suitable for translation, but raise new issues that include processing times, logistics, and contaminant detection.

Keywords: accelerator, actinium, generator, translation

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Introduction

Radionuclide therapy has been a pillar of nuclear medicine for over 80 years. Phosphorus-32 was first applied by Lawrence in 1938 for treatment of leukemia,¹ followed by other agents. Notably, the use of radioiodine for hyperthyroid conditions and cancer has become standard of care.^{2–4} Alpha-particle emitters have long been of interest for systemic applications in oncology and degenerative conditions. These charged helium nuclei deposit MeV of energy along a short path-length of only several cell diameters.⁵ Alpha-particle-emitting radium isotopes localize to sites of bone turnover, and the treatment of ankylosing spondylitis using radium-224 (²²⁴Ra) had been used widely for decades.⁶ The recent approval of radium-213 (²¹³Bi) dichloride for metastatic prostate cancer has stimulated even further interest in the field.⁷

Molecularly specific radioimmunotherapy involves conjugation of radionuclides to targeting vectors, often antibodies or peptides. The first clinical targeted α -particle-emitting radiotherapies developed by McDevitt et al. for bismuth-213 (²¹³Bi) subsequently led to development of actinium-225 (²²⁵Ac)-conjugated antibody and small-molecule therapies.^{8,9} The ²²⁵Ac isotope is considered nearly ideal for antibody-based targeted radiotherapy applications because the biological half-life of a full-length immunoglobulin and the physical half-life of the parent radionuclide ($t_{1/2}$ = 9.92 d) are well matched.^{10,11}

Increased translational efforts have revealed a supply-chain constraint for access to ²²⁵Ac. Production method, i.e., elution of a thorium-229 (²²⁹Th) generator—produces several times less than the estimated need for routine clinical practice.^{12,13} Demand for the isotope has spurred several developmental efforts, including cyclotron, electron, or photon irradiation of radium-226,^{14,15} and spallation of thorium targets by high-energy protons.^{16,17} This latter accelerator-based approach can produce large quantities of actinium. This includes the desired ²²⁵Ac, along with an isotopic mixture of short-lived ^{224,226,228}Ac ($t_{1/2}$ < 2 d) and long-lived ²²⁷Ac ($t_{1/2}$ = 21.8 year). Half-life, infrastructure requirements, and the cost and capabilities of physical separation methods preclude enrichment of the desired ²²⁵Ac. As such, the long-lived ²²⁷Ac is present in this drug formulation reagent.

²²⁷Ac decays predominantly to ²²⁷Th ($t_{1/2}$ = 18.72 d) followed by ²²³Ra ($t_{1/2}$ = 11.43 d), both of which are also alpha-particle emitters of interest for their therapeutic potential (Supplementary Fig. S1).^{18–21} This creates potential issues relating to radiochemical and dosimetric impact of this increasingly complex radionuclide mixture, which includes the concatenated decay progeny of both ^{225/227}Ac. Studies to date have investigated the radiochemical impact of the presence of ²²⁷Ac in chelate radiolabeling.^{16,22,23} At the end of bombardment, the radioisotopic impurity of ²²⁷Ac in the ^{225/227}Ac material is reported to be low (0.2%–0.3%).²⁴ The ratio of isotopes changes rapidly with time as the ²²⁵Ac material decays during chromatographic separation from the target thorium and coproduced isotopes, shipment, and radiopharmaceutical preparation.²⁵

Early evaluation of the radiobiological impact of the impurity is underway in preclinical and modeling studies.^{22,26} These and future studies are important as long-lived impurities in historical formulations of ²²⁴Ra may have contributed to incidences of treatment-induced malignan-

cies,^{27,28} however, evaluation of these data is ongoing.²⁹ As well, regulatory consideration regarding the handling and disposal of the chemical and biological wastes that are contaminated with this long-lived ²²⁷Ac material are a point of continuing discussion.

The authors' interest in the present study was to investigate the implications for quality control and clinical release processing for accelerator-produced actinium used to formulate a therapeutic agent; and the application of an automated synthesis system for such a radiopharmaceutical. They report and discuss the radionuclide and radioisotopic purity impact for this material following good manufacturing processes for labeling, including an automated radiolabeling module, and a variety of analytical methods.

Materials and Methods

Radionuclides

The ²²⁹Th generator-produced ²²⁵Ac and accelerator-produced ^{225/227}Ac sources were supplied by the United States Department of Energy. The accelerator-produced material activity was 79.2 MBq (2.1 mCi) on 07/01/2021, 1 d following purification, and subsequently evaporated and shipped as dried nitrates (Supplementary Fig. S2). The certificate of analysis for this batch indicated an estimated 0.7 MBq (20 μ Ci) of ²²⁷Ac contaminant, as extrapolated from measurements of previously generated material. At receipt, the material in a Wheaton vial (3.0 mL) was measured using a well-style dose calibrator (Capintec CRC25R) set on pre-calibrated ²²⁵Ac setting number (#82) with 55 MBq (1.5 mCi) of starting material (at day 7 postradiomaterial purification, 07/06/2021). A representative certificate of analysis for generator produced material is included (Supplementary Fig. S3).

Radiolabeling

The DOTA-labeled peptide precursor was supplied by Modulation Therapeutics, Inc., (Fig. 1C). The labeling of the peptide was carried out under good manufacturing practice, within a shielded hot cell using a multifunctional automated radiosynthesis module (Trasis, AllinOne mini). The process was controlled remotely using Supervision (v.2.28 Rev. 2, Trasis). In brief, 46.6 MBq (1.27 mCi) of the source dissolved in 0.2 M HCl was loaded under vacuum in the initial vial for radiolabeling with the DOTA-conjugated precursor (200 μ g) on day 5 postsource purification.

The source was transferred to the one-pot radiolabeling reactor cassette, in which the reaction occurred in Tris buffer (1 M, pH 7.2) at 85°C for 70 min in the presence of 20% v/v L-ascorbic acid at pH 6–8. The radiolabeled peptide was transferred in saline and passed through a 0.2 μ m sterilizing filter, resulting in a final volume of 9.7 mL. The final radiolabeled sample was tested for sterility and presence of bacterial endotoxins.

Radio-instant thin-layer chromatography

A sample of the final drug product material (0.4 μ L each) was spotted on glass-microfiber chromatography paper impregnated with a silica gel (115 mm length) and developed using 50 mM DTPA in ultrapure water. Acquisitions of 5 min were conducted at ²²⁵Ac/²¹³Bi equilibrium (>4 h postmigration; typically 6–7 h) utilizing a radioTLC imaging system

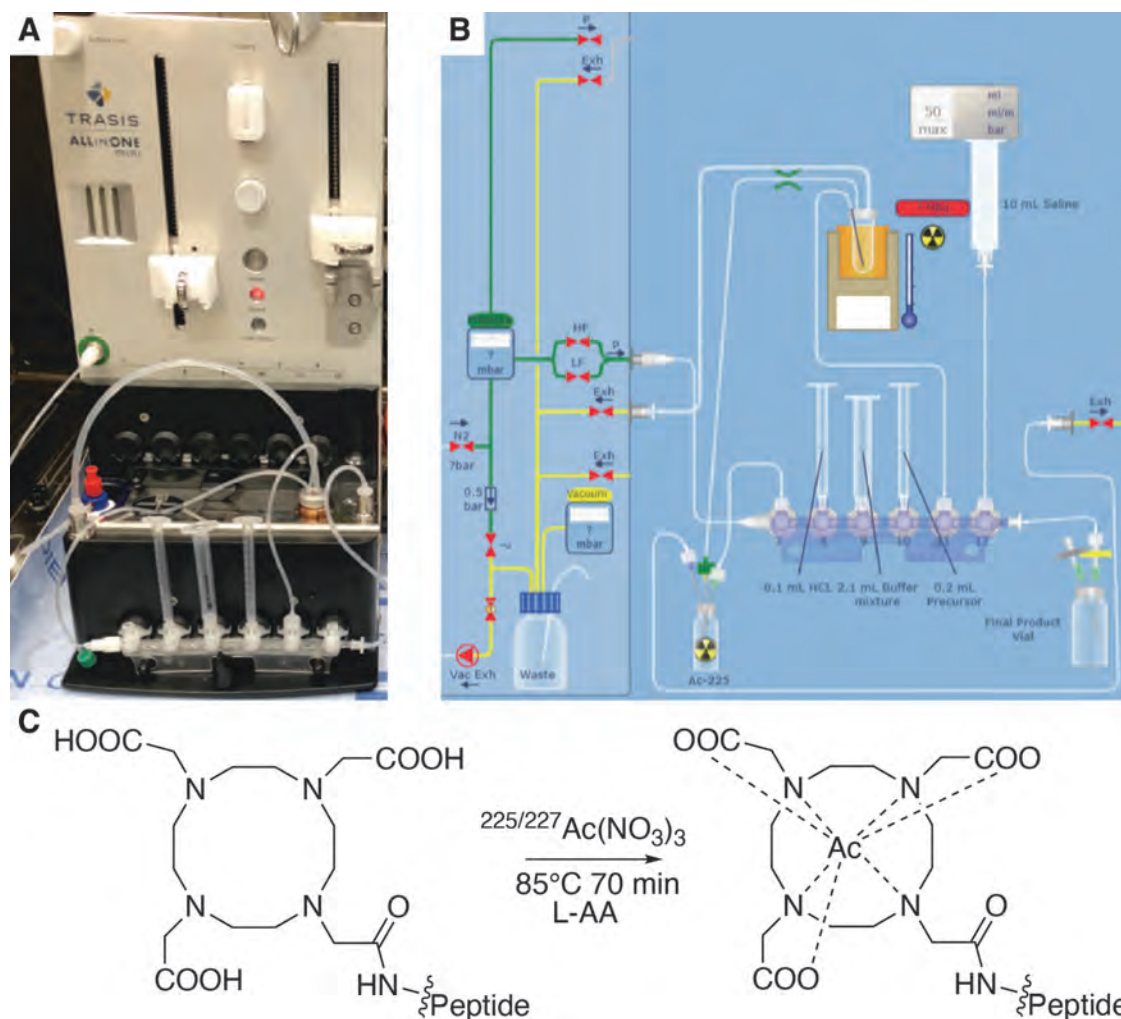


FIG. 1. Automated Radiosynthesis Module and Conditions. **(A)** A software-controlled automated procedure was used to ^{225}Ac -radiolabel the precursor peptide, under GMP conditions in a radiopharmacy hot cell. **(B)** Schematic representation of the conditions and fluid controls. **(C)** Radiolabeling schema for the DOTA-conjugated peptide. GMP, good manufacturing practice.

under default settings and analyzed using vendor software (AR-2000 and Winscan; Bioscan). Free radiometals migrated to the front and the bioactive-labeled material remained at the origin (0–50 mm).

Peptide purification and fractionation

Reverse phase high-pressure liquid chromatography (HPLC) was undertaken utilizing an Agilent system equipped with a HICHROM Vydac 218MS C18 (4.6×250 mm, $5 \mu\text{m}$) column, running with a flow rate of 1 mL/min, and a UV detection set at 220 nm. Twenty microliters of the labeled peptide was injected for analytical chromatography and fractions were collected for radiotracer determination. The total separation was performed over 35 min and fractions were collected every 1 mL. A gradient mobile phase was used consisting of HPLC grade water (Solvent A) and acetonitrile (Solvent B) both with 0.1% trifluoroacetic acid.

Gradient conditions were initially 100% (A), 0% (B); 0–20 min to reach 20% (A), 80% (B); 20–20.5 min was ramped to 100% (A), 0% (B); 20.5–35 min 100% (A), 0% (B). An

elution time of 11.736 min was recorded from the radiolabeled product UV spectra, and 11.779 min was identified to be the unlabeled precursor.

Gamma counting

Collected fractions from the HPLC were measured using a COBRA-2 gamma counter (Packard) after the samples had reached an equilibrium state for ^{225}Ac (>4 h), with an energy window channel setting of 15–800 keV. Acquisitions were recorded 1 min per sample, and the counts per minute were background corrected to determine radiochemical purity defined as the integration of collected fractions 10–14 mL (containing the peptide as determined by UV-trace) over the entire collection (35 mL). Two fractions contain detectable activity, noted as fr12 and fr13, and were submitted for further analyses.

Gamma spectroscopy

A high-purity germanium (HPGe) detector was used to acquire high energy resolution spectra for isotope identification.

The cryocooled system (GEM-50195-S; Ortec) was used with samples placed 5 cm away from the detector enclosed in a 10 cm lead shield (HPLBS1; Ametek). The energy and efficiency calibrations were completed using a gamma ray source traceable to the National Institute of Standards and Technology (Eckert Ziegler Isotope Products) allowing preset calibration of each energy peak and quantification per isotope. The same sample-to-detector geometry was used to measure the HPLC collected fractions of interest and measured over 24 h. Counts were normalized to acquisition time for each gamma spectra, and the isotope quantification was conducted utilizing Gamma-Vision Software (version 8.0, Ametek) accounting for background, half-life, abundance, acquisition time correction, and normalized for each isotope recognized by the edited library.

Safety statement

Handling and evaluation of radiological materials require careful planning, infrastructure, and training. This is all the more important for high linear energy transfer emitters, as used in this work. All experiments were conducted by staff with certified Radiation Safety approvals and dose monitoring by the Washington University School of Medicine Department of Environmental Health and Safety, in approved areas. Samples were handled in either a hot cell or chemical fume hood, and characterization measurements were performed with sealed samples in appropriate and monitored areas. All samples used in this work have half-lives ≥ 10 d, requiring off-site storage and specialized disposal protocols handled by Radiation Safety.

Results

Accelerator produced $^{225/227}\text{Ac}$ was received for radiolabeling using a preprogrammed automated radiosynthesis module (Trasis, AllinOne mini). In brief, the system consists of a volume and time-controlled heating and mixing platform for single-pot radiolabeling of the precursor DOTA-conjugated peptide. The advantages of an automated approach include a systematic and repeatable preparation method with reduced absorbed dose to the radiochemist. In addition, the device is located within an ISO7 hot cell, thereby reducing likelihood of radiocontamination to personnel, and biocontamination to the material. The module was programmed to fully automate the benchtop synthesis used to develop the radiopharmaceutical, see Figure 1. Here, the dry $^{227/225}\text{Ac}$ -nitrate is dissolved in dilute acid, to which antioxidant and peptide is added and heated to 85°C for 70 min. The radiolabeled material was sterilized by filtration, resulting in 9.7 mL of 200 μg of peptide (48.47 MBq) for subsequent quality control analyses.

Initial radiochemical analyses were performed at the time of radiopharmaceutical production and at day 2 postlabeling

(to model time elapsed required to reach and be administered to a patient). The authors first assessed the product using radio-iTLC. Radiolabeled peptide and control (uncomplexed) source radiomaterial were spotted and developed using 50 mM DTPA. The acquisition of the paper chromatographic strip by proportional counter was carried out at >6 h postdevelopment. This time is provided for ^{225}Ac to reach secular equilibrium; and for migrated daughters, primarily ^{221}Fr ($t_{1/2}=4.9$ min) and ^{213}Bi ($t_{1/2}=45.6$ min), to decay. The radio-iTLC analysis indicated $>99\%$ radiochemical purity, Figure 2A and B. The clinical release criterion for this radiopharmaceutical includes $\geq 95\%$ RCP by radio-iTLC, which was met.

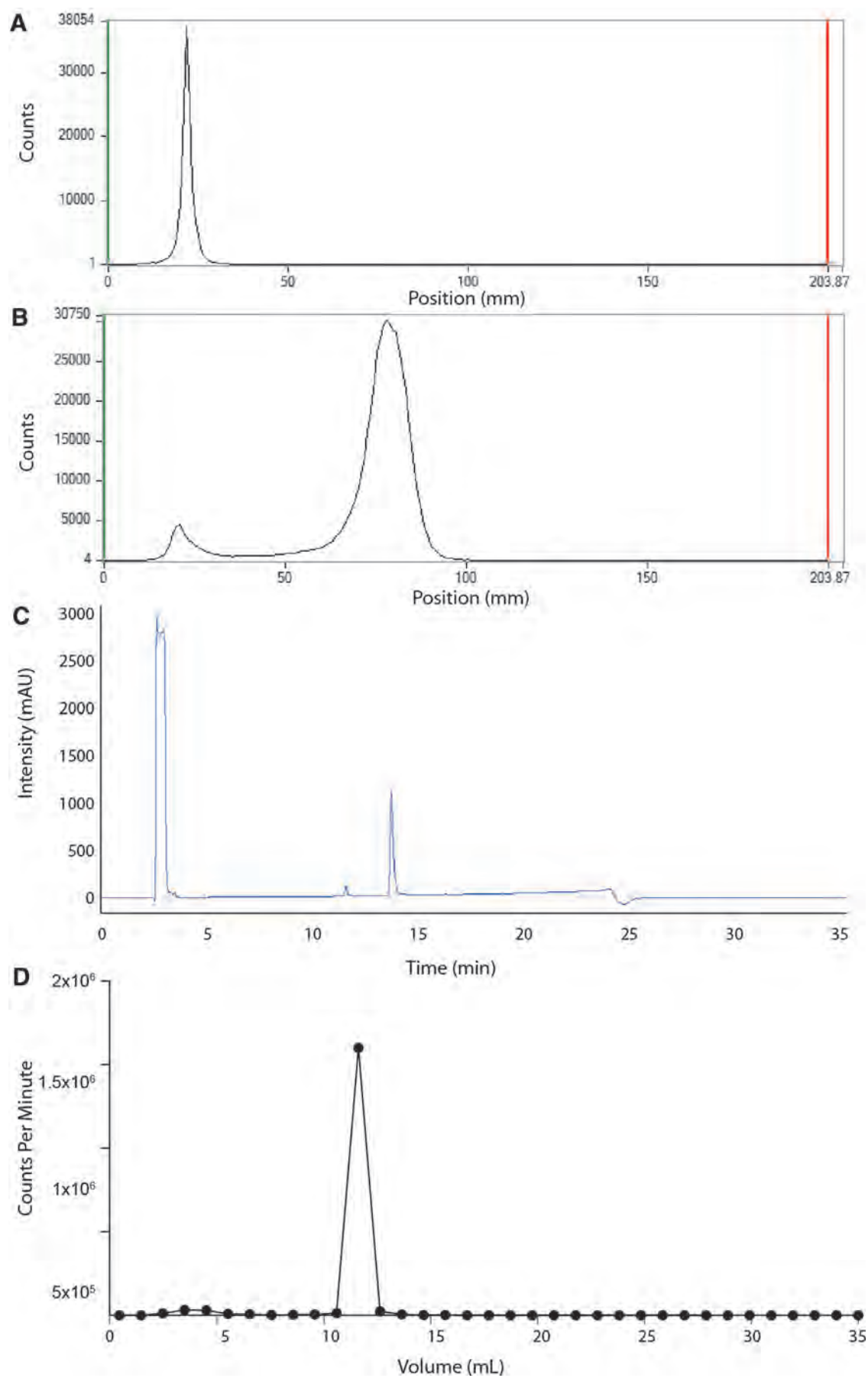
Reverse-phase HPLC was also performed, demonstrating $>90\%$ chemical purity (Fig. 2C). The early (3–5 min) fractions correspond to the low retention of free metal ions (Fig. 2D). The collected peptide fractions were gamma counted, after reaching ^{225}Ac secular equilibrium, and a 94.2% radiochemical identity was observed. This stands in contrast to the automated radiolabeling results from thorium-229 generator produced ^{225}Ac $>95\%$ ($n \geq 5$; Supplementary Fig. S4), analyzed by identical methods.

They next sought to investigate the radioisotopic content of the HPLC-separated peptide to interrogate this discrepancy. High-resolution gamma spectroscopy using cryocooled HPGe was acquired at multiple time points following radiolabeling to identify the peptide-complexed radionuclides. At the earliest analyzed time point, day 2 following radiolabeling, the abundant gamma emissions from ^{221}Fr (218 keV) and ^{213}Bi (440 keV) are prominent (Fig. 3A). The direct detection of ^{225}Ac itself was also observed; and was quantifiable at 188 keV (this gamma ray line is isolated from daughter contributions). The activity determined from this ^{225}Ac peak (99508 Bq) matched that of progeny ^{213}Bi (98086 Bq) at equilibrium (Table 1). The HPGe method was in good agreement with the dose calibrator reading of the labeled radiopharmaceutical, after compensating for the proportion of activity injected by HPLC and that collected in fractions 12 and 13.

Detailed observation also revealed the presence of ^{227}Th in the purified radiolabeled peptide fraction at day 2. The amount measured here indicates that the ^{227}Th present was labeled onto the conjugate at synthesis. In addition, ^{223}Ra is not clearly detected at this early time point. This underlines successful chemical purification by HPLC of this nonchelated rare earth metal, and the direct DOTA-peptide labeling by both $^{225/227}\text{Ac}$ and ^{227}Th (Fig. 3B). The samples were reanalyzed for isotopic content at 82 and 100 d post-radiolabeling (Fig. 3C, D).

Quantification of these isotopes' activities is presented in Table 1, for each of the displayed gamma spectrum measurements. Direct quantification of ^{225}Ac was not successful at later time points due to interfering gamma and X-rays

FIG. 2. Radiochemical Purity Analysis. (A) Radio-instant thin-layer (radio-iTLC) chromatography of $^{225/227}\text{Ac}$ radiolabeled peptide migrated by DTPA (50 mM). (B) Control radio-iTLC of source $^{225/227}\text{Ac}$ material under identical conditions. Peak analysis results show $>99\%$ radiochemical yield by radio-iTLC of (A). (C) UV-chromatogram from HPLC of the peptide post-radiolabeling; DOTA-conjugated radiolabeled peptide peak at 11.7 min is detected. (D) Gamma counting of the HPLC fractionated samples, using an open, 15–800 keV, energy window. Free ions, noncomplexed, ions are detected in fractions 3–4, while the majority of the activity is associated with peptide at 12 mL. Calculation of radiochemical yield by gamma counted HPLC fractions was RCP = 94.2%. HPLC, high-pressure liquid chromatography.



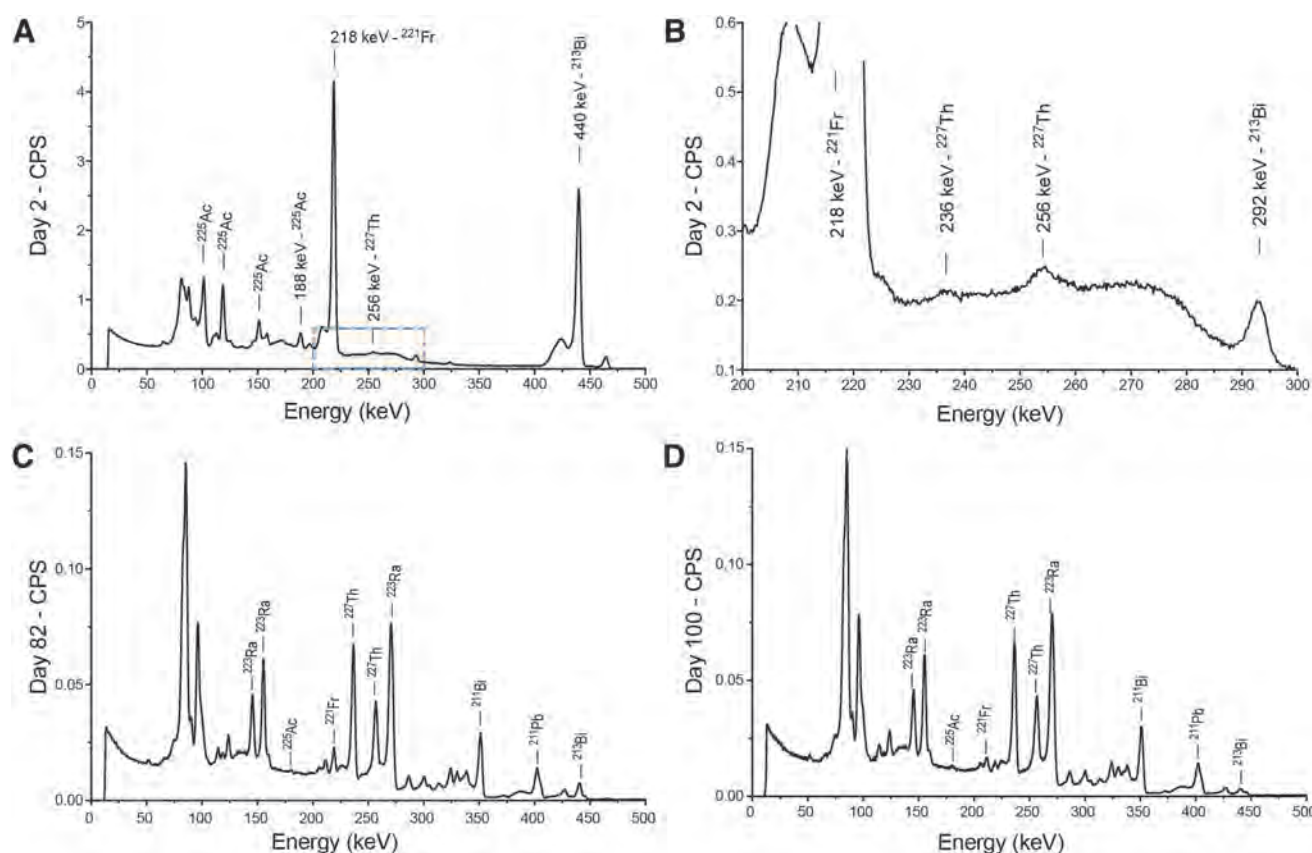


FIG. 3. Gamma Spectroscopy of HPLC-Purified Peptide. **(A)** At day 2 postradiolabeling (day of patient injection) the gamma ray lines from ^{225}Ac and daughters ^{221}Fr and ^{213}Bi are prominent. **(B)** Magnification of dashed region; presence of ^{227}Th associated with the radiolabeled peptide. **(C)** The same fraction evaluated at day 82 and **(D)** day 100 postradiolabeling. The ^{225}Ac contribution to the spectra has diminished, while ^{227}Ac -daughters emissions have reached equilibrium.

from the complex radionuclide mixture. The high-energy 440 keV of the ^{213}Bi daughter could be cleanly assessed, and measured values at 82 and 100 d are within the error of the method as predicted from ^{225}Ac -decay rate from the initial day 2 quantification. In contrast, the ^{227}Th activity in the

peptide fraction does not follow the predicted values for the half-life of this isotope, as it is fed by ^{227}Ac decay.

The 256 keV line used for ^{227}Th analysis has negligible interference from other emissions. Using the activity quantified at 100 d postradiolabeling (for which ^{227}Th has reached >98% equilibrium with its parent), the authors calculated an initial ^{227}Ac activity of 1514.2 Bq in the primary peptide fraction at the proposed day of injection. A further 68.1 Bq in the second largest fraction was measured, Table 2. The

TABLE 1. ACTIVITY (BQ) OF PURIFIED $^{227/225}\text{Ac}$ -LABELED PEPTIDE FRACTIONS MEASURED UTILIZING GAMMA SPECTROMETRY ACQUIRED AT DAY 2, 84, AND 100 POSTRADIOLABELING

	Time postradiolabeling		
	2 d	84 d	100 d
Ac-225 (Bq; 188 keV)			
Measured	99,508.98	n.d	n.d.
Predicted	—	301.33	97.18
Bi-213 (Bq; 440 keV)			
Measured	98,086.32	213.81	77.58
Predicted	—	297.02	95.79
Th-227 (Bq; 256 keV)			
Measured	1824.83	1435.5	1501.64
Predicted	—	87.34	48.26

The predicted activities are calculated for the half-life of each radionuclide from that measured at day 2. The characteristic gamma line for Ac-225 could not be determined (n.d.) after day 2.

TABLE 2. ISOTOPIC PURITY AT THE TIME OF PATIENT INJECTION (DAY 2 POSTLABELING) OF PURIFIED PEPTIDE

	Activity (Bq) at day of injection		Isotopic purity (%)
	Ac-225	Ac-227	
fr12	98,086.32	1514.2	98.46
fr13	4304.06	68.1	98.43
Labeled-peptide	102,390.38	1582.3	98.46

HPLC fraction 12 (11–12 min, the most concentrated fraction) and fraction 13 (12–13 min, the end of the collected peak) constitute the full radiolabeled $^{225/227}\text{Ac}$ -peptide content. Ac-225 was measured by HPGe at day 2; Th-227 was evaluated at 100 d postradiolabeling, reaching equilibrium with Ac-227.

HPLC, high-pressure liquid chromatography; HPGe, high-purity germanium.

resulting isotopic purity of actinium-225 (defined as the percentage of ^{225}Ac to all actinium isotopes present³⁰) of the isolated radiolabeled peptide is 98.46%.

The time elapsed between final $^{225/227}\text{Ac}$ purification from the accelerator bombarded material to the radiopharmaceutical labeling was ~ 7 d. This provides time for ingrowth of ^{227}Ac daughters. The authors have modeled the radionuclidic content using the Bateman equations, and displayed activity over time for all isotopes with a half-life greater than 9 d (Supplementary Fig. S5). The radionuclide content at the day of radiolabeling consists predominantly of ^{225}Ac , as well as ^{227}Ac and ^{227}Th (which increases to the day of injection). The result is a radionuclidic purity for ^{225}Ac of 97.8% at day 2 postlabeling, if they only consider therapeutic isotopes with a half-life greater than 9 d. These data demonstrate the importance of timing both between source purification and radiopharmaceutical administration, as well as controlling the molecular contents of the prepared material.

Discussion

Targeted radiotherapy is an emerging paradigm for the treatment of refractory and disseminated disease. Recent approvals by the European Medicines Agency and the US Food and Drug Administration (FDA), including iodine-131 (^{131}I) meta-iodobenzylguanidine, radium-223 (^{223}Ra) dichloride, and lutetium-177 (^{177}Lu) octreotate, are poised to be the beginning of a trend that will likely see radiotherapies become commonplace in cancer care. These treatments can be considered inherently more complex than many nonionizing agents as issues of handling, time of administration, disposal, and overlapping medical, pharmaceutical, and nuclear regulations must be considered. These issues are relevant both in the preclinical research and development space as well as in the sphere of clinical translation.

Actinium-225 has been evaluated as an α -particle-emitting radionuclide for targeted radiotherapy applications for over 20 years,^{8,30,31} with active clinical assessments ongoing.^{31,32} The majority of the global supply for this work is ^{229}Th -generator-produced material, limited to 20–40 GBq per year.^{17,33,34} Meeting additional supply requirements has been a focus for alternative production methods from the United States Department of Energy (DOE) and others. Large-scale production by proton bombardment using thorium targets is a promising approach.^{24,35} At this time, the DOE has submitted a provisional Drug Master File to FDA for evaluation of ^{225}Ac produced through this process as an active ingredient in radiopharmaceutical drug manufacture.

Special consideration for the coproduced 21.7 year half-life ^{227}Ac and its multiple α -particle-emitting progeny may be required. This includes the radiochemical impact as well as dosimetry and potential radiobiological effects of the contaminants; the handling of long-lived radionuclide materials and wastes; and the long-term contamination of radiochemical equipment and patient treatment spaces.³⁶

The complex mixture of radionuclides present in both starting material and in the final radiolabeled product when using $^{225/227}\text{Ac}$ sources is a potential confounder in radiopharmaceutical quality control and release. Gamma ray spectroscopy can be used to detect isotopes from the ^{227}Ac decay chain (Fig. 2), a technique which may not be accessible at all dispensing academic or tertiary medical centers. The analytical studies undertaken in this work further sought to define differences in peptide labeled with either ^{229}Th generator-produced or accelerator-produced material. The conventional ^{229}Th generator-produced ^{225}Ac passed radiochemical purity quality control levels (defined as >95% as assessed by radio-iTLC) through multiple radiolabeling runs.

Using $^{225/227}\text{Ac}$ starting material for radiolabeling under identical, automated conditions led to similarly high (>99%) yield by TLC, but a lower radiochemical purity of 94.2% by gamma counting. Further, their analysis of the radiopeptide at late time points (100 d postradiolabeling) enables an accurate assessment of the ^{227}Ac content in the labeled radiopharmaceutical. Using this information in concert with the early (2 d postradiolabeling) gamma ray spectroscopy data, they can state that ^{227}Th is initially chelated by the peptide. A limitation of this study is that these results are derived from a single run with the $^{225/227}\text{Ac}$ starting material, which is produced at this time on an infrequent schedule.

However, the authors posit that this would not significantly alter their results as multiple batches of this material have had similar isotopic properties. Additional replicates would strengthen the basis of these observations and these results motivate further testing, and an assessment of the analytical methods leveraged to determine radiopharmaceutical purity and labeling efficiency.

At this time, no standardized methods of analysis or release for clinical use of ^{225}Ac -constructs have been put forward. Techniques developed from clinical development efforts have relied predominantly on TLC of radioconjugate material from ^{229}Th generator-produced sources.^{8,32,37} Here, the francium-221 (^{221}Fr) and ^{213}Bi daughters of ^{225}Ac presenting reasonable distinctive gamma rays (218 and 440 keV) readable using NaI detection or by gas ionization. The authors' analyses of the $^{225/227}\text{Ac}$ material show that such systems are less well suited to deal with multiple α -emitting radionuclides with similar emitted energies. Accelerator-produced material present higher challenges of detection

TABLE 3. ^{227}Ac CONTENT CALCULATED PER ADMINISTERED ^{225}Ac -RADIOPHARMACEUTICAL AT 8 D POSTSOURCE PRODUCTION, 2 D POSTRADIOLABELING, FOR A PATIENT MASS OF 80 KG AND A RADIOCHEMICAL YIELD OF >95%

Radiopharmaceuticals	Dosing (kBq/kg)	Admin. ^{225}Ac (MBq/Cycle)	^{227}Ac (MBq/Cycle)	Clinical trial identifier
^{225}Ac -HuM195	150	12	0.214	NCT02575963
^{225}Ac -PSMA617	100	8	0.144	NCT04597411
^{225}Ac -Peptide	≤ 325	≤ 26	≤ 0.470	—

due to the multiple overlapping α -emitting daughters ^{227}Th (236, 254 keV), ^{223}Ra (145, 154, 270 keV), ^{211}Pb (404 keV), and ^{211}Bi (356 keV), limiting the capacity to clearly visualize and compute radiocontaminants.

In brief, the more complex mixture of isotopes present in the accelerator-produced material brings challenges for radiochemical analysis postlabeling. Radiopharmaceuticals produced using $^{225/227}\text{Ac}$ sources may have to take this into account for pharmacy release criteria. Implied in this statement is that QC evaluation of the radiochemical and -nuclidic purity, along with the isotopic purity, may require systematic specific considerations for this material for each produced batch, including HPLC coupled with radiotracing and HPGe for release.

These data underline that purity considerations are driven by both the time of radiolabeling following the processing of the bombarded material, and the time between radiolabeling and administration. Experimentally derived expiration dates for the material may be required for radiopharmacy validation and release. Previous experience with radionuclide impurities can provide some guidance. Indium-111 (2.8 d) production results in a long-lived isomer, $^{114\text{m}}\text{In}$ (49.5 d). The contaminant produces a highly abundant high-energy beta particle that can significantly contribute to blood dose for leukocyte-labeled $^{111/114\text{m}}\text{In}$.^{38,39} As such, the material should be used as quickly as possible. The beta-particle-emitting lutetium-177 (6.65 d) produced via the ^{176}Lu (n, γ) ^{177}Lu reaction will contain $^{177\text{m}}\text{Lu}$ (160 d).

In the treatment setting, the detectable $^{177\text{m}}\text{Lu}$ may have only a minor contribution to imaging and therapy.^{40,41} Literature, regulatory and manufacturer direction, also exists for the handling and consideration of impurities in yttrium-90, including strontium-90 and yttrium-88, and generator breakthrough for strontium-82, rubidium-82, germanium-68/gallium-68, and molybdenum-99/technetium-99m among others.^{42,43}

Standing-out against the historical considerations of impurities present in the radiopharmaceutical formulations above is the nature of the impurities for the $^{225/227}\text{Ac}$ material. These include high-energy alpha- and beta-particle emitters, concatenated decay of isotopes with long half-lives. Radiopharmaceutical evaluation incorporating accurate information of the $^{225/227}\text{Ac}$, ^{227}Th , and ^{223}Ra and daughters in a formulation is thus necessary to fully evaluate the pharmacokinetic and dosimetric aspects of any drug. The authors have calculated the activity of ^{227}Ac expected for a drug administration using the radiochemical yields and purity from the present work and administered activity levels of ^{225}Ac in ongoing and proposed trials (Table 3). It should be noted that different radiolabeling chemistries have been used (a 2-step DOTA chelation at 65°C for the HuM195 antibody, and direct PSMA-617 chelation at 95°C) and different times of processing which will change the radionuclidic and isotopic purity of the final products.^{31,32}

Consideration of the localization, residency time, stability, and potential redistribution of the mixture of complexed and free radionuclides is likely to be distinct for each agent. Thus, the attendant radiotherapy and dosimetric impact could be evaluated on an individual compounding basis considering yield and timing for each patient. This may be considered onerous compared to current nuclear medicine therapy workflows; however, this is standard of practice for radiation oncology procedures for the deposition of ioniz-

ing radiation through either external beam or brachytherapy interventions.⁴³

In conclusion, this work observed and evaluated potential radiopharmacy quality control and release issues of $^{225/227}\text{Ac}$ in a drug formulation. Standard measures from radio-iTLC and gamma counting of HPLC fractions were performed with generator- and accelerator-produced actinium-labeled peptide. Difference in results between radio-iTLC and gamma counting may be attributed to chemical and radiological properties of the complex radionuclide mixture. The preparation and injection times of therapies using this starting material will be important considerations in the context of radiopharmacy. These are in addition to the careful regulatory guidance required on the application, disposal, and licensing aspects, which will be necessary to a safe framework to both treat patients and to work with these long-lived contaminants.

Disclosure Statement

M.M., T.H., and D.M. are employees of Modulation Therapeutics Incorporated. T.J.W. and D.N.P. own intellectual property on the radiopharmaceutical. The remaining authors declare no conflicts of interest.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S2
Supplementary Figure S4
Supplementary Figure S5

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Article

Establishment of Novel Neuroendocrine Carcinoma Patient-Derived Xenograft Models for Receptor Peptide-Targeted Therapy

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Simple Summary: Gastroenteropancreatic neuroendocrine neoplasms (GEP NENs) are a family of rare cancers with rising incidence in recent years. GEP NEN tumor cells are difficult to propagate, and few cellular and patient-derived xenograft (PDX) models are available for testing new therapies and studying the heterogeneous nature of these cancers. Here, we described the establishment and characterization of two novel NEC cellular and PDX models (NEC913 and NEC1452). NEC913 PDX tumors express somatostatin receptor 2 (SSTR2), whereas NEC1452 PDX tumors are SSTR2 negative. As a proof-of-concept study, we demonstrated how these PDX models can be used for peptide imaging experiments targeting SSTR2 using fluorescently labelled octreotide. The NEC913 and NEC1452 PDX lines represent valuable new tools for accelerating the process of drug discovery for GEP NENs.

Abstract: Gastroenteropancreatic neuroendocrine neoplasms (GEP NENs) are rare cancers consisting of neuroendocrine carcinomas (NECs) and neuroendocrine tumors (NETs), which have been increasing in incidence in recent years. Few cell lines and pre-clinical models exist for studying GEP NECs and NETs, limiting the ability to discover novel imaging and treatment modalities. To address this gap, we isolated tumor cells from cryopreserved patient GEP NECs and NETs and injected them into the flanks of immunocompromised mice to establish patient-derived xenograft (PDX) models. Two of six mice developed tumors (NEC913 and NEC1452). Over 80% of NEC913



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and NEC1452 tumor cells stained positive for Ki67. NEC913 PDX tumors expressed neuroendocrine markers such as chromogranin A (CgA), synaptophysin (SYP), and somatostatin receptor-2 (SSTR2), whereas NEC1452 PDX tumors did not express SSTR2. Exome sequencing revealed loss of *TP53* and *RB1* in both NEC tumors. To demonstrate an application of these novel NEC PDX models for SSTR2-targeted peptide imaging, the NEC913 and NEC1452 cells were bilaterally injected into mice. Near infrared-labelled octreotide was administered and the fluorescent signal was specifically observed for the NEC913 SSTR2 positive tumors. These 2 GEP NEC PDX models serve as a valuable resource for GEP NEN therapy testing.

Keywords: gastroenteropancreatic neuroendocrine neoplasms; patient-derived xenograft; tumor spheroids; somatostatin receptor-2; near infrared-labelled octreotide analog

1. Introduction

Tumors can arise within neuroendocrine cells throughout the body, and some of the most common sites that lead to human morbidity and mortality originate within the GI system, most commonly within the small bowel and pancreas, collectively known as gastroenteropancreatic neuroendocrine neoplasms (GEP NENs). The age-adjusted incidence of GEP NENs increased over sixfold in the United States between 1973 and 2012, with an annual incidence of 3.56 per 100,000 persons [1]. These tumors are typically slow growing, but over 60% of patients present with metastatic disease and survival is greatly diminished [2–4]. In addition to the stage of the disease, both tumor grade and cell morphology/degree of differentiation are also powerful predictors of survival. Based on the 2019 World Health Organization classification of neuroendocrine neoplasms, GEP NENs are comprised of well-differentiated neuroendocrine tumors (NETs) and poorly-differentiated neuroendocrine carcinomas (NECs) [5]. Grade 1, 2, and 3 NETs are all morphologically well-differentiated and have Ki-67 values of <3%, 3–20%, and >20%, respectively. NECs are poorly differentiated tumors with a Ki-67 proliferation index >20% and/or a mitotic rate of over 20 mitoses per 2 mm², and many NECs have Ki-67 indices >50% [5]. In addition to these distinguishing features of poor differentiation and high Ki-67, GEP NECs have frequent mutations in *TP53* and *RB1*, while mutations in NET tumors are less common, and include *MEN1*, *DAXX*, and *ATRX* for pancreatic NETs (PNETs) and *CDKN1B* and 18q loss in small bowel NETs (SBNETs) [5–7].

Clinically, NECs are very aggressive, rapidly dividing tumors. They are associated with high rates of metastatic disease at presentation and poor prognosis. The incidence of NECs is not as well-defined due to changes in the WHO classification over the past decade, but epidemiological studies estimate this to be about 0.4/100,000 person-years [1,8]. The optimal treatment for GEP NECs has not been established due in part to the rarity of these tumors and difficulty with performing randomized trials. As a result, current guidelines for treatment of GEP NEC are based on lower-level evidence (NCCN Guidelines 2021) [9]. Therapeutic strategies are largely derived from experience in management of small cell lung cancer due to the pathologic and immunohistochemical similarities between small cell lung cancer and GEP NECs [10–12]. The appropriateness of this has been called into question as small cell lung cancer differs from NECs in several ways including higher association with smoking, higher rate of brain metastases, and higher sensitivity to platinum-based chemotherapy [11–14]. Patients are generally treated with chemotherapy regimens including platinum-based alkylating agents (carboplatin) and topoisomerase inhibitors (etoposide). Despite treatment, response rates are only 30–50% and median overall survival is 9 to 20 months [13–15].

Current therapies for both NETs and NECs are limited to somatostatin analogues (SSAs), mTOR inhibitors (everolimus), tyrosine kinase inhibitors (sunitinib), limited chemotherapy regimens, and peptide receptor radionuclide therapy (PRRT) [16–18]. One of the biggest barriers to identifying additional active therapeutics has been the inability

to establish GEP NEN cell lines and mouse models that can be grown robustly. Some of these tumor cells can be grown in culture as spheroids, but they grow very slowly and are difficult to propagate as xenografts [19–21]. The two widely used cell lines, BON [22] and QGP1 [23] resemble poorly differentiated NECs [24–26], and unfortunately express low levels of NEN markers such as the somatostatin receptor 2 (SSTR2) [24]. Although well-differentiated GEP NET cell lines, such as the P-STS, GOT1, and NT-3 cells, have been described [24,25,27], difficulties growing these cells in abundance have limited their distribution to other researchers. The paucity of available cell lines has been a significant hurdle towards better understanding NEN biology and to provide theranostic models, and therefore we set out to establish new models to expand these options for NEN research.

2. Methods

2.1. Patient-Derived Xenograft Models and Cell Lines

The inventory of the Washington University PDX center was searched for neuroendocrine tumors and carcinomas, which were collected under an Institutional Review Board-approved protocol (#201708051) of Washington University and cryo-preserved. All peripheral blood and patient tumor tissue were procured on the day of surgery. Peripheral blood was layered onto a Ficoll gradient, peripheral blood mononuclear cells (PBMCs) isolated, rinsed, 5×10^6 /mL transferred to cryovials, and cryopreserved in FBS + 10% DMSO. Tumor tissues were kept cold, cut into multiple small pieces, 5 pieces transferred into each cryovial, and cryopreserved in FBS + 10% DMSO. All cryopreserved samples were progressively cooled in a freezing container at a controlled rate of $-1^\circ\text{C}/\text{min}$ at -80°C . Vials were subsequently transferred to liquid nitrogen for long term storage.

Six patient samples were selected for this study. Tumors were thawed, minced, digested with collagenase and DNase I, and strained to obtain a single-cell suspension [19]. One to ten thousand cells were injected subcutaneously into NOD scid gamma (NSG) mice under an Institutional Animal Care and Use Committee-approved protocol (#9051771). Once visible subcutaneous tumors developed, tumor volume was calculated by multiplying tumor length, width, and depth and expressed as mm^3 . Tumors larger than 1000 mm^3 were harvested, processed, and 1 million tumor cells were injected into another generation of NSG mice, and remaining cells were placed in suspension culture. After 2 days of incubation, mouse fibroblasts from the PDX tumors were easily removed since they adhered to the plastic culture dish while the NEC cells grew in suspension. The floating NEC cells were harvested and transferred into new culture dishes. After 3 passages, the cultures consisted of only NEC cells with no fibroblast contamination was observed. NEC tumor cells can also be seeded in extracellular matrix as 3-dimensional cultures. For optimal NET marker expression, use NEC cells recently isolated from PDX tumors and avoid using cells in culture over 6 months. NEC tumor cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine, insulin, and nicotinamide [19]. BON cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine [22].

2.2. Histology and Immunohistochemistry

Patient samples from surgery were fixed in formalin, embedded in paraffin, and sectioned. Slides were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Slides were immunostained using specific antibodies against chromogranin A (CgA) (Thermo Scientific, Waltham, MA, USA, #MA5-13096), synaptophysin (SYP) (Agilent Dako, Santa Clara, CA, USA, #M7315), achaete-scute family bHLH transcription factor 1 (ASCL1) (BD Pharmingen, San Diego, CA, USA, #556604), p53 (Agilent Dako, #M700101-2), retinoblastoma protein (Rb) (BD Pharmingen, #554136), somatostatin receptor 2 (SSTR2) (Abcam, Waltham, MA, USA, #ab134152), and C-X-C motif chemokine receptor 4 (CXCR4) (Abcam, #ab124824), and Ki-67 (Agilent Dako, #M724001-2). Ki-67 proliferation index was quantified by percentage of positively staining cells in ~500 tumor cells per tumor sample.

2.3. Quantitative PCR

RNA was extracted from tumors grown in mice using the RNeasy Plus Universal Kit (Qiagen, Beverly, MA, USA) and reverse transcribed to cDNA using the qScript cDNA Supermix (QuantaBio, Beverly, MA, USA). Quantitative PCR was performed with gene-specific primers and PerfeCTa SYBR Green Supermix dye (QuantaBio) using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Primer sequences were obtained from PrimerBank (<https://pga.mgh.harvard.edu/primerbank>, accessed on the 8 July 2020) and were purchased from Integrated DNA Technologies (IDT). Primer sequences used for qPCR analysis are shown in Table 1.

Table 1. List of primer sequences used for qPCR experiments.

Gene Symbol	Forward	Reverse
<i>GAPDH</i>	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
<i>CGA</i>	TAAAGGGGATACCGAGGTGATG	TCGGAGTGTCTCAAAACATTCC
<i>SYN</i>	CTCGGCTTTGTGAAGGTGCT	CTGAGGTCACTCTCGGTCTTG
<i>SSTR1</i>	GCGCCATCCTGATCTCTTTCA	AACGTGGAGGTGACTAGGAAG
<i>SSTR2</i>	TGGCTATCCATTCCATTGACC	AGGACTGCATTGCTTGTGACG
<i>SSTR3</i>	AGAACCTGAGAATGCCTCCTC	GCCGCAGGACCACATAGATG
<i>SSTR4</i>	GCATGGTCGCTATCCAGTG	GCGAAGGATCACGAAGATGAC
<i>SSTR5</i>	GTGATCCTTCGCTACGCCAA	CACGGTGAGACAGAAGACGC
<i>CXCR4</i>	ACGCCACCAACAGTCAGAG	AGTCGGGAATAGTCAGCAGGA

2.4. Immunofluorescence

Cells derived from mouse tumors were fixed with 4% paraformaldehyde for 10 min, then stained with primary antibodies against SYN (Abcam, #32127) at 1:600 dilution, CgA (Invitrogen, Waltham, MA, USA, #MA5-13096) at 1:400 dilution, and SSTR2 (Sigma, St. Louis, MO, USA, #HPA007264) at 1:400 dilution overnight. Cells were washed and incubated with an FITC-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA, #115-095-062 and #711-095-152) at 1:500 dilution for 1 h. Immunofluorescent images were taken using a fluorescent microscope at 200 ms exposure time.

2.5. Genomic DNA Analyses

Genomic DNA from PDX tumors, and peripheral blood mononuclear cells (PBMCs) were extracted using a DNA extraction kit (Qiagen). Short tandem repeats (STR) analyses were performed on the DNA samples using the Cell Check9 panel of 9 human STR polymorphisms (IDEXX, Westbrook, ME, USA). Exome sequencings of PDX tumors were performed by submitting genomic DNA from NEC913 and NEC1452 PDX tumors to the Washington University Genome Technology Access Center for analyses with the IDT Exome 150X coverage. Exome sequencing data were analyzed using a DRAGEN processor and compared to the GRCh38 reference genome.

2.6. Imaging of Patient-Derived Xenograft Mouse Model with Bilateral Tumors

Five female NSG mice (Jackson Laboratory, Bar Harbor, ME, USA, Stock no: 005557) were anesthetized with 1% to 2% isoflurane at 10 weeks of age, and were subcutaneously injected with 1×10^6 SSTR2(+) cells in extracellular matrix Matrigel (Corning, Corning, NY, USA, #356235) in the left shoulder, and 1×10^6 SSTR2(−) cells in the right shoulder. When bilateral tumor size reached between 10 to 20 mm in diameter at 5 weeks post-implantation, in vivo and ex vivo near infrared (NIR) fluorescence imaging were conducted with NIR octreotide analog (NIR-TOC) as described by Hernandez-Vargas et al. [28,29]. In brief, 6 nmol of NIR-TOC diluted in 100 μ L PBS was administered via mouse tail-vein injection 24 h prior to imaging studies. NIR fluorescence imaging was acquired using the IVIS Lumina S5 small animal imaging station and Living Image[®] software (PerkinElmer, Waltham, MA, USA) with excitation and emission set to 740 and 790 nm, respectively. Images with favorable contrast-to-noise ratio were obtained using exposure time of 2 s for

in vivo and 0.1 s for ex vivo imaging, with subject height of 1.50 cm, small binning and F/Stop setting of 2, and field of view setting C. After completing in vivo imaging, mice were euthanized and dissection was immediately performed for ex vivo isolation, and imaging of subcutaneous tumors as well as major intraabdominal and intrathoracic organs was performed. Quantification of NIR fluorescent signal was performed using ImageJ version 1.53 a (NIH, Bethesda, MD, USA). Statistical analyses for NIR fluorescent signal were performed using *t*-tests in Prism GraphPad. *p* < 0.05 was depicted with *.

3. Results

Tumor cells from six cryopreserved patient tumors (Table 2) were injected into the flank of NSG mice to generate PDX models (Figure 1A). At 3 months post-tumor cell injection, two mice harboring GEP NEC cells had developed subcutaneous tumors of approximately 1 cm in diameter (NEC913 and NEC1452; Figure 1A) while four GEP NET patient tumor cell samples injected into mice did not form tumors (Table 2). Subcutaneous injection of 1×10^6 NEC913 and NEC1452 cells grew into tumors about 1000 mm³ and 1500 mm³ in size, respectively, after 5 weeks in subsequent passages. The tumor formation rate was 100%. The NEC913 and NEC1452 xenograft tumors were harvested and collected for histological and biochemical analyses. A separate portion of the NEC913 and NEC1452 tumors was collected for tumor cell isolation and injection into another generation of mice for propagation of the PDX models and for establishment of cell lines. Both NEC913 and NEC1452 cells were successfully maintained in culture for months in enriched DMEM/F12 medium. Both PDX tumors stained positive for the neuroendocrine tumor markers chromogranin A (CgA) and synaptophysin (SYP), but only the NEC913 PDX tumor stained positive for somatostatin receptor 2 (SSTR2; Figure 1B). Exome sequencing of the NEC913 and NEC1452 PDX tumors were performed and mutations in *TP53* and *RB1* were confirmed (Figure 1C; Supplementary Tables S1 and S2).

Table 2. List of GEP NEN patient tumor samples used for patient-derived xenograft (PDX) development.

Patient Tumor ID Number	Classification of Tumor	WHO Terminology	Differentiation	Tumor Grade	Ki67 (%)	Establishment of PDX
PNET459	Pancreatic NET	NET Grade 2	Well differentiated	Intermediate	7	no
PNET560	Pancreatic NET	NET Grade 2	Well differentiated	Intermediate	8.4	no
PNET1164	Pancreatic NET	NET Grade 2	Well differentiated	Intermediate	13	no
SBNET1063	Small bowel NET	NET Grade 3	Well differentiated	High	80	no
NEC913	Ampullary NEC	NEC, small and large-cell types	Poorly differentiated	High	80–90	yes
NEC1452	Rectal NEC	NEC, large-cell type	Poorly differentiated	High	80–90	yes

We were able to retrieve the original patient tumor for the NEC913 sample for comparison with the PDX model. The NEC913 tumor came from a patient presenting with jaundice and upper GI bleeding. A biopsy revealed a Grade 3 NEC of the ampulla of Vater, and the patient was treated with carboplatin/etoposide chemotherapy for 4 months, then had a Whipple procedure, where a 0.3 cm primary tumor with multiple involved nodes were also removed (Figure 2A). Hematoxylin and eosin (H&E) staining showed the presence of both small and large NEC cells (Figure 2B). The NEC913 primary tumor stained positive for Ki-67 in over 80% of cells (Figure 2C). An outside pathology report indicated that the specimen was TTF-1 positive and CDX2 negative. We detected positive staining for CgA, SYP, SSTR2, and ASCL1 (Figure 2D–G). A low level of p53 was detected (Figure 2H); however, exome sequencing data identified several stopgain mutations where the first stopgain mutation is located in codon 147 of *TP53* (Figure 1C, Supplementary Table S1) suggesting that the IHC staining detect only the first 146 amino acid fragment of p53. Expression of Rb was lost (Figure 2I). Exome sequencing of the NEC913 PDX tumor revealed an *RB1* frameshift insertion (1091_1092insCG) leading to a premature stop codon

(Figure 1C, Supplementary Table S1). STR analyses confirmed that the NEC913 patient blood sample shared the same alleles with the NEC913 PDX tumor (Supplementary Table S3) and that these samples did not match any of the existing research samples in the IDEXX DSMZ STR database, meaning that they are being reported for the first time.

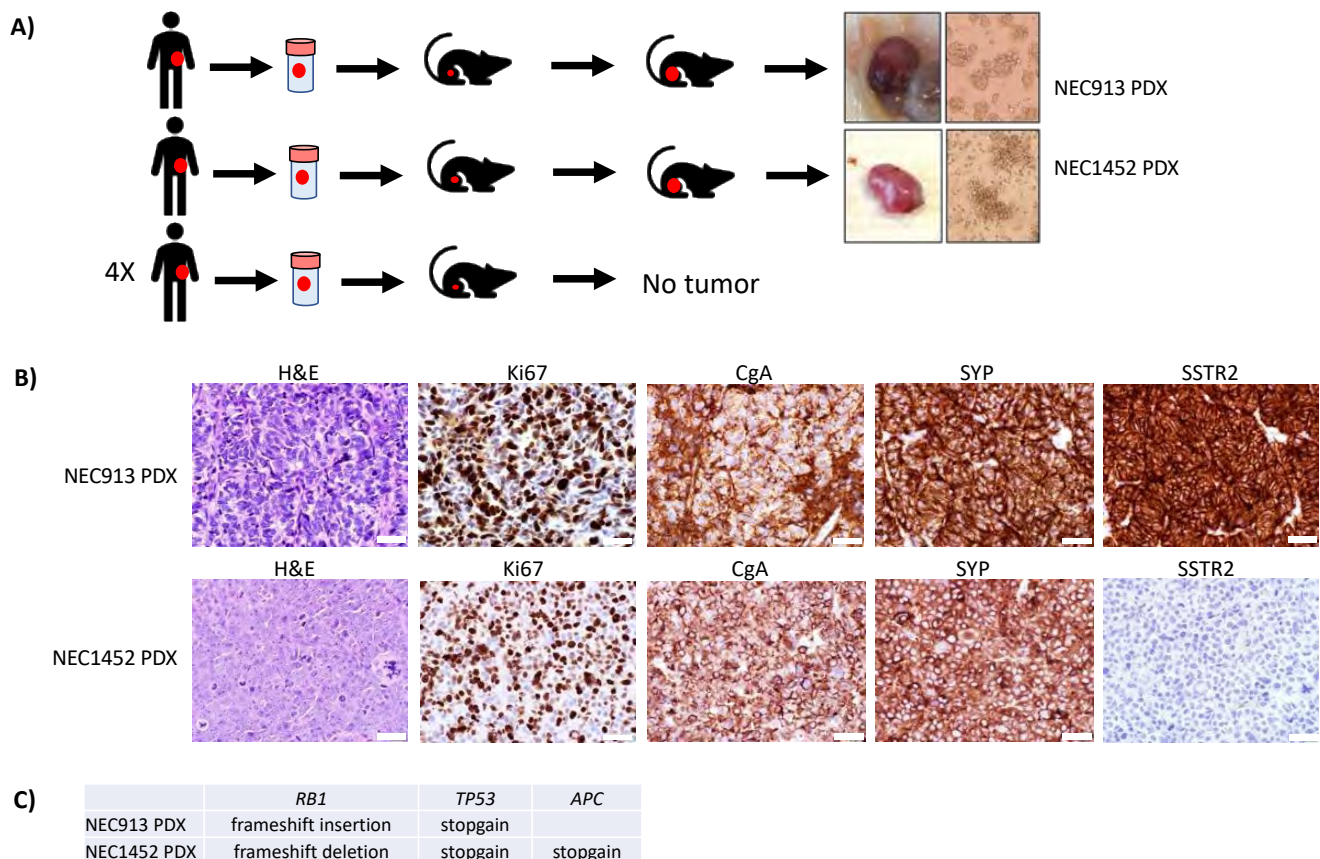


Figure 1. Establishment of neuroendocrine pancreas (NEC) patient-derived xenograft (PDX) models. (A) Tumor samples from NEC patients were cryopreserved, thawed, and injected into the flank of immunocompromised NOD-Scid Gamma (NSG) mice. Two mice developed subcutaneous tumors at three months post-injection (NEC913 and NEC1452 PDX models). Both PDX models have been passed in 6 generations of mice. (B) Formalin-fixed and paraffin-embedded tumor sections are stained with H&E and stained for Ki67 and neuroendocrine tumor markers such as chromogranin A (CgA), synaptophysin (SYP), and somatostatin receptor 2 (SSTR2) by IHC. Scale bar represents 40 μ m. (C) Exome sequencing of NEC913 and NEC1452 PDX tumors demonstrated mutations in *TP53* and *RB1*.

We were able to retrieve the original patient tumor for the NEC913 sample for comparison with the PDX model. The NEC913 tumor came from a patient presenting with supraclavicular masses, as well as liver, pancreatic, retroperitoneal, and rectal lesions that were jaundice and upper GI bleeding. A biopsy revealed a Grade 3 NEC of the ampulla of Vater, and the patient was treated with carboplatin/etoposide chemotherapy for 4 months, then DOTA-octreotate (DOTA-TATE) PET avid. A supraclavicular node biopsy showed a small cell NEC and carboplatin/etoposide were started, followed by FOLFIRI after progression, were also removed (Figure 2A). Hematoxylin and eosin (H&E) staining showed the presence of both small and large NEC cells (Figure 2B). The NEC913 primary tumor stained positive for Ki-67 in over 80% of cells (Figure 2C). An outside pathology report indicated that the specimen was TTF-1 positive and CDX2 negative. We detected positive staining for CgA, SYP, SSTR2, and ASCL1 (Figure 2D–G). A low level of p53 was detected (Figure 2H); however, exome sequencing data identified several stopgain mutations where the first stopgain mutation is located in codon 147 of *TP53* (Figure 1C, Supplementary Table S1) suggesting that the IHC staining detect only the first 146 amino acid fragment of p53.

Expression of Rb was lost (Figure 2I). Exome sequencing of the NEC913 PDX tumor revealed an *RB1* frameshift insertion (1091_1092insCG) leading to a premature stop codon (Figure 1C, Supplementary Table S1). STR analyses confirmed that the NEC913 patient blood sample shared the same alleles with the NEC913 PDX tumor (Supplementary Table S3) and that these samples did not match any of the existing research samples in the IDEXX DSMZ STR database (Supplementary Table S4), and exome sequencing con-

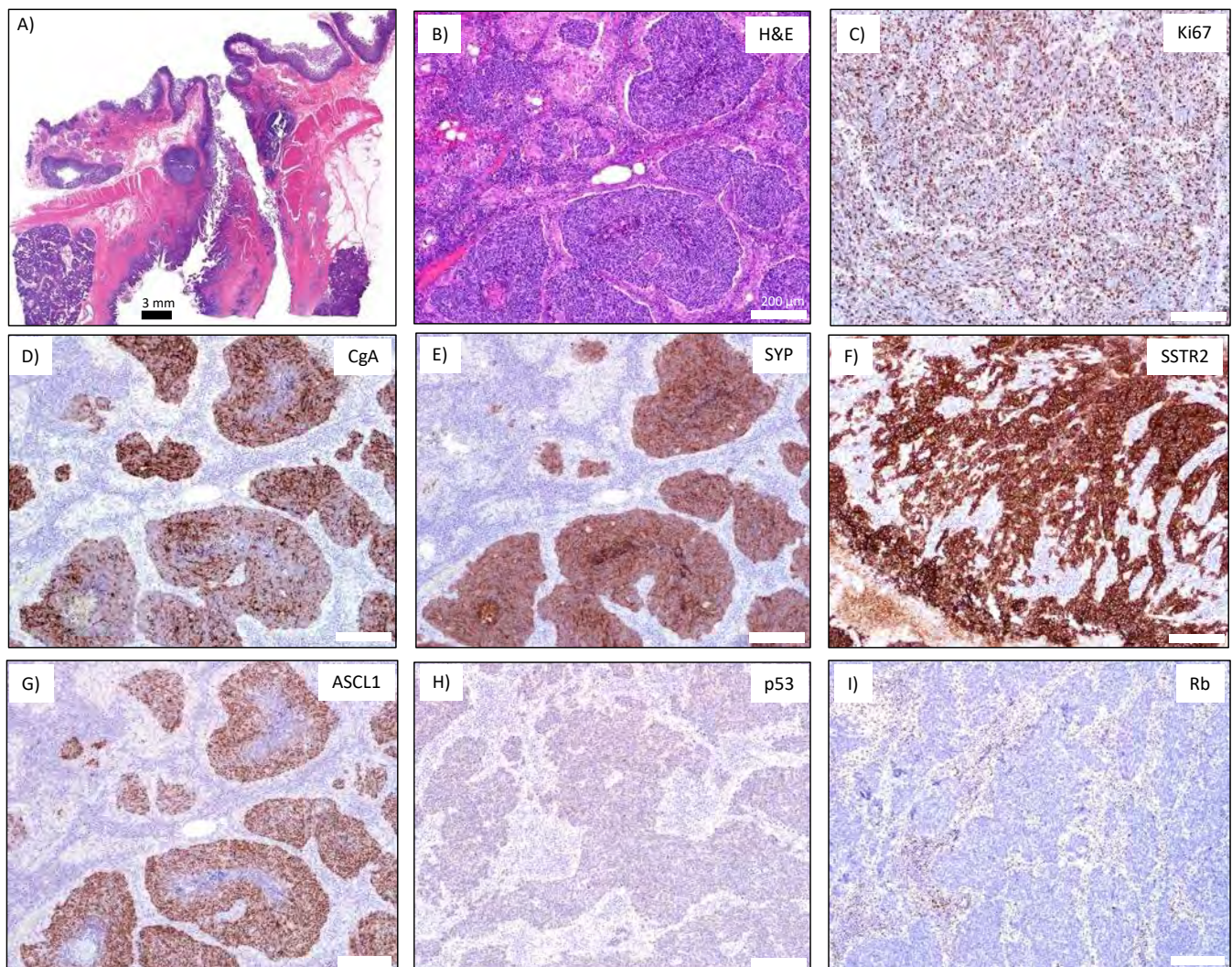
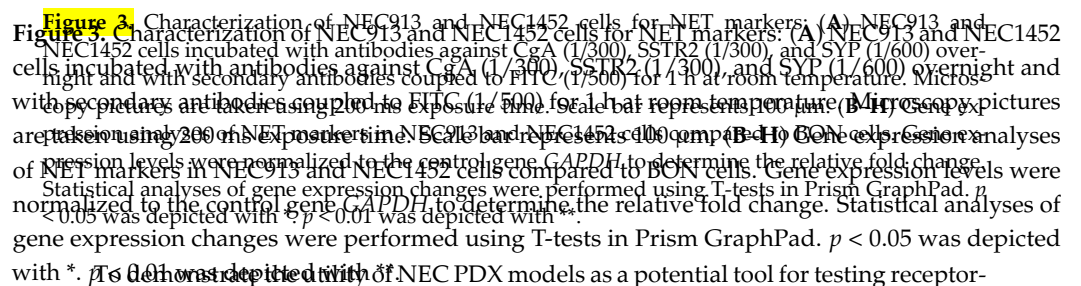


Figure 2. IHC analyses of NEC913 patient sample: (A) Primary NEC tumor at the ampulla of Vater. Scale bar represents 3 mm. (B) H&E staining of primary NEC tumor. (C–I) Staining for Ki67, CgA, SYP, SSTR2, ASCL1, p53, and Rb. Scale bar represents 200 μm.

The patient giving rise to tumor NEC1452 presented with a mediastinal and supraclavicular masses, as well as liver, pancreatic, retroperitoneal, and rectal lesions that were viable cells despite the fact that both patients had been treated with carboplatin/etoposide chemotherapy, suggesting that these NEC cells are resistant to the treatment. Both samples can be robustly passaged as PDXs and tumor cells from these xenografts formed as suspension cultures, or as spheroids embedded in extracellular matrix. By immunofluorescent staining, we confirmed the expression of CgA and SYP in both cell lines and SSTR2 in only the PDX line (Figure 3A). To further characterize these novel cell lines for additional neuroendocrine cancer markers, gene expression analysis using a quantitative RT-PCR was performed (Figure 3B). In comparison and the established BON cell line, the tumor to have significantly increased *SSTR2* and *NEUROD1* expression (Figure 3B). In NEC1452, we determined that the increased *SSTR2* expression (Supplemental Figure 3C) and exome sequencing confirmed *TP53* stopgain mutations and *RB1* frameshift mutation (Figure 1C, Supplementary Table S2).

Tumor cells isolated from the cryopreserved NEC913 and NEC1452 tumors yielded viable cells despite the fact that both patients had been treated with carboplatin/etoposide chemotherapy, suggesting that these NEC cells are resistant to the treatment. Both sam-



Further characterization of the NEC913 PDX tumor by IHC showed low expression of p53, which could be due to the specificity of the antibody for the truncated form of p53, and no expression of Rb (Figure 5A), which is similar to the expression pattern observed in original patient tumor IHC analyses (Figure 2H,I). The expression of ASCL1 was lower in the NEC913 PDX tumor (Figure 5A) when compared to the original tumor (Figure 2G). In addition, the NEC913 PDX tumor expressed CXCR4 (Figure 5A). The CXCR4 expression

in the NEC913 tumor but not NEC1452 (Figure 4C), corroborating the *in vivo* imaging results (Figure 4B). Qualitative assessment of SSTR2-mediated uptake was supported by semi-quantitative image analyses, which revealed an approximately twofold increase and a threefold increase in fluorescent signal intensity of NEC913 compared to NEC1452 tumors in the *in vivo* and *ex vivo* experiments, respectively (Figure 4D,E).

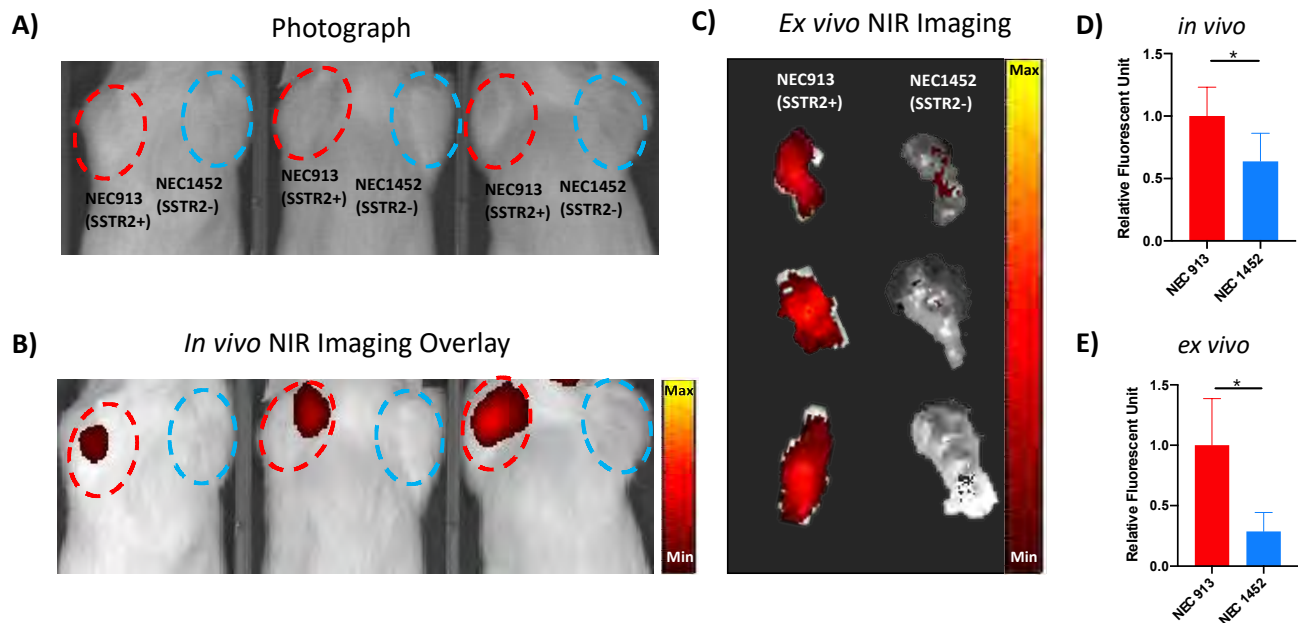


Figure 4. Application of NEC PDX models for SSTR2-targeted imaging: (A) Representative photograph of mice harboring NEC913 and NEC1452 tumors ranging from 1.0 to 2.0 cm in diameter 5 weeks post tumor cell injections from an $n = 5$ mice experiment. (B) Representative Near Infrared (NIR) fluorescence imaging of mice harboring NEC913 and NEC1452 tumors using exposure time of 2 s and excitation and emission wavelengths set at 780 nm. (C) Representative *ex vivo* NIR fluorescence imaging of dissected NEC913 and NEC1452 tumors from an $n = 5$ mice experiment. (D,E) Quantifications of *in vivo* and *ex vivo* NIR fluorescence signal in NEC913 and NEC1452 tumors. T-tests were performed using Prism GraphPad. $p < 0.05$ was depicted with *.

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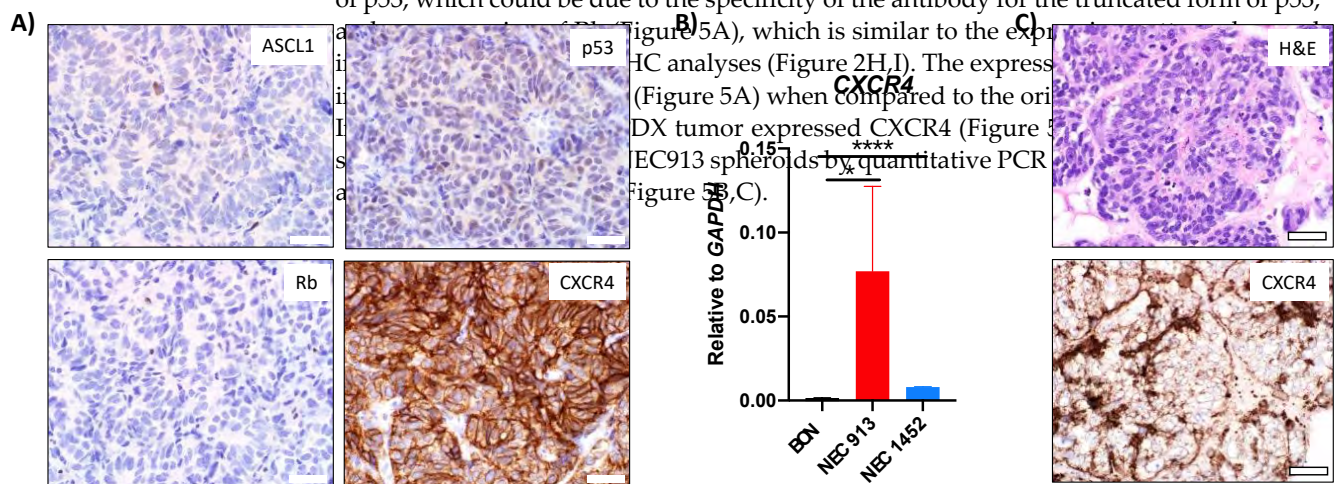


Figure 5. Additional characterization of NEC913 PDX model: (A) IHC analyses of NEC913 PDX tumors for NEC markers such as Rb, p53, ASCL1, and CXCR4. (B) Comparison of the gene expression levels of CXCR4 in BON, NEC913, and NEC1452 cells by quantitative PCR normalized to the control gene GAPDH to determine the relative fold change. Statistical analyses of gene expression changes were performed using T-tests in Prism GraphPad. $p < 0.05$ was depicted with *.

4. Discussion

GEP NETs and NECs are rare cancers with few *in vitro* and *in vivo* models available for therapeutic testing [31]. GEP NET cell lines and spheroids are difficult to propagate as they take approximately 2 to 3 weeks to divide [19,22,24,32]. A limited number of GEP NEC cell and organoid lines have been recently published, but distribution remains lim-

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Because well-differentiated NETs grow slowly, attempts to propagate them long term have not generally been successful. We have shown that these can be grown in culture for up to 9 months, remained well-differentiated, and expressed NET markers such as synaptophysin, chromogranin, and SSTR2 [19]. However, after about 2 weeks, growth remains fairly constant at a low level. In this study, we were unsuccessful at establishing all four frozen NET samples in immunocompromised mice at 3 months post injection (Table 2). Considering that a majority of GEP NETs are generally slow-growing Grade 2 tumors with a Ki-67 index less than 20%, it is possible that a period longer than 3 months is required for tumor formation. Interestingly, even the SBNET sample SBNET1063 (Table 2), which was a WHO Grade 3 tumor, did not generate a subcutaneous tumor, suggesting that a xenograft model may not be ideal for NET PDX development. There have been a few NET cell lines described such as P-ST5, GOT1, NT3 [24,25,27], and a well-differentiated PNET PDX model reported by Chamberlain et al. [33]. Although these appear to be promising GEP NET models, they have not been widely distributed to many investigators. The most likely explanation for this is that they cannot be grown in a large enough quantity to share, or that over time, the cells that do survive could potentially dedifferentiate into NECs.

We established two new NEC cell lines that grow well in culture and can be passaged through several generations of immunocompromised mice. These lines significantly expand the options for study of NECs and were derived from different sites. The NEC913 was derived from an ampullary NEC and NEC1452 from a retroperitoneal node from a patient suspected to have a rectal NEC. Both NEC913 and NEC1452 PDX tumors contain *TP53* and *RB1* mutations that are commonly reported in GEP NECs [6,34,35]. Both lines expressed synaptophysin, chromogranin A, and had Ki-67 of >80%. The NEC913 line expressed SSTR2 and CXCR4 while NEC1452 did not. Tumors from these PDX models can robustly be passaged in more than six generations of mice with a 100% rate of tumor formation. NEC cells from both PDX models can be maintained in culture in media supplemented with insulin and nicotinamide as suspension cultures or spheroids embedded in extracellular matrix.

Several lung NEC lines [36,37] and colon NEC lines such as the HROC47, SS-2, and NEC-DUE1 and 2 [36,38–40] have been established, but few pancreas and rectal NEC lines have been reported [39]. Considering the rarity of GEP NEC PDX and cell models, the NEC913 and NEC1452 PDX models developed in this study could be tremendously valuable for a variety of pre-clinical experiments. Here, we showed that the NEC913 line can be useful in confirming the target specificity of NIR-TOC with fluorescence imaging in a clinically relevant model, suggesting high translational potential (Figure 4). NEC913 PDX model maintains high SSTR2 (Figure 1B) and CXCR4 (Figure 5A,B) expression after six generations of passages. The NEC913 cells grew as spheroids in suspension culture or embedded in extracellular matrix, recapitulated characteristics of the PDX tumor, and stained positive for CXCR4 (Figure 5). This cell line also shows great promise as a potential tool for future investigations involving PRRT including testing of combination therapies or highly potent alpha-emitter PRRT [41]. CXCR4 is also emerging as a valuable target in atypical lung carcinoid and small cell lung cancer, and can be targeted with the radiolabeled ligand Pentixafor and Pentixather [42,43]. Thus, NEC913 could also serve as an effective pre-clinical model for PRRT directed at CXCR4. The development and characterization of these NEC913 and NEC452 PDX lines represent valuable new tools that could overcome

the significant limitations of existing preclinical models in NEN research and accelerate the process of drug discovery.

5. Conclusions

Two novel NEC PDX models (NEC913 and NEC1452) were established from cryopreserved patient tumors. Tumors from these PDX models can robustly be passaged in immunocompromised mice. The NEC913 PDX model maintained SSTR2 expression after six generations of passages and can be visualized using NIR-TOC peptide. In addition, the NEC913 PDX model expressed high level of CXCR4, which makes it potentially useful for CXCR4-targeted theranostics. NEC cells from both PDX models can be maintained in culture in media supplemented with insulin and nicotinamide. Considering the rarity of GEP NEC PDX and cell lines, the NEC913 and NEC1452 PDX models are valuable pre-clinical models for peptide imaging, drug testing experiments, and studying GEP NEC tumor biology.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers14081910/s1>, Table S1: List of curated mutations identified in NEC913 PDX tumors by exome sequencing. Table S2: List of curated mutations identified in NEC1452 PDX tumors by exome sequencing. Table S3: Short Tandem Repeats analyses of NEC913 PDX tumors and patient periphery blood mononuclear cells. Table S4: Short Tandem Repeats analyses of NEC1452 PDX tumors.

Author Contributions: Conceptualization, P.H.E., J.R.H., R.C.F., C.H.F.C., M.S.O., T.O., A.A. and R.G.; Methodology, C.G.T., L.C.B., J.L.M., E.A., S.A., S.C.G., S.H.V., G.L., C.H.F.C., G.V.B., M.M., R.L. and S.A.W.; Software, P.F.C., S.A., S.A.W. and T.J.W.; Formal Analysis, P.H.E., A.M.B., J.R.H., R.C.F., S.A. and P.F.C.; Resources, R.C.F., A.M.B. and A.A.; Data Curation, P.H.E.; Funding acquisition, P.H.E., R.C.F., M.S.O., R.G., J.R.H., A.M.B. and T.O.; Original Draft Preparation, P.H.E.; Writing—Review and Editing, P.H.E., J.R.H., C.G.T., L.C.B., C.H.F.C., T.J.W., S.A.W., S.A., A.A. and J.L.M.; Supervision, P.H.E.; Project Administration, P.H.E., J.R.H., R.C.F. and J.L.M. All authors have read and agreed to the published version of the manuscript.

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The impacts of exosomes on bone metastatic progression and their potential clinical utility

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ABSTRACT

Bone is one of the most common sites of cancer metastasis. Once cancer metastasizes to the bone, the mortality rate of cancer patients dramatically increases. Although the exact mechanisms for this observation remain elusive, recent studies have revealed that the complex crosstalk between bone marrow microenvironment and bone metastatic cancer cells is responsible for the induction of treatment resistance. Consequently, bone metastasis is currently considered incurable. Bone metastasis not only impairs the patients' survival, but also negatively affects their quality of life by causing painful complications. It has recently been implicated the regulatory role of exosomes in cancer development and/or progression as a delivery biomaterial between cancer cells and tumor microenvironment. However, little is known as to how exosomes contribute to the progression of bone metastasis by impact on the crosstalk between bone metastatic cancer cells and bone marrow microenvironment. Here, we highlighted the emerging roles of cancer-derived exosomes in (i) the process of dissemination and bone colonization of bone metastatic cancer cells, (ii) the enhancement of crosstalk between bone marrow microenvironment and bone metastatic cancer cells, (iii) the development of its resultant painful complications, and (iv) the clinical applications of exosomes in the bone metastatic setting.

1. Introduction

Bone metastasis is devastating for cancer patients as in the United States alone, about 350,000 of them die each year from this disease progression (Huang et al., 2020). Additionally, in prostate and breast cancer, which are both known to metastasize to the bone, the median survival time of patients with bone metastases is 25 and 27 months, respectively, with current cancer patients considered incurable when they develop bone metastasis (Huang et al., 2020). However, bone metastases are not only incurable; they also compromise patients' quality of life due to comorbidities known as skeletal related events (SRE) (Orcajo-Rincon et al., 2022; Virk and Lieberman, 2007). These SREs - including severe pain, hypercalcemia, neurological deficits, pathological bone fractures, and spinal cord compression - lead to reduced activity and decreased quality of life (Mercadante, 1997; Zheng et al., 2022; So et al., 2012). Therefore, to immediately reduce the mortality and morbidity of cancer patients as well as improve patient well-being, treatment strategies to abate bone metastases are crucial.

Treatment strategies are relatively unique to bone metastases when compared to treatments for other metastases. In most cases of cancer

metastasis, the treatment strategies for both the primary and secondary tumors are similar, where treatment methods function by either targeting tumors themselves or stimulating the immune system surrounding the tumors. For bone metastases, however, the treatment strategies focus on the function of the bone (an organ that continuously modifies itself throughout life by coupling osteoclast and osteoblast activity in a process called bone remodeling) (Hadjidakis and Androulakis, 2006). It has been established that the cells involved in bone remodeling (e.g., osteoclasts, osteoblasts, and osteocytes) interact with metastatic cancer cells in the bone microenvironment. Here, the crosstalk between metastatic cells and bone-related cells act to further stimulate bone metastatic progression, known as "a vicious cycle of bone metastasis" (Guise, 2002; Cook et al., 2014). It is therefore logical for therapeutic strategies to target the interactions of bone remodeling to interfere with this vicious cycle.

In the clinic, drugs that decrease osteoclastic activity are often given as treatments to help combat the vicious cycle of bone metastases. The most commonly prescribed are bisphosphonates and denosumab, a human monoclonal anti-receptor activator of nuclear factor κ B ligand (RANKL) antibody (Stopeck et al., 2010; Fizazi et al., 2011). These

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treatments have some success reducing some of the painful complications of bone metastases, but unfortunately, they ultimately fail to improve overall patient survival (Stopeck et al., 2010; Fizazi et al., 2011). In terms of targeting hydroxyapatite or osteoblastic bone metastatic lesions, clinical trials indicate that an alpha particle-emitting radiopharmaceutical, radium-223 dichloride ($^{223}\text{RaCl}_2$) can improve overall survival of prostate cancer patients with bone metastases but only by a few months (mean = 3 months) (Parker et al., 2013). Notably, to date, this is the only bone-targeted treatment option known to increase the survival time of patients with bone metastatic cancer. While several combinations of systemic treatments (e.g. hormone therapies, chemotherapies) are known to improve the overall survival of patients, they too only increase survival by a few months in patients with bone metastasis (McCain, 2014). Consequently, extirpating bone metastasis represents one of the greatest challenges of modern health care.

One of the reasons for challenges in developing treatments for bone metastasis is due in part to the intricate interactions between bone metastatic cancer cells and the bone marrow microenvironment, which contain a multitude of cell types, including cells of hematopoietic and mesenchymal origin, with diverging temporal dynamics (Kawai et al., 2018; Satcher et al., 2022). These interactions enable the vicious cycle of bone metastasis, as previously mentioned, where bone metastatic cancer cells stimulate osteolytic and/or osteoblastic activities of bone cells to provide space or growth factors for bone metastatic cancer cells to grow within the marrow (Guisse, 2002). Additionally, the bone marrow microenvironment enhances bone metastatic progression through the high blood flow in the red marrow as well as the production of angiogenic factors, which provide adequate nutrient to support the survival of bone metastatic cancer cells (Suva et al., 2011). These add layers of complexity to understanding the mechanisms of bone metastasis creating a critical need to integrate our current understanding of cancer metastasis with emerging concepts in bone marrow microenvironment to advance our understanding of bone metastasis. Through this integration of knowledge, the overall goal is improving treatment strategies and clinical outcomes, while reducing the financial burden experienced by patients and their families.

Exosome-based cell-cell communication in the tumor microenvironment has recently been appreciated (Li et al., 2019). Exosomes are a type of extracellular vesicles surrounded by a lipid bilayer that carry proteins, DNA, RNA, and lipids that mediate cell communication

(Gurunathan et al., 2019). They range in size from 40 to 120 nm in diameter and are derived from the endolysosomal pathway (El Andaloussi et al., 2013). Indeed, in the tumor microenvironment, exosomes derived from cancer-associated fibroblasts provide metabolites and tricarboxylic acid cycle (TCA) intermediates that can be utilized for metabolism to enhance cancer cell growth under nutrient stressed conditions (Zhao et al., 2016). Additionally, cancer-derived exosomes contribute to priming the secondary site for cancer cells to metastasize, referred to as “pre-metastatic niche formation” (Peinado et al., 2017; Yuan et al., 2021). It has been proposed that exosomes are also involved in the metastatic process of cancer cells by stimulating (i) extravasation of circulating tumor cells (Raskov et al., 2020) and (ii) colonization of metastatic cancer cells through fusion between cancer cells and cells in the metastatic sites (Hoshino et al., 2015). Particularly, prostate cancer-derived extracellular vesicles educate osteoblasts in order to create a favorable environment for further dissemination of prostate cancer to the bone (Furesi et al., 2021). Furthermore, microRNA (miRNA) in cancer-derived exosomes is known to induce osteoblastic differentiation, resulting in the release of growth factors, which initiate the vicious cycle and subsequently enhance the bone metastatic progression (Furesi et al., 2021; Masaoutis and Theocharis, 2019) (Table 1). These findings suggest the exosomes serve as one of the key regulators in the development of bone metastasis.

While the impact of exosomes on the development of bone metastasis has recently been revealed, complete mechanisms whereby exosomes contribute to the formation of the metastatic niche in the bone and how different exosomal-cargo (miRNA, metabolites) impacts bone-specific metastasis still remain to be elucidated. In this review, we therefore will (i) describe the roles of cancer-derived exosomes in bone metastatic progression by focusing on the cancer/bone marrow microenvironment interactions, (ii) introduce the concept that exosomes are involved in the development of SRE by paying special attention to cancer-induced bone pain, and (iii) discuss the potential impacts of exosome functions on the development of therapies and diagnostic tools for bone metastasis.

2. The roles of exosomes in cancer dissemination to bone and colonization in bone

The beginning of the exosome journey starts within cells. Initially, a portion of plasma membrane invaginates carrying cargo into the

Table 1
Exosomal miRNAs and their potential effects on bone metastasis.

Exosomal miRNA	Cancer type	Cell of origin	Recipient cell	Effect	Reference
miR-19a	Breast	Cancer cells	Osteoclasts	Promotes osteoclastogenesis	(Wu et al., 2021)
miR-21	Breast, Lung	Cancer cells	Osteoclasts	Promotes osteoclast differentiation	(Yuan et al., 2021) (Xu et al., 2018)
miR-23b	Breast	Bone marrow-derived mesenchymal stem cells	Cancer cells	Induces tumor dormancy	(Ono et al., 2014)
miR-23c	Prostate	Cancer cells	Human umbilical vein endothelial cells	Induces tumor dormancy by suppressing CXCL12 expression in human umbilical vein endothelial cells	(Sharma et al., 2021)
miR-26a-5p, miR-27a-3p, and miR-30e-5p	Prostate	Cancer cells	Osteoblasts	Inhibits osteoblast differentiation	(Furesi et al., 2022)
miR-92a-1-5p	Prostate	Cancer cells	Osteoclasts, Osteoblasts	Promotes osteoclast differentiation, inhibits osteoblast differentiation	(Yu et al., 2021)
miR-141-3p	Prostate	Cancer cells	Osteoblasts	Enhances osteoblastic metastatic lesions	(Ye et al., 2017)
miR-192	Lung	Cancer cells	Human umbilical vein endothelial cells	Inhibits osteoclastogenesis by impairing tumor-induced angiogenesis	(Valencia et al., 2014)
miR-214-3p	Breast	Cancer cells	Osteoclasts	Promotes osteoclast differentiation	(Liu et al., 2017)
miR-222/223	Breast	Bone marrow-derived mesenchymal stem cells	Cancer cells	Induces tumor dormancy	(Bliss et al., 2016)
miR-503-3p	Breast	Peripheral blood mononuclear cells	Osteoclasts	Inhibits osteoclast differentiation	(Chen et al., 2014; Zhao et al., 2017)
miR-940	Breast	Cancer cells	Bone marrow-derived mesenchymal stem cells	Promotes osteogenic differentiation of bone marrow-derived mesenchymal stem cells	(Hashimoto et al., 2018)
miR-1273 g-3p	Breast	Cancer cells	Bone marrow-derived mesenchymal stem cells	Promotes osteogenic differentiation of bone marrow-derived mesenchymal stem cells	(Sun et al., 2022)

cytoplasm and traffics to the early endosome, where the invaginated plasma membrane's cargo is sorted (Gurung et al., 2021). Thereafter, the early endosome matures into a multivesicular body, or late endosome, through inward budding of its membrane, resulting in the formation of intraluminal vesicles (Zhang et al., 2019b; Alenquer and Amorim, 2015). During this maturation process within the multivesicular body, intraluminal vesicles are known to obtain several cargos (Gurung et al., 2021). Eventually, these multivesicular bodies fuse with the plasma membrane, leading to the secretion of their intraluminal vesicles, which are known as extracellular vesicles, or exosomes, once they are released from the cell (Théry et al., 2009; Zhang et al., 2019b). After being released into the extracellular space, exosomes and their cargo can reach cells in proximal sites by direct interaction, as well as cells in distant sites through circulation (Giuszko et al., 2019). The nature of the encapsulated cargos carried by exosomes is thought to be strongly influenced by the parental cell. Indeed, it has been demonstrated that exosomal miRNA profiles reflect their cells of origin (Corcoran et al., 2014; Wang and Gires, 2019).

The way in which exosomal contents are taken up by target cells is through either (i) direct fusion with the membrane; (ii) receptor-ligand interactions; or (iii) endocytosis (Zhang et al., 2019b). Furthermore, this uptake of exosomal cargo can transfer phenotypic traits of parental cells to recipient cells (Corcoran et al., 2014; Wang and Gires, 2019). For example, normal human bronchial epithelial cells (HBECS) acquired resistance to the chemotherapeutic agent gemcitabine *in vitro*, when they were exposed to exosomes derived from HBECS that were transformed by introducing three common non-small cell lung cancer oncogenic mutations (Lobb et al., 2017). Additionally, exosomes can change the phenotype of recipient cells with their cargo. It has been demonstrated that macrophage migration inhibitory factor within pancreatic ductal adenocarcinomas-derived exosomes induced pre-metastatic niche formation in the liver by (i) inducing transforming growth factor β (TGF- β) production in Kupffer cells, (ii) up-regulating fibronectin expression in hepatic cells, and (iii) recruiting bone marrow-derived macrophages into the liver (Costa-Silva et al., 2015). While this finding suggests that exosomes can be involved in the dissemination process of cancer by influencing the cells in the secondary sites, less is known as to how exosomes contribute to bone metastatic formation.

It has been suggested that bone metastatic cancer cells home to the bone marrow through the C-X-C Motif Chemokine Receptor 4 (CXCR4)/C-X-C Motif Chemokine Ligand 12 (CXCL12) axis (Taichman et al., 2002; Müller et al., 2001). Briefly, CXCR4 (a receptor for CXCL12)-expressing bone metastatic cancer cells migrate towards CXCL12 expressed by cells in the bone marrow (e.g. endothelial cells, stromal cells, osteoblasts) (Wang et al., 2006). Similar to the pre-metastatic niche formation in pancreatic cancer liver metastasis, exosomes also contribute to the priming of the bone marrow microenvironment. In a rodent model, prostate cancer-derived exosomes obtained from prostate cancer patients' serum up-regulated CXCL12 expression in the bone marrow stromal cells by transferring pyruvate kinase M2, resulting in (i) the higher incidence of bone metastasis than lung and kidney metastases and (ii) subsequent bone metastatic progression (Dai et al., 2019). Additionally, an *in vitro* study demonstrated that osteotropic human melanoma LCP-Mel-derived exosomes reprogramed non-osteotropic human melanoma cells (SK-MEL28, WM-266) to acquire the osteotropic ability towards CXCL12-secreting bone fragments by up-regulating their CXCR4 expression (Mannavola et al., 2019). Moreover, miR-192-enriched exosomes derived from human lung cancer A549 cells impaired their bone colonization by preventing angiogenesis in the marrow through suppression of the pro-angiogenic factors CXCL1 and interleukin (IL)-8 (Valencia et al., 2014). These findings suggest that bone metastatic cancer cell-derived exosomes control dissemination of cancer cells to bone and their colonization in bone.

3. The impacts of exosomes on the phenotype of bone metastatic lesions

Under normal physiological conditions, the bone resorption mediated by osteoclasts and the bone formation mediated by osteoblasts are balanced (Li and Wang, 2021), leading to proper bone remodeling. However, when cancer metastasizes to the bone, this well-balanced homeostasis of the bone microenvironment loses this equilibrium becoming dysregulated, resulting in bone metastatic growth. The phenotype of bone metastatic lesions is determined by how bone metastatic cancer cells activate cells involved in bone remodeling, including osteoclasts and osteoblasts. When bone metastatic cancer cells dominantly stimulate the activities of osteoclasts, the lesions of bone metastases become osteolytic, whereas those of bone metastases result in osteogenesis when bone metastatic cancer cells interact more with osteoblasts.

As stated above, the interaction among the bone metastatic cancer cells and cells involved in the bone remodeling is called a vicious cycle of bone metastasis. It has been suggested that this vicious cycle is controlled by several growth factors and/or cytokines [e.g. bone matrix (degraded by osteoclasts)-derived transforming growth factor beta (TGF- β) and endothelin-1 (Furesi et al., 2021; Esposito et al., 2018); osteoblasts-derived RANKL (Esposito et al., 2018); and bone metastatic cancer cells-derived parathyroid hormone-related protein (PTHrP) (Guise et al., 1996)]. However, recent work has demonstrated that exosome-derived bone metastatic cancer cells add another level of complexity in the vicious cycle of bone metastasis by affecting the function and activity of bone-related cells.

Indeed, prostate cancer-derived exosomes induced survival, proliferation, and mineralization of murine pre-osteoblastic cell line MC3T3-E1 cells through the mitogen activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) pathway *in vitro* (Borel et al., 2020; Karagiannis et al., 2009). Along with this concept, in a rodent model, greater osteoblastic bone metastatic burden and lower median survival of the animals inoculated with prostate cancer-derived exosomes over-expressed miR-141-3p were observed, compared to those of animals inoculated with miR-141-3p intact prostate cancer-derived exosomes (Ye et al., 2017). In another rodent study, breast cancer-derived exosomes (which contains long noncoding RNA SNHG3) induced osteolytic bone metastatic lesions by down-regulating exosomal miR-1273 g-3p (Sun et al., 2022). This osteolytic bone metastasis was reversed by differentiating bone marrow stromal cells into osteoblasts through the upregulation of exosomal miR-1273 g-3p when SNHG3 was down-regulated in breast cancer (Sun et al., 2022). Interestingly, when a triple negative breast cancer cell line MDA-MB-231 cells, known to develop osteolytic bone metastatic lesions, were transfected with hsa-miR-940 (known to induce osteoblastic differentiation), exosomal hsa-miR-940 induced the development of osteoblastic bone metastatic lesions in the hsa-miR-940-over-expressing MDA-MB-231 cells-bearing animals (Hashimoto et al., 2018).

Alternatively, exosomes have been shown to enhance or reduce osteoclast activity. Non-small cell lung cancer-derived exosomes that carry amphiregulin (a ligand for epidermal growth factor receptor, EGFR) induce the differentiation of pre-osteoclasts to osteoclasts through EGFR expressed by pre-osteoclasts (Taverna et al., 2017). Metastatic breast cancer cells-derived exosomal miR-21 significantly increased bone metastatic burden at the hind-limbs of cancer-bearing rodents by enhancing osteoclastogenesis through downregulation of programmed cell death 4 (PDCD4), known to inhibit osteoclast differentiation and functions (Yuan et al., 2021). Similarly, exosomal miR-21 derived from human lung adenocarcinoma cell line A549 cells promoted osteoclastic differentiation of murine primary bone marrow monocytes into tartrate-resistant acid phosphatase (TRAP, known to reflect osteoclastic differentiation) positive osteoclasts, by downregulating PDCD4 (Xu et al., 2018). In a rodent study, estrogen receptor (ER) positive breast cancer cells expressing integrin-binding sialoprotein (IBSP)

recruited osteoclast precursors to the bone marrow (Wu et al., 2021). In turn, these osteoclast precursors activated nuclear factor kappa B (NF- κ B) and protein kinase B (AKT) pathways due to the suppression of PTEN expression by exosomal miR-19a-derived from metastatic breast cancer, which ultimately resulted in bone metastatic progression (Wu et al., 2021). Moreover, MDA-MB-231 cells-derived exosomal miR-214-3p induced increased serum bone resorption marker, type I collagen cross-linked C-telopeptide (CTX-1) levels and increased osteoclast numbers in the bones in the MDA-MB-231-bearing mice, by directly downregulating TNF receptor-associated factor 3 [TRAF3, known to limit RNKL-inducing osteoclastogenesis (Boyce et al., 2018)] levels in osteoclasts (Liu et al., 2017).

Intiguently, exosomal miR-92a-1-5p derived from the human prostate cancer cell line MDA PCa 2b cells, known to induce osteoblastic bone metastatic lesions, increased mRNA expression of the osteoclast markers, cathepsin K (CTSK) and RANK, and TRAP activity in murine monocyte/macrophage cell line RAW 264.7 cells *in vitro* (Yu et al., 2021). As expected, this osteoclastic differentiation mediated by exosomal miR-92a-1-5p was reversed with anti-miR-92a-1-5p (Yu et al., 2021). Exosomal miR-92a-1-5p also decreased a mineralization marker Alizarin Red S in MC3T3-E1 cells *in vitro* and degraded bone extracellular matrix of MDA PCa 2b-bearing mice by down-regulation of collagen type I alpha 1 (COL1A1, type I collagen subunit which is a major component of bone extracellular matrix) *in vivo* (Yu et al., 2021). Similarly, exosomes derived from murine prostate cancer cell line RM1-BM cells, diminished expression of osteoblastic differentiation markers, bone morphogenetic protein 2 (BMP2) and RUNX family transcription factor 2 (RUNX2) in osteoprogenitor cells *in vitro* by releasing miR-26a-5p, miR-27a-3p, and miR-30e-5p (Furesi et al., 2022). Conversely, another study demonstrated that prostate cancer-derived exosomes decreased the expression of differentiation markers of monocytic osteoclast precursors (Karlsson et al., 2016).

Collectively, these findings suggest that cancer-derived exosomes are involved in the determination of the phenotype of the bone metastatic lesions, although how exosomal miRNA influence on the phenotype of the bone metastatic lesions are still controversial.

4. Contribution of exosomes to the regulation of tumor cell dormancy in the bone

One of the major challenges in the treatment of bone metastasis is the development of tumor dormancy in the bone. It has been demonstrated that bone metastatic cancer cells utilize the bone marrow microenvironment to acquire a dormant state (Shiozawa et al., 2010). Bone metastatic tumor dormancy is defined as a lag occurring between early cancer bone colonization and full-blown bone metastatic progression (Senft and Ronai, 2016). During this period, bone metastatic cancer cells are thought to arrest in a quiescent-like state, resulting in escaping from the cytotoxic treatments, including chemotherapy and radiation, that mainly target proliferating cells (Attaran and Bissell, 2022). These escaped cells remain undetectable for years or decades, and then eventually re-grow and become incurable.

Recent studies suggest that exosomes are involved in the regulation of tumor dormancy and have a mixed role in this process by promoting or suppressing quiescence. For example, while exosomes derived from bone marrow-derived mesenchymal stem cells induced cell cycle arrest in breast cancer cells (Bliss et al., 2016), bone marrow stromal cell-derived M1 macrophage-secreted and cancer associated fibroblasts-derived exosomes helped breast cancer cells exit from a dormant state (Walker et al., 2019; Sansone et al., 2017). In the contexts of bone metastasis, regucalcin (RGN)-overexpressing human prostate cancer cell line C4-2B and PC-3 cells showed smaller bone metastatic burden than that mediated by control cells through tumor dormancy *in vivo* (Sharma et al., 2021). This tumor dormancy was directly induced by prostate cancer cell-derived exosomes carrying miR-23c and indirectly by miR-23c-induced neoangiogenesis suppression (Sharma et al., 2021). On the

other hand, exosomes-derived bone marrow mesenchymal stem cells, carrying miR-23b induced dormancy of bone tropic MDA-MB-231 cells (BM2 cells), by downregulating myristoylated alanine rich C-kinase substrate (MARCKS, known to promote cell cycling and motility, in the bone of BM2-bearing mice (Ono et al., 2014). Although the roles of exosomes have been appreciated, further studies are clearly warranted to more detailed and precise mechanisms underlying the regulation of tumor dormancy in the bone by exosomes.

5. A potential roles of exosomes in the development of cancer-induced bone pain

Exosomes may not only be involved in the establishment of cancer bone metastasis but also regulate the development of its associated complications, especially cancer-induced bone pain. An estimated 60–84 % of cancer patients with bone metastasis experience a varying degree of bone pain (Zajczkowska et al., 2019), with up to 50 % of them describing the management of pain by present treatments as poor (Mantyh, 2014). Therefore, understanding the molecular underpinnings of cancer-induced bone pain would be of great value to develop therapeutics that are more effective and safer; subsequently, patients' quality of life can be improved. One proposed mechanism of the induction of cancer-induced bone pain is through the tumor microenvironment's harmful stimuli that causes excitation, sensitization, and electrophysiological changes of peripheral primary sensory nerves (Falk and Dickenson, 2014; Mantyh, 2014; Ivanusic, 2017; Yoneda et al., 2021). Interestingly, recent studies demonstrated that cancer-derived exosomes are also involved in this nociception process.

Indeed, a recent study demonstrated that the human lung cancer cell line A549-derived exosomal miRNA, let-7d-5p, induced cancer-induced bone pain by inhibiting the μ -opioid receptor 1 expression in dorsal root ganglia neurons, and the let-7d-5p antagomir inhibited spontaneous flinching of mice inoculated with A549 cells directly into their femur (Li et al., 2021). Additionally, it has been demonstrated that M2 macrophage-derived extracellular vesicles, carrying miR-216A, enhanced antinociception in the mice inoculated with rat breast cancer cell line Walker 256 cells directly into their femur by suppressing high mobility group box 1 (HMGB1) expression, known to be involved in the nociception process in the chronic pains (Klune et al., 2008; Liu et al., 2020) and the toll-like receptor 4 (TLR4)/NF- κ B pathway, known to regulate the expression of a variety of inflammatory cytokine genes (Cai et al., 2021; Kawai and Akira, 2007), in their spinal cord (Cai et al., 2021). Although more comprehensive studies are necessary to fully elucidate the roles of exosomes in cancer-induced bone pain, modulation of proteins in the tumor microenvironment through exosomal-derived cargos can be a new therapeutic strategy to ameliorate cancer-induced bone pain.

6. Exosomes can be used as new theranostic biomaterials for bone metastasis

Since, as discussed above, recent evidence suggest that exosomes are involved in several aspects of the bone metastatic process, exosomes can be used for both treatment and diagnostic options for bone metastasis. Exosomes can (i) influence both cancer cells and cells in the bone microenvironment by delivering cargos and (ii) travel stably throughout the body. Therefore, potential theranostic applications using cancer-derived exosomes for bone metastasis are as follows: (i) a treatment target (Datta et al., 2017); (ii) a treatment derivative (Haney et al., 2020); and (iii) a circulating biomarker (Thakur et al., 2014) (Fig. 1).

6.1. Targeting exosome biogenesis and secretion

Because of the recent findings of exosomal roles in cancer development, targeting exosome biogenesis, secretion, and fusion have been proposed as ways to mitigate cancer progression and metastasis. Indeed,

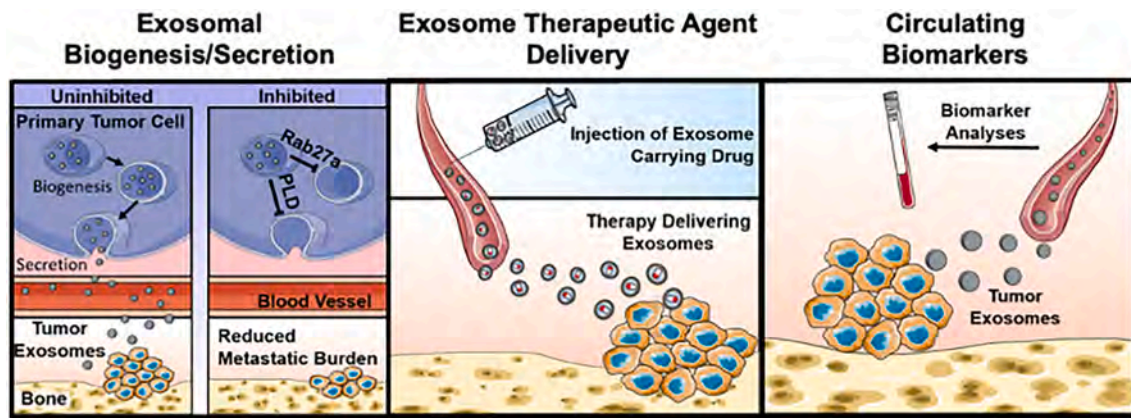


Fig. 1. Exosomes as theranostic options for bone metastasis.

Tumor exosomes derived from primary tumor cells through biogenesis and secretion are thought to contribute to bone metastatic progression in the marrow by traveling through circulation to the bone marrow microenvironment. Due to their roles in enhancing the bone metastatic process, exosomes may also prove to be a useful therapeutic target. As shown in the left panel, targeting exosomal biogenesis through inhibitors such as Ras-related protein (Rab27a) and exosomal secretion via inhibitors such as phospholipase D (PLD) may help inhibit tumor exosomes from carrying out their traditional role as a metastatic catalyst, resulting in a reduced metastatic burden. In the middle panel, the potential use of exosomes as drug delivery vehicles as a new treatment strategy for bone metastasis are described. Since systemic delivery of exosomes originating from cells in the bone marrow has been known to have a homing ability to the bone and cytotoxic agents can be loaded into exosomes, cytotoxic agent-loaded exosomes derived from bone marrow cells can be utilized to specifically target bone metastasis. Finally, the right panel illustrates the potential use of exosomes as circulating biomarkers or a liquid biopsy. Although a specific exosomal biomarker for bone metastasis has not yet been identified, the role of exosomes as biomarkers of other bone-centric diseases has already been demonstrated. Therefore, exosomes may aid in the diagnosis of bone metastasis as bone metastasis also results in bone destruction and an imbalance in osteoblast/osteoclast activity. Graphics adapted from Smart Servier Medical Art (<https://smart.servier.com/>).

in a melanoma mouse study, intratumoral injection of GW4869, an inhibitor of exosome secretion, significantly reduced melanoma (B16-BL6 cells) tumor growth (Matsumoto et al., 2017). Additionally, down-regulation of Ras-related protein Rab27A, known to regulate exosome biogenesis (Ostrowski et al., 2010), in the murine melanoma cell lines B16-F10 and SK-Mel-2 resulted in (i) a 50 % reduction in exosome secretion *in vitro*, and (ii) suppression of primary tumor growth and lung metastasis *in vivo* (Peinado et al., 2012). Moreover, a natural microbial metabolite, Manumycin-A, inhibited exosome biogenesis and secretion in the human castration-resistant prostate cancer cell line C4-2B cells by downregulating the Ras/Raf/ERK1/2 signaling pathway *in vitro* (Datta et al., 2017). Similarly, an inhibitor for a mediator of membrane fusion and exosome secretion, phospholipase D (PLD), decreased exosome secretion and downstream alkaline phosphatase (osteogenic differentiation marker) expression of C4-2B cells (Borel et al., 2020), suggesting that targeting and blocking the generation and release of exosomes, that are associated with contribution to metastasis, may a therapeutic strategy for precluding bone metastasis. More studies including *in vivo* as well as clinical experiments are clearly warranted to further demonstrate this notion.

6.2. Delivering therapeutic agents to bone marrow as a vehicle

Nanoparticles have been used as a drug delivery vehicle, and the drug delivery systems with nanoparticles have been somewhat successful. However, there have still been concerns about its heightened immune response, associated toxicity, and/or lack of therapeutic efficacy (Zolnik et al., 2010). Recent pre-clinical studies have revealed that exosomes can be an alternative drug delivery vehicle due in part to its ability to evade immune clearance (Kalluri and LeBleu, 2020). Interestingly, a more recent study demonstrated that the exosome delivery method can enhance treatment efficacy of chemotherapeutic agents, compared to the conventional delivery methods. In this study, murine triple negative breast cancer cell line T11 cells and human triple negative breast cancer cell line MDA-MB-231 cells were orthotopically inoculated into immunocompetent and immunocompromised mice, respectively, and these mice were treated with either chemotherapeutic agent (paclitaxel for MDA-MB-231 cells and doxycycline for T11 cells)

or exosomes loaded with corresponding chemotherapeutic agent (Haney et al., 2020). The sizes of the tumor treated with chemotherapeutic agent-loaded exosomes were significantly smaller than those treated with chemotherapeutic agent (Haney et al., 2020), suggesting that exosomes, which are derived from the cells that have tumor homing ability, can target cancer specifically even with systemic delivery. This results in enhancing efficacy and minimizing adverse effects of cytotoxic agents. Moreover, exosomes derived from bone marrow mesenchymal stem cells have an ability to preferentially homing to bone (Hu et al., 2021; Naseri et al., 2018). Taken together, these findings suggest that exosomes derived from bone marrow cells can be used to deliver cytotoxic agents straight to the bone that is notoriously known to be a hard-to-reach organ.

6.3. Serving as a circulating biomarkers

Another possible theranostic application of exosomes is through their use as a circulating biomarker (Thakur et al., 2014). The abundance of exosomes and their cargo present in most bodily fluids (e.g., plasma/serum, saliva, urine, fecal matter, etc.) makes them a compelling clinical tool due to the ease of sample collection (Barile and Vassalli, 2017; Zhang et al., 2019a). To date, detecting bone metastasis in its initial stages is extremely difficult. The first sign of bone metastasis in patients is often progressively worsening pain or other SREs, and imaging is then needed to confirm the presence of bone metastatic disease (Jayarangaiah et al., 2022). Since metastatic lesions are detected everywhere in the body when diagnosed and are thus difficult to treat, treatment options left for the patients with bone metastases are primarily palliative. Therefore, early detection of this fatal metastatic disease is much needed. Although plasma exosomal miRNA have been identified as a circulating biomarker for castration resistant prostate cancer (prostate cancer patients with castration resistant prostate cancer likely develop bone metastasis) (Huang et al., 2015), a specific exosomal biomarker for bone metastasis has not been identified yet.

However, exosomes have proven to serve as biomarkers in other bone-centric diseases, although these diagnostic measures have not yet been implemented clinically. In the serum of patients with osteoporosis, exosomal circular RNA Has_circ_0006859 was upregulated and thought

to function as a biomarker (Zhi et al., 2021). This circular RNA, through its role as a miRNA sponge, induces osteoporosis by promoting the differentiation of bone marrow mesenchymal stem cells into adipocytes and inhibit the differentiation of osteoblasts through the down-regulation rho associated coiled-coil containing protein kinase 1 (ROCK1) following the upregulation of miR-431-5p (Zhi et al., 2021). Further, exosomal miR-324-3p, miR-766-3p, miR1247-5p, miR-330-5p, and miR-3124-5p were all found to be differentially expressed in patients with osteoporosis ($n = 16$) when compared with a control group ($n = 18$) suggesting their potential role as biomarkers for this bone degenerative disease (Shi et al., 2022). Similarly, significantly lower exosomal miR-193b-3p, known to regulate chondrocyte formation and metabolism, was found in the plasma of patients with osteoarthritis, compared to that of healthy controls, highlighting its role as a potential diagnostic marker (Meng et al., 2018). Seeing as bone metastasis also results in bone destruction and an imbalance in osteoblast/osteoclast activity, these early exosomal diagnostic techniques used for other bone-tropic diseases (osteoarthritis and osteoporosis) can be useful in the early detection of bone metastasis and may offer opportunity to treat the disease before many of the complications can occur.

7. Conclusion and future perspectives

Our understanding of exosomes and their role in cancer has significantly developed over the past decades. While exosomes were originally thought to be essentially part of a garbage disposal system ridding the cell of waste (H Rashed et al., 2017), recently studies have demonstrated that exosomes play an important role in cancer progression, including the bone metastatic process. As discussed above, exosomes are involved

in (i) the induction of bone tropism in the cancer cells; (ii) the establishment of favorable environment for bone metastatic cancer cells; (iii) the growth of bone metastatic cancer cells; and (iv) the development of complications of bone metastasis (Fig. 2). Currently, when patients are diagnosed with bone metastasis, they are generally considered incurable (Weilbaeche et al., 2011), and the level of complexity of the bone microenvironment proves to be responsible for this clinical obstacle. Since exosomes have begun to be known as part of the regulation of the complex intercellular communication between bone metastatic cancer cells, the cells of hematopoietic origin (Niazi et al., 2020), and the cells of mesenchymal origin (Vakhshiteh et al., 2019), the understating of complete mechanisms whereby exosomes controls these cellular cross-talks may provide new insights into the pathophysiology and treatment of bone metastatic disease.

Despite notable scientific advancements in the early diagnosis and treatment of primary tumors, there is ultimately no curative treatment for bone metastatic disease. Following diagnosis with bone metastasis, patients and their families are subject to enormous financial, emotion, and physical stress as a result of the disease. Financial costs associated with bone metastasis proves to be problematic for the health care system as well as patients with bone metastasis have become increasingly expensive to care for in recent years. As previously mentioned, the current standards of care for treating bone metastasis includes using anti-resorptive bone-targeting agents (e.g. bisphosphonate, denosumab) and/or external beam radiotherapy. While the alpha-emitting pharmaceutical $^{223}\text{RaCl}_2$ can extend overall survival in bone metastatic prostate cancer patients, it is only by a few brief months. Unfortunately, the clinical benefits provided to patients by current therapies are modest. Furthermore, these treatments mainly target bone remodeling.

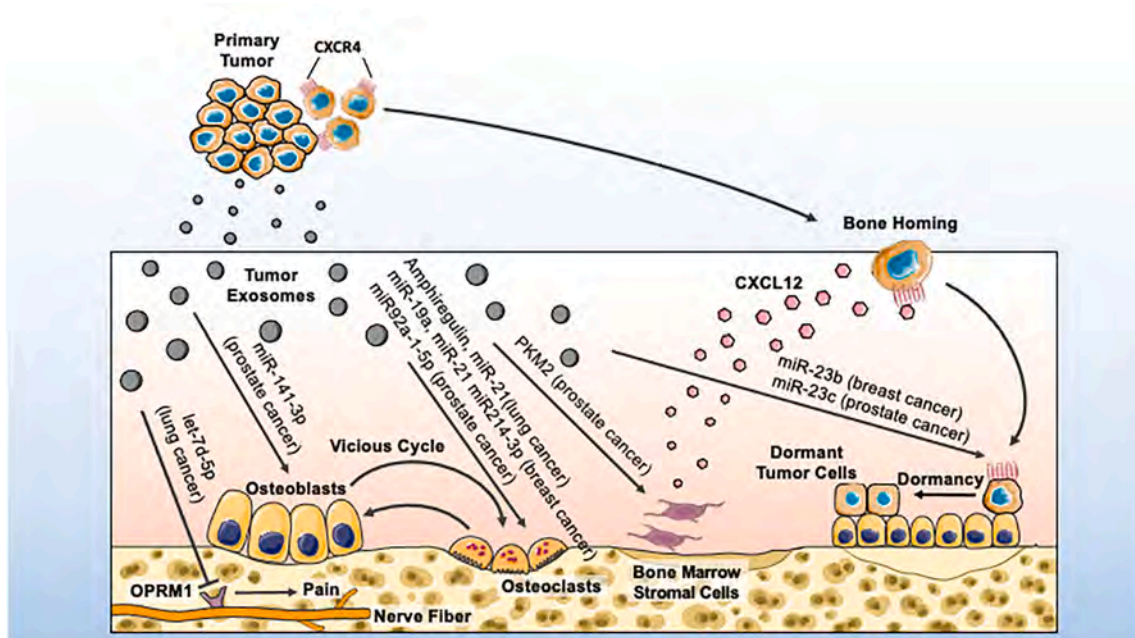


Fig. 2. The Proposed mechanisms where by exosomes contribute to bone metastatic progression.

Exosomes derived primary tumor induce bone tropism in the cancer cells by over-expressing C-X-C Motif Chemokine Receptor 4 (CXCR4), a chemokine receptor known to be associated with cell migration; and these tumor exosomes upregulate of C-X-C Motif Chemokine Ligand 12 (CXCL12) in bone marrow stromal cells through delivering pyruvate kinase M2 (PKM2) to bone marrow stromal cells (observed in prostate cancer). CXCR4-expressing cancer cells are known to migrate towards CXCL12 in the bone marrow as an osteotropic axis, resulting in higher incidence of bone metastasis. Following cancer cell homing to the bone, tumor dormancy is further promoted by exosomes carrying miR-23b derived from bone marrow mesenchymal stem cells (observed in breast cancer) or miR-23c derived from prostate cancer cells (observed in prostate cancer). Exosomal cargo also interacts directly with cells in the bone to create an environment favorable for metastasis. Exosomes carrying miR-141-3p act on osteoblasts contributing to greater osteogenesis and metastatic burden (observed in prostate cancer). Further, amphiregulin (lung cancer), miR-21 (lung cancer), miR-19-a (breast cancer), miR214-3p (breast cancer), or miR92a-1-5p (prostate cancer) from cancer-derived exosomes may act on osteoclasts by inducing their differentiation from pre-osteoclasts to osteoclasts. Following transport to the bone marrow microenvironment, exosomes have also been shown to promote cancer-induced bone pain through release of let-7d-5p, which inhibits the mu (μ) opioid receptor (OPRM1) in sensory nerves (observed in lung cancer). Graphics adapted from Smart Servier Medical Art (<https://smart.servier.com/> (web archive link,)),).

Therefore, new approaches that target factors other than bone remodeling are needed if eradicating bone metastasis is to be an achievable clinical goal. One of the difficulties in treating bone metastasis is that bone metastatic cancer cells acquire dormancy in the marrow. Since exosomes can transfer their cargo to the other cells, they can be used as a new therapeutic option to awake dormant bone metastatic cancer cells by delivering cargos that can reverse cell cycle arrest so that conventional cytotoxic agents, which target rapidly dividing cells, can attenuate these awakened bone metastatic cancer cells. Although further studies are undoubtedly necessary based on evidence supporting the roles of exosomes in bone metastatic development and progression as well as its resultant painful complications, translating therapies targeting and/or utilizing exosomes from bench to bedside may give rise to desperately needed palliative and curative treatment options for cancer patients with bone metastasis.

CRediT authorship contribution statement

J.O., K.F.C., G.D., and Y.S. wrote the manuscript. All authors reviewed and approved the final manuscript.

Declaration of competing interest

Yusuke Shiozawa has received research funding from TEVA Pharmaceuticals, but not relevant to this study. No conflict of interest exists for remaining authors.

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Immunotherapy as a potential treatment approach for currently incurable bone metastasis

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Abstract

Once cancer metastasizes to the bone, the prognosis of cancer patients becomes extremely poor. Unfortunately, the current most successful treatment for bone metastasis can extend their survival by only a few months. Although recent studies have revealed promising impacts of cancer immunotherapies, their treatment efficacy on bone metastatic diseases remains controversial. Therefore, in this review, we discussed (i) preclinical and clinical evidence of the immunotherapeutic strategies for cancer bone metastasis, mainly focusing on cell-based immunotherapy, cytokine-based immunotherapy, and immune checkpoint blockade, and (ii) current shortcomings of immunotherapy for bone metastasis and their potential future directions. Although the evidence on treatment efficacy and safety, as well as long-term effects, is limited, immunotherapies could induce partial or complete remissions in a few cancer patients with bone metastasis. However, there are still hurdles, such as the immunosuppressive nature of the bone marrow microenvironment and poor distribution of cell-based immunotherapies to bone, that need to be overcome to enhance the treatment efficacy of immunotherapies on bone metastasis. While it is apparent that further investigation is needed regarding immunotherapeutic treatment efficacy in patients with bone metastasis, this therapy may prove to be clinically novel in this subset of cancer patients.

Keywords Immunotherapy · Bone metastasis · Cell-based immunotherapies · Immune checkpoint blockade · Bone microenvironment

Introduction

Bone metastasis is one of the major causes of death among cancer patients. Indeed, once cancer cells metastasize to the bone, the 5-year survival rate of cancer patients decreases to 1–13% (e.g., 1–2%: lung cancer, rectum cancer, and cervix cancer; 3–10%: colon cancer and prostate cancer; and 13%: breast cancer) [1]. Additionally, the median survival time of patients with bone metastasis is approximately 12–33 months [1]. Although several combinations of systemic treatments (e.g., hormone therapies, chemotherapies, bone-targeted therapies, radiopharmaceuticals, etc.) are known to enhance the overall survival (OS) of patients with bone metastases, they only increase the OS by several

months [2]. Therefore, bone metastasis is still considered incurable, and there is a clear need to develop new treatment strategies to eradicate bone metastasis.

Recently, cancer immunotherapies have been viewed as the major breakthrough in cancer treatments. For example, pembrolizumab, a humanized antibody targeting programmed cell death protein 1 (PD-1) on T-cells, has significantly improved the OS of cancer patients, compared to those treated with vehicle [e.g., non-small cell lung cancer (NCT02578680, 22 vs. 10.6 months, $p < 0.0001$); head and neck squamous cell carcinoma (NCT02358031, 13 vs. 10.7 months, $p = 0.0067$); and urothelial carcinoma (NCT02256436, 10.3 vs. 7.4 months, $p = 0.004$)] [3]. Although immune cells are produced in the bone marrow and primarily reside there, the efficacy of cancer immunotherapies is not as high as that of the previously stated conventional treatment strategies for bone metastasis. It has recently been suggested that cancer immunotherapies can be potential alternative treatments for patients with bone metastasis as they can induce a complete remission in those patients [4]. However, little is known as to the mechanisms

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whereby bone metastatic cancer cells and/or the bone marrow microenvironment limit the efficacy of cancer immunotherapy on bone metastasis. We must know how to overcome the limitation of its efficacy before applying cancer immunotherapies for bone metastatic disease.

Therefore, in this review, we will (i) highlight the immunotherapeutic strategies for cancer bone metastasis from previous clinical trials or preclinical studies and (ii) discuss the limitations of these therapeutic strategies to direct future studies.

Immunotherapies for bone metastasis

Cancer immunotherapy is a treatment strategy that uses the patients' own immune system to eradicate cancer cells. There are several types of immunotherapies, including cell-based immunotherapy, antibody-based immunotherapy, cancer vaccine, oncolytic virus treatment, cytokine-based immunotherapy, and immune checkpoint blockade (ICB). Although many of these treatments have been tested as therapeutic strategies for bone metastasis, we will focus mainly on the effects of cell-based immunotherapy, cytokine-based immunotherapy, and ICB on bone metastasis in this review.

Cell-based immunotherapies for bone metastasis

Lymphokine-activated killer (LAK) cell therapy

Cell-based immunotherapies (or adoptive immunotherapies) are a type of therapy that uses patients' own immune cells (activated *ex vivo* and then reintroduced into the patient's body) to target cancer cells [5]. In the early development of cell-based immunotherapies, *ex vivo* activated peripheral blood leukocytes derived from cancer patients, referred to as lymphokine-activated killer (LAK) cells, were used. Interestingly, LAK cells were also used to treat bone metastatic patients. LAK cells have been tested to treat advanced renal cell carcinoma (RCC) patients with bone metastases in three clinical trials with greatly differing efficacy: (trial 1) some patients presented with partial remission (> 50% decrease in bone metastatic lesions), although recurrence occurred within 6 months of follow-up [6, 7]; (trial 2) IL-2-activated LAK cells induced a partial response in patients; however, they died 10 months and 28 months after LAK treatment [8]; and (trial 3) when IL-2-activated LAK cell therapy was given with interferon-alpha (IFN- α) [9], patients' bone metastatic lesions progressed [10]. Overall, LAK therapy presents limited efficacy on bone metastasis in addition to renal toxicity.

Autologous cytotoxic T cell therapy

Although LAK cell treatment was somewhat effective, it has been found to induce adverse side effects, such as renal failure, in some patients; therefore, cytotoxic T cells may present as a better option for bone metastasis treatment as they possess specific tumor-killing abilities. It has been demonstrated that when the resting cluster of naïve T cells is treated with anti-CD3 antibodies together with IL-2 *ex vivo*, they differentiate into cytotoxic CD8⁺ T cells [11]. In a preclinical animal study of melanoma bone metastasis, when mice were intracardiacally inoculated with melanoma cells (B16), all the mice developed bone metastatic lesions ($n = 5$) [12, 13]. However, when the mice were given the donor T cells (obtained from tumor bearing mice), which were activated with anti-CD3 antibodies and IL-2 *ex vivo*, the treatment hindered bone metastatic progression and prevented the occurrence of signs of distress, paralysis, or other disturbances in motor activity in recipient mice ($n = 5$) [12, 13]. Moreover, the therapeutic benefit of the activated donor T cells was reversed when mice were treated with anti-CD8 antibodies ($n = 5$) [12, 13]. Cytotoxic T cells were also used to treat a breast cancer patient with bone metastasis, in which the patient's own peripheral blood mononuclear cells (PBMCs) were activated *ex vivo* using a combination of anti-CD3 antibodies, IL-2, and human leukocyte antigen (HLA)-A2-matched GC022588 gastric cancer cell line [14]. In this clinical case report, the patient experienced a return in serum carcinoembryonic antigen levels (which are sometimes increased in breast cancer patients) to normal range and importantly, bone pain relief [14]. However, when RCC patients with bone metastases ($n = 4$) were treated with *ex vivo* activated cytotoxic T cells derived from the patient's PBMCs using the combination of anti-CD3 antibodies and anti-CD28 antibodies, none of them achieved remission, although two patients showed some regression of bone metastatic lesions, and the only adverse events observed were mild to moderate influenza-like symptoms [15].

An alternative method to activate cytotoxic T cells in patients is through infusion of activated dendritic cells (DCs), an antigen-presenting cell type [16]. Briefly, in this process DCs are first stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF) and prostatic acid phosphatase (PAP, known to be specifically expressed on the surface of prostate cancer cells [17]), and the resulting DCs are able to activate PAP-targeting cytotoxic T cells [17]. This concept has been used to treat prostate cancer through products, such as Sipuleucel-T (APC8015) [17]. Sipuleucel-T therapy has improved the survival of metastatic castration-resistant prostate

cancer (CRPC) patients. In two phase III clinical trials of Sipuleucel-T in patients with metastatic CRPC, it was revealed that Sipuleucel-T extended the median survival of treated patients [Trial I ($n=127$), Sipuleucel-T survival: 25.9 months vs. placebo: 21.4 months, $p<0.01$ [18]; Trial II ($n=512$), Sipuleucel-T survival: 25.8 months vs. placebo: 21.7 months, $p=0.03$ [19]]. In addition, adverse events, including anorexia, anxiety, depression, flank pain, and contusion, as well as hydronephrosis, in Sipuleucel-T group were less frequent than those in placebo group (7.1% in placebo group vs. 3.8% in Sipuleucel-T group) [19]. Moreover, APC8015F, a version of Sipuleucel-T salvage treatment that is initiated using cryopreserved cells from patients, has also shown to enhance patient survival. A historical control trial of APC8015F in metastatic CRPC patients selected from the control arm of previous studies (NCT00005947, NCT01133704, and NCT00065442) demonstrated that APC8015F improved the median survival of those patients [APC8015F: 20.0 months vs. historical controls (NCT00005947, NCT01133704, and NCT00065442): 9.8 months, $p<0.001$] [20]. Although these trials were not intended to determine the treatment effects of DC-based autologous cytotoxic T cell therapy on bone metastatic patients, these findings suggest that DC-based autologous cytotoxic T cell therapy can be an effective treatment for prostate cancer patients with bone metastases, since 72.8% of metastatic CRPC patients develop bone metastases [21].

Tumor-infiltrating lymphocyte (TIL) therapy

While LAK cells and autologous cytotoxic T cells used for cell-based immunotherapies are obtained from patients' peripheral blood, the use of T cells existing in the tumor as cell-based immunotherapies has recently been appreciated. This treatment strategy is known as tumor-infiltrating lymphocytes (TIL) therapy. Intriguingly, compared to LAK cell and autologous cytotoxic T cell therapies, TIL therapy appears less toxic and has more specific tumor-killing abilities [22, 23]. When mice inoculated intravenously with MC-38 colon adenocarcinoma were treated with LAK cell therapy and cyclophosphamide, all the mice developed liver metastases [24]. However, when the MC-38-bearing mice were treated with TIL therapy and cyclophosphamide ($n=12$), none of mice developed liver metastases [24]. In a phase I clinical trial of TIL therapy in metastatic malignant melanoma ($n=20$, including one bone metastatic patient), TIL therapy with a single dose of cyclophosphamide induced either a complete or partial remission in 11 patients (the patient with bone metastasis achieved a partial remission for 4 months) [25]. The adverse effects observed in this study were mainly chills (10/20) and gastrointestinal symptoms, including nausea and vomiting (10/20) and diarrhea (9/20) [25]. Importantly, a randomized comparative clinical trial in

cancer patients (lung cancer, breast cancer, or gastric cancer) with bone metastases ($n=20$) using TIL therapy revealed that the patients' median survival time was significantly extended by the combination of TIL therapy and standard of care therapy (183 days vs. 82.3 days, $p<0.05$), compared to that of patients treated with standard of care therapy alone [23]. In addition, TIL therapy produced an analgesic effect on patients with bone metastasis, which ultimately improved quality of life; however, adverse events were not reported [23].

Cytokine-based immunotherapy for bone metastasis

As mentioned above, cytokines have been used to stimulate immune cells *ex vivo* to induce their tumor-killing abilities. However, cytokines themselves have also been known as immune inducers (or immune modulators). IL-2 is well known to enhance tumor-killing abilities of T cells, natural killing (NK) cells, and B cells [8]. When kidney cancer patients with bone metastases ($n=6$) were co-treated with famotidine (antihistamine, known to increase TIL) and high-dose IL-2, three of these patients showed response to this treatment strategy (for 3, 7, and 14 months) and one patient achieved complete remission in two bone sites for 14 months [26]. IFN- α was known to promote the differentiation and activity of host immune cells [9]. Conversely, a phase III clinical trial of RCC patients with metastases ($N=750$) demonstrated IFN- α did not improve OS in patients with or without bone metastases compared to Sunitinib [27].

Immune checkpoint blockade for bone metastasis

Immune checkpoint molecules consist of a receptor and ligand complex, which regulates immune activities [28]. There are two types of immune checkpoint molecules: stimulatory checkpoint molecules [e.g., CD27 (receptor)/CD70 (ligand) and CD28/CD86 (a.k.a. B7-2) or CD80 (a.k.a. B7-1)] and inhibitory checkpoint molecules [e.g., CD279 (a.k.a. PD-1)/CD274 [a.k.a. programmed cell death protein ligand 1 (PD-L1)] and CD152 [a.k.a. cytotoxic T-lymphocyte associated protein 4 (CTLA-4)]/CD86 (a.k.a. B7-2) or CD80 (a.k.a. B7-1)] [28, 29]. The receptors, which are commonly expressed on the surface of T and B cells, are responsible for exerting stimulatory or inhibitory immune responses after binding with their respective ligands.

In the tumor microenvironment, tumor cells and tumor-infiltrating immune cells highly express PD-L1, which upon binding to PD-1 on T cells directly inhibits T cell-mediated immune response to cancer. In addition, since

CD28 binding with CD86 or CD80 results in T cell activation, high levels of CTLA-4 on tumor-infiltrating T regulatory cells (Treg) competing with CD28 for CD86 or CD80 binding results in the inactivation of T cells [30, 31]. Both mechanisms ultimately contribute to cancer cell evasion of immune surveillance [28]. As stated above, blocking inhibitory checkpoint molecule pathways has been somewhat successful to treat cancer and has been appreciated as a breakthrough cancer treatment [32]. Antibodies, which target either the PD-1/PD-L1 pathway or the CTLA-4 pathway, were developed in the clinic for cancer treatment. These antibodies, such as ipilimumab (anti-CTLA-4), nivolumab (anti-PD-1), and atezolizumab (anti-PD-L1), have been FDA approved to treat various types of cancers including melanoma, non-small cell lung cancer (NSCLC), and RCC [32]. However, currently, these treatment strategies have negative treatment effects on patients with bone metastases.

In a two-center retrospective study of the combination of anti-PD-1 therapy and anti-angiogenic therapy in metastatic NSCLC patients ($n = 57$), bone metastasis was considered a negative predictor of prognosis ($p < 0.01$) [33]. Moreover, none of the bone metastatic patients responded to this combination strategy, although its objective response rate was 19.3% [33]. Similarly, a prospective, single-institution study of the second line anti-PD-1/PD-L1 therapy in metastatic NSCLC patients ($n = 66$) revealed that bone metastasis was an independent predictor of shorter survival [hazard ratio = 1.89 (confidence interval (CI) 1.02–3.51, $p = 0.049$)] [34]. For advanced RCC, a phase III clinical trial of nivolumab, an anti-PD-1 antibody, demonstrated that nivolumab ($n = 76$) expanded median OS in bone metastatic patients [18.5 months, 95% CI 10.2—not reached (NR)] compared to everolimus (mTOR inhibitor) chemotherapy ($n = 70$) (13.8 months, 95% CI 7.0–16.4) [35]. No obvious difference in adverse event rates was observed in bone metastatic patients from the nivolumab group or the everolimus group [35]. Another phase III clinical trial compared ipilimumab ($n = 399$), an anti-CTLA-4 antibody, with placebo ($n = 199$) in patients with metastatic (80% bone metastatic) chemotherapy-naïve, CRPC. Unfortunately, no significant difference in median OS was observed between the two groups, though ipilimumab increased median progression-free survival (PFS) for 1.8 months [hazard ratio (HR), 0.67; 95.87% CI 0.55 to 0.81] [36]. Bone pain response was not evaluated due to the small number of patients experiencing pain [36]. Despite limited efficacy of ICB on bone metastasis, ICB may help control bone remodeling. Nivolumab was found to prevent bone destruction by inhibiting TRAP+ osteoclast differentiation in a bone metastatic mouse model, although there was no effect on tumor growth [37].

Current shortcomings of immunotherapy for bone metastasis

As we have discussed, some types of immunotherapies (including cell-based and cytokine-based) have demonstrated moderate treatment efficacy in bone metastatic cancer patients (Table 1); however, bone metastasis overall remains a disease that does not respond well to immunotherapies [20, 33–36, 38]. Although their efficacy has been limited at this point, immunotherapies have potential for the treatment of bone metastasis, since bone marrow harbors various immune cells. To enhance the treatment efficacy of immunotherapies on bone metastasis, we must first identify the key barriers limiting their efficacy. Like other organs, bone marrow contains not only immune-supportive cells, but also immunosuppressive cells. However, bone marrow contains more immunosuppressive cells than other organs [39] in order to protect hematopoietic stem cells, which are crucial for maintaining blood formation from autoimmune insults [40]. For example, mesenchymal stem cells (MSCs) are known as one of the major components of the bone marrow microenvironment, or the “niche,” for hematopoietic stem cells. MSCs were also reported to inhibit T cells’ production of interferon-gamma (IFN- γ) in vitro, which is known as an anti-tumor molecule [41]. This implies MSCs may play a role in the inhibition of immune response to bone metastasis. Moreover, a recent bone marrow single cell sequencing analysis revealed additional contributors to the immunosuppressive environment in bone metastasis. This study compared four groups of patient samples: (i) solid bone marrow prostate cancer metastatic tissue, (ii) liquid bone marrow from the vertebral level of spinal cord compression, (iii) liquid bone marrow from vertebral body distant from the tumor site, and (iv) non-cancer patient bone marrow [42]. After analyzing the sequencing data, they found differences in monocyte and macrophage cell populations that were present. In group (i) with solid bone marrow metastatic tissue, tumor inflammatory monocytes (TIMs) and tumor associated macrophages (TAMs) were observed, while in groups (ii–iv) such populations were not observed [42]. TIMs and TAMs may ultimately contribute to an immunosuppressive microenvironment through support of tumor growth by expansion of the suppressive Treg cells [42]. In the bone marrow, bone metastatic cancer cells escape from immunosurveillance by indirectly inducing anti-inflammatory cytokine transforming growth factor-beta (TGF- β), which is released from the bone resorbed by osteoclasts [43]. TGF- β inhibits T-helper-1 (Th1) development [43], which is known to promote CD8 T cell activity by cytokines such as IL-2 [44]. Overall, current findings suggest that bone metastasis has been difficult to treat with immunotherapies

Table 1 Summary of immunotherapy trials for cancer patients with bone metastasis

Immunotherapy	Study type	Treatment	Cancer	Outcome	Adverse events	Metastatic sites	PMID
Adoptive immunotherapies	Clinical Trial (Phase Unspecified, 1987, USA)	LAK	RCC	5 Bone metastatic patients: 2/5 had PR [Patient 1: <6 months and Patient 2: no recurrence within 6 months]	Malaise, fever, renal failure	Bone, lung, brain, skin, liver, subcutaneous tissue, inferior vena cava	PMID: 2845776
Adoptive immunotherapies	Clinical Trial (Phase Unspecified, 1994, Japan)	LAK	RCC	2 Bone metastatic patients: 2/2 had PR [patient 1: <10 months and patient 2: <28 months]	Hypotension, heart failure, artery occlusion	Bone, muscle, brain, liver, lung, pleura, peritoneum	PMID: 7645136
Adoptive immunotherapies	Clinical Trial (Phase I, 1995, Japan)	LAK + IFN- α	RCC	PD	Fever, anemia, gastric ulcers	Bone, lung, brain	PMID: 7609359
Adoptive immunotherapies	Case Report (2006, Japan)	Autologous T cell	BCa	CEA reduction, bone pain relief	NA	Bone	PMID: 16786141
Adoptive immunotherapies	Clinical Trial (Phase I, 2003, USA)	Autologous T cell	RCC	2 Bone metastatic patients: 2/2 had PR	Influenza-like symptoms	Bone, lung, brain, liver	PMID: 14506142
Adoptive immunotherapies	Clinical Trial (Phase III, 2006, USA)	Sipuleucel-T	CRPC	Extend median survival [25.9 months vs. placebo 21.4 months]	Anorexia, anxiety, depression, flank pain, hydronephrosis	Bone	PMID: 16809734
Adoptive immunotherapies	Clinical Trial (Phase III, 2010, USA)	Sipuleucel-T	CRPC	Extend median survival [25.8 months vs. placebo 21.7 months]	Anorexia, anxiety, depression, flank pain, hydronephrosis	Bone	PMID: 20818862
Adoptive immunotherapies	Clinical Trial (Phase III, 2015, USA)	Sipuleucel-T salvage	CRPC	Extend median survival [20.0 months vs. historical controls 9.8 months]	Chills, nausea, fever	Bone	PMID: 25943532
Adoptive immunotherapies	Clinical Trial (Phase I, 1988, USA)	TIL	Melanoma	CR or PR [10 patients without bone metastasis] and PR [1 patient with bone metastasis: 4 months]	Chills, nausea, vomiting, diarrhea	Bone, lung, brain, subcutaneous tissue, spleen	PMID: 3264384
Adoptive immunotherapies	Clinical Trial (Phase Unspecified, 1994, China)	TIL	Lung cancer, BCa, Gastric cancer	Extend median survival [183 days vs. standard of care therapy 82.3 days]	NA	Bone, lung, brain, liver	PMID: 7956700
Cytokine-based immunotherapy	Clinical Trial (Phase Unspecified, 2006, USA)	Famotidine + IL-2	Kidney cancer	6 Bone metastatic patients: 1/6 had CR [14 months] and 2/6 had PR [Patient 1: 3 months, Patient 2: 7 months]	Hypertension, elevated creatinine, pulmonary edema	Bone, lung, liver	PMID: 17105423

Table 1 (continued)

Immunotherapy	Study type	Treatment	Cancer	Outcome	Adverse events	Metastatic sites	PMID
Cytokine-based immunotherapy	Clinical Trial (Phase III, 2008, USA)	IFN- α	RCC	Did not improve OS compared to Sunitinib	Fatigue, anemia, lymphopenia, nausea	Bone, lung, liver	PMID: 19487381
Immune checkpoint blockade	Retrospective Study (2020, China)	anti-PD-1 + anti-angiogenic agent	NSCLC	ORR 19.3%	Proteinuria, Hypotension, Fatigue	Bone, liver, brain, adrenal gland	PMID: 33039958
Immune checkpoint blockade	Clinical Trial (Phase III, 2017, USA, France, Italy, Spain, Australia, Denmark, Finland, UK, Germany, Japan)	anti-PD-1	RCC	Extend median survival [18.5 months vs. chemotherapy 13.8 months]	Adverse events incidence reduced compared to chemotherapy	Bone, lung, liver	PMID: 28262413
Immune checkpoint blockade	Clinical Trial (Phase III, 2017, USA, Portugal, France, Australia, Canada, Poland, Spain, Germany, Brazil, Mexico, Denmark, the Netherlands)	anti-CTLA-4	CRPC	Increased median PFS by 1.8 months compared to placebo	Diarrhea, rash, pruritus	Bone	PMID: 28034081

LAK lymphokine-activated killer cell therapy, *TIL* tumor-infiltrating lymphocytes therapy, *RCC* advanced renal cell carcinoma, *BCa* breast cancer, *CRPC* castration-resistant prostate cancer, *NSCLC* non-small cell lung cancer, *CR* complete response, *PR* partial response, *PD* progressed disease, *OS* overall survival, *PFS* progression-free survival, *ORR* objective response rate

(Fig. 1, left). Additionally, poor distribution of cell-based immunotherapies to bone has been considered as one of their shortcomings. Indeed, a rodent study revealed that LAK cells distribute thirty times higher to lung, liver, and spleen than femur, muscle, and prostate [45]. Moreover, a study of adoptive cellular therapy for acute myeloid leukemia also unveiled that double negative T cells infused into mice lowly distributed to the bone marrow compared with the heart, liver, kidney, and blood [46]. The distribution of therapeutic chimeric antigen receptor-modified (CAR)-T cells was also found to be much lower in the bone marrow than in the spleens, lungs, livers, and kidneys of mice with acute lymphoblastic leukemia [47]. This phenomenon may be caused by the way of therapeutic cell injection since intravenous administration mainly passes through lung, kidney, and liver.

Future directions and conclusions

The current mainstream treatment strategies for bone metastasis are the combinations of bone-targeted treatments [e.g., bisphosphonates and denosumab (a human monoclonal anti-receptor activator of nuclear factor κ B ligand (RANKL) antibody), and radium-223 dichloride] and systemic treatments (e.g., hormone therapies, chemotherapies). While these strategies have been somewhat effective (improving OS by a few months), they are mainly palliative [48]. Therefore, new treatment strategies other than combination therapies to eradicate bone metastasis are urgently needed. Although, to date, bone metastasis has been considered as an “immune-cold” disease, immunotherapies have potential to become paradigm shift therapies, since bone marrow contains a huge number of immune cells. However, as stated above, there are still several difficulties that need to be overcome before applying immunotherapies for bone metastasis. One example is the immunosuppressive effects of bone marrow. If these effects can be reversed, the efficacy of immunotherapies can be enhanced. Indeed, anti-TGF- β antibody treatment enhanced the efficacy of ICB immunotherapy. In a metastatic CRPC mouse model, treatment with anti-CTLA4 antibody combined with anti-TGF- β antibody significantly expanded intra-tumoral CD8⁺ T cells, whereas single treatment with either one of the two antibodies could not achieve obvious CD8⁺ T cell expansion [43]. Further, anti-TGF- β antibody treatment improved the OS ($p=0.06$) and reduced the burden of bone metastases by approximately 70–80% ($p=0.001$) in a metastatic breast cancer mouse model compared to the control group [49]. Considering that osteoclasts mediate TGF- β release from the bone, combining inhibition of osteoclasts by anti-RANKL antibody with ICBs has induced a dramatic partial response of bone metastasis and complete bone pain relief in a melanoma patient

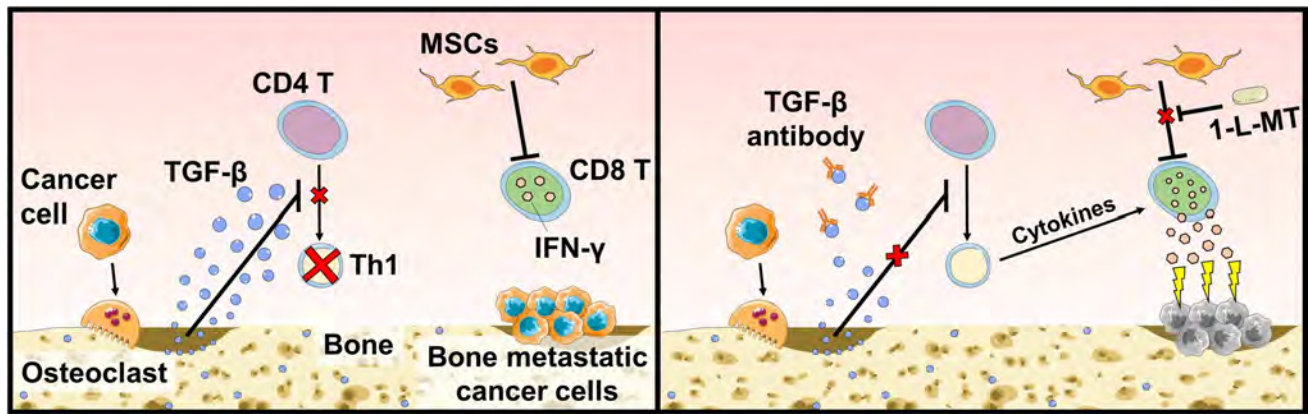


Fig. 1 Models of specific immune evasion in bone metastasis and the potential therapeutic strategies. As shown in the left panel, when bone metastatic cancer cells interact with osteoclasts in the bone marrow, these cancer-associated osteoclasts mediate bone resorption, in which the resorbed bone releases transforming growth factor-beta (TGF- β). TGF- β released from bone is known to inhibit the differentiation of CD4 T cells (CD4 T) into T helper 1 cells (Th1). Additionally, bone marrow mesenchymal stem cells (MSCs) prevent the secretion of interferon-gamma (IFN- γ , a cytokine known to suppress tumor growth) from CD8 T cells (CD8 T) (based on in vitro studies). These bone marrow-specific mechanisms allow bone metastatic cancer cells to evade the immune system, which contributes to the limited treatment efficacy of immunotherapy for bone metastasis. Conversely, as

shown in the right panel, TGF- β that was released from the bone can be blocked with anti-TGF- β antibodies, which allows CD4 T cells to differentiate into Th1 cells. By releasing cytokines, Th1 cells are then able to support CD8 T cells to kill tumor cells. Further, treatment with an indoleamine 2,3-dioxygenase (IDO1) inhibitor, 1-methyl-L-tryptophan (1-L-MT) can prevent the MSC-mediated inhibition of IFN- γ release from T cells (based on in vitro studies). Through activation of CD8 T cells and IFN- γ release, cytotoxicity to bone metastatic cancer cells can be increased, thus enhancing immune response in the bone marrow microenvironment. We propose that by enhancing CD8 T cell tumor-killing ability through anti-TGF- β antibody and 1-L-MT treatment, efficacy of immunotherapies for bone metastatic disease may be improved

with widespread bone metastases [50]. Currently, a phase Ib/II clinical trial (ACTRN12618001121257) comparing combination therapy of RANKL inhibitor and ICB with ICB alone in resectable NSCLC is ongoing [51]. Moreover, the indoleamine-pyrrole 2,3-dioxygenase (IDO1) inhibitor, known as 1-methyl-L-tryptophan (1-L-MT), treatment may be a potential way to inhibit the immunosuppressive effects mediated by MSCs [52]. In a co-culture experiment of MSCs with T cells (exposed to immunostimulants, such as lipopolysaccharides (LPS) or poly(cytidylic-inosinic) acid), 1-L-MT significantly increased T cell proliferation compared to non-treatment control [52]. Although further in vivo experiments are needed, these findings suggest that by combining these anti-immunosuppressive strategies, the efficacy of immunotherapies for bone metastasis can be improved (Fig. 1, right).

To enhance the treatment efficacy and reduce potential off-target effects of cell-based immunotherapies, their bone tropism needs to be improved. Recent gene editing techniques enable us to engineer T cells to recognize specific tumor antigens; these strategies include (i) T-cell receptor (TCR)-T cell technology: stimulates TCR to recognize tumor antigen, and (ii) CAR-T cell technology: artificial antigen receptors to recognize tumor antigen [53]. To engineer TCR-T cells, modified TCRs that specifically recognize tumor antigens are integrated onto the T cell membrane [53]. Compared to TCR-T cells, CAR-T cells use chimeric antigen receptors that include scFv domains derived from

antibody, CD3 ζ , and transmembrane domain, which enable CAR-T cells to recognize cell surface antigens directly without the presentation of major histocompatibility complex (MHC) [53]. Therefore, these technologies may be used to enhance bone tropism of cell-based immunotherapies if TCR or CAR-T cells can be effectively designed to target cells in the bone marrow (e.g., osteoblasts, osteoclasts). By doing so, these T cell engineering technologies have a potential to enhance the treatment efficacy of cell-based immunotherapies on bone metastatic cancer cells. Further studies are warranted prior to applying this idea as a means to improve their bone tropism.

Although recent clinical trials have demonstrated that ICB has limited effects on cancer patients with bone metastases, several case reports have revealed that ICB could induce complete remission in cancer patients with bone metastases (pembrolizumab, an anti-PD-1 antibody, in three bone metastatic lung cancer patients [54, 55]; and ipilimumab in two bone metastatic prostate cancer patients [56]). Additionally, as discussed, therapies using cytotoxic T cells have been somewhat effective for bone metastasis. Therefore, the ICB can be considered as a potential treatment for bone metastasis if the functions of cytotoxic T cells are enhanced. Intriguingly, recent studies revealed that alteration of the gut microbiota of cancer patients can improve the treatment efficacy of ICB by enhancing tumor-killing activities of cytotoxic T cells [32, 57–60]. Thus, strengthening cytotoxic

T cell activities by manipulating the gut microbiota may improve the treatment efficacy of ICB on bone metastatic cancer patients. Additional studies are needed to confirm this hypothesis.

Although further clinical evaluation and improvement of its treatment efficacy are clearly warranted, immunotherapy could be a breakthrough for cancer patients with bone metastases and help alleviate many of the challenges and hurdles faced by bone metastatic patients and their families.

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Declarations

Conflict of interest Yusuke Shiozawa has received research funding from TEVA Pharmaceuticals, but not relevant to this study. No conflict of interest exists for remaining authors.

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