

AWARD NUMBER: W81XWH-17-1-0542

TITLE: Molecular Cross-Talk: Bone Metastatic Prostate Cancer and Nociceptive Neurons

PRINCIPAL INVESTIGATOR: Christopher Peters, Ph.D.

CONTRACTING ORGANIZATION: Wake Forest University Health Sciences
Winston-Salem, NC 27157

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14. ABSTRACT The goal of this project is to determine the roles of angiotensin II and its receptor in prostate cancer induced bone pain and bone metastatic growth. Aim 1 will provide the framework to identify the extent to which the interaction between cancer cells and nociceptive neurons through the angiotensin II and receptor axis affects cancer-induced bone pain. Aim 2 will determine the downstream molecular mechanisms whereby angiotensin II and its receptor axis affects bone pain. Aim 3 will define how nociceptive neuron influence tumor outgrowth. We believe that the insights derived from our investigations will lead to new strategies for reducing cancer-induced bone pain and also the outgrowth of bone metastasis. During this period, we found that prostate cancer (PCa) enhances the sprouting of calcitonin gene-related peptide (CGRP)-expressing sensory nerves both in vitro and in vivo. We also found that the CGRP/its receptor axis controls PCa bone metastatic progression in vitro and human. In addition, we developed the technique to downregulate CGRP expression in mouse nerves that enables us to test effects of CGRP on PCa progression in animals.					
15. SUBJECT TERMS Prostate Cancer; Bone metastasis; Disseminated tumor cells; Cancer-induced bone pain; Sensory nerves; Bone marrow microenvironment					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 81	19a. NAME OF RESPONSIBLE PERSON USAMRMC
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1. INTRODUCTION:

Cancer-related pain, both its causes and its management, poses a tremendous challenge to patients and their caregivers. In the quest for effective cancer therapy, maintaining quality of life can be as crucial as treating the tumor. Seventy-five percent of cancer patients experience pain throughout the course of the disease, and once cancer metastasizes to the bone, the first symptom is often acute bone pain. A full 80% of patients with bone metastasis suffer from cancer-induced bone pain (**CIBP**). Current analgesic strategies for CIBP using nonsteroidal anti-inflammatory drugs (**NSAIDs**) and opioids are effective, but side effects, abuse, and addiction are serious and growing concerns associated with their use. It is therefore essential to develop therapeutic agents that can eradicate bone metastatic diseases and relieve resultant pain.

Metastatic progression of disseminated tumor cells (**DTCs**) to bone may be environmentally directed by influences from the bone marrow microenvironment. Yet a full understanding of the more complex interactions that result in active bone marrow metastasis remains elusive. Our group recently discovered that early in the metastatic process, prostate cancer (**PCa**) cells target and commandeer the specific microenvironment for hematopoietic stem cells (**HSCs**), using mechanisms similar to those involved in HSC homing. Involvement of sympathetic and parasympathetic nerve fibers is crucial for both HSC maintenance and the dissemination of PCa to bone, suggesting that interactions between DTCs and nerves are at least in part responsible for controlling metastatic progression. However, the influence of sensory nerve fibers in the bone on PCa progression and metastasis has not been examined. Although it has been suggested that compression of the soft tissues or peripheral nerve fibers and interference with bone remodeling by bone tumors may be major causes of CIBP, little is known about the molecular interactions of DTCs with bone marrow nerve fibers, and how they affect CIBP.

Elucidating how these interactions influence CIBP is key. Angiotensin II (**Ang II**), a peptide that regulates blood pressure, is also involved in the progression of several types of cancer, including PCa. Ang II is pronociceptive in neuropathic and inflammatory pain models, and elicits sprouting of nociceptive neurons in the skin following inflammation through the angiotensin receptor (**ATR**). In a clinical study of several chronic musculoskeletal pain conditions, a small molecule inhibitor for ATR provided significant pain relief, suggesting that the Ang II/ATR axis controls pain signaling. Our preliminary data suggest that pain-associated neuropeptides can interact directly with PCa cells to cause enhanced proliferation. Therefore, it is critical to explore whether the Ang II/ATR axis is involved in the development of CIBP, and whether pain-associated neuropeptides regulate bone metastatic progression.

We hypothesize that **disseminated PCa stimulates sensory neurons that innervate bone through the Ang II/ATR axis; these cancer-associated sensory neurons then release neuropeptides, resulting in both CIBP and metastatic progression of PCa** (see Fig. 1). Ang II expressed by disseminated PCa induces CIBP (Aim 1) by stimulating sensory neurons to release calcitonin gene-related peptide (**CGRP**) through the ATR/p38 pathway (Aim 2). The nerve-derived CGRP enhances the growth of DTCs via its receptor calcitonin receptor-like receptor (**CRLR**) (Aim 3).

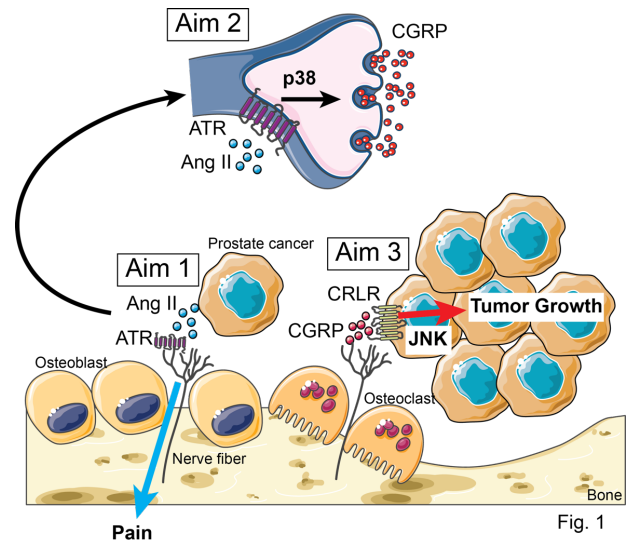


Fig. 1

To address our hypothesis the following aims are proposed:

Aim 1: Determine how disseminated prostate cancer is involved in development of cancer-induced bone pain.

Aim 2: Define the intracellular signaling pathways in peripheral nerves stimulated by disseminated prostate cancer.

Aim 3: Determine the influence of nociceptive neurons on (a) tumor outgrowth in the bone and (b) cancer-induced bone pain.

The proposed studies will focus on fundamental mechanisms behind the novel concept that nerves in the bone marrow microenvironment have a crucial role in PCa bone metastasis. Aim 1 will provide the framework to identify the extent to which the interaction between DTCs and nociceptive neurons affects cancer-induced bone pain. Aim 2 will determine the molecular mechanisms of cancer-induced bone pain. Aim 3 will define how DTC/nociceptive neuron interactions influence tumor outgrowth. We believe that the insights derived from our investigations will lead to new strategies for reducing cancer-induced bone pain and also the outgrowth of DTCs. By promoting understanding of how the crosstalk between nociceptive neurons and DTCs participates in cancer-induced bone pain and tumor outgrowth, this study will lay the foundation for significant improvements in care of PCa patients, allowing development of pain targeting therapies that can also minimize bone metastatic progression. If successful, our study will not only shed new light on the fundamental mechanisms of PCa bone metastasis, but will quickly provide a gateway to novel treatment strategies to lower PCa-associated deaths and suffering, as some of the agents to be tested in this study have been in clinical trials for different diseases.

2. KEYWORDS:

Prostate Cancer; Bone metastasis; Disseminated tumor cells; Cancer-induced bone pain; Sensory nerves; Bone marrow microenvironment

3. ACCOMPLISHMENTS:

What were the major goals and objectives of the project?

The goal of this project is to determine the roles of the interaction between bone metastatic prostate cancer and sensory nerves in cancer-induced bone pain and bone metastatic growth.

Task 1: Determine whether Ang II expressed by disseminated tumor cells affects cancer-induced bone pain using pharmacological interference of Ang II and ATR interactions.

Months 1-11.

- Submit documents for local IACUC review (**Months 1-3; Dr. Shiozawa, Dr. Peters**).
- Submit IACUC approval and necessary documents for ACURO review (**Months 4-6; Dr. Shiozawa**).
- Design and develop an animal model to measure the tumor growth in the marrow, bone remodeling, and pain related behavior within the same animal (**Months 7-8; Dr. Shiozawa, Dr. Peters**).
- Test the effects of Ang II/ATR axis on the tumor growth in the marrow, bone remodeling, and pain related behavior within the same animal (**Months 9-11; Dr. Shiozawa, Dr. Peters**).

Task 2: Determine the effects of disseminated tumor cells on Ang II levels in the marrow and ATR expression in the dorsal root ganglia.

Months 12-14.

- Determine the kinetic pattern of Ang II levels in the tumor inoculated marrow and ATR expression in the tumor inoculated DRG (**Months 12-14; Dr. Shiozawa**).

Task 3: Determine the effects of Ang II expressed by disseminated tumor cells on cancer-induced bone pain by manipulating the tumor-nerve microenvironment.

Months 15-19.

- Design and develop nerve-specific ATR knockdown (KD) mice with adeno-associated viral vectors (**Months 15-16; Dr. Shiozawa, Dr. Peters**).
- Test the effects of Ang II/ATR axis on the tumor growth in the marrow, bone remodeling, and pain-related behavior within the same animal (ATR KD mice) (**Months 17-19; Dr. Shiozawa, Dr. Peters**).

Task 4: Determine whether the p38 pathway influences tumor growth, skeletal remodeling, and cancer-induced bone pain.

Months 20-22.

- Test the effects of p38 inhibitor on the tumor growth in the marrow, bone remodeling, and pain related behavior within the same animal (**Months 20-22; Dr. Shiozawa, Dr. Peters**).

Task 5: Determine the molecular mechanisms whereby Ang II expressed by disseminated tumor cells activates the bone marrow sensory nerves.

Months 23-24.

- Test the effects of ATR antagonist on the expression of pp38 and CGRP in DRG, spinal cord, and bone (**Months 23-24; Dr. Shiozawa, Dr. Peters**).

Task 6: Major Task 6: Define a link between Ang II expression by disseminated tumor cells with activation of the bone marrow sensory nerves in human subjects.

Months 1-34.

- Submit documents for local IRB review (**Months 1-3; Dr. Shiozawa**).
- Submit IRB approval and necessary documents for HRPO review (**Months 4-6; Dr. Shiozawa**).
- Clinical validation of results with bone biopsy samples of confirmed prostate cancer patients (**Months 7-34; Dr. Shiozawa, Dr. Peters**)
- Co-author manuscript on the effects of Ang II/ATR axis on the tumor growth, bone remodeling, and pain related behavior (**Months 23-26; Dr. Shiozawa, Dr. Peters**).

Task 7: Determine whether CGRP released from bone marrow sensory neurons influences tumor growth, skeletal remodeling, and cancer-induced bone pain.

Months 25-29.

- Design and develop nerve-specific CGRP knockdown (KD) mice with adeno-associated viral vectors (**Months 25-26; Dr. Shiozawa, Dr. Peters**).
- Establish CRLR KD PCa cells (**Months 25-26; Dr. Shiozawa**).

- Test the effects of CGRP/CRLR axis on the tumor growth in the marrow, bone remodeling, and pain-related behavior within the same animal (CGRP KD mice) (**Months 27-29; Dr. Shiozawa, Dr. Peters**).

Task 8: Determine whether CGRP promotes bone metastases through JNK pathway.

Months 30-31.

- Test the effects of CGRP antagonist on the expression of JNK in bone metastatic tumor (**Months 30-31; Dr. Shiozawa, Dr. Peters**).

Task 9: Determine whether JNK pathway influences tumor growth, skeletal remodeling, and cancer-induced bone pain.

Months 32-34.

- Test the effects of JNK inhibitor on the tumor growth in the marrow, bone remodeling, and pain related behavior within the same animal (**Months 32-34; Dr. Shiozawa, Dr. Peters**).
- Co-author manuscript on the effects of CGRP/CRLR axis on the tumor growth, bone remodeling, and pain related behavior (**Months 25-26; Dr. Shiozawa, Dr. Peters**).
- Co-author manuscript summarizing all the results (**Months 27-29; Dr. Shiozawa, Dr. Peters**).

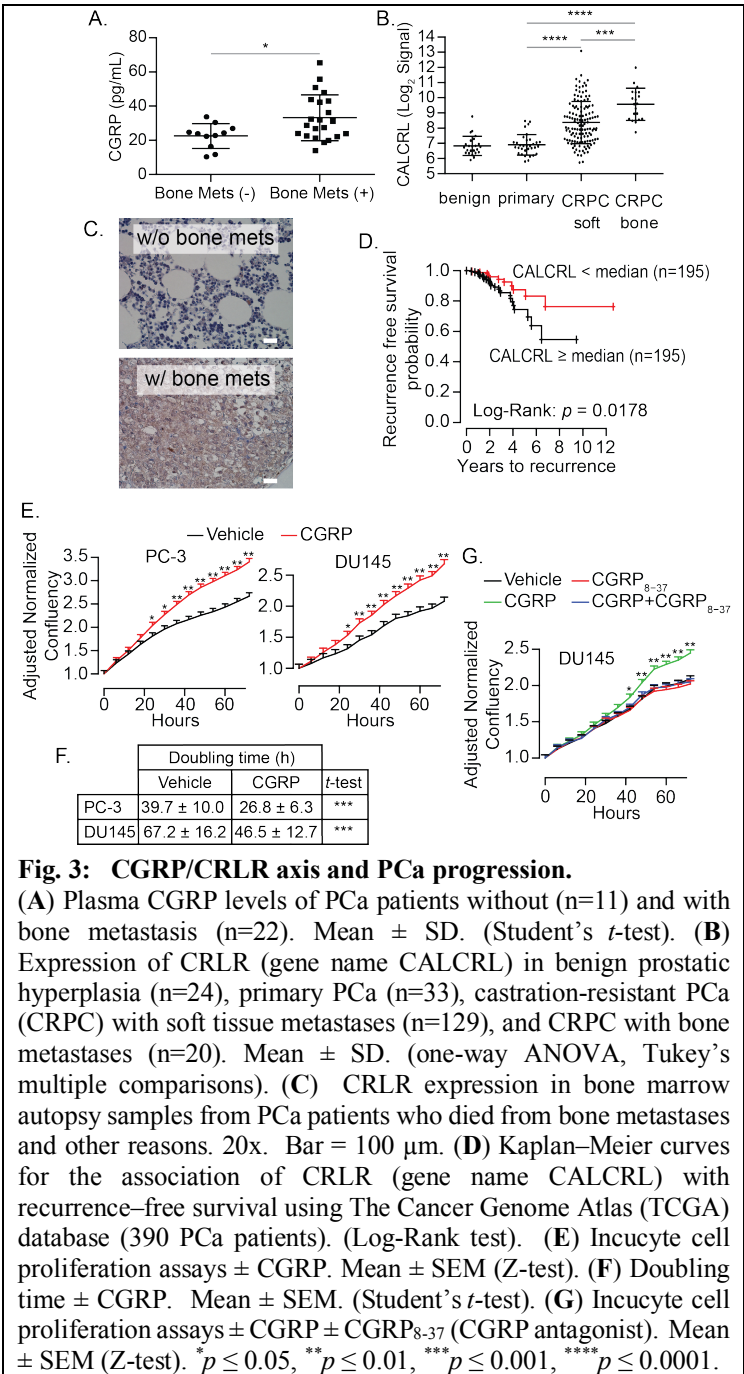
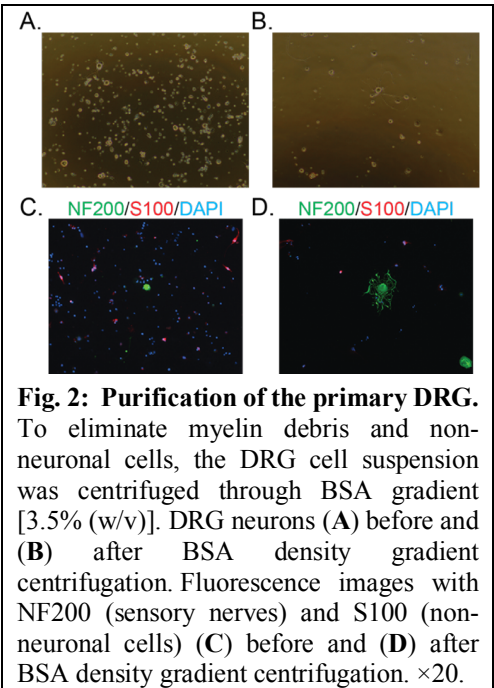
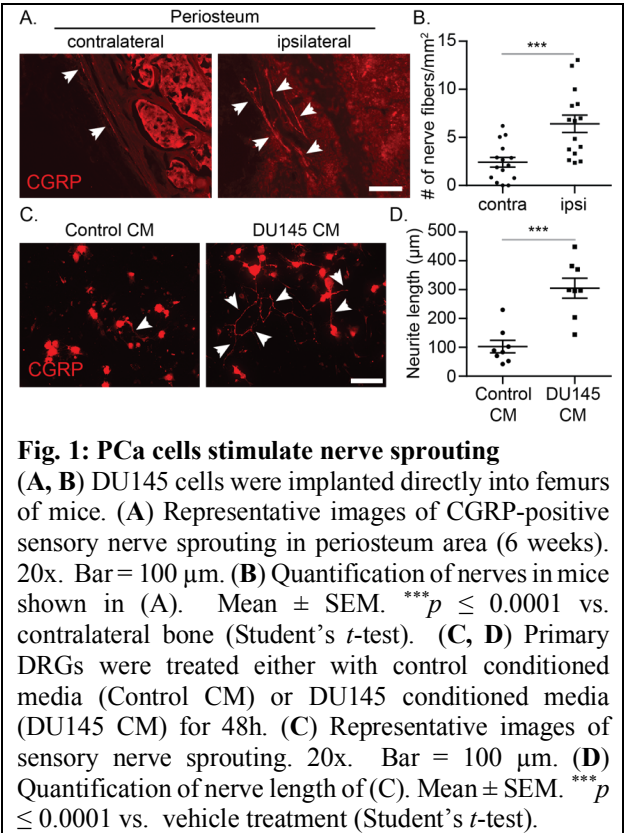
What was accomplished under these goals?

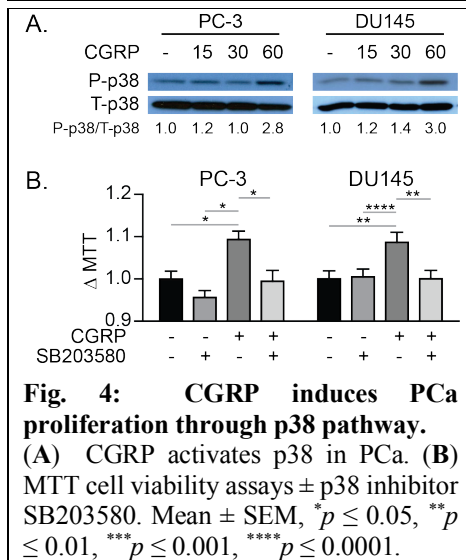
(2017-2018)

Bone metastatic prostate cancer (PCa) causes enriches calcitonin gene-related peptide (CGRP)-expressing sensory nerves in the marrow, and that the CGRP/calcitonin receptor-like receptor (CRLR)/p38 pathway promotes PCa bone metastasis (Dr. Shiozawa).

To test the effects of the bone metastatic tumors on sensory nerves in bone, we developed a bone metastatic mouse model. Briefly, DU145 cells were injected directly into the femurs of immunodeficient mice. After 8 weeks of tumor inoculation, we found more CGRP-expressing sensory nerve fibers in the ipsilateral tumor-bearing bones than the contralateral bones (**Fig. 1A&B**). To further determine the effects of PCa on nerve sprouting, we developed primary dorsal root ganglia (DRG) culture (**Fig. 2**). Similar to the *in vivo* study, when DRGs were treated with conditioned medium from DU145 cells, sprouting of sensory nerve fibers significantly increased (**Fig. 1C&D**). Consistently, PCa patients with bone metastatic disease have elevated CGRP serum levels, compared to those without bone metastases (**Fig. 2A**). In cohorts of clinical samples (**Fig. 2B**) and autopsy samples (**Fig. 2C**), samples from patients with bone metastatic PCa expressed higher levels of the

CGRP receptor, CRLR. CRLR (gene name: CALCRL) expression levels are associated with recurrence-free survival in PCa patients (**Fig. 2D**). In addition, CGRP induced proliferation of PCa cells through CRLR (**Fig. 2E-G**).



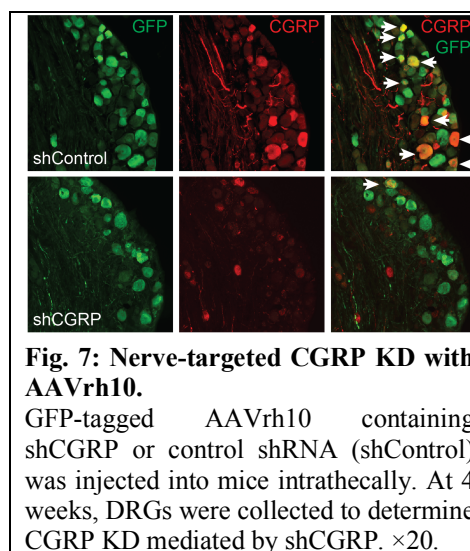
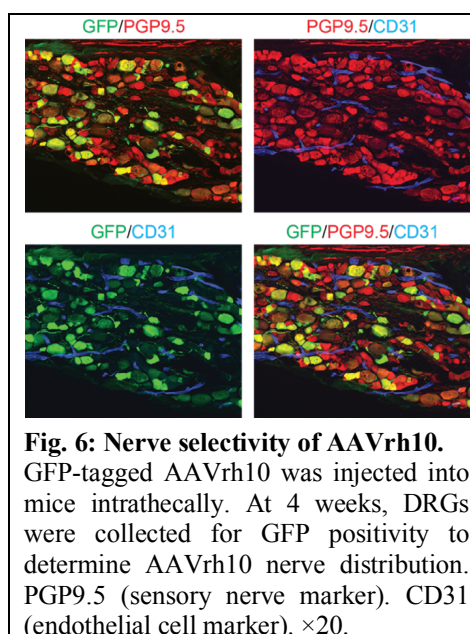
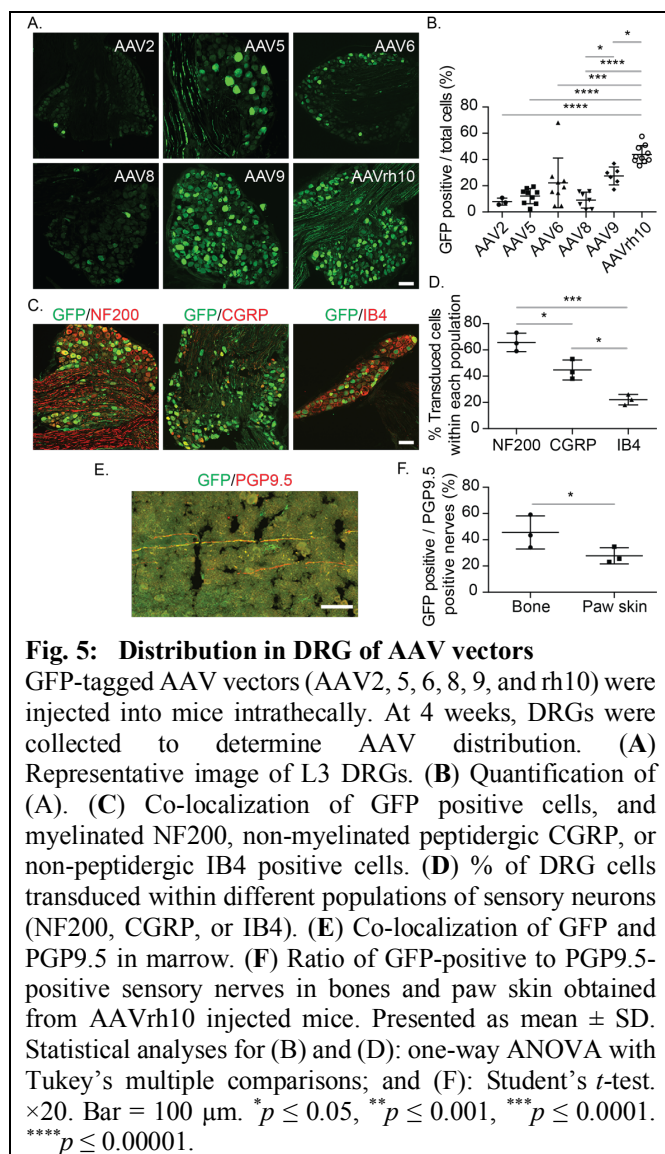


Moreover, CGRP activated p38 in PCa (**Fig. 4A**), and CGRP-mediated cell proliferation was inhibited by a p38 inhibitor (**Fig. 4B**).

CGRP downregulation in sensory nerves that innervate bone using adeno-associated virus serotype rh10 (AAVrh10) (Drs. Shiozawa and Peters).

To determine which AAV serotype has superior gene transduction efficiency to sensory nerves innervating bones, we chose six different serotypes (AAV2, 5, 6, 8, 9, and rh10) which exhibit some degree of nerve tropism *in vivo*. Green fluorescent protein (GFP)-expressing AAVs were inoculated into severe combined immunodeficiency (SCID) mice by intrathecal injection, which is known to be an efficient delivery system to transfer viral vectors into the lumbar spinal cord and dorsal root ganglia (DRG). Four weeks after AAV inoculation, lumbar spinal cord and DRG were dissected for immunohistochemical analyses. We found that 1)

AAVrh10 transduces neurons in the DRG with higher efficacy than AAV2, 5, 6, 8, and 9 (**Fig. 5A&B**); 2) it has higher tropic affinity to large myelinated and small peptidergic sensory neurons that innervate bone, compared to small non-peptidergic sensory neurons that rarely innervate bone (**Fig. 5C&D**); and 3) it has tropism towards sensory neurons in skeletal tissues, while it occasionally reaches the sensory nerve fibers in the mouse footpad (**Fig. 5E&F**). To further validate nerve tropism of AAVrh10, DRGs were stained with both a neuronal marker (PGP9.5) and an endothelial cell marker (CD31), since DRGs are highly vascularized. As expected, GFP signals were mainly overlapped with PGP9.5 positive nerve cell bodies/fibers and there was no co-localization between GFP and CD31 positive blood vessels (**Fig. 6**), suggesting that AAVrh10 has preferential nerve tropism. Importantly, when mice were injected with AAVrh10-containing shRNA targeting CGRP, expression levels of CGRP in DRGs transduced with this virus were down-regulated, compared to those injected with control shRNA (**Fig. 7**). Our results demonstrate that through this technique we can manipulate sensory nerve gene expression in our animal model.



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

(2017-2018)

Dr. Shiozawa was invited to give a seminar at Jikei University School of Medicine (Tokyo, Japan) on October 11, 2017.

Dr. Peters organized a symposium at Wake Forest University Medical School entitled “*Translational Cancer Pain Research: Bridging the Gap Between Basic Science and Clinical Application*”. The symposium consisted of presentations from 3 clinicians (Dr. Francis Walker, Dr. Glenn Lesser, Dr. Roy Strowd) who specialize in clinical research focused on interventional studies for the treatment of cancer related pain and sensory dysfunction as well as 2 preclinical researchers (Dr. Chris Peters, Dr. Mario Danelo Boada)

who are conducted mechanistic research studies in rodent models of cancer pain. The keynote speaker was Dr. Patrick Dougherty from MD Anderson Cancer Center who presented a lecture entitled “Inflammation and Ectopic Spontaneous Activity: New Findings of Chemotherapy Induced Peripheral Neuropathy in Humans & Animals”. This symposium came about largely as a result of the collaborative efforts driven by Dr. Shiozawa between clinicians within Wake Forest Comprehensive Cancer Center and faculty members within the Department of Anesthesiology at Wake Forest Baptist Medical Center.

How were the results disseminated to communities of interest?

There is nothing to report at this time.

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

In the next year of the award, we will continue to determine the roles of renin-angiotensin system in PCa-induced bone pain (Aim 1). We will also further elucidate the molecular mechanisms whereby bone metastatic PCa stimulates the sensory nerves (Aim 2). In addition, we will continue to pursue the effects of sensory nerves on the progression of bone metastatic PCa (Aim 3).

4. IMPACT:

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

(2017-2018)

We found that PCa enhances the sprouting of CGRP-expressing sensory nerves both in vitro and in vivo.

We found that PCa patients with bone metastases have high levels of CGRP in their serum, that PCa in bone metastatic sites expresses high levels of CRLR, and that CRLC expression levels in PCa is associated with their recurrent free survival.

We found that CGRP induces PCa proliferation through CRLR/p38 pathway.

We developed the technique to manipulate gene expression in sensory nerves innervating bones using an AAVrh10 virus vector.

What was the impact on other disciplines?

There is nothing to report at this time.

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?

AWARD: PC160455, PC160455P1

TITLE: Molecular crosstalk: bone metastatic prostate cancer and nociceptive neurons

PI: Yusuke Shiozawa, M.D., Ph.D., Partnering PI: Christopher Peters

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

Nothing to report.

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

(2017-2018)

1. Park SH, Eber MR, Tsuzuki S, Booker ME, Sunil AG, Widner DB, Parker RA, **Peters CM, Shiozawa Y**. Adeno-associated virus serotype rh10 is a useful gene transfer vector for sensory nerves that innervate bone in immunodeficient mice. **Sci Rep**. 2017;7:17428. PMID: 29233995. PMCID: PMC5727257.

Status of Publication: Published

Acknowledgement of federal support: Yes

- **Selected as Papers of the Week (9 Dec 2017- 15 Dec 2017) by Pain Research Forum**

Invited reviews

(2017-2018)

1. Park SH, Keller ET, **Shiozawa Y**. Bone marrow microenvironment as a regulator and therapeutic target for prostate cancer bone metastasis. **Calcif Tissue Int**. 2018;102:152-62. PMID: 29094177. PMCID: PMC5807175.

Status of Publication: Published

Acknowledgement of federal support: Yes

2. Widner DB, Files DC, Weaver KE, **Shiozawa Y**. Preclinical and clinical studies on cancer-associated cachexia. **Front Biol**. 2018;13:11-18. PMID: In Progress. PMCID: In Progress.

Status of Publication: Published

Acknowledgement of federal support: Yes

3. Park SH, Eber MR, Widner DB, **Shiozawa Y**. Role of the bone microenvironment in the development of painful complications of skeletal metastases. **Cancers (Basel)**. 2018;10:141. PMID: 29747461. PMCID: PMC5977114.

Status of Publication: Published

Acknowledgement of federal support: Yes

- **Selected as Featured Paper**

4. Widner DB, Park SH, Eber MR, **Shiozawa Y**. Interactions between disseminated tumor cells and bone marrow stromal cells regulate tumor dormancy. **Curr Osteoporos Rep**. 2018;16:596-602. PMID: 30128835. PMCID: In Progress.
Status of Publication: Published
Acknowledgement of federal support: Yes

Book

(2017-2018)

1. Park SH, Eber MR, **Shiozawa Y**. (2018) Tumor inoculation mouse models of prostate cancer bone metastasis. In Idris A (Ed.), *Bone Research Protocols - 3rd Edition*. In Press. London: Springer Nature.
Status of Publication: Accepted
Acknowledgement of federal support: Yes
2. Park SH, Eber MR, Taichman RS, **Shiozawa Y**. (2018) Determining Competitive Potential of Bone Metastatic Cancer Cells in the Murine Hematopoietic Stem Cell Niche. In Turksen K (Ed.), *Stem cells and Niche - 2nd Edition: Methods and Protocols*, Methods in Molecular Biology. In Press. New York: Humana Press. Methods Mol Biol. In Press. PMID: 30099699. PMCID: In Progress.
Status of Publication: Published
Acknowledgement of federal support: Yes

Presentation

(2017-2018)

1. Williams SN, Eber MR, Tsuzuki S, Park SH, **Shiozawa Y**. Does histamine influence the osteoblastic activities that help regulate the progression of bone metastatic prostate cancer? The 2017 Annual Biomedical Research Conference for Minority Students (ABRCMS), Phoenix, AZ, USA, November 1-4, 2017. Poster.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

AWARD: PC160455, PC160455P1

TITLE: Molecular crosstalk: bone metastatic prostate cancer and nociceptive neurons

PI: Yusuke Shiozawa, M.D., Ph.D., Partnering PI: Christopher Peters

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-17-1-0541

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

Nearest person month worked: 3

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, TEVA Pharmaceuticals

Name: Christopher Peters

Project Role: Partnering PI, W81XWH-17-1-0542

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 1.8

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 training personnel in evoked and non-evoked bone cancer pain behavioral assays. As part of Aims 1 and 3, Dr. Peters will assist with *in vivo* validation of a viral vector approach for sensory neuron selective knockdown of ATR and CGRP. He will also assist with histological and immunohistochemical analysis of skeletal tissue as part of Aims 1-3. Dr. Peters has over 15 years of experience working with preclinical models of skeletal pain including rat and mouse models of bone cancer. He has extensive experience with the behavioral analysis, immunohistochemistry, biochemical analysis and fluorescent, brightfield, and confocal microscopic imaging required for this project. He will meet with Dr. Shiozawa on a weekly basis to discuss progress on projects related to this Partnering PI Prostate Cancer Research Idea Development Award.

Funding Support: National Institutes of Health, Department of Defense

Name: Fang-Chi Hsu

Project Role: Co-Investigator, W81XWH-17-1-0541

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.6

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

Name: Christopher Thomas

Project Role: Co-Investigator, W81XWH-17-1-0541

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.12

Contribution to Project: Dr. Thomas is an Associate Professor in the Department of Hematology/Oncology. On this project, he will assist Dr. Shiozawa's laboratory in tissue procurement, experimental design, and scientific strategy.

Funding Support: Department of Defense

Name: Debra Diz

Project Role: Co-Investigator, W81XWH-17-1-0541

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.6

Contribution to Project: Dr. Diz is the Co-Director of the Cardiovascular Sciences Center and Professor, Department of General Surgery. Her research focuses on cardiovascular neuroscience and neuropharmacology related to the effects of angiotensin II on neuropeptide and release of other transmitters in the context of the renin-angiotensin-system in aging and metabolic regulation. On this project, she will assist Dr. Shiozawa's laboratory in radio binding assay.

Funding Support: National Institutes of Health, Department of Defense

Name: Mark Chappell

Project Role: Co-Investigator, W81XWH-17-1-0541

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.6

Contribution to Project: Dr. Chappell is a Professor in the Department of General Surgery and member of the Cardiovascular Sciences Center. He is an expert in the assessment of the renin-angiotensin-system (RAS) components and is interested in the RAS in hypertension, diabetes, fetal programming and women's health issues. On this project, he will assist Dr. Shiozawa's laboratory in radioimmunoassay.

Funding Support: National Institutes of Health, Department of Defense

Name: Thomas Jeff Martin

Project Role: Co-Investigator, W81XWH-17-1-0542

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.6

Contribution to Project: Dr. Martin is a Professor in the Department of Anesthesiology and Physiology & Pharmacology. Dr. Martin's research interests are on mechanisms of opioid abuse particularly in the setting of chronic pain. He has been instrumental in the development of operant based behavioral assays to monitor the effects of persistent pain on complex animal behaviors. Dr. Martin will assist with training, implementation and analysis of running wheel assays to assess progressive disability in mice inoculated with bone cancer as part of studies outlined in Aims 1-3.

Funding Support: National Institutes of Health, Department of Defense

Name: Juan Miguel Jimenez-Andrade

Project Role: Visiting scholar, W81XWH-17-1-0542

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 3

Contribution to Project: Dr. Jimenez-Andrade is a Professor in the Autonomous University of Tamaulipas in Reynosa, Mexico. Dr. Jimenez-Andrade's laboratory research is focused on mechanisms of skeletal pain and osteoporosis. He conducted a 6 month sabbatical in the lab of Dr. Peters to assist with the development of methods for immunohistochemical analysis of decalcified bone sections as part of studies under this proposal.

AWARD: PC160455, PC160455P1

TITLE: Molecular crosstalk: bone metastatic prostate cancer and nociceptive neurons

PI: Yusuke Shiozawa, M.D., Ph.D., Partnering PI: Christopher Peters

Additionally, he

Funding Support: Department of Defense, National Institutes of Health, UC MEXUS-CONACYT

Name: Enriqueta Munoz-Islas

Project Role: Visiting scholar, W81XWH-17-1-0542

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 3

Contribution to Project: Dr. Munoz-Islas is a Professor in the Autonomous University of Tamaulipas in Reynosa, Mexico. Dr. Munoz-Islas' laboratory research is focused on CGRP and vascular interactions and impact on musculoskeletal pain. She conducted a 6 month sabbatical in the lab of Dr. Peters to assist with behavioral analysis in mouse models of bone cancer being developed under this proposal. She also assisted with the development of methods for immunohistochemical analysis of decalcified bone sections and methods for conducting in situ hybridization in nervous system tissue that will compliment immunohistochemical studies outlined in Aims 1-3.

Funding Support: Department of Defense, National Institutes of Health, UC MEXUS-CONACYT

Name: Ted Bateman

Project Role: Collaborator, W81XWH-17-1-0542

Researcher Identifier (e.g. ORCID ID)

Nearest person month worked: 0.144

Contribution to Project: Dr. Bateman is an Associate Professor in the Department of Radiation Oncology at University of North Carolina at Chapel Hill. His laboratory will perform microCT imaging and analysis as part of experiments in Aims 1-3.

Funding Support: 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?

The University of North Carolina at Chapel Hill

8. SPECIAL REPORTING REQUIREMENTS:

QUAD CHARTS.

9. APPENDICES:

The original copies of manuscript are attached.

Molecular crosstalk: bone metastatic prostate cancer and nociceptive neurons

PC160455, PC160455P1

W81XWH-17-1-0541, W81XWH-17-1-0542

PI: Yusuke Shiozawa, Christopher Peters

Org: Wake Forest University Health Sciences

Award Amount: \$500,001 (Shiozawa)
\$496,403 (Peters)

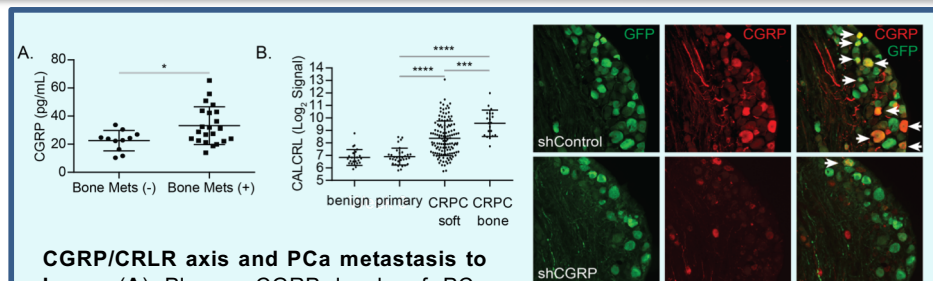


Study/Product Aim(s)

- **Aim 1: Determine how disseminated prostate cancer is involved in development of cancer-induced bone pain.**
- **Aim 2: Define the intracellular signaling pathways in peripheral nerves stimulated by disseminated prostate cancer.**
- **Aim 3: Determine the influence of nociceptive neurons on (a) tumor outgrowth in the bone and (b) cancer-induced bone pain.**

Approach

The goal of this project is to determine the roles of the interaction between bone metastatic prostate cancer and sensory nerves in cancer-induced bone pain and bone metastatic growth using our innovative mouse models that enable us to measure cancer bone pain, tumor growth, and bone dynamics within the same animals. In addition, using biopsy samples from patients, we will verify the results from the animal experiments.



CGRP/CRLR axis and PCa metastasis to bone. (A) Plasma CGRP levels of PCa patients without and with bone metastasis. (B) Expression of CRLR (gene name CALCRL) in benign prostatic hyperplasia, primary PCa, castration-resistant PCa (CRPC) with soft tissue metastases, and CRPC with bone metastases. * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Nerve-targeted CGRP knockdown with AAVrh10. GFP-tagged AAVrh10 containing shCGRP or control shRNA (shControl) was injected into mice intrathecally. At 4 weeks, DRGs were collected to determine CGRP KD mediated by shCGRP. $\times 20$.

- Prostate cancer patients with bone metastases have higher levels of neuropeptide in serum and its receptor in tumor.
- Neuropeptides in nerves innervating bone was downregulated.

Timeline and Cost

Activities	CY	17	18	19	20
Aim 1 (Animal Studies)					
Aim 2 (Animal Studies)					
Aim 2 (Bone sample analyses)					
Aim 3 (Animal Studies)					
Estimated Budget (\$K)		\$100	\$325	\$325	\$246

Updated: 10/18/2018

Goals/Milestones (Example)

CY17 Goal – Team development

- ☒ Set up and organize the monthly meeting

CY18 Goals – Animal studies

- ☒ Develop the technique to manipulate gene in nerves with vector
- ☒ Determine the roles of nerves in cancer progression

CY19 Goal – Bone biopsy sample analyses/Animal studies

- ☐ Develop the system to assess bone biopsy samples
- ☐ Determine the roles of nerves in cancer progression
- ☐ Determine the roles of cancer in cancer-induced bone pain

CY20 Goal – Bone biopsy sample analyses/Animal studies

- ☐ Determine the roles of cancer in cancer-induced bone pain
- ☐ Assess the nerve/cancer interaction in bone biopsy samples

Comments/Challenges/Issues/Concerns

- Nothing to report.

Budget Expenditure to Date

Projected Expenditure: \$400K

Actual Expenditure: \$366,444

SCIENTIFIC REPORTS

OPEN

Adeno-associated virus serotype rh10 is a useful gene transfer vector for sensory nerves that innervate bone in immunodeficient mice

Sun H. Park¹, Matthew R. Eber¹, Shunsuke Tsuzuki¹, Mary E. Booker¹, Aaron G. Sunil^{1,2}, D. Brooke Widner¹, Renee A. Parker³, Christopher M. Peters³ & Yusuke Shiozawa¹ 

Adeno-associated virus (AAV) is frequently used to manipulate gene expression in the sensory nervous system for the study of pain mechanisms. Although some serotypes of AAV are known to have nerve tropism, whether AAV can distribute to sensory nerves that innervate the bone or skeletal tissue has not been shown. This information is crucial, since bone pain, including cancer-induced bone pain, is an area of high importance in pain biology. In this study, we found that AAVrh10 transduces neurons in the spinal cord and dorsal root ganglia of immunodeficient mice with higher efficacy than AAV2, 5, 6, 8, and 9 when injected intrathecally. Additionally, AAVrh10 has tropism towards sensory neurons in skeletal tissue, such as bone marrow and periosteum, while it occasionally reaches the sensory nerve fibers in the mouse footpad. Moreover, AAVrh10 has higher tropic affinity to large myelinated and small peptidergic sensory neurons that innervate bone, compared to small non-peptidergic sensory neurons that rarely innervate bone. Taken together, these results suggest that AAVrh10 is a useful gene delivery vector to target the sensory nerves innervating bone. This finding may lead to a greater understanding of the molecular mechanisms of chronic bone pain and cancer-induced bone pain.

The peripheral ends of sensory nerves express various ion channels and receptors¹. Despite extensive research characterizing the function of these proteins in nociceptive and sensory processing, there is still a lot that is unclear regarding specialized roles of unique ion channels and receptors in different types of tissue. To dissect the role of these proteins in pain signaling it is useful to modulate the level of candidate genes expressed in sensory neurons. Although lentivirus, adenovirus, herpes-simplex virus (HSV), and adeno-associated virus (AAV) have all been widely used to deliver genes or short hairpin RNA (shRNA) to the nervous system^{2–4}, AAV is often considered the preferred and safest method^{5,6}. Several key traits of AAV are largely responsible for this preference: (1) AAV can widely diffuse throughout tissues, since its genome is smaller than other viruses⁷, (2) AAV has the ability to induce robust gene expression in organs/tissues of interest, since high titer virus production and purification are easily achieved⁸, (3) Gene manipulation following a single injection of AAV can induce sustained gene expression for months to years in the targeted organs/tissues^{9–11}, and (4) AAV is associated with only mild immune responses¹².

An important but understudied area of pain biology is cancer-induced bone pain (CIBP). CIBP is the most common complication associated with bone metastatic disease, which occurs in 70–80% of the patients with advanced breast and prostate cancer, and 30–40% of patients with lung cancer^{13,14}, resulting in a significant impairment of quality of life. Unfortunately, currently available treatments for CIBP, including opioids and non-steroidal anti-inflammatory drugs (NSAIDs), come with severe side effects as well as the risk of abuse and addiction^{15–17}. Therefore, it is critical to understand the underlying mechanisms of CIBP in order to develop more effective and safer therapies. Previous studies in rodents demonstrate an extensive innervation of skeletal tissue including bone marrow, mineralized bone, and periosteum by sensory neurons^{18–20}, and growing evidence has demonstrated that cancer growth in bone increases the sprouting of sensory and sympathetic nerves innervating

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bone which may contribute to CIBP^{21–23}. However, the extent to which the interaction between bone metastatic cancer cells and nociceptive neurons influences CIBP or disease progression is still unknown.

To better define the molecular mechanisms of CIBP development, there is a great need for methods that allow gene delivery to the sensory nerves that innervate bone. Several serotypes of AAV have been identified with nerve tropic signatures, but it is still unknown which AAV serotype is capable of or efficiently targets sensory nerves that innervate bone. In this study, we performed intrathecal injections of several serotypes of AAV and found that AAVrh10 has tropism for sensory nerves that innervate the bones of immunodeficient mice. Immunodeficient mice were chosen for this study because of the widespread use of xenograft models in the study of human disease in mice. Although further studies are clearly warranted, the use of AAVrh10 may open new avenues in the understanding of the molecular mechanisms of CIBP.

Results

AAVrh10 has highest tropism to sensory nerves. To determine which AAV serotype has superior gene transduction efficiency to sensory nerves innervating bones, we chose six different serotypes (AAV2, 5, 6, 8, 9, and rh10) which exhibit some degree of nerve tropism *in vivo*^{24–27}. Green fluorescent protein (GFP)-expressing AAVs were inoculated into severe combined immunodeficiency (SCID) mice by intrathecal injection, which is known to be an efficient delivery system to transfer viral vectors into the lumbar spinal cord and dorsal root ganglia (DRG)^{25,28}. Four weeks after AAV inoculation, lumbar spinal cord and DRG were dissected for immunohistochemical analyses. Higher levels of transduction were observed in the dorsal horn of the spinal cord obtained from mice inoculated with AAV9 and rh10, whereas minimal or no virus transduction was detected in mice inoculated with AAV2, 5, 6, or 8 (Fig. 1A). Next, tropism to DRG was assessed in L3 DRGs. As was seen in the spinal cord, less efficient transduction was observed in the DRG obtained from mice inoculated with AAV2, 5, 6, or 8 (Fig. 2A and B). Interestingly, AAVrh10 showed significantly higher transduction efficiency in the DRG when compared to other serotypes, including AAV9 (Fig. 2B). Additionally, higher transduction efficiency was observed in the L2 and L4 DRGs obtained from mice inoculated with AAVrh10, compared to those from mice inoculated with AAV6 and 9, although there were no statistical differences (Fig. 2C and D). We therefore chose AAVrh10 for further analyses.

AAVrh10 predominantly transduces myelinated and non-myelinated peptidergic sensory nerves. It has been shown previously that the mouse femur is preferentially innervated by the myelinated (neurofilament 200 [NF200] positive) and non-myelinated peptidergic (calcitonin gene-related peptide [CGRP] positive) classes of sensory afferents, but not non-peptidergic (isolectin B4 [IB4] positive) sensory neurons^{18,29}. To determine which subpopulation of sensory nerves are transduced by AAVrh10, the lumbar spinal cord and DRG were stained with NF200, CGRP, or IB4 (Fig. 3A). We quantified the percentage of sensory neurons that expressed GFP within each subpopulation (Fig. 3B) and the percentage of total GFP positive cells that co-expressed each marker (Fig. 3C). AAVrh10 demonstrated robust transduction of sensory neurons including both NF200 positive and CGRP positive sensory neurons, but significantly fewer IB4 positive sensory neurons. When we conducted subtype analysis following injection of other serotypes, although we saw a similar pattern of distribution (NF200 > CGRP > IB4), our decision to focus on AAVrh10 was affirmed by the fact that none of the other serotypes had both high transduction of NF200 (>60%) and CGRP (>40%) positive neurons, but significantly reduced transduction of IB4 (<25%) positive neurons (Fig. 3D). Within the spinal cord, GFP expression co-localized with CGRP positive afferent sensory neurons in the dorsal horn, and less with IB4 positive afferent sensory neurons (Fig. 4), similar to the DRG. Since NF200 is highly expressed through the entire spinal cord, we were not able to perform co-localization between NF200 and GFP specific to myelinated sensory nerves in the dorsal horn of spinal cord (Data not shown). These data suggest that AAVrh10 has specific tropism to the NF200 positive nerve fibers and CGRP positive nerve fibers.

AAVrh10 transduces sensory nerve fibers that innervate bone. To investigate whether AAVrh10 can transduce genes in the peripheral sensory nerve fibers innervating bones, we assessed GFP immunoreactivity in femurs. GFP-expressing nerve fibers were observed in the bone marrow and periosteum (Fig. 5). Additionally, these GFP-expressing nerve fibers were co-localized with a specific marker for sensory neurons, PGP9.5 (Fig. 5). Finally, to examine how far AAVrh10 diffuses in peripheral nerve fibers, GFP expression was assessed in the paw skin. Most of the sensory nerve fibers in the paw skin were not transduced by AAVrh10, however GFP-expressing nerve fibers were occasionally observed (Fig. 6A and B), and some of them were co-localized with PGP9.5 positive sensory nerves (Fig. 6A). Analysis of the degree of co-localization between PGP 9.5 and GFP within each tissue revealed that a greater percentage of sensory nerves were transduced by AAVrh10 in the bone compared to the glabrous skin in the hindpaw (Fig. 6C). These data suggest that AAVrh10 can transduce the peripheral sensory nerves, and more predominantly those innervating bone.

Discussion

In this study, we investigated tropism of AAV serotypes to sensory neurons innervating bone. GFP-expressing AAV vectors (AAV2, 5, 6, 8, 9, and rh10) were administered to immunodeficient mice through intrathecal injection. We found that AAVrh10 transduces the sensory neurons in the DRG and spinal cord with higher efficiency than AAV2, 5, 6, 8, and 9. Additionally, AAVrh10 showed high tropism for the NF200 positive large myelinated and CGRP positive small peptidergic sensory neurons that innervate bone. Taken together, our findings suggest that AAVrh10 is a preferred serotype for delivering genes to sensory nerves innervating bone.

It has been appreciated that AAV9 and AAVrh10 have higher transduction efficiency for the central and peripheral nervous systems, compared to other serotypes of AAV^{30–32}. Additionally, these two serotypes do not require mannitol pretreatments to enhance penetration of AAV vectors to the neurons, while others do^{28,33},

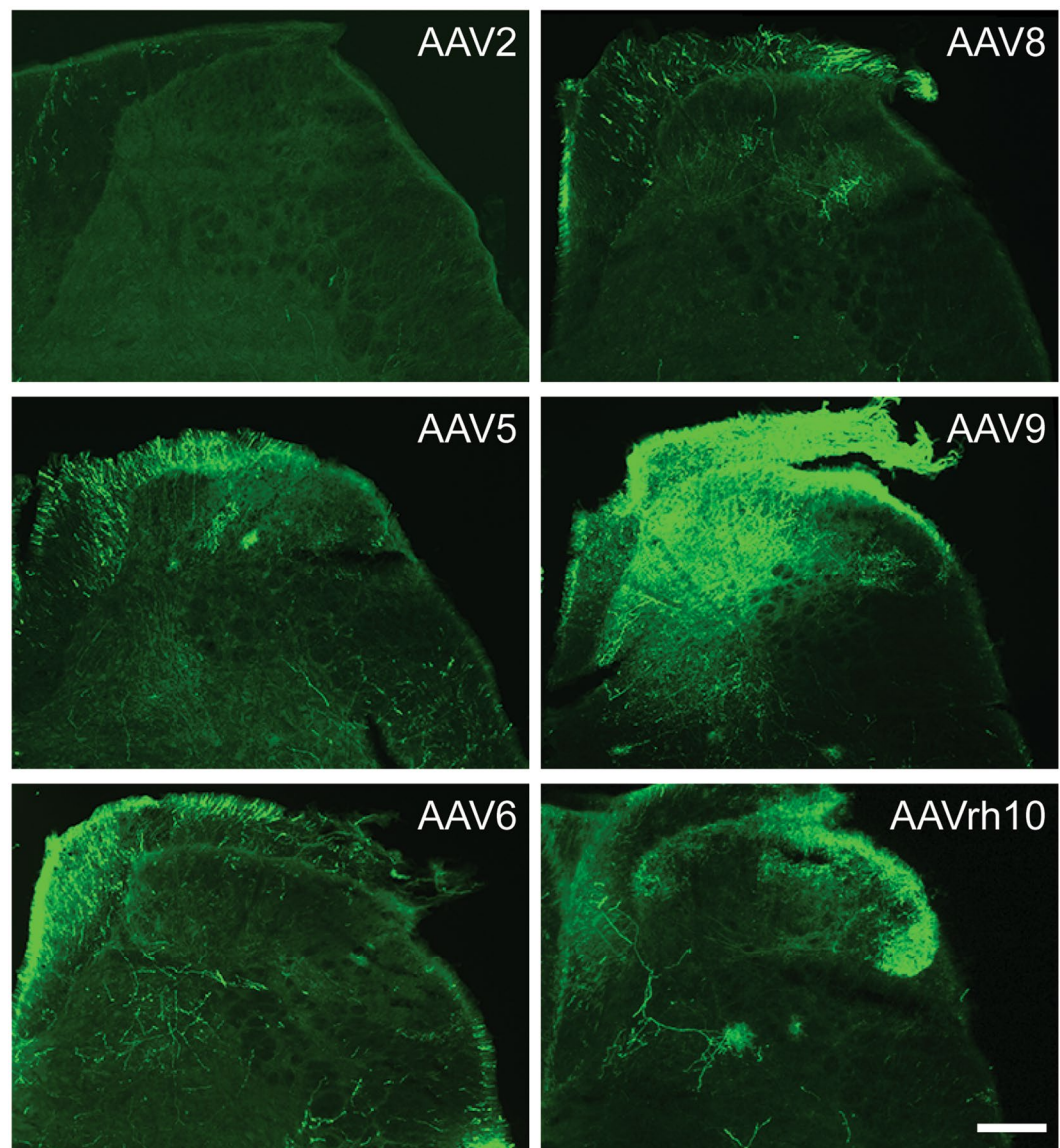


Figure 1. Distribution of AAV vectors in spinal cord. GFP-tagged AAV vectors (AAV2, 5, 6, 8, 9, and rh10) were placed intrathecally between the L4/5 vertebrae of immunodeficient mice by percutaneous lumbar puncture. At 4 weeks, spinal cords were collected for immunofluorescence analyses for GFP positivity to determine AAV distribution. Representative image of spinal cords obtained from AAV injected mice. Magnification, $\times 20$. Scale Bar = 100 μm .

further suggesting their higher affinity to central and peripheral neurons. We also found that transduction of these two vectors were more efficient in immunodeficient mouse spinal cords, when compared to AAV2, 5, 6, and 8. In DRG, there was significantly higher transduction efficiency in AAVrh10 (43.8%) than AAV9 (27.5%). Importantly, in addition to sensory neuron transduction, AAV9 is known to develop robust motor neuron transduction³⁴, and it has been shown that AAVrh10 can also spread to motor neurons when injected intrathecally^{35,36}. Likewise, in this current study, AAVrh10 occasionally transduced motor neurons in the spinal cord, but in those cases transduction efficiency was extremely low (data not shown). Therefore, these findings indicate that in our hands, AAVrh10 was the best AAV vector at targeting sensory nerves in this model.

We are aware that there may be concerns regarding the specificity of gene transduction of AAV vectors to sensory neurons. One strategy to address this issue is to develop sensory nerve specific gene-targeted animals using the Cre-flox system (e.g. crossbreeding between Advillin-Cre animals and animals with a floxed gene of interest)^{37,38}. However, these models might cause off-target effects as changes in gene expression may occur in all sensory nerves. In terms of targeting specific types of sensory nerves (e.g. sensory nerves innervating bones), gene delivery systems using a viral vector may oftentimes be more useful. In fact, AAV delivery of Cre is a strategy that could circumvent this crucial issue of specificity. In this current study, we found that AAVrh10 has higher affinity to myelinated and small peptidergic sensory neurons, compared to small non-peptidergic sensory neurons. On the other hand, it has been shown that AAV9 transduces not only peptidergic sensory neurons, but also

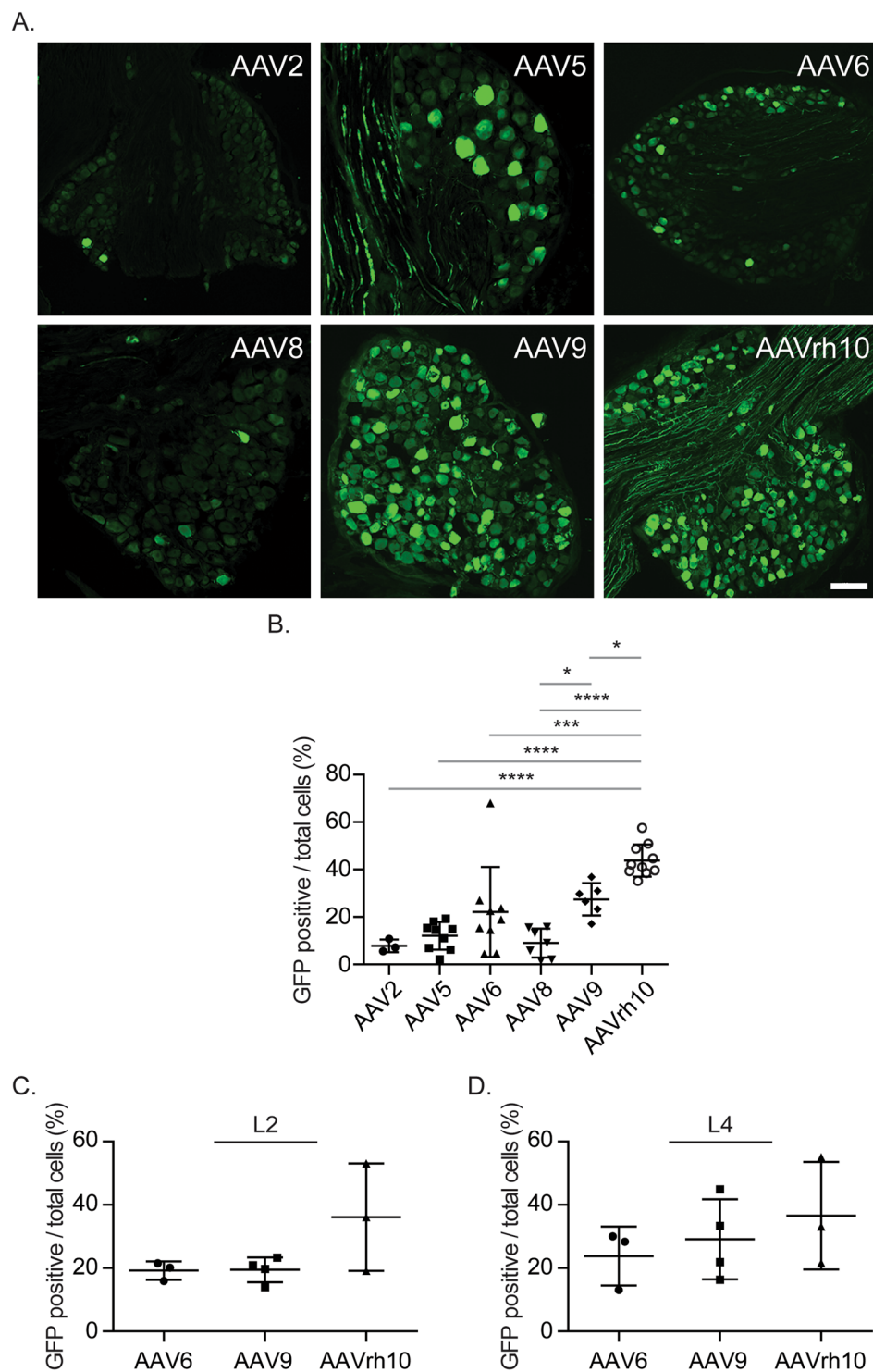


Figure 2. Distribution of AAV vectors in DRG. GFP-tagged AAV vectors (AAV2, 5, 6, 8, 9, and rh10) were placed intrathecally between the L4/5 vertebrae of immunodeficient mice by percutaneous lumbar puncture. At 4 weeks, DRGs were collected for immunofluorescence analyses for GFP positivity to determine AAV distribution. **(A)** Representative image of L3 DRGs obtained from AAV injected mice. Magnification, $\times 20$. Scale Bar = 100 μm . **(B)** Quantifications of **(A)**. **(C,D)** Quantifications of GFP positivity of **(C)** L2 and **(D)** L4 DRGs obtained from AAV injected mice. Presented as mean $\pm$ standard deviation. Significant differences were determined by one-way ANOVA with Tukey's multiple comparisons. $*p \leq 0.05$, $***p \leq 0.0001$, $****p \leq 0.00001$.

non-peptidergic sensory neurons²⁸. Since the fibers that innervate bones are mainly myelinated and small peptidergic sensory neurons, and rarely non-peptidergic sensory neurons^{18,29}, our findings suggest that AAVrh10 can be an alternative strategy for preferential targeting of sensory neurons in bone.

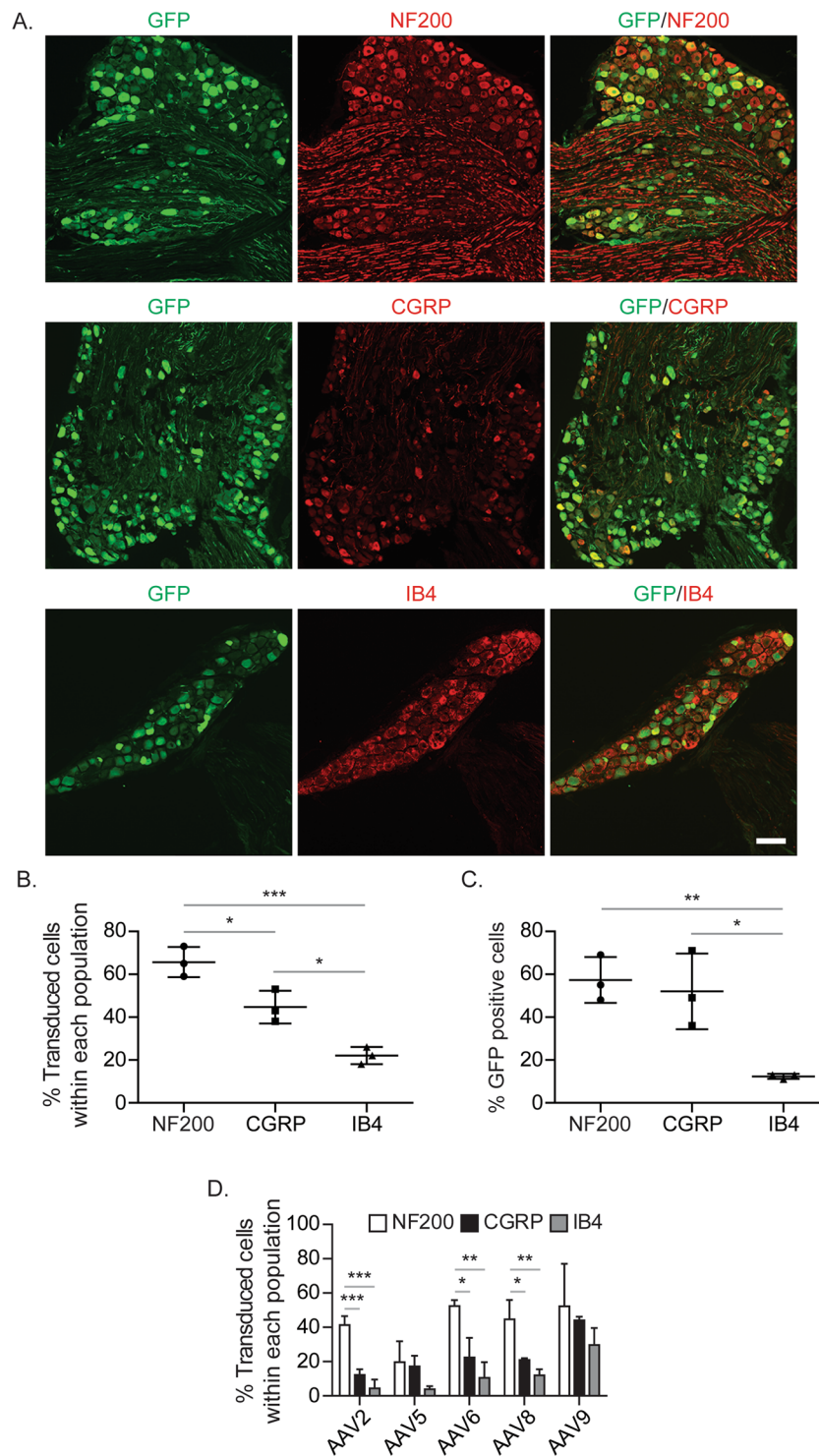


Figure 3. Subpopulations of sensory nerves in DRG transduced by AAVrh10. GFP-tagged AAVrh10 vector was placed intrathecally between the L4/5 vertebrae of immunodeficient mice by percutaneous lumbar puncture. At 4 weeks, L3 DRGs were collected for immunofluorescence analyses to verify expression in subpopulations of sensory neurons. **(A)** Representative image of co-localization of GFP positive cells, and myelinated neurofilament 200 (NF200), non-myelinated peptidergic calcitonin gene-related peptide (CGRP), or non-peptidergic isolectin-B4 (IB4) positive cells. Magnification, $\times 20$. Scale Bar = 100 μm . **(B)** Quantification of the percentage of DRG cells transduced within different populations of sensory neurons (NF200, CGRP, or IB4). **(C)** Quantification of the percentage of total GFP positive DRG cells that co-expressed each marker. **(D)** Quantification of the percentage of DRG cells transduced within different populations of sensory neurons in the L3 DRGs obtained from AAV injected mice. Presented as mean $\pm$ standard deviation. Significant differences were determined by one-way ANOVA with Tukey's multiple comparisons. $*p \leq 0.05$, $**p \leq 0.001$, $***p \leq 0.0001$.

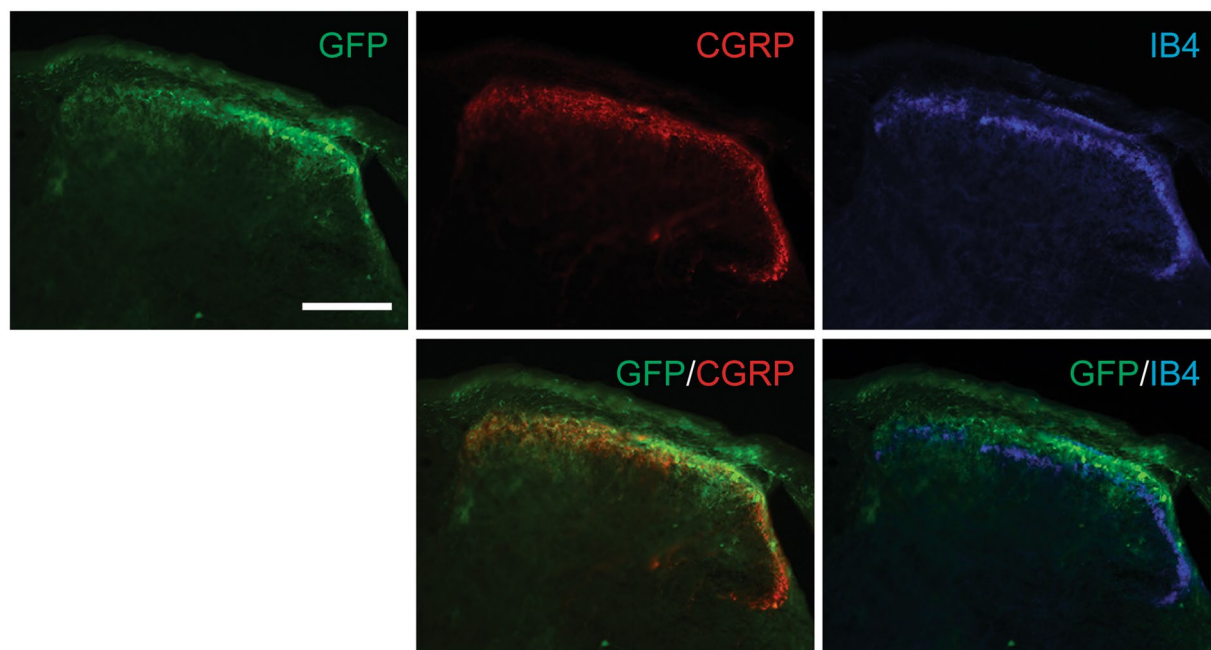


Figure 4. Subpopulations of sensory nerves in spinal cord transduced by AAVrh10. GFP-tagged AAVrh10 vector was placed intrathecally between the L4/5 vertebrae of immunodeficient mice by percutaneous lumbar puncture. At 4 weeks, spinal cords were collected for immunofluorescence analyses to verify expression in subpopulations of sensory neurons. Representative image of co-localization of GFP, CGRP, and IB4 in spinal cord. Magnification, $\times 20$. Scale Bar = $100\ \mu\text{m}$.

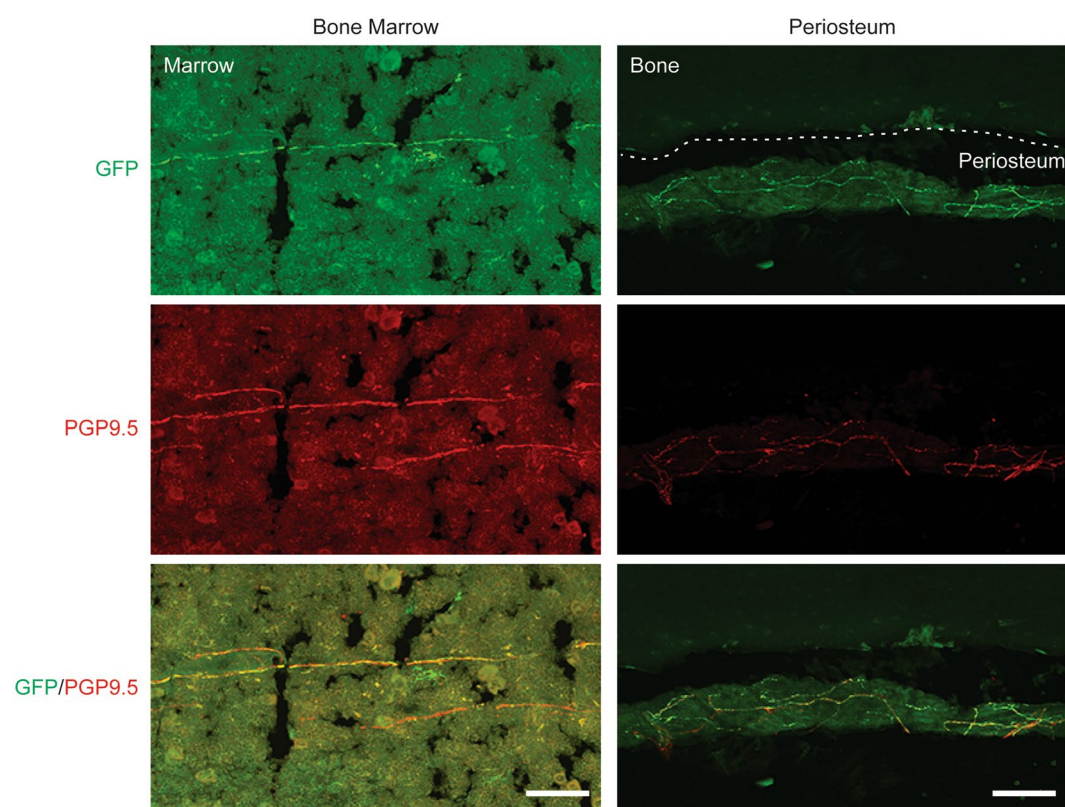


Figure 5. Distribution of AAVrh10 vector to sensory nerves in skeletal tissues. GFP-tagged AAVrh10 vector was placed intrathecally between the L4/5 vertebrae of immunodeficient mice by percutaneous lumbar puncture. At 4 weeks, femurs were collected for immunofluorescence analyses for GFP positivity to determine AAV distribution to sensory neurons. Representative image of co-localization of GFP and PGP9.5 in bone marrow and periosteum. Magnification, $\times 20$. Scale Bar = $100\ \mu\text{m}$.

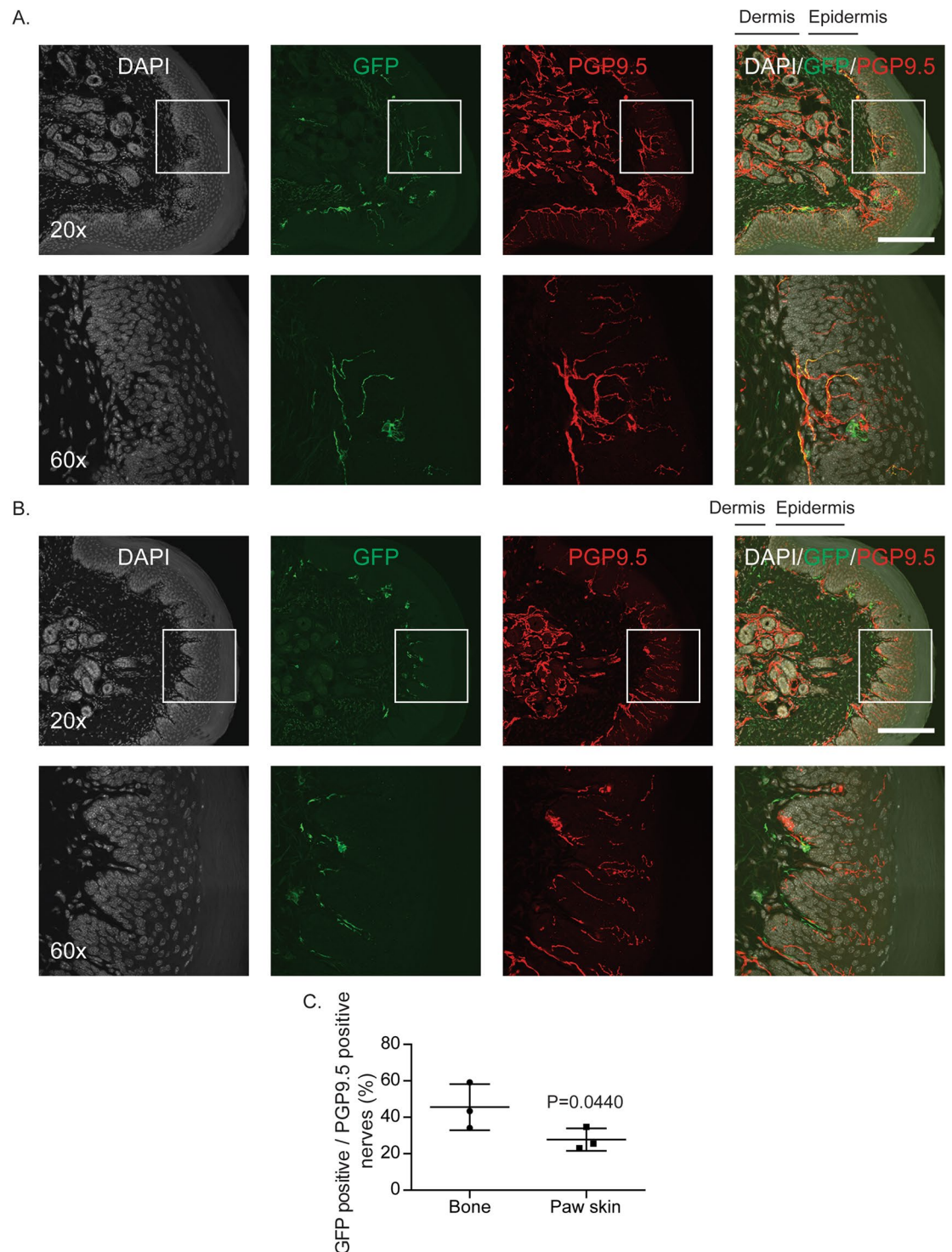


Figure 6. Distribution of AAVrh10 vector to sensory nerves in paw skin compared to those in skeletal tissue. GFP-tagged AAVrh10 vector was placed intrathecally between the L4/5 vertebrae of immunodeficient mice by percutaneous lumbar puncture. At 4 weeks, glabrous skin from the hindpaw was collected for immunofluorescence analyses for co-localization of GFP and PGP9.5 to determine AAV distribution in sensory neurons. (A) Representative images of GFP and PGP9.5 co-localization in paw skin sections. (B) Representative images of GFP and PGP9.5 without co-localization in paw skin sections. Scale Bar = 100 μ m. (C) Quantifications of GFP positive sensory nerves over PGP9.5 positive sensory nerves between bones (Fig. 5) and paw skins (Fig. 6A and B) obtained from AAVrh10 injected mice. Presented as mean $\pm$ standard deviation, Significant differences were determined by Student's *t*-test.

Vector	Titer (VG/ml)
AAV2.CMV.eGFP	6.48×10^{12}
AAV5.CMV.eGFP	1.59×10^{13}
AAV6.CMV.eGFP	3.15×10^{12}
AAV8.CMV.eGFP	5.40×10^{13}
AAV9.CMV.eGFP	5.24×10^{13}
AAVrh10.CMV.PL.eGFP.WPRE.bGH	4.16×10^{12}

Table 1. The titer of injected AAV vector.

Bone pain is a major clinical problem in patients with bone metastases, and the complex interactions between bone metastatic cancer cells and sensory nerves make it difficult to treat. Gene therapy using AAV vectors are currently being tested in patients with neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Canavan disease^{39–41}. Subsequently, AAV gene therapy has been considered a potential alternative treatment for chronic pain without unwanted side effects. Moreover, AAVrh10 is being pursued as an attractive alternative to AAV2, which is the most frequently used serotype in clinical trial settings^{42,43}. AAVrh10 was isolated from the rhesus monkey, while other serotypes including AAV2 were discovered in humans^{11,44–46}. Therefore, neutralizing antibodies against AAVrh10 are less likely to be generated in human serum. Although further studies regarding its efficacy, safety, and long-term effects in a clinical setting are still warranted, a gene delivery system using AAVrh10 to target sensory nerves innervating bones may prove to be a better treatment strategy to improve the quality of life of cancer patients with bone metastases.

Materials and Methods

Adeno-associated virus (AAV). The AAV2, 5, 6, 8, and 9 CMV-eGFP vectors generated using a Sf9/baculovirus system were purchased from the Iowa Viral Vector Core Facility of University of Iowa (Iowa City, IA). The AAVrh10 CMV-eGFP virus generated in HEK293 cells was purchased from the Penn Vector Core in the Gene Therapy Program of the University of Pennsylvania (Philadelphia, PA). The titer of each virus was within a range from 3×10^{12} to 6×10^{13} vector genome (VG)/ml (Table 1).

In vivo intrathecal inoculation of AAV. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Wake Forest School of Medicine, and performed in accordance with their guidelines and regulations. AAV-eGFP virus (10 μ l) was injected intrathecally between the L4/5 vertebrae by percutaneous lumbar puncture into male hairless SCID congenic mice (6–8 weeks old; CB17. Cg-Prkdc^{scid}Hr^{hr}/lcrCrl; Charles River Laboratories, Wilmington, MA), using a 30-gauge needle connected to a connecting joint (Eicom, cat #: JB-30, San Diego, CA) and a Hamilton syringe (Hamilton, Reno, NV). The appropriate insertion of the needle into the intrathecal space was confirmed by tail-flick prior to administration of virus into the subarachnoid space. Four weeks after the virus injection, mice were perfused with 4% paraformaldehyde (PFA); and the lumbar spinal cords, (L2–L4) DRGs, femurs, and hind paw skins were dissected and prepared for immunofluorescence assays.

Tissue processing. The spinal cord, DRG, and paw skin sections were post-fixed with 4% PFA for 6 hours at 4 °C, and cryoprotected with 30% sucrose solution (in phosphate-buffered saline, PBS) for 48 hours at 4 °C. The tissues were then embedded in Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Finetek USA, Torrance, CA), and frozen on dry ice. Frozen spinal cord, DRG, and paw skin were thaw-mounted on Super Frost non-adhesion glass slides (Fisher Scientific, Hampton, NH) at 40 μ m thickness, Super Frost non-adhesion glass slides coated with gelatin (Fisher Scientific) at 16 μ m thickness, and gelatin-coated glass slides at 20 μ m thickness, respectively.

The excess muscles were removed from the dissected femurs, using surgical scissors and a scalpel without disturbing the periosteum around the femur. The femurs were post-fixed with 4% PFA for 6 hours, and then decalcified with 10% ethylenediaminetetraacetic acid (EDTA, in pH 7.3 PBS), for 14 days (the decalcification solution was replaced on day 8) at 4 °C. After decalcification, the femurs were cryoprotected with 30% sucrose solution (in PBS) for 48 hours at 4 °C. The day before freezing on dry ice, the femurs were embedded in OCT compound and incubated overnight at 4 °C. The frozen femurs were thaw-mounted on gelatin-coated glass slides at 20 μ m thickness.

Immunofluorescence assays. Spinal cord was stained using the free-floating method, as previously described²¹, while DRG, bone, and paw skin were stained on the slide. After incubating with blocking buffer (3% Normal Donkey serum [Jackson ImmunoResearch, West Grove, PA], and 0.3% Triton X-100 [Sigma-Aldrich, St. Louis, MO] in PBS) for 1 hour at room temperature; spinal cord, DRG, bone, and paw skin sections were stained with chicken anti-GFP antibody (1:1000, Invitrogen, cat #: A10262, Carlsbad, CA) to enhance the eGFP signal. Bone and paw skin sections were co-stained with anti-PGP9.5 antibody (1:500; Proteintech, cat #: 14730-1-AP, Rosemont, IL). In some cases, spinal cord and DRG sections were co-stained with either mouse anti-NF200 antibody (1:2,000, Abcam, cat #: ab134459, Cambridge, MA), rabbit anti-CGRP antibody (1:10,000, Sigma-Aldrich, cat #: C8198), or biotinylated IB4 antibody (1:2,500, Sigma-Aldrich, cat #: L2140). These primary antibodies were incubated overnight at 4 °C with gentle agitation. Thereafter, secondary antibodies (anti-rabbit CY3 [1:600, Jackson ImmunoResearch], anti-chicken CY2 [1:500, Jackson ImmunoResearch], or anti-streptavidin CY5 [1:400, Jackson ImmunoResearch]) were applied, based on primary antibodies used, for 2 hours at room

temperature. DRG, bone, and paw skin sections were mounted in ProLong™ Gold antifade mount (Fisher Scientific) with DAPI for nuclei labeling. Spinal cord sections were dehydrated using different gradients of ethanol (70, 90, and 100%, for 2 minutes each), cleared with xylene (twice for 2 minutes each), and coverslipped.

Imaging and quantification. Immunofluorescence assays were performed using a Nikon Eclipse Ni fluorescent microscope system (Nikon, Tokyo, Japan) or Olympus FV1200 SPECTRAL Laser scanning confocal microscope (Waltham, MA). For DRG quantification, 4–5 DRG sections from each mouse ($n = 3$) were randomly selected, and GFP, NF200, CGRP, and IB4 positive DRG cells were counted, using an image analysis software, Nikon Elements V4.13 Basic Research. Data were expressed as percentage of the total number of DRG cells that expressed a given marker. For bone and paw skin quantification, 5–6 bone and 3–4 skin sections from each mouse ($n = 3$) were randomly selected, and GFP or PGP 9.5 positive area was determined within the same region of interest, using an image analysis software, Nikon Elements V4.13 Basic Research. The data were expressed as percentage of GFP positive area over PGP9.5 positive area.

Statistical analysis. Numerical data are expressed as mean $\pm$ standard deviation. 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 $P \leq 0.05$.

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Author Contributions

S.H.P. and Y.S. wrote the manuscript with input from all authors. S.H.P., M.R.E., C.M.P., and Y.S. designed study and contributed to data interpretation. S.H.P., M.R.E., S.T., D.B.W., C.M.P., and Y.S. performed animal surgeries. S.H.P., M.R.E., M.E.B., A.G.S., and R.A.P. performed immunohistochemistry and quantification analyses. C.M.P. and Y.S. supervised the study. All authors reviewed and approved the final manuscript.

Additional Information

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Bone Marrow Microenvironment as a Regulator and Therapeutic Target for Prostate Cancer Bone Metastasis

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Abstract Bone is the most common site of prostate cancer metastasis. Once prostate cancer cells metastasize to bone, the mortality rate of prostate cancer patients increases significantly. Furthermore, bone metastases produce multiple skeletal complications, including bone pain that impairs the patients' quality of life. Effective therapies for bone metastatic disease are underdeveloped with most current therapies being primarily palliative with modest survival benefit. Although the exact mechanisms through which prostate cancer metastasizes to bone are unclear, growing evidence suggests that the bone marrow microenvironment, particularly its hematopoietic activity, is a significant mediator of prostate cancer bone tropism. Moreover, the bone microenvironment may regulate metastatic prostate cancer cells between dormant and proliferative states. In this review, we discuss (1) how prostate cancer cells interact with the bone microenvironment to establish bone metastases and (2) current and future potential treatments for prostate cancer patients with bone metastases.

Keywords Prostate cancer · Bone metastasis · Bone-targeted therapy · Bone marrow microenvironment · Skeletal complications

Introduction

The 5-year and 10-year overall survival rates for prostate cancer in the setting of treated non-metastatic local regional disease are nearly 100 and 98%, respectively [1]. This high survival rate has brought attention to the possible over-diagnosis and over-treatment of prostate cancer, resulting in a shift of treatment strategies for patients with low-risk and low-volume prostate cancer. Specifically, patients may be offered active surveillance which entails close observation with frequent prostate-specific antigen (PSA) testing and scheduled biopsies [2]. However, not all prostate cancer patients fit this paradigm and some on active surveillance may progress to metastatic disease. Once prostate cancer metastasizes, the mortality rate of prostate cancer patients significantly increases.

Bone is the most frequent site of prostate cancer metastases with up to 90% of patients with advanced disease having bone metastases [3]. The exact mechanisms that favor bone metastasis versus other sites remain to be elucidated. The prostate is a highly vascularized organ that has a vascular drainage to the bones of the vertebral column. Specifically, the prostatic venous plexus drains into the internal iliac vein, which connects to the vertebral venous plexus. Since the vertebral venous plexus is present throughout the entire spinal column, this may be one of the major routes of prostate cancer bone metastasis [4]. Bone metastatic cells commonly target red marrow, which is typically found in trabecular bones [5]. The highly vascular trabecular structure of the bone provides a welcoming

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environment for bone metastatic cancer cells to colonize the bone including easy access to oxygen and other nutrients [6]. Besides anatomical properties of the bone itself and its relation to the prostate, intrinsic properties of primary tumor such as their unique gene expression profiles are believed to influence bone metastatic process. For example, high levels of cyclin A1 are observed in the bone metastatic lesion of prostate cancer patients which is consistent with the possibility that stem cell-like prostate cancer cells, which overexpress cyclin A1, preferentially metastasize to the bone [7]. In addition, the expression of receptor activator of nuclear factor κ -B (NF κ B) ligand (RANKL) in prostate cancer cells promoted their dissemination and colonization to the bone [8]. While these oncogenic properties of tumor cells are essential for metastatic progression, growing evidence has also suggested that the unique microenvironment of bone (e.g., the cells of hematopoietic origin and the cells that control bone remodeling) plays important roles in prostate cancer bone metastasis [9–11].

Prostate cancer is no longer curable once bone metastases manifest. The median time to death after development of metastases is approximately 5 years [12]. Unfortunately, currently available treatments for bone metastasis are primarily palliative, providing improvement to the patient's quality of life and may provide modest survival benefits. Bone metastases are not only incurable, but also very destructive. Bone metastases can lead to serious complications including hypercalcemia, incapacitating bone pain, pathological fracture, and spinal cord compression [13]. These can manifest as medical emergencies as spinal cord compression can lead to permanent paralysis and loss of limb function and mobility [13]. It is therefore urgent to develop effective therapies for improving both overall survival and quality of life of patients with bone metastases.

In this review, we will discuss the major mechanisms of prostate cancer bone metastasis with a focus on the interaction of prostate cancer with bone marrow microenvironment. Additionally, we will overview the current strategies and future directions in the treatments for prostate cancer patients with bone metastases.

Mechanisms of Prostate Cancer Bone Metastasis

Homing to the Bone

After undergoing epithelial-to-mesenchymal transition (EMT), cancer cells begin to escape from the primary tumor site and enter blood circulation. To extravasate into a secondary site they must first adhere to endothelial cells. During their dissemination to the bone, prostate cancer

cells interact with the bone marrow endothelial cells (BMECs). It has been demonstrated that E-selectin ligand on prostate cancer cells and E-selectin on endothelial cells contribute to the adhesion between the prostate cancer cells and BMECs [14–16]. CD44 expressed by prostate cancer cells has also been demonstrated to be involved in adhesion to endothelial cells by binding to vascular cell adhesion molecule 1 (VCAM-1), and this adhesion can be enhanced by interleukin-17 (IL-17), insulin, and insulin-like growth factor 1 (IGF1) [17]. Another adhesion molecule, the vitronectin receptor $\alpha_v\beta_3$ integrin, is involved in both the interaction with endothelial cells and the bone homing process of prostate cancer [18–20]. Consistent with this notion, $\alpha_v\beta_3$ integrin induces breast cancer bone metastasis, whereas the expression of $\alpha_v\beta_3$ integrin does not affect primary tumor growth [21]. Moreover, C-X-C motif chemokine ligand 12 (CXCL12), a chemokine secreted by bone marrow stromal cells or osteoblasts [22], enhances $\alpha_v\beta_3$ integrin expression in prostate cancer [20, 23], suggesting that the signal from the bone marrow microenvironment may influence bone metastatic ability of prostate cancer. These adhesion molecules, through facilitating binding of prostate cancer cells to BMECs, are important for the dissemination to bone.

In addition to regulating $\alpha_v\beta_3$ integrin expression, CXCL12 is one of the key factors to regulate hematopoietic stem cell (HSC) homing to the bone marrow [24]. Intriguingly, the mechanisms of prostate cancer bone dissemination are similar to the HSC homing. Both HSC and prostate cancer cells express the CXCL12 receptor C-X-C chemokine receptor type 4 (CXCR4) [25, 26] and/or CXCR7 [27], and migrate towards CXCL12-expressing organs, including bone marrow. When the CXCL12/CXCR4 interaction is inhibited with a CXCR4 antagonist AMD3100, the initial colonization to the bone and the growth of prostate cancer are prevented [28]. However, the same treatment fails to diminish the established bone metastatic tumor [28]. This finding suggests that CXCL12/CXCR4 axis may be involved in an early dissemination process of prostate cancer, but not later metastatic growth.

CXCL12 expression derived from tumor microenvironment within the primary tumor has also been suggested to promote the prostate cancer bone metastases. Solid tumors are known to recruit bone marrow-derived mesenchymal stem cells (MSCs) to the primary site to enhance their metastatic potential [29]. CXCL16-expressing prostate cancer cells also recruit bone marrow-derived MSCs which express CXCR6 on their surface [30]. This CXCL16/CXCR6 interaction induces the transition of MSCs to cancer-associated fibroblasts, which is validated by the high levels of CXCL12 expression. The CXCL12 released from the cancer-associated fibroblasts promotes the EMT of prostate cancer cells, resulting in the dissemination to

the bone. On the other hand, prostate cancer cells also express CXCR6. The higher CXCR6 expression levels correlate with the higher Gleason scores, and prostate cancer cells migrate towards CXCL16 [31, 32]. Interestingly, CXCL16 enhances binding of prostate cancer cells to BMECs by upregulating $\alpha_v\beta_3$ integrin expression in prostate cancer cells [31]. Additionally, the higher levels of CXCL16 are detected in bone samples obtained from prostate cancer patients with bone metastases [32, 33]. It has also been recently suggested that factors, including exosome, released from the primary tumor educate bone marrow-derived cells to promote further metastases by establishing a pre-metastatic niche at distant organs [34–36]. In fact, exosome-derived miR-192 regulates bone colonization of lung cancer by influencing endothelial cells in the marrow, leading to enhanced tumor angiogenesis [37]. Interestingly, a greater number of oncosomes, tumor-derived exosomes, in the circulation is associated with the metastatic potential of prostate cancer [38, 39]. These findings suggest that exosomes may also be involved in the dissemination process of prostate cancer to the bone. Further studies are warranted.

Once prostate cancer cells arrive in the bone, they take advantage of a unique environment where HSCs reside, known as the “HSC niche,” to survive. The HSC niche is important for the maintenance of the HSCs [24]. Prostate cancer cells compete for the occupancy of the HSC niche [40]. Once in the niche, the disseminated prostate cancer cells utilize the niche. Cells of the osteoblastic lineage compose a component of the HSC niche and have been shown to play a central role in the early bone colonization of cancer cells [41]. Disseminated prostate cancer cells bind to the osteoblastic HSC niche with the same mechanisms as HSCs. Both prostate cancer cells [42] and HSCs [43] express a receptor for Annexin II and bind to Annexin II expressed by the osteoblastic HSC niche. In addition, Annexin II regulates the CXCL12 expression of the HSC niche [44], and the Annexin II/CXCL12 interaction plays a crucial role in the regulation of prostate bone metastasis [45]. Similar to HSCs [46], niche-associated disseminated prostate cancer cells are mobilized from the niche into the circulation when treated with AMD3100 [40]. In addition to CXCL12, stem cell factor (SCF), another osteoblast-derived factor that regulates HSC function [47], may promote prostate cancer bone metastasis. Indeed, circulating tumor cells [48] and bone metastatic samples [49] obtained from prostate cancer patients express a receptor for SCF, c-Kit, or CD117, which is highly expressed in advanced prostate cancer. The effects of cabozantinib (XL184), a small molecule inhibitor which targets c-Kit, were evaluated in a phase II randomized discontinuation trial in men with castration-resistant bone metastatic prostate cancer [50]. In this study, cabozantinib significantly improved the

median progression-free survival (23.9 weeks) compared with placebo (5.9 weeks, hazard ratio 0.12; $p < 0.001$). 57% of patients treated with cabozantinib had over 50% reduction in bone turnover markers (serum total alkaline phosphatase and plasma cross-linked C-terminal telopeptide of type I collagen), 68% of patients had improvement on bone scan including 12% of complete remission, and 67% of patients had beneficial effects on bone pain. Moreover, it has been proposed that interference with the CXCL12/CXCR4 axis enhances the susceptibility of bone metastatic cancer cells to chemotherapy [51]. These findings suggest that the HSC niche can be a potential therapeutic target for prostate cancer bone metastasis.

Tumor Dormancy Within the Bone

After colonizing bone, disseminated tumor cells must adopt to the new environment to enable long-term survival [52]. In order to do so, cancer cells must control blood supply and escape from the immune system [53]. One mechanism to achieve this is cellular tumor dormancy [53]. In addition to dormancy at the cellular level, dormancy may occur at the tumor level when proliferation and apoptosis of the cells are balanced. This condition is called as “population-level tumor dormancy” (also known as “angiogenic dormancy” and/or “immune-mediated dormancy”) [53]. The tumor microenvironment can influence both population-level dormancy and cellular dormancy [53, 54].

Bone morphogenetic protein 7 (BMP7), a member of transforming growth factor (TGF)- β family, released from bone marrow stromal cells prevents bone metastatic growth by inducing prostate cancer stem cell dormancy [55]. This is achieved, in part, through BMP7’s ability to activate phosphorylation of p38, but not Erk in prostate cancer [55]. The ratio between phosphorylated p38 and phosphorylated Erk has proposed a key regulator of the balance between the dormancy and proliferation of cancer cells [56, 57]. Specifically, when phosphorylated p38 is greater than phosphorylated Erk, tumor cells tend to become dormant. Higher levels of BMP receptor type 2 (BMPR2), a receptor for BMP7, are observed in tumors obtained from prostate cancer patients without bone metastases, compared to those with bone metastases [55], suggesting that BMPR2 negatively correlates with prostate cancer bone metastatic progression. Intriguingly, it has been demonstrated that disseminated prostate cancer cells not only passively receive dormancy signals from bone marrow microenvironment, but also actively educate it to stay dormant [58]. To achieve this, dormant prostate cancer cells release secreted protein acidic and rich in cysteine (SPARC), resulting in increased BMP7 expression from bone marrow stromal cells. Furthermore, methylation in promoter

regions epigenetically silences the SPARC expression in proliferating prostate cancer cells [58].

Growth arrest-specific 6 (GAS6) is a ligand for one of tyrosine kinase receptor families, the TAM receptor family (Tyro3, Axl, MerTK), and prostate cancer expresses all of the TAM receptors. The levels of GAS6 in skeletal organs influence induction of prostate cancer dormancy. Low levels of GAS6 are observed in the bone marrow of the hindlimb where more prostate cancer bone metastases are found, compared to the bone marrow of forelimb where higher levels of GAS6 are found [59]. The GAS6 receptor Axl is upregulated in prostate cancer cells when they bind to Annexin II in the osteoblastic niche [10]. The increased Axl contributes to a dormant state and drug resistance of bone metastatic prostate cancer [10]. In addition, dormant disseminated prostate cancer cells express higher levels of Axl, compared to proliferating cells [60]. However, higher levels of Tyro3 are found in the proliferating prostate cancer cells [60]. Moreover, when the MerTK expression is down-regulated, prostate cancer becomes dormant by decreasing a ratio of phosphorylated Erk to phosphorylated p38 [61]. These findings suggest that GAS6 secreted by the osteoblastic HSC niche is also involved in the induction process of prostate cancer dormancy through its receptors.

Metastatic Outgrowth Within the Bone

To establish clinically detectable metastasis, disseminated prostate cancer cells must exit from dormancy. This may occur after a decade or more of disease-free survival. Although bone metastases of prostate cancer are mainly thought to be osteoblastic, osteoclastic activity is also involved in bone metastatic progression and metastatic outgrowth. Bone is a unique organ that is actively remodeled. In normal physiology, to maintain bone remodeling, osteoblasts and osteoclasts influence one another. Once disseminated tumor cells come in the marrow environment, they interfere with this interaction. This feedback loop among disseminated tumor cells, osteoblasts, and osteoclasts is known as “vicious cycle,” which is believed to be a major mechanism of bone metastatic outgrowth [62].

A major mediator of bone remodeling is RANKL. In the marrow, RANKL, mainly expressed by osteoblasts, osteocytes, and stromal cells, binds to its receptor RANK on the surface of osteoclast progenitors, resulting in osteoclast differentiation and bone resorption [63]. In addition, bone resorption promotes the release of growth factors that activate osteoblastogenesis, including TGF- β and BMPs, from the bone matrix [63]. To balance osteoclast activities, mature osteoblasts and stromal cells also secrete osteoprotegerin (OPG), a soluble decoy RANKL receptor, which prevents RANKL/RANK interaction [63].

It has been demonstrated that disseminated prostate cancer cells enhance RANKL expression of osteoblasts by expressing parathyroid hormone-related protein (PTHrP), leading to osteoclastogenesis [64]. Additionally, in the bone metastatic setting, osteoclasts highly express matrix metalloproteinase-7 (MMP7), which further promotes bone resorption by cleaving membrane binding RANKL on both osteoblasts and prostate cancer [65]. The inhibition of bone remodeling mediated by osteoclastogenesis associated with bone metastatic cancer cells is thought to be an essential process for bone metastatic outgrowth, since it creates space for disseminated tumor cells to grow within the bone marrow. In similar fashion, the inhibition of osteoblastogenesis induces bone metastatic growth of prostate cancer. Dickkopf-1 (DKK-1), an inhibitor of Wnt (a major regulator for osteoblast differentiation), expressed by prostate cancer contributes to the inhibition of osteoblastogenesis, and consequently promotes prostate cancer growth in the marrow [66–68]. Interestingly, RANKL released from osteoblasts also stimulates the growth of prostate cancer through RANK on the prostate cancer [69–71]. Moreover, the RANKL secretion of osteoblasts and the RANK expression of prostate cancer are controlled by IL-6 derived from prostate cancer [71]. As a result, when the IL-6 signal and the RANK/RANKL axis are blocked, bone metastatic growth of prostate cancer is decreased [69–71].

Cancer-induced bone pain is one of the major complications associated with bone metastases [13]. Bone pain is mediated by both sensory and sympathetic nerve fibers that innervate into the marrow [72]. Aside from pain induction, the regulatory roles of nerve systems in bone remodeling have been revealed [73–75]. For instance, it has been indicated that a sensory neuropeptide, calcitonin gene-related peptide (CGRP) promotes osteoblastic differentiation [74, 75]. In addition, norepinephrine released from sympathetic nerve fibers prevents the proliferation of osteoblasts through β 2-adrenergic receptor [74, 75]. The β 2-adrenergic receptor also induces RANKL expression in osteoblasts that leads to the osteoclast activation [74, 75]. Moreover, β 2- and β 3-adrenergic receptors are involved in the development of the HSC niche by controlling its CXCL12 expression [76–78]. Therefore, it has been hypothesized that nerve components within the marrow contribute to bone metastatic progression [79]. Interestingly, prostate cancer cells have a high affinity to nerve fibers [80, 81], and perineural invasion in the primary tumor has been proposed as a predictor for prostate cancer bone metastasis [82, 83]. These findings suggest that the crosstalk between prostate cancer and the central and/or peripheral nervous system might be in part responsible for the bone metastatic process. It has been demonstrated that sympathetic nerve systems play a crucial role in prostate cancer bone metastasis. The high levels of β 2-adrenergic

receptor are observed in prostate cancer samples [84], and treatments with β -blockers lower bone metastatic lesion of prostate cancer [84, 85]. Moreover, when autonomic nervous system is ablated, prostate cancer development and bone metastases are prevented [83]. Importantly, a recent retrospective study with over 3500 prostate cancer patients who receive β -blockers demonstrated that β -blocker treatment improves mortality in high-risk or metastatic prostate cancer patients (adjusted sub-hazard ratio 0.79; 95% confidence interval 0.68–0.91; $p = 0.001$) [86]. Furthermore, bone metastatic prostate cancer cells influence sensory neurons in the marrow. When prostate cancer cells reach to the bone, they enhance sprouting of CGRP-positive sensory neurons in the marrow, resulting in cancer-induced bone pain [87]. Since nerve components in the marrow in part assist the development of a favorable microenvironment for prostate cancer cells to establish bone metastasis, targeting the central and/or peripheral nervous system can be an additional potential therapeutic option for prostate cancer patients with bone metastases.

Treatment Strategies for Men with Bone Metastases

Once prostate cancer has spread beyond the prostate to other parts of body such as bone, it becomes difficult to cure. Therefore, the goals of treatment shift from cure to palliative and disease control. Currently, nearly all prostate cancer patients with bone metastases receive analgesic medications, including opioids, due to painful skeletal-related events (SREs). However, opioid use has become a growing concern. Although opioids provide effective pain relief, the effects are usually partial and therefore the doses to achieve adequate analgesia for bone pain become progressively higher. This causes several side effects, including nausea, vomiting, constipation, pulmonary dysfunction, cognitive dysfunction, and addiction [88]. In addition, a recent study demonstrated that greater opioid requirement may be associated with inferior clinical outcome in advanced prostate cancer patients [89]. Since opioids do not treat the underlying cause of the pain, there is constant need to develop more effective treatments and/or strategies for addressing bone metastatic disease.

Radiotherapy

Radiotherapy offers another palliative pain control option for cancer patients with bone metastases. It has been demonstrated that palliative radiation provides overall pain relief to 50–80% of patients with cancer-induced bone pain [90]. A single fraction of local radiotherapy, repeated if necessary, appears to be the most effective when bone metastatic lesion is single or small. Recent randomized trials revealed that 8 Gy in a single fraction is not inferior

to, but less toxic than 20–30 Gy in multiple fractions [91, 92].

When bone metastases are found in multiple lesions, systemic radiotherapy is generally considered. Historically, β emitter-based radiopharmaceuticals, Strontium-89 and Samarium-153, have been used as palliative treatment for metastatic castration-resistant prostate cancer patients. Although these two agents result in pain relief, neither of them have an impact on survival [93]. An α emitter Radium-223 (Ra-223) is the first and only Food and Drug Administration (FDA)-approved radioisotope for castration-resistant prostate cancer patients with bone metastases. Ra-223, by mimicking calcium, binds to hydroxyapatite enriched in the bone matrix at the area of high bone turnover associated with bone metastases, or osteoblastic bone metastatic lesion [94]. Thereafter, the local emission of high-energy α particles from Ra-223 is thought to induce DNA damage and cell death in bone metastatic cancer cells [94]. Ra-223 has a very high energy deposit, but a very limited path range ($< 100 \mu\text{m}$) because of its atomic weight [95]. Interestingly, Ra-223 provides substantial pain relief to prostate cancer patients with bone metastases, with only minimal myelotoxicity [96]. Therefore, Ra-223 appears to be safe and well tolerated. More importantly, Ra-223 also significantly improves mobility of bone metastatic patients. A phase III double-blind randomized trial in symptomatic metastatic castration-resistant prostate cancer (ALSYMPCA) reported that Ra-223 treatment not only improves bone pain, but also prolongs overall survival, compared to placebo treatment (median survival: 14.9 months vs. 11.3 months; hazard ratio 0.70; 95% confidence interval 0.58–0.83; $p < 0.001$) [97]. This significant improvement in overall survival encourages use of Ra-223 as combination or adaptive treatment strategies for prostate cancer patients with bone metastases. An international, early access, open-label, single-arm phase IIIb trial revealed that combining Ra-223 with other conventional treatments for advanced prostate cancer (e.g., abiraterone, enzalutamide, abiraterone plus enzalutamide, or denosumab) is associated with significantly longer overall survival than Ra-223 alone [98]. Therefore, Ra-223 appears to be a promising treatment for bone metastatic disease; however, there are still financial hurdles that need to be overcome. Currently, in the US, six cycles of Ra-223 treatments cost about \$70,000 [99].

Bone-Targeting Therapy

As stated above, the osteoclastogenesis plays a major role in the development of skeletal metastasis. Therefore, targeting the osteoclast activity seems to be an ideal treatment strategy for bone metastatic disease, and this strategy contributes to delay the onset of SREs. Nitrogen-containing

bisphosphonates are widely used for prevention and treatment of number of osteolytic conditions, including osteoporosis and bone metastases [100]. Bisphosphonates inhibit bone resorption by preventing hydroxyapatite breakdown by binding to hydroxyapatite crystals at the area of active bone turnover [100]. In addition, although clear mechanisms yet remain unknown, it has been suggested that bisphosphonates also directly induce osteoclasts apoptosis [101, 102]. Several different generations of bisphosphonates (e.g., non-nitrogen-containing bisphosphonates, nitrogen-containing bisphosphonates) exist, but only the third-generation bisphosphonate, zoledronic acid show clinical benefit for advanced prostate cancer patients. A placebo-controlled randomized trial in metastatic castration-resistant prostate cancer patients showed that zoledronic acid reduces the incidence of SREs (44.2% vs. 33.2%, $p = 0.021$) [103].

A human monoclonal antibody against RANKL, denosumab, has been emerged as an alternative osteoclast targeting agent [104]. Denosumab can inhibit osteoclast activity mediated by osteoblasts within the bone metastatic lesion by blocking the RANK/RANKL interaction. A phase III randomized trial comparing denosumab and zoledronic acid in metastatic castration-resistant prostate cancer patients indicated that denosumab extends the time to first SREs from 17.1 to 20.7 months (hazard ratio 0.82; 95% confidence interval 0.71–0.95; $p = 0.0002$ for non-inferiority and $p = 0.008$ for superiority) [105]. Another phase III, randomized trials in castration-resistant prostate cancer patients with no sign of bone metastasis, but with rising prostate-specific antigen (PSA), demonstrated that denosumab increases bone metastasis-free survival by a median of 4.2 months over placebo (hazard ratio 0.85; 95% confidence interval 0.73–0.98; $p = 0.028$), and delay the onset of SREs [106]. However, in the same study, no differences in overall survival were observed between denosumab- and placebo-treated patients [106].

Additionally, another potential bone-targeting reagent, OsteoDex has been recently developed. OsteoDex is a bifunctional macromolecular polybisphosphonate which has osteoclast inhibitory function with anti-tumor ability [107, 108]. A phase I safety study performed in castration-resistant prostate cancer patients revealed no serious adverse effects, including renal toxicity [107]. Currently, a phase II trial testing the effect of osteodex on skeletal-related pain in prostate cancer patients with bone metastases is underway (ClinicalTrials.gov: NCT02825628).

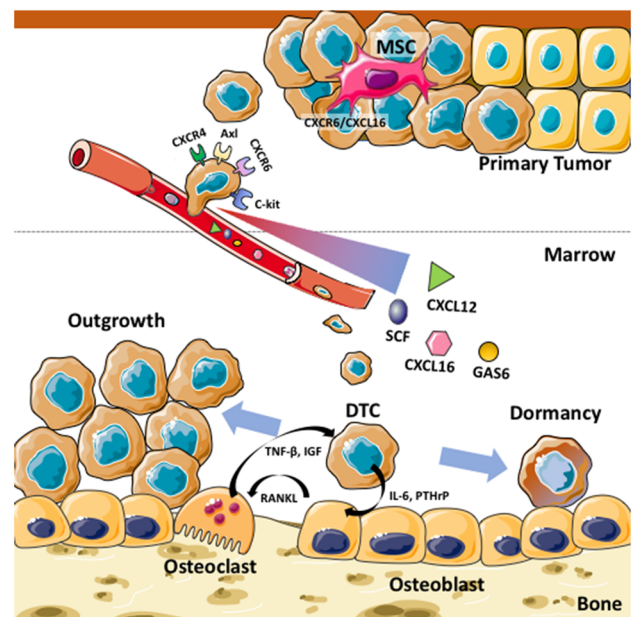


Fig. 1 Crosstalk between bone metastatic prostate cancer and bone marrow microenvironment. The bone marrow microenvironment is involved in the multiple steps of bone metastasis development. Bone marrow-derived mesenchymal stem cells (MSCs) promote primary prostate cancer to disseminate to bone using the CXCL16/CXCR6 axis. The circulating tumor cells that express the high level of CXCR4, CXCR6, c-Kit, and Axl migrate towards bone based on a gradient of the chemokines and ligands (CXCL12, CXCL16, SCF, and GAS6) released from the bone marrow. Once in the bone marrow, the disseminated tumor cells (DTCs) are believed to become dormant by directly interacting with the bone marrow niche (e.g., osteoblast). At the same time, the DTCs influence the osteoblasts and osteoclasts to create a favorable microenvironment to accelerate later outgrowth

Conclusions and Unanswered Questions

Although bone marrow provides a fertile microenvironment for metastatic tumor cells, its role in the process of bone metastasis is largely unknown. The studies discussed in this article suggested that microenvironment for HSCs has a significant influence on the bone metastatic progression of prostate cancer (Fig. 1). It has been evident that bone metastatic prostate cancer cells mimic hematopoietic cells during its dissemination to the bone to establish hold in the marrow, and that once in the marrow, disseminated prostate cancer cells parasitize the microenvironment for HSCs to survive within bone marrow coopting mechanisms that keep HSCs viable. These findings suggest that a specific component of the bone marrow microenvironment (e.g., HSC niche) can serve as a potential therapeutic target for bone metastatic disease.

In addition to pursuing therapeutic targets, palliative strategies are critical for bone metastases patient management. Pain management is vital in improving treatments for patients with advanced prostate cancer, since patients with bone metastases often suffer from significant painful

complications, and cancer bone pain can cause substantially impaired quality of life. Non-steroidal anti-inflammatory drugs (NSAIDs) and opioids are widely used analgesics for cancer bone pain, but can have serious side effects and abuse and addiction of these analgesics are a growing concern. Although bone-targeting agents (bisphosphonate, denosumab) and/or external beam radiotherapy are the standards of care for prevention or delay of SREs, these treatment strategies are not curative. Therefore, new approaches to cure bone metastatic prostate cancer are urgently needed. Immunotherapy has been recently investigated as an alternative therapeutic strategy. Subgroup analysis within the Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT) study found that Sipuleucel-T (Provenge), the first FDA-approved immunotherapy for advanced prostate cancer reduces the risk of death of patients with greater than 10 bone metastases [109]. This finding warrants further investigation into the roles of checkpoint blockade inhibitors (e.g., an anti-PD1 antibody nivolumab and an anti-CTLA4 antibody ipilimumab) in prostate cancer management. Combination therapies may offer the efficacy of the agents we discussed while minimizing their toxicity. Indeed, the combination of Ra-223 with hormone therapy, immunotherapy, chemotherapy, or bone-targeting therapy has been tested in the clinical setting [110–112].

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Conflict of interest Sun H. Park, Evan T. Keller, and Yusuke Shiozawa declare that they have no conflicts of interest.

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Preclinical and clinical studies on cancer-associated cachexia

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BACKGROUND: Cancer cachexia is the wasting condition that is often seen in advanced stage cancer patients. This wasting is largely attributable to a systemic and progressive loss of skeletal muscle mass that greatly hinders performance of normal daily activities, resulting in reduced quality of life. Moreover, it negatively influences the prognosis of cancer patients. A general consensus in the field is that the loss of muscle mass is due both to an increase in protein degradation and a decrease in protein synthesis. Recent studies using preclinical models for studying cachexia have been useful in identifying the contribution of inflammatory cytokines (e.g. tumor necrosis factor- α and Interleukin-6), and myostatin receptors (e.g. the type IIB activin receptor) to cachexia development, and have led to several clinical trials. However, many questions remain about the molecular mechanisms thought to play a role in the development of cachexia.

METHODS: We conducted a literature search using search engines, such as PubMed and Google Scholar to identify publications within the cancer cachexia field.

RESULTS: We summarized our current knowledge of: 1) the driving mechanisms of cancer cachexia, 2) the preclinical models available for studying the condition, and 3) the findings of recent clinical trials.

CONCLUSION: Cancer cachexia is a complex and variable condition that currently has no standard effective therapeutic treatment. Further studies are desperately needed to better understand this condition and develop effective combination treatments for patients.

Keywords cancer cachexia, muscle wasting, bodyweight loss, metabolic changes, increased protein degradation, decreased protein synthesis

Introduction

The term ‘cachexia’ has Greek roots that translate to ‘bad condition’. Descriptions of the condition date back to Hippocrates, around 460 BC (Bennani-Baiti and Walsh, 2009, Lok, 2015). This condition arises frequently with many chronic illnesses, including chronic obstructive pulmonary disorder, chronic kidney disease, and the many forms of cancer (Lok, 2015). Historically, cachexia was characterized by the pale and wasted appearance of patients, but until recently, there were no formal set criterion for its clinical diagnosis (Bennani-Baiti and Walsh, 2009). In 2011, an

international consensus was reached to formally establish diagnostic criterion of cancer cachexia for each of its three stages (Table 1). In the first stage, patients with precachexia have less than 5% premorbid bodyweight loss, but display signs of anorexia and/or metabolic changes. Patients that have progressed to the second stage, cachexia, have weight loss greater than 5% of the their premorbid bodyweight, or alternatively weight loss greater than 2% with signs of sarcopenia or a body mass index under 20. Cachexia patients in this stage are likely to also have systemic inflammation, which may be indicated by high levels of C-reactive protein (CRP) in the serum (Penafuerte et al., 2016). Cachexic patients in the final stage, refractory cachexia, have a life expectancy of less than three months, and their cancer is no longer responsive to treatment (Fearon et al., 2011).

Cachexia is the result of progressive muscle wasting and impaired muscle function. Although one defining character-

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Table 1 Established stages of cancer cachexia.

	Precachexia	Cachexia	Refractory cachexia
Weight loss ^a	< 5%	> 5%	> 5%
Other symptoms	Altered metabolism	Inflammation	Cancer unresponsive to treatment

^a When Body mass index (BMI) is > 20 and there are no signs of sarcopenia related muscle atrophy.

istic of cachexia is weight loss, it differs significantly from illnesses such as anorexia (Table 2). Unlike anorexia, where fat is the main source of initial weight loss, skeletal muscle wasting is a key feature of cachexia occurring early on in the condition (Mueller et al., 2016). Additionally, changes in diet and an increase in nutrient intake do not consistently result in weight gain and increased muscle function in cachexic patients (Onesti and Guttridge, 2014, Lok, 2015).

Table 2 Differences between cachexia and anorexia.

	Cachexia	Anorexia
Classification	Metabolic disorder	Eating disorder
Initially present with	Loss of skeletal muscle mass Possible loss of fat	Loss of adipose tissue No loss of muscle mass
Nutritional supplementation	Does not reverse condition	Reverses condition

Cancer-associated cachexia affects roughly 50% of all cancer patients regardless of their cancer type (Onesti and Guttridge, 2014, Lok, 2015, Fukawa et al., 2016). This percentage increases to about 80% when the cancer is classified as advanced (Onesti and Guttridge, 2014, Lok, 2015, Fukawa et al., 2016). The prevalence of patients developing cachexia does vary between cancer types, but is most common in pancreatic and gastrointestinal cancer patients. For example, roughly 80% of pancreatic or gastrointestinal cancer patients will be classified as cachexic at some point throughout the course of their disease, while a slightly smaller percentage (50-60%) of prostate and lung cancer patients will develop cachexia (Del Ferraro et al., 2012; Argilés et al., 2014). Problematically, cancer patients displaying signs of cachexia are faced with a worsened prognosis and decreased quality of life (Al-Majid and Waters, 2008, Onesti and Guttridge, 2014). However, whether there is a correlation between tumor burden and the development of cachexia is still subject to debate in the field (Consul et al., 2016, Petruzzelli and Wagner, 2016, Shiono et al., 2016). As muscle wasting progresses, patient fatigue and weakness also grow. Performing normal daily activities becomes a constant struggle for cachexia patients. In addition to this, cachexia makes it substantially more difficult to treat their cancer, since chemotherapy treatments are often too toxic for cachexic patients to endure, and major surgeries are too high risk for their weakened state (Lok, 2015). To date, roughly one third of all cancer related deaths are being attributed to cachexia

(Onesti and Guttridge, 2014). Moreover, there is no definitive treatment or cure for cachexia. While ongoing research strives to find an effective therapeutic for cachexia patients and much progress has been made in the field, the molecular mechanisms behind its development are not completely understood, and findings from the models used to study cachexia do not always apply to humans.

In this review, we therefore provide a concise overview of cancer cachexia, encompassing: key molecular mechanisms involved in its development, frequently used preclinical models for its study, and the current standing of recent clinical trials.

Molecular mechanisms contributing to the development of cancer cachexia

Although muscle wasting in cancer cachexia results from the volatile combination of increased protein degradation and decreased protein synthesis within the skeletal muscle, the exact molecular mechanisms that affect both of these processes have yet to be completely discerned. Systemic inflammation has been widely implicated for its role in the development of the cachexic condition, although the systemic levels of inflammatory cytokines have not been consistently correlated with cachexia development in humans (Maltoni et al., 1997; Fearon et al., 2011; Suzuki et al., 2013). Cytokines of the innate immune system such as tumor necrosis factor (TNF)- α and Interleukin 6 (IL-6) have been previously connected to increased muscle deterioration and fat store depletion, which was interpreted as cachexia development in rodent models (Narsale and Carson, 2014; Onesti and Guttridge, 2014; Mueller et al., 2016). These inflammatory cytokines increase activity of the ubiquitin proteasome system which has been suggested to have the most critical role in the breakdown of skeletal muscle proteins contributing to cachexia, and has also been implicated in the cardiac atrophy that can occur with cancer cachexia (Al-Majid and Waters, 2008; Onesti and Guttridge, 2014; Bilodeau et al., 2016; Porporato, 2016). The normal function of the ubiquitin proteasome system is to breakdown poly-ubiquitin tagged protein substrates. To briefly summarize the process, enzymes E1, E2, and E3, the latter of which controls specificity of the targeted substrate, target a specific substrate to tag with a polyubiquitin chain. The ubiquitinated protein is then degraded by the proteasome (Pagan et al., 2013). TNF- α signaling through I κ B kinase (IKK) activates the nuclear transcription factor- κ B (NF κ B) pathway to increase transcription of the ubiquitin proteasome genes, muscle RING-finger protein-1 (MuRF1), which codes for an E3 ligases specific to myofibrillar proteins, such as troponin I, myosin heavy and light chains, and actin (Sandri, 2013; Winbanks et al., 2016), and contributes to increased ubiquitination and consequently proteasome degradation of these myofibrillar proteins (Li and Reid, 2000; Sakuma and Yamaguchi, 2012;

Argilés et al., 2014; Onesti and Guttridge, 2014; Patel and Patel, 2017). Consistent with this notion, TNF- α serum levels are reported to be higher in pancreatic cancer patients with a poor nutritional status (Karayiannakis, Syrigos et al., 2001). IL-6 activates the janus kinase (JAK) (Bonetto et al., 2012)/ signal transducer and activator of transcription (STAT)-3 pathway and mitogen-activated protein kinases (MAPK) cascades (Na et al., 2007) within muscle cells, to promote increased caspase activity, resulting in increased cellular apoptosis (Argilés et al., 2014; Onesti and Guttridge, 2014; Puppa et al., 2014). Additionally, TNF- α , IL-6, and IL-1 β have also been implicated in lipolysis, potentially contributing to fat loss in cancer cachexia (Narsale and Carson, 2014; Petruzzelli and Wagner, 2016; Patel and Patel, 2017). Browning of white fat cells to 'beige' cells has been a focus of cachexia studies involving adipose tissue. In beige cells, there is a higher population of uncoupling proteins that decrease the amount of adenosine triphosphate (ATP) synthesized in the cells' mitochondrion and increases energy expenditure through thermogenesis (Petruzzelli et al., 2014; Petruzzelli and Wagner, 2016). These cytokines likely come from the host's innate immune response to the tumor. Macrophages have been examined as a major source for TNF- α and IL-1 in particular (Onesti and Guttridge, 2014). IL-6 is known to be released from both contracting skeletal muscle and activated innate immune cells (Carson and Baltgalvis, 2010).

Tumor-derived proteolysis inducing factor has also been identified in mouse cachexic models and is thought to play a critical role in the breakdown of skeletal muscle proteins through the ubiquitin proteasome pathway in these models. However, whether the human homolog of this factor is capable of eliciting a cachexic response is currently subject to controversy (Monitto et al., 2004; Wieland et al., 2007; Onesti and Guttridge, 2014). On the other hand, the type IIB activin receptor (ActRIIB) which binds the myokine myostatin, has also been implicated as a potential cachexia target for its role in regulating the transcription of the muscle protein targeting E3 ligases of the ubiquitin pathway. Blocking ActRIIB signaling has shown promising results reducing muscle loss and increasing survival in mice, and has also been reported to improve cardiac atrophy (Zhou et al., 2010; Argilés et al., 2014; Onesti and Guttridge, 2014; Petruzzelli and Wagner, 2016). A recent study evaluated the adeno-associated virus delivery of the gene for the endogenous ActRIIB inhibitor, similar to mothers against decapentaplegic homolog 7 (Smad7), to combat ActRIIB driven muscle atrophy in mice. This strategy shows initial promise for its ability to specifically target striated muscle signaling, potentially cutting down on dangerous off target effects seen with other antagonists (Winbanks et al., 2016). For example, one study using an ActRIIB antagonist in humans with muscular dystrophy was canceled due to unanticipated bleeding in the participants (NCI, 2011; Winbanks et al., 2016). The safety of these strategies for use in patients will therefore have

to be carefully evaluated.

While the above cellular pathways may partially explain the wasting of skeletal muscle, they are not entirely sufficient to explain the loss of protein synthesis that accompanies protein degradation during cachexia. Other factors such as the reduced phosphorylation of the mechanistic target of rapamycin (mTOR) and p70 have been shown to inhibit skeletal muscle protein synthesis in mice with murine adenocarcinoma 16 (MAC16) tumors, contributing to cachexia by preventing repair of weakening muscles, such as the gastrocnemius muscle in the hind limbs of mice (Eley et al., 2007; Al-Majid and Waters, 2008). Additionally, heightened levels of angiotensin II, commonly known for its role as a vasoconstrictor peptide regulating blood pressure as part of the renin-angiotensin system, have been associated with the inhibited synthesis of new proteins in murine myotubes, impaired regeneration of wasting skeletal muscle, and atrophied skeletal muscles as shown in excised muscles and primary satellite cell culture from mice with a cardiotoxin induced injury (Russell et al., 2006; Yoshida et al., 2013). Angiotensin II may simultaneously contribute to cachexia by both impairing protein synthesis and increasing activity of the ubiquitin proteasome system through increased transcription of key muscle protein specific E3 enzymes, as evidenced in murine myotube models (Al-Majid and Waters, 2008). It was even suggested that angiotensin II may be useful as a biomarker for cancer cachexia, based on plasma and whole blood measurements from 122 patients with seven types of cancer (Penafuerte et al., 2016). Although all of the pathways discussed here may contribute to cachexia development, the exact driving mechanisms behind individual cases of cancer cachexia are complex and can differ between patients. For this reason, it is important to have models that can adequately represent cachexia in all of its complexities.

Preclinical models to study cancer

cachexia

Currently, the models available for studying cancer cachexia consist of a combination of *in vitro*, *in vivo*, and *ex vivo* models. There are effective established cell lines for developing an *in vivo* animal model of cachexia. For example, both the cultured colon-26 (C26) carcinoma cell line, and lewis lung carcinoma (LLC) cell line (Choi et al., 2013) are known to induce weight loss and increase mortality when used in a mouse model, providing an effective model of cachexia (Onesti and Guttridge, 2014; Fukawa et al., 2016). The delivery of the cancer cells and the resulting symptoms of cachexia may vary between individual cell lines. C26 colon carcinoma causes significant loss of bodyweight and increased skeletal muscle protein degradation in mice when injected subcutaneously as a cell suspension, or implanted subcutaneously as a solid fragment of a C26 tumor from a

donor mouse (Aulino et al., 2010). LLC cells are typically delivered intramuscularly, and in rarer instances subcutaneously, to cause cachexia in a mouse model (Deboer, 2009; Choi et al., 2013). Tumor-free body mass, as well as excised extensor digitorum longus mass, have been shown to decrease in C57BL/6 mice injected intramuscularly with LLC cells when compared to media-injected mice, without any notable change in diet. The loss in muscle mass corresponded with a loss in measurable tetanic muscle force in an *ex vivo* measurement (Choi et al., 2013). The usefulness of these cell lines as a model for specific studies may depend on the end result that is being measured. For example, high levels of TNF- α are seen in models using LLC cells, but not C26 or MAC16 cells (Mueller et al., 2016). For the C26 model, IL-6 is traditionally viewed as a key mediator of cachexia development (Aulino et al., 2010; Bonetto et al., 2012). Conversely, tumor-derived proteolysis inducing factor (PIF) and lipid-mobilising factor (LMF) have been shown to play a larger role in MAC16 muscle proteolysis, adipolysis, and cachexia development than immune-derived inflammatory cytokines (Bing et al., 2001; Islam-Ali et al., 2001).

Changes in whole bodyweight, daily food intake, total body lean and fat mass composition, cardiac muscle, individual skeletal muscle weights, muscle fiber cross sectional area, and muscle function are key measurements of interest in these *in vivo* models (Deboer, 2009; Winbanks et al., 2016). Key muscles of interest in cachexia studies include the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles located in the hindlimbs (Aulino et al., 2010). These muscles represent multiple muscle fiber types, as the EDL contains predominantly fast twitch fibers and the TA contains a mixture of fast and slow twitch fibers, allowing studies to address how cachexia models may affect specific muscles differently (Aulino et al., 2010; Choi et al., 2013). Repeatable *in vivo* measurements of systemic muscle function include grip strength and force transduction measurements. The former measurement utilizes a grip strength meter (Fig. 1). To do this measurement with the forelimbs, the mice are held by their tails and allowed to grab onto a wire mesh square or bar with only their forepaws. Then they are pulled away from the mesh horizontally by their tails until they release their grip on the mesh or bar. The grip strength meter will then read out the peak force generated from the mouse's grip on the mesh. A second readout uses a force transducer to measure the maximum contractile force able to be elicited from specific muscles or muscle groups stimulated with electrodes (Fig. 2). This technique allows for measurement of both contractile forces and time to fatigue. Individual muscles may also be excised upon sacrifice, weighed, and contractile forces measured as an *ex vivo* model using electrical stimulation and a force transducer (Aulino, et al., 2010; Waning et al., 2015).

To further study the molecular mechanisms where by cancer affects muscle functions, *in vitro* culture systems using primary muscle cell or muscle cell lines can be used.

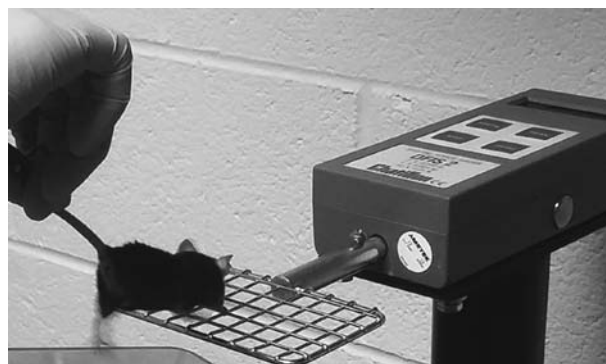


Figure 1 Grip strength measurement. Mouse is held by its tail as it grips the wire mesh bar attached to the grip strength meter. As the mouse is pulled horizontally away from the mesh and loses its grip, the peak force generate from the mouse's grip on the mesh is measured.



Figure 2 Force transducer measurement. The mouse is anesthetized and its foot attached to the pedal of the force transducer. An electrode is placed over the tibialis anterior muscle. A second electrode is placed at the base of the tendon. An electrical current stimulates the muscle to contract. As the muscle contracts, the mouse pushes the pedal, and the resulting force is recorded.

Commonly, both murine and human myotubes are cultured *in vitro*, and their responses to potential cachectic factors added to the media are observed, such as changes in tubule size or diameter, oxidative stress, and protein content (McLean et al., 2014; Bowen et al., 2015; Fukawa et al., 2016). The C2C12 mouse myoblast cell line is able to differentiate into myotubes when its media is supplemented with horse serum. Primary human and mouse myotubes can be generated using isolated

muscle stem cells or satellite cells from individual muscle fibers (Pasut et al., 2013; Fukawa et al., 2016). This provides an alternative that allows for human tissues to be studied and potential differences between the mouse and human muscle regeneration processes to be recorded.

Clinical studies regarding cancer cachexia

Human based studies have been completed for various types of solid tumors, but are relatively small in number when compared with the number of cachexia studies performed using animal models (Mueller et al., 2016). Clinical studies seeking to identify a biomarker for cachexia development have been met with variable results that are difficult to reproduce between multiple patient populations. A recent study attempted to account for this inherent variability by first characterizing their cohort population into three separate groups. The patients were from seven types of cancer and were divided into a 'no cachexia', 'pre-cachexia', or 'cachexia' group. This study complied with the international consensus definition, and cachexia was defined as loss of 5% or more of total bodyweight within 6 months, or loss of more than 2% of bodyweight with a BMI less than 20. Patients in the 'pre-cachexia' group had elevated levels of CRP without cachexic levels of weight loss, patients in the 'no cachexia' group did not present with cachexia or elevated CRP, and patients in the 'cachexia' group had both elevated CRP levels and significant weight loss (Penafuerte et al., 2016). The study then determined the plasma levels of angiotensin II using an ELISA, and the whole blood levels of neutrophil-derived proteases, such as cathepsin B, for each group, using a chip array to measure mRNA expression. This study found significantly higher mRNA expression of both angiotensin II and cathepsin B in the precachexia and cachexia groups when compared to the no cachexia group (Penafuerte et al., 2016). Based on these results angiotensin II and cathepsin B, or similar neutrophil-derived proteases, may serve as usable biomarkers for cachexia, providing the patients are accurately categorized. This characterization of patients based on their stage of cachexia may also be useful in determining which treatments will be most beneficial for individual cancer cachexia patients.

In addition to the work being done to identify suitable biomarkers for cancer cachexia, clinical trials are currently underway to test potential therapeutics for cachexia. To date, these trials have been met with mixed results. Attempts to treat cachexia through exercise and limited bed rest have been largely unsuccessful (Onesti and Guttridge, 2014). The selective androgen receptor modulator, enobosarm is a candidate therapeutic that increased lean body mass in a double blind phase II trial with patients diagnosed with non-small cell lung cancer (NSCLC), colorectal cancer, non-Hodgkin lymphoma, chronic lymphocytic leukemia, or breast cancer (Dobs et al., 2013). Two international double-blind

phase III trials, dubbed the POWER 1 and 2 trials, measured benefits in muscle mass preservation in NSCLC patients receiving enobosarm orally with and without a taxane and chemotherapy, but the results from these trials have not yet been fully published (Crawford et al., 2016). Anamorelin, a mimic of the appetite regulator ghrelin, was found during two phase III trials to increase lean body mass in patients with NSCLC but had no effect on muscle function, measured with handgrip strength (Lok, 2015; Temel et al., 2016). A phase I/II trial with the TNF- α blocker etanercept with gemcitabine did not improve overall survival of participants with advanced cancer, and a phase III study with incurable cancer patients presenting weight loss or impaired appetite showed no benefit in weight or appetite from subcutaneous etanercept injections (Jatoi et al., 2007; Wu et al., 2013; Onesti and Guttridge, 2014; Mueller et al., 2016).

Examples of therapeutic agents targeting inflammation that are being evaluated for their effects on cancer cachexia include Clazakizumab and MABp1. Clazakizumab, or ALD518, is a humanized anti-IL6 monoclonal antibody that has been tested in preclinical trials in NSCLC patients and improves lean body mass preservation (Bayliss et al., 2011). MABp1, also known as Xilonix, is an antibody against IL-1 α . This has been tested in a double-blind phase III clinical trial with advanced colorectal cancer patients. The primary endpoint for this trial was measured as either an overall improvement in lean body mass preservation or a combination of improved pain, fatigue, or anorexia when compared to the initial baseline measurements. Results from this study showed 33% of MABp1 treated patients showed improvement, while only 19% of patients receiving the placebo showed improvement (Hickish et al., 2017). Further evaluation for these agents as cachexia therapeutics will be needed, but indicate that anti-inflammatories are a viable avenue to pursue for cancer cachexia therapy. Much work clearly remains to be done to establish an effective therapeutic strategy for cancer cachexia patients.

Future directions and conclusion

Cachexia is a very complex metabolic syndrome with the capacity to target multiple pathways and organs at once. Much like the cancer that drives the development of cachexia, it is unlikely two cachexic patients will present with their disease in the same way (Mueller et al., 2016). For this reason, it is also unlikely that a single targetable therapeutic will be equally effective in all cachexic patients. If this statement is true, then ongoing clinical trials will help determine why certain patients benefit from specific therapeutics while others do not. Due to this inherent variability among cancer cachexia patients, and the multiple molecular mechanisms involved in its development (Fig. 3), individualized treatment plans and multiple therapeutic options are likely needed to adequately devise effective treatment options

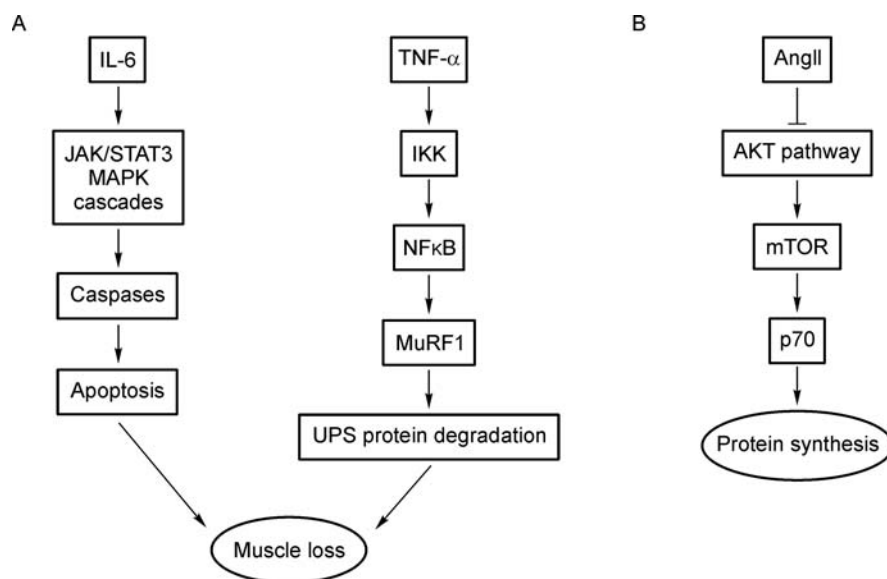


Figure 3 A schematic model of molecular pathways contributing to cancer cachexia. (A) In response to the tumor, the host's immune system releases inflammatory cytokines that can promote cachexia development. Tumor necrosis factor (TNF)- α signaling activates I κ B kinase (IKK) and the nuclear transcription factor- κ B (NF κ B) pathway to upregulate transcription of the E3 ligase, muscle RING-finger protein-1 (MuRF1), increasing ubiquitin mediated degradation of muscle proteins, and leading to muscle loss. Interleukin 6 (IL-6) activates the janus kinase (JAK)/signal transducer and activator of transcription (STAT)-3 pathway and mitogen-activated protein kinases (MAPK) cascades, upregulating caspase activity, and resulting in increased cellular apoptosis in the muscle. (B) Angiotensin II (AngII) can inhibit protein synthesis by inhibiting the Protein Kinase B (AKT) pathway and reducing phosphorylation of mechanistic target of rapamycin (mTOR) and p70.

for cancer cachexia. Combination therapies encompassing multiple therapeutics may also be further explored to uncover an effective treatment for all cachexic patients. Animal models and *in vitro* studies have helped to identify key pathways and potential targets in this complex disease, but unfortunately they are unable to completely mimic all the complexities and variables that are often seen in humans with cachexia. As a result, the findings from these models do not always translate to humans, as evidenced by the unexpected side effects of the ActRIIB inhibitor in clinical trial (NCI, 2011). To overcome these issues, more studies involving human muscle tissue and clinical trials will clearly be needed in order to confirm the translatability of *in vitro* and *in vivo* findings. Combining the knowledge gained from multiple preclinical models with the findings of diverse clinical trials will further knowledge on the complex mechanisms driving cancer cachexia and lead to effective therapeutic options for its treatment.

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Compliance with ethics guidelines

D. Brooke Widner, D. Clark Files, Kathryn E. Weaver, Yusuke Shiozawa declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed. This article does not contain any studies with human subjects performed by any of the authors.

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Review

Role of the Bone Microenvironment in the Development of Painful Complications of Skeletal Metastases

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Abstract: Cancer-induced bone pain (CIBP) is the most common and painful complication in patients with bone metastases. It causes a significant reduction in patient quality of life. Available analgesic treatments for CIBP, such as opioids that target the central nervous system, come with severe side effects as well as the risk of abuse and addiction. Therefore, alternative treatments for CIBP are desperately needed. Although the exact mechanisms of CIBP have not been fully elucidated, recent studies using preclinical models have demonstrated the role of the bone marrow microenvironment (e.g., osteoclasts, osteoblasts, macrophages, mast cells, mesenchymal stem cells, and fibroblasts) in CIBP development. Several clinical trials have been performed based on these findings. CIBP is a complex and challenging condition that currently has no standard effective treatments other than opioids. Further studies are clearly warranted to better understand this painful condition and develop more effective and safer targeted therapies.

Keywords: cancer-induced bone pain; bone marrow microenvironment; osteoclasts; osteoblasts; macrophages; mast cells; stromal cells

1. Introduction

Cancer-induced bone pain (CIBP) is the most common complication of bone metastases, and it significantly reduces the patient's quality of life (QOL) [1]. CIBP poses a tremendous challenge to patients and their caregivers, in both managing it and identifying its underlying cause. In the quest for effective cancer therapy, maintaining QOL can be as crucial as treating the disease itself. Seventy-five percent of cancer patients experience pain throughout their disease [2]. Once cancer metastasizes to the bone, the first symptom is often acute bone pain. A full 80% of patients with bone metastases have CIBP [3].

CIBP remains a therapeutically challenging condition. It includes both spontaneous (ongoing) pain and breakthrough (movement-related) pain, which can present individually or in combination [4]. Unless each component of CIBP is appropriately treated, it cannot be managed. Analgesics for CIBP that target the central nervous system (e.g., opioids, nonsteroidal anti-inflammatory drugs or NSAIDs) are somewhat effective to reduce pain, but have severe side effects and are often extremely addictive [5–7]. External beam radiation, used for patients with bone metastases, is primarily palliative and only half of the patients receiving this therapy achieve partial or complete pain relief [4,8]. Alternatively, bisphosphonate and anti-receptor activator of nuclear factor κ B ligand (RANKL) antibody treatments that decrease bone resorption in cancer patients with bone metastases [9,10], and radium-223 which forms complexes with hydroxylapatite in bone [11–13] can also reduce the onset of pain. However,

even with these well-accepted clinical treatment modalities, 50% of patients with CIBP do not achieve controlled pain status [14].

CIBP is a very complex phenomenon that is uniquely distinct from other forms of chronic pain, such as inflammatory or neuropathic pain [15]. Bone is a richly innervated organ, and it has been suggested that bone metastatic cancer cells interact with sensory nerves in the bone microenvironment, resulting in CIBP development [15]. Mechanical stresses or mass effects from bone metastatic progression can directly induce CIBP, since sensory nerves that express mechanoreceptors reside throughout the interosseous membrane of long bones [16]. However, it has also been suggested that CIBP is not correlated with tumor type, location, number, or size of the metastases [17,18]. This may indicate that CIBP is developed not only during physical contact between bone metastatic cancer cells and sensory nerves, but also from signaling events initiated by cancer-derived factors [15,19–21]. Indeed, the acidic environment surrounding the tumor or cancer secreted growth factors, cytokines, or chemokines can all stimulate receptors on sensory nerves to induce CIBP [15,19,22,23].

The bone marrow provides a unique environment for both hematopoiesis and bone metastatic progression [24–26]. This microenvironment consists of several types of cells, including those that regulate bone remodeling, immune cells, stromal cells, and endothelial cells. It has been appreciated that the crosstalk between the bone microenvironment and bone metastatic cancer cells is crucial for bone metastatic progression [26–28]. However, little is known about how the molecular interactions between metastatic cancer and the bone marrow microenvironment affect CIBP.

Therefore, in this review, we provide a concise overview of the known roles of the bone marrow microenvironment in the development of CIBP and discuss the future directions of research on this topic.

2. The Roles of the Bone Marrow Microenvironment in the Development of Cancer-Induced Bone Pain

2.1. Osteoclasts

During bone metastatic progression, osteolytic cancer cells—originating from primary tumors from breast cancer, lung cancer, renal cancer, sarcomas, and multiple myeloma, etc.—stimulate osteoblasts to release RANKL. The osteoblast-derived RANKL binds to its receptor RANK expressed on osteoclasts. This interaction induces osteoclast maturation and increases osteolytic activity, resulting in enhanced bone resorption. Resorption of the bone matrix then causes the release of growth factors such as transforming growth factor beta (TGF- β) and insulin-like growth factor 1 (IGF-1), leading to further bone metastatic progression. The process whereby bone metastatic cancer cells establish osteolytic lesions to enhance bone metastatic progression is called the “vicious cycle” [27,29–34]. It has been suggested that enhanced osteoclast activity can also lead to CIBP. Consistent with this notion, increased mechanical and thermal hyperalgesia are observed in rodents inoculated with osteolytic osteosarcoma into the bone, compared to animals without tumors [35–37]. Administration of the decoy RANKL receptor osteoprotegerin (OPG) to osteosarcoma-bearing animals significantly decreased spontaneous pain behaviors [35,36]. However, OPG treatments did not affect tumor size.

During the bone resorption process, osteoclasts acidify (pH 4.0–4.5) the extracellular space by releasing protons and chloride ions through membrane transport (V-type H⁺ ATPase) [38]. The low pH condition sensitizes sensory nerves to mechanical, thermal, and chemical stimuli by activating acid sensing receptors such as the acid-sensing ion channels (ASICs) and transient receptor potential cation channel subfamily V member 1 (TRPV1) expressed on sensory nerves [39,40]. In addition, osteoclast activity indirectly causes TRPV1 upregulation in sensory nerves through the release of TGF- β and IGF-1 derived from the resorbed bone matrix. These factors increase the expression of their receptors (TGF- β RI and IGF-1R) on sensory nerves. In a rat CIBP model using mammary gland carcinoma, the upregulation of these growth factor receptors correlates with the upregulation and sensitization of TRPV1 in sensory nerves. The tumor-bearing animals in these studies had significantly increased

mechanical and thermal sensitivity, and observed pain behaviors were reversed with treatments of TGF- β RI and IGF-1R antagonists [41,42].

Osteoclasts mediate bone resorption not only through the creation of acidic conditions, but also through adenosine triphosphate (ATP) production by mitochondrial cytochrome c oxidative activity [43]. Osteoclasts release accumulated intracellular ATP into the extracellular space, which can activate purinergic receptors such as P2X receptors, known as the ATP-gated ion channels [44,45]. It has been demonstrated that: (1) approximately 90% of the peripheral and central sensory neurons express P2X receptors; and (2) the subtypes of P2X receptors including P2X4, P2X3, and P2X7 regulate the development of neuropathic and inflammatory pain [46,47]. Specifically, P2X3 and P2X2/3 expressed on the terminal end of primary afferent neurons innervating bone have been shown to be involved in CIBP development [48–52]. For instance, in C3H/HeJ mice receiving intratibial inoculations of osteolytic fibrosarcoma, increased expression of P2X3 receptor in the peripheral nociceptive fibers is observed, and peri-tumoral injections of receptor antagonist naloxone-methiodide (A-317491) inhibited cancer-mediated thermal hyperalgesia [52]. Moreover, growing tumor cells also generate ATP [53,54], and ATP itself stimulates osteoclast activities through the P2X3 receptor [55,56]. Therefore, ATP can induce CIBP by directly interacting with P2X receptors on sensory nerves or indirectly through enhanced osteoclastic activity. Although further studies are warranted, pharmacologically targeting the ATP/P2X receptors axis may prove to be a potential therapeutic strategy for CIBP.

2.2. Osteoblasts

Unlike osteolytic bone metastatic progression, the mechanisms driving osteoblastic bone metastatic progression are largely unknown. Prostate cancer is one of the primary cancer types that gives rise to developing osteoblastic lesions. It has been demonstrated that prostate cancer cells facilitate new bone formation by secreting parathyroid hormone-related protein (PTHrP) [57], urokinase-type plasminogen activator (uPA) [58], or prostate-specific antigen (PSA) [59]. Endothelin-1 (ET-1), a vasoconstrictor, is also known as an osteoblast inducing factor. It stimulates mitogenesis of osteoblasts when it binds to endothelin A receptor (ETAR) and endothelin B receptor (ETBR) expressed by osteoblasts [60,61]. When the osteolytic human breast cancer cell line ZR-75-1 was made to overexpress ET-1, osteoblastic metastases along with new bone formation were detected after inoculation into murine bone, and treatments with the ETAR selective antagonist (ABT-627) attenuated these osteoblastic lesions [62]. The ET-1/ETAR interaction also plays important roles in CIBP development [20,63]. In fact, the administration of ABT-627 into ET-1 expressing osteosarcoma-bearing mice attenuates CIBP behaviors, but the ETBR selective antagonist, A-192621, does not reduce CIBP behavior [20]. Consistently, phase II and III clinical trials in patients with hormone refractory prostate cancer showed that ABT-627 delayed the time to bone alkaline phosphatase progression and reduced bone pain, compared to those with placebo [64,65]. Additionally, local injection of ET-1 into the peri-tumoral area of a fibrosarcoma bone cancer murine model increased pain behaviors, and the ETAR antagonist BQ-123 inhibited these behaviors [66]. A meta-analysis of 9 clinical studies examining the effects of ETAR antagonists on castration-resistant prostate cancer patients, consistently showed that the ETAR antagonist Atrasentan reduces the relative risk of bone pain [67].

Newly formed bone or woven bone that is mediated by bone metastatic cancer cells is weaker than normal bone. While normal bone has a regular parallel alignment of collagen sheets and is mechanically strong, newly formed immature bone is made of a smaller number of randomly oriented collagen fibers and is mechanically weak, although it forms quickly [68,69]. Therefore, the weakening and instability of new tumor-bearing bone, easily leads to bone damage and CIBP. A canine prostate cancer cell line ACE-1 establishes osteoblastic bone metastatic lesions and develops spontaneous and mechanical pain behaviors in vivo [70]. Since the studies to reveal the impact of tumor-bearing newly formed bone on CIBP are underdeveloped, the use of this model can be helpful to further determine the detailed molecular mechanisms.

2.3. Immune Cells

Inflammation is also involved in the pathobiology of CIBP via several immune cell types (e.g., macrophages and mast cells) [71]. Macrophages are known to contribute to the tumor microenvironment, and they release inflammatory factors upon interacting with tumor cells. These inflammatory factors can induce both disease progression and cancer-related pain [72–74]. Some types of cancer cells that metastasize to the bone (e.g., breast cancer, prostate cancer) highly express and secrete the neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) [75,76]. Macrophages express the functional receptors for NGF [p75 neurotrophin receptor (p75NTR), tropomyosin receptor kinase A (TrkA)] and BDNF [tropomyosin receptor kinase B (TrkB)] [77]. NGF and BDNF derived from bone metastatic cancer cells activate macrophages to release pro-inflammatory cytokines [tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β] and inflammatory regulators [NGF, Prostaglandin E₂ (PGE₂)] that sensitize nociceptors [78,79]. In addition, as their names indicate, NGF and BDNF also directly regulate the survival, development, and function of neurons [80].

IL-1 β can stimulate expression of cyclooxygenase 2 (Cox-2), and Cox-2 facilitates synthesis of prostaglandins in macrophages [81]. Prostaglandins can sensitize or activate the sensory nerves by binding to prostanoid receptors, resulting in CIBP [82]. The administration of a Cox-2 inhibitor into an osteosarcoma CIBP model inhibits pain behaviors as well as bone destruction without affecting tumor burden [82–86]. Since most NSAIDs inhibit both Cox-1 and Cox-2, which are required for PGE₂ synthesis [87], and the major adverse events mediated by NSAIDs are thought to be Cox-1 dependent [88,89], blocking Cox-2 release from macrophages may be a better option for CIBP treatment than NSAIDs. However, a randomized, Phase III trial between a selective Cox-2 inhibitor (rofecoxib) and a non-selective NSAID (naproxen) in patients with rheumatoid arthritis revealed that chronic administration of rofecoxib causes more myocardial infarction than naproxen, while there is no significant difference in treatment efficacy between the two [90]. A more recent pre-clinical study using a lung carcinoma CIBP mouse model demonstrated that when microsomal PGE synthase-1 (mPGES-1), another prostaglandin synthesizing enzyme, is deleted, the onset of pain behaviors mediated by the growth of lung carcinoma in the tibia is delayed [91]. These findings suggest that targeting Cox-2 expression and PGE₂ synthesis in macrophages can be potential therapeutic strategies for CIBP. However, further studies are needed to elucidate the pathophysiology of adverse events before moving into the clinic.

Pre-clinical murine CIBP studies of osteosarcoma, breast cancer, and prostate cancer have demonstrated that blockage of NGF significantly attenuates pain behaviors and bone destruction mediated by bone tumors [92–94]. Additionally, an anti-NGF monoclonal antibody tanezumab was clinically tested in patients with osteoarthritis and diabetic peripheral neuropathy, and overall significant pain relief is observed in patients treated with tanezumab compared to those treated with placebo [95–98]. Although larger clinical trials will be needed, a recent clinical study in patients with bone metastatic prostate cancer, breast cancer, renal cell carcinoma, or multiple myeloma (placebo $n = 30$, tanezumab $n = 29$) demonstrated greater efficacy in pain relief in patients treated with tanezumab than placebo treated patients [99].

Protease-activated receptors (PAR-2), a sub-family of G protein-coupled receptors that are highly expressed on sensory nerves, are known to be involved in the development of inflammatory and neuropathic pain in rodent models [100–102]. PAR-2 is mainly activated by mast cell tryptase and trypsin [103–105]. Mast cells are located near sensory neurons; contain cytoplasmic granules that store inflammatory mediators; and their release of pain transmitters causes pain [106–108]. When the conditioned medium obtained from human squamous cell carcinoma is injected into the mouse hind paw, the skin mast cells are activated and increased pain behaviors are observed [109]. However, this cancer-associated mechanical allodynia is reversed with treatments of the tryptase inhibitor APC-366 or soybean trypsin inhibitor (SBTI) [109]. Activation of PAR-2 has been shown to increase levels of neuropeptides such as calcitonin gene-related peptide (CGRP) and substance

P (SP). Sensory nerve sprouting from CGRP expressing neurons is known to be associated with skeletal pain behaviors [110–113], and levels of plasma CGRP directly correlate with the pain intensity experienced in several pain related conditions [114,115]. It has been demonstrated that bone tumor enhances the PAR-2 expression in sensory nerves [116]. In addition, recent studies have revealed that tumor-infiltrating mast cells in bone metastatic tumors of gastric cancer promote bone metastatic growth, osteolytic lesions, and CIBP by stimulating angiogenesis [117,118].

2.4. Stromal Cells

Bone is a hypoxic tissue (pO₂: 8.1–26.7 mmHg) [119,120], and this hypoxic environment is crucial for controlling angiogenesis [121], bone repair [122], osteoblastogenesis [123], osteoclastogenesis [124], and hematopoiesis [125]. Moreover, under hypoxia, tumor cells generate large amounts of lactate through elevated levels of aerobic glycolysis, leading to a lowering of intracellular pH (pH 6.8). This is known as the Warburg effect [53,126]. To prevent cell death mediated by this intracellular acidification, tumor cells actively pump out the protons and lactate to the extracellular space. This extracellular acidic environment surrounding tumor cells can stimulate the cells of stromal origin in the marrow, such as mesenchymal stem cells (MSCs) and fibroblasts [127]. Bone marrow MSCs and fibroblasts are known to express high levels of acid sensing receptors [acid-sensing ion channel 3 (ASIC3), ASIC4, G protein-coupled receptor 4 (GPR4), and GPR65] [128], and become activated by the acidic environment created by bone metastatic tumor cells. This interaction leads to expression and secretion of inflammatory cytokines [IL-6, IL-8, IL-15, chemokine (C-C motif) ligand 5 (CCL5), IL-1β] as well as nociceptive mediators such as NGF and BDNF [128]. Therefore, it has been suggested that bone metastatic tumor cells induce CIBP by interacting with bone marrow stromal cells.

3. Discussion

Despite the improvement of current cytotoxic treatments, these treatments may not provide survival benefits to all advanced cancer patients. However, most of these patients suffer from symptoms that negatively impact their QOL, such as CIBP. CIBP is a very complex symptom since bone metastatic cancer, sensory nerves, and the bone microenvironment interact together to cause such a painful condition. Therefore, revealing the detailed mechanisms whereby the components that are responsible for bone metastatic progression are involved in the CIBP development will be very important in furthering understanding of this painful symptom and possibly for the development of effective therapies. In this review, we discussed the roles of the cells controlling bone remodeling, immune cells, and stromal cells in the development of CIBP (Figure 1). However, these findings are based on limited evidence. Further studies are therefore clearly needed in this area.

It has been suggested that higher patient QOL may indicate a survival benefit [129]. Along with this notion, recent research suggests that CIBP may be both a reason for decreased QOL and an indicator of survival [130–133]. For instance, the ALSYMPCA trial [134], which investigated the role of radium-223 in patients with prostate cancer and bone metastases, demonstrated that decreased pain levels correlated with increased overall survival. Additionally, the importance of nerves for cancer development has been appreciated [135]. Recent studies revealed that the sympathetic nervous system regulates the metastatic process of prostate cancer to bone [136], and that denervation can suppress tumorigenesis and metastasis [136–139]. However, it remains unclear whether sensory nerves that innervate bone, which are responsible for bone pain, also promote metastatic progression to bone. Therefore, revealing the mechanisms of CIBP may provide a strong foundation for much-needed treatments for bone metastases. Stopping pain signals may be useful to improve both morbidity and mortality.

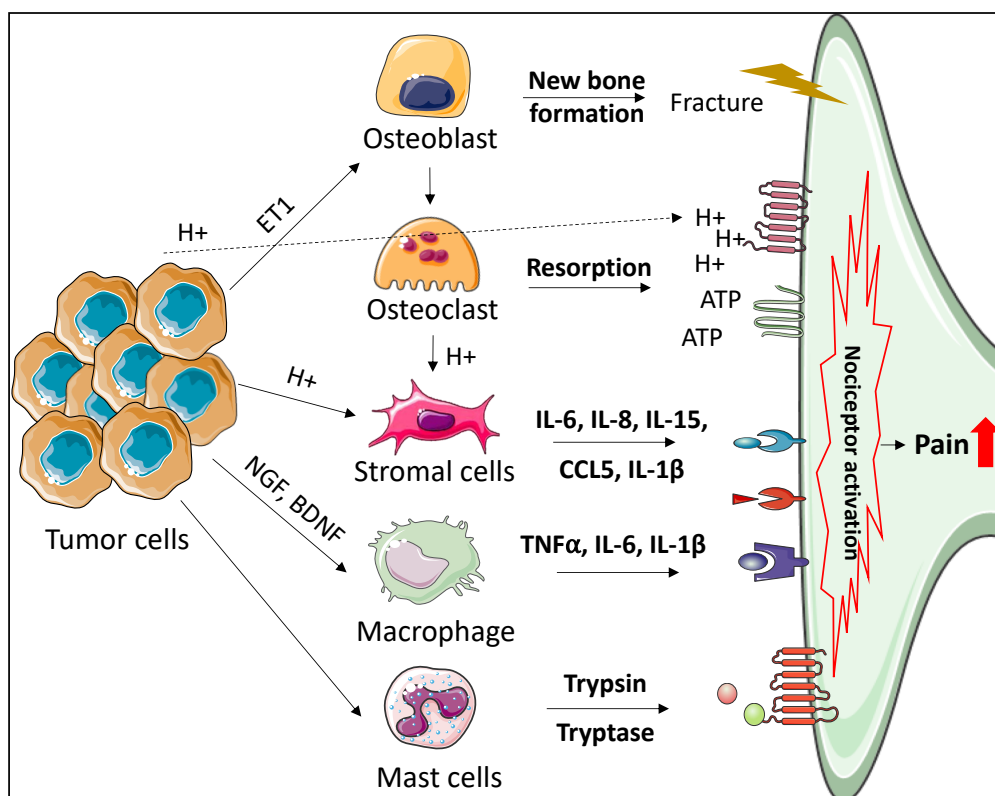


Figure 1. Mechanisms of bone microenvironment involvement in cancer-induced bone pain. Bone-disseminated tumor cells release factors (e.g., ET1) to stimulate the proliferation of osteoblasts (e.g., endothelin A/B receptors), resulting in new bone formation, which is structurally weak and prone to fracture. Active osteoblasts release RANKL to promote osteoclast activity, resulting in increased bone resorption which also weakens bone. During bone resorption, nociceptors become sensitized and activated through osteoclast mediated acidification and ATP accumulation, which activates the acid sensing TRPV1 and ASICs receptors, or the ATP-gated P2X receptors expressed on sensory neurons, respectively. Tumor cell derived H⁺ directly induces nociception via activation of the acid sensing receptors expressed on the sensory neurons. Stromal cells (e.g., fibroblasts and mesenchymal stem cells) also express acid sensing receptors, and acidification of the bone marrow space stimulates release of stromal cell derived pro-inflammatory cytokines (IL-6, IL-8, IL-15, CCL5, IL-1β) and nociceptive mediators (NGF and BDNF). Tumor cells also express NGF and BDNF, which activate macrophages to release pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) and inflammatory regulators (NGF and Prostaglandins) which directly induce pain via binding to their receptors on sensory neurons. Finally, tumor cells interact with peri-neural and tumor-infiltrated mast cells, releasing mast cell derived proteases (trypsin and tryptase) which activate sensory neurons by binding to PAR-2 receptor, resulting in pain and upregulation of pain-related neuropeptides (CGRP and SP).

4. Conclusions

The current first-line treatment for CIBP is still opioids. Opioids are somewhat effective, but can have serious side effects, and abuse and addiction of these analgesics are a growing concern. Therefore, more effective and safer alternative treatment options for CIBP are urgently needed. Unfortunately, there is currently no better treatment for CIBP than opioids, when administered alone. However, several lines of evidence suggest that the combination of non-opioid analgesics with opioids provides synergistic or additive analgesic effects that can lead to decreased opioid dose. In the case of CIBP, the combination treatment of PAR-2 antagonists with morphine can allow the use of significantly lower doses of morphine (1 mg/kg) while maintaining the same levels of analgesia as single high doses of morphine (3 or 10 mg/kg) in a CIBP rat model [140]. Although it might be difficult to immediately

replace opioids with other treatment modalities, we still need to continue efforts to reduce opioid use by discovering potential therapeutic targets for CIBP within the bone marrow microenvironment.

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Interactions Between Disseminated Tumor Cells and Bone Marrow Stromal Cells Regulate Tumor Dormancy

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Abstract

Purpose of Review To succinctly summarize recent findings concerning dormancy regulating interactions between bone marrow stromal cells and disseminated tumor cells.

Recent Findings Recent studies have highlighted roles of the bone marrow microenvironment, including osteoblasts, mesenchymal stem cells (MSCs), and endothelial cells, in inducing or maintaining cancer cell dormancy. Key pathways of interest include: osteoblast-induced transforming growth factor (TGF)- β 2 signaling, transfer of MSC-derived exosomes containing dormancy inducing microRNA, cancer cell cannibalism of MSCs, and endothelial cell secretion of thrombospondin 1 (TSP1).

Summary The bone marrow is a common site of metastatic disease recurrence following a period of cancer cell dormancy. Understanding why disseminated tumor cells enter into dormancy and later resume cell proliferation and growth is vital to developing effective therapeutics against these cells. The bone marrow stroma and the various pathways through which it participates in crosstalk with cancer cells are essential to furthering understanding of how dormancy is regulated.

Keywords Cancer cell dormancy · Bone marrow microenvironment · Bone marrow stromal cells · Osteoblasts · Mesenchymal stem cells · Endothelial cells

Introduction

Although the numbers of long-term cancer survivors are rapidly increasing, many of them are still at risk of recurrence even after decades of disease-free status [1, 2]. It has been suggested that very small numbers of cancer cells can escape from cytotoxic treatments and survive long periods of time either in primary or secondary sites [1, 2]. These cells are not actively proliferating and are inherently resistant to cytotoxic treatments, such as chemotherapies and radiation, that target dividing cells [3]. To make matters worse, these occult cancer cells often remain dormant for a long period of time but are clinically undetectable. Cancer cell dormancy is currently

a key obstacle in effectively treating cancer patients, due to the potential of treatment-resistant dormant cells to recur as lethal metastases [1, 3].

Cancer dormancy is thought to be a reversible low metabolic state, since it is primarily characterized by: (1) prolonged cancer cell survival in cell cycle arrest (spanning from weeks to decades) and (2) their potential ability to leave this state and start proliferating again, ultimately leading to disease recurrence [1, 2, 4–7]. It has been suggested that the following three mechanisms are involved in the development of cancer dormancy: cellular dormancy, angiogenic dormancy, and immune-mediated dormancy [8, 9]. Cellular dormancy is when disseminated tumor cells (DTCs) enter a state of quiescence or growth arrest in the G0 cell cycle phase due to a combination of external and/or internal cellular factors, such as upregulation of p27, a cell cycle inhibitor, to induce cycle arrest [3, 6, 8]. Angiogenic dormancy occurs when tumors lack sufficient vascularization, and consequently sufficient oxygen and nutrients, for further growth [8, 9]. Immune-mediated dormancy is reliant upon cytotoxic activities of T cells and the innate immune system inhibiting tumor growth [8, 10]. As noted above, the tumor microenvironment, such as

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endothelial cells and immune cells, is involved in the establishment of DTC dormancy.

The bone contains a rich tumor microenvironment and is a common site of cancer cell dissemination [1, 6, 11, 12•]. Indeed, DTCs within the bone marrow are often observed when cancer patients are initially diagnosed [1–3, 8]. Most of these DTCs naturally die off or are eradicated by cytotoxic treatments. However, some of them can survive within the harsh bone marrow microenvironment, evade initial resection or treatment, lay dormant for years, and eventually regrow and develop into a full-blown recurrence [4, 6]. Unfortunately, these recurrent tumors are very difficult to treat as they often acquire treatment resistance [6, 11, 12•]. The ability of DTCs to enter and exit dormancy within the bone marrow is thought to be due in part to interactions with specific bone marrow microenvironment stromal cells: osteoblasts, osteoclasts, mesenchymal stem cells (MSCs), endothelial cells, etc. However, how these interactions collectively regulate cancer dormancy is not fully understood to date.

Effectively modeling dormant cancer cells in their microenvironment can be challenging. Cocultures are useful in studying the interactions between cancer and stromal cells; however, 2D cocultures of these cells do not adequately represent natural physiology [7, 13••]. This problem is being overcome through the use of 3D nonadherent cocultures [13••]. The common strategies for measuring dormancy in cultured cells include proliferation assays, such as Ki-67 staining, fluorescent dye staining, and cell cycle analyses, where DNA and RNA markers are used to measure growth arrest at G0 phase [14, 15]. With in vivo animal models, tumor growth over time and histology are typically used to determine if inoculated tagged cancer cells are dormant or actively growing [6, 7]. A combination of in vitro and in vivo models are needed to adequately represent and study the dormancy phenotype [7].

Therefore, this review seeks to provide a concise overview of the current knowledge of the stroma-cancer cell interactions that regulate cancer dormancy within the bone marrow. This will be accomplished by highlighting recent studies addressing the roles of individual classes of bone marrow stroma in this crosstalk and then addressing the clinical significance of this research.

Osteoblasts

Although the primary function of osteoblasts is new bone formation, they also participate in maintaining hematopoiesis homeostasis [16–21]. In the marrow, osteoblasts serve as the specific microenvironment for hematopoietic stem cells (HSCs), or the HSC niche, to control homing to the marrow, quiescence, proliferation, and differentiation of HSCs [16–21]. The HSC niche is also beneficial in recruiting DTCs to the bone marrow and promoting early colonization

of the bone [22–24]. Osteoblasts secrete CXC-chemokine ligand 12 (CXCL12) that binds to the C-X-C motif chemokine receptor 4 (CXCR4) G-protein coupled receptor expressed on both HSCs and DTCs, promoting competitive cellular adhesion of both these cells in the bone marrow [25–28]. Moreover, DTCs hijack the functions of HSC niche that regulate HSC quiescence to become dormant. For example, transforming growth factor (TGF)- β expression in the HSC niche is considered a regulator of HSC quiescence and can potentially regulate DTC quiescence [12•, 29].

Live cell imaging of C4-2B4 prostate cancer cells treated with conditioned media from differentiated and undifferentiated osteoblasts showed that mature osteoblasts release factors that promote cancer cell quiescence [12•]. Specifically, TGF- β 2 and growth differentiation factor (GDF)10 were identified as dormancy-promoting osteoblast-secreted proteins through gene array analysis of the conditioned media, which can promote cell cycle arrest by binding to the TGF- β RIII receptor expressed on the prostate cancer cell lines [12•]. This binding activates p38 mitogen-activated protein kinase (MAPK) to phosphorylate retinoblastoma (RB) at the S249/pT252 site [12•]. This phosphorylation then upregulates p27 expression, and consequently inhibits cancer cell cycle progression and division, indicative of dormancy [6, 11, 12•]. Moreover, TGF- β 2 dormancy signaling was linked to osteoblast-derived ligand growth arrest specific 6 (GAS6) and the tyrosine kinase receptor Axl expression in a coculture model of prostate cancer and osteoblasts [30]. GAS6 is expressed more highly from osteoblasts cocultured with prostate cancer cells, and this high expression correlates with increased prostate cancer cell survival and decreased proliferation [22, 31]. In the coculture condition with osteoblasts, prostate cancer requires the GAS6/Axl axis to respond TGF- β 2-derived dormancy stimulation [30]. Finally, TGF- β is known to be immunosuppressive and aids in immune system evasion of dormant cancer cells through the inhibition of cytotoxic cells of the innate and adaptive immune systems (e.g., T cells and natural killer (NK) cells) [11].

Osteoblasts and osteocytes also secrete leukemia inhibitory factor (LIF), and the importance of DTC LIF receptor (LIFR) expression in maintaining a dormant phenotype in breast cancer cells (e.g., MCF7) disseminated to the bone marrow has recently been illustrated in a mouse model. In this study, inoculated MCF7 cells with LIFR knockdown displayed increased levels of osteoclastogenesis and cancer cell proliferation [32]. As we will discuss in the following section, osteoclasts are known to be a proliferation inducer for dormant DTCs. LIFR is also involved in promoting osteoclastogenesis by inhibiting parathyroid hormone-related protein (PTHrP) expression in cancer [11]. PTHrP promotes pre-osteoclast maturation by enhancing the expression of receptor activator of nuclear factor κ B ligand (RANKL) in osteoblasts [11].

The addition of other factors may be necessary to overcome the dormancy phenotype induced by osteoblasts. When breast

cancer cell dormancy model cell lines, MDA-MB-231BRMS1 and MCF-7, were co-cultured in a 3D model with an osteoblast matrix, they remained dormant until tumor necrosis factor (TNF)- α and interleukin (IL)-1 β were added to the culture [33]. These cytokines, which play a role in bone remodeling signaling, increased cancer cell proliferation by increasing the expression of prostaglandin E2 (PGE2). Cancer cell proliferation mediated by TNF- α and IL-1 β was inhibited by blocking the cyclooxygenase (COX) and PGE2 receptors [33].

Osteoclasts

Just as osteoblasts and osteoclasts have opposing roles in bone turnover (bone formation and resorption, respectively), they also have opposing roles in dormancy regulation. While osteoblasts are primarily associated with dormancy initiation and maintenance, osteoclasts have been reported to play a role in an exit of DTCs from a dormant state and the induction of osteolytic metastases [6, 11, 34]. Indeed, high levels of vascular cell adhesion molecule 1 (VCAM1) by DTCs have also been correlated with early disease recurrence, due to the ability of VCAM1 to bind the integrin $\alpha 4 \beta 1$ on osteoclasts and promote their osteolytic activity [4]. A study using intravital two-photon microscopy monitored the proliferation of 5TGM1 myeloma cells over time by labeling with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD) within murine long bones [34]. DiD is a fluorescent dye that binds the phospholipid membrane of cancer cells [35]. In non-proliferating cells, DiD expression remains high; however, in proliferating cells, this expression will decrease overtime as the cells divide [35]. In this study, the Kal-Col-GFP murine model in which osteoblast-like cells express emerald GFP was used to identify the osteoblastic endosteal niche. Myeloma cells colocalized with the endosteal niche and consistently highly expressed DiD (non-proliferating cells) [34]. Conversely, treatment of mice with the soluble form of RANKL to promote osteoclast maturation significantly decreased the number of DiD-expressing cells in the bone, suggesting that osteoclast activity can promote cellular proliferation of dormant cells [34].

Mesenchymal Stem Cells

MSCs are a type of pluripotent mesenchymal stromal cell, resident within the bone marrow [4, 36]. It has been established that crosstalk occurs between bone marrow MSCs and DTCs [4, 13•, 15, 36]. DTCs secrete factors, such as CXCL12, that act as an attractant for MSCs to initiate their migration to the cancer site, and the MSCs in turn modulate cancer cell growth and proliferation [4, 36, 37]. The exact pathways through which this crosstalk occurs, and the specific impacts it may have on dormancy are still being elucidated. One pathway currently of

primary interest in the field is the transfer of dormancy initiating microRNA (miRNA) from MSCs to cancer cells through exosomes or extracellular vesicles. For example, in a subpopulation of breast cancer cells, exosomes isolated from breast cancer-primed MSCs promoted cell cycle quiescence, ascertained through cell cycle analyses using propidium iodide (a DNA marker), 7-aminoactinomycin D (a DNA marker), and pyronin Y (a RNA marker) labels to track cells in G0 and G1 phase [15]. miR-222 and -223 were upregulated in these exosomes and antagomiR-222/223 could reverse the quiescent phenotype of breast cancer [15]. Additionally, antagomiR-222/223 treatment in a murine model increased the efficacy of carboplatin treatment in tumor-bearing mice inoculated with MSCs compared to control anti-miRNA-treated mice. Likewise, the extracellular vesicles produced by human MSCs reduced proliferation and sphere formation, indicative of dormancy, of MDA-MB-231 breast cancer cells in vitro [38•]. These findings indicated that miRNA from MSC exosomes or extracellular vesicles plays roles in initiating cancer cell dormancy and developing subsequent chemotherapy resistance [15].

Another interesting phenomena and mechanism for MSCs to initiate cancer cell dormancy is cell cannibalism. A 3D hanging drop model containing MD-MBA-231 cells and bone marrow-derived MSCs showed that stressed cancer cells cannibalize surrounding MSCs before entering a state of increased survival, where viability of these cells persisted in nutrient-poor media in vitro [13••]. Moreover, these cancer cells showed decreased tumorigenicity in mice following inoculation [13••].

Endothelial Cells

As previously stated, sufficient blood supply, typically provided through angiogenesis, is necessary to allow for cancer cell growth and progression [11]. Consequently, the absence or presence of angiogenesis can influence cancer cell entry into or exit from dormancy, respectively. The ability of angiogenesis to occur is in turn regulated by other components of the micro-environment. For example, a study of invasive ductal carcinoma (IDC) has shown that thrombospondin 1 (TSP1), a glycoprotein secreted from vascular endothelial cells that contains tryptophan-rich motifs, is associated with reduced IDC proliferation and cell cycle arrest at the G0/G1 phase in vitro [39•, 40]. More notably, this study identified a novel mechanism for IDC-derived interferon- γ (IFN- γ) to promote IDC dormancy evasion by reducing tryptophan expression, and consequently decreasing TSP1 synthesis and total expression levels in a dose-dependent manner [39••]. However, endothelial secreted proteins are not always dormancy promoting. Secretion of TSP1 has also been reported to decrease when endothelial cells are activated, and the proliferation promoting factors TGF- β 1 and periostin are instead secreted and encourage tumor growth [6, 11]. As a result, DTC proliferation can be influenced based on their location near active or inactive endothelial cells.

Other Microenvironment Factors

The bone marrow microenvironment contains an abundance of secreted factors that can inhibit DTC proliferation and promote entry into dormancy [6]. Although their primary sources have not been fully determined, all-trans retinoic acid, or atRA, and bone morphogenetic protein 7 (BMP-7) are both expressed in the bone marrow and are known to promote tumor dormancy [6, 7]. atRA has been reported to increase DTC TGF- β 2 expression and induce dormancy through the pathways previously discussed above (e.g., p38 and p27 activation) [6]. In addition to activating p38 MAPK signaling to inhibit proliferation, BMP-7, secreted from bone marrow stromal cells, has been shown to increase expression of N-myc downstream regulated gene 1 (NDRG1), a metastasis suppressor gene, and treatment of mice with BMP-7 suppresses cancer cell growth in the bone [6, 41, 42]. Notably, prostate cancer-derived secreted protein acidic and rich in cysteine (SPARC) induces bone marrow stromal expression of BMP-7 [42]. SPARC expression is upregulated in indolent cancer cells compared to aggressive cells isolated from murine bone following inoculation into the tibia [42]. Treatment of an in vivo mouse model with recombinant SPARC inhibited tumor growth in the bone [42].

Neurons can also modulate DTC dormancy. The role of sympathetic nervous system signaling through norepinephrine (NE) release in promoting dormancy exit has recently been appreciated [14]. NE promotes PC3 and DU145 human prostate cancer cell proliferation through binding to the β 2-adrenergic receptors expressed on the prostate cancer cells, as well as reducing GAS6 expression by osteoblasts [14]. Prostate cancer cells treated in vitro with NE showed a dramatic increase in proliferation, measured with Ki-67 staining, that was reduced with the addition of a β -adrenergic receptor inhibitor [14]. In a novel ex vivo model, explanted femurs from mice with wild-type or knockout GAS6 expression were injected with prostate cancer cells with and without NE [14]. After 48 h, cell cycle phase of the cancer cells was determined with flow cytometry and showed significant increase in cells in the G2-M phase when treated with NE in GAS6^{+/+} femurs, indicative of cell cycle progression [14].

Clinical Relevance

Cancer cell dormancy and disease recurrence in the bone marrow as metastases remain significant challenges in effectively treating cancer patients. Decades following initial treatment, dormant DTCs can resume proliferation into a potentially

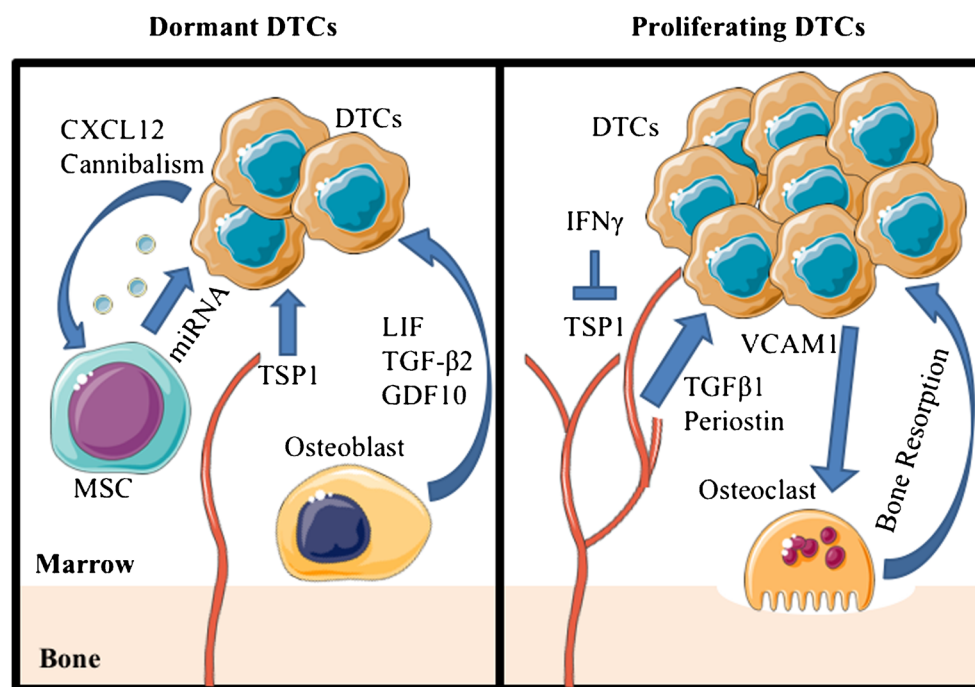


Fig. 1 The roles of the interactions between bone marrow resident disseminated tumor cells (DTCs) and bone marrow stroma in cancer cell dormancy and recurrence. Left panel: mesenchymal stem cells (MSCs) are mobilized to DTCs through CXC-chemokine ligand 12 (CXCL12) and release exosomes containing dormancy promoting miRNA or are cannibalized by the DTCs. Inactive endothelial cells release thrombospondin 1 (TSP1) to promote dormancy and reduce proliferation of DTCs. Osteoblasts secrete transforming growth factor (TGF)- β 2 and growth differentiation factor 10 (GDF10) to inhibit

cellular proliferation, and express leukemia inhibitory factor (LIF) that is recognized by LIF receptor (LIFR) on dormant DTCs. Right panel: DTCs express interferon- γ (IFN- γ) that inhibits TSP1 expression in endothelial cells. Active endothelial cells secrete TGF- β 1 and periostin to promote DTC proliferation. DTCs secrete vascular cell adhesion protein 1 (VCAM1) to promote maturation and activation of osteoclasts. Graphics adapted from Servier Medical Art (<https://smart.servier.com/>)

metastatic lesion [2]. Consequently, there is currently an abundant need for new therapeutics to treat these cancer patients with dormant DTCs [43]. Understanding the dormancy regulating interactions between DTCs and the surrounding bone marrow microenvironment will aid in devising more effective therapeutics that specifically target disseminated dormant cells, either to eradicate them through chemotherapeutics or immunotherapy or to prevent them from exiting dormancy [5, 6, 43, 44]. Manipulation of the microenvironment surrounding DTCs will alter their communication with these stromal cells and potentially make them more susceptible to treatment. One strategy for eliminating DTCs involves mobilizing them from the bone marrow, such as through the disruption of CXCL12/CXCR4 binding, to promote cell cycle progression and increase their susceptibility to established chemotherapeutics, although there is an inherent risk of inducing a potentially chemotherapeutic resistant recurrence with this strategy [3, 44–46]. For example, preclinical and clinical studies are evaluating the efficacy of small molecules, peptides, and monoclonal antibodies in disrupting the CXCL12/CXCR4 axis in patients with acute myeloid leukemia in order to overcome resistance to chemotherapy [47]. To maintain DTCs in a dormant state would likely require administration of dormancy-permissive agents to patients (e.g., TSP1) to prevent the eventual switch to metastatic proliferation and disease recurrence [3, 44]. However, the potential toxicities or off-target effects of chronic treatments to maintain dormancy will have to be carefully evaluated [44]. Alternatively, immunotherapy may be used in replacement of, or in conjunction with, chemotherapy to prevent tumor growth or eliminate DTCs [45]. This may be preferential to chemotherapy alone due to immunotherapy being dependent solely on immunogenicity of the DTCs and not on cell proliferation [45]. Further studies into the pathways and interactions regulating dormancy are needed to identify the most efficacious treatment strategy with the lowest patient risk. Additionally, this knowledge will: (1) provide greater insight into why some patients with DTCs never develop metastases, (2) provide strategies for identifying DTCs prone to resuming proliferation, and (3) potentially prevent overtreatment of patients who are at a lower risk of recurrence [4, 6].

Conclusions

There is much that still remains unknown about the mechanisms driving DTC dormancy and subsequent disease relapse in the bone marrow, and new discoveries in this field can be potentially groundbreaking. For example, it has only recently been shown through a genome wide short hairpin RNA screen that MSK1 gene expression in estrogen receptor-positive breast cancer inhibits metastatic progression, allowing for potentially novel identification of patients with dormant DTCs at a high or low risk of metastatic relapse based on this gene

expression and treatment customization [48]. While the gene alteration or mutation itself can drive cancer cells into a dormancy state, current evidence also supports the role of the tumor microenvironment, including stromal cells, in regulating both of these processes through crosstalk with the DTCs [6] (Fig. 1). As we have highlighted in this review, osteoblasts and MSCs have a role in initiating dormancy, while osteoclasts are primarily associated with metastasis progression [34]. Endothelial cells are able to promote both dormancy and renewed cancer cell growth depending on the activation status of the cells [6, 11]. Further studies are needed in order to take full advantage of the crosstalk with resident bone marrow stromal cells to therapeutically target and/or monitor DTCs prior to metastatic recurrence, potentially preventing disease relapse. Relapse prevention will dramatically improve patient quality of life and overall survival.

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Compliance with Ethical Standards

Conflict of Interest D. Brooke Widner, Sun H. Park, Matthew R. Eber, and Yusuke Shiozawa declare no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- Of importance
- Of major importance

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Determining Competitive Potential of Bone Metastatic Cancer Cells in the Murine Hematopoietic Stem Cell Niche

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Abstract

The ability of cancer cells to compete with hematopoietic stem cells (HSCs) to target the bone marrow microenvironment, or the HSC niche, during the dissemination process is critical for the development of bone metastasis. Here, we describe the methods for testing the relative potential of cancer cells to compete with HSCs for occupancy of the HSC niche by measuring the peripheral blood level of engrafted HSCs by flow cytometry in mice after bone marrow transplantation and tandem cancer cell inoculation. This method is useful for determining the molecular mechanisms for the roles of HSCs in the regulation of bone metastases.

Keywords Bone homing, Bone marrow transplantation, Disseminated tumor cells, Flow cytometry, Hematopoietic stem cell, HSC niche, Mouse model, Niche competition

1 Introduction

Hematopoietic stem cells (HSCs) give rise to mature blood cells (e.g., myeloid and lymphoid lineages) during a process known as hematopoiesis. Besides differentiation potential, to maintain the homeostasis of hematopoiesis HSCs must be capable of self-renewal, similar to other stem cell types. Although most HSCs reside within the bone marrow, it is known that a portion of HSCs circulate throughout the peripheral blood. These circulating HSCs eventually home to the bone marrow [1]. It has been suggested that the specific microenvironment in the marrow, or the “niche,” is an indispensable component for controlling HSC differentiation, self-renewal, and homing [2]. Growing evidence suggests that osteoblasts and their progenitor cells are an important element of the HSC niche, although bone marrow stromal cells (endothelial cells, macrophages, fibroblasts, pericytes, and adipocytes) are also known to serve as HSC niche components [3, 4]. Indeed, chemotactic factors released from osteoblasts and

other niche components [e.g., C-X-C motif chemokine 12 (CXCL12), and stem cell factor (SCF)] help to regulate essential HSC functions through the receptors expressed on HSCs [e.g., C-X-C chemokine receptor type 4 (CXCR4), c-Kit] [2, 5–7].

Interestingly, it has recently been revealed that disseminated tumor cells (DTCs) use bone homing mechanisms similar to HSCs to gain access to the marrow [8]. Moreover, once in the marrow, DTCs directly compete with HSCs to occupy the HSC niche and survive within the bone microenvironment [8]. The HSC niche also induces DTCs to undergo cellular dormancy, a state similar to HSC quiescence. It has been suggested that these dormant DTCs acquire treatment resistance and escape from immune surveillance, eventually developing into full-blown bone metastases [9, 10]. Therefore, the HSC niche is a critical player in bone metastatic progression. However, little is known as to the molecular mechanisms whereby DTCs interact with HSCs to establish bone metastases.

In this protocol, we describe the methods necessary to determine the potential of cancer cells to compete with HSCs for the bone marrow HSC niche by combining cancer cell inoculation techniques with bone marrow transplantation. The workflow of these methods to address specific questions in HSC niche competition is outlined in Fig. 1. These models can be further utilized to understand the mechanisms that control bone marrow stem cell

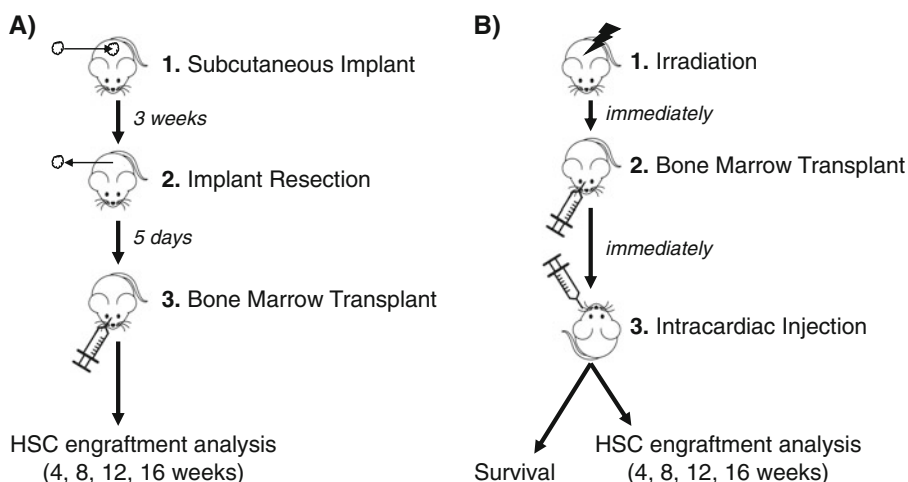


Fig. 1 Hematopoietic stem cell (HSC) niche competition assay workflow. **(a)** In the first model, a cancer cell line is implanted subcutaneously, grown for 3 weeks and then resected. Five days later, a bone marrow transplant is performed. **(b)** In the second model, a bone marrow transplant is performed after total body irradiation, and then a cancer cell line is inoculated systemically with intracardiac injection. In both models, the readout for HSC niche competition is HSC engraftment analysis by flow cytometry of peripheral blood. Additionally, a survival outcome can be used in the second model

niche homing of highly competitive cancer cells in order to promote the development of novel therapeutic strategies for bone metastasis.

2 Materials

2.1 Bone Marrow Transplant

1. Five- to Eight-week-old mice whose leukocytes express the cluster of differentiation (CD)45.2 or Ly5.2, such as C57BL/6J (Jackson Laboratory)
2. Five- to Eight-week-old mice whose leukocytes express CD45.1 or Ly5.1, such as B6.SJL-*Ptprca*^a *Peprc*^b/BoyJ (Jackson Laboratory)
3. CO₂ euthanasia chamber
4. Biosafety cabinet
5. 70% Ethanol solution
6. Sterile surgical instruments (scissors, scalpel, and forceps)
7. 3 mL Syringe with 25-g needle (Becton Dickinson)
8. Dulbecco's phosphate-buffered saline without calcium or magnesium (DPBS): 20 mg/L KCl, 200 mg/L KH₂PO₄, 8.00 g/L NaCl, 2.16 g/L Na₂HPO₄, and pH 7.2–7.4
9. Fetal bovine serum (FBS)
10. Fluorescence activated cell sorting (FACS) buffer: 5% FBS, DPBS
11. 10-cm Tissue culture (TC) dish
12. 40-μm Mesh strainer
13. 50-mL Conical tube
14. Hemocytometer and trypan blue solution, 0.4%
15. 0.9% Sterile saline solution
16. Isoflurane vaporizer
17. A gamma cell 40 cesium source
18. 28-g Insulin syringe

2.2 Cancer Cell Inoculation

1. Cancer cell of interest
2. DPBS
3. Biosafety cabinet
4. 40-μm Mesh strainer
5. 50-mL Conical tube
6. Hemocytometer and trypan blue solution, 0.4%
7. 1.5-mL Microcentrifuge tubes
8. 0.9% Sterile saline solution

9. Five- to Eight-week-old mice
10. Electrical clippers or a depilatory agent
11. Isoflurane vaporizer
12. Povidone-iodine, 5%
13. Sterile alcohol prep pads
14. 28-g Insulin syringe
15. Gelfoam[®] gelatin sponges (Pfizer)
16. 10-cm TC plate
17. Sterile surgical gauze
18. Sterile surgical instruments (scissors and forceps)
19. Surgical wound clips
20. Heating pad

2.3 HSC Engraftment Analysis

1. Heating pad
2. Sterile razor blades
3. 5-mL Round bottom tubes
4. Peripheral blood collection buffer: 5 μ M ethylenediaminetetraacetic acid (EDTA), DPBS
5. Red blood cell (RBC) lysis buffer (BioLegend)
6. DPBS
7. FACS buffer
8. Anti-CD45.1 and CD45.2 antibodies (BioLegend)
9. Flow cytometer

3 Methods

3.1 Bone Marrow Transplant

1. Euthanize and douse the skin of the donor mice in 70% ethanol (*see Note 1*). Remove the hind limbs and store in a 10-cm TC dish containing ice cold sterile DPBS on ice.
2. In a biosafety cabinet, separate the femur from the tibia and remove the excess tissue thoroughly using a scalpel. Be careful to maintain the integrity of the bone in order to maintain sterility of the bone marrow. Store the bare bones in a 10-cm TC dish containing ice cold sterile DPBS.
3. Remove the epiphyses from the femurs and tibias using a scalpel.
4. Flush the marrow from the diaphysis into a new sterile 10-cm TC dish with ice cold sterile FACS buffer using a 25-g needle and 3-mL syringe.

5. Pass the marrow several times through the syringe to dissociate the marrow pieces, and then pass the marrow suspension through a 40- μ m mesh strainer into a 50-ml conical tube.
6. Determine the bone marrow cell number using a hemocytometer and the trypan blue exclusion assay and then pellet the bone marrow cells by centrifugation ($350 \times g$ for 5 min).
7. Aspirate the supernatant and resuspend the bone marrow cells in ice cold sterile 0.9% saline at a concentration of 2×10^5 cells/100 μ L and store on ice (*see Note 2*).
8. If the intracardiac injection model is to be used, the recipient mice must be irradiated prior to bone marrow transplantation. Radiation dose is animal model dependent (*see Note 3*).
9. Anesthetize the recipient mouse and perform retro-orbital sinus injection of 2×10^5 bone marrow cells/100 μ L per each mouse.
10. Allow the animal to recover from anesthesia in a clean cage placed on a heating pad.
11. The engraftment of donor cells will be followed using flow cytometry as described in Subheading 3.3.

3.2 Cancer Cell Inoculation

1. Wash and harvest the cells to be tested under sterile conditions (*see Note 4*).
2. Pass the cells through a 40- μ m cell strainer into a 50-mL conical tube and determine cell number as before with a hemocytometer and the trypan blue exclusion assay and then pellet the cells by centrifugation ($180 \times g$ for 5 min).
3. Aspirate the supernatant and resuspend the cells in ice cold sterile 0.9% saline to an appropriate concentration and volume and transfer to a sterile microcentrifuge tube and store on ice (*see Note 5*).
4. If performing subcutaneous implant of the inoculum, cut the gelatin foam into 3 mm³ squares (scaffolds) using surgical scissors and with forceps place them in a 10-cm TC dish containing ice cold 0.9% saline. This must be performed under sterile conditions.
5. Anesthetize the recipient mouse using isoflurane inhalation and prepare it for the surgery to be performed, either subcutaneous implant and resection (Subheading 3.2.1) or intracardiac injection (Subheading 3.2.2) (*see Note 6*).

3.2.1 Subcutaneous Implant and Resection

1. If the recipient mouse has fur on its back, it must first be removed using electrical clippers or a depilatory agent.

2. Place the anesthetized mouse in the prone position and disinfect the skin of the back using a povidone-iodine scrub and alcohol prep pad.
3. Lift up a tent of skin midway up the back using forceps and make a 1-cm horizontal incision using blunt-tipped surgical scissors.
4. Use blunt-tipped surgical scissors to create a 1–2-cm subcutaneous pouch rostral to the incision site.
5. Blot a 3-mm³ gelatin scaffold on sterile surgical gauze to remove excess liquid and then saturate it with inoculum and place it deep into the subcutaneous pouch using forceps.
6. Release the implant and withdraw the forceps without disturbing the placement of the implant.
7. Close the incision using a surgical wound clip.
8. Allow the animal to recover from anesthesia in a clean cage placed on a heating pad.
9. Remove the wound clip after 7 days or when the incision has healed.
10. Three weeks after implantation, anesthetize the mouse using isoflurane inhalation. If the animal has fur on its back, it must first be removed using electrical clippers or a depilatory agent.
11. Place the anesthetized mouse in the prone position and disinfect the skin of the back using a povidone-iodine scrub and alcohol prep pad.
12. Make an incision near the subcutaneous implant with blunt-tipped surgical scissors.
13. Lift up a tent of skin near the incision/implant and use blunt-tipped surgical scissors to reopen the subcutaneous pouch.
14. Dissect the implant away from the skin and surrounding tissue and remove it entirely.
15. Close the incision using a surgical wound clip.
16. Allow the animal to recover from anesthesia in a clean cage placed on a heating pad.
17. Remove the wound clip after 7 days or when the incision has healed.

3.2.2 Intracardiac Injection

1. If the animal has fur on its chest, it must first be removed using electrical clippers or a depilatory agent.
2. Place the anesthetized mouse in the supine position and disinfect the skin of the chest using a povidone-iodine scrub and alcohol prep pad.
3. Load 100 µL of inoculum into an insulin syringe.

4. Use the index and middle finger of your nondominant hand to secure the limbs of the mouse (*see Note 7*).
5. Using your dominant hand, insert the needle perpendicular about 7–8 mm into the injection point, 1–2 mm left of the midline halfway between the clavicle and xiphoid process.
6. Using your nondominant hand, pull up very slightly on the plunger being careful not to move the needle. If the needle is in the correct position (left ventricle), you will see a pulse of bright red blood flush into the inoculum.
7. Inject the inoculum slowly over about 30 s. During the injection, periodically confirm that the needle is still in the left ventricle by pulling up on the plunger slightly to check for the signature pulse of bright red blood (*see Note 8*).
8. Gently remove the needle.
9. Allow the animal to recover from anesthesia in a clean cage placed on a heating pad.

3.3 HSC Engraftment Analysis

1. Place the cage of animals to be analyzed on the heating pad and allow it to warm up for 10 min.
2. Place a mouse on top of the wire food rack and thread its tail through to the bottom.
3. Make a small nick in the tail vein perpendicular to the tail with a razor blade and collect the blood droplets in a 5-mL round bottom tube containing 4-mL peripheral blood collection buffer (*see Note 9*). Use the lip of the tube to catch the blood droplets and be sure that they mix with the collection buffer by tapping the tube gently in order to prevent coagulation.
4. Apply pressure to the wound with a piece of gauze and confirm hemostasis of the tail wound before placing the mouse back in its cage.
5. Centrifuge the collection tubes ($350 \times g$ for 5 min).
6. Decant the supernatant and resuspend the pellets in 500 μ L RBC lysis buffer and incubate at room temperature for 5 min (*see Note 10*).
7. Fill each tube with 4 mL DPBS and then centrifuge them ($350 \times g$ for 5 min).
8. Decant the supernatant and resuspend the pellets in 500 μ L FACS buffer containing anti-CD45.1 and CD45.2 antibodies (*see Note 11*).
9. Incubate on ice in the dark for 1 h.
10. Fill each tube with 4 mL DPBS and then centrifuge them ($350 \times g$ for 5 min).

11. Decant the supernatant and resuspend the pellets in 500 μL FACS buffer.
12. Analyze the samples for expression of host hematopoietic cells versus expression of donor hematopoietic cells by distinguishing CD45.1 and CD45.2 positive cells by flow cytometry (*see Note 12*).

4 Notes

1. The donor mice must express a different CD45 allele than the recipient mice in order to determine HSC engraftment. We recommend performing the HSC engraftment analysis (Subheading 3.3) on representative naïve donor and recipient mice before transplantation to verify distinguishability by flow cytometry.
2. Instead of mouse donor cells, it is possible to use human HSCs at the same concentration, as long as the recipient animal is immunocompromised. In this case, we would recommend using NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice as a recipient.
3. If greater HSC engraftment is desired, the recipient mice can be irradiated immediately before transplantation. For lethal irradiation levels in immunocompetent mice, we recommend a total body dose of 1140 cGy (two 570 cGy doses, 3 h apart). For lethal irradiation levels in immunocompromised mice, we recommend a total body dose of 700 cGy (two 350 cGy doses, 3 h apart). If human HSC transplant is to be performed in immunocompromised mice, we recommend a sublethal total body dose of 300 cGy (two 150 cGy doses, 3 h apart).
4. In order to remove the variable of implanted cell growth after intracardiac injection, it is possible to irradiate the cell line to be tested immediately before harvest and implantation. In our studies using prostate cancer cells, we first irradiated them with 8 Gy (two 4 Gy doses, 3 h apart). However, irradiation dose will be cell dependent. In this case, a dose that causes cell growth arrest but not apoptosis should be used.
5. The concentration of the cell suspension depends on the aggressiveness of the cell line and the inoculation to be performed. As a general rule, the subcutaneous implant will require 10 μL of inoculum whereas the intracardiac injection requires 100 μL . In our studies using prostate cancer cells, we used 2×10^5 cells/10 μL for the subcutaneous implant and 2×10^6 cells/100 μL for the intracardiac injection. Very aggressive cell lines may not require as many cells, but these are good starting points. For ease of use, we recommend

creating individual tubes containing the inoculum required for each subcutaneous implant.

6. The model chosen depends on the hypothesis to be tested. The subcutaneous implant model relies on the ability of the cell line to disseminate from the primary implant to the bone on its own. While this closely models what happens clinically, the number of disseminated cells in the bone is often quite low and is entirely cell line dependent. The intracardiac model floods the circulation with many cells which increases “dissemination” to the bone, with the caveat that it does not accurately model the dissemination process. Also, using the intracardiac model it is possible to employ an engraftment readout using animal survival if the recipient animals are first lethally irradiated. In this model, longer survival times signify greater HSC engraftment.
7. It is essential that the mouse is flat on its back with its internal organs centered. It is common for the mouse to shift its weight naturally to favor one side over the other, but if this happens it is difficult to reliably locate the left ventricle with the needle. When the mouse is in the correct position, use your hand to secure it in place without using excessive pressure. Pressure on the abdomen causes shifting of the internal organs, again making the injection more challenging.
8. If the action of pulling up on the plunger creates a vacuum and not a pulse of blood, the needle has either drifted too far up or too deep and is no longer in the left ventricle. If this happens, slightly push the needle deeper or withdraw it until the pulse of blood is observed before proceeding with the injection.
9. Create a nick about halfway down the tail so that if the initial cut fails to produce a blood droplet it is possible to make another nick rostral to the initial cut. Do not squeeze or attempt to milk the tail, as this can cause contamination with lymphatic fluid. In our experience, five drops (~10–50 μ L) is sufficient for analysis.
10. This process can be repeated until the pellet is no longer red, or can be skipped entirely if there is concern for lost cells during RBC lysis.
11. It is recommended to use antibodies conjugated to fluorophores that have emission spectrums that do not overlap much, such as fluorescein isothiocyanate (FITC) and allophycocyanin (APC). Concentrations and multiplexing of antibodies require in lab optimization.
12. It is useful to perform HSC engraftment analysis many times over a period of several weeks. We have found that hematopoietic cells of transplanted origin can be detected up to 16 weeks after transplant.

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