AWARD NUMBER: **W81XWH-19-1-0464**

TITLE: Biodegradable Cationic Nanoparticles as a Push Chemodrug Carrier and a Pull cfDNA Scavenger Against Breast Cancer Metastasis

PRINCIPAL INVESTIGATOR: **Piero DALERBA**

CONTRACTING ORGANIZATION: **Columbia University Medical Center (CUMC)**
New York, NY 10032 (USA)

REPORT DATE: **August 2020**

TYPE OF REPORT: **Annual**

PREPARED FOR: **U.S. Army Medical Research and Development Command (USAMRDC)**
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2020	Annual	15Jul2019-14Jul2020

4. TITLE AND SUBTITLE

Biodegradable Cationic Nanoparticles as a Push Chemodrug Carrier and a Pull

cfDNA Scavenger Against Breast Cancer Metastasis

5a. CONTRACT NUMBER
W81XWH-19-1-0464

5b. GRANT NUMBER
BC180904P1

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Piero DALERBA
E-Mail: pdd2109@columbia.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Columbia University Medical Center
Irving Cancer Research Center - Rm 924
1130 St. Nicholas Ave.
New York, NY 10032 (USA)

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command (USAMRDC)
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

This is a revised version (12/23/2020) of the original report, submitted upon request by the USAMRDC.

14. ABSTRACT

The goal of this project is to develop a novel therapeutic approach for the prevention of breast cancer metastasis, by using *nucleic acid binding nanoparticles* (NABNPs) designed to deliver high payloads of cytotoxic drugs to tumor tissues, while simultaneously scavenging the pro-inflammatory *cell-free DNA* (cfDNA) that is released in the blood circulation as a result of tumor progression and/or destruction by chemotherapy. This is a collaborative research project conducted in partnership by two teams, supported by two distinct awards (BC180904, BC180904P1). Among the key aims of the research supported by this award (BC180904P1, partnering team) is the development of experimental models that enable the visualization and quantification of breast cancer dissemination in live animals, using non-invasive imaging techniques that can detect bio-luminescent signals from live cancer cells, and compare them over time, in order to obtain longitudinal measurements of both the anatomical location and overall burden of metastatic tissue. During the first year of the award, we engineered multiple *triple negative breast cancer* (TNBC) cell lines with different lentivirus constructs, each encoding for both fluorescent (EGFP, ZsGreen) and bioluminescent reporters (different variants of Luciferase from the firefly *Photinus pyralis*). We then compared the performance of the various lentivirus constructs in terms of sensitivity and stability of expression over time, in both mouse and human TNBC cells, *in vitro* and *in vivo*. Our experiments led to the generation of 3 independent TNBC cell lines (1 murine, 2 human) expressing high levels of both ZsGreen and Luciferase (*Photinus pyralis*), and can be efficiently monitored in terms of longitudinal growth and spontaneous metastatic dissemination in live animals. Using these models, we performed *in vitro* and *in vivo* experiments that demonstrated the capacity of various NABNP formulations to scavenge the cfDNA released *in vitro* and *in vivo* following tumor exposure to cytotoxic chemotherapy, and thus to dampen its pro-inflammatory and pro-metastatic effects, without compromising anti-tumor activity and systemic tolerability at equimolar doses of cytotoxic drugs.

15. SUBJECT TERMS

Breast cancer, Metastasis, Chemotherapy, Drug formulation, Nanoparticles, Circulating free DNA, DNA scavenging, Anti-inflammatory effects, Prevention of metastasis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	16	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION.

The goal of this project is to develop a novel therapeutic approach for the prevention of breast cancer metastasis, by using *nucleic acid binding nanoparticles* (NABNPs) designed to deliver high payloads of cytotoxic drugs to tumor sites, while simultaneously scavenging the pro-inflammatory *cell-free DNA* (cfDNA) that is released in the blood circulation as a result of tumor progression and/or destruction by chemotherapy. Among the key aims of the project is the development of experimental models that enable the visualization and quantification of breast cancer dissemination in live animals, using non-invasive imaging techniques that are able to detect bio-luminescent signals from live cancer cells and then compare them over time, in order to obtain accurate and longitudinal measurements of both the anatomical location and overall burden of metastatic tissue.

2. KEYWORDS.

Breast cancer, Metastasis, Chemotherapy, Drug formulation, Nanoparticles, Circulating free DNA, DNA scavenging, Anti-inflammatory effects, Prevention of metastasis

3. ACCOMPLISHMENTS.

Major goals of the project. This is a collaborative project conducted in partnership by two research teams (Kam W. Leong, Piero Dalerba), supported by two distinct awards (BC180904, BC180904P1). The project envisions three specific aims, pursued in collaboration by the two research teams:

AIM 1: *To synthesize and optimize nucleic acid binding nanoparticles (NABNPs) with respect to cfDNA-scavenging ability, drug delivery efficiency and low toxicity.* This aim includes only one major task: **Major Task 1** (***Synthesis and characterization of PCL-b- PDACL-b-PEG nanoparticles***).

AIM 2: *To evaluate the anti-metastatic effect of NABNPs as cfDNA scavengers and drug carriers, as well as investigate their anti-metastatic mechanism in vitro*. This aim includes three major tasks: **Major Task 2** (***Evaluation of the capacity of DTXL-loaded PCL-b-PDACL-b-PEG nanoparticles to scavenge cfDNA and inhibit breast cancer cell migration in vitro***), **Major Task 3** (***Evaluation of the disruption of cfDNA related complex and the neutralization of cfDNA related microvesicles by PCL-b-PDACL-b-PEG nanoparticles***) and **Major Task 4** (***Elucidation of the cfDNA-scavenger mechanism of PCL-b-PDACL-b-PEG nanoparticles by tracking endocytosis and intracellular distribution***).

AIM 3: *To evaluate the biodistribution and therapeutic efficacy of NABNPs using in vivo models of breast cancer metastasis.* This aim includes six major tasks: **Major Task 5** (***Regulatory approval of animal research experiments***), **Major Task 6** (***Generation and validation of animal breast cancer models for the in vivo study of spontaneous metastasis***), **Major Task 7** (***Evaluation of the pharmacokinetics, biodistribution and accumulation of PCL-b-PDACL-b- PEG nanoparticles in vivo***), **Major Task 8** (***Evaluation of the therapeutic efficacy of DTXL-loaded PCL-b-PDACL-b-PEG nanoparticles in the 4T1 model of spontaneous metastasis***), **Major Task 9** (***Evaluation of the therapeutic efficacy of DTXL-loaded PCL-b-PDACL-b-PEG nanoparticles in PDX models***), and **Major Task 10** (***Preparation of a manuscript reporting on study results***).

Accomplishments under the goals:

Major Task 1 (***Synthesis and characterization of PCL-b-PDACL-b-PEG nanoparticles***)**.** This major task was, for the most part, to be pursued during the 1st year of the award (months 1-12) by the leading team (Leong). Indeed, during the 1st year of the award, the leading team completed the chemical synthesis and biophysical study of various types of NABNPs **(Subtasks 1.1-1.4).** For a complete description of these experiments, please, refer to the Technical Report related to the leading team's award (BC180904). With specific regard to the contribution to this major task by the partnering team (Dalerba), it was restricted to **Subtask 1.5** (***To evaluate the cytotoxicity of DTXL-loaded PCLb-PDACL-b-PEG nanoparticles on in vitro cell lines***), which required the generation of

a portfolio of *triple-negative breast cancer* (TNBC) cell lines engineered to simultaneously express fluorescent and bio-luminescent reporters, to be transferred from the partnering team (Dalerba) to the leading team (Leong) for joint biochemical and pharmacological studies. This subtask was completed **(100%)** within the first 12 months of the award, as envisioned in the original *statement of work* (SOW). The partnering team (Dalerba) obtained 3 different TNBC cell lines that were identified as experimental models appropriate for the study, based on their capacity to spontaneously metastasize *in vivo*, in animal models: **4T1** (mouse; ATCC #CRL-2539), **MDA-MB-231** (human; ATCC #HTB-26), **MDA-MB-468** (human; ATCC #HTB-132). The partnering team (Dalerba) then used 2 different lentivirus vectors to infect the three cell lines: pLentiLox3.7-mcs-IRES-Luciferase/EGFP (generated in house by modification of the pLentiLox3.7 backbone (Addgene #11795) and the **pHIV-Luc-ZsGreen** (Addgene #39196). Our experimental results led to two important observations:

1) shortly after infection with the pLentiLox3.7-mcs-IRES-Luciferase/EGFP vector, 4T1 cells, which are murine in origin, rapidly and permanently lost EGFP expression, while they retained expression of ZsGreen following infection with the pHIV-Luc-ZsGreen vector; this observation suggested that 4T1 cells, might be able to silence the *Cytomegalovirus* (CMV) promoter (which drives the expression of EGFP in the pLentiLox3.7 backbone), but not of the *Eukaryotic Translation Elongation Factor 1 Alpha* (EF1a) promoter (which drives the expression of ZsGreen in the pHIV-Luc-ZsGreen backbone), in a manner that is reminiscent to what observed in mouse *embryonic stem* (ES) cells (Meilinger *et al.*, *EMBO Reports*, 10:1259-64, 2009);

2) in human TNBC cell lines (MDA-MB-231, MDA-MB-468), the intensity of green fluorescent signals observed following infection with the pHIV-Luc-ZsGreen vector were higher than those observed following infection with the pLentiLox3.7-mcs-IRES-Luciferase/EGFP vector, even after exclusion of un-infected cells, indicating a superior performance of the pHIV-Luc-ZsGreen vector as a reporter for *in vitro* and *in vivo* assays.

The partnering team (Dalerba), therefore, proceeded to use *fluorescence activated cell sorting* (FACS) for the isolation of infected cells (ZsGreen$^+$) from mixed cultures infected with the pHIV-Luc-ZsGreen construct, leading to the generation of sub-lines expressing the reporter genes at 99-100% purity **(Figure 1)**.

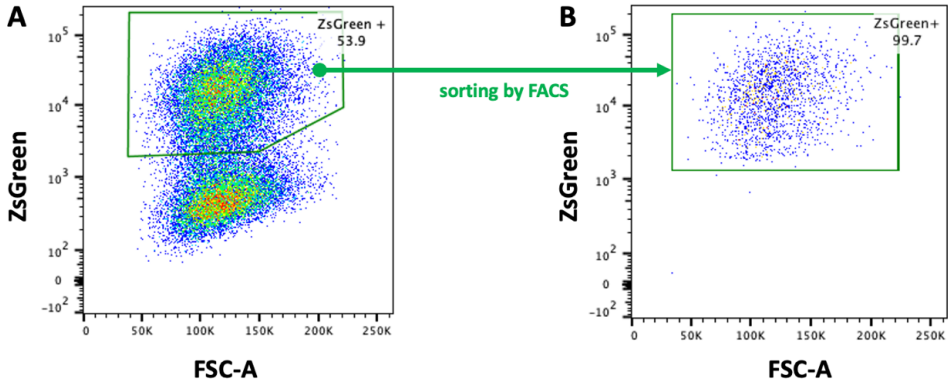

Figure 1. Generation of *triple-negative breast cancer* (TNBC) cell lines engineered to express fluorescent reporters. (A) Following infection with the pHIV-Luc-ZsGreen lentivirus vector, the MDA-MB-231 breast cancer cell line was analyzed by flow cytometry and observed to express high levels of green fluorescence in a substantial percentage (54%) of cells. **(B)** MDA-MB-231 cells expressing high levels of green fluorescence was (ZsGreen$^+$; green gates) were sorted by *fluorescence activated cell sorting* (FACS), and propagated *in vitro* as pure sub-line (>99% ZsGreen$^+$).

All three ZsGreen$^+$ sub-lines generated during the 1st year of the award were then transferred to the leading team (Leong) for joint biochemical and pharmacological studies. The achievement of this technical advancement during the 1st year of the award enabled the leading team (Leong) and partnering team (Dalerba) to move forward with a first round of *in vitro* experiments aimed at testing the anti-tumor activity of NABNPs loaded with cytotoxic agents used in the chemotherapy of TNBCs. The results of this first round of *in vitro* experiments showed that NABNPs loaded with chemotherapy drugs have equal, if not superior anti-tumor activity as compared to equimolar doses of chemotherapy drugs alone **(Figure 2a)**. **For a complete description of these joint experiments, please, also refer to the Technical Report related to the leading team's award** (BC180904)

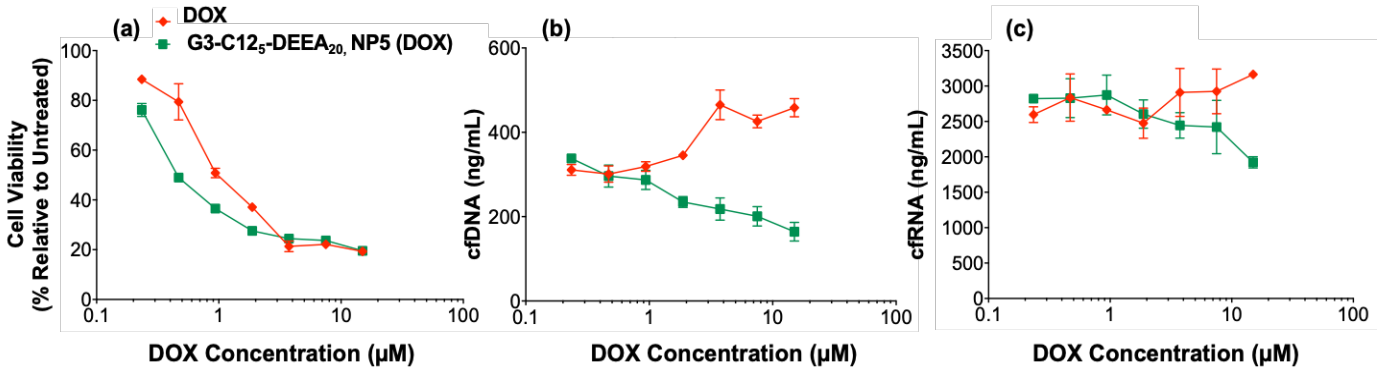

Figure 2. Comparative evaluation of the anti-tumor activity and DNA-scavenging properties of NABNPs loaded with cytotoxic drugs. (A) NABNPs (PAMAM-G3-DEEA20-C125) loaded with doxorubicin (DOX) display similar, if not superior anti-tumor activity against MDA-MB-231 cells as compared to equimolar doses of DOX alone, as demonstrated by a lower IC50 (i.e. a lower value for the DOX concentration necessary to achieve a 50% inhibition of tumor growth. **(B-C)** NABNPs (PAMAM-G3-DEEA20-C125) loaded with doxorubicin (DOX) reduce *cell-free DNA* (cfDNA; b) and *cell-free RNA* (cfRNA; c) content in tissue culture supernatants of MDA-MB-231 cells treated with DOX.

Major Task 2 (*Evaluation of the capacity of DTXL-loaded PCL-b-PDACL-b-PEG nanoparticles to scavenge cfDNA and inhibit breast cancer cell migration in vitro*). This major task is be completed during the 2nd and 3rd years of the award (months 13-36) as a joint collaboration between the leading team (Leong) and the partnering team (Dalerba). However, in order to achieve this major task, especially with regard to **Subtask 2.3 (*To evaluate the capacity of DTXL-loaded PCL-b-PDACL-b-PEG nanoparticles to inhibit the migration and proliferation properties of breast cancer cells*),** which relies on microscope imaging techniques, the availability of TNBC cell lines expressing a green fluorescent reporter is key to ensure a high visual quality of experiments, and to enable the use of computer-assisted, time-lapse, live-imaging platform for the quantitative analysis of their results (e.g. quantitative measurements of cell motility and growth kinetics). As described above, this important technical advancement was achieved during the previous reporting period (please, see description of the generation of TNCB cell lines engineered to constitutively express ZsGreen, a green fluorescence reporter, under Major Task 1; **Figure 1**). The achievement of this technical advancement during the 1st year of the award enabled the leading team (Leong) and partnering team (Dalerba) to perform *in vitro* experiments aimed at testing the effects of NABNPs as scavengers of cfDNA released in tissue-culture supernatants following exposure to cytotoxic drugs, and as inhibitors of cell migration in TNBC cell lines. These experiments confirmed the capacity of NABNPs to act as scavengers of both *cell-free DNA* (cfDNA) and *cell-free RNA* (cfRNA) in tissue culture supernatants of MDA-MB-231 cells treated with cytotoxic agents **(Figure 2b-2c)**, as well as to reduce the pro-migratory effects on TNBC cells of *damage-associated molecular pattern* (DAMP) molecules released in tissue-culture supernatants following exposure to cytotoxic drugs of the taxane family **(Figure 3)**.

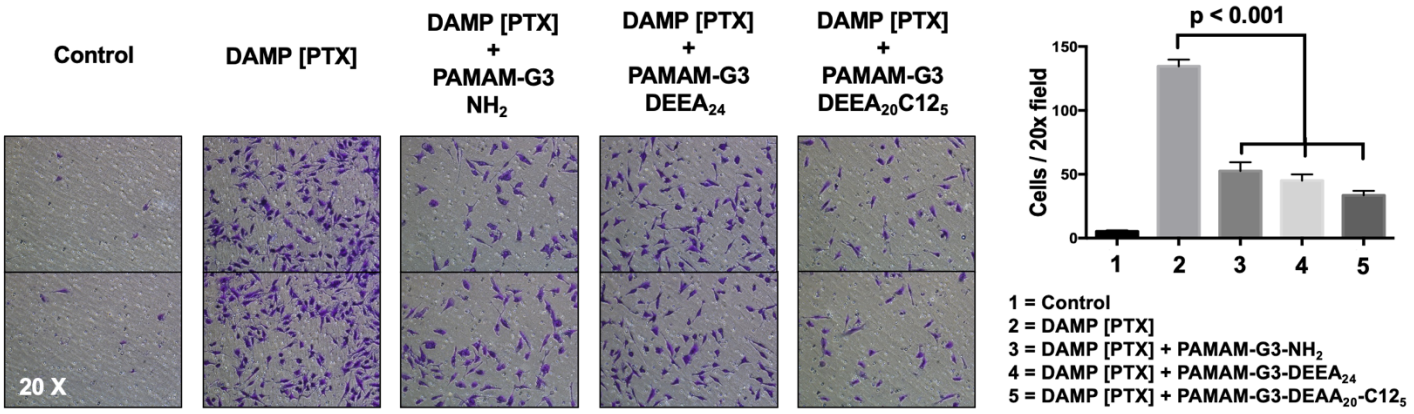

Figure 3. Evaluation of NABNPs as "dampeners" of the cell motility stimulated by *damage-associated molecular pattern* (DAMP) molecules released by tumor cells following chemotherapy. MDA-MB-231 cells were cultured onto a Matrigel-coated porous membrane and exposed to tissue culture supernatants of MDA-MB-231 cells treated with paclitaxel (PTX; 100 nM, 6 hours), either alone or in combination with various NABNP formulations (25 ug/ml). After 24 hours, cells migrated through the porous membrane were visualized using a crystal violet stain and counted.

Taken together, these experiments led to the anticipated, though still partial **(50%)**, completion of Subtask 2Subtask 2.3. **For a complete description of these joint experiments, please, also refer to the Technical Report related to the leading team's award** (BC180904).

Major Task 3 (*Evaluation of the disruption of cfDNA related complex and the neutralization of cfDNA related microvesicles by PCL-b-PDACL-b-PEG nanoparticles*). This major task is to be pursued during the 2nd and 3rd years of the award (months 21-27) by the leading team (Leong).

Major Task 4 (*To elucidate the cfDNA-scavenger mechanism of PCL-b-PDACL-b-PEG nanoparticles by tracking endocytosis and intracellular distribution*). This major task is to be pursued during the 3rd year of the award (months 27-36) as a joint collaboration between the leading team (Leong) and the partnering team (Dalerba). However, in order to achieve all the subtasks of this major task, including **Subtask 4.1 (*To quantify the uptake of PCL-b-PDACL-b-PEG nanoparticles by live cells, including breast cancer cells*), Subtask 4.2 (*To elucidate the role of endocytosis pathways in the uptake of PCL-b-PDACL-b-PEG nanoparticles by living cells, including breast cancer cells*)** and **Subtask 4.3 (*To track the intracellular distribution and bio-degradation of PCL-b-PDACL-b-PEG nanoparticles in living cells, including breast cancer cells*),** all of which rely on microscope imaging techniques, the availability of TNBC cell lines expressing a green fluorescent reporter will be of great help in ensuring the visual quality of the experiments, and in enabling the use of computer-assisted, time-lapse, live-imaging platform for the quantitative analysis of their results (e.g. quantitative measurements of intra-cellular incorporation and sub-cellular trafficking of NABNPs labeled with red fluorescent dyes). This important technical advancement was fully achieved **(100%)** during the previous reporting period (please, refer to the description of the generation of TNCB cell lines engineered to constitutively express ZsGreen, a green fluorescence reporter, under Major Task 1; **Figure 1**).

Major Task 5 (*Regulatory approval of animal research experiments*). This major task was to be completed during the 1st year of the award (months 1-6) by the partnering team (Dalerba), who is responsible for the coordination of the *in vivo* experiments in animals. Indeed, this major task was completed **(100%)** within the 1st year of the award. The animal protocol describing the experiments envisioned under the research proposal was approved by *Columbia University*'s *Institutional Animal Care and Use Committee* (IACUC) on 08/29/2019 **(Subtask 5.1)**. A matching protocol was then submitted for review to the *Department of Defense* (DOD) *Animal Care and Use Office* (ACURO) on 12/13/2019, and finally approved by the DOD's ACURO on 03/17/2020 **(Subtask 5.2)**.

Major Task 6 (*Generation and validation of animal breast cancer models for the in vivo study of spontaneous metastasis*). This major task was intended to be completed during the 1st and 2nd years of the award. During the 1st year of the award, we completed **(100%) Subtask 6.1 (*Engineering of 4T1 cells with lentivirus constructs encoding for Luciferase reporter genes*)**, as described above under Major Task 1, and further detailed below under Section 6 (PRODUCTS). Following the approval of our animal protocol by DOD's ACURO (see above, under Major Task 5), we also actively initiated working on **Subtask 6.2 (*Time-course studies on the kinetics and tissue tropism of spontaneous metastatic dissemination of 4T1 cells engrafted in Balb/cJ mice*)**, and made important progress towards its completion **(50%),** as we were able to confirm that all three TNBC cell lines that we engineered to express a bio-luminescent reporter (i.e. the Luc2P variant of the firefly Luciferase), indeed, could be detected in terms of their anatomical location following *in vivo* injection in mice, based on the emission of bright and specific bio-luminescent signals that can measured using an *IVIS Spectrum* machine PerkinElmer **(Figure 4)**. Unfortunately, however, due to the *Coronavirus disease 2019* (COVID-19) outbreak in New York City (March 2020), we were unable to complete a full time-course study of the metastatic spread of the three cell lines by the end of the 1st year, as also detailed below, under Section 5 (CHANGES/PROBLEMS). We have now resumed experimental operations *in vivo*, and are aggressively pursuing the completion of this sub-task (see also below, under: *Research plans for the next reporting period*). Our preliminary results indicate that all three TNCB lines can form solid tumors at primary injections sites, and also display reproducible

growth kinetics across biological replicates (i.e. equal doses of cells from the same cell line appear to undergo numerical expansion with similar mathematical functions across different animals, when evaluated using quantitative measurements of their global bio-luminescent signal).

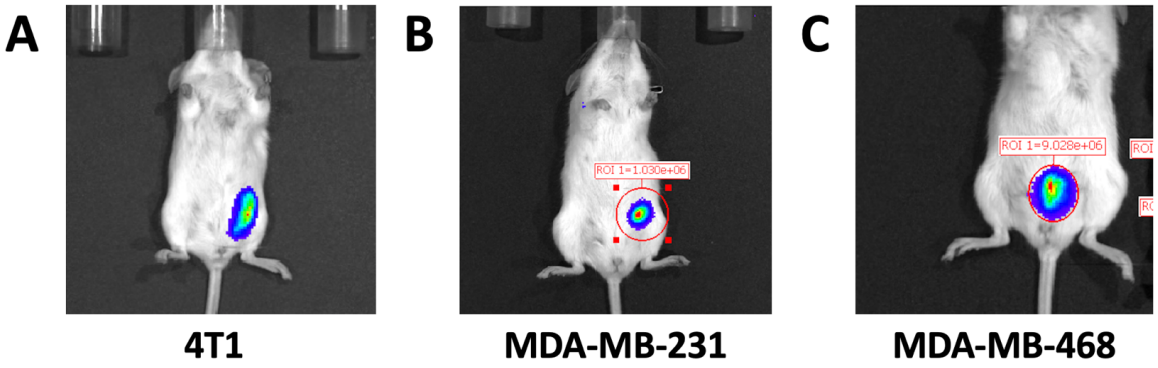

Figure 4. Detection of bio-luminescent signals from breast cancer cell lines infected with the pHIV-Luc-ZsGreen lentivirus vector and injected *in vivo*, in the *sub-cutaneous* (s.c.) tissue of female mice. Following infection with the pHIV-Luc-ZsGreen lentivirus vector and purification by FACS, three TNBC cell lines were injected s.c. in female mice. In all three cases, the anatomical location of injected tumor cells could be readily detected based on the generation of a bio-luminescent signal following intra-peritoneal injection of luciferin (150 mg/kg). **(A)** 4T1 cells (murine) injected in Balb/c mice. **(B)** MDA-MB-231 cells (human) and **(C)** MDA-MB-468 cells (human), injected in NOD/SCID/IL2R$\gamma^{-/-}$ (NSG) mice.

Major Task 7 (*Evaluate the pharmacokinetics, biodistribution and accumulation of PCL-b-PDACL-b-PEG nanoparticles in vivo*). This major task is to be completed during the 2nd year of the award (months 10-18).

Major Task 8 (*Evaluate the therapeutic efficacy of DTXL-loaded PCL-b-PDACL-b-PEG nanoparticles in the 4T1 model of spontaneous metastasis*). This major task is to be pursued during the 2nd and 3rd years of the award (months 21-30). The achievement of this task requires the availability of 4T1 cells expressing a bioluminescent reporter (Luciferase) to enable the use of non-invasive imaging platforms for the prospective monitoring of the metastatic spread of malignant cells in engrafted mice. This technical advancement was achieved **(100%)** during the previous reporting period (please, see description of the generation of TNCB cell lines infected expressing both fluorescent and bioluminescent reporters, under Major Task 1 and Major Task 6; **Figure 1** and **Figure 2**). The achievement of this technical advancement during the 1st year of the award enabled the leading team (Leong) and the partnering team (Dalerba) to initiate a joint pilot *in vivo* experiment aimed at completing **Subtask 8.1 (*Measurement of cfDNA levels and inflammation biomarkers in blood and tumor tissues of mice engrafted with 4T1-Luc cells, before and after treatment with DTXL-loaded PCL-b-PDACL-b-PEG nanoparticles*)**. This experiment confirmed the capacity of NABNPs to reduce the *in vivo* pro-inflammatory effects of cytotoxic drugs of the taxane family, as demonstrated by a reduction in chemotherapy-induced serum cfDNA levels, and a corresponding reduction in the serum's capacity to activate *Toll-like Receptor 9* (TLR9) in reporter cells **(Figure 5)**.

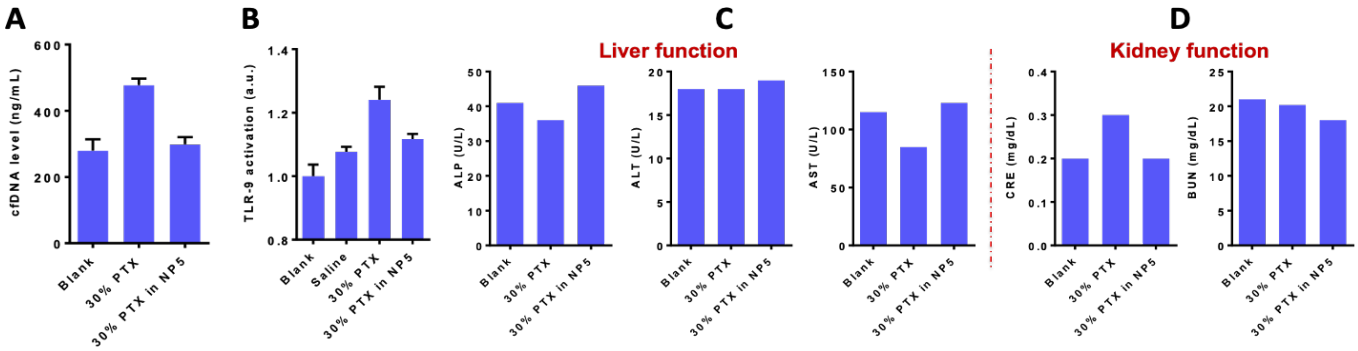

Figure 5. Evaluation of NABNPs as "dampeners" of the pro-inflammatory effects of *in vivo* chemotherapy with taxanes. (A-B) In mice receiving chemotherapy with taxanes, such as paclitaxel (PTX; 6 mg/kg, 30% of maximum dose, 19 days), serum cfDNA levels are increased above baseline, resulting in the augmented capacity of serum samples to stimulate *Toll-like Receptor 9* (TLR9) signaling in reporter cells. However, both effects are dampened, if not abolished, when equimolar doses of PTX are administered as loaded within PAMAM-G3-DEAA$_{20}$-C12$_5$ (NP5) NABNPs. **(C-D)** Importantly, PTX loaded within NP5 NABNPs did not display increased liver or kidney toxicity as compared to free PTX.

This experiment led to the anticipated, though still partial **(50%)**, completion of Subtask 8.1**. For a complete description of this joint experiment, please, also refer to the Technical Report related to the leading team's award** (BC180904).

Major Task 9 (*Evaluate the therapeutic efficacy of DTXL-loaded PCL-b-PDACL-b-PEG nanoparticles in PDX models*). This major task is to be pursued during the 2nd and 3rd years of the award (months 18-33). With specific regard to this Major Task, the experimental plan is to use TNBC *patient-derived xenograft* (PDX) lines that are fully de-identified and commercially available from *The Jackson Laboratory* (JAX), and whose use therefore fulfills the definition of *"Not Human Subjects Research"*. Because the DOD's *Human Research Protection Office* (HRPO) required a formal determination by *Columbia University*'s *Institutional Review Board* (IRB) that, indeed, use of such PDX lines fulfills the definition of *"Not Human Subjects Research"*, we included their use as part of procedures planned under our laboratory's *Human Subjects Research* protocol, which was submitted for review by *Columbia University*'s IRB on 06/18/2020. Please, note that, because our *Human Subjects Research* protocol includes many arms (in addition to the arm related to the DOD grant), our protocol underwent a full review. The first round of the full review was completed by our IRB on 09/02/2020, and resulted in the request for several clarifications and amendments, including a modification of our informed consent document (which is unrelated to the research supported by the present DOD award) in order to update its language with regard to new policies and requirements. A second and revised version of our *Human Subjects Research* protocol was then re-submitted to *Columbia University*'s IRB on 11/05/2020. The second round of review was completed by our IRB on 11/23/2020, and resulted in the request for a second set of relatively minor clarifications and amendments. A third and revised version of our *Human Subjects Research* protocol was then re-submitted to *Columbia University*'s IRB on 12/08/2020 and **received final approval on 12/21/2020.**

Major Task 10 (*Preparation of a manuscript reporting on study results*). This major task is to be pursued during the 3rd year of the award, as the final step of the study (months 33-36).

Opportunities for training and professional development. The personnel who participated in the research project supported by this award includes three trainees:

1) **Junko Mukohyama, MD/PhD** (post-doctoral fellow)
2) **Emily Rinebold, MD** (post-doctoral fellow, resident in general surgery)
3) **William J. Raab, MS** (graduate student; PhD)

At the core of training activities for all trainees, including graduate students and post-doctoral fellows, is the preparation of an ***Individual Development Plan* (IDP),** which is used to help trainees learn how to actively manage the development of their professional career. IDPs are initially developed by the trainees, and subsequently discussed with their primary mentors, on a periodic basis. To provide guidance in the preparation of IDPs, at *Columbia University*, all trainees, including graduate students and post-doctoral fellows, are offered the opportunity to enroll in an institutional IDP program, which is designed to help develop strategies to actively manage their careers, including the preparation of carefully structured IDPs. Program participants are engaged in coursework designed to help them become familiar with a variety of career options, and understand which skills (and professional experiences) are considered necessary to successfully pursue such career paths. As part of their involvement in the educational activities that form the backbone of the IDP program, participants acquire skills relevant to a variety of career paths (e.g. how to write a research proposal, how to prepare for a job talk). More specifically, graduate students and post-doctoral fellows enrolled in the IDP program learn how to: 1) identify potential career options; 2) devise strategies to actively manage their career paths; 3) independently complete an IDP using the "myIDP" or "ImaginePhD" online tools; 4) utilize their IDP to share short-term and long-term career development plans with their mentors; 5) acquire skills that are considered critical to the successful pursuit of a variety of career paths (e.g. effective writing and oral communication skills); and 6) participate in professional "networking" opportunities, designed to enable them to become familiar with the full spectrum of career

opportunities that are most suited to their scholarly and scientific expertise. A description of Columbia University's IDP program can be found at: https://research.columbia.edu/idp-program-2019-2020

Furthermore, as a result of the PI's affiliation with several research programs at *Columbia University*, including the *Tumor Immunology, Microenvironment, and Stemness* program of the *Herbert Irving Comprehensive Cancer Center* (HICCC), the *Columbia Stem Cell Initiative* (CSCI) and the *Digestive Disease Research Center* (DDRC), all three trainees have been constantly exposed to a rich series of scientific seminars by internal and external speakers, on research topics that are closely relevant to their studies. Of particular value to both graduate students and post-doctoral fellows is the *Work-in-Progress* (WIP) seminar series that is coordinated by the CSCI, which provides all trainees with a formal opportunity to discuss the results of their ongoing research in front of a large and qualified scientific audience, consisting of faculty affiliated with the *Columbia University Medical Center* (CUMC). After giving a presentation within the framework of the CSCI-WIP seminar series, all trainees are mandated to attend a *"one-on-one"* mentorship session with a small committee of selected CSCI faculty members, who provides them with feed-back on a variety of topics (e.g. academic trajectory, experimental design, communications skills).

In the laboratory setting, all three trainees had the opportunity to interact with their PI on a weekly (if not daily) basis, discussing the progress of their experiments and possible means to address emerging problems, in order to ensure that the research project proceeds on schedule. Within the PI laboratory, all trainees attended periodic laboratory meetings, during which the trainees presented the results of their experiments, discussed their interpretation with mentors and peers, and received guidance on how to chart their path forward based on the intellectual feed-back received by all members of the research team.

In terms of technical skills, the project also provided the opportunity for one of the post-doctoral fellows recently recruited in the PI's laboratory, Dr. Emily Rinebold, MD, a clinical resident in general surgery who is pursuing an academic career as a physician scientist, to become proficient in relatively a diverse portfolio of research techniques in both cell and molecular biology. More specifically, Dr. Rinebold received "one-on-one" training on laboratory techniques related to: 1) the production of 3rd generation lentivirus vectors and their use for the genetic engineering of cancer cells cultured *in vitro*; 2) the use of *fluorescence activated cell sorting* (FACS) for the purification of cellular subsets engineered to express fluorescent reporters (EGFP, ZsGreen); 3) the injection of cancer cells *in vivo*, in the sub-cutaneous tissue of mice; and 4) the visualization of tumor growth kinetics and metastatic dissemination using bio-luminescent reporters, such as the firefly Luciferase (*Photinus pyralis*).

Dissemination of results to communities of interest. Nothing to report.

Research plans for the next reporting period. Over the next reporting period (07/15/2020-07/14/2021), which corresponds to the 2nd year of the award, we plan to perform a substantial number of the experiments envisioned under Aim 2 and Aim 3 of the funded project, with a specific emphasis on bringing to completion three of their core tasks: **Major Task 2** (*Evaluate the capacity of DTXL-loaded PCL-b-PDACL-b-PEG nanoparticles to scavenge cfDNA and inhibit breast cancer cell migration in vitro*), **Major Task 6** (*Generation and validation of animal breast cancer models for the in vivo study of spontaneous metastasis*) and **Major Task 7** (*Evaluate the pharmacokinetics, biodistribution and accumulation of PCL-b-PDACL-b- PEG nanoparticles in vivo*). With specific regard to **Major Task 2**, which has already been extensively advanced by the leading team (Leong), we plan to implement a new generation of live-imaging technologies aimed at enabling the high-throughput, quantitative analysis of *in vitro* tissue culture experiments by time-lapse, automated digital microscopy. Such technologies have recently become available in our research campus, thanks to the purchase of scientific equipment specifically designed to this effect (*Cytation-5*; BioTek). Over the next year, we aim to perform high-throughput, quantitative measurements of the *in vitro* effects that the various NABNP formulations investigated in this study have on several of the cellular functions

identified as key to understand the pharmacological properties of anti-tumor drugs (e.g. kinetics of drug intra-cytoplasmatic accumulation, effects on cell proliferation and cell migration).

4. IMPACT

Impact on the principal discipline of the project. The results of the experiments conducted so far as part of this study have contributed to important technical advancements in the development of experimental models for the study of breast cancer metastasis. Among the most important observations made during this study are: 1) the observation that lentivirus vectors utilizing the *Cytomegalovirus* (CMV) promoter to drive the expression of fluorescent and/or bioluminescent reporters (e.g. lentivirus vectors based on the pLentiLox3.7 backbone; Addgene #11795) are unsuitable for the genetic engineering of mouse breast cancer cells (e.g. 4T1 cells; ATCC #CRL-2539), as they appear to be rapidly silenced, in a manner similar to what previously observed in mouse ES cells (Meilinger *et al.*, *EMBO Reports*, 10:1259-64, 2009); 2) the observation that lentivirus vectors utilizing the EF1a promoter can be used to obtain stable expression of such reporters, across multiple breast cancer cell lines, both murine (4T1) and human (MDA-MB-231, ATCC #HTB-26; MDA-MB-468, ATCC #HTB-132); 3) the observation that, in human breast cancer cell lines (MDA-MB-231, MDA-MB-468), the fluorescent and bio-luminescent signals obtained using lentivirus vectors based on the pHIV-Luc-ZsGreen backbone (Addgene #39196), which expresses ZsGreen and Luc2P, are higher than those obtained using lentivirus vectors based on the pLentiLox3.7 backbone (Addgene #11795) and engineered to express EGFP and Luc. Taken together, these observations will enable future studies in this same field to proceed in a more rapid and efficient manner, as future investigators will have immediate access to a portfolio of breast cancer cell lines, both human and murine, that have already been genetically engineered to express high and stable levels of both fluorescent and bio-luminescent reporters (please, also refer to Section 6, PRODUCTS).

Impact on other disciplines. The technical advancements that have been achieved as part of this study (e.g. the optimization of fluorescent and bio-luminescent reporter systems used for the study of the *in vivo* growth kinetics and spontaneous metastasis of breast cancer cell lines) are foreseen to enable and accelerate the successful execution of similar studies in other forms of malignancy. For example, future investigations on the biology of metastasis in other types of mouse cancer models will immediately proceed to engineer cancer cells using lentivirus vectors designed to express fluorescent and bio-luminescent reporters under the transcriptional control of the EF1a promoter (as opposed to a CMV promoter).

Impact on technology transfer. Nothing to report.

Impact on society beyond science and technology. Nothing to report.

5. CHANGES/PROBLEMS.

Changes in approach. We decided to add two human *triple-negative breast cancer* (TNBC) cell lines (MDA-MB-231, ATCC #HTB-26; MDA-MB-468, ATCC #HTB-132) to the portfolio of experimental models to be screened for possible use in the *in vivo* experiments envisioned under this project, which originally included only a mouse TNBC cell line (4T1; ATCC #CRL-2539) and a collection of *patient-derived xenograft* (PDX) lines established from human TNBCs. We decided to implement this expansion because it would have improved the project's quality and probability of success, without causing a meaningful increase in associated costs, especially when considering that the two cell lines could be genetically engineered and tested in parallel to 4T1 cells, using the leftovers of previously purchased research reagents. Because we considered this change to represent an improvement upon the previous experimental plan (i.e. an expansion as opposed to a substantial modification) and to have no meaningful impact on the overall budget of the project (i.e. to add no additional costs to the study), we regarded it as "not significant" (and sought no prior written approval).

Problems or delays. Because of the *Coronavirus Disease 2019* (COVID-19) outbreak in New York City (March 2020), our institution (Columbia University) decided to limit research activities conducted on our medical campus to those defined as strictly "essential" (i.e. directly related to COVID-19), in order to avoid endangering the health of our students and staff. As a result of this unforeseen cataclysm, many of the *in vitro* and *in vivo* experiments related to this project had to be temporarily downsized and/or postponed. Fortunately, however, immediately before the outbreak of COVID-19 in New York City, we were able to complete an important set of critical experiments, including the infection with the pHIV-Luc-ZsGreen lentivirus vector and the purification by *fluorescence activated cell sorting* (FACS) of the key breast cancer cell line (4T1) to be used for the first round of calibration experiments envisioned under this project. Therefore, upon formal re-starting of our laboratory activities (June 2020), we were able to immediately transfer these key reagents to the leading team (Leong) for *in vitro* experiments, and then immediately proceed with *in vivo* experiments aimed at obtaining accurate estimates of the kinetics and tissue tropism of metastatic dissemination of the 4T1 cell line (after engineering for expression of ZsGreen and Luc2P). Overall, we estimate that, as a result of the COVID-19 outbreak, our planned research project accumulated a delay of 3 months.

Changes that had a significant impact on expenditures. During the reporting period, one of the post-doctoral fellows working on the project (Dr. Junko Mukohyama, MD/PhD) was awarded a 2-year, full-time post-doctoral fellowship by the *Japanese Society for the Promotion of Science* (JSPS), aimed at pursuing a different project, and eventually facilitate her application for an independent academic position in Japan. Therefore, as of April 1st, 2020, we did not charge the award for the salary Dr. Junko Mukohyama, and decided to recruit into the project a graduate student (PhD) with extensive experience with the use of both lentivirus vectors and animal cancer models (Mr. William J. Raab, MS). It is our opinion that Mr. William J. Raab is fully equipped with all the technical expertise required to successfully support the development of this project. We propose to replace Dr. Junko Mukohyama with Mr. William J. Raab within the research team supporting this project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. Nothing to report.

6. PRODUCTS.

Publications, conference papers, presentations. Nothing to report.

Websites or other internet sites. Nothing to report.

Technologies or techniques. Nothing to report.

Inventions, patent applications, and/or licenses. Nothing to report.

Other Products. Among the important products generated in the course of the first year of the award are key research tools and experimental models, consisting in three (n=3) independent breast cancer cell lines engineered to express high levels of both fluorescent (ZsGreen) and bio-luminescent (Luc2P) reporters. All three cell lines were generated by infection of parent cells with a lentivirus vector based on the *Human Immunodeficiency Virus* (HIV) genomic backbone, and encoding both ZsGreen and Luc2P under the transcriptional control of the *Eukaryotic Translation Elongation Factor 1 Alpha* (EF1a) promoter (Addgene # 39196). For each of the three lines, following lentivirus infection, cells displaying the highest levels of green fluorescence were isolated by *fluorescence activated cell sorting* (FACS) and used to initiate pure cultures. The three cell lines are:

1 - **4T1-ZsGreen/Luc2P** - murine (derived from 4T1 cells; ATCC #CRL-2539);
2 - **MDA-MB-231-ZsGreen/Luc2P** - human (derived from MDA-MB-231 cells; ATCC #HTB-26);
3 - **MDA-MB-468-ZsGreen/Luc2P** - human (derived from MDA-MB-468 cells; ATCC #HTB-132);

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS.

Individuals working on the project.

1 - Name: **Piero DALERBA, MD**

Role: **Principal Investigator (PI)**
Researcher identifier: **ORCID 0000-0002-8815-4981**
Person-months working on the project: **3 person-months**

Contribution to project: **Dr. Dalerba coordinated all aspects of the project, with a special focus on the design of experiments and the statistical analysis of their results. Dr. Dalerba also provided one-on-one training to Dr. Emily Rinebold, with specific regard to the use of animal models (mice) for studies on the biology of cancer metastasis (e.g. sub-cutaneous injection of cancer cells, monitoring of animal health during tumor growth, longitudinal evaluation of *in vivo* metastatic dissemination of cancer cells using bio-luminescent reporters, statistical analysis of growth rates).**

Funding support: **this award**

2 - Name: **Luis VALENCIA, BA**

Role: **Laboratory Technician (Category B)**
Researcher identifier: **n.a.**
Person-months working on the project: **4 person-months**

Contribution to project: **Mr. Valencia provided technical support with regard to the execution of most experiments performed as part of the project, with a special focus on the preparation of high-titer lentivirus supernatants for the permanent transduction of breast cancer cell lines with fluorescent and bioluminescent reporters.**

Funding support: **this award**

3 - Name: **Junko MUKOHYAMA, MD/PhD**

Role: **Post-doctoral fellow**
Researcher identifier: **n.a.**
Person-months working on the project: **5 person-months**

Contribution to project: **Dr. Mukohyama contributed to the execution of most experiments performed as part of the project, with a special focus on the infection of breast cancer cell lines with lentivirus vectors encoding fluorescent and bioluminescent reporters, and the isolation of cells expressing high levels of green fluorescence (ZsGreen^{high}) using *fluorescence activated cell sorting* (FACS). Dr. Mukohyama also provided one-on-one training to Dr. Emily Rinebold, with specific regard to the use of lentivirus vectors to genetically engineer human and murine cell lines and the use of *fluorescence activated cell sorting* (FACS) for the purification of genetically engineered cells based on the expression of fluorescent reporters.**

Funding support: **this award**

4 - Name: **Emily RINEBOLD, MD**

Role: **Post-doctoral fellow**
Researcher identifier: **n.a.**
Person-months working on the project: **5 person-months**

Contribution to project: **Dr. Rinebold contributed to the execution of most experiments performed as part of the project, with a special focus on the infection of breast cancer cell lines with lentivirus vectors encoding fluorescent and bioluminescent reporters, the isolation of cells expressing high levels of green fluorescence (ZsGreen^{high}) using *fluorescence activated cell sorting* (FACS), the *in vivo* injection of cancer cells and the longitudinal monitoring of their metastatic dissemination using bio-luminescent reporters.**

Funding support: **other unrestricted funds, independent post-doctoral fellowship.**

5 - Name: **William J. RAAB, MS**

Role: **Graduate student (PhD)**
Researcher identifier: **n.a.**
Person-months working on the project: **1 person-months**
Contribution to project: **Mr. Raab contributed to *in vivo* experiments aimed at defining the growth kinetics and spontaneous metastatic dissemination of the *triple-negative breast cancer* (TNBC) cell lines used as experimental models in this research project.**
Funding support: **other unrestricted funds, independent post-doctoral fellowship**

Changes in active other support of key personnel:

Principal Investigator (PI): **Piero DALERBA**

Active grants that have been **completed**:

a) 2018 Young Investigator Grant (PI: Dalerba) 02/01/2018-01/30/2020 5% effort
Breast Cancer Alliance (BCA) 0.6 months

The role of SOX10 as a predictor of survival outcomes in familial breast cancer patients. The major goal of this project is to investigate the role of SOX10 protein expression as a predictive biomarker of survival outcomes in familial breast cancer patients from the New York site of the *Breast Cancer Family Registry* (BCFR). **Aim 1:** To test for association between SOX10 protein expression and specific familial breast cancer syndromes. **Aim 2:** To test for association between SOX10 expression, disease relapse, secondary cancer and survival outcomes**. Aim 3:** To test for association between SOX10 expression and BRCA1 mis-sense *variants of unknown significance* (VUS) classified as likely deleterious by computer algorithms. **Overlap with this award:** no

b) CU18-3352 - Research Grant (PI: Dalerba) 12/01/2018-11/30/2020 5% effort
Adenoid Cystic Carcinoma Research Foundation (ACCRF) 0.6 months

Pharmacological manipulation of cell differentiation in human *Adenoid Cystic Carcinomas* (ACCs). The goal of this project is to elucidate the role of *retinoic acid* (RA) signaling pathways in controlling cell fate specification along the ductal and myoepithelial lineages in human *Adenoid Cystic Carcinomas* (ACCs). **Aim 1:** To elucidate the *developmental relationship* between the two sub-types of malignant cells (*ductal-like* and *myoepithelial-like*) of human ACCs. **Aim 2:** To test the *in vivo* therapeutic efficacy of novel drug combinations based on the sequential use of both direct and inverse agonists of RAR/RXR signaling. **Overlap with this award:** no

Pending grants that have been **activated**:

a) R01-DE028961 (PI: Dalerba) 08/01/2019-05/31/2024 50% effort
NIH - National Institute of Dental and Craniofacial Research (NIDCR) 6.0 months

Dissecting cell composition and drug sensitivity in human *Adenoid Cystic Carcinomas* (ACCs). The major goal of this project is to advance our scientific understanding on the molecular mechanisms responsible for the bi-phenotypic cell composition of *Adenoid Cystic Carcinomas* (ACCs), an aggressive sub-type of salivary gland malignancies, and to test the pre-clinical anti-tumor activity against ACCs of novel drug combinations targeting RAR/RXR signaling pathways. **Aim 1**: To elucidate the developmental relationship between the two sub-types of malignant cells (ductal-like and myoepithelial-like) of human ACCs. **Aim 2**: To test the *in vivo* therapeutic efficacy of novel drug combinations based on the sequential use of both direct and inverse agonists of RAR/RXR signaling. **Overlap with this award:** no

b) DOH01-C34925GG-3450000 (PI: Dalerba, Terry) 11/01/2019-10/31/2021 10% effort
New York State Department of Health (NYSDOH) 1.8 months

A novel biomarker to improve risk-prediction in familial breast cancer patients. The major goal of this project is to test the value of a novel biomarker (SOX10) for the identification of subgroups of BRCAX familial breast cancer patients characterized by reduced 10-year survival outcomes. **Aim 1:** To test whether, in familial breast cancer patients, SOX10 expression associates with families characterized by BRCA1 germline mutations or, among BRCAX patients, BRCA1 VUS classified as *"likely deleterious"* or BRCA1 promoter methylation; **Aim 2:** To test whether, in BRCAX patients, SOX10 expression associates with an increased risk of second tumors and reduced survival outcomes, such as *time-to-recurrence* (TTR), *disease-free survival* (DFS) and *overall survival* (OS); **Aim 3:** To test whether SOX10 expression can be integrated within existing risk-assessment algorithms, such as the *Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm* (BOADICEA), in order to improve their predictive performance. **Overlap with this award:** no

c) R01- CA253368 (PI: Gardner) 12/01/2020-11/30/2025 5% effort
NIH - **National Cancer Institute (NCI)** 0.6 months

The linkage between race, Kaiso and the tumor microenvironment in breast cancer health disparities. The goal of this project is to investigate the role played by the transcriptional regulator Kaiso in regulating autophagy and contributing to poor survival outcomes in breast cancer. **Aim 1:** To elucidate how sub-cellular localization regulates the capacity of Kaiso to control autophagy and influence tumor progression. **Aim 2:** To elucidate how sub-cellular localization regulates the capacity of Kaiso to modulate tumor growth, tissue invasion, metastatic spread and the entry into tumor dormancy. **Aim 3:** To test whether a differential sub-cellular localization of Kaiso associates with a different composition of a breast tumor's immune microenvironment and different clinical outcomes, such as response to therapy and patient survival. **Overlap with this award:** no

d) 2019 VELOCITY, Pilot & Feasibility Grant (PI: Dalerba) 09/01/2019-present 5% effort
Columbia University - Herbert Irving Comprehensive Cancer Center (HICCC) 0.6 months

The role of SOX10 as a predictive biomarker of contralateral relapse in BRCAX familial breast cancer patients. The major goal of this project is to test the value of SOX10 as diagnostic biomarker to identify subgroups of BRCAX families who display an increased incidence of TNBCs characterized by BRCA1 promoter methylation. **Aim 1:** To obtain robust measurements of the *positive predictive value* (PPV), *negative predictive value* (NPV) and *receiver operating characteristic* (ROC) curves describing SOX10 as a diagnostic biomarker of *BRCA1* mutation and promoter methylation; **Aim 2:** To test whether, in BRCAX familial breast cancer patients, high SOX10 expression levels are associated with an increased risk of second tumors, such as *contralateral breast cancer* (CBC), and with reduced survival outcomes, such as *time-to-recurrence* (TTR), *disease-free survival* (DFS) and *overall survival* (OS); **Aim 3:** To test whether, in BRCAX familial breast cancer patients, SOX10 expression levels can be integrated with the *Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm* (BOADICEA), in order to improve its diagnostic performance. **Overlap with this award:** no

e) 2019 DSI Seed Grant Program (PI: Wan, Terry, Hu, Dalerba) 04/01/2020-03/31/2021 5% effort
Columbia University - Irving Institute for Cancer Dynamics (IICD) 0.6 months

Modeling the dynamics of young-onset colorectal cancer using big population data. The goal of this project is to aggregate multiple nationwide, large-scale datasets to build a novel model-inference system to identify risk factors underlying the recent increase in young-onset colorectal cancer incidence in the United States. **Aim 1:** To identify epidemiological risk factors associated with the progressive increase in rectal cancer incidence among young adults. **Aim 2:** To develop a *model/data/assimilation* (M/D/A) framework able to reproduce the incidence patterns observed in the US young-adult population starting from a variety of theoretical mechanistic models. **Aim 3:** To

explore the possibility of linking mechanistic models with actual epidemiological variables and formulate hypotheses regarding the causative role of selected exposures and their synergistic effects. **Overlap with this award:** no

Other organizations involved as partners. Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS.

This is a **collaborative award**, consisting of two independent grants **(BC180904, BC180904P1).** The present report relates to work conducted by the partnering team **(BC180904P1).** Please, note that many of the **experiments performed as a joint collaboration between the two research teams have been described in detail as part of the Technical Report** (PHS-398) **submitted by the leading team (BC180904; Kam W. Leong)** in parallel to the present one.

9. APPENDICES.

Nothing to report.