AWARD NUMBER: W81XWH-14-1-0403

TITLE: The Roles of the Bone Marrow Microenvironment in Controlling Tumor Dormancy

PRINCIPAL INVESTIGATOR: Yusuke Shiozawa, M.D., Ph.D.

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

REPORT DATE: October 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                  Fort Detrick, Maryland  21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                  Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and
should not be construed as an official Department of the Army position, policy or decision
unless so designated by other documentation.
The purpose of this study is to identify the mechanisms whereby the bone marrow microenvironment is involved in the regulation of tumor dormancy. Aim1 will identify and explore how disseminated tumor cells (DTCs) stay dormant for long periods of time. We postulate that DTCs drive the bone marrow niche into dormancy through the GAS6 pathway. Aim2 will determine how DTCs escape dormancy, consequently rendering them more susceptible to the chemotherapy.

During this period, we found that a fast growing less differentiated osteoblast may contribute to growth of bone metastatic prostate cancer. In addition, we developed a new and creative in vitro cell culture system that allows us to evaluate the effects of osteoblastic activation on the growth of disseminated prostate cancer cells.
# Table of Contents

1. Introduction.......................................................................................... 4
2. Keywords.............................................................................................. 4
3. Accomplishments..................................................................................... 5
4. Impact...................................................................................................... 11
5. Changes/Problems................................................................................... 12
6. Products.................................................................................................... 13
7. Participants & Other Collaborating Organizations................................ 16
8. Special Reporting Requirements......................................................... 16
9. Appendices............................................................................................. 17
1. INTRODUCTION:

Despite improvements in treatments for primary prostate cancer (PCa), bone metastasis remains a major cause of death in PCa patients. Several studies have shown that disseminated tumor cells (DTCs) shed from a primary tumor may lie dormant in distant tissues for long periods of time, retaining the potential for activation resulting in metastatic growth. Understanding the underlying mechanisms of metastasis is therefore crucial for effective treatment of this disease. Bone marrow has been well established as a regulatory site for hematopoietic function. In the marrow, hematopoietic stem cells (HSCs) are believed to localize to a specific microenvironment, the “niche”, where they reside in a dormant state. Likewise, growing evidence has suggested that disseminated PCa also resides within the marrow niche. In fact, disseminated PCa uses similar mechanisms as HSCs in order to gain access to the marrow microenvironment, and DTCs target and displace HSCs, establishing metastatic foci within the hematopoietic niche. As a result, these cells parasitize the niche to become dormant, utilizing the mechanisms that keep HSCs in a dormant state. Although bone marrow is known as a fertile microenvironment (“soil”) for metastatic tumor cells (“seed”), little is known about how dormancy is established or what leads to re-activation of the dormant cells. Therefore, we hypothesize that once DTCs become dormant within the bone marrow niche, they stay dormant by stimulating the niche to remain dormant, and eventually escape from dormancy when the niche matures.

To address our hypothesis the following aims are proposed:

Aim1: Determine the mechanisms whereby DTCs control the dormancy of the niche cells.  
Sub hypothesis: DTCs drive the niche into dormancy via GAS6 signaling.

Aim2: Determine if the differentiation of the niche cells triggers the regrowth of DTCs.  
Sub hypothesis: Dormant DTCs exit from dormancy when the niche is differentiated via BMP2 signaling.

The proposed studies will provide significant insight into the mechanisms whereby the bone marrow microenvironment is involved in regulation of tumor dormancy. Aim 1 allows us to identify and explore how DTCs stay dormant for long periods of time. We postulate that DTCs drive the bone marrow niche into dormancy through the GAS6 pathway. Aim2 will determine how DTCs escape dormancy, consequently rendering them more susceptible to the chemotherapy. Results from this work will lead to a greater understanding of niche aging effects on metastatic growth, and could result in valuable new treatment approaches.

2. KEYWORDS:

Prostate Cancer; Bone metastasis; Disseminated tumor cells; Bone marrow microenvironment; Tumor dormancy; GAS6; BMP2
3. ACCOMPLISHMENTS:

What were the major goals and objectives of the project?

The goal of this project is to understand the mechanisms of tumor dormancy and metastatic outgrowth of disseminated prostate cancer within the bone marrow microenvironment.

Task 1: Complete the grant transfer from University of Michigan to Wake Forest School of Medicine.

Months 1-3.

- Upon arrival at Wake Forest School of Medicine, the PI will seek to obtain the necessary approvals (IACUC, IRB, IBC) to complete the grant transfer, and then will initiate the proposed research as soon as possible (Months 1-3).

Task 2: Determine the mechanisms whereby DTCs control the dormancy of the niche cells.

Months 4-18.

- To determine the effects of GAS6 on the dormancy of niche cells in vitro, co-culture of bone marrow stromal cells (BMSCs) (pre-stained with DiD fluorescent dye) with either GAS6-downregulated PCa cells (PCa\textsubscript{shGAS6}) or control PCa (PCa\textsubscript{Control}) will be performed. At the termination of experiments, BMSCs will be harvested, and the retention of DiD dye will be measured with FACS (Months 4-7).

To further characterize the difference, gene and protein expression of proliferation markers and cell cycle status will be analyzed using those isolated BMSCs (Months 7-9).

- To determine the effects of GAS6 on the dormancy of niche cells in vivo, we will perform a vertebral body implant (vossicle) experiment. We will implant BrdU-incorporated vossicles directly injected with PCa\textsubscript{shGAS6} or PCa\textsubscript{Control} into immunocompromized mice, and then will determine the effects of GAS6 on the dormancy of the microenvironment by immunohistochemistry for BrdU (Months 9-14).

Additionally, using immunohistochemistry we will also visualize co-localization of PCa cells with the dormant microenvironment cells using these vossicles (Months 14-19).

Task 3: Determine if the differentiation of the niche cells triggers the regrowth of DTCs.

Months 19-36.

- To determine if the differentiation of the niche following exogenous BMP2 treatment stimulates the regrowth of DTCs in vitro, co-culture of BMSCs with G1-Red and SG2M-Cyan co-infected PCa cells will be performed. The differentiation of the niche, and the dormancy, proliferation, and cell
cycle status of PCa cells after treatment with recombinant mouse (rm) BMP2 will be analyzed (Months 19-22).

- To determine if the differentiation of the niche following the exogenous BMP2 treatment stimulates the regrowth of DTCs in vivo, we will implant vossicles directly injected with G1-Red and SG2M-Cyan co-infected PCa cells into immunocompromised mice. The differentiation of the niche, and the dormancy, proliferation, and cell cycle status of PCa cells after treatment with rm BMP2 will be analyzed (Months 22-26).

- To determine whether BMP2 expressed by DTCs is crucial for metastatic progression in vitro, co-culture of BMSCs with BMP2-downregulated PCa (PCa\textsubscript{shBMP2}), upregulated PCa (PCa\textsubscript{BMP2OE}), or control PCa (PCa\textsubscript{Control}) will be performed. Thereafter, the differentiation of the niche, and the dormancy, proliferation, and cell cycle status of PCa cells will be analyzed (Months 27-30).

- To determine whether BMP2 expressed by DTCs is crucial for metastatic progression in vivo, we will implant vossicles directly injected with PCa\textsubscript{shBMP2}, PCa\textsubscript{BMP2OE}, or PCa\textsubscript{Control}. Thereafter, the differentiation of the niche, and the dormancy, proliferation, and cell cycle status of PCa cells will be analyzed (Months 31-36).

What was accomplished under these goals?

(2016)

The Award transfer.
As of 03/01/15, thanks to receiving this Idea Development Award for Young Investigators, the PI, Dr. Yusuke Shiozawa started an independent faculty job as an Assistant Professor at Wake Forest School of Medicine. Upon his arrival at Wake Forest School of Medicine, the PI obtained the necessary institutional approvals (IACUC, IRB, IBC) and submitted the grant transfer request (06/11/2015) to gain approval from the Department of Defense for a transfer of the award from the University of Michigan to Wake Forest School of Medicine. As of 07/01/16, a transfer of the award from the University of Michigan to Wake Forest School of Medicine was completed.

The development of a mouse model to measure tumor growth and bone remodeling.
To evaluate the interaction between the bone marrow niche and disseminated PCa cells, we must be able to measure (i) the growth of bone metastatic PCa and (ii) bone remodeling within the same animal. To address this concern, we first attempted to establish an innovative and powerful mouse model. For this experiment, PCa cells (DU145) were inoculated intrafemorally into severe combined immunodeficient (SCID) mice to establish bone metastases. Thereafter, we measured changes in tumor growth [bioluminescent imaging (BLI), immunohistochemistry (IHC)] and bone remodeling (microCT, IHC). Using this strategy, we found (i) tumor growth by BLI, (ii) tumor burden in the marrow by histology, and decreased bone volume density and connective density in tumor-burdened mice on microCT.

The neuropeptide CGRP expressed by sensory neurons around bone influences PCa proliferation through CRLR/JNK pathway.
We recently reported that PCa cells parasitize the mechanisms whereby HSCs home to the marrow to gain
access to bone. Nerves are a major component of the microenvironment for HSCs, and are also involved in the metastatic process of PCa to bone. Yet whether the interactions between DTCs and sensory neurons in the bone play a crucial role in controlling later bone metastatic progression remains unclear. Interestingly, our preliminary data demonstrated that bone metastatic PCa increases neuronal hypertrophy of calcitonin-gene related peptide (CGRP)-expressing sensory nerves in the periostium in a time-dependent manner. It has been demonstrated that levels of CGRP are increased in the serum of patients with advanced PCa compared to low-grade PCa, and that CGRP induces osteoblastic differentiation. Next, we wondered if CGRP affects PCa progression. We found that CGRP enhances proliferation of PCa cells in vitro. PCa patients with metastases express higher levels of CGRP receptor Calcitonin receptor like receptor (CRLR) (gene name CALCRL), compared to PCa patients without metastases. Additionally, CGRP activates JNK in PCa.

(2016-2017)

**The fast growing less differentiated osteoblasts may contribute more to tumor growth, compared to mature osteoblasts in vitro.**

To test the effects of differentiation status on osteoblast proliferation we used an MTT assay, as well as a live cell imaging device (Incucyte). We used the mouse calvarial preosteoblast cell line MC3T3-E1 transduced to constitutively express green fluorescent protein (eGFP) for these studies. We found that BMP2 stimulated a modest increase in osteoblast proliferation by (Fig. 1A) as well as expression of the osteoblast differentiation marker osteocalcin (Ocn) (Fig. 1C). We used bone mineralization media (BMM) consisting of 0.5mM L-Ascorbic acid and 2mM β-Glycerophosphate as a positive control for osteoblast differentiation and found that only this treatment increased osteoblast proliferation, when measured by Incucyte live cell imaging (Fig. 1B). We were unable to test this by MTT as ascorbic acid interferes with this assay. We also tested the effect of histamine dihydrochloride (HC) and found little to no effect on osteoblast proliferation and differentiation (Fig. 1). Strangely, we found that BMM had no effect on Ocn expression (Fig. 1C). Also, we were surprised to find that another osteoblast differentiation marker, osteopontin (Opn), actually decreased after treatment with BMM, BMP2 and HC (Fig. 1D).

![Fig. 1: The effects of differentiation on osteoblast growth](image-url)

MC3T3-eGFP were seeded into 96-well plates and serum starved for 24 hours before treatment with 25 ng/mL BMP2, 100nM histamine dihydrochloride (HC), or 0.1% DMSO (Vehicle). (A) Relative cell numbers measured at 48 and 72 hours with MTT (500µg/mL) by optical density (OD) at 560nm minus background at 650nm. (B) Confluency change measured for 72 hours using the Incucyte after addition of bone mineralization media (BMM) supplemented with 0.5mM L-Ascorbic acid and 2mM β-Glycerophosphate, or BMP2, HC, or Vehicle. (C) Osteocalcin expression levels were tested by qPCR after 14 day treatments of BMM, BMP2, HC, or Vehicle in complete media (10% FBS). (D) Osteopontin levels were also measured (data presented as fold change using the ∆∆CT method with Gapdh as reference gene). All data presented as mean ± SD, *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0005 (Two-way ANOVA with Bonferroni multiple comparisons)
Next we tested the effects of these treatments on the proliferation of the PCa cell lines PC3 and DU145 alone and in coculture with MC3T3-E1. We used a luciferase reporter to monitor tumor cell growth via bioluminescent imaging (BLI) using the IVIS system. Not surprisingly, we found that PC3 and DU145 grew slower in coculture with osteoblasts (Fig. 2A&D). Additionally, we found that BMM, BMP2 and HC had no effect on PC3 growth when cultured alone (Fig. 2B), whereas BMM slightly increased DU145 growth in the same conditions (Fig. 2E).

A different trend was revealed when the same treatments were tested on the cocultures. There was a significant reduction in PC3 and DU145 growth only when treated with BMM (Fig. 2C&E). We performed the test again using the DiD membrane dye method of tracking cellular divisions and tumor cell dormancy by flow cytometry. We found that coculture of PC3 and DU145 with MC3T3-E1 led to higher retention of DiD, indicating slower cancer cell growth (Fig. 2G&H). Treatment with BMM resulted in even higher retention of DiD in PC3 and DU145 (Fig. 2G&H). Treatment of BMP2 caused a similar effect only in DU145 (Fig. 2H). In addition, we found that the percentage of PC3 and DU145 cells in the cocultures decreased after treatments with BMM and BMP2 (Fig. 2I), although we cannot say whether this is because cancer cell growth was slowed, or osteoblast proliferation was increased. These findings made us reconsider the initial hypothesis as the differentiation status or maturity of osteoblasts may actually slow tumor growth, contributing to dormancy. However, the unexpected result of BMM having no significant effect on Ocn expression, as well as decreasing Opn expression made us question the validity of MC3T3-E1 as a reliable model of osteoblast differentiation.

We thought back to an interesting observation when we perform intracardiac injection of tumor cells as a model of metastasis. Often times these animals develop tumors in the mandible, and sometimes only in the
mandible, as observed by BLI (Fig. 3A). It is not uncommon that these mandible metastases grow faster than those in the hind limbs of the same animal (Fig. 3B&C). It may be that in these cases there was higher seeding of tumor cells in the mandible than the hind limbs, or as we suspect, the mouse mandible microenvironment is less dormant and allows for faster tumor cell growth.

To test this we harvested adult osteoblasts from the mouse mandible and hind limbs using an explant model. Briefly, adult male C57BL/6 mice were sacrificed according to approved protocols and hind limbs and mandibles dissected and muscles completely removed. The epiphyses from the femurs and tibias were cut and the marrow was flushed from the diaphyses and discarded. The empty diaphyses were placed in a tissue culture dish and minced into smaller pieces using a scalpel. The molars, incisors and dental pulp of the mandibles were removed and the remaining pieces (ascending ramus containing the coronoid, condylar and angular processes) were placed in a dish and minced. A 10 minute trypsin digestion was performed on all the bone pieces and discarded. Complete media containing 10nM dexamethasone was added to select for osteoblast outgrowth. The bone cells were allowed to migrate out of the bone and mandible pieces and expand for two weeks before experimentation.

We first compared the proliferation of the mandible osteoblasts (MaOB) and the hind limb osteoblasts (HLOB) using an MTT assay. Indeed, we found the MaOB to be much more proliferative than the HLOB (Fig. 4A). We tested for osteoblast differentiation markers and found the basal expression of Ocn and Opn to be less in the MaOB than the HLOB, however after treatment with BMM we found significantly higher expression of Ocn and a trend toward increased Opn in the MaOB (Fig. 4B&C). These findings suggested to us that the MaOB are more proliferative and less mature, but with a higher potential for differentiation than the HLOB. Finally, we hypothesized that cancer cell proliferation would increase when cocultured with the MaOB, if the microenvironment of the mandible is truly involved in the phenomena observed in Figure 3. Indeed, when we performed this experiment we found that both PC3 and DU145 grew significantly faster in coculture with MaOB compared to HLOB (Fig. 4D&E). Similar to the MC3T3-1 studies in Fig. 2, we found that treatment with BMM decreased this proliferation effect in PC3 (Fig. 4D). Again, the results suggest that a more mature osteoblast may contribute more to tumor dormancy than a fast growing less differentiated osteoblast.
What opportunities for training and professional development did the project provide?

(~2016)
Thanks to receiving an Idea Development Award for Young Investigators, the PI obtained independent status at Wake Forest School of Medicine with lab space, office space, and start-up costs provided.

Thanks to the Department of Defense, the PI attended IMPaCT Young Investigator Meeting, Baltimore, MD, USA, August 4-5, 2016.

The PI was chosen for the 2016-2018 cohort of the Wake Forest Clinical and Translational Science Institute’s Translational Scholar Academy, which supports the scientific and career development of early-stage investigators.

(2016-2017)
The PI was invited to attend The U.S. Bone and Joint Initiative (USBJI) and Bone and Joint Canada (BJC) Young Investigator Initiative Program will be held at Toronto, Canada on 11/10/17-11/12-17.

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 characterize the differences between the mandible and hind limb osteoblasts in order to determine the detailed molecular mechanisms whereby active osteoblasts influence the progression of bone metastatic prostate cancer (Aim 2). We will also further elucidate the role of osteoblastic activation in the progression of prostate cancer bone metastasis in vivo (Aim 2). In addition, we will continue to pursue the effects of the disseminated tumor cells on the dormancy of the bone marrow niche (Aim 1).
4. IMPACT:

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

(~2016)

We developed an innovative and powerful mouse models that enable us to measure within the same animal: (i) the growth of bone metastatic PCa and (ii) bone remodeling.

(2016-2017)

We developed a new and creative tool (mandible vs. hind limb osteoblast) to test the hypothesis that active osteoblasts enhance the re-growth of cancer in the bone.

Using this technique, we found that a fast growing less differentiated osteoblast may contribute to growth of bone metastatic prostate cancer.

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?

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

(~2016)

   Status of Publication: Published
   Acknowledgement of federal support: Yes

   Status of Publication: Published
   Acknowledgement of federal support: Yes

   Status of Publication: Published
   Acknowledgement of federal support: Yes

   Status of Publication: Published
   Acknowledgement of federal support: No
   (2016-2017)

   Status of Publication: Published
   Acknowledgement of federal support: Yes
Status of Publication: Published
Acknowledgement of federal support: Yes

Invited reviews

(~2016)

Status of Publication: Published
Acknowledgement of federal support: Yes

Status of Publication: Published
Acknowledgement of federal support: Yes

Status of Publication: Accepted
Acknowledgement of federal support: Yes

- The Figure 1 is chosen as the Cover Figure of Int J Urol Vol. 23 No. 10

Book

(~2016)

Status of Publication: Accepted
Acknowledgement of federal support: Yes

Presentation

(~2016)


**Website(s) or other Internet site(s)**

Nothing to report.

**Technologies or techniques**

Nothing to report.

**Inventions, patent applications, and/or licenses**

Nothing to report.

**Other products**

Nothing to report.
7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Yusuke Shiozawa
Project Role: PI
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0001-9814-9230
Nearest person month worked: 2.4
Contribution to Project: Dr. Shiozawa provides oversight of the entire program and development and implementation of all policies, procedures, and processes. In this role, Dr. Shiozawa is responsible for the implementation of the specific aims, and for ensuring that systems are in place to guarantee institutional compliance with US laws, including biosafety and animal research, data and facilities. Dr. Shiozawa supervises other personnel on the project to ensure timely and effective studies.
Funding Support: National Cancer Institution, Department of Defense

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

Nothing to report.

What other organizations have been involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS:

N/A.

9. APPENDICES:

The original copies of manuscript and a curriculum vitae are attached.
Mer Tyrosine Kinase Regulates Disseminated Prostate Cancer Cellular Dormancy

Frank C. Cackowski,1,2 Matthew R. Eber,1,3 James Rhee,1 Ann M. Decker,1 Kenji Yumoto,1 Janice E. Berry,1,8 Eunsohl Lee,1 Yusuke Shiozawa,3 Younghun Jung,1 Julio A. Aguirre-Ghiso,4 and Russell S. Taichman1*

1Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, Michigan
2Division of Hematology and Oncology, Department of Medicine, University of Michigan School of Medicine, Ann Arbor, Michigan
3Department of Cancer Biology and Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, North Carolina
4Division of Hematology and Oncology, Tisch Cancer Institute, Departments of Medicine, Otolaryngology, and Black Family Stem Cell Institute, Icahn School of Medicine at Mount Sinai, New York, New York

ABSTRACT
Many prostate cancer (PCA) recurrences are thought to be due to reactivation of disseminated tumor cells (DTCs). We previously found a role of the TAM family of receptor tyrosine kinases TYRO3, AXL, and MERTK in PCA dormancy regulation. However, the mechanism and contributions of the individual TAM receptors is largely unknown. Knockdown of MERTK, but not AXL or TYRO3 by shRNA in PCA cells induced a decreased ratio of P-Erk1/2 to P-p38, increased expression of p27, NR2F1, SOX2, and NANOG, induced higher levels of histone H3K9me3 and H3K27me3, and induced a G1/G0 arrest, all of which are associated with dormancy. Similar effects were also observed with siRNA. Most importantly, knockdown of MERTK in PCA cells increased metastasis free survival in an intra-cardiac injection mouse xenograft model. MERTK knockdown also failed to inhibit PCA growth in vitro and subcutaneous growth in vivo, which suggests that MERTK has specificity for dormancy regulation or requires a signal from the PCA microenvironment. The effects of MERTK on the cell cycle and histone methylation were reversed by p38 inhibitor SB203580, which indicates the importance of MAP kinases for MERTK dormancy regulation. Overall, this study shows that MERTK stimulates PCA dormancy escape through a MAP kinase dependent mechanism, also involving p27, pluripotency transcription factors, and histone methylation. J. Cell. Biochem. 118: 891–902, 2017. © 2016 Wiley Periodicals, Inc.

KEY WORDS: MERTK; AXL; TYRO3; PROSTATE CANCER; DORMANCY; DISSEMINATED TUMOR CELL

Prostate cancer patients often have long time periods between curative intent surgery or radiation therapy until the time of biochemical recurrence or metastatic disease visible with current imaging, which marks incurable disease with current treatment options. For example, in a large series of patients treated with radical prostatectomy, nearly 20% recurrences occurred at least 5 years after surgery [Amling et al., 2000]. Greater than half of prostate cancer patients with no evidence of disease, soon after radical prostatectomy were found to have disseminated prostate tumor cells (DTCs) in their bone marrow, which are thought to be a major source of distant recurrences [Morgan et al., 2009]. This finding implies that many of these tumor cells die, never grow, or grow very slowly. Many investigators refer to this ability of cancer cells to remain viable but not have detectable growth as “cellular dormancy.”

There is significant interest in regulators of cancer cellular dormancy. Several studies have identified a low ratio of phosphorylated MAPK3/MAPK1 (Erk 1/2) to phosphorylated MAPK14 (p38) as marking dormant tumor cells. TGFβ2 (TGF-β2) was proposed to be the major ligand responsible for dormant behavior of head and neck squamous cell carcinoma cells. The cell cycle inhibitor CDKN1B

© 2016 Wiley Periodicals, Inc.
(p27) and transcription factor BHLHE41 (DEC2) were implicated as nuclear signals [Bragado et al., 2013]. More recently, pluripotency associated transcription factors NR2F1, SOX2, SOX9, NANOG, and RARB were identified as transcriptional regulators of dormancy in head and neck, prostate and breast cancers [Sosa et al., 2015]. Similarly, others have shown that TGF-β family member BMP7 maintains prostate cancer dormancy through autocrine SPARC [Kobayashi et al., 2011; Sharma et al., 2016]. A role of the epigenome in regulating cellular dormancy is also becoming apparent. Histone H3 tri-methylated lysine 9 and tri-methylated lysine 27 were shown to identify and to be important for dormant cells, primarily in head and neck cancer [Sosa et al., 2015].

Our group has established a role of the TYRO3, AXL, and MERTK (TAM) family of receptors and one of their ligands, growth arrest-specific 6 (GAS6), in regulation of prostate cancer cell dormancy in the bone marrow [Shiozawa et al., 2010; Jung et al., 2012; Taichman et al., 2013]. We also found that GAS6 and MERTK are important for cancer stem like cell formation [Jung et al., 2016; Shiozawa et al., 2016]. This receptor family has an established role in the regulation of the innate immune system, but more recently has been shown to be important for cancer growth and metastasis as well. For example, MERTK was recently identified in a screen of wild-type kinases as a mediator of prostate cancer metastasis [Faltermeyer et al., 2015]. The TAM family of receptors have a high degree of homology, but have been shown to have different functions, which might relate to differences in ligand binding affinities and downstream pathways [Graham and DeRyckere, 2014]. There are at least four vitamin K dependent γ-carboxylated protein ligands that bind to at least one of the TAM receptors including GAS6, Protein S (PROS1), Tubby (TUB), and Tubby like protein 1 (TULP1) [Caheroy, 2010]. We found that GAS6 decreased prostate cancer proliferation and protected the cells from chemotherapy induced apoptosis [Shiozawa et al., 2010; Lee et al., 2016]. We also found that prostate cancer bone metastases grew larger in the absence of GAS6 [Jung et al., 2012].

However, these studies did not identify which of the TAM receptors are responsible for the ability of GAS6 to slow prostate cancer growth, while also preventing apoptosis—findings which are consistent with cellular dormancy. However, the role of TYRO3 and MERTK remained unclear. To begin to answer this question, we previously studied the relative expression level TYRO3 and AXL in prostate cancer primary tumors, DTCs, and in gross metastases. However, MERTK was not included in these studies. We found that TYRO3 was expressed highly in the primary tumors, but that AXL was expressed highly in disseminated but dormant disease [Taichman et al., 2013]. Based on these results, we hypothesized that TYRO3 might play a role when prostate cancer was actively growing but that AXL might play a role when it is dormant. However, these studies did not include experiments to test this hypothesis further than gene expression. Most recently, in work that is currently in press, we reported that AXL is required for TGF-β2 to induce prostate cancer dormancy [Yumoto et al., 2016]. However, the contributions of MERTK and TYRO3 remain unclear.

In the current study, we took an unbiased approach to discern which of the TAM receptors, including MERTK, are required for prostate cancer dormancy escape. With shRNA and siRNA technology, we knocked down the expression of each of the three receptors in three different prostate cancer cell lines. We found that loss of MERTK, but not the other receptors decreased the ratio of P-Erk to P-p38, increased the expression of p27 and pluripotency associated transcription factors, increased the levels of dormancy associated histone H3 and caused accumulation of cells in the G1 and G0 phases of the cell cycle, and decreased apoptosis, all of which characterize dormant cells. Importantly, the effect of MERTK on the cell cycle and histone H3 post-translational modifications was reversed by altering MAP kinase signaling with a p38 inhibitor. We also found that MERTK knockdown increased metastasis free survival in an intra-cardiac injection mouse prostate cancer xenograft model, but did not inhibit in vitro cell growth or subcutaneous tumor growth showing that it did not compromise global growth characteristics. Thus, our studies implicate MERTK in stimulation of prostate cancer dormancy escape by a mechanism particular to the metastatic microenvironment and involving MAP kinases.

MATERIALS AND METHODS

CELL CULTURE

Human PCa cell lines, PC3, Du145, and LNCaP C4-2B (C4-2B) were obtained from American Type Culture Collection (Rockville, MD [PC3 and Du145]) and UroCor (Oklahoma City, OK [C4-2B]). PCa cells were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in a humidified incubator with 5% CO2. For in vitro assays, unless indicated otherwise, cells were seeded at a density $1 \times 10^5$/ml, allowed to rest for 1 day in 10% serum and then changed to reduced serum concentrations as indicated. For the p38 inhibitor experiments, cells were first cultured for 14 days under routine conditions with 10% serum and 5 μM SB203580 (EMD Millipore #A8254) dissolved in DMSO or 0.05% DMSO control.

TAM RECEPTOR STABLE shRNA KNOCKDOWNS

GFP and luciferase expressing PCa cell lines (PC3 [GFP], Du145 [GFP], and C4-2B [GFP] cells) were first established by lentiviral transduction. Stable knockdowns of the TAM receptors (TYRO3, AXL, and MERTK) were then generated by lentiviral infection. Lentiviruses were constructed by the University of Michigan Vector Core using pGIPZ lentiviral vectors containing either a shRNA targeting one of the TAM receptors or a nonsilencing [shControl] shRNA (Open Biosystems). Stable lines were selected with puromycin. Knockdown of greater than 80% was verified by Western blotting and qRT-PCR. qRT-PCR gene expression data are presented as mean ± SEM of independent cultures.

MerTK TRANSIENT siRNA KNOCKDOWNS

siRNAs targeting MerTK (# s20474, s20473, and s20472) and control siRNA [siControl] (# 4390843) were purchased from Thermo-Fisher Scientific. Transient transfection in C4-2B and PC3 cells was performed using 10 mM of each siRNA with Lipofectamine RNAiMAX reagent (Thermo-Fisher) using the reverse transfection protocol, followed by 3 days incubation. Knockdown was verified by real time qRT-PCR. Data are presented as mean ± SEM of triplicate PCR reactions.
WESTERN BLOTTING
Cells were serum starved overnight unless indicated otherwise. Lysates were prepared in cOmpley’s lysis M (Roche #04 719 956 001) supplemented with proteinase inhibitor Mini cOmplete Tablets (Roche #04705378) and phosphatase inhibitor PhosSTOP EASYpack Tablets (Roche #04 906 837 001). Protein concentration was determined by the BCA method. Twenty micrograms of total protein was added per lane of 4–20% reducing SDS polyacrylamide Tris-Glycine gels after sample preparation in Laemmli sample buffer. The samples were transferred to PVDF membranes and blocked for 1 h in 5% dry milk in TBS with 0.1% Tween-20 (TBST). Antibodies for phosphorylated proteins were applied at 4°C overnight in 5% BSA TBST, washed, and visualized with a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (Cell Signalling #7074S) and SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific #34075). Images were acquired with a ChemiDoc Touch imager (BioRad). Membranes were stripped with Restore PLUS stripping buffer (Thermo Scientific #46430). They were blocked and re-probed for antibodies to total proteins, and again stripped and re-probed for GAPDH or β-actin to normalize for protein loading. All primary antibodies were monoclonal rabbit from Cell Signaling Technology. Catalog numbers and dilutions were as follows; Phosphorylated-Erk 1/2 (P-Erk) Y204(#4377S, diluted 1:500), total Erk (#4695, 1:500), Phospho-p38 (P-p38) T190/Y182 (#4511, 1:500), total p38, (#9212, total Axl (#4939, 1:500), total Tyro3 (#5585, 1:500), total MerTK (#4319, 1:500), Sox2 (# 3579, 1:500), Caspase-9 (#9502, 1:1000), β-actin (#4970, 1:2000), and GAPDH (#2118, 1:2000). Images representative of biological replicates are shown and cropped for presentation. For P-Erk, P-p38, and p27 quantification, images from five independent experiments were quantified relative to each vehicle treated scrambled shRNA control with BioRad ImageLab software and then normalized to housekeeping gene expression. The P-Erk to P-p38 ratio was obtained by dividing the normalized P-Erk and P-p38 values for each independent experiment. All data are shown as fold change from control.

REAL TIME REVERSE TRANSCRIPTASE PCR (qRT-PCR)
Cells were lysed and RNA was harvested using the Qiagen RNAeasy kit followed by reverse transcription using Invitrogen SuperScript II Reverse Transcriptase. Real time qPCR was performed using TaqMan Universal PCR Master Mix and Gene Expression Assays on a Applied Biosystems Viia 7 instrument. TaqMan MGB probes (Applied Biosystems) were as follows: MERTK (Hs00179024_m1), p27/CDKN1B (Hs00153277), SOX2 (Hs01053049_s1), and NANOG (Hs02387400). We designed primers and a probe to specifically detect NR2F1/TFCOUP1: forward; CAAAGCCATGTTGCTTCA, reverse; CCTGCAGGCTCTCAGTGT, and probe; TCA-GACGGCTTGGCCCTG, β-actin (Hs01060665_g1) was used as an internal control for the normalization of target gene expression.

FLOW CYTOMETRY FOR HISTONE POST-TRANSLATIONAL MODIFICATIONS AND Ki67
Cell pellets were fixed and permeabilized with dropwise addition of 1 ml of cold 70% ethanol and then incubated overnight. All steps were at 4°C or on ice. Samples were then washed, blocked, and incubated for 1 h in flow buffer (PBS with 2% FBS and 2 mM EDTA) with each of the following antibodies; Alexa 647 conjugated rabbit anti histone H3 tri-methylated lysine 27 diluted 1:50 (Cell Signaling Technology #12158), unconjugated rabbit polyclonal anti histone H3 tri-methylated lysine 9 (Abcam #8989), or APC conjugated rabbit anti-human Ki-67 antibody (Biolegend #350513). Cells were washed twice with flow buffer. The unconjugated histone H3 tri-methylated lysine 9 antibody was detected with an Alexa 647 conjugated anti-rabbit IgG diluted 1:250 (Cell Signaling Technology #4414). Data were acquired with a three laser (405, 488, and 640 nm) Becton Dickinson FACS Aria llu flow cytometer. Gating was forward scatter versus side scatter, single cells (linear on FSC-A vs. FSC-H), then the 670/30 filter (APC or Alexa 647) versus forward scatter or histogram. An isotype control antibody was used for setting the gates. Negative and dim cells were selected for methylated histones. Negative cells were selected for Ki67. Data are presented as representative plots or mean ± SEM of triplicate wells from replicate experiments.

CELL CYCLE ANALYSIS
Cells were cultured for 3 days as indicated and pulsed with 10 μM bromodeoxyuridine (BrdU) for 30 min. The cells were collected with trypsin as necessary and then fixed and stained for total DNA with 7-AAD and BrdU incorporated into DNA using the Becton Dickinson APC BrdU flow kit (#552598). Data were acquired with a Becton Dickinson FACS Aria llu flow cytometer. Gating was forward scatter versus side scatter, single cells (linear on FSC-A vs. FSC-H), then APC (BrdU) versus 7-AAD (DNA).

LEFT VENTRICLE INTRACARDIAC INJECTION XENOGRAFT MODEL OF PROSTATE CANCER METASTASIS
Stable shRNA infected PC3GFP (1 × 106) or Du145GFP (2 × 105) prostate cancer cells were suspended in 100 μl of PBS and injected into male CB.17. SCID mice (6–8 weeks of age: Charles River Labs) by left ventricle intracardiac injection. For analysis of metastasis free survival, bioluminescence images were acquired after injection of luciferin twice weekly using a PerkinElmer IVIS 2000 system. Animals that had a large portion of the signal in the lungs (indicative of a right ventricle injection) were removed from analysis a priori. After removing mice that had a right ventricular injections or did not survive the procedure, the following numbers of animals were analyzed; PC3 shControl; 6, PC3 shMER; 7, Du145 shControl; 20, and Du145 shMER; 18. Time to metastasis formation visible by bioluminescence (or death in rare cases) was then determined from the images. The data were analyzed by Kaplan–Meier analysis. For analysis of transit to the bone marrow, different mice, five mice per group, were sacrificed 24 h after tumor cell injection, and their pelvis, femora, and tibiae were harvested. The bones were crushed with a mortar and pestle and strained to remove debris. All used PBS buffer with 2% FBS unless otherwise noted. Cells were first depleted of mouse cells with a Mouse Cell Depletion Kit magnetic labeling system (Miltenyi Biotec # 130-104-694) and anti-Biotin MicroBeads and an AutoMACS machine (Miltenyi Biotec). The enriched cells were incubated with an APC-Cy7 conjugated anti-HLA-ABC antibody (BioLegend #311426) and a PerCP-Cy5.5 conjugated anti-mouse lineage cocktail (CD3e, CD11b, B220, Ter-119, Ly-6G, and Ly-6C) (BD Biosciences #561317), for an hour at 4°C, washed and resuspended in

MERTK AND PROSTATE CANCER DORMANCY 893

JOURNAL OF CELLULAR BIOCHEMISTRY
PBS with 2% FBS, 2 mM EDTA and 0.5 μg/ml DAPI. Thereafter, the percentage of disseminated prostate cancer cells (DTCs) was determined by gating on single, viable, lineage negative, HLA+ cells with a FACSAria Ilu flow cytometer. Mice injected with PBS rather than PCa cells were used as a negative control for flow cytometry. Data represents three independent experiments.

PROSTATE CANCER SUBCUTANEOUS TUMOR MODEL
One million prostate cancer cells suspended in 50 μl complete media were mixed with an equal volume of cold collagen solution and then slowly injected under the skin of the back of SCID mice; five mice per group. Bioluminescence images were acquired weekly. Animals were sacrificed before tumors grew to 1 cm³. All experimental procedures were approved by the University of Michigan Committee for the Use and Care of Animals.

PROSTATE CANCER CELL VIABILITY/MTS ASSAY
Prostate cancer cells were seeded at 2000 cells per well in 96 well plates and rested for 1 day in 10% FCS RPMI media. The media was subsequently changed to the indicated serum concentrations and the cells were cultured for an additional 3 days. The total viable cell number was then assayed with the Cell Titer Aqueous One Solution MTS Proliferation Assay System (Promega #G3580) by absorbance at 490 nm. Data represent means of three independent experiments.

STATISTICAL ANALYSES
The type I error rate (α) was set to 0.05 for all analyses. Two-sample, two-tailed Student’s t-tests were used to compare means of two groups. One-way repeated measures analysis of variance (ANOVA) with Bonferroni post-hoc testing was used for data normalized to housekeeping genes (blots and PCR). Standard one-way ANOVA with Tukey’s Honest Significant Difference post-hoc testing was used for multiple comparisons in other experiments. The Log-rank test was used for Kaplan–Meier survival analyses. Growth curves for subcutaneous tumors were analyzed with a mixed design (split plot) ANOVA with repeated measures. All analyses were conducted with SPSS software, except for t-tests, which were performed in Microsoft Excel.

RESULTS
MERTK KNOCKDOWN CAUSES DORMANCY ASSOCIATED CHANGES IN MAPK ACTIVITY AND p27 EXPRESSION
To study the importance of TAM signaling on dormancy, each TAM receptor was stably knocked down with shRNA in PC3, Du145, and
C4-2B prostate cancer cell lines. Protein expression of each receptor was decreased by at least 80% (Fig. S1). A decreased ratio of P-Erk 1/2 to P-p38 MAPK marks cellular dormancy in prostate and other cancers [Kobayashi et al., 2011; Bragado et al., 2013; Chery et al., 2014]. Therefore, we first examined P-Erk1/2 and P-p38 levels in PC3 cells with each of the TAM receptors knocked down. The ratio of P-Erk 1/2 to P-p38 was significantly decreased in serum starved MERTK knockdown cells, but not in TYRO3 or AXL knockdown cells (Fig. 1A and B). Similarly, the cell cycle inhibitor p27 was also previously found to be a dormancy marker [Kobayashi et al., 2011; Bragado et al., 2013]. In agreement with the Erk and p38 data, we found higher basal p27 protein expression in the MERTK knockdown cells (Fig. 1C and D). These data are consistent with a dormant phenotype in prostate cancer cells as a result of chronically reduced expression of MERTK.

**MERTK KNOCKDOWN CAUSES EXPRESSION OF DORMANCY AND PLURIPOTENCY ASSOCIATED TRANSCRIPTION FACTORS**

Transcription factors first studied in embryonic stem cells have been found to promote cancer dormancy [Sosa et al., 2015]. Therefore, we determined the basal expression level of three of these transcription factors in PC3 cells with each of the TAM receptors knocked down by shRNA. In parallel with the MAP kinase and p27 data, we saw marked upregulation of SOX2 message and protein in shMER but not shAXL or shTYRO3 cells (Fig. 2A and B). Similarly, we also observed increased expression of SOX2 in shMER C4-2B cells (Fig. 2A). We also observed increased NR2F1 and NANOG mRNA in shMER, but not shAXL or shTYRO3 cells (Fig. 2C and D). Because of the possibility of off target effects of shRNAs, we performed analogous studies with siRNA rather than shRNA and found that a siRNA targeting MERTK increased expression of SOX2 and NANOG in PC3 and C4-2B cells.

![Fig. 2. TAM receptor knockdown and expression of dormancy and pluripotency associated transcription factors. (A) PC3 cells with each of the TAM receptors knocked down by shRNA or C4-2B cells with MERTK knocked down, analyzed for SOX2 expression by qPCR. (B) Representative SOX2 Western blot of shControl and shMER PC3 cells. (C and D) PC3 TAM receptor shRNA cells with expression of NR2F1 or NANOG respectively, quantified by qPCR. (E) PC3 cells with MerTK knocked down by siRNA and quantified for expression of SOX2 and NANOG by qPCR. (F) C4-2B cells with MERTK knocked down by siRNA and quantified for expression of SOX2 and NANOG by qPCR. All data are presented normalized to control. Error bars represent mean ± SEM. * Represents P < 0.05 compared to shRNA or siRNA control cells.](image-url)
MERTK KNOCKDOWN INDUCES CELL CYCLE CHANGES ASSOCIATED WITH CELLULAR DORMANCY

Cellular dormancy and decreased Erk 1/2 activity are also characterized by arrest in the G1 and G0 phases of the cell cycle [Aguirre-Ghiso et al., 2004]. Therefore, we compared cell cycle characteristics of TAM receptor knockdown PC3 cells cultured in 0.1% serum using flow cytometry to detect antibody labeled pulsed bromodeoxyuridine (BrdU) incorporated into DNA and 7-AAD to quantify total DNA content. This assay identifies BrdU positive cells as S-phase, less than 2n DNA and BrdU negative; Sub-G0 (apoptotic and necrotic), 2n DNA and BrdU negative; G0 and G1 phases, 4n DNA and BrdU negative; G2 and M phases. BrdU positive; S-phase, >4n DNA; polyploid cells. (A) Quantification of the above cell cycle data. The table lists % values for each cell type compared to control with significant comparisons marked with an asterisk. Error bars are shown for the G0/G1 and G2M populations and represent mean ± SEM. * Represents P < 0.05 compared to shRNA control cells. (B) Western blots for total caspase-9 to verify the changes in Sub-G0 cells observed by flow cytometry.

MERTK KNOCKDOWN INDUCES CELL CYCLE CHANGES ASSOCIATED WITH CELLULAR DORMANCY

showed the opposite pattern with fewer cells in G0/G1 and more cells in the G2 and M phases. We did not convincingly see other analogous results for TYRO3 in our other experiments. We did not see differences between the different TAM knockdown cells in the percentage of cells in S-phase, but note that there are very few cells in S-phase in this study because of the low serum conditions. However, we did observe a reduction in the sub-G0 (apoptotic and necrotic) population in the shMER cells. Further, Western blots showed decreased levels of cleaved Caspase-9 in the shMER cells, thus suggesting that this reduced sub-G0 population represented reduced apoptosis. There is precedence in the literature for correlation of reduced apoptosis with a dormant phenotype [Aguirre-Ghiso et al., 2004]. Further, p38 stimulated cellular dormancy and reduced apoptosis have been proposed to be adaptive responses to allow DTCs to survive when conditions are not conducive to growth [Ranganathan et al., 2006].
DEPLETION OF MERTK INCREASES METASTASIS FREE SURVIVAL IN VIVO
We next tested the importance for MERTK for dormancy escape in vivo. GFP and luciferase labeled control shRNA or MERTK shRNA PC3 or Du145 PCa cells were injected in the left ventricle of SCID mice and time to metastases visible by bioluminescence imaging, or death in rare cases, was evaluated with Kaplan–Meier analysis (Fig. 4A). Metastases are primarily to bone with both cell lines. Metastasis free survival in this model is established in the literature as a measure of dormancy [Kobayashi et al., 2011]. In agreement
with the in vitro data, metastasis free survival was prolonged in mice injected with shMER PC3 cells relative to control cells (Fig. 4B, top left). With Du145 cells, the increase in metastasis free survival with MERTK knockdown was small and not statistically significant (Fig. 4B, lower left). However, we noted that shMER Du145 cells appeared to develop metastases to the head more slowly, which approached statistical significance (Fig. 4B, lower right). Time to cranial metastases was again significantly different with PC3 cells. A defect in transit to the bone marrow could also explain delayed metastasis formation but would not involve dormancy escape. Therefore, in separate experiments, we used flow cytometry to quantify the percentage of control or shMER PC3 cells in marrow 1 day after injection and found no difference (Fig. 4C and D). This further supports the conclusion that MERTK is selectively important for dormancy escape rather than transit to the bone marrow in this model.

MERTK KNOCKDOWN DOES NOT DECREASE PROSTATE CANCER GROWTH IN VITRO OR AT A SUBCUTANEOUS SITE IN VIVO

We hypothesized that the role of MERTK was somewhat specific to cellular dormancy and therefore, it should not greatly affect overall cellular growth in vitro or in vivo at a site not dependent on the usual microenvironment, such as a subcutaneous site with an artificial extracellular matrix. Indeed, other groups who have derived dormant and tumorigenic cancer cell lines have observed similar growth in culture [Bragado et al., 2013; Sharma et al., 2016]. In agreement with these expectations, we saw no difference in subcutaneous growth in vivo between control and shMER PC3 cells and saw slightly higher growth of Du145 shMER cells compared to control (Fig. 5A). Similarly, we saw no significant differences in relative cell number, as measured by MTS assay, between any of the TAM shRNA knockdowns in PC3, Du145, and C4-2B cells cultured for 3 days in 0.1%, 1%, or 10% serum (Fig. 5B). The Du145 shMER
cells trended toward higher relative cell number but did not reach statistical significance after multiple comparison testing.

CELL CYCLE CHANGES OF MERTK KNOCKDOWN ARE MAP KINASE DEPENDENT

Because of the well-established role of MAP kinases in regulation of cancer cellular dormancy, we investigated if p38 was required for the dormancy associated cell cycle changes induced by knockdown of MERTK. In appreciation of the known role of epigenetic changes in dormancy regulation and the time period required for these changes to occur, we cultured control and MERTK shRNA PC3 and Du145 cells with p38 inhibitor SB203580 or 0.05% DMSO solvent control for 2 weeks before performing experiments [Sosa et al., 2015]. The expected compensatory increase in P-p38 in response to p38 active site inhibition was observed by Western blot (Fig. S2A). We again observed an increased percentage of G0/G1 and decreased percentage of G2/M cells with MERTK knockdown both in PC3 and Du145 cells (Fig. 6). This change induced by MERTK knockdown was completely reversed by p38 inhibition, thus showing involvement of MAP kinases. Similarly, we also observed a increased percentage of Ki67 negative cells (non-cycling) with MERTK knockdown in PC3 cells, which was also reversed by p38 inhibition (Fig. S2C). Curiously, we did not observe reversal of shMER induced p27 upregulation with p38 inhibition (Fig. S2B). This suggests that not all of the effects of MERTK knockdown are MAP kinase dependent.

MERTK KNOCKDOWN INDUCES DORMANCY ASSOCIATED CHANGES IN HISTONE H3 METHYLATION BY A MAP KINASE DEPENDENT MECHANISM

Lastly, we used flow cytometry with specific antibodies to determine the effect of shRNA knockdown in PC3 cells of each of the TAM receptors on histone H3 tri-methylated lysine 9 (H3 K9 me3) and
histone H3 tri-methylated lysine 27 (H3 K27 me3). Both of these histone marks are increased in dormant cells [Sosa et al., 2015]. Because the majority of cells were positive, we gated on the negative and dim populations rather than the positive population (Fig. 7A). We observed the expected dormancy associated change in H3 K9me3, and a trend toward significance for H3 K27me3 with MERTK knockdown but no significant differences for AXL or TYRO3 knockdown (Fig. 7B). In cells treated with the p38 inhibitor SB203580 or solvent control, we saw significant dormancy associated changes with MERTK knockdown, which were partially reversed by p38 inhibition (Fig. 7C).

**DISCUSSION**

Overall, these studies implicate MERTK in prostate cancer dormancy escape through a MAP kinase dependent mechanism linked to transcriptional and epigenetic regulation. Knockdown of MERTK consistently induced the changes expected for dormant cells; a decreased ratio of P-Erk to P-p38, increased p27 expression, expression of dormancy, and pluripotency associated transcription factors, and G0/G1 arrest. Further, these findings translated to an increased metastasis free survival in vivo. This identifies MERTK as being important for the process whereby one or a few cancer cells progress to a small tumor (i.e., escape from cellular dormancy). As expected, MERTK knockdown did not inhibit growth in culture or growth of subcutaneous tumors implanted in an artificial matrix. This lack of a general growth inhibitory effect of MERTK knockdown suggests specificity for dormancy regulation in bone and a requirement for a signal from the microenvironment rather than dysregulation of multiple cellular processes.

Our data do not identify which signal(s) from the microenvironment interact with MERTK to regulate dormancy. The four other TAM receptor ligands, other than GAS6 (Tubby, Tubby like protein 1, Galectin 2, and Protein S), may also play a role. Although not a MERTK ligand, retinoic acid may be indirectly involved as well because it has been shown to stimulate cancer dormancy through NR2F1 and also interacts indirectly with MERTK in immune cells [Garabuczi, 2015; Sosa et al., 2015]. Because no recombinant protein or other treatment other than low serum conditions was required for
AXL is required for TGF- 
slowly or rapidly cycling [Sharma et al., 2016; Takeishi and these studies and other literature reports that CSCs can be either 
cancer stem-like cells (CSCs) [Jung et al., 2016; Shiozawa et al., 2016]. However, the proliferative rate of CSCs was not examined in 
knockdown might have been insuf 
stimulation of dormancy escape. For example, the percent 
completely unclear. Here, we took an unbiased approach and were 
contribution of MERTK to prostate cancer dormancy remained 
alterations in signaling from autocrine TAM receptor ligands.

Previously, we examined the expression of TYRO3 and AXL, but not MERTK in disseminated tumor cells (DTCs) versus prostate cancer primary tumors and gross metastases [Taichman et al., 2013]. We reported higher expression of AXL in DTCs and higher expression of TYRO3 in primary tumors and gross metastases. Thus, we 
material. When epigenetic reprogramming is induced, MERTK activates p27, pluripotency 
epigenetic reprogramming rather than through a more immediate response to ligands.

Fig. 8. Results summary for MERTK stimulates PCa dormancy escape through a MAP kinase dependent mechanism, also involving p27, pluripotency transcription factors, and histone methylation.

our observed in vitro effects, knockdown of MERTK may have caused epigenetic reprogramming of the cells as a result of chronic

REFERENCES


Takeishi S, Nakayama KI. 2016. To wake up cancer stem cells, or to let them sleep, that is the question. Cancer Sci 107(7):875–881.


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web-site.
The ABC7 regimen: a new approach to metastatic breast cancer using seven common drugs to inhibit epithelial-to-mesenchymal transition and augment capecitabine efficacy

Abstract: Breast cancer metastatic to bone has a poor prognosis despite recent advances in our understanding of the biology of both bone and breast cancer. This article presents a new approach, the ABC7 regimen (Adjuvant for Breast Cancer treatment using seven repurposed drugs), to metastatic breast cancer. ABC7 aims to defeat aspects of epithelial-to-mesenchymal transition (EMT) that lead to dissemination of breast cancer to bone. As add-on to current standard treatment with capecitabine, ABC7 uses ancillary attributes of seven already-marketed noncancer treatment drugs to stop both the natural EMT process inherent to breast cancer and the added EMT occurring as a response to current treatment modalities. Chemotherapy, radiation, and surgery provoke EMT in cancer generally and in breast cancer specifically. ABC7 uses standard doses of capecitabine as used in treating breast cancer today. In addition, ABC7 uses 1) an older psychiatric drug, quetiapine, to block RANK signaling; 2) rifabutin, an antibiotic to block beta-catenin signaling; 3) metformin, a first-line antidiabetic drug to stimulate AMPK and inhibit mammalian target of rapamycin, (mTOR); 4) propranolol, a beta-blocker to block beta-adrenergic signaling; 5) agomelatine, a melatonergic antidepressant to stimulate M1 and M2 melatonergic receptors; and 7) ribavirin, an antiviral drug to prevent eIF4E phosphorylation. All these block the signaling pathways – RANK, TGF-beta, mTOR, beta-adrenergic receptors, and phosphorylated eIF4E – that have been shown to trigger EMT and enhance breast cancer growth and so are worthwhile targets to inhibit. Agonism at MT1 and MT2 melatonergic receptors has been shown to inhibit both breast cancer EMT and growth. This ensemble was designed to be safe and augment capecitabine efficacy. Given the expected outcome of metastatic breast cancer as it stands today, ABC7 warrants a cautious trial.

Keywords: ABC7, breast cancer, agomelatine, capecitabine, metformin, pirfenidone, propranolol, quetiapine, repurposing, ribavirin, TGF-beta

Plain language summary
This article presents the rationale and thinking behind the ABC7 regimen for metastatic breast cancer. Since there is currently no cure for breast cancer once it has spread to bone and other organs beyond the breast itself, the ABC7 regimen was designed to take advantage of ancillary attributes of seven common and readily available noncancer treatment drugs that, in theory, should make current standard cytotoxic chemotherapy with capecitabine more effective. The ABC7 regimen has not been shown to be safe or effective yet. In the current article, we discuss an untested proposal for a new treatment approach to metastatic breast cancer.
**Introduction**

Estrogen-positive metastatic breast cancer cannot be cured currently.\(^1\) One major metastatic site of breast cancer is bone. Once breast cancer metastasizes to bone, the survival rate declines despite recent advances in local treatments of breast cancer. Current treatment strategies for bone metastasis, including bone-targeted agents (bisphosphonate and denosumab), provide only palliation. New and effective therapeutic strategies for this still incurable disease are therefore urgently needed.

This article reviews the attributes of seven currently marketed drugs that, as indicated by prior research data, will block or partially block the escape pathways from current traditional treatments. The seven drugs of ABC7 were chosen by first identifying the basic pathways by which EMT is initiated and maintained. We then reviewed 1000 of the most commonly used drugs\(^2\) for which we have both usual plasma levels and published data showing potential inhibitory interaction with these pathways. The resulting list was reduced by semi-subjective evaluation of the strength of data on their EMT inhibition benefit versus the drugs’ expected tolerability. The better the tolerability, the weaker the data had to be for inclusion.

This ABC7 regimen is designed to block several core breast cancer growth signals in a coordinated manner, thereby augmenting the cytotoxicity of a currently used cytotoxic chemotherapy drug, capecitabine. Figure 1 shows an overview schematic of the biochemistry that ABC7 is designed to influence. This is explained in detail in the respective drug discussions in the “Drugs to inhibit EMT” section. Table 1 gives an overview of the ABC7 drugs and their intended targets in treating breast cancer.

The ABC7 regimen follows the approach of previous cancer treatment regimens, for example, MTZ regimen,\(^3\) COMBAT regimen,\(^4\) MEMMAT regimen, a current trial of Peyrl et al’s seven-drug cocktail (ClinicalTrials.gov Identifier: NCT01356290), and CUSP9 regimen.\(^5,6\) In all of the studies, extensive use is made of drugs not primarily marketed to treat cancer but that have ancillary attributes that research data would enhance the anticancer effect of a cytotoxic, traditional cancer treatment drug. The ancillary drugs exert anticancer effects by blocking various growth-enhancing survival pathways used by the target cancer or as for agomelatine are agonists at growth-retarding receptors.

Similar to other cancers, breast cancer has heterogeneous regions within the same tumor – different areas that depend on or use different growth-signaling pathways. This is related to but distinct from the idea of clonal evolution driven by cytotoxic chemotherapy selection. Both forms of heterogeneity exist in a typical breast cancer, proteomic and genomic. ABC7 aims to inhibit breast cancer by pharmacological manipulation of what genes are expressed and what genes are not, as well as by targeting different clonal variants of the original breast cancer clone.

Because of these limitations, we do not expect testing for molecular markers to be predictive or useful. In addition, further intensifying the spatial and temporal diversity of the molecular status, particularly for EMT markers, are the diversity-driving effects of chemotherapy\(^7\) and discussed in greater detail in the following sections.

Cytotoxic chemotherapy also induces important receptor status changes in a large minority of breast cancer cases.\(^8,9\) Typical findings are as follows: 13% changed from HR+ to HR−, 5% changed from HR− to HR+, 6% changed from HER2+ to HER2, 3% changed from HER2 to HER2+, and 13% changed to triple negative.\(^8\)

Multiple signaling systems have been identified that drive metastatic breast cancer.\(^9,10,11\) These growth-driving receptors can cross cover for each other.\(^1,9–11\) When one is pharmacologically blocked, several parallel growth-driving pathways can become active, taking the place of the blocked pathways. Growth factor signaling converges from a wide variety of outer membrane receptors to more restricted, fewer, intracellular pathways. This is, more elegantly stated, the spatial–temporal genomic and proteomic range, the “genetic collectives [that] dominate the landscape of advanced-stage (malignant) disease.”\(^11,12\) We see this as mandating an integrated, coordinated polypharmacy to successfully address these malignancy attributes.

Capecitabine is intracellularly metabolized to 5-FU; the details are given in the “Capecitabine: 359 Da, half-life <1 hour” section. ABC7 drugs are designed to make 5-FU more effective.

The results of several recent ER+ metastatic breast cancer studies are listed in Table 2. These studies cannot be judged simply by overall survival in that entry requirements were different, with different kinds and number of prior treatments. These numbers in Table 2 are for general idea only.\(^1\) One cannot conclude that one of these is better than another.

In general, post-progression survival durations in recent Phase III studies of combination therapy ranged from approximately 16 to 33 months.\(^1\)

EMT is a feature of cancers generally\(^13\) and breast cancer specifically.\(^14,15\)

Table 3 lists several features and behaviors associated with the two (epithelial and mesenchymal) states. Interestingly, a transcription factor ZEB1, known to control EMT,
EMT is a phase transition, where flat, sessile, mutually adherent epithelioid cells take on a rounded, non-adherent, motile mesenchymal shape and behavior.\textsuperscript{18–20} The reverseless transient state and process, MET, also occurs and is also a feature of robust or aggressive cancer growth.\textsuperscript{21} Post-EMT cells tend to be invasive but proliferation restricted. Post-MET cells tend to be proliferative but have limited invasiveness.\textsuperscript{18–20} Breast cancers develop in proximity to adipose tissue. Adipocytes are a further trigger to EMT.\textsuperscript{22}

**Table 1** The drugs of ABC7, their targets during treatment of breast cancer, and suggested doses

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target in breast cancer treatment</th>
<th>Starting dose</th>
<th>Target dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capecitabine</td>
<td>DNA synthesis</td>
<td>600 mg/m(^2) twice daily.</td>
<td>1250 mg/m(^2) twice daily. 7 days on, 7 days off or 14 days on, 7 off</td>
</tr>
<tr>
<td>Quetiapine</td>
<td>RANK/RANKL</td>
<td>50 mg once at bedtime</td>
<td>300–600 mg once at bedtime</td>
</tr>
<tr>
<td>Pirfenidone</td>
<td>TGF-beta</td>
<td>200 mg three times daily</td>
<td>600 mg three times daily</td>
</tr>
<tr>
<td>Rifabutin</td>
<td>BCL-6; beta-catenin</td>
<td>150 mg/day</td>
<td>300 mg/day</td>
</tr>
<tr>
<td>Metformin</td>
<td>AMPK\textsuperscript{*}; mTOR; mitochondria oxphos</td>
<td>500 mg once daily</td>
<td>1000 mg twice daily</td>
</tr>
<tr>
<td>Propranolol</td>
<td>Beta-adrenergic receptors</td>
<td>10 mg twice daily</td>
<td>Up titrate as tolerated</td>
</tr>
<tr>
<td>Agomelatine</td>
<td>Melatoninergic receptors\textsuperscript{*}</td>
<td>25 mg once at bedtime</td>
<td>50 mg once at bedtime</td>
</tr>
<tr>
<td>Ribavirin\textsuperscript{**}</td>
<td>eIF4E; MNK; IMPDH</td>
<td>600 mg/day</td>
<td>1200 mg/day</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Note that all entries denote inhibition of named target except for metformin that activates AMPK and agomelatine that stimulates melatonin receptors. All drugs, except capecitabine, are given continuously without interruption. Capecitabine is given on 7 days on, 7 days off, or 14 days on, 7 days off cycles.\textsuperscript{**}Ribavirin is likely to give unpleasant side effects and depressed mood but is potentially a beneficial enough drug to try.

**Table 2** Representative recent trials in metastatic HR+ breast cancer

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Months survival</th>
<th>Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anastrozole + fulvestrant</td>
<td>48</td>
<td>SWOG</td>
</tr>
<tr>
<td>Anastrozole + fulvestrant</td>
<td>38</td>
<td>FACT</td>
</tr>
<tr>
<td>Anastrozole + fulvestrant</td>
<td>21</td>
<td>SoFEA</td>
</tr>
<tr>
<td>Letrozole + fulvestrant</td>
<td>52</td>
<td>LEA</td>
</tr>
<tr>
<td>Everolimus + exemestane</td>
<td>31</td>
<td>BOLERO 2</td>
</tr>
</tbody>
</table>

contrtributes to breast cancer osteolytic bone metastasis, but not brain or lung metastasis.\textsuperscript{16,17}
process is also unclear, and the two populations probably largely overlap.

Perhaps our deepest insight into the EMT process in breast cancer came from a study by Bulfoni et al. They showed that all patients with metastatic breast cancer had CTCs. These circulating cancer cells split into four groups: those with epithelial, those with mesenchymal, those with both, and those with neither marker. Patients with higher numbers of circulating cancer cells that expressed both markers had shorter overall survival.

Survival as a function of E-cadherin expression, representative of epithelial state, and fibronectin, representative of mesenchymal state, was examined by immunohistochemistry on 1495 breast cancer biopsies. More E-cadherin and less fibronectin are associated with longer survival. Breast cancer patients whose tissue expresses greater EMT-related protein have shorter survival. EMT drives chemotherapy resistance and other poor prognosis features in breast cancer. In addition, a greater degree of metabolic changes characteristic of EMT in breast cancer predicts shorter overall survival.

Medical research discusses vimentin, fibronectin, and N-cadherin as markers of EMT process, but these proteins would be more accurately viewed as mediators of the attributes we designate EMT.

As indicated in Table 3, fibronectin is a characteristic marker of EMT. Higher breast cancer tissue expression of fibronectin correlates with shorter survival. A range of other characteristic behavioral and morphological attributes of EMT and MET states is also outlined in Table 3.

### Table 3 Characteristic protein markers and mediators of EMT in breast cancer

<table>
<thead>
<tr>
<th>Marker</th>
<th>Epithelial state</th>
<th>Mesenchymal state</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>Increased</td>
<td>Decreased</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Increased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Occludin</td>
<td>Increased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Phenotype</td>
<td>Epithelial state</td>
<td>Mesenchymal state</td>
</tr>
<tr>
<td>Motility</td>
<td>Sessile</td>
<td>Motile</td>
</tr>
<tr>
<td>Shape</td>
<td>Elongated</td>
<td>Rounded</td>
</tr>
<tr>
<td>Adherence</td>
<td>Adherent to neighbors</td>
<td>Non-adherent to neighbors</td>
</tr>
<tr>
<td>Invasion</td>
<td>Noninvasive</td>
<td>Invasive</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Higher proliferation</td>
<td>Lower proliferation</td>
</tr>
<tr>
<td>Microtentacles</td>
<td>Absent</td>
<td>Present</td>
</tr>
</tbody>
</table>

**Abbreviation:** EMT, epithelial-to-mesenchymal transition.

### Chemotherapy triggers EMT

Paclitaxel triggers EMT in breast cancer, increasing mesenchymal markers, vimentin and fibronectin, and decreasing epithelial marker, ZO-1. Experimental inhibitors of TGF-beta signaling block paclitaxel-induced EMT and suppress paclitaxel-induced CSC properties. Paclitaxel also increases EMT markers in mouse breast cancer cell line, MCF-7/PAX. Doxorubicin exposure enhances gastric cancer’s EMT marker expression.

These reports, combined with similar findings in other cancers, allow a general statement of a core principle of oncology: cytotoxic chemotherapies tend to provoke EMT. Such a conjecture is amply supported by the recent work of Yoshimasu et al who reported that cisplatin, 5-FU, gemcitabine, paclitaxel, and vinorelbine show hormesis when tested individually.

### Surgical trauma or fine-needle biopsy triggers EMT

Of concern in current medical practice, there is a tendency for cancers generally, and breast cancer specifically, to be triggered by any kind of tissue disruption – including fine-needle biopsy – to undergo EMT with consequent cancer cell shedding to circulation. Such hematogenous tumor cell dissemination could be the origin of later overt metastases.

Breast cancers in mice release a flood of CTCs after simple fine-needle biopsy. Clinical needle biopsy of breast cancer triggers recruitment of inflammatory cells to the biopsy site and causes increased tumor cell mitoses in the biopsied area. In a second murine breast cancer study, both fine-needle biopsy and surgical resection resulted in the release of a flood of CTCs, but noteworthy in this work is that biopsy triggered by any kind of tissue disruption – including fine-needle biopsy – to undergo EMT with consequent cancer cell shedding to circulation. Such hematogenous tumor cell dissemination could be the origin of later overt metastases.

Breast cancers in mice release a flood of CTCs after simple fine-needle biopsy. Clinical needle biopsy of breast cancer triggers recruitment of inflammatory cells to the biopsy site and causes increased tumor cell mitoses in the biopsied area. In a second murine breast cancer study, both fine-needle biopsy and surgical resection resulted in the release of a flood of CTCs, but noteworthy in this work is that biopsy resulted in greater and longer lasting appearance of circulating cancer cells than did surgical resection. These murine data were replicated by Kaigorodova et al who showed that simple fine-needle biopsy of human breast cancers releases a flood of breast cancer cells into the general circulation. These authors found that although some released CTCs had CSC markers and attributes and some did not, none of them had the particular EMT markers for which they were tested.

This worrisome situation in breast cancer is similar to data collected in other cancers. For example, treatment with radioactive needle insertion into prostate cancer results in significant hematological shedding of tumor cells post procedure. Standard transrectal ultrasound-guided prostate needle biopsy results in detectable prostate cancer cells in the circulation in half of patients. Oral squamous cell carcinoma biopsies result in 16% of patients having post-biopsy...
CTCs. Simple wide excision that does not disrupt the tumor tissue integrity did not result in postoperative CTCs, whereas incisional biopsy did.  

A study is therefore required comparing the long-term outcome potential difference between those having fine-needle biopsy versus those having initial wide lesion excision. If initial excision that leaves the suspicious mass intact does result in fewer later disseminated metastases, it might be worth the iatrogenic morbidity incurred by the consequent excision of some benign masses.

**Radiation triggers EMT**

Above we reviewed some evidence that chemotherapy and mechanical tissue disruption give rise to CTC and EMT in surviving cells. Below we review data showing radiation causes CTC and EMT as well. For a few specific examples, as in other cancers, breast cancers synthesize GM-CSF that then functions as a growth factor for them. Clinically used, radiation treatment not only kills breast cancer cells and prolongs survival in breast cancer but also triggers exposed residual cells that are not killed to undergo EMT, to start migrating, and to synthesize increased amounts of autocrine growth factor, GM-CSF. Radiation also increases IL-6, migration, and EMT markers in murine and human breast cancer cell lines. The subject of radiation-induced EMT and radiation-induced increase in CTCs was recently reviewed by Lee et al.

Clinically, finding greater post-EMT CTCs confers a worse prognosis with more aggressive disease course and greater metastatic proclivity in colon cancer and finding circulating clusters of vimentin-positive gastric cancer cells confers a worse prognosis as did finding circulating cancer cell clusters and vimentin-positive CTC in colon cancer. Surgery for epithelial ovarian cancer causes an increase in both EMT-positive and EMT-negative CTCs, but there is a disproportionate increase in EMT positive. The increase in EMT-positive CTCs was even stronger after platinum-based chemotherapy. As Kolbl et al point out, EMT precedes the release of CTCs but after entering circulation CTC can revert to epithelial or partial epithelial phenotype.

Based on all these evidences, it seems that inhibiting EMT is a worthwhile goal during breast cancer treatment and that current common cancer treatments have elements of cancer growth stimulation inherent to them, or as Niccolo Machiavelli (born 1469–died 1527) said in 1513:

> People should either be caressed or crushed. If you do them minor damage they will get their revenge; but if you cripple them there is nothing they can do. If you need to injure someone, do it in such a way that you do not have to fear their vengeance.

ABC7 regimen was crafted with that in mind.

**Drugs to inhibit EMT**

**Quetiapine: 384 Da, cyp3A4 to norquetiapine, 6-hour half-life**

The RANK, its ligand (RANKL), and the soluble decoy receptor OPG (or bone protector) are central elements in breast cancer’s establishment of metastases to bone. Early indications are that quetiapine inhibits the RANK/RANKL signaling system.

Several forms of pro-RANKL are expressed on osteoblasts. After proteolytic release, RANKL binds to RANK leading to osteoclast syncytium formation then osteoclasts’ resorption of bone. Osteopetrosis results when RANK/RANKL system is nonfunctional. RANK/RANKL also functions in guiding normal breast gland ontogeny. There occurs an ebb and flow of RANK expression in mammary duct epithelial cells during the menstrual cycle, the increase occurring in late luteal phase. RANK/RANKL function is essential to the luminal epithelial proliferation seen particularly where ducts branch. Higher levels of RANK/RANKL in human breast cancer biopsy tissue correlate with higher metastasis likelihood and shorter survival.

PR-negative cells are affected through RANKL-induced paracrine actions leading to proliferation of mammary epithelial PR-negative cells.

RANK/RANKL is a core physiologic signaling system allowing circulating breast cancer cells to metastasize to bone. RANK/RANKL is a principal part of the complex signaling giving rise to breast cancer’s propensity to metastasize to bone.

Since breast cancer commonly metastasizes to bone with consequent bone pain, pathological fractures, vertebral compressions, and hypercalcemia, this process is important to block. Breast cancer cells are continuously shed into circulation from the primary and metastatic sites. Why then do these CTCs choose to establish growth preferentially in bone? This is because these CTCs can establish growth-enhancing communication with bone cells, specifically osteoblasts, and they do so primarily via RANK/RANKL. Muscle, skin, liver, spleen, fat, and other sites of less common breast cancer metastasis cannot so reciprocally communicate.

Osteoblasts receiving RANKL signaling transform to syncytial osteoclasts that resorb bone and increase TGF-beta. TGF-beta is also stored in bone, then released with any bone...
dissolution. This creates room for the CTCs to grow and free TGF-beta signaling prompting them to do so, making the TGF-beta blocking drug pirfenidone, which is discussed in the following sections, an ideal partner drug for quetiapine during metastatic breast cancer treatment.

An initial dose can be 50 mg once at bedtime, uptitrating to a target dose of quetiapine 300 mg or more as tolerated, given once at bedtime. Tiredness for a few hours on awakening is common upon starting quetiapine. It then abates after a week or so but reappears after each dose increase. Some weight increase due to increased appetite can be expected. Otherwise, side effects are not common.

**Pirfenidone: 185 Da, cyplA2, 3-hour half-life**

Pirfenidone is a 185 Da drug approved and marketed to treat idiopathic pulmonary fibrosis. Mild-to-moderate, reversible, nausea, dyspepsia, and rash are side effects in about one-third of treated patients, but these often resolve with continued use. Approximately 2403 mg/day divided into three equal doses is a common pirfenidone dose in treating its marketed indication, idiopathic pulmonary fibrosis.

Pirfenidone blocks TGF-beta signaling. TGF-beta is a 25 kDa signaling protein proteolytically clipped from a larger precursor protein. Carboplatin induces elevation is a 25 kDa signaling protein proteolytically clipped from a larger precursor protein. Carboplatin induces elevation of TGF-beta and triggers EMT in NSCLC, as given in the “Chemotherapy triggers EMT” section, both effects blocked by coadministration with pirfenidone.

TGF-beta signaling is a major driver of EMT in cancer generally and in breast cancer EMT specifically. TGF-beta is a facilitating element of many cancers by promoting angiogenesis and differentiation, by immune suppression, by promoting loss of cell-to-cell contact, and particularly by promoting EMT. Pirfenidone inhibits TGF-beta-induced phosphorylation of SMAD3, p38, and AKT. TGF-beta provides a “get up and go” signal for breast cancer.

In a murine breast cancer model, TGF-beta exposure also enhances normal lung’s ability to better support establishment of breast cancer metastases. TGF-beta drives breast cancer’s EMT and various biochemical, morphological, and behavioral changes characteristic of EMT. The manifold paths by which TGF-beta signaling leads to or enhances EMT specifically in breast cancer were outlined by Tan et al, Chen et al, Nooshinfar et al, and Felipe Lima et al.

TGF-beta dependency for taking on typical mesenchymal morphology, increased motility, and increased vimentin expression after radiation exposure was shown in breast, colon, and lung adenocarcinoma cell lines.

Preclinical studies have shown activity in pirfenidone’s enhancing cisplatin cytotoxicity to NSCLCs. In addition, pirfenidone enhances radiation and sunitinib cytotoxicity in Lewis lung cancer cells and reduces desmoplasia in pancreatic cancer. Growth of human TNBC tissue (ER negative, PR negative, HER2 negative) xenografted to nude mice was inhibited more by pirfenidone and doxorubicin than by doxorubicin alone. In another murine breast cancer model, pirfenidone reduced intratumoral collagen and hyaluronan by TGF-beta inhibition with consequent improvement of doxorubicin efficacy.

Pirfenidone disrupts Hh signaling in parallel with TGF-beta inhibition, a worthwhile added benefit during breast cancer treatment.

The starting dose of pirfenidone is 267 mg three times a day. This is gradually increased at 14-day intervals as tolerated to 801 mg three times daily. Pirfenidone at 400 mg three times daily (1200 mg/day) used to treat potential progression of hepatitis C-related fibrosis reduced circulating TGF-beta and IL-6. Abdominal pain, rash, and nausea were seen in a half of treated patients, but these side effects tended to subside within a month or two and no patient dropped out due to them.

**Ribavirin: 244 Da, 6-day half-life for a single oral dose, up to 12 days after continuous use**

Since its introduction to clinical practice in the late 1970s, ribavirin had been used to treat various viral infections, later becoming central to a now-outmoded hepatitis C treatment. Ribavirin remains useful in treating human respiratory syncytial virus infections and selected other rarer virus infections such as those of the hantavirus group. Ribavirin is commonly used to treat hepatitis C-related fibrosis reduced circulating TGF-beta and IL-6. Ribavirin is currently being investigated in numerous clinical trials for its therapeutic activity in various cancers, particularly acute myeloid leukemia (NCT02109744, NCT02073838), head and neck cancer (NCT01268579), and notably for ABC7, metastatic breast cancer (NCT01056757).

Although ribavirin’s mechanisms of antiviral and anticancer action are uncertain and probably will vary between viruses, several potential mechanisms of action have been identified. One proposes that ribavirin enters the cell via a nucleoside transport mechanism, intermingling itself within the viral RNA, thus inhibiting/altering viral RNA synthesis. However, ribavirin, particularly when paired with interferon-alpha, activates anti-inflammatory responses in various other ways. Alternatively, due to the fact that ribavirin is structurally analogous to GTP, a purine nucleoside, ribavirin can be incorporated into the cell passively, thereafter competitively...
binding to, and inhibiting, RNA polymerase, and RNA synthesis as a whole; ribavirin often achieves this via blocking the IMPDH pathway, among other pathways such as the elf4E pathway. Ultimately, five major mechanisms of action have been proposed:94–96

1. Immunostimulation by upregulating cytokines to shift Th1/2 cell balance to Th1 dominance.
2. Inhibition of 24 kDa elf4E function, thereby inhibiting mRNA capping and translation initiation.
4. Direct inhibition of IMPDH with consequent depletion of intracellular GTP.
5. After triphosphorylation, ribavirin triphosphate is incorporated into replicating RNA viral RNA polymerases with consequent induction of viral mutagenesis.

How ribavirin acts vis-a-vis elf4E is as follows:

elf4E forms part of the multimeric cap-dependent mRNA translation initiation complex. Mammalian cap-dependent translation starts with that complex binding to an RNA methyl-nucleotide. elf4E has many positive and negative control points, two of which are 1) posttranslational phosphorylation and 2) 4E-BPs.97–99 There are several variants of 4E-BP protein, hereafter designated simply as 4E-BP. 4E-BP is in turn controlled by its phosphorylation status.

elf4E non-covalently bound to 4E-BP is inactive in translation initiation. Both currently recognized complexes of mTOR (mTORC1, loosely associated with growth and mTORC2, loosely associated with cell survival and apoptosis resistance) can phosphorylate 4E-BP.100 Unphosphorylated 4E-BP has non-covalent affinity to and prevents transcription initiation activity of elf4E. When phosphorylated, 4E-BP1 loses that affinity and separates from elf4E, thereby allowing elf4E to function in cap-dependent mRNA translation.97–99

MAP kinase interacting kinases (hereafter referred to as MNK) can also phosphorylate 4E-BP, releasing it from elf4E.99,102–105 A wide variety of internal and extracellular events converge on mTOR and/or MNK to enhance or inhibit their activity. Development of resistance to mTOR inhibitors such as everolimus is often caused by elf4E amplification or MNK upregulation.97,105

elf4E overexpression has been identified in 30% of human cancers generally,97,106–110 including in invasive breast cancer where the degree of elf4E, both gene and protein overexpression, has been positively correlated with occurrence, recurrence, and metastasis.111–118 elf4E protein expression was associated with shorter survival, higher tumor mitotic index, and higher-grade breast cancer.115 Increased phosphorylated 4E-BP confers a worse prognosis and faster disease progression in breast, ovary, and prostate cancers.103

A crucially important oddity of elf4E in breast cancer is the homogenous spatial uniformity of phosphorylated elf4E protein overexpression in breast cancer tissues, both metastatic and primary.116 This is particularly notable given the spatial heterogeneity of ER, PR, HER2, mTOR, and other commonly overexpressed markers in breast cancer.

In addition, in 200 patients with Stage 4 breast cancer, immunohistochemistry analysis revealed that greater increase in elf4E phosphorylation in response to chemotherapy with doxorubicin, cyclophosphamide, or FU was correlated with shorter median overall survival,114 4.7 years in patients with a two- to fourfold increase in elf4E phosphorylation versus 3.1 years in patients with a 9–11-fold increase. A second study a few years later found similar results.118 Among patients undergoing primary debulking for a node-positive breast cancer when nodes were positive, after 4-year follow-up, systemic recurrence occurred in 22% of women with low elf4E protein expression, 27% of the intermediate group, and in 49% expressing large amounts of elf4E.119 Even more serious was the presence of multiple distant metastases in 60% of women whose primary expressed large amounts of elf4E but in 15% of women whose primary expressed low amounts of elf4E, again after 4-year follow-up.119

In an unusually exciting and instructive study, Li et al120 studied breast cancer biopsy tissue by immunohistochemistry both before and after chemotherapy. After cytotoxic chemotherapy with doxorubicin, or cyclophosphamide or 5-FU, the expression of phosphorylated elf4E increased in the posttreatment biopsy sample, as given in the “Chemotherapy triggers EMT” section, and chemotherapy-activated Wnt/beta-catenin, as given in the “Rifabutin: 847 Da, 2-day half-life” section, signaling in a phosphorylated elf4E-dependent manner.120

Although the significance of elf4E phosphorylation or its range of functions is not fully understood, some aspects are predominantly the empirical data in the abovementioned paragraph. Regulation of elf4E function is partly achieved through this phosphorylation process. Untreated GBMs show an excess of phosphorylated (unbound) 4E-BP.18,102 Inhibition of 4E-BP phosphorylation with consequent retention of its association with 4E-BP leads to inhibition of protein synthesis, inhibition of glioma cell proliferation in vitro, and tumor growth in vivo, in an orthotopic GBM mouse model.18,102 We know ribavirin gets good brain tissue levels based on the psychiatric morbidities associated with its use in treating hepatitis C. Volpin et al121 suggested using ribavirin to treat GBM based on these considerations.
That metformin inhibits 4E-BP1 phosphorylation via mTOR inhibition makes metformin a good coordinated partner drug to ribavirin. That ribavirin also inhibits MNK and since MNK phosphorylation of eIF4E is an alternate eIF4E activation pathway particularly used during the development of resistance to the mTOR inhibitor everolimus, ribavirin might be combined with everolimus or metformin to advantage. This would be a good example of the phenomenon mentioned in the “Introduction” section that when one growth pathway is pharmacologically blocked other parallel growth-driving pathways can become active, taking the place of blocked paths. mTOR phosphorylates 4E-BP1, or if mTOR is inhibited then MNK can take over, phosphorylating 4E-BP1. This would also explain why and how mTOR inhibitors have not been successful in treating some tumors such as GBM even though they express an overabundance of mTOR. MNK simply takes over when mTOR is blocked.

TGF-beta promotion of EMT that occurs largely through phosphorylation of eIF4E by MNK (with multiple intermediates between the two) makes pirfenidone a good partner drug for both metformin and ribavirin. In addition, experimental MNK inhibitors decrease eIF4E phosphorylation levels in breast cancers, and GBM, where MNK inhibition enhanced temozolomide cytotoxicity. In parallel fashion, in 103 cases of astrocytomas, high expression of phosphorylated eIF4E was significantly correlated with shorter overall survival rates.

All treated breast cancers were found to overexpress phosphorylated (activated) eIF4E, a remarkable and unique finding in any cancer. Decreased eIF4E phosphorylation in breast cancer also resulted in increased E-cadherin and beta-catenin protein levels reflecting a shift from mesenchymal toward epithelial attributes. The abovementioned combined data suggest that ribavirin could be of potential benefit by inhibiting eIF4E in breast cancer. Kentsis et al have demonstrated that ribavirin inhibits mG mRNA cap binding to eIF4E. Ribavirin directly bound to eIF4E with a micromolar affinity at the functional site used by mG mRNA cap, reducing eIF4E/mRNA binding and disrupting the translation process. Of note, not all mRNA translation is eIF4E dependent, but important mRNAs in breast cancer are, for instance, the one coding cyclin D1/3, c-Myc, VEGF, FGFR2/4, and MCL-1. Some preclinical studies in several murine models of breast cancer revealed that ribavirin inhibits breast cancer cell proliferation through eIF4E blockage. Moreover, in these studies, multiple-aspect characteristics of EMT were reversed or diminished by ribavirin.

More recently, two studies demonstrated significant glioma cell killing by ribavirin, confirming a 2014 study showing that ribavirin induced G0/G1 arrest in seven glioma cell lines at a median 55 μM IC50 (range 28–664). This latter study positively correlated mRNA expression of PDGF receptor-alpha, a major driver of GBM growth, with better glioma cell sensitivity (lower IC50) to ribavirin. That PDGF receptor is also a major driver of breast cancer and can cross over for the ER forming one of the many escape paths from aromatase inhibitor suppression of breast cancer growth. This fact favors the possibility of this path contributing to ribavirin’s inhibitory effect in breast cancer as well.

Similar to the abovementioned data on breast cancer, targeting eIF4E using ribavirin to block migration and EMT in NSCLC has been highlighted. In this study, inhibition of eIF4E after ribavirin treatment led to decreased migration, differentiation, and expression of several EMT-related genes such as ESA, SMAD5, NF-κB, cyclin D1, c-MYC, or HIF-1α. As we expect to do but using ribavirin, an engineered short hairpin RNA interfering with eIF4E transcription inhibited breast cancer cell migration, primary tumor growth, and metastasis establishment.

TGF-beta-induced eIF4E phosphorylation enhanced metastases, invasion, and EMT in a mouse breast cancer model, all of which were inhibited when an un-phosphorylatable eIF4E was present. IMPDH is a pivotal enzyme for biosynthesis of GTP and is frequently increased in tumor cells. It has been shown that ribavirin via IMPDH inhibition was effective against chronic lymphocytic leukemia cells. Recently, Isakovic et al demonstrated in glioma cells that ribavirin inhibits IMPDH activity and induces autophagy inhibiting the activity of mTORC1 and the SRC/AKT pathway.

Of deep significance for understanding breast cancer growth and the ABC7 regimen to inhibit it, is the study by Decarlo et al, where they demonstrated a feed-forward amplification loop between TGF-beta and eIF4E (that we intend to block with pirfenidone and ribavirin, respectively). In addition, TGF-beta agonism drives eIF4E activation confirming pirfenidone as a good partner drug for ribavirin.

Ribavirin has also been shown to inhibit mTOR/eIF4E signaling increasing paclitaxel and imatinib activity in squamous cell carcinoma and leukemic cells, respectively.

Discussion here of ribavirin strikes at the heart of why pharmaceutical mTOR inhibitors such as everolimus have not been as clinically useful as the biochemistry of cancer indicates it should be. The data in this section paint a consistent picture of eIF4E as a central element in breast cancer malignancy degree and as such a worthwhile target to inhibit. Ribavirin can be expected to do this effectively but it will be the most difficult of the ABC7 drugs to tolerate. Ribavirin is
the problematic drug of ABC7. When used over months to treat hepatitis C, 1000 mg/day would have been a common dose. Depressed mood, anemia, weight loss, and a severe but ill-defined malaise were common side effects and not rarely required dose reduction or even stopping ribavirin entirely. Given ribavirin’s propensity to give unpleasant side effects, it should be increased with caution from starting dose of 100 mg once daily with frequent mood and CBC evaluations.

Rifabutin: 847 Da, 2-day half-life
Rifabutin is an old antibiotic closely related to the even older drug rifampin (same as rifampicin). Rifabutin is active against Mycobacterium tuberculosis, atypical mycobacteria, staphylococci, group A streptococci, Neisseria gonorrhoeae, Neisseria meningitidis, Haemophilus influenzae, Haemophilus ducreyi, Campylobacter spp., Helicobacter pylori, chlamydia, and Toxoplasma gondii.

In 2016, rifabutin was reported to have blunted the growth of a patient’s NSCLC, subsequently studied in vitro and found to be active in inhibiting lung cancer cell growth and suppressing Ki67 staining. Rifabutin suppressed eIF4E phosphorylation with consequent decreased beta-catenin phosphorylation and increased beta-catenin destruction consequent to that. Thus, rifabutin could coordinate to advantage with ribavirin to thoroughly block eIF4E.

Erlotinib inhibits epidermal growth factor receptors (HER1, EGFR) and is effective initially in stopping some lung cancers’ growth. As resistance to erlotinib develops, EGFR mutations resulting in EGFR affinity to beta-catenin, thereby shifting growth drive to beta-catenin system. The result is a BCL-6-mediated anti-cytotoxicity benefit on multiple accounts during breast cancer treatment. Likewise, Ben Sahra et al demonstrated that metformin cytotoxicity to androgen-sensitive human prostate adenocarcinoma cells was AMPK independent but mTOR inactivation dependent. Furthering complicating delineation of metformin’s mechanism of action in treating cancer, Gui et al showed that metformin’s anticancer effect

Metformin: 129 Da, not metabolized, 6-hour half-life
Metformin is the most prescribed initial drug treatment for type 2 diabetes worldwide. Despite 60 years of use, the mechanism of action in lowering average glucose is not entirely clear. Hepatic gluconeogenesis is decreased by metformin and insulin sensitivity is increased but how that occurs is uncertain. Metformin in vivo and in vitro increases AMPK, a major regulator of energy homeostasis, metabolism, and protein synthesis. Thus, activated AMPK results in inhibition of mTOR. Breast cancer cell expression of beta-catenin was decreased by metformin concomitantly and proportionately to AMPK phosphorylation.

Decreased insulin/insulin-like growth factor-I signaling and inhibition of mitochondrial electron transport chain complex are other documented actions of metformin. Across many cancers, a large chart review has shown decreased mortality in patients treated with metformin. Experimental data support the notions that increased lactate secretion, reduced oxygen consumption, and activated AMPK signaling are plausible mechanisms for metformin’s anticancer effects. Metformin also decreased breast cancer cells’ intracellular adenosine triphosphate, viability, and anti-apoptotic protein expression of others’ in cancers generally and in breast cancer specifically. The result is a BCL-6-mediated anti-apoptosis effect. Breast cancer cells’ survival is enhanced by BCL6. Rifabutin binds to BCL6, preventing its function in translation inhibition. This would be expected to be of benefit on multiple accounts during breast cancer treatment. Interestingly, miR-544 inhibition of BCL6 in TNBC cells inhibited proliferation, migration, and invasion in vitro. BCL6 promoted invasion, migration, and EMT marker expression in breast cancer with indication that greater expression of BCL-6 correlates with shorter overall survival in breast cancer.

Not all malignant cells within a strongly ER+ breast cancer will express ERs. The minority population not expressing ERs is relatively chemotherapy resistant with some of that extra chemotherapy resistance mediated by upregulated BCL-6 specifically in that subpopulation.
was by inhibiting mitochondrial regeneration of oxidized NAD+ regeneration and lowering aspartate levels.

Just in 2016, five extensive reviews appeared recounting evidence favoring the use of metformin as treatment adjunct in cancer generally.166–170

In a study particularly relevant to ABC7 regimen considering that capecitabine metabolizes into 5-FU within cancer cells, Qu et al171 showed that breast cancer cells that had become resistant to 5-FU regained cytotoxic sensitivity to 5-FU by simultaneous exposure with metformin. Metformin synergy with 5-FU could also be demonstrated to breast cancer cells in both the stem and non-stem subpopulations.172

Of central importance to the ABC7 regimen, IC50 of 5-FU to esophageal cancer cells was lowered by metformin173 and correlated with increased AMPK activation and decreased mTOR function and lactate production. Metformin plus 5-FU combination was also active in slowing esophageal cancer growth in a xenotransplant model more than either agent alone.174

YAP is a small protein transcription factor promoting the growth of many cancers. When phosphorylated, it is retained in cytoplasm and therefore nonfunctional in promoting growth or inhibiting apoptosis. Metformin treatment of hepatocellular carcinoma patients increased YAP phosphorylation via AMPK phosphorylation and prolonged survival, half deceased at ~31 months without compared to ~44 months with metformin.175 Adding metformin to exemestane also increased survival in ER+ breast cancers that overexpressed IGF1R.176

An ongoing trial (ClinicalTrials.gov Identifier NCT01589367) is studying potential survival benefits of adding metformin 2000 mg/day to standard antiestrogen aromatase inhibitor, letrozole 2.5 mg/day, in nondiabetic postmenopausal women with ER+ breast cancer.

Preoperative treatment of breast cancer patients with metformin has given mixed results. Some studies showed reduced mitotic rate after metformin 2000 mg/day177 and 1500 mg/day,178 while others showed no reduction using 1500 mg/day.179 A similarly designed study using 1700 mg/day found marginally lower Ki67 only in women with increased insulin resistance.180

A pivotal study supporting metformin use during the treatment of breast cancer was reported back in 2011. In women undergoing primary resection for breast cancer, 1 g twice daily metformin was given 14 days prior to surgery.177 By immunohistochemistry, the diagnostic biopsy was compared to resected tissue for p-AMPK, p-AKT, insulin receptor, cleaved caspase-3, and Ki67. In metformin pretreated, increased p-AMPK and decreased p-AKT were seen compared to those not treated with metformin in the interval between biopsy and surgery. Ki67 and cleaved caspase-3 were diminished in metformin-treated women compared to those not so treated. These changes were not large but were statistically significant and large enough to expect some clinical benefit.177

Although metformin decreases breast cancer cell survival in vitro,160–162,181 the clinical benefit would seem small given the equivocal human trials and evidence that the small benefit seen tended to be restricted to diabetic/prediabetic people. However, small benefit is not no benefit.

Metformin despite being hydrophilic achieves approximately equal plasma and brain tissue levels. In rats, after single-dose oral metformin administration, 28 µmol/L plasma and 14 nmol/g brain tissue (14 µM) were seen.182 Average metformin plasma levels typically seen in asymptomatic diabetes patients were 2.7 ± 7.3 mg/L, ~3 µM. The unusually wide drug range seen, ±7.3 mg/L (±57 µM), reflects metformin’s safety.183 Metformin’s side effects are limited to diarrhea, nausea, and vomiting. Some cases of lactic acidosis could occur but at a low frequency and when metformin is implicated as the cause of lactic acidosis, metformin plasma levels greater than 5 µg/mL are generally found. Target dose of metformin is the standard dose used in past breast cancer studies of metformin ~ 1700–2000 mg/day.

**Propranolol: 259 Da, cyp 1A2, 2D6, 9-hour half-life**

Propranolol was the first beta-blocker introduced to clinical practice. Introduced in the 1960s, it is still in wide use to treat hypertension, migraine, angina, selected arrhythmias, essential tremor, resolution of infantile hematomas, and in reducing the cardiac effects (tachycardia) due to acute anxiety. Propranolol’s general cancer process inhibiting attributes were recently reviewed.184 Below are selected data supporting propranolol’s use specifically as adjunct in breast cancer treatment.

A study of 404 breast cancer patients to compare the proliferation rates of breast cancers in women who had taken beta-blockers compared to those who had not found a clear reduction in Ki67 only in those with Stage 1 disease.185 A single ER+, HER2− patient was treated with 25 days of propranolol 1.5 mg/kg per day after diagnostic biopsy but before resection. Resection of tumor tissue showed a 23% reduction in Ki67 staining compared to biopsy tissue 25 days earlier, before any propranolol.185 Of important note, beta-1-selective beta-blockers did not work to reduce Ki67, only
nonselective beta-blockers did. However, a large European epidemiological study found no survival benefit from propranolol use after a breast cancer diagnosis.186

Of particular interest to ABC7 regimen, Rico et al examined the effects of metformin and propranolol singly and combined in several preclinical TNBC models, finding additive to synergistic growth-inhibiting effects. In a cohort of 800 women with early TNBC, 9% used beta-blockers. The beta-blocker use and nonuse groups were well matched. At 5 years, 19% of the nonusers had died of breast cancer while 8% of beta-blocker users had died of breast cancer.188

In examining a cohort of 1971 multiple myeloma patients, those who took any beta-blocker, had a 24% disease-specific mortality at 5 years. Those who took a beta-blocker plus other cardiac drugs had 32% while those on no cardiac or blood pressure medicines had 41% myeloma-specific mortality at 5 years.189

An interesting study from Choy et al showed that among 1000 breast cancer patients those on a beta-blocker had a lower recurrence rate, and specifically TNBC expressed particularly high levels of beta-adrenergic receptors. Their brain metastases expressed more beta-adrenergic receptors per cell than did the primary tumors.190 This study also gave evidence of propranolol's inhibition of proliferation and migration in breast cancer cells expressing the beta-adrenergic receptor. In reviewing seven epidemiological studies prior to 2015 on beta-blocker use in breast cancer, Childers et al concluded that, although results were mixed between these studies, slightly lower risk of death was associated with beta-blocker use. Beta-blocker use is associated with improved relapse-free survival (but not in overall survival) also in patients with TNBC.192

Bone is richly supplied with sympathetic nerve endings. When specifically osteoblasts' beta-adrenergic receptor is stimulated by norepinephrine from these nerve endings, the osteoblasts secrete RANKL.193 Thus, propranolol should harmonize with quetiapine (vide supra) in treating and preventing bone metastases in breast cancer. CA125 is a high molecular weight mucin commonly elevated in ovarian cancer. Patients given perioperative propranolol showed an 83% CA125 decrease on postoperative day 7 when those given placebo had a 72% decrease.194

Although the data were mixed, a review of 10 studies completed by 2015 of beta-blocker use in breast cancer concluded that specifically propranolol use was indeed associated with slightly reduced breast cancer-specific mortality.195

Propranolol-blocked beta-adrenergic agonist induced increased migration and decreased breast cancer cell-to-cell adhesion.196 Propranolol inhibited breast cancer cell migration in vitro.197 Breast cancer cells express beta-adrenergic receptors. Blocking these with propranolol lowers their glucose uptake.198

Campbell et al demonstrated that beta-adrenergic stimulation of bone increased osteoblasts’ RANKL expression. That induced RANKL increased breast cancer establishment of metastases in bone.199 Thus, the combination with quetiapine might be particularly beneficial.

Beta-adrenergic stimulation did not change the growth of an orthotopic murine breast cancer but did induce a remarkable 30-fold increase in metastases, an effect partially blocked by propranolol.200 Of clinical importance to ABC7, Shaashua et al showed that combining propranolol with a COX-2 inhibitor in perioperative breast cancer decreased EMT, serum IL-6, and C-reactive protein levels.

There is risk of symptomatic iatrogenic hypotension with propranolol. The propranolol dose must therefore be slowly uptitrated as tolerated, monitoring blood pressure.

Capecitabine: 359 Da, half-life <1 hour

Capecitabine is a 359 Da pro-drug giving rise to intracellular release of 130 Da 5-FU.202,203 5-FU inhibits thymidylate synthase, which mediates the synthesis of thymidine monophosphate, the active form of thymidine required DNA synthesis. Despite ~20 years of clinical use in treating breast cancer, there remains some unclarity on the ideal dosing schedule for capecitabine.204–206 A comparison of cycles of 1000 mg/m² twice daily for 14 days, 7 days off with 1250 mg/m² twice daily for 14 days, and 7 days off indicated lower side effect burden with 1000 mg/m² twice daily.207,208

Several reports indicate that dosing capecitabine at just high enough level to generate palmar–plantar erythrodysesthesia might be most effective dosing regimen.209 This would be analogous to erlotinib dosing where titrating to rash might be most effective.210

Capecitabine is best given with Coke™ or fresh squeezed lemon juice to assure low enough gastric pH for adequate and uniform absorption. This would be particularly important for those on proton pump inhibitors.

Principle toxicity is palmar–plantar erythrodysesthesia (synonyms hand-foot syndrome or chemotherapy induced acral erythema), diarrhea, and nausea, although cytopenias, fatigue, dyspnea, or cardiac abnormalities can be seen.211 The common dose for capecitabine in breast cancer is 1250 mg/m² orally twice daily for 14 days, none for 7 days, every 21 days.
Agomelatine: 243 Da, 2-hour half-life

Agomelatine is a 243 Da pharmaceutical melatonergic agonist at both melatonin's receptors, M1 and M2. It has many advantages over the use of melatonin itself. In short, these advantages are: 1) agomelatine is Health Canada and EMA approved and marketed as an antidepressant. As such, it is a well-standardized product, as opposed to over-the-counter melatonin preparations which are exempt from the strict standards of approved medicines; 2) agomelatine has considerably tighter affinity to both M1 and M2 receptors than does the natural ligand (melatonin); 3) agomelatine has a much longer dwell time in the body than does melatonin, and; 4) absorption is more uniform and reliable than is absorption of melatonin.

Although agomelatine is available for import into the USA, it is not FDA approved. Ramelteon is an equally potent melatonergic agonist at M1 and M2 as is agomelatine. Ramelteon is FDA approved and marketed in the USA. It has similar actions and advantages over melatonin as does agomelatine and can be substituted for agomelatine in the ABC7 regimen.

Elevation of hepatic transaminases is of potential concern when using agomelatine. This requires regular monitoring. Elevation is dose dependent, occurring in ~3% of those receiving 50 mg once at bedtime. It is usually reversible.

Work pointing to diminished breast cancer cell malignant behavior during exposure to melatonin dates back at least 3 decades. There are numerous studies about oncostatic effects of melatonin on several tumors as well as recent reviews summarizing the different mechanisms of cancer inhibition by melatonin. These include regulation of estrogen pathway, melatonin as SERM and SEEM, modulation of the cell cycle, differentiation and the induction of apoptosis, inhibition of telomerase activity, inhibition of oxidative stress, inhibition of angiogenesis, regulation of circadian rhythms, avoidance of circadian disruption, inhibition of tumor metastasis, invasiveness and motility decline, and enhancement of immune system and epigenetic regulation.

Briefly and empirically, melatonin has readily demonstrable growth-inhibiting effects in both in vivo animal models, with chemically induced mammary tumors in rodents, and in vitro assays in estrogen-positive human breast cancer cells. Melatonin inhibits invasive and metastatic properties of human breast cancer cells in different xenograft models. Due to the broad spectrum of melatonin's actions, the mechanisms through which it interferes with metastases are varied. These include modulation of cell–cell and cell–matrix interaction, extracellular matrix remodeling by matrix metalloproteinases, cytoskeleton reorganization, EMT, and angiogenesis.

Melatonin shifts human breast cancer cells to a lower invasive status by upregulating E-cadherin and β1-integrin expression and decreasing OCT4, N-cadherin, and vimentin. These findings suggest that melatonin modulates both cell–cell and cell–matrix interactions in breast cancer and reduces the metastatic potential of the tumor. Melatonin also has regulatory actions on matrix metalloproteinases in breast cancer. It has been described that melatonin inhibits the induction, catalytic activity, and expression of MMP-9 and MMP-2. In addition to modulating the metalloproteinase activity, melatonin reduces cancer cell migration through the downregulation of ROCK-1 and MCLCK, two kinases that control the cytoskeletal rearrangement associated with cell–cell and cell–matrix adhesion.

The attenuation of HER2-Rsk2 signaling by melatonin plays a main role in the melatonin-mediated suppression of EMT and late-staged metastasis in breast cancer cells. In tumor angiogenesis, there is a crosstalk between cancer cells and surrounding endothelial cells. Melatonin interferes in the paracrine interactions between malignant epithelial cells and proximal endothelial cells through a downregulatory action on VEGF expression in human breast cancer cells, which decrease the levels of VEGF around endothelial cells. In addition, melatonin directly exerts antiangiogenic actions by reducing endothelial cell proliferation, invasion, migration, and tube formation, through a downregulation of VEGF expression. Melatonin also impedes the EMT process and cancer cell dissemination through downregulatory actions of the p38 pathway and interferences with NF-κB signaling in tumor cells.

Recently, a review of the effects of melatonin and chemotherapeutic agents in combination in cancer treatments has been published. Although the information available is limited, the results obtained suggest that melatonin sensitizes tumor cells to the cytotoxic effects of chemotherapeutic agents.

In addition, in a rat ER+ breast cancer model, melatonin reduced tumor weight, prolonged survival, and increased E-cadherin without giving apparent side effects. In this model, doxorubicin cytotoxicity to the breast cancers was augmented by giving simultaneous melatonin. Melatonin reduced in vitro migration and in vivo growth, proliferation index, and metastases in a murine xenograft model. Of particular relevance to ABC7, earlier in year 2017, melatonin was shown to increase 5-FU inhibition of colon cancer cell proliferation, in vitro colony formation, migration,
and invasion, showing a corresponding in vivo synergy with 5-FU in colon cancer tumor growth inhibition in a xenograft model.239

Similarly, melatonin moderately enhanced cytotoxicity to cisplatin and doxorubicin, while slightly but significantly enhancing 5-FU cytotoxicity to HeLa cells.240 In an in vitro study, in rat pancreatic adenocarcinoma, melatonin augmented cytotoxicity of 5-FU, cisplatin, and doxorubicin.241 Melatonin decreased pancreas cancers in hamsters given a carcinogen (N-nitrosobis-(2-oxopropyl) amine), as did capcitabine. Giving both melatonin and capcitabine decreased this incidence further.242 Melatonin augmented doxorubicin cytotoxicity to lymphocytic leukemia cells without having cytotoxicity to normal lymphocytes.243

Melatonin sensitized human breast cancer cells to radiation via 1) reduction in estrogen-synthesizing proteins, and 2) induction of a twofold change in p53 expression, and 3) downregulation of proteins involved in double-strand DNA break repair, such as RAD51 and DNA-PKcs.244 Melatonin enhanced cytotoxicity of 5-FU to esophageal squamous carcinoma cells both in vitro and in a xenograft model.245 These authors used 20 mg/kg per day melatonin in the xenograft model, corresponding to a nominal 1400 mg/day for a 70 kg adult human. The common over-the-counter melatonin used is 3–20 mg once at bedtime. The tighter affinity to melatonin receptors and much longer half-life of agomelatine compared to melatonin would go some way toward generating a stronger agonist signal to M1 and M2 than today’s commonly used melatonin doses. Another felicitous aspect of melatonergic agonism is a potential increase in NK cell numbers and function.246

A remarkable epidemiological study of cancer-free postmenopausal women showed that higher urinary melatonin levels were associated with a slightly reduced risk of later developing breast cancer,247 although these data are not uncontested. A review of all studies on urinary melatonin would indicate that this matter remains unsettled.248

The suggested dose of agomelatine is 50 mg once at bedtime, twice the EMA and Health Canada recommended dose for treating depression. If ramelteon is used instead 16 mg at bedtime, twice the FDA-approved dose is recommended.

**Conclusion**

Once breast cancer has metastasized to bone, liver, or lungs, the prognosis becomes poor. No current treatment has a reliable and robust disease control rate at that point.

Animal study of the complete ABC7 regimen would be advisable. Based on clinical experience with these drugs individually and in pairs in general medical practice, the predicted safety and tolerability of the ABC7 regimen should be safe. As a further safety measure, the ABC7 drugs should be added one at a time at weekly intervals, thereby catching any unwanted interactions early and the offending drug more easily identified.

In this article, we propose that seven common and already FDA-approved drugs, such as agomelatine (or ramelteon), metformin, pirfenidone, propranolol, quetiapine, ribavirin, and rifabutin, can have the ability to reduce EMT and breast cancer cell tumorigenesis. These ancillary drugs have demonstrated that attributes that we have reason to believe will inhibit EMT and enhance capcitabine’s efficacy. The predicted safety and tolerability of the ABC7 regimen is good. A clinical trial is warranted given the fatal outcome of metastatic breast cancer as things now stand.

**Abbreviations**

ABC7, Adjuvant for Breast Cancer treatment using seven repurposed drugs; AMPK, AMP-activated protein kinase; BCL-6, B-cell lymphoma-6; CBC, complete blood count; COMBAT regimen, combined oral metronomic biodifferentiating antiangiogenic treatment regimen; CSCs, cancer stem cells; CTCs, circulating tumor cells; CUSP9 regimen, coordinating antiangiogenic treatment regimen; CSCs, cancer stem cells; CTCs, circulating tumor cells; CUSP9 regimen, coordinating antiangiogenic treatment regimen; EGFR, epidermal growth factor receptor; EMA, European Medicines Agency; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; ER+, ER positive; 4E-BP, elf4E-binding protein; 4E-BP1, elf4E-binding protein-1; FDA, Food and Drug Administration; 5-FU, 5-fluorouracil; GBM, glioblastoma; GM-CSF, granulocyte–macrophage colony-stimulating factor; GTP, guanosine-5′-triphosphate; HER2, human epidermal growth factor receptor 2; Hh, hedgehog; HR−, hormone receptor negative; HR+, hormone receptor positive; HR−, hormone receptor negative; IGF1R, insulin-like growth factor type 1 receptor; IL-6, interleukin-6; IMPDH, inosine monophosphate dehydrogenase; MCLCK, myosin light-chain kinase; MEMMAT regimen, metronomic and targeted anti-angiogenesis therapy regimen; MET, mesenchymal-to-epithelial transition; MNN, MAP kinase-interacting kinase; mTOR, mammalian target of rapamycin; MTZ regimen, minocycline telmisartan and zoledronic acid regimen; m′G, 7-methylguanosine; NAD+, nicotinamide adenine dinucleotide; NCATS, National Center for Advancing Translational Sciences; NK, natural killer; NSCLC, non-small cell lung cancer; OPG, osteoprotegerin; PDGF, platelet-derived growth factor; PR, progesterone receptor; RANK, receptor activator of nuclear factor-kB; RANKL, RANK ligand; ROCK-1, rho-associated protein kinase; ROS, reactive oxygen species;
SEEM, selective estrogen enzyme modulator; SERM, selective estrogen receptor modulator; TGF-beta, transforming growth factor-beta; Th, T helper; TNBC, triple-negative breast cancer; ZO-1, zonula occludens-1.

Acknowledgments
SC was supported by grants from the Spanish Economy and Competitiveness Ministry (SAF2016-77103-P) and from Instituto de Investigación Sanitaria Valdecilla (IDIVAL) (APG/12). YS was supported by the Department of Defense (W81XWH-14-1-0403), the Wake Forest School of Medicine Internal Pilot Funding, and the Translational Research Academy which is supported by the NCATS, National Institutes of Health, through Grant Award Number UL1TR001420.

Disclosure
The authors report no conflicts of interest in this work.

References


121. Volpin F, Casaos J, Sesen J, et al. Use of an anti-viral drug, ribavirin, as
120. Li Z, Sun Y, Qu M, Wan H, Cai F, Zhang P. Inhibiting the MNK-eIF4E-
beta-catenin axis increases responsiveness of aggressive breast cancer
125. Zhang Y, Zheng XF. mTOR-independent 4E-BP1 phosphorylation
123. Pons B, Peg V, Vázquez-Sánchez MA, et al. The effect of p-4E-BP1
124. Pinto MP, Dye WW, Jacobsen BM, Horwitz KB. Malignant stroma
132. Criscitiello C, Gelao L, Viale G, Esposito A, Curigliano G. Investiga-
126. Liu Z, Sun Y, Wu M, Han H, Cai F, Zhang P. Inhibiting the MNK-eIF4E-
beta-catenin axis increases responsiveness of aggressive breast cancer
124. Pinto MP, Dye WW, Jacobsen BM, Horwitz KB. Malignant stroma
125. Zhang Y, Zheng XF. mTOR-independent 4E-BP1 phosphorylation
123. Pons B, Peg V, Vázquez-Sánchez MA, et al. The effect of p-4E-BP1


Yusuke Shiozawa, MD, PhD
Curriculum Vitae
NAME: Yusuke Shiozawa, M.D., Ph.D.

CURRENT ACADEMIC TITLE: Assistant Professor

ADDRESS: Department of Cancer Biology
Wake Forest School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27157-1010
(336) 716-8743 (office)
(336) 716-0255 (fax)
E-mail: yshiozaw@wakehealth.edu

EDUCATION:
1994-2000 Juntendo University School of Medicine
Tokyo, Japan
M.D.

2002-2006 Juntendo University School of Medicine
Department of Pediatrics
Tokyo, Japan
Ph.D.
Research Advisor: Yuichiro Yamashiro, M.D., Ph.D.
Nobutaka Kiyokawa, M.D., Ph.D.
Thesis: Human osteoblasts support hematopoietic cell development in vitro

2016-2017 Wake Forest School of Medicine
Clinical and Translational Science Institute
Translational Research Academy 2016 Cohort

POSTDOCTORAL TRAINING:
2000-2002 Resident, (Department of Pediatrics)
Juntendo University School of Medicine
Tokyo, Japan

2002-2006 Staff Doctor, (Pediatric Hematology/Oncology)
Juntendo University School of Medicine
Tokyo, Japan

2004-2006 Predoctoral Research Fellow,
National Research Institute for Child Health and Development,
Department of Pediatric Hematology and Oncology Research
Research Advisor: Nobutaka Kiyokawa, M.D., Ph.D.
Research Project: Human osteoblasts support hematopoietic cell development in vitro

2006-2010 Postdoctoral Research Fellow,
University of Michigan School of Dentistry,
Yusuke Shiozawa, M.D., Ph.D.
Department of Cancer Biology

Department of Periodontics & Oral Medicine.
Research Project: Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bone marrow

PROFESSIONAL LICENSURE:

2000-present Japanese Medical License

EMPLOYMENT:

Academic Appointments:

Wake Forest School of Medicine:

2015-Present Assistant Professor, Department of Cancer Biology

University of Michigan School of Dentistry:

2010-2014 Research Investigator, Department of Periodontics & Oral Medicine
2014-2015 Assistant Research Scientist, Department of Periodontics & Oral Medicine

OTHER PROFESSIONAL APPOINTMENTS AND INSTITUTIONAL SERVICE:

Institutional Committee Service:

Wake Forest School of Medicine:

2016, 2017 The Cancer Center Protocol Review Committee
2016-2019 The Radiation Safety Committee
2017 Application reviewer for Medical Student Research Program

University of Michigan School of Dentistry:

2014, 2015 Judge for Dental School Research day

Departmental Committee Service:

Wake Forest School of Medicine:

2015, 2016, 2017 Molecular and Cellular Bioscience PhD Track 4 Recruiting committee (Interview, Application Review)
2016, 2017 Molecular and Cellular Bioscience MD/PhD Track 4 Recruiting committee (Interview, Application Review)
**EXTRAMURAL APPOINTMENTS AND SERVICE:**

**Journal Reviewer:**

<table>
<thead>
<tr>
<th>Year Range</th>
<th>Journal Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008-Present</td>
<td>Journal of Gene Medicine</td>
</tr>
<tr>
<td>2008-Present</td>
<td>Biochemical Pharmacology (reviewed 16 times)</td>
</tr>
<tr>
<td>2010-Present</td>
<td>Journal of Bone and Mineral Research (reviewed 4 times)</td>
</tr>
<tr>
<td>2010-2016</td>
<td>Journal of Pediatric Biochemistry (reviewed 1 time)</td>
</tr>
<tr>
<td>2010-Present</td>
<td>Journal of Pediatric Epilepsy</td>
</tr>
<tr>
<td>2010-Present</td>
<td>Journal of Pediatric Neuroradiology</td>
</tr>
<tr>
<td>2010-Present</td>
<td>Journal of Pediatric Intensive Care</td>
</tr>
<tr>
<td>2011-Present</td>
<td>Journal of Dental Research (reviewed 1 time)</td>
</tr>
<tr>
<td>2012-Present</td>
<td>Asian Journal of Andrology (reviewed 1 time)</td>
</tr>
<tr>
<td>2012-Present</td>
<td>Clinical Cancer Research (reviewed 1 time)</td>
</tr>
<tr>
<td>2013-Present</td>
<td>Pediatric Oncall</td>
</tr>
<tr>
<td>2014-Present</td>
<td>The journal Minerva Pediatrica (reviewed 4 times)</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Oncotarget (reviewed 5 times)</td>
</tr>
<tr>
<td>2015-Present</td>
<td>PLOS ONE</td>
</tr>
<tr>
<td>2015-Present</td>
<td>Cancer Research Frontiers (reviewed 1 time)</td>
</tr>
<tr>
<td>2015-Present</td>
<td>International Journal of Urology</td>
</tr>
<tr>
<td>2017-Present</td>
<td>Journal of Child Science</td>
</tr>
</tbody>
</table>

**Ad-hoc Journal Reviewer:**

<table>
<thead>
<tr>
<th>Year</th>
<th>Journal Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>Blood</td>
</tr>
<tr>
<td>2009</td>
<td>American Journal of Pathology</td>
</tr>
<tr>
<td>2009</td>
<td>Journal of Clinical Investigation</td>
</tr>
<tr>
<td>2009</td>
<td>Acta Pediatrca</td>
</tr>
<tr>
<td>2011</td>
<td>International Medical Case Reports Journal</td>
</tr>
<tr>
<td>2012</td>
<td>Clinical &amp; Experimental Metastasis</td>
</tr>
<tr>
<td>2012</td>
<td>Current Psychiatry Reviews</td>
</tr>
<tr>
<td>2012</td>
<td>Molecular Cancer Therapeutics</td>
</tr>
<tr>
<td>2013</td>
<td>African Journal of Biotechnology</td>
</tr>
<tr>
<td>2013</td>
<td>Expert Opinion On Biological Therapy</td>
</tr>
<tr>
<td>2013</td>
<td>Hematology/Oncology and Stem Cell Therapy</td>
</tr>
<tr>
<td>2013</td>
<td>International Journal of Nutrition and Metabolism</td>
</tr>
<tr>
<td>2013</td>
<td>Letters in Drug Design &amp; Discovery</td>
</tr>
<tr>
<td>2013</td>
<td>Journal of Experimental &amp; Clinical Cancer Research</td>
</tr>
<tr>
<td>2013</td>
<td>Tissue Engineering</td>
</tr>
<tr>
<td>2013</td>
<td>Journal of Immunoassay and Immunochemistry</td>
</tr>
<tr>
<td>2013</td>
<td>Topics in Anticancer Research (BSP-Patent Book Series)</td>
</tr>
<tr>
<td>2014</td>
<td>Expert Opinion On Biological Therapy</td>
</tr>
<tr>
<td>2014</td>
<td>International Journal of Endocrinology</td>
</tr>
<tr>
<td>2014</td>
<td>BioMed Research International</td>
</tr>
<tr>
<td>2014</td>
<td>Oncotarget</td>
</tr>
<tr>
<td>2014</td>
<td>Current Pharmaceutical Design</td>
</tr>
<tr>
<td>Year</td>
<td>Journal/Conference/Review</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>2014</td>
<td>Stem Cells International</td>
</tr>
<tr>
<td>2014</td>
<td>International Journal of Nanomedicine</td>
</tr>
<tr>
<td>2014</td>
<td>Cancer Informatics</td>
</tr>
<tr>
<td>2015</td>
<td>Current Cancer Drug Targets (reviewed 2 times this year)</td>
</tr>
<tr>
<td>2015</td>
<td>Frontiers in Drug Design and Discovery (eBook Series)</td>
</tr>
<tr>
<td>2015</td>
<td>Evidence-Based Complementary and Alternative Medicine</td>
</tr>
<tr>
<td>2015</td>
<td>Medical Journals-Journal of Bone Marrow Research</td>
</tr>
<tr>
<td>2015</td>
<td>Expert Opinion On Drug Discovery</td>
</tr>
<tr>
<td>2015</td>
<td>BBA - Molecular Cell Research</td>
</tr>
<tr>
<td>2015</td>
<td>Medical Journals-Angiology (reviewed 2 times this year)</td>
</tr>
<tr>
<td>2015</td>
<td>Medical Journals-Integrative Oncology</td>
</tr>
<tr>
<td>2015</td>
<td>Cancers (reviewed 2 times this year)</td>
</tr>
<tr>
<td>2015</td>
<td>Calcified Tissue International and Musculoskeletal Research</td>
</tr>
<tr>
<td>2015</td>
<td>Cancer Biology &amp; Medicine</td>
</tr>
<tr>
<td>2015</td>
<td>Medical Journals-Palliative Care &amp; Medicine</td>
</tr>
<tr>
<td>2015</td>
<td>Jacobs Journal of Dentistry and Research</td>
</tr>
<tr>
<td>2015</td>
<td>Neoplasia</td>
</tr>
<tr>
<td>2015</td>
<td>Medical Journals-Journal of Pain Management &amp; Medicine</td>
</tr>
<tr>
<td>2015</td>
<td>Cancer Growth and Metastasis</td>
</tr>
<tr>
<td>2015</td>
<td>Medical Journals-Journal of Pain Management &amp; Medicine</td>
</tr>
<tr>
<td>2015</td>
<td>Gastroenterology Research and Practice</td>
</tr>
<tr>
<td>2015</td>
<td>Stem Cells and Cloning: Advances and Applications</td>
</tr>
<tr>
<td>2016</td>
<td>Medical Journals-Integrative Oncology</td>
</tr>
<tr>
<td>2016</td>
<td>Anesthesiology</td>
</tr>
<tr>
<td>2016</td>
<td>Medical Journals-Nuclear Medicine &amp; Radiation Therapy</td>
</tr>
<tr>
<td>2016</td>
<td>The Journal of Cellular Biochemistry</td>
</tr>
<tr>
<td>2016</td>
<td>Medical Journals-Nuclear Medicine &amp; Radiation Therapy</td>
</tr>
<tr>
<td>2016</td>
<td>Medical Journals-Journal of Prostate Cancer (reviewed 2 times this year)</td>
</tr>
<tr>
<td>2016</td>
<td>Stem Cell International (reviewed 2 times this year)</td>
</tr>
<tr>
<td>2016</td>
<td>International Journal of Molecular Sciences (reviewed 2 times this year)</td>
</tr>
<tr>
<td>2016</td>
<td>Cancer Growth and Metastasis</td>
</tr>
<tr>
<td>2016</td>
<td>Journal of Urology and Renal Diseases</td>
</tr>
<tr>
<td>2016</td>
<td>Journal of Spine &amp; Neurosurgery</td>
</tr>
<tr>
<td>2017</td>
<td>International Journal of Molecular Sciences (reviewed 3 times this year)</td>
</tr>
<tr>
<td>2017</td>
<td>Nature Communications</td>
</tr>
<tr>
<td>2017</td>
<td>Journal of Clinical &amp; Experimental oncology (reviewed 3 times this year)</td>
</tr>
<tr>
<td>2017</td>
<td>Marine Drugs</td>
</tr>
<tr>
<td>2017</td>
<td>Molecular Medicine Reports</td>
</tr>
<tr>
<td>2017</td>
<td>Urologic Oncology: Seminars and Original Investigations</td>
</tr>
<tr>
<td>2017</td>
<td>BBA - Molecular Cell Research</td>
</tr>
</tbody>
</table>

**Funding Agency Reviewer:**

The NY Stem Cell Research Program, Washington DC
(Ad hoc Reviewer, October 15-17, 2008)

Cancer Research UK Program
(Ad hoc Reviewer (remote, online), February, 2009)

The Fund for Scientific Research (F.R.S.-FNRS)
The Fund for Scientific Research (F.R.S.-FNRS)  
(Ad hoc Reviewer (remote, online), September, 2013)

The Fund for Scientific Research (F.R.S.-FNRS)  
(Ad hoc Reviewer (remote, online), September, 2014)

The Fund for Scientific Research (F.R.S.-FNRS)  
(Ad hoc Reviewer (remote, online), September, 2015)

Prostate Cancer Foundation, 2017 Young Investigator Award  
(Ad hoc Reviewer (remote, online), April, 2017)

Editorial Boards:

<table>
<thead>
<tr>
<th>Year</th>
<th>Journal Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011-Present</td>
<td>Journal of Clinical Medicine and Research</td>
</tr>
<tr>
<td>2011-Present</td>
<td>Case Reports in Hematology (edited 61 times)</td>
</tr>
<tr>
<td>2012-Present</td>
<td>International Journal of Experimental Dental Science</td>
</tr>
<tr>
<td>2012-Present</td>
<td>Case Reports in Pediatrics (edited 13 times)</td>
</tr>
<tr>
<td>2013-Present</td>
<td>JSM Dentistry</td>
</tr>
<tr>
<td>2013-Present</td>
<td>Genetic Disorders &amp; Gene Therapy</td>
</tr>
<tr>
<td>2013-Present</td>
<td>Cancer Studies</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Pharmacologia</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Austin Journal of Dentistry</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Journal of Stem Cell Research and Transplantation (reviewed 4 times)</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Journal of Hematology, Blood Transfusion and Disorders</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Journal of Stem Cells Research, Development and Therapy</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Journal of Clinical Studies and Medical Case Reports</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Journal of Cancer Science and Clinical Research</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Journal of Dentistry and Clinical Research</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Journal of Stem Cells Research, Reviews &amp; Reports</td>
</tr>
<tr>
<td>2014-Present</td>
<td>Sarcoma Research - International (reviewed 5 times)</td>
</tr>
<tr>
<td>2014-Present</td>
<td>International Journal of Hematology Research</td>
</tr>
<tr>
<td>2015-Present</td>
<td>Medical Oncology</td>
</tr>
<tr>
<td>2015-2016</td>
<td>Journal of Molecular Cytotherapy</td>
</tr>
<tr>
<td>2016-Present</td>
<td>Journal of Cancer Biology and Treatment (edited 2 times)</td>
</tr>
<tr>
<td>2016-Present</td>
<td>JSM Bone Marrow Research</td>
</tr>
</tbody>
</table>

Ad-hoc Journal Editor for Special Issue:

2014 BioMed Research International

Session Chair:

19th Annual Prostate Cancer Foundation Scientific Retreat
Session 1: Field Cancerization & the Tumor Microenvironment
October 25-27, 2012
San Diego, CA

PROFESSIONAL MEMBERSHIPS:

2008-Present American Association for Cancer Research

2008-2016 American Society of Hematology

2011-Present International Bone and Mineral Society
Yusuke Shiozawa, M.D., Ph.D.
Department of Cancer Biology

2015-Present
Metastasis Research Society

2017-Present
Cancer and Bone Society

2017-Present
Society for Basic Urologic Research

HONORS AND AWARDS:

2006
Pediatric Oncology Research Fellowship

2007
Best Paper Award from the Japan Cytometry Society

2009
Department of Defense Prostate Cancer Physician Research Training Award

2009
4th place in the 2009 University of Michigan Cancer Center Fall Research Symposium Poster Session (out of 78 posters)

2011
Prostate Cancer Foundation Young Investigator Award

2017
Accepted Participant of The U.S. Bone and Joint Initiative (USBJI) and Bone and Joint Canada (BJC) Young Investigator Initiative Program

INVITED PRESENTATIONS AND SEMINARS:


GRANTS:

Current:

W81XWH-14-1-0403, Idea Development Award (Shiozawa, PI, 25% effort) 03/01/2015-02/28/2018
Department of Defense $75,000/year direct cost

The roles of the bone marrow microenvironment in controlling tumor dormancy
The goal of this project is to provide significant insight into the mechanisms whereby the bone marrow microenvironment is involved in regulation of tumor dormancy. Aim 1 allows us to identify and explore how disseminated tumor cells (DTCs) stay dormant for long periods of time within the bone marrow microenvironment through the GAS6 pathway. Aim 2 will determine whether exogenous BMP2 triggers the activation of dormant DTCs through the bone marrow microenvironment. This work will inform greater understanding of microenvironmental aging’s effects on metastatic growth, and could result in valuable new treatment approaches.

Cancer Center, Tumor Progression and Recurrence Program Pilot Funding (Shiozawa, PI) 06/01/2017-05/31/2018
Wake Forest Baptist Comprehensive Cancer Center $10,000/year direct cost

The roles of renin-angiotensin system in cancer-induced bone pain
In this pilot project we will generate preliminary data for future external grant applications, which will determine the mechanisms of cancer-induced bone pain development.

Multi-Investigator Prostate Cancer Research Internal Pilot Funding (Shiozawa, PI) 06/01/2017-05/31/2018
Wake Forest Baptist Comprehensive Cancer Center $20,000/year direct cost

Development of theranostic strategies for prostate cancer bone metastases
In this pilot project we will develop a theranostic strategy that targets pain signaling in bone metastatic disease.

W81XWH-17-1-0541, Idea Development Award (Shiozawa, PI, 20% effort, Peters, Partnering PI) 09/01/2017-08/31/2020
Department of Defense $333,333/year direct cost

Molecular crosstalk: bone metastatic prostate cancer and nociceptive neurons
The proposed studies will focus on fundamental mechanisms behind the novel concept that nerves in the bone marrow microenvironment have a crucial role in prostate cancer bone metastasis. Aim 1 will provide the framework to identify the extent to which the interaction between disseminated tumor cells (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.

Cancer Center, Tumor Progression and Recurrence Program Push Pilot Funding (Shiozawa, PI) 09/01/2017-08/31/2018
Wake Forest Baptist Comprehensive Cancer Center $50,000/year direct cost

The roles of the stem cell factor/its receptor c-Kit axis in cancer-induced bone pain
In this pilot project we will generate preliminary data for future external grant applications, which will determine the mechanisms whereby the stem cell factor/its receptor c-Kit axis controls cancer-induced bone pain development.
Feasibility study for in vivo gene manipulation with adeno-associated viral vectors

In this pilot project, we will optimize and demonstrate the feasibility of the generation of nerve-specific c-Kit knockdown mice using adeno-associated virus (AAV) vectors in vivo. Our other studies will continue to investigate the influence of SCF on neurite outgrowth and neuropeptide expression in sensory nerves.

Pending:
None

PAST GRANT HISTORY:

Pediatric Oncology Research Fellowship
(Shiozawa, PI) 2006-2007 (¥4,000,000/year direct cost)

Department of Defense
Role and Function of the Chemokine CXCL16 and its Receptor CXCR6 in Prostate Cancer
(Shiozawa, PI) 01/01/2009-07/31/2011 ($60,000/year direct cost)

Prostate Cancer Foundation
Molecular dissection of the premetastatic niche in prostate cancer
(Shiozawa, PI) 01/01/2012-12/31/2014 ($75,000/year direct cost)

National Cancer Institution
1U54CA163124, Tumor Microenvironment Network U54
Mechanisms of Prostate Cancer Dormancy In The Bone Marrow Niche
(Taichman, PI: Shiozawa, Co-PI) 09/16/2011-07/31/2016 ($87,233/year direct cost to YS)

Wake Forest School of Medicine CTSI
Wake Forest School of Medicine Internal Pilot Funding
Molecular Crosstalk of Bone Metastatic Prostate Cancer and Nociceptive Neurons
(Shiozawa, PI) 07/01/2016-06/30/2017 ($10,000/year direct cost)

PATENTS:


The invention provides methods and compositions for the repair or regeneration of osteochondral tissue. The methods and compositions provide an effective amount of isolated differentiable human Very Small Embryonic Like Stem Cells (hVSELs) sufficient for regeneration or repair of an osteochondral tissue. The compositions can be administered directly to the tissue or administered systemically.
Yusuke Shiozawa, M.D., Ph.D.
Department of Cancer Biology

BIBLIOGRAPHY:

Peer-Reviewed Journal Articles:


- **Comment in**


- **Comment in**


Invited Reviews:


- The Figure 1 is chosen as the Cover Figure of *Int J Urol* Vol. 23 No. 10

Invited Book Chapters:


Miscellaneous:

Letters to the Editor

1. **Shiozawa Y**, Taichman RS. Erythropoietin and Bone Remodeling. [e-Letter], *Blood* (http://bloodjournal.hematologylibrary.org/content/117/21/5631.full/reply#bloodjournal_el_2584). Published July 25, 2011.
Editorial


Abstracts/Scientific exhibits/Presentations at national meetings:


27. Tsuzuki S, Eber MR, Miler SF, Park SH, Widner DB, **Shiozawa Y**. The effects of neuropeptides on the prostate cancer progression. IMPaCT Young Investigator Meeting, Baltimore, MD, USA, August 4-5, 2016. Poster.


29. 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.
GRADUATE STUDENTS/RESIDENTS/FELLOWS ADVISED:

Wake Forest School of Medicine:

Courses taught:
- Metastasis, Cancer Cell Biology (CABI 705), Lecture, (2015: 3h, 2016: 3h, 2017: 3h) (directed by Dr. William Gmeiner)
- Prostate Cancer: Oncogenesis and Progression, Carcinogenesis, DNA Damage and Repair (MCB721), Lecture, (2015: 1.5h, 2016: 1.5h, 2017: 1.5h) (directed by Dr. Hui-Wen Lo)
- Prostate Cancer Etiology and Treatment, Cancer Cell Biology (CABI 705), Lecture, (2015: 3h, 2016: 3h, 2017: 3h) (directed by Dr. William Gmeiner)
- Prostate cancer / Bone metastatic niche / Cancer dormancy, Advanced Topics in Cancer Biology (CABI 711), Facilitator, (2015: 1h, 2016: 1h, 2017: 1h) (directed by Dr. Ravi Singh (2015, 2016), and by Dr. Neveen Said (2017))
- Prostate cancer / Bone metastatic niche / Cancer dormancy, Advanced Topics in Cancer Biology (CABI 712), Facilitator, (2016: 1h, 2017: 1h) (directed by Dr. Ravi Singh)
- Prostate cancer diagnosis and treatment, North Carolina Summer Undergraduate Prostate Cancer Research Program, Lecture, (2016: 1h, 2017: 1h) (directed by Dr. Kounosuke Watabe)
- A small group discussion using the Problem-Based Learning (PBL) case-based method, Scientific Integrity (Grad 713), Facilitator, (2016: 8h) (directed by Dr. J. Charles Eldridge)
- A small group discussion using the Problem-Based Learning (PBL) case-based method, Scientific Integrity (Grad 714), Facilitator, (2017: 18h) (directed by Dr. J. Charles Eldridge)
- Bone metastasis and treatment, Molecular Pathogenesis of Cancer (MCB722), Lecture, (2017: 1.5h) (directed by Drs. Thaddeus J. Wadas and Bethany Kerr)
- Tumor Metastasis, Oncology Core Curriculum Lecture Series (CABI 707), Lecture, (2017: 1h) (directed by Dr. Susan Melin)

Student research:

Undergraduate Students: 2015-2017
Sloan Miler (Wake Forest University)  
Cancer Biology  
Research mentor (BIO 391)
- Undergraduate Student Stipend for Participation in International Conferences/Activities

2016-2016
Caroline Warren (Wake Forest University)  
Cancer Biology  
Research mentor

2016-2016
Mahder Mamo (North Carolina A&T State University)  
Cancer Biology  
Research mentor
- 2016 North Carolina Summer Undergraduate Prostate Cancer Research Program
<table>
<thead>
<tr>
<th>Year</th>
<th>Researcher</th>
<th>Institution</th>
<th>Program</th>
</tr>
</thead>
<tbody>
<tr>
<td>2016-2016</td>
<td>Taryn Strickland (Johnson C. Smith University)</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>The CRUISE program</strong></td>
</tr>
<tr>
<td>2016-</td>
<td>Sara Nakamura-Peek (Wake Forest University)</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td>2016-</td>
<td>L. Daniela Smith (Wake Forest University)</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td>2017-2017</td>
<td>Claudia Thornton (Wake Forest University)</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td>2017-2017</td>
<td>Aaron Sunil (Wake Forest University)</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td>2017-</td>
<td>Ethan Green (Wake Forest University)</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td>2017-2017</td>
<td>Selena Williams (North Carolina A&amp;T State University)</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>2017 North Carolina Summer Undergraduate Prostate Cancer Research Program</strong></td>
</tr>
<tr>
<td>2017-</td>
<td>Amy Medford (Wake Forest University)</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td>2017-</td>
<td>Molly Sohn (Wake Forest University)</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td>2017-</td>
<td>Sarah Sharp (Wake Forest University)</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td>Medical Students:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2017-2017</td>
<td>Mary Booker</td>
<td>Cancer Biology</td>
<td>Research mentor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>The 2017 Medical Student Research Program, provided by Laura Scales Cancer Fund</strong></td>
</tr>
<tr>
<td>Graduate Students:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2015-2016</td>
<td>Amanda Hyre</td>
<td>Cancer Biology</td>
<td>Research mentor (rotation)</td>
</tr>
</tbody>
</table>
2016-  D. Brooke Widner
        Cancer Biology
        Research mentor

Postdoctoral Fellows:
2015-  Shunsuke Tsuzuki
        Cancer Biology
        Research mentor

2016-  Sun Hee Park
        Cancer Biology
        Research mentor
University of Michigan School of Dentistry:

Courses taught:

- Bone marrow transplantation, Advanced Topics in Oral Pathology OP 711, Lecture, (2010: 1h, 2011: 1h, 2012: 1h, 2013: 1h) (directed by Dr. Theodora Danciu)

- Bone marrow niche and prostate cancer bone metastasis, Advanced Cellular and Molecular Biology course MCDB 440 Cell Cycle Control and Cancer, Guest Lecture, (2014: 2h) (directed by Dr. Laura Buttitta)

Student research:

High School Students:

2013-2013
Jacob Scherba
Periodontics and Oral Medicine
Lab supervisor

Undergraduate Students:

2006-2010
Elisabeth A. Pedersen
Periodontics and Oral Medicine
Lab supervisor

2007-2008
Michael J. Pienta
Periodontics and Oral Medicine
Lab supervisor

2010-2014
Samantha J. McGee
Periodontics and Oral Medicine
Lab supervisor

2012-2015
Matthew R. Eber
Periodontics and Oral Medicine
Lab supervisor
* 4th Prize Poster Award - 6th Annual Prostate Cancer Program Retreat (2013).

2013-2013
Felicia Scharf
Periodontics and Oral Medicine
Lab supervisor

2013-2015
Lulia Kana
Periodontics and Oral Medicine
Lab supervisor
* Third place award in Undergraduate, DDS, DH, MS/Certificate - Basic Science and Research - the University of Michigan School of Dentistry 2014 Research Day (2014).

2013-2013
Alexis N. Witkin
Periodontics and Oral Medicine
Lab supervisor

2013-2013
James Rhee
Periodontics and Oral Medicine
Lab supervisor
Visiting Undergraduate Students (from Brazil):
2012-2013 Eric Spinetti
   Periodontics and Oral Medicine
   Lab supervisor

Undergraduate/Dental Students:
2006-2010 Anne M. Zeigler
   Periodontics and Oral Medicine
   Lab supervisor
   - Grand Prize - ADA Dentsply Award in the University of

Undergraduate/Master Students:
2006-2013 Aaron M. Havens
   Periodontics and Oral Medicine
   Lab supervisor
   - Second prize - the Edward Hatton Competition of the
   American Associations for Dental Research (2013).

Dental hygiene/Dental Students:
2008-2015 Janet L. Zalucha
   Periodontics and Oral Medicine
   Lab supervisor
   - Audience Choice Award - the University of Michigan

Dental Students:
2010-2013 Alexandra Forest
   Periodontics and Oral Medicine
   Lab supervisor
   - Grand Prize - ADA Dentsply Award - the University of
   - Young Investigator Award - the University of Michigan
   1st symposium on Head & Neck cancer stem cells
   (2012).
   - Third place award in basic science research - 2012 the
   53rd annual American Dental Association/Dentsply
   Student Clinician Research Award Program (2013).

2013-2015 Baxter Jones
   Periodontics and Oral Medicine
   Lab supervisor

Master Students:
2009-2012 Lalit R. Patel
   Urology and Internal Medicine
   Lab supervisor

Graduate Students (rotation):
2014-2014 Ann Delker
   Periodontics and Oral Medicine
   Lab supervisor

Graduate Students:
2008-2011 Amy Hsiao
Biomedical Engineering
Lab supervisor

Postdoctoral Fellows:
2010-2012

Jeena Joseph
Periodontics and Oral Medicine
Lab supervisor

2010-2011

Anjali Mishra
Periodontics and Oral Medicine
Lab supervisor

2010-2011

Jinkoo Kim
Periodontics and Oral Medicine
Lab supervisor

2012-2012

Monika Verma
Periodontics and Oral Medicine
Lab supervisor

2012-2012

Biao Nie
Periodontics and Oral Medicine
Lab supervisor

2012-2015

Kenji Yumoto
Periodontics and Oral Medicine
Lab supervisor

2013-2014

Jacqueline Jones
Periodontics and Oral Medicine (Dr. McCauley’s lab)
Lab supervisor

• DOD Postdoctoral Training Award (2014).
• Assistant Professor at Troy University.

2014-2015

Frank Cackowski
Periodontics and Oral Medicine
Lab supervisor