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14. ABSTRACT

A major problem in prostate cancer is finding and eliminating the non-proliferating or "quiescent" cancer cells. This is because early in prostate cancer, a small number of cancer cells metastasize to other tissues such as the bone, where they can lay dormant for years. Most chemotherapies target actively dividing cancer cells causing primary tumor shrinkage, but leave behind quiescent cancer cells which may seed new, more aggressive and chemo-resistant cancers at a later date. During this second year of funding, we have discovered that PCa cells that metastasize to the bone exhibit dramatically different cell cycle characteristics from those in the liver, suggesting signals from the bone are key to regulating PCa cell cycle and dormancy. We therefore tested signals from the marrow environment and determined how they influence the proliferation vs. quiescence decision in PCa cells. During the no cost extension period we are continuing experiment to examine how these signals may modulate the effect of chemotherapies on PCa cell cycle regulation.

15. SUBJECT TERMS

tumor dormancy, prostate cancer cell quiescence, cancer recurrence

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Targeting Quiescence in Prostate Cancer

W81XWH-15-1-0413 PC140656

INTRODUCTION:

Prostate cancer (PCa) is characterized by the early spreading of a small number of tumor cells to other tissues, termed disseminated tumor cells (DTCs). DTCs in the bone are problematic because they may lie dormant for months or even years, yet a percentage of patients will later develop recurrent cancer with significant bone metastases from these cells, which often become resistant to treatment. Understanding how DTCs reside undetected in the marrow for long periods of time and finding ways to eliminate or minimize them, is an important issue in prostate cancer research and treatment.

Hypothesis: We hypothesize that dormant DTCs enter a state of cellular quiescence in the bone marrow, which renders them insensitive to chemotherapies designed to target actively proliferating cancer cells. Recent work has revealed that quiescence may encompass multiple "depths" that impact the speed and ability of dormant cancer cells to re-enter the cell cycle. Our goal is to examine whether dormant DTCs enter into a deep quiescent state known as G0 or arrest in a quiescent but "alert" and ready to re-enter state more similar to G1 in the bone marrow and test whether the disruption of signaling from the marrow that promotes DTC quiescence may reduce tumor burden and improve treatment outcomes by sensitizing quiescent cancer cells to chemotherapies.

Aims: To address this hypothesis we pursued two aims: Aim 1, we developed PCa cell lines expressing novel cell cycle reporters which will allow us to determine the cell cycle state of DTCs during dormancy in a xenograft prostate cancer model. In Aim 2, we address how signals from osteoblasts, osteoclasts or chemotherapy treatments alter DTC quiescence, to determine the impact on tumor dormancy, recurrence and response to current treatments.

Summary of results to date: We have successfully generated prostate cancer cell lines carrying fluorescent cell cycle sensors compatible with live imaging and flow cytometry, that together distinguish G0 and G1. We verified that these sensors accurately indicate the cell cycle state of the cells *in vitro* without disrupting their dynamics, and that these cell lines respond to signals from the bone marrow thought to promote dormancy by increasing cell cycle arrest. In our xenograft model, we have confirmed that these cell lines can form tumors and metastasize to the bone marrow. However, in the process of optimizing their recovery from the marrow, we discovered that over time in the mouse the reporters become silenced. In year 2 we therefore took an alternate strategy to incorporate a constitutive fluorescent nuclear marker to facilitate the recovery and imaging of these cells from the bone marrow. We also performed a proteomic screen to identify cell surface markers that can be used to distinguish quiescent cancer cells in G0 from cycling cells in G1. Despite this unexpected challenge, we

successfully performed *in vitro* and shorter-term *in vivo* experiments (<1 week) with our cell lines. These experiments revealed that DTCs in the bone marrow accumulate in the G1-phase of the cell cycle within 48h of injection, suggesting that entry into a quiescent but "alert" state may be a very early event for DTCs in the marrow. We have since extended this result to 1 week and find that while a percentage of cells still lose reporter expression, this G1 arrest is consistent. Using *in vitro* co-culture and single cell assays we have also been able to show that signals from osteoblasts such as Gas6 promote quiescence and entry into G0, consistent with our hypothesis that the bone marrow environment promotes quiescence of DTCs over time. We have now confirmed this *in vivo* as well. By contrast, signaling from the bone marrow via Transferrin or signaling from osteoclasts induces proliferation of PCa in the bone, a result which we have also been able to confirm *in vivo*. In year 1 we provided evidence that treatment with Docetaxel enhanced the effects of Gas6 in promoting PCa quiescence and an increased entry into arrests in G0 and G1. Critically we also showed that entry into cell cycle arrests in G0 or G1 phase could increase cell survival in the presence of Docetaxel, suggesting current chemotherapies enhance PCa quiescence and this cooperates to increase chemo-resistance and PCa survival during treatment. During the no- cost extension period, we will complete our current experiments that test *in vivo* in the mouse xenograft model whether chemotherapies enrich for cancer cell quiescence and survival in the bone. We will also test whether and how this impacts rates of tumor recurrence *in vivo*.

KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

GAS6	Growth arrest specific 6
HSC	Hematopoietic stem cells
HSC Niche	Hematopoietic Stem Cell Niche
PC3	Prostate cancer cell line
PCa	Prostate Cancer
Docetaxel	Chemotherapy agent
G0/quiescence	A reversible quiescent or non-cycling state
Transferrin	Iron transporting protein, high in blood and marrow
FACS	Fluorescence activated cell sorting
G1	Gap1 phase of the cell cycle
S-phase	DNA synthesis phase of the cell cycle
G2	Gap2 phase of the cell cycle
Proliferation	Actively replicating and dividing cells
Osteoblasts	Cells that secrete matrix for bone formation
Osteoclasts	Cells that remodel and resorb bone

ACCOMPLISHMENTS: **Major goals of the project**

STATEMENT OF WORK

START DATE: Sept 15, 2016

Revised SOW (Major tasks 2 and 3) approved Dec. 19 2017

Major Task 1: Generate prostate cancer cell lines stably expressing G0/G1 cell cycle reporters.	Months	status	**Summary of progress**
Subtask 1: Generate Lentivirus and transduce cells	1	completed	-
Subtask 2: Select stable cells lines	2	completed	-
Subtask 3: Verify reporters are correctly expressed	3	completed	-
Milestone(s) Achieved : PC-3 prostate cancer cell lines containing fluorescent cell cycle reporters		completed	PC3 labeled cell lines behave as expected in vitro. This was established in year 1
Major Task 2: Implant labeled cancer cells (or controls) into SCID mice to monitor dormancy			**Major Task 2: Implant labeled cancer cells (or controls) into SCID mice to monitor dormancy**
Subtask 1: Insert labeled cancer cells by i.c. injection or implants	1-4 months	Completed	Subtask 1: Insert labeled cancer cells by i.c. injection or implants
Subtask 2: Remove Collagen Implants if used	1		Subtask 2: Remove Collagen Implants if used
Subtask 3: Isolate tissues and test for metastasis to bone using QPCR of Alu Repeats* and detection of fluorescent labels (Timepoints throughout 1-7 months will be examined)	1-7 months	completed	Subtask 3: Isolate tissues and test for metastasis to bone using QPCR of Alu Repeats* and detection of fluorescent labels (Timepoints throughout 1-7 months will be examined)
Subtask 4: Isolate tissues and perform flow cytometry to identify cell cycle distributions of metastatic populations* (Timepoints throughout 1-7 months will be examined)	1-7 months	Early timepoints complete. Later timepoints in progress	Subtask 4: Isolate tissues and perform flow cytometry to identify cell cycle distributions of metastatic populations* (Timepoints throughout 1-7 months will be examined)
Subtask 5: Isolate tissues and perform immunofluorescence to identify cell cycle distributions of cancer cells in the Hematopoietic Stem cell niche* (Timepoints throughout 1-7 months will be examined)	1-7 months	In progress	Subtask 5: Isolate tissues and perform immunofluorescence to identify cell cycle distributions of cancer cells in the Hematopoietic Stem cell niche* (Timepoints throughout 1-7 months will be examined)
Subtask 6: Isolate tissues and test for metastasis to bone using QPCR of Alu Repeats and detection of fluorescent labels under chemotherapy treatments* (Timepoints throughout 1-7 months will be examined)	1-7 months	In progress	Subtask 6: Isolate tissues and test for metastasis to bone using QPCR of Alu Repeats and detection of fluorescent labels under chemotherapy treatments* (Timepoints throughout 1-7 months will be examined)
Subtask 7: Isolate tissues and perform flow cytometry and immunofluorescence	1-7 months	Optimization in progress	Subtask 7: Isolate tissues and perform flow cytometry and

to identify cell cycle distributions of metastatic populations under chemotherapy conditions* (Timepoints throughout 1-7 months will be examined)			immunofluorescence to identify cell cycle distributions of metastatic populations under chemotherapy conditions* (Timepoints throughout 1-7 months will be examined)
Milestone(s) Achieved: Measurement of PC3 cell cycle dynamics during dormancy with or without chemotherapy treatment		In progress	Milestone(s) Achieved: Measurement of PC3 cell cycle dynamics during dormancy with or without chemotherapy treatment
Major Task 3: Implant labeled cancer cells (or controls) into SCID mice to monitor tumor recurrence			**Major Task 3: Implant labeled cancer cells (or controls) into SCID mice to monitor tumor recurrence**
Subtask 1: Insert stably labeled cancer cells by i.c. injection or implants. Remove implants if used.	1mo	In progress	Subtask 1: Insert stably labeled cancer cells by i.c. injection or implants. Remove implants if used.
Subtask 2: Monitor tumor formation in PC3 controls labeled with fluorescent reporters under conditions with vs. without chemotherapy	7-9 months	In progress	Subtask 2: Monitor tumor formation in PC3 controls labeled with fluorescent reporters under conditions with vs. without chemotherapy
Subtask 3: Isolate recurrent cancers and perform flow cytometry to identify cell cycle distributions of recurrent tumor populations*	3-4 months	Not yet started	Subtask 3: Isolate recurrent cancers and perform flow cytometry to identify cell cycle distributions of recurrent tumor populations*
Subtask 4: Isolate recurrent tumors and perform immunofluorescence to identify cell cycle distributions of cancer cells *	3-4 months	Not yet started	Subtask 4: Isolate recurrent tumors and perform immunofluorescence to identify cell cycle distributions of cancer cells *
Milestone(s) Achieved: Measurement of PC3 cell cycle dynamics during tumor recurrence- with or without chemotherapy treatment		In progress	Milestone(s) Achieved: Measurement of PC3 cell cycle dynamics during tumor recurrence- with or without chemotherapy treatment

ACCOMPLISHMENTS & GOALS (detailed):

Major activities in year 1:

- Generate PC3 prostate cancer cell lines containing two sets of fluorescent cell cycle reporters
- Verify correct cell cycle reporter expression and cell cycle behavior of cell lines by 3 approaches - flow cytometry, live cell imaging and expression of cell cycle phase molecular markers.
- Examine the cell cycle response of cells *in vitro* to signals thought to promote tumor dormancy in bone
- Examine the cell cycle response of cells *in vitro* to chemotherapeutic agents
- Confirm that cell cycle indicator cell lines form tumors in vivo and metastasize to bone

Major activities in year 2:

- Encountered unexpected challenge that cell cycle reporters in PC3 cells implanted in mice become silenced during timecourse experiments longer than 1 month to model tumor dormancy and recurrence.

- Attempted an CRISPR/Cas9-based strategy to target reporters to endogenous gene loci in PC3 and C4-2B cells
- Determined that cell cycle indicator cell lines that metastasize to bone vs. liver in the short term (less than 1 week) exhibit dramatically different cell cycle characteristics, suggesting signals from the bone are key to regulating PCa cell cycle and dormancy.
- Discovered using single cell tracking assays (described in year 1) that PCa cells *in vitro* undergo asymmetric cell divisions where one daughter enters quiescence while the other re-enters the cell cycle.
- Determined that signals from the marrow environment (Gas6 and GM-CSF) influence the frequency of asymmetric cell divisions and the proliferation vs. quiescence decision in PCa cells providing a possible mechanism for how the bone marrow environment may promote PCa dormancy.
- Performed transcriptome analysis on mouse bone marrow cells with dormant PCa DTCs vs. recurrent PCa to identify secreted host marrow signals that may promote dormancy in PCa cells.

Major activities in year 3:

- Performed in vivo experiments up to 1 week using DiI pre-labeling of cancer cells carrying cell cycle reporters to facilitate recovery. (e.g. Fig 4)
- Followed up on our *in vitro* results on asymmetric division in PCa cells with an *in vivo* test. We found that similar to our *in vitro* study, treatment with GM-CSF increases the number of PCa cells in the bone and that these cells are poised to cycle in a G1 state. (e.g. Fig 4)
- Completed a proteomic screen to identify cell surface markers that can be used via FACS to distinguish quiescent (G0) cancer cells from cycling (G1) cells to facilitate cell cycle analysis of recovered cells. (e.g. Fig 1)
- Identified interactions with osteoclasts that promote PCa proliferation while interactions and signals from osteoblasts inhibit PCa proliferation and promote PCa survival. (e.g. Figs.2-7)

-- Use of PC3 cell lines carrying fluorescent cell cycle indicators *in vivo*:

We used gene delivery via lentivirus, to stably integrate two different combinations of cell cycle reporters in the PC3 prostate cancer cell line (described in detail in year 1 report). Together these reporters were designed to allow us to monitor cell cycle dynamics, including G0, G1, S and G2 phases. In year 1 we successfully generated and validated *in vitro* a cell line to distinguish G0 and G1 and a second, complimentary line to monitor G0/G1, S and G2-phases. In year 2 we discovered that when we performed in vivo xenograft metastasis assays with these cell lines, the cells we recovered from the bone marrow at timepoints beyond 1 week lacked detectable reporter expression (described in detail in year 2 report). To address this challenge, we performed a "pre-label" of the PCa cells with the lipophilic fluorescent dye DiD (in the far red spectrum to avoid overlap with our cell cycle reporters), and confirmed that indeed we could recover the pre-labeled cells from the mouse bone marrow at short timepoints. Importantly in year 2, we also established that these cells metastasize to the bone and in the longer-term, form tumors. We therefore proposed a CRISPR/Cas9-based strategy and in parallel pursued alternative strategies to identify additional molecular markers to verify the cell cycle status of PCa cells recovered from the bone marrow in our in vivo xenograft model of bone metastasis.

Alternative approach to identify molecular markers for quiescent PCa cells:

We undertook a proteomic screen to identify cell surface proteins that could validate the cell cycling status of PCa cells isolated from the bone marrow. To do this we cultured PC3 cells containing G0 and G1 reporters in conditions to promote proliferation or quiescence and sorted cells into cycling G1 and quiescent G0 populations. We then isolated membranes and performed protein preps followed by GC-MS/MS and comparative quantification to identify cell surface proteins that may serve as novel markers for quiescent vs proliferating PCa cells. In sum, 3,791 proteins were identified with 20 showing significant differences between G0 and G1 cells. Eight could be validated to be expressed on the PCa cell surface by FACS. Two (CD146 and CD340) could be confirmed to correlate with cell cycle status using double labeling with Ki67, an intracellular proliferation marker (**Fig. 1**). CD146 which is more abundant in cycling G1 cells, is also known as the melanoma cell adhesion molecule (MCAM). CD340, which is more abundant in quiescent G0 cells is also known as Receptor tyrosine-protein kinase erbB-2 or HER2. Over-expression of CD340 is strongly associated with increased disease recurrence and a poor prognosis in breast cancer. While high levels of HER2 are associated with MAPK

induced proliferation, it is unclear whether high levels of HER2 specifically retained on the cell surface may be associated with non-signaling receptor and quiescence. We are pursuing the use of these new markers to confirm our in vivo results with the xenograft model with and without chemotherapy during the no-cost extension period.

-- PCa cells *in vitro* undergo asymmetric cell divisions that can be influenced by osteoblasts, osteoclasts and secreted signals in the bone marrow

Using our cell tracking methodology and single-cell in vitro assays (described in detail in year 1 report), we discovered that PC3 cells exhibit 3 different patterns in the proliferation-quiescence decision after completing mitosis. Daughter cells from the same mitosis can make "asymmetric" decisions, where one enters the into the next cell cycle proceeding through G1 while the other enters into a prolonged quiescent G0. Alternatively both daughters can make a "symmetric" decision to enter the cell cycle proceeding through G1, or both can symmetrically enter into a quiescent state of G0. By monitoring these cell cycle decisions over time we have found that on average about 20% of PC3 cells under normal serum conditions perform asymmetric divisions where one daughter cell enters quiescent G0 (described in detail in year 2 report). This is of interest since asymmetric divisions and entry into G0 are hallmarks of stem cells and may be a feature of cancer stem cells. Consistent with this idea, we find that the majority of PC3 Venus-Cherry cells that are double positive for the cancer stem cell markers CD133 and CD44 are in a quiescent G0 state (described in detail in year 2 report). This suggested to us a possible mechanism by which secreted signals in the bone marrow environment could modulate dormancy vs. cell cycle entry and recurrence. We propose that secreted signals may influence the frequency of symmetric vs. asymmetric cell cycle decisions in daughter cells as well as impact the level of proliferation. During dormancy we expect signals to lead to alterations in the rate of symmetric decisions that lead to G0 entry or G1 arrest over time to promote dormancy. Then a change in the marrow signaling environment later could promote symmetric decisions to enter into the cell cycle and proceed through G1 without arrest driving tumor recurrence. Consistent with this hypothesis, we previously showed that a signal from osteoblasts, Gas6, which we showed previously in year 1 [4,5]can induce a G0/G1 arrest in prostate cancer cells, induces quiescence by promoting symmetric divisions where both daughter cells enter into quiescent G0. We further confirmed this with co-culture and recombinant Gas6 assays (**Fig. 2 A-C**). However, Gas6 is just one of multiple signals in the bone marrow environment that may impact cell cycle arrest and the complex signaling environment may lead to complex dynamics such as PCa transitions between G0 and G1. Indeed, when we assayed PCa cell cycle status when cultured with conditioned medium from osetoclasts (OCs) we found a decrease in cells in G0 consistent with osteoclasts promoting PCa proliferation (**Fig 2 D**).

To investigate further how osteoclasts may do this, we co-cultured PC3 cells containing our G0/G1 cell cycle reporters with GFP-labeled osteoclasts. Using our live imaging techniques (described in year 1 report) we observed a striking correlation between the transition of PC3 cells from G0 to G1 and contact with GFP-labeled osteoclasts via tunneling nanotubes (TNTs). In sum, we observed that after a G0 PC3 cells has made contact with an osteoclast, it enters into G1 (**Fig. 3A**). We went on to quantify this and we confirmed that these cells entered the cell cycle and proceed to S, G2 and M phases. Finally, we used the inhibitor of actin polymerization, Cytochalasin D to inhibit TNT formation and showed that this partially blocked the ability of osteoclasts to promote PC3 cell proliferation. (**Fig.3B**). In sum this work suggests that complex signals both secreted and dependent on cell-cell contact from osteoclasts and osteoblasts act to regulate PCa proliferation in the bone.

PC3 cells that metastasize to bone exhibit a cell cycle status consistent with a poised quiescent G1 or "G alert" state.

In year 2 we established that we could use our PC3 cells expressing cell cycle reporters to examine the short-term consequences of metastasis to bone vs. other sites. We found that the vast majority of cells in the bone marrow exhibited reporter activity indicating G1 phase, while cells isolated from the liver (where dormancy is rare) exhibited reporter activity indicating a mixture of G0, G1 and S/G2 and M-phases, consistent with asynchronous active proliferation (described in detail in year 2 report). Since nearly all of the PCa cells recovered from the bone marrow are in G1 (rather than progressing through multiple cell cycle phases), we suggest these cells most likely have become arrested in G1. The finding that this can occur within 48h of injection is striking and suggested signals from the bone environment strongly impact the cell cycle in PCa cells. In year 3 we have confirmed this is also the case for up to 1 week after metastasis (**Fig. 4**). Recent work has demonstrated that quiescent stem cells may exist in two states; the traditionally quiescent G0 or a state now

termed G alert which is more similar to early G1-phase where cell cycle genes are still highly expressed and cells are poised to enter the cell cycle [3]. Our new results suggest that DTCs in the bone marrow may be induced to enter a state similar to G alert with features of a poised G1 arrest rather than the strongly quiescent G0 state that we initially expected. This is a surprising finding and has altered our interpretation of how signals from the marrow impact the cell cycle in PCa.

Following on this *in vivo* result and the results with osteoclasts described in Fig 3, we investigated how modulation of osteoclast activity might affect the cell cycle status of PCa cells in the mouse bone. To do this we performed shorter term in vivo xenograft studied using luciferase labeled PC3 cells, to monitor cell number over time in the bone for weeks and our PC3 cells containing G0/G1 cell cycle reporters, to monitor cell cycle status when the cells are recovered from the marrow after 1 week. We found that promoting osteoclast activity with GM-CSF increased PCa cell metastasis to the bone and pushed PC3 cells toward cell cycle entry and G1 within 1 week, while inhibitors of osteoclast activity (Zoledronic Acid or Osteoprotegerin) effectively reduced PCa metastasis to the bone (**Fig 4**). This work demonstrates that we can effectively use our PCa cell lines carrying cycle reporters to monitor PCa cell cycle status in the bone during metastasis and cancer progression and demonstrates a potentially effective role for modulating osteoclast activity in reducing PCa cell proliferation in the bone.

Transferrin promotes PCa proliferation.

One of our major goals is to identify signals in the bone marrow that mediate proliferation vs. quiescence of PCa cells. Toward this goal in year 3 we identified the iron binding protein Transferrin (TF) as a potential modulator of PCa proliferation-quiescence decision in the bone marrow. Transferrin levels are high in the blood and can also be high in the bone marrow. We confirmed that the Transferrin receptors are expressed in PCa cells (**Fig.5**) and found their levels to be higher in PCa cells than in normal prostate epithelium. We next showed in cell culture that Transferrin stimulated proliferation of PCa cells, but not normal prostate epithelium (**Fig 5**). We next examined the cell cycle effects of TF exposure on the proliferation-quiescence decision in our PC3 cell cycle reporter cell line. To measure the effect that TF exposure might have on quiescent (G0) PC3 cells in the marrow, we isolated quiescent G0 PC3 cells by FACS and cultured them the presence of TF for 3 days. We observed that TF exposure promoted PC3 cells to transition from quiescence (G0) and into G1 and subsequent cell cycle stages consistent with promoting full proliferation (**Fig 6**). Importantly, using Luciferase-labeled PC3 cells in our mouse xenograft assay, we observed that PC3 cell metastasis to the bone increased TF levels in the bone marrow, while having no effect on serum levels (**Fig 7**). Altogether this data suggests that there may be a feedback relationship with PCa cells in the marrow and transferrin, such that low, dormant levels of PCa cells promote PCa dormancy by not stimulating TF expression, while high levels of proliferating PCa cells stimulate further PCa cell proliferation via a positive feedback relationship through transferrin signaling.

Significant results and key outcomes

Over the 3 years of funding we have encountered unexpected challenges, but also made important discoveries regarding cell cycle status of PCa cells in the bone and the proliferation-quiescence decision in those cells. We have uncovered signals from osteoclasts and osetoblasts that regulate quiescence vs cell cycle entry in PCa. We have also identified the secreted signal Transferrin which may play a role in amplifying the switch between doemancy and proliferation in PCa. We have shown that quiescence in PCa cells increases survival and promotes chemoresistance, and we will complete the in vivo studies on this during the no cost extension period. Finally, we have uncovered that PCa cells that metastasize to the bone exhibit dramatically different cell cycle characteristics than those that metastasize to the liver. Since the bone is a major site of PCa dormancy and recurrence, we hypothesize that signals from the bone marrow induce cell cycle changes to promote dormancy.

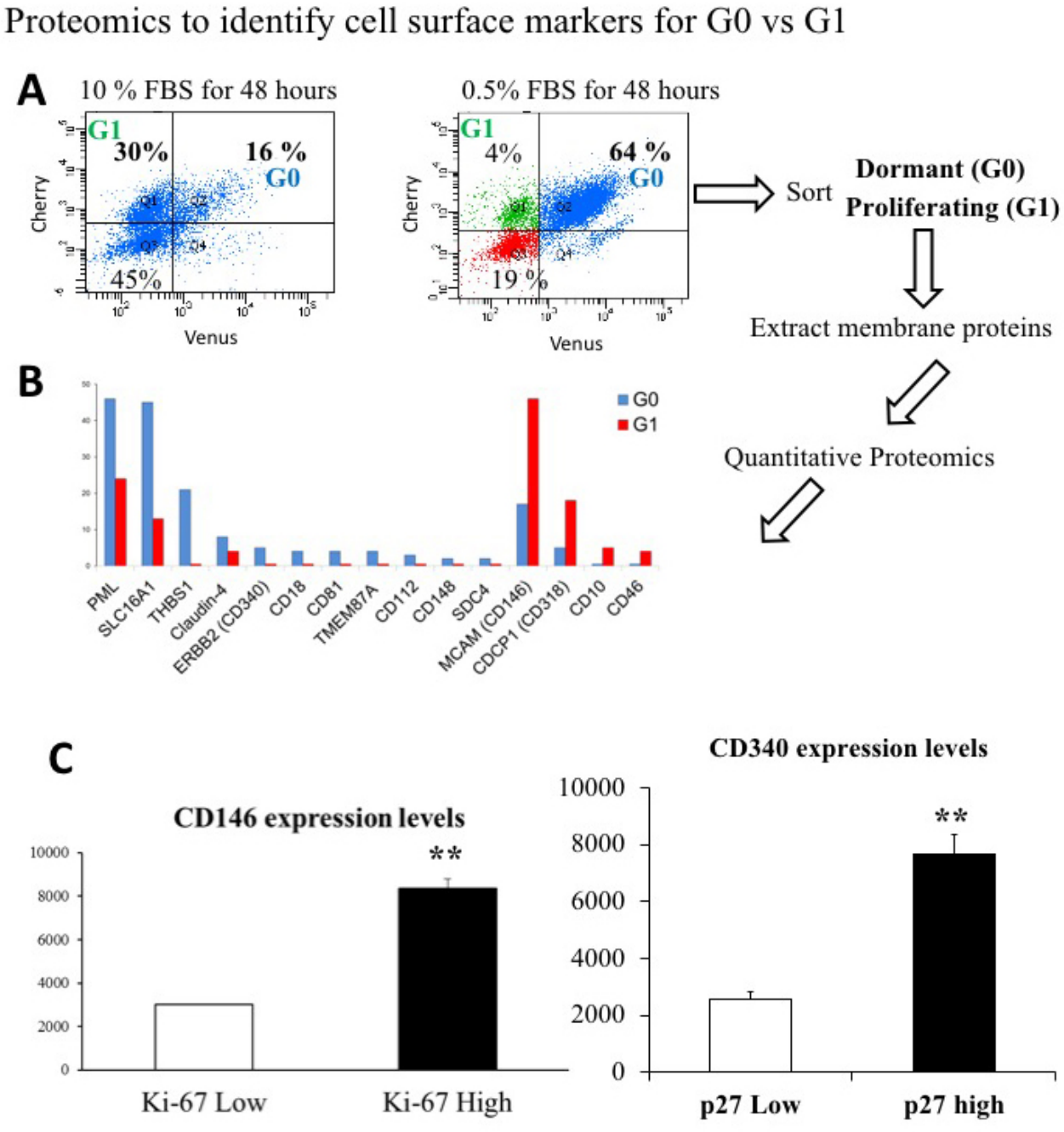


Fig.1. (A) Outline of proteomic approach to identify cell surface markers for PC3 cells carrying G0-Venus and G1-Cherry reporters to identify dormant (G0) vs proliferating (G1) molecular markers. (B) Cell surface proteins identified as significantly enriched in either G0 or G1 cells. (C) CD146 surface expression correlates with proliferation while CD340 cell surface expression correlates with cell cycle arrest when CDKN1B (p27) levels are high.

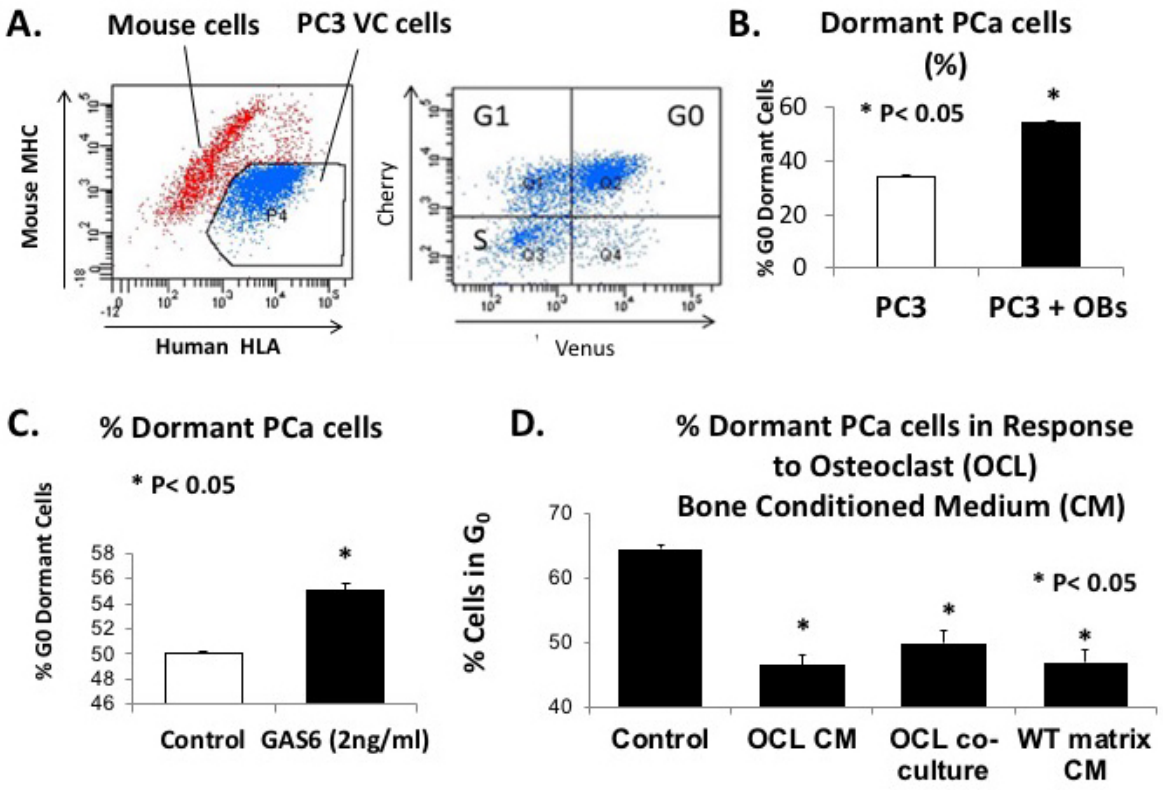


Fig.2. (A) PC3 cells carrying G0-Venus and G1-Cherry reporters (PC VC) were co-cultured with mouse osteoblasts (OBs) to identify osteoblast signals that impact proliferation vs quiescence of PCa. Mouse OBs can be eefectively separated from the human PC3 cells by FACS using anti-human HLA. All subsequent panels show data for the human cells only. (B) Co-culture with mouse OBs increases the percentage of PCa cells in G0. (C) The effect of co-culture with mouse OBs can be mimicked by recombinant Gas6. (D) Co-culture with Osteoclasts (OCL) reduces the fraction of PCa cells in G0.

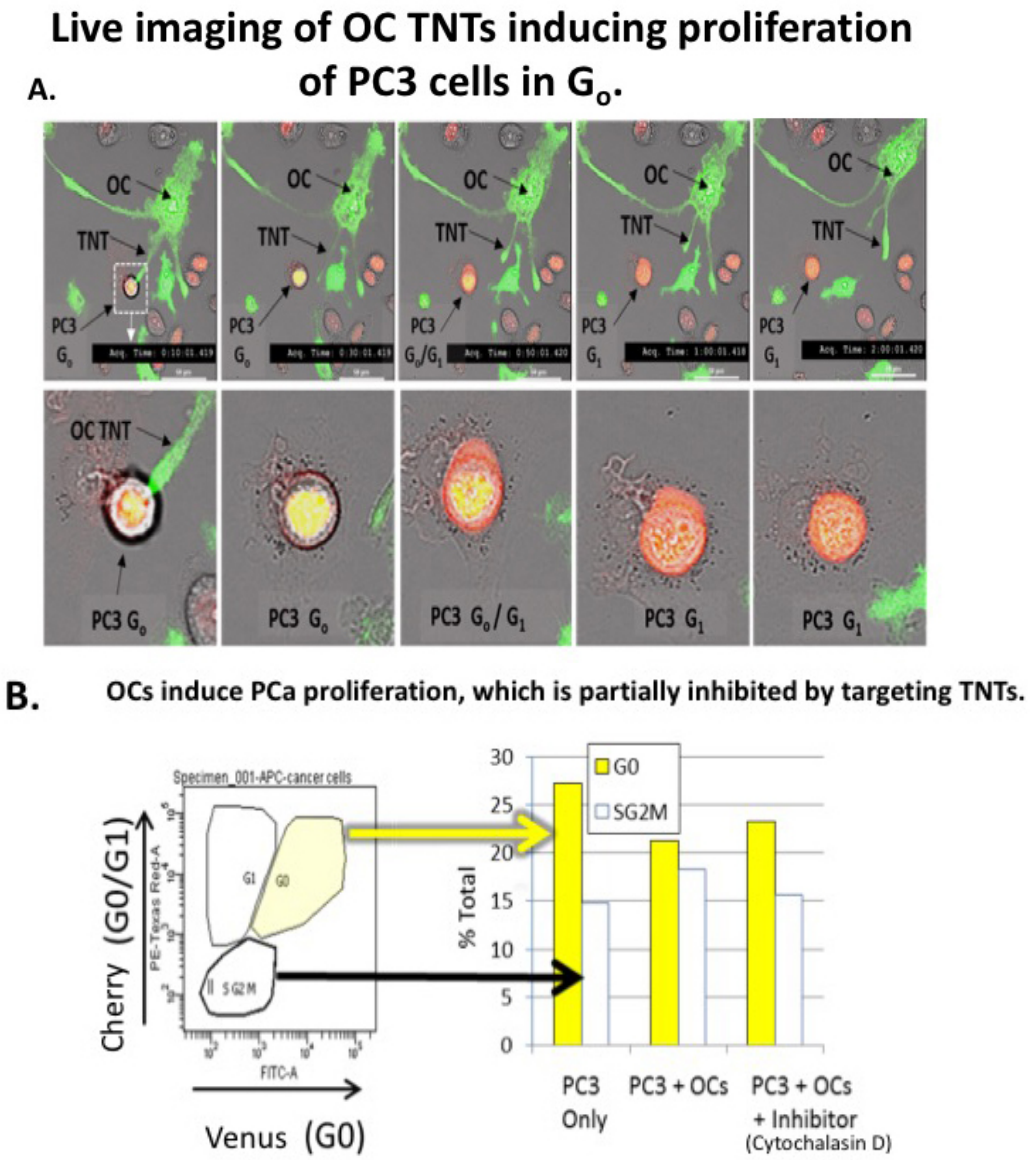
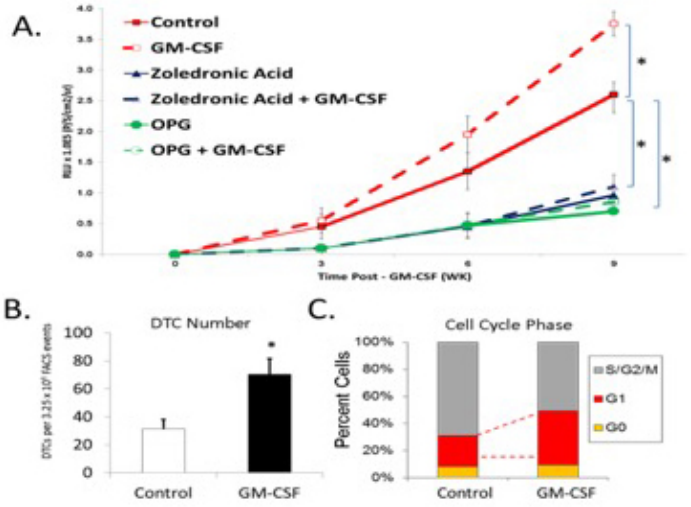


Fig.3. (A) Live imaging reveals that co-culture of PC3 VC cells with GFP-labeled Osteoclasts (OC) promotes PCa cell cycle entry (transition from yellow (G0) to red (G1)) after contact with tunneling nanotubes (TNTs). (B) Quantification via FACS reveals that co-culture with OCs promotes PCa exit from quiescence (reduced G0) and cell cycle entry (transition into G1, S and G2,M phases). This effect of OCs on PCa cells can be reduced when pre-treated with the TNT inhibitor Cytochalasin D.

Inhibition of Osteoclastic activities limits DTC proliferation and decreases time to metastasis.



(**A**) i.c. injection of PC3 cells in animals pretreated with the OC inhibitors zoledronic acid or osteoprotegerin (OPG) and/or OC activity induced by GM-CSF. (**B**). Repeat of A, using PC3 cells containing cell cycle reporters. DTCs recovered at 6 days. GM-CSF induces more DTCs and more cells in G1 than vehicle treated controls.

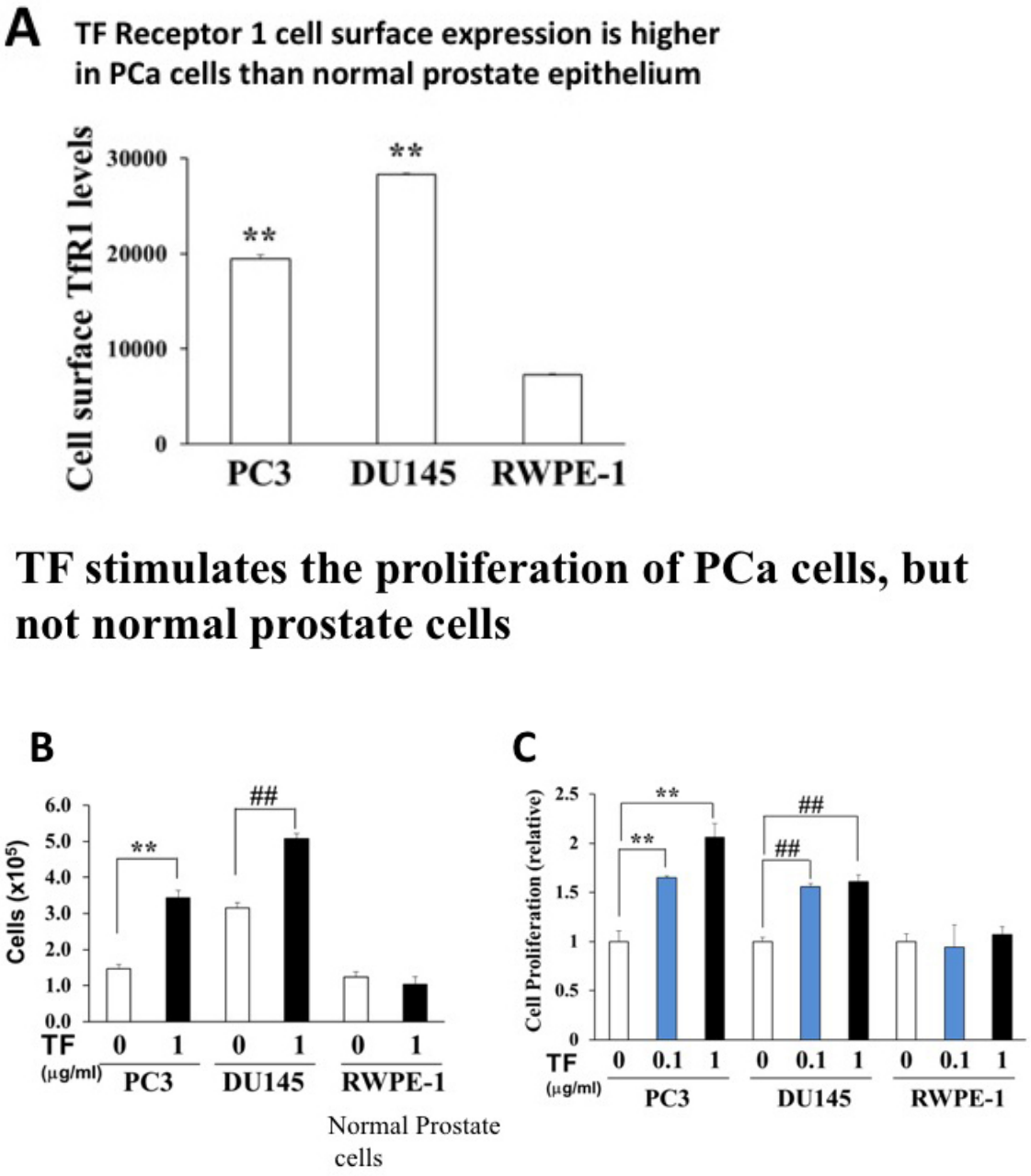


Fig.5. (A) Transferrin Receptor (TFR1) expression is higher in PCa cells than in normal prostate epithelium (RWPE-1). (B,C) Transferrin (TF) stimulates proliferation of PCa cells but not normal prostate epithelium (as measured by total cell number and relative proliferation rate).

TF stimulates PCa cells to leave G0 and enter G1 and S phase

Quiescent (G0) PC3 cells were isolated by FACS and cultured in the presence of Tf for 3 days

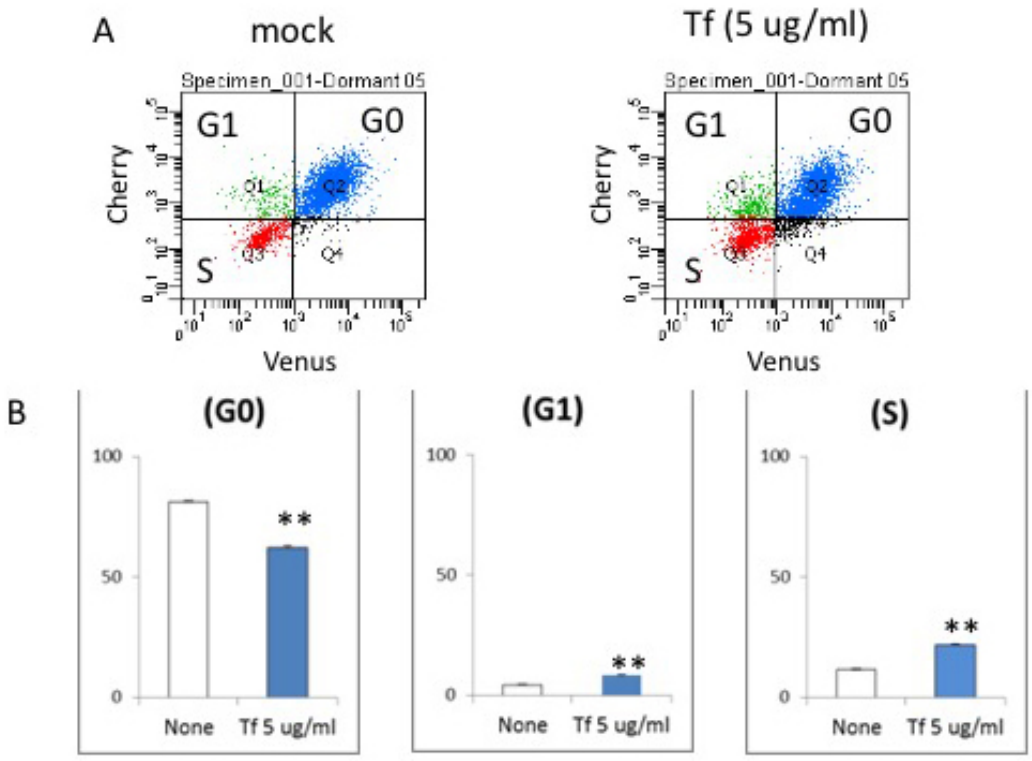


Fig.6 (A) PC3 cells carrying G0-Venus and G1-Cherry reporters (PC VC) were induced into quiescent G0, isolated by FACS and then cultured in the absence or presence of Transferrin (TF). (B) TF exposure induced PC3 cells to leave G0 and enter the cell cycle into G1 and S-phases, consistent with full proliferation.

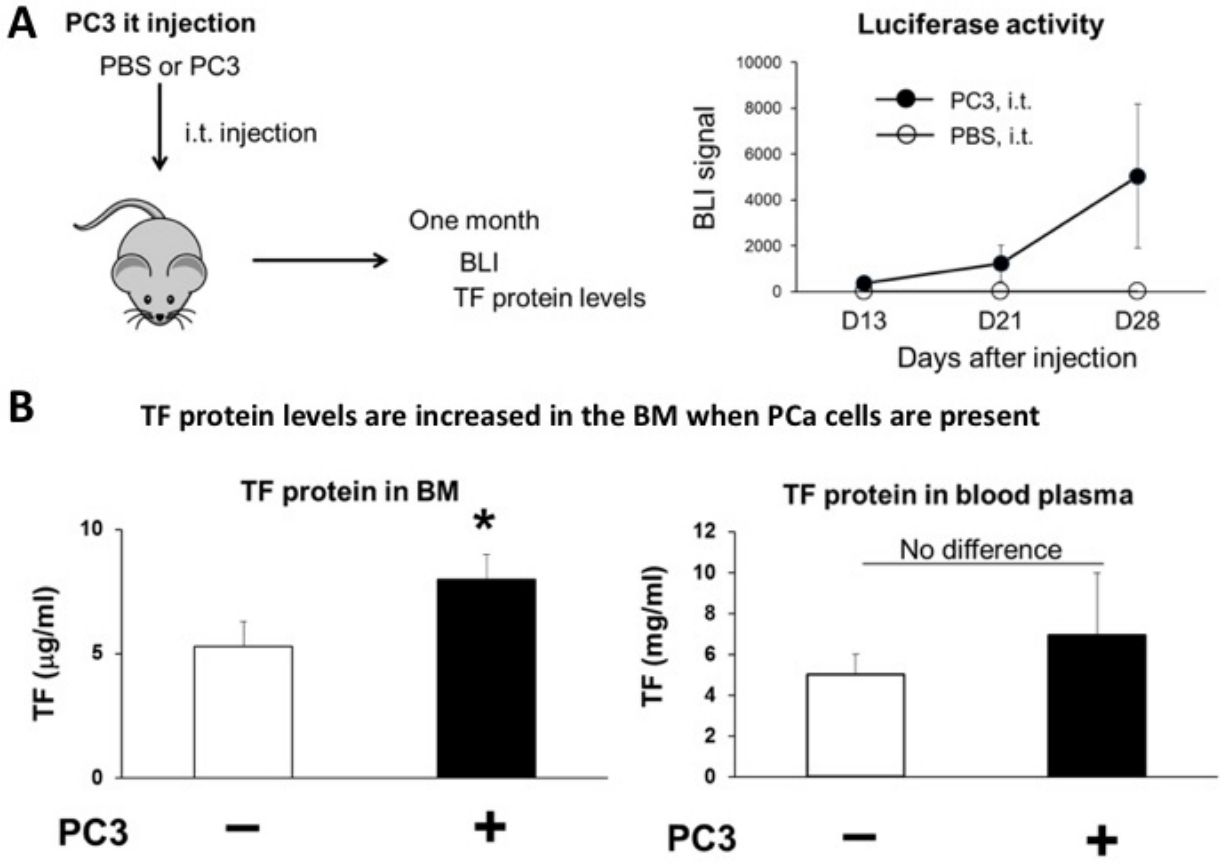


Fig.7 (A) The xenograft model was used to generate bone metastases of PCa cells monitored through luciferase labeling. (B) TF levels were measured in bone marrow isolated with and without PCa cells present.

Other achievements
We have continued to routinely verify that our cell lines are indeed PC3 by sequencing. We feel this is an important precaution, as recent work has emphasized the importance of independently verifying selected cell lines. We also routinely test for mycoplasma contamination and treat when necessary.

Stated goals not yet met

- *Monitoring tumorigenesis by timecourse in the in vivo metastatic model treated with chemotherapy:* As described in the year 2 progress report, we encountered unexpected challenges with using our cell cycle labeled PC3 lines. We have taken alternative approaches to resolve these issues (described here and in year 2 report) and we will perform the experiments described in Major Task 3 during the no cost extension period.

Opportunities for training and professional development.
This project has provided several opportunities for training and professional development as outlined below: *Professional development activities for Dr. Buttitta under PCRP award*: Dr. Buttitta attended the 2018 and 2017 Prostate Cancer Foundation Retreats. This was a great networking opportunity to meet others in the prostate cancer field (which she is relatively new to) and also to learn about the newest work in various realms of prostate cancer research. In addition the work described in year one on the single cell tracking and analysis platform was presented at the AACR 2017 meeting as a poster titled "A computational and statistical approach for interpreting real-time in-vitro gene reporter data"

Dr. Taichman continues to serve as an incredibly valuable co-mentor and facilitator for Dr. Buttitta's emerging work in the prostate cancer field. Dr. Buttitta was also promoted to Associate Professor with tenure at the University of Michigan during year 2 of this award.

- *Collaboration between the Buttitta and Taichman Labs*: This project has provided support and a platform for continued collaboration between the Buttitta and Taichman Labs. Dr. Buttitta's lab is relatively new to the prostate cancer field, while Dr. Taichman's group has extensive experience with models for prostate cancer metastasis and recurrence. The Taichman Lab has developed protocols and techniques that have been approved by the University of Michigan University Committee on the Use and Care of Animals (UCUCA), and students and postdocs working on this project, work in close collaboration with Dr. Taichman's lab members to learn these protocols. In turn, Dr. Buttitta's lab provides the expertise and protocols for the cell cycle studies and the live cell tracking methodology. As described in further detail below, we have had three young scientists, at varying levels of experience, co-mentored by both labs through this project and an additional graduate student and junior faculty has been added this year.

1. *Co-mentorship of undergraduate student Lulia Kana*: Lulia joined the Taichman lab in 2012 where she worked on a project looking at the effects of Gas6 signaling on PC3 cells in vitro. As the collaboration led to development of new tools such as the PC3-FUCCI cell line, Lulia transitioned to being co-mentored by both labs in 2014, resulting in her work being approximately 50% in each lab. Lulia was an excellent and productive student and is now a co-author on two shared publications with the Buttitta and Taichman Labs [1, 10]. Lulia has since graduated with her BS degree and is now in medical school.
2. *Co-mentorship of graduate PhD student Dan Sun*: Dan Sun joined the Buttitta Lab as a PhD student in 2011. After completion of her first project on cell cycle regulation in *Drosophila* in early 2014, she asked to transition to working on mammalian cancer cells, since her future research interests lie in the problem of tumor dormancy. Dan has worked closely with members of the Taichman Lab to learn techniques and helped to generate the cell lines for this project as well as learning FACS protocols, cell sorting etc. This award provided tuition and stipend support for Dan in 2015. Dan was an essential member of the team that developed the cell-tracking pipeline we use for the live image analysis of the cell cycle reporters. In July of 2016 Dan successfully defended her PhD thesis and she is now a postdoc studying cancer in Dr. Julio Aguirre-Ghiso's lab at Mt. Sinai.
3. *Co-mentorship of graduate student Ajai Pullianmackal*: Ajai joined the Buttitta lab in 2015. He has worked on our alternate strategies to deal with the challenges of the cell cycle reporters in PC3 cells.

Ajai performed the single-cell experiments shown in the year 2 report and has learned flow cytometry and cell sorting with the assistance of the Taichman Lab.

4. *Co-mentorship of Postdoctoral Fellow Kenji Yumoto*: Dr. Kenji Yumoto, a senior postdoctoral fellow in the Taichman Lab, has contributed to the training of the students on this shared project and helped to acquire the majority of the data presented here. This award has provided salary support for Dr. Yumoto since the summer of 2016. Dr. Yumoto has provided essential work on the FACS protocols for selecting cell lines, confirming the proper reporter behavior via FACS and use of the cell lines in the mouse model, as well as the studies of Gas6, Transferrin and contributed to studies of osteoclast effects on PCa cell cycle. Dr. Yumoto obtained most of the data shown in this progress report. As a senior scientist Dr. Yumoto has also provided valuable mentorship and training to the students contributing to this project. Dr. Yumoto is currently a co-author on two shared publications between the Buttitta and Taichman Labs [1, 10]. Our labs are located immediately across the street from each other on the University of Michigan campus, which facilitates frequent interactions between our lab members. In addition we have established formal scheduled meetings for the entire research group with both Dr. Buttitta and Dr. Taichman every other week that alternate between the School of Dentistry and Dr. Buttitta's department of Molecular, Cellular and Developmental Biology.
5. *Co-mentorship of Junior Faculty Dr. Frank Cackowski, MD PhD*: Dr. Cackowski is a Clinical Lecturer at University of Michigan working with patients and also performing research with Dr. Taichman's group. Dr. Cackowski is working to identify new molecular markers of DTCs has become an expert in working with the mouse xenograft model in the Taichman Lab to model dormancy and recurrence. Dr. Cackowski performed work shown in progress report for year 2.

- **How were the results disseminated to communities of interest?**

Dr. Buttitta attended the IMPaCT 2016 conference in Baltimore, MD where she presented a poster on this project to the prostate cancer research community. In addition Dr. Buttitta and collaborators presented a portion of this work as a poster at the 2017 AACR meeting. Dr. Yumoto presented this work at the University of Michigan Research day in a poster. Dr. Buttitta presented a portion of this work at the ASCB meeting cell cycle minisymposium in December 2017 and recently presented a portion of this work at the Prostate Cancer Foundation U.Michigan 2018 site visit.

- **What do you plan to do during the next reporting period to accomplish the goals?**

N/A Final progress report

Technical challenges leading to changes in approach:

The SOW was revised and approved after year 2 (in Dec. 2017) and has not been changed.

2. **IMPACT:**

- **Impact on the development of the principal discipline(s) of the project**

1. We are generated PC3 cell lines carrying cell cycle indicators that can be used to monitor cell cycle dynamics of cancer cells *in vitro* and *in vivo*. We expect this to be a useful tool for the scientific community and these cell lines have already been used in 4 published papers and two more currently in preparation.

2. We have also obtained results supporting the hypothesis that prostate cancer cells exposed to specific signals in the bone marrow alter their cell cycle dynamics. This is significant, as we have also shown that prostate cancer cells in G0 exhibit increased resistance to Docetaxel induced cell death. Our results suggest that prostate cancer metastasis to the bone and treatment with traditional chemotherapeutic agents such as Docetaxel likely leads to a relative increase in the fraction of cancer cells in a quiescent or G0 population which may seed recurrent tumors later.

3. We have demonstrated that the bone marrow environment has dramatic effects on the cell cycle state of PCa cells and we have identified candidate signaling pathways that may mediate these effects.

- **What was the impact on other disciplines?**

The automated cell tracking method we have developed to monitor cell cycle reporters in PC3 cells can be applied to other cancer and non-cancer cell types. We expect this will be useful pipeline for other academic scientists using these cell cycle indicators in other cell types. We have a paper in revision for publication in *Scientific Reports* on this methodology. We expect our results on candidate signals from the bone marrow that promote cancer dormancy and recurrence to have impacts on the research of other cancers that also metastasize to the bone such as breast cancer.

- **What was the impact on technology transfer?**

Nothing to Report.

- **What was the impact on society beyond science and technology?**

Nothing to Report

3. **CHANGES/PROBLEMS:**

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

The challenges with using our cell cycle reporter cell lines in the xenograft model (described in detail in year 2 report) pushed back our expected timeline for major tasks 2 and 3. We will complete the experiments for major task 3 during the no cost extension period.

Changes that had a significant impact on expenditures

The delays in performing the *in vivo* metastasis assays with recovery from the bone marrow shifted a portion of our anticipated animal costs for period 1 to periods 2 and 3 and into a no cost extension period. However there was no change in the overall budget.

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

- **Significant changes in use or care of human subjects**

None

- **Significant changes in use or care of vertebrate animals.**

None.

- **Significant changes in use of biohazards and/or select agents**

None

4. **PRODUCTS:**

- **Publications, conference papers, and presentations**.

Nothing to report

- **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**

The automated cell tracking method we have developed to monitor cell cycle reporters in PC3 cells can be applied to other cancer and non-cancer cell types. We expect this will be useful pipeline for other academic scientists using these cell cycle indicators in other cell types. We have a paper in preparation on this methodology, which we expect to be published in the next year. Once this work is published we will make all customized software scripts available to academic not-for-profit researchers as described in our data sharing plan.

5. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

Name:	*Laura Buttitta*
Project Role:	*PI*
ORCID ID	0000-0002-5064-0650
Nearest person month worked:	2.0
Contribution to Project:	*Dr. Buttitta is the directing PI for this project and is involved in all aspects of project management, student and postdoc mentorship and writing for publications and reports under this award.*
Funding Support:	*Dr. Buttitta is supported by her nine-month appointment in the College of LS&A at University of Michigan as well as a Scholar Award from the American Cancer Society and a collaborative PCF grant with Dr. Taichman. Dr. Buttitta obtained an NIH R01 award during the no cost extension phase of this project.*
Name:	*Russell Taichman*
Project Role:	*Senior/Key Personnel*
ORCID ID	*0000-0002-7890-0020*

Nearest person month worked:	*0.2*
Contribution to Project:	*Dr. Taichman is a senior co-mentor for Dr. Buttitta and a collaborator for this project. He assists with co-mentorship of all working on this project and provides input and advice on project management and publications.*
Funding Support:	*3P01CA093900-06 (PI: Keller) (PI Project 3: Taichman)* *The Biology of Prostate Cancer Skeletal Metastases* *Project 3: Regulation of the PCa Metastatic Phenotype by the HSC Niche* *05/1/15-04/30/20 NIH/NCI* *Targeting quiescence in prostate cancer* *PI: L. Buttitta, Collaborator: Taichman* *CDMRP, W81XWH-15-1-0413* *8/28/2015-8/30/2018* *Sympathetic Nervous System Control of Disseminated Tumor Cell (DTCs) Dormancy* *PI: Taichman* *CDMRP/ DOD, PC140665 9/1/15-8/31/18* *Mechanisms of PCa Relapse in Marrow (new project)* *Prostate Cancer Foundation (Russell Taichman, PI)* *08/22/2016 – 08/22/2018* *Effort: 1.2 Cal.*
Name:	*Kenji Yumoto*
Project Role:	*Postdoctoral Fellow*
ORCID ID	*None*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Yumoto is the senior scientist on this project and peforms the majority of the experiments and helps to provide training for students working on this project. Dr. Yumoto also contributes to project management and writing of publications. Dr. Yumoto is an expert in the mouse xenograft model and using shRNA approaches.*
Funding Support:	*Dr. Yumoto is currently supported by this award*
Name:	*Dan Sun*
Project Role:	*Graduate Student Research Assistant*
ORCID ID	*None*
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Sun completed her PhD in the Buttitta Lab in 2016 and worked on this project together with Dr. Yumoto.*
Funding Support:	*Dr. Sun was supported by this award as well as a Scholar Award (PI: Buttitta) from the American Cancer Society*
Name:	*Lulia Kana*

Project Role:	*Undergraduate research assistant*
ORCID ID	*None*
Nearest person month worked:	*2*
Contribution to Project:	*Ms Kana was an undergraduate co-mentored by the Taichman and Buttitta labs from 2013-2016. She worked for research credit and assisted Dr. Sun and Dr. Yumoto with experiments. She graduated in 2016.*
Funding Support:	*Ms. Kana's position was an unpaid position for research credit.*
Name:	*Frank Cackowski MD PhD*
Project Role:	*Clinical Lecturer and Research Associate*
ORCID ID	*None*
Nearest person month worked:	*0.2*
Contribution to Project:	*Dr. Cackowski is a new collaborator on this project and has worked with Dr. Yumoto on the in vivo bone metastasis assays*
Funding Support:	*Dr. Cackowski is supported by a collaborative PCF Challenge Grant on which he is a New Investigator*
Name:	*Ajai Pullianmackal*
Project Role:	*Graduate student Research Assistant*
ORCID ID	*None*
Nearest person month worked:	*1*
Contribution to Project:	*Mr. Pullianmackal has worked with Dr. Yumoto on optimizing the flow cytometry and single cell live imaging assays.*
Funding Support:	*Mr. Pullianmackal is supported by a Scholar Award (PI: Buttitta) from the American Cancer Society*

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

New Funding for Dr. Buttitta (PI) since Sept. 2017:
Dr. Buttitta obtained an NIH R01 on an unrelated project studying cellular quiescence in Drsosophila wings.

New Funding for Dr. Taichman (Senior/Key Personnel) since Sept. 2017:
None

6. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** Not applicable
- **QUAD CHARTS:** Not applicable

7. **APPENDICES:**

None