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14. ABSTRACT

In prostate cancer patients with disease apparently localized to the prostate, cancer cells can spread early in the disease process, lie dormant in distant sites and then grow decades after a patient was thought to be cured. In the application for this proposal, we hypothesized that; inhibition of hippo signaling or YAP1 and TAZ activation stimulates dormancy escape in prostate cancer. We proposed to; Aim 1: Determine which hippo pathway components stimulate or inhibit dormancy escape in vivo, Aim 2: Delineate how tissue mechanics regulate prostate cancer dormancy through the hippo pathway, and Aim 3: Investigate how non-coding RNAs regulate prostate cancer dormancy through the hippo pathway. Thus far, we have found that culture of prostate cancer cells on medium stiffness matrix induces maximal cell cycle activity and transcription of YAP/TAZ target genes. Knockdown of the non-coding RNA, SNHG1, induces quiescence, but unexpectedly decreases YAP1 and TAZ mRNA levels. We have also have institutional approval for our animal protocol and have created stable cell lines.

15. SUBJECT TERMS
Hippo, dormancy, prostate cancer recurrence, non-coding RNA, mechanobiology

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1. **INTRODUCTION:**

Though some patients with prostate cancer are cured by surgical removal or radiation of the prostate, some patients have recurrences as long as decades after initial treatment[1]. These recurrences are usually incurable if they occur at distant sites (outside the pelvis). Prostate cancer disseminated tumor cells (DTCs) display a dormant phenotype and act as an important reservoir for distant recurrence after curative intent surgery or radiation[2-4]. However, there are no specific treatments able to inactivate or eradicate these cells. In the preliminary data for this proposal, we used RNA-seq to compare macroscopic vs. microscopic prostate cancer tumors, analyzed TCGA data, and predicted the cell cycle phase in single cell RNA-seq data; and found that the hippo pathway and its downstream transcriptional coactivators YAP1 and TAZ appear to regulate prostate cancer dormancy and recurrence. We hypothesized that; **inhibition of hippo signaling or YAP1 and TAZ activation stimulates dormancy escape in prostate cancer**, and organized the proposed studies around the following specific aims.

Specific Aim 1: *Determine which hippo pathway components stimulate or inhibit dormancy escape in vivo*.
Specific Aim 2: *Delineate how tissue mechanics regulate PCa dormancy through the hippo pathway*.
Specific Aim 3: *Investigate how non-coding RNAs regulate PCa dormancy escape through the hippo pathway*.

Because this is an early career award, the training and career development of the principal investigator (Dr. Cackowski) is also particularly important. As guided by primary mentor, Dr. Evan Keller, co-mentors and consultants, Dr. Cackowski's career development will be structured around the following training objectives:

Training Objective 1: *Develop expertise in analyzing cell signaling of prostate cancer DTCs in vivo*.
Training Objective 2: *Learn techniques to study signaling modulation by mechanical forces and non-coding RNAs*.
Training Objective 3: *Hone skills in scientific communication and laboratory management*.

2. KEYWORDS:

Hippo kinases	MST1/MST2; Protein products of *STK4* and *STK3* genes
PCa	Prostate Cancer
PCR	Polymerase chain reaction
qRT- PCR	Real-time reverse transciptase polymerase chain reaction
s.c.	Subcutaneous
SCID	Severe Combined Immune Deficient
YAP1	Product of *YAP1* gene, a hippo pathway downstream effector
TAZ	Product of the *WWTR1* gene, a hippo pathway downstream effector
Myc-CaP	Prostate cancer cell line derived from the FVB high myc mouse
PC3	Human patient derived prostate cancer cell line
RNA-Seq	RNA sequencing
scRNA-Seq	Single cell RNA sequencing
SNHG1	A long non-coding RNA gene
LATS1	Large tumor suppressor kinase 1, negatively regulates YAP1 and TAZ
LATS2	Large tumor suppressor kinase 2, negatively regulates YAP1 and TAZ
DTC	Disseminated tumor cell
DCC	Disseminated cancer cell (synonym of DTC)
Young's modulus	Physics and engineering term to describe the stiffness of a substance
lncRNA	Long non-coding RNA
PCCTC	Prostate Cancer Clinical Trials Consortium
SPORE	Specialized Programs of Research Excellence
AACR	American Association for Cancer Research
TEAD2	TEA Domain Transcription Factor 2, a co-TF for YAP1 and TAZ

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Below are the tasks in the current statement of work, proposed timeline and the current status for each.

Training-Specific Tasks

Major Task 1: Training and career development in prostate cancer research	Months	Status
Subtask 1: Learn techniques to modify gene expression in prostate cancer disseminated tumor cells after the cells home to metastatic sites such as bone marrow.	1-24	Ongoing: approx. 20% completed, including establishment of tetracycline inducible TEAD2 wild type and dominant negative PC3 cell lines.
Subtask 2: Learn how to use archived patient samples to assess a potential biomarker	1-12	Pending
Subtask 3: Learn how to use acoustic tweezing cytometry to modulate signaling pathways.	13-36	Pending
Subtask 4: Learn techniques to vary strength an composition of the extracellular matrix to modulate signaling pathways	13-36	Ongoing; approx. 30% completed, February, 2021
Subtask 5: Learn ultrasound imaging techniques to measure mechanical properties of tissues.	25-36	Pending
Subtask 6: Obtain proficiency with analysis of bulk and single cell RNA-sequencing data including statistics	1-48	Ongoing; approx. 60% completed, including manuscript published in *Frontiers in Cell and Developmental Biology*
Subtask 7: Learn how to study how the cell cycle is controlled by non-coding RNAs	13-48	Ongoing, approx. 50% completed. Poster presented at AACR 2022
Subtask 8: Learn how to use reporter constructs to assess signaling pathway activity	13-48	Pending
Subtask 9: Peer review at least two manuscripts per year with mentor feedback	1-48	Completed for years 1 and 2
Subtask 10: Complete Wayne State University "Academic Leadership Academy"	25-36	Pending
Subtask 11: Attend at least one national meeting per year	1-48	Completed for years 1 and 2 (virtual); Prostate Cancer Foundation (10/2020, 10/2021), ASCO (6/2021) and Prostate SPORE retreat (3/2022)
Subtask 12: Attend Wayne State University seminar: "Planning and Writing Successful Grant Proposals for Biomedical Research"	24-36	Pending
Milestone 1: Submit first manuscript from this proposal	*24*	Completed: including corresponding author manuscript published in *J Bone Oncology*[5] and Co-Corresponding author manuscript published in *Frontiers in Cell Dev Biology*.[6]
Milestone 2: Obtain NIH R01, DoD IDEA or equivalent	*36-48*	Ongoing: approximately 10% completed. New grants; PI on Pilot

funding		grant from Michigan Prostate Cancer SPORE and Co-I on DoD Clinical Consortium Research Site Award (PCCTC)

Research-Specific Tasks:

Major Task 1: Determine which hippo pathway components stimulate or inhibit dormancy escape *in vivo*.	Months	Status
Subtask 1: Submit animal protocol to Wayne State IACUC and make any necessary revisions for approval. Also applies to Major tasks 2, 3 and 4.	1-3	Completed, approved 10/2020
Subtask 2: Obtain ACURO approval. Will be completed before any animal work. Also applies to Major tasks 2, 3 and 4.	4-6	Completed: Approved by ACURO 8/3/2021
Subtask 3: Finish preparation and testing of dominant negative and constitutively active hippo pathway constructs. For all experiments, established, de-identified human cell lines will be used: C42B, LNCaP, PC3 and Du145. The mouse prostate cancer cell line FVB MyC-CaP may also be used.	1-12	Ongoing: approx. 10% completed, establishment of tetracycline inducible TEAD2 wild type and dominant negative PC3 cell lines.
Subtask 4: Complete testing of which hippo pathway members affect the cell cycle *in vitro*. This will include analysis of single cell RNA sequencing data and use of the hippo pathway constructs	1-12	Ongoing: approx. 20% complete. Have at least one working siRNA sequence for each gene.
Subtask 5: Perform *in vivo* experiments using the constructs tested in Subtasks 3 and 4. Mice: 120 male CB17 SCID.	7-24	Pending
Subtask 6: Establish immunofluorescence methods for tissue micro-arrays.	1-6	Pending
Subtask 7: Use tissue microarrays from PCBN, corresponding patient data and immunofluorescence to test if YAP and TAZ protein expression or localization predict time to biochemical recurrences	7-12	Ongoing: approx. 20% complete. 3/25/2022: Wayne State University and ORP Determination that work is not Human Participants Research
Subtask 8: Prepare inducible CRISPR-Cas9 cell lines if necessary depending on results from Subtask 1.	12-15	Pending
Milestone: Publication submission	24	Pending
Major Task 2: Delineate how mechanics of the tumor microenvironment regulate PCa dormancy through the hippo pathway	**Months**	**Status**
Subtask 1: Use acoustic tweezing cytometry to assess the effect of mechanical stress and strain on the hippo pathway and cell cycle. Cell lines are as described for Major Task 1.	13-24	Pending
Subtask 2: Determine the effect of substrate stiffness on the hippo pathway and cell cycle	13-24	Ongoing; approx. 30% completed, February, 2021
Subtask 3: Validate techniques to modulate the stiffness of the extracellular matrix *in vivo*. Mice: 30 FVB and 10 CB17 SCID	13-18	Pending
Subtask 4: Determine the effect of ECM stiffness on	19-24	Pending

prostate cancer dormancy escape. Mice: 30 CB17 SCID		
Subtask 5: Determine if DTCs' location and cell cycle status correlates with ECM stiffness *in vivo*. Uses tissues from Subtask 4.	28-36	Ongoing: 5% completed; Reagents purchased
Subtask 6: Determine if the hippo pathway is required for the effects of ECM stiffness on cell cycle *in vitro*.	13-18	Pending
Subtask 7: Determine if the hippo pathway is required for the effects of ECM stiffness on dormancy escape *in vivo*. Mice: 30 CB17 SCID	31-36	Pending
Subtask 8: Determine if the actin cytoskeleton mediates the effect of mechanics on proliferation	24-30	Pending
Subtask 9: Use Inducible CRISP/Cas9 or shRNA to determine if *RHOA* is involved in mechanical stimulation of dormancy escape *in vivo*. Mice: 60 CB17 SCID	24-36	Pending
Milestone: Publication submission	36	Pending
Major Task 3: Investigate how non-coding RNAs regulate PCa dormancy escape through the hippo pathway.	**Months**	**Status**
Subtask 1: Determine how SNHG1 knockdown and overexpression alters hippo signaling, and the cell cycle *in vitro*.	13-24	Ongoing; 75% completed
Subtask 2: Determine if other candidate non-coding RNAs interact with the hippo pathway in the experimental systems used.	16-36	Pending
Subtask 3: Create an SNHG1 inducible knockdown or over-expressing PCa cell line	31-36	Pending
Subtask 4: Determine the effect of SNHG1 modulation on prostate cancer recurrence *in vivo*. Mice: 30 CB17 SCID	37-48	Pending
Milestone: Publication submission	48	Ongoing: Poster presented at AACR 2022
Major Task 4: Ensure publication of at least one manuscript per specific aim	**Months**	**Status**
Subtask 1: Additional experiments requested by reviewers	24-48	Pending
Subtask 2: Resubmission of manuscripts(s)	24-48	Pending

What was accomplished under these goals?

Training-Specific Tasks:
Major Task 1: Training and career development in prostate cancer research

We have made significant progress in <u>Subtask 4: Learn techniques to vary strength an composition of the extracellular matrix to modulate signaling pathways</u>. On culture with silicone matrices of varying stiffness coated with either collagen or fibronectin, we observed changes in both cell cycle and transcription of YAP/TAZ target genes (see also Figure 2).

Much progress has been made under <u>Subtask 6: Obtain proficiency with analysis of bulk and single cell RNA-sequencing data including statistics</u>. Our analysis of bulk RNA-sequencing data is now peer reviewed and published in Frontiers in Cell and Developmental Biology.[6] This data was included in the proposal for this project as part of the rationale for the study of hippo pathway. Additionally, during the second year of this award (current progress report period) I received a pilot grant from the Michigan Prostate Cancer SPORE entitled "Profiling of Single Quiescent Prostate Cancer Cells Using a Neural Network Model." This project focuses on single cell RNA-sequencing data and is a collaboration with mathematical modeler, Dr. Suzan Arslanturk. We have alread acquired the majority of the raw data for this project, which has greatly increased my skill in working with this type of data.

Similarly, we are pleased to report experiential learning and experimental progress under <u>Subtask 7: Learn how to study how the cell cycle is controlled by non-coding RNAs</u> (see also Figures 4-9). Prior to this proposal, I had no experience in this complex and rapidly changing research area. We also presented this data as a poster at 2022 AACR meeting.[7]

<u>Subtask 9: Peer review at least two manuscripts per year with mentor feedback is completed for years</u> 1 and 2. Journals reviewed for include; *Cancers, Cell Death & Disease, Clinical Genitourinary Cancer, eLife, European Urology, Medical Oncology, Molecular Therapy Nucleic Acids, PeerJ,* and *Pharmaceutics*.

Although travel was severely limited by the COVID-19 pandemic, I fulfilled <u>Subtask 11: Attend at least one national meeting per year</u>, by attending the Prostate Cancer Foundation Scientific Retreat and the American Society for Clinical Oncology Annual Meeting in the virtual format for both years. As travel restrictions are beginning to be eased, I also attended the Prostate SPORE Research Retreat in person for 2022.

We have completed *Milestone 1: Submit first manuscript from this proposal.* We have thus far published two primary research articles and two review articles significantly supported by this proposal, all of which were accepted in year two of the grant period; 1) *Use of FVB MycCaP Cells as an Immune Competent, Androgen Receptor Positive, Mouse Model of Prostate Cancer Bone Metastasis* in *Journal of Bone Oncology*[5] as the corresponding author, and 2) *Monitoring spontaneous quiescence and asymmetric proliferation-quiescence decisions in prostate cancer cells*, to *Frontiers in Cell and Developmental Biology*[6] as the co-corresponding author along with Co-Mentor, Dr. Laura Buttitta, 3) *Prostate Cancer Dormancy and Recurrence* in *Cancer Letters* as the first author[8], and 4) *Wnt and beta-Catenin Signaling in the Bone Metastasis* of *Prostate Cancer* in *Life*[9]. The first article (*Use of FVB Myc-CaP cells…*) includes data presented in Figure 4 of the initial application and figures prominently into most of the animal studies for the entire project. This manuscript describes a new model rather than findings concerning hippo signaling. However, establishment of the model in a publication first will greatly expedite subsequent studies from this project because it will allow subsequent publications to focus on the "science" rather than description and validation of the experimental model. The second manuscript (*Monitoring spontaneous quiescence…*) includes data presented in figures 2 and 3 of the original proposal. This manuscript focuses on cell cycle decisions and differential regulation of quiescent (G0 cell cycle phase) cells and describes the hippo pathway as being differentially regulated in quiescence and differentially regulated between macroscopic and microscopic tumors. We found that the mRNA for multiple genes affecting the hippo pathway in both positive and negative directions are increased in G0, but that the pathway appears to be held in check by protein phosphorylation and degradation. This manuscript establishes the rationale for the study of the hippo pathway in prostate cancer dormancy and recurrence so the future publications from this study can focus on how the pathway works and cite the article in *Frontiers in Cell and Developmental Biology* as the Reason for studying hippo signaling in prostate cancer dormancy. This award also supported three additional published articles, which acknowledge its support.[10-12]

I have obtained additional funding a ground work for *Milestone 2: Obtain NIH R01, DoD IDEA or equivalent funding*. I am PI on a $50,000 pilot grant funded by the Michigan Prostate SPORE entitled "Profiling of Single Quiescent Prostate Cancer Cells Using a Neural Network Model" and Co-I on the recently renewed clinical trials grant Department of Defense Prostate Cancer Research Program "Prostate Cancer Research Program Clinical

Consortium Research Site Award (PCCTC)." Although these grants do not qualify as the large, multi-year, laboratory based research grant intended by this milestone, they do show a track record of funding at my new institution. Additionally, the SPORE pilot grant is designed to provide seed funding for larger grants such as NIH R01 or DoD IDEA.

Research-Specific Tasks:
Major Task 1: Determine which hippo pathway components stimulate or inhibit dormancy escape *in vivo*.

Subtask 1: Submit animal protocol to Wayne State IACUC and make any necessary revisions for approval. Completed. Our protocol for the studies in this proposal was approved by the Wayne State IACUC in October, 2020. We then had a minor revision for change in personnel. For the current version, please see APPENDIX p 43-64.

Subtask 2: Obtain ACURO approval. Will be completed before any animal work: Completed. The protocol was approved by ACURO on August 3, 2021. Please see APPENDIX p 23-24 for the approval letter.

Subtask 3: Finish preparation and testing of dominant negative and constitutively active hippo pathway constructs. Tetracycline inducible TEAD2-WT and TEAD2-dominant negative constructs are established, expanded, mycoplasma checked and frozen down in PC3 cells also expressing GFP and luciferase. To compliment the serine – alanine mutants, we will also perform transient RNAi transfections of the genes of the hippo pathway. To prepare for these experiments, we tested various RNAi sequences for the necessary genes of the hippo pathway: *WWTR1* (encodes TAZ protein), *LATS1*, *LATS2*, *STK4* (encodes MST1 protein), and *STK3* (encodes MST2 protein) and have at least one sequence for each gene with at 67% mRNA knockdown in PC3 cells (**Figure 1**). We previously showed knockdown efficacy for *YAP1* (the remaining core gene of the pathway) in the application for this award.

Subtask 4: Complete testing of which hippo pathway members affect the cell cycle *in vitro*. This will include analysis of single cell RNA sequencing data and use of the hippo pathway constructs: In figure 3 of the initial application for this project, we presented data that knockdown of *YAP1* increases the number of cells in the G0 phase of the cell cycle in PC3 prostate cancer cells. Subsequently, we have confirmed and extended this finding using alternative techniques and showed that total cell number and growth rate are also decreased by *YAP1* knockdown by siRNA and measurement of total viable cell number in PC3 cells and also in a second cell line, C42B. (**Figure 2**). We seeded cells at 50,000 cells per ml and transiently transfected with control or *YAP1* targeting siRNAs (Thermo-Fisher) using Lipofectamine RNAi MAX reagents. We trypsinized triplicate wells and changed the culture media to RPMI with 10% FCS after one day. We trypsinized triplicate wells each day for 7 days and counted total viable cell number with a Countess™
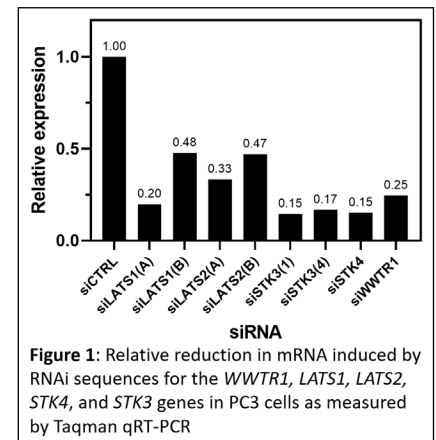
Figure 1: Relative reduction in mRNA induced by RNAi sequences for the *WWTR1, LATS1, LATS2, STK4,* and *STK3* genes in PC3 cells as measured by Taqman qRT-PCR
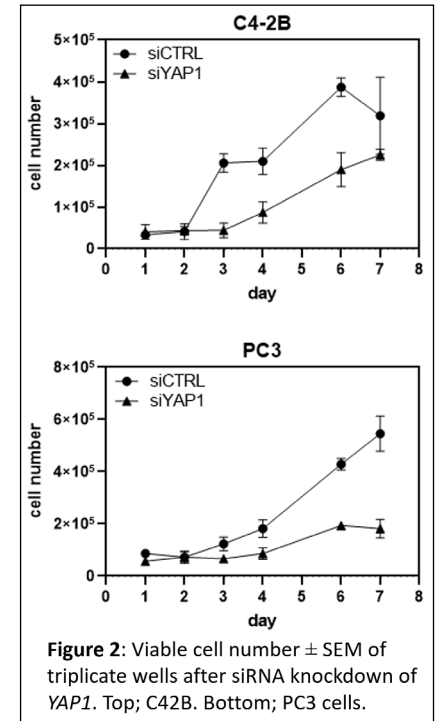
Figure 2: Viable cell number ± SEM of triplicate wells after siRNA knockdown of *YAP1*. Top; C42B. Bottom; PC3 cells.
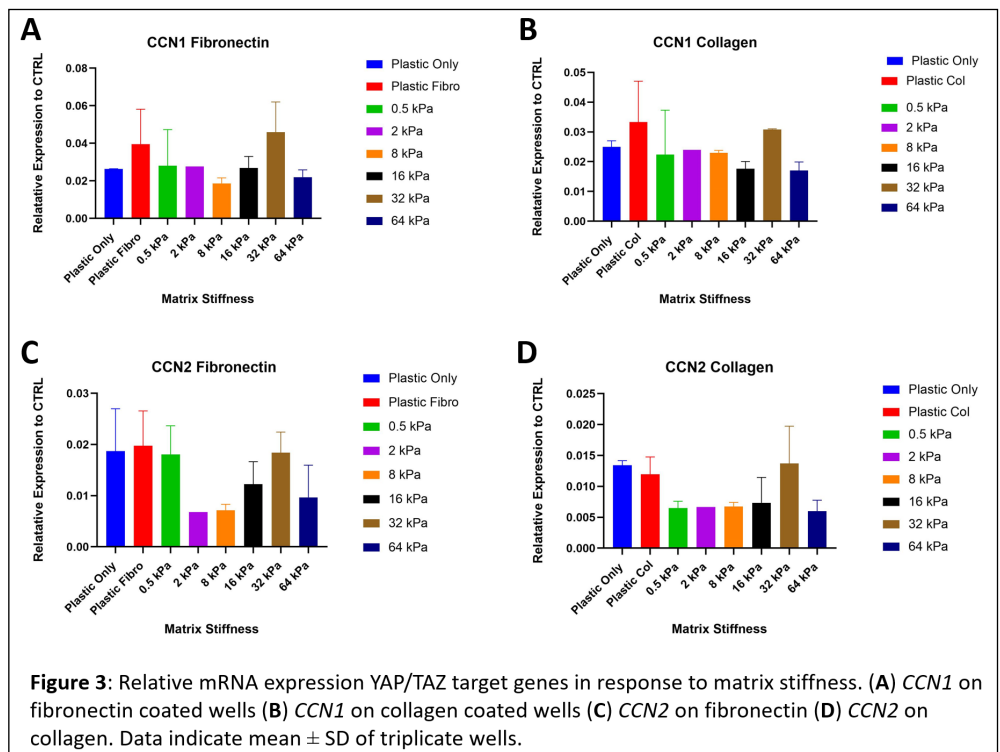
Figure 3: Relative mRNA expression YAP/TAZ target genes in response to matrix stiffness. (**A**) *CCN1* on fibronectin coated wells (**B**) *CCN1* on collagen coated wells (**C**) *CCN2* on fibronectin (**D**) *CCN2* on collagen. Data indicate mean ± SD of triplicate wells.

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automated cell counter.

Major Task 2: Delineate how mechanics of the tumor microenvironment regulate PCa dormancy through the hippo pathway

Subtask 2: Determine the effect of substrate stiffness on the hippo pathway and cell cycle:

We began our studies of the effects of the stiffness of the extracellular matrix on hippo signaling in prostate cancer cells. We were able to purchase (Advanced Biomatrix) pre-made six well culture plates coated with silicone of defined stiffness (Young's modulus) ranging from 0.5 to 64 kilo-Pascals (kPa). To allow cell attachment, we coated these substrates with two common matrix molecules; collagen or fibronectin. We seeded PC3 cells on these protein coated matrices, cultured for 2 days in RPMI with 1% FCS, lysed the cells, isolated RNA and conducted qRT-PCR with Taqman primer probes for the YAP/TAZ target genes; *CCN1* (CYR61) and *CCN2* (CTGF). For genes and for both protein coatings, we saw increased mRNA expression at only 32 kPa (**Figure 3**). Curiously, this is well within the expected range for the stiffness of pre-calcified bone (the matrix surrounding pre-osteoblasts), which is 20-50 kPa[13]. We also note that investigators have also proposed pre-calcified bone and the osteoblastic niche to be an especially important location for homing of prostate cancer disseminated tumor cells[14]. We think that these future studies will be especially important for studies on the mechanism of prostate cancer recurrence.

Major Task 3: Investigate how non-coding RNAs regulate PCa dormancy escape through the hippo pathway.

Subtask 1: Determine how *SNHG1* knockdown and overexpression alters hippo signaling, and the cell cycle in vitro.

*Effect of SNHG1 on hippo signaling*

To determine how *SNHG1* alters the hippo pathway, we used RNAi to knockdown expression of *SNHG1* in prostate cancer cell lines, then measured expression of hippo pathway genes by RT-qPCR. As a surrogate for hippo pathway signaling, we measured expression levels of the downstream hippo-regulated genes *CCN1* and *CCN2* (*CTFG*). In PC3 cells, we obtain a 51-64% knockdown of SNHG1 over a 3 day period. Knockdown resulted in increased expression of CCN1 and CCN2, up to 1.9-fold and 4.5-fold, respectively (**Figure 4**). This indicates reduced hippo pathway signaling resulting in transcriptional activation by YAP1/TAZ/TEAD proteins. When we analyzed the expression of other hippo pathway genes following *SNHG1* knockdown, we found increased expression of *LATS1, LATS2, STK3, STK4, and YAP1* (2.1-4.0-fold increase), while *WWTR1* (gene for TAZ protein) expression was unchanged. Similar results were obtained in C4-2B cells. Investigations into the mechanisms used by *SNHG1* to affect hippo pathway signaling are ongoing.

Effect of SNHG1 on cell cycle

We analyzed the effect of *SNHG1* on cell cycle first by following growth of PC3 and C4-2B cells *in vitro* after *SNHG1* knockdown. Little to no proliferation was observed in PC3 and C4-2B cells deficient in *SNHG1* over a 7 d period (**Figure 5**). However, in control cells, cell counts increased 6-11-fold over 7 d. This was not due to greater cell death, as viability was similar to controls cells following siRNA transfection, and cell counts reported are viable cell counts.
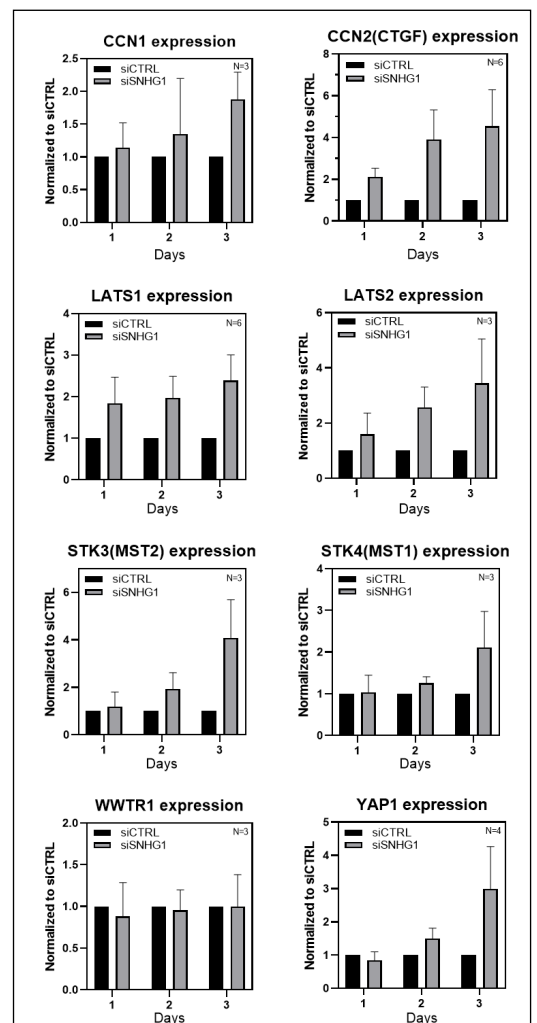

Figure 4: Expression of hippo pathway gene mRNA following *SNHG1* knockdown in PC3 cells. Mean ± SD.
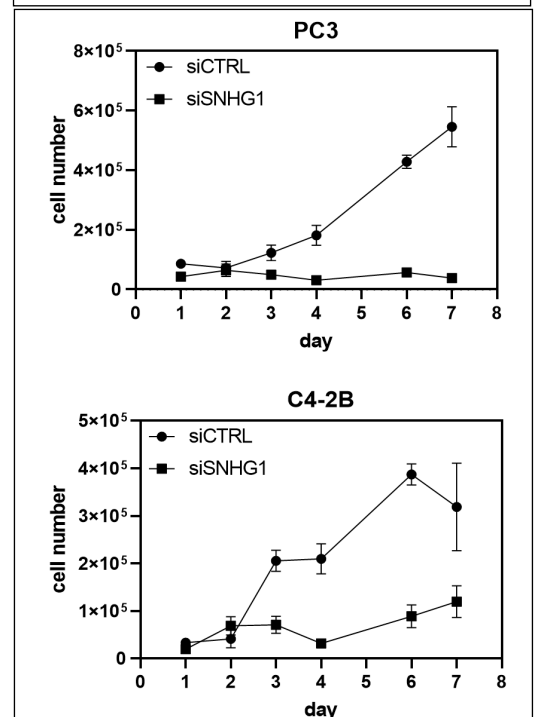

Figure 5: Proliferation of PC3 and C4-2B cells following *SNHG1* knockdown.

The lack of proliferation of *SNHG1*-deficient cells led us to investigate cell cycle status of these cells. Non-proliferating cells may be senescent, or in the G_0 or G_1 phase of the cell cycle. We used PC3 cells with "venus / cherry" reporters (PC3-VC) engineered to doubly fluoresce Venus and mCherry fluorescent proteins when they are in the G_0 cell cycle phase (quiescent) based on expression of fluorescent protein reporters for CDKN1B (p27) and CDT1. G1 phase cells

Figure 6: Effect of SNHG1 knockdown on the cell cycle as measured by reporters for CDT1 and p27 (venus-cherry cells). (**A**) Example flow cytometry plots. (**B**) Quantified data for two different *SNHG1* targeting siRNA sequences. Mean ± SD of three independent experiments.

are singly positive for CDT1-mCherry. The double negative population contains cells in the S, G2 and M phases. This is the same cell cycle reporter system that we presented in figure 3 of the preliminary data in the application for this project. It was originally tested in mouse fibroblasts[15] and has been validated for use in prostate cancer cells by our group and collaborators, including a publication characterizing these cells in 2021[6,16]. On analysis by flow cytometry, SNHG1 knockdown by siRNA induced a four-fold increase in the percentage of cells in G_0 (**Figure 6**).
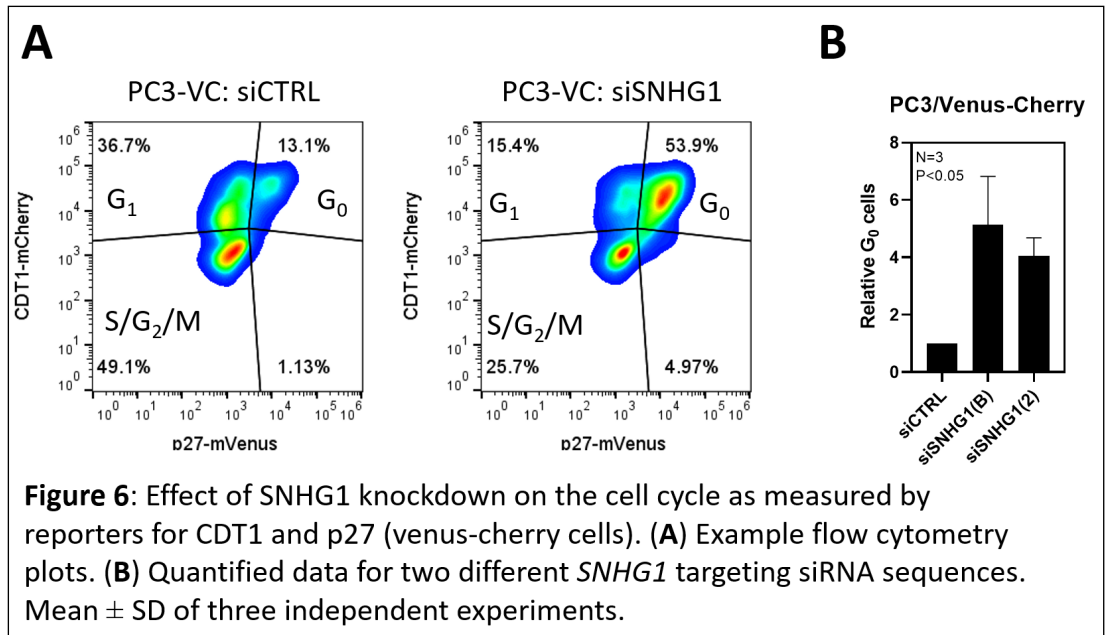
To examine the effect of SNHG1 on knockdown on the cell cycle, we used flow cytometry to analyze PC3, Du145, C425 and LNCaP cells labeled with EdU (for DNA synthesis) and propidium iodide (for DNA content). The percentage of EdU positive (S-phase) was lower in each cell line after SNHG1 knockdown – again suggesting that *SNHG1* stimulates cell cycle progression (**Figure 7**).
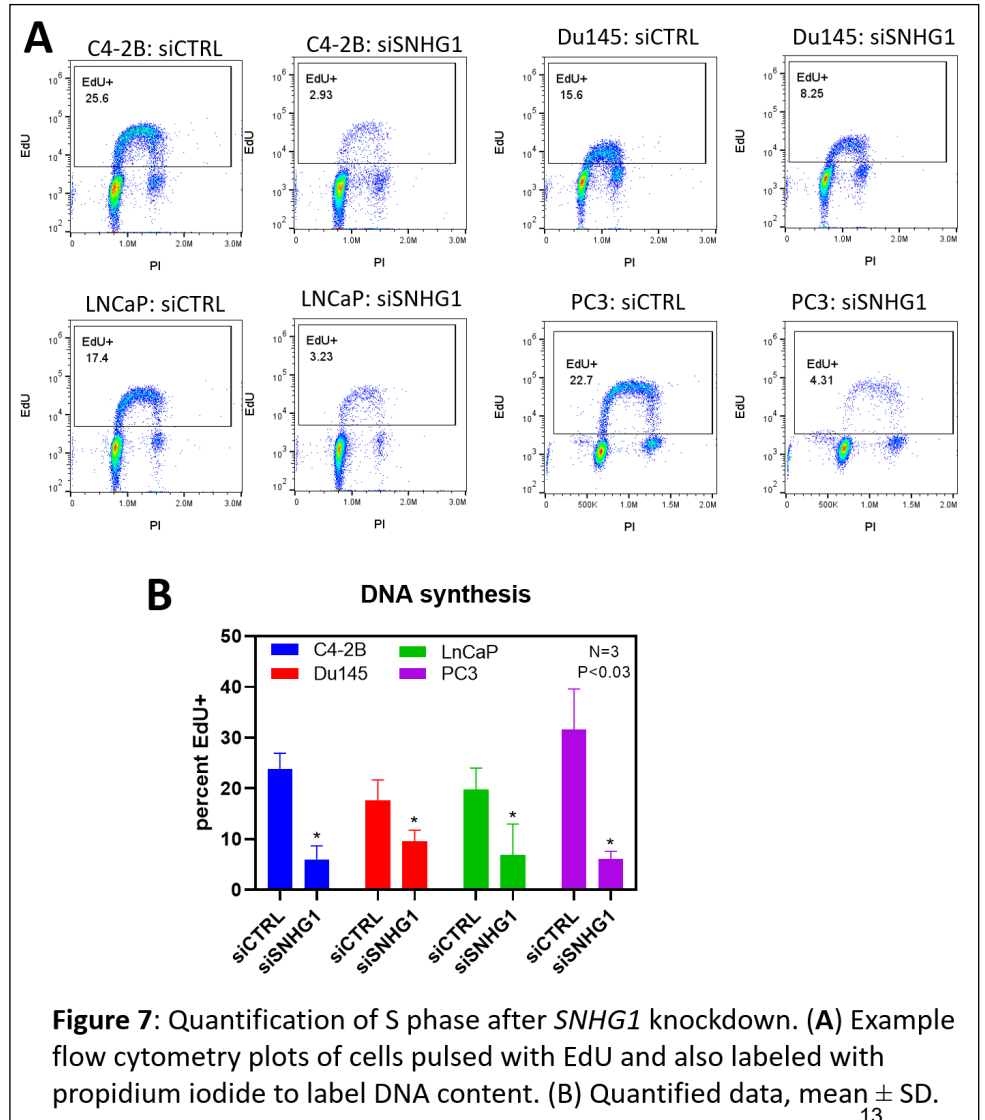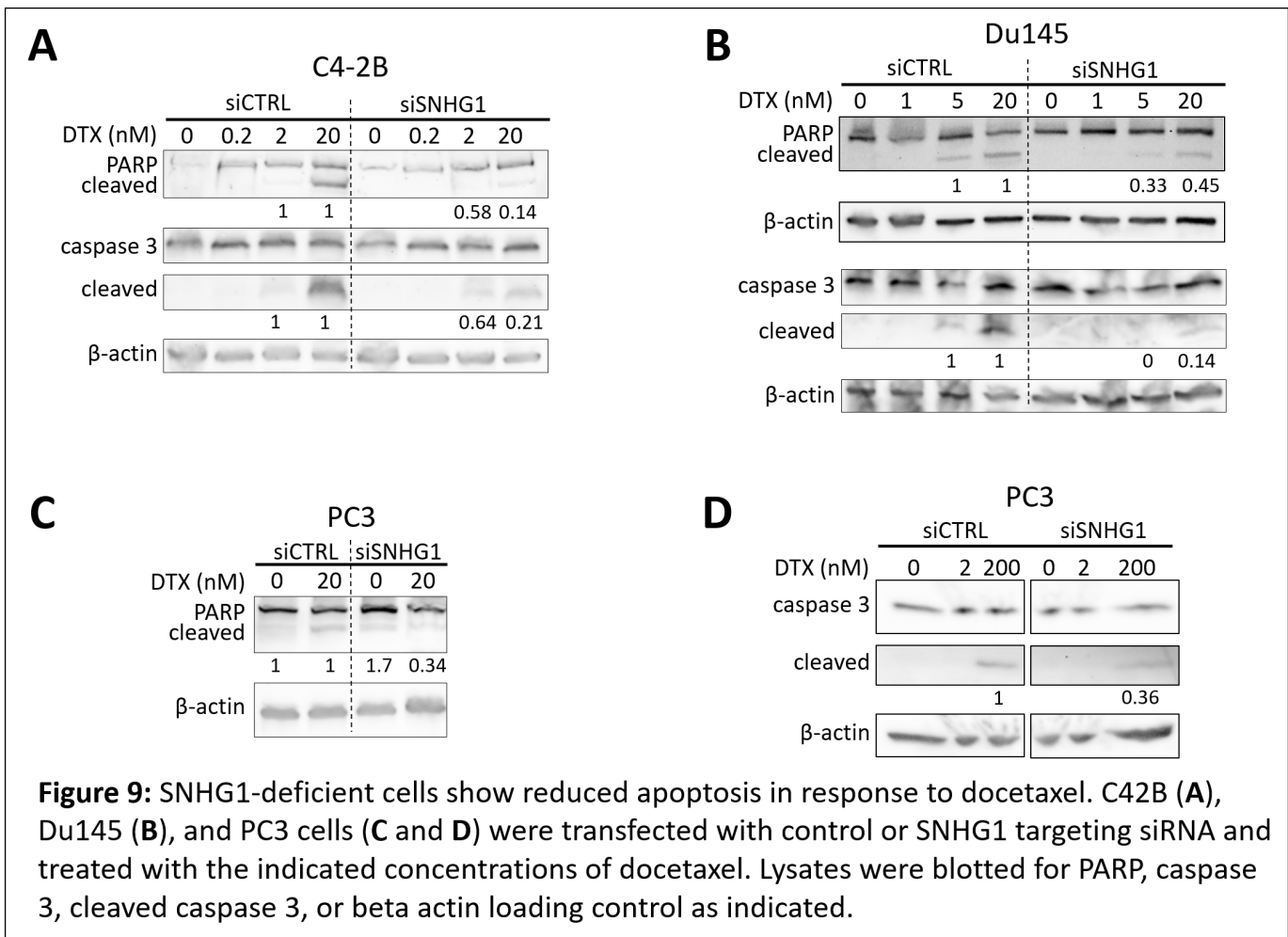

Figure 7: Quantification of S phase after *SNHG1* knockdown. (**A**) Example flow cytometry plots of cells pulsed with EdU and also labeled with propidium iodide to label DNA content. (B) Quantified data, mean ± SD.
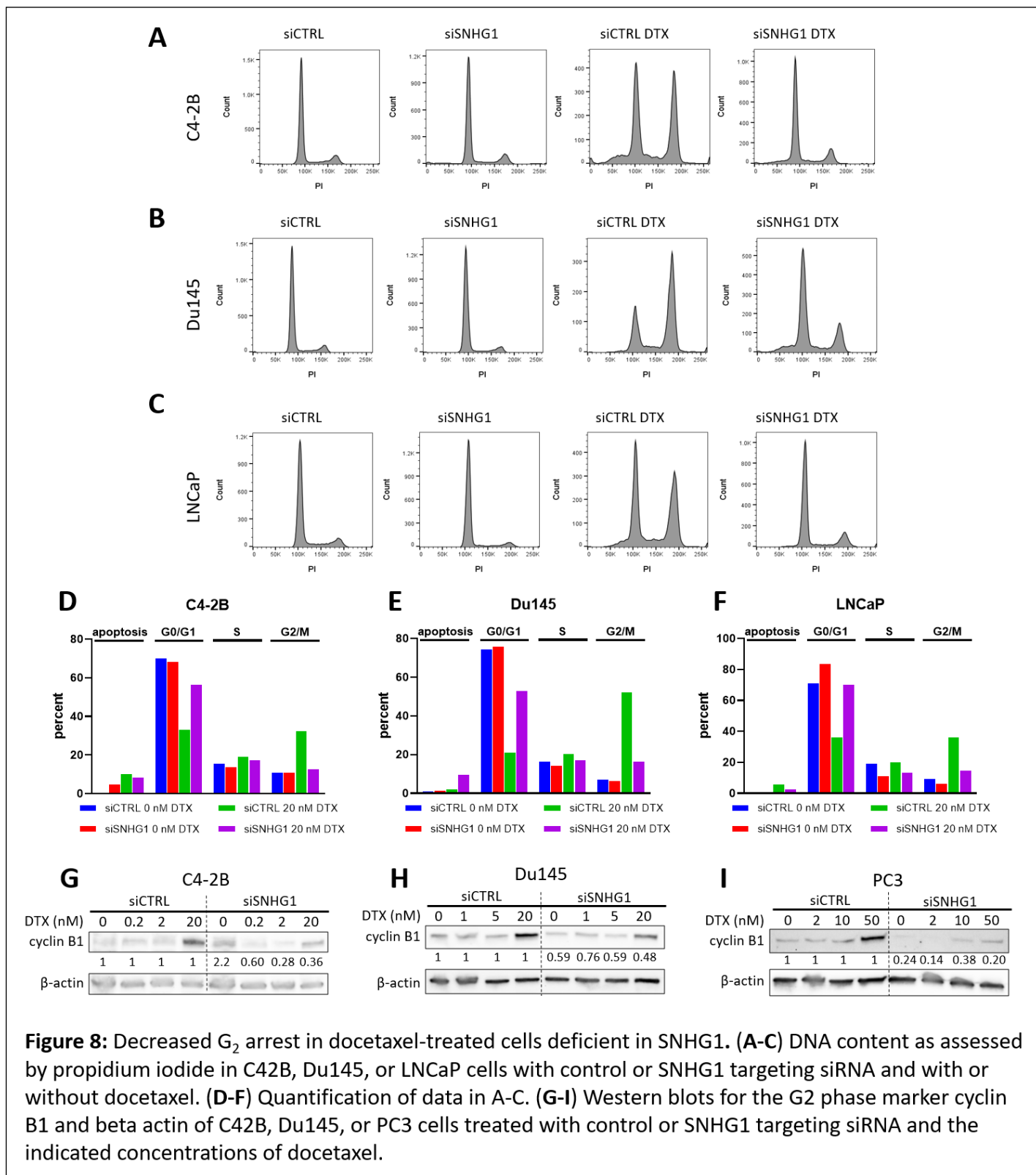
Figure 8: Decreased G_2 arrest in docetaxel-treated cells deficient in SNHG1**. (A-C)** DNA content as assessed by propidium iodide in C42B, Du145, or LNCaP cells with control or SNHG1 targeting siRNA and with or without docetaxel. **(D-F)** Quantification of data in A-C. **(G-I)** Western blots for the G2 phase marker cyclin B1 and beta actin of C42B, Du145, or PC3 cells treated with control or SNHG1 targeting siRNA and the indicated concentrations of docetaxel.

We also examined the effect of SNHG1 on prostate cancer cell cycle using three different cell lines in the presence of docetaxel – the most commonly used cytotoxic chemotherapy drug in prostate cancer. As expected, docetaxel treatment induced a G2 arrest. Surprisingly, this was almost completely blocked by knockdown of *SNHG1* as assessed by flow cytometry for DNA content. Note the disappearance of the right most peak (4n DNA) on the histograms in cells treated with both docetaxel and *SNHG1* knockdown (**Figure 8A – 8F**). To further validate these findings, we evaluated the accumulation of the G2 phase marker; cyclin B1. This marker accumulated at the higher docetaxel concentrations in control siRNA treated cells and was greatly reduced in cells treated with *SNHG1* siRNA. (**Figure 8G – 8I**).

Figure 9: SNHG1-deficient cells show reduced apoptosis in response to docetaxel. C42B (**A**), Du145 (**B**), and PC3 cells (**C** and **D**) were transfected with control or SNHG1 targeting siRNA and treated with the indicated concentrations of docetaxel. Lysates were blotted for PARP, caspase 3, cleaved caspase 3, or beta actin loading control as indicated.

We also evaluated docetaxel induced apoptosis in the context of SNHG1 depletion. For three cell lines, we saw that SNHG1 knockdown reduced the levels of apoptotic markers cleaved PARP and cleaved caspase 3 induced by docetaxel (**Figure 9**). Taking all of our SNHG1 data in context shows interesting but complex roles for SNHG1 in prostate cancer biology and treatment. Activity of SNHG1 stimulates exit from quiescence and cell cycle entry but also increases apoptosis in response to docetaxel. One potential explanation for these findings is that SNHG1 affects chemotherapy induced apoptosis through its cell cycle effects – because it decreases the number of cells in G_0 which are resistant to apoptosis.

Subtask 4: Determine the effect of *SNHG1* modulation on prostate cancer recurrence *in vivo*.

We have acquired protocol approval by the Wayne State Institutional Animal Care and Use Committee (IACUC) for and Department of Defense ACURO to perform these experiments.

Major Task 4: Ensure publication of at least one manuscript per specific aim.

Thus far, we have published two primary research articles which included preliminary data from the application for this award: Wang *et al* in *J Bone Oncology* and Pulianmackal *et al* in *Frontiers in Cell and Developmental Biology*.[5,6] Wang *et al* describes a new model of prostate cancer bone metastases. Pulianmackal et al use the p27 and CDT1 (venus-cherry) cell cycle markers to describe the potential cell cycle fates after a mitotic division and includes the bulk RNA-seq data which was much of our rationale for the study of the hippo pathway, and the qRT-PCR array data which suggested there is specific regulation of hippo pathway mRNA in quiescent prostate cancer cells (specific aim 3). Five other peer reviewed articles acknowledge support from this award and are reviews or primary research articles less directly related to the award.[8-12] Within the next year, we expect to publish the first primary research publication wholly from this award – on the role of SNHG1 in regulating prostate cancer quiescence. The manuscript will be similar in scope to the poster we presented at the AACR meeting this year on the same topic.[7]

What opportunities for training and professional development has the project provided?

In addition to the specific skills acquired, this project has been instrumental in my career development in other critical ways – especially in the assistance it provided in achieving promotion, an independent research laboratory and a competitive start-up package. When I received the positive funding decision for this award, I was a Clinical Lecturer at University of Michigan and did not have independent laboratory space. The Clinical Lecturer title at University of Michigan is intended to give new physician researchers time to develop a research portfolio and is limited to five years. Before receiving the positive funding decision on this award, I had an active Prostate Cancer Foundation Young Investigator Award for $225K of direct costs over three years, which was crucial to my career development. However, laboratory based researchers almost always need a career development award over twice this size in order to negotiate a physician scientist appointment, competitive start-up package, and workable clinic schedule for long term independent success. With the support of this Department of Defense Physician Research Award, I was able to successfully begin my independent career at Wayne State University and Karmanos Cancer Institute; with an appointment on the Clinical Scholar (tenure) Track, 750 square feet of laboratory space, a $750,000 start-up package, and a 25% clinical commitment; consisting of 1.5 days per week of genitourinary medical oncology clinic and not more than six weeks of hospital consult rounding per year. In my experience and the experience of my peers, this position would not have been have been possible without my DoD Physician Research Award or another similarly sized grant.

With this position have come other invaluable opportunities not specifically listed in the objectives of the grant. In addition to the expected opportunities at a research university and NCI Designated Comprehensive Cancer Center, Wayne State and Karmanos Cancer Institute (WSU / KCI) have unique opportunities beneficial to a prostate cancer researcher. We are a site for the DoD funded Prostate Cancer Clinical Trials Consortium (PCCTC), which greatly eases translating laboratory based discoveries to clinical trials. I am now a Co-Investigator on this grant, with salary support. Along with this are opportunities to design investigator initiated trials under the mentoring of our GU disease group lead and PCCTC site PI, Dr. Elisabeth Heath. Also, there are multiple advantages afforded by the geographic proximity to my previous institution (University of Michigan). Perhaps the most important of these is that University of Michigan and WSU / KCI share a Prostate SPORE grant. This relationship has allowed my first grant as principal investigator not geared toward career development – a pilot grant entitled "Profiling of Single Quiescent Prostate Cancer Cells Using a Neural Network Model." The geographic proximity also allows easy transfer of samples between me and my mentors and collaborators at University of Michigan which benefits the pilot grant and the current award. In the future, the geographic proximity will also be useful when I require hands on teaching and use of equipment at University of Michigan. As a further illustration of collaboration between the institutions, I published a co-first author publication with Dr. Arul Chinnaiyan and others at University of Michigan[11] (see also APPENDIX p 75-81). Lastly, WSU/KCI would not be so strong in prostate cancer research if not for the leadership and insight from our Genitourinary disease group lead, Dr. Elisabeth Heath. Her presence has also created multiple opportunities for both collaborative projects and funding. For example, along with Dr. Heath I was first author on a chapter published in the 2021 ASCO Educational Book[12] (APPENDIX p 25-36) and a review article on postate cancer dormancy and recurrence. Also, through Dr. Heath's mentoring and contacts, I am a member of and site PI for the PROMISE consortium – a multi-institutional collaborative focused on the effects of clinical genomic data on prostate cancer treatment.[8] See APPENDIX p 82-88 for a description of the consortium.[17]

Although not specifically listed in the Statement of Work, a major career development goal during this award period was setting up my independent research laboratory at Wayne State University and Karmanos Cancer Institute. I began laboratory set-up after my appointment began on May 1, 2021 and was ready to begin experiments in August. Laboratory set-up was critical for the experiential learning of laboratory management described in the Researcher Development Plan. During laboratory setup and hiring of personnel, I frequently consulted with members of my mentoring team, especially, Dr. Russell Taichman, who is now a Dental School Dean.

How were the results disseminated to communities of interest?

We have published work partially funded by this award in seven peer reviewed articles,[5,6,8-12] and at two scientific meetings.[7,18] Both of these meetings also included patient advocates. Please also see the "PRODUCTS" section below.

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

We expect continued progress during the next reporting period. Dr. Steven Zielske has made steady progress on Research Major Task 3 and will submit results for publication before the end of 2022. This progress on Research Major Task 3 will allow Dr. Zielske to devote more effort towards Research Major Task 1. Shortly before submission

of this report, rotating graduate student, Mr. Alexander Ullrich began working on Research Major Task 1, as guided by Drs. Zielske and Cackowski. Research Associate, Ms. Kristina Ibrahim, will devote about half of her time to Research Major Task 2.

4. **IMPACT:**

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

   Nothing to Report

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

   Nothing to Report

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

   Nothing to Report

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

   Nothing to Report

5. **CHANGES/PROBLEMS:**

   o **Changes in approach and reasons for change**

   Nothing to Report

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

   Wet bench productivity was mildly reduced due to COVID-19 safety precautions primarily during reporting period 1. However, institutional safety requirements dictated that only one person be present in the laboratory at a given time, which did reduce the amount of benchwork that could be conducted. Fortunately, wet bench activities have now returned mostly to normal.

   o **Changes that had a significant impact on expenditures**

   Nothing to report

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

   o **Significant changes in use or care of human subjects:** Nothing to report

   o **Significant changes in use or care of vertebrate animals:** Nothing to report

   o **Significant changes in use of biohazards and/or select agents:** Nothing to report

6. **PRODUCTS:**

   o **Publications, conference papers, and presentations**

   - **Journal publications.**

     Kaplan Z, Zielske SP, Ibrahim KG, Cackowski FC. Wnt and beta-Catenin Signaling in the Bone Metastasis of Prostate Cancer. *Life (Basel)* 2021; **11**(10).
     > Status: published
     > Federal support acknowledged: yes

     Cackowski FC, Heath EI. Prostate cancer dormancy and recurrence. *Cancer Lett* 2022; **524**: 103-8.
     > Status: published
     > Federal support acknowledged: yes

     Koshkin VS, Patel VG, Ali A, et al. PROMISE: a real-world clinical-genomic database to address knowledge gaps in prostate cancer. *Prostate Cancer Prostatic Dis* 2021.
     > Status: published
     > Federal support acknowledged: no

     Pulianmackal AJ, Sun D, Yumoto K, Li Z, Chen YC, Patel M, Wang Y, Yoon E, Pearson A, Yang Q, Taichman R, Cackowski FC, and Buttitta L. Monitoring Spontaneous Quiescence and Asynchronous Proliferation-Quiescence Decisions in Prostate Cancer Cells. *Front Cell Dev Biol* 2021; **9**: 728663.
     > Status: published
     > Federal support acknowledged: yes

     Wang Y, Zielske S, Ellis L, Taichman R, and Cackowski FC. Use of FVB Myc-CaP Cells as an Immune Competent, Androgen Receptor Positive, Mouse Model of Prostate Cancer Bone Metastases. *J Bone Oncol* 2021; **30**: 100386.
     > Status: published
     > Federal support acknowledged: yes

     Jung Y, Cackowski FC, Yumoto K, et al. Abscisic acid regulates dormancy of prostate cancer disseminated tumor cells in the bone marrow. *Neoplasia* 2021; **23**(1): 102-11.
     > Status: published
     > Federal support acknowledged: yes

     Cackowski FC, Kumar-Sinha C, Mehra R, Wu YM, Robinson D, Alumkal J, and Chinnaiyan A. Double-Negative Prostate Cancer Masquerading as a Squamous Cancer of Unknown Primary: A Clinicopathologic and Genomic Sequencing-Based Case Study. *JCO Precis Oncol* 2020; **4**.
     > Status: published
     > Federal support acknowledged: yes

   - **Books or other non-periodical, one-time publications.**

     Cackowski FC, Mahal B, Heath EI, Carthon B. Evolution of Disparities in Prostate Cancer Treatment: Is This a New Normal? *Am Soc Clin Oncol Educ Book* 2021; **41**: 1-12.
     > Status: published
     > Federal support acknowledged: yes

   - **Other publications, conference papers, and presentations.**

     Zielske SP, and Cackowski, F. C. . The lncRNA SNHG1 modulates quiescence and chemoresistance of prostate cancer. The American Association for Cancer Research; 2022 April 8-13; New Orleans, LA; 2022.
     > Status: presented (poster)
     > Federal support acknowledged: yes

Pulianmackal AJ, Sun, D., Yumoto, K., Li, Z., Chen, YC., Patel, M., Wang, Y., Yoon, E., Pearson, A., Qiong, Yang., Taichman, R.S., Cackowski, F.C., Buttitta, L. . Monitoring spontaneous quiescence and asynchronous proliferation-quiescence decisions in prostate cancer cells. Prostate SPORE Research Retreat; 2022; Los Angeles, CA; 2022
     Status: presented (poster)
     Federal support acknowledged: yes

- o **Website(s) or other Internet site(s)**
  Nothing to Report

- o **Technologies or techniques**
  Nothing to Report

- o **Inventions, patent applications, and/or licenses**
  Nothing to Report

- o **Other Products**
  Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Frank Cackowski, MD, PhD
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-0075-3745
Nearest person month worked:	6
Contribution to Project:	Supervision, overall direction, benchwork, student mentoring
Funding Support:	This grant, Prostate Cancer Foundation Young Investigator Award, and Karmanos Cancer Institute start-up funds

Name:	Steven Zielske, PhD
Project Role:	Scientist
Researcher Identifier (e.g. ORCID ID):	none
Nearest person month worked:	4
Contribution to Project:	Benchwork predominantly on Major Task 3, and student mentoring
Funding Support:	This grant, Prostate Cancer Foundation Young Investigator Award, and Karmanos Cancer Institute start-up funds

Name:	Alexis Wilson
Project Role:	Graduate student
Researcher Identifier (e.g. ORCID ID):	none
Nearest person month worked:	2
Contribution to Project:	Benchwork predominantly on Major Task 2
Funding Support:	NCI T32 training grant

Name:	Kristina Ibrahim, MS
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	none
Nearest person month worked:	6
Contribution to Project:	Cell culture maintenance, ordering supplies, bench work on Major Task 2
Funding Support:	Karmanos Cancer Institute start-up funds, this grant

Name:	Alexander Ullrich, MS
Project role:	Graduate student
Researcher Identifier (e.g. ORCH ID):	0000-0003-3720-1212
Nearest person month worked:	0
Funding Support	NCI T32 training grant

Has there been change in active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to Report

o **What other organizations were involved as partners?** Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**: Nothing to Report

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2. Cackowski FC, Taichman RS. Minimal Residual Disease in Prostate Cancer. *Adv Exp Med Biol* 2018; **1100**: 47-53.
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5. Wang Y, Herroon MK, Zielske SP, et al. Use of FVB Myc-CaP cells as an immune competent, androgen receptor positive, mouse model of prostate cancer bone metastasis. *J Bone Oncol* 2021; **30**: 100386.
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9. Kaplan Z, Zielske SP, Ibrahim KG, Cackowski FC. Wnt and beta-Catenin Signaling in the Bone Metastasis of Prostate Cancer. *Life (Basel)* 2021; **11**(10).
10. Jung Y, Cackowski FC, Yumoto K, et al. Abscisic acid regulates dormancy of prostate cancer disseminated tumor cells in the bone marrow. *Neoplasia* 2021; **23**(1): 102-11.
11. Cackowski FC, Kumar-Sinha C, Mehra R, et al. Double-Negative Prostate Cancer Masquerading as a Squamous Cancer of Unknown Primary: A Clinicopathologic and Genomic Sequencing-Based Case Study. *JCO Precis Oncol* 2020; **4**.
12. Cackowski FC, Mahal B, Heath EI, Carthon B. Evolution of Disparities in Prostate Cancer Treatment: Is This a New Normal? *Am Soc Clin Oncol Educ Book* 2021; **41**: 1-12.
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9. **APPENDICES:**

DEPARTMENT OF THE ARMY

HEADQUARTERS, U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
810 SCHREIDER STREET
FORT DETRICK, MD 21702-5000

August 03, 2021

Director, Office of Research Protections
Animal Care and Use Review Office (ACURO)

Subject: Approval of Proposal Number PC190093, Award Number W81XWH-20-1-0394 entitled, "Regulation of Prostate Cancer Dormancy and Recurrence by Hippo Signaling"

Dr. Frank Cackowski, MD, PhD
Wayne State University
Detroit, MI, US

Dear Dr. Frank Cackowski, MD, PhD:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Conducted and Supported Research and Training"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"

In accordance with the above references, ACURO protocol PC190093.e001 entitled, "The Hippo pathway in prostate cancer dormancy and recurrence," IACUC protocol number IACUC-20-07-2485, Protocol Principal Investigator Dr. Frank Cackowski, MD, PhD, is approved by ACURO as of 07/29/2021 for the use of mice and will remain so until modification, expiration or cancellation. This protocol was approved by the Wayne State University IACUC on 09/15/2020; IACUC approval expires 09/14/2023.

Required Actions:

A. Submit to ACURO for review and approval prior to implementing:

• IACUC-approved de novo reviews of the protocol

• IACUC-approved significant changes to this protocol (see guidance document)

B. Notify ACURO within 5 business days of any of the following:

• Any noncompliance, suspensions or adverse events (see guidance document)

• Receipt of notification that the institution is under investigation by USDA

• AAALAC, International accreditation status change

For further assistance, please contact ACURO at (301) 619-6694, FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

Krinon Moccia, DVM, MPH, DACLAM
LTC, VC, USA
Director, Animal Care and Use
 Review Office

Copies Furnished:
Dr. Frank Cackowski, MD, PhD
Asa R. Mulligan
IACUC Wayne State University
Michael Bradley
Dr. Lymor R. Barnhard
Asa Mulligan

Evolution of Disparities in Prostate Cancer Treatment: Is This a New Normal?

Frank C. Cackowski, MD, PhD[1]; Brandon Mahal, MD[2]; Elisabeth I. Heath, MD[1]; and Bradley Carthon, MD, PhD[3]

overview

Despite notable screening, diagnostic, and therapeutic advances, disparities in prostate cancer incidence and outcomes remain prevalent. Although commonly discussed in the context of men of African descent, disparities also exist based on socioeconomic level, education level, and geographic location. The factors in these disparities span systemic access issues affecting availability of care, provider awareness, and personal patient views and mistrust. In this review, we will discuss common themes that patients have noted as impediments to care. We will review how equitable access to care has helped improve outcomes among many different groups of patients, including those with local disease and those with metastatic castration-resistant prostate cancer. Even with more advanced presentation, challenges with recommended screening, and lower rates of genomic testing and trial inclusion, Black populations have benefited greatly from various modalities of therapy, achieving comparable and at times superior outcomes with certain types of immunotherapy, chemotherapy, androgen receptor–based inhibitors, and radiopharmaceuticals in advanced disease. We will also briefly discuss access to genomic testing and differences in patterns of gene expression among Black patients and other groups that are traditionally underrepresented in trials and genomic cohort studies. We propose several strategies on behalf of providers and institutions to help promote more equitable care access environments and continued decreases in prostate cancer disparities across many subgroups.

Prostate cancer continues to be the leading cancer among men in the United States, with 33,000 deaths and 191,000 cases occurring in 2020.[1] One in nine men overall in the United States develops prostate cancer in his lifetime. In Black men, however, this rate is one in seven, and the incidence rate among Black men is 2.2 times that in White men, with the death rate 1.7 times higher.[1] Moreover, although Black men are diagnosed at younger ages, they present routinely with more advanced disease and higher prostate-specific antigen (PSA) levels at presentation than other groups.[2-7]

Disparities have been shown to exist not only between ethnic groups but also based on socioeconomic level,[8,9] education level,[10] and rural versus urban residency.[11] Here, we will examine common areas of disparity in prostate cancer. We will also address some of the systemic and personal patient views that contribute to these disparities. We will show how equitable access to care may alleviate some of these areas of disparity, especially in the context of challenges brought on by the COVID-19 pandemic. Lastly, we will examine molecular signatures and the trends and roles they may play in disparate outcomes in patients with prostate cancer.

CHANGING TRENDS

Over the last few decades, technologic and screening advances have led to a decrease in some of the noted prostate cancer disparities.[12] U.S. Surveillance, Epidemiology, and End Results database analyses show that at a peak in 1992, there were approximately 237 new cases per 100,000 patients. This decreased markedly over time to a nadir of 102 cases per 100,000 patients.[13,14] Five-year overall survival increased during the same period of time, from 96% to greater than 98% for all patients registered.[13,15] Moreover, the disparities between Black and White patients have narrowed over time, with recent analyses of prostate cancer survival rates between 2001 and 2016 showing Black men with metastatic prostate cancer are surviving at a similar rate to White men, with approximately 31% of men alive at 5 years.[12,15] There are likely several reasons for this, including screening increases as well as treatment advances being available. The rates and any differences in incidence and mortality in the future remain to be seen. Changes in screening recommendations by the U.S. Preventive Services Task Force in 2012 against general PSA screening and later in 2018 to favor open-ended discussion on screening for most men at general risk of prostate cancer may have played a role in a disproportionate effect within Black populations, which had a 29% decrease in screening rates after the recommendation changes, despite being at higher risk.[16] Decreases in screening have previously been shown to have an effect on later presentation patterns. Indeed, in the 5 years after the initial

PRACTICAL APPLICATIONS

- Prostate cancer disparities exist in the context not only of race and ethnicity but also of socioeconomic level, education level, and geographic location.
- Systemic hurdles to access with regard to screening, diagnostics, and therapeutics, as well as personal patient views and medical hesitancy, may play a role in noted disparities.
- Despite presenting with more advanced disease, Black patients demonstrate favorable outcomes in trial formats and equal access environments with regard to multiple types of therapy.
- Genomic trial data and information useful for all patients depend on diverse patient inclusion to ensure applicability to a wide population.

decreased PSA screening recommendation, there was a decrease in incidence of local stage disease but increases in regional and distant metastases.[17-19] Drops in screening rates have also become more problematic during the COVID-19 pandemic. Data demonstrate that prostate cancer screening rates dropped approximately 60% to 75% in different series during the COVID-19 pandemic when compared with prior time periods.[20,21] Prostate cancer pathologic diagnoses have also decreased in several series examined, with declines ranging from 20% to 39% lower than the preceding year.[20,22] The total effects of the COVID-19 pandemic on overall outcomes are not yet clear, but early analyses paint a challenging picture. A recent case control analysis of more than 73 million electronic medical records showed that Black patients with prostate cancer were five times more likely to be infected with COVID-19 (odds ratio, 5.10; 95% CI, 4.34–5.98; p < .001). There was a synergistic adverse effect of COVID-19 and cancer with regard to patient rates of hospitalization (Black patients, 55.56% vs. White patients, 43.24%; p = .003).[23] The life expectancy for all men in the United States is estimated to have dropped by approximately 1.13 years during the early parts of the COVID-19 pandemic, but the life expectancy for Black men decreased by approximately 2.10 years and the life expectancy for Latino men decreased by approximately 3.05 years during the same period.[24] This rapid decrease in life expectancy has been suggested to be due in large part to COVID-19 effects. The long-term effects of delayed care with regard to the most common medical comorbidities, such as cardiovascular disease, chronic kidney disease, diabetes, and cancer, present in these groups remain to be seen, but indeed, these groups seem to have increased COVID-19–related mortality in the short term in meta-

analyses.[25] Moreover, loss of insurance stemming from jobs that were disproportionally affected during the pandemic can amplify the effects of normal medical visits and screening to detect these comorbid conditions and cancers, especially among Black and Hispanic workers.[26]

Whereas some of these changes in prostate cancer epidemiology are related to policy changes on screening and recent pandemic-related factors, there remains a host of other underlying problems affecting clinical care and even beneficial measures such as clinical trial participation. Standard-of-care benchmark treatments and clinical trials are vitally important parts of cancer care progress. Limited access to care, language barriers, transportation, and cost of care, even among insured patients, can also play a role in trial participation.[27] Patients who live farther from cancer centers have been shown to be less likely to enroll in clinical trials as well.[28] Underserved patients have also been shown to be less likely to regularly visit their physicians or enroll in interventional clinical trials.[29-32] Importantly, hurdles remain because of patient perception and medical mistrust among many affected high-risk groups.

Engaging local resources within respective communities may help provide culturally appropriate screening strategies and methods to address prostate cancer treatment. Surveyed men noted insufficient information regarding prostate cancer, medical mistrust, poor relationships with medical providers, and lack of sustained relationships from within the community as barriers to an emphasis on prostate health.[33] Embarrassment and fear of a positive diagnosis, reluctance to talk about sexually related complications, and beliefs that prostate cancer may stem from sexual behavior have also been cited during focus groups, which may affect continuity of cancer education and care.[34] Strong emphasis on a role for information from cancer survivors was noted.[33,35,36] Successful outreach efforts have used community institutions such as churches and barbershops along with trusted community educators and local patients with cancer and contacts.[37-41] Indeed, familiarity with presenters has been shown to lead to more highly rated and effective engagement during educational ventures.[42,43] Partners and spouses are also highly useful participants in educational efforts.

DIFFERENCES IN PATTERN OF AND RESPONSE TO TREATMENT: NOT JUST RACE

In the last several years, patients, the research community, and the press have appropriately paid an increasing amount of attention to disparities in outcomes based on race for prostate cancer and other malignancies. However, prostate cancer disparities also exist based on other factors, including rural versus urban location, socioeconomic status, ethnicity, clinical trial participation, and country of treatment. Here, we briefly discuss the multiple demographic

determinants of disparities in prostate cancer and focus on treatment and response to treatment rather than purely on outcomes. These disparities are complex in etiology and may be addressed in part by providers familiarizing themselves with where disparities exist and options for care,[44] improving communication skills,[42] and standardizing treatment options.[45] Health care entities may also help by providing more broadly based support with navigators and other support mechanisms for clinical choices and presenting trial opportunities for patients.[46-48]

Race and Ethnicity

Patients with prostate cancer may receive different treatments based on stage, but there is a striking racial disparity in the treatment of localized disease. Using U.S. Surveillance, Epidemiology, and End Results data, Moses et al[45] reported that Black men were less likely than White men to receive definitive treatment by either prostatectomy or definitive radiation therapy for localized prostate cancer (odds ratio, 0.73; 95% CI, 0.71–0.75; $p < .001$). Although of a smaller magnitude, a significant disparity also existed between Hispanic and White patients, with fewer Hispanic patients receiving definitive treatment (odds ratio, 0.95; 95% CI, 0.92–0.98; $p < .001$). The racial disparity also seems to extend to the use of molecular imaging. In a single-center study, Black patients were more likely than White patients to receive the older fluciclovine PET/CT as opposed to the newer gallium-68 prostate-specific membrane antigen PET/CT to evaluate biochemically recurrent prostate cancer.[49] Although direct comparison studies do not exist, prostate-specific membrane antigen–based PET scans are generally agreed to be more sensitive than fluciclovine PET scans.[50] Although on average Black patients with prostate cancer receive less intensive treatment of localized disease, they unfortunately are more likely to have decisional regret afterward and may later wish they had opted for more aggressive approaches to their treatment.[51] Lastly, even if assigned the same treatment, Black patients might be less likely to complete it. In a study of 25,727 Black and 126,199 White patients, 12.8% of Black men did not complete definitive radiation therapy for localized prostate cancer as compared with 11.8% of White men (odds ratio, 1.14; 95% CI, 1.09–1.19; $p < .001$).[52]

Geographic Location

Other factors besides race and ethnicity are associated with disparities in prostate cancer treatment. Patients in nonurban areas are more likely than patients in urban areas to receive no treatment of prostate cancer. Conversely, patients in urban areas are more likely to receive radical prostatectomy than patients in nonurban areas.[53] Many researchers might expect that underserved patients treated at academic centers might receive more optimal or perhaps more aggressive care for prostate cancer than those patients treated at nonacademic centers. However, this does not consistently seem to be the case. Black, Hispanic, and uninsured patients were all more likely to experience treatment delays than White patients at both academic and nonacademic centers.[54] Delays were actually slightly longer at academic centers, although academic and nonacademic centers were not directly compared. However, in a different study, patients at an academic center were more likely to be treated with radical prostatectomy (odds ratio, 2.57; 95% CI, 2.45–2.69; $p < .001$) as compared with community sites.[55]

Financial and Socioeconomic Status

Another key area affecting treatment of prostate cancer comprises financial concerns and socioeconomic status. Sayyid et al[56] found in a U.S. population that high socioeconomic status was associated with an increased odds ratio for receipt of definitive treatment of localized disease. Socioeconomic status is interrelated with race, and the two can be difficult to disentangle. In a large series of patients from Detroit, Michigan, Black patients with prostate cancer had lower survival, but adjustment for treatment and socioeconomic status removed the survival difference.[57] However, even in a more homogenous population (Geneva, Switzerland), patients with prostate cancer of lower socioeconomic status had shorter survival. The survival difference was attributed to delayed diagnosis, different diagnostic workup, and less invasive treatment.[58] Prostate cancer treatment also differs on a wider scale, between rich and poor countries, as opposed to between patients of different socioeconomic status within a country. As an example, clinical trial participation varies greatly between countries. A recent study estimated that 96% of participants in clinical trials are White and that only 3% of African and Caribbean countries are even included in any clinical trials.[59] Cooperative group trials in the United States are somewhat better, but Black patients make up only approximately 9% of participants, less than the overall population and less than the overall percentage of patients with prostate cancer.[60,61] The relatively low population of patients in clinical trials across many trial formats makes it challenging to apply trial results to a diverse population.

RESPONSE TO PROSTATE CANCER TREATMENT AND MITIGATION OF DISPARITIES

Despite the disparities in outcome discussed earlier and the differing treatment patterns outlined, one may argue that meaningful differences can be made by the practice patterns of individual physicians.[44,62] Other points of view would support that often-touted biologic differences are minimal as opposed to large-scale societal issues that may address disparities.[63] Despite the complexity and multiple factors at play, mitigation of prostate cancer disparities will likely involve efforts such as enrollment in clinical trials that contribute toward better outcomes. Importantly, Black men in

particular seem to respond at least as well as White men to standardized prostate cancer treatments.

Black Patients Have Equal or Better Treatment Response

In the metastatic setting, Black men seem to respond better than or similar to White men to multiple different treatments. Among those with metastatic castration-resistant prostate cancer, Black patients have better and more durable PSA responses.[64,65] In the area of immunotherapy, which has overall been disappointing in prostate cancer, the survival data in Black men are more encouraging. In a registry analysis, Black men treated with sipuleucel-T had a significant reduction in risk of death, with hazard ratios of 0.81 (95% CI, 0.68–0.97; p = .03) for all patients and 0.70 (95% CI, 0.57–0.86; p < .001) in patients with matched PSA values.[66] This pattern was corroborated on additional analyses.[67] Black men treated with docetaxel for metastatic castration-resistant prostate cancer had a similar survival to that of White men by raw numbers but had a lower risk of death when adjusted in a multivariable fashion within a large meta-analysis (HR, 0.81; 95% CI, 0.72–0.91; p < .001).[68] Differences have also been examined in the use of radium-223, a radiopharmaceutical agent approved in the castration-resistant prostate cancer setting. In a retrospective analysis of 318 patients receiving this agent, Black men had a lower risk of death from the time of radium-223 initiation (HR, 0.75; 95% CI, 0.57–0.99; p = .045).[69] Differences in treatment response have also been well elucidated with regard to oral novel androgen receptor pathway inhibitors. In the prechemotherapy castration-resistant prostate cancer setting, Black patients had a 53% rate of PSA decrease to greater than 90%, whereas only 31% of corresponding White patients had a similar reduction with abiraterone, a CYP-17 lyase inhibitor.[70] Statistical power in this study was limited by the low number of Black patients (28 of 1,088 total patients).[70] Patients treated in an equal access center had similar patterns of PSA decline with abiraterone, with 68.9% of Black patients demonstrating PSA level decline of 50% or greater versus 48.9% of White patients (p = .028).[64] A separate analysis of abiraterone or enzalutamide in castration-resistant prostate cancer showed improved overall survival of 918 days for Black patients versus 781 days over their White counterparts (HR, 0.826; 95% CI, 0.732–0.933).[71] Importantly, this benefit of androgen-based therapies has also been analyzed in a prospective fashion, where PSA progression-free survival was approximately 16.6 months in Black patients (95% CI, 11.5 to not reached) versus 11.5 months in non-Black patients (95% CI, 8.5–19.3) treated for castration-resistant prostate cancer.[65] These various outcomes in prostate cancer for Black patients are summarized in Table 1.[72]

The reason for similar or enhanced responses in Black patients versus White patients with metastatic castration-resistant prostate cancer is not completely understood. Genomic differences that may play a role in effects on various treatment pathways are explored more in depth later in this review. For example, in small cohorts of patients with metastatic castration-resistant prostate cancer, Black men demonstrated more tumor mutations in androgen receptor and DNA repair genes compared with other men.[73] Lastly, Black patients may have an equal or better response to therapy in localized disease as well, if they receive the same treatment as their White counterparts. As mentioned, in selected U.S. Surveillance, Epidemiology, and End Results analyses, Black men were more likely to receive no treatment at all or to receive radiotherapy versus radical prostatectomy when compared with White men (HR, 1.03; p = .041).[62] The patients with similar risk profiles analyzed in this study had no difference in overall survival. In a separate analysis of data from the U.S. Veterans Administration, however, Black men had a lower 10-year all-cause mortality than White men who received definitive radiation therapy for localized prostate cancer.[74] The mortality racial differences by type of primary treatment seen in some series of patients with prostate cancer but not others require more study and analysis. Most analyses have been retrospective and may have been affected by various types of bias. Additional prospective analyses for localized prostate cancer treatment outcomes by race are necessary.

Treatment in Clinical Trials Mitigates Disparities

Black men and patients from other minority groups have a lower rate of participation in prostate cancer clinical trials, both in the United States[75] and globally.[59] Additionally, rural residents are less likely to enroll in clinical trials overall, although there are limited analyses specific to prostate cancer.[76] However, treatment in a clinical trial has been shown to eliminate differences in overall survival, both for Black patients with metastatic castration-resistant prostate cancer[77] and for patients with cancer in rural areas overall.[76] Several problematic issues exist even with large clinical trials that may confound trial availability and outcomes for marginalized patients. These may include site selection that favors more affluent populations, criteria that neglect comorbidities with real-world populations, distance from trial sites, and lack of insurance.

Importantly, additional confounding issues may also affect clinical trial availability. Lack of accrual has been shown to be a major reason for clinical trial closure and affects the presence of clinical treatment options for future patients.[78] Poor performance status is often a major reason for patients not qualifying for or being excluded from clinical trials.[28] Analyses of large phase III clinical trials demonstrated that 96% of patients enrolled across 600 analyzed trials had an Eastern Cooperative Oncology Group performance status of 0 or 1.[79] Analysis of clinical trial enrollment in

TABLE 1. Analyses Demonstrating Therapeutic Efficacy in Black Patients With Metastatic Prostate Cancer

Author	Agent Investigated	Trial and Analysis Type	Number of Patients	Endpoint	Outcomes
Halabi et al[68]	Docetaxel	Meta-analysis	8,820 (White, 7,528 [85%]; Black, 500 [6%])	Median OS and risk of death	Median OS, 21.0 vs. 21.2 months; (multivariable HR, 0.81; 95% CI, 0.72–0.91; p < .001)
Ramalingam et al[64]	Abiraterone	Case control analysis	135 (White, 90 [66%]; Black, 45 [33%])	PSA response	68.9%; ≥ 50% PSA level decline in Black patients vs. 48.9% in White patients (p = .028)
Efstathiou et al[70]	Abiraterone	Retrospective subset analysis	28 Black patients (of 1,088 total patients in COU-AA-302)	PSA response, radiographic PFS	> 90% PSA in 53% of Black patients vs. 31% of White patients; radiographic PFS, 16.6 months in Black patients vs. 11.1 in White patients
McNamara et al[71]	Abiraterone or enzalutamide in CRPC	Retrospective medical record review of VA database	787 Black patients and 2,123 White patients with CRPC	Median OS and risk of death	Median OS, 918 days for Black patients and 781 days for White patients (multivariable HR, 0.826; 95% CI, 0.732–0.93; p = .0020)
George et al[65]	Abiraterone in metastatic CRPC	Prospective parallel group study	50 Black patients and 50 White patients	PSA, PFS, PSA response	Median PSA PFS, 16.6 months for Black patients vs. 11.5 for White patients; > 90% PSA decline in 48% of Black patients vs. 38% of White patients
Sartor et al,[66] Higano et al[67]	Sipuleucel-T	Registry cohort analysis	1,976 (White, 1,649 [83.4%]; Black, 221 [11.1%])	Median OS and risk of death	Median OS, 25.8 vs. 35.3 months (HR, 0.81; 95% CI, 0.68–0.97; p = .03) in all patients (HR, 0.70; 95% CI, 0.57–0.86; p < .001) in PSA-matched set (HR, 0.60; 95% CI, 0.48–0.74; p < .001)
Zhao et al[69]	Radium-223	Retrospective medical record review of VA database	87 Black patients (27%) of 318 patients treated with radium-223	Risk of death	Black race was associated with decreased risk of mortality (HR, 0.75; 95% CI, 0.57–0.99; p = .045)

Abbreviations: OS, overall survival; PSA, prostate-specific antigen; PFS, progression-free survival; CRPC, castration-resistant prostate cancer; VA, Veterans Affairs.

Data adapted from Carthon et al.[72]

a predominantly Black population also showed that these patients were often unable to take part in clinical trials because of comorbid conditions.[80] Although large clinical trials may help answer pertinent and relevant questions, they often exclude the very groups that may benefit most. These limitations in clinical trial design highlight the importance of including a diverse population of patients who are able to safely enroll.

Outreach, Communication, and Action Items

Individual physicians and other health care providers have the capacity to educate and help mitigate disparities in prostate cancer treatment and outcomes in their practice. Given that Black men have had similar or better responses to treatment than White men for both localized and metastatic prostate cancer, we recommend treatment for Black men should be as intensive as is safe and supported by the literature. This contrasts with a potential pitfall of treating Black patients more conservatively to avoid doing harm. Second, we recommend treatment in a clinical trial for any patient, but especially for underserved populations. Because Black men in particular have higher mistrust of the medical system in general and of clinical trials in particular, special efforts in communication may be required for Black patients. Although working to help rectify entrenched societal differences may seem daunting for an individual practitioner, recognizing that there are existing organizations in place can be the first step in countering systemic obstacles to care. These include the National Cancer Institute Geographic Management of Cancer Health Disparities and National Cancer Institute Center to Reduce Cancer Health Disparities.[81,82] These entities not only support research but also provide assistance to cancer health professionals and patients and work to diversify the cancer care workforce.

Patient beliefs may also play a role in outcomes, both during screening and also once a patient is actually diagnosed with cancer and must undergo therapy. The exact contribution of patient beliefs (versus implicit or explicit bias) to systemic access issues is difficult to determine.[83,84] Race alone has not been shown to be predictive of poor outcomes when examining multiple forms of treatment, including watchful waiting,[85] prostatectomy,[85] or even definitive radiation therapy.[86,87] Various studies suggest that hurdles such as more adverse presenting features, negative patient beliefs, and systemic access issues may be overcome to some degree by receiving care in more equitable access environments or via other mechanisms.

Patient navigation is an opportunity to make use of nursing and other support professionals for better health outcomes. This also has the potential to provide increased access to clinical trial enrollment among diverse populations. This approach has been used in several cancers and has been shown to help with screening and treatment planning in Black patients with prostate cancer. Men benefited by having more timely and increased rates of follow-up.[43,88] Standard use of patient navigators may improve patient outcomes with regard to disparities in screening and overall outcomes in prostate cancer.

As we discussed, treatment in a clinical trial can help eliminate racial disparities,[89] but Black patients are less likely than White patients to participate in prostate cancer clinical trials.[75] Therefore, we encourage prostate cancer physicians to offer clinical trials to patients in all demographic groups. Additionally, the U.S. Food and Drug Administration has studied potential solutions to disparate clinical trial enrollment in patients from multiple demographic groups in part through workshops.[90] Multiple solutions were proposed in a nonbinding U.S. Food and-Drug Administration guidance document,[91] including loosening exclusion criteria throughout clinical trials, not copying overly restrictive phase II exclusion criteria in phase III trials, making trials less burdensome for patients by including more telehealth visits and reimbursing trial participants for travel expenses, conducting community outreach events, and including trial sites with a high percentage of underrepresented populations in patient pools. Although Black people make up approximately 12% of the U.S. population and approximately 22% of patients with cancer in the United States,[60] they constitute a small proportion of patients enrolled in pharmaceutical industry–sponsored trials (approximately 3%) and are enrolled less frequently in general in cooperative group (approximately 9%) and other types of trials.[61] Therefore, in addition to individual physicians, authors of clinical trial protocols in academia and industry have an opportunity to lessen prostate cancer health disparities through thoughtful trial design.

PROSTATE CANCER DISPARITIES: GENOMIC DATA

A historical and persistent difficulty in the management of prostate cancer is being able to identify and distinguish indolent from aggressive disease.[92-94] Prostate cancer incidence and mortality rates vary widely by ancestry, with men of African ancestry experiencing the greatest burden of disease, likely because of the interplay of socioeconomic factors, environmental exposures, and biologic/epigenetic phenomena.[95-100] Decades of evidence have clearly established that Black men have a much greater burden of prostate cancer than men of European ancestry, including a nearly two-fold increased risk of developing prostate cancer and a more than 2.2-fold increased risk of dying as a result of prostate cancer.[1] As noted earlier, Black men present at earlier ages and with more advanced disease but are less likely to have access to screening and guideline-concordant care, when compared with other men.[101]
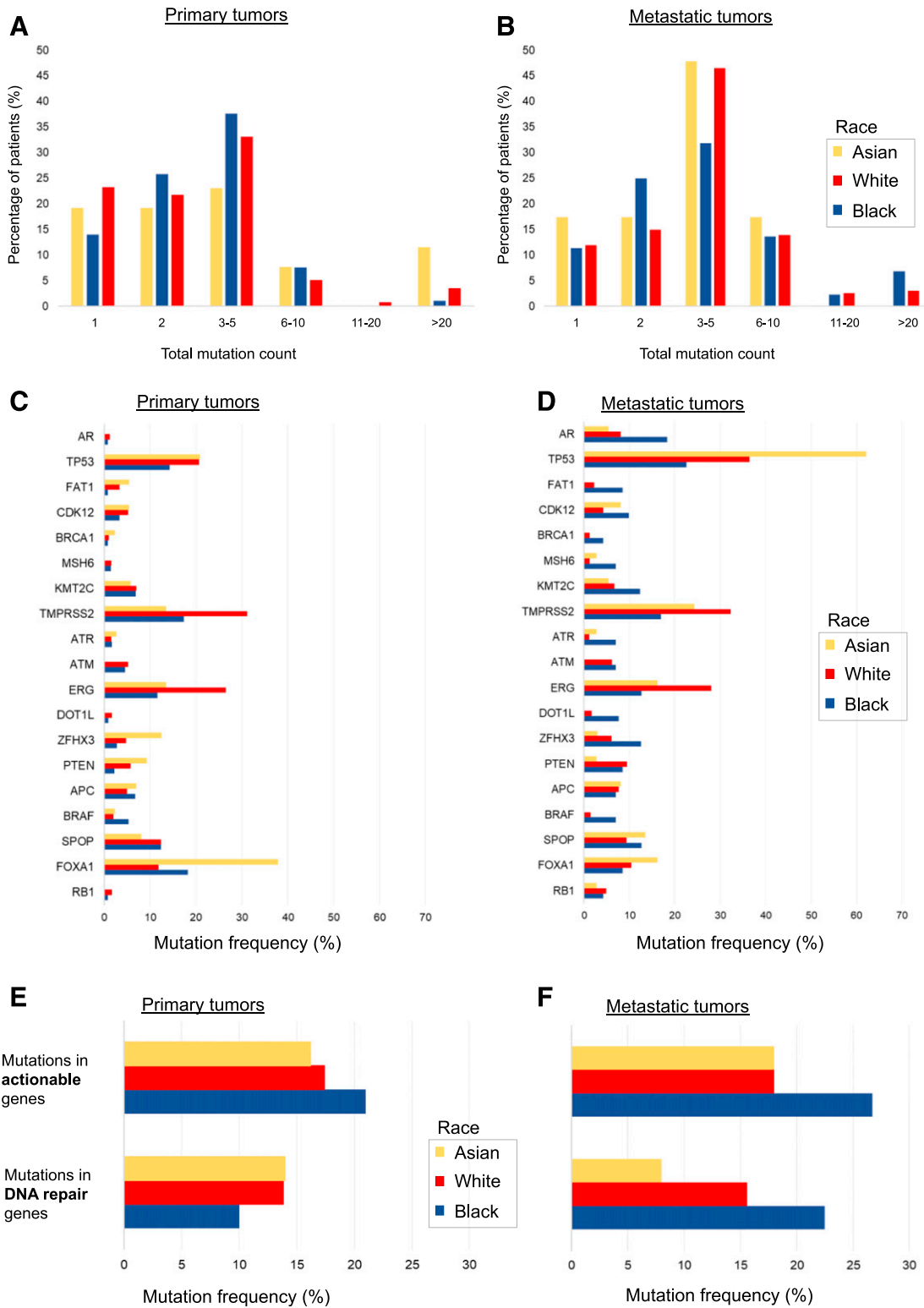
FIGURE 1. Tumor Mutation Profiles by Race in Primary and Metastatic Prostate Cancer

Tumor mutation profiles (MSK-IMPACT and Dana-Farber Sequencing) by race in 2,393 patients (2,109 White; 204 Black; 80 Asian) with primary (1,484 patients [1,308 White; 133 Black; 45 Asian]) or metastatic prostate cancer (909 patients [801 White; 71 Black; 37 Asian]). (A, B) Total mutation count was calculated in the MSK-468 cohort. (C, D) Mutation frequency in primary

FIGURE 1. (Continued). and metastatic tumors. (E, F) DNA repair genes include *ERCC5, MRE11, TP53BP1, POLE, RAD21, MSH2, MSH6, BRCA1/2, ATR, and ATM*. In metastatic cases, DNA repair gene mutations occurred more often in Black men (22.5%) compared with White men (15.6%; p = .05). Mutations in *ATR* and *MSH6* occurred more often in Black compared with White men (7.0% vs. 1.1%; p = .0002 for both). Actionable mutations include *ABL1, EGFR, ERBB2, BRAF, BRCA1/2, FGFR2/3, KIT, NTRK1/2/3, PDGFRA, RET, ROS1, ALK*, and *PIK3CA*. In metastatic cases, actionable gene mutations occurred more often in Black (26.7%) compared with White men (18.0%; p = .05).

The increasing use of precision genomics and medicine has the potential to improve outcomes for all men with prostate cancer; however, genomic efforts and clinical studies have been highly Eurocentric, and there is a risk of widening disparities in the precision medicine era if efforts continue to not include cohorts that are representative of local, national, and global populations.[102-106] Efforts to address racial differences in prostate cancer outcomes have largely been focused on investigating the contribution of social versus biologic factors in high-risk populations.[107-109] Now, there is increasing evidence that many genomic prognostic models and subsequent targeted therapies are most likely to benefit White patients and least likely to benefit Black patients with cancer and other diseases.[102-106] Furthermore, there are no specific tumor-based biomarkers or tailored nomograms to guide workup and management of prostate cancer among the subset of patients of African descent who are known to have a higher risk of death resulting from prostate cancer.[110,111] This is largely due to insufficient minority participation in cancer research, hence the present lack of validated predictive tools for this patient population.

This lack of inclusion of men of African descent is a particular potential hazard in prostate cancer, given the disease has some of the greatest disparities recorded to date. Clinically relevant alterations may occur at different frequencies across race that could have implications for prognosis, therapy response, and enrollment of minority populations in clinical trials and precision oncology studies. An alternative possibility for outcome disparities would suggest that outcome differences could be accounted for by differences in environment, socioeconomic differences, and/or contributors from structural racism. However, there may be a more complex biologic component to these outcomes as well. Preliminary studies in small cohorts of men with metastatic prostate cancer have demonstrated that Black men were more likely to have tumor mutations in androgen receptor, actionable mutations, and DNA repair genes compared with other men (Fig. 1).[73,110,111] Nevertheless, conclusions cannot be drawn until appropriate studies are conducted with large numbers of non-White patients and that include data on environmental exposures.

Of note, only 13% of the samples from a recent study examining genomic variants in prostate cancer were from Black patients.[112] Several issues complicate the availability of genomic clinical trials and next-generation sequencing to all patients. Insurance issues and cost may make these items financially unavailable. Patients may also refuse genomic testing because of a misunderstanding of terminology and hesitancy regarding what is believed to be genomic research.[113] Availability of appropriate and standard genetic counseling may also provide a major impetus to overcoming such hurdles. Adherence to recent consensus meeting recommendations regarding genomic testing and utility of navigation may provide a way to definitely decrease the disparity in genomic analyses,[114] but this will require resources to implement.

FUTURE DIRECTIONS

Despite the progress in prostate cancer survival, treatment, and disparity, much work remains. Because the etiology of these disparities is multifactorial and based not only on ethnicity, geographic location, and socioeconomics, the approach to address these disparities must also be multifaceted. We encourage and recommend:

1. More studies of novel therapies stratified by race
2. Use of patient navigation with clinical therapies and trial enrollment in underserved patient settings
3. Enhanced and guideline-driven use of genomic testing to personalize therapies
4. Standardization of treatment such that care is delivered in more equal access environments and pathways for optimal outcomes

It is hoped that ongoing community-based educational efforts using invested and familiar partners will continue to address the advances made in cancer therapies and approaches that have led to progress in eliminating prostate cancer disparities.

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Prostate cancer dormancy and recurrence

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ABSTRACT

Prostate cancer can progress rapidly after diagnosis, but can also become undetectable after curative intent radiation or surgery, only to recur years or decades later. This capacity to lie dormant and recur long after a patient was thought to be cured, is relatively unique to prostate cancer, with estrogen receptor positive breast cancer being the other common and well-studied example. Most investigators agree that the bone marrow is an important site for dormant tumor cells, given the frequency of bone metastases and that multiple studies have reported disseminated tumor cells in patients with localized disease. However, while more difficult to study, lymph nodes and the prostate bed are likely to be important reservoirs as well. Dormant tumor cells may be truly quiescent and in the G0 phase of the cell cycle, which is commonly called cellular dormancy. However, tumor growth may also be held in check through a balance of proliferation and cell death (tumor mass dormancy). For induction of cellular dormancy, prostate cancer cells respond to signals from their microenvironment, including TGF-β2, BMP-7, GAS6, and Wnt-5a, which result in signals transduced in part through p38 MAPK and pluripotency associated transcription factors including SOX2 and NANOG, which likely affect the epi-genome through histone modification. Clinical use of adjuvant radiation or androgen deprivation has been modestly successful to prevent recurrence. With the rapid pace of discovery in this field, systemic adjuvant therapy is likely to continue to improve in the future.

1. Natural history of prostate cancer dormancy and recurrence

Unlike most cancers, prostate cancer (PCa) can recur many years after the patients' disease was thought to be cured by prostate radiation or surgery. In the largest case series of PCa recurrence after radical prostatectomy surgery (1997 patients total, 304 with recurrence), in 25% of patients, the initial sign of recurrence occurred ≥5 years after surgery [1]. Also, unlike most other cancers, PCa has a sensitive and specific biomarker for recurrent disease – the prostate specific antigen (PSA). Therefore, most recurrences of PCa are initially PSA only – or biochemical recurrence (BCR) and do not represent enough cancer to be detectable on physical exam or imaging, or to cause symptoms. Therefore, the time to a recurrence capable of causing symptoms is even longer. In the same large series, though 25% of patients with recurrence had their PSA rise ≥5 years after surgery, it took an additional median of eight more years for a clinically meaningful metastatic recurrence [2]. These late recurrences were not merely indolent cancers, but also resulted in patient deaths, though at a slightly lower frequency than more rapid recurrences. Although characteristic of PCa, late recurrences occur in other diseases as well. In a meta-analysis of nearly 63,000 patients with localized hormone receptor positive breast cancer, there was no decrease in the risk of recurrence out to 20 years [2]. Curiously, although hormone receptor negative breast cancers have a high risk of early recurrence and death the recurrence risk subsequently abates and is lower than the recurrence risk for hormone receptor positive cancers at ten years or later [3]. The observations of continued risk of recurrence in some cancers are consistent with a model where cancer cells spread distantly early in the disease process, some of which later reactivate, start growing and lead to metastatic disease. Researchers subsequently began to try to understand this process and have subsequently gained significant insight into the cellular and molecular mechanisms. Below, we summarize the current state of knowledge of PCa dormancy and recurrence, describe how this knowledge has been applied to treatment thus far, and discuss future areas for study.

2. Reservoirs for later recurrence

The spread of cancer cells while a prostate tumor is still small could

occur through invasion into the surrounding stroma, lymphatic spread, hematogenous spread, or perhaps more likely a combination of these processes. For example, a cancer cell could invade the tumor stroma, migrate to a lymph vessel, then enter the blood stream. It is difficult or impossible to determine the anatomic location where most cancer cells lie dormant prior to recurrence experimentally in patients. However, it seems reasonable to presume that the most important "reservoirs of dormancy" are where recurrences are later found clinically. In an autopsy series of 631 patients with stage IV prostate cancer, 66% of patients had involved lymph nodes and 90% of patients had bone metastases. Lung and liver metastases were next most common, with 46% and 25% respectively [4]. Similarly, in a study of biochemically recurrent PCa patients evaluated with Choline C [11] PET/CT scans, disease was found most commonly in lymph nodes, bones and the prostate bed [5]. This population of patients with biochemical recurrence (elevated prostate specific antigen but no detectable disease on physical exam and most imaging tests) can sometimes have a low level of recurrent PCa visualized on Choline C [11] PET/CT or other "molecular imaging" tests. Lesions detected on these scans represent the initial sites of relapse and are presumably where previously dormant PCa cells exited quiescence and began to grow – unless they migrated to an additional anatomic site while still in a dormant state.

Therefore, the prostate bed, lymph nodes, and bones appear to be the most important sites for PCa to lie dormant and undetectable after initial treatment of the primary tumor with radiation or surgery. Of these sites, only bone has been the subject of patient samples research into the detection and biology of rare disseminated PCa cells in patients without detectable disease outside their prostate tumors. Presumably, this is because bone marrow aspirates can be obtained with appropriate patient informed consent, but research samples of lymph nodes or prostate bed tissue are very difficult to obtain. Evidence for the presence of these cells, termed disseminated tumor cells (DTCs) or disseminated cancer cells (DCCs) has been found in bone marrow aspirates of patients with localized PCa in multiple reports over the past three decades in frequencies ranging from about 30% to 70% of patients [6–11]. Techniques included RT-PCR for the *KLK3* (PSA) gene [12], immunocytochemistry [13], immune-magnetic beads and single cell isolation [6], and fluorescence activated cell sorting (FACS) [14].

3. Cell biology and genomics

Investigators have found evidence for several non-mutually exclusive cellular processes to explain the clinical observations that PCa and other cancers can remain undetected for many years after curative intent therapy but then relapse with fatal consequences [15,16]. Most investigators refer to the processes which keep DTCs from growing into clinically detectable tumors, yet fail to totally eradicate the cancer as "dormancy maintenance." Conversely, the processes that convert DTCs from rare and asymptomatic to clinically detectable and problematic are referred to as "dormancy escape." Conceivably, the phenomenon of dormancy could be accomplished through one or more of several patterns of cell growth or lack thereof, or interaction with the microenvironment. DTCs might divide very slowly or not at all – termed "cellular dormancy." This lack of cell division is thought to be the result of interactions with the microenvironment and may be accompanied by reversible epigenetic reprogramming of the DTCs [11,16]. Alternatively, dormancy might result from the balance of cell division and cell death, so that very little change in the total number of DTCs occurs – termed "tumor mass dormancy [12]." In addition, dormancy might result from "angiogenic dormancy" where DTC or micrometastasis growth is held in check by lack of a blood supply, or "immunologic dormancy" where the immune system prevents DTC or micrometastasis outgrowth [12].

In PCa and most other cancers, "cellular dormancy" has been the most studied mode of dormancy maintenance. Curiously, many of the initial observations of cellular dormancy were not in PCa, but in head and neck squamous cell carcinoma. Aguirre-Ghiso and colleagues developed head and neck squamous cell carcinoma sub-lines from a growth permissive site (lung) or an inhibitory/dormant site (bone marrow). After isolation from the mice, these sub-lines retained the expected growth patterns for weeks to months; i.e. the lung sub-lines grew much more rapidly than the bone marrow derived sub-lines. This maintenance of phenotype after removal from the microenvironment suggested that there is an important epigenetic component to dormancy regulation. With these dormant vs. aggressive sub-lines, they were able to interrogate the signaling pathways and epigenetic modifications [17, 18], including the importance of p38 vs. Erk MAP kinase signaling, which we discuss below.

One labor intensive study built on the concept of cellular dormancy and found evidence that at least some DTCs in patient samples cycle slowly or not at all. Chery et al. isolated single DTCs from PCa patients with either "no evidence of disease (NED)"; defined as no sign of recurrence after prostatectomy, or "advanced disease (ADV)"; defined as metastases at the time of prostatectomy or PSA recurrence after prostatectomy or radiation, and then analyzed the cells by microarrays [19]. After excluding cells thought to be contaminating normal marrow cells using an erythroid gene signature, they analyzed 44 cells from 10 patients. They compared cells from NED vs. ADV patients to discover difference between dormant and active cells. However, they subsequently found that about half of the cells from ADV patients had gene expression that clustered with NED patients, whereas the other half of cells from ADV patients did not. This data supports a hypothesis that most of the DTCs in patients with dormant disease are quiescent, whereas patients with active disease have some cells that are quiescent, but others that are actively cycling. In a different study of microarray analysis of isolated DTCs some of which had their DNA analyzed by comparative genome hybridization, Guzvic et al. observed an unexpectedly high number of transcripts for hematopoietic genes and hypothesized that DTCs had a very plastic phenotype – akin to an extreme form of epithelial mesenchymal transition [20]. The study of DTC dormancy is difficult to adapt to model systems. Therefore, the literature directly examining the cell cycle status of PCa DTCs in *in vivo* model systems is limited. Owen et al. used a label retention based approach and single cell RNA sequencing of bone marrow DTCs isolated from an animal model and found evidence for the importance of another mode of dormancy maintenance; immunologic dormancy [21]. In the current review, we concentrate on recent advances. Other authors have reviewed literature specific to PCa prior to the past five years – which provide complimentary discussions to the present work [22,23].

A factor potentially affecting PCa dormancy particularly difficult to study in model systems is the impact of genomics (DNA sequence changes). Most investigators would agree that the time period for model systems is too short to study genomic changes in dormancy regulation, though innovative investigators might solve this problem in the future. Using genomic hybridization techniques, Holcomb et al. investigated large genomic changes (megabase and chromosomal level) in DTCs from metastatic vs. non-metastatic patients. They observed changes characteristic of PCa including 8p loss, 8q amplification and amplification of Xq, which contains the androgen receptor gene. They observed more genomic changes in metastatic than non-metastatic patients, which is consistent with a hypothesis that acquired genomic changes in DTCs could be important for metastatic recurrence. To again borrow from another malignancy, Werner-Klein and colleagues sequenced tumor cells from melanoma tumor cells and DTCs isolated from the sentinel lymph nodes. They found more differences between primary tumors and DTCs than expected. These included differential presence of typical driver mutations such as the *BRAF* gene, which are usually thought to be clonal [24]. They concluded that it was very likely that DTCs were spreading to the draining lymph nodes when the primary tumor was very small (0.5 mm thick) and continuing to acquire genomic changes at the lymph node site.

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4. Microenvironment and intracellular signaling

In a similar fashion as the work with DTCs from patient samples, the majority of the research into PCa dormancy regulation has focused on the bone marrow microenvironment, though other sites are very likely to be important as well. Presumably, this is likely due to practical considerations; i.e. the difficulty of studying lymph nodes in a model organism the size of a mouse. The bone marrow is a complex organ with multiple disparate cell types including; precursors and mature hematopoietic cells (myeloid, lymphoid, erythroid, and thrombocytic), fat, sinusoids, arterioles, nerves, osteoblasts, and osteoclasts. Functionally, Shiozawa et al. found that PCa DTCs appear to share the same environment, or niche, as hematopoietic stem cells by showing that PCa cells displace hematopoietic stem cells from the bone marrow and vice versa [25]. However, the precise microanatomic location of this niche is poorly defined. The components of the hematopoietic stem cell niche have been hotly debated, with the likely conclusion that many different cells can function as niche components [26].

Given that DTCs are integrated into the bone microenvironment, it may not be surprising that they are regulated by protein ligands that are also important in other bone and bone marrow processes, most notably proteins of the tumor growth factor beta (TGFβ) and bone morphogenetic protein (BMP) families. Kobayashi and colleagues reported that BMP-7 signals through BMPR2 to keep PCa DTCs dormant. They focused their analyses on a sub-population purported to have cancer stem cell like characteristics. They found that BMP-7/BMPR2 signaling regulated the quiescence marker, senescence associated β-galactosidase, *in vitro* and lengthened metastasis free survival using xenograft animal models. They also showed that BMPR2 inversely correlated with metastasis free survival in patient specimens [27]. Subsequently, Sharma et al. found that secreted protein acidic and rich in cysteine (SPARC) stimulated BMP-7 expression in bone marrow to maintain dormancy [28]. TGF-β2 signaling was first shown to maintain dormancy in in other malignancies [17]. Then, Yumoto and colleagues showed that TGF-β2 also acted to maintain dormancy in PCa and that it required AXL, a tyrosine kinase receptor from another family [29]. AXL is a member of the TAM family of receptor tyrosine kinases, named for the three members; TYRO3, AXL, and MERTK. The TAM family has multiple protein ligands with varying affinities for the three receptors, with the most studied ligands being GAS6 and Protein S (PROS1). GAS6 signaling through AXL promotes PCa cell survival and causes a G1/G0 arrest during docetaxel treatment [30,31]. In agreement with this, Taichman et al., profiled the cell surface expression of AXL and TYRO3 in subcutaneous tumors, bone marrow DTCs, and bone metastatic tumors. They found that AXL was expressed more highly on DTCs, whereas TYRO3 was expressed more highly on macroscopic tumors at either the subcutaneous or metastatic sites. This is consistent with a model where AXL signaling regulates dormancy maintenance and TYRO3 regulates dormancy escape. However, they did not examine MERTK in this study and did not perform functional studies [32]. Subsequently, we showed that MERTK knockdown inhibited cell cycle progression *in vitro* and lengthened metastasis free survival after intra-cardiac left ventricle injection *in vivo* [33]. Therefore, AXL seems to promote dormancy maintenance and TYRO3 and MERTK promote dormancy escape. In related studies, norepinephrine was shown to stimulate PCa dormancy escape directly by signaling through the β2-adrenergic receptor and indirectly by decreasing GAS6 expression in bone marrow stroma [34]. More recently, WNT5A was shown to maintain PCa dormancy. Ren and colleagues found that WNT5A induced Siah E3 Ubiquitin Protein Ligase 2 (SIAH2), which repressed β-catenin signaling to maintain dormancy [35]. This study showed an unexpected signaling pathway because WNT5A, typically a ligand for non-canonical (β-catenin independent) WNT signaling, acted indirectly to affect canonical (β-catenin dependent) WNT signaling.

However, with regard to downstream intracellular signaling, the bulk of the literature points to MAP kinases rather than β-catenin. Largely in head and neck squamous cell carcinoma model systems, p38

MAPK (*MAPK14* gene) was found to be a key node regulating dormancy related intracellular signaling. A key component of this signaling module is the relative abundance of phosphorylated (active) p38 vs. phosphorylated ERK1/2 kinases (*MAPK3* and *MAPK1* genes). In this model, phosphorylated p38 favors dormancy maintenance, while phosphorylated ERK1/2 favors dormancy escape, proliferation and relapse [17,18, 36]. In line with these studies, Ingenuity Pathway Analysis predicted p38 to be a key node regulating a quiescent vs proliferative phenotype in bone marrow DTCs isolated from PCa patients. In this study, Chery et al. isolated single DTCs from either patients with active PCa or patients with resected localized PCa and no evidence of recurrence for at least seven years. They then amplified the cDNA and performed microarray analysis on the single cell samples. By comparing gene expression in patients with inactive vs. active PCa, they derived a 21-gene signature that converged on p38 by Ingenuity Pathway Analysis [19]. The balance of phosphorylated p38 vs phosphorylated ERK1/2 or p38 alone has been shown to be important in model system studies of PCa dormancy as well, downstream of multiple ligands and receptors including; TGF- β2, GAS6/AXL, MERTK, BMP-7/BMPR2, SPARC and norepinephrine/β2-adrenergic receptor [27–29,33,34]. Recently, another intracellular pathway regulating dormancy was discovered; PPARγ signaling through mTOR to maintain dormancy. Curiously, this was stimulated by the plant phytohormone abscisic acid [37]. Continuing further downstream to the nucleus, less is known about how transcription factors and epigenome modifying enzymes regulate PCa dormancy. In other malignancies, the embryonic stem cell transcription factors SOX2 and NANOG, and the orphan transcription factor NR2F1 appear to be important for dormancy maintenance, at least in part through histone H3 post-translational modifications [17,18]. This discovery prompted study of the potential use of all trans retinoic acid and 5-azacitidine as a treatment approach to revert cycling PCa cells to a dormant phenotype in and ongoing clinical trial discussed more below. In keeping with these findings, we showed that MERTK knockdown delayed dormancy escape and induced higher levels of NR2F1, SOX2 and NANOG and induced higher levels of the inactivating histone H3 marks; trimethylated lysine 9 and lysine 27[33].

Much of the work discussed above focuses on "cellular dormancy," i. e. the lack of cell division of DTCs induced by paracrine signaling in the microenvironment, often accompanied by epigenetic reprogramming. There has been comparatively little work on the role of the immune system (immunologic dormancy) on PCa dormancy maintenance and escape. This may be due partly to the fact than many of these studies were conducted using immune compromised animals. A recent study used single cell RNA sequencing to compare slowly vs. rapidly cycling DTCs in an immune competent mouse PCa model and discovered a novel mechanism of PCa dormancy maintenance involving both the immune system and epigenetic reprogramming [21]. They found that tumor cell intrinsic (as opposed to microenvironmental) type 1 interferon was critical for PCa dormany maintenance and observed the expected effects on the immune system and reversal of dormancy by administration of systemic interferon. They also saw reversal of the effect with a histone deacetylase inhibitor, which highlights a potential interaction between immune regulated and epigenetic regulated dormancy. It seems likely that investigators will continue to unearth interactions between different dormancy regulatory mechanisms in the near future. Please see Fig. 1 for an illustration of the key molecular mechanisms regulating PCa dormancy.

5. Adjuvant treatment to prevent recurrence

A major practical application of cancer dormancy research is to design adjuvant therapies – to prevent recurrence in patients with treated primary tumors and no current clinically detectable cancer. In PCa there has been some progress in adapting existing therapies to the adjuvant setting, though no molecular targeted therapies have been approved in this setting thus far. As an illustration that the prostate bed
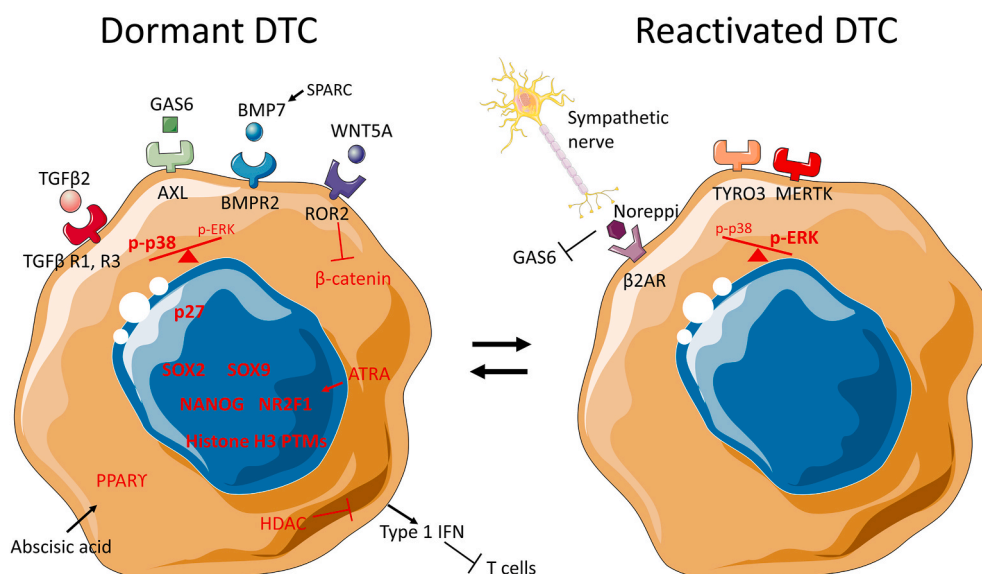
Fig. 1. Illustration of key molecular mechanisms regulating prostate cancer dormancy.

and lymph nodes can serve as a reservoir of residual disease, adjuvant radiation therapy after radical prostatectomy can increase event free survival. However, early salvage radiation (soon after initial PSA relapse) appears to be as effective as salvage radiation [38]. Indefinite androgen deprivation therapy (ADT) with medical castration improves survival if given immediately to patients with involved lymph nodes discovered at the time of radical prostatectomy [39]. However, this therapy has significant side effects, especially if continued life long. Similarly, adjuvant ADT is beneficial in localized PCa patients treated with definitive radiation rather than surgery. While ADT is often given concurrently with radiation to sensitize the cancer cells to the effects of radiation, if continued long term, ADT also acts as an adjuvant therapy in addition to its effect as a radiosensitizer. Three years of ADT (radiosensitizer and adjuvant) given with prostate radiation results in longer overall survival than only six months (radiosensitizer only) of ADT [40]. In a later trial, an intermediate period of radiation (18 months) was shown to have superior prostate cancer specific mortality to six months of ADT when given with radiation [41].

However, clinical trials of PCa systemic adjuvant therapy other than ADT with medical castration have met with much less success. The first randomized trial of a chemotherapy drug for prostate cancer adjuvant therapy after curative intent radical prostatectomy randomized patients to ADT or ADT plus mitoxantrone – a drug much less commonly used today as when the first patients were enrolled to the trial in 1999 [42]. Unfortunately, the trial needed to be stopped early by the data safety monitoring board because of concern for an increased number of cases of acute myeloid leukemia in the mitoxantrone group. In longer-term follow-up, the monitoring board's concerns appear justified. At a median follow-up of 11.2 years, there was no improvement in overall survival for the mitoxantrone group, but there was an increased risk of death from other cancers. A later trial of a contemporary chemotherapy drug, docetaxel, for PCa adjuvant therapy was also unsuccessful. The trial, named TAX-3501, was terminated early when the pharmaceutical company sponsor withdrew support – citing enrollment below expectations [43]. Most recently, the Radiation Therapy Oncology Group (RTOG) sponsored a trial to test if addition of docetaxel improved survival for high risk localized PCa when added to definitive radiation with concurrent ADT. Initially there appeared to be an overall survival advantage with addition of docetaxel, prompting listing this approach as an option in the United States National Comprehensive Cancer Network (NCCN) guidelines. However, the survival curves converged with longer follow-up [44,45]. Therefore, with the modest success of PCa systemic

adjuvant therapy, new agents and approaches are needed.

One such approach is an ongoing randomized crossover pilot trial of the addition of all trans retinoic acid and 5-azacitadine to ADT in patients with PCa biochemical recurrence [46]. In biochemical recurrence, patients initially have no clinical evidence of disease after treatment of localized PCa with surgery or radiation, but then have an increase in prostate specific antigen, but do not have enough disease to be detected by physical exam or imaging. These cases of biochemical recurrence are presumably from reactivation of residual PCa in the prostate bed or DTCs in metastatic sites such as bones or lymph nodes. The purpose of addition of 5-azacitadine and all trans retinoic acid to ADT is to restore the reactivated residual cancer to a dormant state rather than to necessarily eradicate it completely. This approach was largely based on work highlighting the importance of the transcription factor NR2F1 in maintaining cancer dormancy [18]. In this study, NR2F1 was induced by all trans retinoic acid and by histone H3 post-translational modifications. In their treatment model, all trans retinoic acid was sufficient to convert active cells to a dormant state temporarily, but addition of 5-azacitadine was required for a lasting effect. We anxiously await results of this clinical trial, which are expected in August 2022 and expect that this will be just the first of many trials of targeted agents for PCa adjuvant therapy.

6. Conclusions

Investigators have made great progress in understanding how PCa can disseminate early in the disease course and relapse months or decades later. Consistent with bone as a common site of PCa metastasis, researchers have found evidence of disseminated tumor cells (DTCs) in bone marrow of many, and in some cases the majority, of patients with clinically localized PCa. Laboratory researchers, predominantly in model systems, have gained a significant amount of insight in to the molecular mechanisms controlling PCa dormancy. TGF-β2, GAS6, BMP-7, and WNT5A are extracellular molecules that maintain dormancy, whereas adrenergic signaling stimulates dormancy escape. Dormant DTCs also have increased histone H3 post-translational modifications and have increased activity of the transcription factors; SOX2, SOX9, NANOG, and NR2F1. A recent work found importance for tumor intrinsic type 1 interferon as maintaining dormancy and lack of type 1 interferon for stimulating dormancy escape. This work brings up the importance of immunologic dormancy in addition to cellular dormancy, which was the subject of most of the other work. Although clinical

researchers have had modest gains thus far, much more work is needed to refine the basic science and translate this knowledge to the clinic.

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Frank C. Cackowski: Conceptualization, Writing – original draft. **Elisabeth I. Heath:** Conceptualization, and, Writing – review & editing.

Declaration of competing interest

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[**EXTERNAL**]

SUBJECT: HRPO Concurrence with the Determination of Research Not Involving Human
Subjects for the Protocol, "Regulation of Prostate Cancer Dormancy and Recurrence by
Hippo Signaling," Submitted by Frank Cackowski, MD, PhD, Wayne State University, Detroit,
Michigan, Proposal Log Number PC190093, Award Number W81XWH-20-1-0394, HRPO Log
Number E01284.1a

1. The subject protocol and supporting documents received on 13 March 2022 in the U.S.
Army Medical Research and Development Command, Office of Research Protections,
Human Research Protection Office (ORP HRPO) have been reviewed for applicability of
human subjects protection regulations.

2. The research involves analysis of deidentified samples obtained from the Prostate Cancer
Biorepository Network.

3. The Wayne State University Institutional Review Board (IRB) determined that the protocol
does not constitute human subjects research per 45 CFR 46.102(e) as the
investigators conducting research will not obtain information or biospecimens through
intervention or interaction with the individual, and uses, studies, or analyzes the information
or biospecimens or obtain, use, study, analyze, or generate identifiable private information
or identifiable biospecimens.

4. As required by DoD Instruction 3216.02, the ORP HRPO concurs with the research not
involving human subjects determination made by the Wayne State University IRB Office. The
project may proceed with no further requirement for review by the HRPO. The HRPO
protocol file will be closed.

5. The HRPO determined the role of the collaborator at the University of Michigan also
comprises research not involving human subjects as it does not involve a living individual
about whom an investigator (whether professional or student) conducting research obtains
information or biospecimens through intervention or interaction with the individual, and
uses, studies, or analyzes the information or biospecimens; or obtains, uses, studies,
analyzes, or generates identifiable private information or identifiable biospecimens [32 CFR
219.102]. The collaborator's work may proceed with no further requirement for review by

the HRPO. The HRPO protocol file will be closed.

6. In the event that there is a change to the subject research or statement of work (SOW), the Principal Investigator must notify the Contracting Officer's Representative (COR) or Grant Officer's Representative (GOR) and send a description of the change to the HRPO at usarmy.detrick.medcom-usamrmc.other.hrpo@mail.mil referencing both the proposal log number and the HRPO log number listed in the "SUBJECT" line above. The HRPO will re-open the protocol file if necessary.

Any changes to the SOW that the COR or GOR determines could affect the exemption status of the project must be reviewed by the HRPO prior to approval by the Contracting Officer/Grants Officer.

7. Do not construe this correspondence as approval for any contract or grant/cooperative agreement funding. Only the Contracting Officer/Grants Officer can authorize expenditure of funds by notice of official award documentation. It is recommended that you contact the appropriate contract/grants specialist or Contracting/Grants Officer regarding the expenditure of funds for your project.

8. Further information regarding this review may be obtained by contacting Mrs. Katelyn Murter, Human Subjects Protection Scientist, at 301-619-7839 or katelyn.d.murter.ctr@mail.mil.

ANDREA J. KLINE, MS, CIP
Deputy Director
Human Research Protection Office
Office of Research Protections
U.S. Army Medical Research and Development Command

PROTOCOL
IACUC
Wayne State University

Protocol # IACUC-20-06-2465
Date Printed:
06/06/2022

e-PROTOCOL

June 06, 2022

Wayne State University
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)
Animal Research Protocol

Protocol #
IACUC-20-06-2465

Protocol Title:	Regulation of Prostate Cancer Dormancy and Recurrence by Hippo Signaling
Protocol Type:	IACUC
Approval Period:	Draft
Important Note:	This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.

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* * * Personnel Information * * *

Principal Investigator

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E-mail Address:	hf7160@wayne.edu	**Laboratory Phone:**	313-576-8321
Emergency Phone:			

Training Details			
Course ID	Course	Course Completion Date	Course Expiration Date
100	Ani Con Questionnarire	2020-07-22 00:00:00	
73559	Animal Allergy Exposure Reduction	2020-07-22 10:50:00	2023-07-22
27185	Aseptic surgery	2020-07-22 14:06:00	
27086	Biomedical Investigators	2020-05-14 18:04:00	2023-05-14
27090	Biomedical Responsible Conduct of Research Course 1.	2020-05-15 14:42:00	
99756	Biosafety/Bloodborne Pathogens	2021-11-02 17:54:00	2022-11-02
27089	CITI Good Clinical Practice Course	2020-05-14 16:42:00	2023-05-14
27177	CITI Health Information Privacy and Security (HIPS) for Clinical Investigators	2020-05-15 11:49:00	
62527	Conflicts of Interest	2020-05-15 13:56:00	2024-05-14
203	DLAR Mouse	2022-03-04 00:00:00	
119473	GS0900 RCR Core Topics Course	2020-05-15 15:13:00	2024-05-14
99755	Laboratory Safety Training	2021-11-02 17:59:00	2022-11-02
130575	Principal Investigators: NIH Guidelines involving Recombinant or Synthetic Nucleic Acid Molecules	2020-07-17 13:34:00	2023-07-17
132965	Radiation Awareness (Non-users)	2021-01-13 16:36:00	
133087	Radiation Generating Machine	2022-04-21 12:53:00	2023-04-21
27184	Reducing Pain and Distress in Laboratory Mice and Rats	2020-07-22 11:45:00	

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27187	Working with Mice in Research	2020-07-22 14:56:00	
27182	Working with the IACUC	2020-07-22 12:57:00	2023-07-22

Working with animal models?

Describe Previous Experience and Responsibilities for this Protocol: Identify the responsibilities of this individual, his/her experience with the procedures and the animal species, and who will train personnel on the procedures for work specific to this protocol.

Is this person an emergency contact? Emergency contacts need to be able to authorize treatment or euthanasia of sick animals.

     Co-Investigator

     Research Staff

     Non-WSU (or Affiliate) Collaborator

A proxy may be listed if the Chair is the PI or a member of the research team.

Emergency Contacts

Name   Emergency Phone

_____

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* * * Species * * *

NUMBER OF ANIMALS - If this is an initial submission of a multi-year grant beyond the three year protocol period, all the work and number of animals must be included in this protocol application. For all other submissions, list the total number of animals to be used over the 3 YEAR PERIOD of this protocol (or for the life of the project if less than 3 years)

Species to be used

Please review the detailed Explanation of USDA Reporting Codes.

Brief examples:

Category B: Animals being bred but not used for experimental purposes.

Category C: Experimental animals that will experience no pain or distress.

Category D: Experimental animals where anesthetic or analgesic agents are used to avoid pain or distress.

Category E: Experimental animals where anesthetic or analgesic agents cannot be used to avoid pain or distress.

1. USDA CATEGORY E: Identify the condition that places the animals in Category E and provide scientific justification for withholding alleviation of pain/distress. Describe any non-pharmaceutical methods that will be used to minimize pain and distress.

NOTE: If animals may die as a result of experimental procedures (e.g., infectious disease or oncology studies), or because an endpoint is used that allows the animals to experience significant pain or distress, justify why an alternate endpoint (e.g.,weight loss, clinical signs, tumor size) cannot be used prior to death or pain or distress.

2. Indicate how the total number of animals needed for this study was reached for each USDA category (group size X groups in each experiment X number of experiments). Provide the number and type of experimental and control groups in each experiment, the number of experiments planned, and the number of animals in each group. Include all animals in each USDA category, including those that will be needed for training and those that will be culled.

The number and category of animals in this section must match the animal tables above.

DO NOT cut and paste your experimental aims from your grant proposal.

Details of each procedure are to be described in the appropriate section, NOT here.

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* * * General Questions * * *

General Questions

1. **Is this protocol replacing an expiring protocol?**

   The number of the expiring protocol is:

   a. Provide a brief summary of the work completed under the expiring protocol. It may be helpful to include the number of animals that were used/bred related to how many were approved in the expiring protocol; DLAR can provide you with a report, call 577-1107.

   b. Describe any unexpected adverse events that resulted in increased pain, distress or death rates to animals that were not described in the original protocol. Include how these were managed and what steps were taken to prevent recurrence (if applicable). Please make sure that any additional adverse effects, expected mortality, pain category changes, humane endpoints, etc. have been incorporated into this application.

   c. Do you have animals currently in-house that will be transferred to this renewal protocol upon approval?

      If yes, please include them in species section

2. **Is this VA research (i.e. conducted in VA facilities, funded by the VA)?**

   Please affirm that you understand that this research cannot be initiated until after the John D. Dingell VAMC, Detroit, MI (station number 553) R&D Committee approval.

3. **Will this protocol be submitted to the VA Central Office for approval (formerly submitted on an ACORP)?**

4. **Will this research involve students/visitors (not listed as Research Staff)?**

   If yes, the guideline will need to be affirmed

   Describe the nature of the potential participation:

5. **Is this a teaching protocol?**

   How many classes are held per year?

   Approximately how many students participate in each class?

6. **Is this a collaboration protocol with another institution via a WSU Memorandum of Understanding?**

   Institution Name:

   Institution Protocol Number:

   The institution's protocol and approval letter must be attached to this application

   Describe the experimental procedures that will be conducted at WSU in this protocol application

7. **Is this wildlife field research?**

   Wildlife Field Research

   1. List all State, Federal and International permits required for your research.

      Note: Permits must be obtained before research is initiated (attach copies to this application).

   2. Describe precautions taken to ensure the health and safety of personnel working in the field and handling wild animals (e.g. rabies immunization, Lyme disease, Hanta virus)

   3. List the study site location(s). Include country, state, county.

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4.   Does this research involve live capture and release?

     a.   List the method(s) of capture:

     b.   What precautions will be used to minimize injury and/or mortality?

     c.   What precautions will be taken to reduce non-target captures?

     d.   Will marking procedures to be used?
         Describe the marking procedures:

         How will the animal be monitored during recovery from these procedures?

     e.   Will blood or other tissue samples be taken?
         Describe procedures to be taken to prevent infection at the sample site.

         How will the animal be monitored during recovery from these procedures?

     f.   Will animals be held longer than 12 hours?
         Indicate the type of confinement and the length of time they will be confined.

         How often will the captive animals be observed during daily care?

         Will food and water will be withheld?
         Explain the circumstances and justify

     g.   Will live animals be transported?
         Indicate method of transportation

5.   Does this research involve non-survival collection?

     a.   Describe procedure(s) to be used.

     b.   Describe precautions that will be taken to prevent non-target mortalities.

     c.   How will you ensure that the animal will not revive (e.g. removal of heart, induction of bilateral pneumothorax, observation of cessation of heart beat and respiration accompanied by fixed and dilated pupils and loss of corneal reflex, etc.)?

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* * * Are you using? * * *

Are you using?

1. BIOLOGICAL HAZARDS - Does this research involve the use of any recombinant DNA, mammalian viruses, biological toxins,infectious agents, human blood, human cell lines, human tissue, and/or transgenic animals?

   a. Transgenic/Knockout/Knockin/GEM Animals

   Detailed information regarding transgenic animals must be provided in the "Animal Breeding, Housing and Care" section.

   b. CDC Select Agents and Biotoxins (see the list of CDC select agents)

   Note : Use of Select agents and Biotoxins requires an Institutional Biosafety Committee(IBC) Protocol application.
   IBC Protocol ID:

   c. Other Toxins (non CDC select agent toxin with LD50<100ng/kg)

   Note : Use of toxins requires an Institutional Biosafety Committee(IBC) Protocol application.
   IBC Protocol ID:

   d. Human or non-human primate cell lines, tumors, blood, tissues, etc.

   e. Infectious Agents ONLY (if you will be using viral vectors to express rDNA proceed to the next question)

   Note : Use of Infectious Agents requires an Institutional Biosafety Committee(IBC) Protocol application.
   IBC Protocol ID:

   f. Non-plasmid rDNA, shRNA

   Note : Use of Non-plasmid rDNA shRNA requires an Institutional Biosafety Committee(IBC) Protocol application.
   IBC Protocol ID:

   g. Plasmid recombinant (NIH Exempt) DNA
   Type:

   (Please verify by reviewing the NIH exempt explanation)

2. CHEMICAL HAZARDS - Does this research involve the use of any hazardous chemicals (e.g. CFA), chemotherapeutic drugs or gas anesthetic agents (e.g. isoflurane)? (If you are uncertain, you can refer to Chemical Hazards Definition and Examples)

   a. Hazardous chemicals/chemotherapeutic drugs

   b. Gas anesthetic agents

   b1. Gas scavenging system?

   If No, please explain

3. Radiation Hazard - Does this research involve the use of ionizing/non-ionizing equipment, radioisotopes in vivo or the use of irradiators?

   a. Non-Ionizing radiation equipment-Examples include: Lasers, Infrared, Microwaves, MRI.

   b. Ionizing radiation equipment-Examples include: PET scanner,CT scanner, SPECT, Fluoroscopy, Radiography and other imaging equipment

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Note: Use of Ionizing Radiation Equipment requires a Radiation Safety Committee(RSC) Protocol application.

RSC Protocol ID:

     c.   Sealed Source / X-ray irradiators

     d.   Radioisotopes

Note: Use of Radioisotopes requires a Radiation Safety Committee(RSC) protocol application.

RSC Protocol ID:

4.   PHYSICAL or OTHER HAZARDS - Does this research involve the use of any physical/other hazards (e.g. nanoparticles, noise, cryogens)?

5.   CONTROLLED SUBSTANCES - Does this research involve the use of Controlled Substances (if you are uncertain you can check the DEA CS list: Controlled Substances - by CSA Schedule)?

     Note : Use of Controlled Substance requires a Controlled Substance(CS) Committe Protocol application.

     CS Protocol ID:

6.   OCCUPATIONAL HEALTH CONCERNS - Are there any non-routine measures, such as special vaccines or additional health screening techniques that would potentially benefit staff (e.g. research, husbandry, veterinary) that participate in or support this project? Routine measures included in the Occupational Health and Safety Program (vaccination for tetanus, rabies, and hepatitis B, and TB screening) need not be mentioned here.

     Describe:

7.   **Use of Non-Pharmaceutical Grade Compounds**

     a.   **Are any of the drugs, biologics or reagents being used in these procedures non-pharmaceutical grade (neither human nor veterinary)**

         Identify the non-pharmaceutical grade drugs, biologics or reagents that will be administered to animals. Provide scientific justification for their use and describe methods that will be used to ensure appropriate preparation and administration.

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* * * Funding * * *

Funding Checklist

Funding - Other

June 06, 2022

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Animal Research Protocol

Protocol #
IACUC-20-06-2465

Protocol Title:	Regulation of Prostate Cancer Dormancy and Recurrence by Hippo Signaling
Protocol Type:	IACUC
Approval Period:	Draft
Important Note:	This Print View may not reflect all comments and contingencies for approval. Please check the comments section of the online protocol.

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* * * Purpose and Value * * *

PURPOSE AND POTENTIAL VALUE OF STUDY

Official Project Title

Regulation of Prostate Cancer Dormancy and Recurrence by Hippo Signaling

In non-technical, everyday language that a senior high school student would understand, BRIEFLY state the research or development question to be addressed in this protocol. Also, explain the potential value of this study and the ways the proposed animal use might benefit human or animal health, the advancement of knowledge, education and training, or the good of society.

A scientific abstract from a grant or funding proposal is not acceptable. Do not describe experiments or procedures, or use abbreviations. The information provided in this section could be used for possible press release.

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* * * Animal Use Justification * * *

Animal Use Justification

The US Animal Welfare Act (AWA) and USDA Policy #12 regulations require principal investigators to consider alternatives to procedures that may cause more than momentary or slight pain or distress to animals, and provide a written narrative of the methods used and sources consulted to determine the availability of alternatives, including refinements, reductions, and replacements (the 3Rs).

Examples of Refinement: The use of most appropriate anesthetics and analgesics, the use of remote telemetry to increase the quality and quantity of data gathered, and humane endpoints.

Examples of Reduction: The use of shared control groups, preliminary screening in non-animal systems, innovative statistical packages or a consultation with a statistician.

Examples of Replacement: Alternatives such as tissue culture models, or computer-based simulations. Alternative animal models lower on the phylogenetic scale (i.e. using a mouse model in lieu of a non-human primate model).

1. Consideration of Alternatives and the Prevention of Unnecessary Duplication. Complete items below. Keep copies of computer database search results in your files to demonstrate your compliance with the law if regulatory authorities or the IACUC should choose to audit your project.

    The USDA webpage Literature Searching and Databases contains links to excellent resources that can help you better understand the requirements and organize your search for alternatives.

    WSU Medical School Contact Shiffman Medical Library via askmed@wayne.edu or 313-577-1094

    WSU General Libraries visit ASK-A-LIBRARIAN; subject specialists are available.

a. Investigators must consider less painful or less stressful alternatives to procedures, and provide assurance that proposed research does not unnecessarily duplicate previous work. You should perform one or more database searches to meet these mandates unless compelling justifications can be made without doing so. Complete the table below for each database search you conduct to answer the questions below.

    The literature search must not be older than 3 months at time of submission of this protocol application.

b. Could any of the animal procedures described in this protocol be replaced by non-animal models, such as mathematical models, computer simulations, or in vitro biological systems? Indicate below if such replacement is or is not possible, and provide a narrative as on how you came to your conclusion.

    _____

c. Could a smaller, less sentient mammalian species or a non-mammalian species (e.g. fish, invertebrates) substitute for the mammals in any of the experiments planned? Indicate below if such substitution is or is not possible and provide a narrative on how you came to your conclusion.

    _____

    i. Describe the biological characteristics that make each species, strain and sex selected the most appropriate for this project. If you will use transgenic, knockout or knockin animals, describe the unique feature(s) of each. Cost is not an acceptable consideration.

    _____

d. Could a different animal model or different animal procedure that involves (1) less distress, pain, or suffering, or (2) fewer animals substitute for any proposed animal model or animal procedure planned? Indicate below if such replacement is or is not possible, and provide a narrative on how you came to your conclusion:

    _____

e. Does the proposed research unnecessarily duplicate previous work? Indicate below if the proposed work unnecessarily duplicates previous work and provide a narrative on how you came to your conclusion:

    _____

2. **Indicate the METHOD(S) used to determine the group size of animals needed for this study.**

    Note: The Guide states that whenever possible, the number of animals requested should be justified statistically. A power analysis is strongly encouraged to justify group sizes when appropriate. Please provide this information.

a. Group sizes determined statistically. State what statistical analysis was performed and give the power function. The variance may be estimated from similar previously published studies. Software such as that available at www.poweranalysis.com or www.statistics.com may be helpful.

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www.statistics.com may be helpful.

b. Group sizes based on quantity of harvested cells or amount of tissue required. Elaborate. (Note: A statement such as "The study requires 50 experiments" is not sufficient.)

c. Pilot study or preliminary project, group variances unknown at present. Minimal number of animals should be requested. You must provide justification for the number of animals you are requesting. State the basis for your request.

d. Other Elaborate and justify criteria used to determine group size.

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* * * Animal Breeding, Housing & Care * * *

Animal Breeding, Housing and Care

1.   Breeding: Will animals be bred in-house?

     YES

     NO

     All animals bred in-house must be listed in the "Species" section including any excess or unsuitable animals that will not be used for experiments. For complicated breeding schemes, please consider including an attachment (see "Attachments" tab above)

     a.   Review the Rodent Breeding and Weaning Policy and complete the table below.

	Pair mating		Pups weaned at 21 days
	Trio mating		Other (describe below):
	Other (describe below):		

     b.   Will the offspring be genotyped?

	Tail biopsy	Review Rodent Tail Biopsy
	Toe clipping	Review Rodent Toe Clipping (please justify below)
Toe clip ping ena ble		
	Other:	

2.   Rodent Identification Method (e.g. ear punch, tattoo, ear notch) See Rodent Identification for guidance.

     Not Applicable

     None

     List: [ ]

3.   Will Transgenic, Knockout and Knockin animals be used?

     YES (review the Genetically-Modified Animals Guideline)

     NO

     a.   Describe any special care or monitoring that the animals will require, or need for special breeding systems.

         No special care required

         Special care required (describe below):

         [ ]

     b.   For each strain: what is the inserted or knocked-out gene (avoid abbreviations; for an inserted gene, indicate the source species, wild-type or mutant; if mutant, indicate how) and what is the function of the wild-type gene product?

         [ ]

     c.   Will these modifications to the genome cause an increased risk for the animal to shed intentionally introduced infectious agents, biological toxins, hazardous chemical agents, radioisotopes or create other hazards for the animal handlers and research staff?

         YES Explain the hazard the animal will present to staff handling the animals and provide safety precautions required to be observed in housing and handling these animals in the space below.

         NO

         [ ]

     d.   Are two or more different strains of transgenic animals being bred?

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YES

NO

i.   Which strains are being bred?

ii.   What is the expected biological characteristic(s) or outcome of the novel strain(s)?

iii.   Will the novel strain(s) pose any additional risks to staff? If yes, please explain.

4.   **Housing Outside DLAR Facilities: Will animals need to be maintained outside the DLAR facilities for more than 12 hours?**

        YES (Review Overnight and Long-Term Housing of Animals in Investigator Laboratories)

        NO

             >12 hours but <=24 hours

             >24 hours

        If animals are housed more than 24 hours in a laboratory, the room is designated as a satellite animal housingfacility and must comply with all pertinent regulations as if it was a DLAR facility. If this room has not been set up as such, contact the IACUC office immediately.

        Building:

        Room:

             DLAR will provide all husbandry and oversight.

             DLAR and PI will share the responsibilities for husbandry and oversight.

             PI will be responsible for all husbandry and oversight. Provisions for care and housing, animal monitoring and environmental monitoring will meet or exceed standard DLAR SOPs.

             All outside housing requests require a Husbandry Agreement between the DLAR and PI. A scanned signed agreement must be attached to this protocol, See attachments tab in the Protocol information.

             In the Training Checklist section, please select the persons reponsible for taking care of animals outside of DLAR facilities.

      a.   Which animals will be housed outside of the DLAR facilities? Please include species and specific information about which animals will be housed (e.g. post-op animals, animals undergoing behavioral testing).

      b.   How many animals will be housed outside the DLAR facilities at one time?

      c.   How long will the animals be maintained outside of the DLAR facilities?

      d.   Justify why it is necessary to house animals outside of the DLAR facilities?

5.   **Caging Requirements**

        Standard housing (appropriate for species, including sterile for immunocompromised animals)

        Special housing needs required (e.g. suspended wire mesh flooring, non-standard size) for some or all animals on this protocol. Provide justification and describe circumstances below:

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6. **Social Housing: The Guide states: "Single housing of social species should be the exception and justified based on experimental requirements or veterinary-related concerns about animal well-being."**

   Standard social housing

   Single housing will be required for some or all animals on this protocol. Provide justification and describe circumstances below; include the duration of time animals will be singly housed:

   [ ]

7. **Environmental Enrichment: The Guide states: "The primary aim of environmental enrichment is to enhance animal well-being by providing animals with sensory and motor stimulation, through structures and resources that facilitate the expression of species-typical behaviors and promote psychological well-being through physical exercise, manipulative activities, and cognitive challenges according to species-specific characteristics"**

   Species-specific enrichment will be provided (see Environmental Enrichment and Behavioral and Social Management of Research Animals Policy/Guideline)

   Enrichment will not be provided for some or all animals on this protocol. Provide justification and describe circumstances below:

   [ ]

8. **State the period of time animals will be allowed to acclimate following arrival at WSU and prior to the initiation of experimental or breeding procedures (Review the Acclimation of Animals Guideline).**

   [ ]

9. **Will photographs and/or videos of animals be taken in an animal holding facility (i.e. DLAR)? Review the Security Policy/Guideline.**

   YES, list building(s) and room number(s) and describe:

   [ ]

   NO

10. **Will animals be transported between buildings for procedures?**

    YES Review the Transportation of Animals Policy/SOP

    NO

    To ensure humane animal handling and protect against disease spread, IACUC/DLAR requires that special provisions be met regarding the transportation of animals between WSU buildings or off campus locations. Transportation arrangements can be made through DLAR by calling 313-577-1343.

    1. State the species and number of animals to be transported at one time:

       [ ]

    2. Identify the building and room numbers involved in the transport:

    Note: If animals will be taken into a medical center area hospital for a procedure, you must have prior approval from the authorizing persons at that hospital.

    3. State the purpose of the transportation, indicate if it may be necessary to do this more than once with the same or different groups of animals, and the length of the stay at each site (e.g., 1 hour, 6 hours, overnight, permanent).

       [ ]

    4. Authorization to bring animals into WSU locations such as hospitals, clinics or access equipment in the WSU campus area used for human patients. Provide details of authorization to use the facilities by the person responsible for the area(s). Include the name and title of the individual(s), and the date authorization was obtained. Also describe how animal use locations/equipment will be cleaned following use. An authorization letter can be attached in the "Attachments" tab above.

       NA

       [ ]

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5. If animals are being transported to WSU buildings, will they come into contact with, or be housed in the same room as other animals already residing at the destination?

> Yes (provide details below):

> ☐

> No

6. Will the animals need to be sedated or anesthetized prior to or during transportation? Note that large animals (e.g. dogs) usually require sedation prior to transportation. Please consult a veterinarian if this was not previously approved by the IACUC.

> Yes (provide details below):

> No

7. **Will the animals be transported by DLAR?**

You are encouraged to make arrangements with DLAR to transport your animals free of charge by calling 313-577-1452 if you are considering using a personal vehicle.

> Yes

> No, provide details below. You will be required to select the person(s) responsible for transporting the animals in the Training Checklist.

a. **If you and/or your staff will be transporting the animals, please assure the Committee that:**

- Animals will be transported in an appropriate climate controlled vehicle. (i.e. air conditioned/heated). The use of personal vehicles is discouraged, as it can result in allergen exposure to the occupant and future occupants of the car, as the car can serve as a potential reservoir of animal pathogens. During regular business hours, arrangements can be made with DLAR to transport animals free of charge.
- Animals will be transported expeditiously in a draped cage or cart by an approved route (out of public view avoiding personnel areas such that no one is aware that an animal is being taken into the hospital area).
- Animals will be hand carried between WSU buildings (the use of carts is discouraged due to uneven pavement conditions on walkways).
- Rodents will be transported in clean filtered microisolator cages and water bottles Inverted to prevent leakage.
- Rodent cages will be sanitized at the destination. Rodent cage exteriors must be sprayed with bleach solution (1 part bleach to 20 parts water) when they reach their destination. Cages cannot be opened until the bleach solution has been on the cage for 10 minutes.
- DLAR facility leaders will be notified at least 24 hrs in advance of the return of the animals.

> I will comply with the transportation requirements outlined above and have reviewed the Transportation of Animals Policy/SOP

b. **Briefly describe transportation route below.**

> ☐

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* * * Non-Surgical Procedure Details * * *

Non-Surgical Procedure Details

DESCRIBE ALL NON-SURGICAL PROCEDURES: Summarize in a narrative what procedures will be done. Include only those experiments where animals are directly involved. When animals are used as donors of organs, tissues, or cells, only describe how the organs, tissues or cells will be obtained. Do not describe what will be done with those organs, tissues or cells once they have been removed from the animal.

1. Describe every procedure.

2. How will the animals be monitored for adverse effects? Describe any likely effects.

   Indicate what parameters will be used to determine the need for additional doses of anesthesia.

3. Post-Anesthetic Care of Rodents

   Review and affirm the Post Operative/ Post Anesthetic Care of Rodents in the Guidelines section. If it will not be followed then the variance must be justified in that section.

4. Post-Anesthetic Care of Non-Rodents

   Describe supportive care and monitoring provided during immediate anesthetic recovery period (from cessation of anesthesia until sternal recumbency is regained) and intermediate recovery period (from sternal recumbency until the animal is able to walk).

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* * * Surgery Relationships * * *

1.   Will this project include multiple survival surgeries on the same animal?

     1a.   Multiple survival surgeries will be conducted on an animal, review the Multiple Survival Surgeries Policy and provide justification below.

         [   ]

2.   Will this project include multiple major survival surgeries on the same animal?

3.   Describe the sequence and timing of the surgeries and how they relate to each other. If multiple surgeries will be conducted on some or all of the animals use enough details to allow the reviewers to understand what each animal will undergo.

     [   ]

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* * * Schedule of Procedures * * *

Schedule of procedures for experimental groups: State or list in chronological order all procedures for each experimental group, their frequency, and time points over the course of the experiment. Details of each procedure are to be described in the appropriate sections, NOT here. A diagram or chart may be helpful to explain complex designs, which can be added in the "Attachments" tab above.

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* * * Euthanasia * * *

1. **STATE the SPECIFIC CRITERIA for the euthanasia of abnormal or moribund animals (assume someone may have to euthanize animals IN YOUR ABSENCE). Review the Defining Humane Endpoints and End-stage Illness Guideline.**

   Not Applicable (e.g.animals are used for tissue harvesting only and will not undergo any procedures prior to death)

   Weight loss of 20% or more

   Other conditions (examples may include, but are not limited to: a clinical condition that does not respond to treatment, such as an infected surgical site; any condition that a veterinarian deems severe enough to warrant euthanizing the animal). Please describe below:

   PHS Policy on Humane Care and Use of Laboratory Animals requires the IACUC to use the recommendations of the AVMA Guidelines for the Euthanasia of Animals: 2020 Edition; please refer to it when necessary. If anesthetic overdose or CO2 narcosis is used, a secondary procedure such as bilateral pneumothorax, severing the aorta, or removal of a critical organ must be used to assure that the animal will not recover.

   Euthanasia

2. **Is the method consistent with the AVMA Guidelines for the Euthanasia of Animals: 2020 Edition**

   If no, please provide scientific justification for the use of the method below.

3. **Who will be responsible for performing euthanasia?**

   Personnel Details

4. **Does this research include the euthanasia of mouse and/or rat fetuses and neonates?**

   Review and affirm the Euthanasia of Mouse and Rat Fetuses and Neonates in the Guidelines section. If it will not be followed then the variance must be justified in that section.

5. **Will all animals be euthanized at the end of this study?**

   If no, state their final disposition:

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* * * Attachments * * *

Research Paper

Use of FVB Myc-CaP cells as an immune competent, androgen receptor positive, mouse model of prostate cancer bone metastasis

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ARTICLE INFO

ABSTRACT

Prostate cancer (PCa) metastasis research has been hamstrung by lack of animal models that closely resemble the disease present in most patients – that metastasize to bone, are dependent on the androgen receptor (AR), and grow in an immune competent host. Here, we adapt the Myc-CaP cell line for use as a PCa androgen dependent, immune competent bone metastases model and characterize the metastases. After injection into the left cardiac ventricle of syngeneic FVB/NJ mice, these cells formed bone metastases in the majority of animals; easily visible on H&E sections and confirmed by immunohistochemistry for Ar and epithelial cell adhesion molecule. Mediastinal tumors were also observed. We also labeled Myc-CaP cells with tdTomato, and confirmed the presence of cancer cells in bone by flow cytometry. To adapt the model to a bone predominant metastasis pattern and further examine the bone phenotype, we labeled the cells with luciferase, injected in the tibia and observed tumor formation only in tibia with a mixed osteolytic/osteoblastic phenotype. The presence of Myc-CaP tumors significantly increased tibia bone volume as compared to sham injected controls. The osteoclast marker, TRAcP-5b was not significantly changed in plasma from tibial tumor bearing animals vs. sham animals. However, conditioned media from Myc-CaP cells stimulated osteoclast formation *in vitro* from FVB/NJ mouse bone marrow. Overall, Myc-CaP cells injected in the left ventricle or tibia of syngeneic mice recapitulate key aspects of human metastatic PCa.

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1. Introduction:

Prostate cancer (PCa) causes over 33,000 deaths per year in the United States [1]. However, there are relatively few models available to study its pathophysiology, especially for metastatic disease, and with characteristics that closely mimic the disease of most patients [2]. Genetically engineered mouse models of PCa have been developed based on expression of the SV40 large T antigen; (transgenic adenocarcinoma of the mouse prostate (TRAMP)), or deletion of *Pten* targeted to the prostate gland [3–5]. Xenograft models metastasize to bone and other tissues but lack a normal immune system and are almost completely devoid of signaling downstream of the androgen receptor (AR) [6–8].

A basis for improvement in PCa mouse models came with the development of the Myc-CaP cell line [9]. This line was initially developed from a spontaneous prostate tumor of a c-myc overexpressing mouse and shows overexpression of wild type *Ar* [9] and also *Ar* splice variants [10]. Mammary fat pad tumors developed from this cell line partially regress after animals are castrated [9]. Additionally, because they are syngeneic, they have been used in studies of tumor immunology and immunotherapy when injected subcutaneously or as an intraosseous injection into the femur, but with minimal characterization of bone tumors formed after femur intraosseous injection [11–13]. However, adaptation of the FVB Myc-CaP cell line to model disseminated PCa has progressed at a slower pace. A recent report describes use of the cell line to induce liver tumors after injection in the spleen [14]. The same cell line on the C57BL/6 rather than the original FVB/NJ

background was recently shown to form bone tumors after systemic (intra-cardiac) inoculation, but only from a single cell suspension of an existing tumor – which limits the studies that can be performed with the model [15]. Here, we inject FVB Myc-CaP cells maintained under routine culture conditions in the left cardiac ventricle of syngeneic mice and observe metastases to multiple bones. To better study the bone phenotype of the cells, we directly injected the cells in the tibia of mice and observed formation of large mixed osteolytic/osteoblastic tumors and characterized their bone phenotype. Therefore, we expect these models to be invaluable for future studies of PCa bone metastases.

2. Materials and methods

2.1. Cell culture and in vitro assays

The Myc-CaP cell line on the FVB background (#CRL-3255) was obtained from ATCC and cultured as recommended in DMEM with 10% FCS. PC3 cells were obtained from ATCC (#CRL-1435) and cultured in RPMI with 10% FCS. Myc-CaP cells were labeled by lentiviral transduction with either; tdTomato (CloneTech Lenti-LVX-IRES) and selected by FACS, or labeled with firefly luciferase (Genecopoeia hLUC-Lv105) and selected with puromycin. To model mouse osteoclast (OCL) formation, bone marrow cells from FVB/NJ mice not adherent to tissue culture plastic were used as previously described [16]. The cells were cultured for 7 days in α-MEM with 10% FCS and 10 mg/ml of rhM-CSF (R&D Systems) and rhRANKL (R&D Systems) at a sub-maximal concentration of 10 ng/ml to allow for stimulation by added conditioned media (CM). $2x10^5$ cells were seeded per well in 200 μl volume in 96 well plates. Media was changed every other day. For conditioned media collection, $5x10^5$ Myc-CaP or PC3 cells were seeded in 10 cm dishes in their usual growth media (DMEM or RPMI respectively) containing 10% FCS and cultured for one day. For CM collection, the media was changed to α-MEM with 10% FCS and cells were cultured for an additional day prior to CM collection. CM was added to OCL cultures at 25% of the total volume (50 μl per well). Cultures were fixed with 10% neutral buffered formalin, and stained for tartrate resistant acid phosphate (TRAP/Acp5) as previously described [17]. TRAP^{+} cells with three or more nuclei were counted as OCLs. To assay TRAP activity secreted into culture media, 100 μl of media collected after 5 days of culture was combined with 200 μl of TRAP substrate and absorbance at 562 nm was determined with a microplate reader [17]. Bone resorption and formation markers were evaluated by ELISA of plasma samples; TRAP-5b (Immunodiagnostic Systems #SB-TR103), type 1 collagen pro-peptide (Immunodiagnostic Systems #AC-33F1) and osteocalcin (Novus Biologicals #NBP2-68151). Osteoclastogenic factors were measured from conditioned media of Myc-CaP cells; soluble Rankl (R&D Systems # MTR00) and Cxcl15/Il-8 (RayBiotech #ELM-CXCL15).

2.2. Animal models and imaging

All studies were approved by the Institutional Animal Care and Use Committee of the University of Michigan. 5×10^5 or 5×10^4 cells recovered from *in vitro* culture were injected in the cardiac left ventricle or left tibia respectively of male syngeneic FVB/NJ mice (Jackson Labs) as previously described [18]. Bioluminescence *in vivo* imaging or fluorescence *ex vivo* imaging were conducted with a Perkin Elmer IVIS 2000 instrument. For bioluminescence imaging, 200 mg/kg of Promega VivoGlo™ luciferin was injected 10 min prior to imaging under 2% isofluorane in oxygen anesthesia. Mice were euthanized by CO_2 asphyxiation if they became moribund from disease, had tumors > 1 cm, were not using the affected leg, or at experiment endpoint. After euthanasia, blood was collected into EDTA tubes and centrifuged to collect plasma. Tissues were drop fixed in 10% neutral buffered formalin for one day at 4 °C and then stored in 70% ethanol. Bone specimens were imaged with a microCT system (μCT100, Scanco Medical, Bassersdorf, Switzerland). Scan settings were: voxel size 12 μm, 70 kVp, 114 μA, 0.5 mm AL filter, and integration time 500 ms. Analysis was performed using the manufacturer's evaluation software, and a fixed global threshold of 18% (180 on a grayscale of 0–1000) was used to segment bone from non-bone. A 6 mm region of bone was analyzed beginning immediately below the growth plate for 500 slices. Bone volume and tissue mineral density were generated and compared with the control tibiae for each animal.

2.3. Histology and immunohistochemistry

Bones were then decalcified in multiple changes of 10% EDTA pH 8 for 3 weeks at 4 °C and embedded in paraffin. 5 μm sections stained with hematoxylin and eosin were used for general morphologic assessment. Blue staining on Masson's Trichrome was used to assess collagen. TRAP (Acp5) cytochemistry was used to visualize OCLs, followed by hematoxylin counterstaining and aqueous mounting as described [17]. PCa origin of tumors was confirmed by immunohistochemistry. After de-waxing and permeabilization with PBS with 0.1% Triton X-100 (PBT), immunohistochemistry (IHC) conditions were as follows: For Ar and Epcam; antigen retrieval was performed with pepsin (Invitrogen #003009) for 15 min at 37 °C. Endogenous peroxidases were blocked with 3% hydrogen peroxide in PBS for 15 min at room temperature. Slides were blocked with 5% goat serum in PBT overnight at 4 °C. Primary antibodies for androgen receptor (Millipore antibody #06–680, diluted 1:50) and Epcam (Abcam antibody #71916, diluted 1:250) or the corresponding concentrations of rabbit IgG were diluted in PBT and applied at room temperature for one hour. Slides were washed with PBT and visualized using reagents provided with the Vector rabbit ABC Elite™ kit (#PK-6101). Slides were subsequently counterstained with hematoxylin, dehydrated and mounted. For F4/80, antigen retrieval was with 10 mM pH 6 sodium citrate with 0.05% tween heated to 125 °C for 30 s, then 90 °C for 10 s at 15 psi in a Biocare medical DC2002 Decloaking Chamber. The primary antibody was a monoclonal rabbit anti-mouse (Cell Signaling Technology #99940) diluted 1:200. For Cd3ε, antigen retrieval was with Antigen Unmasking Solution Tris pH 9 (Vector Labs #H-3301) heated in a pressure cooker as above and the primary antibody was monoclonal rabbit anti-mouse diluted 1:100 (Cell Signaling Technology #99940). For F4/80, and Cd3ε, peroxidase staining was with the Vector Laboratories NovaRED kit (#SK-4805).

2.4. FACS and flow cytometric analysis

All analyses were conducted on a BD FACS AriaIIu cell sorter and analyzer with 405 nm, 488 nm, and 630 nm lasers and non-colinear detectors. After transduction, successive rounds of FACS for tdTomato (PE channel) were performed until a uniform population was obtained. Unlabeled Myc-CaP cells were used as a negative control. To detect tdTomato positive tumor cells *in vivo*, lungs or bones (tibia, femur and lumbar vertebrae) were disrupted with a mortar and pestle and strained. The cells were labeled with DAPI and mouse PE/Cy7 conjugated Ter119 (Biolegend #116222) and APC conjugated Cd45 (Biolegend #103112) antibodies. Putative tumor cells were defined as single viable cells, negative for Ter119, and successively negative for Cd45 but positive for tdTomato.

2.5. Statistical analyses

Student's unpaired equal variance *t*-test was used to compare two means. Multiple means were analyzed with one-way ANOVA with Tukey post-hoc testing. The log-rank test was used for survival analyses. All analyses were conducted with GraphPad Prism software.

3. Results

We injected half a million Myc-CaP cells into the left cardiac ventricle of eight FVB/NJ mice and injected 4 mice with phosphate buffered saline (PBS) as sham controls. The animals injected with Myc-CaP cells died or were moribund at a median of 30 days after injection (Fig. 1A). Upon necropsy of these animals, with the aid of H&E histology, we observed tumors in bones of 4 of 7 animals and in the chest of all 7 animals analyzed (Fig. 1B). One animal was not analyzed by histology due to inadequate preservation of the specimen. We did not observe tumors in the kidneys, livers, gastrointestinal tracts, or genitourinary tracts by gross analysis or H&E histology. We confirmed the presence of bone metastases by immunohistochemistry (IHC) for both androgen receptor and epithelial cell adhesion molecule (EPCAM) (Fig. 1C). At the level of gross anatomic analysis, the tumors in the chest cavities,
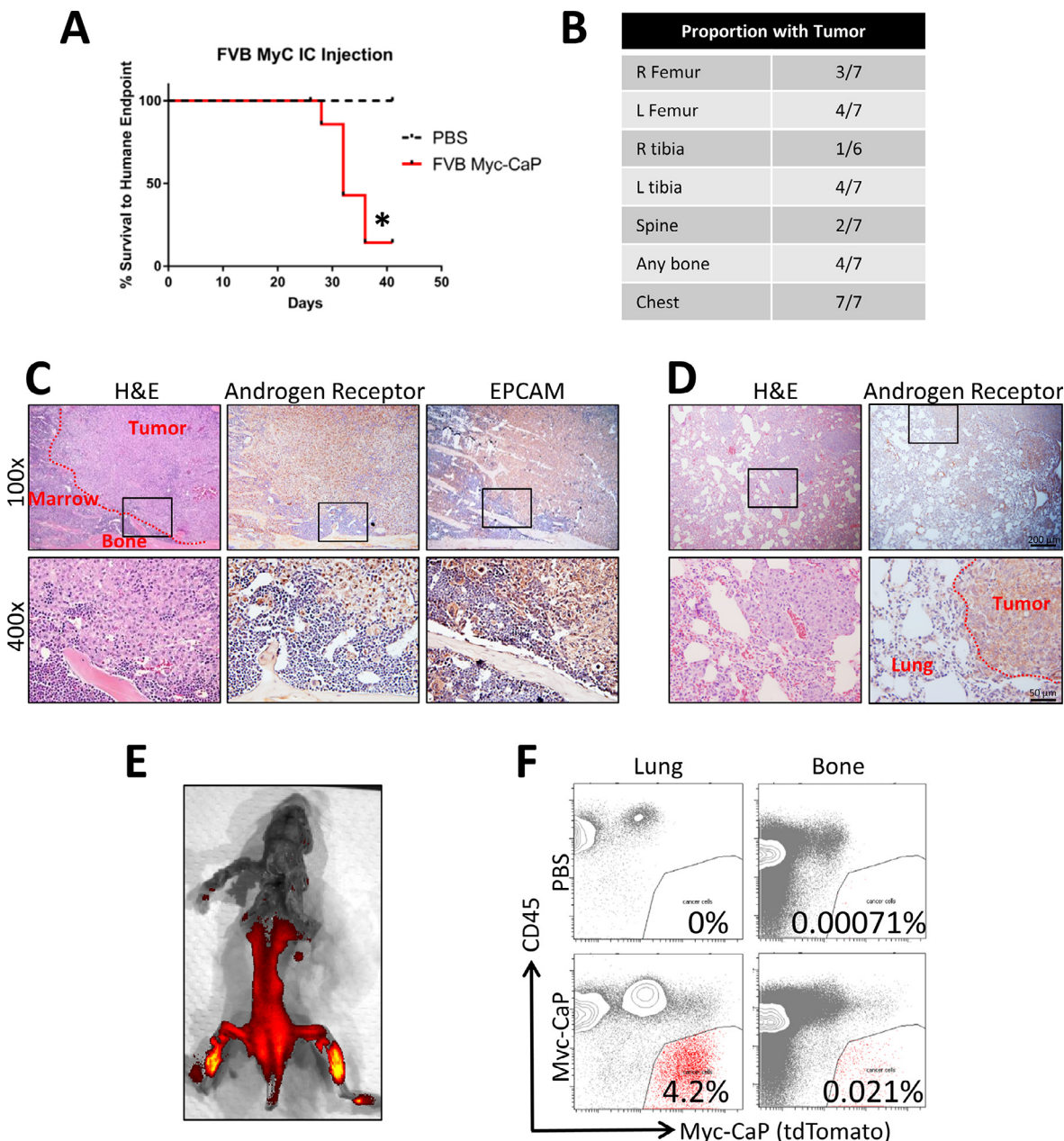
Fig. 1. Distribution of tumors formed by FVB Myc-CaP cells after left ventricle injection by gross analysis, histology, fluorescence imaging, and flow cytometry. (A) Kaplan-Meier curve of time to death or humane endpoint of FVB mice injected with parental FVB Myc-CaP cells (*n* = 8) or PBS as a negative control (*n* = 4). * indicates *p* < 0.05 by the log rank test. (B) Number of animals injected with tumor cells with tumor visible on H&E sections at each listed anatomic site. (C) Immunohistochemical (IHC) validation of tumor identity of suspected bone metastases. Representative images for sections stained with H&E or IHC staining against either androgen receptor or Epcam (brown) with hematoxylin counterstain (blue). Original magnification 100x (10x objective), or 400x (40x objective). Area of higher magnification is indicated by the boxes. (D) IHC validation of tumor identity of suspected lung metastases. (E) *ex vivo* fluorescent imaging of the CNS and MSK systems of a mouse injected in the left ventricle with tdTomato labeled FVB Myc-CaP cells. (F) Flow cytometry plots showing detection of tumor cells at metastatic sites (lung or bone) from sham (PBS) or tumor injected animals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
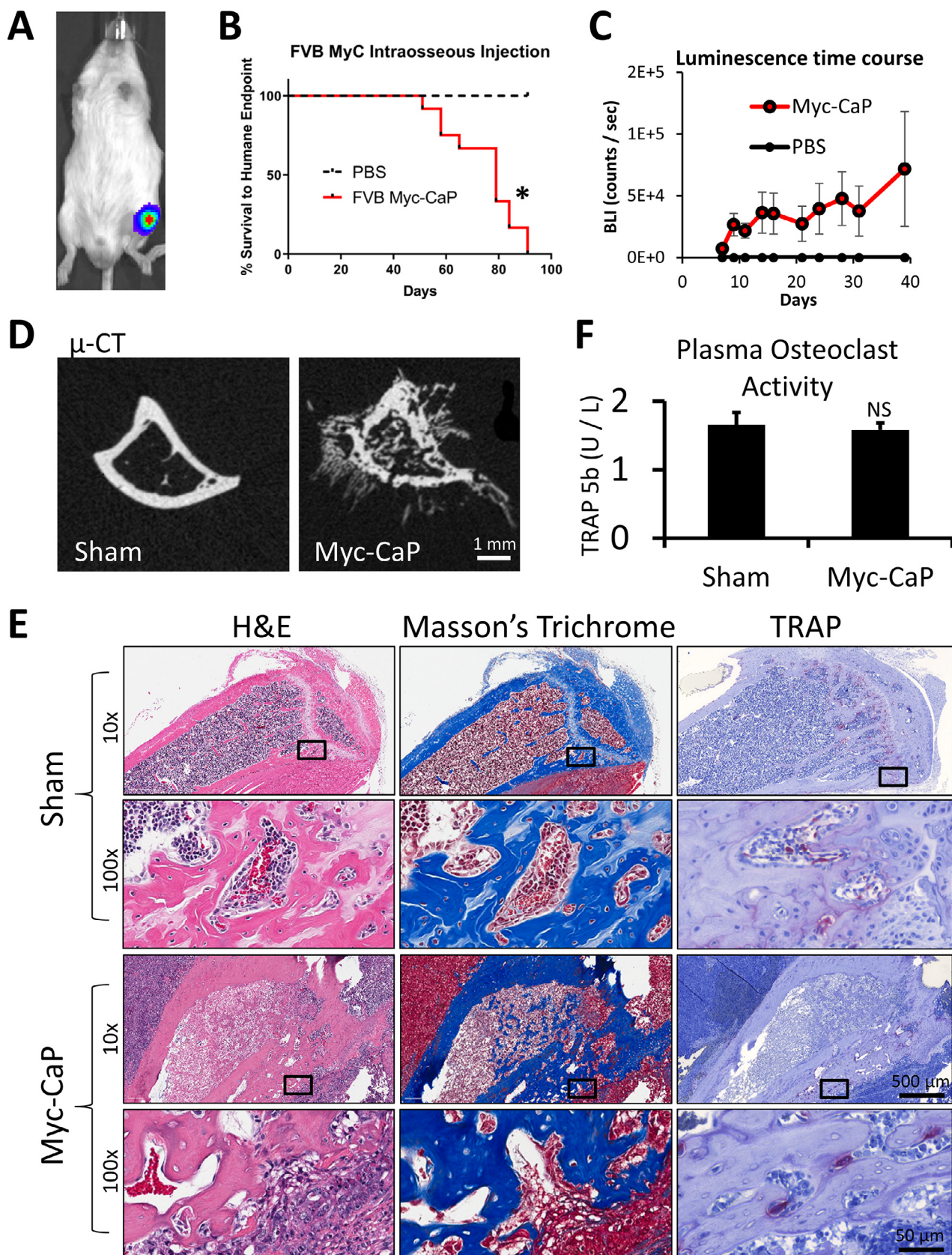
Fig. 2. Myc-CaP tumor formation after intra-tibial injection. (A) Example bioluminescence image of mouse injected with luciferase labeled Myc-CaP cells. (B) Kaplan-Meier curve of time to humane endpoint of FVB mice injected with parental FVB Myc-CaP cells (*n* = 14) or PBS (*n* = 4) as a negative control.. (C) Mean bioluminescence ± SEM over time of Myc-CaP or PBS (sham) tumors. (D) Sample axial micro-CT images of tibia injected with PBS or Myc-CaP cells. (E) Sample sections stained with hematoxylin and eosin (left), Masson's trichrome (middle), or tartrate resistant acid phosphatase (TRAP) with hematoxylin counterstain (right) of sham (top) or Myc-CaP injected mouse tibiae (bottom). (F) Osteoclast activity as measured by TRAcP-5b ELISA of peripheral blood plasma from sham injected or Myc-CaP intra-tibial tumor bearing animals. Data is presented as mean ± SEM. * indicates *p* < 0.05.
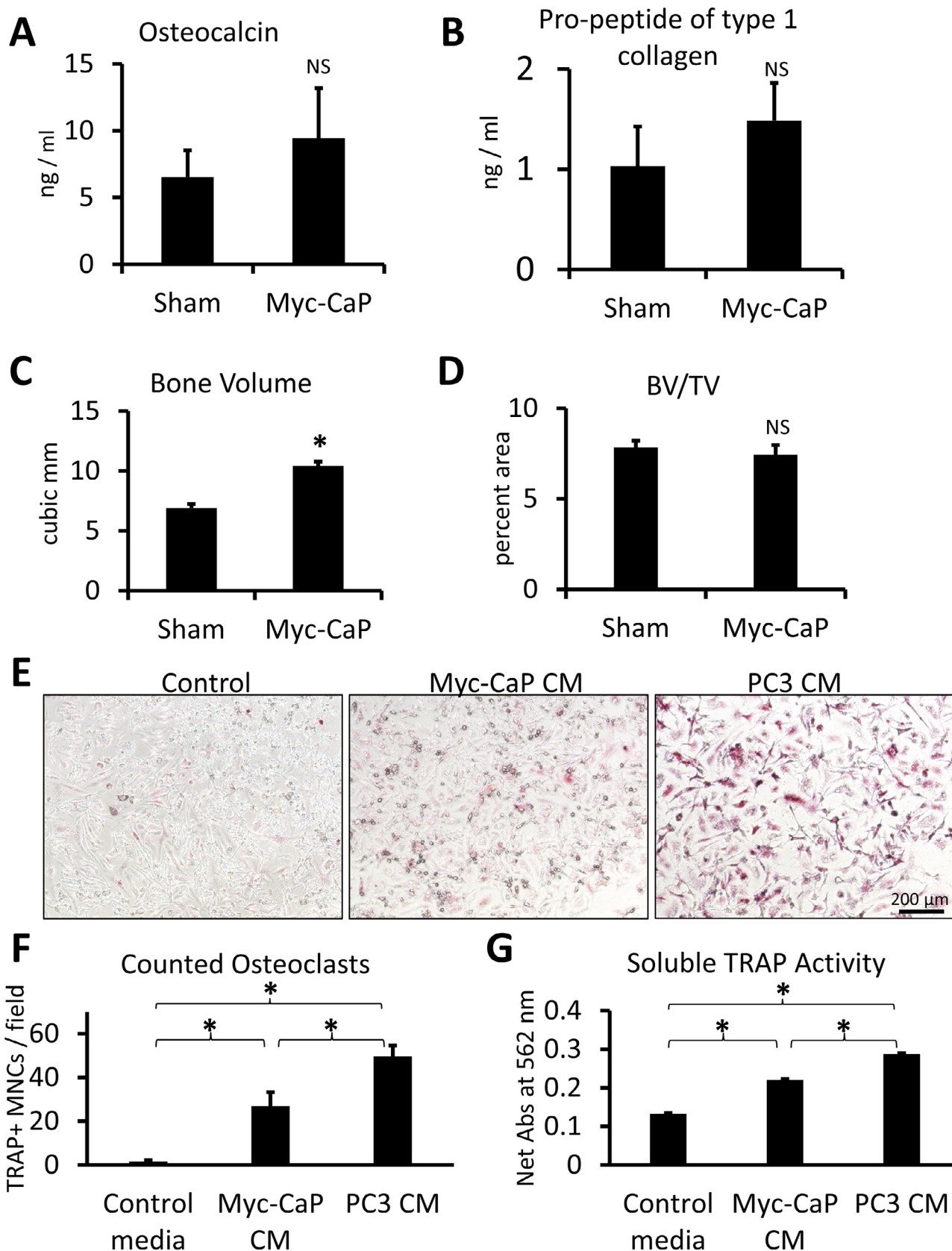
Fig. 3. Quantification of the Myc-CaP bone tumor phenotype. (A) and (B) Osteocalcin or type 1 collagen pro-peptide concentration in plasma of mice injected in the tibia with luciferase labeled Myc-CaP cells ($n = 14$) or PBS ($n = 4$). (C) and (D) Bone volume or the ratio of bone volume to total volume (BV/TV) in tibia injected with sham or luciferase Myc-CaP cells. (E) 100 × original magnification images of osteoclast cultures stained for TRAP (red). (F) TRAP + multinucleated cells per low power field of mouse OCL cultures with or without addition of conditioned media from Myc-CaP or PC3 cells. (G) Colorimetric assay of secreted TRAP activity in the mouse OCL cultures. Data is represented as mean ± SEM. * indicates $p < 0.05$. Data from *in vitro* studies represents quadruplicate wells from one of four independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

appeared to be in the mediastinum rather than arising from heart or lung. Histologically, the majority of the tumor volume was in the mediastinum, but we did observe small tumors in lung parenchyma as well (Fig. 1D). To further validate the distribution of metastases in the left cardiac ventricle injection model, we labeled Myc-CaP cells with tdTomato for *in vivo* imaging and isolation and performed left cardiac ventricle injections. Gross analysis, as aided by *ex vivo* fluorescent imaging, showed tumor cells in the bone; with putative tumor cells defined as tdTomato positive and negative for Ter119 (erythroid marker) and Cd45 (leukocyte marker) (Fig. 1F). We consistently found in excess of an order of magnitude more events in the tumor cell gate in Myc-CaP injected animals as compared to PBS injected controls.

To explore the potential of Myc-CaP cells as a bone metastasis model and to quantify the bone phenotype, we next examined their growth after intra-tibial intraosseous injection. To increase the sensitivity of *in vivo* imaging, we labeled the Myc-CaP cells with luciferase (Fig. 2A) before performing intra-tibial injections of Myc-CaP cells (14 FVB/NJ mice) or sham injections of PBS into 4 mice. These animals required euthanasia for limping, not using the affected hind limb, or tumor diameter >1 cm after about two months and had large tibial tumors at the time of necropsy (Fig. 2B). All 14 Myc-CaP injected mice developed grossly visible tumors. Tumor growth as measured by bioluminescence was detectable 5 days after injection and was only detectable in the left legs of Myc-CaP injected – but not sham injected animals (Fig. 2C). On micro computed tomography (μ-CT) analysis, the lesions had areas of both bone loss and apparent new bone formation including areas outside of the prior boundary of bone cortex (Fig. 2D). Because of the surprising finding of suspected extra-cortical bone formation, we analyzed the bones histologically. The extra-cortical bone areas stained as expected (pink) on hematoxylin and eosin (H&E) stains, and also as expected (blue) on Masson's tri-chrome stain, which selectively stains collagen (Fig. 2E). Therefore, from the histologic findings and high attenuation on μ-CT analyses, we concluded that Myc-CaP bone tumors induce extra-osseous calcification in addition to abnormal bone formation within the existing bone cavity.

To further evaluate the presence of abnormal bone remodeling, we labeled sections from these specimens for tartrate resistant acid phosphatase (TRAP/Acp5), as a marker of mouse osteoclasts and observed increased TRAP staining of osteoclasts (OCLs) at many of the areas of extra-cortical ossification (Fig. 2E). However, we did not detect a difference in TRAP-5b in plasma from control compared to Myc-CaP tumor bearing animals (Fig. 2F).

To better determine if the Myc-CaP bone tumor phenotype is osteosclerotic, osteolytic, or mixed, we quantified bone remodeling parameters. The concentration of bone formation markers, osteocalcin and pro-peptide of type 1 collagen, trended higher in the plasma of mice injected with luciferase labeled Myc-CaP cells but was not statistically significant (Fig. 3A and 3B). Therefore, we quantified μ-CT images of the sham or Myc-CaP tumor bearing tibiae (Fig. 2D). Presence of the Myc-CaP tumor increased the bone volume in a statistically significant fashion (Fig. 3C). However, the ratio of the bone volume to total volume (BV/TV) remained unchanged (Fig. 3D). Therefore, we concluded that the volume of new bone induced by the tumors was approximately balanced by the increased bone area caused by extra-cortical ossification. To further assess the contribution of Myc-CaP cells to OCL formation, we assayed formation of OCLs *in vitro* from bone marrow of FVB/NJ mice. As assessed by microscopy, counted TRAP$^+$ multi-nuclear cells, and activity of TRAP secreted into the media of the OCL cultures; conditioned media (CM) from Myc-CaP cells significantly induced OCL formation as compared to control media, but induced less OCL formation than the strongly osteolytic cell line, PC3 (Fig. 3E – 3G).
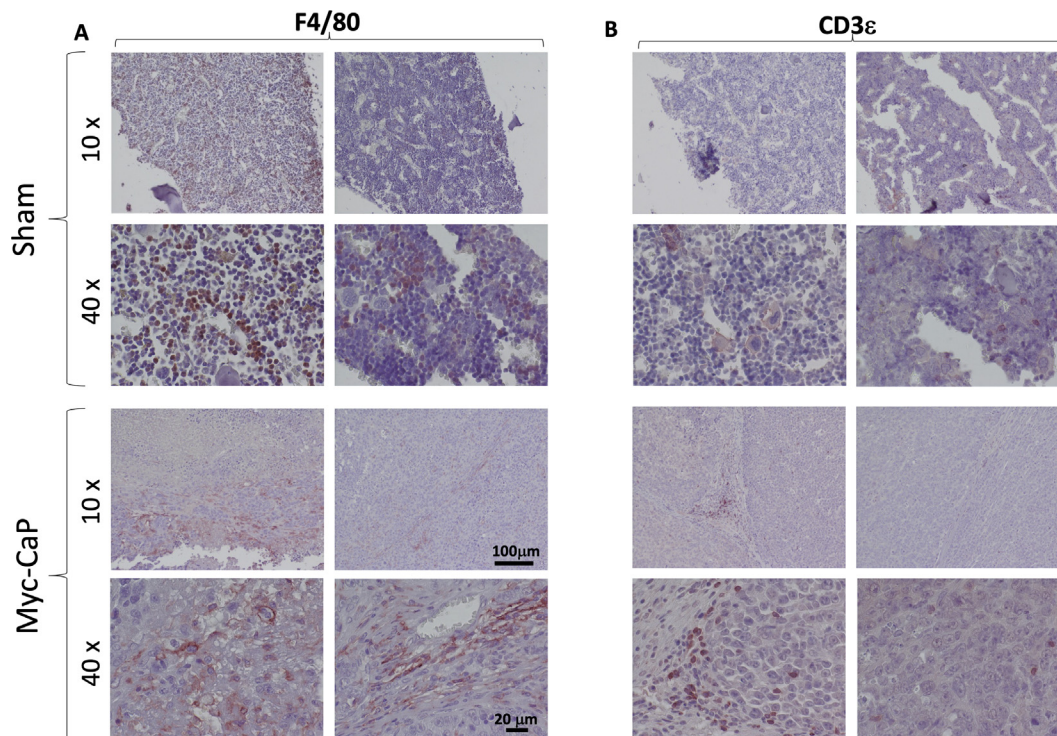
Fig. 4. Immune cell infiltration in Myc-CaP intra-tibial tumors. (A) IHC for macrophage marker, F4/80 (B) IHC for T-cell marker Cd3ε. Antigens are labeled in red. Nuclei are counter-stained with hematoxylin (blue). Two representative images acquired using either 10x or 40x objectives from either sham injected (top panels) or MycCaP tumor bearing animals (bottom panels) are shown for each condition as indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Lastly, as an aid to researchers using this model in future studies of cancer immunity, we examined immune infiltration of luciferase labeled Myc-CaP tibial tumors. Upon immunohisto-chemistry (IHC) for F4/80, we observed a moderate macrophage infiltration, predominantly at the edge of the tumors (Fig. 4A). With the aid of labeling for Cd3ε, we observed a much more sparse T-cell infiltration, again predominantly at the periphery of the tumors (Fig. 4B). This low number of T-cells is in keeping with findings of multiple investigators that PCa is an immunologically "cold" tumor with low numbers of tumor infiltrating lymphocytes (TILs)[19]. Although various definitions and quantification methods have been used for TILs, CD3^{+} total T-cells and subsets, especially CD8^{+} T-cells are shown in other cancers to correlate with survival [20,21].

4. Discussion

Here we describe a new mouse model of PCa bone metastases, which shares important features of the disease of many metastatic prostate cancer patients. This model utilizes the Myc-CaP prostate cancer cell line inoculated into syngeneic FVB/NJ mice and therefore produces a model that requires androgen signaling and is immune competent. The FVB background Myc-CaP cell line models the most common type of deadly PCa; disease which initially responds to androgen deprivation, then becomes castration resistant (can grow in the presence of a low concentration of testosterone), but continues to require transcription regulated by the androgen receptor. Subcutaneous FVB Myc-CaP tumors established in intact FVB/NJ mice initially shrink after medical or surgical castration, but then uniformly progress within two months [22]. In patients, selective pressure of continual hormonal based therapies can induce the development of small cell neuroendocrine prostate cancer, or alternatively "double negative" prostate cancer which has neither androgen signaling nor neuroendocrine markers. However, even in modern patients, androgen receptor positive PCa remains the most common subtype of castration resistant disease [23]. One of the ways that prostate cancers become castration resistant while retaining androgen receptor related signaling is through ligand independent splice variants of the *AR* gene including *AR-v7*. FVB background Myc-CaP cells express splice variants of the *Ar* gene, whereas B6 background Myc-CaP cells do not [10]. This might explain why FVB Myc-CaP cells rapidly become castrate resistant *in vivo*, and in the current study, FVB Myc-CaP cells formed bone tumors after routine cell culture. But in another study using B6 background Myc-CaP cells, the investigators only observed bone tumor formation after preparing a single cell suspension of an existing tumor [15]. We expect the tumor growth from routine culture, which we present here, to expedite future mechanistic studies using FVB background Myc-CaP cells as a bone metastasis model.

Furthermore, because of the syngeneic and immune competent host, FVB Myc-CaP models are also well suited to studies of the immune system and immune therapy in prostate cancer [22]. This is unlike the more commonly used xenograft based models of metastatic PCa [6]. These studies are especially of interest because modern immune checkpoint inhibitors have not been effective enough in most PCa patients to garner approval from the U.S. F.D.A. or regulatory bodies in other countries, though many investigators are trying to understand the mechanisms of resistance. Prostate cancer tumors usually have a "cold" immune phenotype with scant numbers of tumor infiltrating lymphocytes, and induction of tolerance in many of the lymphocytes that are present [19]. In keeping with this literature, in this model we performed Cd3ε IHC of intra-tibial tumors and observed only rare infiltrating T-lymphocytes, predominantly near the edges of the tumors. Of unique interest to PCa, androgens are reported to partially regulate tumor immunology, with androgen suppression favoring a more robust immune response [24]. Therefore, the previously reported ability of the FVB Myc-CaP model to respond to castration and later progress makes the model particularly attractive in studies of immunotherapy.

Lastly, development of a bone metastatic model of PCa is also notable because bone is the most common metastatic site for the disease. Indeed, 90% of patients who die of PCa have bone metastases [25]. Our studies showed that FVB Myc-CaP cells form bone metastases when administered both systemically (left ventricle intracardiac injection) and in a directed fashion when injected in the tibia. The results of our characterization of these tumors resembled the appearance of bone metastases in many PCa patients. We characterize the tumors as mixed osteosclerotic/osteolytic and quantitatively demonstrated new calcified bone formation, which curiously included areas outside of the prior cortical boundary. Although PCa bone metastases are predominantly osteosclerotic, mixed osteolytic/osteosclerotic PCa bone metastases, like our model, are not rare either and comprised 12.7% of patients in one series [26]. We also found that Myc-CaP conditioned media induced osteoclast formation *in vitro*, though not to the extent induced by the purely osteolytic cell line, PC3 [8]. While others have begun to use a femoral intraosseous injection adaptation of FVB Myc-CaP cells, there has been minimal description of the bone phenotype and no reports of bone metastases after systemic (intracardiac) administration as we describe here [12,13].

Useful further characterization of this model system could include which secreted factors are responsible for induction of osteoclast formation and new bone formation in this system. To begin these investigations, we measured the concentrations of soluble Trance (RANKL) and Cxcl15 (Il-8) in biologic triplicates of Myc-CaP conditioned media. However, the concentration of each was below the detection limit of the ELISA assays we used (<5 pg/mL for Rankl and 0.8 ng/mL for Il-8 – data not shown). However, we think that our demonstration of reliable bone tumor formation and characterization of the bone tumor phenotype will give investigators the necessary information to add this to their portfolio of PCa models. Overall, the FVB Myc-CaP model promises to be a clinically relevant and practical model of PCa bone metastases.

5. Conclusions

In these studies, we found that androgen receptor positive, murine FVB Myc-CaP cells form bone metastases in syngeneic FVB/NJ hosts with a mixed osteolytic/osteosclerotic appearance after systemic and localized intraosseous injection of cells cultured *in vitro*. The model therefore promises great utility for prostate cancer research involving bone, androgen signaling, or the immune system.

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CRediT authorship contribution statement

Yu Wang: Investigation, Visualization, Methodology, Writing - review & editing. **Mackenzie K. Herroon:** Investigation, Visualization, Methodology, Writing - review & editing. **Steven P. Zielske:** Investigation, Visualization, Writing - review & editing. **Leigh Ellis:** Conceptualization, Methodology, Resources, Writing - review & editing. **Izabela Podgorski:** Investigation, Visualization, Methodology, Supervision, Writing - review & editing. **Russell S. Taichman:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Frank C. Cackowski:** Conceptualization, Supervision, Investigation, Visualization, Methodology, Funding acquisition, Writing – original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Double-Negative Prostate Cancer Masquerading as a Squamous Cancer of Unknown Primary: A Clinicopathologic and Genomic Sequencing-Based Case Study

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Case Description

In January 2018, a 79-year-old man was referred to our genitourinary medical oncology clinic for management of his prostate adenocarcinoma metastatic to multiple bones. His case was complicated by a concurrent diagnosis of melanoma metastatic to a distant skin site, for which he had started pembrolizumab immunotherapy the week previously. His prostate cancer had originally been diagnosed in November 2011 after a transurethral resection of the prostate performed for urinary obstruction revealed Gleason score 7 prostate adenocarcinoma. In June 2015, he was found to have biopsy-proven prostate adenocarcinoma from a rib metastasis. The patient elected to defer all medical therapy and had his rib metastasis treated with radiation alone. He then had definitive radiation to the prostate in June 2016. His prostate cancer spread to additional bony sites, which were likewise treated with radiation, including radiation to the thoracic spine in July 2017 and the proximal humerus in August 2017, rather than any systemic therapy. In December 2017, he had prophylactic nipple irradiation to prevent gynecomastia in preparation for noncastrating medical therapy with single-agent bicalutamide. On meeting the patient for the first time in January 2018, we elected to continue with the plan for single-agent bicalutamide 50 mg per day, given the patient's preference and the uncertain prognosis from his metastatic melanoma. His prostate-specific antigen (PSA) was 30.8 ng/mL at the time of treatment initiation and responded rapidly to bicalutamide, reaching a nadir of 1.1 ng/mL in September 2018 (Fig 1A).

His melanoma was diagnosed from a shave biopsy of the right superior lateral lower back in August 2015 as localized ulcerated malignant melanoma, unclassified, with nevoid features. It was invasive to at least 1.45 mm and at least Clark level IV, and it had five mitoses/mm². He subsequently had a microstaging excision and a right back excision with a negative sentinel lymph node in the right groin. In 2017, the patient noticed a new lump on his right lower back scar in 2017. In November 2017, excisional biopsies of his right lower back and right groin both showed metastatic melanoma, as confirmed by immunohistochemistry (IHC) showing positive staining for SOX-10 and MART-1. A 7-mm lesion was also detected on his posterior scalp and was also visible on magnetic resonance imaging of his brain and metabolically active on positron emission tomography (PET)/computed tomography (CT); hence, it was diagnosed as stage IV melanoma. Mutation testing showed an atypical N581I mutation in the *BRAF* gene, but wild-type status at the *BRAF* V600 codon. His PET/CT showed extensive osteosclerotic lesions, most of which were not fluorodeoxyglucose (FDG) avid, consistent with metastatic prostate cancer. The most avid lesion in the right ischium had a maximum standardized uptake value (SUV max) of 7.2 but was biopsied and found to be metastatic prostate adenocarcinoma, as confirmed by IHC assessment that demonstrated NKX3.1 expression. He began treatment of his metastatic melanoma with pembrolizumab 200 mg intravenous every 3 weeks and received four treatments before the pembrolizumab was stopped because of the development of pneumonitis. This treatment resulted in a complete response in his melanoma as assessed by physical examination of his scalp lesion. His pneumonitis was treated with a taper of prednisone, which was reduced to physiologic dosing by August 2018.

However, despite evidence of response in his melanoma by physical examination, there was concern for progression on PET/CT in June 2018, with FDG update in the descending colon/small bowel wall, perisplenic region, a mildly FDG-avid left internal iliac lymph node, and a right external iliac nodal conglomerate encasing the right ureter, 4.1 cm in diameter and with an SUV max of 11.7. By December 2018, the right pelvic mass had increased in size to 4.4 cm and in metabolic activity to SUV max 19.8 (Fig 1B). At this time, he was also noted to have multiple small lung nodules of unclear etiology. Because the appearance of the right

FIG 1. Clinical timeline and cross-sectional imaging. (A) Summary of the clinical course and systemic therapy. (B) Positron emission tomography (PET)/computed tomography (CT) showing the right pelvic mass before treatment in December 2018. Dark orange/white color indicates metabolic activity. (C) CT with oral and intravenous contrast showing the right pelvic mass after treatment with carboplatin and gemcitabine (Gem-carbo) chemotherapy. Dashed red circle indicates the metabolically active lesion in the previous PET scan. (D) Noncontrast chest CT before docetaxel chemotherapy in September 2019. (E) Noncontrast chest CT after three cycles of docetaxel chemotherapy. Red arrows in (D) and (E) indicate a lesion responsive to docetaxel. PSA, prostate-specific antigen.
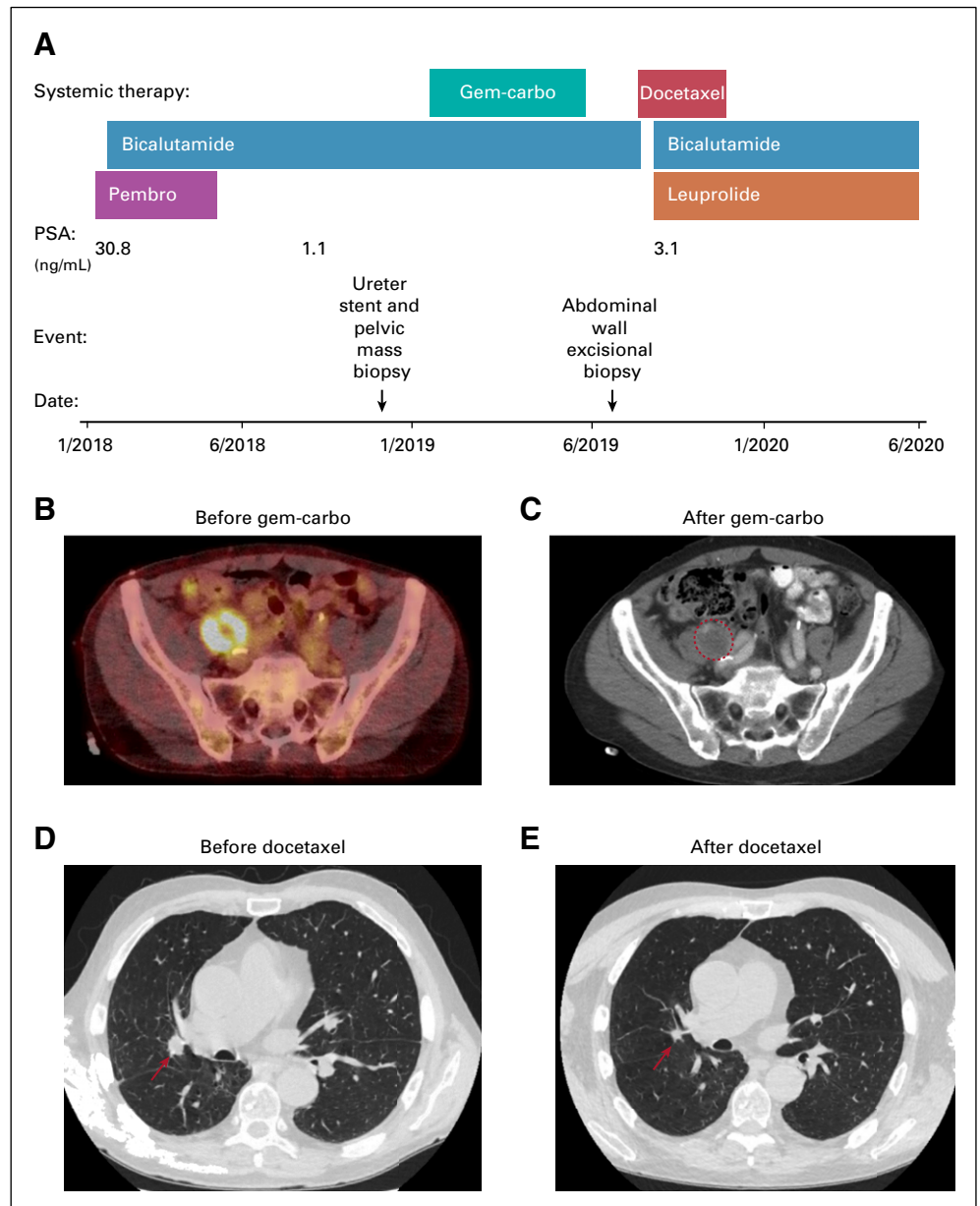
pelvic mass was judged to be atypical for either prostate cancer or melanoma, it was biopsied by pelvic laparoscopy in November 2018 and was found on surgical pathology assessment to be consistent with a high-grade carcinoma with squamous features (Fig 2A). IHC assessment demonstrated neoplastic cells positive for p63 and GATA3, but negative for PSA and NKX3-1 expression. Urine cytology also showed atypical urothelial cells. We performed exome sequencing using the University of Michigan OncoSeq panel (MI-OncoSeq) and whole transcriptome analysis on formalin-fixed paraffin-embedded tissue from this biopsy. All patients enrolled in the MI-OncoSeq study provided written informed consent approved by the University of Michigan Institutional Review Board. Consent is inclusive of publishing information and/or

images from participants (or their designate). However, results were limited because of low tumor content, estimated to be less than 10%. The variant allele fraction of mutations spanned 1% to 6%, and no copy number aberrations were detected (Table 1). Because the pelvic mass was causing pain and urinary obstruction, we elected to treat it with a platinum doublet active in urothelial carcinoma and carcinoma of unknown primary, despite not knowing the tissue of origin. In January 2019, we began carboplatin area under the curve 5 mg/mL/minute on day 1 and gemcitabine 1,000 mg/m² on days 1 and 8 of 21-day cycles, and we completed six cycles before stopping because of progressive fatigue. The patient experienced some improvement in pain, but the size of the right pelvic mass was largely unchanged (Fig 1C).

FIG 2. Histology and immunohistochemistry (IHC). (A) Hematoxylin and eosin (H&E) histology of pelvic mass biopsy showing squamous cell carcinoma. (B) H&E histology of the excisional biopsy of an abdominal (Abd) wall nodule, also showing squamous cell carcinoma. (C) H&E histology of the patient's (untreated) prostate tumor at initial diagnosis showing prostate adenocarcinoma. (D) IHC for ERG of the primary prostate tumor showing strong labeling. (E) H&E histology of the Abd wall nodule biopsy specimen used for molecular analysis. (F) IHC for ERG of the Abd wall nodule showing much weaker labeling.
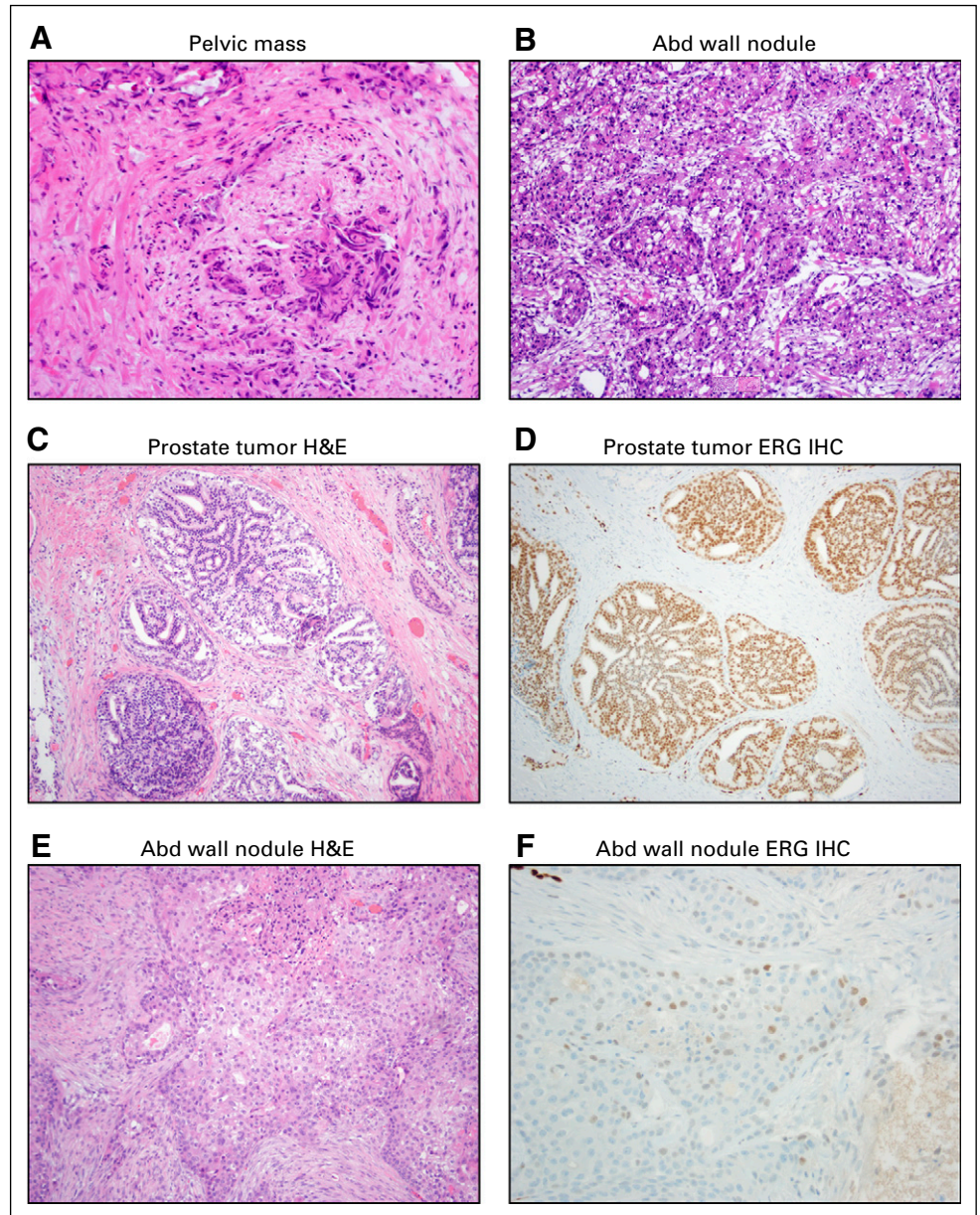
In June 2019, while being treated only with bicalutamide, the patient was noted on CT scan as having a 1.4-cm nodule in the superficial anterior abdominal wall, which subsequently became easily palpable and caused skin erythema. This nodule, together with smaller adjacent nodules, was removed by excisional biopsy in July 2019, where surgical pathology assessment demonstrated tumor features consistent with metastatic squamous cell carcinoma (Fig 2B). A fresh portion of this specimen was sent for analysis using the MI-OncoSeq platform. This specimen showed a much higher tumor content of 54%, allowing the discovery of additional molecular alterations.

Precision Medicine Tumor Board Discussion

Results were discussed at the University of Michigan Precision Medicine Tumor Board in September 2019.

Somatic aberrations detected in the previous sample were detected in the new biopsy specimen, suggesting clonal relatedness, and additional alterations consistent with the increased tumor content were noted as well (Table 2). Most curiously, we noted a few reads of chimeric transcripts supporting a gene fusion between TMPRSS2 exon 1 and ERG exon 2, accompanied by a focal deletion located in the intergenic region between the two genes and breakpoint visible on the copy number profile (Table 2 and Fig 3A). Fusions between the TMPRSS2 locus and Ets family transcription factors occur in nearly one half of prostate cancers in the United States and, to our knowledge, do not occur in other cancers.[1,2] Given the presence of this pathognomonic gene fusion and the patient's history of prostate adenocarcinoma, we concluded that his squamous cell carcinoma had arisen from his prostate cancer.

TABLE 1. Summary of OncoSeq Findings From the Pelvic Mass

Mutation Class	Gene/Aberration
Somatic point mutations (Total: 4) 1.3 Mutations/Mb	*PIK3CA*: p.E542K, activating *SF3B1*: p.K666Q (hotspot, also detected in blood) Mutations of uncertain significance: *ETV3* (p. L90P), *HOXA3* (p. P295L)
Somatic indels (Total: 2)	*ELK4:* Frameshift deletion, p.L353fs *NFKB1*: Frameshift deletion, p.E63fs
Copy-number aberrations	Insufficient tumor content for analysis
Gene fusions	No gene fusion detected
Outlier gene expression	Insufficient tumor content for analysis
Germline variants for disclosure	No pathogenic variants detected

To further confirm the findings, we subsequently performed IHC analysis, which showed positive ERG expression on the patient's initial prostate transurethral resection, which was strongly positive for ERG protein (Figs 2C and 2D). We further performed ERG IHC assessment on the patient's recent abdominal biopsy, which demonstrated focal and weak-to-moderate ERG protein expression, supporting a clonal phenotypic origin and evolution from the patient's original conventional (acinar) prostatic adenocarcinoma (Figs 2E and 2F).

Armed with the knowledge that this patient's squamous cell carcinoma was either a trans-differentiated or metaplastic variant of prostate cancer, we discussed the possible therapeutic implications at this point, when the patient was taking only single-agent bicalutamide. For prostate adenocarcinoma, the addition of medical castration, likely in addition to either abiraterone and prednisone or a non-steroidal second-generation antiandrogen, would have been a reasonable next line of therapy. However, the transcriptomics data of the MI-OncoSeq platform showed low expression of the androgen receptor and androgen responsive genes *KLK2*, *KLK3* (PSA), *TMPRSS2*, *ACPP*, and *SLC45A3* (Fig 3B). In keeping with these findings, his PSA level was only 3.1 ng/mL at this point. Therefore, we concluded that additional therapy targeting androgens or the androgen receptor was unlikely to be successful. Similarly, we examined markers for neuroendocrine carcinoma, the most common nonadenocarcinoma type of prostate cancer. However, expression of the neuroendocrine markers *SYP*, *CHGA*, *CHGB*, and *NCAM1* were also low (Fig 3C). Therefore, we did not plan chemotherapy with a regimen such as carboplatin and etoposide, which is active against small-cell neuroendocrine carcinomas.

We examined the remainder of the molecular results in an effort to find alternative, clinically actionable molecular targets. We noted two alterations associated with PTEN and Akt signaling: an activating mutation in *PIK3CA* and a homozygous deletion in *PTEN* itself (Table 2). Inhibitors of mammalian target of rapamycin (mTOR) have been suggested for use in *PTEN*-deficient tumors. However, overall, the results for mTOR inhibitors in prostate cancer have been disappointing.[3] Some randomized data support the use of PI3K inhibitors in metastatic castration-resistant prostate cancer.[4] However, this was in combination with abiraterone, which made PI3K inhibitors less attractive in this case, given the patient's near total lack of androgen signaling. Last, the homozygous deletion of *CDKN2B* could theoretically sensitize to CDK4/6 inhibitors. However, we decided against this option because CDK4/6 inhibitors have thus far shown disappointing results in prostate cancer.[5] Therefore, we elected to treat with docetaxel, the most common cytotoxic chemotherapy drug for castration-resistant prostate cancer.

At this point, the patient's lung nodules, which were previously indeterminate, had enlarged greatly (Fig 1D). We started treatment with docetaxel at 75 mg/m^2 for one cycle, then decreased the dose to 60 mg/m^2 because of fatigue, and completed three more cycles. We also attempted bicalutamide withdrawal for the adenocarcinoma component of his disease but resumed bicalutamide and added

TABLE 2. Summary of OncoSeq Findings From the Abdominal Wall Nodule

Mutation Class	Gene/Aberration
Somatic point mutations (Total: 14) 3.9 mutations/Mb	*PIK3CA* p.E542K, activating 13 nonrecurrent SNVs of unknown significance
Somatic indels	*ELK4*: Frameshift deletion, p.L353fs *NFKB1*: Frameshift deletion, p.E63fs
Copy-number aberrations	Extensive polyploidy, UPDs, and focal deletions: *CDKN2A* homozygous deletion *PTEN*; homozygous deletion; copy gain: chr1q, 2q, 4q, 3, 7, 8q, 12 to 16, 17q, 19 to 22 UPD: chr1p, 2p, 6, 8p, 9 to 11, 17p, 18
Gene fusions	*TMPRSS2* (exon 1) – *ERG* (exon 2), low (8) chimeric reads; minimal expression of both genes
Outlier gene expression	No expression of AR signaling genes and *ERG*; no expression of neuroendocrine markers; high expression of keratins KRT4, 5, 13, 14, 15, 19, 8; high expression of TP63 (basal cell marker)
Germline variants for disclosure	No cancer associated pathogenic variants

Abbreviations: SNV, single-nucleotide variant; UPD, uniparental disomy.
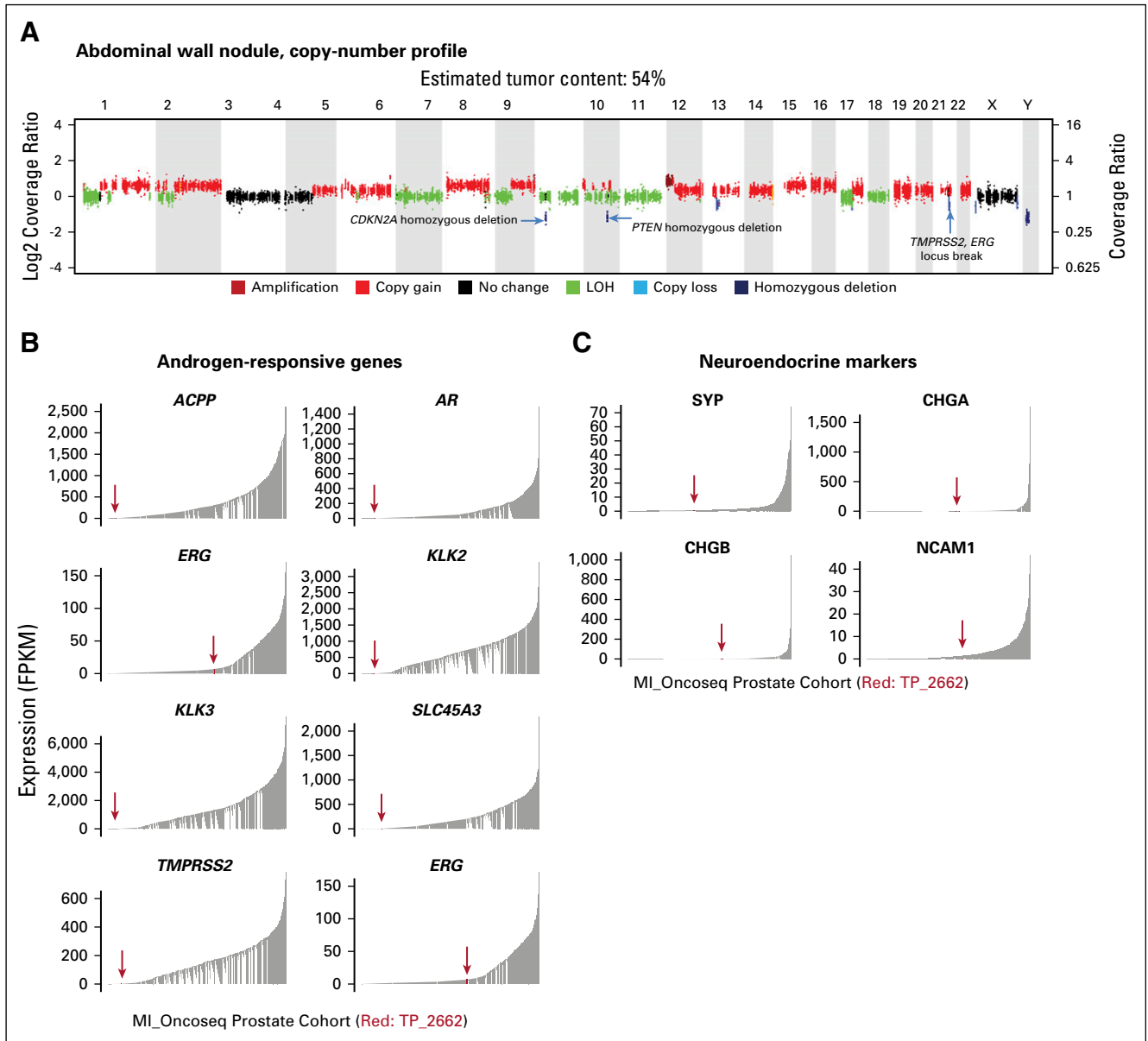
FIG 3. Next-generation sequencing. (A) Copy-number profile from the abdominal wall nodule highlighting the *TMPRSS2 ERG* locus break, a *PTEN* homozygous deletion, and a *CDKN2A* homozygous deletion. (B) Expression of androgen-responsive genes in the patient's second sample OncoSeq transcriptomics data. (C) Expression of neuroendocrine marker genes in the patient's second sample OncoSeq transcriptomics data. FPKM, fragments per kilobase of transcript per million mapped reads; LOH, loss of heterozygosity.

leuprolide a month later after his PSA continued to rise. The patient reported improvement in right groin pain shortly after initiation of docetaxel. CT completed after three cycles showed an interval decrease in the size of multiple bilateral lung nodules, and a decrease in size of his right pelvic mass, compatible with a therapeutic response (Fig 1E).

Discussion

In this case, we used next-generation sequencing to determine that the patient's squamous cell carcinoma was actually of prostate origin, because of the presence of a gene fusion between *TMPRSS2* and *ERG*. Transcriptomic

and histologic analysis showed minimal evidence of androgen receptor signaling, but it also showed a lack of evidence of neuroendocrine differentiation. Such prostate cancers without evidence of androgen receptor signaling and without neuroendocrine markers have been termed "double-negative" prostate cancers.[6] In more recent work profiling rapid autopsy specimens, investigators identified a squamous subtype of double-negative prostate cancer present in eight of 98 patients.[7] Squamous histology prostate cancer was reported previously but was a rare finding before the development of advanced antiandrogens.[8] However, the detection of squamous and other nonadenocarcinoma

prostate cancer subtypes has become more common since the development of abiraterone and nonsteroidal second-generation antiandrogens,[6] possibly as a means to escape continual selective pressure against androgen signaling, a phenomenon that had been described rarely in the past.[9] Some of these double-negative prostate cancer (DNPC) tumors seem to be driven by fibroblast growth factor (FGF) alterations, and trials of FGF inhibitors have recently begun in advanced prostate cancer.[10] Our patient did not have an FGF abnormality. Platinum-based chemotherapy is also commonly used to treat squamous cell neoplasms. In the current case, despite having squamous differentiation, this patient had only stable disease in response to platinum-based chemotherapy.

After using whole exome sequencing and RNA sequencing to identify this tumor as a squamous neoplasm of prostate origin, we elected to treat him with an agent approved for prostate cancer (docetaxel), an agent we would not have elected to use without knowing the tissue of origin. We elected not to pursue the therapies that could target molecular alterations in his tumor because of the known survival benefit of docetaxel in men with advanced prostate cancer, but therapies targeting his tumor's molecular alterations remain options down the road if his disease progresses. In summary, this report demonstrates a case of transdifferentiation of a prostate adenocarcinoma to a DNPC tumor with squamous differentiation without evidence of an FGF alteration. Consideration should be given to docetaxel in patients with tumors of a similar phenotype.

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Collection and assembly of data: All authors
Data analysis and interpretation: All authors
Manuscript writing: All authors
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REVIEW ARTICLE   OPEN

PROMISE: a real-world clinical-genomic database to address knowledge gaps in prostate cancer

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PURPOSE: Prostate cancer is a heterogeneous disease with variable clinical outcomes. Despite numerous recent approvals of novel therapies, castration-resistant prostate cancer remains lethal. A "real-world" clinical-genomic database is urgently needed to enhance our characterization of advanced prostate cancer and further enable precision oncology.
METHODS: The Prostate Cancer Precision Medicine Multi-Institutional Collaborative Effort (PROMISE) is a consortium whose aims are to establish a repository of de-identified clinical and genomic patient data that are linked to patient outcomes. The consortium structure includes a (1) bio-informatics committee to standardize genomic data and provide quality control, (2) biostatistics committee to independently perform statistical analyses, (3) executive committee to review and select proposals of relevant questions for the consortium to address, (4) diversity/inclusion committee to address important clinical questions pertaining to racial disparities, and (5) patient advocacy committee to understand patient perspectives to improve patients' quality of care.
RESULTS: The PROMISE consortium was formed by 16 academic institutions in early 2020 and a secure RedCap database was created. The first patient record was entered into the database in April 2020 and over 1000 records have been entered as of early 2021. Data entry is proceeding as planned with the goal to have over 2500 patient records by the end of 2021.
CONCLUSIONS: The PROMISE consortium provides a powerful clinical-genomic platform to interrogate and address data gaps that have arisen with increased genomic testing in the clinical management of prostate cancer. The dataset incorporates data from patient populations that are often underrepresented in clinical trials, generates new hypotheses to direct further research, and addresses important clinical questions that are otherwise difficult to investigate in prospective studies.

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INTRODUCTION

Despite advances in treatment, the median overall survival from onset of metastatic castrate-resistant prostate cancer (mCRPC) remains dismal [1]. In this subset of patients, there is a variable response to currently available therapies, and treatment in these men has not historically been guided by molecular biomarkers. Over the last decade, advancements in genomic sequencing technologies have allowed for a deeper understanding of the molecular complexity of this disease. Many potentially actionable alterations are now identified, fueling biomarker-based clinical trials of novel molecularly targeted agents as well as standard therapies. An important example of genomically tailored therapy in prostate cancer is the utility of Poly-(ADP ribose) polymerase (PARP) inhibitors in patients whose tumors harbor Homologous Recombination Repair (HRR) defects. The demonstrated clinical efficacy of olaparib [2] and rucaparib [3] in metastatic CRPC with HRR defects and their subsequent approval by the US Food and Drug Administration (FDA) marked an important milestone in the implementation of precision medicine for this disease.

The approvals of PARP inhibitors make it imperative to obtain somatic sequencing for all men with advanced prostate cancer. In addition, germline testing is also recommended for all men with

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metastatic prostate cancer per National Comprehensive Cancer Network guidelines, and in selected men with localized disease based on clinical risk and histologic subtypes as well as family history [4]. In fact, germline mutations in HRR genes exists in ~5% of localized prostate cancer [5]. In addition, multiple guideline panels recommend genomic sequencing for all patients with advanced prostate cancer. With this shifting paradigm and advancements in technology, there will be a rise in availability, breadth, and scope of genomic data derived from CLIA-based testing platforms. Linkage of such data with granular patient outcomes can be leveraged to improve our understanding of the molecular mechanisms that lead to variable clinical outcomes across prostate cancer disease states over time, identify additional subgroups of patients whose tumors are more vulnerable to specific therapies, and help guide decisions of optimal sequencing of therapies in prostate cancer.

The Prostate Cancer Precision Medicine Multi-Institutional Collaborative Effort (PROMISE) is a consortium of academic cancer centers with the goal of better defining the clinical-genomic features across the entire prostate cancer disease spectrum. Herein, we outline the rationale, design, and objectives of our multi-institutional, retrospective clinical-genomic database of advanced prostate cancer patients that addresses this important need.

MOLECULAR LANDSCAPE OF ADVANCED PROSTATE CANCER

Large-scale genomic analyses of metastatic prostate tumors have demonstrated a high frequency of germline and somatic alterations in several cancer-specific genes that are actionable or currently being investigated as candidate predictive biomarkers (Fig. 1) [6–9]. Some are more commonly seen in localized disease such as *SPOP* mutations and ETS family gene fusions [10]. The most common gene alterations enriched in CRPC, compared to earlier disease states, include *AR* and *TP53*, which are present in >50% of cases [6, 9]. Other commonly affected pathways with important clinical and therapeutic implications include the PI3K pathway genes such as *PTEN-AKT-mTOR*, and HRR genes such as *BRCA1/2*, *ATM* which are altered in ~45%, ~25%, ~7-10% of CRPC cases, respectively [6, 8]. Additional altered genes in CRPC include those involved in the cell cycle (~30%) such as *RB* loss, *CDKN1B*, and *CCND1*; epigenetic regulator genes (~25%) such as *KMT2C*, *KMT2D*, and *CHD1*; WNT pathway genes (~15%) such as *APC* and *CTNNB1*, *CDK12* (~7%); and MAP kinase pathway genes (~5%) [6, 8, 9]. These alterations in the context of drug development and clinical decision-making are discussed in further detail in the next section. Many of these alterations occur concurrently, and based on prior reports, about 65–85% of analyzed CRPC tumors harbor potentially actionable alterations beyond AR, defined as the ability to predict response to an available drug based on existing preclinical data [6]. Structural variants in the non-protein-coding regions also have a potential impact on the activity of important regulators of cancer progression [9]. A recent whole-genome sequencing analysis, the first of its kind in CRPC, identified that 81% of cases harbored amplification of a putative enhancer of *AR*, which may drive androgen resistance [9, 11]. Structural variants were also demonstrated near the *MYC, TP53, CDK12, FOXA1*, and *BRCA2* genes with potential biological and clinical implications.

In metastatic hormone-sensitive prostate cancer (mHSPC), treatment options have rapidly expanded, yet decision-making is based mainly on clinical factors without integration of genomic biomarkers. A recent study describes potential biomarkers of disease aggressiveness [7]. Specifically, alterations involving AR, TP53, cell cycle, and MYC pathways predict for a shorter time to castration resistance, while alterations in *SPOP* gene and WNT pathway are associated with lower rate and longer time to castration resistance [7]. Furthermore, in tumors with high-volume (≥ 4 bone metastases and/or visceral metastasis) mHSPC, higher
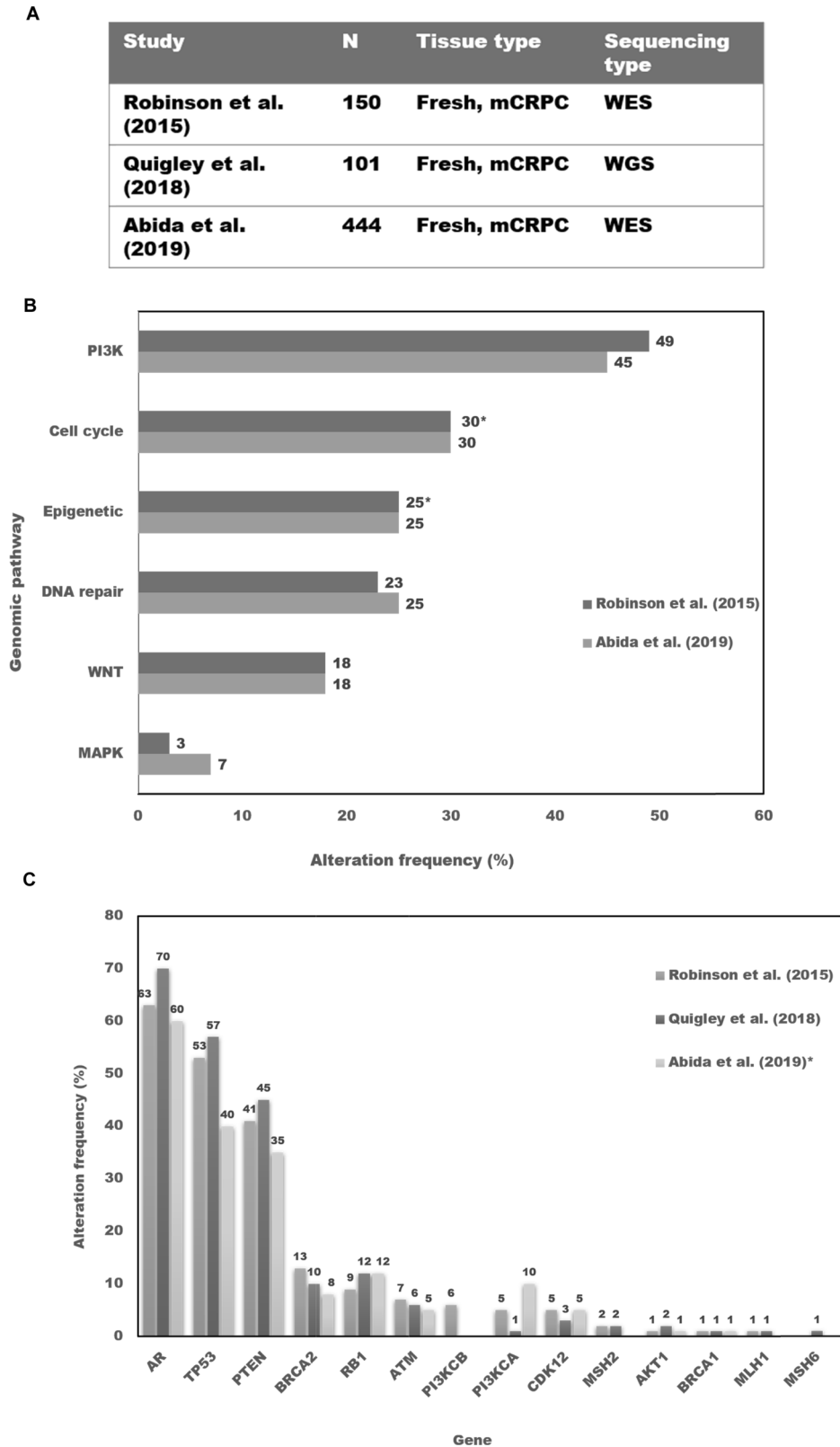
fraction of alterations indicative of genomic instability, and alterations involving cell cycle, epigenetic regulation, and NOTCH pathways are seen. Thus, the development of a clinical-genomic model for both mHSPC as well as CRPC represents a major unmet medical need, for which a large number of patients are needed with careful clinical and genomic annotation.

IMPLEMENTATION OF PRECISION MEDICINE IN PROSTATE CANCER

Our understanding of the molecular landscape of advanced prostate cancer provides a platform for the development of targeted therapies, and for optimizing therapeutic combination strategies and sequencing. Excitingly, intense investigation is already taking place to leverage this knowledge and expand therapeutic options for men with advanced prostate cancer. Increasingly, genomic sequencing is also becoming a pre-requisite for clinical trials, thus availability of routine genetic testing becomes imperative to ensure patients have access to biomarker-driven therapies and clinical trials.

Approximately 20–25% of men with mCRPC have germline or somatic alterations involving HRR genes, such as *BRCA1/2* and *ATM*, which may predict for sensitivity to PARP inhibitors and platinum agents [12]. Large clinical trials such as PROfound and TRITON2 have led to the FDA approval of two PARP inhibitors, olaparib and rucaparib, respectively, in men with mCRPC that harbor deleterious somatic or germline mutations in HRR genes [2, 3]. In May 2020, olaparib was approved for men with HRR gene-altered CRPC while the rucaparib approval was restricted to patients with tumors harboring alterations in *BRCA1/2*. Despite these approvals, many questions remain about the relevance of PARP inhibitors in patients harboring non-*BRCA* HRR genes. Many of these questions can be answered with a real-world database such as PROMISE. In addition to PARP inhibitors, exceptional responses to platinum-based chemotherapy have also been observed in *BRCA2*-mutant CRPC cases [13, 14]. However, not much is known in this population about the utility of platinum agents and PARP inhibitors in combination or as sequential therapy. Other HRR gene targets under clinical development include ATR inhibitors and ATM inhibitors. Of specific interest within the HRR pathway is *CDK12* loss, which has shown to correspond with high focal tandem duplications and high neoantigen burden, potentially sensitizing *CDK12*-altered tumors to immune checkpoint blockade [15–17]. Thus, knowledge from real-world datasets on the appropriate sequencing and outcomes of taxane chemotherapy with PARP inhibitors in less common subgroups of men with mCRPC such as *ATM* mutations or less common HRR gene alterations is needed.

About 45% men with mCRPC harbor pathogenic genomic alterations within the PI3K pathway. The majority of alterations involving this pathway occur in the *PTEN* gene (~40%), which has previously proven to be a difficult target with single agents and is associated with poor prognosis [18]. Preclinical data suggest that PTEN/PI3K and AR pathways have reciprocal crosstalk, thus targeting both pathways in combination may enhance therapeutic efficacy [19]. A phase II study of abiraterone acetate and AKT inhibitor, ipatasertib, showed a clinical benefit particularly in *PTEN*-deficient mCRPC [20]. In the primary analysis of the phase III study of this combination (IPATential150, NCT03072238), combined AR and AKT blockade provided an improved progression-free survival in patients with mCRPC with *PTEN* loss, compared to AR blockade alone [21]. Another target within this pathway is *AKT1* gene, which occurs in about 1% of mCRPC patients. AKT inhibitors exhibit clinical activity in AKT-mutated breast cancer and other solid tumors [22] and are currently in development for mCRPC (NCT04087174). Other alterations of interest in this pathway involve *PIK3CA, PIK3C2B, BRAF, MAP2K1*, and *KRAS* genes. Recently the first PI3KCA inhibitor, alpelisib, was approved for

A

Study	N	Tissue type	Sequencing type
Robinson et al. (2015)	150	Fresh, mCRPC	WES
Quigley et al. (2018)	101	Fresh, mCRPC	WGS
Abida et al. (2019)	444	Fresh, mCRPC	WES

B

C

Fig. 1 Large-scale genomic sequencing analyses of mCRPC. A List of three prospective genomic analyses of mCRPC samples. **B** Distribution of non-androgen-receptor, actionable genomic pathway alterations. **C** Distribution of commonly expressed and actionable genomic alterations. *Represent estimates based original article.

PIK3CA-mutated breast cancer [23], a development that can inform future clinical trial designs and treatment options for advanced prostate cancer as well.

About 3–5% of patients with CRPC also have evidence of DNA mismatch repair deficiency (dMMR) and/or microsatellite instability (MSH)-high that are found through immunohistochemistry to evaluate for loss of MMR protein and/or DNA sequencing [24]. These patients tend to have tumors with a higher tumor mutational burden (TMB), predicting response to immune checkpoint blockade [25–28]. In 2017, based on data from five multi-cohort, single-arm studies of MSH-H or dMMR advanced solid tumors [29, 30], pembrolizumab received a tumor-agnostic approval for patients who progressed on prior treatment and did not have satisfactory alternative treatment options. Although this study marked the first time in oncology drug development that a therapy was approved based on a specific biomarker irrespective of tumor histology, very few patients with prostate cancer were enrolled. Furthermore, it is not clear whether routine testing of dMMR and/or MSI is being performed in most clinical practices to identify this subset of prostate cancer patients. More recently, tumor-agnostic pembrolizumab approval was expanded to include tumors with high TMB [31].

In addition to potential actionable alterations discussed, multi-institution clinical trials such as Targeted Agent and Profiling Utilization Registry (TAPUR, NCT02693535) by American Society of Clinical Oncology highlight the aims of understanding the safety and efficacy of novel targeted agents in advanced prostate cancer and other malignancies. In addition, large registries of real-world data are being built to identify novel targets. The American Association of Cancer Research (AACR) has implemented Project GENIE (Genomics Evidence Neoplasia Information Exchange), which consist of real-world data among 19 leading cancer centers in the world. Despite the robustness of these pan-cancer platforms, the nuances of different clinical states of prostate cancer will not be captured. To complement these efforts, our consortium was developed to leverage the wealth of existing clinical-genomic data to expand our understanding of prostate cancer biology and to improve current therapeutic approaches. In the remaining sections, we highlight some key clinical questions that are present in routine practice to provide context into the design, structure, and objectives of PROMISE.

CURRENT CHALLENGES IN PROSTATE CANCER AND POTENTIAL SOLUTIONS

The last two decades have seen significant new advancements in the treatment of metastatic prostate cancer. Much wider availability of next-generation sequencing (NGS) panels has made it possible to individualize treatment options for a subset of prostate cancer patients with specific tumor markers or alterations. These developments have created a plethora of new questions, many of which may be answered though accumulation of clinical and genomic data in a "real-world" dataset. The following examples highlight current challenges in the treatment of advanced prostate cancer as well as potential solutions and strategies to overcome barriers to the realization of the true promise of precision medicine in prostate cancer.

Sequencing of therapies in prostate cancer
With the evolution of multiple novel treatment options in mCRPC, including novel hormonal agents and PARP inhibitors, as well as the emergence of novel therapeutic classes such as PSMA-targeted radioligand therapies, there is increasingly a wealth of options to offer patients in a disease space where only a couple of decades ago there were relatively few [32, 33]. In this setting, the questions about appropriate sequencing of therapies become

especially relevant, especially as most patients will not be able to receive all available treatment options and decisions of which therapies to prioritize earlier as opposed to later in the disease course must be made by the treating physician. For instance, in molecularly selected patients able to receive PARP inhibitors, what is the most optimal way to sequence these agents with taxane chemotherapy and should the sequencing of therapies be impacted by mutation status? The PROMISE clinical-genomic platform can help to generate initial data on potential positive and negative predictive biomarkers to guide these types of therapeutic questions.

Novel treatment paradigms in mHSPC and impact on subsequent treatment options
As novel treatment options are also introduced earlier in the disease course of prostate cancer, such as novel androgen receptor signaling inhibitors (ARSIs) [34–37], the natural history of many patients with mCRPC progressing on these therapies earlier in the disease course may also be altered. Do patients who develop castrate-resistant disease while being treated with ARSIs in the hormone-sensitive space subsequently have more aggressive and rapidly progressing CRPC? Do these patients subsequently respond to the standard of care treatment options available for mCRPC similarly to prior populations of patients not treated with these agents in the hormone-sensitive setting? Are these patients more likely to develop treatment-emergent neuroendocrine or small cell prostate cancer [38]? It is incumbent on the prostate cancer research community to better understand this natural history in light of new treatment paradigms. In this dynamic and rapidly changing treatment space much of the available clinical trial data used to guide treatment decisions may reflect a very different patient population than the patients being treated now. Serial cell-free DNA (cfDNA) is a cutting-edge solution to temporally understand therapeutic resistance mechanisms and is expected to be widely adopted in the future. Therefore, the vast genomic data gathered for each patient will be invaluable in our efforts to answer questions related to treatment exposure and acquisition of resistance.

Natural history and treatment options for novel molecular subtypes in prostate cancer
As treatment for prostate cancer is increasingly individualized and new biological subsets are identified through increased use of NGS, it is also important to better define the natural history of these patient populations. The molecular characterization of exceptional responders and non-responders to standard of care therapies will help better define molecular predictive biomarkers. In addition, as of 2020 there are now two agents, olaparib and rucaparib, specifically approved for the treatment of mCRPC with HRR alterations [2, 3] as well as multiple clinical trial options for patients with advanced prostate cancer and various genomic alterations. This is particularly important since response to standard of care treatments will serve as a benchmark for the efficacy of novel therapies that specifically target these genomic alterations. These questions need to be answered in order for precision medicine to truly fulfill its promise. More individualized treatment, implying the division of the broad diagnosis of prostate cancer into ever smaller molecularly defined subsets, will increasingly help guide the rational design of clinical trials and therapy selection. One early example of this approach is the IND.234 trial (NCT03385655) from the Canadian Clinical Trials group that uses a cfDNA platform to select therapy in prostate cancer. The improved understanding of these molecularly defined subsets through hypothesis-generating retrospective studies can help improve the design of biomarker-driven clinical trials of prostate cancer.

Impact of race/ethnicity or socioeconomic status on prostate cancer treatments and outcomes

Many studies focusing on characterizing the molecular alterations in prostate cancer have included a very limited number of ethnically diverse subsets of patients. Understanding how race/ethnicity impacts genomic profile and response to therapy is critical to bridging the disparities gap in men with prostate cancer. Real-world data from this platform can fill this gap. Black men remain underrepresented in phase 3 trials in prostate cancer despite being disproportionately impacted by lethal prostate cancer [39] and despite evidence of perhaps superior outcomes when treated with sipuleucel-T or docetaxel [40, 41]. Do these factors impact the diagnostic decisions made, such as the choice of imaging in advanced prostate cancer [42]? Are certain treatment options chosen preferentially over others and does this create healthcare inequities? Among the treatment options chosen, do certain options work better for certain subsets of patients? Such questions are difficult to answer with clinical trials which historically underrepresent many of the populations in question [43]. However, it is absolutely vital to answer these questions in order to help us improve the care of all patients with advanced prostate cancer. The collection of germline data for the patients in a real-world dataset will also help to both understand the landscape of somatic and germline mutations in underrepresented patients and the general availability of germline testing in this patient population [44, 45].

UTILITY OF REAL-WORLD DATA AND THE UNMET NEED FOR THE PROMISE CONSORTIUM

All of the above clinical questions and scenarios in advanced prostate cancer illustrate the importance and potential utility of a large and well-maintained real-world database that serves as a repository for clinical and genomic data of prostate cancer patients.

There are several pertinent reasons necessitating such a database as an important clinical research tool in advanced prostate cancer.

Prospective randomized clinical trials remain the gold standard in clinical cancer research and shape the current standard of care. While prospective clinical trials capture data on efficacy, observational studies can help to better understand effectiveness of the treatments in the real-world setting. Importantly, most prostate cancer patients do not get their cancer treatment as part of a clinical trial. A recent study estimated that only about 8% of oncology patients enroll in a clinical trial [46]. Consequently, the experiences and treatment outcomes of most prostate cancer patients are not systematically recorded or analyzed. In addition, most clinical trial datasets do not capture the entirety of patient experience from diagnosis until final treatments, focusing rather on one specific treatment outcome, and thus provide only a limited window into the outcomes of patients in the real-world setting. Retrospective studies of real-world patient datasets that include well-annotated clinical and genomic data can shed light on important questions that may be difficult to address in prospective clinical trials.

The inclusion of underrepresented minorities in this dataset is one of the important missions of this consortium. Data from retrospective series can more extensively capture clinical outcomes and other information of more diverse populations, including minority populations underrepresented in clinical trials. The low participation rates of patients who identify as underrepresented minorities in clinical trials of prostate cancer have been well documented and remain a significant challenge in clinical research and the conduct of trials [43]. The reasons for this are complex and multifactorial but it remains a significant challenge that is yet to be addressed. The selection for this consortium of diverse clinical sites representing different geographic regions of the United States that serve unique underrepresented patient populations is certainly an important step toward bridging the cancer genomics disparities gap in prostate

A

PROMISE CONSORTIUM					
ADMINISTRATIVE HEADQUARTERS	**BIO-INFORMATICS COMMITTEE**	**EXECUTIVE COMMITTEE**	**DATA COMMITTEE**	**DIVERSITY & INCLUSION COMMITTEE**	**PATIENT ADVOCACY COMMITTEE**
▪ Maintain RedCap database ▪ Manage the development of the consortium	▪ Format somatic and germline alterations to standardized criteria ▪ Perform quality control of genomic data	▪ Select project proposals through scientific review	▪ Independently perform approved analyses	▪ Raise important clinical questions to emphasize and address racial disparities	▪ Incorporate perspectives of patient advocacy groups to improve clinical research feasibility and to serve as a reminder on improving patients' quality of life and outcomes

B

Data collection → Quality control → Project development → Data analysis → Release data Abstract/manuscript
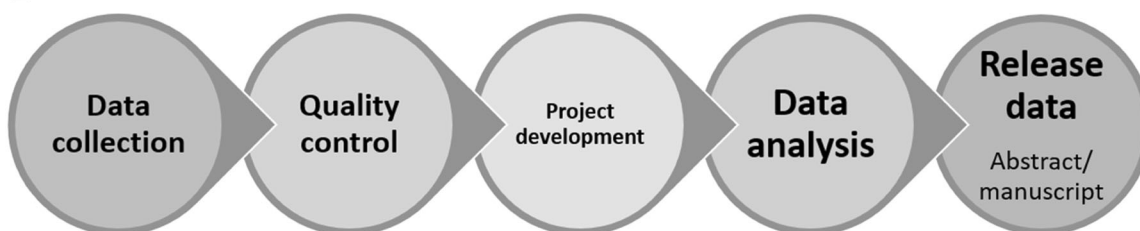
Fig. 2 PROMISE consortium structure and workflow. A Structure consisting of administrative headquarters, bio-informatics committee, executive committee, data committee, diversity & inclusion committee, and patient advocacy committee. Responsibilities of each committed listed. **B** Project workflow from data collection to release of data in abstract/manuscript form.

cancer. Many of the initially proposed projects focus on questions related to racial disparities.

It is also important to keep in mind that although randomized clinical trials remain the gold standard for generating clinical data, many relevant clinical questions are initially asked in hypothesis-generating retrospective studies. Important descriptive data of the natural history of disease in these specific subgroups can be obtained through retrospective chart review. Many important prognostic and predictive markers and biomarkers of interest can initially be identified through analyses of real-world data and are subsequently validated prospectively. Furthermore, in contrast to prospective clinical trials where treatment options are prespecified, retrospective observational studies can also attempt to capture clinical decision-making, such as decisions made based on the results of genomic testing. In databases with large numbers of patients and well-annotated clinical and genomic data, important insights can be gleaned from this approach.

As treatments are increasingly individualized for molecularly selected subsets of patients with mCRPC, the careful collection of genomic data in large real-world cohorts can also help shed light on tumor biology and help advance questions that produce important validation studies. The availability of multiple tissue or blood samples from the same patient can also be particularly instructive. This information can help better define the evolution of molecular alterations or other biomarkers in the tumor throughout the course of disease progression. The understanding of such changes obtained from serial sampling of either repeat solid biopsies or of repeated cfDNA sampling can also help define the changes in the tumor that happen during progression on specific treatments. Further important comparisons can be drawn in the molecular alterations and other biomarkers detected in solid biopsies and cfDNA in the same patient.

Overall, our long-term goal is to develop a large real-world dataset collected from institutions including both academic and large community-based hospital systems, which by definition will be both heterogeneous and also more representative of real-world trends. Clinical and genomic information from many patients who are unable or unwilling to be treated on a clinical trial due to clinical, logistical or other reasons will also be included. This will make potential findings more generalizable relative to the data obtained from carefully selected clinical trial populations. The patient population included in a real-world dataset will also reflect the diversity of treatment options and strategies employed, diagnostic and genomic testing used, and the diverse patient populations across the different geographic regions.

DEVELOPMENT OF PROMISE, ITS AIMS, AND THE INCLUSION OF A DEDICATED BIO-INFORMATICS COMMITTEE

The PROMISE consortium was formed with the recognition of the significant clinical and research needs of linking clinical and genomic data to outcomes, in order to better inform treatment decisions and outcomes for patients with advanced prostate cancer. Data collected in this consortium will address significant knowledge gaps that come about as the underlying biology of prostate cancer becomes better understood and multiple novel agents and classes of drugs emerge as treatment options. The structure of the PROMISE consortium including all of the committees and the flow chart of data analysis are shown in Fig. 2.

PROMISE is currently a consortium of 16 academic institutions with the common goal of understanding the real-world data in patients with advanced prostate cancer. The goal is to expand the reach of this database to include academic institutions and community practices. The consortium has two main aims:

(1) To establish a large, diverse, inclusive, and well-annotated repository of completely de-identified clinical and genomic

Table 1. Examples of clinical and genomic data collected in the PROMISE consortium.

Clinical data elements				Genomic data elements		
Demographics	Disease characteristics	Lab values	Treatment characteristics	Pathology status	Gene information	Class of alteration
Age	Prior prostatectomy	PSA	ECOG PS	Specimen source	Type of sequencing test	Copy number variation
Race	Histology	Hemoglobin	Systemic therapies	Specimen date	Gene name	Structural variant (SV)
Veteran status	Gleason score	Platelets	Duration of Treatment	MSI	Somatic vs germline	Single nucleotide variant (SNV)
Marital status	Stage at diagnosis	ANC/ALC	Imaging obtained	MMR	Type of alteration	Insertion/Deletion
Smoking status	De novo metastasis	Alkaline phosphatase	Time to progression or death from diagnosis, metastatic disease, and CRPC	PD-L1	Zygosity	Allele frequency (for cfDNA)

Table 2. Current Institutions Participating in the PROMISE Consortium.

Institution name	Location
City of Hope	Duarte, CA
Duke Cancer Institute	Durham, NC
Emory University	Atlanta, GA
Henry Ford Health System	Detroit, MI
Icahn School of Medicine at Mount Sinai	New York, NY
Johns Hopkins University School of Medicine	Baltimore, MD
Karmanos Cancer Center	Detroit, MI
Medical College of Wisconsin	Milwaukee, WI
Tulane University	New Orleans, LA
University of California San Diego	San Diego, CA
University of California San Francisco	San Francisco, CA
University of Iowa	Iowa City, IA
University of Michigan	Ann Arbor, MI
University of Oklahoma	Oklahoma City, OK
University of Washington	Seattle, WA
Weil Cornell Medical Center	New York, NY

patient data that is linked to clinical characteristics and disease-related outcomes.

(2) To investigate prognostic and potentially predictive biomarkers in patients with advanced prostate cancer treated with approved therapies including genomic, transcriptomic, and proteomic biomarkers.

Inclusion criteria for patients in this dataset consist of having advanced prostate cancer with either mHSPC or castrate-resistant prostate cancer (CRPC). In addition, patients should have undergone prior germline and/or somatic tumor molecular profiling studies including, but not limited to, one or more of the following blood or tissue-based assays: tumor DNA sequencing (e.g., cell-free circulating tumor DNA or tumor tissue sequencing), germline DNA sequencing and transcript profiling studies (e.g., RNA-seq, qRT-PCR). In total, consecutive patients with available clinical and genomic data at each site will be included to limit selection bias. Clinical and treatment data is collected from the time of initial prostate cancer diagnosis (Table 1). All data are maintained in a secure RedCap database and is completely deidentified without any protected health information. Genomic information entered into the database from both solid tissue and liquid biopsy testing and inclusive of both somatic and germline alterations are formatted to standardized criteria and reviewed by the bioinformatics committee. We will only include the entries that have all available data related to clinical outcomes and genomic sequencing.

PROMISE CONSORTIUM INSTITUTIONS, DATA ACQUISITION PLAN, AND MISSION STATEMENT

The PROMISE consortium participating institutions are listed in Table 2. The consortium was formed in the first half of 2020 and data entry to the consortium has since risen steadily as sites have come onboard and over 1000 patient records are anticipated by early 2021 (Fig. 3). Genomic data are being audited for accuracy at each institution.

The mission of the PROMISE consortium is to investigate the outcomes of patients with advanced prostate cancer who have genomic and molecular profiling information and therefore to bridge the knowledge gap between real-world genomic data and clinical outcomes in patients with advanced prostate cancer.
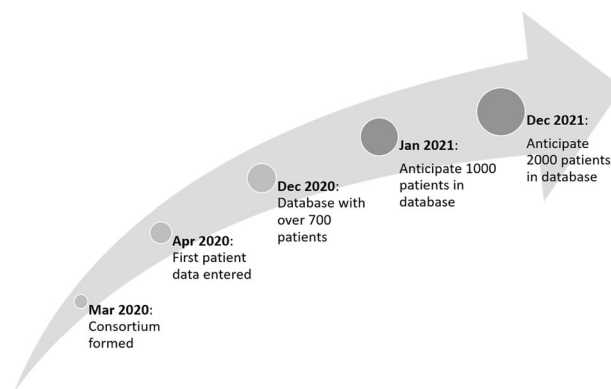
Fig. 3 Project milestones and future timeline. The PROMISE consortium was formed in March 2020. At the end of 2020, we collected clinical data of over 700 patients. We anticipate clinical data collection of 2000 patients by the end of 2021.

Consortium structure will also provide significant opportunities for mentorship of junior investigators and trainees. The consortium will plan to pursue multiple projects addressing specific questions which are proposed by the participating site investigators and selected through scientific review by the executive committee with priority given to projects proposed by junior investigators and trainees. Given the important potential contribution of evaluating outcomes in a more diverse population than that represented in clinical trials, a subcommittee for diversity and inclusion has been created with a plan to implement strategies to increase diversity if the database fails to maintain a balanced population.

FUTURE DIRECTIONS

In a short period of time, we have gathered vast amounts of clinico-genomic information to answer important clinical questions. We are looking forward to expand our database to include many more academic institutions and community practices to make our patient population more representative of the real-world setting. In addition, once the database becomes robust for large-scale analyses, these data may be made publicly available for future research, following efforts such as cBioportal. Lastly, there is a long-term plan to develop infrastructure for blood- and tissue biorepository to perform molecular analyses.

CONCLUSIONS

Precision medicine holds significant promise for helping to individualize treatment approaches for patients with advanced prostate cancer and to help understand the underlying mechanisms of this heterogeneous disease. The PROMISE consortium is an important new collaboration among leading academic centers in the United States that aims to bridge the gap between clinical and genomic information for patients with metastatic prostate cancer and available genomic data from commercial and institutional assays. The well-annotated, de-identified patient data collected for this consortium will be leveraged to help answer specific questions and guide projects proposed by the consortium members. Genomic data entered into the database will be vetted by a bioinformatician and individual projects approved by the executive committee as part of the consortium structure. This organized approach will help to address important questions at the intersection of clinical and genomic data that are most optimally addressed using a real-world dataset. It is our hope that this approach will eventually help to improve outcomes for patients with advanced prostate cancer.

8

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Monitoring Spontaneous Quiescence and Asynchronous Proliferation-Quiescence Decisions in Prostate Cancer Cells

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The proliferation-quiescence decision is a dynamic process that remains incompletely understood. Live-cell imaging with fluorescent cell cycle sensors now allows us to visualize the dynamics of cell cycle transitions and has revealed that proliferation-quiescence decisions can be highly heterogeneous, even among clonal cell lines in culture. Under normal culture conditions, cells often spontaneously enter non-cycling G0 states of varying duration and depth. This also occurs in cancer cells and G0 entry in tumors may underlie tumor dormancy and issues with cancer recurrence. Here we show that a cell cycle indicator previously shown to indicate G0 upon serum starvation, mVenus-p27K-, can also be used to monitor spontaneous quiescence in untransformed and cancer cell lines. We find that the duration of spontaneous quiescence in untransformed and cancer cells is heterogeneous and that a portion of this heterogeneity results from asynchronous proliferation-quiescence decisions in pairs of daughters after mitosis, where one daughter cell enters or remains in temporary quiescence while the other does not. We find that cancer dormancy signals influence both entry into quiescence and asynchronous proliferation-quiescence decisions after mitosis. Finally, we show that spontaneously quiescent prostate cancer cells exhibit altered expression of components of the Hippo pathway and are enriched for the stem cell markers CD133 and CD44. This suggests a hypothesis that dormancy signals could promote cancer recurrence by increasing the proportion of quiescent tumor cells poised for cell cycle re-entry with stem cell characteristics in cancer.

Keywords: quiescence, dormancy, prostate cancer, cell cycle, live imaging

INTRODUCTION

Cycling cells tend to enter quiescence, a reversible, non-cycling state in response to contact inhibition, reduced levels of mitogens, or under various stress conditions. Quiescent cells retain the ability to re-enter the cycle upon the addition of serum or under favorable conditions (Coller et al., 2006; Yao, 2014). However, studies of mammalian cells in the past few years have found that many cells enter a spontaneous reversible G0-like state in cell culture even in the presence of mitogens and abundant nutrients (Spencer et al., 2013; Overton et al., 2014; Min and Spencer, 2019). This suggests that the proliferation-quiescence decision is constantly regulated—even under optimal growth conditions.

The relative percentage of cells that enter a temporary G0-like state after mitosis varies with cell type and culture conditions, suggesting many signaling inputs influence the proliferation-quiescence decision (Spencer et al., 2013). This is also consistent with findings in several cancer cell lines, where some cells enter a temporary quiescent state while others do not (Dey-Guha et al., 2011). This leads to heterogeneity in cell culture, with a subpopulation of cells entering and leaving temporary quiescent states (Overton et al., 2014). This proliferative heterogeneity may underlie states of dormancy in cancer and has been shown to be related to cancer therapeutic resistance (Recasens and Munoz, 2019; Risson et al., 2020; Nik Nabil et al., 2021). This is particularly relevant in prostate cancer, where it is thought that early spreading of tumor cells to the bone marrow and other tissues may provide signals leading to quiescence and tumor dormancy (Chen et al., 2021). Prostate cancer dormancy in tissues such as the bone are problematic as a percentage of patients will later develop recurrent cancer with significant metastases from these cells, which are often also resistant to treatment (Lam et al., 2014). Understanding how and why quiescent cancer cells reside in environments such as the bone marrow for long periods of time, and finding ways to eliminate them, is an important ongoing challenge in prostate cancer research and treatment.

The difficulties in monitoring the proliferation-quiescence decision and distinguishing different states and lengths of G0 has limited our ability to understand how signals impact the heterogeneity of quiescence in cell populations. Most assays for cell cycling status use immunostaining of cell cycle phase markers or nucleotide analogue incorporation, both of which assess static conditions in fixed samples (Matson and Cook, 2017). Cell cycle reporters such as the FUCCI system (Fluorescent Ubiquitination-based Cell Cycle Indicator), have become widely used to track cell cycle dynamics live in individual cells (Sakaue-Sawano et al., 2008). The FUCCI system and related systems such as CycleTrak and others including a constitutive nuclear marker are able to differentially label cells in G1, S and G_2/M phases, allowing us to visualize the G_1-M transition, however G0 cannot be distinguished from G1 in these approaches (Ridenour et al., 2012; Chittajallu et al., 2015).

Recent methods to monitor quiescence heterogeneity have used live cell imaging with sensors for Cdk2 activity, Ki-67 expression, and expression of Cdk inhibitors such as p21 and p27 (Spencer et al., 2013; Overton et al., 2014; Stewart-Ornstein

and Lahav, 2016; Miller et al., 2018; Zambon et al., 2020). Here we take advantage of the cell cycle indicator, mVenus-p27K$^-$, which was generated to work in combination with the G0/G1 FUCCI reporter mCherry-hCdt1 (30/120), to specifically label quiescent cells (Oki et al., 2014). This probe is a fusion protein consisting of a fluorescent protein mVenus and a Cdk binding defective mutant of p27 (p27K-). p27 accumulates during quiescence and is degraded by two ubiquitin ligases: the Kip1 ubiquitination-promoting complex (KPC) at the G0-G1 transition, and the SCF^{Skp2} complex at S/G2/M phases (Kamura et al., 2004). When used in combination with the G0/G1 FUCCI reporter, cells can be tracked from a few hours after mitosis until early S phase with distinct colors. This allows us to examine the dynamics of the proliferation-quiescence transition after mitosis on a single-cell level without artificial synchronization.

Prior work with a Cdk2 sensor and monitoring p21 levels revealed that both non-transformed and cancer cells in culture can enter "spontaneously" quiescent states of variable length, even under optimal growth conditions (Spencer et al., 2013; Overton et al., 2014; Yang et al., 2017; Min and Spencer, 2019). The proportion of spontaneously quiescent cells in a population and their variability in the length of quiescence leads to cell cycle heterogeneity (Overton et al., 2014), which may in part also underlie cell cycle heterogeneity within clonal tumors (Dey-Guha et al., 2011; Dey-Guha et al., 2015). This led us to examine whether we could monitor spontaneous quiescence using the mVenus-p27K$^-$ G0 reporter. Here we show that by tracking the trajectory of this reporter activity, we can monitor spontaneous quiescence in non-transformed mouse fibroblasts and prostate cancer cells. While measuring the heterogeneity of spontaneous quiescence, we also observed that a pair of daughter cells resulting from a single mitosis can make asynchronous proliferation-quiescence decisions. In this type of asynchronous decision, one daughter from a mitosis enters G0, while the other enters G1, further increasing cell cycle heterogeneity within a clonal population (Dey-Guha et al., 2011). We find that signals associated with promoting or releasing tumor dormancy can influence quiescence and asynchronous proliferation-quiescence decisions in prostate cancer cells. Using the mVenus-p27K$^-$ G0 reporter, we isolate populations containing quiescent cancer cells and find they are enriched for a subpopulation expressing stem cell markers and express high levels of Hippo pathway signaling components, but with inactivated YAP, which may indicate a state poised for cell cycle re-entry. Finally, we provide evidence that the expression of immune recognition signals may be decreased in populations containing quiescent cancer cells, suggesting a hypothesis for how these cancer cells may preferentially evade the immune system.

RESULTS

mVenus-p27K$^-$ Based G0/G1 Cell Cycle Indicators Track Spontaneous Quiescence

To characterize the proliferation-quiescence transition at single-cell resolution in mouse 3T3 cells under full serum conditions without synchronization, we used the G0 cell cycle indicator
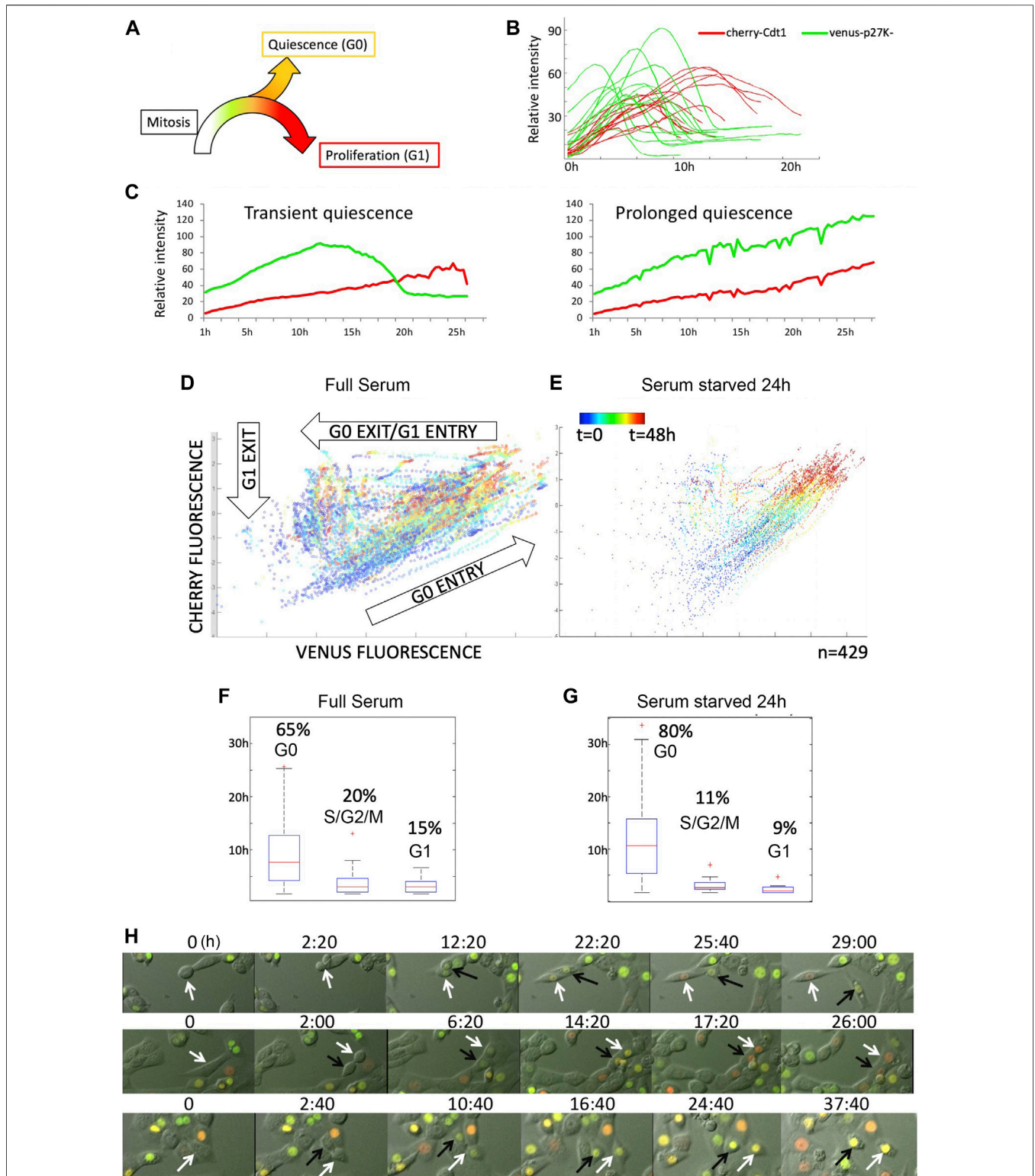
FIGURE 1 | The G0 sensor, mVenus-p27K-, can be used to monitor spontaneous quiescence and asynchronous proliferation-quiescence decisions in untransformed cells. **(A)** The proliferation-quiescence decision as monitored with the G0 sensor, mVenus-p27K- (G0-Venus) and G1 sensor hCdt1$_{(30–120)}$ -Cherry (G1-Cherry). For NIH/3T3 cells, on average, 2–4 h after cytokinesis, G0-Venus expression begins increasing, followed approximately 3–4 h later by G1-Cherry expression. For cells entering G1, the Venus/Cherry double-positive phase lasts 5–10 h. For quantification purposes we define a Venus/Cherry double-positive phase prolonged beyond 14 h as spontaneous G0. **(B)** Example traces of G0-Venus/G1-Cherry reporter dynamics in cells entering the cell cycle. 0 h is relative time, aligned to

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mVenus-p27K⁻ combined with the G0/G1 reporter from the FUCCI cell cycle system, mCherry-hCdt1 (30/120) to distinguish G0 cells from G1 cells as previously described (Oki et al., 2014). We first manually examined movies of asynchronously proliferating 3T3 cells stably expressing these reporters to monitor reporter dynamics (**Supplementary Movie S1, S2**). With this combination of cell cycle reporters, mVenus-p27K⁻ expression begins approximately 2–6 h after cytokinesis is complete, followed by mCherry-hCdt1expression approximately 2–6 h later. Most cells then exhibit a rapid reduction in mVenus-p27K⁻ within approximately 3 h, signaling G1 entry followed by mCherry-hCdt1 degradation at G1 exit (**Figures 1A,B**). However, for a fraction of cells (ranging between 20–65% in different movies) we observed both mVenus-p27K⁻ and mCherry-hCdt1 to both continue to accumulate for up to 14 h and beyond, without division or evidence of S/G2/M entry for 20 h or more, signaling spontaneous G0 entry (**Figure 1C**). This progression of reporter expression in order from mVenus-p27K⁻ positive to mVenus-p27K⁻ and mCherry-hCdt1 double positive to mCherry-hCdt1 was invariant in the movies, although we did observe some cell to cell variation in reporter expression intensity, despite using a clonal cell line.

To monitor and quantitatively measure the dynamic transitions of cell cycle states—from cytokinesis to S phase entry, we developed an Automated temporal Tracking of Cellular Quiescence (ATCQ) analysis platform. This platform includes a computational framework for automated cell segmentation (identification of individual cells in an image), tracking, cell cycle state identification, and quantification from movies (**Supplementary Figure S1**). The cell segmentation and tracking allows us to record the fluorescent reporter intensity changes within individual cells in real-time imaging, without the aid of a constitutive nuclear marker. The single-cell fluorescence changes over time, in turn, are used to obtain cell cycle state identification (G0, G1, or early S phase) and quantification, which allows us to examine the kinetics of the proliferation-quiescence transition. The single-cell traces of fluorescent reporters, mVenus-p27K⁻ and mCherry-hCdt1, graphed by ATCQ is consistent with trajectories of G0 entry (increasing Venus and Cherry), G0 exit/G1 entry (degradation of Venus, increasing Cherry), and G1 exit/S-phase progression (degradation of Cherry) we manually observed in movies (**Figure 1D**).

To confirm that the Venus/Cherry-double positive population represents G0 phase, we performed a short-term (24 h, 1%FBS) serum starvation treatment followed by 48 h of live imaging. As expected in low serum, the reporter trajectories collapsed into predominantly G0 entry (**Figure 1E**). When we measure the

timing of the reporter trajectories we find that the timing of G0 is heterogeneous compared to G1 entry and G1 exit and becomes further prolonged under low serum (**Figures 1F,G**).

We next examined whether the mVenus-p27K⁻/mCherry-hCdt1 double positive population under full serum conditions exhibits molecular markers of G0. To do this, we sorted cells into Venus/Cherry double-positive, Cherry single-positive, and Venus/Cherry double-negative populations and performed western blots for markers of G0 vs G1 phase. As a positive control for G0, cells cultured under serum deprivation were sorted in a similar manner (**Supplementary Figure S2**). We found that Venus/Cherry double-positive cells under full serum conditions exhibited hypo-phosphorylated pocket proteins RB and p130, increased endogenous p27, and reduced phosphorylation of Cdk2 on the activating T-Loop (Jeffrey et al., 1995; Sherr and Roberts, 1999; Tedesco et al., 2002). We also confirmed reduced expression of cell cycle genes, and upregulated expression of genes associated with G0 in the double positive cells in full serum by qRT-PCR on sorted cells (**Supplementary Figure S2**) (Oki et al., 2014). Taken together, our tracking and molecular data suggests that many of the Venus/Cherry double-positive cells under full serum conditions enter a temporary G0 of variable length. We therefore conclude that this reporter combination also captures temporary, spontaneous quiescence in a fraction of asynchronously proliferating cells.

Asynchrony in the Proliferation-Quiescence Decision

In the manual tracking of dividing cells, we noticed several instances where pairs of daughters, born of a single mitosis, make different proliferation-quiescence decisions. In this situation, one daughter will remain G0, while the other daughter born at the same time will degrade the mVenus-p27K⁻ reporter and enter G1, followed by S/G2 and mitosis (**Figure 1H**). Under normal culture conditions we observe this in 20–40% of 3T3 cells entering G0, with the differences in the timing of G1 entry between asynchronous daughters varying from 1–15 h. We also find that daughters can exhibit varying lengths of the Venus/Cherry double-positive state (from 5—24 h) before one from the pair enters G1. We next examined whether such asynchrony in the cell cycle progression of two daughters born of the same mitosis could be observed in other cell types. We manually examined movies of published live cell imaging and observed instances of cell cycle asynchrony in pairs of daughters in BT549 and MCF10A cells (**Supplementary Table S1**). Cell cycle asynchrony in daughters born of the same mitosis has also been reported in MCF7 and HCT116 cells and referred to as "asymmetric" cell divisions, accompanied by differences in AKT
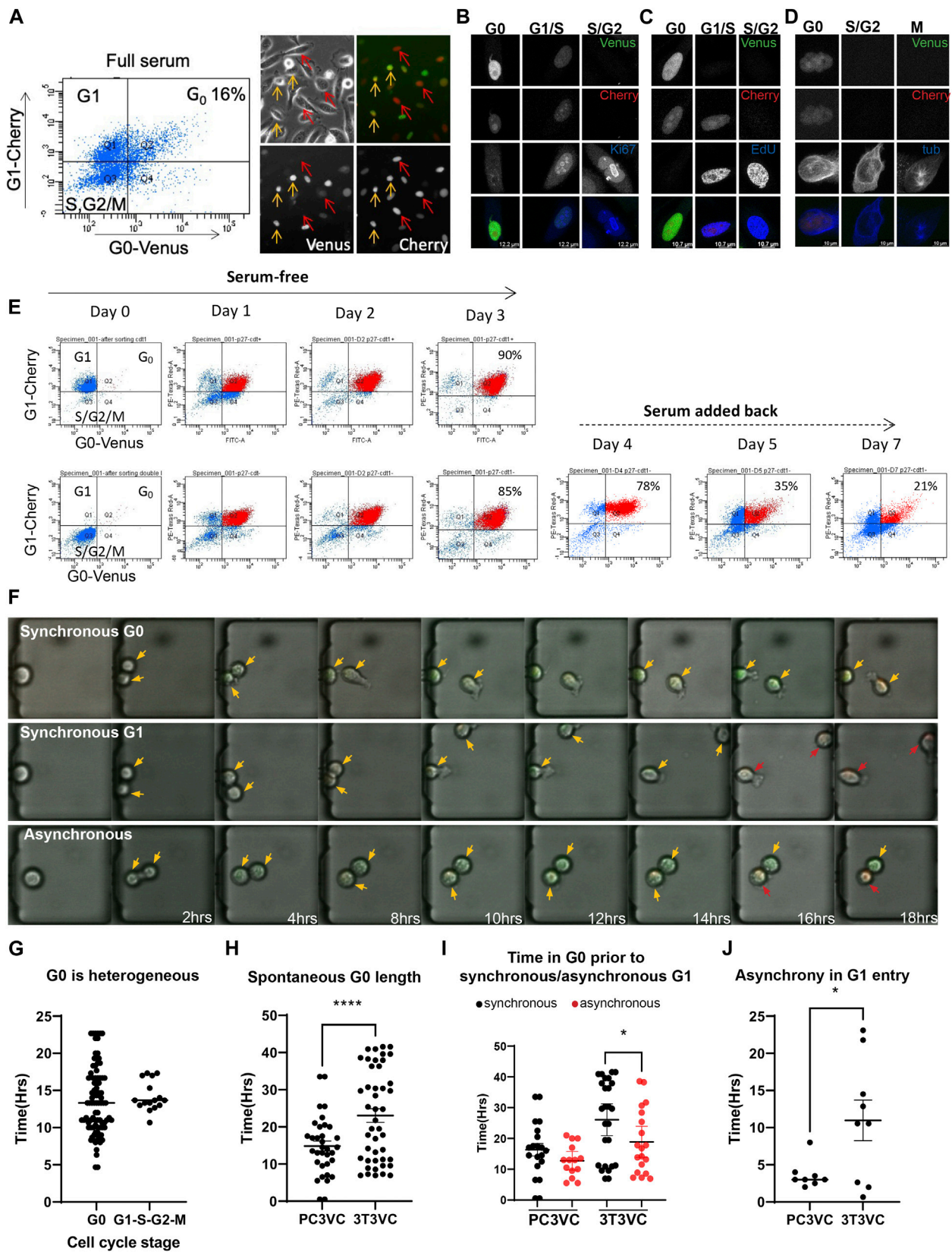
FIGURE 2 | Spontaneous quiescence and asynchronous proliferation-quiescence decisions occur in PC3 cells. **(A)** G0-Venus and G1-Cherry reporters were transduced into PC3 cells and a clone exhibiting normal growth rate and strong, stable reporter expression was isolated. PC3 Venus/Cherry cells exhibit a fraction of cells double positive for G0-Venus/G1-Cherry under full serum conditions. Imaging reveals Venus/Cherry double positive cells (orange arrows), Cherry only positive cells (red

(Continued)

signaling between daughters after telophase (Dey-Guha et al., 2011; Dey-Guha et al., 2015). We suggest that both spontaneous G0 and asynchronous proliferation-quiescence decisions in pairs of daughters after mitosis both contribute to cell cycle heterogeneity in clonal cell populations.

PC3 Prostate Cancer Cells Exhibit Spontaneous Quiescence and Asynchrony in the Proliferation-Quiescence Decision

Cellular quiescence in prostate cancer is thought to contribute to tumor dormancy and issues with metastatic cancer recurrence. However, it is not well understood how and why prostate cancer cells enter and exit quiescence. We wondered whether spontaneous quiescence and asynchronous proliferation-quiescence decisions may, in part, underlie cell cycle heterogeneity in prostate cancer cells. To examine this, we transduced the mVenus-p27K⁻ G0 and mCherry-hCdt1 G1 reporters into PC3 cells. We initially selected pools of transduced cells expressing both reporters under reduced serum conditions by FACS. We found that sorted, pooled cells quickly lost expression of one or the other reporter after a limited number of passages. We therefore isolated clones and selected a clonal PC3 Venus-Cherry cell line, stably expressing both reporters at visible levels with normal cell cycle dynamics (i.e a cell doubling time similar to parental PC3). In this line, we readily observe double positive Venus/Cherry cells under normal full-serum culture conditions (**Figure 2A**) that are negative for EdU incorporation, negative for Ki-67 and both reporters are silent in cells that progress through the cell cycle into mitosis (**Figures 2B–D**). We also confirmed that the reporters exhibited the expected G0/G1 dynamics during serum withdrawal and serum re-addition in PC3 cells (**Figure 2E**).
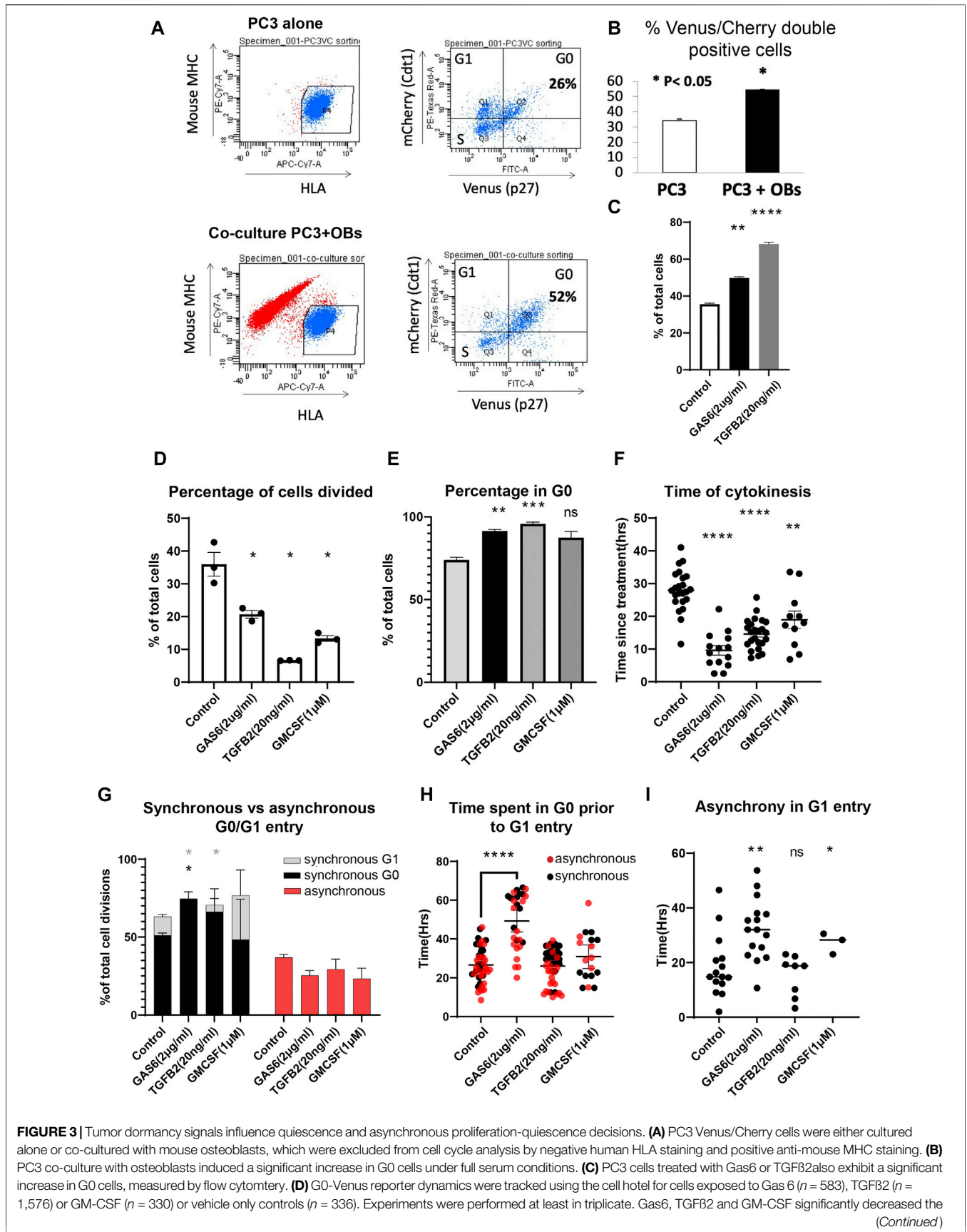
We next attempted to track the reporter dynamics in PC3 cells with live cell imaging and found that these cancer cells were too motile to be tracked accurately for more than a few hours. We therefore used a microfluidic device we term the "cell hotel," to capture one or a few cells and trap them in a chamber, to allow for manual tracking of individual cells and their daughters (Cheng et al., 2016). Each cell hotel slide allows simultaneous recording of up to 27 chambers under ×10 magnification. We confirmed that

the PC3 Venus-Cherry cells in the cell hotel exhibited similar growth and cell doubling times as previously reported for PC3 cells in bulk cell culture. In addition we repeated measurements of 3T3 Venus/Cherry cells in the cell hotel for all comparisons to PC3 (**Figures 2F–J**).

Similar to the 3T3 cells, we observed a nearly invariant reporter progression of mVenus-p27K⁻ expression ~2 h after cytokinesis, followed by mCherry-hCdt1 expression approximately 2 h later. Cells that enter the cell cycle, degrade mVenus-p27K⁻ within approximately 4 h, followed by mCherry-hCdt1 degradation and ultimately cell division (**Figure 2F**). As in 3T3 cells, we observed 20% of cells with stabilized mVenus-p27K⁻ and mCherry-hCdt1 for 14 h and beyond, without division or evidence of S/G2/M entry for 20 h or more, suggestive of spontaneous G0 entry in PC3 cells (**Figures 2G,H**). Notably, spontaneous quiescence in PC3 cells tends to be more rare and shorter than in 3T3 cells (**Figure 2H**). This could reflect the important role for p53 signaling in spontaneous quiescence (Arora et al., 2017; Yang et al., 2017), as PC3 cells lack functional p53 (Carroll et al., 1993). We also observed evidence of asynchronous proliferation-quiescence decisions, with 30% of daughters making asynchronous G0/G1 decisions within 1–6 h of each other (**Figures 2I,J**). Interestingly, the asynchronous proliferation-quiescence decisions were also rarer and the difference in timing between asynchronous daughters was less dramatic in PC3 cells (**Figure 2J**).

Tumor Dormancy Signals can Influence Quiescence and Asynchronous Proliferation-Quiescence Decisions

Bone is a common site for prostate cancer metastasis and work from our group and others have shown that signals from osteoblasts can influence prostate cancer dormancy and PC3 cell cycle dynamics (Jung et al., 2016; Lee et al., 2016; Yumoto et al., 2016). Our previous work on PC3 cell cycle dynamics used the FUCCI cell cycle reporters, which could not distinguish between G0 and G1 arrest (Jung et al., 2016). We therefore examined whether PC3 Venus-Cherry cells co-cultured with osteoblasts increased entry into G0 quiescence. We found that

FIGURE 3 | Tumor dormancy signals influence quiescence and asynchronous proliferation-quiescence decisions. **(A)** PC3 Venus/Cherry cells were either cultured alone or co-cultured with mouse osteoblasts, which were excluded from cell cycle analysis by negative human HLA staining and positive anti-mouse MHC staining. **(B)** PC3 co-culture with osteoblasts induced a significant increase in G0 cells under full serum conditions. **(C)** PC3 cells treated with Gas6 or TGFß2also exhibit a significant increase in G0 cells, measured by flow cytomtery. **(D)** G0-Venus reporter dynamics were tracked using the cell hotel for cells exposed to Gas 6 (n = 583), TGFß2 (n = 1,576) or GM-CSF (n = 330) or vehicle only controls (n = 336). Experiments were performed at least in triplicate. Gas6, TGFß2 and GM-CSF significantly decreased the

(Continued)

co-culture with mouse MC3T3-E1 pre-osteoblasts under full serum conditions significantly increased the fraction of double positive PC3 Venus-Cherry cells consistent with increased entry into G0 (**Figures 3A,B**). We next examined whether Gas6 and TGFß2, signals from osteoblasts we have previously shown to induce a G0/G1 cell cycle arrest (Jung et al., 2016; Lee et al., 2016; Yumoto et al., 2016), induced entry into G0. Indeed, exposure to Gas6 or TGFß2 significantly increased the fraction of double positive PC3 Venus-Cherry cells after 48 h, suggesting the G0/G1 arrest we previously observed was indeed arrest in G0 (**Figure 3C**).
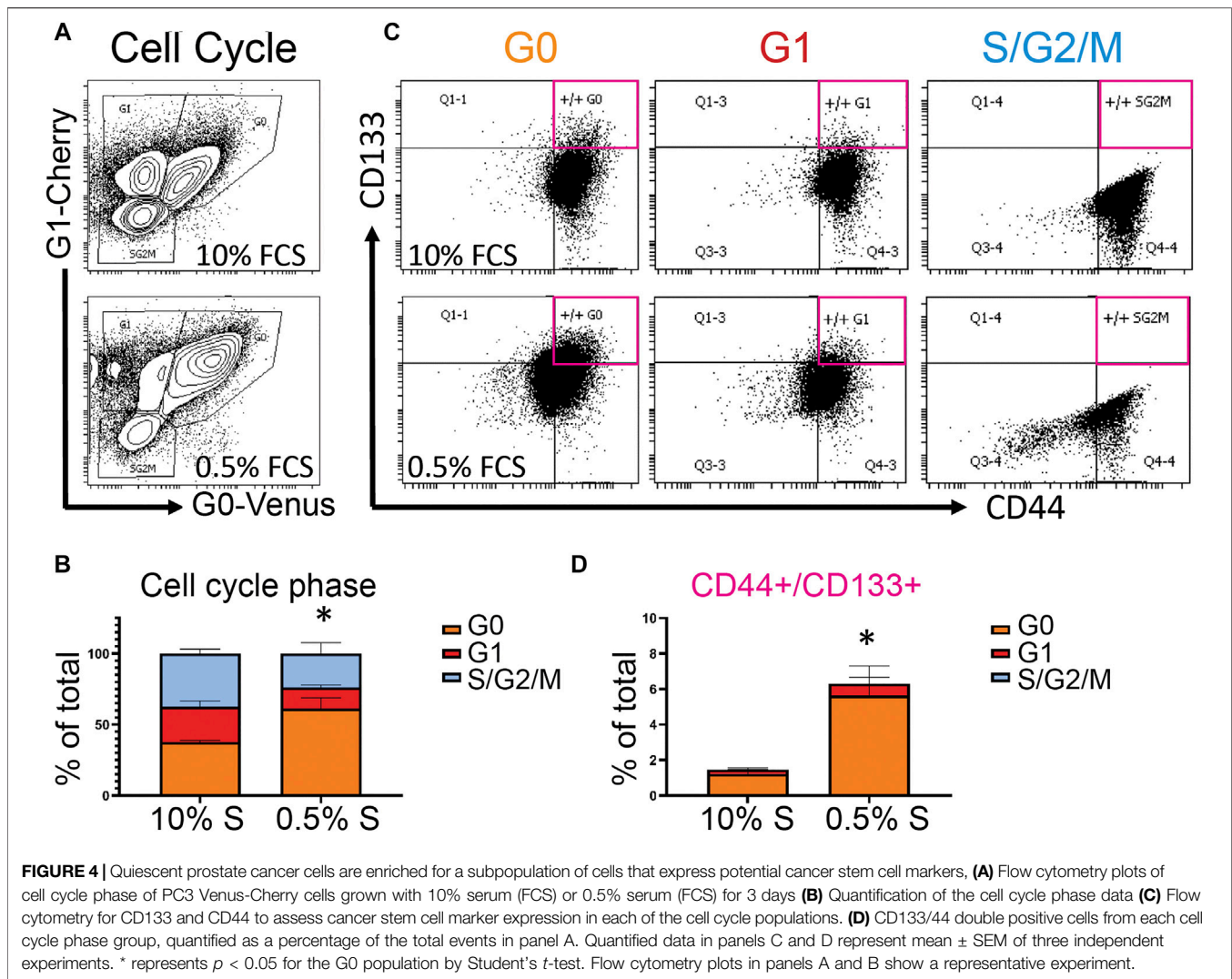
In the bone marrow, Gas6 and TGFß2 are thought to promote prostate cancer dormancy (Jung et al., 2012; Taichman et al., 2013; Ruppender et al., 2015; Yumoto et al., 2016) while GM-CSF promotes stem cell release from the bone marrow, which may provide cues for metastatic cancer cells in the bone marrow to exit from dormancy and proliferate (Dai et al., 2010). While some of the role for GM-CSF is thought to be due to indirect effects on the bone marrow stem cell niche, studies of GM-CSF directly added to cultured PC3 cells also show increased S-phase entry, proliferation and clonogenic growth consistent with exit from dormancy (Lang et al., 1994; Savarese et al., 1998). We therefore wanted to test whether GM-CSF impacted the proliferation-quiescence decision in PC3 cells and compare this to the effects of the dormancy associated signals Gas6 and TGFß2 on G0. We used the cell hotel to track cell cycle and mVenus-p27K⁻ and mCherry-hCdt1 dynamics in PC3 cells 2 h after the addition of Gas 6, TGFß2 or GM-CSF. In response to Gas6 and TGFß2 we observed a significant decrease in the number of cells undergoing divisions during the 72 h live imaging period, consistent with an increase in G0 entry (**Figures 3D,E**). Unexpectedly, we also observed a similar decrease in cell divisions for cells treated with GM-CSF, and an increase in Venus-Cherry double positive cells, suggesting GM-CSF also promotes G0 entry. For the fraction of cells undergoing divisions during the live imaging, we tracked when these cell divisions occur. We found that control cells asynchronously proliferate throughout the live imaging time course, while most cells treated with Gas6, TGFß2 or GM-CSF divide within the first 24 h of imaging (**Figure 3F**). These data suggest that cells uncommitted to the cell cycle either enter or remain in quiescence in response to Gas6, TGFß2 or GM-CSF. Cells that are past the restriction point when the treatment begins, and therefore committed to cycle, must make a subsequent proliferation-quiescence decision after mitosis that may be tipped toward quiescence. To confirm this, we examined the proliferation-quiescence choices made by pairs of daughters that divide under each treatment, broken down into: synchronous

entry into G0, synchronous entry into G1 or asynchronous entry with one daughter entering G1 while the other remains in G0. For Gas6 and TGFß2 treated cells, we observed a significant increase in synchronous entry into G0 at the expense of synchronous entry into G1, with little impact on the proportion of cells that exhibit asynchronous proliferation-quiescence decisions (**Figure 3G**). This suggests that Gas6 and TGFß2 continue to promote quiescence in cells that are already committed to cycle after treatment addition. By contrast, GM-CSF treatment increased the proportion of divisions resulting in synchronous entry into G1, suggesting prolonged GM-CSF exposure may eventually promote cell cycle entry in a subset of the population (**Figure 3G**). Of note the pro-proliferative effects previously reported were seen in experiments performed on much longer timescales of at least 3–4 days (Lang et al., 1994; Savarese et al., 1998), suggesting the response to GM-CSF may be complex. Our data suggests GM-CSF treatment initially promotes quiescence entry in cells that are prior to the restriction point, but for a subset of cells past the restriction point it promotes their daughters to preferentially enter G1.

We next tracked pairs of daughters from the dividing cells under treatment and measured how long they spent in a Venus/Cherry double positive G0 state after mitosis prior to entering a Cherry-only G1 state. The goal was to determine whether each treatment also impacted the heterogeneity of transient quiescence, a feature that may initially promote tumor dormancy, but also lead to later recurrence. We found that only treatment with Gas6 impacted cells that entered transient quiescence, by significantly prolonging the time spent in G0 prior to the next G0-G1 transition (**Figure 3H**). This prolonged G0 occurred whether the pairs of daughters entered into G1 synchronously or asynchronously, and increased the differences in timing of G1 entry between pairs of asynchronous daughters (**Figure 3I**). This suggests that Gas6 promotes quiescence, but also promotes quiescence heterogeneity in cells that retain the ability to re-enter the cell cycle.

Quiescent Cancer Cells are Enriched for Stem Cell Markers and Express High Levels of Hippo Pathway Signaling Components

Identifying molecular markers of quiescent cancer cells that could be assayed in patient samples is an attractive approach to identify those at risk for metastasis and recurrence. Toward this goal, we used the PC3 Venus-Cherry cells to isolate populations enriched for quiescent cancer cells by FACS, to examine their cell surface markers and gene expression changes. (**Figures 4,5**). We first

FIGURE 4 | Quiescent prostate cancer cells are enriched for a subpopulation of cells that express potential cancer stem cell markers, **(A)** Flow cytometry plots of cell cycle phase of PC3 Venus-Cherry cells grown with 10% serum (FCS) or 0.5% serum (FCS) for 3 days **(B)** Quantification of the cell cycle phase data **(C)** Flow cytometry for CD133 and CD44 to assess cancer stem cell marker expression in each of the cell cycle populations. **(D)** CD133/44 double positive cells from each cell cycle phase group, quantified as a percentage of the total events in panel A. Quantified data in panels C and D represent mean ± SEM of three independent experiments. * represents $p < 0.05$ for the G0 population by Student's t-test. Flow cytometry plots in panels A and B show a representative experiment.
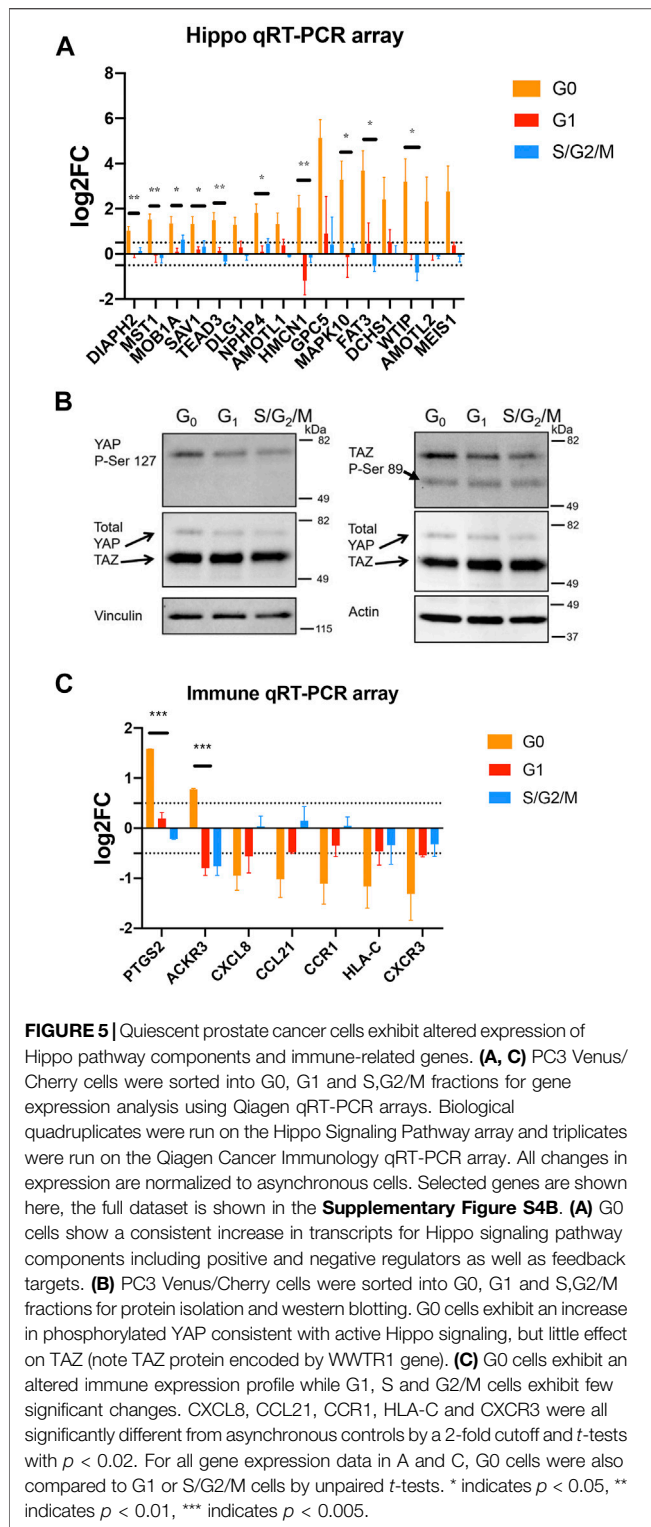
assayed the prostate cancer stem cell markers CD133 and CD44 to determine whether increasing cellular quiescence could increase the fraction of CD133/CD44 double positive potential cancer stem cells (Jung et al., 2016). We cultured PC3 Venus/Cherry cells under normal 10% serum conditions, or reduced 0.5% serum conditions for 72 h. We confirmed an increase in the G0 population under reduced serum (**Figure 4A, B**) and compared the fraction of CD133/CD44 double positive cells in the G0, G1 and S/G2/M populations (**Figure 4C**). We observed the majority of the CD133/CD44 double positive cells to be in the G0 population, with a much smaller fraction in G1 and almost none in the S/G2/M population (**Figures 4C,D**). This suggests that signals in the tumor environment that increase the quiescent population in prostate cancer may also increase the number of potential cancer stem cells that could underlie recurrence.

We previously established a mouse xenograft model of prostate cancer bone metastasis using Du-145 cells, that recapitulates aspects of dormancy and recurrence (Cackowski et al., 2017). We attempted to use the PC3 Venus/Cherry cells in a

similar xenograft model, but found that the cells quickly silenced the cell cycle reporters *in vivo*. We therefore used the xenograft model with Du-145 cells as a tool to compare gene expression profiles for cells in actively growing bone metastases as assessed by bioluminescence imaging ("involved") vs bones without imaging detected metastases, but which still contained cancer cells that were fewer in number and presumably more slowly growing ("uninvolved"). Use of this approach of comparing cancer cells from high burden/involved vs low burden/uninvolved sites was previously used in a breast cancer model and showed that cancer cells from the uninvolved sites had more stem-like properties (Lawson et al., 2015). We isolated cells from mouse marrow under both conditions and performed bulk RNA-seq to compare global gene expression changes in the growing vs dormant state. Due to the small number of human cells recovered from uninvolved bones, we were only able to accurately assign differences in expression for 117 genes (**Supplementary Figure S3**). Nonetheless using DAVID and the KEGG database to perform pathway analysis of differentially expressed genes, we found an enrichment of genes involved in extracellular matrix

FIGURE 5 | Quiescent prostate cancer cells exhibit altered expression of Hippo pathway components and immune-related genes. **(A, C)** PC3 Venus/Cherry cells were sorted into G0, G1 and S,G2/M fractions for gene expression analysis using Qiagen qRT-PCR arrays. Biological quadruplicates were run on the Hippo Signaling Pathway array and triplicates were run on the Qiagen Cancer Immunology qRT-PCR array. All changes in expression are normalized to asynchronous cells. Selected genes are shown here, the full dataset is shown in the **Supplementary Figure S4B**. **(A)** G0 cells show a consistent increase in transcripts for Hippo signaling pathway components including positive and negative regulators as well as feedback targets. **(B)** PC3 Venus/Cherry cells were sorted into G0, G1 and S,G2/M fractions for protein isolation and western blotting. G0 cells exhibit an increase in phosphorylated YAP consistent with active Hippo signaling, but little effect on TAZ (note TAZ protein encoded by WWTR1 gene). **(C)** G0 cells exhibit an altered immune expression profile while G1, S and G2/M cells exhibit few significant changes. CXCL8, CCL21, CCR1, HLA-C and CXCR3 were all significantly different from asynchronous controls by a 2-fold cutoff and t-tests with $p < 0.02$. For all gene expression data in A and C, G0 cells were also compared to G1 or S/G2/M cells by unpaired t-tests. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.005$.

which is a key regulator of cell cycle exit (Zheng and Pan, 2019). We therefore decided to examine whether components of Hippo signaling may be altered in quiescent prostate cancer cells.

To examine differences in gene expression between G0, G1, and S/G2/M populations, we sorted populations and examined gene expression differences using a Hippo signaling Pathway qRT-PCR array (**Figure 5A**). In G0 cells, we noted a widespread increase in transcripts for Hippo signaling pathway components (e.g., DCHS1, FAT3, MST1, MOB1A, SAV1), transcriptional regulators (TEAD3, MEIS1) as well as targets (AMOTL1, AMOTL2) (Wang et al., 2018). The increased expression of some transcriptional targets of Hippo signaling is surprising, since these cells are in G0 and therefore would be expected to have Hippo signaling on. Hippo signaling acts via phosphorylation to suppress the activity of the downstream transcriptional effectors YAP1 and TAZ (encoded by the WWTR1 gene in humans) (Zheng and Pan, 2019). We therefore examined whether YAP and TAZ are suppressed via phosphorylation in G0 cells through active Hippo signaling. We performed westerns for the inactivating phosphorylations on YAP1 and TAZ in G0, G1 and S/G2/M sorted cells (**Figure 5B**) and found a small increase in phosphorylated YAP, but no effect on TAZ. We hypothesize the increased expression of Hippo pathway components may poise quiescent cells to re-enter the cell cycle upon receipt of a dormancy escape signal, but that during G0, active Hippo signaling restrains YAP transcriptional activity through inhibitory phosphorylation. (**Figure 5** and **Supplementary Figure S4**).

Hippo signaling in cancer has been associated with restraining proliferation (Zheng and Pan, 2019), but also has been shown to alter immune response, with active Hippo signaling suppressing tumor immunogenicity (Moroishi et al., 2016; Yamauchi and Moroishi, 2019). We therefore next examined whether G0 cells exhibited alterations in expression of immune response-associated signals. Using the Qiagen Cancer Immunology array on sorted G0, G1 and S/G2/M PC3 cells vs the mixed population as a reference, we observed a moderate but widespread decrease in the expression of immune-related genes including signals known to target cancer cells for host immune destruction, such as CXCR3, CXCL8, and HLA-C (**Figure 5C**) in G0 cells. This suggests that spontaneously quiescent cancer cells may exhibit altered immunoreactivity. Interestingly, one pro-inflammatory gene, PTGS2 (Cox-2), was strongly upregulated in G0 cells (**Figure 5C**), consistent with the previous work showing this target to be de-repressed when upstream Hippo signaling is active and YAP/TAZ are suppressed by phosphorylation (Zhang et al., 2018). Taken together, our results suggest the inherent cell cycle heterogeneity of metastatic prostate cancer includes a fraction of spontaneously quiescent cells that are enriched for cells expressing cancer stem cell markers and exhibit gene expression changes consistent with a state poised to re-enter the cell cycle, but potentially less visible to the host immune system.

DISCUSSION

Several cancers contain heterogeneous populations with varying levels of proliferation (Davis et al., 2019). Some studies suggest

interactions and the TGF-beta signaling pathway, factors known to impact prostate cancer dormancy and dormancy escape (White et al., 2006; Bragado et al., 2013; Ruppender et al., 2015). We noted that several of the genes falling into these enriched categories also interface with Hippo signaling,

that quiescent tumor cells contribute to drug-resistance, by providing a population of non-cycling cells that survive cytotoxic chemotherapy (De Angelis et al., 2019; Talukdar et al., 2019; Hen and Barkan, 2020). Understanding the molecular basis of proliferative heterogeneity therefore may assist in developing better therapeutic approaches for cancer. Here, we show that untransformed 3T3 cells and PC3 prostate cancer cells show spontaneous quiescence and heterogeneous G0 lengths under pro-proliferative culture conditions. We propose that spontaneous quiescence may be related to quiescence in cancer, since quiescent cancer cells must leave the cell cycle in the presence of pro-proliferative growth factor and oncogenic signaling.

Spontaneous quiescence has been shown to underlie clonal cell cycle heterogeneity (Overton et al., 2014) and may in part underlie cell cycle heterogeneity in tumors. Here we show an additional mechanism to create heterogeneity, asynchronous G0/G1 decisions, where one daughter from a mitotic event remains in G0, while the other enters G1. These asynchronous decisions are somewhat surprising, since recent work has suggested the signals that influence the proliferation-quiescence decision are integrated over the previous cell cycle phases prior to mitosis and therefore would be expected to be inherited equally in daughters after mitosis (Yang et al., 2017; Min et al., 2020). The asynchrony in asynchronous PC3 cell divisions is often only a few hours but can extend to over 20 h or more in the presence of the dormancy inducing factor, Gas6 (**Figure 3I**). Small differences in asynchronous pairs of daughters may possibly be explained by fluctuations resulting in unequal protein and transcript inheritance at mitosis. However, this is a less satisfying hypothesis for differences in G0 exit between asynchronous daughters longer than 10 h. Previous work in MCF7 breast and HCT116 colon cancer cells has shown a population of dormant cells resulting from asymmetric Akt signaling after cell divisions (Dey-Guha et al., 2011). In this example, about 1% of cell divisions exhibit asymmetry, resulting in a daughter with low Akt signaling. Importantly, elimination of Akt prevented proliferative heterogeneity in these lines in cell culture (Dey-Guha et al., 2015), and inhibition of asymmetric Akt signaling reduced tumor recurrence after treatment in a xenograft model (Alves et al., 2018). It is worth noting that PC3 cells lack functional PTEN (Huang et al., 2001; Dubrovska et al., 2009) and therefore would be expected to have higher endogenous Akt signaling that suppresses some degree of asymmetry. In addition, Gas6 and other TYRO3/AXL/MERTK ligands signal in part through Akt, and therefore may also impact Akt asymmetry (Cosemans et al., 2010; Kasikara et al., 2017). Inhibition of Akt signaling can lead to up regulation of p27 in PC3 cells (Van Duijn and Trapman, 2006), while over expression of p27 can also inhibit Akt signaling (Chen et al., 2009). Further work will be needed to determine if asymmetric Akt signaling may be a cause or consequence of asynchronous proliferation-quiescence decisions in prostate cancer.

The relationship of dormancy and cellular quiescence remains unclear. Here we show that dormancy-associated signals in prostate cancer, Gas6 and TGFß2, rapidly within a few hours, induce quiescence entry in prostate cancer cells after mitosis. This

is in part because these signals tip the balance of proliferation-quiescence decisions in favor of synchronous G0 entry. By contrast, a presumed pro-proliferative signal for PC3 cells, GM-CSF tips the balance in favor of synchronous divisions into G1 for the cells that divide after initial exposure. Thus, although GM-CSF promotes G0 entry initially, sustained signaling may promote cell cycle re-entry in the longer term. Gas6 also has a complex effect on the proliferation-quiescence decision. In addition to promoting G0 in cells that are uncommitted to the cell cycle, Gas6 also prolongs G0 in cells that retain the ability to eventually re-enter the cell cycle. This suggests that the quiescence response to Gas 6 is not an all or nothing response and it can be graded, resulting in varying lengths of G0 to promote quiescence heterogeneity. While none of the signals we tested significantly altered the frequency of asynchronous cell cycle entry in pairs of daughters after mitosis, Gas6 significantly increased the asynchrony in G0 exit and G1 entry. We suggest this could be another source of quiescence heterogeneity in cancer.

Understanding the gene expression changes in dormant cancer cells will be essential to understanding their biology, but will also be useful tools as molecular markers for identifying them in patient samples. Here we show that quiescent PC3 cells are enriched for prostate cancer stem cell markers CD133 and 44 and that driving quiescence entry through serum starvation significantly increases the population of CD133/44 double positive cells in the population. Quiescent prostate cancer cells also exhibit increased expression of some Hippo pathway components, while the Hippo pathway remains on to restrain cell cycle entry. This finding in cell culture was also supported by our gene expression analysis in an *in vivo* xenograft model for prostate cancer tumor dormancy (**Supplementary Figure S3**). Interestingly, this is correlated with suppressed levels of mRNA for immune targeting factors, and may suggest a mechanism by which quiescent cancer cells evade host immune attack. Whether there is a direct or indirect relationship between the Hippo signaling status and expression of immune targets in quiescent cells remains to be examined.

MATERIALS AND METHODS

Cells and Cell Culture

The mouse embryonic fibroblast 3T3 cell line containing the G0 and G1 cell cycle reporters were kindly provided by Dr. Toshihiko Oki (University of Tokyo). These cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Serum levels were reduced as indicated in the figures and text for serum starvation experiments. PC3 prostate cancer cells were cultured in RPMI medium with 10% serum and 1% penicillin-streptomycin and transduced with the G0 and G1 cell cycle reporters as previously described (Takahashi et al., 2019).

Live Cell Imaging

NIH/3T3 cells were cultured at low density (to avoid contact inhibition) on 12-well plates in phenol red-free DMEM/10%FBS

or 1%DMEM. Experiments in **Figure 1** (and **Supplementary Figure S1**) were performed using an EVOS FL cell imaging system with a ×20 objective lens or an IncuCyte Zoom at 37°C, 5% CO_2. The imaging intervals were 20–30 min.

For experiments using the "Cell Hotel" (**Figures 2, 3**), 10,000 PC3 cells in RPMI medium with 10% serum were loaded into the inlet of the microfluidic chamber. The chamber loading was monitored until most of the chambers were occupied with single cells (~5 mins). Remaining cells were then removed from the inlet and the outlet and replenished with fresh media. Imaging was performed using a Leica DMI 6000 with a Tokai Hit stage-top environmental chamber at 37°C, 5% CO_2. TGFß2, Gas6 and GM-CSF (R&D systems) were reconstituted according to the manufacturer's guidelines (R&D systems). For Gas6, TGFß2 and GM-CSF treatments, 10,000 PC3 cells were mixed with media containing the ligand (2 µg/ml for Gas6, 20 ng/ml for TGFß2 and 1 µM for GM-CSF) and introduced into the chamber. Cells were then incubated in media with the indicated ligand for 2 h of pre-equilibration prior to imaging every 30 min.

Fabrication of the Microfluidic Device for Single-Cell Tracking

The microfluidic device used for single-cell tracking was developed in our previous work (Cheng et al., 2016). The device was built by bonding a PDMS (Polydimethylsiloxane, Sylgard 184, Dow Corning) layer with microfluidic patterns to a glass slide. The PDMS layer was formed by standard soft lithography. The SU-8 mold used for soft-lithography was created by a 3-layer photolithography process with 10 µm, 40 µm, and 100 µm thick SU-8 (Microchem) following the manufacturer's protocol. PDMS was prepared by mixing with 10 (elastomer): 1(curing agent) (w/w) ratio, poured on SU-8 molds, and cured at 100°C overnight. Inlet and outlet holes are created by biopsy punch cutting. The PDMS with microfluidic channel structures and the glass slide were treated using oxygen plasma (80 W for 60 s) and bonded. The devices after bonding were heated at 80°C for an hour to ensure bonding quality. The microfluidic chips were sanitized using UV radiation and primed using a either a Collagen solution (1.45 ml Collagen (Collagen Type 1, 354,236, BD Biosciences) or Fibronectin solution, 0.1 ml acetic acid in 50 ml DI Water) overnight before use.

Flow Cytometry Analysis and FACS

For cell sorting and flow cytometry assays in **Figures 2–5**, cells were cultured in RPMI supplemented with either 10% FBS or 1% as indicated and subpopulations were sorted according to the intensity of their fluorescent reporters, using a BD FACS Aria II system. Cells were sorted into SDS-PAGE loading buffer or RLT (Qiagen) for immediate protein extraction or RNA isolation. A minimum of ~10^5 cells were collected for each experiment. Antibodies used for PC3 isolation from osteoblasts co-culture in **Figure 2** were APC/Cy7 anti-human HLA A,B,C antibody (Biolegend #311426).

For **Figure 4**, we assessed PC3 cells for dual positivity for CD44 and CD133 as we previously described (Shiozawa et al.,

2016). PC3 cells were seeded at 10^5 cells per well of six well plates in RPMI with 1% penicillin/streptomycin and either 10% or 0.5% serum, then cultured for 3 days. Both adherent and floating cells were analyzed for flow cytometry using a four laser BD LSR II instrument and FACSDiva™ software. We plotted G0-Venus vs G1-Cherry from the single, viable (DAPI negative) population and drilled down from each cell cycle phase group (G0, G1, or S/G/M) to analyze the percent CD133+/CD44 + cells from each population. Antibodies were PE-vio770 conjugated CD133/1, clone AC133 (Miltenyi Biotech #130–113–672) diluted 1:50 and APC conjugated CD44 (BD #559942) diluted 1:5.

Western Blotting

Cleared cell lysates in SDS loading buffers were separated on 4–20% SDS PAGE gels under reducing conditions and transferred to PVDF membranes. Membranes were blocked with 5% milk in TBST and probed with primary antibodies diluted 1:1,000 in 5% BSA TBST; YAP1 phospho-serine 127 (Cell Signaling Technology #4911) and TAZ phospho-serine 89 (Cell Signaling Technology #59971). The secondary antibody was Cell Signaling #7074 diluted 1:1,000 in 1% milk TBST. Blots were developed in Pierce Supersignal Pico ECL substrate and visualized with a Biorad Image Doc Touch system. The membranes were subsequently stripped and reprobed for total YAP1 (CST #14074), total TAZ (CST #83669), beta actin (CST #4970), or vinculin (CST #13901) as indicated.

qRT-PCR Arrays

PC3 Venus/Cherry cells were seeded at 10^5 cells per dish in 10 cm dishes and cultured for 3 days in RPMI media with 1% FCS. Cells were seeded on different days for biologic triplicate or quadruplicate samples. After 3 days of culture, cells were released by tripysinization, stained with DAPI for viability and sorted by FACS into either the total (mixed) viable cell population, G0, G1, or S/G2/M phases using the Venus/Cherry markers. 10^5 viable cell events for each population were collected directly into Qiagen RLT buffer containing β-mercaptoethanol. Total RNA was isolated with Qiagen RNeasy kits. The samples were analyzed with the Human Hippo Signaling RT2 Profiler PCR array (Qiagen #PAHS-172ZA) or Human Cancer Inflammation and Immunity Crosstalk array (Qiagen #PAHS-181ZA) using the recommended cDNA synthesis and PCR reagents. Data are presented as biologic quadruplicate or triplicate samples of expression relative to the total viable population sample. Visualization and hierarchical clustering was prepared with Morpheus software (Broad Institute).

Additional Methods and details for ATCQ and Supplemental Figures are included in the Supplemental Data file.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Michigan Committee for the Use and Care of Animals.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.728663/full#supplementary-material

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