Fibroblast Growth Factor Receptor 1 Drives the Metastatic Progression of Prostate Cancer

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Article info

Article history:
Received 7 July 2021
Received in Revised form 16 September 2021
Accepted October 4, 2021

Associate Editor:
Gianluca Giannarini

Keywords:
Bone metastasis
Fibroblast growth factor receptor 1
Ladinin 1
Prostate cancer

Abstract

Background: No curative therapy is currently available for metastatic prostate cancer (PCa). The diverse mechanisms of progression include fibroblast growth factor (FGF) axis activation.

Objective: To investigate the molecular and clinical implications of fibroblast growth factor receptor 1 (FGFR1) and its isoforms (α/β) in the pathogenesis of PCa bone metastases.

Design, setting, and participants: In silico, in vitro, and in vivo preclinical approaches were used. RNA-sequencing and immunohistochemical (IHC) studies in human samples were conducted.

Outcome measurements and statistical analysis: In mice, bone metastases (chi-square/Fisher’s test) and survival (Mantel-Cox) were assessed. In human samples, FGFR1 and ladinin 1 (LAD1) analysis associated with PCa progression were evaluated (IHC studies, Fisher’s test).

Results and limitations: FGFR1 isoform expression varied among PCa subtypes. Intracardiac injection of mice with FGFR1-expressing PC3 cells reduced mouse survival (α, p < 0.0001; β, p = 0.032) and increased the incidence of bone metastases (α, p < 0.0001; β, p = 0.02). Accordingly, IHC studies of human castration-resistant PCa (CRPC) bone metastases revealed significant enrichment of FGFR1 expression compared with treatment-naïve, nonmetastatic primary tumors (p = 0.0007). Expression of anchoring filament protein LAD1 increased in FGFR1-expressing PC3 cells and was enriched in human CRPC bone metastases (p = 0.005).

Conclusions: FGFR1 expression induces bone metastases experimentally and is significantly enriched in human CRPC bone metastases, supporting its prometastatic effect in PCa. LAD1 expression, found in the prometastatic PCa cells expressing FGFR1, was also

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https://doi.org/10.1016/j.euo.2021.10.001
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Please cite this article as: E. Labanca, J. Yang, Peter D.A. Shepherd et al., Fibroblast Growth Factor Receptor 1 Drives the Metastatic Progression of Prostate Cancer, Eur Urol Oncol (2021), https://doi.org/10.1016/j.euo.2021.10.001
enriched in CRPC bone metastases. Our studies support and provide a roadmap for the development of FGFR blockade for advanced PCa.

**Patient summary:** We studied the role of fibroblast growth factor receptor 1 (FGFR1) in prostate cancer (PCa) progression. We found that PCa cells with high FGFR1 expression increase metastases and that FGFR1 expression is increased in human PCa bone metastases, and identified genes that could participate in the metastases induced by FGFR1. These studies will help pinpoint PCa patients who use fibroblast growth factor to progress and will benefit by the inhibition of this pathway.

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1. Introduction

Metastatic prostate cancer (PCa) that progresses after androgen ablation therapy (castration-resistant PCa [CRPC]) remains incurable [1]. The fibroblast growth factor (FGF) axis was implicated in PCa development and progression [2–5]. We previously reported that FGFR receptor (FGFR) blockade with dovitinib (TKI258) has clinical activity in a subset of men with CRPC and bone metastases [6]. Subsequent reports support our findings implicating the FGF axis in PCa pathogenesis [7,8]. Currently, a phase 2 clinical trial using erdafitinib, a specific FGFR inhibitor, is underway for CRPC (Clinicaltrials.gov ID NCT03999515). Therefore, a deeper understanding of the FGF-mediated mechanisms underlying PCa progression is needed to refine FGFR blockade as a therapy for PCa.

The FGF axis consists of numerous receptor-binding ligands, receptor tyrosine kinases, and their isoforms. The FGFR1 extracellular ligand-binding region comprises two or three Ig-like domains, resulting from alternative splicing of the α-exon, leading to FGFR1α (containing the α-exon) and FGFR1β (lacking the α-exon) isoforms. These isoforms were associated with glioblastoma and pancreatic, breast, and bladder cancer [9–13], and were suggested to have different cellular effects: while FGFR1α was associated with tumorigenesis and poor survival [9–16], FGFR1β was implicated in cell differentiation [14,17]. Only FGFR1α was found in the nucleus, suggesting that IgI might be important for its nuclear targeting [18].

The focus of this study is to investigate the role of FGFR1 and its isoforms, α/β, in PCa bone growth and metastasis. Here, we report for the first time that the expression of FGFR1 isoforms varies among PCa cases and is associated with different gene signatures, and that FGFR1 accelerates PCa metastatic dissemination. We also found that ladinin 1 (LAD1), a relatively uncharacterized anchoring filament protein, is induced by FGFR1 expression and enriched in human PCa bone metastases. Results from our studies provide a framework for developing FGFR-targeted therapies for PCa and identification of markers of progression.

2. Patients and methods

2.1. Cell lines, patient-derived xenografts, and treatment

PC3 and C4-2B cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 (Millipore Sigma, Burlington, MA, USA) supplemented with 10% FBS (Millipore Sigma).

FGFR1α and FGFR1β isoforms were developed from parental cell lines by stably transfecting them with pcDNA3.1-FGFR1α-P2A-eGFP (PC3-FGFR1α and C4-2B–FGFR1α) or pcDNA3.1-FGFR1β-P2A-eGFP (PC3-FGFR1β and C4-2B–FGFR1β) plasmids (GenScript, Piscataway, NJ, USA). Control sublines (transfected with empty vectors, PC3-V and C4-2B–V) were also generated. Transfected cell lines were selected by treatment with G418 (Geneticin, Sigma-Aldrich, St. Louis, MO, USA) in vitro and subsequent cell sorting for GFP expression by fluorescence-activated cell sorting (FACS).

 Luciferase-expressing sublines of C4-2B–FGFR1α, C4-2B–FGFR1β, and V were generated by infection with CMV-luciferase (firefly)-2A-RFP virus vector (AMSBIO, Abingdon, UK) followed by selection with puromycin (Thermo Scientific, Waltham, MA, USA) and FACS as described above.

LAD1 silencing in PC3-V and PC3-FGFR1β was performed by using pRFP-CB-shLenti shRNA lentiviral particles (shLAD1) or Scramble shRNA (Scr) as control (Origene, Rockville, MD, USA), followed by blasticidin (InvivoGen, San Diego, CA, USA) selection.

2.1.1. Patient-derived xenograft

MDA PCA 118b was previously developed in our laboratory [19].

2.1.2. Cell treatment

PC3, C4-2B stably expressing FGFR1 isoforms, and empty vector controls were serum starved for 3 h, and 50 ng/ml heparan sulfate proteoglycan (Sigma-Aldrich) was added during the last hour. Then we added 100 μg/ml FGF2 or FGF9 (Peprotech, Cranbury, NJ, USA) for 45 min to activate the FGFR pathway. Untreated cells were used as controls.

2.2. Western blot analysis

Cells treated as outlined above were harvested at the end of treatment, and total cell lysates were used for immunoblot analysis as described previously [6]. Antibodies used are described in the Supplementary material.

2.3. Reverse-phase protein array

Reverse-phase protein array (RPPA) was performed as described previously [20].

2.4. Animals

2.4.1. Intrabone assays

Cells were injected into the distal end of femurs of 6- to 8-wk-old male CB17.SCID mice, as described previously [3]. All femurs of injected mice were monitored by x-ray imaging. Quantification of the radiolucent
areas of the x-ray analyses were obtained as the ratio of radiolucent area to the total tissue area (whole bone) using BioQuant Osteo (BIOQUANT, Nashville, TN, USA).

2.4.2. Intracardiac injection

Cells were injected into the left ventricle of 6- to 8-wk-old male CB17. SCID mice. Bone reaction and bone metastasis development were monitored by x-ray and micro–computed tomography (μCT).

Additional details are described in the Supplementary material.

All practices involving laboratory animals were approved by the Institutional Animal Care and Use Committee of MD Anderson, Houston, TX, under the regulation of the Animal Welfare Committee (IACUC), and conform to the NIH Policy on Humane Care and Use of Laboratory Animals.

2.5. Bone histomorphometric analysis

Histomorphometric analyses were performed at the Bone Histomorphometry Core Laboratory, The Bone Disease Program of Texas, Houston, TX, USA, as described previously [3]. Bone parameters (bone mass, osteoblast, and osteoclast) were obtained using tartrate-resistant acid phosphatase stains and Harris-modified hematoxylin counterstain of decalcified bones.

2.6. Immunohistochemical analyses in human prostate and PCA specimens

We performed immunohistochemical (IHC) analyses of FGFR1 and LAD1 expression in PCA samples and of FGFR1 expression in normal prostate-derived tissue, obtained from the Prostate Tissue Bank, Department of Pathology, MD Anderson Cancer Center, Houston, TX, USA, under an Institutional Review Board–approved protocol. PCA tissue specimens were derived from the peripheral zone of nonmetastatic, untreated, primary PCAs (seven, Gleason score 8; 23, Gleason score 9 [ten, pT2 and 20, pT3]), and from CRPC bone metastases.

Bone metastasis specimens were decalcified, and formalin fixed and paraffin embedded; all samples were stained with an anti-FGFR1 antibody (Cat# ab76464; Abcam, Cambridge, UK) and an anti-LAD1 antibody (Cat# HPA028732; Sigma-Aldrich), as described elsewhere [6]. Slides were read independently by two investigators and classified according to staining intensity, with – being negative stain and +++ being the most intense staining. Positive/negative (+/–) expression refers to heterogeneous expression, that is, some areas positive and some areas negative.

2.7. Bioinformatics analysis

Human RNA-sequencing data from the following datasets were used: TCGA-PRAD, containing gene expression data from primary PCA samples [21], and SU2C-PCF that has mRNA expression (FPKM capture) for CRPC samples [22].

TCGA-PRAD was mined for expression of FGFR1 isoforms and their molecular correlates (476 samples, last access: December 2018) [23]. Search was performed using FGFR1 isoforms (α NM_023110.2; β NM_023105.2). FGFR1 score was defined as the ratio of FGFR1α to the sum of FGFR1α and FGFR1β [24]. Gene set enrichment analysis (GSEA) was used to determine FGFR1-associated pathways [25].

SU2C-PCF detailed analysis is provided in the Supplementary material.

2.8. Statistical analysis

Two experimental groups were compared with the two-tailed Student’s t test for unpaired data, unless otherwise indicated. Differences in FGFR1 isoform expression between metastatic tissue sites in SU2C were assessed by one-way analysis of variance followed by Dunnett’s multiple comparisons test.

3. Results

3.1. Different human PCa samples express different FGFR1 isoforms

FGFR1 has the highest expression among all FGFRs in human PCa samples [6]. Herein, we show that different FGFR1 isoforms are expressed in different PCA samples (Supplementary Table 1). We focused this study on the two best characterized FGFR1 isoforms, α (containing the α-exon; NM_023110.2; 822 aa) and β (lacking the α-exon; NM_023105.2; 733 aa) [27,28]. When mining TCGA-PRAD and SU2C [22], results confirmed that both isoforms are expressed at different levels in different PCA samples (Supplementary Fig. 1A and 1B).

3.2. FGFR1 isoforms are associated with different gene expression

The TCGA-PRAD analysis revealed that FGFR1α and FGFR1β are associated with the expression of different gene transcripts, with FGFR1β being associated with a larger number of genes than FGFR1α (Fig. 1A and 1B). Accordingly, GSEA indicated that FGFR1β is associated with a larger number of pathways than FGFR1α (Fig. 1C, and Supplementary Tables 2 and 3). When the stringency of prioritized pathways was increased, we identified only one pathway associated with FGFR1α (Fig. 1D), while 50 pathways were associated with FGFR1β (Fig. 1E).

Supporting our in silico finding that the MAPK cascade is significantly associated with the β isoform (Fig. 1E), we used PC3 (hormone insensitive) and C4-2B (hormone sensitive) cell lines stably expressing FGFR1 isoforms (PC3-FGFR1α, PC3-FGFR1β; C4-2B-FGFR1α, C4-2B-FGFR1β) or empty vector (V) and treated with FGF (Fig. 1F, and Supplementary Fig. 2A and 2B), and observed greater induction of P-MAPK in FGFR1β-expressing cells (Fig. 1G). This was confirmed by RPPA of C4-2B cells stably expressing FGFR1 isoforms (Fig. 1H).

3.3. FGFR1 alters the bone phenotype induced by PCa cells in tumor-bearing femurs

Next, we evaluated tumor growth and bone reaction induced by PC3-FGFR1α or PC3-FGFR1β after direct femur injection (Fig. 2A). After 4 wk, radiolucent areas (α; p = 0.009; β; p = 0.0001; Fig. 2B) and tumor volume (α; p = 0.0006; β; p = 0.002; Fig. 2C) were increased and bone volume was reduced (α; p = 0.01; β; p = 0.007; Fig. 2D) in femurs injected with PC3-FGFR1 isoform tumors compared with PC3-V–injected femurs, indicating that FGFR1 expres-
sion in PCa cells induces tumor growth and bone resorption. Bone mass reduction in PC3-FGFR1 isoform tumor-bearing femurs was confirmed by histomorphometry ($\alpha$, $p = 0.0006$; $\beta$, $p = 0.004$; Fig. 2E); with a significant increase in osteoclast parameters only in PC3-FGFR1$\beta$-injected femurs (Fig. 2E).

We next evaluated the effect of FGFR1 isoform expression in C4-2B cells (Fig. 3A and 3B). No difference in tumor
volume was observed between C4-2B–FGFR1 isoforms and C4-2B–V groups (Fig. 3C). Bone volume decreased only in femurs injected with C4-2B–FGFR1β compared with controls (p = 0.02; Fig. 3D), with a concomitant increase in osteoclast parameters (p = 0.036; Fig. 3E).

These results suggest that FGFR1β-expressing PCa cells are more suited to activate osteoclasts.

### 3.4. FGFR1 significantly increases PCa bone metastases in vivo

In a survival study, mice injected intracardially with either PC3-FGFR1α or PC3–FGFR1β had reduced survival compared with controls (p < 0.0001 or p = 0.032, respectively; Fig. 4A).

Bone metastases after intracardiac injection of these sublines were monitored by x-ray and confirmed by histology when the study concluded (Fig. 4B–D). An increased number of mice injected with PC3-FGFR1α and PC3-FGFR1β developed bone metastases, compared with controls (p = 0.00005 and p = 0.02, respectively; Fig. 4C [bottom]). These results suggest that in androgen receptor (AR)-negative cells (PC3), FGFR1 mediates PCa progression.

FGFR1 isoform expression in C4-2B cells (AR expressing) did not increase bone metastases (Fig. 4E–F), indicating different FGFR1 prometastatic effects in different cell lines.

Taken together, these results suggest that both the isoforms and the genetic background of cells modulate the FGFR1 effect in PCa.

### 3.5. FGFR1 expression is significantly increased in human PCa metastases and negatively correlated with AR

FGFR1 enrichment in human CRPC bone metastases (11/26) versus untreated, nonmetastatic primary PCa (two out of 29) was detected by IHC analyses (p = 0.0007; Fig. 5, Supplementary Table 4, and Supplementary Fig. 2C), supporting that FGFR1 induces the PCa metastatic cascade. We found an increase in FGFR1 transcripts after androgen deprivation therapy (ADT) when assessing locally advanced/metastatic PCa patient–paired samples (GSE51005–GSE48403, p < 0.001; Supplementary Fig. 3A). Of note, FGFR1 mRNA and AR score were inversely associated (r = –0.42, p < 0.0001; Supplementary Fig. 3B) in SU2C [22]. Accordingly, we ascertained a negative correlation between FGFR1 and AR (r = –0.38, p < 0.0001; Supplementary Fig. 3C) in 39 CRPC MDA PCa patient-derived xenografts (PDxS) [19,29]. Strikingly, in SU2C, FGFR1β transcript expression was increased in bone metastasis compared with liver and lymph node (p = 0.006 and p = 0.0007, respectively; Supplementary Fig. 1B). These results suggest that cells expressing FGFR1β have more affinity for bone or that, once these cells are in the bone, they upregulate the expression of this isoform.

### 3.6. FGFR1 induces LAD1, an anchoring filament protein, in PCa cells

To understand the mechanism of FGFR1-induced metastases, we performed RPPA of PC3–FGFR1 cells (Fig. 6A) and found that FGFR1 modulates genes associated with cellular movement (Fig. 6B), prioritizing those genes implicated in cancer progression and/or PCa (LAD1, CDH1, and GLS) [30–34]. We confirmed LAD1 upregulation in PC3–FGFR1 cells (Fig. 6C). LAD1 was enriched in human CRPC bone metastases compared with treatment-naïve, nonmetastatic primary tumors (p = 0.005; Fig. 6D and Supplementary Table 4), supporting that LAD1 mediates, at least in part, PCa metastasis.

### 3.7. LAD1 silencing in FGFR1β-overexpressing tumors reduces bone metastases

We assessed bone metastases in mice intracardially injected with PC3 cells overexpressing FGFR1β and with silenced
Fig. 2 – FGFR1 alters the bone phenotype induced by PC3 cells in tumor-bearing femurs. (A) Schematic representation of femur injection of PC3-FGFR1α, PC3-FGFR1β, or control empty vector (V) cells and monitoring. Western blot analysis and immunocytochemistry results of FGFR1 expression in cells used in these studies. GAPDH was used as a loading control in the Western blot analysis. Injected mice (n = 6 per group) were monitored by x-ray and MRI. (B) Representative radiographs (left panel) and quantification of radiolucent areas (right panel) of the x-ray analysis of PC3-FGFR1α, PC3-FGFR1β, and V tumor-bearing femurs at 4 wk after injection. Student’s t test; error bars indicate SD. (C) Representative sagittal MR images of femurs acquired with a 4.7-T scanner using a T2-weighted fast spin (T2-FS) echo sequence with fat suppression (upper panel). Arrows indicate tumor, which appears as an area of increased signal on T2-weighted images. Tumor volume of PC3-FGFR1α, PC3-FGFR1β, or V tumor-bearing femurs was assessed by MRI analysis (lower panel). Student’s t test; error bars indicate SD. (D) Representative two-dimensional slices of specimens analyzed by high-resolution μCT analysis at the end of study (left panel). Bone volume results assessed by μCT analysis (right panel). Student’s t test; error bars indicate SD. (E) Representative photomicrographs of decalcified tumor-bearing femur sections stained with HE (left panel) and tartrate-resistant acid phosphatase (TRAP; middle panel; 20× magnification, left; 40× magnification, right). Bone histomorphometry analyses indicated a reduced ratio of bone volume to tissue volume (BV/TV) in PC3-FGFR1 tumor-bearing mice (upper right panel). Bone histomorphometry analyses of TRAP-stained sections indicate an increase in osteoclast (OC) surface/bone surface in PC3-FGFR1 tumor-bearing mice (lower right panel). Scale bar, 100 μm (20×) or 50 μm (40×). Student’s t test; error bars indicate SD. FGFR = fibroblast growth factor receptor; HE = hematoxylin and eosin; μCT = micro–computed tomography; MRI = magnetic resonance imaging; OC/BS = osteoclast surface/bone surface; SD = standard deviation.
LAD1 or controls (Supplementary Fig. 4A and 4B). As expected, the number of mice with bone metastases was higher among those injected with PC3-FGFR1b than among controls (Supplementary Fig. 4C, left). Further, a higher number of bone metastases per mouse was detected in the same group ($p = 0.03$; Supplementary Fig. 4C, right), which decreased with LAD1 silencing (PC3-FGFR1b shLAD1 vs PC3-FGFR1b Scr, $p = 0.048$; Supplementary Fig. 4D).

Furthermore, survival curves were associated negatively with LAD1 levels in the injected cells ($p = 0.03$; Supplementary Fig. 4E, left). We confirmed the contribution of LAD1 expression in mice survival by comparing this parameter in PC3-FGFR1b Scr (highest LAD1 levels by Western blot [WB]; 6.51)-injected mice with the combined survival data from the other three groups (PC3-V shLAD1, PC3-FGFR1b shLAD1, PC3-V Scr; LAD1 levels by WB: 0.06-1; $p = 0.041$; Supplementary Fig. 4E, right).

All these results support that FGFR1 reduces mice survival and increases bone metastasis, and suggest that LAD1 is one of the players in the FGFR1-induced PCA metastatic process.
A. Kaplan-Meier survival curves of mice injected intracardially with PC3 sublines

B. Intracardiac injection of cells and monitoring

C. Representative X-ray images of mice injected intracardially with PC3 sublines

D. Representative radiolucent areas at the X-ray with corresponding microscopic analysis demonstrating tumor

E. Cells injected intracardially and monitoring

F. Representative MR images of bioluminescence area in E. with corresponding microscopic analysis demonstrating tumor

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Please cite this article as: E. Labanca, J. Yang, Peter D.A. Shepherd et al., Fibroblast Growth Factor Receptor 1 Drives the Metastatic Progression of Prostate Cancer, Eur Urol Oncol (2021), https://doi.org/10.1016/j.euo.2021.10.001
4. Discussion

Our comprehensive study of the biological role of two FGFR1 isoforms (α/β) and their associated signaling pathways in PCa underlies the importance of defining FGFR1 mediators of PCa progression and markers of FGFR1 signaling. This knowledge will help develop/optimize effective strategies for targeting FGFR1 as PCa therapy.

In addition to reporting that FGFR1 isoforms are associated with different genes and pathways in PCa, we show,
for the first time, that FGFR1 expression in PCa cells enhances their metastatic behavior. These findings are in alignment with our discovery that FGFR1 expression is increased in CRPC bone metastases compared with primary, untreated disease.

Alterations in FGFR-downstream pathways (PI3K/Akt and Src) have been implicated in CRPC progression [35,36]. Furthermore, FGFR1 overcomes AR-dependent inhibition of cell proliferation in epithelial cells derived from differentiated Dunning R3327 adenocarcinomas [37].

Primary hormone-naïve/CRPC matched pairs showed FGFR1 transcript upregulation in CRPC [38]. This was confirmed (1) in vitro, where high FGFR1 expression was detected in bicalutamide-resistant LNCaP established by long- and short-term treatment; and (2) in clinical samples, by IHC analyses in hormone-naïve/CRPC matched samples, and associated with shorter time to relapse and reduced survival in CRPC [38].

A more recent report demonstrated that FGF signaling is capable of bypassing PCa AR dependence, in particular, in a subpopulation of patients following ADT and/or second-generation androgen blockade, characterized as “double-negative” due to the absence of both AR and neuroendocrine markers [8]. Notably, upregulation of the FGFR pathway occurred after AR ablation in PCa cells and PDX.

Our and others’ findings [7,8,38] support the concept that, under selective pressure, FGFR1 pathway activation occurs later in the progression of the disease, mediating...
therapy resistance. Therefore, the time frame of initiation of FGFR1 blockade therapy is of upmost relevance as a secondary prevention strategy.

Another important contribution of our studies was the identification of LAD1 as a downstream target of FGFR1. LAD1, a relatively uncharacterized protein, was implicated in mammary cancer cell motility [30]. Increased LAD1 predicted poor prognosis in patients with high-grade breast tumors [30], and was suggested as a new therapeutic target for triple-negative breast and ovarian cancers [31,32].

5. Conclusions

Our studies highlight the complexity of the FGFR pathway, and report for the first time the prometastatic effect of FGFR1 in AR-negative PCa cells and the enrichment of FGFR1 expression in CRPC bone metastases. Importantly, we identified LAD1 as a putative mediator of PCa metastases and/or marker of signaling activation (Fig. 6E). These findings are essential for the effective development of FGFR blockade as a therapy for advanced PCa.

The prometastatic effects of fibroblast growth factor receptor 1 (FGFR1) in prostate cancer emphasize the need to develop FGFR blockade as therapy for prostate cancer. FGFR1 isoforms and associated pathways reported in this study are essential to define the mediators of pathway activation and therapy resistance.

Author contributions: Nora M. Navone had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Labanca, Yang, Navone.

Acquisition of data: Labanca, Yang, Wang, Shepherd, Dong, Bizzotto, Anselmino, Chinnaiyan, Broom.

Analysis and interpretation of data: Labanca, Starbuck, Guerra, Bizzotto, Anselmino, Ravoori, Kundra, Gueron, Troncoso, Navone.

Drafting of the manuscript: Labanca, Gueron, Navone.

Critical revision of the manuscript for important intellectual content: Corn, Logothethis.

Statistical analysis: Labanca, Broom, Navone.

Obtaining funding: Navone.

Administrative, technical, or material support: Navone.

Supervision: Navone.

Other: None.

Financial disclosures: Nora M. Navone certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (e.g., employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

Funding/Sponsor and role of the sponsor: This work was supported in part by the Prostate Cancer Foundation, generous philanthropic contributions to The University of Texas MD Anderson Moon Shot Program, National Center Institute (NCI) Cancer Center Support Grant (P30CA16672), Cancer Center Prostate Cancer SPORE (National Institutes of Health [NIH]/NCI P50 CA140388-08), DOD-PCRP (W81XWH-14-1-0554), Janssen Pharmaceutical Companies of Johnson & Johnson, and the David H. Koch Center for Applied Research in Genitourinary Cancers at MD Anderson, Houston, TX. Bradley M. Broom was supported in part by NIH/NCI grants SU24CA199461, 5P30CA016672, and 2P50CA140388-06A1.

Data sharing: Materials are available through a material transfer agreement, and data are freely available.

Acknowledgments: We thank Charles V. Kingsley for technical assistance in the i.c. injection, the Small Animal Imaging Facility personnel at MDACC for technical support, Brian C. Dawson for CT analysis, Sarah E. Townsend for editing the manuscript, Elba S. Vazquez for helpful advice and criticism, Jordan T. Pietz for scientific illustrations, and the Rolanette and Berdon Lawrence Bone Disease Program of Texas.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.euo.2021.10.001.

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