Bone Metastasis of Prostate Cancer Can Be Therapeutically Targeted at the TBX2–WNT Signaling Axis


Identification of factors that mediate visceral and bone metastatic spread and subsequent bone remodeling events is highly relevant to successful therapeutic intervention in advanced human prostate cancer. TBX2, a T-box family transcription factor that negatively regulates cell-cycle inhibitor p21, plays critical roles during embryonic development, and recent studies have highlighted its role in cancer. Here, we report that TBX2 is overexpressed in human prostate cancer specimens and bone metastases from xenograft mouse models of human prostate cancer. Blocking endogenous TBX2 expression in PC3 and ARCaPM prostate cancer cell models using a dominant-negative construct resulted in decreased tumor cell proliferation, colony formation, and invasion in vitro. Blocking endogenous TBX2 in human prostate cancer mouse xenografts decreased invasion and abrogation of bone and soft tissue metastasis. Furthermore, blocking endogenous TBX2 in prostate cancer cells dramatically reduced bone-colonizing capability through reduced tumor cell growth and bone remodeling in an intratibial mouse model. TBX2 acted in trans by promoting transcription of the canonical WNT (WNT3A) promoter. Genetically rescuing WNT3A levels in prostate cancer cells with endogenously blocked TBX2 partially restored the TBX2-induced prostate cancer metastatic capability in mice. Conversely, WNT3A-neutralizing antibodies or WNT antagonist SFRP-2 blocked TBX2-induced invasion. Our findings highlight TBX2 as a novel therapeutic target upstream of WNT3A, where WNT3A antagonists could be novel agents for the treatment of metastasis and for skeletal complications in prostate cancer patients.

Abstract

Introduction

Prostate cancer is the second leading cause of cancer-related death in men in the United States. Most patients who suffer from prostate cancer do not die from the tumor at the primary site but rather due to complications arising from the spread of the tumor to bone and other visceral organs. Evidence indicates that only 25% of patients with metastatic and invasive prostate cancer live 5 years subsequent to the initial diagnosis of metastasis. Therefore, early detection and treatment before the tumors colonize the bone, and visceral organs are critical for reducing the mortality and morbidity of prostate cancer patients.

The TBX genes belong to the T-box family of transcription factors reported to play critical roles during embryonic development (1–3). Several reports have implicated TBX2 in the cell cycle and cancer; TBX2 has been shown to transcriptionally repress p14 arf/p19 arf (4, 5) and p21 expression (6) and to be associated with retinoblastoma (7). In prostate cancer, the T-box gene TBX2 has been associated with advanced stage adenocarcinomas and 31% of metastases (8).

The Wnt signaling pathway mediates a myriad of biological processes, including cell proliferation and survival. Canonical Wnt signaling results in the stabilization and translocation of β-catenin to the nucleus and concomitant activation of downstream targets associated with an invasive phenotype. Several reports have documented the crucial roles played by canonical Wnt signaling in prostate cancer tumorigenesis and metastasis.

In this report, we analyzed the specific role played by TBX2 in human prostate cancer. By a combination of in vitro assays and in vivo xenograft experimental approaches, we focused our investigation on the biology of TBX2 in prostate cancer progression, especially local invasion to lymph nodes and metastasis to bone, and the subsequent bone remodeling events that follow...
colonization and growth of prostate cancer cells in the bone microenvironment. We found that blocking endogenous TBX2 reduces prostate cancer cell proliferation and invasion in vivo, abrogates prostate cancer metastasis to the bone, and reduces the ability of prostate cancer cells to grow in the bone microenvironment in vivo. Furthermore, we found that WNT3A signaling is an essential mediator for the effects of TBX2 in prostate cancer metastasis, raising the possibility of targeting WNT3A by employing WNT3A-neutralizing antibodies or WNT antagonist secreted frizzled receptor-2 (SFRP-2) for the management of men with locally invasive and metastatic prostate cancer.

Materials and Methods

Tissue specimens

Thirty-five formalin-fixed, paraffin-embedded tissues from patients with prostatic disease, including 9 with benign prostatic hyperplasia, 9 with grade 3, 10 with grade 4, including 7 with Gleason score 8, and 7 with bone metastasis, were used to determine the protein expression of TBX2 by IHC. Usage of clinical specimens was approved by the Institutional Research Board (IRB Protocol 00021228).

Cells and reagents

Human prostate cancer PC3 and RWPE1 cell lines were obtained from ATCC. Human prostate cancer LNCaP, C4-2, C4-2B (9), ARCaP, and ARCaPM cells (10, 11) were established or obtained from ATCC. Human prostate cancer LNCaP, C4-2, C4-2B (9), ARCaP, and ARCaPM cells (10, 11) were established in our laboratory between 1994 and 1996. These cell lines have been authenticated and can be obtained through characterized cell core at the University of Texas MD Anderson Cancer Center (Houston, TX) or through Novicure Biotechnology. Luciferase-tagged parental PC3 and ARCaP cells that were stably transduced with either the control Neo or p-BABE-puro-TBX2 dominant-negative (DN) retroviral vectors were used for this study. These cells were maintained in T-medium (Invitrogen) supplemented with 5% FBS. LN Cancer cells were maintained in RPMI medium supplemented with 10% FBS and were stably transduced with either the control Neo or LZRS-TBX2-ires GFP vectors. p-BABE-puro-TBX2 DN construct was provided by Colin Goding (Nufield Department of Medicine, University of Oxford, Oxford, United Kingdom), and TBX2 overexpression construct (LZRS-TBX2-iresGFP) was provided by Maarten van Lohuizen (NKI, Amsterdam, the Netherlands). Active WNT3A vector (43810) containing a neomycin-resistant gene was acquired through a Material Transfer Agreement from Addgene.

Animal studies

All animal procedures were performed according to an approved protocol from the Institutional Animal Care and Use Committee. Male 4- to 6-week-old athymic nude mice, SCID-beige purchased from Charles River Laboratories were housed at Cedars-Sinai Medical Center (Los Angeles, CA) and fed a normal chow diet. Cells were inoculated intracardially or intratibially, or grafted beneath the kidney capsule (subrenal capsular model) or orthotopically. For metastatic studies, 1 × 10^6 ARCaP or 2 × 10^5 PC3 cells tagged with a firefly luciferase reporter construct (Promega) were injected into the left cardiac ventricle of anesthetized mice as described previously (11). Anesthetized mice were injected intraperitoneally with 30 mg of d-luciferin per mouse (Gold Biotechnology), and development of metastases was monitored by bioluminescence imaging (BLI) on a weekly basis. Bioluminescence images were acquired using Xenogen IVIS Spectrum Imaging System (PerkinElmer), and analysis was performed with Living Image software (PerkinElmer) by the measurement of photon flux in whole mice bodies. For intratibial studies, 1 × 10^5 PC3 cells were injected in the tibia of mice, and the tibia were harvested after 5 weeks. For orthotopic grafts, 1 × 10^6 PC3 cells were engrafted in the anterior lobe of the mouse prostate and harvested after 10 weeks of implantation. For the subrenal capsule xenograft model, 3 × 10^6 cells were implanted beneath the kidney capsule of mice and harvested after 8 weeks.

Microcomputed tomography analysis

To determine the 3-dimensional architecture of the bone following tumor cell inoculation, mice were euthanized, and the tibia were harvested and fixed overnight in 10% neutral-buffered formalin for 24 hours, followed by 70% ethanol. Bone samples were scanned using a microcomputed tomography (μCT) system (Scanco μCT 40). The scan was composed of 129 slices, with a threshold value of 263. A 3-dimensional reconstruction of the image was performed with the region of interest consisting of trabecular bone area. Regions of interest were drawn on each of the 100 slices in such a way that only the trabecular bone and the marrow were included.

IHC

Immunohistochemical analysis of tumor xenograft samples followed a previously published protocol (12) using primary antibodies against TBX2, WNT3A, matrix metalloproteinase 9 (MMP9), and FOXA1. Briefly, formalin-fixed, paraffin-embedded sections (4 μm) were deparaffinized, rehydrated, and subjected to antigen retrieval. Following incubation with Dual Endogenous Enzyme Block solution (Dako) for 10 minutes, the section was treated with primary antibody of varying dilutions using Antibody Diluent solution (Dako) at 4°C overnight. The section was then washed 3 times with PBS (PBS containing 0.2% Tween-20) for 5 minutes per wash. The section was then treated with EnVision + Dual Link System-HRP (Dako) for 30 minutes to detect specific staining. The sections were then washed 3 times for 5 minutes each and developed with 3'3''-diaminobenzidine (Dako). Image acquisition was performed using a Nikon camera and software (magnification, ×500). Immunohistochemical staining intensity was scored using the combined intensity and percentage of positive-scoring cells as reported previously (13). Strong intensity was scored as 3, intermediate as 2, weak as 1, and negative as 0. Each intensity score was then summed with the percentage of cells that were stained, with >50% of the cells as 2, <50% of the cells as 1, and none as 0.

In vitro cell proliferation, colony formation, and invasion assays

For cell proliferation assays, cells were seeded on 24-well plates. Cell numbers from triplicate wells were counted. For colony formation assays, 200 viable cells were seeded in 6-well plates and cultured for 10 to 14 days. The cell colonies were stained with crystal violet and then counted. To determine the invasive ability of prostate cancer cells, the upper sides of the transwell polycarbonate membrane filters, with 8-mm pore size (Corning Inc.), were coated with diluted Matrigel (BD Biosciences). A total of 50,000 cells were seeded in the upper chamber with serum-free media, and the bottom chamber of the
apparatus contained media with 10% FBS. Cells were incubated for 48 hours at 37°C. Following incubation, the cells that had invaded and attached to the lower surface of the membrane were fixed with 100% methanol and stained with 0.5% crystal violet. All experiments were repeated 3 times with cells grown at 37°C with 5% CO₂. Cell numbers were counted and quantified in five randomly chosen macroscopic fields per membrane using an inverted microscope. WNT expression was antagonized through adenoviral expression of SFRP-2 at 10⁵ virus particles/ml for 24 hrs prior to replacing the standard culture medium. WNT3A (cat. nr. MAB1324) and IL6 (cat. nr. MAB-206) neutralizing antibodies were obtained from R&D Systems.

Biochemical analyses

Total RNA from cells was isolated using an RNasy Mini kit (Qiagen) and reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Details of primers and methods used for qPCR are provided in Supplementary Materials and Methods. For immunobLOTS, proteins (30 μg) were resolved on a 4% to 12% Bis-Tris gradient SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane. The primary antibodies were TBX2, p21, WNT3A, and HA. Chromatin immunoprecipitation (ChIP) assay in PC3 cells to determine the binding of TBX2 on WNT3A promoter was performed using ChIP Assay Kit (Upstate Biotechnology) following the manufacturer’s protocol. ChIP DNA was analyzed by PCR analysis using primers encompassing the regions of interest on WNT3A promoter. ChIP in LNCaP cells was performed using the Zymo-Spin Kkit (Zymo Research) following the manufacturer’s protocol. Details of primers used for ChIP are provided in Supplementary Materials and Methods.

Statistical analysis

GraphPad Prism 6 was used for graphs and statistics. Data were expressed as mean ± SE. All data were analyzed using the Student t test for comparison of two groups or one-way ANOVA for three groups or more. Differences were considered statistically significant when the P value was <0.05.

Results

TBX2 is overexpressed in human prostate cancer cell lines, tumor xenografts, and clinical specimens, and its expression correlates with the potential to metastasize to bone and soft tissues

We evaluated TBX2 expression in human prostate cancer cell lines, prostate cancer tumor xenografts, and prostate cancer clinical specimens with the goals of assessing the relationship between TBX2 expression and the ability of prostate cancer cells to home to bone and soft tissues. In addition, we examined the potential correlation of TBX2 expression in pathologic prostate cancer specimens collected from patients with various stages of the disease. Three bone metastatic human prostate cancer cell lines and tumor xenografts with lineage relationships, LNCaP-C4-2-B, ARCaP₁–ARCaP₂, and PC3-PC3M (Supplementary Fig. S1). We found that TBX2 expression was higher in human prostate cancer clinical specimens and increased with disease progression (Fig. 1B and C). In addition, we evaluated TBX2 expression in a number of human prostate cancer microarray datasets using Oncomine 4.4. Using Cancer Outlier Profile Analysis, a methodology that has been validated for the identification of candidate oncoprogneses, such as ERG (17), we found that TBX2 is significantly overexpressed in a subset of tumor samples in 11 of 23 available datasets (gene rank, top 20%; fold change > 2; P < 1 × 10⁻³; Supplementary Table S1). Our results are consistent with a previous report in which TBX2 was found to be highly overexpressed in osteoblastic bone metastases in patients with castration-resistant prostate cancer (CRPC; ref. 18). Taken together, these results show that TBX2 has a preferential expression in prostate cancer bone metastases and that TBX2 expression increases with human prostate cancer progression.

Identification of WNT3A and MMP9 as downstream effectors of the TBX2 signaling axis

To understand the biological role of TBX2 in prostate cancer growth and metastasis, we took a genetic approach by blocking endogenous TBX2 expression in prostate cancer cells using a DN construct (TBX2 DN) that comprises of a deletion mutant that includes amino acids 1-301. This TBX2 DN construct contains the T-box DNA-binding domain, but lacks the carboxy-terminal residues necessary for transcriptional repression. A previous study assessing the functional role of TBX2 in melanoma successfully used the TBX2 DN construct and reported that TBX2 DN works in concurrence with TBX2 siRNA and upregulates p21, a known TBX2 downstream signal (6). Furthermore, deletion mutants designed in this manner have been previously demonstrated to act as DN for several other genes of the T-box family in a variety of tissues and organisms (19, 20). TBX2 DN expression in prostate cancer cells decreased the ability of PC3 and ARCaP₂ cells to proliferate and to form soft agar colonies (Fig. 2A and B). Cells transduced with TBX2 DN construct had greater expression of p21 (Fig. 2D), in agreement with the function of this construct in human melanoma cells (6). We screened the expression of both canonical and noncanonical WNTs in prostate cancer cells expressing TBX2 DN. Results showed that the TBX2 DN construct decreased a canonical WNT, WNT3A, in both PC3 and ARCaP₂ cells (Fig. 2C). Furthermore, the expression of MMP9, a known target downstream from WNT3A signaling, was decreased in prostate cancer cells expressing TBX2 DN construct (Fig. 2C). In addition, utilizing the converse approach, we found that LNCaP human prostate cancer cells transduced with TBX2 overexpression construct (LNCaP-TBX2) displayed increased WNT3A and MMP9 levels compared with control LNCaPneo cells (Supplementary Fig. S2A). These results taken together suggest that the TBX2 downstream signaling axis involves WNT3A, MMP9, and p21 gene expression in prostate cancer cells.

TBX2 contributes to local tumor invasion and distant metastasis

The functional effects of the TBX2 DN construct on the invasion and metastasis of PC3 and ARCaP₂ cells in vitro and in vivo were
TBX2 is overexpressed in human prostate cancer cell lines, tumor xenografts, and clinical specimens, and its expression correlates with the potential to metastasize to bone and soft tissues. 

**A,** Immunohistochemical staining showing correlation of TBX2 with ARCaP<sub>e</sub> – ARCaPM, LNCaP – C4-2, and LNCaP<sup>Neo</sup> – LNCaP<sup>DNKEL</sup> bone metastasis (Bone Met) models. Prim Tum, primary tumor. 

**B and C,** Increased TBX2 expression in high Gleason grade prostate cancer compared with low Gleason grade prostate cancer and benign prostatic hyperplasia (BPH). Original magnification, ×250.

**D,** Extracts from prostate cancer cell lines RWPE1, LNCaP, C4-2B ARCaP<sub>e</sub>, ARCaP<sub>Neo</sub>, PC3, and PC3-M were analyzed for the expression of TBX2 by qPCR (mean ± SEM, n = 3). 

**E,** Extracts from prostate cancer cell lines RWPE1, LNCaP, C4-2B ARCaP<sub>e</sub>, ARCaP<sub>Neo</sub>, PC3, and PC3-M were analyzed for the expression of TBX2 and WNT3A by immunoblotting.

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**Figure 1.**

TBX2 is overexpressed in human prostate cancer cell lines, tumor xenografts, and clinical specimens, and its expression correlates with the potential to metastasize to bone and soft tissues. A, Immunohistochemical staining showing correlation of TBX2 with ARCaP<sub>e</sub> – ARCaPM, LNCaP – C4-2, and LNCaP<sup>Neo</sup> – LNCaP<sup>DNKEL</sup> bone metastasis (Bone Met) models. Prim Tum, primary tumor. B and C, Increased TBX2 expression in high Gleason grade prostate cancer compared with low Gleason grade prostate cancer and benign prostatic hyperplasia (BPH). Original magnification, ×250. 

****, P < 0.0001. Further analysis showed that TBX2 in benign prostatic hyperplasia and Gleason score 6 (3+3) is lower than in Gleason 7 (3+4 or 4+3) and Gleason 8 (4+4), P < 0.05. Bone metastasis was positive in 5 of 7 specimens. Original magnification, ×500. D, Extracts from prostate cancer cell lines RWPE1, LNCaP, C4-2B ARCaP<sub>e</sub>, ARCaP<sub>Neo</sub>, PC3, and PC3-M were analyzed for the expression of TBX2 by qPCR (mean ± SEM, n = 3). E, Extracts from prostate cancer cell lines RWPE1, LNCaP, C4-2B ARCaP<sub>e</sub>, ARCaP<sub>Neo</sub>, PC3, and PC3-M were analyzed for the expression of TBX2 and WNT3A by immunoblotting.
Figure 2.
Identification of WNT3A and MMP9 as downstream effectors of the TBX2 signaling axis. 

A, Growth curves of PC3 and ARCaPm cells (Neo and TBX2 DN; mean ± SEM, n = 3). 

B, Colony formation of PC3 and ARCaPm cells (Neo and TBX2 DN; mean ± SEM, n = 3). 

C, qPCR analysis of WNT3A and MMP9 in PC3 and ARCaPm cells (Neo and TBX2 DN; mean ± SEM, n = 3). 

D, Immunoblots of PC3 and ARCaPm cells (Neo and TBX2 DN) for p21 and HA. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.
investigated. TBX2 DN stable infection dramatically reduced the invasive behavior of PC3 and ARCaP cells. Modified Boyden chamber assays showed dramatically reduced invasive capabilities of these cells when compared with respective cells stably transduced with the control vector (Fig. 3A; Supplementary Fig. S3). These results were corroborated by the in vivo behaviors of subrenal implanted PC3 cells transduced with TBX2 DN construct (PC3^{TBX2 DN}). To differentiate the tumor xenograft from

![Figure 3](image-url)

**Figure 3.** Blocking endogenous TBX2 in prostate cancer cells reduces local tumor invasion and metastasis. A, PC3 cells (Neo and TBX2 DN) were assessed for their ability to invade. Data normalized to control and represent the mean ± SEM (n = 3). B, H&E and immunohistochemical analysis of FOXA1 in subrenal capsule xenografts of PC3 cells (Neo and TBX2 DN). Arrows, tumor–kidney interface. Representative images from six different samples are shown. Original magnification, ×250. C, Immunohistochemical analysis of WNT3A and MMP9 in subrenal capsule xenografts of PC3 cells (Neo and TBX2 DN). Representative images from six different samples are shown. Original magnification, ×250. D, Top, immunohistochemical analysis of FOXA1 in primary orthotopic xenograft tumors of PC3 cells (Neo and TBX2 DN). Arrows, tumor xenograft. Bottom, immunohistochemical analysis of FOXA1 in lymph nodes obtained from mice orthotopically xenografted with PC3 cells (Neo and TBX2 DN). Representative images from six different samples are shown. Original magnification, ×250. ***P ≤ 0.001. Prim Tum, primary tumor; Lym node met, Lymph node metastasis.
the kidney tissue, we stained the tumor xenograft sections with FOXA1, as PC3 tumors stain positive for FOXA1 while there is no detectable staining in the kidney tissue. We observed that the TBX2 DN construct dramatically reduced the invasive front of the tumor as seen in the boundaries clearly demarcated in tumor and kidney (Fig. 3B, highlighted by arrows and a dotted line). Interestingly, although tumor invasiveness was greatly decreased, the TBX2 DN construct did not affect the growth and the size of PC3 tumors in mice (data not shown). As expected, PC3TBX2 DN tumors lost the expression of WNT3A and MMP9, two of the downstream target genes of TBX2. Although strong WNT3A and MMP9 staining was evident in control PC3Neo tumors, PC3TBX2 DN tumors demonstrated a significant loss of WNT3A and MMP9 (Fig. 3C). To further examine the functional significance of TBX2 in prostate cancer invasion in the prostate microenvironment, we compared local invasion and lymph node metastases in an orthotopic xenograft model where mouse prostate glands were inoculated with either TBX2 DN or control-transduced PC3 cells. Orthotopically implanted PC3 cells have been observed to invade locally and metastasize readily to adjacent lymph nodes (21–23). Consistent with data from the kidney capsule tumor xenograft study, orthotopically implanted PC3TBX2 DN cells exhibited drastically reduced local invasion with no evidence of lymph node metastases (Fig. 3D highlighted by arrows), whereas control PC3Neo tumors showed local invasion and regional lymph node metastasis. Taken together, these results further support the finding that TBX2 plays a critical role in prostate cancer local invasion and distant metastases to lymph nodes in mouse models.

**DN TBX2 expression in prostate cancer cells abrogates cancer bone metastasis**

As bone is the predominant metastatic site in human prostate cancer, we next investigated whether TBX2 expression had a role in the manifestation of prostate cancer bone metastasis. ARCaPm and PC3 cells known for their ability to metastasize spontaneously to mouse skeleton were used as models to evaluate the functional role of TBX2 in prostate cancer cells. Intracardiac inoculation of control vector–transduced ARCaPm cells (ARCaPmNeo) resulted in a bone metastatic frequency of 80% during an 8-week observation period (Fig. 4A), whereas control PC3Neo cells resulted in a bone metastasis frequency of 80% during an 8-week observation period (Fig. 4A), whereas control PC3Neo tumors demonstrated a significant loss of WNT3A and MMP9 (Fig. 3C). To further examine the functional significance of TBX2 in prostate cancer invasion in the prostate microenvironment, we compared local invasion and lymph node metastases in an orthotopic xenograft model where mouse prostate glands were inoculated with either TBX2 DN or control-transduced PC3 cells. Orthotopically implanted PC3 cells have been observed to invade locally and metastasize readily to adjacent lymph nodes (21–23). Consistent with data from the kidney capsule tumor xenograft study, orthotopically implanted PC3TBX2 DN cells exhibited drastically reduced local invasion with no evidence of lymph node metastases (Fig. 3D highlighted by arrows), whereas control PC3Neo tumors showed local invasion and regional lymph node metastasis. Taken together, these results further support the finding that TBX2 plays a critical role in prostate cancer local invasion and distant metastases to lymph nodes in mouse models.

**In vitro**

To further tease out the in vivo biological consequences of WNT3A rescue in the context of blocking endogenous TBX2, we injected all three types of cells, PC3Neo, PC3TBX2 DN, and PC3TBX2 DN + WNT3A, by intracardiac inoculation in immunodeficient mice. BLI revealed that mice injected with PC3TBX2 DN + WNT3A cells had partially restored bone and soft tissue metastatic potential (Fig. 6C), when compared with mice injected with PC3Neo cells, the growth and size of trabecular bone at the site of tumor cell inoculation (Fig. 5).

In contrast, PC3Neo cells gave rise to mild osteolysis as seen by X-ray and confirmed by μCT analysis (Fig. 5). Furthermore, significantly smaller tumors were observed in mouse skeletal inoculated intrasosseously with PC3Neo cells when compared with PC3Neo tumors (Fig. 5). We performed a similar experiment with ARCaPm cells, which form a mixture of osteolytic and osteoblastic lesions. Consistent with the PC3 tumors in mouse skeleton, when compared with ARCaPmNeo cells, ARCaPmTBX2 DN cells also formed smaller tumors with significantly reduced potential to remodel the bone microenvironment, manifested by reduced osteolysis and osteoblastic lesions (Supplementary Fig. S4).

**WNT3A, a downstream target gene of TBX2, mediates prostate cancer bone metastasis**

As we observed downregulated WNT3A in prostate cancer cells transduced with TBX2 DN, we determined the possible functional role of WNT3A in mediating prostate cancer metastasis. We therefore generated stable WNT3A-expressing PC3 cells that had previously been transduced with TBX2 DN (PC3TBX2 DN + WNT3A cells). To examine the phenotypic behavior of these cells with respect to the behavior associated with WNT3A or its downstream effectors, we treated all three types of cells: PC3Neo, PC3TBX2 DN, and PC3TBX2 DN + WNT3A with (i) WNT3A-neutralizing antibody, (ii) SFRP-2, or (iii) IL6-neutralizing antibody, and examined the invasive behavior of the cells. In **vitro** Boyden chamber assays showed that (i) PC3Neo cells displayed significantly increased invasion compared with PC3TBX2 DN cells and (ii) although treatments with WNT3A-neutralizing antibody or SFRP-2 or IL6-neutralizing antibody drastically reduced the invasive behavior of PC3Neo and PC3TBX2 DN + WNT3A cells, the invasive behavior of PC3Neo and PC3TBX2 DN + WNT3A cells did not change significantly (Fig. 6A; Supplementary Fig. S5). Quantitative real-time RT-PCR confirmed that WNT3A expression as well as MMP9, MMP2, and IL6, genes known to be downstream of WNT3A were rescued in PC3Neo and PC3TBX2 DN + WNT3A compared with PC3Neo and PC3TBX2 DN cells (Fig. 6B).

To further tease out the in vivo biological consequences of WNT3A rescue in the context of blocking endogenous TBX2, we injected all three types of cells, PC3Neo, PC3TBX2 DN, and PC3TBX2 DN + WNT3A, by intracardiac inoculation in immunodeficient mice. BLI revealed that mice injected with PC3TBX2 DN + WNT3A cells had partially restored bone and soft tissue metastatic potential (Fig. 6C), when compared with mice injected with PC3Neo cells, which, as shown previously, did not show any evidence of metastasis.

We next investigated the molecular mechanisms by which TBX2 regulated WNT3A. We used ChIP assay to determine whether there is a differential recruitment of TBX2 to WNT3A promoter in PC3Neo and PC3TBX2 DN cells. The results showed that binding of TBX2 to WNT3A promoter was significantly diminished in PC3TBX2 DN cells when compared with control PC3Neo cells (Fig. 6D). These results collectively suggest that the diminished binding of TBX2 on WNT3A promoter that occurs in PC3TBX2 DN cells leads to decreased expression of WNT3A. To further validate this finding, we examined whether
Figure 4.  
DN TBX2 expression in prostate cancer cells abrogates cancer bone metastasis.  

A, Representative BLI (week 8) images of 5 of 10 mice in each group from mice intracardially injected with ARCaP\(_m\) cells (Neo and TBX2 DN).  

B, Representative BLI (week 5) images of 5 of 10 mice in each group from mice intracardially injected with PC3 cells (Neo and TBX2 DN).  

C, Normalized BLI curves of metastasis development for each experimental group from B.  

Data, mean ± SEM (n = 10).  

D, H&E analysis of bone and lymph node metastases from B (mice injected with PC3-Neo cells).
TBX2 activation led to the recruitment of RNA polymerase II (Pol II) to the WNT3A promoter. We observed that recruitment of Pol II to the WNT3A promoter was decreased in PC3\textsuperscript{TBX2 DN} cells compared with control PC3\textsuperscript{Neo} cells (Fig. 6D; Supplementary Fig. S6A), implying that blocking TBX2 leads to a significant decrease in WNT3A promoter activity. Utilizing an alternative approach, we observed enhanced TBX2 binding on WNT3A promoter in LNCaP\textsuperscript{TBX2} cells compared with LNCaP\textsuperscript{Neo} cells (Supplementary Fig. S6A and S6B). Overall, the ChIP data reveal a mechanism of WNT3A regulation by TBX2, and the experiments with rescue of WNT3A expression in the context of blocking endogenous TBX2 demonstrate that (i) the expression and phenotypic properties, \textit{in vitro} invasion, associated with WNT3A and its downstream effectors like MMP9, MMP2, and IL6 are restored in PC3\textsuperscript{TBX2 DN + WNT3A} cells compared with PC3\textsuperscript{TBX2 DN} cells and (ii) \textit{in vivo} metastatic behavior is partially rescued in PC3\textsuperscript{TBX2 DN + WNT3A} cells when compared with PC3\textsuperscript{TBX2 DN} cells.

In summary, our data suggest that TBX2 expression is increased in prostate cancer bone metastatic tumor xenograft samples and in clinical high Gleason grade prostate cancer samples. Our data further show that increased TBX2 in prostate cancer activates an invasive and metastatic behavior in prostate cancer cells that is mediated by WNT3A and its downstream effectors, MMP9, MMP2, and IL6 (Fig. 7).

**Discussion**

The widespread clinical manifestation of bone metastases in prostate cancer patients is the leading cause of mortality as...
Figure 6.

WNT3A, a downstream target gene of TBX2, mediates prostate cancer bone metastasis. **A**, PC3 cells (Neo, TBX2 DN, and TBX2 DN + WNT3A) were assessed for their ability to invade with control (IgG ab), or addition of WNT3A-neutralizing antibody (WNT3A neu Ab; 10 μg/mL), or IL6-neutralizing antibody (IL6 neu Ab; 150 ng/mL). Data, mean ± SEM (n = 3). **B**, qRT-PCR analysis of WNT3A, MMP9, MMP2, and IL6 in PC3 cells (Neo, TBX2 DN, and TBX2 DN + WNT3A; mean ± SEM, n = 3). **C**, Representative BLI (week 5) images of 5 of 10 mice in each group from mice intracardially injected with PC3 cells (Neo, TBX2 DN, and TBX2 DN + WNT3A). **D**, ChIP analysis of PC3 cells (Neo, TBX2 DN) showing in vivo binding of TBX2 on WNT3A promoter (mean ± SEM, n = 3). *, P ≤ 0.05; **", P ≤ 0.01; ", P ≤ 0.0001.
In patients with CRPC (18). In agreement with that study, we found TBX2 to be highly overexpressed in osteoblastic bone metastases in patients with CRPC (18). WNT3A, MMPs, and IL6.

Of relevance to our study, a previous report identified TBX2 as a key regulator of prostate cancer bone metastasis mediated by TBX2 through its downstream effectors WNT3A, MMPs, and IL6.

Several studies have shown that TBX2 is induced in the bone microenvironment as demonstrated by the intratibial xenograft model utilizing both PC3 and ARCaPM cells. Although prostate cancer bone metastases are predominantly osteoblastic, several studies have strongly suggested that the successful colonization of the bone by prostate cancer cells encompasses both osteolytic and osteoblastic events, and that the initial colonization step leads to a lytic lesion (24–28). These observations are in accord with and appropriately reflected by the models we used in our study; while the bone lesions formed by PC3 cells are highly osteolytic, ARCaPM cells on the other hand form a mixture of lytic and blastic lesions in the bone.

Work done in the past decade has shown that bone metastasis in prostate cancer is a complex process that broadly involves three steps: (i) malignant progression of the primary tumor and invasion through the surrounding extracellular matrix to reach the blood stream; (ii) prostate cancer cells traveling in the blood stream and homing to the bone through extravasation; and (iii) interaction of the colonized prostate cancer cells with the bone microenvironment to produce mixed lesions, bone resorption or osteolysis along with concomitant new bone formation or osteopetrosis. Utilizing a series of xenograft mouse models, our results consistently show that TBX2 plays a seminal role in mediating all three steps in the metastatic cascade. Furthermore, our results from the orthotopic xenograft technique (that physiologically mimics the primary organ microenvironment) showing that blocking TBX2 expression abrogates prostate cancer lymph node metastasis are consistent with our data from the experimental metastasis model.

In addition to understanding prostate cancer cell homing to the bone, it is paramount to decipher the molecular mechanisms that determine prostate cancer cell interactions with the bone microenvironment. It is well known that reciprocal interactions between the bone microenvironment and the colonizing tumor cells brings about bone remodeling, a process that leads to the painful complications associated with bone metastasis in prostate cancer patients. Our data show that blocking endogenous TBX2 greatly reduces growth in the bone microenvironment as demonstrated by the intratibial xenograft model, and targeting IL6 signaling has been shown to inhibit prostate cancer bone metastasis through the regulation of BMPs (36). WNT3A levels are upregulated in human prostate cancer cell lines, and WNT3A has been reported to induce prostate cancer bone metastasis through the regulation of BMPs (37, 38). WNT3A-neutralizing antibodies injected intraperitoneally have been shown to decrease proliferation and induce apoptosis in a prostate cancer mouse model (39), and the WNT antagonist SFRP-2 has been shown to inhibit WNT3A in vivo (40). Furthermore, WNT3A is reported to induce IL6 expression (41). High circulating levels of IL6 have been associated with adverse clinical outcomes in patients with metastatic CRPC (42), and targeting IL6 signaling has been shown to inhibit prostate cancer growth in the bone (43). In addition, WNT3A is
reported to regulate MMP2 and MMP9 (44), the extracellular matrix components that bring about the degradation of base-ment membrane and are known to play seminal roles in prostate cancer invasion and metastasis (45). In addition, high levels of MMP2 and MMP9 in the plasma and urine of prostate cancer patients have been shown to correlate with prostate cancer metastasis (46, 47); and IL6 has been shown to regulate MMP9 expression (48). Taken together, these reports are in congruence with our findings that (i) addition of WNT3A-neutralizing antibodies, or the WNT antagonist SFRP-2, or IL6 neutralizing antibodies decreased the in vitro invasiveness of prostate cancer cells in the context of high TBX2 or WNT3A expression; and (ii) blocking TBX2 abrogates prostate cancer bone metastasis in mouse xenograft models in the context of decreased expression of WNT3A, MMPs, and IL6 and that rescuing WNT3A levels in these cells partially rescues their metastatic ability with concomitant increase in the expression of MMPs and IL6.

Our results highlight a new and specific function of TBX2 in regulating WNT3A at the transcriptional level. In congruity with our findings, a previous study identified WNT3A as a target of Brachyury (T), a transcription factor that shares the same consensus T-box-binding element with TBX2 (49). Of note, TBX2 has been reported to function both as a strong repressor and a relatively weak activator (50). In our studies, as we are examining the function of TBX2 as an activator of WNT3A transcription, we think this could explain the observed weak binding of TBX2 on the WNT3A promoter. However, our data demonstrate that despite the relatively weak activation, WNT3A mediates the biological function of TBX2 in the manifestation of prostate cancer metastasis, as rescuing WNT3A levels in the context of blocking endogenous TBX2 levels in PC3 cells partially restores the metastatic ability of these cells.

It is important to underscore the reported correlation that genes and signaling pathways that play vital roles during embryonic development are often found to be dysregulated in tumor progression and metastasis. TBX2 seems to fit in to the profile of these genes as previous reports have reported the role played by TBX2 in embryonic development. Interestingly, we have observed that TBX2 expression is detected very early in mouse prostate development, as early as E16 (data not shown). In addition, interestingly, our studies point to the fact that TBX2 primarily mediates invasion and metastasis, rather than tumor growth in vivo. This is because despite differences in proliferation of prostate cancer cells in vitro upon blocking endogenous TBX2, our observations both in the kidney capsule and orthotopic xenograft mouse models demonstrate that genetically modulating TBX2 has no statistical differences in the in vivo tumor size. We speculate that this anomaly could be due to the presence of additional factors in the ecosystem of in vivo tumors, such as reciprocal interactions between the tumor cells and the cells of the tumor microenvironment.

Designing novel therapeutic approaches against prostate cancer bone metastasis based on the inhibition of WNT signaling offers potent advantages given WNT’s widely reported role in prostate cancer bone metastasis and bone remodeling events. Our model suggests that increased expression of TBX2 promotes bone metastasis and growth in the bone microenvironment through the regulation of downstream WNT3A. We posit that this newly uncovered TBX2–WNT3A signaling pathway represents a novel and exciting opportunity for the development of novel therapies targeting prostate cancer bone metastasis.

Disclosure of Potential Conflicts of Interest
N.A. Bhowmick is a researcher at Veterans Administration Greater Los Angeles Healthcare. No potential conflicts of interest were disclosed by the other authors.

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References


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