Title # PITX2 and the non-canonical Wnt pathway in the development of metastatic prostate cancer

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Abstract

The non-canonical Wnt pathway, a regulator of cellular motility and morphology, is increasingly implicated in cancer metastasis. In a quantitative PCR array analysis of 84 Wnt pathway associated genes, both non-canonical and canonical pathways were activated in primary and metastatic tumors relative to normal prostate. Expression of the Wnt target gene PITX2 in a prostate cancer (PCa) bone metastasis was strikingly elevated over normal prostate (over 2000 fold) and primary prostate cancer (over 200 fold). The elevation of PITX2 protein was also evident on tissue microarrays, with strong PITX2 immunostaining in PCa skeletal and, to a lesser degree, soft tissue metastases. PITX2 is associated with cell proliferation and migration during normal tissue morphogenesis. Here, overexpression of individual PITX2A/B/C isoforms stimulated PC-3 PCa cell proliferation and motility, with the PITX2A isoform imparting a specific motility advantage in the presence of non-canonical Wnt5a stimulation. Furthermore, PITX2 specific shRNA inhibited PC-3 cell migration toward bone cell derived chemoattractant. These experimental results support a pivotal role for PITX2A and non-canonical Wnt signaling in enhancement of PCa cell motility, suggest PITX2 involvement in homing of PCa to the skeleton, and are consistent with a role for PITX2 in PCa metastasis to soft and bone tissues. Our findings, which significantly expand previous evidence that PITX2 is associated with risk of PCa biochemical recurrence, indicate that variation in PITX2 expression accompanies and may promote prostate tumor progression and metastasis.

Keywords: Prostate cancer, metastasis, non-canonical Wnt pathway, PITX2, bone

Introduction

It is widely acknowledged that malignancy subverts biologic and developmental processes and pathways during cancer progression and metastasis. Pathways that normally act as tumor suppressors or biologic gatekeepers are transmuted to provide positive signals for cellular survival, proliferation or motility. One pathway increasingly described as having such duality of function in normal and malignant cells is the Wnt pathway, which denotes a set of related but functionally distinct canonical and non-canonical pathways. The canonical Wnt response is mediated via nuclear β-catenin transcriptional activity and has been implicated in both prostate cancer progression and control of normal bone function (1-4). The major non-canonical Wnt pathways are not β-catenin mediated (5), but instead involve planar cell polarity (PCP), calcium, Rho GTPases and JNK signaling (6-8). Recently, accumulating evidence implicates non-canonical Wnt signaling in the control of cellular movement during embryologic development and in cancer-related events such as epithelial to mesenchymal transition (EMT) and the metastatic process (7-9).

The Wnt-associated gene PITX2, originally identified in the pituitary, encodes a transcription factor carrying a bicoid-related homeodomain (10). There are three major PITX2 protein isoforms in humans (10). PITX2A and PITX2B are translated from alternatively spliced transcripts originating from a promoter designated P2, whereas the PITX2C encoding transcript originates at an alternative upstream promoter, P1 (10, 11). Point mutations in this gene are responsible for the Axenfeld Rieger Syndrome, which is characterized by ocular, umbilical/body wall and cardiovascular abnormalities, tooth agenesis, and facial dysmorphism including cleft palate (11-13). In mice, PITX2 plays a role in the left-right determination of various structures such as gut and heart, and controls cellular migration and motility during this process (10, 14, 15). Consistent with clinical and experimental evidence for its involvement in tissue development, PITX2 has been observed to interact with the canonical Wnt (12, 16-20) and non-canonical Wnt (21, 22) pathways and also to act as a downstream effecter of developmental regulatory pathways involving Sonic Hedgehog, Lefty and Nodal (11, 13-15).

Recent reports suggest that PITX2 might act as a tumor suppressor. Hypermethylation of the PITX2 gene in primary PCa was associated with an increased risk of biochemical recurrence following primary therapy, potentially indicating a tumor suppressor role (23-25). Furthermore, PITX2 was also associated with increased risk of disease recurrence in node negative, hormone receptor positive, tamoxifen-treated breast cancer (26-31). In contrast to these reports, we present evidence

from both quantitative PCR array and immunohistochemistry analyses that PITX2 is elevated in Gleason score 3, albeit in a minority of samples, with a loss of expression at Gleason score 4 followed by reappearance of considerable expression in metastatic samples, particularly in bone metastases. In light of this new information and given the human and mouse genetic evidence about PITX2 involvement in cell migration during development, we conducted a functional analysis to determine whether PITX2 may be directly involved in control of cellular motility in a prostate cancer cell line, specifically testing for roles in the non-canonical Wnt response and in bone cell-directed migration.

Material and methods

Frozen tissue samples and PCR arrays

All studies were carried out following approval by the local Human Ethics Review Panel with consented patients at the Princess Alexandra Hospital (Brisbane, Australia) providing specimens. The qRT-PCR Wnt array study used fresh and fresh frozen archived human prostate normal and primary PCa specimens obtained either at radical prostatectomy, transrectal ultrasound guided biopsy or transurethral resection of the prostate. In addition, one fresh clinical human PCa bone metastasis was obtained with patient consent at time of elective orthopedic fixation of an impending pathologic fracture of the femur. Trizol reagent RNA extraction from fresh frozen tissue was performed with further purification using the RNeasy Minikit including DNase I treatment (Qiagen).

Wnt pathway related gene expression was quantitatively assessed using the RT^2 Profiler PCR Wnt Pathway Array System (SA Bioscience). Real time PCR analysis was performed using Sybr Green technology (ABI 7300 Thermal Cycler, Applied Biosystems). Array data were normalized to housekeeping genes utilising the ΔCt method and analyzed statistically by one-way analysis of variance followed by Student's T-test using the spreadsheet provided by SA Bioscience at http://www.sabiosciences.com/pcrarraydataanalysis.php.

Clinical tissue microarrays and immunohistochemistry (IHC)

Human tissue microarrays (TMA) of fixed paraffin embedded primary and metastatic PCa tissues were obtained as part of the University of Washington (UW) PCa Research Program and the UW

Medical Center PCa Donor Rapid Autopsy Program, as approved by the UW Institutional Review Board with written informed consent obtained for all patients. Radical prostatectomy TMA series consisted of cores taken from 47 total patients; 67 cores of non-neoplastic prostate (from 24 patients), 61 cores of Gleason score 3 (from 27 patients), and 44 cores of Gleason score 4 PCa (from 18 patients). The PCa metastases were obtained from 42 patients participating in the Prostate Cancer Donor Rapid Autopsy Program (32). The PCa metastasis array consisted of 90 soft tissue cores (24 patients) and 141 bone cores (24 patients). Five-μm sections of TMAs were stained with either H&E or by IHC using a protocol we have described previously (33), using antibodies against PSA 1:30,000 (Dako) or P2R10 for PITX2 1:100-200 (Capra Bioscience, Angelholm, Sweden) after pH 6 antigen retrieval (Dako). The P2R10 antibody recognizes all known PITX2 protein isoforms except PITX2D (34).

Cell lines and cultures

PC-3 prostate cancer and SaOS-2 osteosarcoma human cell lines, the mouse fibroblastic L cell and Wnt5a over-expressing L cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in appropriate growth media. SaOS-2 conditioned media (CM) was prepared from confluent monolayers cultured for 48 h in serum free DMEM, sterile filtered (0.22 μm) and stored at 4˚C. Conditioned media from L cell and Wnt5a over-expressing cell lines (L-CM and Wnt5a-CM) were produced as per ATCC instructions. Co-culture experiments to assess osteoblast effects on PITX2 expression in PCa cells were conducted in experimental medium (ExpM) consisting of 50% DMEM and 50% RPMI plus 1% FBS and 1% P/S using transwell inserts (0.4 μM pore) in 6 well plates (BD Biosciences). SaOS-2 cells were seeded in the bottom well (1 x $10^4/cm^2$) and PC-3 cells were seeded in the insert (1 x $10^4/cm^2$) cultured separately for 48 h in standard growth media containing 10% FBS prior to bringing the two co-culture components together in the ExpM. PC-3 cells were collected from the insert 48 h later for RNA preparation.

Plasmids and transient transfection of PC-3 cells

Plasmids for over expression of individual PITX2 isoforms (human cDNA clones of transcript variants 1 (NM_153427), 2 (NM_153426) and 3 (NM_000325)) in a MYC- and FLAG-tagged pCMV6 entry vector were purchased from Origene (TrueORF, Origene Technologies). PITX2 specific shRNA plasmids targeting all three variants (Sure Silencing Plasmids, SA Bioscience) were

screened for ability to reduce production of all three PITX2 isoforms (Fig S.1); the SH3, SH4 and negative control (NC) plasmids were chosen for subsequent studies (Table 1). PC-3 cells were transfected with shRNA or expression plasmids using Lipofectamine 2000 (Invitrogen). Co-transfection of each PITX2 isoform plasmid (0.5 μg) with each shRNA plasmid (0.5 μg) was used to validate individual isoform effects on cell motility in scratch assays.

Proliferation and viable cell assays

BrdU cell proliferation ELISA (Roche) was performed to assess effects of PITX2 over expression or knockdown on PC-3 cell proliferation. In separate experiments, changes in cell number were estimated by Syto 60 assay to assess effects of transient PITX2 over expression or knockdown in PC-3 cells. Results were analyzed using the plug-in Odyssey software following scanning on the Odyssey Infrared scanner (LI-COR Biosciences).

Scratch motility assay

Effects of PITX2 over expression or knockdown on PC-3 cell motility were analyzed using a scratch or wound-healing assay. Cells were seeded in externally marked 6 well plates (5.25 x $10^4/cm^2$), transfected in serum free RPMI, cultured a further 24 h, then treated for 5 h with mitomycin C (15 μg/ml) prior to scratching, washing in PBS and refeeding of the confluent monolayers. Wells were imaged at 0, 4, 8, 12 and 24 h post scratch using external reference lines to ensure reproducibility. Images were analyzed using Image J software (NIH) with closure of the scratch calculated as a percentage of the original wound area over time. To assess PITX2 involvement in the non-canonical Wnt response, scratch motility assays were performed on transfected cultures with PITX2 over expression or knockdown, initially in RPMI culture medium supplemented with 10% L-CM or 10% Wnt 5a-CM, and subsequently in serum free RPMI supplemented with purified recombinant Wnt 5a protein (50 ng/ml) (R&D Systems).

Transwell migration assay

Migration assays were also performed in 12 well cell culture inserts with 8 μm pores (BD Biosciences). PC-3 cells were transfected with shRNA plasmids in the absence of FBS as described

for scratch assays. After 24 h transfected cells were seeded into the well insert (5.25 x 10^4/cm^2) in serum free medium plus 0.1% BSA (Sigma) and at 5.25 x 10^4/cm^2 in 200 µL of serum free medium plus 0.1% BSA (Sigma). The seeded inserts were subsequently placed into wells containing culture medium supplemented with 10% FBS or 10% SaOS-2 CM. Mock transfected (control) cells in inserts above medium containing 0% FBS were used to establish a baseline level of random migration. Cell migration was assessed after 12 h incubation by the standard crystal violet quantification procedure and normalized back to serum free control levels (35).

Quantitative reverse transcription polymerase chain reaction

First strand cDNA synthesis for qRT-PCR was performed using the Superscript III First-Strand Synthesis System (Invitrogen). Specific transcript levels were analyzed on the Rotor-gene RG-6000 thermocycler (Corbett Research, Sydney) or ABI 7900HT Thermal cycler (Applied Biosystems). All primers were designed using Primer 3 software (36).

Statistics

Data analysis was performed using Microsoft Excel 2007 (Microsoft Corp, Redmond, WA, USA) and R version 2.7.2, pgirmess library (Team RDC, Vienna, Austria). Data were analyzed using ANOVA and the Student's T test to evaluate the significance of the difference in mean values between cell lines, treatments and specimens. P values <0.05 were considered to indicate statistically significant differences. Data are represented as mean ± standard error (SE) unless otherwise stated.

To compare the staining profile of PITX2 in the nucleus and cytoplasm of the primary prostate and PCa metastases, GEE regression was used, keeping track that cores from a same site of a same patient tend to be more closely correlated with each other than others. The GEE regression model used:

Model $\quad E(Score_i) = 1.32 - 0.44N_i - 0.5SOFT_i,$

95% Confidence interval $\quad (-0.69,-0.19) \quad (-0.71,-0.3)$

where $N_i = 1$ if tissue from nucl, 0 else; $SOFT_i = 1$ if tissue from soft tissues, 0 else. The baseline group is cytoplasm staining of tissue from bone.

Results

In an initial investigation of altered expression of Wnt pathway genes in prostate tumor tissues at various stages of cancer progression, transcript abundance levels were profiled in a small number of archived and fresh frozen tissue samples using quantitative RT-PCR arrays. Multiple differences in Wnt pathway and associated gene expression levels (at least 2 fold change) were noted between specimens of normal (benign) prostate (n = 2) and malignant prostate (n = 9). Interestingly, data for a single available PCa bone metastasis suggested that these changes might even be increased in metastasis vs primary PCa. Analyses of these changes indicated that similar sets of genes were altered in primary and metastatic PCa compared to normal prostate, with the number and magnitude of changes greater in metastatic disease. Specifically, cellular growth, proliferation and cell survival/viability pathways were increased whereas genes involved with apoptosis and differentiation were down regulated in malignant tissues; in metastatic disease a strong increase in genes involved in cellular motility was also noted (Table S.1).

One of the large, consistent changes we detected in our preliminary analysis was alteration of PITX2 expression, with marked differences among normal, primary cancer and metastatic cancer samples in terms of both fold change and P value (Fig. 1A). PITX2 expression in the bone metastatic deposit exceeded the mean levels in normal prostate by 2252 fold and in primary PCa by 227 fold. The array results were confirmed by gene-specific qPCR analysis using universal PITX2 primers, which amplify transcripts encoding the three major PITX2 isoforms (Fig. 1B). Total PITX2 transcripts were 5 fold higher in primary tumor samples and over 1000 fold higher in the bone metastatic specimen compared to normal prostate tissue.

These preliminary analyses were of total tissue extracts, which would be affected by the cellular composition and proportion of stroma to cancer in each sample. Nevertheless the data suggested a potential role for PITX2 in PCa progression. To explore this possibility, an investigation of PITX2 protein patterns in early stages of PCa was conducted by immunohistochemistry staining of a TMA composed of samples from normal (benign) prostate, primary PCa Gleason score 3 and score 4 (Fig. 2). Although PITX2 functions as a nuclear transcription factor, our analysis did not reveal any significant differences in nuclear PITX2 immunoreactivity when normal prostate, Gleason score 3 and Gleason score 4 samples were compared. In all of these samples we detected only a few nuclei with intense immunoreactivity, while at least 75% of nuclei were negative (Fig. 2A). In comparison, there was evidence that cytoplasmic staining was significantly different between benign, Gleason score 3 and Gleason score 4 specimens ($p = 0.025$), with the average cytoplasmic staining score in

Gleason score 3 cores estimated to be 0.11 higher than in benign cores (95% confidence interval, -0.08 to 0.32). In contrast, cytoplasmic staining was reduced in cores from Gleason score 4 tumors, estimated to be -0.19 lower than scores for benign cores (95% confidence interval -0.39 to 002) and -0.30 lower than Gleason score 3 cores (95% confidence interval -0.50 to -0.10). Representative histological sections show the nuclear and cytoplasmic staining observed in these specimens (Fig. 2B).

Next, given our finding of >1000-fold elevation of PITX2 expression in the single bone metastasis sample of the PCR array analysis, we extended our investigations to more advanced stages of the disease by evaluation of PITX2 immunoreactivity using a TMA consisting of a large cohort of matched bone and soft tissue metastases. These metastatic samples were from 24 different patients and totaled 141 bone cores and 90 soft tissue cores, the latter including lymph nodes, liver and other non-skeletal metastases. Of an original 256 cores, a total of 231 were scored for PITX2 staining in both nucleus and cytoplasm as 25 cores either had no cancer cells or sections were lost from slides (9.8%). Representative sections show the generally increased PITX2 signal observed in the bone metastases compared to soft tissue metastases (Fig. 3B).

Variation in PITX2 expression profile was specifically assessed between bone metastases and soft tissue metastases (Fig. 3A). Our analysis suggests that the nuclear staining score of soft tissues (liver, lymph node and other soft tissues) is different from bone ($p=0.02$) with the average soft tissue score estimated to be -0.26 lower than that of bone (95% confidence interval, -0.48 to -0.05). Despite notable diversity between patients, there is strong evidence that the staining score in bone metastases was greater than the score for metastases in soft tissues ($p<0.01$), with the score for soft tissue metastases estimated to be -0.5 lower than that for bone metastases (95% confidence interval, -0.71 to -0.3). For cytoplasmic staining, there is strong evidence that the staining score of soft tissues (liver, lymph node and other soft tissues) is different from bone ($p=0.01$), with the average score of soft tissue estimated to be -0.74 lower than that of bone (95% confidence interval, -1.01 to -0.48)

Although cytoplasmic staining has previously been observed, the functional significance of this distribution is not known. Variation in nuclear and cytoplasmic staining patterns could potentially relate to differences in PITX2 isoform expression profile. To explore this concept, individual levels of transcripts encoding each isoform as well as total PITX2 transcripts were assessed in the clinical specimens previously interrogated on PCR transcript arrays. Consistent with the earlier findings (Fig. 1) total PITX2 transcript levels in tumor samples were elevated compared to normal prostate,

and transcript levels of individual isoforms also rose incrementally from normal prostate tissue, to primary cancer, to metastasis (Table 3). Specifically, PITX2A transcripts were not detectable in normal tissue but were present at a fairly low level in primary tumors and elevated over 1500 fold in the bone metastasis sample compared to the primary tumors. Likewise, levels of PITX2B and PITX2C transcripts were low in normal tissue, elevated somewhat in primary tumors, and markedly elevated (2500 and 4200 fold, respectively) in the bone metastatic deposit.

These consistent indications from both mRNA and protein analyses that PITX2 expression was higher in more advanced cancers led us to investigate its involvement in characteristic cellular functions that are altered in metastatic cancer cells. A role for PITX2 in cell proliferation and migration was therefore tested in the PC-3 PCa cell line, which expresses a low level of endogenous transcripts encoding each of the three PITX2 isoforms. In tests using over-expression of individual PITX2 isoforms, cell proliferation increased significantly in cells transiently over expressing any one of the three isoforms (Fig. 4A). In contrast, effects of isoform over expression on cell number were less uniform, with PITX2A over expression causing an increase in cell number but PITX2C transfection leading to a modest but significant decline in cell number (Fig. 4C). Knockdown of all PITX2 isoforms, however, did not significantly alter cell proliferation or number (Fig. 4B, D) despite evidence that each PITX2-specific shRNA construct can effect a knockdown of at least 25% for all PITX2 isoforms in the PC-3 cell line (Fig. S.1). Together, these over expression and knockdown studies indicated that all three PITX2 isoforms have modest but positive effects on cell proliferation and mixed effects on cell number.

The effect of increasing levels of individual PITX2 isoforms on cell migration was assessed using scratch wound healing assays in non-proliferating (mitomycin C treated) PC-3 cultures. Over expression of any single PITX2 isoform significantly increased cellular motility compared to control cultures (Fig. 5A). Conversely, knockdown of endogenous PITX2 significantly decreased cellular motility, with both gene-specific shRNA constructs reducing wound closure compared to the negative control shRNA (Fig. 5B). Knockdown of individual over-expressed isoforms by co-transfection with shRNA constructs validated these results (Fig. 5C-E). Together these findings strongly support a role for PITX2 in promotion of cellular motility in PC-3 PCa cells.

Given prior evidence of PITX2 involvement in cell migration during development (14, 15, 37) and PITX2 interactions with both Wnt pathways (12, 17-19, 38), as well as of non-canonical Wnt involvement in cellular motility and EMT, a potential interaction between PITX2 and non-canonical Wnt in regulating cancer cell motility was investigated in PC-3 PCa cells, which secrete high

baseline levels of the canonical Wnt antagonist DKK1 (2). In the scratch wound closure assay, the motility of PC-3 cells exposed to Wnt5a-CM for 24 h was increased by 30% (p=0.038) compared to cultures treated with control L-CM (Fig. 6A, grey vs white circles). This stimulatory effect of Wnt5a-CM was evident at earlier time points (t= 4 h and thereafter, grey vs white diamonds) in cultures transfected with the negative control NC shRNA plasmid (e.g., 50% increase at 24 h, p=0.0008) in spite of a general decrease in wound closure in the transfected control cultures. PITX2 involvement in the Wnt5A-associated motility increase was confirmed by knockdown of cellular PITX2, with transfection of the SH4 construct abolishing the motility increase observed with Wnt5a-CM treatment observed in the NC cultures (grey vs white triangles). Transfection with the SH3 construct yielded similar results (data not shown). The specificity of the Wnt5a effect was confirmed in cultures treated with purified recombinant Wnt5a protein (Fig. 6B). Together these data support a critical role for PITX2 in the motility changes associated with Wnt5a induction of non-canonical Wnt pathway activation.

To investigate roles of individual PITX2 isoforms in the non-canonical Wnt motility effects, transient over expression of individual isoforms and a wound healing scratch assay in the presence of Wnt5a-CM were performed. Over expression of PITX2A significantly increased PC-3 cell motility in Wnt5a-CM treated cultures (Fig. 6C, grey vs white squares). By contrast, in the empty vector control cells the increase in motility after Wnt5a-CM treatment was more modest and reached statistical significance only at the 24 h time point (grey vs white circles). In PITX2B or PITX2C over expressing cells (grey vs white triangles and diamonds, respectively), no further increases in motility were observed after Wnt5a-CM treatment. Thus, our data suggest that PITX2A is responsible for the effects on migration, while PITX2B and PITX2C do not appear to be directly involved in the non-canonical Wnt signaling effects on cellular motility.

Given the high levels of PITX2 expression in the bone metastases, the possibility that PITX2 expression in cancer cells is subject to paracrine regulation by the bone environment was investigated through trans-well co-culture experiments with human osteoblastic SaOS-2 cells. PITX2 transcription in PC-3 cells grown in the presence of the SaOS-2 culture was at least 3-fold higher than in control PC-3 cultures (Fig. 7A), indicating that SaOS-2 cells secrete a soluble factor(s) that stimulates PITX2 expression in cancer cells. This result suggests that PITX2 may be an active participant in the interaction between metastatic PCa cells and target skeletal tissue.

To test whether PITX2 may be involved in homing of cancer cells to bone, we assessed PC-3 cell migration toward osteoblastic SaOS-2 conditioned medium using PITX2 knockdown. The

chemotactic response by cancer cells transfected with either SH3 or SH4 plasmid was significantly reduced (by 59% or 92% respectively) relative to cultures transfected with the NC shRNA (Fig. 7B, black bars). In contrast, transfection of either gene-specific or negative control shRNA plasmid did not significantly affect the migratory response to medium supplemented with FBS (Fig. 7B, white bars). These results are in agreement with those from the scratch motility assay analysis and also support a positive role for PITX2 in migration of the PC-3 cancer cells, particularly toward soluble factors produced by bone cells *in vitro*.

Discussion

PITX2 has previously been investigated in prostate cancer. Hypermethylation of the gene in primary PCa was an independent prognostic indicator for biochemical recurrence (23, 25, 39) and predictive of increased risk of local recurrence in a stratified patient cohort (24). Promoter hypermethylation has also been associated with reduced PITX2 transcription in primary tumors (39). To a certain extent our immunohistochemistry analysis of early PCa samples is concordant with those published epigenetic findings, showing decreased levels of PITX2 protein in malignant versus normal prostate. Conversely, however, our analysis of advanced disease in prostate cancer metastases detected increased PITX2 levels, indicating re-expression of this highly methylated gene during cancer progression. We also observed higher levels of PITX2 protein in bone vs soft tissue metastases, which suggests that advanced PCa progression in the skeleton may occur through altered regulation of PITX2 expression and function caused by crosstalk between tumor cells and the bone microenvironment. Results of our in vitro investigations into mechanism support this possibility with evidence that a factor(s) secreted by osteoblastic cells stimulated PITX2 gene expression and was chemotactic for PCa cell migration. Our investigations further suggest a specific functional interaction between non-canonical Wnt pathway activity and the PITX2A isoform.

PITX2 is a homeodomain-containing transcription factor. Our immunostaining data clearly show increased nuclear PITX2 signal in bone metastasis, again substantiating a claim of its involvement in bone metastasis progression. In addition, the strong cytoplasmic staining clearly indicates that the PITX2 gene promoter is not hypermethylated in these patient samples, especially in the bone metastases. This altered staining may reflect a specific change of PITX2 function secondary to metastatic niche interactions. The data from the SaOS-2 osteoblastic co-culture and conditioned media experiments (discussed further below) suggest that this differential subcellular distribution and level of PITX2 could be indicative of particular isoform interactions with secreted osteoblast-derived factors. Because the antibody used in this study does not distinguish between the major PITX2 isoforms (34) however, it is not possible to discern from our data whether the levels of individual protein isoforms change differentially with PCa progression.

To gain insight into the roles of the PITX2 isoforms in PCa we analyzed levels of the individual isoform-encoding transcripts in a small subgroup of clinical samples. The PITX2A transcript was detected only in malignant tissue and not in normal prostate specimens whereas PITC2B and C transcripts were detected in all samples. In both normal and malignant prostate, the PITX2B transcript was expressed at a higher level than either A or C, making PITX2B the major expressed

isoform in this sample set. The differential expression of the A and B isoforms is interesting as both transcripts arise from the P2 promoter of the PITX2 gene (10, 34), far upstream of the P1 promoter region (51) which was the focus of the previous hypermethylation studies (25). Although the various PITX2 isoforms have been studied in normal development (12, 15, 16, 40, 41) and relative levels of isoform-specific transcripts have been seen to vary between tissues (34), our results showing expression of PITX2B and C in the absence of PITX2A transcripts in normal but not malignant prostate is to our knowledge the first observation of this pattern in any tissue. The manifestation of the pattern in the present context suggests a particular role for PITX2A in prostate malignancy.

Our functional analyses in cultured PC-3 cells support significant roles for all three PITX2 isoforms in control of PCa cells, with both similarities and differences between the isoforms detected. Over expression of any of the three isoforms increased PC-3 cell proliferation modestly but significantly. A similar stimulatory effect of over expressed PITX2A on cell proliferation has also been observed in ovarian cancer cells (42), likely associated with activation of the Wnt/β-catenin pathway which was induced in ovarian cancer cells by all three PITX2 isoforms *in vitro* (43). Our experiments in PC-3 PCa cells did not investigate potential PITX2 involvement with canonical Wnt regulation in PCa, however, because this cell line produces a high level of Dkk1, a secreted antagonist of canonical Wnt activation (2). In contrast to the uniform stimulatory effects of the three isoforms on PC-3 proliferation, the PITX2 isoform effects on cell number varied. Over expressed PITX2A increased but PITX2C decreased viable cell numbers, highlighting the potential for PITX2 involvement in PCa cell survival or apoptosis. In concept, this divergence between effects of the isoforms on cell proliferation and number in cancer cells is compatible with PITX2 involvement in multiple aspects of organogenesis and development including cell proliferation, differentiation, motility and apoptosis (14, 17, 44-46).

In the scratch wound healing assays, each individual PITX2 isoform significantly increased cellular motility when over-expressed in PC-3 cells, but a difference between the isoforms emerged with concurrent non-canonical Wnt activation which uniquely enhanced the PITX2A stimulatory effect on motility. Thus far there is limited literature in this area (37), but it is feasible that Rho GTPase family (47) or ATF/CREB (21) signaling may be involved in interactions between PITX2A and non-canonical Wnt. In contrast, the absence of a non-canonical Wnt effect on the PITX2B and C isoform responses indicates interaction with other signaling pathway(s) associated with cell motility. Given frequent evidence for interactions between PITX2 and TGF β superfamily members (21, 48-50) and in light of the chemotactic response of PC-3 cells to SaOS-2 secreted factor(s), a

likely possibility is involvement of TGF β or bone morphogenetic proteins (BMPs), which are both abundant in bone matrix. Upon its release by bone resorption in the initial stages of skeletal metastases, TGF β is thought to provide multiple cancer promoting signals that enhance bone resorption and lead to further liberation of growth factors. Given the importance of Wnt signaling in adult bone tissues (2), it seems likely that PITX2 may play an integrative role between Wnt and TGF β pathways in PCa bone metastasis. Our evidence that PITX2 expression in PC-3 cells was stimulated by an osteoblast secreted factor(s) suggests a scenario in which PITX2 may play a central role in synergistic interactions between bone and cancer cells in late stage PCa bone metastasis.

No documented increase in cancer formation has been observed in patients with Axenfeld Rieger syndrome, in which loss of function mutation of the PITX2 gene leads to developmental abnormalities. This argues against a potential role for PITX2 solely as a tumour suppressor gene. In light of this lack of genetic association as well as recent evidence that elevated PITX2 expression occurs in other carcinomas (42, 43, 45, 52) and given the results of our study, it now appears that the literature associating PITX2 hypermethylation with a higher risk of prostate and breast cancer recurrence (23-25, 28-31, 53-56) may be a partial account of the complex involvement of PITX2 in these cancers. There is precedent that other cancer-associated pathways act as both tumor suppressors and promoters depending on their temporal activation. In the context of the preceding discussion, a notable example is the TGF β pathway which can have tumor suppressor effects early in cancer progression but promote metastasis and tumor growth in later disease (57, 58). PITX2 may exhibit a similar duality of function, acting as a tumor suppressor early in disease (consistent with hypermethylation as a negative prognostic event for progression) and subsequently providing tumor and metastasis promoting facilities including interaction with the metastatic microenvironment in bone and potentially other Wnt regulated tissues.

Conclusion

The research presented here provides preliminary evidence for involvement of PITX2 in prostate cancer progression and metastasis. Clinically and experimentally PITX2 shows potential as a factor involved in the promotion of the metastatic phenotype and as a novel therapeutic target. The applicability of PITX2 to these areas mandates further investigation.

Acknowledgments

We would like to thank the patients and their families who were willing to participate in the Prostate Cancer Donor Program, for without them research of this nature would not be possible. We would like to acknowledge Robert Vessella, and Lisha Brown and the rapid autopsy teams in the Urology Department at the University of Washington. This material is the result of work supported by resources from the VA Puget Sound Health Care System, Seattle, Washington, by the Pacific Northwest Prostate Cancer SPORE (P50CA97186), the PO1 NIH grant (PO1CA085859), and the Richard M. Lucas Foundation. CM is a recipient of the Career Development Award from the Pacific Northwest Prostate Cancer SPORE (P50CA097186). These studies were supported by the Australian National Health and Medical Research Council (Project Grant 455854), the UQ Diamantina Institute, Queensland Health, the Australasian Urologic Foundation, the Queensland Government Smart State PhD Funding Program,

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Table 1. Short hairpin RNA plasmids

Clone ID	Insert Sequence
shRNA clone 3 (SH3)	GTCTCAACAGCCTGAATAACT
shRNA clone 4 (SH4)	GACATGTCCACACGCGAAGAA
Negative control (NC)	GGAATCTCATTCGATGCATAC

Purchased shRNA plasmids and sequences of their inserts, in 5' to 3' orientation.

Table 2. Primer pairs for quantitative polymerase chain reaction.

Gene	Primer Sequence Forward	Primer Sequence Reverse
PITX2 (U)	CAGCCTGAGACTGAAAGCA	GCCCACGACCTTCTAGCAT
PITXA	GCGTGTGTGCAATTAGAGAAAG	CCGAAGCCATTCTTGCATAG
PITXB	GCCGTTGAATGTCTCTTCTC	CCTTTGCCGCTTCTTCTTAG
PITXC	ACTTTCCGTCTCCGGACTTT	CGCGACGCTCTACTAGTCGTCCT
Housekeeping gene		
Cyclophilin	GCCGATGACGAGCCCTTGGGCC	ACCAGTGCCATTATGGCGTGTG

Sequences of primer sets for quantitative real time PCR, all in 5' to 3' orientation. The universal primer set (U) recognizes all three PITX2 isoforms.

Table 3. PITX2 transcript levels in clinical specimens.

Sample	PITX2	PITX2A	PITX2B	PITX2C
Normal Prostate (n=2)	8.4E-04	0	2.7E-05	1.6E-06
Primary Prostate Cancer (n=9)	3.4E-03	2.2E-05	6.8E-05	2.0E-05
Prostate Cancer Bone Metastasis (n=1)	9.6E-01	3.5E-02	1.7E-01	8.4E-02

Levels of PITX2 encoding transcripts in clinical tissue samples as assessed using quantitative PCR with Universal and isoform-specific primers, values normalized to the cyclophilin housekeeping gene.

A

Gene	Normal prostate (n=2)	Primary PCa (n=9)	PCa bone metastasis (n=1)
PITX2	1.5E-04##	1.5E-03#	3.5E-01

B

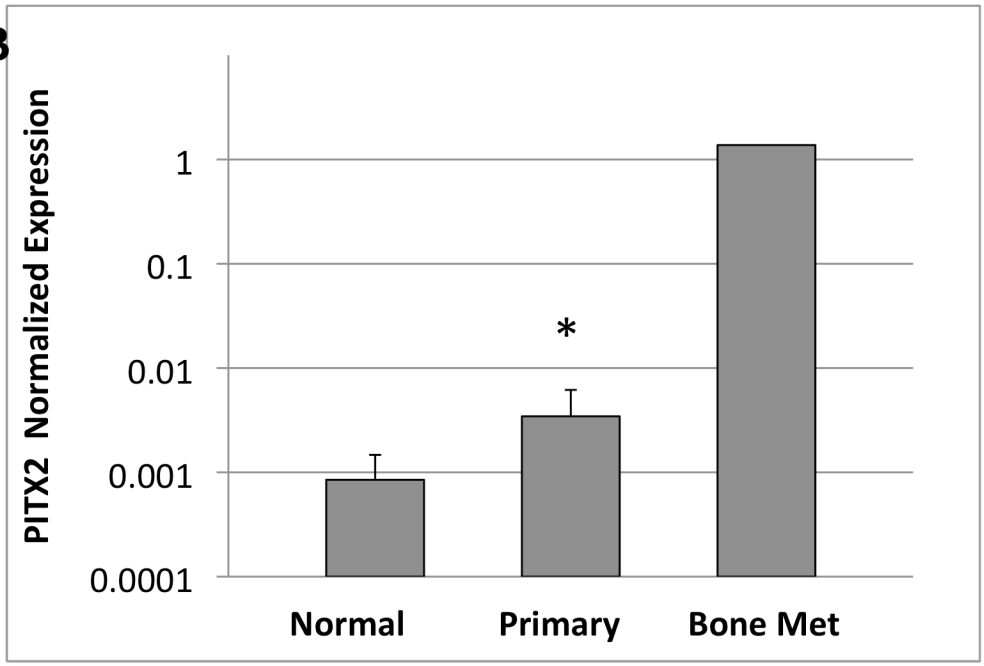
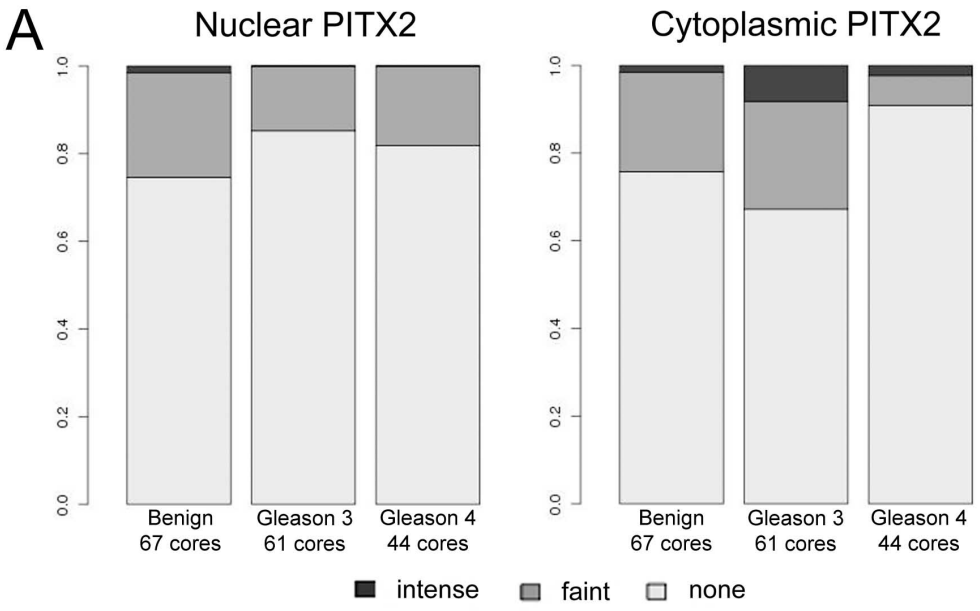


Figure 1. PITX2 expression levels in clinical prostate tissue and tumor specimens. (A) Data from qPCR array, normalized to housekeeping gene expression. #$p<0.001$, ##$p<0.0005$, difference vs metastatic sample. (B) Independent confirmation of array results using the PITX2 universal primer pair for qPCR analysis. Mean values for cancer samples were normalized to mean value for normal prostate tissue. *$p<0.05$ vs normal prostate. All values are mean ± S.D. of clinical samples.


A
Nuclear PITX2
Cytoplasmic PITX2
0.0
0.2
0.4
0.6
0.8
1.0
Benign
67 cores
Gleason 3
61 cores
Gleason 4
44 cores
intense
faint
none

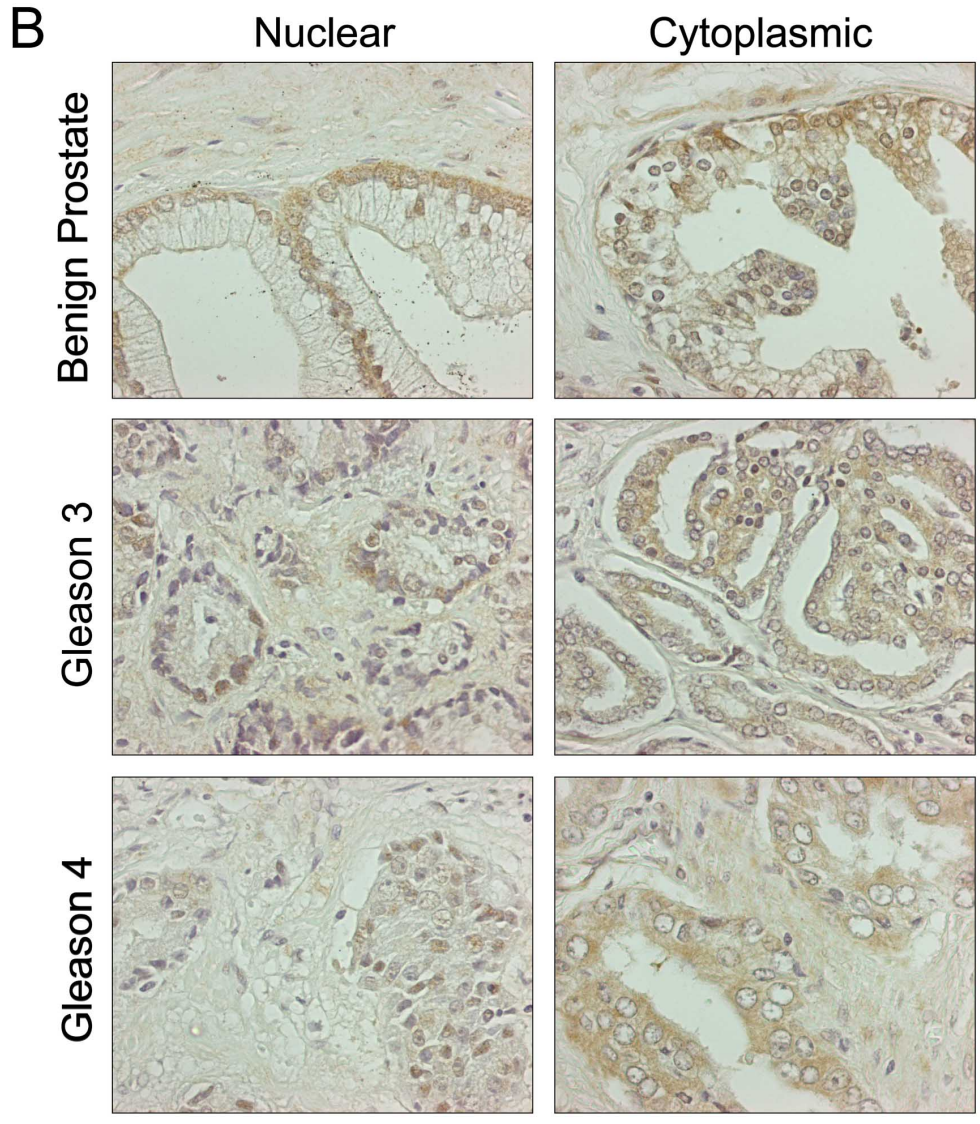
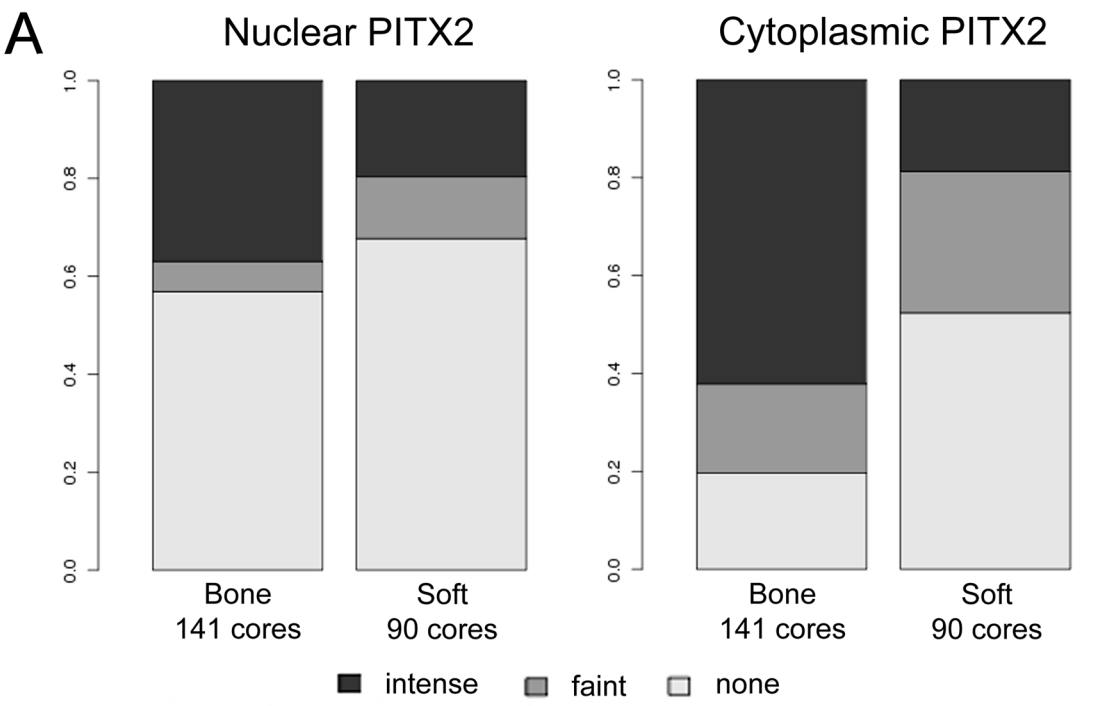

B
Nuclear
Cytoplasmic
Benign Prostate
Gleason 3
Gleason 4

Figure 2: Tissue microarray analysis of PITX2 in benign prostate tissue and primary tumors. Profiles of PITX2 nuclear and cytoplasmic staining intensities (A), and representative examples of PITX2 staining (B) in benign (normal) prostate, and primary prostate cancer Gleason scores 3 and 4. Magnification x200.


A
Nuclear PITX2
Cytoplasmic PITX2
0.0
0.2
0.4
0.6
0.8
1.0
Bone
141 cores
Soft
90 cores
Bone
141 cores
Soft
90 cores
intense
faint
none

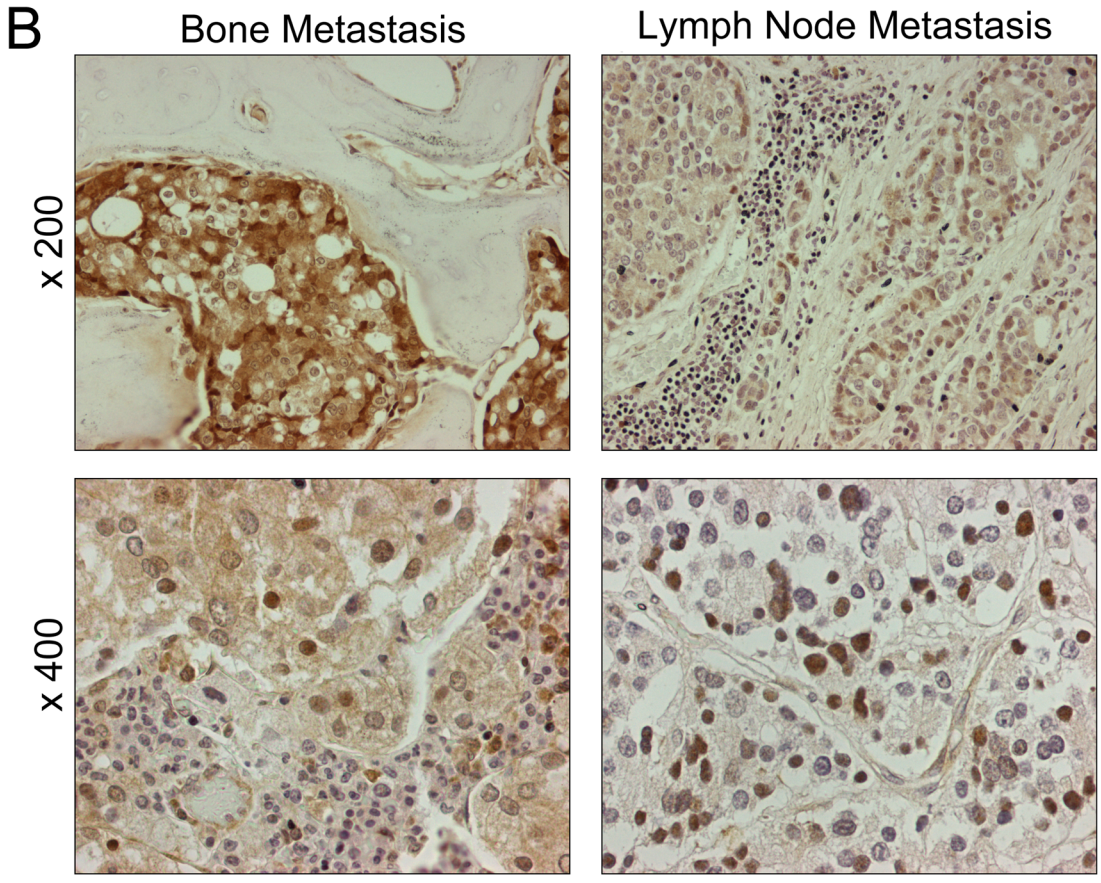

B
Bone Metastasis
Lymph Node Metastasis
x 200
x 400

Figure 3: Comparison of PITX2 cytoplasmic and nuclear staining profiles for bone and soft tissue metastases. (A) There is strong evidence that staining score of soft tissues (liver, lymph node and others) is lower than bone scores. See text for statistical details. (B) Tissue microarray examples stained for PITX2 protein. Generally more intense signal was seen in the metastatic samples compared to the primary prostate in Figure 2 with bone metastases exhibiting stronger signal compared to the soft tissue metastases.

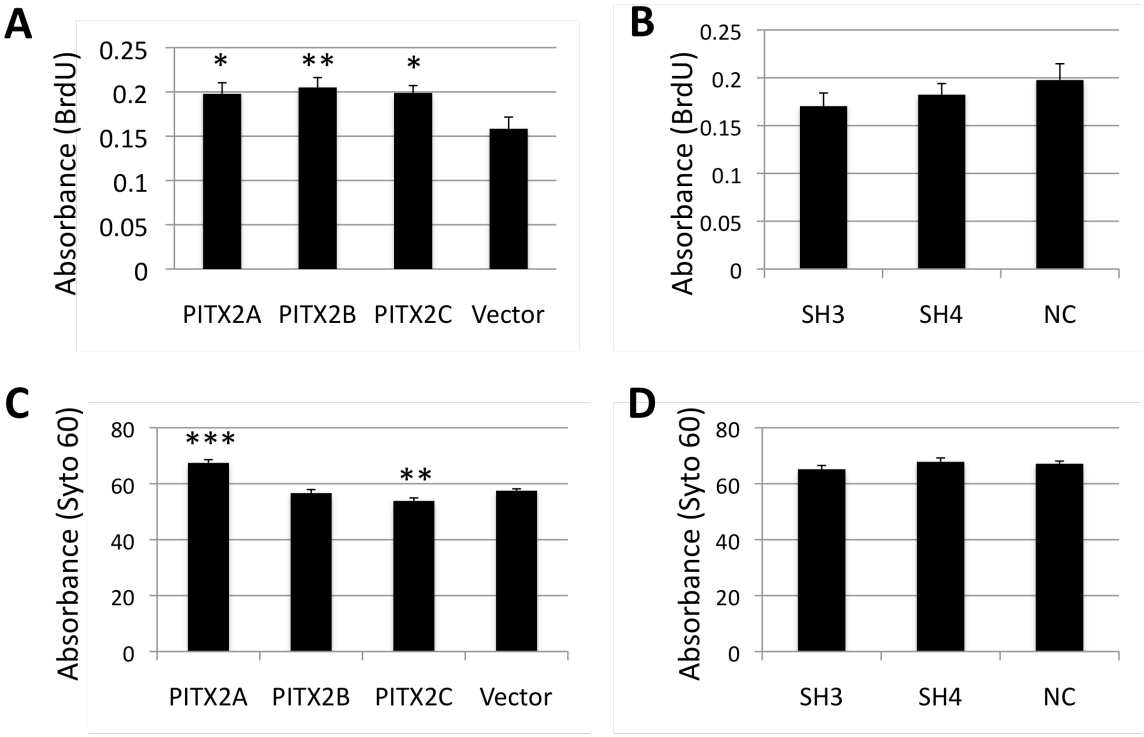


Figure 4: Changes in cell proliferation and number with altered PITX2. PC-3 cells transiently transfected with PITX2 isoform-specific expression vectors (**A, C**) or shRNA plasmid (**B, D**) and assayed after 48 h for incorporation of BrdU to measure cell proliferation (**A, B**) or Syto 60 to assess viable cells (**C, D**). Bars show normalized absorbance from respective assays, mean ± SE. Significance *p<0.05, **p<0.01, ***p<0.001 vs empty vector (Vector) or NC control as relevant. N= 12 wells /condition.

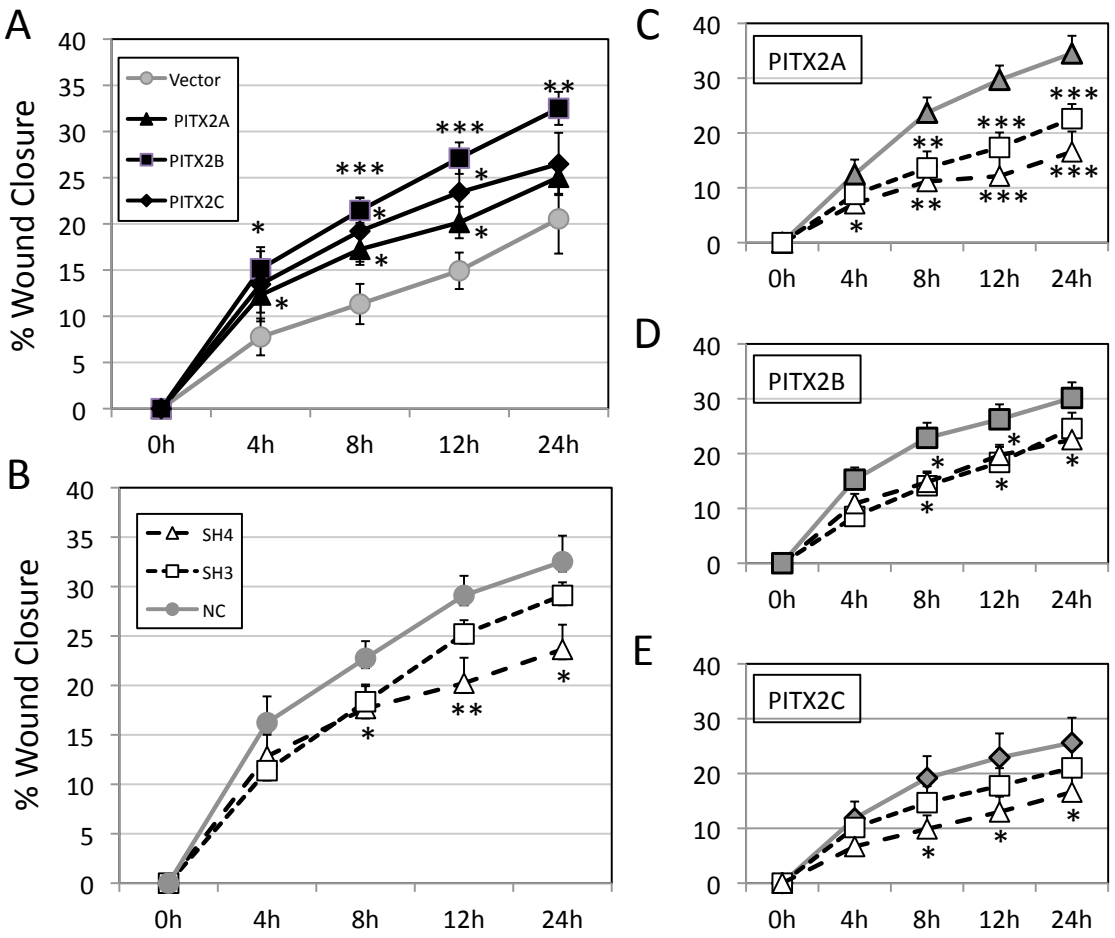


Figure 5: PITX2 over expression promotes cancer cell migration. **(A)** Effects of individual PITX2 isoform over expression on cellular motility as assessed by scratch assay closure in mitomycin C-treated PC-3 cultures. **(B)** Effects of knocking down endogenous PITX2 using two gene-specific shRNA constructs (SH3, SH4) or nonspecific control (NC). **(C-E)** Knockdown of individually overexpressed PITX2 isoforms confirms specificity of the effects on motility. Grey symbols indicate individual isoform vector (specified in legend) co-transfected with NC plasmid. White symbols denote co-transfection with particular shRNA construct as indicated in panel **B**. Data are mean±SE; significance *p<0.05, **p<0.005, ***p<0.0005 vs mean value at the same time point post scratch (X-axis) for control samples (A,B) or for single isoform co-transfected with NC plasmid (C-E). Y axis indicates % wound closure in all panels.

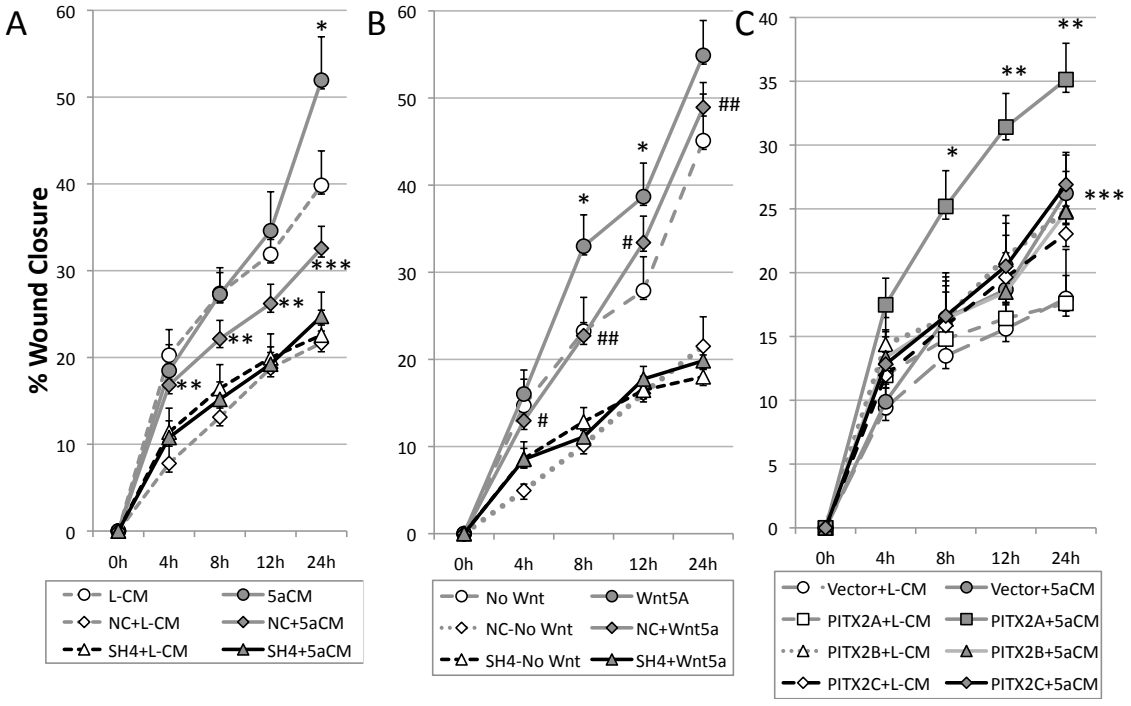


Figure 6. Effects of PITX2 knockdown or over expression on scratch wound closure in presence of Wnt5a. In vitro assay of cell migration in PC-3 monolayer cultures, transiently transfected with shRNA constructs, without (A, B) or with (C) individual PITX2 isoform expression constructs. Transfected monolayers were treated with mitomycin C, scratches were applied and cultures were supplemented with conditioned medium (CM) from Wnt5a-transfected (5aCM) or control L cells (A, C), or recombinant Wnt 5a protein (50 ng/ml) (B). L-CM from control L cells or 5aCM from Wnt5a-expressing L cells; SH4, PITX2-specific or NC, negative control shRNA; Wnt5a, recombinant Wnt5a protein. White and grey symbols denote L-CM and 5aCM (A, C) or No Wnt5a and recombinant Wnt5a (B) treatments respectively, as indicated. *$p<0.05$; **$p<0.01$; ***$p<0.001$, #$p<0.0005$, ##$p<0.00005$, vs L-CM (A, C) or vehicle (B) treated cultures at same time point. Y axis indicates % wound closure; note scale change in panel C.

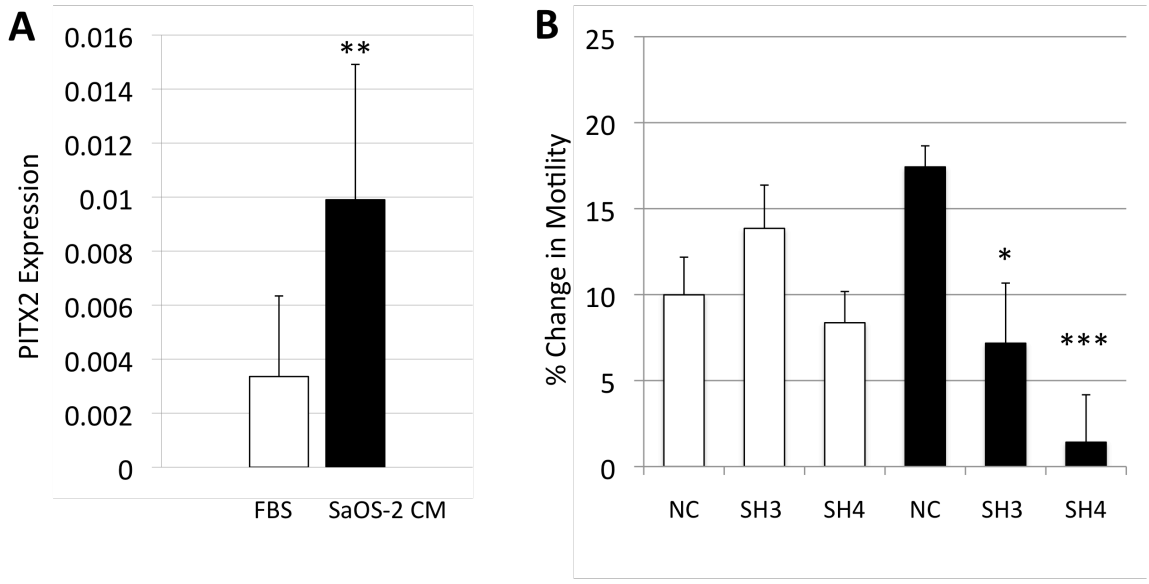


Figure 7. PITX2 implicated in the PC-3 motility response to osteoblast-conditioned medium. (A) PITX2 expression in PC-3 cells was increased after 48 h co-culture with SaOS-2 osteoblastic cells. PITX2 transcripts normalized to cyclophilin expression using the PITX2 universal primer set; **p= 0.01, significant difference vs PC-3/FBS controls. (B) PC-3 migration toward osteoblastic chemoattractant(s) was decreased by PITX2 knockdown with SH3 or SH4 (gene specific) vs NC negative control shRNA plasmid. Increase in motility of transfected cells toward control medium (10% FBS, white bars) or osteoblast-conditioned medium (10% SaOS-2 CM, black bars) relative to control cultures (mock transfected then exposed to 0% FBS, baseline). *p<0.05, ***p<0.001, difference vs NC transfected cultures of the same group. Values are mean±SE.

	Primary CaP /Normal Prostate		Hu CaP MET/Normal Prostate	
	Fold Difference	P value	Fold Difference	P value
Transcription Factors				
PITX2	10.20	0.0634	2252.14	0.0005
T	3.47	0.3847	4.33	0.0175
MYC	2.42	0.2726	3.74	0.0204
LEF1	2.26	0.2120	3.23	0.1539
FOSL1	2.02	0.4169	12.48	0.1405
TLE1	2.02	0.4056	4.68	0.0613
FOXN1	1.36	0.7901	-4.08	0.0270
SOX17	1.28	0.6944	3.24	0.1175
EP300	1.21	0.6796	-1.22	0.1244
CTNNB1	-1.12	0.8053	-1.30	0.1834
TCF7	-1.17	0.8082	-1.72	0.1982
PYGO1	-1.27	0.6571	2.14	0.0565
JUN	-1.27	0.7644	3.88	0.0389
CCND2	-1.31	0.7349	-1.36	0.4350
CCND1	-1.37	0.6320	-3.96	0.0507

TLE2	-1.47	0.6056	-1.35	0.2441
CCND3	-1.68	0.3794	-1.70	0.3490
CSNK1A1	-2.10	0.2129	-2.80	0.0153
Signal Transduction Factors				
APC	-1.59	0.4446	1.28	0.4787
FZD8	2.64	0.1541	3.37	0.0302
FZD7	1.49	0.6569	1.24	0.4922
DVL1	1.18	0.6811	4.06	0.0019
FZD2	1.11	0.9021	-4.89	0.1323
FZD3	-1.06	0.9218	-1.93	0.0748
LRP5	-1.09	0.8562	-1.58	0.0178
FBXW11	-1.14	0.7622	1.22	0.1546
FZD4	-1.17	0.8080	1.57	0.0471
AES	-1.24	0.6211	-1.18	0.0978
WISP1	-1.30	0.7465	2.57	0.0522
FZD5	-1.34	0.5244	-3.89	0.0264
FZD6	-1.40	0.4451	-4.53	0.0157
DIXDC1	-1.46	0.5816	-1.67	0.1018

SLC9A3R1	-1.65	0.1151	-3.08	0.0255
FZD1	-1.76	0.3368	-2.64	0.0739
DVL2	-1.76	0.2626	-3.14	0.0115
Canonical and Non-canonical Wnts				
WNT1	3.08	0.2452	409.38	0.0062
WNT5A	2.72	0.2550	1.79	0.1554
WNT9A	2.23	0.2857	9.79	0.0029
FGF4	2.02	0.6038	30.40	0.0423
WNT11	1.77	0.6048	5.36	0.0487
WNT7B	1.69	0.3979	-2.42	0.2114
WNT2	1.54	0.6437	-3.96	0.1880
WNT8A	1.50	0.6616	3.35	0.0509
WNT2B	1.47	0.6071	-4.66	0.1040
WNT16	1.11	0.9023	1.47	0.0341
WNT3	1.04	0.9537	-1.14	0.3081
WNT7A	-1.02	0.9895	1.09	0.8917
WNT4	-1.03	0.9638	-2.33	0.0426
WNT5B	-1.21	0.7780	-6.68	0.0099

WNT10A	-1.47	0.6703	-1.61	0.5156
WNT6	-1.56	0.6703	-2.19	0.0136
WNT3A	-1.83	0.4476	-4.63	0.0178
Protein Modification Factors				
CTBP2	1.30	0.8733	-1.01	0.9799
CSNK1G1	1.24	0.6062	1.25	0.3025
CTBP1	-1.05	0.8733	1.49	0.0504
FBXW2	-1.09	0.8410	-1.84	0.0410
LRP6	-1.15	0.7569	-1.05	0.8358
FBXW4	-1.18	0.7499	1.39	0.0135
CSNK2A1	-1.23	0.6434	1.56	0.0071
CSNK1D	-1.31	0.3662	1.04	0.7817
BTRC	-1.42	0.3978	-1.41	0.0515
NLK	-1.43	0.3654	-1.19	0.3083
FRAT1	-2.11	0.1760	-1.33	0.2520
RHOU	-2.12	0.1074	-1.41	0.1270
Wnt Pathway Antagonists				
SFRP4	4.30	0.2124	-1.52	0.6190

FSHB	2.37	0.1928	3.76	0.3647
AXIN1	1.78	0.2785	1.95	0.2671
CXXC4	1.54	0.5992	-3.07	0.1550
DKK1	1.54	0.6974	3.96	0.0543
PPP2R1A	1.27	0.5708	1.82	0.0666
GSK3B	1.12	0.7705	1.87	0.0042
BCL9	1.11	0.7665	-2.73	0.0207
FRZB	1.06	0.9532	-2.39	0.3826
PORCN	1.04	0.9458	1.03	0.9501
SFRP1	-1.04	0.9655	-8.62	0.0003
GSK3A	-1.24	0.5604	1.10	0.5060
DAAM1	-1.27	0.5407	-2.70	0.0053
PPP2CA	-1.39	0.4604	1.38	0.0954
NKD1	-1.45	0.6823	-2.87	0.0947
KREMEN1	-1.51	0.5416	1.38	0.1835
TCF7L1	-1.60	0.5948	-1.43	0.1828
CTNNBIP1	-2.06	0.0908	-3.32	0.0326
WIF1	-17.89	0.0039	-4.68	0.0182

Supplementary Table 1: Wnt pathway array analysis of clinical samples. Fold difference values for each gene were calculated for primary tumor vs normal prostate and bone metastasis vs normal prostate comparisons. Genes are grouped in categories defined by manufacturer. RNA from fresh frozen clinical samples was analyzed using the SA Bioscience RT^2 Profiler PCR Wnt Pathway array system and assessed statistically using one-way ANOVA followed by Student's T-test using the analysis template provided by SA Bioscience, as detailed in methods. Sample $n = 2$ normal prostate, 9 primary CaP tumor and 1 CaP metastasis to bone. P values <0.05 were considered to indicate significant differences from normal prostate values.

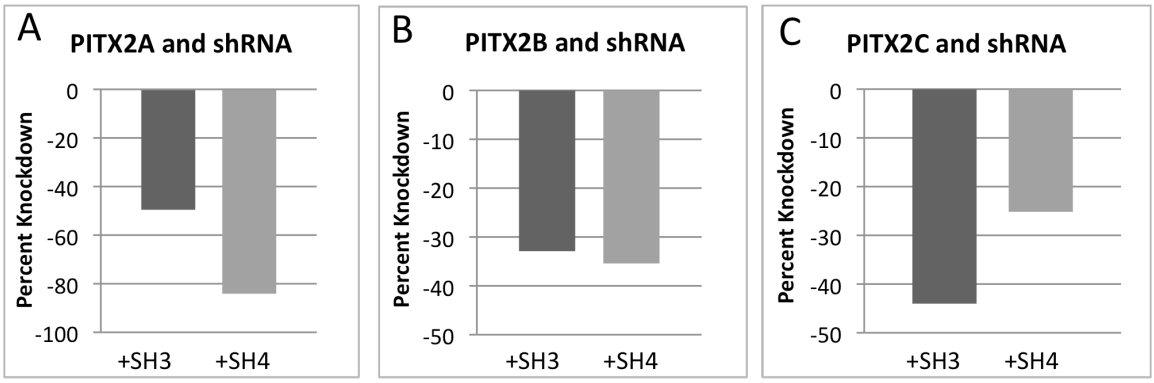


Figure S.1: Knockdown of individual PITX2 isoforms by shRNAs employed in this study. Specific FLAG-tagged PITX2 isoforms were over-expressed in PC-3 cells by transient transfection without or with individual SH3, SH4 or NC plasmid. **(A)** PITX2A + shRNA, **(B)** PITX2B + shRNA, **(C)** PITX2C + shRNA. Protein expression of the FLAG epitope tag was assessed by Western blot, with quantification of specific signal intensity normalized to β tubulin signal. Knockdown efficacy was normalized to expression of each PITX2 isoform co-transfected with NC (zero value). Effective and consistent knockdown of all three PITX2 isoforms was observed with over expression of SH3 or SH4.

Method for assessment of shRNA efficacy.

To verify the functionality of shRNA plasmids (SA Bioscience), PC-3 cells were transiently co-transfected with each individual PITX2 isoform clone (0.5μg) along with each individual shRNA clone (0.5μg). Whole cell lysates were resolved by SDS-PAGE and transferred to Immobilon-FL (Millipore). Primary antibody was anti-DYKDDDDK FLAG (Cell Signaling Technology) antibody (1:5000) or monoclonal anti-β-tubulin (1:5000). Secondary fluorescent conjugated antibodies (Rockland) was diluted in PBS:Odyssey blocking buffer (1:10000). Membranes were scanned and analyzed using the LI-COR Odyssey Infrared Imaging System and proprietary software.