Oncogene (2008) 27, 596–603

www.nature.com/onc

ORIGINAL ARTICLE

Low-density lipoprotein receptor-related protein 5 (LRP5) mediates the prostate cancer-induced formation of new bone

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The tendency of prostate cancer to produce osteoblastic bone metastases suggests that cancer cells and osteoblasts interact in ways that contribute to cancer progression. To identify factors that mediate these interactions, we compared gene expression patterns between two bone-derived prostate cancer cell lines that produce osteoblastic (MDA PCa 2b) or osteolytic lesions (PC-3). Both cell lines expressed Wnt ligands, including WNT7b, a canonical Wnt implicated in osteogenesis. PC-3 cells expressed 50 times more Dickkopf-1 (DKK1), an inhibitor of Wnt pathways, than did MDA PCa 2b cells. Evaluation of the functional role of these factors (in cocultures of prostate cancer cells with primary mouse osteoblasts (PMOs) or in bone organ cultures) showed that MDA PCa 2b cells activated Wnt canonical signaling in PMOs and that DKK1 blocked osteoblast proliferation and new bone formation induced by MDA PCa 2b cells. MDA PCa 2b cells did not induce bone formation in calvaria from mice lacking the Wnt co-receptor *Lrp5*. In human specimens, WNT7b was not expressed in normal prostate but was expressed in areas of high-grade prostate intraepithelial neoplasia, in three of nine primary prostate tumor specimens and in 16 of 38 samples of bone metastases from prostate cancer. DKK1 was not expressed in normal or cancerous tissue but was expressed in two of three specimens of osteolytic bone metastases ($P = 0.0119$). We conclude that MDA PCa 2b induces new bone formation through Wnt canonical signaling, that LRP5 mediates this effect, and that DKK1 is involved in the balance between bone formation and resorption that determines lesion phenotype.
Oncogene (2008) **27,** 596–603; doi:10.1038/sj.onc.1210694; published online 13 August 2007

Keywords: WNT; WNT7b; prostate cancer; bone metastases; osteoblasts; DKK1

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Received 30 July 2006; revised 12 March 2007; accepted 1 June 2007; published online 13 August 2007

Introduction

Prostate cancer has a high tropism for bone and produces typical osteoblastic bone metastases (Cook and Watson, 1968). Bone metastases are responsible for most of the mortality and morbidity of advanced prostate cancer. These observations suggest that interactions between prostate cancer cells and osteoblasts (the bone-forming cells) contribute to the lethal progression of the disease. Understanding the mechanisms underlying these interactions will provide insights into the pathogenesis of prostate cancer progression in bone and will open opportunities for rational therapy development.

Several factors released by prostate cancer cells affect osteoblast biology, and are thought to participate in the pathophysiology of prostate cancer progression in bone (Logothetis and Lin, 2005). WNTs are a family of secreted proteins that affect aspects of skeletal development and tumor biology (van Es *et al*., 2003; Westendorf *et al*., 2004). The WNT pathway consists of 19 WNT ligands, 10 Frizzle receptors and two low-density lipoprotein receptor-related protein (LRP) co-receptors, LRP5 and LRP6 (van Es *et al*., 2003). Binding of WNT proteins to their receptors activates one of three intracellular pathways—the planar cell polarity pathway, the WNT/Ca++ pathway or the WNT canonical signaling pathway. In the canonical pathway, WNT signaling stabilizes free cytosolic β-catenin that translocates to the nucleus and activates the expression of specific genes. WNT signaling is regulated by a diverse group of antagonists, including Dickkopf 1 (DKK1). DKK1 prevents activation of WNT signaling by interacting with extracellular domains in LRP5/6 proteins (Mao *et al*., 2001; Westendorf *et al*., 2004).

We previously showed that the prostate cancer cell line MDA PCa 2b, derived from a bone metastasis of prostate cancer, induced increases in osteoblast proliferation and differentiation *in vitro* and new bone formation *in vivo* (Yang *et al*., 2001; Fizazi *et al*., 2003). The paracrine factors that mediate the bone-forming phenotype of MDA PCa 2b cells are not known. We reasoned that comparing the genes

expressed by MDA PCa 2b and PC-3 cells (also derived from bone metastases but producing osteolytic rather than osteoblastic lesions) could provide clues as to which paracrine factors are involved in the stimulation of new bone. Here, we report for the first time that Wnt canonical signaling is involved in the MDA PC 2b-induced formation of new bone. We also detected the WNT canonical ligand WNT7b in specimens of human prostate cancer osteoblastic lesions and DKK1 in some specimens that did not display an osteoblastic phenotype. These findings contribute to the hypothesis that signaling mediated by the WNT canonical pathway modulates osteoblast function and is involved in the induction of the osteoblastic phenotype in prostate cancer bone metastasis.

Results

Bone-derived prostate cancer cell lines influence primary mouse osteoblast proliferation

We compared two bone-derived prostate cancer cell lines, MDA PCa 2b and PC-3. Implanted in the bones of immunodeficient mice, MDA PCa 2b cells produce bone-forming effects, whereas PC-3 cells produce predominantly bone-destroying effects (Yang *et al*., 2001; Fizazi *et al*., 2003). Coculture of these cells with primary mouse osteoblasts (PMOs) had distinctly different effects: MDA PCa 2b cells (or conditioned medium from those cells) enhanced osteoblast proliferation, but PC-3 cells (or their medium) suppressed osteoblast proliferation (Figures 1a and b). These results suggest that interactions between prostate cancer cells and osteoblasts are mediated by soluble factors.

DKK1 is expressed by PC-3 cells but not by MDA PCa 2b cells

Next, we looked for genes expressed at different levels by MDA PCa 2b and PC-3 cells, with an eye toward genes implicated in osteoblast proliferation. Analysis of 30 000 genes showed that *DKK1* was upregulated in PC-3 cells (by 50 times relative to MDA PCa 2b cells; Supplementary Table 1). These findings were confirmed by reverse transcriptase–PCR (RT–PCR) of cell lysates and western blot analysis of conditioned medium (Figures 2a and b). Immunohistochemical staining showed high DKK1 levels in tumors produced by PC-3 cells grown in the femurs of SCID mice, but not in tumors produced by MDA PCa 2b cells grown in this way (Figure 2c). These results establish an association between the effects of prostate cancer cells on osteoblast proliferation and DKK1 expression.

MDA PCa 2b cells express Wnt ligands and activate Wnt canonical signaling in osteoblasts

Because DKK1 is an extracellular inhibitor of WNT canonical signaling, we focused on the expression of WNT-associated genes by MDA PCa 2b and PC-3 cells by testing a second gene array containing the sequences of 96 genes in the WNT signaling pathway. Levels of *DKK1* transcript in PC-3 cells were 135 times higher than those in MDA PCa 2b cells, confirming the results of the previous gene array analysis. The WNT ligands *WNT1*, *WNT7b* and *WNT8a* were expressed by both cell lines in the gene array, but only *WNT7b* (not *WNT1* or *WNT8a*) was confirmed by RT–PCR in both cell lines (Figure 2d). Interestingly, *WNT7b* was detected in two prostate cancer cell lines not derived from bone metastases (LNCaP and DU145) but not in normal prostatic epithelial cells (Figure 2d). Immunohistochemical analysis also confirmed WNT7b protein expression by MDA PCa 2b and PC-3 cells grown in the femurs of SCID mice (Figure 2e).

To see if MDA PCa 2b cells could activate canonical Wnt signaling in osteoblasts, we cocultured MDA PCa 2b cells and PMOs that had been transfected with a TCF reporter gene construct (TOP-flash) (Korinek *et al*., 1997). Luciferase activity in PMOs was higher when those cells were cocultured with MDA PCa 2b cells than when grown alone (Figure 2f). These results suggest that soluble factors produced by MDA PCa 2b cells induce canonical Wnt activity in osteoblasts.

DKK1 inhibits the prostate cancer-induced proliferation of primary mouse osteoblasts

Next, to clarify the effect of DKK1 in the MDA PCa 2b-induced osteoblast proliferation, we treated PMOs with medium conditioned by MDA PCa 2b cells, with or

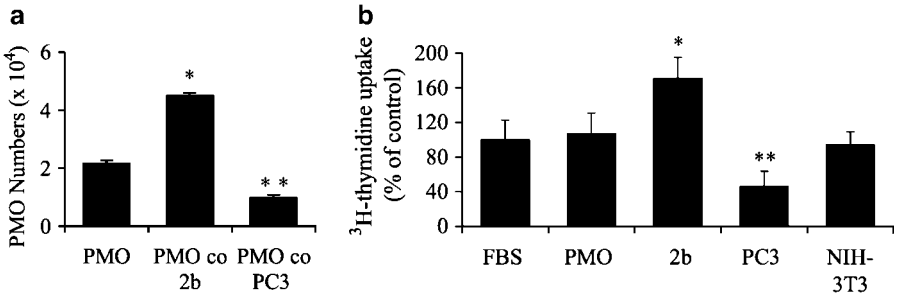

Figure 1 (**a**) Osteoblastic (MDA PCa 2b) prostate cancer cells, but not PC-3 (osteolytic) cells, induce osteoblast proliferation. Similar results were found in two independent experiments. *,**$P<0.001$ vs control (primary mouse osteoblasts (PMOs) monoculture). (**b**) ^{3}H-thymidine incorporation into PMOs after 48 h of culture alone (fetal bovine serum (FBS)) or with medium conditioned by PMOs, MDA PCa 2b, PC-3 or NIH-3T3 cells. Results are expressed as a percentage of the control (FBS) (set at 100%). Similar results were found in two independent experiments. *,**$P<0.01$ vs FBS.

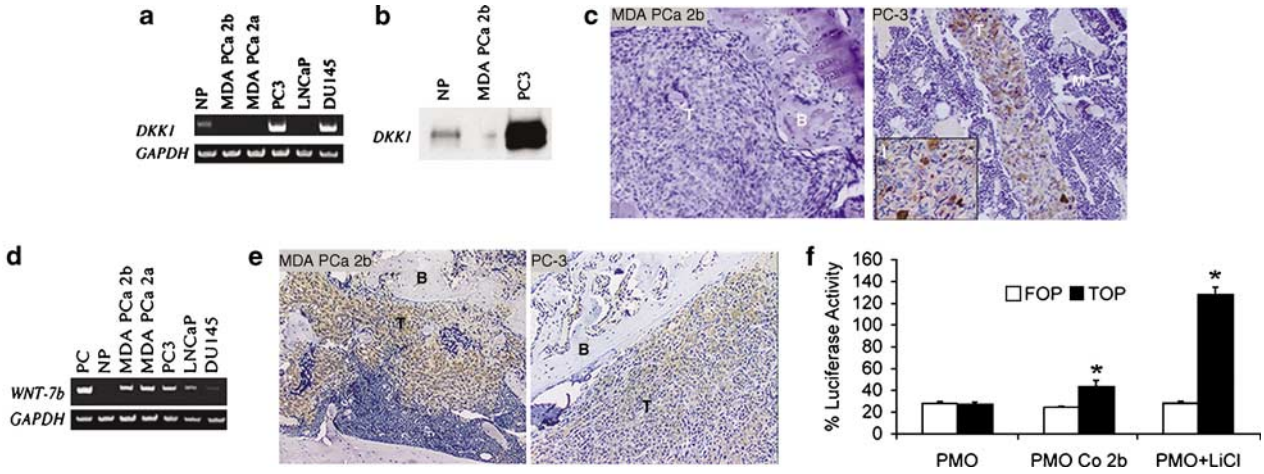

Figure 2 Dickkopf-1 (DKK1) and WNT7b expression in prostate cancer cell lines and canonical WNT signaling in osteoblasts. (**a**) RT–PCR shows DKK1 expression in PC-3 and DU145 prostate cancer cells; normal prostatic (NP) epithelial cells. (**b**) Western blots show DKK1 in conditioned medium from PC-3 cells but not from MDA PCa 2b cells. (**c**) Immunohistochemical stains show DKK1 in SCID mouse femurs implanted with PC-3 cells but not in those with MDA PCa 2b cells. T, tumor; B, bone; M, bone marrow; magnification, $\times 100$. Inset (I), DKK1 in PC-3 cells at $\times 400$. (**d**) RT–PCR shows Wnt7b expression in MDA PCa 2a, MDA PCa 2b, PC-3 and LNCaP prostate cancer cells and in cDNA from day 11 mouse embryos (positive control (PC)); NP epithelial cells. (**e**) Immunohistochemical stains show WNT7b in SCID mouse femurs implanted with MDA PCa 2b or PC-3 cells. T, tumor; B, bone; magnification, $\times 100$. (**f**) TOP-flash reporter activity in PMOs grown alone or in coculture with MDA PCa 2b cells; PMOs treated with LiCl was a positive control and FOP-flash reporter activity a negative control. Similar results were obtained in an independent experiment. $^*P<0.01$.

without recombinant human DKK1 (rhDKK1), and assessed DNA synthesis in the osteoblasts by thymidine incorporation. DNA synthesis in the osteoblasts was enhanced by conditioned medium, but this enhancement was reversed by rhDKK1 (Figure 3a), confirming that WNT signaling is involved in MDA PCa 2b-induced new bone formation and suggesting that modulation by DKK1 may account for the non-blastic phenotype of PC-3 cells.

DKK1 inhibits prostate cancer-induced new bone formation

To extend these results, we used a bone organ culture assay and found that mouse calvaria treated with medium conditioned by MDA PCa 2b cells had more total bone area, new bone area and osteoblasts than did the untreated controls (Figure 3b), indicating that factors in MDA PCa 2b-conditioned medium induce new bone formation. To see if DKK1 could reverse this effect, we established a DKK1-overexpressing MDA PCa 2b transfectant (with the goal of mimicking physiological conditions more closely than use of rhDKK1) and confirmed the synthesis of DKK1 by immunofluorescence and its secretion into the culture medium by western blotting (Figure 3c). Coculture experiments demonstrated that PMO proliferation was blocked by MDA PCa2b-DKK1 cells (Figure 3d). Similar effects were evident in the bone organ culture assay; calvaria treated with medium conditioned by MDA PCa 2b-vector-control cells showed increases in total bone ($P=0.0244$), new bone ($P=0.0372$) and osteoblast numbers ($P=0.0060$) relative to untreated control calvaria (Figure 3e (black vs white bars on bottom)), but medium conditioned by the MDA PCa 2b–DKK1 transfectants did not affect bone formation (Figure 3e). Collectively, these findings demonstrate that MDA PCa 2b cells produce factors that lead to osteoblast proliferation and increased bone mass; moreover, because DKK1, which interacts with LRP5 or LRP6 (Tamai *et al*., 2000; Bafico *et al*., 2001; Mao *et al*., 2001), reversed this effect, these factors are probably ligands for the LRP5 or LRP6 receptors.

Lrp5 mediates prostate cancer-induced new bone formation

To confirm that the LRP5/6 Wnt co-receptors mediate prostate cancer-induced new bone formation and to see if either receptor predominates in this effect, we treated the calvaria of *lrp5*-knockout mice and their wild-type littermates with medium conditioned by MDA PCa 2b cells. (As a quality control, we also used the calvaria of wild-type CD1 mice, which were used for most experiments.) As expected, calvaria of the CD1 mice treated with MDA PCa 2b-conditioned medium had more total bone area ($P=0.0067$), new bone area ($P=0.0001$) and osteoblasts ($P=0.0171$) than did the untreated controls (Figure 4b, black vs white bars). Moreover, calvaria of $lrp5^{+/+}$ mice treated with MDA PCa 2b-conditioned medium also had more total bone area ($P=0.0380$), new bone area ($P=0.0133$) and osteoblasts ($P=0.0040$) than did the untreated controls, but the same conditioned medium had no effect on any of these variables in the calvaria of $lrp5^{-/-}$ mice

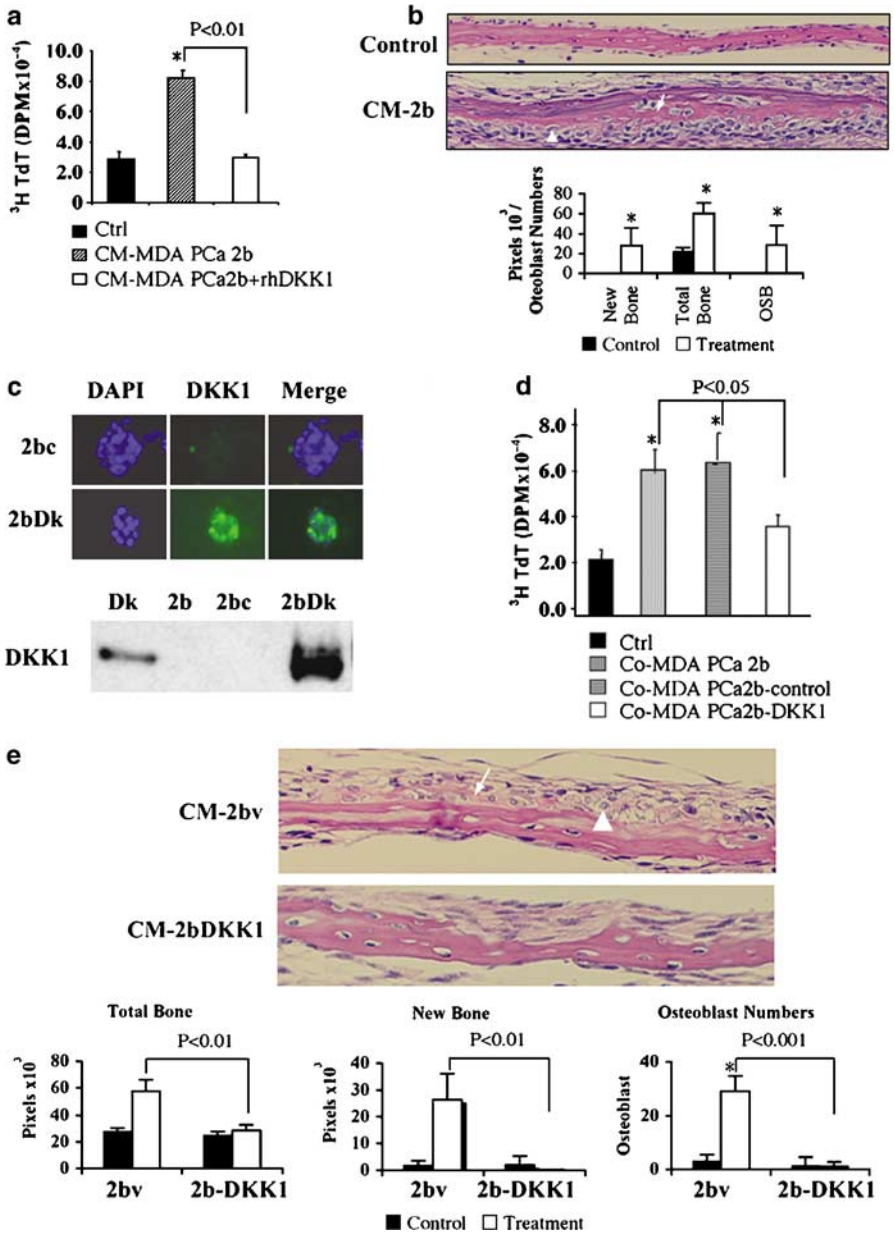

Figure 3 Dickkopf-1 (DKK1) blocks prostate cancer-induced osteoblast proliferation and new bone formation. (**a**) ^{3}H-thymidine incorporation into PMOs after a 48-h culture with medium only (ctrl), medium conditioned by MDA PCa 2b cells only (CM-MDA PCa 2b) or CM-MDA PCa 2b plus recombinant human DKK1 (rhDKK1). Results were confirmed in an independent experiment. *$P<0.01$. (**b**) Top, hematoxylin- and eosin-stained cross-sections of calvaria cultured alone (Control) or treated with 1 × conditioned medium from MDA PCa 2b (CM-2b) show increases in new bone (arrow) and osteoblasts (arrowhead) in the treated condition. Each condition involved calvaria from three different mice. Magnification, ×200. Bottom, new bone area and total bone area (quantified from stained sections by the NIH Image J program) and osteoblasts (OSBs) (quantified with the Osteomeasure system (OsteoMetrics, Atlanta, GA, USA)) show increased bone formation from exposure to CM. *$P<0.0125$. (**c**) Top, micrograph of MDA PCa 2b cells (2bc) and MDA PCa 2b cells transfected with a DKK1 expression vector (2b Dk1). Bottom, western blot of medium containing rhDKK1 (Dk) or medium conditioned by MDA PCa 2b cells (2b), MDA PCa 2b cells transfected with a control vector (2bc) or PCa 2b-DKK1 transfectants (2bDk). (**d**) ^{3}H-thymidine incorporation in PMOs grown alone (Ctrl) or in coculture with MDA PCa 2b cells (Co-MDA-PCa2b), MDA PCa 2b-transfected with a control vector or MDA PCa 2b–DKK1 transfectants. Results were confirmed in an independent experiment. *$P<0.05$. (**e**) Cross-sections and quantitative results of bone organ cultures treated with 1 × conditioned medium from MDA PCa 2b-vector control cells (CM-2bv) or 1 × conditioned medium from MDA PCa 2b–DKK1 cells (CM-2bDKK1); treatment conditions and analyses were as described in (**b**). Arrow indicates new bone; arrowhead indicates osteoblasts. *$P<0.0125$.

(Figures 4a and b). Differences between the wild type and knockout conditions were also significant for total bone formation, new bone formation and osteoblast numbers (Figures 4a and b). These results implicate LRP5 in the formation of new bone induced by MDA PCa 2b prostate cancer cells.

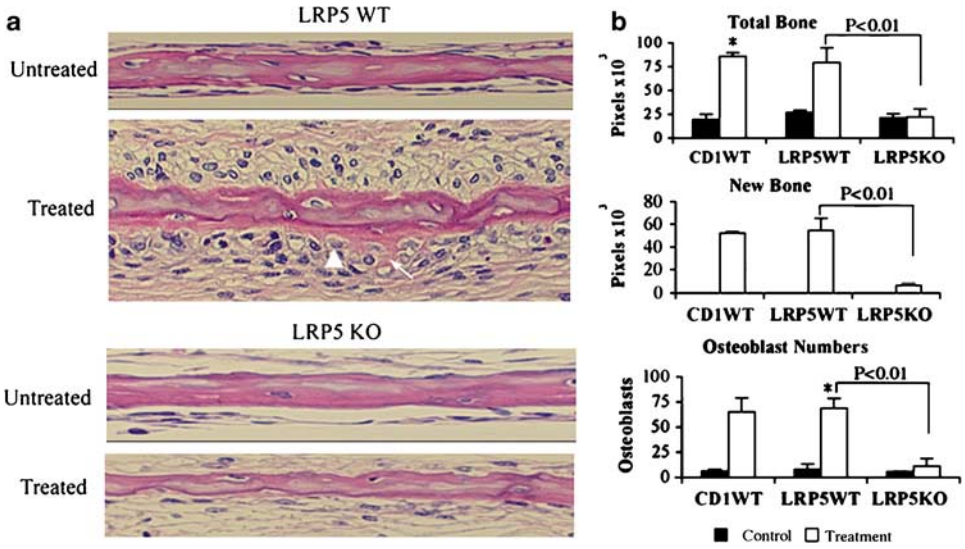

Figure 4 MDA PCa 2b prostate cancer cells do not induce bone formation in *lrp5*$^{-/-}$ mice. (**a**) Hematoxylin- and eosin-stained cross-sections of calvaria derived from *Lrp5*$^{-/-}$ (knockout, KO) or *Lrp5*$^{+/+}$ (wild type, WT) mice treated with medium conditioned by MDA PCa 2b cells. Arrow indicates new bone; arrowhead indicates osteoblasts. (**b**) Total bone area, new bone area and osteoblast numbers were quantified on stained sections as described in Figure 3b. *$P<0.0125$.

WNT7b and DKK1 expression in human prostate cancer specimens

To explore the clinical relevance of these findings, we used immunostaining to detect WNT7b and DKK1 in samples of bone metastases from prostate cancer and in specimens of normal prostate and primary prostate cancer. WNT7b was not found in normal prostate specimens (Figure 5) but was present (at intensity 2) in some areas of high-grade prostate intraepithelial neoplasia, in three of nine primary prostate cancer specimens (Gleason grades 7–9) (33%) (not shown) and in 16 of 38 bone metastasis specimens (42%) (Figure 5). These observations suggest that expression of WNT7b in prostate cancer increases during cancer progression. No association was found between WNT7b expression and bone phenotype in patients with bone metastases. However, WNT7b is known to activate Wnt canonical signaling in osteoblasts (Zhang *et al*., 2004; Hu *et al*., 2005), and prostate cancer bone metastases is known to be heterogeneous (Shah *et al*., 2004), and so WNT7b could be involved in prostate cancer-induced new bone formation in a subpopulation of patients.

Immunostaining for DKK1 was negative in both normal prostate and primary prostate cancer specimens (Figure 5); of the 26 bone-metastasis lesions examined, DKK1 was detected in two of three with osteolytic features but was negative in seven osteoblastic and thirteen mixed lesions (no radiological reports were available from three patients) (Figure 5). χ^2-tests showed that bone phenotype was associated with DKK1 expression ($P=0.0119$). These results agree with our finding that DKK1 staining was negative in osteoblastic MDA PCa2b cells but positive in osteolytic PC-3 cells; they further suggest that some prostate cancer cells may secrete DKK1 and that DKK1 could negatively regulate the formation of new bone induced by prostate cancer cells.

Discussion

Our results indicate that prostate cancer cells activate Wnt canonical signaling in osteoblasts: MDA PCa 2b cells expressed WNTs and induced TCF reporter activity in osteoblasts; and the effects of MDA PCa 2b cells on bone formation could be blocked by DKK1 and inhibited by genetic blockade of the Wnt co-receptor Lrp5. We further found that prostate cancer cells in bone metastases express at least one Wnt canonical ligand (WNT7b) and that DKKI (an inhibitor of WNT canonical signaling) was expressed in some *osteolytic* prostate cancer metastases. Thus, WNT7b and DKKI may contribute to the phenotype of at least some prostate cancer bone metastases by modulating the balance between bone formation and bone resorption.

DKK1 was initially identified as an inhibitor of WNT-induced axis duplication in *Xenopus* development (Glinka *et al*., 1998). Subsequent studies implicated DKK1 in the pathogenesis of osteolytic lesions in patients with multiple myeloma (Tian *et al*., 2003). During our study, Hall *et al*. (2005) reported that PC-3 prostate cancer cells express DKK1 and showed by genetic manipulation of DKK1 in C4-2b prostate cancer cells that WNT signaling participates in prostate cancer-induced new bone formation. Our observations are consistent with these findings and extend further to the identification of the expression of WNT7b and DKK1 in human prostate cancer specimens. Our discovery of DKKI in some osteolytic specimens is of particular interest and suggests that increased expression of WNT ligands or DKK1 by prostate cancer cells could contribute to the metastatic phenotype, further underscoring how heterogeneity in pathogenesis of prostate cancer bone metastases could account for clinical variations in response to therapy and prognosis.

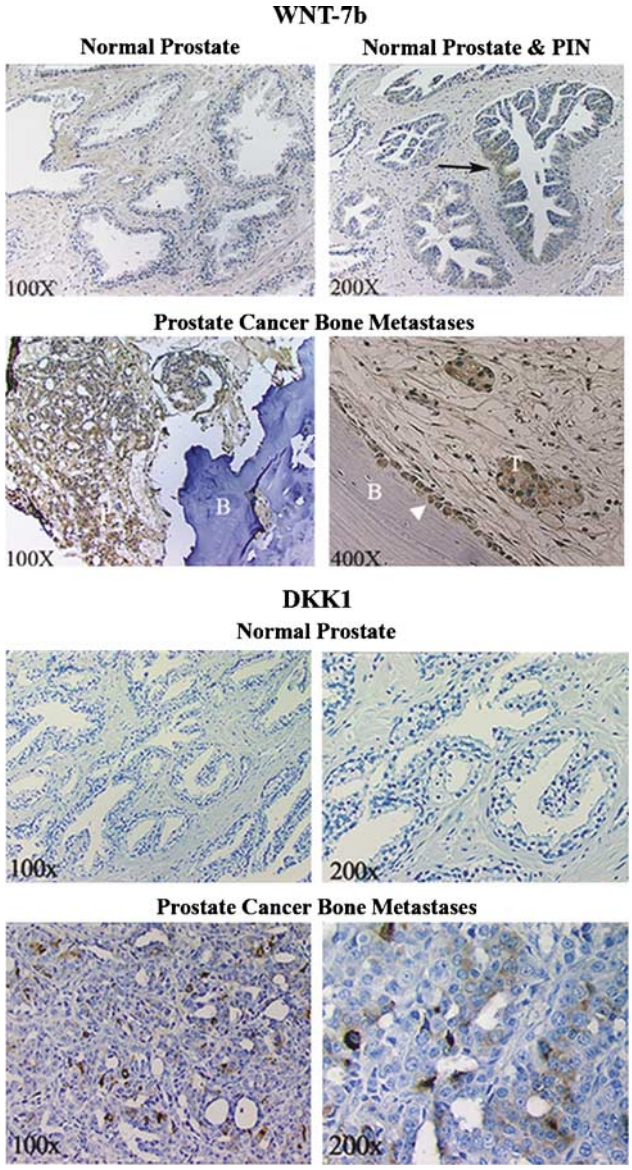

Figure 5 Immunohistochemical stains for WNT7b (top) and Dickkopf-1 (DKK1) (bottom) in human specimens. Arrow indicates area of prostate intraepithelial neoplasia (PIN) with WNT7b positive staining; arrowhead indicates osteoblasts: T, tumor; B, bone.

WNT canonical signaling has been implicated in the regulation of bone mass during development and adulthood in humans and mice (Hu *et al*., 2005; Kolpakova and Olsen, 2005). The specific pathway activated depends on the WNT/FRIZZLE combination (with or without the LRP5/6 co-receptors). WNT7b, an effective activator of the canonical pathway, has been implicated in osteoblast differentiation and identified as a potential endogenous ligand that regulates osteogenesis *in vivo* (Zhang *et al*., 2004; Hu *et al*., 2005). WNT7b is a strong candidate for involvement in prostate cancer bone metastasis because it is expressed by prostate cancer cells and is a known LRP5/6 ligand. Studies of the murine embryonic mesenchymal cells suggest that WNT7b signaling mediates osteogenesis induced by hedgehog proteins, another pathway implicated in prostate cancer initiation and progression (Karhadkar *et al*., 2004). We found here that WNT7b was expressed in prostate intraepithelial neoplasia and in a subgroup of primary prostate cancer specimens. Activation of hedgehog signaling during prostate cancer development could upregulate WNT7b, thereby favoring the progression of prostate cancer to bone metastases; another possibility is that WNT7b may participate in an autocrine loop that favors prostate cancer growth.

LRP5 and LRP6 share high homology (71% identical amino acids) and both interact with DKK1; however, no conclusive information is available as to how these co-receptors differ in mediating WNT canonical signaling (Westendorf *et al*., 2004). Indeed, they seem to have overlapping roles in the accrual of bone mass, although LRP5 may preferentially regulate cortical bone and LRP6 trabecular bone mass (Ferrari *et al*., 2005). We provide here evidence that LRP5 mediates the prostate cancer-induced formation of new bone in a bone organ culture assay, implying that LRP5 blockade might control prostate cancer-induced new bone formation. So far no study has addressed the role of *lrp6* in prostate cancer, and thus additional studies are necessary to better understand the pathogenesis of prostate cancer growth within bone.

In conclusion, laboratory and clinical evidence have mechanistically implicated the bone-forming phenotype of prostate cancer bone metastasis in disease progression (Logothetis and Lin, 2005). Current treatment strategies for prostate cancer bone metastases have focused on inhibiting the growth of both prostate cancer cells and bone (Loberg *et al*., 2005). Factors that have been implicated in osteoblastic prostate cancer metastases include bone morphogenetic proteins (Dai *et al*., 2005; Feeley *et al*., 2005) and endothelin-1 (Nelson *et al*., 2003). Our findings suggest that the WNT canonical pathway, specifically Lrp5, is also involved in osteoblastic metastases of prostate cancer. Further development of strategies to inhibit this pathway to treat prostate cancer bone metastases is warranted.

Materials and methods

Cell cultures

Human MDA PCa 2b cells (Navone *et al*., 1997) were propagated in BRFF-HPC1 medium (Athena Enzyme Systems, Baltimore, MD, USA) with 20% fetal bovine serum (Sigma Aldrich, St Louis, MO, USA). The prostate cancer cell lines PC-3, LNCaP and DU145 were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum. Human prostate epithelial cells were purchased from Clonetics-BioWhittaker (Walkersville, MD, USA) and grown according to the manufacturer's instructions. Primary cultures of osteoblasts were established from calvaria of CD1 mouse pups and grown alone or in coculture with prostate cancer cells in a bicompartmental system in which the cells share medium but are not in physical contact (Yang *et al*., 2001).

Conditioned medium
Prostate cancer cells grown to 60–70% confluence were placed in serum-free BRFF-HPC1 medium for 48 h. Conditioned medium was then collected, filtered and concentrated with a Centrifugal Filter Unit with low binding and a 10-kDa cut-off membrane (Millipore, Billerica, MA, USA). Untreated, concentrated BRFF-HPC1 medium was used as a control.

Mitogenic assays
PMOs were grown in α-minimal essential medium with 10% fetal bovine serum alone or in coculture with prostate cancer cells; cultures were supplemented with conditioned medium from MDA PCa 2b cells (with or without rhDKK1 (R&D Systems, Minneapolis, MN, USA)) or PC-3 cells. Proliferation and DNA synthesis were assessed by adding [^{3}H]-thymidine (NEN Life Science Products, Boston, MA, USA) during the final 3 h of culture, and incorporation was measured as described elsewhere (Freshney, 1994).

Gene array analyses
Total RNA was isolated with TRIzol reagent (Invitrogen). Differences in genes expressed by MDA PCa 2b and PC-3 cells were analysed with cDNA gene arrays containing the sequences of 30 000 genes and expressed sequence tags (Millennium Pharmaceuticals, Cambridge, MA, USA). A second analysis of differences in the expression of WNT-related genes was done with a cDNA gene array containing sequences of 96 genes in the WNT signaling pathway (SuperArray Bioscience Corporation, Frederick, MD, USA). Pair-wise comparisons of gene expression were made with normalized data.

Reverse transcriptase–PCR
RT–PCR was done by standard procedures. Gene-specific primers used for cDNA amplification for DKK1 were 5′-caac gctatcaagaacctgc-3′ (sense) and gatcttggaccagaagtgtc (antisense), resulting in a 510-bp product; for wnt7b, cgtgtttctctgc tttggcg (sense) and tggctgcaggcagcggtgac (antisense), resulting in a 341-bp product.

Western blot analysis
For western blotting, 15 μg of protein from concentrated conditioned medium was separated on 4–20% Tris-glycine polyacrylamide gels and transferred to nitrocellulose membranes (Novex, San Diego, CA, USA). DKK1 was detected by electrochemiluminescence (Amersham, Arlington-Heights, IL, USA) with goat anti-human DKK1 antibody (R&D Systems).

In vivo *prostate cancer intrabone model*
MDA PCa 2b and PC-3 cells were injected into the femurs of male SCID mice (Charles River Laboratories, Wilmington, MA, USA) as previously described (Yang *et al*., 2001).

Immunohistochemical analysis of bone specimens
Tissue sections (4 μm thick) were boiled in 0.01 M sodium citrate (pH 6.0) with 0.1% NP-40 for 10 min, treated with 3% H_2O_2 in methanol for 15 min and then incubated at 4°C overnight with anti-DKK1 (R&D Systems) (1:100 dilution) or -WNT7b (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:75 dilution). Antibody binding was detected with an LAB kit with 3,3′-diaminobenzidine as the chromogen (DAKO, Carpinteria, CA, USA). Sections were counterstained with hematoxylin.

TCF luciferase reporter assay and transfections
We used a TOP-flash reporter gene construct containing four consensus TCF binding sites, a minimal Fos binding site and a luciferase reporter (Korinek *et al*., 1997). A FOP-flash construct with a mutated TCF binding site was used as a negative control. PMOs were transfected using Lipofectamine 2000 (Invitrogen) and 2 μg of plasmid DNA. *Renilla* was used as a co-reporter vector to normalize transfection efficiency. PMOs were then cocultured with MDA PCa 2b cells for 48 h, after which PMOs were harvested and luciferase activity measured with a Dual-Luciferase Assay System (Promega, Madison, WI, USA). MDA PCa 2b cells were transfected with a human *DKK1* expression vector or an empty vector (gifts from Dr Vincent J Hearing, NIH, Bethesda, MD, USA) by standard procedures (Sambrook *et al*., 1989). Pooled G418-resistant transfectants were used to avoid bias from clonal selection. Transfection efficiency was verified by immunofluorescence staining and western blotting.

Organ culture bone-formation assay
Bone formation was assessed as described elsewhere (Garrett, 2003). Briefly, calvaria from 4-day-old CD1 mouse pups (Charles River Laboratories) or *lrp5*$^{-/-}$ or *lrp5*$^{+/+}$ mice (kindly provided by Dr G Karsenty, Columbia University, New York, NY, USA) were excised, cut in half and cultured for 7 days in six-well plates. Half of each calvarium was placed in BGJ medium (Sigma Aldrich) containing 0.1% bovine serum albumin and 1× (that is, not concentrated) conditioned medium derived from MDA PCa 2b cells that were untreated, transfected with empty vector or transfected with DKK1. The other halves were placed in BGJ medium containing 0.1% bovine serum albumin and used as a control. Medium was changed every 2 days, and the experiment was terminated at the end of 7 days. At that time, the calvaria halves were fixed, decalcified, paraffin-embedded, sectioned, stained with hematoxylin and eosin and photographed with a high-resolution video camera (Sony 3CCD) linked to a Nikon microscope. Total bone area, new bone area and numbers of osteoblasts were quantified in the stained sections as described elsewhere (Garrett, 2003).

Human prostate and bone metastasis specimens
We tested six samples of normal prostate tissue from non-tumorous areas of prostatectomy specimens; two sections showed high-grade prostate intraepithelial neoplasia. Another nine samples of prostate cancer (two Gleason 6, three Gleason 7, two Gleason 8 and two Gleason 9) were from radical prostatectomy specimens from men with organ-confined disease and no prior therapy. Another 38 samples were from bone metastases of prostate cancer: two were derived from men that had not received any systemic therapy and the other 36 had experienced relapse after androgen-deprivation therapy. Of those 36 men, 32 had undergone androgen-deprivation therapy plus chemotherapy and four androgen-deprivation only. Radiologic evaluation of the bony areas of the specimens revealed 16 samples to be osteoblastic, 16 mixed type and three osteolytic. No radiological reports were available for three patients. All samples were selected from a tissue bank supported by the Prostate Cancer Specialized Program of Research Excellence at MD Anderson Cancer Center. All sections were from formalin-fixed, paraffin-embedded tissue specimens; sections were decalcified in formic acid. Slides were read independently by two investigators (FVL and NMN). Evaluations were concordant in 90% of the readings; differences were resolved by consensus after joint review. For WNT7b immunostaining, sections were classified according to staining intensity (0, 1 or 2) and the percentage of cells showing

positive immunostaining. Tissue specimens were considered positive for WNT7b expression if more than 30% of cells showed staining intensity of 1 or 2. Positive controls for Wnt7b and DKK1 were MDA PCa 2b cells or PC-3 cells, respectively, grown in the bones of SCID mice.

Statistical analyses

Correlations between DKK1 expression and bone lesion type were analysed with Fisher's exact tests. Two-sample *t*-tests for equal variance were used to identify differences between the means of different treatment groups. *P*-values <0.05 were considered statistically significant. For organ culture experiments, paired *t*-tests were used to compare control and treatment in each treatment group, and two-sample *t*-tests for equal variance were used to compare the treatment groups between two independent groups (for example, 2bc treatment vs 2bDKK1 treatment). Because of the multiple comparisons in the bone-formation studies, we adjusted the significance level to $0.05/4 = 0.0125$; thus, results were considered significant if *P* was less than 0.0125.

Acknowledgements

This work was supported by grants from the US National Institutes of Health (R01CA096797, P50CA90270 and R01CA111479) and the Prostate Cancer Foundation to NMN and SHL, and Postdoctoral Traineeship Award PC050182 from the Department of Defense Congressionally Directed Medical Research Program to ZGL.

References

Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA. (2001). Novel mechanism of Wnt signaling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat Cell Biol* **3**: 683–686.

Cook GB, Watson FR. (1968). Events in the natural history of prostate cancer: using salvage curves, mean age distributions and contingency coefficients. *J Urol* **96**: 87–96.

Dai J, Keller J, Zhang J, Lu Y, Yao Z, Keller ET. (2005). Bone morphogenetic protein-6 promotes osteoblastic prostate cancer bone metastases through a dual mechanism. *Cancer Res* **65**: 8274–8285.

Feeley BT, Gamradt SC, Hsu WK, Liu N, Krenek L, Robbins P *et al*. (2005). Influence of BMPs on the formation of osteoblastic lesions in metastatic prostate cancer. *J Bone Miner Res* **20**: 2189–2199.

Ferrari SL, Deutsch S, Antonarakis SE. (2005). Pathogenic mutations and polymorphisms in the lipoprotein receptor-related protein 5 reveal a new biological pathway for the control of bone mass. *Curr Opin Lipidol* **16**: 207–214.

Fizazi K, Yang J, Peleg S, Sikes CR, Kreimann EL, Daliani D *et al*. (2003). Prostate cancer cells-osteoblast interaction shifts expression of growth/survival-related genes in prostate cancer and reduces expression of osteoprotegerin in osteoblasts. *Clin Cancer Res* **9**: 2587–2597.

Freshney RI. (1994). *Culture of Animal Cells. A Manual of Basic Techniques*. Wiley-Liss Inc: New York.

Garrett R. (2003). Assessing bone formation using mouse calvarial organ culture. In: Helfrich MH and Ralston SH (eds). *Bone Research Protocols: Methods in Molecuar Medicine*. Humana Press: Totowa, NJ,pp 183–198.

Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**: 357–362.

Hall CL, Bafico A, Dai J, Aaronson SA, Keller ET. (2005). Prostate cancer cells promote osteoblastic bone metastases through Wnts. *Cancer Res* **65**: 7554–7560.

Hu H, Hilton MJ, Tu X, Yu K, Ornitz DM, Long F. (2005). Sequential roles of Hedgehog and Wnt signaling in osteoblast development. *Development* **132**: 49–60.

Karhadkar SS, Steven Bova G, Abdallah N, Dhara S, Gardner D, Maitra A *et al*. (2004). Hedgehog signaling in prostate regeneration, neoplasia and metastasis. *Nature* **431**: 707–712.

Kolpakova E, Olsen BR. (2005). Wnt/[beta]-Catenin—a canonical tale of cell-fate choice in the vertebrate skeleton. *Dev Cell* **8**: 626–627.

Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW *et al*. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC−/− colon carcinoma. *Science* **275**: 1784–1787.

Loberg RD, Logothetis CJ, Keller ET, Pienta KJ. (2005). Pathogenesis and treatment of prostate cancer bone metastases: targeting the lethal phenotype. *J Clin Oncol* **23**: 8232–8241.

Logothetis CJ, Lin S-H. (2005). Osteoblasts in prostate cancer metastasis to bone. *Nat Rev Cancer* **5**: 21–28.

Mao J, Wang J, Liu B, Pan W, Farr I, Gist H *et al*. (2001). Low-density lipoprotein receptor-related protein-5 binds to axin and regulates the canonical Wnt signaling pathway. *Mol Cell* **7**: 801–809.

Navone NM, Olive M, Ozen M, Davis R, Troncoso P, Tu SM *et al*. (1997). Establishment of two human prostate cancer cell lines derived from a single bone metastasis. *Clin Cancer Res* **3**: 2493–2500.

Nelson J, Bagnato A, Battistini B, Nisen P. (2003). The endothelin axis: emerging role in cancer. *Nat Rev Cancer* **3**: 110–116.

Sambrook J, Fritsch EF, Maniatis T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Shah RB, Mehra R, Chinnaiyan AM, Shen R, Ghosh D, Zhou M *et al*. (2004). Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res* **64**: 9209–9216.

Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y *et al*. (2000). LDL-receptor-related proteins in Wnt signal transduction. *Nature* **407**: 530–535.

Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B *et al*. (2003). The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. *N Engl J Med* **349**: 2483–2494.

van Es JH, Barker N, Clevers H. (2003). You Wnt some, you lose some: oncogenes in the Wnt signaling pathway. *Curr Opin Genet Dev* **13**: 28–33.

Westendorf JJ, Kahler RA, Schroeder TM. (2004). Wnt signaling in osteoblasts and bone diseases. *Gene* **341**: 19–39.

Yang J, Fizazi K, Peleg S, Sikes CR, Raymond AK, Jamal N *et al*. (2001). Prostate cancer cells induce osteoblast differentiation through a Cbfa1-dependent pathway. *Cancer Res* **61**: 5652–5659.

Zhang Y, Wang Y, Li X, Zhang J, Mao J, Li Z *et al*. (2004). The LRP5 high-bone-mass G171V mutation disrupts LRP5 interaction with Mesd. *Mol Cell Biol* **24**: 4677–4684.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).