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Runx2 Association with Progression of Prostate Cancer in Patients: Mechanisms Mediating Bone Osteolysis and Osteoblastic Metastatic Lesions

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Abstract

Runx2, a bone-specific transcriptional regulator, is abnormally expressed in highly metastatic prostate cancer cells. Here we identified the functional activities of Runx2 in facilitating tumor growth and osteolysis. Our studies demonstrate that negligible Runx2 is found in normal prostate epithelial and non-metastatic LNCaP prostate cancer cells. In the intra-tibial metastasis model, high Runx2 levels are associated with development of large tumors, increased expression of metastasis-related genes (MMP9, MMP13, VEGF, Osteopontin), and secreted bone resorbing factors (PTHrP, IL-8) promoting osteolytic disease. Runx2 siRNA treatment of PC3 cells decreased cell migration and invasion through Matrigel in vitro, and in vivo shRunx2 expression in PC3 cells blocked their ability to survive in the bone microenvironment. Mechanisms of Runx2 function were identified in co-culture studies demonstrating that PC3 cells promote osteoclastogenesis and inhibit osteoblast activity. The clinical significance of these findings is supported by human tissue microarray studies of prostate tumors at stages of cancer progression, where Runx2 is expressed in both adenocarcinomas and metastatic tumors. Together these

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findings indicate that Runx2 is a key regulator of events associated with prostate cancer metastatic bone disease.

Keywords

Runx2; bone metastasis; prostate cancer tissue-arrays

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer in men, with occurrence of metastasis to the bone in almost 80% of advanced metastatic disease (Mundy, 2002; Kingsley *et al*., 2007; Guise *et al*., 2006). Although clinically PCa metastases have been associated primarily with osteoblastic lesions, there is also an osteolytic component to metastatic bone disease (Roudier *et al*., 2008; Keller and Brown, 2004). The mechanisms by which prostate cancers are induced to metastasize to bone and successfully grow tumors rely on a tight interplay between the tumor and the tissue micro-environment. Both breast and prostate cancer cells that metastasize to bone express several classes of bone matrix and signaling proteins involved in adhesion and migration that contribute to osteomimetic properties (Huang *et al*., 2005; Zayzafoon *et al*., 2004; Kingsley *et al*., 2007; Li *et al*., 2008b). Among these are osteocalcin (OC), osteopontin (OP), bone sialoprotein (BSP), matrix metalloproteinases (MMPs) and Wnt factors (Pratap *et al*., 2006; Deryugina and Quigley, 2006; Selvamurugan *et al*., 2006), which are linked to disease prognosis. Interrogating the mechanisms that contribute to prostate tumor growth and metastasis is important for early detection.

Growth of prostate tumors in bone and the accompanying metastatic bone disease presents an imbalance in the normal process of bone remodeling (formation and resorption) as a result of factors secreted by the tumor cells (Guise *et al*., 2006). These signaling proteins include the receptor activator of N_F-κB ligand (RANKL) (Brown *et al*., 2001; Armstrong *et al*., 2008), parathyroid hormone related protein (PTHrP) and interleukin 8 (IL8) (Bendre *et al*., 2005; Araki *et al*., 2007), that promote bone resorption, while endothelin and Wnt pathway factors promote osteoblastic lesions (Li *et al*., 2008b; Clines *et al*., 2007).

Transcription factors that promote bone formation are also highly expressed in tumor cells that metastasize to bone, including Msx2 which supports pathological calcification (Shao *et al*., 2005) Runx2, a master regulator of bone formation reviewed in (Pratap *et al*., 2006; Barnes *et al*., 2003; Shao *et al*., 2005; Blyth *et al*., 2005) Runx2 is abnormally and highly expressed in MDA-MB-231 breast cancer cells that metastasize to bone and form osteolytic lesions (Pratap *et al*., 2008). However, Runx2 is not significantly detected in normal breast or prostate epithelial cells (Barnes *et al*., 2004; Pratap *et al*., 2006; Pratap *et al*., 2005; Inman and Shore, 2003). Runx2 functions in many regulatory processes in osteoblasts including epigenetic control of genes during mitosis (Young *et al*., 2007), suppression of cell growth (Pratap *et al*., 2003), cellular senescence (Zaidi *et al*., 2007a), and bone turnover. A unique property of Runx2 is its sub-nuclear targeting to foci recruiting co-regulatory factors that mediate transduction of Wnt, Src, BMP and TGFβ signaling (Zaidi *et al*., 2007b; Javed *et*

al., 2005; Kingsley *et al*., 2007). These pathways are activated in tumor cells (Kingsley *et al*., 2007; Hall *et al*., 2006; Li *et al*., 2008a). A key question is the extent to which Runx2 expression contributes to progression of PCa and is functionally related to formation of osteolytic and osteoblastic lesions at the bone metastasis site. By combining in vitro and in vivo strategies, we demonstrate that Runx2-mediated gene expression is associated with increased motility and invasiveness of PCa cells, and the aggressiveness of osteolytic bone disease that accompanies PCa metastasis to bone. We provide compelling evidence from human prostate tissue arrays and the intra-tibial mouse model of bone metastasis that suggests Runx2 can be a viable therapeutic target for reducing prostate cancer metastasis.

MATERIALS AND METHODS

Cell culture

PCa PC3 sublines, LNCaP, C4-2B and RWPE from non-tumorigenic prostate cells were maintained as follows. Media and supplements were obtained from Invitrogen (Carlsbad, CA). PC3-H were cultured in T-medium with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) (Huang *et al*., 2005). PC3-M were grown in DMEM-F12 with 10% FBS according to ATCC. PC3-L cells (Fornaro *et al*., 2003) and non-metastatic LNCaP and C4-2B cells were maintained in RPMI, 10 mM non-essential amino acids, 2 mM sodium pyruvate and 10% FBS. RWPE cells were maintained in reduced keratinocyte medium, and RAW 264.7 in DMEM with 10% FBS (Atlanta Biologicals).

Western blot analysis

Proteins were isolated from whole cells in RIPA buffer containing 25 μM MG132, and protease inhibitor cocktail (Roche, Nutley, NJ). Nuclear extracts were prepared for Runx2 detection as previously described (Hassan *et al*., 2004). Proteins were separated and immobilized on PVDF membranes (Millipore, Billerica, MA). Blots were probed with Runx1 rabbit polyclonal antibody (Calbiochem, San Diego, CA), Runx2 (MBL, Woburn, MA) and Runx3 (MBL) monoclonal antibodies. αTubulin (1:25,000) (Sigma) and CDK1 (1:1000) monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used as loading controls as described (Pratap *et al*., 2008). Secondary antibodies conjugated to HRP (Santa Cruz Biotechnology) were used to detect immunoreactive proteins.

Runx2 knockdown

Small interfering RNA (siRNA). Control (non-silencing) and siRNA targeting Runx2 (Smartpool On Target Plus®) (Dharmacon, Lafayette, CO) were used to transfect PC3-H cells at 30-50% confluence using Oligofectamine® (Invitrogen). Knockdown of endogenous Runx2 and its effect on metastatic markers and survivin were examined after 48 h. *Short hairpin RNA (shRNA)*. PC3-H cells expressing scrambled or Runx2 shRNA were generated for *in-vivo* studies as described (Pratap *et al*., 2008). Runx2 knockdown by siRNA and shRNA was confirmed by western blot and qRT-PCR.

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA from PCa cells was primed with oligo(dT) or random hexamers (histone H4) to synthesize cDNAs (Invitrogen). Human specific primer pairs using SYBR green and Assay

on Demand® primer mixes (Taqman; Applied Biosystems, Foster City, CA) for mouse genes are shown in supplemental table S1 Amplicon quantities were normalized to mouse or human GAPDH.

Adenovirus infections

Adenoviral delivery of vectors containing cDNA of Runx2-IRES-GFP under the control of the CMV5 promoter were used as described (Pratap *et al*., 2005). Preparation and purification of virus were performed according to the manufacturer's protocols (Promega, Madison, WI). For control of infection efficiency, the same vector carrying GFP was used. Expression of Runx2, metastatic, and osteolytic genes was analyzed by qRT-PCR.

Invasion and adhesion assays

Invasion: PC3-H cells were treated with siRNA and analyzed for invasion or migration using Boyden chambers (BD Biosciences, Bedford, MA) as previously described (Pratap *et al*., 2005; Javed *et al*., 2005). Cells were counted and results are presented as percentages of invasion (invasion/migration × 100). *Adhesion:* PC3-H cells (1×10^5) were treated with Runx2 or control siRNA, plated on fibronectin (10 μg/ml per well). Ninety-six well plates were blocked with medium containing 1% BSA for 1 h at 37°C. PC3-H cells were incubated for 3 h at 37°C, fixed with 3% paraformaldehyde (PFA) and stained with 0.5% crystal violet (Sigma) and absorbance read at 630 nm.

Animal protocols

Animal studies were conducted in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols and the NIH Guide for Care and Use of Laboratory Animals. 1×10^5 cells (PC3-H, PC3-M, and PC3-L) were injected into tibiae of SCID mice (n=12). 3 mice per group were used as representatives for analysis. Tumors were allowed to grow for a period of 4 or 6 weeks. In other experiments, PC3-H alone and PC3-H transduced with scrambled or Runx2 shRNA sequences were injected into tibiae and allowed to grow for 3 or 4 weeks. Bone lesions were analyzed weekly by radiography. using Faxitron MX-20 (Faxitron X-ray, Wheeling, IL).

Immunological and histological analysis of tissue sections

Tumors from injected tibiae were harvested and processed as described (Rubin *et al*., 2000), then stained with H&E and Toluidine blue. Consecutive tissues were stained for tartrate resistant acid phosphatase (TRAP) activity (Barnes *et al*., 2004) or incubated with Runx2 (PEPB2αA rabbit IgG, M-70) (Santa Cruz) and cytokeratin (AE1/AE3) (Dako Cytomation, Carpinteria, CA) antibodies following antigen retrieval with citrate buffer (pH 6.0) at 95°C.

Tissue microarrays

Case selection: Tissue microarrays (TMAs) were developed from samples obtained through radical prostatectomy and from the Rapid Autopsy Program within the Michigan Prostate SPORE Tissue Core as previously described (Rubin *et al*., 2000). The TMA consisted of 37 non-neoplastic prostate tissues; 15 prostatic intra-epithelial neoplasia (PIN) tissues; 25 primary prostate tumors (Gleason scores 5-8) and 24 prostate metastases. All tissue

procurement and analysis was approved by the University of Michigan Institutional Review Board. *Immunohistochemical evaluation:* TMA slides were deparaffinized, rehydrated to water, and antigen retrieved in citrate buffer, pH 6.0 for 10 min with microwaving. After peroxidase blocking, the slides were incubated with 1:400 dilution of goat anti-Runx2 antibody [Runx2 (27-K); Santa Cruz Biotechnology] on an Auto Stainer using the LSAB+ detection kit and counterstained with Hematoxylin. Each section was evaluated as either positive or negative staining for Runx2. Staining intensity was also scored as negative [0], weak [1], moderate [2], or strong [3] based on the amount of stain detected (Fu *et al*., 2006). Examples are shown in supplementary figure 1.

Co-culture studies

Boyden chambers (1micron inserts; BD Biosciences, Bedford, MA) were used to test the influence of PC3-H cells on osteoclasts using RAW 264.7 mouse monocytes. Cells (0.4×10^6) were plated in 6 wells for 3 days before adding either RANKL (5 ng/ml) (control) or PC3-H cells (4×10^5 cells/well). Osteoclast formation was monitored by qRT-PCR analysis. For osteoblast co-culture studies, conditioned medium (CM) was harvested from PC3-H cells and added to MC3T3 cells at confluency (day 3). MC3T3 cells were cultured with 10% or 20% CM, added to MEM with 50 μg/ml ascorbate and 10 mM β-glycerolphosphate (osteogenic media) and cultured for 14 and 21 days. MC3T3 cells were fixed in 2% PFA and stained for alkaline phosphatase activity (Sigma).

RESULTS

Selective expression of the Runx2 transcription factor in metastatic prostate cancer cells and functional activities

We compared four widely studied prostate cancer cell lines with distinct metastatic and tumor growth potential to characterize Runx2 expression in relation to their phenotypic properties. These included highly metastatic PC3 cells isolated from bone metastases, non-metastatic LNCaP cells that do not grow in bone and C4-2B cells that are derived from LNCaP cells and form osteoblastic lesions in the bone (Thalmann *et al*., 1994). PC3 cells express the highest level of Runx2 compared to LNCaP, C4-2B and RWPE by both qRT-PCR and western blot analysis (Fig. 1A). Because prostate cell lines exhibit genomic instability and centrosome defects that lead to gene alterations (Glinsky *et al*., 2006), we further analyzed Runx2 among metastatic PC3 sublines, maintained in distinct media (see methods). PC3 cells were designated PC3-H (high), PC3-M (moderate), and PC3-L (low) according to Runx2 mRNA expression levels, determined by qRT-PCR (Fig. 1B, top panel). Runx2 mRNA levels in PC3-H cells are 2 fold higher than PC3-M and 15 fold greater in PC3-L cells. Because protein levels in whole cell lysates are low in PC3-M and PC3-L cells, nuclear extracts were examined demonstrating highest levels in the PC3-H line. Because Runx factors recognize the same regulatory sequence (Blyth *et al*., 2005), we compared Runx2 expression to hematopoietic Runx1 and nerve related Runx3 factors in all sublines (Supplementary figure 2). Runx1 protein was very low in all sublines, while Runx3 mRNA and protein levels were significantly higher in PC3-L cells. These results show that the three PC3 sublines can be examined for further studies for relative levels of Runx2 influencing bone metastatic properties of prostate cancer cells.

To determine if Runx2 target genes are functionally linked to metastatic properties of PC3-H cells, we selectively inhibited Runx2 expression by RNA interference. Runx2 protein was completely inhibited (Fig. 1C) and RNA reduced by 80% compared to controls (Fig. 1E). Metastatic genes established to be regulated by Runx2 (e.g., VEGF, MMP9) and bone related genes (e.g., OC) were reduced by 80-90% compared to the non-silencing oligo control (Fig. 1E). Runx2 siRNA treated PC3-H cells exhibited a 30% increased adhesion to fibronectin and significantly decreased migration and invasion through Matrigel (Fig. 1D). Thus, increased adhesion is consistent with decreasing invasive properties of PC3-H cells by deletion of Runx2. Zaidi et al (32) reported a correlation between Runx2 expression and either cell survival or tumorigenesis depending on the cellular context. We addressed if Runx2 contributed to survival of PC3-H cells and show that the cell survival marker survivin is highly expressed in PC3-H cells. A 50% decrease occurred in response to Runx2 knockdown (Fig. 1F). These results suggest that Runx2 regulates cellular activities associated with tumor cell invasion and survival of prostate cancer cells.

Runx2 mediates prostate tumor-induced osteolytic bone disease

To identify the functional activities of Runx2 in the PC3 sublines in-vivo, cells were inoculated into the intramedullary cavity of SCID mice (Fig. 2). Osteolysis was first evident in PC3-H and PC3-M tumors by radiography at 3 weeks (data not shown) and by 4 weeks osteolysis of the bone cortex was evident, along with tumor cell invasion in muscle by 6 weeks (Fig. 2A). PC3-L cells however, produced a mixed lesion phenotype of mild osteolysis at 3 and 4 weeks, followed by later osteoblastic disease observed more prominently by 6 weeks in tumor tissue (Fig. 2A). Histologically, active osteoclasts were found at the tumor-bone interface resorbing bone by the PC3-H (Fig. 2B) and PC3-M cells (not shown). PC3-L tumors showed unorganized woven bone, especially at the interface of the cortical bone and tumor growing in the muscle (Fig. 2B). This is consistent with the irregular cortical bone surface in the PC3-L radiographs showing woven bone, in contrast to radiographs of the eroded cortical surface of PC3-H and PC3 M tumors (Fig. 2A, 6 week). Immunohistochemistry (IHC) of PC3-H tumor showed robust Runx2 expression in the majority of cells positive for human cytokeratin (Fig. 2B), which was used to distinguish between implanted human prostate cancer cells and mouse marrow cells. In addition, Runx2 expression in PC3 sublines was analyzed post implantation verifying that differences were maintained (Fig 2C). In summary, PC3-H cells resulted in the most aggressive osteolysis, PC3-M cell-mediated osteolytic disease occurred at a slower rate, while PC3-L derived tumors exhibited limited osteolysis (that is, smaller lesions at 3 and 4 weeks), concurrent with osteoblastic disease. These findings indicate a positive correlation of Runx2 mRNA with severity of osteolytic bone disease induced by PC3 tumor cells.

To provide further support that Runx2 promotes osteolytic disease, we analyzed expression of IL8 and PTHrP (Fig. 2D), signals known to activate osteoclast activity (Bendre *et al*., 2005; Liao and McCauley, 2006). Robust expression of PTHrP in PC3-H and IL8 in PC3-M cells was found, consistent with their aggressive osteolysis. PC3-L cells exhibited low expression of both bone resorbing factors. Analysis of expression levels of Runx2 and these factors post implantation, revealed that the PC3 sublines maintained their unique

characteristics in vivo (supplementary figure 3) Thus, expression of Runx2 in PC3 cells appears to promote activity of secreted osteolytic factors.

PC3-H cells contribute to proliferation of osteoclast precursors and osteoclastogenesis

To delineate the mechanism(s) for tumor-related resorption, PC3-H cells were co-cultured with osteoclast precursors (RAW 264.7). PC3-H cells were cultured for 7 days in the upper compartment of a Boyden chamber, while RAW 264.7 cells were seeded in the bottom chamber to allow for the exchange of secreted factors that generate osteoclasts. Three groups were compared as indicated in figure 3A. A 3-7 fold increased expression of genes associated with activation of osteoclasts (TRAP, Cathepsin K, MMP9, RANK) and cell growth (histone H4) was observed when RAW 264.7 cells were co-cultured with PC3-H cells, compared to control groups (RAW 264.7 and RAW 264.7 with RANKL). These results indicate that the aggressive stimulation of osteolytic disease is due in part to proliferation of osteoclast precursors that differentiate to active osteoclasts under the influence of secreted factors PTHrP and IL8 as shown in figure 2D. This finding is consistent with Runx2 positive PC3-H and PC3-M cells promoting osteolytic bone disease, but not PC3-L cells which lack expression of these factors.

Given the osteomimetic properties of prostate cancer cells (Huang *et al*., 2005; Guise *et al*., 2006), we next addressed if PC3-H cells influenced the activity of osteoblasts. CM (10% and 20%) from PC3-H cells was used to supplement MC3T3 osteoprogenitor cell cultures during osteoblast differentiation. We find reduced alkaline phosphatase staining on days 14 and 21 reflecting an inhibition of the activity of osteoblasts and competency further to differentiate to mature osteoblasts (Fig. 3B). In summary, prostate tumor cells expressing high levels of Runx2 influence both osteoclast and osteoblast activities at the tumor-bone interface (in vivo studies) by modulating target genes.

Exogenous expression of Runx2 promotes activation of metastatic target genes in prostate cancer

Runx2 increases expression of numerous genes essential for cell growth, angiogenesis, and metastasis in breast cancer cells. In order to address if Runx2 can alter the metastatic properties of PCa cells, we first evaluated basal expression of metastatic Runx2 target genes in the cell lines (Fig 4A). We found each PC3 subline exhibited a distinct profile of metastasis related genes. PC3-H cells preferentially expressed MMP9, PC3-M cells expressed MMP13 and PC3-L cells had high MMP2 levels. LNCaP cells did not express MMPs at significant levels, but showed high levels of VEGF. Notably, the bone matrix proteins osteocalcin and osteopontin were expressed at much lower levels than the MMPs in all cell lines.

From these results, we selected PC3-M, PC3-L and LNCaP cells to address mechanisms directly related to Runx2 activities in PCa cells by adenoviral delivery of Runx2 (Fig. 4B). Our results reveal that only those MMP genes which are differentially expressed at low endogenous levels (Fig. 4A) become induced by Runx2 (Fig. 4B). For example in PC3-M cells, MMP9, but not MMP13, was induced because basal MMP13 levels are very high and not further stimulated by Runx2. Likewise PC3-L cells, which only express endogenous

MMP2, and LNCaP cells, which do not express MMP2, both showed robust induction of MMP9 and MMP13 by Runx2. In summary, these studies reveal that prostate cells differentially express genes that are Runx2 targets and that Runx2 can activate metastatic genes in PCa cells that are expressed at low levels, suggesting Runx2 can contribute to metastatic properties of PCa cells. Our findings provide an insight to the specific genes that are regulated by Runx2 in the various prostate cancer cells.

Knockdown of Runx2 expression in PC3 cells blocks tumor growth and metastatic lesions formed by PC3-H cells in vivo

To determine the direct roles of Runx2 in formation of bone metastatic lesions, parental PC3-H cells were infected with scrambled or Runx2 shRNA for intra-tibial injections. Radiography at 4 weeks showed that mice (n=12; 3 representative mice) with PC3-H cells expressing scrambled shRNA formed aggressive osteolysis, similar to mice tibiae with parental PC3-H cells (Fig. 5A top and center panels). In contrast, PC3-H cells infected with Runx2 shRNA (Fig. 5A lower panel) showed no evidence of osteolytic disease in 2 of 3 mice, while one mouse exhibited the onset of mild bone lysis (arrows). Knockdown of Runx2 by shRNA was confirmed by western blot and qRT-PCR revealing an almost complete knockdown of bone resorbing factors IL8 and PTHrP (Fig 5B). These studies indicate that Runx2 expression is essential for tumor survival in the bone microenvironment and formation of osteolytic lesions. Figure 5C summarizes the activities of Runx2 regulation and contributions to prostate cancer metastasis and formation of bone lesions.

Runx2 expression in human prostate cancer tissue is associated with tumor metastasis

To address the clinical significance of our in vivo findings, we analyzed a prostate cancer tissue microarray to determine if Runx2 expression correlates with prostate cancer progression by immunohistochemistry. The tumor tissue was classified as either negative or positive for Runx2 (Table 1A). The majority of non-neoplastic tissues and PIN lesions were negative for Runx2, while 48% and 46% of the primary tumors and metastases were Runx2 positive. The staining intensity determined by immunohistological scoring (see methods and supplementary figure 1) was evaluated in Table 1B. We find that the majority (92-93%) of non-neoplastic and PIN lesions had a staining intensity of zero, while half (almost 48%) of the advanced primary tumors had a staining intensity of 1 and 2, and metastatic lesions (45.8%) showed intensities of 1 to 3. Thus Runx2 expression is detected in human prostate tumors at advanced stages of the disease.

DISCUSSION

A key finding of the present studies is that the Runx2 transcription factor mediates the expression of multiple genes that promote osteolytic bone disease during metastatic growth of prostate tumors. Studies in human prostate tumor tissue arrays indicate that nearly 50% of the samples exhibited robust Runx2 expression in advanced lesions. Runx2 may therefore represent a determinant of a subpopulation of cells in the prostate tumors for metastasis to bone. A functional relationship between Runx2 and expression of a metastatic phenotype is demonstrated by Runx2 overexpression and knockdown studies that directly show an enhancement and reduction, respectively, of prostate tumor growth and osteolytic disease.

These striking findings suggest involvement of Runx2 in tumor growth, invasion and metastasis, as a result of Runx2 activation of genes in PC3 cells that include VEGF, OP, MMPs and survivin. The inhibition of survivin by Runx2 knockdown is consistent with reduced tumor cell viability and the role of survivin in protecting prostate cancer cells from apoptosis (Roca *et al*., 2008; Altieri, 2008). Taken together our studies indicate that Runx2 is a component of a mechanism that promotes prostate cancer progression and metastatic bone disease.

Many investigators have reported variable results from studies carried out using prostate cancer cell lines that are attributed to genetic drift. It is likely that the three PC3 sublines were obtained from different sources that originated from a heterogeneous population that was selected during different long-term culture conditions. We maintained the cells in their respective media for growth and found them to retain their unique expression profile through many passages and after in vivo implantation. Taking advantage of their properties, our in vitro and in vivo findings suggest that expression of Runx2, MMPs and PTHrP are interrelated and provide a signature for responsiveness of prostate cancer cells to the bone microenvironment. While each subline expresses predominantly one of the MMPs prominently, these MMPs are linked to metastasis (reviewed in (Deryugina and Quigley, 2006)), verified by clinical data (Egeblad and Werb, 2002; Morgia *et al*., 2005) and are regulated by Runx2 (Selvamurugan *et al*., 2006; Pratap *et al*., 2006). This implies that perhaps Runx2 does not regulate all MMPs in the PC3 sublines because some MMPs are already maximally expressed. It is noteworthy that the PC3-L cells which promote osteoblastic lesions have undetectable expression of IL8 and PTHrP. However, the low Runx2 protein and the robust expression of MMP2 in these cells could account for the mild osteolysis observed concomitant with osteoblastic lesions.

The gene expression signature of PC3-H cells that express maximal levels of Runx2 include target genes include factors related to tissue invasion and destruction. VEGF facilitates tumor growth and osteolytic disease by enhancing osteoclast survival (Yang *et al*., 2008). VEGF is also associated with osteoblastic disease (Dai *et al*., 2004), consistent with our finding in PC3-L cells which form osteoblastic lesions. Both PC3-H and PC3-M express mediators of the “vicious cycle” of tumor growth and bone destruction, IL8 and PTHrP. Runx2 expression has also been associated with osteolysis in multiple myeloma and inhibited osteoblastogenesis (Edwards *et al*., 2008), as we show for PC3-H cells. Thus a pattern of expression of Runx2 and Runx2 target genes emerges from three types of cancers that are accompanied by osteolytic disease; breast cancer, multiple myeloma and prostate cancer. In our studies, we have established using PC3-H cells that inhibition of Runx2 by shRNA nearly prevented metastatic bone disease, analogous to studies of breast cancer metastatic disease (Javed *et al*., 2005; Pratap *et al*., 2008). The evidence that Runx2 is a key mediator of metastasis related genes in two different solid tumor types, suggests that Runx2 in the primary tumor could be an important diagnostic for bone metastasis. Indeed, we detected strong Runx2 staining in advanced human prostate tumor tissue compared to PIN lesions.

Osteoblastic lesions were observed in the PC3-L line with new woven bone formation in the medullary cavity replacing resorbed bone. Prostate cancer cells secrete factors that stimulate

the proliferation of osteoprogenitor cells, or recruit circulating or bone marrow cells into the osteoblast lineage in the tumor environment. Notch, Wnt, BMP, FGF and VEGF signaling in metastatic prostate cancer cells have all been reported to contribute to the osteoblastic phenotype in mice (Li *et al*., 2008b; Zayzafoon *et al*., 2004; Clines *et al*., 2007; Dai *et al*., 2004; Li *et al*., 2008a). These pathways are linked to Runx2 bone promoting properties (reviewed in (Pratap *et al*., 2006)). Our findings contribute to the concept that human prostate tumors exist as a heterogeneous population of prostate cancer cells which may express different signature genes that enable them to respond to the bone microenvironment in a distinct manner that determines the characteristics and extent of the metastatic bone disease.

PCa metastasis remains a debilitating complication experienced by patients with advanced disease. Expression of Runx2 in the prostate tumor may be a target for reversing the metastasis and accompanying bone disease. The absence of Runx2 protein in early PIN lesions suggests that Runx2 is induced after transformation and is upregulated during advanced progression of adenocarcinomas. Numerous signaling pathways, e.g integrin, FGF9, BMP, TGFβ1, and Wnt are upregulated in prostate cancer cells that increase Runx2 expression and may activate metastasis-related genes in the primary prostate tumors. Thus, targeting Runx2 for inhibition in prostate tumors is a potentially viable strategy for blocking prostate cancer metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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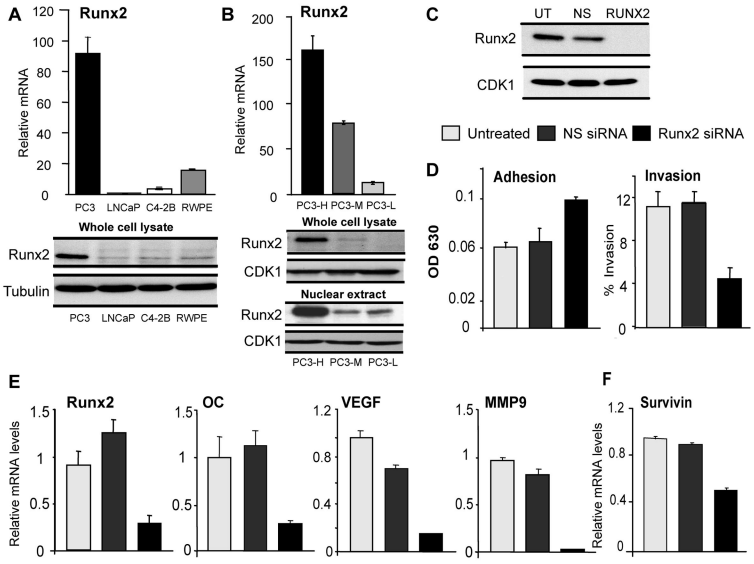


Figure 1. Runx2 expression in prostate cancer cell lines regulates metastatic properties

(A) Runx2 mRNA levels in PC3, LNCaP, C4-2B and non-tumorigenic RWPE cells were analyzed by qRT-PCR (upper panel, see Methods). Protein levels by western blot analysis (lower panel). **(B)** PC3 sublines (PC3-H, PC3-M, PC3-L) were analyzed for Runx2 mRNA and protein in whole cell lysates and nuclear extracts. (**C**) Western blot shows 80% knockdown of endogenous Runx2 in PC3-H cells; CDK1 is used as internal control. (**D**) Invasion through matrigel is reduced and adhesion increased in Runx2 siRNA treated PC3-H cells. (**E**) Expression of Runx2 and target genes (OC, VEGF and MMP9) by qRT-PCR following knockdown (48hr). Values are mean ± SD of n=3 samples analyzed in triplicate. (**F**) Reduced expression of survivin by Runx2 siRNA. E, F panels are representative of triplicate assays of n=4 experiments. Error bars are mean ± SD.

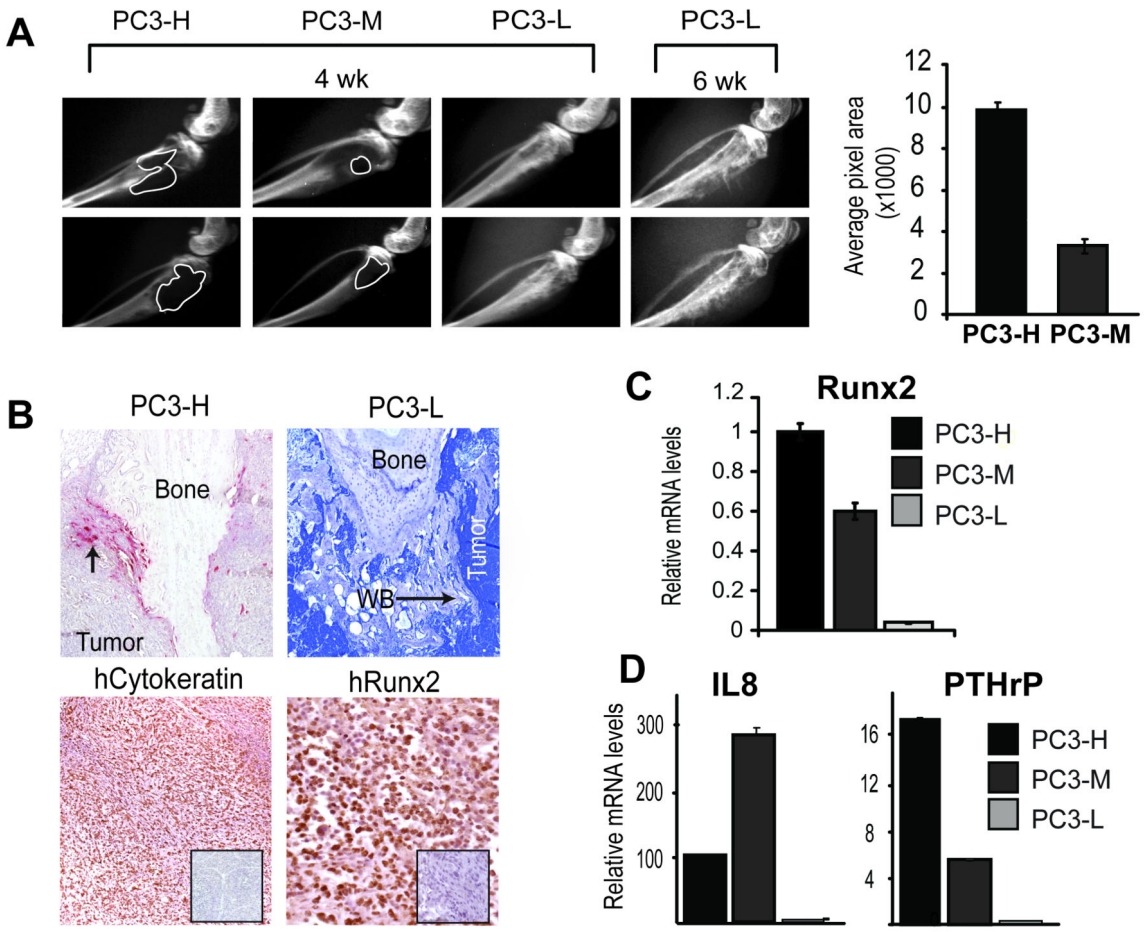


Figure 2. Runx2 expression in PC3 sublines promotes osteolytic bone disease in vivo and osteoclast activation in vitro

(**A**) Mouse tibiae at 4 and 6 weeks expressing different Runx2 levels (PC3-H, PC3-M, PC3-L) as indicated. Aggressive osteolysis in tumors from PC3-H cells (at 4 weeks); near complete erosion through cortical bone also occurs in PC3-M. PC3-L induced tumors have minimal lysis at 4 weeks and increased radiopaque areas at 6 weeks which fill in the eroded areas and represent osteoblastic lesions. Quantification of extent of osteolysis marked areas in PC3-H and PC3-M is shown **(B)** PC3 cells induce osteolytic lesions shown by TRAP staining of the bone resorbing cells (arrow shows osteoclasts). PC3-L tumors induced woven bone (WB) formation, shown here at the tumor-bone interface. Immunohistochemistry of the tumor area for Runx2 expression in PC3 tumors. The human specific marker cytokeratin (10x) and Runx2 is shown (40x). Insets show IgG antibody control. (**C**) Expression of Runx2 by qRT-PCR showing mRNA levels in PC3 sublines post-implantation. Data shown are mean ± SD of samples analyzed in triplicates. (**D**) PTHrP and IL8 expression in PC3 sublines measured by qRT-PCR.

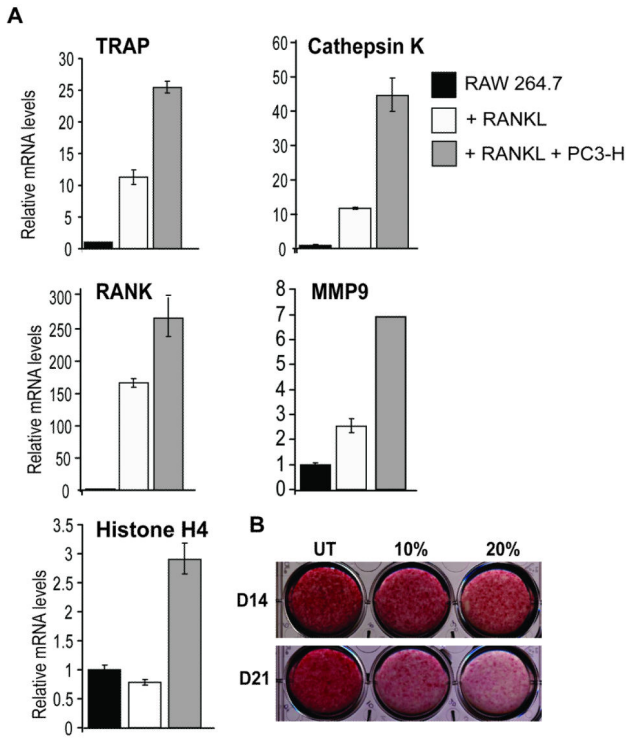
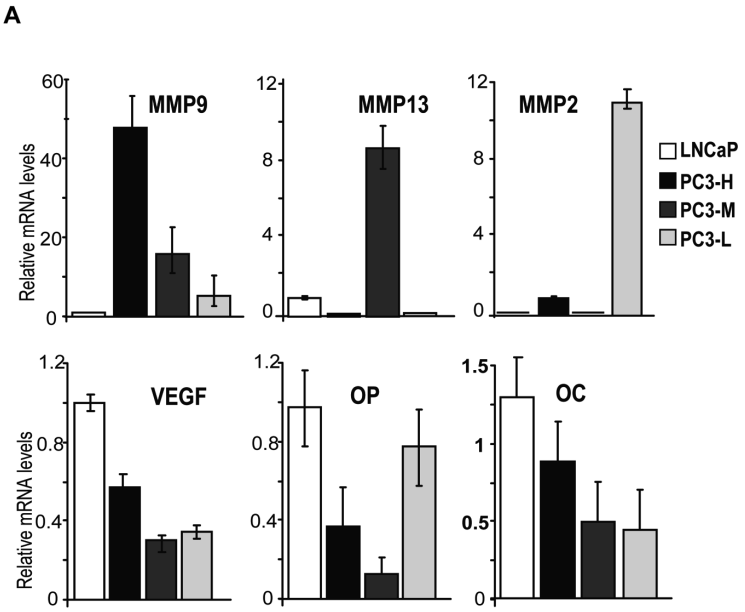


Figure 3.

(**A**) Gene expression of markers for osteoclast differentiation detected by qRT-PCR after co-culturing PC3-H cells with RAW 264.7 cells. **(B)** Histochemical staining of MC3T3 cells for alkaline phosphatase shows inhibited osteoblast differentiation when medium from PC3-H cells (10% or 20%) was added to cultured osteoblasts and analyzed after 14 and 21 days.



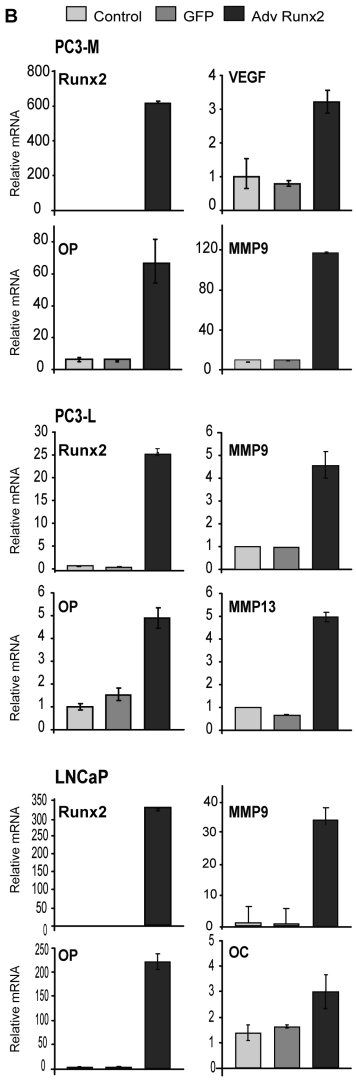


Figure 4. Forced expression of Runx2 leads to activation of metastatic markers

(A) Expression of MMP9, MMP13, MMP2, VEGF, OP and OC in PC3 sublines by qRT-PCR. GAPDH was used for internal control for qRT-PCR. Data shown are mean ± SD of samples analyzed in triplicates (**B**) Expression levels of metastasis related genes in PC3-M, PC3-L and LNCaP cells after viral transduction are shown and were normalized to GAPDH. Runx2 regulation of MMP2, MMP9, MMP13, OC and OP is shown. Data from one representative of n=4 experiments is shown. Values are mean ± SD.

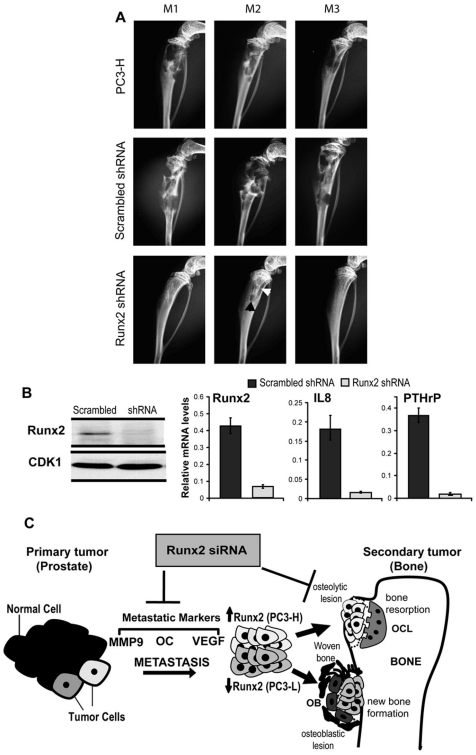


Figure 5. Runx2 knockdown reduces metastatic bone disease

(**A)** Radiographs of tibiae 3 weeks post-injection of tumor cells show osteolytic lesions caused by parental PC3-H cells (top panel) and slightly more aggressive lesions by PC3-H cells infected with scrambled shRNA (center), but absence of tumor activity (M1, M3) or evidence of onset of lysis (M2) in the Runx2 shRNA group (lower panels, arrows point to onset of lysis). (**B**) Western blot showing Runx2 protein levels of whole cell extracts from PC3-H infected with scrambled or Runx2 shRNA (left). qRT-PCR showing knockdown of Runx2, IL8 and PTHrP in tumors from implanted PC3-H cells infected with scrambled or Runx2 shRNA (right). (**C**) Runx2 mediated mechanisms of metastasis and formation of osteolytic and osteoblastic bone lesions. Prostate cancer progression from primary prostate tumor to metastatic cancer occurs through Runx2 regulation of metastasis related genes (e.g. MMP9, OC, VEGF, see Figs 1 and 4). At the onset of bone metastasis, tumor cells become heterogeneous, expressing different levels of Runx2 [high Runx2 (PC3-H) and low Runx2 (PC3-L)]. Within the tumor-bone interface, PC3-H cells release factors (PTHrP, IL8, see Fig 2D) that activate osteoclasts (OCL) resulting in bone resorption and osteolytic lesions. PC3-L cells form osteoblastic lesions induce new bone formation potentiated by osteoblasts (OB**)** via several possible pathways [reviewed in (Keller and Brown, 2004), and in discussion]. Runx2 knockdown (siRNA; shRNA in vivo) blocks the expression of metastasis promoting genes and reduces the formation of osteolytic lesions.

Table 1

Runx2 expression in primary and metastatic prostate tumor tissue microarrays

A. Presence of Runx2 positive cells in prostate cancer.

	Histological Diagnosis			
Staining	**Non-neoplastic**	**PIN**	**Primary Tumor***	**Metastases***
Negative	34 (91.9)	14 (93.3)	13 (52.0)	13 (54.2)
Positive	3 (8.1)	1 (6.7)	12 (48.0)	11 (45.8)

B. Staining intensity for Runx2 in prostate cancer.

Staining Intensity	**Histological Diagnosis**			
	Non-neoplastic	**PIN**	**Primary Tumor***	**Metastases***
0	34 (91.9)	14 (93.3)	13 (52.0)	13 (54.2)
1	2 (5.4)	1 (6.7)	10 (40.0)	8 (33.3)
2	1 (2.7)	0 (0)	2 (8.0)	2 (8.3)
3	0 (0)	0 (0)	0 (0)	1 (4.2)
TOTAL	37 (100)	15 (100)	25 (100)	24 (100)

(**A**) Indicates the number of sections stained (and percent in parentheses) as either negative or positive for each histological diagnosis. **(B)** Indicates the number of sections stained (and percent in parentheses) as either negative or positive by staining intensity for each histological diagnosis. Statistical analyses for TMAs were performed using Fisher's exact test. Statistical significance was determined as $P<0.05$.

* $P<0.01$ distribution of staining intensity versus Normal or PIN.

* $P<0.01$ distribution of positivity versus Normal or PIN.