ARTICLE
Journal of Cellular Biochemistry 109:828–837 (2010)

Journal of Cellular Biochemistry

The Cancer-Related Runx2 Protein Enhances Cell Growth and Responses to Androgen and TGFβ in Prostate Cancer Cells

Margaretha van der Deen,[1] Jacqueline Akech,[1] Tao Wang,[2,3,4] Thomas J. FitzGerald,[2,3,4] Dario C. Altieri,[3,4,5] Lucia R. Languino,[3,4,5] Jane B. Lian,[1,3,4] Andre J. van Wijnen,[1,3,4] Janet L. Stein,[1,3,4] and Gary S. Stein[1,3,4]*

[1]*Department of Cell Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655*

[2]*Department of Radiation Oncology, University of Massachusetts Medical School, Worcester, Massachusetts*

[3]*Prostate Cancer Discovery and Development Program, University of Massachusetts Medical School, Worcester, Massachusetts*

[4]*Cancer Center, University of Massachusetts Medical School, Worcester, Massachusetts*

[5]*Department of Cancer Biology, University of Massachusetts Medical School, Worcester, Massachusetts*

ABSTRACT

Prostate cancer cells often metastasize to bone where osteolytic lesions are formed. Runx2 is an essential transcription factor for bone formation and suppresses cell growth in normal osteoblasts but may function as an oncogenic factor in solid tumors (e.g., breast, prostate). Here, we addressed whether Runx2 is linked to steroid hormone and growth factor signaling, which controls prostate cancer cell growth. Protein expression profiling of prostate cell lines (i.e., PC3, LNCaP, RWPE) treated with 5α-dihydrotestosterone (DHT) or tumor growth factor β (TGFβ) revealed modulations in selected cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors that are generally consistent with mitogenic responses. Endogenous elevation of Runx2 and diminished p57 protein levels in PC3 cells are associated with faster proliferation in vitro and development of larger tumors upon xenografting these cells in bone in vivo. To examine whether TGFβ or DHT signaling modulates the transcriptional activity of Runx2 and vice versa, we performed luciferase reporter assays. In PC3 cells that express TGFβRII, TGFβ and Runx2 synergize to increase transcription of synthetic promoters. In LNCaP cells that are DHT responsive, Runx2 stimulates the androgen receptor (AR) responsive expression of the prostate-specific marker PSA, perhaps facilitated by formation of a complex with AR. Our data suggest that Runx2 is mechanistically linked to TGFβ and androgen responsive pathways that support prostate cancer cell growth. J. Cell. Biochem. 109: 828–837, 2010.

KEY WORDS: CELL CYCLE; BONE; STEROID HORMONE RECEPTORS; PROLIFERATION; CELL SIGNALING

Prostate cancer is the most common cancer in men in Western countries and the second leading cause of cancer death [Buijs and Van der Pluijm, 2009]. Treatment involves anti-androgen therapy, orchiectomy, radiation, and/or chemotherapy. One main problem for prostate cancer therapy is that, at relapse, tumors often become androgen independent and insensitive to therapy-induced apoptosis. Metastasis to bone is common (~70% of cases), and cancer cells form osteolytic lesions in bone [Buijs and Van der Pluijm, 2009]. The Runt-related transcription factor Runx2 (CBFA1/AML3) is a critical regulator of bone formation, based on studies demonstrating that Runx2 null or Runx2ΔC mice do not form bone due to maturational arrest of osteoblasts [Komori et al., 1997; Choi et al., 2001]. Runx2-deficient cells escape senescence and proliferate faster than Runx2-expressing osteoblasts, indicating that Runx2 acts as a growth suppressor in a normal cellular context [Pratap et al., 2003; Kilbey et al., 2007; Zaidi et al., 2007]. Furthermore, Runx2 function is coupled to cell-cycle progression and Runx2 levels oscillate during the cell cycle in osteoblasts [Galindo et al.,

Abbreviations: CDK, cyclin-dependent kinase; CKI, CDK inhibitor; PSA, prostate-specific antigen; TGFβ, tumor growth factor β; DHT, 5α-dihydrotestosterone; ELISA, enzyme-linked immuno-sorbent assay; TGFβRII, TGFβ receptor II; AR, androgen receptor.

Grant sponsor: NIH; Grant numbers: CA082834, AR048818, CA078810, HL054131, CA090917, DK032520.

*Correspondence to: Gary S. Stein, Department of Cell Biology and Cancer Center, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655. E-mail: gary.stein@umassmed.edu

Received 6 November 2009; Accepted 20 November 2009 • DOI 10.1002/jcb.22463 • © 2010 Wiley-Liss, Inc.
Published online 15 January 2010 in Wiley InterScience (www.interscience.wiley.com).

2005], but this cell-cycle regulatory function may be altered in osteosarcoma cells [Kansara and Thomas, 2007; Nathan et al., 2009; Pereira et al., 2009; San Martin et al., 2009].

Runx proteins are expressed in prostate tissue and in prostate cancer cells, but their biological functions in prostate have not been resolved [Yeung et al., 2002; Brubaker et al., 2003; Fowler et al., 2006; Akech et al., 2009; Baniwal et al., 2009]. Previous studies have demonstrated that Runx2 and Runx1 interacts with the prostate-specific antigen (PSA) promoter [Fowler et al., 2006], an important marker for progression of prostate cancer. This finding suggests that Runx2 plays a role in progression of the disease. Furthermore, several studies have indicated that Runx2 may increase the oncogenic potential of cancer cells (e.g., breast, prostate, and bone) and that Runx2 regulates many genes involved in metastasis and invasion, such as matrix metalloproteinases (MMP2, MMP9, and MMP13) and vascular endothelial growth factor (VEGF) [Pratap et al., 2005, 2006; Blyth et al., 2006]. These studies together suggest that Runx2 acts as an oncogene in the context of tumor cells and that Runx2 function is deregulated in prostate tumors resulting in increased metastatic potential.

Runx2 is an important mediator of TGFβ/SMAD and steroid hormone signaling [Zaidi et al., 2002; Paredes et al., 2004; Kang et al., 2005; Javed et al., 2008], which are both implicated in cancer progression. TGFβ functions as a tumor suppressor in many cell types and is a potent growth inhibitor by, for example, inducing the CDK inhibitor p21. However, TGFβ receptors are frequently mutated or downregulated in prostate cancer to render cells insensitive to TGFβ signaling [Williams et al., 1996; Kim et al., 1998]. TGFβ/BMP signaling promotes interactions of Runx2 with SMADs, a mechanism that integrates biological cues required for osteoblastogenesis and tumor progression [Pratap et al., 2006; Javed et al., 2008]. Runx2 is also associated with androgen receptor (AR) activity in prostate cancer cells [Baniwal et al., 2009]. In addition, there is cross-talk between TGFβ signaling and the AR but the mechanisms are poorly understood. Exposure to androgen (5α-dihydrotestosterone, DHT) and TGFβ may have different effects on cell-cycle progression in prostate cancer cells, depending on their expression of androgen and TGFβ receptors [Danielpour, 2005; van der Poel, 2005]. Also, DHT reduces expression of TGFβ receptor II (TGFβRII), leading to suppression of the ability of TGFβ to induce apoptosis [Song et al., 2008].

In this study, we have examined the functional interrelationships of Runx2, DHT, and TGFβ with proliferation in prostate cancer cells that express either AR or TGFβRII. Our data indicate that both DHT- and TGFβ-signaling modulate the levels of cell-cycle regulatory proteins and the transcriptional activity of Runx2, and that Runx2 levels are coupled with growth of prostate cancer cells in vitro and in vivo.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Two sub-cultured lines of PC3 prostate cancer cells (PC3-a and PC3-b), as well as LNCaP, C4-2B, RWPE1, and HeLa cells were maintained as follows. PC3-a cells were cultured in T-medium with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) [Huang et al., 2005]. PC3-b cells were grown in DMEM-F12 with 10% FBS. LNCaP cells were cultured in RPMI supplemented with 10 mM non-essential amino acids, 2 mM sodium pyruvate, and 10% FBS. C4-2B cells, which promote formation of osteoblastic lesions [Wu et al., 1998], were cultured in RPMI supplemented with 10% FBS, and HeLa cells were maintained in DMEM with 10% FBS. The above cell lines were also supplemented with 2 mM L-glutamine and penicillin–streptomycin. RWPE1 cells were maintained in reduced keratinocyte medium and supplements [Bello et al., 1997]; these immortalized prostate cells are TGFβRII positive and express AR at low levels in standard culture conditions (without androgen supplementation) [Bello et al., 1997]. Cell cultures were incubated at 37°C with 5% CO_2. All media and supplements were purchased from Gibco/Invitrogen (Grand Island, NY).

For treatment with DHT and TGFβ, normal growth medium was replaced with medium containing 2% charcoal stripped (CCS) FBS for 24 h, followed by incubation with DHT (10 or 20 nM) (Sigma, St. Louis, MO) or porcine TGFβ (10 or 50 ng/ml) (R&D Systems, Minneapolis, MN) or vehicle (ethanol for DHT and incomplete growth medium for TGFβ) for 24 h. Cells were harvested for protein and mRNA detection (see below).

CELL GROWTH ASSAYS

For growth curves, PC3-a and PC3-b cells were plated at equal densities in six-well plates (5×10^4 cells per well). Cells were harvested with trypsin/0.25% EDTA and counted in quadruplicate on days 1–6 after plating using Trypan Blue exclusion and a hemocytometer. For siRNA experiments PC3-a cells were plated at ~30–40% confluence and transfected using Oligofectamine® (Invitrogen, Carlsbad, CA). Commercially available siRNA oligonucleotides for Runx2 and non-silencing oligonucleotides (Smartpool ON-TARGETplus) (Dharmacon, Lafayette, CO) were used, and treatment (50 nM) was carried out for 48 h, after which cells were re-plated in 10 cm plates at day 0 and counted in triplicate on days 1–4.

WESTERN BLOT ANALYSIS

Cells were lysed in RIPA buffer supplemented with protease inhibitors (complete, EDTA-free, Roche Diagnostics, Mannheim, Germany), and MG132 (25 μM). Equal amounts of protein lysates in SDS sample buffer were loaded on a 10% acrylamide gel and subjected to electrophoresis and immunoblotting (Bio-Rad System, Hercules, CA).

Immunoblots were incubated for 1 h with the following antibodies (purchased from Santa Cruz Biotechnology, Santa Cruz, CA): p57 (C-20), p27 (C-19), p21 (H-164), cyclin D1 (DCS-6), cyclin A (C-19), CDK4 (C-22), AR (441), and CDK2 (M-2). Antibodies against cyclin E (HE-12) (Becton Dickinson, Franklin Lakes, NJ), tubulin (DM1A) (Sigma), and Runx2 (mouse monoclonal clone 8G5) (MBL International, Woburn, MA) were purchased from the indicated vendors. Peroxidase-labeled goat-anti-rabbit or goat-anti-mouse antibodies (Santa Cruz Biotechnology) were used as secondary antibodies and were visualized with enhanced chemiluminescence (ECL) chemistry (PerkinElmer, Waltham, MA) on BioMax film (Kodak, Rochester, NY).

LUCIFERASE ASSAYS

PC3 and LNCaP cells were plated in six-well plates in normal growth medium. The next day, cells were incubated for 24 h in medium containing 2% charcoal stripped (CCS) FBS. Transient transfections were then performed in fresh 2% CCS FBS containing medium using FuGENE6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Cells were transfected with one of the three different reporters in which the luciferase gene is driven by promoters containing tandemly repeated regulatory elements: six binding sites for Runx2 (6x-Runx2-luc, 500 ng), three TGFβ responsive elements each spanning a Smad and Runx2 binding site (3x-TGFβRE-luc, 500 ng), or multiple androgen responsive elements (ARE-luc, 2 μg). Co-transfections were performed with plasmids expressing HA epitope-tagged Runx2 under control of the CMV promoter. A promoterless luciferase vector construct (pGL2-Luc) was used as a negative control. The same total amount of DNA was used in every transfection. After 16 h of transfection, cells were treated with DHT (10 nM) or TGFβ (10 or 50 ng/ml) or vehicle (ethanol for DHT and incomplete growth medium for TGFβ) for 24 h. Cell lysates were analyzed for firefly luciferase activity with the dual-luciferase reporter assay system (Promega, Madison, WI). Firefly activity was normalized to Renilla (phRL-null, 25 ng per well) (Promega).

QUANTITATIVE RT-PCR

Total RNA purified using Trizol (Invitrogen) was subjected to DNase I digestion, and cDNA was prepared with the qScript cDNA synthesis kit (Quanta BioSciences, Gaithersburg, MD). Relative PCR quantitation was determined using a 7300 sequence detection system (Applied Biosystems, Foster City, CA) with SYBR Green chemistry (Bio-Rad System). The relative mRNA expression was calculated with the $\Delta\Delta C_T$ method. Real-time primer pairs were used to amplify human mRNA (in 5′–3′ direction): p57 forward AAG AGA TCA GCG CCT GAG AA and reverse TGG GCT CTA AAT TGG CTC AC; p27 forward AGA TGT CAA ACG TGC GAG TG and reverse TCT CTG CAG TGC TTC TCC AA; p21 forward GAC TCT AGG GTC GAA AAC G and reverse GGA TTA GGG CTT CCT CTT GG; RB forward CAC ATT CCT CGA AGC CCT TA and reverse TTT TTG TTG GTG TTG GCA GA; p107 forward ATG GAT GCT CCA CCA CTC TC and reverse GAG CGC TTC TTG GTG TAA GG; p130 forward ATT ACG CCG TCT CCA AGA TG and reverse ATG CAT TAA AGG CTG CTG CT; CDK4 forward GGA ACT CTG AAG CCG ACC AG and reverse ACA TCT CGA GGC CAG TCA TC; cyclin D1 forward GAT CAA GTG TGA CCC GGA CT and reverse TCC TCC TCC TCT TCC TCC TC; cyclin A2 forward CCT GCA AAC TGC AAA GTT GA and reverse AAA GGC AGC TCC AGC AAT AA; cyclin B1 forward CAA GCC CAA TGG AAA CAT CT and reverse GGA TCA GCT CCA TCT TCT GC; cyclin B2 forward ACT GCT CTG CTC TTG GCT TC and reverse TTT CTC GGA TTT GGG AAC TG; H4n/o forward AGC TGT CTA TCG GGC TCC AG and reverse CCT TTG CCT AAG CCT TTT CC; GAPDH forward ATG TTC GTC ATG GGT GTG AA and reverse TGT GGT CAT GAG TCC TTC CA; Runx2 forward CGG CCC TCC CTG AAC TCT and reverse TGC CTG CCT GGG GTC TGT A.

TUMOR CELL INJECTION INTO MOUSE TIBIAE

Animal studies were conducted in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols and the National Institutes of Health Guide for Care and Use of Laboratory Animals. PC3-a and PC3-b (1×10^5 cells) were injected into tibiae of SCID mice (three mice per group) as described previously [Lee et al., 2003; Barnes et al., 2004]. Tumors were allowed to grow for a period of 6 weeks and images were acquired for tumor growth analysis.

IMMUNOPRECIPITATION

LNCaP cells were infected with an adenoviral vector-expressing Runx2 and allowed to grow for 48 h. Cells were harvested and lysed in 800 μl of Nonidet P-40 buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40, 1× complete protease inhibitor (Roche), and 25 μM MG132 (Sigma)). Following centrifugation of the lysate for 15 min, the supernatant was transferred to a clean microcentrifuge tube and precleared with 20 μl of protein A/G plus-agarose beads (Santa Cruz Biotechnology) at 4°C for 30 min. Beads were collected by centrifugation at 1,000*g* for 5 min at 4°C. Runx2 antibody (PEBP2αA M-70; 3 μg) (Santa Cruz Biotechnology) was added to the pre-cleared cell lysate followed by incubation at 4°C for 1 h. To precipitate immunocomplexes, a suspension of protein A/G plus agarose beads (50 μl) was added and further incubated at 4°C with agitation for 1 h. The beads were washed twice with 1 ml wash buffer (20 mM Tris, pH 8.3, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 50 mM NaCl, 2 mM EDTA, 1× complete protease inhibitor, and 25 μM MG132) and the bead pellet was subsequently suspended in an equal volume of 2× SDS sample buffer. AR proteins were analyzed by Western blot analysis using mouse monoclonal antibodies (H-300, Santa Cruz Biotechnology) and secondary HRP-conjugated goat anti-mouse antibodies.

DETECTION OF PROSTATE-SPECIFIC ANTIGEN (PSA) IN CELL CULTURE

Supernatants were collected from LNCaP cells stimulated with DHT and transfected with Runx2-HA expression plasmids (see the Luciferase Assays Section). Supernatants from untransfected cells and vehicle-treated cells were collected as negative controls. For measuring PSA protein levels, a PSA enzyme-linked immunosorbent assay (ELISA) kit was used (United Biotech, Inc., Mountain View, CA). PSA levels were calculated using the standard curve provided in the kit.

RESULTS

RESPONSIVENESS OF CELL-CYCLE REGULATORY PROTEINS TO ANDROGEN AND TGFβ SIGNALING IN PROSTATE CANCER CELLS

We initially investigated the expression of cell-cycle regulators in selected prostate cancer cell lines upon exposure to androgen (DHT) and TGFβ to determine the contributions of DHT and TGFβ signaling to cell-cycle control. PC3 cells are AR negative but express TGFβRII, whereas LNCaP cells express AR but are TGFβRII negative. As a control line, we used immortalized RWPE prostate cells that are TGFβRII positive and appear to express AR at levels below detection based on our immunoblotting data (Fig. 2A), although it has been reported that these cells are AR positive [Bello et al., 1997]. Incubation of PC3 and RWPE cells with 10 nM DHT for 24 h does not change the expression of cell-cycle-related proteins,

including cyclins D1, E, and A, CDK4 and CDK2, or the CDK inhibitors p57, p27, and p21 as examined by Western blotting (Fig. 1A). However, in androgen-responsive LNCaP cells, DHT incubation increases protein expression of cyclin A and CDK2, while p21 levels are moderately decreased (up to twofold). Because expression of cyclin A and CDK2 is linked to progression through the S and G2 phases of the cell cycle, our results are consistent with proliferation upon DHT stimulation of AR-positive LNCaP cells, but not of PC3 and RWPE cells.

Treatment of the TGFβ responsive PC3 cells with 10 ng/ml of TGFβ for 24 h stimulates protein expression of cyclins E and A, as well as CDK2 and p21, but not for the TGFβRII-negative LNCaP cells, indicating that TGFβ may accelerate cell-cycle progression in PC3 but not LNCaP cells. In contrast, in the immortalized prostate cell line RWPE, TGFβ incubation results in downregulation of cyclin D1 and concomitant upregulation of p21, reflecting an anti-mitogenic response. Quantitative reverse transcriptase PCR (qRT-PCR) was applied to assess whether changes in the protein levels of cell-cycle regulators in PC3 or LNCaP cells are mediated by either transcriptional or post-transcriptional mechanisms (Fig. 1B). In general, we find that mRNA levels for p21, cyclin A2, and cyclin D1 do not change appreciably upon DHT or TGFβ treatment in either cell type, suggesting that protein translation or stability and not mRNA levels determine the levels of these cell-cycle regulators in prostate cancer cells.

Surprisingly, TGFβ interferes with the mitogenic response to DHT in TGFβRII-negative LNCaP cells, because combined treatment with TGFβ and DHT blocks the modest suppression of p21 observed with DHT alone and enhances CDK4 protein expression. In comparison, DHT does not alter the pro-proliferative effects of TGFβ in AR-negative PC3 cells, albeit that a modest decrease in CDK4 is observed. Hence, TGFβRII-negative LNCaP cells appear to employ compensatory mechanisms to support TGFβ signaling (e.g., through alternate TGFβ receptors), while AR-negative PC3 cells do not have

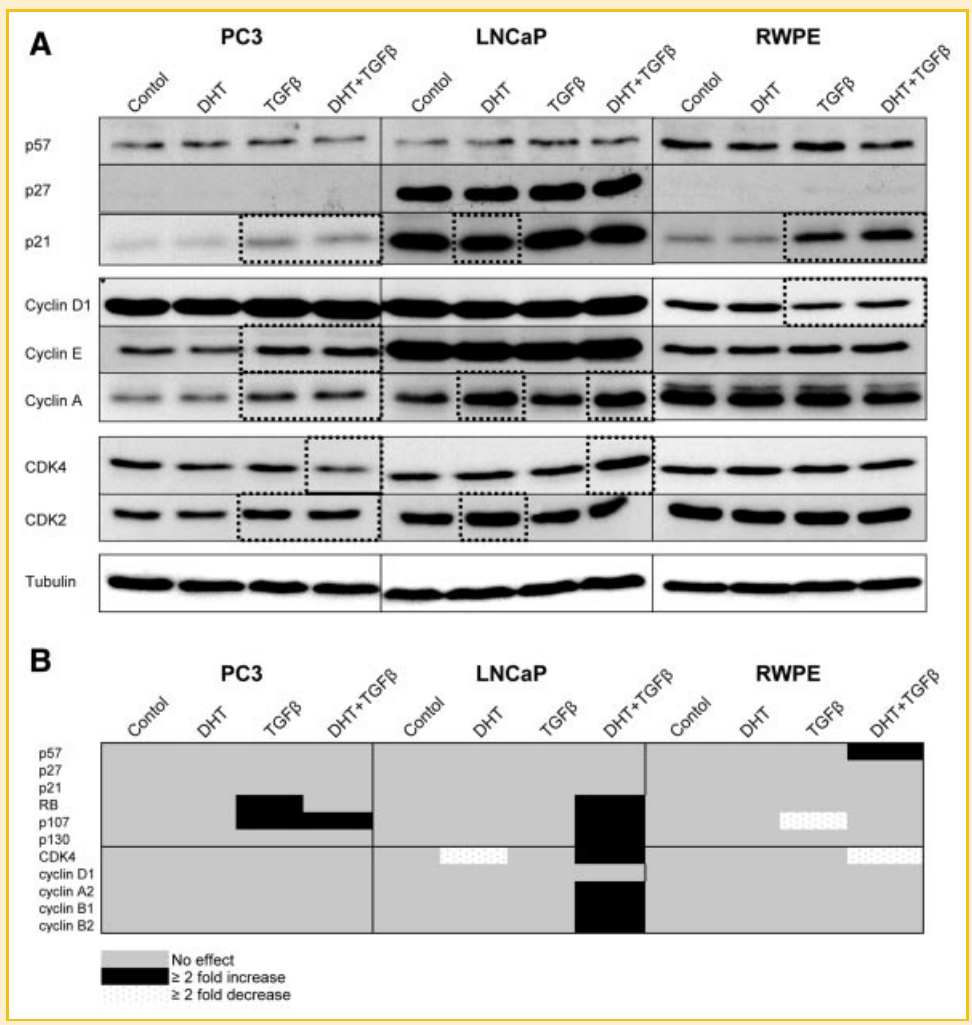

Fig. 1. Cell-cycle responses of prostate cancer cells to androgen and TGFβ. Prostate cancer cell lines PC3-b and LNCaP, and immortalized prostate cells RWPE were treated for 24 h with DHT (10 nM) or TGFβ (10 ng/ml) in 2% CCS medium. A: Cell lines respond differently to treatment dependent on the presence or absence of a functional androgen or TGFβ pathway. Equal amounts of protein were subjected to Western blot analysis and stained for cell-cycle markers. Dotted boxes indicate proteins that show more than twofold increase or decrease compared to control treatments. B: mRNA detection under the same treatment conditions. Gray boxes indicate no effect upon treatment, black and dotted boxes indicate, respectively, more than twofold increase or decrease in mRNA levels. Transcript levels were normalized to GAPDH mRNA levels.

a substitute receptor to mediate DHT signaling. We note that elevations of CDK4 and cyclin A1 protein levels that are observed in LNCaP cells upon combined treatment with both DHT and TGFβ are directly reflected by increased mRNA levels and occur concomitant with increased mRNA expression of cyclins B1 and B2, as well as the pocket proteins pRB, p107, and p130, consistent with cell-cycle-related cross-talk between DHT and TGFβ signaling.

ENDOGENOUS LEVELS OF Runx2 AND AR TRANSCRIPTION FACTORS IN PROSTATE CANCER CELLS

We assessed expression patterns of the bone-related transcription factor Runx2, which is ectopically expressed in metastatic breast cancer cells [Pratap et al., 2003, 2005; Galindo et al., 2005; Blyth et al., 2006], cell-cycle related protein and the AR, which is frequently lost in recurring prostate cancer, in a panel of five prostate cell lines. Expression levels were correlated with cell growth rate and metastatic capacity that are known for each cell line. We compared two different sub-lines of PC3 cells (PC3-a and PC3-b), the prostate cancer cell line LNCaP and its derivative cell line C4-2B (derived from LNCaP, grown in vivo in mice) [Wu et al., 1998], as well as the immortalized prostate cell line RWPE and HeLa cervical carcinoma cells (Fig. 2). PC3-a cells express relatively high Runx2 protein and mRNA levels, whereas PC3-b cells express Runx2 protein and mRNA at or below the level of detection (Fig. 2A,B). In all other prostate cell lines, Runx2 protein expression was not evident (Fig. 2A) and mRNA levels were only detectable at relatively low levels (Fig. 2B). As expected, LNCaP and C4-2B express high protein levels of AR (Fig. 2A). However, there is no appreciable expression of AR in the two PC3 sub-lines, nor in HeLa and RWPE cells under basal (non-DHT stimulated) conditions. It appears that the robust expression of Runx2 in one of the PC3 sub-lines is a sporadic event that may occur in a subset of prostate cancer cells.

The differences in Runx2 and AR expression in selected prostate cancer cell lines correlate with expression profiles of cell-cycle proteins. We find that PC3-a, PC3-b, LNCaP, C4-2B, RWPE, and HeLa cells each have distinct expression signatures for cell-cycle regulatory proteins (Fig. 2). For example, in LNCaP and C4-2B cells, the expression of p27 and p21 is significantly higher compared to PC3 cells. In RWPE cells, p57, p27, and p21 are expressed at relatively low levels. Cyclin D1 protein levels are higher in PC3-b cells compared to PC3-a cells. Because cyclin D1 plays a role in degradation of Runx2 [Shen et al., 2006], elevation of cyclin D1 may further prevent accumulation of Runx2 protein in combination with the low expression of Runx2 mRNA in PC3-b cells. Strikingly, expression of the CDK inhibitor p57 is clearly elevated in PC3-b cells (Fig. 2) (also presented in Fig. 1) compared to PC3-a cells and other prostate cell lines. The p57 level in PC3b cells is comparable to the level observed in HeLa cells that are known to express high levels of p57 [Mitra et al., 2009]. Expression of p57 is often silenced in prostate cancer due to methylation of the p57 promoter [Lodygin et al., 2005]. It is possible that the p57 promoter may have been re-activated (e.g., by demethylation) in PC3-b cells to support ordered cell-cycle progression.

In conclusion, the expression levels of Runx2 and other cell-cycle-related proteins are variable in different AR-positive and -negative prostate cancer cell types. There is an inverse relationship between Runx2 and p57 expression in two sub-lines of PC3 cells, which may be related to different levels of cyclin D1 expression. Furthermore, LNCaP and C4-2B cells express relatively high p27 and p21 levels, perhaps related to the slower growth rate of these cell lines compared to PC3 cells.

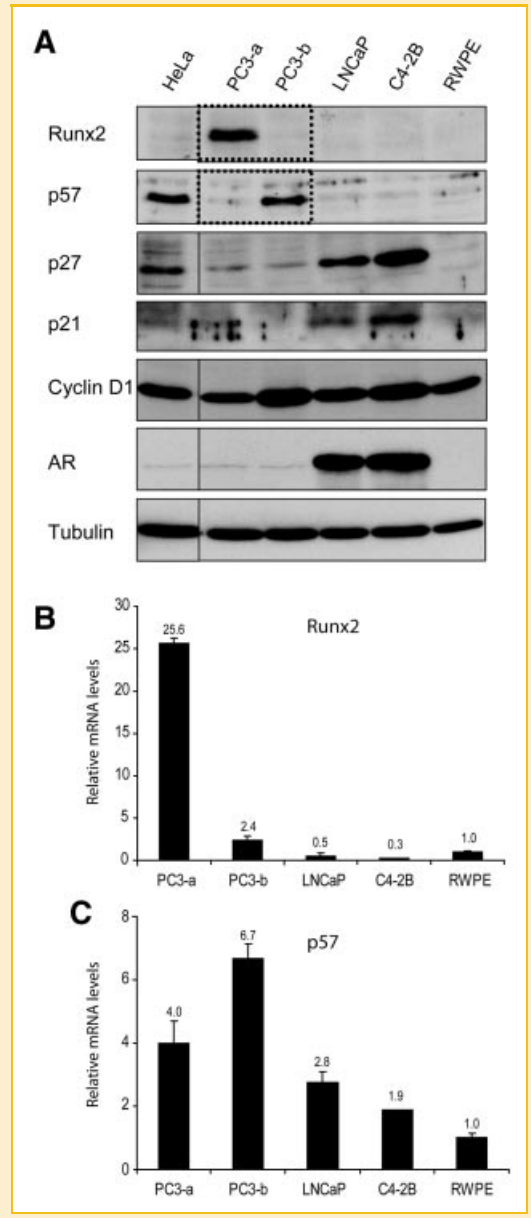

Fig. 2. Endogenous levels of Runx2, cell-cycle proteins, and AR in prostate cancer cells. A: Prostate cancer cells were analyzed for protein expression with Western blot for Runx2, p57, p27, and p21, cyclin D1, and AR. Equal amounts of protein were loaded for all cell lines, with tubulin as a loading control. HeLa cells were included as a control cell line. Dotted boxes indicate interesting differences in Runx2 and p57 expression in two PC3 sub-lines (PC3-a and PC3-b). For comparison, mRNA levels for Runx2 (B) and p57 (C) are shown in the lower panels. The graphs show data from representative and reproducible experiments.

ELEVATED Runx2 EXPRESSION IS RELATED TO INCREASED TUMOR VOLUME AND CELL GROWTH RATE OF PC3 CELLS

Runx2 expression has been shown to correlate with expression of genes that augment the metastatic capacity of breast and prostate cancer cells [Pratap et al., 2005; Akech et al., 2009]. At a gross anatomical level, PC3-a cells expressing high Runx2 levels appear to form larger bone tumors than PC3-b cells upon xenografting by tibial injection (Fig. 3A). Histological analysis revealed an apparent increase in Ki67 staining in tumor cells derived from PC3-a cells suggesting a higher proliferation rate (data not shown). We tested whether elevated Runx2 expression correlates with increased cell growth of PC3-a cells compared to PC3-b cells. Indeed, PC3-a cells grow faster than PC3-b cells in vitro (Fig. 3B). To address whether Runx2 plays a direct role in this higher proliferation rate, we performed RNA interference using Runx2 siRNA in PC3-a cells. Downregulation of Runx2 in PC3-a cells inhibits cell growth at day 4 by ~25–30% (Fig. 3C). Thus, the higher proliferation rate of PC3-a cells expressing high levels of Runx2 is associated with the larger tumor volume observed in vivo and is consistent with increased Ki67 staining and low p57 levels.

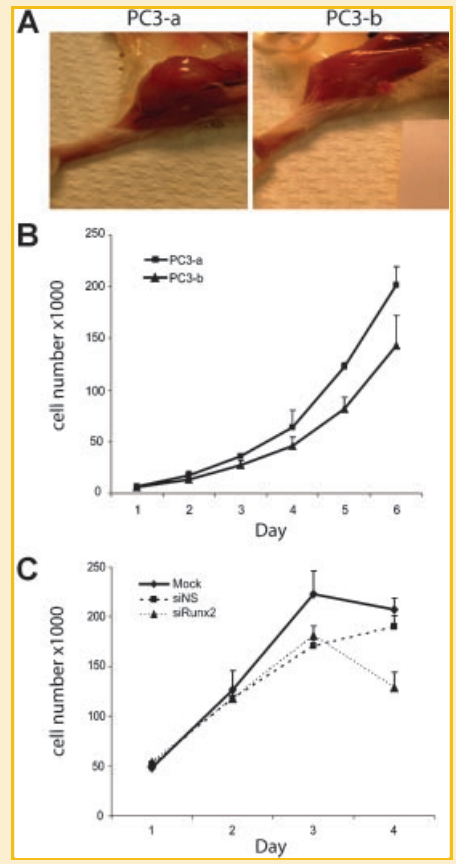

Fig. 3. Runx2 expression in PC3 cells is related to higher cell growth rate and larger tumor volume. A: Upper panel depicts tumor size resulting from PC3-a and PC3-b cells. Cells were injected in tibiae of SCID mice ($n = 3$), and photographs were taken 6 weeks after injection. Representative images are shown. B: Cell growth studies were performed with PC3 sub-lines a and b. PC3-a cells with higher Runx2 expression grow faster than PC3-b cells that express higher p57 levels (see Fig. 2). Data shown are from averages of two independent growth curves counted in quadruplicate. C: Growth curves in which PC3-a cells were subjected to Runx2 siRNA. At day 4 there is less cell growth compared to control treated cells (non-silencing, NS) and mock-treated cells. Data shown are from averages of cells counted in triplicate.

TRANSCRIPTIONAL CO-OPERATION BETWEEN Runx2 AND TGFβ/SMAD SIGNALING IN PC3 CELLS

Because Runx2 integrates growth factor and steroid hormone dependent cell signaling responses and positively correlates with cell growth in prostate cancer cells, we focused on the role of Runx2 in TGFβ/Smad and AR signaling. We first investigated whether TGFβ or DHT affect Runx2 expression in PC3-a prostate cancer cells which express functional TGFβRII. While TGFβ treatment increases p21 protein levels in PC3 cells as expected, Runx2 protein and mRNA levels did not change significantly (Fig. 4A and data not shown). We also investigated the effect of TGFβ on Runx2 activity in functional assays using two different reporters with multimerized Runx2-binding sites (6x-Runx2-luc) or tandemly repeated composite regulatory elements containing a TGFβ responsive motif flanked by a Runx2 motif (3x-multimerized TGFβRE-luc reporter). Runx2 co-transfection stimulates the 6x-Runx2-luc reporter in PC3 cells, but TGFβ treatment by itself had no effect. However, addition of TGFβ increases transcriptional activity in cells transfected with Runx2 (Fig. 4B). The increase may be mediated by protein/protein interactions between Runx2 and TGFβ dependent Smads (Smad2/3) [Afzal et al., 2005; Javed et al., 2008].

TGFβ administration and Runx2 each separately stimulates activity of the 3x-multimerized TGFβRE-luc reporter as expected. Interestingly, Runx2 expression synergizes with the TGFβ response to increase promoter activity in PC3-a cells (Fig. 4C). Taken together, these data indicate that Runx2 is transcriptionally active and that its functional co-operation with TGFβ is maintained and enhances transcriptional responses in prostate cancer cells.

Runx2 INCREASES ANDROGEN RESPONSIVENESS IN LNCaP CELLS

Runx2 co-operates with steroid hormone signaling in the osteogenic lineage; therefore, we analyzed whether the transcriptional activities of AR and Runx2 are functionally coupled in prostate cancer cells. We first examined whether exposure of LNCaP cells, which express AR, to DHT and/or TGFβ modulates protein levels of Runx2 and AR. However, neither ligand affects AR or Runx2 protein levels in LNCaP cells (Fig. 5A). Thus, Runx2 expression is constitutive and independent of androgen and TGFβ stimulation in both PC3-a and LNCaP prostate cancer cell lines (Figs. 4A and 5A).

We investigated whether Runx2 activity is affected by androgen, and/or if androgen responsiveness is regulated by Runx2 in prostate cancer cells transfected with the 6x-Runx2-luc or with a construct containing a tandemly repeated ARE fused to the luciferase gene (ARE-luc reporter). Runx2 transfection stimulates the 6x-multimerized Runx2-luc reporter in both LNCaP (Fig. 5B) and PC3-a cells (data not shown), but addition of DHT does not significantly change the activity in either cell line. In the absence of Runx2 (EV ± DHT), DHT mediates an androgen response of about sixfold using the ARE-luc reporter (Fig. 5C). Forced expression of Runx2 in LNCaP cells increases ARE activity in the absence or presence of DHT. The

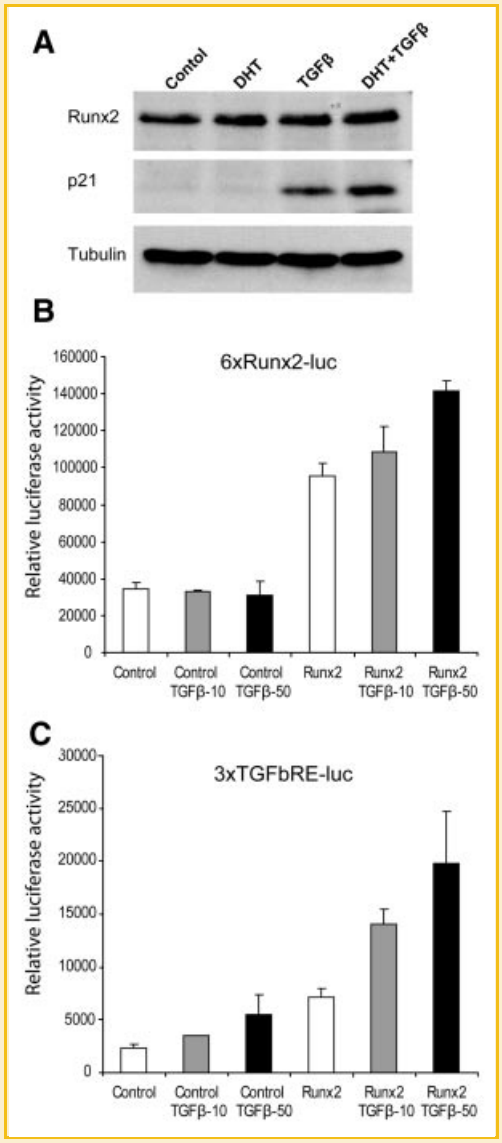

Fig. 4. Runx2 and TGFβ responses cooperate in PC3 cells. A: Treatment of PC3 (sub-line a) cells with TGFβ (10 ng/ml) for 24 h increased p21 levels but did not significantly affect Runx2 protein levels. B: In functional luciferase reporter assays, TGFβ (10 or 50 ng/ml) stimulated Runx2 activity in a dose-dependent manner. Empty vector plasmid (control) was transfected or Runx2-HA plasmid (250 ng). C: Runx2 enhances the TGFβ response in PC3 cells. Averages were taken from duplicate experiments and firefly luciferase activity was normalized to Renilla values.

Runx2-dependent stimulation in the absence of DHT may reflect effects on the unliganded AR or perhaps low endogenous levels of androgen present in cell cultures. In PC3-a cells that lack the AR, the opposite effect was observed, that is, Runx2 expression suppresses the ARE-luc reporter with no effect of DHT (data not shown). Sequence analysis of the ARE-luc reporter identified a putative Runx2 binding site (5′-CGCGGTC-3′) 4 bp upstream from the tandemly repeated androgen binding sites. Runx2 may either bind directly to this site or may physically interact with AR, in both cases resulting in an activated androgen response. Therefore, we performed immunoprecipitation experiments to investigate whether Runx2 directly binds to AR. The Runx2 antibody we used is very effective in precipitating Runx2 from cell lysates (data not shown). Importantly, as shown in Figure 5D, AR protein is enriched in immunoprecipitates with Runx2 antibody in lysates obtained with LNCaP cells that endogenously express AR. This finding indicates that Runx2 physically interacts with AR in these cells.

Previous studies have demonstrated Runx2 occupancy on the PSA promoter enhancing its activity [Fowler et al., 2006]. Because Runx2 interacts with AR (Fig. 5D), we assessed whether PSA production changes upon Runx2 expression. As a positive control, we treated LNCaP cells with DHT and detected >10-fold elevated PSA protein levels as measured by ELISA (Fig. 5E). When LNCaP cells were transfected with increasing amounts of Runx2, PSA production was significantly increased in a dose-dependent manner (Fig. 5F). We conclude that Runx2 is functionally related to AR activity and consequently increases production of PSA.

DISCUSSION

In this study, we provide evidence that Runx2 enhances androgen and TGFβ responses in prostate cancer cells. This finding, together with the observation that Runx2 is related to increased cell growth in PC3 cells, suggests that expression of Runx2 may play an important role in progression of prostate cancer. Runx2 is an essential transcription factor for bone formation and suppresses cell growth in normal osteoblasts. However, we and others have shown that Runx2 may be an oncogenic factor in tumor cells (breast, prostate, lymphoma) and regulates genes involved in migration and invasion [Blyth et al., 2005, 2006; Pratap et al., 2005, 2008]. Runx2 is expressed at low but detectable levels in normal prostate epithelial cells and has been detected in selected prostate cancer cell lines [Yeung et al., 2002; Brubaker et al., 2003; Fowler et al., 2006; Baniwal et al., 2009], but the biological function of Runx2 in prostate has not been resolved. Importantly, we find that depletion of Runx2 in cultured prostate cancer cells results in a lower cell growth rate, indicating that Runx2 may have a growth promoting function in cells in which proliferation is deregulated. Xenograft experiments presented in this study show that PC3 cells expressing high levels of Runx2 grow faster and form larger tumors in bone than PC3 cells with low Runx2 levels. Taken together, these findings are consistent with a pro-mitogenic role for Runx2 in prostate cancer.

Prostate cancer frequently becomes refractory to hormone therapy, because the AR is often mutated or absent in recurrent tumors [So et al., 2003; Danielpour, 2005] thus rendering prostate cancer cells androgen independent. The androgen response normally suppresses TGFβ-induced cell death [So et al., 2003; van der Poel, 2005], and androgen independence thus requires aberrations in TGFβ signaling to promote proliferation of prostate cancer epithelial cells. We investigated the effects of both DHT and TGFβ on cell-cycle regulators in cell lines that differ in expression of Runx2, AR, and TGFβRII. We observed that expression of cyclins, CDKs, and CKIs is distinct in different prostate cancer cell lines; DHT

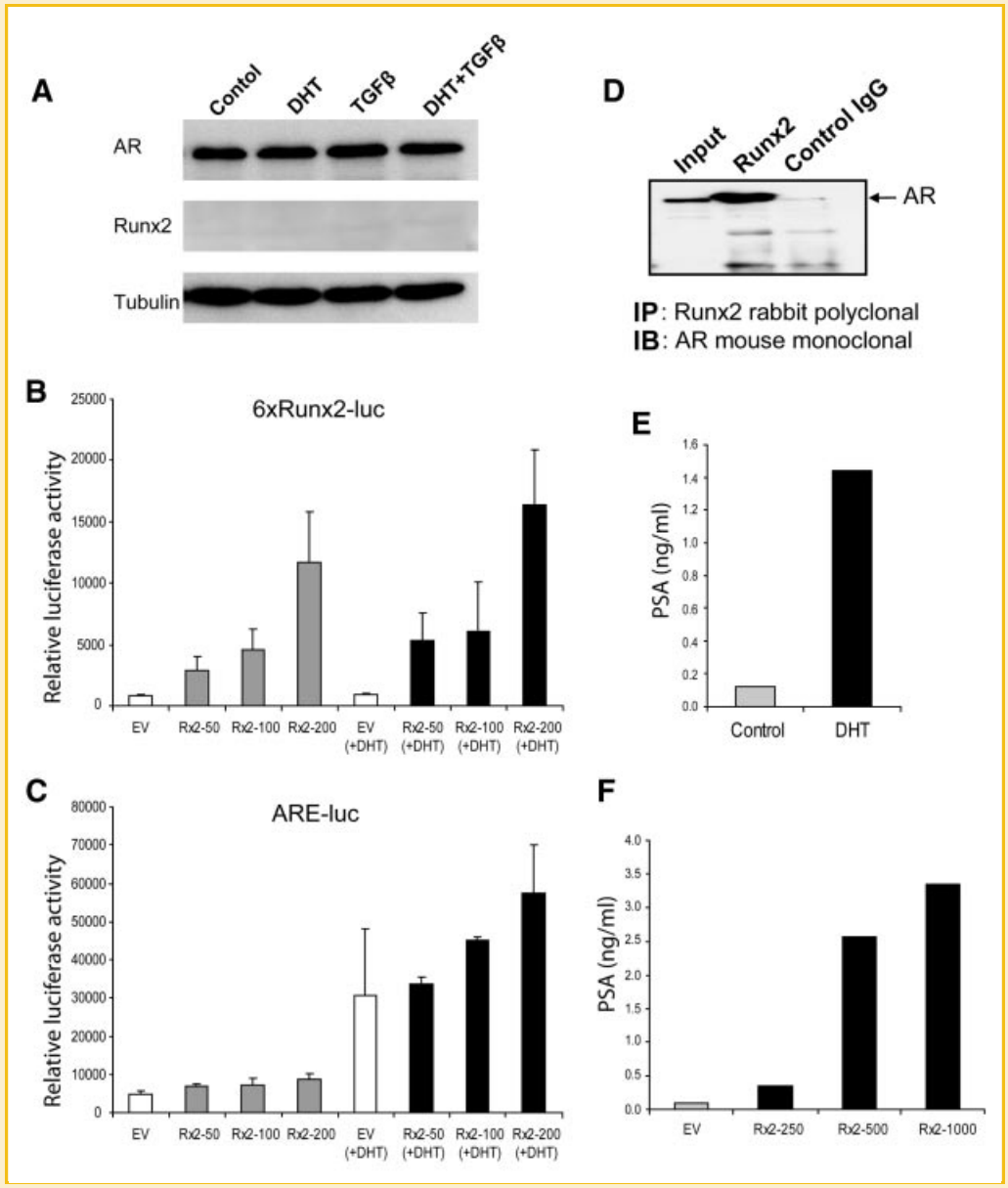

Fig. 5. Runx2 interacts with AR and increases androgen activity and PSA production in LNCaP cells. A: Treatment of LNCaP cells with DHT (10 nM) and/or TGFβ (10 ng/ml) for 24 h did not affect AR or Runx2 expression at the protein level. B: DHT (20 nM) does not significantly increase Runx2 activity in LNCaP cells. Cells were transfected with 50, 100, and 200 ng Runx2-HA plasmid DNA or empty factor (EV) as a negative control. Luciferase activity was normalized to Renilla values. A representative experiment is shown from three independent experiments performed in triplicate. C: Runx2 enhances ARE activity in LNCaP cells in a dose-dependent manner. A representative experiment is shown from three independent experiments performed in triplicate. D: Pulldown with Runx2 antibody in immunoprecipitation assay using LNCaP cell lysate demonstrates that Runx2 and AR physically interact. E: DHT incubation (10 nM) for 24 h stimulates PSA protein production in LNCaP cells as measured by ELISA. F: Forced Runx2-HA expression (250, 500, and 1,000 ng) in LNCaP cells strongly increases PSA production after 24 h of DHT (20 nM) treatment. A representative experiment is shown performed in triplicate.

and TGFβ generally modulate the levels of these cell-cycle factors as would be predicted from mitogenic or pro-survival effects. For example, the levels of several cell-cycle regulators increase with TGFβ treatment in PC3 cells that are androgen independent but express a functional TGFβRII, consistent with a pro-proliferative effect. Interestingly, TGFβ upregulates p21 in PC3 cells, although these cells do not express p53; expression of p21 might instead be stimulated through a TGFβ-Smad2/3-Runx2 pathway in PC3 cells expressing Runx2. We also observed that distinct sub-lines of PC3 cells exhibit differences in p57 levels. The promoter of p57 is CpG methylated in ~56% cases of prostate cancer and in several prostate cancer cell lines including PC3 cells [Lodygin et al., 2005], and p57 expression may be stimulated by demethylation of the promoter. The levels of p57 are reciprocal with Runx2 levels, although causal relationships between p57 and Runx2 were not further explored.

Runx2 activity is known to synergize with steroid hormone signaling (including vitamin D and ERalpha signaling) in different biological contexts. The data we present here show that Runx2 also functionally cooperates with androgen signaling as reflected by enhancement of DHT-stimulated and AR-mediated transcriptional activation upon Runx2 co-expression. Furthermore, immunoprecipitations reveal that Runx2 and AR physically interact. Consistent with this finding, Runx2 and AR were reported to colocalize in osteoblasts and repress each other's functional activity [Kawate et al., 2007]. While our studies were in progress, Baniwal et al. [2009] also detected AR–Runx2 complexes in prostate cancer cells. Their results suggest that AR represses Runx2 activity by blocking DNA binding and that DHT treatment inhibits Runx2 activity in COS7 and PC3 cells in which AR and Runx2 are overexpressed. However, our data did not reveal a DHT-dependent reduction in Runx2 activity in functional reporter assays in LNCaP cells that endogenously express AR. While additional studies will be required to resolve the effects of DHT on Runx2 in different cell types and experimental conditions, both studies support the concept that AR and Runx2 form complexes that modulate their respective transcriptional functions.

TGFβ acts as a tumor suppressor in many cell types and TGFβRII is often mutated in prostate tumors resulting in resistance to TGFβ-induced cell death. Runx2 is an important mediator in TGFβ signaling and binds Smad proteins that mediate downstream effects of TGFβ [Zaidi et al., 2002; Afzal et al., 2005; Javed et al., 2008]. We observed increased Runx2 activity upon TGFβ treatment, but no effect on Runx2 protein expression in PC3 and LNCaP cells. Thus, in contrast to mesenchymal cells in which Runx2 protein levels and activity are both stimulated by TGFβ/Smad signaling, our results indicate that Runx2 activity but not its protein levels are enhanced by TGFβ signaling in prostate cells. Because of the pro-mitogenic function of Runx2 we observed in prostate cancer cells, the responsiveness of Runx2 activity to TGFβ/Smad signaling would provide a selective advantage to cells that retain TGFβRII expression during prostate tumor progression.

PSA is an important biomarker that monitors disease progression in prostate cancer. Both Runx1 and Runx2 have been reported to regulate PSA gene transcription in cooperation with prostate-derived Ets factor (PDEF) [Fowler et al., 2006]. Consistent with their findings, we observe that DHT-dependent production of secreted PSA protein (as measured with ELISA) is increased by Runx2. Hence, our results and those obtained by Fowler and colleagues suggest that Runx2 is a key regulator of PSA as a prognostic factor in prostate cancer.

In conclusion, our results indicate that prostate cancer cells have unique expression signatures of cell-cycle regulatory factors and sporadically silence or activate expression of key proteins including Runx2 and the CDK inhibitor p57. We show that expression of Runx2 in PC3a cells is associated with increased cell growth, and that Runx2 activity is functionally linked to TGFβ signaling and the androgen response in prostate cancer cells. Cross-talk with androgen signaling is reflected by protein/protein interaction between Runx2 and AR, and the resulting enhanced androgen response correlates with increased PSA production. Taken together, our results suggest that Runx2 may be a novel molecular marker and a component of the pathological molecular mechanisms that contribute to development of prostate cancer.

ACKNOWLEDGMENTS

We thank Dr. Leland Chung (Mount Sinai, Los Angeles) for providing PC3-a cells, and Dr. Theresa Guise (University of Indiana, Indianapolis) for providing the PC3-b cells. Runx2 antibodies (mouse monoclonal) were a generous gift from Yoshiaki Ito (National University of Singapore). We also thank the members of our laboratories, and specifically Jitesh Pratap, Tripti Gaur, Mohammad Hassan, and Kaleem Zaidi for stimulating discussions throughout these studies. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

REFERENCES

Afzal F, Pratap J, Ito K, Ito Y, Stein JL, van Wijnen AJ, Stein GS, Lian JB, Javed A. 2005. Smad function and intranuclear targeting share a Runx2 motif required for osteogenic lineage induction and BMP2 responsive transcription. J Cell Physiol 204:63–72.

Akech J, Wixted JJ, Bedard K, van der Deen M, Hussain S, Guise TA, van Wijnen A, Stein JL, Languino LR, Altieri DC, Pratap J, Keller E, Stein GS, Lian JB. 2009. Runx2 association with progression of prostate cancer in patients: mechanisms mediating bone osteolysis and osteoblastic metastatic lesions. Oncogene [Epub ahead of print].

Baniwal SK, Khalid O, Sir D, Buchanan G, Coetzee GA, Frenkel B. 2009. Repression of Runx2 by androgen receptor (AR) in osteoblasts and prostate cancer cells: AR binds Runx2 and abrogates its recruitment to DNA. Mol Endocrinol 23:1203–1214.

Barnes GL, Hebert KE, Kamal M, Javed A, Einhorn TA, Lian JB, Stein GS, Gerstenfeld LC. 2004. Fidelity of Runx2 activity in breast cancer cells is required for the generation of metastases associated osteolytic disease. Cancer Res 64:4506–4513.

Bello D, Webber MM, Kleinman HK, Wartinger DD, Rhim JS. 1997. Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18:1215–1223.

Blyth K, Cameron ER, Neil JC. 2005. The runx genes: Gain or loss of function in cancer. Nat Rev Cancer 5:376–387.

Blyth K, Vaillant F, Hanlon L, Mackay N, Bell M, Jenkins A, Neil JC, Cameron ER. 2006. Runx2 and MYC collaborate in lymphoma development by suppressing apoptotic and growth arrest pathways in vivo. Cancer Res 66: 2195–2201.

Brubaker KD, Vessella RL, Brown LG, Corey E. 2003. Prostate cancer expression of runt-domain transcription factor Runx2, a key regulator of osteoblast differentiation and function. Prostate 56:13–22.

Buijs JT, Van der Pluijm G. 2009. Osteotropic cancers: From primary tumor to bone. Cancer Lett 273:177–193.

Choi J-Y, Pratap J, Javed A, Zaidi SK, Xing L, Balint E, Dalamangas S, Boyce B, van Wijnen AJ, Lian JB, Stein JL, Jones SN, Stein GS. 2001. Subnuclear targeting of Runx/Cbfa/AML factors is essential for tissue-specific differentiation during embryonic development. Proc Natl Acad Sci USA 98:8650–8655.

Danielpour D. 2005. Functions and regulation of transforming growth factor-beta (TGF-beta) in the prostate. Eur J Cancer 41:846–857.

Fowler M, Borazanci E, McGhee L, Pylant SW, Williams BJ, Glass J, Davis JN, Meyers S. 2006. RUNX1 (AML-1) and RUNX2 (AML-3) cooperate with prostate-derived Ets factor to activate transcription from the PSA upstream regulatory region. J Cell Biochem 97:1–17.

Galindo M, Pratap J, Young DW, Hovhannisyan H, Im HJ, Choi JY, Lian JB, Stein JL, Stein GS, van Wijnen AJ. 2005. The bone-specific expression of RUNX2 oscillates during the cell cycle to support a G1 related anti-proliferative function in osteoblasts. J Biol Chem 280:20274–20285.

Huang WC, Xie Z, Konaka H, Sodek J, Zhau HE, Chung LW. 2005. Human osteocalcin and bone sialoprotein mediating osteomimicry of prostate cancer cells: Role of cAMP-dependent protein kinase A signaling pathway. Cancer Res 65:2303–2313.

Javed A, Bae JS, Afzal F, Gutierrez S, Pratap J, Zaidi SK, Lou Y, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2008. Structural coupling of Smad and Runx2 for execution of the BMP2 osteogenic signal. J Biol Chem 283:8412–8422.

Kang JS, Alliston T, Delston R, Derynck R. 2005. Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. EMBO J 24:2543–2555.

Kansara M, Thomas DM. 2007. Molecular pathogenesis of osteosarcoma. DNA Cell Biol 26:1–18.

Kawate H, Wu Y, Ohnaka K, Takayanagi R. 2007. Mutual transactivational repression of Runx2 and the androgen receptor by an impairment of their normal compartmentalization. J. Steroid Biochem Mol Biol 105: 46–56.

Kilbey A, Blyth K, Wotton S, Terry A, Jenkins A, Bell M, Hanlon L, Cameron ER, Neil JC. 2007. Runx2 disruption promotes immortalization and confers resistance to oncogene-induced senescence in primary murine fibroblasts. Cancer Res 67:11263–11271.

Kim IY, Ahn HJ, Lang S, Oefelein MG, Oyasu R, Kozlowski JM, Lee C. 1998. Loss of expression of transforming growth factor-beta receptors is associated with poor prognosis in prostate cancer patients. Clin Cancer Res 4:1625–1630.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao Y-H, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Lee Y, Schwarz E, Davies M, Jo M, Gates J, Wu J, Zhang X, Lieberman JR. 2003. Differences in the cytokine profiles associated with prostate cancer cell induced osteoblastic and osteolytic lesions in bone. J Orthop Res 21:62–72.

Lodygin D, Epanchintsev A, Menssen A, Diebold J, Hermeking H. 2005. Functional epigenomics identifies genes frequently silenced in prostate cancer. Cancer Res 65:4218–4227.

Mitra P, Ghule PN, van der Deen M, Medina R, Xie R, Holmes WF, Ye X, Nakayama KI, Harper JW, Stein JL, Stein GS, van Wijnen AJ. 2009. CDK inhibitors selectively diminish cell cycle controlled activation of the histone H4 gene promoter by p220(NPAT) and HiNF-P. J Cell Physiol 219:438–448.

Nathan SS, Pereira BP, Zhou YF, Gupta A, Dombrowski C, Soong R, Pho RW, Stein GS, Salto-Tellez M, Cool SM, van Wijnen AJ. 2009. Elevated expression of Runx2 as a key parameter in the etiology of osteosarcoma. Mol Biol Rep 36:153–158.

Paredes R, Arriagada G, Cruzat F, Olate J, van Wijnen A, Lian J, Stein G, Stein J, Montecino M. 2004. The Runx2 transcription factor plays a key role in the 1a,25-dihydroxy vitamin D3-dependent upregulation of the rat osteocalcin (OC) gene expression in osteoblastic cells. J Steroid Biochem Mol Biol 89–90:269–271.

Pereira BP, Zhou Y, Gupta A, Leong DT, Aung KZ, Ling L, Pho RW, Galindo M, Salto-Tellez M, Stein GS, Cool SM, van Wijnen AJ, Nathan SS. 2009. Runx2, p53, and pRB status as diagnostic parameters for deregulation of osteoblast growth and differentiation in a new pre-chemotherapeutic osteosarcoma cell line (OS1). J Cell Physiol 221:778–788.

Pratap J, Galindo M, Zaidi SK, Vradii D, Bhat BM, Robinson JA, Choi J-Y, Komori T, Stein JL, Lian JB, Stein GS, van Wijnen AJ. 2003. Cell growth regulatory role of Runx2 during proliferative expansion of pre-osteoblasts. Cancer Res 63:5357–5362.

Pratap J, Javed A, Languino LR, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2005. The Runx2 osteogenic transcription factor regulates matrix metalloproteinase 9 in bone metastatic cancer cells and controls cell invasion. Mol Cell Biol 25:8581–8591.

Pratap J, Lian JB, Javed A, Barnes GL, van Wijnen AJ, Stein JL, Stein GS. 2006. Regulatory roles of Runx2 in metastatic tumor and cancer cell interactions with bone. Cancer Metastasis Rev 25:589–600.

Pratap J, Wixted JJ, Gaur T, Zaidi SK, Dobson J, Gokul KD, Hussain S, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2008. Runx2 transcriptional activation of Indian hedgehog and a downstream bone metastatic pathway in breast cancer cells. Cancer Res 68:7795–7802.

San Martin IA, Varela N, Gaete M, Villegas K, Osorio M, Tapia JC, Antonelli J, Mancilla E, Pereira BP, Nathan SS, Lian JB, Stein JL, Stein GS, van Wijnen AJ, Galindo M. 2009. Impaired cell cycle regulation of the osteoblast-related heterodimeric transcription factor Runx2-Cbfb in osteosarcoma cells. J Cell Physiol 221:560–571.

Shen R, Wang X, Drissi H, Liu F, O'Keefe RJ, Chen D. 2006. Cyclin d1-Cdk4 induce Runx2 ubiquitination and degradation. J Biol Chem 281:16347–16353.

So AI, Hurtado-Coll A, Gleave ME. 2003. Androgens and prostate cancer. World J Urol 21:325–337.

Song K, Wang H, Krebs TL, Kim SJ, Danielpour D. 2008. Androgenic control of transforming growth factor-beta signaling in prostate epithelial cells through transcriptional suppression of transforming growth factor-beta receptor II. Cancer Res 68:8173–8182.

van der Poel HG. 2005. Androgen receptor and TGFbeta1/Smad signaling are mutually inhibitory in prostate cancer. Eur Urol 48:1051–1058.

Williams RH, Stapleton AM, Yang G, Truong LD, Rogers E, Timme TL, Wheeler TM, Scardino PT, Thompson TC. 1996. Reduced levels of transforming growth factor beta receptor type II in human prostate cancer: An immunohistochemical study. Clin Cancer Res 2:635–640.

Wu TT, Sikes RA, Cui Q, Thalmann GN, Kao C, Murphy CF, Yang H, Zhau HE, Balian G, Chung LW. 1998. Establishing human prostate cancer cell xenografts in bone: Induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. Int J Cancer 77:887–894.

Yeung F, Law WK, Yeh CH, Westendorf JJ, Zhang Y, Wang R, Kao C, Chung LW. 2002. Regulation of human osteocalcin promoter in hormone-independent human prostate cancer cells. J Biol Chem 277:2468–2476.

Zaidi SK, Sullivan AJ, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2002. Integration of Runx and Smad regulatory signals at transcriptionally active subnuclear sites. Proc Natl Acad Sci USA 99:8048–8053.

Zaidi SK, Pande S, Pratap J, Gaur T, Grigoriu S, Ali SA, Stein JL, Lian JB, van Wijnen AJ, Stein GS. 2007. Runx2 deficiency and defective subnuclear targeting bypass senescence to promote immortalization and tumorigenic potential. Proc Natl Acad Sci USA 104:19861–19866.