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The Cancer-related Runx2 Protein Enhances Cell Growth and Responses to Androgen and TGF β in Prostate Cancer Cells

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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., PC-3, LNCaP, RWPE) treated with 5 α -dihydrotestosterone (DHT) or TGF β revealed modulations in selected cyclins, CDKs and CDK inhibitors that are generally consistent with mitogenic responses. Endogenous elevation of Runx2 and diminished p57 protein levels in PC-3 cells are associated with faster proliferation *in vitro* and development of larger tumors upon xenografting these cells in bone *in vivo*. To examine whether TGF β or DHT signaling modulates the transcriptional activity of Runx2 and vice versa, we performed luciferase reporter assays. In PC-3 cells that express TGF β RII, TGF β 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 TGF β and androgen responsive pathways that support prostate cancer cell growth.

Keywords

cell cycle; bone; steroid hormone receptors; proliferation; cell signaling

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Introduction

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 Runx-related transcription factor Runx2 (CBFA1/AML3) is a critical regulator of bone formation, based on studies demonstrating that Runx2 null or Runx2 Δ C 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; Zaidi et al., 2007; Kilbey 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., 2005], but this cell cycle regulatory function may be altered in osteosarcoma cells [Nathan et al., 2009; Pereira et al., 2009; San Martin et al., 2009; Kansara and Thomas, 2007].

Runx proteins are expressed in prostate tissue and in prostate cancer cells, but their biological functions in prostate have not been resolved [Baniwal et al., 2009; Fowler et al., 2006; Yeung et al., 2002; Brubaker et al., 2003; Akech 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) [Blyth et al., 2006; Pratap et al., 2005; Pratap 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 TGF β /SMAD and steroid hormone signaling [Javed et al., 2008; Zaidi et al., 2002; Kang et al., 2005; Paredes et al., 2004], which are both implicated in cancer progression. TGF β functions as a tumor suppressor in many cell types and is a potent growth inhibitor by, for example, inducing the CDK inhibitor p21. However, TGF β receptors are frequently mutated or down-regulated in prostate cancer to render cells insensitive to TGF β signaling [Kim et al., 1998; Williams et al., 1996]. TGF β /BMP signaling promotes interactions of Runx2 with SMADs, a mechanism that integrates biological cues required for osteoblastogenesis and tumor progression [Javed et al., 2008; Pratap et al., 2006]. Runx2 is also associated with androgen receptor (AR) activity in prostate cancer cells [Baniwal et al., 2009]. In addition, there is cross-talk between TGF β signaling and the androgen receptor but the mechanisms are poorly understood. Exposure to androgen (5 α -dihydrotestosterone, DHT) and TGF β may have different effects on cell cycle progression in prostate cancer cells, depending on their expression of androgen and TGF β receptors [Danielpour, 2005; van der Poel, 2005]. DHT reduces expression of TGF β RII, leading to suppression of the ability of TGF β to induce apoptosis [Song et al., 2008].

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

Material and Methods

Cell culture and reagents

Two sub-cultured lines of PC-3 prostate cancer cells (PC-3-a and PC-3-b), as well as LNCaP, C4-2B, RWPE1, and HeLa cells were maintained as follows. PC-3-a cells were cultured in T-medium with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) [Huang et al., 2005]. PC-3-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 TGF β RII 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% CO₂. All media and supplements were purchased from Gibco/Invitrogen (Grand Island, NY).

For treatment with DHT and TGF β , 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 TGF β (10 or 50 ng/ml) or vehicle (ethanol for DHT and incomplete growth medium for TGF β) for 24 h. Cells were harvested for protein and mRNA detection (see below).

Cell growth assays

For growth curves, PC-3-a and PC-3-b cells were plated at equal densities in 6-well plates (5×10^4 cells per well). Cells were harvested with trypsin/0.25% EDTA and counted in quadruplicate on days 1 to 6 after plating using Trypan Blue exclusion and a hemocytometer. For siRNA experiments PC-3-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 to 4.

Western blot analysis

Cells were lysed in RIPA buffer supplemented with protease inhibitors (Complete, EDTA-free, Roche Diagnostics, Mannheim, Germany), and MG132 (25 μ M). Equal amounts of protein lysates in SDS sample buffer were loaded on a 10% acrylamide gel and subjected to electrophoresis and immunoblotting (BioRad system).

Immunoblots were incubated for one hour 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) were used as secondary antibodies and were visualized with enhanced chemiluminescence (ECL) chemistry (PerkinElmer, Waltham, MA) on BioMax film (Kodak, Rochester, NY).

Luciferase assays

PC-3 and LNCaP cells were plated in 6-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 three different reporters in which the luciferase gene is driven by promoters containing tandemly repeated regulatory elements: six binding sites for Runx2 (6×Runx2-luc, 500 ng), three TGFβ responsive elements each spanning a Smad and Runx2 binding site (3×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 promoter-less 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, Carlsbad, CA) 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, Hercules, CA). The relative mRNA expression was calculated with the $\Delta\Delta CT$ 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.

Immunological and histological analysis of tumors in xenografts

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. PC-3-a and PC-3-b (1×10^5 cells) were injected into tibiae of SCID mice (3 mice per group) as described previously [Barnes et al., 2004; Lee et al., 2003]. Tumors were allowed to grow for a period of 6 weeks and images were acquired for tumor growth analysis. Tumors from injected tibiae were harvested and processed for immunohistochemistry as described [Rubin et al., 2000]. Consecutive tissue sections were hydrated for antigen retrieval carried out in citrate buffer (pH 6.0) at 95°C. After peroxidase

blocking, sections were incubated with Ki67 rabbit polyclonal antibodies (C-19, Santa Cruz) followed by HRP conjugated secondary antibodies (Dako Cytomation, Carpinteria, CA).

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 μ l of Nonidet P-40 buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40, 1 $\times$ Complete protease inhibitor (Roche) and 25 μ M MG132 (Sigma). Following centrifugation of the lysate for 15 min, the supernatant was transferred to a clean microcentrifuge tube and precleared with 20 μ l of protein A/G plus-agarose beads (Santa Cruz) at 4°C for 30 min. Beads were collected by centrifugation at 1,000 g for 5 min at 4°C. Runx2 antibody (PEBP2 α A M-70; 3 μ g) (Santa Cruz) 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 μ l) 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 $\times$ Complete protease inhibitor and 25 μ M MG132) and the bead pellet was subsequently suspended in an equal volume of 2 $\times$ SDS sample buffer. AR proteins were analyzed by western blot analysis using mouse monoclonal antibodies (H-300, Santa Cruz) 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 luciferase assays). Supernatants from untransfected cells and vehicle treated cells were collected as negative controls. For measuring PSA protein levels, a PSA enzyme-linked immuno-sorbent 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 TGF β 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 TGF β to determine the contributions of DHT and TGF β signaling to cell cycle control. PC-3 cells are AR negative but express TGF β RII, whereas LNCaP cells express AR but are TGF β RII negative. As a control line, we used immortalized RWPE prostate cells that are TGF β RII positive and appear to express AR at levels below detection based on our immunoblotting data (Fig. 1A), although it has been reported that these cells are AR positive [Bello et al., 1997]. Incubation of PC-3 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 (Figure 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 2 fold). 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 PC-3 and RWPE cells.

Treatment of the TGF β responsive PC-3 cells with 10 ng/ml TGF β for 24 h stimulates protein expression of cyclins E and A, as well as CDK2 and p21, but not for the TGF β RII-negative LNCaP cells, indicating that TGF β may accelerate cell cycle progression in PC-3 but not LNCaP cells. In contrast, in the immortalized prostate cell line RWPE, TGF β

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 PC-3 or LNCaP cells are mediated by either transcriptional or post-transcriptional mechanisms (Figure 1B). In general, we find that mRNA levels for p21, Cyclin A2 and Cyclin D1 do not change appreciably upon DHT or TGF β 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, TGF β interferes with the mitogenic response to DHT in TGF β RII-negative LNCaP cells, because combined treatment with TGF β 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 TGF β in AR negative PC-3 cells, albeit that a modest decrease in CDK4 is observed. Hence, TGF β RII-negative LNCaP cells appear to employ compensatory mechanisms to support TGF β signaling (e.g., through alternate TGF β receptors), while AR negative PC-3 cells do not have 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 TGF β 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 TGF β 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; Pratap et al., 2005; Blyth et al., 2006; Galindo et al., 2005], and the Androgen Receptor, 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 sublines of PC-3 cells (PC-3-a and PC-3-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 (Figure 2). PC-3-a cells express relatively high Runx2 protein and mRNA levels, whereas PC-3-b cells express Runx2 protein and mRNA at or below the level of detection (Figures 2A and 2B). In all other prostate cell lines, Runx2 protein expression was not evident (Figure 2A) and mRNA levels were only detectable at relatively low levels (Figure 2B). As expected, LNCaP and C4-2B express high protein levels of AR (Figure 2A). However, there is no appreciable expression of AR in the two PC-3 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 PC-3 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 PC-3-a, PC-3-b, LNCaP, C4-2B, RWPE and HeLa cells each have distinct expression signatures for cell cycle regulatory proteins (Figure 2). For example, in LNCaP and C4-2B cells, the expression of p27 and p21 is significantly higher compared to PC-3 cells. In RWPE cells, p57, p27 and p21 are expressed at relatively low levels. Cyclin D1 protein levels are higher in PC-3-b cells compared to PC-3-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 PC-3-b cells. Strikingly, expression of the CDK inhibitor p57 is clearly elevated in PC-3-b cells (Figure 2) (also presented in Figure 1) compared to PC-3-a cells and other prostate cell lines. The p57 level in PC-3b 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 PC-3-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 sublines of PC-3 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 PC-3 cells.

Elevated Runx2 expression is related to increased tumor volume and cell growth rate of PC-3 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, PC-3-a cells expressing high Runx2 levels appear to form larger bone tumors than PC-3-b cells upon xenografting by tibial injection (Figure 3A). Histological analysis revealed an apparent increase in Ki67 staining in tumor cells derived from PC-3-a cells suggesting a higher proliferation rate (data not shown). We tested whether elevated Runx2 expression correlates with increased cell growth of PC-3-a cells compared to PC-3-b cells. Indeed, PC-3-a cells grow faster than PC-3-b cells *in vitro* (Figure 3B). To address whether Runx2 plays a direct role in this higher proliferation rate, we performed RNA interference using Runx2 siRNA in PC-3-a cells. Downregulation of Runx2 in PC-3-a cells inhibits cell growth at Day 4 by ~25-30% (Figure 3C). Thus, the higher proliferation rate of PC-3-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.

Transcriptional cooperation between Runx2 and TGF β /Smad signaling in PC-3 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 TGF β /Smad and AR signaling. We first investigated whether TGF β or DHT affect Runx2 expression in PC-3-a prostate cancer cells which express functional TGF β RII. While TGF β treatment increases p21 protein levels in PC-3 cells as expected, Runx2 protein and mRNA levels did not change significantly (Figure 4A and data not shown). We also investigated the effect of TGF β on Runx2 activity in functional assays using two different reporters with multimerized Runx2 binding sites (6 $\times$ Runx2-luc) or tandemly repeated composite regulatory elements containing a TGF β responsive motif flanked by a Runx2 motif (3 $\times$ -multimerized TGF β RE-luc reporter). Runx2 co-transfection stimulates the 6 $\times$ -Runx2-luc reporter in PC-3 cells, but TGF β treatment by itself had no effect. However, addition of TGF β increases transcriptional activity in cells transfected with Runx2 (Figure 4B). The increase may be mediated by protein/protein interactions between Runx2 and TGF β dependent Smads (Smad2/3) [Javed et al., 2008; Afzal et al., 2005].

TGF β administration and Runx2 each separately stimulates activity of the 3 $\times$ -multimerized TGF β RE-luc reporter as expected. Interestingly, Runx2 expression synergizes with the TGF β response to increase promoter activity in PC-3-a cells (Figure 4C). Taken together, these data indicate that Runx2 is transcriptionally active and that its functional cooperation with TGF β is maintained and enhances transcriptional responses in prostate cancer cells.

Runx2 increases androgen responsiveness in LNCaP cells

Runx2 cooperates 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 TGF β modulates protein levels of Runx2 and AR. However, neither ligand affects AR or Runx2 protein levels in LNCaP cells (Figure 5A). Thus, Runx2 expression is constitutive and independent of androgen and TGF β stimulation in both PC-3-a and LNCaP prostate cancer cell lines (Figure 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 6 $\times$ Runx2-luc or with a construct containing a tandemly repeated androgen responsive element (ARE) fused to the luciferase gene (ARE-luc reporter). Runx2 transfection stimulates the 6 $\times$ -multimerized Runx2-luc reporter in both LNCaP (Figure 5B) and PC-3-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 $\pm$ DHT), DHT mediates an androgen response of about 6 fold using the ARE-luc reporter (Figure 5C). Forced expression of Runx2 in LNCaP cells increases ARE-activity in the absence or presence of DHT. The 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 PC-3-a cells that lack the androgen receptor, the opposite effect was observed, i.e., 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') four basepairs 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 (Figure 5E). When LNCaP cells were transfected with increasing amounts of Runx2, PSA production was significantly increased in a dose-dependent manner (Figure 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 TGF β responses in prostate cancer cells. This finding, together with the observation that Runx2 is related to increased cell growth in PC-3 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 [Pratap et al., 2005; Blyth et al., 2006; Blyth et al., 2005; Pratap et al., 2008]. Runx2 is expressed at low but detectable levels in normal prostate epithelial cells and has been detected in selected prostate cancer cell lines [Fowler et al., 2006; Brubaker et al., 2003; Baniwal et al., 2009; Yeung et

al., 2002], 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 PC-3 cells expressing high levels of Runx2 grow faster and form larger tumors in bone than PC-3 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 androgen receptor is often mutated or absent in recurrent tumors [Danielpour, 2005; So et al., 2003] thus rendering prostate cancer cells androgen-independent. The androgen response normally suppresses TGF β induced cell death [So et al., 2003; van der Poel, 2005], and androgen-independence thus requires aberrations in TGF β signaling to promote proliferation of prostate cancer epithelial cells. We investigated the effects of both DHT and TGF β on cell cycle regulators in cell lines that differ in expression of Runx2, AR and TGF β RII. We observed that expression of cyclins, CDKs and CKIs is distinct in different prostate cancer cell lines; DHT and TGF β 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 TGF β treatment in PC-3 cells that are androgen-independent but express a functional TGF β RII, consistent with a pro-proliferative effect. Interestingly, TGF β upregulates p21 in PC-3 cells, although these cells do not express p53; expression of p21 might instead be stimulated through a TGF β -Smad2/3-Runx2 pathway in PC-3 cells expressing Runx2. We also observed that distinct sub-lines of PC-3 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 PC-3 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 ER α 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, immuno-precipitations reveal that Runx2 and AR physically interact. Consistent with this finding, while our studies were in progress, Baniwal and colleagues also detected AR-Runx2 complexes in prostate cancer cells [Baniwal et al., 2009]. Their results suggest that AR represses Runx2 activity by blocking DNA binding and that DHT treatment inhibits Runx2 activity in COS7 and PC-3 cells in which AR and Runx2 are over-expressed. 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.

TGF β acts as a tumor suppressor in many cell types and TGF β RII is often mutated in prostate tumors resulting in resistance to TGF β induced cell death. Runx2 is an important mediator in TGF β signaling and binds Smad proteins that mediate downstream effects of TGF β [Javed et al., 2008; Zaidi et al., 2002; Afzal et al., 2005]. We observed increased Runx2 activity upon TGF β treatment, but no effect on Runx2 protein expression in PC-3 and LNCaP cells. Thus, in contrast to mesenchymal cells in which Runx2 protein levels and activity are both stimulated by TGF β /Smad signaling, our results indicate that Runx2 activity but not its protein levels are enhanced by TGF β signaling in prostate cells. Because of the pro-mitogenic function of Runx2 we observed in prostate cancer cells, the

responsiveness of Runx2 activity to TGF β /Smad signaling would provide a selective advantage to cells that retain TGF β RII expression during prostate tumor progression.

Prostate Specific Antigen (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 signatures of cell cycle regulatory factors and sporadically silence or activate key proteins including Runx2 and the CDK inhibitor p57. We show that expression of Runx2 in PC-3a cells is associated with increased cell growth, and that Runx2 activity is functionally linked to TGF β 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.

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Abbreviations

CDK	Cyclin Dependent Kinase
CKI	CDK inhibitor
PSA	Prostate Specific Antigen
TGFβ	Tumor Growth factor β
DHT	5 α -dihydrotestosterone
ELISA	Enzyme-Linked Immuno-Sorbent Assay
TGFβRII	TGF β receptor II
AR	Androgen Receptor

Figure 1. Cell cycle responses of prostate cancer cells to androgen and TGFβ

Prostate cancer cell lines PC-3-b and LNCaP, and immortalized prostate cells RWPE were treated for 24 h with DHT (10 nM) or TGFβ (10 ng/ml) for 24 h 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 2 fold increase or decrease compared to control treatments. (B) mRNA detection under the same treatment conditions. Grey boxes indicate no effect upon treatment, colored boxes indicate, respectively, more than 2 fold increase (red) or decrease (blue) in mRNA levels. Transcript levels were normalized to GAPDH mRNA levels.

Figure 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 PC-3 sublines (PC-3-a and PC-3-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.

Figure 3. Runx2 expression in PC-3 cells is related to higher cell growth rate and larger tumor volume

(A) Upper panel depicts tumor size resulting from PC-3-a and PC-3-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 PC-3 sublines a and b. PC-3-a cells with higher Runx2 expression grow faster than PC-3-b cells that express higher p57 levels (see Figure 2). Data shown are from averages of two independent growth curves counted in quadruplicate. (C) Growth curves in which PC-3-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.

Figure 4. Runx2 and TGFβ responses cooperate in PC-3 cells

(A) Treatment of PC-3 (subline 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 PC-3 cells. Averages were taken from duplicate experiments and firefly luciferase activity was normalized to Renilla values.

Figure 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 TGF β (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 ng, 100 ng 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 3 independent experiments performed in triplicate. (C) Runx2 enhances ARE-activity in LNCaP cells in a dose-dependent manner. A representative experiment is shown from 3 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 ng, 500 ng,

1000 ng) in LNCaP cells strongly increases PSA production after 24 h of DHT (20 nM) treatment. A representative experiment is shown performed in triplicate.