Stromal-Epithelial Cell Interactions and Androgen Receptor-Coregulator Recruitment Is Altered in the Tissue Microenvironment of Prostate Cancer

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Abstract

Tissue recombination experiments show that prostate mesenchyma directs prostate epithelial cell growth and development in an androgen-dependent manner, and that functional differentiation of prostate epithelium requires androgen-driven processes in both epithelia and stroma. The androgen induction of target genes in primary cultures of prostate stromal and epithelial cells was determined using an adenoviral expression system, which employed the MMTV-enhancer driven luciferase reporter as an androgen receptor (AR)–mediated transcription assay. These studies indicate that both cell types contain functional AR. Androgen induction of luciferase reporter activity is 3-fold in stromal cells compared with 10-fold in epithelial cells. AR-mediated transcription activity in stroma cells was enhanced by coculture with epithelial cells or epithelial cell–conditioned media. The elevated AR-mediated transcription activity in stromal cells that were exposed to epithelial factors correlated with increased recruitment of coactivators to the AR transcriptional complex. Epithelial cells facilitated interactions of AR with SRC-1 in an androgen-dependent manner. However, AR-mediated transcriptional activity in stromal cells isolated from prostate cancer was reduced compared with stromal cells isolated from benign prostate and continued to be reduced when cocultured with tumor-derived prostate epithelial cells. The occupancy of AR and coregulators on target genes showed that androgen-bound AR in prostate cancer stromal cells was associated with the corepressor silencing mediator for retinoid and thyroid hormone receptor. Thus, the ability of epithelial cells to modulate coregulator recruitment to the AR transcriptional complex on androgen-responsive genes seems altered in the stromal microenvironment of prostate cancer. [Cancer Res 2007;67(2):511–9]

Introduction

The prostate gland is organized into luminal epithelial and stromal compartments. Androgens and stromal-epithelial cell interactions are key regulators involved in prostate growth, development, and differentiation (1–9). The effects of androgens on the prostate are mediated by the androgen receptor (AR). Prostate development requires functional AR in stroma, but not epithelia; therefore, the growth-promoting effects of androgens on the developing epithelium are mediated through an AR-positive stroma. Full development of differentiated and functional secretory prostatic epithelia growth requires AR in both cellular compartments (10–13). Stromal and epithelial cells synthesize and respond to growth factors in a reciprocal and dynamic manner to develop and maintain prostate function, a process that depends on androgens. Therefore, androgen-driven processes in the stromal and epithelial compartments are important for the normal development and physiology of glandular epithelia.

Disruption of the androgen-regulated pathways in both cellular compartments may affect the development and progression of prostate cancer. The development of human prostate cancer occurs in the presence of AR-positive stroma (7, 9, 14–17). Transformed epithelial cells actively influence stromal cells, including inflammatory cells, vascular endothelial cells, and fibroblasts, to generate a microenvironment that fosters carcinogenesis. Myofibroblasts are found in the stromal microenvironment of most invasive tumors and are often referred to as "activated" fibroblasts or "reactive stroma." Studies on the molecular mechanisms by which AR in the stromal and epithelial cells influence AR function in normal prostate tissue are essential to delineate the role of androgens and stromal-epithelial cell interactions in the signaling molecules and pathways that are involved in prostate carcinogenesis (9, 16–18).

AR is a ligand-inducible transcription factor that binds specific DNA sequences and stabilizes the RNA polymerase II (pol II) transcription initiation complex to enhance or diminish the expression of androgen-regulated genes. Coregulators (coactivators and corepressors) are nuclear proteins that modulate AR function by regulating the interactions between the receptor and the pol II transcription initiation complex. Coactivators increase androgen-regulated gene expression and corepressors decrease androgen-regulated gene expression. The p160 family of coactivators (SRC-1, GRIP/TIF-2, and AIB1/ACTR/pCIP) recruit histone acetyl-transferases (p300/CBP and pCAF) and methyl-transferases (CARM1 and PRMT) to the AR-promoter complex and enhance transcription by modifying localized chromatin structure on androgen-regulated promoters (19–22). Other coactivators include TRAP220, which is part of the multi-subunit TRAP/DRIP/SMCC mediator complex that contacts the basal transcription machinery to activate transcription (23). Corepressors include the silencing mediator for retinoid and thyroid hormone receptor (SMRT) and the nuclear receptor corepressor (NCoR). These corepressors interact with histone deacetylases to repress transcription, either directly or indirectly.

This study examines AR-mediated transcriptional activity in prostatic stromal and epithelial cells that were isolated from benign and malignant human prostate tissue specimens and shows that epithelial cells modulate the AR-mediated transcriptional

Note: This work is in partial fulfillment for P. Cano to obtain a doctoral degree under the advisors Dr. R. Maldonado and S.A. Onate.

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activity in stromal cells in a coculture system. As assessed by the chromatin immunoprecipitation assay, epithelial cells were found to enhance AR-mediated recruitment of the SRC-1 coregulator to the AR transcriptional complex in an androgen-dependent manner. When compared with stromal cells isolated from benign prostate, AR-mediated transcriptional activity in stromal cells isolated from prostate cancer was reduced because of an altered interaction with coregulators. Specifically, occupancy of AR and coregulators on target genes in prostate cancer stromal showed that ligand-bound AR in prostate cancer stromal cells associates with the corepressors SMRT and NCoR. Thus, stromal-epithelial cell interactions that modulate AR-coregulator recruitment and AR function seem altered in the stromal cell microenvironment of prostate cancer.

Materials and Methods

Tissue digestion and prostatic cell isolation. Prostate tissue was obtained from the Genitourinary Tissue Resource and Morphologic Core at the University of Pittsburgh and from the Tissue Procurement Resource at the Roswell Park Cancer Institute. All tissue procurement was conducted following standards and procedures approved by the internal review board, which are consistent with the guidelines established by the NIH. All prostate tissue samples are routinely analyzed by the histology/pathology core laboratory and classified as normal, hyperplastic, preneoplastic, or neoplastic. Tissue was obtained from organ donors free from other medical complications or treatments for prostate cancer. Tissue was also obtained from radical prostatectomy specimens. Prostate tissue was cut into 3-mm pieces under sterile conditions and subjected to collagenase/DNase digestion at 37°C (24). Stromal and epithelial cells were separated using Percoll gradient. Cells were plated in chemically defined media (ISOMS) containing 10% bovine serum to promote cell attachment. Cocultures were done using a Transwell insert of 0.4-μm pore size (Costar, Cambridge, MA). The medium conditioned by epithelial cells was obtained from cells seeded in six-well plates and cultured in ISOMS supplemented with insulin-like growth factor at 37°C for 48 h. Conditioned medium was collected, centrifuged at 1,500 × *g* for 10 min at 4°C, and stored at −20°C.

Immunohistochemistry. Prostate tissue specimens were formalin fixed and paraffin embedded. Cultured cells were fixed *in situ* with 4% (w/v) paraformaldehyde for 30 min at room temperature. The endogenous peroxidase activity of xylene-deparaffinized and rehydrated sections and of fixed cells was inhibited with 0.3% (v/v) H_2O_2 in methanol. Nonspecific binding was blocked with 3% (w/v) bovine serum albumin for 30 min at room temperature. Histologic sections and fixed cells were immunostained using anti-AR antibody PG21 (1:100; Upstate, Charlottesville, VA), anti-cytokeratin-18 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), anti–prostate-specific antigen (anti-PSA; 1:50; Abcam, Cambridge, MA), anti–smooth muscle actin (1:100; DakoCytomation, Carpinteria, CA), anti-vimentin (1:200; Santa Cruz Biotechnology), or anti-desmin (1:200; Santa Cruz Biotechnology). Horseradish peroxidase–conjugated anti-rabbit IgG or anti-mouse IgG (1:100; DakoCytomation) was used as a secondary antibody. Peroxidase activity was developed using 3,3-diaminobenzidine tetrahydrochloride (1 μg/mL; Sigma-Aldrich, St. Louis, MO) and H_2O_2 (1 μL/mL; VWR International, West Chester, PA). Hematoxylin was used for nuclear counterstaining. For immunofluorescence studies, cells were incubated with Cy2-conjugated affinity-purified donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). 4′,6-Diamidino-2-phenylindole was used to visualize nuclei. Negative controls were provided by using immunocytochemistry in the absence of primary antibodies, or using pre-immune serum.

Luciferase assays. Epithelial and stromal cells (200,000 per well in 24-well plates) in culture were infected with MMTV-promoter driven luciferase reporter adenoviral expression vector (5 μL/mL, $\sim 10^{-13}$ adenoviral particles per mL) for 3 h in an RPMI without serum and then incubated for 36 h in ISOMS containing 5% (v/v) dextran/charcoal–treated fetal bovine serum supplemented with androgens, as indicated in figure legends. Cells lysates (25 μL) were assayed for luciferase enzyme activity using the Luciferase Assay System (Promega, Madison, WI) and Lumat LB 9501 luminometer or Veritas microplate luminometer (Turner BioSystems, Sunnyvale, CA). Luciferase values were normalized to total protein measured using Bradford assays (Bio-Rad, Hercules, CA).

Chromatin immunoprecipitation assay. Stromal and epithelial cells in culture were infected with Ad-MMTV-luc and Ad-his-AR expression vectors and incubated at 37°C for 36 h to allow protein expression. Following the addition of hormone for 1 h at 37°C, cells were cross-linked with 1% formaldehyde for 1 h at room temperature. Cross-linking was stopped with glycine buffer (0.125 mol/L final concentration). Cell pellets were suspended in a 50 mmol/L HEPES (pH 8) lysis buffer containing 1 mmol/L EDTA, 0.5 mmol/L EGTA, 140 mmol/L NaCl, 10% glycerol, 0.5% NP40, 0.25% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 0.7× proteinase inhibitor cocktail (Roche, Indianapolis, IN) and nutated for 10 min at 4°C. The crude nuclei were collected by centrifugation (600 × *g* for 5 min at 4°C) and resuspended in 3 cpv wash buffer (10 mmol/L Tris-HCl at pH 8, 1 mmol/L EDTA, 0.5 mmol/L EGTA,

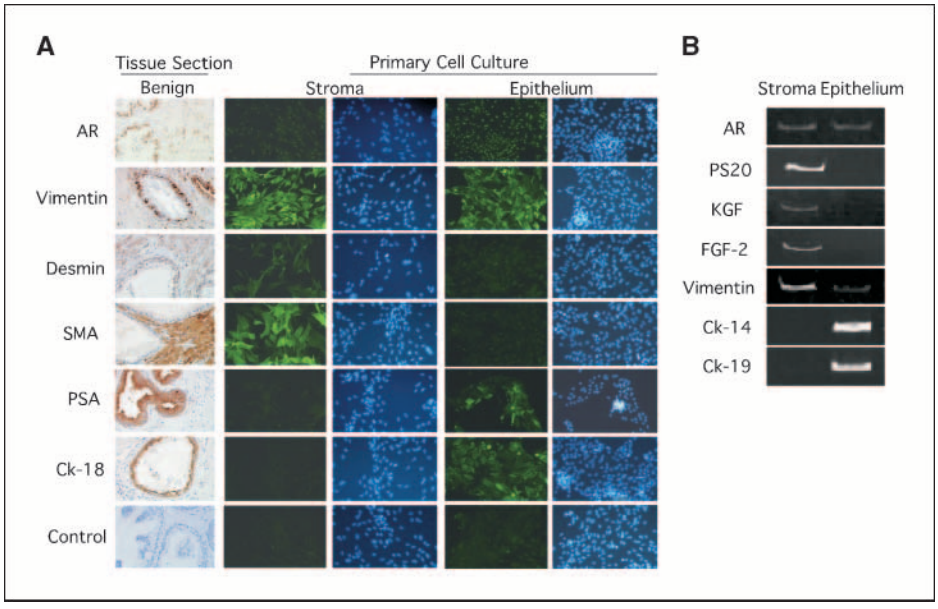

Figure 1. Immunocytochemical analysis of prostatic epithelial and stromal cell markers. Expression of AR and cellular markers in prostate stromal and epithelial cells. *A,* formalin-fixed, paraffin-embedded tissue sections and primary cell cultures. Tissue sections and primary cell cultures of stromal and epithelial cells were stained for AR, vimentin, desmin, smooth muscle actin (*SMA*), PSA, and cytokeratin 18 (*Ck-18*). Negative controls included immunocytochemical assays without primary antibody or with nonspecific IgGs. Stromal cells express markers consistent with stroma. Epithelial cells express markers consistent with epithelia. *B,* gene expression of AR and cellular markers by PCR total RNA from primary cultured stromal and epithelial cells were isolated. The expression of stromal and epithelial cell markers were determined using standard reverse transcription-PCR and primers specific for AR, ps20, KGF, FGF-2, vimentin, cytokeratin-14, and cytokeratin-19. Cells cryopreserved in liquid nitrogen for 1 yr were recovered with ~90% viability.

200 mmol/L NaCl, 1 mmol/L PMSF, and 0.7× proteinase inhibitor cocktail) and nutated again. Washed nuclei were centrifuged and suspended in 500 μL of 1× radioimmunoprecipitation assay (RIPA) buffer (10 mmol/L Tris-HCl at pH 8, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 140 mmol/L NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 1 mmol/L PMSF, and 0.7× proteinase inhibitor cocktail). Samples were sonicated at power setting 4 (Fisher Sonicator Dismembrator 100) with a 10-s burst and 1 min of ice cooling for a total sonication time of 1 min per sample. Protein extracts (10 μg) were diluted in RIPA buffer for immunoclearing with 2 μg sheared salmon sperm DNA and protein A-Sepharose for 30 min at 4°C. Immunoprecipitation of AR transcriptional complexes was done overnight at 4°C with specific antibodies (as described in the figure legends). Protein-DNA immunocomplexes were boiled 30 min to reverse cross-linking; DNA fragments were purified with a QIAquick Gel Extraction kit (Qiagen, Carlsbad, CA); and PCR was amplified using primers forward (TGGTTACAAACTGTTCTTAAAACAAGG) and reverse (AACACTAA-GAGCTCAGATCAGAACATTT) specific for MMTV-LTR.

Results

Primary cultures of human prostatic epithelial and stromal cells. Primary cell cultures of prostatic cells were characterized for epithelial and stromal cellular markers. Paraffin-embedded and formalin-fixed human prostate tissue sections exhibited AR staining confined to the nuclei of the secretory epithelia and stroma microenvironment. Epithelial cells that were isolated using collagenase enzyme digestion of prostate tissue samples and cultured in chemically defined media were positive for AR, PSA, and cytokeratin-18, indicating the presence of luminal epithelial cells in primary cultures (Fig. 1*A*). Stromal cells that were isolated using Percoll gradient or serum-containing media were positive for vimentin and desmin, indicating the presence of myoblasts in the isolated stroma (Fig. 1*A*). Other stromal cell markers [keratinocyte growth factor (KGF), fibroblast growth factor-2 (FGF-2), ps20, and vimentin] and epithelial cell markers (cytokeratin-14 and cytokeratin-18) were detected in stromal and epithelial cell cultures using reverse transcription-PCR (Fig. 1*B*). Thus, epithelial and stromal cells were isolated and cultured successfully from fresh human prostate tissue specimens.

Functional characterization of endogenous AR in the human prostate. An adenoviral expression vector for the luciferase reporter under the control of the mouse mammary tumor virus (MMTV) hormone-responsive DNA element (hereafter termed Ad-MMTV-Luc) was used to determine the ability of AR to activate gene expression in both compartments. Epithelial and stromal cells were infected independently with Ad-MMTV-Luc in the absence or presence of increasing concentrations of R1881. Ad-MMTV-Luc reporter activity in stromal cells from three independent experiments was induced 3-fold, compared with the 10-fold induction observed in epithelial cells (Fig. 2*A*). Radioligand assays indicated that AR expression is similar in both cellular compartments with specific ligand-binding activity of 17.1 ± 4.4 fm/mg protein in epithelial cells and 16.4 ± 1.7 fm/mg protein in stromal cells (Fig. 2*B*). Thus, AR is expressed in prostatic epithelial and stromal cells at similar levels, but AR differentially regulates target gene expression in each cellular compartment (Fig. 2). The transcriptional activity of various transcription factors was examined in stromal and epithelial cells to determine whether the general transcriptional machinery required to transactivate the Ad-MMTV-Luc reporter was compromised in stromal cells. Both stromal and epithelial cells contain functional glucocorticoid receptor (GR). Both cell types are equally capable of initiating transcription of the adenoviral-mediated reporter gene (Fig. 2*C*).

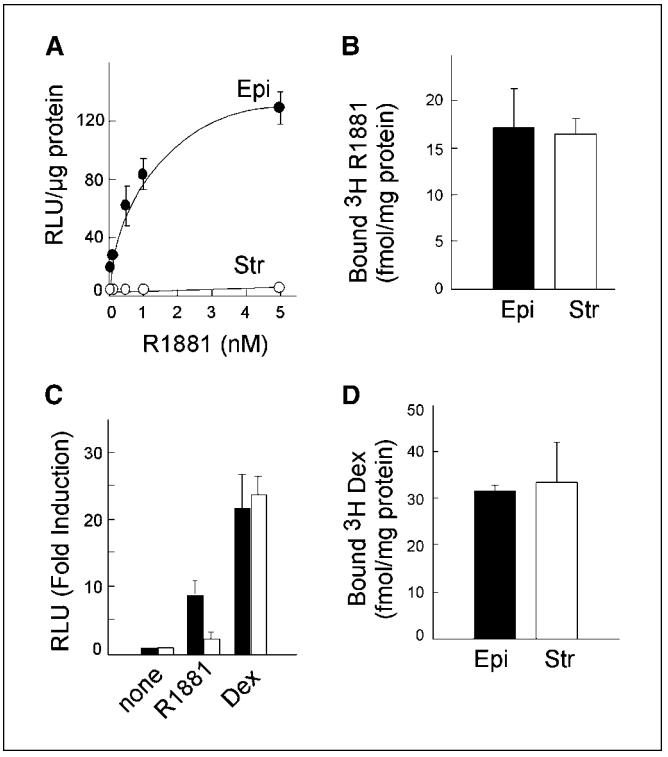

Figure 2. *A,* AR-mediated transcriptional in epithelial and stromal cells. Isolated epithelial (*Epi*) and stromal (*Str*) cells were infected with Ad-MMTV-Luc reporter expression vector in the presence of increasing concentrations of R1881. The relative luciferase activity in the epithelial (●) and stromal (○) cells was determined. *B,* levels of androgen-binding activities of AR were determined using hormone-binding assays in whole cells. Stromal (*white columns*) and epithelial (*black columns*) cells were incubated in media containing 1 nmol/L [^{3}H]R1881 (specific activity = 85 Ci/mmol) in the absence and presence of 200 nmol/L excess of unlabeled R1881. The specific hormone binding activity was determined by subtracting the nonspecific (200 nmol/L excess unlabeled R1881) from the total [^{3}H]R1881 binding and the amount of receptors expressed in fmol/mg of protein. *C,* AR and GR-mediated transcriptional activity. Human prostate epithelial (*black columns*) and stromal (*white columns*) cells were isolated and infected with Ad-MMTV-Luc reporter expression vector in the absence (none) or presence of 10 nmol/L R1881 or dexamethasone (*Dex*) and relative luciferase activity determined. *D,* levels of glucocorticoid-binding activities of GR were determined using hormone-binding assays in whole cells. Stromal (*white columns*) and epithelial (*black columns*) cells were incubated in media containing 1 nmol/L [^{3}H]dexamethasone (specific activity = 85 Ci/mmol) in the absence and presence of 200 nmol/L excess of unlabeled dexamethasone, and the specific hormone binding activity was determined as above.

The glucocorticoid dexamethasone elicited comparable levels of ligand-inducible transcriptional activity of the Ad-MMTV-Luc reporter in both epithelial and stromal cells (Fig. 2*C*). Hormone-binding assays indicate that the specific [^{3}H]dexamethasone-binding activity of GR is similar in stromal cells (33.7 ± 8.6 fm/mg protein) and epithelial cells (31.7 ± 1.7 fm/mg protein; Fig. 2*D*). Thus, the inability of stromal cells to induce transcriptional activity efficiently in the presence of androgens is AR specific and unrelated to general changes in the basic transcription initiation machinery. An adenovirus β-galactosidase expression vector that encoded the nuclear-targeted β-galactosidase enzyme activity (Ad-β-gal) was used as a control for the effect of adenovirus infection on infected cells and to determine the relative infection efficiencies among experiments. Ad-β-gal activity in primary prostate cultures is present in at least 98% of both epithelial and stromal cells. β-Galactosidase enzyme activity is qualitatively similar in both cell types (data not shown).

Vector encoding human AR (Ad-his-AR) was used as a control to determine the relative efficiency of exogenous AR-mediated transactivation in stromal and epithelial cells. Ad-his-AR was coinfected with the Ad-MMTV-Luc reporter. AR-mediated transactivation was determined in the absence and presence of increasing concentrations of androgens. Differences in the ability of Ad-his-AR to activate the luciferase reporter in these two cellular compartments was observed under increasing concentrations of exogenous AR in stromal and epithelial cells (Fig. 3*A*) despite similar expression of the receptor (Fig. 3*B*). His-tagged AR was found to be functional because it bound its cognate ligands, translocated to the nucleus, and activated target gene expression in a ligand-dependent manner (data not shown). A radioligand-binding assay indicated that AR was expressed in both cell types at similar levels. Specific AR ligand-binding activity was 2,036 ± 417 fm/mg of protein in epithelial cells and 1,531 ± 267 fm/mg of protein in stromal cells (Fig. 3*B*). The difference in the AR-mediated transcriptional activity of the luciferase reporter by exogenous AR was also observed at different concentrations of androgens (Fig. 3*C*). Therefore, differences in the expression or recruitment of coregulators may contribute, at least in part, to the mechanism by which AR differentially regulates androgen target gene expression in stromal and epithelial cells of the human prostate.

Stromal cells modulate AR activity in prostate epithelia. Because stromal-epithelial interactions in the prostate are well characterized, a trans-well coculture system that allows the continuous exchange of factors in the media without direct cellular contact was used to measure ligand-inducible transcriptional activity of AR in epithelial cells in the absence and presence of stromal cells. AR-mediated transcriptional activity in epithelial cells isolated from benign prostate increased when cocultured with stromal cells isolated from either benign (BAS) or prostate cancer–associated (CAS) stromal cells (Fig. 4*A*). In epithelial cells isolated from the primary tumor, AR-mediated transcriptional activity was not increased when cocultured with either BAS or CAS (Fig. 4*A*). However, in cell lines isolated from metastatic prostate cancer, such as LAPC-4 (Fig. 4*B*) and LNCaP (Fig. 4*C*), ligand-inducible transcriptional activity of AR increased significantly in the presence of either CAS or BAS (Fig. 4). These findings provide evidence that epithelial cells in prostate cancer exhibit altered AR-mediated transactivation function, and that stromal-epithelial interactions alter androgen signaling in both cellular compartments.

Epithelial cells modulate AR activity in the stroma. AR-mediated transcriptional activity in BAS increased 10-fold in the presence of epithelial cells (Fig. 5*A*). Basal transcriptional activity of the TATA-luc reporter that lacked the hormone-responsive DNA element was affected minimally in BAS cocultured with epithelial cells (2-fold induction; Fig. 5*A*). Conditioned media from epithelial prostatic cells was found to increase AR transcriptional activity in stromal cells (data not shown). However, AR-mediated transcriptional activity in CAS was affected minimally when cocultured with epithelial cells from either benign or malignant prostate tissue (data not shown). A variety of other prostatic cells, including LNCaP, LAPC4, PC3, and DU145, failed to modulate AR transactivation in stromal cells (data not shown). Protein factor(s) produced by epithelial cells in coculture are presumably responsible, at least in part, for increased AR-mediated gene transcription in stromal cells. The signal(s) involved in stromal-epithelial cell interactions and AR and coregulator function of the prostate that are missing in prostate cancer remain to be identified. In addition, the ability of prostatic epithelial cells to modulate AR transcriptional activity in prostate stroma is cell type specific.

The molecular mechanisms by which epithelial cells modulate AR transcriptional activity in the stroma was investigated using a

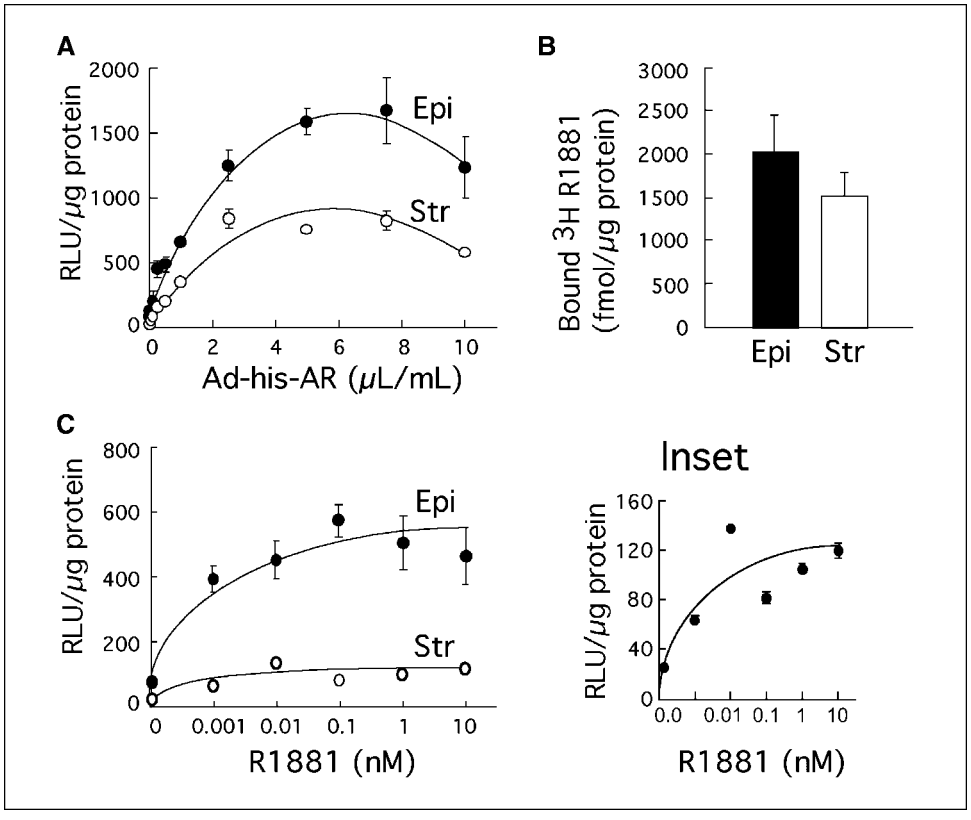

Figure 3. Functional characterization of Ad-his-AR in prostatic cells. *A,* transcription activity mediated by Ad-his-AR stromal (*white columns*) and epithelial (*black columns*) cells maintained in dextran/charcoal–treated serum-containing media for 36 h were infected with Ad-MMTV-Luc reporter and increasing concentrations of Ad-his-AR virus (stock: 1×10^{12} particles per mL). Relative luciferase activity was determined in the absence (none) or presence of 5 nmol/L R1881 (R1881). *B,* ligand binding of Ad-his-AR. The relative [^{3}H]R1881-binding activity of AR in epithelial (*white columns*) and stromal (*black columns*) cells infected with Ad-his-AR was determined using hormone-binding assay. The specific hormone binding activity was determined by subtracting the nonspecific (200 nmol/L excess unlabeled R1881) from the total [^{3}H]R1881 binding and the amount of receptors expressed in fmol/mg of protein. *C,* ligand-dependent transcription activity mediated by Ad-his-AR relative luciferase activity in the epithelial (●) and stromal (○) cells was determined in increasing concentrations of R1881. *Points,* average of three independent experiments; *bars,* SE.

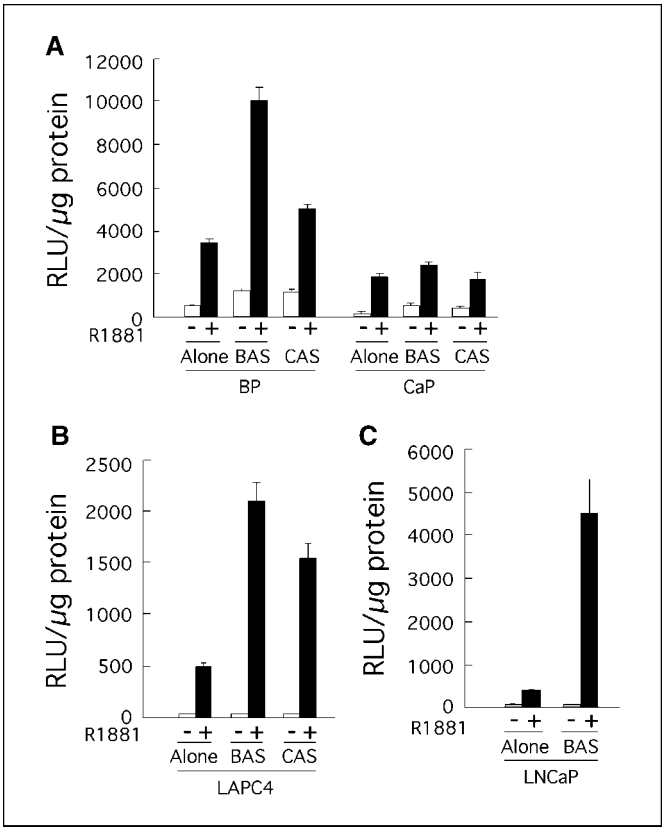

Figure 4. Altered AR and coregulator function in prostate cancer epithelia. *A,* altered stromal-epithelial interactions in prostate cancer primary cultures of prostate epithelial cells isolated from benign (*BP*) and malignant (*CaP*) prostate tissue were infected with 10^{-10} adenoviral particles/mL of Ad-MMTV-Luc reporter expression and incubated alone or in coculture with stromal cells isolated from benign or prostate cancer tissue, as indicated. Relative luciferase activity (*RLU*) was determined in the absence (*white columns*) or presence (*black columns*) of 5 nmol/L R1881. *Columns,* average of three independent experiments; *bars,* SE. *B* and *C,* stromal-epithelial interactions in prostate cancer cell lines metastatic prostate epithelial cells, including LAPC-4 and LNCaP cells, were infected with 10^{-10} adenoviral particles per mL of Ad-MMTV-Luc reporter expression and incubated alone or in coculture with stromal cells from benign (BAS) or prostate cancer (CAS). Relative luciferase activity was determined in the absence (*white columns*) or presence (*black columns*) of 5 nmol/L R1881.

chromatin immunoprecipitation assay to analyze for the occupancy of AR and coactivators on target genes in stromal cells *in vivo*. Chromatin immunoprecipitation assay immunoprecipitates cross-linked protein-DNA complexes bound to a specific gene promoter with antibodies specific for a known DNA-interacting protein. The gene is detected using PCR and primers specific for the MMTV-LTR region. In stromal cells, un-liganded AR had limited occupancy on the MMTV-luciferase reporter and was associated with the corepressor SMRT (Fig. 5*B*). Although the corepressor SMRT is associated with un-liganded AR, NCoR was not part of the complex in the absence of androgens in stromal cells that were isolated from benign prostate tissue (Fig. 5*B*). The addition of R1881 released the corepressor SMRT from the AR-DNA complex and allowed recruitment of the coactivator SRC-1 (Fig. 5*B*). Ligand-induced association of AR with SRC-1 on the DNA element in stromal cells increased further when the assay was done in the presence of epithelial cells (Fig. 5*B*). Consistent with increased recruitment of SRC-1, overexpression of SRC-1 (Ad-his-SRC-1) in stromal cells increased transcriptional activity mediated by AR (Fig. 5*C*). Thus, when SRC-1 was transfected into stromal cells using an adenoviral expression system, it mimicked the effect of epithelial cells in coculture. These data support the concept that AR-mediated transcriptional activity in prostatic stromal cells is influenced by the associated epithelium through recruitment of coregulators for AR.

AR-mediated transcriptional activity and coregulator recruitment is altered in the prostate cancer microenvironment. Prostatic stromal cells isolated from BAS or from CAS were used to determine the effect of androgens on AR-mediated transcriptional activity. Endogenous and exogenous AR-mediated transcriptional activity was reduced in cultured CAS (Gleason score 6-7) compared with cultured BAS from the same human prostatectomy specimen (Fig. 6*A* and *B*, respectively). AR-mediated transcriptional activity from 14 clinical samples was analyzed. In seven samples, AR activity in CAS was decreased 67.4 $\pm$ 16.4%. In six samples, AR activity in CAS was increased 1.87 $\pm$ 0.54-fold. In one sample, AR activity in CAS was similar to BAS. Therefore, AR expression and function in primary cell cultures from human clinical samples is heterogeneous. To standardize the variation, a cohort of a mix of at least 10 clinical specimens should be used to maintain the heterogeneity observed *in vitro* and *in vivo* between individual samples. In addition, AR-mediated transcriptional activity in CAS was affected minimally when cocultured with epithelial cells from either benign (BP-Epi) or malignant (CaP-Epi) prostate samples. The occupancy of AR and AR coregulators on target genes was determined using a chromatin immunoprecipitation assay for AR and coregulator complexes (Fig. 6*C*). When un-liganded AR in BAS was complexed with the corepressor SMRT, addition of R1881 decreased SMRT association (Fig. 6*C*). Androgens increased AR DNA-binding activity and recruitment of SRC-1 to the promoter (Fig. 6*C*). Unexpectedly, NCoR, SMRT, and SRC-1 were recruited by androgens to the AR transcriptional complex in CAS cells (Fig. 6*C*). The overexpression of SRC-1 increased AR-mediated reporter activity in BAS cells. However, overexpression of SRC-1 failed to increase AR activity in CAS cells (Fig. 6*D*). The low efficiency of SRC-1 to increase AR-mediated transcriptional activity in CAS cells may be due, at least in part, to the continuous association of AR with the corepressors NCoR and SMRT in the presence of androgens. These findings provide evidence that altered AR coregulator function and altered stromal-epithelial interactions in the cellular microenvironment of the prostate change the androgen-signaling axis in prostate cancer.

Discussion

Paracrine factors, including growth factors produced by the stroma, regulate proliferation of the developing epithelia, and influence epithelia differentiation. Reciprocal interactions are equally relevant in the development and function of prostate stroma. Fully differentiated secretory prostatic epithelial cells require the expression of functional AR in both stroma and epithelial compartments of the adult prostate (13, 24–28). However, the biological consequences of AR activity differ in each cellular compartment. It is difficult to elucidate the molecular mechanisms of androgen action that underlie the biology and pathology of the prostate because several hormonally regulated factors interact in complex ways at various times during prostate development. Studies of the mechanism of androgen action in the prostate have been conducted primarily *in vitro*, which ignores the tissue microenvironment. Primary cultures of epithelial and stromal cells provide with the opportunity to explore how AR and coregulators

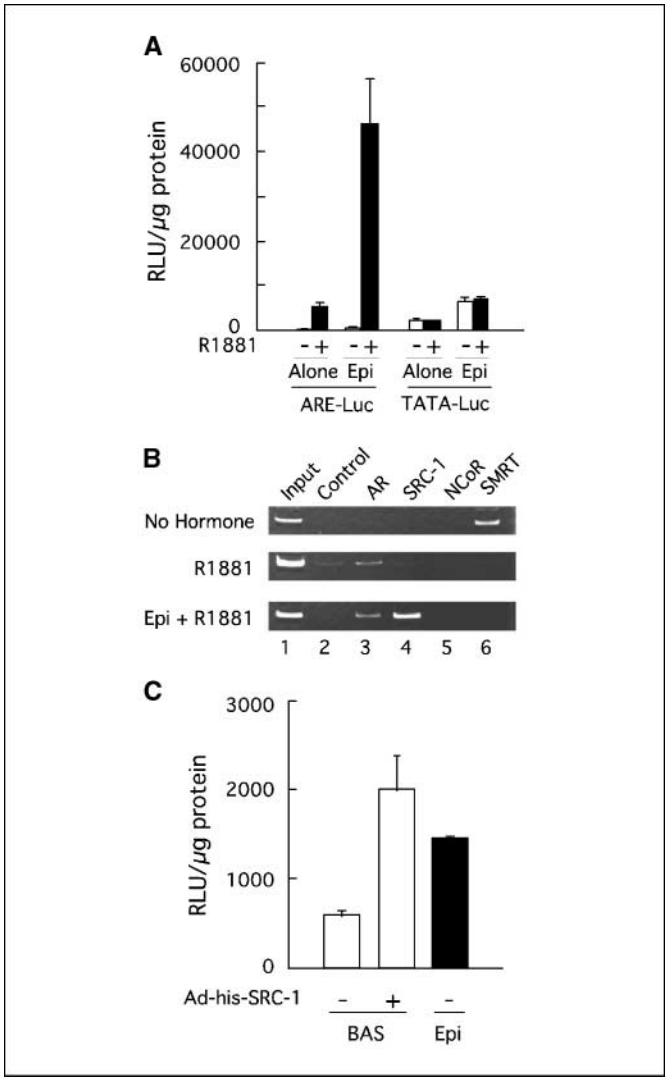

Figure 5. Stromal-epithelial cell interactions modulate coregulator recruitment. *A,* AR-mediated transcription activity in stromal cells in coculture primary cultures of stromal cells were coinfected with adenovirus expression vectors for AR (Ad-his-AR) at 1×10^9 particles per mL and Ad-ARE-luc (HRE-Luc) or TATA-Luc (TATA-luc) adenoviral vectors and grown alone or in coculture with epithelial cells, as indicated. Luciferase activity was determined in the absence (*white columns*) or presence (*black columns*) of 5 nmol/L R1881. Epithelial cells increased AR-mediated transcriptional activity of the luciferase reporter in stromal cells. *B,* recruitment of SRC-1 to the MMTV hormone response DNA element *in vivo*. The effect of prostate epithelial cells on the occupancy of AR and coregulators in the MMTV promoter in stromal cells in the absence or presence of R1881 and epithelial cells was analyzed using chromatin immunoprecipitation assay. Primary cultures of stromal cells were coinfected with Ad-his-AR, Ad-his-SRC-1, and Ad-MMTV-Luciferase reporter and cross-linked with 1% formaldehyde. Cross-linked chromatin complexes were prepared using sonication and immunoisolated with antibodies against AR, SRC-1, NcoR, and SMRT. Immunoisolated protein-DNA complexes were analyzed using PCR and MMTV primers (forward, TGGTTACAAACTGTTCT-TAAAACAAGG; reverse, AACACTAAGAGCTCAGATCAGAACATTT). An aliquot of chromatin complexed before immune isolation was used as Input (*lane 1*). Nonspecific binding was assessed using rabbit anti-mouse IgG (*RAM, lane 2*). AR (*lane 3*) interacted with SMRT corepressor (*lane 6*) in the absence of ligand. Addition of R1881 decreased SMRT signaling. Addition of R1881 increased recruitment of AR (*lane 3*) and SRC-1 (*lane 4*) to MMTV-promoter in stromal cells. AR and SRC-1 occupancy increased in presence of R1881 in stromal cells cocultured with epithelial cells. *C,* SRC-1 enhances AR-mediated transcriptional activity in stromal cells primary cultures of stromal cells isolated from benign tissue (BAS) and epithelial (Epi) cells were grown and infected with Ad-his-AR and Ad-MMTV-luc reporter in the absence (−) and presence (+) of Ad-his-SRC-1 expression vector. Ligand-induced luciferase activity was compared in stromal (*white columns*) and epithelial cells (*black columns*).

function in an *in vitro* system that models prostate tissue microenvironment. To study stromal-epithelial interactions, the AR-coregulator complex was analyzed in both cellular compartments using a coculture approach. Both cell types express mRNA encoding AR and AR protein at comparable levels. AR-mediated transcriptional activity differs markedly in epithelial cells compared with stromal cells. Differences in AR-mediated transactivation between the two cellular compartments are specific for AR because differences in androgen-induced reporter activity are observed at increasing concentrations of exogenous AR. Moreover, dexamethasone efficiently induces GR-mediated transcriptional activity in both compartments. Therefore, differences in AR-mediated transactivation are receptor and cell type specific.

Differences in AR-mediated transactivation were observed in stromal cells isolated from BAS and CAS cells. Expression of AR in human prostate clinical samples is heterogenous and varies during prostate cancer progression (29). Olapade-Olaopa et al. reported substantial decreased in AR expression during tumor progression to androgen independence (30). However, other laboratories observed slight changes in AR expression during prostate cancer progression (29). Moderate changes in AR expression occurred in both epithelia and stroma (31). In our studies, AR expression in benign and malignant clinical samples was heterogeneous. However, hormone binding indicates that AR expression in BAS and CAS was similar. Therefore, primary cell cultures from BAS and CAS may represent a subfraction of AR-positive stromal cells.

Differential expression or activity of coregulators may allow steroid receptors to achieve specificity when expressing hormonally regulated genes in a particular endocrine target tissue. Several coactivators, including SRC-1 (32, 33), the AR-specific coactivator FHL2 (34), AR-interacting protein-three (35), RAP250 coactivator (36), and peroxisome proliferator-activated receptor-γ coactivator-one (37), are differentially expressed in endocrine target tissues. The differential expression of AR-regulated genes in epithelial and stromal compartments may result when AR forms active transcriptional complexes with coregulators that differ between the two compartments. Epithelial cells have consistently higher relative gene expression of SRC-1, RAC-3, TIF2, and p300/CBP coactivators. For example, experiments conducted to determine the precise expression pattern of coactivators revealed that SRC-1 is expressed prominently in the stromal compartment of the mouse mammary gland, and that the AR coactivator FHL2 is primarily expressed in the epithelial compartment of the prostate. Therefore, differential expression of coregulators that are relevant for androgen action is an important mechanism for AR to regulate target gene expression in a cell-specific manner in prostate tissue and between different endocrine target tissues.

Tissue recombination studies using testicular feminized (Tfm) urogenital tissue that lacks functional AR shows that AR is required in the stroma but not in the epithelia for early development and prostatic growth (13, 38, 39). When Tfm (mutated AR) urogenital sinus mesenchyme (UGM) was recombined with wild-type urogenital sinus epithelia (UGE) and grown under the kidney capsule, the resulting recombinant tissue formed stratified epithelia similar to that found in the vagina. When wild-type UGM was recombined with Tfm UGE, the recombinant tissue exhibited ductal morphology similar to immature prostate tissue (13, 38, 39). Thus, the growth-promoting effect of androgens on the epithelium during development is mediated through an AR-positive stroma. Fully differentiated secretory prostatic epithelial

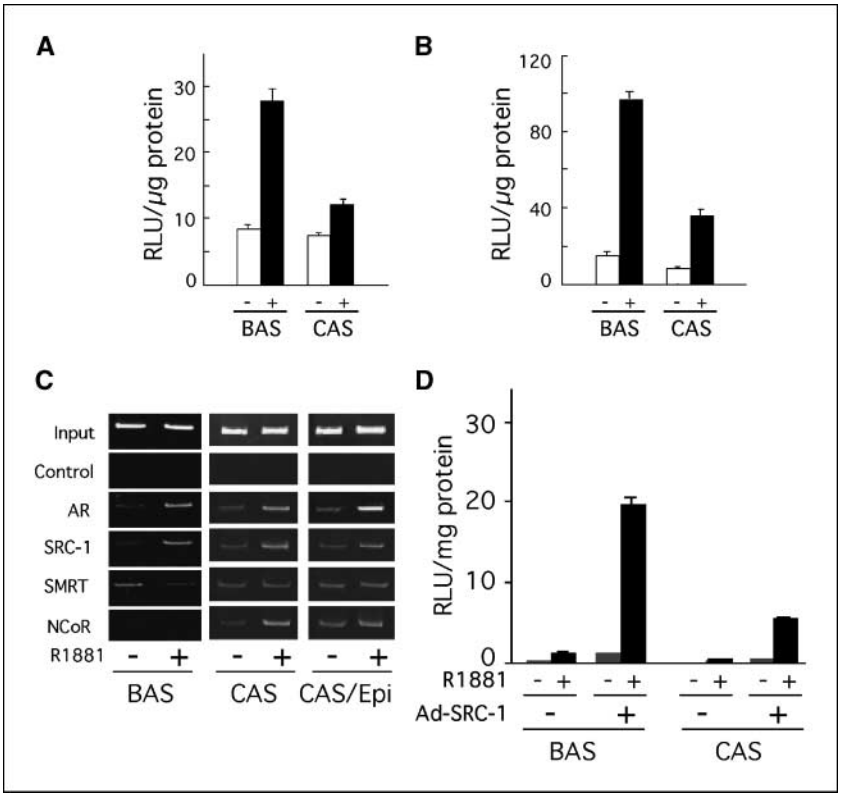

Figure 6. Altered AR and coregulator function in prostate cancer stroma. *A,* reduced AR-mediated transactivation in stromal prostate cancer cells prostate stromal cells isolated from benign (BAS) and malignant (CAS) prostate tissue were grown and infected with 10^{-10} adenoviral particles per mL of Ad-MMTV-Luc reporter expression vector in the absence (*white columns*) or presence (*black columns*) of 5 nmol/L R1881. The relative luciferase activity was determined. *Columns,* average of three independent experiments; *bars,* ± SE. *B,* altered exogenous AR-mediated transcriptional activity in stromal prostate cancer. Prostate stromal cells isolated from benign (BAS) and malignant (CAS) prostate tissue were coinfected with 10^{-10} adenoviral particles per mL of AD-his-AR and Ad-MMTV-Luc reporter expression vector in the absence (*white columns*) or presence (*black columns*) of 5 nmol/L R1881, and the relative luciferase activity determined as in (*A*). *C,* recruitment of coregulators to target genes prostate stromal cells isolated from benign (BAS) and malignant (CAS) prostate tissue, either alone or in the presence of epithelial cells (*CAS/Epi*), were coinfected with Ad-his-AR, Ad-his-SRC-1, and Ad-MTV-Luciferase reporter and a chromatin immunoprecipitation assay done in the absence (−) and presence (+) of 5 nmol/L R1881. The protein-DNA complexes were immune isolated antibodies against AR, SCR-1, NcoR, and SMRT. An aliquot of the chromatin complexes before immune isolation was used as input (*Input*). Nonspecific binding was assessed using rabbit anti-mouse IgG as a control (*Control*). *D,* reduced SRC-1 coactivation function in malignant prostatic cells. Stromal cells from either benign (BAS) or malignant (CAS) prostate tissue were grown and coinfected with Ad-his-AR and Ad-MMTV-Luc reporter. The AR-mediated reporter activity in the absence (−) or presence (+) of Ad-SRC-1 was determined in androgen presence (*black columns*) and absence (*white columns*) of 5 nmol/L R1881.

cells require the expression of functional AR in both the stromal and epithelial compartments of the adult prostate (13, 25, 26, 39–42). Therefore, stromal cells are required to maintain epithelial cell differentiation and to repress proliferation in an androgen-dependent manner. Conversely, prostatic epithelia maintain stromal cells in a differentiated state. However, the molecular mechanisms of androgen-driven processes in stromal and epithelial cells are poorly understood in the context of stromal-epithelial cell interactions. The coculture system, which allows for interaction between these two cell types, shows that epithelial cells isolated from benign prostate tissue modulate AR function in stromal cells by promoting the recruitment of AR coactivators to androgen-responsive gene promoters that harbor a simple androgen-responsive DNA element in a ligand-dependent manner, minimally affecting the basal activity of the promoter under the control of the TATA box DNA element. The combination of adenoviral expression vectors and luciferase reporter activity permits numerous gene expression studies with relatively few cultured prostate cells. MMTV is a classic and well-characterized hormone-responsive DNA element model system used to study steroid hormone-mediated gene transcription. It allows study of the basic molecular mechanisms of AR and coregulator function and specificity in the different compartments of the prostate during tumor progression from androgen-sensitive to castration-resistant prostate cancer. However, the MMTV reporter may not reflect the activity of AR on endogenous androgen response DNA elements in the stromal and epithelial compartments of the prostate. A classic example of differential regulation of androgen-mediated genes is *PSA* and *KGF/FGF7*. These two genes are present in the genome of every cell of the prostate; yet, mature epithelia express PSA and not KGF/FGF7, and mature stroma produce KGF and not PSA. Therefore, an extension of these studies will be to determine changes in the AR transcriptional complex in the PSA and KGF/FGF7 cell type–specific gene promoters in the context of stromal-epithelial cell interactions.

Tissue recombination experiments using stroma isolated from Tfm mice showed that AR in stroma is a key player in the growth and function of the epithelium in both normal and diseased states (13–17, 38, 39, 43). However, Gao et al. (44) showed that human tumor cell lines isolated from prostate cancer metastases, including LNCaP and LAPC4, are able to form tumors in the presence of stroma isolated from Tfm mice (44). This result indicate that tumor cell lines isolated from prostate cancer metastasis already acquired a "stromal cell–like"

phenotype that allow tumor growth as xenografts independent of an AR-positive stroma. The finding that primary cultures of prostate cancer epithelial cells and transformed tumor cell lines failed to modulate AR activity and coregulator recruitment in BAS and CAS cells further highlights the role of stromal-epithelial cell interactions in the *in vitro* coculture system used in these studies.

The deregulation of coregulators has been associated with cancer. AIB1 coactivator is overexpressed or amplified in several pancreatic, ovarian, and mammary cancer cell lines (45). The monoallelic inactivation of the *CBP* gene in CBP null-heterozygous ($CBP^{+/-}$) mice having a gene dose-dependent decrease of CBP expression resulted in hematologic neoplasia (46). Spleen and bone marrow cells transplanted from $CBP^{+/-}$ mice into sublethally irradiated mice results in tumor formation of hematologic origin (46). The altered expression of AIB1 and CBP coactivators has been implicated in the development and progression of cancer (45). The AR coactivator ARA55 is consistently overexpressed in some prostate cancer cells (47). In addition, expression levels of SRC-1 increase in more poorly differentiated prostate cancer and in castration-resistant prostate cancer (48, 49). Thus, several reports support the concept that alteration in coregulator expression plays a key role in carcinogenesis. This report indicates that AR transcriptional activity is reduced in stromal cells isolated from prostate cancer tissue compared with stromal cells isolated from benign prostate. Because AR mutations are found in prostate cancer, the transcriptional activity of wild-type human AR was evaluated in prostate cancer–associated stromal cells. The results showed that the reduced AR activity in these cells was not due to AR itself; other factors were involved in the down regulation of AR-mediated transactivation. NCoR in combination with AR was bound to DNA in the presence of ligands in prostate cancer–associated stromal cells. These data support the concept that growth factors produced by stromal cells under androgen regulation act on epithelial cells, causing abnormal growth of epithelial cells. The finding that AR interacts with corepressors (SMRT and NCoR) in a ligand-dependent manner in prostate cancer–associated stromal cells raises the possibility that AR may recruit NCoR and SMRT to androgen-regulated promoters in response to androgens. Thus, the balance of overall AR activity in the presence of androgens may be regulated by competition between p160s and corepressors for the same AR AF-2 surface. Thus, SMRT/NCoR corepressors could play a role in prostate microenvironment during the development of prostate cancer. The factor(s) secreted by epithelial cells that have such effect on stromal cells should be identified because they may regulate androgen action and modulate the development and maintenance of prostate homeostasis. Taken together, the altered expression or activity of coregulators in the prostate stroma microenvironment may be important for the development and progression of prostate cancer. Isolated primary prostate cell cocultures may provide an innovative alternative and physiologically relevant *in vitro* model for studying the molecular mechanisms by which AR in the stroma influences AR function in the secretory epithelia during prostate cancer progression from androgen-dependent to castration-resistant prostate cancer. If the mechanism of differential AR trans-activation in the stromal and epithelial compartments of prostate cancer can be understood, these findings can be used to develop new therapies for prostate cancer.

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