Differential Effects of RUNX2 on the Androgen Receptor in Prostate Cancer: Synergistic Stimulation of a Gene Set Exemplified by SNAI2 and Subsequent Invasiveness

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Abstract
Changes to androgen signaling during prostate carcinogenesis are associated with both inhibition of cellular differentiation and promotion of malignant phenotypes. The androgen receptor (AR)-binding transcription factor RUNX2 has been linked to prostate cancer progression but the underlying mechanisms have not been fully defined. In this study, we investigated the genome-wide influence of RUNX2 on androgen-induced gene expression and AR DNA binding in prostate cancer cells. RUNX2 inhibited the androgen response partly by promoting the dissociation of AR from its target genes such as the tumor suppressor NKX3-1. However, AR activity persists in the presence of RUNX2 at other AR target genes, some of which are cooperatively stimulated by androgen and RUNX2 signaling. These genes are associated with putative enhancers co-occupied by AR and RUNX2. One such gene, the invasion-promoting Snail family transcription factor SNAI2, was co-activated by AR and RUNX2. Indeed, these two transcription factors together, but neither alone stimulated prostate cancer cell invasiveness, which could be abolished by SNAI2 silencing. Furthermore, an immunohistochemical analysis of SNAI2 in archived primary prostate cancer specimens revealed a correlation with the RUNX2 histoscore, and simultaneous strong staining for SNAI2, RUNX2, and AR (but not any pair alone) was associated with disease recurrence. Overall, our findings suggest cooperation between AR and RUNX in the stimulation of oncogenes such as SNAI2, which might be targeted for individualized prostate cancer therapy. Cancer Res; 74(10); 2857–68. ©2014 AACR.

Introduction
Contrasting their role in prostate epithelial cell differentiation and physiological functions, androgens acquire oncologic roles during prostate carcinogenesis, including promotion of cellular proliferation, survival, and aerobic glycolysis (1–4). These changes are associated with redistribution of the androgen receptor (AR) across the prostate cancer cell genome and alterations to its transcriptional regulatory properties (5, 6). Contributing to changes in its genomic locations and activities are AR coactivators and collaborating DNA-binding proteins such as FOXA1, NKX3-1, GATA2, RUNX2, and members of the ETS family of transcription factors (6–9).

The mammalian RUNX family consists of 3 transcription factors with well-established roles in both development and cancer (10–12). RUNX2, best known for its roles in skeletal development (13, 14), has also been implicated in carcinogenesis, including the promotion of breast and prostate cancer metastasis (8, 15–20). RUNX2 activity in prostate cancer is negatively regulated by PTEN through a FOXO1-dependent mechanism (21), RUNX2 expression progressively increases during prostate cancer development in the PTEN conditional knockout mouse model (22) and its immunoreactivity is higher in human prostate cancer than in prostatic intraepithelial neoplasia (PIN) and normal prostate epithelium (16, 23, 24). Furthermore, manipulation of RUNX2 in tissue culture and xenograft mouse models of prostate cancer metastasis alters invasiveness and tissue destruction (16, 17).

RUNX2 directly interacts with and influences the activity of other transcription factors, including members of the nuclear hormone receptor family. In both breast cancer cells and osteoblasts, RUNX2 and estrogen signaling modulate each other’s activity in a locus-specific manner, with implications for the regulation of both breast cancer progression and bone mass control (12, 20, 25–27). In osteoblasts, RUNX2 interacts...
with and augments the transcriptional activity of the vita-
mín D receptor at the osteocalcin gene (28). Finally, RUNX2
directly binds the AR, and this interaction is potentially
important for both modulating and interpreting androgen
signaling in various physiologic and pathologic contexts
including bone metabolism and prostate cancer progression
(8, 27, 29).

In prostate cancer and other cell types, physical interac-
tion between AR and RUNX2's DNA-binding domain inhibits
RUNX2's recruitment to and activation of target genes
(8, 27, 29, 30). Limited investigations of the reciprocal effects,
those of RUNX2 on AR, led to apparently conflicting results
indicating either inhibition (29, 31) or stimulation (30, 32) of
AR activity. To address the hypothesis that RUNX2 influ-
ences AR activity in a locus-dependent manner, we set out to
categorize genome-wide the influence of RUNX2 on AR-
regulated gene expression by comprehensive mRNA profiling
of C4-2B/Rx2\textsuperscript{dox} prostate cancer cells after activation of the
AR with dihydrotestosterone (DHT) and/or induction of
RUNX2 by doxycycline (dox). As previously described, dox
increases RUNX2 expression in these cells from hardly
detectable to levels normally seen in other cell lines (17).
The gene expression profiles, in combination with chromatin
immunoprecipitation-sequencing (ChIP-seq) analyses of
RUNX2 and AR, demonstrate complex remodeling of the
AR-regulated gene network. Whereas RUNX2 generally
attenuated recruitment of AR and stimulation of target
genes, AR remained bound and active upon a specific subset
of genes and even synergized with RUNX2 in some cases.
Here we pursued the mechanistic basis of these diverse
interactions and then investigated the significance of the
synergistic activation of SNAI2 by RUNX2 and AR.

Materials and Methods

Reagents

Dox and DHT, both from Sigma-Aldrich, were used at final
concentrations of 10 nmol/L and 0.25 μg/mL, respectively. AR
(N-20), RUNX2 (M70), and glyceraldehyde-3-phosphate dehy-
drogenase (V-18) antibodies were from Santa Cruz Biotechnol-
y; Flag (M2) and SNAI2 (C19G7) antibodies were from Sigma-
Aldrich and Cell Signaling Technology, respectively. RUNX2
(ab76956) and AR (F.39.4.1) antibodies for immunohistochem-
istry were from Abcam and Biogenex Laboratories, respectively.
Protein-A dynabeads were from Invitrogen. Dulbecco’s Modifi-
ced Eagle Medium (DMEM) and RPMI-1640 media were from
Mediatech, Inc. FBS was from Omega Scientific. Charcoal
dextran stripped serum (CSS) was from Gemini Bio Products.

Cell culture and immunofluorescence

COS7 cells and the human prostate cancer cell lines C4-2B/
Rx2\textsuperscript{dox}, 22Rv1/Rx2\textsuperscript{dox}, and LNCaP/Rx2\textsuperscript{dox}
were previously described (8, 17) and have been passaged for less than 6
months. Prostate cancer cells were maintained in RPMI-
1640 supplemented with 10% FBS and COS7 cells were main-
tained in DMEM with 5% FBS. Hygromycin (50 μg/mL) and
puromycin (1 μg/mL) were used to select cells that had
incorporated the Rx2\textsuperscript{dox} and the shSNAI2 lentiviral vectors,
respectively. Two days before initiation of hormone treatment,
10% FBS was replaced with 5% CSS, and all experiments were
performed in the absence of any selection marker. AR and
RUNX2 immunofluorescence studies were performed using
the N20 and M70 primary antibodies and fluorescein- and
rhodamine-conjugated secondary antibodies, respectively.
Cells were mounted using Vectashield mounting medium with
4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories
Inc.) and viewed using an LSM 510 Zeiss confocal microscope
(Carl Zeiss). Fluorescence recovery after photobleaching
(FRAP) was carried out as previously described (8).

ChIP, mRNA, DNA, and protein assays

AR ChIP and Flag-RUNX2 ChIP were performed essentially as
described previously (9, 33). Processing and quantification of
mRNA and ChIP by quantitative PCR (qPCR) was as described
(33) using the primers listed in Supplementary Table S1. Western
blot analyses were carried out essentially as described (33).

Invasion assay

C4-2B/Rx2\textsuperscript{dox}/Luc cells, expressing RUNX2 conditionally
and firefly luciferase constitutively (17) were suspended in
serum-free medium and seeded in 24-well plates for morphol-
ogy assessment, or in Matrigel-coated inserts (BD Biosciences)
for evaluating invasiveness. The inserts were placed for 24
hours in wells containing 5% CSS, and nonmigrating cells were
removed. Results are presented as invasion indices, defined as
the ratio between the luciferase activity in cells that invaded
through Matrigel-coated membranes and the respective values
obtained from cells plated in control inserts with uncoated
membranes. Treatment with DHT and/or dox commenced
48 hours before seeding in the inserts and lasted throughout
the experiment. Silencing of SNAI2 was performed as described
(20).

Bioinformatics

Gene expression profiling was performed as described pre-
viously (17, 33) and in the Supplementary Methods. Briefly,
total RNA from C4-2B/Rx2\textsuperscript{dox} cells was extracted in biological
triplicates and hybridized to BeadChip HumanHT-12 v4 (Illumi-
na Inc.).

For RUNX2 and AR genomicChip, read coordinates
(aligned to hg18) for RUNX2 and AR ChIP-seq experiments
were obtained from our recent paper (33) and from Massie
and colleagues (3), respectively. A total of 36,698 RUNX2
peaks and 10,949 AR peaks were detected using MACS (34)
with a P-value threshold of $P \leq 1 \times 10^{-10}$. Scoring profiles were
constructed as described previously (35). Detailed method-
ologies and the combinatorial effects of AR and RUNX2 were
described in the Supplementary Methods. Microarray gene
expression data have been uploaded to GEO, accession no.
GSE52627.

Immunohistochemistry

A series of 95 prostate cancer patients with lymph node
involvement who had undergone radical prostatectomy for
locally advanced disease were selected from the institutional
database at the University of Southern California. Clinical
characteristics are described in Supplementary Table S2. Detailed methodologies are described in Supplementary Methods. Scoring of the SNAI2 (0.1.2.3), AR (high/low), and RUNX2 (high/low) immunoreactivity was performed under the supervision of a certified prostate cancer pathologist, and only regions of invasive carcinoma were considered. The Institutional Review Board of USC approved the tissue procurement protocol for this study (IRB approval HS-08-00590). Appropriate written informed consent was obtained from all patients.

Results

RUNX2 antagonizes AR recruitment to and stimulation of the majority (type I) of DHT-stimulated genes

Three cell lines were used in this study to investigate the influence of RUNX2 on AR-driven gene expression in prostate cancer cells. All 3 lines are essentially RUNX2 negative and each was engineered with the Rx2^flox^ lentiviral system, which facilitates RUNX2 induction upon dox treatment (8). The LNCaP and the C4-2B cell lines require presence of androgens for AR activation, whereas the 22Rv1 cell line also expresses AR variants that are active independent of ligand (36). We first analyzed global mRNA profiles of C4-2B/Rx2^flox^ cells treated with DHT to activate the AR and/or with dox to induce RUNX2 expression (Fig. 1A). DHT significantly upregulated 2002 genes (false discovery rate-adjusted P < 0.01). To illustrate the global influence of RUNX2 on the DHT response, we plotted the normalized gene expression values from cells cotreated with DHT plus dox against the respective values from cells treated with DHT alone (Fig. 1B). Approximately half (1,148) of the genes responded in a similar manner to DHT alone and to DHT plus dox (Fig. 1B and C, gray). The remaining 854 genes responded differently to DHT plus dox compared with DHT alone, and of these, 751 (88%, henceforth type I) were less strongly stimulated in the presence of RUNX2 (Fig. 1B and C, open circles). Similarly, in the reciprocal orientation, the predominant influence of DHT was attenuation of RUNX2-mediated stimulation of gene expression (Supplementary Fig. S1). Thus, in prostate cancer cells, AR and RUNX2 are generally antagonistic, consistent with the expression patterns of hand-picked genes previously investigated in this and other cell types (29, 30). Interestingly, however, 12% of the DHT-stimulated genes whose expression was modified by RUNX2 were further stimulated, rather than inhibited when RUNX2 was induced. These 103 genes were designated type II (Fig. 1B and C, triangles). Supplementary Tables S3 and S4 list the DHT-stimulated genes, whereby the response to DHT is attenuated (type I) or augmented (type II) in the presence versus absence of RUNX2. Reverse transcription (RT)-qPCR analysis essentially confirmed the expression pattern of several type I and type II genes (Fig. 2A and Supplementary Tables S3 and S4). RT-qPCR analysis of these genes in another prostate cancer cell line, LNCaP/Rx2^flox^, demonstrated similar locus-dependent effects of RUNX2 on DHT-stimulated genes (Fig. 2A, bottom panels and Supplementary Tables S3 and S4). We also investigated the effects of RUNX2 on DHT-stimulated genes by RT-qPCR in the 22Rv1/Rx2^flox^ cell line, a model of castration-resistant prostate cancer (CRPC). Interestingly, the results from 22Rv1 cells were unlike those in LNCaP and C4-2B cells, with type II behavior representing the most common mode of interaction in this CRPC model (Supplementary Fig. S2). These results suggest that the interaction between RUNX2 and AR signaling is not only locus dependent, but may also be modified during the transition from androgen dependent prostate cancer to CRPC. This speculation is the focus of an ongoing investigation, which is outside the scope of the present study.

The functional interrelationships between AR and RUNX2 could be related to the physical interaction between these two transcription factors. Indeed, similar to PC3-AR, COS7, SaOS-2, and MC3T3-E1 cells (8, 29), the two transcription factors seem to physically interact in the C4-2B/Rx2^flox^ culture model as well. This is suggested by colocalization of AR and RUNX2 within distinct nuclear domains (Fig. 3A) as well as alteration to AR cellular distribution, from a relatively uniform nuclear staining in the absence of RUNX2 to textural staining that includes nuclear speckles co-occupied by the two proteins once RUNX2 is expressed (Fig. 3A). Furthermore, RUNX2 modified the FRAP of GFP-AR (Fig. 3B), indicating that RUNX2 influenced AR intranuclear mobility, consistent with physical interaction between the two proteins in living cells. As control, mobility of the AR-A573D mutant, which binds neither DNA nor RUNX2 (8), was not influenced by RUNX2 (Fig. 3B). Although binding of RUNX2 to AR within distinct subnuclear domains may underlie the modification of the androgen response by RUNX2, it cannot explain the...
locus-dependent interaction observed in type I versus type II genes (Figs. 1 and 2A).

Because recruitment of AR is central to androgen-mediated stimulation of target genes, we measured AR occupancy by ChIP-qPCR at known androgen response elements (ARE) associated with the type I genes NKX3-1 and TMPRSS2 and the type II genes PIP and PGC (6, 30, 37, 38). As expected, treatment of either C4-2B/Rx2\textsuperscript{lox} or LNCaP/Rx2\textsuperscript{lox} cells with DHT alone resulted in AR recruitment to AREs of both type I and type II genes (Fig. 2B). When RUNX2 was induced along with DHT treatment, we observed differing behaviors of the AR in both these cell lines. Although RUNX2 attenuated AR recruitment to the type I genes (Fig. 2B, black), likely contributing to their blunted DHT response, RUNX2 did not attenuate (PGC) and even enhanced (PIP) the recruitment of AR to AREs near type II genes (Fig. 2B, gray). Thus, RUNX2 influences DHT-mediated AR recruitment to and activation of target genes in a locus-dependent manner.

**Regions doubly occupied by AR and RUNX2 are found near type II genes**

Although RUNX2-mediated attenuation of DHT responsiveness in type I genes was attributable in part to lesser AR recruitment, the uninhibited DHT response of type II genes, and in particular the enhanced response of PGC, could not be explained simply based on AR recruitment (Fig. 2). We tested, initially by RUNX2 ChIP-qPCR, the alternative and nonmutually exclusive hypothesis that RUNX2 itself is recruited along with AR to type II genes. Indeed, RUNX2 was readily
genes (Fig. 4C). These genome-wide aggregate peaks associated with type II genes in cells treated with DHT plus dox as compared with DHT alone is attributable to regulation by enhancers capable of recruiting both AR and RUNX2, and that the binding of RUNX2 to these enhancers allows them to escape RUNX2-mediated attenuation of the androgen response. Seeking further support for this view, we mapped the doubly-occupied presumptive enhancers with respect to the TSSs of type II versus type I genes. Enumeration of the doubly-occupied enhancers as a function of distance from their respective nearest TSSs revealed many more doubly-occupied enhancers near type II as compared with type I TSSs (Fig. 4D). Remarkably, 27% of the 100 type II genes with mapped Refseq coordinates had doubly occupied enhancers between positions −30 and +30 kb, compared with only 7.6% (60/792) of type I genes having corresponding doubly occupied enhancers (Fig. 4D). Although the enrichment for doubly occupied enhancers near type II compared with type I genes dramatically dropped as a function of distance from the respective TSSs, it remained significantly higher at distances exceeding 200-kb (Fig. 4D), likely reflecting looping of doubly-occupied enhancers onto type II target genes located many kilobases away.

**RUNX2 and AR synergistically stimulate a subset of type II genes that includes SNAI2**

We had initially defined type II genes based on stronger stimulation by DHT plus dox as compared with DHT alone (Fig. 1). Because we observed more RUNX2 occupancy near type II as compared with type I genes (Fig. 4), the high enrichment of type II genes in cells treated with DHT plus dox compared with DHT alone could simply reflect the summed stimulatory effects of AR and RUNX2. Close examination of the expression profiles of type II genes, however, revealed cases of synergistic, rather than additive stimulation by DHT and RUNX2. Indeed, a scatter plot of the RUNX2 response of type II genes in the presence versus absence of DHT (Fig. 5A) demonstrates that many (61%) of the type II genes, hereafter referred to as ENRs, were synergistically stimulated by DHT and dox. One of the clearest examples of synergism was SNAI2 (see Fig. 5A and Supplementary Table S5). Consistent with previous investigations (17, 39), RT-qPCR analysis shows that each of DHT and RUNX2 increases SNAI2 mRNA levels in prostate cancer cells (Fig. 5B). More importantly, and consistent with the microarray analysis, the simultaneous induction of RUNX2 (by dox) and activation of AR (by DHT) results in cooperative stimulation of SNAI2 transcription in three different prostate cancer cell lines, with particularly strong synergism in C4-2B cells (Fig. 5B). Western blot analysis confirmed the synergism between AR and RUNX2 in stimulating SNAI2 expression at the protein level (Fig. 5C and Supplementary Fig. S3).

The landscape of AR and RUNX2 occupancy at the SNAI2 locus, derived from the aforementioned ChIP-seq datasets (3, 33) suggested recruitment of both RUNX2 and AR to a putative composite enhancer approximately 4-kb upstream of the SNAI2 TSS (Fig. 5D). ChIP-qPCR analysis of C4-2B/Rx2M transduced cells with DHT and/or dox confirmed occupancy as well as mutual enhancement of the RUNX2 and AR recruitment (Fig. 5E and F).
RUNX2 and AR signaling cooperatively induce invasiveness in a SNAI2-dependent manner

SNAI2 promotes invasiveness and other metastatic properties in various cancers (40). We therefore asked whether the synergistic stimulation of SNAI2 by AR and RUNX2 in C4-2B/Rx2dox cells might influence invasiveness. Coactivation of RUNX2 and AR induced an elongated cell morphology and dendrite-like processes (Fig. 6A) often associated with invasiveness and metastasis (41). Matrigel invasion assays showed that combined AR activation and RUNX2 induction, but neither alone, led to a remarkable increase in cell invasiveness (Fig. 6B), and Western blot analysis confirmed the synergistic stimulation of SNAI2 by AR and RUNX2 under the conditions employed during the invasion assay (Supplementary Fig. S4). Finally, to test the role of SNAI2 in this increased invasiveness, we knocked down its expression using each of two shRNAs (Fig. 6C). Both the morphologic changes (Fig. 6D) and the synergistic stimulation of cellular invasiveness (Fig. 6E) in response to DHT and dox were diminished with shRNA#1, which robustly knocked down SNAI2 expression. Somewhat weaker diminution of the invasiveness was observed with shRNA#2, which decreased SNAI2 expression to a lesser extent. These results indicate that synergistic stimulation of SNAI2 expression by RUNX2 and androgen signaling is required for the increased invasiveness observed when the two pathways are simultaneously activated.

Strong SNAI2 expression in prostate cancer biopsies with high nuclear levels of both AR and RUNX2 predicts disease recurrence

In pursuit of evidence for potential costimulation of SNAI2 by AR and RUNX2 in a clinical setting, we assessed immunohistochemical staining expression of the respective proteins in 95 primary prostate cancer tumors using a tissue microarray (TMA) representing tumors from 73 patients who remained free from clinical recurrence and 22 who relapsed. Consistent with published data (39), most of the
tissue samples were stained for SNAI2 only weakly, but four sections were assigned the highest SNAI2 histoscore of 3 (Supplementary Table S6). Each of these four sections, for example, case 1 in Fig. 7A, was also assigned high histoscores for both RUNX2 and AR (Supplementary Table S6). Reciprocally, absence or low expression of either nuclear RUNX2 or nuclear AR was most commonly associated with low or lack of detectable SNAI2 (e.g., Fig. 7A, cases 2 and 3, respectively). Overall, there was a strong correlation between the SNAI2 histoscore and the sum histoscores for AR and RUNX2 ($r = 0.26$, $P = 0.003$, based on Kendall’s $\tau$ measure of correlation), with RUNX2 making the major contribution to the correlation (Supplementary Table S7). However, a minor yet sizable proportion of the SNAI2-negative tumors stained strongly for both nuclear RUNX2 and nuclear AR (Supplementary Table S6), possibly reflecting conditions in these cases that limit the transcriptional activity of RUNX2, AR, or the cooperation between them. Taken together, the TMA data suggest that, similar to our in vitro results, cooperation between AR and RUNX2 in stimulating SNAI2 expression exists in the majority of human prostate cancer tumors in vivo. In our cohort, however, none of the AR, RUNX2, or SNAI2 histoscores in isolation significantly correlated with disease recurrence (Fig. 7B).

Because a minority of the tumors did not exhibit evidence for cooperation between AR and RUNX2 in stimulating SNAI2, we asked whether they differed from the majority of tumors (with evidence of cooperation) in terms of disease recurrence. Indeed, tumors with evidence of cooperation (RUNX2 high /AR high /SNAI2 high) recurred more frequently than those expressing high SNAI2, but low AR or RUNX2. Association between SNAI2 and recurrence risk was significant when RUNX2 and AR were both high ($P = 0.011$) but not when either was low (Fig. 7C and Supplementary Table S8). These results suggest that tumors in which AR and RUNX2 can interact to stimulate SNAI2 expression are more likely to recur after resection.

Discussion

Expression of the osteoblast master regulator RUNX2 in prostate cancer cells was originally investigated in the context
of the osteomimetic properties displayed by these bone-seekers (42). More recent studies demonstrate that RUNX2 stimulates various pro-metastatic genes and phenotypes that include, but are not limited to such related to the high predilection of prostate cancer for bone (16, 17). Here we further demonstrate that RUNX2 modulates activity of the AR. This modulation primarily entails inhibition of androgen-stimulated expression of genes, including such that mediate cellular differentiation and tumor suppression. Examples include inhibition of the NKX3-1 and SPDEF tumor suppressor genes (43, 44) and the epithelial marker KRT19 (Supplementary Table S3). However, a small subset of the AR transcriptome was resistant to attenuation by RUNX2, and in some cases RUNX2 even augmented the expression of androgen-stimulated genes. Examples for these so-called type II genes include the anti-apoptotic genes EGFR, ITSN1, and CRYAB, the pro-proliferative gene PRKCD, the prometastatic gene SNAI2 and additional genes implicated in various aspects of prostate cancer progression such as HIPK2, SOX9, and RAB3B (Supplementary Table S4). Thus, the ectopic expression of RUNX2 during prostate cancer progression may reshape the androgen response by attenuating expression of AR-regulated tumor suppressor genes while sparing and even augmenting expression of AR-regulated oncogenes.

Attenuation of the androgen response by RUNX2 at most androgen-stimulated (type I) genes, as well as the reciprocal attenuation of the RUNX2 response by androgens (Supplementary Fig. S1), are attributable to the direct interaction between the two transcription factors, demonstrated previously by coimmunoprecipitation and GST pull-down assays (8, 29) and reiterated herein based on colocalization in C4-2B/Rx2dox cells and alteration to AR intranuclear mobility in response to RUNX2. Consistent with the involvement of the respective DNA-binding domains in their physical interaction (8, 29), attenuation of the androgen response after

Figure 6. RUNX2 and androgen signaling cooperatively induce invasiveness of prostate cancer cells via SNAI2. A, phase contrast images of C4-2B/Rx2dox cells treated with DHT and/or dox as indicated. B, C4-2B/Rx2dox cells constitutively expressing luciferase were treated with dox and/or DHT and invasion index was assessed based on luciferase activity in cells that had invaded through Matrigel-coated versus noncoated membranes as described in Materials and Methods. C, C4-2B/Rx2dox cells were transduced with control (shCtrl) or SNAI2-targeting shRNA lentiviruses (shSNAI2 #1, #2) and SNAI2 silencing was assessed by Western blotting (top and middle) and by RT-qPCR (bottom). D and E, effects of DHT and dox on cell morphology (D) and invasiveness (E) were determined as in A and B, respectively, after transduction of C4-2B/Rx2dox cells with shSNAI2#1, shSNAI2#2, or shCtrl. A, B, D, and E are representative of three independent experiments. Bars in C are mean ± SEM of three experiments.

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RUNX2 induction was associated with decreased recruitment of AR to type I genes (this study); and, attenuation of the RUNX2 response by androgens was associated with compromised recruitment to its targets (8). In breast cancer cells, a similar relationship of reciprocal attenuation has been documented for most RUNX2- and most estrogen-responsive genes (25, 26). Interestingly, however, RUNX2-stimulated SNAI2 expression in breast cancer cells followed the global trend and was attenuated by estradiol, potentially contributing to the anti-RUNX2 and protective effects that estradiol had with regard to breast cancer cell invasiveness (20). Unlike in breast cancer cells, the present work with prostate cancer cells demonstrates that SNAI2 in this cancer type is subject to an unusual mechanism whereby androgens and RUNX2 signaling cooperate to stimulate gene expression.

How a minority of AR-stimulated genes, for example, PIP, PGC (Fig. 2), and SNAI2 (Fig. 5) escape RUNX2-mediated attenuation remains to be fully elucidated. We observed retention of AR and recruitment of RUNX2 itself to AR-occupied regions (AROR) near these so-called type II genes (Fig. 4). At first glance, the recruitment of RUNX2 could be interpreted as tethering to these ARORs via contacting DNA-bound AR. Arguing against such a tethering mechanism, RUNX2 was recruited to the doubly occupied regions even in cells not stimulated by DHT (Figs. 4A and 5E). Furthermore, the doubly occupied regions near type II genes are enriched for sequence elements resembling the RUNX consensus motif TGTGGT.

Figure 7. Evidence that RUNX2 and AR cooperate to induce SNAI2 in prostate cancer tumors and the potential clinical significance. A prostate cancer TMA was subjected to immunohistochemical staining of AR, RUNX2, and SNAI2. A, the relationships between SNAI2 staining and nuclear AR and RUNX2 staining, summarized in Supplementary Table S6, are represented here by three cases. Case 1 is strongly stained for SNAI2 (score = 3) as well as for nuclear RUNX2 and AR. Case 2, with high nuclear AR, but no nuclear RUNX2 staining lacks SNAI2 staining; case 3, with a low level of nuclear AR and high level of nuclear RUNX2 also lacks SNAI2 staining. B, ORs and 95% confidence intervals for association of each of RUNX2, AR, and SNAI2 with recurrence. C, ORs and 95% confidence intervals for association of SNAI2 with recurrence for each combination of high (hi) or low (lo) RUNX2 and AR.

ns, not significant.
(91% contain such a motif, compared with 43% of AR-only peaks). Our working model therefore suggests that AR and RUNX2 bind individual elements at composite enhancers of type II genes, and that proximity between these elements permits each transcription factor to remain bound in the presence of the other. We do not know, however, why some type II genes merely escape attenuation of the androgen response by RUNX2 (e.g., SGK1; see Supplementary Fig. S5), whereas others are further stimulated by AR and RUNX2 in a synergetic manner (e.g., SNAI2). We speculate that certain spatial configurations of AR- and RUNX2-binding elements render composite enhancers of the so-called type IIA genes exceptionally attractive to coactivators, which promote the observed transcriptional synergism.

SNAI2 is a major player in cancer metastasis (20, 40). Knockdown of endogenous SNAI2 in prostate cancer cells results in reduced expression of mesenchymal markers, corresponding morphologic changes, and decreased cell invasiveness (45, 46). In frozen sections of prostate cancer biopsies, SNAI2 mRNA was higher in microdissected metastatic lesions compared with primary prostate cancer (39, 47). Recent studies also demonstrated positive correlation between SNAI2 immunohistochemical staining in primary tumors and disease progression (16, 39, 48). The regulation of SNAI2 by each of AR and RUNX2 has been independently reported (17, 20, 39), and here we show that the two regulatory pathways intersect to cooperatively promote SNAI2 expression and prostate cancer cell invasiveness in vitro. Clinically, we observe that the minority of primary prostate cancer tumors that are strongly immunostained for SNAI2 are typically highly positive for both AR and RUNX2 nuclear immunostaining; low or no nuclear staining of either AR or RUNX2 is usually associated with lack of SNAI2 staining. Perhaps most significantly, high SNAI2 expression in our series of primary tumor biopsies correlated with disease recurrence, but only when it was associated with strong AR and strong RUNX2 immunohistochemical staining. These AR<sup>high</sup>/RUNX2<sup>high</sup>/SNAI2<sup>high</sup> tumors may represent an aggressive prostate cancer subtype with a high recurrence rate. In contrast, many tumors where high SNAI2 expression was associated with low AR or low RUNX2 had low recurrence rates. If AR<sup>high</sup>/RUNX2<sup>high</sup>/SNAI2<sup>high</sup> primary tumors were reproducibly found aggressive in additional patient cohorts, efforts would be warranted to screen for such patients and develop drugs, for example, AR/RUNX2 disruptors, which may spare them the dire consequences of disease recurrence.

In conclusion, RUNX2 remodels androgen signaling in prostate cancer cells in a locus-dependent manner. It usually attenuates AR-driven transcription, but a minority of genes remain androgen responsive in the presence of RUNX2. Some of them, for example, SNAI2, exhibit synergistic stimulation and recruitment of both AR and RUNX2 to composite enhancers. Targeting the AR–RUNX2 interaction presents an opportunity for the development of novel therapeutic approaches that would retain expression of androgen-stimulated tumor suppressors while preventing synergistic interaction between AR and RUNX2 at prostate cancer–driving genes. Such novel therapeutic approaches would be particularly suited to prevent disease recurrence in patients whose primary tumor biopsies exhibit high expression of AR, RUNX2, and SNAI2.

Disclosure of Potential Conflicts of Interest
J. Pinski has received honoraria from the speakers’ bureaus of Janssen, Astellas, Beyer, and Durand and has ownership interest (including patents) from OncoNaturals Solutions, MDOracle, and Biscayne Pharma. J. Pinski is a consultant/advisory board member of Janssen, Astellas, and Durand. No potential conflicts of interest were disclosed by the other authors.

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