Signaling and Regulation

HOXC8 Inhibits Androgen Receptor Signaling in Human Prostate Cancer Cells by Inhibiting SRC-3 Recruitment to Direct Androgen Target Genes

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Abstract

HOX (homeobox) genes encode homeodomain-containing transcription factors critical to development, differentiation, and homeostasis. Their dysregulation has been implicated in a variety of cancers. Previously, we showed that a subset of genes of the HOXC cluster is upregulated in primary prostate tumors, lymph node metastases, and malignant prostate cell lines. In the present study, we show that HOXC8 inhibits androgen receptor (AR)-mediated gene induction in LNCaP prostate cancer cells and HPr-1 AR, a nontumorigenic prostate epithelial cell line. Mechanistically, HOXC8 blocks the AR-dependent recruitment of the steroid receptor coactivators steroid receptor coactivator-3 (SRC-3), and CREB binding protein to the androgen-regulated prostate-specific antigen gene enhancer and inhibits histone acetylation of androgen-regulated genes. Inhibition of androgen induction by HOXC8 is reversed upon expression of SRC-3, a member of the SRC/p160 steroid receptor cofactor family. Coimmunoprecipitation studies show that HOXC8 expression inhibits the hormone-dependent interaction of AR and SRC-3. Finally, HOXC8 expression increases invasion in HPt-1 AR nontumorigenic cells. These data suggest a complex role for HOXC8 in prostate cancer, promoting invasiveness while inhibiting AR-mediated gene induction at androgen response element–regulated genes associated with differentiated function of the prostate. A greater understanding of HOXC8 actions in the prostate and its interactions with androgen signaling pathways may elucidate mechanisms driving the onset and progression of prostate cancer. Mol Cancer Res; 8(12); 1643–55. ©2010 AACR.

Introduction

Androgen ablation therapy for prostate cancer exploits the characteristic androgen dependence of prostate epithelium for survival and growth and is the first-line strategy for treatment of surgically refractory or recurrent prostate cancer. However, despite sustained androgen ablation, the majority of men eventually cease responding and "castration-resistant prostate cancer" (CRPC) claims the life of the patient. Thus, a major objective of prostate cancer research is to better understand the mechanisms underlying the transition from androgen-dependence to a castration-resistant state. Previously, our laboratory reported that certain HOX (homeobox) genes are overexpressed in primary and metastatic prostate cancer (1). These observations led us to explore a potential cross talk between HOX genes and androgen receptor (AR)-mediated signaling.

There are 39 human HOX genes, arranged in 4 chromosomal clusters designated HOXA, HOXB, HOXC, and HOXD. HOX genes encode a large family of homeodomain-containing transcription factors considered “master regulators” in embryonic development because of their key involvement in body patterning, growth, and differentiation in vertebrates and invertebrates (2–5). As many of these processes critical to development are also known to be dysregulated in carcinogenesis, it is not unexpected then that aberrant HOX gene regulation has been implicated in a variety of cancers including leukemias (6–12), and cervical (13), colorectal (14), breast (15, 16), brain (17), renal (18), lung (19, 20), and esophageal cancers (21).

In the androgen-responsive, normal mature prostate, little or no expression of HOXC genes is seen, but any of a subset of these genes (HOXC4, HOXC5, HOXC6, or HOXC8) is upregulated in primary prostate tumors, lymph node metastases, and malignant prostate cell lines (1). HOXC8 overexpression has been correlated with loss of differentiation/higher Gleason grade in human prostate cancer cell lines and tissues (22). siRNA-mediated
knockdown of HOXC8 and its transcriptional binding partner, PBX1, results in a decrease in androgen-independent growth of DU-145 prostate cancer cells (23). Loss of HOXC6 expression in prostate cancer cells by siRNA induces apoptosis, suggesting a protective role for HOXC6 in prostate cancer cell survival (24). Our present studies show that HOXC8 overexpression promotes invasiveness in a nontumorigenic prostate epithelial model, suggesting a role for HOXC genes in cancer progression.

A link between androgen signaling and HOX gene expression is suggested by a report indicating that a subset of HOX proteins bind to the steroid receptor coactivators CBP (CREB binding protein) and p300, inhibiting the histone acetyltransferase (HAT) activity of the coactivators (25). A direct interaction of HOXB13 and AR has been reported (26, 27). This interaction can have positive and negative effects on AR- and HOXB13-regulated transcription (26). In the present studies directed at understanding the consequences of the overexpression of HOXC genes in prostate, we show that the overexpression of HOXC8 inhibits androgen induction at promoters regulated by androgen response elements (ARE). Investigation into the mechanistic basis for this inhibition indicates that HOXC8 inhibits the loading of steroid receptor coactivator-3 (SRC-3), a transcriptional coactivator critical in AR-mediated gene transcription, at direct androgen target genes, and that inhibition of AR-induced transcription is abrogated by SRC-3 overexpression. Significantly, SRC-3 amplification and/or overexpression have been observed in many hormone-sensitive tumors including prostate (28), breast (29), endometrial (30), and ovarian cancers (31), as well as many nonsteroid targeted tumors such as pancreatic cancer (32, 33), gastric cancer (34), colorectal carcinoma (35), and hepatocellular carcinoma (36).

SRC-3 is expressed in prostate cancer cell lines and prostate tumors, and its expression is correlated with tumor grade and stage in prostate cancer patients (37, 38). It is overexpressed in tissue samples from prostate cancer patients, and its overexpression correlates with prostate cancer proliferation and inversely correlates with apoptosis (28). Knockdown of SRC-3 by siRNA results in decreased cell proliferation, delays in the G1-S transition, and increased apoptosis in prostate cancer cell lines, as well as decreased tumor growth in nude mice (28). Other studies show that SRC-3, along with the AR, is required for androgen-dependent and -independent proliferation of prostate cancer cells and for xenograft tumor growth (39). Furthermore, AR and SRC-3 are recruited to gene promoters involved in cell-cycle control in an androgen-independent manner (39). Taken together, these data suggest that SRC-3 is required for prostate cancer cell proliferation and survival, and together with the AR, plays a central role in progression to CRPC.

The present work ties together 3 major players in prostate cancer, AR, SRC-3, and HOXC8, and explores the consequences of their interaction. We also provide evidence implicating HOXC8 overexpression in prostate cancer progression and discuss the role of HOXC gene overexpression in prostate cancer despite its inhibitory actions on AR-mediated gene expression.

**Materials and Methods**

**Cell lines and culture**

The human prostate cell lines were obtained from the following sources: LNCaP (40, 41), Dr. J. Horoszewicz (Roswell Park Memorial Institute, Buffalo, NY), and HPr-1 AR (42), Dr. K. Yamamoto (University of California San Francisco). LNCaP cells were maintained in RPMI 1640 supplemented with 10% FBS (HyClone), penicillin G (100 U/mL), and streptomycin (100 µg/mL). HPt-1 AR cells were maintained in keratinocyte serum-free media (Invitrogen) supplemented with penicillin G (100 U/mL), streptomycin (100 µg/mL), gentamicin (50 µg/mL), and the manufacturer-supplied supplements epidermal growth factor, and bovine pituitary extract.

**HOXC8 retrovirus construction and infection**

pMSCV-IREs-GFP-HOXC8 was constructed by standard subcloning techniques. HOXC8 cDNA was cloned into the EcoRI site within the multiple cloning site of pMSCV-IREs-GFP (gift from Dr. James DeGregori) and confirmed by sequencing. Virus preparation and cell infection were performed as previously reported (43). Expressing cell lines were selected by sorting for GFP expression and represent many independent integrants.

**RNA isolation and PCR analysis**

Total RNA was isolated using RNeasy Plus (Qiagen) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega). Real-time reverse transcriptase-PCR (RT-PCR) for HOXC8, SRC-3, PSA (prostate-specific antigen), and 18S was performed on 1/10 (HOXC8, SRC-3, PSA) or 1/20 (18S) of the synthesized cDNA by using primer and probe sets and TaqMan Fast Universal Master Mix (Applied Biosystems). Amplification signals were detected with the ABI Prism 7500 Fast Sequence Detection System (Applied Biosystems). Fold change in expression was calculated using the comparative Ct method (44) following confirmation that the amplification efficiencies of the target (HOXC8, SRC-3, or PSA) and endogenous control (18S) were approximately equal.

**Luciferase reporter assays**

Cells were seeded at 3 x 10^5 cells per well in 3 mL of RPMI 1640 supplemented with 10% FBS in 6-well plates. Twenty-four hours later, cells were cotransfected with 2 µg of probasin-, MMTV (mouse mammary tumor virus)-, or PSA-luciferase (luc) reporter constructs (45; gift from Dr. Carlos Perez-Stable, University of Miami School of Medicine) and pCMV6c-HOXC8 expression plasmid and/or empty vector control pCMV6c to ensure that the same amount of DNA was transfected into each well, using TransIT-LT1 Transfection Reagent (Mirus Bio). After 24 hours, cells were treated with 10 nmol/L of R1881.
mediated by HOXC8 expression alone without. The average inhibition of hormone induction the normalized luciferase activity with hormone minus that primer: 5'-ACACCTTTTTCCTTTCTGGATTTGTTG-3';

Western blot analysis
Cell extracts were separated on NuPAGE 4% to 12% Bis-Tris gel (Invitrogen) and transferred to polyvinylidene difluoride membrane. Following incubation with antibody, signals were detected by enhanced chemiluminescence (PerkinElmer) and autoradiography. Bands were quantified using Image J software (NIH) and normalized to β-actin loading control.

Chromatin immunoprecipitation
Cells were grown to near confluency in 15-cm dishes in phenol red–free RPMI 1640 supplemented with 5% dextran-coated, charcoal-treated FBS for 3 to 5 days. For histone acetylation analyses, cells were transfected with 2 μg/mL of MMTV-luc and 200 ng/mL of pCMV6c-HOXC8 or pCMV6c-Empty vector 18 hours before hormone treatment. Cells were treated with 10 nmol/L of R1881 or ethanol control for 24 hours. Coimmunoprecipitation (co-IP) was performed as previously described by Gnanapragasam et al. (38), with an additional step added to preclar the lysate with 100 μL of protein A agarose. Immune complexes were collected by centrifugation, washed, and analyzed by Western blot as described.

Glutathione-S-transferase pull-down assay
HOXC8 cDNA was cloned into pGEX-4T1 (GE Healthcare) and purified using the glutathione-S-transferase (GST) Purification Kit (Clontech) according to the manufacturer’s instructions. To assess interaction between GST-HOXC8 and SRC-3, baculovirus-expressed, Flag-tagged SRC-3 protein was used. To assess interaction between GST-HOXC8 and AR, cell extract from a confluent 15-cm dish of LNCaP cells treated for 24 hours with 10 nmol/L of R1881 was used. Cells were harvested in cold PBS and lysed in 1 mL of GST lysis buffer. GST pull-down assay was performed as previously described (47).

Invasion
Invasion was assayed using Cell Biolabs Cytoselect 96-well Cell Migration and Invasion Assay (Cell Biolabs, Inc.) according to manufacturer’s directions. LNCaP or HPt-1 AR cells were starved for 18 hours and then 5 × 10^4 cells in serum-free RPMI (LNCaP) or keratinocyte serum-free medium without supplements (HPt-1 AR) were added to the upper invasion chamber. Cells were incubated for 48 hours at 37°C, and cell invasion was assessed. Invasion values are reported as mean RFU (relative fluorescence units) of quadruplicate samples.

Results
HOXC8 inhibits AR-mediated transcription
It has been previously shown that HOX proteins bind to the steroid receptor coactivators CBP and p300, inhibiting the HAT activity of the coactivators (25). We therefore asked whether overexpression of HOXC8 might inhibit AR-mediated transcription via inhibition of CBP/p300. These experiments were performed in LNCaP cells, as they express functional AR and very low endogenous HOXC8 mRNA.
(Supplementary Fig. S1A) and protein levels (Supplementary Fig. S1B). A progressive increase in the expression of HOXC8 (Fig. 1A) decreased androgen-dependent activity of a probasin-luc reporter vector (Fig. 1B). This cannot be ascribed to a general squelching or inhibitory effect of HOXC8 overexpression, as expression of neither pCMV-βgal (Fig. 1C) nor pRSV-luc (Fig. 1D) is affected.

Many HOX proteins share overlapping function. Our laboratory previously observed the overexpression of HOXC genes, including HOXC6, in primary tumors, metastases, and in prostate cancer cell lines, including LNCaP (1). We asked whether further overexpression of HOXC6 in LNCaP cells also inhibits androgen-mediated gene induction. Similar to the results of overexpressing HOXC8, HOXC6 expression inhibits androgen-induced transcription in a dose-dependent manner (Supplementary Fig. S2A). HOXB13, important in normal prostate function as well as in prostate cancer, exhibited a similar inhibitory effect (Supplementary Fig. S2B). These data support the notion that, among the subset of HOX proteins tested, there may exist functional redundancy with respect to inhibition of AR-mediated signaling. The availability of an adequately specific commercial antibody for the detection of HOXC8 and the null background in LNCaP prompted us to focus further studies on HOXC8.

To validate the transient transfection reporter assays and to investigate additional activities of HOXC8, an LNCaP cell line stably overexpressing HOXC8 was constructed by retroviral transduction. HOXC8 mRNA (Supplementary Fig. S3A) and protein (Supplementary Fig. S3B) expression levels are increased in LNCaP-HOXC8 compared with the negative control LNCaP-Empty cell line. Although many prostate cancer cell lines exhibit low levels of HOXC8, the expression of HOXC8 in LNCaP-HOXC8 cells is comparable to the levels seen in the prostate cancer cell line, PC-3, that endogenously expresses HOXC8 (Supplementary Fig. S1). These HOXC8- and empty vector–transduced LNCaP stable cell lines were transiently transfected with the androgen-responsive MMTV-luc or PSA-luc reporter plasmids. The HOXC8 expressing lines exhibit diminished AR-mediated promoter activity of both reporters (Fig. 2A and B).

To determine whether this effect was specific to prostate cancer cells, or to LNCaP cells in particular, we performed similar experiments in the immortalized, nontumorigenic prostate epithelial cell line HPr-1 AR, which was transduced with a retrovirus to stably overexpress HOXC8 or empty vector control. Real-time RT-PCR and Western blot analyses confirm that HOXC8 mRNA (Supplementary Fig. S3C) and protein (Supplementary Fig. S3D) expression levels are increased in HPr-1 AR-HOXC8 cells compared with the negative control HPr-1 AR-Empty cells. Androgen-induced activity directed by the MMTV-luc (Fig. 2C) and PSA-luc (Fig. 2D) reporter plasmids was diminished in HPr-1 AR cells stably overexpressing HOXC8. These data indicate that the inhibitory effect of HOXC8 on androgen-mediated induction of different direct androgen target promoters is observed in both tumorigenic and nontumorigenic prostate cancer cell lines. Our results are consistent with the notion that at least some HOX proteins can inhibit androgen action and that this activity is present in LNCaP cells.

Figure 1. HOXC8 overexpression specifically inhibits AR-mediated gene induction in LNCaP prostate cancer cells. A and B, LNCaP cells were transfected with a probasin-luc reporter vector and increasing amounts of HOXC8 expression vector. Total transfected plasmid was held constant by balancing with empty expression vector. Cells were treated with 10 nmol/L of R1881 for 18 hours. A, Western blot for HOXC8 (60s exposure) and actin (1s exposure). B, luciferase activity normalized to total protein. LNCaP cells were transfected with (C) pRSV-luc or (D) pCMV-βgal along with the HOXC8 expression vector or the empty vector control. Luciferase data shown are from a single experiment and are representative of 3 (B) or 2 (C and D) separate experiments. Values represent mean ± SD (B, n = 4; C and D, n = 3).
genic prostate cell lines and when HOXC8 is transiently or stably overexpressed.

We also tested whether androgen induction of an endogenous gene was inhibited by HOXC8 expression. We compared the androgen induction of the endogenous PSA gene in LNCaP-HOXC8 and LNCaP-Empty as well as in HPr-1 AR-HOXC8 and HPr-1 AR-Empty cells. In both comparisons, the androgen induction of the gene was reduced in the HOXC8-expressing line (Fig. 3).

HOXC8 inhibition of AR-mediated transcription is not due to downregulation of the AR

To rule out the possibility that HOXC8 inhibits AR-mediated transcription by downregulating AR expression levels, real-time RT-PCR and Western blot analyses of AR mRNA and protein levels was performed in LNCaP-HOXC8 and LNCaP-Empty cells and compared with that of the empty vector control cell line. LNCaP-HOXC8 and LNCaP-Empty cells express nearly identical AR mRNA (Supplementary Fig. S4A) and protein (Supplementary Fig. S4B) levels, indicating that the inhibition of AR-mediated transcription by HOXC8 cannot be attributed to downregulation of AR expression levels. AR protein levels are also unchanged in HPr-1 AR-HOXC8 cells when compared with the empty vector control cell line (data not shown).

Overexpression of SRC-3, but not CBP, reverses the HOXC8-induced inhibition of AR-mediated transcription

The report of Shen et al. (25) that HOX genes interact with the transcriptional coactivator CBP and inhibit its HAT activity suggests a possible mechanistic basis for the HOXC8-mediated inhibition of androgen-induced gene expression. If suppression of CBP coactivator function were responsible for the inhibition of AR action, we reasoned that overexpression of CBP might overcome the inhibition by HOXC8. We overexpressed CBP and other cofactors that are major coregulators of steroid signaling, the CBP relative p300 and the 3 members of the p160 steroid receptor coactivator family of coregulators SRC-1, SRC-2, and SRC-3.

LNCaP prostate cancer cells were cotransfected with the probasin-luc reporter, 100 ng of HOXC8 expression vector (or empty vector control) and 100 or 500 ng of the indicated coactivator expression vector (or empty vector control). Western blot analysis of coactivator protein expression levels following transfection is shown in Supplementary Figure S5. The results of the coactivator coexpression experiment are presented in Figure 4. The average inhibition of the hormone induction by HOXC8 from duplicate experiments is 58% without added coactivators. This is the control inhibition (Fig. 4, leftmost bar), and the inhibition

Figure 2. AR-mediated transcription is inhibited in prostate cells stably overexpressing HOXC8. Retroviral transduction was employed to stably overexpress HOXC8 in prostate cancer cells (LNCaP; A and B) and nontumorigenic prostate (HPr-1 AR; C and D) cell lines. Paired control cell lines were created by transduction of the empty vector. HOXC8-expressing and control cell lines were transfected with MMTV-luc (A and C) or PSA-luc (B and D) reporter and treated with 10 nmol/L of R1881 for 18 hours. Luciferase activity is normalized to total protein. Data shown are from a single experiment and representative of 3 independent experiments. Values represent mean ± SD (n = 4; *, P < 0.05).
imposed by HOXC8 when coactivators are expressed is compared with this standard. Contrary to our expectation, overexpression of CBP only modestly counters the HOXC8-mediated inhibition of the hormone induction (Fig. 4, second and third bars from the left). Both p300 and SRC-1 enhance the HOXC8-mediated inhibition of AR-mediated transcription somewhat (Fig. 4, from left, bars 4–7), increasing the inhibition from 58% to about 70% to 80%. Overexpression of SRC-2 weakly diminishes the HOXC8 inhibition (Fig. 4, from left, bars 8–9). In contrast to the other coactivators, overexpression of SRC-3 reverses the HOXC8-mediated inhibition. At the highest level of expression, SRC-3 completely restores the hormone induction despite the overexpression of HOXC8 (Fig. 4, last 2 bars from left). These data suggest that SRC-3, rather than CBP, may be the critical target for HOXC8 inhibition of AR-mediated signaling. This result is of particular interest, as will be discussed, and directed subsequent efforts toward elucidating the interplay between HOXC8 and SRC-3 in the context of AR signaling in prostate cells.

**SRC-3 expression in LNCaP prostate cancer cell lines**

In consideration of the finding that overexpression of SRC-3, but not CBP, abrogates the HOXC8-mediated inhibition of AR transcriptional activity, we asked whether SRC-3 mRNA or protein levels are altered with HOXC8 overexpression. SRC-3 mRNA and protein levels were compared in LNCaP-Empty and LNCaP-HOXC8 prostate cancer cell lines by real-time RT-PCR and Western blot analysis. LNCaP-Empty and LNCaP-HOXC8 cells express very similar SRC-3 mRNA (Supplementary Fig. S6A) and protein (Supplementary Fig. S6B) levels. Therefore, HOXC8-mediated changes in overall SRC-3 expression levels are not a contributing factor in the HOXC8 inhibition of AR-mediated signaling.

**HOXC8 overexpression inhibits the hormone-dependent interaction of AR and SRC-3**

Gnanapragasam et al. (38) showed that SRC-3 binds AR in a ligand-dependent manner in LNCaP prostate cancer cells and that this interaction is important in enhancing transcriptional activity in prostate cancer cells. We, therefore, tested whether overexpression of HOXC8 might alter the physical interaction between AR and SRC-3.

**Figure 3.** Induction of the endogenous PSA gene is inhibited when HOXC8 is stably overexpressed. A, LNCaP and B, HPr-1 AR cells stably expressing HOXC8 and paired control cells were serum starved for 48 hours and then treated with 10 nmol/L of R1881 for 6 hours. PSA mRNA levels were analyzed by real-time RT-PCR analysis. Data shown are from a single experiment and representative of 3 separate experiments. Values represent mean ± SD (n = 3; *, P < 0.01, **, P < 0.05).

**Figure 4.** SRC-3 overexpression reverses the HOXC8-induced inhibition of AR-mediated transcription. LNCaP cells were cotransfected with a probasin-luc reporter, 100 ng of HOXC8 expression vector or empty vector control and 100 or 500 ng of the indicated coactivator expression vector. Total transfected plasmid was held constant by balancing with empty expression vector. Cells were treated with 10 nmol/L of R1881 for 18 hours. The inhibition of the hormone induction by HOXC8 was 58% without added coactivators. This is the control inhibition (leftmost bar), and the inhibition imposed by HOXC8 when coactivators are expressed is compared with this standard. Data shown are the average from 2 independent experiments in which each condition was tested in duplicate. Values represent mean ± range.
Figure 5. The hormone-dependent interaction of AR/SRC-3 is inhibited in LNCaP-HOXC8 prostate cancer cells. A. LNCaP-Empty and LNCaP-HOXC8 cells were cultured in 10 μM of R1881 or ethanol for 24 hours. SRC-3 was immunoprecipitated and the association with AR was assessed by Western blot analysis by using an antibody directed against AR. AR protein levels were similar in all samples as shown in the input (bottom). B, quantitation of AR bound to SRC-3 (data from A, top, normalized to SRC-3 input, middle). Data shown are from a single experiment and representative of 3 separate experiments.

Co-IP experiments were performed to examine binding interactions between SRC-3 and AR, using LNCaP cells stably overexpressing HOXC8 and LNCaP control cells. LNCaP-Empty cells exhibit an increase in the interaction of SRC-3 and AR upon hormone treatment (Fig. 5A, top left). In contrast, LNCaP-HOXC8 cells exhibit diminished interaction of SRC-3 and AR following treatment with R1881 (Fig. 5A, top-right bands), showing that HOXC8 expression disrupts the ligand-enhanced interaction between SRC-3 and AR. This is not due to a decrease in SRC-3 or AR protein levels (Fig. 5A, middle and bottom, respectively). Although a slight decrease is observed in SRC-3 protein levels after treatment with androgen (Fig. 5A, middle), this decrease is seen in both the LNCaP-Empty and LNCaP-HOXC8 cell lines. Quantitation of these data normalized to input is presented in Figure 5B.

HOXC8 expression inhibits recruitment of SRC-3 and CBP and acetylation of histones

ChIP analyses were performed to assess whether the recruitment of SRC-3 to the endogenous AR target gene PSA is also inhibited by HOXC8 overexpression. It has been previously shown that in the presence of hormone, AR predominately occupies the distal enhancer region, not the proximal promoter region, of the PSA gene in LNCaP cells (48–50). In LNCaP-HOXC8 cells, the occupancy by SRC-3 at the PSA enhancer region is greatly inhibited when compared with that observed in the LNCaP-Empty control cell line (Fig. 6A). The Srcs interact directly with steroid receptors bound to target DNA elements and serve as a platform to recruit additional coregulators including the HAT coactivator CBP (51, 52). Recruitment of these factors by Src/p160 proteins is critical for steroid receptor-mediated local chromatin remodeling and assembly of transcriptional machinery around target gene promoters (53, 54). We, therefore, also assessed recruitment of CBP and found its androgen-dependent recruitment to be diminished in LNCaP-HOXC8, consistent with the idea that the inhibition of SRC-3 recruitment in turn diminishes CBP recruitment (Fig 6B).

A potential explanation for reduced recruitment of SRC-3 is that HOXC8 inhibits recruitment of AR to target response elements. However, we observe no significant change in either the basal or hormone-induced occupancy of AR at the PSA enhancer region in LNCaP-HOXC8 cells when compared with that of the LNCaP-Empty control cell line (Fig. 6C). This suggests that HOXC8 imposes its inhibitory effects on AR signaling independent of AR binding to target genes by inhibiting further recruitment of cofactors, including SRC-3 and CBP, to AR target genes.

The SRC-3 loading experiments show that basal as well as hormone-induced levels of SRC-3 loading are diminished by HOXC8 expression. SRC-3 is a coactivator for many factors acting at many promoters, and HOXC8 may be inhibiting SRC-3 at many or all of these. This suggestion implies that HOXC8 would impose pleiotropic actions beyond that of androgen-regulated genes through its inhibitory effect on SRC-3.

Both CBP and SRC-3 possess intrinsic HAT activity (51, 55, 56). Coactivator-dependent chromatin modification is a critical step in hormone receptor-mediated induction of gene expression. We predicted that the HOXC8-mediated inhibition of coactivator recruitment would be reflected in reduced acetylation of histones at target promoters. As can be seen in Figure 7A, androgen-induced acetylation of histones H3 and H4 at the MMTV promoter is indeed suppressed by HOXC8. Quantitation of these data normalized to input is shown in Figure 7B.

HOXC8 does not directly interact with SRC-3, AR, or CBP

To further assess the mechanism of HOXC8-mediated inhibition of AR signaling, experiments were conducted to test for direct interactions between HOXC8 and SRC-3. In vitro GST pull-down assays were performed using the fusion protein GST-HOXC8 as bait along with Flag-tagged SRC-3 purified from baculovirus expression vector-infected insect cells. No interaction was detected between GST-HOXC8 and purified SRC-3 above the minimal levels observed with the GST control (Supplementary...
Interaction between HOXC8 and AR was also examined, but no binding above background was detected between GST-HOXC8 and AR by using extracts from hormone-treated LNCaP as a source of AR (Supplementary Fig. S7B) or extracts from untreated cells (data not shown). We also examined binding between HOXC8 and AR, SRC-3, or CBP by co-IP by using LNCaP cellular extracts, but no binding interactions were detected either in the presence or absence of hormone (data not shown). All in vitro binding assays have limitations; therefore, the possibility of interactions between HOXC8 and SRC-3 or AR in the context of the cell cannot be completely discounted.

HOXC8 effects on in vitro surrogate markers of tumorigenesis and progression

Overexpression of HOXC genes is associated with prostate cancer (1) and, in the case of HOXC8, increased Gleason grade (22). Recent studies have implicated HOXC8 in the regulation of cell cycle and proliferation (57–60), apoptosis (58, 61), cell differentiation (62–67), cell adhesion (58, 61, 68), cytoskeleton/motility/migration (61), and tumorigenesis (58, 60, 61, 68, 69). On the basis of these reports and the present lack of understanding as to HOXC8 function in prostate carcinogenesis, its effects on in vitro surrogate markers of prostate cell tumorigenicity were examined.

Malignant transformation is associated with certain phenotypic changes including dysregulation of cell cycle and cell death pathways, loss of contact inhibition and anchorage independence, and increased motility and invasiveness. We observed no dramatic changes in anchorage-independent growth, apoptosis, or migration in conjunction with HOXC8 overexpression in prostate cancer cells or non-tumorigenic prostate cell lines. A mild inhibition of growth of LNCaP and HPt-1 AR cells was seen with an accompanying increase in G1 phase of the cell cycle (data not shown).
shown). None of these assays suggested a critical role of HOXC8 in prostate cancer tumorigenesis or progression.

**HOXC8 expression increases invasiveness in HPr-1 AR prostate epithelial cells but not in LNCaP prostate cancer cells**

Invasive capacity is the single most important trait that distinguishes benign from malignant lesions. Invasion assays *in vitro* require cells to migrate through an extracellular matrix or basement membrane extract barrier by first enzymatically degrading the matrix barrier in order to become established in a new location. HOXC8 has been implicated as a regulator of cadherin-11 and osteopontin (58, 61). Cadherin-11 has been shown to produce significant changes in the invasive capacity of cancer cells (70), and cumulative evidence suggests that osteopontin functions in the regulation of tumor metastasis and invasion (71, 72).

The ability of LNCaP prostate cancer and HPr-1 AR nontumorigenic prostate epithelial cells overexpressing HOXC8 to invade through a coated membrane was assessed. LNCaP-HOXC8 cells invade to a similar degree as the LNCaP-Empty control cell line (Fig. 8A). HPr-1 AR-Empty cells invade to a level that is less than 20% as that of LNCaP cells, but in contrast to paired LNCaP cell lines, invasion by HPr-1 AR-HOXC8 cells is nearly double that of HPr-1 AR-Empty control cells (Fig. 8B). These data suggest that HOXC8 expression may direct protumorigenic actions in prostate carcinogenesis by promoting increased invasive capacity.

**Discussion**

Inappropriate expression of HOX genes is associated with a number of cancers (6–10, 14–20). In particular, any of the 4 genes of the HOXC cluster may be overexpressed in prostate cancer (1). The present study suggests a complex role for HOXC8 in prostate cancer, promoting invasiveness while inhibiting AR-mediated gene induction at ARE-regulated genes associated with differentiated function of the prostate. HOXC8 overexpression may well affect the participation of SRC-3 at promoters in addition to ARE-driven promoters. SRC-3 serves as a cofactor for many transcription factors, and a general inhibition of its activity by HOXC8 would be expected to have pleiotropic disruptive effects on cellular homeostasis.

**Mechanism of HOXC8-dependent inhibition of androgen-mediated gene expression**

Although a report that HOX genes inhibit the HAT activity of CBP (25) suggested that HOXC8 might inhibit AR-mediated gene induction through this mechanism, we find evidence that it is SRC-3, rather than CBP, p300, SRC-1, or SRC-2, that seems to be the key target of HOXC8. SRC-3 is an intriguing candidate because like HOX genes, it is overexpressed in many cancers. Indeed, one of its many names (NCoA-3/AIB-1/pCIP/ACTR/RAC3/TRAM-1), AIB-1 (amplified in breast cancer-1), came from its identification as 1 of 3 gene amplifications frequently observed in human breast carcinomas (73). The involvement of SRC-3 overexpression in prostate cancer seems to be especially critical in AR-mediated gene induction. Among the p160 family of coactivators, SRC-3 is the preferred coactivator for hormone-activated AR. SRC-3 is selectively phosphorylated when cells are treated with androgen (74), its binding affinity for AR is 10- to 100-fold stronger than that of SRC-1 or SRC-2 (75), and it activates AR more potently than other SRCs in the presence of 5α-dihydrotestosterone (DHT; ref. 75). Thus, SRC-3 represents an attractive target for HOXC8 inhibition of AR function.

**Inhibition of androgen action—a conundrum?**

Because prostate growth and maintenance are androgen dependent, then why doesn’t HOXC8 expression serve a tumor suppressor function rather than being associated with
prostate cancer and/or worse prognosis? One possibility is that any inhibition of androgen signaling by HOXC gene overexpression is sufficiently countermanded by concomitant overexpression of SRC-3 to maintain androgen-driven growth of prostate cancer. Another possibility is that maximal AR activity is not favorable to growth promotion in prostate cancer. LNCaP cells, widely used as a model of androgen-dependent prostate cancer growth, exhibit a biphasic response to androgens. Maximal growth promotion androgen-dependent prostate cancer growth, exhibit a biphasic response to androgens. Maximal growth promotion is seen at levels of androgen not only below the biphasic response to androgens. Maximal growth promotion is seen at levels of androgen not only below the Kd for the receptor, but also below those considered to be normal physiologic levels of androgens (Daddario Axlund and Nordeen, unpublished results). Thus, to the extent that LNCaP growth responses reflect prostate cancer in vivo, partial suppression of AR activity at physiologic androgen levels may effectively shift the growth response to a more growth-promoting place on the dose–response curve.

Alternatively, HOXC genes may be acting differentially on critical genes in the prostate. PSA is a gene representing a differentiated function of the prostate. Perhaps HOXC expression inhibits genes associated with prostate differentiation but has no effect, or a stimulatory effect, on genes involved in tumorigenesis. Our data showing that HOXC8 expression inhibits AR-mediated induction of the PSA gene, coupled with the finding that HOXC8 expression increases invasiveness in prostate epithelial cells, and recent work on HOXB13 (26) suggest this may in fact be the case.

Norris et al. (26) have recently shown that HOXB13 regulates AR-target gene activity in both positive and negative manners. HOXB13 interacts with AR and inhibits the transcription of genes that contain an ARE, whereas, in complex together, AR-HOXB13 confers androgen responsiveness to genes whose promoters contain a HOXB13 response element. Furthermore, they show that in the case of genes that contain a HOXB13 element adjacent to an ARE, HOXB13 and AR synergistically enhance the transcription of these genes (26). These observations support the notion that HOX proteins may play a dynamic role in the normal prostatic development and tumorigenesis through both the repression and activation of AR-mediated signaling. Like HOXC8 or HOXC6, we see that HOXB13 inhibits AR-mediated transcription at ARE-driven promoters. Although it is plausible that HOXB13 and HOXC8 act in a similar fashion to inhibit direct AR target genes, we have been unable to detect an interaction between HOXC8 and AR in contrast to what has been previously reported for HOXB13 and AR (27). To assess whether HOXC8 modulates AR function in both positive and negative manners via direct or indirect interaction with AR, SRC-3, or other coregulators important in AR signaling, further investigations will be required.

A final mechanism, which is not mutually exclusive, to explain a role of HOXC overexpression in prostate cancer in the context of a suppressive action on androgen action invokes the concept that HOXC overexpression is critical to disruption of cellular homeostasis at an early stage of prostate tumorigenesis. The developing tumor is forced to adapt to diminished androgen signaling that accompanies other protumorigenic actions of HOXC expression, including increased invasion by HOXC8 expression, thereby predisposing the tumor to survive in the face of a subsequent androgen withdrawal through androgen deprivation therapy (ADT). This model could account for the relatively rapid failure of ADT by suggesting that an adaptation to diminished androgen signaling has already occurred before androgen ablation by ADT is imposed.

There is precedence for the notion that dysregulated HOXC expression may be an early event in carcinogenesis. Oct-4, a homeobox gene critical in the genesis of testicular germ cell tumors, is expressed in precursor lesions (76, 77), whereas homeobox genes NKX3.1 and CDX2 are downregulated at the earliest stages of carcinogenesis of the prostate and colon, respectively (78, 79).

**HOXC gene expression and prostate cancer**

Additional studies are required to distinguish between the different explanations to reconcile the apparent paradox that HOXC8 overexpression is associated with prostate cancer yet can inhibit androgen-dependent signaling. Our studies reveal that HOXC8 promotes invasiveness in prostate epithelial cells but does not further promote invasiveness in tumorigenic prostate cancer cells, consistent with an early role of HOXC genes in promoting prostate cancer progression and suggesting that HOX genes play an important role in the disruption of cellular homeostasis leading to cancer. The findings of this work are summarized in the model presented in Figure 9 depicting the inhibition of direct AR target genes by HOXC8 through its inhibition of SRC-3 recruitment. The model also suggests that effects of HOXC8 acting as a transcription factor in its own right along with its transcription partner Pbx may be mediating increased invasion and other activities promoting tumorigenesis. Preliminary microarray studies implicate HOXC8 in the regulation of various genes involved in matrix degradation including MMP1, MMP2, TIMP1, PLAU, and SERPINE1 (data not shown). Other studies identifying putative transcriptional targets of HOXC8 have implicated it in the regulation of cell cycle (57–60), apoptosis (58, 61), cell adhesion (58, 61, 68), migration (61), and tumorigenesis (58, 60, 61, 68, 69). Confirmation of these and other HOXC8 targets implicated in cancer may prove extremely valuable and help achieve a greater understanding of the role of HOXC8 in prostate tumorigenesis.

An estimated 91% of men with newly diagnosed prostate cancer are likely to have stage 1 or stage 2 disease, in which case the 5-year relative survival is nearly 100% (80). With such a favorable prognosis, many question the benefit of exposing men with early-stage and low- or moderate-grade prostate cancer to radical surgery or radiation therapy (81). It is, therefore, critical to be able to identify men whose histologic early-stage prostate cancer will progress and who would, therefore, benefit from aggressive therapy (82, 83). Hence, there is a great need for reliable prostate cancer markers that can predict cancers requiring aggressive therapy, thereby enabling better informed treatment options. Given the correlation of HOXC8 with increased Gleason grade (22) and data herein showing that HOXC8 increases...
invasiveness of nontumorigenic prostate cells, further analysis of the consequences of HOXC gene expression and its use as a prognostic marker is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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