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The Role of HOX Proteins in Androgen-Independent Prostate Cancer

PRINCIPAL INVESTIGATOR:
Sunshine Daddario

CONTRACTING ORGANIZATION:
University of Colorado Health Sciences Center
Aurora, CO 80045-0508

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14. ABSTRACT

HOX genes encode a large family of transcription factors involved in key developmental decisions, and are often aberrantly expressed in cancer. Our laboratory has previously shown that a subset of genes of the HOXC cluster are overexpressed in primary prostate tumors, metastases, and prostate cancer (PCa) cell lines¹. Increasing transient expression of HOXC8 in LNCaP PCa cells as well as HPr-1 AR non-tumorigenic prostate epithelial cells results in a progressive suppression of androgen responsive promoters. Transcription from both the mouse probasin promoter and the MMTV promoter is inhibited at levels of HOXC8 expression comparable to those seen in PCa cell lines. Other members of the HOX family also inhibit androgen signaling. We have created various tumorigenic and non-tumorigenic prostate cell lines that stably overexpress HOXC8 and show that signaling through androgen responsive promoters is inhibited, and PSA mRNA levels are decreased in these cell lines. HOX proteins block the histone acetyltransferase activity of the coactivators CBP and p300. As these are key mediators of steroid-dependent transcription, our original hypothesis proposed that inhibition of these coactivators may account for the HOX-dependent suppression of androgen receptor (AR)-mediated transcription. However, recent data suggests that overexpression of CBP only partially relieves the inhibition of androgen receptor-mediated transcription by HOXC8. We therefore expanded our search for other cofactors potentially involved in androgen signaling, and found that SRC-3 selectively and completely restores the inhibition of androgen receptor-mediated transcription by HOXC8. In order to determine the mechanism of action of HOXC8 inhibition of androgen signaling, various immunoprecipitation experiments were performed using an LNCaP cell line stably overexpressing HOXC8. We found that HOXC8 inhibits the interaction between SRC-3 and AR, but not the interaction of SRC-3 and CBP or p300, suggesting that HOXC8 specifically disrupts the interaction of SRC-3 and AR. SRC-3 and AR levels are unchanged in LNCaP cells stably overexpressing HOXC8. Further, chromatin immunoprecipitation demonstrates that HOXC8 expression inhibits hormone-induced histone acetylation at MMTV. These data indicate that our original hypothesis suggesting that HOX overexpression impacts intracellular receptor activity through the inhibition of CBP/p300 is, at best, only part of the story. We propose that HOXC8 competes with SRC-3 for a common cofactor, AR. This competition results in decreased signaling through AR via SRC-3 when HOXC8 is overexpressed. SRC-3 has been recently implicated in various cancers, yet its function in prostate cancer is still unclear. Further studies involving the mechanism and consequences of this interaction between HOXC8 and SRC-3 may lead to a much greater understanding of the pathogenesis of prostate, breast and many other cancers. In the context of prostate cancer specifically, this may ultimately lead to a better understanding of the mechanism underlying the failure of endocrine intervention and in turn lead to the design of therapeutic modalities that would prolong the efficacy of androgen ablation therapy.

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INTRODUCTION

In the androgen-responsive, normal prostate, essentially no expression of HOXC genes is seen. Our laboratory has previously shown that a subset of genes of the HOXC cluster are overexpressed in primary prostate tumors, metastases, and PCa cell lines². Although little is known regarding the consequences of HOXC overexpression in the prostate, we have shown that HOXC6 or HOXC8 overexpression inhibits androgen receptor (AR)-mediated transcription in LNCaP cells. The goal of this work is to explore further the interplay between HOXC expression and steroid receptor signaling, investigating both the underlying mechanisms and the consequences of HOXC overexpression on androgen action in prostate cells.

BODY

Two tasks were listed in the approved Statement of Work:

Task one: To characterize the consequences of HOXC expression on steroid signaling in human PCa cell lines.

Task two: To dissect the molecular mechanism of HOXC inhibition of androgen signaling.

Task One: To characterize the consequences of HOXC expression on steroid signaling in human PCa cell lines.

We have previously shown that HOXC8 and HOXC6 transient overexpression inhibits AR-mediated transcription of the androgen-responsive MMTV and probasin promoters in LNCaP and PC-3 AR PCa cells. We created a series of cell lines by viral transduction which stably overexpress HOXC8 and have shown that HOXC8 inhibits AR-mediated signaling when stably overexpressed in LNCaP, DU-145, PC-3-AR, and ALVA-31 cells.

In order to further characterize the consequences of HOXC expression in the context of PCa, we utilized various tumorigenicity assays in LNCaP PCa cells stably overexpressing HOXC8. These assays include cell proliferation (Hoechst staining), invasion and migration (Boyden chamber), and soft agar colony formation (anchorage-independent growth). Thus far we have been unable to detect any significant difference between the HOXC8 overexpressing cell lines and control cell lines in these assays (data not shown). Because HOXC8 overexpression may be involved in early tumorigenesis, we reasoned that it would be important to perform these tumorigenicity assays in cell lines derived from non-tumorigenic “normal” prostate epithelial cells. We therefore created cell lines overexpressing HOXC8 using RWPE-I and PWR-IE non-tumorigenic prostate epithelial derived cell lines, both of which have been reported to express AR. In our hands however, no androgen receptor protein was detected by western analysis after chronic treatment with the synthetic androgen R1881 for up to 4 weeks (data not shown), and there was no detectable induction of androgen signaling in reporter assays using the androgen responsive reporters PSA-luciferase, MMTV-luciferase and probasin-luciferase (data not shown). These cell lines were therefore not useful for our studies of the interaction of HOXC8 and AR signaling.

We therefore obtained another non-tumorigenic prostate epithelial derived cell line, HPr-1 AR. These cells express androgen receptor (Fig. 1A) and are induced upon androgen treatment through the probasin promoter in luciferase reporter assays (Fig. 1B). We virally transduced these cells and confirmed overexpression of HOXC8 protein levels by western analysis (Fig. 2). In reporter assays, induction of the PSA-luciferase promoter is inhibited in HPr-1 AR HOXC8 cells when compared with empty vector transduced control cells (Fig. 3). HOXC8 overexpression has been shown to correlate with higher Gleason grade PCa³. Our studies suggest that HPr-1 AR-HOXC8 are more invasive *in vitro* (Fig. 4). Further characterization studies of HPr-1 AR HOXC8 have shown no difference in cell proliferation, cell cycle, soft agar colony formation or migration between HPr-1 AR HOXC8 cells and those transduced with empty vector.

Task two: To dissect the molecular mechanism of HOXC inhibition of androgen signaling.

Our initial findings that HOXC8 and HOXC6 overexpression inhibit AR-mediated transcription in PCa cells, coupled with reported data demonstrating that homeodomain-containing proteins interact with and inhibit the histone-acetyltransferase (HAT) activity of the steroid receptor coactivators CBP and p300¹, originally lead us to hypothesize that HOXC proteins inhibit AR-mediated signaling through inhibition of CBP/p300 HAT activity. Further, we have demonstrated by chromatin immunoprecipitation (ChIP) that hormone-induced histone acetylation (H3 and H4) at the androgen-responsive MMTV promoter is inhibited upon overexpression of HOXC8 in LNCaP and PC-3 PCa cells. Contrary to our hypothesis, however, we found that increased expression of CBP only partially relieves HOXC8 induced inhibition of AR-mediated transcription of the androgen-responsive MMTV promoter in LNCaP PCa cells (Fig. 5). This led us to expand our search for other possible cofactors involved in the HOXC8 mediated-inhibition of AR signaling. We performed luciferase reporter assays using the androgen responsive MMTV-luciferase construct, and found that SRC-3 strongly restores HOXC8 mediated inhibition of signaling through the MMTV promoter in LNCaP PCa cells (Fig. 6).

In light of this data, we performed co-immunoprecipitation assays using LNCaP cells stably overexpressing HOXC8 in order to investigate those interactions involving SRC-3 that are critical to androgen signaling in the prostate. When precipitating with an antibody directed against SRC-3 and immunoblotting with an antibody directed against, AR, we demonstrate that the interaction between SRC-3 and AR is greatly diminished in cells overexpressing HOXC8 in the presence of hormones (Fig. 7). We also observed that the interaction between SRC-3 and CBP is not inhibited in cells overexpressing HOXC8 suggesting that the inhibition of the SRC-3 AR interaction is specific. SRC-3 (Fig. 7) and AR (data not shown) levels remain relatively unchanged in the various treatment conditions.

KEY RESEARCH ACCOMPLISHMENTS

- Development and characterization of several HOXC8 overexpressing PCa and non-tumorigenic prostate epithelial cell lines by viral transduction
- Successful siRNA knockdown of HOXC8 protein levels in LNCaP PCa cells

- Successful implementation of ChIP analysis using transiently transfected target DNA
- Demonstration that non-tumorigenic HPr-1 AR-HOXC8 cells are more invasive *in vitro*
- Discovery and validation of the inhibition of the SRC-3/AR interaction in LNCaP PCa cells stably overexpressing HOXC8 by co-immunoprecipitation

REPORTABLE OUTCOMES

Abstract: **Androgens in Prostate Cancer Cells: Implications for hormone-based therapy and derivation of androgen-independent cell lines.** Daddario SN, Hedlund, T, Johannes W, Lambert JR, Nordeen SK. Poster presentation at the 88th Annual Meeting of the Endocrine Society. June 2006. Boston, Massachusetts.

Abstract: **HOX Gene Expression Modulates Androgen- and Vitamin D-Mediated Actions in Human Prostate Cancer Cells.** Daddario SN, Lambert JR, Lucia MS, Nordeen SK. Poster presentation at the 88th Annual Meeting of the Endocrine Society. June 2007. Toronto, Canada.

Abstract: **HOX Gene Expression Modulates Androgen- and Vitamin D-Mediated Actions in Human Prostate Cancer Cells.** Daddario SN, Lambert JR, Lucia MS, Nordeen SK. Poster presentation at the Innovative Minds in Prostate Cancer Today (IMPACT) Meeting. September 2007. Atlanta, Georgia.

Abstract: **HOX Gene Expression Modulates Androgen- and Vitamin D-Mediated Actions in Human Prostate Cells.** Daddario SN, Lambert JR, Lucia MS, Nordeen SK. Poster presentation at the Keystone Nuclear Receptor Symposium. March 2008. Whistler, BC.

CONCLUSIONS

We have extended upon our initial observations demonstrating that HOXC6 and HOXC8 inhibit AR-mediated transcription in LNCaP PCa cells in transient reporter assays to include congruent data from LNCaP PCa cells as well as non-tumorigenic HPr-1 AR cells stably overexpressing HOXC8, all further demonstrating HOXC8 inhibition of AR-mediated transcription in prostate cells. We have demonstrated by ChIP analysis that hormone-induced histone acetylation at the androgen-responsive MMTV promoter is inhibited upon overexpression of HOXC8 in both LNCaP and PC-3 AR PCa cell lines.

We have also performed various tumorigenicity assays in HOXC8 overexpressing cell lines, including cell proliferation, migration, invasion and soft agar colony formation (anchorage independent growth). We were unable to detect any significant difference between the HOXC8 overexpressing PCa cell lines and control cell lines in these experiments, which led us to believe that HOXC8 overexpression may be involved in an early step in tumorigenesis, and therefore it is critical to perform these assays in non-tumorigenic prostate epithelial cell lines. We have therefore created cell lines stably overexpressing HOXC8 using HPr-1 AR non-tumorigenic prostate epithelial derived cell lines. These studies have shown successful as we have demonstrated that signaling through the PSA promoter is inhibited, and overexpression of HOXC8 in these cells leads to increased invasiveness in Boyden chamber/Matrigel *in vitro*

invasion assays. We believe that further characterization and analysis of these cell lines will prove extremely informative in elucidating the role of HOXC8 in androgen receptor-mediated signaling and prostate tumorigenesis.

We initially hypothesized that HOX proteins inhibit AR-mediated signaling through inhibition of the HAT activity of the steroid receptor coactivators CBP/p300. However, data demonstrating that CBP overexpression only partially rescues HOXC-8 mediated inhibition of AR-mediated signaling compelled us to analyze additional steroid receptor cofactors for potential involvement in the mechanism of HOXC8 inhibition of AR signaling. We demonstrate that SRC-3 selectively and completely restores HOXC8-induced inhibition of AR-mediated transcription in LNCaP PCa cells in a dose dependent manner.

SRC-3 has been recently implicated in various cancers, including breast and gastric, yet its function in prostate cancer is still unclear. SRC-3 interacts with multiple nuclear receptors, including AR, in a ligand-dependent manner and significantly enhances nuclear receptor-dependent transcription. SRC-3 also recruits CBP/p300 to receptor-coactivator transcriptional activation complex. SRC-3 is expressed in PCa cell lines and tumors, and its mRNA and protein levels correlate with tumor grade and stage (but not with serum PSA levels)⁴.

We then hypothesized that HOXC8 and SRC-3 may share a similar cofactor, perhaps AR, and that overexpression of HOXC8 depletes the cofactor that was once available to interact with SRC-3, resulting in an inhibition of AR-mediated signaling. We performed co-immunoprecipitation assays in LNCaP cells stably overexpressing HOXC8 and have demonstrated that the interaction of SRC-3 and AR (but not that of SRC-3 and CBP) is inhibited in LNCaP-HOXC8 cells.

In order to further elucidate the mechanism of action of SRC-3 and HOXC8 in prostate cancer, future work would include the following:

1. ChIP analysis of endogenous androgen receptor target genes (such as PSA), in order to investigate the occupancy of key proteins including AR and SRC-3 in the presence and absence of HOXC8.
2. Perform MMTV-luciferase reporter assays utilizing either of the following SRC-3 constructs (both of which have been published) in AR-negative PCa cell lines PC-3 and DU-145 (both express low levels of SRC-3), in order to determine if SRC-3 overexpression is still able to restore HOXC8-induced inhibition of AR-signaling through the androgen responsive reporter construct. This would directly suggest that in addition to SRC-3, AR or CBP/p300 are involved and necessary for the HOXC8 inhibition of AR-mediated signaling.
 - a. containing a mutated nuclear receptor binding domain
 - b. containing a mutated CBP/p300 binding domain

Further studies involving the mechanism and consequences of this interaction between HOXC8 and SRC-3 may be vital to gaining a greater understanding of the pathogenesis of prostate, gastric, breast and many other cancers. In the context of prostate cancer specifically,

this may ultimately lead to a better understanding of the mechanism underlying the failure of endocrine intervention and in turn lead to the design of therapeutic modalities that would prolong the efficacy of androgen ablation therapy.

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APPENDICES- none

SUPPORTING DATA

A)

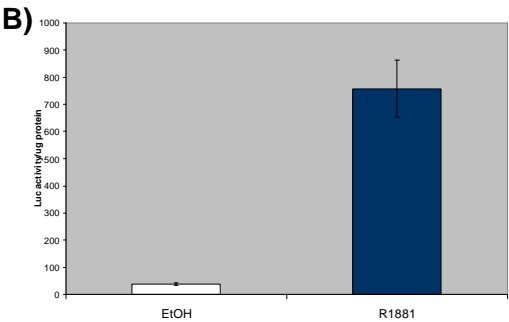
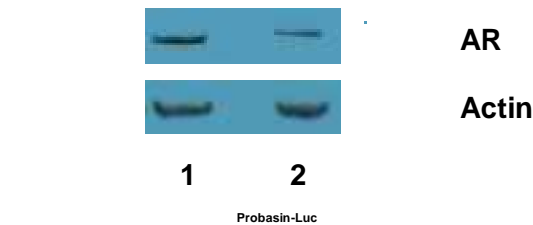


Fig. 1A. HPr-1 AR (1) express AR by western analysis. LNCaP (2) positive control.

1B. HPr-1 AR show induction through the probasin-luciferase reporter when treated with R1881 for 18 hours.

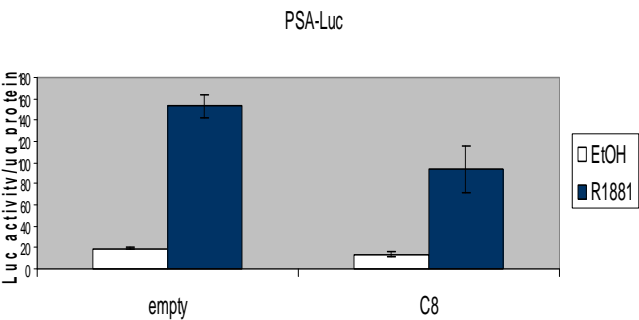


Fig. 3. Induction of signaling through the PSA promoter is inhibited in HPr-1 AR cells stably overexpressing HOXC8 in luciferase reporter assays.

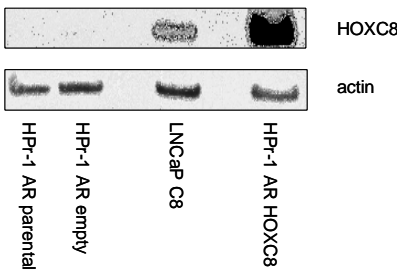
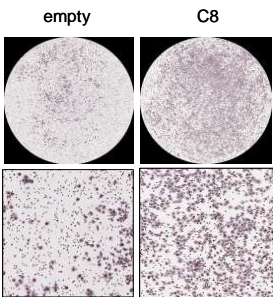


Fig. 2. HPr-1 AR HOXC8 overexpress HOXC8 protein by western analysis.

A)



B)

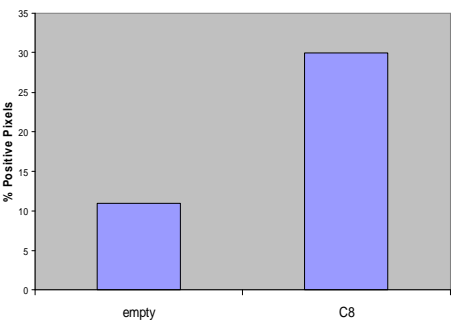


Fig. 4. HPr-1 AR HOXC8 are more invasive in vitro when compared with empty vector control HPr-1 AR cells. 3 day Matrigel Boyden chamber assay.

A) Scanned images of stained cells which have invaded through Matrigel coated filters.

B) Quantification of invading cells.

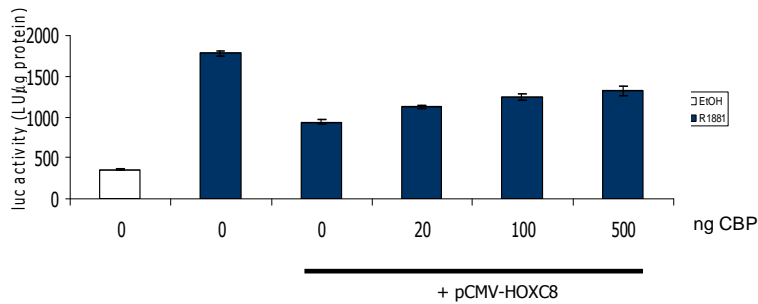


Fig. 5. Overexpression of CBP only partially restores HOXC8-induced inhibition of AR-mediated transcription of MMTV-luciferase. LNCaP cells transfected with HOXC8 expression plasmid were co-transfected with increasing amounts of CBP expression plasmid. Only partial restoration of transcription is achieved transfecting up to 500ng CBP expression plasmid.

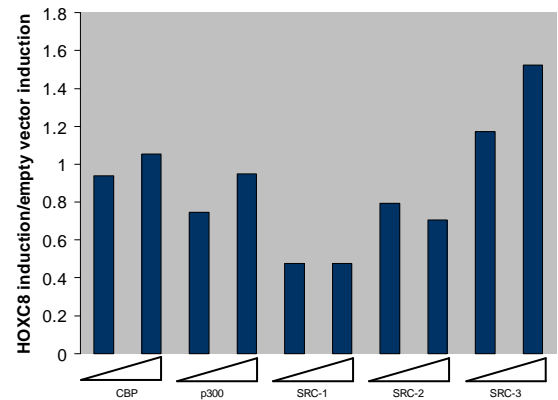


Fig. 6. SRC-3 selectively restores the HOXC8-mediated inhibition of AR-mediated transcription through the MMTV-promoter in LNCaP PCa cells. Restoration of HOXC8 inhibited transcription levels is normalized to those of empty vector transfected levels in order to normalize for any increase in basal transcription.

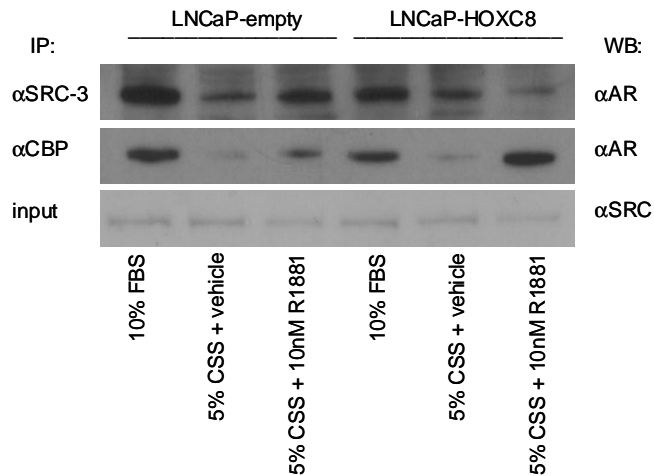


Fig. 7 The interaction of SRC-3 and AR, but not SRC-3 and CBP, is inhibited in the presence of hormone in LNCaP-HOXC8 cells. Co-immunoprecipitation was performed after 24 hours treatment. SRC-3 and AR (data not shown) levels remain relatively unchanged across treatment conditions.