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Roles of the HOXA10 gene during castrate-resistant prostate cancer progression

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Abstract

Homeobox A10 (HOXA10) is an important transcription factor that regulates the development of the prostate gland. However, it remains unknown whether it modulates prostate cancer (PCa) progression into castrate-resistant stages. In this study, we have applied RNA *in situ* hybridization assays to demonstrate that downregulation of HOXA10 expression is associated with castrate-resistant PCa. These findings are supported by public RNA-seq data showing that reduced HOXA10 expression is correlated with poor patient survival. We show that HOXA10 suppresses PCa cell proliferation, anchorage colony formation and xenograft growth independent to androgens. Using AmpliSeq transcriptome sequencing, we have found that gene groups associated with lipid metabolism and androgen receptor (AR) signaling are enriched in the HOXA10 transcriptome. Furthermore, we demonstrate that HOXA10 suppresses the transcription of the fatty acid synthase (FASN) gene by forming a protein complex with AR and prevents AR recruitment to the FASN gene promoter. These results lead us to conclude that downregulation of HOXA10 gene expression may enhance lipogenesis to promote PCa cell growth and tumor progression to castrate-resistant stage.

Key Words

- ▶ castrate-resistant prostate cancer
- ▶ HOXA10
- ▶ FASN
- ▶ lipid synthesis and androgen receptor

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Background

The homeobox A10 (*HOXA10*) gene is a member of the superfamily of homeobox genes that are master regulators of anterior-posterior (AP) axis positioning and tissue determination during development (Mark *et al.* 1997, Wellik 2009). These genes encode for transcription factors that have highly conservative ~60aa homeodomains to recognize specific regulatory DNA elements in target promoters (Mark *et al.* 1997). Because of gene duplication

and divergence, four *HOX* gene clusters (A, B, C and D) and a total of 39 *HOX* genes exist in chromosomal clusters in humans (Krumlauf 1994). Along the AP axis, *HOX* genes are expressed in a 3' to 5' pattern, where the 5' genes are associated to more caudal and distal structures (Shah & Sukumar 2010). This spatial collinearity is exemplified by the fact that 5' genes, such as *HOXA10*, 11 and 13 (there is no *HOXA12* gene), are necessary for the development

of the prostate gland, a structure that develops distally (Satokata *et al.* 1995, Podlasek *et al.* 1999, Dhanasekaran *et al.* 2005). *HOX* gene expression also shows temporal collinearity. For example, the 3' *HOX* genes are mainly expressed in the earlier structures, such as main stem bronchi during pulmonary embryogenesis, while the 5' genes are expressed in later developed structures, such as the alveoli (Maeda *et al.* 2007). Therefore, the primary function of *HOX* genes during development is to specify the identity of body segments along the AP axis. To date, this has been demonstrated by phenotypical alterations in *HOX*s knockout mouse models (Podlasek *et al.* 1999, Post & Innis 1999, Villavicencio-Lorini *et al.* 2010) as well as the established association of germline mutations of *HOX* genes with inherited human diseases (Quinonez & Innis 2014).

HOXA10 is located in the most 5' cluster of the *HOX* genes that are expressed in the posterior domains of the spinal cord, digestive tract and urogenital sinus (Warot *et al.* 1997, Podlasek *et al.* 1999). Studies using knockout mouse models have shown that *HOXA10* is necessary for the formation of reproductive organs including the prostate, epididymis and seminal vesicle in males and the oviduct and uterus in females (Satokata *et al.* 1995, Podlasek *et al.* 1999). *HOXA10* expression has been reported to be low at birth in the rat ventral prostate, but increase after day 10 to a two-fold higher-level until and throughout adulthood (Huang *et al.* 2007). In both human and mouse, persistent *HOXA10* expression in fully developed adult prostate suggests that *HOXA10* may be important to maintain terminal differentiation of prostatic cells or regulate phenotype plasticity of differentiated cells by interacting with intracellular signaling (Shah & Sukumar 2010). This is supported by the findings that while *HOXA10* is continually expressed in the endometrium, its levels fluctuate during the menstrual cycle and peak at the proliferative phase via the estrogen signaling (Taylor *et al.* 1998, Bagot *et al.* 2001). Female *HOXA10*-knockout mice have serious blastocyst implantation defects due to aberrant endometrium function (Benson *et al.* 1996). However, it remains to be determined whether *HOXA10* exerts any biological functions in fully developed prostate in males, and whether *HOXA10* regulates signal pathways during prostate cancer (PCa) development or tumor progression into the castrate-resistant stage.

PCa is the most commonly diagnosed cancer in males. The primary treatment for metastatic PCa is androgen deprivation therapy that either blocks androgen synthesis or prevents androgens from activating the androgen receptor (AR) (Ferraldeschi *et al.* 2015).

However, tumors will inevitably relapse and most of them restore their AR signaling and resume tumor progression to castrate-resistant prostate cancers (CRPC) (de Bono *et al.* 2011, Scher *et al.* 2012). This re-activated AR signaling modulates gene transcription to accelerate cell cycling and develop anti-apoptotic mechanisms for cell growth and tumor progression. AR also regulates genes to adapt multiple lipid metabolism signaling in order to (i) increase phospholipid synthesis for membrane production to cope with increased cell proliferation; (ii) generate and store sufficient energy required for tumor growth and (iii) modulate intracellular signaling to survive unfavorable microenvironments including hypoxia and lack of vascularity (Qi *et al.* 2013, Ackerman & Simon 2014). Androgen stimulation of lipid metabolism involves lipid synthesis and storage as well as lipid intake from dietary lipoproteins in circulation or lipid release from adjacent adipocytes in tumor tissues (Butler *et al.* 2016). It had been reported that androgen regulation of *de novo* lipogenesis involves multiple enzymes including fatty acid synthase (FASN), acetyl-coA carboxylase (ACAC), sterol regulatory element-binding proteins (SREBPs), SREBP cleavage-activating protein (SCAP), acyl-coA synthase long-chain family members (ACSLs) and ATP citrate lyase (ACLY) (Swinnen *et al.* 1997, Welsh *et al.* 2001, Singh *et al.* 2002, Vanaja *et al.* 2003, Wallace *et al.* 2008, Han *et al.* 2018).

In this study, we confirmed that *HOXA10* gene is predominantly expressed in prostate epithelial cells and its levels are reduced in tumors at the CRPC stage. *HOXA10* suppresses PCa cell proliferation, colony formation and xenograft growth independent to androgens. Whole transcriptome profiling assays revealed that *HOXA10* regulates lipid metabolism and AR signaling. We demonstrate that *HOXA10* can form a protein complex with AR, preventing AR from being recruited to the *FASN* gene promoter and thereby inhibiting *FASN* gene transcription. These findings indicate that reduced *HOXA10* expression may contribute to CRPC progression by enhancing AR-mediated lipogenesis.

Materials and methods

PCa cell lines, transfection and lentivirus transduction

LNCaP, VCaP, PC-3 and DU145 PCa cell lines were purchased from American Type Culture Collection (ATCC). C4-2, MR49F, LNAI and 293T cell lines were generously provided by Drs Rennie, Gleave and Buttyan at the Vancouver Prostate Centre, University of British

Columbia. LNCaP95 and BPH1 cells were a kind gift from Dr Alan Meeker (Johns Hopkins University) and Dr Simon Haywards (Vanderbilt University). Cell culture conditions have been previously described (Liu *et al.* 2014, Palmeri *et al.* 2015, Li *et al.* 2016, Lee *et al.* 2017). STR assays confirmed cell line authentication. LNCaP cells within 40–50 passages were applied in this study. VCaP, PC-3 and DU145 lines were within 15 passages upon purchased from ATCC. C4-2, MR49F, LNAI, LNCaP95 and 293T lines were within 12 passages upon the dates when received from collaborators. Lipofectamine 3000 (Invitrogen) and SuperFect Transfection Reagents (Qiagen) were used for plasmid and siRNA transfection.

Lentivirus vectors for HOXA10, 11 and 13 were constructed as previously reported (Liu *et al.* 2014, Li *et al.* 2016, Lee *et al.* 2017). Plasmids containing the cDNA of HOXAs were kindly provided by Drs Hugh Taylor and Gunter Wagner (Yale School of Medicine). These plasmids were used as templates to clone HOXAs into the pCMV2 for transient transfection, and the pDONR201 and pFUGWBW vectors for lentivirus infections (Yu *et al.* 2013). Sanger sequencing was used to validate all expression vectors. LNCaP cells were infected with lentivirus encoding a control as well as the HOXA genes, followed by blasticidin selection to generate LNCaP(CTL), LNCaP(HOXA10), LNCaP(HOXA11) and LNCaP(HOXA13) cell lines. Vectors encoding shRNA against HOXA10 were purchased from Dharmacon (RHS4533-EG3206).

PCR and immunoblotting assays

Real-time qPCR and immunoblotting assays were performed as previously described (Liu *et al.* 2014, Li *et al.* 2016, Lee *et al.* 2017). Primer sets used include HOXA10 (F: TGGCTC A CGGCAAAGAGTG; R: GCTGCGGCTAATCTCTAGGC), FASN (F: AAGGACCTGT CTA GGTGTGATGC; R: TGGCTTCATAGGTGACTTCCA), SREBF-1 (F: CGGAACCATCTTG GCAACAGT; R: CGCTTCT CAATGGCGTTGT), SCAP (F: CTGAGGATGAGGAACCTT GGAG; R: TTGGCCAGTGTGATGTTGTAAT), GAPDH (F: GGACCGACCTGCCGTCTA GAA; R: GGTGTCGCTG TTGAAGTCAGAG). Antibodies used in immunoblotting include the anti-HOXA10 antibody (sc17158, Santa Cruz), the anti-FASN antibody (sc20140, Santa Cruz), the anti-SREBP1 antibody (ab28481, Abcam), the anti-AR antibody (ab9474, Abcam), the anti-Flag-tag antibody (F4042, Sigma-Aldrich) and the anti-Actin antibody (A5316, Sigma-Aldrich). All assays were carried out using three technical replicates and three independent biological

replicates. Only one representative immunoblotting results from the three repeats is shown.

Tissue microarrays (TMAs)

Prostate tumor samples were extracted from the Vancouver Prostate Centre (VPC) tissue bank and used to build several PCa TMAs as we previously reported (Yu *et al.* 2015, Li *et al.* 2016). There are 65 cores from 40 benign prostate tissues, 157 cores from 42 primary tumors from patients with no prior hormonal therapies and 83 cores from 32 patients who had received hormonal therapy, chemotherapy or radiotherapy. The recurred tumors were removed by transurethral resection prostatectomy to relieve the lower urinary tract symptoms. Patient information is listed in the Supplementary tables (data not shown). All patients had given informed consent to a protocol that was reviewed and approved by the UBC Clinical Research Ethics Board (Certificate #: H09-01628).

RNA *in situ* hybridization (RISH) and immunohistochemistry (IHC)

The RISH probe targeting the 909–1270bp of NM_018951.3 for HOXA10 was designed by Advanced Cell Diagnostic (Hayward, USA). A probe targeting the *dapB* gene of bacteria was used as a negative control probe. RISH assays were performed using the BaseScope assay kit following manufacture's instruction. IHC was performed by Ventana Discovery XT (Ventana) using a DAB MAP kit, as we have previously reported (Yu *et al.* 2015, Li *et al.* 2016). A Leica SCN400 scanner to form digital images scanned all stained slides. Positive RISH signals were presented as red dots under 40× magnification. RISH signals were scored as 0 if no positive signal; one if RISH signal was positive in ≤20% of all cells within a core; two if RISH signal was positive in >20% of the cells throughout the core and the dots were not merged into dot clusters; and three if RISH signal was positive in >20% of the cells and dots were merged into dot clusters. IHC signals were scored by both the percentage of stained cells (0–16, 17–33, 34–66 and 67–100%, as 0–3 scores) and the staining intensity (no staining, low, moderate and high intensity staining, as 0–3 scores). The histology index of HSCORE = $\sum p_i(i+1)$, where i = the intensity of staining, and p_i = the percentage of stained cells as reported (Yu *et al.* 2015, Li *et al.* 2016, 2018). Antibodies used in immunoblotting include the anti-Ki67 antibody (RM9106, Thermo Fisher Scientific)

and the anti-cleaved caspase3 antibody (9661s, Cell Signaling Technology).

Cell proliferation and colony formation assays

Cell proliferation rates were measured by the CellTiter 96 AqueousOne kit (Promega) or crystal violet assays as described (Gillies *et al.* 1986). In the colony formation assays, approximately 2×10^4 cells were seeded in 0.7% soft agar in a six-well plate, with a 1% soft agar bottom base coating. Cells were allowed to grow for 14 days to form colonies. Colonies were stained with crystal violet and imaged by stitching $5 \times$ field images together to capture the entire well (Zeiss light microscope (Carl Zeiss)). Colony numbers were counted if their diameters were $>100 \mu\text{m}$. Three independent biological replicates were performed for all assays.

Human prostate cancer xenografts

LNCaP xenograft studies follow the protocols we have previously reported (Palmeri *et al.* 2015, Li *et al.* 2016). A total of 1×10^6 LNCaP(CTL) or LNCaP(HOXA10) cells in 0.1 mL Matrigel (BD Labware) were inoculated subcutaneously in the bilateral flanks of 6- to 8-week-old male athymic nude mice ($n=8/\text{group}$; Harlan Sprague Dawley Inc.). Tumor volume ($V=L \times W \times D \times 0.5236$) and body weight were measured weekly. Serum PSA levels were determined by ELISA. When tumor volumes reach 200 mm^3 , mice were castrated. Mice were killed when tumor volume was $>10\%$ of bodyweight or when there was $>20\%$ loss of bodyweight. Tumors were harvested to evaluate gene expression using real-time PCR and immunoblotting assays. All animal procedures were under the guidelines of the Canadian Council on Animal Care.

Luciferase reporter assay

Cells were transfected with PSA-luciferase reporter plasmid with the Renilla reporter as a control for transfection efficiency. Luciferase activities were determined using the luciferin reagent (Promega) according to the manufacturer's protocol. Transfection efficiency was normalized by Renilla luciferase activity.

AmpliSeq transcriptome sequencing

LNCaP(CTL) and LNCaP(HOXA10) cells were cultured in medium containing 10% charcoal stripped serum for 2 days before they were treated with vehicle or 10 nM DHT

for 24 h. Total RNA was extracted by using the mirVana RNA Isolation Kit (Ambion) according to the manufacturer's protocol. Two independently repeated experiments were performed for each experimental condition. The quantity and quality of the RNA samples was assessed by NanoDrop 2000 as well as Agilent 2100 Bioanalyzer (Caliper Technologies Corp., Canada) before being sent for AmpliSeq transcriptome sequencing. Library preparation, sequencing and primary analyses were performed by UBC-DMCBH Next Generation Sequencing Centre following the protocol described by Li *et al.* (Choo *et al.* 2008). In summary, cDNA was synthesized from 100 ng of total RNA using the SuperScript VILO cDNA Synthesis kit and amplified with Ion AmpliSeq technology. Barcoded cDNA libraries were diluted to 100 pM, equally pooled and amplified on Ion Torrent OneTouch2 instrument using emulsion PCR. Template libraries were subjected for sequencing of $>20,000$ RefSeq transcripts using the Ion Torrent Proton sequencing system. Primary analysis and normalization were performed using the AmpliSeq RNA plugin available through the Ion Torrent suite Software (Choo *et al.* 2008).

Oil red O staining

Oil red O staining was performed as previously described (Ramirez-Zacarias *et al.* 1992). Briefly, formalin-fixed cells were immersed in working solution of Oil red O (Sigma). The dye was then extracted by isopropyl alcohol and quantified by spectrophotometry at a wavelength of 510 nm.

Co-immunoprecipitation (Co-IP) and chromatin immunoprecipitation (ChIP)

Co-IP and Ch-IP assays were performed as we have previously reported (Li *et al.* 2014, Palmeri *et al.* 2015, Xie *et al.* 2015). Briefly, cell lysates were extracted by NETN buffer containing 0.5% NP40, 1 mM of EDTA, 50 mM of Tris and 150 mM of NaCl plus proteinase and phosphatase inhibitor (Roche). Pre-cleared lysates were incubated with either control IgG or AR/HOXA10 antibody, and the associated proteins were immunoblotted by AR and HOXA10 antibody. In ChIP assays, chromatin was cross-linked with 1% formaldehyde for 10 min at 37°C and sonicated in the lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.0, plus protease inhibitor cocktail). After centrifugation, 10 μL of the supernatants was used as input, and the remaining lysate was subjected to a ChIP assay using the AR or HOXA10 antibody. The primers

used to amplify AREs on the *FASN* and *PSA* promoters include *FASN* (F: TATGACACCCAGGGCTTTCGTTCA; R: TAACGTTCCCTGCGCGTTTACAGA), *PSA* (F: ACCTGCTCAGCCTTTGTCTCTGAT; R: AGATCCAGGCTTGCTTACTGTCCT).

Statistics

Statistical analysis was performed using R software. Public RNA-seq data were analyzed by one-way ANOVA followed by one-side Welch's *t*-test. Kaplan–Meier curves and log-rank tests were used to compare the overall survival and disease-free survival. Cox regression analyses were used to analyze the hazard ratio. The contingency table of counts of the *HOXA10* RISH scores and *FASN* IHC scores were compared by Fisher's exact test. Spearman correlation analyses were used to test the correlation of *HOXA10* RISH scores with Gleason groups. One-way ANOVA followed by Tukey tests were used in pairwise comparisons among different experimental groups. Student *t*-tests were used to compare results between the two experimental groups. Functional annotation assays with Benjamini adjustment of *P* values were performed using the DAVID 6.8 software. Pearson correlation analyses were used to test the correlation of AR and *FASN* expressions with *HOXA10* levels. The level of significance was set at $P < 0.05$ as *, $P < 0.01$ as ** and $P < 0.001$ as ***.

Results

Downregulation of *HOXA10* expression associates with CRPC

Since *HOXA10*, *11*, and *13* are all located in the most 5' cluster of *HOX* genes that regulate prostate development, we transduced all three *HOXA* genes into the LNCaP PCa cell line (Fig. 1A). However, only *HOXA10* significantly suppressed cell proliferation, leading our following studies to focus on this *HOXA* member. Genomic profiling of PCa patients (Chandran *et al.* 2007, Taylor *et al.* 2010, Grasso *et al.* 2012) showed that while *HOXA10* mRNA levels are similar between benign prostate and primary tumors, they are statistically lower in both the metastatic tumors from the Chandran 2007 cohort (Fig. 1B) and the CRPC from the Grasso 2012 cohort (Fig. 1C). Consistent to these public data, TCGA provisional data indicated that patients with relatively lower *HOXA10* expression have shorter total patient survival and disease-free survival rates (Fig. 1D). *HOXA10* is expressed in several PCa cell lines (Fig. 1E). Except for the VCaP cell line that has gene

amplifications, all PCa cell lines expressed lower levels of *HOXA10* than the benign BPH1 cell line. Androgen-independent LNCaP95 and enzalutamide-resistant MR49F cells were established by treating LNCaP cells with pronged androgen depletion or enzalutamide treatments, respectively. Both LNCaP95 and MR49F cells are *HOXA10* negative, in contrast to the *HOXA10*-positive LNCaP cells.

Currently, commercially available *HOXA10* antibodies only show cytoplasmic staining in PCa tissues that do not reflect the precise localization of *HOXA10* protein in PCa cells. Therefore, we utilized RISH assays on TMAs to measure *HOXA10* expression in patients. RISH signals appeared predominantly in luminal epithelial cells and were rarely present in prostate stroma. *HOXA10* was highly expressed in 26% (17/65) of the benign tissue cores that have RISH scores=3. It is moderately expressed in 69% (45/65) of the benign tissue cores that have RISH scores=2. No benign prostate samples were *HOXA10* negative (Fig. 2A). While similar *HOXA10* expression was observed in primary tumors comparing with benign prostate, its levels were significantly lower in CRPC (Fig. 2B). Among all 83 tissue cores, no CRPC samples had strong *HOXA10* staining. There were 65% (54/83) of cores that moderately expressed *HOXA10*, 26% (22/83) of cores that weakly expressed *HOXA10* and ~10% of CRPC samples that were *HOXA10* negative. The mean *HOXA10* RISH score was 2.22 ± 0.087 in benign prostate and 2.09 ± 0.156 in primary tumors, but 1.57 ± 0.209 in CRPC. Among primary tumors, *HOXA10* expression was negatively correlated with Gleason groups (Spearman correlation $r = -0.253$, $P = 0.001$) (Fig. 2C). In summary, both public data and our RISH results indicate that reduced *HOXA10* expression associates with CRPC progression and poor patient survival.

HOXA10 suppresses PCa cell growth and xenograft progression

Cell proliferation assays showed that gain of function of *HOXA10* inhibited and *HOXA10* depletion enhanced LNCaP cell proliferation rates independent to androgen depletion (Fig. 3A). Consistently, *HOXA10* also inhibited proliferation of androgen-independent LNCaP95 cells. Furthermore, *HOXA10* downregulated anchorage colony formation rates in both LNCaP and LNCaP95 cells (Fig. 3B). Using the LNCaP xenograft model, we showed that *HOXA10* inhibited tumor growth at not only the hormone-sensitive stage, but also the castrate-resistant stage (Fig. 3C). The CRPC stages were defined by the time of recurrence of serum PSA levels to the pre-castrate levels in mice. Immunoblotting assays confirmed *HOXA10*

Figure 1

HOXA10 expression in PCa patients and cell models. (A) LNCaP cells were stably transduced with HOXA10, 11 and 13 by lentivirus. Cell proliferation rates were measured using MTS assay and presented as relative fold change to that at day 0. (B) Affymetrix microarray (GSE6919 – Yu, *et al.* 2004) compared *HOXA10* mRNA levels among benign prostate, primary, and metastatic prostate tumors. (C) RNA-seq analyses (GSE35988 – Grasso *et al.* 2012, GSE21032 – Taylor *et al.* 2010) compared *HOXA10* mRNA levels among benign prostate, primary prostate tumors, and CRPC. One sided Welch's t-test is used to compare results among different groups (B and C). (D) RNA-seq data from TCGA provisional database were analyzed using cBioportal software (<http://www.cbioportal.org/>). Z-scores were used to divide PCa patients into *HOXA10* low and high groups. Kaplan-Meier curves plot overall survival and disease-free survival of PCa patients by *HOXA10* expression. Cox regression was used to calculate the hazard ratio (HR) of overall survival and disease-free survival between *HOXA10* low and high patient groups. (E) Multiple prostatic cell lines were used to measure *HOXA10* expression in both protein and mRNA levels by immunoblotting and real-time PCR assays. Data were presented as mean $\pm$ standard error (s.e.m.). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0465>.

expression in xenograft samples (Fig. 3D). Both IHC and luciferase results confirmed the suppressive effect of HOXA10 to PSA secretion (Fig. 3D and E). Ki-67 and cleaved caspase3 IHC staining indicated that HOXA10 inhibited tumor cell proliferation, but increase cellular apoptosis. These results indicate that HOXA10 inhibits PCa cell proliferation, colony formation, and xenograft growth.

HOXA10 regulates lipid metabolism in PCa cells

We have used Ampliseq to profile the HOXA10 transcriptome in PCa cells (Fig. 4). Under androgen depletion conditions, 1040 genes were upregulated and 621 genes were downregulated by HOXA10 (fold change >1.5 , $P < 0.05$) (Fig. 4A). In the presence of DHT, there were

738 genes upregulated and 611 genes downregulated by HOXA10. Because HOXA10 inhibited PCa cell proliferation independent to androgens, we focused on the 267 genes that were upregulated and the 178 genes that were downregulated by HOXA10 regardless of DHT treatment. DAVID 6.7 analyses showed that HOXA10-induced genes that have diverse functions such as cell morphogenesis and organ formation. The genes that were induced are consistent with how HOXA10 functions in AP axis positioning during development (Supplementary Fig. 1). However, HOXA10 suppressed genes are limited to genes that regulate lipid metabolism (Fig. 4A). These findings are supported by GSEA analyses showing that lipid metabolism gene sets are highly suppressed by HOXA10 ($P < 0.001$) (Fig. 4B). Additionally, androgen response genes were also inhibited (Fig. 4C). Together, these

Figure 2

Application of RISH assays to measure HOXA10 expression during PCa progression. (A) RISH assays were performed on PCa TMA using a HOXA10 specific probe as described in the Materials and methods section. Representative images of RISH staining are shown. (B) Distributions of HOXA10 RISH scores in benign prostate, primary PCa tumors and CRPC were plotted. Fisher's exact tests were performed to compare HOXA10 expression among the three groups. (C) The association of *HOXA10* expression with tumor Gleason groups was calculated by Spearman's correlation test. Data were presented as mean $\pm$ S.E.M. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0465>.

results revealed an association of HOXA10 with both cell lipid metabolism and the AR signaling.

It has been reported that several lipid biosynthetic genes are regulated by the AR signaling (Han *et al.* 2018). Among these genes, the *FASN* gene is the most significantly suppressed gene by HOXA10 defined by *P* values under both CSS and DHT condition (Fig. 4D). It had been demonstrated that AR can bind directly the *FASN* gene promoter to activate *FASN* transcription (Norris *et al.* 2009). AR can also activate *SCAP* gene transcription, which in turn stimulates SREBPs to induce a wide range of enzymes including *FASN* for *de novo* lipogenesis (Heemers *et al.* 2004; Fig. 4E). These findings demonstrate that HOXA10 is a repressor of lipid metabolism in PCa cells, partially through the inhibition of AR-mediated *FASN* expression. Our results were further supported by clinical data showing that HOXA10 mRNA levels are negative associated with both *FASN* and AR in both TCGA provisional and SU2C CRPC datasets (Fig. 4F).

To further validate the prediction of Ampliseq analyses, our real-time PCR and immunoblotting assays showed that HOXA10 inhibited *FASN*, but not *SCAP*

mRNA levels independent to androgens (Fig. 5A and B). Additionally, HOXA10 depletion in LNCaP cells resulted in increased *FASN* mRNA and protein levels (Fig. 5C and D). HOXA10 had no impact on SREBP1 expression, which supports the hypothesis that HOXA10 inhibition to *FASN* expression is not through the SCAP-SREBP1-*FASN* pathway. The inhibitory effects of HOXA10 to *FASN* have also been demonstrated by fatty acid synthesis assays using Oil red O staining (Fig. 5E). Furthermore, we found that the suppressive effects of HOXA10 to *FASN* expression were in AR positive LNCaP95 cells, but not AR negative PC3 and DU145 cells (Fig. 5F and G). This suggests that HOXA10 suppression of *FASN* transcription relies on the AR (Fig. 5F and G). Co-IP assays confirmed that both full-length AR and AR-v7 can form a protein complex with HOXA10 in LNCaP cells and 293T cell transfected with AR-v7 (Fig. 5H, I and J). ChIP assays demonstrated that HOXA10 prevents the recruitment of AR to the *FASN* and PSA promoters (Fig. 5K). Additionally, HOXA10 does not bind the regions next to the AREs of *FASN* and PSA gene promoters (Fig. 5L). These results indicate that HOXA10 suppresses *FASN* expression by forming a protein complex

Figure 3

HOXA10 inhibits PCa cell proliferation, colony formation and xenograft growth. (A and B) Gain- and loss-of-function of *HOXA10* were applied to LNCaP and LNCaP95 cells by lentivirus. LNCaP cells were also treated with vehicle or 10 nM DHT. Cell proliferation rates were measured by crystal violet assays and presented as relative fold change to that at day 0. Student *t*-test was used to compare the results from day 4 between CTL and HOXA10 treatments under either DHT or CSS conditions. (A). LNCaP and LNCaP95 cells were used to perform colony formation assays. Colony numbers were counted if their diameters were >100 μ m. Three independent biological replicates were performed (B). (C) LNCaP xenografts were established as described in the Materials and methods section. Tumor volumes and serum PSA concentrations were measured weekly and plotted. Student *t*-tests were used to compare tumor volume or PSA levels between CTL and HOXA10 groups. (D) LNCaP xenografts were collected at endpoint. IHC assays were performed to measure PSA, Ki-67 and Cleaved Caspase3 expression. Represented images were shown. Real-time PCR and immunoblotting assays confirmed HOXA10 expression in LNCaP xenografts. (E) LNCaP cells were co-transfected with PSA-luc and increased doses of HOXA10 expression plasmid for 24 h. Luciferase assays were performed as described in Materials and methods section. Data were presented as the mean $\pm$ standard deviation (s.d.) with * denotes $P < 0.05$. One-way ANOVA followed by Tukey test was used in pairwise comparisons among different groups. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0465>.

with AR and preventing AR from activating the *FASN* promoter activity.

The expression of *FASN* is upregulated in PCa

Reduced HOXA10 expression in CRPC (Figs 1 and 2) permits AR-mediated induction of *FASN* expression in PCa cells (Fig. 5). These findings are consistent to previous reports that *FASN* expression is upregulated in high-grade primary tumors and metastatic tumors (Rossi *et al.* 2003, Montgomery *et al.* 2008, Migita *et al.* 2009). To measure *FASN* protein expression in benign prostate and prostate tumors, we applied IHC assays on our CRPC TMAs.

Compared to benign prostate, *FASN* staining was higher in primary tumors and even further upregulated in CRPC (Fig. 6). The average H-score of *FASN* was 1.28 ± 0.216 in benign prostate, 1.96 ± 0.144 in primary tumors and 2.18 ± 0.206 in CRPC. Fisher's exact tests indicated that both primary and CRPC tumors expressed significantly higher *FASN* protein levels when compared to benign prostate ($P < 0.0001$) and that *FASN* protein levels were significantly higher in CRPC when compared to primary tumors ($P = 0.0294$). These results were similar to the public database (Taylor *et al.* 2010), which showed that *FASN* mRNA levels were upregulated in primary tumors and CRPC and that there was a trend of increased HOXA10

Figure 4

HOXA10 represses lipid metabolism genes and the AR signaling. (A) A Venn diagram summarized the Amplicon results comparing transcriptomes between LNCaP(CTL) and LNCaP(HOXA10) cells in the presence of $\pm$ DHT treatment (fold change >1.5 and $P < 0.05$). Top-ranked gene functions suppressed by HOXA10 were analyzed by DAVID software (version 6.8). Benjamini adjustment of P values were shown. (B and C) GSEA analyses showed negative correlations of HOXA10 with cellular lipid metabolism (B) and androgen response gene sets (C). (D) A volcano diagram showed P values of cellular lipid metabolism genes regulated by AR signaling describe in Han *et al.* (2018). (E) A schematic diagram summarized the mechanisms by which AR regulates FASN expression. (F) The correlation of FASN and AR expression with HOXA10 levels in PCa patients was calculated by using TCGA provisional and SU2C database. To dilute noise for expression correlation analyses between HOXA10 and AR or FASN, we ranked patients by expression values of HOXA10, divided patients into individual groups that each contain 20 and 8 neighboring patients from the TCGA and SU2C cohorts, respectively, calculate average expression value for each gene in each group, and finally determined the Pearson correlation coefficient between a pair of genes across individual patient groups. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0465>.

mRNA levels in CRPC in comparison to primary tumors (Fig. 6C). These results supported that increased FASN expression is associated with prostate cancer progression.

Discussion

This study demonstrates that downregulation of HOXA10 enhances lipogenesis through AR signaling in PCa cells to promote prostate cancer progression to the castrate-resistant stage. This HOXA10 function adds to its multiple roles in urogenital organ formation during development. We report that HOXA10 inhibits the expression of

lipogenesis genes including FASN, which is known for its role in accelerating cell proliferation as well as and energy generation and storage during CRPC progression. Mechanically, HOXA10 abolishes AR recruitment to the FASN promoter, thereby preventing AR from activating FASN gene transcription. Importantly, we confirm that HOXA10 downregulation and FASN upregulation are both correlated with CRPC, emphasizing that HOXA10 is a key regulator linking the AR signaling and lipogenesis in promoting tumor progression to CRPC.

HOX genes are key regulators of normal organogenesis. Therefore, a balanced HOX gene expression and function

Figure 5

HOXA10 represses FASN expression through the AR. (A and B) LNCaP(CTL) and LNCaP(HOXA10) cells were treated with either vehicle or 10 nM DHT. Real-time PCR and immunoblotting measured FASN and SCAP mRNA levels in (A) and FASN, AR, SREBP1, HOXA10 protein levels by immunoblotting in (B). (C and D) LNCaP(shCTL) and LNCaP(shHOXA10) cells were treated with either vehicle or 10 nM DHT. Real-time PCR and immunoblotting measured FASN and SCAP mRNA levels in (C) and FASN, AR, SREBP1, HOXA10 protein levels by immunoblotting in (D). (E) LNCaP(CTL), LNCaP(HOXA10), LNCaP(shCTL) and LNCaP(shHOXA10) cells were used for Oil red O staining. Neutral lipid levels were quantified using spectrophotometry with 510 nm wavelength. (F and G) LNCaP, LNCaP95, PC3 and DU145 cells were transfected with control or HOXA10 plasmid (F). HOXA10 expression was depleted in LNCaP and DU145 cells (G). FASN mRNA and protein levels were measured by Real-time PCR and immunoblotting assays, respectively. (H, I and J) Whole cell lysates were extracted from LNCaP (H), 293T cells transfected with AR-v7 plasmid (I), or LNCaP cells with HOXA10 knockdown (J) and were used to perform immunoprecipitation assays with either control IgG or an AR antibody. Precipitated proteins were immunoblotted with AR and HOXA10 antibodies. (K and L) LNCaP(CTL) and LNCaP(HOXA10) cells were treated with vehicle or DHT, and used to perform ChIP assays with the AR antibody (K) or the HOXA10 antibody (L). Eluted DNA fragments were used as templates for real-time qPCR to measure the enrichment of AR or HOXA10 to androgen response elements in FASN and PSA promoters. Signals were calculated as percentage of input. All results were derived from three independent experiments performed in triplicate. Data were presented as the mean $\pm$ s.d. Statistical analyses used one-way ANOVA test followed by Tukey test with $P < 0.05$ as * and $P < 0.01$ as **.

must be delicately controlled in both spatial and temporal manners. The 5' posterior cluster of *HOX* genes regulates prostate development by inducing mature morphogenesis and terminal differentiation of prostatic cells. Downregulation of these *HOX* genes in fully developed prostate is predicted to disrupt terminal differentiation and likely switch on cell proliferation. This hypothesis is consistent with our findings that enhanced HOXA10 expression suppresses PCa cell growth and tumor

progression. Previous studies have demonstrated that the posterior *HOX* gene, *HOXB13*, is also critical for prostate development (Economides & Capecchi 2003). Enhanced HOXB13 expression inhibits AR signaling, as well as PCa cell and xenograft growth (Jung *et al.* 2004). Similarly, HOXA10 had been demonstrated to be suppressive to estrogen receptor-negative breast cancer cells by blocking *TP53* gene expression (Chu *et al.* 2004). These findings support the idea that the dysregulation of *HOX* genes can

Figure 6

FASN protein expression in PCa. (A) IHC with the FASN antibody was performed on PCa TMA. IHC scores were evaluated as described in the Materials and methods section. Representative IHC images were presented. (B) Distribution of FASN IHC scores in benign, primary prostate tumor and CRPC groups were plotted. The primary tumor samples were first ranked by H-scores, then were divided into 4 groups with equal sample sizes. H-Score cutoffs were retrieved and applied to benign prostate and CRPC sample groups. Fisher's exact tests were used to compare FASN expression among the three tissue groups. (C) RNA-seq analyses (GSE21032 – Taylor *et al.* 2010) compared *FASN* mRNA levels among benign prostate, primary prostate tumors, and CRPC. One sided Welch's t-test is used to compare results among different groups. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-18-0465>.

promote tumor development and disease progression by regulating intracellular signaling.

Although the AR signaling plays an important role in driving the differentiation of luminal epithelial cells during prostate development, there is a paradigm shift of AR signaling away from maintaining cell differentiation in hormone naïve PCa (Wang *et al.* 2009) to accelerating cell cycling and mitosis in CRPC (Wang *et al.* 2009, Cai *et al.* 2011). This conclusion is demonstrated by several genome-wide profiling studies showing that not only the AR cistrome, but also the AR transcriptome is re-programmed to activate mitosis in CRPC (Wang *et al.* 2009, Cai *et al.* 2011). Accompanying the re-activation of AR signaling is the alteration of lipogenesis. Our results show that *FASN* is upregulated in CRPC, concurrent with the downregulation of *HOXA10*. *HOXA10* suppresses *FASN* transcription by interfering with AR functions. Therefore, *HOXA10* downregulation could contribute to CRPC progression by re-activating AR signaling and enhancing AR driven lipogenesis.

HOXA10 is a transcriptional factor that recognizes the 'TAAT' motif within the target promoters to control gene transcription (Gehring *et al.* 1994). The new finding from our study indicated that *HOXA10* can also form a protein complex with AR and indirectly regulate gene transcription through interfering AR transcriptional activity. Because *HOXA10* also inhibits the transcriptional activities mediated by AR-v7 and *HOXA10* do form a protein complex with AR-v7, it is likely that *HOXA10* associates with AR are at the AR DNA-binding domain or the N-terminus. *HOXA10* does not recognize the regions next to AREs in the *FASN* and *PSA* promoters, suggesting that the *HOXA10*-AR complex is not recruited to the AR regulated promoter.

We did not observe any negative associations between *HOXA10* RISH scores and *FASN* IHC scores from the same TMA. This discrepancy from our *in vitro* studies using cell models could be explained by: (1) *FASN* protein levels are regulated by multiple mechanisms at not only transcriptional (e.g. *HOXA10*

and AR), but also post-transcriptional and translational levels. A copy of HOXA10 mRNA can be translated into multiple copies of HOXA10 protein, which can result in discordance between HOXA10 RISH signal and FASN IHC signal; (2) compared to *in vitro* cell models, tumor microenvironments and the heterogeneous nature of cancer cells within patient tumors also make it challenging to establish associations between HOXA10 and FASN; (3) most importantly, the principles of IHC and RISH assays, and how the scoring methods used differ from each other. In contrast to the IHC signal that was scored by not only the intensity of IHC staining but also the percentile of positive stained cells, the intensity of RISH signal is indistinguishable among tumor cells. However, regardless of these factors that prevent us from establishing a negative correlation during statistical analyses on tissues, they did not preclude us from applying IHC and RISH signals to compare FASN and HOXA10 expression between primary PCa and CRPC, nor do they impede us from drawing the conclusion that the downregulation of HOXA10 and the upregulation of FASN are both associated with CRPC.

Conclusion

Reduced HOXA10 gene expression enhances lipogenesis through AR signaling in PCa cells and may promote prostate tumor progression to the castrate-resistant stage.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ethics approval and consent to participate

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of British Columbia. All patients had given informed consent to a protocol that was reviewed and approved by the UBC Clinical Research Ethics Board (Certificate #: H09-01628).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contribution statement

Concept and design: Dong X. Acquisition of data: Long Z, Li Y, Gan Y, Ning X, Zhao D. Analysis and interpretation of data: Long Z, Li Y. Drafting the manuscript: Li Y, Lovnicki J, Dong X. Critical revision of the manuscript: Dong X, Cao Q, Chen K. Statistically analysis: Li Y, Zhao D, Wang G. Obtain funding: Dong X. Administrative, technical or material support: Fazli L.

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