Genome-wide Analysis of the Homeobox C6 Transcriptional Network in Prostate Cancer

Colleen D. McCabe¹, Demetri D. Spyropoulos², David Martin¹,³, and Carlos S. Moreno¹,³

¹ Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322
² Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425
³ Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia

Abstract

Homeobox (HOX) transcription factors are developmentally regulated genes that play crucial roles in tissue patterning. Homeobox C6 (HOXC6) is overexpressed in prostate cancers and correlated with cancer progression, but the downstream targets of HOXC6 are largely unknown. We have performed genome-wide localization analysis to identify promoters bound by HOXC6 in prostate cancer cells. This analysis identified 468 reproducibly bound promoters whose associated genes are involved in functions such as cell proliferation and apoptosis. We have complemented these data with expression profiling of prostates from mice with homozygous disruption of the Hoxc6 gene to identify 31 direct regulatory target genes of HOXC6. We show that HOXC6 directly regulates expression of BMP7, FGFR2, IGFBP3, and PDGFRA in prostate cells and indirectly influences the Notch and Wnt signaling pathways in vivo. We further show that inhibition of PDGFRA reduces proliferation of prostate cancer cells, and that overexpression of HOXC6 can overcome the effects of PDGFRA inhibition. HOXC6 regulates genes with both oncogenic and tumor suppressor activities as well as several genes such as CD44 that are important for prostate branching morphogenesis and metastasis to the bone microenvironment.

Keywords

Prostate Cancer; Homeobox; HOXC6; Transcription; Systems Biology

INTRODUCTION

Homeobox (HOX) transcription factors are developmentally regulated genes that play crucial roles in tissue patterning (1). However, HOX gene expression has also been observed in many adult tissues with possible roles in cellular differentiation. Dysregulation of HOX genes has been observed in many human cancers including leukemias, and solid tumors of the breast, colon, lung, kidney, ovary, and prostate (2). HOX family member HOXC6 is expressed in osteosarcomas, medulloblastomas, as well as carcinomas of the breast, lung, and prostate, and is overexpressed in the LNCaP prostate cancer cell line (3). In a recent prostate cancer study,
HOXC6 was identified as the gene most strongly correlated with Gleason grade out of a newly identified sixteen gene signature (4).

Previously we have shown that siRNA knockdown of HOXC6 expression induces apoptosis and overexpression of HOXC6 results in increased proliferation and decreased apoptosis in LNCaP cells (5). This previous analysis identified T-cell receptor Alternate Reading Frame Protein (TARP), Insulin-like growth factor binding protein 3 (IGFBP3), and neutral endopeptidase/membrane metallo-endopeptidase (NEP/MME) as biologically relevant HOXC6 target genes due to their change in expression levels in prostate tumor compared to normal tissue as well as when HOXC6 levels were perturbed in LNCaP cells. However, to date few direct targets of HOXC6 are known and none have been identified in prostate cancer cells. Indeed there is evidence that HOX genes function differently in different tissues so targets identified in one cell type might not reflect the targets in another (6).

Here we have developed a comprehensive transcriptional network of genes under the control of HOXC6 in prostate cancer. We developed a stable LNCaP cell line that expresses epitope-tagged HOXC6-HA protein and used these cells in chromatin immunoprecipitation (ChIP) followed by microarray analysis (ChIP-chip). This analysis identified 468 reproducibly bound promoters whose associated genes are involved in functions such as cell proliferation and apoptosis. We correlated HOXC6 induced gene expression changes with gene promoters bound by HOXC6 and identified several key developmental ligands and receptors under direct transcriptional regulation by HOXC6. We further demonstrated the biological relevance of many identified direct target genes by performing gene expression profiling on mature prostates of Hoxc6−/− and Hoxc6+/+ mice. Bone Morphogenic Protein 7 (BMP7), Fibroblast Growth Factor Receptor 2 (FGFR2), and Platelet-derived Growth Factor Receptor α (PDGFRα), among others, were identified in these studies as in vivo direct regulatory targets of HOXC6 in prostate tissue. Along with elucidation of direct targets of HOXC6, we have also identified several biological pathways, including the Notch and Wnt signaling pathways that are indirectly affected by HOXC6 expression and are important in cancer progression. Additionally, several of the targets and pathways regulated by HOXC6 impact prostate duct branching morphogenesis and bone development, and may facilitate metastasis of prostate cancers to the bone microenvironment.

**MATERIALS AND METHODS**

**Lentiviral Vectors and Cell Culture**

An HA tag was inserted by restriction enzyme digestion onto the 3′ end of a HOXC6 cDNA clone. The resulting HOXC6-HA open reading frame was subcloned into pUBQ-IRES-eYFP-ΔU3 (a gift from Dr. Hihn Ly, Emory University) producing pUBQ-HOXC6-IRES-eYFP-ΔU3. The virus producing cell line, HEK293FT, was transfected with either empty vector or the HOXC6-HA containing construct along with viral packaging vectors, pVSVG (Clontech) and pCMV_R8.2. At 48 hours post-transfection, virus-containing supernatant was collected, filtered through a 45-micron filter and used to infect either LNCaP or RWPE-1 cells. Infected cells were isolated from non-infected cells by flow sorting for YFP expression at the Emory University flow core.

**Immunoprecipitation and Western Blot**

Cells and tissues were lysed by mechanical disruption in IP Lysis Buffer (20mM Tris pH 8.0, 10% glycerol, 137mM NaCl, 10mM EDTA, 1% NP40) and quantitated using Bradford Protein Assay (Biorad). Immunoprecipitations were performed using a mouse monoclonal antibody

1http://corelabs.emory.edu/fcm
to the 12CA5 HA tag or normal mouse IgG and protein-G sepharose beads. Immunoblots were probed with polyclonal antibodies to HOXC6 (5).

**Chromatin Immunoprecipitation**

Two 150mm dishes each of HOXC6-HA-LNCaP or -RWPE-1 stable and YFP-LNCaP or -RWPE-1 control cell lines were grown to 80% confluency. The cells were fixed with formaldehyde, lysed, and sonicated as previously described (7). Sonicated chromatin was immunoprecipitated with 12CA5 ascites or mouse IgG overnight and collected with Dynal M280 sheep anti-mouse IgG magnetic beads for 2 hours at 4°C. The beads were washed, complexes eluted, and the DNA was purified as previously described (7). Purified DNA was subjected to PCR using the primers in Supplementary Table 1.

**ChIP-chip data analysis**

DNA isolated from ChIP with 12CA5 mAb from the HOXC6-HA-LNCaP and YFP-LNCaP stable cell lines, along with input control, was amplified through linker-mediated PCR as previously described (8). Amplified DNA was labeled and hybridized to the Nimblegen 25K human promoter array, which tiles approximately 5kb upstream of 25,000 human transcribed sequences. The data from each hybridization was Z-score normalized and ratios of IP to input signal were determined. ChIPOTle analysis, which identifies peaks using a sliding window approach, was performed for each array (9). The window was set to the average shear length of the DNA, 500bp, and the step was set to the probe size, 50bp. The peaks of signal over the input DNA background identified anti-HA 12CA5 enriched DNA. Each cell line was analyzed in duplicate. Chip-chip data have been submitted to the GEO repository, accession number GSE9772.

**Luciferase Assays**

Promoters were amplified from human genomic DNA and directionally cloned into pGL3-basic (Promega) using restrictions sites incorporated into the primers. LNCaP cells were plated at 70% confluence and transfected using DMRIE-C (Invitrogen) with luciferase expression vector, Renilla expression control vector, and either pCDNA3.1 or pCDNA3.1-HOXC6 expression vectors. Forty-eight hours post-transfection cells were rinsed with 1xPBS followed lysis in Passive Lysis buffer (Promega). 50 μg of each lysate was used in a Dual-Luciferase assay according to the manufacturer (Promega), and normalized for transfection efficiency by Renilla light units. Fold change upon HOXC6 expression was computed relative to the pcDNA control and normalized to the fold change observed on the empty pGL3-basic vector.

**Mouse Tissue Collection and Genotyping**

All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at Emory University. C57Bl/6 Hoxc6 knockout mice are null for HOXC6 expression due to disruption of the Hoxc6 gene homeodomain (10). Hoxc6+/− and Hoxc6+/- male mice were generated from intercrosses of heterozygous parents. Mice were genotyped using PCR amplification from tail DNA as previously described (10). Prostates were dissected from Hoxc6+/− and Hoxc6+/- male mice at postnatal week 32 and immediately snap frozen in liquid nitrogen and stored at −80°C prior to RNA and protein isolation.

**Microarray Analysis**

Total RNA was extracted from three Hoxc6+/− and three Hoxc6+/- mouse prostates using RNeasy kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. All RNA samples were DNase treated (Rnase-free, Invitrogen) to eliminate residual genomic DNA. RNA was submitted to the Winship Cancer Institute Microarray Core Facility at Emory University, subjected to quality control analysis, labeled using the TotalPrep RNA labeling kit
(Ambion), and hybridized to the Illumina Mouse-6 BeadChip for analysis of 47,000 transcripts. After data normalization, genes with uniformly low expression (detection p-value > 0.05) were removed from consideration, leaving 23,386 probe sets for analysis using Significance Analysis of Microarrays (SAM 2.23) software (11). Relevant parameters for the SAM analysis were: two-class, unpaired data (wildtype and knockout prostates), Imputation engine, 10-Nearest Neighbor; Number of Permutations, 200; RNG Seed, 1234567; Delta, 1.345; Fold-Change, 1.7; False Discovery Rate < 1%. Microarray data are available at the GEO repository, accession numbers GSE9182 (Illumina) and GSE9207 (Affymetrix).

**Bioinformatic Analysis of Transcription Factor Binding Sites (TFBS)**

The in vivo PWM for HOXC6 we used THEME software (12) using the homeobox (PF00046) matrix as an initial hypothesis and the 21 confirmed peaks. Co-occurring TFBS were identified using CONFAC software\(^2\) which identifies transcription factor binding sites that are conserved between mouse and human sequences (13). A total of 515 promoter fragments that were bound by HOXC6 in LNCaP cells were analyzed together with 10 sets of 515 promoter sequences that were not bound by HOXC6 in LNCaP cells. Mann-Whitney tests were used to identify transcription factor binding sites that were significantly enriched in the HOXC6 bound fragments compared to the 10 random sets of fragments.

**QRT-PCR**

Total RNA was extracted from and treated as above. cDNA was synthesized by reverse transcription (Superscript III, Invitrogen), and transcript levels were quantitated by QRT-PCR using SYBR Green (Molecular Probes). Cycle threshold differences were determined using an I-cycler (Bio-Rad, Hercules, CA, USA) relative to 18s to control for total starting RNA levels in each prostate sample. All primers used in this analysis are listed in Supplementary Table 2.

**Apoptosis Assay**

1×10\(^4\) LNCaP cells were plated in a 96-well dish and allowed to adhere overnight. Cells were transiently transfected with pcDNA-HOXC6 expression vector or pcDNA empty vector using DMRI-C (Invitrogen) per the manufacturer’s directions. Twenty-four hours post-transfection cells were treated with 25 ng/ml recombinant BMP7 (Leinco Technologies) or PBS. At 72 hours post-treatment the Caspase 3/7 activity was measured using the Caspase 3/7 kit (Promega) and luminescence was read using a Bio-Tek 96-well plate reader luminometer.

**Proliferation Assay**

1×10\(^4\) LNCaP cells were plated in a 96-well dish and allowed to adhere overnight. Cells were transiently transfected with pcDNA-HOXC6 expression vector or pcDNA empty vector using DMRI-C (Invitrogen) per the manufacturer’s directions. 24 hours post-transfection cells were treated with 25 ng/ml recombinant BMP7 (Leinco Technologies), 5 μM Imatinib (a generous gift from Dr. Jing Chen) or vehicle. At 72 hours post-treatment 20 μl of 5 μg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma) was added to each well, incubated for 2 hours at 37°C, supernatant removed and precipitate was dissolved in DMSO and absorbance read at 570 nm.

**RESULTS**

**HOXC6-HA associates with the IGFBP3 and TARP promoters in LNCaP cells**

Although HOXC6 expression is upregulated in human prostate cancers, little is known about the role it plays in disease progression (3–5). To understand the molecular role that HOXC6

---

\(^2\)http://confac.emory.edu
plays in prostate cancer progression it is essential to identify its direct downstream targets. Because antibodies able to specifically immunoprecipitate HOXC6 are not available, we created HOXC6-HA expressing stable LNCaP cells that could be used in chromatin immunoprecipitation (ChIP) experiments. A bicistronic HOXC6-HAIRE-eyFP construct under an ubiquitin promoter was used in a lentiviral infection system to create LNCaP cells that stably express HOXC6-HA (Figure 1A). The ubiquitin promoter allows for low, physiological levels of constitutive HOXC6-HA expression. The infected cells were isolated from the non-infected cells using flow cytometry for yellow fluorescent protein (YFP) expression (Figure 1B). In addition, a control LNCaP cell line that stably expressed YFP alone was created (data not shown). The resulting stable cell lines were tested for expression by immunoprecipitation (IP) with monoclonal anti-HA 12CA5 antibodies followed by immunoblot with polyclonal anti-HOXC6 (Figure 1C).

To ensure the ability of the stable cell lines to identify HOXC6 bound promoters, ChIP experiments were performed for promoter regions of candidate HOXC6 target genes. We previously identified IGFBP3 as a repression target of HOXC6 and TARP as an activation target of HOXC6 (5). Primers were designed to amplify regions in the promoters of IGFBP3 and TARP that contain consensus HOX family binding sites (Supplementary Table 1). Enrichment was seen in the anti-HA sample from the HOXC6-HA stable LNCaP cells compared to IgG control IPs from these same cells. No enrichment was observed in anti-HA IPs from the control YFP LNCaP cell line (Figure 1D). Thus, IGFBP3 and TARP, whose transcript levels are altered upon perturbation of HOXC6 expression, are direct downstream targets of HOXC6 in LNCaP cells. Additionally, these results justify the use of stable LNCaP HOXC6-HA and YFP cell lines to globally identify HOXC6 bound promoters.

**Global identification of novel gene promoters bound by HOXC6**

To globally identify promoters bound by HOXC6 in LNCaP cells, genome-wide localization (ChIP-chip) was performed using the HOXC6-HA-LNCaP and YFP-LNCaP stable cell lines. Each ChIP sample was collected and analyzed in two independent replicates. Isolated DNA from each sample and total input DNA were amplified by ligation-mediated PCR. To identify the promoters bound by HOXC6, the anti-HA specific DNA and total input control DNA samples from the HOXC6-HA stable cells were hybridized to the Nimblegen 25k human promoter microarray set. The same analysis was performed using the anti-HA IP DNA sample and its total input control from the YFP alone stable cells to identify promoters that were enriched due to non-specific DNA that co-precipitated with the 12CA5 anti-HA antibody, thus eliminating false positive results.

We used ChIPOTle analysis software (9) to identify 514 reproducible peaks, representing 468 different probe sets, that were specific to the HOXC6-HA ChIP samples (Supplementary Table 3). To determine which annotated functional pathways were overrepresented by these 468 HOXC6 direct target genes, we performed an Ingenuity Pathways Analysis (IPA) to identify the canonical pathways and gene functions that are significantly enriched relative to random sets of genes. Interestingly, the 468 HOXC6 direct target genes were enriched for gene functions involved in apoptosis (p = 5.17E-5) and cellular development (p = 1.71E-4) (Table 1). This finding supports a possible role in prostate cancer progression for these newly identified transcriptional targets of HOXC6.

To validate the ChIP-chip data set, PCR primers (Supplementary Table 1) were designed to 24 genomic regions representing HOXC6-HA bound peaks and conventional ChIP-PCR was performed. The twenty-four peaks were chosen based on the function of their associated genes being relevant to cancer progression. Twenty-one of twenty-four peaks tested (87.5%) were reproducibly enriched by anti-HA 12CA5 ChIP in the HOXC6-HA cells compared to the YFP control (Figure 2A). Thirteen of twenty-four genes, including BMP7, FGFR2, HOXC6,
caspase recruitment domain family, member 8 (CARD8), caspase 1 (CASP1), and Runt-Related Transcription Factor 1 (RUNX1) were also confirmed as direct targets in RWPE-1 cells that stably express HOXC6-HA (Figure 2A). To quantitate the enrichment of HOXC6-HA anti-HA DNA over YFP anti-HA DNA in LNCaP cells quantitative real time PCR (QRT-PCR) was performed for seven peaks (Figure 2A). All seven promoters showed 2 to 7-fold enrichment over QRT-PCR from control YFP cells in LNCaP cells, and five of seven promoters were confirmed in RWPE-1 cells. These results verify the validity of the ChIP-chip analysis of HOXC6-HA bound promoters in LNCaP and RWPE-1 cells. Furthermore, they show that HOXC6 binds to its own promoter.

**HOXC6 activates expression from the PDGFRA and CARD8 promoters and represses expression from the CASP1 promoter**

To explore the ability of HOXC6 to regulate transcription from target gene promoters identified by ChIP-chip, we cloned the promoters of three genes whose HOXC6 bound peak was relatively close to the start of transcription, PDGFRA (1225 bp), CARD8 (736 bp), and CASP1 (1298 bp) into a luciferase expression vector. The cloned promoters, at a minimum, contained the region bound by HOXC6 in LNCaP cells as well as the transcription start site of the target gene. Co-expression of HOXC6 with PDGFRA and CARD8 reporter plasmids increased luciferase activity while coexpression of HOXC6 with CASP1 reporter plasmid decreased luciferase activity when compared to control transfections lacking HOXC6 (Figure 2B). The direct interaction of HOXC6 protein with these promoters as well as the ability of HOXC6 to regulate reporter expression from these promoters indicates that HOXC6 is functionally capable of regulating gene expression through direct promoter interaction in living cells.

**HOXC6 target promoters identify a novel in vivo position weight matrix**

The HOX family of transcription factors has little sequence specificity in vitro (14) which results in a position weight matrix (PWM) in the TRANSFAC database that is general to all HOX family members. To develop an in vivo PWM for HOXC6 we used THEME software (12). THEME uses a principled statistical method to test hypotheses about the binding site specificity of a transcription factor given a set of input sequences. We used the sequences for the 21 verified HOXC6 bound peaks as input for THEME analysis and the homeobox family PWM as an initial hypothesis.

To determine the significance of the THEME discovered motif an enrichment score, \(-\log_{10}(p\text{-value})\), and a cross-validation error (CV), a measure of how often THEME’s discovered motif correctly predicts binding sites in a subset of input sequences, were calculated. CV values less than 0.4 are considered significant. To determine the degree of similarity between the THEME discovered motif and the initial hypothesis a Beta score was calculated. The Beta score can range from 0–1 with higher values representing a motif more similar to the initial hypothesis. The most significant HOXC6 predicted PWM has an Enrichment score of 1.66 and a CV value of 0.32. This motif is highly different from the initial hypothesis with a Beta score of 0.1 by showing non-specificity at the 3rd position (Figure 2C). This analysis identified an in vivo derived PWM for HOXC6 and the first in vivo PWM available for any HOX family protein. Sequence analysis of the 468 promoters bound by HOXC6 determined that 263 (56%) contained matches to this motif.

**PDGFRA, FGFR2, and RUNX1 expression are significantly increased by HOXC6 overexpression in LNCaP cells**

To begin to address the functional role that HOXC6 plays when bound to its target promoters we cross-referenced our previously generated HOXC6 gene expression microarray data (5) with our ChIP-chip data (Supplementary Table 3). We identified 50 genes with bound promoters that were significantly altered in our previously reported gene expression data, five
of which were verified in ChIP-PCR experiments (Figure 2A and Supplementary Table 4). Expression of FGFR2, PDGFRα, and RUNX1 were upregulated while p21-activated kinase 2 (PAK2) and RAB9A were downregulated by HOXC6 overexpression in LNCaP cells. Upregulation of PDGFRα upon overexpression of HOXC6 is consistent with our luciferase reporter assays demonstrating HOXC6 activation of the PDGFRA promoter region (Figure 2B). While CARD8 and CASP1 did not demonstrate gene expression changes upon overexpression of HOXC6, reporter assays using their promoters demonstrated < 2-fold changes, suggesting a level of regulation by HOXC6 that may be below the level of detection of the microarray assay. Alternatively, in LNCaP cells the genomic promoters may be constrained from regulation by HOXC6 due to chromatin structure or cell-specific co-factor differences.

To identify potential DNA-binding co-factors that could be interacting with HOXC6 on its target gene promoters we performed CONFAC analysis. CONFAC identifies transcription factor binding sites that are conserved between mouse and human sequences (13). We input the 515 promoter fragments that were bound by HOXC6 in LNCaP cells. We also generated 10 sets of 515 promoter sequences that were not bound by HOXC6 in LNCaP cells and performed a similar analysis. Mann-Whitney tests were used to identify transcription factor binding sites that were significantly enriched in the HOXC6 bound fragments relative to at least 9 of the 10 random sets of sequences. This analysis identified 18 enriched position weight matrices (Figure 2D). The PWM identified several homeodomain sites and forkhead sites, suggesting that HOXC6 may interact with one or more forkhead transcription factors.

To determine if these targets are perturbed in human prostate cancers, publicly available microarray expression data were downloaded (15) and integrated with our own public microarray expression data (16) to identify genes significantly changed in localized and metastatic prostate cancers (q-value < 0.01). Of the 50 identified direct target genes of HOXC6, 33 have previously been shown to be altered in prostate cancer relative to benign prostate epithelium (Supplementary Table 4) including FGFR2, PDGFRα, Presenilin-1 (PSEN1), and RUNX1. These data suggest that HOXC6 directly regulates the expression of these genes in LNCaP cells and prostate tumors.

To identify biologically relevant targets of Hoxc6 in vivo, we utilized mice homozygous for a targeted disruption of Hoxc6 DNA binding domain (10). Prostates were isolated from three Hoxc6+/+ and three Hoxc6−/− mice at 32 weeks of age, assayed for Hoxc6 RNA and protein expression (Figure 3A and 3B) and subsequently used for whole-genome expression profiling. We identified 1213 genes with differential gene expression between these groups using SAM analysis (Figure 3C and Supplementary Table 3). Eight of these target genes were confirmed by QRT-PCR (Figure 3D). Comparison of microarray data from Hoxc6+/+ relative to Hoxc6−/− prostates revealed that expression of Pdgfra, Fgfr2, Cd44, Hey1, Wif1, and Dkk3 was upregulated while Bmp7 expression was downregulated.

Thirty-one genes that demonstrated mRNA expression changes in Hoxc6−/− compared to Hoxc6+/+ mouse prostates were also bound by HOXC6 in LNCaP cells (Table 2). Of these 31 genes, 21 were positively correlated with Hoxc6 expression and 10 were negatively correlated. We believe that this list of genes represent the most biologically relevant direct targets of HOXC6 in normal prostate tissue. Additionally, of those 31 direct targets of Hoxc6 in mouse prostate, 23 have previously been shown to be significantly altered in prostate cancers,

Gene expression changes upon knockout of HOXC6 in mouse prostate overlap with HOXC6 direct target genes identified in LNCaP cells

To identify biologically relevant targets of Hoxc6 in vivo, we utilized mice homozygous for a targeted disruption of Hoxc6 DNA binding domain (10). Prostates were isolated from three Hoxc6+/+ and three Hoxc6−/− mice at 32 weeks of age, assayed for Hoxc6 RNA and protein expression (Figure 3A and 3B) and subsequently used for whole-genome expression profiling. We identified 1213 genes with differential gene expression between these groups using SAM analysis (Figure 3C and Supplementary Table 3). Eight of these target genes were confirmed by QRT-PCR (Figure 3D). Comparison of microarray data from Hoxc6+/+ relative to Hoxc6−/− prostates revealed that expression of Pdgfra, Fgfr2, Cd44, Hey1, Wif1, and Dkk3 was upregulated while Bmp7 expression was downregulated.

Thirty-one genes that demonstrated mRNA expression changes in Hoxc6−/− compared to Hoxc6+/+ mouse prostates were also bound by HOXC6 in LNCaP cells (Table 2). Of these 31 genes, 21 were positively correlated with Hoxc6 expression and 10 were negatively correlated. We believe that this list of genes represent the most biologically relevant direct targets of HOXC6 in normal prostate tissue. Additionally, of those 31 direct targets of Hoxc6 in mouse prostate, 23 have previously been shown to be significantly altered in prostate cancers,
including FGFR2 and PDGFRA (Table 2), making them strong candidates for regulation by HOXC6 in prostate cancer progression. Furthermore, 7 of these 31 genes, including FGFR2 and PDGFRA, were also affected by overexpression of HOXC6 in LNCaP cells (Table 2).

**Recombinant BMP7 decreases proliferation and increases apoptosis in LNCaP cells**

Previously we reported that overexpression of HOXC6 leads to increased proliferation and decreased apoptosis in LNCaP cells (5). Because BMP7 is a downstream repression target of HOXC6, we predicted that treatment of LNCaP cells with exogenous recombinant BMP7 could prevent the proliferative and anti-apoptotic effects of HOXC6 overexpression. To test this prediction, we transiently transfected LNCaP cells with a HOXC6 expression vector and treated them with 25 ng/ml BMP7 or PBS vehicle for 72 hours. Reaching this post-treatment we observed that recombinant BMP7 eliminates the reduction in apoptosis and increased proliferation caused by exogenous HOXC6 expression, as expected (Figure 4A). These data suggest that one mechanism by which HOXC6 exerts effects on proliferation and apoptosis is through repression of BMP7.

**PDGFRA inhibition blocks the HOXC6-induced increase in prostate cancer cell proliferation**

Imatinib is a potent inhibitor of cell proliferation through inhibition of PDGFRA activity (17). Because PDGFRA is a downstream activation target of HOXC6, we predicted that overexpression of HOXC6 might overcome the anti-proliferative effects of Imatinib by increasing PDGFRA levels. To test this prediction, we treated LNCaP cells with 5 μM Imatinib with or without HOXC6 overexpression. After 72 hours of Imatinib treatment, LNCaP cells transfected with vector alone showed a significant decrease in proliferation (Figure 4B). In contrast, LNCaP cells transiently transfected with a HOXC6 expression vector and treated for 72 hours with 5 μM Imatinib showed no reduction in proliferation by Imatinib treatment. Interestingly, Imatinib treatment did block the proliferative effect of HOXC6 in LNCaP cells, suggesting that HOXC6 exerts its proliferative effect partially through PDGFRA (Figure 4B).

**HOXC6 directly and indirectly regulates multiple genes involved in cell proliferation, cell death, and tissue development**

To better understand the role that HOXC6 plays in prostate cancer progression, we performed Ingenuity Pathways Analysis (IPA) on those genes with at least two lines of evidence supporting their transcriptional regulation by HOXC6 in prostate cells (Supplementary Table 3). Those genes with promoters bound by ChIP experiments and whose expression is perturbed in *in vitro or in vivo* expression profiling experiments were considered direct targets of HOXC6. Those genes whose promoters were not bound in the ChIP experiments but whose expression was changed in both *in vitro* and *in vivo* expression profiling experiments were considered indirect targets of HOXC6. The 115 direct and indirect targets of HOXC6 were significantly enriched for cellular growth (p = 5.3e-9), apoptosis (p = 1.7e-6), development (p = 3.5e-6) and cancer (p = 8.3e-6) biological functions (Supplementary Table 5).

Some HOXC6 activation targets such as FGFR2 and CD44 have crucial roles in promoting prostate ductal branching morphogenesis (18,19). To determine if prostate ductal branching was also reduced in *Hoxc6*−/− mice, we microdissected mature prostates from *Hoxc6*−/− and *Hoxc6*+/+ mice at 12 weeks. Although we did not observe a statistically significant quantitative difference in the number of prostate branches between these two groups of mice, we did observe that prostates from *Hoxc6*−/− exhibited a variable phenotype of fewer and thicker ducts with some aborted branches (Supplementary Figure 1). The reduced penetrance of this phenotype may be due to compensatory activity from other HOX family members such as Hoxb6 (Supplementary Table 3), which is common among HOX transcription factors.
DISCUSSION

Here we have developed a comprehensive transcriptional network of genes under control of the developmental transcription factor HOXC6 in the mature prostate gland. Using a variety of complementary approaches, we have identified several direct targets of HOXC6, as well as a number of indirect targets that are affected by loss of HOXC6 in vivo. A major finding of this study is that several key developmental ligands and receptors are under direct regulation of HOXC6, including IGFBP3, BMP7, RUNX1, FGFR2, and PDGFRA. Increases in HOXC6 expression results in repression of IGFBP3 and BMP7 and activation of RUNX1, FGFR2, and PDGFRA.

IPA analysis of the direct and indirect targets of HOXC6 identified several pathways affected by changes in HOXC6 target genes expression level. The PI3K/Akt pro-proliferative pathway is regulated by the HOXC6 direct targets BMP7 (20), IGFBP3 (21), and PDGFRA (22) (Figure 4C). This suggests that one way HOXC6 may exert its pro-survival function is through the PI3K/Akt pathway. Moreover, our functional analysis of BMP7 and PDGFRA indicate that these genes are key effectors that mediate part of the proliferative and anti-apoptotic effects of HOXC6 in LNCaP cells.

Since HOXC6 expression increases with tumor aggressiveness and metastasis in human prostate cancers, one would expect that the downstream targets of HOXC6 would favor proliferation and inhibit differentiation. Targets such as IGFBP3, PDGFRA, RUNX1, and BMP7 do support this aspect of HOXC6’s role. For example, over-expression of RUNX1 accelerates cell cycle progression in a myeloid progenitor cell line (23). Our identification of these ligands as direct targets of HOXC6 suggests that their expression changes may work together to regulate the proliferative and anti-apoptotic effects of HOXC6.

Interestingly, there were also a number of tumor suppressor targets of HOXC6 activation that are believed to inhibit proliferation and promote differentiation. The most definitive of these targets is FGFR2 (18,24), which is directly activated by HOXC6 and whose expression is reported most often to be lost in prostate cancers (25). Other indirect tumor suppressive genes that were activated by HOXC6 included CD44 and four inhibitors of Wnt signaling: WIF, DKK3, SFRP1, and SFRP2. Importantly, although HOXC6 activates expression of these genes in normal mouse prostates, all six of these genes have been previously shown to be silenced by hypermethylation in tumors and cancer cell lines (26–31). Thus, epigenetic hypermethylation of these tumor suppressor genes via alternative mechanisms likely prevents their activation when HOXC6 is overexpressed in prostate cancers.

Many of the direct targets of HOXC6 have also been demonstrated to play roles in bone morphogenesis (Figure 4D), and metastatic prostate cancer is characterized by a strong predisposition for metastasis to the bone. BMP7 is a direct repression target of HOXC6 that has osteogenic properties that can produce ectopic bone formation (32). Importantly, BMP7 has recently been shown to inhibit growth of prostate cancer cells in mouse bone suggesting it plays a role in inhibiting prostate cancer bone metastases (33). Furthermore, BMP7 represses Notch signaling and Hey1 during prostate development (20). Thus, our results support a model in which HOXC6 directly represses BMP7, indirectly activating the Notch pathway and Hey1 expression.

HOXC6 direct target PDGFRA and indirect target CD44 have also been shown to regulate bone morphogenesis. PDGFRA is a direct HOXC6 activation target that is essential for normal neural crest, vertebrae, and rib development (34) and has been shown to be a significant predictor of tumor progression in osteosarcomas (35). PDGFRA expression is increased in prostate cancer bone metastases (36) and correlates with propensity of prostate cancer cell lines to metastasize to the bone (37). The HOXC6 indirect activation target CD44 is critical for
adhesion of prostate cancer cells to bone marrow endothelial cells (38). Moreover, CD44+
prostate cancer cells are enriched for metastatic and tumorigenic progenitor cells (39). Thus,
HOXC6 appears to affect multiple genes that influence metastasis of prostate cancers to the
bone microenvironment. HOXC6 may promote prostate tumor progression and metastasis to
the bone through repression of BMP7 and activation of PDGFRA, CD44, and Hey1.

Another method by which HOXC6 may be exerting its pro-survival phenotype is through
prostate ductal branching morphogenesis. HOXC6 activation targets FGFR2 (18) and CD44
(19) have demonstrated crucial roles in promoting prostate ductal branching morphogenesis
(Figure 4D). The HOXC6 repression target BMP7 (Figure 4D) has been shown to inhibit
branching morphogenesis and Notch signaling in the prostate (20). Notch signaling is crucial
for branching morphogenesis during prostate development (40). Hoxc6−/− mice have reduced
thoracic mammary gland branching and ductal development in newborn pups (10). Thus,
HOXC6 may promote branching morphogenesis by direct repression of BMP7, direct
induction of FGFR2, and indirect induction of CD44 and Notch signaling.

Taken together, these data suggest a model in which HOXC6 is a critical regulator of normal
prostate development, directly controlling expression of BMP7, FGFR2, and PDGFRA,
indirectly inhibiting Wnt signals and activating Notch signals. However, in prostate cancers,
epigenetic silencing of Wnt-suppressing target genes enables HOXC6 to activate Notch signals
without interfering with Wnt signaling. Our data show that the transcriptional network
regulated by HOXC6 plays crucial roles in proliferation, survival, and metastasis of prostate
cancer cells. Further studies will be required to determine whether HOXC6 represents a
potential therapeutic target in prostate cancer.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgements**

**Grant Support:** This research was supported by NCI R01 CA106826 to CSM. CDM was supported by DOD CDMRP
Prostate Cancer Postdoctoral Training Fellowship PC060114.

The authors wish to thank Dr. Maja Kodani in the WCI Microarray Core Facility, the Robert Karaffa at the Emory
University FlowCore, Dr. Hinh Ly for the IRES-eYFP lentiviral vector, Dr. Jing Chen for Imatinib, and the WCI
Transgenic Core Facility for help with prostate microdissections.

**References**


Figure 1.
Creation of stable cells expressing HOXC6-HA/YFP or YFP only. (A) Transient virus producing cell lines were created using a lentiviral vector modified with a bicistronic HOXC6-HA-IRES-eYFP expression cassette or IRES-eYFP alone. (B) YFP-based FACS sorting isolated near-pure populations of cells expressing the HOXC6-HA/YFP transgene. (C) Anti-HA antibody specifically immunoprecipitates HOXC6-HA. Lysates from LNCaP cells stably expressing HOXC6-HA/eYFP or eYFP alone were immunoprecipitated with anti-HA or anti-IgG and immunoblotted with HOXC6 antisera. (D) ChIP using anti-IgG or anti-HA were performed with stable LNCaP cell lines expressing HOXC6-HA/eYFP (HOXC6-HA) or eYFP alone (eYFP) followed by PCR using primers to the candidate target promoter regions of TARP (2 sets) and IGFBP3.
Figure 2.
(A) ChIP-ChIP experiments were ~86% (21/24) accurate as determined by conventional ChIP. PCR verification was performed with input DNA, water control, anti-HA ChIP DNA from LNCaP cells and RWPE-1 cells stably expressing HOXC6-HA/YFP (HOXC6-HA) or YFP alone. *Genes whose expression was changes by HOXC6 expression in transient expression assays. Quantitative Real-time PCR demonstrates fold enrichment of HOXC6 promoter targets in HOXC6-HA expressing cells versus YFP expressing cells. The results are consistent with what is observed using traditional PCR. (B) PDGFRA, CARD8, and CASP1 promoters were cloned into the pGL3-basic reporter vector and cotransfected in LNCaP cells with pDNA3.1 or pCDNA3.1-HOXC6. HOXC6 addition resulted in significant increase in luciferase reporter activity from the PDGFRA and CARD8 promoters and decrease from the CASP1 promoter compared to pCDNA3.1 vector alone. All fold changes were normalized to the HOXC6 effect on empty pGL3-basic vector. (C) Sequence for the 21 verified HOXC6 bound promoters were input for THEME analysis using the homeobox family position weight matrix as an initial
hypothesis. The most significant motif is pictured along with the cross-validation, significance, and Beta, similarity, scores. The HOXC6 predicted motif varies from the initial hypothesis by showing non-specificity at the 3rd position. (D) CONFAC Analysis of the 515 HOXC6 bound sequences compared to 10 random sets of 515 unbound sequences of similar size resulted in 18 enriched PWM. The significant PWM include homeodomain sites and forkhead protein sites.
Figure 3.
Three prostates from 32 week old Hoxc6−/− and Hoxc6+/+ mice were isolated and used for gene expression profiling. (A) RT-PCR for Hoxc6 demonstrates expression of Hoxc6 in each wildtype (WT) prostate and absence of expression in each knockout (MUT) prostate. (B) Western blot analysis demonstrates protein expression of HOXC6 in the WT mouse prostates and absence in the MUT mouse prostates. (C) Microarray analysis of each mouse prostate sample resulted in 1213 genes that were significantly differentially expressed between WT and MUT mice. (D) Quantitative RT-PCR verified gene expression changes for 8 genes in the WT vs. KO mouse prostates. Bmp7 demonstrates decreased expression in the WT vs. KO mice prostates while Pdgfra, Fgfr2, Gas2l3, Dkk3, Cd44, Hey1, and Wif1 demonstrate increased expression in WT vs. KO mouse prostates.
Figure 4.
(A) LNCaP cells were transiently transfected with empty vector of HOXC6 expression vector and treated with 25 ng/ml recombinant BMP7 or PBS vehicle. At 72 hours post treatment cells were tested for proliferation by MTT assay or apoptosis by Caspase 3/7 activation assay. Transient expression of HOXC6 results in significant increase in proliferation and decrease in apoptosis (* p<0.05 compared to vector + PBS) in LNCaP cells. Treatment of cells with recombinant BMP7 results in a significant increase in apoptosis and decrease in proliferation in LNCaP cells (* p<0.05 compared to Vector +PBS; ** p<0.05 compared to HOXC6 + PBS). BMP7 blocks the anti-apoptotic and pro-proliferative effect of increased HOXC6 levels. (B) LNCaP cells were transiently transfected with HOXC6 expression vector or vector alone and treated with 5 μM Imatinib or DMSO. At 72 hours cells were tested for proliferation by MTT assay. Imatinib largely inhibited the proliferative effects of HOXC6 transient expression in LNCaP cells (*p<0.05 compared to vector + DMSO; **p<0.05 compared to vector + Imatinib).
(C) Ingenuity Pathway Analysis for genes demonstrating at least 2 lines of evidence for direct or indirect regulation by HOXC6. A subset of the direct and indirect target genes of HOXC6 create a network that affects Proliferation and Survival through Akt, PI3K, and Caspase pathways. Asterisk (*) denotes direct transcriptional regulation from this study. (D) An overlapping subset of HOXC6 targets affects Bone Morphogenesis and Branching Morphogenesis. The upregulated and downregulated genes are highlighted in red and green respectively and the intensity of color reflects the level of expression change.
Table 1
GO Biological Processes Significantly Enriched in HOXC6 Direct targets

<table>
<thead>
<tr>
<th>Category</th>
<th>Process Annotation</th>
<th>Significance</th>
<th># Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>apoptosis of tumor cell lines</td>
<td>5.71E-05</td>
<td>31</td>
</tr>
<tr>
<td>Cellular Development</td>
<td>development of cells</td>
<td>1.71E-04</td>
<td>28</td>
</tr>
<tr>
<td>Cell Morphology</td>
<td>morphogenesis of cells</td>
<td>4.76E-04</td>
<td>16</td>
</tr>
<tr>
<td>Cellular Movement</td>
<td>cell movement</td>
<td>5.04E-04</td>
<td>36</td>
</tr>
<tr>
<td>Tissue Development</td>
<td>developmental process of tissue</td>
<td>3.34E-03</td>
<td>21</td>
</tr>
</tbody>
</table>
### Table 2

HOXC6 Direct Targets affected by loss of Hoxc6 expression in normal mouse prostates. Also shown are genes affected by overexpression of HOXC6 in LNCaP cells. HOXC6−/− FC represents the fold change of normal mouse prostates lacking HOXC6 relative to wt prostates, and thus, HOXC6 activation targets show negative fold changes for that column. Affymetrix HOXC6 FC indicates fold change from overexpression of HOXC6 in LNCaP cells, and thus, HOXC6 activation targets have positive fold changes in that column. Those genes with concordant effects in mouse prostate and LNCaP cells are shown in **bold.** Also indicated are microarray expression data from oncomine and our own previous data (16) indicating studies that showed changes in these genes in localized and metastatic prostate cancers.

<table>
<thead>
<tr>
<th>Hs Symbol</th>
<th>Hs Entrez ID</th>
<th>Mm Entrez ID</th>
<th>Illumina Probe ID</th>
<th>HOXC6−/− FC</th>
<th>Affymetrix HOXC6 FC</th>
<th>Up in PCa*</th>
<th>Up in Mets*</th>
<th>Down in PCa*</th>
<th>Down in Mets*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHRNA2</td>
<td>1135</td>
<td>110902</td>
<td>5050315</td>
<td>6.58</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMELY</td>
<td>266</td>
<td>11832</td>
<td>2940722</td>
<td>3.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDE4DIP</td>
<td>9639</td>
<td>83679</td>
<td>4730131</td>
<td>2.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCDO2</td>
<td>83875</td>
<td>170752</td>
<td>4560279</td>
<td>2.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP7</td>
<td>655</td>
<td>12162</td>
<td>100360181</td>
<td>2.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLClA3</td>
<td>6507</td>
<td>20512</td>
<td>1500070</td>
<td>2.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEPT7</td>
<td>989</td>
<td>235072</td>
<td>105570062</td>
<td>1.93</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIPA1L1</td>
<td>26037</td>
<td>217692</td>
<td>50292</td>
<td>1.92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRAK4</td>
<td>51135</td>
<td>266632</td>
<td>6130594</td>
<td>1.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFXDC2</td>
<td>64864</td>
<td>319758</td>
<td>10011095</td>
<td>1.75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERRF1</td>
<td>54206</td>
<td>74155</td>
<td>3450273</td>
<td>–6.65</td>
<td></td>
<td>LV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAM49A</td>
<td>81553</td>
<td>76820</td>
<td>102190458</td>
<td>–1.76</td>
<td></td>
<td>LV</td>
<td>LY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SENP8</td>
<td>123228</td>
<td>71599</td>
<td>2350184</td>
<td>–1.82</td>
<td></td>
<td>V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCN</td>
<td>1634</td>
<td>13179</td>
<td>106110022</td>
<td>–1.82</td>
<td></td>
<td>V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH3D19</td>
<td>152503</td>
<td>27059</td>
<td>6020520</td>
<td>2.49</td>
<td></td>
<td>L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZBTB7C</td>
<td>201501</td>
<td>207259</td>
<td>2970609</td>
<td>–1.86</td>
<td></td>
<td>V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGFRα</td>
<td>5156</td>
<td>18595</td>
<td>104670375</td>
<td>–1.87</td>
<td></td>
<td>Y</td>
<td>MV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pak1</td>
<td>5058</td>
<td>18479</td>
<td>4540315</td>
<td>–1.88</td>
<td></td>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF2</td>
<td>2263</td>
<td>14183</td>
<td>6940377</td>
<td>–1.9</td>
<td></td>
<td>L</td>
<td>L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC4A41</td>
<td>23446</td>
<td>100434</td>
<td>2510164</td>
<td>–1.96</td>
<td></td>
<td>L</td>
<td>L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APBB1</td>
<td>322</td>
<td>11785</td>
<td>104070044</td>
<td>–2.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL23A1</td>
<td>91522</td>
<td>237759</td>
<td>2003058</td>
<td>–2.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFAIP8</td>
<td>25816</td>
<td>106869</td>
<td>2760551</td>
<td>–2.08</td>
<td></td>
<td>Y</td>
<td>L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCAM1</td>
<td>7412</td>
<td>22329</td>
<td>101580309</td>
<td>–2.11</td>
<td></td>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLXDC1</td>
<td>57125</td>
<td>72324</td>
<td>540132</td>
<td>–2.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAS2L3</td>
<td>283431</td>
<td>237436</td>
<td>105703979</td>
<td>–2.47</td>
<td></td>
<td>VY</td>
<td>DY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZNF536</td>
<td>9745</td>
<td>243937</td>
<td>4920008</td>
<td>–2.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14orf57</td>
<td>145407</td>
<td>67419</td>
<td>5860195</td>
<td>–2.71</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAM101A</td>
<td>144347</td>
<td>73121</td>
<td>5570040</td>
<td>–2.94</td>
<td></td>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNT1</td>
<td>1272</td>
<td>12805</td>
<td>3800204</td>
<td>–3.15</td>
<td></td>
<td>LMW</td>
<td>V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEGF10</td>
<td>84466</td>
<td>70417</td>
<td>101990463</td>
<td>–3.71</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

* Key
D = Dhanasekaran (41)
T = Tomlins (42)
L = LaPointe (43)
V = Varambally (44)
M = Liu (16)