Aberrant HOXC Expression Accompanies the Malignant Phenotype in Human Prostate

Gary J. Miller, Heidi L. Miller, Adrie van Bokhoven, James R. Lambert, Priya N. Werahera, Osvaldo Schirripa, M. Scott Lucia, and Steven K. Nordeen

Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado 80262

ABSTRACT

Dysregulation of HOX gene expression has been implicated as a factor in malignancies for a number of years. However, no consensus has emerged regarding specific causative genes. Using a degenerate reverse transcription-PCR technique, we show up-regulation of genes from the HOX cluster in malignant prostate cell lines and lymph node metastases. When relative expression levels of the four HOX clusters were examined, lymph node metastases and cell lines derived from lymph node metastases exhibited very similar patterns, patterns distinct from those in benign cells or malignant cell lines derived from other tumor sites. Specific reverse transcription-PCR for HOX4, HOX5, HOX6, and HOX8 confirmed overexpression of these genes in malignant cell lines and lymph node metastases. Laser capture microdissection and examination of paired tumor/normal prostate epithelial cells also indicated overexpression of these HOX genes in primary tumor cells. Our data indicate a possible link between expression of HOXC genes and malignancy in prostate cells. Overexpression of HOX8 in LNCaP prostate cancer cells suppressed transactivation by androgen receptors. We speculate that HOX overexpression may predispose tumor cells to androgen independence by necessitating adaptation to diminished androgen signaling.

INTRODUCTION

Prostate cancer is the most common male cancer and the second leading cause of male cancer deaths in the United States (1). Autopsy data indicate nearly 30% of men 30–39 years old have small foci of carcinoma in their prostate (2), and it is believed nearly all men develop at least one small focus with age (3). Thus, although more men die with prostate cancer than of it, progression of the cancer in an individual usually results in a poor outcome. Metastasis to a site other than prostate has long been a problem poorly understood and usually leading to shortened life span. Although relatively little is known of the underlying molecular events that lead to progression of a cancer, clearly cellular functions such as proliferation, cell death, motility, signal transduction, and microenvironment interactions must play a role. The homeobox superfamily of genes and the HOX subfamily contain members that are transcription factors involved in controlling and coordinating complex functions during development via spatial and temporal expression patterns. There are growing lines of evidence that dysregulation of HOX gene expression plays important roles in cancer (see Refs. 4–6 for review).

Dysregulation of a variety of HOX genes has been implicated in several human cancers including leukemias (7–11); colorectal (12), breast (13, 14), and renal (15) carcinomas; melanomas (16); and squamous carcinomas of the skin (17). Because the genes implicated show little consensus, the dysregulation may be a tissue-specific perturbation of the existing HOX expression pattern rather than a single causative gene. Tissue-specific expression patterns have been reported in kidney and colon, by Northern blot analysis (12, 15). Primary tumors in both kidney and colon showed variations in specific HOX gene expression from the corresponding normal tissue, but overall expression patterns for individual tumors were not reported. Only primary kidney tumors were examined (15), but liver metastases from colon tumors reportedly displayed expression of specific HOX genes similar to that seen in either primary colon tumors or normal colon but not in normal liver (12). Translocations involving several HOXA, HOXC, and HOXD genes and the NUP98 nucleoplin gene have been reported in hematopoietic malignancies (11, 18–22); however, translocations have not been reported for solid tumors.

In humans, HOX genes comprise the largest of several families of genes containing a sequence motif termed the homeobox. The homeobox was first identified as a motif shared among the Drosophila homeotic genes. HOX genes represent their human counterpart. HOX genes are highly conserved across a very broad range of animal organisms from Caenorhabditis elegans and Drosophila through humans. A uniform nomenclature for vertebrates was established in 1992 using naming conventions with uppercase letters for human genes (HOXAI) and lowercase letters for mouse genes [Hoxa1 (23)]. In humans, the 39 members of the HOX family are organized into four clusters (A–D) on chromosomes 7, 17, 12, and 2, respectively, which contain between 2 and 4 members. The sequence similarities, as well as overlapping expression in developing embryos (25), have suggested possible functional redundancy. Gene disruption studies in mice indicate some degree of functional cooperation during development (25, 26), and these studies are being extended into adult tissues (27). However, the relationships between paralogs appear complex, and the relative importance of individual HOX genes is currently unknown. Indeed, evidence is accumulating that the same HOX gene functions differently in different tissues (28, 29) and that distinct portions of the protein structure are important for this specificity (30). This may also prove to be the case between developing and adult tissues.

Very little has been published on HOX gene expression in human prostate. Detectable expression of HOXD10 and HOXA9 RNA was reported in normal prostate (31). Regulation of two unidentified homeobox-containing genes by extracellular matrix and androgens in prostatic carcinoma cells has been reported (32). One recent publication correlated HOXC8 expression with higher Gleason grades in prostate tumors (33). A divergent homeobox gene, NKX3.1, is also expressed in normal human prostate and has been implicated as a tumor suppressor gene (34). This conclusion is bolstered by data from

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3 Present address: Department of Clinical Cancer Genetics, City of Hope National Cancer Center, Duarte, CA 91010.

4 To whom requests for reprints should be addressed, at Department of Pathology, B216, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262. E-mail: steve.nordeen@uchsc.edu.
mice with targeted disruption of the Nkx3.1 gene, in which age-
dependent hyperplastic and dysplastic lesions resembling prostate
intraepithelial neoplasia are seen (35–37). Our laboratory has recently
completed a large screen of expression patterns of the HOX family of
genes in benign and malignant prostate cells in vitro, as well as in
benign and malignant tissue specimens, to determine whether shifts in
relative expression levels might provide evidence of a role for HOX
genes in prostate cancer development or progression. Our findings
indicate changes in HOX gene expression patterns with malignancy and,
in particular, an up-regulation of HOXC cluster genes. In particu-
lar, lymph node metastases present a distinct pattern of HOX expres-
sion. Surprisingly, this pattern is maintained in established cell lines
derived from lymph node metastases. Finally, we also show that
overexpression of one of the HOXC genes, HOXC8, suppresses an-
drogen-dependent transcription in prostate cancer cells, suggesting
that dysregulation of HOX gene expression has important conse-
quences in prostate cancer.

MATERIALS AND METHODS

Cell Lines and Culture. The human prostate cancer cell lines were ob-
tained from the following laboratories: LAPC-4 (38), Dr. C. Sawyers (Uni-
versity of California at Los Angeles, Los Angeles, CA); LNCaP (39, 40), Dr.
J. Horoszewicz (Roswell Park Memorial Institute, Buffalo, NY); ALVA-31
(41), Dr. S. Loop (American Lake VAMC, Tacoma, WA); PCC-1 (42), Dr. A.
Brothman (University of Utah, Salt Lake City, UT), 22Rv1 (43), Dr. J.
Jacobsberger (Case Western Reserve University, Cleveland, OH); and PC-346C
(44), Dr. W. van Weerden (Erasmus University, Rotterdam, the Netherlands).
The cell lines PC-3 (45) and DU 145 (46) were obtained from the American
Type Culture Collection (Manassas, VA). Stromal cells were from primary
cultures established from BPH1 nodules obtained from radical prostatectomy
specimens. Primary cultures of PrECs from three independent origins were
obtained from BioWhittaker (San Diego, CA) and grown in PrEGM supple-
manted as per the manufacturer’s instructions. LAPC-4 cells were grown in
Iscove’s modified Dulbecco’s medium (Invitrogen, Carlsbad, CA) with 7.5%
FBS (Omega Scientific, Tarzana, CA) and 10−8 M mithromelienolone (R1881;
Perkin-Elmer Life Sciences, Boston, MA). PC-346C cells were grown in
DMEM/Ham’s F-12 plus 10% FBS (HyClone, Logan, UT). All other cell lines
were grown in RPMI 1640 (Invitrogen) plus 10% FBS (HyClone). The authen-
ticity of each was verified by comparison with the original karyotype as well
as by DNA profiling using the AMPF/STR Profiling Plus Kit [Applied Bio-
systems, Foster City, CA (47)]. While this work was in progress, ALVA-31
and PCC-1 were shown by our laboratory to be derivatives of PC-3 (48).

Tissue Samples. Samples were snap frozen in liquid nitrogen immediately
after procurement and stored there until harvest of RNA. Normal prostate
tissue was obtained from organ donors of ages 1.5, 13, 15, 18, 20, 44, and 61
years. BPH specimens were obtained from patients undergoing radical pro-
statectomy. Lymph node metastases were obtained from two patients with
primary prostate tumors. Highly epithelial areas, devoid of inflammation, were
selected from all tissue used for whole tissue RNA extraction after examination
of H&E-stained sections. Prostate tissue containing normal and malignant
epithelium was obtained from two radical prostatectomy patients and one
research organ donor.

LCM. LCM was performed using a PixCell II System (Archerus Engi-
neering, Inc., Mountain View, CA) on 8-μm-thick frozen sections of normal
prostate from one prepubertal (1.5 year), two pubertal (13 and 15 years), and
two adult specimens (18 and 20 years) to separately capture epithelial and
stromal cells for RNA isolation. Eight micrometer frozen sections were also
used to capture the paired tumor/normal epithelium samples.
The cryostat blade was wiped with RNase Zap (Ambion, Austin, TX) before
each specimen was cut, and solutions were made with RNase-free water.

5 The abbreviations used are: BPH, benign prostatic hyperplasia; FBS, fetal bovine
serum; FISH, fluorescence in situ hybridization; LCM, laser capture microdissection;
PrEC, benign prostate epithelial cell; RT, reverse transcription; RT-PCR, reverse tran-
scription-PCR; PSA, prostate-specific antigen; dNTP, deoxynucleotide triphosphate;
PAC, PI-derived artificial chromosome; CBP, cAMP-responsive element binding protein
(CREB)-binding protein.

Tissue sections were fixed in 70% ethanol for 30 s, washed in water for 10 s,
and then stained in Gill’s hematoxylin (Electron Microscopy Sciences, Fort
Washington, PA) for 1–2 min. After another water wash, they were put in 1%
ammonia for 30–60 s and then in 70% and 95% ethanol washes for 10 s each.
After staining in eosin for 1–2 min, they were dehydrated in 95% and 100%
ethanol washes for 10 s each, washed in xylene for 5–10 min, and then air dried
for 60 min before capture of cells.

RNA Isolation. Total RNA was isolated by the guanidinium isothiocya-
nate/acid phenol method of Chomczynski and Sacchi (49), with minor modi-
fications.

Cultured cells were grown to 70–80% confluence before harvest. Briefly,
cells were scraped from the flasks, washed in PBS, and then lysed in guani-
dinium isothiocyanate buffer. After extraction with acid-phenol, the aqueous
layer was precipitated overnight with an equal volume of isopropanol. The
precipitate was dissolved in guanidinium buffer and reprecipitated overnight
with isopropanol. The pellet was washed twice with 75% ethanol and then
dried briefly before resuspending in diethylpyrocarbonate-treated H2O.

Frozen tissue was weighed and manually pulverized before addition of the
appropriate volume of guanidinium buffer. Samples were homogenized using
a Polytron 2000 (Brinkmann Instruments Inc., Westbury, NY). RNA isolation
was as performed for cell cultures with the addition of a second phenol
extraction step. Analysis of RNA was done by 260/280 absorbance ratio and
electrophoresis on a nondenaturing 1% agarose gel.

Laser-captured specimens were extracted with additional slight modifica-
tions. Two hundred μl of guanidinium extraction buffer containing 1.6 μl of β-
mercaptoethanol per CapSure prep (Arcturus Engineering, Inc.) were used
with 2-min tissue lysis times. Centrifugation times were 30 min each. One μl of
glycogen (10 μg/μl in water) was added to each sample before precipitation in
isopropanol at −20°C overnight followed by one 75% ethanol wash. RNA was
redissolved in diethylpyrocarbonate-treated water and used for reverse
transcription in volumes used for 1 μg of RNA.

RT and PCR. RT was preceded by treatment with DNase I (Invitrogen) as per
the manufacturer’s instructions. For each microgram of RNA, the volume
was adjusted to 20 μl containing 2.5 μM random hexamers, dNTPs (1 mM
each), 1 unit of RNasin (Promega, Madison, WI), and 1 × RT buffer (Prom-
ega). A mock RT sample of 10 μl was removed, and 50 μl of Moloney murine
leukemia virus reverse transcriptase (Promega) were added to the
actual RT tube. Samples were incubated at 22°C for 10 min, 42°C for 45 min,
95°C for 5 min, and 4°C for 5 min. The equivalent of 250 ng of starting RNA
from the RT or mock sample were used for each degenerate PCR reaction.

Degenerate PCR primers corresponded to the conserved peptide motifs ELE-
KEF and KIWFQN. Sequences of all primers used are listed in Table 1. Degenerate
PCR reaction volumes were 25 μl each, containing the following:
template or mock; 2 μM of each primer; 200 μM each dNTP; 4.5 mM MgCl2;
and 0.625 unit of Taq polymerase (Promega) in 1 × reaction buffer. Cycle
number was 32 for the degenerate primers. PCR products were electrophoreted
on gels of 2% NuSieve 3:1 agarose (BioWhittaker Molecular Applications,
Rockland, ME), and the appropriate size band was cut out and extracted with
QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). All PCR assays included
negative controls using mock RT templates as well as a water template sample.
Only samples showing no PCR amplicons in the corresponding mock tubes
were used for subsequent cloning and sequencing.

Amplification volumes for nondegenerate RT-PCR reactions were 25 μl
containing template (equivalent to 100 ng of starting RNA), 200 μM each
dNTP; 200 nM each primer, 0.625 unit of Taq polymerase (Promega), and
1.5–2.5 mM MgCl2 (optimized for each primer pair) in 1 × reaction buffer (see
Table 1). Specific HOXC reactions with tissue templates ran for 35 cycles,
whereas those from cell line templates ran for 32 cycles. Reactions for β-actin
were cycled 30 times, and reactions for PSA were cycled 32 times.

Cloning and Sequencing. Purified PCR products were cloned using a
TOPO TA Cloning Kit (Invitrogen) as per the manufacturer’s instructions.
After overnight incubation of plates, colonies were selected for each sample
and checked for presence of the correct-sized insert by direct PCR with the
degenerate primers. Bacterial cultures were grown overnight, and plasmids
were isolated using QIAwell 8 Ultra Kits (Qiagen). Sequencing was done by
the University of Colorado Cancer Center DNA Sequencing Core using
dye-terminator chemistry on ABI 373A and ABI 377 automated sequencing
machines (Applied Biosystems). Resulting sequences were compared with the

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nonredundant nucleotide database and the SwissProt protein database of GenBank using the BLAST search algorithm (50).

**RNA Amplification.** RNA from LCM-captured malignant and normal epithelial cells was amplified through two rounds using the RiboAmp RNA Amplification Kit (Arcturus Engineering, Inc.) as per the manufacturer’s instructions. Optional maximum times for the in vitro transcription incubations were used in both rounds to increase yield. Amplified RNA was subjected to an additional DNase I treatment before RT for RT-PCR.

**FISH.** FISH analysis was performed by the Cytogenetics Core Laboratory of the University of Colorado Cancer Center. Cells were blocked in metaphase with Colcemid (0.05 μg/ml) for 1–2 h before hypotonic swelling in a 4:1 mixture of 0.075 M KCl and 1% sodium citrate. Fixation was done using a 3:1 mixture of methanol and glacial acetic acid. Two PAC clones that map to 12q12–14 were used as probes; one contains the complete HOX cluster, and the other contains the vitamin D receptor gene, which is located proximal to the HOX cluster. The probes were labeled using the Vysis nick translation kit (Vysis, Downers Grove, IL). The HOX PAC was labeled with dUTP Spec-trumGreen, and the vitamin D receptor PAC was labeled with dUTP-Specific Orange (Vysis, Downers Grove, IL). The probes were denatured for 10 min in 1% formaldehyde at room temperature for 10 min. Two hundred ng of each probe DNA, dissolved in 50% formamide hybridization solution levels within each specimen. A summary of the raw data collected is presented in Table 2. Divergence in the conserved sequences in the homeodomain prevented detection of eight of the HOX genes (HOXA1, HOXA3, HOXB13, HOXC11, HOXC12, HOXD11, and HOXD13). Three HOX genes (HOXB1, HOXB2, and HOXD3) were not detected by the degenerate primers in any of these samples, despite retention of the conserved sequences and theoretical match with the primers. A total of 548 HOX inserts from benign sources and 342 HOX inserts from malignant sources were identified from cell lines, whole tissue, and LCM-captured epithelium and stroma.

We first examined overall expression patterns of the four HOX clusters (A–D) in the broad categories of benign and malignant epithelium (Fig. 1). Benign stroma, from laser capture-microdissected specimens, is shown for comparison because whole tissue specimens, by definition, contained some stromal components. However, the highly epithelial areas selected for extraction were approximately 2:1 epithelial to stromal cells. We noted that less total RNA was isolated from stromal cells and that overall HOX expression seemed lower from stromal samples (data not shown). We thus felt the overall contribution of stromal expression in whole tissue samples to be minimal. Additionally, when we examined epithelium and stroma separately, we found the overall expression patterns to be remarkably similar. The malignant epithelial pattern shows definite changes in the relative expression of the HOXA, HOXB, HOXC, and HOXD clusters when compared with the benign expression pattern (P < 0.001). Benign epithelial cells showed approximately 70% of the HOX genes expressed to be from the A cluster, with virtually no expression of C cluster genes. However, malignant cells expressed A and C cluster genes at approximately equal rates (roughly 40% of total). Malignant
epithelium also showed a decrease in expression of the D cluster compared with benign epithelium \( (P < 0.001) \), and particularly HOXD10 \( (P < 0.05) \).

As can be seen in Fig. 2, the change in relative expression level of A and C cluster genes is not simply the result of substitution of HOXC paralogs for HOXA paralogs. The increased expression in the C cluster is concentrated in C4, C5, and C6, although C8, C9, and C10 are also expressed, whereas the main decrease in the A cluster appears to be in A9 and A10.

**Hox Expression Patterns in Prostate Cell Lines.** Because the differences between benign and malignant Hox expression patterns were quite clear, in view of the heterogeneous nature of prostate cancer, we decided to examine subsets of the malignant cell lines. Based on originating tumor location, the malignant prostate cell lines could be divided into three groups. LNCaP and LAPC-4 are derived from lymph node metastases. 22R\textsuperscript{v}1 and PC-346C are derived from primary prostate tumors xenografted into mice and then established as cell lines. PC-3 and DU 145 are both metastases from other sites, specifically bone and brain, respectively (because ALVA-31 and PPC-1 are clonal derivatives of PC-3 and not unique cell lines, we have excluded them from this analysis). For statistical analyses, we chose to group the cell lines into the three pairs mentioned above to compare expression patterns in lymph node metastasis-derived cell lines, primary tumor-derived cell lines, and cell lines from other metastatic sites. Fig. 3 shows expression of the four Hox clusters in these categories and in lymph node metastases. Interestingly, breaking out the malignant subgroups revealed that the patterns of relative expression of the Hox clusters demonstrated by lymph node metastases and prostate cell lines derived from lymph node metastases were very similar. In contrast, the relative expression of the HoxB and HoxC clusters in lymph node-derived cell lines differed significantly \( (P < 0.001) \) from that observed in cell lines derived from primary tumors (22R\textsuperscript{v}1 and PC-346C) or other metastatic sites (PC-3 and DU 145).

**Table 2. Number of various Hox genes identified from different sample types**

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<th>Malignant</th>
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**Fig. 1. Relative expression of the four Hox clusters (A–D) in benign and malignant cell types.** Benign epithelium (ben.epl) includes PrECs, normal prostate tissue, and LCM epithelium. Benign stroma (ben.str.) includes LCM stroma. Malignant epithelium (malign.epl) includes eight malignant cell lines and lymph node metastases. Error bars represent 95% confidence intervals for sample sizes. Differences in relative expression of the four Hox clusters between benign and malignant epithelium are significant \( (P < 0.001) \).
Examination of expression patterns within each HOX cluster also revealed differences characteristic of the origin of the cell lines (Fig. 4). We focused our attention on the HOXC cluster, which was overexpressed by each of the malignant cell line sub-groupings. When the expression pattern of individual HOXC genes was compared in lymph node metastases and cell lines derived from lymph node metastases, the patterns appeared very similar, particularly with respect to HOXC4, HOXC5, and HOXC6 (Fig. 5). In contrast, the pattern of genes expressed in primary tumor-derived cell lines or other metastases-derived cell lines exhibited low expression of HOXC4, HOXC5, and HOXC6 but stronger expression of HOXC9 or HOXC8, respectively. The differences in expression of HOXC6 were highly significant between the lymph node metastases-derived cell lines and either primary tumor-derived or other metastases-derived cell lines (P < 0.001). Due to sample sizes, the smaller differences detected in HOXC8 and HOXC9 expression between all three subgroupings do not reach statistical significance. However, the similarities between HOX expression patterns in lymph node metastases and cell lines derived from lymph node metastases and the differences between these samples and primary tumor-derived or other metastases-derived cell lines suggest that these patterns relate to the origination site of the cell line.

Fig. 2. Relative gene expression within the four HOX clusters in benign and malignant cells. Benign epithelium includes PrECs, normal prostate tissue, and LCM epithelium. Malignant epithelium includes eight malignant cell lines and lymph node metastases. Percentages are based on total HOX genes identified within each sample category.

Fig. 3. Relative expression levels of the four HOX clusters in malignant cells. l.n.mets, lymph node metastases. Percentages are based on total for each category.

Fig. 4. Relative expression of genes within the four HOX clusters by malignant cell line groups. Differences in expression of HOXC6 between lymph node-derived cell lines and primary tumor-derived or other metastases-derived cell lines are statistically significant (P < 0.001).
Expression of Specific HOXC Genes. Of 548 inserts examined by the degenerate technique from all benign sources (whole tissue, cultured cells, and LCM samples of both epithelium and stroma) only 4 were HOXC genes. Two of these were HOXCl0 from the prepubertal stromal sample, and two were HOXC6 from the whole BPH sample. In contrast, 134 HOXC inserts were identified out of 342 inserts examined from malignant sources (malignant cell lines and lymph node metastases). The degenerate data provide information on the relative expression of HOX clusters and individual genes within a particular sample, based on sampling. Thus we can compare relative expression within a sample, but we do not have information regarding absolute HOX expression level either overall or for specific genes.

To confirm the implications of the results seen by degenerate RT-PCR, we performed RT-PCR for specific HOXC genes on several different benign and malignant human tissue samples (Fig. 6A). Little or no expression of HOXC4, HOXC5, or HOXC6 was detected in normal tissue, and very low HOXC8 expression was seen in two of the four normal samples. BPH samples showed very low level expression of HOXC4, HOXC5, and HOXC6, whereas both lymph node metastasis samples expressed all four of the HOXC genes examined.

Specific RT-PCR on cell lines confirmed that HOXC products were overexpressed in malignant lines compared with cultures of normal epithelial and stromal cells (Fig. 6B). Except in the derivative cell lines ALVA-31 and PPC-1, HOXC4, HOXC5, HOXC6, and HOXC8 were broadly expressed in the malignant prostate cell lines examined. The benign stromal cells did not express detectable levels of these HOXC genes, whereas PrECs show some HOXC4, HOXC6, and HOXC8.

Some samples, which did not show HOXC expression by degenerate RT-PCR, did show faint products with specific primers. This was not totally unexpected, due to the increased sensitivity of RT-PCR using specific primers. Whereas the degenerate primers should amplify all matching HOX gene products, those with low representation might not be detected by our sampling unless much larger numbers of inserts were examined for each specimen. Pooling data from all of the malignant cell lines and the malignant lymph node tissue was designed to minimize this effect by increasing the total number of inserts examined for each specimen. Relative expression levels were similar to those shown in Fig. 6, A and B.

To further examine HOXC gene expression in malignancy, we performed LCM on frozen sections from biopsy punch specimens of three prostates to collect corresponding benign and malignant epithelium. Due to limited tumor availability, RNA from both tumor and normal cells was extracted and first amplified to provide sufficient template to allow examination of HOXC4, HOXC5, HOXC6, HOXC8, and β-actin in all specimens. This technique has been used for microarray assays and maintains differential mRNA expression (54), although not necessarily identical to unamplified levels (55). Fig. 6C shows a representative gel showing the specific RT-PCR products. HOXC5 was expressed in all six samples. Two of the tumors showed increased expression over their normal component, and one showed decreased expression. The relatively strong expression in normal cells

Fig. 5. Comparison of relative expression of HOXC genes in malignant cells. \textit{benign}, PrECs, normal donor prostate tissue, and LCM epithelium and stroma; \textit{l.n.mets}, lymph node metastases; other categories are malignant prostate cell lines.

Fig. 6. M, molecular weight marker lane. A, RT-PCR analysis of specific HOXC genes from tissue samples. Normal 1–4, donor prostate tissues; BPH 1 and 2, epithelial BPH nodules; l.n.met 1 and 2, lymph node metastases. B, RT-PCR analysis of specific HOXC genes from cell line samples. STR, primary stromal culture; other names refer to specific malignant prostate cell lines. C, RT-PCR analysis of specific HOXC genes from tumor/normal epithelial cells selected by LCM. T, tumor; N, normal. Numbers indicate paired material from the same patient. D, Western analysis for HOXC8 protein expression in the same cell lines used in B.
was somewhat unexpected, based on the degenerate data as well as on the absence of expression seen with specific primers on normal primary-culture cells and normal whole tissue templates. As a control, we then isolated RNA from 8-μm sections of normal tissue from two of the samples used for the tumor/normal pairs, and we performed RT-PCR using the HOXC5 primers without initial RNA amplification. A faint product could be detected in the sample with the strongest β-actin signal. Thus, it would appear the HOXC5 expression seen after two rounds of amplification may be enhanced but does not appear to be solely an artifact of amplification. One hypothesis is that the HOXC5 expression in these normal samples is related to the presence of tumor within the same prostate because the previously examined normal samples were taken from patients without tumors. A larger sample size designed specifically to address this question will be needed before drawing further conclusions. Neither HOXC6 nor HOXC8 was expressed in any of the normal specimens, and their absence despite RNA amplification appears to confirm lack of expression. One tumor sample expressed both HOXC5 and HOXC6 strongly, whereas the other two tumors expressed HOXC5 and HOXC8. No expression of HOXC4 was seen from any of these tumor or normal cells (data not shown).

To test whether RNA expression levels of HOXC genes were reflected in protein expression, Western analysis was performed on extracts of the same prostate cell lines analyzed for HOX RNA using an antibody to a unique peptide in HOXC8. The cell lines showing the highest levels of expression of HOXC8 RNA (PC-3, DU 145, PC-346C, 22Rv1, and LAPC-4) also showed the highest levels of HOXC8 protein expression (Fig. 6D). Lower to undetectable levels of HOXC8 were seen in LNCaP, ALVA-31, and PPC-1, consistent with the RT-PCR data.

**Reciprocal Regulation of HOXC8 and Androgen Signaling.** Homeodomain proteins have been reported to inhibit the related transcriptional coactivators CBP and p300 by inhibiting their intrinsic histone acetyltransferase activity (56). CBP and p300 are two of the best-studied coactivators of the steroid receptor family of hormone-dependent transactivators (57, 58). Because CBP is an androgen receptor coactivator (59, 60), we reasoned that overexpression of HOXC genes might abrogate androgen-dependent gene activation. HOXC8 cDNA was cloned into an expression vector, and the vector was transfected into LNCaP cells along with an androgen-responsive luciferase reporter (Fig. 7A). Increasing expression of HOXC8 resulted in a progressive decrease of reporter gene induction by the synthetic androgen R1881. At the highest levels of HOXC8 expression, the hormone induction was completely abolished. Thus, overexpression of HOXC8 results in a major alteration in a critical signaling pathway in prostate cancer cells. Additional studies will investigate the influence of other members of the HOX family on androgen signaling.

Conversely, we examined the consequences of androgen signaling on HOXC8 gene expression. LNCaP cells in medium containing charcoal-stripped serum were treated with R1881 or hydroxyflutamide. After isolation of RNA, RT-PCR was used to assess levels of HOXC8, HOXC5, and PSA. Expression of PSA was induced as expected by R1881 and also by hydroxyflutamide (Fig. 7B). Although hydroxyflutamide is an androgen antagonist, it displays agonist activity with the mutated androgen receptor found in LNCaP cells (61). In contrast to PSA, both ligands inhibited expression of HOXC8 (Fig. 7B). HOXC5 expression showed no significant response to either ligand (data not shown). Thus, HOXC8 and androgens exhibit reciprocal inhibitory actions.

**Analysis of Chromosomal Abnormalities in Prostate Cell Lines.** We performed FISH analysis on the prostate carcinoma cell lines to determine whether there was a correlation between HOXC gene expression and chromosomal abnormalities such as amplification and/or translocation. No such correlation was found. Three lines (LNCaP, PC-346C, and 22Rv1) displayed only normal copies of chromosome 12 equivalent to the modal ploidy for this line (tetraploid; diploid and mixed tetraploid/diploid, respectively). Two triploid lines (LAPC-4 and DU 145) both showed two normal copies of chromosome 12 plus one differing abnormal chromosome in each cell line exhibiting rearrangement outside the 12q12–14 region. The final three triploid cell lines (PC-3, ALVA-31, and PPC-1) showed complex rearranged karyotypes, as we have published previously (48). These lines exhibited from two to five different derivatives of chromosome 12. Interestingly, two of these cell lines (PPC-1 and ALVA-31) show very low HOXC gene expression, even though they are derived from the parental PC-3 line (47). Whether a connection exists between the rearranged chromosomes and the apparent decrease in HOXC expression seen in the clonal derivatives of PC-3 needs further study. The existence of small deletions or mutations within individual HOXC genes as potential contributing factors cannot be ruled out.

**DISCUSSION**

Comparison of expression patterns of benign and malignant prostate cells indicates overexpression of HOXC cluster genes in malignancy. RT-PCR using degenerate primers that detect 31 of the 39 HOX genes revealed that expression from the HOXC cluster was low compared with the other HOX clusters in benign cells. RT-PCR for several specific HOXC genes confirmed very low expression in be-
nign primary cultures, benign whole tissue, and laser-captured normal tissue. Malignant cell lines and lymph node metastases demonstrate substantially higher expression of these genes. Because the HOXC genes we examined do not appear to be uniformly up-regulated but show varied expression levels by gene in each malignant sample, it appears that independent control mechanisms are operating rather than a single switch for the entire HOX cluster.

A recent study also found little or no expression of HOXC8 in normal tissue by in situ hybridization (33). However, increased HOXC8 expression in tumors correlated with higher Gleason score (decreasing differentiation). These investigators also detected HOXC8 in PC-3 and DU 145 cells by RT-PCR but found no expression in LNCaP. We also find little or no expression of HOXC8 in LNCaP and more robust expression in PC-3 and DU 145. Moreover, we have detected expression of HOXC8 protein in both of the latter cell lines.

Detectable expression of HOXA9 and HOXD10 has been reported in normal prostate tissue by Northern blot (31). We found HOXA9 to be broadly expressed in almost all prostate samples, both benign and malignant, although individual samples showed some variation. We also found HOXD10 to be expressed rather widely in normal prostate cells, but not in malignant ones. Adult mouse prostate has been shown to express Hoxb13 (62) and Hoxd13 (63). Due to sequence divergence in the region of the degenerate primers, we do not have expression information for these genes in our human samples. Exploration of expression of the paralogous groups 11–13 will require a more individualized approach.

Mammalian downstream targets of HOX genes have proven difficult to identify. The mouse homologue of the Drosophila tumor suppressor l(2)gl, called mgl-1, was identified as a target of Hoxc8, and a possible inverse relationship was postulated (64). Increasing evidence links various HOX genes to cell adhesion molecules. Hoxc6 and Hoxb9 have been reported to increase NCAM promoter activity, whereas Hoxb8 repressed this activity in NIH 3T3 fibroblasts (65, 66). HOXD9 was found to increase expression of the L-CAM enhancer in the same cell type (67). Human prostate tissue has been shown to contain NCAM-like molecules (68). Other investigators (69) have reported that HOXD3 down-regulates E-cadherin expression, up-regulates integrins αs and βs, and produces de novo expression of N-cadherin and integrin αs in HOXD3-transfected A549 lung cancer cells. Mobility and invasion assays indicated increased activity in the transfected cells compared with the parental line or control transfec-tants. Transfected cells also produced a greater number of metastatic foci compared with the parental cells or control transfec-tants when injected into nude mice. Decreased E-cadherin expression in human prostate cancers has been correlated with the degree of differentiation of the tumor and the presence of metastases (70). Taken together, these studies indicate the possibility that dysregulation of HOX genes in the malignant prostate could play a role in metastasis by facilitating migration away from the original tissue through modulation of hemostatic cell adhesion molecules. Whether expression of specific cell adhesion molecules could also play a role in selecting metastatic sites remains to be demonstrated.

In view of the vastly different cellular environments between in vivo and in vitro conditions, particularly the lack of stromal cell components in vitro, the similarity in relative HOX cluster expression between lymph node metastases and lymph node metastasis-derived cell lines is quite remarkable. Several investigators (71, 72) have suggested the importance of stroma in prostatic tissue homeostasis as well as cancer progression. An interesting question is whether prostatic stromal signaling could be responsible for modulating epithelial HOX expression. The similarity in relative expression of the four HOX clusters, as well as genes within the C cluster, between lymph node metastases and lymph node metastases-derived cell lines, two extra-prostatic environments both lacking prostatic stromal components, suggests this not to be the case. Alternatively, stromal influences may have permanently entrained a constitutive pattern of HOX gene expression. The observed similarities between cell lines and tumors provide supplemental evidence that some in vivo characteristics of cells can be maintained in culture, further validating the use of cell lines as specific investigative tools, especially for initial studies.

The complexity of expression patterns of HOX genes in prostate tissue means that much work remains to clarify the role played by these genes in prostate cancer. We have attempted to examine HOX expression simultaneously as an overall pattern rather than simply focusing on single genes. The lack of expression of HOXC genes in normal prostate and up-regulation in cultured malignant cell lines, lymph node metastases, and primary prostate tumors suggests a role in prostate cancer. No correlation between chromosomal abnormalities and HOX expression was found. Similarity of expression patterns between lymph node metastases and cell lines derived from lymph node metastases suggests possible links between HOX expression and metastatic site. An intriguing speculation is whether the HOX expression patterns within a malignant cell could be instrumental in determining where that cell might lodge and metastasize. Whereas this first broad study of HOX gene expression in human prostate has exposed some intriguing clues, much more work remains to elucidate the role of HOX genes in prostatic malignancy.

One functional consequence of overexpression of HOXC genes was suggested by work reporting that homeodomain proteins inhibit the histone acetyltransferase activity of the transcriptional coactivator, CBP (56). In androgen-responsive LNCaP cells, we have demonstrated that increasing expression of HOXC8 progressively inhibited transactivation by the androgen receptor. It may seem paradoxical to suggest that a gene whose expression is associated with malignancy would inhibit a signaling pathway that is growth-promoting. However, if overexpression of HOXC occurs at a relatively early stage of prostate tumorigenesis, then the tumor must adapt to the diminished androgen signaling that accompanies expression of HOXC genes, thereby predisposing the tumor to survive in the face of a subsequent withdrawal of androgens. Thus, we postulate that the tumor would already be at least partially androgen resistant at the onset of ablation therapy, allowing some tumor cells to escape therapy and eventually progress. This speculation suggests an important role for HOXC in the pathogenesis of androgen-resistant prostate cancer.

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Hox expression in human prostate cancer


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Aberrant $HOXC$ Expression Accompanies the Malignant Phenotype in Human Prostate

Gary J. Miller, Heidi L. Miller, Adrie van Bokhoven, et al.


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