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Aberrant *HOXC* Expression Accompanies the Malignant Phenotype in Human Prostate[1]

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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 *HOXC* 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 *HOXC4*, *HOXC5*, *HOXC6*, and *HOXC8* 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 *HOXC* genes in primary tumor cells. Our data indicate a possible link between expression of *HOXC* genes and malignancy in prostate cells. Overexpression of *HOXC8* in LNCaP prostate cancer cells suppressed transactivation by androgen receptors. We speculate that *HOXC* 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 *NUP*98 nucleoporin 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 (*HOXA1*) 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 arose through reduplication and divergence of the ancestral cluster (24). "Paralogy" is used to describe the relationship between genes from different clusters that demonstrate the greatest sequence similarity as well as the same linear arrangement along the chromosomes (*e.g.*, *HOXA5*, *HOXB5*, and *HOXC5*). Whereas 13 potential paralogy groups exist, no HOX cluster has members in all 13 groups, and each group therefore contains 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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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 particular, lymph node metastases present a distinct pattern of *HOX* expression. 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 androgen-dependent transcription in prostate cancer cells, suggesting that dysregulation of *HOX* gene expression has important consequences in prostate cancer.

MATERIALS AND METHODS

Cell Lines and Culture. The human prostate cancer cell lines were obtained from the following laboratories: LAPC-4 (38), Dr. C. Sawyers (University 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); PPC-1 (42), Dr. A. Brothman (University of Utah, Salt Lake City, UT); 22R*v*1 (43), Dr. J. Jacobberger (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 BPH[5] 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 supplemented 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 methyltrienolone (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 authenticity of each was verified by comparison with the original karyotype as well as by DNA profiling using the AMPF*l*STR Profiling Plus Kit [Applied Biosystems, Foster City, CA (47)]. While this work was in progress, ALVA-31 and PPC-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 prostatectomy. 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 (Arcturus Engineering, 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. 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 isothiocyanate/acid phenol method of Chomczynski and Sacchi (49), with minor modifications.

Cultured cells were grown to 70–80% confluence before harvest. Briefly, cells were scraped from the flasks, washed in PBS, and then lysed in guanidinium 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 H_2O.

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 modifications. Two hundred μl of guanidinium extraction buffer containing 1.6 μl of β-mercaptoethanol per CapSure cap (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 (Promega). A mock RT sample of 10 μl was removed, and 50 units 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 ELEKEF 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 $MgCl_2$; and 0.625 unit of Taq polymerase (Promega) in 1× reaction buffer. Cycle number was 32 for the degenerate primers. PCR products were electrophoresed 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 $MgCl_2$ (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

[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 transcription-PCR; PSA, prostate-specific antigen; dNTP, deoxynucleotide triphosphate; PAC, P1-derived artificial chromosome; CBP, cAMP-responsive element binding protein (CREB)-binding protein.

Table 1 *Primer sequences and PCR cycling conditions*

Gene	Sequence	PCR conditions	Product size (bp)
Degenerate *HOX*	Forward: 5′-gctctagarytngaraargartt-3′ Reverse: 5′-ggaattcrttytgraaccadatytt-3′	94°C-1 min, 40°C-1 min, 72°C-1 min	125
HOXC4	Forward: 5′-caccaccccgagaaatcaca-3′ Reverse: 5′-aattggggtttcaccgtgctaac-3′	94°C-30 s, 55°C-30 s, 72°C-30 s	187
HOXC5	Forward: 5′-cagcctgctagctagctcaact-3′ Reverse: 5′-caggaaggacccagagtcaata-3′	94°C-30 s, 56°C-30 s, 72°C-30 s	159
HOXC6	Forward: 5′-ctgagcagggcaggact-3′ Reverse: 5′-gcgttaggtagcgattgaag-3′	94°C-30 s, 58°C-30 s, 72°C-1 min	186
HOXC8	Forward: 5′-cagtggacggcaaacttac-3′ Reverse: 5′-ccttttttccacttcatccttc-3′	94°C-30 s, 53°C-30 s, 72°C-30 s	170
β-Actin	Forward: 5′-atctggcaccacaccttctacaatgagctgcg-3′ Reverse: 5′-cgtcatactcctgcttgctgatccacatctgc-3′	94°C-45 s, 60°C-45 s, 72°C-2 min	838
Alternate *β-actin*	Forward: 5′-acacaggggaggtgatagcatt-3′ Reverse: 5′-atctcaagttgggggacaaaaa-3′	94°C-30 s, 52°C-30 s, 72°C-30 s	150
PSA	Forward: 5′-gcctctcgtggcagggcagt-3′ Reverse: 5′-gggtgaacttgcgcacacac-3′	94°C-1 min, 60°C-1 min, 72°C-1 min	440

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 *HOXC* cluster, and the other contains the vitamin D receptor gene, which is located proximal to the *HOXC* cluster. The probes were labeled using the Vysis nick translation kit (Vysis, Downers Grove, IL). The *HOXC* PAC was labeled with dUTP SpectrumGreen, and the vitamin D receptor PAC was labeled with dUTP-SpectrumRed. The prehybridization process consisted of cleaning the slides with 70% acetic acid and then rinsing them, followed by an ethanol dehydration series. Next the slides were digested in 0.008% pepsin/0.01 M HCl at 37°C for 10 min and fixed in 1% formaldehyde at room temperature for 10 min. Two hundred ng of each probe DNA, dissolved in 50% formamide hybridization mix, were added to the selected hybridization area, which was covered with a 177-mm^2 glass coverslip. Probe and chromosomal DNA were codenatured for 8 min at 80°C and then hybridized at 37°C for 48–72 h. Approximately 13 metaphase cells were analyzed per cell line. Additionally, red and green signals were scored in 100 interphase nuclei. Analysis and imaging were performed using an Olympus BX-60 epifluorescence microscope coupled with SmartCapture software (Vysis).

Transfection/Luciferase Assays. Sixteen h before transfection, 3×10^5 LNCaP cells were seeded into each well of a 6-well dish in medium containing charcoal-stripped serum. Plasmids pAHluc (51), pCMV6c (52), and pCMVHOXC8 were transfected into LNCaP cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The total amount of DNA per well was 3 μg, with pCMV6c being used as empty vector to normalize DNA amounts per transfection. The day after transfection, the cells were treated with 10 nM R1881 for 24 h, and luciferase assays were performed as described previously (53). Protein concentration of the extracts was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA) and used to normalize the luciferase activity of the corresponding extract. The plasmid pCMVHOXC8 was constructed by RT-PCR amplification of *HOXC8* cDNA and cloning the amplicon into the expression vector pCMV6c. The insert was sequenced to affirm that no enzymatic errors had been introduced.

Western Analysis. Total cellular extracts were made by resuspending the cell pellets in radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 7.5)] containing protease inhibitors (Protease Inhibitor Cocktail; Sigma-Aldrich, St. Louis, MO). After 10 min on ice, the extracts were cleared by microcentrifugation for 10 min, the supernatants were transferred to a new tube, and the protein concentration of each extract was determined by Bradford assay. Equivalent amounts of extract were separated on a 4–12% NuPAGE gel (Invitrogen) and transferred to polyvinylidene difluoride membrane. HOXC8 was detected with monoclonal antibody C953-7E (Covance Research Products, Berkeley, CA). Western blots were developed using enhanced chemiluminescence Western Lightning (Perkin-Elmer Life Sciences). Blots were stripped and reprobed for β-actin using monoclonal antibody AC-15 (Sigma).

Statistical Analysis. Statistical analyses were performed using the Intercooled Stata 5.0 software package (Stata Corp., College Station, TX). χ^2 and Fisher 's exact test results were considered to be statistically significant when $P \leq 0.05$.

RESULTS

Relative Expression of *HOX* Clusters in Benign and Malignant Prostate. We used degenerate RT-PCR combined with cloning and sequencing to simultaneously detect expression of multiple *HOX* genes. This sampling method permits examination of relative expression 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 (*HOXA11*, *HOXA13*, *HOXB13*, *HOXC11*, *HOXC12*, *HOXC13*, *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

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

	Benign					Malignant	
	Cell lines		LCM tissue[a]		Whole tissue	Cell lines[b]	Whole tissue
HOX gene	$n = 3$[c] Epithelial[d]	$n = 1$ Stromal[e]	$n = 5$ Epithelial	$n = 5$ Stromal	$n = 5$ Normal donor	$n = 8$ Epithelial	$n = 2$ l.n. mets[f]
A1	8					6	
A2						1	
A3	6	2	4	4	11	13	5
A4	2	2	1		1	4	3
A5			1	2	2	1	
A6	1	5	3	6	6	4	1
A7	6	24	5	14	9	4	3
A9	36	3	52	37	27	61	1
A10	4	7	38	32	28	35	2
B1							
B2							
B3	5	1	1	14	10	4	1
B4				1			
B5						4	
B6	4			1		17	1
B7	3			4		11	1
B8						2	
B9	2	1			1	14	1
C4						18	7
C5						12	5
C6					2	51	13
C8						10	
C9						10	
C10				2		7	1
D1			7		10	2	2
D3							
D4			3	2	1	1	
D8	1		5	1	1	1	
D9	6		1	3	3		2
D10	18	1	18	23			
D12			2		1		
Total *HOX*	102	46	141	146	113	293	49

[a] Paired epithelial and stromal cells from prepubertal, pubertal, and adult prostates.
[b] Includes LNCaP, LAPC-4, DU 145, PC-3, ALVA-31, PPC-1, 22R*v*1, and PC-346C.
[c] *n*, number of cell lines or patients.
[d] PrEC.
[e] Primary stromal culture.
[f] l.n. mets, lymph node metastases.

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*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*v*1 and PC-346C) or other metastatic sites (PC-3 and DU 145).

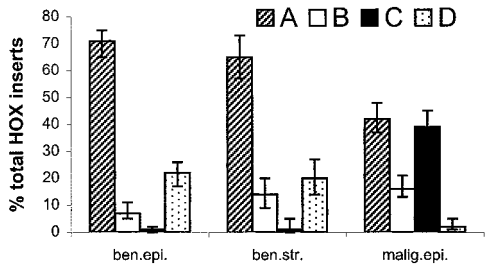

Fig. 1. Relative expression of the four *HOX* clusters (A–D) in benign and malignant cell types. Benign epithelium (*ben.epi.*) includes PrECs, normal prostate tissue, and LCM epithelium. Benign stroma (*ben.str.*) includes LCM stroma. Malignant epithelium (*malig.epi.*) includes eight malignant cell 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 ($P < 0.001$).

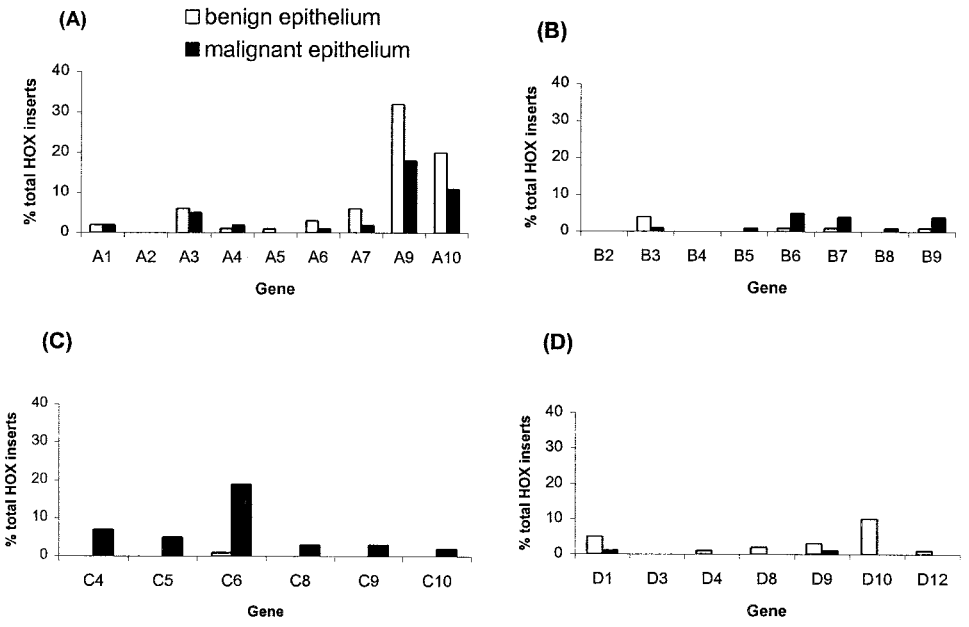

Fig. 2. Relative gene expression within the four *HOX* clusters in benign and malignant cells. Benign epithelium includes PrECs, normal prostate tissue, and LCM epithelium. Malignant epithelium includes eight malignant cell lines and lymph node metastases. Percentages are based on total *HOX* genes identified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

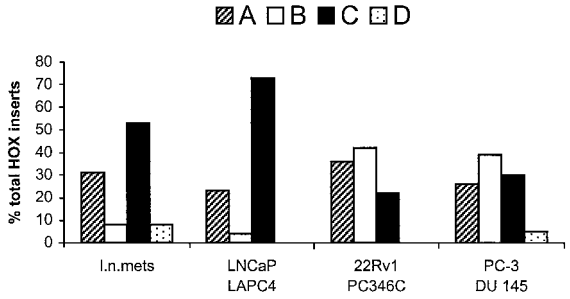

Fig. 3. Relative expression levels of the four *HOX* clusters in malignant cells. *l.n.mets,* lymph node metastases. Percentages are based on total for each category.

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 subgroupings. 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.

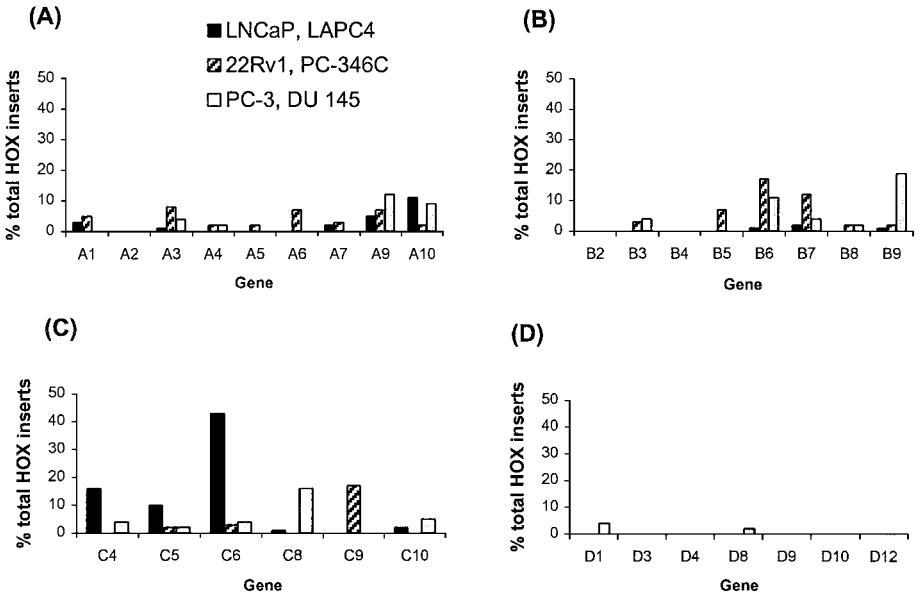

Fig. 4. Relative expression of genes within the four *HOX* clusters by malignant cell line groups. Differences in expression of *HOXC6* between lymph node-derived cell lines and primary tumor-derived or other metastases-derived cell lines are statistically significant ($P < 0.001$).

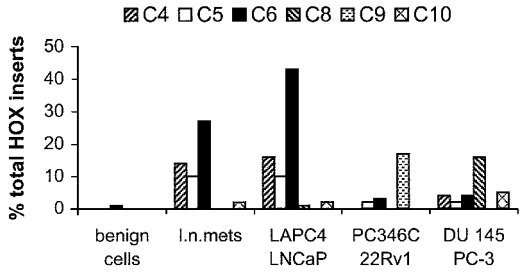

Fig. 5. Comparison of relative expression of *HOXC* genes in malignant cells. *benign*, PrECs, normal donor prostate tissue, and LCM epithelium and stroma; *l.n.mets*, lymph node metastases; other categories are malignant prostate cell lines.

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 *HOXC10* 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. 6*A*). 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. 6*B*). 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 the malignant category and allowing comparison of benign and malignant expression.

We also confirmed expression of *HOXC5* and *HOXC8* on the cell lines and whole tissue samples using intron-spanning primers that gave products of 333 and 388 bp, respectively. 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. 6*C* 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

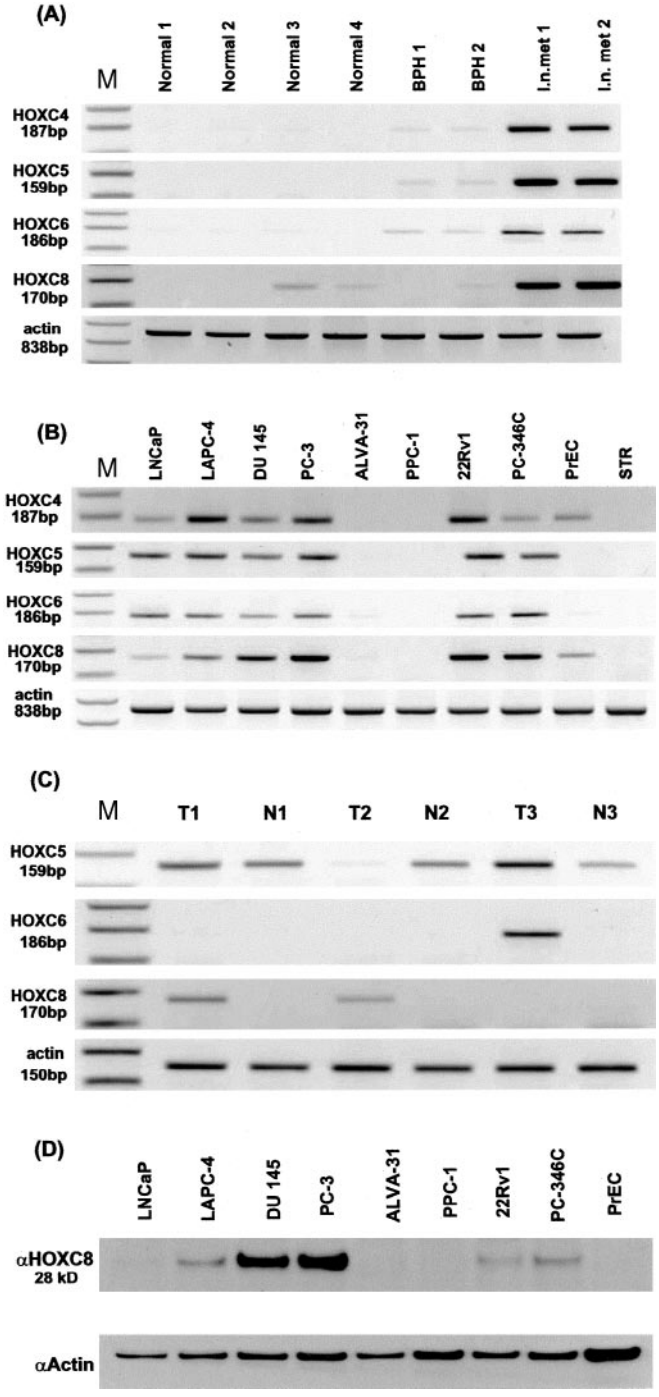

Fig. 6. *M,* molecular weight marker lane. *A,* RT-PCR analysis of specific *HOXC* genes from tissue samples. *Normal 1–4,* donor prostate tissues; *BPH 1* and *2,* epithelial BPH nodules; *l.n.met 1* and *2,* lymph node metastases. *B,* RT-PCR analysis of specific HOXC genes from cell line samples. *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, 22R*v*1, and LAPC-4) also showed the highest levels of HOXC8 protein expression (Fig. 6*D*). 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. 7*A*). 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. 7*B*). 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. 7*B*). *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 see whether there was a correlation between *HOXC* gene expression

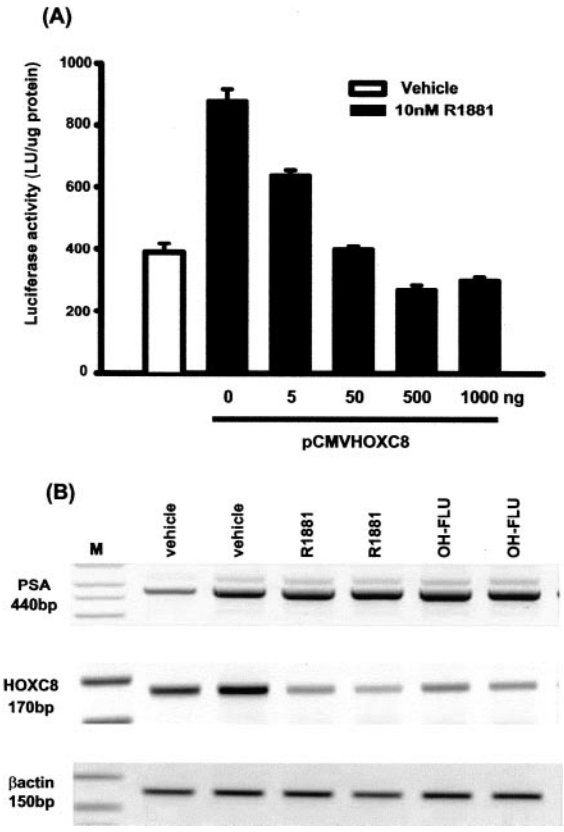

Fig. 7. Mutual inhibition by HOXC8 and androgen receptor in prostate cancer cells. *A,* LNCaP cells were transfected with increasing levels of an expression vector for HOXC8 along with an androgen-responsive mouse mammary tumor virus-luciferase reporter, pAHluc. After treatment with the synthetic androgen R1881 for 24 h, cells were harvested for assay of luciferase activity. A representative of three identical experiments is shown. *Error bars* represent the SE of triplicate wells. *B,* LNCAP cells were treated with 10 nM R1881 or 10 μM hydroxyflutamide (*OH-FLU*) for 24 h and then harvested for RT-PCR to analyze gene expression of PSA and *HOXC8*. *M,* molecular weight marker lane.

and chromosomal abnormalities such as amplification and/or translocation. No such correlation was found. Three lines (LNCaP, PC-346C, and 22R*v*1) displayed only normal copies of chromosome 12 equivalent to the modal ploidy for that 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 *HOXC* 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 α_3 and β_3, and produces *de novo* expression of N-cadherin and integrin α_4 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 transfectants. Transfected cells also produced a greater number of metastatic foci compared with the parental cells or control transfectants 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 homeostatic 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 extraprostatic 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 *HOXC* 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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REFERENCES

1. Jemal, A., Murray, T., Samuels, A., Ghafoor, A., Ward, E., and Thun, M. J. Cancer statistics, 2003. CA Cancer J. Clin., *53:* 5–26, 2003.
2. Sakr, W. A., Haas, G. P., Cassin, B. F., Pontes, J. E., and Crissman, J. D. The frequency of carcinoma and intraepithelial neoplasia of the prostate in young male patients. J. Urol., *150:* 379–385, 1993.
3. Guileyardo, J. M., Johnson, W. D., Welsh, R. A., Akazaki, K., and Correa, P. Prevalence of latent prostate carcinoma in two U.S. populations. J. Natl. Cancer Inst. (Bethesda), *65:* 311–316, 1980.
4. Cillo, C., Cantile, M., Faiella, A., and Boncinelli, E. Homeobox genes in normal and malignant cells. J. Cell. Physiol., *188:* 161–169, 2001.
5. Abate-Shen, C. Deregulated Homeobox gene expression in cancer: cause or consequence? Nat. Rev. Cancer, *2:* 777–785, 2002.
6. Owens, B. M., and Hawley, R. G. HOX and non-HOX homeobox genes in leukemic hematopoiesis. Stem Cells, *20:* 364–379, 2002.
7. Lawrence, H. J., Stage, K. M., Mathews, C. H. W., Detmer, K., Scibienski, R., MacKenzie, M., Migliaccio, E., Boncinelli, E., and Largman, C. Expression of HOXC homeobox genes in lymphoid cells. Cell Growth Differ., *4:* 665–669, 1993.
8. Celetti, A., Barba, P., Cillo, C., Rotoli, B., Boncinelli, E., and Magli, M. C. Characteristic patterns of HOX gene expression in different types of human leukemia. Int. J. Cancer, *53:* 237–244, 1993.

9. Lawrence, H. J., Sauvageau, G., Ahmadi, N., Lopez, A. R., LeBeau, M. M., Link, M., Humphries, K., and Largman, C. Stage-and lineage-specific expression of the HOXA10 homeobox gene in normal and leukemic hematopoietic cells. Exp. Hematol., *23:* 1160–1166, 1995.
10. Perkins, A., Kongsuwan, K., Visvader, J., Adams, J. M., and Cory, S. Homeobox gene expression plus autocrine growth factor production elicits myeloid leukemia. Proc. Natl. Acad. Sci. USA, *87:* 8398–8402, 1990.
11. Borrow, J., Shearman, A. M., Stanton, V. P., Jr., Becher, R., Collins, T., Williams, H. J., Dube, I., Katz, F., Kwong, Y. L., Morris, C., Ohyashiki, K., Toyama, K., Rowley, J., and Housman, D. E. The t(7;11)(p15;p15) translocation in acute myeloid leukemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. Nat. Genet., *12:* 159–167, 1996.
12. DeVita, G., Barba, P., Odartchenki, N., Givel, J-C., Freschi, G., Bucciarelli, G., Magli, M., Boncinelli, E., and Cillo, C. Expression of homeobox-containing genes in primary and metastatic colorectal cancer. Eur. J. Cancer, *29A:* 887–893, 1993.
13. Chariot, A., and Castronovo, V. Detection of HOXA1 expression in human breast cancer. Biochem. Biophys. Res. Commun., *222:* 292–297, 1996.
14. Chariot, A., Castronovo, V., Le, P., Gillet, C., Sobel, M. E., and Gielen, J. Cloning and expression of a new HOXC6 transcript encoding a repressing protein. Biochem. J., *319:* 91–97, 1996.
15. Cillo, C., Barba, P., Freschi, G., Bucciarelli, G., Magli, M. C., and Boncinelli, E. *HOX* gene expression in normal and neoplastic human kidney. Int. J. Cancer, *51:* 892–897, 1992.
16. Caré, A., Silvani, A., Meccia, E., Mattia, G., Stoppacciaro, A., Parmiani, G., Peschle, C., and Colombo, M. P. HOXB7 constitutively activates basic fibroblast growth factor in melanomas. Mol. Cell. Biol., *16:* 4842–4851, 1996.
17. Rieger, E., Bijl, J. J., van Oostveen, J. W., Soyer, H. P., Oudejans, C. B., Jiwa, N. M., Walboomers, J. M., and Meijer, C. J. Expression of the homeobox gene HOXC4 in keratinocytes of normal skin and epithelial skin tumors is correlated with differentiation. J. Investig. Dermatol., *103:* 341–346, 1994.
18. Nakamura, T., Largaespada, D. A., Lee, M. P., Johnson, L. A., Ohyashiki, K., Toyama, K., Chen, S. J., Willman, C. L., Chen, I. M., Feinberg, A. P., Jenkins, N. A., Copeland, N. G., and Shaughnessy, J. D., Jr. Fusion of the nucleoporin gene *NUP*98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukemia. Nat. Genet., *12:* 154–158, 1996.
19. Fujino, T., Suzuki, A., Ito, Y., Ohyashiki, K., Hatano, Y., Miura, I., and Nakamura, T. Single-translocation and double-chimeric transcripts: detection of *NUP98-HOXA9* in myeloid leukemias with *HOXA11* or *HOXA13* breaks of the chromosomal translocation t(7;11)(p15;p15). Blood, *99:* 1428–1433, 2002.
20. Arai, Y., Kyo, T., Miwa, H., Arai, K., Kamada, N., Kita, K., and Ohki, M. Heterogenous fusion transcripts involving the *NUP*98 gene and HOXD13 gene activation in a case of acute myeloid leukemia with the t(2;11)(q31;p15) translocation. Leukemia (Baltimore), *14:* 1621–1629, 2000.
21. Taketani, T., Taki, T., Shibuya, N., Kikuchi, A., Hanada, R., and Hayashi, Y. Novel NUP98-HOXC11 fusion gene resulted from a chromosomal break within exon 1 of HOXC11 in acute myeloid leukemia with t(11;12)(p15;q13). Cancer Res., *62:* 4571–4574, 2002.
22. Panagopoulos, I., Isaksson, M., Billström, R., Strömbeck, B., Mitelman, F., and Johansson, B. Fusion of the *NUP*98 gene and the homeobox gene *HOXC13* in acute myeloid leukemia with t(11;12)(p15;q13). Genes Chromosomes Cancer, *36:* 107–112, 2003.
23. Scott, M. P. Vertebrate homeobox gene nomenclature. Cell, *71:* 551–553, 1992.
24. Ruddle, F. H., Bartels, J. L., Bentley, K. L., Kappen, C., Murtha, M. T., and Pettaway, C. A. Evolution of HOX genes. Annu. Rev. Genet., *28:* 423–442, 1994.
25. Maconochie, M., Nonchev, S., Morrison, A., and Krumlauf, R. Paralogous HOX genes: function and regulation. Annu. Rev. Genet., *30:* 529–556, 1996.
26. Greer, J. M., Puetz, J., Thomas, K. R., and Capecchi, M. R. Maintenance of functional equivalence during paralogous Hox gene evolution. Nature (Lond.), *403:* 661–665, 2000.
27. Chen, F., and Capecchi, M. R. Paralogous mouse *Hox* genes, *Hoxa69*, *Hoxb9*, and *Hoxd9*, function together to control development of the mammary gland in response to pregnancy. Proc. Natl. Acad. Sci. USA, *96:* 541–546, 1999.
28. Zhao, Y., and Potter, S. S. Functional comparison of the *Hoxa 4, Hoxa 10*, and *Hoxa 11* homeoboxes. Dev. Biol., *244:* 21–36, 2002.
29. van den Akker, E., Fromental-Ramain, C., de Graaff, W., Le Mouellic, H., Brûlet, P., Chambon, P., and Deschamps, J. Axial skeletal patterning in mice lacking all paralogous group 8 Hox genes. Development (Camb.), *128:* 1911–1921, 2001.
30. Chauvet, S., Merabet, S., Bilder, D., Scott, M. P., Pradel, J., and Graba, Y. Distinct Hox protein sequences determine specificity in different tissues. Proc. Natl. Acad. Sci. USA, *97:* 4064–4069, 2000.
31. Redline, R. W., Hudock, P., MacFee, M., and Patterson, P. Expression of AbdB-type homeobox genes in human tumors. Lab. Investig., *71:* 663–670, 1994.
32. Robbins, S. E., Shu, W-P., Kirschenbaum, A., Levine, A. C., Miniati, D. N., and Liu, B. C-S. Bone extracellular matrix induces homeobox proteins independent of androgens: possible mechanism for androgen-independent growth in human prostate cancer cells. Prostate, *29:* 362–370, 1996.
33. Waltregny, D., Alami, Y., Clausse, N., de Leval, J., and Castronovo, V. Overexpression of the homeobox gene *HOXC8* in human prostate cancer correlates with loss of tumor differentiation. Prostate, *50:* 162–169, 2002.
34. Dong, J-T. Chromosomal deletions and tumor suppressor genes in prostate cancer. Cancer Metastasis Rev., *20:* 173–193, 2001.
35. Bhatia-Gaur, R., Donjacour, A., Sciavolino, P., Kim, M., Desai, N., Young, P., Norton, C., Gridley, T., Cardiff, R., Cunha, G., Abate-Shen, C., and Shen, M. Roles for *NKX3.1* in prostate development and cancer. Genes Dev., *13:* 966–977, 1999.
36. Kim, M. J., Bhatia-Gaur, R., Banach-Petrosky, W. A., Desai, N., Wang, Y., Hayward, S. W., Cunha, G., Cardiff, R., Shen, M. M., and Abate-Shen, C. Nkx3.1 mutant mice recapitulate early stages of prostate carcinogenesis. Cancer Res., *62:* 2999–3004, 2002.
37. Abdulkadir, S. A., Magee, J. A., Peters, T. J., Kaleem, Z., Naughton, C. K., Humphrey, P. A., and Milbrand, J. Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. Mol. Cell. Biol., *22:* 1495–1503, 2002.
38. Klein, K. A., Reiter, R. E., Redula, J., Moradi, H., Zhu, X. L., Brothman, A. R., Lamb, D. J., Marcelli, M., Belldegrun, A., Witte, O. N., and Sawyers, C. L. Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. Nat. Med., *3:* 402–408, 1997.
39. Horoszewicz, J. S., Leong, S. S., Chu, T. M., Wajsman, Z. L., Friedman, M., Papsidero, L., Kim, U., Chai, L. S., Kakati, S., Arya, S. K., and Sandberg, A. A. The LNCaP cell line: a new model for studies on human prostatic carcinoma. Prog. Clin. Biol. Res., *37:* 115–132, 1980.
40. Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, T. M., Mirand, E. A., and Murphy, G. P. LNCaP model of human prostatic carcinoma. Cancer Res., *43:* 1809–1818, 1983.
41. Loop, S. M., Rozanski, T. A., and Ostenson, R. C. Human primary prostate tumor cell line, ALVA-31: a new model for studying the hormonal regulation of prostate tumor cell growth. Prostate, *22:* 93–108, 1993.
42. Brothman, A. R., Lesho, L. J., Somers, K. D., Wright, G. L., and Merchant, D. J. Phenotypic and cytogenetic characterization of a cell line derived from primary prostatic carcinoma. Int. J. Cancer, *44:* 898–903, 1989.
43. Sramkoski, R. M., Pretlow, T. G., II, Giaconia, J. M., Pretlow, T. P., Schwartz, S., Sy, M. S., Marengo, S. R., Rhim, J. S., Zhang, D., and Jacobberger, J. W. A new human prostate carcinoma cell line, 22R*v*1. In Vitro Cell. Dev. Biol. Anim., *35:* 403–409, 1999.
44. Romijn, J. C., Erkens-Schulze, S., and Schröder, F. H. Perspectives of the use of tissue culture methods as an alternative to human prostate cancer xenografts in nude mice. *In:* W. Arnold, P. Köpf-Maier, and B. Micheel (eds.), Immunodeficient Animals: Models for Cancer Res., Vol. 51, pp. 209–213. Basel: Karger, 1996.
45. Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F., and Jones, L. W. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Investig. Urol., *17:* 16–23, 1979.
46. Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H., and Paulson, D. F. Isolation of a human prostate carcinoma cell line (DU 145). Int. J. Cancer, *21:* 274–281, 1978.
47. van Bokhoven, A., Varella-Garcia, M., Korch, C., Hessels, D., and Miller, G. J. Widely used prostate carcinoma cell lines share common origins. Prostate, *47:* 36–51, 2001.
48. Varella-Garcia, M., Boomer, T., and Miller, G. J. Karyotypic similarity identified by multiplex-FISH relates four prostate adenocarcinoma cell lines: PC-3, PPC-1, ALVA-31, and ALVA-41. Genes Chromosomes Cancer, *31:* 303–315, 2001.
49. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.
50. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res., *25:* 3389–3402, 1997.
51. Nordeen, S. K., Moyer, M. L., and Bona, B. J. Modulation of glucocorticoid-regulated transcription by purines: novel characteristics and implications for tissue specificity of steroid responses. Endocrinology, *136:* 1120–1127, 1995.
52. Chapman, B. S., Thayer, R. M., Vincent, K. A., and Haigwood, N. L. Effect of intron A from human cytogmegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. Nucleic Acids Res., *19:* 3979–3986, 1991.
53. Nordeen, S. K., Bona, B. J., and Moyer, M. L. Latent agonist activity of the steroid antagonist, RU486, is unmasked in cells treated with activators of protein kinase A. Mol. Endocrinol., *7:* 731–742, 1993.
54. Phillips, J. and Eberwine, J. H. Antisense RNA amplification: a linear amplification method for analyzing the mRNA population from single living cells. Methods, *10:* 283–288, 1996.
55. Luzzi, V., Hotschlag, V., and Watson, M. A. Expression profiling of ductal carcinoma *in situ* by laser capture microdissection and high-density oligonucleotide arrays. Am. J. Pathol., *158:* 2005–2010, 2001.
56. Shen, W., Krishnan, K., Lawrence, H. J., and Largman, C. The HOX homedomain proteins block CBP histone acetyltransferase activity. Mol. Cell. Biol., *21:* 7509–7522, 2001.
57. McKenna, N. J., Xu, J., Nawaz, Z., Tsai, M. J., and O'Malley, B. W. Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. J. Steroid Biochem. Mol. Biol., *69:* 3–12, 1999.
58. Dilworth, F. J., and Chambon, P. Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. Oncogene, *20:* 3047–3054, 2001.
59. Aarnisalo, P., Palvimo, J. J., and Janne, O. A. CREB-binding protein in androgen receptor-mediated signaling. Proc. Natl. Acad. Sci. USA, *95:* 2122–2127, 1998.
60. Fronsdal, K., Engedal, N., Slagsvold, T., and Saatcioglu, F. CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. J. Biol. Chem., *273:* 31853–31859, 1998.
61. Berrevoets, C. A., Veldscholte, J., and Mulder, E. Effects of antiandrogens on transformation and transcription activation of wild-type and mutated (LNCaP) androgen receptors. J. Steroid Biochem. Mol. Biol., *46:* 731–736, 1993.
62. Sreenath, T., Orosz, A., Fujita, K., and Bieberich, C. J. Androgen-independent expression of *hoxb-13* in the mouse prostate. Prostate, *41:* 203–207, 1999.

63. Oefelein, M., Chin-Chance, C., and Bushman, W. Expression of the homeotic gene Hox-d13 in the developing and adult mouse prostate. J. Urol., *155:* 342–346, 1996.
64. Tomotsune, D., Shoji, H., Wakamatsu, Y., Kondoh, H., and Takahashi, N. A mouse homologue of the *Drosophila* tumour-suppressor gene *l(2)gl* controlled by Hox-c8 *in vivo*. Nature (Lond.), *365:* 69–72, 1993.
65. Jones, F. S., Prediger, E. A., Bittner, D. A., De Robertis, E. D., and Edelman, G. M. Cell adhesion molecules as targets for *Hox* genes: neural cell adhesion molecule promoter activity is modulated by cotransfection with *Hox-2.5* and *-2.4*. Proc. Natl. Acad. Sci. USA, *89:* 2086–2090, 1992.
66. Jones, F. S., Holst, B. D., Minowa, O., De Robertis, E. D., and Edelman, G. M. Binding and transcriptional activation of the promoter for the neural cell adhesion molecule by *HoxC6 (Hox-3.3)*. Proc. Natl. Acad. Sci. USA, *90:* 6557–6561, 1993.
67. Goomer, R. S., Holst, B. D., Wood, I. C., Jones, F. S., and Edelman, G. M. Regulation *in vitro* of an L-CAM enhancer by homeobox genes *HoxD9* and HNF-1. Proc. Natl. Acad. Sci. USA, *91:* 7985–7989, 1994.
68. Lipford, G. B., and Wright, G. L., Jr. Comparative study of monoclonal antibodies TURP-27 and HNK-1: their relationship to neural cell adhesion molecules and prostate tumor-associated antigens. Cancer Res., *51:* 2296–2301, 1991.
69. Hamada, J-I., Omatsu, T., Okada, F., Furuuchi, K., Okubo, Y., Takahashi, Y., Tada, M., Miyazaki, Y. J., Taniguchi, Y., Shirato, H., Miyasaka, K., and Moriuchi, T. Overexpression of homeobox gene *HOXD3* induces coordinate expression of metastasis-related genes in human lung cancer cells. Int. J. Cancer, *93:* 516–525, 2001.
70. Umbas, R., Isaacs, W. B., Bringuier, P. P., Schaafsma, H. E., Karthaus, H. F. M., Oosterhof, G. O. N., Debruyne, F. M. J., and Schalken, J. A. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Res., *54:* 3929–3933, 1994.
71. Hayward, S. W., Cunha, G. R., and Dahiya, R. Normal development and carcinogenesis of the prostate. Ann. N. Y. Acad. Sci., *784:* 50–62, 1996.
72. Tuxhorn, J. A., Ayala, G. E., and Rowley, D. R. Reactive stroma in prostate cancer progression. J. Urol., *166:* 2472–2483, 2001.