American Journal of Pathology, Vol. 158, No. 3, March 2001
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HOX Genes in Human Lung

Altered Expression in Primary Pulmonary Hypertension and Emphysema

Heiko A. Golpon,*† Mark W. Geraci,*† Mark D. Moore,*† Heidi L. Miller,‡ Gary J. Miller,‡ Rubin M. Tuder,*†‡ and Norbert F. Voelkel*†

From the Pulmonary Hypertension Center, and the Departments of Pathology‡ and Respiratory and Critical Care Medicine,† University of Colorado Health Sciences Center, Denver, Colorado*

HOX genes belong to the large family of homeodomain genes that function as transcription factors. Animal studies indicate that they play an essential role in lung development. We investigated the expression pattern of HOX genes in human lung tissue by using microarray and degenerate reverse transcriptase-polymerase chain reaction survey techniques. HOX genes predominantly from the 3′ end of clusters A and B were expressed in normal human adult lung and among them HOXA5 was the most abundant, followed by HOXB2 and HOXB6. In fetal (12 weeks old) and diseased lung specimens (emphysema, primary pulmonary hypertension) additional HOX genes from clusters C and D were expressed. Using *in situ* hybridization, transcripts for HOXA5 were predominantly found in alveolar septal and epithelial cells, both in normal and diseased lungs. A 2.5-fold increase in HOXA5 mRNA expression was demonstrated by quantitative reverse transcriptase-polymerase chain reaction in primary pulmonary hypertension lung specimens when compared to normal lung tissue. In conclusion, we demonstrate that HOX genes are selectively expressed in the human lung. Differences in the pattern of HOX gene expression exist among fetal, adult, and diseased lung specimens. The altered pattern of HOX gene expression may contribute to the development of pulmonary diseases. ***(Am J Pathol 2001, 158:955–966)***

Homeodomain genes are a superfamily of genes encoding transcription factors, which are well known for their important functions regulating developmental processes such as body patterning and organogenesis.[1–3] Homeodomain genes are widely distributed in all animal groups including mammals and eukaryotes, accounting for ~0.1% of the vertebrate genome.[4] The family of vertebrate homeodomain genes can be divided into two subfamilies: the clustered homeodomain genes known as *HOX* genes or class I homeobox genes; and the nonclustered or divergent homeodomain genes such as *PAX, EMX, OTX*, and *MSX*, named after their homologs in Drosophila (paired, empty spiracles, orthodenticle, and muscle segment homeobox genes, respectively).[5] The protein products of the HOX genes are helix-turn-helix transcription factors that share a highly conserved 60-amino acid DNA-binding region, known as the homeobox. In humans, 39 HOX genes are known, which are organized into four physical clusters (HOXA, HOXB, HOXC, and HOXD).

Most studies regarding HOX gene expression have focused on embryonic development and, hence, little information is available regarding their expression in adult tissues. Recently, some functions of HOX genes in adult tissues have been described. For example, deficits in group 9 paralogous genes (Hoxa-9, Hoxb-9, Hoxd-9; mouse nomenclature) resulted in abnormal cellular proliferation and differentiation in the mammary gland of adult mice.[6] It has also been reported that the size of the prostate was decreased by inactivation of Hoxd-13.[7] Moreover, abnormal homeobox gene expression has been described in a number of leukemias.[8,9] HOX gene expression in leukemic cell lines seems to be lineage-restricted, and a regulatory role for HOX genes in blood cell maturation has been proposed. HOXA5 might function as an important regulator of hematopoietic cell lineage determination and maturation.[10,11]

Presently, very little information is available regarding the role of HOX genes in the adult respiratory system.[12] Apart from the description of HOX gene expression in lung cancer cell lines,[13,14] HOX gene expression has only been reported in cultured human bronchial fibroblasts, where retinoic acid caused an induction of

Supported by the Hart Chronic Obstructive Pulmonary Disease Research Fund, grants from the National Institutes of Health [RO1 HL60913-01 (to N. F. V.), RO1 HL60195-01 and 1RO1 HL66554-01 (to R. M. T.), and CA84269 (to G. J. M.)] and a CaP CURE grant (to G. J. M.). H. A. Golpon received a training stipend (Go 902/1-1) from the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, Germany.

Accepted for publication December 1, 2000.

Address reprint requests to Norbert F. Voelkel, M.D., Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Health Sciences Center, Box C 272, 4200 E. Ninth Ave., Denver, CO 80262. E-mail: norbert.voelkel@uchsc.edu.

HOXA5 expression.[15] It is also known that 16 homeobox genes are differentially expressed in embryonic and newborn mouse lungs,[2,16,17] and here again the pattern of gene expression in the developing lung is affected by retinoic acid.[16,18] Because some mouse Hox mutants have severe disruption of lung morphogenesis, Hox genes might play an important role in branching morphogenesis, epithelial cell fate, and the differentiation of conducting airway epithelium.[19] Specifically, Hoxa-1 mutants failed to initiate respiration[20] and Hoxa-3 mutants die shortly after birth, likely because of pulmonary failure.[21] A smaller lung was also noted for Hoxa-1/Hoxb-1 double mutants.[22] Homozygous mutants for Hoxa-5 showed perinatal lethality because of improper tracheal and lung morphogenesis that caused tracheal occlusion and respiratory distress.[23] Loss of Hoxa-5 function may also impair the production of surfactant-associated proteins.[23] Despite the growing evidence linking homeobox genes to murine lung embryogenesis and development, information regarding the expression of HOX genes in the human adult lung is still lacking. In the present study, we surveyed HOX gene expression in normal and diseased human adult lungs. The most abundant HOX genes identified by us in normal lung, HOXA5, HOXB2, and HOXB6, were the subject of further expression studies in the normal lung and in the lungs from patients with emphysema and pulmonary hypertension.

Materials and Methods

Microarray Analysis

Preparation of RNA

Total RNA was extracted from the lung tissues by using the Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). Polyadenylated (poly A+) RNA was purified from total RNA using oligo-dT-linked Oligotex Resin (Qiagen, Valencia, CA).

Reverse Transcription

One microgram of poly A+ RNA was converted to double-stranded cDNA (ds-cDNA) by using Superscript Choice System (Life Technologies, Inc., Rockville, MD). An oligo-dT primer containing a T7 RNA polymerase promoter (Promega Corp., Madison, WI) was used. After second strand synthesis, the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol and the ds-cDNA was recovered by ethanol precipitation.

In Vitro *Transcription*

In vitro transcription, to generate biotin-labeled cRNA, was performed using a T7 Megascript kit by Ambion (Austin, TX). ds-cDNA template (1.5 μl) was transcribed in the presence of a mixture of unlabeled ATP, CTP, GTP, and UTP. In addition, biotin-labeled CTP and UTP (bio-11-CTP and bio-16-UTP) from Enzol (Farmingdale, NY) was added to the mixture. Biotin-labeled cRNA was purified using an RNeasy affinity column by Qiagen. To ensure optimal hybridization to the oligonucleotide array, the cRNA was fragmented. Fragmentation was performed by incubating the cRNA at 94°C for 35 minutes to produce cRNA fragments between 35 to 200 bases in length. The sample was then added to a hybridization solution containing 100 mmol/L MES, 1 mol/L Na^+, and 20 mmol/L of ethylenediaminetetraacetic acid in the presence of 0.01% Tween 20 to a final cRNA concentration of 0.05 mg/ml.

Hybridization of the Gene Chip

Hybridization was performed for 18 to 20 hours by incubating 200 ml of the sample to the Affymetrix gene chip (Affymetrix, Santa Clara, CA). After hybridization, the hybridization solutions were removed and the gene chips were washed and stained with streptavidin-phycoerythrin. Chips were read at a resolution of 6 μm with a Hewlett-Packard gene array scanner (Hewlett-Packard Co., Boise, ID).

Data Analysis

Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described.[24,25] Briefly, each gene is represented by the use of ~20 perfectly matched and mismatched control probes. The mismatched probes act as specificity controls that allow the direct subtraction of both background and cross-hybridization signals. The number of instances in which the perfectly matched hybridization signal is larger than the mismatched signal is computed along with the average of the logarithm of the perfectly matched:mismatched ratio (after background subtraction) for each probe set. These values are used to make a matrix-based decision concerning the presence or absence of an RNA molecule. To determine the quantitative RNA abundance, the average of the differences representing perfectly matched minus mismatched for each gene-specific probe family is calculated, after discarding the maximum, the minimum, and any outliers beyond three standard deviations. All of the aforementioned calculations are functions performed by the Gene Chip Analysis Suite (Affymetrix). The Gene Chip Analysis Suite is a program that is used to analyze expression data from all Affymetrix expression arrays. This analysis software is specifically designed to determine intensity of hybridization from all features on the array. Specifically, this program determines the intensity of expression for all genes on the array. One chip with adult mouse lung (pooled sample of five FVB/N mice), one chip of human fetal lung (pooled sample of at least three lungs with a gestation age of 12 weeks), three chips with normal human adult lung tissue, three chips with emphysematous human lung tissue, and three chips with primary pulmonary hypertension (PPH) human lung tissue were hybridized and analyzed for the expression of HOX genes.

Degenerate Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

To survey expression of HOX genes in normal human lung tissue, we used a degenerate RT-PCR technique.[16] Because the homeobox domain of most of the HOX proteins is flanked by the highly conserved ELEKEF and KIWFQN motifs, we used a set of degenerate PCR primers recognizing these motifs.[26] The sequence of the degenerate primers were (International Union of Pure and Applied Chemistry Code used): 5′-GCT CTA GAR YTN GAR AAR GAR TT-3′; 5′-GGA ATT CRT TYT GRA ACC ADA TYT T-3′ (for the ELEKEF and KIWFQN motifs, respectively). Total RNA from human fetal lung (pooled sample of at least three lungs with a gestation age of 12 weeks), normal human adult lung ($n = 2$), emphysematous lungs ($n = 3$), and lungs from patients with PPH ($n = 3$) was isolated using the RNeasy mini kit (Qiagen) and converted to ds-cDNA using the Reverse Transcription System kit (Promega Corp.). Five μl of the cDNA mixture served as a template for PCR amplification in a 25-μl reaction using 2 μmol/L each of sense and antisense degenerate oligonucleotide primers (200 μmol/L of dNTP, 4.5 mmol/L of $MgCl_2$, 0.65 U of *Taq* polymerase). The reaction conditions were initial denaturation at 94°C for 4 minutes, followed by 32 cycles of 94°C for 1 minute, 40°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 5 minutes. PCR products were separated in 2% NuSieve 3:1 agarose by gel electrophoresis. The 125-bp band containing homeobox amplicons was excised and purified with the QIAquick gel extraction kit from Qiagen. The concentration of PCR products was estimated by comparison to DNA mass standards. Approximately 20 ng of the PCR product was ligated into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and expanded in One Shot (Invitrogen) cells. An efficient cloning reaction produced ~200 positive (>95%) colonies on each plate, which were easily identified by white color on X-Gal-containing medium. Only colonies of efficient cloning reactions were sequenced. The cloned inserts from overnight minipreps were isolated using the QIA well 8 ultra plasmid kit (Qiagen). Twenty-seven colonies originating from human fetal lung (normal adult lung = 47, emphysema = 51, PPH = 47 sequenced colonies) were sequenced at the Cancer Center Core Lab., University of Colorado Health Sciences Center (Denver, CO), using the dideoxy sequencing method of Sanger and colleagues.[27] Sequences were compared to those in the nonredundant database of the National Center for Biotechnology Information using the BLAST search algorithm.

Quantitative RT-PCR Analysis for HOXA5, HOXB2, HOXB5, HOXB6, and HOXC5

Lung tissue samples were obtained from six patients with PPH (three males, three females; 36 ± 5.47 [SEM] years). Two of the lung specimens were obtained at autopsy—three patients were undergoing open lung biopsy for diagnostic purposes and one patient was undergoing lung transplantation. All of these patients had severely elevated pulmonary artery pressure as documented by right heart catheterization. We also obtained lungs from six patients [three males, three females; 59.17 ± 4.32 (SEM) years] with severe emphysema who were either undergoing lung transplantation, lung volume reduction surgery, or lobectomy. All six patients were chronic cigarette smokers with pulmonary obstructive changes documented by lung function studies. Normal lung tissue was obtained from six patients (three males, three females; 62.5 ± 6.28 [SEM] years) undergoing open lung biopsy for diagnostic purposes [localized inflammation ($n = 1$) or primary or metastatic malignancies ($n = 5$)]. Histologically, these tissues did not show any histological abnormalities. Total RNA from human embryonic lung tissues at 12 weeks of age (embryonic period/pseudoglandular stage) was obtained from ViroGen (Watertown, MA).

Quantitative RT-PCR was performed using the SYBR Green PCR core reagents (Perkin-Elmer, Foster City, CA). Direct detection of the polymerase chain reaction product was monitored with the Gene Amp 5700 sequence detection system (ABI Prism, Perkin-Elmer) by measuring the increase in fluorescence caused by the binding of SYBR Green to ds-DNA. The primers used were *HOXA5* (forward 5′-CGCCCAACCCCAGATCTA-3′; reverse, 5′-GGCCGCCTATGTTGTCATG-3′; 66-bp band product), *HOXB2* (forward 5′-TCTCCCCTAGCCTACAGGGTTC-3′; reverse, 5′-GGTGAAAAAATCCAGCTCTTCCT-3′; 71-bp band product), *HOXB5* (forward 5′-GAGATCGCCCACGCACTCT-3′; reverse, 5′-TTCATGCGCCGGTTCTG-3′; 68-bp band product), *HOXB6* (forward 5′-GCCCCCTGCCCCAAT-3′; reverse, 5′-CCCAATCTTCTTCTATCTCCTAACTGA-3′; 67-bp band product), *HOXC5* (forward 5′-CCCAGAGTCAATAAATAGTTGGACAA-3′; reverse, 5′-CCATAGTCCCTGCCACGAAT-3′; 71-bp band product). We confirmed that the primer pairs amplified the expected HOX genes by cloning and sequencing the PCR products. The absence of nonspecific amplification was confirmed either by running dissociation curves or examining PCR amplification products by agarose gel electrophoresis. To determine the optimal primer concentrations, which gave the highest levels of specific products and minimal nonspecific DNA and primer-dimer products, we performed a primer optimization as described in the manufacturer's protocol. RT and amplification were performed by using one-step RT-PCR. A 50-μl reaction mix contained 5 μl of 10× SYBR PCR buffer, 5 μl of template, 6 μl of 25 mmol/L $MgCl_2$, 6 μl of 10 mmol/L dNTPs, 1 μl of each primer, 1 μl of RNase inhibitor, and 25 μl of RNase-free H_2O. All reagents used in the RT-PCR were purchased from Perkin-Elmer. The RT reaction was performed for 30 minutes at 48°C using MultiScribe reverse transcriptase (final concentration, 1.25 U). AmpliTaq Gold Polymerase (final concentration, 1.25 U) was activated by incubating the reactions at 95°C for 10 minutes followed by 40 cycles of amplification (95°C for 15 seconds and 60°C for 1 minute). The TaqMan ribosomal RNA control reagents (Perkin-Elmer) were used as a standard to quantify the initial concentration and ascertain the quality of the total RNA and to assure reproduc-

ibility of the RT-PCR. Human total RNA dilutions from 10 ng/μl down to 0.0001 ng/μl were used as templates to create a standard curve. Each experiment was run twice in duplicate and the data reported are the average results for duplicate samples from each of the two experiments. The initial amount of rRNA in the sample was assessed using a threshold cycle (Ct) value. The Ct value is defined as the first cycle at which a statistically significant increase is detected in the difference between the emission intensity of the reporter dye in the sample and the emission intensity of the controls without RNA. This value is then used to compare one sample value to another. To quantitate the copy numbers of HOX genes present in the lung samples, we used plasmids (pGEM-T vector systems, JM 109 cells; Promega Corp.) containing cloned HOXA5, HOXB2, HOXB5, HOXB6, and HOXC5 sequences in concentrations ranging from 0.1 ng to 0.0001 ng/μl. We compared the Ct of the unknown samples against the standard curve of the plasmid with known copy numbers. Plasmids and lung samples were run in duplicate and a control reaction without reverse transcriptase was also run for each sample. In addition, reactions without RNA were used to establish baseline levels for fluorescence. Five μl (300 ng of total RNA from lung tissue samples) was used as a template for the one-step RT-PCR.

In Situ *Hybridization*

We used fresh adult lung fragments frozen in Tissue Tek embedding medium (Sakura, Torrance, CA) and maintained at −70°C until sectioning, and paraffin-embedded fetal lung tissue (pseudoglandular and canalicular stage). Seven-mm cryostat sections were mounted onto Superfrost slides (Fisher Scientific, Pittsburgh, PA). At least three different sections of each lung were processed for *in situ* hybridization. HOXA5, HOXB2, and HOXB6 were PCR amplified as described before and cloned into the pGEM T vector (Promega Corp.). Digoxigenin-labeled antisense and sense cRNA probes were synthesized from the T7 and SP6 polymerase promoters, respectively (Genius IV kit; Boehringer Mannheim Biochemicals, Indianapolis, IN). Paraffin sections (fetal lung) and frozen sections of normal human adult lung ($n = 3$), emphysematous lungs ($n = 3$), and lungs with severe pulmonary hypertension ($n = 3$) were submitted to *in situ* detection of HOXA5, HOXB2, and HOXB6 mRNA according to the protocol by Crabb and colleagues[28] and Boehringer Mannheim's guide for filter hybridization. The slides were prehybridized with 50% formamide hybridization cocktail (Amresco, Solon, OH) for 2 hours at room temperature. Hybridization (probe concentration, 400 ng/ml) was performed at 42°C overnight. Immunodetection of the hybridized product was performed with a sheep anti-digoxigenin antibody at 1:2,000 dilution (Boehringer Mannheim). The color solution consisted of 4-tetrazolium chloride, 4.5 μg/ml, and X-phosphate, 3.5 μl in buffer 3, according to the manufacturer's protocol (Boehringer Mannheim). The reaction was allowed to develop for ~7 hours. The slides were then mounted in Immuno-mount (Shandon, Pittsburgh, PA).

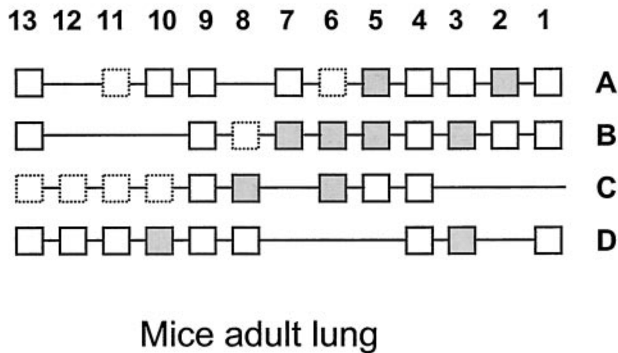
Statistics

All data underwent variance analysis and significant differences between two groups were consecutively compared using Student's *t*-tests. Differences were considered significant at $P < 0.05$ and the values represent the mean ± SD.

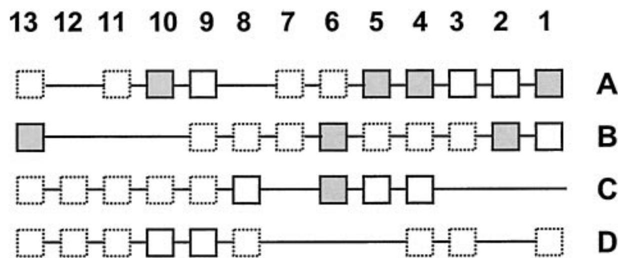
Results

To survey the expression of HOX genes in human lung tissue, we used a microarray gene chip technique (Hu GeneFL array, Affymetrix gene chip). We screened three normal human adult lungs, three emphysematous lungs, three lungs with severe pulmonary hypertension, and a sample of pooled fetal lung tissue (12 weeks of gestation age). To compare the expression of HOX genes in human and mouse lung, we additionally screened lung tissue from adult mice (pooled samples of five FVB/N mice) using a murine-specific microarray (Mu 6500, Affymetrix gene chip). The human microarray contained probes for 17 of the 39 known HOX genes (murine microarray contains 32 out of 39 known Hox genes). The 17 HOX genes represented on the human microarray chip were also represented on the murine microarray, allowing thus a direct comparison of the corresponding HOX gene lung expression between species. Figure 1 demonstrates the expression pattern of HOX genes in normal adult human and mouse lung tissue. Of the 17 HOX genes only three HOX genes were detected in both mice and human lungs (HOXA5, HOXB6, HOXC6). However, five HOX genes were detected in human lungs but not in mice lungs (HOXA1, HOXA4, HOXA10, HOXB2, and HOXB13). Three Hox genes (Hox-A2, Hox-C8, and Hox-D10) were only detected in the mouse but not in the normal human-adult lung tissue. Table 1 shows the expression pattern by microarray technique of HOX genes in human normal adult lung, fetal, emphysematous, and PPH lung tissue. Of the 17 HOX genes represented on the chip, six HOX genes (HOXA4, HOXA5, HOXB2, HOXB6, HOXC6, and HOXC8) were expressed in fetal lung tissue. In normal human-adult lung tissue, eight HOX genes were detected. Compared to the fetal lung, HOXC8 was not expressed in adult lung tissue and three additional HOX genes (HOXA1, HOXA10, and HOXB13) were present in adult but not fetal lung tissue. Interestingly, the expression pattern of HOX genes in normal adult lung tissue was not consistent in all three examined lungs. For example HOXA1 was detectable in one lung sample but it was below the detection level in the other two samples. HOXA4, HOXA5, and HOXC6 were detected in two out of three samples of normal adult lung tissue. A variation of HOX gene expression was also noted in the samples of emphysematous and PPH lung tissue. In lung emphysema only HOXC8 was present in each of three examined tissue samples. HOXA5, HOXB2, and HOXB13 were detected in two tissue samples whereas HOXA4 was present in only one of the samples. In PPH lung tissue HOXB2 was detected in two of three samples (HOXA5, HOXA10, HOXB13 = one of three samples). Compared

Microarray-survey



Mice adult lung



Human normal adult lung

Figure 1. HOX gene expression pattern in normal mouse lung (**top**) and human adult lung tissue (**bottom**) assessed by the microarray technique (Affymetrix gene chip). Each **solid box** marks a HOX gene represented on the array, whereas the **dotted line boxes** represent the genes that are not included on the array. The order of genes within a cluster is depicted in the 5′ to 3′ orientation and paralogous genes are aligned vertically. The **shaded boxes** represent the genes that were expressed.

Table 1. HOX Genes in PPH and Emphysema

	Fetal lung (12 weeks) pooled samples	Normal adult lung $n = 3$	Emphysema $n = 3$	PPH $n = 3$
HOXA1	a	p (1/3)	a	a
HOXA2	a	a	a	a
HOXA3	a	a	a	a
HOXA4	p	p (2/3)	p (1/3)	a
HOXA5	p	p (2/3)	p (2/3)	p (1/3)
HOXA9	a	a	a	a
HOXA10	a	p (3/3)	a	p (1/3)
HOXB1	a	a	a	a
HOXB2	p	p (3/3)	p (2/3)	p (2/3)
HOXB6	p	p (3/3)	a	a
HOXB13	a	p (3/3)	p (2/3)	p (1/3)
HOXC4	a	a	a	a
HOXC5	a	a	a	a
HOXC6	p	p (2/3)	a	a
HOXC8	p	a	p (3/3)	a
HOXD9	a	a	a	a
HOXD10	a	a	a	a

a, absent; p, present; *n*, number of samples.

to the normal adult lung, a reduction in the number of detectable HOX genes was noted for fetal, emphysematous, and PPH lung tissue. Only four of the 17 represented HOX genes were detectable in PPH lung tissue (emphysema, 5 of 17; fetal lung, 6 of 17) whereas eight HOX genes were detected in normal adult lung tissue. Among those eight HOX genes, only HOXA5 and HOXB2 were detectable in all examined adult lungs.

To provide an independent confirmation of the expression pattern found by the microarray gene chip technique, we used a degenerate RT-PCR technique followed by cloning and DNA sequencing. Figure 2 shows the expression pattern of HOX genes found in fetal lung tissue (pooled sample, 12 weeks of gestation age), human normal adult lung ($n = 2$), emphysematous lung ($n = 3$), and PPH lung tissue ($n = 3$). Twenty-seven individual clones derived from fetal lung tissue were sequenced revealing the expression of seven different genes from clusters A, B, C, and D. The most frequently detected transcript was HOXA5 (11 of 27). Forty-seven clones originating from normal adult lung were sequenced (emphysema = 51, PPH = 47 sequenced clones). In normal adult lung tissue, nine HOX genes were detected belonging to clusters A and B. Among these genes, HOXA5 (21 of 47), HOXB2 (13 of 47), and HOXB6 (7 of 47) were the most frequently detected transcripts. HOX genes of the 5′ end (HOX10 to HOX13) were not detected by the degenerate RT-PCR technique. Similar to the normal adult lung, HOXA5 and HOXB2 were the most frequently detected transcripts in PPH and emphysematous lung tissue but HOX genes of the 5′ end (HOX10 to HOX13) were not detected in these tissues. In contrast to the normal adult lung, 19% of the cloned inserts derived from emphysematous lung tissue revealed HOX genes from clusters C and D (PPH = 14%). In this respect PPH and emphysematous lungs shared similarities to fetal lung tissue because 22% of the cloned inserts in fetal lung tissue represent HOX genes from clusters C and D.

Since using the degenerate RT-PCR survey-technique, HOXA5, HOXB2, and HOXB6 emerged as the HOX genes with the highest expression in the normal human adult lung, we focused on these HOX genes for subsequent studies. We quantified the level of mRNA expression in human fetal and adult lung tissue by quantitative RT-PCR. Expression was displayed as copy numbers per 300 ng total RNA of extracted lung tissue. In fetal lung tissue (12 weeks) HOXA5 was most abundantly expressed (1,082,000 copies), followed by HOXB2 (642,600 copies), and HOXB6 (327,000 copies) (Figure 3; A, B, and C). In the adult lung (normal, PPH, and emphysema, $n = 6$ each) the highest expression was again noted for HOXA5, followed by HOXB2 and HOXB6. HOXA5 expression was 2.5-fold increased in PPH lungs (663,400 copies) when compared to normal adult lung tissue (251,400 copies). The lowest expression for HOXA5 was detected in emphysematous lungs (164,800 copies). However, a wide variation of HOXA5 expression

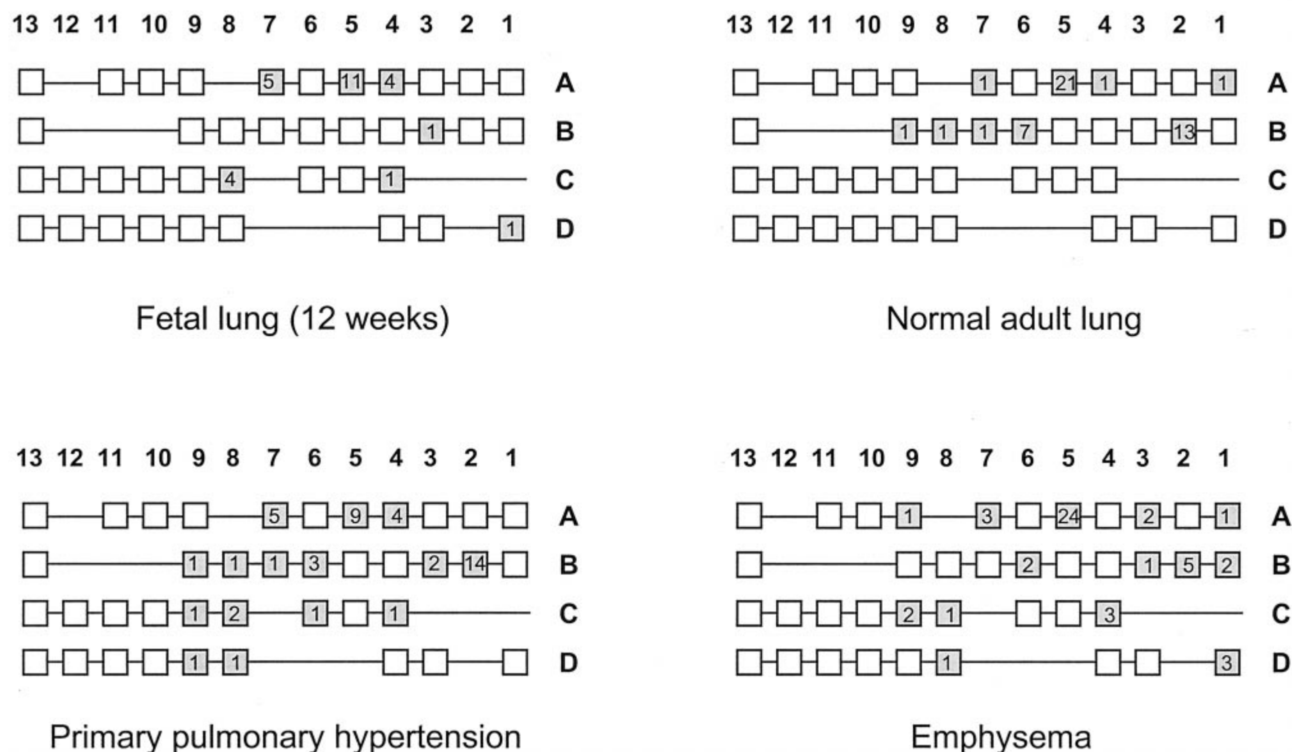


Figure 2. HOX gene expression pattern in the fetal lung (12 weeks of gestation age, pooled sample), normal adult (n = 2), and diseased human lungs (emphysema, n = 3; PPH, n = 3) assessed by RT-PCR using degenerate primers for the HOX domain. The products were cloned into bacteria and sequenced. Each box represents a known HOX gene. The order of genes within a cluster is depicted in the 5′ to 3′ orientation and paralogous genes are aligned vertically. The **open boxes** represent HOX genes that were not identified by RT-PCR, whereas the **shaded boxes** represent genes that were detected. The frequency at which positive clones were identified is expressed by the number in the **shaded box**. Twenty-seven clones originating from the fetal lung were sequenced (normal adult lung, 47 clones; emphysema, 51 clones; PPH, 47 clones).

was noted within the groups of lung samples. The number of HOXA5 transcripts ranged from 333,230 to 1,271,000 copies/300 ng total RNA in PPH lungs (emphysema, 10,630 to 434,400 copies; normal adult lung, 58,590 to 725,300 copies). The number of HOXB2 transcripts in adult lung tissue (normal, PPH, emphysema) was slightly lower than that for HOXA5. Similar to HOXA5, highest expression for HOXB2 was noted in PPH lungs (398,100 copies) followed by normal adult lung (181,500 copies) and emphysematous lung tissue (104,000 copies) (Figure 3B). In contrast to HOXA5 and HOXB2, transcripts for HOXB6 were below 100,000 copies/300 ng total RNA either in normal or diseased lung tissue (Figure 3C).

Because it is known that a functional relationship exists between paralogous HOX genes in that their protein products are functionally equivalent,[29,30] we investigated the expression pattern of the paralogous genes HOXA5, HOXB5, and HOXC5 by quantitative PCR. The number of transcripts for HOXB5 and HOXC5 in human adult lung was <50,000 copies/300 ng total RNA (data not shown). However, 532,440 copies/300 ng total RNA were noted for HOXB5 in fetal lungs (HOXC5, 73,000 copies).

The expression of HOXA5 mRNA in fetal lung (pseudoglandular and canalicular stage), normal human adult lung (n = 3), emphysematous lungs (n = 3), and lungs with severe pulmonary hypertension (n = 3) was evaluated by *in situ* hybridization using a digoxigenin-labeled, antisense cRNA (Figures 4, 5, and 6). The hybridization control consisted of serial lung sections hybridized with the sense cRNA probe (Figures 4D and 5B). Consistent with the overall pattern of HOXA5 expression using quantitative PCR, we found a strong signal for HOXA5 mRNA in pseudoglandular and canalicular stage of fetal lungs by *in situ* hybridization (Figure 4, A to E).

Normal lungs exhibited a high-level HOXA5 hybridization signal in alveolar and bronchial epithelial cells (Figures 5A and 6A). A weaker signal was noted in arterial smooth muscle cells (Figure 6B). However, the endothelial cells exhibited a strong hybridization signal (Figure 6B). Compared to normal lungs, an overall reduction of HOXA5 signal intensity was noted in the examined emphysematous lungs (Figure 5C). However, the cellular pattern of mRNA expression for HOXA5 did not differ from that in normal lungs. An intense hybridization signal for HOXA5 was exhibited in PPH lungs (Figure 5D); similar to normal lungs, the hybridization signal was mainly located to alveolar septal cells (Figure 5D). A strong hybridization signal was also noted in plexiform and concentric lesions (Figure 6C). HOXB2 mRNA was expressed less abundantly than HOXA5 in normal septal

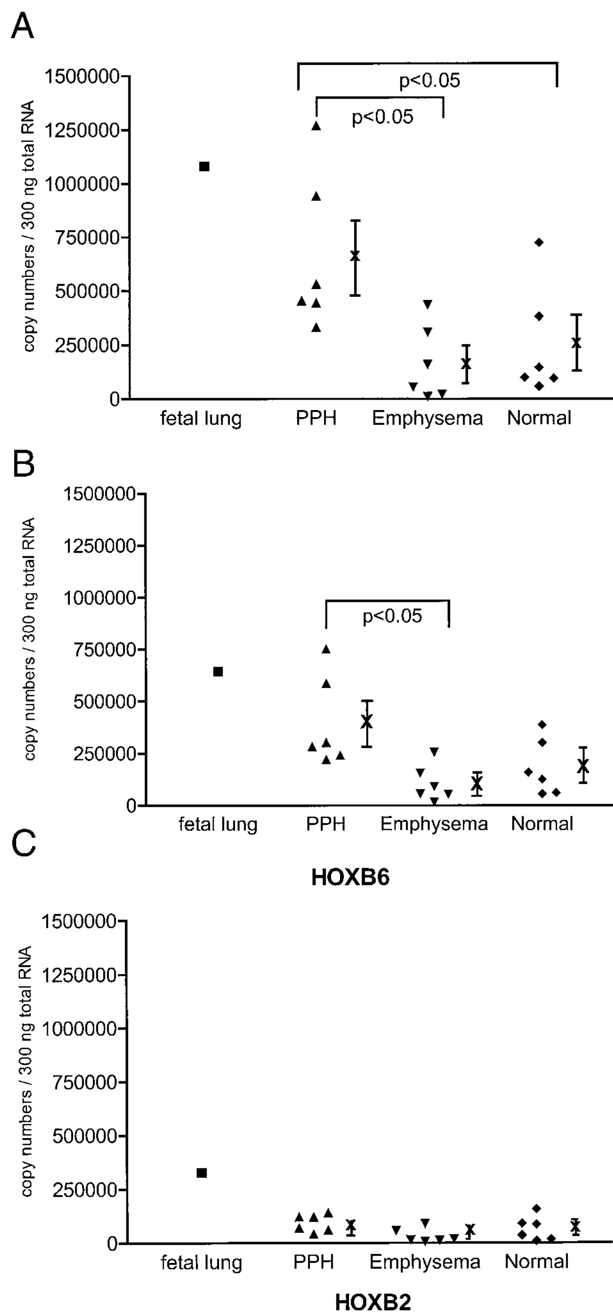


Figure 3. Expression of HOXA5 (**A**), HOXB2 (**B**), and HOXB6 (**C**) in human lung tissues assessed by quantitative RT-PCR. Each data point represents the average result for duplicate samples from the same tissue. The initial amount of RNA was assessed using TaqMan ribosomal RNA control reagents. Samples were quantified by comparison against standard curves with known copy numbers (plasmids containing cloned HOXA5, HOXB2, and HOXB6 sequences). Fetal lung (pool of lungs with gestation age of 12 weeks), PPH ($n = 6$), emphysema ($n = 6$), normal (histologically normal human adult lung tissue, $n = 6$). The mean ± SD of expression for each group is also shown.

epithelial cells (Figure 5E). HOXB6 mRNA was expressed in low levels in similar cellular sites that also expressed HOXA5 and HOXB2 (Figure 5F).

To assess whether the HOXA5, HOXB2, and HOXB6 transcripts were also translated in the lung we performed immunoblots and immunohistochemistry. We used commercially available polyclonal antibodies generated against the peptides derived from the human homeobox gene sequence. However, we became concerned about the specificity of these antibodies because a positive staining signal in the immunoblots was observed in the molecular weight standard carbonic anhydrase. This staining signal was identical to the size of the putative HOX bands when analyzing lung protein extracts. All three antibodies detected in the lung tissue samples, the carbonic anhydrase protein, and the band corresponded to the putative HOX protein bands (data not shown).

Discussion

In the present study, we demonstrate the expression of HOX genes in fetal and adult human lung tissues by using both microchip array and degenerate RT-PCR survey techniques. Differences in the pattern of HOX gene expression were detected by comparing human with mouse lung tissue. In normal human adult lung tissue, predominantly HOX genes from the 3′ end of clusters A and B were expressed. Among these, HOXA5 was the most abundant HOX gene expressed followed by HOXB2 and HOXB6. Additional HOX genes from clusters C and D were expressed in fetal, emphysematous, and PPH lung tissue. We further demonstrated a 2.5-fold increase in HOXA5 expression in PPH lungs when compared to normal lungs. HOXA5 transcripts were mainly located in alveolar and bronchial epithelial cells but also vascular smooth muscle and endothelial cells demonstrated a positive staining signal.

Little information is available regarding the function of homeobox genes in the lung.[12] Most data are derived from animal studies that investigated the expression of homeobox genes during lung development. Approximately 16 Hox genes, predominantly from clusters A and B are expressed in embryonic and newborn mouse lungs, and this expression is affected by the gestational age and retinoic acid.[2,16] Further insights into the function of Hox genes in lungs came from studies of mice carrying disrupted Hox alleles.[20–23] In Hoxa-1/Hoxb-1 double mutants a hypoplasia of lung formation was observed with variable changes ranging from five smaller than normal lobes to the formation of only two lung lobes.[22] Hoxa-3 mutants die shortly after birth, likely because of respiratory failure. They exhibit smaller tracheae and bronchi. The cells of the tracheal lining are disorganized and have lost their columnar appearance.[21] The viability of homozygous Hoxa-5 mutant mice is markedly reduced, with 50% of the mutant animals dying at birth or shortly thereafter. The mutant mice suffer from severe obstruction of the laryngotracheal airways and display respiratory distress at birth with a marked decrease in the production of surfactant proteins. Histologically, a profound disorganization of the tracheal epithelium was observed.[23]

It is not known whether homeobox genes also play an important role in human lung organogenesis and respiratory tract function, although induction of HOXA5 expression by retinoic acid has been demonstrated for cultured human bronchial fibroblasts.[15] Recent studies

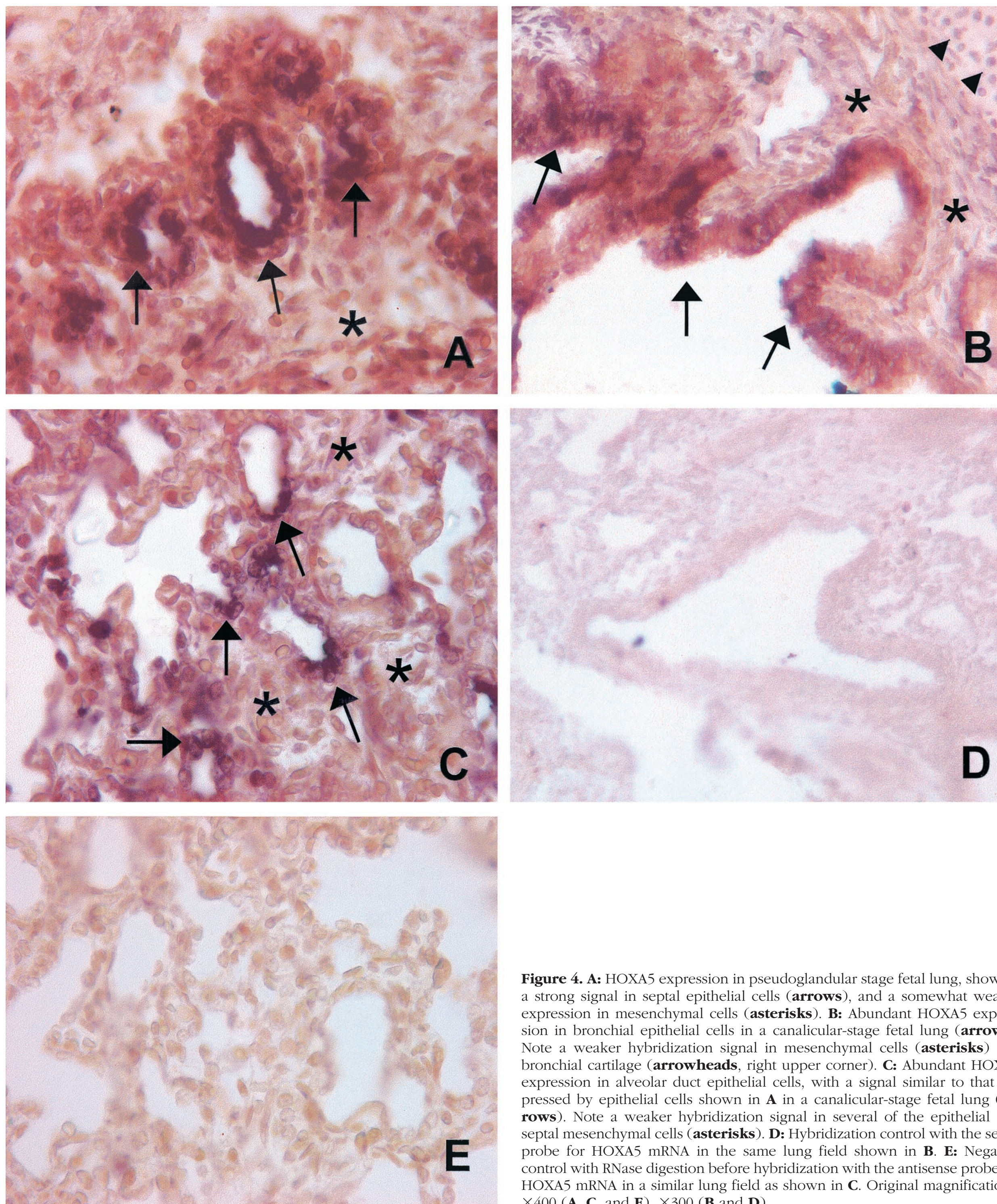


Figure 4. A: HOXA5 expression in pseudoglandular stage fetal lung, showing a strong signal in septal epithelial cells (**arrows**), and a somewhat weaker expression in mesenchymal cells (**asterisks**). **B:** Abundant HOXA5 expression in bronchial epithelial cells in a canalicular-stage fetal lung (**arrows**). Note a weaker hybridization signal in mesenchymal cells (**asterisks**) and bronchial cartilage (**arrowheads**, right upper corner). **C:** Abundant HOXA5 expression in alveolar duct epithelial cells, with a signal similar to that expressed by epithelial cells shown in **A** in a canalicular-stage fetal lung (**arrows**). Note a weaker hybridization signal in several of the epithelial and septal mesenchymal cells (**asterisks**). **D:** Hybridization control with the sense probe for HOXA5 mRNA in the same lung field shown in **B**. **E:** Negative control with RNase digestion before hybridization with the antisense probe for HOXA5 mRNA in a similar lung field as shown in **C**. Original magnifications: ×400 (**A**, **C**, and **E**), ×300 (**B** and **D**).

suggest that HOX genes could be involved in tumorigenesis in the lung.[13,14] Tiberio and colleagues[14] conducted complete surveys of the expression of HOX genes in small-cell lung cancers that had been xenografted into nude mice. The authors also studied the expression pattern of HOX genes in a normal lung sample using Northern blotting techniques. In keeping with our survey data, almost all expressed HOX genes detected were from the 3′ end of clusters A and B.[14] This expression pattern is not surprising because the expression domains of these HOX genes have been localized to cervical and thoracic regions. As in our investigation, HOXA5 was most abundantly expressed, followed by HOXB2, HOXB6, and HOXA3.[14]

To examine the expression pattern of HOX genes in human lungs, we applied two independent survey tech-

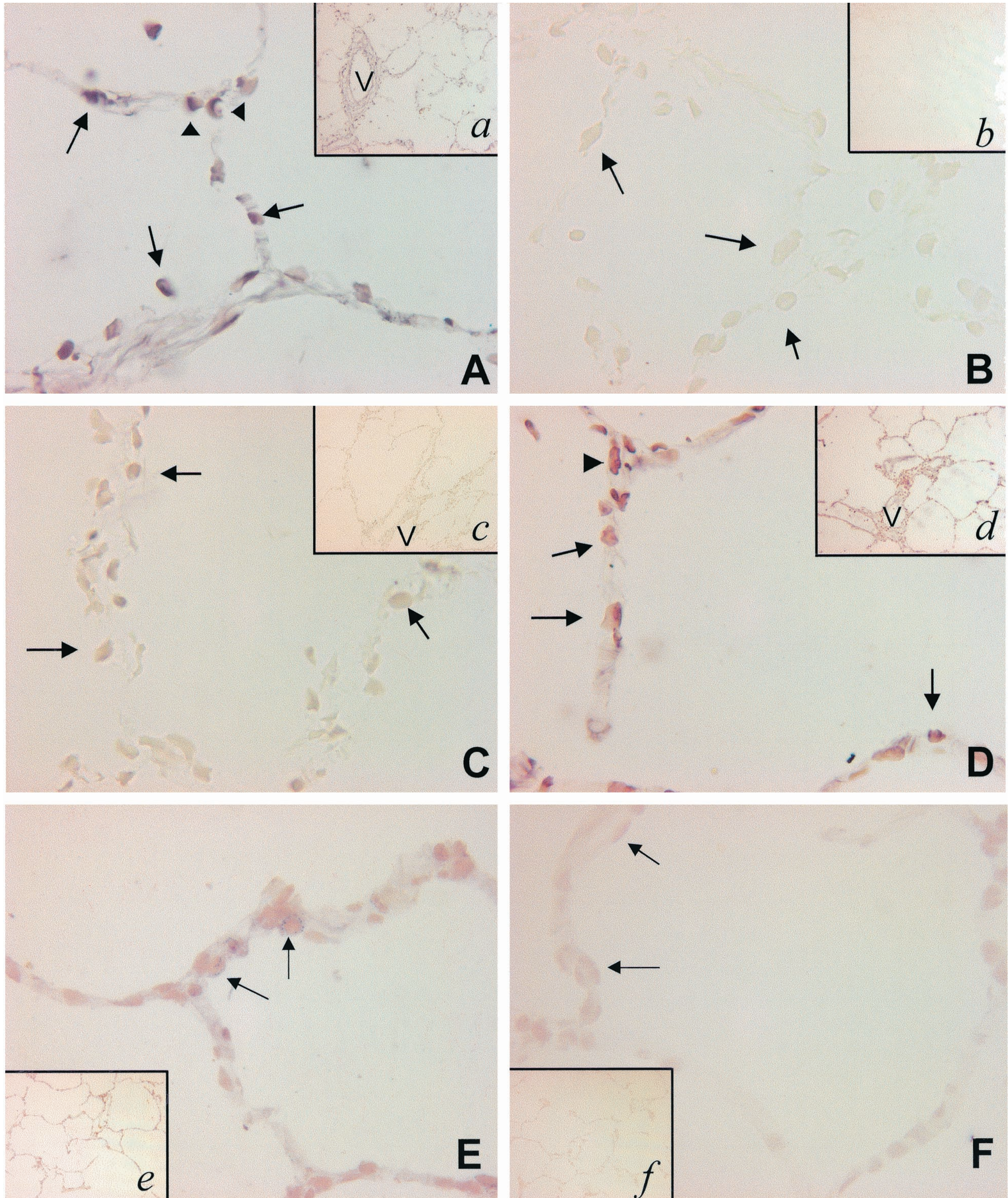


Figure 5. A: Normal lung shows HOXA5 mRNA expression in septal epithelial cells (**arrows**), including type II pneumocytes (**arrowhead**). The hybridization signal appears as a dark-blue reaction in both the cytoplasm and overlying the nuclei. **Inset a** shows a low-power view, which highlights the overall HOXA5 expression both in alveolar septa and pulmonary vascular wall (V). **B:** Hybridization control with the sense probe for HOXA5 mRNA in the same lung field shown in **A**. **C:** Emphysema lung shows overall decreased HOXA5 mRNA expression, both in alveolar septal cells (**arrows**), and in vascular wall (**inset c**). **D:** PPH lung demonstrates intense hybridization signal for HOXA5 along alveolar septa (**arrows**), including type II pneumocytes (**arrowhead**), and vascular structures (**inset d**). Original magnification: ×400; **insets**: ×100 (**A**, **C**, **D:** antisense, digoxigenin-labeled probe; **B:** sense). **E:** HOXB2 mRNA expression in alveolar septal cells in normal adult lung tissue. **Inset e** shows the overall expression pattern of HOXB2 mRNA. The expression pattern is similar to that seen with HOXA5. **F:** HOXB6 mRNA expression in alveolar septal cells in normal adult lung tissue. **Inset f** shows the overall expression pattern of HOXB6 mRNA, which is markedly reduced when compared with that shown for HOXA5 and HOXB2 mRNA. Original magnifications: ×400; **insets**, ×100 (**A**, **C**, **D**, **E**, **F:** antisense, digoxigenin-labeled probe; **B:** sense).

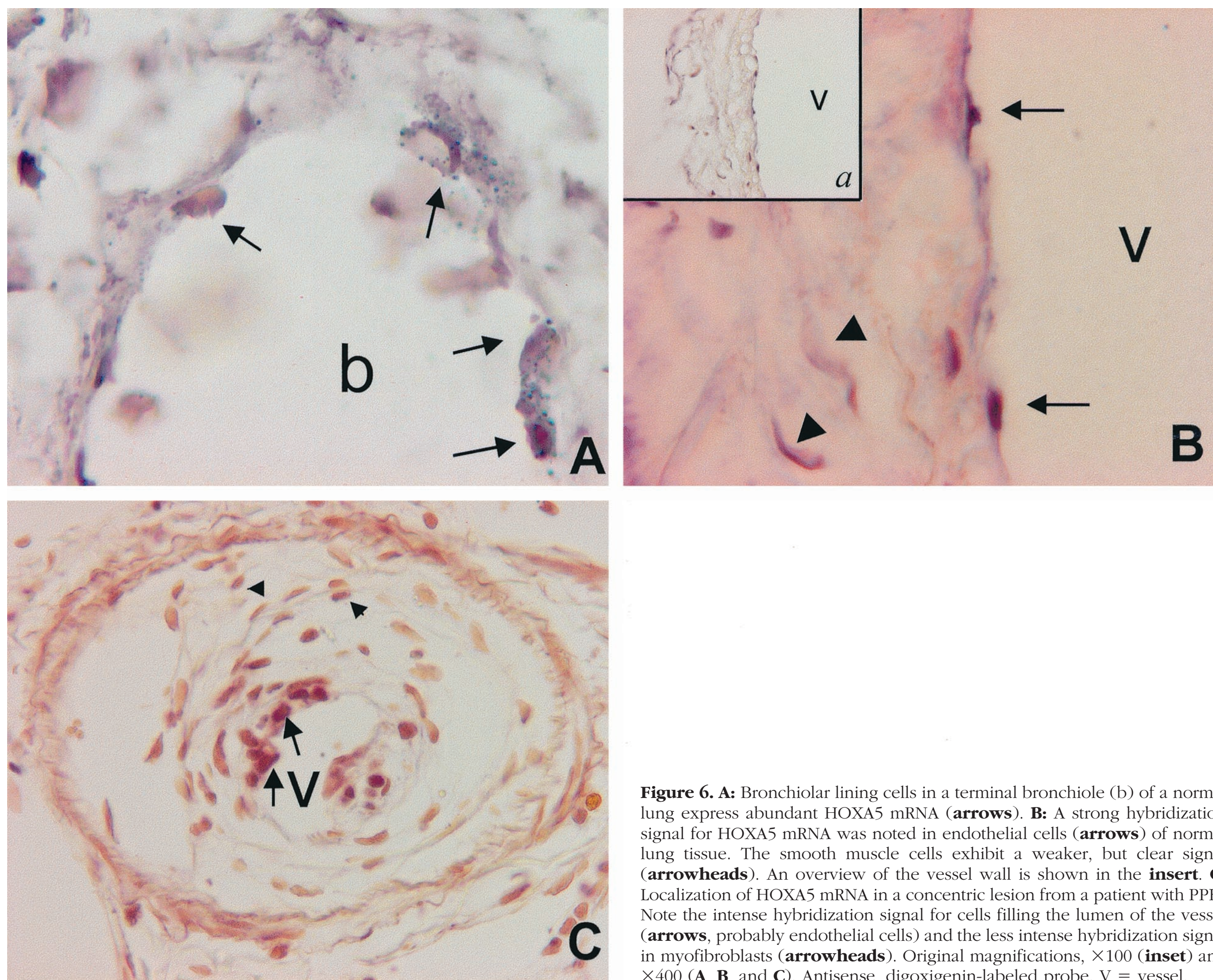


Figure 6. A: Bronchiolar lining cells in a terminal bronchiole (b) of a normal lung express abundant HOXA5 mRNA (**arrows**). **B:** A strong hybridization signal for HOXA5 mRNA was noted in endothelial cells (**arrows**) of normal lung tissue. The smooth muscle cells exhibit a weaker, but clear signal (**arrowheads**). An overview of the vessel wall is shown in the **insert**. **C:** Localization of HOXA5 mRNA in a concentric lesion from a patient with PPH. Note the intense hybridization signal for cells filling the lumen of the vessel (**arrows**, probably endothelial cells) and the less intense hybridization signal in myofibroblasts (**arrowheads**). Original magnifications, ×100 (**inset**) and ×400 (**A**, **B**, and **C**). Antisense, digoxigenin-labeled probe. V = vessel.

niques. We started with a microarray technique using a human-specific gene chip from Affymetrix, which provided information about 17 of the 39 known clustered HOX genes. We additionally performed a microarray survey of mouse lung tissue using a murine-specific microarray from Affymetrix. This gene chip contains probes for 32 of the known 39 mice Hox genes. Because all of the 17 HOX genes from the human chip were also represented on the murine chip, we compared the HOX gene expression pattern in human and mouse lungs. Even if the microarrays do not provide information about the complete set of the 39 HOX genes, it is evident that HOX gene expression differs in human and mouse lung tissue. Only three of the 17 HOX genes were detected both in human and mouse lungs. However, five HOX genes were detected in human but not in mouse lungs and three genes were expressed in mouse but not in human lung tissue. These results raise the question whether Hox data derived from animal studies can be translated to the human lung.

We then used a degenerate RT-PCR technique with subsequent cloning of the PCR amplicons and sequencing of the cloned inserts to validate the data generated by the microarray technique. This technique has been used in previous gene expression studies and has provided information about the expression of some of the 39 HOX genes.[16,26] Depending on the number of developed clones, this method also allows a quantitative estimate of gene expression. Both microarray and degenerate RT-PCR studies predominantly detected HOX genes from the A and B clusters in normal human adult lungs. In addition, both techniques identified the expression of HOX genes from the C cluster in fetal and emphysematous lung tissues. However, there were also differences in the pattern of HOX gene expression when we compared both survey techniques. In contrast to the microarray, no HOX transcripts from the 5′ end of clusters A and B were detected with the degenerate RT-PCR technique. It is possible that there is a bias toward preferential amplification of selected HOX genes using degenerate primers. Indeed, the microarray detected HOXA10 and HOXB13 transcripts in all of the examined human normal adult lungs, whereas no bacterial clones containing these HOX genes were detected in our degenerate PCR assays.

Our finding of lung expression of HOXA10 and HOXB13 transcripts is remarkable because the expression domains of these HOX are not localized to cervical and thoracic regions. Interestingly, these HOX genes were not detected in the fetal lung tissue and they were

expressed only in some of the examined emphysematous and PPH lungs. Perhaps the expression of HOXA10 and HOXB13 characterizes the normal adult lung phenotype and altered expression is a feature of a diseased lung. In contrast, the absence of expression of genes from the C and D clusters may characterize the phenotype of the normal adult lung whereas fetal and diseased lung specimens do express these HOX genes. The expression of HOX genes from clusters C and D has been previously demonstrated by Tiberio and colleagues[14] in lung small-cell cancer cells. In preliminary experiments, we also investigated the expression pattern of C cluster HOX genes in fetal, normal, and diseased lung specimens by quantitative RT-PCR. In these studies we found practically no expression of C cluster HOX genes in normal adult lungs but a specific expression pattern in diseased (emphysema, PPH, lung cancer) and fetal lungs (data not shown). Because HOXA5, HOXB2, and HOXB6 emerged as the HOX genes with the highest expression in the human adult lung, we focused on these HOX genes for subsequent studies. To assess whether HOXA5, HOXB2, and HOXB6 transcripts were also translated in the lung and to localize the proteins, we did perform immunoblots and immunohistochemistry. However, we noticed that the commercially available antibodies directed against these HOX proteins lacked specificity because they clearly bind to carbonic anhydrase that is abundantly expressed in lung tissue. At present other commercial sources for antibodies against these HOX proteins are not available. Therefore immunoblots and immunohistochemistry are not being presented in this communication but quantitative RT-PCR and *in situ* hybridization studies, instead. We found the highest copy numbers of HOXA5, HOXB2, and HOXB6 transcripts in fetal lungs. This underscores the important function of these genes in lung development.

Expression of these HOX genes was also detected in human fetal and adult lung tissues suggesting an important role in the development and maintenance of human lung tissue. Using *in situ* hybridization, we localized HOXA5 transcripts predominantly to bronchial and alveolar epithelial cells. This result is in contrast with the finding that Hoxa-5 mRNA is only expressed by mesenchymal cells in the developing mouse lung.[23] We believe that the discrepancy in HOXA5 expression noted for fetal mice and human lung tissues might be explained by species variations or methodological differences in the *in situ* hybridization protocols. However, by using quantitative RT-PCR, we detected HOXA5 transcripts in A549 cells—a human lung epithelial-derived cell line (data not shown). A strong hybridization signal was also noted in type II cells suggesting perhaps a role for HOXA5 in surfactant metabolism. This finding is in accordance with the observation that Hoxa-5 mutant mice die shortly after birth because of a respiratory distress syndrome and these animals show a decreased expression of surfactant-associated proteins.[23] If HOXA5 expression is essential for the expression of surfactant-proteins, we wonder why we found a wide variation in the HOXA5 expression levels in human adult lung specimens ranging from 5 to 67% of the corresponding HOXA5 expression in the fetal lung. None of the patients from whom the normal lung tissue was obtained suffered from respiratory distress. Probably, the lack of HOXA5 expression can be compensated for by other paralogous HOX genes. Recently, it has been postulated that HOX gene products from the same paralogous group are functionally equivalent, despite the divergent protein composition. Therefore, it has been hypothesized that the quantity of proteins within a paralogous group, rather than their quality, modulates the biological action.[29,30] We therefore investigated whether a reduced HOX expression in samples of lung tissue was compensated for by HOX genes from the paralogous group 5 (HOXB5, HOXC5). However, our results show that only HOXA5 was appropriately expressed among the paralogous group 5 genes and variations of HOXA5 mRNA expression were not compensated for by either HOXB5 or HOXC5.

Interestingly, when compared with normal lung tissue, HOXA5 was overexpressed in lungs from patients with PPH, as shown by quantitative RT-PCR. In addition, we noted an intense hybridization signal for HOXA5 mRNA in PPH lungs. Similar to normal lungs, the hybridization signal was mainly located to bronchial and alveolar cells but a strong hybridization signal was also noted in plexiform and concentric lesions. The increased expression of HOXA5 in PPH lungs may be the result of overexpression in endothelial cells. Because HOX genes control fundamental developmental processes such as cell proliferation, differentiation, motility, adhesion, and apoptosis,[3,12,31,32] it is possible that HOXA5 participates in the proliferation of endothelial cells and vascular remodeling in PPH lungs.

In conclusion, we believe that the assessment of microarray and degenerate RT-PCR survey techniques combined with quantitative RT-PCR and *in situ* hybridization gives valid information about the expression of HOX genes in the human lung. We demonstrate that HOX genes predominately from the 3′ end of clusters A and B are expressed in the normal adult human lung. We propose that these genes may have a role in the differentiation and maintenance of the lung structure and that an altered pattern of HOX gene expression may be part of pulmonary diseases.

Acknowledgments

We thank Lori Alger for advice and technical help in performing *in situ* hybridization, Dr. Gail Deutsch for providing us with fetal lung tissue, Tracy Gesell for reading the manuscript, and the thoracic surgery team of the University of Colorado Health Sciences Center, in particular Dr. Marvin Pomerantz and Dr. James Mault for the lung specimens.

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