Growth factors and dexamethasone regulate Hoxb5 protein in cultured murine fetal lungs

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Chinoy, Mala R., Maryann V. Volpe, Robert E. Cilley, Steven E. Zgleszewski, Robert J. Vosatka, Ana Martin, Heber C. Nielsen, and Thomas M. Krummel. Growth factors and dexamethasone regulate Hoxb5 protein in cultured murine fetal lungs. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L610–L620, 1998.—Studies on lung morphogenesis have indicated a role of homeobox (Hox) genes in the regulation of lung development. In the present study, we attempted to modulate the synthesis of Hoxb5 protein in cultured murine fetal lungs after mechanical or chemical stimuli. Murine fetuses at gestational stages in gestation (GD14) were removed from pregnant CD-1 mice, and lungs were excised and cultured for 7 days in BGJb media. The experimental groups were 1) untreated, unligated; 2) tracheal ligation; 3) supplemented media with either epidermal growth factor (EGF; 10 ng/ml), transforming growth factor (TGF)-β1 (2 ng/ml), dexamethasone (10 nm), EGF + TGF-β1, or EGF + TGF-β1 + dexamethasone. After 3 or 7 days, the cultured lungs were compared with in vivo lungs. Immunoblotting analyses showed that ligation, EGF, TGF-β1, and EGF + TGF-β1 downregulated Hoxb5 protein to ~20–70% of Hoxb5 protein levels in unligated, untreated cultured lungs. Furthermore, dexamethasone alone or in combination with EGF and TGF-β1 downregulated Hoxb5 protein by >90% (P < 0.05) signal strength, similar to that seen in GD19 or in neonatal lungs. Immunostaining showed that Hoxb5 protein was expressed strongly in the lung mesenchyme at early stages in gestation. However, by GD19 and in neonates, it was present only in specific epithelial cells. A persistent level of Hoxb5 protein in the mesenchyme after EGF or TGF-β1 treatments or tracheal ligation was noted. Hoxb5 protein was significantly downregulated by EGF + TGF-β1, and it was least in lungs after dexamethasone or EGF + TGF-β1 + dexamethasone treatment. The decrease in Hoxb5 protein was significant only in the groups with dexamethasone added to the media. Thus immunostaining results parallel those of immunoblotting. The degree of Hoxb5 downregulation by dexamethasone or EGF + TGF-β1 + dexamethasone was similar to that seen in vivo in very late gestation, which correlated to the advancing structural development of the lung.

The relevance of Hox genes to lung development is becoming increasingly evident; however, a role for Hox transcription factors in regional specification and/or tissue-specific cell differentiation in lung development remains to be characterized. Hox genes of the paralogous groups 2–6 have been detected in embryonic lungs and the lung proper. The lung arises from the foregut in a region where Hoxb3–Hoxb5 are highly expressed, thus suggesting that all three genes are important in the initiation of lung morphogenesis. Aberrant expression of Hox genes has been associated with both morphological abnormalities and oncogenesis (38).

Recently, Shephard et al. (34) and Bogue et al. (7) demonstrated by Northern blot analysis of both fetal mouse and rat lung that mRNA levels for Hox genes a5, b5, b6, and b8 are gestationally regulated and decrease with advancing gestational age. Recent data have also indicated that expression of Hox genes during lung development in the rat and mouse is temporally and spatially regulated in a manner that is colinear with gene order on each chromosome (39, 40). Furthermore, Bogue et al. (8) confirmed earlier findings (22, 41) that the expression of Hox genes early in lung development is restricted to mesenchymal cells. They focused on four genes that are sequentially located in the Hoxb cluster: Hoxb2–Hoxb5. They demonstrated that an embryonic day 9.5 of development in the mouse, Hoxb2–Hoxb5 are...
expressed in the branchial arches and developing foregut such that Hoxb2 was expressed in more rostral regions that develop into the pharynx, whereas Hoxb3, Hoxb4, and Hoxb5 were localized progressively caudally along the foregut axis. In the region of the prospective lung bud, Hoxb2 expression was weak, whereas Hoxb3, Hoxb4, and Hoxb5 expressions were stronger. By embryonic day 10.5 of development, Hoxb3 and Hoxb4 were expressed in proximal and distal rat lung, whereas Hoxb5 was restricted to the distal lung. Volpe et al. (39) evaluated the level of Hoxb5 protein in mouse lungs from embryonic day 13.5 to postnatal day 2 and showed that, with advancing gestation, Hoxb5 protein became restricted to subepithelial fibroblasts and adjacent columnar and cuboidal epithelial cells of conducting airways. These results suggest a function for Hox genes in the mesenchyme and possibly in mesenchymal-epithelial cell interactions. Interactions of the mesenchyme (mesodermal origin) and epithelial cells (endodermal origin) are believed to be critically important for the differentiation of epithelial cell types in the lung (37, 42).

Despite a growing number of experiments providing insight into the crucial role of Hox genes in development and differentiation of tissues, virtually nothing is known about the regulatory mechanisms involving Hox genes in the developing lung. For more than three decades, proximal lung mesenchyme and distal lung mesenchyme have been known to differ in their ability to support branching morphogenesis in vitro (2, 3). Although the specific molecular interactions that occur between lung mesenchyme and epithelium are not known, it has been suggested that there is a different combination of Hox genes expressed in the mesoderm of the trachea and main stem bronchi than in the mesoderm of the distal lung. A functional role for mesenchymal-epithelial cell interactions in lung development was suggested as early as 1970 (37, 42), when it was indicated that the interactions of the mesenchyme and epithelial cells were critically important for the differentiation of lung epithelial cell types. Several recent reports support these early observations.

We have previously demonstrated that murine lung development in whole organ culture may be accelerated through the use of mechanical stimuli (increased airway pressure secondary to bronchial or tracheal ligation) (6) and by the application of combinations of growth factors and hormones [epidermal growth factor (EGF)+transforming growth factor (TGF)-β1 and EGF + TGF-β1 + dexamethasone] (12, 15). In the present study, we hypothesized that in early lung development Hoxb5 is involved in tissue-specific differentiation of the lung mesoderm and epithelia and that later in lung development Hoxb5 protein is downregulated under conditions that induce maturation.

Recently, Bogue et al. (7) reported distinct patterns of expression of various Hox genes that suggest the role of Hox genes in specification of differentiation of proximal versus distal development of the lung. Distinct regulatory elements may control the expression of Hox genes in specific regions of the developing lung. Studies are warranted to isolate and identify these regulatory elements. The identification of factors that enhance or repress the spatial and temporal expression of Hox genes in the lung will then open avenues for molecular investigation of the components that may be involved in the regulation of lung development.

In this study, our goal was to identify factors responsible for the downregulation of Hoxb5 in cultured lungs and their relationship to lung development and maturation. We compared the effects of these factors in cultured lungs with normal in vivo lung development.

**MATERIALS AND METHODS**

**Animals**

Time-dated pregnant CD-1 mice (Charles River Laboratories, Wilmington, MA) were euthanized by an overdose of halothane on gestational day 14 (GD14). The finding of a vaginal plug was counted as day 0. Animal use and method of euthanasia were approved by the Institutional Animal Care and Use Committee and conform to federal guidelines for animal care and euthanasia. The fetuses were harvested by laparotomy under sterile conditions and placed in cold Hanks’ solution (on ice). The fetuses were dissected free of embryonic membranes, and a median sternotomy was performed under a dissecting stereomicroscope. The heart–lung units along with the trachea and larynx were excised. The hearts were then removed, and the tracheas were transected, allowing free egress of airway fluid (unligated). In some animals, the trachea was ligated with a 10.0 nylon suture, preventing egress of airway fluids during the period of organ culture.

**Culture Techniques**

The lungs were placed individually onto 0.45-µm pore size 5-mm² sterile membranes (Gelman Sciences, Ann Arbor, MI) and placed on sterile stainless steel wire mesh screens suspended in an organ culture dish filled with BGJb (GIBCO, Grand Island, NY) culture medium (Fitton-Jackson modification). The screens were placed in the culture wells such that each lung was at the air-medium interface, allowing surface tension to continually bathe the organ in a thin film of fluid. The BGJb culture medium was supplemented with penicillin G (100 U/ml), streptomycin (0.1 mg/ml), amphotericin B (0.25 mg/ml), and sodium ascorbate (1 mg/ml), pH 7.4. The organ cultures were incubated for 3 or 7 days in a 95% air–5% CO₂ environment at 37°C. Lungs were cultured in the following groups: 1) unligated, untreated lung (control) in BGJb media with no added factors, 2) tracheal ligation, 3) EGF, 4) TGF-β1, 5) dexamethasone, 6) both EGF and TGF-β1, or 7) the combination of EGF + TGF-β1 + dexamethasone.

Fresh medium was added daily. Most of our previous experiments were done on lungs at 7 days in culture; therefore, a 7-day period was chosen as an experimental time point, and another time point at 3 days was chosen because most of the morphological alterations are distinct by 3 days in culture.

**Growth Factors and Dexamethasone**

TGF-β1 (2 ng/ml, porcine; R&D Systems), EGF (10 ng/ml, EGF culture grade; Collaborative Biomedical Products), or dexamethasone (10 nM; Sigma) alone or in combination was added to the culture media daily for 3 or 7 days. For the combination treatments, the doses of each growth factor and hormone were the same as for the individual treatments. The doses chosen were based on our earlier studies (12), which
have shown that 10 ng/ml of EGF have a maximal proliferative effect on lungs in culture and that 2 ng/ml of TGF-β1 have inhibitory effects on branching morphogenesis of airways in lungs. A higher dose of TGF-β1 could be very inhibitory. The dose for dexamethasone treatment was also based on our earlier studies (14, 15), in which it was known to increase surfactant proteins and increase maturation of type II cells.

After 3 or 7 days in culture, the lungs were removed and processed for immunohistochemical and immunoblotting analyses. The results are summarized from three separate experiments.

Antibody Production

An affinity-purified rabbit polyclonal anti-mouse antibody to Hoxb5 protein was used as the primary antibody in the immunostaining and Western blot studies. The cDNA encoding the glutathione-S-transferase (GST)-Hoxb5 fusion protein used to develop this antibody was generously contributed by Drs. Nancy Wall and Brigid Hogan of Vanderbilt University. The protocol used was as described earlier by Volpe et al. (39), a modification of a method by Wall et al. (41). Plasmid pGex/c2.1A was transfected into HB 101 and grown in Luria-Bertani broth. The GST-Hoxb5 fusion protein was induced with isopropylthiogalactoside. Bacteria were harvested by centrifugation. The bacterial pellets were then lysed by sonication in 10% Triton X-100, and the Hoxb5 fusion protein was purified by affinity chromatography on a glutathione-agarose column (Bio-Rad, Richmond, CA). The purified protein was eluted with 5 mM glutathione. After preimmune serum was obtained and low background immunoreactivity to murine tissue was screened for by ELISA, New Zealand White rabbits were injected subcutaneously with 1 mg of Hoxb5 fusion protein homogenized in Freund's complete adjuvant. Subsequent injections were with incomplete Freund's adjuvant and 0.3 mg of Hoxb5 protein. Test bleed was obtained until the animals were producing high-titer antisera to the Hoxb5 fusion protein.

Production bleed provided quantities of antisera that were then partially purified by precipitation with 30% saturated ammonium sulfate. The antisera were affinity purified by first removing antibodies reactive to the GST moiety by flowing the antisera over a column to which the GST moiety had been affixed (Affigel 10; Bio-Rad). The resulting flow-through was then affinity purified on a column to which the immunizing antigen had been affixed. After extensive washing, the Hoxb5-specific immunoglobulins were eluted with 1 M glycine, pH 2.5, and rapidly neutralized by titration with 1 M Tris, pH 8.5. The purified immunoglobulins were reactive to the Hoxb5 fusion protein. On Western blots of murine lung proteins, two Hoxb5-specific bands with apparent molecular masses of 38 and 40 kDa reacted with antibody to the Hoxb5 protein. The specificity and characteristics of this Hoxb5 antibody were tested and were identical to the Hoxb5 antibody originally produced from this Hoxb5 fusion protein (41) (see Fig. 1C).

Immunoblotting

Three lungs from each condition were pooled and homogenized on ice in PBS (pH 7.5) with protease inhibitors (1 µM of aprotinin, 2 µM of antipain, and 2 µM of leupeptin). Adult mouse heart tissue was also similarly homogenized to act as a negative control because the murine heart does not express Hoxb5 protein (26). Total protein concentrations were analyzed by protein microassay (9). The homogenates were then mixed with lysis buffer (62 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, and 5% β-mercaptoethanol) (39, 41) and heated at 80°C for 5 min to denature proteins. Ten micrograms of total lung protein from each sample were separated on a 12% polyacrylamide gel by PAGE (27). The separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then incubated with 1% gelatin to block nonspecific binding sites and incubated overnight at 4°C with a 1:200 dilution of rabbit polyclonal anti-mouse Hoxb5 IgG antibody. Control blots were incubated simultaneously with preimmune rabbit sera at the same IgG concentration as the primary antibody. The polyvinylidene difluoride membranes were then reacted with goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN), and the antigen-antibody complexes were detected with 4-nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate. Relative changes in the expression of Hoxb5 protein were evaluated during in vivo development and with different culture conditions. Results were quantified by densitometry, normalized to GD14 lung samples on each blot, and then compared with the untreated, unligated lung samples on each blot as control.

Immunohistochemistry

Immunostaining of Hoxb5 protein was performed with a standard protocol used in our laboratory (13). Briefly, the lungs, either freshly harvested or after 3 or 7 days of culture, were fixed in freshly prepared, filtered 4% paraformaldehyde for 3 h at 4°C. They were then transferred to 30% sucrose prepared in PBS and stored overnight at 4°C. The lungs were individually embedded in CRYOform (International Equipment, Needham, MA), quick-frozen in liquid nitrogen, and stored at −80°C until the cryosections were ready to be cut. Serial 6-µm coronal cryosections were cut, mounted on slides, air-dried for 5 min, and fixed in cold acetone for 2 min. The slides were stored at −80°C until immunostaining was performed. For immunostaining, the slides were rinsed in PBS for 5 min. Endogenous peroxidase activity was quenched with hydrogen peroxide, and then the sections were blocked with normal goat serum (1:20). The primary antibody to Hoxb5 protein, an affinity-purified rabbit polyclonal anti-mouse IgG, was used at a dilution of 1:200. The sections were incubated for 90 min with primary antibody. The slides were then rinsed in PBS supplemented with Triton X-100. Biotinylated goat anti-rabbit IgG was used as the secondary antibody at a concentration of 0.5% (Vector Laboratories, Burlingame, CA). After the slides were rinsed with PBS, immunoperoxidase staining was performed using the Vectastain Elite avidin-biotinylated horseradish peroxidase complex (ABC) kit (Vector Laboratories). Sections were incubated for 5 min with the Vectastain Elite ABC reagent. After this, the sections were rinsed two times with PBS for 2 min each. For chromogen, the 3,3′-diaminobenzidine (DAB) substrate kit for peroxidase (Vector Laboratories) was used, which gives a black reaction product. Immuno- and DAB substrate solutions were used at room temperature. The sections were incubated with the DAB substrate solution for 12 min. After being rinsed with PBS two times for 2 min each, the sections were counterstained with methyl green for 20 min. They were then dehydrated in 100% ethanol two times for 30 s each and cleared in xylene two times for 20 s each. The sections were coverslipped with Permount. The stained sections were viewed under the light microscope for analysis.

Statistical Analysis

Lungs for each of the seven experimental culture groups (control, ligated, EGF, TGF-β1, EGF + TGF-β1, dexamethasone, and EGF + TGF-β1 + dexamethasone) were obtained as
follows. Ten lungs for each of the seven experimental lung culture groups were obtained from six simultaneously bred pregnant mice. Each pregnant mouse provides a litter of at least 10–14 fetuses. Fetuses from each pregnant mouse were randomly allocated to all of the experimental groups.

After 3 days, three of the lungs were removed and pooled from each cultured condition for immunoblotting. The remaining seven lungs from each condition were kept in culture for a total of 7 days, at which time three more lungs were removed and pooled for immunoblotting. In addition, two lungs from each condition were fixed for immunohistochemical analysis. Remaining lungs from each of the conditions after 7 days in culture were frozen for other studies. Freshly excised lungs from 14-, 16-, and 19-day fetuses and newborns were also processed for immunoblotting and immunohistochemistry.

The entire process was repeated in triplicate. The immunoblotting results were averaged from the three experiments. Immunoblotting quantification was performed densitometrically, and the values were normalized to those of GD14 (time 0) lungs on each immunoblot. Comparisons for immunoblotting analyses were made by ANOVA followed by Bonferroni multiple comparisons. The level of significance was taken as $P < 0.05$. Semiqualitative data are presented for immunohistochemistry. Serial sections were cut from a minimum of three lungs from each condition. Images were stored from randomly picked areas from at least 10 different sections from each condition. Numbers of stained cells present in the field of view were compared, and although no statistical analysis was carried out on the counted cells, the qualitative data are reported in the results. The grading was based on degrees of staining: no staining and minimum, mild, moderate, and heavy staining for Hoxb5 protein.

RESULTS

Immunoblotting

GD14 lungs cultured for 3 days. The Western blots revealed that the level of Hoxb5 protein in the time 0 (GD14) lungs was taken as 100%, which decreased slightly to 70% in unligated, untreated GD14 lungs cultured for 3 days compared with in vivo lungs processed at GD14 or GD16. After 3 days in culture, Hoxb5 level was reduced to 40% in TGF-$\beta$1-treated lungs and to 35% in lungs treated with the combination of EGF + TGF-$\beta$1 compared with the untreated group. In addition, Hoxb5 protein level in lungs treated with EGF alone was reduced to 45% compared with the time 0 lungs and was almost 25–30% less than in cultured untreated, unligated lungs. The level of Hoxb5 protein in ligated lungs was one-third of that seen in the unligated, untreated group. These proportionally large changes in expression fell short of statistical significance because of increased variability in the GD14 unligated controls, whereas Hoxb5 protein was almost completely downregulated in lungs cultured in the presence of dexamethasone alone or the combination of EGF + TGF-$\beta$1 + dexamethasone, i.e., reduced to 10% Hoxb5 protein ($P < 0.05$). The reduction was similar to that seen in GD19 lungs (Fig. 1, A and B).

GD14 lungs cultured for 7 days. After 7 days in culture, a $\geq 50%$ reduction in Hoxb5 protein was seen in control untreated, unligated lungs compared with the respective culture group at 3 days. Hoxb5 protein in lungs from all the experimental groups was reduced to 60–80% from the untreated, unligated group except for the treatments with dexamethasone alone or in combination with both growth factors, i.e., EGF + TGF-$\beta$1 + dexamethasone, which showed near-complete downregulation of Hoxb5 protein, i.e., reduced to 10% compared with untreated, unligated group ($P < 0.05$).

Overall, Hoxb5 protein was less in lungs at 7 days in culture compared with lungs at 3 days in culture. The difference was statistically significant under all conditions except for cultured ligated lungs. No protein was detected in neonatal lungs or in adult lungs. These results are demonstrated in Fig. 1, B and D, and summarized in Table 1.

Adult mouse heart does not express Hoxb5 protein (26). Therefore, in our experiments, we prepared Western blots with Hoxb5 fusion protein and total protein from adult mouse heart, which were immunoblotted with Hoxb5 antibody or preimmune sera. Adult mouse heart did not show any bands when reacted with Hoxb5 antibodies, whereas Hoxb5 fusion protein and fetal lung were positive for Hoxb5 protein. No reaction was seen on immunoblots when treated with preimmune sera (Fig. 1C).

Immunostaining

Lungs from various gestational ages and those cultured for 7 days with or without additional growth factors and hormones were immunostained for Hoxb5 protein. Immunostaining results were evaluated on the basis of the localization pattern in the epithelia and mesenchyme as well as on the intensity of staining and were graded (Table 1). This grading was based on degree of staining: no staining and minimum, mild, moderate, and heavy staining for Hoxb5 protein.

Sections of uncultured murine fetal lungs at different gestational ages showed that Hoxb5 protein decreased as lung structural development progressed from the pseudoglandular and canalicular periods and finally to the terminal sac period of lung development. During the early canalicular period of lung development at GD16, sections incubated with Hoxb5 antibody (Fig. 3, a and b) showed reduced Hoxb5 protein compared with GD14 (time 0) lungs. Significant staining was seen around the main stem bronchus, and a very specific pattern consisting of a single layer of stained mesenchymal cells was seen around distal airway tubules. By GD19, i.e., during the terminal sac period of lung development, Hoxb5 protein was localized only in specific epithelial cells in the distal air spaces of the lung (Fig. 4, a and b). In neonatal lungs, the staining for Hoxb5 protein was further reduced, but when present, it was localized only to distal epithelial cells of the lung (Fig. 5, a and b). There was an absence of Hoxb5 protein in the adult lung except for rare stained cells (Fig. 6, a and b).

The sections incubated with the preimmune serum in the absence of primary antibody for Hoxb5 showed no staining. Hoxb5 was localized in the mesenchyme as
seen more clearly at higher magnification (Fig. 7, a and b). These representative sections are from GD16 lungs.

The localization of Hoxb5 protein was reduced in the mesenchyme of untreated, unligated lungs cultured for 7 days (Fig. 8, a and b) compared with GD14 (time 0) lungs (Fig. 2) and was further reduced in the airway of ligated lungs cultured for the same time period (Fig. 9, a and b). When treated with EGF for 7 days, lungs in culture showed hypertrophied airways. At this time point, the development of distal airways with low cuboidal to flattened epithelia was apparent. They did not stain; however, significant mesenchymal tissue was present. Lung sections from EGF-treated lungs incubated with Hoxb5 antibody showed positive staining for Hoxb5 protein in the mesenchyme (Fig. 10, a and b). The localization was comparable with that of the untreated, unligated lungs (Fig. 8, a and b). Despite a reduction in mesenchymal tissue observed in TGF-β1-treated lungs (Fig. 11, a and b), Hoxb5 staining was present. This localization pattern was comparable with that seen in the ligated lungs (Fig. 9). Hoxb5 protein was greatly reduced after the combination treatment with EGF + TGF-β1 (Fig. 12, a and b) and was almost negligible in lungs treated with growth factors + dexamethasone (Fig. 13, a and b) or dexamethasone alone (Fig. 14, a and b). The reduction in Hoxb5 protein was not statistically significant unless dexamethasone was present (Fig. 2).

**DISCUSSION**

Approximately 38 Hox genes have been identified in the mouse and human, one-half of which are expressed in lung tissue (reviewed in Ref. 25). Transcripts containing some of these Hox sequences appear in specific regions of the embryo or fetus at precise times during murine development (29). Experimental evidence showed that the Hox genes function as DNA sequence-specific transcription factors, in some cases with both self- and cross-regulatory properties (1). In the mouse embryo, Hox gene expression has been most exten-

![Image](image-url)
high levels of Hox gene expression are found in a number of nonneural tissues including the lung, which strongly suggests a role for these genes in lung development. Current evidence supports the possibility that these genes may be of developmental importance in fetal mouse lungs, yet their exact role(s) remains unknown.

A variety of hormones and growth factors influence lung morphogenesis and differentiation of the epithelium, including EGF (12, 30), TGF-β1 (12, 33, 43), fibroblast growth factors (35), retinoic acid (11, 16), and dexamethasone (14, 15, 24). We chose to study EGF and TGF-β1 because these have frequently been used in similar studies of lung development to model contrasting signals in positive and negative regulation of branching morphogenesis and cell differentiation. Dexamethasone was also studied because the most is known about glucocorticoid influence on lung maturation and because glucocorticoid treatment is used clinically to influence lung maturation. EGF and dexamethasone promote branching morphogenesis, mesenchymal remodeling, and production of surfactant phospholipid and surfactant protein C, whereas TGF-β1 inhibits branching, mesenchymal remodeling, and surfactant phospholipid and surfactant protein C production. Studies were done evaluating each treatment separately in an effort to ascertain their individual effects, and then, because the developing lung is exposed to these factors simultaneously, we also studied the effects of combinations on Hoxb5 protein in lung development.

Previous studies of embryonic mouse lung morphogenesis have shown that Hoxb5 protein is gestationally regulated, suggesting that unique patterns of expression of Hoxb5 in the fetal mouse lung may help control specific elements of airway branching and epithelial cell fate (39, 40). Using antisense oligonucleotides for Hoxb5, Volpe et al. (40) demonstrated that the inhibition of Hoxb5 caused abnormal orientation and delayed growth of monopodial branches from proximal airways and abnormal dipodial branching in distal airways associated with disorganized or absent cleft formation. Inhibition of Hoxa5 using antisense oligonucleotides prevented the development of monopodial branching and cleft formation to form dipodial branches. Volpe et al. (40) speculated that the patterns of Hoxa5 and Hoxb5 expression defined distinct elements of airway structure and function through epithelial cell orientation and mesenchymal-epithelial interactions. Other studies by Volpe et al. (39) demonstrated that the cellular specificity and quantitative expression of Hoxb5 protein change with advancing in vivo lung development in the mouse, suggesting a role for Hoxb5 in the regulation of proximal airway development.

We extended these studies in our cultured murine fetal lung model in the presence and absence of growth factors and dexamethasone and demonstrated their potential regulatory effects on the Hoxb5 protein in the lung. Western blot analyses of the lungs in culture for 3 or 7 days with or without growth factors and dexamethasone corroborated the immunostaining results. In general, signals for Hoxb5 protein were stronger in each condition after 3 days in culture compared with those seen after 7 days in culture. As described in MATERIALS AND METHODS, the two bands for Hoxb5 protein represent two isoforms of the protein as previously reported in brain and lung (39, 40). It is not unusual for Hox genes to have multiple transcripts and multiple translation products. In fact, for many Hox genes, two proteins have been described. It is not known whether one or both of these products are active and/or inactive in different tissues or at different times in gestation (5, 17, 21, 26, 28, 31, 36). Earlier, we demonstrated that airway ligation accelerated development of lungs in culture compared with the unligated lungs, with increased protein and DNA contents and more mature-appearing epithelial cells and increased numbers of lamellar bodies observed ultrastructurally (6). In the present experiment, the untreated, unligated lung appeared, histologically, to be structurally less developed compared with the ligated lung, with higher Hoxb5 protein. However, quantitatively, no statistically significant differences were seen in any of these cultured lungs except those cultured in presence of dexamethasone. In lungs cultured for 3 or 7 days, a significant progressive diminishing pattern of Hoxb5 protein was noted after treatment with dexamethasone to the culture media, suggesting that as the development and maturation of fetal lungs progressed, the Hoxb5 was reduced. Thus these factors may play a direct or indirect role in the downregulation of the Hoxb5 gene. The effects of these growth factors with advancing lung maturation may be mediated in part by downregulation of Hoxb5. Earlier in lung development, Hoxb5 may direct the structural development of specific airways,
whereas once the fate of these airways is determined, downregulation of Hoxb5 at this point in development may allow for different combinations of developmentally controlled genes to be expressed, thus allowing lung maturation to progress with the differentiation of alveolar structures in the terminal saccular period of lung development. These observations were supported by our earlier studies on the effects of growth factors and dexamethasone on lung development in organ culture (12, 15). It is evident from our earlier results as well as from the data presented in the current paper that the acceleration of development induced by the growth factors and dexamethasone may be accompanied by mesenchymal thinning in these developing lungs as the airways or distal tubules develop. The reduction of Hoxb5 staining observed when treated with the above agents may be attributed in part to the thinning of mesenchyme as the lung develops.

Fig. 2. Sections of fetal lung at GD14 (time 0). In all figures, cryosections of lungs taken in coronal plane (6-µm thick) were immunostained for Hoxb5 protein (stained black) and counterstained with methyl green; sections immunostained without addition of primary antibody are termed controls. a: Intense staining for Hoxb5 protein was seen in mesenchyme of GD14 lung. However, no staining for Hoxb5 protein was observed in epithelial cells (arrows). Original magnification, ×125. b: At higher magnification (×1,125), Hoxb5 was localized in mesenchyme, and there was no epithelial localization of Hoxb5 seen.

Fig. 3. a: Representative section of GD16 lung (original magnification, ×62.5) shows reduced Hoxb5 staining compared with GD14 lung (see Fig. 2). Significant staining was seen around main stem bronchus (arrows), and a very specific pattern of a single layer of stained mesenchymal cells was seen around distal airway tubules (small arrows). b: Cells stained positively for Hoxb5 protein. Original magnification, ×1,125.

Fig. 4. By GD19, Hoxb5 protein was localized only in specific alveolar acinar cells (arrows) in primitive alveoli of lung. Original magnifications, ×125 (a) and ×1,125 (b).

Fig. 5. In neonates, staining for Hoxb5 protein was further reduced, and, when seen, it was localized only in alveolar acinar cells of lung (arrows). Original magnifications, ×125 (a) and ×1,125 (b).

Fig. 6. There was an absence of Hoxb5 protein in adult lung except for a few very rare cells that were positive (arrows). Original magnifications, ×125 (a) and ×1,125 (b).
We also employed immunotechniques to localize Hoxb5 protein in uncultured lungs at different stages of development and also in lungs in vitro with or without additional factors. The immunohistochemistry clearly revealed that Hoxb5 protein was localized only to mesenchymal cells in pseudoglandular lungs at GD14. With progression in development and thinning of the mesenchyme at GD19 and in neonates, the protein was localized in specific epithelial cells. We speculate that with advancing maturation of the lung (e.g., in the presence of dexamethasone), the cellular specificity of Hoxb5 changes, and therefore its role in development may also be different than it was at earlier time points. These findings agree with those of Volpe et al. (39), who found that the cellular specificity of Hoxb5 protein changes to include expression in the subepithelial fibroblasts and adjacent epithelial cells on GD15, whereas later in development (postnatal days 1 and 2), Hoxb5 protein is localized to specific epithelial cells and wanes significantly in the adjacent mesenchyme. Cell-cell and cell-matrix interactions, which are crucial to developmental processes, may be related to the patterns of Hox gene expression.

As discussed earlier, experiments with cultured day 11 and 13 embryonic mouse lungs showed that treatment with antisense oligonucleotides for Hoxb5 resulted in branching alterations (8, 39, 40). It is possible that the abrupt interruption of Hoxb5 protein during early branching morphogenesis in the absence of stimulatory signals for mesenchymal-epithelial interactions may have resulted in abnormal branching patterns. In support of this, the current study examines lung development at later stages, demonstrating downregulation of Hoxb5 protein by the addition of a combination of growth factors and/or hormones such as dexamethasone. These treatments also resulted in a reduction of mesenchyme and flattening of distal epithelial cells, structural events similar to those observed during in vivo lung development. In our study, the developmental alterations were associated with a downregulation of Hoxb5 protein, but no obvious abnormality of airway branching was observed. The downregulation of Hoxb5 protein seen from 3 to 7 days in cultured lungs corresponded with their developmental progression. In vitro, downregulation of Hoxb5 was similar to that observed in vivo developing lungs from similar embryonic stages. We speculated that at those gestational stages, the intrinsic growth factors and an increase in cortisol during late gestation may be responsible for the diminished Hoxb5 protein. This further supports the possibility that downregulation of Hoxb5 in late lung development may be a necessary developmental process, whereas early in lung development, downregulation of Hoxb5 may lead to abnormal developmental patterns of...
airway branching. To the best of our knowledge, to date, no loss or gain of function experiments on the Hoxb5 gene have been reported in mouse embryo with reference to the lung.

We conclude that 1) the downregulation of Hoxb5 was more pronounced after 7 days than after 3 days for all conditions except ligated lungs; 2) the addition of dexamethasone alone or along with both growth factors dramatically downregulated Hoxb5 protein in the cultured lungs to a level similar to that seen in neonatal lungs; 3) dexamethasone alone or in combination with both growth factors recapitulated, in culture, the regional and cellular changes in Hoxb5 protein seen in vivo (i.e., expression wanes with the reduction in mesenchyme); 4) with normal progression of lung development, the expression of Hoxb5 was seen only in

Fig. 10. Lungs in culture for 7 days treated with EGF. Despite development of large airways (hypertrophied) after EGF treatment, significant mesenchymal tissue was present that stained for Hoxb5 protein (arrows). Amount of Hoxb5-positive tissue was comparable with that seen in untreated, unligated lung (Fig. 8a) but was more than that seen in ligated lungs (Fig. 9a). Original magnifications, ×125 (a) and ×1,125 (b).

Fig. 11. Despite a reduction in mesenchymal tissue of lungs in culture for 7 days treated with TGF-β1, Hoxb5 staining was significant. Staining patterns of Figs. 11b and 9b are comparable. Original magnifications, ×125 (a) and ×1,125 (b).

Figs. 12–14. Lungs in culture after treatments with EGF+TGF-β1 (Fig. 12), EGF+TGF-β1+Dex (Fig. 13), and Dex alone (Fig. 14). Hoxb5 protein was significantly reduced in EGF+TGF-β1-treated lungs (arrows) and was almost negligible in mesenchyme after treatment with Dex alone or in combination with both growth factors (arrows). Original magnifications, ×125 (a) and ×1,125 (b).
apparent low cuboidal epithelial cells, which remain to be identified; and 5) downregulation of Hoxb5 protein is apparently correlated to advancing lung development at these later times in gestation.

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