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HoxB-5 down regulation alters Tenascin-C, FGF10 AND HoxB gene expression patterns in pseudoglandular period fetal mouse lung

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1. ABSTRACT

Organ-specific patterning is partly determined by Hox gene regulatory interactions with the extracellular matrix (ECM), cell adhesion and fibroblast growth factor (FGFs) signaling pathways but coordination of these mechanisms in lung development is unknown. We have previously shown that Hox b-5 affects airway patterning during lung morphogenesis. We hypothesize that Hoxb-5 regulation in fetal lung affects ECM expression of tenascin-C and alters FGF10 spatial and cellular expression. To test this hypothesis, gestational day 13.5 (Gd13.5) fetal mouse lung fibroblasts and whole lungs were cultured with Hoxb-5-specific small interfering RNA (siRNA). Western blots showed that siRNA-down regulation of Hoxb-5 led to decreased tenascin-C and FGF10 and was associated with increased Hoxb-4 and decreased Hoxb-6 protein levels. Hoxa-5 protein levels were not affected. Hoxb-5 siRNA-treated whole lung cultures had a significant decrease in total lung and peripheral branching region surface area. Immunostaining showed negligible levels of Hoxb-5 protein and tenascin-C, and loss of FGF10 spatial restriction. We conclude that Hoxb-5 helps regulate lung airway development through modulation of ECM expression of tenascin-C. ECM changes induced by Hoxb-5 may affect mesenchymal-epithelial cell signaling to alter spatial and cellular restriction of FGF10. Hoxb-5 may also affect lung airway branching indirectly by cross regulation of other Hoxb genes.

2. INTRODUCTION

2.1. Hox Genes

Numerous studies have identified an essential role for Hox genes in the orchestration of the embryonic body plan, organ-specific development and cell-specific fate (1,2). These genes code for "master regulatory" transcription factor proteins that regulate subordinate genes during embryogenesis and in the adult. In vertebrates, Hox genes comprise a subset of the homeobox gene family, characterized by a conserved homeobox region that codes for the homeodomain region responsible for DNA binding. They are organized into four clusters that are thought to have arisen by duplication and divergence from an ancestor Hox cluster. The four Hox gene clusters, termed Hoxa-d in mouse (HoxA-D in human) are located on four different chromosomes. In mouse and human, the 39 Hox genes are organized into 13 paralogous groups within the four clusters based on sequence similarity to each other and to their counterpart in drosophila. The 3' to 5' chromosomal order in each cluster corresponds to the anterior to posterior and temporal expression patterns in the developing embryo, called spatial and temporal colinearity of Hox genes (2). The necessity of tight control of Hox gene expression patterns and developmental regulation is now evidenced by multiple studies revealing that both spontaneously occurring and experimentally induced alteration of Hox gene expression leads to specific developmental anomalies corresponding to the expression domain of the particular Hox gene (2,3,4,5). This tight control of Hox gene expression is also influenced by auto and cross regulation between and within the Hox clusters with 3' genes in general being more likely to regulate 5' genes (2,8). In recent years, ongoing research on Hox gene control of developmental processes has shown that the downstream targets of Hox genes are most likely other transcription factors as well as other molecules called "realizators" or "realizator genes" including extracellular matrix (ECM) and cell adhesion molecules (6,7,8). The characteristics of the ECM or "cellular environment" helps to localize the cellular response to secreted growth factors and other substances that ultimately influence cell behavior, cell migration, proliferation and apoptosis, processes known to be essential for appropriate lung airway branching morphogenesis (9,10,11). Several ECM and cell adhesion molecules are known to be important to lung development, and in other systems are regulated by Hox genes, but the interaction of Hox genes and the ECM during lung airway branching has not been studied.

The predominant Hox genes expressed during lung morphogenesis are from the Hoxa and Hoxb clusters. Notably, they are expressed in developmentally specific patterns suggesting a "Hox code" for the developing lung (12,13,14,15,16). The timing and anterior expression pattern of Hoxb-5 in thoracic mesenchyme with the initiation of lung budding suggests that it is one of the more important Hox genes involved in lung morphogenesis (1, 13,14, 17). The absence of an overt lung phenotype in the Hoxb-5 knockout mouse does not rule out potential morphoregulatory effects of altered Hoxb-5 expression (18). Indeed, the necessity of Hoxb-5 function to normal lung development may have lead to the development of compensatory mechanisms in evolution thus preventing obvious identification of morphological and functional abnormalities in the Hoxb-5 experimentally induced knockout mouse. It is clear from results of other Hox gene mutation studies showing less severe malformations than predicted by the expression pattern of the gene under study that compensation or interdependence among Hox genes exists to conserve normal function in developing organisms (1, 2). Our studies on the temporal and spatial expression pattern of Hoxb-5 in both mouse and human lung development and in vitro and in vivo abnormal airway branching with altered Hoxb-5 expression indicates that Hoxb-5 is important for control of airway branching during lung morphogenesis. Specifically, we have shown that Hoxb-5 is strongly expressed in mesenchyme immediately adjacent to branching airways during the pseudoglandular period of lung development and becomes down regulated as bronchiolar airway branching is completed (14). Up regulation of Hoxb-5 in embryonic mouse whole lung cultures leads to elongated airway branches whereas down regulation led to a paucity of and foreshortened branch generations of the mainstem bronchi. This in vitro phenotype is in line with our observations of up regulated and altered developmental expression of Hoxb-5 in human bronchopulmonary sequestration (BPS) and congenital cystic adenomatoid malformation (CCAM), congenital lung lesions with aberrant airway branching (5). This ability of Hoxb-5 to control airway patterning suggests that mesenchymal expression of Hoxb-5 helps orchestrate the regulation of downstream morphoregulatory or "realizator genes" involved in branching morphogenesis in lung development.

2.2. Tenascin-C

Tenascin-C, also called cytotactin, is a large ECM glycoprotein belonging to a family of at least five members. It is highly expressed during development including lung development with much lower levels being seen in adult tissues. Tenascin-C, like Hoxb-5, is expressed in mesenchyme surrounding branching airways during the pseudoglandular period of lung development. Similar to Hoxb-5, tenascin-C is up regulated by transforming growth factor beta (TGFbeta), down regulated by dexamethasone and its expression decreases to lower levels after airway branching is completed (14, 19-21). The expression pattern, regulation of tenascin-C and airway development in tenascin-C deficient mice suggests that tenascin-C is one of the ECM molecules important for the coordination of airway branch development (19, 22-25). The tenascin-C promoter contains homeobox binding sites and in vitro, can be activated by homeobox containing transcription factors but the relationship of Hox genes and tenascin-C in lung morphogenesis is not known (22,26-28).

2.3. Fibroblast Growth Factors (FGFs) and FGF10

FGF's have been implicated as potential regulators of tenascin-C expression in muscle tendon and CNS development (29,30). Studies also suggest, however that FGF's and Hox genes interact to control limb and vertebral patterning during embryogenesis (31-33). In some cases, Hox gene activation modulates FGF activity whereas

in other situations FGF expression is necessary for Hox gene expression (36). The relationship between Hox genes and FGF regulation appears to be influenced by temporal, spatial, developmental and organ-specific control (34,35). Thoracic mesoderm expression of FGF10 and the presence of the FGF10 receptor, FGFR2b are crucial for lung bud formation and appropriate airway branching, with resulting lung agenesis in the absence of either FGF10 or its epithelial receptor (36,37).

Despite the evidence for the identified importance of Hox genes, tenascin-C, and FGFs in lung development and the identifiable relationship of these pathways in coordination of limb, vertebral and CNS development, the interaction of these regulatory pathways during lung–specific patterning has not been studied. The objective of our study was to determine if Hoxb-5 effects on airway patterning are related to changes in the ECM and spatial restriction of FGF10. We hypothesized that Hoxb-5 regulation in pseudoglandular period mouse lung would affect ECM expression of tenascin-C and alter FGF10 spatial and cellular expression. To study this we used Hoxb-5 specific small interfering RNA's (siRNA) methodology to inhibit Hoxb-5 protein expression in Gd13.5 pseudoglandular period fetal mouse lung fibroblast and whole lung cultures to determine if Hoxb-5 down regulation leads to altered expression of tenascin-C in conjunction with changes in airway branch development and spatial and cellular restriction of FGF10.

3. MATERIALS AND METHODS

The animal study protocol was approved by the Tufts-New England Medical Center Institutional Animal Research Committee. Principals of laboratory animal care were followed according to the National Institute of Health Guidelines for Care and Use of Laboratory Animals. Timed pregnant Swiss Webster mice obtained from Charles River Laboratories (Wilmington, MA, USA), with morning of the vaginal plug defined as Gestation day 0.5 (Gd0.5).

3.1. Reagents

Six well culture plates used for Gd13.5 fetal lung fibroblast cultures were purchased from BD Biosciences (San Jose, CA) and culture dishes (35 mm) used for Gd13.5 whole fetal lung cultures purchased from Corning Glass Works (Corning, NY). Dulbecco's modified eagle's medium (DMEM) was purchased from Sigma (St. Louis, MO) and Optimem medium from Gibco Brl (Grand Island, NY). Affinity purified rabbit anti-mouse Hoxb-5 antibody was prepared and characterized as previously described (14). Goat anti-mouse antibodies to tenascin-C, Hoxa-5, Hoxb-4, Hoxb-6 and FGF10 were obtained from Santa Cruz (Santa Cruz, CA), Mouse monoclonal antibody to GAPDH was obtained from Research Diagnostics (Flanders, NJ). Donkey anti-goat secondary antibodies used for Western blot analysis were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Immunostaining reagents were obtained from Vector (Burlingame, CA). All other reagents were from Sigma (St. Louis, MO) unless otherwise specified.

3.2. SiRNA Methodology

All siRNA molecules were chosen and prepared by published methods used for siRNA transfection of mammalian cells (38). Optimal siRNA sequence and dose for Hoxb-5 inhibition without non specificity or toxicity was determined using four different siRNAs from different regions of the Hoxb-5 cDNA, each being tested at several incremental doses (10 nM-400 nM). The specificity of the Hoxb-5 siRNA sequences was confirmed by screening candidate sequences by Blast Search (www.ncbi.nlm.nih.gov/Blast/) for uniqueness to the Hoxb-5 gene (39). Results for both the Gd13.5 fetal mouse lung fibroblast and whole lung cultures are shown for most optimal dose (50 nM) and most optimal Hoxb-5 siRNA sequence for inhibition of Hoxb-5 protein expression corresponding to a region within exon 2 of the Hoxb-5 cDNA (Hoxb-5: sense sequence 5'GGAUGAUGGCUGUAUUUUGtt3' and antisense sequence 3'CAAAAUACAGCCAUCAUCCTT3') (Ambion, Austin, TX) (39). The sequence of the commercially prepared scrambled siRNA was predetermined to not have sequence similarity to any known gene. The scrambled (negative control) and GAPDH (positive control) siRNA were purchased from Ambion (Austin, TX). The Transit-TKO transfection vehicle was purchased from Mirus (Madison, WI). Controls included no treatment, transfection vehicle alone (TKO), scrambled siRNA in the same concentration as the Hoxb-5 siRNA, and GAPDH siRNA (positive control). Immediately prior to transfection of Gd13.5 fetal lung fibroblast or whole lung cultures, siRNAs molecules were complexed to Transit-TKO reagent as recommended by manufacturer (Mirus, Madison, WI) and adapted from the method of Elbashir (38). Fluorescein-labeled siRNA, used in preliminary experiments to confirm uptake of siRNA into fetal lung cells, was prepared according to manufacturers recommendations using fluorescein label IT siRNA tracker reagent (Mirus, Madison, Wisconsin).

3.3. Gestational day (Gd) 13.5 Fetal Lung Fibroblast and Whole Lung Cultures

Fetal mouse lung fibroblast and whole lung cultures were prepared, as we have previously described. Briefly, timed pregnant Swiss Webster mice were sacrificed by $C0_2$ inhalation at Gd13.5 (morning of vaginal plug is Gd0.5, term is 19 days, Theiler stage 21-22). After sacrifice, the uterus is removed, placed in ice-cold Hanks balanced salt solution. Under a dissecting microscope (Mag. 20x), embryos were individually removed and fetal lung dissected free of surrounding thoracic tissue and foregut using jewelers forceps. Fetal lungs were then prepared for either fibroblast cultures or whole lung cultures as described below (40,41)

Gd13.5 fetal mouse lungs used for fibroblast culture were washed in additional ice cold Hank's balanced salt solution, minced with a razorblade and stirred at 37^0C (12 min) in Hank's balanced salt solution with DNAse (1mg/100ml) + trypsin (0.25%) (Gibco Brl, Grand Island, NY). Trypsinization was stopped by mixing cell suspension with equal volumes of DMEM with 10% charcoal-stripped fetal calf serum (FCS-) (Hyclone, Logan, UT). The cell

suspension was then filtered (100 μM) by gravity, centrifuged, resuspended in DMEM containing 10% FCS- with PCN (50 units/Streptomycin (50μg)/Amphotericin (0.25 migrogram/ml) and plated in 6 well plates (25 x 10^4 cells/well) for 60 minutes at 37^0C in a 21% 0_2/5%$C0_2$ atmosphere to permit differential adherence of fibroblasts. After one hour, epithelial cells were removed and fibroblasts cultured in the same environment in fresh medium as above until 50% confluence (approximately 18 hours). All cultures were plated at the same cell density as noted above and confirmed by microscopy to have similar cell confluence at Time 0 hrs and at time of siRNA transfection. At 50% confluence, siRNA transfection was performed in DMEM/10% FCS- with 25% original antibiotic/antifungal concentration. Fibroblasts were cultured for an additional 72 hours in presence of siRNA treatments, with daily monitoring of cell cultures by light microscopy. At the end of the transfection period, fetal lung fibroblasts were harvested and prepared for Western blot analysis as described below. Overall, culture confluence was approximated at 80% at end of culture period as judged by light microscopy and was similar for all culture conditions.

Gd13.5 fetal mouse lungs used for whole lung cultures were prepared as we have previously described with modifications to account for siRNA transfection (41). Gd13.5 whole embryonic lungs were placed on GVWP membranes (Millipore, Bedford, MA) suspended at the air-liquid interface on metal grids in 35 mm culture dishes and cultured (37^0C, 21% 0_2 / 5%$C0_2$) overnight in DMEM/containing 10% FCS- with PCN/Streptomycin/ Amphotericin as noted above. At approximately 18 hours of culture, siRNA transfection as noted above was accomplished in DMEM/10% FCS- with 25% original antibiotic/antifungal concentration. Whole fetal lungs were examined at beginning of culture and at 24-hour intervals using a Nikon inverted diaphot microscope at 40X magnification with phase contrast. Descriptive evaluations of lung morphology and characteristics of airway branching were made by observing the progression of monopodial and dipodial branching in each explant for each culture condition. The degree of airway branching was quantified by lung morphometry as described below. After 72 hours of culture in the presence of the siRNA treatments, all lungs were harvested and prepared for immunostaining reactions as noted below.

Culture conditions for both the Gd13.5 fetal lung fibroblasts and whole lung cultures were as follows: (1) no treatment (C); (2) Transit TKO transfection vehicle alone (TKO) (8ul/well); (3) 50 nM scramble siRNA + TKO 8ul/well (scramble); (4) 50 nM Hoxb-5-specific siRNA + 8ul TKO/well (b5). In preliminary experiments, using these same culture conditions, uptake of siRNA into cells was confirmed using fluorescein labeled siRNA tracker molecules prepared with a commercially available kit from Mirus (Madison, WI). Additionally, to confirm the efficiency and specificity of the siRNA methodology, GAPDH siRNA (in the same concentration as scramble and Hoxb-5 siRNA) was used as a positive control in preliminary experiments.

3.4. Lung morphometry of Gd13.5 fetal mouse whole lung cultures

Cultured fetal mouse lungs used for lung morphometry were confirmed at Time 0 hours of culture to be complete whole lungs, defined as whole lungs with complete complement of lobular regions (N = 5 lungs per condition). Lung morphometry using an eyepiece micrometer was performed after 72 hours of siRNA transfection without knowledge of treatment condition. Total lung surface area and surface area of peripheral branching regions was evaluated in following manner. Each whole lung was magnified identically to micrometer grid followed by importing of lung and micrometer microscopic images into an imaging program. In this manner each box on micrometer grid measured 1 mm^2. Total lung surface area and surface area of peripheral branching regions was performed by counting the number of 1 mm^2 boxes occupied by each total lung and its corresponding peripheral branching regions. Peripheral branching regions were predefined for each lung by microscopically denoting the demarcation zone between the central less arborized branch generations (corresponding to first 4 branch generations off mainstem bronchi) and peripheral regions with multiple arborizations giving rise to the densely packed peripheral branches of the respiratory tree.

3.5. Western Blot Analysis

After 72 hours of siRNA treatment, fetal lung fibroblast cultures (N=4) were washed with ice-cold PBS and cells lysed in lysis buffer (Hepes 20mM pH7.4, 150mM NaCl, 1mM EDTA, 1% NP40) + 1mM Na3V04, 1 mM PMSF, and 4 mg/ml each of antipain, aprotinin, leupeptin and pepstatin, followed by microcentrifugation. Protein concentration was determined by Lowry assay (42). Protein gel electrophoresis and Western blots of Hoxb-5 protein were prepared and evaluated as we have previously described (14) For evaluation of tenascin-C , FGF10, Hoxa5, Hoxb-4, Hoxb-6, and FGF10, 40 μg protein aliquots from same samples used for Hoxb-5 protein determination were separated by either 12% (for Hoxa5, b4, b6 and FGF10) or 6% (for tenascin-C) SDS-polyacrylamide gel electrophoresis followed by transfer to a nitrocellulose membrane. The membranes were blocked in 3% donkey serum in Tris-buffered saline (Tris Base 10mM, NaCl 150mM with 0.1% Tween (TBST) for 2 hours at room temperature followed by overnight incubation (4^0C) in primary antibody at 1/200 concentration and 1 hour room temperature incubation in HRP-linked donkey anti-goat secondary antibody (1/25,000). Protein bands were then detected by chemiluminescence (Western Lightning Chemiluminescence Reagent, Perkin Elmer, Boston, MA) detection, and quantified by densitometry (Alpha Innotech, San Leandro, CA). Each blot was subsequently washed in TBST, reblocked with 3% donkey serum, reacted with a mouse monoclonal antimouse GAPDH antibody (1/500), followed by donkey anti-mouse secondary antibody (1/30,000) with chemiluminescence detection. The resulting GAPDH Western blot analysis and densitometry of each blot was used as an internal control with protein levels of Hoxb-5, tenascin-C, FGF10, Hoxb-4, Hoxb-6, and Hoxa-5 normalized to GAPDH.

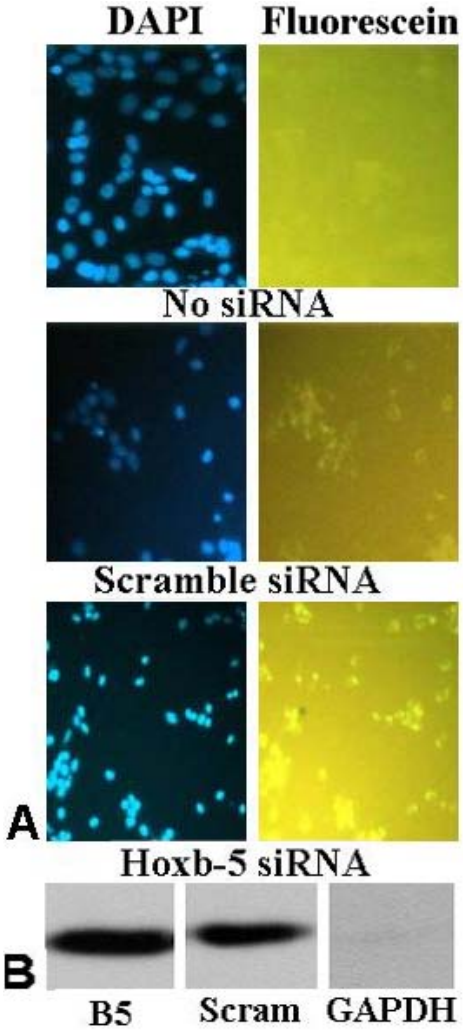

Figure 1. A. Top Panel: Cells not treated with siRNA show dapi stained blue nuclei, with same cells with absence of fluorescein-labeled cells in absence of siRNA. Middle Panel: control siRNA treated cells with DAPI stained nuclei and same cells showing uptake of fluorescein-labeled siRNA designated by green fluorescence. Bottom Panel: Cells treated with Hoxb-5 specific siRNAs with DAPI stained nuclei and same cells with green fluorescence showing uptake of fluorescein-labeled Hoxb-5 siRNA. B. Representative Western blot from Gd13.5 fetal lung fibroblasts cultures treated with Hoxb-5 siRNA (50 nM), scramble siRNA (50 nM) or GAPDH siRNA (50 nM). The efficiency and specificity of the siRNA methodology was confirmed by GAPDH Western blot demonstrating the ability of GAPDH siRNA (50 nM) to down regulate GAPDH protein levels.

3.6. Immunostaining

After 72 hours culture with siRNA or control treatments, cultured Gd13.5 fetal mouse whole lungs from 3 experiments were prepared as we have previously described using 4% paraformaldehyde fixation followed by immersion in 30% sucrose and embedding in OCT frozen specimen embedding medium (Miles, Elkhart, IN). Lung specimens were cryosectioned in the coronal plane to 6 microns at -24^{0}C and mounted onto room temperature superfrost plus slides (Fisher Scientific, Pittsburgh, PA). The primary antibody against Hoxb-5 and immunostaining techniques used in these experiments have been previously described in detail (14). Briefly, after sectioning, slides are either immediately prepared for immunostaining or rinsed for 2 minutes in ice-cold acetone and stored at -70^{0}C for later experiments. At beginning of immunostaining reaction, slides are washed in Phosphate Buffered Saline with 0.1% Tween 20 (PBST) for 30 minutes followed by blocking of nonspecific sites with avidin, biotin and blocking serum according to manufactures recommendations (Vector, Burlingame, CA). Sequential lung sections of lungs from each of the treatment conditions within each experiment are incubated overnight at 4^{0}C with a 1/200 concentration of either Hoxb-5, tenascin-C, or FGF10 antibody or negative control (PBST or nonimmune serum). The following day, slides are warmed to room temperature and incubated sequentially with secondary antibody (1:1000), with avidin-biotin complex conjugated to alkaline phosphatase, followed by incubation with blue alkaline phosphatase as the chromagen. Sections are counterstained with nuclear fast red, dehydrated through graded alcohol, coverslips applied and sequential sections analyzed by light microscopy.

3.7. Statistical Analysis

Western blot densitometry results for Hoxb-5siRNA treated Gd13.5 fetal lung fibroblast cultures versus control treated cultures (no tko, tko, scramble siRNA treatments) were statistically analyzed by ANOVA with level of significance of $P < 0.05$. Whole mouse fetal lung morphometry results of Hoxb-5 siRNA treated lungs versus control treated explants were also statistically analyzed by ANOVA.

4. RESULTS

4.1. Confirmation of siRNA uptake into fetal lung fibroblasts and effect of GAPDH positive control siRNA treatment

Fluorescein labeled siRNA molecules were used to confirm the ability of fetal lung fibroblasts to be transfected with siRNA. Gd13.5 fetal lung fibroblasts were cultured in the identical manner and in parallel to fibroblast cultures used for experimental evaluations and transfected with fluorescein labeled siRNA or fluorescein marker without siRNA (Figure 1A). As expected, no fluorescein staining is seen in absence of siRNA molecules (Figure 1A, top panel). Fluorescein stained nuclei correlate with DAPI stained nuclei in the cells transfected with either scramble siRNA (Figure 1A, middle panel) or Hoxb-5 siRNA (Figure 1A, bottom panel). GAPDH siRNA treatment was used to confirm efficiency of siRNA transfection. GAPDH Western blots (Figure 1B) were performed from the same protein lysates that demonstrated decreased Hoxb-5 protein (discussed below, see Figure 2). A representative GAPDH Western blot is shown from scrambled, Hoxb-5 and GAPDH siRNA treated Gd13.5 fetal lung fibroblasts (Figure 1B). Densitometry (not shown) confirmed that GAPDH protein levels were decreased 60% to 90% by

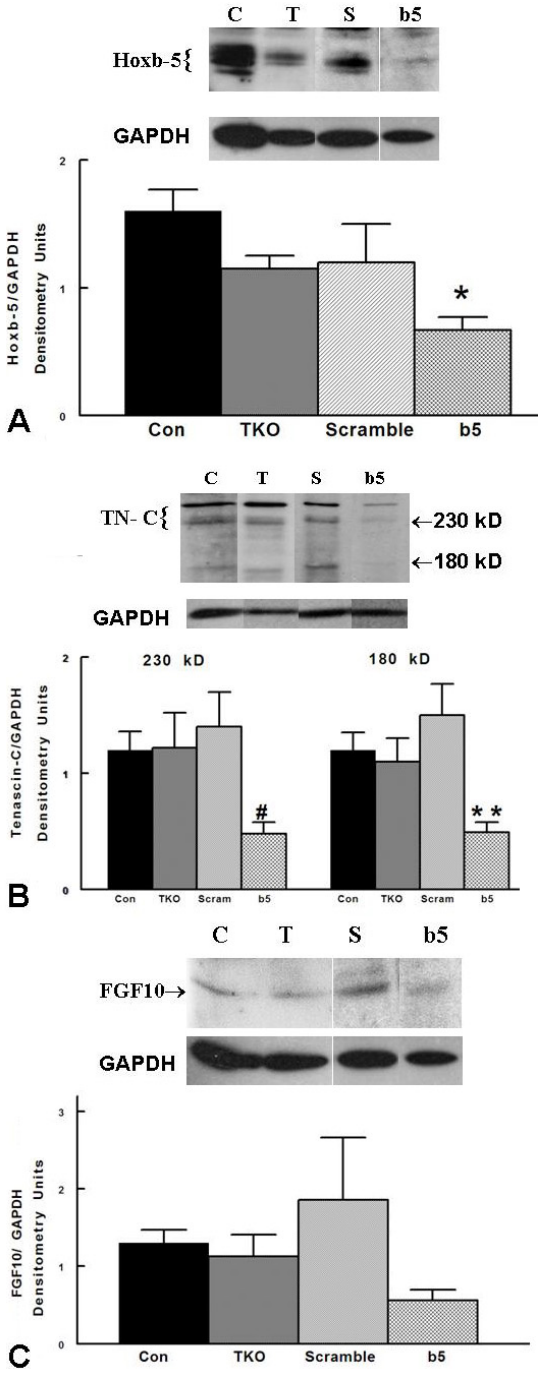

Figure 2. Representative Western blots and densitometry of Hoxb-5(A), tenascin-C (B), and FGF10 (C) protein levels in Gd13.5 fetal lung fibroblast cultures treated as no treatment control (C, Con), or with transfection vehicle alone (T, TKO), scrambled siRNA (S, Scram), or Hoxb-5 siRNA (b5). Corresponding GAPDH Western blot shown below representative Western blots. Western blots and densitometry analysis from four individual experiments demonstrated significantly decreased Hoxb-5 protein levels (A) in Hoxb-5 siRNA-treated versus scramble siRNA-treated Gd13.5 fetal mouse lung fibroblasts cultures. (* $P < 0.01$, N= 4, Mean ± S.E.M) Down regulation of Hoxb-5 led to a significant decrease in tenascin-C (TN-C) protein levels (B) (Hoxb-5 siRNA-treatment versus scrambled siRNA treatment, $P < 0.01$ for tenascin-C 230 kD isoform (#) and the 180 kD isoform (**), N=4, Mean ± S.E.M.). Down regulation of Hoxb-5 protein expression caused a trend towards decreased FGF10 protein levels (C) by 30% in Hoxb-5 siRNA treated fetal lung fibroblasts compared to scramble siRNA treated fibroblasts ($p = 0.2$).

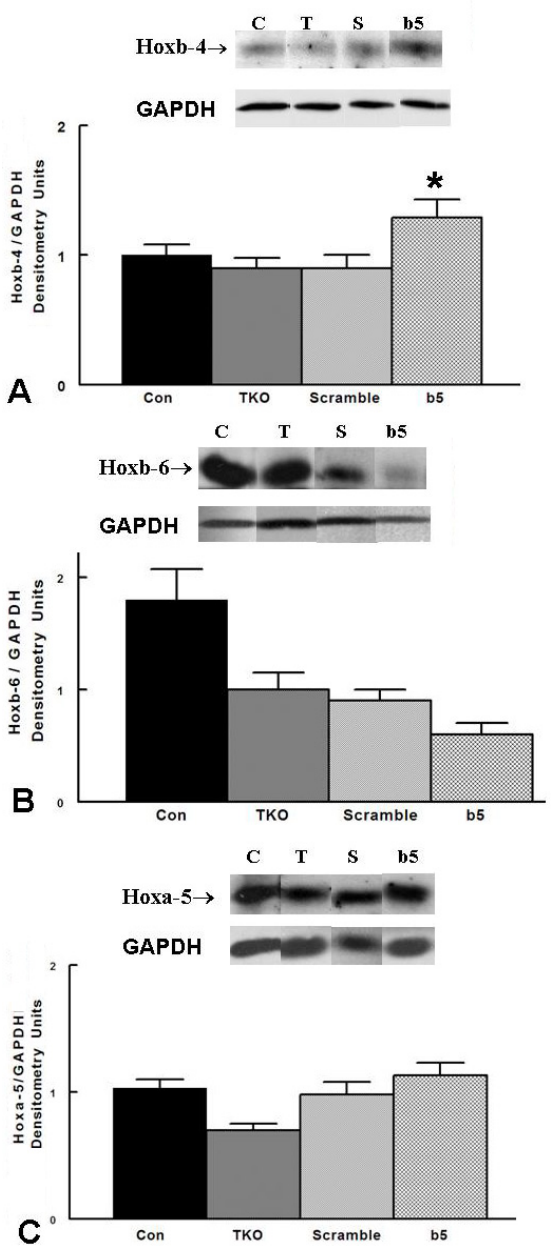

Figure 3. Representative Western blots and densitometry analysis of Hoxb-4 (A), Hoxb-6 (B), and Hoxa-5 (C) protein levels in Gd13.5 fetal lung fibroblast cultures treated as no treatment control (C, Con), or with transfection vehicle alone (T, TKO), scrambled siRNA (S, Scramble), or Hoxb-5 siRNA (b5). Corresponding GAPDH Western blot is shown below each representative Western blot. Western blots and densitometry analysis from four individual experiments demonstrated that as compared to scramble siRNA treated cultures, inhibition of Hoxb-5 led to a significant increase (40%) in Hoxb-4 protein levels (A) (Hoxb-5 siRNA treatment versus scrambled siRNA treatment, $P < 0.05$, $N = 4$, Mean +SEM). There was a trend towards decreased Hoxb-6 protein levels (B) by 30% in these same samples ($p = 0.17$). Hoxa-5 protein levels (C) were not affected by siRNA down regulation of Hoxb-5 protein expression.

GAPDH siRNA treatment but not decreased by scramble or Hoxb-5 siRNA treatment.

4.2. Effect of Hoxb-5 down regulation on Hoxb-5, tenascin-C and FGF10 protein levels in Gd13.5 fetal mouse lung fibroblast cultures

To determine if siRNA inhibition of Hoxb-5 protein expression affected tenascin-C and FGF10 protein levels in Gd13.5 fetal mouse lung fibroblast cultures, Western blot analyses with densitometry were performed. In Gd13.5 fetal mouse lung fibroblasts treated with Hoxb-5 siRNA, Hoxb-5 protein levels were decreased by 50% compared to scrambled siRNA treated cells (Figure 2A). In cells from these same experiments, this inhibition of Hoxb-5 led to a significant decrease in tenascin-C protein levels (Figure 2B). Tenascin-C is a large glycoprotein. Multiple isoforms of tenascin-C are detected at different levels in different organs. The antibody used in these studies is raised against the carboxy terminus of tenascin-C and detects the more commons isoforms of 260, 230 and 180 kD seen in fetal lung (19,20). Densitometry analysis is shown for the 230 and 180 kD bands. Compared to scramble siRNA treated cells, siRNA down regulation of Hoxb-5 led to a 66% decrease in the 230 kD isoform and 180 kD isoform of tenascin-C (Figure 2B). The levels of 260 kD isoform of tenascin-C were decreased by 40% in Hoxb-5 siRNA treated cells compared to controls (data not shown). Western blot analysis of FGF10 protein from these same experimental samples showed that in vitro down regulation of Hoxb-5 in Gd13.5 fetal lung fibroblasts was associated with a trend towards decreased FGF10 protein levels compared to scramble siRNA treated fetal lung fibroblasts (Figure 2C).

4.3. Effect of Hoxb-5 down regulation on Hoxb-4, Hoxb-6 and Hoxa-5 protein levels in Gd13.5 fetal mouse lung fibroblast cultures

Hox gene regulation of developmental processes necessitate auto and cross regulation between Hox genes to allow for appropriate developmental expression patterns during embryogenesis and organogenesis (1,35, 43). Therefore, we investigated whether Hoxb-5 inhibition in cultured fetal lung fibroblasts would alter the protein levels of the 3' and 5' homologues Hoxb-4 and Hoxb-6 and the Hoxb-5 parolog, Hoxa-5. Western blots and densitometry showed that as compared to scramble siRNA treated cultures, Hoxb-4 protein levels were significantly increased by 40% in the presence of Hoxb-5 down regulation (Figure 3A). This Hox gene is the 3' homolog of Hoxb-5 in the Hoxb gene cluster. In contrast, protein levels of Hoxb-6, the Hoxb homolog located 5' to Hoxb-5 in the Hoxb gene cluster, showed a trend towards decreased levels by 30% in Hoxb-5 siRNA treated fibroblasts compared to scramble siRNA treated cells (Figure 3B). In these same samples, Hoxa-5 protein levels were not affected by siRNA down regulation of Hoxb-5 (Figure 3C).

4.4. Effect of Hoxb-5 down regulation on in vitro lung airway branching in Gd13.5 fetal mouse whole lung cultures

To understand the impact of down regulation of Hoxb-5 expression in fetal lung fibroblasts on the

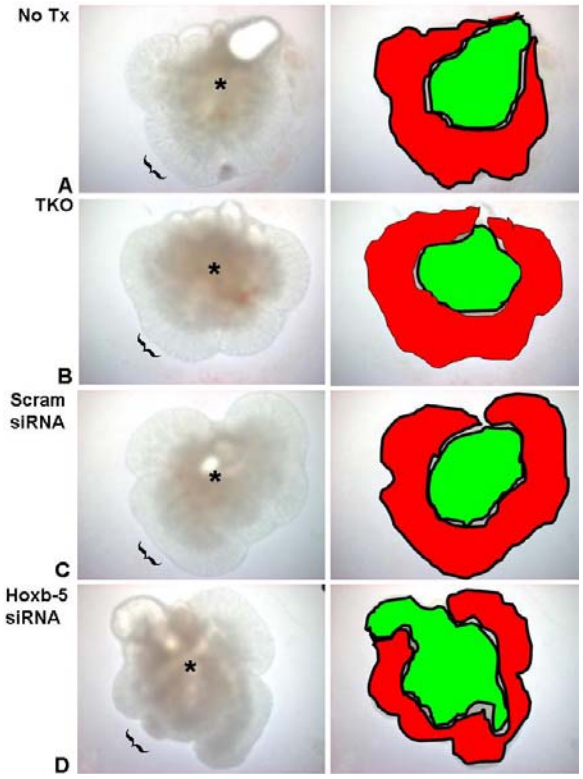

Figure 4. Gd13.5 fetal mouse lungs cultured for 4 days including 3 days of treatment as (A) no treatment control; or with (B) TKO control; (C) Scramble siRNA control; (D) Hoxb-5-specific siRNA. Representative lung shown in left panels with same lungs shown in right panels but with cartoon outline showing central less branched regions (green) and peripheral branching regions (red) for each lung. Over the time in culture, control treated lungs (A, B, C) developed very densely packed branches (brackets, red regions) of the different lobes of each lung organized in the periphery around the central (*, green regions) appropriately less branched regions. In contrast, Hoxb-5 siRNA treatment (D) led to smaller appearing lungs with less well organized peripheral branching regions (bracket, red regions). The area occupied by these peripheral branching regions (*, red regions) appeared decreased. Lobe structure appeared less well organized. These findings were observed on each day of siRNA treatment.

progression of airway branching morphogenesis during the pseudoglandular period of lung development, Gd13.5 fetal mouse whole lungs were cultured with Hoxb-5 siRNA or as control and airway branching morphogenesis was observed by microscopy and quantified by lung morphometery. All lungs showed progression of branching on each day of culture but this process appeared blunted and less organized in the Hoxb-5 siRNA-treated lungs. After four days of culture, including three days exposure to treatment conditions, the no treatment control (Figure 4A), TKO vehicle (Figure 4B) and scrambled siRNA (Figure 4C) treated lungs had very densely packed peripheral branches (See { and red regions in Figure 4) within the different lobes of each lung. The peripheral branches were organized around the centrally located and appropriately less branched airways (see * and green regions in Figure 4). In contrast, Hoxb-5 siRNA-treated lungs (Figure 4D) appeared smaller and arborization of peripheral branching regions was less well organized. The area occupied by these peripheral branching regions also appeared decreased and lobe structure appeared altered. The visual interpretation of smaller appearing lungs with less branching in the Hoxb-5 siRNA-treated lungs was quantified using lung morphometry (Figure 5). Compared to scrambled siRNA treated lungs, lungs treated with Hoxb-5 siRNA showed a significant decrease in total lung surface area (20%) and surface area of peripheral branching regions (30% decrease) (Figure 5A). There was a trend towards increased surface area (18% increase) of central less arborized regions of Hoxb-5 siRNA treated lungs compared to scramble siRNA treated lungs (Figure 5B). To determine if the decreased lung size in Hoxb-5 siRNA treated lungs was caused by decreased surface area of peripheral branching regions or secondary to the overall decreased size of these lungs, changes in surface area of peripheral branching regions of each lung was normalized to corresponding total surface area (Figure 5C). This analysis showed that even when accounting for overall decreased lung size in Hoxb-5 siRNA treated lungs the surface area of peripheral branching regions remained significantly diminished.

4.5. Effect of Hoxb-5 down regulation on lung histology and spatial and cellular expression of Hoxb-5, Tenascin-C, and FGF10 protein

Coronal lung sections from Gd13.5 fetal mouse whole lung cultures used for lung morphometry were immunostained for Hoxb-5, Tenascin-C and FGF10 protein to evaluate the effect of Hoxb-5 inhibition on lung histology and tenascin-C and FGF10 cell-specific and spatial expression patterns. The lung sections evaluated for Hoxb-5, tenascin-C and FGF10 are representative of lung sections taken from the same TKO, scramble siRNA, or Hoxb-5 siRNA treated lungs. The top of each picture corresponds to the periphery and bottom of each picture to more central regions of each lung and corresponding lung section. Lungs cultured with TKO vehicle (Figure 6A, D, G) or scrambled siRNA (Figure 6B, E, H) showed normal lung histology of columnar lined bronchioles and cuboidal lined more distal airways of varying size. However, lung histology in Hoxb-5 siRNA-treated lungs (Figure 6C, F, I) was abnormal with large airway lumens reaching close to the periphery of lung (top of each picture). In lung sections from TKO vehicle (Figure 6A) and scramble siRNA-treated lungs (Figure 6B), we saw the expected pattern of Hoxb-5 protein localization that we have previously demonstrated {Volpe 1997}(41). Hoxb-5 protein was seen in nuclei of mesenchymal cells surrounding developing airways lined by columnar and cuboidal epithelial cells. In contrast, lungs sections from Hoxb-5 siRNA-treated lungs (Figure 6C) had minimal to absent Hoxb-5 protein expression and minimal to absent tenascin-C protein expression (Figure 6F). Compared to the peripheral mainly mesenchymal immunostaining pattern of FGF10 protein noted in TKO control (Figure 6G) and scramble-siRNA-

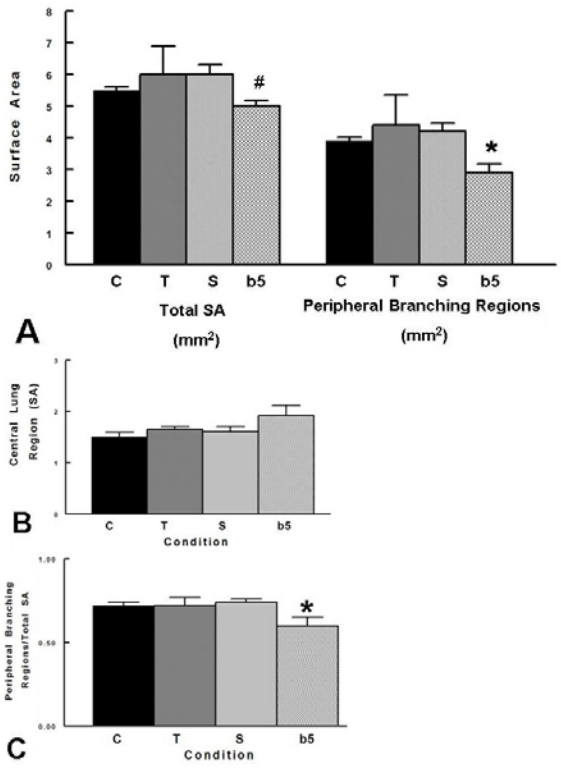

Figure 5. Lung morphometry of GD13.5 whole fetal mouse lung cultures. Lung morphomentry demonstrated that total lung surface area (left graph in A) and surface area of peripheral branching regions (right graph in A) were significantly decreased by 20% and 30% respectively in Hoxb-5 siRNA treated lungs compared to scramble siRNA treated lungs. There was a trend towards increased surface area of central regions by 18% in Hoxb-5 siRNA treated lungs compared to scramble siRNA treated lungs (B). When controlling for total lung surface area (C), the decreased peripheral branching region surface area in the Hoxb-5 siRNA treated lungs remained significant. (Bars correspond to C, no treatment control; T, TKO alone treatment; S, scrambled siRNA treatment; b5, Hoxb-5 siRNA treatment)(N =5, Mean ± S.E.M., $P < 0.05$).

reated lung section (Figure 6H), Hoxb-5 inhibition (Figure 6I) was associated with more diffuse FGF10 protein expression in mesenchyme around proximal and distal airways and epithelial expression of FGF10 appeared increased. In the absence of each primary antibody, no blue immunostaining reaction was seen (data not shown).

5. DISCUSSION

We have previously reported a series of studies on the temporal and spatial expression pattern and postulated function of Hoxb-5 protein in mouse and human lung morphogenesis. These studies suggested to us that Hoxb-5 helps control lung airway branching patterns by modulating morphoregulatory signals through mesenchymal-epithelial cell adhesion and cell-cell communication in mesenchyme surrounding branching airways. (5,14,41,44). In our current study, we found that in fetal mouse lung fibroblast cultures, down regulation of Hoxb-5 led to decreased tenascin-C and FGF10 protein expression in association with altered expression levels of Hoxb-5 homologs, Hoxb-4 and Hoxb-6. In whole lung cultures this decreased Hoxb-5 protein expression altered cellular expression of tenascin-C and FGF10 in association with decreased lung surface area of branching regions.

There is growing evidence for regulated interactions between Hox genes, cell adhesion and FGF signaling in organ-specific development (31-33). In particular, the hierarchy of Hox genes, tenascin-C and FGF interactions in developmental processes depends on the temporal/spatial expression domains and organ or region of the embryo being studied (29, 30,34,45). The importance of tenascin-C and the FGF signaling pathways in lung morphogenesis has been established but their relationship to Hox gene expression has not been studied (25,36,46). To begin to decipher this regulatory hierarchy between Hoxb-5, cell adhesion and FGF expression during lung branching morphogenesis, we used Hoxb-5-specific siRNA gene inhibition strategy to down regulate Hoxb-5 in cultured Gd13.5, pseudoglandular period fetal lung fibroblasts and whole lung explants. To our knowledge this is the first study that addresses the interaction of a Hox gene, Hoxb-5 with tenascin-C and FGF10 expression patterns and regulation within the developing lung. The pseudoglandular period in lung development was chosen for evaluations in these current studies as Hoxb-5 protein is highly expressed in this phase of lung development where it appeared in our previous work to help regulate bronchiolar airway branching patterns. Primary fetal lung fibroblasts cultures were used to isolate the effect of Hoxb-5 down regulation on fibroblast expression of tenascin-C and FGF10, thus eliminating potential fibroblast-epithelial interactions that might be affected by Hoxb-5 regulation. The whole lung explant cultures allowed us to further elucidate the effects of Hoxb-5 down regulation on cellular and spatial expression patterns of tenascin-C and FGF10. A significant down regulation of total Hoxb-5 protein levels was only seen in the Hoxb-5 siRNA treated fetal lung fibroblast or whole lung cultures and not seen in the scramble siRNA treated control samples. This demonstrates that the ability of Hoxb-5 siRNA to down regulate Hoxb-5 protein levels was indeed specific and not related to non-specific effects of siRNA treatment (38). Further, in vitro airway branching was only altered by treatment with Hoxb-5 siRNA and not by other treatment conditions. Our conclusions, therefore, are that the observed effects on down stream mechanisms of lung development seen in our study are related to the modification in Hoxb-5 protein synthesis and subsequent altered Hoxb-5-related regulation of lung morphogenesis.

These experiments suggest that one of the mechanisms by which Hoxb-5 modulates airway branching is through regulation of tenascin-C expression in the ECM of developing lung. Our current study cannot determine if this interaction in developing lung is direct as we harvested fibroblasts after 72 hours of siRNA inhibition of Hoxb-5. However, other studies show that tenascin-C has promoter binding sites for Hox genes and is, in part, regulated by

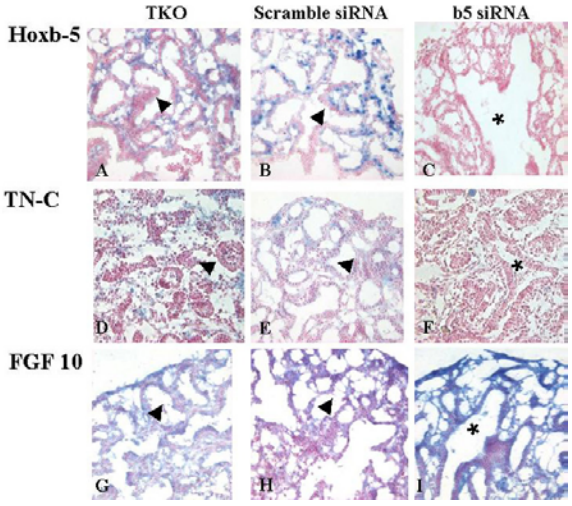

Figure 6. Immunohistochemistry in representative lung sections from cultured Gd13.5 fetal mouse lungs treated for 72 hours with transfection vehicle TKO alone (A, D, G); with scramble siRNA (B, E, H); or with Hoxb-5 specific siRNA (C, F, I). Magnification x 100. Coronal lung sections with top of each panel corresponding to periphery of lung and bottom of each panel corresponding to central region of lung. In lungs treated with TKO alone (A) or scramble siRNA (B) there is the expected pattern of Hoxb-5 protein localization in nuclei of mesenchymal cells surrounding columnar and cuboidal lined airways of varying size (arrowhead). In contrast, in Hoxb-5 siRNA treated lungs (C), Hoxb-5 protein expression is minimal to absent and airways have large lumens with apparent decreased side branches. These large airways reach close to periphery of lung section (*). SiRNA inhibition of Hoxb-5 was associated with decreased to absent tenascin-C (TN-C) protein localization (compare F verses D, E). FGF10 protein expression was more diffuse in mesenchyme and increased in epithelial cells in Hoxb-5 siRNA treated lungs (I) compared to the more peripheral immunostaining pattern noted in the scramble siRNA (H) or TKO control (G) treated lungs.

Hox genes (11,27). Additionally, the ability of Hoxb-5 inhibition to cause a significant down regulation of tenascin-C in cultured fetal mouse lung fibroblasts suggests a direct cellular event not mediated by mesodermal-epithelial cell interactions. Therefore, our results along with previously published studies on the interaction of Hox genes and tenascin-C, suggest that that Hoxb-5 may be one of the direct transcriptional regulators of tenascin-C in fetal lung fibroblasts during mouse lung branching morphogenesis. It would be expected that Hoxb-5 would act in cooperation with other transcriptional regulators of tenascin-C, possibly other Hox genes including Hoxb-4 and Hoxb-6, whose expression levels were altered by Hoxb-5 down regulation in our study (22). Other studies reveal that the paired related homeobox gene (Prx1) is expressed in conjunction with tenascin-C around developing vascular networks in late lung development. Prx1 can regulate tenascin-C in vitro in the RFL6 lung mesodermal cell line (28) As the expression of tenascin-C is dynamically regulated during lung development it is plausible that different transcriptional regulators of tenascin-C may demonstrate a predominant effect at different times in lung morphogenesis.

In the fetal mouse whole lung cultures, we also demonstrated down regulation of tenascin-C in association with decreased expression of Hoxb-5 protein. These finding were accompanied by significantly decreased development of peripheral branching regions of the cultured lungs as confirmed by lung morphometric measurements along with visual interpretation of altered organization of lobe structure. In our study, the complexity of lung structure prevented counting all airway subdivisions off the mainstem bronchi to the periphery. However, our lung morphometry suggests that lack of appropriate branching of proximal more central regions in the Hoxb-5 siRNA-treated lungs led to decreased and more disorganized branching of peripheral regions. This correlates with our previous work on Hoxb-5 down regulation in Gd11.5 embryonic mouse lung cultures showing decreased elongation and numbers of terminal branch buds in association with Hoxb-5 down regulation (41).

Previous studies have shown that lung explant cultures from tenascin-C homozygote or heterozygote null mice were smaller than wild-type cultured lungs and had decreased airway branch generation. This was dependent on the amount of tenascin-C present and not on changes in cell proliferation or cell death (25). In our current study, we cannot comment on changes in cell proliferation and differentiation but growth-arrest and differentiation in mammary cell lines and vascular smooth muscle cells accompanies decreased tenascin-C expression (22). Our studies along with information from the tenascin-C knockout mouse model suggest that Hoxb-5 control of tenascin-C expression caused changes in ECM composition and attachments altering appropriate movement of epithelial cells into the surrounding mesenchyme. Altered adhesive and deadhesive properties due to decreased tenascin-C may have altered the ability of epithelial cells to migrate into the mesenchyme during branching morphogenesis. The effect of decreased tenascin-C on interaction with its ligands, including but not limited to, integrins, fibronectin, heparin sulfate proteoglycans, and certain growth factors may have also played a role in altered coordination of branch development (22,47-50).

In the Gd13.5 fetal lung fibroblast cultures, Hoxb-5 down regulation was also associated with a trend towards decreased fibroblast protein levels of FGF10. The trend towards decreased FGF10 in association with down regulation of Hoxb-5 is worth noting as previous studies looking at spinal and vertebral patterning in the developing embryo suggest a hierarchy of interactions between retinoic acid, Hox gene and FGF regulatory pathways. In lung as well in the developing spinal cord, retinoic acid and FGF10 compete for the modulation of Hox gene expression with the resultant effects being dependent on the region of lung or spinal cord being examined and the timing in development. This differential spatial and temporal effect is

in part modified by anterior-posterior expression patterns of Hox genes (34,51-53). In the developing chick CNS, expression of 3' anteriorly expressed Hox genes (Hoxb-1, Hoxb-3-b-5) are affected by retinoic acid but refractory to FGF (54,55). Conversely, 5' more posteriorly expressed Hoxb genes (Hoxb-6-Hoxb-9) are up regulated by exogenous FGF but refractory to retinoic acid. Further, this effect of FGF on posterior, 5' Hox genes is dependent on expression of CDX homeobox gene activity (34,53). Additionally, retinoic acid treatment of fetal rat lung explants down regulates mesodermal expression of FGF10 (46). As retinoic acid is a known regulator of Hox gene expression, it is plausible that endogenous retinoic acid regulation of Hoxb genes, including Hoxb-5, and subsequent effect of Hoxb genes on FGF10 expression in fibroblasts around branching epithelia may be part of the mechanism to set the pattern of proximal to distal airway branching during lung morphogenesis. This is in line with our current results showing that down regulation of Hoxb-5 alters localized cellular and spatial expression of FGF10 in the fetal mouse whole lung cultures. These findings also correlate nicely with what we have previously shown with retinoic acid modulation of proximal-distal airway branching being in part dependent on Hoxb-5 expression, indicating that Hoxb-5 is one of the Hox genes needed to set the proximal-distal airway axis during branching morphogenesis (41). Taking into account our current and previous studies on Hoxb-5 regulation of airway branching and the studies of RA, Hoxb genes and FGF interactions in the developing CNS and gastrointestinal system, 3' Hoxb genes including Hoxb-5 may set up the "proximal" regions of the lung where as more 5' Hoxb genes potentially regulated by FGFs may pattern the distal regions of the lung. These possibilities remain to be investigated.

Although, we saw a trend towards decreased FGF10 protein levels in the mouse fetal lung fibroblast cultures, our immunostaining data demonstrate that Hoxb-5 inhibition was associated with more diffuse mesenchymal FGF10 expression and apparent increased epithelial expression of FGF10. This suggests that Hoxb-5 inhibition may have led to increased FGF10 release from lung fibroblasts allowing FGF10 to bind to epithelial cell receptors. FGF10 binding to its epithelial receptor may then have helped to alter the coordination of branch elongation and distal branch budding (46,51). Altered tenascin-C expression in mesenchyme and subsequent modified composition of the ECM around developing airways may have altered the FGF10 gradient adjacent to airway epithelial cells (55). This possibility is particularly interesting when taking into account a recent study by Izvolsky and colleagues, showing that FGF10 interactions with heparin sulfate proteoglycans helps to localize the effect of FGF10 and direct FGF10 to airway epithelium where it interacts with FGFR2 receptors (56). Tenascin-C also interacts with ECM via heparin sulfate proteoglycans facilitating interactions with fibronectin as well. Thus, tenascin-C, proteoglycan, fibronectin assembly in ECM may be part of scaffolding matrix around developing airways to allow FGF10 to appropriately reach epithelial cell targets in areas of branch budding. Conversely, we cannot rule out that in the whole mouse lung cultures that altered spatial restriction of FGF10 helped to modulate tenascin-C expression along with Hoxb-5 (29,30).

There is evidence that Hox genes also modulate their developmental effects by specific auto and cross regulation within and between the four vertebrate Hox clusters. Typically, 5' Hox genes dominate over 3' Hox genes, a phenomenon termed "posterior prevalence" (43,57). Therefore, in the fetal mouse lung fibroblast cultures we evaluated the effect of Hoxb-5 inhibition on the protein levels of the Hoxb-5 paralog Hoxa-5 and the Hoxb-5, 3' homolog Hoxb-4 and its 5' homolog Hoxb-6. Hoxb-5 inhibition led to a significant increase in Hoxb-4 protein levels while causing a trend towards decreased Hoxb-6 expression. Hoxa-5 protein levels, however, were not affected. This suggests that in the mesenchyme of the developing lung a negative feedback loop exists between Hoxb-5 and Hoxb-4 and that Hoxb-5 may subsequently regulate levels of Hoxb-6. Our data correlate with previous studies showing a cross-regulatory loop within the Hoxb cluster, with Hoxb-4 inducing its own and Hoxb-5 expression, whereas, induction of Hoxb-6 and other 5' located Hoxb genes was dependent on protein synthesis. In our study down regulation of Hoxb-5 may have removed negative cross regulation of Hoxb-5 on Hoxb-4 and positive cross regulation of Hoxb-5 on Hoxb-6. In other systems Hoxb-4 cannot induce expression of Hoxb-6 through Hoxb-9 without protein synthesis, suggesting that in developing lung the effect of Hoxb-5 inhibition on Hoxb-6 is likely direct and not related to up regulation of Hoxb-4 (43). Altered balance of expression between Hoxb-4, Hoxb-5 and Hoxb-6 may have contributed to modulation of tenascin-C and FGF10 interactions and subsequent effects on airway branching. Interestingly, during embryonic and early pseudoglandular period fetal mouse lung development Hoxb-4 is expressed throughout lung mesoderm whereas Hoxb-5 and Hoxb-6 are expressed in distal lung mesoderm (13,16). Altered levels of Hoxb-4 may have contributed to abnormal proximal to distal airway branching patterns. Further, studies suggest that Hoxb-5 and Hoxb-6 cooperate together to determine axial patterning in the thoracic region of the developing embryo (18). Thus, in developing lung, increased Hoxb-4 in association with decreased Hoxb-5 and Hoxb-6 may have shifted the developmental program within the fetal lung fibroblasts.

In summary, our current findings in conjunction with our previous studies on Hoxb-5 expression and regulation in mouse and human lung development, suggest that Hoxb-5 helps regulate expression of tenascin-C in pseudoglandular period fetal mouse lung fibroblasts. Hoxb-5 may modulate airway branch elongation and budding by helping control ECM expression of tenascin-C potentially aiding in appropriate and coordinated epithelial cell migration into surrounding mesenchyme during branching morphogenesis. Hoxb-5 effects may be part of the mechanism that localizes expression and effects of FGF10 on epithelial branching and migration. Finally, Hoxb-5 regulation of airway branching may be modified or fine-tuned by Hoxb-5 regulation or feedback on expression of neighboring Hoxb homologs.

6. ACKNOWLEDGEMENTS

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Abbreviations: ECM: extracellular matrix, FGF: fibroblast growth factor, siRNA: small inhibitory RNA, GD: gestational day, TN-C: tenascin-C

Key Words: Lung development, branching morphogenesis, Hox genes in lung, Hoxb-5, Hox a5, Hoxb-4, Hoxb-6, FGF10, tenascin-C, siRNA

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