Perinatal increases in TGF-α disrupt the saccular phase of lung morphogenesis and cause remodeling: microarray analysis

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Kramer EL, Deutsch GH, Sartor MA, Hardie WD, Ikegami M, Korfhagen TR, Le Cras TD. Perinatal increases in TGF-α disrupt the saccular phase of lung morphogenesis and cause remodeling: microarray analysis. Am J Physiol Lung Cell Mol Physiol 293: L314–L327, 2007. First published April 27, 2007; doi:10.1152/ajplung.00354.2006.—Transforming growth factor-α (TGF-α) and its receptor, the epithelial growth factor receptor (EGFR), have been associated with lung remodeling in premature infants with bronchopulmonary dysplasia (BPD). The goal of this study was to target TGF-α overexpression to the saccular phase of lung morphogenesis and determine early alterations in gene expression. Conditional lung-specific TGF-α bitransgenic mice and single-transgene control mice were generated. TGF-α overexpression was induced by doxycycline (Dox) treatment from embryonic day 16.5 (E16.5) to E18.5. After birth, all bitransgenic pups died by postnatal day 7 (P7). Lung histology at E18.5 and P1 showed abnormal lung morphogenesis in bitransgenic mice, characterized by mesenchymal thickening, vascular remodeling, and poor apposition of capillaries to distal air spaces. Surfactant levels (saturated phosphatidylcholine) were not reduced in bitransgenic mice. Microarray analysis was performed after 1 or 2 days of Dox treatment during the saccular (E17.5, E18.5) and alveolar phases (P4, P5) to identify genes induced by EGFR signaling that were shared or unique to each phase. We found 196 genes to be altered (≥1.5-fold change; P < 0.01 for at least 2 time points), with only 32% similarly altered in both saccular and alveolar phases. Western blot analysis and immunostaining showed that five genes selected from the microarrays (egr-1, SP-B, SP-D, S100A4, and pleiotrophin) were also increased at the protein level. Pathological changes in TGF-α-overexpressing mice bore similarities to premature infants born in the saccular phase who develop BPD, including remodeling of the distal lung septae and arteries.

bronchopulmonary dysplasia; epidermal growth factor receptor; lung remodeling; lung development; transforming growth factor-α

WHEN INFANTS ARE BORN PREMATURELY, a myriad of health problems often result, including respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD) (68). BPD represents a major public health problem as more and increasingly immature babies survive premature birth due to improvements in neonatal care and the use of surfactant therapy for RDS. BPD is a chronic lung disease of premature newborns, associated with fibrosis, inflammation, and disrupted development (27, 28). Morbidity and mortality in infants with BPD can be severe, resulting in death or costly long-term care (68). The long-term sequelae of BPD reflect the profound and complex impact of the disease: a predisposition to infections, cor pulmonale, and abnormal lung growth (1, 28).

Premature infants who deliver at 26–36 wk of gestation are born in the saccular phase of lung development and are particularly susceptible to BPD (33). Lung development during the saccular phase involves a number of vital morphological processes, including growth and widening of distal airways, differentiation of type I and type II cells, and thinning of the air-blood barrier (6). The saccules subsequently divide dichotomously, giving rise to terminal air spaces. In addition, as the interstitial tissue thins, the distal air spaces expand and the capillary networks encompassing them reorganize to lie in close apposition to the epithelium. Hence, the saccular phase is a critical stage, which prepares the distal lung for the subsequent formation of alveoli (the alveolar phase) and gas exchange at birth. In premature infants who develop BPD, disruption of the saccular phase results in lung remodeling and altered lung morphogenesis. The molecular mechanisms responsible are unclear but may include epidermal growth factor receptor (EGFR) activation (61, 62).

The EGFR is a tyrosine kinase receptor with multiple ligands, including EGF, transforming growth factor-α (TGF-α), heparin-bound EGF, and amphiregulin. The EGFR is involved both in the regulation of normal cellular processes and in lung cancer and other diseases (7, 25, 57). Some EGFR ligands, such as TGF-α, are released from the cell membrane by sheddingases before activating EGFR signaling (53). EGFR signaling has been shown to be downstream of proinflammatory pathways, reactive oxygen species, and hyperoxia, which are believed to be major contributors to the etiology of BPD (46, 67). Reactive oxygen species may also activate EGFR directly through tyrosine phosphorylation (13, 14). In addition, EGFR signaling has been shown to be downstream of inflammatory cytokines and mediators such as IL-13 and leukotrienes (5, 30, 58, 66). Other activators of EGFR signaling include acrolein, cigarette smoke, bacterial products, and mechanical damage (10, 31, 37, 52, 63). These and other studies suggest that EGFR signaling plays a central role in lung remodeling (3, 4, 17, 49, 61, 62).

TGF-α and the EGFR have been shown to be increased in lungs of infants with BPD (61, 62). Although the mechanism(s) responsible for increases in TGF-α and EGFR in BPD is uncertain, TGF-α levels are increased in experimental models by exposure to hyperoxia, inflammatory cytokines, and neu-
EGFR signaling that were shared or unique to each phase.

induction during the saccular phase was compared with TGF-β microarray analysis. The transcriptional profile after TGF-β overexpression was assessed before and after birth. To identify downstream genes, epithelial maturation, and surfactant levels were measured at E18.5. Sat PC was isolated from lipid extract of lung tissue using osmium tetroxide and quantified as previously described (39). For histology, lungs from single-transgene control and transgenic littermates were fixed at E17.5, E18.5, and P1 following treatment. Embryonic lungs were fixed by immersion in fixative (4% paraformaldehyde in 1× PBS, pH 7.4); postnatal lungs were fixed at 25 cmH₂O pressure after tracheal installation of the fixative (35). Lung sections were paraffin-embedded, and hematoxylin and eosin staining was performed on 5-μm paraffin sections from control and transgenic mice. Immunostaining for mucin 5AC (Mac5AC, chicken polyclonal antibody, kindly provided by Dr. Samuel Ho, University of Minnesota) was performed to determine whether prenatal TGF-β overexpression caused goblet cell hyperplasia. CCSP (rabbit polyclonal, kindly provided by Dr. Barry Stripp, University of Pittsburgh) immunostaining was used to detect Clara cells lining the conducting airways. Immunohistochemistry for prosurfactant protein-C (pro-SP-C; rabbit polyclonal AB3428; Chemicon, Temecula, CA) was performed to assess type II cell differentiation and numbers. Type II cells were then counted in five × 20 images from each animal, acquired from gas exchange regions of the lung, excluding conducting airway and large vessels (n = 5 per group). A Nikon Microphot FXA EPI-FL3 microscope was used to acquire digital images, which were then analyzed in MetaMorph imaging software (v6.2; Universal Imaging). Pixel density for each × 20 digital image was 0.266 μm²/pixel. Type II cells labeled by the pro-SP-C stain were then counted. Counts were normalized to the area of tissue present in each field. Tissue area was determined by subtracting air space area using the thresholding function of MetaMorph from the total area of the field (92,711 μm², area of a ×20 microscope field). Pan-cytokeratin (CK; C1801; Sigma-Aldrich, St. Louis, MO) immunostaining was used to assess overall epithelial morphology. Fractional areas for air spaces, total tissue,

The goal of the present study was to determine the impact of increases in TGF-β-induced EGFR signaling during the saccular phase of lung morphogenesis, since premature infants born at 26–36 wk of gestation are born in this phase. We hypothesized that, based on previous studies in neonatal and adult animals, overexpression of TGF-β during the saccular phase would cause lung remodeling and disrupt lung morphogenesis. To address this question, we used conditional transgenic mice to target TGF-β overexpression to the early saccular phase of lung development. Neonatal mortality, lung morphogenesis, epithelial maturation, and surfactant levels were assessed before and after birth. To identify downstream genes that may play a role in the disease process, we performed gene microarray analysis. The transcriptional profile after TGF-β induction during the saccular phase was compared with TGF-β induction in the alveolar phase to identify genes induced by EGFR signaling that were shared or unique to each phase.

**METHODS AND MATERIALS**

**Animal study protocols.** All animal procedures and protocols were approved by the Animal Care and Use Committee at the Cincinnati Children’s Hospital Research Foundation (Cincinnati, OH). All the mice used were on the FVB/N strain genetic background. For these studies, CCSP-rtTA transgenic activator mice were used, which express the reverse tetracycline transactivator (rtTA) transcription factor driven by a 2.3-kb sequence from the rat Clara cell secretory protein (CCSP) gene promoter (47, 65). In this CCSP-rtTA line, rtTA is expressed in the alveolar type II cells and occasionally in Clara cells (respiratory epithelium) (47). CCSP-rtTA+/−/activator mice were mated to conditional doxycycline (Dox)-regulated transgenic mice containing the human TGF-β cDNA under the control of seven copies of the tetracycline operon [(tetO) −/TGF-β−/−]+ plus a minimal cytomegalovirus promoter (19). Single-transgenic (CCSP-rtTA−/−) pups and transgenic [CCSP-rtTA+/−/(tetO)−/TGF-β−/−] pups were produced in the same litter by mating homozygous CCSP-rtTA−/− mice to hemizygous (tetO)−/TGF-β−/−/ mice. Single-transgene mice (CCSP-rtTA−/−) exposed to Dox served as controls throughout this study. Dox-containing chow (625 mg/kg) was given to time-mated pregnant dams from embryonic days 16.5 to 17.5 (E16.5–E17.5) or E16.5–E18.5, which represents the beginning of the saccular phase of lung morphogenesis in mice. When Dox is present, rtTA is activated and binds to the tet operator DNA binding element in the (tetO)−/TGF-β−/− transgene in bitransgenic mice, driving TGF-β overexpression (19, 35, 47). In the absence of Dox, no TGF-β is expressed by the TGF-β transgene (35). PCR analysis of tail DNA was performed to genotype pups for the presence of the CCSP-rtTA and (tetO)−/TGF-β transgenes. Postnatal survival of pups after Dox E16.5–18.5 was monitored and evaluated using Kaplan-Meier analysis. When neces-

sary, mice were killed for further analysis using pentobarbital sodium (65 mg/ml) solution (Fort Dodge Animal Health, Fort Dodge, Iowa).

**TGF-β ELISA.** TGF-β levels in lung homogenates after Dox induction from E16.5–E18.5 were measured at E18.5 and P3 using a human TGF-β ELISA kit (Oncogene Research Products, San Diego, CA) as previously described (35). TGF-β levels (pg/ml) were corrected to total lung protein levels (mg/ml).

**Surfactant analysis, histology, epithelial maturation, elastin staining, and cell proliferation.** Saturated phosphatidylcholine (Sat PC) levels were measured at E18.5. Sat PC was isolated from lipid extract of lung tissue using osmium tetroxide and quantified as previously described (39). For histology, lungs from single-transgene control and transgenic littermates were fixed at E17.5, E18.5, and P1 following treatment. Embryonic lungs were fixed by immersion in fixative (4% paraformaldehyde in 1× PBS, pH 7.4); postnatal lungs were fixed at 25 cmH₂O pressure after tracheal installation of the fixative (35). Lung sections were paraffin-embedded, and hematoxylin and eosin staining was performed on 5-μm paraffin sections from control and transgenic mice. Immunostaining for mucin 5AC (Mac5AC, chicken polyclonal antibody, kindly provided by Dr. Samuel Ho, University of Minnesota) was performed to determine whether prenatal TGF-β overexpression caused goblet cell hyperplasia. CCSP (rabbit polyclonal, kindly provided by Dr. Barry Stripp, University of Pittsburgh) immunostaining was used to detect Clara cells lining the conducting airways. Immunohistochemistry for prosurfactant protein-C (pro-SP-C; rabbit polyclonal AB3428; Chemicon, Temecula, CA) was performed to assess type II cell differentiation and numbers. Type II cells were then counted in five × 20 images from each animal, acquired from gas exchange regions of the lung, excluding conducting airway and large vessels (n = 5 per group). A Nikon Microphot FXA EPI-FL3 microscope was used to acquire digital images, which were then analyzed in MetaMorph imaging software (v6.2; Universal Imaging). Pixel density for each × 20 digital image was 0.266 μm²/pixel. Type II cells labeled by the pro-SP-C stain were then counted. Counts were normalized to the area of tissue present in each field. Tissue area was determined by subtracting air space area using the thresholding function of MetaMorph from the total area of the field (92,711 μm², area of a ×20 microscope field). Pan-cytokeratin (CK; C1801; Sigma-Aldrich, St. Louis, MO) immunostaining was used to assess overall epithelial morphology. Fractional areas for air spaces, total tissue,
epithelial, and mesenchymal compartments were then determined from images acquired on the Nikon microscope and imported into MetaMorph (45). Area measurements were performed on digital images of five fields per animal from distal gas exchange regions (n = 5 per group). Air space area was measured using the thresholding function of MetaMorph. Tissue area was determined by subtracting air space area from the total area of each field (92,711 m²). Epithelial area measurements were determined by thresholding on the pan-CK stain and determining staining density. Mesenchymal area was determined by subtracting epithelial area from the total tissue area. Cell proliferation was assessed by immunostaining with an anti-phospho-histone H3 antibody (H5110-14B rabbit polyclonal, US Biological, Swampscott, MA) and Western blot analysis for proliferating cell nuclear antigen (PCNA; 1:20,000; BD Pharmingen, San Diego, CA). Elastic fibers were detected with a modified Hart’s stain by using resorcin-fuchsin and counterstaining with 0.25% tartrazine in saturated picric acid (Poly Scientific, Bay Shore, NY) (35).

Wet-to-dry lung weight and heart-to-body weight ratios. Bitransgenic animals and control littersmates were treated with Dox from E16.5 to E18.5 and then collected at P1. Lungs and hearts were collected from animals after body weights were recorded. Total heart weights were recorded. Wet lung weights were measured, and then the lungs were baked overnight at 60°C. Dry lung weights were then measured. Wet-to-dry weight ratios were calculated.

Electron microscopy. Transmission electron microscopy of control and bitransgenic lungs was performed at E18.5 (after Dox from E16.5–E18.5) as previously described (35), except that lungs were fixed in 3% glutaraldehyde-0.15 M cacodylate buffer, pH 7.4, at 4°C for 24 h. Tissue blocks were collected from all lobes and post-fixed in 1% osmium tetroxide, dehydrated in propylene oxide, and embedded in LX112 resin (Ladd, Burlington, VT). Uranyl acetate and lead citrate counterstaining of 80-nm sections cut with a diamond knife was performed. Photographs were taken with a Hitachi H-7600 transmission electron microscope (Hitachi, Kyoto, Japan).

RNA collection for microarray. For microarray analysis, total lung RNA was isolated at E17.5 and E18.5 after Dox induction from E16.5 to E17.5 (1 day) and E16.5 to E18.5 (2 days) and also at P4 and P5 after Dox induction from P3 to P4 (1 day) or P3 to P5 (2 days). Total RNA was isolated using the Qiagen Mini RNeasy kit following the manufacturer’s instructions (Qiagen, Valencia, CA). For the saccular phase time points (E17.5 and E18.5), each array represented total RNA from a single animal as the phenotype was more consistent...
(n = 5 arrays for single-transgene controls and n = 5 arrays for bitransgenics for each of the prenatal time points). For the alveolar phase time points (P4 and P5), total RNA from three mice was pooled for each array, because there was more variability between litters (n = 4 arrays for single-transgene controls and n = 4 arrays for bitransgenics for each of the postnatal time points).

**Microarray hybridization.** The microarray experiments were carried out as previously described (16, 29, 56). The mouse 70-mer oligonucleotide library version 3.0 (31,775 optimized oligos; Qiagen, Alameda, CA) was suspended in 3 × SSC at 30 μM and printed at 22°C and 65% relative humidity on aminosilane-coated slides (CEL Associates, Pearland, TX) using a high-speed robotic Omnimgrid machine (GeneMachines, San Carlos, CA) with Stealth SMP3 pins (Telechem, Sunnyvale, CA). Spot volumes were 0.5 nl, and spot diameters were 75–85 μm. The oligonucleotides were cross-linked to the slide substrate by exposure to 600 mJ of ultraviolet light. Fluorescence-labeled cDNAs were synthesized from total RNA by using an indirect amino allyl labeling method via an oligo(dT)-primed reverse transcriptase reaction. The cDNA was decorated with monofunctional reactive cyanine-3 and cyanine-5 dyes (Cy3 and Cy5; Amersham, Piscataway, NJ). The microarrayed DNA probes were incubated in prehybridization buffer (5× SSC, 0.1% SDS, and 1% bovine serum albumin) at 42°C for 90 min. For hybridization, the microarray slides were spotted with 42 μl of hybridization buffer [25% formamide, 5× SSC, 0.1% SDS, 5 μg/μl COT1-DNA, 5 μg/μl poly(A)-DNA, and 2 μg/μl yeast tRNA]. Fluorescence-labeled target cDNA was added, and then the slides were covered with glass coverslips (Fisher, Pittsburgh, PA) and placed in humidified hybridization chambers (Corning, Acton, MA). The hybridization chambers were placed in a water bath at 48°C for 66 h. The slides were washed in 1× SSC with 0.2% SDS for 4 min at 48°C with agitation and then transferred to 0.1× SSC and 0.2% SDS and washed with agitation for 4 min at room temperature and two times for 4 min each in 0.1× SSC at room temperature. The slides were spun-dried immediately after

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**Fig. 4.** Epithelial maturation and differentiation after TGF-α induction from E16.5 to E18.5. Immunostaining was performed on E18.5 lung sections for mucin 5AC (Muc5AC), Clara cell secretory protein (CCSP), prosurfactant protein-C (pro-SP-C), and cytokeratin (CK). Muc5AC staining was, for the most part, restricted to the upper airways (bronchus and conducting airways). Muc5AC and CCSP staining was similar in distribution between bitransgenic and control mice. Staining for pro SP-C at E18.5 indicated increased numbers of type II cells in the lung parenchyma of bitransgenic mice compared with controls (see insets). Quantification of CK staining (black) showed increased epithelial area in bitransgenic animals (see high-power insets). Bars, 100 μm.
washing. The complete gene list and more details of cDNA labeling and slide preparation can be found at http://microarray.uc.edu.

Imaging and data acquisition were carried out using a GenePix 4000A and GenePix 4000B and associated software (Axon Instruments, Foster City, CA). The microarray slides were scanned with dual lasers with wavelength frequencies to excite Cy3 and Cy5 fluorescence emittance. Images were captured in JPEG and TIFF files, and DNA spots were captured using the adaptive circle segmentation method. Information extraction for a given spot was based on the median value for the signal pixels minus the median value for the background pixels to produce a gene set data file for all the DNA spots.

**Microarray data normalization and analysis.** Data representing background subtracted spot intensities generated by GenePix Pro version 5.0 software were analyzed to identify differentially expressed genes. Data normalization was performed in two steps for each microarray separately (16, 29, 56). First, background adjusted intensities were log-transformed, and the differences (R) and averages (A) of log-transformed values were calculated as $R = \log_2(X1) - \log_2(X2)$ and $A = (\log_2(X1) + \log_2(X2))/2$, where X1 and X2 denote the Cy5 and Cy3 intensities after subtraction of local backgrounds, respectively. Second, normalization was performed by fitting the array-specific local regression model of R as a function of A. Normalized log-intensities for the two channels were then calculated by adding one-half of the normalized ratio to A for the Cy5 channel and subtracting one-half of the normalized ratio from A for the Cy3 channel. The statistical analysis was performed for each time point comparison and for each gene separately by fitting the following mixed-effects linear model (12): $Y_{ijk} = \mu + A_i + S_j + C_k + e_{ijk}$.

### Table 1. Fractional area of tissue and space

<table>
<thead>
<tr>
<th></th>
<th>Tissue, %</th>
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<tbody>
<tr>
<td></td>
<td>Airspace, %</td>
</tr>
<tr>
<td>Control</td>
<td>26.7±2.3</td>
</tr>
<tr>
<td>TGF-α overexpressing</td>
<td>9.4±2.1*</td>
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The MetaMorph program was used to estimate fractional areas of air space, tissue, and epithelial and mesenchymal compartments at embryonic day 18.5 (E18.5) in distal gas exchange regions of transforming growth factor-α (TGF-α)-overexpressing animals compared with controls. Epithelial area was identified using pan-cytokeratin staining. *P < 0.05 compared with control.

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**Fig. 5.** Cell proliferation after TGF-α induction (E16.5–E18.5). A: Western blot analysis shows increased levels of proliferating cell nuclear antigen (PCNA) in lungs of bitransgenic vs. control mice at E18.5. *P < 0.05 vs. control. B: immunostaining for phosphohistone shows increased phosphohistone-positive cells in the distal lung parenchyma and adventitia around the bronchovascular tissue in bitransgenic mice compared with controls. Bars, 100 μm.
where $Y_{ik}$ corresponds to the normalized log-intensity on the $i$th array, with the $j$th treatment condition and labeled with the $k$th dye ($k = 1$ for Cy5 and 2 for Cy3), $\mu$ is the overall mean log-intensity, $A_i$ is the effect of the $i$th array, $S_j$ is the effect of the $j$th treatment, and $C_k$ is the gene-specific effect of the $k$th dye. Assumptions about model parameters were the same as previously described (69), with array effects assumed to be random and treatment and dye effects assumed to be fixed. Resulting $t$-statistics from each contrast were modified using an empirical Bayesian moderated-$T$ method (59). This method uses variance estimates from all genes to improve the variance estimates of each individual gene. Estimates of degree of change were calculated, and genes with $P$ values $<0.01$ and greater than 1.5-fold change for at least two of the four comparisons were considered to be significantly differentially expressed. In addition, we required that the gene be measurable on at least three arrays at each time point to be considered significant for that time point. Cluster analysis was performed using the Bayesian infinite mixture models (40) as implemented in the GIMM software (http://eh3.uc.edu/gimm). Clustering results were visualized using Java TreeView (54). The EASE program was used to determine which Gene Ontology categories were overrepresented among altered genes from the array ($P < 0.005$ using Fisher’s exact test) (24). Array data comply with MIAME standards and is available for access on ArrayExpress (accession no. E-MEXP-698, http://www.ebi.ac.uk/arrayexpress/).

Assessment of protein levels for selected genes detected by microarray and verification of EGFR activation. A sample of three genes from the saccular phase array (SP-B, SP-D, egr-1) and three genes from the alveolar phase array (egr-1, pleiotrophin, S100A4) were verified at the protein level by performing Western blot analysis or immunohistochemistry. In addition, EGFR activation was confirmed by assessing the levels of phosphorylated EGFR (pY1086) from single-transgene controls and bitransgenic littermates (TGF-α) by using primary antibodies against phospho-EGFR (1:1,000, rabbit monoclonal, pY1086; Epitomics, Burlingame, CA), total EGFR (1:5,000, rabbit polyclonal; kind gift from Dr. B. Warner, Cincinnati Children’s Hospital Medical Center), egr-1 (1:400 dilution, C19; Santa Cruz Biotechnology, Santa Cruz, CA), SP-D (1:1,000, rabbit polyclonal; Seven Hills Bioreagents, Cincinnati, OH), SP-B (1:5,000, rabbit polyclonal; Chemicon), pleiotrophin (1:1,000; R&D Systems, Minneapolis, MN), and pan-actin (1:20,000, MAB1501, clone C4; Chemicon). Goat anti-rabbit or goat anti-mouse (Santa Cruz Biotechnology) secondary antibodies were used, and chemiluminescence detection was performed using the ECL Plus system (Amersham Biosciences). In addition, an anti-S100A4 antibody (1:200; Lab Vision, Fremont, CA) was used for immunostaining of lung sections.

Statistical analysis. Data are means ± SE. The Prism 4 software package was used to perform data analyses (GraphPad Software, San Diego, CA). Western blots were analyzed using ImageQuant (Molecular Dynamics, Sunnyvale, CA). Statistical comparisons were made using unpaired $t$-tests, one-way ANOVA, and the log-rank test to analyze the Kaplan-Meier survival analysis. $P < 0.05$ was considered significant, except as described above in the microarray analysis.

RESULTS

TGF-α expression, neonatal mortality, body weight, total lung protein, and surfactant analysis in conditional TGF-α transgenic mice. TGF-α levels were increased in the lungs of bitransgenic mice [CCSP-rtTA$^{+/-}$/tetO$^{-/-}$]-TGF-α$^{+/+}$] at E18.5 compared with single-transgene controls after Dox treatment from E16.5 to E18.5 ($n = 6–7$ per group; $P < 0.05$) (Fig. 1). All bitransgenics survived ($P < 0.05$) (Fig. 1). After Dox was removed at E18.5, TGF-α levels decreased in the lungs of bitransgenic mice at P3 compared with levels at E18.5 ($n = 3–7$ per group; $P < 0.05$) (Fig. 1). All bitransgenic pups died by P6, whereas most of the single-transgene controls survived ($n = 14$ bitransgenic, $n = 16$ control; Fig. 2). Kaplan-Meier survival analysis with the log-rank test showed a significant reduction in the survival of bitransgens compared with controls ($P < 0.05$). Body weights of bitransgenic (1.239 ± 0.022 g) and single-transgene control mice (1.226 ± 0.032 g) at E18.5 (after Dox from E16.5

![Fig. 6. Abnormal elastic fiber deposition in bi-transgenic mice at E18.5 and P1. Filled arrows show deposition of elastic fibers in control animals; open arrows indicate elastic fibers in bi-transgenic animals at E18.5 and P1. In control mice at P1, elastin fibers were abundant and aligned with the alveolar structure in the lung parenchyma. Elastin fibers were reduced in the distal air spaces of bitransgenic mice at P1, despite the hypercellularity of these regions. Elastic fibers were detected using the modified Hart’s stain. Bars, 50 μm.](http://ajplung.physiology.org/ by 10.22032/32.246 on November 6, 2017)
to E18.5) were similar (P > 0.05). Total lung protein content was 20% higher in bitransgenic mice compared with controls (86.0 ± 4.2 vs. 71.5 ± 3.2 mg protein/g lung wt, respectively; n = 5 bitransgenic, n = 4 control; P < 0.05) at E18.5. Sat PC levels in bitransgenic and control littermates were similar (48.10 ± 2.473 μmol/kg body wt in controls and 57.92 ± 5.531 μmol/kg body wt in bitransgenics; n = 15 bitransgenic, n = 9 control; P > 0.05).

Fig. 7. Induction of TGF-α during the saccular phase of lung morphogenesis causes thickening of the distal parenchyma characterized by abundant lipofibroblasts and interposition of the capillaries in the alveolar septae. Electron microscopy of control (left) and bitransgenic (right) lungs at E18.5 shows lipofibroblasts (asterisks), characterized by lipid inclusions. Bitransgenic lungs show more conspicuous lipofibroblasts in the distal septae (inset shows lipofibroblast from bitransgenic lung). Capillaries apposed to the distal air spaces are indicated by arrows, whereas capillaries interpositioned in the septae are marked with a “C”. Some of the capillaries in the bitransgenic sample show poor apposition to the alveolar space. Increased extracellular matrix can also be seen in the parenchyma of the bitransgenic lung.

Fig. 8. TGF-α activation in the saccular vs. alveolar phases of lung morphogenesis induces different transcriptional profiles. Microarray analysis was performed on single and bitransgenic mice at E17.5 and E18.5 after Dox from E16.5 to E17.5 and E16.5 to E18.5, respectively. In addition, microarray analysis was also performed at P4 and P5 after Dox from P3 to P4 and P3 to P5. Cluster analysis of the 1- and 2-day time points shows different transcriptional profiles induced by TGF-α in the saccular vs. alveolar phases of lung morphogenesis. Saccular 1 or 2 days indicates Dox administration from E16.5 to E17.5 or E16.5 to E18.5, respectively; alveolar 1 or 2 days indicates Dox administration from P3 to P4 or P3 to P5, respectively. Arrows indicate the positions of verified genes in the heat map.
Lung histology of conditional TGF-α transgenic mice. Lung histology at E17.5, after 1 day of Dox treatment, showed an increase in parenchymal cellularity in bitransgenic mice compared with controls (Fig. 3). By E18.5, there was extensive thickening of the septae in the sacculles, as well as thickening of the mesenchyme surrounding the arteries and central airways in bitransgenic mice. In contrast, control animals showed thinning of the septae in the lung parenchyma from E17.5 to P1 (Fig. 3, insets). At P1, histology of bitransgenic pups that did not die at birth showed persistent thickening of the septae and vascular remodeling (Fig. 3, insets).

Epithelial morphology and differentiation in lungs of conditional TGF-α transgenic mice. To determine whether there were any abnormalities in epithelial morphology and differentiation in the bitransgenic mice, epithelial specific marker proteins were assessed and compared with controls at E18.5 (Fig. 4). The number and distribution of Muc5AC-positive cells in the bitransgenic pups was similar to that in controls (Fig. 4). CCSP-positive cells (Clara cells) lining the distal conducting airways had a normal distribution in bitransgenic pups compared with controls (Fig. 4, arrows). Immunostaining for pro-SP-C at E18.5 (Fig. 4, inset) and type II cell counts showed more type II cells in TGF-α-overexpressing animals compared with controls. Type II cell counts corrected to tissue area were as follows: 70.2 ± 5.2 cells per 0.1 mm² lung tissue in bitransgenic animals vs. 43.4 ± 2.2 in controls (n = 5 mice in each group; P < 0.01). A pan-CK antibody was used to stain all epithelial cells in the lung (Fig. 4). Fractional areas of air space, tissue, pan-CK-positive epithelium, and mesenchyme are presented in Table 1. Percent total tissue area present in each microscope field picture was increased 24% in bitransgenic animals compared with controls (P < 0.05), whereas air

Table 2. EASE ontology analysis of gene categories overrepresented in genes found to be altered by microarray analysis

<table>
<thead>
<tr>
<th>Gene Ontology Category</th>
<th>Saccular 1 Day</th>
<th>Saccular 2 Day</th>
<th>Alveolar 1 Day</th>
<th>Alveolar 2 Day</th>
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<tr>
<td></td>
<td>Inc</td>
<td>Dec</td>
<td>Inc</td>
<td>Dec</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>4%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Behavior</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Blood vessel development</td>
<td>4%</td>
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<td></td>
<td></td>
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<td>Bone remodeling</td>
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<td>Catalytic activity</td>
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<tr>
<td>Collagen</td>
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<tr>
<td>Defense/immunity protein activity</td>
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<tr>
<td>Development</td>
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<tr>
<td>Exonuclease activity</td>
<td></td>
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<td>3%</td>
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<tr>
<td>Extracellular activity</td>
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<td>25%</td>
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<tr>
<td>Extracellular matrix</td>
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<td></td>
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<td>8%</td>
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<tr>
<td>Extracellular matrix structural constituent</td>
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<td>6%</td>
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<tr>
<td>Extracellular matrix structural constituent conferring tensile strength</td>
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<tr>
<td>Extracellular space</td>
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<tr>
<td>Glycosaminoglycan binding</td>
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<tr>
<td>Growth factor activity</td>
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<tr>
<td>Hematopoietin/interferon-class (d200-domain) cytokine receptor activity</td>
<td></td>
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<td>4%</td>
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<td>Heparin binding</td>
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<td>Hormone activity</td>
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<tr>
<td>Hydrolase activity</td>
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<tr>
<td>Hydrolase activity, acting on ester bonds</td>
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</tr>
<tr>
<td>Learning</td>
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<tr>
<td>Learning and/or memory</td>
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<tr>
<td>Morphogenesis</td>
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<tr>
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<tr>
<td>Ossification</td>
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<tr>
<td>Phosphoric ester hydrolase activity</td>
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<tr>
<td>Phosphoric monoester hydrolase activity</td>
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<td>Protein-tyrosine-phosphatase activity</td>
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<tr>
<td>Receptor binding</td>
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<tr>
<td>Regulation of angiogenesis</td>
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<td>Respiratory gaseous exchange</td>
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<td>RNA methyltransferase activity</td>
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<tr>
<td>Signal transducer activity</td>
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<td>Skeletal development</td>
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<tr>
<td>Surfactant activity</td>
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<tr>
<td>tRNA (guanine) methyltransferase activity</td>
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<tr>
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<tr>
<td>tRNA processing</td>
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The EASE program was used to find Gene Ontology categories overrepresented among genes altered on the TGF-α microarray. Values indicate the percentage of genes that fall into the specified Gene Ontology category within the corresponding time point and alteration grouping (for example, 4% of genes increased after 2 days of TGF-α induction during the saccular phase fall into the angiogenesis category). Inc, increase; Dec, decrease. Categories listed have ≥1 members and P < 0.005.
space area was decreased 65% in bitransgenic animals compared with control animals ($P < 0.05$). Both epithelial and mesenchymal area were higher in bitransgenic animals compared with control animals ($P < 0.05$); however, the ratio of epithelial-to-mesenchymal area was not different ($P > 0.05$). To examine whether increased cell proliferation in the bitransgenic mice was contributing to the parenchymal thickening, lung PCNA levels were measured by Western blot analysis and immunostaining was performed for phosphohistone-positive cells (Fig. 5). PCNA levels were increased 1.6-fold in lung homogenates of E18.5 bitransgenic mice compared with controls ($n = 4–5$ per group; $P < 0.05$) (Fig. 5A). Phosphohistone-positive cells were abundant in bitransgenic pups, especially in the distal lung parenchyma and in the mesenchyme around the bronchovasculature compared with controls (Fig. 5B). Because TGF-α induction during the alveolar phase was previously shown to result in abnormal elastin deposition, a modified Hart’s stain for elastin was performed on E18.5 lungs after Dox from E16.5 to E18.5 (35). The Hart’s stain showed a paucity and disorganization of elastic fibers in bitransgenic mice compared with control littermates (Fig. 6).

Wet-to-dry lung weight and total heart-to-body weight ratios in bitransgenic mice. Wet-to-dry lung weight and total heart-to-body weight ratios were determined in newborn mice (P1) after Dox induction from E16.5 to E18.5 ($n = 9$ control animals, $n = 10$ bitransgenic animals). No significant difference was found in the ratios of wet to dry lung weights between control and bitransgenic mice ($5.11 \pm 0.14$ vs. $5.4 \pm 0.08$, respectively; $P > 0.05$). Total heart weight (mg)-to-body weight (g) ratios in bitransgenic mice were similar to those of controls ($7.47 \pm 0.33$ vs. $7.00 \pm 0.48$ mg/g, respectively; $P > 0.05$).

Ultrastructural changes in the lung parenchyma of TGF-α-overexpressing transgenic mice. Electron microscopy at E18.5 after Dox from E16.5 to E18.5 demonstrated increased cellu-

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**Fig. 9.** Assessment of protein levels of genes selected from the saccular phase microarray analysis. Western blot analysis for egr-1, surfactant protein B (SP-B), and SP-D was performed on lung homogenates of control and bitransgenic mice. Egr-1 protein levels were increased in bitransgenic mice at E17.5, after Dox from E16.5 to E17.5. SP-B and SP-D levels were measured in lung homogenates at E18.5 after Dox treatment from E16.5 to E18.5. Both SP-B and SP-D protein levels increased in bitransgenic mice compared with single-transgene controls. *$P < 0.05$ vs. control.
larity and expansion of the interstitium in the distal lungs of bitransgenic mice compared with controls (Fig. 7). Lipofibroblasts were more conspicuous in the distal septae of bitransgenic mice (Fig. 7, asterisks). In addition, there was interposition of capillaries in the septae and poor apposition of some capillaries relative to the distal air spaces (Fig. 7, arrows).

Identification of transcriptional profiles after TGF-α induction in the saccular phase and alveolar phase. To identify genes downstream of TGF-α-induced EGFR signaling in the saccular versus alveolar stages, microarray analysis was performed on lung RNA from single-transgene controls and bitransgenic mice after Dox treatment from E16.5 to E17.5 (saccular 1 day), E16.5 to E18.5 (saccular 2 days), P3 to P4 (alveolar 1 day), or P3 to P5 (alveolar 2 days) (n = 4–6 arrays per group) (Supplemental Table 1). Analysis of the microarray was restricted to genes that were altered >1.5-fold and where P < 0.01 compared with control for at least two of the four time points. Of the 196 genes that were found to be significant using these criteria, only 62 (32%) were similarly altered in both the prenatal and postnatal time points. Clustering analysis highlighted the differences in the transcriptional profiles after saccular versus alveolar induction of TGF-α-induced EGFR signaling (Fig. 8). Eleven major clusters of similarly regulated genes were found (Fig. 8 and Supplemental Table 2). By using the EASE Gene Ontology analysis program (24), categories overrepresented among altered genes were identified (Table 2). After 1 and 2 days of TGF-α induction during the saccular phase, genes with surfactant activity were overrepresented among increased genes. In both the saccular and alveolar phase arrays, extracellular matrix/space genes were overrepresented among decreased genes.

Assessment of protein levels for selected genes detected by microarray analysis and verification of EGFR activation. Candidate genes identified from the microarray were verified at the protein level by Western blot analysis or immunostaining. Consistent with our hypothesis that neonatal death in the TGF-α-overexpressing mice was not due to surfactant deficiency, SP-B protein levels were increased 1.2-fold in bitransgenic mice at E18.5 after Dox from E16.5 to E18.5 (n = 4–5 animals per group; P < 0.05) (Fig. 9). In addition, SP-D protein levels were also increased 3.6-fold at E18.5 in bitransgenic mice (n = 4–5 animals per group; P < 0.05). The zinc finger transcription factor egr-1 was the most highly upregulated gene on the saccular phase array. Western blot analysis showed that egr-1 protein levels were increased 4.2-fold in the lungs of bitransgenic pups treated with Dox for 1 day in the saccular phase (n = 4–5 animals per group; P < 0.05) (Fig. 10). Activation of EGFRs was verified at P4 after 1 day of Dox treatment (P3–P4) by Western blot analysis. Phosphorylated EGFR (pY1086) levels corrected to total EGFR levels were increased 7.6-fold in bitransgenic mice compared with controls (n = 4–5 animals per group; P < 0.05) (Fig. 10). Egr-1 protein levels were also increased 1.9-fold after 2 days of Dox induction of TGF-α during the alveolar phase (n = 4–5 animals per group; P < 0.05) (Fig. 11). The mRNAs of pleiotrophin and S100A4 were increased on the microarray after alveolar phase TGF-α induction. The protein levels of pleiotrophin were increased 9.7-fold in bitransgenic animals as determined by Western blot analysis (n = 4–5 animals per group; P < 0.05) (Fig. 11). Immunostaining showed increased S100A4 in the smooth muscle and adventitial layers of small pulmonary arteries in bitransgenic animals compared with controls (Fig. 11).

DISCUSSION

Premature infants that are born between 26 and 36 wk of gestation are in the saccular stage of lung morphogenesis (33). This stage precedes the alveolar phase of lung development, when the final gas exchange structures of the lung, the alveoli, are formed (6). The present study demonstrates that increased TGF-α causes severe lung remodeling in the prenatal lung.
Increased expression of TGF-α during the saccular phase disrupted lung morphogenesis, caused mesenchymal and vascular remodeling, and led to neonatal mortality within the first week of life in bitransgenic mice.

Surfactant analysis and immunostaining for markers of epithelial morphology and differentiation indicated that neonatal mortality in the bitransgenic mice was not due to surfactant deficiency or severe defects in epithelial maturation. In fact, SP-B expression and type II cell counts were increased in bitransgenic mice. In accordance with our data, previous studies have shown that EGFR signaling increases both maturation of type II cells and surfactant secretion (48, 60). Lung histology and electron microscopy showed that there was extensive mesenchymal thickening, vascular remodeling, and abundant lipofibroblasts in the lung parenchyma of bitransgenic mice. Electron microscopy also showed that there was interposition of the capillaries with poor apposition to the alveolar space. An expanded mesenchyme, increased interstitial cellularity, vascular remodeling, and altered lung function are characteristic of the pathology and pathophysiology of infants with BPD (9, 28).

Elastin is a critical component of the lung structure that allows the alveoli to regain their shape after stretching during inhalation. Reductions in elastin, as occurs in emphysema, can result in tissue damage (23). Furthermore, elastin is believed to be vital for the formation as well as the structure of alveoli (44). Elastin mRNA levels were decreased on the microarray after saccular phase TGF-α induction. Using a modified Hart’s
stain for elastin, we found evidence of disorganization and a paucity of elastic fibers in bitransgenic mice. Disorganized elastic fiber structure was also seen after TGF-α induction during the alveolar phase (35).

The role of TGF-α-induced EGFR signaling in lung remodeling was previously studied by our group using transgenic mice that overexpress TGF-α in the distal epithelium throughout pre- and postnatal lung development under the direct control of the surfactant protein C promoter (SPC-TGF-α mice) (32). Chronic overexpression of TGF-α caused pulmonary fibrosis, thickening of alveolar septae, disrupted vascular and alveolar development, vascular remodeling, and pulmonary hypertension (18, 32, 36). Expression of dominant negative mutant EGFR and breeding to EGFR hypomorph mice (wa-2 mutant) inhibited these pathological processes in SPC-TGF-α mice (18, 36). Subsequent studies using conditional TGF-α mice showed that even transient increases in TGF-α in the alveolar phase of lung morphogenesis were sufficient to disrupt alveolar and vascular morphogenesis and cause pulmonary hypertension but not pulmonary fibrosis (35). In contrast, longer term increases in TGF-α in adult mice, using the same conditional system as used in the present study, did cause progressive pulmonary fibrosis (21).

To identify genes that might be playing a role early in the disease process, we performed RNA microarray analysis after just 1 and 2 days of Dox-induced TGF-α overexpression in the saccular phase. In addition, we determined the transcriptional profile after 1 and 2 days of TGF-α induction in the alveolar phase of lung morphogenesis. Western blot analysis of phosphorylated EGFR levels showed potent activation after just 1 day of Dox-induced TGF-α expression. The goal of these microarray studies was to identify genes that act early in the disease process and to determine which genes were common or specific to each developmental phase. Of the 196 genes that were detected as significantly altered by our microarray analysis in bitransgenic mice compared with control mice, only 62 (32%) were similarly altered during both the saccular and alveolar phases. Nineteen genes (10%) were increased at one developmental phase and decreased in the other phase. Gene Ontology analysis using the EASE program also showed that many of the genes altered at the saccular and alveolar phases belonged to different functional categories. It is important to mention that a reliance solely on microarray analysis may lead to the omission of important mechanisms and that changes in gene expression may only detect alterations in growth factors and proteins that are affected at the transcriptional level by TGF-α overexpression.

We examined the expression of some of the most highly increased genes identified by the microarray. The most highly upregulated gene in the saccular phase arrays was the zinc finger transcription factor egr-1. Egr-1 (also known as NGFI-A, Krox-24, and zif268) has also been shown to be induced in the adult lung by EGF treatment (38). Egr-1 was rapidly and highly induced in the saccular phase of TGF-α-overexpressing mice and, to a lesser extent, in the alveolar phase. Egr-1 has been shown to be associated with and to contribute to the pathogenesis of several diseases, including atherosclerosis, hypoxic and ischemic stress, IL-13- and TGF-β-induced lung remodeling, chronic obstructive pulmonary disease (COPD), and acute respiratory distress syndrome (ARDS) secondary to acute pancreatitis (8, 20, 26, 42, 43, 70).

Interestingly, antisense oligonucleotides to egr-1 inhibited the autonomous growth of pulmonary artery adventitial fibroblasts induced by hypoxia in neonatal calves (2). Egr-1 is induced by a number of cytokines and growth factors, including IL-13, fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and growth hormone (GH), as well as EGF (8, 21, 22, 38, 55, 64). In addition, egr-1 is induced by cigarette smoke and in the lungs of smokers with COPD (43, 50). Induction of egr-1 in this model is most likely downstream of EGFR signaling; however, we cannot exclude a role for other factors altered in the TGF-α-overexpressing mice, although there were no detectable changes on the microarray in other known regulators of egr-1 expression such as IL-13, FGF-2, VEGF, or PDGF.

S100A4 (also known as Mts-1) and pleiotrophin (HB-GAM, HARP) were increased in the alveolar phase array and were chosen for further study. S100A4 expression is increased in remodeling arteries in both a mouse model of hypoxia-induced pulmonary hypertension and in human patients with pulmonary arterial hypertension (15, 34). In this study, increases in S100A4 were localized to remodeling vessels in neonatal TGF-α-overexpressing mice, which have been previously shown to develop pulmonary hypertension (35). Pleiotrophin is a member of the protein family that also includes midkine (41). Overexpression of midkine in the developing lung increases muscularization of small pulmonary arteries (51).

In summary, we examined the role of increases in TGF-α in the saccular phase of lung morphogenesis. Increases in TGF-α during the saccular phase disrupted lung morphogenesis, causing mesenchymal thickening, vascular remodeling, and neonatal mortality. Interestingly, saccular phase induction of TGF-α caused a different phenotype and gene expression profile compared with increases in TGF-α during the alveolar phase and adult lung. Thus increases in TGF-α and EGFR signaling can cause different lung disease phenotypes depending on the developmental phase and/or the length of exposure. Future studies need to examine the role of genes identified by microarray analysis in the pathogenesis of lung disease induced by TGF-α and EGFR signaling.

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REFERENCES

3. Baughman RP, Lower EE, Miller MA, Bejarano PA, Heffelfinger SC. Overexpression of transforming growth factor-alpha and epidermal growth


