CTGF disrupts alveolarization and induces pulmonary hypertension in neonatal mice: implication in the pathogenesis of severe bronchopulmonary dysplasia

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Overexpression of CTGF in AT II cells results in lung pathology similar to those observed in infants with severe BPD and that ILK/GSK-3β/β-catenin signaling may play an important role in the pathogenesis of severe BPD.

Chen S, Rong M, Platteau A, Hehre D, Smith H, Ruiz P, Whitsett J, Bancalari E, Wu S. CTGF disrupts alveolarization and induces pulmonary hypertension in neonatal mice: implication in the pathogenesis of severe bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 300: L330–L340, 2011. First published January 14, 2011; doi:10.1152/ajplung.00270.2010.—The pathological hallmarks of bronchopulmonary dysplasia (BPD), one of the most common long-term pulmonary complications associated with preterm birth, include arrested alveolarization, abnormal vascular growth, and variable interstitial fibrosis. Severe BPD is often complicated by pulmonary hypertension characterized by excessive pulmonary vascular remodeling and right ventricular hypertrophy that significantly contributes to the mortality and morbidity of these infants. Connective tissue growth factor (CTGF) is a multifunctional protein that coordinates complex biological processes during tissue development and remodeling. We have previously shown that conditional overexpression of CTGF in airway epithelium under the control of the Clara cell secretory protein promoter results in BPD-like architecture in neonatal mice. In this study, we have generated a doxycycline-inducible double transgenic mouse model with overexpression of CTGF in alveolar type II epithelial (AT II) cells under the control of the surfactant protein C promoter. Overexpression of CTGF in neonatal mice caused dramatic macrophage and neutrophil infiltration in alveolar air spaces and perivascular regions. Overexpression of CTGF also significantly decreased alveolarization and vascular development. Furthermore, overexpression of CTGF induced pulmonary vascular remodeling and pulmonary hypertension. Most importantly, we have also demonstrated that these pathological changes are associated with activation of integrin-linked kinase (ILK)/glucose synthesis kinase-3β (GSK-3β)/β-catenin signaling. These data indicate that overexpression of CTGF in AT II cells results in lung pathology similar to those observed in infants with severe BPD and that ILK/GSK-3β/β-catenin signaling may play an important role in the pathogenesis of severe BPD.

inflammation; vascular development; vascular remodeling; GSK-3β; β-catenin

Despite recent advances in neonatal intensive care and surfactant therapy, bronchopulmonary dysplasia (BPD) continues to be one of the most common long-term pulmonary complications associated with preterm birth (29). Exposure to mechanical ventilation and supplemental oxygen for initial respiratory distress induces inflammatory responses that play a key role in the pathogenesis of BPD (29, 46). The inflammatory processes disrupt normal lung development and lead to chronic lung structure damage characterized by arrested alveolarization, abnormal vascular growth, and variable interstitial fibrosis (8, 23, 28, 46). Severe BPD is often complicated by pulmonary hypertension characterized by excessive pulmonary vascular remodeling and right ventricular hypertrophy that significantly contributes to the mortality and morbidity of these infants (14, 47, 48). However, there remains a significant gap in our knowledge regarding the interplay of growth factors, transcription factors, and inflammatory mediators that orchestrate normal lung development, the inflammatory response to injury, and the pathogenesis of BPD.

Connective tissue growth factor (CTGF) is a prototypical member of the CCN family of modular proteins that coordinate complex biological processes during tissue development and remodeling (34). Expression of CTGF is upregulated by several factors involved in tissue remodeling, including transforming growth factor-β (TGF-β), mechanical ventilation, and oxygen exposure (7, 17, 18, 49, 50). Upon stimulation, CTGF is secreted into the extracellular environment, where it interacts with distinct cell surface receptors, growth factors, and the extracellular matrix (ECM) (34). The principal CTGF receptor is the heterodimeric cell surface integrin complex whereas integrin-linked kinase (ILK) is a key mediator of integrin signaling that interacts with the cytoplasmic domain of β integrins (22, 25, 40). Activated ILK subsequently phosphorylates AKT, also known as protein kinase B and glucose synthesis kinase-3β (GSK-3β) thus leading to activation of a diverse array of cellular processes (22, 37, 45). In addition to its binding to integrins, CTGF can also bind to low-density lipoprotein receptor-related protein 5/6, thus modulating Wnt signaling (38, 53). Furthermore, CTGF also binds to various growth factors in the ECM, thereby modifying their activity. The binding of CTGF to TGF-β enhances TGF-β dimerization with its receptors, thus facilitating TGF-β signaling (1). In contrast, binding of CTGF to vascular endothelial growth factor (VEGF) decreases VEGF availability to its receptors, thus inhibiting VEGF-induced angiogenesis (11, 24). Overexpression of CTGF is associated with many forms of adult lung fibrosis and pulmonary vascular remodeling (3, 44). Increasing data suggest that CTGF also plays an important role in neonatal lung injury. Our laboratory and others have demonstrated that mechanical ventilation and hypoxia exposure induce CTGF expression in lungs of neonatal animals (2, 7, 50). Furthermore, our most recent study has demonstrated that inducible overexpression of CTGF in airway epithelium under the control of the
Clara cell secretory protein (CCSP) gene promoter disrupts alveolarization, decreases pulmonary vascular development, and induces interstitial fibrosis in the neonatal mouse lungs, which are similar to the lung pathology seen in BPD (51).

Because BPD is a distal lung disease, we have recently generated doxycycline (Dox)-inducible transgenic mice with overexpression of CTGF in alveolar type II epithelial (AT II) cells under the control of the surfactant protein C (SP-C) gene promoter. We report here that overexpression of CTGF in AT II cells of neonatal mice not only severely disrupts alveolarization and pulmonary vascular remodeling but also induces excessive pulmonary vascular remodeling and pulmonary hypertension. Furthermore, these lung structural changes were associated with activation of ILK/GSK-3β/β-catenin signaling in vivo and in vitro. Taken together, these data strongly support an important role for CTGF and ILK/GSK-3β/β-catenin signaling in the pathogenesis of severe BPD.

MATERIALS AND METHODS

Generation of Dox-inducible transgenic mice with overexpression of CTGF in AT II cells under the direction of the SP-C gene promoter. The study protocols were reviewed and approved by the Animal Care and Use Committee at the University of Miami (Miami, FL). The conditional and tissue specific overexpression of CTGF was achieved by mating two lines of transgenic mice, the SP-C-rtTA mice (line 2), bearing the reverse tetracycline responsive transactivator (rtTA) under the control of the 3.7-kb rat SP-C gene promoter (41), and the TetO-CTGF mice, containing tetracycline operator (TetO) and minimal CMV promoter and CTGF transgene. The SP-C-rtTA mice were provided by Dr. Jeffrey Whitsett (Cincinnati Children’s Hospital, Cincinnati, OH), and the TetO-CTGF mice were generated in the University of Miami Transgenic Facility and have been previously described (51). To generate double transgenic mice, the homozygous SP-C-rtTA mice were mated with the heterozygous TetO-CTGF mice. The newborn mice were genotyped by PCR of tail DNA with previously described (51). The single transgenic SP-C-rtTA mice were used as control mice, and the double transgenic SP-C-rtTA/Teto-CTGF mice were used and referred to as CTGF mice. To induce CTGF expression in the lungs of newborn pups, the nursing dams were fed with Dox-containing water (1 mg/ml) from postnatal day (P) 1. Additional litters were given regular drinking water to determine whether CTGF is induced in the absence of Dox treatment.

Wild-type mice and hyperoxia exposure. Time pregnant wild-type FVB mice were raised in the animal facility at the University of Miami School of Medicine. Within 24 h after birth mouse pups were randomized to receive normoxia (21% O2) or hyperoxia (90% O2) for 14 days. Continuous 90% O2 exposure was achieved in a Plexiglas chamber by a flow-through system and the oxygen levels inside the Plexiglas chamber were monitored continuously with a Ceramatec MAXO2 oxygen analyzer. Nursing dams were rotated between normoxia and hyperoxia every 48 h to prevent oxygen toxicity in the dams.

Tissue preparation. Mice were killed on P7 and P14, and the lungs were infused with 4% paraformaldehyde via a tracheal catheter at 20 cmH2O of pressure for 5 min and then fixed in 4% paraformaldehyde solution overnight at 4°C. Fixed lung tissues were paraffin embedded, and 5-μm sections were processed. Additional lungs were collected for total RNA and protein isolation.

Lung histology and morphometry. Lung tissue sections were immunostained in FE colonized mice with expression of CTGF under control of the 3.7-kb rat SP-C gene promoter. A rabbit anti-von Willebrand factor (vWF) antibody from Dako (Carpinteria, CA); a rat anti-Mac3 antibody from BD Biosciences (San Jose, CA); a rabbit anti-ILK antibody from Cell Signaling Technology (Danvers, MA). The immunostaining and double immunofluorescence staining were performed as previously described (51). The tissue sections were deparaffinized in xylene and rehydrated through graded ethanol into PBS. The sections were incubated with the respective primary antibodies overnight at 4°C. For immunostaining, the tissue sections were then incubated with biotinylated secondary IgGs for 1 h at room temperature. The cell-bound biotinylated secondary antibodies were detected with either streptavidin-biotin-alkaline phosphatase complexes and substrates or streptavidin-biotin-peroxidase complexes and diaminobenzidine substrates (Vector Laboratories, Burlingame, CA). For double immunofluorescence staining, the tissue sections were then incubated with Alexa Fluor 488 and/or Alexa Fluor 594-labeled secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h at room temperature. After being washed with PBS, the tissue sections were counterstained with 4,6-diamidino-2-phenylindole (Vector Laboratories) and mounted with glycerol.

Pulmonary vascular morphometry. Immunofluorescence staining for vWF, an endothelial specific marker, was performed for assessing vascular density. Ten random images were taken with the ×20 objective on each vWF-stained slide. The vascular density was expressed as the average number of vWF-positive vessels (15–50 μm) per high-power field (HPF).

Assessment of pulmonary vascular muscularization and remodeling. The degree of muscularization and medial wall thickness of peripheral pulmonary vessels (15–50 μm) were determined on α-SMA-stained tissue sections. Muscularized vessels were defined by the presence of smooth muscle cells positively stained with α-SMA antibody in 50% or more of the vessel circumference of intraacinous arterioles as previously described (15, 55). The total number of vessels and muscularized vessels were counted from 10 random images taken with the ×20 objective on each slide for calculation of the percentage of muscularized vessels. For medial wall thickness assessment, random images containing 25 vessels were taken with the ×40 objective for each slide. The external diameter and internal diameter of the α-SMA-stained lamina were measured, and the medial wall thickness was determined by the average external diameter minus the average internal diameter and divided by the average external diameter from each slide. For assessing proliferation of vascular smooth muscle cells, double immunofluorescence staining for PCNA and α-SMA was performed. The number of vessels with at least one PCNA-positive nucleus in smooth muscle cells was counted from 25 vessels (15–50 μm) on each slide.

Assessment of pulmonary hypertension. Right ventricular systolic pressure (RVSP) and right ventricle (RV) to left ventricle (LV) plus septum weight ratio (RV/LV+S) were determined as indexes for
pulmonary hypertension in 2-wk-old mice as previously described (54). Briefly, mice were sedated, tracheotomized, and ventilated with a Harvard Mini-Vent. After thoracotomy, a 25-gauge needle fitted to a pressure transducer was inserted into the RV. RVSP was measured and continuously recorded on a Gould polygraph. Immediately after RVSP measurements, hearts were dissected for RV free wall separation from LV + S for RV/LR + S ratio measurements.

**BAL and analysis.** Bronchoalveolar lavage (BAL) was performed in 4-wk-old control and CTGF mice by instilling 0.5 ml cold normal saline into the airway through a tracheal catheter and gently withdrawing the fluid. The lavage was repeated four times to recover a total volume of 1.5–2 ml. The cells were washed with Trypan blue to determine viability and with Turk solution to obtain total nucleated cell counts by use of a hemocytometer. Cytospin (Cytospin 2; Shandon, Waltham, MA) slides were prepared from the BAL fluid and were then fixed and stained by using a Neat Stain hematology kit (Polysciences, Warrington, PA). Differential cell counts were determined by counting 300 cells per each slide.

**Assessment of lung inflammation.** Lung inflammation was assessed by histology and immunostaining for Mac3, a macrophage-specific marker. The number of Mac3-positive cells in the alveolar air spaces was counted from 10 random images taken with the ≥40 objective on each slide. To determine the potential mechanisms causing macrophage infiltration, double immunofluorescence staining for MCP-1 and Mac3 as well as MCP-1 and pro-SP-C was performed.

**AT II cell isolation and culture.** AT II cells were isolated and cultured from 4-wk-old control and CTGF mice as previously described with some modification (42, 56). Mice were euthanized by intraperitoneal injection of pentobarbital and tracheotomized. The lungs were infused with dispase solution followed by low-melting agarose via a tracheal catheter and were covered by ice to allow the agarose to polymerize. The lungs were then dissected and digested in dispase and digested from the bronchi in DNase solution. The cell suspension was filtered through gradient cell strainers (100 μm to 20 μm), and the cells were pelleted by centrifugation and then placed on tissue culture plates precoated with anti-CD 45 and anti-CD 32 antibodies (BD Biosciences). After incubation for 2 h in a humidified 37°C incubator with 21% O₂ and 5% CO₂, the AT II cells (nonadhered) were collected, centrifuged, and placed on Matrigel (70%)-coated eight-well chamber slides or six-well plates (BD Biosciences).

The AT II cells were cultured overnight in bronchial epithelial cell growth medium minus hydrocortisone (Lonza, Portsmouth, NH) with 5% charcoal-stripped fetal calf serum and keratinocyte growth factor. The media were changed to fresh media containing Dox (1 μg/ml), and the cells were cultured for additional 72 h. Live cells were imaged and immunofluorescence staining was performed on these cells. The purity of AT II cells was typically >90%, as assessed by pro-SP-C staining.

**RNA isolation and quantitative real-time RT-PCR.** Total RNA was isolated from frozen lung tissues and AT II cells and treated with DNase to remove possible DNA contamination as described (51). One microgram of total RNA was reverse-transcribed in a 20-μl reaction by using a first-strand cDNA synthesis kit according to manufacturer’s protocol (Invitrogen). The real-time RT-PCR was performed on an ABI Fast 7500 System (Applied Biosystems, Foster City, CA).

Each reaction included diluted first-strand cDNA, specific primers, and master mix containing enzymes and TaqMan probes according to the manufacturer’s instruction (Applied Biosystems). Real-time RT-PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. RNAse-free water was used as a negative control. For each target gene, a standard curve was established by performing a series of dilutions of the first-strand cDNA. The mRNA expression levels of target genes were determined from the standard curve and normalized to GAPDH.

**Western blot analysis.** Total protein was extracted from frozen lung tissues with a RIPA buffer according to manufacturer’s protocol (Santa Cruz). The protein concentrations were measured by a BCA protein assay using a commercial kit from Pierce Biotechnology (Rockford, IL). Total proteins (50 μg/sample) were fractionated by SDS-PAGE on 4–12% Tris-glycine precast gradient gels (Invitrogen) and then transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). The membranes were incubated overnight at 4°C with respective primary antibodies and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. Antibody bound proteins were detected by ECL chemiluminescence methodology (Amersham). Membranes were then stripped with 0.2 N NaOH and reincubated with primary antibodies reactive with a normalization protein, β-actin. The intensities of protein bands were quantified by Quantity One Imaging Analysis Program (Bio-Rad, Hercules, CA).

**RESULTS**

**Overexpression of CTGF disrupts alveolarization.** After mice were exposed to Dox from P1 to P14, high levels of CTGF expression were detected by Western blot analysis and lung homogenates from CTGF mice (Fig. 1A). Double immunofluorescence staining colocalized CTGF in AT II cells (Fig. 1B). This increased CTGF expression was similar to that observed in lungs from hypoxia-exposed newborn mice for 14 days (Fig. 1, C and D). On histological examination at P7 and P14, the control lungs displayed normal alveolar development with numerous small alveoli and secondary septa (Fig. 1, E and G). In contrast, there were larger and simplified alveoli with fewer secondary septa in CTGF lungs (Fig. 1, F and H). Further morphometric analysis demonstrated a 30% increase at P7 and a 50% increase at P14 in MLI in CTGF lungs compared with control lungs (Fig. 1I). The RAC was reduced by near 60% in CTGF lungs at P14 (Fig. 1J). There was no difference in CTGF expression and alveolarization between CTGF lungs and control lungs without Dox exposure (data not shown). Thus overexpression of CTGF in AT II cells severely disrupted alveolarization in neonatal mice.

**Overexpression of CTGF inhibits pulmonary vascular development.** To determine whether epithelial overexpression of CTGF disrupts pulmonary vascular development, vascular density was quantified by immunofluorescence staining for vWF. There was a 60% decrease in vWF-positive intracellular vessels in CTGF lungs compared with control lungs at P14 (Fig. 2, A–C), indicating severe inhibition of pulmonary vascular development. Because VEGF plays a key role in pulmonary vascular development, VEGF expression and VEGF receptor 2 (VEGFR2) phosphorylation were assessed in lung homogenates. VEGF expression was significantly decreased in CTGF lungs compared with control lungs (Fig. 2, D and E). Correlating with decreased VEGF expression, there was a decreased VEGFR2 phosphorylation in CTGF lungs (Fig. 2, D and E). We also examined VEGF gene expression in primary cultured AT II cells and found that it was significantly decreased in CTGF lungs (Fig. 2F). These results indicate that decreased VEGF expression and its signaling may mediate CTGF inhibition of lung vascular development.

**Overexpression of CTGF induces pulmonary vascular remodeling.** To determine the effect of overexpression of CTGF on pulmonary vascular remodeling, we performed immunostaining for α-SMA, a smooth muscle marker. Abnor-
mally extensive muscularization of peripheral pulmonary vessels was detected in CTGF lungs compared with control lungs, which showed little or partial staining for \( \alpha \)-SMA (Fig. 3, A and B). There was a 70% increase in muscularized vessels in CTGF lungs (Fig. 3, C). There was also a more than twofold increase in medial wall thickness of these vessels in CTGF lungs (Fig. 3, D). Correlating with increased wall thickness, there was significantly increased vessels with proliferating cells in CTGF lungs (Fig. 3, E and F). These data indicate that epithelial overexpression of CTGF results in excessive pulmonary vascular remodeling.

**Overexpression of CTGF causes pulmonary hypertension.** To determine whether CTGF-induced inhibition of pulmonary vascularization and vascular remodeling leads to pulmonary hypertension, RVSP and RV/LV+S were assessed. The RVSP was significantly higher in CTGF mice compared with control mice (Fig. 4A). The CTGF mice also had a significant increase in RV/LV+S (Fig. 4B), suggesting right ventricular hypertrophy. These results support that epithelial overexpression of CTGF causes pulmonary hypertension in neonatal mice.

**Overexpression of CTGF causes pulmonary inflammation.** On histological examination, there was increased inflammatory cell infiltration in alveolar air spaces and perivascular regions in CTGF lungs (Fig. 5B). Immunostaining with a Mac3 antibody demonstrated a marked increase in CTGF expression in hyperoxia-exposed lungs compared with normoxia-exposed lungs. Hematoxylin and eosin (HE)-stained lung tissue sections from control lungs at postnatal day (P) 7 (E) and P14 (G) demonstrated normal alveolar development. However, overexpression of CTGF disrupted alveolar development with larger and simplified alveoli at P7 (F) and P14 (H). Mean linear intercept (MLI) was significantly decreased in CTGF lungs (J). \( N = 4 \) /group. *\( P < 0.01 \). **\( P < 0.001 \). Magnification: \( \times 40 \) (B, D), \( \times 20 \) (E–H). Scale bars: 50 \( \mu \)m (D, G, H) and 100 \( \mu \)m (E, F).
there was a more than twofold increase in TNF-α gene expression in CTGF lungs (Fig. 5G), although there was no significance difference in gene expression of IL-1β and IL-6 between control and CTGF lungs (data not shown). Furthermore, macrophage and neutrophil counts were drastically increased in BAL fluids from CTGF mice (Fig. 5, J–L). There were numerous multinucleated giant cells in BAL fluids from CTGF mice, which were rarely seen in control mice (Fig. 5J).

To determine the potential mechanisms of overexpression of CTGF-induced macrophage infiltration, we examined MCP-1 expression. Double immunofluorescence staining demonstrated increasing MCP-1 expression in infiltrated macrophages (Fig. 5F) as well as in AT II cells (data not shown) in CTGF lungs compared with control lungs (open bars). Real-time RT-PCR demonstrated a significant decrease in VEGF gene expression in CTGF lungs (Fig. 5H). These results suggest that overexpression of CTGF induces MCP-1 expression in macrophages and AT II cells that causes macrophage infiltration.

Overexpression of CTGF induces GSK-3β phosphorylation and β-catenin nuclear translocation in vivo. To determine the potential mechanisms by which overexpression of CTGF causes lung pathology, we performed double immunofluorescence staining for β-catenin and CTGF in control and CTGF lungs. As demonstrated in Fig. 6, A and C, CTGF was undetectable and β-catenin was localized in the cytoplasm in control lungs. In contrast, β-catenin-positive nuclei were co-localized with CTGF-expressing cells in CTGF lungs (Fig. 6, B and D), suggesting that overexpression of CTGF induces β-catenin nuclear translocation. Because GSK-3β plays an role in the regulation of β-catenin stability, the phosphorylation of GSK-3β could cause the nuclear translocation of β-catenin.
important role in modulating β-catenin degradation and nuclear translocation. Western blot was performed to determine GSK-3β expression and phosphorylation. Overexpression of CTGF did not change GSK-3β expression but increased Ser9 GSK-3β phosphorylation, which is known to lose the ability to phosphorylate β-catenin, thus leading to β-catenin accumulation and nuclear translocation (52) (Fig. 6, E and F).

**Overexpression of CTGF induces β-catenin nuclear translocation in primary AT II cells in vitro.** Primary AT II cell culture was performed to further identify the cellular and molecular mechanisms by which CTGF causes β-catenin nuclear translocation. When cultured on Matrigel, the AT II cells from control lungs grew in clusters and formed alveolar-like cysts (Fig. 7A). In contrast, the AT II cells from CTGF lungs appeared larger and were spread out (Fig. 7B). Double immunofluorescence staining demonstrated that the AT II cells from control lungs express high level of pro-SP-C but no CTGF (Fig. 7C). However, multinucleated gain cells (black arrow) and neutrophils (white arrow) were detected in BAL cytospin from CTGF mice (J). Quantification of macrophages (K) and neutrophils (L) were both significantly increased in CTGF lungs. N = 4/group. **P < 0.001 and ***P < 0.05. Magnification: ×40. Scale bars: 50 μm.
consistent with the in vivo data suggesting that overexpression of CTGF in AT II cells activates ILK/GSK-3β pathway, which may play an important role in CTGF-induced β-catenin nuclear translocation and lung pathology.

**DISCUSSION**

In the present study, we provide direct evidence that overexpression of CTGF in AT II cells results in severe BPD-like architecture in neonatal mice. We demonstrate that inducible overexpression of CTGF in AT II cells during the alveolar stage of lung development induces pulmonary inflammation, disrupts alveolarization and pulmonary vascularization, and results in pulmonary vascular remodeling and pulmonary hypertension. The pulmonary inflammation was associated with increased MCP-1 expression in macrophages and AT II cells. The disruption of alveolarization and vascularization were associated with decreased VEGF expression and VEGFR2 activation. Furthermore, overexpression of CTGF activates ILK/GSK-3β pathway and results in β-catenin nuclear translocation. This study, therefore, provides important insights into the interplay of growth factors, transcriptional factors, and inflammatory mediators that orchestrates the epithelial-mesenchymal cross talk and the development of BPD.
We have demonstrated in this study that inducible overexpression of CTGF in AT II cells, under the control of SP-C promoter, severely disrupts alveolarization and pulmonary vascular development; this is similar to that observed in our previous study with overexpression of CTGF in airway epithelium, under the control of CCSP promoter (51). CTGF has been implicated in both angiogenesis and antiangiogenesis. Recent data demonstrate that CTGF knockout mice have normal angiogenesis, suggesting that endogenous CTGF is not essential for angiogenesis (31). However, increasing data indicate that CTGF is angiogenic through its interactions with VEGF, a key mitogenic and survival factor for endothelial cells produced by many cell types including AT II cells (33). CTGF inhibits VEGF expression in tumor cells through degrading hypoxia-inducible factor, which is responsible for VEGF gene transcription (6). CTGF can also form a complex with VEGF in ECM of aortic endothelial cells that inhibits the binding of VEGF to VEGF receptor 2 (VEGFR2), thus inhibiting VEGF angiogenesis (11, 24). Increasing data indicate that disruption of normal lung vascular development plays a critical role in the pathogenesis of BPD. Inhibition of VEGF or disruption of VEGFR2 results in severe disruption of air spaces and vasculature in experimental BPD and pulmonary hypertension (19, 33). In this study, overexpression of CTGF not only decreased VEGF expression but also impaired VEGFR2 activation. These results suggest that epithelial overexpression of CTGF may inhibit alveolarization and pulmonary vascular development through a VEGF-dependent mechanism.

Besides disruption of alveolarization and pulmonary vascular development, overexpression of CTGF in AT II cells also caused excessive pulmonary vascular remodeling. The pulmonary vascular remodeling is demonstrated by abnormally extensive muscularization and increased medial wall thickness of peripheral pulmonary vessels in CTGF lungs. Moreover, we have also demonstrated that there is increased cell proliferation of these vessels. There is compelling evidence that suggests that CTGF plays an important role in vascular remodeling in both pulmonary and systemic vasculatures. CTGF gene expression is upregulated in pulmonary artery smooth muscle cells (PASMC) in monocrotaline-induced pulmonary hypertension (35). TGF-β/CTGF signaling promotes progressive neointimal hyperplasia in vein grafts (27). Furthermore, CTGF is the downstream mediator of TGF-β-induced adventitial remodeling in carotid angioplasty (32). This study provides for the first time the direct evidence that epithelial overexpression of CTGF causes excessive pulmonary vascular remodeling in the developing lung.

The development of pulmonary hypertension remains a significant cause of morbidity and mortality in severe BPD (14, 47, 48). In this study, overexpression of CTGF not only impaired vascular development but also induced pulmonary vascular remodeling that led to significant increase in both pulmonary artery pressure and right ventricular hypertrophy. In a rodent model of hypoxia-induced BPD, pulmonary hypertension and right ventricular hypertrophy are associated with both impaired vascular development and increased vascular remodeling (4). The pulmonary hypertension may be caused by decreased pulmonary vasculature that limits vascular surface area, thus leading to elevation of pulmonary vascular resistance (47). The excessive pulmonary vascular remodeling may further contribute to high pulmonary vascular resistance through narrowing of the vessel diameter and decreased vascular compliance (47). However, the molecular mechanisms as well as the interplay of vascular development and remodeling in the pathogenesis of neonatal pulmonary hypertension are poorly understood. Our model provides the advantage for further exploring the individual role of vascular development and remodeling in the pathogenesis of neonatal hypertension.

An important finding of this study is that overexpression of CTGF results in significant inflammation in the neonatal lung. There was increased inflammatory cell infiltration in both air spaces and perivascular regions in CTGF lungs. Lung inflammation, whether induced prior to birth or during the early postnatal period, is considered the key mediator of alveolar and vascular injury in BPD. Clinical studies have demonstrated the presence of increased neutrophils, activated macrophages, and high concentrations of inflammatory mediators such as TNF-α, IL-1β, and TGF-β in tracheal fluid from preterm infants who subsequently developed BPD (46). Animal models further support the key role of inflammation in the pathogenesis of BPD. Transgenic overexpression of IL-1β induced neutrophilic and monocytic infiltration, increased expression of chemokines such as macrophage inflammatory protein-2 (CXCL2) and MCP-1, and disrupted alveolar septation and capillary development in neonatal mice (5). A recent study has demonstrated that bone marrow stromal cells attenuate alveolar damage and pulmonary vascular remodeling in hypoxia-induced murine BPD, and this correlated with prevention of neutrophil and macrophage infiltration (4). It is suggested that the protective effects of bone marrow stromal cells on alveolar structure may be caused by the immunomodulatory proteins produced by these cells (4). In contrast to CTGF’s well-known ability to induce tissue remodeling, the inflammatory potential is just beginning to be understood. A recent study has demonstrated that systemic administration of CTGF increased renal expression of chemokines (MCP-1 and RANTES) and cytokines

![Diagram](http://ajplung.physiology.org/)

**Fig. 8. Proposed model for CTGF inhibition of alveolarization and vascularization and induction of pulmonary hypertension in neonatal lungs.** Overexpression of CTGF in AT II cells via an autocrine mechanism induces β-catenin nuclear translocation, and that may decrease VEGF expression in epithelium leading to decreased VEGFR2 phosphorylation in endothelium, thus disrupting alveolar and pulmonary vascular development. Epithelium-produced CTGF may also induce β-catenin nuclear translocation via a paracrine mechanism in pulmonary artery smooth muscle cells (PASMC), thus leading to excessive pulmonary vascular remodeling. The combination of poor alveolar and vascular development as well as increased vascular remodeling results in pulmonary hypertension. The CTGF-induced infiltration via β-catenin-dependent and/or independent mechanisms further disrupts alveolar and vascular development and induces pulmonary hypertension.
inhibit GSK-3 in lung fibroblasts and stimulating proliferation and migration of AT II cells. We speculate that MCP-1 and other potential chemokines produced by macrophages and AT II cells may play a key role in CTGF-induced lung inflammation.

Although dysregulation of many growth factors and inflammatory mediators has been linked to the pathogenesis of BPD, the underlying molecular mechanisms are poorly understood. Our study identified for the first time that overexpression of CTGF induces β-catenin nuclear translocation. β-Catenin is a membrane-associated protein that is an effector of the canonical Wnt signaling (26, 30). β-Catenin also binds to cadherin in the adherens junction where it stabilizes cadherin-mediated cell-cell contact (26, 30). GSK-3β plays a key role in β-catenin degradation and nuclear translocation. Nonphosphorylated GSK-3β is the active form and can phosphorylate β-catenin and lead to β-catenin degradation in the cytoplasm (26, 30). Activation of ILK and/or Wnt signaling results in GSK-3β phosphorylation, thus leading to GSK-3β inactivation. Subsequently, β-catenin phosphorylation is inhibited, and its degradation is attenuated. Accumulated β-catenin then undergoes nuclear translocation, and that regulates target gene expression and cellular function (26, 30). The Wnt/β-catenin signaling is essential for lung morphogenesis and its dysregulation has been recently linked to the pathogenesis of many lung diseases, in particular lung fibrosis and hypoxia-induced neonatal lung injury (10, 30). Less is known about ILK/GSK-3 and Wnt signaling in various pathological conditions. CTGF induces human proximal tubular epithelial cells to undergo epithelial-mesenchymal transition via an ILK-dependent pathway (36). CTGF induces primary mesangial cell migration and cytoskeletal rearrangement, and this is associated with GSK-3β phosphorylation. Furthermore, CTGF promotes tumorigenicity of esophageal squamous cell carcinoma by inducing Ser9 GSK-3β phosphorylation and β-catenin nuclear translocation (12). In our transgenic model, overexpression of CTGF induces β-catenin nuclear translocation in AT II cells as well as other cells and this is associated with increased GSK-3β phosphorylation in vivo. Most importantly, we have also demonstrated that overexpression of CTGF causes β-catenin nuclear translocation in AT II cells in vitro and this is associated with increased ILK expression. These in vivo and in vitro data strongly support that the ILK/GSK-3β/β-catenin signaling plays a key role in CTGF disruption of neonatal lung structure. Further studies will be done to investigate the functional significance of this pathway in CTGF-induced neonatal lung injury and in other experimental models of BPD.

In summary, our data reveal a novel role of CTGF in the pathogenesis of severe BPD. This transgenic model replicates the pathological hallmarks of severe BPD, namely pulmonary inflammation, impaired alveolarization and vascularization, and excessive pulmonary vascular remodeling and pulmonary hypertension. Most importantly, this study provides both in vivo and in vitro evidence that overexpression of CTGF activates the ILK/GSK-3β pathway and induces β-catenin nuclear translocation. We propose that epithelial overexpression of CTGF activates the ILK/GSK-3β/β-catenin via autocrine and/or paracrine mechanisms leading to epithelial and endothelial cell dysfunction and PASMC remodeling, thus resulting in lung structural changes and pulmonary hypertension. Furthermore, the inflammatory response induced by CTGF via β-catenin-dependent and/or independent mechanisms potentiates CTGF-induced lung structural change (Fig. 8). This work provides important insights into the interplay of growth factors, transcriptional factors, and inflammatory processes orchestrating normal lung development and the pathogenesis of BPD, and this could potentially lead to the development of novel therapies aimed at anti-inflammation, promoting lung development and preventing pulmonary hypertension in neonates.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

CTGF DISRUPTS ALVEOULARIZATION AND INDUCES PULMONARY HYPERTENSION


