Deregulation of the lysyl hydroxylase matrix cross-linking system in experimental and clinical bronchopulmonary dysplasia

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BRONCHOPULMONARY DYSPLASIA (BPD) is a significant complication of prematurity birth that, since the first description of BPD by Northway and colleagues in 1967 (28), remains a key cause of morbidity and mortality in neonatal intensive care units worldwide. The diagnosis of clinical BPD currently relies exclusively on the degree of oxygen dependence at a defined postmenstrual age (21). A key pathophysiological feature of infants afflicted with BPD is a dysmorphic pattern of lung development, including an arrest of alveolarization, where secondary septation is limited, and thus the formation of the alveolar gas exchange units is impeded (19). The resultant general reduction in the gas exchange surface area of the lung has both immediate and long-term consequences for affected infants that extend beyond childhood (29). Interestingly, improvements in the medical management of premature infants have led to improved survival of extremely premature infants and, with that, a concomitant increase in the prevalence of BPD. However, the pathogenesis of BPD, in particular, the molecular basis of blunted septation and the consequent impaired alveolarization, is very poorly understood (20, 24).

An emerging area of interest in BPD pathogenesis is the possible role played by aberrant remodeling of the extracellular matrix (ECM). In particular, several studies have pointed to improper deposition and maturation of elastin and collagen fibers in the developing septa (1, 5, 6, 8, 23, 30, 43). This has been attributed to two possible phenomena. First, the abundance of total collagen and elastin may be altered in the injured lung. Abnormal collagen and elastin deposition has been reported in premature lambs (1, 6, 30), in mechanically ventilated newborn mice (5, 8), and mouse pups in which late lung development has been arrested by exposure to high oxygen levels (23, 49). Second, a growing body of evidence indicates that the cellular machinery that is responsible for the posttranslational processing of ECM components (7), in particular, the proteolytic processing and covalent cross-linking of ECM structures, is dysregulated in the lungs of patients with BPD, as well as in animal models of BPD. Notable among these ECM maturation systems is the lysyl oxidase family of amine oxidases, which catalyze the covalent cross-linking of lysine and hydroxylysine residues in collagen and elastin and thereby promote stability of the ECM. Recent reports have indicated that lysyl oxidase expression and activity are increased both in lungs of patients affected with BPD (23), as well as in two rodent models of BPD that rely on mechanical ventilation and hyperoxia to induce lung damage (5, 23). It has been proposed that an aberrantly active ECM cross-linking system, as would be expected with elevated activity of lysyl oxidases, would generate a lung matrix structure that was “over cross-linked” or too stable, which may resistant the normal remodeling of the developing lung that must take place to allow new alveolar units to form (23).

Although the lysyl oxidases have been studied in BPD, lysyl oxidases do not act alone to covalently cross-link ECM structures in the lung and other organs. Lysyl oxidases require lysine or hydroxylysine residues in substrate ECM molecules,
which serve as cross-linkable residues. Hydroxylsine residues are generated by another family of enzymes: the lysyl hydroxylase, or procollagen-lysine, 2-oxoglutarate 5-dioxigenase (PLOD) (EC 1.14.11.4) family (26). The lysyl hydroxylase family comprises three members, all of which are products of separate genes, designated PLOD1, PLOD2, and PLOD3, which are mixed-function oxygenases that catalyze the hydroxylation of peptidyllysine (usually in procollagen, as well as other proteins with collagen-like domains) to peptidylhydroxylsine (26). The hydroxylsine residues generated serve as substrates for lysyl oxidase, which convert hydroxylsine to hydroxylysine, which is a precursor for a covalent cross-link. Additionally, the hydroxylsine residues serve as acceptors for sugars, permitting glycosylation of ECM proteins (26).

Indeed, PLOD3, in addition to lysine hydroxylation activity, also possesses glucosyltransferase and galactosyltransferase activities. Thus lysyl hydroxylases promote ECM structural stability and maturation by promoting inter- and intramolecular cross-links and the addition of carbohydrate moieties to ECM molecules (26).

These lysyl hydroxylases have been implicated in connective tissue disorders, exemplified by Ehlers-Danlos Syndrome type VI, where mutations in the PLOD1 gene cause a heritable disorder characterized by kyphoscoliosis, joint laxity, skin fragility, and muscle hypertonia (9, 50). PLOD2 is implicated in pathological processes, where increased PLOD2 expression in fibroblasts is associated with systemic sclerosis (dermal fibroblasts) (39) and fibrosis (fibroblasts isolated from keloid, hypertrophic scars, and the palmar fascia of patients with Dupuytren’s disease) (46), and mutations in the PLOD2 gene are associated with Bruck’s Syndrome, a recessively inherited ECM disorder presenting with skeletal changes comparable to osteogenesis imperfecta with contractures of the large joints (18). More recently, mutations in PLOD3 have been linked to a severe connective tissue disorder reminiscent of Stickler Syndrome (36). However, to date, no lysyl hydroxylase has been implicated in normal or pathological processes in the lung. Given the emerging importance of perturbed ECM remodeling in arrested lung development, the authors hypothesized that lysyl hydroxylase expression was deregulated during aberrant late lung development associated with BPD.

**MATERIALS AND METHODS**

**Mouse model of BPD.** All animal procedures were approved by the Regierungspräsidium Gießen (which houses the functional equivalent of an Institutional Animal Care and Use Committee in Germany) under approval 22/2000, for animal studies conducted in Germany. An arrest of alveolarization was induced in mouse pups by exposure to normobaric hyperoxia (85% O₂), exactly as described previously (3). Mouse pups were randomized to two groups, one group exposed to normobaric normoxia (21% O₂) and the other group exposed to normobaric hyperoxia (85% O₂), within 12 h of birth [postnatal day (P) 0.5]. This model has previously been described and carefully characterized by the investigators (3, 23) and other groups (10, 14, 27), where a pronounced arrest of lung development is seen in response to hyperoxia exposure.

**Cells.** The A549 cell line was obtained from the American Type Culture Collection. Primary mouse lung fibroblasts and alveolar type II cells were isolated from the lungs of adult C57Bl/6J mice, and primary lung fibroblasts were isolated from human lungs, exactly as described previously (2, 25). Primary human lung microvascular endothelial cells and primary human pulmonary artery smooth muscle cells were purchased from Promocell and maintained as recommended by the manufacturer.

**Gene and protein expression analysis.** By convention, mouse genes and proteins are indicated in lower case (for example, *plod1* and *Plod1*, respectively), and human genes and proteins are indicated in upper case (for example *PLOD1* and *Plod1*, respectively). Real-time RT-PCR was undertaken exactly as described previously (2, 25), using mouse lung, human lung, and whole cell mRNA pools as a template, with the primers listed in Table 1. For TGF-β stimulation, cells were exposed to TGF-β1 (2 ng/ml final concentration; R&D Systems) for 18 h. This represents a dose well within the standard range (0.2–10.0 ng/ml) for in vitro TGF-β1 stimulation studies (3, 23). Immunoblotting was undertaken exactly as described previously, using the following primary antibodies: goat anti-Plod1 (SC-50062, 1:200; Santa Cruz Biotechnology), goat anti-Pold2 (SC-50067, 1:200; Santa Cruz Biotechnology), rabbit anti-Plod3 (11027–1-AP, 1:200; Santa Cruz Biotechnology), goat anti-Plod2 (SC-50067, 1:200; Santa Cruz Biotechnology). Immune complexes were detected with the following secondary antibodies: donkey anti-goat IgG horseradish peroxidase conjugate (SC-2020, 1:1,000; Santa Cruz Biotechnology) and goat anti-rabbit IgG horseradish peroxidase conjugate (31460, 1:3,000; Pierce), employing enhanced chemiluminescence.

**Immunohistochemistry.** Lungs from mouse pups were pressure fixed at 20 cm H₂O pressure, embedded in paraffin, and 3-μm sections were prepared from mouse and human lungs and were processed for the detection of lysyl hydroxylases exactly as described previously (2,
Lysyl hydroxylases were detected using the following primary antibodies: goat anti-Plod1 (SC-50062, 1:50; Santa Cruz Biotechnology), goat anti-Plod2 (SC-50067, 1:25; Santa Cruz Biotechnology), rabbit anti-Plod3 (11027–1-AP, 1:25; Protein Tech). Staining specificity was assessed by preadsorbing with a 100-fold molar excess of a competing peptide C-19 (SC-50062P; Santa Cruz Biotechnology) for Plod1, competing peptide N-15 (SC-50067P; Santa Cruz Biotechnology), and a glutathione-S-transferase (GST)-Plod3 fusion protein (AgI480; Protein Tech) for Plod3. Immune complexes were detected with biotinylated Histostain secondary antibodies: biotinylated rabbit anti-mouse (95–6543B, “ready to use”; Invitrogen) and biotinylated rabbit anti-goat (A10518, 1:1,000; Invitrogen), followed by a Streptavidin-horseradish peroxidase complex colorimetric detection system.

Cloning of the mouse plod2 and human PLOD2 promoters. The mouse plod2 promoter was cloned by PCR amplification of a 2,767 base-pair fragment using the forward and reverse primers: 5’-GAGCTCTCTTCAACCTCACCTACTCAGT-3’ (forward; SacI) and 5’-CTCGAGGGGAGGGGCTGGGCGGGACT-3’ (reverse; XhoI), containing built-in restriction sites (in bold type) and mouse lung genomic DNA as a template. The mouse plod2 promoter sequence has been deposited in the GenBank database under accession number KC788822. Sequences were initially TA cloned into pGEM T-Easy (Promega) and then subcloned into pGL3-basic (Promega) using the restriction sites built into the primers to create pGL3-plod2 (for mouse plod2 promoter) and pGL3-PLOD2 (for human PLOD2 promoter).

Dual luciferase reporter assay. The dual luciferase reporter assay was performed exactly as described previously (2, 25), using the firefly luciferase-expressing pG3L3-plod2 and pG3L3-PLOD2 constructs described above, along with the Renilla luciferase-expressing pRLTK (Promega) normalization construct.

Table 2. Clinical characteristics of patients with BPD or at risk for the development of BPD

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Birth Weight, g</th>
<th>Sex</th>
<th>Gestational Age, wk</th>
<th>CAD, days</th>
<th>Duration FiO2 &gt;0.50, days</th>
<th>Mechanical Ventilation, days</th>
<th>Cause of Death/autopsy Diagnosis and Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>720</td>
<td>M</td>
<td>26</td>
<td>62</td>
<td>13</td>
<td>62 (c, hf)</td>
<td>BPD, IRDS, Staphylococcus aureus sepsis. Drugs: surfactant, inotropes, tobramycin, fluocoxacinil, cortisone</td>
</tr>
<tr>
<td>2</td>
<td>1,055</td>
<td>M</td>
<td>32</td>
<td>18</td>
<td>18</td>
<td>18 (c)</td>
<td>BPD, ventricular septal defect, Edwards syndrome. Drugs: inotropes</td>
</tr>
<tr>
<td>3*</td>
<td>835</td>
<td>M</td>
<td>26</td>
<td>65</td>
<td>27</td>
<td>65 (c, hf)</td>
<td>BPD, cerebral bleeding, ductus arteriosus. Drugs: surfactant, inotropes, dexamethasone, theophylline</td>
</tr>
<tr>
<td>4*</td>
<td>930</td>
<td>M</td>
<td>26</td>
<td>99</td>
<td>98</td>
<td>99 (c, hf)</td>
<td>BPD, IRDS, pneuomothorax, subependymal hemorrhage. Drugs: surfactant, inotropes, dexamethasone, tobramycin, penicillin, amphotericin</td>
</tr>
<tr>
<td>5*</td>
<td>1,250</td>
<td>F</td>
<td>28</td>
<td>34</td>
<td>34</td>
<td>34 (c, hf)</td>
<td>BPD, IRDS, Staphylococcus epidermis sepsis. Drugs: surfactant, furosemide, amoxicillin, erythromycin</td>
</tr>
<tr>
<td>6*</td>
<td>1,220</td>
<td>M</td>
<td>31</td>
<td>35</td>
<td>35</td>
<td>35 (c, hf)</td>
<td>BPD, IRDS, right ventricular hypertrophy, anemia, rickets. Drugs: furosemide, amoxicillin, vancomycin</td>
</tr>
</tbody>
</table>

Median 930
Mean ± SE 976 ± 77
P value vs. CAD (Table 3) 0.0016
P value vs. GAB (Table 3) 0.593 (NS) 0.1776 (NS) 0.0001

Inotropes included dopamine, dobutamine, and adrenaline. *Patient had clinically-defined BPD. †By unpaired Student t-test. In the case of chronological age at death (CAD), the postmenstrual ages at death, rounded to the nearest full week, were compared. BPD, bronchopulmonary dysplasia; c, conventional ventilation; hf, high-frequency ventilation; GAB, gestational age at birth; IRDS, infant respiratory distress syndrome; NS, not significant.
and plod3 (Fig. 1C) was dramatically downregulated by 3–4 ΔCt units, comparing P14.5 with P9.5 (comparing normoxia-treated groups only, for normally developing lungs). In sum, lysyl hydroxylase gene expression was progressively downregulated in normally developing lungs of mouse pups over the first 14 days of life. This trend is strongly impacted (and to a degree, reversed) by exposure to 85% O2, where levels of all three genes were comparable between the 21% O2 and 85% O2 groups at P2.5, but by P9.5 both plod1 (Fig. 1A) and plod3 (Fig. 1C) mRNA levels were increased in the lungs of mouse pups exposed to 85% O2. By P14.5, the levels of plod1 (Fig. 1A), plod2 (Fig. 1B), and plod3 (Fig. 1C) mRNA were all elevated by 2–4 ΔCt units in the 85% O2 group, compared with the 21% O2 group. The ability of 85% O2 to maintain elevated lysyl oxidase expression was also evident in immunoblots, where an increase in Plod1 and Plod2 protein levels was evident in the lungs of 85% O2-exposed mouse pups, compared with 21% O2-exposed littermates (Fig. 1D). In sum, although lysyl hydroxylase expression is gradually downregulated over the course of normal alveolarization, exposure to 85% O2 leads to abnormally elevated levels of lysyl hydroxylases, over the course of aberrant alveolarization in mouse pups.

Table 3. Clinical characteristics of control patients

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Birth Weight, g</th>
<th>Sex</th>
<th>Gestational Age, wk</th>
<th>CAD, days</th>
<th>Duration FiO2</th>
<th>Mechanical Ventilation, days</th>
<th>Cause of Death/autopsy Diagnosis and Medication/material Available</th>
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<tr>
<td>7</td>
<td>1,625</td>
<td>M</td>
<td>33</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>Congenital heart malformation. Drugs: atropine, prostaglandin A1 (r)</td>
</tr>
<tr>
<td>8</td>
<td>2,350</td>
<td>M</td>
<td>35</td>
<td>&lt;1</td>
<td>0</td>
<td>&lt;1 h</td>
<td>Perinatal asphyxia. Drugs: atropine, adrenaline (h+r)</td>
</tr>
<tr>
<td>9</td>
<td>1,740</td>
<td>F</td>
<td>34</td>
<td>&lt;1</td>
<td>0</td>
<td>0</td>
<td>Intrauterine death (ventriculomegaly) (r)</td>
</tr>
<tr>
<td>10</td>
<td>1,800</td>
<td>M</td>
<td>32</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>Meningoencephalitis (h+r)</td>
</tr>
<tr>
<td>11</td>
<td>1,190</td>
<td>M</td>
<td>31</td>
<td>&lt;1</td>
<td>0</td>
<td>0</td>
<td>Placental abruption (h+r)</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>1,741 ± 186</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Control group matched to the BPD group (Table 2) for CAD

Control group matched to the BPD group (Table 2) for birth weight and GAB

Fig. 1. Expression of lysyl hydroxylases during normal and aberrant late lung development. Expression levels of mRNA encoding plod1 (A), plod2 (B), and plod3 (C) were assessed by real-time RT-PCR in mRNA pools from lung homogenates from mouse pups at postnatal days (P) 2.5, 9.5, and 14.5, after exposure to 21% O2 (●) or 85% O2 (○) from P0.5. The 18S rRNA was used as a reference. Data reflect means ± SE (n = 5). The P values, indicated above pairs of data sets, were assessed by 1-way ANOVA with Tukey’s post hoc test.

D: protein expression of Plod1, Plod2, and Plod3 was assessed by immunoblot of protein extracts from lung homogenates from mouse pups over the course of late lung development, during exposure to 21% O2 or 85% O2 from P0.5. A single representative series is illustrated that is representative of at least 2 other series.
Lysyl hydroxylases have specific expression patterns in the developing mouse lung. To assess where deregulated lysyl hydroxylase expression may impact late lung development, the localization of the three lysyl hydroxylases was determined by immunohistochemistry. Plod1 could be localized to the parenchyma, where Plod1 staining was observed in developing septa of mouse pups at P7 exposed to 21% O2 (Fig. 2A) and 85% O2 (Fig. 2B). Plod1 staining was also observed in the blood vessel walls, appearing to localize to the muscular layer of the vessel wall in the lungs of mouse pups exposed to 21% O2 (Fig. 2C) and 85% O2 (Fig. 2D). Staining for Plod1 in the lung vessel walls appeared to be more intense in the 85% O2-treated mouse pups (Fig. 2D) compared with the 21% O2-treated mouse pups (Fig. 2C) although the authors do not consider the relative intensity of immunohistochemical staining to be quantitative. Plod2 was also detected in the developing septa of mouse pups at P7 (Fig. 3, A and B), with staining again appearing more intense in the 85% O2-treated group (Fig. 3B) compared with the 21% O2-treated group (Fig. 3A). In contrast to Plod1, no Plod2 staining was evident in the vessel wall of mouse pups at P7 exposed to 21% O2 (Fig. 3C) and 85% O2 (Fig. 3D). Staining for Plod3 revealed a very specific and discrete expres-
sion pattern, specifically at the base of the developing septa (Fig. 4, A and B), which localizes Plod3 to a place of intense activity in the developing lung and, hence, in a position where Plod3 may influence alveolar development. No appreciable staining for Plod3 was evident in the muscular layer of the vessel walls in the lungs of mouse pups at P7 exposed to 21% O2 or 85% O2 from P0.5. The airways and vessels are indicated. E: staining for Plod2 in a neonatal mouse lung at P7.5, after exposure to 85% O2 from P0.5, after preadsorption of the primary anti-Plod2 antibody with a competing peptide, to demonstrate specificity. F: low magnification staining for Plod2 in a neonatal mouse lung at P7.5 after exposure to 85% O2 from P0.5.

Fig. 3. Localization of Plod2 expression in the parenchyma (A and B) and vessel walls (C and D) of the lungs of mouse pups at P7.5, after exposure to 21% O2 or 85% O2 from P0.5. The airways and vessels are indicated. E: staining for Plod2 in a neonatal mouse lung at P7.5, after exposure to 85% O2 from P0.5, after preadsorption of the primary anti-Plod2 antibody with a competing peptide, to demonstrate specificity. F: low magnification staining for Plod2 in a neonatal mouse lung at P7.5 after exposure to 85% O2 from P0.5.

sion pattern, specifically at the base of the developing septa (Fig. 4, A and B), which localizes Plod3 to a place of intense activity in the developing lung and, hence, in a position where Plod3 may influence alveolar development. No appreciable staining for Plod3 was evident in the muscular layer of the vessel walls in the lungs of mouse pups at P7 exposed to 21% O2 (Fig. 4C) and 85% O2 (Fig. 4D) although staining in the endothelium of the latter group was evident (Fig. 4D). Together, these data reveal that lysyl hydroxylases have discrete expression patterns in the developing mouse lung and that the detection of all three lysyl hydroxylases in the developing septa places all three members of this family of matrix cross-linking enzymes in a position to influence secondary septation and, hence, alveolarization.

TGF-β regulates mRNA levels of lysyl hydroxylases in constituent cells types of the developing lung. TGF-β is acknowledged as a key regulator of late lung development and alveolarization. TGF-β is also a potent profibrotic growth factor and has been ascribed a key role in the pathogenesis of BPD. Furthermore, we identified multiple Smad-binding elements (which confer TGF-β responsiveness) in the plod2 and PLOD2 promoters, by in silico analysis. For these
reasons, the ability of TGF-β to impact lysyl hydroxylase expression in constituent cell types of the developing alveolus was assessed. TGF-β exhibited a broad spectrum of effects on lysyl hydroxylase gene expression, where an 18-h exposure of A549 cells, a commonly used model of the human lung epithelium, to a dose of 2 ng/ml TGF-β, upregulated PLOD2 expression (Fig. 5A). In contrast, in primary mouse alveolar type II cells, TGF-β was without effect on plod2 mRNA levels but did upregulate plod3 mRNA expression (Fig. 5B). TGF-β dramatically increased PLOD2 mRNA levels in primary human lung fibroblasts (Fig. 5C) and dramatically increased plod2 mRNA levels in primary mouse lung fibroblasts (Fig. 5D). TGF-β also upregulated PLOD3 mRNA levels in primary human lung microvascular endothelial cells (Fig. 5E) although TGF-β was without any impact on lysyl hydroxylase mRNA levels in primary human pulmonary artery smooth muscle cells (Fig. 5F). Thus TGF-β appears to be a mediator of plod2 and plod3 expression in constituent cells types of the alveolus.

TGF-β regulated PLOD2 promoter activity in human lung fibroblasts and epithelial cells. TGF-β increased the activity of the human PLOD2 promoter in human lung fibroblasts (Fig. 5G).
The specificity of the effect of TGF-β was confirmed by the observation that a pan-TGF-β-neutralizing antibody (1D11) was able to abrogate the effects of TGF-β, while an isotype-matched control antibody (MOPC21) was not (Fig. 6, A and B). These data support the observation that TGF-β increased the abundance of PLOD2 mRNA in human lung fibroblasts (Fig. 5C) and in A549 cells (Fig. 5A) and support the contention that TGF-β is a regulator of PLOD2 expression in constituent cell types of the developing alveolus.

Fig. 5. Regulation of lysyl hydroxylase mRNA levels by TGF-β. PLOD1, PLOD2, and PLOD3 expression was assessed by real-time RT-PCR using the primers listed in Table 1, in mRNA pools from human A549 cells (A), as well as in primary human lung fibroblasts (C), microvascular endothelial cells (E), and pulmonary artery smooth muscle cells (F) after stimulation with vehicle alone or TGF-β (2 ng/ml; 16 h). The HPRT gene was used as a reference. Similarly, plod1, plod2, and plod3 expression was assessed by real-time RT-PCR using the primers listed in Table 1, in mRNA pools from primary mouse lung alveolar type II cells (B), and mouse lung fibroblasts (D) after stimulation with vehicle alone or TGF-β (2 ng/ml; 16 h). The hprt gene was used as a reference. Data reflect means ± SD (n = 3). The P values (above the horizontal line) represented compare vehicle- vs. TGF-β-treated groups and were assessed by unpaired Student’s t-test.

Fig. 6. Regulation of PLOD2 promoter activity by TGF-β. The activity of the human PLOD2 promoter was assessed by dual luciferase assay (DLR) in primary human lung fibroblasts (A), as well as human lung A549 cells (B), as a transfectable model of the alveolar epithelium, after preincubation (30 min, 37°C; in stimulation medium) of TGF-β with a pan-TGF-β-neutralizing antibody (1D11) or an isotype-matched control (MOPC21) antibody. Data reflect means ± SD (n = 5). The P values (above the horizontal line) represented compare vehicle- vs. TGF-β-treated groups, or MOPC21 vs. 1D11 control groups and were assessed by unpaired Student’s t-test.
Exposure to 85% O₂ drove aberrant plod2 expression in the developing mouse lung via TGF-β. Neutralization of TGF-β signaling in vivo in mouse pups that were exposed to 85% O₂ partially normalized elastin structures in the developing septa (Fig. 7C; arrowheads) because these structures largely resembled elastin structures in the developing septa of mouse lungs exposed to 21% O₂ (Fig. 7A; arrowheads), compared with the perturbed elastin structures seen in the septa of developing lungs in mice that were exposed to 85% O₂ that received a control MOPC21 antibody (Fig. 7B; arrows). These data support previous observations (23, 27) that neutralization of TGF-β signaling in the hyperoxia model of BPD partially restores normal structure to the developing lung.

Neutralization of TGF-β signaling in vivo in mouse pups that were exposed to 85% O₂ did not impact plod1 expression in the lungs of mouse pups (Fig. 8A); however, TGF-β neutralization did restore normal plod2 expression in the lungs of mouse pups (Fig. 8B). As with plod1, no impact of TGF-β neutralization on plod3 expression was noted (Fig. 8C).
though the authors do not consider the intensity of immunohistochemical staining to be quantitative, staining intensity for Plod2 in the developing septa of mouse pup lungs exposed to 85% O2 that received a TGF-β/H9252-neutralizing antibody (Fig. 9D) appeared less intense when compared with mouse pup lungs exposed to 85% O2 that received a control (nonimmune) MOPC21 antibody (Fig. 9B). Together, these data suggest that TGF-β mediated the effects of hyperoxia exposure on Plod2 expression in the aberrantly developing mouse lung.

Elevated PLOD2 expression is associated with BPD. The mRNA levels of PLOD1 (Fig. 10A), PLOD2 (Fig. 10B), and PLOD3 (Fig. 10C) were assessed in mRNA pools from the lungs of patients that had died with BPD or at risk for BPD, as well as in two control groups, one control group age matched to the BPD group for gestational age at birth (GAB) and another control group age matched to the BPD group for chronological age at death (CAD). No appreciable differences were observed comparing the expression of PLOD1 (Fig. 10A)}
and PLOD3 (Fig. 10C) across all three groups. However, the mRNA levels of PLOD2 were appreciably elevated in the lungs of patients with or at risk for BPD, compared with both the GAB and CAD groups (Fig. 10C). These data demonstrate that the expression of lysyl hydroxylases, namely the PLOD2 gene, is deregulated in the lungs of infants with or at risk for BPD. All three PloD proteins were detected in the developing lungs of human neonates by immunohistochemistry, with Ploid evident in the airway epithelium, vascular smooth muscle, and in the septa (Fig. 11, A and B). PloD2 was evident in the endothelium, airway epithelium, and developing septa (Fig. 11C), whereas PloD3 was evident in the septa (Fig. 11D). It is evident from these data that PloD2, which has emerged from this study as a candidate player in the pathogenesis of BPD, is present at sites of intensive ECM production and remodeling in the developing human lung.  

**DISCUSSION**

The proper deposition and posttranslational modification and remodeling of the lung ECM is critical to normal lung development, including the formation of the alveolar air sacs, the principal gas exchange unit of the lung, which takes place during late lung development. Perturbations to the proper deposition, processing, and remodeling of the lung ECM are credited as being a key underlying cause of disturbed late lung development. Clinically, disturbed late lung development is exemplified in humans by BPD, also called chronic lung disease of early infancy. BPD results from the mechanical ventilation and/or oxygen supplementation of premature infants, which then exhibit a pronounced arrest of late lung development (19), where alveolar simplification is evident, as well as blunted lung microvascular development (41) and dysangiogenesis (13). The pathomechanisms at play in the lung of affected infants are not well understood. However, several histopathological observations have pointed to dramatically malformed matrix structures in patients with BPD, including collagen fibers in the alveolar septa that were described to be “thickened, tortuous, and disorganized” (43). These observations have also been made examining lungs of experimental animals where BPD has been modeled, including in ventilated preterm lambs (1, 6, 30), ventilated mouse pups (5, 8), and mouse pups exposed to elevated oxygen concentrations (23, 49). To date, little information is available about how and why ECM structures are deformed in lungs in which alveolarization has been disturbed and whether these perturbed matrix structures play a causal role in arresting the alveolarization process. One theory put forward is that the matrix processing and maturations systems, which remodel the ECM structures, may be disturbed. Along these lines, deregulated expression and activity of matrix metalloproteinases, which remodel ECM structures, have been implicated in BPD. Another theory for-
Lysyl hydroxylases are ideally positioned to influence late lung development, as they convert lysine to hydroxylysine, generating a substrate for cross-linking. All three lysyl hydroxylases—Plod1, Plod2, and Plod3—are abundantly expressed in the lung and other tissues, and their expression is critical for normal lung development.

In the context of bronchopulmonary dysplasia (BPD), Plod3 expression has been shown to be upregulated by TGF-β in a rodent model. These data make a strong case for a role for perturbed matrix cross-linking in arrested lung development.

The ability of TGF-β to regulate lysyl hydroxylase activity in the lung epithelial basement membrane is particularly interesting, as it suggests a potential role for Plod3 in the pathogenesis of BPD. Further studies are needed to determine the role of Plod3 in the development of BPD and to elucidate the mechanisms by which TGF-β regulates lysyl hydroxylase activity.
lysyl hydroxylases in bronchopulmonary dysplasia

lungs. These data add to a growing body of evidence that points to a role for TGF-β in driving perturbed ECM remodeling in animal models of BPD. It is important to note that observations were made using RNA pools from lung tissue homogenates, and, as such, the effects of TGF-β neutralization on lysyl hydroxylase expression in specific cellular compartments of the developing lung would not have been detected in this approach and cannot be ruled out.

In support of a role for PLOD2 in aberrant late lung development, PLOD2 expression was also elevated in patients with BPD or at risk for BPD, irrespective of whether the patient group was matched for gestational age at death or chronological age at death. As such, PLOD2 is an exciting patient group was matched for gestational age at death or at risk for BPD, irrespective of whether the mouse lung expression, TGF-β neutralization, and clinical BPD studies examined changed in lungs of newborn mice. Prelude to defective alveolar septation during lung development? Am J Physiol Lung Cell Mol Physiol 294: L3–L14, 2008.


