Lysyl Oxidase Activity Is Dysregulated during Impaired Alveolarization of Mouse and Human Lungs

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Rationale: Disordered extracellular matrix production is a feature of bronchopulmonary dysplasia (BPD). The basis of this phenomenon is not understood.

Objectives: To assess lysyl oxidase expression and activity in the injured developing lungs of newborn mice and of prematurely born infants with BPD or at risk for BPD.

Methods: Pulmonary lysyl oxidase and elastin gene and protein expression were assessed in newborn mice breathing 21 or 85% oxygen, in patients who died with BPD or were at risk for BPD, and in control patients. Signaling by transforming growth factor (TGF- β) was preemptively blocked in mice exposed to hyperoxia using TGF- β -neutralizing antibodies. Lysyl oxidase promoter activity was assessed using plasmids containing the *lox* or *lox1* promoters fused upstream of the firefly luciferase gene.

Measurements and Main Results: mRNA and protein levels and activity of lysyl oxidases (Lox, LoxL1, LoxL2) were elevated in the oxygen-injured lungs of newborn mice and infants with BPD or at risk for BPD. In oxygen-injured mouse lungs, increased TGF- β signaling drove aberrant *lox*, but not *lox1* or *lox2*, expression. Lox expression was also increased in oxygen-injured fibroblasts and pulmonary artery smooth muscle cells.

Conclusions: Lysyl oxidase expression and activity are dysregulated in BPD in injured developing mouse lungs and in prematurely born infants. In developing mouse lungs, aberrant TGF- β signaling dysregulated lysyl oxidase expression. These data support the postulate that excessive stabilization of the extracellular matrix by excessive lysyl oxidase activity might impede the normal matrix remodeling that is required for pulmonary alveolarization and thereby contribute to the pathological pulmonary features of BPD.

Keywords: lung development; septation; TGF- β ; transforming growth factor

Bronchopulmonary dysplasia (BPD) is a consequence of prolonged mechanical ventilation of premature infants with

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The formation of extracellular matrix structures is perturbed in bronchopulmonary dysplasia (BPD), which is believed to contribute to the arrested alveolarization seen in “new BPD.”

What This Study Adds to the Field

The expression and activity of lysyl oxidases, which regulate collagen and elastin cross-linking, are dysregulated in BPD in mice and humans. These data could suggest that overstabilization of the extracellular matrix may impede normal matrix remodeling, thereby creating a less plastic lung structure.

supplemental oxygen. The syndrome was first described by Northway and colleagues in 1967 (1) and remains a significant complication of premature birth, affecting nearly 10,000 newborns annually in the United States. BPD causes long-term respiratory consequences that reach beyond childhood (2). Today, BPD—which is also called “new BPD” or chronic lung disease of early infancy—is believed to result from an arrest of late lung development that is characterized by alveolar simplification (3, 4) and an arrest of microvascular development (5, 6) or microvascular dysangiogenesis (7, 8). Thus, BPD leads to a long-term reduction in the total number of alveoli, and hence, reduces the surface area available for gas exchange.

The pathogenesis of new BPD is not fully understood; however, several lines of evidence indicate that a severely perturbed extracellular matrix (ECM) metabolism contributes to this disorder. For example, an accumulation of elastic fibers has been observed in patients with classic BPD (9, 10), and secondary collagen fibers in the saccular wall of patients who died with new BPD are “thickened, tortuous, and disorganized” (11). Excessive production and accumulation of elastin has also been reported in animal models of BPD. For example, in the injured lungs of premature lambs, increased deposition of elastic fibers has been associated with thickened walls of the terminal respiratory units (12). In addition, in the injured lungs of mouse pups with BPD, increased elastin synthesis has also been reported (13, 14). These observations are important, because the ECM plays a pivotal role in late lung development, and any perturbations to the formation and remodeling of matrix structures would affect the development of the immature lung (15).

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Although many studies have examined the synthesis and production of ECM molecules in BPD, few studies to date have addressed the subsequent post-translational processing of these molecules, or the remodeling of the matrix itself (11, 12). Therefore, in this study the expression and activity of lysyl oxidases, which play a key role in regulating the stability of the ECM, have been examined in an animal model of BPD, as well as in ventilated human neonates who have died either with BPD or at risk for BPD.

Five lysyl oxidases have been described: the archetypical member, Lox, and four closely related, or Lox-like (LoxL) members, named LoxL1–LoxL4 (16, 17). All lysyl oxidases catalyze the oxidative deamination of lysine and hydroxylysine residues to peptidyl α -amino adipic- δ -semialdehydes, generating reactive semialdehydes that condense to form intramolecular and intermolecular covalent cross-links in elastin and collagen molecules. This process drives fiber maturation and thus imparts structural stability to the ECM (16, 17).

In this study it was hypothesized that lysyl oxidase expression and activity are dysregulated in BPD. The data reported here demonstrate that lysyl oxidase expression and activity are indeed up-regulated in the injured lungs of newborn mice and human infants, and that this up-regulation is driven, in part, by the excessive transforming growth factor (TGF)- β signaling associated with oxygen injury to the lung. It is proposed that the persistence and overabundance of elastin fibers and the formation of thickened, tortuous, and irregularly distributed collagen fibers observed in BPD may result from “overstabilization” of these important matrix molecules through excessive lysyl oxidase activity, making them resistant to normal remodeling processes in the developing lung. This “locked” lung structure may well underlie the arrested development of immature lungs and lead to the impaired septation and vascularization observed in BPD.

METHODS

Animal and Tissue Treatment

The animal ethics authority of the government of the State of Hessen and the Subcommittee for Research Animal Studies of the Massachusetts General Hospital approved all animal procedures. The newborn mouse model of BPD used in this study, in which newborn mice are exposed to normoxic (21% O₂) or hyperoxic (85% O₂) gas mixtures for the first 28 days after birth, has been defined and characterized by others (14, 18, 19) and described previously by our own laboratory (20, 21). Mice were killed at days 1, 7, 14, 21, and 28 after birth for analysis, which spans the period of late lung development in mice. The processing of lung tissue from this model for RNA and protein analysis, as well as immunohistology, has been described previously (20, 21), as has the inhibition of TGF- β signaling in mouse pup lungs with a TGF- β 1,2,3-neutralizing IgG₁ antibody (1D11; Genzyme, Cambridge, MA) (22). In these studies, an isotype-matched nonimmune IgG₁ antibody (MOPC21; Sigma, St. Louis, MO) did not modulate pulmonary TGF- β signaling.

Human Tissue

Human material was used with the approval of the Human Subjects Review Committees of the Erasmus University Medical Centre and the University of Giessen Lung Center, which use human research protection principles espoused in the Declaration of Helsinki. Neonatal human lung tissue was retrieved from archived autopsy material at the Erasmus University Medical Centre and the University of Giessen Lung Center. The clinical characteristics of the patients from which the specimens were derived are presented in Table 1 (two groups of control patients) and Table 2 (patients with BPD or at risk for developing BPD, called the “BPD group”). Two groups of control patients were used: control group 1, which included patients with the gestational age at birth and birth weight matched to the BPD group; and control group 2, which included patients with the chronological age at death matched to the chronological age at death of the BPD group. Tissue samples were collected at autopsy, within 24 hours of death. The average time

TABLE 1. CLINICAL CHARACTERISTICS OF CONTROL PATIENTS

Patient (Material)*	Birth Weight, g	M/F	Gestational Age, wk	Chron. Age at Death, d	Combined Lung Weight, g	Days FiO ₂ >0.50	Days Mechanical Ventilation	Cause of Death/Autopsy Diagnosis and Medication
Control group 1, matched to BPD group (Table 2) for birth weight and gestational age at birth								
1 (h, r)	954	M	26	<1	14.6	0	0	Intracranial hemorrhage
2 (h, r)	914	M	27	<1	15.5	0	0	DiGeorge syndrome (22q11.2 deletion); intrauterine infection
3 (r)	1,210	M	28	<1	ND	0	0	Hypoxic-ischemic encephalopathy
4 (h, r)	826	M	29	<1	ND	0	0	Hydrocephalus, Arnold Chiari malformation
5 (r)	852	M	27	<1	24.0	0	0	Hypoxic-ischemic encephalopathy
6 (h, r)	995	M	27	<1	19.0	0	0	Placental abruption
7 (h, r)	758	M	26	<1	17.0	0	0	Intrauterine infection
Median	914		27					
Mean $\pm$ SE	929 $\pm$ 55		27 $\pm$ 0.4					
Control group 2, age matched to BPD group (Table 2) for chronological age at death								
8 (h)	2,334	M	33	<1	ND	0	0	Intrauterine death, no congenital abnormalities evident
9 (h)	1,904	F	34	<1	34.0	0	0	Intrauterine death (chorioamnionitis)
10 (r)	1,625	M	33	3	ND	0	0	Congenital heart malformation. Drugs: atropine, prostaglandin A1
11 (h, r)	2,350	M	35	<1	30.0	0	<1 h	Perinatal asphyxia. Drugs: atropine, adrenaline
12 (h)	2,040	M	33	<1	ND	0	<1 h	Multiple congenital anomalies (VACTERL). Drugs: adrenaline
13 (r)	1,740	F	34	<1	ND	0	0	Intrauterine death (ventriculomegaly)
14 (h, r)	1,800	M	32	5	ND	0	5	Meningoencephalitis
15 (h, r)	1,190	M	31	<1	ND	0	0	Placental abruption
Median	1,852		33					
Mean $\pm$ SE	1,873 $\pm$ 135		33 $\pm$ 0.44					

Definition of abbreviations: BPD = bronchopulmonary dysplasia; Chron. = chronological; F = female; M = male; ND = not determined; VACTERL = vertebra/anus/cardiac/trachea/esophagus/radius/renal/limb anomalies.

* Material includes RNA (r) and histological sections (h).

TABLE 2. CLINICAL CHARACTERISTICS OF PATIENTS WITH BPD OR AT RISK FOR BPD

Patient (Material)*	Birth weight, g	M/F	Gestational age, wk	Chron. age at death, d	Combined lung weight, g	Days $\text{FiO}_2 > 0.5$	Days Mechanical Ventilation†	Cause of Death/Microbiology/Autopsy Diagnosis/Drugs‡
16 (h)	650	M	27	9	33.3	9	9 (c, hf)	BPD, IRDS, sepsis. Drugs: surfactant, inotropes, tobramycin, vancomycin, cortisone
17 (r)§	720	M	29	62	59.0	13	62 (c, hf)	BPD, IRDS, <i>Staphylococcus aureus</i> sepsis. Drugs: surfactant, inotropes, tobramycin, flucloxacillin, cortisone
18 (h, r)	1055	M	32	18	ND	18	18 (c)	BPD, ventricular septal defect, Edwards syndrome (trisomy 18). Drugs: inotropes
19 (h, r)	825	F	27	6	35.0	6	6 (c, hf)	BPD, IRDS, bronchopneumonia, intracranial hemorrhage. Drugs: surfactant, inotropes, tobramycin, penicillin
20 (h)	920	M	26	19	ND	17	19 (c, hf)	BPD, retinopathy, patent ductus arteriosus; <i>Streptococcus</i> , <i>Staphylococcus</i> and <i>Ureaplasma</i> infection. Drugs: surfactant, indomethacin, dexamethasone, inotropes, vancomycin, penicillin, erythromycin
21 (h)§	650	M	27	32	44.0	16	32 (c)	BPD, IRDS, <i>Staphylococcus epidermidis</i> infection. Drugs: surfactant, dexamethasone, inotropes, penicillin, vancomycin
22 (r)§	835	M	26	65	ND	27	65 (c, hf)	BPD, cerebral bleeding, ductus arteriosus. Drugs: surfactant, inotropes, dexamethasone, theophylline
23 (h, r)§	930	M	26	99	186.0	98	99 (c, hf)	BPD, IRDS, pneumothorax, subependymal hemorrhage. Drugs: surfactant, inotropes, dexamethasone, tobramycin, penicillin, amphotericin
24 (h, r)§	1250	F	28	34	86.0	34	34 (c, hf)	BPD, IRDS, <i>Staphylococcus epidermidis</i> sepsis. Drugs: surfactant, furosemide, amoxicillin, erythromycin
25 (r)§	1220	M	31	35	95.0	35	35 (c, hf)	BPD, IRDS, right ventricular hypertrophy, anemia, rickets. Drugs: furosemide, amoxicillin, vancomycin
Median	878		27	33		18	33	
Mean $\pm$ SE	906 $\pm$ 68		28 $\pm$ 0.8	38 $\pm$ 8		27 $\pm$ 8	38 $\pm$ 9	
P value vs. CTRL1	0.111 (NS)		0.403 (NS)	0.016				
P value vs. CTRL2	<0.001		<0.001	0.949 (NS)				

Definition of abbreviations: BPD = bronchopulmonary dysplasia; Chron. = chronological; CTRL = control; IRDS = infant respiratory distress syndrome; F = female; M = male; ND = not determined; NS = not significant.

* Material includes RNA (r) and histological sections (h).

† c, conventional ventilation; hf, high-frequency ventilation.

‡ Inotropes include dopamine, dobutamine, and adrenaline.

§ Patients have clinically defined BPD.

|| By paired Student *t*-test, compared with the control 1 (CTRL1) and control 2 (CTRL2) groups in Table 1. In the case of chronological age at death, the postmenstrual ages at death, rounded to the nearest full week, were compared.

between death and tissue processing was similar in all three patient groups.

Cell Culture

The NIH/3T3 fibroblast-like cell line and primary human fibroblasts and primary human pulmonary artery smooth muscle cells (paSMC) were cultured as described previously (20, 23). The human fibroblast cell line HFL1 was passaged as recommended by the American Type Culture Collection (ATCC). Gas tension in the culture media was monitored daily. When the headspace gas contained 21% oxygen, the cell culture media exhibited a PO_2 of 142.4 ± 4.9 mm Hg for NIH/3T3 cells and 137.4 ± 8.3 mm Hg for paSMC. With the hyperoxic 85% oxygen-containing headspace gas, the cell culture media exhibited a PO_2 of 506.3 ± 16.7 mm Hg for NIH/3T3 cells and 466.9 ± 29.4 mm Hg for paSMC over the experimental time course (maximum 96 h). The pH and PCO_2 values did not appreciably change over the experimental time course.

Immunohistochemistry

Hematoxylin staining, Hart's elastin staining, picrosirius red, and Masson's trichrome staining for collagen, and staining for the expression of lysyl oxidase isoforms was performed on 3- μm tissue sections as described previously (20), with goat anti-Lox (SC-32409; blocking peptide SC-32409P), goat anti-LoxL1 (SC-48720; blocking peptide SC-48720P), goat anti-LoxL2 (SC-48723; blocking peptide SC-487243), and goat anti-LoxL3 (SC-48728; blocking peptide SC-48728P). All anti-

bodies were used at 1:50 dilution, and were from Santa Cruz (San Francisco, CA). In the antibody blocking studies, the antibodies were preadsorbed with a 10-fold molar excess of the peptides described above, for 30 minutes at room temperature, before application to lung sections. Immune complexes were visualized with a Histostain Plus Kit (Zymed, San Francisco, CA).

Analysis of Gene Expression

Total RNA (collected from whole mouse lungs as described above or from peripheral regions of human lungs) was screened by semiquantitative or quantitative real-time reverse transcription (RT) polymerase chain reaction (PCR) with the primers listed in Table 3, as described previously (20). For quantification of relative mRNA expression by semiquantitative RT-PCR, band intensities from specific samples were normalized for loading using the constitutively expressed *hspa8* or *gapdh* band amplified from the same sample. Densitometric analysis of amplicon bands generated in the linear range of product amplification was performed using a GS-800 model densitometer with Quantity One software (both from Bio-Rad Laboratories, Munich, Germany). For real-time PCR, changes in mRNA expression were evident comparing ΔCt values, and using the *hmbs* gene as an internal control (23).

Protein Isolation and Immunoblotting

Protein was collected, transferred to charged membranes, and immunoblots were probed as described previously (20) with the antibodies

TABLE 3. PRIMERS USED FOR REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Gene	Forward Primer	Reverse Primer	Amplicon Size, bp	Number of Cycles	Annealing Temperature, °C
Semiquantitative real-time RT-PCR					
Mouse					
<i>eln</i>	5'-GGAGTCCCGGTGGAGTCTATT-3'	5'-ACCAGGAATGCCACCAACACCTG-3'	1,079	25	60.0
<i>emilin1</i>	5'-AACCGTGTCTCCACTCATGA-3'	5'-GGTGTCCAGTCGTTCCGACA-3'	1,470	26	60.0
<i>fbn5</i>	5'-CTAGATATTGATGAATGCCG-3'	5'-TGCCTCTGAAGTTGATGACA-3'	1,180	26	60.0
<i>gapdh</i>	5'-AACTTTGGCATTGTGGAAGG-3'	5'-ACACATTGGGGGTAGGAACA-3'	222	25	60.0
<i>lox</i>	5'-ATGCGTTTCGCTGGGCTGTGC-3'	5'-CTAATACGGTGAAATTGTGCAGCC-3'	630	31	60.0
<i>lox1</i>	5'-AGCTTGCTCAACTCGGGCTCTGAG-3'	5'-TCAGGACTGGACGATTTTGCAG-3'	1,668	32	57.5
<i>lox2</i>	5'-GGTGTCTGAAGAATGGAGAATGGG-3'	5'-TACTGTACAGAGAGCTGGTTATTT-3'	1,295	32	57.5
<i>lox3</i>	5'-ATGAGAGCTGTCAAGTGTGTGG-3'	5'-GCACCGAACTTCACTCAAGTGG-3'	1,132	32	57.0
<i>lox4</i>	5'-ATGATGTGGCCCCAACACC-3'	5'-TCAGATCAAGTTGTTCTGAGTCGCT-3'	2,273	32	57.0
Human					
<i>hspa8</i>	5'-TGGGTGGAGAAGATTTTGAC-3'	5'-ACCACAGGGAGGAGCTCCAC-3'	1,219	24	60.0
<i>lox</i>	5'-CCTGCTGATCCGCGACAACC-3'	5'-CCTGAGGCATACGCATGATGTCC-3'	937	31	60.0
<i>lox1</i>	5'-CCGGACCTCAGCGCTCCGAGAGTAGC-3'	5'-CCACGTTGTTGGTGAAGTCAGACTCC-3'	1,031	31	60.0
<i>lox2</i>	5'-GGTCTGCAGAGAGCTGGGCTTTGG-3'	5'-GGCAGTCGATGTCATGGCCGTACATG-3'	1,000	31	60.0
<i>lox3</i>	5'-GGCTTCTGACGGCTGGTCGCAAG-3'	5'-GTGCTGAGTGCAGCAACAGATCTGATG-3'	1,034	31	60.0
<i>lox4</i>	5'-GCACAGAGAGCTCCTTGACCAG-3'	5'-GCACAGAGAGCTCCTTGACCAG-3'	1,284	31	60.0
Quantitative real-time RT-PCR					
Mouse					
<i>hmbs</i>	5'-GGTCCAGAAGATGACCCACA-3'	5'-AAGCTGCCGTGCAACATCCA-3'	120	—	—
<i>Lox</i>	5'-GCACTGCACACACAGGGA-3'	5'-TTAGTGTAGTCTGATTGAGG-3'	120	—	—
Human					
<i>hmbs</i>	5'-TTGCTGCTGTCCAGTGCTTA-3'	5'-AGATGAAGCCCCACATACT-3'	130	—	—
<i>lox</i>	5'-TGCCAGTGGATTGATATTAC-3'	5'-TACGGTGAAATTGTGCAGCC-3'	130	—	—
<i>lox1</i>	5'-CTGCTATGACACCTACAATG-3'	5'-TGTGTAGTGAATGTTGCATC-3'	120	—	—
<i>lox2</i>	5'-TCGGCCTCAGCCGCGCAGAC-3'	5'-CCTCCATGCTGTGGTAGTGC-3'	120	—	—
<i>lox3</i>	5'-ATCAGGATGTGAGCCAGG-3'	5'-TGAAGGCATCAACATGTGG-3'	120	—	—
<i>lox4</i>	5'-AAGTGGCAGAGTCAGATTTC-3'	5'-TGTTCTGAGACGCTGTTC-3'	110	—	—

Definition of abbreviation: RT-PCR = reverse transcriptase-polymerase chain reaction.

used for immunohistochemistry (1:750), as well as goat anti-LoxL4 (SC-48732; Santa Cruz, 1:500) and mouse α -tubulin (SC-58667; Santa Cruz, 1:2,000). Immune complexes were detected using peroxidase-conjugated secondary antibodies: rabbit anti-mouse (R&D Systems, Wiesbaden, Germany; 1:2,500) and donkey anti-goat (Santa Cruz, 1:1,000). Densitometric analysis of bands was performed as described above.

Elastin and Desmosine Measurements

Soluble elastin levels were determined using the FASTIN elastin assay (BioColor, County Antrim, UK) as per the manufacturer's recommendations. Desmosine was measured using the procedures of Starcher and colleagues (24) as modified by Hornstra and colleagues (25). The levels of these ECM components were related to the total protein levels in the samples.

Lysyl Oxidase Activity Assay

Lysyl oxidase activity is reported as β -aminopropionitrile-sensitive activity assessed using Amplex Red (Invitrogen, Karlsruhe, Germany) detection and 1,5-diaminopentane (Sigma, Taufkirchen, Germany) as substrate, as described previously (26).

Luciferase-based Promoter Reporter Assay

The construction of luciferase-linked promoter reporter plasmids containing the -2,073/+1434 proximal region of the murine *lox* gene or the -712/-1 proximal region of the human *lox1* gene in pGL2 (Promega, Madison, WI) has already been described (27, 28), as has the construction of a luciferase-linked promoter reporter plasmid containing 1,547 bp of 5' flanking sequence from the human *lox* gene with a pGL3 backbone (29).

Statistical Treatment of Data

Unless otherwise indicated, data are presented as mean $\pm$ SD. Differences between groups were analyzed by analysis of variance with the Student-Newman-Keuls *post hoc* test for multiple comparisons,

or by Student *t*-test, with *P* values less than 0.05 regarded as significant.

RESULTS

Elastin and Desmosine Metabolism Is Perturbed in Oxygen-injured Mouse Lungs

Neonatal mice breathing 21% oxygen from Postnatal Day (P)1 exhibited typical elastin deposition in developing alveoli that appeared to be condensed into punctate foci in the tips of developing septa at days P7 and P28 (Figure 1A, *arrows*). In contrast, mice exposed to 85% oxygen from P1 for similar durations exhibited disorganized elastin fiber networks in the developing septa. Changes in septal collagen distribution were not evident by Masson's trichrome staining (Figures 1Ba and 1Bb), but septal collagen abundance appeared to be increased, or collagen fibers were thickened, in lungs from 85% oxygen-exposed mice examined by picrosirius red staining under polarized light (Figures 1Bc and 1Bd). Soluble elastin protein levels were comparable in normoxia- and hyperoxia-exposed pups at P14, but were elevated at P28 in hyperoxia-exposed pups (Figure 1C). Furthermore, levels of desmosine, a marker of elastin cross-linking by lysyl oxidases, were elevated in the lungs of hyperoxia-exposed pups both at P14 and P28 (Figure 1D). Although the expression of elastin (*eln*) mRNA normally peaked by P21, it remained high at P28 in mice breathing 85% oxygen (Figure 1E). In addition to elastin, levels of mRNA encoding fibulin-5 and emilin-1, two important elastic fiber components, also appeared to be elevated in oxygen-injured lungs of mouse pups (Figure 1E), indicating that in addition to elastin, the expression of other components of the elastic fibers are affected in lungs exposed to 85% oxygen. Together, these data indicate that elastin metabolism is dysregulated in the 85%

Figure 1. Elastin production and cross-linking are dysregulated in the injured developing mouse lung. (A) Hart's stain for elastin in air-exposed mouse pup lungs, indicating punctate elastin foci (arrows) in the developing septa at Postnatal Day (P)7 and P28 in the lungs of pups exposed to 21% O₂ that are absent in the lungs of pups exposed to 85% O₂. (B) Assessment of collagen morphology by Masson's trichrome stain (a and b) in the developing septa of P7 mouse pups exposed to 21% O₂ or 85% O₂. Collagen was also assessed by picrosirius red staining observed under polarized light in mouse pups exposed to (c) 21% O₂ or (d) 85% O₂. Both (C) soluble elastin and (D) desmosine were elevated in 85% O₂-exposed pups (open bars) compared with 21% O₂-exposed pups (solid bars) at P28 (n = 5). (E) Expression of elastin (the *eln* gene), fibulin-5 (the *fbn5* gene), and emilin-1 (the *emilin1* gene) mRNA monitored by semiquantitative reverse transcriptase–polymerase chain reaction in the first month of postnatal life of pups exposed to 21% O₂ or 85% O₂. The constitutively expressed *hspa8* and *gapdh* genes served as controls for loading equivalence. *P < 0.01.

oxygen-injured developing mouse lung, and that this dysregulation is associated with altered elastin turnover or cross-linking, as revealed by elevated desmosine levels.

Lysyl Oxidase Expression and Activity Are Elevated in Oxygen-injured Mouse Lungs

The elevated desmosine levels in the 85% oxygen-exposed mouse pup lungs (Figure 1D) suggested that hyperoxia disrupts the mechanisms responsible for ECM maturation. Because elastin cross-linking is primarily performed by lysyl oxidases, the expression and function of these enzymes were examined in the injured developing lung. Lox was weakly expressed at P1 in the air-breathing mouse pup lung, and was not detected at all between P7 and P28 (Figure 2). In contrast, pronounced *lox* gene expression (Figure 2A; quantified in Figure 2B) and Lox protein expression (Figure 2C; quantified in Figure 2D) was observed in the lungs of hyperoxia-exposed mouse pups between P7 and P28. The expression of *lox1* and *lox2* mRNA was progressively increased between P7 and P28 in normoxia-exposed pups, and although a similar trend was observed in hyperoxia-exposed pups, *lox1* and *lox2* mRNA expression was

higher in hyperoxia-exposed pups at P21 and P28 compared with age-matched normoxia-exposed pups (Figures 1A and 1B). This trend in expression was confirmed at the protein level for LoxL2 (Figures 2C and 2D). Strong LoxL1 protein expression was observed in hyperoxia-exposed pups throughout the period P7 to P28 compared with relatively weak expression in normoxia-treated pups over the same time-frame. No appreciable changes in the trends in *lox3* and *lox4* gene expression (Figure 2A) or LoxL3 or LoxL4 protein expression (Figure 2C) were evident in normoxia- versus hyperoxia-exposed pups over the period P7 to P28. Consistent with the trend of increased expression of some lysyl oxidases in oxygen-injured mouse pup lungs, lysyl oxidase activity was increased in the lungs of P14 and P28 hyperoxia-exposed pups compared with age-matched litter mates (Figure 2E).

The most pronounced changes in lysyl oxidase expression in the lungs of hyperoxia-exposed pups were seen on P21 and P28. Therefore, P21 mouse lungs were stained for Lox, LoxL1, LoxL2, and LoxL3 to assess expression and localization of lysyl oxidases in the developing septa (Figure 3). No immunoreactivity was observed for LoxL4 (indeed, LoxL4 appears to have

Figure 2. Lysyl oxidase mRNA and protein expression is dysregulated in the injured developing mouse lung. (A) Expression of lysyl oxidase mRNA was monitored by semiquantitative reverse transcriptase-polymerase chain reaction where the constitutively expressed *hspa8* gene served as a loading control. These data were quantified by densitometric analysis in B. (C) Expression of lysyl oxidase protein was monitored by immunoblot, using constitutively expressed α -tubulin as a loading control. These data were quantified by densitometric analysis in D. (E) Lysyl oxidase activity, assessed by an Amplex Red-based assay, was elevated in whole lung extracts from 85% O₂-exposed pups (open bars) compared with 21% O₂-exposed pups (solid bars) at P14 and P28. * $P < 0.01$ ($n = 4$ for A and C, and $n = 5$ for E). AFU = arbitrary fluorescence units.

the lowest expression of all lysyl oxidases in the lung; Figures 2A and 2B), and these images have therefore been omitted. Staining specificity was confirmed by preadsorption of antibodies with a competing peptide (Figure 3; right column). Consistent with the mRNA and protein expression data (Figure 2), pronounced immunoreactivity for Lox, LoxL1, and LoxL2 was observed in the developing alveolar septa of hyperoxia-exposed pups, whereas immunoreactivity was less pronounced for the normoxia-treated group (Figure 3; central vs. left column), and no differences in staining intensity or localization for LoxL3 were observed between the normoxia- and hyperoxia-treated groups.

Lysyl Oxidases Are Induced by Transforming Growth Factor- β and Hyperoxia

Although these studies indicate that lysyl oxidase expression and activity are increased in the hyperoxic mouse pup lung, the mechanisms regulating these enzymes are unknown. TGF- β has been broadly implicated in regulating the expression and activity of lysyl oxidases (30, 31), and TGF- β signaling is dynamically regulated during mouse and human lung alveola-

rization (32). Furthermore, TGF- β signaling is abnormally increased in oxygen-injured mouse lungs (20, 22). Therefore, the effects of TGF- β , hyperoxia, and combinations thereof, on the expression of lysyl oxidases in fibroblasts (both mouse fibroblast-like NIH/3T3 cells and primary human lung fibroblasts) and paSMC were assessed, because fibroblasts and smooth muscle cells are the primary lysyl oxidase-producing cells (33). In mouse fibroblast-like NIH/3T3 cells, the expression of the *lox* gene was not detected under baseline conditions in a 21% oxygen environment. However, exposure of these cells to 85% oxygen increased *lox* mRNA expression, as did stimulation of NIH/3T3 cells with 2 ng/ml TGF- β (20, 23). A combination of 85% oxygen and TGF- β had a synergistic effect, causing pronounced *lox* mRNA expression (Figure 4A). In contrast, hyperoxia alone was unable to drive *loxl1* gene expression in NIH/3T3 cells; however, stimulation with 2 ng/ml TGF- β increased *loxl1* gene expression in NIH/3T3 cells, and this induction was increased when cells were maintained in an 85% oxygen environment (Figure 4A). No *loxl2* gene mRNA was detected in NIH/3T3 cells, and neither 85% oxygen nor TGF- β had any impact on *loxl3* and *loxl4* gene expression by NIH/3T3 cells

Figure 3. Lysyl oxidase protein expression is dysregulated in the septa of oxygen-injured mouse pup lungs. Increased staining intensity is observed for Lox, LoxL1, and LoxL2 in the septa of mice exposed to 85% O₂ compared with 21% O₂-exposed mouse pups at P14. Antibody specificity was confirmed by preadsorption of antibodies with a competing peptide, which had been used as the immunogen for antibody generation. Results for LoxL4 are omitted as no immunoreactivity was observed with the anti-LoxL4 antibody on mouse lung sections.

(Figure 4A). Identical trends were observed in primary human lung fibroblasts (data not shown).

In human paSMC, 2 ng/ml TGF- β increased mRNA expression of the *lox* gene, both in 21% oxygen and 85% oxygen environments (Figure 4B). However, no synergistic effect of 85% oxygen and TGF- β was observed, as had been observed in NIH/3T3 cells and human fibroblasts. Neither 85% oxygen nor TGF- β , or combinations thereof, impacted the expression of *lox11*, *lox12*, *lox13*, or *lox14* (Figure 4B). Taken together, these data indicate that *lox* and *lox11* can be induced in fibroblasts by both hyperoxia and TGF- β , and that these stimuli can act synergistically. In contrast, TGF- β , but not hyperoxia alone, could drive *lox* expression in paSMC.

To further explore the mechanism of hyperoxia-induced *lox* and *lox11* gene expression, the NIH/3T3 mouse fibroblast cell line and the HFL1 human fibroblast cell line were transfected with luciferase-linked promoter reporter plasmids, in which the *lox* and *lox11* promoter regions were located upstream of a firefly luciferase gene, and activity could be assessed in the cells by measuring the expression of firefly luciferase. Consistent with the NIH/3T3 cell data presented in Figure 4A, TGF- β stimulated *lox* promoter activity, and this effect was enhanced in an 85% oxygen environment (Figure 5A, *solid bars*). Similarly, TGF- β also stimulated *lox11* promoter activity in NIH/3T3 cells (Figure 5A, *open bars*); however, stimulation with TGF- β in an

85% oxygen environment did not have an additive effect on *lox11* promoter activity (Figure 5A, *open bars*). This contrasts with data presented in Figure 4A, however, might be explained by the use of a human *lox11* promoter reporter construct in a mouse fibroblast-like cell line. Therefore, studies were also performed in the transfected human HFL1 fibroblast cell line, where TGF- β (2 ng/ml) could stimulate *lox11* promoter activity, and this effect was enhanced in an 85% oxygen environment (Figure 5A; *left panel, open bar*).

Incubation of NIH/3T3 cells harboring a murine *lox* luciferase-linked promoter reporter plasmid in an 85% oxygen environment caused a fivefold increase in *lox* promoter activity without addition of exogenous TGF- β (Figure 5B). This effect was abrogated when a TGF- β -neutralizing antibody was added to the cell-culture medium, suggesting that hyperoxia drove autocrine stimulation of the *lox* promoter through TGF- β production by NIH/3T3 cells. No autocrine activation of the human *lox11* promoter was observed in NIH/3T3 cells. Interestingly, although a hyperoxia environment was able to augment *lox11* promoter activity in response to exogenously applied TGF- β (Figure 5A, *right panel*), no autocrine activation of the human *lox11* promoter was observed in HFL1 cells either (Figure 5B, *right panel*). This probably indicates that although the *lox11* promoter can be activated by exogenously applied TGF- β , the levels of TGF- β or the increased activity of the

Figure 4. Lysyl oxidase mRNA expression can be modulated *in vitro* by oxygen and transforming growth factor (TGF)- β . (A) Murine NIH/3T3 fibroblast-like cells and (B) human pulmonary artery smooth muscle cells were maintained under hyperoxic (85% O₂) or normoxic (21% O₂) conditions for 24 hours, before addition of TGF- β (2 ng/ml) for an additional 24 hours, after which mRNA was isolated and assessed for lysyl oxidase gene expression. Whole-lung mRNA from mouse or human lungs, respectively, served as a positive control for the polymerase chain reactions (PCR) (Pos.), whereas expression of the *hspa8* gene was used as a loading control. The PCR amplicons derived from two separate cell cultures are represented for each condition. For quantification, densitometric data for amplicons derived from six different cell cultures per condition were averaged; † $P < 0.01$ comparing 85% O₂ versus 21% O₂ exposures in the presence of vehicle; § $P < 0.01$ comparing TGF- β -stimulated versus unstimulated cells with 21% oxygen or 85% oxygen exposure; * $P < 0.01$ comparing 85% O₂ versus 21% O₂ exposures 24 hours after TGF- β stimulation.

TGF- β pathways caused by exposure to hyperoxia are not sufficient to activate the *loxl1* promoter. Taken together, these data suggest that increased oxygen tension augments Lox, and perhaps LoxL1 production by lung fibroblasts, through induction of the *lox* and *loxl1* promoter activity by TGF- β .

Attenuating TGF- β Signaling *In Vivo* Prevents Induction of *lox* Expression by Hyperoxia

Baseline TGF- β signaling is increased in the lungs of mouse pups exposed to 85% oxygen (20). To explore whether hyperoxia induced abnormal lysyl oxidase expression in the lungs of mouse pups through TGF- β -dependent signaling mechanisms, pups were treated with TGF- β 1,2,3-neutralizing antibodies before exposure to 85% oxygen. This has previously been demonstrated to dampen TGF- β signaling in the lungs of 85% oxygen-exposed pups, to improve elastin biogenesis and assembly, and to limit the deleterious effects of hyperoxia on alveologenesis and vasculogenesis (22). Treatment of pups with TGF- β 1,2,3-neutralizing antibodies before exposure to 85% oxygen reduced pulmonary *lox* mRNA expression in the lung, as assessed by semiquantitative RT-PCR (Figure 6A), as well as quantitative real-time RT-PCR (Figure 6B). No effects on the mRNA levels of *loxl1*, *loxl2*, *loxl3*, or *loxl4* were observed (data not shown). Moreover, Lox protein immunoreactivity in the lungs of pups exposed to 85% oxygen appeared to be less intense in lung tissue from pups treated with TGF- β 1,2,3-neutralizing antibodies, compared with tissue

from pups treated with an isotype-matched nonimmune control antibody (Figure 6C). Additionally, improved elastin deposition was evident in the lungs of mouse pups exposed to hyperoxia that were treated with TGF- β 1,2,3-neutralizing antibodies (Figure 6D).

Lox and LoxL1 Expression Is Increased in Human Infants with BPD or at Risk for BPD

The data presented above suggest that lysyl oxidase expression is increased in the injured developing mouse pup lung. To assess whether the expression of lysyl oxidases is similarly modulated in the injured developing lung of humans, the immunoreactivity and mRNA levels of lysyl oxidases were examined in 10 prematurely born infants who died either with BPD or had been at risk for the development of BPD (Table 2; the "BPD group"). The BPD group probably represents a mixture of new BPD and classic BPD. The use of surfactant in 8 out of 10 patients places these patients in the post-surfactant era, and the extremely low birth weight (median, 878 g) and gestational age at birth (27 wk) argue in favor of new BPD, although some degree of fibrosis can be observed in some patients, which is more characteristic of classic BPD. Two groups of control patients were used, which included either seven patients with the gestational age at birth and birth weight matched to the BPD group (control group 1; Table 1), or eight patients with the

Figure 5. Hyperoxia induced autocrine activation of lysyl oxidase promoters in NIH/3T3 through transforming growth factor (TGF)- β . (A) Murine NIH/3T3 fibroblast-like cells and human HFL1 fibroblast-like cells were transfected with plasmid constructs in which the mouse *lox* or human *lox1* promoters had been inserted, upstream of a firefly luciferase gene. The cells were maintained under hyperoxic (85% O₂) or normoxic (21% O₂) conditions for 24 hours, before addition of TGF- β (2 ng/ml) for an additional 12 hours, after which cell extracts were assessed for firefly luciferase activity. To control for the effects of ligand stimulation and hyperoxia on the baseline transcriptional activity of cells, values were normalized for the transcriptional activity of the pGL3-control vector (20). (B) Murine NIH/3T3 fibroblast-like cells and human HFL1 fibroblast-like cells were treated as described in A, except that medium was supplemented with nonimmune IgG or anti-TGF- β 1,2,3-neutralizing antibodies over the entire time course of the experiment (10 μ g/ml). **P* < 0.01 (*n* = 5). ALU = arbitrary luminescence units; n.i. = nonimmune.

chronological age at death matched to the chronological age at death of the BPD group (control group 2; Table 1).

Lung sections from control patients (Figures 7A, 7B, 7E, and 7F) exhibit normal lung expansion with loose extracellular matrix in the interalveolar septa without inflammatory cells. The epithelial lining of the alveoli contains a single cell layer without epithelial defects, and hyaline membranes are not evident. In contrast, unequal lung expansion and broadened intraalveolar septa are evident in lung sections from patients with BPD (Figures 7C, 7D, 7G, and 7H), with desquamated epithelial lining and casts evident in the larger airways, along with eosinophilic infiltrates and an increased abundance of neutrophilic cells in the intraalveolar septa.

Pronounced immunoreactivity was observed for LoxL1 in the smooth muscle layer of blood vessels in control infants (age-matched for chronological age at death with the BPD group) who died without apparent lung disease (Figures 7A and 7B) and in patients with BPD (Figures 7C and 7D). LoxL1 immunoreactivity was also observed in the alveolar tissue of patients with BPD (Figures 7C and 7D) but not those of control patients (Figures 7A and 7B). A similar pattern of staining for Lox was observed. Interestingly, although LoxL1 appeared to stain the entire vascular smooth muscle layer, Lox expression appeared to be confined to the inner smooth muscle layer (Figure 7B vs. Figures 7F and 7H). Lox staining was evident exclusively in the pulmonary vascular smooth muscle of control patients (Figures 7E and 7F), whereas lung sections from the

group of patients with BPD revealed additional staining in cells within the alveolar lumen and in cells lining the alveolar walls. The source of Lox and LoxL1 in the alveolar lumen and cells lining the alveolar walls has not been clarified. Both Lox and LoxL1, which are secreted enzymes, may be derived from interstitial fibroblasts, or from the lung epithelium itself, because Lox and LoxL1 have now been detected in prostate, mammary, and retinal epithelial cells (34–36).

The antibody specificity was confirmed by preadsorption of anti-Lox and anti-LoxL1 antibodies with a competing peptide (Figures 7I and 7J). No changes in the immunoreactivity of LoxL1, LoxL2, LoxL3, or LoxL4 were seen in lung sections from any patients with BPD compared with control patients (data not shown).

A more quantitative assessment of lysyl oxidase expression was obtained by screening whole-lung RNA from patients with BPD and control patients by quantitative real-time RT-PCR. Consistent with the immunohistochemical data, an increase in lung *lox* mRNA expression levels was seen in patients with BPD versus both the control group with the gestational age at birth and birth weight matched to the BPD group (control group 1), and the control group with the chronological age at death matched to the chronological age at death of the BPD group (control group 2) (Figure 7K). In contrast, lung *lox1* mRNA expression levels were increased in patients with BPD compared with the control group 2, but not when compared with control group 1 (Figure 7K). The mRNA expression levels

Figure 6. Treatment of neonatal mice with transforming growth factor (TGF)-β1,2,3-neutralizing antibodies suppressed the induction of *lox* gene expression by hyperoxia. Neonatal mice were treated either with control IgG or anti-TGF-β1,2,3-neutralizing antibodies before exposure to hyperoxic (85% O₂) or normoxic (21% O₂) conditions for 10 days, and then killed. The expression of lysyl oxidases was assessed in mRNA from mouse lungs by (A) semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR), where expression of the *hspa8* gene was used as a loading control; or (B) real-time quantitative RT-PCR (n = 5); or (C) by immunohistochemistry. Antibody specificity was confirmed by preadsorption of antibodies with a competing peptide, which had been used as the immunogen for antibody generation (*insets*). Additionally, (D) lung sections from mice were screened for assessment of elastin deposition in the developing septa by Hart's elastin stain. *P < 0.01.

for *lox12*, *lox13*, and *lox14* were unchanged between the BPD and both control groups. As 6 out of 10 patients with BPD or at risk for developing BPD received corticosteroids, these two subgroups of patients (patients 17, 22, and 23 vs. patients 18, 19, 24, and 25; Table 2) were assessed for differences in *lox* mRNA expression. This comparison is relevant as corticosteroids have powerful effect on gene expression. No difference in the expression level of any lysyl oxidase mRNA was observed between the corticoid-treated and untreated groups of patients (results not shown).

DISCUSSION

The production and remodeling of the ECM are critical processes required for the secondary septation of the developing lung and the production and maturation of functional pulmonary gas exchange units (37, 38). The pathogenic mechanisms that result in the perturbed deposition and remodeling of the ECM observed in important developmental lung diseases such as BPD (12, 15) are poorly understood. Although several

studies performed to date have examined the production of ECM molecules in BPD, few have addressed the subsequent processing of these molecules, which can facilitate the remodeling of the matrix itself. Therefore, in this study, the expression and activity of lysyl oxidases, a family of enzymes that play a crucial role in regulating the stability of the ECM, were examined in an animal model of BPD, and in prematurely born infants who have died with BPD or at risk for BPD.

The data presented here demonstrate the up-regulation of three elastin/collagen cross-linking enzymes (Lox, LoxL1, and LoxL2) in newborn animals with hyperoxic lung injury and dysregulated ECM development, and up-regulation of at least two elastin/collagen cross-linking enzymes (Lox and LoxL1) in patients with BPD. Interestingly, two of these enzymes, Lox and LoxL1, have already been identified as being temporally regulated during normal mouse lung development (39). Additionally, both *lox*^{-/-} (25, 40) and *lox11*^{-/-} (41) mouse pups exhibit disturbed respiratory tract development, and although *lox*^{-/-} embryos develop to term, they die within hours of birth with evidence of pronounced cardiopulmonary

Figure 7. The expression of Lox and LoxL1 was elevated in the lungs of neonatal patients who died with BPD or were at risk for BPD. The expression of lysyl oxidases was assessed in the lungs of patients with BPD or at risk for BPD, as well as in control lungs. A low-power (200-μm scale) view of representative sections from (A) patients in control group 2 (the histopathology of six patients [Table 1] was examined; in this case, sections from patient 11 are illustrated) and from (C) patients in the BPD group (the histopathology of seven patients [Table 2] was examined; in this case, sections from patient 24 are illustrated), stained for LoxL1. (B, D) High-power views (50-μm scale) are also illustrated for LoxL1 staining in the same sections (the magnified area is demarcated in the low-power view by a black box). Representative medium-power views (100-μm scale) are illustrated for the same two patients, stained for Lox (E, F), with the corresponding high-power views (50-μm scale) illustrated in F and H (the magnified area is demarcated in the low-power view by a black box). (I, J) Antibody specificity was confirmed by preadsorption of antibodies with a competing peptide, which had been used as the immunogen for antibody generation, before staining a section of lung tissue from the same patient with BPD. Staining was consistently more intense in patients with BPD, and the sections illustrated are representative of the trends observed in a total of five patients assessed per group (as indicated in Tables 1 and 2). The expression of lysyl oxidase mRNA was also assessed in mRNA isolated from the lungs of seven patients in control group 1 (red), seven patients with BPD or at risk for BPD (green) and five patients in control group 2 (yellow) by quantitative real-time reverse transcriptase–polymerase chain reaction (K). The bars represent the data range and the boxes represent lower and upper quartiles. The line within the quartile box indicates the median; **P* < 0.01.

failure (25, 40). These data suggest a key role for this family of enzymes in the development of the respiratory tract. Additional studies in these knockout mice have revealed key roles for lysyl oxidases in imparting tensile strength to connective tissue, because they exhibit pathologies consistent with a loss of structural stability of the ECM. For example, soon after parturition, *lox*^{-/-} pups exhibit ruptured arterial aneurysms and ruptured diaphragms (25), whereas adult *lox11*^{-/-} mice develop pelvic organ and rectal prolapse and excessive skin laxity (41). In addition to the induction of lysyl oxidase gene expression by hyperoxia reported in this study, mechanical ventilation of mice with room air or with 40% oxygen gas mixtures for 8 hours was also able to increase *lox*, but not *lox11* mRNA expression (13); however, a similar modulation of lysyl oxidase gene expression has not been observed in premature lambs mechanically ventilated with high (F_IO₂ ≈ 0.26) levels of oxygen (42). Variable responses of elastin gene expression to oxygen levels has also been reported. Although a mild increase in *eln* mRNA levels was observed in response to 85% oxygen exposure of mice in this study, Bruce

and colleagues (43) reported a decrease in elastin gene expression in the lungs of rats exposed to 95% oxygen between P4 and P14. This paradox might be explained by the higher oxygen concentrations used in the rat model, or by the very different sensitivities to high oxygen concentrations displayed by mammals, even between different mouse strains (44).

The transcriptional regulation of lysyl oxidases is poorly described; however, several growth factors are known to drive lysyl oxidase expression (16, 17). Notable among these are members of the TGF-β family of peptide growth factors, which are also key regulators of late lung development (45). Recently, excessive TGF-β activity has been identified as a key pathogenic factor in animal models of BPD (20, 22, 46), which suggested that dysregulated TGF-β signaling might underlie the aberrant expression of lysyl oxidases in BPD. Indeed, dampening of TGF-β signaling in the newborn lung injury model used in this study reduced the expression of the *lox* gene, although expression of other members of the lysyl oxidase family was unaffected. Thus, in the case of oxygen injury to the

lungs of mouse pups, elevated Lox levels are attributable to increased TGF- β signaling in the lungs of affected animals, which most likely drives Lox production by lung fibroblasts. Along these lines, elevated levels of TGF- β 1 have also been detected in bronchoalveolar lavage fluids of ventilated neonates who went on to develop new BPD, versus ventilated neonates who did not (47). This observation might suggest that elevated *lox* mRNA levels seen in infants who died with BPD or were at risk for BPD may be attributable to elevated TGF- β levels in these neonates.

The increased expression of lysyl oxidase family members reported here is consistent with the increased levels of tissue desmosine, an enzymatic product of lysyl oxidase, that are observed in animal models of BPD (this study and Reference 12). Patients who develop BPD exhibit increased urinary desmosine levels (48), which have been attributed to increased elastin degradation due to proteolysis in association with infection and prolonged exposure of infants to hyperoxia (49). However, data presented here suggest that the increased desmosine levels may be due, at least in part, to an overactive cross-linking system in affected lungs, which could promote excessive cross-linking of elastin and collagen fibers, perhaps resulting in the accumulation of elastic fibers in peripheral lung tissue (9, 10). Such mechanisms could give rise to the "thickened, tortuous and disorganized" collagen fibers that are often observed in patients who have died with BPD (11). Such disorganized collagen fibrils have been observed in the skin of patients with amyotrophic lateral sclerosis who have excessive lysyl oxidase activity. In such patients, the thickness of collagen fibers directly correlated with the number of stable collagen cross-links (50), further supporting the notion that elevated lysyl oxidase activity might generate the thickened collagen fibers observed in the lungs of patients with BPD. This idea is also supported by the observation that elastic fibers in the lungs of *lox*^{-/-} pups are thinner than in wild-type age-matched pups, and that collagen fibers in *lox*^{-/-} pups were dispersed and loose (51), contrasting with the thickened, tightly bundled fibers observed in patients with BPD (11). Clearly, other mechanisms of elastin fiber assembly may also be affected in BPD. Mechanical ventilation of mice with 40% oxygen gas mixtures for 8 hours decreased the expression of fibulin-5 and emilin-1 (13), which are critical players in elastin fiber dynamics. In this study, both fibulin-5 and emilin-1 expression appeared to be dysregulated at the mRNA level, with increased mRNA abundance evident in oxygen-injured lungs. Dysregulated expression levels of both molecules would most likely affect elastin fiber assembly, and suggests an avenue independent of lysyl oxidases, which may impact elastin fiber formation and maintenance in oxygen-injured lungs.

Changes in lysyl oxidase activity and expression have not been causally implicated in the development of BPD in this study. The early perinatal death of the *lox*^{-/-} knockout animals prevents their use in studies of postnatal lung injury. However, with the data presented in mind, the authors would like to propose a novel hypothesis of how disordered elastin and collagen cross-linking and deposition might inhibit alveolarization in the injured developing lung. It is generally believed that as the lung develops, a continuous cycle of ECM deposition and remodeling occurs: matrix scaffolding must be erected to support the developing structures of the lung and in some locations the matrix must be removed and/or otherwise remodeled to permit extension or reshaping of existing structures (15). The authors propose that excessive elastin and collagen cross-linking by overabundant lysyl oxidases might overstabilize elastin and collagen and make them chemically resistant to the normal proteolytic and other remodeling events that are required for successful ECM remodeling and alveolar and microvascular development.

This hypothesis is supported by studies in which it has been demonstrated that the degree of elastin and collagen cross-linking determines the susceptibility of ECM fibers to proteolytic degradation, and hence, remodeling. Disruption of elastin cross-linking through dietary copper restriction (which down-regulates the activity of Cu²⁺-dependent lysyl oxidases) reduced the accumulation of elastin in the aorta of experimental animals (52), which was directly attributed to increased proteolytic degradation of elastin molecules in copper-restricted animals, because elastin had become more susceptible to proteolysis as a result of reduced cross-linking (52). Indeed, the inability to cross-link tropoelastin renders this molecule sensitive to proteolysis by trypsin-like proteases (53). Conversely, hyper-crosslinking of collagen fibers in tissue-engineered arteries through stimulation of lysyl oxidase activity increased the stability of collagen fibers to proteolysis (54). Thus, elevated lysyl oxidase activity observed in animal models of BPD—and in patients with BPD—may generate excessively cross-linked elastin and collagen fibers that are resistant to proteolysis and degradation-dependent remodeling.

Clearly, the impact of an overstabilized matrix would not be limited to the division of the alveolar septa, but could also extend to pulmonary vascular development. In addition to decreased alveolar septation, patients with BPD exhibit pronounced disruption of pulmonary vascular development (5–8). An inability to remodel the vascular matrix, as might occur in an overstabilized vascular matrix, would also have implications for vascular development. Remodeling of the ECM by proteases promotes cell migration, which is critical for vascular development, and matrix-bound growth factors that are released during vessel matrix remodeling, promoting proper angiogenesis by regulating endothelial migration and growth (reviewed in Reference 55). These processes would be impeded by an overstabilized ECM. Furthermore, Lox has already been implicated in several vascular pathologies (56) in which, when overexpressed, Lox can promote local neointimal thickening and vessel muscularization. These observations are interesting, because in addition to dysregulated angiogenesis, the pulmonary vasculature in patients with BPD undergoes remodeling that includes medial hypertrophy (thus, vessel thickening) and muscularization of small arteries.

Thus, it is proposed here that as a consequence of up-regulated expression of ECM cross-linking enzymes such as lysyl oxidases, the ECM is less plastic and resists remodeling that facilitates lung growth. This may, at least in part, underlie the impaired septation and vascular development seen in patients with BPD and animal models of BPD.

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