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Lysyl Oxidase Is Essential for Normal Development and Function of the Respiratory System and for the Integrity of Elastic and Collagen Fibers in Various Tissues

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Lysyl oxidases, a family comprising LOX and four LOX-like enzymes, catalyze crosslinking of elastin and collagens. Mouse Lox was recently shown to be crucial for development of the cardiovascular system because null mice died perinatally of aortic aneurysms and cardiovascular dysfunction. We show here that Lox is also essential for development of the respiratory system and the integrity of elastic and collagen fibers in the lungs and skin. The lungs of E18.5 *Lox*^{-/-} embryos showed impaired development of the distal and proximal airways. Elastic fibers in E18.5 *Lox*^{-/-} lungs were markedly less intensely stained and more disperse than in the wild type, especially in the mesenchyme surrounding the distal airways, bronchioles, bronchi, and trachea, and were fragmented in pulmonary arterial walls. The organization of individual collagen fibers into tight bundles was likewise abnormal. Similar elastic and collagen fiber abnormalities were seen in the skin. Lysyl oxidase activity in cultured *Lox*^{-/-} skin fibroblasts and aortic smooth muscle cells was reduced by ~80%, indicating that Lox is the main isoenzyme in these cells. LOX abnormalities may thus be critical for the pathogenesis of several common diseases, including pulmonary, skin, and cardiovascular disorders. (*Am J Pathol* 2005, 167:927–936)

Lysyl oxidases are extracellular copper-dependent enzymes that catalyze the formation of lysine and hydroxylysine-derived cross-links in collagens and lysine-derived

cross-links in elastin. These cross-links are essential for the tensile strength of collagens and the rubber-like properties of elastin, both abundant extracellular matrix proteins that are necessary for the structural integrity and function of connective tissues.^{1–3} In addition to the first characterized lysyl oxidase, LOX,^{4,5} four LOX-like proteins are currently known: LOXL1, LOXL2, LOXL3, and LOXL4.^{6–14} All four are likely to catalyze cross-link formation in collagens and elastin, as has been shown so far for LOXL1 and LOXL4,^{15–17} but the specific functions of these isoenzymes are as yet unknown.

Expression of LOX is markedly increased in fibrotic tissues, including models of dermal, lung, liver, and arterial fibrosis.¹⁸ Although no human disease caused by a primary LOX deficiency has been identified so far, at least three vertebrate conditions are associated with reduced lysyl oxidase activity. Administration of β -amino-propionitrile (β -APN), a lysyl oxidase inhibitor, leads to abnormal cross-linking of collagens and elastin and results in lathyrism, a disease characterized by kyphoscoliosis, bone deformities, weakening of the epiphyseal plates, tendons, ligament attachments, skin and cartilage, dislocation of joints, loss of teeth, hernias, and vascular rupture.¹⁹ A deficiency in copper, a lysyl oxidase cofactor, leads to manifestations closely resembling those of lathyrism.¹⁹ Lysyl oxidase activity is also reduced in two X-linked recessively inherited disorders, Menkes disease and its milder form, occipital horn syndrome, both caused by mutations in a copper transporter gene and characterized by neurological and connective tissue symptoms.²⁰ LOX has also been reported to have novel biological roles as a tumor suppressor, affecting

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cell adhesion and growth control and having intracellular and intranuclear functions.^{2,18,21}

Inactivation of the mouse *Lox* gene has recently been shown to lead to perinatal death caused by aortic aneurysms, cardiovascular dysfunction, and diaphragmatic rupture.^{22,23} The wall of the aorta in *Lox*-null (*Lox*^{-/-}) embryos was significantly thicker and the aortic lumen markedly smaller than in wild-type (WT) embryos, and the elastic fibers in the smooth muscle cell layers of their aortic walls were highly fragmented and discontinuous.^{22,23} These results suggested that *Lox* has an essential role in the development and function of the cardiovascular system. Elastin cross-links and immature collagen cross-links were decreased in amount in the aorta and lungs of the *Lox*^{-/-} embryos by ~60% and 40%, respectively.²³ Some of the *Loxl* proteins thus appear to participate in the cross-linking of collagens and elastin, but they cannot fully compensate for the absence of *Lox* activity. Inactivation of the mouse *Loxl1* gene is not lethal, but *Loxl1*^{-/-} mice show disturbed regeneration of elastic fibers in the postpartum intrauterine tract and also develop pelvic prolapse, enlarged air spaces in the lung, laxity of the skin, and vascular abnormalities that coincide with accumulation of the elastin precursor tropoelastin.²⁴ In contrast to *Lox*, *Loxl1* was localized to sites of elastogenesis and interacted with fibulin-5, which is known to bind tropoelastin.²⁴

The phenotypes of the *Lox*^{-/-} and *Loxl1*^{-/-} knockout mice suggest that members of the *Lox* family are likely to show functional differences *in vivo*. In the current study we examined the *Lox*^{-/-} mouse line further. The distal and proximal airways of the lungs in embryos were found to be enlarged and their numbers were found to be decreased, leading to a condition resembling adult emphysema. Histochemistry and electron microscopy of the lungs demonstrated generalized elastinopathy and structurally abnormal collagen bundles, which were also seen in the skin. The amount of lysyl oxidase activity was reduced by ~80% in cultured *Lox*^{-/-} skin fibroblasts and aortic smooth muscle cells, the *Loxl* proteins evidently being responsible for the remaining 20% of the activity. This is the first demonstration at the level of lysyl oxidase activity that the *Loxl* proteins also contribute to the total activity *in vivo*, although *Lox* is responsible for the majority of it, at least in the cell types studied. Thus, in addition to proper development of the cardiovascular system,²² *Lox* activity is essential for normal development of the respiratory system and the skin.

Materials and Methods

Histology

Tissue samples and whole embryos were fixed overnight in 10% buffered formalin or in Bouin's solution or Carnoy's solution for 2 to 12 hours, depending on the fixation method used, and embedded in paraffin. The lung samples used in the morphometric analysis were fixed under a 50-mbar vacuum. Sections were stained with hematox-

ylin and eosin (H&E), Masson's trichrome, and Hart's modified elastin stain. The WT and *Lox*^{-/-} samples were processed and sectioned in parallel and analyzed without knowledge of the genotype.

Immunohistochemistry

Paraffin sections (5 μ m) from the WT and *Lox*^{-/-} animals were used for immunohistochemical analyses. Antibodies against tropoelastin domains 7 to 26 (Elastin Products Co. Inc., Owensville, MO), type I collagen (Rockland, Gilbertsville, PA), type III collagen (Rockland), type IV collagen (Chemicon Int. Inc., Temecula, CA), and smooth muscle α -actin (α -SMA) (Sigma, St. Louis, MO) were used at a dilution of 1:50 to 1:200 in phosphate-buffered saline (PBS). Samples stained with the collagen antibodies were pretreated with 0.4% pepsin in 0.01 mol/L HCl, pH 2.0, at 37°C for 5 minutes. Before application of the primary antibodies, the sections were treated with 0.3% H₂O₂ in methanol to suppress endogenous peroxidase activity. The primary antibody binding was visualized using the Histomouse SP (AEC) kit (Zymed Laboratories, Inc., San Francisco, CA) according to the manufacturer's instructions. Sections were counterstained with hematoxylin.

Morphometric Analysis of the Lungs

Digital images with appropriate magnification were captured from H&E-stained paraffin sections of the left lobe of the lung at E15.5 and E18.5, E15.5 being the earliest time point analyzed morphometrically. Immunostaining with α -SMA antibody was used to identify the pulmonary arteries. Eight to ten randomly selected paraffin sections from different parts of the left lobe of the lung of at least six embryos per genotype were analyzed. The numbers of distal airways, bronchioles, and arteries per mm² were counted, and also the numbers of epithelial cells in the bronchioles, and the thicknesses of the bronchial epithelium and pulmonary arterial walls were measured. In the latter case, the thicknesses of arteries within the typical 70- to 130- μ m circumference range at E18.5 were measured using 8 to 10 randomly selected sections from three individual embryos per genotype. Statistical analyses were performed using the *F*- and *t*-tests.

Electron Microscopy

Lung and skin biopsies from E18.5 embryos were fixed in a mixture of 1% glutaraldehyde and 4% formaldehyde in 0.1 mol/L phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Epon EMBed 812. For elastin contrasting, specimens treated with osmium tetroxide were immersed in 2% aqueous tannic acid for 1 hour and further processed as described above. Thin sections were cut with a Reichert Ultracut ultramicrotome and examined in a Philips CM100 transmission electron microscope (FEI Co., Eindhoven, The Netherlands). Images were captured with a charge-coupled device camera equipped with TCL-EM-Menu

version 3 from Tietz Video and Image Processing Systems GmbH (Gaunting, Germany).

Samples for immunoelectron microscopy were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer with 2.5% sucrose for 2 hours at room temperature, after immersion in 2.3 mol/L sucrose, and frozen in liquid nitrogen. Thin cryosections were cut with a Leica Ultracut UCT microtome. Sections for immunolabeling were first incubated in 0.05 mol/L glycine in PBS, followed by incubation in 5% bovine serum albumin (BSA) with 0.1% cold water fish skin gelatin (Aurion, Wageningen, The Netherlands) in PBS. The sections were then incubated with a polyclonal antibody against tropoelastin (Elastin Products Co. Inc.) for 60 minutes, followed by a protein-A gold complex. The antibodies and the gold conjugate were diluted in 0.1% BSA-C (Aurion) in PBS and all of the washing steps were performed with 0.1% BSA-C in PBS. The controls were prepared by performing the labeling procedure without the primary antibody. The sections were embedded in methylcellulose and examined as described above.

Isolation of Aortic Smooth Muscle Cells and Skin Fibroblasts from E18.5 Mouse Embryos

The aortas were aseptically removed from E18.5 WT, *Lox^{+/-}* and *Lox^{-/-}* embryos and placed in an AmnioMAX C-100 complete medium (Invitrogen Corp., Grand Island, NY). Fat tissue surrounding the aorta and the adventitia were grossly stripped away. The aorta was positioned longitudinally and scraped to remove the intima and the cleaned aorta was cut into pieces, which were placed into individual wells of a plastic six-well culture dish containing AmnioMAX C-100 medium supplemented with penicillin/streptomycin. The cultures of smooth muscle cells that formed around the tissue were trypsinized (0.05% trypsin, 0.53 mmol/L ethylenediamine tetraacetic acid), passaged, expanded, and genotyped. The cells were stained with α -SMA (Sigma) and analyzed for the classic hill-and-valley morphology to confirm their smooth muscle cell status.

Skin fibroblasts were isolated from E18.5 embryos by placing 1- to 2-mm skin biopsies into a cell culture plate with Dulbecco's modified Eagle's medium (Invitrogen Corp.) containing 20% fetal bovine serum (v/v), 1% non-essential amino acids, and 100 U/ml penicillin and streptomycin. Cultures of the fibroblasts that formed around the tissue were trypsinized (0.05% trypsin, 0.53 mmol/L ethylenediamine tetraacetic acid), passaged, expanded, and genotyped.

Lysyl Oxidase Activity Assay

Lysyl oxidase activity was measured in the conditioned medium of the aortic smooth muscle cell and skin fibroblast cultures using a tritiated recombinant human tropoelastin as a substrate.²⁵ The cell culture medium was changed to a serum-free Dulbecco's modified Eagle's medium (Life Technologies Inc., Grand Island, NY) con-

taining 0.5% bovine serum albumin, 1% nonessential amino acids, and 100 U/ml penicillin and streptomycin at 70% confluency, and the cells were incubated for 16 hours. The 0.8-ml reaction mixtures contained 600 μ l of the conditioned media, 0.1 mol/L borate, 0.15 mol/L NaCl, pH 8.0, and 300,000 cpm of tritiated tropoelastin in the presence or absence of 50 μ g/ml β -APN (Sigma). The reactions were incubated for 3 hours at 37°C, followed by distillation under a vacuum. The radioactivity in 0.5-ml aliquots of the distillates was determined by liquid scintillation spectrometry. Protein concentrations in the cell layers were analyzed by the method of Bradford²⁶ to normalize the enzyme activities between samples.

Immunofluorescence Staining of the Cultured Smooth Muscle Cells and Skin Fibroblasts

Aortic smooth muscle cells and skin fibroblasts isolated from WT and *Lox^{-/-}* E18.5 embryos were seeded onto glass coverslips and grown for 9 to 12 days after confluency to ensure extracellular matrix production, fixed for 8 minutes in 2% paraformaldehyde at room temperature, and incubated in 2% BSA/PBS (pH 7.2) for 30 minutes. The samples were incubated with a polyclonal anti-tropoelastin antibody (Elastin Products Company Inc.) at a 1:100 dilution for 1 hour, washed with PBS, and incubated with a tetramethylrhodamine B isothiocyanate-conjugated goat anti-rabbit secondary antibody (DAKO A/S, Glostrup, Denmark) diluted according to the manufacturer's instructions for 1 hour at room temperature. After extensive washing with PBS, the coverslips were mounted onto microscope slides with Immu-Mount (Thermo Shandon, Pittsburgh, PA) and analyzed by fluorescence microscopy.

Results

**Lox^{-/-}* Lungs Show Impaired Development of Distal and Proximal Airways*

H&E staining of the E18.5 *Lox^{-/-}* embryonic lungs showed enlarged distal and proximal airways (Figure 1, a and b). The *Lox^{-/-}* distal airways (ie, acinar tubule derivatives, including alveolar ducts, sacs, and primitive alveolar structures) and bronchioles were dilated and the walls of the distal airways were thickened, but no destruction of the walls was observed. These abnormalities were even more severe at P0, the distal airways of *Lox^{-/-}* lungs being extremely dilated with further thickening of their walls, and numerous atelectatic areas being observed (Figure 1, c and d). The number of proximal airways, ie, developing bronchioles, in the E18.5 *Lox^{-/-}* lungs (122 ± 25 , $n = 8$) was ~22% lower ($P < 0.05$) than in the WT siblings (156 ± 44 , $n = 12$), indicating defective branching of the airways. No differences in the gross morphology of the lungs were apparent between the null and WT embryos until E17.5, when a marked decrease in the staining of elastic fibers was observed in the *Lox^{-/-}* lungs (see below). The weight of the E18.5 *Lox^{-/-}* lungs,

Figure 1. Enlarged distal and proximal airways in E18.5 and P0 *Lox*^{-/-} lungs. **a** and **b**: Masson's trichrome staining of the left lobe of E18.5 WT (**a**) and *Lox*^{-/-} (**b**) lungs. The distal airways in the *Lox*^{-/-} lungs are dilated and have thicker walls than in the WT, and the bronchioles are enlarged (**arrowheads**). **c** and **d**: H&E staining of the left lobe of P0 WT (**c**) and *Lox*^{-/-} (**d**) lungs. The distal airways in the *Lox*^{-/-} lungs are dramatically dilated with further thickening of their walls, and various sites of atelectasis are observed (**arrowheads**). Scale bars: 100 μ m (**a**, **b**); 1 mm (**c**, **d**).

ie, 1 day before birth, was reduced by $\sim 17\%$ ($P < 0.01$) relative to the WT, with concomitant decreases of 23% ($P < 0.05$) and 21% ($P < 0.001$), respectively, in the numbers of bronchioles and distal airways (Table 1). The epithelial cells in the *Lox*^{-/-} bronchioles were disorganized (data not shown) and reduced in number by 8% ($P < 0.01$), the thickness of the epithelium also being significantly reduced ($P < 0.001$) (Table 1). The number of pulmonary arteries was reduced by $\sim 11\%$, but this was not statistically significant (Table 1).

Elastic and Collagen Fibers in *Lox*^{-/-} Lungs are Abnormal

Histochemical elastin staining showed less prominent staining of the elastic fibers in the E18.5 *Lox*^{-/-} lung sections than in the WT (Figure 2, a and b), and staining was virtually absent in the *Lox*^{-/-} lung mesenchyme surrounding the distal airways and less prominent in the bronchioles (Figure 2, a and b),

bronchi, and trachea (data not shown). Individual elastic fibers in the *Lox*^{-/-} lung sections appeared thinner than in the WT, and were irregular with a hazy appearance. The intensity of elastin staining in the *Lox*^{-/-} pulmonary arterial walls was not as markedly reduced relative to the WT as in the lung mesenchyme, but the elastic fibers seemed to be partially destroyed (Figure 2, a and b). The staining of elastic fibers was decreased to an even greater extent in the lungs of *Lox*^{-/-} neonates than in the E18.5 *Lox*^{-/-} lungs (data not shown). Immunohistochemical analysis with a tropoelastin antibody likewise showed reduced staining of elastic fibers in the E18.5 *Lox*^{-/-} lungs (Figure 2; c to f). Furthermore, the elastic lamellae around the *Lox*^{-/-} pulmonary arteries were highly fragmented (Figure 2, c and d). No such fragmentation was observed in the *Lox*^{-/-} bronchiolar lamina propria, but the staining intensity of elastin was slightly decreased relative to the WT (Figure 2, e and f). Electron microscopy of the E18.5 *Lox*^{-/-} lungs showed a dramatic decrease in the staining of individual elastic fibers, with very little if any amorphous material, the individual fibers being highly dispersed (data not shown). Staining of elastic fibers was essentially absent from the *Lox*^{-/-} walls of the distal airways, but was prominent in the WT walls (Figure 2, g and h).

Immunohistochemical analysis with type I and IV collagen antibodies showed prominent staining in both the E18.5 *Lox*^{-/-} and WT lungs (Figure 3; a to d). Likewise, no obvious differences were observed in the immunostaining of type III collagen (data not shown). However, electron microscopy analysis showed clear differences in the organization of collagen fibers between the *Lox*^{-/-} and the WT E18.5 lungs (Figure 3, e and f). The individual collagen fibers in the *Lox*^{-/-} lungs were dispersed, short, and did not form tight bundles as observed in the WT lungs (Figure 3, e and f).

Diaphragmatic Hernias Are Not a Primary Cause of the Pulmonary Changes

As described previously, diaphragmatic hernias are occasionally found in E18.5 *Lox*^{-/-} embryos and more

Table 1. Morphometric Analysis of WT and *Lox*^{-/-} Lungs at E18.5

	WT	-/-
Body weight (g)	1.246 $\pm$ 0.157 (n = 14)	1.161 $\pm$ 0.149 ^{NS} (n = 18)
Lung weight/body weight (g)	0.0391 $\pm$ 0.006 (n = 14)	0.0324 $\pm$ 0.006 [†] (n = 18)
Number of bronchioles/mm ²	8.66 $\pm$ 1.97 (n = 6)	6.67 $\pm$ 0.77* (n = 6)
Number of distal airways/mm ²	229.37 $\pm$ 8.00 (n = 6)	180.31 $\pm$ 27.10 [†] (n = 6)
Number of arteries/mm ²	15.93 $\pm$ 0.96 (n = 6)	14.25 $\pm$ 2.48 ^{NS} (n = 6)
Thickness of the bronchial epithelium (μ m)	13.08 $\pm$ 1.15 (n = 33 [§])	11.75 $\pm$ 0.75 [†] (n = 25 [§])
Number of epithelial cells in bronchioles (cells/mm)	166.42 $\pm$ 17.94 (n = 32 [§])	153.52 $\pm$ 16.26 [†] (n = 25 [§])
Thickness of the pulmonary arterial walls versus diameter (%)	29.85 $\pm$ 4.93 (n = 37 [§])	31.44 $\pm$ 4.94 ^{NS} (n = 49 [§])

* $P < 0.05$; [†] $P < 0.01$; [‡] $P < 0.001$.

[§]n refers to the number of bronchioles or arterioles calculated from randomly selected sections of three embryos. Otherwise n refers to the number of embryos included in the analysis.

Figure 2. Analysis of elastic fibers in E18.5 lungs. **a** and **b**: Hart's elastin staining of a WT E18.5 lung (**a**) showed a strong staining of elastic fibers throughout the lung mesenchyme, especially in the areas surrounding the distal airways (Da) and in the lamina propria of the bronchioles (Br) (arrowheads), whereas the staining was diminished or even lacking in the $Lox^{-/-}$ lungs (**b**). The elastic fibers in the walls of the $Lox^{-/-}$ pulmonary arteries (Ar) were fragmented (**b**, arrows), but the reduction in staining was not as obvious as in other parts of the lung. **c** and **d**: Immunostaining of elastin showed continuous, uninterrupted elastic fibers in the WT pulmonary arteries (**c**, Ar), whereas they were irregular and highly fragmented (arrowheads) in the arterial walls of the $Lox^{-/-}$ embryos (**d**). **e** and **f**: Elastic fibers are continuous in the lamina propria of the bronchioles in WT lungs (**e**, Br), but those in the $Lox^{-/-}$ lamina propria (**f**, arrowheads) were slightly disrupted and staining was less marked. No obvious differences were seen in the staining of the $Lox^{-/-}$ and WT pulmonary arteries and bronchioles with the α -SMA antibody (insets in **c**–**f**). **g** and **h**: Electron micrographs of the wall of the distal airway stained with tannic acid show prominent staining of elastic fibers in the WT embryos (**g**, arrowheads), whereas the staining is essentially missing in the $Lox^{-/-}$ embryos (**h**). Scale bars: 100 μ m (**a**–**f**); 2 μ m (**g**, **h**).

frequently at P0.^{22,23} The diaphragmatic rupture occurs in the E18.5 $Lox^{-/-}$ embryos at the site of the collagen-rich diaphragmatic central tendon, allows abdominal contents to enter the thoracic cavity and therefore disturbs the respiratory movements (Figure 4). Electron microscopy (data not shown) showed clear differences in the organization of collagen fibers in the diaphragmatic muscle of the E18.5 $Lox^{-/-}$ embryos, similar to those shown in the lung and skin, and the surfaces of individual fibers contained undefined loosely associated material, which was also seen in other tissues studied (see below).

Figure 3. Analysis of collagen fibers in E18.5 lungs. **a**–**d**: Immunostaining of type I (**a** and **b**) and IV collagen (**c** and **d**) showed no obvious difference in its distribution and abundance between the genotypes. **e** and **f**: Electron micrographs of the E18.5 lung mesenchyme showed that the individual collagen fibers are organized into bundles in the WT (**e**), but the fibers are dispersed and do not form tight bundles in the $Lox^{-/-}$ embryos (**f**). Scale bars: 100 μ m (**a**–**d**); 1 μ m (**e**, **f**).

Because diaphragmatic herniation has been shown to have a negative effect on the development of the lungs and branching of the alveobronchial tree in early embry-

Figure 4. Analysis of the diaphragmatic rupture that occasionally occurs in E18.5 $Lox^{-/-}$ embryos. Masson's trichrome staining shows that the rupture occurs at the collagen-rich diaphragmatic central tendon region (arrowheads in insets), thus allowing some of the abdominal contents to enter the thoracic cavity (arrows). L, liver; Lu, lung; H, heart; I, intestine.

Figure 5. Analysis of elastic fibers in E18.5 skin. **a** and **b**: Hart's staining showed abundant long, clearly defined elastic fibers (arrowheads) in the WT skin (**a**), whereas the fibers were rarer, shorter, and had an abnormal appearance in the *Lox*^{-/-} skin (**b**). **c** and **d**: Immunoelectron microscopy with a tropoelastin antibody showed abundant labeling of elastic fibers in the WT skin (**c**), whereas they were difficult to detect in the *Lox*^{-/-} skin, were sparsely labeled, and had very little if any amorphous material within the microfibrils (**d**). Scale bars: 50 μ m (**a**, **b**); 200 nm (**c**, **d**).

os,²⁷⁻²⁹ the occurrence of diaphragmatic hernias and ruptures was analyzed carefully in E13.5 to E17.5 *Lox*^{-/-} embryos. Diaphragmatic hernias were not observed in the *Lox*^{-/-} embryos before E18.5. Because abnormalities in the lungs of these embryos are observed earlier, and because they occur regardless of diaphragmatic herniation, the diaphragmatic hernias cannot be the primary cause of the observed pulmonary changes.

Elastic and Collagen Fibers Are Also Abnormal in the *Lox*^{-/-} Skin

To study whether the abnormalities seen in the elastic and collagen fibers in the lungs and previously in the elastic fibers of the aortic walls^{22,23} are also present in other tissues, elastic fibers in sections of E18.5 *Lox*^{-/-} embryonic skin were analyzed by histochemical staining. The staining was clearly reduced in the *Lox*^{-/-} dermis and the individual fibers had an abnormal appearance and seemed to be shorter than in the WT (Figure 5, a and b). Reduced staining of elastic fibers in the *Lox*^{-/-} skin was also seen in immunoelectron microscopy with a tropoelastin antibody, suggesting sparse occurrence in the null skin as compared with the WT (Figure 5, c and d). The majority of the elastic fibers in the *Lox*^{-/-} skin were also considerably smaller in size (data not shown), and very little, if any, amorphous elastin was seen within the microfibrils (Figure 5, c and d).

Immunohistochemical analysis with collagen-specific antibodies showed that the staining intensities and distributions of collagen types I (Figure 6, a and b), III (data not shown), and IV (Figure 6, c and d) were approximately equal in the E18.5 *Lox*^{-/-} and WT skin sections, but electron microscopy showed that the collagen fibers in the *Lox*^{-/-} skin were not arranged in tight bundles but

Figure 6. Analysis of type I and IV collagen in E18.5 skin. **a-d**: Immunostaining of type I (**a** and **b**) and IV collagen (**c** and **d**) showed no obvious differences between the WT and *Lox*^{-/-} skin samples. **e** and **f**: Electron micrographs of the WT skin showed clearly defined, large bundles consisting of numerous individual collagen fibers (**e**), whereas the collagen bundles in the *Lox*^{-/-} skin were less well organized and contained fewer individual fibers (**f**). The collagen fibers generally had uncovered, clean surfaces in the WT skin (**g**), whereas the surfaces were surrounded by a precipitate-like material of undefined character in the *Lox*^{-/-} skin (**h**). Scale bars: 100 μ m (**a-d**); 1 μ m (**e**, **f**); 200 nm (**g**, **h**).

were loose and dispersed, and the number of individual collagen fibers also seemed to be reduced (Figure 6, e and f). Thin collagen filaments were occasionally seen within the dispersed collagen bundles in the *Lox*^{-/-} skin (data not shown). In addition, loosely associated material of undefined character was present on the surface of individual collagen fibers, such material being rare in the WT fibers (Figure 6, g and h).

Lysyl Oxidase Activity Is Markedly Reduced in *Lox*^{-/-} Skin Fibroblasts and Aortic Smooth Muscle Cells

Lysyl oxidase activity was assayed in the conditioned medium of cultured WT, *Lox*^{+/-}, and *Lox*^{-/-} skin fibro-

Figure 7. Lysyl oxidase activity and synthesis of elastic fibers in cultured fibroblasts and aortic smooth muscle cells. **a** and **b**: Lysyl oxidase activity was measured in the conditioned medium of WT, *Lox*^{+/-}, and *Lox*^{-/-} fibroblasts (**a**) and aortic smooth muscle cells (**b**) at 70% confluency. Activities are presented as means ± SD for six independent experiments, each assayed in quadruplicate. **c-f**: Immunofluorescence staining of aortic smooth muscle cells (**c**, **d**) and skin fibroblasts (**e**, **f**) at 9 days after confluency with a tropoelastin antibody showed no obvious differences between genotypes. Scale bars, 100 μm (**c-f**).

blasts and aortic smooth muscle cells at 70% confluency by a method based on the measurement of tritiated water released from [L-4,5-³H]lysine-labeled recombinant human tropoelastin used as a substrate.²⁵ The lysyl oxidase activity level in *Lox*^{-/-} skin fibroblasts was 19.8% of that in the WT fibroblasts, whereas the *Lox*^{+/-} fibroblasts retained 89.4% of the activity (Figure 7a). The activity level in the *Lox*^{-/-} aortic smooth muscle cells was 18.6% of that in the WT cells, the level in the *Lox*^{+/-} cells being 51.9% (Figure 7b). Synthesis of tropoelastin by the WT and *Lox*^{-/-} fibroblasts and aortic smooth muscle cells was studied by immunostaining at days 9 to 12 after confluence to ensure the accumulation of extracellular matrix components. A dense elastic fiber network was stained by the tropoelastin antibody around the cultured cells, no obvious differences being observed between the WT and null cells (Figure 7; c to f).

Discussion

It has been shown previously that Lox activity is essential for the proper functioning of the embryonic cardiovascular system and for the structural durability of the diaphragm, the abnormalities found in *Lox*^{-/-} mice, which die at the end of gestation or as neonates due to aortic aneurysms and cardiovascular dysfunction, most probably being due to highly decreased cross-linking of elastin and collagens.^{22,23} Our results show that, in addition to

the cardiovascular system, Lox activity is also essential for the integrity of elastic and collagen fibers in other mouse tissues, and for the normal development and functioning of the respiratory system.

Histological analysis of E18.5 *Lox*^{-/-} lungs showed a markedly less intense staining of elastic fibers than in the WT, especially in the mesenchyme in areas surrounding the distal airways, bronchioles, bronchi, and trachea, and a fragmentation of the fibers in pulmonary arterial walls. Histological and immunohistological analyses revealed no obvious differences in the staining intensities of collagen types I, III, and IV between the *Lox*^{-/-} and WT lungs, but ultrastructural analysis demonstrated that the arrangement of individual collagen fibers into well-ordered, tight bundles was highly unsuccessful in *Lox*^{-/-} lungs, these fibers being mostly disperse and loose. Furthermore, the surfaces of individual collagen fibers were frequently covered by additional loose material of an undefined character. The surfaces of fibril-forming collagens are known to be decorated by various proteoglycans, glycoproteins, and other types of collagen molecules classified as fibril-associated collagens,^{1,3,30} and such components may well be involved in the additional material observed. On the other hand, it is also possible that the unidentified material reflects reduced strength of the collagen fibers in *Lox*^{-/-} tissues and is due to their increased susceptibility to proteolytic digestion. Similar abnormalities in the elastic and collagen fibers were also seen in the skin of the E18.5 *Lox*^{-/-} embryos.

The overall phenotype of the *Lox*^{-/-} embryonic lungs resembles that found in human patients suffering from emphysema with dilated distal airways.^{19,31-34} However, the emphysema-like condition in these embryos is not due to destruction of the alveolar walls but rather the impaired branching of the airways during development caused by alterations in extracellular matrix. Type I collagen and elastin are the major components of the lung, comprising 50 to 60% and 18% of its extracellular matrix, respectively,^{35,36} and alterations in both elastic and collagen fibers are hallmarks of the pathogenesis of pulmonary emphysema.^{19,31-34} Elastin-null mice suffer from an emphysema-like condition associated with impaired distal airway development at birth, but at E18.5 their lungs are indistinguishable from those of the WT.³⁷ In mice lacking platelet-derived growth factor (PDGF-A), myofibroblasts do not form at the tips of the alveolar septae, and consequently elastin is not produced.³⁸ Therefore, *Pdgfr-a*^{-/-} mice essentially lack elastin in the lung parenchyma, but lung development appears to be normal from gestation to P4, after which the failure of alveogenesis becomes evident, and by P10 the lungs are grossly abnormal.³⁹ Reduced branching of the bronchiolar tree in *Lox*^{-/-} embryos is observed at E15.5, which was the earliest time point analyzed morphometrically, and by birth the lungs are hypoplastic and structurally abnormal. This more severe lung phenotype than in elastin- or PDGF-A-null mice is most probably due to the additional defects found in the arrangement and integrity of collagen fibers. The importance of collagen types I and III during lung maturation has been shown by several groups.⁴⁰⁻⁴⁴ At the pseudoglandular stage of lung mat-

uration in the rat (E9.5 to E14.2 in the mouse embryo), collagen fibers are concentrated at the sites of bifurcations of primitive bronchial primordia, giving rise to further branching of the primitive bronchi and bronchioles at later stages of development. This localization of interstitial collagens suggests a mechanical as well as regulatory role for them in the formation of new bronchial trees^{44–46} and their role may be disturbed in developing *Lox*^{−/−} lungs. In mature lungs, collagen types I and III are found in the walls of pulmonary arteries, the interstitium of the bronchial tree, the interlobular septa, the bronchial lamina propria, and the alveolar interstitium, where the pathological changes involved in emphysema are known to occur.⁴⁷ Collagen fibers confer tensile strength to the lungs, which is necessary for the maintenance of alveolar interdependence and thus helps to preserve the stability of the small pulmonary airways.^{47,48} Furthermore, a transgenic mouse line expressing the collagen-degrading human MMP-1 in the lungs, develops emphysema with no effect on lung elastin.⁴⁹

Respiratory movements mediated by diaphragmatic muscles have been shown to be crucial for proper development of the lungs during embryogenesis.^{28,29} In animal models with induced congenital diaphragmatic hernias, as also in human patients suffering from this condition, the number of bronchial divisions is reduced leading to a smaller number of alveoli and to acinar hypoplasia.^{27,50} Diaphragmatic herniation in *Lox*^{−/−} embryos most probably occurs due to the altered tensile strength of the irregularly organized collagen bundles at the site of the diaphragmatic central tendon. Diaphragmatic hernias were occasionally found in the *Lox*^{−/−} embryos, but because they did not occur before E18.5 and because the first signs of defective branching of the bronchial tree were already seen at E15.5, they cannot be a primary cause of the abnormal development of the lungs. The possibility of dysfunction of the diaphragmatic muscle cannot be excluded, however, and this may have an additional role in the developmental defects found in the lungs.

The amounts of elastin cross-links are decreased in the aorta and lung of E18.5 *Lox*^{−/−} embryos by ~60%, and those of collagen cross-links in the whole body and lung by 40%.²³ The cross-links are formed spontaneously after the lysine- or hydroxylysine-derived aldehydes have been generated by the action of Lox or the other lysyl oxidase isoenzymes, and thus their amount is not a direct measure of lysyl oxidase activity. We therefore isolated and cultured aortic smooth muscle cells and skin fibroblasts to measure the level of lysyl oxidase activity, using tritiated tropoelastin as a substrate. Our results show that Lox is likely to be the main lysyl oxidase isoenzyme in these cells because the lack of its activity led to a reduction of ~80% in the overall amount of lysyl oxidase activity in both cases. However, the effect of confluency of the cultured cells on the enzyme activity cannot be excluded, because different forms of lysyl oxidase may have differential expression patterns and activities at different cell densities and may be differentially regulated *in vivo*. In our experiment the other four Loxl proteins are most likely responsible for the remaining 20% of the activity, but it is

currently unknown which of them contribute to the residual activity in these particular cell types and what their substrate specificities are. It has been shown recently that Loxl1 has a critical role in the renewal of elastic fibers because *Loxl1*^{−/−} mice are viable and fertile but do not deposit normal elastic fibers in the uterine tract postpartum and also develop a pelvic prolapse.²⁴ These mice also show emphysematous changes, loose skin, and vascular abnormalities.²⁴ The amounts of elastin cross-links were reduced by 40 to 53% in the postpartum uterus of adult *Loxl1*^{−/−} mice, but not the virgin uterus, and in the skin and lungs. Furthermore, Loxl1 was localized specifically to sites of elastogenesis, overlapping entirely with elastic fibers, whereas Lox had a broad, diffuse distribution in the aorta, uterus, and skin.²⁴ Therefore, based on these and our current findings, Loxl1 is likely to function specifically in elastin cross-linking during elastogenesis and renewal of elastic fibers, whereas Lox has an essential role in the cross-linking of both elastin and collagens. The actual role of Loxl1 in collagen cross-linking remains to be determined.

The importance of lysine and hydroxylysine-derived crosslinks in maintaining the integrity, physical properties, and durability of elastic and collagen fibers is evident from the present findings and from previous studies.^{22–24,51–53} Despite the marked reduction in the amount of lysyl oxidase activity, the *Lox*^{−/−} aortic smooth muscle cells and fibroblasts were able to generate a fibrous matrix containing tropoelastin. This is in accordance with a recent observation that lysyl oxidase activity is not required for the initial deposition of tropoelastin onto pre-existing elastic fibers or microfibrils.⁵⁴ Therefore, it is most likely that the reduced cross-linking of elastic fibers, as also of collagen fibers, in the *Lox*^{−/−} embryos leads to diminished physical durability of the fibers and/or increased susceptibility to proteolysis, eventually leading to the histological and pathological abnormalities observed.

Because the manifestations of Menkes disease, occipital horn syndrome, and copper deficiency in humans^{19,20} include various connective tissue defects similar to those observed in *Lox*^{−/−} embryos in the current work and elsewhere,^{22,23} LOX is likely to have a major role in the development of the connective tissue abnormalities in these diseases. No human mutations in the LOX gene are currently known, but it is interesting that reduced lysyl oxidase activity has been reported in two cases of cutis laxa with normal copper metabolism.⁵⁵ The histological and pathological findings in the *Lox*^{−/−} embryos strongly suggest that a reduction in LOX activity for either primary or secondary reasons may have a critical effect on the pathogenesis of several common diseases, including pulmonary diseases, diaphragmatic hernias, skin deformities, and vascular defects.

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