

Proteoglycan and collagen expression during human air conducting system development

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Abstract

The lung is formed from a bud that grows and divides in a dichotomous way. A bud is a new growth center which is determined by epithelial-mesenchymal interactions where proteins of the extracellular matrix (ECM) might be involved. To understand this protein participation during human lung development, we examined the expression and distribution of proteoglycans in relation to the different types of collagens during the period in which the air conducting system is installed. Using light microscopy and immunohistochemistry we evaluate the expression of collagens (I, III and VI) and proteoglycans (decorin, biglycan and lumican) between 8 to 10 weeks post fertilization and 11 to 14 weeks of gestational age of human embryo and fetus lungs. We show that decorin, lumican and all the collagen types investigated were expressed at the epithelium-mesenchymal interface, forming a sleeve around the bronchiolar ducts. In addition, biglycan was expressed in both the endothelial cells and the smooth muscle of the blood vessels. Thus, the similar distribution pattern of collagen and proteoglycans in the early developmental stages of the human lung may be closely related to the process of dichotomous division of the bronchial tree. This study provides a new insight concerning the participation of collagens and proteoglycans in the epithelial-mesenchymal interface during the period in which the air conducting system is installed in the human fetal lung.

Introduction

The human lung undergoes dramatic changes in embryo-fetal life that are the result of growth and maturation. The formation of the air conducting system is the base of lung development and its dysmorphogenesis invol-

ved lung malformations. The lung *primordium* begins around days 26-27 post-fertilization (pf), when the embryo has between 23-25 pairs of somites with the generation of a bud that grows and divides in a dichotomous way, giving rise to the entire bronchial tree in a process called branching morphogenesis.^{1,2} Initially, each lung bud, called primary bronchus, divides within the mesenchyme to form a new generation of lung buds by week 5 of pf time, which after elongation and budding originate three secondary bronchi on the right lung and two on the left. These growth steps are the basis for the mature lobar airway organization of the lung. Thereafter, each lobar bronchus is divided dichotomously, generating the segmental bronchi organization.³ By the end of the pseudoglandular stage (week 15 of gestation), with the installation of the terminal bronchiole,⁴ the air conducting system is definitively installed. In this generational process each bud represents a new growth center in which epithelial-mesenchymal interactions are produced, and where extracellular matrix proteins (ECM) and growth factors are involved.⁵

Observations of human lung fetuses indicate that the branching pattern of the airway and its division are determined by epithelial-mesenchymal interaction in which the ECM plays a leading role in the regulation of the growth and in the type design of the ramification.⁶ In this sense, the ECM molecules not only have a supporting role in cell architecture, but also an active role modulating cell proliferation and differentiation.⁷

In the opinion of several authors, the interaction of proteoglycans with collagen fibrils is important in the assembly and maintenance of the ECM during tissue growth and development.⁸⁻¹² Among the proteoglycans, decorin, biglycan and lumican, three small leucine rich proteoglycans (SLRPs) that show several structural and molecular similarities, are involved in the organization of the collagen fibril in the extracellular spaces.¹³⁻¹⁵

Gene ablation experiments have shown that mice lacking these SLRPs have collagen fibrillogenesis abnormalities leading to fragile skin, corneal opacity, osteoporosis or osteoarthritis.¹⁶⁻¹⁸ There is evidence that both decorin and biglycan bind to transforming growth factor β (TGF- β), modulating its activity as mediator in growth inhibition.¹⁹⁻²¹ Moreover, while decorin is capable of directly suppressing cell proliferation,^{22,23} lumican can regulate collagen fibril organization, epithelial cell migration, and tissue repair.²⁴ These results reinforce the concept that these molecules have a role in several biological processes related to growth, morphogenesis, and maturation of the extracellular matrix organization.

It is well known that epithelium-mesenchy-

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mal interactions are key processes during the generation of the air conducting system of lung development, where ECM molecules such as collagen and proteoglycans are involved. Collagen distribution has been investigated,²⁵ while proteoglycan expression and distribution is a matter not yet resolved in humans. The aim of our study was to evaluate the expression and distribution of SLRPs's proteoglycans in relation to the different types of collagens during the period in which the air conducting system becomes installed in human lungs.

Materials and Methods

Human embryos ranging from 8 to 10 weeks (wk) pf and fetuses from 11 to 14 wk of gestational age were used. Embryonic and fetal developmental age was determined based on the date of the last menstrual period and post collection measurements of limb size and shape. The embryos aged wk 8 (n=2), wk 9 (n=2), and wk 10 (n=2) of pf time, were obtained from tubal ectopic pregnancies; the fetuses aged wk 11 (n=3), wk 12 (n=2), wk 13 (n=2) and wk 14 (n=2) of pregnancy were obtained from spontaneous abortion and con-

served in 10% formalin in buffer phosphate at the Human Embryo Collection of the Universidad de Santiago de Chile. After fixation, lung samples were dehydrated and embedded in paraffin. Histological sections were processed for immunohistochemical analysis.

The study was approved by the local University Ethics Committee at the Universidad de Santiago de Chile (Authorization No. C-1004).

Immunohistochemistry

Immunohistochemistry was performed according to a previously established protocol.²⁶ Briefly, in order to block endogenous peroxidase activity, sections were treated with 3% (v/v) H₂O₂ in phosphate buffered saline (PBS) for 30 min. Each of the succeeding steps was followed by three PBS rinses. All steps were performed in a humid chamber in order to avoid dehydration of the sections. Antigen retrieval was carried out for all antibodies including the samples where the enzymatic treatment was performed,^{10,26-28} by immersing the slide in sodium citrate solution (10 mM, pH 6.0) for 15 min at 95°C. Non-specific staining was blocked by incubating the slide with Cas-Block solution (Zymed Laboratories, South San Francisco, CA, USA) for 10 min.

Primary antibody incubation (Table 1) was achieved by subjecting the diluted sections in PBS containing 0.3% (v/v) Tween 20, overnight at 4°C. After rinsing in PBS, all sections were incubated for 1 h at room temperature with specific secondary antibody (Vector, Burlingame, CA, USA). Sections were incubated with RTU Vectastain kit (Vector) for 1 h at room

temperature. Peroxidase reaction was observed using the NovaRED kit (Vector). Finally, the immunostaining sections were lightly stained with Mayer's haematoxylin (Merck, Darmstadt, Germany). For each immunohistochemical reaction, negative controls were used by incubating sections in normal rabbit or goat serum or by omitting the primary antibody. Human placenta was used as positive control for collagens. Cartilage of the lung samples was used as internal positive control for proteoglycans. Sections were examined under an IX81-Olympus microscope, and images were captured using a digital camera (Olympus DP-71) and the Olympus DP-BSW software.

Results

During all the study period, collagen I, III, VI, decorin and lumican were expressed in the basement membrane region of the bronchial tree (Figures 1 and 2). However, the immunostaining was more concentrated in the proximal airway ducts. Moreover, these collagens and proteoglycans were expressed in the epithelial-mesenchymal interface of the branch site, forming a sleeve around the bronchiolar ducts. The immunoreaction was more evident in the clefts of branching ducts. However, it was not continuous along the basement membrane region of the terminal bud. The proteoglycan biglycan was not expressed in the epithelial-mesenchymal interface. Collagens I, III and VI were expressed in the subendothelial region of arteries, veins and capillaries (Figure 1). Decorin and lumican were not observed in

blood vessels under any condition studied (Figure 2).

The expression and intensity of decorin, biglycan and collagen types III and VI in the lung mesenchyme retained their distribution during the study period. In contrast, lumican and collagen type I showed low immunoreaction from wk 11 of gestational age onward. Biglycan (Figure 2g) was observed at wk 8 pf in the endothelial cells and in the blood vessel smooth muscle cells surrounding the terminal bud, but it was not detected in the mesenchymal cell layer beneath the epithelial tubules. The biglycan immunostaining was observed only in blood vessels (Figure 2 g-i) located near the epithelium. Between wk 9 and 14 a network of blood vessels was observed in the whole lung, maintaining a close relationship with the airway structures. This immunoreactivity pattern was generally observed to be uniform throughout the capillary network of the lung.

The results of each of the individual structures have been summarized in the Table 2.

Discussion

The results of the present study demonstrated the expression and distribution of collagens and proteoglycans at the epithelial-mesenchymal interface of bronchiolar duct and around blood vessels during the period in which the air conducting system of the human lung install. Decorin, lumican and all collagens types investigated were expressed in the epithelial-mesenchymal interface, forming a sleeve around each bronchiolar duct from wk 8 to 14 of lung development, while collagens

Table 1. Immunohistochemical markers.

| Primary antibody | Specificity | Enzymatic pretreatment | Working dilution |
|--|---|---|------------------|
| Rabbit polyclonal (Rockland) Cat No. 600-401-103-01 | Anti-human collagen I Human procollagen 1 (I), mature chain and heterotrimer collagen I | _____ | 1:1000 |
| Rabbit polyclonal (Rockland) Cat No. 600-401-105-01 | Anti-human collagen III Human procollagen α1 (III) | _____ | 1:2000 |
| Rabbit polyclonal (Chemicon, Int.) Cat No. AB7821 | Anti-human collagen VI Human procollagen 1 (VI) | _____ | 1:2000 |
| Goat polyclonal (R&D System) Cat No. AF 143 | Anti-human decorin aa 17-359 of human decorin core protein | Chondroitinase ABC (Seikagaku, Corp) 0.2 U/mL in Tris-HCl 20 mmol, pH 6.0 1 h at 37°C | 1:100 |
| Goat polyclonal (R&D System) Cat No. AF 2745 | Anti-mouse lumican mouse lumican shares 88% aa sequence identity human mature lumican | _____ | 1:200 |
| Goat polyclonal (R&D System) Cat No. AF 2667 | Anti-human biglycan aa 18-368 of human biglycan core protein | Chondroitinase ABC (Seikagaku, Corp) 0.2 U/ml in Tris-HCl 20 mmol, pH 6.0 1 h at 37°C | 1:500 |

were expressed in the subendothelial region of blood vessels. Our observations give new additional information to the results of studies done on the macromolecular organization of the ECM during human lung development.

Using an immunohistochemical approach, Wright *et al.*²⁵ studied human lung develop-

ment from 10 to 42 weeks of gestation, finding the expression of collagens I, III, IV, V and VI. They found that collagens were consistently present at branching points of the airways, suggesting that collagen would have a role in the initiation and stabilization of airway branches during lung development. Our

results are similar and complementary to previous descriptions made by Wright *et al.*²⁵ showing that collagens type I, III and VI are present very early in the epithelial-mesenchymal interface of the branching process. Moreover, during the study period we showed that not all types of collagen were present in

Table 2. Proteoglycan and collagen distribution in different region during human air conducting system development.

| Extracellular matrix molecules | Epithelial-mesenchymal interface | Endothelial cells | Subendothelial region |
|--------------------------------|----------------------------------|-------------------|-----------------------|
| Collagen I | + | - | + |
| Collagen III | + | - | + |
| Collagen VI | + | - | + |
| Lumican | + | - | - |
| Decorin | + | - | - |
| Biglycan | - | + | + |

+, presences of the molecule; -, absences of the molecule.

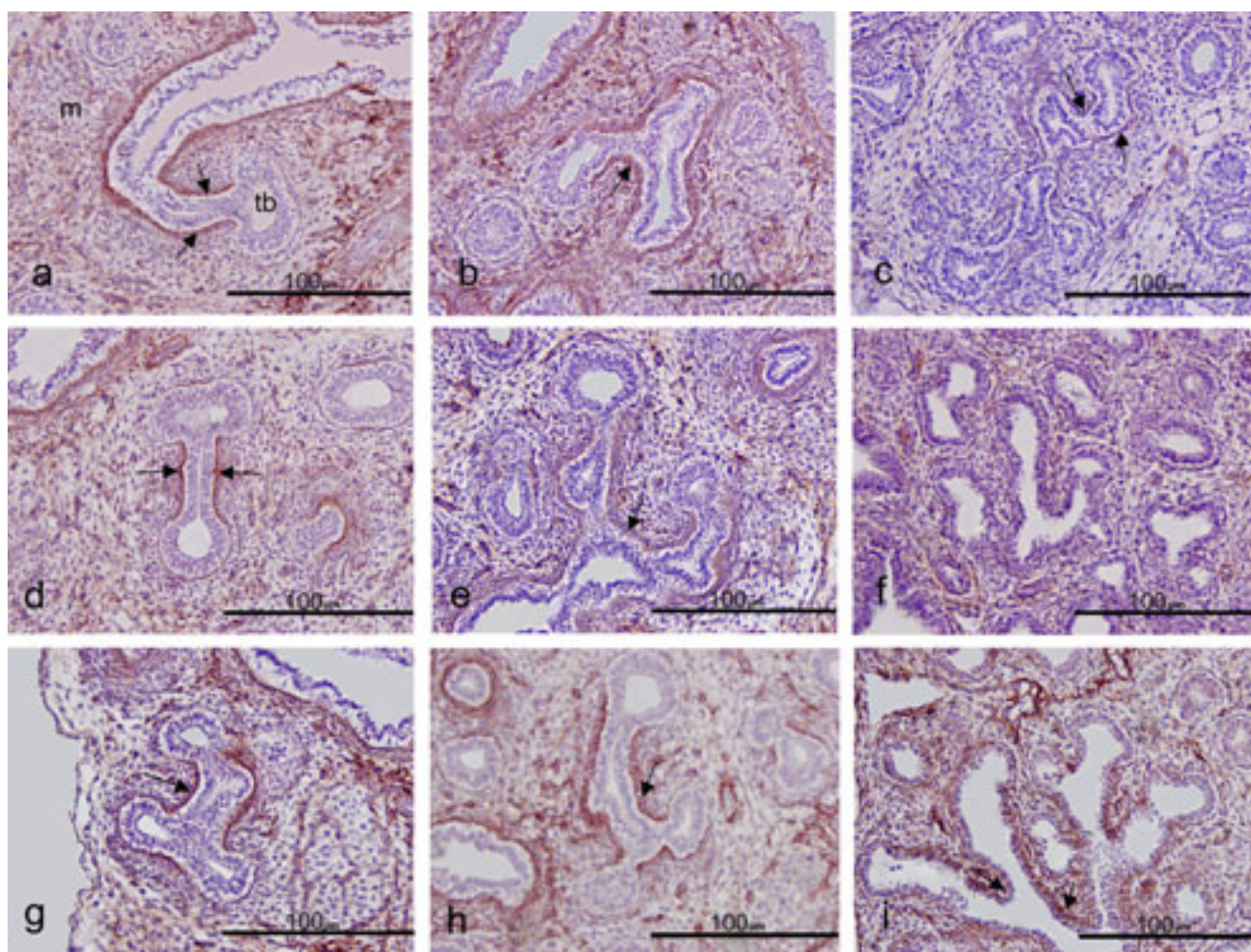


Figure 1. Immunoperoxidase stain for collagen type I (a-c), collagen type III (d-f) and collagen type VI (g-i). From wk 8 post-fertilization time (a, d, g) collagen is expressed in the epithelial-mesenchymal interface in the branch site and forms a sleeve around bronchiolar ducts (arrows). The surface of the terminal buds is not stained. At 10 wk pf time (b, e, h) the immunoreaction is present in the branching ducts clefts (arrows). At wk 14 of gestational age low immunoreaction for collagens type I is detected (c), but collagens type III (f) and VI (i) retain their distribution in the mesenchyme. M, mesenchyma; tb, terminal bud.

the terminal bud surface, suggesting that the absence of collagen in this region may be necessary for the normal growth of the bronchial tree. The low immunostaining of collagen I from wk 11 gestational age onwards showed in the present work is different to the results obtained by Wright *et al.*,²⁵ who report an increase in immunostaining as gestation progresses. However, our results agree with those described by Kaarteenaho-Wiik *et al.*²⁹ where a varied expression of precursors protein and mRNA of collagen type I, going from weak to moderate, was observed at the walls of bronchioles to 12 at 40 week of gestation on human fetal lung.

A different expression pattern for collagens I and III has been reported in chicken³⁰ and mice embryos lung.³¹ In the early stages they

were present in all areas of the lung primordium, concentrated in the proximal region as branching progressed. Concerning the expression pattern of collagen I, our results were similar to previously published data.^{30,31} It has been mentioned that inhibition of collagen synthesis inhibits lung branching.³² However, lung branching was totally normal in collagen I-deficient mice,³³ showing that collagen I is not essential in the branching of budding regions, because other types of collagens, such as types III and V, replace the collagen type I function.¹

In other models where branching morphogenesis occurs, as is the case of the submandibular glands of the mouse, a central role of collagen III in the epithelial-mesenchymal interactions and in cleft initiation in glands

has been reported by Nakanishi *et al.*³⁴ We suggest that this model of branching morphogenesis, where collagens play an important role in the process of division of submandibular glands, might be similar to human lung branching.

Using rat lungs, Amenta *et al.*³⁵ studied the distribution of collagen III, IV and VI, which was expressed in the interstitium between the major airways and blood vessels. They found a close association between collagen I, III, VI and the epithelial basal membrane, suggesting that collagen VI may bind components of the pulmonary ECM and integrate them into a functional unit. These findings suggest an association of collagen I and III with collagen VI which is also located at the epithelium-mesenchyma interface in the human lung. It has

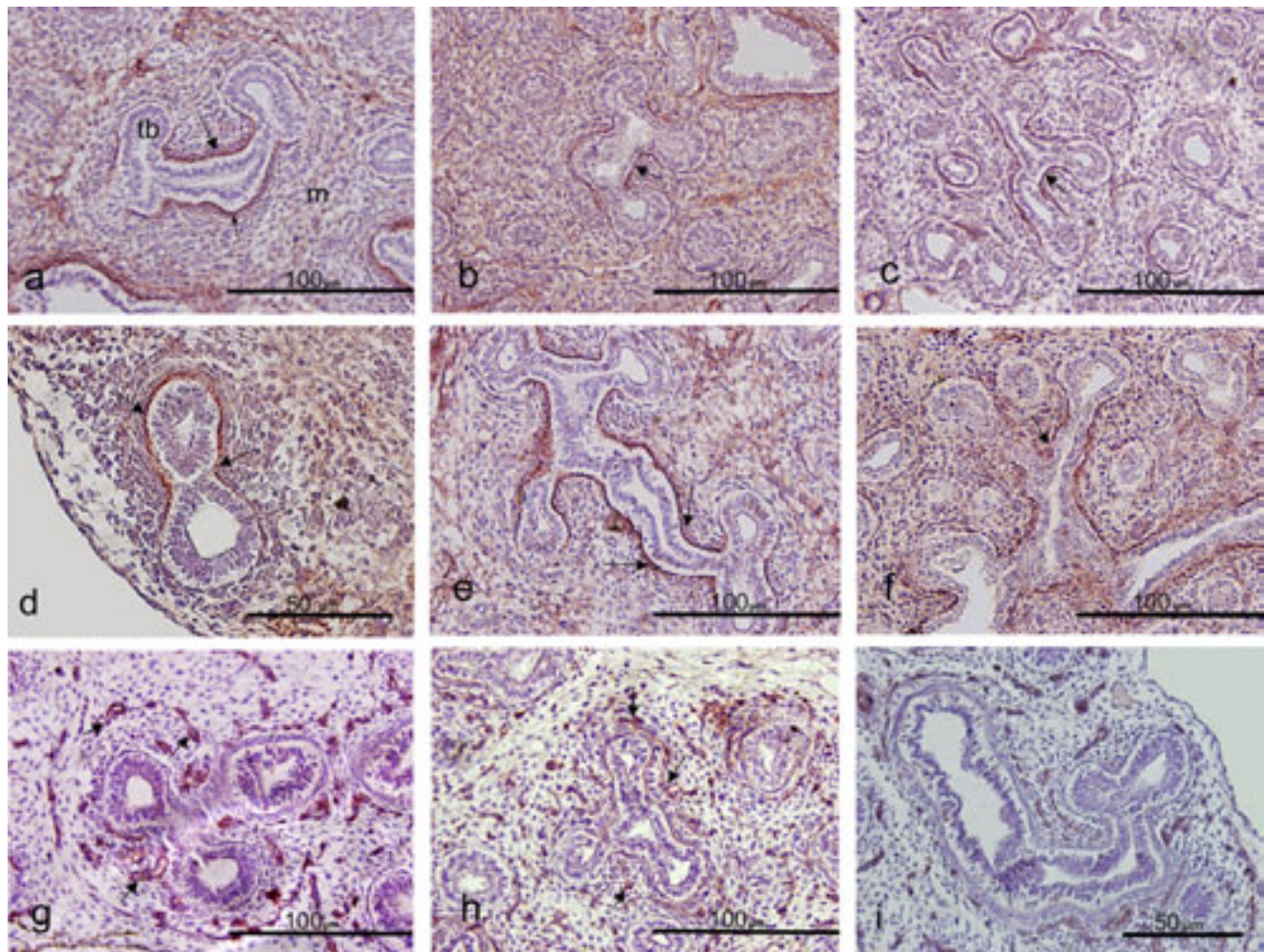


Figure 2. Immunoperoxidase stain for lumican (a-c), decorin (d-f) and biglycan (g-i). From wk 8 post-fertilization time (a, d) proteoglycans were found in the epithelial-mesenchymal interface at the branching site, and it forms a sleeve around bronchiolar ducts (arrows). The surface of the terminal buds is not stained. Between 10 wk pf time (b, e) to 14 wk of gestational age (c, f) the immunoreaction was present in the branching duct cleft. Lumican (c) showed a low immunoreaction at 14 wk of gestational age. Biglycan (g-i) was found in the blood vessels (arrows) surrounding the terminal bud at 8 (g), 10 (h) and 14 (i) wk. M, mesenchyma; tb, terminal bud.

been suggested^{35,36} that the contribution of collagen I, III and VI to blood vessel development indicates an important role of these molecules in the mechanical stability of the circulatory system. Therefore, and in agreement with our results, the presence of collagens in the sub-endothelial region of the blood vessels may indicate their involvement in the early development of blood vessels.

The distribution of the proteoglycans decorin, lumican and biglycan is described here for the first time during the pseudoglandular phase of human lung development. These macromolecules are involved in several functions such as the regulation of protease and antiprotease activity, cell response to growth factors, cell-cell interaction and their interactions with the epithelial ECM molecules, and also in the regulation of ECM assembly.³⁷ Experiments with mouse embryonic lung cell cultures using beta-xyloside, a proteoglycan biosynthesis disrupter, reveal that in the absence of chondroitin sulfate the ECM is disturbed and branching morphogenesis is compromised.³⁸ Hall *et al.*³⁹ studied the expression of versican in human lungs between 28 and 98 days of development and found that this proteoglycan was present in the mesenchyme, but not in the endodermal epithelium and capillaries.

Decorin belongs to the SLRP family and consists of a core protein linked to the glycosaminoglycan chains of chondroitin sulfate or dermatan sulfate, and it is closely related structurally to the proteoglycan biglycan.⁴⁰ Decorin as a component of connective tissue fibers is associated with collagen I and VI, and plays an important role in the assembly of the extracellular matrix.^{41,42} Our results confirm this association between decorin and collagen, where a similar distribution pattern of decorin, biglycan, collagen I and VI was observed in the branching duct clefts, suggesting that they may have a similar role during lung development.

Biglycan consists of a core protein and two chains of chondroitin or dermatan sulfate. The role of this proteoglycan on blood vessel formation is a matter that has not been studied deeply. Biglycan is expressed by human endothelial cells⁴³ and in this location it has been reported that it plays a role in the regulation of some type-specific cell functions of endothelial and smooth muscle cells.⁴⁴ In our study we found that the expression of biglycan in the endothelial cells and vascular smooth muscle are of interest and may suggest a similar function related with the early development of blood vessels of the lung during the period analysed.

Lumican is another member of the SLRP family that binds to collagen I through highly charged hydrophilic glycosaminoglycans regu-

lating interfibrillar spacing.^{14,42} In fact, Vogel and Trotter¹⁵ showed that decorin and lumican link to fibrillar collagen *in vitro*, stabilizing thin fibrils. We also found in this study a similar distribution pattern of lumican, decorin and collagen I in the branching duct clefts of the airway that suggests a similar fibril stabilizing role of collagen at the interface.

In agreement with previous observations conducted by others,^{25,45} we concluded that the distribution pattern of collagens and proteoglycans at the epithelial-mesenchymal interface and in the cleft sites in the developing human lung might indicate that these molecules may play a role in the regulation of the initiation and stabilization of airway bud formation during the branching process. This process may be very important in the airway conducting system of the human fetal lung during the establishment of the pattern for the process of dichotomous division of the bronchial tree.

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