Chondroitin sulfate proteoglycans are required for lung growth and morphogenesis in vitro

John M. Shannon, Kathleen McCormick-Shannon, Michael S. Burhans, Xiaofei Shangguan, Kalpana Srivastava, and Brian A. Hyatt
Division of Pulmonary Biology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio 45229

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Shannon, John M., Kathleen McCormick-Shannon, Michael S. Burhans, Xiaofei Shangguan, Kalpana Srivastava, and Brian A. Hyatt. Chondroitin sulfate proteoglycans are required for lung growth and morphogenesis in vitro. Am J Physiol Lung Cell Mol Physiol 285: L1323–L1336, 2003.—Proteoglycans (PGs) have been shown to play a key role in the development of many tissues. We have investigated the role of sulfated PGs in early rat lung development by treating cultured tissues with 30 mM sodium chlorate, a global inhibitor of PG sulfation. Chlorate treatment disrupted growth and branching of embryonic day 13 lung explants. Isolated lung epithelium (LgE) migrated toward and invaded lung mesenchyme (LgM), and chlorate irreversibly suppressed this response. Chlorate also inhibited migration of LgE toward beads soaked in FGF10. Chlorate severely decreased branching morphogenesis in tissue recombinants consisting of LgM plus either LgE or tracheal epithelium (TrE) and decreased expression of surfactant protein C gene (SP-C). Chlorate also reduced bone morphogenetic protein-4 expression in cultured tips and recombinants but had no effect on the expression of clara cell 10-kDa protein (CC10), sonic hedgehog (Shh), FGF10, and FGF receptor 2IIIb. Chlorate reduced the growth of LgE in mesenchyme-free culture but did not affect SP-C expression. In contrast, chlorate inhibited both rudiment growth and the induction of SP-C in mesenchyme-free cultured TrE. Treatment of lung tips and tissue recombinants with chondroitinase ABC abolished branching morphogenesis. Chondroitinase also suppressed growth of TrE in mesenchyme-free culture. Chondroitinase treatment, however, had no effect on the induction of SP-C expression in any of these cultures. These results demonstrate the overall importance of sulfated PGs to normal lung development and demonstrate a dynamic role for chondroitin sulfate PGs in embryonic lung growth and morphogenesis.

LUNG ORGANOGENESIS REQUIRES the coordinated temporal and spatial expression of multiple genes, both prenatally and after birth. Deviations from the normal developmental program resulting from improper or insufficient gene expression can have serious consequences, such as pulmonary hypoplasia, pulmonary agenesis, congenital alveolar proteinosis, and neonatal respiratory distress syndrome. Studies in both mammals (3, 67, 75) and birds (13, 52) have repeatedly shown that normal lung morphogenesis and differentiation are dependent on reciprocal interactions between the endodermal epithelium and mesenchyme derived from the splanchnic mesoderm. These interactions are responsible not only for patterning of the lung but also for the specification of the many specialized cell types that populate both the epithelial and mesenchymal tissue compartments. Elucidation of the molecular basis of these interactions has been the subject of intense recent interest. Although much remains to be learned, the results thus far demonstrate that these interactions are highly complex, involving the interplay of numerous hormones, growth factors, and downstream effectors such as transcription factors (9, 60, 72).

In addition to soluble factors, the extracellular matrix (ECM) also plays an important role in lung morphogenesis, not only by providing the physical milieu in which patterning occurs, but also by serving as a reservoir for growth factors and mediating the effects of morphogens. Perturbations in the normal expression and organization of insoluble ECM components such as laminin (55, 57), nidogen (20), and the collagen (1, 2, 66, 76) have been shown to significantly disrupt normal lung morphogenesis. Another class of molecules, the proteoglycans (PGs), has the potential to influence lung development on more than one level. PGs consist of a protein core to which different glycosaminoglycan (GAG) chains [e.g., heparan sulfate (HS) or chondroitin sulfate (CS)] are covalently linked. These GAG chains are modified during synthesis by a series of epimerization and sulfation reactions that generate structural diversity among them. Differences in the amino acid sequences of the core proteins, along with number, size, type, and degree of sulfation of the GAG chains provide the potential for vast heterogeneity among PGs, which is thought to account for their functional differences. PGs are present in the ECM, where they can serve as structural molecules, as well as regulators of growth factor and morphogen diffusion and activity (5, 24, 25, 28, 51). In addition, PGs are abundantly expressed on cell surfaces, where they mediate cell-cell adhesion and cell-ECM binding (12, 31, 44), and also serve as low-affinity growth factor receptors (11, 43, 48, 53, 79).
To further investigate the role of PGs in lung development we have examined the effects of sodium chlorate, which inhibits sulfation of all types of GAG chains, on lung epithelial morphogenesis and differentiation in vitro. Our results show that sulfated PGs are required for lung growth and branching, morphogenetic movement, and specification of the distal lung epithelial phenotype. We further demonstrate that one class of PGs, the chondroitin sulfate proteoglycans (CSPGs), plays a critical role in lung patterning but does not appear to be necessary for induction of surfactant protein C (SP-C), a marker of distal lung epithelial differentiation.

MATERIALS AND METHODS

Animals and isolation of tissues. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Children’s Hospital Research Foundation. Timed-pregnant Sprague-Dawley rats were obtained from Taconic (Germantown, NY); the day on which a sperm-positive vaginal plug was detected was considered day 0 of gestation. Pregnant dams were killed on embryonic day 13 (E13) of gestation, and the fetuses were removed by hysterotomy; only fetuses weighing between 80 and 90 mg and whose lungs were at the pseudoglandular stage of development (Fig. 1A) were used in these experiments. Lung-trachea complexes were dissected with Moria microsurgery knives (Fine Science Tools, Foster City, CA) in ice-cold Hank’s balanced salt solution (GIBCO-BRL, Gaithersburg, MD) containing 100 units penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (all from GIBCO-BRL) and used as described below.

Lung explant culture. Whole lung explants were cultured on a semisolid culture medium consisting of DMEM/F-12 (GIBCO-BRL) containing 0.5% agarose, 5% fetal bovine serum (FBS; Sigma, St. Louis, MO), and antibiotics as previously described (59). Cultures were generated from lung-trachea complexes by removing the trachea above the bifurcation and placing the lungs on the surface of 2.5 ml of semisolid medium in six-well dishes; liquid medium (four drops) was added to maintain explants at an air-liquid interface, and cultures were maintained for up to 5 days. Sodium chlorate (30 mM, Sigma) was added to half of the cultures at the time of explanting as an addition to the both liquid and semisolid media.

For determination of branching by distal lung signaling centers, the caudal tip of the left lung and the cranial, middle, accessory, and caudal tips of the right lung were removed and cultured as described above. The four culture conditions evaluated were DMEM/F-12 + 5% FBS (DF5), DF5 + 30 mM sodium chlorate, DF5 + 200 ng/ml human recombinant bone morphogenetic protein-4 (BMP-4; R&D Systems, Minneapolis, MN), and DF5 + sodium chlorate, and BMP-4. Lung tips were cultured for 3 days, at which time they were photographed and the number of terminal buds was counted; buds were scored positive if the evagination from the lung epithelial wall comprised at least one-half of a sphere (29).

Separation and recombination of tissues. Tissue recombinants composed of lung mesenchyme (LgM) plus either lung epithelium (LgE) or tracheal epithelium (TrE) were prepared and cultured as previously described (62). Briefly, E13 distal lung tips were removed and incubated with dispase (BD Biomedical Products, Bedford, MA) for 30 min at 37°C then separated into purified epithelial and mesenchymal components with tungsten needles. E13 tracheae were bisected, incubated for 45 min at 37°C in DMEM/F-12 containing 0.05% collagenase IV (Worthington Biochemicals, Freehold, NJ) and 1% FBS, then separated into epithelial and mesenchymal components with tungsten needles. Both lung and tracheal epithelia were treated a second time with dispase for 10 min to remove any residual adherent mesenchymal cells. Tissues were recombined on the surface of a semisolid medium consisting of DMEM/F-12 containing 0.5% agarose and 5% FBS; in some experiments the medium also contained other additions as described below. Dishes containing tissue recombinants were placed in humidified 150-mm diameter dishes and cultured for up to 5 days.

Mesenchyme-free culture of embryonic lung and tracheal epithelium. Purified E13 lung and tracheal rudiments were prepared as described above and placed into mesenchyme-free culture as previously detailed (19, 61). Briefly, several pads of growth factor-reduced Matrigel (BD Biomedical) were created on the bottom of a 35-mm culture dish by spreading 15 μl to a diameter of 0.5 cm. Epithelial rudiments (three to four per pad) were transferred to the surface of the matrix, then enrobed in an additional 20 μl of Matrigel that was subsequently gelled at 37°C. The tissues were then covered with BFGM medium, which consists of DMEM/F-12 containing 3% charcoal-stripped FBS, 10 μg/ml insulin (BD Biomedical), 1 μg/ml cholera toxin (ICN Pharmaceuticals, Irvine, CA), 25 ng/ml human recombinant epidermal growth factor (R&D Systems), 10 ng/ml human recombinant hepatocyte growth factor, 25 ng/ml human recombinant FGF7 (PeproTech, Rocky Hill, NJ), and 100 ng/ml bovine brain FGF1 (R&D Systems). Tissues were cultured in this medium alone, or with the addition of sodium chlorate, for up to 6 days. As controls, some cultures were treated with both 30 mM sodium chlorate and 10 mM sodium sulfate (Sigma).

Epithelial chemoattraction in vitro. Purified E13 distal lung epithelium and mesenchyme were prepared and enrobed in growth factor-reduced Matrigel as described above. One piece of epithelium was positioned ~200 μm away from one or two pieces of mesenchyme and in the same plane of focus. For bead experiments, ~100 heparin-coated acrylic beads (Sigma) were soaked for 2 h in 25 μl of phosphate-
buffered saline (PBS) containing 1% bovine serum albumin (BSA) and either 1, 10, or 100 μg/ml human recombinant FGFI0 (R&D Systems). The total amount of FGFI0 was 5, 50, or 500 ng, respectively. Beads soaked in PBS + BSA served as controls. The beads were rinsed briefly, then placed ~200 μm from the epithelium. Tissues were cultured in DMEM/F-12 + 5% PBS in the presence or absence of 30 mM sodium chlorate.

Treatement of cultured tissues with GAG-degrading enzymes. Intact distal lung tips, along with LGM + LG and LGM + TRG recombinants, were cultured as described above on semisolid medium containing either 0.7 U/ml each heparinase I (Sigma H-2519) and III (Sigma H-8891), or 0.7 U/ml chondroitinase ABC (SIGMA-C-2905); enzymes were added to both the semisolid and liquid media. Cultures were maintained for 3 days.

To determine the effects of degradation of specific classes of GAGs on the induction of distal lung epithelial differentiation, we cultured isolated TRG mesenchyme-free in BFGM that contained either heparinases I and III or chondroitinase ABC. Experiments were also done in which TRG was cultured for 3 days in BFGM + chondroitinase ABC, washed, then cultured for an additional 3 days in BFGM alone.

Immunohistochemistry. The efficacy of heparinases in removing HS GAGs in cultured lung explants and recombinants was assessed by immunostaining paraformaldehyde-fixed sections with monoclonal antibody 3G10 (1:2,000). This antibody specifically recognizes HS fragments displaying a nonreducing terminal unsaturated hexuronic acid that remain attached to PG core proteins following heparinase digestion (14). Secondary antibody treatment and color development were done with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). As a positive control, some sections were pretreated for 2 h at 37°C with 1 U/ml heparinase III before incubation with the primary antibody.

DNA content. Lung explants were harvested into 1 ml of 2 M NaCl, 2 mM EDTA in PBS, sonicated on ice, and then DNA content assayed fluorimetrically by the method of Labarca and Paigen (35). Lung explants were assayed individually, and DNA content values for each lung were determined in duplicate.

In situ hybridization. Tissues for whole mount in situ hybridization (WM-ISH) were fixed in freshly prepared 4% paraformaldehyde in PBS. Lung explant cultures and tissue recombinants were fixed directly in the dish. Early attempts at WM-ISH on epithelial rudiments cultured in Matrigel, however, were confounded by high background staining of the matrix. To circumvent this problem, we first freed the epithelial rudiments from the Matrigel by incubating them for 1 to 2 h in Matrisperse (BD Biomedical) on ice, then transferring the naked rudiments to a 12-well cluster dish containing fixative. WM-ISH was performed essentially as described by Wilkinson (77), using a digoxigenin-labeled antisense or sense RNA probes transcribed from a full-length rat SP-C cDNA (23) using a commercially available kit (Promega, Madison, WI). Tissue-section ISH was done on 4-μm sections as previously described (19), with the exception that [α32P]UTP (2,000–3,000 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA) was used to radiolabel the RNA probe. Hybridized slides were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY), developed after an appropriate exposure period, and counterstained with hematoxylin.

Real-time PCR. Samples for real-time PCR were harvested into 4 M guanidinium isothiocyanate, 0.5% N-laurylsarcosine, and 0.1 M 2-mercaptoethanol in 25 mM sodium citrate buffer. Total RNA was isolated using the acidified guanidinium method (10), treated with DNase I (DNA-free; Ambion, Austin, TX), and reverse transcribed using a cDNA cycle kit (Invitrogen, Carlsbad, CA). Levels of gene expression were assessed by real-time PCR (Smart Cycler; Cepheid, Sunnyvale, CA). PCR reaction mixes consisted of 50 ng of cDNA template, 0.5 μM of each primer (except SP-C, 1.0 μM of each primer), 2.5 mM MgCl2, and 1× DNA Master SYBRgreen I (Roche Molecular Biochemicals) containing Taq polymerase, dNTPs, SYBRgreen dye, and buffer. Reaction conditions differed slightly depending on primers used but generally were 95°C for 150 s followed by 25–35 cycles of amplification (95°C for 6–10 s, 53–61°C for 6–20 s, and 72°C for 10–25 s). Amplification product size and forward and reverse primer sequences were as follows: SP-C (207 bp): 5’-CATCGTGTGTATGACTCACGCG-3’, 5’-AATCGGACTCGGAAACAGATTC-3’, 5’-TCTCTTGTTGTC-TCCGTCCCTGC-3’, 5’-CGCTGACCTGCGCTTTCTCTC-3’; FGF receptor (R) IIb (162 bp): 5’-AAGGTITACAGCATCGCCAG-3’, 5’-TCTCGTACATTTAAGACACG-3’; BMP-4 (147 bp): 5’-CTGTTAATCCAGGGAGTTGGAATG-3’, 5’-TCTTATCTCTCTCTGAGGACCCTG-3’; sonic hedgehog (Shh, 260 bp): 5’-GGCACTATATGAAAGAGGAAGAT-3’, 5’-ACTGCTGACCCATACTGTGG-3’; gene for Clara cell 10-kDa protein (CC10, 281 bp): 5’-CAGGTGTCATGCTGCTCAT-3’, 5’-CCAGTGAACCTCAGACCTTCA-3’; β-actin (350 bp): 5’-TGGAACTCCTGTGGACATCATACG-3’, 5’-TAAACCGACGTCAGTAAACGCT-3’.

Measurement of amplified product was made for 6 s every cycle at a temperature above that of the melting temperature of nonspecific products (e.g., primer-dimers) and 1–2°C below the melting temperature of the specific product. Melt curve analyses were performed after every run to assure that a single amplified product was produced. Input levels of cDNA were assessed by optical density and values for SP-C, CC10, Shh, BMP-4, FGF10, and FGF2IIb were normalized to the level of β-actin expression. The quantitative data presented are the result of at least three independent experiments. For the visual representations shown in Figs. 3 and 5, parallel real-time PCR was done, with the modification that all reactions were terminated when the sample(s) with the highest level of expression reached plateau.

Statistics. Data were analyzed with InStat version 3.0 (GraphPad Software, San Diego, CA). Values are presented as means ± SE. Multiple comparisons were made by ANOVA using the Tukey–Kramer multiple-comparisons test. Paired samples were analyzed by t-tests. In both cases, statistical significance was defined as P < 0.05.

RESULTS

Sulfated PGs are required for normal lung growth and morphogenesis. When cultured in DF5 control medium, explants of day 13 lung (Fig. 1A) showed an increase in the number distal tips that was obvious by day 3 of culture, and the appearance of some of the distal tips was disrupted (Fig. 1B). Disrupted morphogenesis was most apparent after 5 days of culture (Fig. 1C), when branching was significantly reduced and the epithelium was disorganized, forming large sacs throughout the explants. We also observed that chlorate-treated explants appeared smaller than controls. This
was confirmed by measuring the DNA content of the explants. Day 13 lungs contained 1.52 ± 0.07 μg (n = 16) of DNA at the time of isolation. DNA content increased significantly (P < 0.001) after 3 days of culture in both control medium (6.98 ± 0.24 μg; n = 18) and in medium plus chlorate (4.91 ± 0.34 μg; n = 20), but the extent of increase was significantly (P < 0.001) greater in control vs. chlorate-treated cultures.

To quantitate the inhibitory effects of chlorate on distal lung branching, we removed individual distal lung tips from day 13 lungs (Fig. 2A) and cultured them separately in the presence and absence of chlorate. The epithelium in distal tips cultured in DF5 for 3 days (Fig. 2B) branched to form structures that had a larger central lumen surrounded by numerous distal buds (19.7 ± 0.8 buds/cultured tip, n = 50). Addition of 30 mM chlorate to distal tip cultures (Fig. 2C) inhibited both growth and branching, and the number of distal buds was significantly reduced (5.2 ± 0.3 buds/cultured tip; P < 0.001, n = 49). The morphology of chlorate-treated tips was also aberrant, with some of the epithelium forming elongated unbranched structures. Because it has been reported (8, 63) that BMP-4 stimulates lung branching in mice, we tested its effects on rat distal lung tips cultured in the absence or presence of chlorate. Somewhat surprisingly, we found that addition of 200 ng/ml BMP-4 to distal lung tips had no qualitative (Fig. 2D) or quantitative (20.4 ± 0.8 buds/cultured tip; P > 0.05, n = 47) effect on the branching of distal buds. The concentration of BMP-4 we used was the same as that used in the previous mouse studies and is one that effectively suppresses the growth of isolated rat lung and tracheal epithelium in mesenchyme-free culture (J. M. Shannon, unpublished observations). Furthermore, addition of BMP-4 to chlorate-treated distal tip cultures did not overcome either the suppression of growth and branching (4.8 ± 0.4 buds/cultured tip; P < 0.001, n = 20) or the disrupted morphogenesis (Fig. 2E). Using real-time PCR to compare levels of BMP-4 in control and chlorate-treated tips and tissue recombinants, we found (Fig. 5) that BMP-4 was expressed in the presence of chlorate, but at reduced levels in LgM + LgE (47% decrease, n = 4) and LgM + TrE recombinants (72% decrease, n = 3).

Because the distal tips of E13 lungs have already been induced by LgM, the effects of chlorate on these tissues show the importance of sulfated PGs for maintenance of the distal lung epithelial phenotype. To examine the effects of chlorate on the induction of the distal lung epithelial phenotype, we made heterotypic tissue recombinants using distal LgM and TrE, which we have previously shown (61) can be reprogrammed to differentiate as distal lung. Intact distal lung tips and homotypic recombinants of LgM and LgE served as controls. The results showed that recombined LgE (Fig. 2F) as well as TrE (Fig. 2H) branch in a lung-like pattern under the influence of LgM. Addition of chlorate to the medium diminished branching in intact tips (Fig. 2C), and in LgM + LgE recombinants (Fig. 2G) but had a much more severe effect LgM + TrE recombinants (Fig. 2I) in which branching was completely inhibited.

Sulfated PGs are required for distal LgE differentiation. We next examined the effects of chlorate on the induction and maintenance of SP-C, which is a specific marker of the distal LgE, in cultured intact lung tips and tissue recombinants. WM-ISH demonstrated that intact lung tips (Fig. 3A) and LgM + LgE recombinants (Fig. 3B) cultured for 3 days in DF5 showed maintenance of SP-C expression and that LgM induced ectopic SP-C expression when recombined with TrE (Fig. 3C). SP-C expression was limited to the distal acini of the epithelium. Addition of chlorate to the medium resulted in a clear diminution of SP-C expression in all tissues, but the magnitude of the response was variable. All of the intact lung tips treated with chlorate (Fig. 3D) showed some SP-C content, which was expressed in a spatially correct pattern at the distal tips. Chlorate treatment of LgM + LgE recombinants (Fig. 3E) gave a more variable response, with some recombinants showing moderate SP-C expression, whereas others had clearly diminished levels (arrow, Fig. 3E). The spatial distribution of SP-C expression was also altered in LgM + LgE recombinants, with some distal...
Sodium chlorate inhibits induction and maintenance of surfactant protein C gene (SP-C) expression. Whole mount in situ hybridization shows that SP-C is readily detectable in the distal epithelium of cultured intact lung tips (A) and in tissue recombinants made from LgM + LgE (B) and LgM + TrE (C). Chlorate treatment of intact tips causes a reduction in SP-C signal intensity (D). The response of LgM + LgE recombinants to chlorate shows some variability, with most showing extremely low SP-C signal (arrow, E), whereas some show an intensity comparable to that seen in chlorate-treated intact tips. Tissue-section in situ hybridization of LgM + LgE recombinants (G, H) shows a patchy distribution of SP-C-positive cells, with some areas of distal epithelium being completely devoid of signal. Whole mount (F) and tissue-section (I, J) in situ hybridization of LgM + TrE recombinants show that chlorate treatment inhibits SP-C expression almost completely, with only a few cells (arrow in F, arrowheads in J and J) being positive. A–F are at the same magnification; bar = 700 μm. Hybridization conditions and color development times were identical for all samples. G–J are at the same magnification; bar = 130 μm. Exposure times for the autoradiographs were identical. Real-time PCR confirms the in situ hybridization results (K). Equivalent amounts of input cDNA were analyzed in parallel real-time PCR reactions. In 1 set, all reactions were terminated when the highest expressing sample(s) reached plateau and run on a gel to give a qualitative image that accurately reflects the real-time PCR data. The products shown here are the result of 25 cycles to 35 allowed the visual detection of SP-C expression in all 3 tissues. Culture in the presence of chlorate suppresses SP-C expression. Although this effect is apparent in intact tips (lane 4), it is more pronounced in LgM + LgE (lane 5) and LgM + TrE (lane 6) recombinants. Washing chlorate from the cultures after 3 days then culturing in control medium for an additional 3 days results in recovered SP-C expression in intact tips (lane 7), LgM + LgE (lane 8) and LgM + TrE (lane 9) recombinants. Lane 10 is a no-template control.

The recovery in intact tips (107%) was significantly greater than that seen in LgM + LgE recombinants (69%) and particularly in LgM + TrE recombinants (26%). This most likely reflects the difference in the extent of TrE induction achieved in 3 (recovery) vs. 6 (control) days. It is also possible, however, that some of the TrE cells lose their competence (61) to respond to PG sulfation on chondroitin sulfate proteoglycans in LgM induction during the 3-day exposure to chlorate. Although chlorate significantly diminished expression of SP-C, it did not inhibit expression of the proximal lung epithelial marker CC10 (Fig. 5).

Sodium chlorate inhibits chemoattraction of LgE by LgM. Complex signaling centers regulate morphogenesis in the developing lung. One key aspect of this process is the initiation and expansion of distal lung buds, which is thought to be mediated by the chemoattractant effects of FGF10 (45, 73). Because chlorate treatment disrupted morphogenesis so severely, we evaluated the effects of inhibiting PG sulfation on morphogenetic movement in vitro. When placed ~150–200 μm from LgM (Fig. 4A), LgE was reproducibly (44 positive responses out of 49) induced to migrate toward it over the next 24 h (Fig. 4B). Upon contacting the LgM, the LgE invaded it and branched in a typical lung-like pattern (Fig. 4C). Adding chlorate to these cultures (Fig. 4D) prevented chemoattractive movement in every case (0 positive responses out of 53). This inhibitory effect of chlorate on chemoattraction was irreversible: when chlorate was washed out of the cultures and replaced with fresh DF5, chemoattractive movement was not reinitiated after 24 (Fig. 4E) or 96
The amount of FGF10 was increased to 50 ng, however, further deleterious effect on the LgE (Fig. 4). Addition of chlorate to these cultures had no effect on either growth or chemoattraction (Fig. 4H). When the amount of FGF10 is increased to 50 ng, the LgE moves by chemoattraction and contacts bead by 3 days (Fig. 4I). Addition of chlorate to these cultures did not inhibit the chemoattractive movement of LgE toward the bead (Fig. 4L). To address the possibility that chlorate might have decreased FGF10 expression by LgM, we examined the effect of chlorate on FGF10 expression in cultured distal lung tips and recombinants and found that, unlike SP-C, FGF10 levels were unaffected (Fig. 5). We also found no significant difference in expression of the mRNA for the IIIb splice variant of FGFR2, which is the primary receptor for FGF10 (16).

Sodium chlorate directly inhibits growth of lung and TrE in mesenchyme-free culture. The results above demonstrated that sulfated PGs are necessary for the growth and morphogenesis of explanted lung tissues comprising both epithelium and mesenchyme. Because PGs are synthesized and secreted by both epithelial and mesenchymal cells, we next tested the effects of chlorate on the growth of isolated embryonic epithelia maintained in mesenchyme-free culture. We have previously demonstrated that both lung (18, 19) and trauma-tation of the LgE ensued (Fig. 4H), resembling that seen when LgM was used (Fig. 4B). Addition of chlorate to these cultures completely inhibited chemoattraction (Fig. 4K). Increasing the amount of FGF10 to 500 ng resulted in a more robust chemoattractive response and also increased the size of the LgE rudiments (Fig. 4I). Interestingly, addition of chlorate to these cultures did not inhibit the chemoattractive movement of LgE toward the bead (Fig. 4L). When the amount of FGF10 is increased to 50 ng, the LgE moves by chemoattraction and contacts bead by 3 days (Fig. 4I). Similar results were found when LgM was used (Fig. 4L). This result may have its basis in previous observations from our laboratory (unpublished) that embryonic LgE cultured for 24 h in medium containing only serum loses its competence to respond to induction when switched to BFGM.

As noted above, FGF10, which is produced by LgM, is a strong chemoattractant for LgE. LgE did not migrate toward a control heparin bead (data not shown) or toward a bead that had been soaked in a small (25 μl) volume of PBS containing a total of 5 ng of FGF10 (Fig. 4G). Addition of chlorate to these cultures had no further deleterious effect on the LgE (Fig. 4J). When the amount of FGF10 was increased to 50 ng, however, (Fig. 4F) h. This result may have its basis in previous observations from our laboratory (unpublished) that embryonic LgE cultured for 24 h in medium containing only serum loses its competence to respond to induction when switched to BFGM.

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Fig. 4. Sodium chlorate inhibits chemoattraction of lung epithelum by LgM. Embryonic LgE cultured for 24 h in medium containing a total of 5 ng of FGF10 is not induced to move toward the bead (A). Addition of chlorate to these cultures had no effect on either growth or chemoattraction (B). When the amount of FGF10 is increased to 50 ng, the LgE moves by chemoattraction and contacts bead by 3 days (C). This result may have its basis in previous observations from our laboratory (unpublished) that embryonic LgE cultured for 24 h in medium containing only serum loses its competence to respond to induction when switched to BFGM.

Fig. 5. Gene expression in intact lung tips and tissue recombinants cultured in the absence or presence of chlorate. Intact distal lung tips (lanes 1 and 4), LgM + LgE recombinants (lanes 2 and 5), and LgM + TrE recombinants (lanes 3 and 6) are cultured without (lanes 1–3) or with (lanes 4–6) chlorate for 3 days. Lane 7 is a no-template control. Equivalent amounts of input cDNA template were analyzed in parallel real-time PCR reactions. In 1 set, the reactions were terminated when the highest-expressing samples reached plateau and electrophoresed to give a qualitative representation of the differences among the samples. The results shown are from 23–25 cycles of PCR. Chlorate significantly decreases expression of SP-C but has no effect on expression of genes for Clara cell 10-kDa protein (CC10), sonic hedgehog (Shh), FGF10, FGFR1 receptor (R) IIIb, and β-actin. BMP-4 expression appears decreased in chlorate-treated LgM + TrE recombinants (lane 7) vs. controls (lane 3). Parallel quantitation by real-time PCR analysis of these same samples showed that, compared with controls, chlorate decreased BMP-4 by 20, 55, and 75% in tips, LgM + LgE, and LgM + TrE, respectively.
chelal epithelium grown in a complex growth medium (BFGM) will differentiate to form mature alveolar type II cells; FGF1 and FGF7 are necessary, but not sufficient, to elicit this response.

Isolated distal tip LgE (Fig. 6A) cultured in BFGM for 3 (Fig. 6B) or 6 (Fig. 6C) days exhibited dramatic cell proliferation and an increase in size. LgE cultured in BFGM containing chlorate still showed significant growth after 3 (Fig. 6D) or 6 (Fig. 6E) days, but the extent of expansion was clearly diminished. To assess the possible toxicity of chlorate, we performed washout studies. Rudiments were cultured for 3 days in BFGM in the presence of chlorate, washed four times with DMEM/F-12, then cultured an additional 3 days in BFGM alone. The results showed that chlorate inhibition of rudiment growth was not due to a general toxicity, since rudiments cultured with chlorate for 3 days (Fig. 6F) that were subsequently washed and cultured in BFGM for an additional 3 days (Fig. 6G) showed a renewed and rapid expansion. Isolated TrE (Fig. 6H) showed a similar vigorous growth response to BFGM after 3 (Fig. 6I) and 6 (Fig. 6J) days. Addition of chlorate to these cultures decreased the amount of rudiment expansion after 3 (Fig. 6K) and 6 (Fig. 6L) days, and washing chlorate from cultures after 3 days resulted in increased expansion of the epithelium (Fig. 6, M and N). Rudiments cultured in medium containing both 30 mM chlorate and 10 mM sodium sulfate showed no inhibition of growth over 6 days (data not shown).

**Sodium chlorate inhibits the reprogramming of embryonic TrE to a distal lung phenotype.** We next examined the effects of chlorate on the expression SP-C by lung and tracheal epithelium in mesenchyme-free cultures. We have previously shown that embryonic lung epithelial cells will progress to full type II cell differentiation in mesenchyme-free culture (19) and that embryonic TrE can be reprogrammed to express a distal lung epithelial phenotype, including the expression of SP-C (61). LgE cultured in BFGM showed widespread expression of SP-C (Fig. 7A); samples hybridized with sense RNA probe showed no signal (Fig. 7C). Treatment with chlorate inhibited LgE growth but did not diminish expression of SP-C (Fig. 7B). TrE cultured in BFGM was positive for SP-C expression after 3 days in culture (Fig. 7D), and the intensity of expression was increased after 6 days (Fig. 7E). Tissue-section in situ hybridization using a radioactive probe, which is more sensitive than with a nonradioactive probe, showed that most of the cells in the BFGM-treated TrE were positive for SP-C (Fig. 7, F and G). Addition of chlorate to TrE cultured in BFGM resulted in no detectable SP-C by WM-ISH after 3 (Fig. 7H) or 6 (Fig. 7I) days. Tissue-section in situ hybridization, however, revealed that SP-C was induced in a few cells, similar to the results seen in chlorate-treated LgM + TrE recombinants (Fig. 3, I and J). TrE cultured in BFGM plus chlorate for 3 days, washed, then cultured an additional 3 days in BFGM alone showed strong expression of SP-C (Fig. 7, L–N), indicating that the competence to respond to the inductive components of BFGM was not irreversibly inhibited by chlorate.

**Effects of chondroitinase ABC and heparinase treatment on lung growth, morphogenesis, and differentiation.** Sodium chlorate inhibits sulfation of all PGs and thus does not allow the determination of the relative importance of different classes of PGs. We therefore treated cultured intact lung tips, or LgM + LgE, or LgM + TrE recombinants with GAG degrading enzymes to assess how disruption of specific classes of PGs affected lung growth and morphogenesis. Compared with control tissues (Fig. 8, A–C), treatment with heparinas I and III had a modest inhibitory effect on branching in intact lung tips (Fig. 8D), LgM + LgE recombinants (Fig. 8E), and LgM + TrE recombinants (Fig. 8F). To confirm that enzyme treatment in vitro was effective, we stained heparinase-treated lung tips with a 3G10, a monoclonal antibody that specifically reacts with desaturated hexuronate residues remaining on HS chains after heparinase digestion. As shown in Fig. 8G, untreated explants did not stain with 3G10, but incubating sections of these tissues with heparinas I and III resulted in 3G10 staining (Fig. 8H). Treatment of explants with heparinas I and III during the culture period effectively digested HS

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**Fig. 6. Sodium chlorate inhibits the growth of lung and tracheal epithelium in mesenchyme-free culture.** E13 LgE enrobed in growth factor-reduced Matrigel (A) and cultured in BFGM medium (see MATERIALS AND METHODS for contents) shows extensive growth after 3 (B) and 6 (C) days of culture. Addition of chlorate to the medium partially suppresses growth after 3 (D) and 6 (E) days. The effects of chlorate are not toxic and are reversible, since washing chlorate from the cultures after 3 days (F) results in the reinitiation of robust growth after 3 (G) or 6 (H) days. Removal of chlorate from the medium after 3 days (I) results in increased growth (J). B and C, D and E, and F and G are sequential micrographs of the same LgE rudiments. E13 TrE gives a similar response. The initial epithelial tube (H) shows rapid growth in response to BFGM after 3 (I) and 6 (J) days of culture, and this is partially suppressed by chlorate on days 3 (K) and 6 (L). Removal of chlorate from the medium after 3 days (M) results in increased growth (N). I and J, K and L, and M and N are sequential micrographs of the same TrE rudiments. All panels are at the same magnification; bar = 500 μm.

AJP-Lung Cell Mol Physiol • VOL 285 • DECEMBER 2003 • www.ajplung.org
chains in both the epithelium and mesenchyme (Fig. 8I). In contrast to the limited effects seen with heparinases, treatment with chondroitinase ABC had a dramatic effect on the growth and morphogenesis of all three types of explants. Branching was completely inhibited and the epithelia formed small cysts within the mesenchyme (Fig. 8J–L).

We next examined cultured lung tips and tissue recombinants treated with GAG degrading enzymes for SP-C expression. Consistent with the modest effects we observed on morphogenesis, treatment with heparinases I and III had no apparent effect on the intensity of SP-C signal or on its spatial distribution (Fig. 9D–F) compared with controls (Fig. 9A–C). The effects we observed with chondroitinase ABC treatment were striking: in spite of the significantly reduced growth and lack of branching, SP-C was still maintained in distal epithelium, either in the intact tips (Fig. 9G) or in LgM + LgE recombinants (Fig. 9H). Furthermore, the inhibitory effects of chondroitinase ABC on growth and morphogenesis did not prevent LgM from reprogramming TrE to express SP-C (Fig. 9I). Combined treatment with heparinases and chondroitinase ABC had no more effect on SP-C expression than either treatment alone (data not shown).

Chondroitinase ABC acts directly on the epithelium. Because CSPGs are present in both epithelial and mesenchymal tissue compartments, we examined the effects of chondroitinase ABC on purified TrE cultured under conditions that induce an alveolar type II cell phenotype. When cultured in BFGM for 6 days, embryonic tracheal epithelial cells proliferated rapidly and the size of the rudiments increased dramatically (Figs. 10A and 6H–J). Addition of chondroitinase ABC to this medium resulted in a clear decrease in the amount of growth (Fig. 10B), although the rudiments still increased in size. These effects of chondroitinase ABC were reversible, since washing the enzyme out after 3 days of culture followed by 3 days in BFGM alone resulted in increased epithelial expansion (Fig. 10C). Examination of these cultures for the induction of SP-C showed that chondroitinase ABC, unlike chlorate, had no apparent effect on the reprogramming of TrE to express a distal lung phenotype (Fig. 10D–F).

DISCUSSION

Sulfated PGs are required for lung growth and morphogenesis. Given the widespread distribution of PGs and their diverse activities, it is not surprising that...
they have been shown to be important mediators of the epithelial-mesenchymal interactions governing the growth and differentiation of many tissues (78, 80). Whereas the importance of epithelial-mesenchymal interactions in lung development has long been appreciated (37, 52, 67), the involvement of PGs in lung organogenesis has been largely unexplored. In this paper we have shown a requirement for PG sulfation in the
growth, morphogenesis, and differentiation of lung explants, tissue recombinants, and embryonic respiratory epithelia in vitro. Importantly, we have demonstrated that one class of PGs, the CSPGs, plays a critical role in supporting normal growth and branching but is not involved in inducing distal lung epithelial differentiation.

Sodium chlorate competes with sulfate in the synthesis of phosphoadenosine 5'-phosphosulphate, which is the sulfate donor utilized by all of the sulphotransferases that catalyze the sulfation of polysaccharides (22). Cells treated with chlorate produce PG core proteins to which GAG side chains are linked, but the GAG chains are not sulfated and therefore have altered charge characteristics. Treatment of embryonic lungs with chlorate significantly disrupted normal branching morphogenesis and had a modest, but significant, inhibitory effect on overall lung DNA content. That this disruption occurred prominently in distal signaling centers was confirmed when we cultured individual distal tips, where chlorate not only decreased the number of distal lung buds but also caused significant changes in gross morphology.

Some insight into how chlorate disrupts distal lung signaling centers can be gained from the results of our chemoattraction experiments. Experiments from other groups have established that FGF10, which is produced by the distal LgM (6) and is absolutely required for lung development (39, 58), is also a potent chemoattractant for LgE (45, 73). One well-documented function of HSPGs is that the HS component can bind FGFs. This binding induces or stabilizes the formation of tyrosine kinase FGFR dimers, or a ternary complex of ligand, FGFR, and HSPG, which are necessary for the initiation of the signaling cascade (38, 46). Our observation that chlorate completely abolished the migration of LgE toward LgM suggests that chlorate rendered critical HSPGs and, perhaps, CSPGs (see below) nonfunctional, thereby suppressing FGF10-induced signaling and thus disrupting the spatial regulation of budding of the LgE. The possibility that chlorate affected the expression of either FGF10 itself or its predominant receptor, FGFR2IIIb, was eliminated by our real-time PCR data. Our observations are in agreement with recent studies (32, 33) showing that the regional pattern of HSPG sulfation critically influenced FGF10 induced budding in embryonic mouse lungs. Our FGF10 bead chemoattraction experiments further underscored this conclusion and also provided additional insights. Whereas at a low concentration of FGF10 we saw no chemoattraction, increasing the concentration of FGF10 10-fold resulted in chemoattraction that appeared similar to that seen when LgM was used as the chemoattractive source; that is, the epithelium elongated and migrated toward the bead. Like the LgM chemoattraction experiments, addition of chlorate to these cultures completely inhibited chemoattraction. A further 10-fold increase in FGF10 concentration resulted in a more robust chemoattraction, and the addition of chlorate to these cultures had no effect on chemoattraction. The most likely explanation for the different effects of chlorate in these experiments is that high levels of FGF10 obviate the need for PG coreceptors. This conclusion is supported by observations in Drosophila embryos (36), where overexpression of branchless, the Drosophila homolog of FGF10, can overcome the requirement for HSPG sulfation in FGF-mediated tracheal development.

Sodium chlorate has been shown to inhibit ureteric bud branching in the developing kidney (15), which is associated with decreases in the expression of Wnt-11 (34) and BMP-7 (26). BMP-4 plays a role in lung morphogenesis (7, 74), has been reported to increase lung branching (8, 63) and is induced by FGF10 (73). Because treatment of cultured lung tips with chlorate both inhibited morphogenesis and partially suppressed BMP-4 expression, we hypothesized that treatment with exogenous BMP-4 might circumvent the inhibitory effects of chlorate in distal tip cultures. We found that 200 ng/ml BMP-4, the concentration used to increase branching in mouse lungs, did not overcome the effects of chlorate on rat distal lung tip branching. Furthermore, we did not observe a BMP-4-induced increase in branching in control cultures. We do not know the basis for the difference between our results and those seen in mouse lung explants. Our results, however, suggest that downstream mediation of FGF signaling in the lung is more complex than the activation of BMP-4 signaling.

Although chlorate had a substantial effect on the growth and branching of cultured intact distal lung tips, it had an even more pronounced effect on tissue recombinants. Chlorate treatment of LgM + LgE recombinants severely inhibited epithelial branching and prevented branching in LgM + TrE recombinants altogether. We believe that we observed a more severe effect of chlorate in tissue recombinants because the proteolytic treatment (dispase) required for clean tissue separation degrades PGs in both the ECM and on cell surfaces. Therefore, only PGs produced de novo may be available in tissue recombinants, and those synthesized in the presence of chlorate lack the proper sulfation necessary for normal PG function. In contrast, intact tips not treated with dispase contain pre-synthesized PGs that can support normal development for a limited amount of time in the presence of chlorate.

Our experiments using mesenchyme-free cultures demonstrated that PGs produced by embryonic lung and tracheal epithelia themselves are required for responsiveness to inductive signals, since growth was suppressed when either type of epithelium was cultured in BFGM containing chlorate. Our observation that the inhibition of growth was reversed upon washing chlorate out of the cultures showed that chlorate was not generally cytotoxic. Importantly, addition of 10 mM sodium sulfate to the medium also overcame the inhibitory effects of chlorate, indicating that growth suppression by chlorate was due to its effects on sulfation. The inhibitory effect of chlorate on the growth of LgE and TrE in mesenchyme-free culture, however, was not as dramatic as that seen in tissue recombinants, where growth was completely inhibited. This is
probably due to the fact that BFGM contains higher concentrations of FGFs than are normally present in vivo, which may be able to partially circumvent the requirement for low affinity HSPG coreceptors. Alternatively, sulfated PGs may be required for the production of inductive signals by LgM; this would not affect epithelia in mesenchyme-free cultures, where the factors necessary for both growth and differentiation are supplied exogenously.

**Sulfated PGs are required for the induction of SP-C.** In addition to its inhibitory effects on epithelial growth, chlorate also significantly suppressed expression of the distal lung epithelial marker, SP-C. Like its effects on growth and branching, this was more apparent in tissue recombinants than in cultured intact distal lung tips. LgM + TrE recombinants were the most affected, where only a few cells expressed detectable SP-C. This demonstrates that sulfated PGs are not only required for the maintenance of distal lung epithelial differentiation, but are particularly important for the induction of SP-C. The requirement for sulfated PGs in SP-C induction is underscored by the observation that only a few cells were induced to express SP-C when TrE was cultured mesenchyme-free in BFGM in the presence of chlorate. This inhibition of SP-C induction occurred despite the fact that chlorate did not completely suppress TrE growth. These observations indicate that sulfated PGs in the TrE are necessary for its competence to respond to the signals that induce SP-C. These signals are most likely FGFs, which we have shown are necessary, but not sufficient, to reprogram tracheal epithelial cells to adopt a distal lung fate (61). The loss of response to FGFs by chlorate-treated TrE was not permanent and not due to toxicity, however, since washing chlorate from the medium after 3 days allowed SP-C to be induced in almost all of the cells after an additional 3 days of culture. The need for PG sulfation in the induction of SP-C in TrE stands in contrast to our results in mesenchyme-free cultures of LgE, which showed little diminution of SP-C expression in response to chlorate. As noted above, this may occur because the concentrations of growth factors, especially FGFs, in BFGM exceed those produced by LgM. It appears, therefore, that the requirement for sulfated PGs in the induction of SP-C is particularly stringent but, after induction has occurred, can be overcome by high levels of FGFs.

**HSPGs in lung development.** HSPGs are multifunctional molecules that are involved in diverse processes such as cell adhesion and the binding of growth factors, which are mediated by their HS chains. HSPG binding may protect growth factors from protease degradation (27), sequester them near their site of function (24), or maintain them in active or inactive states (48). Evidence is accumulating that the binding of growth factors to HS chains is highly specific and is regulated by changes in sugar epimerization and variable sulfation (42). For example, FGF2 and FGF4, which bind the same high-affinity tyrosine kinase receptors, show tissue-specific differences in their ability to bind HS chains and assemble into FGF/FGFR complexes (4).

Such differences might explain why FGF7 and FGF10 have such dramatically different effects on lung morphogenesis and differentiation, even though they both bind only the FGFR2IIIb splice variant in LgE and TrE.

The ability of HSPGs to bind FGFs predicts that they might play a role in lung development. This has been demonstrated in the rat lung by disrupting normal HSPG function by heparin treatment in vitro. Heparin, which is a highly sulfated form of HS, decreased branching morphogenesis but had no effect on cell proliferation (50). These effects were thought to be due to heparin preventing the effective binding of heparin binding growth factors to their receptors. Recent in vitro studies by Izvolsky and colleagues (32, 33) have refined this concept by demonstrating that varying O-sulfation levels of HSPGs are important for different aspects of FGF10 signaling. HSPGs also play an important role in the developing lung by interacting with laminin to facilitate assembly of the basement membrane at epithelial-mesenchymal interfaces, which is critical for establishing a polarized epithelium (56).

A role for HSPGs in lung development in vivo has been recently demonstrated by two groups (21, 49) who generated mice with a targeted deletion of the bifunctional enzyme N-deacetylase/N-sulfotransferase-1 (NDST-1), which plays a primary role in determining the final structure of the HS polysaccharide. NDST-1 null animals died neonatally from respiratory distress, and morphological examination revealed that the distal lung acini were atelectatic and had immature alveolar type II cells. The retarded maturation of type II cells occurred even though overall lung size appeared the same as in wild-type littermates.

The foregoing observations suggest that HSPGs may play a critical role in type II cell differentiation but may not be irreplaceable in lung growth and morphogenesis. This is consistent with our observations when we treated distal lung tips or tissue recombinants with heparinase. Our results are in agreement with those of Toriyama et al. (68), who reported that mouse lung explants treated with heparinase continued to branch, although the number of distal buds decreased somewhat. They also observed that inhibition of branching in their cultures could be overcome by the addition of exogenous FGF2, again suggesting that the need for sulfated PGs may be circumvented if concentrations of ligand are sufficiently high.

Given the known involvement of HSPGs in signaling by FGFs and other ligands, we expected a pronounced effect of heparinase treatment but saw only a slight decrease in branching. Several explanations for these observations are possible. First, although 3G10 immunostaining for desaturated hexuronate residues showed that our heparinase preparations were active, we do not know quantitatively how much HS was degraded; an earlier study (68) showed that treatment of 35S-labeled mouse lung explants with heparinase decreased labeling in the HS fraction by only 40%. It should also be noted that the 3G10 antibody will react with desaturated hexuronate residues at any point
along the HS chain, depending on the extent of heparinase digestion. It is therefore possible that we were detecting HS chains that had only been partially degraded and that these might still retain sufficient activity to modulate growth factor binding and signaling. Indeed, heparinase sequences containing as few as eight saccharides promote FGF2-FGFR binding and activation (41). Second, since it is likely that heparinase activity declined over the 3-day culture period, HS chains in HSPGs generated de novo may not have been digested, and these may have allowed normal HSPG function. Incompletely degraded HSPGs might also explain why we did not see the inhibition of SP-C expression in tissues treated with heparinase that we observed with chlorate treatment, which effectively disrupts function of all PGs. An alternative explanation that cannot be discounted, however, is that chlorate affects the sulfation of bioactive molecules other than PGs that are necessary for the induction and maintenance of SP-C expression.

**CSPGs in lung development.** Because of their ability to bind and modulate the function of growth factors, most studies on the role of PGs in lung development have focused on HSPGs. CSPGs, however, are also abundantly present in the developing (71) and adult (54) lung as components of the ECM and basement membrane. Furthermore, nominal HSPGs such as the syndecans (47) carry both HS and CS chains, and it has been suggested (30) that most, if not all, HSPGs may contain both HS and CS side chains. The possibility that CSPGs may affect lung development was suggested by studies in which cultured lung explants were treated with p-nitrophenyl-β-D-xylopyranoside (β-xyloside), which competes with xylosylated PG core proteins for the addition of GAG chains at the level of the first galactosyltransferase (40). β-Xyloside treatment of E11.5 mouse lungs resulted in decreased branching (64), and a subsequent study (65) revealed that β-xyloside treatment inhibited CSPG synthesis in lung explants while leaving HSPG synthesis unaffected. These results are in agreement with our observations, which are the first to directly demonstrate a primary role for CSPGs in lung morphogenesis. Chondroitinase ABC treatment inhibited both growth and branching in all types of cultures. The effects of chondroitinase on cultures of intact distal tips were actually more pronounced than those seen with chlorate treatment. This may have occurred because intact tips contained residual CSPGs that may have been able to sustain a reduced amount of growth in the presence of chlorate, but treatment with chondroitinase effectively eliminated CS side chains and suppressed branching completely. Considered together with the results from β-xyloside-treated lung explants, our data suggest that CSPGs play a critical role in lung growth and branching morphogenesis. Furthermore, the CSPGs important for growth must be produced by the epithelium, since chondroitinase acted directly on the TrE to suppress its growth in mesenchyme-free culture, and washing chondroitinase from the medium allowed growth to resume.

A potential mechanism by which CSPGs are involved in lung morphogenesis is suggested by the recent demonstration (17) that the oversulfated chondroitin sulfate, CS-E [GlcUAβ1–3GalNAc (4S, 6S)], specifically binds a number of heparin binding growth factors, including FGF10. CS-E is found in the developing rat brain in significant amounts (70) and is also a component of syndecan-1 purified from mammary epithelial cells (69). The embryonic mouse LgE contains syndecan-1, but it cannot be assumed that CS-E is also present, because GAG side chain modifications may be tissue specific. If the disaccharide composition of CSPGs in the embryonic lung does include CS-E, however, the lack of growth and branching we observed resulting from chondroitinase treatment would be consistent with a blockade of FGF10 signaling.

Although CSPGs may play an important role in lung growth and morphogenesis, they are apparently not involved in specification of the distal lung epithelial phenotype. Chondroitinase treatment clearly inhibited the growth of TrE that was recombined with LgM but did not prevent the induction of SP-C. Similar to the tissue recombinants, chondroitinase treatment of TrE in mesenchyme-free culture significantly suppressed growth but did not affect its reprogramming by BFGM to express a distal lung marker.

In summary, we have shown that sulfated PGs are necessary for lung growth, branching, and morphogenetic movement, as well as for the induction of the distal lung epithelial marker SP-C. Our data demonstrate that CSPGs are dynamically involved in lung growth and branching and that these CSPGs are associated with the epithelium. The mechanism for how CSPGs affect lung growth and morphogenesis is not yet understood, but our results are consistent with a role for CSPGs in mediating aspects of FGF signaling. Understanding the mechanisms by which CSPGs affect lung growth and morphogenesis awaits the identification and full characterization of the CSPGs present in the developing lung.

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**DISCLOSURES**

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