Mesenchymal maintenance of distal epithelial cell phenotype during late fetal lung development

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Deimling J, Thompson K, Tseu I, Wang J, Keijzer R, Tanswell AK, Post M. Mesenchymal maintenance of distal epithelial cell phenotype during late fetal lung development. Am J Physiol Lung Cell Mol Physiol 292: L725–L741, 2007. First published November 17, 2006; doi:10.1152/ajplung.00221.2006.—Classical tissue recombination experiments have reported that at early gestation both tracheal and distal lung epithelium have the plasticity to respond to mesenchymal signals. Herein we examined the role of epithelial-mesenchymal interactions in maintaining epithelial differentiation at late (E19–E21, term = 22 days) fetal gestation in the rat. Isolated distal lung epithelial cells were recombined with mesenchymal cells from lung, skin, and intestine, and the homotypic or heterotypic recombinant cell aggregates were cultured for up to 5 days. Recombining lung epithelial cells with mesenchyme from various sources induced a morphological pattern that was specific to the type of inducing mesenchyme. In situ analysis of surfactant protein (SP)-C, SP-B, and Clara cell secretory protein (CCSP) expression, as well as SP-C and CCSP promoter transactivation experiments, revealed that distal lung epithelium requires lung mesenchyme to maintain the alveolar, but not bronchiolar, phenotype. Incubation of lung recombinants with an anti-FGF7 antibody resulted in a partial inhibition of mesenchyme-induced SP-C promoter transactivation. Immunoreactivity for Delta and Lunatic fringe, components of the Notch pathway that regulates cell differentiation, was downregulated in the heterotypic recombinants. In contrast, Hes1 mRNA expression was increased in these recombinants. Cumulatively, these results suggest that at late fetal gestation, distal lung epithelial cells are not fully committed to a specific phenotype and still have the plasticity to respond to various signals. Their alveolar phenotype is likely maintained by Notch/Notch ligand interactions and mesenchymal factors, including FGF7.

epithelial-mesenchymal interactions; cell differentiation; Notch signaling; fibroblast growth factors

DURING LUNG MORPHOGENESIS numerous different cell phenotypes are formed along the anterior-posterior axis of the developing epithelial airways, each with different morphologies and patterns of gene expression. This anterior-posterior epithelial patterning of airways is characterized by distinct boundaries and is likely controlled by epithelial-mesenchymal interactions (37). Classical tissue recombination experiments have shown that epithelial-mesenchymal interactions are required for branching of the lung epithelium in both avian and mammalian systems (27, 34, 44, 48). When distal lung mesenchyme is grafted onto tracheal epithelium, branching occurs in a pattern that is remarkably similar to that seen in the developing lung (4). However, studies in which tracheal epithelium were cultured with grafted mesenchyme from intestine or skin demonstrated simple bud formation without significant branching (51). Furthermore, the grafting of tracheal mesenchyme to distal epithelium denuded of its own mesenchyme inhibits all distal epithelial branching (51). These studies clearly demonstrate a key difference in the instructive capabilities of embryonic lung and tracheal mesenchyme and suggest a specific requirement of lung epithelial cells for lung mesenchyme in order for proper branching morphogenesis to occur.

More recent studies indicate that epithelial-mesenchymal interactions are also important for lung epithelial cell differentiation. Tracheal graft studies have demonstrated that distal lung mesenchyme is a potent inducer of distal epithelial phenotype when recombined with tracheal epithelium. The induced tracheal epithelium adopted an alveolar type II cell phenotype as determined by the presence of lamellar bodies and expression of surfactant protein C (SP-C), a specific marker for type II cell differentiation (36). Similarly, tracheal mesenchyme induces a tracheal epithelial phenotype when grafted to distal lung epithelium (39). These data suggest that during early lung development, epithelium of the respiratory lineage has the plasticity to respond to various mesenchymal cues originating from the respiratory tract. The regulatory molecules involved in epithelial-mesenchymal signaling during lung development include ECM proteins, cell-associated signaling molecules, and various diffusible factors (19, 37, 50, 54).

Although the importance of epithelial-mesenchymal interactions in early lung development is now appreciated, less is known about the role of these interactions in lung cell morphogenesis and differentiation at late fetal gestation. In the present study, we examined the effect of both homotypic and heterotypic mesenchyme on lung epithelial morphology and differentiation during relatively late fetal gestation. We employed a cell recombination model to study these cell-cell interactions in vitro. This system allows studying the importance of epithelial-mesenchymal interactions at both early and late gestation, as well as the effect of mesenchyme, from both the same and alternate sources, on lung epithelial cell morphology and differentiation. The results reported herein suggest that, at late gestation, distal epithelial cells, particularly type II...
Fig. 1. Morphology of homotypic and heterotypic cell recombinants. A: 4,6-diamidino-2-phenylindole nuclear staining (a–f) was performed on E13 (a–c) and E19 (d–f) lung epithelial (a and d) and mesenchymal (b and e) cells cultured alone or as recombinants (c and f). E13 and E19 recombinants (c and f), but not epithelial or mesenchymal cells alone, adopted a lung-like appearance. B: light microscopy of E19 epithelial cells alone (a) or recombined with either E19 lung (b), skin (c), or intestinal (d) mesenchymal cells, illustrating the appearance of the structures (arrows) after 5 days of culture.
cells, are not committed to a specific phenotype but rather have the plasticity to respond to mesenchymal signals of various origins (i.e., lung, skin, and intestine).

MATERIALS AND METHODS

Materials. Animal protocols were in accordance with Canadian Council of Animal Care guidelines and were approved by the Animal Care and Use Committee of the Hospital for Sick Children (Toronto, ON, Canada). Female (200–250 g) and male (250–300 g) Wistar rats were obtained from Charles River (St. Constant, QC, Canada). The animals were kept in a controlled light-dark cycle and were supplied food and water ad libitum. Rats were mated overnight, and the finding of a sperm-positive vaginal smear was designated as day 0 of gestation. Rats were euthanized at E13, E19, and E21 of gestation (term = 22 days). Culture media, antibiotics, fetal bovine serum (FBS), and ascorbic acid were from GIBCO (Grand Islands, NY). Cell culture plates (24 wells) were from Nunc (Intermed, Denmark), and 4-μm Whatman Nuclepore polycarbonate membranes were from Integra Environmental (Burlington, ON, Canada). Mouse monoclonal anti-vimentin (V9 clone) was from Serotec (Mississauga, ON, Canada).
whereas mouse monoclonal anti-cytokeratin (AE1/AE3 clone) was from Roche (Laval, QC, Canada). Rabbit polyclonal antibodies against pro-SP-C and Clara cell secretory 10-kDa protein (CCSP) were kind gifts from Drs. J. Whitsett (Cincinnati, OH) and S. L. Kaytal (Pittsburg, PA), respectively. Goat polyclonal antibodies against Jagged1, Delta, and Lunatic fringe were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit, donkey anti-goat, and sheep anti-mouse biotinylated secondary antibodies were obtained from Calbiochem (LaJolla, CA) and Santa Cruz Biotechnology. A kit for avidin-biotin peroxidase complex immunostaining was purchased from Vector Laboratories (Burlingame, CA). Lipo-fectamine was from GIBCO. The Phospha-Light kit was from Tropix (Bedford, MA), and the β-galactosidase enzyme assay system was obtained from Promega (Madison, WI). The SP-C promoter, a gift from Dr. J. Whitsett (Cincinnati, OH), was subcloned in the pSEAP2-Basic vector (Clontech, CA). The CCSP promoter-secreted alkaline phosphate (SEAP)2 construct was obtained from Dr. J. Hu (Toronto, ON). FGF7 and anti-FGF7 antibody were both purchased from R&D (Minneapolis, MN).

Cell isolation. Timed-gestation Wistar rats were euthanized by exposure to diethyl ether. The fetuses were delivered by caesarean section using aseptical surgical techniques. Lungs were dissected from embryos at E13 and transferred to Hanks’ balanced salt solution (HBSS-). The E13 lungs were treated with 20% (vol/vol) dispase for 30 min at 37°C to loosen the tissue layers. Following neutralization of enzyme activity with FBS, the endodermal and mesodermal compo-

Fig. 3. Expression of surfactant protein (SP)-C and Clara cell secretory protein (CCSP) in E13 homotypic and heterotypic cell recombinants. Nonradioactive in situ hybridization using digoxigenin-labeled SP-C (A,a and B,a,c,d) and CCSP (A,b and B,b,d,f) riboprobes was performed on E13 lung epithelium/E13 lung mesenchyme (A, a and b) and E13 lung epithelium/E19 lung mesenchyme (B, a and b) homotypic recombinants and E13 lung epithelium/E19 skin mesenchyme (B, c and d) and E13 lung epithelium/ E19 intestinal mesenchyme heterotypic recombinants (B, e and f). Dark blue color indicates positive staining. Sections were counterstained with methyl green. E13/E13 homotypic recombinants showed weak SP-C expression; however, SP-C transcripts were readily detected in E13/E19 homotypic recombinants. No SP-C mRNA was detected in the E13/E19 heterotypic recombinants. No CCSP mRNA expression was noted in any of the recombinants.
nents at the tips of the lung buds were separated manually under a
dissection microscope using 27-gauge needles. The separated rudiment layers were broken up into a single cell suspension by gentle
agitation. At E19 and E21, lungs, skin, and intestine were dissected
from the fetuses in HBSS, and epithelial and mesenchymal cells
were separated by using standard primary culture cell techniques, as
previously described (8). The purity of each cell type was ×90%. All
recombination experiments were performed within 24–48 h of cell
isolation. At the time of recombination E19, but not E13, epithelial
cells expressed SP-C mRNA.

Transfection of epithelial cells. E19 fetal lung epithelial cells were
transiently transfected with 0.9 μg of SP-C-SEAP2 or Clara cell 10
kDa protein (CCSP)-SEAP2 plasmid and 0.1 μg of internal control
(pCMV-β-galactosidase) plasmid per 10-cm² dish, using cationic
liposomes (Lipofectamine) and plasmid DNA at a ratio of 12:1.
Transfected cells were incubated for 24 h before recombination with

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**Fig. 4.** Expression patterns of SP-C, SP-B, and CCSP mRNA in the developing rat lung. Section in situ hybridization was used to determine SP-C (A–E), SP-B (F–J), and CCSP (K–O) mRNA levels in the developing rat lung. Positive staining is indicated by the dark blue color. Sections were counterstained with methyl green. All 3 genes were expressed at late fetal rat gestation.
mesenchymal cells. In some cases, the transfected cells were incubated for another 24 h with and without 100 ng/ml FGF7.

**Cell recombinations.** Transfected and control (untransfected) fetal lung epithelial cells were cultured alone or recombined in ratios of 1:1 with lung, skin, or intestinal mesenchymal cells of the same or a different gestation. Mesenchymal (E13) cells and E19 fibroblasts were also cultured alone. To recombine the cells, individual cell populations were trypsinized from tissue culture flasks and counted with a Coulter cell counter. Approximately $3 \times 10^6$ endodermal (E13) or epithelial (E19, E21) cells were mixed with $3 \times 10^6$ mesenchymal (E13) cells or fibroblasts (E19, E21), and the mixtures were then centrifuged at 300 g for 5 min. Excess medium was removed, and the cell aggregates were incubated at 37°C in an atmosphere of 5% CO$_2$ in air for 1 h. Aggregates were then gently loosened by stirring with the tip of a micropipet and transferred to porous membranes (4-μm pore size) in 24-well tissue culture plates. Membranes were presoaked in DMEM for 1 h before addition of cells. A 250-μl aliquot of DMEM containing 5% (vol/vol) heat-inactivated FBS was then added to each well, such that it just moistened the surface of the cell aggregates but without the aggregates being submerged. The cell recombinants were then cultured at 37°C in an atmosphere of 5% CO$_2$ in air for 5 days. In some experiments, 10 μg of either rabbit anti-FGF7 antibody or nonimmune rabbit IgG were added to the cultures after the first 24 h of incubation.

**Mesenchyme-free lung endodermal bud culture.** Lungs were dissected from E12 embryos and transferred to HBSS$.\textsuperscript{-}$$. The lungs were treated with 20% (vol/vol) dispase (GIBCO) for 30 min at 37°C to loosen the tissue layers. Following neutralization of enzyme activity with FCS, the endodermal and mesodermal components at the tips of the lung buds were separated manually under a dissection microscope using 27-gauge needles. The distal endodermal buds were then embedded in 300 μl growth factor-reduced Matrigel (BD Biosciences, Bedford, MA), diluted 1:1 in DMEM with and without 100 ng/ml FGF-7. For the sectioning, lung bud cultures were fixed overnight in ice-cold 70% (vol/vol) ethanol, dehydrated, and embedded in paraplast. Sections (7 μm) were stained with Carrazzi hematoxylin or used for in situ hybridization.

**Reporter gene assays.** After 5 days in culture, the medium from the recombinants was collected and frozen at $-20$°C until further use in the SEAP assay. The cell aggregates were then washed with phosphate-buffered saline (PBS), lysed, and sonicated, and β-galactosidase activity was measured. The SEAP assay was performed as described in the Phospha-Light kit (Tropix). Briefly, the collected medium was diluted and heated for 1 h at 65°C to inactivate endogenous alkaline

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**Fig. 5.** Immunocytochemistry, using anti-cytokeratin (A–C) and anti-vimentin (D–F) monoclonal antibodies, was performed on E19 homotypic and heterotypic cell recombinants to analyze tissue morphology. Brown color indicates positive immunoreactivity. Sections were counterstained with hematoxylin. Lung epithelial cells in homotypic recombinants (A and D) were organized into structures that had a saccular, alveolar-like appearance, whereas lung epithelial cells in heterotypic recombinants (B–F) adopted a morphology that was specific to the associated mesenchyme, i.e., skin- (B and E) and intestine-like (C and F) morphologies.
phosphatases. Equal volumes of sample, assay buffer, and reaction buffer were added to the wells of a luminometer plate, and chemiluminescence was measured. SEAP2 luminescence levels were normalized to β-galactosidase activity to account for variations in transfection efficiency. All promoter analyses were carried out in duplicate for four separate experiments.

**Immunohistochemistry.** Cell recombinants of epithelial and mesenchymal cells were fixed in 4% (vol/vol) paraformaldehyde, embedded in paraplast, cut in 5-μm sections, and mounted on Superfrost slides (Fisher Scientific, Unionville, ON, Canada). Tissue sections were dehydrated, endogenous peroxidase activity inactivated, and nonspecific binding blocked with 5% (wt/vol) normal goat serum (NGS) and 1% (w/v) bovine serum albumin (BSA) in PBS. Tissue sections were then incubated with first antibody solution in NGS-BSA at 21°C for 1 h and biotinylated secondary antibody solution in NGS-BSA for an additional hour, with extensive washing with PBS in each interval. Slides were incubated for another 2 h with a strepavidin-biotinylated peroxidase complex, followed by color development using 3,3'-diaminobenzidine as substrate. Tissue sections were then counterstained with Carazzi hematoxylin, dehydrated, and prepared for viewing. Anti-cytokeratin, anti-vimentin, anti-pro-SP-C, anti-CCSP, anti-jagged1, anti-Delta, and anti-Lunatic fringe were diluted 1:5, 1:2, 1:500, 1:2,000, 1:25, 1:25, or 1:400, respectively, and used as primary antibodies. The biotinylated secondary antibodies to rabbit (goat IgG), goat (donkey IgG), and mouse (ovine IgG) were diluted 1:300.

**Synthesis of riboprobes.** Rat specific SP-B, SP-C, and CCSP cDNA fragments were generated by RT-PCR and inserted into the EcoRI site of PCR 2.1 vector (Invitrogen, San Diego, CA). The identity and orientation of the cloned fragments were confirmed by sequencing. Notch receptor (Notch 1 and 3), Notch ligand (Jagged1, Delta1), and Hes1 probes in pGEM-T vectors were kind gifts from Dr. Sean Egan (Toronto, ON, Canada). The plasmid DNA was linearized with appropriate restriction enzyme and in vitro transcribed with SP6 or T7 RNA polymerase and digoxigenin-labeled according to a protocol provided by the manufacturer (Roche).

**In situ hybridization.** Tissue sections were rehydrated and washed in PBS. Pretreatment included postfixation in 4% (wt/vol) paraformaldehyde (20 min), followed by proteinase K digestion (20
mg/ml, 17 min, 25°C) and acetylation [0.1 M triethanolamine, 0.25% (vol/vol) acetic anhydride, 10 min, 25°C]. Sections were then dehydrated and air-dried before addition of the hybridization solution. Digoxigenin-labeled probes were then added to freshly prepared hybridization solution [50% (wt/vol) deionized formamide, 10% (wt/vol) dextran sulfate, 1.5 × Denhardt’s reagent, 0.5 mg/ml yeast tRNA, 0.3 M NaCl, 0.005 M EDTA, and 0.025 M Tris (pH 7.5)] at a concentration of 1 ng/μl. Following denaturation at 80°C, probe solution was added to the tissue section and incubated overnight at 55°C. After brief washes with 5 × SSC and 50% (wt/vol) formamide at 55°C, the tissue was treated with RNaseA (10 μg/ml) for 30 min at 37°C, followed by PBS-T (PBS containing 0.1% Tween-20) washes. Subsequently, the sections were incubated with anti-digoxigenin alkaline phosphatase (1:1,000) in blocking solution (Roche) at 4°C. The next day, sections were washed in PBS-T, followed by washes in NTM [100 mM NaCl, 100 mM Tris (pH 9.5), and 50 mM MgCl2] and then incubated with nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyolphosphate (Roche) at room temperature until purple color ap-

Fig. 7. Expression of SP-B and SP-C in E19 homotypic and heterotypic cell recombinants. Section in situ hybridization for SP-B (G–I) and SP-C (A–C) was performed on E19 lung epithelial cells recombined with either E19 lung (A and G), skin (B and H), or intestinal (C and I) mesenchymal cells. Dark blue color indicates positive staining. Sections were counterstained with methyl green. Both SP-B and SP-C transcripts were present in E19 homotypic recombinants but absent in E19 heterotypic recombinants. Immunostaining for pro-SP-C (D–F) was performed on all tissues to assess protein expression. Brown color indicates positive immunoreactivity. Sections were counterstained with hematoxylin. Pro-SP-C protein was detected in E19 epithelial cells of all recombinants.
peared (4 to 5 h). All color reactions were arrested at the same time to make comparisons at different stages of development possible. After color development, sections were washed in distilled water, counterstained with either methyl green or nuclear fast red, dehydrated in a graded series of ethanol and xylene, and mounted with coverslips using Permount (Fisher Scientific). Pictures were taken by using a Leica digital imaging system.

Statistical analysis. All recombination experiments were repeated at least four times. The promoter activity data are presented as means ± SE. Statistical significance was determined by one-way analysis of variance followed by assessment of differences using Student-Newman-Keuls test. Significance was defined as $P < 0.05$.

RESULTS

Inductive capabilities of late lung mesenchyme. Since the epithelium of the early lung anlage has been shown to respond to various cues (37), we recombined E13 distal lung endodermal cells with E19 lung and nonlung mesenchymal cells to investigate their inducing capabilities. Recombinations with

![Fig. 8. Expression of SP-B, SP-C, and CCSP in E21 homotypic and heterotypic cell recombinants. Section in situ hybridization for SP-B (D–F), SP-C (A–C), and CCSP (G–I) was performed on E21 lung epithelial cells recombined with either E19 lung (A, D, and G), skin (B, E, and H), or intestinal (C, F, and I) mesenchymal cells. Dark blue color indicates positive staining. Sections were counterstained with methyl green. E21/E19 homotypic recombinants expressed all 3 genes. SP-C and SP-B transcripts were absent in the E21/E19 heterotypic recombinants, whereas CCSP mRNA was readily detected in these recombinants.](image)
E13 distal lung mesenchymal cells served as control. The morphology of homotypic (lung epithelium + lung mesenchyme) and heterotypic (lung epithelium + skin or intestinal mesenchyme) recombinants was assessed by 4,6-diamidino-2-phenylindol staining and immunostaining using monoclonal antibodies against cytokeratin, an epithelial cell marker, and vimentin, a mesenchymal cell marker. Immediately after recombination, no specific epithelial cell organizations were apparent, but, after 2 to 3 days in culture, organization of epithelial cells into patterns specific to the inducing mesenchyme was evident by light microscopy (not shown). After 5 days in culture, E13 and E19 lung epithelial or mesenchymal cells alone did not display any lung-like appearance (Fig. 1, A,a,b,d,e and B,a). In contrast, E13 or E19 lung cell recombinants had adopted a lung-like morphology (Fig. 1, A,c,f and B,b; and Fig. 2). E13 distal lung endodermal cells combined with E13 or E19 distal lung mesenchymal cells organized in open, saccular structures that were lined with cytokeratin-positive cells (Fig. 2, A,a and B,a, respectively). In these homotypic recombinants, vimentin immunostaining showed the presence of mesenchymal cells in the interstitial matrix of the tissue recombinants (Fig. 2, A,b and B,b). These data suggest that E19 lung mesenchyme is a strong morphogenetic inducer similar to E13 lung mesenchyme (4, 22, 24, 34, 51).

When E13 distal lung endodermal cells were combined with E19 skin mesenchymal cells, organization of the epithelial cells into a skin-like morphology was apparent after 5 days in culture. Cytokeratin staining in these recombinants revealed an epithelial cell patterning reflecting epidermal cell layers (Fig. 2B,c). In contrast, E13 lung endodermal cells recombined with E19 intestinal mesenchymal cells organized into cytokeratin-positive structures that somewhat resembled intestinal villi (Fig. 2B,e). The mesenchymal cells surrounding the epithelial structures in both skin and intestinal heterotypic recombinants stained positively for vimentin (Fig. 2B, d and f). Similar to lung cells, intestinal and skin mesenchymal cells alone were void of any morphogenic appearance resembling skin or intestine (not shown). The heterotypic recombinant data suggest that the E13 distal lung epithelium responds to cues of nonlung mesenchyme, in agreement with previous reports (51). In subsequent experiments we investigated whether E19 lung mesenchyme is able to induce proximal or distal epithelial cell differentiation in E13 endodermal cells. Nonradioactive in situ hybridization (ISH) revealed that CCSP transcripts, a marker for proximal epithelial cell differentiation (20), were undetectable in the E13 epithelial cells of all homotypic and heterotypic recombinants (Fig. 3, A,b and B,b,d,f). SP-C, a distal epithelial cell marker (18), expression was weakly induced in E13 epithelial cells after recombination with E13 mesenchymal cells (Fig. 3A,a). In contrast, recombination of E13 epithelial cells with E19 lung mesenchymal cells resulted in a strong induction of SP-C mRNA expression (Fig. 3B,a). SP-C transcripts were undetectable in E13 epithelial cells after recombination with E19 skin (Fig. 3B,c) and intestinal (Fig. 2B,e) mesenchymal cells. Although lactase mRNA, a marker for intestinal epithelial differentiation (31), was detected in E19 intestinal epithelial cells, no expression was noted in E13 lung epithelial cells after recombination with E19 intestinal mesenchymal cells (not shown). In addition, E19 skin mesenchymal cells did not induce expression of loricrin mRNA, a marker for the epidermal differentiation (23), in the E13 epithelial cells (not shown). Taken together, the data suggest that E19 lung mesenchyme strongly affects E13 distal epithelial patterning and differentiation. However, despite differences in structural organization, E13 distal lung epithelial cell cannot be induced to a nonlung cell phenotype, as assessed by tissue-specific markers.

Late lung mesenchyme maintains distal lung epithelial differentiation. Since E19 lung mesenchyme was a strong inducer of early lung epithelial morphogenesis and differentiation, we then investigated whether E19 lung mesenchymal cells also control the patterning and differentiation of E19 lung epithelial cells. Figure 4 shows the developmental expression patterns of both CCSP and SP-C mRNA in intact fetal rat lung using nonradioactive ISH. We used SP-B as an additional marker for
distal alveolar epithelial cells, although it has also been found to be expressed in more proximal bronchiolar epithelial cells (45). In agreement with previous reports (18, 42, 45, 52), SP-C and SP-B mRNA expression localized to the distal epithelial cell populations, whereas CCSP transcripts were detected in more proximal epithelial cells lining the major airways of the lung. Transcripts for all three genes were detected at the time of isolation, at 19 days of gestation, suggesting that the epithelial cells at this time point were committed to their lung cell phenotype (Fig. 4). Based on these findings, we hypothesized that the E19 rat lung epithelial cells are committed to their phenotype but morphogenically could be altered depending on

Fig. 10. Effects of FGF7 on E12 endodermal cells. Mesenchyme-free endodermal lung buds were cultured in growth factor-reduced Matrigel in the presence (A and B,b,d,f) or absence (B, a, c, and e) of 100 ng/ml FGF7. FGF7 stimulated branching (A and B,b) and induced SP-C (B,f), but not CCSP (B,d), mRNA expression as assessed by in situ hybridization.
their associated mesenchyme. To test this hypothesis, we employed the same recombination strategy by recombining E19 epithelial lung cells with E19 homotypic (lung) or heterotypic (skin or intestinal) mesenchymal cells. After 5 days in culture, epithelial cells of E19 homotypic recombinants had a sacculary appearance (Fig. 1A,f) with the saccules staining positively for cytokeratin (Fig. 5A). Lung epithelial cells recombined with skin mesenchyme, however, formed an epidermal-like layer of cytokeratin-positive epithelial cells that rested on multiple layers of mesenchymal cells (Fig. 5B), whereas those lung epithelial cells that were combined with intestinal mesenchymal cells formed cytokeratin-positive structures which remarkably resembled intestinal villi (Fig. 5C). Occasional saccular epithelial structures were also noted in some heterotypic recombinants. Immunostaining for vimentin supported the anti-cytokeratin morphogenetic findings for both homo- and heterotypic recombinants (Fig. 5, D–F). Nonradioactive ISH results demonstrate that CCSP is expressed in the epithelial cells of all E19 homotypic and heterotypic recombinants (Fig. 6, A–C). Immunostaining, with the use of a specific anti-CCSP polyclonal antibody, confirmed the in situ hybridization results (Fig. 6, D–F). ISH analysis of SP-C and SP-B mRNA expression in E19 recombinants yielded more surprising results (Fig. 7). Epithelial cells of homotypic recombinants expressed high levels of SP-C and SP-B mRNA (Fig. 7, A and G, respectively). However, in E19, epithelial cells recombined with either E19 skin or intestinal mesenchymal cells expression of both surfactant proteins fell below detectable levels (Fig. 7, B and C, for SP-C; and H and I for SP-B). Immunohistochemical analysis of pro-SP-C protein yielded positive staining in patterns identical to mRNA localizations in both E19 homotypic and heterotypic recombinants, indicating the presence of residual protein remaining after transcription has ceased in heterotypic recombinants (Fig. 7, D–F). Similar to the E19 heterotypic recombinants, E19, epithelial cells cultured without mesenchymely expressed CCSP but not SP-C (results not shown). Based on cytokeratin and vimentin immunostaining, the starting epithelial cell cultures were >90% pure and free of fibroblast contamination (not shown). Thus the recombinant findings are not greatly influenced by residual mesenchyme. To determine whether the findings were specific to lung epithelial cells of the canalicular stage (E19) of development, we repeated the recombination experiments with lung epithelial cells of the saccular stage (E21) of development. As can be seen in Fig. 8, homotypic and heterotypic recombinants of E21 epithelial cells and E19 mesenchymal cells exhibited similar in situ hybridization patterns as E19 recombinants. CCSP mRNA was expressed in all homotypic and heterotypic recombinants (Fig. 8, G–I), whereas SP-C and SP-B transcripts were present in the homotypic recombinants (Fig. 8, A and D, respectively) but absent in the heterotypic recombinants (Fig. 8, B and C, and E and F, respectively). Although only in situ hybridization patterns in E21 lung epithelial cells recombined with E19 mesenchyme are shown, comparable expression patterns were seen in E21 epithelial cells when cultured in the presence of both E21 homotypic and heterotypic mesenchymal cells (data not shown). Finally, we determined whether associated mesenchyme affects the promoter activities of CCSP and SP-C in epithelial cells. The effect of varying mesenchyme on the transactivation of the CCSP and SP-C promoters was determined by measuring the activity levels of SEAP after transfection of E19 epithelial cells with a SEAP2 reporter gene construct under the control of either the SP-C or CCSP promoter. Following transfection, epithelial cells were recombined with E19 homotypic and heterotypic mesenchyme. If epithelial promoter activities were to be controlled by associated mesenchyme, we expected that the transactivation patterns of the promoters would mimic the expression profiles for both genes in the recombinants. Indeed, CCSP promoter activity was comparatively consistent between E19 epithelial cells alone and both homotypic and heterotypic E19 recombinants (Fig. 9A). SP-C promoter activity was low in E19 epithelial cells alone, as well as in E19 epithelial cells in both E19 skin and E19 intestinal heterotypic recombinants. In contrast, SP-C promoter activity was significantly greater (>10-fold) in E19 lung epithelial cells after recombination with E19 lung mesenchymal cells (Fig. 9B). Taken together, the data indicate that, at late gestation, distal epithelial cells have the plasticity to respond to mesenchymal cues and that maintenance of the alveolar, but not bronchiolar, epithelial cell phenotype is dependent on the associated mesenchyme.

FGF7 stimulates and maintains alveolar epithelial cell phenotype. Several studies have reported that FGF7 induces a distal lung epithelial phenotype (41) and enhances the maturation of fetal rat lung type II cells (9). Since FGF7 is produced by the fetal lung mesenchyme (14, 26), we investigated whether FGF7 plays a role in maintaining the alveolar phenotype in the late lung recombinant cultures. We observed that 100 ng/ml FGF7 stimulated branching (Fig. 10, A and B,b) as well as SP-C (Fig. 10B,f) but not CCSP (Fig. 10B,d), mRNA expression in E12 endodermal lung buds, in line with previous
Fig. 12. Epithelial Notch signaling depends on mesenchyme. A: expression patterns of Notch1 (a, b, and c) and Notch3 (d, e, and f) mRNA in the developing rat lung. B: expression patterns of Jagged1 (a and b) and Delta1 mRNA (d and e) and protein (c and f) in the late rat lung. Section in situ hybridization was used to determine mRNA expression of Notch molecules mRNA levels at E16, E19, and E22. Dark blue color indicates positive signals. Sections were counterstained with nuclear fast red. Brown color indicates positive immunoreactivity. Sections for immunostaining were counterstained with hematoxylin. C: Notch signaling in E19 homotypic [lung (L) Epi/L Mes] and heterotypic [L. Epi/intestinal (I) Mes] recombinants. Immunostaining for Delta (a and d) and Lunatic fringe (b and e) as well as section in situ hybridization for Hes1 (c and f) was performed on E19 lung epithelial cells recombined with either E19 lung (d-f) or intestinal (a-c) mesenchymal cells.
inducing mesenchyme present. These results are consistent with many previous studies which established that recombining distal lung epithelium with mesenchyme from another source, early in lung development, resulted in a cessation of normal epithelial branching (51). Similar to lung epithelial cells of the early respiratory system, fetal lung epithelial cells in lung homotypic recombinants of late gestation displayed a lung-like morphology, whereas fetal lung epithelial cells in skin and intestine mesenchymal heterotypic recombinants of the same late gestation adopted a more skin- and intestine-like morphology, respectively. To our knowledge this is the first evidence that suggests that late gestation lung epithelium can be programmed to organize into different structures not resembling normal lung morphology. Moreover, it indicates that lung mesenchyme not only directs lung branching morphogenesis at early gestation but also is actively involved in saccular and alveolar epithelial morphogenesis, which takes place at late gestation and postnatally in the rat. Recent coculture experiments have shown that adult lung fibroblasts maintain adult alveolar type II cell differentiation, but the inducing capabilities of the fibroblasts were not investigated (40). Second, we showed that lung mesenchyme affects epithelial lung cell differentiation at late fetal gestation. Expression patterns of SP-B, SP-C, and CCSP in both homotypic and heterotypic recombinants revealed that CCSP mRNA and protein were expressed in the epithelial cells of all three recombinants. In contrast, SP-B and SP-C mRNA were only expressed in epithelial cells that were cultured in the presence of lung mesenchyme, even though both SP-B and SP-C mRNA expression was detected in distal lung epithelial cells at the time of their isolation, suggesting that they were committed to their phenotype. SP-C and SP-B mRNA expression was measured by nonradioactive ISH, and the absence of mRNA signals in the heterotypic recombinants may be due to the lower detection limit of nonradioactive ISH. However, the ISH data suggest that SP-C and SP-B mRNA levels are significantly reduced in the heterotypic recombinants. Promoter activation studies also revealed that CCSP promoter activity in epithelial cells was unchanged in heterotypic recombinants, whereas SP-C promoter activity levels were significantly reduced in skin and intestine recombinants compared with their homotypic counterparts. Together, the results suggest that at relatively late fetal gestation, lung epithelial-mesenchymal interactions do not affect CCSP expression or perhaps the bronchiolar epithelial cell phenotype. In contrast, interactions between the epithelium and specifically lung mesenchyme are critical for the maintenance of SP-C expression or, more importantly, the support of the alveolar epithelial cell phenotype at relative late fetal gestation.

**Table 1. Summary of SP-C and CCSP expression in homotypic and heterotypic recombinants**

<table>
<thead>
<tr>
<th>Recombinant</th>
<th>Morphology of Recombinant</th>
<th>SP-C Expression</th>
<th>CCSP Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>E13lung</td>
<td>Lung</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E13lung</td>
<td>Lung</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>E13lung</td>
<td>Intestine</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E13lung</td>
<td>Skin</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E19lung</td>
<td>Lung</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>E19lung</td>
<td>Intestine</td>
<td>++/–</td>
<td>++/–</td>
</tr>
<tr>
<td>E19lung</td>
<td>Skin</td>
<td>+/–</td>
<td>+/–</td>
</tr>
</tbody>
</table>

SP-C, surfactant protein C; CCSP, Clara cell secretory protein.
Mesenchymal transactivation of epithelial genes is not unique for the developing lung and has been demonstrated for the pepsinogen promoter in developing avian stomach (15).

The present data were surprising since we expected that, based on differentiation marker expression at late gestation, lung epithelial cells would be committed to a specific phenotype. Hence, it may be appropriate to hypothesize that lung alveolar epithelial cells, particularly type II cells, require specific factors provided only by lung mesenchyme to maintain the alveolar type II phenotype. Furthermore, these cells may never reach what may be referred to as a terminally differentiated state; that is, these cells may maintain the plasticity to respond to specific signals from surrounding mesenchymal and epithelial cells indefinitely. There is evidence that suggests that indeed the alveolar type II cell functions as a sort of stem cell in lung injury and repair (1, 2, 30, 49). Upon lung injury, the damaged alveolar type I cells undergo cell death and are shed from the epithelial basement membrane. The remaining type II cells in the area then begin to proliferate, thereby reconstituting the region of injury with alveolar type II cells (47). During the resolution phase of the repair process, hyperplastic type II cells undergo extensive apoptosis (6, 13), and a portion of the type II cells undergo differentiation into new alveolar type I cells.

If we hypothesize that lung alveolar type II cells are maintained in a state of plasticity, even in the mature lung, the important question we must then ask is, What are the factors involved in the maintenance of this state? Are they of mesenchymal origin, epithelial origin, or both? Clearly, some essential factor(s) are provided by the lung mesenchyme for the maintenance of the alveolar type II cell phenotype. There are a number of studies that support the concept that the differentiated state of lung epithelial cells requires the presence of specific components of the ECM. Alveolar type II cells in culture lose their cell-specific phenotype unless cultured on either collagen gels (11, 12), type I collagen/fibronectin matrix enriched with laminin (16), or an ECM extracted from either the Engelbreth-Holm-Swarm tumor (33, 38) or Madin-Darby canine kidney cells (3). These findings suggest that not only does the ECM function as an architectural component, but it is also involved in signal transduction. With the assumption that the heterotypic recombinants produce and deposit a different ECM than homotypic recombinants, the loss in surfactant protein expression in the lung epithelial cells in the heterotypic recombinants may be due to altered ECM-mediated signaling.

Others have reported the importance of various soluble growth factors. Members of the FGF family, especially FGF7 (16, 46), are thought to be important factors in maintaining the alveolar type II cell phenotype. Additionally, FGF7 has been shown to be involved in alveolar differentiation (9, 41). Herein, we confirm that FGF7 induces a distal lung epithelial phenotype in embryonic lung endodermal cells. Moreover, we show that FGF7 is involved in maintaining the alveolar phenotype, based on SP-C promoter transactivation experiments, in the homotypic lung recombinants at late gestation. However, both fetal skin (26) and intestinal (14) mesenchyme produce FGF7. Thus it is unlikely that the loss of epithelial surfactant protein expression in the heterotypic recombinants is due to a lack of FGF7. It has been suggested that a causal relationship between ECM and growth factors may be of fundamental importance in control of morphogenesis and differentiation during development (25). Recent data (15, 33) showing that both ECM and FGF7 are required for optimal maintenance of the alveolar type II cell phenotype in vitro support such a relationship.

The Notch signaling pathway is a conserved pathway involved in cell fate control (5, 7). Recent studies suggest that this pathway may regulate epithelial cell differentiation in the lung (10, 35). Herein we found that expression of Notch3, Jagged1, and Delta was restricted to the epithelial lining cells of distal Airways at the canalaric stage of lung development. With advancing gestation, the expression of the three Notch molecules disappeared. We have reported similar temporal and spatial expression patterns for two other Notch molecules, namely Lunatic fringe and Hes1 (54). The present study shows that expression of Lunatic fringe and Delta1 in lung epithelial cells depends on the type of inducing mesenchyme present. Homotypic, but not heterotypic, mesenchyme was able to maintain expression of both Notch molecules. The decrease in Lunatic fringe and Delta expression was accompanied by increased Hes1 expression in the heterotypic recombinants. Since Hes1-deficient mice have reduced numbers of Clara cells (17), elevated Hes1 expression may maintain the Clara cell phenotype in these heterotypic cell recombinants. Whether Hes1 affects the alveolar epithelial phenotype remains to be elucidated, but expression of active Notch3 (and therefore Hes1) in distal epithelial cells using the SP-C promoter has been shown to inhibit alveolar epithelial differentiation (10). Lunatic fringe and Hes1 expression in dental epithelium during tooth development also depends on mesenchymal signals (29), such as FGFs and bone morphogenetic factors. FGF10 stimulated Lunatic fringe expression in dental epithelium, and this stimulatory effect was antagonized by bone morphogenetic factor 4 (29). FGF and Notch interactions also affect pancreatic development since FGF10 impairs differentiation of pancreatic precursors by maintaining Notch activation (28). In contrast, in NIH3T3 cells, FGF1 inhibited Notch signaling by suppressing target protein RBP-Jk (43). Thus, although the exact nature remains obscure, it appears that the interplay between Notch and FGF receptor signaling pathways may be important for maintaining the alveolar epithelial type II phenotype at late gestation.

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REFERENCES


