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Induction of alveolar type II cell differentiation in embryonic tracheal epithelium in mesenchyme-free culture

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SUMMARY

We have previously shown that fetal lung mesenchyme can reprogram embryonic rat tracheal epithelium to express a distal lung phenotype. We have also demonstrated that embryonic rat lung epithelium can be induced to proliferate and differentiate in the absence of lung mesenchyme. In the present study we used a complex growth medium to induce proliferation and distal lung epithelial differentiation in embryonic tracheal epithelium. Day-13 embryonic rat tracheal epithelium was separated from its mesenchyme, enrobed in growth factor-reduced Matrigel, and cultured for up to 7 days in medium containing charcoal-stripped serum, insulin, epidermal growth factor, hepatocyte growth factor, cholera toxin, fibroblast growth factor 1 (FGF1), and keratinocyte growth factor (FGF7). The tracheal epithelial cells proliferated extensively in this medium, forming lobulated structures within the extracellular matrix. Many of the cells differentiated to express a type II epithelial cell phenotype, as evidenced by expression of *SP-C* and osmiophilic lamellar bodies. Deletion studies showed that serum, insulin, cholera toxin, and FGF7 were necessary for maximum growth. While no single deletion abrogated expression of *SP-C*, deleting both FGF7 and FGF1 inhibited growth and prevented *SP-C* expression. FGF7 or FGF1 as single additions to the medium, however, were unable to induce *SP-C* expression, which required the additional presence of serum or cholera toxin. FGF10, which binds the same receptor as FGF7, did not support transdifferentiation when used in place of FGF7. These data indicate that FGF7 is necessary, but not sufficient by itself, to induce the distal rat lung epithelial phenotype, and that FGF7 and FGF10 play distinct roles in lung development.

Key words: Rat, Lung development, Alveolar epithelium, Epithelial-mesenchymal interactions, Surfactant proteins, Fibroblast growth factor

INTRODUCTION

Lung development in the embryonic rat begins on gestational day 11 as paired evaginations from the floor of the foregut endoderm into mesenchyme derived from the splanchnic mesoderm. A series of repetitive lateral and terminal branchings of the epithelium then gives rise to the pulmonary tree. Branching morphogenesis is accompanied by the differentiation of epithelial cell types that serve specific functions in the lung postnatally; these include alveolar type II cells, Clara cells, mucous secretory cells, and ciliated cells. Studies in birds (Rudnick, 1933; Dameron, 1961) and mammals (Sampaolo and Sampaolo, 1959; Spooner and Wessells, 1970; Wessells, 1970; Masters, 1976) have shown that branching morphogenesis and cytodifferentiation of the embryonic lung epithelium occur in response to specific signals produced by lung mesenchyme. The inductive properties of lung mesenchyme are most strikingly demonstrated by in vitro experiments in which a portion of mesenchyme is surgically removed from the embryonic trachea and replaced by mesenchyme from the distal lung tips. In these experiments the tracheal epithelium is both induced to branch in a lung-like pattern (Alescio and Cassini, 1962) and reprogrammed to express an alveolar type II cell phenotype (Shannon, 1994). Conversely, grafting of tracheal mesenchyme onto distal lung epithelium results in the cessation of branching (Wessells, 1970) and the reprogramming of distal lung epithelium to express a tracheal phenotype (Shannon et al., 1998). Thus at early stages of development the entire respiratory epithelium, from the trachea to the distal lung tips, can adopt any respiratory epithelial phenotype, depending on the type of mesenchyme with which it is associated.

Induction of lung epithelium by lung mesenchyme requires a coordinated interplay among hormones, growth factors and other signaling molecules, as well as cell-extracellular matrix interactions (for recent reviews see Minoo and King, 1994; Hilfer, 1996; Shannon and Deterding, 1997). The signaling molecules that drive lung epithelial morphogenesis and differentiation are diffusible, since induction will occur across a Millipore filter (Taderera, 1967). In vitro culture of purified embryonic lung epithelial rudiments has demonstrated that soluble factors can replace lung mesenchyme. Using a complex growth medium, we have shown that the epithelium from the distal tips of day-13 embryonic rat lungs will proliferate in the absence of lung mesenchyme (Deterding and Shannon, 1995). Distal lung epithelial cell differentiation was maintained in

these cultures, as shown by the expression of mRNA for surfactant protein C (*SP-C*), which is a specific marker for the distal lung epithelium. Furthermore, the epithelial cells in these cultures progressed from an undifferentiated, columnar morphology to one seen in late-gestation alveolar type II cells; this included the elaboration and secretion of osmiophilic lamellar bodies, the storage organelle of pulmonary surfactant. In a subsequent study (Deterding et al., 1996) we determined that members of the fibroblast growth factor (FGF) family, notably acidic FGF (FGF1) and keratinocyte growth factor (FGF7), play a pre-eminent role in this permissive induction. Similar experiments using mesenchyme-free culture of mouse embryonic lung rudiments have also demonstrated an important role for FGF1 and FGF7 (Nogawa and Ito, 1995; Cardoso et al., 1997), as well as for FGF10 (Bellusci et al., 1997).

Because lung mesenchyme is able to reprogram embryonic tracheal epithelium to express an alveolar type II cell phenotype (Shannon, 1994; Shannon et al., 1998), we have employed mesenchyme-free culture to determine if embryonic tracheal epithelium can be reprogrammed to express a distal lung epithelial phenotype. We report here that embryonic tracheal epithelium can be induced to express an alveolar type II cell phenotype, but the competence of tracheal epithelium to respond is temporally restricted. Furthermore, not all of the medium components that are necessary for maximum growth are required to induce *SP-C*. Finally, FGF7 is necessary, but not sufficient, to effect the transdifferentiation of tracheal epithelium to a distal lung phenotype, and cannot be replaced by FGF10.

MATERIALS AND METHODS

Isolation and culture of purified embryonic tracheal epithelium

Timed-pregnant rats were obtained from Charles Rivers Laboratories (Raleigh, NC); the day on which a sperm-positive vaginal plug was found was considered day 0 of gestation. For most experiments, pregnant dams were killed on day 13 of gestation; in this case only fetuses weighing 80-90 mg, whose lungs are at the pseudoglandular stage of development, were used. In one series of experiments fetuses were obtained on days 15 and 17. Fetuses were decapitated and transferred to a Maximov depression slide containing ice-cold Hank's balanced salt solution (HBSS; GIBCO/BRL, Gaithersburg, MD) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotercin B (all from GIBCO/BRL), and 10 μg/ml gentamicin sulfate (Sigma Chemical Co., St. Louis, MO). The lungs and trachea were dissected intact using Moria microsurgery knives (Fine Science Tools Inc., Foster City, CA). The trachea was removed from the lung/trachea complex just above the bifurcation and bisected. Epithelial and mesenchymal tracheal rudiments were isolated by incubating the tracheal halves in HBSS containing 0.05% collagenase (CLS IV; Worthington Biochemicals, Freehold, NJ) plus 1% fetal bovine serum (FBS; Sigma) for 45 minutes at 37°C. The tracheae were washed twice with HBSS + 10% FBS, treated briefly with 1-2 drops of a 1 mg/ml solution of DNase I (Sigma) in HBSS, and separated into epithelial and mesenchymal components with tungsten needles. Epithelial rudiments were then treated with dispase for 10 minutes to remove any potential residual mesenchymal cells, washed with HBSS + 10% FBS, then washed and stored on ice in serum-free DMEM/F12 (GIBCO/BRL) containing antibiotics.

Mesenchyme-free culture of tracheal epithelial rudiments was performed essentially as described previously for embryonic lung epithelium (Deterding and Shannon, 1995). Briefly, 10 μl of growth factor-reduced Matrigel (Collaborative Biomedical Products, Bedford, MA) was spread in a small circle (approximately 0.5 cm diameter) in a 35 mm culture dish and allowed to gel. Epithelial rudiments were transferred to the surface of the matrix in a small volume of medium, then covered with 15 μl of Matrigel. The rudiments were gently mixed into the matrix with an iridectomy knife to ensure complete enrobement of the rudiments, then the Matrigel was gelled at 37°C. The day of rudiment isolation and enrobement was considered day 0 of culture. The enrobed rudiments were covered with 3 ml of medium and cultured for up to 7 days.

Culture medium

Based on our earlier results with embryonic lung epithelial rudiments in mesenchyme-free culture (Deterding et al., 1996), along with our previous observations in culturing adult type II cells (Leslie et al., 1985, 1990, 1993; Sugahara et al., 1995), initial experiments utilized DMEM/F12 containing antibiotics and the following additions: 3% charcoal-stripped (Yoshizato et al., 1980) FBS (CSS), insulin (I; Collaborative Biomedical Products) 10 μg/ml, cholera toxin (CT; ICN Pharmaceuticals, Irvine, CA) 1 μg/ml, human recombinant epidermal growth factor (EGF; R & D Systems, Minneapolis, MN) 25 ng/ml, human recombinant hepatocyte growth factor (HGF; R & D Systems) 10 ng/ml, human recombinant FGF7 (Promega Biotech, Madison, WI) 25 ng/ml, and bovine brain FGF1 (R & D Systems) 100 ng/ml; in one series of experiments human recombinant FGF1 from two different manufacturers (Promega, R & D Systems) 100 ng/ml was used in place of bovine brain FGF1. A series of experiments was also conducted in which human recombinant FGF10 (R & D Systems) 1-1000 ng/ml was used in place of FGF1 and FGF7. In some experiments CT was replaced by either 8-bromo-adenosine 3′,5′-cyclic monophosphate (8-Br-cAMP; Sigma) at concentrations ranging from 25-500 μM, or by 10 μM forskolin plus 10 μM 3-isobutyl-1-methylxanthine (IBMX; both from Sigma).

Analysis of tracheal epithelial proliferation

To estimate of the degree of epithelial cell proliferation, in some experiments cultured rudiments were pulsed for 4 hours with 2 μCi [^{3}H]thymidine (specific activity 6.7 Ci/mmol; NEN Life Science Products, Boston, MA) on day 5 of culture. Cultures were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) and embedded in paraffin. Sections 4 μm thick were deparaffinized, rehydrated through water, and dipped in NTB-2 nuclear track emulsion (Eastman Kodak Co, Rochester, NY). Autoradiograms were developed after 7 days exposure, and the sections stained with hematoxylin and eosin, and the labeling index determined by counting a minimum of 500 cells in 6 independent fields.

In situ hybridization

As a marker of the distal lung epithelial phenotype we examined the expression of *SP-C*, which was transcribed from a full length rat cDNA (Fisher et al, 1989). As a marker of the proximal respiratory epithelial cell phenotype we examined the expression of *CC10*, the 10 kDa Clara cell secretory protein, using a 275 bp fragment of the rat cDNA (provided by Dr Arun Rishi). Cultures were fixed in freshly prepared 4% paraformaldehyde and embedded in paraffin. In situ hybridization was performed on 4 μm sections as previously described (Deterding and Shannon, 1995), with the exception that [^{33}P]UTP (2000-4000 Ci/mmol; NEN Life Science Products) was used in the transcription of RNA probes. Slides were dipped in NTB-2 emulsion and developed after an exposure appropriate for each probe. Hybridizations with radiolabeled sense RNA probes were done as a control in all experiments.

Electron microscopy

Samples of cultured tracheal epithelium were fixed in 2% glutaraldehyde-1% paraformaldehyde, post-fixed in 1.5% osmium tetroxide, stained en bloc with uranyl acetate, and embedded in Lufts

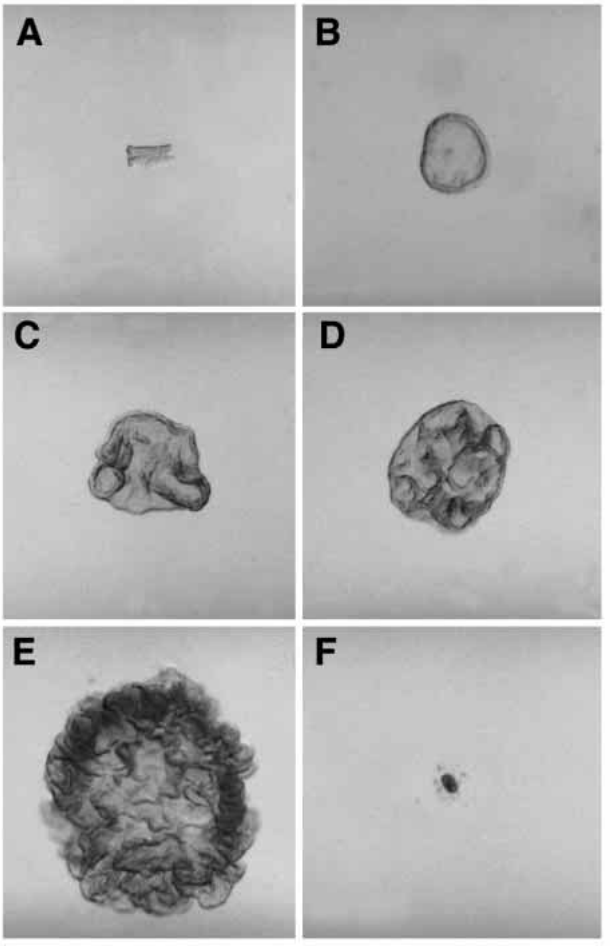

Fig. 1. Growth of day-13 embryonic rat tracheal epithelium in mesenchyme-free culture. Tracheal epithelial rudiments were isolated by collagenase digestion and manual dissection, enrobed in growth factor-reduced Matrigel, and cultured in BFGM medium (A-E), or DMEM/F12 medium containing 3% charcoal-stripped FBS (CSS) (F). BFGM contained 3% CSS, insulin (10 μg/ml), cholera toxin (1 μg/ml), EGF (25 ng/ml), HGF (10 ng/ml), bovine FGF1 (100 ng/ml) and FGF7 (25 ng/ml). (A) Purified tracheal epithelium immediately after enrobement. (B) After 1 day in culture the epithelium has grown and formed a cyst. (C) After 2 days in culture the epithelium has expanded further, and irregular folding of the surface is seen. (D) Further growth and increased folding on the surface are seen after 3 days in culture. (E) After 5 days in culture the tracheal epithelium has undergone substantially more growth, and the epithelium is extensively folded. (F) In contrast, tracheal epithelium cultured for 5 days in medium containing only CSS shows no evidence of growth and expansion, instead forming a small nest of cells within the matrix. All panels are at the same magnification (original magnification, 56×).

3:7 mixture of LX-112 (Ladd Research Industries, Burlington, VT). Sections, 1-1.5 μm thick, were stained with Mallory's azure II-methylene blue. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Phillips 400 electron microscope.

Histochemistry

The presence of glycogen was detected in paraformaldehyde-fixed tissues by comparing periodic acid-Schiff (PAS) staining in sequential sections that were or were not pretreated with diastase (Sheehan and Hrapchak, 1980).

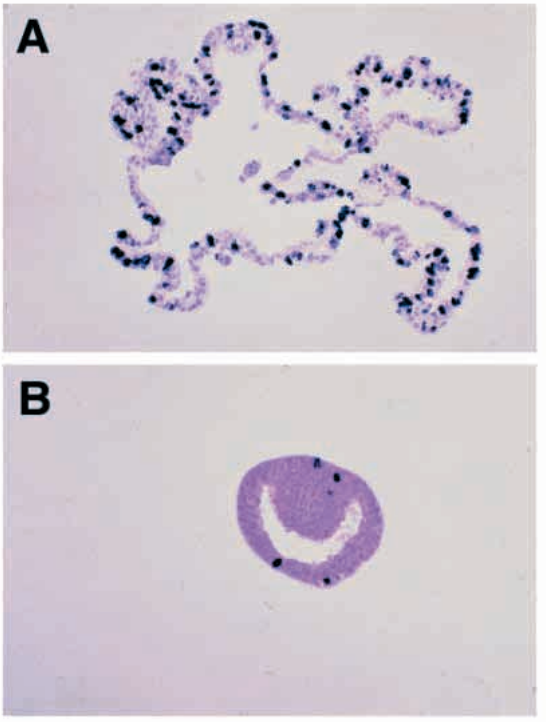

Fig. 2. Cell proliferation in cultured embryonic tracheal epithelium. Tracheal epithelial rudiments were enrobed in Matrigel and cultured for 5 days in either BFGM or in DMEM/F12 containing 3% CSS. [^{3}H]thymidine (2 μCi/ml) was added to the media for the final 4 hours of culture and autoradiograms prepared. (A) BFGM induces widespread proliferation throughout the tracheal epithelium, with a labeling index of approximately 34%. Only a small, representative portion of the entire rudiment is shown in this photomicrograph. (B) Tracheal epithelium cultured in BFGM from which FGF1 and FGF7 have been deleted shows significantly less labeling. A cross section through the entire rudiment is shown in this photomicrograph. Both panels are at the same magnification (original magnification, 200×).

RESULTS

Proliferation and induction of lung epithelial differentiation in tracheal epithelium grown in mesenchyme-free culture

As shown in Fig. 1, purified day-13 tracheal epithelium that was enrobed in growth-factor-reduced Matrigel and cultured in a medium containing CSS, I, CT, EGF, HGF, FGF1 and FGF7 (hereafter BFGM) exhibited robust growth over the 5-day culture period. The tracheal epithelial tube (Fig. 1A) expanded to form a cystic structure after 24 hours in culture (Fig. 1B). This dilation of the epithelium appears similar to that which we have observed when day-13 tracheal epithelium is recombined with day-13 distal lung mesenchyme (Shannon et al., 1998). On day 2 of culture the cyst began to show folding in the epithelial wall (Fig. 1C), which was more pronounced on day 3 (Fig. 1D). This gross morphology is identical to that previously described by us (Deterding and Shannon, 1995; Deterding et al., 1996) and others (Bellusci et al., 1997) for distal lung epithelial rudiments grown in mesenchyme-free culture in the presence of FGF7. By day 5 of culture the size of the rudiment had increased dramatically (Fig. 1E), with the epithelial wall

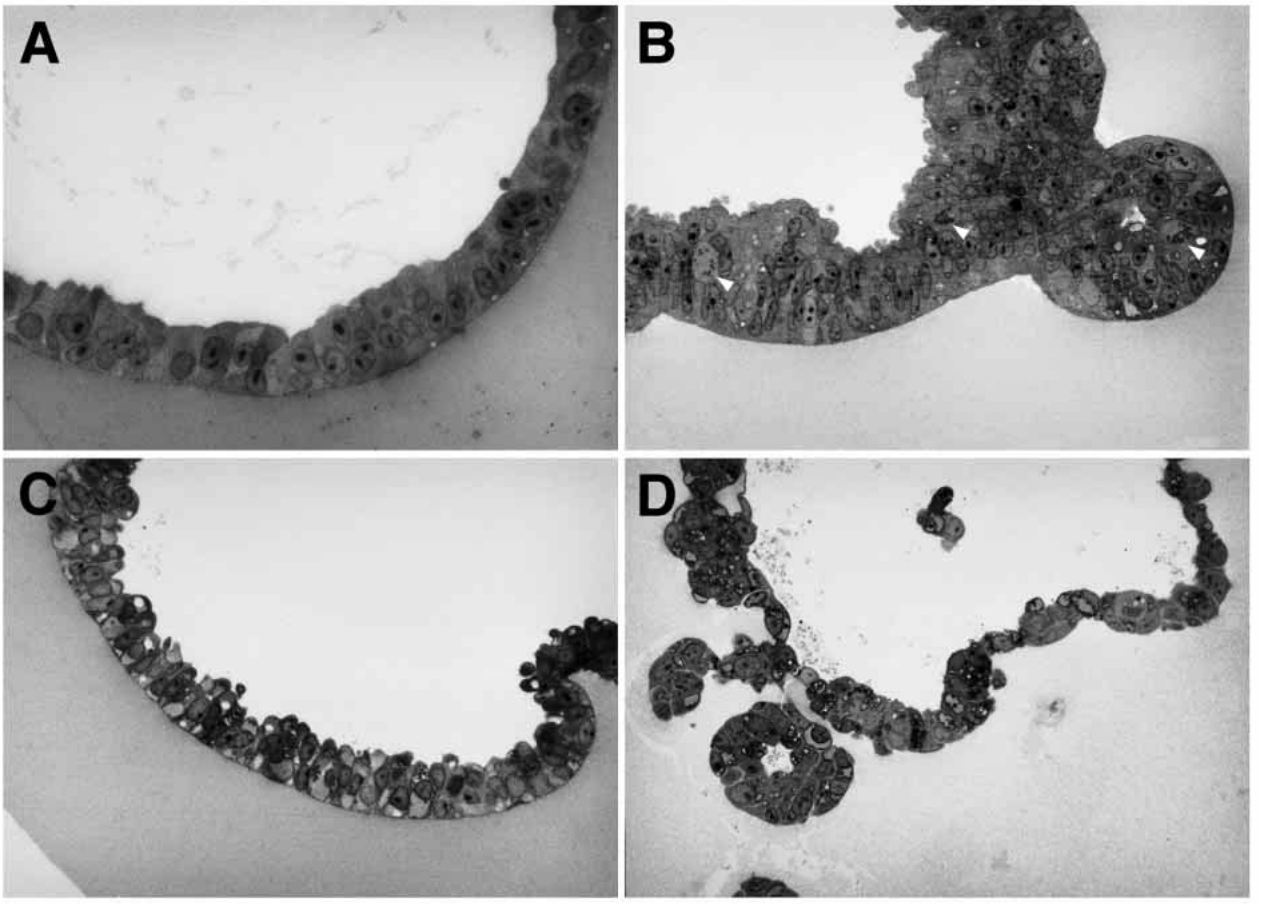

Fig. 3. Light microscopic analysis of embryonic tracheal epithelium enrobed in Matrigel and cultured in BFGM. (A) After 2 days in culture the tracheal epithelial cells exist in a single or double-layer, with the constitutive cells being cuboidal to columnar. (B) After 3 days in culture the epithelium has become multilayered in some areas. Mitotic figures (white arrowheads) are commonly observed. (C) After 5 days in culture many of the epithelial cells contain pale-staining areas in their cytoplasm, which were shown to be glycogen by histochemistry. (D) Significant morphological changes are apparent after 7 days in culture. Many of the cells contain darkly stained inclusion bodies; darkly stained bodies are also seen in the lumen in close proximity to the apical epithelium. All panels are at the same magnification (original magnification, 425×).

showing extensive folding over the entire surface of the rudiment; this pattern of epithelial expansion and surface folding continued in rudiments cultured for 7 days (data not shown). In contrast, tracheal epithelial rudiments cultured in the presence of CSS alone showed no evidence of growth whatsoever (Fig. 1F), and actually appeared smaller than the starting epithelial tube. The sustained proliferative response of day-13 tracheal epithelium to BFGM was underscored by the observation that these rudiments had a labeling index of 34% when pulsed with [^{3}H]thymidine for 4 hours on day 5 of culture. Labeled cells did not appear localized to particular areas of the rudiments cultured in BFGM, but rather were evenly distributed throughout the epithelium (Fig. 2A).

When examined by light microscopy, tracheal rudiments cultured for 24 hours in BFGM appeared as a single layer of cuboidal to columnar epithelial cells lining a lumen that contained a lightly staining amorphous material and some cellular debris (Fig. 3A). This appearance did not change appreciably by the third day of culture, with the exception that the epithelial wall was multilayered in some areas; mitotic figures were commonly observed (Fig. 3B). By day 5 of culture the tracheal epithelial rudiments had undergone significant changes, with many areas of the epithelial wall containing multiple layers of cells. Some epithelial cells also exhibited significant alterations in morphology, with the cells containing pale areas consistent with extracted glycogen (Fig. 3C). The presence of glycogen was confirmed by histochemical identification of PAS-staining material in these cells that was abolished by pretreatment with diastase (data not shown). The morphological changes seen on day 5 became much more pronounced on day 7, when many of the cells contained large accumulations of glycogen, along with densely stained inclusion bodies (Fig. 3D). The presence of large amounts of glycogen in these cells is significant, since glycogen may be an important source of glucose that is used in surfactant phospholipid biosynthesis in late-gestation alveolar type II cells (Farrell and Bourbon, 1986). When examined by electron microscopy, the epithelial cells constituting tracheal rudiments cultured for 7 days in BFGM appeared indistinguishable from late-gestation alveolar type II cells (Fig. 4). The inclusion bodies were revealed to be osmiophilic lamellar bodies, which serve as the storage organelle for pulmonary surfactant. Taken together, these morphological changes indicated that the tracheal epithelium had been induced to express a distal lung

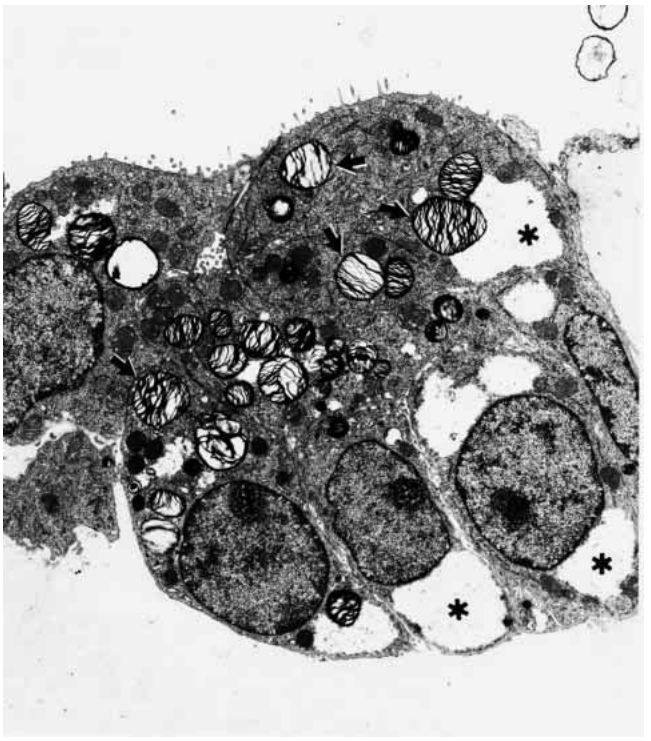

Fig. 4. Ultrastructural analysis of embryonic tracheal epithelium cultured for 7 days in BFGM. The tracheal epithelial cells in this electron micrograph contain numerous osmiophilic multilamellar bodies (arrows) and areas that contained glycogen (asterisks), which was extracted during tissue processing. These morphological features are identical to those seen in late-gestation alveolar type II cells (original magnification, 4300×).

epithelial cell phenotype. Morphological evidence of induction of the tracheal epithelial phenotype was absent, since ciliated cells, goblet cells and mucous cells were not observed.

Because the presence of osmiophilic lamellar bodies is not necessarily diagnostic of the type II cell phenotype, we also examined cultured tracheal epithelium for the expression of *SP-C*. SP-C is an alveolar type II cell-specific marker in the adult lung, and is seen only in the most distal epithelium during lung development (Kalina et al., 1992; Wert et al., 1993). *SP-C* was not detected by in situ hybridization in uncultured day-13 tracheal epithelium, even after prolonged exposure of the autoradiograms (Fig. 5A,B). After 24 hours of culture in BFGM, some epithelial cells in day-13 tracheal rudiments were positive for *SP-C*, although the level of expression was low (Fig. 5C,D). Many of the epithelial cells in tracheal rudiments were clearly positive for *SP-C* after 48 hours of culture in BFGM (Fig. 5E,F). Rudiments cultured for 3 days (Fig. 5G,H) appeared similar to 48 hour cultures. Rudiments cultured for 5 days (Fig. 5I,J) showed the most intense expression of *SP-C*, but the pattern of expression was not uniform throughout the epithelium. Whereas some clusters of cells were intensely positive for *SP-C*, other cells were completely devoid of transcripts. We also examined cultured tracheal epithelial rudiments for the expression of *CC10*, the 10 kDa Clara cell secretory protein, which in the adult is predominantly expressed in the bronchiolar epithelium and in the epithelium proximal to the bronchioles (Singh and Katyal, 1997). *CC10* was expressed in virtually every cell of the cultured rudiments (Fig. 5K,L), but the intensity of expression was not equivalent among all cells. It was also evident that cells containing *SP-C* transcripts co-expressed *CC10*, as has been described in the cultured embryonic rat lung explants (Shannon et al., 1998). Control sense SP-C and CC10 probes gave no hybridization signal (data not shown).

The ability to induce alveolar type II cell differentiation in embryonic tracheal epithelium is temporally restricted

In a previous study (Shannon, 1994) we demonstrated that day-13 distal lung mesenchyme was able to reprogram day-13 tracheal epithelium to express an alveolar type II cell phenotype, but that the competence of tracheal epithelium to respond to the inductive signals provided by the lung mesenchyme was lost by day 16 of gestation. When tracheal epithelial rudiments isolated from day-15 fetuses were cultured in BFGM the rudiments showed a clear increase in size. However, when these rudiments were examined for the presence of *SP-C* they were uniformly negative (Fig. 6A,B), but were positive for *CC10* (Fig. 6D,E). Tracheal epithelial rudiments isolated from day-17 fetuses and cultured in BFGM gave identical results (data not shown).

Effects of medium deletions on growth and transdifferentiation of tracheal epithelium in mesenchyme-free culture

We next examined the effects of deleting the various components of the medium on cultured tracheal epithelial rudiments. Single deletions of CSS (Fig. 7B), I (Fig. 7C), CT (Fig. 7D), and FGF7 (Fig. 7H) all resulted in a reduction in the size of the cultured rudiments; deleting EGF (Fig. 7E), or HGF (Fig. 7F) or FGF1 (Fig. 7G) did not have a dramatic effect on rudiment size. The most pronounced effect on rudiment growth was seen when both FGF1 and FGF7 were deleted from the medium (Fig. 7I). In this case the rudiment formed a small epithelial cyst that showed little increase in size over the 5-day culture period, although some cells did incorporate [^{3}H]thymidine (Fig. 2B).

The effects of deleting components from BFGM on the induction of *SP-C* expression was evaluated by in situ hybridization (Fig. 8). *SP-C* transcripts were detected in tracheal rudiments cultured in BFGM from which CSS (Fig. 8C,D), I (Fig. 8E,F), CT (Fig. 8G,H), EGF (Fig. 8I,J), HGF (Fig. 8K,L), FGF1 (Fig. 8M,N), or FGF7 (Fig. 8O,P) had been singly deleted. Rudiments cultured in BFGM from which both FGF1 and FGF7 had been deleted, however, were uniformly negative for *SP-C* (Fig. 8Q,R), but virtually every cell was positive for *CC10* transcripts (Fig. 8S,T).

FGF7 is necessary, but not sufficient, to reprogram tracheal epithelium to express a distal lung epithelial phenotype in mesenchyme-free culture

The deletion data suggested that CSS, I, CT, FGF7 and perhaps FGF1 were the components of BFGM critical for rudiment growth, and that the induction of a distal lung epithelial phenotype required FGF7 and/or FGF1. When tested as single medium additions, CSS (Fig. 9A), I (Fig. 9B), or CT (Fig. 9C)

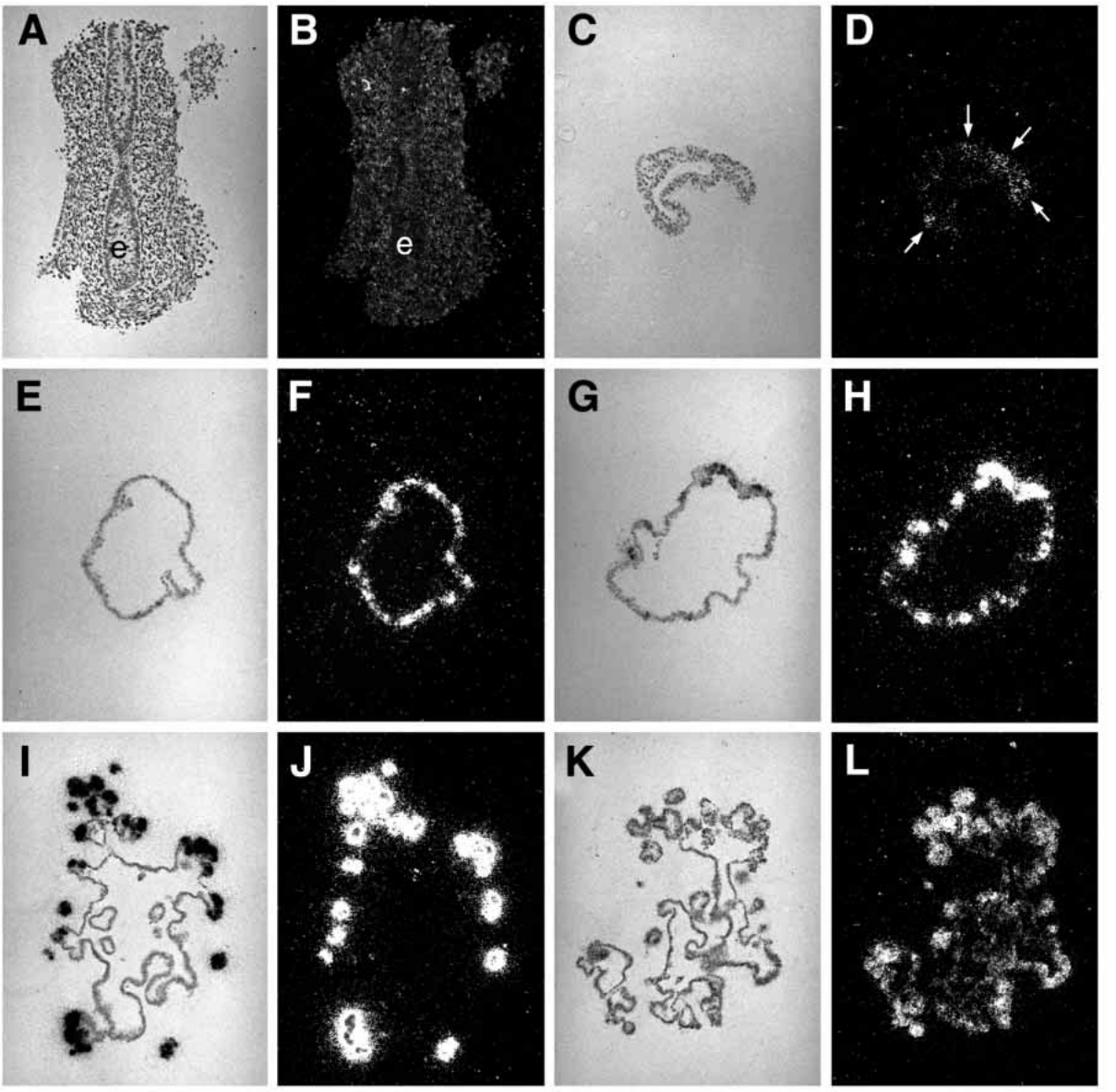

Fig. 5. Expression of distal and proximal lung epithelial markers in tracheal epithelium cultured in BFGM. In situ hybridization for *SP-C* (A-J) or *CC10* (K,L) was performed on uncultured day-13 trachea or on day-13 tracheal epithelium that had been enrobed in Matrigel and cultured in BFGM for 1 to 5 days. Exposure times of the autoradiographs are identical for each probe. Brightfield and darkfield images are paired; darkfield images were photographed and printed identically. (A,B) Day 13 embryonic trachea probed for *SP-C*; no signal is present in the epithelium (e). (C,D) Day 13 embryonic tracheal epithelium after 1 day of culture in BFGM probed for *SP-C*. Some of the epithelial cells have begun to express *SP-C* (arrows). (E,F) After 2 days of culture a significant number of cells are strongly positive for *SP-C*. This pattern of *SP-C* expression continues through day 3 of culture (G,H). (I,J) After 5 days of culture intense *SP-C* expression is observed, predominantly in the most distal aspects of the epithelium. (K,L) A section adjacent to that in I,J that has been probed for *CC10*. Note that most cells of the rudiment are positive for *CC10*, including the most distal epithelial cells. All panels are at the same magnification (original magnification, 132×).

did not stimulate rudiment growth. FGF7 given as a single addition at 25 ng/ml had a slight effect on rudiment growth (Fig. 9D) that did not change when the concentration of ligand was increased to 200 ng/ml (data not shown). The combination of CSS, I, CT and FGF7 (25 ng/ml), however, stimulated significant growth of the tracheal epithelium (Fig. 9E), although not to the extent seen when rudiments were cultured in BFGM (Fig. 9F). The fact that the combination of CSS, I, CT and FGF7 did not support rudiment expansion to the same extent as complete BFGM suggests that missing components – EGF and HGF – have some stimulatory effect on proliferation.

When evaluated by in situ hybridization, the combination of CSS, I, CT and FGF7 induced expression of *SP-C* in tracheal epithelium (Fig. 10A,B). Single additions of CSS, or I, or CT failed to induce expression of *SP-C* (data not shown), as did the combination of CSS, I and CT (Fig. 14A,B). Importantly, FGF7 by itself also did not induce *SP-C* expression (Fig. 10C,D). FGF7 in combination with either CSS, or I, or CT induced *SP-C* expression in some tracheal epithelial cells, but

their distribution was not as widespread as seen with the combination of all four factors (data not shown).

Our previous experiments on day-13 embryonic rat lung epithelium maintained in mesenchyme-free culture demonstrated that FGF1 and FGF7 were equivalent in their ability to support proliferation (Deterding et al., 1996). In the present study, however, a consistent observation from the deletion experiments was that FGF1 did not support tracheal rudiment growth as well as FGF7: whereas rudiment expansion was not dramatically reduced when FGF1 was deleted from BFGM (Fig. 7G), deletion of FGF7 resulted in a significant reduction in rudiment size (Fig, 7H). FGF1 was not completely ineffective in supporting growth, however, since rudiment size was reduced even more when both FGF1 and FGF7 were

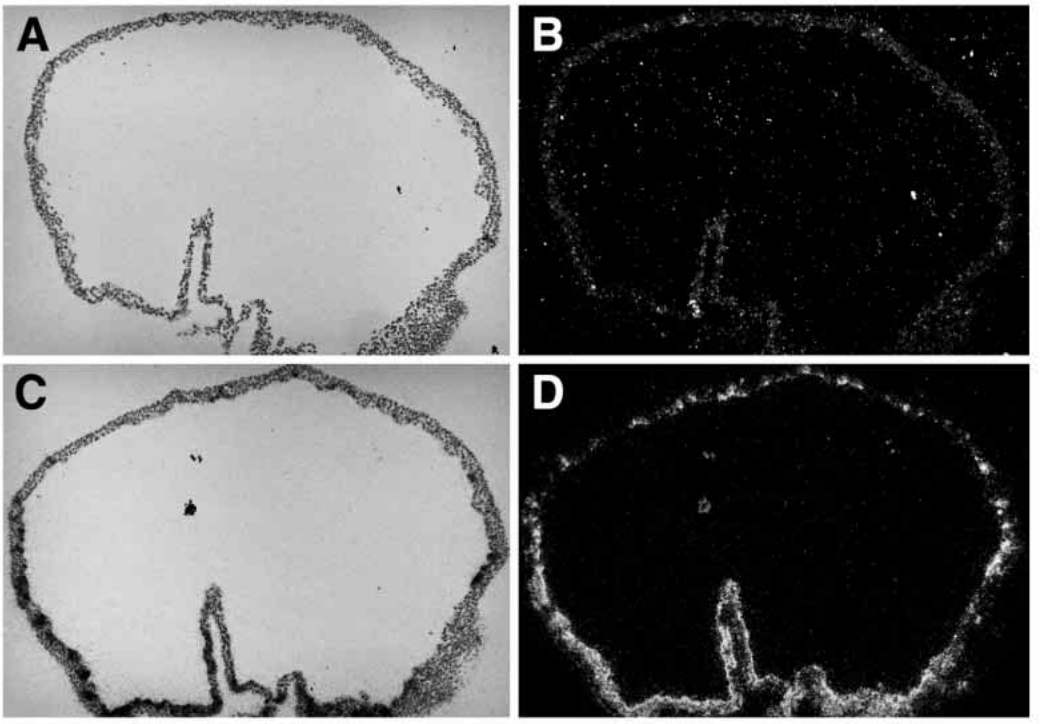

Fig. 6. The ability of tracheal epithelium to express a distal lung phenotype is temporally restricted. Tracheal epithelium was isolated from day-15 rat fetuses, enrobed in Matrigel, and cultured for 5 days in BFGM. (A,B) The epithelium has expanded, but expression of *SP-C* is not detected. (C,D) In contrast, *CC10* is detected in virtually every cell. All panels are at the same magnification (original magnification, 132×).

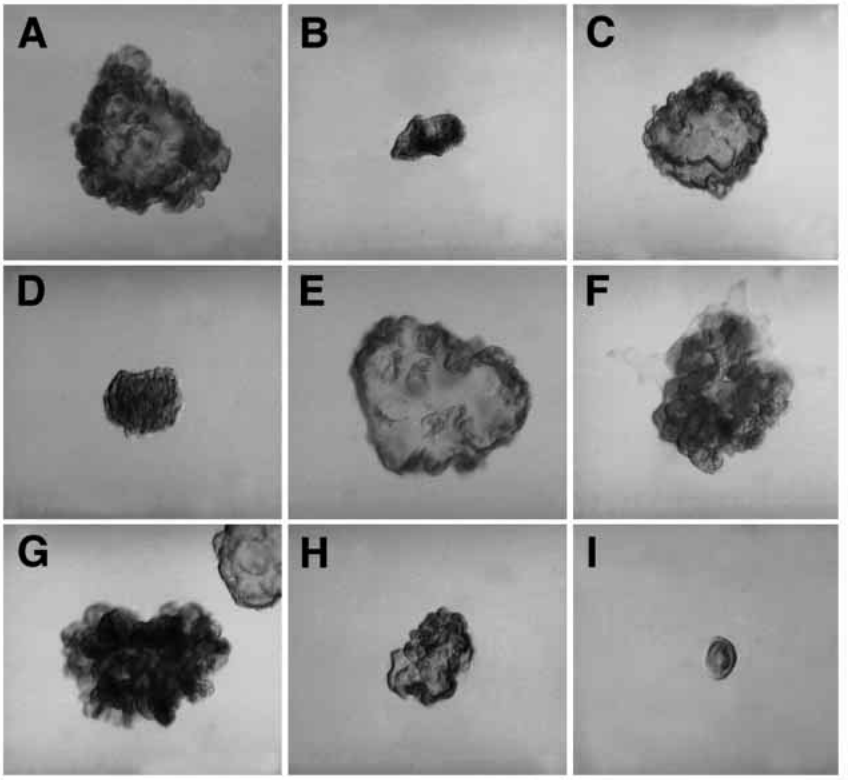

Fig. 7. Effects of deleting components from BFGM on embryonic tracheal epithelial growth. Day 13 embryonic tracheal epithelium was enrobed in Matrigel and cultured for 5 days in BFGM (A), or in BFGM from which specific components had been deleted. Deleting CSS from the medium (B) results in a significant reduction in growth, as does the deletion of cholera toxin (D). Deleting insulin (C) noticeably reduces growth, but not to the same extent as deleting CSS or cholera toxin. Deleting EGF (E), or HGF (F), or FGF1 (G) does not have a pronounced effect on rudiment growth. Deleting FGF7 (H) from the medium substantially reduces growth. The greatest reduction in growth is seen when both FGF1 and FGF7 are deleted from the medium (I), where the epithelium forms a small cyst. All panels are at the same magnification (original magnification, 56×).

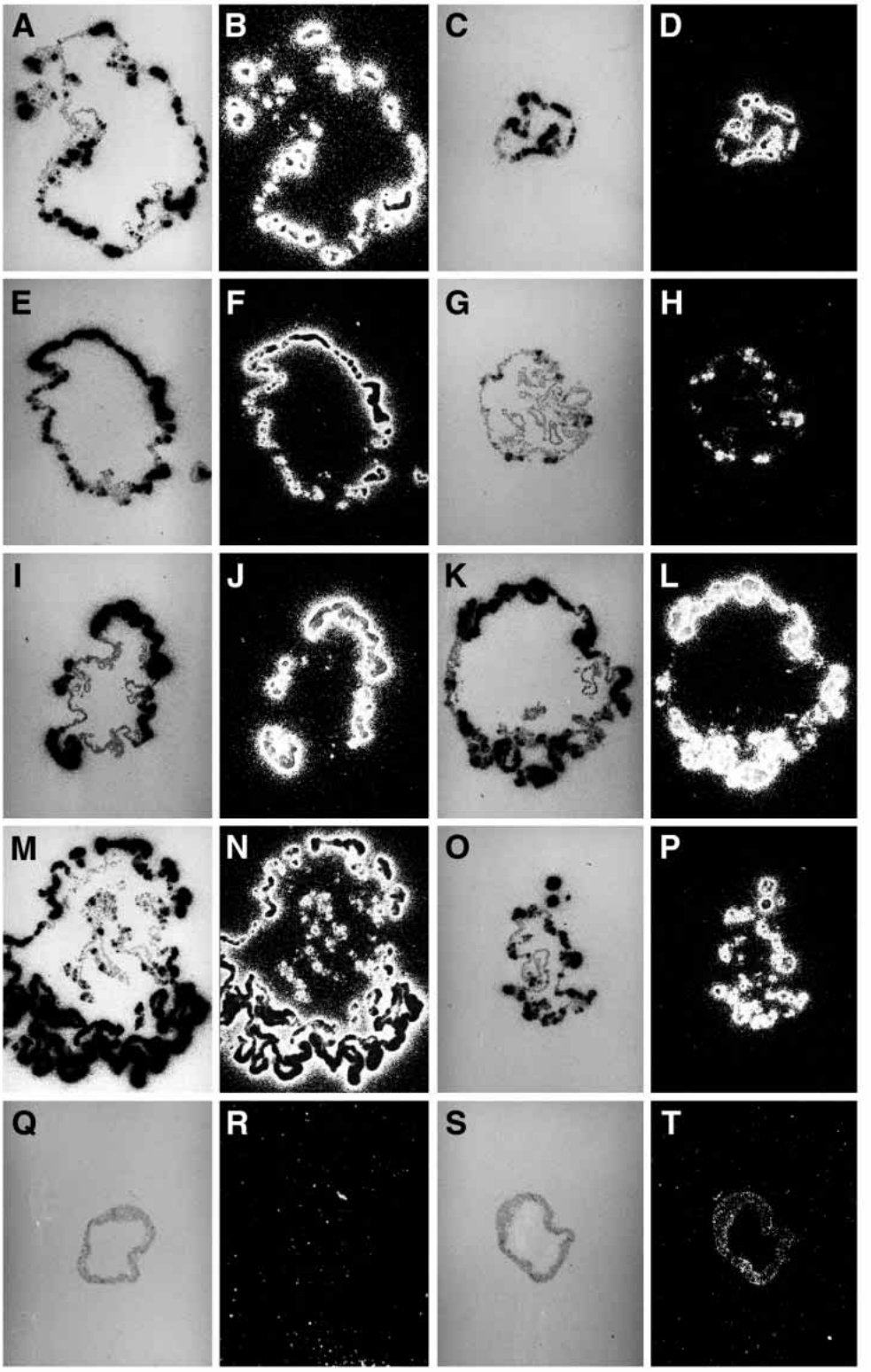

Fig. 8. Effects of deleting components from BFGM on expression of *SP-C* and *CC10* in embryonic tracheal epithelium. In situ hybridization was performed on day-13 embryonic tracheal epithelium that was enrobed in Matrigel and cultured for 5 days in BFGM from which specific components had been deleted. Exposure times of the autoradiographs is the same for each probe. Corresponding darkfield images of each sample were also taken. (A,B) Tracheal epithelium cultured for 5 days in BFGM and probed for *SP-C*. Widespread expression of *SP-C* is seen in clusters of cells throughout the rudiment. Tracheal epithelium cultured in BFGM from which CSS (C,D), or insulin (E,F), or cholera toxin (G,H), or EGF (I,J), or HGF (K,L), or bovine FGF1 (M, N), or FGF7 (O,P) have been singly deleted have still been induced to express *SP-C*. Deleting both FGF1 and FGF7 from the medium (Q,R) eliminates induction of *SP-C*; expression of *CC10*, however, is seen in virtually every cell within the epithelial cyst (S,T). All panels are at the same magnification (original magnification, 132×).

deleted (Fig. 7I). Because the FGF1 we used was purified from bovine brain and was 97% pure, we were concerned that the response to bovine FGF1 was due to a contaminant(s) that was eliciting a response at the concentration (100 ng/ml) used. To test this possibility, we compared bovine FGF1, human recombinant FGF1 from two different manufacturers, and FGF7 for their ability to support both tracheal epithelial growth and the induction of *SP-C*. The combination of CSS, I, CT and 100 ng/ml bovine FGF1 elicited a small amount of rudiment expansion (Fig. 11B) that was substantially less than that seen in the combination of CSS, I, CT, and 25 ng/ml FGF7 (Fig. 11A). Substitution of 100 ng/ml human recombinant FGF1 for bovine FGF1 resulted in no rudiment growth whatsoever (Fig. 11C).

FGF10 is a recently described member of the FGF family

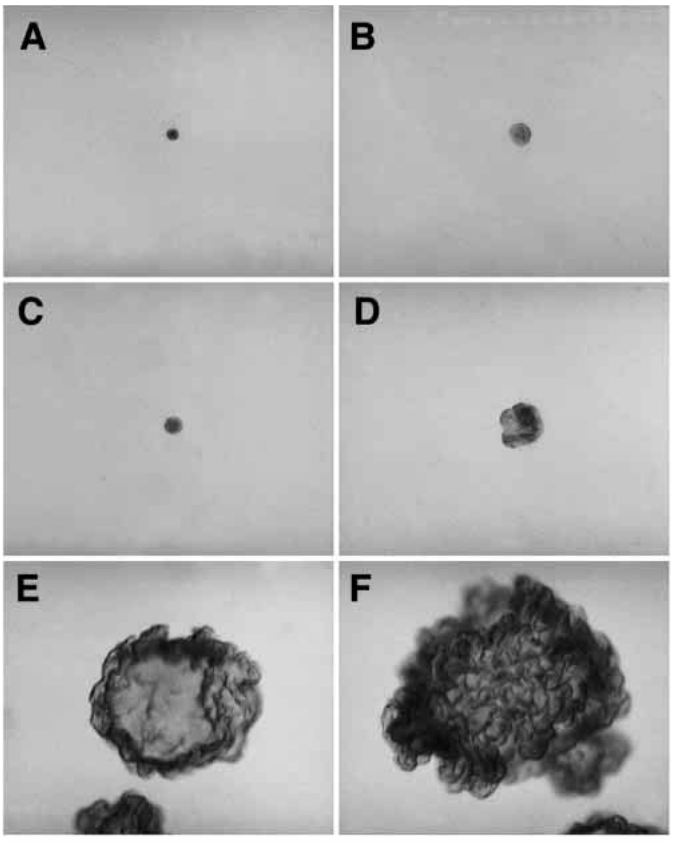

Fig. 9. Effects of single medium components on growth of embryonic tracheal epithelium enrobed in Matrigel and cultured for 5 days. (A) Day 13 tracheal epithelium cultured in medium containing only CSS; little or no growth has occurred. Tracheal epithelium cultured in medium containing only insulin (B) or only cholera toxin (C) also show little growth. (D) Tracheal epithelium cultured in medium containing only FGF7 (25 ng/ml) shows a small amount of growth; increasing the FGF7 concentration to 200 ng/ml had no further effect. The combination of CSS, insulin, cholera toxin and FGF7 (E) causes a much more pronounced stimulation of growth, but to an extent less than that seen in rudiments cultured in BFGM (F). All panels are at the same magnification (original magnification, 56×).

that is present in mesenchymal cells subtending the distal embryonic mouse lung epithelium (Bellusci et al., 1997) and that plays a key role in lung development (Min et al., 1998). However, FGF10 had no marked effect on tracheal epithelial expansion when given in combination with CSS, I and CT. Some of the rudiments were dilated as shown in Fig. 11D, but a morphology similar to that seen in Fig. 11C was equally prevalent. FGF10 given by itself had even less effect on the rudiments, in that dilation was never observed (data not shown). These observations were true for concentrations of FGF10 ranging from 1 to 1000 ng/ml.

As noted above, the combination of CSS, I, CT and FGF7 induced *SP-C* expression in many of the epithelial cells (Fig. 12A,B). Substituting bovine FGF1 for FGF7 resulted in cells being induced to express *SP-C* transcripts (Fig. 12C,D), but the percentage of *SP-C*-positive cells was far less than that seen with FGF7. Substituting human recombinant FGF1 for FGF7, however, did not result in induction of *SP-C* expression (Fig. 12E,F). Substitution of FGF10 for FGF7 (Fig. 12G,H) also did not induce expression of *SP-C* in tracheal epithelium.

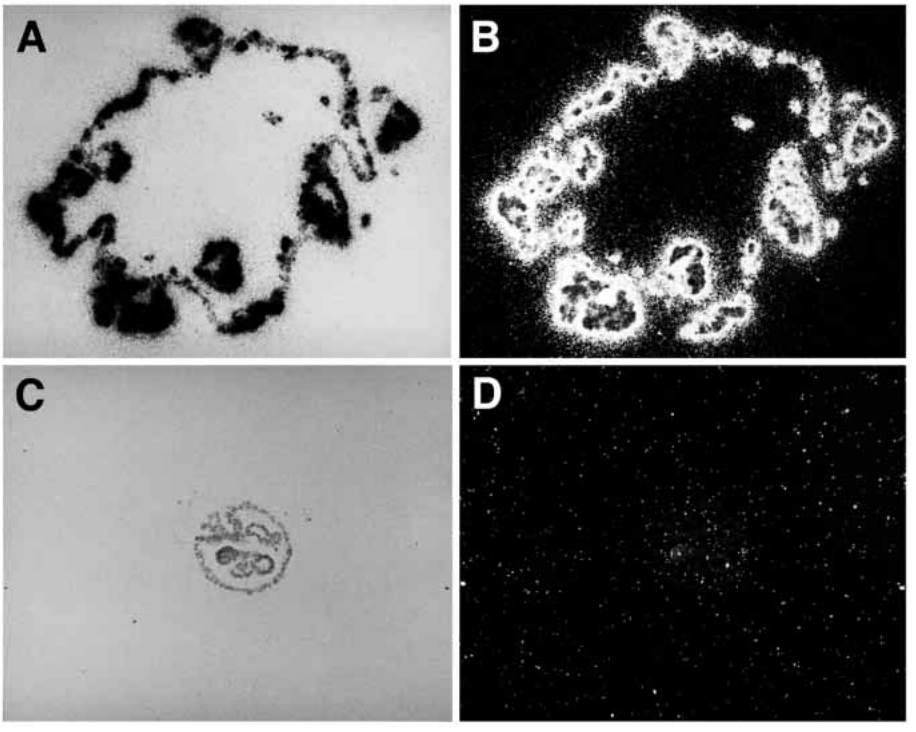

Fig. 10. Effect of FGF7 in inducing *SP-C* expression in cultured day-13 embryonic tracheal epithelium. (A,B) The combination of CSS, insulin, cholera toxin, and FGF7 induces *SP-C* expression in a pattern and intensity similar to that seen with BFGM. (C,D) FGF7 (25 ng/ml) added as a single addition to the medium fails to induce *SP-C* expression. Single additions of CSS, insulin, or cholera toxin also failed to induce *SP-C*. All panels are at the same magnification (original magnification, 132×).

The effects of cyclic AMP on tracheal epithelial rudiment growth and differentiation

Because one major effect of CT is to increase intracellular levels of cyclic AMP (cAMP) to very high levels, we next determined if CT could be replaced by other methods of increasing intracellular cAMP levels. Neither the combination of CSS, I and CT (Fig. 13A), nor CSS, I and forskolin/IBMX (Fig. 13C) supported rudiment expansion. The further addition of FGF7 to both combinations induced rudiment expansion, and demonstrated that forskolin/IBMX (Fig. 13D) was able to replace CT (Fig. 13B). Results using the cAMP analogue 8-bromo-cAMP (8-Br-cAMP) to replace CT were somewhat different. The combination of CSS, I, and 8-Br-cAMP also did not support rudiment growth (Fig. 13E). Further addition of FGF7 to the medium resulted in only a modest increase in rudiment expansion (Fig. 13F), that was less that seen when

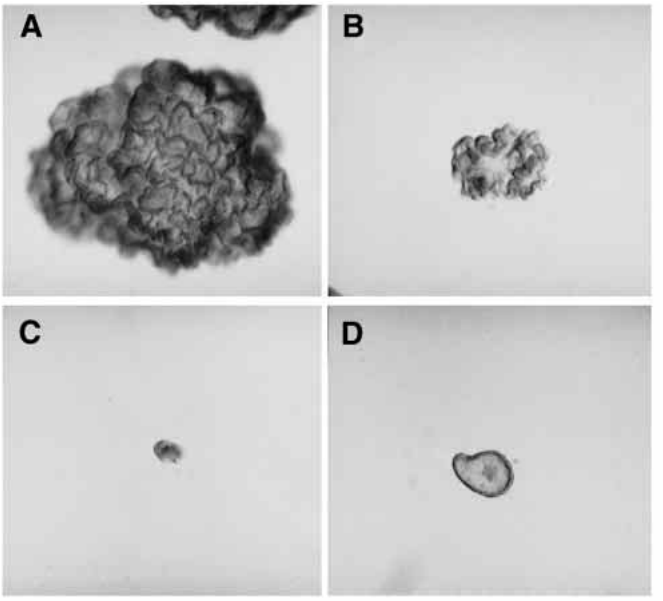

Fig. 11. Differential effects of human recombinant FGF7, bovine FGF1, human recombinant FGF1 and human recombinant FGF10 on growth of embryonic tracheal epithelium. (A) Day-13 embryonic tracheal epithelium enrobed in Matrigel and cultured for 5 days in medium containing CSS, insulin, cholera toxin and FGF7 (25 ng/ml) exhibits significant growth and surface folding. Rudiment growth occurs when bovine FGF1 (100 ng/ml) is substituted for FGF7 (B), but the extent of expansion is significantly reduced. Substitution of human recombinant FGF1 (100 ng/ml) (C) results in no rudiment growth whatsoever. When human recombinant FGF10 (100 ng/ml) (D) is substituted for FGF7, a small amount of rudiment expansion is seen. Some rudiments appear dilated as shown here, while others are not dilated. All panels are at the same magnification (original magnification, 56×).

either CT (Fig. 13B) or forskolin/IBMX (Fig. 13D) was used in the growth medium; this was true when 8-Br-cAMP was used at concentrations from 25 to 500 μM.

When cell diffentiation was examined, the combination of CSS, I and CT did not induce expression of *SP-C* (Fig. 14A,B). Addition of FGF7 to this combination resulted in the expression of *SP-C* (Fig. 14C,D). Substituting 8-Br-cAMP for CT in this combination resulted in the induction of *SP-C* (Fig. 14D,E), but the number of positive cells and the signal intensity was substantially less than that seen in the combination of CSS, I, CT and FGF7 or CSS, I, forskolin/IBMX and FGF7 (data not shown). Why 8-Br-cAMP was unable to mimic the effects of cholera toxin is unclear. It may be that 8-Br-cAMP is unable to attain a sufficiently high level within the cells to effect a maximal response, or that the brominated form of cAMP is less effective in signaling in this system.

DISCUSSION

In the present study we have utilized mesenchyme-free culture to demonstrate that embryonic tracheal epithelium can be reprogrammed to express an alveolar type II cell phenotype in the absence of lung mesenchyme, and that FGF7 is necessary, but not sufficient by itself, to effect this transdifferentiation. Like embryonic lung epithelium in mesenchyme-free culture, tracheal epithelium cultured in BFGM traversed the full pathway to distal lung epithelial cell differentiation, resulting in cells that were ultrastructurally indistinguishable from late-gestation alveolar type II cells. This commitment to distal lung epithelial cell differentiation was apparently at the exclusion of tracheal differentiation, since we did not observe any ciliated or mucous secretory cells. This lack of morphologically identifiable tracheal epithelial cells is in agreement with our observation (Shannon et al., 1998) that tracheal epithelial differentiation requires specific inductive signals elaborated by tracheal mesenchyme. As noted above, however, not all tracheal epithelial cells were induced to express an alveolar type II cell phenotype. Furthermore, we observed cells that expressed both *CC10*, a marker of proximal pulmonary epithelium, and *SP-C*, which is specific to the distal epithelium. These cells may represent progenitor cells that are not yet committed to a proximal or distal epithelial fate. We have previously observed cells positive for both *SP-C* and *CC10* in explant cultures of intact embryonic lung and in tissue recombinants that were constructed with lung mesenchyme (Shannon et al., 1998). Cells positive for *SP-C* and *CC10* have also been observed in adult mouse lung epithelium that is undergoing repair following bleomycin injury (Daly et al., 1998), supporting the possibility that these cells are multipotential cells.

The ability of tracheal epithelium to be reprogrammed by the inductive signals present in BFGM is, however, temporally restricted. Although tracheal epithelium from day-15 and -17 embryos grew in BFGM, by day 15 of gestation the tracheal epithelium had lost its competence to express *SP-C* in response to BFGM. This observation is in accord with our earlier observation (Shannon, 1994) that day-13 lung mesenchyme, which will induce lung-like branching and type II cell differentiation when recombined with day-13 tracheal epithelium, could not elicit a similar response in day-16 tracheal epithelium. It appears, therefore, that rat tracheal epithelium is restricted between gestational days 13 and 15, probably by inductive cues from tracheal mesenchyme. Furthermore, this restriction of the tracheal epithelium is not dependent on sustained induction by tracheal mesenchyme, since 5 days of culture in BFGM did not reprogram mesenchyme-free day-15 or -17 tracheal epithelial rudiments.

Studies on mesenchyme-free culture of embryonic rat (Deterding et al., 1996) and mouse (Nogawa and Ito, 1995; Cardoso et al., 1997; Bellusci et al., 1997) lung epithelium have suggested a critical role for members of the FGF family in lung development. Of particular interest are FGF1, FGF7, and FGF10, all of which have been shown to bind the IIIb splice variant of the FGF receptor 2 (FGFR2IIIb). Because *Fgf7* (Mason et al., 1994; Finch et al., 1995) and *Fgf10* (Bellusci et al., 1997) are expressed in lung mesenchyme, and because *FgfR2IIIb* is expressed in the developing lung epithelium (Peters et al., 1992; Orr-Urtreger et al., 1993; Finch et al., 1995) these ligands are attractive candidate mediators of mesenchyme-directed lung morphogenesis and differentiation. The importance of the FGF signaling pathway to normal lung development is emphasized by experiments in which a truncated form of the FGFR2IIIb was targeted to the lung using the *SP-C* promoter; the resultant overexpressed dominant-negative receptor completely blocked branching morphogenesis

(Peters et al., 1994). Similarly, broadly expressed soluble dominant-negative FGFR2IIIb results in pulmonary agenesis (Celli et al., 1998). Antisense oligonucleotides to *FgfR2IIIb* also inhibit branching morphogenesis of embryonic rat lung explants in vitro (Post et al., 1996).

A significant observation in the experiments of Peters et al. (1994) was that the most distal regions of the lung epithelium in transgenic mice expressing a dominant-negative form of FGFR2IIIb were negative for *SP-C*, but positive for *CC10*. Since *SP-C* is found only in the distal epithelium of the developing lung (Kalina et al., 1992; Wert et al., 1993), this suggests that ligands binding FGFR2IIIb are not only important for supporting branching morphogenesis, but also in specifying the distal lung epithelial phenotype. Our data support this possibility, since transdifferentiation of the tracheal epithelium only occurred in the presence of FGF7 or bovine FGF1, and both FGF1 and FGF7 bind FGFR2IIIb (Ornitz et al., 1996).

Whereas our data suggest an important role for FGF7 in specification of the distal lung epithelial phenotype, transgenic mice null for the *Fgf7* gene show no abnormalities in lung development (Guo et al., 1996). This suggests that another member of the FGF family that binds FGFR2IIIb can serve as a functionally redundant ligand in the developing lung. Given the results seen in mesenchyme-free cultures of embryonic mouse lung epithelium, FGF1 would seem to be a good candidate molecule. Although FGF1 does not contain a signal peptide for classical secretion, several studies have shown that FGF1 can be secreted by non-classical means that do not require cell death (Guillonneau et al., 1997). *Fgf1* is not seen in either the epithelium or mesenchyme in the early (day 11.5) embryonic mouse lung by in situ hybridization, but is detectable 2 days later in both tissue compartments (Bellusci et al., 1997). Immunodetectable FGF1 is present in the day-14 embryonic rat lung, but only in the mesenchyme (Fu et al., 1991), similar to the distribution of *Fgf7* (Mason et al., 1994; Finch et al., 1995).

Our results regarding the role of FGF1 in specification of the distal lung epithelial phenotype, however, are somewhat unresolved. Whereas our data demonstrate a clear effect of human recombinant FGF7, human recombinant FGF1 was completely ineffective in inducing either growth or *SP-C* expression in tracheal epithelial rudiments. A possible

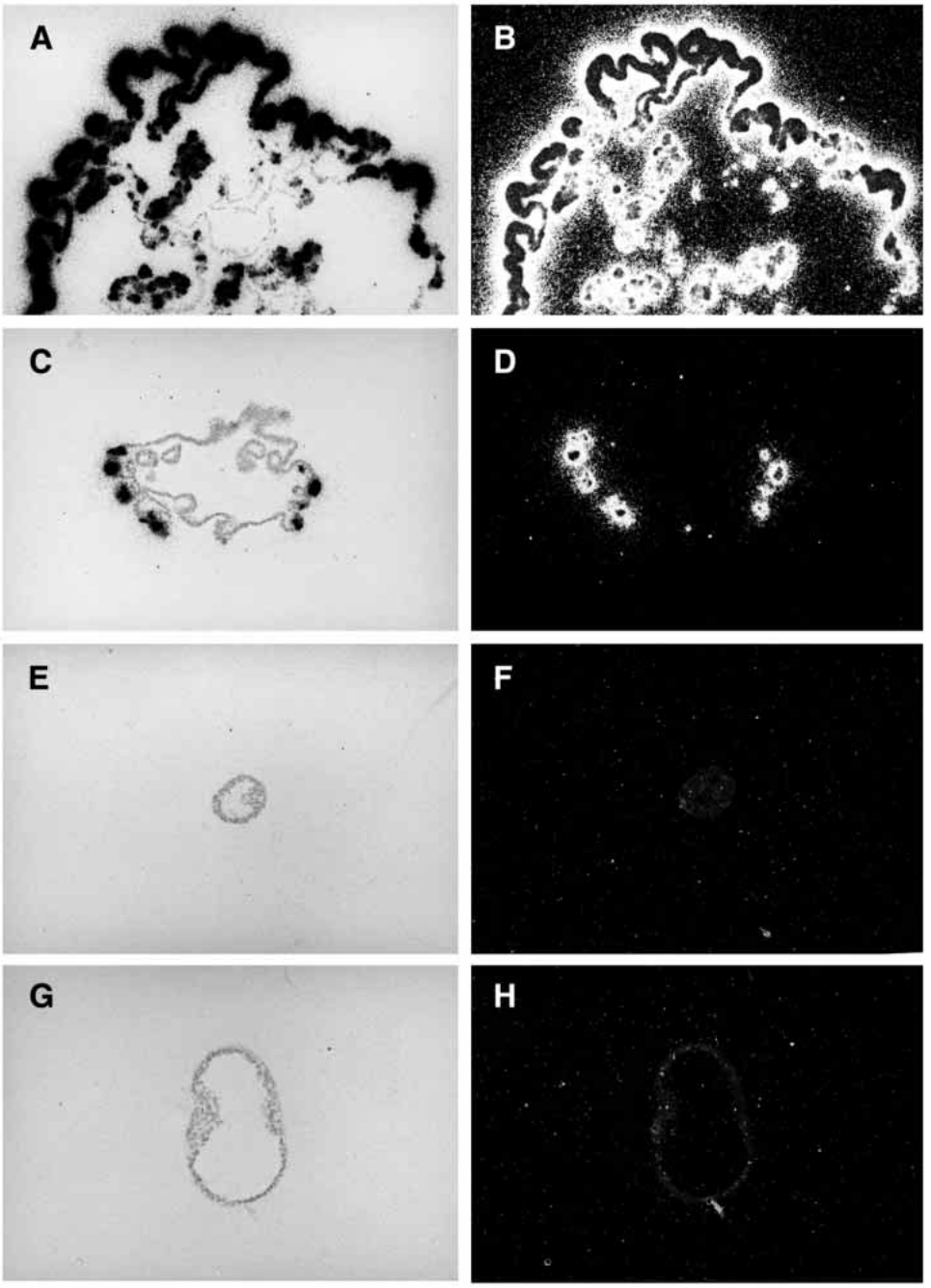

Fig. 12. FGF7, bovine FGF1, human recombinant FGF1 and human recombinant FGF10 differ in their ability to induce *SP-C* expression in cultured embryonic tracheal epithelium. Cultures were prepared as described for Fig. 11 and sections probed for *SP-C*. (A,B) The combination of CSS, insulin, cholera toxin and FGF7 induces extensive *SP-C* expression. (C,D) Substitution of bovine FGF1 for FGF7 results in the induction of *SP-C* in small clusters of cells in the distal aspects of the rudiment. (E,F) Substitution of human recombinant FGF1 for FGF7 fails to induce *SP-C* in embryonic tracheal epithelium. FGF10 also fails to induce *SP-C* expression when substituted for FGF7 (G,H). All panels are at the same magnification (original magnification, 132×).

explanation is that there is a significant species difference in the ability of rat cells to respond to human FGF1. FGF1 extracted from bovine brain did not give as great a growth response as FGF7, but did induce *SP-C* in the tracheal epithelium, albeit with less widespread distribution than that seen with FGF7. Since, as noted above, the bovine FGF1 preparation was extracted from tissue and not completely (97%) pure, we considered the possibility that the observed results were due to a highly active contaminating factor in this bovine FGF1 preparation. In a previous study on mesenchyme-free culture of embryonic rat lung epithelium (Deterding et al., 1996), however, we demonstrated that bovine FGF1 and human recombinant FGF7 were quantitatively equal and not additive in their ability to support growth and differentiation of embryonic rat lung epithelium in mesenchyme-free culture. These embryonic lung epithelial rudiments were *SP-C* positive at the time of isolation, indicating that they had already been directed towards distal lung differentiation. Considering these observations with the present data, it appears that embryonic lung and tracheal epithelium differ fundamentally in their response to FGF1. This implies that FGF7 and FGF1 may have distinct roles in the specification and maintenance of the distal lung epithelial phenotype during development. This is supported by the observation that embryonic mouse lung epithelium grown in mesenchyme-free culture exhibits different patterns of growth in response to FGF1 and FGF7 (Cardoso et al., 1997). Although the differences we observed in the response of rat tracheal epithelium to human recombinant and bovine FGF1 do not provide definitive evidence for FGF1 as a compensating growth factor for FGF7, we cannot exclude the possibility that the endogenous rat ligand might be a more effective substitute.

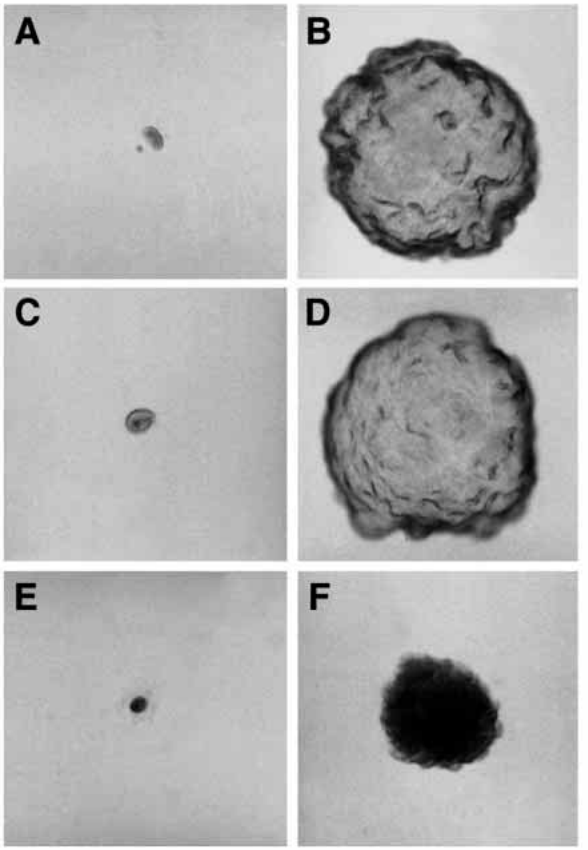

Fig. 13. The effects of cholera toxin can be mimicked by increasing intracellular cAMP. Day-13 embryonic tracheal epithelium was enrobed in Matrigel and cultured for 5 days. (A) The combination of CSS, insulin and cholera toxin does not support rudiment growth, which requires the further addition of FGF7 (B). Forskolin plus isobutylmethylxanthine (IBMX) increases intracellular cAMP. The combination of CSS, insulin and forskolin/IBMX does not support rudiment growth (C); however, the further addition of FGF7 (D) results in rudiment growth identical to that seen with CSS, insulin, cholera toxin and FGF7. (E) The combination of CSS, insulin and 8-Br-cAMP does not support growth of tracheal epithelium; addition of FGF7 to this combination (F) results in rudiment expansion, but not to the extent seen in combinations containing cholera toxin or forskolin/IBMX. All panels are at the same magnification (original magnification, 56×).

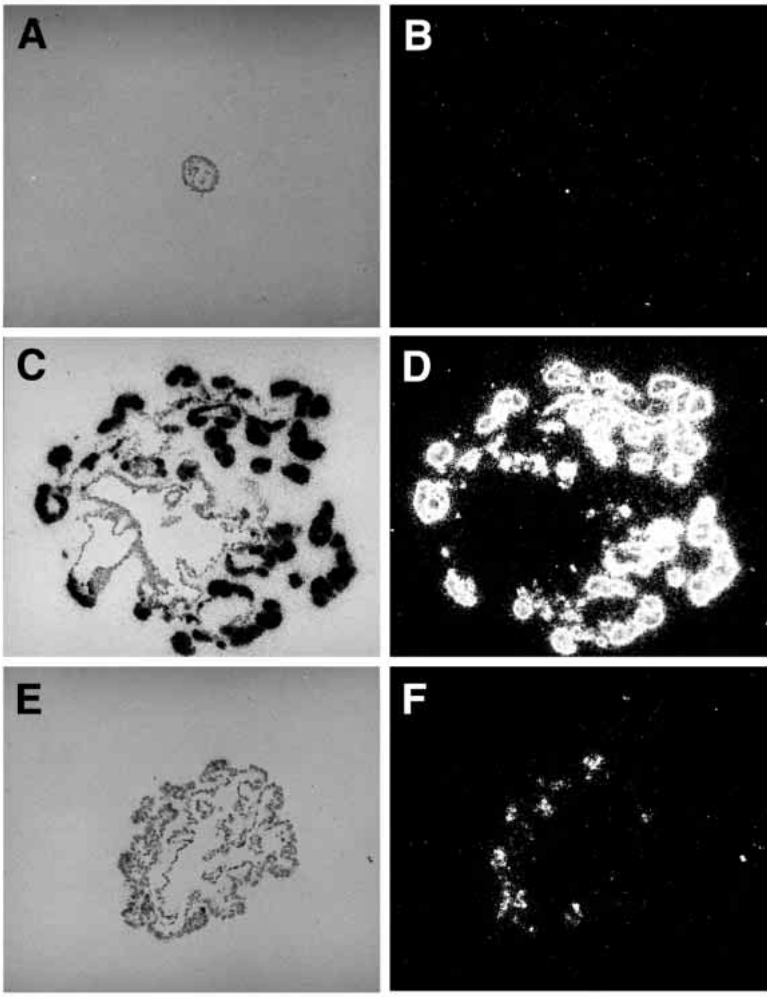

Fig. 14. Comparison of cholera toxin and 8-Br-cAMP in inducing *SP-C* expression in cultured tracheal epithelium. Exposure times are identical for the autoradiographs. Corresponding darkfield images are also shown. (A,B) The combination of CSS, insulin, and cholera toxin does not induce *SP-C*, which requires the further addition of FGF7 (C,D). The combination of CSS, insulin, 8-Br-cAMP and FGF7 also induces *SP-C* (E,F), but the intensity of expression is less than that seen in the combination containing cholera toxin. All panels are at the same magnification (original magnification, 132×).

Another member of the FGF family that is known to bind FGFR2IIIb is the recently described FGF10 (Yamasaki et al., 1996; Tagashira et al., 1997; Beer et al., 1997; Emoto et al., 1997), which contains a classical signal peptide for secretion, and is highly expressed in distal lung mesenchyme but not in tracheal mesenchyme (Yamasaki et al., 1996; Bellusci et al., 1997). Furthermore, addition of FGF10 to mesenchyme-free cultures of embryonic lung epithelium results in rudiment expansion and budding (Bellusci et al., 1997). The key role that FGF10 plays in lung development is shown by the observation that mice deficient for *Fgf10* lack tracheal bifurcation and lung branching (Min et al., 1998). Recent data (Park et al., 1998) suggest that FGF10 may act as a chemotactic factor in the developing lung that promotes epithelial ingrowth into the mesenchyme as opposed to acting as a mitogen. This is reminiscent of the role played by the *Drosophila* FGF homologue *branchless* (Sutherland et al., 1996).

Embryonic tracheal epithelium has been shown to express *FgfR2IIIb* by in situ hybridization, which, given its ability to respond to FGF7, must be functional. Since FGF10 also binds FGFR2IIIb (Igarashi et al., 1998), we tested FGF10 for its ability to substitute for FGF7 in the transdifferentiation of competent tracheal epithelium. Somewhat to our surprise, FGF10 acted as a weak mitogen that did not support an appreciable amount of tracheal rudiment expansion, and was also unable to induce *SP-C* expression. These data, along with the observations of others (Park et al., 1998), suggest that the roles played in lung development by FGF7 and FGF10 are clearly different. FGFR2IIIb is the only receptor splice variant reported that binds FGF7 and FGF10. Since we observed such disparate effects of FGF7 and FGF10 on tracheal epithelium, our data suggest that the ligand-receptor interaction and subsequent intracellular signaling must be more complex than is currently understood. These in vitro observations about the different roles for FGF7 and FGF10 in lung development is supported by observations made in vivo: mice null for *Fgf7* have normal lungs (Guo et al., 1996), whereas mice null for *Fgf10* lack lungs entirely (Min et al., 1998).

Tracheal epithelium maintained in mesenchyme-free culture in the presence of FGF7 alone showed a very limited amount of expansion over the 5-day culture period, and no detectable expression of *SP-C*. This indicates that FGF7 is insufficient by itself to effect transdifferentiation of tracheal epithelium to a distal lung phenotype. This is confirmed by the observation that heparin beads soaked in FGF7 and grafted onto a stretch of tracheal epithelium that has had its own mesenchyme removed does not induce branching (J. M. S. unpublished observations). Our observation that FGF7 alone had little effect on rat tracheal epithelium in mesenchyme-free culture differs from the response of embryonic mouse lung epithelium in culture for 1 to 3 days; in these experiments FGF1, FGF7 and FGF10 were all able to support some degree of rudiment expansion by themselves (Nogawa and Ito, 1995; Bellusci et al., 1997; Cardoso et al., 1997). Since lung mesenchyme can reprogram tracheal epithelium to express a type II cell phenotype (Shannon et al., 1998), these observations suggest that lung mesenchyme produces another factor(s) in addition to a ligand(s) that binds FGFR2IIIb which is involved in specification of the lung epithelial phenotype. Since the response of tracheal epithelium to FGF7 was greatly potentiated by CT or forskolin/IBMX, a likely candidate would be a factor(s) that causes an increase in intracellular cAMP, which serves as a second messenger for a number of hormones and other factors. The identity of this factor in the embryonic lung is presently unknown. Although tracheal mesenchyme expresses *Fgf7* (Finch et al., 1995), it may lack an additional factor(s) produced by lung mesenchyme whose presence is continuously required to sustain lung differentiation. This could explain why tracheal mesenchyme will not support continued differentiation of lung epithelium (Shannon et al., 1998). Alternatively, tracheal mesenchyme may produce a factor(s) that actively induces tracheal differentiation and suppresses the distal lung phenotype, or redirects the response to FGF7. Our present data do not allow us to distinguish among these possibilities.

As noted above, the combination of CSS, I, CT and FGF7 did not completely reproduce the effects of BFGM in inducing epithelial expansion. This suggests that two components deleted from BFGM, EGF and HGF, contribute to the overall growth response of the epithelium. This is consistent with the observations that mice null for the EGF receptor have pulmonary hypoplasia (Miettinen et al., 1995; Sibilia and Wagner, 1995), and that HGF, which by itself does not promote growth of embryonic lung epithelium in mesenchyme-free culture, can synergize with FGF1 or FGF7 to promote growth (Ohmichi et al., 1998). Neither EGF nor HGF appear to be crucial to induction of the distal lung epithelial phenotype in tracheal epithelium, however, since reprogramming occurred in their absence.

In summary, we have demonstrated that early embryonic tracheal epithelium can be reprogrammed to express a distal lung phenotype in the absence of lung mesenchyme. FGF7 is necessary, but not sufficient, to mediate this instructive induction, and cannot be replaced by FGF10. Since this transdifferentiation occurs within 24 hours of the exposure of the tracheal epithelium to the inducing medium, we believe that this system is ideally suited for studying early events in the specification of the distal lung epithelial phenotype.

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