Phenotype and Differentiation Potential of a Novel Rat Tracheal Epithelial Cell Line

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In this report we described the establishment and characterization of a continuous rat tracheal epithelial (RTE) cell line spontaneously derived from secondary RTE cell cultures. Designated SPOC1, this cell line is nontumorigenic and maintains a diploid karyotype with specific, nonrandom chromosomal alterations involving chromosomes 1, 3, and 6. SPOC1 cells demonstrate decreased requirements for peptide growth factors, compared with primary RTE cells. Upon inoculation into denuded rat tracheas, which are then implanted into syngeneic hosts, SPOC1 cells initially form a stratified squamous epithelium, which becomes less stratified with time and forms glandlike invaginations into the surrounding lamina propria. No evidence of ciliated cell differentiation is detected. The epithelium formed by SPOC1 cells in tracheal grafts reacts with antibodies specific for keratin 14, 13, and 19 (but not keratin 18) at both early and late time points, although the localization of antibody staining changes as the epithelium becomes less stratified with time. The suprabasal epithelial cells become positive for alcian blue–periodic acid-Schiff staining at later time points. The near-normal karyotype and differentiation potential of SPOC1 cells make this cell line a unique window into early changes occurring during immortalization of airway epithelial cells and will allow studies of relationships between differentiation state and neoplastic transformation.

The development of cancer is a multistep process in which molecularly altered progenitor cells clonally expand in response to proliferative stimuli (1, 2). Both genetic and epigenetic influences on this altered population lead to progressive loss of key growth regulatory features and neoplastic transformation. Lung neoplasms are the most frequent human cancers, and primary lung cancer is divided into four major histologic subtypes. The predominant forms of bronchogenic carcinoma include squamous cell carcinoma, adenocarcinoma, and adenosquamous carcinoma. It is not known, however, which airway cells are the progenitors for these lung carcinomas. The major cell types in the tracheal and proximal bronchial epithelium are basal, ciliated, and

secretory (mucous and serous) cells, and in the bronchioles, Clara and ciliated cells. Cells capable of cell division, and therefore candidates for neoplastic transformation include basal, secretory, and Clara cells (3, 4). Identifying the target cells for lung cancers would greatly facilitate our understanding of the cellular and molecular mechanisms involved in lung cell neoplastic progression.

In vitro models have been widely used to study better the molecular and cellular alterations in the various forms of lung cancer. Immortalized human cell lines have been established from lung tumor tissue (5) and by transformation of primary tracheobronchial epithelial cells with DNA tumor viruses, such as SV40 (6) and cooperating oncogenes (7, 8). However, there are only a few reports that describe human airway epithelial cell lines that maintain their differentiated phenotype (9, 10). Such cell lines would be valuable for studying the progenitor cells of airway neoplastic disease.

Our laboratory has been studying neoplastic progression in rat tracheal epithelial (RTE) cells in vitro. Carcinogen exposure of primary RTE cells results in the development of enhanced growth variants (EGV) (11). A certain percentage of EGV can be expanded into cell lines, and some of these cell lines form tumors upon injection into nude mice. Characterization of growth requirements for EGV cell lines reveals that they are less dependent on exogenous growth factors than are primary RTE cells (12, 13), and they have

alterations in expression and organization of extracellular matrix proteins (14). Aberrations in chromosome number and structure, some of which are nonrandom, have been noted in all EGV cell lines (15, 16). Of particular interest is the disparity in differentiation potential between primary RTE cells and EGV cell lines. Primary RTE cells repopulate denuded tracheas with a normal pseudostratified mucociliary epithelium, whereas EGV cell lines from a keratinizing squamous epithelium and neoplastic EGV cells form invasive squamous cell tumors when transplanted (17). Thus, the RTE transformation system apparently selects for cells with a squamous pattern of differentiation, which is a phenotype seen in preneoplastic and neoplastic tracheobronchial lesions (11).

In this report we describe the establishment and characterization of a continuous RTE cell line spontaneously derived from secondary RTE cell cultures. Designated SPOC1 (*spo*ntaneously derived in *c*omplete serum-free medium), this cell line is nontumorigenic and, unlike any other RTE cell line, maintains a diploid karyotype with specific, nonrandom chromosomal alterations. Although similar to other RTE cell lines in having reduced growth factor requirements compared with primary RTE cells, SPOC1 cells are unique in their ability to differentiate into a pseudostratified epithelium with glandlike structures in denuded tracheal grafts. The near-normal karyotype and differentiation potential of SPOC1 cells make this cell line a useful tool for studying early cellular and molecular alterations associated with airway epithelial cell multistep carcinogenesis.

Materials and Methods

Primary RTE Cell Culture

Epithelial cells were isolated from the tracheas of 10- to 14-wk-old Fischer 344/NCR rats by protease digestion as previously described (12). Primary RTE cells were plated at a density of 1×10^4 cells/60-mm dish in complete serum-free medium (CSFM) consisting of Ham's F-12 media containing 5 ng/ml epithelial growth factor (EGF) and 5 μg/ml transferrin (Collaborative Research, Waltham, MA), 5 μg/ml insulin, 0.1 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 50 mM ethanolamine and phosphoethanolamine, 0.8 mM $CaCl_2$, 1.5 mg/ml bovine serum albumin, essentially globulin free (Sigma Chemical Co., St. Louis, MO), 0.1 μg protein/ml of bovine pituitary extract (BPE) and 15 mM Hepes, pH 7.3 (Sigma), cultured in a humidified 5% CO_2/95% air environment at 37°C. Medium was changed 1 d after plating and subsequently every other day.

Secondary RTE Cell Culture and Establishment of Cell Line

The scheme for generation of the SPOC1 cell line is shown in Figure 1. On Day 41 of primary culture, cells were trypsinized, centrifuged to remove trypsin, and counted. After counting, cells were seeded into 60-mm dishes at a density of 1000 cells/dish (clonal density). Cells were grown under the same conditions as primary cultures.

When secondary cultures were 1 mo old, the cells from five dishes were pooled (10 to 20 colonies visible per dish) and plated at a density of 4×10^5 cells in a 25-cm² flask and grown to 80% confluency at which time cells were pas-
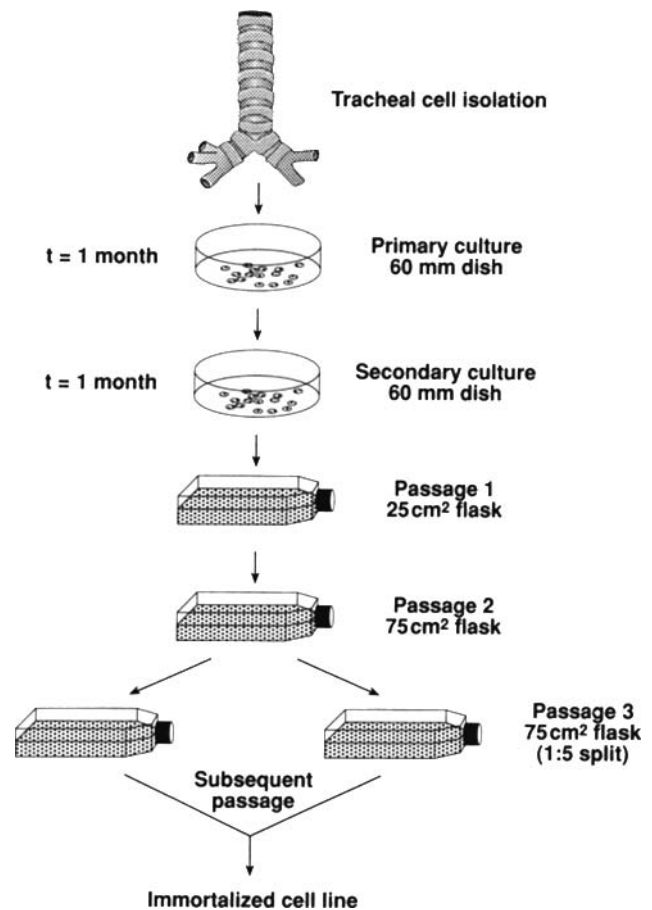

Figure 1. Diagram of the steps taken in establishing the SPOC1 cell line.

saged into 75-cm² flasks (1:1) and designated as SPOC1, passage 1. At 80% confluence, the cells were split (1:5) into 75-cm² flasks. Cells were continuously passaged at 80% confluence, 1:10 split, for > 31 passages (> 100 population doublings) and were considered a continuous cell line.

For some experiments, cells were plated at 2×10^4 cells/well onto Transwell-Col tissue culture inserts (Costar, Cambridge, MA), which have been shown to promote differentiation of primary RTE cells *in vitro* (18). Culture methods were essentially the same as those on plastic, except that cells were directly plated on inserts in medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 medium and the supplements described previously (18). In addition, some cultures received 5×10^{-8} M retinoic acid, which promotes mucociliary differentiation in primary RTE cells (18). When SPOC1 cultures were confluent, medium was removed from the apical surface, and cells received fresh medium every other day in the basolateral compartment only.

To determine colony forming efficiency of SPOC1 cells, cells were plated on plastic at clonal density at passages 3, 5, 10, 15, 20, and 25. The colony-forming efficiency was determined from the average number of colonies on three dishes at Day 10 divided by the number of cells plated per dish.

Growth curves for different passage SPOC1 cells were

generated by plating cells on 60-mm culture dishes at a cell density of 1 to 5 × 10⁴ cells/dish. At the indicated times, triplicate dishes were washed in phosphate-buffered saline (PBS) and trypsinized, and the cells were counted using a Coulter counter. Some cells were plated in CSFM medium, but changed to medium without insulin, EGF, BPE, or both EGF/BPE 1 d after plating. For all growth conditions, medium was then replaced every other day for the duration of the experiment. Growth curve experiments were repeated at least 2 times.

Cytogenetics and Karyotyping

Logarithmically growing cells from passages 4 and 28 were incubated with colcemid (0.05 μg/ml, final concentration) in CSFM for 15 to 45 min. Cells were detached by trypsinization, transferred to a 15-ml centrifuge tube, and collected by centrifugation. Supernatant was decanted, and cells were resuspended in hypotonic solution. Cells were fixed in a 3:1 methanol:glacial acetic acid fixative and after fixation, spread on slides and allowed to air dry. For G-banding, slides were immersed in trypsin solution (0.025% in PBS, pH 6.8), rinsed in PBS, and stained in 5% Giemsa, pH 6.8. Chromosome analysis was made according to the standardized nomenclature for rat chromosomes (19). At least 30 metaphases were analyzed to determine the modal karyotype of SPOC1 cells at passages 4 and 28. Recurrent chromosome abnormalities were noted.

Tumorigenicity Analysis

At passage 28, cells were injected into anesthetized nude mice (Nu/Nu Balb/c). Two sites per mouse were inoculated with 200 μl of Ham's F-12 containing either 4 or 2 × 10⁶ cells per inoculum. Control animals were injected with 200 μl of F-12. Mice were monitored weekly for 1 yr for the appearance of tumor nodules.

Tracheal Grafts

Tracheas to be inoculated with SPOC1 cell suspensions were obtained from male F344 rats 8 to 16 wk of age. The tracheas were frozen at −20°C and thawed 1 h later. This was repeated 4 times. Before use, frozen tracheas were thawed, and the distal ends were sealed with a silk suture. Each trachea was then inoculated with 1 × 10⁴ cells resuspended in 50 μl of Ham's F-12 medium, and the proximal end was tied with another suture. Transplants were placed subcutaneously onto the backs of syngeneic rats.

Tracheal grafts were harvested on Day 7 and Day 21 after implantation and prepared for histology and immunohistochemistry as previously described (20). Briefly, grafts were cut into rings and fixed with OmniFix II (An-Con Genetics, Melville, NY) or 4% PFA in PBS, embedded in paraffin and stained with hematoxylin and eosin (H&E), alcian blue (pH 2.5)–periodic acid–Schiff-hematoxylin (AB-PAS), or cell type–specific monoclonal antibodies (mAb). Sections were deparaffinized, rehydrated, and stained as previously described (20), and peroxidase activity was visualized using a 6-min incubation in diaminobenzidine (DAB)-H_2O_2 solution (20 mg DAB and 100 μl of 3% H_2O_2 in 100 ml of 0.05 M Tris buffer, pH 7.6). The slides were counterstained with 1% methyl green. For negative controls, hybridoma supernatants were replaced with equal dilutions of NS 1

myeloma cell–conditioned medium plus normal mouse serum immunoglobulin G (50 μg/ml).

Five different mAb, whose reactivity with normal RTE cells has been characterized (18, 21, 22), were used to determine cell type–specific reactivity of SPOC1 cells in grafts. mAb LL002 (kindly provided by Dr. E. B. Lane, Dundee, UK) reacts with human keratin 14 and is specific for basal cells in many epithelia, including the rat trachea. Keratin 13 was detected with mAb AE8 (ICN, Costa Mesa, CA) and is a marker for squamous epithelium (23). mAb CK18-2 (kindly provided by Dr. F. C. S. Ramaekers, Maastricht, The Netherlands) reacts with human keratin 18 and strongly stains columnar-type epithelial cells in the rat trachea and in primary RTE cell cultures (18, 22). Keratin 19 was detected with mAb RTE 1, which has previously been shown to react with most epithelial cells in intact adult rat tracheas (21, 22). The presence of ciliated cells was assessed with mAb RTE 3, previously shown to be specific for an epitope expressed by ciliated cells *in vivo* (22).
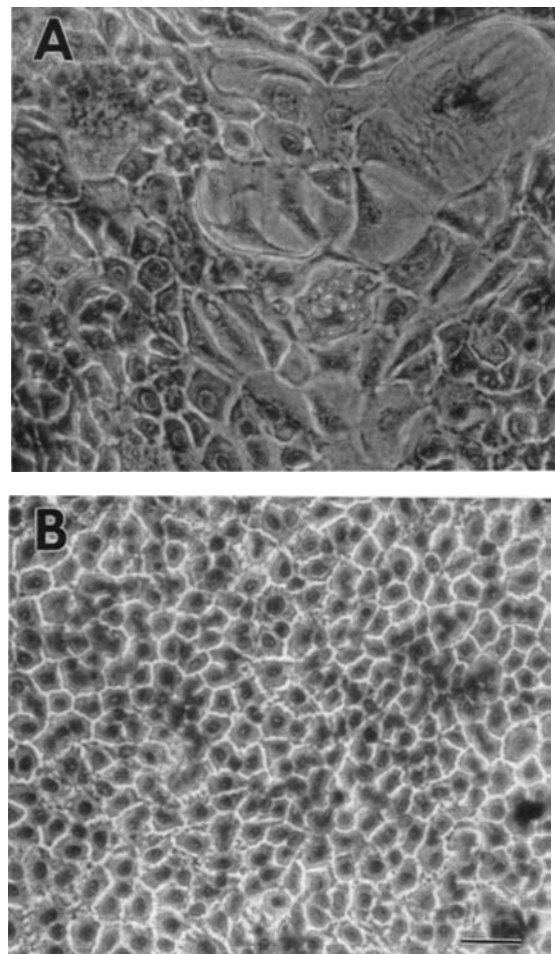

Figure 2. Phase-contrast microscopy of primary (*A*) and secondary (*B*) RTE cell culture morphology. Primary RTE cells propagated for 1 mo in CSFM were trypsinized and replated into secondary culture under the same growth conditions. At the same stage in culture, primary cells appear larger and more heterogeneous than cells in secondary culture. Bar = 50 μm.

Electron Microscopy

Before fixation for electron microscopy, SPOC1 cells (passage 8) were plated onto four-well, plastic Lab-Tek tissue culture slides in CSFM medium and allowed to proliferate until they were almost confluent. Cultured cells were fixed overnight with a 3% glutaraldehyde solution in CSFM at 37°C and postfixed in 1% osmium tetroxide in 0.1 M Sorensen's phosphate buffer for 1 h and transferred to 30% ethanol for 5 min. Cells were stained in 2% uranyl acetate in 30% ethanol and dehydrated and embedded in Polybed 812 (Polysciences, Inc., Warrington, PA). Ultrathin sections were cut *en face* at 70 nm using an LKB Ultramicrotome (Leica), mounted on uncoated copper grids and poststained with 4% aqueous uranyl acetate and 0.4% lead citrate. All sections were examined with a Zeiss 10A transmission electron microscope at 80 kV.

Bioelectric Properties of SPOC1 Cells

Transepithelial electrical potential difference (V_t) and resistance (R_t) of confluent SPOC1 cultures grown on 24-mm Transwell-Col inserts (Costar) in the presence of retinoic acid were measured daily. V_t was determined by placing the tips of sterile calomel electrodes in the media, bathing the apical and basolateral sides of the cultures. Calomel electrodes were coupled to an electrometer (custom unit, input impedance $> 10^{10}$ Ω). Constant transepithelial current, generated by a stimulus isolation unit (World Precision Instruments, 305-B) was passed from platinum electrodes, which were wrapped around the tips of the calomel electrodes. Values of R_t were corrected for the resistance of the culture insert and media by subtracting the voltage drop associated with the insert alone (0.7 mV, 100 Ω) from the total change in V_t determined when passing current.

Analysis of mRNA Expression

Total RNA was isolated from passage 8 SPOC1 cells by the method of Chirgwin and colleagues (24), and the level of fibronectin (Fn) RNA determined by Northern analysis (25). Total RNA (10 to 20 μg) was electrophoresed through 1% agarose/formaldehyde gels, transferred to Nitroplus 2000 (MSI Inc., Westboro, MA) and hybridized to ^{32}P-α-dCTP labeled cDNA probes as described previously (26). Rat Fn cDNA was an ECOR1 0.5-kb fragment, which includes the coding sequence for the cell, heparin, and fibrin-binding domains (27). Equal loading of RNA samples was assessed by photographing ethidium bromide–stained gels before transfer and using a laser densitometer to scan the negatives. Autoradiographs of the Northern blots were also scanned by the densitometer, and gene expression was normalized to RNA loading.

Results

In Vitro Growth Characteristics and Morphology of SPOC1 Cells

After 4 wk in primary culture, RTE cell size and shape were heterogeneous, and the cultures appeared squamous (Figure 2A). These cultures were replated in CSFM at clonal density and maintained in secondary culture for an additional 4 wk. Cells grew in tightly packed colonies (10 to 20 per dish), and the resulting cell population is shown in Figure 2B. Secondary cultures had a greater nuclear-to-cytoplasmic ratio than primary RTE cells, and cell shape was more homogeneous than the parent culture. The secondary cultures of RTE cells were similar in appearance to EGV colonies, which arose after MNNG treatment of primary RTE cells (28).

Early-passage (before passage 10) SPOC1 cells were separated by wide intercellular spaces, except for occasional filopodia connecting cells, as shown in the electron micrograph in Figure 3. Desmosomes and tonofilament bundles could be identified but not with the frequency seen in proliferating primary RTE cells in culture (29). Although tight junctions were not apparent in cells grown on plastic, SPOC1 cells (passages 8 through 15) grown on permeable Transwell-Col supports in the presence of retinoic acid developed a significant transepithelial resistance. Figure 4 shows the development of V_t and R_t as a function of time in culture for three successive passages. Both bioelectric parameters were generally low at the day of confluence and developed over a period of 5 to 7 d. The mean V_t and R_t for Day 11 through 15 SPOC1 cultures was -0.42 ± 0.01 mV and 385 ± 21 Ωcm^2, respectively $(n = 3)$. The development of the bioelectric parameters coincided with development of the original confluent monolayer of cells into three to four layers of cuboidal cells, with some areas of each culture exhibiting
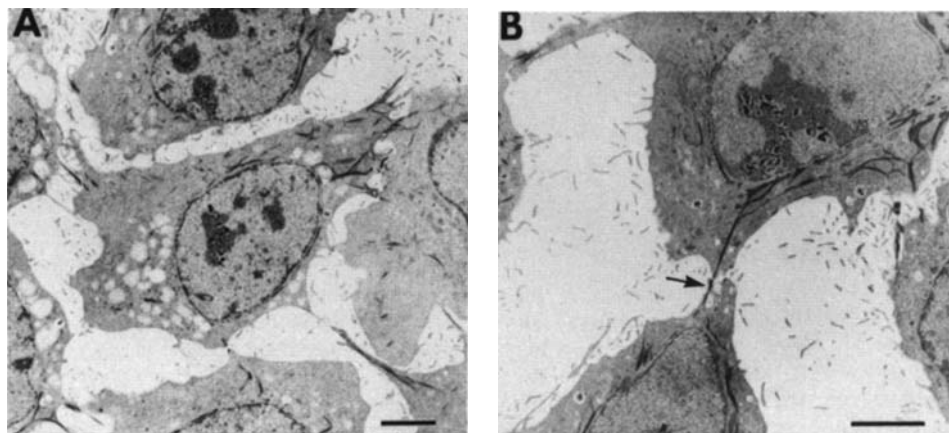

Figure 3. Panels A and B: Electron micrographs of passage 8 SPOC1 cells in culture. Cells were propagated on plastic in CSFM and fixed for electron microscopy on Day 6 in culture. Cells have a high nuclear-to-cytoplasmic ratio and display few intercellular contacts, with only occasional cell extensions forming cellular junctions (*arrow*). Bar = 4 μm.

Figure 4. Bioelectric parameters of SPOC1 cells propagated on Transwell-Col inserts at an air-liquid interface. Transepithelial resistance (R_t) and membrane potential (V_t) were measured in serial passages of SPOC1 cultures for 25 d, beginning at confluence. Both bioelectric parameters were generally low at the day of confluence and developed over a period of 5 to 7 d. The mean V_t and R_t for Days 11 through 15 SPOC1 cultures was -0.42 ± 0.01 mV and 385 ± 21 Ωcm^2, respectively ($n = 3$).
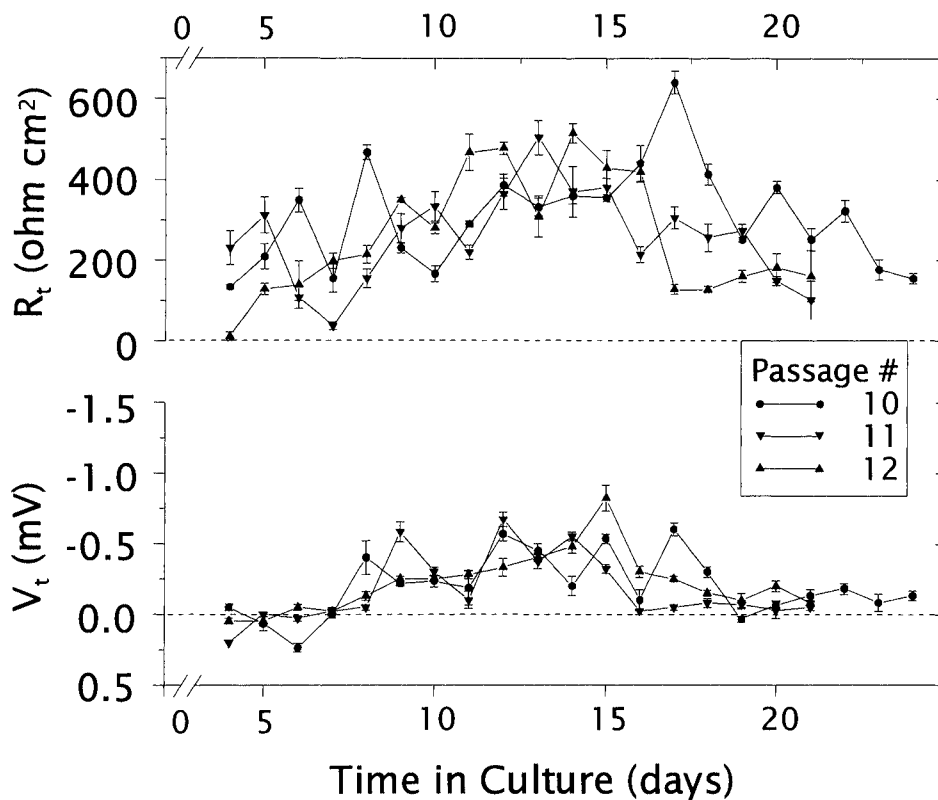

Figure 5. Growth curves for passage 9 and passage 27 SPOC1 cells. Cells were plated at 5×10^4 cells/dish in CSFM. On Day 1, cells were refed with CSFM or medium lacking the indicated growth factors. Early-passage cells have similar maximum cell densities regardless of medium composition; however, cells grown in CSFM reach plateau phase sooner than cells grown in other media. Similar doubling times ($\sim$ 24 h) and maximum cell densities are seen regardless of the medium composition with later-passage cells. Repeats of this experiment yielded similar results.
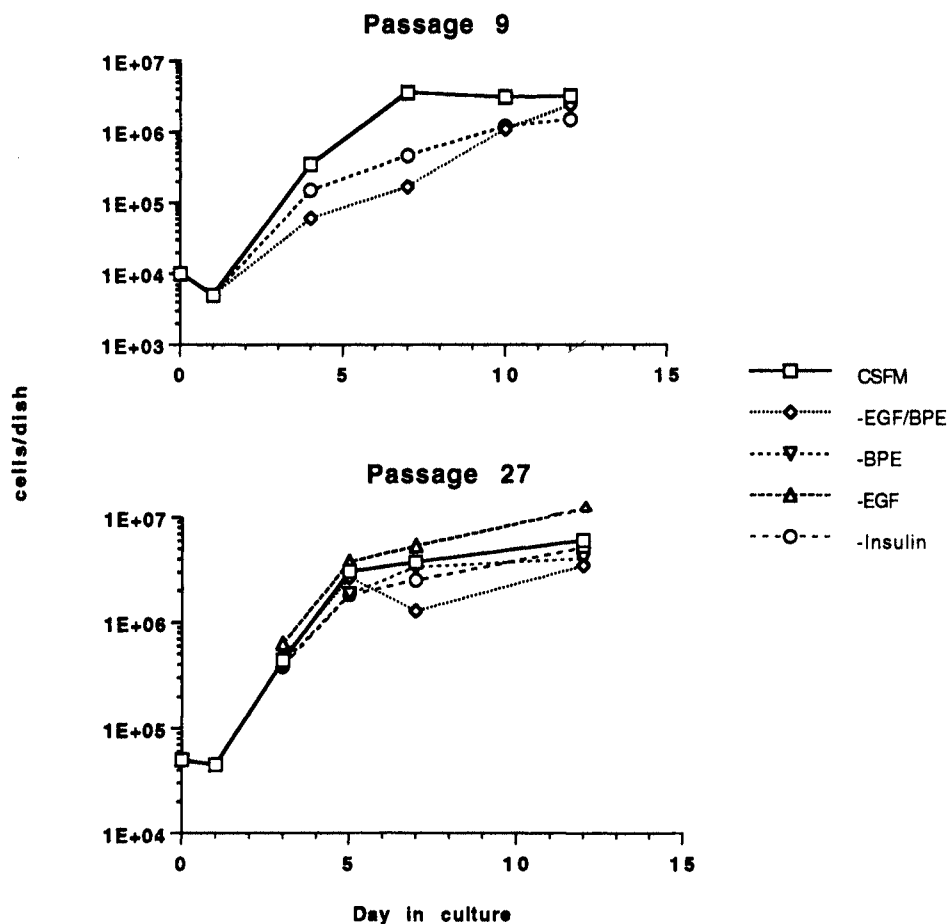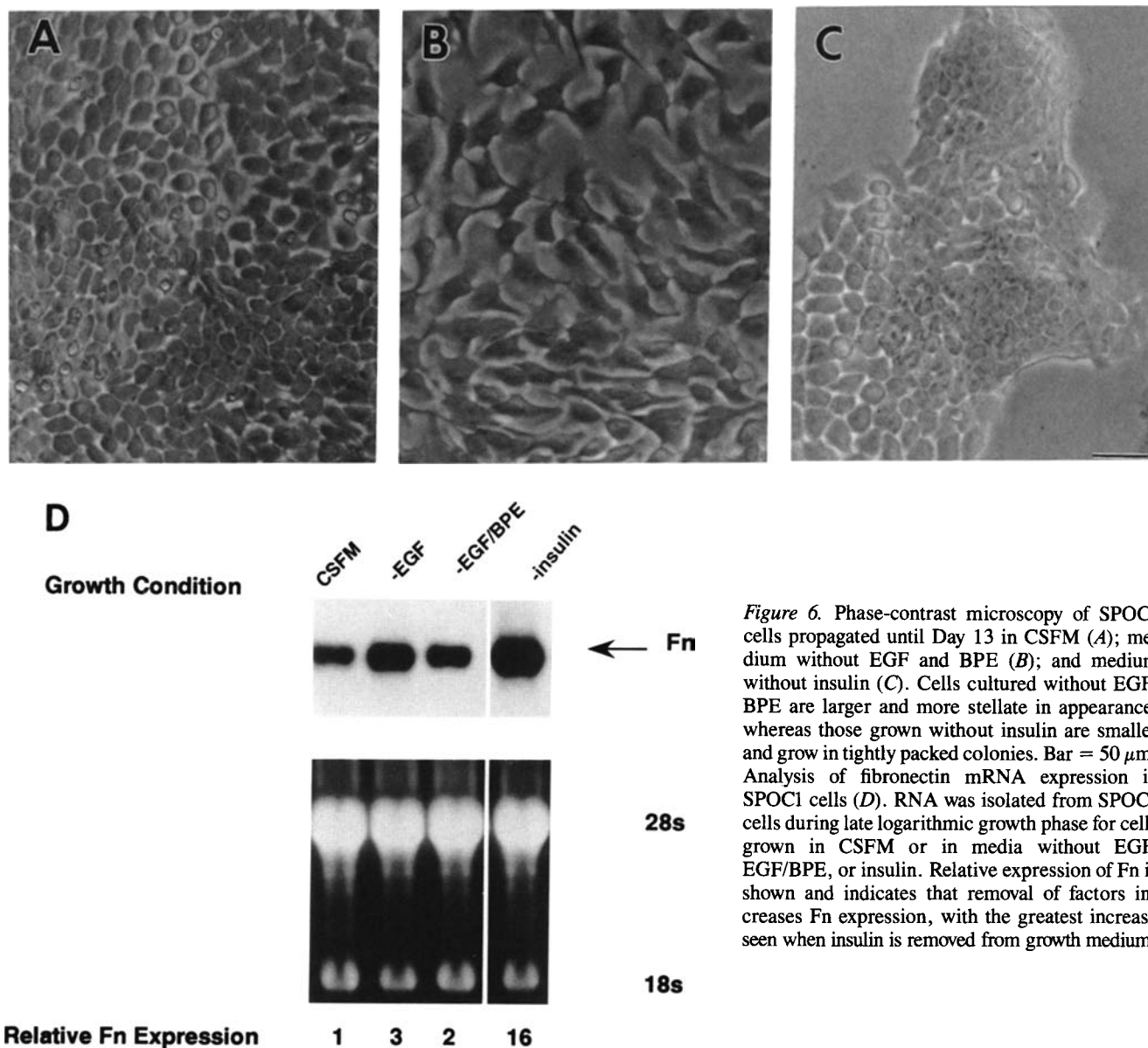
Figure 6. Phase-contrast microscopy of SPOC1 cells propagated until Day 13 in CSFM (*A*); medium without EGF and BPE (*B*); and medium without insulin (*C*). Cells cultured without EGF/BPE are larger and more stellate in appearance, whereas those grown without insulin are smaller and grow in tightly packed colonies. Bar = 50 μm. Analysis of fibronectin mRNA expression in SPOC1 cells (*D*). RNA was isolated from SPOC1 cells during late logarithmic growth phase for cells grown in CSFM or in media without EGF, EGF/BPE, or insulin. Relative expression of Fn is shown and indicates that removal of factors increases Fn expression, with the greatest increase seen when insulin is removed from growth medium.

squamous cell differentiation (data not shown). SPOC1 cells grown on these supports did not develop cilia, but cuboidal cells stained positive for AB-PAS and contained mucous granulelike structures, as shown by TEM (data not shown). These data indicate that SPOC1 cells display both squamous and mucosecretory (but not ciliated) cell differentiation potential *in vitro*.

Colony-forming efficiency was determined at passages 3, 5, 10, 15, 20, and 25 for SPOC1 cells grown on plastic. Efficiency increased with passage number and was stable by passage 25 (data not shown). These replating efficiencies are similar to those seen in EGV cell lines.

Growth curves comparing proliferation of passages 9 and 27 SPOC1 cells propagated in CSFM or in medium lacking certain growth factors are shown in Figure 5. These growth factors have previously been shown to be required for maximum growth of primary RTE cells but not EGV cell lines

(12, 26). Removal of insulin, EGF, and/or BPE from the growth medium 1 d after plating had no significant effect on the maximal cell number achieved by passage 9 SPOC1 cells. However, the time it took to reach maximum cell density was increased when the growth factors were removed from passage 9 cells. Statistics on replicate experiments showed significant differences between the control and minus insulin or minus EGF/BPE conditions on Day 7 in culture (Student's *t* test, $P < 0.05$, $n = 3$). Differences in logarithmic growth were not observed with passage 27 SPOC1 cells.

Although growth factor removal did not affect maximum cell density, the morphology of SPOC1 cells was affected by growth factor removal, as shown in Figure 6. Figure 6A shows a phase-contrast micrograph of passage 28 SPOC1 cells grown in CSFM for 13 d. Cell size and shape were homogeneous. Figure 6B shows SPOC1 cells grown in medium lacking EGF and BPE, and Figure 6C shows cells grown in

Figure 7. Ploidy of SPOC1 cells at passages 4 and 31. At both early and late passages, SPOC1 cells have a diploid modal chromosome complement. At the early passage, there is a greater frequency of random chromosome loss, whereas at the later passage, a greater frequency of tetraploidy and random extra chromosomes occurs.
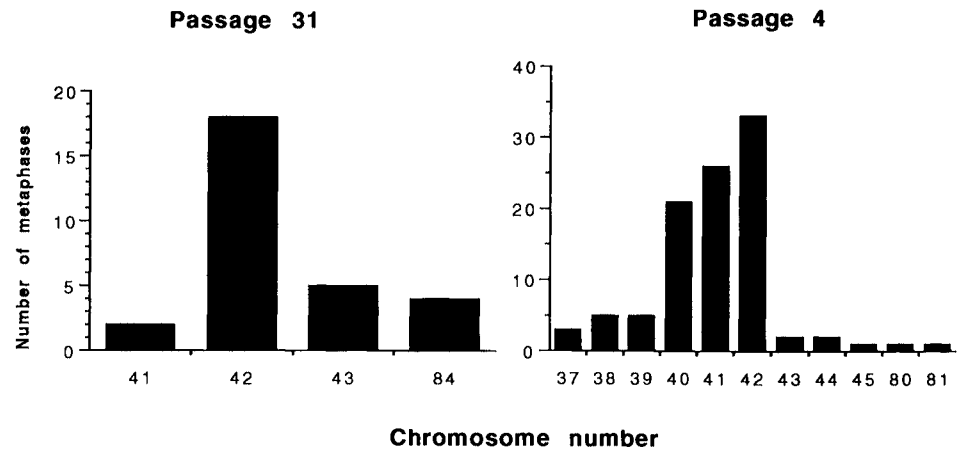
medium lacking insulin. Cells propagated without EGF and BPE were larger and more stellate in appearance. Cells grown in medium without insulin grew in tightly associated colonies of cells rather than spreading and forming a confluent monolayer. We have previously shown that the morphology of primary RTE cells is altered by growth factor removal (26) and that Fn is a major cellular glycoprotein regulating RTE cell spread and morphology (30). We have also shown that Fn expression and organization are altered in SPOC1 cells relative to primary RTE cells (14). To determine whether growth factor effects on morphology are mediated by changes in Fn levels, we assessed whether Fn levels were altered by growth factor removal from SPOC1 cells. RNA was isolated from SPOC1 cells grown in CSFM, medium without EGF and BPE, and medium without insulin and hybridized to a Fn cDNA. As shown in Figure 6D, Fn RNA levels were significantly increased in cells grown without insulin, but in contrast to primary RTE cells, were not altered by removal of EGF or BPE.

Cytogenetic Analysis of SPOC1 Cells

Chromosome counts were performed on cells from cultures at passages 4, 17, and 28. Results from each passage were similar in that all passages had a modal chromosome number of 42, which indicates that SPOC1 cells retain a diploid karyotype (Figure 7). Polyploidy was evident in only a few cells.

Karyotyping of passage 31 cultures demonstrated several nonrandom chromosome alterations, which are summarized in Table 1. All cells examined contained structural alterations involving chromosomes 1, 2, 6, and 17. Chromosomes 1 and 2 were translocated, with breakpoints at p13 and q12, respectively. A second translocation involving the long arm of chromosome 6 and the short arm of chromosome 17 was also seen in 100% of cells examined. In 38% of metaphase spreads examined, a translocation of chromosome 3 was seen, which resulted in an isochromosome. Marker chromosomes and double minute chromosomes were occasionally seen in cells.

Tumorigenicity and Regeneration Potential of SPOC1 Cells

Late-passage SPOC1 cells were injected into nude mice to determine whether they had acquired neoplastic potential *in*

vivo. Subcutaneous injections of two concentrations of passage 28 SPOC1 cells failed to produce any tumors after 1 yr.

The regenerative and differentiation potential of SPOC1 cells was determined by inoculating denuded Fischer 344 rat tracheas with cell suspensions of SPOC1 cells. Samples were prepared from tracheas on Days 7 and 21 for morphologic characterization at the light-microscopic level. Figure 8 shows H&E-stained sections of tracheas repopulated with passage 8 SPOC1 cells. By Day 7, a confluent epithelium was established, which was predominantly multilayered and composed of cuboidal basal and suprabasal layers. The superficial layers resembled a stratified squamous epithelium (Figures 8A and 8B). The cytoplasm of the cells in the apical stratified layers was highly eosinophilic, but areas of dense cornification were not seen. The epithelium of Day 21 tracheas had thinned considerably and had an overall pseudostratified rather than a squamous appearance (Figures 8C and 8D). Glandlike invaginations into the submucosal tissue were frequently seen. Cells situated at the basal lamina maintained a basal cell–like appearance. The more columnar cells contained a clear, frothy cytoplasm suggestive of secretory granules. This apparent secretory differentiation was also seen in the glandular structures. The frothy cytoplasmic areas of the columnar cells of both the surface epithelium and the glands were AB-PAS positive (Figure 9). No AB-PAS staining was observed in the Day 7 epithelium. There was no evidence of ciliated cell differentiation in grafts.

Expression of Differentiation Markers by Regenerated Tracheal Epithelium

Tracheal grafts repopulated with SPOC1 cells were stained with a panel of antikeratin antibodies. The results are shown

TABLE 1

Nonrandom structural chromosome alterations in SPOC1 cells

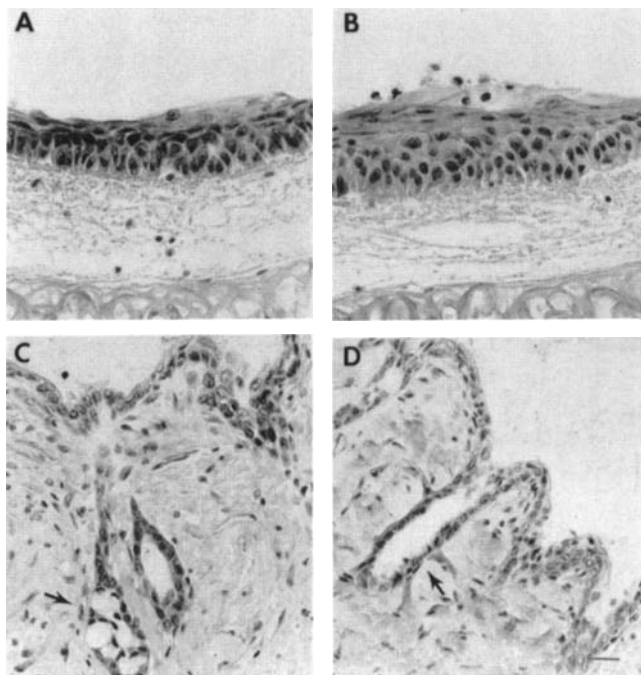| Passage No.	Modal Chromosome No.	Abnormality*	Frequency (%)
4, 31	42	T(1; 2)(p13; q12)	100
31	42	T(6; 17?)(q11.1; p11)	100
31	42	i(3q)	38

* See text.

Figure 8. Light microscopy of denuded tracheal grafts repopulated with passage 8 SPOC1 cells. *Panels A and B*: One week after implantation, cells form a multilayered stratified squamous epithelium, with evidence of keratinization. *Panels C and D*: Three weeks after implantation, epithelium is less multilayered and more cuboidal and occasionally forms glandular infiltrates into the surrounding tissue (*arrows*). Tracheal grafts were stained with H&E. Bar = 20 μm.

in Figure 9 and summarized in Table 2. Keratin 13, a marker of squamous differentiation (23), was heterogeneously expressed in focal areas by suprabasal cells in both Day 7 and Day 21 grafts. Therefore, SPOC1 cells in Day 21 tracheal grafts showed evidence of mixed differentiation potential, with some cells displaying a secretory phenotype and others expressing markers of squamous cells. Day 7 grafts showed diffuse staining of the entire epithelium for keratin 14, which is a basal cell marker in normal trachea. Keratin 14 staining in Day 21 grafts was restricted primarily to the basal cell layer. Keratin 19, which is expressed in most normal rat RTE cells, was expressed primarily in suprabasal cells in both Day 7 and Day 21 graft sections, although staining was more uniform at the later time point. Tracheal grafts did not react with the ciliated cell marker RTE3 or with mAb against keratin 18, which stain columnar cells in the intact trachea and differentiated primary RTE cells (data not shown).

Discussion

Neoplastic and preneoplastic RTE cell lines derived from carcinogen-exposed primary RTE cells have a number of alterations, compared with primary RTE cells. RTE cell lines express decreased requirements for exogenous growth factors and have numerous chromosomal alterations and various degrees of tumorigenicity. All RTE cell lines examined thus far exhibit a keratinizing, stratified, squamous morphology when inoculated into tracheal grafts. SPOC1 cells also

have increased proliferative capacity and decreased requirements for exogenous growth factors. Both SPOC1 and the other RTE cell lines have alterations in expression and localization of extracellular matrix proteins (14). However, unlike previously characterized RTE cell lines, SPOC1 cells are unique in having a diploid karyotype and, potentially, the capacity to differentiate into both secretory and squamous cell phenotypes both in culture and in tracheal grafts. Therefore, the SPOC1 cell line should prove to be a useful tool for studying both differentiation and neoplastic progression of airway epithelial cells.

Transformed cells *in vitro* often have altered growth factor requirements compared with their normal counterparts (31). The present data indicate that alterations in growth factor requirements occur early during immortalization of RTE cells. Comparison of early- versus late-passage SPOC1 cell growth factor requirements indicates that there may be selection for growth factor independence with time in culture. Therefore, the SPOC1 cell line will be useful in examining the alterations that give rise to changes in growth factor requirements in airway epithelial cells.

We have previously found that exogenous growth factors alter primary RTE cell morphology and extracellular matrix gene expression (13, 30). SPOC1 cell morphology is altered by withdrawal of growth factors, although not in the same way that primary RTE cells are. Primary RTE cell morphology changes dramatically and Fn RNA levels decrease with removal of EGF and BPE (13). Changes in SPOC1 cell morphology and Fn levels, however, are minimal with removal of these factors. On the other hand, withdrawal of insulin from SPOC1 cells significantly alters morphology and correlates with increased Fn RNA levels. Insulin has been found to affect extracellular matrix expression in different ways. In 3T3 cells, insulin has been shown to increase secretion of Fn protein (32). Fn secretion in other cell types is inhibited by the addition of insulin (33). It will be of interest to determine the role insulin and related growth factors play in regulation of extracellular matrix in SPOC1 cells.

Although normal diploid karyotypes have been identified in enhanced growth variants of RTE cells exposed to carcinogens (16), RTE cell lines derived from these enhanced variants are aneuploid (reference 15 and unpublished observations). The modal chromosome number of SPOC1 cells is diploid at both early and late passages. However, all SPOC1 cells possess nonrandom chromosome changes involving chromosomes 1, 2, and 6, and a smaller percentage of cells have alterations in chromosome 3. Since SPOC1 cells are not tumorigenic, these chromosome alterations can be associated with immortalization of RTE cells. Previous studies have found that structural and/or numerical alterations in chromosomes 1 and 3 are frequent occurrences in transformed rat cells (15, 34, 35). Treatment of SPOC1 cells with carcinogens or transfection with transforming oncogenes may help identify molecular alterations associated with neoplastic progression and tumor formation in tracheal epithelial cells.

A frequent criticism of *in vitro* cell culture models is that dissociation of normal cells and propagation on plastic often renders cells de-differentiated, with growth characteristics very dissimilar from their *in vivo* state. Although many cells express an altered phenotype *in vitro*, several systems have
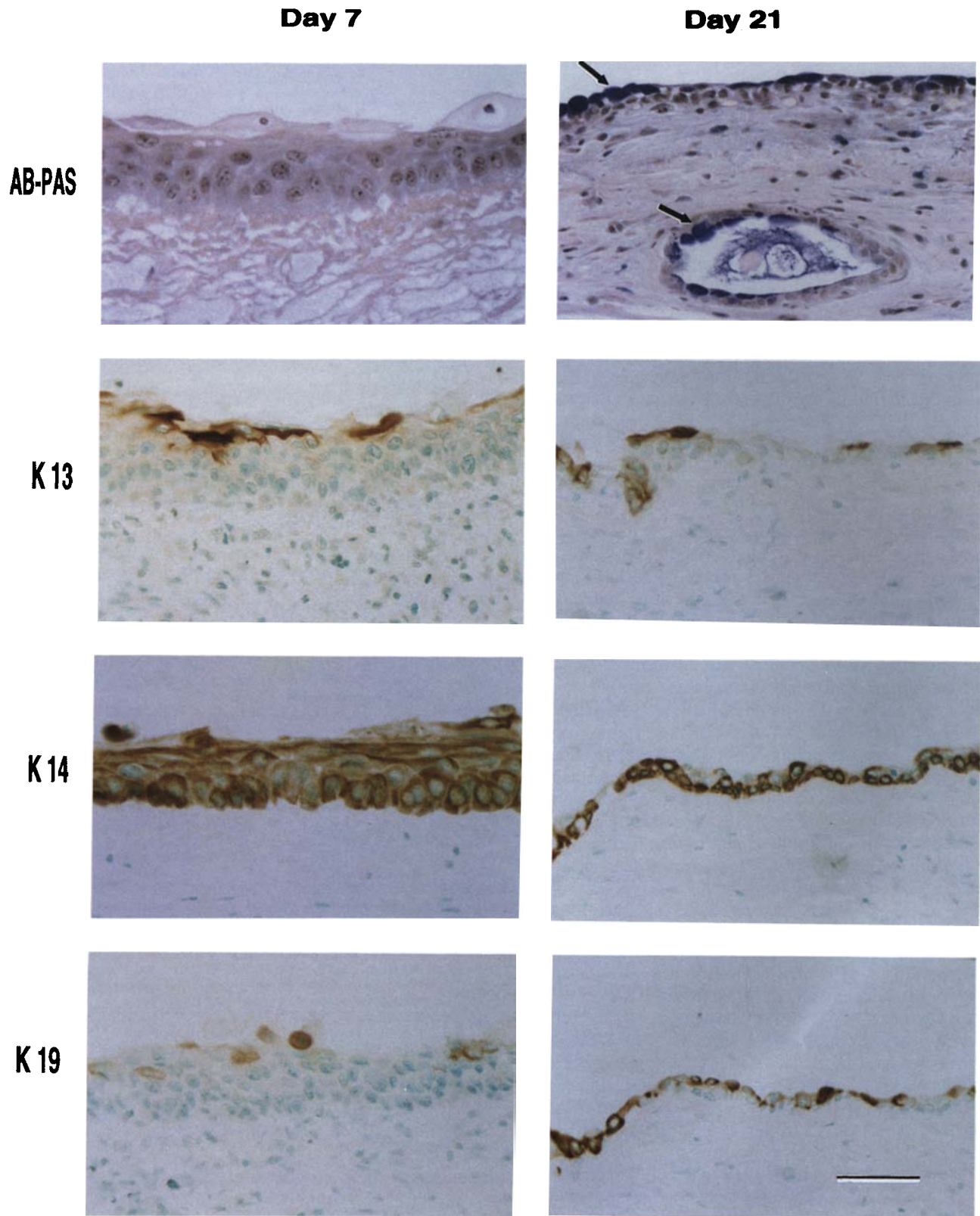
Day 7   **Day 21**

Figure 9. Staining of SPOC1 cells in Day 7 and Day 21 tracheal grafts with AB-PAS and antibodies for keratins 13, 14, and 19. Positive AB-PAS staining is seen only in Day 21 grafts and is localized in cells of the surface epithelium as well as in cells in the glandlike structures (*arrow*). Keratin 13, 14, and 19 staining is observed in both early and late grafts; however, the localization and intensity of staining changes with time (see text). Bar = 50 μm. These results are summarized in Table 2.

TABLE 2

Summary of SPOC1 repopulated tracheal graft reactivity with cytochemical markers

Marker Expression*	AB-PAS: Secretory Granules	K13: Squamous Differentiation	K14: Basal Cells	K18: Columnar Cells	K19: All Cells	RTE3: Ciliated Cells
Day 7	−	+	+ +	−	+/−	−
Day 21	+	+	+	−	+	−

* In normal rat trachea (22).
+ +, strongly expressed in most cells; +, strongly expressed in some cells; +/−, staining in few cells; −, no staining.

been developed in which some aspect of cellular differentiation is maintained. Cultures of tracheobronchial epithelial cells from a variety of species, including rat (18), maintain structural and functional features of differentiation under the appropriate culture conditions (10, 36–40). However, there are few instances of immortalized airway epithelial cell lines that maintain the ability to differentiate into mucociliary epithelium (9, 10, 37). RTE cells lose their differentiated phenotype (29) as well as their capacity to regenerate denuded tracheas with a pseudostratified epithelium upon culture on plastic for extended periods. Transformed RTE cells re-epithelialize tracheas with a keratinizing squamous epithelium containing abundant cellular atypia, which in some cases form invasive squamous cell tumors (17). Repopulation of tracheas with SPOC1 cells yields a unique pattern of differentiation for RTE cell lines. These cells initially form a squamous epithelium, which subsequently gives rise to a more cuboidal, pseudostratified glandular epithelium that reacts with both AB-PAS and keratin 13 mAb. Cells also show positive staining with an antibody that recognizes keratin 19, which has been shown to be widely expressed in both stratified and simple epithelia (41). SPOC1 cells show no evidence of ciliated cell differentiation and have lost K18 antigenicity, a marker of columnar epithelial cells in rat trachea (20, 22). These characteristics make SPOC1 cells a unique system with which to study the molecular and cellular events associated with airway epithelial cell differentiation.

In conclusion, the SPOC1 cell line expresses characteristics that are similar to those seen in other RTE cell lines, including decreased growth factor requirements and alterations in extracellular matrix expression. However, these cells also have traits not expressed by other RTE cell lines, including a diploid karyotype and the ability to form both squamous and glandular epithelium. Therefore, SPOC1 cells may provide a window into very early changes occurring during multistep neoplastic progression of airway epithelial cells. Injury (either physical or chemical) of the tracheobronchial epithelium can result in squamous cell metaplasia, and similar changes in differentiation pathways are seen in preneoplastic tracheobronchial lesions (11). Since the molecular alterations responsible for modulation of mucosecretory and squamous differentiation in airway epithelium remain uncertain, the SPOC1 cells should prove to be a valuable model system with which to study the relationship between airway cell differentiation state and neoplastic transformation.

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