American Journal of Pathology, Vol. 129, No. 3, December 1987

Differentiation of Tracheal Mucociliary Epithelium in Primary Cell Culture Recapitulates Normal Fetal Development and Regeneration Following Injury in Hamsters

ELIZABETH M. McDOWELL, BVet Med, PhD, THERESA BEN, BS, CARNELL NEWKIRK, MS, SEUNG CHANG, MS, and LUIGI M. DE LUCA, PhD

From the Department of Pathology, University of Maryland School of Medicine, Baltimore, Maryland, and Differentiation Control Section, Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

Hamster tracheal epithelial cells were grown in primary culture for 8 days on a collagen gel substrate, in hormone-supplemented serum-free medium (Ham's F-12). On Days 1–3 in culture, the colonies were composed of a monolayer of poorly-differentiated flattened cells. Most of these were large cells which appeared to be altered secretory (mucous) cells. On Days 4 and 5, many of the epithelial cells were cuboidal. The rough endoplasmic reticulum was moderately developed, and mucous granules were seen at the cell apices. Preciliated and newly formed ciliated cells were observed on Day 6, and a differentiated mucociliary epithelium was established by Day 7 in culture. The study shows that in the hamster tracheal epithelium, the stages of normal fetal development and regeneration following injury, which have been characterized previously *in vivo*, are recapitulated *in vitro*. Formation of a mucociliary tracheal epithelium occurs within 7 days *in vito* and *in vitro*. (Am J Pathol 1987, 129:511–522)

THE MUCOCILIARY tracheal epithelium of Syrian golden hamsters provides a satisfactory model for studying the normal and injured human bronchial epithelium. Hamster tracheal epithelial cells resemble those of the normal human bronchus,[1–3] and the spectrum of respiratory tract tumors induced experimentally in hamsters following instillations of benzo(a)pyrene-Fe_2O_3[4,5] shares striking similarities with that of bronchogenic carcinomas in humans.[6]

Epithelial regeneration, a basic response to injury, is essential for the sustained existence of all multicellular organisms. This process is fundamental to the maintenance of normal epithelia and to pathologic processes, including neoplasia. Moreover, regenerating epithelia of mature adult tissues display properties and structures that are essentially similar to those of the corresponding immature fetal tissues.[7] This is true for the hamster trachea, and regeneration of the mature mucociliary epithelium following an injury shares many similarities with the sequence of normal fetal development.[8–15]

Biochemical and molecular approaches are now required for further probing into the mechanisms of tracheobronchial epithelial development and regeneration. These mechanisms are difficult to study *in vivo* because of the many variables involved, which include complex interactions between epithelial and mesenchymal cells, and, during regeneration following injury, the multiple effects of the inflammatory-immunologic cascades. It is essential, therefore, that suitable *in vitro* models of development and regeneration be developed. Many laboratories have cultured tracheobronchial epithelial cells in an attempt to reconstitute a differentiated and functional mucociliary epithelium.[16,17] Success has been achieved recently by

Supported in part by USPHS Grants HL-24722 and HL-37640.

Accepted for publication July 7, 1987.

Address reprint requests to Dr. Elizabeth M. McDowell, Department of Pathology, University of Maryland, School of Medicine, 10 S. Pine Street, Baltimore, MD 21201.

Wu and co-workers[18] with the use of hamster tracheal epithelial cells grown on a collagen gel in a serum-free hormone-supplemented medium. This method provides an exciting new approach for the study of respiratory mucociliary function and may also provide a tool for biochemical and molecular studies of tracheobronchial epithelial development, regeneration, and carcinogenesis.

We undertook the present study to determine the extent to which growth of hamster tracheal epithelial cells in primary cell culture recapitulates normal epithelial development and regeneration following injury, as occurs *in vivo* in fetuses and adults.

Materials and Methods

Cell Culture

Male and female 6-week-old Syrian golden hamsters (strain CR : RGH) were obtained from the NCI-Frederick Cancer Research Facility. The tracheal epithelial cells were isolated by overnight protease digestion at 4 C and cultured in serum-free hormone-supplemented medium closely following the procedures of Wu et al.[18] The yield per trachea was about 1×10^5 cells. Ham's F-12 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml, obtained from GIBCO, Grand Island, NY), was supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (25 ng/ml), hydrocortisone (1 μM), bovine hypothalamus extract (22 μg/ml), cholera toxin (40 ng/ml), and retinotic acid (10^{-7} or 10^{-8} M, dissolved in DMSO). Insulin, transferrin, epidermal growth factor, and hydrocortisone were obtained from Collaborative Research, Inc., Lexington, Massachusetts. Cholera toxin and retinoic acid were obtained from Sigma Chemical Co., St. Louis, Missouri, and Hoffman LaRoche, Nutley, New Jersey, respectively. Bovine hypothalamus was obtained from Pel-Freeze, Rogers, Arizona; the extract was prepared according to the method of Groelke et al.[19] The collagen gel substrate (0.6–1.0 ml per 35-mm culture dish) was prepared according to Reen Wu (personal communication) as follows: 0.133 ml×5 concentrated minimal essential medium (GIBCO), 0.067 ml 0.5N NaOH, made sterile by filtration, and 0.80 ml Vitrogen-100 (Collagen Corporation, Palo Alto, Calif) were mixed and poured into the culture dishes. The gel was polymerized by incubation at 37 C for 30–60 minutes in a CO_2 incubator. The tracheal cells were plated onto the gel in 35-mm culture dishes in 2 ml of Ham's F-12 medium containing all supplements. Cells were plated at 15,000, 20,000, or 60,000 cells per 35-mm dish and incubated at 37 C in a humidified atmosphere containing 95% air and 5% CO_2. In experiments where the cells were counted, the medium was changed daily. In other experiments, the medium was changed every 2 or 3 days.

Cell Counts

Cells that were attached to the collagen substrate were released for counting by incubation for 1 hour at 37 C in 0.35% collagenase (Worthington Biochemicals, Malvern, Pa), followed by treatment with 0.1% trypsin and 0.02% EDTA for 15 min. The cells were counted with a particle counter (Coulter Electronics, Hialeah, Fla).

Morphological Studies

In all experiments, the morphology of the cell colonies was monitored by phase contrast microscopy over the first 8 days in culture. Representative specimens were prepared for high-resolution light microscopy and transmission electron microscopy on Days 2–8. Cells growing on the collagen gel substrate were fixed by adding 2 ml of 4% formaldehyde–1% glutaraldehyde in a 200 mOsm phosphate buffer[20] to 2 ml of the culture medium. After 10–15 minutes, full-strength fixative replaced the half-strength mixture, and the cells and the collagen gel substrate were stored in the same until they were processed for high-resolution light microscopy and transmission electron microscopy.

High-Resolution Light Microscopy

The colonies and the collagen gel substrate were embedded in glycol methacrylate.[21,22] The colonies were cut vertically with glass knives on a JB-4 rotary microtome (Dupont-Sorvall, Wilmington, Del), set at 2 μ, and mounted onto glass slides. The sections were stained with periodic acid–Schiff (PAS)–lead hematoxylin[22] and by hematoxylin and eosin (H&E).

Transmission Electron Microscopy

The colonies and the collagen gel substrate were postfixed at room temperature for 1 hour in 1% osmium tetroxide, buffered with *s*-collidine, pH 7.4. The specimens were washed three times (5 minutes each wash) in sucrose–cacodylate buffer and treated with 1% thiocarbohydrazide (TCH) for 5 minutes at room temperature, to make TCH-osmium bridges.[23] This treatment facilitated visualization of the colonies during subsequent processing steps, and will also allow parts of the same specimens to be prepared for scanning electron microscopy for study in the future. The specimens were then washed three times (5 minutes each wash) in sucrose-cacodylate buffer, to re-

move excess TCH which was not bound. The specimens were treated further in 1% buffered osmium tetroxide (10 minutes), and washed again in sucrose–cacodylate buffer. They were then dehydrated in a series of alcohols, followed by propylene oxide, and embedded in epoxy resin.

The colonies were oriented and cut in horizontal and vertical planes. Semithin sections, cut at 0.5 μ with glass knives, were stained with toluidine blue for light-microscopic evaluation, prior to thin sectioning. Ultrathin sections were cut with diamond knives and mounted on copper grids. The sections were examined in a JEOL 100B electron microscope after they had been stained with uranyl acetate and lead citrate on the grids.

Results

Normal Hamster Tracheal Epithelium

The structure of the normal epithelium warrants brief description to facilitate comparisons of the component cells prior to and during culture. The pseudostratified epithelium was composed of columnar secretory (mucous) and ciliated cells and pyramidal basal cells (Figure 1A and B). In young hamsters the proportions of secretory, ciliated, and basal cells are about 34%, 40%, and 26%, respectively.[24] The bases of all cells rested upon the basal lamina, but only the apices of the columnar cells reached to the airway. Basal cells, the smallest cells and least frequent cell type, did not form a continuous cell layer, but existed as single cells interspersed between the basilar portions of the columnar secretory and ciliated cells (Figure 1A and B). The basal cells had a very scant cytoplasm, which contained tonofilament bundles; the endoplasmic reticulum and Golgi apparatus were very poorly developed. The basilar membrane of basal cells was anchored to the basal lamina by well-developed hemidesmosomes (Figure 1B). In contrast, the cytoplasm of the secretory cells was abundant. Rough endoplasmic reticulum (RER) and Golgi apparatus were well-developed, and PAS-positive mucous granules were clustered in the apical cytoplasm (Figure 1A and B). The apical cell membrane extended into blunt microvilli, and the cells were joined apically by junctional complexes. A few small desmosomes joined the simple lateral membranes (Figure 1B). Further comparison of basal and secretory cell morphology is given elsewhere.[13,25]

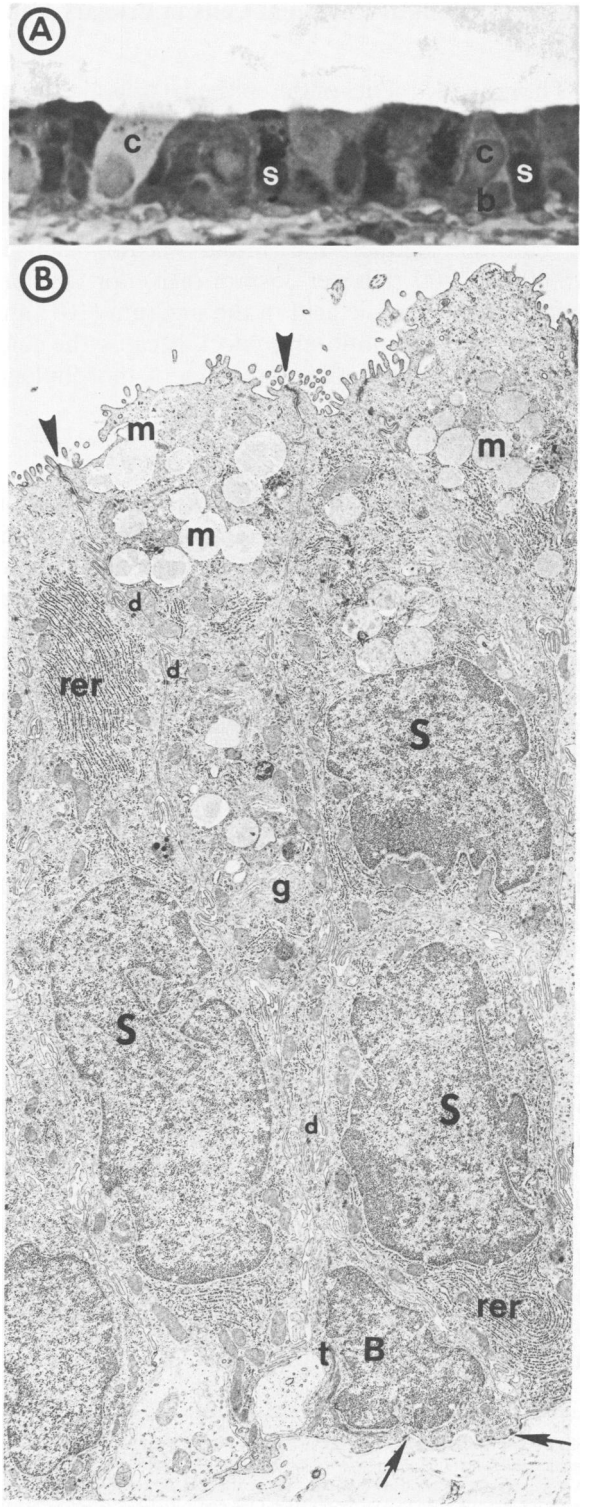


Figure 1—Hamster tracheal epithelium before culture. **A**—The epithelium consists of columnar ciliated (*c*) and secretory cells (*s*), and a few small basal cells (*b*). The apices of the secretory cells contain clusters of PAS-positive mucous granules. (Glycol methacrylate section, PAS–lead hematoxylin, ×950) **B**—Ultrastructure of the tracheal epithelium. Secretory cells (*S*) and a basal cell (*B*) rest on the basal lamina. The cytoplasm of the columnar secretory cells is abundant. It contains well-developed RER (*rer*) and Golgi apparatus (*g*). Mucous granules (*m*) are clustered in the apical cytoplasm. The apical membrane extends into blunt microvilli and the cells are joined by junctional complexes (*arrowheads*). Small desmosomes (*d*) join the lateral membranes. The scant cytoplasm of the short pyramidal basal cell contains tonofilament bundles (*t*). The basal cell is attached to the basal lamina by hemidesmosomes (*arrows*). (×5000)

Hamster Tracheal Epithelial Cells in Primary Cell Culture

The growth kinetics of the epithelial cells are shown in Figure 2. The stationary phase was reached after 6 days in culture.

The morphology of the cell colonies modulated over 8 days and changed characteristically with time in culture. Neither the initial seeding density (15,000–60,000 cells per 35-mm dish) nor the concentration of retinoic acid in the medium (10^{-7} and 10^{-8} M), had any noticeable effect, because the colonies underwent a similar sequence of morphologic change with time in all experiments.

Days 1–3

Apart from colony size, which increased, the morphology of the colonies appeared quite similar during the first 3 days. Ciliated cells were rare, and most of each colony was composed of large flat nonciliated cells, each with an abundant cytoplasm and phase-lucent nucleus (Figure 3A). The large cells, which were seen in mitotic division on days 2 and 3 (Figure 3A and B) formed a simple (one-layered) squamous epithelium (Figure 3B and C). The colonies were examined ultrastructurally on Days 2 and 3. On Day 2, the cytoplasm of the large cells contained sparse tonofilament bundles and numerous dispersed cytoplasmic ribosomes; the RER was very scant, but the Golgi apparatus was large (Figure 3D). The RER was a little better developed on Day 3 and the cytoplasmic ribosomes were aggregated into polyribosomes (Figure 3C). A few granules were seen apically in a few of the large cells on Day 2, similar to mucous granules of the normal epithelium (Figure 3E). These granules were presumably carried over from *in vivo*, because mucous granules were not seen in the large cells on Day 3 of culture. The lateral cell membranes were simple and joined by small desmosomes (Figure 3D). Cell membranes that interfaced with the culture medium, extended into blunt microvilli and were joined by junctional complexes (Figure 3D and E), similar to those seen at the apices of columnar secretory cells in the normal tracheal epithelium.

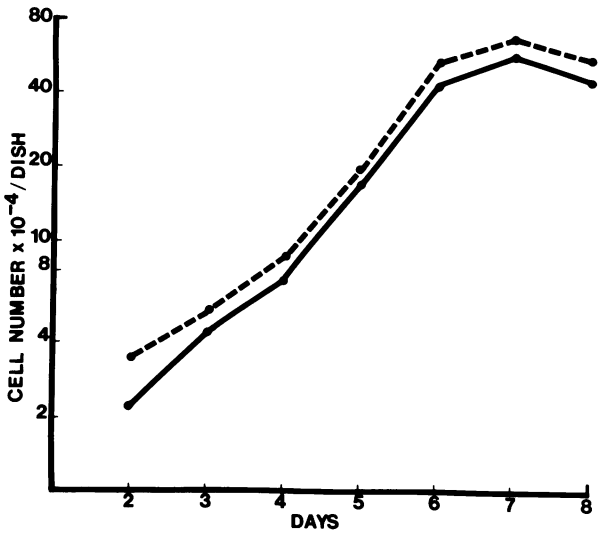


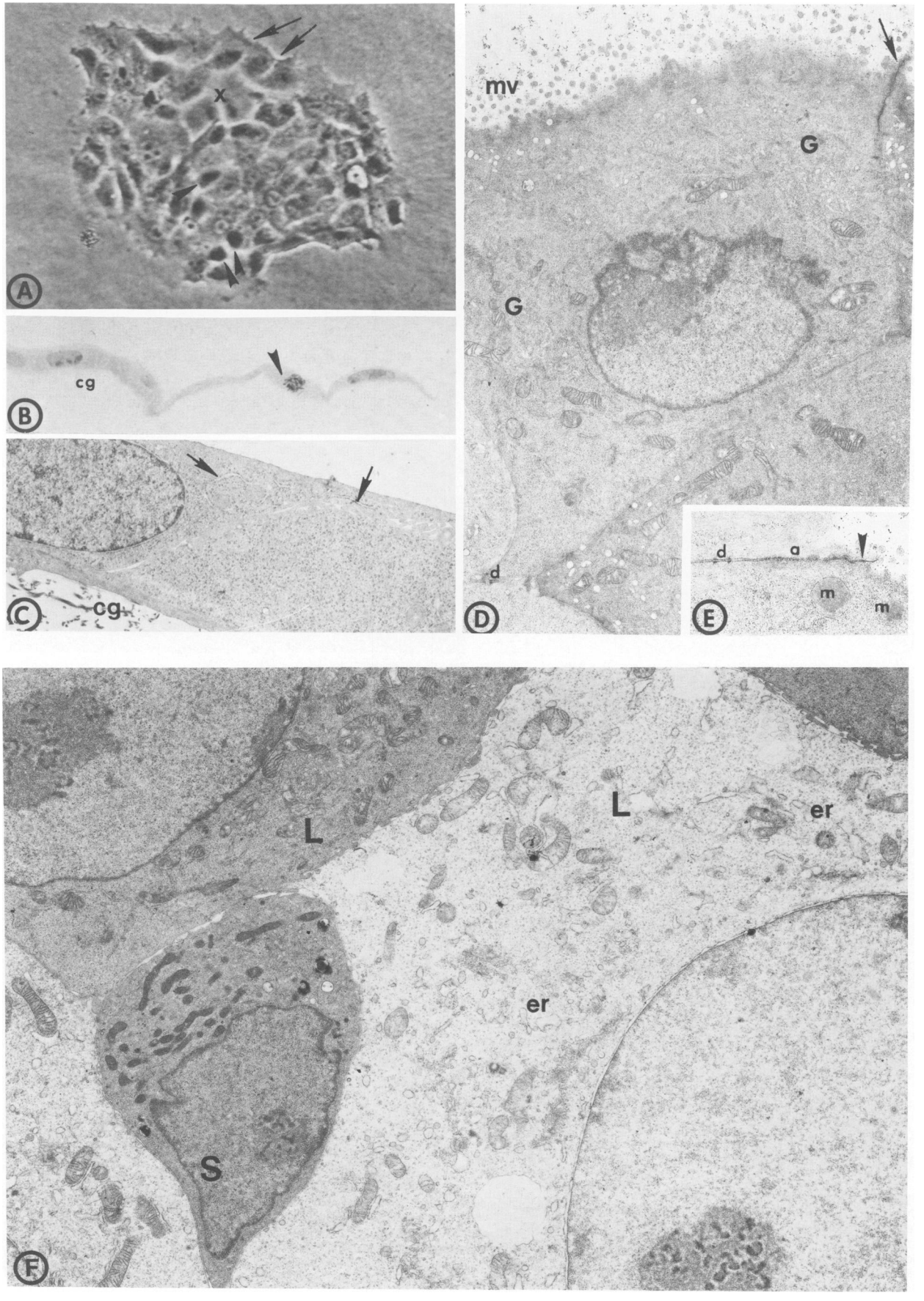
Figure 2—Growth kinetics of hamster tracheal epithelial cells in primary cell culture. On Day 0, 15,000 cells were plated into 35-mm culture dishes, each coated with 0.6 ml of collagen gel. The medium was changed daily, starting on Day 2. The concentration of retinoic acid was 10^{-8} M. Cells attached to the collagen gel substrate were collected and counted after treatment with collagenase and trypsin. Exfoliated cells were also collected and counted at each medium change. The *dotted line* represents the total number of cells in each dish (ie, attached cells plus exfoliated cells). The continuous line represents the number of cells attached to the substrate. On Days 5–8, the attached cell count includes cells in those parts of the colonies that had lifted from the collagen gel, as continuity was maintained between the attached and lifted parts of the confluent cell sheet, ie, cells in the lifted sheet were still "attached" to the substrate indirectly and had not exfoliated into the medium. Duplicate dishes were used to determine the mean number at each time point. Cell numbers were determined with a particle counter (Coulter).

A minority of nonciliated cells were small. The cytoplasm was scant, and the nuclei were phase-dense (Figure 3A) and relatively electron-dense (Figure 3F). The small cells were seen in mitosis by phase-contrast microscopy on Days 2 and 3 of culture. On Day 3, small clusters of the small cells were seen scattered within wide fields of large cells.

Days 4 and 5

Apart from size, which increased with time, the phase-contrast appearance of the colonies was quite similar on Days 4 and 5, but some differences were noted. Ciliated cells were very rare on Day 4 and absent on Day 5. Moreover, it was very difficult on

Figure 3—Hamster tracheal epithelial cells during the first 3 days of culture. **A**—Phase-contrast appearance of a colony at 2 days. Most of the cells are large, each with an abundant cytoplasm and phase-lucent nucleus. Cells forming the edge of the colony are joined tightly (*arrows*). One large cell is in division (*x*). A few cells are small; their cytoplasm is scant and the nucleus is phase-dense (*arrowheads*). (×250) **B**—Vertical section through a 2-day-old colony. The large squamous cells form a monolayer. One cell is in division (*arrowhead*). Collagen gel (*cg*). (Glycol methacrylate section, H&E, ×250) **C**—Vertical section through two squamous cells of a 3-day-old colony. The cytoplasm is abundant and contains numerous aggregates of polyribosomes and a few chains of RER (*arrows*). *cg*, collagen gel. (×6000) **D**—Horizontal section through large squamous cells at the edge of a 2-day-old colony (similar to area at *arrows* in **A**). The cell membranes, which interface with the medium, extend into microvilli (*mv*) and are joined by a junctional complex (*arrow*). The cells are joined laterally by small desmosomes (*d*). The Golgi apparatus (*G*) is large, but rough endoplasmic reticulum is very scant, and ribosomes are freely dispersed throughout the abundant cytoplasm. (×6000) **E**—Junctional complex between two large cells of a 2-day-old colony. *Arrowhead*, occluding junction; *a*, zonula adherens *d*, desmosomes. Mucous granules (*m*) are present at the cell apex. (×9000) **F**—Horizontal section through a 2-day-old colony. The large cells (*L*) have abundant cytoplasm; the nuclei are electron-lucent and contain large nucleoli. The cytoplasm of the small cell (*S*) is scant and the nucleus is relatively electron-dense. The endoplasmic reticulum (*er*) is scant in large and small cells, and cytoplasmic ribosomes are dispersed. The morphology of the small cell (*S*) is similar to that of a basal cell (*B*), shown in Figure 1B. (×6000)


A
x
B
cg
C
cg
D
mv
G
G
d
E
d
a
m
m
F
L
L
er
er
S

Day 4 and impossible on Day 5 to discriminate between the large and small nonciliated cells, because all cells appeared to be of similar size when viewed from above (Figure 4C). This resulted from a change in the shape of the large cells from flat (Days 1–3) to cuboidal (Figure 4A). Other important changes occurred in the large cells at these times. Notably, the RER became increasingly better developed over Days 4 and 5 (Figure 4B and D). A few autophagic vacuoles were seen in the abundant cytoplasm (Figure 4B and D). Small membrane-bounded mucous granules (Figure 4B), which stained PAS-positive (Figure 4A), accumulated at the cell apices. They were quite abundant in some cells on Day 5. The epithelium was simple (one-cell-thick) on Day 4 (Figure 4A and B), but cytoplasmic layering was seen on Day 5 (Figure 4D), and two layers of nuclei were seen focally. On Day 5, cytoplasmic glycogen was present, either dispersed or aggregated into lakes (Figure 4D). The first hint of lysis of the collagen gel substrate was seen on Day 5. This process began as a few small cell-free circular foci, which appeared as "holes" punched out of the cell sheet. Around the circumference of each hole, the cells lifted from the substrate and began to curl back.

Days 6 and 7

Vertical sections through the colonies showed the epithelial morphology to vary from simple cuboidal to areas where up to four layers of nuclei were observed (Figure 5A and D). Although the epithelium in these regions appeared to be stratified, a proportion of cells were clearly columnar and reached from the col-

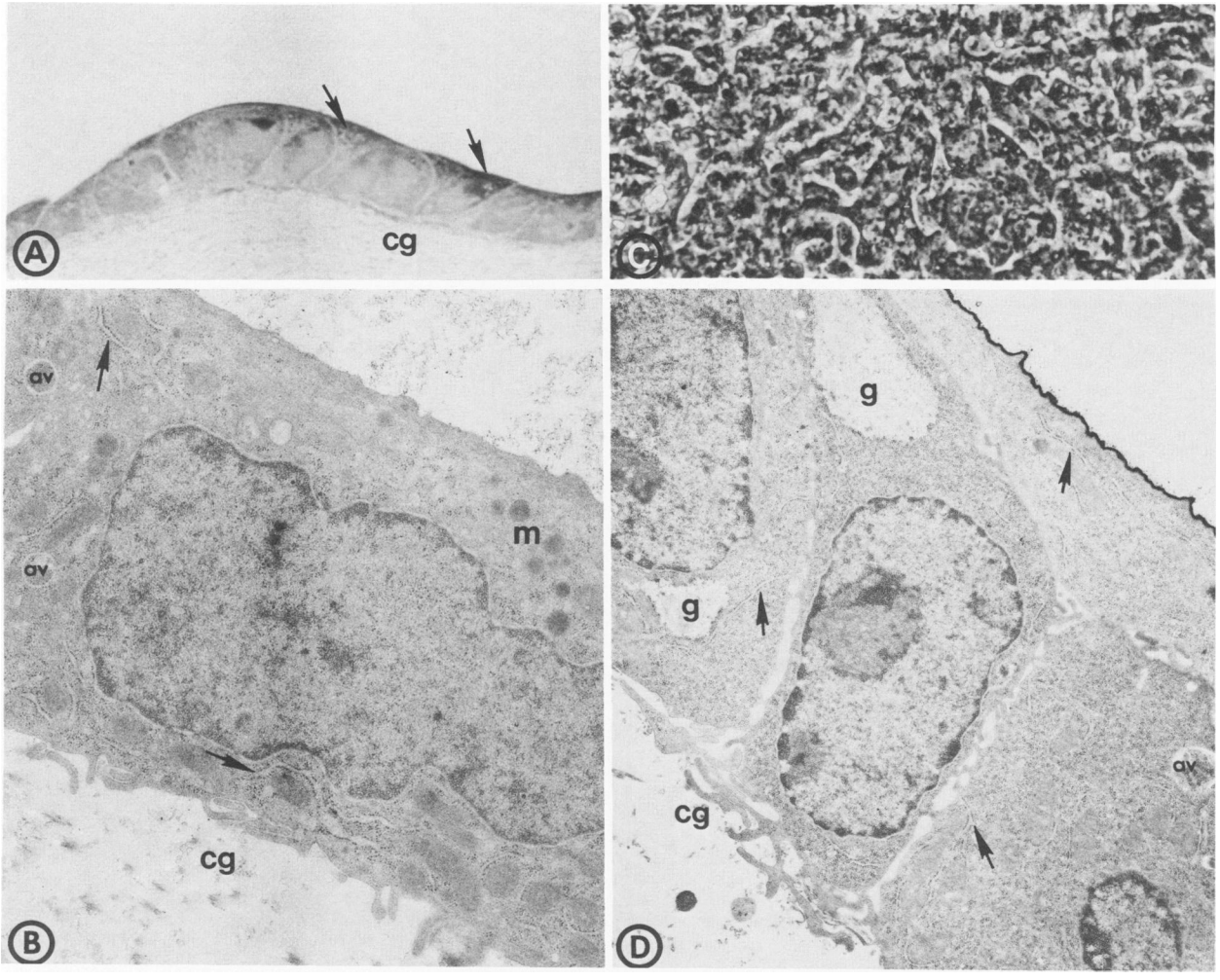


Figure 4—Hamster tracheal epithelial cells during Days 4 and 5 of culture. **A**—Vertical section through a 4-day-old colony. The large cells are cuboidal and form a monolayer. PAS-positive granules are clustered apically (*arrows*). *Cg*, collagen gel. (Glycol methacrylate section, PAS–lead hematoxylin, ×400) **B**—Vertical section through a 4-day-old colony. The RER (*arrows*) is moderately well developed, and mucous granules (*m*) are clustered in the apical cytoplasm. *av*, autophagic vacuoles; *cg*, collagen gel. (×10,000) **C**—Phase-contrast appearance of a 5-day-old colony. All cells are of similar size and phase contrast when viewed from above. (×100) **D**—Vertical section through a 5-day-old colony shows two layers of cytoplasm. The RER is fairly well developed (*arrows*). *g*, lakes of glycogen; *av*, autophagic vacuole; *cg*, collagen gel. The electron-dense deposit on the apical membrane is an artefact resulting from thiocarbohydrazide–osmium binding. (×7600)

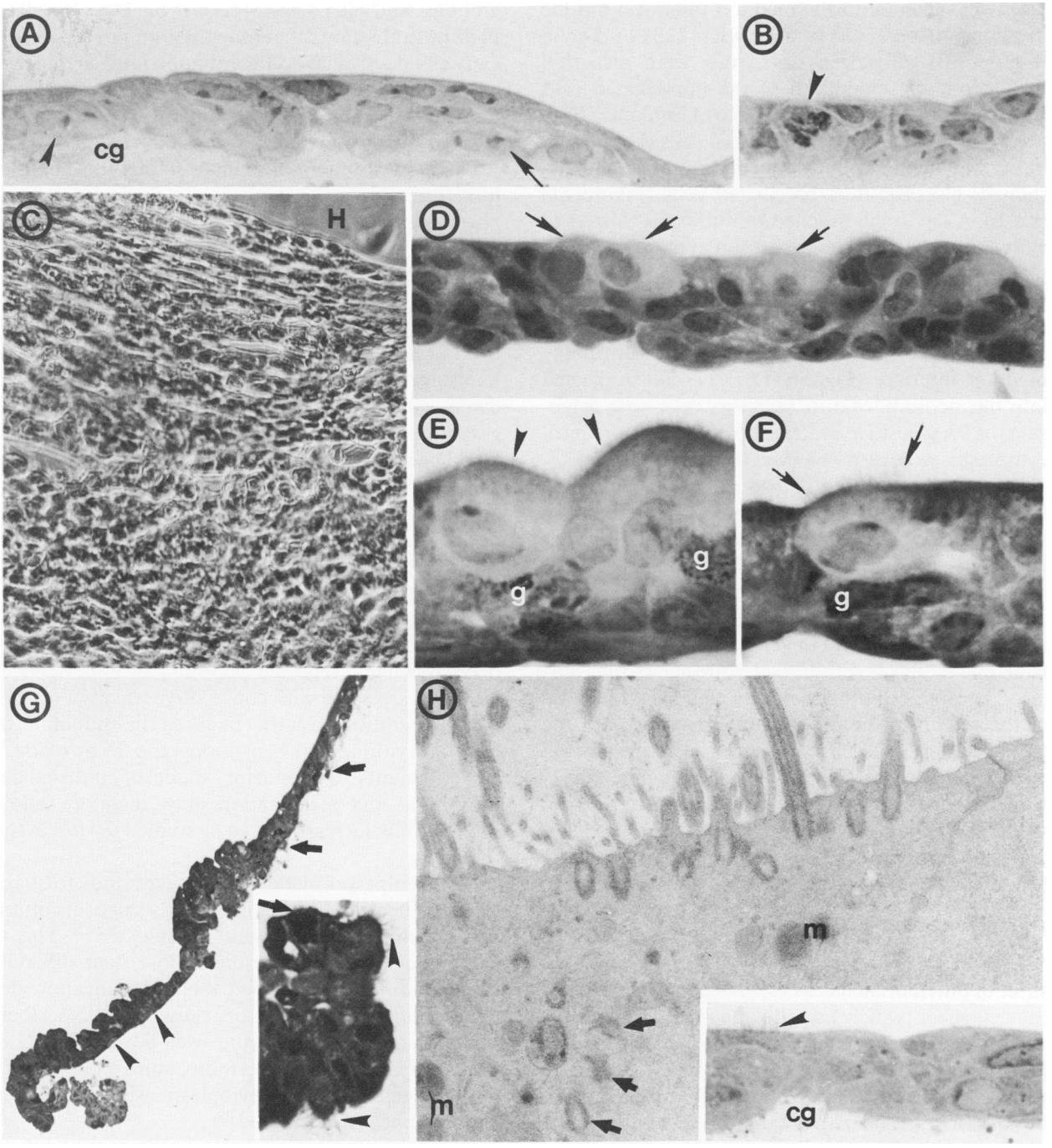


Figure 5—Hamster tracheal epithelial cells during Days 6 and 7 of culture. **A**—Vertical section through the epithelial cell sheet after 6 days in culture. The morphology varies from simple cuboidal (one nuclear layer at *arrowhead*) to an area which has three nuclear layers (at *arrow*). *cg*, collagen gel. (Glycol methacrylate section, PAS–lead hematoxylin, ×360) **B**—Vertical section through 6-day-old epithelium. A columnar cell is in mitosis (*arrowhead*). (Glycol methacrylate section, H&E, ×360) **C**—Phase-contrast appearance of the epithelial cell sheet as it lifts from the collagen gel after 6 days in culture. A large circular hole (*H*) was present at the center of the sheet of epithelial cells. (×100) **D**—Vertical section shows this part of the epithelium to be multilayered after 6 days in culture. Large pale-stained preciliated cells have formed in the upper stratum (*arrows*). Cells of the lowest stratum have smooth basilar surfaces, indicating that this area had already lifted from the collagen gel. (Glycol methacrylate section, H&E, ×300) **E** and **F**—Large pale-stained preciliated cells (**E**), and an immature ciliated cell (**F**), have formed in the upper stratum, after 6 days in culture. Note the well-developed microvillus border (*arrowheads*) and short cilia (*arrows*). These cells are glycogen-free, but the surrounding nonciliated cells contain aggregates of glycogen (*g*). (Glycol methacrylate sections, PAS–lead hematoxylin, ×600) **G**—Vertical section through epithelial cell sheet after 7 days in culture. The basilar surfaces of the cells at upper right are irregular because they penetrate into the collagen gel substrate (*arrows*), but basilar surfaces of the cells at lower left are smooth (*arrowheads*), because this part of the sheet had lifted from the gel (as shown in **C**. (×150) **Inset**—Enlargement of the curled end of the lifted sheet of cells, shown in G. Note cilia of mature ciliated cells (*arrowheads*) and PAS-positive mucous granules (*arrow*) in a secretory cell. (×600) (Glycol methacrylate section, PAS–lead hematoxylin) **H**—Vertical section through an immature ciliated cell after 7 days in culture. Note mucous granules (*m*) and a cluster of basal bodies (*arrows*) in the apical cytoplasm. (×1300) **Inset**—Adjacent semithin epoxy section shows the same ciliated cell (*arrowhead*). Irregular processes project from the basilar surfaces of the cells into the collagen gel (*cg*). (Toluidine blue, ×750)

lagen gel substrate to the medium interface; mitotic division was observed in a very few cells on Day 6 (Figure 5B). Glycogen aggregates were dispersed throughout the cytoplasm of the non-ciliated cells (Figure 5E and F). The appearance of immature (Figure 5F and H) and mature ciliated cells (Figure 5G, inset) was heralded by the formation of large pale-stained, glycogen-free, preciliated cells (Figure 5D and E). A few small mucous granules and clusters of cytoplasmic basal bodies were seen in the apical cytoplasm of the immature ciliated cells (Figure 5H). The polarity of the ciliated cells was normal; and even in areas where the cells appeared to be stratified, the ciliated cells arose from the uppermost stratum, at the medium interface (Figure 5D). The cilia projected into the medium. Mature secretory cells, with aggregates of PAS-positive mucous granules in their apical cytoplasm, were also observed (Figure 5G, inset).

Lysis of the collagen spread rapidly (Figure 5C). The holes became more numerous and coalesced, so that by Day 7, the center of each culture dish contained one large circular hole. Meanwhile, the cells at the periphery of the large central colony remained attached to the collagen gel substrate. This phenomenon could be visualized in vertical sections. Cells still adherent to the substrate had irregular basilar surfaces, because cell processes projected from the bases of the cells into the gel (Figure 5H, inset), but the basilar surfaces of cells that had lifted from the gel were quite smooth (Figure 5D and G). The phase-contrast appearance of cells still attached to the substrate at the periphery of the large central colonies appeared similar to that on Day 5, except that by Day 6, some ciliated cells were present.

Day 8

Lysis of the collagen gel and lifting of the confluent sheet of differentiated cells progressed. Large areas of the cell sheet (which had previously lifted from the collagen gel) became folded and puckered and formed long broad cords of cells, which attached to the plastic surface of the dish. After the cords attached, cells began to migrate from them, thereby starting a second wave of epithelial regeneration. By Day 8, extensive sheets of new cell growth composed of large flat cells, covered the "hole" at the center of each culture dish.

Discussion

Wu et al[18] reported that hamster tracheal epithelial cells grown on a collagen gel substrate, in serum-free hormone-supplemented medium, underwent mucociliary differentiation in late confluent cultures. No mucous granules were detected on Day 5, but mucous granules and cilia were observed on Day 21.[18] The present study shows that mucous granules are seen as early as 4 days, ciliary differentiation is occurring by 6 days, and most of the stages of normal fetal development and regeneration following injury are recapitulated in the primary cell cultures by 7 days.

Fetal development of the hamster tracheal epithelium has been studied in detail *in vivo*.[14,15,26] This normal process shares many similarities with regeneration as it occurs in the adult hamster tracheal epithelium following injury.[8–13] Both processes are implicated in the mechanisms of tumorigenesis. For example, tumors of the tracheal epithelium developed in large numbers in adult hamsters if the same hamsters had been treated as fetuses *in utero* with the carcinogen diethylnitrosamine (DEN) on Day 15 of gestation. Fewer tumors arose if they were treated on fetal Days 12–14, and none arose if they were treated earlier.[27] This was because DEN was metabolized to an active carcinogen by enzymes of the RER in the developing tracheal secretory cells only after Day 12. The RER and these enzyme systems are not sufficiently developed *in vivo* on fetal Day 11 and are not fully developed until fetal Day 15.[27] In regard to regeneration of the tracheal epithelium, it has recently been shown that repeated wounding of adult hamster trachea, associated with consecutive waves of epithelial regeneration, greatly enhances the induction of respiratory tumors by benzo(a)pyrene–ferric oxide.[28] Development of a satisfactory model of epithelial development and regeneration may, therefore, allow observations such as these to be studied *in vitro* in the future.

No attempt was made in the present study to quantify the different cell types in the early stages of culture because this is fraught with difficulty.[18,29,30] This is because cells in culture quickly lose their differentiated features upon which their identification depends. For example, secretory cells rapidly expel their mucous granules, and the well-developed RER largely disappears. Furthermore, some ciliated cells retract their cilia into the cytoplasm, which causes the cells to appear non-ciliated.[29]

Of the three cell types which constitute the tracheal epithelium, only basal cells and secretory cells divide.[31] A dogma still lingers that basal cells of the trachea are progenitors for secretory and ciliated cells, but evidence against this premise is mounting. During fetal development, the basal cells are the last cell type to differentiate.[32,33] Moreover, during regeneration of the tracheobronchial epithelium following diverse types of injury, secretory cells clearly play a major progenitor role, and the rate of division of basal cells is not increased.[8–13,34–36] Collectively, evidence is accu-

mulating to suggest that secretory cells divide to provide new secretory cells and ciliated cells, whereas basal cells divide to replenish themselves.

In the present study, a differentiated mucociliary epithelium was established in the cultures on Day 7. Basal cells appeared to play an insignificant role in this process. On the first 3 days of culture a few basal cells were identified by phase-contrast microscopy as small cells with phase-dense nuclei. Thereafter, their fate could not be traced. The function of tracheal basal cells *in vivo* is unknown, but they may play a role in epithelial adhesion. Of all cell types in the tracheobronchial epithelium only basal cells bear hemidesmosomes on the basilar cell membranes.[25,37] Hemidesmosomes are functional organelles specialized for adhesion. At these sites, the cytokeratin–desmosome system of the epithelial cells is continuous with the anchoring fibril–collagen system of the underlying mesenchyme.[38] In the present study, hemidesmosomes were not observed at the basilar surface of any cell at the interface with the collagen gel substrate.

Apart from a few basal cells (discussed above) and small numbers of ciliated cells, which had declined to zero on Day 5 of culture, confirming the earlier report,[18] the young colonies were composed of altered secretory cells. The columnar secretory cells of the normal epithelium flattened when seeded onto the collagen gel, to form a simple squamous epithelium composed of poorly differentiated large cells. Cell apices were joined by junctional complexes, and the lateral cell membranes were joined by small desmosomes, but the cytoplasm lacked specialization and the RER was poorly developed. Loss of differentiated functions commonly occurs during the first 3 days of cell culture following the isolation and dissociation of specialized cells.[39]

Signs of the return of secretory specialization were obvious by Day 4 and increased progressively until Day 7. Concurrently, the large cells, which were differentiating into recognizable secretory cells, changed their shape from squamous to cuboidal–columnar on Day 4. The RER developed, and mucous granules accumulated in the apical cytoplasm of the cells. Cytoplasmic glycogen was observed in the secretory cells from Day 5 onward. These morphologic changes agree closely with reports of the production of high-molecular-weight mucous glycoproteins by hamster tracheal cells in culture, which began to increase on Day 4 and peaked on Day 7.[40]

Ciliated cells were absent from the cultures on Day 5 but were present on Days 6 and 7. Evidence that ciliated cells had been generated in the cultures from nonciliated progenitors was provided on Day 6 by the presence of preciliated and immature ciliated cells, which contained mucous granules and clusters of basal bodies in the apical cytoplasm. Preciliated cells arise in large numbers during tracheobronchial epithelial development[14,32,33] and in the adult epithelium regenerating after injury.[11–13,34,35,41,42] They also occur, albeit less frequently, in the mature epithelium as part of the process of normal maintenance.[25] Evidence that preciliated cells arise from secretory cells and detailed descriptions of their characteristic light- and electron-microscopic morphologic features are given in detail elsewhere.[11–15,25,35,41,42]

In the hamster trachea, there are similarities between normal epithelial development in the fetus and epithelial regeneration following injury in the adult.[13,15] These processes encompass many significant aspects of the normal and abnormal biology of the tracheobronchial epithelium. In each case, a period of rapid cell proliferation is followed by a decline in the mitotic rate. As cell division wanes, ciliated cells and secretory cells become differentiated and a mucociliary epithelium is established (see Figure 6). In the present study, the growth and differentiation of tracheal cells in culture followed a similar pattern. Figure 2 shows that the log phase of growth continued until Day 6; then the cells entered the stationary phase. The preciliated cells first appeared on Day 6, coincident with the diminished rate of cell growth in culture.

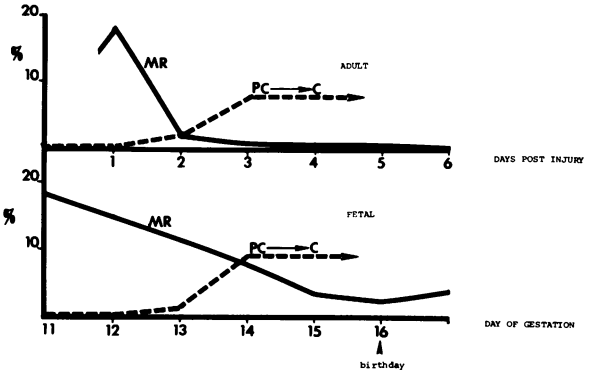


Figure 6—Similarities between normal fetal development and regeneration following injury in hamster tracheal epithelium. (Top graph adapted from data from regenerating dorsal tracheal epithelium.[11] Lower graph adapted from data from developing dorsal tracheal epithelium[14,15] and day after birth from reference.[44]) Data are presented as mean percentages of the total number of epithelial cells counted. In the normal adult epithelium the mitotic rate (*MR*) is very low, about 0.1%; but 1 day after mechanical injury the mitotic rate peaks at the wound site and is equivalent to the rates of proliferation on the 11th and 12th gestational days. The extent and timing of cell replication in relation to the production of preciliated cells (*PC*) is very similar in both situations. Preciliated cells rapidly mature into ciliated cells (*C*). In the adult epithelium regenerating after mechanical injury, the mitotic rate represents the percentage of cells in metaphase arrest after a 6-hour colchicine block. In the fetal epithelium the original data were collected as a mitotic index (ie, without colchicine). Here the original fetal data are multiplied by 6 to equate with a 6-hour colchicine block.

Fetal development of the hamster tracheal epithelium occurs from Day 10 to Day 16 of gestation, the day of birth. During this 7-day period, a simple epithelium composed of very poorly differentiated *columnar* cells changes into one that is differentiated and mucociliary. Reconstitution of the mucociliary epithelium in primary cell culture also occurs in 7 days, and a simple epithelium composed of very poorly differentiated *squamous* cells changes into one that is differentiated and mucociliary. The similarities are striking (Table 1). However, there are differences regarding basal cells and endocrine cells between fetal development and primary cell culture. Basal cells do not appear in the hamster fetal trachea until Day 13,[14,15,26] whereas in the cultures, the basal cells were present initially but were not recognized with certainty after Day 3. Endocrine cells appear during development on fetal Day 12[14] but were not observed in the cultures at any time.

After cessation of mechanical or nutritional (vitamin A deficiency) insult to adult hamster trachea, a nearly normal mucociliary epithelium is regenerated in 5–7 days.[9–13] In several respects, growth of hamster tracheal epithelial cells in primary cell culture also recapitulates this process. In our experimental model of mechanical injury *in vivo*, all the epithelial cells were carefully scraped away from a focal area.[9–12] Within the first 12 hours, columnar secretory cells and basal cells adjacent to the wounded area flattened and migrated over the wound site. By 24 hours the wounds were covered by simple squamous epithelium, composed in large part of altered secretory cells.[8,12] However, loss of differentiation was less marked *in vivo* than *in vitro*, and the degree of differentiation in the altered secretory cells that covered the wounds *in vivo* resembled that seen in the cell colonies after 3 days in culture. One day after mechanical injury *in vivo*, the flattened secretory cells in the wound site proliferated rapidly, piled up, and formed a stratified metaplastic epidermoid epithelium. The keratinized metaplastic cells also contained mucous granules, consistent with their origin from secretory cells. Epidermoid metaplasia was well-developed *in vivo* at the wound site 2 and 3 days following injury.[8,11,12] This stage of regeneration was recapitulated *in vitro* on Days 5 and 6 of culture. However, tonofilament bundles were not well developed in the cultured cells, so that although the epithelium appeared to be stratified and superficially resembled epidermoid metaplasia (Figure 5A), typical keratinizing metaplasia was not reproduced *in vitro*. Three days after mechanical injury *in vivo*, some of the most heavily keratinized cells sloughed,[12] and proliferation of less keratinized secretory cells provided new columnar secretory cells and preciliated cells, which rapidly matured into ciliated cells.[11,12] A nearly normal mucociliary epithelium was restored *in vivo*, 5–7 days after the injury. During vitamin A deficiency *in vivo*, prior to the development of keratinizing epidermoid metaplasia, the number of ciliated cells was reduced, preciliated cells were virtually absent, and the mitotic activity of the secretory cells was greatly reduced.[13] If the deficiency is prolonged, a keratinizing epidermoid metaplasia develops. Following restoration of vitamin A to

Table 1—Comparison of Hamster Tracheal Epithelial Cells During Fetal Development and Growth in Primary Cell Culture

	Days of gestation/Days of culture				
	10 and 11/1 and 2	12/3	13/4	14/5	15 and 16*/6 and 7*
Shared characteristics	Very poorly differentiated	Poorly differentiated, but RER more developed than on previous days	Poorly moderately differentiated; RER developing in cuboidal/columnar cells	Moderately differentiated; glycogen; RER developed in secretory cells	Moderately well differentiated; glycogen; RER developed in secretory cells; mucous granules; preciliated/ciliated cells
Fetal characteristics†	Simple columnar	Simple columnar; endocrine cells first appear	Pseudostratified, due to appearance of basal cells; first preciliated/ciliated cells in dorsal epithelium	Pseudostratified; preciliated/ciliated cells in dorsal and ventral epithelia	Pseudostratified
Culture characteristics	Simple squamous	Simple squamous	Simple cuboidal; some mucous granules	Simple cuboidal–stratified‡; mucous granules	Simple cuboidal–stratified‡

*Day 16 is the day of birth.

†Fetal data taken from McDowell et al.[14,15]

‡At focal areas, the epithelium appeared to be stratified, but this was not substantiated by serial sectioning.

the diet, mitotic activity in secretory cells was restored to the control level; and shortly thereafter, preciliated cells (which contained secretory granules) appeared in the epithelium and rapidly matured into ciliated cells. The normal tracheal mucociliary epithelium was restored in 7 days.[13] The present study shows that a mucociliary epithelium is also formed in primary cell culture in 7 days.

Lysis of the collagen gel substrate began in the cultures on Day 5, and was well under way on Day 6. Nevertheless, the present study shows that the progressive stages in formation of the mucociliary epithelium occurred in synchrony during the first 7 days of culture, and many of the well-defined stages of normal fetal development and regeneration following injury *in vivo* were recapitulated *in vitro* by Day 7, before lysis of the collagen gel was widespread. Unless lysis of the collagen gel is prevented, large areas of the confluent cell sheet lift from the substrate and attach to the bottom of the culture dish in broad cords. Cells migrate from these cords, and the process of development/regeneration is repeated, but now the dynamic process becomes asynchronous, because the cell sheet began to lift on Day 5 and continued to do so progressively until Day 8. Therefore, the second and following waves of regeneration start at different times focally within areas of the same culture dish, spaced apart by several days. Attempts are being made to prevent lysis of the substrate.[43] When this difficulty is overcome, this model[18] promises to be of great value for the study of normal structure and function, including development, and for studies of pathology of the tracheobronchial epithelium, including hyperplasia, metaplasia, and neoplasia.

References

1. Kennedy AR, Desrosiers A, Terzaghi M, Little JB: Morphometric and histological analysis of the lungs of Syrian golden hamsters. J Anat 1978, 125:527–553
2. McDowell EM, Barrett LA, Galvin F, Harris CC, Trump BF: The respiratory epithelium: I. Human bronchus. J Natl Cancer Inst 1978, 61:539–549
3. Becci PJ, McDowell EM, Trump BF: The respiratory epithelium: II. Hamster trachea, bronchus and bronchioles. J Natl Cancer Inst 1978, 61:551–561
4. Saffiotti U, Cefis F, Kolb L: A method for the experimental induction of bronchogenic carcinoma. Cancer Res 1968, 28:104–124
5. Becci PJ, McDowell EM, Trump BF: The respiratory epithelium: VI. Histogenesis of lung tumors induced by benzo[a]pyrene-ferric oxide in the hamster. J Natl Cancer Inst 1978, 61:607–618
6. McDowell EM, McLaughlin JS, Merenyi DK, Kieffer RF, Harris CC, Trump BF: The respiratory epithelium: V. Histogenesis of lung carcinomas in the human. J Natl Cancer Inst 1978, 61:587–606
7. Willis RA: Regeneration and repair: Resumed embryonic growth, The Borderland of Embryology and Pathology. 2nd edition. Washington, Butterworths, 1962, pp 495–518
8. McDowell EM, Becci PJ, Schürch W, Trump BF: The respiratory epithelium: VII. Epidermoid metaplasia of hamster tracheal epithelium during regeneration following mechanical injury. J Natl Cancer Inst 1979, 62:995–1008
9. Keenan KP, Combs JW, McDowell EM: Regeneration of hamster tracheal epithelium after mechanical injury: I. Focal lesions: Quantitative morphologic study of cell proliferation. Virchows Arch [Cell Pathol] 1982, 41:193–214
10. Keenan KP, Combs JW, McDowell EM: Regeneration of hamster tracheal epithelium after mechanical injury: II. Multifocal lesions: stathmokinetic and autoradiographic studies of cell proliferation. Virchows Arch [Cell Pathol] 1982, 41:215–229
11. Keenan KP, Combs JW, McDowell EM: Regeneration of hamster tracheal epithelium after mechanical injury: III. Large and small lesions. Virchows Arch [Cell Pathol] 1982, 41:231–252
12. Keenan KP, Wilson TS, McDowell EM: Regeneration of hamster tracheal epithelium after mechanical injury: IV. Histochemical, immunocytochemical and ultrastructural studies. Virchows Arch [Cell Pathol] 1983, 43:213–240
13. McDowell EM, Keenan KP, Huang M: Restoration of mucociliary tracheal epithelium following deprivation of vitamin A: A quantitative morphologic study. Virchows Arch [Cell Pathol] 1984, 45:221–240
14. McDowell EM, Newkirk C, Coleman B: Development of hamster tracheal epithelium: I. A quantitative morphologic study in the fetus. Anat Rec 1985, 213:429–447
15. McDowell EM, Newkirk C, Coleman B: Development of hamster tracheal epithelium: II. Cell proliferation in the fetus. Anat Rec 1985, 213:448–456
16. Wu R: In vitro differentiation of airway epithelial cells, In Vitro Models of Respiratory Epithelium. Edited by LJ Schiff. Boca Raton FL, CRC Press, 1986, pp 1–26
17. Van Scott MR, Yankaskas JR, Boucher RC: Culture of airway epithelial cells: Research techniques: Minireview. Exp Lung Res 1986, 11:75–94
18. Wu R, Nolan E, Turner C: Expression of tracheal differentiated functions in serum-free hormone-supplemented medium. J Cell Physiol 1985, 125:167–181
19. Groelke JW, Coalson JJ, Baseman JB: Growth requirements of ferret tracheal epithelial cells in primary culture. Proc Soc Exp Biol Med 1985, 179:309–317
20. McDowell EM, Trump BF: Histologic fixatives suitable for diagnostic light and electron microscopy. Arch Path Lab Med 1976, 100:405–414
21. Ruddell CL: Embedding media for 1-2 micron sectioning. Stain Technol 1967, 42:253–255
22. Sorokin SP, Hoyt RF: PAS-lead hematoxylin as a stain for small-granule endocrine cell populations in the lungs, other pharyngeal derivatives and the gut. Anat Rec 1978, 192:245–259
23. Aoki M, Tavassoli M: OTO method for preservation of actin filaments in electron microscopy. J Histochem Cytochem 1981, 29:682–683
24. Sigler RE, Jones RT, Hebel JR, McDowell EM: Hamster tracheal organ culture in serum-free media: A quantitative comparison of *in vitro* epithelial morphology with that of *in vivo* controls. In Vitro 1987, 23: 100–110
25. McDowell EM, Combs JW, Newkirk C: A quantitative light and electron microscopic study of hamster tracheal epithelium with special attention to so-called "intermediate cells." Exp Lung Res 1983, 4:205–226
26. Emura M: Development of Syrian golden hamster tracheal epithelium during prenatal and immediate post-

natal stages, Respiratory System. Edited by TC Jones, U Mohr, RD Hunt. Heidelberg, Springer-Verlag, 1985, pp 11–27
27. Schüller HM: Perinatal carcinogenesis in hamsters, Comparative Perinatal Carcinogenesis. Edited by HM Schüller. Boca Raton, FL, CRC Press, 1984, pp 13–22
28. Keenan KP, Saffiotti U, Stinson SF, Riggs CW, McDowell EM: Enhancement of respiratory carcinogenesis by tracheal wounding from cannulation (Abstr). Fed Proc 1987, 46:432
29. Chang LY, Wu R, Nettesheim P: Morphological changes in rat tracheal cells during the adaptive and early growth phase in primary cell culture. J Cell Sci 1985, 74:283–301
30. Wu R: Growth and differentiation of airway epithelial cells in a serum-free, hormone-supplemented medium, Growth and Differentiation of Cells in Defined Environment. Edited by H Murakami, I Yamane, DW Barnes, et al. Tokyo, Kodansha, 1985, pp 187–192
31. Boren HG, Paradise LJ: Cytokinetics of the Lung, Pathogenesis and Therapy of Lung Cancer. Edited by CC Harris. Marcel Dekker, New York, 1978, pp 369–418
32. Plopper CC, Alley JL, Weir AJ: Differentiation of tracheal epithelium during fetal lung maturation in the Rhesus Monkey *Macaca mulatta*. Am J Anat 1986, 175:59–71
33. Cutz E: Cytomorphology and differentiation of airway epithelium in developing human lung, Lung Carcinomas. Edited by EM McDowell. Edinburgh, Churchill Livingstone, 1987, pp 1–41
34. Wilson DW, Plopper CG, Dungworth DL: The response of the Macaque tracheobronchial epithelium in acute ozone injury. Am J Pathol 1984, 116:193–206
35. Evans MJ, Shami SG, Cabral-Anderson LJ, Dekker NP: Role of nonciliated cells in renewal of the bronchial epithelium of rats exposed to NO_2. Am J Pathol 1986, 123:126–133
36. Jeffery PK, Ayers M, Rogers D: The mechanisms and control of bronchial mucus cell hyperplasia. Adv Exp Med Biol 1982, 144:399–409
37. Kawanami O, Ferrans VJ, Crystal RG: Anchoring fibrils of the normal canine respiratory system. Am Rev Respir Dis 1979, 120:595–611
38. Garrod DR: The adhesions of epithelial cells, Cellular and Molecular Control of Direct Cell Interactions. Edited by HJ Marthy. New York and London, Plenum Press, 1984, pp 43–83
39. Wolffe AP, Tata JR: Primary culture, cellular stress and differentiated function. FEBS Lett 1984, 176:8–15
40. Kim KC, Rearick JI, Nettesheim P, Jetten AM: Biochemical characterization of mucous glycoproteins synthesized and secreted by hamster tracheal epithelial cells in primary culture. J Biol Chem 1985, 260:4021–4027
41. Gordon RE, Lane BP: Ciliated cell differentiation in regenerating rat tracheal epithelium. Lung 1984, 162:233–243
42. Gonzalez S, van Bassewitz DB, Grundmann E, Nakhosteen JA, Müller K-M: The ultrastructural heterogeneity of potentially preneoplastic lesions in the human bronchial mucosa. Pathol Res Pract 1986, 181:408–417
43. Niles RM, Kim KC, Brody JS: Long-term culture of hamster tracheal epithelial cells which retain their differentiated phenotype (Abstr). J Cell Biol 1986, 103:201a
44. Otani EM, Newkirk C, McDowell EM: Development of hamster tracheal epithelium: IV. Cell proliferation and cytodifferentiation in the neonate. Anat Rec 1986, 214:183–192

Acknowledgment

The authors thank Janet S. Folmer for her willing cooperation and expertise in preparing sections of the monolayer cultures for electron microscopy, Perry Comegys for his help and expertise in preparing the photomicrographs for publication, and Michael J. Evans for his critical reading of the manuscript.