Scanning Microscopy

Volume 2 | Number 3 Article 45

5-22-1988

Polarized Epithelial Cysts in vitro: A Review of Cell and Explant Culture Systems that Exhibit Epithelial Cyst Formation

J. A. McAteer
Indiana University

G. S. Dougherty
Indiana University

K. D. Gardner Jr.
University of New Mexico

A. P. Evan
Indiana University

Follow this and additional works at: https://digitalcommons.usu.edu/microscopy

Part of the Biology Commons

Recommended Citation

McAteer, J. A.; Dougherty, G. S.; Gardner, K. D. Jr.; and Evan, A. P. (1988) "Polarized Epithelial Cysts in vitro: A Review of Cell and Explant Culture Systems that Exhibit Epithelial Cyst Formation," *Scanning Microscopy*: Vol. 2 : No. 3 , Article 45.
Available at: https://digitalcommons.usu.edu/microscopy/vol2/iss3/45

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Scanning Microscopy by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.

Scanning Microscopy, Vol. 2, No. 3, 1988 (Pages 1739-1763) 0891-7035/88$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

POLARIZED EPITHELIAL CYSTS IN VITRO: A REVIEW OF CELL AND EXPLANT CULTURE SYSTEMS THAT EXHIBIT EPITHELIAL CYST FORMATION

J.A. McAteer*[1], G.S. Dougherty[1],
K.D. Gardner Jr.[2] and A.P. Evan[1]

[1]Department of Anatomy Indiana University School of Medicine
Indianapolis, Indiana
[2]Department of Medicine, University of New Mexico
Albuquerque, New Mexico

(Received for publication June 25, 1987, and in revised form May 22, 1988)

Abstract

The purpose of this paper is to review in vitro cell and explant culture systems that exhibit epithelial cyst formation and that are used as models of polarized epithelial function. We examine a number of culture systems derived from a variety of cell and organ types, briefly describe the methodology and conditions used to establish these cultures and discuss aspects of the experimental application of each system. We conclude that the characteristics of epithelial cyst-forming cultures are dependent upon the origin and identity of the cell population, as well as the multiple factors that define the culture environment. Culture systems in which epithelial cyst development occurs provide tools to study fundamental problems in epithelial biology, such as the establishment and maintenance of cell polarity, cell recognition and cell sorting, also cell-specific functions involving solute and water transport and the production and modification of secreted products. In addition, epithelial cyst culture systems offer useful models to better understand cellular behavior in various pathologic conditions of cyst formation in man.

Key Words: epithelial cysts, cell culture, explant culture, cell polarity, fluid transport, model systems, experimental pathology.

*Address for Correspondence:
Department of Anatomy
Indiana University School of Medicine
635 Barnhill Dr.
Indianapolis, IN 46223 Phone No. (317) 274-7935

Introduction

Cyst formation occurs in cultures derived from a wide variety of organs and cell types, established by both common as well as rather novel cell and explant culture methodologies (Table 1). In these cultures some or all of the cells participate to form a continuous, polarized epithelium (simple or stratified) that fully surrounds and encloses a fluid-filled space. Such in vitro cysts are in contrast to the multicellular hemicysts that form when fluid transporting epithelia are cultured to confluency atop a non-permeable substrate (47,67).

Included in this broad definition of an "in vitro cyst", are structures that may be spherical, tubular or irregular in form. The orientation of cell polarity does not influence this definition, and no distinction is made between the terms "cyst" and "follicle". Cyst shape and cell orientation are important features that may be of critical importance in a given line of investigation, however, we have chosen not to limit discussion on this basis. Instead, this paper will consider the wide variety of cell and explant culture systems in which epithelial cells organize to form "cysts". In this context, we have described selected examples of the many epithelial cyst-forming culture systems that have been reported. This review is not intended to be all-inclusive of the many cyst culture systems that have been described by so many excellent laboratories. Instead, we have selected specific systems to serve as illustrative examples of cyst formation by various types of epithelia in culture. It is hoped that this will help demonstrate the unique characteristics of these cultures, highlight the range of problems to which these novel culture systems have been applied (Table 2) and underscore their usefulness as experimental models.

Epithelial Cyst Formation in Cell Culture

Epithelial cyst formation in vitro cannot be ascribed to a common series of events, but, is characterized by a number of processes, including 1) cell sorting following the aggregation of dissociated cells, 2) the outgrowth of histotypic structures from cells or explants seeded within a three-dimensional matrix and 3) the reorganiza-

TABLE 1

EXAMPLES OF CELL AND EXPLANT CULTURE SYSTEMS THAT EXHIBIT EPITHELIAL CYST FORMATION

	References
Lung & Airways	
o Alveolar-like-structures from fetal lung cells in Gelfoam sponge	(15-17,87)
o Rotation-mediated aggregation of dissociated cells in suspension	(59)
o Submersion cultured whole lung	(58,60)
o "Alveolization" of collagen-embedded lung explants	(57)
o Fetal tracheal explants	(56)
o Type II epithelial cell cysts from adult lung	(84)
Thyroid Epithelium	
o Follicle formation by dissociated cells in suspension culture	(35,54)
o Thyroid cell clusters form follicles in suspension culture	(33,76-79)
o Cloned FRT cells form follicles in suspension culture	(25)
o Follicle formation by dissociated cells in collagen gel	(12)
o Collagen-induced follicular reorganization of thyroid monolayers	(12)
o Thyrotrophin stimulation of follicle formation by monolayers	(51)
Kidney	
o Aggregation of chick kidney cells seeded to collagen sponge	(2)
o Cystic dilation of proximal tubules in chick mesonephric explants	(13)
o Tubular cyst formation in mouse metanephric explants	(3-5,7)
o Human polycystic kidney cyst wall explants form solitary cysts	(63)
Mammary Epithelium	
o Aggregation of dissociated cells embedded in collagen gel	(93-95)
o Rama 25 forms duct-like outgrowths at surface of collagen gel	(8,9,80)
Submandibular Gland: Tubules and cysts form in collagen gels	(92)
Prostatic Epithelium: Acinar-like structures form in collagen gel	(40)
Vaginal Epithelium: Tubular outgrowths within collagen gel	(38)
Endometrium: Cyst formation by explants in submersion culture	(43)
Small Intestine: Cyst formation in response to collagen overlay	(70)
Choroid Plexus: Epithelial vesicles develop in submersion culture	(1)
Urinary Bladder Tumors: Epithelial cysts in submersion culture	(90)
Vascular Endothelium	
o Capillary-like outgrowths from explants in fibrin & collagen gels	(68,74,75)
o Collagen-induced capillary-like networks in monolayers	(69)
Preimplantation Mouse Embryo: Blastocyst from 4-cell embryo	(86)
Teratocarcinoma: Cystic embryoid bodies from cell aggregates	(18,53)
Renal Cell Lines	
o LLC-PK1 cysts	(61,91)
o Collagen overlay causes lumen formation by MDCK monolayers	(32)
o MDCK-cysts form in fibrin or collagen gels	(45-48,62)

TABLE 2

EXPERIMENTAL APPLICATIONS OF SELECTED EPITHELIAL CYST CULTURE SYSTEMS

	References
Thyroid Biology	
o Epithelial-mesenchymal interactions influence the capacity of thyroid cells to form *in vitro* follicles	(35)
o *In vitro* follicles are morphologically responsive to thyrotrophin	(51)
o Thyrocyte mitotic activity is stimulated by thyrotrophin	(77)
o Basal lamina formation is stimulated by extracellular matrix	(28)
o Polarity reversal occurs in response to manipulation of the extracellular environment	(26,27,78)
o Cell shape is influenced by intraluminal pressure	(79)
o Dynamics of transepithelial fluid movement	(79)
o Vesicular transcytosis is regulated by thyrotrophin	(33,34)
o TSH and prostaglandins influence cell polarity and iodine concentrating ability	(54)
o Electrical and morphological polarity influences capacity to concentrate iodine	(88)
o Follicle formation is stimulated by collagen overlay	(12)
Respiratory System Development	
o *De novo* basal lamina formation and establishment of an epithelial-mesenchymal interface in fetal lung RMA	(59)
o Production and metabolism of pulmonary surfactant in fetal lung organotypic cultures	(15-17,19)
o Endocrine control of epithelial morphogenesis	(56,58,59)
o Alveolar septum formation in the peripheral lung	(57)
Mammary Biology	
o Modeling mammary morphogenesis	(8,80,81)
o Sustained growth of a primary cell population	(94,95)
Embryogenesis	
o Regulation of inner cell mass formation in the mouse	(86)
o Cystic embryoid bodies derived from mouse teratocarcinoma cells model events in early mammalian development	(53)
o Human teratocarcinoma cells form cystic embryoid bodies with morphology similar to the preimplantation embryo	(18)
Renal Cystic Disease	
o Proximal tubule cyst formation in response to hormones and agents	(3-5,7)
o Modeling the cellular events in renal cyst enlargement	(62,66)
Other Applications	
o Angiogenesis and neovascularization	(68,69,74)
o Epithelial events in morphogenesis: response of an established cell line (MDCK) to an interstitial environment	(32)
o Human endometrium: long term culture with retention of hormone responsiveness	(43)
o Urinary bladder tumors: *in vitro* cyst formation correlates with pathology	(90)
o Vaginal epithelium: sustained cell growth in a model to test hormonal regulation of growth and differentiation	(38)

tion or repolarization of an established epithelium in response to manipulation of the culture environment.

Cyst Formation That Is Dependent Upon Cell Aggregation and/or Cell Sorting. A number of cell culture systems have been described in which _in vitro_ cyst formation involves the establishment of a polarized epithelium following the aggregation of dissociated cells. Cell aggregation, although largely dependent upon the physical conditions of the culture environment, is exhibited by a wide variety of cell types representing each of the basic tissues (epithelium, muscle, connective tissue, nerve). Since cells for primary culture are most commonly derived from an organ or organ fragment that is composed of more than one tissue, the aggregates that form from such a preparation may possess the cells of more than a single tissue type. Such heterogeneous cell aggregates often exhibit tissue-specific cell sorting in which cells of the same tissue type establish predictable associations with one another. Commonly, cell-cell interactions also develop between the cells of different tissues, such as the formation of an epithelial-mesenchymal interface (15-17). When tissue-specific sorting results in the formation of an organized multicellular structure that resembles or has functional characteristics of the organ from which the cells were derived, the structure is described as being organotypic or histotypic (also, histiotypic) (71,72).

Cell aggregation _in vitro_ clearly is influenced by the nature of the culture environment and is facilitated by conditions that promote cell-cell contact. A variety of three-dimensional substrates such as collagen sponge (15-17), collagen coated sponge (50) and collagen gel (55,94) have been used to prepare cultures in which histotypic epithelial cell sorting occurs. The fibrillar lattice of a collagen gel or plasma fibrin clot, or the trabeculae of a sponge may promote cell-cell association by compartmentalizing the environment. Single cells seeded to such a substrate, instead of dispersing, settle within the interstices of the lattice and are held close to one another, thereby increasing the chances for cell-cell contact.

Although cell aggregation is promoted by a three-dimensional substrate, cell aggregation and subsequent histotypic cell sorting can occur under appropriate conditions in suspension culture. A variety of methods have been devised to hold cells in suspension at high population density to facilitate cell-cell contact. One example is rotation-mediated aggregation (RMA) (71). In RMA, dissociated cells are seeded to a culture vessel (dish or flask) that is placed atop a gyrotory shaker. Centripetal forces produced by the motion of the shaker cause the cells to drift toward the center of the culture vessel, where they contact one another and aggregate. RMA has been used for a variety of applications in developmental biology to study cell-cell recognition and epithelio-mesenchymal interactions (71-73).

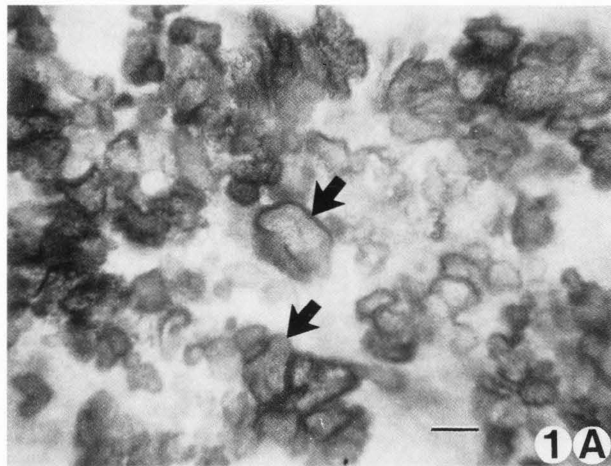
Fetal Lung "Alveolar-like-structures". Histotypic Aggregation Within Collagen Sponge. One example of cyst formation under such conditions is the formation of histotypic "alveolar like structures" (Fig 1) by dissociated fetal lung cells (15-17). Douglas and coworkers (15-17) found that fetal peripheral lung from a variety of species including man (87) formed polarized epithelial cysts when seeded within medium hydrated Gelfoam™ collagen sponge. Initially, cells aggregated to form randomly oriented cell clusters. Within 48 h (or less) these clusters exhibited epithelial-specific sorting to form small cysts that increased in size (final diameter 200-300 µm) with continued culture. These "alveolar-like-structures" (Fig 1) were composed primarily of cuboidal epithelial cells that differentiated to become alveolar type II cells. Although the type II cell was observed to be the predominant epithelial cell type present in these cultures, various airway cells were also observed. In addition, non-epithelial cells, chiefly lipofibroblasts, were present subjacent to the epithelial basal lamina. The type II cells in these cultures have been shown to synthesize and secrete pulmonary surfactant phospholipids into the cyst lumen (19). This culture system has been used to examine the metabolism of the differentiating type II cell and to study the factors that control the maturation of the pulmonary surfactant system (14-17,19).

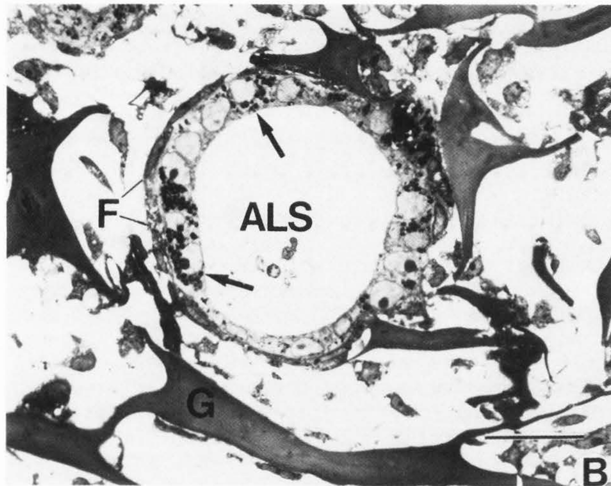
Histotypic Aggregation By Embryonic Kidney Cells. A physically similar culture system has been described by Ansevin and Lipps (2) in which enzymatically dissociated embryonic chick kidney cells were seeded into pieces of synthetic sponge. In these cultures, the cells formed irregularly shaped cysts lined by a simple cuboidal epithelium. The cells were polarized so that the apical surface bordered the lumen. The authors did not describe the growth characteristics of the cyst population, although it was indicated that cultures remained viable for a period of at least 6 weeks.

Mammary Epithelial Cells in Collagen Gel: Mammary epithelial cells also exhibit histotypic aggregation and tubule formation when dissociated cells are mixed within collagen gel. Yang et al. (93-95) studied collagen gel embedded cultures of cells derived from several sources including mouse primary mammary tumors, hyperplastic mammary alveolar nodules and normal mammary gland. Their collagenase dissociation procedure produced a cell suspension comprised of single cells and small clusters of cells. This cell preparation was then mixed with fluid collagen gel and plated to prepare a dispersed cell population. Alternatively, cells were seeded to the surface of gelled collagen then overlaid with fluid collagen so that the majority of cells occupied the same region of the gel. The investigators observed tubule formation in both preparations from each source of mammary tissue. They described that the cells first aggregated to form enlarged clusters which produced thick duct-like structures that projected into the

surrounding collagen gel. These outgrowths consisted of polarized cuboidal cells surrounding a lumen. The cells were oriented with their apical surface facing the lumen. The cells of tubular outgrowths showed considerable mitotic activity and a labeling index for ^{3}H-thymidine incorporation of greater than 50%. The micrographs in their paper show different patterns of tubule formation for each of the source tissues. It was not indicated, however, whether growth pattern was characteristic of tissue type. These studies are of particular value in mammary biology since they provide a culture system that allows sustained growth of a primary cell population. Primary mammary cells cultured under conventional substrate-dependent conditions have a relatively short in vitro life span. The authors speculate that culture within collagen gel offers a means to study endocrine regulation of mammary epithelial proliferation.

Submandibular Gland Epithelium Within a Collagen Lattice. When submandibular gland epithelial cells are cultured within collagen gel, they exhibit morphogenetic behavior similar to that observed for mammary gland cells. Yang et al. (92) observed that submandibular cell clusters fully surrounded by collagen gel formed elongated branching tubules. The morphology of these outgrowths could be altered by changing the composition of the culture medium. Elongated tubules formed in cultures established using a complex serum-free medium, and in medium containing 0.1% BSA but without 1 µg/ml cortisol. The cysts were roughly spherical in form, and greatly enlarged, when BSA was deleted from the medium. When both BSA and cortisol were absent from the medium, the cells formed neither tubules nor cysts. The authors speculate that the





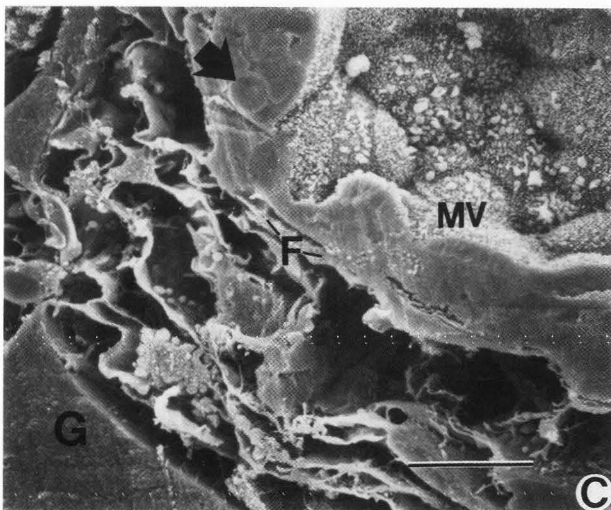


Figure 1. FETAL RAT LUNG ORGANOTYPIC CULTURES

A: This light micrograph shows an intact living culture (7 days in vitro) stained with triphenyl tetrazolium chloride. Numerous irregular shaped cystic structures (arrows) are visible against the unstained Gelfoam collagen sponge substrate. Bar = 200 µm

B: This light micrograph of a tissue section shows an "alveolar-like" structure (ALS) formed by the histotypic aggregation of fetal lung epithelial cells. The wall of this structure contains a number of cuboidal type II pneumocytes (arrows) possessing osmiophilic inclusions. Fibroblastic cells (F) underlie the epithelium. Gelfoam sponge (G). Bar = 50 µm

C: This scanning electron micrograph shows a portion of a cystic "alveolar-like" structure lined by a simple cuboidal epithelium. The apical cell surface possesses numerous microvilli (MV). One cell (arrow) contains cytoplasmic inclusions suggestive of lamellar bodies. Attenuated processes of fibroblastic cells (F) occupy the region between the cyst wall and trabeculae of the Gelfoam sponge (G) substrate. Bar = 5 µm

presence of adhesion-promoting molecules such as laminin or fibronectin in the BSA might influence the interaction between cells and substrate, thus preventing cyst formation. Cyst formation in this system clearly involves fluid accumulation, possibly mediated by hormones in the medium. This concept is supported by the observation that cortisol promotes transepithelial fluid movement by monolayer cultured MDCK cells (65). A possible role for corticosteroids in in vitro epithelial cyst formation is further suggested by the observation that hydrocortisone induces cyst development within renal tubules of fetal mouse kidney organ explants (3) (discussed below).

Prostatic Epithelium Forms Epithelial Cysts in Collagen Gel Primary Culture. Kawamura and Ichihara (40) observed epithelial cyst formation by cells of dissociated rat ventral prostate seeded to collagen gel. Cells suspended in collagen showed vigorous proliferative activity to form multicellular aggregates that developed three-dimensional "acinar-like" form by 10 days in vitro. The cells were polarized with apical surface facing the lumen.

Vaginal Epithelium Embedded Within Collagen Gel. Vaginal epithelial cells also exhibit lumen formation when explanted to collagen gel. Iguchi et al. (38) prepared small pieces of mouse vaginal epithelium, stripped cleanly from the subjacent stroma, for culture within or atop collagen gel. Cells embedded within collagen formed tubular outgrowths after 12 h. The cells of tubules were polarized with apical surface facing the lumen. These highly epithelial cell-enriched primary cultures appeared to form histotypic structures in the absence of fibroblastic cells. Cultures grown atop the collagen gel did not form tubules but instead grew as monolayers. These cultures formed a keratinized stratum corneum when raised under conditions in which the collagen gel was released from the dish to float near the air-medium interface (floating collagen gels). Fully submerged monolayers did not keratinize. Micrographs indicate that the cells of tubular structures, likewise, did not keratinize (38). This paper suggests, therefore, that both tubule formation and a morphologic expression of differentiation (keratinization) are influenced by physical culture conditions.

In Vitro Follicle Formation by Cultured Thyroid Epithelium. Thyroid cells in culture have provided a number of valuable model systems to study a variety of aspects of epithelial cell behavior including in vitro follicle (or cyst) formation. Hilfer (35) was the first to demonstrate that dissociated thyroid epithelial cells retain the capacity to form histotypic follicles in vitro. He devised a system to test the influence of environmental factors, including the role of the thyroid fibroblast, on in vitro follicle formation. In this system, Hilfer dissociated embryonic chick thyroid with trypsin and versene, then seeded the cell suspension to the surface of lens paper rafts (a substrate that does not promote cell attachment and spreading) positioned at the air-medium interface. Cells cultured under these conditions formed three-dimensional follicles that accumulated PAS positive colloid and synthesized thyroid hormone precursors. Hilfer found that if the thyroid epithelium was first seeded to substrate-dependent culture the cells lost the ability to form in vitro follicles when subsequently dissociated and seeded to aggregate culture. However, in vitro follicles did form if thyroid fibroblasts were combined with the epithelium harvested from monolayer culture. This suggests a mesenchymal influence on epithelial behavior.

Thyroid Follicle Fragments in Suspension Culture. Thyroid cells in suspension culture provide a fascinating system for the study of epithelial cell behavior. Nitsch and Wollman (76) demonstrated that fragments of rat thyroid follicles isolated by digestion with collagenase form in vitro follicles when maintained in fluid suspension. In their preparation, the initial cell isolate consisted of clusters of cells derived from broken follicles. Single cells were excluded by filtration-separation. Thyroid cell clusters were then cultured in fluid medium within dishes coated with agarose to prevent cell attachment. They observed that by one day in culture complete follicles had formed in which a polarized simple epithelium enclosed a lumen filled with PAS positive colloid material. The ultrastructure of in vitro follicles showed characteristics of the unstimulated thyroid in vivo. The cells had apical tight junctions and microvilli bordering the lumen. Lysosomes occupied the basal cytoplasm. Treatment of in vitro follicles with thyrotropin caused the cells to exhibit morphology of the stimulated thyroid. In response to thyrotropin administration lysosomes migrated to the apical cytoplasm, pseudopodia formed at the apical surface and colloid was depleted from the lumen. Nitsch and Wollman (77) also observed that mitotic activity of these cultured thyrocytes was stimulated by thyrotropin administration. Thus, thyroid follicles cultured in suspension were shown to exhibit characteristics of the thyroid in vivo.

This culture system has further been used to examine a variety of fundamental problems in epithelial biology. Nitsch and Wollman (76) observed that in vitro follicles under routine media conditions (medium F12, 0.5%FBS; supplemented with insulin, transferrin and trace elements) did not possess a basal lamina. Subsequently, Garbi and Wollman (28) found that basal lamina formation by these cells could be induced by the addition of soluble extracellular matrix (ECM) components to the medium. They observed that basal lamina formed in response to laminin but not type I collagen. Laminin and type I collagen together, however, resulted in deposition of a thicker basal lamina. In related studies, Garbi et al. (26) examined the role that ECM components play in the maintenance of epithelial polarity within in vitro follicles. They observed that raising the concentration of

fetal bovine serum in the medium from 0.5 to 5.0% caused follicles to reverse polarity so that the apical cell surface faced the surrounding medium. They tested a variety of ECM components and matrices and found that culture within type I collagen gel or fibrin clot stabilized follicles in an apex-in configuration in the presence of elevated serum. Suspension culture in the presence of laminin, cellular or plasma fibronectin, gelatin, soluble type I collagen, methylcellulose or agarose did not stabilize polarity, nor did culture within an agarose gel.

Garbi and Wollman (29) have characterized the ultrastructure of apex-out *in vitro* thyroid follicles, while Nitsch and Wollman (78) have documented the morphological changes that take place during the process of polarity reversal. When *in vitro* thyroid follicles undergo polarity reversal, apical surface features form at the cell surface facing the medium, cytoplasmic organelles move away from the luminal surface and tight junctions form at the newly formed apical surface. Nitsch and Wollman (78) indicated that tight junctions and microvilli were not abolished from the luminal surface until cytoplasmic organelles had redistributed. Complete reversal of cell polarity was accompanied by loss of colloid from the lumen. Apex-out follicles also increased dramatically in size. Increase in follicle diameter was accompanied by cell attenuation. Although some cells apparently retained cuboidal morphology, many cells became highly attenuated, and many of these lost apical and lateral-basal appendages. Garbi and Wollman (29) report some further observations on the behavior of apex-out thyroid follicles. They purposely punctured highly expanded follicles with a sharp probe. Punctured follicles collapsed within seconds and the previously attenuated cells rapidly acquired cuboidal morphology and typical cell surface appendages.

Fluid Accumulation Within *In Vitro* Thyroid Follicles. The accumulation of fluid within the lumen of apex-out *in vitro* thyroid follicles likely involves regulated transepithelial transport. Nitsch and Wollman (79) have demonstrated that apex-out follicles periodically collapse and re-expand. They used video-enhanced image intensification of a high resolution dark-field image to quantitate changes in follicle diameter with time. They observed that follicles commonly exhibited episodes of rapid shrinkage (within 8 seconds) in which follicle diameter decreased by 10-40%. This was usually followed (10 min lag), by a period of re-expansion or dilation that was linear with time and almost always much slower than the shrinkage phase. The authors suggest that follicle shrinkage is likely caused by the breakage of tight junctions allowing luminal fluid to leak out. Re-expansion of the follicle occurs after tight junctions have been reestablished. This culture system may provide a means to examine aspects of fluid transport by the thyroid epithelium and possibly model fluid accumulation by an epithelial cyst.

***In Vitro* Thyroid Follicles as a Model of Endocytosis/Transcytosis.** Apex-out thyroid follicles prepared from hog thyroid (33) have been used to study the intracellular events in endocytosis and transcytosis. Herzog (34) has demonstrated that transcytosis in cultured thyrocytes is regulated by thyrotropin. These cultures prove to be a superb model with which to follow endosome movement and the trafficking of cell membranes during the transcytosis of macromolecules.

***In Vitro* Thyroid Follicles From Dissociated Cells.** Studies by Mauchamp et al. (54) have demonstrated that dissociated thyroid cells also exhibit the capacity to form *in vitro* follicles in stationary suspension culture. The process of follicle formation, therefore, involves aggregation and cell sorting in the establishment of a polarized epithelium. Mauchamp et al. prepared a single cell dissociate from hog thyroid by sequential treatment with trypsin and EGTA. Cells were plated in non-culture grade polystyrene dishes in stationary culture. Cell aggregation and follicle formation were observed within hours. The polarity of the *in vitro* follicle epithelium was found to be influenced by the composition of the culture medium. Cells cultured in medium (Eagle MEM) containing 10% newborn bovine serum formed apex-out follicles. These were initially irregular in shape but with continued culture (8 days) formed spherical follicles 20-200μm in diameter. The authors indicate that addition of thyroid stimulating hormone, prostaglandin E_1, PGE_2 or gelatin to the medium led to formation of apex-in follicles that exhibited the capacity to concentrate iodine in the lumen.

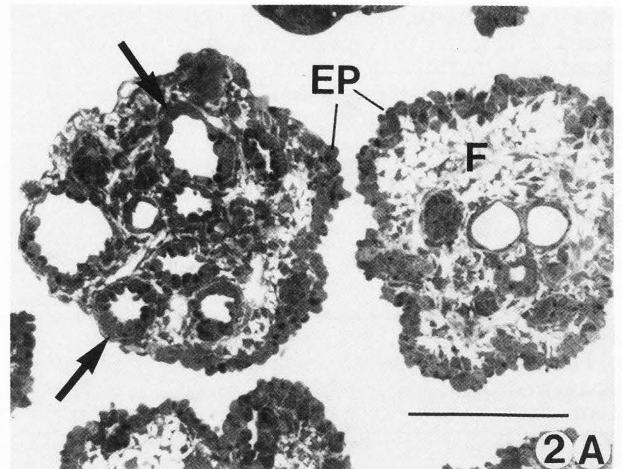
Takasu et al. (88) have examined iodine metabolism and the electrophysiological and morphologic features that characterize cell polarity in this system. They demonstrated that the electrophysiologic polarity of apex-out follicles is reversed compared to apex-in follicles. They note that apex-out follicles do not incorporate iodine, and suggest that cell polarity may influence the capacity for concentration of iodine by the thyroid epithelium.

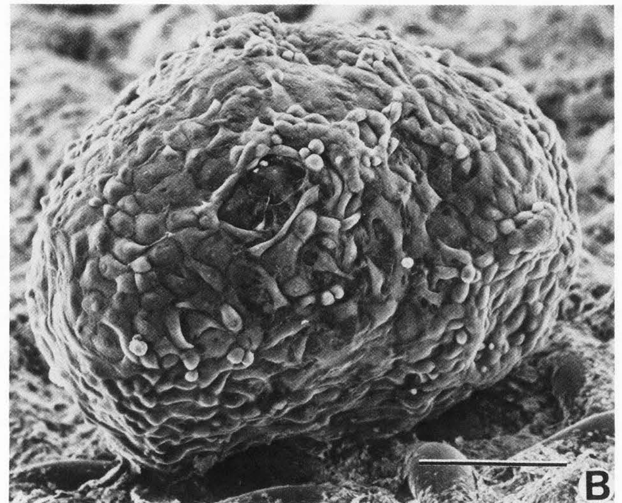
In vitro follicle formation is also exhibited by a clonally-derived thyroid epithelial cell line. Garbi et al. (25) cultured Fischer rat thyroid (FRT) cells within agarose-coated dishes. The dissociated cells formed aggregates (within 8 h) in which the cells subsequently (within 72 h) organized to form a solitary lumen bordered by a simple epithelium. The epithelium was polarized, with its apical surface facing the surrounding medium. When these apex-out follicles were embedded within collagen, gelatin or agarose they reorganized to form multiple lumina in which the cells were polarized with apex-in orientation.

Thyroid Follicle Formation Within Collagen Gel. The histotypic aggregation of dissociated thyroid cells to form polarized *in vitro* follicles has also been shown to occur when the cells are embedded within collagen gel. Chambard et al.

(12) observed that hog thyroid epithelial cells cultured within type I collagen gel first aggregated to form cell cords that subsequently organized to form apex-in follicles. This behavior occurred in medium with 1% serum and appeared to occur more rapidly when serum was raised to 10%. The authors suggest that the process of follicle formation by cells embedded within collagen gel may be similar to the lumen formation that occurs when a confluent thyroid epithelial monolayer atop collagen is overlaid with collagen (discussed below).

Fetal Lung Cells in Rotation-Mediated Aggregation. Another example of histotypic organization of a dissociated cell population to form cyst-like structures is the rotation-mediated aggregation of fetal lung cells. We have observed that enzyme dissociated fetal rat lung cells form multicellular aggregates when seeded to rotation-suspension culture in fluid medium (Fig 2). Dissociated cells are seeded ($1\text{-}5 \times 10^6$ cells/ml) to bacteriological grade dishes which are placed atop a gyrotory shaker within a CO_2 incubator. The gyrotory motion of the platform forces the cells toward the center of the dish, where they contact one another and aggregate. The aggregates are made up of epithelial and non-epithelial (mesenchymally derived) cells. Initially, the cell population is randomly oriented. With continued culture, however, cell type-specific sorting occurs, resulting in formation of small clusters of epithelial cells surrounded by the non-epithelial cell population. These cells subsequently organize to establish a simple





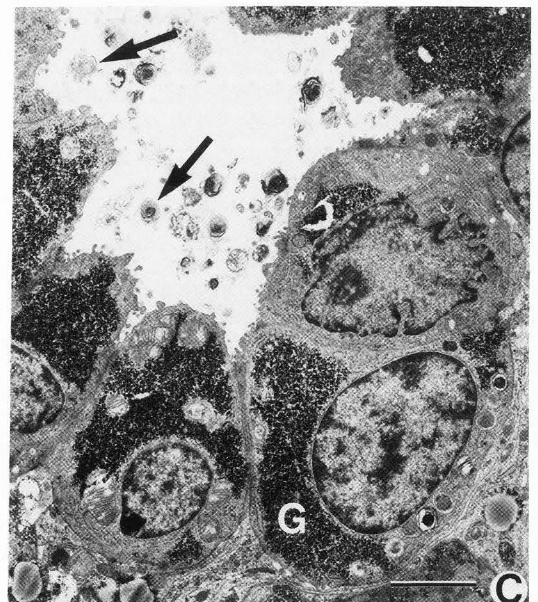


Figure 2. FETAL RAT LUNG AGGREGATES IN ROTATION-MEDIATED AGGREGATION

A: This light micrograph of a tissue section shows portions of several aggregates approximately 48 h following the seeding of dissociated 18 day fetal rat lung cells to fluid suspension atop a gyrotory shaker. The aggregates possess a number of cystic structures (arrows) lined by a simple epithelium. Fibroblastic cells (F) surround these epithelial cysts and lie subjacent to epithelial cells (EP) lining the surface of the aggregates. Bar = 50 µm

B: This scanning electron micrograph shows the form of a fetal lung cell aggregate. The surface of the aggregate is lined by a heterogeneous epithelium. Bar = 5 µm

C: This transmission electron micrograph shows a portion of an epithelial cyst within a fetal lung cell aggregate. These cuboidal epithelial cells are undifferentiated and bear large deposits of glycogen (G). Although the epithelial cells in this field do not appear to possess the structural features of the alveolar type II pneumocyte the cyst lumen contains numerous lamellar bodies and tubular myelin figures (arrows) characteristic of secreted pulmonary surfactant. Bar = 50 µm

epithelium, lining small epithelial cysts. Histotypic cell sorting and cyst formation in these cultures is evident within 24 h. The cysts enlarge somewhat with continued culture but rarely exceed 50-100µm in diameter. Such cysts are frequently irregular in form and numerous cysts may occupy a single aggregate.

Epithelial differentiation occurs in fetal lung cell aggregates. When cultures are established from 15-16 day fetal rat lung in which the pulmonary epithelium is undifferentiated, the initial aggregates that form by 24 h are likewise composed of glycogen-rich undifferentiated cells. By 48 h some ciliated cells are present and small numbers of type II cells bearing osmiophilic inclusions can be identified. The number of ciliated and granulated cells increases with continued culture. Type II cells release lamellar bodies which accumulate within the lumen. Ciliary activity can be observed in living cultures, especially in those aggregates that develop an epithelium lining their outer surface. This culture system which involves cell aggregation, cell sorting, establishment of an epithelium and the formation of an epithelial-mesenchymal interface may provide a means to examine aspects of cell-cell interactions in lung development including events in basal lamina formation.

LLC-PK1 Cysts. The porcine proximal tubule epithelial cell line LLC-PK1 (37) has been observed to form free-floating cysts when cultured under conditions that prevent attachment to the substrate. Wohlwend et al. (91) seeded LLC-PK1 cells to agarose coated dishes. The cells aggregated within several hours and organized within 20 h to form fluid-filled cysts in which the epithelium was polarized with apical cell surface facing the surrounding medium. Cysts subsequently grew to 50-250 µm in diameter. We also have studied the cyst forming behavior of LLC-PK1 cells (61). We have found that apex-out cysts develop spontaneously in substrate-dependent culture (Fig 3) or will form at the site of injury of a confluent monolayer when cells are plated in bacteriological grade polystyrene dishes. Such cysts can be fractured during processing for SEM to provide a view of the lateral-basal cell surface (Fig 3D).

Wohlwend et al. (91) studied the response of LLC-PK1 cysts to embedment within collagen gel. They observed that cysts collapsed upon embedment and that the cells underwent polarity reversal. Polarity reversal in response to contact with collagen at the apical surface appears to be a common behavior exhibited by a variety of epithelial cyst culture systems, many of which are cited above (26,27,43,65,91). In each of these examples, the cells of the apex-out cyst undergo a progressive alteration in the expression of polarized morphology, such that they establish an apex-in orientation. Once polarity reversal is completed, cysts in most systems apparently stabilize but do not continue to grow in diameter. One exception is the growth of MDCK-cysts formed by planting apex-out MDCK-"follicles" within collagen gel (65). We have found that MDCK cells seeded to rotation-suspension culture will aggregate to form small (< 50µm dia) apex-out "follicles". When these "follicles" are embedded within collagen gel the cells undergo polarity reversal. The resultant MDCK-cysts subsequently enlarge and may exceed 1000 µm in diameter (discussed below).

Histotypic "Cystic" Outgrowth of Cells Into a Three-dimensional Matrix

A three-dimensional culture substrate may also serve to promote epithelial outgrowth from an explant or organized cell aggregate resulting in the formation of epithelial cysts, tubules or duct-like structures.

Outgrowth From Peripheral Lung. Rosenbaum et al. (84) seeded collagen-coated sponge with small (< $1mm^3$) fragments of terminal airway prepared from adult rabbit lung. They observed that epithelial cells migrated from the cut edges of the fragments to line the sponge lattice. Cells of the outgrowth formed polarized cysts (apex-in) comprised largely of type II cells. Micrographs in their report show a basal lamina underlying the epithelium, and the presence of lipofibroblasts subjacent to the cells. The authors did not indicate whether these outgrowth cysts increased in size with continued culture.

Angiogenesis In Vitro. Vascular endothelial cells have been shown to form tubular outgrowths from the cut edges of organ explants embedded in plasma fibrin or collagen gels (68,75). Nicosia et al. (75) used the histophysiologic gradient culture system developed by Leighton et al. (49) to demonstrate the angiogenic capacity of the vascular tunica intima. They embedded explants of rat aorta within plasma fibrin and observed the outgrowth of solid cords of endothelial cells radiating into the surrounding substrate. These endothelial cords subsequently canalized to form a patent lumen. Recently, Nicosia (74) applied similar culture conditions (plasma fibrin or collagen gel) to support explants of rat thoracic duct. He observed the formation of endothelial channels that were in continuity with the tunica intima of the explant and, therefore, of lymphatic origin. Montesano et al. (68), also, have used a fibrin or collagen substrate to study the formation of elaborate branched (anastamosed) vascular channels from rat muscle and adipose explants. These structures were histotypic of blood capillaries and possessed a polarized, slightly attenuated epithelium that had the ultrastructural appearance of capillary endothelial cells. The authors indicated that some of the tubular structures were in continuity with the vasculature of the explant. This suggests that the structures were direct outgrowths of the vessel wall.

Mammary Tumor Cells Form Duct-Like Outgrowths in Collagen Gel. Dissociated cells seeded to the surface of collagen gel can form polarized epithelial tubules that invade the collagen lattice. Bennett (8) observed that a rat mammary tumor epithelial line (Rama 25) formed invasive

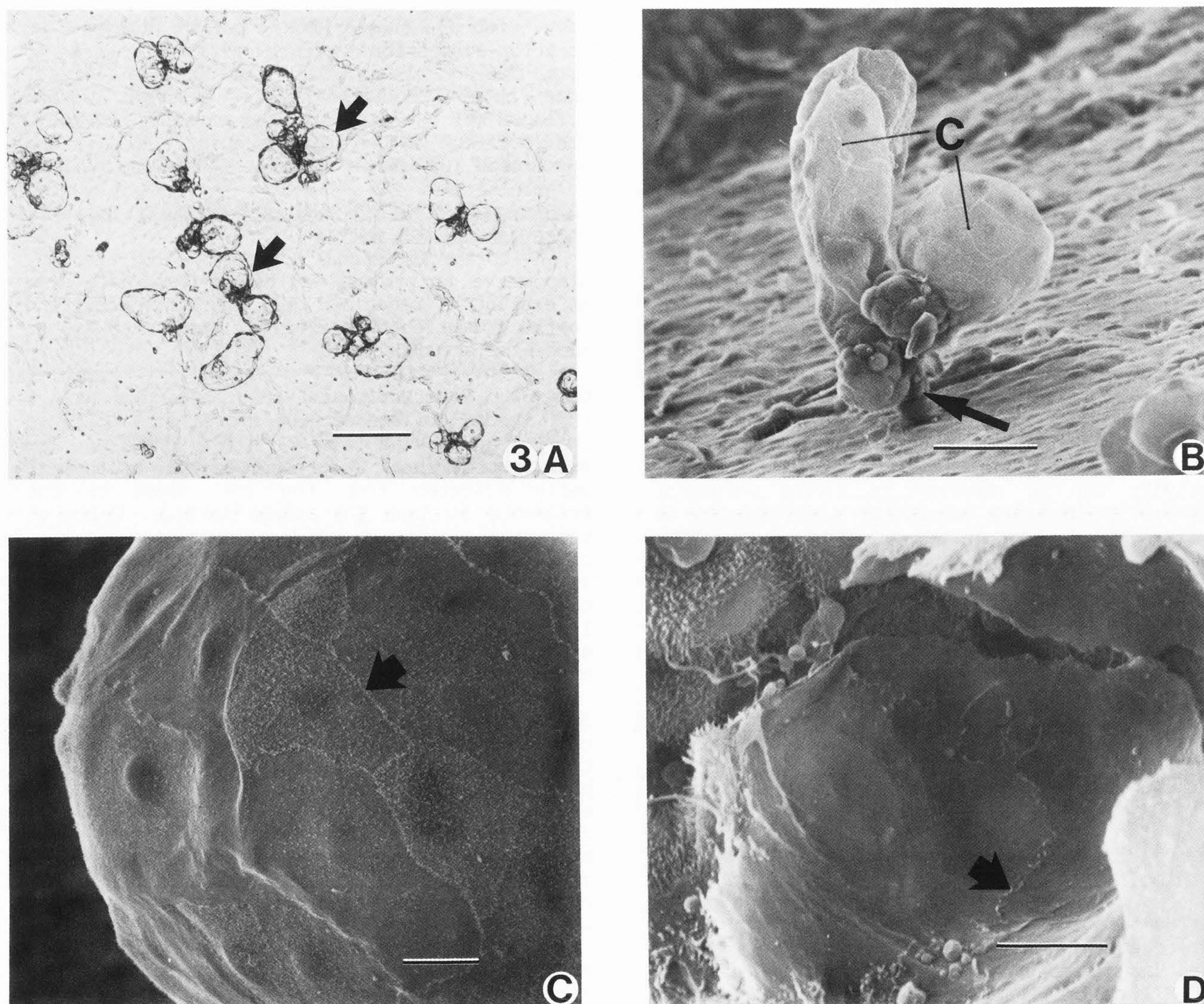


Figure 3. LLC-PK1 CELL CYSTS

A: This light micrograph of a living culture shows numerous irregular shaped cysts (arrows) resting above a substrate-adherent cell sheet. Bar = 200 µm

B: This scanning electron micrograph shows a pair of LLC-PK1 cell cysts (C) similar to those in frame A. These structures are attached to the underlying cell sheet by a narrow stalk (arrow). Bar = 50 µm

C: This scanning electron micrograph shows the apical surface of an LLC-PK1 cell cyst. The cells are highly attenuated. Intercellular margins (arrow) appear distinct. Bar = 10 µm

D: This scanning electron micrograph shows the interior aspect of an LLC-PK1 cell cyst that was fractured during processing. Although intercellular boundaries are visible (arrow) the basal cell surface appears to lack prominent appendages. Bar = 5 µm

---

blunt duct-like outgrowths that extended within the gel. The Rama 25 cell line exhibited a cobblestone pattern when grown on plastic but formed "hyperplastic-like" papillae and small apex-out cysts when grown atop collagen. Bennett and coworkers (9) further determined that invasive tubule formation was a property of subpopulations of the line. Interestingly, two cloned populations of Rama 25, neither of which showed invasive tubule formation alone, formed duct-like outgrowths when grown in combination. In monolayer culture, the two populations showed

different morphology, one cuboidal and the other elongate. The authors speculated that the elongated cell type may be a myoepithelial cell, and that in vitro tubule formation involves some level of interaction between these cells and glandular epithelial cells.

Ormerod and Rudland (80,81) have shown that the pattern of tubule formation by Rama 25 seeded to the surface of collagen gel (floating collagen gels) is influenced by the collagen concentration and physical form of the gel, and by hormone and growth factor supplements to the medium. They observed that an elevated collagen concentration (0.6% w/v) promoted the formation of long tubules bearing an extensive lumen, while cells cultured in lower collagen concentrations formed narrow tubules and invasive spikes of elongated cells. Spindle-shaped cells also predominated when monolayers of Rama 25 atop collagen gel were overlaid with collagen. They found that the tubules that developed in floating collagen gels commonly followed the path of wrinkles in the gel, and that tubule formation could be stimulated by folding the gel back upon itself. Collagen gels that were prevented from contracting did not support tubule formation. Hydrocortisone and insulin added to serum-supplemented medium stimulated tubule formation. Cholera toxin or epidermal growth factor preferentially stimulated the formation of tubules with a broad lumen. The addition of hyaluronic acid to the collagen gel had an inhibitory effect on the formation of these broad tubules, but did not influence the formation of a population of phenotypically distinct (narrow lumen) tubules that also develop in this system. Gels prepared from human epidermal keratin did not support tubule formation. These data indicate that lumen formation by the Rama 25 line is influenced by both the physical and macromolecular composition of the extracellular matrix.

Reorganization of an Epithelial Monolayer to Form Cystic Structures

Several culture systems have been described in which a confluent population of cells in monolayer culture responds to manipulation of the culture environment by forming epithelial cysts.

Thyrotrophin Stimulates Thyroid Epithelial Cyst Formation. Thyroid epithelial cells seeded to monolayer culture can be induced to form three-dimensional follicles by the addition of thyrotrophin to the medium. In companion papers, Lissitizky et al. (51) and Fayet et al. (23) demonstrated that thyrotrophin stimulates the synthesis of RNA that is likely involved in the production of proteins associated with cell-cell recognition. Under the influence of thyrotrophin, cells in substrate-dependent monolayer culture reorganized to form lumena bordered by microvilli, and with apical tight junctions. In unstimulated cells, intracellular organelles occupied a perinuclear position. In vitro follicles showed an apical to basal distribution of organelles characteristic of follicular cells in vivo.

In Vitro Thyroid Cysts Form in Response to Collagen Overlay. Chambard et al. (12) overlaid a confluent monolayer of thyroid epithelial cells with type I collagen gel. Under these conditions, the monolayer reorganized to form numerous small follicular structures in which the cells were polarized with apical surface facing the lumen. This expression of cell behavior appears to be a response to conditions in which the apical cell surface is challenged by an "interstitial environment".

Lumen Formation By Intestinal Epithelial Cells. Montgomery (70) has observed that epithelial cells of dissociated fetal rat small intestine form villus-like multicellular aggregates when seeded to either a plastic or collagen gel substrate. When cultures atop collagen are subsequently overlaid with collagen gel, the cells exhibit a morphogenetic reorganization, to form multiple lumena in which the cells are polarized with their apical surface facing the lumen. The cells bear a microvillus brush border that stains PAS and alkaline phosphatase positive.

Lumen Formation by MDCK Cells in Monolayer Culture. Hall et al. (32) described lumen formation by MDCK cells in response to collagen overlay. They prepared a confluent monolayer of MDCK cells atop a collagen substrate then overlaid the cell sheet with type I collagen gel. The cells of the monolayer responded by reorganizing into numerous multicellular cysts in which apical cell surface faced the lumen. Cell behavior in this system, as in others in which an epithelial cyst is embedded within collagen, indicates that an epithelium is sensitive to the environment at its apical surface. This suggests that epithelial cells can recognize an "interstitial" environment and that an "interstitial" environment is incompatible with the apical cell surface. This also demonstrates that cells have the capacity to reorganize the cell surface so that lateral-basal membrane interfaces with the interstitium.

Vascular Endothelial Cells Form Capillary-like Networks in Response to Collagen Overlay. Montesano et al. (69) have observed that clonally derived adrenal cortical capillary endothelial cells grown atop a collagen substrate will undergo histotypic organization to form capillary-like networks when overlaid with collagen gel. The cells were polarized with apex-in orientation and possessed a basal lamina at the interface with the surrounding collagen gel. This work complements studies in which vascular outgrowths form at the cut surface of organ explants (discussed above) and demonstrates that endothelial cells retain in vitro angiogenic capacity following repetitive subculture.

Other Examples of Epithelial Cyst Formation From Multicellular Aggregates

Additional in vitro systems have been described in which epithelial cells undergo a spontaneous (de novo) "morphogenetic" reorganiza-

tion that results in cyst formation. These include cultures of the preimplantation mammalian embryo, aggregate cultures of teratocarcinoma cells and cultures in which epithelial fragments from various sources are explanted to submersion culture.

Culture of Preimplantation Mouse Embryos; Blastocyst Formation <u>In Vitro</u>. Blastocyst formation during embryogenesis involves epithelial cyst formation. Cavitation of the morula is a dynamic morphogenetic event in which blastomeres organize to surround the developing blastocoel. The first polarized simple epithelium to develop in the embryo is the wall (trophoblast) of the newly formed blastocyst. The cells of the trophoblast (trophectoderm) are joined by tight junctions and are polarized so that the apical surface faces away from the lumen. The events that occur leading to blastocyst formation are critical to subsequent development, and are not fully understood. Questions remain regarding fundamental events such as cell polarization in the morula, cavitation and the production of blastocoel fluid, the regulation of cell differentiation and the formation of the pluripotent inner cell mass. Cell culture has been used to examine these and other problems in early development.

Culture conditions have been developed under which embryos at the 4 to 16-cell stage can be nurtured through blastocyst formation. One example of such a system is that described by Shirayoshi et al. (86) for studies on the role of cell-cell contact in the regulation of inner cell mass formation. Mouse embryos at the 4-cell stage were collected, treated with acidic saline to remove the adherent zona pellucida and then transferred to submersion culture within 96-well plates (non-adhesive substrate) containing serum-supplemented medium. Embryos cultured under control conditions over a 48 h period progressed through identifiable developmental stages (8, 16, 32-cell) to the formation of a blastocyst. The authors found that the development of an inner cell mass could be manipulated. Embryos cultured in medium containing a monoclonal antibody that inhibits Ca^{++}-dependent cell-cell adhesion in teratocarcinoma cells did not develop an inner cell mass. Instead, blastocysts developed as simple epithelial cysts containing only trophectoderm. The authors speculated that loss of cell-cell adhesion in response to experimental treatment disrupted communication between cells that was necessary for the differentiation of multiple cell lineages in the embryo.

Cystic Embryoid Bodies Derived From Teratocarcinoma Cells <u>In Vitro</u>. Investigators have demonstrated that cell populations clonally derived from teratocarcinoma cells exhibit morphogenetic behavior to form multicellular cystic structures that resemble the mammalian blastocyst (18,53). Such cultures have been used as <u>in vitro</u> model systems to study various aspects of the cellular events involved in early embryonic development. The morphology of these cystic embryoid bodies is dependent upon the specific cell population of origin and is influenced by culture conditions. Martin et al. (53) have described the formation of cystic embryoid bodies from clonal cell lines derived from a transplantable mouse tumor. These embryonal carcinoma cell lines were propagated as undifferentiated cells by subculturing them atop non-mitotic feeder layers. The cells were then passaged to culture-grade plastic without a feeder layer. Within three days, the cells formed aggregates that were loosely adherent to the substrate. Subsequently, the aggregates were collected and transferred to submersion culture within bacteriological-grade dishes. Under these conditions the cells organized (by 48 h) to form a polarized surface lining epithelium (endoderm). By 4-6 days, cavitation was evident with formation of a fluid-filled cavity. Continued development resulted in the formation of an expanded epithelial cyst (embryoid body) with a wall composed of two cell layers, a visceral and a parietal endoderm. The authors point out that this morphology is not analogous to the structure of the mouse blastocyst which bears a single layer of trophectoderm. Indeed, they stress that the cystic embryoid bodies which formed under these culture conditions did not possess a trophectoderm.

Ducibella and coworkers (18) have studied the formation of embryoid bodies from a human teratocarcinoma cell line. They observed that the HT-H line cultured on plastic exhibited spontaneous formation of embryoid bodies at confluency. The cells formed aggregates attached to the substrate by a narrow stalk. These "morula-like" bodies eventually broke loose to float freely within the medium. The aggregates subsequently cavitated to form a fluid-filled cyst lined by a simple epithelium, polarized with lateral-basal surface bordering the lumen. The authors point out that human embryoid bodies possessed a number of characteristics in common with the preimplantation embryo. These included the presence of an eccentrically positioned mass of inner cells and a polarized simple epithelial wall that possessed apical tight junctions.

Human Endometrial Explants. Kirk and Alvarez (43) observed the development of polarized epithelial cysts in explant cultured fragments of human endometrium. They explanted small tissue fragments (1-3mm^3) to submersion culture in hormone-supplemented serum-free medium. Following one week in culture, the explants formed small epithelial cysts or vesicles that subsequently separated from the explant to float freely in the medium. Free floating vesicles increased in size to reach 500-1000 µm in diameter, and remained morphologically stable for up to three months. These vesicles were spherical to irregular in form and consisted of a heterogeneous simple cuboidal to squamous epithelium polarized with the apical surface facing the surrounding medium. Mitotic activity in these cultures was hormone sensitive, depressed by treatment with progesterone or by progesterone and estradiol in combination. The authors point out that the extended <u>in vitro</u> life span and hormone

sensitivity of cells cultured using serum-free medium in this system may offer advantages for experimental studies of the uterine epithelium.

Choroid Plexus in Submersion Culture. Agnew et al. (1) used similar culture conditions to study the behavior of explants derived from rat choroid plexus. These explants formed bulbous fluid filled vesicles at their surface. Vesicles were made up of a simple polarized cuboidal epithelium with the histotypic orientation of apical surface facing the surrounding medium. These cultures were morphologically stable for several weeks and incorporated labeled leucine and thymidine, indicating active protein and DNA synthesis.

Urinary Bladder Tumors. Tchao and Leighton (90) have observed epithelial cyst formation by fragments of human urinary bladder tumor collected at biopsy by transurethral resection. Multiple explants ($1mm^3$) were placed in submersion culture within a flask atop a gyrotory shaker. Cyst formation occurred usually within 4-5 days and was stimulated by the addition of dibutyryl cAMP to the medium. The authors observed two types of cysts, those that were lined by a simple epithelium in which the cells were polarized with apical surface facing the surrounding medium, and multilayered cysts bearing apical tight junctions adjacent to the lumen. The authors also demonstrated a correlation between the histological grade of the tumor and the development of epithelial cysts _in vitro._ Low grade tumors exhibited cyst formation while high grade tumors did not.

Cyst Formation In Explant Culture

There are a number of examples of explant culture systems in which epithelial cyst formation occurs. Cyst formation in organ explants usually involves the enlargement of an existing epithelial structure such as a tubule or glandular acinus (or alveolus) and always involves fluid accumulation. In some systems, cyst enlargement is accompanied by structural changes of the lining epithelium and/or the surrounding parenchyma.

Cyst Formation by the Chick Mesonephros. Perhaps the first demonstration of epithelial cyst formation _in vitro_ appeared in a report by Chambers and Kempton (13) published in 1933. They performed a study to examine secretory function of the renal tubule, using explant cultured chick mesonephros as the model. They prepared tissue fragments of 9 day embryonic chick kidney (mesonephros) and cultured these explants by the hanging drop method. They observed that cut ends of the renal tubule healed and that proximal tubule segments of the nephron showed signs of dilation by 24 h. Cystic tubules continued to dilate and achieved a diameter many times that of the tubule at explantation. The epithelium of extremely dilated tubules was attenuated. The authors indicate that these cells returned to cuboidal form after lumenal fluid was aspirated with a micropipet. The authors demonstrated secretory function in these cultures by observation of lumenal accumulation of phenol red added to the culture medium. Only proximal tubules accumulated phenol red and only proximal tubules formed cysts.

Fetal Rat Lung in Submersion Culture. Our laboratory has observed that epithelial lumen enlargement (i.e., cyst formation) is a common feature of a variety of systems for culture of the fetal lung and trachea (56-60). The pattern of _in vitro_ development exhibited by the fetal lung, and the degree of lumen enlargement that occurs is influenced by both the age of the tissue at explantation and the physical conditions of the culture environment. In conventional interface culture in which explants (lung fragments or intact lobes) are incubated supported at the gas-fluid interface, fetal lung maintains a glandular morphology and tends to attenuate atop the substrate (64). Enlargement of the lumen of the developing pulmonary space (airway/alveolar space) is minimal and most regions of the parenchyma retain a glandular or acinar appearance. However, when fetal lung (intact lung or isolated lobes) is cultured fully submerged in medium it develops as an expanded hollow (fluid filled) organ (58) (Fig 4). The explant accumulates a substantial amount of fluid that distends the pulmonary space. The pattern of the developing airways is lost and the pulmonary parenchyma comes to occupy only a narrow margin at the periphery of the organ. This degree of expansion of the explant not seen in culture at the gas-fluid interface may occur in part because fluid tension forces at the surface of the explant are minimal. This response to culture conditions is also influenced by the age of the fetal tissue. Rat lung only in early stages of development (fetal days 13-15) exhibit extensive lumenal expansion in submersion culture.

Although _in vitro_ development in submersion culture disrupts the form of the developing lung it does not interfere with epithelial differentiation. Both airway and alveolar cell types differentiate in these cultures. This form of explant culture of the fetal lung may provide a means to examine cellular events in development such as the role of the epithelium in the production and modification of pulmonary fluid. We have used these cultures to demonstrate that cytodifferentiation _in vitro_ occurs in the absence of exogenous hormones (59). In addition, fetal lung cultures in this form present an ideal specimen for analysis of cell surface changes by SEM in the development of both the airway and alveolar epithelium (58).

"Alveolization" of Fetal Rat Lung Explants Embedded in Collagen Gel. Another example of how physical culture conditions may influence fetal lung explant development is seen in studies we have performed using medium hydrated collagen gel as a substrate in submersion culture. We prepared tissue fragments ($1mm^3$) of 16-18 day fetal rat lung and embedded these explants in type I collagen gel (57). The explants were then submerged in fluid medium. As with intact fetal lung in submersion culture, the collagen embedded

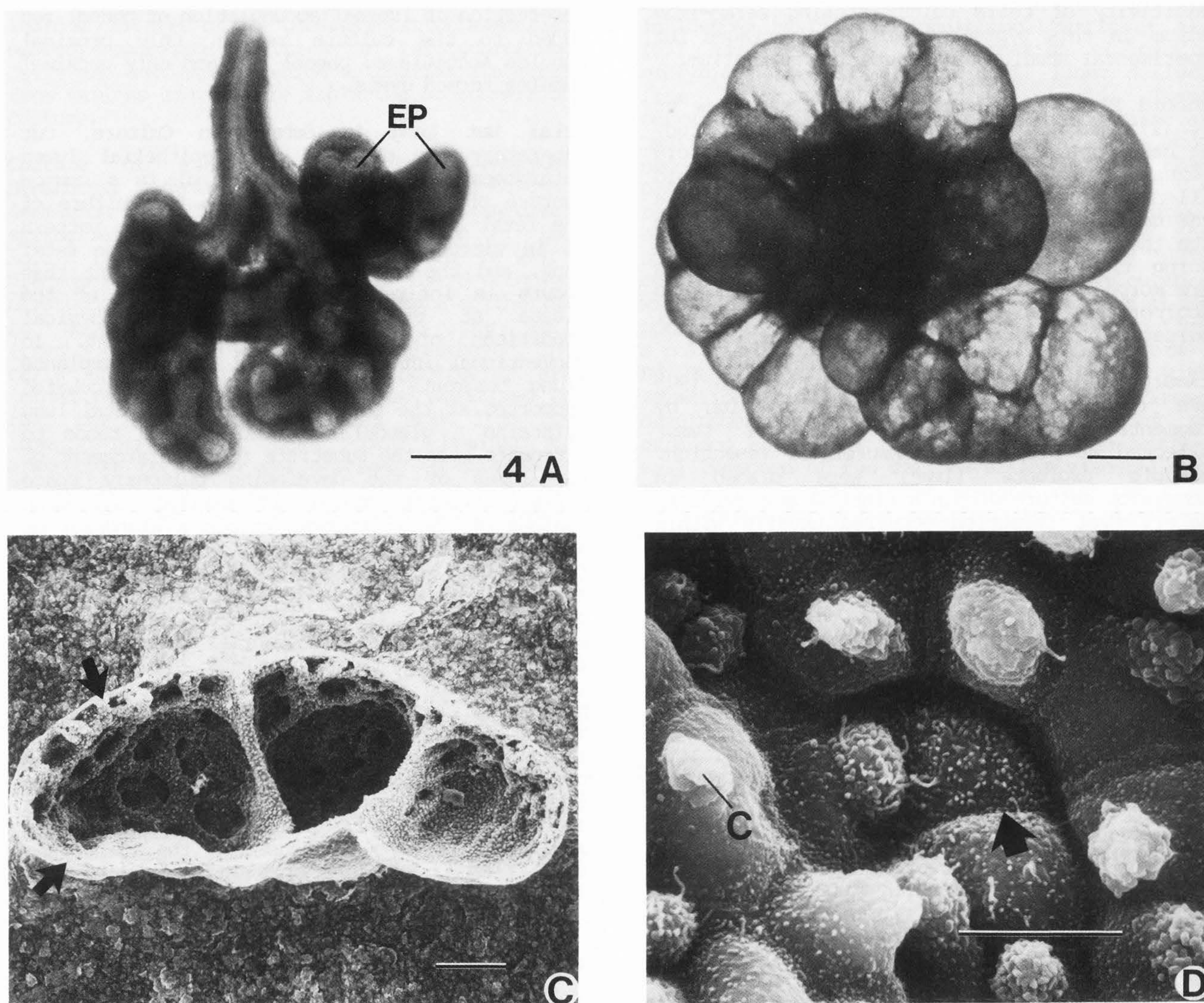


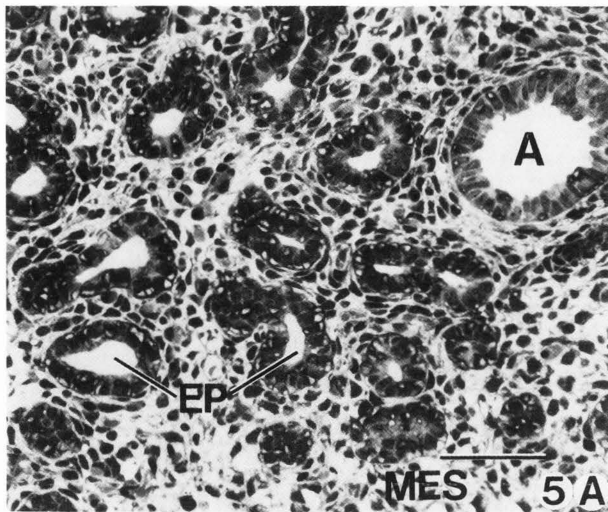
Figure 4. SUBMERSION CULTURED FETAL RAT LUNG

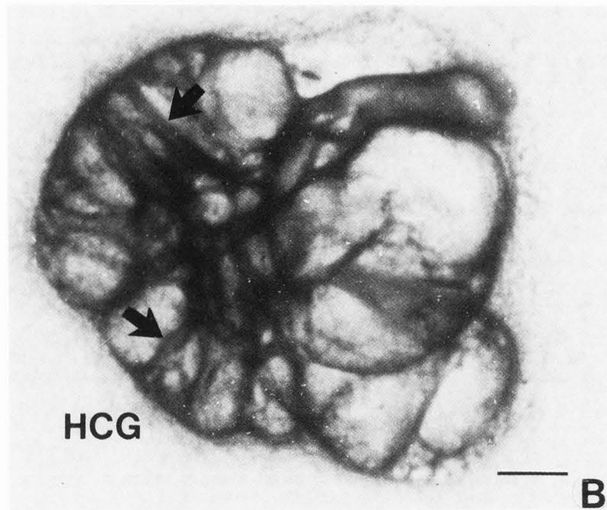
A: This light micrograph shows the morphology of an isolated intact 16 day fetal rat lung immediately following dissection. Individual lobes are distinct and the simple branching pattern of the pulmonary epithelium (EP) is visible. Bar = 500 µm

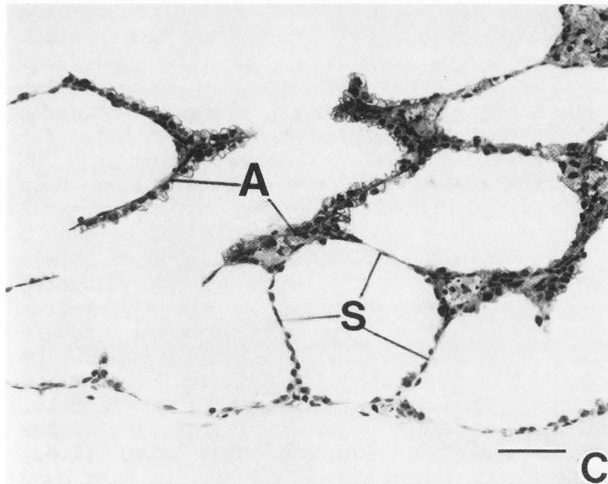
B: This light micrograph shows a 16 day fetal rat lung following 7 days in submersion culture. The explant has expanded dramatically due to fluid accumulation and has lost its organotypic form. Bar = 500 µm

C: This scanning electron micrograph shows a cultured (5 days in vitro) fetal lung cut to reveal the interior of the explant. The lung parenchyma (arrows) occupies only a narrow margin at the periphery of the explant. Bar = 100 µm

D: This scanning electron micrograph shows the surface features of cells from a cultured explant. The majority of cells in this region of the explant are still undifferentiated. The prominent apical cytoplasmic cap (C) seen atop most of these cells will be lost during subsequent differentiation. A cell at the center of the field (arrow) is likely at a more advanced stage of differentiation and bears a population of apical microvilli. Bar = 5 µm







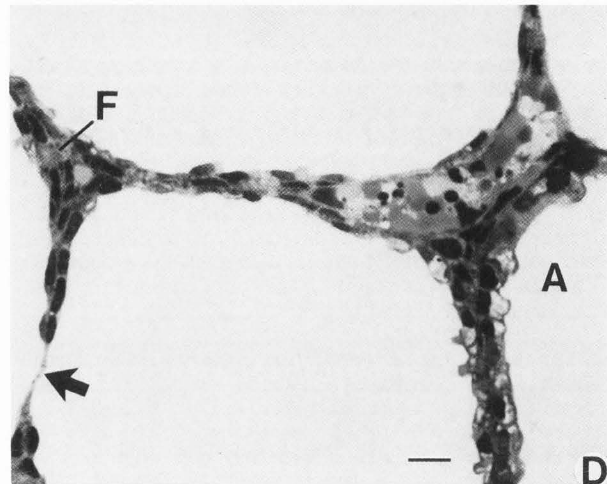


Figure 5. "ALVEOLIZATION" OF FETAL LUNG CULTURED WITHIN COLLAGEN GEL

A: This light micrograph of a tissue section from 18 day fetal rat lung shows the structure of the pulmonary parenchyma at the time of explantation to culture. The tissue is glandular in appearance. Epithelial elements (EP) are surrounded by lung mesenchyme (MES). Airway (A). Bar = 50 µm

B: This light micrograph shows a lung explant cultured for 5 days embedded within collagen gel (HCG). Although some areas are highly expanded, much of the explant has retained a reticular network of tissue septae (arrows). Bar = 500 µm

C: This light micrograph of a tissue section shows the structure of a fetal lung explant 5 days in culture. The lung parenchyma now occupies only narrow septae (S) separating adjacent open (fluid-filled) spaces. Tissue bearing a thicker wall (A), at the center of the field, is derived from a pulmonary airway. Bar = 50 µm

D: This light micrograph of a tissue section shows the histologic structure of tissue septae from a 5 day explant. The septae are extremely narrow. In some areas (F) they have a fibroblastic core. The lining epithelial cells are low cuboidal to squamous in form. In some regions (arrow) continuity of the epithelium cannot be resolved. Cuboidal cells line an airway (A). Bar = 10 µm

explants accumulated fluid and exhibited expansion of the developing pulmonary space (Fig 5). These explants, however, showed unique morphogenetic behavior. The expansion of glandular elements of the parenchyma was accompanied by septation and the formation of structures reminiscent of the alveolar wall. The surrounding collagen gel lattice restricted the overall expansion of the explants. This apparently preserved parenchyma throughout the explant but induced changes within this tissue. It is possible that the wall of the developing alveolar spaces was then under increased lumenal pressure leading to compression of the interstitium and attenuation of the lining epithelium. Not all epithelial cells in these cultures attenuated. In some areas, ciliated cells characteristic of the peripheral airways were observed. Many of the epithelial cells lining the septae did attenuate. These cells maintained tight junctions with adjacent cuboidal or attenuated cells and commonly had narrow cytoplasmic processes (<0.5μm in thickness) possessing numerous pinocytotic vesicles. In this respect, the cells bore a striking resemblance to the attenuated type I alveolar epithelial cell. It is not clear whether the processes of septation of the parenchyma and attenuation of the lining epithelium in these cultures is at all related to the developmental events involved in the morphogenesis of the alveolar wall. What is clear is that fluid accumulates in these cultures. We believe that this accumulation of fluid causes the epithelial elements to expand in a manner suggestive of epithelial cyst enlargement.

Tubular Explants of Fetal Rat Trachea. The fetal trachea also exhibits lumenal expansion when maintained in submersion culture (Fig 6). We have cultured 13-16 day fetal rat trachea intact or as short tubular explants (56). The cut ends of the explants heal, thus, enclosing the lumen. With continued culture, the explants accumulate fluid and expand, sometimes dramatically. Tracheal explants undergo differentiation of both the lining epithelium and elements of the mesenchymal wall. Cartilage bars form and smooth muscle develops as a prominent feature of the original posterior tracheal wall. That portion of the tracheal wall bearing developing cartilage (pars cartilagina) appears to be structurally rigid, while the remaining muscular portion of the wall (pars membranacea) becomes extremely thin. The form that these explants take make them well suited for analysis of mucociliary epithelial development by scanning electron microscopy (56).

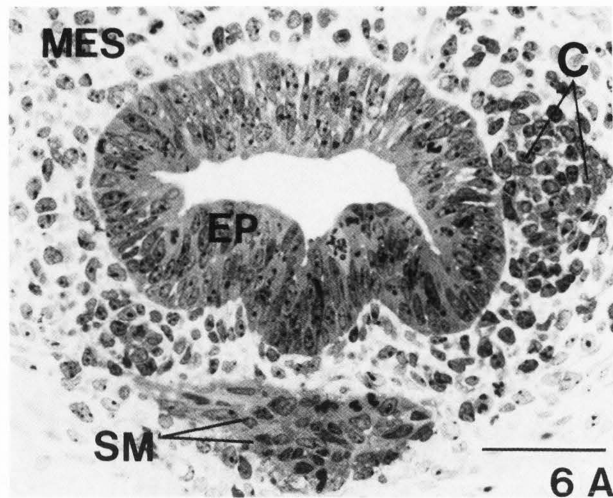
Application of Epithelial Cyst Culture to Renal Cystic Disease

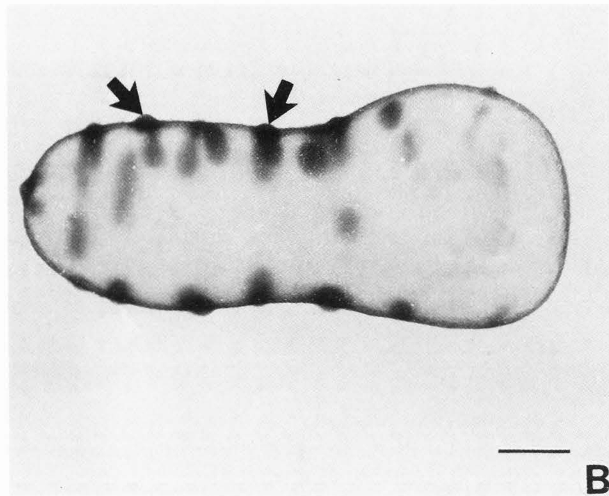
Pathologic cyst formation in the kidney is a characteristic of renal cystic disease (10). Various forms of renal cystic disease have been defined including polycystic kidney disease (PKD) (10). PKD is genetically transmitted (82) and at least two forms are recognized, autosomal dominant PKD (ADPKD) and an autosomal recessive infantile form (10). The predominant lesion in PKD is tubular cyst formation. Renal cyst enlargement has been shown to involve epithelial hyperplasia accompanied by fluid accumulation. The mechanism responsible for cyst formation is not known but may be multifactorial. A variety of animal models have been developed to allow experimental study of PKD (11,20-22,30,39,42,85). Each has proven useful to help better understand the epigenetic factors that influence cyst development. None, however, has adequately provided a means to investigate the apparent relationship between abnormal epithelial hyperplasia, fluid accumulation and cyst growth. *In vitro* cell and explant culture systems may be a means to do this.

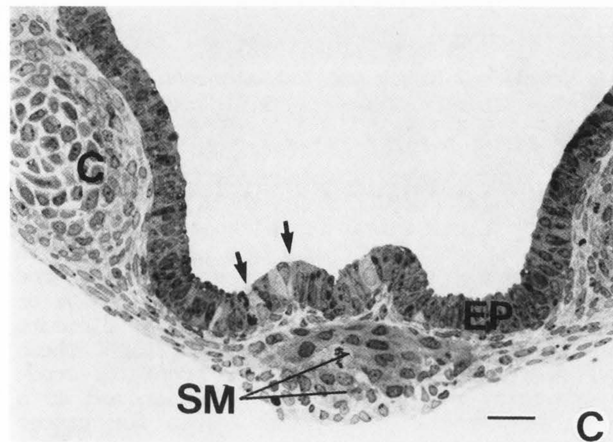
Several culture systems have been reported that offer great promise toward improving our understanding of the cellular events in renal cyst enlargement (Table 3).

Mouse Metanephric Organ Culture. Avner et al. (3-5,7) have described a fetal mouse metanephric organ culture system in which tubular cyst formation can be induced by altering the composition of the culture medium. Kidney slice explants were maintained at the gas-fluid interface in serum-free medium F12 containing insulin, T_3, hydrocortisone, PGE_1, transferrin and sodium selinite. Avner et al. (4) demonstrated first that addition of cis-dichlorodiammineplatinum II (CP) to this hormone-supplemented serum-free medium caused a progressive dilation of the renal tubule over a 120 h culture period. Subsequent studies showed that elevated hydrocortisone (3) or elevated L-3,5,3'-triiodothyronine (7) (in serum-free medium as above) induced proximal tubule dilation. Tubule dilation was preceded by ultrastructural changes of the epithelium including expansion of intercellular channels, suggesting some effect involving the lateral-basal cell membrane. This model (i.e., elevated hydrocortisone or T_3) was in turn used to study the relationship between Na^+/K^+ ATPase activity and tubule dilation (3,7). Tissue assay for Na^+/K^+ ATPase was higher for hydrocortisone or T_3-treated (*in vitro* cystic) than for control cultures, and preceded morphological changes characteristic of cyst formation in this system. The addition of ouabain to the medium prevented cyst formation without disrupting metanephric development in the cultures. The authors concluded that elevation of Na^+/K^+ ATPase is a primary event in tubule dilation in this model (3,7). This is compelling experimental evidence to suggest that an alteration in the cellular apparatus involved in ion movement may be linked to fluid accumulation in the enlarging tubular cyst.

In a related culture system, Avner et al. (6) further examined how environmental factors influence the expression of cystic disease *in vitro.* They studied the CPK mutant strain of the C57BL/6J mouse, a model of autosomal recessive PKD. Affected CPK offspring develop cystic abnormalities of the kidney soon after birth, and succumb to renal failure usually within one month. Avner et al. established kidney explants







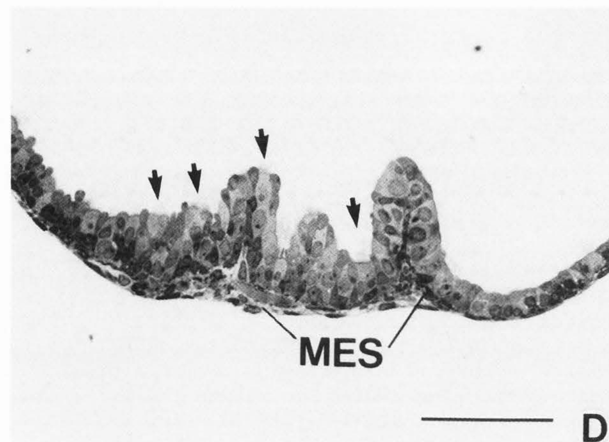


Figure 6. TUBULAR TRACHEAL EXPLANTS

A: This light micrograph of a tissue section shows the morphology of 16 day fetal rat trachea at the time of explantation to culture. The epithelium (EP) is undifferentiated and appears to be pseudostratified in form. The surrounding mesenchyme (MES) shows evidence of differentiation to smooth muscle cells (SM) forming part of the posterior wall, and to a population of primitive chondrocytes (C) of a developing cartilage bar. Bar = 50 µm

B: This light micrograph shows the form of a living tubular tracheal explant 4 days in submersion culture. The tracheal lumen is highly expanded due to fluid accumulation. Portions of developing cartilage bars are visible (arrows). Bar = 500 µm

C: This light micrograph of a tissue section shows the structure of a portion of the wall from a 4 day tracheal explant. The epithelium (EP) shows evidence of continued differentiation, with the presence of a small number of ciliated cells (arrows). A bundle of smooth muscle cells (SM) remains a prominent feature of the posterior wall of the explant. Also, a focus of cells subjacent to the epithelium are identifiable as chondrocytes (C). Bar = 50 µm

D: This light micrograph of a tissue section shows the posterior wall of a tracheal explant 8 days in culture. The epithelium has further differentiated and numerous ciliated cells (arrows) are present. The mesenchyme (MES) is highly attenuated, likely in response to enlargement of the explant due to continued fluid accumulation. Bar = µm

TABLE 3

CELL & EXPLANT CULTURE MODELS OF RENAL CYST ENLARGEMENT

		References
Mouse Metanephric Organ Culture	Progressive dilation of proximal tubule, induced by cis-dichlorodiammineplatinum II	(4)
	Hydrocortisone or T_3 induced proximal tubule dilation preceded by ultrastructural changes in lateral-basal membrane, morphological changes preceded by elevated Na^+/K^+ ATPase	(3,5,7)
CPK Mouse	Cystic abnormality present at birth regresses in explant culture	(6)
Human ADPKD Kidney	Cyst wall explants develop solitary mural cysts	(63)
MDCK-CYSTS	Morphogenetic clonal growth of MDCK cells (strain 2) seeded within collagen gel, cyst fluid accumulation and cyst enlargement influenced by culture conditions	(62,66)

from CPK cystic newborns. When explants were cultured in serum-free medium (as above, but without hydrocortisone) the tubular cysts regressed. Control cultures did not develop cysts. The authors suggest that cyst regression _in vitro_ may be analogous to regression of the cystic lesion produced in chemically induced models of renal cystic disease _in vivo_ when the cystogen is witheld from the diet. The authors further suggest that cyst regression _in vitro_ demonstrates environmental modulation of the expression of genetically determined PKD.

Human ADPKD Cyst Wall in Explant Culture. Our laboratory has observed tubular cyst formation and growth within explants of human ADPKD cyst wall epithelium (63). Renal cysts that develop within the outer cortex of the polycystic kidney commonly protrude above the surface of the kidney. These surface cysts often have a very thin wall that makes an advantageous specimen for explant culture. We have used explanted segments of cyst wall as a source for the outgrowth of cyst lining epithelium. We excise pieces of cyst wall and embed these explants within collagen gel. The cyst epithelium migrates onto the surface of the collagen and can then be propagated. Cyst wall explants frequently contain segments of renal tubule, some of which have normal morphology and others that are dilated. We have observed that upon extended culture (>2wk) some explants develop cysts. These mural cysts are usually less than 200 μm in diameter, although some cysts are larger. Mural cysts appear to be solitary and are not in continuity with the surface epithelium of the cyst wall explant. The cells lining mural cysts are cuboidal but usually do not appear to be morphologically characteristic of identifiable renal cell types. In this respect, they resemble cells of the lining epithelium of ADPKD surface cysts (31). We do not know if mural cyst formation _in vitro_ represents the enlargement of ADPKD-affected renal tubules or perhaps involves the cystic enlargement of segments of normal nephron. Clearly, however, fluid accumulation is involved.

The MDCK-Cyst Model of Epithelial Cyst Enlargement. Leighton (46) observed that MDCK cells seeded to plasma fibrin or collagen-coated sponge (49) formed multicellular cysts or follicles in which the epithelium was polarized with the apical surface facing the lumen. The glandular or cystic histological structure of these elements was suggestive of adenomatous tumor (48). These cultures appeared promising as a model for study of cellular events in tumor formation and as a possible means of screening agents for cancer chemotherapy (45). We similarly have studied cyst development by the MDCK line (62). Our investigation has focused on the cellular events involved in cyst formation and growth. We believe that the MDCK-cyst may be an appropriate model to examine the cellular level mechanism(s) involved in tubular cyst enlargement in renal cystic disease.

We have found that when MDCK cells are seeded within collagen gel, a sub-population of these cells exhibit clonal growth to form spherical multicellular cysts (61,62). We have termed this process "morphogenetic clonal growth" to indicate the establishment of a polarized epithelium organized in histotypic form.

The cells of MDCK-cysts are polarized (Fig 7). They bear apical microvilli, a solitary cilium and apical tight junctions. The cells produce a scant (apparently incomplete) basal lamina that stains with ruthenium red. The basal cell surface possesses few appendages and rests in contact with the collagen fibril lattice of the surrounding substrate. Contact with collagen at the basal surface is necessary for maintenance of polarity. MDCK-cysts harvested by dissection or digestion with collagenase and returned to stationary suspension culture will collapse and undergo polarity reversal (65).

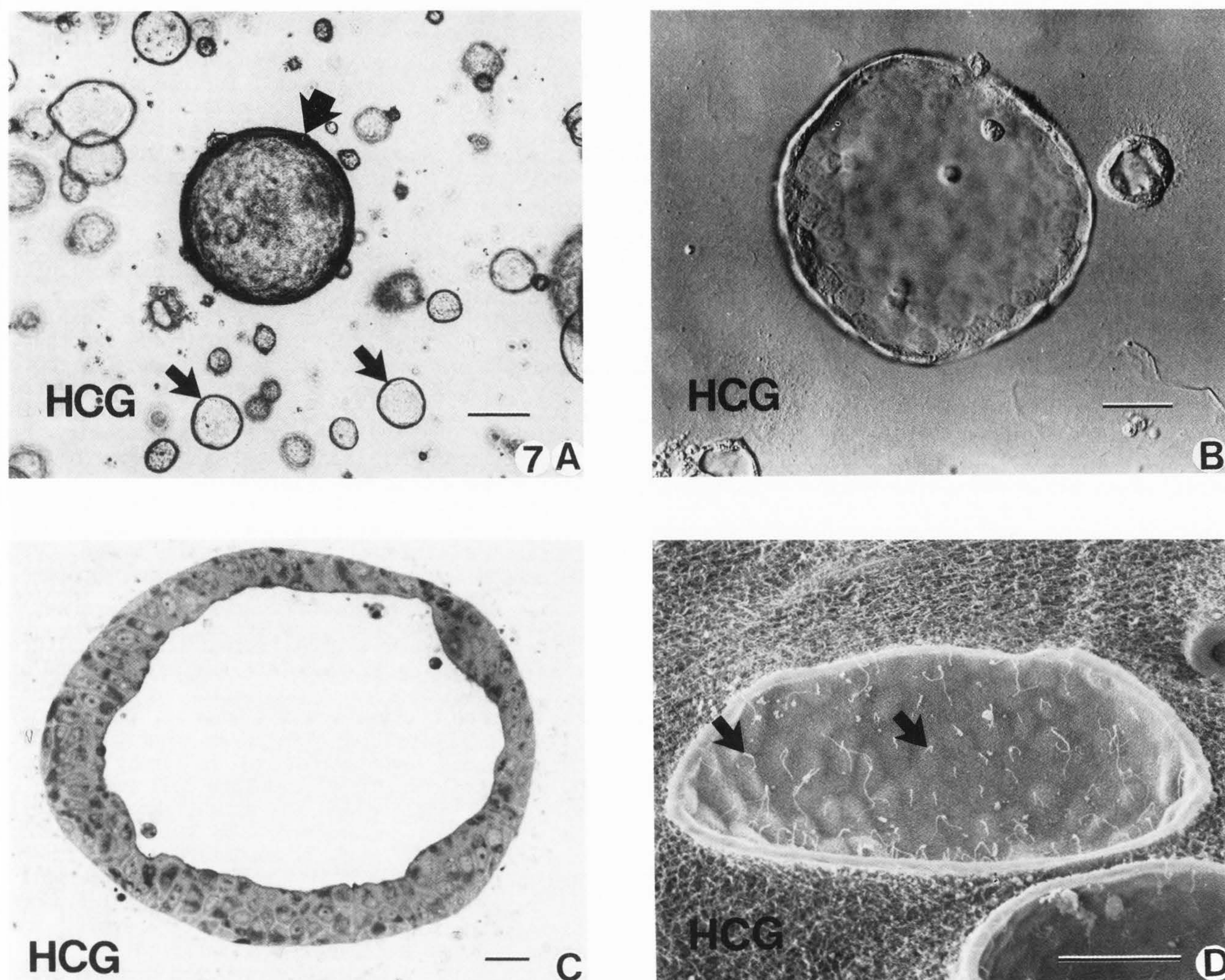


Figure 7. MDCK-CYSTS WITHIN COLLAGEN GEL

A: This light micrograph of a living culture shows numerous MDCK-cysts suspended within a collagen gel substrate (HCG). The majority of cysts (small arrows) in this region of the culture have a thin wall. One cyst (large arrow) at the center of the field has a noticeably thicker wall. Bar = 200 µm

B: This differential interference contrast light micrograph shows two MDCK-cysts in a living culture. These cysts bear a simple epithelium surrounding a fluid-filled lumen. Collagen gel (HCG). Bar = 50 µm

C: This light micrograph of a plastic embedded tissue section shows the morphology of a thick walled MDCK-cyst. The height of the cyst wall is irregular. Some regions of the epithelium appear to be non-uniformly stratified. Collagen gel (HCG). Bar = 10 µm

D: This scanning electron micrograph shows a fractured MDCK-cyst surrounded by collagen gel (HCG). The apical cell surface facing the lumen is characterized by microvilli and solitary cilia (arrows). Bar = 50 µm

The growth of MDCK-cysts involves fluid accumulation and cell proliferation (62). Growth in diameter of individual cysts can be followed morphologically and quantitated (66). We have found that MDCK-cyst growth can be influenced by altering the composition of the collagen substrate (66). Elevated collagen content of the surrounding substrate slows the rate of cyst growth but does not influence maximal diameter attainable. Seeding density, also, influences cyst growth. Cultures seeded at lower population density exhibit continued growth over a longer period, and reach a substantially larger maximal diameter. In one experiment, cultures seeded at 2×10^5 cells/ml(collagen gel) reached maximal diameter at about 21 days. Most cysts were < 800 µm in diameter. Cultures seeded at $1\text{-}2 \times 10^4$ cells/ml were still growing at 46 days, with some cysts > 2000 µm in diameter. Composition of the culture medium, as well, influences cyst growth. In one study, cultures were seeded at 1×10^4 cells/ml and the number of cysts > 50 µm in diameter was quantitated at day 24. In cultures receiving serum-free medium k-1 of Taub et al. (89), 1.45% of the cells formed cysts. Cells

cultured in DMEM supplemented with 5% fetal bovine serum showed 5.75% efficiency of cyst formation.

The majority of MDCK-cysts bear a simple cuboidal epithelium. Recently, however, we have observed that a small percentage (likely <1%) of cysts appear to have a pseudostratified or possibly multilayered epithelium. This observation indicates that the cyst-forming cell population of the strain 2 MDCK line is heterogeneous. We have yet to define the growth characteristics of this population of "thick-walled" cysts.

Clonal cyst formation in vitro is not a common cell characteristic. We have been unable to identify any other epithelial cell type that exhibits this behavior. Cell lines we have tested include LLC-PK1 (37), MDBK (52), BS-C-1 (36), OK (44), HT-29 (24) and PK-15 (American Type Culture Collection #CCL 33). In addition, we have tested subcultured human umbilical vein endothelial cells and epithelial cell-enriched primary cultures derived from normal human renal cortex (41) and from surface cysts of human adult autosomal dominant polycystic kidney (63). Moreover, we have found that not all MDCK cell populations exhibit cyst formation. Two strains of the MDCK line have been characterized (83). The so called high resistance strain (strain 1) is an avid dome former in monolayer culture and develops a relatively high transepithelial potential difference (PD) (4.16 ± 0.24 KΩ·cm^{-2}) (83) when cultured to confluency on a fluid permeable filter. Strain 1 MDCK cells do not form clonal cysts. Low resistance (PD 71 ± 6Ω·cm^{-2}) (83), strain 2 MDCK cells are infrequent dome formers in monolayer culture but do form clonal cysts. At this time, we have no experimental data to suggest why strain 2 cells are cyst formers while strain 1 cells are not. The capacity for morphogenetic clonal growth may or may not be related to the quality of intercellular junctions established by these cells. Cyst formation may instead be dependent upon some feature such as the disposition of ion and solute transport systems that result in net fluid secretion. This suggestion poses an interesting experimental problem. Elucidation of the mechanism by which MDCK-cysts form and enlarge may provide a key to understanding the apparent relationship between fluid accumulation and epithelial hyperplasia that exists in renal cyst formation and perhaps in other forms of pathologic cyst formation in man.

Summary

A number of cell and explant culture systems, representing a variety of epithelia, exhibit the phenomenon of cyst formation. In all such cultures, epithelial cells organize to enclose a fluid-filled lumen or cavity. The polarity of the epithelium characterizes in vitro cysts as two types, those that are "apex-in" and those that are "apex-out". "Apex-in" cysts are those in which the apical surface of the epithelium faces the lumen. In "apex-out" cysts, the lateral-basal cell surface borders the cyst cavity. The orientation of cell polarity ("apex-in" vs "apex-out") appears to be determined by culture conditions. "Apex-out" cysts form when the culture environment does not provide a compatible substrate with which the lateral-basal cell surface can form an interface. Such a condition exists in suspension culture within fluid medium that is not supplemented with the appropriate soluble extracellular matrix components. "Apex-in" cysts form when cells are cultured within an extracellular matrix such as a collagen gel, or when the apical surface of an already established epithelium is "challenged" by certain types of extracellular components (e.g. collagen).

"Apex-in" and "apex-out" cysts exhibit certain features distinct from one another. "Apex-in" cysts tend to be considerably more stable (less likely to dissociate, or reverse polarity) than "apex-out" cysts. Also, it is "apex-in" cysts that exhibit the capacity to increase in size, while "apex-out" cysts have a limited potential for enlargement.

The mechanism(s) by which epithelial cells in culture organize to form cysts, and subsequently increase in diameter are not clearly known. Cyst formation and cyst growth in vitro are separate, but related events that appear to involve separate mechanisms, possibly directed by different factors. Cyst formation in culture requires that cells associate (aggregate), and/or proliferate to form a population of cells that can further organize to form a contiguous, polarized epithelium (in cyst configuration). This process (cyst formation) is likely governed by cellular events that regulate cell recognition, cell sorting and the establishment of cell polarity. Cyst enlargement, on the other hand, characterized by fluid accumulation in the lumen, and an increase in cell number in the cyst wall, is probably controlled by factors that influence transepithelial ion and solute transport, and those that regulate cell proliferation.

All cysts accumulate fluid, and growing cysts accumulate increasing amounts of fluid. Indeed, fluid accumulation may be the one principal feature that cysts derived from various epithelia share in common. The mechanism by which fluid accumulates within epithelial cysts is unknown, but may be dependent upon cell type. The epithelium of in vitro cysts frequently consists of differentiated cells that express structural and/or functional characteristics of their tissue or organ of origin. In many cases, cells that form cysts in culture are derived from epithelia (e.g. thyroid follicle, mammary gland, submandibular gland, prostate) that function normally by secreting an exocrine product from their apical surface. In cysts derived from these epithelia, normal secretory function may contribute to fluid within the lumen. However, "apex-in" cysts that exhibit significant enlargement, regardless of epithelial type, accumulate a far greater amount of fluid than could be contributed by the release of secretory products. Moreover, the group of epithelia that form progressively growing "apex-in" cysts, includes "non-secretory" epithelia (e.g. vaginal epithelium, small intestine) that do not produce a voluminous secretory product. Still, these cysts accumulate a large amount of fluid. This suggests that

transepithelial fluid transport may play an important role in cyst enlargement.

Cysts in culture also are formed by cells that function principally in fluid transport (e.g. kidney explants, renal cell lines). Fluid transporting epithelia, particularly cells of the renal tubule, move fluid in an apical to basal direction. If such cells form an "apex-in" cyst, and the cells continue to transport fluid in an apical to basal direction, the cyst may not accumulate fluid, and may not enlarge. If, however, the transport function of the cells is abnormal, such that net fluid movement is in a basal to apical direction, fluid accumulation will likely occur. So it is, that altered fluid transport may be involved in fluid accumulation within cultured cysts.

Fluid accumulation alone, does not fully explain cyst enlargement. It is probably more correct to say that fluid accumulation, in itself, can lead to cyst distension, but that cyst enlargement requires both fluid accumulation and cell proliferation. When cysts enlarge in vitro (or in vivo), the lining epithelium proliferates. This increased cell division may be stimulated by factors unrelated to fluid accumulation, or, alternatively, these two events (fluid accumulation and cell proliferation) may be linked. A relationship between fluid accumulation and cell proliferation in cyst growth can be explained in at least two ways. First, fluid accumulation may be a primary event that acts, possibly by increasing intralumenal pressure, to stimulate a compensatory proliferation in the cyst lining epithelium. Second, cell proliferation may be the primary event, and fluid accumulation may occur by passive transport, in response to enlargement of the lumen.

An understanding of the relationship between epithelial hyperplasia and fluid accumulation in cyst formation and growth may be essential in determining the mechanism(s) that underly diseases of cyst formation. Cell culture systems in which cyst formation occur, offer an excellent opportunity to establish if these events are associated. The use of tissue culture techniques makes it possible to simulate the in vivo conditions that lead to cyst formation, but under controlled experimental conditions. Thus, cell and explant culture provides a useful experimental tool to help us better understand the pathogenesis of epithelial cystic diseases.

Acknowledgements

This study was supported by grants AM33003 and HD19263 from the National Institutes of Health, and by the PKR Foundation (Kansas City). The authors wish to thank Ellen Vance, Patty Summerlin and Kathy Welborn-Craig for their expert technical assistance.

References

1. **Agnew WF,** Alvarez RB, Yuen TGH, Abramson SB, Kirk D. (1984) A serum-free culture system for studying solute exchanges in the choroid plexus. In Vitro, 20, 712-722.
2. **Ansevin KD,** Lipps BV. (1973) Tissue plate: A new technique for long-term organotypic culture. In Vitro 8, 483-488.
3. **Avner ED,** Piesco NP, Sweeney WE, Studnicki FM, Fetterman GH, Ellis D. (1984) Hydrocortisone-induced cystic metanepheric maldevelopment in serum-free organ culture. Lab. Invest. 50, 208-218.
4. **Avner ED,** Sweeney WE, Ellis D. (1983) Cyst formation in metanepheric organ culture induced by cis-dichlorodiammineplatinum (II). Experientia 39, 74-76.
5. **Avner ED,** Sweeney WE, Finegold DN, Piesco NP, Ellis D. (1985) Sodium-potassium ATPase activity mediates cyst formation in metanepheric organ culture. Kidney Int. 28, 447-455.
6. **Avner ED,** Sweeney WE, Piesco NP, Ellis D. (1986) Regression of genetically determined polycystic kidney disease in murine organ culture. Experientia 42, 77-80.
7. **Avner ED,** Sweeney WE, Piesco NP, Ellis D. (1987) Triiodothyronine-induced cyst formation in metanephric organ culture: the role of increased Na-K ATPase activity. J. Lab. Clin. Med. 109, 441-454.
8. **Bennett DC.** (1980) Morphogenesis of branching tubule in cultures of cloned mammary epithelial cells. Nature 285, 657-659.
9. **Bennett DC,** Armstrong BL, Okada SM. (1981) Reconstitution of branching tubules from two cloned mammary cell types in culture. Dev. Biol. 87, 193-199.
10. **Bernstein J.** (1973) The classification of renal cysts. Nephron 11, 91-100.
11. **Carone FA,** Rowland RG, Perlman SG, Ganote CE. (1974) The pathogenesis of drug-induced renal cystic disease. Kidney Int. 5, 411-421.
12. **Chambard M,** Gabrion J, Mauchamp J. (1981) Influence of collagen gel on the orientation of epithelial cell polarity: Follicle formation from isolated thyroid cells and from preformed monolayers. J. Cell Biol. 91, 157-166.
13. **Chambers R,** Kempton RT. (1933) Indications of function of the chick mesonephros in tissue culture with phenol red. J. Cell. Comp. Physiol. 3, 131-167.
14. **Douglas WHJ,** Hitchcock KR. (1979) Organotypic cultures of diploid type II alveolar pneumocytes. Surfactant associated esterase activity. J. Histochem. Cytochem. 27, 852-856.
15. **Douglas WHJ,** McAteer JA, Cavanagh T. (1978) Organotypic culture of dissociated lung cells on a collagen sponge matrix. Tiss. Cult. Assn. Man. 4, 749-753.
16. **Douglas WHJ,** Moorman GW, Teel RW. (1976) The formation of histotypic structures from monodisperse fetal rat lung cells cultured on a three-dimensional substrate. In Vitro 12, 373-381.
17. **Douglas WHJ,** Teel RW. (1976) An organotypic in vitro model system for studying pulmonary surfactant production by type II alveolar pneumonocytes. Am. Rev. Respir. Dis. 113, 17-23.

18. Ducibella T, Anderson D, Aalberg J, DeWolf WC. (1982) Cell surface polarization, tight junctions and eccentric inner cells characterize human teratocarcinoma embryoid bodies. Dev. Biol. 94, 197-205.
19. Engle MJ, Sanders RL, Douglas WHJ. (1980) Type II alveolar cells in organotypic culture. A model system for the study of surfactant synthesis. Biochim. Biophys. Acta 617, 225-236.
20. Evan AP, Gardner Jr KD. (1976) Comparison of human polycystic and medullary cystic kidney disease with diphenylamine-induced cystic disease. Lab. Invest. 35, 93-101.
21. Evan AP, Gardner Jr KD. (1979) Nephron obstruction in nordihydroguaiaretic acid-induced renal cystic disease. Kidney Int. 15, 7-19.
22. Evan AP, Gardner Jr KD. (1985) Morphology of polycystic kidney disease in man and experimental models. In: Chronic Renal Disease. Causes, Complications and Treatment, Cummings NB, Klahr S (eds.), Plenum Medical Book Co., New York, 55-68.
23. Fayet G, Michel-Bechet M, Lissitzky S. (1971) Thyrotrophin-induced aggregation and reorganization into follicles of isolated porcine-thyroid cells in culture. 2. Ultrastructural studies. Eur. J. Biochem. 24, 100-111.
24. Fogh J, Trempe G. (1975) New human tumor cell lines. In: Human Tumor Cells In Vitro, Fogh J (ed.), Plenum Press, New York, 115-159.
25. Garbi C, Mascia A, Nitsch L. (1987) Cell polarity and morphogenetic properties of Fischer rat thyroid cells (FRT) cultured in suspension or embedded in different gels. Cell. Molec. Biol. 33, 293-305.
26. Garbi C, Nitsch L, Wollman SH. (1984) Embedding in a collagen gel stabilizes the polarity of epithelial cells in thyroid follicles in suspension culture. Exp. Cell Res. 151, 458-465.
27. Garbi C, Tacchetti C, Wollman SH. (1986) Change of inverted thyroid follicle into a spheroid after embedding in a collagen gel. Exp. Cell Res. 163, 63-77.
28. Garbi C, Wollman SH. (1982a) Basal lamina formation on thyroid epithelia in separated follicles in suspension culture. J. Cell Biol. 94, 489-492.
29. Garbi C, Wollman SH. (1982b) Ultrastructure and some other properties of inverted thyroid follicles in suspension culture. Exp. Cell Res. 138, 343-353.
30. Goodman T, Grice HC, Becking C, Salem FA. (1970) A cystic nephropathy induced by nordihydroguaiaretic acid in the rat. Lab. Invest. 23, 93-107.
31. Grantham JJ, Geiser JL, Evan AP. (1987) Cyst formation and growth in autosomal dominant polycystic kidney disease. Kidney Int. 31, 1145-1152.
32. Hall GH, Farson DA, Bissell MJ. (1982) Lumen formation by epithelial cell lines in response to collagen overlay: A morphogenetic model in culture. Proc. Natl. Acad. Sci. (USA). 79, 4672-4676.
33. Herzog V. (1983) Preparation of inside-out thyroid follicles for studies on transcellular transport (transcytosis). Meth. Enzymol. 98, 447-458.
34. Herzog V. (1983) Transcytosis in thyroid follicle cells. J. Cell Biol. 97, 607-617.
35. Hilfer SR. (1962) The stability of embryonic chick thyroid cells in vitro as judged by morphological and physiological criteria. Dev. Biol. 4, 1-21.
36. Hopps HE, Bernheim BC, Nisalak A, Tjio JH, Smadel JE. (1963) Biologic characteristics of a continuous kidney cell line derived from African green monkey. J. Immunol. 91, 416-424.
37. Hull RN, Cherry WR, Weaver GW. (1976) The origin and characteristics of a pig kidney strain LLC-PK1. In Vitro 12, 670-677.
38. Iguchi T, Uchima FDA, Ostrander PL, Bern HA. (1983) Growth of normal mouse vaginal epithelial cells in and on collagen gels. Proc. Natl. Acad. Sci. (USA) 80, 3743-3747.
39. Kanwar YS, Carone FA. (1984) Reversible changes of tubular cell and basement membrane in drug-induced renal cystic disease. Kidney Int. 26, 35-43.
40. Kawamura H, Ichihara I. (1987) Primary culture of epithelial cells derived from the rat ventral prostate: formation of three-dimensional acinus-like structure in collagen gel. Prostate 10, 151-161.
41. Kempson SA, McAteer JA, Dougherty GS, Evan AP. (1986) Proximal tubule characteristics of cultured human renal cortex epithelium. In Vitro 22, 23A. (abstract)
42. Kime Jr SW, McNamara JJ, Luse S, Farmer S, Silbert C, Bricker NS. (1962) Experimental polycystic renal disease in rats: electron microscopy, function, and susceptibility to pyelonephritis. J. Lab. Clin. Med. 60, 64-78.
43. Kirk D, Alvarez RB. (1986) Morphologically stable epithelial vesicles cultured from normal human endometrium in defined media. In Vitro Cell. Develop. Biol. 22, 604-614.
44. Koyama H, Goodpasture C, Miller MM, Teplitz RL, Riggs AD. (1978) Establishment and characterization of a cell line from the American opossum (Didelphys virginiana). In Vitro 14, 239-246.
45. Leighton J. (1969) Propagation of cancer: Targets for future chemotherapy. Can. Res. 29, 2457-2465.
46. Leighton J. (1981) Anatomic evidence of transport function by adenocarcinomas and a suggested role of transport in the spread of cancer. Ann. N.Y. Acad. Sci. 372, 455-464.
47. Leighton J, Brada Z, Estes LW, Justh G. (1969) Secretory activity and oncogenicity of a cell line (MDCK) derived from canine kidney. Science 163, 472-473.
48. Leighton J, Estes LW, Mansukhani S, Brada Z. (1970) A cell line derived from normal dog kidney (MDCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium. Cancer 26, 1022-1028.
49. Leighton J, Mark R, Justh G. (1968) Patterns of three-dimensional growth in vitro in collagen-coated cellulose sponge: Carcinomas and embryonic tissues. Cancer Res. 28, 286-296.
50. Leighton J, Tchao R, Johnson W, Abaza N. (1980) Histokinetic responses of epithelial cells in histophysiologic gradient culture. In: Cell Movement and Neoplasia. DeBrabander M (ed.), Pergamon Press, New York, 61-64.

51. Lissitzky S, Fayet G, Giraud A, Verrier B, Torresani J. (1971) Thyrotrophin-induced aggregation and reorganization into follicles of isolated porcine-thyroid cells. 1. Mechanism of action of thyrotrophin and metabolic properties. Eur. J. Biochem. 24, 88-99.
52. Madin SH, Darby Jr NB. (1958) Established kidney cell lines of normal adult bovine and ovine origin. Proc. Soc. Exp. Biol. Med. 98, 574-576.
53. Martin GR, Wiley LM, Damjanov I. (1977) The development of cystic embryoid bodies in vitro from clonal teratocarcinoma stem cells. Dev. Biol. 61, 230-244.
54. Mauchamp J, Margotat A, Chambard M, Charrier B, Remy L, Michel-Bechet M. (1979) Polarity of the three-dimensional structure derived from isolated hog thyroid cells in primary culture. Cell Tiss. Res. 204, 417-430.
55. McAteer JA, Cavanagh TJ. (1982) Medium hydrated collagen gel as an explant support in organ culture. J. Tiss. Cult. Meth. 7, 117-122.
56. McAteer JA, Cavanagh TJ. (1983a) Differentiation of the airways epithelium of fetal rat tracheal organ explants in serum-free culture: SEM observations. In: Proceedings EMSA, Bailey GW (ed.), San Francisco Press, San Francisco, 478-479.
57. McAteer JA, Cavanagh TJ. (1983b) Type I alveolar epithelial cell differentiation in fetal rat lung organ explants. In Vitro 19, 272. (abstract)
58. McAteer JA, Cavanagh TJ, Evan AP. (1983a) Submersion culture of intact fetal lung. In Vitro 19, 210-218.
59. McAteer JA, Cavanagh TJ, Evan AP. (1983b) Pulmonary epithelial cytodifferentiation in vitro occurs in the absence of key hormones implicated in fetal lung development. Anat. Rec. 205, 115A-116A. (abstract)
60. McAteer JA, Cavanagh TJ, Evan AP. (1983c) Submersion organ culture of preglandular fetal lung. J. Tiss. Cult. Meth. 8, 59-62.
61. McAteer JA, Dougherty GS, Gardner KD, Evan AP. (1986) Scanning electron microscopy of kidney cells in culture: Surface features of polarized epithelia. Scanning Electron Microsc. 1986; III: 1135-1150.
62. McAteer JA, Evan AP, Gardner KD. (1987) Morphogenetic clonal growth of kidney epithelial cell line MDCK. Anat. Rec. 217, 229-239.
63. McAteer JA, Evan AP, Grantham JJ, Carone FA, Gardner Jr KD. (1986) Human polycystic kidney in primary culture: growth characteristics and ultrastructure of cyst-derived epithelium. Kidney Int. 29, 323. (abstract)
64. McAteer JA, Hegre OD. (1978) A continuous-flow method of organ culture. In Vitro 14, 795-803.
65. McAteer JA, Vance EE, Gardner KD, Evan AP. (1985) Polarity reversal by MDCK Cells in vitro. In Vitro Cell. Dev. Biol. 21, 8A. (abstract)
66. McAteer JA, Welling DJ, Evan AP, Connors BA, Welling LD. (1985) Determination of growth rate for MDCK-cysts cultured within collagen gel. Fed. Proc. 44, 1039. (abstract)
67. Misfeldt DS, Hamamoto ST, Pitelka D. (1976) Transepithelial transport in cell culture. Proc. Natl. Acad. Sci. (USA) 73, 1212-1216.
68. Montesano R, Mouron P, Orci L. (1985) Vascular outgrowths from tissue explants embedded in fibrin or collagen gels: a simple in vitro model of angiogenesis. Cell Biol. Internat. Rep. 9, 869-875.
69. Montesano R, Orci L, Vassalli P. (1983) In vitro rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. J. Cell Biol. 97, 1648-1652.
70. Montgomery RK. (1986) Morphogenesis in vitro of dissociated fetal rat small intestinal cells upon an open surface and subsequent to collagen gel overlay. Dev. Biol. 117, 64-70.
71. Moscona AA. (1961) Rotation-mediated histogenic aggregation of dissociated cells: a quantifiable approach to cell interactions in vitro. Exp. Cell Res. 22, 455-475.
72. Moscona AA. (1962) Analysis of cell recombination in experimental synthesis of tissues in vitro. J. Cell. Comp. Physiol. 60, (Suppl. 1), 65-80.
73. Moscona AA. (1974) Surface specification of embryonic cells: lectin receptors, cell recognition and specific cell ligands. In: The Cell Surface in Development, Moscona AA (ed.), John Wiley & Sons, New York, 67-99.
74. Nicosia RF. (1987) Angiogenesis and the formation of lymphaticlike channels in cultures of thoracic duct. In Vitro Cell. Dev. Biol. 23, 167-174.
75. Nicosia RF, Tchao R, Leighton J. (1982) Histotypic angiogenesis in vitro: Light microscopic, ultrastructural, and radioautographic studies. In Vitro 18, 538-549.
76. Nitsch L, Wollman SH. (1980a) Suspension culture of separated follicles consisting of differentiated thyroid epithelial cells. Proc. Natl. Acad. Sci. (USA) 77, 472-476.
77. Nitsch L, Wollman SH. (1980b) Thyrotropin preparations are mitogenic for thyroid epithelial cells in follicles in suspension culture. Proc. Natl. Acad. Sci. (USA) 77, 2743-2747.
78. Nitsch L, Wollman SH. (1980c) Ultrastructure of intermediate stages in polarity reversal of thyroid epithelium in follicles in suspension culture. J. Cell Biol. 86, 875-880.
79. Nitsch L, Wollman SH. (1986) Sudden volume changes of the lumen of inverted thyroid follicles in suspension cultures. Exp. Cell Res. 162, 278-283.
80. Ormerod JE, Rudland PS. (1982) Mammary gland morphogenesis in vitro: formation of branched tubules in collagen gels by a cloned rat mammary cell line. Dev. Biol. 91, 360-375.
81. Ormerod JE, Rudland PS. (1988) Mammary gland morphogenesis in vitro: extracellular requirements for the formation of tubules in collagen gels by a cloned rat mammary epithelial cell line. In Vitro Cell. Dev. Biol. 24, 17-27.
82. Reeders ST, Bruening MH, Davies KE, Nicholls RD, Jarman AP, Higgs DR, Pearson PL, Weatherall DJ. (1985) A highly polymorphic DNA marker linked to adult polycystic kidney disease on chromosome 16. Nature 317, 542-544.
83. Richardson JCW, Scalera V, Simmons NL. (1981) Identification of two strains of MDCK cells which resemble separate nephron tubule segments. Biochim. Biophys. Acta 673, 26-36.

84. Rosenbaum RM, Picciano P, Kress Y, Wittner M. (1977) Ultrastructure of in vitro type 2 epithelial cell cysts derived from adult rabbit lung cells. Anat. Rec. 188, 241-262.
85. Safouh M, Crocker JFS, Vernier RL. (1970) Experimental cystic disease of the kidney. Lab. Invest. 23, 392-400.
86. Shirayoshi Y, Okada TS, Takeichi M. (1983) The calcium-dependent cell-cell adhesion system regulates inner cell mass formation and cell surface polarization in early mouse development. Cell 35, 631-638.
87. Stratton CJ, Douglas WHJ, McAteer JA. (1978) The surfactant system of human fetal lung organotypic cultures: Ultrastructural preservation by a lipid-carbohydrate retention method. Anat. Rec. 192, 481-492.
88. Takasu N, Handa Y, Shimizu Y, Yamada T. (1984) Electrophysiological and morphological cell polarity and iodine metabolism in cultured porcine and human (normal and Graves') thyroid cells. J. Endocrinol. 101, 189-196.
89. Taub M, Chuman L, Saier Jr MH, Sato G. (1979) Growth of Madin-Darby canine kidney epithelial cell (MDCK) line in hormone-supplemented, serum-free medium. Proc. Natl. Acad. Sci. (USA) 76, 3338-3342.
90. Tchao R, Leighton J. (1981) 3':5'-stimulation of cyst formation by dibutyryl cyclic adenosine monophosphate in human urinary bladder tumors cultured in vitro. Cancer Res. 41, 635-639.
91. Wohlwend A, Montesano R, Vassalli J-D, Orci L. (1985) LLC-PK1 cysts: A model for the study of epithelial polarity. J. Cell. Physiol. 125, 533-539.
92. Yang J, Larson L, Nandi S. (1982) Three-dimensional growth and morphogenesis of mouse submandibular epithelial cells in serum-free primary culture. Exp. Cell Res. 137, 481-485.
93. Yang J, Nandi S. (1983) Growth of cultured cells using collagen as substrate. Int. Rev. Cytol. 81, 249-286.
94. Yang J, Richards J, Bowman P, Guzman R, Enami J, McCormick K, Hamamoto S, Pitelka D, Nandi S. (1979) Sustained growth and three-dimensional organization of primary mammary tumor epithelial cells embedded in collagen gels. Proc. Natl. Acad. Sci. (USA) 76, 3401-3405.
95. Yang J, Richards J, Guzman R, Imagawa W, Nandi S. (1980) Sustained growth in primary culture of normal mammary epithelial cells embedded in collagen gels. Proc. Natl. Acad. Sci. (USA) 77, 2088-2092.

Discussion with Reviewers

E. Avner: The culture of fetal lung cells in rotation-mediated aggregation, as you have described, would be an interesting model to test the potential effects of various growth factors on epithelial development. Have you examined epithelial differentiation using serum-free, totally characterized culture medium?
Authors: We have observed that *in vitro* morphogenesis of the fetal pulmonary epithelium occurs in the complete absence of hormones and growth factors. We have established fetal lung RMA cultures from rat fetuses as early as day 14-15, using basal medium F12K or DMEM without serum or supplemental hormones. Under these conditions, the undifferentiated cells aggregate, organize to form lumena, and then progressively develop ultrastructural characteristics typical of differentiated cell types, including the type II cell (lamellar bodies, apical microvilli) and the ciliated cell.

E. Avner: In your studies on submersion and collagen gel culture of fetal rat lung and trachea, have you characterized the biochemical (i.e., enzymatic changes or surfactant production) or immunologic (i.e., development of apical/basal antigenic markers) differentiation of the pulmonary epithelium? Such studies would provide a powerful tool for analysis of developmental events involved in the polarized morphogenesis of alveolar walls or bronchopulmonary cilia.
Authors: Our studies, so far, have been limited to morphologic analysis of the airway and alveolar cell types.

E. Avner: In the MDCK cyst model, you note that fluid accumulation is an important factor in cellular growth, and that the composition of the culture medium influences cell growth. Given the distal nephron origin of this epithelial cell line, can you speculate on altered epithelial transport processes which may be operative in this model? Do ultrastructural studies show significant differences between the intercellular junctions of strain 1 and 2 MDCK cells or suggest whether ion and solute transport in clonal cyst growth may be transcellular or paracellular?
Authors: At this time, we can, indeed, only speculate on the mechanism by which MDCK cysts accumulate fluid. There are several possible mechanisms that might explain this phenomenon, 1) Na^+ and K^+ transport may be altered, involving the activity and/or the polarity of the Na^+/K^+ ATPase, 2) the cells of MDCK cysts may be active chloride ion secretors, chloride representing the primary osmotically active solute involved in net fluid movement across the cell, and 3) the cells of the MDCK cyst may have increased mitotic activity independent of fluid accumulation, such that fluid enters the cyst passively to accommodate lumen enlargement.

Intercellular junctions may play a role in fluid movement and cyst enlargement. We have not performed the detailed analysis (freeze fracture) that would be necessary to identify and characterize differences in the junctions among the cyst-forming subline, and the two "parent" lines, strains 1 and 2.

G. Stoner: What do you think is (are) the principal mechanism(s) of epithelial cyst formation *in vitro?* Is (are) the mechanism(s) common to different epithelial cell types?
Authors: There is, unfortunately, no data that

would allow a comparison of the mechanism(s) by which various epithelial cell types form cysts or how cysts, once formed, continue to enlarge. Cyst formation and cyst growth are related events in that one must precede the other, however, they probably involve different mechanisms. Cyst formation _in vitro_ commonly involves processes in the establishment of cell polarity. For example, cell aggregation and cell sorting, to produce a polarized epithelium, involve cell-cell interactions of the plasma membrane. These processes by which some cell types initially form cysts, may not be related to the events by which a cyst epithelium mediates the accumulation of fluid in the lumen. Fluid accumulation is a characteristic in common among cysts of various cell types, however, the mechanism by which fluid enters and is retained in the lumen is as yet unknown.

G. Stoner: Are factors intrinsic to the epithelial cells (e.g. junctional complexes) more important for cyst formation than extrinsic factors (e.g. hormones)?
Authors: It may not be possible to dissect cyst formation along these lines. Intrinsic factors are essential to the formation of a cyst. For example, in our reading of the literature, apical tight junctions are described for all cyst culture systems in which an ultrastructural analysis was performed. Therefore, tight junctions appear to be necessary for cyst formation. However, extrinsic factors may be required to allow functional tight junctions to be established. This reasoning may, likewise, explain the relationship between the presence and activity of ion and solute transport systems that are likely necessary to mediate fluid accumulation in the cyst lumen. The cell must possess the apparatus to facilitate fluid accumulation, but the presence and activity of the transport system may possibly be controlled by extracellular factors.

G. Stoner: Are cysts formed _in vitro_ by a specific epithelial cell type similar to those observed _in vivo_ by the same cell type?
Authors: Morphological similarities are common. For example, _in vitro_ thyroid cysts, under appropriate conditions, accumulate colloid and show certain ultrastructural features typical of the thyroid follicle.

G. Stoner: Please speculate as to the reasons why many epithelial cell types appear to lose their ability to form cysts _in vitro._
Authors: It strikes us as intriguing that so many epithelia do retain the capacity to form cysts, even under the influence of an "artificial" _in vitro_ environment. Those epithlelia that do not exhibit cyst formation in cell culture may have specific requirements, such as the nature of the extracellular matrix, that are not met by the _in vitro_ conditions that have been attempted.

S. Wollman: I wish to provide a clarification of data that you have cited in your paper. Recently, Garbi and at least one other member of the lab have independently tried to reproduce the induction of basal lamina formation as reported in your reference no. 28. They were completely unsuccessful. The original studies lasting over a period of a year reproduced the original observation many times with no failures, but all studies were done with aliquots of a single sample of laminin. The later work was done with two or three different samples of laminin. The reason for the discrepancy in the results is unknown.

J. Yang: Do hormones, growth factors or metabolic inhibitors influence the polarity reversal of apex-out "follicles" of MDCK cells embedded in collagen gel?
Authors: We have not examined this phenomenon in detail. We have observed, however, that polarity reversal of MDCK "follicles" does occur in Taub's serum-free medium K-1.

J. Yang: Dome formation by MDCK in monolayer culture is influenced by various factors. Do similar factors influence cyst formation of MDCK cells in collagen gel?
Authors: Unlike dome formation, in which fluid movement across the epithelium occurs in an apical to basal direction, cyst formation by MDCK cells within collagen gel involes net basal to apical fluid transport. For this reason, it may be difficult to compare the two phenomena.

J. Yang: Do isolated primary cells, derived from kidney, exhibit cyst formation when cultured within collagen gel?
Authors: No. We have tested the influence of a collagen gel environment on enzyme dissociated cells from rabbit, rat and human renal cortex and medulla. None of these cells, and, indeed, no other cell population we have encountered, exhibits the phenomenon of morphogenetic clonal growth that we have observed with MDCK strain 2 cells.