Comparison of the Ability of Basement Membranes Produced by Corneal Endothelial and Mouse-derived Endodermal PF-HR-9 Cells to Support the Proliferation and Differentiation of Bovine Kidney Tubule Epithelial Cells In Vitro

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ABSTRACT The proliferation and morphological differentiation of bovine kidney collecting-tubule epithelial cells has been examined as a function of substrata and plasma factors. Collecting kidney tubule explant maintained in vitro gave rise to two distinct cell populations; one was composed mostly of fibroblastic cells whereas the other was epithelioid (EP cells). The proliferation of fibroblastic cells when exposed to serum-supplemented medium was best expressed when cells were maintained on a basement membrane produced by bovine corneal endothelial cells. This basement membrane has a composition, which in previous studies has been shown to favor the proliferation of mesenchymal cells. In contrast, the proliferation of EP cells was best expressed when cells were maintained on a basement membrane produced by the mouse-derived endodermal cell line PF-HR-9 (HR-9-BM). This basement membrane has a biochemical composition very similar to the basement membrane underlying the kidney tubules. Although the fibroblast confluent monolayer maintained on bovine corneal endothelial cell extracellular matrix did not undergo morphogenesis, the confluent monolayer of EP cells maintained on HR-9-BM shows hemicyst formation, suggesting that they were capable of vectorial fluid transport. They also built a complex three-dimensional kidney tubulelike network. Some tubules became grossly visible and floated into the tissue culture medium, remaining tethered to the cell monolayer at either end of the tubule. On an ultrastructural level, the tubules consisted of cells held together with junctional complexes arranged so as to form a lumen. The smallest lumen were bordered by 2–3 cells, and the largest ones by 8–15 cells. The lumens of the larger tubules did contain granular fibrillar and amorphous debris.

Low-density EP cell cultures maintained on HR-9-BM could be induced to proliferate at a rate approaching that of cultures exposed to serum when they were exposed to medium supplemented with high-density lipoprotein (HDL, 750 μ g protein/ml) and transferrin (50 μ g/ml). When exposed to HDL concentrations equal or lower than 250 μ g protein/ml, low-density cultures proliferated at a slow rate and readily formed tubulelike structures. This observation indicates that EP cells do not need to reach confluence to undergo morphogenesis, and that HDL, which in the presence of transferrin supports the cell proliferation, can favor their differentiation into tubulelike structures once its concentration becomes limiting for mitogenesis.

An intact basement membrane scaffold is required *in vivo* for the maintenance of orderly tissue structure and regeneration. By its presence, the membrane scaffold defines the spatial relationships among similar and dissimilar types of cells. Such a substrate plays an important role not only in cell attachment and migration (1–3), but also in the cell response to various growth-promoting agents present in plasma, lymph, or interstitial fluid (4). It enables multicellular organisms to reconstitute histological structures of most tissue and organs to what they were before loss of cells (5). In the early stage of embryonic development, when different tissues composing a given organ are formed as a result of strictly timed and spatially interrelated proliferative and differentiative events, interaction of cells with newly formed basement membrane has been shown to result in cell proliferation and the expression of new phenotypes (6–8). Such is the case in the formation of the kidney nephron resulting from the invasion of the ureter bud into the metanephric mesenchyme. Metanephric mesenchymal cells are embedded in an extracellular matrix composed in part of collagen isotypes I and III and of fibronectin (9–12). Under the directive influence of the invading ureter bud, mesenchymal cells condense and synthesize a basement membrane composed mainly of collagen type IV, heparan sulfate proteoglycan, and laminin (9–12). This closely correlates with the synchronized development of the epithelial part of the glomeruli and associated nephron tubule (9–12). Induction by the ureter bud therefore stimulates the production of laminin, type IV collagen, and heparan sulfate proteoglycan, resulting in the formation of a proper substratum for epithelial cell attachment and further differentiation.

In the present studies, we have compared the ability of basement membrane produced by cultured bovine corneal endothelial cells versus that produced by a mouse teratocarcinoma-derived endodermal cell line PF-HR-9, to support the growth and differentiation of kidney epithelial cells derived from collecting tubules isolated from the outer medulla of adult bovine kidney. Of those two types of basement membrane, only that produced by the PF-HR-9 cell line, which has a composition similar to that of the kidney tubule basement membrane, fully supports the growth of the bovine kidney epithelial cells. Among the serum components required for optimal proliferation of the cells when maintained in serum-free conditions, are the high-density lipoproteins (HDL)¹ and transferrin. Once confluent, bovine kidney epithelial cells maintained on HR-9 basement membrane (HR-9-BM) started to form tubular structures, reminiscent of those seen *in vivo*. The present study therefore further supports the concept of specificity of basement membranes in regard to their ability to support the proliferation of kidney epithelial cells into tubules, as well as their differentiation into complex tubular structures.

MATERIALS AND METHODS

Materials: Dulbecco's modified Eagle's medium (DME) and F-12 medium were obtained from Grand Island Biological Co. (Grand Island, NY).

¹ *Abbreviations used in this paper:* BCE, bovine corneal endothelial (cells); BM, basement membrane; DF medium, mixture of Dulbecco's modified Eagle's medium and F12 medium; DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EP cells, epithelioid cells; FB cells, fibroblastic cells; FCS, fetal calf serum; FGF, fibroblast growth factor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDCK, Madin-Darby canine kidney; STV solution; sodium phosphate, trypsin, and EDTA solution.

Calf serum and fetal calf serum (FCS) were obtained from Hy Clone (Sterile Systems Inc., Logan, UT). Tissue culture dishes were from Falcon Plastics (Oxnard, CA), Gentamicin from Schering Co. (Kenilworth, NJ), and Fungizone from Squibb (Princeton, NJ). BSA and cholera toxin were obtained from Schwarz-Mann Div., Becton, Dickinson & Co., (Orangeburg, NY). Dextran T-40, insulin, hydrocortisone, triiodothyronine, cholesterol, and vasopressin were obtained from Sigma Chemical Co. (St. Louis, MO).

Fibronectin was purified from bovine plasma as described by Engvall et al. (13). When analyzed by SDS PAGE under reduced conditions, the purified bovine plasma fibronectin ran as a doublet with a molecular weight in the range of 220,000. Brain fibroblast growth factor (FGF) was purified by isoelectric focusing, as already described (14). FGF-2 (14) was used in the present study. When the homogeneity of that fraction was analyzed by slab gel electrophoresis, a single band was observed (14). Epidermal growth factor (EGF) was purified as described by Savage and Cohen (15).

Human low-density lipoprotein (LDL, $1.019 < d < 1.063 \text{ g/cm}^3$) and high-density lipoproteins ($1.07 < d < 1.21 \text{ g/cm}^3$) were obtained from human plasma by differential ultracentrifugal flotation (16). To remove possible contamination by fractions of higher density, each lipoprotein was washed by flotation through a solution corresponding to its respective upper limiting density. Protein concentrations were determined by the method of Lowry et al. (17), as modified by Maxwell et al. (18).

Each lipoprotein fraction migrated as a homogeneous band on agarose electrophoretic gels. The purity of HDLs and LDL was also analyzed by double immunodiffusion using rabbit antisera directed against the α_1 - and β_1 -apoprotein (anti-HDL and anti-LDL, respectively), and PAGE as previously described (19, 20). The degree of cross-contamination between the lipoprotein fractions was <0.2%, and no extraneous plasma proteins were detectable in either fraction. Contamination by transferrin in HDL preparations was on the basis of radioreceptor assays $2 \times 10^{-4}\%$.² The degree of somatomedin C contamination was estimated to be $6 \times 10^{-5}\%$.³ That of EGF was, as determined by radioreceptor assays using A-431 cells, $<5 \times 10^{-5}\%$. Contamination by glucocorticoids could not be detected (21). Contamination by FGF was, on the basis of bioassay using bovine vascular endothelial cells (22), below the level of detection (20 pg/ml) when an HDL concentration as high as 1 mg protein/ml was tested. Contamination by platelet-derived growth factors would not have been expected inasmuch as HDL is purified from plasma. This was supported by the fact that the addition of 1 mg of HDL protein/ml to bovine vascular smooth muscle cells exposed to plasma-supplemented medium did not stimulate cell proliferation.

Cell Culture Conditions: Cultures of bovine corneal endothelial (BCE) cells were established from steer eyes as already described (23, 24). Stock cultures were maintained on tissue culture dishes in DME supplemented with 10% FCS, 5% calf serum, 50 $\mu\text{g/ml}$ Gentamicin, and 0.25 $\mu\text{g/ml}$ Fungizone. Before being used, all media were passed through a Millipore filter (0.2 μm ; Millipore Corp., Bedford, MA). Brain FGF (50 ng/ml) was added every other day until the cells were nearly confluent. The endodermal cell line PF-HR-9 originally derived from the mouse endodermal carcinoma line PF-HR-9 was generously provided by Dr. R. Kramer (University of California, San Francisco). PF-HR-9 cells were maintained on tissue culture dishes in DME supplemented with 10% FCS and passaged upon reaching confluence.

Preparation of Extracellular Matrix-coated Dishes: For production of basement membrane (BM)-coated dishes, BCE cells were seeded at an initial density of 4×10^4 cells per 35-mm dish and grown in the presence of DME supplemented with 10% calf serum, 5% Dextran T-40, and FGF (50 ng/ml added every other day) (25). HR-9 cells were seeded at high density (2×10^5 cells per 35-mm dish). To reinforce the adhesion of the HR-9 BM to the plastic, we seeded the cells on fibronectin-coated dishes. Coating was done as previously described (26, 27), and cells were grown in the presence of DME supplemented with 10% calf serum. Once cultures had been confluent for 5 d (HR-9 cell cultures) or 7 d (BCE cell cultures), the media were removed and the confluent cultures were washed once with PBS. The cells were then exposed to 0.02 M NH_4OH in distilled water for 5 min, followed by washing with PBS (25, 28). This results in the denudation of the BM produced by both types of cells. While cellular elements are absent from the BM produced by BCE cells (25), little cellular debris remains closely associated with the HR-9-BM (Fig. 1) (29). These debris were only seen in areas where PF-HR-9 cells grew in small papillomalike structures. It probably reflects cell death with necrotic debris left strongly attached to the BM.

Initiation of Primary Bovine Kidney Tubule Cell Cultures: Bovine kidneys were obtained from a local slaughterhouse. The outer medullary region from one pyramid was isolated and put into a 10-cm plastic dish, where it was minced into 3-mm fragments. The tissue fragments were

² J. Lepine, personal communication.

³ J. Van Wyk and P. Chatelain, personal communication.

FIGURE 1 Scanning and transmission micrographs of HR-9 cell cultures and their extracellular matrix. (A) Scanning electron micrograph of a confluent culture of HR-9 cells before treatment with 20 mM NH_4OH ($\times 600$). (B) After treatment, the cell monolayer has disappeared and the BM produced by the cells is denuded ($\times 2,000$). A bit of cellular debris adhering firmly to the BM can be seen. The arrows point to a hole in the BM where the plastic can be seen. (C) Transmission electron micrograph showing a cross section of a part of an HR-9 cell resting on its BM. p, plastic. ($\times 6,400$). (D) BM after removal of the cell layer by treatment with 20 mM NH_4OH in water. A bit of cell debris can be seen on top of the matrix (arrowheads). p, plastic. ($\times 23,300$).

then suspended in PBS and pipetted up and down five times in a 10-ml plastic pipette. The suspension was then left to sediment for 5 min at room temperature, the cloudy supernatant was discarded, and the same process repeated five times. The supernatant of the last suspension was then put in a 10-cm tissue culture dish and observed using a Nikon phase-contrast microscope (Nikon Inc., Garden City, NY). Single segments of collecting kidney tubules were isolated using an automatic pipette equipped with a 200- μl pipette tip. Alternatively, segments of collecting kidney tubules were microdissected from the outer medulla. The isolated segments (Fig. 2A) were then put in a 35-mm BCE-BM-coated dish containing 2 ml of a 50:50 mixture of DME and F12 medium (DF medium) supplemented with 10% FCS, 50 $\mu\text{g}/\text{ml}$ Gentamicin, 0.25 $\mu\text{g}/\text{ml}$ Fungizone, and 5×10^{-8} M selenium. Cultures were kept in a humidified 37°C incubator in a 5% CO_2 /95% air environment. After 1 wk cells migrated out of the explant and the medium was renewed on a weekly basis. After 3 wks in culture, the cultures became confluent (Fig. 2B). To isolate the island of cells with an epithelioid morphology (EP cells), the primary cultures were washed with PBS and then exposed for 5 min at 37°C to a solution containing 0.9% NaCl, 0.01 M sodium phosphate (pH 7.4), 0.05% trypsin, and 0.02% EDTA (STV solution). Within 5 min most of the fibroblastic cells had rounded up and could be removed by washing the dishes with PBS. Cells with a fibroblastic appearance (FB cells) were passaged on BCE-BM-coated dishes in DF medium supplemented with 10% FCS, 50 $\mu\text{g}/\text{ml}$ Gentamicin, 0.25 $\mu\text{g}/\text{ml}$ Fungizone, and 5×10^{-8} M selenium (Fig. 2C). The isolated islands of EP cells were mechanically removed using an automatic pipette equipped with a 200- μl pipette tip. EP cell aggregates were seeded onto HR-9-BM-coated dishes in DF medium supplemented with 10% FCS, 50 $\mu\text{g}/\text{ml}$ Gentamicin, 0.25 $\mu\text{g}/\text{ml}$ Fungizone, and 5×10^{-8} M selenium (Fig. 2D). Both cell types were passaged weekly as described below at a split ratio of 1 to 40.

Cell Seeding: FB cell monolayers from stock plates grown in the

presence of serum-supplemented medium were washed with PBS and then exposed to STV solution as described above. After 5 min at 37°C the STV solution was removed and the rounded cells were resuspended in DF medium supplemented with 10% FCS. Aliquots of the cell suspension were counted in a Coulter counter (Coulter Electronics Inc., Hialeah, FL). Cells were seeded in 2 ml of DF medium at the initial density described in the legends to Figs. 2, 3, and 10–12 on 35-mm plastic tissue culture dishes coated or not with a BCE- or HR-9-BM. To trypsinize EP cells, we increased the EDTA concentration to 0.3% and the cells' monolayer was exposed to STV solution for 10–15 min at 37°C . In contrast to FB cells which trypsinize easily, EP cells dissociated in clumps of three to five cells. EP cell aggregates were resuspended in DF medium supplemented with either 10% FCS (for cultures exposed to serum-supplemented medium) or with 2 mg/ml BSA (for cultures exposed to serum-free medium). The cell aggregates were spun down and resuspended in DF medium supplemented with or without 10% FCS. EP cell density was determined using an hemacytometer. Cell viability was assessed by trypan blue exclusion test. Only free blue nuclei were observed. All cells within aggregates did not stain. Similar results were obtained when cell survival was assessed by cellular uptake and hydrolysis of fluorescein diacetate, as described earlier (30, 31). Aliquots containing the appropriate cell number were then seeded onto uncoated or coated 35 mm-tissue culture dishes.

Cell Growth Measurement and Culture Lifetime Determination: For cell growth measurements and culture lifetime determinations, cells were seeded, as described above, on 35-mm plastic, BCE-BM- or HR-9-BM-coated dishes. When cultures were to be grown in serum-free medium, seeding of the cultures was done in the presence of 2 ml of DF medium supplemented with 50 $\mu\text{g}/\text{ml}$ Gentamicin, 0.25 $\mu\text{g}/\text{ml}$ Fungizone, 5×10^{-8} M selenium, and with various factors being analyzed for their ability to support cell growth. When cultures were to be grown in serum-supplemented medium,

FIGURE 2 Cell types growing out of a segment of collecting kidney tubule dissected from the medullary region of an adult bovine kidney. (A) Collecting kidney tubule explants were seeded as described in Materials and Methods on 35-mm BCE-BM-coated dishes and exposed to DF medium supplemented with 50 $\mu\text{g/ml}$ Gentamicin, 0.25 $\mu\text{g/ml}$ Fungizone, 5×10^{-8} M selenium, and 10% FCS. (B) After 3 wk in culture, two distinct cell populations have migrated from the kidney tubule explant, one cell type forming a uniform monolayer composed of cells with a fibroblastic appearance in which a few islands (arrows) of epithelioid cells growing in isolated colonies can be seen. (C) First-passage kidney tubule cells with a fibroblastic appearance (FB cells) were seeded as described in Materials and Methods on BCE-BM-coated dishes and grown in the presence of serum-supplemented medium. Cells at confluency form a monolayer from which islands of epithelioid cells are now absent. (D) First-passage kidney tubule cells with an epithelioid appearance (EP cells) were seeded as described in Materials and Methods on HR-9-BM matrix and grown in the presence of serum-supplemented medium. At confluency a monolayer composed of small epithelioid cells from which fibroblastic cells are absent can be observed. Phase-contrast micrograph. ($\times 100$).

cells were seeded in DF medium supplemented with 10% FCS, 50 $\mu\text{g/ml}$ Gentamicin, 0.25 $\mu\text{g/ml}$ Fungizone, and 5×10^{-8} M selenium. At appropriate times triplicate plates were trypsinized for 5 min at 37°C for FB cell cultures, and 30 min at 37°C for EP cell cultures. The prolonged trypsin exposure in the case of EP cells resulted in their dissociation, after vigorous pipetting, into single cells. Cell number was determined in both cases using a Coulter counter. The morphological appearance of the cultures was observed by phase-contrast microscopy, and pictures were taken once the cultures became confluent.

Culture lifetime determinations were performed as already described (28, 29). An important factor in continuous passage of EP cells grown in transferrin and HDL-supplemented media was to avoid excessive trypsinization of confluent cultures. The cells were therefore never exposed for more than 5 min at 37°C to the STV solution. This treatment resulted in the formation of cell aggregates containing from 3 to 10 cells. The aggregates were resuspended in DF medium supplemented with BSA (2 mg/ml), and cell density was assessed using a hemacytometer. Aliquots containing the desired cell number were then seeded onto 35-mm HR-9-BM-coated dishes containing 2 ml of DME supplemented with transferrin (50 $\mu\text{g/ml}$) and HDLs (750 μg of protein/ml). To assess the plating efficiency, we trypsinized triplicate plates 12 h later as described above and counted the cells in a Coulter counter. Plating efficiency was consistently between 80 and 95%.

Morphological Studies: For scanning electron microscopy the cultures, or denuded BM-coated dishes were fixed for 1 h in 1.5% paraformaldehyde-glutaraldehyde fixative in 0.2 M sodium cacodylate (pH 7.4), 0.2% calcium chloride, and 0.05% potassium chloride. The plates were then washed in a 1:1 rinse solution of 0.5 M sucrose and 0.2 M sodium cacodylate buffer. The samples were critically point dried using the Polaron Instruments E-300 (Doylestown, PA) and CO_2 . The samples were coated with gold using the Polaron sputter coater model E-5100. A JEOL scanning microscope (model 35 U, JEOL USA, Electron Optics Div., Peabody, MA) was used to examine and photograph the samples.

For electron microscopy the culture medium was removed from the dishes and replaced with a 1.5% paraformaldehyde-glutaraldehyde fixative in 0.2 M sodium cacodylate buffered at pH 7.4 and 0.2% calcium chloride and 0.05% potassium chloride. The cells were fixed for 1 h in the paraformaldehyde-glutaraldehyde mixture, washed in a 1:1 rinse solution of 0.5 M sucrose and 0.2 M sodium cacodylate buffer, and postfixed for 2 h in 1% osmium tetroxide buffered with 0.2 M sodium cacodylate. After postfixation with osmium, the cells were washed three to six times in the rinse solution before being stained en bloc in Kellenberger's uranyl acetate stain for 1 h, dehydrated in graded 50–100% alcohol solution, and embedded in Araldite 502. Sections of 600–800 Å were cut with a Sorvall MT-2B Ultramicrotome (DuPont Instruments, Sorval

Div., Newtown, CT) and stained with uranyl acetate and lead citrate. A JEOL electron microscope (model 100C) was used to examine and photograph the samples.

For light microscopy 1- μ m sections were placed on microslides. Sections were then stained with toluidine blue and basic Fuchsin. Photomicrographs were taken with a Zeiss photomicroscope (Carl Zeiss, Inc., New York).

RESULTS

Maintenance and Propagation of Primary Bovine Kidney Cell Cultures: Substrate Requirement

Bovine kidney tubule explant (Fig. 2A) maintained on BCE-BM-coated dishes attach to the BM within 1 wk. Within 2–3 wk the dishes become covered with a cell monolayer composed of two distinct cell populations. The major cell population had a fibroblastic appearance (FB cells). The minor cell population composed of small epithelioid-looking cells (EP cells) did form discrete islands within the confluent monolayer of tightly packed fibroblastic cells (Fig. 2B). The FB cell population was propagated successfully on BCE-BM-coated dishes (Fig. 2C), whereas the islands of EP cells were best propagated on HR-9-BM-coated dishes (Fig. 2D). The

behavior of the two cell types on both types of substrate, was further analyzed by looking at their respective growth rate as a function of substrate and as a function of initial cell density.

As shown in Fig. 3A, second-passage FB cells seeded at an initial cell density of 2×10^4 cells per 35-mm dish and exposed to serum-supplemented medium did not grow appreciably over a period of 1 wk when maintained on plastic dishes. The cells adopted the morphological appearance of large and flattened cells (Fig. 3a). In contrast, when maintained under similar conditions on either HR-9- or BCE-BM, the cells grew rapidly, and became confluent within 1 wk. The average doubling time of the cultures during their logarithmic growth phase was 27 h for cells maintained on HR-9-BM and 19 h for cells maintained on BCE-BM. The final density of cultures maintained on BCE-BM, after 5 d (8×10^5 cells/35-mm dish) was higher than that of cultures maintained on HR-9-BM (3×10^5 cells/35-mm dish). While cultures maintained on BCE-BM were composed of small tightly apposed cells (Fig. 3b), cultures maintained on HR-9-BM were composed of larger cells (Fig. 3c). One could therefore conclude that BCE-BM, when compared to HR-9-BM, supports the growth of FB cells

FIGURE 3 Proliferation of kidney tubule epithelial cells on BCE-BM versus HR-9-BM-coated dishes. (A) 2×10^4 FB cells were seeded on 35-mm plastic tissue culture dishes or on either BCE-BM- or HR-9-BM-coated dishes. Cultures were exposed to DF medium supplemented with 50 μ g/ml Gentamicin, 0.25 μ g/ml Fungizone, 5×10^{-8} M selenium, and 10% FCS. Triplicate plates were trypsinized at regular intervals and cells were counted with a Coulter counter. Standard deviation was $<10\%$. In the micrographs, a–c show the final morphology of the culture after 8 d. (a) Plastic dishes. (b) BCE-BM. (c) HR-9-BM. $\times 100$. (B) 4×10^4 EP cells were seeded on 35-mm plastic tissue culture dishes or on either BCE-BM or HR-9-BM-coated dishes. Cultures were exposed to DF medium supplemented with 50 μ g/ml Gentamicin, 0.25 μ g/ml Fungizone, 5×10^{-8} M selenium, and 10% FCS. Triplicate plates were trypsinized at regular intervals, and cells were counted with a Coulter counter. In the micrographs, d–f show the final morphology of the cultures after 8 d. (d) Plastic dishes. (e) BCE-BM. (f) HR-9-BM. $\times 100$. (C) Proliferation of EP cells on HR-9-BM-coated dishes as a function of their initial cell density. EP cells were seeded at an initial cell density ranging from 5×10^3 to 2×10^5 cells per 35-mm dish on plastic dishes ($\blacktriangle$) or dishes coated with BCE-BM ($\circ$) or HR-9-BM ($\bullet$). Cultures were exposed to DF medium supplemented with 50 μ g/ml Gentamicin, 0.25 μ g/ml Fungizone, 5×10^{-8} M selenium, and 10% FCS. After 8 d in culture for cells seeded at an initial cell density of 50, 100, or 200 $\times 10^3$ cells per 35-mm dish, or after 14 d for cells seeded at an initial cell density of 5, 10, or 20 $\times 10^3$ cells per 35-mm dish, cultures were trypsinized and cells were counted in a Coulter counter. Standard deviation was $<15\%$.

best. Completely opposite results were obtained when the proliferation of EP cells was analyzed as a function of substrate (Fig. 3*B*). EP cells seeded at low density (1.5×10^4 cells/35-mm dish) on BCE-BM started to proliferate after a lag time of 4–6 days. Their growth rate was very slow (average doubling time of 48–64 h), and cells grew in small islands composed of extremely small rounded and tightly packed cells (Fig. 3*e*). When EP cells were seeded on plastic, after a lag phase of 4–6 days they started to proliferate with an average doubling time of 29 h. The culture, however, did not reach the state of confluence; instead, the cells formed discrete islands composed of very large and flattened cells with prominent ridges (Fig. 3*d*). In contrast, when seeded on HR-9-BM, EP cells started to proliferate without a lag phase, with an average doubling time of 29 h. After 8 d in culture, the cells formed a confluent monolayer (9×10^5 cells/35-mm dish). Cell proliferation continued over the following week, albeit at a diminished rate and cultures reached a final cell density by day 14 of 2×10^6 cells/35-mm dish. By that time the confluent monolayer was composed of extremely small, rounded, and tightly packed cells (Fig. 3*f*).

Cell proliferation *in vitro* is not only a function of substrate but also a function of the initial cell density to which cultures are initiated. To see whether EP cells seeded at high density could grow better on either plastic or BCE-BM, we analyzed the ability of EP cells seeded at densities ranging from 5×10^3 cells to 2×10^5 cells/35-mm dish to reach confluence as a function of the substrate upon which the cells were maintained (Fig. 3*C*).

While EP cells maintained on HR-9-BM and seeded at densities ranging from 5×10^3 to 2×10^5 cells per 35-mm dish became confluent within a period of 8 or 14 d (depending on their initial seeding density), neither culture maintained on BCE-BM or plastic became confluent, even when they were seeded at a cell density as high as 2×10^5 cells/35-mm dish. This observation therefore confirms that, irrespective of the initial cell density to which EP cell cultures were started, HR-9-BM better supported the growth of EP cells than either plastic or BCE-BM.

Morphological Differentiation of EP Cells Maintained on HR-9-BM

Light microscopy examination of confluent EP cell monolayers indicate that the cells were cuboidal or slightly elongated in shape (Fig. 4*A*). Although EP cells grew on the HR-9-BM as a monolayer, they could be present in collections of two or more cells in thickness. Occasionally they were arranged as very small papillary growths of four to six cells deep. Ultrastructural study using thin-section perpendicular to the culture plane revealed that the cuboidal EP cells have a luminal surface studded with short microvillilike extensions directed toward the medium (Fig. 4*B*). The cells were attached to one another by intracellular occluding junctions consistently pres-

FIGURE 5 Functional differentiation of EP cells maintained on HR-9-BM and grown in the presence of serum-supplemented medium. (A and B) After 1 wk at confluence, hemicyst formation can be observed in the monolayer, indicating that cells have retained their ability to transport fluid. (A) Phase-contrast micrograph with focus on the monolayer. (B) Phase-contrast micrograph with focus on the apical part of the dome. (C and D) A region where dome and tubule formation coexist. Pictures were taken when the monolayer was in focus (C) or when the apical part of the dome was in focus (D). Phase-contrast micrographs. ($\times 100$).

ent at the border between the luminal and basolateral surfaces (Fig. 4, *B* and *C*). EP cells interdigitated with one another on their lateral surfaces by complex infoldings and widened intercellular spaces were frequent and often located in the junction vicinity (Fig. 4, *B* and *C*). Interconnecting desmosomal and puncta adhering types of junctions could be observed on the lateral and abluminal surfaces (Fig. 4*C*). The nuclei containing a single prominent nucleolus was located in the central or basal part of the cells (Fig. 4*C*). Organelles consisting mainly of elongated mitochondria and cisternae of the endoplasmic reticulum were randomly distributed in the cytoplasm; numerous free ribosomes made up the bulk of the

FIGURE 4 Morphological organization and ultrastructural organization of confluent EP cells grown on HR-9-BM. (A) Low-power view of a confluent monolayer of EP cells composed of small, closely apposed, nonoverlapping cuboidal cells ($\times 960$). (B) Low-magnification electron micrograph with four cells in a monolayer formation. Microvilli are seen along their surface (a), interdigitations of the cell membrane along the basal side (b). Arrowheads indicate the widened intercellular space. Dense core granules (c) are seen in every cell ($\times 7,656$). (C) Two types of junctions are seen in this illustration. One desmosomal (a) and two puncta adhering junctions (b). The cytoplasm contains glycogen deposits (arrows), a Golgi complex (c), mitochondria (d), and rough endoplasmic reticulum (e). Prominent interdigitation of the cell membrane is seen at the basal side (arrowheads) ($\times 12,240$).

FIGURE 6 Scanning electron micrograph of confluent monolayer of EP cells showing tubular formation. (A) Confluent monolayer of EP cells maintained on HR-9-BM in the presence of serum-supplemented media and showing an extensive network of tubules ($\times 30$). (B) An area from which the tubules originate. Numerous rounded cells are in the process of forming tubules ($\times 300$) which in (C) can be seen floating in the media above the cell monolayer ($\times 400$). (D) Three tubules have formed and start to intertwine ($\times 1,600$); while in E the tubules still attached to the cell monolayer run, as in vivo, in parallel ($\times 1,000$). (F) Point of attachment of tubules floating free in the medium to the cell monolayer ($\times 1,200$). (G) Intertwined tubules ($\times 860$) with numerous villi (H) present on their abluminal surface ($\times 3,600$).

cytoplasm. A prominent Golgi complex could be observed in the basal part of the cells (Fig. 4C). Numerous dense core (secretory) granules and glycogen granules could be seen distributed evenly in the cytoplasm (Fig. 4C). A basement membrane underlying the abluminal cell surface could be seen readily. It could be either the original HR-9-BM coating the dishes, or newly formed basement material secreted by EP cells in response to their close contact with the HR-9-BM.

1 wk after reaching the stage of a cell monolayer, the EP cells started to show signs of functional differentiation. Hemicyst formation indicative of vectorial fluid transport was

observed (Fig. 5, A and B), together with the formation of a complex network of tubular structures (Fig. 5, C and D). When observed by scanning electron microscopy (Fig. 6), this network was arranged as branching strands that varied in diameter and appeared randomly distributed throughout the culture dish. In places the tubular structures were closely associated with the luminal surface of the cell monolayer, and tubules ran in parallel strands thereby adopting a morphological configuration reminiscent of that shown by collecting kidney tubules in vivo (Fig. 6E). In other areas individual strands detached partially from the monolayer, and floated in

the medium (Fig. 6C). They either remained single or intertwined forming even larger collections of tubules, which could be observed visually (Fig. 6, D and G). Light microscopy examination of perpendicular thin sections of the large tubules floating in the medium revealed that their lumen, bordered by 8–12 cuboidal cells, was filled up with amorphous material (Fig. 7). The cells forming tubules had numerous tight junctions, an interdigitated plasmalemma, and formed junctional complexes (Figs. 8 and 9). Indications that the polarity of the cells forming large tubular structures was reversed from that seen *in vivo* could be deduced from the presence of a basement membrane on their luminal surface and from the presence of numerous microvillilike extensions on the abluminal surface facing the medium (Fig. 9). In the case of small tubules, however, the cell polarity seems to be the same as *in vivo* because the lumen was free of extracellular material other than a small amount of cell debris and the cells had microvillilike extensions on their luminal surface (Fig. 8). Cells organized within large tubular structures floating in the medium were not terminally differentiated. When structures were isolated with a pipetman equipped with a 200- μ l pipette tip, tubular structures could be propagated in cultures seeded on

HR-9-BM-coated dishes. Recapitulation of the events shown in Fig. 2 was observed with the difference that only EP cells were present in the first passage.

Growth Requirement of EP Cells Maintained on HR-9-BM

Previous studies (32) have shown that HDL and transferrin could support the growth and the long-term passage under serum-free conditions of kidney epithelial cell lines such as the Madin-Darby canine kidney (MDCK) cell line. Others (33) have reported that, in the case of primary cultures of rabbit kidney epithelial cells expressing proximal tubule function, a combination of transferrin, insulin, hydrocortisone, triiodothyronine, and EGF could support cell growth. Slightly different growth requirements were reported for the porcine proximal kidney tubule epithelial cell line LLC DK-1 which required transferrin, insulin, hydrocortisone, vasopressin, and cholesterol in order to grow at a rate approaching that observed when cells were exposed to serum-supplemented medium (34). We therefore have looked at the ability of these various factors to support the proliferation of EP cells.

FIGURE 7 Organization at various stages of tubule formation of confluent EP cell cultures maintained on HR-9-BM. (A) In the first step in the formation of a tubule, the cells have started overgrowing each other and formed a multiple cell layer ($\times 960$). (B) A lumen formed by apposition of cell processes has appeared between the cells ($\times 960$). (C) A cross section of a fully formed tubule floating in the media over the cell monolayer. The inside of the lumen is filled with amorphous material ($\times 960$). (D) Tubular formation bordered by two cells. Cytoplasmic extensions of one cell engulf the lumen (A). The cell membrane (b) with an intercellular junction (c) near the cell surface opens to the lumen (d). The luminal contents consists of amorphous (e) and some electron dense material (f). Dense core granules are present in both cells (g) ($\times 7,953$).

As shown in Fig. 10A, low-density (4×10^4 cells/35-mm dish) EP cells previously maintained in the presence of medium supplemented with 10% FCS did not proliferate actively over a period of 7 d when seeded on HR-9-BM and exposed to medium supplemented with transferrin alone (50 μ g/ml), or HDL alone (750 μ g protein/ml). Addition of both transferrin and HDL resulted in active cell proliferation, and cultures after 7 d in culture each had a final cell density that was 72% of that found in control cultures exposed to serum-supplemented medium. Neither the addition of EGF (20 ng/ml), nor of insulin (5 μ g/ml) to cultures exposed to both transferrin and HDL had any significant effect on cell proliferation. Addition of cholera toxin (10 mg/ml) had a small but significant effect which was potentiated by the presence of EGF (20 mg/ml). One could therefore conclude that the two major plasma factors involved in the control of proliferation of EP cells are HDL and transferrin.

The proliferation of FB cells (4×10^4 cells/35-mm dish) exposed to serum-free medium was also markedly improved by the addition to the medium of 50 μ g of transferrin/ml and 750 μ g of HDL protein/ml. The requirements for a proper substrate in order for FB cells to proliferate actively when exposed to serum-free conditions were even more stringent than when they were exposed to serum-supplemented medium. Their final cell density after 8 d in culture were 5.64×10^5 cells for cultures maintained on BCE-BM versus 1.6×10^5 cells and 3.2×10^4 for cultures maintained on HR-9-BM and plastic, respectively (unpublished observation).

The effect of increasing concentrations of HDL on the proliferation of low-density (2×10^4 cells/35-mm dish) EP cells maintained on HR-9-BM-coated dishes and exposed to medium supplemented with transferrin (50 μ g/ml) is shown in Fig. 10B. HDL had a minimal effect when present at concentrations of 100 μ g of protein/ml or less. In that range of HDL concentration, the cells differentiated readily forming small tubular structures. Concentrations of HDLs in the range of 250–2,000 μ g of protein/ml induced active cell proliferation, with saturation at 1,000 μ g of protein/ml. When the growth-promoting effect of transferrin was analyzed using low-density EP cell cultures maintained on HR-9-BM-coated dishes and exposed to medium supplemented with 750 μ g of protein/ml, it had a marginal mitogenic effect at concentrations of 1 μ g/ml and saturated at concentrations of 50 μ g/ml (Fig. 10C).

In order to confirm the ability of HDL and transferrin to support the growth of EP cells, we have compared their growth rate and final cell density when maintained on HR-9-BM and exposed to medium supplemented with serum versus medium supplemented with either HDL and transferrin, or transferrin alone (Fig. 10D). Cultures exposed to serum supplemented medium divided during their logarithmic growth phase with a doubling time of 29–32 h and reached a final cell density of 2×10^6 cells/35-mm dish after 8 d in culture. A slower growth rate (48 h) was observed when cultures were exposed to medium supplemented with HDL and transferrin, but, after

10 d in culture, the final cell density was the same as that of cells exposed to serum. When cells were exposed to transferrin alone they did not divide and started to die after 4 d in culture. The ability of transferrin and HDL to support the proliferation of EP cells was not limited in time in that cells could be passaged repeatedly in absence of serum. While cultures exposed to serum-supplemented medium had a limited lifespan in culture of 50 generations, the lifespan of EP cells exposed to HDL and transferrin was 32 generations (Fig. 11). Organization into large tubular structure could be observed until passage 12 (43 generations) with cultures propagated in the presence of serum-supplemented medium and passage 9 (30 generations) with cultures passaged in serum-free medium.

The growth promoting effect of HDL could not be replaced by a combination of a variety of factors including insulin (10 μ g/ml), EGF (20 ng/ml), hydrocortisone (2×10^{-7} M), triiodothyronine (10^{-9} M), vasopressin (10 μ g/ml), and cholesterol (10^{-8} M) (Fig. 12). Likewise, prostaglandin E_1 (25 ng/ml) substituting for vasopressin had no mitogenic effect (unpublished observation), and FGF (20 ng/ml) had no effect either (unpublished observation). It was only when HDL was added on top of the factors listed above that EP cells started to proliferate actively. Since one of the roles of HDL could be to indirectly increase the cell cholesterol supply by activating 3-hydroxy-3-methylglutaryl coenzyme A reductase, we have analyzed whether or not delivery of cholesterol by LDL (50 μ g protein/ml) to the cells could replace the requirement for HDL. As shown in Fig. 12 it did not; instead, it markedly reduced the growth stimulus provided by HDL.

DISCUSSION

The present study emphasizes the role of basement membrane produced by cultured cells in the control of proliferation and differentiation of kidney tubule epithelial cells. While the BM produced by cultured BCE cells favors the proliferation of the fibroblastic population, the BM produced by the mouse teratocarcinoma-derived endodermal cell line PF-HR-9 favors the proliferation of the epithelioid population.

The behavior of both cell types on these BMs correlates with their known composition. The basement membrane produced by BCE cells is mostly composed of collagens, proteoglycans, laminin, fibronectin, and elastin (35–37). The type of collagen present is mostly type III, with smaller amounts of collagen type I and BM collagen type IV and V associated with it (35). Proteoglycans are mostly heparan and chondroitin sulfate proteoglycans (36). This type of BM has been shown to best support the proliferation and differentiation of squamous epithelial cells such as vascular and corneal endothelial cells (38–40), as well as fibroblast and vascular smooth muscle cells. In contrast, the HR-9-BM is mostly composed of basement membrane collagen type IV, heparan sulfate proteoglycans, laminin, and entactin (41–43). It therefore has a composition very similar to that of the kidney epithelial cell BM and would have been expected to support

FIGURE 8 Ultrastructural characteristics of EP cells lining the lumen of a small tubule. (A) A small tubule (a) surrounded by the cytoplasm of two cells. The dividing intercellular membrane opens to the lumen on two sides (b). Junctions along the cell membrane are indicated (arrows). Vesicular debris is seen in the lumen (arrowheads). Dense core granules (c) and bundles of filaments (d) are abundant ($\times 7,326$). (B) High-magnification electron micrograph, showing a detail of a tubular wall with the cytoplasm of an adjacent cell. Microvilli with radiating filaments are lining the lumen. Dense core granules (a), rough-surfaced endoplasmic reticulum (b), mitochondria (c), and bundles of filaments (d) are seen adjacent to the nucleus (e) ($\times 25,896$).

FIGURE 9 Ultrastructural characteristics of EP cells lining the lumen of a large tubule. (A) Transmission electron micrograph showing a large tubular structure. Microvilli are covering the surface of the cells (a) that are lining the tubular lumen. The contents of the luminal space are composed of amorphous, fine granular and vesicular material (b). Material of similar consistency is deposited on the basal side of the tubule (c) ($\times 6,600$). (B) A higher-magnification electron micrograph with a cell portion lining the tubular lumen (a). The material within the lumen consists of filamentous material (b) and fine granular substances (c). A few pinocytotic vesicles are also present (d) ($\times 17,500$).

FIGURE 10 Proliferation of kidney epithelial cells (EP cells) in serum-supplemented medium versus medium supplemented with transferrin, HDL, EGF, insulin, or cholera toxin. (A) 4×10^4 EP cells were seeded as described in Materials and Methods onto 35-mm HR-9-BM-coated dishes in DF medium supplemented with 10% FCS or supplemented with 50 $\mu\text{g/ml}$ Gentamicin, 0.25 g/ml Fungizone, 5×10^{-8} M selenium, and either 10% FCS or one of the following combination of factors: (i) transferrin alone (T, 50 $\mu\text{g/ml}$); (ii) transferrin and 750 μg HDL protein/ml, (TH); (iii) transferrin, HDL, and EGF (20 ng/ml) (THE); (iv) transferrin, HDL, and insulin (5 $\mu\text{g/ml}$) (THI); (v) transferrin, HDL, insulin, and cholera toxin (20 ng/ml) (THI + CT); (vi) transferrin, HDL, insulin, EGF, and cholera toxin (THIE + CT). Triplicate plates were counted after 8 d in culture. Standard deviation was $<10\%$. (B and C) 2.5×10^4 EP cells were seeded onto 35-mm HR-9-BM-coated dishes in DF medium supplemented with 50 $\mu\text{g/ml}$ Gentamicin, 0.25 g/ml Fungizone, 5×10^{-8} M selenium, and either 50 μg of transferrin/ml (B) or 500 μg of HDL protein/ml (C). Increasing concentrations of HDL (ranging from 10 μg to 2 mg of HDL protein/ml) (B) or increasing concentrations of transferrin (ranging from 0.5 to 100 $\mu\text{g/ml}$) (C) were then added to triplicate dishes. Cells were counted after 8 d in culture. Standard deviation was $<10\%$. In the case of plates exposed to low-HDL concentration (10–250-HDL protein/ml), differentiation into tubules occurred after 3 d in culture. (D) 7×10^4 EP cells were seeded onto 35-mm HR-9-BM-coated dishes and exposed to DF medium supplemented with 50 $\mu\text{g/ml}$ Gentamicin, 0.25 $\mu\text{g/ml}$ Fungizone, 5×10^{-8} M selenium, and either 10% FCS (10% FCS, Δ), or a combination of 50 μg of transferrin/ml and 750 μg of HDL protein/ml (TH, $\bullet$), or 50 μg of transferrin/ml alone (T, $\circ$). Triplicate plates were trypsinized and counted at regular intervals. Standard deviation was $<10\%$.

FIGURE 11 Comparison of the EP cell lifespan when maintained on HR-9-BM and exposed to medium supplemented with either 10% fetal calf serum or transferrin and HDL. EP cells seeded at 4×10^4 cells/35-mm HR-9-BM-coated dish were exposed to DF medium supplemented with 50 $\mu\text{g/ml}$ Gentamicin, 0.25 $\mu\text{g/ml}$ Fungizone, 5×10^{-8} M selenium, and either 10% FCS ($\bullet$), or 750 μg of HDL protein/ml and 50 μg of transferrin protein/ml ($\circ$). The number of generations was determined from the initial cell density 12 h after seeding and the number of cells harvested at each transfer. Each point represents a single transfer. Roman numerals indicate the passage number. Cultures were passaged at weekly intervals.

the proliferation and differentiation of that cell type.

Among the plasma factors involved in the control of proliferation of kidney epithelial cells maintained on HR-9-BM are transferrin and high-density lipoproteins. Both of these factors are normal plasma components and have been shown in previous studies to support the proliferation of the MDCK cell line (32) originally derived from the distal part of the kidney tubule (44, 45). Although it is likely that the role of transferrin is to deliver iron to the cells (46), that of HDL could be its ability to stimulate 3-hydroxy-3-methylglutaryl coenzyme A reductase activity (47), thereby resulting in increased mevalonate production. This metabolite is a precursor of nonsterol isoprene products such as dolichols, ubiquinones, and isopentenyl tRNA (48–51), which could be critical for cell growth (52, 53) as well as for cell shape maintenance (54). A requirement for transferrin in order for kidney nephrons to appear within the metanephric mesenchyme maintained in organ culture and induced by the spinal cord has recently been reported by Eckblom et al. (55, 56). Whether the acquisition of transferrin responsiveness is caused by the de novo appearance of transferrin receptors or whether it is related to changes in basement membrane composition during the induction process is not known (56). It nevertheless raises the possibility that one of the mechanisms by which the HR-9-BM supports the growth of EP cells could be through its ability to stimulate the appearance of the transferrin receptor.

FIGURE 12 Proliferation of EP cells in medium supplemented with various combinations of transferrin, insulin, hydrocortisone, triiodothyronine, vasopressin, cholesterol, HDL, and LDL. 4×10^4 EP cells were seeded on either 35-mm HR-9-BM, or BCE-BM-coated dishes or on plastic dishes. Cells exposed for 8 d to DF medium supplemented with 50 μ g/ml Gentamicin, 0.25 μ g/ml Fungizone, 5×10^{-8} M selenium, 50 μ g/ml transferrin, 10 μ g/ml insulin, 10^{-9} M triiodothyronine, 2×10^{-7} M hydrocortisone 10 μ U/ml vasopressin, and 10^{-8} M cholesterol (medium K). In the case of cultures maintained on HR-9-BM-coated dishes, cholesterol present in medium K was replaced by either 500 μ g of HDL protein/ml (MED-K-Chol + HDL) or by 50 μ g of LDL protein/ml alone (MED-K-Chol + LDL) or by a combination of 500 μ g of HDL protein/ml and 50 μ g of LDL protein/ml (MED-K-Chol + LDL). After 8 d in culture, cultures were trypsinized and cells counted in a Coulter counter. Standard deviation was <10%.

Alternatively, since previous studies have outlined the importance of cell shape in the proliferative response of cultured cells to plasma or serum factors (57–59), it is possible that only on HR-9-BM would the EP cells adopt a shape which would let them respond to the growth stimulus provided by both HDL and transferrin.

The growth requirement of bovine kidney epithelial cells derived from the collecting tubules differed from that shown by other kidney tubule segments. Taub and Sato (60) have reported that insulin, transferrin, prostaglandin E_1 , triiodothyronine, and hydrocortisone support the growth of baby mouse kidney epithelial cells, a similar requirement has also been shown by MDCK cells (61), a cell line of canine origin. Chuman et al. have reported (34) that in the case of proximal tubular kidney epithelial cells of porcine origin (LLC-PK₁ cell line) transferrin, triiodothyronine, vasopressin, hydrocortisone, insulin, and cholesterol were required. Similar requirements, although quantitatively different, were reported by Chung et al. (33) to support the growth of primary rabbit kidney cultures that express proximal tubule function in a hormonally defined medium. In our hands, none of these factors supported the growth of EP cells. Proliferation was only observed when HDL was added to the various combinations of factors cited above. This could be due to the origin of the EP cells, which are derived from collecting tubules, versus cells derived from proximal and distal segments of the kidney tubule. It could also reflect a species difference, in that EP cells are from bovine origin in contrast to other kidney

epithelial cells, which are from species as different as rodents (60), dogs (61), lagomorphs (33), or pigs (34).

Once reaching confluence, EP cells maintained in serum supplemented medium did show signs of differentiation as reflected by their morphological polarity unique to transporting epithelia with apical microvilli extending upward into the medium and occluding junctions joining adjacent cells at the apical and basolateral membrane border. Evidence from vectorial transport of fluid can be inferred from the presence of hemicyst formation similar to those formed by MDCK cell cultures (62).

The unexpected finding, however, was that EP cells maintained on HR-9-BM can be induced to mold into a tissue by themselves, building a complex three-dimensional kidney tubule network. Although in cultures exposed to serum-supplemented medium, this network formed only when the cells reached the stage of a confluent monolayer, in the case of cultures maintained in transferrin-supplemented medium and exposed to concentrations of HDL below 250 μ g of protein/ml, it did appear even in sparse cultures. This indicates that cells do not need to become confluent in order to form tubular structures. It is more likely that these structures can form only when the rate at which EP cells divide starts to decrease. This would occur either at confluency in the case of cultures exposed to serum-supplemented medium, or in sparse cultures in the case of cells exposed to medium supplemented with low HDL concentrations. The importance of the composition of the BM, on which cells rest, to induce them to form tubules is evident, inasmuch as no such formation was observed when EP cells were maintained on either plastic or BCE-BM, despite the fact that cells proliferated at a slow rate, a condition that did favor tubule formation for cells maintained on HR-9-BM.

Others have reported that MDCK cells growing as a monolayer on collagen gel can undergo reorganization when overlaid with another collagen layer and coalesced to form large cavities comparable in size to a tubule (63). No tubule formation was, however, observed (63). This is therefore the first report of kidney tubule formation from single cells in vitro.

Two types of tubules can be observed in the cultures. Those with a small lumen bordered by three to four cells had their lumen free of amorphous material and have microvilli on the luminal surface as well as BM material closely associated with their abluminal surface. Such tubules therefore exhibit a morphological polarity similar to that seen in vivo. In contrast, the larger tubules had an inverse polarity, their lumen bordered by 8–12 cells held together with junctional complexes and an interdigitating plasma membrane was filled with amorphous material. BM material could be seen closely associated with their luminal surface while their abluminal surface were covered with microvilli. Initial attempts to reverse the polarity of such tubules by adding gelatin to the medium as described by Mauchamp et al. (64) for thyroid follicles has been unsuccessful. The reverse polarity of the cells forming large tubules is not unlike that observed for capillary, venous, or arterial endothelial cells forming tubules in vitro (65–67). Cells forming large tubular structures were not terminally differentiated because isolated tubules when seeded on HR-9-BM matrix gave rise to homogeneous EP cell cultures. Such cultures could be successfully passaged, and did reorganize at confluency into tubular structures.

In conclusion, the present study demonstrates that when maintained on the proper substratum, kidney epithelial cells

still retain morphogenetic capabilities that can be analyzed in culture. This relatively simple model may prove valuable for the study of the various steps involved in cellular organization into tissues and the role of the various components of the BM in that process.

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