Kidney International, Vol. 33 (1988), pp. 677–684

# Glomerular epithelial, mesangial, and endothelial cell lines from transgenic mice

KAREN MACKAY, LILIANE J. STRIKER, SHARON ELLIOT, CARL A. PINKERT, RALPH L. BRINSTER, and GARY E. STRIKER

*Metabolic Disease Branch, National Institute of Diabetes, Digestive and Kidney Disease, Bethesda, Maryland, and School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA*

**Glomerular epithelial, mesangial, and endothelial cell lines from transgenic mice.** The culture of glomerular cells has represented an important tool in the understanding of individual glomerular cell functions. However, the complexity of the glomerulus has made it difficult to obtain pure cell populations. It has also been difficult to culture glomerular endothelial cells, even as mixed cell populations. At present there are no established glomerular cell lines from any source. We have established permanent cell lines of cloned glomerular epithelial, mesangial, and endothelial cells from a line of mice transgenic for the early region of simian virus 40 (SV40). These mice appear normal at birth but by three to four months of age have sclerosis affecting a variable percentage of their glomeruli. The cells maintain features characteristic of their normal counterparts despite their transformed phenotype. These cell lines could be useful tools in understanding the pathogenesis of glomerulosclerosis in this transgenic mouse model and in studying those features of normal glomerular cell biology which are not altered by a transformed phenotype.

The complexity of the intact glomerulus has made glomerular cell culture an attractive tool for use in the study of glomerular cells. Two obstacles in the use of such cultures are the difficulty of obtaining pure cell populations and their finite in vitro life span [1, 2]. It has also been extremely difficult to obtain and maintain glomerular endothelial cells in culture [3].

Because of these problems, the establishment of lines of glomerular cells could be advantageous in the study of glomerular cell biology. We have recently identified a line of mice transgenic for the early region of the DNA tumor virus simian virus 40 (SV40) that develops a progressive glomerulosclerosis which closely resembles the lesions seen in human focal glomerulosclerosis [4, 5]. These animals appear normal at birth, but develop choroid plexus tumors and sclerosis involving a variable percentage of their glomeruli by three to four months of age.

We have isolated glomerular cells from these transgenic mice and from their normal litter mates. We have cloned and maintained glomerular epithelial, mesangial and endothelial cells from the transgenic mice in culture for over 40 passages at a 1:10 split ratio (indicating over 130 population doublings). The cells from the transgenic mice appear to have an infinite culture life-span. Despite their transformed phenotype they maintain several characteristics of their normal counterparts.

Received for publication September 11, 1986
and in revised form August 10, 1987



## Methods

### *Isolation and cloning of transgenic glomerular cells*

Seven, 10-week-old transgenic mice (C57B1/6J × SJL/J) were sacrificed with an overdose of ether. Kidneys were removed using sterile technique and the cortex diced into small (2 $mm^3$) pieces which were forced through a 200 grade, stainless steel mesh. Unencapsulated glomeruli, which pass through this mesh, were allowed to sediment by gravity in a sterile tube filled with a defined MEM/F12 medium (3:1) [6, 7, as modified by P. Rabinovitch, personal communication] supplemented with 1 mm glutamine (Gibco, Grand Island, New York, USA) 100 $\mu$/ml penicillin, and 100 $\mu$g/ml streptomycin (Gibco) (hereafter referred to as basal medium). After sedimentation the upper portion of the medium, which contained single cells and small tubular fragments, was aspirated off. This sedimentation was repeated several times until the glomerular preparation was free of single cells. The resulting preparation contained approximately 30% tubular fragments.

Glomeruli were plated in 100 mm culture dishes (Nunc, PGC Scientifics Corp., Gaithersburg, Maryland, USA) which were coated with a thin layer of a solution containing 200 $\mu$g/ml human fibronectin (Collaborative Research, Lexington, Massachusetts, USA). Culture medium consisted of basal medium supplemented with 10% Nu serum$^R$ (Collaborative Research), and 30 ng/ml epidermal growth factor (EGF) (Collaborative Research). Approximately 100 glomeruli were plated in each dish.

Plates were examined after 7 to 14 days of undisturbed growth at 37°C in 5% $CO_2$ in air. Individual glomeruli and their surrounding outgrowth were selectively trypsinized using a cloning ring (to avoid trypsinization of tubules and their outgrowth) and passed to 16 mm fibronectin coated dishes. Cells were trypsinized by first gently washing the cell layer with versene (1:5000) (Gibco) followed by incubation with trypsin (0.5 g/100 ml) (Gibco) until the majority of cells had rounded up. At confluence cells in the 16 mm wells were trypsinized and passed to a fibronectin coated, 96 well plate. The cell suspension was diluted so that approximately 100 cells were plated in

each 96 well plate. The plates were examined after 12 hours of incubation and wells containing only one cell were circled and served as the source of clones.

After cloning the cells were maintained in culture with twice weekly passage at a 1:10 split ratio. The cells were found not to require EGF for growth or fibronectin for efficient plating. The concentration of these components was decreased and then discontinued between passages 5 and 8. As the normal cells adhered better in the presence of fetal bovine serum (FBS) than in Nu serum$^R$ the transgenic cells were gradually changed to FBS (Gibco) supplementation so as to maintain the transgenic and normal cells under more similar culture conditions. This was done during passages 8 to 10. Five percent FBS was chosen as all three cell lines appeared to do well in this serum concentration.

### *Isolation of glomerular cells from normal mice*

The techniques used were the same as for the transgenic mice except that six 4-week-old mice (C57B1/6J × SJL/J) were used. We were unsuccessful in cloning these glomerular cells using 96 well plates. Instead, 500 to 1000 homogenous appearing cells were plated in a 100 mm dish. Isolated patches were selectively trypsinized two to four weeks later and served as the source of normal glomerular cell populations.

Normal populations were found not to require EGF for proliferation, so its use was discontinued during passages 5 to 8. The use of fibronectin was continued, as these cells plated very poorly without it. After isolation of the patch clones it was noted that both epithelial and mesangial cells detached from the culture dishes when near confluence. This did not occur when the cultures were changed to supplementation with 10% FBS in place of Nu serum$^R$. This change was gradually accomplished between passages 4 to 9. Cultures were passaged one to two times per week at a 1:4 split in basal medium supplemented with 10% FBS in fibronectin coated flasks. The normal cells were maintained in 10% FBS rather than the 5% FBS in which the transgenic cells were maintained because the normal cells did not tolerate a lower serum concentration.

### *Immunofluorescence staining*

Ten thousand to 20,000 cells were plated in each well of fibronectin coated, 8 well Lab Tek$^R$ chamber slides (Miles Scientific, Naperville, Illinois, USA) in basal medium supplemented with 5% FBS for transgenic cells or 10% FBS for normal cells. Slides were incubated at 37°C in 5% $CO_2$ in air overnight. Slides were washed once with phosphate buffered saline (PBS), fixed at 37°C with 2% paraformaldehyde in PBS, and permeabilized with 0.1% triton in PBS. Cells were incubated with normal goat serum (Serotec, Indianapolis, Indiana, USA), followed by the unlabeled first antibody of interest. Cells were washed, incubated with the appropriate FITC conjugated goat antibody, washed and covered with coverslips mounted with several drops of a solution of 50 mg p-phenylenediamine (Sigma, St. Louis, Missouri, USA), 5 ml PBS, 45 ml glycerol (Sigma).

The antibodies used were: rabbit anti-human factor VIII related antigen (Behring Diagnostics, La Jolla, California, USA), mouse monoclonal anti-human cytokeratin (Enzo Biochem, New York, New York, USA), mouse monoclonal anti-T-antigen (Oncogene, Mineola, New York, USA), FITC conjugated goat anti-rabbit IgG and anti-mouse IgG, and IgM (Tago, Burlingame, California, USA), FITC conjugated goat anti-rat IgM (Cappel, Cochranville, Pennsylvania, USA). Rhodamine conjugated phalloidin (Molecular Probes, Junction City, Oregon, USA) was used to detect actin.

SSEA3 is an antibody raised in rat which recognizes mouse distal tubules cells and not glomeruli [8]. Cells stained with this antibody were not permeabilized with triton.

### *Angiotensin II contractility*

Cells were plated on 35 mm dishes coated with a 1:1000 dilution of Hydron Polymer (Hydro Med Science, New Brunswick, New Jersey, USA) as described [9]. After eight hours of incubation at 37°C in 5% $CO_2$ in air, dishes were washed in serum free medium then exposed to fresh serum-free medium either with or without $10^{-6}$ M angiotensin II (Boehringer Mannheim, Indianapolis, Indiana, USA). Phase contrast photographs of the same field with the same magnification were taken immediately after the addition of the fresh medium and again after 10 minutes of incubation at room temperature, and were compared.

### *Growth in soft agar*

Growth of the transgenic cells in soft agar was assessed as described [10], except that the cells were tested in the presence of 5% FBS. Balb 3T3 cells (ATCC CCL 163), used as a negative control, did not form colonies when tested under these conditions.

### *Histochemical assays for gamma-glutamyl transpeptidase and alkaline phosphatase activity*

Confluent 35 mm dishes of glomerular epithelial cells and LLC-PK1 cells (ATCC CRL 1392) were washed in PBS then frozen in liquid nitrogen. They were tested for gamma-glutamyl transpeptidase activity as described [11].

Confluent monolayers of glomerular epithelial cells and LLC-PK1 cells were assayed for alkaline phosphatase activity using a modification of the procedure described by Wachsmuth and Torhorst [12]. In brief, monolayers were washed in PBS then incubated for 15 minutes at room temperature in a freshly made and filtered solution containing 50 mM Tris-HCl (pH 9.0), 0.33 mM naphthol AS-BI phosphate (Sigma), and 3 mM fast blue BB. The reaction was stopped by incubation in 100 mM phosphate buffer (pH 8.0). Monolayers were then rinsed in PBS and examined.

### *Growth curves*

Cells were plated at 8 to 15,000 cells per well of 24 well plates in basal medium with 5% FBS for the transgenic cells and in basal medium with 10% FBS on fibronectin coated dishes for the normal mesangial cells. After 48 hours the cells were washed with serum-free medium, and the medium replaced with 1 ml of serum-free medium, in which they were incubated for an additional 48 hours. Medium was then changed to basal medium containing 5%, 1%, or 0% FBS. Cell counts were assessed by trypsinizing triplicate wells and counting them with an Elzone$^R$ cell counter (Particle Data Inc., Elmhurst, Illinois, USA). The thoroughness of trypsinization was checked by examination of culture wells following trypsinization using phase contrast

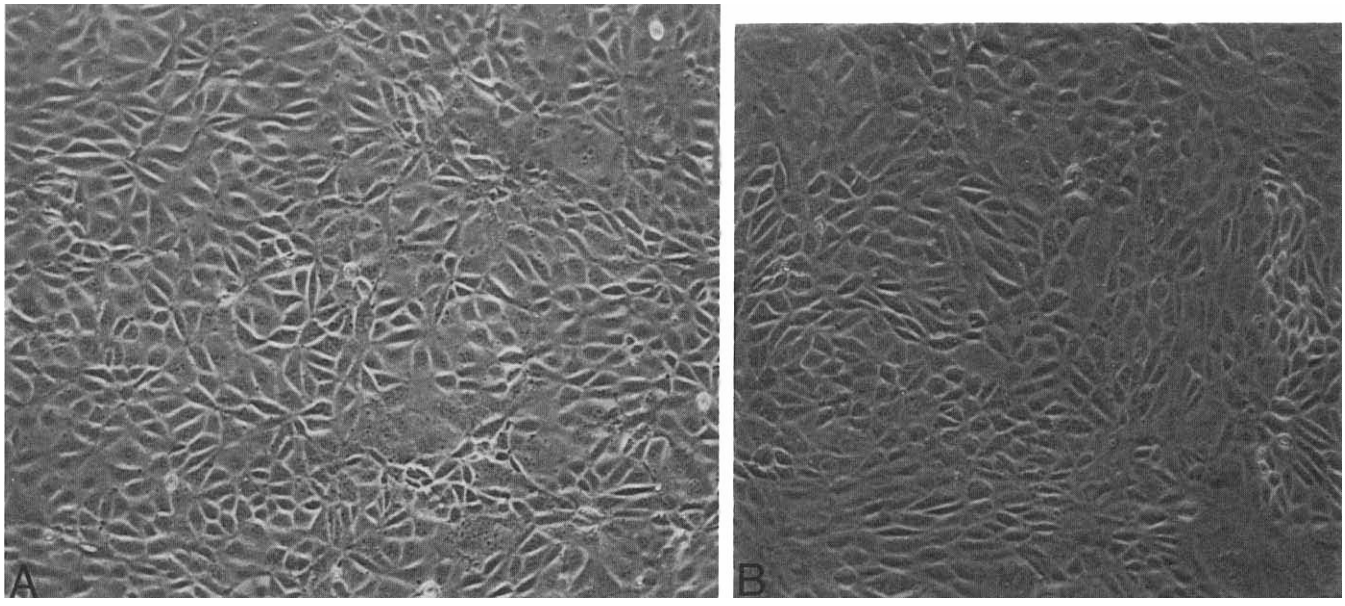


**Fig. 1.** *Transgenic (A) and normal (B) glomerular epithelial cells.* Both cell types grow as a monolayer of cobblestone appearing cells. (×100)

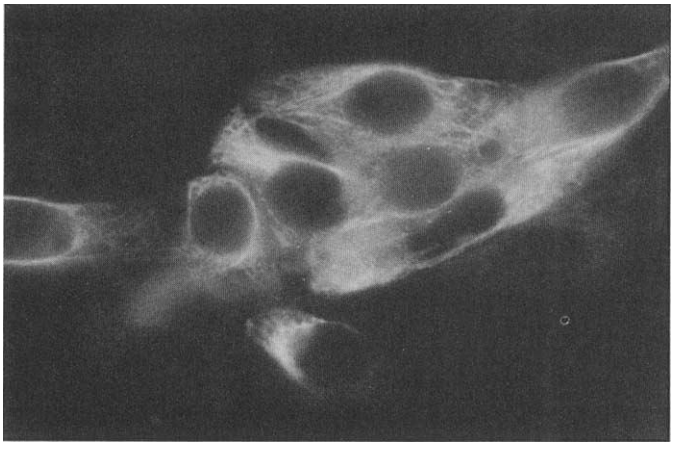

**Fig. 2.** *Immunofluorescence staining of transgenic mouse epithelial cells with monoclonal antibody against human cytokeratin.* (×630)

microscopy. Complete trypsinization was achieved for all cell types.

## Results

### *Glomerular epithelial cells*

Glomerular epithelial cells isolated from transgenic and normal mice were identified by their cobblestone morphology at confluence (Fig. 1), their cytoskeletal staining with anti-cytokeratin antibody (Fig. 2), negative staining for factor VIII related antigen, and failure to contract in the presence of angiotensin II.

Since the initial glomerular isolates were heavily contaminated with tubules, great care was taken to selects structures and their outgrowths that were unquestionably glomerular for trypsinization and passage. Nonetheless, the possibility remained that a tubular fragment could resemble a glomerulus and serve as the source of epithelial cell clones. This possibility was evaluated by histochemical determination of gamma-glutamyl transpeptidase and alkaline phosphatase activity. These enzymes are present in proximal tubular cells and absent in

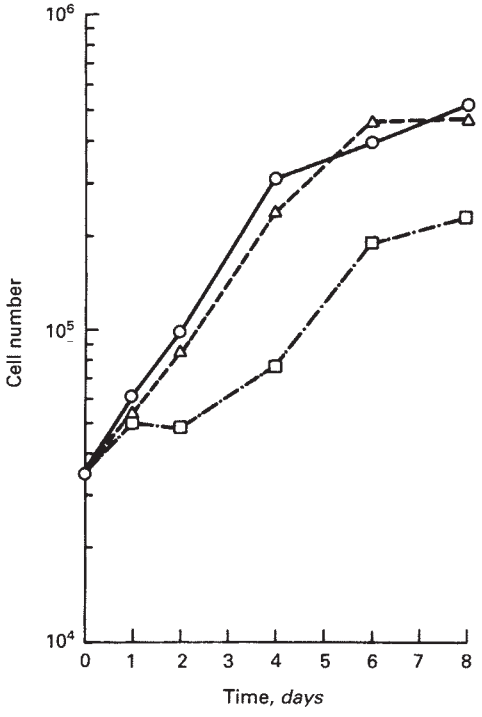


**Fig. 3.** *Growth of transgenic epithelial cells in varying concentrations of FBS.* These cells continued to proliferate after confluence was reached and were able to proliferate in serum free medium. Symbols are: (○) 5%; (△) 1%; (□) 0%.

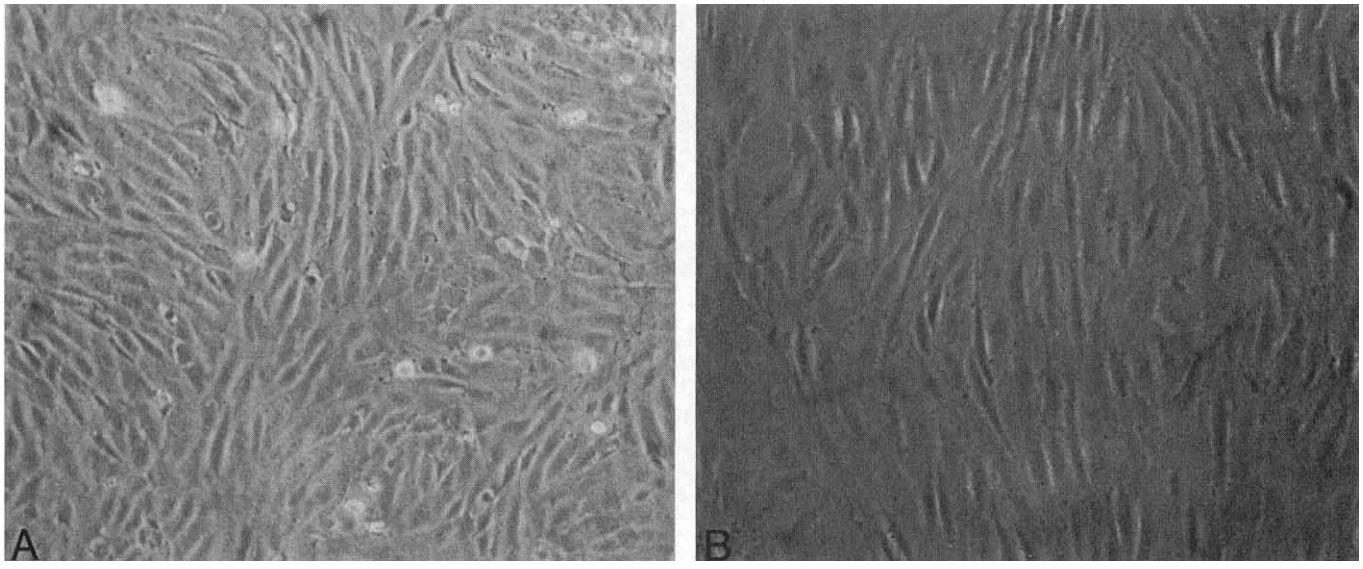


**Fig. 4.** *Transgenic* (**A**) *and normal* (**B**) *mesangial cells.* Both cell types grew as a monolayer of spindle shaped cells. (× 100)

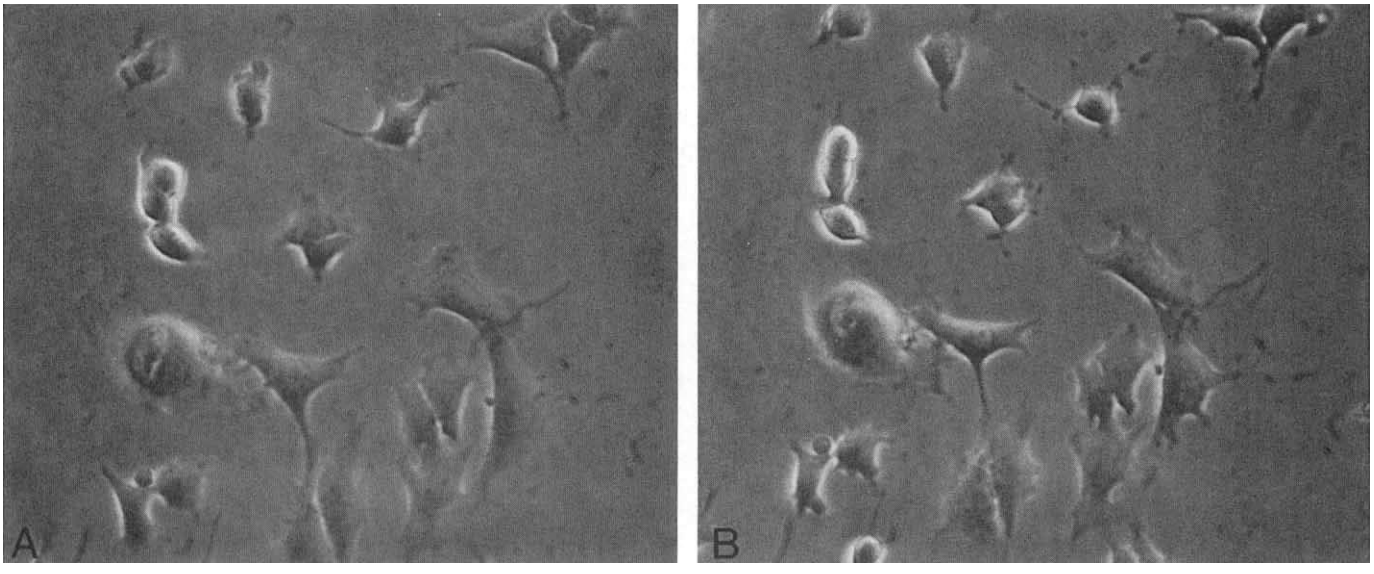


**Fig. 5.** *Contraction of transgenic mesangial cells in response to angiotensin II.* (**A**) Cells immediately after the addition of $10^{-6}$ M angiotensin II. (**B**) Same cells 10 minutes later. The majority of cells display changes in cell shape in response to angiotensin. (×250)

glomerular epithelial cells [11, 12]. The normal and transgenic glomerular epithelial cells were negative, while LLC-PK1 cells were strongly positive in both assays (data not shown). Cells were also stained with SSEA-3 antibody, which recognizes determinants on mouse distal tubular cells [8]. The normal and transgenic glomerular epithelial cells did not stain with this antibody.

While the normal cells initially appeared to represent a homogenous population of epithelial cells, we noted after additional passages, and freezing and thawing, that the cells appeared more elongated. The cells were restained and were found to be cytokeratin negative, factor VIII antigen negative, and to have a distribution of actin typical of mesangial cells. Thus, the technique of "patch cloning" normal mouse glomerular epithelial cells from dilute plates yielded populations of epithelial cells which were apparently contaminated with small numbers of mesangial cells which became the predominant cell type with serial passages. In contrast, the cloned transgenic epithelial cells maintained their original morphology and immunofluorescence staining patterns after multiple passages and freeze/thawings.

The growth characteristics of transgenic glomerular epithelial cells in varying concentrations of FBS are shown in Figure 3. The cells had a doubling time of 30 hours in 5% FBS, were only

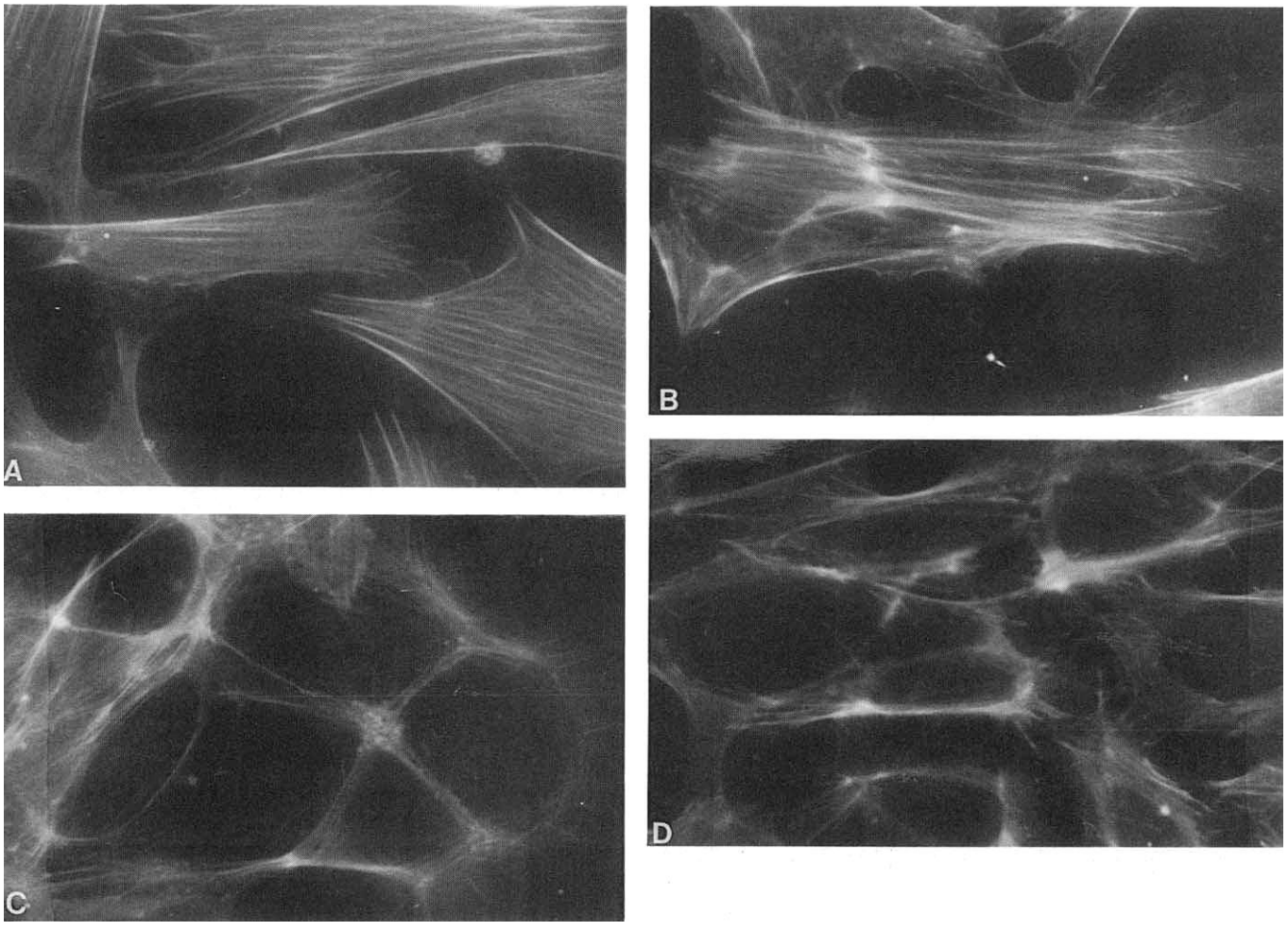


**Fig. 6.** *Staining of transgenic* (**A**) *and normal* (**B**) *mesangial cells for actin using rhodamine conjugated phalloidin.* Mesangial cells have actin filaments running throughout the cytoplasm while actin in both transgenic glomerular epithelial (**C**) and endothelial (**D**) cells is distributed predominantly about the cell periphery. (×630)

partially contact inhibited, and were able to proliferate in the absence of serum. The cells formed small colonies in soft agar and displayed nuclear staining for the SV40 gene product, large T-antigen.

### *Glomerular mesangial cells*

Glomerular mesangial cells were isolated from both transgenic and normal mice. The cells were identified by their appearance as elongated cells growing in parallel arrays (Fig. 4), their failure to stain with antibodies to cytokeratin or factor VIII related antigen, and their contraction in response to $10^{-6}$ M angiotensin II (Fig. 5). Mesangial cells displayed prominent cytoskeletal staining for actin with abundant parallel fibrils throughout the cytoplasm. In contrast, epithelial and endothelial cell actin was located predominantly in the cell periphery (Fig. 6).

While early passage cultures displayed a morphology of elongated spindle shaped cells, later passage cultures (over twenty) from both normal and transgenic mice became less elongated. Despite this morphologic variation, the immunofluorescence pattern of actin staining remained unchanged. The cells also remained cytokeratin and factor VIII related antigen negative.

The transgenic cells grew more rapidly than the normal cells, with doubling times in 5% FBS of 26 and 40 hours, respectively (Fig. 7). In addition, proliferation of mesangial cells derived from normal mice slowed as the cells reached confluence (approximately $8 \times 10^5$ cells/well) while the transgenic cells continued to proliferate after the cells reached confluence. To test whether the observed differences in proliferative characteristics of the transgenic and normal mesangial cells were secondary to the different conditions in which the transgenic and normal mesangial cells were maintained and plated, we maintained the transgenic mesangial cells in fibronectin coated flasks in 10% FBS for several passages. These cells were then plated in 10% FBS on plastic or fibronectin. The results for cells plated on plastic or fibronectin were identical. The cells did not display contact inhibition of proliferation but did have a slight prolongation of their doubling time to 30 hours (data not shown). The

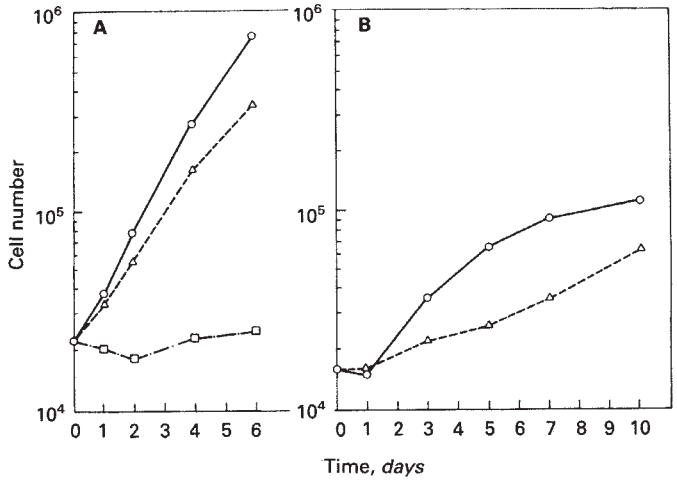


**Fig. 7.** *Growth of transgenic* (**A**) *and normal* (**B**) *mesangial cells in varying concentrations of FBS.* While proliferation of normal mesangial cells is inhibited at culture confluence the transgenic cells continue to proliferate despite culture confluence. Symbols are: (○) 5%; (△) 1%; (□) 0%.

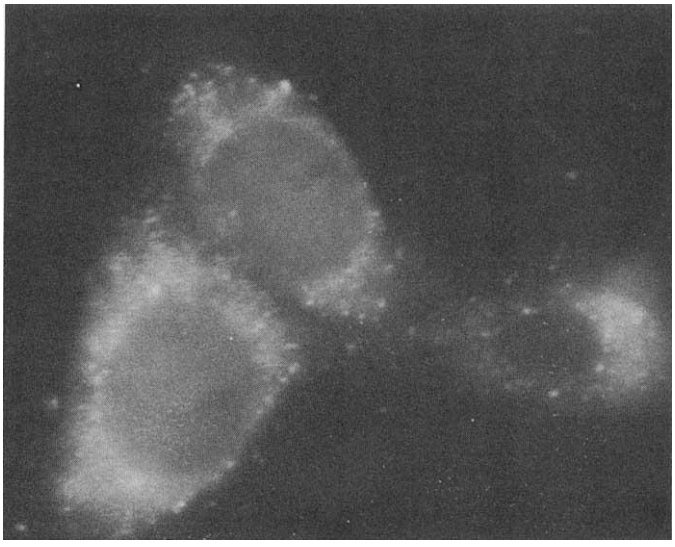

**Fig. 9.** *Staining of transgenic endothelial cells with antiserum against human factor VIII related antigen.* (×630)

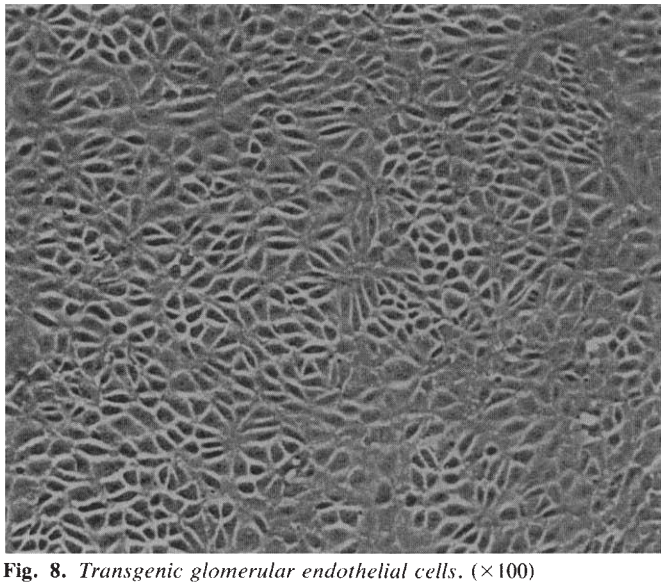

**Fig. 8.** *Transgenic glomerular endothelial cells.* (×100)

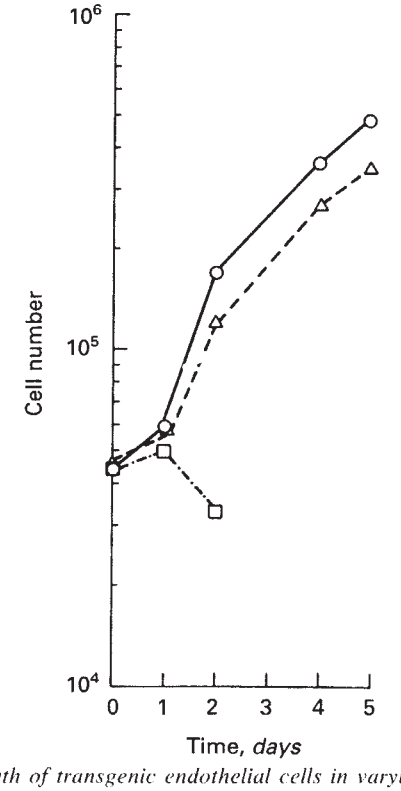


**Fig. 10.** *Growth of transgenic endothelial cells in varying concentrations of FBS.* Proliferation continued despite culture confluence. Cells maintained in no serum began to detach from the culture dish at day 2. Symbols are: (○) 5%; (△) 1%; (□) 0%.

transgenic mesangial cells were able to form small colonies in soft agar, and stained for large T-antigen.

### *Glomerular endothelial cells*

We were successful in isolating glomerular endothelial cells only from the transgenic mice. Morphologically, they were very similar to glomerular epithelial cells except that the endothelial cells were slightly smaller (Fig. 8). The cells were further identified by their staining for factor VIII related antigen (Fig. 9), their failure to stain with anti-cytokeratin antibody, and their lack of contractile response to angiotensin II.

These cells did not exhibit any change in morphology or immunofluorescence staining for actin, cytokeratin and factor VIII related antigen after multiple passages and freezing and thawings. The cells had a doubling time of 15 hours in 5% FBS (Fig. 10), were not contact inhibited, formed small colonies in soft agar, and stained for large T-antigen.

## Discussion

The cellular complexity of the kidney and glomerulus makes it difficult to be certain that an uncloned population of glomerular cells is not contaminated with a small number of a different cell type [1, 2]. In the present study, we observed that populations of normal epithelial cells, obtained by dilute plating and patch cloning, initially appeared to be homogeneous by light and immunofluorescence microscopy. However, during subsequent passages, they gradually became overgrown by mesangial cells. Contamination of cultures with an undesired cell type is especially important in view of reports detailing modification in the behavior of one cell type by the presence of other cells or their products [13–15]. A major difficulty in establishing pure cultures has been the finite life span of cultured glomerular cells. At present, there are no cloned lines of glomerular cells from any source.

We chose to use mice transgenic for the early region of SV40 in our attempt to establish permanent cloned glomerular cell lines for several reasons. First, since cells cultured from a number of different tissues taken from these mice appeared to be immortal in vitro [16], it seemed likely that glomerular cells from these mice would also be immortal. Secondly, we found that younger animals (4 weeks) had normal renal histology [5]. The presence of a normal glomerulus demonstrated that normal glomerular development could, and did, occur despite the presence of foreign (SV40) DNA in each of the mouse's cells. This offers a theoretic advantage over cells transfected in vitro with transforming genes and over cells which spontaneously transform in vitro. In the latter circumstances, it is not possible to be certain that the mechanisms involved in transformation do not prohibit the expression of a normal glomerular cell phenotype.

A major reason for choosing the SV40 transgenic mice as a source of glomerular cell lines was the renal lesions which the older (6 to 16 weeks) mice developed. The majority of mouse lines established by injecting fertilized ova with constructs of DNA coding for the large T-antigen gene along with its enhancer developed renal disease [4, 5]. However, the lesions differed somewhat from construct to construct and from line to line [5]. We chose the SV Tag 188(8-1) line because mice from this line developed a renal disease which resembled human focal glomerulosclerosis. As there are no evident extrarenal sources of injury, and as large T-antigen expression has been demonstrated in the kidneys of this and similar lines of mice [5, 16, 17], we suspect that the sclerotic lesion may be caused by an alteration in resident glomerular cell behavior induced by large T-antigen. If the presence of large T-antigen in vivo is responsible for triggering abnormal glomerular cell behavior which leads to sclerosis, it is possible that glomerular cells from these mice would display the same abnormal behavior in vitro, since they express large T-antigen in culture. These cells could therefore be useful in dissecting the role(s) of resident glomerular cells in the development of glomerulosclerosis.

The lines of glomerular cells obtained from the transgenic mice are clones derived from a single cell, and are thus unquestionably homogeneous. Despite their transformed phenotype, evidenced by a lack of contact inhibited growth and growth in soft agar, they maintained features of their normal counterparts in vitro. The epithelial cells grew as a monolayer of cobblestone appearing cells at confluence, contained cytokeratin, did not contract in response to angiotensin II, did not contain factor VIII related antigen, gamma-glutamyl transpeptidase or alkaline phosphatase activity, and did not stain with SSEA-3 antibody. The mesangial cells grew in parallel arrays of elongated cells, had a cytoskeleton containing actin filaments distributed in a parallel design throughout the cytoplasm, contracted in response to angiotensin II, and did not stain with antibodies directed against cytokeratin or factor VIII related antigen. The endothelial cells grew as a monolayer of smaller cobblestone appearing cells, contained factor VIII related antigen, and neither stained for cytokeratin nor contracted in response to angiotensin II.

The most commonly observed alteration in cell behavior found in cells transfected in vitro with SV40 is an increase in proliferative capacity [18–20]. As expected, glomerular cells derived from these transgenic mice exhibited decreased doubling times and an indefinite culture lifespan. However, expression of the SV40 DNA does not appear to be the only factor controlling cell growth. This is evidenced by the different doubling times of the three glomerular cell lines. In 5% FBS the doubling time of endothelial cells is 15 hours, of mesangial cells 26 hours, and of epithelial cells 30 hours. This relationship corresponds to that found in vivo in rats using $^{3}H$ thymidine injection followed by autoradiography [21]. In these studies, glomerular endothelial cells were labeled most frequently. Mesangial cells were labeled less frequently and glomerular epithelial cells were rarely labeled. It is thus possible that elements which normally regulate glomerular cell proliferation are still partially operative in these cells despite their transformed phenotype.

In summary, we have established clonal lines of mouse glomerular epithelial, mesangial, and endothelial cells which maintain characteristics of normal glomerular cells despite their transformed phenotype. These lines could be useful in the study of the pathogenesis of the glomerular lesions in this transgenic model of glomerulosclerosis and in the study of other features of glomerular cell biology which are not altered by a transformed phenotype.

## Acknowledgments

Portions of these studies were presented at the 18th annual meeting of the American Society of Nephrology, New Orleans, Louisiana, December, 1985, and were published in abstract form. SSEA3 antibody used in this study was a gift from Barbara B. Knowles, M.D. Portions of these studies were supported by National Institutes of Health Grant CA38635. Dr. MacKay is a recipient of an Individual National Research Service Award 1 F32 AM07843 01.

*Reprint requests to Dr. Karen MacKay, Building 10 Room 3N110, National Institutes of Health, Bethesda, Maryland 20892, USA.*

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