

Establishment of Conditionally Immortalized Mouse Glomerular Parietal Epithelial Cells in Culture

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ABSTRACT

Parietal epithelial cells (PEC) are major constituents of crescents in crescentic glomerulonephritis. The purpose of these studies was to establish an immortalized PEC cell line with similar characteristics to PEC *in vivo* for use in future mechanistic studies. Glomeruli were isolated from H-2Kb tsA58 transgenic mice (ImmortoMouse) by standard differential sieving, and several candidate PEC cell lines were obtained by subcloning outgrowths of cells from capsulated glomeruli. One clone, designated mouse PEC (mPEC), was extensively characterized. mPEC exhibited a compact cell body with typical epithelial morphology when grown in permissive conditions, but the cell shape changed to polygonal after 14 d in growth-restrictive conditions. mPEC but not podocytes used as a negative control expressed claudin-1, claudin-2, and protein gene product 9.5, which are proteins specific to PEC *in vivo*, and did not express the podocyte-specific proteins synaptopodin and nephrin. The junctional proteins zonula occludens-1 and β -catenin stained positively in both mPEC and podocytes, but the staining pattern at cell–cell contacts was intermittent in mPEC and linear in podocytes. Finally, mPEC had thin bundled cortical F-actin filaments and no F-actin projections compared with podocytes, which exhibited thick bundled cortical F-actin filaments and interdigitating F-actin projections at cell–cell contacts. We conclude that immortalized mPEC in culture exhibit specific features of PEC *in vivo* and that these cells are distinct from podocytes, despite having the same mesenchymal origin. This mPEC line will assist in future mechanistic studies of PEC and enhance our understanding of glomerular injury.

J Am Soc Nephrol 19: 1879–1890, 2008. doi: 10.1681/ASN.2007101087

The role of glomerular parietal epithelial cells (PEC) in glomerular diseases is becoming increasingly recognized. PEC make up a large part of glomerular crescents in certain forms of ANCA and non-ANCA-related glomerulonephritis. Moreover, in disease, they express several cytokines and chemokines, such as TGF- β 1,¹ PDGF-B,² and CTGF,³ and more recent studies suggested that they might also be precursors to podocytes.⁴ Several animal models of glomerulonephritis characterized by PEC proliferation have enhanced our understanding of these cells in disease. Although human studies and animal models might be considered the gold standards in the study of glomerular disease, they provide limited mechanistic opportunities for detailed studies of PEC. In contrast to studies in mesangial cells, podocytes, and tubular epithelial

cells, PEC studies have been hampered by the lack of a reliable and robust cell culture model that resembles these cells *in vivo*. Despite excellent efforts, PEC cell lines available in culture are typically primary rat cultures.³ Unfortunately, they proliferate under basal conditions and thus do not truly represent their *in vivo* counterparts under nonstressed states.

Received October 10, 2007. Accepted April 15, 2008.

Published online ahead of print. Publication date available at www.jasn.org.

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Proteins specifically expressed in PEC are critical to distinguish these cells from other glomerular cells, specifically podocytes. More recently, several proteins have been reported to be expressed specifically in the PEC and thus serve as useful tools to establish and characterize PEC in culture. For example, the expression profile of two proteins from the claudin family of tight junction proteins were recently described by Kiuchi-Saishin *et al.*⁵ in the kidney. In their report, immunostaining for claudin-1 and -2 localizes exclusively to the PEC in the mature glomerulus.

Another protein recently described in the kidney is protein gene product 9.5 (PGP9.5), also known as ubiquitin carboxyl-terminal esterase L1, which was reported many years ago to be highly specific to neurons.⁶ Recently, Shirato *et al.*⁷ showed that PGP9.5 is also expressed in PEC. Expression during kidney development was also reported. PGP9.5 is expressed in all cells in the comma- and early S-shaped body stages of development; however, by late S-shaped body and the capillary loop stage, PGP9.5 was exclusively expressed in PEC. Furthermore, PGP9.5 was not expressed in proximal tubular cells at the urinary pole in the mature glomerulus.

These recently described proteins provide valuable new markers to identify PEC in culture. Accordingly, the ultimate purpose of this study was the establishment and characterization of an immortalized PEC cell line in culture, with similarities and characteristics of PEC *in vivo*, so we and others can better understand PEC biology in health and disease.

RESULTS

Establishing Immortalized Mouse PEC

Isolated glomeruli were plated onto type I collagen-coated 10-cm plates (at a concentration of 6 glomeruli/cm²) and maintained in medium containing 5% FBS. Cellular outgrowths were observed daily by an inverted light microscope. On average, 40 glomeruli adhered to each 10-cm plate. Of these, <10% were capsulated glomeruli; the remainder were decapsulated. Although a small fraction of tubules were also present in the isolated glomerular fraction and therefore seeded at the same time, they did not attach to the cell culture plate under these culture conditions and were thus readily removed by changes of medium (Figure 1).

Adherent capsulated glomeruli, which comprise PEC and Bowman’s capsule basement membrane, remained intact for the first 3 d, with no cells growing in their vicinity (Figure 2A). At day 5, 50 to 70% of capsulated glomeruli showed evidence for the first time of cellular outgrowths (Figure 2B). At day 7, there were progressive increases in the number of cells adjacent to these glomeruli (Figure 2C). These cells were irregularly shaped, uniform in size, and convex and tended to grow in clusters (rather than as isolated cells). By day 10, outgrowing cells formed a distinct monolayer around the individual glomeruli from which they likely derived (Figure 2D). Outgrowing cells formed a dense monolayer, and the cell bodies were compact. There was no evidence of cells shaped differently

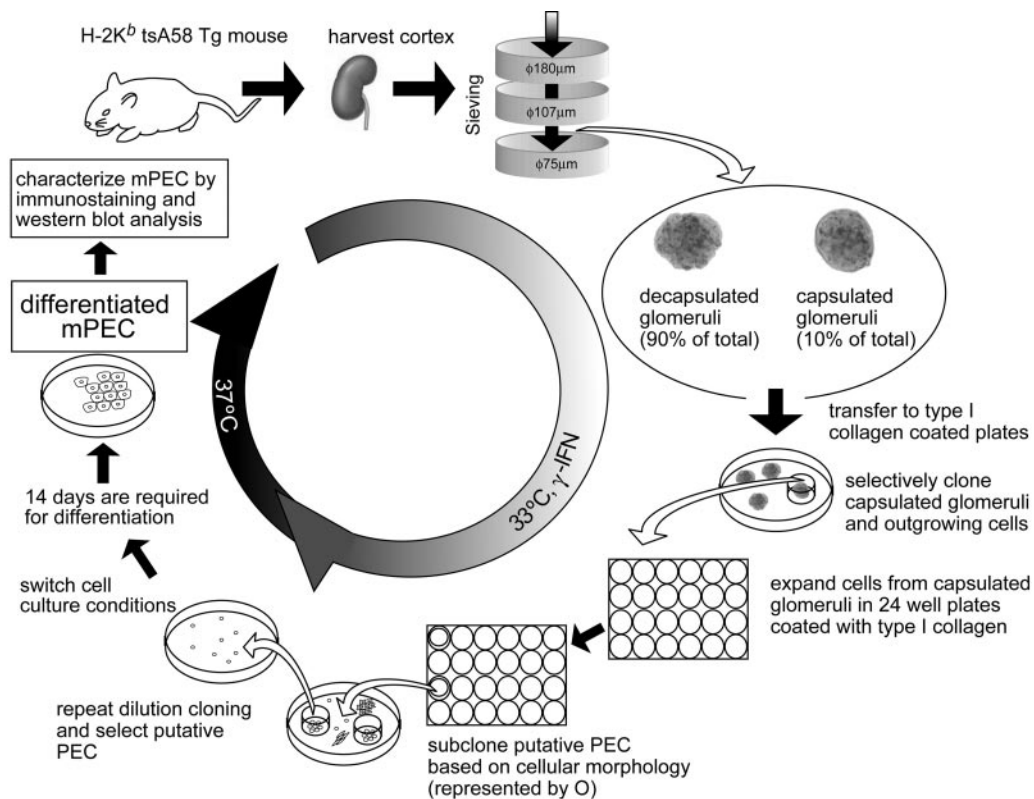


Figure 1. Protocol for isolating mPEC in culture. See text for details.

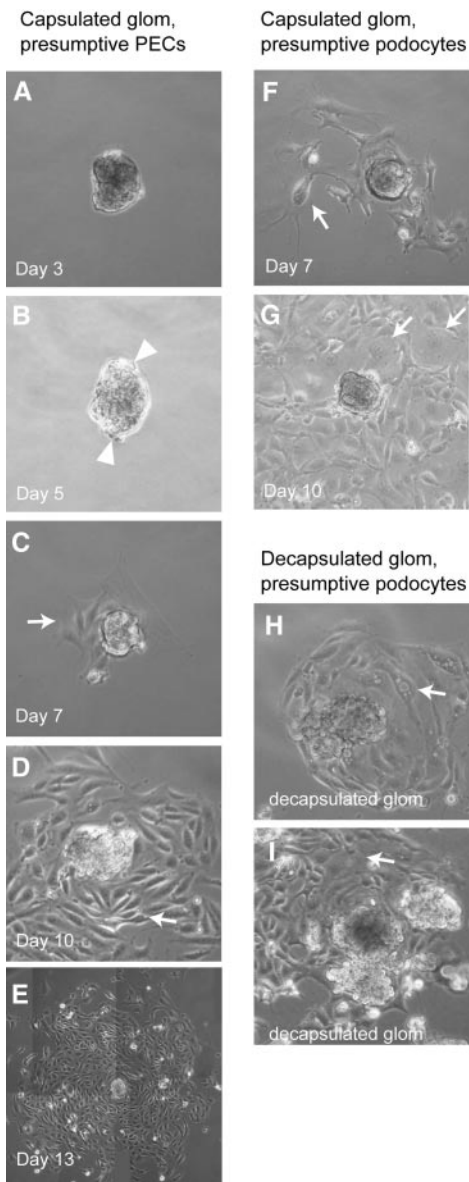


Figure 2. Cell outgrowths from capsulated glomeruli. Adherent isolated capsulated glomeruli were grown in culture under growth-permissive conditions and monitored daily by inverted microscopy. (A) No cellular outgrowths were observed in this attached glomerulus after 3 d in culture. (B) The first evidence of cells emerging from the glomerulus was after 5 d in culture (examples represented by arrow). (C) The number of cells increased by day 7 (examples represented by arrow). (D) By day 10, cells with compact bodies were abundant and surrounded the “parent” glomerulus. (E) By day 13, this merged image illustrates that several of the parent capsulated glomeruli were completely surrounded by a large colony of cells, all uniform in shape and size. These cells were used for dilution cloning to propagate putative mPEC. (F and G) A minority of capsulated glomeruli, such as the one represented, had cell outgrowths characterized by larger size, with distinct processes emerging from the cell body. The morphology of these cells was consistent with podocytes, not PEC. (H and I) For a comparison, representative images of decapsulated glomeruli with cellular outgrowths are shown. Arrows indicate the arborized putative podocyte.

from the ones described in the vicinity of capsulated glomeruli. We concluded that these cells were likely PEC, and these presumptive PEC were therefore selected for dilutional subcloning. At day 13, cells emerging and growing from capsulated glomeruli were compact and dense and formed a large colony. Having identified these as presumptive PEC, they were isolated using a cloning cylinder, trypsinized, and transferred to a 24-well plate.

After repeated dilution cloning, six single cell lines, which grew robustly and showed a compact form, were chosen. Finally, on the basis of their ability to replicate and their morphology, a single cell line was subjected to further analysis. We called this line mouse PEC (mPEC).

Not all capsulated glomeruli gave rise to putative PEC. Figure 2, F and G, shows that certain capsulated glomeruli (approximately 50%) showed outgrowing cells that were distinct from the cells described. These cells were larger in size, more irregular in shape, and flatter; had multiple cellular processes; and grew at a slower rate. On the basis of our experience,⁸ the morphology of these cells was consistent with podocytes (Figure 2, F and G), similar to those that we⁸ and others previously characterized.^{9,10} Decapsulated glomeruli were easily distinguished from capsulated glomeruli under the microscope by their characteristic shape (Figure 2, H and I).

Induction of Differentiation and Quiescence in Cultured Cells

For induction of mPEC differentiation and induce quiescence, similar to their *in vivo* counterparts, the cell culture conditions were switched from growth permissive to growth restrictive because these cells contain the SV40 large T-antigen that is thermosensitive. When confluent in growth-permissive conditions, cells exhibited a similar morphology to the outgrowths described previously (Figure 3A). In contrast, cells displayed a very different morphology when grown under growth-restrictive conditions: At day 3, they were larger and flatter and had more processes. By day 6, individual cells increased in size 10-fold, and the edges of the cells were smoother and had substantially fewer processes. The actin cytoskeleton was easily detectable by light microscopy. At day 13, individual cells had increased further in size, up to 20-fold compared with cells grown in permissive conditions. They stopped replicating by day 13, and the cell borders were smooth, linear, and well demarcated (Figure 3, B through E). These results are consistent with a differentiated morphology of the mPEC when grown under these conditions.

To confirm that mPEC exited the cell cycle and were quiescent when grown under growth-restrictive conditions, we performed immunostaining for proliferating cell nuclear antigen (PCNA) and Ki-67 in cells grown in both growth-permissive and growth-restrictive conditions. As shown in Figure 4A, as expected, mPEC stained positive for Ki-67 during growth-permissive conditions. In contrast, Ki-67 staining was not detected in mPEC after 14 d in growth-restrictive conditions (Figure 4D). This was not a false-negative result, because the

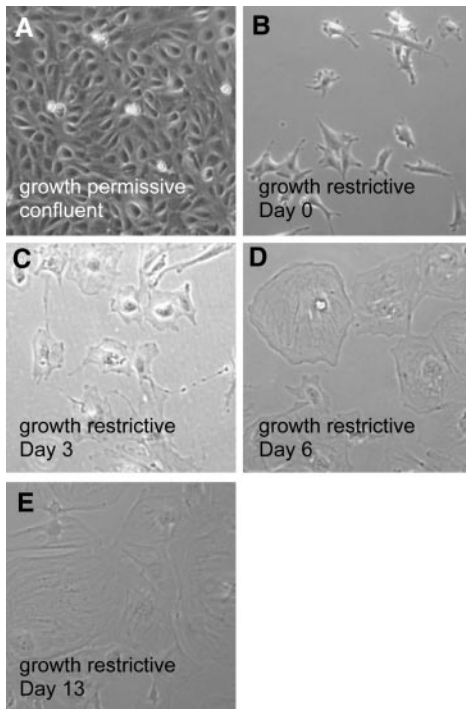


Figure 3. Differentiation of mPEC. (A) After dilutional cloning, putative mPEC were initially cultured under growth-permissive conditions to enhance cell proliferation and thus expansion of the cell colony. The figure shows cells with small bodies in a confluent monolayer. For induction of cell differentiation and quiescence, similar to the *in vivo* phenotype, cells were replated and grown under growth-restrictive conditions, and the results are shown in B through E. (B) mPEC show small cell bodies and had small processes. (C) By day 3 of growth-restrictive condition, mPEC started to spread out, with an increase in cell size. (D) At day 6, mPEC spread out further, and the edges of cells were polygonal. (E) At day 13, mPEC had spread out even more and were uniformly polygonal.

cells grown under growth-permissive cells were positive. Similar results were shown with PCNA staining (data not shown). As expected, mPEC stained positive for PCNA during growth-permissive conditions; in contrast, PCNA staining was absent in mPEC grown in growth-restrictive conditions. To quantify changes in cell number, we also performed the 3-[4,5] dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay. MTT assay revealed that cell number increases during growth-permissive conditions; however, the cell number plateaued by day 6 in growth-restricted conditions and no further increase in cell number was observed (Figure 4E). Taken together, these data support the notion that, like their *in vivo* counterparts in normal glomeruli, immortalized mPEC do not proliferate under growth-restrictive culture conditions.

Verifying the Expression of PEC and Podocyte-Specific Proteins in Kidney Tissue In Vivo

To characterize the isolated cells in culture as mPEC, we began by staining normal mouse kidneys with antibodies to candi-

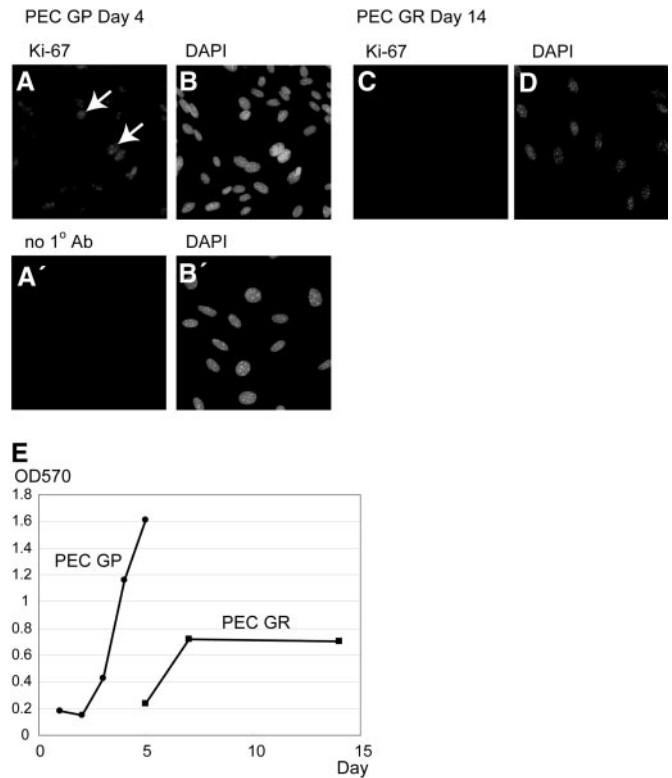


Figure 4. PEC proliferation decreased in growth-restrictive conditions. PEC were grown in growth-permissive (GP; 33°C + IFN) and growth-restrictive (GR; 37°C) conditions. Immunofluorescence for Ki-67, a marker of cell proliferation, and DAPI as a cell marker were performed. (A and B) GP cells at day 4 showed positive Ki-67 staining in the majority of cells. (C and D) GR PEC at day 14 revealed minimal Ki-67 staining. (A' and B') No primary antibody revealed lack of background staining. Not shown here are similar results obtained with PCNA. (E) MTT assay (quantification of cell viability) demonstrated lower cellular activity in GR cells than GP PEC. These data demonstrate that *in vitro* PEC proliferate in GP conditions whereas they lack proliferation in GR conditions.

date antigens/proteins that are specific for PEC and others that are specific for podocytes. The results are shown in Figure 5.

Kiuchi-Saishin *et al.*⁵ showed that the tight junctional proteins claudin-1 and claudin-2 were expressed specifically in PEC. Our data shown in Figure 5, A through F, demonstrate membrane staining for both claudin-1 and -2 in cells lining Bowman’s basement membrane. Shirato *et al.*⁷ showed that PGP9.5, a neuron-specific ubiquitin C-terminal hydrolase, is also expressed in PEC. Our data (Figure 5, G through J) also show that PGP9.5 staining localized to the cytoplasm of cells lining Bowman’s membrane. No staining was observed when the primary antibodies were omitted, used as negative controls. Taken together, our results show that claudin-1, claudin-2, and PGP9.5 are expressed specifically in mature and differentiated PEC in the normal kidney *in vivo*, and, thus, these antigens can be used to identify putative PEC in culture.

Two antibodies were used to identify the presence of spe-

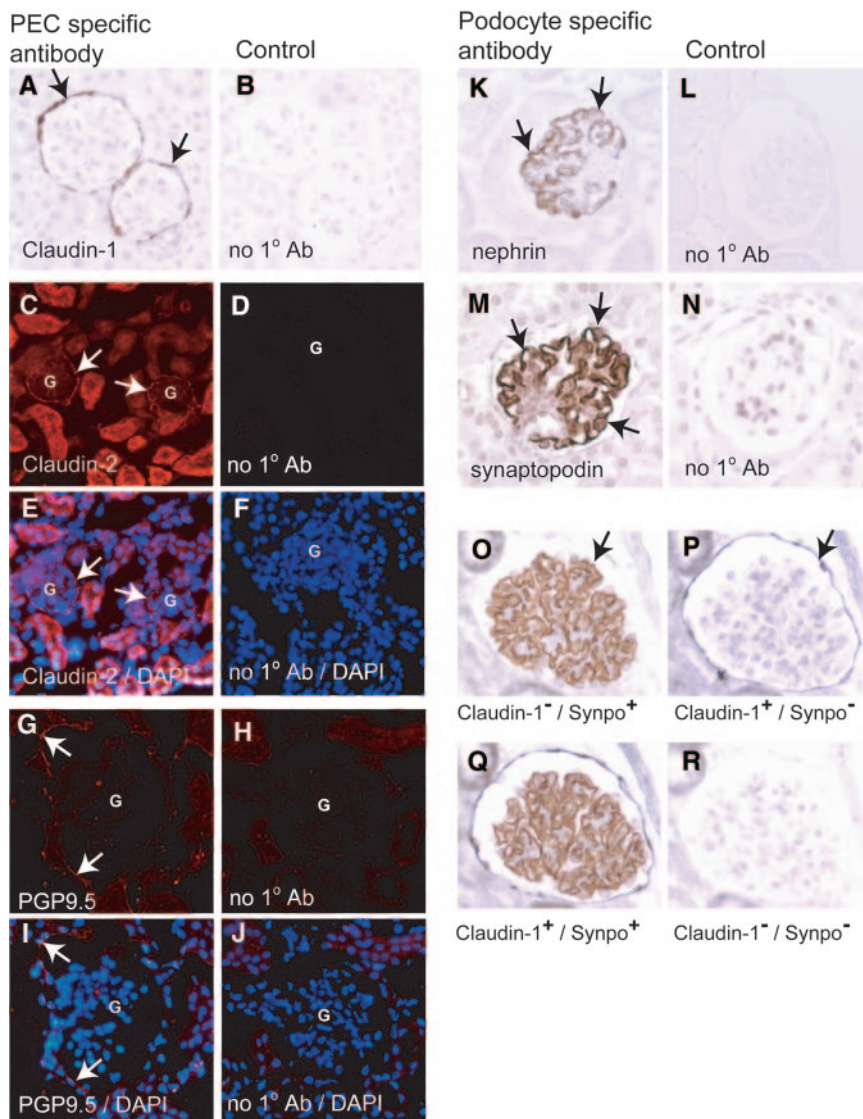


Figure 5. Immunostaining for PEC- and podocyte-specific proteins in normal adult mouse kidneys. To ensure that the antibodies used in culture stained specific cells *in vivo*, we performed immunostaining on normal adult mouse kidney sections. Three PEC markers (claudin-1, claudin-2, and PGP9.5) and two podocyte markers (nephrin and synaptopodin) were examined. Examples of positive staining are illustrated with arrows. (A through F) claudin-1 (A and B) and claudin-2 (C through F) showed a cytoplasmic staining pattern in PEC along with Bowman's basement membrane. (G through J) Cytoplasmic staining for PGP9.5 was confined to PEC along Bowman's basement membrane. (K through N) Nephrin (K and L) and synaptopodin (M and N) staining was abundant and restricted to podocytes. (O through R) Double staining of claudin-1 and synaptopodin is shown. For ruling out the possibility that signals are produced by nonspecific reaction, primary antibody–omitted staining is shown (O and P), in addition to no primary antibody control (R). These results show that PEC and podocytes express unique proteins in normal mice *in vivo*, which can be used to distinguish specific glomerular epithelial cells *in vivo* and *in vitro*.

cific podocyte antigens. Nephrin, a protein localizing specifically to the slit diaphragm of podocytes, showed positive staining in glomeruli in a podocyte distribution. Synaptopodin, an actin-associated protein expressed in podocytes, also stained positively in glomeruli in a podocyte distribution (Figure 5, K

through N). These results verify that nephrin and synaptopodin were expressed in podocytes but not in PEC and thus serve as excellent markers to differentiate PEC from podocytes in culture. To demonstrate further the localization and immunostaining specificity of each glomerular epithelial cell type more clearly, we performed double immunostaining for claudin-1 (PEC protein) and synaptopodin (podocyte protein), and the results are shown in Figure 5, O through R. As expected, claudin-1 and synaptopodin stained only PEC and podocytes, respectively, in the normal glomerulus. Negative controls ensured antibody specificity (Figure 5).

Verifying the Expression of PEC-Specific Proteins in Cultured mPEC by Immunostaining

Having demonstrated glomerular epithelial cell specificity with the antibodies described, we next performed immunostaining for the tight junction protein claudin-1 in putative mPEC in culture. Claudin-1 stained strongly positive in an intermittent pattern along the cell border and linearly in the cytoplasm of mPEC (Figure 6, B and C). In contrast, claudin-1 immunostaining was very faint and barely detected in culture podocytes (Figure 6D); <5% of podocyte showed very weak staining for claudin-1. mPEC in growth-permissive conditions (which are immature and not fully differentiated) showed faint but positive staining for claudin-1 (Figure 6A). The staining for claudin-2 was also robust along the cell borders in putative cultured mPEC (Figure 6, F and G). In contrast, claudin-2 immunostaining was negative in cultured podocytes (Figure 6H). Staining for claudin-2 was not detected in mPEC grown in growth-permissive conditions. Taken together, the presence of claudin-1 and claudin-2 staining in differentiated cultured mPEC is consistent with the expression pattern for these two tight junction proteins in the mature PEC *in vivo*. Finally, as shown in Figure 6, J and K, immunostaining for PGP9.5 was also present at the cell borders of putative mPEC in culture. In contrast, PGP9.5 was extremely faint and barely detected in the cytoplasm of cultured podocytes (Figure 6L). mPEC

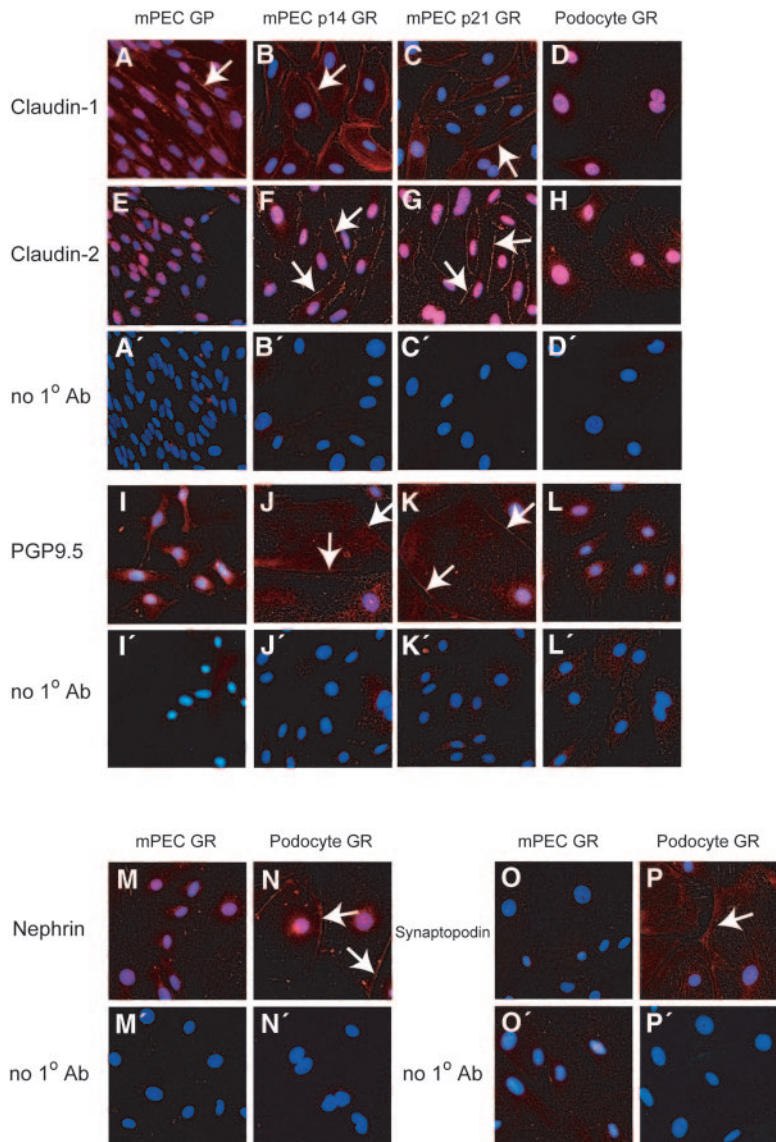


Figure 6. Specific indirect immunofluorescence immunostaining characterizes and distinguishes mPEC from podocytes in culture. (A through J) Putative mPEC and previously characterized podocytes were grown separately under different cell culture conditions and stained with antibodies that specifically stained either PEC or podocyte *in vivo*. Examples of positive staining are illustrated by arrows. (A through D) Claudin-1 staining was abundant in mPEC along the cell border and in the cytoplasm in both growth-permissive and growth-restrictive conditions; this was absent in podocytes. (E through H) Claudin-2 staining was detected in mPEC along the cell membrane only in growth-restrictive conditions; in contrast, claudin-2 staining was not detected in podocytes. (I through L) PGP9.5 was restricted to the cell membrane of mPEC in growth-restrictive conditions. In contrast, staining was not detected in podocytes for PGP9.5. These results demonstrate that the cells isolated in culture were indeed mPEC. mPEC did not show the positive staining for nephrin (M) or synaptopodin (O), whereas podocytes showed positive linear staining of nephrin at cell borders (N, arrow) and positive linear staining of synaptopodin in cytoplasm and cell border (P, arrow). These results demonstrate that the mPEC grown in culture are distinct from podocytes.

in growth-permissive conditions showed negative staining of PGP9.5. Again, staining for PGP9.5 in differentiated mPEC is consistent with the expression seen in PEC *in vivo*.

on Western blots consistent with the predicted molecular weights for the PEC-specific proteins claudin-1, claudin-2, and PGP9.5. A monoclonal and polyclonal antibody was

These data show that mPEC in culture but not podocytes stain positively for claudin-1 and -2 and PGP9.5. Finally, to ensure that the cells did not transform in culture under the conditions described, we performed immunostaining for these proteins regularly over several passages. Our data showed positive staining for claudin-1, claudin-2, and PGP9.5 in mPEC up to passage 21.

Podocyte-Specific Protein Immunostaining Is Absent in Cultured mPEC

To demonstrate that mPEC do not exhibit features of differentiated podocytes in culture, immunostaining for nephrin, a protein shown to be specifically expressed at the slit diaphragm of the mature podocyte, was performed. As shown in Figure 6M, staining for nephrin in mPEC was absent. As a positive control for nephrin staining, cultured podocytes were stained, and Figure 6N demonstrates the typical staining for nephrin in differentiated podocytes in culture, which localizes to the cortical actin along the cell borders. In addition to nephrin, synaptopodin was immunostained in both differentiated mPEC and podocytes. As with nephrin, synaptopodin was not detected in mPEC (Figure 6O). In contrast, there was intense granular staining for synaptopodin in association with cortical and cytoplasmic actin in cultured podocytes (Figure 6P), consistent with the typical expression pattern seen in these cells *in vivo*.

Validating the Expression of PEC and Podocyte-Specific Proteins by Western Blot Analysis

To further verify the aforementioned immunostaining of proteins specifically expressed in either differentiated mPEC or podocytes in cell culture, we also performed Western blot analysis on protein extracts from mPEC and podocytes in culture. Our results showed the presence of intense bands

used for Western blot analysis for PGP9.5 to ensure specificity. In contrast, Western blot analysis showed barely detectable or absent bands in protein extracts obtained from cultured podocytes (Figure 7, A through D). To verify that mPEC do not express podocyte-specific protein and that the podocytes used in these studies express only podocyte-specific proteins, we also examined nephrin and synaptopodin. As expected, nephrin and synaptopodin were detected specifically in protein extracts from podocytes. In contrast, synaptopodin was not detected in mPEC, and nephrin exhibited a very faint band (Figure 7, E and F).

Staining for internal control protein Na-K-ATPase (for nephrin) or glyceraldehyde-3-phosphate dehydrogenase (for claudin-1, claudin-2, PGP9.5, and synaptopodin) was performed to demonstrate equal loading of protein in each lane. These data further demonstrate that differentiated mPEC in culture express specific proteins similar to their *in vivo* counterparts and that when grown under the conditions described here express podocyte proteins with a very low level of signal intensity.

Actin Cytoskeleton and Cell Junction Staining Differs in mPEC from Podocyte

To determine further the morphologic differences between mPEC and podocytes in culture, we stained for the cytoskeletal

protein F-actin and the junctional proteins zonula occludens-1 and β -catenin. Although these proteins were detected in both cell types, the expression pattern was different and thus distinguished these cells. mPEC showed thin linear staining of F-actin in cytoplasm and along the cell borders, reflecting the simpler cytoskeletal structure of PEC; however, podocytes showed thick bundled cortical actin at the edge of cells similar to the microtubules of the primary processes of podocytes, and staining was also detected at cell–cell junction bridging adjacent cells similar to the microfilaments of the foot processes of podocytes (Figure 8, A and B). Staining for ZO-1 revealed significant differences in the cell–cell junctions between mPEC *versus* podocytes. Whereas mPEC showed a strikingly intermittent pattern of ZO-1 staining, podocytes showed a very linear staining pattern (Figure 8, C and D), indicating distinct differences in the tight junctions formed between neighboring mPEC *versus* podocytes in culture.

Likewise, β -catenin revealed an intermittent pattern at the cell borders of mPEC (Figure 8G), whereas cultured podocyte showed a mixture of linear and intermittent staining patterns at cell borders (Figure 8H). There were also signals for β -catenin at the cell–cell junctions bridging adjacent podocytes (Figure 8H, inset).

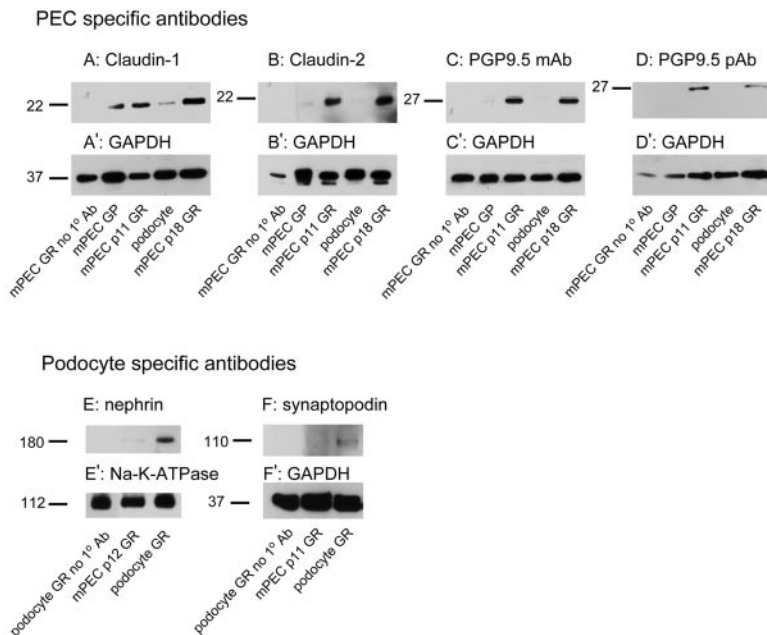


Figure 7. Western blot analysis for mPEC- and podocyte-specific proteins. For ensuring antibody and cell specificity, protein was extracted from mPEC and podocytes for Western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control for all proteins except nephrin, for which Na-K-ATPase was used. The molecular weight (kD) is shown on the left of each Western blot. mPEC but not podocytes expressed PGP9.5. The abundance of claudin-1 and claudin-2 was substantially greater in mPEC than in podocytes. In contrast, synaptopodin was detected only in podocytes. There was a very slight band detected for nephrin in mPEC. In contrast, nephrin was abundant in podocytes. These results validate the staining results and provide further proof of the establishment of an mPEC-specific cell line in culture.

DISCUSSION

A role for PEC participation in crescent glomerulonephritis has been well documented,^{2,3,11,12} and more recent studies suggested an additional possible role in collapsing glomerulopathy.¹³ Although there are several animal models available for study, they are not primarily PEC specific. Since the establishment of conditionally immortalized mouse podocytes,⁹ other conditionally immortalized mouse glomerular cell lines have been reported, including endothelial and mesangial cells,^{14–16} all of which have significantly enhanced our understanding of the biology of these cells under normal and diseased states. The principle behind these cell lines is the use of the same H-2K^b-tsA58 transgenic mice to generate cells in culture that are not transformed either spontaneously or with SV40 and will more accurately reflect a more differentiated and less tumor-like phenotype. Thermolabile large tumor (T) antigen (TAg) encoded by SV40 virus mutant temperature sensitive (ts) strain tsA58 is harbored under the control of the mouse major histocompatibility complexes H-2K^b class I

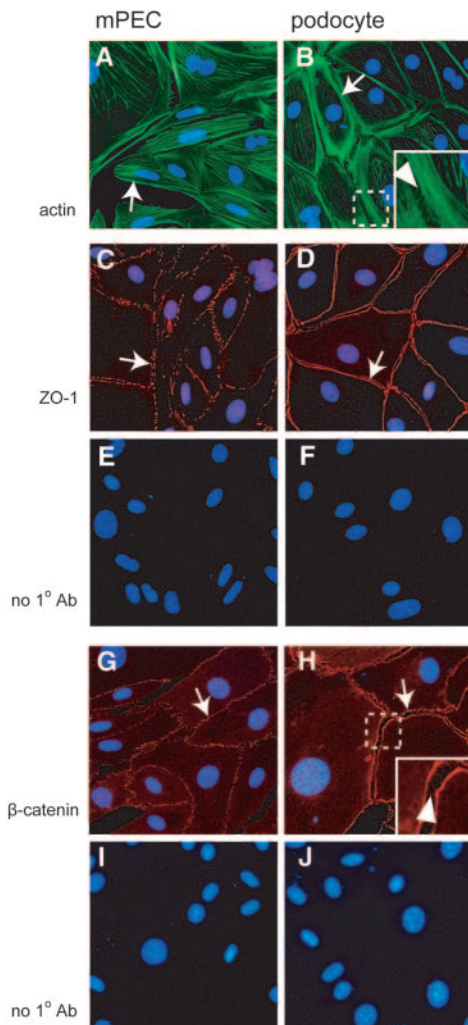


Figure 8. Characterizing and distinguishing cytoskeletal and junctional proteins in cultured mPEC and podocytes. Staining with specific antibodies was used to delineate the cytoskeletal architecture of mPEC and to determine the possibility that mPEC express junctional proteins distinct from podocytes. Examples are illustrated with arrows, and magnified insets highlight the pattern of staining. (A) F-actin staining was abundant in the cytoplasm of mPEC in a linear distribution. (B) Podocytes are characterized by thick bundled cortical actin and thin bridging structures between adjacent cells (arrowhead). (C) ZO-1 staining was readily detected at the cell border of mPEC in a punctuated distribution. (D) ZO-1 staining was abundant at the cell membrane of podocytes in a continuous and linear pattern. (G) β -Catenin staining was intermittent and restricted to the cell border in mPEC. (H) Positive staining was seen in podocyte with a mixture of linear and intermittent patterns at cell borders (arrow) and with thin bridging signals between adjacent cells (arrowhead).

promoter in these mice. Thus, the expression of tsA58 TAg will be enhanced by exposure of cells to interferons. When grown under the permissive temperature (33°C) with IFN, tsA58 TAg is activated and will immortalize the cells by inactivating the tumor suppressor gene p53.¹⁷ We report here for the first time

the establishment of a reliable and well-characterized immortalized PEC cell line that can be used for *in vitro* studies.

In these studies, we used H-2kb-tsA58 transgenic mice as a source for the mPEC cell line and expanded the cells from the capsulated glomeruli isolated by the standard sieving technique. We initially isolated cells using the criteria that the cells with compact cell bodies were distinct from mesangial cells, endothelial cells, podocytes, or fibroblasts by the morphology. This approach allowed us to obtain cells growing out from the isolated capsulated glomeruli that we believed were similar to the PEC reported by Yaoita *et al.*¹⁰; until fully characterized, we considered these cells to be presumptive PEC.

Although the number of reported PEC-specific proteins is limited, a combination of immunocytochemistry and Western blot analysis revealed that mPEC specifically express claudin-1, claudin-2, and PGP9.5. We began our studies by verifying their expression on normal mouse tissue sections so that our *in vitro* results could then be compared with those *in vivo*. Moreover, because PEC and podocytes derive from the same mesenchymal cell, we used cultured podocytes as a negative control to ensure PEC specificity. There are several lines of evidence to support our claim that the cells generated in culture are indeed immortalized mPEC: (1) They were isolated only from capsulated glomeruli, not from decapsulated glomeruli; (2) the cell shape and size were distinct from other glomerular cells in culture, especially when directly compared with cultured podocytes; (3) they acquire a quiescent phenotype when grown under growth-restrictive conditions; (4) both immunostaining and Western blot analysis show that they express proteins (claudin-1, claudin-2, and PGP9.5) that are specific for PEC *in vivo*; (5) by immunostaining and Western blotting, they express either no or low levels of proteins specific to podocytes (synaptopodin, nephrin) *in vivo* or *in vitro*; (6) there were marked differences in the expression and distribution of cytoskeletal and junctional proteins; (7) they maintained cell-specific staining up to passage 21, the latest passage studied at the time this article was submitted; (8) the concentration of serum in the culture medium required for survival of mPEC was 5%. mPEC did not survive in a concentration of 10% serum, which are used for podocyte culture studies in order for them to survive. These results indicate a difference in growth requirements between these two cell types.

Podocytes display actin cytoskeleton with thick bundled cortical actin and with thin bridging actin between cell-cell contacts. In contrast, mPEC did not show these features; (9) there were marked differences in the expression and distribution of junctional proteins. Podocytes showed a very linear staining pattern of ZO-1 and β -catenin with a gap between each cell, whereas mPEC showed intermittent staining at the cell-cell junctions. These results indicate that the foot processes characteristic of podocytes were not present in mPEC in culture. Furthermore, these stains indicate that mPEC seem to have more typical epithelial cell-like characteristics than podocytes. (10) Finally, like their *in vivo* counterparts, growth-restrictive mPEC are quiescent, as judged by the combination of

a cell proliferation assay and the absence of immunostaining for Ki-67 and PCNA.

Our result showed that synaptopodin protein was absent in mPEC by immunocytochemistry and Western blot analysis. A very faint band was detected for nephrin by Western blot analysis (detected by the sensitive ECL technique) in mPEC. Nephrin staining was absent in mPEC but abundant in podocytes. We also observed a faint band for claudin-1 by Western blot analysis in podocytes. Given the shared embryonic origin of mPEC and podocytes, these results lead one to speculate on the shared expression of proteins between mPEC and podocytes.

One might ask, “What is the utility of an immortalized mPEC line in culture?” Although primary PEC have been reported, they are not representative of their *in vivo* counterparts with regard to phenotype and are therefore likely less valuable. We recognize that *in vivo* studies in humans and animal models are ideal; however, mechanistic studies are limited and, like most nonrenal cells, require the use of cells in culture to ask such questions. For example, the study of podocyte biology has “exploded” since the generation of podocytes in culture by the pioneering work of Reiser and Mundel.⁹ This almost certainly would not have been possible if researchers relied on *in vivo* studies alone. Given that PEC are injured by mechanisms that seem to be primarily non-PEC in origin, such as the glomerular basement membrane in anti-glomerular basement membrane disease, podocytes in collapsing glomerulopathy, and endothelial cells in ANCA glomerulonephritis, we believe that PEC grown in culture alone or in co-culture with other glomerular cells will, in the future, substantially enhance our understanding of cell-cell communication within glomeruli. By identifying genes that are specific to PEC in culture, there is the possibility for the generation of a PEC-specific floxed mouse for future studies. These studies are planned for the future. Finally, more functional studies delineating normal PEC functions and responses to injury in disease can be performed.

In summary, we have generated the first immortalized parietal epithelial cell culture that is distinct from other glomerular cells and is very representative of their *in vivo* counterpart. Although studies should incorporate *in vivo* experiments, we believe that encompassing cells in culture is an invaluable and powerful tool to further the knowledge of enigmatic PEC in health and disease.

CONCISE METHODS

Establishing Temperature-Sensitive Immortalized mPEC

To establish a conditionally immortalized mPEC line in culture, we used H-2Kb-tsA58 mice, also called ImmortoMice (Jackson Laboratory, Bar Harbor, ME).¹⁷ Kidney cortex from ImmortoMice was removed, minced, and subjected to standard differential sieving under sterile conditions to isolate glomeruli as previously reported.^{8,18,19} All animal procedures were conducted in accordance with the Institutional Animal Care and Use Committee. Isolated capsulated (contains

the glomerular tuft, Bowman’s membrane, and PEC) and decapsulated (contains the glomerular tuft only) glomeruli were plated onto 10-cm primaria plates (BD Biosciences, San Jose, CA) coated with type I collagen (BD Biosciences). Culture media consisted of RPMI 1640 containing 5% FBS (Summit Biotechnology, Ft. Collins, CO), penicillin (100 U/ml), streptomycin (100 mg/ml), sodium pyruvate (1 mmol/L; Irvine Scientific, Santa Ana, CA), HEPES buffer (10 mmol/L), sodium bicarbonate (0.075%; Sigma-Aldrich, St. Louis, MO), and 50 U/ml IFN- γ (Roche Molecular Biochemicals, Indianapolis, IN). After 11 d in culture at 33°C, glomeruli that had intact Bowman’s capsules and that contained outgrowing cells with compact cell bodies growing at high density were selectively isolated with cloning cylinders (PGC Sciences, Frederick, MD), trypsinized, and replated onto 24-well plates coated by type I collagen for further growth and expansion. After another 13 d of growing in culture, four wells containing candidate PEC were selected on the basis of cell morphology for limited dilution cloning. Two cycles of limited dilution cloning were performed to obtain pure cell colonies. A total of six cell clones of PEC candidates were obtained, from which one cell clone was subjected to further analysis and was named “mPEC.” The others were frozen for future use.

Induction of mPEC Differentiation

The initial studies performed in the previous section were conducted under growth-permissive conditions (grown at 33°C, in the presence of 50 U/ml IFN- γ) for cells to proliferate and be used for study. For inhibition of cell growth and induce mPEC differentiation such as occurs *in vivo*, culture conditions were switched from the growth-permissive conditions to growth-restrictive conditions (grown at 37°C, in the absence of IFN- γ). mPEC were then maintained in growth-restrictive conditions for 13 to 15 d, at which time a detailed cell characterization was performed.

Immunostaining of Kidney Sections

Although the primary focus of these studies was to generate immortalized mPEC in culture, we wanted to ensure that the cultured cells expressed antigens similar to their counterparts *in vivo*. Accordingly, immunostaining was also performed on kidneys from normal mice by indirect immunoperoxidase methods and by immunofluorescence. For indirect immunoperoxidase staining, renal biopsies from C57Bl/6 mice (Jackson Laboratory) were fixed in neutralized formalin and embedded in paraffin. For synaptopodin and nephrin staining, kidney was perfusion fixed with neutralized formalin, transferred to 10% sucrose, and embedded in paraffin. Indirect immunoperoxidase staining was performed on 4- μ m sections as previously reported²⁰ with minor modifications.

In brief, paraffin was removed using Histoclear (National Diagnostics, Atlanta, GA), and sections were rehydrated in ethanol. Antigen retrieval was performed by boiling sections in the microwave in 10 mM citric acid buffer (pH 7.0; for synaptopodin), 1 mM EDTA (pH 8.0; for claudin-1), or 1 mM EDTA (pH 6.0; for nephrin).²¹ Endogenous peroxidase activity was quenched with 3% hydrogen peroxidase, and nonspecific protein binding was blocked with Background Buster (Accurate Chemical & Scientific Corp., Westbury, NY) and endogenous biotin activity was quenched with the Avidin/biotin blocking kit

(Vector Laboratories, Burlingame, CA). After blocking, tissue sections were incubated overnight at 4°C with the primary antibodies listed as immunohistochemistry in Table 1. The appropriate biotinylated secondary antibodies anti-rabbit IgG or anti-mouse IgG (Vector Laboratories) were applied followed by R.T.U. Vectastain kit (Vector Laboratories).

For synaptopodin staining, the mouse on mouse (M.O.M.) kit (Vector Laboratories) was used for additional blocking according to the manufacturer's protocol. Staining was visualized by precipitation of diaminobenzidine (Sigma Chemical).

For immunofluorescence staining for claudin-2, normal mouse kidney biopsies were snap-frozen and embedded in 22-oxalcaltriol compound (Tissue-Tek O.C.T. compound; Sakura Finetek, Torrance, CA). Sections (4 μm) were then fixed in 100% methanol at -20°C. Primary antibody for claudin-2 was incubated on frozen sections.

For immunofluorescence staining for PGP9.5, normal mouse kidney was perfusion-fixed with neutralized formalin, transferred to 10% sucrose, snap-frozen, and embedded in 22-oxalcaltriol compound (Tissue-Tek O.C.T. compound, Sakura Finetek). After, the sections (4 μm) were processed and hydrated. Primary antibody for PGP9.5 was incubated on frozen sections. For immunofluorescence staining for both claudin-2 and PGP9.5, the appropriate biotinylated secondary antibodies were applied and specific signals were visualized by Alexa Fluor 594-conjugated streptavidin (Invitrogen, Carlsbad, CA).

For double staining of claudin-1 and synaptopodin, kidney with perfusion fixation was processed, and, after rehydration, antigen retrieval was performed with 1 mM EDTA (pH 8.0) solution similar to what has previously been reported.²¹ After the blocking step mentioned previously, claudin-1 was stained first and the black signal was developed by diaminobenzidine with nickel. After color development, avidin/biotin blocking was performed again, and tissue sections were incubated with synaptopodin primary antibody. A brown color was then developed for synaptopodin.

Immortalized Mouse Podocyte Cell Line

Podocytes and PEC both have the potential to grow from isolated glomeruli in culture. Because it is critical to ensure that the targeted cell population generated was indeed mPEC and not podocytes, separate studies were performed in parallel on a well-characterized con-

ditionally immortalized mouse podocyte cell line in culture also derived from ImmortoMice^{8,19} for comparisons with mPEC.

Immunocytochemistry of mPEC and Podocytes

Candidate mPEC or well-characterized podocyte cell lines were plated simultaneously onto SecureSlips (Sigma-Aldrich) coated with Poly-D-lysine (Sigma) and type IV mouse collagen (BD Biosciences) as recommended by the manufacturer. After 14 d in culture under growth-restrictive conditions, cells were fixed with 2% formaldehyde containing 4% sucrose for 10 min, then permeabilized with 0.3% Triton-X100 in PBS for 10 min. For PGP9.5 staining, cells were fixed with 100% methanol for 30 min at -20°C. After a wash with 0.1% Triton X-100 in PBS, cells were incubated with the primary antibodies listed as immunocytochemistry in Table 1 overnight at 4°C. Biotinylated anti-rabbit IgG, anti-mouse IgG, or anti-guinea pig IgG antibodies were applied, followed by an Alexa Fluor 594-conjugated streptavidin. The cells were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). F-actin was visualized by incubation with FITC-conjugated phalloidin at room temperature for 1 h after fixation and permeabilization.

Western Blot Analysis

To ensure the specificity of the immunostaining results and to quantify specific proteins by a second method, we also performed Western blot analysis to analyze the expression levels of proteins specific for either mPEC or podocytes. Different conditions were required to detect specific proteins as follows. Cells were suspended in RIPA buffer (claudin-1, claudin-2, synaptopodin; 50 mM Tris HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% NP-40, containing proteinase inhibitor cocktail [Roche Molecular Biochemicals], with 1 mM sodium orthovanadate) or TG buffer (for PGP9.5; 1% Triton X-100, 10% glycine, containing proteinase inhibitor cocktail [Roche Molecular Biochemicals], with 1 mM sodium orthovanadate). Lysates were cleared by centrifugation, and protein concentrations were determined with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol. As for nephrin, we performed subcellular fractionation on the basis of multiple

Table 1. List of primary antibodies used in the studies^a

Target Protein	Host (Clone)	Vendor	Application
Claudin-1	Rabbit	Invitrogen (Carlsbad, CA)	WB, IHC, IC
Claudin-2	Rabbit	Abcam (Cambridge, MA)	WB, IC, IF
PGP9.5	Mouse (13C4)	Biomedica (Foster City, CA)	IF, WB
PGP9.5	Rabbit	Chemicon (Temecula, CA)	WB, IC
Nephrin	Guinea pig	Fitzgerald (Concord, MA)	WB, IC, IHC
Synaptopodin	Mouse (G1D4)	Fitzgerald	WB, IHC, IC
ZO-1	Rabbit	Invitrogen	IC
β-catenin	Mouse (14)	BD Biosciences (San Jose, CA)	IC
Ki-67	Rabbit (SP6)	Lab Vision (Fremont, CA)	IC
GAPDH	Mouse (6C5)	Abcam	WB
Na-K-ATPase	Mouse (464.6)	Abcam	WB
PCNA	Mouse (PC10)	Sigma-Aldrich (St. Louis, MO)	IC
F-actin (phalloidin)		Sigma-Aldrich	IC

^aGAPDH, glyceraldehyde-3-phosphate dehydrogenase; IC, immunocytochemistry; IF, immunofluorescent staining; IHC, immunohistochemistry; WB, Western blot.

centrifugation steps with increasing speed as previously reported.²² All steps were performed at 4°C. Cells were lysed in 10 mM Tris buffer (pH 7.5) containing 300 mM sucrose, 1 mM EDTA (all from Sigma-Aldrich), protease inhibitors (Roche), 50 mM NaF (Sigma-Aldrich), 1 mM Na-orthovanadate (Sigma-Aldrich). The cell homogenate was centrifuged for 5 min at 800 × g to clear the lysates from cellular debris and unlysed cells. The supernatant was further centrifuged for 20 min at 10,000 × g to separate cellular organelles (pellet) from cytosolic proteins (supernatant). The supernatant was further centrifuged for 60 min at 100,000 × g, resulting in a cytosolic protein fraction and a membranous fraction. All protein fractions were stored at -80°C. Na⁺/K⁺-ATPase (Abcam, Cambridge, MA) was stained at the same time as membranous protein markers.

Reduced protein samples (5 to 10 μg, for claudin-1, nephrin, and synaptopodin) or nonreduced protein samples (10 μg for claudin-2) were electrophoresed on 8, 10, or 12% SDS-polyacrylamide gel and subsequently transferred to polyvinylidene difluoride membranes (Hybond-P). After blocking for 30 min with either 5 or 0.5% nonfat dried milk, membranes were incubated overnight (4°C) with the primary antibodies listed as Western blot in Table 1. After washing in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), membranes were incubated with either biotinylated anti-mouse IgG or biotinylated anti-rabbit IgG (GE Healthcare Bio-Sciences, Piscataway, NJ) or biotinylated anti-guinea pig IgG (Abcam, Cambridge, MA). Bands were visualized with ECLplus detection reagent for all proteins (GE Healthcare Bio-Sciences), and membranes were exposed to Classic Blue autoradiography film BX (MidSci, St. Louis, MO) and developed.

Proliferation Assay

To examine the proliferative capacity of mPEC in growth-permissive and -restrictive conditions, we performed immunostaining for Ki-67 and PCNA and also used the MTT assay. mPEC were cultured in growth-permissive and growth-restrictive conditions. Cells in growth-restrictive conditions were fixed at day 14 and permeabilized and incubated with antibodies as described previously in the methods of immunocytochemistry. Primary antibodies for Ki-67 and PCNA are listed in Table 1.

mPEC activity was assessed by MTT assay (Promega, Madison, WI) as published previously.²³ Briefly, cells were plated into a 96-well plate; allowed to adhere overnight; and cultured in growth-permissive condition for 1, 2, 3, 4, or 5 d or in growth-restrictive conditions for 5, 7, or 14 d. A labeling dye included in the kit was added 4 h before the end point. A stop solution was added to each well to stop the reaction and solubilize the cells, and the absorbance was read at 570 nm.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (DK60525, DK56799, DK51096, F32 DK070434, and K08DK076970) and by the American Diabetes Association. S.J.S. is also an Established Investigator of the American Heart Association. T.O. was supported by Sankyo Foundation of Life Science.

DISCLOSURES

None.

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