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A Conditionally Immortalized Human Podocyte Cell Line Demonstrating Nephrin and Podocin Expression

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Abstract. Recent molecular insights have established the podocyte as a key component of the glomerular filtration barrier, and hence an important common pathway in proteinuric diseases. A conditionally immortalized human podocyte cell line has been developed by transfection with the temperature-sensitive *SV40-T* gene. These cells proliferate at the "permissive" temperature (33°C). After transfer to the "nonpermissive" temperature (37°C), they entered growth arrest and expressed markers of differentiated *in vivo* podocytes, including the novel podocyte proteins, nephrin, podocin, CD2AP, and synaptopodin, and known molecules of the slit diaphragm ZO-1, α-, β-, and γ-catenin and P-cadherin. The differentiation was accompanied by a growth arrest and the upregulation of cyclin-dependent kinase inhibitors, p27 and p57, as well as cyclin D_1, whereas cyclin A was downregulated. These data are consistent with cell cycle protein expression during podocyte maturation *in vivo*. In conclusion, the development of this cell line provides a new tool in the study of podocyte biology, which will enable accurate assessment of the behavior of these complex cells in health and disease.

Podocytes are highly specialized, terminally differentiated cells, with cell bodies, major processes, and foot processes interlinked by slit diaphragms. During renal development, podocyte precursor cells arise from induced mesenchymal renal stem cells and subsequently undergo a considerable change in phenotype before reaching the mature, differentiated state (1,2). In the "S shaped body stage," podocytes have the characteristics of epithelial cells with apically located tight junctions (3) and prominent mitotic activity (4). During the capillary loop stage of glomerular development, the cells begin to establish their complex architecture, including the characteristic foot processes (3), and express markers of maturity, such as vimentin, an intermediate filament protein (4), and synaptopodin, an actin-associated protein that is present specifically in process-bearing differentiated podocytes (5,6).

In the mature state, podocytes lose their proliferative capacity and develop specialized junctions between the cell body and the glomerular basement membrane, known as the focal adhesion complex, and junctions between interdigitating foot processes, known as the slit diaphragm or membrane. The functional basis for the maintenance of this cell in its specialized state and its ability to sustain the filtration barrier are poorly understood, and it is hoped that an understanding at the molecular level will begin to unravel the podocyte's properties. To unravel the functional pathways in which these proteins have a role and to further our comprehension of the cellular mechanisms intrinsic to this complex cell, cell culture experiments will become increasingly important.

For a long time, the selective analysis of podocytes *in vitro* was difficult, because only rather undifferentiated "podocytes" of questionable cellular origin were available in culture. Cultivation of podocytes under standard conditions leads to dedifferentiation reflected by the loss of processes. It is also accompanied by a loss of synaptopodin (5,7). We therefore established cell culture conditions that allow the differentiation of podocytes *in vitro* (8). We modified the cell culture conditions for rat and human podocytes by avoiding repeated subcultivation. This led to profound phenotypic changes in podocytes *in vitro*. We directly observed the conversion of cobblestones into arborized cells by documenting a series of intermediate phenotypes (8). The cells converted within 2 to 3 wk from typical cobblestone appearance into individual arborized cells more closely resembling podocytes *in vivo*. Arborized cells are frequently binucleated and can reach a size of up to 500 μm. Both cobblestone and arborized cells originated from podocytes, as evidenced by the expression of a podocyte-specific O-acetylated ganglioside (9) and the WT-1 protein, which is restricted to podocytes in the adult kidney (8). The differentiation of arborized cells was reflected by the formation of processes and the expression of synaptopodin, which was never detected in cobblestones (8). As in nephrogenesis, as soon as cobblestones begin to form processes, they stop dividing. Concomitantly, synaptopodin emerges and is found in a punctuated pattern alongside actin filaments extending into cell

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processes (8). These data are supportive for a potential role of synaptopodin in the development and maintenance of podocyte processes.

The differentiation of human and rat podocytes *in vitro* is associated with an irreversible growth arrest. To circumvent this problem we derived conditionally immortalized podocyte cell lines from the immortomouse carrying a temperature-sensitive T antigen as transgene (10). These cells can be maintained either in a proliferative state at 33°C or be induced to differentiate at 37°C. Both proliferating and differentiating podocytes express the WT-1 protein (7). The differentiated cell showed an ordered array of actin filaments and microtubules extending into the forming processes during differentiation, reminiscent of podocyte processes *in vivo*. These cytoskeletal rearrangements and process formation were accompanied by the onset of synaptopodin synthesis (7).

On the basis of our previous experience with primary human podocyte cultures (8), we have now created a cell line of human podocytes that are derived from a nephrectomy specimen transfected with a tsA58 T antigen using a retroviral vector. At the nonpermissive temperature, these cells show growth arrest and key features of podocyte differentiation and function. We report the expression and distribution in these cells of the newly described podocyte proteins, nephrin (11) and podocin (12), for the first time in cultured podocytes. The availability of differentiated human cells to study, in defining functional characteristics of important new podocyte specific proteins, will provide a powerful new tool in this area of biology.

Materials and Methods

Primary Culture of Podocytes

A nephrectomy specimen was obtained from a 3-yr-old child with a minimally functioning (10% differential function) kidney, which had been detected antenatally as hydronephrosis. The other kidney appeared normal on imaging, with a calculated GFR within the normal range. Histology on the specimen revealed a small scarred kidney with glomerulosclerosis, tubular atrophy, and large scars over dilated calyces. No specific histologic features of dysplasia were seen. The features were consistent with a unilateral antenatal obstructive/reflux nephropathy, and no primary glomerular disease is described or predicted in this condition; therefore, isolated podocytes from this kidney were taken to be normal. This was to circumvent the logistical and ethical difficulty in obtaining fresh normal human kidney specimens. Local ethics committee approval was obtained for this purpose.

Glomeruli were isolated as described previously (8) and cultured in 25-cm^2 flasks (Falcon; BD Biosciences, Bedford, MA) in RPMI 1640 medium with added penicillin, streptomycin, insulin, transferrin, selenite (Sigma Chemicals, Dorset, UK) and 10% fetal calf serum. Epithelial cell outgrowths appeared and grew to confluence at 10 to 14 d, and the cells were passaged at this point and transfected with the tsSV40 gene construct.

Retroviral Construct and Virus Infection

The retroviral construct consisted of a SV40 large T antigen gene containing both the tsA58 and the U19 mutations inserted into the single *Bam*HI restriction site of the vector, pZipNeoSV(X)1 (13). Cultures of primary human podocytes were infected with retrovirus-containing supernatants from the packaging cell line (PA317). Cells in log-phase growth were exposed to freshly thawed filtered (0.45 μm) supernatant mixed 1:1 with growth medium plus 8 μg/ml polybrene. After 24 h, cultures were refed with fresh growth medium and grown for an additional 7 d to confluence. The culture medium was then supplemented with 0.5 mg/ml G418 (Life Technologies BRL, Life Technologies, Paisley, UK) until selection was complete (7 to 10 d). Infection, selection, and continuous culture were carried out at 33°C.

Subcloning of Cell Lines

The transfected cells were subcloned twice as follows. Confluent cells were trypsinized, spun down, and counted on a hemocytometer. The cells were then seeded at densities of 100, 200, 300, and 400 cells per 25-cm^2 flask and grown at 33°C in growth medium as described. Irradiated NIH 3T3 mouse fibroblast cells were added to each flask at a density of 0.5×10^6 cells/flask as nondividing feeder cells. Single cell clones were picked when visible to the naked eye (21 to 28 d) by using plastic cloning rings and transferred to individual wells of a 24 well plate. When grown to confluence, these were transferred to larger flasks, and a single clone was used for all the experiments described. Cells were used for experiments between passages 10 to 20.

Propagation of Cells

Cells were grown to confluence at 33°C, at which point they were trypsinized and reseeded in fresh flasks at a dilution of between 1:3 and 1:5. Before thermoswitching to 37°C, cells were grown to 70 to 80% confluence. At both temperatures, cells were fed with fresh medium 3 times per week.

Induction of Differentiation

Cells were subsequently grown on type I collagen–coated flasks layered with glass cover slips for purposes of immunostaining. Cells were then plated onto the flasks and grown either at the permissive temperature of 33°C (in 5% CO_2) to promote cell propagation as a cobblestone phenotype (undifferentiated) or at the nonpermissive temperature of 37°C (in 5% CO_2) to inactivate the SV40 T antigen and allow the cells to differentiate.

Antibodies

Primary antibodies: monoclonal p27, p57, cyclin A, cyclin D_1, and polyclonal proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal WT-1 (Santa Cruz Biotechnology); polyclonal ZO-1 and p-cadherin (Zymed, San Francisco, CA); monoclonal synaptopodin (Progen, Heidelberg, Germany); polyclonal α-, β- and γ-catenins (Santa Cruz Biotechnology). Secondary antibodies: FITC-conjugated anti-mouse IgG_1, anti-rabbit IgG, and anti-goat IgG (Jackson Immunoresearch, Philadelphia, PA); Rhodamine-conjugated anti-mouse (Jackson Immunoresearch). Controls used were rabbit, mouse, or goat serum (as appropriate) for polyclonal primary antibodies and mouse IgG_1 or IgG_2 (Sigma) for monoclonal antibodies. FITC- and Rhodamine-conjugated secondary antibody alone were used in all experiments as additional controls.

Monoclonal mouse anti-nephrin IgG_1 was a kind gift of Dr. K. Tryggvason, Karolinska Institute, Stockholm, Sweden. This was raised against recombinant protein representing the complete intracellular nephrin domain produced in *Escherichia coli*. Mouse polyclonal anti-CD2AP antibody was a kind gift of Dr A. Shaw, St. Louis, MO.

The polyclonal antibody against podocin was generated by immunizing rabbits with a keyhole limpet hemocyanin-conjugated peptide (single letter code, SKPVEPLNPKKKDSPML) corresponding to the C-terminus of mouse and human podocin, which are 100% identical

in this region of the molecule (authors' not shown). The antiserum was affinity-purified with the corresponding peptide linked to Ultralink (Pierce, Rockford, IL) according to the manufacturer's instructions. The specificity of the podocin antibody for the podocin C-terminus was verified by Western blot analysis with crude bacterial lysates from cells expressing either glutathione s-transferase (GST), the podocin N-terminal fragment or the podocin C-terminal fragment. After induction of fusion protein expression, only the antigen containing C-terminal fragment showed a signal on Western blots, whereas GST alone or the N-terminal fusion protein were negative (data not shown). Moreover, the antibody did label podocytes in normal human kidney but not in patients with the R138Q podocin mutation (data not shown), thereby confirming the specificity of the antibody against the podocin C-terminus.

Immunostaining

The immunolabeling was done as described previously (7). Briefly, cover slips were fixed with 2% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS) for 10 min and then permeabilized with 0.3% Triton X-100 (Sigma) in PBS for 10 min. Nonspecific binding sites were blocked with 4% fetal calf serum + 0.1% Tween 20 (Sigma) in PBS for 30 min. Primary and secondary antibodies were applied at the appropriate dilutions according to standard techniques, and the cover slips were mounted on glass slides with 15% Mowiol (Calbiochem, La Jolla, CA), 50% glycerol in PBS. Double-staining was achieved by incubating with primary antibody and FITC-conjugated secondary antibody as above and then incubating further with Texas Red preconjugated second antibody for 20 min at room temperature. Further washes and mounting were as above. Images were obtained by using a Leica photomicroscope attached to a Spot 2 slider digital camera (Diagnostic Instruments Inc., Sterling Hts. MI) and processed with Adobe Photoshop 5.0 software.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was done by using "environmental" scanning electron microscopy (ESEM), which allows observation of the cells without freezing (14). The cells were fixed for 2 h in 5% glutaraldehyde in 0.1 M phosphate buffer. They were rinsed in buffer three times and stored at 4°C. For ESEM, the coverslips were dipped briefly into filtered (0.22 μm) distilled water. The underside was blotted dry and attached to an ESEM stub with double-sided conductive tape. The cells were cooled to 5°C and the chamber pumped down at 6.8 torr initially to reduce evaporation. The pressure in the ESEM chamber was slowly reduced to evaporate water and expose the cells.

Polymerase Chain Reaction

Reverse transcription-PCR (RT-PCR) for nephrin was done with the following sequence-specific primers (from Interactive Biotechnology): forward 5'–TGG CGA TTC CTG CCT CCG TT–3' (from exon 25); reverse 5'–TTC TGC TGG GAG CCC TCG TT–3' (from exon 27). Total RNA from undifferentiated and differentiated immortalized podocytes and primary cultured proliferating podocytes was used. Positive control cDNA was from a human kidney library (Clonetics, Walkersville, MD), and negative controls included a no RT lane and a lane run without cDNA in the reaction mix. Sequencing of nephrin PCR product was carried out by using standard preparation techniques for an ABI prism automatic sequencer (Applied Biosystems, Foster City, CA). Sequence specific primers for CD2AP were: forward 5'–CTGTCAGCTGCAGAGAAGAAA; reverse 5'–TTGGGTTGGAGAATGTCCAC.

Protein Extraction and Western Blotting

Cell proteins were extracted by addition of a lysis buffer containing 1% saponin (Sigma), 75 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 50 mM Tris (pH 7.7), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na vanadate at 4°C. The suspension was centrifuged at 14000 $\times$ *g*, and the supernatant containing cellular protein was collected. For Western blotting, An 8% sodium dodecyl sulfate–polyacrylamide gel was run under standard conditions, loading 100 μg of total protein in each lane. The gel was placed in transfer buffer for 15 min and set up for transfer to a polyvinylidene fluoride membrane at 200 mA for 1 h. The membrane was rinsed in Tris-buffered saline followed by rinsing in blocking buffer (5% milk powder) for 5 min. The membrane was then immersed in blocking buffer for 1 h before incubation with primary antibodies at appropriate dilutions. After rinsing in wash buffer, horseradish peroxidase–conjugated secondary antibody (Amersham Biotech, Buckinghamshire, UK) was used for 1 h at 1:2500 dilution. After final washing, the membrane was developed using ECL chemiluminescence reagent (Amersham Biotech).

Results

Generation of Conditionally Immortalized Human Podocyte Cell Lines

Of the primary cultures immortalized, approximately 80% of cells expressed the podocyte-specific marker, WT-1 (15) (data not shown). After two rounds of subcloning, eight clones were picked, all of which were phenotypically identical under light microscopy. Of the clones examined, 100% of cells expressed WT-1, and one of these clones was used for all subsequent experiments. At the permissive temperature of 33°C, the cells grew in a typical (of epithelial cells) cobblestone morphology (Figure 1A, left panel). Shifting the cells to 37°C resulted in arrest of proliferation of 100% of cells within 14 d, and over a period of 7 to 14 d, the cell bodies enlarged in an irregular shape, with the formation of processes both short and more rounded and also long, spindle-like projections (Figure 1A, right panels) similar to those previously described for primary human cultures (8). On SEM, undifferentiated cells had a smooth shape with no projections (Figure 1B, left panel), whereas differentiated cells demonstrated thin processes of between 3 to 10 μm in length (arrows in Figure 1B, right panel). These were seen to form interdigitations with slit diaphragm–like structures compatible with the *in vivo* appearance (Figure 1B, right panel).

Expression of Podocyte-Specific Markers

Immunohistologic markers were used to compare the phenotype of the cells under these two different temperatures. In line with previous results obtained for nonimmortalized primary human podocytes (7), WT-1 was expressed at comparable levels in all cells at both permissive and nonpermissive temperatures (Figure 1C). These findings provide the first line of evidence that the cell lines generated are of podocyte origin. One of the most sensitive and specific markers for differentiated podocytes so far described is synaptopodin. This has been found to be expressed in mature foot processes and to colocalize with the actin cytoskeleton of foot processes (5). As in primary human cultures (8) and in the conditionally immortal-

ized murine cell lines (7), there was no expression of synaptopodin in the undifferentiated human cell lines at 33°C (Figure 1D, left panel). By day 14 under nonpermissive conditions, we saw strong expression in mature cells, particularly in the cytoplasm and extending into cell processes (Figure 1D, right panel).

Formation of the Slit Diaphragm Complex

It was recently demonstrated that the slit diaphragm may represent a P-cadherin–based, modified adherens junction, also found *in vitro* in the differentiated murine cell line (16). Here we demonstrated the same components at the cell-cell junctions in our human cell line. P-cadherin expression was not seen in undifferentiated cells (Figure 2A, left panel), but was expressed at the cell surface in the differentiated cells, particularly at cell-cell contacts (arrow in Figure 2A, right panel). As in the murine cells (16), P-cadherin was also strongly expressed in the nucleus of differentiated podocytes. The nuclear staining for P-cadherin is specific, because it was not seen with the anti-mouse secondary antibody alone (Figure 2E, right panel). Nephrin is a newly described transmembrane protein located at podocyte slit diaphragm (11). We detected mRNA

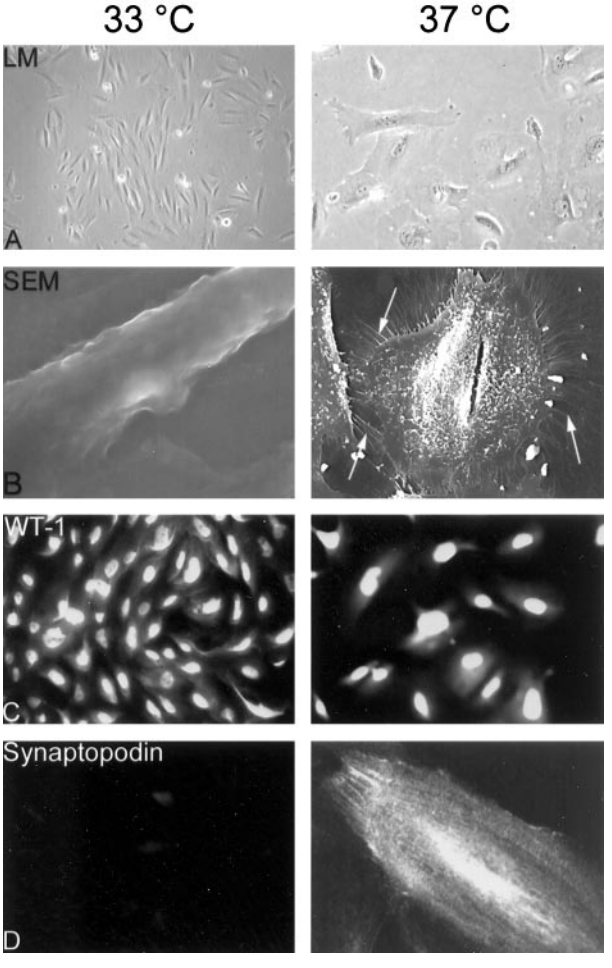

Figure 1. Morphology of cultured podocytes and expression of podocyte-specific markers. Undifferentiated (left panels) and differentiated (right panels) cultured podocytes. (A) Light microscopy images. (B) Scanning electron microscopy showing undifferentiated cell without processes and differentiated cell demonstrating fine processes (arrows) with interdigitations between cells and presumed slit-diaphragm–like structures. (C) WT-1 showing nuclear expression in both undifferentiated and differentiated cells. (D) Synaptopodin, absent in undifferentiated cells, and appearing along actin filaments in differentiated cells. Magnifications: ×40 in A and C; ×60 in D.

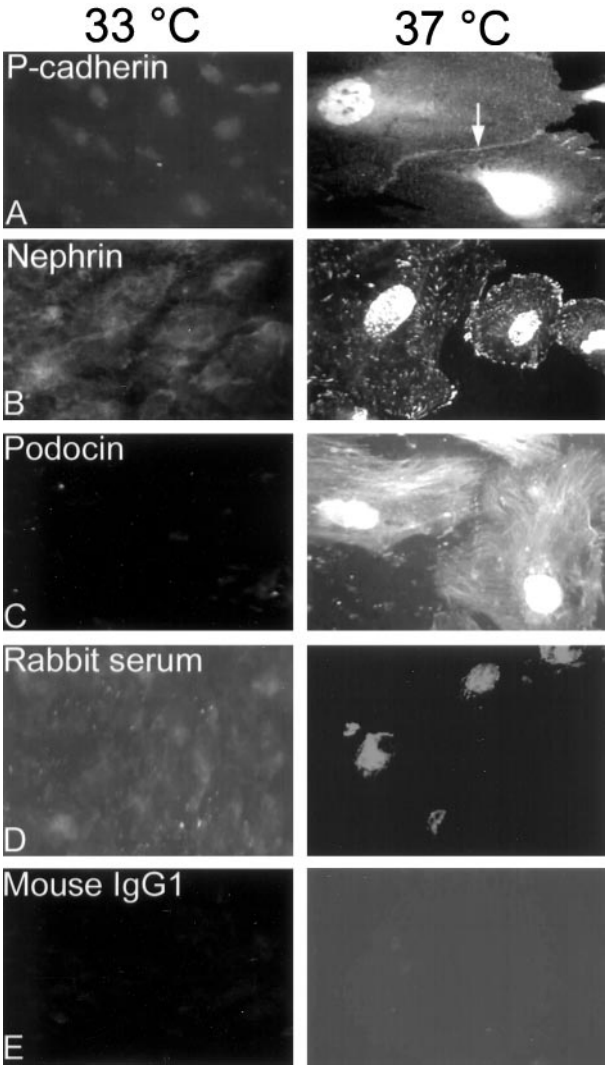

Figure 2. Immunofluorescence microscopy of slit diaphragm-associated proteins. Undifferentiated (left panels) and differentiated (right panels) podocytes. Immunofluorescence staining for podocyte-specific proteins. (A) Weak nuclear expression of P-cadherin in undifferentiated cells (left panel), but clear expression at cell-cell junction (arrow) and in the nucleus of differentiated cells (right panel). (B) Weak diffuse cytoplasmic expression of nephrin in undifferentiated cells (left panel), changing to punctuated cell surface and cytoplasmic distribution in differentiated cells. (C) Podocin, no expression in undifferentiated cells; filamentous and cell surface (arrows) expression in differentiated cells. (D and E) Controls without primary antibodies. (D) Rabbit serum for podocin antibody, showing unspecific nuclear staining. (E) Mouse IgG_1 control for nephrin and P-cadherin antibodies, showing specificity of nuclear nephrin and P-cadherin expression. Magnification, ×40

expression of nephrin and CD2AP, a cytoplasmic binding partner of nephrin (17,18), by RT-PCR (Figure 3A) and confirmed these results by sequencing the PCR products (data not shown). Western blotting demonstrated nephrin protein expression in undifferentiated and differentiated cells (Figure 3B, left panel). On immunofluorescence, a weak diffuse nephrin staining was seen in undifferentiated cells (Figure 2B, left panel). In differentiated cells, we observed nephrin expression at the cell periphery, as would be expected, but also cytoplasmic distribution reminiscent of a focal contact pattern (Figure 2B, right panel). As for P-cadherin, a specific nuclear expression was seen for nephrin, which was not detected with the anti mouse IgG_1 secondary antibody alone (Figure 2E). Podocin is the protein product of the *NPHS2* gene, discovered as the affected gene in familial focal segmental glomerulosclerosis (FSGS) (12). Podocin was detected by Western blotting in differentiated cells and was absent in undifferentiated cells (Figure 3B, right panel). This pattern was repeated on immunofluorescence, with no podocin seen in undifferentiated cells. In differentiated cells, we observed podocin distribution in a filamentous and cell surface distribution (Figure 2C, right panel). The nuclear staining observed in Figure 2C is unspecific, because it was also seen with the anti-rabbit secondary antibody alone (Figure 2D, right panel). As previously described in the mouse podocyte model (7), we also found expression of the slit diaphragm proteins, ZO-1 and α-, β-, and γ-catenin, and the focal adhesion proteins, paxillin, vinculin, and α_3-integrin (data not shown),

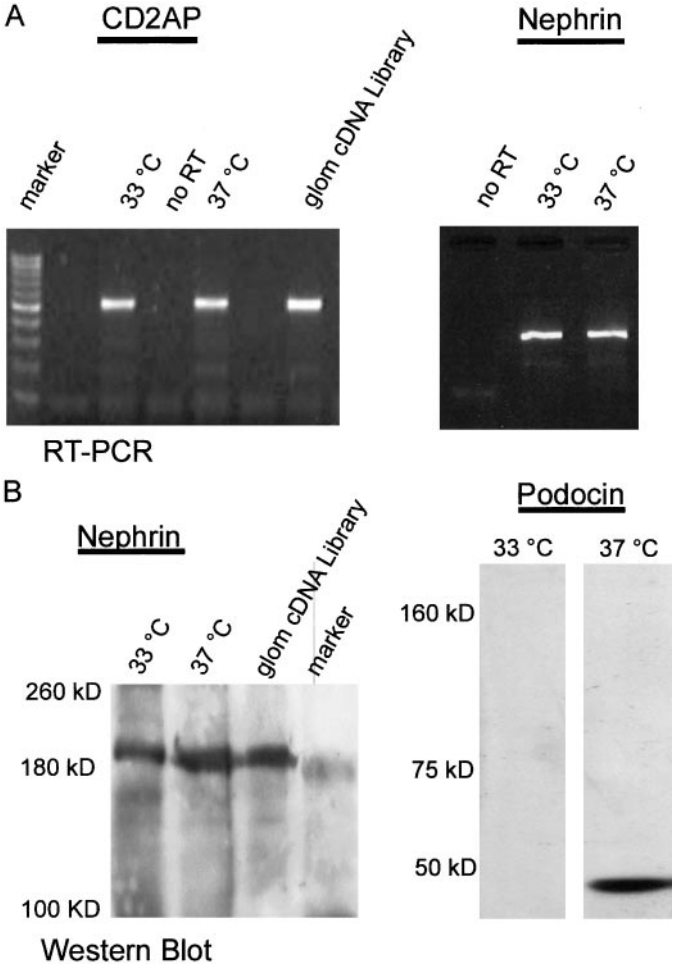

Figure 3. Reverse transcription-PCR (RT-PCR) and Western blot analysis of slit diaphragm–associated proteins. (A) RT-PCR for CD2AP and nephrin using cDNA from undifferentiated (33°C) and differentiated (37°C) immortalized podocytes. Control lanes are from reactions without reverse transcription (no RT). As a positive control, CD2AP was also amplified from a mouse glomerular cDNA library). CD2AP mRNA is expressed in both undifferentiated and differentiated cells (left panel); nephrin mRNA is expressed in differentiated and in undifferentiated podocytes (right panel). (B) Western blot analysis of nephrin (left panel) and podocin (right panel) using total protein extracts from undifferentiated and differentiated immortalized podocytes. Nephrin is detected in undifferentiated and differentiated cells at a size of approximately 180 kD; podocin is expressed only in differentiated cells at a size of approximately 45 kD.

The Cell Cycle Regulation of Cultured Podocytes is Comparable to the In Vivo Situation

The ability of podocytes to proliferate depends on their state of differentiation. During glomerulogenesis, immature podocyte precursor cells undergo marked proliferation (19). However, as podocytes mature, they become terminally differentiated, a phenotype that is quiescent (4,19,20). Normal mature podocytes express cyclin kinase inhibitors (CKI), p27 and p57, and cyclin D_1, indicating that they are in a state that precedes the first checkpoint in the cell cycle (4). To look for evidence that cell cycling is switched off in the differentiated cells, we immunostained them with PCNA, a nuclear marker of cell proliferation, and cyclin A, known to regulate the G2/M transition. At 33°C, there was strong nuclear expression of both PCNA (Figure 4A, left panel) and cyclin A (Figure 4B, left panel), which were gradually downregulated and were absent by day 14 (Figure 4, A and B, right panels). In contrast, the expression of the CKI, p27 (Figure 4C) and p57 (data not shown), and of cyclin D_1 (Figure 4D), expressed in the late G_1 phase of the cell cycle (4), was in the opposite direction, with no expression at 33°C (Figure 4D, left panel), changing to much stronger expression after 14 d at 37°C (Figure 4D, right panel), as in the *in vivo* podocyte (4). Hence, the cell lines provide a useful tool to analyze the mechanisms regulating podocyte cell cycle progression.

Discussion

In this study, we have transfected primary cultures of human podocytes with a temperature-sensitive gene construct and shown that they display properties of differentiation seen for the first time in a cultured human podocyte cell line. This coincides with the discovery of novel podocyte-specific genes that have established the podocyte as a crucial component of glomerular filtration, and promise to transform our knowledge of this poorly understood process. Expression of nephrin is described for the first time in cultured podocytes. Until recently, the nature of the podocyte slit pore, the intercellular junction that regulates the passage of macromolecules at the podocyte level, had largely remained a mystery. A crucial

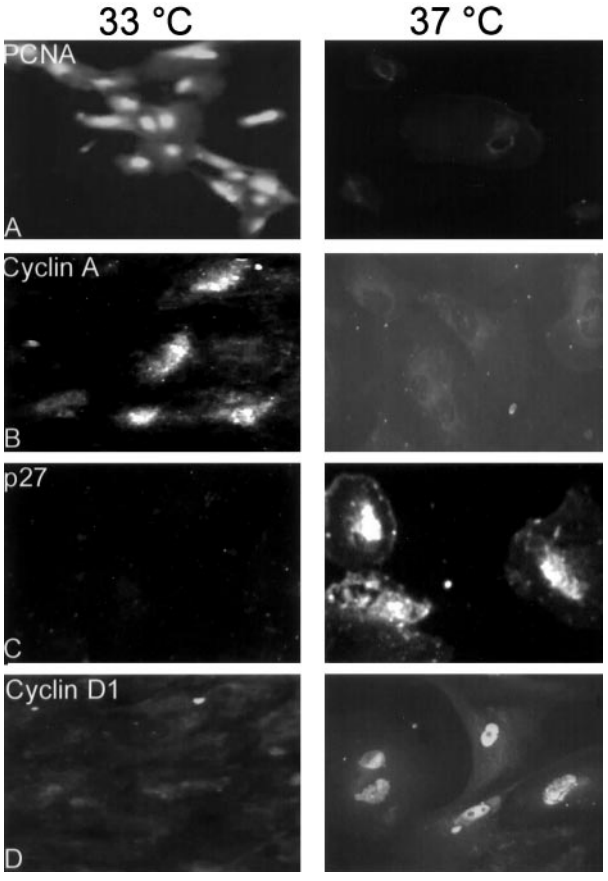

Figure 4. Immunofluorescence microscopy of cell cycle–associated proteins. Undifferentiated (left panels) and differentiated (right panels) cultured podocytes. Nuclear staining of proliferating cell nuclear antigen (PCNA) (A) and cyclin A (B) in undifferentiated cells, switching off in differentiated cells. (C and D) Absence of p27 (C) and cyclin D_1 (D) in undifferentiated cells (left panels), and strong expression in differentiated cells (right panels). Magnifications: ×40 in A and D; ×60 in B and C.

recent development in the understanding of the molecular constituents of this junction has been the discovery of the gene encoding the novel transmembrane protein, nephrin (11). This appears to be a podocyte-specific molecule within the kidney, which has been localized to the intercellular slit pore by immunogold electron microscopy studies (11,21), and the possibility of intracellular expression has also been raised (22). We show nephrin localizing at the cell membrane as expected in a discontinuous pattern and also cytoplasmic filamentous distribution. Intracellular nephrin localization has not been detailed previously. Immunogold electron microscopy studies reveal gold particles at most foot process slit diaphragms (11).

It may be that the latter technique is not sufficiently sensitive to detect intracellular nephrin, as immunofluorescence has long been established as a highly sensitive technique. It might also be argued that full maturity of the cell was not achieved in our culture model, and we were seeing an intermediate stage in which nephrin is being transported intracellularly to its final functional site. We consider this unlikely because, developmentally, nephrin appears at the early capillary loop stage (21) (*i.e.*, concurrent with or slightly earlier than synaptopodin) and hence would be unlikely to be incompletely expressed in a cell in which synaptopodin is fully apparent. Immunogold electron microscopy on tissue sections of mouse developing glomerulus at the capillary loop stage also showed nephrin distribution at the basal margins of podocytes, reflecting the mature distribution (21). Finally, the clinical observation in Finnish congenital nephrotic syndrome is that proteinuria is detectable early *in utero*, indicating that nephrin is required intact at the slit diaphragm at an early stage of development to prevent fetal protein losses, which would not correspond to a model of relatively late maturity of nephrin expression. Therefore, we suggest that this intracellular distribution of nephrin is related to its intracytoplasmic portion associating with actin filaments, as part of the mechanism regulating the physical structure of the foot process. This is being further studied. The intracellular portion of nephrin contains nine tyrosines (11), some of which could become phosphorylated during ligand binding and mediate important signals to the intracellular compartment. Of particular interest would be the relay of messages to the cytoskeleton as there is morphologic data suggesting that cytoskeletal abnormalities are responsible for the podocyte foot process effacement seen in all types of nephrotic syndrome (23). Intriguingly, it has been suggested that there is a decreased expression of nephrin in glomeruli of patients with minimal change nephrotic syndrome (24) and altered distribution in experimental nephrosis (25). However, functional studies on how nephrin regulates slit pore porosity or is itself regulated have not yet been reported and would be greatly facilitated by representative *in vitro* podocyte models.

Two other gene mutations have recently come to light as separate and specific causes of familial FSGS. These are the genes coding for α-actinin–4 (26), a cytoskeletal linking protein, and the gene termed *NPHS2*, encoding the novel protein podocin (12). Within the glomerulus, both are expressed exclusively in the podocyte. In mouse knockout models of the genes for CD2AP (18) and s-laminin/laminin-β2 (27), which are podocyte/glomerular basement membrane–expressed proteins, there is massive proteinuria. Additionally, the knockout of α3-integrin results in abrogation of foot process formation (28). Other genes mutated in nephrotic diseases are currently being sought and will all contribute to the information required to analyze podocyte function in health and disease.

The study of podocytes in culture to date has for a long time been hampered by the unrepresentative phenotype of proliferating epithelial cells, a problem recently overcome by using the immortomouse transgenic mouse to grow podocytes that lose their proliferative phenotype under nonpermissive conditions (7). Podocytes in culture differ from those *in vivo* in two crucial respects. First, initiation of podocyte differentiation is intimately linked to these cells leaving the cell cycle and stopping cell division (19). Dividing primary culture cells or those immortalized with constitutively active SV40 T antigen (29) are, therefore, unable to exit the cell cycle and to display the features of mature (postcapillary loop stage) podocytes. They will therefore display markers of cell proliferation, such

as PCNA and Ki67 antigens, and will not express the proteins that downregulate the cell cycling process, namely the cyclin dependent kinases (4). Cell cycle control of podocytes is a key issue in the biology of this cell, as their inability to replicate and regenerate in most forms of glomerular injury is thought to be a major factor in the progression of renal injury. The underlying premise of conditionally immortalizing cells is that they are able to achieve a growth-arrested state akin to the *in vivo* mature cell phenotype. During development, podocytes express PCNA, a marker of proliferation, until the capillary loop stage, at which point they stop dividing (4,19). The CKI, p27 and p57, are first detectable at this stage and are coexpressed with podocyte differentiation markers, such as synaptopodin (4) and CR1 (C3b receptor). Haseley *et al.* (30) have shown in murine tsSV40 podocytes that differentiation of these cells is associated with upregulation of p27 and p57 but not of p21. Our cells reflected this progression of maturity, with nuclear expression of PCNA in the cobblestone stage and no expression of p27 and p57. Under nonpermissive conditions, the mirror image situation was seen, with little or no PCNA expression and upregulation of p27 and p57 CKI. Additionally, the cyclins, cyclin A and cyclin D_1, altered their expression commensurate with their known role in controlling the cell cycle (31). We also showed that the cells express the cyclin-dependent kinases, p27 and p57, in the differentiated form, indicating that these regulators of the cell cycle process are important in growth arrest in mature podocytes and strengthening a previous observation of enhanced expression of these molecules in experimental glomerulonephritis (32). It has now been shown that podocytes can proliferate in the human diseases, HIV nephropathy and collapsing glomerulopathy (33). This is important in that lack of podocyte regeneration in glomerular disease is a key factor in progressive glomerular degeneration and ultimately end-stage renal failure. In human diseases, such as focal segmental glomerulosclerosis, hypertension, or diabetes loss of podocytes can lead to progressive glomerular degeneration, as the supporting and filtering role of podocytes is lost, and these cells appear unable to regenerate *in vivo* (34). Study of the factors controlling podocyte cell cycle arrest in the mature state and how this might be reversed is therefore highly desirable and more achievable with this cell line.

We then looked at expression of proteins known to be important in podocyte structure. In the differentiated state, we observed localization of known components of the slit pore, focal contacts, and cytoskeleton as would be appropriate for mature podocytes *in vivo*. We demonstrated expression of P-cadherin and nephrin, which are transmembrane components of the slit diaphragm. We also showed filamentous organization of nephrin and podocin. Hence we have observed in podocytes *in vitro* a process of maturation analogous to the developmental maturation of podocytes *in vivo*, with the final stage being a nondividing arborized cell expressing the known antigenic markers of terminally differentiated cells.

Previously constitutively immortalized (SV40) human podocyte cell lines exhibit a regular, cobblestone-like polygonal phenotype (29,35). They maintain the morphologic features of transformed cells, and express cytokeratin, an epithelial cytoskeletal protein, with no evident pattern of filaments. They have not been reported to express specialized features of mature podocytes, such as synaptopodin or nephrin. Second, proliferating podocytes in culture display the properties of typical cobblestone epithelial cells in culture, with (nonspecific) tight junctions. In addition, human cell lines will provide a more accurate phenotype in studying proteins and pathways involved in human disease. This is well illustrated in the case of nephrin, where the murine protein has only about 83% sequence identity with human nephrin (36).

In summary, we have developed a tool in the study of human glomerular disease that will help circumvent some of the practical problems so far encountered in podocyte culture experiments and will also allow for the exploitation of some of the molecular data that is now coming to light in podocyte biology. The observations that these cells in culture follow a pattern of maturation similar to that seen in the stages of embryonic development gives us confidence that this is an accurate human podocyte model with which to proceed to further studies. Also, with recent interest focusing on the precise nature of the slit diaphragm and its derangement in disease, it is clearly important to study cells that recapitulate *in vitro* the molecular anatomy of mature cell-cell junctions. It should be emphasized that, although our detailed characterization in this study reveals features consistent with considerable *in vitro* differentiation, there is not at present any means to prove that this process is complete. Finally, the immortalizing technique used here allows us to isolate podocyte cell lines from patients with congenital nephrotic syndromes (which we are now successfully doing), which will provide cell lines of human podocytes with naturally occurring gene mutations (37,38). These will be valuable in functional studies of newly discovered podocyte genes and in defining the effects of specific human mutations. Podocyte biology is on the verge of being rapidly unraveled at the molecular level, and we feel this cell line has the potential to provide an integral tool in that process.

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