Conditionally immortalized human podocyte cell lines established from urine

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1Kidney Disease Section, Kidney Disease Branch, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland; 2Department of Rheumatology and Applied Immunology, Saitama Medical University, Saitama, Japan; and 3Department of Neurodegenerative Diseases, Institute of Neurology, University College London, London, United Kingdom

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Sakairi T, Abe Y, Kajiyama H, Bartlett LD, Howard LV, Jat PS, Kopp JB. Conditionally immortalized human podocyte cell lines established from urine. Am J Physiol Renal Physiol 298: F557–F567, 2010. First published December 9, 2009; doi:10.1152/ajprenal.00509.2009.—Evidence suggests that loss of podocytes into urine contributes to development of glomerular diseases; shed podocytes are frequently viable and proliferate in culture conditions. To determine the phenotypic characteristics of viable urinary cells derived from human subjects, we established long-term urinary cell culture from two patients with focal segmental glomerulosclerosis and two healthy volunteers, via transformation with the thermosensitive SV40 large T antigen (U19tsA58) together with human telomerase (hTERT). Characterization of arbitrarily selected two clonal cell lines from each human subject was carried out. mRNA expression for the podocyte markers synaptopodin, nestin, and CD2AP were detected in all eight clones. Podocin mRNA was absent from all eight clones. The expression of nephrin, Wilms’ tumor 1 (WT1), and podocalyxin mRNA varied among the clones, which may be due to transformation and/or cloning. These results suggest that podocyte cell lines can be established consistently from human urine. The generation of podocyte cell lines from urine of patients and healthy volunteers is novel and will help to advance studies of podocyte cell biology. Further improvements in the approaches to cell transformation and/or cell culture techniques are needed to allow cultured podocytes to fully reproduce in vivo characteristics.

focal segmental glomerulosclerosis; SV40 large T antigen; human telomerase

There is strong evidence that podocyte damage and loss contribute to the initiation of glomerulosclerosis and progression of renal disease (21, 28). Mature podocytes are highly differentiated cells; it remains controversial as to whether they undergo replenishment under normal or pathological conditions (1, 21, 32). Podocyte depletion leads to nephron degeneration and renal tubular fibrosis that are closely correlated with decline in renal function (17, 18), and a reduction of the podocyte number is related to severity of glomerular injury (19, 20, 23, 27, 41).

Measurement of podocyte number or mRNA of podocyte proteins in urine sediment has been suggested as a useful tool for monitoring glomerular disease activity. Hara et al. (8–10), Nakamura et al. (25), and Habara et al. (7) measured the number of urinary podocytes using an antibody recognizing podocalyxin and reported that the excretion of podocalyxin-positive cells was increased in active glomerular diseases. Wang et al. (39, 40) and Szeto et al. (36) reported that mRNA expression of nephrin, podocin, and synaptopodin in urine sediment was correlated with proteinuria as well as reduced renal function in patients suffering from glomerular diseases. More recently, Sato et al. (34) investigated mRNA in urine sediment of a rat glomerular disease model and lupus nephritis patients and reported that upregulation of podocin and nephrin mRNA in urine sediment mirrored disease activity.

Urine-derived podocytes undergo proliferation under cell culture conditions. Vogelmann et al. (38) cultured urinary cells from humans, and Petermann et al. (29) and Yu et al. (44) studied proliferating urinary cells from rats. They detected podocyte markers in cultured urinary cells. Further study of urinary podocytes offers an open window to an understanding of glomerular disease. Several laboratories have established conditionally immortalized podocyte cell lines derived from isolated mouse or human glomeruli using the thermosensitive SV40 T antigen tsA58 (13, 24, 33, 43). Under growth-restricted conditions, these cells display the differentiated phenotype which mimics physiological status and have been widely used to study the biology of podocytes.

We have analyzed the phenotype of human urinary cells in primary culture and following transformation, using urine obtained from two focal segmental glomerulosclerosis (FSGS) patients and two healthy volunteers. After transformation of urinary cells by a thermosensitive SV40 T antigen (U19tsA58) and with human telomerase (hTERT), we carried out antibiotic selection and limiting dilution cloning, which yielded several clones for each subject. Notably, all of the clones and primary urinary cells analyzed in the current study were positive for several podocyte markers but negative for tubular cell markers, indicating that podocyte-derived cells are the major cell type in human urine that survives and proliferates in long-term cell culture. In further experiments, we found that immortalization with the SV40 T antigen (U19tsEl89-97) that was not capable of benznidazole 1 homolog (Bub-1) binding, together with hTERT, produced podocyte cell lines with a more differentiated podocyte phenotype.

METHODS

Human subjects. Patients with FSGS and healthy volunteers gave informed consent to provide urine for research studies. The research protocol, Pathogenesis of Glomerulosclerosis (94-DK-0129), was approved by the National Institute of Diabetes and Digestive and Kidney Diseases Institutional Review Board (National Institutes of Health, Bethesda, MD). Estimated glomerular filtration rate (eGFR) was calculated using the four-variable Modification of Diet in Renal Disease equation. Renal diagnosis was made by renal biopsy carried out for clinical indications. We studied two patients: a male, age 48, with human immunodeficiency virus-associated collapsing glomerulopathy (eGFR 34 ml·min−1·1.73 m−2; proteinuria 1.99 g/day) and a...
male, age 46, with idiopathic FSGS (eGFR 55 ml·min⁻¹·1.73 m⁻², proteinuria 2.58 g/day). Healthy volunteers, two males (ages 36 and 29), were shown to lack proteinuria by urine dipstick. In confirmatory studies, we also studied urinary cells from another patient with idiopathic FSGS [male, age 38 (eGFR 103 ml·min⁻¹·1.73 m⁻², proteinuria 0.6 g/day)] and two additional healthy volunteers (a male, age 36, and a female, age 29).

Primary urinary cell culture. Urinary cell culture was performed as previously described with minor modifications (38). Midstream urine was collected in sterile containers and centrifuged at 1,000 rpm for 5 min. Pelleted cellular material was washed with PBS twice and suspended in RPMI medium supplemented with 10% heat-inactivated FBS, insulin-transferrin-selenium G supplement (Invitrogen, Carlsbad, CA), and penicillin and streptomycin (Invitrogen). Cells were seeded on 75 cm² type I collagen coated tissue culture flasks. After ~3 wk, urine-derived cells were subcultured once by trypsin digestion and prepared for retrovirus infection, quantitative (q)RT-PCR, or immunofluorescence.

Additional podocyte cultures. Mouse podocyte cell line AI cells were cultured as described previously (13). Two additional mouse podocyte cell lines, provided from Peter Mundel (24) and Masanori Kitamura (43), were cultured in RPMI, including 10% heat-inactivated FBS with IFN-γ in 33°C under growth-permissive (GP) conditions or without IFN-γ in 37°C [growth-restrictive (GR) conditions]. A human podocyte cell line (AB8/13; provided by Moin Saleem) (33) was cultured in RPMI supplemented with 10% heat-inactivated FBS and insulin-transferrin-selenium G supplement (Invitrogen) under GP or GR conditions.

Retroviral infection. Urinary cells were infected with retrovirus carrying the SV40 T antigen mutants U19tsA58 or U19del89-97 in combination with retrovirus encoding hTERT at 37°C (3, 12, 26). Beginning 72 h after infection with U19tsA58 or hTERT, cells were cultured at 33°C with 0.2 mg/ml G418 (selection for T antigen); after 7 days, the selected antibiotic was changed to 0.05 mg/ml hygromycin (selection for hTERT) for a further 7 days. Cells infected with U19del89-97 were cultured at 37°C under an identical selection regimen. Surviving cells were propagated, subcultured several times, and subjected to RT-PCR, qRT-PCR, magnetic bead separation with podocalyxin antibody, or limiting dilution cloning. Limiting dilution yielded several clones of transformed cells, from which two clones were randomly chosen and prepared for RT-PCR, Western blotting, or immunofluorescent staining.

RT-PCR and qRT-PCR. Total RNA of the urinary cells (primary cells, transformed bulk cells, or clones) for each podocyte cell line was purified with an miRNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). RT-PCR was performed with Platinum PCR SuperMix High-Fidelity (Invitrogen). Corresponding primers are shown in Table 2. The absence of genomic DNA contamination was confirmed by observation of no amplification following cDNA synthesis and PCR using primers for β-actin in the absence of reverse transcriptase (RT−).

In separate experiments, cDNA synthesis was performed with a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA), and qRT-PCR was carried out in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) with Power-SYBR green PCR Master Mix (Applied Biosystems). Relative amounts of indicated genes were calculated by the delta-delta Ct method and normalized to β-actin.

Western blotting. Urinary cell clones were harvested by trypsin digestion and washed with PBS twice by centrifugation. Cell pellets were lysed in RIPA lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 2.5% deoxycholic acid, 1% NP-40, 1 mM EDTA) with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). Total cell lysate (equal to 20 μg of protein) was separated on NuPAGE 7% Tris-acetate gels (Invitrogen) under reducing conditions. The following primary antibodies were used: mouse monoclonal anti-SV40 T antigen antibody, Ab-2 (EMD Biosciences, Gibbstown, NJ) at 1:1,000; mouse monoclonal anti-Wilms’ tumor 1 (WT1) antibody, clone 6F-H2 (Millipore, Billerica, MA) at a dilution of 1:500; mouse monoclonal anti-podocalyxin antibody, clone 18.29 (Millipore) at 1:200; rabbit polyclonal anti-CD2AP antibody (provided from Dr. Andrey S. Shaw) at 1:2,000; and mouse monoclonal anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO). Signal was detected with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Immunofluorescent staining and phalloidin staining. Primary urinary cells forming colonies grown on type I collagen-coated tissue culture dishes or urinary cell clones cultured on type I collagen-coated coverslips in six-well dishes were fixed by ethanol-acetone (1:1) for 20 min at −20°C or 4% paraformaldehyde in PBS for 30 min at room temperature followed by permeabilization with 0.2% Triton X-100 in PBS for 10 min. After incubation with a blocking solution (2% FBS, 2% BSA, 0.2% gelatin in PBS) for 30 min, cells were probed with mouse monoclonal anti-syntaphodin antibody, clone G1D4 (Fitzgerald Industries International, Concord, MA) ready to use, rabbit polyclonal anti-nestin antibody (Millipore) at a dilution of 1:200, mouse monoclonal anti-WT1 antibody (Millipore) at 1:100, mouse monoclonal anti-podocalyxin antibody (Millipore) at 1:50, or rabbit polyclonal anti-von Willebrand factor (Dako) at 1:400 as primary antibodies for 1 h at room temperature. Signals were visualized by incubating cells with Alexa Fluor 488-conjugated anti-mouse or anti-rabbit secondary antibody (Invitrogen). To visualize F-actin, cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and incubated with Alexa Fluor 594-phalloidin (Invitrogen) at a dilution of 1:100 for 1 h.

Cell selection by magnetic beads. Immortalized bulk urinary cells were subjected to magnetic bead separation using a miniMACS separation unit (Miltenyi Biotec, Auburn, CA) with a monoclonal mouse anti-podocalyxin antibody (Millipore) according to the manufacturer’s instructions. Positive and negative selection were carried out twice in series, to maximize cell purity. Separated cells were expanded, passaged twice, and prepared for qRT-PCR.

Proliferation assay. Urinary cells (10 × 10⁴) were plated on six-well culture plates coated with type I collagen. At various time points, the number of viable cells was counted in a hemocytometer under the microscope by trypan blue dye exclusion.

Statistics. Statistical analyses were carried out using Prism 5 software (Graphpad Software, San Diego, CA), and significance was evaluated by one-way ANOVA, using Bonferroni testing for intergroup comparisons. A P value of <0.05 was taken as significant.

RESULTS

Urinary podocytes from healthy volunteers and FSGS patients are viable and proliferate in culture. We cultured urine sediment cells to confirm that podocytes are present in urine and can proliferate in vitro. As shown in Fig. 1A, small cell colonies with typical epithelial cell morphology appeared at 1–2 wk after the start of culture. These cells typically stopped proliferating after 4–5 wk. At 3–4 wk of culture, we characterized these cells and carried out subculture and transformation. These proliferating cells were obtained consistently from two healthy volunteers and two FSGS patients. Immunofluorescent staining of cell colonies of a healthy volunteer revealed that podocyte markers WT1 (Fig. 1B), nestin (Fig. 1C), and syntaphodin (Fig. 1D) were expressed in urine-derived cells. These results indicate that podocyte-derived cells constitute a sizeable fraction of the cells that survive in long-term human urinary cell cultures.
Conditionally transformed urinary cells from FSGS patients express podocyte markers. We infected cultured urinary cells from two FSGS patients with retrovirus vectors carrying a thermosensitive SV40 T antigen (U19tsA58) and hTERT. After antibiotic selection and limiting dilution cloning, we obtained several conditionally transformed urinary cell clones. The growth curve of a representative urinary cell clone obtained from each of two FSGS patients is presented in Fig. 2A, and Western blotting for SV40 T antigen is shown in Fig. 2B. At 33°C (growth-permissive condition; GP), cell numbers increased over time (Fig. 2) and SV40 T antigen was expressed at high levels (Fig. 2B). As expected, at 37°C (GR condition), cell numbers gradually fell and immunodetectable-SV40 T antigen was present at much lower levels.

Under GP conditions, cells from both patients exhibited cobblestone morphology, typical of epithelial cells; Fig. 2C.
shows the morphology of representative cell clones. Under GR conditions, the cell clones from patient A maintained a cuboidal morphology, whereas cell clones from patient B developed a more irregular shape than from patient A. Some cells retained a cuboidal shape whereas others exhibited polygonal or multipolar shapes. Under GR conditions, compared with GP conditions, cells from both patients were enlarged and certain cells showed pyknotic nuclei suggestive of apoptosis (Fig. 1C, arrows).

F-actin staining for cells under GR conditions was carried out (Fig. 2C). Cells from each patient manifested thick cortical actin bundles with moderate stress fiber formation, whereas some cells, especially cells with polygonal or multipolar shapes from patient B, manifested diminished cortical actin.

We next analyzed expression of podocyte markers in transformed urinary cell clones by RT-PCR (Fig. 3A and Table 1). Nephrin mRNA was expressed in all four cell lines, although the expression level was low. Podocin mRNA was not detected in any cell line. WT1 mRNA was absent from cell lines from patient A, whereas both cell lines from patient B expressed WT1 mRNA strongly. Podocalyxin and CD2AP mRNA were positive in all clones tested. Surprisingly, podocytes were not consistently more differentiated under GR conditions. While nephrin mRNA expression rose in both clones from patient A under GR conditions, nephrin mRNA was unaffected in both clones from patient B under GR conditions. Nestin mRNA expression rose to some extent in all cells under GR conditions, although the technique is not quantitative. The other markers were unaffected by the shift to GR conditions. By Western blotting, WT1 protein was absent in cell lines from patient A, whereas it was strongly expressed in both cell lines from patient B (Fig. 3B). Podocalyxin protein was expressed in cell lines from both patients, at lower levels in cell lines from patient B. CD2AP was also detected in cell lines from both patients. Thus mRNA and protein expression were congruent. Immunofluorescent staining revealed that synaptopodin was distributed in a partially filamentous and partially homogenous pattern, and nestin was stained as fine filaments in cytoplasm (Fig. 3C). Podocalyxin was expressed on the cell surface in both patients (Fig. 3C; only cells from patient A are shown), and WT1 was detected in the nucleus of a cell line from patient B (Fig. 3C).

In summary, conditionally transformed urinary cell lines from two FSGS patients expressed various characteristic podocyte markers at the RNA and protein levels.

Conditionally transformed urinary cell lines from healthy volunteers exhibit a similar phenotype to those from FSGS patients. We next established two urinary cell lines from healthy volunteer A and B; 1B, healthy volunteers A and B; all supplementary material for this article is available on the journal web site) by RT-PCR (15, 16). Surprisingly, aquaporin-1 (AQP1) mRNA, a water channel expressed in proximal tubules, was amplified in all the clones. Uromodulin (UMOD), also known as Tamm-Horsfall glycoprotein, and the Na-K-2Cl cotransporter (NKCC2) are localized in the thick ascending limb of Henle; mRNAs were not detected in any cell clones. The thiazide-sensitive NaCl cotransporter (TSC; SLC12A3) is identified in the distal convoluted tubule; mRNA was not amplified in any clones. AQP2 is localized in the collecting duct; mRNA was not amplified in any clone. von Willebrand factor (VWF), a marker for endothelial cells, mRNA was not expressed in any clones. We also performed immunofluorescent staining for VWF in transformed bulk urinary cells from each human subject and confirmed the absence of this protein (data not shown).

We analyzed expression of these tubular cell markers except AQP1 in urinary primary cells and transformed bulk cells (i.e., before cloning) as well as clones from the FSGS patients to evaluate the possibility that transformation or single cell cloning diminished the tubular cell marker expression (Supplementary Fig. 1C, patient A, and 1D, patient B). Primary cell cultures, transformed bulk cells, and two clones from both patients did not express the tubular cell markers or VWF, suggesting that tubular epithelial cells or endothelial cells were absent in urine from these individuals or did not survive and proliferate in culture.

In vivo, AQP1 expression has been reported in proximal tubules and mesangial cells but not in podocytes (6, 22). We next examined AQP1 mRNA expression in three mouse conditionally immortalized podocyte cell lines (Supplementary Fig. 2A) and one human cell line (Supplementary Fig. 2B) generated from isolated glomeruli, all of which have been previously published by other investigators and which were generously provided to us (13, 24, 33, 43). AQP1 mRNA was detected in all four podocyte cell lines, suggesting that podocyte-like cells in vitro express AQP1 mRNA.
Fig. 3. Characteristic podocyte molecules were identified in urinary cell clones from FSGS patients. Two urinary cell clones from each FSGS patient under GP or GR conditions were analyzed for indicated podocyte molecules by RT-PCR (A). Nephrin mRNA was weakly detected in each clone from both patients. WT1 mRNA was absent in urinary cells from patient A in contrast to high expression in patient B. Synaptopodin, nestin, CD2AP, and podocalyxin mRNA was detected in each clone examined. A human kidney was used as a positive control for each RT-PCR reaction. /H-Actin served as an internal control. Reaction for /H-actin without RT (RT-) was performed to rule out the possibility of genomic DNA contamination. One representative clone from each FSGS patient was selected, and Western blotting for indicated podocyte-associated markers was performed (B). WT1 was negative in cells from patient A, whereas strongly positive in cells from patient B. The nonspecific bands are marked with an asterisk (*). Podocalyxin was detected in both cells, although expression in cells from patient B is weak. CD2AP was expressed in each. A human kidney and mouse kidney served as a positive control, and /H-actin served as a loading control. Lack of WT1 in the human kidney is probably due to low sensitivity of the assay. Anti-podocalyxin antibody and anti-CD2AP antibody used in this study do not react in the mouse. Immunofluorescent staining for synaptopodin, nestin, podocalyxin, or WT1 in one representative clone from each of patient A or patient B under GR condition is displayed (C). Synaptopodin was detected with a pattern that is partially homogenous and partially filamentous. Nestin was expressed with a filamentous pattern. Podocalyxin was identified on the cell surface, and WT1 was located in nuclei. Original magnification for synaptopodin, nestin, and podocalyxin: ×600; WT1, ×400. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole in podocalyxin staining.
Podocalyxin were used for both RT-PCR and qRT-PCR. The other primer pairs were used only for RT-PCR. The non-exon-spanning primer pair for ACTB (*) was used for qRT-PCR.

Table 2. Pairs of primers used in this study

<table>
<thead>
<tr>
<th>Gene Name (Alternative Name)</th>
<th>Accession No.</th>
<th>Sequence (5’-3’)</th>
<th>Exon Position</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPHS1 (Nephrin)</td>
<td>NM_004646.2</td>
<td>F: CGGAGGAGGGAGGTGTCTTTATTC</td>
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<td>234</td>
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<tr>
<td>NPHS2 (Podocin)</td>
<td>NM_014625.2</td>
<td>R: CGGTTTCAGAGGGGGTGTTTGGAG</td>
<td>E28</td>
<td>223</td>
</tr>
<tr>
<td>WT1</td>
<td>NM_000378.3,</td>
<td>F: CAAGAATGAGATGGGGTGTTTGGAG</td>
<td>E7</td>
<td>127</td>
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<tr>
<td>CD2AP</td>
<td>NM_012120.2</td>
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<td>E8</td>
<td>136</td>
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<td>AQP2</td>
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<tr>
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<td>163</td>
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<td>SLC11A2 (TSC)</td>
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<td>ACTB+ (β-Actin)</td>
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<td>R: GCCAGGAGATGAGGAGGAC</td>
<td>E4</td>
<td>175</td>
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Shown are the primer pairs used for RT-PCR and quantitative (q)RT-PCR. F, forward; R, reverse; AQPI, aquaporin-1. The primer pairs for nephrin, WT1, and podocalyxin were used for both RT-PCR and qRT-PCR. The other primer pairs were used only for RT-PCR. The non-exon-spanning primer pair for ACTB (*) was used for RT-PCR including an RT(–) reaction to rule out genomic DNA contamination. The exon-spanning primer pair for ACTB (+) was used for qRT-PCR.

Transformation by SV40 T antigen U19tsA58 and hTERT alters gene expression of urinary cells. As described above, there was heterogeneity of podocyte marker expression across all eight cell lines studied. We next evaluated the role of the viral oncogene transformation and cell cloning in producing this heterogeneity. SV40 T antigen transformation is associated with genomic instability (30, 31), and furthermore the procedure of cloning by limiting dilution may select for cells that do not represent faithfully their parent populations. To evaluate this possibility, we compared podocyte marker mRNA expression by RT-PCR from cells obtained from FSGS patients at three stages of selection: primary urinary cell cultures, transformed bulk cell cultures, and podocyte cell clones. Nephrin mRNA and WT1 mRNA were expressed in primary urinary cells of patient A (Fig. 5A) and patient B (Fig. 5B), but expression was reduced in immortalized bulk cells. WT1 mRNA disappeared after single-cell cloning in patient A clones but was preserved in patient B clones. Podocin mRNA was consistently negative among cells at each stage. These results indicate that SV40 T antigen transformation and single-cell cloning did indeed affect podocyte marker expression, with reduced expression of nephrin and WT1 mRNA. By contrast, podocin mRNA was consistently absent in all the urinary cells analyzed, including primary cells. We performed RT-PCR for podocin using urinary primary cell cultures from two additional healthy volunteers and one FSGS

Two cell lines were obtained from subjects with focal segmental glomerulosclerosis (FSGS) and healthy volunteers. WT1, Wilms’ tumor 1. In cells cultured under growth-restricted conditions, mRNA expression was determined by PCR, and protein expression was determined by Western blotting and/or immunostaining. Results are presented as present (+), absent (−), or not determined (ND).

Table 1. Podocyte marker expression

<table>
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<tr>
<th>Subject</th>
<th>Cell Line</th>
<th>CD2AP mRNA</th>
<th>Protein</th>
<th>Nestin mRNA</th>
<th>Protein</th>
<th>Synaptopodin mRNA</th>
<th>Protein</th>
<th>Nephrin mRNA</th>
<th>Protein</th>
<th>Podocalyxin mRNA</th>
<th>Protein</th>
<th>WT1 mRNA</th>
<th>Protein</th>
<th>Podocin mRNA</th>
<th>Protein</th>
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<tr>
<td>FSGS patient A</td>
<td>I</td>
<td>+</td>
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<td>ND</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>FSGS patient B</td>
<td>I</td>
<td>+</td>
<td>ND</td>
<td>+</td>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Healthy volunteer A</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
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<td>−</td>
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<td>−</td>
</tr>
<tr>
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Podocalyxin expression is not related to WT1 or nephrin mRNA expression by urinary cells. We next examined cell heterogeneity in transformed bulk cultures (there were insufficient cells in the primary cultures for this technique). We performed magnetic bead separation with podocalyxin antibody, as this technique requires a cell surface protein. As shown in Fig. 6, podocalyxin mRNA was considerably increased in the podocalyxin-positive fraction compared with the podocalyxin-negative fraction in urinary cells from both patients (Fig. 6A, patient A; 6B, patient B), confirming that the cell fractionation was successful. On the other hand, the expression level of nephrin mRNA was not significantly different between podocalyxin-positive cells and podocalyxin-negative cells from both FSGS patients. Furthermore, unexpectedly, WT1 mRNA expression in podocalyxin-positive cells was significantly lower than in podocalyxin-negative cells from patient A and confirmed the absence of podocin mRNA (data not shown), whereas the expression of other podocyte molecules was generally similar to the two primary cultures described herein.

Fig. 4. Podocyte markers are also expressed in urinary cell clones from healthy volunteers. Four urinary cell clones from 2 healthy volunteers grown under GP or GR conditions were analyzed with RT-PCR for podocyte markers (A). Nephrin mRNA was weakly expressed in 1 of 2 clones of healthy volunteer A and 2 clones from healthy volunteer B. Low expression of WT1 mRNA was exhibited in both healthy volunteers. Synaptopodin, nestin, and CD2AP mRNA was expressed in each healthy volunteer. Podocalyxin mRNA was absent from cells from healthy volunteer A but present in healthy volunteer B. A human kidney served as a positive control for each RT-PCR. RT-PCR for β-actin was used as an internal control. Reaction for β-actin without RT (RT−) was carried out to confirm the absence of genomic DNA contamination. Immunofluorescent staining for synaptopodin or nestin in 1 representative clone from each of healthy volunteer A or healthy volunteer B is shown (B). Synaptopodin was stained in each cell clone with a pattern that is partially homogenous and partially filamentous. Nestin was exhibited in both human subjects with a filamentous pattern. Original magnification: ×600.

Fig. 5. U19tsA58 alters gene expression of urinary cells. Primary urinary cells, transformed bulk urinary cells, and 2 representative urinary cell clones from patient A (A) or patient B (B) were examined by RT-PCR for indicated podocyte components. In cells from both patients, nephrin mRNA was strongly expressed in primary cells, but expression was reduced in transformed bulk cells and clones. WT1 mRNA disappeared after single cell cloning in urinary cells from patient A but persisted in patient B in both clones. Podocin mRNA was negative at each step, from primary cells to clones in cells from both patients. Human kidney tissue was used as a positive control, and RT− served as a loading internal control. Contamination of genomic DNA was excluded by no observation of amplification in reaction for β-actin without RT (RT−).
Fig. 6. Expression of podocalyxin is not related to nephrin or WT1 expression in urinary cells. Immortalized bulk urinary cells from FSGS patient A (A) or FSGS patient B (B) were divided into a podocalyxin-positive fraction (PODXL +) and negative fraction (PODXL −) by magnetic bead separation with anti-podocalyxin antibody. Each fraction was propagated and subcultured twice. RNA samples were collected under GP and GR conditions and analyzed with quantitative (q) RT-PCR for podocalyxin, nephrin, or WT1. Podocalyxin mRNA was significantly higher in the PODXL-positive fraction than in the PODXL-negative fraction in cells from both human subjects, indicating that the separation was successfully performed. The expression of nephrin mRNA was not significantly different between PODXL-positive and PODXL-negative fractions in cells from both patients. WT1 mRNA in the PODXL-positive fraction was downregulated compared with the PODXL-negative fraction in healthy volunteer A, whereas no significant difference was observed between the PODXL-positive and PODXL-negative fractions in cells from healthy volunteer B. Data were normalized to expression of β-actin mRNA, and means ± SD, relative to PODXL-negative cells cultured under GR conditions, are presented.

**DISCUSSION**

In the present study, we have established conditionally transformed urinary podocyte cell lines from FSGS patients and healthy volunteers by transducing the cells with thermo-sensitive SV40 T antigen and hTERT. Previously, four groups have established transformed podocyte cell lines from isolated human glomeruli (4, 5, 33, 37). These cell lines were originated from kidneys surgically removed for cancer, obstructive nephropathy, and Denys-Drash syndrome, whereas in the present study podocyte cell lines were derived from urine obtained from patients with glomerular disease and also healthy volunteers. Vogelmann et al. (38) established primary cell cultures from human urine and showed expression of podocyte markers; Petermann et al. (29) and Yu et al. (44) did similar studies of rat urine. In the present work, we demonstrate the ability to reproducibly culture podocyte-like urinary cells from FSGS patients (who would be expected to shed increased numbers of podocytes in urine) and from healthy volunteers (from whom the shedding of podocytes is perhaps more surprising), and to generate transformed podocyte cell lines. This is the first report to establish and characterize urinary podocyte cell lines. The ability to routinely establish podocyte cell lines, using urine obtained from particular patients and individuals with distinct genotypes, will provide a useful tool for studies in podocyte biology, including biomarker analysis and medication sensitivity.

Synaptopodin, nestin, and CD2AP were expressed by all cell lines. However, podocin was consistently absent in every urinary cell, and expression of other markers were diverse among the urinary cell clones. Urinary podocytes may undergo irreversible dedifferentiation under the artificial conditions of culture. Petermann et al. (29) reported that cultured rat urinary cells expressed WT1 and podocin mRNA, although nephrin and synaptopodin mRNA were absent. In contrast, we detected synaptopodin mRNA in all of urinary cell clones, nephrin and WT1 in some clones, but did not observe podocin mRNA in primary cells, transformed bulk cells, or cell clones. These
There were certain differences among the cell lines that we studied. Podocytes from patient A lacked WT1 protein and mRNA expression in both clones in contrast to podocytes from patient B, but primary podocytes from patient A expressed WT1 mRNA. Both urinary cell clones from the healthy volunteer A lacked podocalyxin RNA expression, and one of the clones from volunteer A did not express nephrin mRNA, in contrast to volunteer B. However, there is no reason to believe that there is any marked podocyte phenotype difference in vivo between these healthy volunteers. We believe that expression differences for nephrin, WT1, and podocalyxin among podocyte clones most commonly emerge as a result of transformation and/or cloning, rather than reflecting a persistence of an in vivo phenotype.

All of the urinary cell clones were positive for AQP1 mRNA, raising the possibility that these cells could be proximal tubular cells. Although we cannot completely exclude this possibility, our findings that several podocyte cell lines previously reported also expressed AQP1 mRNA support our interpretation that podocytes express AQP1 mRNA in culture.

Mesangial cells and glomerular endothelial cells, the other two cell types in glomeruli are separated from the urinary space by the glomerular basement membrane (GBM). As expected, urinary cells did not express the endothelial cell marker VWF. Mesangial cells are reported to form multilayers in culture (42) and manifest typical F-actin stress fiber formation (35). In this study, primary and transformed urinary cells manifested typical monolayers (Figs. 1A and 2C), and most transformed urinary cells showed cortical F-actin (Fig. 2D) rather than stress fibers. These findings suggest that mesangial cells are not a source of urinary cells.

In an attempt to obtain more homogeneous podocyte cultures, we used podocalyxin expression to sort bulk-transformed urinary cells but found no consistent differences in nephrin or WT1 mRNA expression in podocalyxin-positive and podocalyxin-negative cell populations. These findings suggest that the podocalyxin expression level varies among cells that express other podocyte markers and suggest the limitations of relying on podocalyxin expression to identify podocytes, at least in cell culture.

In summary, we have demonstrated that podocyte cell lines can be routinely established from urine of both FSGS patients and healthy volunteers. mRNA and protein of synaptopodin, nestin, and CD2AP were consistently expressed in all urinary cell clones most commonly emerging as a result of transformation and/or cloning, rather than reflecting a persistence of an in vivo phenotype.

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