Human Urine-Derived Renal Progenitors for Personalized Modeling of Genetic Kidney Disorders

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ABSTRACT

The critical role of genetic and epigenetic factors in the pathogenesis of kidney disorders is gradually becoming clear, and the need for disease models that recapitulate human kidney disorders in a personalized manner is paramount. In this study, we describe a method to select and amplify renal progenitor cultures from the urine of patients with kidney disorders. Urine-derived human renal progenitors exhibited phenotype and functional properties identical to those purified from kidney tissue, including the capacity to differentiate into tubular cells and podocytes, as demonstrated by confocal microscopy, Western blot analysis of podocyte-specific proteins, and scanning electron microscopy. Lineage tracing studies performed with conditional transgenic mice, in which podocytes are irreversibly tagged upon tamoxifen treatment (NPHS2::CreER; mT/mG), that were subjected to doxorubicin nephropathy demonstrated that renal progenitors are the only urinary cell population that can be amplified in long-term culture. To validate the use of these cells for personalized modeling of kidney disorders, renal progenitors were obtained from (1) the urine of children with nephrotic syndrome and carrying potentially pathogenic mutations in genes encoding for podocyte proteins and (2) the urine of children without genetic alterations, as validated by next-generation sequencing. Renal progenitors obtained from patients carrying pathogenic mutations generated podocytes that exhibited an abnormal cytoskeleton structure and functional abnormalities compared with those obtained from patients with proteinuria but without genetic mutations. The results of this study demonstrate that urine-derived patient-specific renal progenitor cultures may be an innovative research tool for modeling of genetic kidney disorders.


The incidence of AKI and CKD is rising and reaching epidemic proportions. In patients with CKD, the progressive decline in renal function is multifactorial and attributable to a variety of mechanisms. In particular, the critical role of genetic factors in the etiology, pathogenesis, and progression of many renal disorders is gradually becoming clear, especially in children. Indeed, the advent of high-throughput sequencing techniques has fostered the identification of novel causative genes and allows the continuous discovery of genetic variants of unknown clinical significance, often raising the problem of the functional testing of their pathogenic role. However, emerging evidence suggests that influence of the

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genomic background of the patient and epigenetics are critical for determining the clinical phenotype. With such complexity, the need for disease models that recapitulate human kidney disorders in a personalized manner is paramount. The generation of induced pluripotent stem cell (iPSC)–derived kidney cell lines from patients that maintain their genotype and phenotype may provide a valuable tool. However, iPSC reprogramming erases somatic epigenetic signatures and establishes epigenetic marks of embryonic stem cells. As more iPSC lines are produced, it has become clear that variability between cell lines emerges after reprogramming. Differences between clonal lines have been detected at multiple levels, including variable expression profile levels, X-inactivation status on female lines, genetic instability, partial reprogramming, and differentiation potential. In addition, these procedures are still laborious and time consuming.

An alternative to create a "personalized disease model in a dish" is to use renal progenitor cell (RPC) cultures obtained from the affected patient. Indeed, RPCs display the inherent patient specificity at a genomic level as well as kidney-specific epigenetic changes. In addition, they can be easily amplified, maintained in culture, and induced to differentiate into tubular cells as well as podocytes, as described in many studies. Unfortunately, the fundamental inaccessibility of the human RPCs from patients with kidney disorders prevents their isolation for in vitro studies of disease. However, loss of renal cells in urine naturally occurs in patients, and, therefore, urine may represent a potential RPC source. Thus, in this study, we aimed to establish RPC cultures from the urine of children with glomerular disorders in order to use them as a reliable tool for personalized modeling of renal diseases.

**RESULTS**

**Set Up of a Method to Culture RPCs from Urine**

To establish RPC cultures from the urine of patients, we used the medium previously optimized to culture RPCs from human renal tissue, EGM-MV. Because a high frequency of bacterial contamination is associated with urine cultures, the sterility of cultures was checked by performing PCR for bacterial 16S ribosomal RNA. In a preliminary set of experiments, Sanger sequencing of the PCR product and alignment against sequences present in 16S ribosomal RNA database was performed in cultures. This showed frequent contamination with bacteria belonging to the *Enterococcus* group as well as staphylococci, which can cause intracellular infection. For this reason a combination of penicillin (100 U/ml), streptomycin (1 mg/ml), and rifampcin (8 μg/ml) that can cover a broad range of Gram-positive and Gram-negative bacteria, including those able to give intracellular infection, was added to the medium. This combination of antibiotics allowed us to obtain sterile cultures, as confirmed by analysis for 16S ribosomal RNA repeated again after 2 weeks, and was thus applied in all successive experiments.

The established procedure is detailed in Figure 1. Fresh urine samples were collected from 60 pediatric patients with different glomerular diseases and from 10 healthy children (Table 1). In children with glomerular disorders, rare urine-derived cells could generate clones that grew exponentially and achieved confluence in about 2–3 weeks. By contrast, no cell culture could be obtained from the urine of healthy children. In our patients, the success rate for achieving a culture was 33% for each sampling. The success rate increased to 51% if samples were collected when patients were proteinuric (urinary protein-to-creatinine ratio >0.2 mg/mg), whereas if samples were collected from patients in the remission phase of the disease (urinary protein-to-creatinine ratio ≤0.2 mg/mg), the success rate decreased to 8%. At confluence, cells were detached and flow cytometry analysis demonstrated that they co-expressed CD133 and CD24, which are specific markers for human RPC, in a range of 28%–70%; the remaining cells did not express CD133 and variably expressed CD24 (Figure 2A). The cultures were then separated through CD133-immunomagnetic bead sorting and the recovered populations were analyzed (Figure 2A). CD133+ cells exhibited a significantly higher growing (Figure 2B) and clonogenic potential (Figure 2C) than CD133− cells. Clones derived from CD133− cells were clonogenic and could exponentially grow in culture for 2–3 months, yielding about 30–40 million cells for each patient (Figure 2D). Of all cells present in fresh urine (median: 11,333 cells/100 ml), only rare cells (median: 5 cells/100 ml), which were mostly CD133+ (median: 4 cells/100 ml), were clonogenic and could exponentially grow in culture. The established procedure is detailed in Figure 1. Fresh urine samples were collected from 60 pediatric patients with different glomerular diseases and from 10 healthy children (Table 1). In children with glomerular disorders, rare urine-derived cells could generate clones that grew exponentially and achieved confluence in about 2–3 weeks. By contrast, no cell culture could be obtained from the urine of healthy children. In our patients, the success rate for achieving a culture was 33% for each sampling. The success rate increased to 51% if samples were collected when patients were proteinuric (urinary protein-to-creatinine ratio >0.2 mg/mg), whereas if samples were collected from patients in the remission phase of the disease (urinary protein-to-creatinine ratio ≤0.2 mg/mg), the success rate decreased to 8%. At confluence, cells were detached and flow cytometry analysis demonstrated that they co-expressed CD133 and CD24, which are specific markers for human RPC, in a range of 28%–70%; the remaining cells did not express CD133 and variably expressed CD24 (Figure 2A). The cultures were then separated through CD133-immunomagnetic bead sorting and the recovered populations were analyzed (Figure 2A). CD133+ cells exhibited a significantly higher growing (Figure 2B) and clonogenic potential (Figure 2C) than CD133− cells. Clones derived from CD133− cells maintained CD133 expression over time. By contrast, the rare clones that did not express CD133 never acquired this marker (Figure 2D). Of all cells present in fresh urine (median: 11,333 cells/100 ml), only rare cells (median: 5 cells/100 ml), which were mostly CD133+ (median: 4 cells/100 ml), were clonogenic and could exponentially grow in culture for 2–3 months, yielding about 30–40 million cells for each patient (Figure 2D). Consistently, after about 1 month of culture, urine-derived cells represented a homogeneous population and strongly expressed surface markers CD133, CD24, and CD106, as assessed by flow cytometry analysis (Figure 2F). Interestingly, the expression of these markers is characteristic of the most immature RPCs, which exhibit resistance to death, exponential growth, and clonogenic ability and can differentiate into tubular cells as well as podocytes. In agreement with their renal origin, urine-derived cells did not express the urothelial cell marker uroplakin III (Figure 2F). Taken together, these results demonstrate that long-term cultures derived from urine of children with glomerular disorders can be obtained, and they result from rare RPC that are amplified and progressively selected.

**RPC, but Not Fully Differentiated Podocytes, Can Be Cultured Long-Term from the Urine of Mice**

Many studies described the presence of podocytes in urine, and some studies also reported the setup of short-term podocyte cultures from the urine of patients with glomerular disorders. However, podocytes are terminally differentiated cells that cannot divide, and thus it was hypothesized that podocyte mitosis occurred upon their dedifferentiation in culture. Indeed, urine-derived podocyte cultures exhibit features of undifferentiated cells and share markers with parietal epithelial...
cells. Thus, to evaluate the origin of urine-derived cell cultures ruling out dedifferentiation, we obtained cultures from the urine of NPHS2.iCreER;mT/mG conditional transgenic mice. In this model, podocytes are irreversibly tagged with green fluorescent protein (GFP) upon induction with tamoxifen, while all the other kidney cells, including RPCs, express Tomato Red (Figure 3A). Thus, using an anti–claudin-1 antibody, which specifically tags RPCs in the Bowman capsule in the mouse, RPCs can be identified as Tomato Red–positive and claudin-1-expressing cells (Figure 3A).

To evaluate whether RPCs or podocytes are lost in the urine during glomerular disease, NPHS2.iCreER;mT/mG were treated with tamoxifen and, after a washout period of at least 1 week, were injected with doxorubicin hydrochloride to induce doxorubicin nephropathy, which is considered as a model of human FSGS. Upon doxorubicin hydrochloride treatment, these mice developed podocyte loss (Figure 3B) and proteinuria (urinary albumin-to-creatinine ratio at day 10, 4.2 ± 1.3 mg/mg). Because in NPHS2.iCreER;mT/mG transgenic mice GFP is specifically and permanently expressed by podocytes independently of their differentiation status, podocytes can be identified in the urine of mice, and they will remain as GFP positive (green), even if they undergo dedifferentiation in culture. Similarly, RPCs will be identified as Tomato Red–positive (red) cells that co-express claudin-1 and will remain red even if they should undergo differentiation into podocytes in culture. In fresh mouse urine, >90% of the cells appeared red and lacked a nucleus, indicating their advanced death status (Figure 3C). These cells were not costained with claudin-1 (blue), indicating that they were not of RPC origin (Figure 3C). Interestingly, very rare cells, which represented <1% of all the red urinary cells, costained in blue, indicating their RPC origin (Figure 3, C and c’). Rare live green cells were also observed, indicating that they represented detached podocytes (Figure 3D). Urinary cells were then cultured and after about 2 weeks, few cells were attached to the plate and exhibited a modest growth. None of the attached cells were green, indicating that fully differentiated podocytes cannot undergo cell division in culture. However, some red cells exhibited synaptopodin expression when stained with an anti-synaptopodin antibody, indicating a podocyte phenotype (Figure 3E). These cells also costained with an anti–claudin-1 antibody (Figure 3E), thus demonstrating a transitional phenotype. Because such cells were not found in fresh urine, this observation suggests that at least a subset of RPC upregulated podocyte markers in culture, in agreement with recent findings. Transitional cells exhibited a limited growth potential in culture, undergoing few rounds of cell division. However, a rare subset of Tomato Red+ cells that did not exhibit expression of podocyte markers but costained with claudin-1 underwent repeated cell divisions and generated clones that could be amplified over 1–2 months (Figure 3, F and G). Similar results were obtained when the RPC-optimized medium endothelial cell growth medium-microvascular (EGM-MV) was used, as well as when media previously reported for setup of podocyte cultures from mice were used.

Taken together, these results demonstrate that (1) fully differentiated podocytes cannot divide in culture, (2) urinary podocyte cultures are derived from RPCs, (3) immature RPCs are the only urinary cells that can undergo numerous rounds of cell division in culture and exhibit clonogenic and amplification potential over time.

RPCs Obtained from Urine of Patients Exhibit the Same Phenotypic and Functional Features of RPCs Obtained from Human Kidney Tissue

To evaluate whether RPCs cultured from urine of patients (which were renamed u-RPCs) maintain the same phenotypic features of RPCs obtained from renal tissue, a comparison of gene expression profile of a total of 472 mRNA as well as of 168 microRNA samples was performed, demonstrating that u-RPCs and RPCs exhibit an almost identical transcriptome
Table 1. Urine-derived RPC culture achievement at first sampling in relation to clinical features of patients

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To then evaluate whether u-RPCs also displayed functional properties of RPCs, we analyzed their capacity to engraft into injured renal tissue using a model of doxorubicin nephropathy, which can be achieved by intravenous injection of doxorubicin hydrochloride and is characterized by podocyte depletion and tubular damage. To this end, u-RPCs, RPCs, or CD133<sup>2</sup> cells were labeled with the red fluorescent dye PKH26 and then injected into doxorubicin hydrochloride–treated severe combined immunodeficiency (SCID) mice. Ten days after doxorubicin hydrochloride injection, labeled u-RPCs (Figure 4, A–D) and labeled RPCs (Figure 4E) localized to glomerular structures, where they acquired the podocyte-specific markers podocin and nephrin, as previously described. Relevant numbers of PKH26-labeled u-RPCs were also observed in tubular structures (Supplemental Figure 2, A and B), while PKH26 red labeling was never observed in mice injected with CD133<sup>2</sup> cells (Figure 4F) or saline solution (Figure 4G). Injection of RPCs or u-RPCs significantly reduced proteinuria, while CD133<sup>2</sup> cells had no significant effect (Figure 4H). The number of cells that showed PKH26 labeling was equal to 8.3% ± 1.4% of all podocytes for u-RPCs and 9.1% ± 2.7% for RPCs (P = NS). The number of cells that showed PKH26 labeling was equal to 6.7% ± 1.9% of all proximal tubular cells for u-RPCs and 7.1% ± 1.8% for RPCs (P = NS), consistent with previous reports.

We then evaluated the capacity of u-RPCs and RPCs to differentiate in vitro into tubular cells and podocytes. Differentiation toward tubular cells resulted in a strong upregulation of mRNA characteristic of different tubular portions of the nephron, such as the Na/H exchanger (Na/H), aquaporin 3, Na/K/Cl transporter (Na/K/Cl), and amino acid transporter (SLC3A1) (Supplemental Figure 2C). In addition, differentiated u-RPCs acquired the property to bind LTA and started to express Epithelial Membrane Antigen-1 (EMA-1) (Supplemental Figure 2D). Tubular differentiation was obtained in 79% ± 8.7% of the total number of u-RPCs plated.

More important, differentiation into podocytes resulted in upregulation of nephrin (NPHS1), podocin (NPHS2), podocalyxin (PODXL), and Kruppel-like factor 15 (KLF15) mRNA (Figure 5A) in u-RPCs and RPCs to levels similar to those observed in cultured podocytes (Supplemental Figure 3), as well as in protein expression of nephrin, podocin and synaptopodin, as evaluated through Western blot analysis (Figure 5, B and C) and confocal microscopy (Figure 5D). Moreover, u-RPCs acquired a complex cytoskeleton architecture, characteristic of fully differentiated podocytes, as demonstrated by performing phalloidin staining (Figure 5E). Podocyte differentiation was obtained in 71% ± 11.7% of the total number of u-RPCs plated.

To further evaluate the ultrastructure of u-RPCs and to compare it with that of RPCs, we performed scanning electron microscopy before and after their differentiation into podocytes. Undifferentiated RPCs appeared as small cells with a simple ultrastructure and a polygonal shape (Figure 6, A and B). After their differentiation, RPCs completely modified their structure, acquiring an octopus-like structure resembling foot processes (Figure 6, C and D). A nearly identical ultrastructure was also observed in u-RPCs (Figure 6, E and F).

### Table 1. Continued

<table>
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<th>Participants</th>
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<th>Protein-to-Creatinine Ratio (mg/mg)</th>
<th>Hematuria</th>
<th>Serum Creatinine (mg/dl)</th>
<th>Success in Achieving u-RPC Culture</th>
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The table summarizes the clinical features of patients on the day of the first urine sample collection. CNS, congenital nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome; IgAN, IgA nephropathy; HSP, Henoch-Schönlein purpura; AlpS, Alport syndrome; HUS, hemolytic uremic syndrome; aHUS, atypical hemolytic uremic syndrome; LN, lupus nephritis; MPGN, membranoproliferative GN; NA, not applicable.
was observed before and after differentiation of u-RPCs (Figure 6, E–H).

Urine-Derived RPC Cultures Can Be Used for Personalized Modeling of Genetic Kidney Disorders

We then evaluated the possibility of using u-RPCs to model genetic kidney disorders. Urine-derived RPCs were obtained from three children with nephrotic syndrome carrying pathogenic mutations in genes encoding for podocyte cytoskeleton proteins or transcription factors (see patients 1–3 in Table 1), as well as from children without genetic alterations, as assessed by next-generation sequencing (Figure 7A). Once u-RPCs obtained from these patients were induced to differentiate into podocytes, they acquired high levels of nephrin mRNA and protein (Figure 7, B and C). Although no significant difference was observed at mRNA levels in all patients analyzed (Figure 7D), podocin expression was significantly reduced in podocytes obtained from u-RPCs isolated from patients 1 and 2 carrying mutations in NPHS2 in comparison to a patient without genetic mutations but proteinuric (control) (Figure 7E). In particular, podocin was nearly absent in patient 1, in agreement with the presence of a homozygous frameshift leading to a STOP codon, and extremely reduced in patient 2. In the latter patient, a heterozygous compound mutation in NPHS2 was determined by a missense mutation in an allele and a frameshift leading to a STOP codon in the other allele, as evaluated by confocal microscopy and Western blot analysis (Figure 7E) using an antipodocin antibody that specifically recognizes the C-terminus of the protein. By contrast, in patient 3 a heterozygous missense mutation in the dominantly transmitted LMX1B gene did not seem to impair the synthesis of nephrin or podocin protein (Figure 7, C and E). However, in this patient, podocin appeared as mislocalized and the cell structure was abnormal.

Because a recent study showed that pathogenic mutations in LMX1B disrupt the cytoskeleton structure of the podocyte, we analyzed actin distribution and cytoskeleton architecture of podocytes obtained after differentiation of u-RPCs by performing phalloidin staining. In all three patients carrying pathogenic mutations, we observed the loss of a proper organization of the cytoskeleton architecture with an altered distribution of actin filaments, while the cytoskeleton structure of the nonmutated but proteinuric patient (control) was perfectly maintained (Figure 7F). In agreement with the presence of severe cytoskeleton abnormalities, podocytes obtained from all three mutated patients exhibited a significant increase in detachment (Figure 7G) and death (Figure 7H) upon differentiation, in comparison to controls. Moreover,
PKH26-labeled u-RPCs obtained from patient 1 injected into SCID mice with doxorubicin nephropathy could engraft within glomeruli, where they acquired some synaptopodin, but almost no podocin, consistent with in vitro studies (Supplemental Figure 4A). Interestingly, no significant improvement in proteinuria was observed (Supplemental Figure 4B).

Taken together, these results demonstrate that u-RPCs may be used as tools for studying the functional consequences of potentially pathogenic mutations identified through next-generation sequencing on podocyte structure and function.

**DISCUSSION**

A major challenge for experimental research of human diseases and drug discovery is the use of biologically relevant methods of investigation. A substantial amount of the current knowledge about the mechanisms underlying human diseases has been derived from the study of mouse models. Mice can be genetically manipulated and combine the advantage of being phylogenetically close to humans with that of being free of both the costs of large-animal models and the additional ethical concerns with using primates. However, these advantages can be offset by species-specific differences between mice and humans in their biochemical, physiologic, and anatomic characteristics. Furthermore, many mouse models of human disease do not reliably reproduce the human phenotypes. Indeed, the human kidney is difficult to study because of its anatomic and functional complexity, being constituted by 26 different cell types that interact to build the nephrons and by the surrounding interstitium. Different cell types contribute to different disease states requiring cell type–specific modeling of disease-specific phenotypes and pharmacologically relevant strategies for drug screening.

Microimmunodissection techniques have made purification, isolation and culture of specific kidney cells possible, providing controlled environments for the elucidation of disease mechanisms. Disadvantages exist, however, in primary cell cultures, whereby certain cells, such as podocytes, exhibit rapid growth arrest, losing their ability to replicate and maintain certain characteristics as effective models. Advances in immortalization of cells have overcome the detrimental growth arrest characteristics, facilitating their use in the in vitro dissection of physiologic and disease-specific mechanisms. Despite this, immortalization of cell lines invariably leads to a failure to conserve the native characteristics of the original cell type. More important, both primary renal cell cultures and immortalized cell lines cannot be used for personalized modeling of kidney disorders.

In this study, we describe a new method that allows to obtain personalized RPC cultures from the urine of patients with genetic kidney disorders. Urine-derived RPCs exhibit the potential to differentiate into tubular cells as well as podocytes and have long-term growth capacity in culture, thus obviating the need for immortalization. Lineage tracing studies in models of doxorubicin nephropathy performed in conditional transgenic mice (NPHS2.1CreERTm/mG), where podocytes are irreversibly tagged upon tamoxifen treatment, confirmed that RPCs are the only urinary cell population that can be amplified long term in culture. These studies also demonstrated that podocytes amplified from urine are derived from RPCs. These observations are consistent with previous studies demonstrating that urinary
podocyte cultures also express parietal epithelial cell markers and that parietal epithelial cells can upregulate podocyte markers upon culture.

Taken together, the results of this study demonstrate that u-RPCs may represent an innovative tool for modeling of kidney disorders in the context of the individual’s overall genetic and epigenetic background. Indeed, patient-specific cells were obtained from children with genetic forms of steroid-resistant nephrotic syndrome (SRNS), differentiated into podocytes after exposure to specific medium and then used to functionally evaluate the role of known pathogenic mutations in two patients carrying homozygous or compound heterozygous mutations of the NPHS2 gene, that were indeed characterized by a reduced or absent synthesis of podocin. In addition, this method allowed establishment of the role of a variant of unknown clinical significance in the LMX1B gene, a crucial controller of podocyte cytoskeleton integrity, in inducing functional alterations of the podocyte cytoskeleton in a child with SRNS. This method may similarly apply to other inherited disorders of the kidney, such as tubulopathies, and used for functional studies of potentially pathogenic mutations of unknown significance to complement diagnostic of genetic kidney disorders.

Nonetheless, u-RPC technology provides a valuable tool to interrogate human disease mechanisms and offers an unprecedented opportunity to develop innovative models that will facilitate mechanistic studies of disease. Urine-derived RPCs maintain the patient genotype and will be a valuable tool for better understanding how mutations cause diseases, and for developing disease-modifying assays. In addition, patient-specific u-RPCs can be used for screening of new drugs in terms of safety and efficacy. Indeed, compounds are typically not tested against diseases, and for developing disease-modifying assays. In addition, patient-specific u-RPCs can be used for screening of new drugs in terms of safety and efficacy. Indeed, compounds are typically not tested in a relevant patient population until phase II clinical trials have been carried out. By contrast, u-RPC technology may provide the means to screen drug efficacy and toxicity, which are strongly influenced by the genetic background and by epigenetic factors, at the earliest stages of drug development in a patient-specific manner.

In conclusion, u-RPCs provide an innovative tool for personalized modeling of kidney disorders that has the potential to increase our knowledge of the pathogenesis of renal diseases and of patient-specific biology.

CONCISE METHODS

Patients, Urine Samples, and Tissues
Urine samples (10–100 ml) were collected immediately after the first urination in the morning from 60 pediatric patients with different glomerular diseases and from 10 healthy pediatric patients. These patients had been referred to the Nephrology Unit of Meyer Children’s Hospital of Florence, Italy. Patients 1–3 had SRNS with a biopsic diagnosis of FSGS.

The local ethics committee of the Meyer Children’s Hospital approved the study, and the parents or legal guardians of each study participant provided written informed consent.

RPCs were obtained, as previously described, from normal kidney fragments of eight patients who underwent nephrectomy...
Figure 5. u-RPCs exhibit the same in vitro properties as RPCs. (A) mRNA levels of podocyte-specific markers in HaCaT (n=3), RPCs (n=8) and u-RPCs (n=8) after differentiation toward podocytes. Data are obtained as mean±SD of at least eight separate experiments. *P<0.05 by Mann-Whitney test. (B) Western blot for GAPDH, podocin, nephrin, and synaptopodin on RPCs and u-RPCs before (T0) and after podocyte differentiation (VRAD). Protein bands are at the expected size of 180 kD for GAPDH, 180 kD for nephrin, 42 kD for podocin, and 110 kD for synaptopodin. One representative out of four independent experiments is shown. (C) Densitometric analysis of protein bands. The relative density of nephrin, podocin, and synaptopodin was normalized on GAPDH band. Results (mean±SD) are expressed as ratio of differentiated versus undifferentiated cells as obtained in four independent experiments. (D) Expression of podocyte markers (green) before (day 0) and after (day 2) culture of u-RPCs in podocyte differentiation medium VRAD. One representative out of four independent experiments is shown. To-pro-3 counterstains nuclei (blue). Bars=20 μm. (E) Phalloidin staining (green) before (day 0) and after (day 2) differentiation of u-RPCs. A representative experiment out of four independent experiments is shown. To-pro-3 counterstains nuclei (blue). Bars=20 μm.

because of kidney and urinary tract carcinomas, in accordance with the recommendations of the Ethical Committee of the Azienda Ospedaliero-Universitaria Careggi of Florence.

Standardized Protocol for the Isolation and Purification of Urine-Derived Cells

Urine samples were centrifuged at 1400 rpm for 10 minutes, the supernatant was removed, and the pellet washed with PBS (Lonza Sales Ltd., Basel, Switzerland) and centrifuged again at 1400 rpm for 5 minutes. After removal of the supernatant, cells were plated in a 24-well dish with EGM-MV (Lonza Sales Ltd.) supplemented with 20% FBS (HyClone Laboratories, South Logan, UT). Most cells from urine did not attach to culture plates and were removed when the culture medium was changed after 6 days. A few days after plating, one single cell or a few cells grew and formed a cluster of small, compact, and uniform cells able to achieve confluence in about 2–3 weeks, thereby allowing us to perform flow cytometry analysis. To test bacterial contamination, all the primary cultures obtained were screened for the presence of bacterial 16S ribosomal RNA gene. To this aim, DNA was extracted and the presence of 16S ribosomal RNA gene verified by PCR with the following primers: forward 5′ AGA GTT TGA TCC TGG CTC AG 3′; reverse 5′ ACG GCT ACC TTG TTA CGA GTT 3′.

Flow Cytometry and Immunomagnetic Cell Sorting

Flow cytometry triple staining for CD133, CD24, and CD106 on cultured cells was performed as described elsewhere.14,16,17 Each area of positivity was determined by gating on the same cells stained with isotype-matched monoclonal antibodies (mAbs). A total of 10^6 events for each sample was acquired with MACSQuant Analyzer Flow Cytometer and analyzed with MACSQuantify software (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The following antibodies were used: FITC-conjugated mAb anti-CD14 (clone SN3, Santa Cruz Biotechnology, Santa Cruz, CA), PE-conjugated mAb anti-CD106 (IE10, R&D Systems, Minneapolis, MN), allophycocyanin-conjugated mAb anti-CD133 (clone 293C3), mouse IgG2b-APC (clone IS6–11E5.11), mouse IgG2a-PE (clone S43.10), and mouse IgG1-FITC (clone IS5–21F5) (all from Miltenyi Biotec GmbH). CD133+ and CD133− cells were isolated by high-gradient magnetic cell separation using a CD133 Cell Isolation Kit obtained from Miltenyi Biotec.14 Cell death was evaluated using propidium iodide staining (Invitrogen, Carlsbad, CA) with MACSQuant Analyzer Flow Cytometer.

Urine-derived CD133+ and CD133− cells were maintained in culture as bulk, and routine cell passaging was performed. Medium was changed twice a week. The cell counts and cellular dilution factor were recorded at each passage. Clones from urine-derived CD133+ and CD133− cells were generated by limiting dilution in 96-well plates in EGM-MV+20% FBS (Lonza Sales Ltd.). To evaluate the number of clonogenic cells present in urine samples, clones were isolated using cloning cylinders (Sigma-Aldrich) and analyzed for the expression of CD133 and CD24 by flow cytometry analysis.

Taqman Low-Density Array and Real-Time Quantitative RT-PCR

A total of eight u-RP and eight RPC samples were analyzed. Briefly, total RNA was extracted using an RNasy Microkit (Qiagen, Hilden, Germany) and retrotranscribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Life Technologies Ltd, Paisley, UK),
tissue-derived RPC

Figure 6. u-RPCs exhibit the same ultrastructural features as RPC. (A) Scanning electron microscopy on RPCs before differentiation into podocytes. Original magnification, ×744; bar=20 μm. (B) Higher magnification of scanning electron microscopy on RPCs before differentiation into podocytes. Original magnification, ×2.70K; bar=10 μm. (C) Scanning electron microscopy on RPCs after exposure to podocyte differentiating medium (VRAD). Original magnification, ×1.13K; bar=20 μm. (D) Scanning electron microscopy on RPCs after exposure to podocyte differentiating medium (VRAD). Original magnification, ×1.13K; bar=10 μm. (d’) Detail of the image showed in D. (E) Scanning electron microscopy on u-RPCs before differentiation into podocytes. Original magnification, ×819; bar=20 μm. (F) Higher magnification of scanning electron microscopy on u-RPCs before differentiation into podocytes. Original magnification, ×2.70K; bar=10 μm. (G) Scanning electron microscopy on u-RPCs after exposure to podocyte differentiating medium (VRAD). Original magnification, ×969; bar=20 μm. (h’) Scanning electron microscopy on u-RPCs after exposure to podocyte differentiating medium (VRAD). Original magnification, ×740; bar=20 μm. (h’, h”) Detail of the image showed in H. A representative experiment out of two independent experiments is shown.

while microRNA was extracted and retrotranscribed using the Taqman microRNA Cells-to-CT Kit (Ambion, Life Technologies Ltd., Austin, TX). Predesigned TaqMan low-density array for 472 mRNA of human G protein–coupled receptor (catalog no. 4367785) and inflammation (catalog no. 4378707) genes and 168 microRNA (catalog no. 4342265) were obtained from Applied Biosystems and processed following manufacturer’s instructions. TaqMan low-density array analysis were performed using the RQ Manager and Data Assist software (Applied Biosystems). Data were shown as a volcano plot. In this type of plot, each point represents an RNA, the x-coordinate represents the (log2) fold change in expression between RPCs and u-RPCs, and the y-axis represents the –log10 of the P value from a t test. RNA with statistically significant differential expression according to the specific t test will lie above a horizontal threshold line (P<0.05).

NPHS2 Taq-Man quantitative PCR required preamplification of NPHS2 cDNA. To this aim, total RNA was extracted using an RNasey Microkit (Qiagen) and quantified with the Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Five hundred nanograms of total RNA was retrotranscribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer’s instructions, in a final volume of 20 μl. A multiplex PCR preamplification of NPHS2 and the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using TaqMan PreAmp Master Mix Kit (Applied Biosystems) following manufacturer’s instructions. The TaqMan probe for GAPDH (Applied Biosystems) and the two Assays on Demand (Hs00922492_m1 and Hs00387817_m1, Applied Biosystems) for NPHS2 were pooled together at 0.2X final concentration. The preamplified products were then diluted with TE buffer at a ratio of 1:5 for NPHS2 and 1:10000 for GAPDH, and were used as templates for TaqMan RT-PCR performed as previously described.16,17

Taq-Man RT-PCR was performed as described.16,17 Na/H, aquaporin 3, Na/K/Cl, SLC3A1, NPHS1, PODXL and KLF15 quantification were performed using commercially available Assay on Demand kits (Applied Biosystems).

Urine Cell Cultures from NPHS2.iCreER;mT/mG Transgenic Mice

NPHS2.iCreER22 mice (here abbreviated NPHS2.iCreER) were a kind gift from Dr. Farhad Danesh, Bayor College of Medicine (Houston, TX), and B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP) Lai/J (here abbreviated mT/mG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The NPHS2.iCreER; mT/mG mice were developed to stably and irreversibly tag podocytes upon induction with tamoxifen (Sigma-Aldrich). Animal experiments were performed in accordance with institutional, regional, and state guidelines and in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male mice, 5–6 week old, were intraperitoneally injected with 2 mg of tamoxifen per mouse for 8 consecutive days, followed by a 1-week washout period. These mice express strong red (Tomato Red) fluorescence in all tissues and cell types, but the podocin promoter drives the expression of the Cre recombinase in podocytes, allowing the expression of the membrane-targeted enhanced GFP specifically in podocytes that will appear as green cells. Following tamoxifen withdrawal, no new podocyte will be labeled.

To establish a model of doxorubicin nephropathy, NPHS2.iCreER; mT/mG mice (n=16) were injected with a first retro-orbital injection of doxorubicin hydrochloride (18 mg/kg in PBS, Sigma-Aldrich)
followed by a second one (18 mg/kg) 7 days later. Injury was confirmed by measurement of urinary albumin-to-creatinine ratio. For all mice, urinary albumin and creatinine were determined in spot urine with the Albuwell M kit (Exocell, Philadelphia, PA) and Creatinine Assay kit (Cayman Chemical, Ann Arbor, MI). Normal range of urinary albumin or creatinine in our experiments was measured in eight additional untreated mice.

On day 10 after the second injection, mice were perfused with 4% paraformaldehyde and kidneys were collected, left for 2 hours in 15% sucrose at 4°C, and left overnight in 30% sucrose at 4°C. Six additional healthy mice were processed identically as controls.

Fresh spot urine samples were collected every day after second injection of doxorubicin hydrochloride. Urine samples were centrifuged at 1400 rpm for 5 minutes. The supernatant was removed, and the pellet was washed with EGM-MV+20%FBS and either was cytospinned or was cultured in EGM-MV+20%FBS+penicillin (100 U/ml), streptomycin (1 mg/ml), and rifampicin (8 μg/ml) (all from Sigma-Aldrich).

Xenograft in SCID Mouse Model of Doxorubicin Nephropathy

Doxorubicin nephropathy was induced in female SCID mice (CB17/ScN-Scid, Balb/c background, Harlan, Udine, Italy) at the age of 6 weeks by a single intravenous injection of doxorubicin hydrochloride (6 mg/kg in PBS, Sigma-Aldrich) on day 0 in the tail vein. Animal experiments were performed in accordance with institutional, regional, and state guidelines and in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. On days 1 and 4 after doxorubicin hydrochloride injection, four groups of mice received intravenous administration as follows: group 1, saline (n=16 mice); group 2, tissue-derived CD133+ cells (n=12 mice; 0.75×10^6 cells/d); group 3, RPCs (n=16 mice; 0.75×10^6 cells/d); group 4, u-RPCs (n=16 mice; 0.75×10^6 cells/d). An additional group of healthy mice (n=12) was used as control. Before injection cells were labeled with the PKH26 Red Fluorescence Cell Linker Kit following manufacturer’s instructions (Sigma-Aldrich). CD133+, RPCs, and u-RPCs were obtained from three different donors each. For all mice, urinary albumin and creatinine were determined in 24-hour urine with an Albuwell M kit (Exocell) and Creatinine Assay kit (Cayman Chemical). Normal range of urinary albumin or creatinine in our experiments was measured in eight additional untreated mice.
measured in eight additional untreated mice. Moreover, in an additional experiment, on days 1 and 4 after doxorubicin hydrochloride injection, three groups of mice received intravenous administration as follows: group 1, saline (n=4 mice); group 2, PKH26-labeled u-RPCs (n=4 mice; 0.75×10⁶ cells/d); group 3, PKH26-labeled u-RPCs obtained from patient 1 (n=4 mice; 0.75×10⁶ cells/d). Mice of all the experiments were euthanized on day 10 after doxorubicin hydrochloride injection and kidneys were removed for morphologic assessment.

**u-RPC In Vitro Differentiation**

For the differentiation toward the tubular lineage, u-RPCs (n=8) and RPCs (n=8) were cultured in the tubular differentiating medium renal epithelial cell growth medium (Lonza) supplemented with 50 ng/ml of hepatocyte growth factor (Peprotech, Rocky Hill, NJ) for 21 days. For the differentiation toward podocyte, u-RPCs (n=8) and RPCs (n=8) and human keratinocyte cell line (HaCaT; Life Technologies) were cultured in the podocyte-differentiating medium VRAD, composed of DMEM/F12 (Sigma-Aldrich) supplemented with 10% FBS (Hyclone), 100 nM vitamin D₃, and 100 μM retinoic acid, for 48 hours (all from Sigma-Aldrich). Cultured podocytes (n=8) were obtained as previously described.37

**Immunofluorescence and Confocal Microscopy**

Confocal microscopy was performed on 5-μm sections of renal frozen tissues, on cells cultured on chamber slides as described16,17 or in centrifuged samples using a Leica TCS SP5-II laser confocal microscope (Leica, Germany). FITC-labeled LTA (Vector Laboratories, Burlingame, CA), anti-α–actinin mAb (Dako, Glostrup, Denmark), anti-E-cadherin mAb (Chemicon, Cambridge, UK), anti-synaptopodin mAb (Progen, Heidelberg, Germany), FITC-labeled IFA (Vector Laboratories, Burlingame, CA), and anti–claudin-1 pAb (Invitrogen). Alexa Fluor goat anti-mouse IgG1 488 and 647, goat anti-rabbit 488, goat anti-mouse IgG2a 488, and rabbit anti-goat 488 were used as secondary antibodies (Invitrogen). For the detection of cytoskeleton, staining of cells with Alexa Fluor 488 phalloidin (Molecular Probes from Invitrogen) was performed. To-pro-3 (Invitrogen) and DAPI (Sigma-Aldrich) were used for counterstaining nuclei.

**Protein Extraction and Western Blot**

Urine-derived RPCs obtained from patients (n=3) without mutations or u-RPCs obtained from patients (n=3) carrying genetic mutations and RPCs (n=3) were cultured in the podocyte-differentiating medium VRAD. After 48 hours, cell proteins were extracted by addition of RIPA buffer (Sigma-Aldrich) containing 150 mM NaCl, 1% IGE-PAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH, 8.0) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail 2 following manufacturer’s instructions (Sigma-Aldrich) at 4°C. The suspension was centrifuged at 14,000 g, and the supernatant containing cellular proteins was collected. For Western blot, an 8% SD–PAGE (NuPAGE 3%–8% Tris-Acetate gel; Life Technologies) was run under standard conditions, loading 25 μg of total protein in each lane. The gel was placed in transfer buffer and set up for transfer to a polyvinylidene fluoride membrane (Sigma-Aldrich) at 30V overnight at 4°C. The membrane was rinsed in Tris-buffered saline-Tween buffer and immersed in blocking buffer (5% milk powder) for 1 hour; it was then incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: anti-α–actinin mAb (Dako, Glostrup, Denmark), anti-podocin pAb (directed against C-terminus; Abcam, Inc., Cambridge, UK), anti-synaptopodin mAb (Progen, Heidelberg, Germany), FITC-labeled IFA (Vector Laboratories, Burlingame, CA), and anti–claudin-1 pAb (Invitrogen). Alexa Fluor goat anti-mouse IgG1 488 and 647, goat anti-rabbit 488, goat anti-mouse IgG2a 488, and rabbit anti-goat 488 were used as secondary antibodies (Invitrogen). For the detection of cytoskeleton, staining of cells with Alexa Fluor 488 phalloidin (Molecular Probes from Invitrogen) was performed. To-pro-3 (Invitrogen) and DAPI (Sigma-Aldrich) were used for counterstaining nuclei.

**Nonmutated, but Proteinuric, Patients as Controls**

Nonmutated, but proteinuric, patients as controls, after differentiation into podocytes. Results are expressed as mean ± SD obtained in at least three separate experiments. P<0.05 by Mann–Whitney test for patients 1 and 2 versus controls. (F) Altered cytoskeleton architecture of podocytes obtained from three patients carrying genetic mutations (patients 1–3) and from u-RPCs obtained from nonmutated, but proteinuric, patient (control) before (T0) and after (VRAD) podocyte differentiation using an anti-podocin antibody that specifically recognizes the C-terminus of the protein. A representative experiment out of four independent experiments is shown. To-pro-3 counterstains nuclei (blue). Bars=20 μm. Bottom left: A representative Western blot for podocin on u-RPCs obtained from the three patients carrying genetic mutations (patients 1–3) and from u-RPCs obtained from nonmutated, but proteinuric, patient (control) by using an anti-podocin antibody that specifically recognizes the C-terminus of the protein. A representative experiment out of four independent experiments is shown. To-pro-3 counterstains nuclei (blue). Bars=20 μm. Bottom left: A representative Western blot for podocin on u-RPCs obtained from the three patients carrying genetic mutations (patients 1–3) and from u-RPCs obtained from nonmutated, but proteinuric, patient (control) before (T0) and after (VRAD) podocyte differentiation using an anti-podocin antibody that specifically recognizes the C-terminus of the protein. Protein bands are at the expected size of 42 kD for podocin in patient 3 and in control, whereas in patient 1 and 2 the bands were absent. Protein bands are at the expected size of 36 kD for GAPDH. A representative experiment out of four independent experiments is shown. Bottom right: densitometric analysis of protein bands. Results (mean±SD) are expressed as relative ratio of podocin/GAPDH in differentiated versus undifferentiated cells as obtained in four independent experiments. *P<0.05 by Mann–Whitney test for patients 1 and 2 versus controls. (F) Altered cytoskeleton architecture of podocytes obtained from u-RPCs of three patients carrying genetic mutations (patients 1–3) in comparison to u-RPCs obtained from nonmutated patient (control), as demonstrated by phalloidin staining (green). The details below the pictures show the distribution of actin filaments at membrane level. One representative out of four independent experiments is shown. Top: To-pro-3 counterstains nuclei (blue). Bars=20 μm. (G) Number of adherent cells of u-RPCs obtained from three patients carrying genetic mutations (patients 1–3) and from three u-RPCs obtained from nonmutated, but proteinuric, patients as controls, after differentiation into podocytes. Results are expressed as mean ± SD obtained in at least five separate experiments. *P<0.05 by Mann–Whitney test for patients 1–3 versus controls. (H) Percentage of dead cells following differentiation into podocytes of u-RPCs obtained from three patients carrying genetic mutations (patients 1–3) and from u-RPCs obtained from nonmutated, but proteinuric, patients as controls, as assessed by spontaneous uptake of propidium iodide (PI). Left: graph representing mean ± SD of PI-positive cells, obtained in at least four separate experiments. *P<0.05 by Mann–Whitney test for patients 1–3 versus controls. Right: Flow cytometry analysis that shows one representative of four independent experiments for each patient.
water, pH 2.2). After rinsing in wash buffer, horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (GE Healthcare, UK) was used at 1:2000 dilution and incubated for 1 hour at room temperature. After final washing the membrane was developed using Luminol chemiluminescence reagent (EMD Millipore, Darmstadt, Germany). Western blot images were quantified using ImageJ, a Java-based image-processing software (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Background signal was subtracted before normalization. Four separate Western blot experiments were used to obtain semiquantitative data for statistical analysis.

**Scanning Electron Microscopy Analysis**

For scanning electron microscopy analysis, RPCs (n=2) and u-RPCs (n=2) were seeded on Thermanox slides (Thermo Fisher Scientific, Waltham, MA) and cultured in the podocyte-differentiating medium VRAD. After 48 hours, cells were fixed in 2.5% glutaraldehyde (buffered with 0.1 M sodium cacodylate buffer, pH of 7.4) for 1 hour, postfixed in 1% osmium tetroxide, and dehydrated through an increasing ethanol series. Cell monolayers were then dried with pure hexamethyldisilazane (Fluka Chemie AG, Buchs, Switzerland) (twice for 30 minutes), sputter-coated with gold, and observed at scanning electron microscopy (Supra 55; Carl Zeiss, Oberkochen, Germany).

**DNA Sequencing**

DNA was extracted from peripheral blood using a QIAamp DNA Mini Kit (Qiagen). The DNA libraries were prepared and were hybridized to the exon sequencing array in a solution-based method according to the manufacturer’s protocol (NimbleGen Arrays User’s Guide: 454 Optimized Sequence Capture). The probes were designed by Roche NimbleGen Inc. to capture all coding exons and flanking regions of 46 genes, 19 known genes responsible for SRNS, and 27 candidate genes associated with onset of proteinuria in animal models and expressed in the glomerular filtration barrier (Supplemental Table 1). To simultaneously characterize more patients, Roche FLX Titanium MID-Adapter oligonucleotides were ligated to the ends of the library fragments. The enriched libraries were sequenced using the Roche 454 sequencing FLX platform according to the Roche manufacturer’s protocols (emPCR Method Manual-Lib-L IV-Sequencing Method Manual). Reads (120.331bp mapped reads per sample) were mapped to the reference human genome (UCSC build hg19) with the GS Mapper software version 2.6 (Roche Diagnostic, Basel, Switzerland). Coverage statistics (88% of bases had at least 10 reads) and mean depth (79×) were extracted from the mapping output files using custom scripts. The poorly covered regions were successively resequenced by Sanger method. On the basis of our analysis we found the following: in patient 1, a homozygous mutation in NPHS2 gene (c.[419delG]+[419delG]; p.[Gly140Aspfs*41]+[Gly140Aspfs*41]); a frameshift leading to a STOP codon previously recognized cause of SRNS2,3; in patient 2, a heterozygous compound mutation in the NPHS2 gene (c.413G>A)+[467_468insT]; that p.[Arg138Gln]+[Leu156Phefs*11]) is determined by a missense mutation in an allele and a frameshift leading to a STOP codon in the other allele, both previously recognized cause of SRNS2,3; and, in patient 3, a heterozygous missense mutation in the dominantly transmitted LMX1B gene (c.[833C>T]+[=]; p.[Ala278Val]+[=]). This variant was not previously reported but was localized in highly conserved domains of the protein. It was predicted to be pathogenic by in silico analysis and was consistent with the phenotype of the patient and considered as potentially pathogenic in agreement with the American College of Medical Genetics and Genomics guidelines,38 as also detailed elsewhere.34 The variants detected in three patients with SRNS by 454 FLX analyzer were selected and confirmed by Sanger sequencing (Supplemental Table 2).

**Statistical Analyses**

The results are expressed as mean±SD. Comparison between groups was performed by the Mann–Whitney test. P<0.05 was considered to represent a statistically significant difference. ANOVA with Bonferroni correction was used for multiple comparison.

In the volcano plot, the −log10-transformed P values from the gene-specific t test was plotted against the log2 fold change in RNA expression between RPCs and u-RPCs. RNA with statistically significant differential expression according to the specific t test will lie above a horizontal threshold line (P<0.05).

The Pearson product-moment correlation coefficient (Pearson r) was used for linear correlation (dependence) between two variables. A value of 1 was considered a total positive correlation, 0 as no correlation, and −1 as total negative correlation.

**ACKNOWLEDGMENTS**

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**DISCLOSURES**

E.L., L.L., and P.R. are authors of a patent that is property of the public pediatric hospital Meyer Children’s Hospital.

**REFERENCES**


See related editorial, “From Patient to Dish and Back Again: Are We There Yet?,” on pages 1757–1759.
Supplementary Figure 1: u-RPC exhibit the same transcriptome of RPC

A) Volcano plot showing 472 mRNA and 168 microRNA data. Each point represents a RNA; x-axis is the fold change (in log2 scale) of RNA expression between RPC (n=8) and u-RPC (n=8). The y-axis is the statistical significance (-log10 of p-value). The horizontal line shows where p is equal to 0.05. Points above the line have p<0.05 and points below the line have p>0.05.

B) Correlation between 472 mRNA and 168 microRNA expression levels of RPC (y-axis) versus u-RPC (x-axis) with best fit line curve, showing high positive correlation (Pearson’s r= 0.88). The relative amount of each mRNA and miRNA was computed using the equation $2^{-\Delta CT}$, where $\Delta CT=(CT_{miRNA or mRNA}-CT_{housekeeping})$; CT values were defined as the cycle number in which the fluorescence crossed the fixed threshold. Each point represents a RNA.
Supplementary Figure 2: u-RPC exhibit the same *in vivo* and *in vitro* properties of RPC

A) Representative micrograph of kidney sections of mice with Adriamycin nephropathy after injection of PKH26-labeled u-RPC (red), stained with LTA (green). To-pro-3 counterstains nuclei (blue). Bar 20µm.

B) Detail of a tubular structure of a mouse with Adriamycin nephropathy showing integration of u-RPC labeled with PKH26 (red). To-pro-3 counterstains nuclei (blue). Bar 20µm.

C) mRNA levels of tubular-specific markers in RPC (n=8) and in u-RPC (n=8) after differentiation toward tubular phenotype. Data are obtained as mean ± SD of at least eight separate experiments. NS by Mann-Whitney test.

D) Expression of tubular markers before (day 0) and after (day 21) culture of u-RPC in tubular differentiation medium. A representative experiment out of three independent experiments is shown. To-pro-3 counterstains nuclei (blue). Bars 20µm.
Supplementary Figure 3: RPC and u-RPC exhibit comparable levels of podocyte markers to those observed in cultured podocytes

A) Relative expression of NPHS1 mRNA levels in RPC (n=8) and in u-RPC (n=8) before (T0) and after differentiation (VRAD) vs cultured podocytes (n=8). Data are obtained as mean ± SD of at least eight separate experiments. Undifferentiated RPC or u-RPC vs differentiated RPC or u-RPC p<0.001 by Mann-Whitney test. Differentiated RPC or u-RPC vs cultured podocytes NS by Mann-Whitney test.

B) Relative expression of NPHS2 mRNA levels in RPC (n=8) and in u-RPC (n=8) before (T0) and after differentiation (VRAD) vs cultured podocytes (n=8). Data are obtained as mean ± SD of at least eight separate experiments. Undifferentiated RPC or u-RPC vs differentiated RPC or u-RPC p<0.001 by Mann-Whitney test. Differentiated RPC or u-RPC vs cultured podocytes NS by Mann-Whitney test.

C) Relative expression of PODXL mRNA levels in RPC (n=8) and in u-RPC (n=8) before (T0) and after differentiation (VRAD) vs cultured podocytes (n=8). Data are obtained as mean ± SD of at least eight separate experiments. Undifferentiated RPC or u-RPC vs differentiated RPC or u-RPC p<0.05 by Mann-Whitney test. Differentiated RPC or u-RPC vs cultured podocytes NS by Mann-Whitney test.
D) Relative expression of KLF15 mRNA levels in RPC (n=8) and in u-RPC (n=8) before (T0) and after differentiation (VRAD) vs cultured podocytes (n=8). Data are obtained as mean ± SD of at least eight separate experiments. Undifferentiated RPC or u-RPC vs differentiated RPC or u-RPC p<0.001 by Mann-Whitney test. Differentiated RPC or u-RPC vs cultured podocytes NS by Mann-Whitney test.

Supplementary Figure 4: *In vivo* functional properties of u-RPC obtained from patient 1 with *NPHS2* mutation

A) Representative micrograph of kidney section of mice with Adriamycin nephropathy after injection of PKH26-labeled u-RPC (red) obtained from a patient with *NPHS2* mutation (patient 1), stained with anti-podocin antibody (green) and with anti-synaptopodin antibody (blue). To-pro-3 counterstains nuclei (white). Bar 20µm.

B) Albumin/creatinine ratio (day 10) in mice with Adriamycin nephropathy (ADR) injected with saline (n=4), u-RPC (n=4) and u-RPC obtained from patient 1 (n=4). * p<0.05 by Mann-Whitney test.
### Supplementary Table 1: Genes included in the sequencing array. The detailed procedure for this diagnostic sequencing array is reported elsewhere.\(^\text{34}\)

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### Supplementary Table 2: Primer sequences used to confirm pathogenic mutations identified with next generation sequencing.