Several kidney diseases including congenital nephrotic syndrome, Alport syndrome, and diabetic nephropathy are linked to podocyte dysfunction. Human podocytopathies may be modeled in either primary or immortalized podocyte cell lines. Human induced pluripotent stem cell (hiPSC)-derived podocytes are a source of human podocytes, but the existing protocols have variable efficiency and expensive media components. We developed an accelerated, feeder-free protocol for deriving functional, mature podocytes from hiPSCs in only 12 days, saving time and money compared with other approaches.
Accelerated protocol for the differentiation of podocytes from human pluripotent stem cells

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https://doi.org/10.1016/j.xpro.2021.100898

SUMMARY
Several kidney diseases including congenital nephrotic syndrome, Alport syndrome, and diabetic nephropathy are linked to podocyte dysfunction. Human podocytopathies may be modeled in either primary or immortalized podocyte cell lines. Human induced pluripotent stem cell (hiPSC)-derived podocytes are a source of human podocytes, but the existing protocols have variable efficiency and expensive media components. We developed an accelerated, feeder-free protocol for deriving functional, mature podocytes from hiPSCs in only 12 days, saving time and money compared with other approaches.

BEFORE YOU BEGIN
Development of the protocol
Podocytes are highly differentiated cells with long foot processes that wrap around the renal capillaries to provide glomerular filtration. Most glomerular disorders are associated with phenotype alterations in proliferating podocytes whose malfunction leads to proteinuria (Barisoni et al., 1999). Animal models have been extensively used in research to better understand the genesis and progression of podocytopathies as well as to find possible drug targets and treatments (Pippin et al., 2009). Despite this, animal models do not always replicate human analogs or disease characteristics, necessitating the development of robust and reproducible methods for the in vitro culture of human podocytes. The reprogramming of human induced pluripotent stem cells (hiPSCs) from adult human cells has opened up new techniques to generate various cell types in vitro through directed differentiation (Takahashi et al., 2007). Utilizing a strong basis of knowledge gained from studies of embryonic kidney development, multiple approaches for generating podocytes from hiPSCs have been developed (Musah et al., 2017; Qian et al., 2019; Rauch et al., 2018; Song et al., 2012).

The embryonic development of the kidney starts with the generation of primitive streak. The primitive streak is an elongating groove-like structure that forms in the posterior region of the blastula (Downs, 2009). The cells then migrate from the late primitive streak to form the mesoderm which consists of paraxial, intermediate, and lateral plate cells. The anterior region of the primitive streak forms the paraxial mesoderm whereas the posterior region forms the lateral plate mesoderm (Wilson and Beddington, 1996). The intermediate mesoderm (IM) lies between the paraxial and lateral plate mesoderm. IM cells are the source of kidney progenitor populations. The two major progenitors present in the kidney are the ureteric epithelium that develops into the collecting duct/ureter (Mendelsohn, 2009) and the metanephric mesenchyme that differentiates into the remaining structures of the nephron including the glomerular podocytes (Kobayashi et al., 2008). Within the intermediate
mesoderm, the anterior region differentiates to the ureteric epithelium and the posterior region differentiates into the nephron progenitor cells (NPCs) (Taguchi et al., 2014). Since podocytes arise from the cap mesenchyme, metanephric mesenchyme induction from iPSCs is required for efficient podocyte production.

The type of extracellular matrix (ECM) is critical for the support and adhesion of iPSCs and also for the efficient differentiation to podocytes. Interaction between cells and ECM is mediated via integrins, which are the transmembrane receptors consisting of $\alpha$- and $\beta$-subunits. Integrins $\beta 1$ and $\alpha v \beta 5$ are highly expressed in both human iPSC and in a human podocyte cell line (Musah et al., 2017). $\beta 1$ integrins are essential for podocyte function in vivo (Kanasaki et al., 2008; Pozzi et al., 2008). Laminin 5-11 (LN-511), which consists of $\alpha 5, \beta 1,$ and $\gamma 1$ chains, has been reported to promote greater adhesion of hESCs and hiPSCs than matrigels and other matrices (Rodin et al., 2010). Taking each of these factors into consideration, we developed the protocol described below. This protocol produces podocytes from human iPSC with ~70% efficiency, comparable to most of the existing protocols, in a short time period.

### Primitive streak induction

Most accelerated protocols for podocyte differentiation either employ the embryoid body (EB) formation method or use media containing serum. The use of EBs in other protocols gives rise to cell-to-cell heterogeneity, while serum-containing media results in inconsistent results due to batch effects. The existing serum-free protocols require longer culture time which is usually undesirable. Therefore, our goal was to generate an accelerated protocol containing serum-free media to generate podocytes (Figure 1). The first step of differentiation is the induction of the late primitive streak. Wnt and TGF-$\beta$/nodal/activin signaling are simultaneously required for the generation of the Brachyury+ primitive streak population (Gadue et al., 2006; Tam and Loebel, 2007). These signals can efficiently generate posterior primitive streak from hPSCs (Morizane and Bonventre, 2017; Musah et al., 2017; Taguchi et al., 2014; Takasato et al., 2014, 2015). The cells are cultured under monolayer conditions to achieve more precise anterior-posterior cell fate. Activin A has also been reported to differentiate hPSCs to primitive streak (Takasato et al., 2014). Therefore, we used a combination of a low dose of canonical WNT signaling and Activin A for the first 2 days to generate primitive streak. These cells exhibited changed morphology (Figure 2) and stained positive for the primitive streak marker MIXL1 (Figure 3).

### Intermediate mesoderm induction

The second stage of podocyte differentiation is the differentiation of primitive streak cells into the intermediate mesoderm. ESCs and iPSCs require canonical Wnt signaling activation for posterior primitive streak and intermediate mesoderm induction (Kreuser et al., 2020; Lindsley et al.,...
Alternative protocols used a combination of BMP7 and Wnt to derive intermediate mesoderm from iPSCs (Musah et al., 2017). Therefore, we treated primitive streak cells with a high dose of the Wnt activator CHIR for three days to induce intermediate mesoderm formation. The cells began to proliferate and started to form thick layers at this stage of differentiation (Figure 2). These cells expressed the intermediate mesoderm cell marker paired box gene 8 protein (PAX8) (Figure 3), which is a critical regulator of the nephric lineage specification (Bouchard et al., 2002).

**Nephron progenitor induction**

Because the primitive streak differentiates spontaneously into the lateral plate mesoderm, exogenous factors to direct differentiation to the medial plate are necessary. At this stage, cells include progenitors of the ureteric epithelium, metanephric mesenchyme, renal interstitium, and endothelium (Mugford et al., 2008). The metanephric mesenchyme is derived from the posterior intermediate mesoderm (PIM) so the next step is to induce nephron progenitors from these cells. Most existing protocols used a combination of three or four factors to induce nephron progenitor formation. Morphogens such as BMP4 and FGF9 induce the medial-lateral patterning of the trunk mesoderm. BMP4 is expressed in the lateral plate mesoderm (James and Schultheiss, 2005) whereas FGF9 is expressed in the intermediate mesoderm (Colvin et al., 1999). When starting from iPSCs, FGF9 alone is sufficient to specify the intermediate mesoderm and generate nephron progenitors (Ciampi et al., 2016; Low et al., 2019). FGF9 can also maintain nephron progenitors in vitro (Barak et al., 2012). Therefore, in order to simplify the procedure we relied upon FGF9 alone to generate nephron progenitors (Figure 1). Heparin causes oligomerization of FGFs and supports their binding to FGF receptors, resulting in their activation (Spivak-Kroizman et al., 1994). Therefore, heparin is used together with FGF9 in many existing kidney differentiation protocols (Morizane et al., 2015; Takasato et al., 2015). Compared to other protocols, the nephron progenitor formation using FGF9 required a short window of only 2 days. We added FGF9 together with heparin until day 7 to induce the generation of nephron progenitors. The cells continued to proliferate and thick cobblestone-like morphology was seen in the culture (Figure 2), as previously reported (Pleniceanu et al., 2018). The cells derived stained positive for the nephron progenitor cell markers SIX2 and CITED1 at day 7 (Figure 3).

**Podocyte induction**

Growth factor cocktails including retinoic acid, BMP7, vascular endothelial growth factor (VEGF), and activin A are effective for generating podocytes from iPSCs (Rauch et al., 2018). Retinoic acid (RA) encourages the differentiation of podocytes (Dai et al., 2017; Mallipattu and He, 2015; Vaughan et al., 2005), whereas BMP7 is both a podocyte differentiation and survival factor (Mitu et al., 2007). VEGF is crucial for endothelial cell development. But VEGF also supports the survival of podocytes both in vitro and in vivo (Harper et al., 2001).
differentiation of podocytes from the nephron progenitors, we used a cocktail of these factors. We dissociated the nephron progenitors and plated them onto laminin 5-11 coated plates to support the adhesion and maturity of podocytes. The recombinant laminin-511 E8 fragment used in our protocol is composed of the C-terminal regions of the alpha, beta, and gamma chains which bind to integrin $\alpha_6\beta_1$, located on the cell surface. The matrix has been reported to be essential for integrin-receptor-mediated glomerular basement membrane (GBM) signaling (Maier et al., 2021; Suleiman et al., 2013) and has been used for podocyte differentiation (Musah et al., 2017). Most existing protocols require 6–10 days of differentiation to derive podocytes from nephron progenitors (Ciampi et al., 2016; Qian et al., 2017; Rauch et al., 2018). However, this protocol has reduced time required for the generation of podocytes from nephron progenitors to 5 days. The cell morphology changed into a large arborized structure with a large cell body (Figures 4A and 4C). Scanning electron microscopy (SEM) showed that the cells exhibit prominent primary thin processes (Figure 5B) and cells visualized with transmission electron microscopy (TEM) displayed tight junctions (Figure 5C). The resulting cells expressed podocyte lineage specification markers including: Synaptopodin (SYNPO), Podocalyxin (PODXL), MAF BZIP transcription factor (MAFB), and NPHS1 Adhesion Molecule (Nephrin) (Figure 5A).
Figure 4. Characterization of podocyte cells derived from iPSCs

(A) Lower and higher magnification brightfield images of the day 12 podocytes derived from the DYR0100 iPSC cell line.

(B) Flow cytometry analysis of the DYR0100-derived podocytes for podocyte markers MAFB and PODX.

(C) Lower and higher magnification brightfield images of the day 12 podocytes derived from MAFB:mTagBFP2/GATA3:mCherry iPSC cell line.

(D) Flow cytometry analysis of the derived podocytes for expression of podocyte marker MAFB and the MAFB promoter-driven mTagBFP2 stained with a FITC secondary antibody. The scale bar is 50 μm.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rat Monoclonal anti-OCT-4 (1:200) | R&D systems | Cat# MAB1759SP |
| Rabbit Polyclonal anti-MIXL1 (1:200) | Proteintech | Cat#22772-1-AP |
| Rabbit Polyclonal anti-PAX8 (1:200) | Proteintech | Cat#10336-1-AP |
| Goat Polyclonal anti-GATA3 (1:200) | R&D systems | Cat#AF2605 |
| Mouse Monoclonal anti-CITED1 (1:200) | Fisher Scientific | Cat#89-335-107 |
| Rabbit Polyclonal anti-SIX2 (1:200) | Proteintech | Cat#11562-1-AP |
| Rabbit Polyclonal anti-MAFB (1:200) | Abcam | Cat#ab223744 |
| Rabbit Polyclonal anti-PODX (1:200) | Proteintech | Cat#1815O-1-AP |
| Rabbit Polyclonal anti-WT1 (1:200) | Proteintech | Cat#12609-1-AP |
| Sheep Polyclonal anti-NEPHRIN (1:200) | R&D systems | Cat#AF4269 |
| Rabbit Polyclonal anti-Synaptopodin (1:200) | Abcam | Cat#ab224491 |
| Alexa 488, goat anti-mouse IgGa (1.400) | Life Technologies | Cat#A-21131 |
| Alexa 488, goat anti-rat IgG (1.400) | Life Technologies | Cat#A-11006 |
| Alexa 488, donkey anti-goat IgG (1.400) | Life Technologies | Cat#A-11055 |
| Alexa 594, goat anti-rabbit IgG (1.400) | Life Technologies | Cat#A-11037 |
| Alexa 594, donkey anti-sheep IgG (1.400) | Life Technologies | Cat#A-11016 |
| **Chemicals, peptides, and recombinant proteins** | | |
| mTeSR medium | STEMCELL Technologies | Cat#05825 |
| Y27632 ROCK inhibitor | STEMCELL Technologies | Cat#72304 |
| Accutase | Fisher Scientific | Cat#A1110501 |
| DMEM/F12 with GlutaMAX supplement | Thermo Fisher Scientific | Cat#10565042 |
| B27 serum-free supplement | Thermo Fisher Scientific | Cat#17504044 |
| Laminin-511 (BG iMatrix-511) | PeproTech | Cat#R511 |
| Human Activin A | PeproTech | Cat#120-14P-10ug |
| ChIR99021 | Reagents Direct | Cat#27-H76 |
| Human BMP7 | Fisher Scientific | Cat#354-BP |
| Human VEGF | Millipore Sigma | Cat#V7259 |
| All-trans retinoic acid | Stem Cell Technologies | Cat#72262 |

(Continued on next page)
| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| Fetal bovine serum       | Life Technologies | Cat#26140079 |
| Penicillin-Streptomycin  | Mediatech/CellGro-Corning | Cat#30002CI |
| BSA                      | Sigma Aldrich | Cat#A7030-50g |
| DMSO                     | Sigma Aldrich | Cat#D2650 |
| Phosphate-buffered saline (PBS), 1X w/o Calcium & Magnesium | Mediatech/CellGro-Corning | Cat#21-04OCV |
| Phosphate-buffered saline (PBS), 10X w/o Calcium & Magnesium | Mediatech/CellGro-Corning | Cat#MT46013CM |
| Cell culture grade water | Millipore Sigma | Cat#W3500-500ML |
| Deionized water          | n/a     | n/a        |
| FGF9                     | PeproTech | Cat#100-23-50ug |
| Heparin                  | Sigma Aldrich | Cat#H3149-100KU |
| Geltrax™, LDEV-Free      | Fisher Scientific | Cat#A1413202 |
| Matrigel                 | Fisher Scientific | Cat#CB-40230A |
| Trypsin EDTA, 0.05%      | Thermo Fisher Scientific | Cat#25-300-054 |
| CryoStor CS-10 CRYOPRESERVATION MEDIUM | STEMCELL Technologies | Cat#07959 |
| Paraformaldehyde         | Fisher Scientific | Cat#S0-980-487 |
| Tween 20                 | Sigma-Aldrich | Cat#P9416 |
| Sodium Azide             | Sigma-Aldrich | Cat#S2002 |
| DAPI (6-Diamidino-2-Phenylindole Dihydrochloride) | Sigma-Aldrich | Cat#D9542-5MG |
| Methanol                 | Sigma Aldrich | Cat#D2650 |
| FITC albumin             | Thermo Fisher Scientific | Cat#A23015 |
| F-actin (Phalloidin 594) | Molecular grade Probe | Cat#A12381 |

**Experimental models: Cell lines**

| Cell line | Source | Identifier |
|-----------|--------|------------|
| Human iPSC | ATCC | Cat#DYR0100 |
| MAFB:mTagBFP2/GATA3:mCherry human iPSC | NIH RBK | https://www.rebuildingakidney.org/cell-lines/ |

**Software and algorithms**

| Software | Source | Identifier |
|----------|--------|------------|
| Image J | NIH | https://imagej.nih.gov/ij/ |
| FlowJo | BD Biosciences | https://www.flowjo.com/ |

**Others**

| Item | Source | Identifier |
|------|--------|------------|
| Biological safety cabinet | NUAIRE, Class II Type A2 | n/a |
| Water bath, 37°C | Fisher Scientific, Isotemp 2332 | n/a |
| Benchtop centrifuge | Eppendorf, Centrifuge 5424 | n/a |
| CO₂ incubators | NUAIRE | Cat#NU5500 |
| Conical tubes (15,50 mL) | VWR | Cat#490001-621, Cat#490010-627 |
| Cryogenic Storage Vials | Greiner Bio-One | Cat#12263 |
| Cryovial Freezer | BioExpress | Cat#BSC-4050 |
| Inverted contrasting tissue culture microscope | Zeiss, Primovert | n/a |
| Laser confocal microscope | Nikon, Spinning Disk | n/a |
| Transmission Electron Microscope | FEI, Tecnai T12 | n/a |
| Scanning Electron Microscope | FEI, Quanta TM250 FEG | n/a |
| Flow Cytometer | BD, BD LSRFortessa | n/a |
| Media storage bottle | Fisher Scientific | Cat#14-389-136TU |
| Pipette Controller | Fisher Scientific | Cat#FBE00002 |
| Pipette Set | Fisherbrand (P2, P10, P200 and P1000 Elite) | Cat#FBE00002, Cat#FBE00010, Cat#FBE00200, Cat#FBE10000 |
| Serological pipettes | VWR | Cat#2050-482, Cat#2050-478 |
| Steriflip 0.22 µm filter unit | Fisher Scientific | Cat#SGP0052S |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Sterile filter pipette tips (10, 20 mL) | VWR | Cat#76322-132, Cat#76322-134 |
| Sterile filter pipette tips (200, wide bore 200, 1000 µL) | Fisher Scientific | Cat#NC1915695, Cat#14222730, Cat#NC1915694 |
| Sterile microcentrifuge tube | Fisher Scientific | Cat#02-707-352 |
| Swing-out rotor centrifuge | Thermo IEC, Centra CL3R | n/a |
| Tissue culture-treated plates (12-well and 6-well) | Fisher Scientific | Cat#08-772-29, Cat#07-200-83 |

**MATERIALS AND EQUIPMENT**

**Geltrex**

First, thaw the Geltrex by placing it on ice. To prepare 1% Geltrex, add 400 µL of Geltrex into a 50 mL tube containing 40 mL of chilled DMEM/F-12. Make 1 mL aliquots and store them at −20°C or −80°C until ready to use. The aliquots can be stored for up to 36 months. Add 1 mL of aliquot to each well of the 6-well plate and keep it at 37°C for at least 1 h to allow Geltrex to coat the surface. Use the coated plate within 48 h, storing at 4°C.

⚠️ CRITICAL: Handle Geltrex on ice as it solidifies when warmed over 16°C. The pipette tips used for dilution should be pre-chilled by keeping the pipette tips at −20°C for 30 min before use.

Coated plates must be used within 48 h. Plates kept longer will dry out and the cells will not attach to the plates. Do not use dry plates.

**Matrigel**

First, thaw Matrigel by placing it on ice. To prepare 1:30 dilution of Matrigel, add 1 mL of Matrigel into a 50 mL tube containing 29 mL of chilled DMEM/F-12. Make 1 mL aliquots and store them at

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Figure 5. Immunofluorescence staining and electron micrograph images of human podocytes derived from iPSCs

(A) Immunostaining of derived podocytes showing expression of podocyte lineage characterization markers WT1, MAFB, Synaptopodin, PODX, Podocin, Nephrin, and F actin for cytoskeleton. Scale bar is 25 µm.

(B) Scanning electron microscopy images of iPSC podocytes showing cell bodies with cytoplasmic projections extending to adjacent cells (white arrows). Scale bar is 25 µm.

(C) TEM images of the iPSC-derived podocytes showing tight junction-like structures between adjacent cell types (black arrows). Scale bar is 400 nm.
–20°C until ready to use. The aliquots should be valid until the expiration date on the sample. Add the 1 mL aliquot to each well of a 6-well plate and keep it at 37°C for at least 1 h to allow the Matrigel to coat the surface of the well. Use the coated plate within 48 h, storing at 4°C.

△ CRITICAL: Handle Matrigel on ice as it solidifies when warmed over 16°C. The pipette tips used for dilution should be pre-chilled by keeping the pipette tips at –20°C for 30 min before use.

Coated plates must be used within 48 h. Plates kept longer will dry out and the cells will not attach to the plates. Do not use dry plates.

**mTeSR medium**

Combine 400 mL of mTeSR basal medium, 100 mL of 5x supplement, and 5 mL of 100x Penicillin/Streptomycin to make the stock media. Aliquot this prepared media into 50 mL conical tubes and store at –20°C for up to six months. Thawed aliquots may be kept in the refrigerator at 4°C and used within two weeks.

**Y27632 (10 mM)**

Resuspend 1 mg of Y27632 in 312 µL of PBS (pH 7.2) or cell culture grade water to make a 10 mM stock. Prepare 50 µL aliquots and store at –20°C for up to six months. Avoid freeze-thaw cycles.

**Human Activin A (100 µg/mL)**

Centrifuge the tube briefly before opening. Reconstitute to 100 µg of Activin A in 1 mL of sterile cell culture grade water containing 0.1% BSA. Prepare 20 µL aliquots and store at –20°C for six months. Avoid freeze-thaw cycles.

**CHIR99021 (10 mM)**

Centrifuge the tube briefly before opening. Reconstitute the 2 mg vial by adding 430 µL of DMSO to make a 10 mM stock. Prepare 10 µL aliquots and store at –20°C or –80°C for up to 12 months. Avoid repeated freeze-thaw cycles. The reconstituted vial can be stored in 2°C–8°C for a week.

**FGF9 (200 µg/mL)**

Centrifuge the tube briefly before opening. Reconstitute to 50 µg in 250 µL of sterile cell culture grade water containing 0.1% BSA. Prepare 10 µL aliquots and store at –20°C or –80°C for up to three months. Avoid freeze-thaw cycles. The reconstituted vial can be stored in 2°C–8°C for one week.

**Heparin solution (180 USP/mL)**

Reconstitute in 180 USP of heparin in 1 mL sterile cell culture grade water and filter sterilize it through a polyethersulfone (PES) 0.22 µm syringe-driven filter unit to derive the 180 USP/mL heparin stock. Prepare 10 µL aliquots and store. Stocks are stable at 2°C–8°C for at least 12 months.

**Human BMP7 (100 µg/mL)**

Prepare the reconstitution solution by making a solution of 4 mM HCl containing 0.1% (wt/v) BSA and sterilize using a syringe-driven filter unit. To make the 100 µg/mL stock of BMP7, add 100 µL of reconstitution solution to 10 µg of BMP7. Prepare 10 µL aliquots and store at –20°C or –80°C for up to three months. Avoid freeze-thaw cycles. The reconstituted vial can be stored in 2°C–8°C for one month.

**Human VEGF (50 µg/mL)**

Reconstitute 50 µg in 1 mL sterile cell culture grade water to make 50 µg/mL stocks. Prepare 10 µL aliquots and store at –20°C or –80°C for up to 12 months. Avoid repeated freeze-thaw cycles. The reconstituted vial can be stored at 2°C–8°C for one week.
All-trans retinoic acid
To prepare a 100 µM stock solution, resuspend 100 µg of all-trans retinoic acid in 3.33 mL of sterile DMSO. Prepare the stock solution fresh before use, or aliquot into working volumes and store at –20°C for up to 1 year. Avoid repeated freeze-thaw cycles.

Paraformaldehyde (PFA) (4%)
Inside a fume hood, carefully break the glass vial containing 10 mL of a 16% PFA solution. Add the 10 mL of 16% PFA to a 50 mL conical vial. Add 4 mL of 10xPBS and 26 mL of deionized water to make 40 mL of a 4% (wt/vol) paraformaldehyde in PBS solution. The 4% PFA solution can be stored at room temperature (20°C–22°C) for 1–2 weeks or at 4°C for 3 weeks. For long-term storage, aliquot and keep at ~20°C for up to a year.

△ CRITICAL: Paraformaldehyde is toxic and must be handled inside a fume hood. Personnel should wear the appropriate personal protective equipment such as gloves, a lab coat, a face mask, and goggles.

1X Phosphate-Buffered Saline, 30 mg/mL BSA, 0.1% Tween 20 detergent (PBS-BT)
To prepare PBS-BT, add 6 g BSA to 20 mL 10xPBS. To this, add 2 mL of a 10% solution of Tween 20 and 2 mL of a 2% solution of sodium azide. Add sterile deionized water to bring the total volume of the solution to 200 mL. Filter sterilize and store at 4°C for 1 week.

△ CRITICAL: Sodium azide is a toxic preservative and must be handled inside a fume hood. Personnel should wear the appropriate personal protective equipment such as gloves, a lab coat, a face mask, and goggles.

2% bovine serum albumin (BSA)
To prepare 2% BSA in PBS, dissolve 0.8 g of BSA in 40 mL of 1x PBS.

5% bovine serum albumin (BSA)
To prepare 5% BSA in PBS, dissolve 2.0 g of BSA in 40 mL of 1x PBS.

4',6-diamidino-2-phenylindole (DAPI) stock solution
Dissolve 5 mg of DAPI in 250 mL of sterile deionized water. Solution should be kept at 4°C and remains stable for up to 3 weeks. Alternatively, 20 µL aliquots can be stored at ~20°C up to 1 year.

Laminin-511-coated plates
Add 9.6 µL of iMatrix-511 to 1.99 mL of PBS. To coat one well of a 12-well plate add 1 mL of the diluted iMatrix-511 solution. Incubate the plate for 1 h at 37°C, 3 h at room temperature, or 24 h at 4°C. Use the plate within 24 h and do not let the plate dry. Parafilm may be used to seal the plate, but is not typically necessary. Aspirate the laminin-511 coating solution before adding cells.

iPSC passage media
iPSC passage media consists of mTeSR medium supplemented with 10 µM Y27632. For example, add 3 µL of 10 mM Y27632 to 3 mL of mTeSR medium. iPSC passage medium should be prepared fresh before each use.

iPSC cryopreservation media
iPSC cryopreservation media consists of 90% FBS and 10% DMSO. Alternatively, commercially available cryopreservation media such as Cryostor can be used.
**Podocyte culture base media**
The base media consists of DMEM/F12 with GlutaMax containing 1x B27 serum-free supplement and 1% (v/v) of penicillin-streptomycin. Once prepared, podocyte culture base media can be stored at 4°C up to 1 month.

DMEM/F12 with GlutaMax can be stored at 2°C–8°C for up to 12 months. The B27 serum-free supplement can be stored at –20°C for up to 12 months. Penicillin-streptomycin can be stored at –20°C for up to 12 months.

**Primitive streak induction media**
The mesoderm induction medium can be prepared by adding 100 ng/mL Activin A and 3 μM CHIR99021 to the podocyte culture base medium. Once prepared, primitive streak medium can be stored at 4°C for up to 1 week.

**Intermediate mesoderm induction media**
The mesoderm induction medium can be prepared by adding 8 μM CHIR99021 to the base media. Once prepared, intermediate mesoderm induction medium can be stored at 4°C for a week.

**Nephron progenitor induction medium**
The intermediate mesoderm induction medium can be prepared by adding 200 ng/mL FGF9 and 0.180 USP/mL Heparin to the base media. Once prepared, nephron progenitor induction medium can be stored at 4°C for one week.

**Podocyte induction media**
The podocyte media can be prepared by adding 100 ng/mL BMP7, 100 ng/mL Activin A, 50 ng/mL VEGF, 3 μM CHIR99021, and 0.1 μM all-trans retinoic acid to the base media. Once prepared, podocyte induction media can be stored at 4°C for one week.

| Reagent                              | Final concentration | Amount  |
|--------------------------------------|---------------------|---------|
| DMEM/F12 with GlutaMAX supplement    | n/a                 | 4.894 mL|
| B27                                  | 1X                  | 50 μL   |
| Penicillin-Streptomycin              | 1X                  | 50 μL   |
| Activin A                            | 100 ng/mL           | 5 μL    |
| CHIR99021                            | 3 μM                | 1.5 μL  |
| **Total**                            | n/a                 | 5 mL    |

| Reagent                              | Final concentration | Amount  |
|--------------------------------------|---------------------|---------|
| DMEM/F12 with GlutaMAX supplement    | n/a                 | 4.896 mL|
| B27                                  | 1X                  | 50 μL   |
| Penicillin-Streptomycin              | 1X                  | 50 μL   |
| CHIR99021                            | 8 μM                | 4 μL    |
| **Total**                            | n/a                 | 5 mL    |

| Reagent                              | Final concentration | Amount  |
|--------------------------------------|---------------------|---------|
| DMEM/F12 with GlutaMAX supplement    | n/a                 | 4.890 mL|
| B27                                  | 1X                  | 50 μL   |
| Penicillin-Streptomycin              | 1X                  | 50 μL   |
| FGF9                                 | 200 ng/mL           | 5 μL    |
| Heparin                              | 0.180 USP/mL        | 5 μL    |
| **Total**                            | n/a                 | 5 mL    |
Use the suggested amount of media per well of the plate following the manufacturer’s instructions. Add e.g., 2 mL of media per well of a 12-well plate.

**STEP-BY-STEP METHOD DETAILS**

**Growing human iPSC in feeder-free culture with mTeSR medium**

**Note:** The timing for human iPSC thawing is around 30 min.

1. Prepare Geltrex or Matrigel coated plates (see materials and equipment).
2. Transfer 5 mL of warmed mTeSR media (see materials and equipment) into a 15 mL tube.
3. Thaw a frozen vial of hiPSC containing at least 1.5–2.0 $10^6$ cells in a 37°C water bath with gentle shaking until the ice starts to melt.
4. Transfer hiPSCs in a drop-wise manner into the 15 mL conical tube containing 5 ml prewarmed media (iPSC passage media). Gently rotate the tube to mix the cells with the media. Centrifuge the cells for 5 min at 300 g at room temperature.
5. Remove the supernatant and add 3 mL of fresh iPSC passage media (see materials and equipment).
6. Seed the cells onto plates coated with Geltrex or Matrigel i.e., 1.5 $10^6$ into one well of a 6-well plate. Make sure that cells are distributed evenly by rocking back and forth gently to distribute the cells and incubate at 37°C in a 5% CO₂ incubator.
7. After 24 h, change the iPSC passage media to regular mTeSR media.
8. Change the media every day.

△ CRITICAL: Changing media every day is very crucial since iPSCs proliferate quickly. Lack of nutrients in the exhausted media will lead to cell detachment and death.

**Note:** Different iPS cell lines are grown in different starting media such as StemFlex or Essential 8. We have used StemFlex and Essential 8 in substitution for mTeSR for cell lines requiring these media types. Cells should be approximately 70%–80% confluent after 7 days of culture. If cells do not reach this confluency, allow them to grow until they reach confluency. The cells can be cryopreserved or differentiated when they reach confluency. Follow the instructions below for cryopreservation or differentiation.

### Human iPSC passage

**Note:** Timing: 30 min

9. Prepare Geltrex or Matrigel coated plates (see materials and equipment).
10. Remove the media from the hiPSCs in the 6-well plate.
1. Wash the cells with 3 mL of PBS three times. Aspirate PBS.
2. Add 1 mL per well of Accutase to cells and incubate at 37°C for 5 min.
3. Add 2 mL per well of warm mTeSR to the cells, mix, and ensure cells have lifted off from the plastic surface.

△ CRITICAL: Do not pipette cells more than twice as hiPSCs are very sensitive to mechanical perturbation.

4. Collect cells in a 15 mL tube. Count the cell number using a hemocytometer.

△ CRITICAL: Automatic cell counters should be validated with a hemocytometer before use. Some commercial machines do not accurately count iPSC due to their unique shape.

5. Calculate required volume to achieve 1.5 × 10^6 iPSC per well of the 6-well plate. Aliquot the iPSC to be plated into a 15 mL tube and centrifuge at 300 × g for 5 min.
6. Remove the supernatant and gently resuspend the cells in iPSC passage media (see materials and equipment).
7. Seed the cells onto the Geltrex or Matrigel coated plates. Make sure that cells are distributed evenly by gently rocking back and forth and incubate at 37°C in a 5% CO₂ incubator for 24 h.
8. The iPSC will attach and grow colonies and will be 20%–30% confluent on Day 1. If the cells do not attach and grow after 24 h, do not attempt to use the cells for differentiation. Lower cell density leads to spontaneous differentiation of iPSCs.

**Human iPSC cryopreservation**

⊙ Timing: 30 min

9. Rinse the cells 3 times with PBS and add 1 mL of Accutase per 6-well plate.
10. Incubate the cells at 37°C and 5% CO₂ for 5 min or until the cells start to detach. Incubate for 2–3 min more if the cells do not start to detach.

△ CRITICAL: Do not overdigest the cells with Accutase, as this will result in cell death (Figure 6).

11. Add 3 mL of fresh mTeSR media to the dissociated cells, mix, and ensure cells have lifted off from the plastic surface.

**Figure 6.** Brightfield images of human iPSC after Accutase treatment

(A) Prolonged Accutase treatment results in over-digestion resulting in single cells with more debris (white arrows) when resuspended.

(B) Cells treated for the optimal time are in clumps (black arrows) after resuspension. Scale bar is 200 μm.
CRITICAL: Do not pipette cells more than twice as hiPSCs are very sensitive to mechanical perturbation.

22. Collect cells in a 15 mL tube. Count the cell number using a hemocytometer.
23. Centrifuge the cells for 5 min at 300 × g at room temperature.
24. Remove the supernatant and gently resuspend 2 × 10⁶ cells per mL of a cryopreservation medium consisting of 90% FBS and 10% DMSO. Alternatively, commercially available cryopreservation media such as Cryostor can be used.
25. Add 1 mL of cell suspension per cryopreservation tube.
26. Place the tubes into a cryovial freezer box and freeze at −80°C for 24 h.
27. Transfer the tubes to a liquid nitrogen cell storage tank for long-term cryopreservation.

Note: To start a live culture from a frozen vial, follow steps 1–8.

**hiPSC plating for differentiation**

© Timing: ~1 day, day 0

28. Follow steps from 9–14.

Note: Earlier passage cells (P1–P25) are better for differentiation. If the morphology looks different while passaging, check for pluripotency before beginning differentiation.

29. Calculate required cell volume to achieve 1 × 10⁵ iPSC per well of the 12-well plate.
30. Aliquot the iPSC to be plated to a 15 mL tube and centrifuge at 300 × g for 5 min.
31. Remove the supernatant and gently resuspend the cells in iPSC passage media (see materials and equipment).
32. Seed the cells on the Geltrex/Matrigel plates. Make sure that cells are distributed evenly by rocking back and forth and incubate at 37°C in a 5% CO₂ incubator for 24 h.
33. iPSC cells will attach and grow colonies and will be 30%–40% confluent on Day 1 (Figure 2). If the cells have not reached the desired confluency, they may grow for one additional day prior to proceeding.

**Differentiation of human iPSCs into the posterior primitive streak**

© Timing: 2 days, day 1–2

34. Prepare primitive streak induction media (see materials and equipment).
35. Aspirate the iPSC passage media from cells and add 2 mL primitive streak media per well of a 12-well plate.
36. Culture the cells in primitive streak media for 2 days without changing the media.
37. Primitive streak cells have a triangular shape morphology are 40%–50% confluent (Figure 2).

**Induction of intermediate mesoderm**

© Timing: 3 days, day 3–5

38. Prepare intermediate mesoderm induction media (see materials and equipment).
39. Aspirate the primitive streak media from the cells and add 2 mL per 12-well plate intermediate mesoderm media.
40. Refresh medium every day and let the induction of mesoderm continue until day 5.
41. The intermediate mesoderm cells will start to proliferate and reach 55%–60% confluency (Figure 2).
Critical: Since the cells start to proliferate, the rate of consumption of growth factors and supplements will increase. Make sure to add sufficient media (i.e., 2 mL for each well of a 12-well plate), since the lack of nutrients will lead to cell death.

**Induction of nephron progenitor cells**

- **Timing:** 2 days, day 6–7

42. Prepare nephron progenitor differentiation medium (see materials and equipment).
43. Aspirate the medium from the intermediate mesoderm cells and incubate with 2 mL per 12-well plate of nephron progenitor medium.
44. Refresh medium daily.
45. Culture cells in nephron progenitor medium for 2 days.
46. The intermediate mesoderm cells will start to form clumps and will have 60%–70% confluency (Figure 2).

Critical: As the cells start to proliferate, the rate of consumption of growth factors and supplements will increase. Make sure to add sufficient nephron progenitor media, since the lack of nutrients will lead to cell death.

**Note:** FGF9 concentration below 200 ng/mL may result in an inefficient induction of the nephron progenitors.

**Derivation of mature kidney podocytes on laminin-coated plates**

- **Timing:** 5 days, day 8–12

47. Prepare laminin-511-coated plates (see materials and equipment).
48. Prepare podocyte induction medium (see materials and equipment).
49. Remove the intermediate mesoderm differentiation medium from cells and wash cells one time with PBS.
50. Add Accutase (0.5 mL per well for 12-well plates) to cells and incubate for 5 min at 37°C.
51. Visualize cells under the microscope to ensure the cells are properly dissociated into either individual cells or small clumps.

Critical: It is important to avoid bigger clumps since it leads to poor podocyte differentiation.

52. Pipette the cells gently 3 times and transfer the cells to a 15 mL tube. Add fresh nephron progenitor media to the tube to quench the Accutase.
53. Centrifuge cells at 300 × g for 5 min and aspirate the supernatant. Resuspend the cells with podocyte induction media at a 1:4 dilution ratio to plate 2.5 × 10^5 cells per well of a laminin-511-coated 12-well plate.
54. Change the podocyte induction media daily.
55. The derived podocytes are characterized by a large cell body and arborized morphology (Figure 2). The iPSC-derived podocytes may be used for further studies at this point.

**Endpoint analysis of iPSC-derived podocytes by immunostaining**

- **Timing:** 2 days, day 13

56. Remove the media from the podocytes and wash the cells with PBS twice.
57. Fix the cells in 4% paraformaldehyde (PFA) in PBS by adding 1 mL per well of a 12-well plate for 15 min. Then wash the cells with PBS twice.

58. Block the samples by adding 1 mL per well of 12-well plate in PBS-BT for 30 min.

59. Stain with the desired primary antibodies diluted in 2% BSA for either 4 h at room temperature (RT) or 24 h at 4°C (Table 1).

60. Wash the cells with 500 μL PBS per well of 12-well plate twice by aspirating and adding fresh PBS. Incubate the cells with the corresponding secondary antibody (Table 1).

61. Stain the nuclei with DAPI diluted 1:1000 in PBS for 5 min and visualize using a fluorescent microscope.

62. For F-actin staining, fixed cells may be stained with phalloidin and DAPI instead (Figure 5).

### Endpoint analysis of iPSC-derived podocytes by flow cytometry analysis

© Timing: 1–2 days, day 13

63. Remove the media from the podocytes and wash the cells with PBS twice.

64. Add 1 mL of 0.05% trypsin per well of 12-well plates and incubate at 37°C for 5 min.

65. Add 2 mL of warm DMEM/F12 to the cells, mix, and ensure cells have lifted off from the plastic surface.

66. Collect cells in a 15 mL tube and centrifuge at 300 × g for 5 min.

67. Wash the cells with 1 mL of PBS twice. Aspirate PBS.

68. Fix the cells in 1 mL of 4% paraformaldehyde (PFA) in PBS for 15 min. Then wash the cells with 500 μL of 2% BSA twice by centrifugation at 400 × g for 2 min after each wash. Remove the supernatant.

69. For intracellular staining, permeabilize the cells with 100% cold ethanol for 10 min at 4°C, then centrifugate at 400 × g for 2 min. Remove the supernatant and wash the cells with 2% BSA for twice with centrifugation. Remove the supernatant.

70. Block the samples by adding 1 mL of 5% BSA in PBS for 30 min, then centrifugate briefly at 400 × g for 2 min. Remove the supernatant and wash the cells with 2% BSA for twice with centrifugation. Remove the supernatant.

71. Depending on the secondary antibodies being used, separate the desired isotope controls. These controls are samples that will only be treated with secondary antibody and no primary antibody.

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**Table 1. List of antibodies and dilutions**

| Cell type             | Primary antibody | Origin/Isotype | Dilution |
|-----------------------|------------------|----------------|----------|
| Undifferentiated      | OCT-4            | Rat IgG2b      | 1:200    |
| Primitive streak      | MIXL1            | Rabbit IgG     | 1:200    |
| Intermediate mesoderm | PAX8             | Rabbit IgG     | 1:200    |
|                       | GATA3            | Goat IgG       | 1:200    |
| Nephrin Progenitors   | CITED1           | Mouse IgG2a    | 1:200    |
|                       | SIX2             | Rabbit IgG     | 1:200    |
| Podocyte              | MAFB             | Rabbit IgG     | 1:200    |
|                       | PODOX            | Rabbit IgG     | 1:200    |
|                       | Nephrin          | Sheep IgG      | 1:200    |
|                       | Synaptopodin     | Rabbit IgG     | 1:200    |
| Pathway               | F-actin          | Phalloidin 594 | 1:100    |
| Secondary             | Alexa 488, goat anti-mouse IgG | 1:200 |
|                       | Alexa 488, goat anti-rat IgG | 1:400 |
|                       | Alexa 488, donkey anti-goat IgG | 1:400 |
|                       | Alexa 594, goat anti-rabbit IgG | 1:400 |
|                       | Alexa 594, donkey anti-sheep IgG | 1:400 |
72. Stain with the desired primary antibodies (200 μL) diluted in 2% BSA for either 4 h at RT or 24 h at 4°C (Table 1).
73. Wash with 2% BSA and aspirate the supernatant. Incubate the cells with 200 μL of the corresponding secondary antibody diluted in 2% BSA for 2 h at RT or for 24 h at 4°C (Table 1). Wash with PBS twice and resuspend the cells in 200 μL PBS.
74. Analyze the cells using a flow cytometer and compare to the negative isotype controls using FlowJo software (Figure 3C).

Endpoint analysis of iPSC-derived podocytes by FITC albumin uptake assay

© Timing: 1 day, day 13
75. Remove the media from the podocytes and incubate with podocyte induction medium for 24 h.
76. Wash the cells with PBS two times by aspirating and replacing with fresh PBS.
77. To evaluate the temperature-dependent endocytosis of albumin in podocytes add 50 μg/mL FITC-conjugated bovine serum for either 1 h at 4°C or 37°C for 24 h to the podocytes.
78. Visualize endocytosis with a fluorescent microscope.

EXPECTED OUTCOMES

This protocol allows the serum-free production of functional podocytes from human iPSC in a fast and efficient manner. The differentiation process begins with the generation of primitive streak cells from iPSC via two days of exposure to activin A and a low dose of Wnt signaling activator CHIR (3 μM). The cells at this stage have a spiky, triangular morphology (Figure 2). Subsequent treatment of these cells with high-dose CHIR (8 μM) for 3 days produces intermediate mesoderm (Figure 3). Treatment of the intermediate mesoderm cells with FGF9 for two days permits the generation of nephron progenitors (Figure 3). Finally, podocytes are derived from nephron progenitor cells through incubation in podocyte induction media containing VEGF for the survival of podocytes, retinoic acid to support the generation of glomerular transition cells, and BMP7 for differentiation and survival.

Higher magnification brightfield images of the derived podocytes show the main body surrounded by structures resembling elongated foot processes (Figure 4A). Additionally, flow cytometry analysis of the podocytes revealed high expression of markers MAFB (77%) and PODX (58%) indicating efficient differentiation (n=3) (Figure 4B). To further confirm the reproducibility of the protocol, we used a MAFB:mTagBFP2/GATA3:mCherry blue fluorescent protein and mCherry iPSC cell line (Vanslambrouck et al., 2019). The differentiated podocytes from this line exhibited the same arborized morphology (Figure 4C). Flow cytometry analysis found that 37% the cells from this line were BFP+ and 57% cells were MAFB+ (Figure 4D). The difference in the expression of MAFB suggests that this cell line may require further optimization for efficient differentiation.

To confirm the phenotype we performed electron microscopy on the derived podocytes. The cells at day 7 were plated onto coverslips and grown until day 12 and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate for SEM imaging. SEM analysis confirmed the arborized structure with thin processes extending to the adjacent cells (Figure 5B). TEM imaging was performed on 50%–60% confluent cells at day 12 grown in a 60 mm dish that was fixed with 2.5% glutaraldehyde in 0.1 M cacodylate. TEM images showed tight junctions present between the adjacent cells (Figure 5C). Immunostaining with podocyte markers was performed to evaluate the efficient differentiation of podocytes from iPSCs. The podocytes derived from iPSC stained positive for mature podocyte markers (Figure 5A). The functionality of the hiPSC-derived podocytes was evaluated by FITC-albumin uptake assay. The results showed that the iPSC-derived podocytes endocytosed albumin in intracellular vesicles at 37°C, whereas the cells at 4°C displayed inhibited endocytosis (Figure 7). Together, this protocol provides a feeder-free robust method for the derivation of podocytes from iPSCs.
in twelve days with an additional one week period after differentiation for experiments. The cells start to lose their proliferating efficiency and lose the structure after a week.

Applications
Our protocol recapitulates the natural process of podocyte development, resulting in a culture of cells expressing podocyte-specific markers beginning from human iPSC. The phenotype of the iPSC-derived podocytes is similar to those of terminally differentiated podocytes. Therefore, this protocol could be developed further in order to model podocytopathies, for toxicity screening, or to study podocyte biology. Diseases affecting podocytes include autoimmune disorders (Koffler et al., 1967; Radford et al., 1997; Wilson and Smith, 1972), bacterial endocarditis (Neugarten et al., 1984), HIV (Barisoni et al., 1999), Alport syndrome (Kashtan, 1998), and diabetic nephropathy (Sassy-Prigent et al., 2000). Starting iPSC of different genetic backgrounds could be derived from fibroblasts or other cells isolated from patients. Alternatively, CRISPR-mediated genetic mutation to generate mutant iPSC lines can model genetic kidney diseases (Freedman et al., 2015; Kim et al., 2017).

LIMITATIONS
Compared to existing methods, this protocol provides a comparable method for podocyte differentiation. Although some protocols report somewhat higher efficiency, they require prolonged culture time. Nevertheless, the podocytes derived have not matured to the level of the adult kidney, suggesting that further adjustments could improve maturation. Podocytes are the epithelial lining of Bowman’s capsule which filters the blood, but our protocol lacks the presence of the vasculature so this may limit the functional maturity of the iPSC-derived podocytes. It is possible that other iPSC lines may need optimization. For example, the concentration and duration of the growth factors may need modification to achieve maximal efficiency.

TROUBLESHOOTING
Problem 1
Cells do not grow well after thawing. Step 7

Potential solution
Unhealthy cells after thawing can result from unhealthy cells at cryopreservation (Figure 8). Only cryopreserve healthy cells or use a different cryopreservation reagent (CryoStor). Because this is a relatively common problem, it is recommended to freeze early passage cells on multiple days for backup purposes.
Problem 2
Cells detach during the intermediate mesoderm stage. Step 41

Potential solution
The reason for the cell detachment is either very high or very low cell density (Figure 9). Please follow the cell number mentioned in step 29 for plating.

Problem 3
No intermediate mesoderm marker expression at day 6. Step 41

Potential solution
Depending on the cell line used, the intermediate mesoderm induction will need to be adjusted. Optimize the number of days (2–4) and the concentration of CHIR for the cell line used. The reporter cell lines need different exposure time with lower concentration of CHIR for generating posterior intermediate mesoderm while generating kidney organoids (Vanslambrouck et al., 2019). The same principle may be also necessary for the podocyte differentiation protocol.

Problem 4
Nephron progenitors do not attach and differentiate into podocytes at Day 9. Step 55

Potential solution
Pipetting too vigorously while dissociating the progenitors will result in cell damage. Pipette gently while dissociating. Adding 10 μM Y27632 to the podocyte media for the first 24 h may help if gentle pipetting does not solve the problem.

Problem 5
Failure to visualize podocyte markers. Step 61

Potential solution
The reason for this is failed differentiation of the podocytes. Thaw another iPSC vial for the next protocol attempt and confirm the presence of nephron progenitor markers at day 7 by immunostaining. Negative staining for nephron progenitors at day 7 means that the iPS cells may have become aberrantly differentiated, preventing their directed differentiation into the desired podocyte cell type.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lauren Elizabeth Woodard, Ph.D. (Lauren.Woodard@vumc.org).
Materials availability
This study did not generate new unique reagents.

Data and code availability
Original data files are available from the corresponding author upon request.

ACKNOWLEDGMENTS
L.E.W. was supported by the Department of Veterans Affairs (BX004845), as well as a pilot and feasibility study from the Vanderbilt O’Brien Kidney Center (VCKD) (SP30DK114809-02). This material is the result of work supported with resources and use of facilities at the VA Tennessee Valley Healthcare System. Images were acquired with the resources of the Vanderbilt Cell Imaging Shared Resource, supported by NIH grants (CA68485, DK20593, DK58404, DK59637, and EY08126). Flow Cytometry experiments were performed in the VMC Flow Cytometry Shared Resource. The VMC Flow Cytometry Shared Resource is supported by the Vanderbilt Ingram Cancer Center (IP30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404). The project described was supported by CTSA award (UL1 TR002243) from the National Center for Advancing Translational Sciences to the Vanderbilt Institute for Clinical and Translational Research. Its contents are solely the responsibility of the authors and do not necessarily represent official views of the National Center for Advancing Translational Sciences or the National Institutes of Health. We thank Dr. Melissa Little, NIH (Re)Building a Kidney, and Dr. Sanjay Jain of Washington University’s Kidney Translational Research Center for providing the MAFB:mTagBFP2/GATA3:mCherry iPSC line.

AUTHOR CONTRIBUTIONS
Conceptualization, methodology, validation, writing – original draft, and visualization, J.B; investigation, J.B. and E.S.Q.; resources, project administration, and funding acquisition, J.B. and L.E.W.; writing – review & editing and supervision, L.E.W.

DECLARATION OF INTERESTS
L.E.W. and J.B. are co-inventors on a provisional patent application for the podocyte differentiation protocol.

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