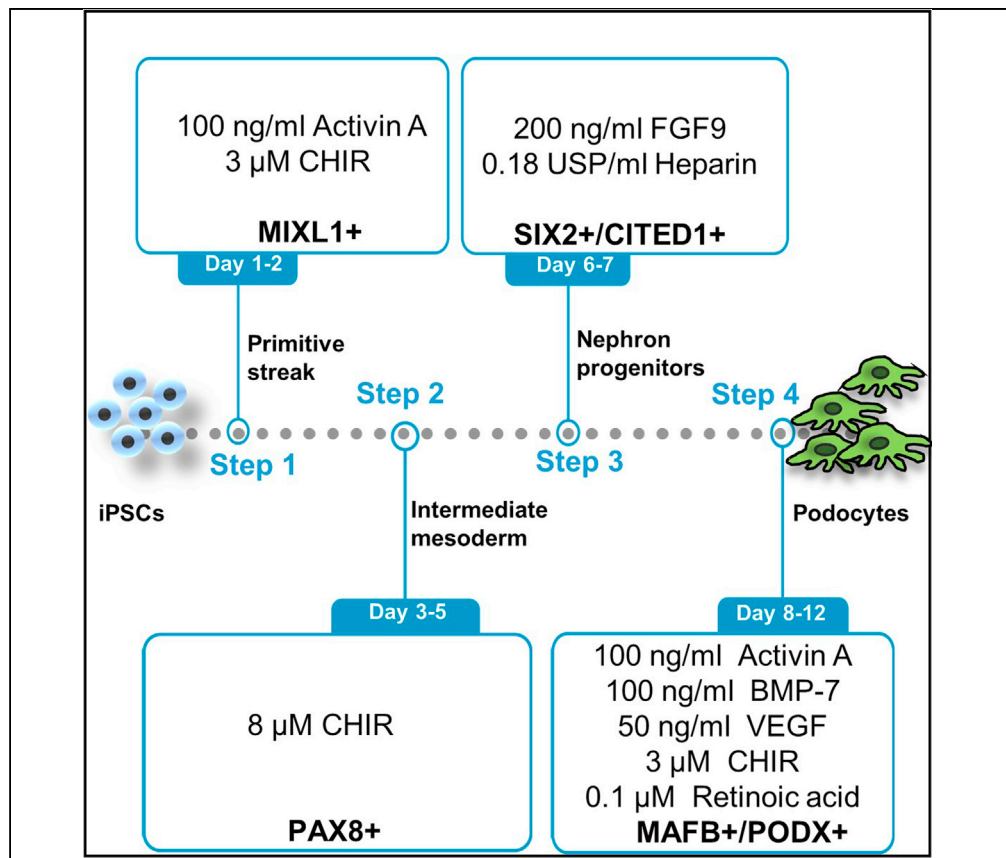# STAR Protocols

Protocol

Accelerated protocol for the differentiation of podocytes from human pluripotent stem cells

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Highlights

Specific steps with media formulations to differentiate podocytes from human iPSCs

The accelerated process mimics the phases of kidney development

Instructions for marker analysis of the defined cell types present at each stage

Validation of podocyte function includes marker analysis and FITC-albumin uptake

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.

Protocol

Accelerated protocol for the differentiation of podocytes from human pluripotent stem cells

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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
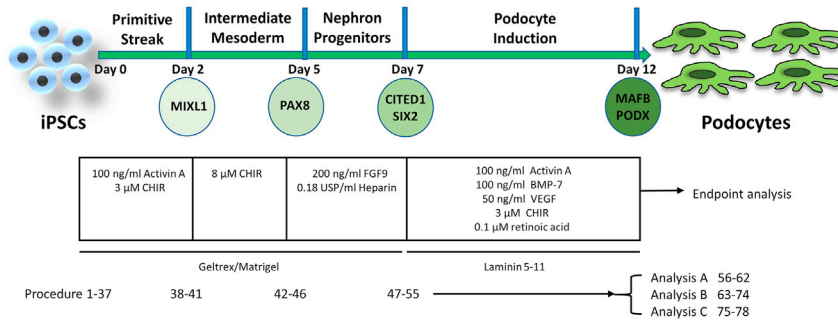
Figure 1. Schematic diagram of the protocol for generating podocytes from hiPSCs
The protocol begins with differentiation of hiPSC to primitive streak using Activin A and CHIR, followed by induction of mesoderm with CHIR alone. Then, intermediate mesoderm was induced using FGF9. As final step to differentiate to podocytes, the intermediate cells were treated with a combination of podocyte inducing factors including VEGF, retinoic acid, BMP-7, CHIR, and Activin A.

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 α- and β-subunits. Integrins β1 and αvβ5 are highly expressed in both human iPSC and in a human podocyte cell line (Musah et al., 2017). β1 integrins are essential for podocyte function *in vivo* (Kanasaki et al., 2008; Pozzi et al., 2008). Laminin 5-11 (LN-511), which consists of α5, β1, and γ1 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-β/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.,
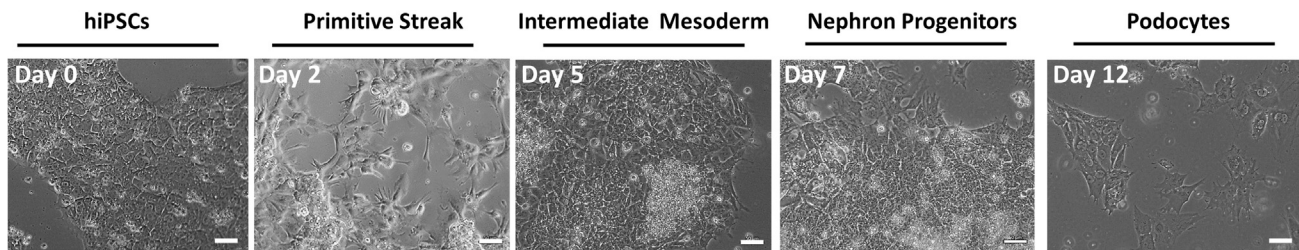
hiPSCs	Primitive Streak	Intermediate Mesoderm	Nephron Progenitors	Podocytes

Figure 2. Morphological changes during each stage of differentiation
Brightfield images show differentiation of human iPS cells into primitive streak cells at day 2, intermediate mesoderm cells at day 5, nephron progenitors at day 7 and differentiated podocytes at day 12. The derived podocytes have a main body surrounded by elongated foot processes. The scale bar is 50 μm.

2006; Mae et al., 2013). 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). To induce the
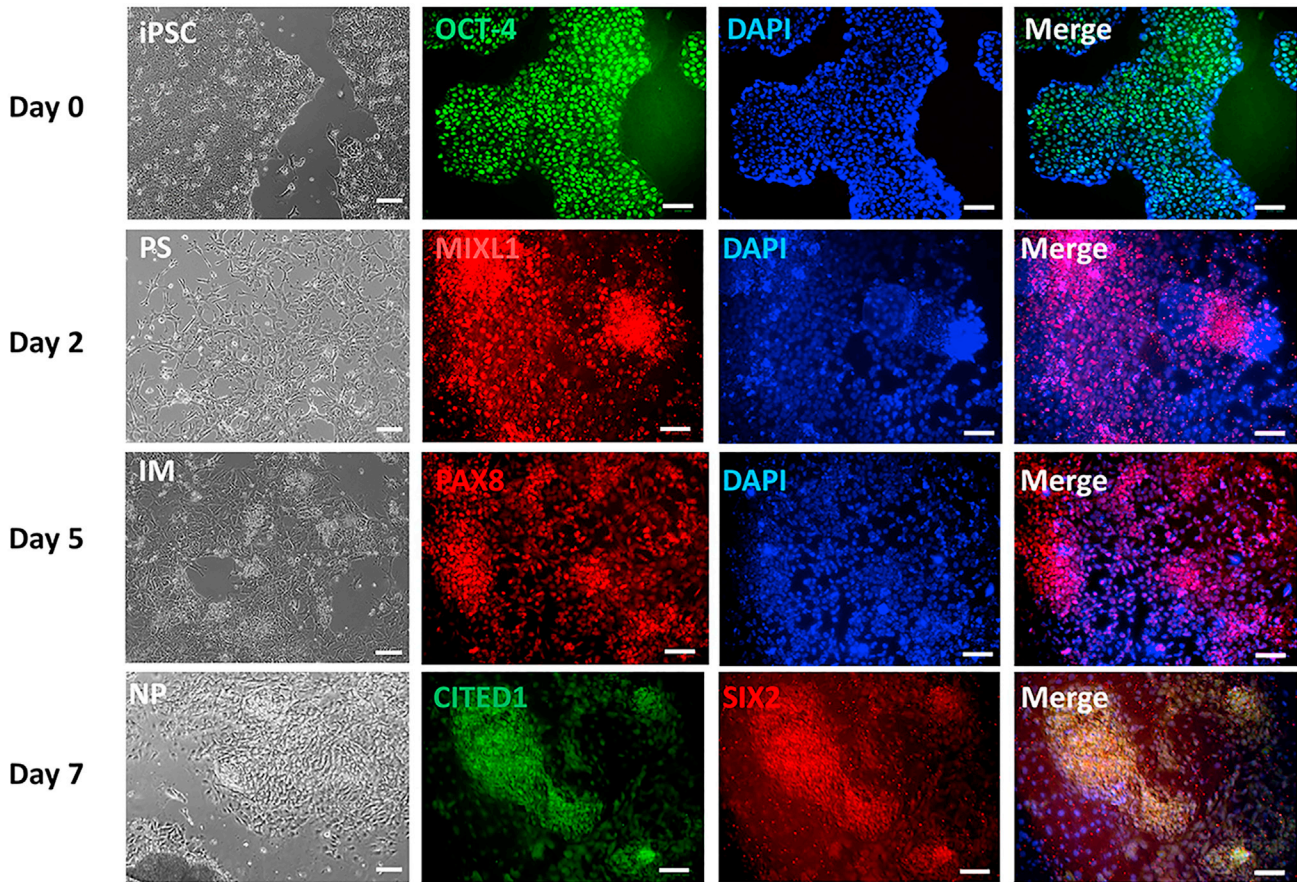
Figure 3. Brightfield and immunostaining images of the intermediate stages of podocyte differentiation: From human iPS cells to nephron progenitors

Undifferentiated human iPS cells stained positive for pluripotency marker OCT-4. iPSC differentiated into primitive streak cells at day 2 were positive for MIXL1. Day 5 intermediate mesoderm cells stained positive for PAX8. The nephron progenitors derived at day 7 stained positive for nephron progenitor markers CITED1 and SIX2. Scale bar is 100 μm. iPSC: induced pluripotent stem cells; PS: primitive streak; IM: intermediate mesoderm; NP: nephron progenitors.

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 α6ß1, 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).
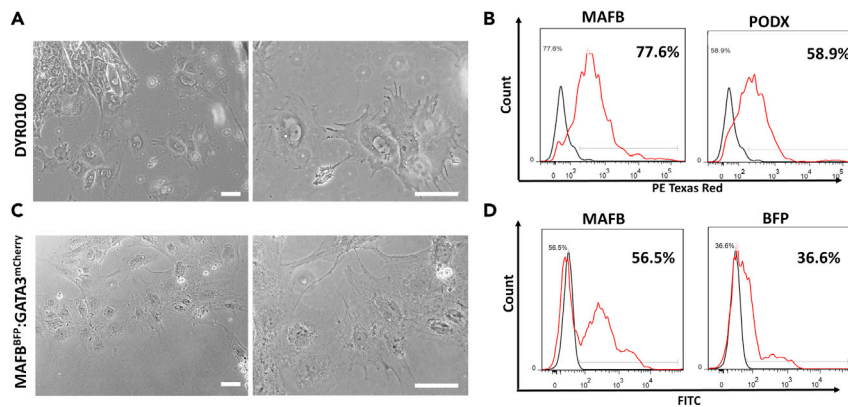
Figure 4. Characterization of podocyte cells derived from iPSCs

(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#18150-1-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#RL511
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

REAGENT or RESOURCE	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#21-040CV
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
Geltrex™, 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#50-980-487
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		
Human iPSC	ATCC	Cat#DYR0100
MAFB:mTagBFP2/GATA3:mCherry human iPSC	NIH RBK	https://www.rebuildingakidney.org/cell-lines/
Software and algorithms		
Image J	NIH	https://imagej.nih.gov/ij/
Flow Jo	BD Biosciences	https://www.flowjo.com/
Others		
Biological safety cabinet	NUAIRE, Class II Type A2	n/a
Water bath, 37°C	Fisher Scientific, Isotemp 2332	n/a
Benchtop centrifuge	Eppendrof, Centrifuge 5424	n/a
CO_2 incubators	NUAIRE	Cat#NU5500
Conical tubes (15,50 mL)	VWR	Cat#490001-621 Cat#490001-627
Cryogenic Storage Vials	Greiner Bio-One	Cat#122263
Cryovial Freezer	BioExpress	Cat#BCS-405O
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, QuantaTM 250 FEG	n/a
Flow Cytometer	BD, BD LSRFortessa	n/a
Media storage bottle	Fisher Scientific	Cat#07-201-600
Media storage bottle	Fisher Scientific	Cat#431531
Pipette Controller	Fisher Scientific	Cat#14-389-136TU
Pipette Set	Fisherbrand (P2, P10, P200 and P1000 Elite)	Cat#FBE00002 Cat#FBE00010 Cat#FBE00200 Cat#FBE10000
Serological pipettes	VWR	Cat#82050-482 Cat#82050-478
Steriflip 0.22 μm filter unit	Fisher Scientific	Cat#SCGP00525

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
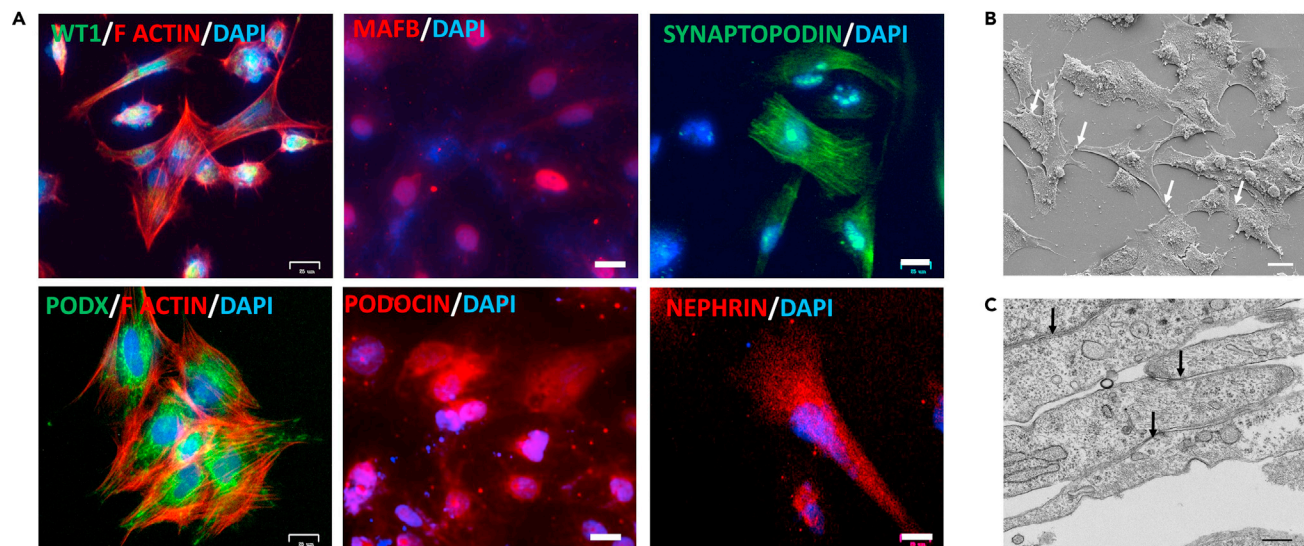

Figure 5. Immunofluorescence staining and electron micrograph images of human podocytes derived from iPSCs
(A) Immunostaining of derived podocytes showing expression of podocyte lineage characterization markers WT1, MAFB, Synaptopodin, PODX, Podocin, Nephrin, and F actin for cytoskeleton. Scale bar is 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.

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

Intermediate mesoderm induction media

The mesoderm induction media 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.

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

Nephron progenitor induction medium

The intermediate mesoderm induction media 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.

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

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	4.878 mL
B27	1X	50 μL
Penicillin-Streptomycin	1X	50 μL
CHIR99021	3 μM	1.5 μL
Activin A	100 ng/mL	5 μL
BMP-7	100 ng/mL	5 μL
VEGF	50 ng/mL	5 μL
all-trans retinoic acid	0.1 μM	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

⏱ Timing: ∼ 7 days

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% CO_2 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

⏱ 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.
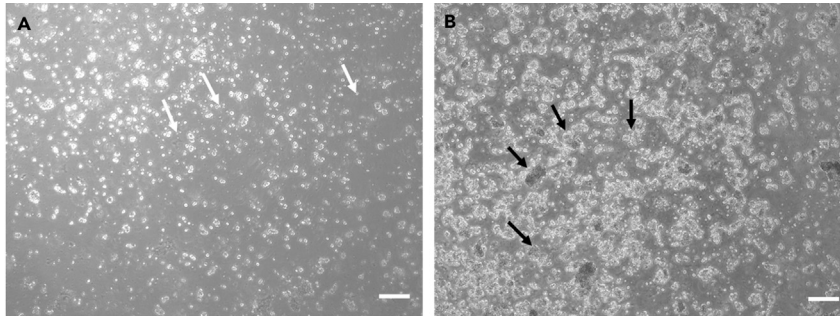
Figure 6. Brightfield images of human iPSC after Accutase treatment
(A) Prolonged Accutase treatment results in over-digestion resulting in single cells with more debris (white arrows) when resuspended.
(B) Cells treated for the optimal time are in clumps (black arrows) after resuspension. Scale bar is 200 μm.

11. Wash the cells with 3 mL of PBS three times. Aspirate PBS.
12. Add 1 mL per well of Accutase to cells and incubate at 37°C for 5 min.
13. 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.

14. 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.

15. 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 $300 \times g$ for 5 min.
16. Remove the supernatant and gently resuspend the cells in iPSC passage media (see materials and equipment).
17. 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% CO_2 incubator for 24 h.
18. 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

19. Rinse the cells 3 times with PBS and add 1 mL of Accutase per 6-well plate.
20. Incubate the cells at 37°C and 5% CO_2 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).

21. Add 3 mL of fresh mTeSR media to the dissociated 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.

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 2×10^6 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 1×10^5 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% CO_2 incubator for 24 h.
33. iPS 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.

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
Nephron 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 IgGa		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

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 centrifuge 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 centrifuge 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 isotype controls. These controls are samples that will only be treated with secondary antibody and no primary antibody.

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 endocytose albumin in a temperature-dependent manner. 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
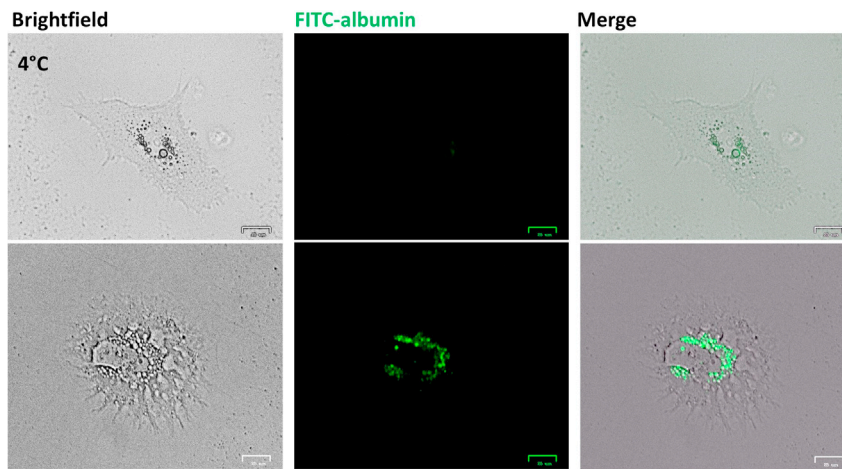
Figure 7. Functional validation of iPSC-derived podocytes

iPSC-derived podocytes were incubated with FITC-albumin for 1 h at 4°C showing inhibition of endocytosis whereas 24 h at 37°C showing endocytosis of the labeled albumin. Scale bar is 25 μm.

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.
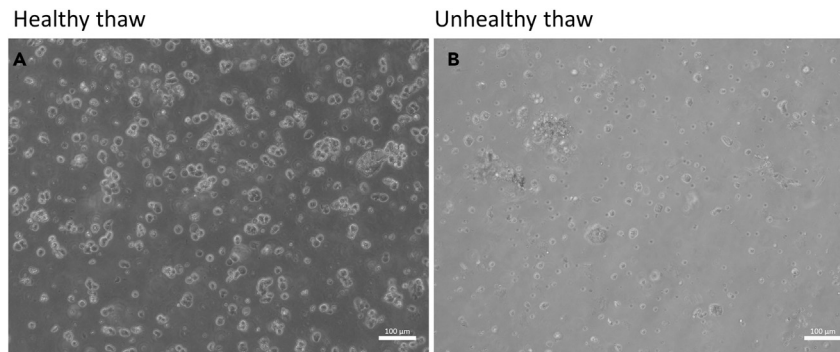
Figure 8. Brightfield images of a healthy vs unhealthy thaw of iPSC
A desirable healthy thaw (A) has cells with a round morphology having clear edges whereas a thaw of unhealthy iPS cells (B) have mostly debris. Scale bar is 100 μm.

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).
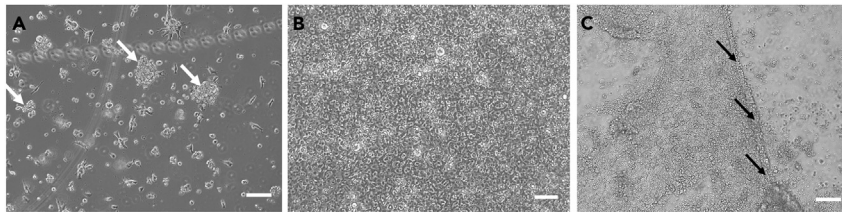
Figure 9. Undesirable plating densities of human iPSC at day 0

(A) iPSC plated at too low density will fail to differentiate. Cells plated such low density will detach (white arrows) and start to die following the primitive streak media treatment.

(B and C) (B) High density plating of iPSCs at day 0 will result in detachment of the cells (black arrows) after the primitive streak media treatment (C). Scale bar is 100 μm.

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.

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.

REFERENCES

Barak, H., Huh, S.H., Chen, S., Jeanpierre, C., Martinovic, J., Parisot, M., Bole-Feysot, C., Nitschké, P., Salomon, R., Antignac, C., et al. (2012). FGF9 and FGF20 maintain the stemness of nephron progenitors in mice and man. Dev. Cell 22, 1191–1207.

Barisoni, L., Kriz, W., Mundel, P., and D'Agati, V. (1999). The dysregulated podocyte phenotype: a novel concept in the pathogenesis of collapsing idiopathic focal segmental glomerulosclerosis and HIV-associated nephropathy. J. Am. Soc. Nephrol. 10, 51–61.

Bouchard, M., Souabni, A., Mandler, M., Neubüser, A., and Busslinger, M. (2002). Nephric lineage specification by Pax2 and Pax8. Genes Dev. 16, 2958–2970.

Ciampi, O., Iacone, R., Longaretti, L., Benedetti, V., Graf, M., Magnone, M.C., Patsch, C., Xinaris, C., Remuzzi, G., Benigni, A., et al. (2016). Generation of functional podocytes from human induced pluripotent stem cells. Stem Cell Res. 17, 130–139.

Colvin, J.S., Feldman, B., Nadeau, J.H., Goldfarb, M., and Ornitz, D.M. (1999). Genomic organization

and embryonic expression of the mouse fibroblast growth factor 9 gene. Dev. Dyn. 216, 72–88.

Dai, Y., Chen, A., Liu, R., Gu, L., Sharma, S., Cai, W., Salem, F., Salant, D.J., Pippin, J.W., Shankland, S.J., et al. (2017). Retinoic acid improves nephrotoxic serum-induced glomerulonephritis through activation of podocyte retinoic acid receptor α. Kidney Int. 92, 1444–1457.

Downs, K.M. (2009). The enigmatic primitive streak: prevailing notions and challenges concerning the body axis of mammals. BioEssays 31, 892–902.

Freedman, B.S., Brooks, C.R., Lam, A.Q., Fu, H., Morizane, R., Agrawal, V., Saad, A.F., Li, M.K., Hughes, M.R., Werff, R.V., et al. (2015). Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. Nat. Commun. 6, 8715.

Gadue, P., Huber, T.L., Paddison, P.J., and Keller, G.M. (2006). Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc. Natl. Acad. Sci. U S A 103, 16806–16811.

Harper, S.J., Xing, C.Y., Whittle, C., Parry, R., Gillatt, D., Peat, D., and Mathieson, P.W. (2001). Expression of neuropilin-1 by human glomerular epithelial cells in vitro and in vivo. J. Clin. Sci. 101, 439–446.

James, R.G., and Schultheiss, T.M. (2005). Bmp signaling promotes intermediate mesoderm gene expression in a dose-dependent, cell-autonomous and translation-dependent manner. Dev. Biol. 288, 113–125.

Kanasaki, K., Kanda, Y., Palmsten, K., Tanjore, H., Lee, S.B., Lebleu, V.S., Gattone, V.H., Jr., and Kalluri, R. (2008). Integrin beta1-mediated matrix assembly and signaling are critical for the normal development and function of the kidney glomerulus. Dev. Biol. 313, 584–593.

Kashtan, C.E. (1998). Alport syndrome and thin glomerular basement membrane disease. J. Am. Soc. Nephrol. 9, 1736–1750.

Kim, Y.K., Refaeli, I., Brooks, C.R., Jing, P., Gulieva, R.E., Hughes, M.R., Cruz, N.M., Liu, Y., Churchill, A.J., Wang, Y., et al. (2017). Gene-edited human kidney organoids reveal mechanisms of disease in podocyte development 35, 2366–2378.

Kobayashi, A., Valerius, M.T., Mugford, J.W., Carroll, T.J., Self, M., Oliver, G., and McMahon, A.P. (2008). Six2 defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development. Cell Stem Cell 3, 169–181.

Koffler, D., Schur, P.H., and Kunkel, H.G. (1967). Immunological studies concerning the nephritis of systemic lupus erythematosus. J. Exp. Med. 126, 607–624.

Kreuser, U., Buchert, J., Haase, A., Richter, W., and Diederichs, S. (2020). Initial WNT/β-Catenin activation enhanced mesoderm commitment, extracellular matrix expression, cell aggregation and cartilage tissue yield from induced pluripotent stem cells. Front. Cell Dev. Biol. 8, 581331.

Lindsley, R.C., Gill, J.G., Kyba, M., Murphy, T.L., and Murphy, K.M. (2006). Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. Development 133, 3787–3796.

Low, J.H., Li, P., Chew, E.G.Y., Zhou, B., Suzuki, K., Zhang, T., Lian, M.M., Liu, M., Aizawa, E., Rodriguez

Esteban, C., et al. (2019). Generation of human PSC-derived kidney organoids with patterned nephron segments and a de novo vascular network. Cell Stem Cell 25, 373–387.e9.

Mae, S.-I., Shono, A., Shiota, F., Yasuno, T., Kajiwara, M., Gotoda-Nishimura, N., Arai, S., Sato-Otubo, A., Toyoda, T., Takahashi, K., et al. (2013). Monitoring and robust induction of nephrogenic intermediate mesoderm from human pluripotent stem cells. Nat. Commun. 4, 1367.

Maier, J.I., Rogg, M., Helmstädter, M., Sammarco, A., Schilling, O., Sabass, B., Miner, J.H., Dengjel, J., Walz, G., Werner, M., et al. (2021). EPB41L5 controls podocyte extracellular matrix assembly by adhesome-dependent force transmission. Cell Rep. 34, 108883.

Mallipattu, S.K., and He, J.C. (2015). The beneficial role of retinoids in glomerular disease. Front Med. (Lausanne) 2, 16.

Mendelsohn, C. (2009). Using mouse models to understand normal and abnormal urogenital tract development. Organogenesis 5, 306–314.

Mitu, G.M., Wang, S., and Hirschberg, R. (2007). BMP7 is a podocyte survival factor and rescues podocytes from diabetic injury. Am. J. Physiol. Renal Physiol. 293, F1641–F1648.

Morizane, R., and Bonventre, J.V. (2017). Generation of nephron progenitor cells and kidney organoids from human pluripotent stem cells. Nat. Protoc. 12, 195–207.

Morizane, R., Lam, A.Q., Freedman, B.S., Kishi, S., Valerius, M.T., and Bonventre, J.V. (2015). Nephron organoids derived from human pluripotent stem cells model kidney development and injury. Nat. Biotechnol. 33, 1193–1200.

Mugford, J.W., Sipilä, P., McMahon, J.A., and McMahon, A.P. (2008). Osr1 expression demarcates a multi-potent population of intermediate mesoderm that undergoes progressive restriction to an Osr1-dependent nephron progenitor compartment within the mammalian kidney. Dev. Biol. 324, 88–98.

Musah, S., Mammoto, A., Ferrante, T.C., Jeanty, S.S.F., Hirano-Kobayashi, M., Mammoto, T., Roberts, K., Chung, S., Novak, R., Ingram, M., et al. (2017). Mature induced-pluripotent-stem-cell-derived human podocytes reconstitute kidney glomerular-capillary-wall function on a chip. Nat. Biomed. Eng. 1, 0069.

Neugarten, J., Gallo, G.R., and Baldwin, D.S. (1984). Glomerulonephritis in bacterial endocarditis. Am. J. Kidney Dis. 77, 297–304.

Pippin, J.W., Brinkkoetter, P.T., Cormack-Aboud, F.C., Durvasula, R.V., Hauser, P.V., Kowalewska, J., Krofft, R.D., Logar, C.M., Marshall, C.B., Ohse, T., et al. (2009). Inducible rodent models of acquired podocyte diseases. Am. J. Physiol. Renal Physiol. 296, F213–F229.

Pleniceanu, O., Omer, D., Harari-Steinberg, O., and Dekel, B. (2018). Renal lineage cells as a source for renal regeneration. Pediatr. Res. 83, 267–274.

Pozzi, A., Jarad, G., Moeckel, G.W., Coffa, S., Zhang, X., Gewin, L., Eremina, V., Hudson, B.G., Borza, D.B., Harris, R.C., et al. (2008). Beta1 integrin expression by podocytes is required to maintain glomerular structural integrity. Dev. Biol. 316, 288–301.

Qian, T., Hernday, S.E., Bao, X., Olson, W.R., Panzer, S.E., Shusta, E.V., and Palecek, S.P. (2019). Directed differentiation of human pluripotent stem cells to podocytes under defined conditions. Sci. Rep. 9, 2765.

Qian, T., Maguire, S.E., Canfield, S.G., Bao, X., Olson, W.R., Shusta, E.V., and Palecek, S.P.J.S.a. (2017). Directed differentiation of human pluripotent stem cells to blood-brain barrier endothelial cells. Sci. Adv. 3, e1701679.

Radford, M.G., Donadio, J.V., Bergstralh, E.J., and Grande, J.P. (1997). Predicting renal outcome in IgA nephropathy. J. Am. Soc. Nephrol. 8, 199–207.

Rauch, C., Feifel, E., Kern, G., Murphy, C., Meier, F., Parson, W., Beilmann, M., Jennings, P., Gstraunthaler, G., and Wilmes, A. (2018). Differentiation of human iPSCs into functional podocytes. PLoS One 13, e0203869.

Rodin, S., Domogatskaya, A., Ström, S., Hansson, E.M., Chien, K.R., Inzunza, J., Hovatta, O., and Tryggvason, K. (2010). Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat. Biotechnol. 28, 611–615.

Sassy-Prigent, C., Heudes, D., Mandet, C., Bélair, M.-F., Michel, O., Perdereau, B., Bariéty, J., and Bruneval, P. (2000). Early glomerular macrophage recruitment in streptozotocin-induced diabetic rats. Diabetes 49, 466–475.

Song, B., Smink, A.M., Jones, C.V., Callaghan, J.M., Firth, S.D., Bernard, C.A., Laslett, A.L., Kerr, P.G., and Ricardo, S. (2012). The directed differentiation of human iPS cells into kidney podocytes. PLoS One 7, e46453.

Spivak-Kroizman, T., Lemmon, M.A., Dikic, I., Ladbury, J.E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., and Lax, I. (1994). Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. Cell 79, 1015–1024.

Suleiman, H., Zhang, L., Roth, R., Heuser, J.E., Miner, J.H., Shaw, A.S., and Dani, A.J.E. (2013). Nanoscale protein architecture of the kidney glomerular basement membrane. Elife 2, e01149.

Taguchi, A., Kaku, Y., Ohmori, T., Sharmin, S., Ogawa, M., Sasaki, H., and Nishinakamura, R. (2014). Redefining the in vivo origin of metanephric nephron progenitors enables generation of complex kidney structures from pluripotent stem cells. Cell Stem Cell 14, 53–67.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S.J.c. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872.

Takasato, M., Er, P.X., Becroft, M., Vanslambrouck, J.M., Stanley, E.G., Elefanty, A.G., and Little, M.H. (2014). Directing human embryonic stem cell differentiation towards a renal lineage generates a self-organizing kidney. Nat. Cel. Biol. 16, 118–126.

Takasato, M., Er, P.X., Chiu, H.S., Maier, B., Baillie, G.J., Ferguson, C., Parton, R.G., Wolvetang, E.J., Roost, M.S., Chuva de Sousa Lopes, S.M., et al. (2015). Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. Nature 526, 564–568.

Tam, P.P., and Loebel, D.A. (2007). Gene function in mouse embryogenesis: get set for gastrulation. Nat. Rev. Genet. *8*, 368–381.

Vanslambrouck, J.M., Wilson, S.B., Tan, K.S., Soo, J.Y.-C., Scurr, M., Spijker, H.S., Starks, L.T., Neilson, A., Cui, X., Jain, S., et al. (2019). A toolbox to characterize human induced pluripotent stem cell–derived kidney cell types and organoids. J. Am. Soc. Nephrol. *30*, 1811–1823.

Vaughan, M.R., Pippin, J.W., Griffin, S.V., Krofft, R., Fleet, M., Haseley, L., and Shankland, S.J. (2005). ATRA induces podocyte differentiation and alters nephrin and podocin expression in vitro and in vivo. Kidney Int. *68*, 133–144.

Wilson, C.B., and Smith, R.C. (1972). Goodpasture's syndrome associated with influenza A2 virus infection. Ann. Intern. Med. *76*, 91–94.

Wilson, V., and Beddington, R.S. (1996). Cell fate and morphogenetic movement in the late mouse primitive streak. Mech. Dev. *55*, 79–89.