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Directed differentiation of human induced pluripotent stem cells into mature kidney podocytes and establishment of a Glomerulus Chip

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Protocols have been established to direct the differentiation of human induced pluripotent stem (iPS) cells into nephron progenitor cells and organoids containing many types of kidney cells, but it has been difficult to direct the differentiation of iPS cells to form specific types of mature human kidney cells with high yield. Here, we describe a detailed protocol for the directed differentiation of human iPS cells into mature, post-mitotic kidney glomerular podocytes with high (>90%) efficiency within 26 d and under chemically defined conditions, without genetic manipulations or subpopulation selection. We also describe how these iPS cell–derived podocytes may be induced to form within a microfluidic organ-on-a-chip (Organ Chip) culture device to build a human kidney Glomerulus Chip that mimics the structure and function of the kidney glomerular capillary wall in vitro within 35 d (starting with undifferentiated iPS cells). The podocyte differentiation protocol requires skills for culturing iPS cells, and the development of a Glomerulus Chip requires some experience with building and operating microfluidic cell culture systems. This method could be useful for applications in nephrotoxicity screening, therapeutic development, and regenerative medicine, as well as mechanistic study of kidney development and disease.

Introduction

Human pluripotent stem cells, which include embryonic stem (ES) cells[1] and iPS cells[2], have expanded the capabilities of cell culture models and their applications in tissue engineering, regenerative medicine, and disease modeling[3,4]. The ability of human iPS cells to self-renew indefinitely and differentiate into many cell types also makes them attractive for studying the mechanisms of organ development and function. However, development of defined methods for the generation of human kidney cells from iPS cells with specificity and high efficiency has remained elusive. By considering the roles of multiple factors within the cellular microenvironment—including the extracellular matrix (ECM) and soluble signaling factors—we developed a feeder-free and a serum-free protocol for efficient derivation of kidney glomerular podocytes from both human ES and iPS cells[5,6]. This protocol produces human iPS cell–derived podocytes with >90% efficiency, without genetic manipulations or subpopulation selection during the differentiation procedure, and the resulting cells express lineage-specific markers and exhibit morphological characteristics consistent with the mature phenotype. Additional characterization data based on a global transcriptomic analysis of the human iPS cell–derived podocytes is also presented. Finally, we describe an in vitro method for integrating the iPS cell–derived podocytes into one channel of a two-channel microfluidic Organ Chip device, along with human glomerular endothelial cells in the second channel, to recapitulate the specialized tissue–tissue interface and selective molecular filtration function of the kidney glomerulus.

Development of the protocol

There is mounting evidence that multiple factors within the cellular microenvironment can influence cell fate decisions. These include cell interactions with neighboring cells, soluble growth factors, and

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both mechanical and chemical interactions between cells and the ECM[7–15]. Our strategy was to first identify ECM molecules that could robustly support the adhesion of human iPS cells, as well as their differentiated derivatives, as they transition from the pluripotent state to the differentiated podocyte lineage. We examined the expression of cell surface integrin receptors on human iPS cells and an immortalized human podocyte cell line (PCL) (Supplementary Fig. 1 and Supplementary Methods). We found that the integrins β1 and $\alpha_v\beta_5$ were highly expressed on both human iPS cells and the PCL (Supplementary Fig. 1b–d). Given the importance of β1 integrins in glomerular development and podocyte function in vivo[16,17], we used the ECM proteins laminin-511 and laminin-511-E8 fragment for our differentiation studies, as these ligands exhibit strong binding affinity for this integrin receptor subtype[18,19].

We confirmed that human iPS cells cultured on the laminin-coated surfaces and treated with a medium containing activin A; the small molecule CHIR99021, which activates canonical Wnt signaling[20]; and the Rho-associated kinase (ROCK) inhibitor Y27632 (ref. [10]) can differentiate into mesoderm cells, as indicated by the expression of HAND1, goosecoid, and brachyury (Figs. 1–3). By treating the mesoderm cells with an intermediate mesoderm-inducing medium containing bone morphogenetic protein 7 (BMP7) and CHIR99021 (ref. [20]), we obtained cells that express the nephron progenitor cell markers Wilm's tumor 1 (WT1), odd-skipped related 1 (OSR1), and paired box gene 2 protein (Pax2). We optimized the timing of the intermediate mesoderm induction and found that all of the human iPS cell (PGP1, IMR-90-1, and IISH3i-CB6) and ES cell (H9) lines examined in this study express lineage-specific markers within 16 d of differentiation, and the resulting cells can be expanded in culture for 6–8 weeks or cryopreserved (Figs. 1 and 2, Boxes 1 and 2).

To induce differentiation into mature, terminally differentiated podocytes, we treated the intermediate mesoderm cells for 4–5 d with a novel medium consisting of BMP7, activin A, vascular endothelial growth factor (VEGF), retinoic acid, and CHIR99021 (refs [5,6]) (Fig. 1). Contrary to previous speculations based on the development of kidney organoids[21,22], and in accordance with in vivo studies on the development of glomerular capillaries[23–25], we found that induction of the mature differentiated podocyte phenotype does not require supplementation with fibroblast growth factors (FGFs) such as FGF2 or FGF9. By following our differentiation method (Figs. 1 and 2), we obtained cells that exhibit morphological, molecular, and functional characteristics of mature kidney glomerular podocytes[5], including the expression of nephrin, podocin, and WT1 proteins (Fig. 3c) and the podocyte-lineage-specification genes *SYNPO*, *PODXL*, *MAF*, and *EFNB2*, with a corresponding decrease in the expression of the nephron progenitor genes *PAX2*, *SALL1*, and *EYA1* and the

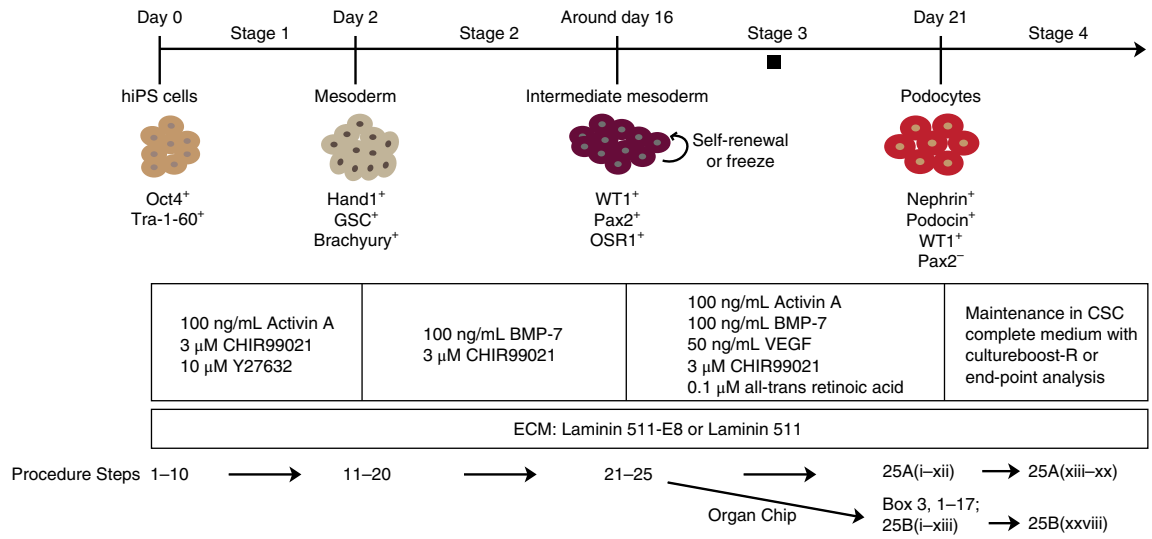

Fig. 1 | Schematic overview of the protocol for derivation of mature kidney glomerular podocytes from human iPS cells. The diagram shows sequential differentiation stages in the protocol. The concentrations of growth factors and signaling molecules are shown. In addition, the markers used to characterize the cells at each stage of the differentiation protocol are shown. Black square indicates a pause point, at which the protocol can be paused and the intermediate mesoderm cells can be stored by cryopreservation. BMP-7, bone morphogenetic protein 7; CSC, Cell Systems Corporation; ECM, extracellular matrix; GSC, goosecoid; hiPS cells, human induced pluripotent stem cells; OSR1, odd-skipped-related transcription factor 1; Pax2, paired box gene 2 protein; VEGF, vascular endothelial growth factor; WT1, Wilm's tumor 1. Adapted with permission from Musah et al.[5], Macmillan Publishers Limited.

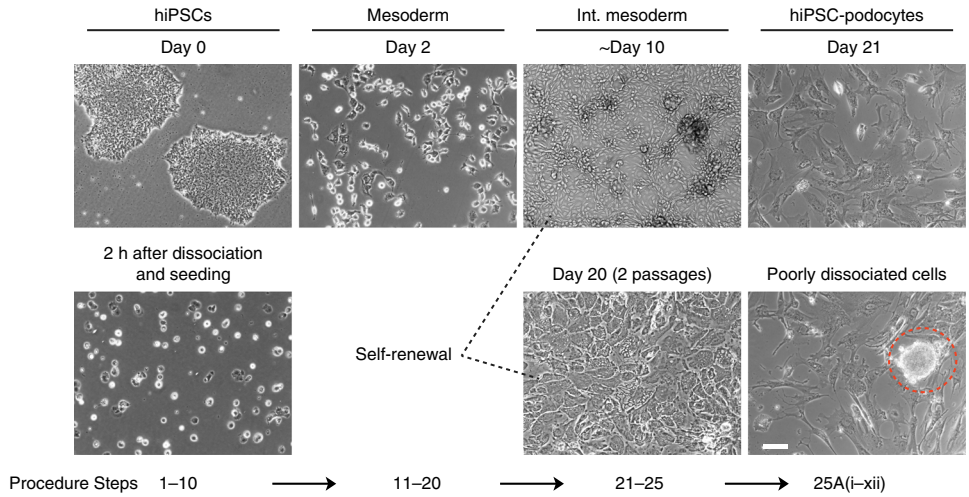

Fig. 2 | Morphological changes of human iPS cells at each stage of differentiation. Representative bright-field images show human iPS cells before and after dissociation on day 0 (when differentiation is initiated), mesoderm cells after 2 d of differentiation, intermediate mesoderm cells at ~10 d and after 20 d of culture with two passages, as well as the terminally differentiated podocytes. An example of an image of podocytes derived from a population of cells containing a poorly dissociated colony (dotted red circle) is also shown. hiPS-podocyte, human iPS cell–derived podocyte, Int. mesoderm, intermediate mesoderm. Scale bar, 100 μm.

pluripotency genes *POU5F1*, *SOX2*, *MYC*, and *NANOG* (Fig. 4). The human iPS cell–derived podocytes also developed primary and secondary foot processes[5] (Fig. 3d), similar to those seen in functional kidney glomerular podocytes in vivo[26]. After differentiation, the iPS cell–derived podocytes can be maintained in culture for up to 4 weeks in vitro using a commercially available medium (Complete Medium Kit with CultureBoost-R, Cell Systems) (Fig. 1).

To develop an in vitro model of the kidney glomerulus, we engineered a two-channel microfluidic Organ Chip device[27,28] that recapitulates the tissue–tissue structure, mechanochemical properties, and functional characteristics of a small portion of the human kidney glomerular capillary wall (Figs. 5 and 6, Box 3). The microfluidic device is made from a flexible poly(dimethylsiloxane) (PDMS) elastomer and it contains two parallel microchannels (1 × 1-mm top and 1 × 0.2-mm bottom channels) separated by a porous flexible PDMS membrane (50 μm thick, with 7-μm-diameter pores with 40-μm spacing), as previously described[5] (Fig. 5). We culture human iPS cell–derived intermediate mesoderm cells in the top channel and differentiate them into podocytes in situ. Primary human glomerular microvascular endothelial cells are seeded on the opposite side of the same porous ECM-coated membrane in the bottom channel to recreate the podocyte–endothelial interface (Fig. 6), in which the glomerular basement membrane normally forms in vivo, thereby mimicking the urinary and capillary compartments of a functional kidney glomerulus.

To mimic the dynamic mechanical stretching and relaxation motion observed in living glomeruli in vivo in response to the cyclic pulsations of renal blood flow[29], we included two hollow chambers on each side of the central microfluidic channels and applied cyclic suction (1 Hz, −85 kPa) to facilitate stretch (10% strain) and relaxation of the PDMS side walls along with the attached horizontal flexible PDMS membrane with its adherent cell layers. By co-culturing human iPS cell–derived podocytes with a layer of primary human kidney glomerular endothelial cells in the microfluidic device, we developed a human kidney Glomerulus Chip that mimics the tissue–tissue interface (Fig. 5, top right and left) and differential molecular filtration functions of the human glomerular capillary wall, as well as Adriamycin-induced podocyte injury and albuminuria in vitro[5]. Together, our protocol enables the derivation of mature terminally differentiated podocytes from human iPS cells, as well as their integration into a functional microfluidic device with an adjacent endothelium-lined vascular circuit to recreate the structure, function, and specific drug (Adriamycin) responses of the living human kidney glomerulus in vitro.

Applications

The podocyte differentiation protocol has applications in modeling the development and function of the kidney glomerulus, understanding the mechanisms of podocyte injury in glomerulopathies, and

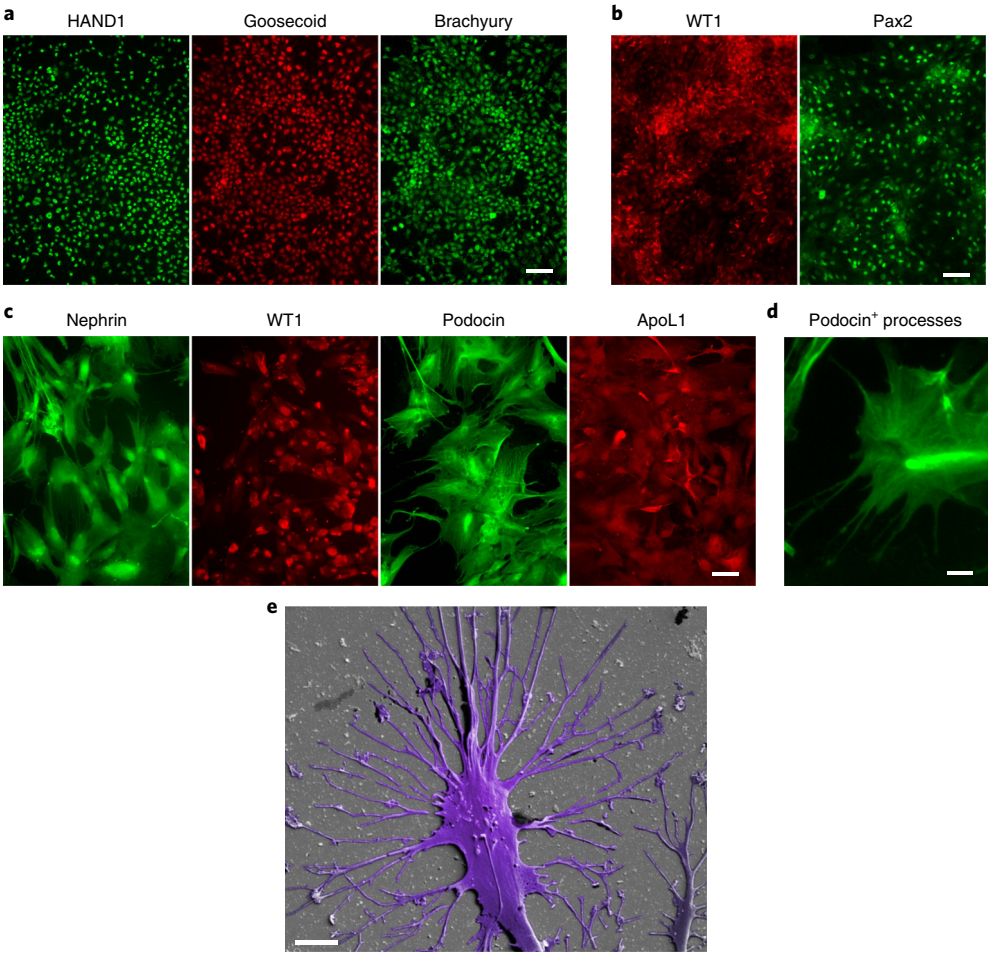

Fig. 3 | Immunofluorescence staining and scanning electron micrograph of cells during the differentiation process. **a**–**c**, Human iPS cell-derived mesoderm cells expressing HAND1, goosecoid, and brachyury (**a**), intermediate mesoderm cells expressing WT1 and Pax2 markers (**b**), and podocytes expressing the lineage-characterization markers nephrin, WT1 and podocin, as well as the associated protein ApoL1 (**c**). **d**, High-magnification image of iPS cell-derived podocytes showing the development of foot processes that are positive for podocin. **e**, Scanning electron micrograph of human iPS cell-derived podocytes showing the development of primary and secondary foot processes. Scale bars, (**a**–**c**) 100 μm, (**d**) 25 μm, and (**e**) 2 μm. **d**,**e**, adapted with permission from Musah et al.[5], Macmillan Publishers Limited.

the establishment of in vitro systems for nephrotoxicity screening and drug discovery. Although recent methods in stem cell differentiation have provided insights into the development of nephron progenitor cells[30–33], the mechanisms underlying podocyte-lineage specification and maturation remain largely unknown. This approach can be used to examine the factors that determine cell fate and tissue morphogenetic decisions in terminal differentiation of the kidney glomerulus. Thus, this protocol also provides opportunities for studying both early- and late-onset kidney diseases such as congenital nephrotic syndrome (CNS) of the Finnish type and steroid-resistant nephrotic syndrome[34,35]. Mutations in podocyte genes have been implicated in many forms of kidney diseases[36–38], but animal models often fail to recapitulate human physiological responses[3,39]. This method can therefore be used in combination with genome editing technologies such as CRISPR/Cas9 (ref. [40]) to produce isogenic human iPS cell lines that differ only by specific mutations and then differentiate them into kidney podocytes to examine disease phenotype and facilitate therapeutic discovery. The microfluidic human kidney Glomerulus Chip advances the capabilities of current tissue culture methods by providing a unique platform to simultaneously investigate the roles of multiple factors on glomerular capillary wall function, including cell–cell interactions, fluid shear stress, and mechanical deformation forces in kidney development and pathophysiology. It also provides opportunities to build more complex in vitro structures of the kidney by fluidically linking the Glomerulus Chip to functional microfluidic models of other subunits of the human kidney, such as the kidney proximal

Box 1 | Passaging of human iPS cell–derived intermediate mesoderm cells ● Timing ~3 h

We recommend that the cells be cultured in the intermediate mesoderm (Fig. 1) induction medium for a minimum of 7 d before passaging onto the newly prepared laminin 511-E8–coated plates. It is acceptable for these cells to reach 100% confluence before passaging.

Procedure

1 Prepare the laminin 511-E8–coated plates (Reagent setup).
2 Prepare the intermediate mesoderm differentiation medium (Reagent setup).
3 Rinse the cells three times with pre-warmed DMEM/F12 and add 0.5 mL of 0.05% (vol/vol) trypsin-EDTA per 12-well plate.
4 Incubate the cells at 37 °C and 5% CO_2 for ~3 min or until the cells begin to dissociate or ball up. ▲ **CRITICAL STEP** Colonies with defined borders may begin to roll up at the edge. Timing depends on the cell density.
5 Add ~2 mL of trypsin-neutralizing solution to each well and gently scrape the cells using a cell lifter.
6 Pipette the cell suspension several times to form a mixture of single cells and small clumps (<200 μm).
7 Transfer the cell suspension to a 15-mL conical tube and bring to volume (15 mL) with pre-warmed DMEM/F.
8 Centrifuge for 5 min at 201*g* at room temperature (20–23 °C).
9 Remove the supernatant by aspiration. Be careful not to aspirate the cell pellet.
10 Resuspend the cells in an appropriate volume of the intermediate mesoderm differentiation medium for seeding at a 1:4 or 1:6 splitting ratio.
11 Add 1 mL of the cell suspension to each well of the newly prepared laminin 511-E8–coated 12-well plates.
12 Incubate the cells at 37 °C and 5% CO_2, and refresh the medium daily.

Box 2 | Cryopreservation of human iPS cell–derived intermediate mesoderm cells ● Timing ~30 min

We recommend a minimum of 16 d of differentiation (and one passaging, if necessary) in the intermediate mesoderm stage (Fig. 1) before cryopreservation. It is also advisable to cryopreserve the cells while they are growing in the mitotic phase (within ~5 d after passaging). Growth rate depends on the seeding density and the hPSC line used.

Procedure

1 Prepare intermediate mesoderm differentiation medium (Reagent setup).
2 Rinse the cells three times with pre-warmed DMEM/F12 and add 0.5 mL of 0.05% trypsin-EDTA per 12-well plate.
3 Incubate the cells at 37 °C and 5% CO_2 for ~3 min or until the cells begin to dissociate or ball up. Colonies with defined borders may begin to roll up at the edge. Timing depends on cell density.
4 Add ~2 mL of trypsin-neutralizing solution to each well and gently scrape the cells using a cell lifter.
5 Pipette the cell suspension several times to form a mixture of single cells and small clumps (<200 μm).
6 Transfer the cell suspension to a conical tube and bring to volume (15 mL) with pre-warmed DMEM/F. Take a small volume to count the cells.
7 Centrifuge the cells for 5 min at 201*g* at room temperature.
8 Remove the supernatant by aspiration. Be careful not to aspirate the cell pellet.
9 Resuspend the cells in an appropriate volume of a cryopreservation medium consisting of 50% (vol/vol) FBS, 40% (vol/vol) intermediate mesoderm differentiation medium, and 10% (vol/vol) dimethyl sulfoxide (DMSO) to obtain ~1 × 10^6 cells per mL.
10 Add 1 mL of the cell suspension to each cryopreservation tube.
11 Plate the tubes in a Mr. Frosty freezing container and freeze at −80 °C for 24 h.
12 Move the tubes to a liquid nitrogen cell storage tank for long-term cryopreservation.
13 To initiate live cultures, slowly thaw the cells using a water bath. Then resuspend the cells in intermediate mesoderm differentiation medium and centrifuge for 5 min at 201*g* at room temperature. Remove the supernatant and resuspend the cells in intermediate mesoderm differentiation medium. Plate the cells on the newly prepared laminin 511-E8–coated plates and refresh the medium daily. Cells can also be directly seeded in the microfluidic cell culture devices described in this protocol (Step 25B).

tubule, which have been described previously[41,42], to study the filtration and reabsorption of molecules, or by interconnecting the Glomerulus Chip to other similarly vascularized Organ Chips (e.g., with lung[28,43], liver[3,44–46], gut[47], and bone marrow[48]) to develop a human 'body-on-a-chip' for physiologically based prediction of pharmacokinetic/pharmacodynamic parameters in vitro[49–51]. Together, this protocol may be useful for tissue engineering, 3D bioprinting of organs, and regenerative medicine[52–55]. Ultimately, this approach could enable analysis of both hereditary and chronic forms of human kidney disease, as well as facilitate therapeutic development. The methods could also provide an alternative to animal models for studying the pathogenesis of human kidney disease. Because current approaches for propagating primary glomerular podocytes often lead to loss of differentiated phenotype[56,57], it is possible that the factors we identified for inducing mature podocyte

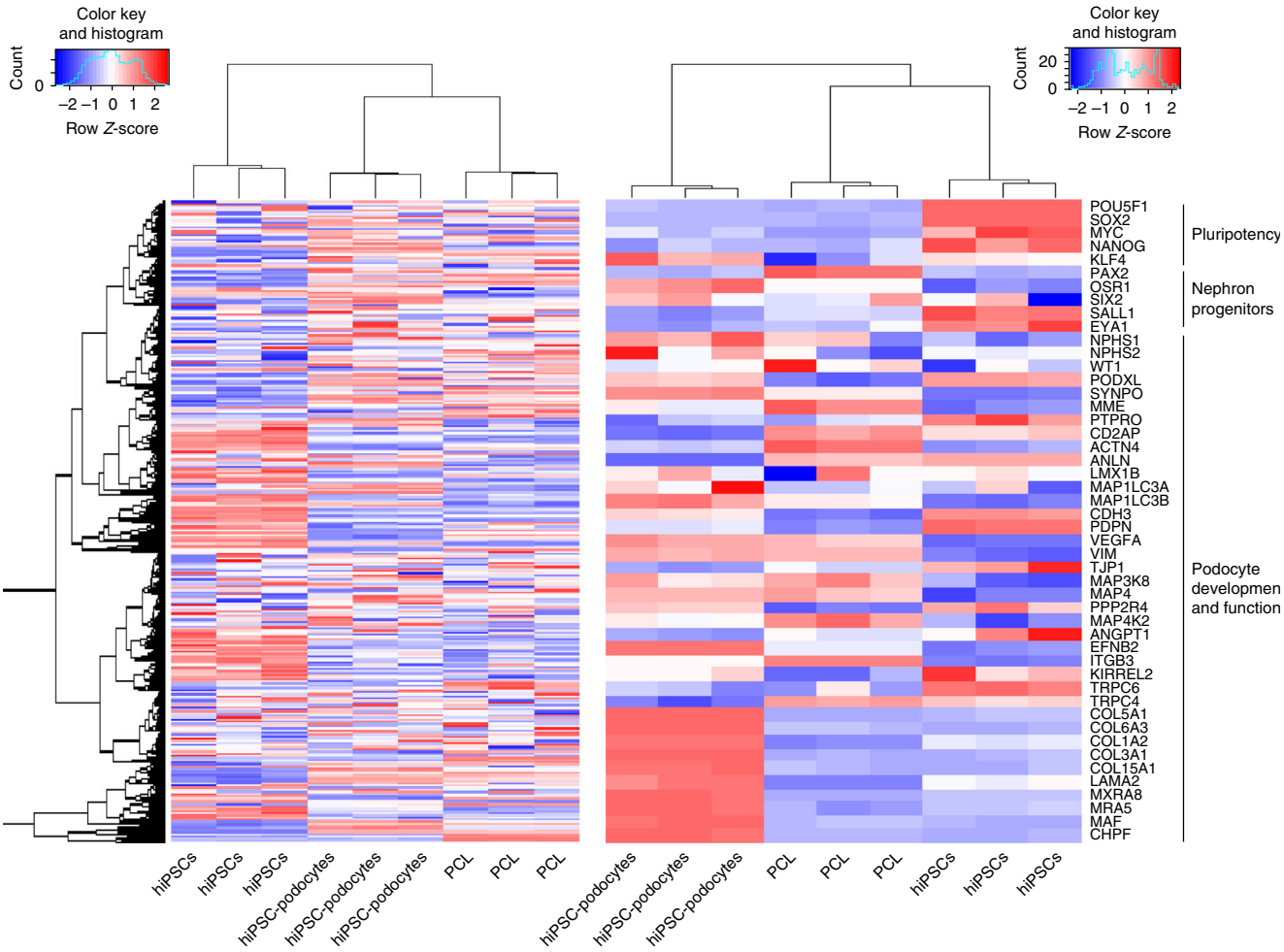

Fig. 4 | Whole-transcriptome analysis using Affymetrix Human Gene 2.0 ST gene array. a, Global gene expression profile of triplicate samples of undifferentiated human iPS cells (hiPSCs), iPS cell-derived podocytes (hiPSC-podocytes), and an immortalized human glomerular podocyte cell line (PCL). **b**, Expression levels of genes involved in pluripotency, development of nephrogenic progenitors, or lineage specification and functional maturation of kidney glomerular podocytes. Each replicate represents an independent experiment. See also Supplementary Data 1 and 2. Heatmaps depict genes that are upregulated (red), downregulated (blue), and unchanged (white).

phenotype from human iPS cells could be used to improve cell culture methods for primary tissues isolated from human kidney. Finally, cell-based therapy for human kidney glomerular tissue has remained largely unexplored because of several factors, including the lack of functional human kidney glomerular podocytes and a method to precisely deliver these cells to injured glomerular capillaries. We hypothesize that it also may be possible to use the human iPS cell–derived podocytes as an injectable form of cell therapy for the treatment of diseases that are characterized by podocyte loss or dysfunction.

Comparison with other methods

Our differentiation protocol is specific for the derivation of only one type of human kidney cell—mature podocytes—with extremely high (>90%) efficiency. Other stem cell differentiation strategies, such as those used previously to generate kidney organoids[21,22,32], produce mixed populations of cells and therefore require additional optimization strategies to guide lineage specification into podocytes. This limits their application in cases in which pure populations of podocytes are desired, such as in injectable cell-based therapies, targeted drug screening, or the development of tissue-specific models of glomerular function, which we do here by using Organ Chip technology. It also remains unclear whether the podocyte-like cells produced by kidney organoid cultures can be isolated, maintained in culture, or used for applications in different experimental systems without loss of cell phenotype and functionality, as this frequently occurs in primary cultures of kidney podocytes[57,58]. By contrast, we

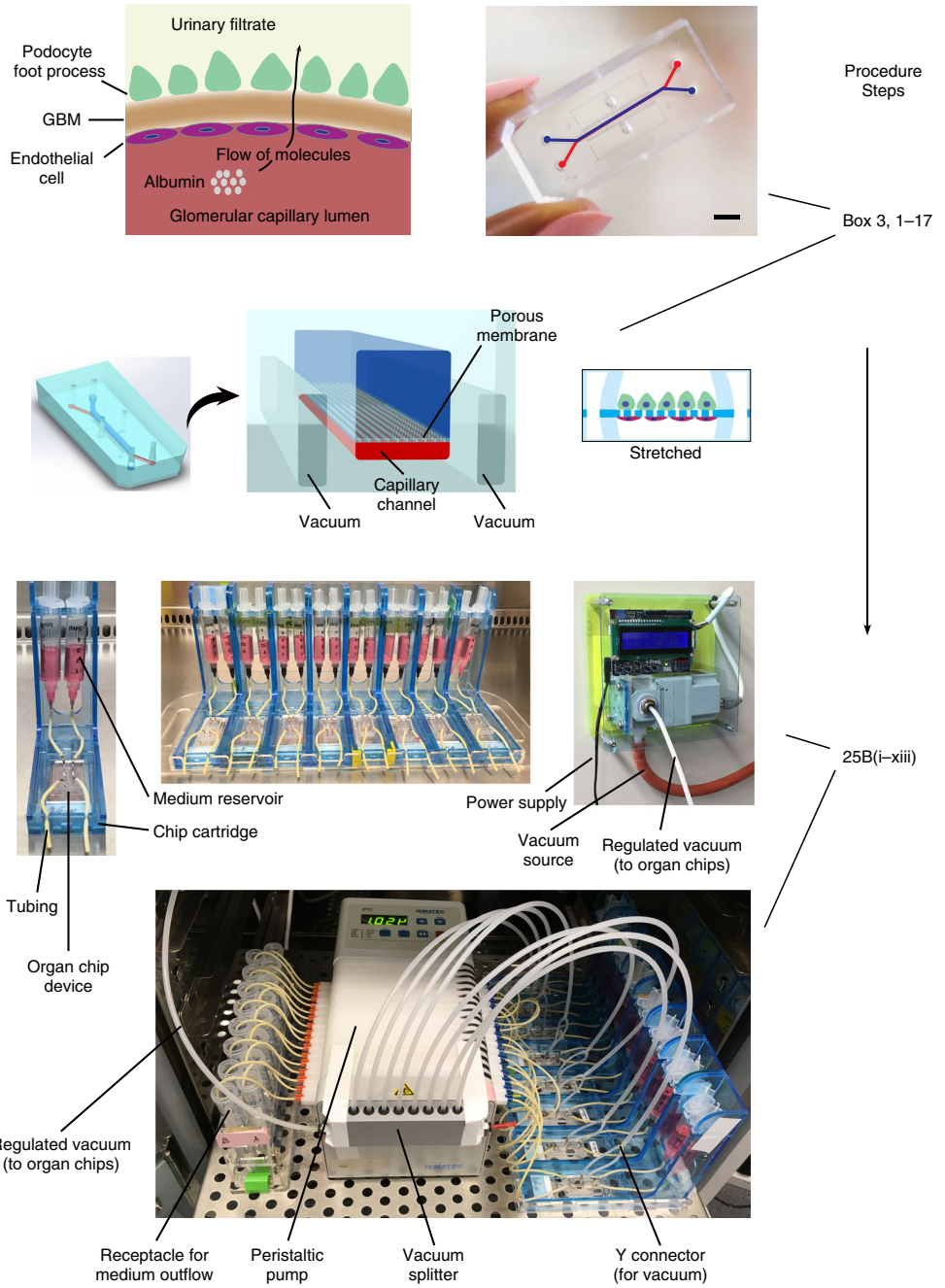

Fig. 5 | Design of microfluidic Organ Chip device to recapitulate the structure and function of the kidney glomerular capillary wall. (Top left) Schematic representation of the glomerular capillary wall, showing podocyte and endothelial cell layers separated by the GBM to form capillary and urinary compartments. (Top right) Photograph of the microfluidic device engineered from PDMS. Scale bar, 5 mm. (Second row) Schematic representation of the microfluidic kidney Glomerulus Chip, with microchannels replicating the urinary and capillary compartments of the glomerulus. The GBM is mimicked by using a porous and flexible PDMS membrane functionalized with the protein laminin. Cyclic mechanical strain was applied to cell layers by stretching the flexible PDMS membrane using a vacuum. (Third row) Example photographs of the experimental setup for Glomerulus Chip cultures show (left) a single Organ Chip microfluidic device connected to two reservoirs containing cell culture media for the urinary (right) and capillary (left) channels, (center) multiple Organ Chips placed on a cartridge built-in-house for handling of microfluidic devices, and (right) a photograph of the programmable vacuum regulator system built in-house. (Bottom) Complete setup of the microfluidic cell culture system with the Glomerulus Chips connected to media reservoirs, inlet and outlet tubing, vacuum lines for stretching, and the peristaltic pump, as well as receptacles for media outflow. Adapted with permission from Musah et al.[5], Macmillan Publishers Limited.

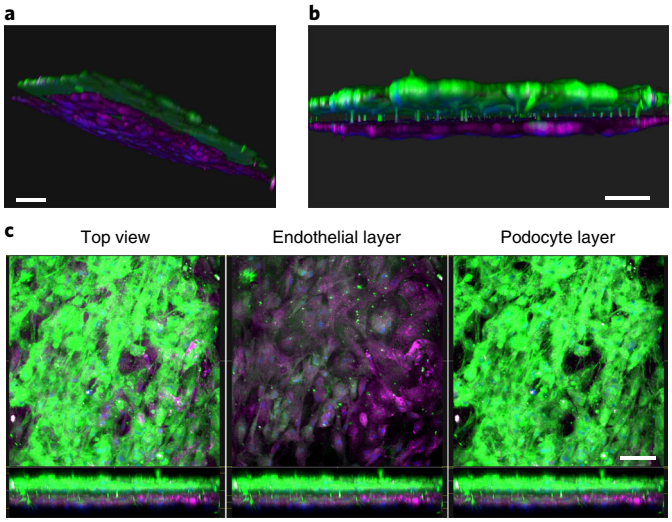

Fig. 6 | Fluorescence microscopy images of the human kidney Glomerulus Chip established from iPS cell–derived podocytes and primary human glomerular endothelial cells. a,**b**, Side (**a**) and cross-sectional (**b**) views of 3D reconstructed confocal images of the human Glomerulus Chip, showing the iPS cell–derived podocytes and endothelial cells in their respective layers after differentiation and co-culture on opposing sides of the flexible ECM-coated PDMS membrane. **c**, Additional immunofluorescence confocal images showing a top view of both cell layers (left), the endothelial cell layer only (middle), and the human iPS cell–derived podocyte layer (right). Scale bars, 100 μm. Adapted with permission from Musah et al.[5], Macmillan Publishers Limited.

demonstrate high levels of differentiation and functionality in our model, independent of genetic manipulation or subpopulation selection, which have not been achieved previously.

Another method that has been previously used to differentiate human iPS cells into podocytes involved the formation of embryoid bodies (EBs) in serum-containing medium[59]. As with organoids, the inherent heterogeneity of EBs results in a very low (<1%) yield of podocyte-like cells with immature phenotype, which limits their utility in applications in which a specific cell type (such as neurons or podocytes) is desired. By contrast, >90% of cells produced with our method have morphological and molecular phenotypes associated with mature glomerular podocytes. In addition, the use of serum components and poorly defined animal-derived ECM such as Matrigel and Geltrex in previous reports[59,60] makes it unclear as to which signaling components contribute specifically to the differentiation of human kidney podocytes. Our protocol uses defined soluble signaling factors and ECM proteins that bind to specific cell surface integrins and robustly support the adhesion and induction of podocyte lineage from human iPS cells. Our protocol may therefore be better suited for studying the molecular mechanisms of podocyte development and lineage specification.

We induced the differentiation of human iPS cell–derived podocytes in our microfluidic Organ Chip device, which is not possible with previously published methods. We also engineered a functional in vitro model of the human kidney glomerular capillary wall by co-culturing the iPS cell–derived podocytes with a layer of primary human glomerular endothelial cells to form a functional endothelium-lined vasculature, as required for glomerular filtration studies. Our microfluidic model of the glomerular capillary wall also recapitulates the tissue–tissue structure and partitioning of the urinary and vascular compartments of living glomeruli, and enables the filtration of molecules such as inulin from the vascular channel into the urinary space while retaining albumin in the vasculature, much as expected in a functional human kidney glomerulus. Our microfluidic human kidney Glomerulus Chip also serves as a useful platform for studying the role of mechanical forces in glomerular development, as we demonstrated by uncovering a role for mechanical strain in podocyte differentiation and differential deposition of the glomerular basement membrane (GBM) component Collagen IV by podocytes and endothelial cells[5]. This could not be accomplished using traditional cell culture models or organoids, as they lack the tissue structure and endothelium-lined vascular circuit necessary for kidney glomerular filtration studies.

Limitations

Our differentiation method worked well for all the human ES (H9) and iPS (PGP1, IISH3i-CB6, and IMR-90-1) cell lines examined in this study. Owing to intrinsic variability in human iPS and ES cell

Box 3 | Microfabrication of microfluidic organ-on-a-chip device ● Timing ~5–6 d

▲ CRITICAL We previously published a detailed method for making Organ Chip microfluidic devices[27]. For this section of the protocol, we recommend that researchers refer to the published protocol for additional details and step-by-step instructions for engineering the microfluidic organ-on-a-chip device, and follow the considerations outlined below.

Assembly of microfluidic devices ● Timing 4–5 d

1 Develop the mold out of ProtoTherm 12120, using stereolithography.
2 Cast the 'urinary' and 'microvascular' channels of the devices from PDMS at a 10:1 (wt/wt) ratio of base to curing agent. Degas the prepolymer mixture and then cure for 4 h to overnight at 60 °C.
3 Cast the Glomerulus Chip so that it contains two fluidic channels (a 1 × 1-mm urinary channel and 1 × 0.2-mm microvascular channel), two vacuum channels parallel to the fluidic channels, and ports for all fluidic and vacuum channels. Ensure that the urinary and microvascular channels of the device are separated by a porous PDMS membrane, which can be developed by casting against a deep reactive-ion etching (DRIE)-patterned silicon wafer (50 × 50 mm) consisting of 50-μm-high and 7-μm-diameter posts spaced 40 μm apart.
4 To produce through holes in the membrane using the microengineered post array, pour 100 μL of PDMS onto the wafer and then compress a polycarbonate backing against the post array, followed by baking at 60 °C for 4 h.
5 Bond the porous PDMS membrane to the top component of the device by using oxygen plasma treatment (40 W, 800 mbar, 40 s).
6 Bond the top-membrane assembly to the bottom component containing the microvascular channel (1 mm wide × 0.2 mm high) and matching vacuum channels.
7 For handling, place each microfluidic chip on a 25 (width) × 75 (height) × 1-mm (depth) microscope glass slide.
■ PAUSE POINT Microfluidic chips can be used immediately or stored at room temperature for up to 6 months.

Preparation of microfluidic devices for cell culture ● Timing 1 d

▲ CRITICAL Microfluidic devices should be activated with oxygen plasma immediately before use.

8 Place microfluidic Organ Chip devices in a 150-mm cell culture dish, which fits about eight chips. Avoid stacking of the chips. Activate/sterilize the chips by treatment with oxygen plasma (100 W, 15 standard cubic centimeters per minute, 30 s). Quickly move the chips to sterile biosafety cabinet for ECM coating. Activated chips should be used within 15 min.
9 Functionalize the activated chips with full-length laminin 511 protein (Reagent setup).
? TROUBLESHOOTING
10 Prepare primary human glomerular endothelial cells as described in Reagent setup. Dissociate the cells from a T25 flask, incubate them with 2 mL of 0.05% trypsin-EDTA for 1 min at 37 °C, and stop the reaction by adding ~5 mL of trypsin-neutralizing solution (Reagent setup).
11 Transfer the cell suspension to a 15-mL conical tube and bring the volume to 15 mL with Complete Medium containing serum and CultureBoost-R. Centrifuge the cells for 5 min at 201*g* at room temperature and aspirate the supernatant.
12 Resuspend the endothelial cells in an appropriate volume of complete medium with serum and CultureBoost-R to achieve a concentration of ~2 × 10^6 cells/mL.
13 Using a 100-μL pipette with a barrier tip, gently add 10 μL of the cell suspension (~2 × 10^4 cells) to the bottom (vascular) channel of the microfluidic device. We found that a cell seeding density of 2 × 10^4 to 4 × 10^4 cells per channel works well.
? TROUBLESHOOTING
14 Before incubation, fill the top (urinary) channel of the device with DMEM/F12 to minimize evaporation in the chip and prevent the laminin-coated surface in the top channel from drying out. Incubate the chips in an inverted position at 37 °C and 5% CO_2 for 3 h.
▲ CRITICAL STEP It is important that the chip be incubated in an inverted position to ensure that endothelial cells adhere to the flexible PDMS membrane separating the two channels, as this also positions the cells in a way that allows for the establishment of the endothelial–basement membrane–podocyte interface in vitro.
15 Perform visual inspection and ensure that a complete monolayer of endothelial cells is achieved before proceeding to the next step. The chip can now be used in the original orientation such that the endothelium-lined channel is at the bottom.
? TROUBLESHOOTING
16 Remove non-adherent endothelial cells by gently pipetting the medium from the bottom channel of the microfluidic device. Then add 10 μL of fresh medium to the endothelium-lined channel.
? TROUBLESHOOTING

lines[61], however, researchers may need to adjust the protocol depending on the cell line they are using. Some of the modifications may include the use of different concentrations of the signaling factors, adjusting the timing of induction for each stage of differentiation, and altering the type of cell-culture substrate or ECM component. The overall timing of our protocol is adequate for the creation of the Glomerulus Chip, and given that it typically takes several months to obtain functional cell types such as motor neurons[62] and astrocytes[63] from human iPS and ES cell lines, the derivation of mature glomerular podocytes in <30 d is considered rapid. Still, a faster protocol may be desired in some

situations, and it is conceivable that the pathways targeted by the signaling factors that we have identified for lineage specification of kidney podocytes could inform future studies focused on the establishment of more rapid methods for podocyte differentiation using technologies such as transcriptional activation[64,65] and mechanotransduction[13,14,66,67] in a synergistic or independent manner.

Clinical applications of the human iPS cell–derived podocytes may also require differentiation on a larger scale. Although differentiation of human podocytes at an industrial scale was not the goal of this work, we achieved podocyte differentiation in both the microfluidic Organ Chip device (to model glomerular development and function in a multiplexed manner) and in standard tissue culture plates, which should enable podocyte differentiation at a larger scale in the future.

Our microfluidic kidney Glomerulus Chip device also has some limitations. First, the PDMS material used in the fabrication of our model may absorb small hydrophobic molecules, which could compromise their utility in measuring the toxicity and efficacy of certain drugs. However, we and others have identified elastomers[68,69] that do not absorb small molecules and could potentially be used as alternatives to PDMS for engineering microfluidic Organ Chips. It is also possible to use analytical techniques such as mass spectrometry to examine the real dose of drugs within the microfluidic devices for more accurate detection of effective concentrations. With these PDMS drug absorption data in hand, it is then possible to carry out pharmacokinetic modeling using these types of Organ Chips[51]. The flexible PDMS membrane with engineered pores is also thicker (50 μm) than the GBM in vivo, which is usually <1 μm thick[70], and it lacks the curvature of glomerular capillaries. These differences are primarily due to technical challenges in engineering thin sheets of PDMS with optimal structural integrity and ease of handling. Although we achieved retention of albumin in the vascular channel and excretion of inulin across the glomerular filtration barrier, the 7-μm-diameter pores of the engineered membrane are large and allow large proteins to enter the urinary channel if cells are seeded sparsely, and thus cultures must be confluent before studies can be initiated. We found that the human iPS cell–derived podocytes and primary endothelial cells used in the microfluidic device can produce GBM components, including collagen IV[5] and laminin (S.M. and D.E.I., unpublished data), which could potentially form a more physiologically relevant barrier between the two cell layers. Results of control studies also showed that acellular chips, or chips lined by endothelium alone or by irrelevant cell types such as fibroblasts and renal proximal tubule epithelial cells (all coated with similar ECM), fail to recapitulate the selective filtration of the Glomerulus Chip[5]. Thus, our results could not be explained simply by differences in diffusion coefficients alone. In the future, it might be possible to engineer the microfluidic device such that it comprises a porous membrane made with degradable ECM molecules[71], which would allow the cells to further remodel the basement membrane it forms and produce a barrier that would more closely resemble the GBM in vivo. This could also help overcome some of the technical challenges associated with the processing of cell-lined PDMS materials for endpoint analyses by transmission electron microscopy and scanning electron microscopy, which may be necessary to visualize podocyte foot process development and interdigitating networks.

Finally, although the Glomerulus Chip recapitulates many key functions of the glomerular capillary wall, it does not mimic all the functionalities of a whole kidney glomerulus. For example, our microfluidic device contains only a subset of cells, as compared with a whole living glomerulus. The fluid flow rate (60 μL/h) and shear stresses (0.0007 and 0.017 dyn/cm^2) in the urinary and vascular channels, respectively, are also lower than those observed in vivo. Overcoming this problem may require the use of a large volume of medium per experiment, which may not be practical for most laboratory settings. It is also possible to modify the experimental setup to enable fluid recirculation at higher flow rates or to use pressure-driven flow of smaller volumes of cell culture medium.

Materials

Reagents

Cells

- Human iPS cells or ES cells. This protocol can be performed on any human iPS or ES cell line. We used PGP1 (The Personal Genome Project and Church Laboratory at Harvard Medical School, available from the authors upon request), IISH3i-CB6 (WiCell Research Institute, lot no. DB0005), IMR-90-1 (WiCell Research Institute, lot no. iPS(IMR90)-1-DL-01), and H9 (WA09, WiCell Research Institute, lot no. RB40917) **! CAUTION** The use of human iPS and ES cells requires adherence to appropriate

national laws and institutional regulatory board and funding agency guidelines All cell lines were obtained under appropriate material transfer agreements and approved by the institutional review board (IRB) and institutional embryonic stem cell research oversight (ESCRO) committee at Harvard University **! CAUTION** The cell lines in your research should be regularly tested to ensure that they are karyotypically normal and not contaminated with mycoplasma All cell lines mentioned in this protocol were tested for mycoplasma contamination before use.

- Conditionally immortalized human podocyte cell line (Mundel laboratory at Massachusetts General Hospital and Harvard Medical School; requests for cell line should be addressed to M.A. Saleem (M.Saleem@bristol.ac.uk))
- Primary human glomerular microvascular endothelial cells (Cell Systems, cat. no. ACBRI 128 V)

Growth factors and media supplements

- mTeSR medium (Stem Cell Technologies, cat. no. 05850)
- DMEM/F12 (Thermo/Life Technologies, cat. no. 12634028)
- DMEM/F12 with GlutaMAX supplement (Thermo/Life Technologies, cat. no. 10565042)
- Complete Serum-free Medium Kit with recombinant proteins and CultureBoost-R (Cell Systems, cat. no. SF-4Z0-500-R)
- Complete Medium Kit with CultureBoost-R (Cell Systems, cat. no. 4Z0-500-R)
- Human activin A (Thermo/Life Technologies, cat. no. PHC9564)
- Human BMP7 (Thermo/Life Technologies, cat. no. PHC9544)
- Human VEGF (Thermo/Life Technologies, cat. no. PHC9394)
- B27 serum-free supplement (Thermo/Life Technologies, cat. no. 17504044)
- Y27632 ROCK inhibitor (Tocris, cat. no. 1254)
- CHIR99021 (2 mg; Stemgent, cat. no. 04-0004) **▲ CRITICAL** We have observed differences in the quality of cells cultured with CHIR99021 compound from different suppliers or manufacturers We therefore recommend lot testing, especially for compounds purchased elsewhere.
- All-trans retinoic acid (500 mg; Stem Cell Technologies, cat. no. 72262)
- Heat-inactivated FBS (Thermo/Life Technologies, cat. no. 10082147)
- Penicillin–streptomycin, liquid (100×; Thermo/Life Technologies, cat. no. 15140-163)

Antibodies and fluorescent stains

- Oct4 (R&D Systems, cat. no. AF1759)
- TRA-1-60 (Abcam, cat. no. ab16288)
- Goosecoid (R&D Systems, cat. no. AF4086)
- Brachyury (Abcam, cat. no. ab20680)
- HAND1 (Abcam, cat. no. ab196622)
- Pax2 (Invitrogen, cat. no. 71-6000)
- OSR1 (Novus Biologicals, cat. no. H00130497-M04
- WT1 (Millipore, cat. no. MAB4234)
- Nephrin (Progen, cat. no. GP-N2)
- Podocin (Abcam, cat. no. ab50339)
- VE-cadherin (Santa Cruz Biotech, cat. no. sc-9989)
- Collagen type IV (Abcam, cat. no. ab6586)
- Integrin β1 (Abcam, cat. no. ab24693)
- ApoL1, anti-apolipoprotein L1 (Abcam, cat. no. ab85795)
- 4′,6-Diamidino-2-phenylindole (DAPI; Thermo/Life Technologies, cat. no. D1306)
- Oct4 conjugated to phycoerythrin (Oct4-PE; Stem Cell Technologies, cat. no. 60093PE)
- Alexa Fluor 488– and Alexa Fluor 594–conjugated secondary antibodies (Thermo/Life Technologies)
- WTI conjugated to APC (WT1-APC; LifeSpan Biosciences, cat. no. LS-C224662/5744)
- Nephrin conjugated to PE-Cy5 (Nephrin-PE-Cy5; Bioss, cat. no. bs-10233R-PE-Cy5)

ECM molecules

- Matrigel hESC-qualified matric, 5-mL vial (BD Biosciences, cat. no. 354277). This reagent shows lot-to-lot variation. Lot testing for successful human iPS and ES cell culture is recommended. Keep Matrigel cold and use a manufacturer-recommended dilution factor for iPS or ES cell culture
- iMatrix-511 Laminin-E8 (LM-E8) fragment (Iwai North America, cat. no. N-892012)
- Human recombinant Laminin 511 protein (Biolamina, cat. no. LN511-02)

Enzymes and other reagents

- Accutase (Thermo/Life Technologies, cat. no. A1110501)
- Enzyme-free cell dissociation buffer, Hank's balanced salt (Thermo/Life Technologies, cat. no. 13150016)

- Trypsin-EDTA, 0.05% (Thermo/Life Technologies, cat. no. 25300-120)
- MycoAlert Mycoplasma Detection Kit (Lonza, cat. no. LT07-318)
- Phosphate-buffered saline (PBS; Thermo/Life Technologies, cat. no. 14190-250)
- PBS with calcium and magnesium (Thermo/Life Technologies, cat. no. 14040182)
- Saponin (Sigma-Aldrich, cat. no. 47036)
- Triton X-100 (VWR, cat. no. 97062-208)
- BSA (Sigma-Aldrich, cat. no. A9418)
- Paraformaldehyde (PFA; Thermo/Life Technologies, cat. no. 28906) **! CAUTION** PFA should be handled in a chemical fume hood with proper personal protection equipment, including gloves, lab coat, and safety eye glasses. Avoid inhalation and contact with skin.
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D2438) **! CAUTION** DMSO is toxic and should be handled in chemical safety hood while using personal protection equipment
- Cell counting kit 8 (CCK-8; Dojindo, cat. no. CK04-01)
- Inulin-FITC (Sigma-Aldrich, cat. no. F3272)
- Human albumin conjugated to Texas Red (Rockland, cat. no. 009-0933)

Equipment

Tissue culture and microengineering supplies

- T25 flask with canted vented cap (Corning, cat. no. 353108)
- Filter system, 0.22 μm, PES 500 mL (Corning, cat. no. 431097)
- Steriflip, 0.22 μm, PES (EMD Millipore, cat. no. SCGP00525)
- Tissue culture–treated six-well plates (Corning, cat. no. 353046)
- Tissue culture–treated 12-well plates (Corning, cat. no. 353043)
- Falcon 150-mm cell culture dish (Corning, cat. no. 353025)
- Wide-beveled cell lifter (Corning, cat. no. 3008)
- Cryogenic vial rack and tray (Corning, cat. no. 430525)
- Conical centrifuge tube, 15 mL (Corning, cat. no. 352097)
- Conical centrifuge tube, 50 mL (Corning, cat. no. 352098)
- CryoTube vials (Nunc, cat. no. 377267)
- Aspirating pipettes, individually wrapped (Corning, cat. no. 29442-462)
- P10 precision barrier pipette tips (Denville Scientific, cat. no. P1096-FR)
- P20 barrier pipette tips (Denville Scientific, cat. no. P1121)
- P100 barrier pipette tips (Denville Scientific, cat. no. P1125)
- P200 barrier pipette tips (Denville Scientific, cat. no. P1122)
- P1000 barrier pipette tips (Denville Scientific, cat. no. P1126)
- Serological pipette, 5 mL, individually wrapped (Corning, cat. no. 356543)
- Serological pipette, 10 mL, individually wrapped (Corning, cat. no. 356551)
- Serological pipette, 25 mL, individually wrapped (Corning, cat. no. 356525)
- Stainless-steel disposable safety scalpels (Integra Miltrex, cat. no. 4-510)
- Kimwipes, small (VWR, cat. no. 21905-026)
- Kimwipes, large (VWR, cat. no. 21905-049)
- Kimberly-Clark nitrile gloves, long cuff (VWR, cat. no. 50601)
- Kimber-Clark basic plus lab coat (VWR, cat. no. 37000-922)
- Tyvek isoclean sleeves (VWR, cat. no. 89127-350)
- Ethanol solution, 70% (vol/vol), biotechnology grade (VWR, cat. no. 97065-058) **! CAUTION** Ethanol is toxic and flammable. Use personal protection equipment while handling.

Microengineering and microfluidic cell culture supplies

- Poly(dimethylsiloxane) (PDMS) Sylgard 184 Silicone Elastomer Kit (Dow Corning, cat. no. 3097358-1004)
- ProtoTherm 12120 (Proto Labs, cat. no. 12120)
- Blunt needles (18-gauge; VWR, cat. no. KT868280)
- Silicone tubing (Saint-Gobain Tygon sanitary tubing, i.d. = 1/32 inches; Fisher Scientific, cat. no. 02-587-1a)
- 2-Stop PharMed BPT tubing with i.d. = 0.25 mm (Cole-Parmer, cat. no. EW-95713-12)
- PharMed BPT extension tubing, 0.25 mm diameter (Cole-Parmer, cat. no. EW-95809-12)
- Dakin fully frosted microscope slide glass, 25 × 75 × 1 mm (Thermo Scientific, cat. no. 50-949-382)
- Blunt needles (18 gauge; VWR, cat. no. KT868280)

- Disposable 5-mL syringes with Luer-Lok tips (BD Bioscience, cat. no. 309646)
- Y connectors (Cole-Parmer, cat. no. 30703-90)
- Silicon wafers (50 × 50 mm; University Wafer, cat. no. 1080)
- Tweezers (SPI Supplies, cat. no. 2WFG.SA)
- Biosafety cabinet, Series 1300, Type A2 (Thermo Fisher, cat. no. 1333)
- CO_2 incubator, Forma Steri-Cycle CO_2 Incubator (Thermo Fisher, cat. no. 201370)
- Light microscope (Nikon, model no. EclipseTS100-F, equipped with a Zeiss, model no. AxioCam MRc 5)
- Hemacytometer chamber with cover glass (VWR, cat. no. 15170-172)
- Epifluorescence microscope (Zeiss, model no. Axio Observer Z1 microscope, equipped with a Photometrics, model no. CoolSnap HQ2 camera and Carl Zeiss Zen software)
- Confocal microscope (Leica, model no. SP5 X MP inverted microscope with a 25×/0.95 water objective)
- Plate reader, Synergy NEO HTS Multi Mode microplate reader (BioTek Instruments, cat. no. 12817142)
- Balance (Mettler Toledo, cat. no. XS204)
- Dual chamber dry oven (FinePCR, cat. no. combi-D24)
- PlasmaEtcher (Plasma Etch, cat. no. PE-100)
- Peristaltic pump, Ismatec low-speed planetary gear-driven peristaltic pump with 16 channels (Cole-Parmer, cat. no. EW-78001-30)
- Programable vacuum regulator system. Our vacuum regulator was built in-house, and it consists of a vacuum regulator (SMC Corporation of America, cat. no. ITV0091-2BL) that is electronically controlled by a Leonardo microcontroller board (Arduino, code A000057) and a MAX517 digital-to-analog converter (DigiKey Electronic, cat. no. MAX517BCPA+-ND). The regulator outputs a sinusoidal vacuum profile with a user-defined amplitude and frequency.
- Centrifuge, 5810R Benchtop Centrifuge (Eppendorf, cat. no. 022625501)
- Flow cytometer, LSRFortessa (BD Biosciences, cat. no. 647465)

Reagent setup

Human pluripotent stem cells

Before initiating differentiation, the human iPS or ES cells should be adapted to feeder-free culture systems with mTeSR medium in Matrigel-coated plates. We use human iPS and ES cells at ~70% confluency (~4 d after passaging) for differentiation experiments.

Defined mTeSR medium

Combine basal medium and supplement to make a 500-mL stock. Prepare 45-mL aliquots in 50-mL conical tubes and store at −20 °C for up to 6 months. Thaw the aliquots in the refrigerator overnight and use within 2 weeks.

Matrigel

It is essential that all items that come in contact with Matrigel be kept cold to prevent it from polymerizing at room temperature (20–25 °C). To thaw Matrigel, place it on ice and keep it refrigerated overnight. Prepare aliquots on ice according to the dilution factor provided by the manufacturer for each lot. Store the aliquots at −20 °C until ready to use and no longer than the expiration date indicated by the manufacturer.

Matrigel-coated plates

Dilute the appropriate amount of Matrigel in 25 mL of cold DMEM/F12 in a 50-mL conical tube and mix well. Add 1 mL of Matrigel to each well of a six-well plate and incubate at 37 °C for 2 h or at 4 °C for a minimum of 24 h. The coated plates can be used immediately or stored at 4 °C for up to 2 weeks.

Laminin 511-E8–coated plates

All 2D differentiation steps were carried out in 12-well plates. For each 12-well plate, dilute an appropriate amount of laminin 511-E8 in 9 mL of sterile distilled water to achieve a concentration of 5 μg/mL. Coat the plates by adding 700 μL per well and incubate for 2 h at room temperature or refrigerate at 4 °C overnight. The laminin 511-E8–coated plates can be stored for up to 1 week at 4 °C.

Activin A

Reconstitute in sterile PBS with 0.1% (wt/vol) BSA to make a 100 μg/mL stock. Prepare working aliquots (e.g., 100 μL) and store at −20 °C for up to 6 months; avoid freeze–thaw cycles.

BMP7
Reconstitute in sterile distilled water containing 0.1% (wt/vol) BSA to make a 100 μg/mL stock. Prepare working aliquots (e.g., 100 μL) and store at −20 °C for up to 6 months; avoid freeze-thaw cycles.

VEGF
Reconstitute in sterile PBS with 0.1% (wt/vol) BSA to make 100 μg/mL stocks. Prepare working aliquots (e.g., 100 μL) and store at −20 °C for up to 6 months; avoid freeze-thaw cycles.

CHIR99021
Prepare a 30 mM stock solution by dissolving 2 mg of CHIR99021 in 143.3 μL of sterile DMSO. Prepare working aliquots (e.g., 5 μL) and store at −20 °C for up to 1 month.

Y27632
Dissolve 10 mg of Y27632 in 3.079 mL of sterile distilled water to obtain a 10 mM stock solution. Prepare working aliquots (e.g., 100 μL) and store at −20 °C for up to 6 months.

All-trans retinoic acid
Prepare a 10 mM stock solution by dissolving 10 mg in 3.33 mL of sterile DMSO. Prepare working aliquots (e.g., 500 μL) and store protected from light at −20 °C for up to 6 months.

Complete Medium with CultureBoost-R
Prepare the stock by adding CultureBoost-R supplement to basal medium per the manufacturer's guidelines. Store at 4 °C and use within 2 weeks.

Mesoderm differentiation medium
Immediately before initiating the differentiation process or dissociating cells, prepare fresh medium consisting of DMEM/12 with GlutaMax, 100 ng/mL activin A, 3 μM CHIR99021, 10 μM Y27632, and 1× B27 serum-free supplement. As an option, 1% (vol/vol) penicillin–streptomycin may be added, in which case the volume of DMEM/12 with GlutaMax should be adjusted accordingly. Prepare the medium at a volume suitable for the scale of the experiment. We typically prepare 50 mL of medium for experiments with two 12-well plates.

Intermediate mesoderm differentiation medium
Prepare medium consisting of DMEM/12 with GlutaMax, 100 ng/mL BMP7, 3 μM CHIR99021, and 1× B27 serum-free supplement. An optional 1% (vol/vol) penicillin–streptomycin may be added, in which case the volume of DMEM/12 with GlutaMax should be adjusted accordingly. This medium may be prepared in a large batch (e.g., 500 mL) divided into 50-mL working aliquots, and stored at −20 °C for up to 3 months. Frozen aliquots can be thawed overnight at 4 °C before use.

Podocyte induction medium
Prepare induction medium, consisting of DMEM/F12 with GlutaMax supplemented with 100 ng/mL BMP7, 100 ng/mL activin A, 50 ng/mL VEGF, 3 μM CHIR99021, 1× B27 serum-free supplement, and 0.1 μM all-trans retinoic acid. An optional 1% (vol/vol) penicillin–streptomycin may be added, in which case the volume of DMEM/12 with GlutaMax should be adjusted accordingly. It is crucial that this medium be protected from light. The medium may be prepared in a large batch (e.g., 500 mL), divided into 12-mL working aliquots, and stored protected from light at −20 °C for up to 3 months. Thaw frozen aliquots overnight at 4 °C before use.

Trypsin-neutralizing solution
Freshly prepare a solution consisting of DMEM/F12 with GlutaMax and 10% (vol/vol) FBS. Filter the medium by using a filter system or a Steriflip filter.

Laminin 511–coated chips
Dilute the laminin 511 protein in sterile PBS containing calcium and magnesium to obtain a 50 μg/mL solution. A volume of 500 μL is sufficient for an experiment with ten microfluidic chips. Fill both channels of the microfluidic device with the freshly prepared solution and incubate the chips

overnight at 37 °C. ECM-coated chips should be used within 24 h. Before cell seeding, gently remove the ECM solution by pipetting and rinse the chips three times with prewarmed DMEM/F12.

Permeabilization buffer
Prepare a solution consisting of 0.125% (vol/vol) Triton X-100 in PBS. This solution can be prepared in a large batch (e.g., 500 mL) and stored at room temperature for up to 1 month.

Procedure

▲ **CRITICAL** We have optimized multiple parameters in this differentiation protocol, including the type of ECM used, soluble factors and their concentrations, timing of differentiation, and the frequency of medium change. We strongly recommend that researchers follow this protocol as described.

Maintenance of human iPS cells in feeder-free culture with mTeSR medium ● Timing ~7 d

▲ **CRITICAL** All iPS cell maintenance culture described here uses defined mTeSR medium and six-well tissue culture–treated plates coated with human ES cell–qualified Matrigel.

1 Before passaging, when iPS cell colonies are ~70–80% confluent, inspect the condition of the iPS cell cultures and determine if there are spontaneously differentiated cells. Good undifferentiated iPS cells exhibit round colony morphology with defined boundaries. Some merged colonies are acceptable, especially for feeder-free culture conditions.
2 Prepare the Matrigel solution (Reagent setup).
3 Prepare a Matrigel-coated six-well plate (Reagent setup).
4 Aspirate Matrigel from the plates and rinse three times with prewarmed DMEM/F12.
5 Aspirate the mTeSR medium from the human iPS cells and rinse the cells twice with DMEM/F12. Add 1 mL of Accutase to each well and incubate at 37 °C for 1 min or until the edges of the colonies begin to roll up or loosen. Perform a visual check and aspirate the Accutase.
! CAUTION It is important to avoid exposure of iPS cells to Accutase for too long, as this could lead to complete dissociation and loss of the cells during aspiration. Timing may vary depending on iPS cell density or the cell line. For all cell lines used in this study, 1–2 min was sufficient to achieve cell dissociation.
? TROUBLESHOOTING
6 Add 3 mL of mTeSR to each well and gently scrape the cells using a cell lifter.
7 Pipette several times to obtain a cell suspension with small clumps. Add 0.5 mL of the iPS cell suspension to each well of the newly prepared Matrigel-coated six-well plates. Add an additional 2 mL of mTeSR medium to each well and shake the plates gently to distribute the cells.
8 Maintain the iPS cells at 37 °C in a 5% CO_2 incubator. Replace the medium daily.
9 Passage the cells approximately every 6 d or when they reach ~80% confluency.

Preparation of human iPS cells for differentiation ● Timing ~4 d

▲ **CRITICAL** We recommend starting all differentiation studies with iPS cell cultures that are in the mitotic phase. This is typically achieved when human iPS cells are within the first 4 d of passaging or when the cells are no more than 80% confluent. We use cells after 3–4 d of passaging and at a maximum of ~70% confluence.

10 Perform visual inspection of the iPS cells colonies that have been cultured for 3–4 d after passaging and are ~70% confluent to make sure that there are no spontaneously differentiated cells. If necessary, remove differentiated cells by scraping or aspiration under sterile conditions. Add 2 mL of mTeSR medium to each well and place the cells at 37 °C in a 5% CO_2 incubator until needed for mesoderm differentiation as described in Step 13.

Differentiation of human iPS cells into mesoderm cells ● Timing 2 d

11 Prepare laminin 511-E8–coated plates (Reagent setup).
12 Prepare the mesoderm induction medium (Reagent setup).
13 Working in a biosafety cabinet and under sterile conditions, aspirate mTeSR medium from iPS cells cultured on Matrigel-coated plates (Step 10). Rinse the cells three times with prewarmed DMEM/F12.
14 Dissociate the iPS cells by incubating them with enzyme-free cell dissociation buffer (1 mL per well of a six-well plate) for ~10 min in a 37 °C incubator. Timing may depend on the density of the iPS cell

colonies, as well as on the cell line. Visually inspect the iPS cells to ensure that the colonies are dissociated. It is normal to have some floating cells.

15 Using a cell lifter, gently scrape the cells and pipette the cell suspension into a conical tube. For example, transfer up to two wells of dissociated iPS cells to a 15-mL conical tube. Pipette the cells few times to individualize the iPS cells. Bring the conical tube up to a 15-mL volume by using prewarmed DMEM/F12 and centrifuge the cells for 5 min at 290*g* at room temperature (20–23 °C). Remove the supernatant and resuspend the cells again in prewarmed DMEM/F12 for another round of centrifugation under the same conditions. The second round of centrifugation should minimize the retention of residual components such as Matrigel (from scraping the plates) and cell dissociation reagent in the final cell suspension for seeding in the plates coated with the defined laminin 511-E8.

16 Aspirate the supernatant and resuspend the cells first in a small volume (~1 mL) of mesoderm induction medium. Using a hemocytometer or other cell-counting device, determine the number of cells and add an appropriate volume of mesoderm induction medium to achieve a concentration of ~10×10^4 cells per mL.

17 Aspirate the ECM solution from the newly prepared laminin 511-E8–coated plates (Step 11) and rinse the plates twice with DMEM/F12. Remove residual DMEM/F12 before adding the cell suspension in the following step.

18 Mix the iPS cell suspension well by pipetting a few times and then transfer 1 mL of the cell suspension to each well of a 12-well plate coated with laminin 511-E8. Shake the plate gently to distribute the cells and incubate at 37 °C in a 5% CO_2 incubator.

19 Refresh the medium the next day.

20 Culture the cells for 2 d of differentiation, after which the human iPS cell–derived mesoderm cells should be ready for intermediate mesoderm induction as described below.
? TROUBLESHOOTING

Differentiation of human iPS cell–derived mesoderm into intermediate mesoderm cells

● **Timing minimum of 14 d**

21 Prepare intermediate mesoderm differentiation medium (Reagent setup).

22 Aspirate the medium from the mesoderm cells and incubate with prewarmed intermediate mesoderm differentiation medium at 1 mL per well of a 12-well plate.

23 Refresh the medium daily.
▲CRITICAL STEP It is important that the medium be refreshed daily, as the cells are metabolically active and quickly deplete the growth factors and supplements. If the medium becomes too acidic (typically yellow in appearance), increase the volume of the medium (e.g., to 1.3 mL per well of a 12-well plate). Some researchers may wish to experiment with growth factors that are packaged for controlled release into the cell culture medium, but this will need to be tested for feasibility and effectiveness in the differentiation of each iPS cell line.
? TROUBLESHOOTING

24 Culture the cells for 14 d to reach intermediate mesoderm differentiation. Follow Box 1 for a detailed protocol for passaging of the human iPS cell–derived intermediate mesoderm cells for up to 1 month, or see Box 2 for a protocol for cryopreservation of human iPS cell–derived intermediate mesoderm cells.
? TROUBLESHOOTING

Derivation of podocytes

25 To generate podocytes using tissue culture plates, follow option A; to generate podocytes using an organ-on-a-chip microfluidic device, follow option B.

(A) Derivation of mature kidney podocytes using laminin-coated tissue culture plates

● **Timing 4–5 d**

(i) Prepare a new laminin 511-E8–coated plate (Reagent setup).

(ii) Prepare podocyte induction medium (Reagent setup) at a volume suitable for the scale of your experiment. A total of 50 mL of the podocyte induction medium is sufficient for an experiment using a 12-well plate.
▲CRITICAL STEP It is important to protect this medium from light. The podocyte induction medium container can be wrapped with foil during prewarming, handling, and storage.

(iii) Prepare 25 mL of trypsin-neutralizing solution (Reagent setup) for each 12-well plate.

(iv) Rinse the intermediate mesoderm cells three times with prewarmed DMEM/F12.

(v) Dissociate the cells by incubating them with 0.05% trypsin-EDTA at 0.5 mL per well of a 12-well plate for ~3 min at 37 °C. Timing will depend on cell density and the human iPS cell line used. We typically passage the intermediate mesoderm cells when they are ~100% confluent, and ~3 min of incubation with trypsin was sufficient for dissociation of cells derived from all the human iPS and ES cell lines used in the previous study.

(vi) Pipette the cells several times, using a pipette with a P1000 barrier tip. Perform a visual check to ensure that the cells are properly dissociated into either individual cells or small clumps. Avoid clumps that are larger than 200 μm in size, as they may not spread out well or could fail to exhibit differentiated podocyte morphology at the end of the induction time line (Fig. 2, dotted circle).

(vii) To stop the enzymatic activity of the trypsin, add ~2 mL of the trypsin-neutralizing solution to each well.

(viii) Transfer the cell suspension to a 50-mL conical tube and bring the volume to 50 mL with DMEM/F12.

(ix) Centrifuge the cells for 5 min at 201*g* and 22 °C.

(x) Aspirate the supernatant and resuspend the cells with the podocyte induction medium at a volume appropriate for a 1:4 splitting ratio.

(xi) Add the cell suspension to the newly prepared laminin 511-E8–coated plate and shake the plate gently to distribute the cells.

(xii) Incubate the cells at 37 °C and 5% CO_2. Refresh the medium daily. The iPS cell–derived podocytes can be used for further studies or maintained in culture by feeding them every 2 d with Complete Medium with CultureBoost-R (with or without serum, see Reagents). We recommend using the human iPS cell–derived podocytes for additional experiments within 1–2 weeks after differentiation.

? TROUBLESHOOTING

(xiii) After 4 or 5 d of induction, fix the cells by incubating them with 4% (vol/vol) PFA in PBS for 30 min at room temperature.

▲ **CRITICAL STEP** Immunostaining and end-point analysis of human iPS cell–derived podocytes differentiated in tissue culture plates (Step 25A(xiii–xx) take 2 d.

(xiv) Rinse the cells three times with PBS

■ **PAUSE POINT** Fixed cells may be stored in PBS at 4 °C for up to 2 weeks without adverse effects.

(xv) Permeabilize the cells with permeabilization buffer (Reagent setup) for 5 min at room temperature.

(xvi) Incubate the cells with a blocking solution consisting of 2% (wt/vol) BSA and 0.125% (vol/vol) Triton X-100 in PBS for 30 min at room temperature.

(xvii) Incubate the cells with primary antibodies in permeabilization buffer overnight at 4 °C.

(xviii) Wash the cells three times with permeabilization buffer and then incubate them with secondary antibodies (1:1,000) for 1 h in permeabilization buffer. If immunostaining for multiple markers (such as nephrin, WT1, and podocin) in a single well, incubate the cells with one secondary antibody at a time.

(xix) Counterstain the cells with DAPI (1:1,000) in distilled water for 5 min. Wash the cells three times with PBS.

(xx) Observe the cells by using an immunofluorescence microscope. Images can be analyzed using standard software such as Fiji or ImageJ[72,73]. Cells may also be analyzed by flow cytometry[5], which will also enable the evaluation of differentiation efficiency (Fig. 3).

▲ **CRITICAL STEP** For flow cytometry analysis, we recommend that the differentiated podocytes be characterized for dual expression of lineage-identification markers such as nephrin and podocin. Because the expression of associated markers of podocytes such as WT1 is not restricted to kidney podocytes, we recommend that the expression of this marker be examined in combination with that of either nephrin or podocin, as we previously described[5].

(B) Differentiation of human iPS cell–derived podocytes in microfluidic devices, and the establishment and analysis of the Glomerulus Chip ● Timing ~12 d

▲ **CRITICAL** To generate podocytes in an organ-on-a-chip microfluidic device, follow Box 3 to make and prepare the chip for cell culture 5 d before the cells reach intermediate mesoderm differentiation.

(i) Dissociate the iPS cell–derived intermediate mesoderm cells from the laminin 511-E8–coated plates by first rinsing them three times with DMEM/F12, followed by incubation with 0.05%

trypsin-ETDA (0.5 mL per well of a 12-well plate) for ~3 min. The timing depends on cell density and the iPS cell line.

(ii) Scrape the cells with a cell lifter/scraper. Using a P1000 pipette with barrier tip, pipette the cell suspension several times to dissociate the cells. Individualized cells or small clumps (<200 μm) are acceptable for seeding into the microfluidic chip (see Box 3 for guidance on fabrication and preparation of the microfluidic chip).

(iii) Add ~2 mL of trypsin-neutralizing solution to each well and transfer the cell suspension to a 15-mL conical tube. Bring the tube to a 15-mL volume by adding DMEM/F12 and centrifuge at 201*g* for 5 min at 22 °C.

(iv) Resuspend the cells in DMEM/F12 and centrifuge once again at 201*g* for 5 min at 22 °C.

(v) Aspirate the supernatant and add an appropriate volume of intermediate mesoderm medium to achieve a cell suspension of ~2×10^6 cells/mL.

(vi) Add ~30 μL of the cell suspension to fill the top (urinary) channel of the device and inspect to ensure that there are no air bubbles trapped in the fluidic channel. This step can be repeated until there are no air bubbles observed in the device.

? TROUBLESHOOTING

(vii) Place the microfluidic chips at 37 °C in a 5% CO_2 incubator for 3–4 h. Perform visual inspection to ensure that a complete monolayer of iPS cell–derived intermediate mesoderm cells is achieved.

? TROUBLESHOOTING

(viii) Aspirate the medium, along with non-adhered cells, from the top channel of the device and add fresh intermediate mesoderm medium to the same microfluidic channel.

(ix) Incubate the microfluidic devices overnight at 37 °C and 5% CO_2.

? TROUBLESHOOTING

(x) Remove the media from both channels of the device and add 10 μL of Complete Medium with CultureBoost-R to the bottom channel of the device, which is lined by endothelial cells.

? TROUBLESHOOTING

(xi) To start the differentiation of the human iPS cell–derived intermediate mesoderm cells into podocytes, fill the top channel of the device with 30 μL of podocyte induction medium, while minimizing exposure of the chips to light. Incubate the chips again in static (no fluidic flow) conditions at 37 °C and 5% CO_2 for 24 h.

(xii) Connect the cell-lined microfluidic chips to presterilized reservoirs with the respective medium for each channel and continuously perfuse at a volumetric flow rate of 60 μL/h using a peristaltic pump. Then apply cyclic stretching (−85 kPa and 1 Hz to achieve ~10% strain) by connecting the vacuum lines to the dedicated ports of the microfluidic chips. Replenish the medium in the reservoirs every 2 d. Culture the devices under these conditions for a minimum of 4 d. See Fig. 5 (third row and bottom)for example images of the experimental setup for microfluidic Organ Chip cultures.

? TROUBLESHOOTING

(xiii) After a total of ~5 d of culture in podocyte induction medium, culture the human iPS cell–derived podocytes (in the top channel of the microfluidic device) with the same Complete Medium with CultureBoost-R as the endothelial cells cultured in the bottom channel. We typically use the chips for secondary applications such as immunofluorescence and filtration studies after they have been cultured under continuous perfusion and mechanical stain for a total of 12 d or more.

▲ CRITICAL STEP Perform end-point analysis of the kidney Glomerulus Chip by immunostaining (Step 25B(xiv–xxv); 2 d) or skip to Step 25B(xxvi) to perform analysis by molecular filtration (1 d).

? TROUBLESHOOTING

(xiv) Disconnect the microfluidic devices from the reservoirs and the peristaltic pump of the chip. The microfluidic devices can be placed in a Petri dish for handling.

(xv) Aspirate the medium from the microfluidic channels and fix the cells by incubating with 4% (vol/vol) PFA in PBS for 1 h at room temperature.

(xvi) Rinse the cells three times with PBS.

■ PAUSE POINT Fixed microfluidic devices may be stored in PBS at 4 °C for up to 2 weeks without adverse effects.

(xvii) Permeabilize the cells with permeabilization buffer for 10 min at room temperature.

(xviii) Incubate the cells with a blocking solution consisting of 2% (wt/vol) BSA and 0.125% (wt/vol) Triton X-100 in PBS for 2 h at room temperature.

(xix) Incubate the cells with primary antibodies in permeabilization buffer overnight at 4 °C. For example, the top channel containing the human iPS cell–derived podocyte layer can be immunostained for nephrin or podocin, and the bottom channel containing the endothelial cells can be immunostained for VE-cadherin.

▲ **CRITICAL STEP** As WT1 alone is not a definitive marker for kidney podocytes, we recommend immunostaining for multiple lineage-identification markers, such as nephrin and podocin, at the end of the podocyte differentiation protocol.

(xx) Wash the cells three times (10 min each) with permeabilization buffer and then incubate them with secondary antibodies (1:1,000) for 1 h in permeabilization buffer at room temperature. If immunostaining for multiple markers (such as nephrin and podocin) in a single microfluidic device, incubate the cells with one secondary antibody at a time.

(xxi) Counterstain the cells with DAPI (1:1,000) in distilled water for 5 min. Wash the cells three times (5 min each) with PBS.

(xxii) Observe the cells by using a confocal microscope. Images can be analyzed using standard software such as Fiji or ImageJ[72,73]. 2D and 3D-reconstructed images can also be generated by using IMARIS software[5].

▲ **CRITICAL STEP** In the following steps, culture both the top and the bottom channels of the cell-lined microfluidic device with Complete Medium containing CultureBoost-R without phenol red.

▲ **CRITICAL STEP** Ensure that the microfluidic chips have been cultured for a minimum of 8 d under continuous perfusion and mechanical strain before proceeding to molecular filtration studies.

(xxiii) Prepare a solution consisting of 10 μg/mL inulin-FITC and 100 μg/mL human albumin–Texas Red in complete medium with CultureBoost-R.

(xxiv) Infuse the bottom (vascular) channel of the microfluidic device with culture medium containing the fluorescently labeled inulin and albumin. The top (urinary) channel should be cultured with the regular Complete Medium with CultureBoost-R.

(xxv) Continuously perfuse the microfluidic chips for 6–8 h while applying cyclic strain. For each fluidic channel of the microfluidic device, collect the medium outflow in a separate tube.

(xxvi) Transfer 100 μL of the outflow from each channel to a clear-bottom plate appropriate for fluorescence measurements and measure the fluorescence intensity of FITC and Texas Red using a plate reader. We typically take three measurements for each condition and use a minimum of three independent replicates per experimental condition.

(xxvii) Calculate the amount of inulin-FITC or albumin–Texas Red filtered from the bottom microvascular channel to the top urinary channel by using the equation for renal clearance[5]:

$$\text{Urinary clearance} = ([U]x\text{UV})/[P]$$

Where $[U]$ = urinary concentration (podocyte channel), UV = urinary volume, and $[P]$ = plasma or microvascular concentration (endothelial channel). The percentage of urinary clearance can be calculated from a ratio of urinary clearance to urinary volume.

(xxviii) Data can be analyzed and visualized with GraphPad Prism or Microsoft Office Excel. We used GraphPad Prism v7 and Microsoft Excel 2016 for data analysis. Additional information on data analysis using GraphPad Prism and Microsoft Excel can be found at www.graphpad.com and mva.microsoft.com, respectively.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Solution
5	The human iPS cells completely dissociated from the Matrigel-coated plate after treatment with Accutase	If most of the human iPS cells no longer adhere to the Matrigel-coated plate, they can be pulled from suspension and transferred to a conical tube, followed by centrifugation at 290*g* for 5 min at 22 °C. Aspirate the supernatant and then resuspend the cells in prewarmed mTeSR medium for passaging. To improve cell survival, supplement the mTeSR medium with 10 μM of Y27632 for the first 24–48 h of culture
20, 23, and 24	Cell death	Test reagents for lot-to-lot variability. In addition, check and calibrate CO_2 and humidity levels in the incubator
24	Overgrowth of differentiating intermediate mesoderm cells	The cells can be dissociated with 0.05% trypsin-EDTA and plated at a 1:4 splitting ratio onto newly prepared laminin 511-E8–coated plates
23 and 24	Intermediate mesoderm differentiation medium turns yellow during culture	Refresh the medium daily and, if necessary, increase the volume of the medium (e.g., to ~1.3 mL of medium per 12-well plate)
	Detachment of intermediate mesoderm cells from laminin 511-E8–coated plate	This can be avoided by passaging the intermediate mesoderm cells every 10–14 d to a newly prepared laminin 511-E8–coated plate. We have also found that the cells are sensitive to fluctuations in the level of CO_2 in the incubator. We recommend checking and calibrating the incubator CO_2 and humidity levels regularly (at least once a week)
25A(xii)	Cell detachment after 4–5 d of podocyte induction	Replace the induction medium with Complete Medium with CultureBoost-R and refresh the medium every 2 d
25B(vii, ix, and x) and Box 3, step 9	Fluid in microfluidic channels dries up during ECM coating or cell seeding	Check that appropriate temperature and humidity levels are maintained in the incubator. In addition, add water to sterile conical tube caps and place them in the Petri dish containing the microfluidic chips
25B(xii)	Temperature rises in the incubator while the pump is running	Some fluidic pumps get very hot during operation. We have previously observed this problem while using syringe pumps for perfusion but have not experienced a similar issue with the peristaltic pump listed in the 'Equipment' section. We recommend that the incubator's temperature be monitored before and during operation of the pump
25B(xiii)	Medium flows too quickly or slowly during perfusion	Calibrate the peristaltic pump and check that the 2-stop tubing and cartridge are properly secured to the pump
25B(vi) and Box 3, steps 9 and 13	Trapped air bubbles are seen during ECM coating or cell seeding in the microfluidic chips	Remove the ECM solution or cell suspension by pipetting and repeat the step slowly
Box 3, step 9	Hydrophobic microfluidic chip	Check that the plasma treatment machine is working properly
25B(vii) and Box 3, step 16	Cells fail to attach to the ECM-functionalized chip	Either start the experiment with new chips or decellularize the used chip, followed by baking at 60 °C for a minimum of 4 h, and then repeat the plasma activation step. In addition, check that the plasma machine is working properly
25B(xii)	Medium reservoirs are not flowing equally	Inspect the chips for ruptured membranes or blockage by debris. In addition, check that the 2-stop tubing on the pump and the cartridge is properly secured to the pump
25B(vi) and Box 3, steps 15 and 16	Uneven cell seeding	Cells were too clumpy during seeding or the cell seeding density was too low. In addition, consider double-seeding of the cells or prolonging the incubation time for seeding. For human iPS cell-derived intermediate mesoderm cells, consider adding 10 μM Y27632 for first 24 h of culture

Timing

Steps 1–9, maintenance of human iPS cells in feeder-free culture with mTeSR medium: ~7 d
Step 10, preparation of human iPS cells for differentiation: ~4 d
Steps 11–20, differentiation of human iPS cells into mesoderm cells: 2 d
Steps 21–24, differentiation of human iPS cell–derived mesoderm into intermediate mesoderm cells: ~14 d
Step 25A(i–xx), derivation of mature kidney podocytes using laminin-coated tissue culture plates: 4–5 d
Step 25B(i–xxviii), differentiation of human iPS cell–derived podocytes in microfluidic devices, and the establishment and analysis of the Glomerulus Chip: ~12 d
Box 1, passaging of human iPS cell–derived intermediate mesoderm cells: ~3 h

Box 2, cryopreservation of human iPS cell–derived intermediate mesoderm cells: ~30 min
Box 3, steps 1–7, microfabrication of microfluidic organ-on-a-chip devices: ~4–5 d
Box 3, steps 8–16, preparation of microfluidic devices for cell culture: 1 d

Anticipated results

This protocol provides a detailed procedure for directed differentiation of human PSCs (including ES and iPS cell lines) into mature kidney glomerular podocytes and their application in a microfluidic Organ Chip device to create a functional in vitro model of the human kidney glomerular capillary wall. With this protocol, human iPS cells can be differentiated first into mesoderm cells within 2 d, followed by the generation of intermediate mesoderm cells in ~16 d, and finally induction into mature glomerular podocytes by 21 d of directed differentiation (Figs. 1 and 2). The mesoderm cells express the lineage markers HAND1, goosecoid, and brachyury (Fig. 3a). The intermediate mesoderm cells express WT1 and Pax2 (Fig. 3b), and although they transiently express OSR1 within the first 10 d of differentiation, they should not express the mesodermal marker brachyury after 16 d of differentiation[5]. Subsequent treatment of the intermediate mesoderm cells with the podocyte induction medium described in this report enables the derivation of kidney podocytes with morphological and molecular characteristics of the mature phenotype (Fig. 3c–e).

Whole-transcriptome analysis (Supplementary Methods) revealed that the human iPS cell–derived podocytes are more similar to an established human PCL than the undifferentiated iPS cells based on a global gene expression profile (Fig. 4a and Supplementary Fig. 2). Consistent with the functional characterization[5], the human iPS cell–derived podocytes express higher levels of genes that have critical roles in the lineage specification and functional maturation of kidney glomerular podocytes in vivo, which include *SYNPO*, *MAF*, *PODXL*, *MAP1LC3B* and *EFNB2* (which encode synapopodin, Maf, podocalyxin, microtubule associated protein 1, and ephrin B2, respectively) (Fig. 4b). Given the substantial contribution of podocytes to the GBM composition in vivo[74–76], it is intriguing that the human iPS cell–derived podocytes also exhibit significant (adjusted P value <0.001 and false discovery rate <0.05) upregulation of several genes, including *COL5A1*, *COL6A3*, *LAMA2*, *MXRA8*, *MRA5*, and *CHPF*, that are involved in the synthesis and remodeling of the GBM. This differentiation protocol might therefore provide an opportunity to model human kidney development and disease pathogenesis in vitro.

To develop an in vitro model of the glomerular capillary wall using microfluidic Organ Chip technology, we recommend that researchers differentiate the podocytes in situ, rather than terminally differentiating the cells on traditional tissue culture plates and then transferring them to the microfluidic cell culture system. In addition to enhancing the differentiation phenotype (when fluid flow and mechanical strain are applied), this also prevents damage to the podocytes that typically results from treatment with cell dissociation enzymes such as trypsin. Differentiation of the human iPS cell–derived podocytes and co-culture with primary human glomerular endothelial cells on opposing sides of the flexible PDMS membrane in the microfluidic device (Fig. 5, second row) enable the establishment of a human kidney Glomerulus Chip (Fig. 3a,b), in which the interfaced podocyte and endothelial cell layers express nephrin and VE-cadherin, respectively (Fig. 6c). Together, this protocol provides a chemically defined method for the derivation of mature podocytes that recapitulate the structure and function of the kidney glomerular capillary wall in a microfluidic Organ Chip. Depending on the goal of the experiment, the cell-lined microfluidic device can be maintained in culture for at least a month. This microfluidic model can be used to study the selective molecular filtration function, as well as drug toxicities of the human kidney glomerulus, in vitro[5]. This protocol may also be useful for 3D bioprinting of organs, tissue engineering, and regenerative medicine in the future.

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Author contributions
S.M., G.M.C., and D.E.I. conceived the strategy for this study; S.M. designed and performed the experiments; S.M. and D.E.I. wrote the manuscript; N.D. and D.M.C. independently analyzed the microarray data; S.M. interpreted the results; and N.D. generated heatmaps and corresponding statistical datasets. All authors discussed the results and commented on the manuscript.

Competing interests
D.E.I. and S.M. declare that they are authors on a patent pending for methods for the generation of kidney glomerular podocytes from pluripotent stem cells (US patent application 14/950859). D.E.I. declares that he is a founder, holds equity and chairs the scientific advisory board at Emulate Inc. The remaining authors declare no competing interests.

Additional information
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