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Generating kidney organoids from human pluripotent stem cells

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Abstract

The human kidney develops from four progenitor populations; nephron progenitors, ureteric epithelial progenitors, renal interstitial progenitors and endothelial progenitors; resulting in the formation of maximally 2 million nephrons. Until recently, methods differentiating human pluripotent stem cells (hPSCs) into either nephron progenitor or ureteric epithelial progenitor had been reported, consequently forming only nephrons or collecting ducts, respectively. Here, we detail a protocol that simultaneously induces all four progenitors to generate kidney organoids within which segmented nephrons are connected to collecting ducts and surrounded by renal interstitial cells and an endothelial network. As evidence of functional maturity, proximal tubules within organoids display megalin-mediated and cubilin-mediated endocytosis, and respond to a nephrotoxicant to undergo apoptosis. This protocol consists of 7 days of monolayer culture for intermediate mesoderm induction followed by 18 days of three-dimensional culture to facilitate self-organising renogenic events leading to organoid formation. Personnel experienced in culturing hPSCs are required to conduct this protocol.

Keywords

Kidney organoid; Human pluripotent stem cell; Directed differentiation

INTRODUCTION

Directed differentiation of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), is one of the most promising approaches to recreate organs for regenerative medicine. As human embryonic stem cells represent the epiblast stage of embryogenesis¹, such directed

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AUTHOR CONTRIBUTIONS

MT and MHL wrote the manuscript. MT, PXE and HSC performed the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests (see the HTML version of this article for details). MT and MHL are named inventors on a patent relating to this methodology.

differentiation is undertaken in a stepwise manner designed to recapitulate the developmental process from the epiblast to a specific tissue cell type. This methodology works more efficiently than single-step based methods² and has been widely used to successfully induce various human tissues such as hematopoietic, cardiac, lung, pancreatic, hepatic, intestinal, cerebral and renal endpoints^{3–9}. While a variety of cell types can be induced *in vitro* via stepwise differentiation protocols, three-dimensional structures are further required to recreate complex multicellular and functional organs. Generation of such three-dimensional mini-organs, called organoids¹⁰, from hPSCs has been reported in recent years, including organoids of brain, optic cup, stomach, intestine and liver^{11–15}. In this protocol, we describe the methodology we have recently published for generating kidney organoids from hPSCs¹⁶. This expands on the brief step-by step protocol describing kidney organoid generation we previously uploaded to Protocol Exchange¹⁷.

Development of the protocol

The stepwise differentiation of hPSCs to kidney begins with the induction of the primitive streak which is the progenitor population for both endoderm and mesoderm. While the anterior primitive streak gives rise to the endoderm, the posterior primitive streak has potential to develop into the mesoderm, including the axial, paraxial, intermediate and lateral plate mesoderm^{18,19}. The intermediate mesoderm differentiates to the ureteric epithelium and the metanephric mesenchyme, which are two key kidney progenitor populations subsequently undergoing a reciprocal interaction to form the kidney²⁰. The ureteric epithelium develops into the collecting duct of the kidney and the ureter connecting the kidney with the bladder²¹. The metanephric mesenchyme gives rise to the cap mesenchyme which has been shown via lineage tracing to differentiate into all other epithelial cell types of the nephrons²². In addition to these two progenitors, endothelial and renal interstitium progenitors also arise from the intermediate mesoderm although it is not yet clear if these are subsets of the metanephric mesenchyme or distinct outcomes from intermediate mesoderm²³.

Primitive streak induction—The first stage of differentiation in this protocol is induction of the posterior primitive streak. As previously investigated^{24,25}, the posterior primitive streak can be differentiated from mouse embryonic stem cells by activating BMP, Nodal and canonical WNT signaling in two-dimensional culture methods. This method can also be successfully applied to hPSCs²⁶. At this stage, we also culture cells under a monolayer culture condition to control anteroposterior cell fate of the primitive streak more precisely than in embryoid bodies. Cell-autonomous effects and cell-cell interactions promote spontaneous differentiation within embryoid bodies whereas specific conditions of growth factors, concentration, and timing can be chosen in monolayer culture to produce more robust and uniform differentiation to a specific lineage. We previously demonstrated that the posterior primitive streak was induced by canonical WNT signalling or the combination of high and low doses of BMP4 and Activin A, respectively, in 2 days. In contrast, high Activin A with low BMP4 concentrations differentiated hPSCs into the anterior primitive streak⁹.

Intermediate mesoderm induction—The second stage is differentiation of posterior primitive streak cells into the intermediate mesoderm. Our previous study showed that hESC-derived posterior primitive streak spontaneously gives rise to the lateral plate mesoderm under APEL medium culture conditions⁹. As the intermediate mesoderm develops medial to the lateral plate mesoderm during embryogenesis, it is necessary to control the medial nature of the differentiation process using exogenous factors. Thus, again, we keep a monolayer culture condition to control M-L cell fate. There are only a few morphogens that have been proven to regulate M-L patterning in trunk mesoderm. These are BMP4 and FGF9. BMP4 is expressed in the lateral plate mesoderm and promotes lateral plate mesoderm development, whereas noggin (NOG)-mediated antagonism of BMP signaling is required for paraxial mesoderm while a low concentration of BMP4 induces the intermediate mesoderm²⁷. FGF9 is specifically expressed in the intermediate mesoderm from the caudal through to the rostral trunk region²⁸ and effectively directs the differentiation of hPSC-derived primitive streak to the intermediate mesoderm⁹. In our protocol, FGF9 is sufficient to specify the intermediate mesoderm without using NOG (Fig. 1).

Kidney organoid—The kidney functions as a three-dimensional organ, hence the culture conditions for differentiation need to switch from monolayer to three-dimensional for the cells to form intact renal structures. While continued 2D culture may be adequate to induce specific target cell types, as we have previously demonstrated⁹, the process of aggregation provides an increased cell density and volume within which cells are able to positionally reorganise with respect to each other. We chose day 7 of differentiation to transfer cells from a culture plate onto a transwell filter as an aggregate grown at an air-media interface. Day 7 represents the stage of intermediate mesoderm which is considered to include not only progenitors of ureteric epithelium and metanephric mesenchyme but also progenitors of renal interstitium and endothelium²³. This methodology of aggregation culture has been previously optimized for *ex vivo* culture of mouse embryonic kidneys²⁰ and reaggregations of mouse embryonic kidney cells²⁹. A proper three-dimensional environment allows this kidney progenitor mixture to undergo self-renogenesis to form a kidney organoid.

Alternative methods for generating kidney tissues

Several studies in which kidney progenitors were induced from hPSCs have been reported. Xia et al. generated CK8-positive ureteric bud progenitor-like cells from hiPSCs and showed that those cells could integrate into ureteric epithelium in a re-aggregate with mouse embryonic kidney cells³⁰. Other studies differentiated monolayer hPSCs into SIX2-positive metanephric mesenchyme that developed to renal tubules^{31–33}. Taguchi and colleagues carefully investigated the developmental process of the metanephric mesenchyme commitment in mice and used that knowledge to obtain metanephric mesenchyme cells from mouse ESCs and human iPSCs based on an embryoid body culture method³⁴. Their protocol focused on inducing the posterior intermediate mesoderm in order to obtain only the metanephric mesenchyme without collecting duct, renal interstitial and endothelial cells. These induced metanephric mesenchyme cells could differentiate into not only renal tubules but also glomeruli by being combined with mouse dorsal spinal cord, a source of WNT signals known to induce nephrogenesis from the metanephric mesenchyme. Their follow-up

study showed that these nephrons could become vascularized by incorporating host blood vessels when transplanted under a mouse renal capsule³⁵. Morizane et al. optimized the differentiation protocol using monolayer hPSCs to maximally induce the metanephric mesenchyme with 90% efficiency³⁶. Hence, similarly to the above protocol, this did not induce collecting ducts and other non-epithelial renal cell types. The induced metanephric mesenchyme cells were also able to develop into nephron structures including renal tubules and glomeruli when stimulated by canonical WNT signaling using CHIR99021, a WNT agonist. Generated renal tubules revealed cell death in response to nephrotoxicants, cisplatin and gentamicin. Freedman et al. employed an approach in which they started the differentiation from hPSC-derived epiblast spheroids by sandwiching hPSCs between two layers of dilute Matrigel³⁷. Epiblast spheroids underwent epithelial-to-mesenchymal transition (EMT) to form a monolayer, followed by a mesenchymal-to-epithelial transition (MET) when reaggregated, resulting in the formation of renal tubules, glomeruli and endothelial cells.

While all these studies exclusively induced either the ureteric epithelium or metanephric mesenchyme and their derivative cell types, our unique methodology generates both cell types at the same time⁹. In addition, our optimized protocol enables the generation of kidney organoids that contain not only the collecting duct and nephrons but also renal interstitium and an endothelial network¹⁶. Within the organoids, individual nephrons segment into distal tubules, early loops of Henle, proximal tubules and glomeruli containing podocytes that elaborate foot processes and can undergo vascularization. Such segmented nephrons are connected with collecting ducts and surrounded by renal interstitium and an endothelial network. This protocol is clearly distinguished from others, as all anticipated cell types are simultaneously developed in the organoids.

Applications and limitations of the protocol

The differentiation of hPSCs using this protocol recapitulates the developmental process of human kidney organogenesis. Therefore, this protocol can be used as a platform for a variety of studies such as understanding human development, modeling renal disease and nephrotoxic drug screening. For instance, we have used this protocol to investigate mediolateral (M-L) and anteroposterior (A-P) patterning during trunk mesoderm development^{9,16,38}. Also, as kidney organoids contain all components of the kidney, kidney organoids can be used for studying development of each distinct cell type present within the human kidney.

Kidney organoids can be also utilized to test renal tubular damage in response to nephrotoxic drugs. We have confirmed proximal tubule specific cell death in kidney organoids by adding 5-20 μ M cisplatin to the culture medium for 2 days¹⁶. This demonstrated the presence of proximal tubules which are sufficiently mature to appropriately respond to cisplatin, presumably as a result of the presence of basolateral organic cation transporter 2 (OCT2) and copper transporter 1 (CTR1) that mediate uptake of cisplatin into these tubular cells^{39,40}. Hence, kidney organoids should be of value for the screening of pharmaceuticals.

Modeling genetic renal disease using kidney organoids is another useful application. Kidney organoids using iPSCs that are generated from readily accessible somatic cells (e.g. skin fibroblasts or leucocytes) from inherited kidney disease patients may recapitulate features of these renal disorders *in vitro*³⁷. Such disorders include autosomal dominant / recessive polycystic kidney disease (PKD), medullary cystic kidney (MCKD), nephronophthisis (NPHP), Alport syndrome and Bartter syndrome⁴¹. However, considering the likely variability in forming kidney organoids between iPSC lines, it will be desirable to compare mutant iPSC to a proper isogenic control iPSC line. This can be done using genome-editing tools like CRISPR/Cas9 system to artificially generate a specific genetic mutation in an iPSC line³⁷. It may be further ideal to correct the mutation in patient-derived iPSCs, again using CRISPR/Cas9 technology, in order to generate an isogenic control iPSC line. This will only be possible in instances where the underlying mutation has been identified.

Despite significant possibilities, the protocol has limitations and is likely to benefit from further protocol improvements. Kidney organoids at day 18 in three-dimensional culture, while transcriptionally similar to the first trimester human kidney¹⁶, have not matured to the level of the adult kidney. As consistent with a previous study of Cadherin expression in the developing mouse kidney^{42,43}, we showed that more mature proximal tubules in kidney organoids expressed ECAD¹⁶. Cultured human proximal tubule cells also express ECAD⁴⁴, however, some reports suggest that terminally differentiated proximal tubules in adult human kidney reveal no or very low ECAD expression⁴⁵. This protocol has not generated kidney organoids that reach an adult stage of maturation. This is evident by a lack of expression of some mature renal tubule markers and capillary loops do not form in most glomeruli. Hence, it is highly likely that organoid-culture conditions may require further optimization to reach maturation. This immaturity may be problematic when studying disease modelling because most aforementioned inherited kidney diseases develop after birth, some not until adulthood. Also, it is important to note that the kidney organoid is not a kidney in miniature form from the anatomical and functional point of view. The mature kidney filters the blood to form a urinary filtrate before reclaiming the majority of this fluid to finally produce 1 to 1.5 litres of urine a day. During this process, the kidney also removes nitrogenous and toxic wastes, regulates electrolytes, maintains acid–base balance and regulates blood pressure, both via selective tubular reabsorption, secretion and hormone production. To recapitulate such diverse functions would require hPSC-derived kidney organoids not only to be reasonably big and comprised of mature cell types, but also develop a vascular access and a single exit path for urine, both of which are currently missing in current kidney organoids.

Experimental Design

hPSC culture for differentiation—This protocol can be applied to both hESC and hiPSC. We have successfully applied the method described here to both hESCs (HES-3, commercially available) and hiPSCs (CRL1502 clone C32) that were authenticated and tested for mycoplasma infection⁴⁶. Cells were maintained on mouse embryonic fibroblast (MEF) feeder layer and passaged using TrypLE Select. However, before the differentiation began, cells were adapted to feeder-free conditions with MEF-conditioned medium and Matrigel-coated culture dishes. This is to eliminate MEFs from the differentiation culture to avoid any unknown influence of MEFs to the differentiation. The differentiation begins with

hPSC density at approximately 40-50% confluency (step 24), which can be obtained by plating about 15,000 cells / cm² the day before (step 23). This plating cell number depends on the cell cycle speed and recovery rate, hence, should be adjusted for each hPSC line/clone.

Intermediate mesoderm induction—Although the intermediate mesoderm is the common origin of all known kidney progenitors, different regions in the intermediate mesoderm along with A-P axis contribute to distinct types of kidney progenitor. The anterior intermediate mesoderm (GATA3⁺) gives rise to the ureteric epithelium and the posterior intermediate mesoderm (HOXD11⁺) develops into the metanephric mesenchyme^{34,47,48}. In an embryo, A-P patterning in the intermediate mesoderm is associated with primitive streak cell migration in which cells migrate from caudal to rostral during which they are exposed to canonical WNTs, followed by FGF9 and RA³⁸. Hence, earlier migrating cells, which are exposed to WNTs for a shorter period of time, give rise to the anterior intermediate mesoderm, whereas cells migrating late are exposed to WNTs for longer and form the posterior intermediate mesoderm. In the protocol, hPSCs are treated with GSK-3 inhibitor, CHIR99021 to induce canonical WNT signaling (Fig. 1). This differentiates hPSCs into the posterior primitive streak that expresses *T* and *MIXL1* with minimum expression of *SOX17*, a marker of the anterior primitive streak⁹. CHIR99021 is added to hPSC culture for 3 to 4 days, which is an intermediate duration designed to induce both the anterior and the posterior intermediate mesoderm at the same time (Fig. 1). In this protocol, we employ a CHIR99021 Conversely, a shorter (less than 3 days) or longer (more than 4 days) phase of CHIR99021 results in the predominant induction of the anterior or posterior intermediate mesoderm, respectively. The medium period of CHIR99021 may vary depending on hPSC clones and lines to use, therefore it needs to be optimized not to obtain one-sided intermediate mesoderm population of either GATA3⁺ or HOXD11⁺ at day 7 of the differentiation¹⁶. As generally seen in human hPSC differentiation, each hPSC line or clone has its own propensity to develop into a certain cell fate easier than to the other lineages⁴⁹ and we experienced in the initial step of the differentiation that distinct clones respond to CHIR99021 differently. To generate kidney organoids reproducibly, it is recommended to test the protocol once with distinct hES/iPSC lines or distinct hiPSC clones even when derived from the same individual, and choose the most responsive lines or clones to use thereafter. Hence, an optimal CHIR99021 concentration and differentiation time period may be required for each line. e.g. a range of 2-5 days of CHIR99021 period first, then concentrations between 6 to 10 μ M CHIR99021 if necessary.

Kidney organoid formation—The kidney progenitor aggregate includes the ureteric epithelium and the metanephric mesenchyme, hence their reciprocal interaction spontaneously initiates nephrogenesis. However, to maximize nephron formation, the protocol stimulates the organoids with a high concentration CHIR99021 for 1 hour. After the CHIR99021 pulse, the organoids should be supplemented with FGF9 to maximize the development of nephrons. We found 5 additional days of FGF9 supplementation was sufficient to obtain maximal nephrogenesis. Subsequently, the organoids are cultured in growth factor-free APEL medium⁵⁰ for further self-organization (Fig. 1). The resulting

organoids are regarded as successful based upon the presence of appropriately segmented nephrons and surrounding stromal and endothelial cell populations.

MATERIALS

REAGENT

- Human embryonic stem cells. We have used HES-3 (Wicell Research Institute Inc, cat. no. ES03) ! **CAUTION** Experiments using hPSCs must conform to all relevant governmental and institutional regulations relating to human ethics, biosafety and genetic modification This work was approved by the MCRI Institutional Biosafety Committee (212-2014PC2).It is recommended to check regularly to ensure cells are chromosomally stable and are not infected with mycoplasma.
- Human induced pluripotent stem cells. We have used CRL1502 clone C32 (gifted from Dr. Ernst Wolvetang, The University of Queensland, Australia) ! **CAUTION** Experiments using hPSCs must conform to all relevant governmental and institutional regulations relating to human ethics, biosafety and genetic modification. This work was approved by the Royal Childrens Hospital human ethics committee (HREC/14/QRBW/34; HREC/15/QRCH/126) and the MCRI Institutional Biosafety Committee (212-2014PC2). It is recommended to check regularly to ensure cells are chromosomally stable and are not infected with mycoplasma.
- 2-Mercaptoethanol (55 mM) (Thermo Fisher Scientific, cat. no. 21985-023)
- Antibiotic-Antimycotic (Thermo Fisher Scientific, cat. no. 15240-062)
- bFGF (Merck, cat. no. GF003-AF)
- BSA (Sigma Aldrich, cat. no. A3311-10G)
- CHIR99021 (R&D, cat. no. 4423/10)
- DMEM high glucose (Thermo Fisher Scientific, cat. no. 11995-073)
- DMEM/F-12 (Thermo Fisher Scientific, cat. no. 11320-082)
- DMSO (Sigma Aldrich, cat. no. D5879)
- DTT, 100 mM (Promega, cat. no. P1171)
- Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific, cat. no. 14190-144)
- FGF9 (R&D, cat. no. 273-F9-025)
- Foetal Bovine Serum (FBS) (Interpath Services, cat. no. SFBSF)
- Gelatin (Sigma Aldrich, cat. no. G9391)
- GlutaMAX Supplement (Thermo Fisher Scientific, cat. no. 35050-061)

- Glycerol (Sigma Aldrich, cat. no. G9012-500ML)
- Heparin (Sigma Aldrich, cat. no. H4784-250MG)
- hESC-qualified Matrigel, LDEV-Free (Corning, cat. no. 354277)
- Knockout Serum Replacement (Thermo Fisher Scientific, cat. no. 10828-028)
- Non-Essential Amino Acids (Thermo Fisher Scientific, cat. no. 11140-050)
- Penicillin/Streptomycin (Thermo Fisher Scientific, cat. no. 15070-063)
- Recombinant human serum albumin, 10% (wt/vol) (Novozymes, 230-005)
- STEMdiff APEL Medium (Stem Cell Technologies, cat. no. 5210)
- TrypLE Select (Thermo Fisher Scientific, cat. no. 12563-029)
- Trypsin EDTA, 0.05% (Thermo Fisher Scientific, cat. no. 25300-054)
- Paraformaldehyde (Sigma-Aldrich, cat. no. 158127)
- Triton X-100 (TritonX) (Sigma-Aldrich, cat. no. T9284-500ML)
- Donkey serum (Merck Millipore, cat. no. S30-100ML)
- DAPI (6-Diamidino-2-Phenylindole Dihydrochloride) (Thermo Fisher Scientific, cat. no. D1306)
- Appropriate antibodies to detect proteins of interest (see Table 1 for primary antibodies we have successfully used).

EQUIPMENT

- 175 cm² tissue culture flask (Nunc, cat. no. 159910)
- 25 cm² tissue culture flask (Nunc, cat. no. 156367)
- 6-well transwell cell culture plate, 0.4 µm pore polyester membrane (Corning, cat no. 3450)
- Benchtop centrifuge (Thermo Scientific, Heraeus Pico 17)
- Biological safety cabinet (LAF Technologies, TOP-SAFE 1.2)
- CO₂ incubators (Thermo Scientific, Heracell 150i)
- Conical tubes, 15 ml (Corning, cat. no. 352096)
- Conical tubes, 50 ml (Corning, cat. no. 352070)
- Freezing container (Nalgene, Mr. Frosty)
- Glass-Bottom Dish, 35mm (Mattek, cat. no. P35G-0-14-C)
- Inverted contrasting tissue culture microscope (Nikon, TS100)
- Laser confocal microscope (Zeiss, LSM780)

- Media storage bottle, 500 ml (Corning, cat. no. 430282)
- Pipetboy (Integra Biosciences, PIPETBOY pro)
- Pipettes (Gilson, P10, P200 and P1000 PIPETMAN Classic)
- Serological pipettes, 10 ml (Corning, cat. no. 357551)
- Stericup 0.22 μ m filter unit (Merck Millipore, cat. no. SCGPU05RE)
- Sterile filter pipette tips (1000, 200, wide bore 200 and 10 μ l; Molecular BioProducts, cat. nos. 3581, 3551, 3531 and 3501, respectively)
- Sterile microcentrifuge tube, 1.5 ml (Eppendorf, cat. no. 3810X)
- Swing-out rotor centrifuge (Eppendorf, Centrifuge 5702)
- Syringe-driven 0.22 μ m filter unit (Merck Millipore, cat. no. SLMP025SS)
- Ultrapure water generation system (Sartorius, arium pro)
- Water bath, 37 °C (Thermoline, TWB-24D)

REAGENT SETUP

Matrigel-coated 25 cm² tissue culture flask—To prepare Matrigel-coated 25 cm² tissue culture flask, add 30 μ l of hESC-qualified Matrigel into a 15 ml tube containing 3 ml of chilled DMEM/F-12. Mix it well and transfer it into a 25 cm² tissue culture flask. Keep the flask at room temperature, 15 to 25 °C, for at least 30 min to allow Matrigel to coat the surface. Use the flask within a day.

▲ **CRITICAL STEP** Handle Matrigel on ice as it solidifies when warmed over 16 °C. P200 pipette tips should be also pre-chilled before use.

bFGF (10 μ g ml⁻¹)—Centrifuge the tube briefly before opening. Reconstitute to 10 μ g ml⁻¹ in a filtered solution of 0.5% (wt/vol) BSA, 1 mM DTT and 10% (vol/vol) glycerol in DPBS. Aliquot it into appropriate amounts and store them at –80°C for up to 6 months. bFGF can be stored at 4 °C for up to 2 weeks once it is thawed.

FGF9 (100 μ g ml⁻¹)—Centrifuge the tube briefly before opening. Reconstitute to 100 μ g/ml in filtered DPBS containing 0.1% (wt/vol) human serum albumin. Aliquot it into appropriate amounts and store them at –80°C for up to 6 months. FGF9 can be stored at 4 °C for up to 2 weeks once it is thawed.

Heparin solution (1 mg ml⁻¹)—Reconstitute to 1 mg ml⁻¹ in ultrapure water and filter sterilize it through a polyethersulfone (PES) 0.22 μ m syringe-driven filter unit. Heparin solution can be stored at 4 °C for more than 12 months.

CHIR99021 (10 mM)—Centrifuge the tube briefly before opening. Reconstitute 10 mg of CHIR99021 into 2.149 ml of DMSO to make 10 mM stock. Aliquot it into appropriate amounts and store them at –20°C.

Gelatin solution (0.1%, wt/vol)—For 500 ml of 0.1% gelatin solution, dissolve 0.5 g of gelatin in 500 ml of ultrapure water or DPBS and autoclave it. The solution can be stored at 4 °C for up to 3 months.

Mouse Embryonic Fibroblast (MEF) culture medium—For preparing 500 ml of MEF culture medium, combine 442.5 ml of DMEM high glucose, 50 ml of foetal bovine serum, 5 ml of GlutaMAX-1 and 2.5 ml of Penicillin/Streptomycin. Filter sterilize the medium through a polyethersulfone (PES) 0.22 µm vacuum-driven filter unit and store it at 4 °C for up to 2 weeks.

hESC medium—For preparing 500 ml of hESC medium, combine 386.5 ml of DMEM/F-12, 100 ml of Knockout serum replacement, 5 ml of GlutaMAX-1, 5 ml of Non-Essential Amino Acids, 2.5 ml of Penicillin/Streptomycin and 1 ml of 2-Mercaptoethanol (55 mM). Filter sterilize media through a polyethersulfone (PES) 0.22 µm vacuum-driven filter unit and store it at 4 °C for up to 2 weeks. To ensure bFGF is fresh, only supplement the medium with 10 ng ml⁻¹ bFGF just before use for hPSC maintenance.

MEF-conditioned hESC medium—Seed 1×10^7 mitotically inactivated MEFs onto a 175 cm² tissue culture flask containing 40 ml of MEF culture medium. Next day, change the culture medium to 40 ml of hESC medium without bFGF. After overnight culture, collect the conditioned medium into a 500 ml bottle and store it at 4 °C. Feed MEFs again with 40 ml of fresh hESC medium. Repeat this cycle another 6 times to pool 280 ml of the conditioned medium in total. Filter sterilize the medium through a polyethersulfone (PES) 0.22 µm vacuum-driven filter unit and aliquot it into 50 ml tubes. Store tubes at -20°C for up to 6 months. MEF-conditioned hESC medium can be stored at 4 °C for up to 2 weeks once it is thawed. To ensure bFGF is fresh, only supplement the medium with 10 ng ml⁻¹ bFGF just before use for hPSC maintenance.

APEL medium—Once a bottle of APEL (100 ml volume) is opened, add 0.5-1 ml of Antibiotic-Antimycotic and store it at 4 °C for up to 2 weeks.

Frozen stock of human pluripotent stem cells (hPSCs)—For expanding hPSCs before cryopreserving them, cells are cultured in hESC medium supplemented with 10 ng ml⁻¹ bFGF on mitotically inactivated MEF feeder layer by a single-cell culture method in which cells are passaged using TrypLE Select, as described in procedure steps 1-9. Once hPSCs reach the desired number, harvest cells using TrypLE Select, centrifuge (×400g for 3 min) and resuspend them in 10% DMSO / 90% FBS. Split cell-suspension into cryo-vials for 1 ml per vial. Typically, hPSCs of 100% confluent in a 75 cm² tissue culture flask are split into 9 cryo-vials, so that each cryo-vial contains $1.5\text{--}2.0 \times 10^6$ cells. Freeze vials in a freezing container at -80 °C overnight and subsequently store them in liquid nitrogen.

▲ **CRITICAL** We have experienced a huge variability in differentiation success rate between experiments when cells were obtained from a continuously maintained cell culture pool. To minimize this variation, hPSCs should be thawed one by one from a cryopreserved stock for each experiment.

2% (wt/vol) paraformaldehyde (100 ml)—Add 2 g of PFA and 10 μ l of 10 N NaOH to 80 ml of DPBS at 60 °C and mix it until the powder dissolves. Adjust the final volume up to 100 ml by adding DPBS. Aliquot it into appropriate amounts and store them at –20 °C for up to one month. **! CAUTION** Paraformaldehyde is toxic and must be handled inside a fume hood with masks and goggles.

Blocking buffer (50 ml)—Dissolve 5 ml of donkey serum and 30 μ l of TritonX in 45 ml of PBST. Aliquot it into appropriate amounts. Blocking buffer can be stored at –20 °C for up to one year.

PROCEDURE

MEF feeder seeding • TIMING 2 h—1. Coat a 25 cm² tissue culture flask with 3ml of 0.1% gelatin solution. Incubate the flask in a 37 °C CO₂ incubator for 1h.

2. Prepare 10 ml of prewarmed MEF culture medium in a 15 ml conical tube.

3. Thaw a frozen vial of mitotically inactivated mouse embryonic fibroblasts (MEFs) in 37 °C water bath until a small ice pellet remains. Transfer MEFs into a 15 ml conical tube containing prewarmed MEF culture medium in a drop wise manner and centrifuge at $\times 400g$ for 3 min.

4. Remove supernatant and resuspend MEFs in MEF culture medium. Count cell number using a hemocytometer. Make cell-suspension of 3×10^5 cells in 5 ml of MEF culture medium. Remove gelatin and seed cells onto a gelatin coated 25 cm² tissue culture flask to obtain the density at 12×10^3 cells / cm² and incubate it overnight in a 37 °C CO₂ incubator.

Human PSC thawing • TIMING 30 min—5. Prepare 10 ml of prewarmed hESC medium in a 15 ml conical tube.

6. Thaw a frozen vial of hPSC containing $1.5\text{--}2.0 \times 10^6$ cells in 37 °C water bath until a small ice pellet remains. Transfer hPSCs into a 15 ml conical tube containing prewarmed DMEM/F-12 in a drop wise manner and centrifuge at $\times 400g$ for 3 min.

7. Remove supernatant and resuspend cells in 5 ml of hESC medium supplemented with 10 ng ml^{–1} bFGF.

8. Remove MEF culture medium from the flask containing mitotically inactivated MEFs and plate above hPSCs suspension. Incubate it overnight in a 37 °C CO₂ incubator.

Growing hPSCs on MEF feeder layer • TIMING 4-5 d—9. Change 5 ml of hESC medium supplemented with 10 ng ml^{–1} bFGF daily for 4-5 days. **▲ CRITICAL STEP** For optimal results, cells should be approximately 80-100% confluent after 4-5 days culture. If cells do not reach this confluency, allow them to grow for another day.

? TROUBLESHOOTING

Growing hPSCs on Matrigel • TIMING 2 d—10. Prepare Matrigel-coated 25 cm² tissue culture flask.

11. To passage hPSCs onto Matrigel, wash hPSCs on MEF feeder layer in 25 cm² tissue culture flask with 3 ml DPBS twice. Aspirate DPBS.

12. Add 1 ml of TrypLE Select to cells and incubate the flask at 37 °C for 3 min.

13. Pipette 11 ml of DMEM/F-12 to cells, mix and ensure cells have lifted off from the plastic surface. ▲ **CRITICAL STEP** Pipette cells no more than twice as hPSCs are very sensitive.

14. Collect 4 ml of cell suspension in a 15 ml tube to obtain a 1:3 split ratio and centrifuge it at $\times 400g$ for 3 min. Alternatively, a 1:2 split ratio can be chosen to achieve 70-100 % confluency at step 17.

15. Remove supernatant and add 5 ml of MEF-conditioned hESC medium supplemented with 10 ng ml⁻¹ bFGF to cells. Mix it gently.

16. Aspirate Matrigel-containing DMEM/F-12 from a prepared Matrigel-coated 25 cm² tissue culture flask and seed cells from step 15. Culture them in a 37 °C CO₂ incubator for 2 days with daily changing MEF-conditioned hESC medium supplemented with 10 ng ml⁻¹ bFGF.

Plating hPSCs for differentiation • TIMING 1 h—17. Wash hPSCs on Matrigel in 25 cm² tissue culture flask with 3 ml of DPBS twice. Aspirate DPBS.

18. Add 1 ml of TrypLE Select to cells and incubate at 37 °C for 3 min.

19. Pipette 10 ml of DMEM/F-12 to cells, mix and ensure cells have lifted off from the plastic surface. ▲ **CRITICAL STEP** Do not pipette cells more than twice as hPSCs are very sensitive.

20. Collect cell suspension into 15 ml tube. Count cell number using a hemocytometer.

21. Calculate cell suspension volume to achieve 375,000 cells.

22. Aliquot cells into a 15 ml tube. Centrifuge the tube at $\times 400g$ for 3 min.

23. Remove supernatant and resuspend cells in 4 ml of MEF-conditioned hESC medium supplemented with 10 ng ml⁻¹ bFGF. Seed cells onto a prepared Matrigel-coated 25 cm² tissue culture flask to obtain the density at 15×10^3 cells / cm². Culture them overnight in a 37 °C CO₂ incubator.

Inducing the intermediate mesoderm • TIMING 7 d—24. Aspirate MEF-conditioned hESC medium from the 25 cm² tissue culture flask.

▲ **CRITICAL STEP** Cells should reach to 40-50% confluent on this day. If not, adjust cell number of plating at Step 21.

25. Add 4 ml of APEL medium containing 8 μ M CHIR99021 to hPSCs.

26. Culture them in a 37 °C CO₂ incubator for 2-5 days, refreshing APEL medium containing 8 μ M CHIR99021 every 2 days.

▲ **CRITICAL STEP** Duration of CHIR99021 determines the ratio of collecting duct/nephron in the organoid. Shorter periods of CHIR99021 phase induce more anterior intermediate mesoderm whereas a longer periods results in the more posterior part. To obtain both compartments, 3 or 4 days of CHIR99021 is recommended.

? TROUBLESHOOTING

27. After CHIR99021 phase, change culture medium to 8 ml of APEL medium supplemented with 200 ng ml⁻¹ FGF9 and 1 μ g ml⁻¹ Heparin.

▲ **CRITICAL STEP** A shortage of FGF9 concentration and/or media volume causes an inefficient induction of the intermediate mesoderm.

28. Culture them in a 37 °C CO₂ incubator, refreshing medium every 2 days until day 7 of the differentiation counting step 24 as day 0 this includes both CHIR99021 phase and FGF9 phase

Forming aggregates for developing kidney organoids • TIMING 11-18 d—29.

Aspirate the culturing medium and wash with 3 ml of DPBS. Aspirate DPBS.

30. Add 1 ml of trypsin EDTA (0.05%) to cells and incubate them at 37 °C for 3 min.

31. Monitor under the microscope to make sure all cells have lifted from the surface after 2 min. If the cells are still attached to the surface, gently pipette the cells with trypsin and place back into the incubator for further 1 min.

32. Transfer cell suspension to a 15 ml tube containing 9 ml of MEF culture medium to neutralize trypsin. Centrifuge the tube at $\times 400g$ for 3 min.

33. Aspirate the supernatant and resuspend the cells with 3 ml of APEL medium.

34. Take out 10 μ l of cell suspension and perform a cell count with a hemocytometer.

35. Each organoid will have roughly 5×10^5 cells. Aliquot the required amount of cell suspension into a 1.5 ml microcentrifuge tube. Centrifuge the tube at $\times 400g$ for 2 min to make a cell pellet.

36. Aliquot 1.2 ml of APEL containing 5 μ M CHIR99021 into a 6-well transwell cell culture plate. The transwell filter attaches on the surface of the medium.

37. Pick a pellet up by using a P1000 or P200 wide bore tip.

? TROUBLESHOOTING

38. Carefully place pellets onto the 6-well transwell filter with minimal APEL medium carry over. Incubate pellets at 37 °C for 1 h.

39. After 1 h incubation, remove the medium of APEL containing 5 µM CHIR99021, and use 1.2 ml of APEL medium supplemented with 200 ng ml⁻¹ FGF9 and 1 µg ml⁻¹ Heparin.

40. Culture pellets for 5 days, refreshing FGF9 and Heparin-containing APEL medium every two days.

? TROUBLESHOOTING

41. After 5 days, change the culture medium to APEL medium without FGF9 and Heparin supplemented.

42. Culture the organoids for further 6 to 13 days, refreshing APEL medium every two days. If you wish to proceed to whole mount immunofluorescence, proceed to the next section.

Whole mount immunofluorescence • TIMING 3 d—43. After 18 days of organoid culture, if desired, fix the pellets in the transwell cell culture plate with 2% (wt/vol) paraformaldehyde at 4 °C for 20 minutes.

44. Remove the paraformaldehyde and wash three times with DPBS.

■ **PAUSE POINT** Once fixed and washed, organoids can be stored at 4 °C for several months before immunofluorescence staining.

45. Aliquot around 150 µl of blocking buffer (10% donkey serum / 0.3% TritonX / DPBS) into the MatTek Glass Bottom Dishes.

46. Carefully cut the organoids off the filter and submerge the filter into the blocking buffer.

47. Block the organoids at room temperature for 2-3 h, gently keep shaking the dish using a rocker during incubation.

48. Prepare primary antibodies of choice in blocking buffer (0.3% TritonX / 10% Donkey serum / DPBS) with appropriate dilutions (Table 1).

49. Aspirate the blocking buffer off the MatTek dish, and aliquot 150 µl of blocking buffer containing primary antibodies into the MatTek dish.

50. Incubate the organoids with primary antibody solution at 4 °C over-night.

▲ **CRITICAL STEP** Make sure the organoid is completely submerged into the solution. Preferably, gently keep shaking the dish using a rocker during incubation.

51. Aspirate the primary antibody solution off the dish and wash with PBTX (0.3% TritonX / DPBS) for 6 times, 10 minutes each with gentle shaking.

52. Prepare secondary antibodies (1:400 dilution) of choice in PBTX.

- 53.** Aspirate PBTX off the MatTek dish, and aliquot 150 μ l of PBTX containing secondary antibodies into the MatTek dish.
- 54.** Incubate organoids in secondary antibody solution at 4 °C over-night.
- 55.** Remove secondary antibody solution and incubate with DAPI (1:1000 dilution) in DPBS for 3 h.
- 56.** Wash organoids with DPBS for 10 min, 3 times.
- 57.** Image organoids using a laser confocal microscope.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

• TIMING

Steps 1-4, MEF feeder seeding: 2 h (Begin 9 days before hPSC differentiation at step 24)

Steps 5-8, Human PSC thawing: 30 min

Step 9, Growing hPSCs on MEF feeder layer: 4-5 d

Steps 10-16, Growing hPSCs on Matrigel: 2 d

Steps 17-23, Plating hPSCs for differentiation: 1 h

Steps 24-28, Inducing the intermediate mesoderm: 7 d (Day 0 to 7)

Steps 29-42, Forming aggregates for developing kidney organoids: 11-18 d (Day 7 to 25)

Steps 43-57, Whole mount immunofluorescence: 3 d

ANTICIPATED RESULTS

This protocol produces kidney organoids from hPSCs with a high success rate. Success is defined as organoids containing segmented nephrons comprised of collecting duct, renal interstitium and endothelium. We have successfully generated organoids from both human ES cell lines (HES-3) and human iPSC lines (CRL1502 clone C32) and a number of patient-derived iPSC lines derived in house (unpublished). In addition, there is little variation between organoids within an experiment¹⁶.

When the differentiation starts with CHIR99021 (Fig. 1 **day 0**), hPSCs on Matrigel should retain pluripotency as indicated by expression of stem cell markers such as OCT4 and NANOG, and with the anticipated morphology of a big nucleus, although defined borders of colonies are typically not seen at this stage (Fig. 2a,b). After 2 days of CHIR99021 exposure, colonies break apart into single cells with the morphology of spiky, typically triangular, shape (Fig. 2c). This is evidence of the epithelial-to-mesenchymal transition (EMT) expected of hPSCs differentiating into the primitive streak. Once the 3-4 days of

CHIR99021 phase ends (Fig. 2d) and the culture medium contains FGF9, cells start growing rapidly to reach confluence by day 7 of differentiation. The surface of the cell-layer at day 7 typically looks ‘hilly’ due to the contrast between thin and thick cell-layers (Fig. 2e). Occasionally cells become balls by day 7, which indicates the initial CHIR99021 duration or CHIR99021 concentration should be slightly shortened or reduced for an optimal result. If immunofluorescence on this day shows predominant population of either GATA3 or HOXD11-positive cells, adjust days of CHIR99021 to obtain both populations (Fig. 3a)¹⁶. The size of pellets comprised of 5×10^5 cells is approximately 2 mm in diameter (Fig. 2f). This pellet initiates nephrogenesis with developing renal vesicles in 3 days under the three-dimensional culture (organoid culture) conditions. The presence of renal vesicles can be confirmed under a tissue culture microscope (Fig. 2g,h). In the case of a failure of nephrogenesis, organoids look like a pancake without formation of any complex structures. After 11 days of organoid culture, if the differentiation has worked properly, obvious formation of glomeruli as well as tubular structures can be observed (Fig. 2i,j). The resulting kidney organoid will grow up to 6 mm in diameter by 18 days of organoid culture (Fig. 1 day 25).

To evaluate tissues in kidney organoids, whole mount immunofluorescence can be performed (**steps 44-58**). Within the organoid, a number of different kidney cell types are visible (Table 1), including collecting ducts marked by PAX2, GATA3 and ECAD staining (Fig. 3b), loops of Henle marked by UMOD staining (Fig. 3c), proximal tubules marked by LTL and cubilin (CUBN) staining (Fig. 3d), the podocyte of the glomerulus marked by WT1 and NPHS1 staining (Fig. 3f) and basement membrane in glomeruli marked by LAM (Fig. 3g **arrowheads**), early mesangial cells marked by PDGFRA staining (Fig. 3h), distal tubule marked by ECAD staining in the absence of co-staining with either LTL or GATA3 (Fig. 3f), medullary interstitium marked by MEIS1 alone and cortical interstitium marked by MEIS1 and FOXD1 staining⁵¹ (Fig. 3e) and an endothelial network marked by CD31 (PECAM), KDR and SOX17 staining (Fig. 3h,i). Endothelial networks tend to run in between nephrons and also on the surface of the organoid (Fig. 3j). 4-color staining using NPHS1, LTL, ECAD and GATA3 antibodies/lectin enables to identify segmented nephrons in the kidney organoid (Fig. 3k). Typically, bigger sized kidney organoids develop nephrons densely in the peripheral region and have renal interstitium and endothelial network to grow in the middle (Fig. 3l).

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Editorial summary

This protocol describes stepwise differentiation of human pluripotent stem cells into 3D kidney organoids that contain segmented nephrons connected to collecting ducts, surrounded by renal interstitial cells and an endothelial network

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Figure 1. Schematic diagram of the timeline for generating kidney organoids from hPSCs
The protocol is based upon a step-wise differentiation protocol with sequential changing of culture media. hPSCs are cultured in MEF-CM (MEF-conditioned hESC medium). Differentiation begins in APEL/CHIR99021 (APEL medium supplemented with 8 μ M CHIR99021) followed by APEL/FGF9/Heparin (APEL medium supplemented with 200 ng ml⁻¹ FGF9 and 1 μ g ml⁻¹ heparin) and all growth factors are withdrawn in the final step. A pulse of CHIR99021 (5 μ M) stimulates nephrogenesis in the organoids.

Figure 2. Bright field images of hPSCs differentiation to kidney organoids

Bright field images of the cells during differentiation. **a**, hPSC colonies on MEF feeder layer at day -3, before transfer onto Matrigel. **b**, hPSCs on Matrigel at day 0 before adding CHIR99021 to the medium. **c**, hPSCs differentiated into the posterior primitive streak cells with CHIR99021 induction showed a triangular shaped cell morphology. **d**, Monolayer culture at day 4 of differentiation **e**, Cells at day 7 of differentiation. Ideally, cultures nearly reach confluence at this time and form distinct areas of thick and thin layers across the surface. **f**, At day 7 of differentiation, cells were trypsinized and centrifuged to form an aggregate. This panel represents an aggregate at 30 min after transfer to transwell filter. **g,h**, A whole kidney organoid at day 10 of differentiation (3 days in organoid culture). A black square indicates the field of the right panel (h), which shows the formation of small renal vesicles. **i,j**, A whole kidney organoid at day 25 of differentiation (18 days in organoid culture). A black square indicates the field of the right panel (j). If the differentiation has been successful, glomerular structures (gl) can be recognized under microscope. Scale bars, 200 μ m (**a-e**), 1 mm (**f,g,i**), 100 μ m (**h,j**).

Figure 3. Immunological characterization of structures within kidney organoids

a, Staining for anterior intermediate mesoderm cells (GATA3) and posterior intermediate mesoderm cells (HOXD11) on day 7 of the differentiation (Step 29). **b-l**, Whole mount immunostaining of kidney organoids (Step 57). **b**, Co-staining for markers of collecting ducts (PAX2, GATA3 and ECAD). **c**, Staining for loop of Henle (UMOD and ECAD). **d**, Staining for proximal tubule markers (LTL and CUBN) shows evidence of an apical brush border (CUBN). **e**, Staining for cortical interstitium (MEIS1 and FOXD1, a white arrowhead) and medullary interstitium (MEIS1 only). FOXD1 also marks podocytes (WT1). **f**, Evidence for the formation of segmenting nephrons showing staining for early podocytes (WT1 and NPHS1), distal tubules (ECAD) and intervening unstained segments at day 18 of differentiation. An involute cluster of podocytes can be seen at the end of a forming nephron (center). **g**, At day 25 of differentiation, maturing glomeruli develop glomerular basement membrane (LAM, blank arrowheads) in the middle of podocytes (NPHS1). **h**, Staining for early mesangial cells (PDGFRA) invaginating into podocytes (NPHS1). **i**, Staining for the endothelial network (CD31 and SOX17). **j**, Staining of a representative whole mount kidney organoid illustrating the endothelial network (CD31) and glomerular podocytes (NPHS1). Cell nuclei stained by DAPI are shown in the inset panel. **k**, An example of a small whole kidney organoid (< 3 mm). **l**, An example of a larger kidney organoid (> 4 mm). Scale bars, 50 μm (**a-i**), 1 mm (**j-l**).

Table 1

Antibodies for tissue characterization

Cell type	Antigen	Host	Supplier	Cat. no.	Dilution
Posterior IM	HOXD11	Mouse	Santa Cruz Biotechnology	sc-192	1:300
Anterior IM and collecting duct	GATA3	Goat	R&D Systems	AF2605	1:300
Renal tubules and collecting duct	PAX2	Rabbit	Zymed Laboratories	71-6000	1:300
Maturing proximal, tubule, distal tubule and collecting duct	ECAD	Mouse	BD Biosciences	610181	1:300
Loop of Henle	UMOD	Rabbit	Biomedical Technologies	BT-590	1:300
Proximal tubule	LTL	Biotin-conjugated	Vector Laboratories	B-1325	1:300
Proximal tubule	CUBN	Rabbit	Santa Cruz Biotechnology	sc-20607	1:150
Podocyte	WT1	Rabbit	Santa Cruz Biotechnology	sc-192	1:100
Podocyte	NPHS1	Sheep	R&D Systems	AF4269	1:300
Endothelium	CD31	Mouse	BD Pharmingen	555444	1:300
Endothelium	KDR	Rabbit	Cell Signaling Technology	2479	1:300
Endothelium	SOX17	Goat	R&D Systems	AF1924	1:300
Early mesangium	PDGFRA	Mouse	BD Pharmingen	556001	1:200
Basement membrane	LAM	Rabbit	Sigma-Aldrich	L9393	1:300
Renal interstitium	MEIS1/2	Mouse	Activemotif	ATM39795	1:300
Podocyte and renal interstitium	FOXD1	Goat	Santa Cruz Biotechnology	sc-47585	1:200

Table 2

Troubleshooting table

Step	Problem	Possible reason	Solution
9	Cells do not recover or grow slowly after thawing	Unhealthy cells at cryopreservation.	Cryopreserve healthy cells. Alternatively, Rho kinase inhibitor (Y-27632) can be supplemented to hESC medium at 10 μ M for 24 h post thawing.
26	Cells come off the plastic during CHIR99021 phase	Cell density is too low	Plate more cells at Step 23.
37	Pellets break up during a transfer	Not enough centrifuging	Perform two sequential centrifugations of $\times 400g$ for 2 min. Rotate the tube 180° before the second spin at Step 35. Up to four sequential centrifugations can be performed.
40	No nephrogenesis happens	Damaged cells due to centrifuge	If centrifugations are repeated multiple times at Step 37, reduce the number of times or duration.
		Intermediate mesoderm induction fails	Confirm FGF9 activity is intact. Use freshly thawed FGF9. Use more than 0.32 ml of FGF9/Heparin/APEL medium per cm^2 at Step 27.
		Differentiation fails	Confirm if posterior intermediate mesoderm markers, HOXD11, are positive at step 29 (Fig. 3a). If they are negative, modify culture conditions for your cells; e.g. initial cell density at Step 23, CHIR99021 concentration or CHIR99021 duration at Step 25, 26.