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PROTOCOL

An efficient method to generate kidney organoids at the air-liquid interface

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Abbreviations used: AF488, alexa flour 488; CKD, chronic kidney disease; ESKD, end stage kidney disease; hESCs, human embryonic stem cells; PFA, paraformaldehyde; TCA, time-dependent cell-fate acquisition

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ABSTRACT

The prevalence of kidney dysfunction continues to increase worldwide, driving the need to develop transplantable renal tissues. The kidney develops from four major renal progenitor populations: nephron epithelial, ureteric epithelial, interstitial and endothelial progenitors. Methods have been developed to generate kidney organoids but few or dispersed tubular clusters within the organoids hamper its use in regenerative applications. Here, we describe a detailed protocol of asynchronous mixing of kidney progenitors using organotypic culture conditions to generate kidney organoids tightly packed with tubular clusters and major renal structures including endothelial network and functional proximal tubules. This protocol provides guidance in the culture of human embryonic stem cells from a National Institute of Health-approved line and their directed differentiation into kidney organoids. Our 18-day protocol provides a rapid method to generate kidney organoids that facilitate the study of different nephrological events including *in vitro* tissue development, disease modeling and chemical screening. However, further studies are required to optimize the protocol to generate additional renal-specific cell types, interconnected nephron segments and physiologically functional renal tissues.

Keywords: air-liquid interface, organotypic culture, kidney organoids, kidney progenitors, proximal tubule

BACKGROUND

Chronic kidney disease (CKD) is a worldwide healthcare problem with 13.4% global estimated prevalence [1]. Approximately 10% of the United States adult population has CKD [2]. CKD strongly predisposes to end stage kidney disease (ESKD) when kidney function falls below the threshold for maintenance of basic physiological function. There is no curative treatment available for patients with ESKD except renal transplantation. The lack of availability of transplantable organs warrants research into technologies to understand how new kidney tissues can be generated. In recent years, procedures have been reported to generate kidney organoids [3,4] from human embryonic stem cells (hESCs) and human induced pluripotent cells, but differentiation into off-target cells [5,6] and lack of dense tubules within kidney organoids limit their use in modeling renal diseases, development of *in vitro* chemical screening systems and generation of transplantable renal tissue.

We followed a previously published protocol to generate kidney progenitors from hESCs [3]. The kidney functions as a 3D organ and an appropriate 3D environment allows kidney progenitors to self-organize to form a kidney organoid [7]. A 3D organotypic culture condition was selected to generate kidney organoids because it supports vigorous growth and differentiation of mammalian embryonic kidneys [8]. Using this

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approach, hESCs derived kidney progenitors were aggregated at the air-liquid interface, to provide a 3D organotypic culture environment for differentiation.

During kidney development, several stages of differentiating cells coexist within the embryonic kidney [9], and their differentiation fate and spatial patterning along the nephron depend on the timing of their recruitment. To establish such a culture condition *in vitro*, a method was developed for asynchronous mixing of kidney progenitors generated by directed differentiation [3]. Asynchronous mixing refers to the combination of two progenitor populations that are at different stages of differentiation. Directed differentiation cultures were staggered two days apart and newly differentiated cells were mixed with cells that have been cultured as aggregates in organotypic conditions for 2 d. Heterochronic mixing of two cell batches improve the fidelity of pluripotent stem cell-derived organoids [10].

Here, we provide an efficient method of asynchronous mixing of the kidney progenitors at the air-liquid interface that potentiates nephrogenesis to produce tightly packed nephron epithelia with more tubular clusters in kidney organoids. These kidney organoids were filled with glomerular podocytes, proximal tubules, distal tubules, stromal cells, connecting tubule or collecting ducts. The protocol yields a complex and extensive network of endothelial cells. In addition, proximal tubules in kidney organoids were mature and functional, showing endocytic function confirmed by alexa flour 488 (AF488) labeled dextran uptake. In this protocol, a step by step methodology of asynchronous mixing of kidney progenitors to generate kidney organoids is presented which we recently published elsewhere [10].

MATERIALS

Cell line and reagents

- ✓ Geltrex (Thermo fisher Scientific, Cat. # A1413301)
- ✓ DMEM/F12 (Thermo fisher Scientific, Cat. # 11330-032)
- ✓ StemFit (amsbio, Cat. # SFB-500)
- ✓ FGF2 (R&D systems, Cat. # 234-FSE-025)
- ✓ Rock inhibitor Y-27632 (EMD Millipore, Cat. # 688002-1mg)
- ✓ H9 cell line (WiCell, Cat. # WA09)
- ✓ Accutase (STEMCELL Technologies, Cat. # 7920)
- ✓ Advanced RPMI 1640 (Thermo fisher Scientific, Cat. # 12633-012)
- ✓ Glutamax (Thermo fisher Scientific, Cat. # 35050061)
- ✓ CHIR99021 IN SOLUTION (Reprocell, Cat. # 04-0004-10)
- ✓ Activin A (R&D systems, Cat # 338-AC-010)
- ✓ FGF9 (R&D systems, Cat. # 234-F9-025)
- ✓ Heparin, (Sigma-Aldrich, Cat. # H3393-25KU)
- ✓ TrypLe express ((Thermo fisher Scientific, Cat. # 12563029)
- ✓ FBS (Atlanta Biologicals, Cat # S11550)
- ✓ APEL2 (STEMCELL Technologies, Cat. # 5270)
- ✓ PFHM II (Thermo fisher Scientific, Cat. # 12040077)
- ✓ BMP7 (R&D systems, Cat. # 354-BP-010)
- ✓ Reconstitution Buffer 4, BSA/HCl (R&D systems, Cat. # RB04)
- ✓ Dextran, AF 488, 10000 MW (Thermo fisher Scientific, Cat. # D22910)
- ✓ Paraformaldehyde (Sigma-Aldrich, Cat. # P6148-500G)
- ✓ DPBS (Thermo fisher Scientific, Cat. # 14190144)
- ✓ Triton X-100 (Sigma-Aldrich, Cat. # X100-100ML)
- ✓ Normal Donkey Serum (Jackson ImmunoResearch, Cat. # 017-000-121)
- ✓ Normal goat Serum (Jackson ImmunoResearch, Cat. # 005-000-121)
- ✓ BRN1 (Santa Cruz Biotechnology, Cat. # sc-6028-R)
- ✓ CDH1 (Abcam, Cat. # ab11512)
- ✓ CD31 (Cell Signaling Technology, Cat. # 3528)
- ✓ DBA (Vector Laboratories, Cat. # B-1035)
- ✓ GATA3 (Cell Signaling Technology, Cat. # 5852S)
- ✓ HNF4A (Cell Signaling Technology, Cat. # 11F12)
- ✓ LTL (Vector Laboratories, Cat. # B-1325)
- ✓ Meis1 (Active Motif, Cat. # 39795)
- ✓ PDGFRB (Abcam, Cat. # ab32570)
- ✓ Podocalyxin (R&D systems, Cat. # AF1658)
- ✓ DAPI (Thermo Fisher Scientific, Cat. # D1306)
- ✓ Donkey anti-Goat IgG, Alexa Fluor 568 (Thermo Fisher Scientific, Cat. # A-11057)
- ✓ Donkey anti-rat IgG, Alexa Fluor 488 (Thermo Fisher Scientific, Cat. # A-21208)
- ✓ Streptavidin, Alexa Fluor (Thermo Fisher Scientific, Cat. # S21374)
- ✓ Goat anti-Mouse IgG1, Alexa Fluor 568, (Thermo Fisher Scientific, Cat. # A-21124)
- ✓ Donkey anti-Rabbit IgG, Alexa Fluor 488 (Thermo Fisher Scientific, Cat. # A-21206)

Equipment

- ✓ 6 well plate (VWR, Cat. # 29442-042)
- ✓ 15 ml falcon tube (VWR, Cat. # 62406-200)
- ✓ 50 ml falcon tube (VWR, Cat. # 21008-940)
- ✓ Strainer 40 micron (VWR, Cat. # 21008-949)
- ✓ Isopore membrane (EMD Millipore, Cat. # VCTP01300)
- ✓ 24 well plate (VWR, Cat. # 29443-952)
- ✓ 96 well Clear Round Bottom Ultra-Low Attachment Microplate (Corning, Cat. # 7007)
- ✓ Refrigerated Benchtop Centrifuge (Beckman Coulter, Type: X-15R)
- ✓ Inverted Phase Contrast microscope (Nikon, Type: Eclipse TS100)
- ✓ CO_2 incubator (Thermo fisher Scientific, Type: HERAcell 150i)
- ✓ Sunflower Mini-Shaker (Grant-bio, Type: PS-3D)
- ✓ Confocal microscope (Leica Microsystems, Type: SP8)

PROCEDURE

Cell line WA09 (H9) was approved by the National Institutes of Health (registration number 0062) and tested negative for mycoplasma infection.

1. 1. Medium and plate preparation for hESCs culture
    - 1.1. Dilute the matrix (reduced growth factor basement membrane *e.g.*, Geltrex) in DMEM/F12 (1:100) and add 1 ml/well in 2 wells of a 6 well plate.
    - 1.2. Incubate coated plate undisturbed at 37°C for 1–2 h for effective coating.
    - 1.3. Prepare 7 ml of culture medium by adding prewarmed basic culture medium (*e.g.*, StemFit) supplemented with 100 ng/ml FGF2 and 10 μM Rock inhibitor Y-27632.
2. hESCs thawing and culture
    - 2.1. Thaw a frozen vial containing 1–1.5 × 10^6 H9 cells. Rapidly swirl the vial in a 37°C water bath until just a small sliver of ice is left. This takes approximately 90–120 s.
    - 2.2. Triturate once with 1 ml micropipette and transfer to a 15 ml conical tube. Slowly drip 2 ml of the medium prepared in step 1.3, dropwise into the thawed cell suspension.
    - 2.3. Spin the cell suspension at 1000 *g* for 3 min to pellet the cells.
    - 2.4. Discard the supernatant and resuspend the cell pellet in 1 ml of fresh culture medium (as prepared in step 1.3) and triturate 2 times. Afterwards, add 3 ml of additional culture medium making a total volume of 4 ml.
    - 2.5. Add 2 ml of the cell suspension into each of the matrix coated wells of the 6 well plate and culture at 37°C, 5% CO_2 in the incubator.
    - 2.6. Change the medium after 48 h with fresh basic culture medium supplemented with 50 ng/ml FGF2 only (now Y-27632 is not required). To bring down FGF2 concentration to 10 ng/ml, supplement medium with a 50% reduced FGF2 concentration every 48 h until cells become 70% confluent.
    - 2.7. Change the medium after 48 h with basic culture medium supplemented with 25 ng/ml FGF2 and then after next 48 h with 10 ng/ml FGF2.
    - 2.8. Split cells once 70% confluency is reached.
    - 2.9. Passage the cells at 1:10 ratio (*i.e.*, distribute cells from 1 well into 10 wells of 6-well-plate) by following steps 3.5 to 3.8 for the cell culture maintenance.

    CAUTION: Follow step 2.2 carefully and gently to maximize cell viability.

3. Plating hESCs for directed differentiation
    - 3.1. Dilute the matrix in DMEM/F12 (1:100) and add 1 ml/well into 3 wells each of two 6 well plates.
    - 3.2. Incubate the coated plate, undisturbed at 37°C for 1–2 h for effective coating.
    - 3.3. Prepare 18 ml of prewarmed basic culture medium supplemented with 100 ng/ml FGF2 and 10 μM Rock inhibitor Y-27632.
    - 3.4. Remove the medium from 1 well of the 6 well hESCs culture plate and then wash the cells once with DPBS. After washing, add 1 ml prewarmed cell detachment solution (*e.g.*, Accutase) to detach the cells from the plate. The 2nd well can be used to freeze cells or to propagate cell culture.
    - 3.5. Incubate in a 37°C incubator for 10 min.
    - 3.6. Triturate 3–4 times gently and then transfer the cells into a 15 ml conical tube.
    - 3.7. Adjust the volume to 3 ml with medium. Take 10 μl cell suspension to count cells.
    - 3.8. Spin the cell suspension at 1000 *g* for 3 min to pellet the cells.
    - 3.9. Discard the supernatant and resuspend the cell pellet in 1 ml of medium and triturate 2 times.
    - 3.10. For the 1st batch of asynchronous mixing, plate 1.7 × 10^5 hESCs /well in 2 ml medium (Plate 1) and culture at 37°C, 5% CO_2.
    - 3.11. For the 2nd batch of asynchronous mixing, plate 0.45 × 10^5 hESCs/well in 2 ml medium in a 6 well plate (Plate 2) and culture at 37°C, 5% CO_2.
    - 3.12. Change the medium of both plates after 48 h with fresh basic culture medium supplemented with 50 ng/ml FGF2 without Rock inhibitor Y-27632.
    - 3.13. After 72 h, ensure that cells in Plate 1 are ~50% confluent. This is the correct time to start directed differentiation (proceed to section 4).

3.14. Ninety-six hours after seeding, change the medium in Plate 2 with fresh basic culture medium supplemented with 25 ng/ml FGF2.

3.15. One hundred and twenty hours after seeding, ensure that cells of the Plate 2 are ~50% confluent. This is the correct time to start directed differentiation (proceed to section 4).

4. Directed differentiation of hESCs into kidney progenitors (perform this procedure on both batches of cells staggered 2 d apart) (**Fig. 1A**)

4.1. The hESCs should be ~50% confluent at the start of differentiation. Remove the medium and wash the cells once with DPBS.

4.2. Add 2 ml of advanced RPMI 1640 containing 1× L-glutamine supplement (*e.g.*, GlutaMax) and 8 µM CHIR into each well of the 6 well plate and culture at 37°C, 5% CO_2. This will be day 0 of differentiation for Plate 1.

4.3. On day 2, of CHIR 99021 treatment, hESCs colonies may break apart and disperse into single cells throughout the well of the 6 well plate. Change the medium with fresh advanced RPMI 1640 containing 1× L-glutamine and 8 µM CHIR very carefully to avoid dislodging the cells that still adherent to the plate.

4.4. On day 4, change the medium with fresh advanced RPMI 1640 containing 1× L-glutamine and 10 ng/ml Activin A.

4.5. On day 6, change the medium with fresh advanced RPMI 1640 containing 1× L-glutamine and 10 ng/ml Activin A.

4.6. On day 7, change the medium with fresh advanced RPMI 1640 containing 1× L-glutamine and 10 ng/ml FGF9.

4.7. After 9 d of this treatment protocol (day 9), ensure that the cells are differentiated to kidney progenitors and adopt renal vesicle-like morphology (**Fig. 2F** and **2G**). Now this 2D culture is ready for transition into 3D culture at the air-liquid interface to generate kidney organoids. For Plate 1—proceed to section 5. For Plate 2—proceed to section 6.

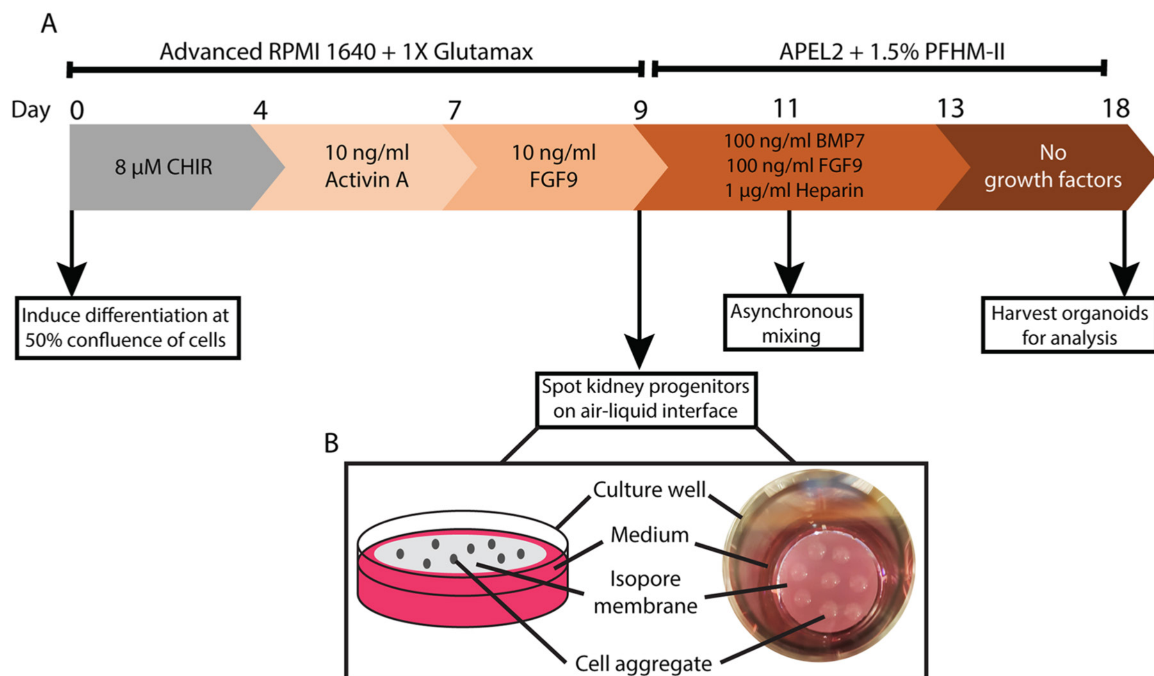

Figure 1. Schematic illustration of the protocol to generate kidney organoids by asynchronous mixing at the air-liquid interface. A. Schematic representation showing directed differentiation of hESCs to kidney progenitors. hESCs were treated with 8 µM CHIR for four days, three days with 10 ng/ml activin A and then two days with 10 ng/ml FGF9 to differentiate into kidney progenitors. **B.** On day 9, cell aggregates were spotted at the air-liquid interface (membrane floating on medium containing 100 ng/ml FGF9, 100 ng/ml BMP7 and 1 µg/ml heparin) for the next two days. On day 11, fresh progenitors (two days staggered from first batch of progenitors) were mixed with differentiating progenitors and spotted again at the air-liquid interface. Growth factors were removed from the medium during day 13 to day 18 of differentiation.

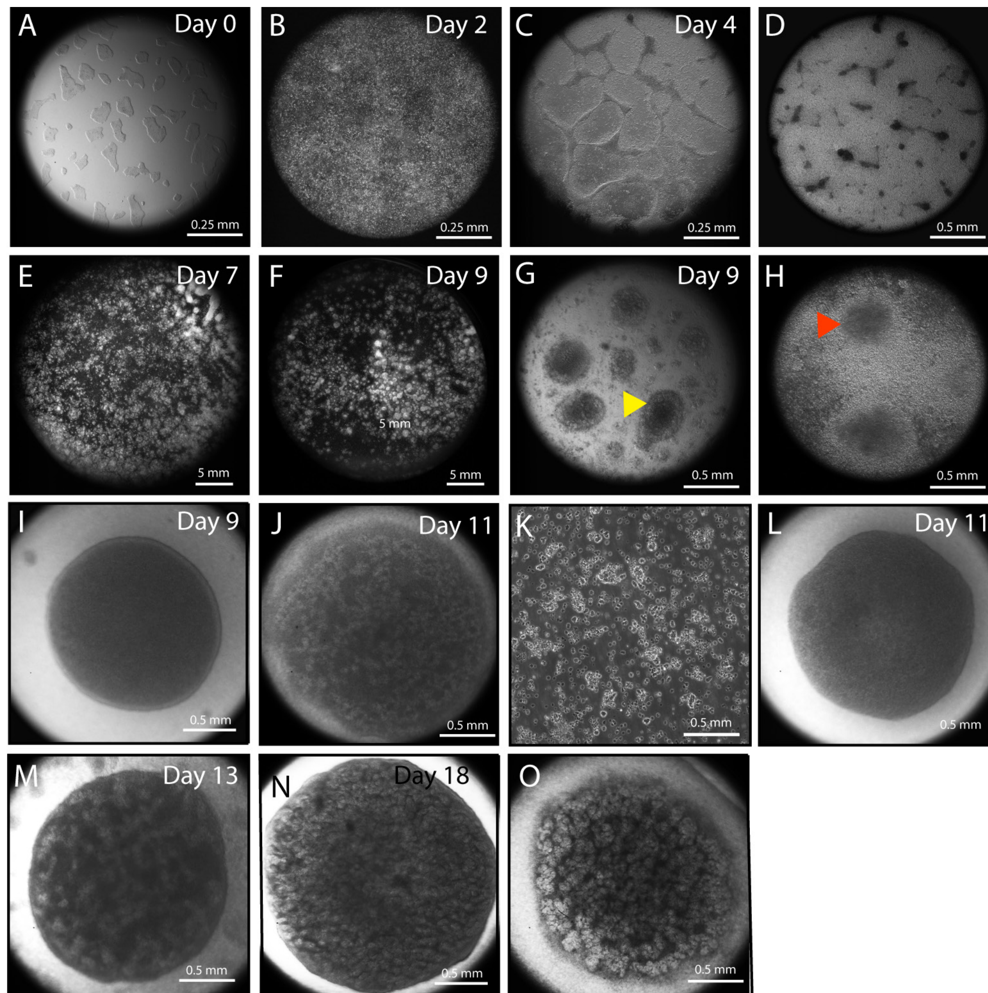

Figure 2. Representative images showing morphological changes of hESCs during differentiation. A. Directed differentiation of hESCs was started on day 0 when cell culture attains ~50% confluency. **B.** On day 2, cells spread throughout wells and on day 4 (C), they formed small loosely dense cell clusters. **D.** An example of too dense clusters formed on day 4. **E.** On day 7, cells start to form small clusters. **F** and **G.** Adopt renal vesicle-like clusters (yellow arrowhead). **H.** An example of no renal vesicle-like clusters on day 9 (red arrowhead). **I.** On day 9, kidney progenitors were aggregated at the air-liquid interface. **J.** On day 11, cell .aggregates were broken into small fragments and asynchronous mixing was performed. **K.** An example of small fragments of the 5 cell aggregates in 2 ml medium to mix with fresh kidney progenitors. **L.** Mixed progenitors were spotted again at the air-liquid interface. **M.** Cell aggregates started to show tubule formation. **N.** Cell became densely filled with tubular clusters on day 18. **O.** An example of the absence of densely packed tubular clusters. At this stage differentiation can be continued for the next 3–4 d to get more densely packed tubular clusters in the kidney organoids.

CRITICAL STEP: Follow step 4.1 only when cells reach ~50% confluence. On day 4, cells should form loosely dense clusters. If cells reach the "too dense clusters" stage (**Fig. 2D**), directed differentiation should be started again. Aggregate cells on floating membrane only when cells form renal vesicle-like morphology (**Fig. 2F** and **2G**). In the absence of this morphology (**Fig. 2H**), differentiation should be started again with a new culture.

CAUTION: Change medium with growth factors carefully and gently during differentiation. Harsh pipetting may dislodge cells from the culture plate surface and once cells start to float in the medium, they subsequently die.

5. Making kidney progenitor cell aggregates at the air-liquid interface

5.1. Prepare a 24 well plate for culture of cells at the air-liquid interface.

5.1.1. Prepare the medium containing APEL2, 1.5% PFHM II, 100 ng/ml BMP7, 100 ng/ml FGF9 and 1 µg/ml Heparin and add 1 ml/well into the 24 well plate.

5.1.2. Float polycarbonate membrane on the medium in each well of the 24 well plate, using sterile forceps. Make sure not to flood the filters—wells with partially or entirely submerged filters should not be used. Keep plate aside in the hood for later use.

5.2. Wash the wells of the Plate 1 two times with DPBS and add 1 ml of cell dissociation enzyme (*e.g.*, TrypLe express) into each well of the 6 well plate.

5.3. Incubate the plate for 5 min at 37°C. Triturate 3–4 times to disperse cell clusters into single cell suspension.

5.4. Neutralize the cell dissociation enzyme by adding 8 ml/well of advanced RPMI 1640 with 1 ml of FBS (total 10% FBS) in a 50 ml conical tube.

5.5. Strain the cells through a 40 µm strainer and use 10 µl of the strained cell suspension to count the cells.

5.6. Centrifuge the cells at 300 *g* for 5 min.

5.7. Resuspend the cells at 2.5×10^5 cells/µl in APEL2 medium containing 1.5% PHFM II.

5.8. Spot 2 µl of the cell suspension onto the polycarbonate membrane floating on the medium in the 24 well plate. Spot 6–8 cell aggregates/membrane.

5.9. Culture for 2 d at 37°C, 5% CO_2 in the incubator.

CAUTION: Follow step 5.1.2 very carefully to float membranes at the surface of the medium.

TIPS/HINTS: If there is air bubble below the floating membrane, remove the bubble with 1 ml pipette or replace membrane with a new one.

6. Asynchronous mixing of kidney progenitors to generate kidney organoids

6.1. Prepare a 24 well plate to spot cells following step 5.1.

6.2. Harvest and count the kidney progenitors in the Plate 2 following steps 5.2–5.7.

6.3. Remove membranes one at a time from the 24 well plate seeded in step 5.8 and break the cell aggregates into small fragments using a 200 µl micropipette by triturating 7–10 times (**Fig. 2K**).

6.4. Mix these small fragments with fresh kidney progenitors from Plate 2 at a 1:1 ratio. (*e.g.*, mix fragments from 1 cell aggregate of 5×10^5 cells with 5×10^5 cells of newly differentiated kidney progenitors from Plate 2, resuspend in 4 µl and make two cell aggregates on the membranes).

6.5. Spot the mixed cells onto the membranes floating at the surface of the medium in a 24 well plate. 6–8 cell aggregates/the membrane can be spotted.

6.6. Change the medium on day 13 with fresh prewarmed APEL2 containing 1.5% PHFM II without any growth factors.

6.7. Change the medium every 48 h with APEL2 containing 1.5% PHFM II.

6.8. On day 18, image the organoids under a stereo microscope and proceed to section 8 for marker expression analysis. In the absence of densely packed tubular clusters (**Fig. 2O**) differentiation can be continued for the next 3–4 d.

7. Evaluation of dextran uptake by proximal tubule cells in kidney organoids

7.1. On day 18, remove the membrane from the wells of the 24 well plate and transfer the kidney organoids such that 1 organoid is placed in each well of a "U" bottom low attachment 96 well plate.

7.2. Replace the medium with 200 µl of fresh prewarmed APEL2 containing 1.5% PHFM II and 10 µg/ml of 10000 MW dextran conjugated with AF488.

7.3. Incubate the organoids at 37°C, 5% CO_2 in the incubator for next 24 h on a shaker.

7.4. Stain the organoids using a "whole mount staining technique" described in section 8 for proximal tubule specific markers to evaluate dextran uptake in proximal tubule cells.

8. Whole mount immunofluorescence staining on kidney organoids

8.1. On day 18 of the differentiation, remove the membrane from the wells and transfer each organoid to a "U" bottom 96 well plate.

8.2. Fix the organoids with 150 µl 4% paraformaldehyde (PFA) for 15 min at room temperature.

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8.3. Remove the PFA and wash the organoids 3 times with DPBS.

8.4. Add 150 µl of 1% Triton X-100 and incubate for 10 min at 4°C on a shaker.

8.5. Remove the Triton X-100 and wash the organoids 3 times with DPBS.

8.6. Add 150 µl blocking buffer (5% serum from a species that matches the secondary antibody's host species in DPBS) and incubate for 1 h at room temperature.

8.7. Incubate the organoids with primary antibodies diluted in blocking buffer at 4°C overnight on a shaker.

8.8. Remove the primary antibodies and wash the organoids with DPBS for the next 8 h on a shaker.

8.9. Incubate the organoids with secondary antibodies diluted in blocking buffer containing 0.01 µg/ml DAPI at 4°C overnight on shaker.

8.10. Remove the secondary antibodies and wash the organoids with DPBS for the next 8 h on a shaker.

8.11. Mount the organoids on a glass slide and image under a fluorescent microscope.

CAUTION: Follow step 8.1 very carefully to avoid damage to the kidney organoids. Pipette tips can be cut to a wider bore to avoid damaging the organoids during transfer.

ANTICIPATED RESULTS

This protocol describes asynchronous mixing of kidney progenitor cells differentiated from hESCs (H9) at the air-liquid interface to generate kidney organoids with reproducible results and high success rates. We followed a previously published protocol to differentiate hESCs into kidney progenitors [3] (**Fig. 1**). The mix of cells that arises from the directed differentiation process is believed to represent the repertoire of developmental kidney progenitors that gives rise to different nephron segments, stromal cells, and vasculature of the fetal kidney. Further, we followed an organotypic culture condition *i.e.*, air-liquid interface that was developed to culture rodent kidney tissues, which served as a logical starting point to generate human kidney tissue from hESCs. It has been reported that there is gradual contribution of the renal progenitors at various stages of differentiation process to the developing nephron [11]. The differentiation fate of each progenitor cell depends on the timing of its recruitment, dictating the spatial patterning of each cell along the nephron; time-dependent cell-fate acquisition (TCA) [11,12]. To establish such a culture condition *in vitro*, we mixed epithelializing kidney progenitors with newly differentiated kidney progenitors to improve epithelialization and tubule formation.

In our experience, it is crucial to start directed differentiation at ~50% confluence of hESCs (**Fig. 2A**). Extensive cell death was observed when we started differentiation at less than 50% confluence. Differentiating cells became over confluent if we started differentiation at more than 50% confluence. On day 0, we started directed differentiation by treating cell cultures with CHIR 99021. On day 2 of CHIR 99021 treatment, hESCs colonies were broken apart and dispersed into single cells throughout the well of the 6 well plate (**Fig. 2B**). On day 4, cells formed loosely dense clusters that represent an optimum time point to start activin A treatment to differentiate cells into intermediate mesoderm (**Fig. 2C**). On day 7, cells started to form small clusters (**Fig. 2E**) and on day 9, *i.e.*, two days after FGF9 treatment, renal vesicle-like clusters were formed (**Fig. 2F** and **2G**). FGF9 treatment helped in the survival and self-renewal of progenitors. We observed that the quantity of these renal vesicle-like clusters are predictive of the potential of a culture to generate high quality kidney organoids. At this point, the kidney progenitors became ready to be transferred to the air-liquid interface to form 3D kidney organoids. On day 9, cells were harvested and spotted as cell aggregates on isopore membranes floating on medium in a 24 well plate to generate air-liquid interface (**Fig. 1** and **2I**). On day 11, these cell aggregates (**Fig. 2J**) were broken into small fragments (**Fig. 2K**) and mixed with fresh kidney progenitors, *i.e.*, asynchronous mixing of progenitors, and re-spotted at the air-liquid interface (**Fig. 2L**). On day 13, these cell aggregates start to show tubularization (**Fig. 2M**) and became filled with tubular structures by day 18 (**Fig. 2N**). We supplemented the air-liquid interface culture medium with FGF9 and BMP7, which has been reported to facilitate nephron progenitor cell survival and self-renewal [13].

These kidney organoids derived from hESCs were densely packed with tubular clusters (**Fig. 3A**) and H&E staining confirmed that the organoids were packed with tubules containing lumens (**Fig. 3B** and **3C**) and presumptive glomerular structures with Bowman's spaces (**Fig. 3C**). We found that asynchronous mixing of progenitors at the air-liquid interface generated kidney organoids filled with CDH1$^+$ epithelial structures connected with LTL$^+$ proximal tubules and PODXL$^+$ presumptive podocytes (**Fig. 3D** and **3G**). Lumens were also observed in LTL$^+$ & HNF4A$^+$ proximal (**Fig. 3H**) and CDH1$^+$ & BRN1$^+$ distal tubules (**Fig. 3I**). Asynchronous mixing of kidney progenitors at the air-liquid interface supports development of a CD31$^+$ endothelial network (**Fig. 3E**) around presumptive PODXL$^+$ podocytes (**Fig. 3J**) but capillary loops were absent which is a classical feature in the glomeruli. MEIS1$^+$ & PDGFRB$^+$ renal stromal cells were observed between tubular structures (**Fig. 3F** and **3K**). In addition to nephron structures, we also found GATA3$^+$ DBA$^+$ & CDH1$^+$connecting tubule or collecting ducts in these kidney organoids (**Fig. 3L**). Proximal tubules play an important role in selective absorption of solutes, vitamins, hormones, and amino acids. Therefore, we performed an *in vitro* dextran uptake assay to examine whether proximal tubules in the kidney organoids exhibit physiologically relevant characteristics. We found that LTL$^+$ & HNF4A$^+$ proximal tubules were able to absorb AF 488 labeled dextran (**Fig. 4B**).

Furthermore, we followed this asynchronous mixing protocol to generate kidney organoids in submerged culture condition. Kidney organoids generated under submerged culture condition had fewer LTL$^+$ proximal tubules, CDH1$^+$ epithelial structures, PODXL$^+$podocytes and CD31$^+$ endothelial networks (**Fig. 5A-5D**) in comparison to kidney organoids generated at the air-liquid interface. To quantify the effect of the air-liquid interface culture, we compared the degree of staining for molecular markers of proximal tubule glycoproteins (LTL), podocytes

(PODXL), and distal tubules (CDH1) between organoids derived from submerged versus air-liquid interface culture condition. Kidney organoids generated at the air-liquid interface displayed higher number of structures stained for each molecular marker (**Fig. 5E-5G**). Comparing kidney organoids generated using both culture conditions, submerged culture condition was less efficient than air-liquid interface culture.

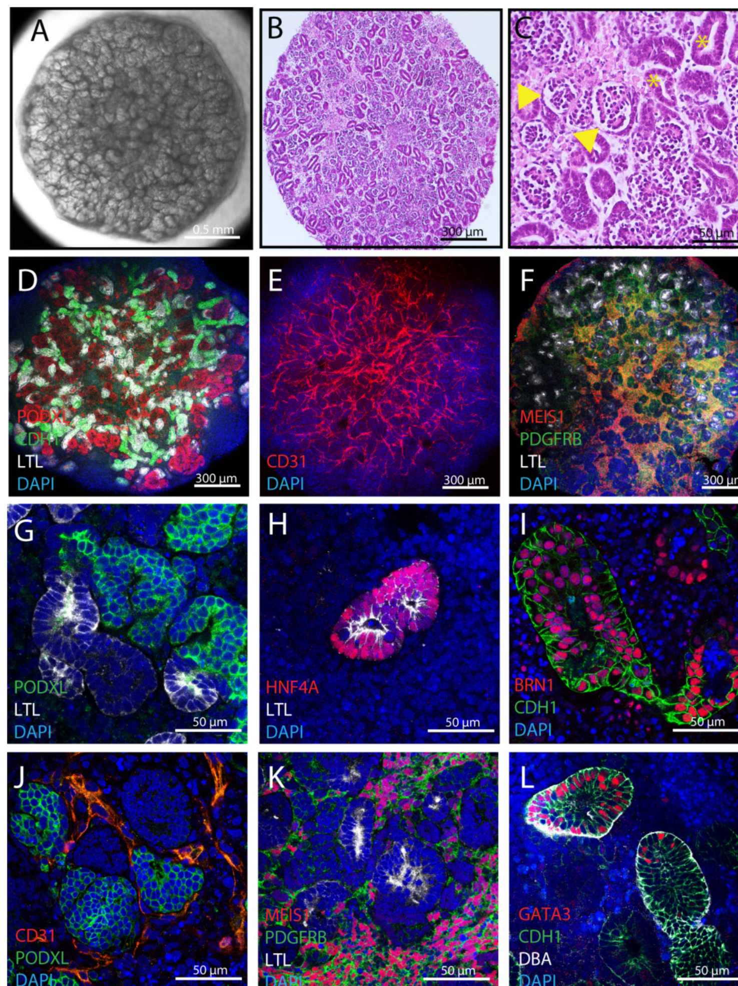

Figure 3. Representative images showing morphology and immunological characterization of kidney organoids on day 18. A. Representative stereo microscope image showing densely packed kidney organoid with tubular clusters. **B** and **C.** Hematoxylin-eosin (H&E) staining of kidney organoid showing tubular structures with lumens (yellow star) and presumptive glomerular structures with Bowman's spaces (yellow arrow head). **D.** Immunofluorescence staining showing PODXL$^+$ podocytes, CDH1$^+$ epithelial structures and LTL$^+$ proximal tubules. **E.** CD31$^+$ endothelial networks were also observed throughout the kidney organoids. **F.** Further, tubular structures were surrounded by MEIS1$^+$ PDGFRB$^+$ renal stromal cells. **G.** High magnification immunofluorescence image showing an interconnection between LTL$^+$ proximal tubules and PODXL$^+$ presumptive glomerular structures. **H.** LTL$^+$ tubules also expressed HNF4A, another marker for proximal tubules. **I.** Distal tubules expressed BRN1 and CDH1 together. **J.** CD31$^+$ endothelial networks were detected around PODXL$^+$ glomerular structures but capillary loops were absent. **K.** MEIS1$^+$ PDGFRB$^+$ Renal stromal cells were found around tubular structures. **L.** In addition, GATA3$^+$ DBA$^+$ & CDH1$^+$ connecting tubule or collecting ducts were also present in the kidney organoids.

DISCUSSION

Asynchronous mixing of progenitors at the air-liquid interface presents an efficient method to generate kidney organoids from hESCs. Although this method to generate kidney organoids from hESCs is reproducible, cell line specific variation can be observed. Genetic background, reprogramming methodology, cell line origin and culture conditions may contribute to the differentiation variability between different human pluripotent stem cell (hPSC) lines. So a researcher may need to make adjustment to the protocol to get efficient results between different hPSC lines. Previously, we reported that kidney organoids generated from asynchronous mixing exhibited approximately double the number of structures stained for proximal tubule, podocytes and distal tubule markers [10]. This work describes stepwise protocols to thaw and culture hESCs, their directed differentiation to kidney progenitors, cell aggregation at the air-liquid interface, asynchronous mixing to generate kidney organoids tightly packed with tubular clusters and major renal structures including endothelial network and functional proximal tubules, dextran uptake by proximal tubule and whole mount immunostaining of kidney organoids. Together, this protocol provides guidance on the

culture of human embryonic stem cells and their directed differentiation into kidney organoids. Our 18-day protocol provides a rapid method to generate kidney organoids that will facilitate the study of different features of kidney biology, including *in vitro* tissue development, disease modeling and chemical screening.

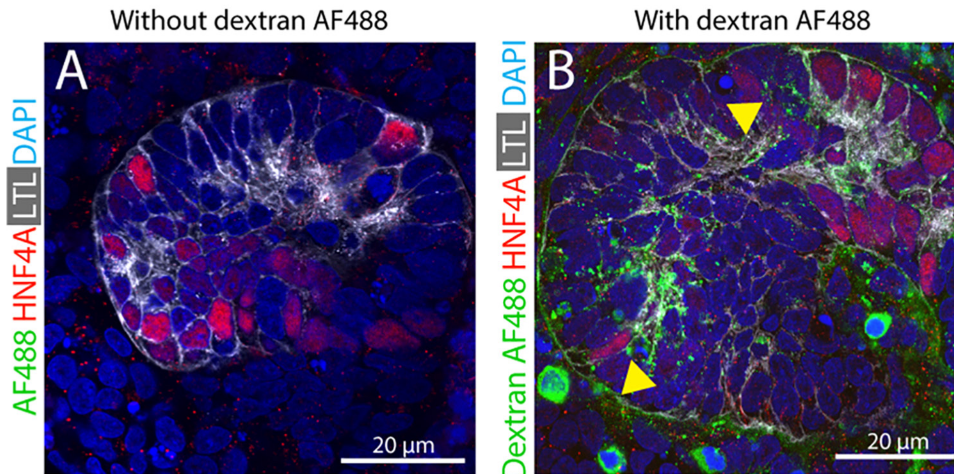

Figure 4. Representative images showing fluorescent dextran uptake by proximal tubules in kidney organoids on day 18. Kidney organoids were incubated with dextran AF488 or AF488 only (control). **A.** AF488 was not detectable in control organoids where AF488 was mixed with medium. **B.** Dextran AF488 vesicles (yellow arrowhead) were observed in kidney organoids, 24 h after incubation with dextran AF488 supplemented medium showing endocytic uptake by HNF4A⁺ LTL⁺ proximal tubules.

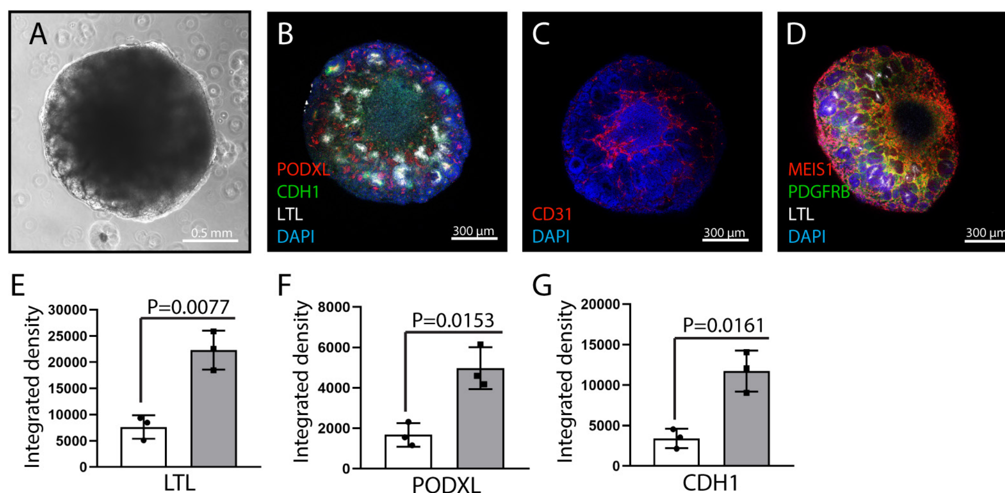

Figure 5. Representative images showing morphology and immunological characterization of organoids on day 18 generated under submerged culture condition. Kidney organoids were generated using submerged culture condition to see the effect of culture at the air-liquid interface. In brief, kidney progenitors were aggregated on day 9 in U bottom low attachment 96 well plate by centrifugation at 300 g for 15 s and were asynchronously mixed on day 11. Cell count, medium composition, and timeline were kept same as in the air-liquid interface protocol. **A.** Stereo microscope image of kidney organoid generated under submerged culture condition showing fewer tubular clusters. **B.** Immunofluorescence image showing fewer LTL⁺ proximal tubule, CDH1⁺ epithelial structures and PODXL⁺ Podocytes. **C.** Furthermore, endothelial networks were not developed as we had found in the kidney organoids cultured at the air-liquid interface. **D.** MEIS1⁺ PDGFRB⁺ renal stromal cells were widespread in the kidney organoids. Integrated density was calculated using ImageJ software for LTL (E), PODXL (F), and CDH1 (G) in the kidney organoids generated from submerged (open bar) and air-liquid interface culture condition (gray bar). Values calculated from n = 3 independent biological replicates and expressed as mean ± SD. Unpaired t test with Welch's correction was applied to calculate P value using GraphPad Prism 8 software.

Although our protocol efficiently generates high quality kidney organoids that can be used for *in vitro* modeling, certain aspects require further development. Epithelial structures such as proximal tubules, distal tubules, connecting tubules, or collecting ducts were densely represented, but markers for other nephron segments such as the ascending and descending limbs of the loop of Henle were absent. Bowman's spaces were also found in presumptive glomerular structures, but capillary loops were absent despite the widespread endothelial network. Interconnection

between tubular segments were also primitive. Hence, it is important to further optimize this protocol to achieve more mature renal tissues with interconnected nephron segments and other kidney-specific cell types.

Most of the previous protocols employ the treatment with CHIR 99021 to induce the mesenchyme-to-epithelial transition [3,4,13,14]. CHIR 99021 is a potent inhibitor of GSK3 and is expected to have many effects on cellular pathways apart from activating Wnt/β-catenin signaling [10]. Our protocol omits the application of CHIR 99021 at the kidney progenitor epithelialization step, thus reducing the scope for unintended effects that may skew differentiation. In previous work, we evaluated the time frame for asynchronous mixing and found that mixing cells two days after differentiation of the first batch is the optimum time to add fresh progenitors [10].

During kidney development, the ureteric bud tip acts as the epithelial inducer for metanephric mesenchyme, ensuring appropriate placement of new nephrons within the collecting duct system. Taguchi *et al*. recreated this developmental interaction by inducing stem cells to differentiate into metanephric mesenchyme and ureteric bud cells separately [15]. In contrast, the mixture of cells generated through our directed differentiation strategy does not require addition of ureteric bud or small molecule substitutes for Wnt signaling to induce nephron differentiation. We hypothesize that the GATA3$^+$DBA$^+$&CDH1$^+$structures that differentiate in our organoids, may act as an inducer for nephron epithelial differentiation, and further studies will be required to determine if this is indeed the source of Wnt induction in our differentiation system.

Certain critical points should be considered while generating kidney organoids using this protocol. Visual monitoring of cultures is important to ensure preparation of high quality organoids. At day 4 of directed differentiation, cultures should be visually confirmed to have loosely dense clusters before Activin A treatment is started. In the absence of these loosely dense clusters, it is recommended that directed differentiation to be started again from a new hESCs culture. Similarly, on day 9, it is important that cultures display renal vesicle-like cell aggregates for efficient generation of kidney organoids. In the absence of this morphology, FGF 9 treatment should be continued for another 2 d until the culture displays the appropriate morphology. On day 18, organoids should be evaluated under a stereo microscope and differentiation can be continued for the next 3–4 days in the absence of densely packed tubular clusters. These considerations are important and should be kept in mind to improve the fidelity of hESCs-derived organoids.

TROUBLESHOOTING

Possible problems and their troubleshooting solutions are listed in **Table 1**.

Table 1. Troubleshooting.

Step	Problem	Solution
2.6	Cell death or slow growth after thawing	• Freeze cells once they are ~70% confluent • Add medium drop by drop into the thawed cells at step 2.2
4.3	Abundant cell death and detachment from the surface of the plate during first 4 days of CHIR treatment	• Start CHIR treatment (step 4.2) once cells reach ~50% confluence • Change medium gently
4.4	No “loosely dense clusters” on day 4	• Wait 2–4 h to get “loosely dense clusters” • OR start new differentiation with fresh cell culture
4.7	Renal vesicle-like morphology absent	• Wait 12–24 h to get “renal vesicle-like morphology” • OR start new differentiation with fresh cell culture
5.1.2	Membrane submerged in medium	• Remove any visible air bubble below the membrane • Use fresh membrane if partially or entirely submerged
6.8	Air bubble or few tubular clusters in the organoids	• Cell suspension should not have any visible air bubble at step 6.5 • Wait 3–4 d more to get densely packed tubular clusters

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References

1. Lv JC, Zhang LX. Prevalence and disease burden of chronic kidney disease. Adv Exp Med Biol. 2019;1165:3–15. https://doi.org/10.1007/978-981-13-8871-2_1 PMID:31399958
2. Oxburgh L, Carroll TJ, Cleaver O, Gossett DR, Hoshizaki DK, Hubbell JA, et al. (Re)Building a Kidney. J Am Soc Nephrol. 2017 May;28(5):1370–8. https://doi.org/10.1681/ASN.2016101077 PMID:28096308
3. Morizane R, Lam AQ, Freedman BS, Kishi S, Valerius MT, Bonventre JV. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. Nat Biotechnol. 2015 Nov;33(11):1193–200. https://doi.org/10.1038/nbt.3392 PMID:26458176
4. Takasato M, Er PX, Chiu HS, Maier B, Baillie GJ, Ferguson C, et al. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. Nature. 2015 Oct;526(7574):564–8. https://doi.org/10.1038/nature15695 PMID:26444236
5. Wu H, Uchimura K, Donnelly EL, Kirita Y, Morris SA, Humphreys BD. Comparative Analysis and Refinement of Human PSC-Derived Kidney

Organoid Differentiation with Single-Cell Transcriptomics. Cell Stem Cell. 2018 Dec;23(6):869–881.e8. https://doi.org/10.1016/j.stem.2018.10.010 PMID:30449713

6. Gupta AK, Coburn JM, Davis-Knowlton J, Kimmerling E, Kaplan DL, Oxburgh L. Scaffolding kidney organoids on silk. J Tissue Eng Regen Med. 2019 May;13(5):812–22. https://doi.org/10.1002/term.2830 PMID:30793851
7. Takasato M, Er PX, Chiu HS, Little MH. Generation of kidney organoids from human pluripotent stem cells. Nat Protoc. 2016 Sep;11(9):1681–92. https://doi.org/10.1038/nprot.2016.098 PMID:27560173
8. Grobstein C. Inductive epitheliomesenchymal interaction in cultured organ rudiments of the mouse. Science. 1953 Jul;118(3054):52–5. https://doi.org/10.1126/science.118.3054.52 PMID:13076182
9. Oxburgh L. Kidney Nephron Determination. Annu Rev Cell Dev Biol. 2018 Oct;34(1):427–50. https://doi.org/10.1146/annurev-cellbio-100616-060647 PMID:30125139
10. Kumar Gupta A, Sarkar P, Wertheim JA, Pan X, Carroll TJ, Oxburgh L. Asynchronous mixing of kidney progenitor cells potentiates nephrogenesis in organoids. Commun Biol. 2020 May;3(1):231. https://doi.org/10.1038/s42003-020-0948-7 PMID:32393756
11. Lindström NO, De Sena Brandine G, Tran T, Ransick A, Suh G, Guo J, et al. Progressive Recruitment of Mesenchymal Progenitors Reveals a Time-Dependent Process of Cell Fate Acquisition in Mouse and Human Nephrogenesis. Dev Cell. 2018 Jun;45(5):651–660.e4. https://doi.org/10.1016/j.devcel.2018.05.010 PMID:29870722
12. Saxén L, Sariola H, Lehtonen E. Sequential cell and tissue interactions governing organogenesis of the kidney. Anat Embryol (Berl). 1986;175(1):1–6. https://doi.org/10.1007/BF00315450 PMID:3799983
13. Brown AC, Muthukrishnan SD, Oxburgh L. A synthetic niche for nephron progenitor cells. Dev Cell. 2015 Jul;34(2):229–41. https://doi.org/10.1016/j.devcel.2015.06.021 PMID:26190145
14. Subramanian A, Sidhom EH, Emani M, Vernon K, Sahakian N, Zhou Y, et al. Single cell census of human kidney organoids shows reproducibility and diminished off-target cells after transplantation. Nat Commun. 2019 Nov;10(1):5462. https://doi.org/10.1038/s41467-019-13382-0 PMID:31784515
15. Taguchi A, Nishinakamura R. Higher-Order Kidney Organogenesis from Pluripotent Stem Cells. Cell Stem Cell. 2017 Dec;21(6):730–746.e6. https://doi.org/10.1016/j.stem.2017.10.011 PMID:29129523

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