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Supplemental Information

Renal Subcapsular Transplantation of PSC-Derived Kidney Organoids Induces Neo-vasculogenesis and Significant Glomerular and Tubular Maturation *In Vivo*

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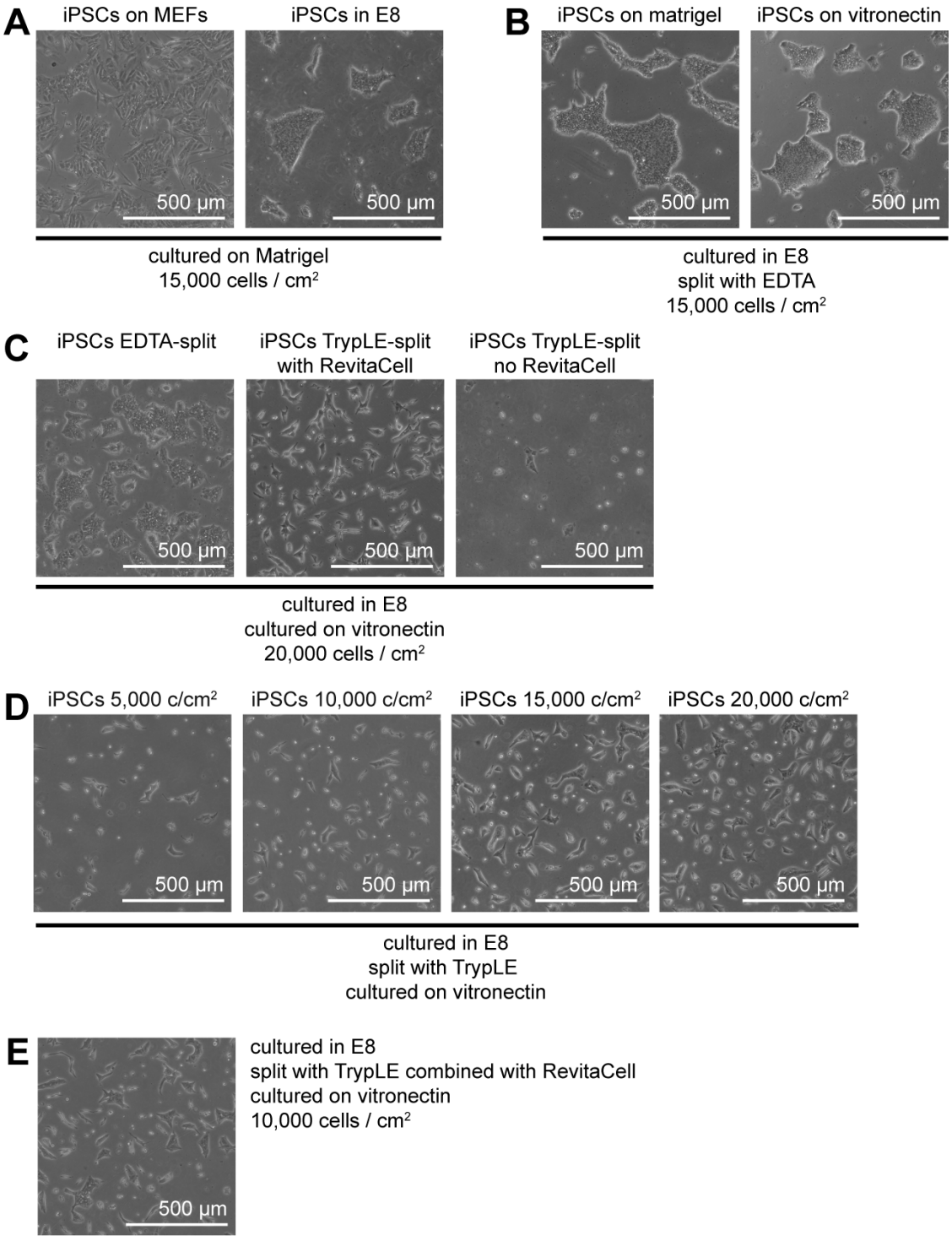

Figure S1: Adaptation of Culture Protocol for Differentiation of Pluripotent Stem Cells to Kidney Organoids using Defined Medium

Numerous variables were investigated for optimal differentiation procedure: **(A)** culture medium, **(B)** matrix, **(C)** passaging reagents, and **(D)** cell density.

(A) Pluripotent stem cells were initially enzymatically passaged on MEFs and were adapted to Essential 8 (E8) cultures. Cells were passaged using EDTA and plated on matrigel using 15,000 cells/cm^2.

(B) Cell matrix was changed from matrigel to more defined vitronectin for better support of differentiation in the early differentiation stages.

(C) Single cell passaging and estimating precise cell numbers is possible using a combination of TrypLE Select and RevitaCell.

(D) Seeding density for pluripotent stem cells was optimized for organoid differentiation.

(E) Final parameters for differentiation of pluripotent stem cells to kidney progenitor cells.

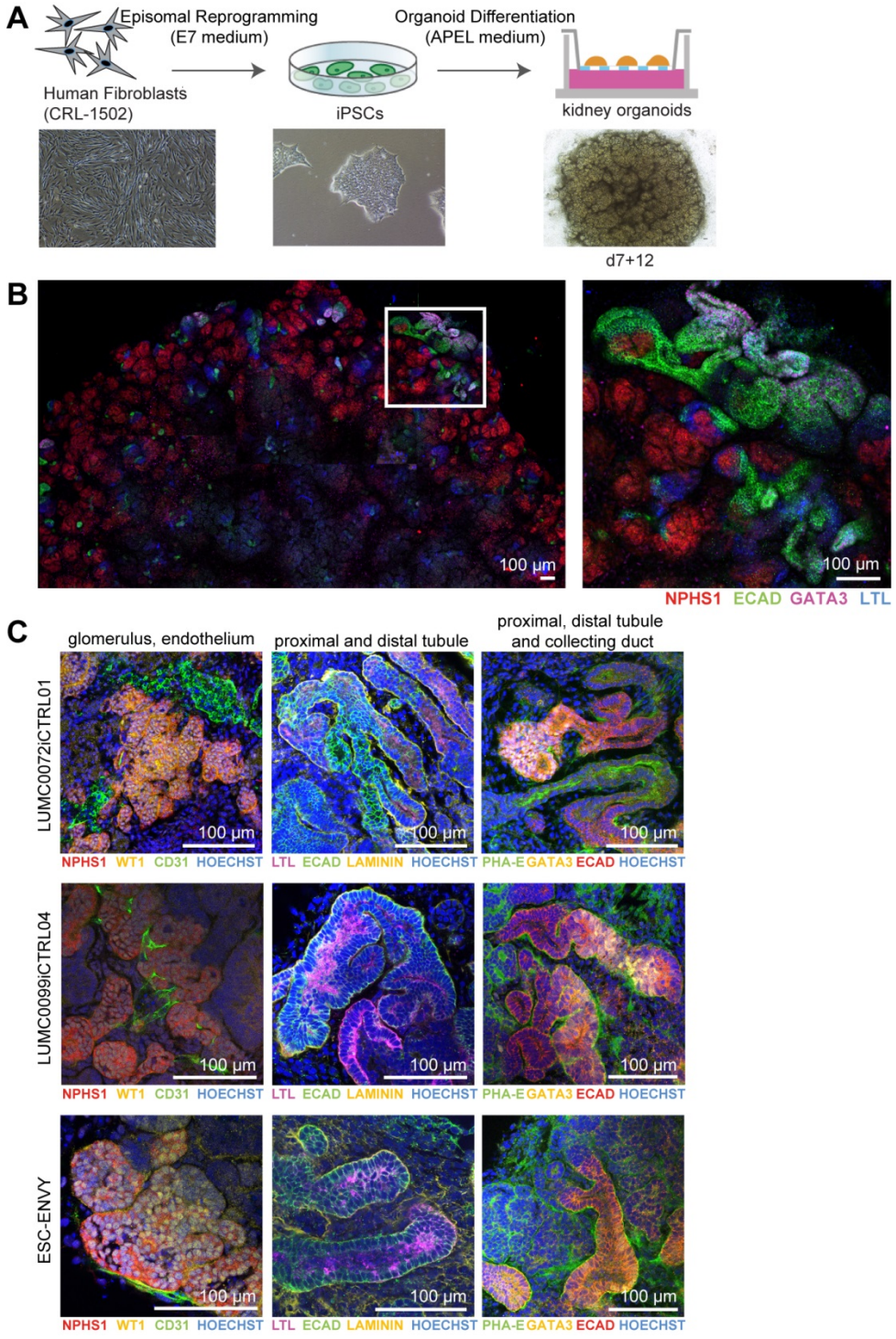

Figure S2: Human Kidney Organoids Derived from Multiple Pluripotent Stem Cell Lines Contain All Nephron Structures

(A) Kidney organoids can be derived from hiPSCs that were originally generated by episomal reprogramming of human fibroblasts in Essential 7 (E7) medium without culture on mouse embryonic fibroblasts.

(B) Tile scan of a kidney organoid generated from hiPSCs derived in E7 medium displays all characteristic compartments of a nephron: glomerulus ($NPHS1^+$), proximal (LTL^+) and distal tubular ($ECAD^+$) segments and collecting duct ($GATA3^+$) on day 7 + 12 of differentiation. Close-up view of the boxed area displays the structures in detail.

(C) Kidney organoids derived from one additional embryonic (ESC-ENVY) and two induced pluripotent stem cell lines (LUMC0072iCTRL01 and LUMC0099iCTRL04) display characteristic glomerular structures ($NPHS1^+$, $WT1^+$), endothelial cells ($CD31^+$), proximal tubules ($PHA\text{-}E^+$, LTL^+), distal tubules ($ECAD^+$) and collecting duct ($GATA3^+$) on day 7 + 18.

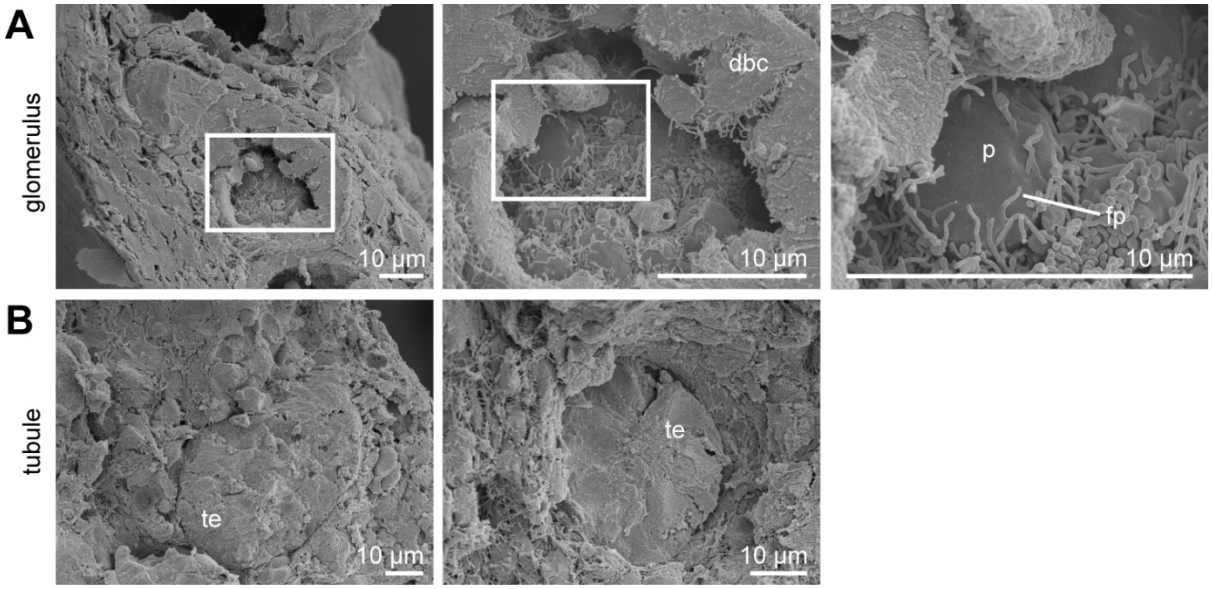

Figure S3: Scanning Electron Microscopy (SEM) Analysis of Kidney Organoids on Day 7 + 18
(A) Developing Bowman's capsule is surrounding the glomerular structure. Boxed areas show close-up views of the glomerular structure and podocytes with primitive foot processes.
(B) SEM showing tubular structures in kidney organoid.
dbc, developing Bowman's capsule; p, podocytes; fp, foot processes; te, tubular epithelium.

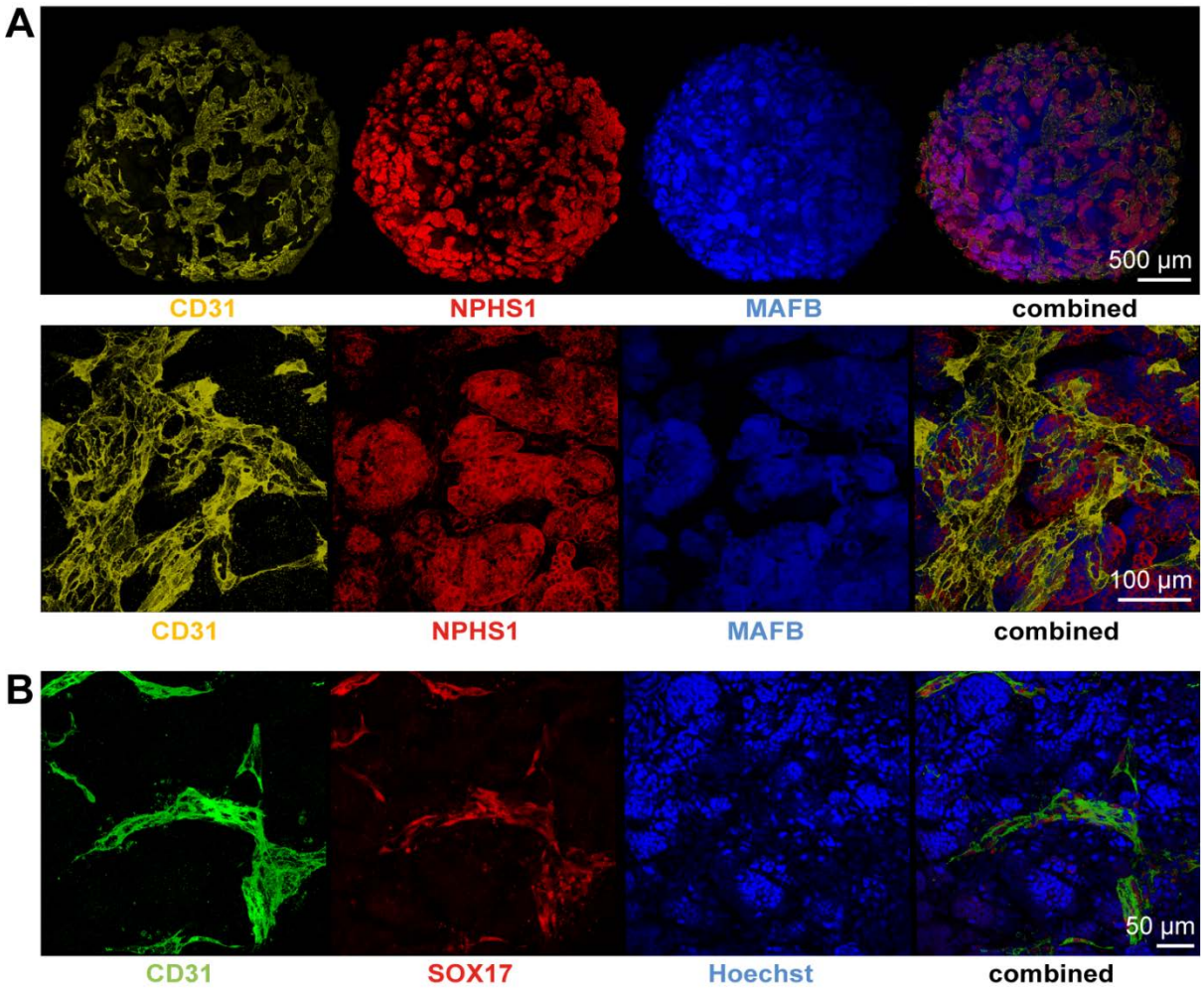

Figure S4: Characterisation of Kidney Organoids Derived from hiPSC-MAFB-BFP and hESC-SOX17-mCherry Reporter Lines
(A) Kidney organoids derived from hiPSC-MAFB-BFP reveal co-localisation of $NPHS1^{+}$ and mTagBFP2 reporter expression in the podocyte population in developing glomeruli surrounded by $CD31^{+}$ endothelial cells. **(B)** hESC-SOX17-mCherry derived kidney organoids display co-localisation of $CD31^{+}$ endothelial cells and SOX17-$mCherry^{+}$ cells.

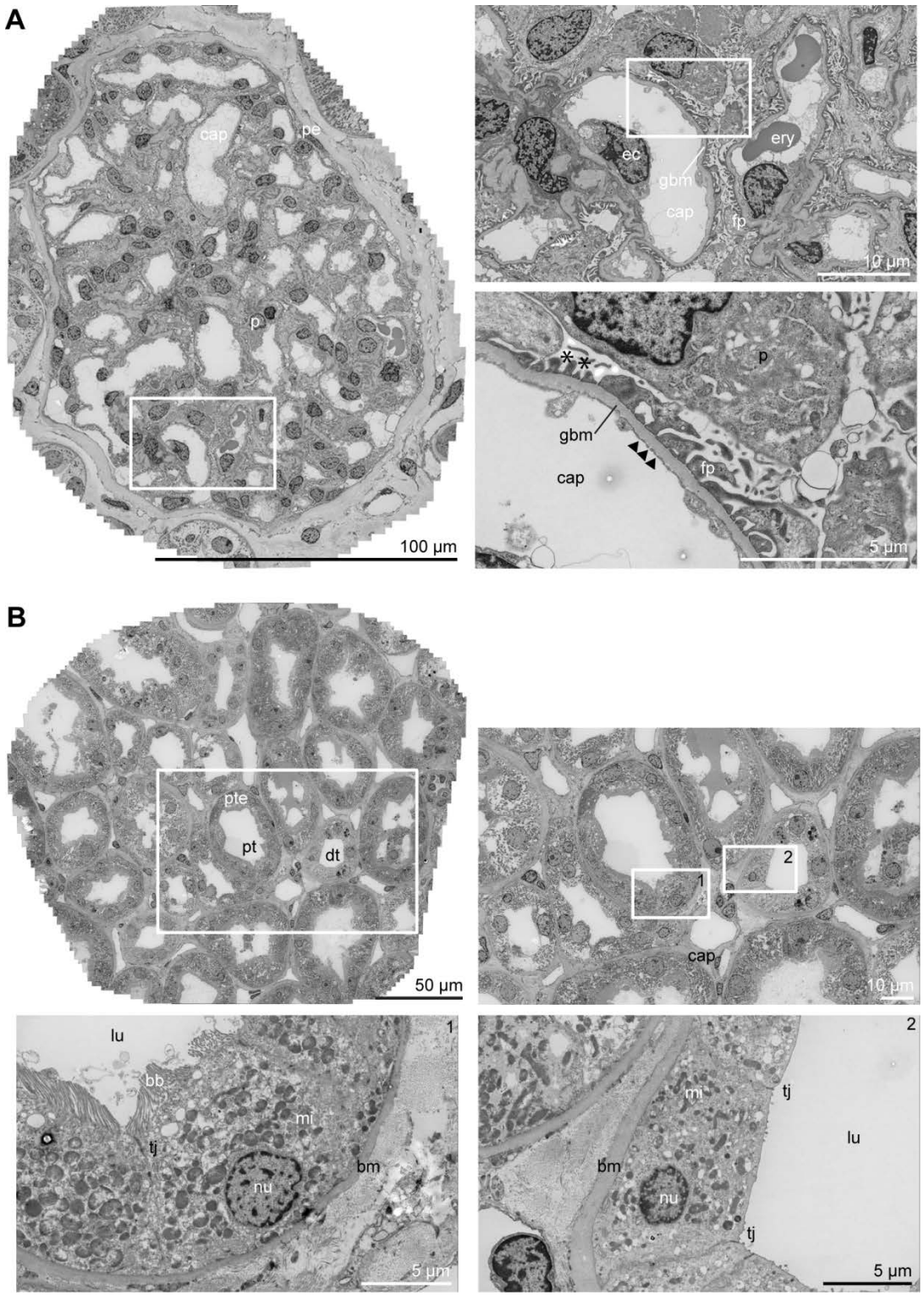

Figure S5: Ultrastructural Reference of Glomerulus and Tubular Structures in the Human Kidney
(A) Transmission electron microscopy overview of a glomerulus with boxed areas displaying close-up views of the glomerular structure and foot processes. Boxed areas correspond with close-up views.
(B) Various cortical tubular structures in a human kidney. Numbered boxed areas display close up views of distinct tubular structures.
p, podocytes; cap, capillary; pe, parietal epithelium; ec, endothelial cell; ery, erythrocyte; fp, foot process; gbm, glomerular basement membrane; asterisk, slit diaphragm; arrow head, fenestrae; pte, proximal tubular epithelium; pt, proximal tubule; dt, distal tubule; lu, lumen; bb, brush border; mi, mitochondria; nu, nucleus; bm, basement membrane; tj, tight junction.

Movie S1:
***In Vivo* Imaging (z Stack) of Vascularized hiPSC-MAFB-BFP Organoids after Labelling of Blood Plasma with 2,000 kDA FITC-labelled Dextran after 7 and 14 Days of Transplantation**

Movie S2:
***In Vivo* Imaging (z Stack) of Vascularized hESC-SOX17-mCherry Organoids after Labelling of Blood Plasma with 2,000 kDA FITC-labelled Dextran after 14 Days of Transplantation**

Supplemental Experimental Procedures

Maintenance of Human Pluripotent Stem Cells

hESC and hiPSC lines were maintained in Essential 8 medium (E8, Thermo Fisher Scientific) according to the manufacturers protocol. hESC-ENVY (Costa et al., 2005), H9 hESC line *SOX17*$^{mCHERRY/w}$*RUNX1C*$^{GFP/w}$ (hESC-SOX17-mCherry) (Ng et al., 2016) and hiPSC-CRL1502 clone C32 (Briggs et al., 2013) were initially maintained on irradiated mouse embryonic feeders (MEFs) and hESC medium (Costa et al., 2008) before transfer to feeder free culture conditions.

Also numerous other hiPSC lines were investigated. An extra hiPSC-CRL1502 line was generated without the use of MEFs using episomal reprogramming plasmids (Chen et al., 2011) including a plasmid encoding miR302-367 (Howden et al., 2015) and mRNA encoding a truncated version of EBNA1 (Chen et al., 2011). Electroporation was performed using a Neon Transfection Device (Thermo Fisher Scientific) (1,400 V, 20 ms, 2 pulses). Cells were initially maintained in fibroblast medium (DMEM/F12 (Thermo Fisher Scientific) supplemented with 15% FCS and 1× NEAA (Thermo Fisher Scientific)) followed by E7 medium (Essential 8 medium without transforming growth factor β) and further cultured in E8 medium using EDTA for passaging. Reporter hiPSC *MAFB*:mTagBFP2 (hiPSC-MAFB-BFP) was simultaneously reprogrammed and gene-edited using CRISPR/Cas9 in E8 medium (unpublished).

Two other hiPSC lines, LUMC0072iCTRL01 and LUMC0099iCTRL04, were generated on MEFs from fibroblasts using Simplicon RNA Reprogramming Kit (Millipore) (iPSC core facility, LUMC) and further cultured in TeSR-E8 medium (Stem Cell Technologies). Pluripotency and spontaneous differentiation to the three germlayers was verified by immunofluorescence. All cell lines were negative for mycoplasma. All hiPSCs were transferred to culture in E8 medium on vitronectin and were maintained for several passages as small clumps using 0.5 mM UltraPure EDTA (Thermo Fisher Scientific) before transfer to E8 culture as single cells using TrypLE Select (Thermo Fisher Scientific) and the addition RevitaCell Supplement (ThermoFisher Scientific) for 24 hr. The E8 medium was supplemented with 0.5% Penicillin-Streptomycin (Thermo Fisher Scientific) and hPSCs were passaged twice a week.

Differentiation and Organoid Formation

hPSCs were plated for differentiation on vitronectin coated culture dishes at 10,000 cells / cm^2 in E8 medium supplemented with RevitaCell. The cells were cultured until the dish was about 10–20% confluent (usually 24 hr) on the day that the differentiation was started (day 0). Cells were incubated for 4 days in 8 μM CHIR99021 (R&D Systems) in STEMdiff APEL medium (APEL) or STEMdiff APEL2 medium (Stem Cell Technologies) supplemented with 1% Protein Free Hybridoma Medium II (PFHMII, Thermo Fisher Scientific) and Antibiotic-Antimycotic (Thermo Fisher Scientific). From day 4–7, cells were treated with 200 ng mL^{-1} rhFGF9 (R&D Systems) and 1 μg mL^{-1} heparin (Sigma Aldrich) in APEL medium. After 7 days, cells were switched from monolayer culture to 3D culture after a 1 hr pulse with 5 μM CHIR to stimulate nephrogenesis. Differentiated cells were dissociated using Trypsin-EDTA (0.25%, Thermo Fisher Scientific) and centrifuged at 400 × *g* containing 500,000 cells per tube. The pellets were transferred to a Transwell 0.4 μM pore polyester membrane and further cultured on an air-liquid interface for another 5 days in APEL medium containing 200 ng mL^{-1} rhFGF9 and 1 μg mL^{-1} heparin (only bottom compartment). For the remaining days, the organoids were further cultured in medium without FGF9 and heparin and medium was changed every 2 days. Organoids were maintained on the transwell membranes until day 7 + 18 to day 7 + 53.

Animal Experiments

All animal experimental protocols were approved by the animal welfare committee of the Leiden University Medical Center. Eight week old recipient mice (n = 8, non-obese diabetic/severe combined immunodeficiency (NOD/SCID), Charles River Laboratories) were anesthetized with isoflurane and injected with temgesic (buprenorphine) for pain relief before surgery. Core body temperature was maintained at 37 °C. Via flank incisions, the kidneys were exteriorized and a small incision was made in the renal capsule. Kidney organoids, cultured for 7 + 18 days were bisected and transplanted under renal capsule in the left and right kidney. The mice were anesthetized and sacrificed after 7 and 28 days and the kidneys were collected.

Intravital Microscopy

For Intravital microscopy, 8 week old recipient mice (n = 10, NOD/SCID, Charles River Laboratories) were anesthetized with isoflurane and injected with temgesic (buprenorphine) for pain relief before surgery. Core body temperature was maintained at 37 °C. Organoids derived from hiPSC-MAFB-BFP and hESC-SOX17-mCherry were transplanted under the renal capsule as described above. A titanium abdominal imaging window was implanted on top of the left kidney as described (Ritsma et al., 2012; van Gurp et al., 2016). In short, a purse-string suture was placed in the intra-abdominal muscle, the kidney was exposed and then fixed in the subcutaneous space by tightening of the suture around the stalk while blood flow and ureter remained intact. A second purse-string suture was made along the edge of the incision in the skin and an abdominal window was fitted, placing the skin in the window groove. Tightening the suture fixed the window in the skin with the kidney and organoid directly underneath it. After surgery, mice were housed separately and provided with food and water at libitum. Seven and 14 days after surgery the mice were intravitally imaged on a Zeiss LSM 710 NLO upright multiphoton microscope equipped with a tunable Mai Tai Deep See multiphoton laser (690–1040 nm). Mice were anesthetized with isoflurane and their core body temperature was kept at 37 °C during the imaging session using a temperature-controlled heat mat connected to a rectal probe. Mice were intravenously injected in the tail vein with 2,000 kDa FITC-Dextran (100 µL of 20 mg/mL, Sigma FD2000S) before the imaging started. The animals were placed on their side on a custom-made microscope insert with the window stably fixed in an upward horizontal position using a custom made window holder. Imaging was performed through a W Plan-Apochromat 20×/1.0 DIC M27 75 mm objective. Fluorophores in iPSC-MAFB-BFP organoids were excited at 800 nm and emission was collected in two LSM PMTs: FITC (522–600 nm) and BFP (440–500 nm). Fluorophores in hESC-SOX17-mCherry organoids were excited using single photon at 488 (FITC) and 568 (mCherry) and emission was collected in two LSM PMTs: FITC (500–558 nm) and mCherry (578–700 nm). Time lapse series were collected and Z stacks were taken with a Z-step of 1 or 2 µm.

Immunohistochemistry

Organoids (day 7 + 18) were stained for kidney structures as described previously (Takasato et al., 2015, 2016). Briefly, organoids were fixed in 2% paraformaldehyde (PFA) at 4 °C for 20 minutes. The organoids were permeabilized and blocked in 10% donkey serum in 0.3% TritonX in PBS for 2 hr. Primary antibodies were incubated overnight and were detected by secondary antibodies incubated for 2 hr at room temperature or overnight at 4 °C. Organoids under the mouse renal capsule were snapfrozen in TissueTek or fixed for 20 min in 2% PFA and stored in PBS for whole mount analysis. Frozen kidney sections (5–10 µm thick) were fixed in 2% PFA for 10 minutes at room temperature and permeabilized in 0.3% TritonX in PBS for 15 minutes. Mouse on Mouse Basic Kit was used to detect structures in the human organoid and mouse kidney.
Immunofluorescence characterisation of the transplanted and non-transplanted organoids was performed using antibodies for NPHS1 (AF4269, R&D Systems), WT1 (SC-192, Santa Cruz Biotechnology), CUBILIN (SC-20607, Santa Cruz Biotechnology), CD31 (555444, BD Biosciences), ECAD (610181, BD Biosciences), MECA-32 (553849, BD Biosciences), LTL-biotin-conjugated (B-1325, Vector Laboratories), FITC-conjugated PHA-E (FL-1121, Vector Laboratories), Tamm Horsfall (BT85-9500-54, Biotrend) thiazide-sensitive NaCl cotransporter (NCC, AB3553, Millipore), aquaporin-2 (AQP2) (kindly provided by Joost Hoenderop and Peter Deen, Nijmegen), GATA3 (AF2605, R&D Systems), PDGFR-β (MAB1263, R&D Systems), MEIS1/2/3 (39795, Active Motif), LAMININ (L9393, Sigma Aldrich) and human nuclei (abin361360, antibodies-online). Primary antibodies were detected with donkey-α-sheep Alexa Fluor 568 (A-21099) and 647 (A-21448), donkey-α-rabbit Alexa Fluor 568 (A-10042) and 647 (A-31573), donkey-α-mouse Alexa Fluor 488 (A-21202), goat-α-mouse Alexa Fluor 488 (A-21121), donkey-α-goat Alexa Fluor 568 (A-11057) and 647 (A-21447), donkey-α-rat Alexa Fluor 488 (A-21208), goat-α-guinea pig Alexa Fluor 647 (A-21450), streptavidin Alexa Fluor 532 (S11224, all Thermo Fisher Scientific) and donkey-α-rat Alexa Fluor 568 (ab175475, Abcam).
Organoids and sections were counterstained with Hoechst33258 (Thermo Fisher Scientific) and embedded in ProLong Gold Antifade Mountant (Thermo Fisher Scientific) respectively in 35 mm glass bottom dishes (MatTek corporation) or adhesive microscope slides (StarFrost, Knittel glass). Tissues were examined using the Leica White Light Laser Confocal Microscope TCS SP8 using LAS-X Image software with 3D module (Leica) or the LSM 780 confocal microscope (Zeiss).

Cytokine Analysis

Cell culture supernatant of the organoids was collected weekly from day 7 + 10 until day 7 + 52 from 1 to 3 wells with 3 organoids during 3 independent differentiations. The levels of VEGF released into the supernatant were assessed using the Human Premixed Magnatic Luminex Assay for VEGF according to the manufacturer's protocol. The Bio-Plex Luminex system (Bio-Rad) was used for readout and VEGF concentration was expressed as pg mL^{-1}.

Transmission and Scanning Electron Microscopy

Tissue samples of organoids at day 7 + 18 and organoids under the mouse renal capsule were fixed overnight at 4 °C in 1.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium-cacodylate buffered solution, pH 7.4. Subsequently, the tissue was rinsed twice with 0.1 M sodium cacodylate-buffered solution, and fixed for 1 hr on ice in 1% osmium tetroxide (Electron Microscopy Sciences) in sodium cacodylate buffer. Tissue probes were further washed 2× with sodium cacodylate buffer, dehydrated overnight in 70% ethanol, then in 80% ethanol (10 min), 90% ethanol (10 min), and 100% ethanol absolute (twice 15 min; once 30 min).

For TEM, the tissue samples were further infiltrated with a mixture of epon LX-112 (Ladd Research) and propylene oxide (Electron Microscopy Sciences) (1:1) for 1 hr, followed by pure epon for 2 hr, embedded in pure epon, mounted in BEEM capsules (Agar Scientific) and polymerized for 48 hr at 60 °C. Ultrathin sections (100 nm) were mounted on copper slot grids (Storck Veco B.V.), covered with formvar film and carbon layer, and then stained with an aqueous solution of 7% uranyl acetate for 20 minutes, followed by Reynold's lead citrate for 10 minutes. Organoids at day 7 + 18 and organoids under the mouse renal capsule were analyzed at an acceleration voltage of 120 kV using an FEI Tecnai 12 (BioTWIN) transmission electron microscope (FEI), equipped with an FEI 4k Eagle CCD camera. Virtual slides showing glomerular and tubular structures that allow for an unbiased assessment of the whole organoids were recorded using automated large-scale data acquisition combined with stitching software (Faas et al., 2012). Images were captured at 13,000× or 18,500× magnification, respectively corresponding to a 1.66 or 1.22 nm pixel size at the specimen level.

For SEM, dehydration of the tissue samples was immediately followed by critical point drying. Samples were mounted on 0.5" SEM pin stubs (Agar Scientific) covered with conductive carbon discs (Agar Scientific) and then coated with gold-palladium. SEM images were acquired on a JEOL JSM-6700F Field Emission Scanning Electron Microscope (JEOL Europe B.V.).

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