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

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https://doi.org/10.1016/j.stemcr.2018.01.041

SUMMARY

Human pluripotent stem cell (hPSC)-derived kidney organoids may facilitate disease modeling and the generation of tissue for renal replacement. Long-term application, however, will require transferability between hPSC lines and significant improvements in organ maturation. A key question is whether time or a patent vasculature is required for ongoing morphogenesis. Here, we show that hPSC-derived kidney organoids, derived in fully defined medium conditions and in the absence of any exogenous vascular endothelial growth factor, develop host-derived vasculization. In vivo imaging of organoids under the kidney capsule confirms functional glomerular perfusion as well as connection to pre-existing vascular networks in the organoids. Wide-field electron microscopy demonstrates that transplantation results in formation of a glomerular basement membrane, fenestrated endothelial cells, and podocyte foot processes. Furthermore, compared with non-transplanted organoids, polarization and segmental specialization of tubular epithelium are observed. These data demonstrate that functional vasularization is required for progressive morphogenesis of human kidney organoids.

INTRODUCTION

The adult kidney is a highly complex organ, containing more than 20 specialized cell types, arranged in a spatial architecture critical to its function. Each human kidney contains approximately one million functional units: the nephrons. These regulate the plasma composition by glomerular filtration, active tubular secretion, and reabsorption of waste products and useful substances, respectively, in addition to the contribution of metabolic, hemodynamic, and endocrine functions. Worldwide, the number of patients with end-stage kidney disease necessitating dialysis or transplantation is reaching epidemic proportions (Hill et al., 2016). Therefore, innovative models that can advance our understanding of kidney disease and the potential for endogenous kidney regeneration are warranted. Human pluripotent stem cells (hPSCs) represent one possible source for such replacement kidney tissue.

Human kidney cell types have recently been generated from hPSCs (Freedman et al., 2015; Morizane et al., 2015; Taguchi et al., 2014; Takasato et al., 2015; Xia et al., 2013), and strategies to control and guide the resulting development of these hPSC-derived kidney tissues will be crucial for future regenerative medicine applications. Each of the available protocols varies slightly in the growth factors used, timing, and format of cell culture. As such, each protocol generates slightly different subsets of renal cell types (Morizane and Bonventre, 2017). Xia et al. (2013) generated ureteric bud progenitor cells that contribute to the collecting duct after co-culture with embryonic kidney, while Taguchi et al. (2014), working first with mouse embryonic stem cells, showed the exclusive induction of nephrons with strong evidence for podocyte formation. This group has subsequently provided a separate protocol for the generation of collecting duct from mouse PSC (Taguchi and Nishinakamura, 2017). Takasato et al. developed a differentiation protocol that simultaneously induces all four renal progenitors (nephron progenitors, ureteric bud progenitors, renal interstitial progenitors, and endothelial progenitors) from human induced pluripotent stem cells (hiPSCs) to generate what are referred to as kidney organoids (Takasato et al., 2015, 2016). In these human kidney organoids, segmented nephrons are connected to collecting ducts, surrounded by renal interstitial cells and an endothelial network (Takasato et al., 2015, 2016). While this and some other protocols (Freedman et al., 2015) show evidence for the formation of a vasculature, not all protocols contain endothelial cells (Morizane et al., 2015) and, when vascular tissue is present,
peritubular and glomerular capillary beds are not yet appropriately patterned (Freedman et al., 2015; Takasato et al., 2015). Although each of these approaches can generate cell types and structures recognizable as elements of a developing human kidney, they represent protocols spanning no more than a few weeks of culture. As such, the degree of resulting maturation in vitro is universally low, as demonstrated at the transcriptional and morphological level, while the anatomical structure is also inaccurate. This raises the question of whether maturation requires additional time, changes in culture conditions or a patent blood supply.

The formation of the glomerulus in mice commences with the involution of the proximal end of the elongating nephron to form an outer layer of parietal epithelial cells and an internal group of pre-podocytes (Quaggin and Kreidberg, 2008). This occurs without an apparent need for inductive signals from mesangial cells or the vascular endothelium. However, development of a glomerular filtration barrier and formation of slit diaphragms is known to require the presence of vascularization (Quaggin and Kreidberg, 2008). In the embryonic mouse kidney, SCL+ progenitors have been shown to be essential in initiating the development of the glomerular microcirculation with the FOXD1-derived stroma giving rise to all mural populations with these being essential for appropriate vascular patterning (Dekel et al., 2003, 2004; Sequeira-Lopez et al., 2015; Sequeira Lopez and Gomez, 2011). While these progenitor populations are present in the developing organ, the major blood vessels are thought to arise via angiogenic ingrowth from outside of the developing organ (Dekel et al., 2003). Multiple studies have shown that, upon transplantation of embryonic human and mouse kidney tissue in vivo, vascular precursors within the transplanted tissue can give rise to the glomerular capillaries (donor-derived vasculogenesis), but also that host-derived endothelial cells can migrate into the graft to form blood vessels within the transplanted tissue (Dekel et al., 1997, 2002; Hammerman, 2004; Rogers et al., 1998). Sharmin et al. (2016) showed evidence for vascularization and podocyte slit diaphragm formation in hPSC-derived kidney structures treated with vascular endothelial growth factor (VEGF) and placed under the renal capsule of recipient mice. However, while endothelium was shown to be essential in initiating the formation of small clumps, making precise seeding of cell numbers difficult resulting in lower-differentiation reproducibility. We therefore switched our cultures to dissociation withTrypLE Select, which provided the opportunity to passage the hPSCs as single cells. However, survival of these cells post dissociation was very low and we therefore supplemented the medium with RevitaCell, a commercially available Rho-kinase inhibitor, for 24 hr after plating, which substantially increased survival and attachment of the cells (Figure S1C). We were now able to plate a more precise and reproducible number of cells per well leading to a more reproducible on vitronectin (Figure S1B). Cell confluence at commencement of differentiation was very important. Passing the hPSCs with EDTA resulted in the formation of small clumps, making precise seeding of cell numbers difficult resulting in lower-differentiation reproducibility. We therefore switched our cultures to dissociation with TrypLE Select, which provided the opportunity to passage the hPSCs as single cells. However, survival of these cells post dissociation was very low and we therefore supplemented the medium with RevitaCell, a commercially available Rho-kinase inhibitor, for 24 hr after plating, which substantially increased survival and attachment of the cells (Figure S1C). We were now able to plate a more precise and reproducible number of cells per well for the differentiation. We next investigated a range of 5,000–20,000 cells/cm² (Figure S1D). Differentiations that were initially seeded in E8 medium at a density of 10,000 cells/cm² using TrypLE Select with RevitaCell on vitronectin were highly reproducible with high differentiation...
The CD31+ cells do not invade glomerular structures (NPHS1+) and pericytes (PDGFR-LTL+), Lis of Henle (Tamm Horsfall+), distal tubule (ECAD+), collecting duct (AQP-2+), and stromal cells (MEIS1/2/3+) visualized as 3D

Endothelial cells (CD31+) are mainly localized around glomerular structures (NPHS1+) and not around tubular structures (LTL+).

Close-up view of the boxed area displays the structures in detail.

Tile scan of an immunofluorescent organoid demonstrating anatomically correct interconnected segments of the nephron: glomerulus, proximal tubule, distal tubule, and collecting duct. Observed by the formation of small vesicles in the air-liquid interface. On day 7 + 5, FGF9 and heparin were pulsed with CHIR99021 before dissociation, aggregation, and transfer to transwell filters to form 3D structures and continued to differentiate supported by the air-liquid interface. On day 7 + 5, FGF9 and heparin were removed from the medium and nephrogenesis was visualized by the formation of small vesicles in the organoids (Figure 1A). To evaluate the outcome, organoids were cultured until day 7 + 18 of differentiation, and characterized for the presence of appropriate structures using immunofluorescence. NPHS1 and WT1 marked the podocytes in the glomerular structures (Figure 1B). Furthermore, proximal tubular (CUBN+, PHA-E+, LTL+), loop of Henle (Tamm-Horsfall+), distal tubular (ECAD+, GATA3−), collecting duct (ECAD+, GATA3+, AQP2+) structures, and interstitial cells (MEIS1/2/3+) were identified (Figure 1B). Successful differentiation was observed in six independent cell lines (four hiPSCs and two hESCs) (Figures S2B and S2C). We also show the presence of the sodium chloride co-transporter (NCC) on both the basolateral and apical membrane of the distal convoluted tubule cells (Figure 1C). Whole organoid immunofluorescence showed the presence of appropriately segmenting nephron structures with glomeruli (NPHS1+), proximal tubule (LTL+), and distal tubule/collecting duct (ECAD+) (Figure 1D). As previously reported, day 7 + 18 kidney organoids contained NPHS1+ glomerular structures surrounded by CD31+ endothelial cells and PDGFR-β+ pericytes (Figures 1E and 1F). However, formation of the capillary loop inside the glomerular structures was not overtly evident.

Organized Glomerular and Tubular Structures Observed by Electron Microscopy
To further evaluate the organization and maturity of the glomerular and tubular structures, we performed both scanning and transmission electron microscopy (SEM, TEM) analysis of organoids at day 7 + 18. SEM showed a glomerular structure in the organoid that was surrounded by a putative Bowman’s capsule. The podocytes inside the Bowman’s capsule were aligned and showed small, immature foot processes (Figure S3A). To allow for an unbiased assessment of ultrastructural features of the organoids, we used tiling of adjacent TEM fields to produce virtual slides providing a wide field of view at nanometer-scale resolution (Faas et al., 2012). Such unbiased wide-field nanomicroscopy revealed the presence of primitive foot processes in between the glomerular cells (Figures 2A and 2B). At this time point, the tubular structures were multilayered epithelial structures with no or a small lumen, with some evidence of apical microvilli forming (Figures 2A, 2B, and S3B). In addition, mitochondria were present in these cells. The multi-layered or pseudo-stratified epithelium, and the lack of a clear tubular basement membrane, suggested incomplete polarization. As suggested by the immunofluorescence, there was no evidence of glomerular vasculature in these organoids.

Prolonged Time in Culture Does Not Influence Organoid Maturation
To evaluate the influence of prolonged culture on these kidney organoids, we evaluated the organoids after 7 + 53 days in culture on the air-liquid interface using nanomicroscopy. We observed lack of evidence for glomerular

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Figure 1. Kidney Organoids Derived from Induced Pluripotent Stem Cells Display Structures Characteristic of a Nephron on Day 7 + 18 of Differentiation

(A) Bright-field images and schematic of the experimental timeline for the generation of kidney organoids.

(B) Immunofluorescence analysis of the different segments of the nephron: podocytes (WT1+, NPHS1+), proximal tubule (CUBN+, PHA-E+, LTL+), Lis of Henle (Tamm Horsfall+), distal tubule (ECAD+), collecting duct (AQP2+), and stromal cells (MEIS1/2/3+) visualized as 3D structures.

(C) Presence of the sodium chloride (NCC) symporter in the kidney organoid.

(D) Tile scan of an immunofluorescent organoid demonstrating anatomically correct interconnected segments of the nephron: glomerulus, proximal tubule, distal tubule, and collecting duct. Close-up view of the boxed area displays the structures in detail.

(E) Endothelial cells (CD31+) are mainly localized around glomerular structures (NPHS1+) and not around tubular structures (LTL+).

(F) The CD31+ cells do not invade glomerular structures (NPHS1+) and pericytes (PDGFR-β+) surround glomerular structures.
Figure 2. Ultrastructural Evaluation Shows Lack of Advanced Maturation of Kidney Organoids In Vitro over Time at Day 7 + 18 and Day 7 + 53

(A) Low-magnification transmission electron microscopy (TEM) tile scan of kidney organoid cultured for 7 + 18 days on the air-liquid interface displaying glomerular and tubular structures.

(B) High-magnification TEM images of boxed areas in (A) demonstrate characteristic structures, such as podocytes with primitive foot processes in glomerulus (top) and brush border with microvilli in the open lumen of a tubular structure (bottom).

(C) Low-magnification TEM image of a kidney organoid cultured for prolonged time (7 + 53 days) on the air-liquid interface.

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capillaries, extraglomerular podocytes, and substantial deposition of glomerular extracellular matrix, little evidence of tubular maturation, the presence of ectopic stromal tissues, and cell death (Figures 2C and 2D). We focused on the presence of CD31+ cells in the organoids after prolonged culture and observed progressive loss of these cells around day 7 + 25 (data not shown). This is consistent with our previous profiling of the differentiation protocol where vascular gene expression decreased between 7 + 11 and 7 + 18 days (Takasato et al., 2015). One possible cause for a lack of robust vascularization of these developing glomeruli would be the absence of VEGF production by the podocytes. We therefore evaluated the production of VEGF by these organoids and found increased VEGF levels across time in culture, peaking by day 7 + 17 and remaining at this level even after culture for 7 + 52 days (Figure 3A). Given the evidence for VEGF production within the kidney organoids, it was possible that even more prolonged time in culture may have been required for increased instances of capillary loop formation in vitro. The immature phenotype of the tubules may also have reflected insufficient time for tubular elongation and segment differentiation. However, we conclude that prolonged culture in this format does not facilitate onward differentiation of kidney organoids, whereas, likely due to the large size of these structures, a progressive pathology arises resulting from hypoxia and metabolic deficit.

Kidney Organoids Become Functionally Vascularized upon Transplantation In Vivo

As has been studied previously with transplantation of human and mouse metanephric tissues (Dekel et al., 2002, 2003, 1997; Hammerman, 2004), we aimed to examine the degree of organoid maturation achieved upon transplantation under the renal capsule of recipient immunocompromised mice. Biased day 7 + 18 kidney organoids were transplanted under the renal capsule for up to 28 days. The size of the organoid increased progressively with time of transplantation (Figure 3B), and toluidine blue staining demonstrated presence of glomerular and tubular structures within the organoid after transplantation (Figure 3C). SEM analysis suggests the presence of vasculature inside the organoid and the glomerular structures (Figure 3D). Indeed, host-derived mouse endothelial cells (MECA-32+) were evident within the hPSC-derived transplanted organoid (Figure 3E). Further analysis on the location of mouse endothelial cells, showed an invasion of host cells inside glomerular-like structures (NPHS1+ and WT1+) (Figure 3F). In addition, we observed peritubular vascularization in association with tubular epithelium (Figure 3G).

To study functionality of the vasculature we used repeated intravital multiphoton imaging of the neo-vascularized transplanted organoids. We employed the combinatorial approach of organoid transplantation and an abdominal imaging window (AIW) (Ritsma et al., 2012) to serially image the transplanted organoids in vivo through imaging windows that were surgically implanted in the flank of the animal (Figure 4A). Kidney organoids (day 7 + 18) were generated using our previously described reporter hiPSC line in which blue fluorescent protein (mTAG-BFP) was inserted into one copy of the MAFB gene, a transcription factor expressed in the podocytes of the glomerulus (Howden et al., unpublished data). In vitro, this results in the clear co-localization of BFP and the glomerular-expressed protein NPHS1 (Figure S4A). These iPSC-MAFB-BFP kidney organoids were bisected and transplanted under the renal capsule of the left kidney of recipient mice. Immediately afterward, the kidney was secured in the subcutaneous space by tightening the abdominal muscle tissue around the kidney stalk without restricting the vascular flow. The AIW was implanted in the skin on top of the kidney. At both day 7 and 14 after transplantation, mice were intravenously injected with 2,000 kDa fluorescein isothiocyanate (FITC)-labelled dextran just prior to imaging to visualize host-derived blood flow. Intravital imaging revealed the presence of host-derived blood flow (FITC-labelled dextran, green) through glomerular structures (MAFB-BFP, blue) within the organoids (Figures 4B–4E; Movie S1). To test whether the hPSC-derived endogenous vascular plexus present within the transplanted organoids connects to the invading host vasculature, similar experiments were performed using transplanted organoids derived from the hESC-SOX17-mCherry reporter line (Ng et al., 2016). This allowed us to visualize the organoid-derived mCherry+ endothelial cells (Figure S4B).

In contrast to the loss of endothelial cells in organoids maintained in culture, we noted that the pre-existing human vascular plexus in the organoids was still present after the transplantation procedure (Figures 4F and 4G). Using intravital microscopy we showed that part of this human-derived vascular plexus was perfused by the injected FITC-labelled dextran, suggesting that the plexus functionally connects to the ingrowing host-derived renal vasculature (Figure 4F; Movie S2). Indeed, immunofluorescence

(B) Detailed TEM images of boxed areas in (C) from glomerular structure (top) and tubular structure (bottom) demonstrate features of maturation, such as foot processes, formation of glomerular basement membrane, and microvilli, respectively. However, the structures are more disorganized.

p, podocyte; fp, foot processes; te, tubular epithelium; mi, mitochondria; bb, brush border.
Figure 3. Kidney Organoids Become Vascularized upon Transplantation for 7 and 28 Days
(A) Concentration of VEGF (pg mL⁻¹) determined by Luminex assay in the supernatant of three cultured organoids measured weekly from day 7 + 10 until day 7 + 52. Data are represented as means ± SEM.

(B) Transplanted human kidney organoid under renal capsule of mice on the day of transplantation (tx), after 7 and 28 days showing growth upon vascularization.

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showed the combined presence of human CD31+ and mouse MECA-32+ endothelial cells in these organoids at transplantation day 14 (Figure 4G), showing the contribution from both host and donor to the vascular network.

**DISCUSSION**

In this study, we have directly compared the effect of prolonged in vitro culture versus transplantation on the forward maturation of hPSC-derived organoids. We show the development of a functional host-derived vascular network that invades the developing glomerular structures in the organoids upon transplantation. With time, transplantation resulted in progressive maturation of the glomerular filtration barrier together with the deposition of a GBM, the development of fenestrated glomerular endothelium, and apical-basal polarization of the podocytes. Time-matched non-transplanted organoids do not show any of these phenomena, but remain structurally immature and disorganized. Similarly, with time post transplantation, tubular epithelial maturation is observed with the development of a polarized single-layer epithelium, widened lumina with areas that display an extensive brush border, as can be seen in proximal tubules, and areas with intratubular segmental specification, such as can be observed in the collecting duct in the adult kidney.

These experiments establishing the capacity of a PSC-derived model of the human kidney recapitulate earlier work by Dekel et al. (2003) and Harari-Steinberg et al. (2013), where human fetal kidney tissue was transplanted under the kidney capsule. In the work of Dekel et al., early fetal, but not mature, human kidney tissue was able to recruit host-derived endothelial cells to form a host-derived glomerular circulation (Dekel et al., 2003). Here we show that the vascular network originating from the hPSC lines and present within the organoids contribute to graft vascularization via anastomosis to the host-derived endothelial plexus. This appears to recapitulate the embryonic development of the kidney vasculature, where both angionic hemangioblast precursor cells, as well as vasculogenic endothelial precursors within the organ itself, are required for development of the glomerular microcirculation (Halt et al., 2016; Robert et al., 1998; Sequeira Lopez and Gomez, 2011). To explore whether the pre-existing vascular network in the organoids connects to the ingrowing host circulation, we transplanted organoids derived from hESC-SOX17-mCherry under the kidney capsule, thus
Figure 4. *In Vivo* Imaging of Vascularized Kidney Organoids Shows Glomerular Vascularization and Chimeric Organoid Circulation after 7 and 14 Days of Transplantation

(A) Mouse with the abdominal imaging window (AIW) on the left kidney and a close-up view of a kidney window after 14 days of implantation. Right picture shows the mouse with the AIW installed for microscope analysis.

(B and C) *In vivo* imaging of transplanted organoids derived from hiPSC-MAFB-BFP after 7 days (B) and 14 days (C) of transplantation. Circulating plasma was visualized with 2,000 kDa FITC-labelled dextran and flow was detected in BFP⁺ glomerular structures.

(D and E) Kymographs from marked positions demonstrating the dynamic blood flow through the capillary in the organoid (D) and inside the glomerular structure (E) after 14 days of transplantation.

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allowing fate mapping of donor-derived endothelium. Our results show that there is still a SOX17-mCherry+ vascular network present in the kidney organoids for at least 14 days after transplantation. Part of this network becomes connected to the host circulation upon transplantation, as indicated by the dextran-FITC perfusion, which underscores the vasculogenic potential of this plexus. The apparent lack of ingrowth of the pre-existing vascular network into glomeruli of the cultured organoids may to some extent reflect a culture condition less permissive for vasculogenic activity within organoids. On the other hand, podocytes were shown to produce high amounts of VEGF, even after prolonged in vitro culture in APEL medium, which should have facilitated endothelial cell migration into the glomerular structures. Our data suggest that angiogenic ingrowth from the host was either required to drive vasculogenesis of the endogenous endothelium in the kidney organoids or simply facilitated the survival of these cells.

It is well known that both the formation of the GBM as well as the attainment of the fenestrated endothelial phenotype depend upon iterative growth factor cross-communication between podocytes and endothelial precursor cells (Quaggin and Kreidberg, 2008). This process is critical to the development of a functioning glomerular filtration unit (Scott and Quaggin, 2015), and the subsequent developmental organization of the kidney. The ultrastructural analysis of the glomerular filtration barrier in the transplanted organoids shows an advanced state of maturation and is indicative of functional endothelial-podocyte crosstalk. The peritubular microcirculation primarily derives from the efferent arteriole of the glomerular capillary network (Sequeira Lopez and Gomez, 2011). After organoid transplantation, host-derived peritubular capillaries were also observed particularly at 28 days of transplantation, whereas the CD31+ cells that were originally present in the organoids did not show such an organization. This further indicates the progressive development of a renal microcirculatory network.

Previous studies using human and mouse PSC-derived nephrons (Sharmin et al., 2016; Taguchi et al., 2014) demonstrated vascularization of iPSC-derived podocytes upon transplantation under the kidney capsule. The model developed by Sharmin et al. however, used a distinct differentiation protocol (Taguchi et al., 2014), which generates significant evidence of glomerular/proximal nephron segments, but appears to lack more distal nephron cell types. Their transplantation approach also required the addition of VEGF to the transplant and resulted in excessive growth of non-renal stromal cells during prolonged transplantation. In this study, we show that the kidney organoids themselves actively secrete VEGF and did not require any

(F) Low and high magnification of hESC-SOX17-mCherry derived kidney organoids after transplantation for 14 days revealing mCherry+ endothelial cells perfused with FITC-labelled dextran (arrowhead) and host-derived vasculature (asterisk). Note that not all mCherry+ endothelial cells were perfused (open arrowhead).
(G) Immunofluorescence of human CD31+ and mouse MECA-32+ in the transplanted kidney organoid.
further stimulation to induce host-derived angiogenic vascularization upon transplantation.

To date, a substantial amount of published kidney organoid characterization has been performed at the level of immunofluorescence. It is important to note that, while this approach can identify the presence of protein markers for various renal segments from early on, the apparent presence of these markers remained present even in long-term in vitro cultures and have not been useful as markers for maturation. To assess tissue and cellular maturation in this study, we used virtual nanomicroscopy (Faas et al., 2012), an approach that employs TEM, rapid automated data collection, and stitching, to create large virtual slides with a relatively large field of view at nanometer-scale resolution. Having access to data at this scale and resolution allows for a genuine and unbiased representation of ultrastructural events at the level of several nephron structures. The virtual nanomicroscopy approach allowed for assessment of maturation by ultrastructural mapping of cellular processes, such as apicobasal polarization of epithelium and the formation and structure of the glomerular filtration barrier.

It is possible that the prolonged in vitro culture of kidney organoids in the current format (floating filter at an air-liquid interface) was unsuccessful at supporting maturation for reasons other than a lack of blood flow. By day 7 + 25, kidney organoids are 5–7 mm in diameter and potentially reaching a size unsupported by the culture system itself.

While changes to the in vitro culture approach may improve outcomes, it would appear that the capacity to visualize a perfused transplanted organoid in vivo using the abdominal window approach may provide a far superior approach to evaluating progress in vivo. Indeed, this may be a notable approach for disease modeling where maturation of appropriate tubular compartments is likely to be crucial.

We also demonstrate here the feasibility of transferring the existing protocol by Takasato et al. (2016) from hPSCs cultured on feeders to E8 culture such that both the culture of the undifferentiated hiPSCs and the differentiation procedure itself are fully defined and serum free. Despite these changes, this protocol resulted in highly reproducible and efficient differentiations from multiple starting cell lines and did not compromise the potential to derive all renal progenitor lineages. This revised protocol will enhance the broad utility of kidney organoids by increasing the transferability between different hPSC lines and different laboratories.

In summary, kidney organoids may provide a suitable technology for drug screening, disease modeling, and studying kidney regeneration. Most of the current developments in this field are still solely focused on kidney (proximal) tubular epithelial cells. However, renal toxicity can also primarily occur in the glomerulus (Barri et al., 2004; Musu et al., 2011; Naesens et al., 2009; Semeniuk-Wojtas et al., 2016). Nephrotoxic drugs such as bisphosphonates, cyclosporine, NSAIDs, antihypertensive drugs, and anti-angiogenesis inhibitors have been shown to predominantly lead to glomerulopathies by compromising endothelial-podocyte crosstalk or podocyte integrity (Radhakrishnan and Perazella, 2015). Similarly,
most kidney diseases are primarily glomerulus-associated diseases. The vascularized kidney organoids, and in particular the potential to develop a glomerular filtration unit in these organoids, may constitute a more faithful model for screening of nephrotoxicity, as well as renal disease modeling.

EXPERIMENTAL PROCEDURES

Full details are provided in Supplemental Experimental Procedures.

Maintenance of hPSCs

hESC and hiPSC lines were transferred to culture on vitronectin in E8 medium (Thermo Fisher Scientific) and maintained for several passages as small clumps using 0.5 mM UltraPure EDTA (Thermo Fisher Scientific) before transfer to culture as single cells using TrypLE Select (Thermo Fisher Scientific) and the addition RevitaCell Supplement (Thermo Fisher Scientific) for 24 hr.

hiPSC-CRL1502 clone C32 (Briggs et al., 2013) were initially maintained on irradiated MEFs in hESC medium (Costa et al., 2008). An extra hiPSC-CRL1502 line was generated without culture on MEFs using episomal reprogramming plasmids (Chen et al., 2011; Howden et al., 2015). Reporter hiPSC MAFBmTagBFP2 (hiPSC-MAFB-BFP) was simultaneously reprogrammed and gene-edited using CRISPR/Cas9 (Howden et al., unpublished data). LUMC0072iCTRL01 and LUMC0099iCTRL04 were generated on MEFs from fibroblasts using a Simplicon RNA Reprogramming Kit (Millipore) (iPSC core facility, LUMC) and further cultured in TeSR-E8 medium (Stem Cell Technologies).

Figure 7. Wide-Field Ultrastructural Evaluation of Transplanted Organoids Demonstrates Peritubular Vascularization and Intratubular Specification

(A) Transmission electron microscopy stitch showing tubular structures after 28 days of transplantation displaying an open lumen with a single layer of epithelial cells. The tubule has a brush border, tight junctions, and a peritubular capillary. (B) Transmission electron micrographs showing tubular dilation with micro projections that suggest the presence of intercalated cells existing in the collecting duct-type structure.

Boxed areas correspond with numbered close-up views. te, tubular epithelium; ptc, peritubular capillary; bb, brush border; ec, endothelial cell; tj, tight junction; ic, intercalated cell; ci, cilium; ce, centriole; mv, microvilli; mi, mitochondria; mp, micro projections.

Differentiation and Organoid Formation

hPSCs were plated on vitronectin-coated culture dishes at 10,000 cells/cm² in E8 medium supplemented with RevitaCell. The differentiation was started (day 0) when the dish was 10%–20% confluent (usually 24 hr). Cells were incubated for 4 days in 8 µM CHIR99021 (R&D Systems) in STEMdiff APEL medium (APEL). On day 4 medium was switched to APEL medium containing 200 ng mL⁻¹ rhFGF9 (R&D Systems) and 1 µg mL⁻¹ heparin (Sigma-Aldrich). Cells were switched (day 7) from monolayer culture to 3D culture on Transwell 0.4 µM pore polyester membranes.
in the same medium after a 1 hr pulse with 5 μM CHIR. On day 7 + 5, growth factors were removed and APEL 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. Recipient mice (n = 8, non-obese diabetic/severe combined immunodeficiency, 8 weeks, Charles River Laboratories) were anesthetized with isoflurane and injected with temgesic (buprenorphine) for pain relief. 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 (day 7 + 18) were bisected and transplanted under renal capsule of both kidneys. Samples were collected after 7 and 28 days.

Intravital Microscopy
For intravital microscopy, hiPSC-MAFB-BFP and hESC-SOX17-mCherry organoids were transplanted (n = 10), and a titanium AIW was implanted on top of the left kidney as described previously (Ritsma et al., 2012; van Gurp et al., 2016). Seven and 14 days after surgery, mice were intravitally imaged on a Zeiss LSM 710 NLO upright multiphoton microscope equipped with a Mai Tai Deep See multiphoton laser (690–1040 nm). Mice were injected in the tail vein with 2,000 kDa FITC-Dextran (100 μl of 20 mg/mL, Sigma FD2000S) and 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 20x/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 were stained for kidney structures as described previously (Takasato et al., 2015, 2016). Organoids under the mouse renal capsule were snapfrozen in TissueTek or fixed in 2% paraformaldehyde and stored in PBS. Mouse on Mouse Basic Kit was used to detect structures in the transplanted human organoid and mouse kidney. Tissues were examined using the White 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 were assessed using the Human Premixed Magnetic Luminex Assay for VEGF. The Bio-Plex Luminex system (Bio-Rad) was used for readout and VEGF concentration was expressed as pg mL⁻¹.

TEM and SEM
Tissue samples of organoids and organoids under the mouse renal capsule were analyzed for TEM 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 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, images were acquired on a JEOL JSM-6700F Field Emission Scanning Electron Microscope (JEOL Europe).

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and two movies and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.01.041.

AUTHOR CONTRIBUTIONS
C.W.v.d.B. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. L.R., M.C.A., L.E.W., B.M.v.d.B., D.G.L., E.L., M.K., and J.M.V. performed experiments and acquired and/or interpreted data. S.E.H generated and characterized reporter lines, acquired data, and interpreted data. A.J.K. facilitated electron microscopy research. M.T. provided advice and contributed intellectually. T.J.R. and M.H.L. designed experiments, interpreted data, and wrote the manuscript.

ACKNOWLEDGMENTS
We thank Christian Freund (hiPSC core facility, LUMC, Leiden, the Netherlands) for providing two hiPSC lines (LUMC0072CTRL01 and LUMC0099CTRL04), Christine Mumery (LUMC, Leiden, the Netherlands) for providing hES3 ENVY, and Andrew Elefanty and Edouard Stanley (MCRI, Melbourne, Australia) for providing reporter hESC-SOX17-mCherry. We acknowledge the support of Maaike Hanegraaf, Angela Koudijs, Dorien Ward-van Oostwaard, Manon Zuurmond (LUMC, Leiden, the Netherlands), Caro Overmars-Bos and Joost Hoenderop (Radboud UMC, Nijmegen, the Netherlands), and Pei Er, Joanne Soo and Irene Ghobrial (MCRI, Melbourne, Australia). M.H.L. is a Senior Principal Research Fellow of the National Health and Medical Research Council of Australia (GNT1042093). Funding is from the European Community’s Seventh Framework Program (FP7/2007-2013) (stem cell-based therapy for kidney repair, STELLAR, grant agreement number 305436) and RECellularizing ORgan Donors for KIDney bioengineering (RECORD KID, Dutch Kidney Foundation, 15RN02), the NIH (DK107344), and the National Health and Medical Research Council (NHMRC, GNT1100970). C.W.v.d.B. is supported by the Wiyadharma fellowship (Bontius Stichting, LUMC). L.R. is supported by a Veni-grant from the Netherlands Organisation for Scientific Research (NWO, 016.176.081), the Gisela Thier grant (LUMC), and a subsidy from the Leids Universiteits Fonds (LUF, CWB 7204). MCRI is supported by the Victorian Government’s Operational Infrastructure Support Program.
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Title:
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Date:
2018-03-13

Citation:
van den Berg, C. W., Ritsma, L., Avramut, M. C., Wiersma, L. E., van den Berg, B. M., Leuning, D. G., Lievers, E., Koning, M., Vanslambrouck, J. M., Koster, A. J., Howden, S. E., Takasato, M., Little, M. H. & Rabelink, T. J. (2018). Renal Subcapsular Transplantation of PSC-Derived Kidney Organoids Induces Neo-vasculogenesis and Significant Glomerular and Tubular Maturation In Vivo. STEM CELL REPORTS, 10 (3), pp.751-765. https://doi.org/10.1016/j.stemcr.2018.01.041.

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