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Exploring kidney organoid vascularization

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CHAPTER 3

Co-culture of kidney organoids with a perfusable endothelial cell network in a scalable organ-on-chip system

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Abstract

Kidney organoids cultured in vitro contain some endothelial cells (ECs), but lack a functional vasculature. This is an important limitation for disease modeling and regenerative medicine. Transplantation under the kidney capsule in mice has been shown to induce vascularization by mainly host-derived ECs, which are attracted by VEGF-A produced by the organoid podocytes. This is consistent with the process of vascularization in embryology, which is thought to be at least partly based on sprouting angiogenesis from previously formed blood vessels. We therefore hypothesized that in vitro vascularization of kidney organoids might require an external source of ECs. Here, we used the commercially available OrganoPlate Graft to co-culture kidney organoids with an EC network. This plate contains culture chips that each consist of 1 graft chamber flanked by 2 perfusable lanes and can be placed on a rocker to mimic shear stress. Human umbilical vein endothelial cells (HUVECs) or human induced pluripotent stem cell (hiPSC)-derived ECs seeded into the perfusion lanes formed a continuous endothelium from which vessels sprouted towards the kidney organoid after placing it in the graft chamber. This vascular network was perfusable, as shown by injection of fluorescently labeled dextran. As expected, podocyte-derived VEGF-A was a major inducer of angiogenic sprouting, and blocking of VEGF-A activity by VEGF neutralizing antibody strongly inhibited vessel growth. Unfortunately, although the vessels moved towards the organoid, a connection between the vascular network and the kidney organoid did not form. Adaptation of the extracellular matrix, the differentiation stage of the organoids, the location of the ECs and addition of pericytes to the culture system did not improve the results. In conclusion, co-culture of kidney organoids and ECs in the Organoplate Graft results in the formation of a perfusable vascular network that sprouts towards the kidney organoid, but fails to invade it. This implies that an essential unknown cue for vascularization is still missing in the organoid and culture environment.

Introduction

Kidney organoids derived from human induced pluripotent stem cells (hiPSCs) offer great possibilities for disease modeling and regenerative medicine. They contain glomerular, tubular, stromal and endothelial cells (ECs) and have been shown at a transcriptional level to resemble third trimester fetal kidneys[1]. However, to harness their full potential, limitations with regard to maturity and functionality will have to be overcome. Vascularization is essential for kidney maturation and functionality. It provides the developing kidney with oxygen and nutrients and enables the interaction between ECs and podocyte precursors that leads to the formation of the glomerular basement membrane and the maturation of podocytes and ECs[2-4]. Unfortunately, the ECs present in kidney organoids fail to form the blood vessels and glomerular capillary tufts that are required for this interaction. Transplantation of kidney organoids in mice has been shown to induce functional vascularization and even enables glomerular filtration[5-8]. Interestingly, the blood vessels in these transplanted organoids are mainly host-derived, formed through sprouting angiogenesis in response to vascular endothelial growth factor (VEGF-A) produced by the organoid. This matches the process of kidney vascularization in embryology, which is believed to be at least partly based on angiogenesis from existing vasculature induced by podocyte-derived VEGF-A[9-11]. Although transplantation in mice is an effective method for functional vascularization, variability is difficult to control and it lacks scalability. We therefore aimed to develop a model for in vitro vascularization of kidney organoids. Based on the in vivo findings, we hypothesized that this might require a pre-formed vascular network that could function as a source for sprouting angiogenesis.

The development of microfluidic chips has enabled high-throughput 3D culture of ECs as perfusable microvessels that display more resemblance to in vivo vasculature than 2D monolayer cultures[12]. In this study, we used the Organoplate Graft, a microfluidic chip system specifically designed for co-culture of a graft with perfused microvessels. We demonstrate that kidney organoids, through production of VEGF-A, induce the formation of angiogenic sprouts from the microvessels towards the organoids. Unfortunately, we did not observe the formation of a vascular network within the kidney organoids.

Results

Kidney organoids induce angiogenesis upon co-culture with endothelial cells

The Organoplate Graft contains 64 chips that each consist of a graft chamber supported by an extracellular matrix (ECM) channel, flanked by 2 perfusion lanes (Fig. 1a). The ECM

channel, perfusion lanes, and graft chamber can be accessed separately to add ECM, seed ECs, and insert a graft. By placing the Organoplate on a rocker, bidirectional flow can be generated in the perfusion channels. We aimed to use this system to enhance kidney organoid vascularization by co-culturing kidney organoids with ECs. Kidney organoids were generated from hiPSCs using a previously published protocol[1,6]. This entails differentiation of hiPSCs in monolayer culture in APEL2 medium for 7 days, followed by the formation of aggregates that are further maintained at an air-liquid interface on transwell filters (Fig. 1b). The characteristics of the Organoplate Graft required adaptation of several aspects of the protocol: The size of the graft chamber necessitates sectioning of the organoids, the chip only allows for submerged culture, and the culture medium has to support co-culture. To analyze the effects of these modifications on kidney organoid morphology, we evaluated the impact of each adaptation separately in a regular 24 well plate with transwell inserts (supplementary Fig. 1). Sectioning organoids did not have a major impact on organoid morphology. Organoids in submerged culture in the organoid medium APEL2 maintained their structure, although they appeared slightly more dense. The EC medium MV2, however, had a detrimental effect on the organoids in submerged as well as air-liquid interface culture, with organoids losing recognizable structures (supplementary Fig. 1). In the Organoplate Graft, we therefore decided to use APEL2 medium in the graft chamber and MV2 medium in the perfusion lanes, to provide optimal support to both organoids and ECs.

For co-culture experiments, the ECM channel was loaded with Collagen I followed by incubation for 15 min (Fig. 1b, bottom, day -2). Subsequently, red fluorescent protein expressing human umbilical vein endothelial cells (RFP-HUVECs) were seeded in the perfusion lanes, positioning them against the collagen I, and allowed to attach before placing the Organoplate on the rocker (Fig. 1b, bottom, day -2). Once the ECs had formed a confluent lining of the perfusion channels, usually 2-3 days after seeding, a kidney organoid fragment was added to the graft chamber (Fig. 1b, bottom, day 0). Kidney organoids were prepared for co-culture by generating and maintaining them under standard conditions until day 7+12 or day 7+18 of differentiation (Fig. 1b, top), when they were sectioned using a surgical knife and added to the Organoplate. These timepoints were based on the previously demonstrated increasing production of VEGF by kidney organoids between d7+10 and d7+17 of differentiation[6]. In contrast to standard culturing conditions, in which the organoids are maintained directly on a polyester transwell membrane, the Organoplate contains Collagen I in the ECM channel, a suitable matrix to support ECs in microfluidic culture systems[12]. To evaluate the optimal ECM for kidney organoids, we compared the effect of using no additional ECM to placing a layer of Matrigel (Growth factor reduced, phenol red free) or Collagen I in the graft chamber before adding the organoid.

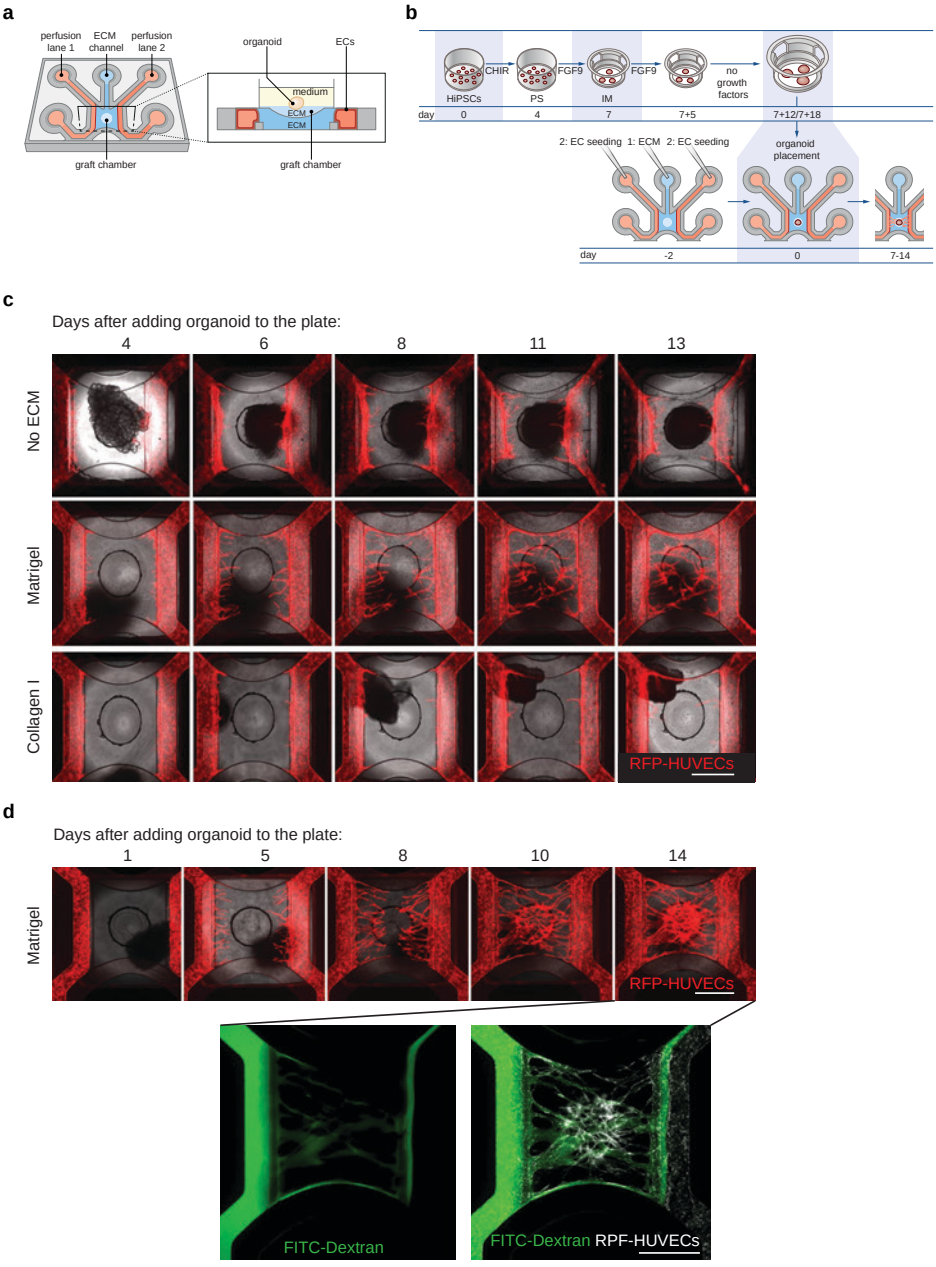

Figure 1: Kidney organoids induce angiogenesis upon co-culture with endothelial cells. a Schematic of culture chips in the Organoplate Graft. Each chip consists of a graft chamber supported by an extracellular matrix (ECM) channel and flanked by 2 perfusion lanes. ECM channel, both perfusion lanes and the graft chamber can be accessed separately for gel loading, cell seeding, graft placement and medium change. **b** Timeline of differentiation of hiPSCs to kidney organoids (top), and preparation of the Organoplate followed by co-culture of organoids and ECs on the Organoplate Graft (bottom). Differentiating iPSCs are cultured as a monolayer for 7 days, followed by aggregation

to organoids that are maintained at an air liquid interface. On day 7+12 or day 7+18 of differentiation, organoids are sectioned and an organoid fragment is added to the graft chamber of the Organoplate Graft, that has been prepared 2-3 days earlier by loading the ECM channel with Collagen I and seeding ECs in the perfusion lanes. Kidney organoids and ECs are co-cultured for 7-14 days. HiPSC human induced pluripotent stem cells PS primitive streak IM intermediate mesoderm**. c** Co-culture of kidney organoids with ECs in the Organoplate. Top: Without additional ECM in the graft chamber. Middle: With Matrigel coating of the graft chamber. Bottom: With Collagen I coating of the graft chamber. Matrigel coating of the graft chamber yields optimal results, with abundant sprouting of ECs from the perfusion lanes into the graft chamber. RFP-HUVECs are shown in red. **d** Upon injection of FITC-labelled dextran into the left perfusion lane, dextran flows through the EC network to the opposite perfusion lane, demonstrating that the angiogenic sprouts connect to each other and are perfusable. RFP-HUVECs are shown in white, FITC-labelled dextran in green.

In co-culture experiments without ECM in the graft chamber, the ECs displayed sprouting angiogenesis from day 6 to 8 after adding the organoid, before the sprouts deteriorated (Fig. 1c, top panel). The addition of Collagen I to the graft chamber yielded similar results (Fig. 1c, bottom panel). Adding Matrigel to the graft chamber resulted in extensive sprouting of ECs from the perfusion channels (Fig. 1c, middle panel). These newly formed vessels appeared to connect to each other in the middle of the chip (Fig. 1d, top panel). Further experiments were therefore performed with Matrigel in the graft chamber. To evaluate connectivity and perfusability of the vessels, FITC-labeled Dextran (150 kDa, Sigma-Aldrich 46946) was added to one side of the perfusion channel. The Dextran remained inside the lumen of the blood vessels and travelled through them to reach the perfusion channel on the opposite side (Fig. 1d, bottom panel). Not all of the vascular network was perfusable, as RFP-positive, FITC-negative HUVECs were also visible. These might be the youngest sprouts that have yet to become functional.

VEGF-A is an important driver of organoid-induced angiogenesis

We hypothesized that the angiogenic sprouting that occurred upon co-culture of kidney organoids with ECs was induced by factors secreted by the organoids. To test this, conditioned APEL2 medium was collected from kidney organoids cultured under standard conditions in transwells, and added to the graft chamber of a chip with RFP-HUVECs seeded in the perfusion lane. As a negative control, unconditioned APEL2 medium was used. Indeed, in the chips with conditioned medium, sprouts appeared after 4 days and expanded over time, whereas in the control chips, no sprouting was observed (Fig. 2a). A Luminex Assay was then performed to assess the levels of several known modulators of angiogenesis in conditioned medium from the Organoplate Graft: Vascular Endothelial Growth Factor A (VEGF-A), Angiopoietin-1 (Ang1), Angiopoietin-2 (Ang2), and platelet derived growth factor BB (PDGF-BB) (Fig. 2b). Medium was collected from the perfusion lanes and graft chambers of chips with organoid monoculture or organoid-EC co-culture on day 5 and 12 after adding the organoid.

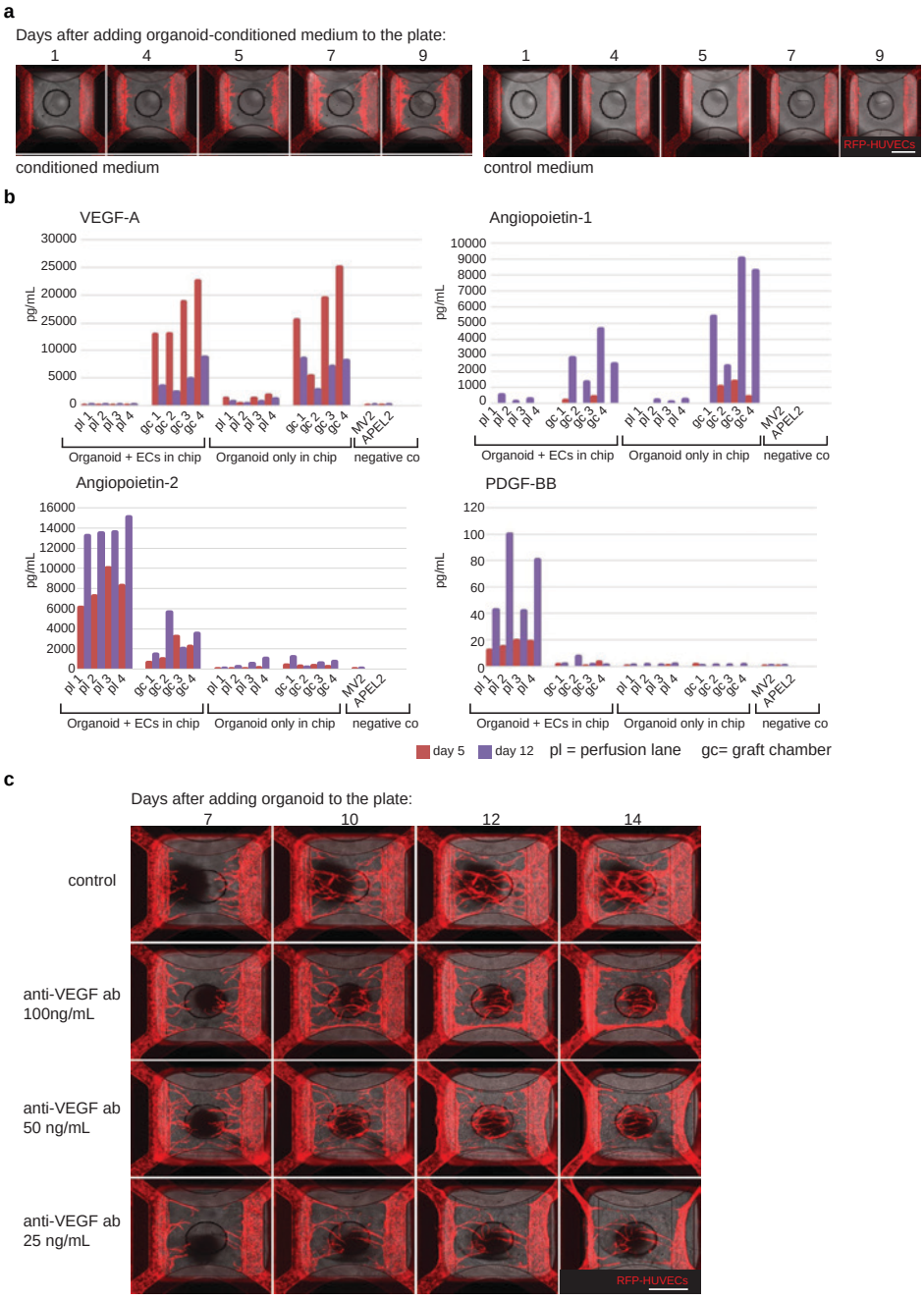

Figure 2: VEGF-A secretion is an important driver of organoid-induced angiogenesis. a Organoid-conditioned medium induces sprouting of ECs in the Organoplate (left), while control APEL2 medium does not (right), indicating that organoids secrete an angiogenic factor. **b** Levels of Angiopoietin-1, Angiopoietin-2, VEGF-A, and PDGF-BB measured by Luminex screening in conditioned medium from the perfusion lanes and the graft chamber of Organoplate chips after 5 and 12 days and in unconditioned MV2 and APEL2 medium. Kidney organoids produce VEGF-A at day 5 and at a lower

level at day 12 and ANGPT1 at day 12. ECs produce ANGPT2 and PDGF-BB with the highest levels detected at day 12. pl perfusion lane gc graft chamber. **c** VEGF-blockade with anti-VEGF antibody starting on day 7 after adding the organoid to the Organoplate leads to demise of the vascular network.

On day 5, the graft chambers contained high levels of VEGF-A in organoid monoculture as well as organoid-EC co-culture. In the perfusion lanes of chips containing ECs, Ang-2 was detected at this timepoint. On day 12, VEGF-A levels in the graft chambers were still detectable although clearly lower than at day 5, and a striking increase in Ang-1 concentration occurred in the graft chambers. In the perfusion lanes, Ang-2 expression was even higher than at day 5. In addition, PDGF-BB was detected (Fig. 2b). VEGF-A and Ang-1 were clearly produced by the organoids, as these factors were detected in the graft chamber irrespective of the presence of ECs. This fits with their role in embryonic kidney development, in which both factors are known to be secreted by podocyte precursors. VEGF-A is essential for the attraction of ECs into the vascular cleft of the S-shaped body stage nephron[2,13]. Ang-1 is produced by podocytes and mural cells and promotes EC survival and quiescence[14]. Ang-2 and PDGF-BB appear to be secreted by ECs, since they are mainly detected in medium from the perfusion lanes of chips with organoid-EC co-culture. The effect of EC-derived Ang-2 on ECs is context-dependent, stimulating angiogenesis and vascular remodeling in the presence of VEGF-A while inducing vessel regression in its absence[15,16]. In this co-culture model with high VEGF-A levels, it is expected to support the formation of the vascular network. PDGF-B is released by angiogenic ECs to attract pericytes for vessel stabilization[17,18]. To confirm the role of VEGF-A in the formation of a vascular network in the Organoplate Graft, we blocked its activity through the addition of an anti-VEGF antibody in 3 different concentrations (25, 50 and 100ng/mL) 7 days after placing the organoid in the graft chamber (Fig. 2c). This led to the regression of formed sprouts and affected the confluency of the ECs in the perfusion lanes over the following week (Fig. 2c).

Endothelial cells do not invade kidney organoids

Although the VEGF-A and ANGPT-1 produced by the organoids induced EC sprouting, 3D reconstruction of whole mount stained and imaged organoids revealed that the ECs did not invade the organoids (Fig. 3a). A connection between the newly formed microvessels and the endogenous organoid ECs did not occur. The explanation for the limited success of this co-culture method could lie in any one or multiple components of the system: ECs, organoids, proximity between organoids and ECs, the lack of mural cells supporting the microvessels, the characteristics of the flow in the chip, or suboptimal interplay between any of these elements. In an attempt to enhance our experimental set-up, we made stepwise adaptations to several of these components, namely the ECs, the proximity and the mural cells. ECs in vivo are heterogeneous and display organ specificity. Even within the kidney, ECs are phenotypically

diverse to support organ functionality[19-21]. HUVECs, although frequently used as an easily accessible EC source, display their own tissue-specific phenotype[22-24].

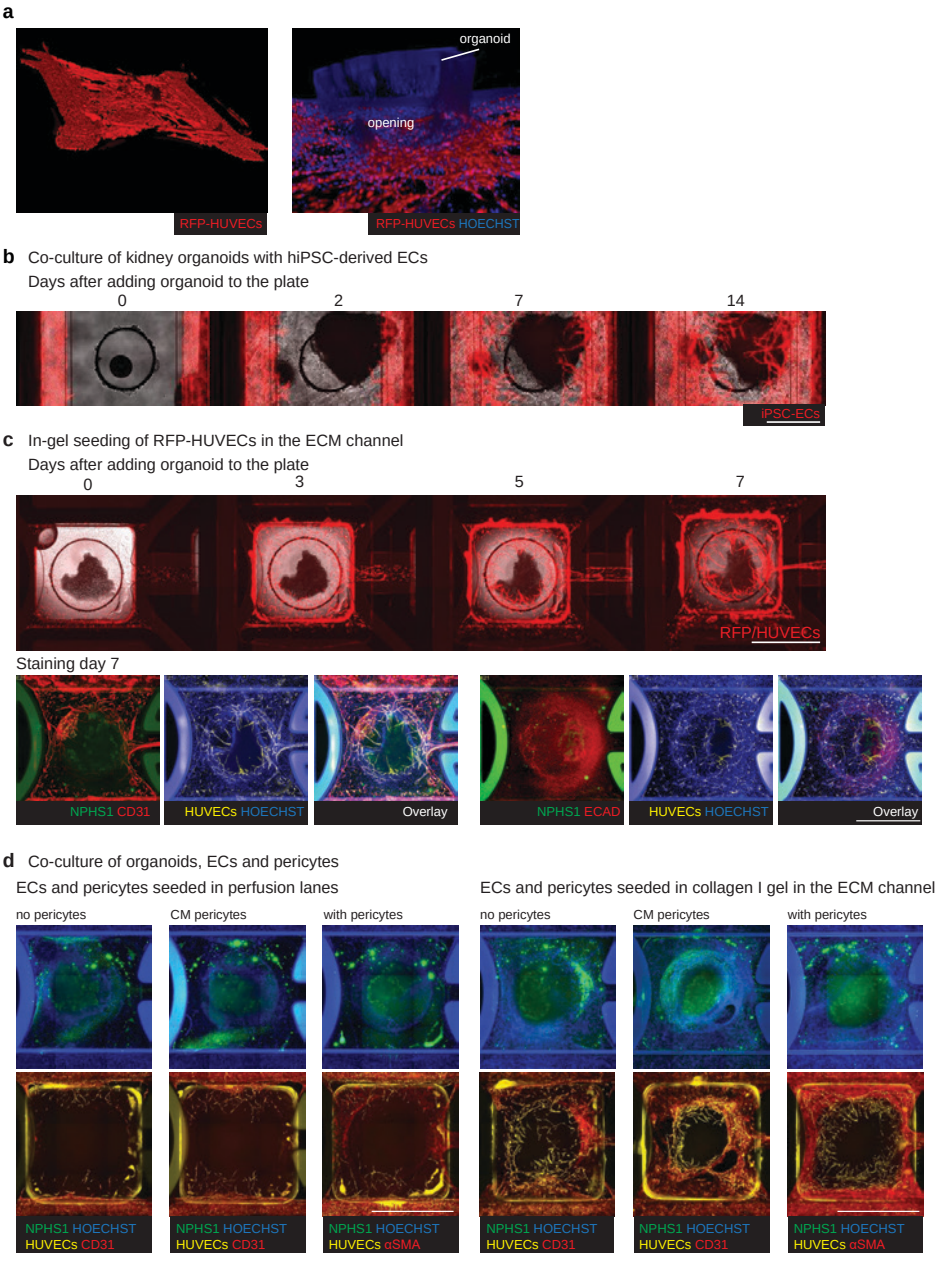

Figure 3: strategies to improve co-culture. a 3D reconstruction of kidney organoid and EC co-culture demonstrates that, although ECs move towards the organoid, they fail to invade them. **b** Co-culturing kidney organoids with hiPSC-ECs instead of HUVECs leads to similar results, with the induction of sprouting angiogenesis without invasion of kidney organoids by the hiPSC-ECs.

c Seeding HUVECs in hydrogel in the ECM channel to minimize the distance between ECs and organoid does not enhance vascularization after 7 days of co-culture. Left: Organoids were stained for NPHS1 (green), CD31 (red), HOECHST (blue). Right: Organoids were stained for ECAD (green), CD31 (red), HOECHST (blue). RFP-HUVECs are depicted in yellow. Overlay of RFP-HUVECs and CD31 is shown in white. **d** Co-culture of organoids and ECs in pericyte conditioned medium (CM pericytes) or with pericytes does not induce invasion of ECs into the organoid. Left: ECs and pericytes seeded in the perfusion lanes. Right: ECs and pericytes seeded in collagen I in the graft chamber. Organoids were stained for NPHS1 (green), CD31 (red), HOECHST (blue). RFP-HUVECs are depicted in yellow.

They might therefore lack the characteristics required for optimal interaction with renal cells as well as the plasticity to acquire these. As an alternative EC source we used hiPSC-derived ECs, generated through an established protocol[25]. Similar to the experiments with HUVECS, co-culture of organoids with hiPSC-ECs led to sprouting of ECs towards the organoid, but did not result in vascularization (Fig. 3b). Next, we enhanced the proximity between organoid and ECs by seeding HUVECs in hydrogel in the ECM channel directly below the organoid instead of in the perfusion lanes. Although the ECs formed microvessels, invasion of the organoids did not occur (Fig. 3c). Finally, we addressed the lack of mural cells in our system. In vivo, pericytes play an important role promoting vessel stabilization and integrity[26]. For practical reasons, we used commercially available brain pericytes. However, addition of pericytes or pericyte-conditioned medium to the co-culture system, either in the perfusion lanes or the ECM channel, did not induce the development of a more extensive vascular network or the establishment of a connection to the organoid (Fig. 3d). Also, the αSMA positive pericytes did not appear to support the newly forming blood vessels, but remained instead within the perfusion lanes or ECM channel depending on where they were seeded.

Discussion

In this study, we developed a method to co-culture kidney organoids and ECs in a microfluidic chip. The design of the Organoplate Graft enabled the maintenance of 64 organoids per plate, each flanked by 2 perfusion lanes. ECs seeded in the perfusion lanes formed a continuous lining within days, and upon addition of an organoid fragment to the graft chamber gave rise to perfusable angiogenic sprouts. Unfortunately, a connection between the microvessels and the organoids was not established.

Angiogenesis in vivo is regulated by the balance between angiogenic and quiescent stimuli. Quiescent ECs are maintained under the influence of autocrine and pericyte-derived signals such as Ang-1, VEGF-A, FGF and NOTCH. Upon sensing an angiogenic signal like VEGF-A, VEGF-C or Ang-2 released from hypoxic cells, vessel permeability is increased and ECs migrate toward the angiogenic signal, one EC forming the tip cell

and the others following as stalk cells[27]. The VEGF family plays an essential role in angiogenesis, and deficiency of either VEGF-A or its receptor VEGFR2 results in defects in vascular development and embryonic lethality[28-30]. It is therefore not surprising that in our model VEGF-A secretion by kidney organoids was indispensable for inducing angiogenesis, with VEGF blockade leading to the demise of the vascular network. Also, the increase over time of Ang-2 in the perfusion lanes matches the in vivo situation, in which Ang2 is released by sprouting ECs to enhance pericyte detachment, vessel permeability and further sprouting[27]. Despite the presence of angiogenesis promoting factors in the co-culture system, vascularization of the organoids did not occur, illustrating the complexity of angiogenesis regulation. Sprouting angiogenesis is induced by a gradient of proangiogenic factors that is established due to secretion by hypoxic and nutrient deprived cells. Once these cells are vascularized, the gradient diminishes and ECs obtain a quiescent phenotype [31]. It is perceivable that our culture method did not provide the optimal conditions to support the development of a gradient. As demonstrated in the Luminex assay, the organoids released VEGF-A into the medium in the graft chamber. Organoid medium was refreshed 3 times a week, allowing accumulation of proangiogenic factors in the medium. This may have led to the formation of a gradient with the highest concentration of VEGF-A in the medium instead of the organoid itself, explaining the development of microvessels into the graft chamber but not the organoid.

Once sprouting angiogenesis has occurred, functionality of newly formed blood vessels requires their stabilization and maturation. For this purpose, ECs attract pericytes through the production of PDGF-B[27]. The ECs in the co-culture system secreted low levels of PDGF-B which increased over time, implying the acquisition of a quiescent cell state by a proportion of the ECs. However, the absence of mural cells in the Organoplate Graft precluded coverage of the vessels by pericytes in response to PDGF-B. Upon addition of brain pericytes to the chip, these did not encase the ECs, possibly due to a mismatch in origin of ECs and pericytes. The inability to stabilize the microvascular network in the co-culture system could have contributed to the failure to establish a connection to the organoid.

ECs display extensive phenotypic diversity between and within different tissues[32-34]. This allows them to optimally support the diverse physiological functions of the organs and tissues. In this study, we utilized HUVECs and human iPSC-ECs as accessible EC sources, with the added advantage that fluorescently labeled cell lines are available. As discussed, neither cell type invaded the kidney organoids. Since HUVECs are terminally differentiated umbilical cord specific ECs, their lack of plasticity might play a role in the failure to integrate into the kidney organoids. On the contrary, the limitation of hiPSC-ECs is their immaturity, lacking a functional glycocalyx and displaying reduced

mitochondrial function[35]. Beside the characteristics of the ECs that were used, the type of flow in the Organoplate Graft likely influenced the outcome. One of the advantages of the Organoplate Graft is the possibility to subject cells and tissues to flow without an extensive pump and tubing system, by placing the plate on a rocker. In contrast to the in vivo situation, in which ECs are exposed to unidirectional flow, this induces alternating flow in opposite directions. In vivo, shear stress plays an important role in arterialization of ECs[36]. In addition, it enables the synthesis of the glycocalyx by inhibiting PFKFB3 through increasing the inhibitory effects of Krüppel-like factor 2 (KLF2) and nitric oxide[37,38]. It is unclear to what extent alternating flow such as in our system can mimic these effects. Laminar shear stress has been applied to kidney organoids in vitro in different forms[39-41]. Application of flow over the top of organoids increased organoid ECs compared to static conditions, but with only limited development of perfusable vessels[39]. Co-culture of kidney organoids with HUVECs subjected to laminar flow in different designs of microfluidic chips resulted in angiogenesis from the microvessel to the organoid[40] or, when adding VEGF to the medium in the microvessel, from the organoid to the microvessel[41]. In both cases, a connection between organoid and microvessels was established. Unfortunately, glomerular vascularization did not occur. Recently, a new Organoplate Graft became available that allows for unidirectional flow without the need for a tubing system. Utilization of this or a similar microfluidic system combined with more frequent medium changes to maintain an angiogenic gradient, and optimization of EC type and timing of co-culture might contribute to optimization of kidney organoid vascularization in vitro.

In conclusion, the Organoplate Graft enables the co-culture of kidney organoids with a perfusable EC network that is formed in response to VEGF-A secreted by the organoids. Unfortunately, although the microvessels and organoids come in close proximity, they do not form a connection. This study identifies possible culprits, such as an insufficient gradient of proangiogenic factors, suboptimal EC characteristics, lack of laminar flow and of mural cells, that can be addressed to enhance methods for in vitro vascularization of kidney organoids.

Methods

iPSC maintenance and differentiation to kidney organoids

HiPSC lines hiPSC-CRL1502 clone C32, and LUMC0072iCTRL01 were maintained in Essential 8 medium (E8, Thermo Fisher Scientific) with 0.5% Penicillin-Streptomycin (Thermo Fisher Scientific) on recombinant human Vitronectin (Thermo Fisher Scientific). Cell lines were mycoplasma free. hiPSCs were passaged twice a week using 0.5 mM UltraPure EDTA (Thermo Fisher Scientific). They were differentiated to kidney organoids using a previously published protocol.[6] Briefly this entails monolayer culture for 7 days

in STEMdiff APEL2 medium (Stem Cell Technologies) containing 1% PFHMII (Life Technologies) and Antibiotic-Antimycotic solution supplemented with 8 µM CHIR99021 (Tocris) for 4 days, followed by 200 ng mL^{-1} rhFGF9 (R&D Systems) and 1 µg mL^{-1} heparin (Sigma-Aldrich) for 3 days. On day 7, the cells were treated with a CHIR pulse, dissociated to single cells and centrifuged to form aggregates of 500,000 cells, which were placed on Transwell 0.4 µM pore polyester membranes and further cultured at an air-liquid interface. Until day 7+5, the developing organoids were cultured in APEL2-medium supplemented with 200 ng mL^{-1} rhFGF9 and 1 µg mL^{-1} heparin (only bottom compartment), and from day 7+5 onwards in APEL2 without supplements.

Endothelial cell maintenance

RFP-HUVECs (VeraVec, Angiocrine Bioscience or Alphabioregen, #RFP4) were cultured in Endothelial Cell Growth Medium MV2 (Promocell). ECs derived from NCRM1 hiPSCs with constitutive mCherry and Venus reporter, kindly provided by Valeria Orlova (LUMC), were cultured in Endothelial cell serum-free medium (EC-SFM, Gibco, Thermo Fisher Scientific). All ECs were maintained until 80% confluency in T75 tissue culture flask. The cells were dissociated with TrypLE™ Express Enzyme (Thermo, #12604021), before introducing them to the OrganoPlate Graft.

Co-culture in the Organoplate Graft

Co-culture of kidney organoids and ECs was performed using the OrganoPlate Graft with 400 µm x 220 µm (w x h) channels (Mimetas BV, the Netherlands). The ECM channel was loaded with 2.5 µL of gel composed of 4 mg/mL Collagen I (AMSbio Cultrex 3D Collagen I Rat Tail, 5 mg/mL, #3447-020-01), 100 mM HEPES (Life Technologies, #15630-122) and 3.7 mg/mL NaHCO3 (Sigma, #S5761) followed by incubation for 15 min at 37 °C. HUVECs or hiPSC-ECs were dissociated to single cells using TrypLE™ Express Enzyme (Thermo, #12604021), and 20000 cells suspended in 2 µL were seeded in each perfusion channel through the inlet. Subsequently, 50 µL of MV2 (HUVECs) or EC-SFM (hiPSC-ECs) medium was added to each inlet and the OrganoPlate Graft was incubated at 37 °C for at least 1 hour until the ECs attached to the bottom of the perfusion channels. 50 µL of MV2 (HUVECs) of EC-SFM (hiPSC-ECs) medium was then added to the perfusion channel outlets. The OrganoPlate Graft was placed in the incubator (37°C and 5% CO2) on a rocker switching between a +14° and -14° inclination every 8 min (OrganoFlow S, Mimetas) allowing bi-directional flow. Medium (50 µL in each inlet and outlet) was refreshed 3 times per week. ECs were maintained in monoculture for ~2-3 days until confluent vessels were formed in the perfusion channels. The graft chambers were

then prepared for the organoids by aspirating the medium, and adding 10µL of Matrigel (growth factor reduced, phenol red free, Corning) followed by incubation at 37°C for 20 min. Kidney organoids at day d7+12 or 7+18 of differentiation were sectioned using a surgical knife and one organoid fragment was added to each graft chamber, suspended in 30µL STEMdiff APEL2 medium (Stem Cell Technologies). Alternatively, an organoid fragment suspended in 10ul Matrigel was introduced to the graft chamber and incubated for 20 min before 30µL STEMdiff APEL2 medium was added. Sectioning of the organoids was required due to the size mismatch between organoids and graft chamber. Medium in the graft chamber was refreshed 3 times per week. For the experiments with EC in-gel seeding in hydrogrel, 3D Life ToGro Hydrogel from Cellendes (cat G94-1) was used. ECs were suspended in the hydrogel before loading the gel in the ECM channel.

Immunofluorescence analysis

Organoids and ECs were imaged periodically during the experiments using the ImageXpress Confocal Imaging system (Molecular Devices, ImageXpress Micro Confocal). HUVECs and hiPSC-ECs were fluorescently labeled, allowing for their identification during culture without prior fixation and staining. For more detailed analysis of organoid structures and the interaction between organoids and ECs, they were fixed and stained for immunofluorescence analysis within the Organoplate Graft after completing the experiment. Organoids and ECs were fixed in 2% paraformaldehyde (PFA) at 4°C for 20 min. They were permeabilized and blocked in 0.3% TritonX in PBS containing 10% donkey serum for 2 h. Primary antibodies were diluted in blocking solution and incubated for 24 h. Upon washing, secondary antibodies were incubated for 2–4 h at room temperature. Kidney organoids were characterized for NPHS1 (AF4269), ECAD (610181), CD31 (555444), α-SMA (ab5694). Primary antibodies were detected with donkey-α-sheep Alexa, donkey-α-mouse Alexa, donkey-α-rabbit Alexa conjugated with Fluorofores 488, 568 and 647. Nuclei were stained with Hoechst33258 (Thermo Fisher Scientific). All antibodies and isotype controls were validated in human kidney samples. Image Xpress Confocal Imaging system and Leica White Light Laser Confocal Microscope TCS SP8 using LAS-X Image software with 3D module (Leica) was used for analysis of the tissues.

Growth factor analysis

Cell culture supernatant of organoids cultured in transwells was collected during each media change between day 7+7 and day 7+18. It was pooled together and added to the graft chamber of the Organoplate Graft to evaluate its potential to induce angiogenesis.

For the measurement of growth factor concentrations in medium from the Organoplate Graft, cell culture supernatant was collected from the perfusion lanes and graft chamber of 4 chips containing organoids and ECs and 4 chips containing organoids only. Unconditioned MV2 and APEL2 medium was used as a negative control. Medium was collected 5 and 12 days after adding the organoid to the chips. The levels of VEGF-A, Ang1, Ang2 and PDGF-BB were assessed using the Human Luminex Discovery Assay. The Bio-Plex Luminex system (Bio-Rad) was used for readout and growth factor concentration was expressed as pg/mL

Blockade of VEGF-A activity was performed through the addition of human VEGF165 antibody (R&D Systems AF-293-NA) in 3 different concentrations (25, 50 and 100ng/mL), 7 days after placing the organoid in the graft chamber.

Pericyte culture

Primary human brain pericytes (Sciencell, 1200) were cultured on Poly-L-lysine (Cultrex, 3438-100-01) coated flask in pericyte medium (Pericyte Medium Kit (PM, cat#1201) including FBS (2%) (#0010), PGS (#1252) and P/S (#0503)). Cells were detached by use of TrypLE™ Express Enzyme (Thermo, #12604021), counted and pelleted (5 min, 300 x g), before addition to the OrganoPlate Graft.

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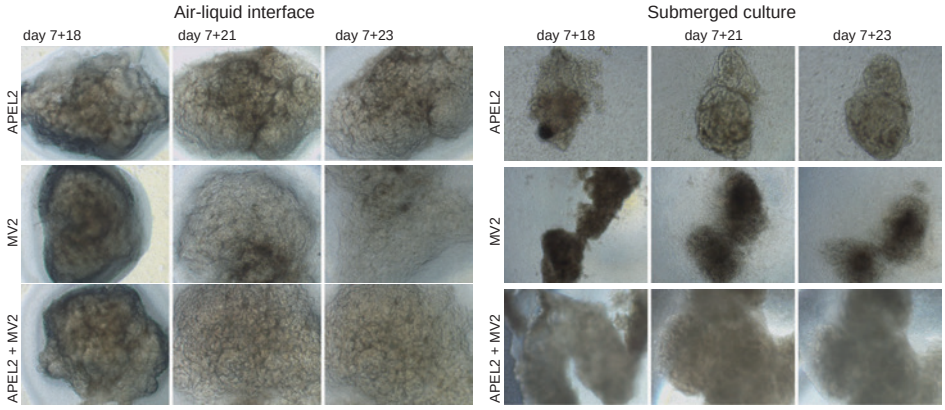
Supplementary Figures



Supplementary Fig. 1: Culturing kidney organoid fragments at air-liquid interface (left) versus submerged culture (right) in APEL2, MV2 or mixed medium