Vascularization of organoid microenvironments: perfusable networks for organoid growth and maturation

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Vascularization of organoid microenvironments: Perfusable networks for organoid growth and maturation

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Generation of functional vasculature within organoids is considered important for their development and maturation. However, direct differentiation of endothelial cells (ECs) in organoids remains challenging so that creating fully perfusable vasculature often still requires transplantation into host animals. This review discusses recent strategies for generating pre-vascularized human pluripotent stem cell (hPSC)-derived organoids, that include co-differentiation of ECs using growth factors or (an inducible transcription factor) ETV2, controlled assembly of tissue organoids with hPSC-derived ECs or Blood Vessel Organoids (BVOs), and 3D bioprinting. Additionally, the potential and key challenges of organ-on-chip technology for creating perfusable and functional vascular networks in organoids are explored, highlighting their implications for advancing research and improving experimental models of human tissue and disease.

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Introduction

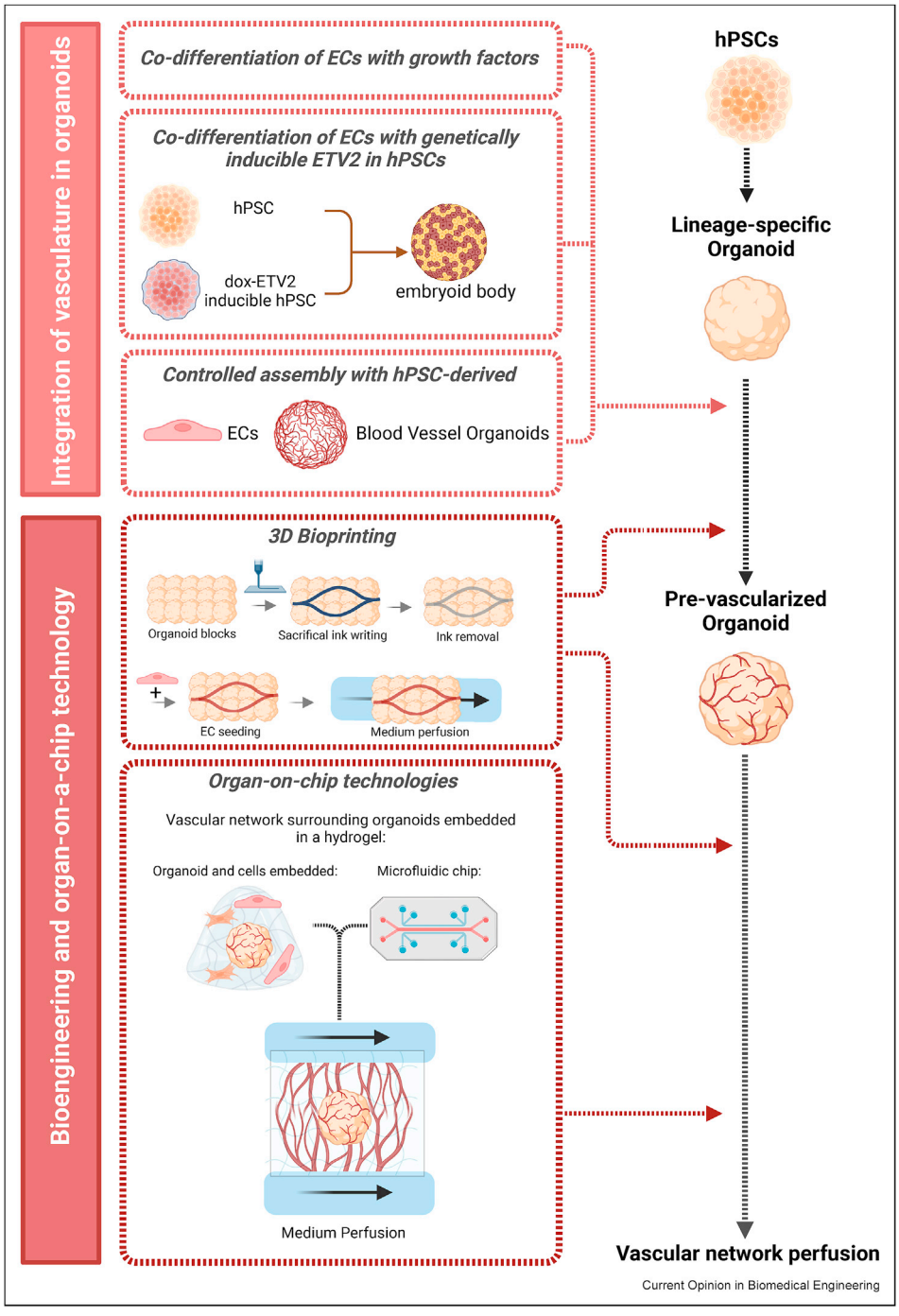
Organoids are three-dimensional (3D), self-organizing structures that mimic essential aspects of *in vivo* tissue architecture and function [1]. Originally derived from adult stem cells (ASCs), organoid models can now be derived from human pluripotent stem cells (hPSCs), including human induced PSCs (hiPSCs) and human embryonic stem cells (hESCs). These hPSC organoids recapitulate many features of human tissue including the stroma and not just the epithelial organ component as ASCs. In principle this means both healthy and diseased tissues can be created from donors, allowing the study of individual cell populations *in vitro* in a physiologically relevant context. However, a significant challenge in current organoid models is their lack of vasculature.

Integrating functional vasculature is important for advanced organoid development, as it not only facilitates oxygen and nutrient delivery but also supports tissue maturation [2]. Endothelial cells (ECs), which form the inner lining of all blood- and lymphatic vessels, originate from the mesoderm. This makes it challenging to co-differentiate ECs in ectoderm- and endoderm-derived organoids, as these require distinct signaling pathways and little or no mesoderm may form under those conditions [2,3]. Additionally, even though mesoderm- and some endoderm-derived organoids contain ECs, they are often few in number and they tend to regress over time [4–6]. The lack of fluid perfusion is another major challenge, as it is essential not only for maintaining vessel network stability and EC survival but also for the efficient delivery of oxygen, nutrients, and drugs [2,3].

To address these challenges, several strategies have been developed to incorporate vasculature into organoids. Several of these have resulted in the formation of a naïve vascular network that is typically coincident with improved tissue structure and functionality [7–15]. Achieving efficient perfusion and the formation of functional vasculature often requires *in vivo* transplantation to further enhance organoid maturation [3]. Alternatively, organ-on-chip (OoC) platforms support integration of pre-vascularized organoids into microfluidic devices [16,17], enabling perfusion and providing greater physiological relevance.

In this review, we discuss current strategies for generating pre-vascularized hPSC-derived organoid models and methods for establishing perfusable vascular networks using OoC technologies (Figure 1). Finally, we discuss future directions and challenges to the field.

Figure 1



Schematic of strategies to generate perfusable and functional vascular networks in organoid systems.
Integration of vascular networks into organoids to create vascularized tissue models derived entirely from hiPSC. This can be achieved in several ways, including co-differentiating ECs within the organoid using growth factors or hPSCs in which ETV2 is inducible, and co-assembly of pre-differentiated hPSC-ECs or Blood Vessel Organoids (BVOs) with tissue organoids. Bioengineering and OoCs support further development of perfusable and functional vascular networks through 3D bioprinting or the integration of pre-vascularized organoids into OoC systems.

Current approaches for integrating vasculature into organoids

Co-differentiation of ECs in organoids

In several hPSC-derived organoid models, especially those derived from mesoderm-like kidney and heart, the presence of ECs has been described during early stages of differentiation [7,8,18–21]. For example, kidney organoids derived from hiPSCs developed not only nephrons and collecting ducts but also networks of CD31+KDR+SOX17+ ECs that were surrounded by PDGFRA+ pericytes when the duration of WNT signaling was carefully controlled and followed by FGF9 supplementation [7]. In another study, Low et al. demonstrated that podocyte-produced VEGFA at an early stage of differentiation was sufficient to sustain resident vasculature [18]. Heart forming organoids, or cardioids, are another example. These organoids contain not only cardiomyocytes but also associated cardiac fibroblasts and ECs. Rossi et al. demonstrated that adding a combination of bFGF, ascorbic acid and VEGFA promoted vascular network formation and supported EC maturation over time in mouse ESC-derived cardiac gastruloids [9]. Alternatively, Hofbauer et al. showed that supplementing with low WNT and low ACTIVIN during cardiac mesoderm induction led to upregulation of VEGFA expression in cardiomyocytes, promoting EC differentiation in cardioids [19]. Another example is the heart forming organoids that co-develop not only myocardial but also formation of vessel-like structures and endocardial cells, as well as foregut endoderm tissue [20]. More recently, heart forming organoids were described with venous, arterial and hemogenic ECs and the ability to generate mature hematopoietic progenitors [8]. A different approach used spatially micropatterned hPSCs to generate cardiac vascular organoids by screening conditions that enhance differentiation of CMs, ECs, and VSMCs. As a result, addition of FGF2, SB, ANG1, ANG2, VEGF, PDGF-BB, and TGFb1 resulted not only in efficient EC- and VSMC differentiation but also supported the formation of branched networks and lumen and vessel maturation [21].

Protocols for differentiating endoderm-derived organoids often include mesoderm lineages with the potential to generate ECs. However, the first protocols frequently lacked ECs due to incompatibility of differentiating conditions for epithelial cells with EC development. Recently, new methods have been developed to increase EC numbers in intestinal and lung organoids. Holloway et al. modified the hPSC-derived intestinal organoid protocol to support the differentiation, expansion and maintenance of ECs [5]. By supplementing with VEGFA, BMP4 and FGF2 during the intestinal organoid patterning stage (day 3–6) and continuing with VEGFA during the organoid expansion phase (from day 6 and up to two months), they achieved a 9-fold increase in ECs [5]. Similarly, Miao et al. developed vascularized lung and intestine organoids by first co-differentiating mesoderm and endoderm lineages (day 0–3), followed by anterior-posterior patterning to specify lung and intestine lineages (day 3–7) [6]. Supplementing with VEGFA and ANG1 during the organogenesis stage (day 7–21) enhanced EC differentiation without disrupting the lung and intestine lineages. Childs et al. showed in a different approach that multi-lineage differentiation, including the formation of neurons, smooth muscle and ECs, could be promoted in intestine organoids by the addition of EPIREGULIN, a EGF-like family member important for intestinal development [22].

Despite these efforts, ectoderm-derived organoids, which lack mesoderm lineages, cannot be vascularized using the same strategies. As a result, alternative methods are required, as discussed below.

Co-differentiation of ECs using genetically inducible ETV2 in hPSCs

ETS Variant Transcription Factor 2 (ETV2) is a key regulator of endothelial and hematopoietic cell lineages during early development. Brief activation of ETV2 is sufficient to convert both fibroblasts and hPSCs into ECs [23]. Moreover, integrating constitutive- or inducible ETV2 into mature adult ECs reprograms them into embryonic-like ECs with enhanced vascularization capacity. This approach has been used to engineer vascularized ASC-derived mouse and human intestinal organoids and human pancreatic islets [24]. Notably, incorporating hESCs that have been engineered to express inducible ETV2 into aggregates called embryoid bodies prior to cortical organoid patterning promoted the formation of vascular networks in human cortical brain organoids [10]. These vascularized cortical brain organoids not only supported the acquisition of a blood–brain-barrier (BBB)-like identity in ECs, as indicated by increased expression of TJP1, CLDN5, ABCB1 and GLUT1, but also facilitated the appearance of pericytes and astrocytes, which were absent in non-vascularized cortical organoids [10]. More recently, Maggiore et al. developed an entirely hiPSC-derived kidney organoid that incorporates a transgenic inducible ETV2-hiPSC line [**11]. In this model, an extensive EC network developed that interacted with podocyte clusters, promoting the expression of basement membrane and glomerular development pathways [**11]. The model also showed EC specification, producing both glomerular- and venous-specific EC subpopulations [**11]. Another notable study by Yang et al. developed an entirely hESC-derived pancreatic islet organoid that integrated both hESC-derived macrophages and ETV2-inducible ECs, resulting in improved insulin production and enhanced β cell maturity [12].

Controlled assembly of organoids with hPSC-derived ECs and Blood Vessel Organoids (BVOs)

Controlled self-assembly that involves compaction of soft matrixes by mesenchymal cells was first used by Takebe to vascularize hiPSC-derived human liver buds [25]. However, initial studies used either primary adult endothelial cells or hiPSC-derived ECs that failed to acquire liver sinusoidal endothelial cell (LSEC) identity [25,26]. Recently, however, Takebe reported a protocol to differentiate LSECs from hiPSCs and a modified controlled self-assembly technique, called "inverted multilayered air-liquid interface" (IMALI) 3D culture [**13]. The IMALI method was used to generate functional, vascularized liver bud organoids [**13] in which a branched endothelial network formed with porous, fenestrae-like structures typical of sinusoidal ECs. This innovation not only established organ-specific vasculature but also enhanced the expression and activity of peri-central hepatocytic metabolic enzymes, outcomes that could not be achieved with standard hiPSC-ECs [26] [**13].

Another example of controlled assembly includes the use of assembloids with BVOs, that has proven effective for integrating both hPSC-derived ECs and perivascular cells into organoids. Wimmer et al. demonstrated the direct differentiation of hiPSCs into self-assembling BVOs, forming branched, lumenized EC networks tightly interacting with mural cells and a continuous basement membrane [27,28]. By day 14, BVO cells resemble early-stage human ECs and perivascular cells [28]. Currently, most assembloids combine BVOs with hPSC-derived cerebral or neural-like organoids, likely because brain organoids have a limited capacity to support the development of mesodermal derivatives. Recently, a similar approach was extended to kidney by co-assembling hPSC-derived kidney organoids with hPSC-derived endothelial organoids [29]. Protocols for creating vascularized assembloids often involve embedding them in Matrigel, alone or in combination with Collagen I or hyaluronic acid (HA)-based hydrogel droplets [*14], [29,30]. Alternatively, suspension culture has also been used [15,29,31]. These methods consistently show EC tube-like structures infiltrating brain organoids, along with the expression of BBB specific markers such as CLDN5 and TJP1, as well as transport proteins like GLUT1 [15,30,31], [*14]. Additionally, brain organoids co-cultured with BVOs have proven valuable for studying diseases like SARS-CoV-2 [31] and recapitulating genetic disease-specific phenotypes, such as those seen in hereditary cerebral cavernous malformations [*14].

Bioengineering and organ-on-a-chip technology

Bioprinting

3D organoid bioprinting enables precise control over the spatial and temporal deposition of cells, matrices, and sacrificial inks, facilitating the fabrication of organoids with pre-defined vascular architectures [32]. The Lewis lab developed a biomanufacturing method called sacrificial writing into functional tissue (SWIFT), where a sacrificial gelatin ink is embedded within organoid blocks via 3D bioprinting (Figure 1) [33]. Once the ink is removed, it creates endothelial channels. They further advanced this method by combining orthogonal induced differentiation with 3D bioprinting to create a vascularized cortical organoid model [34]. This model features spatially organized lineage differentiation of ECs and neurons through the overexpression of ETV2 and NGN1, showcasing the potential of this approach for creating complex tissue structures [34]. In another recent study, SWIFT bioprinting was combined with coaxial embedding to generate perfusable channels lined with smooth muscle cells and ECs to form an interconnected, perfusable vessel network in a hiPSC-derived cardiac tissue model [35]. Under continuous perfusion, the cardiac tissue maintained its contractility and exhibited an effective drug response [35]. Additionally, Xu et al. used two-photon polymerization (TPP) to bioprint an artificial mesh-like vessel networks within brain organoids [36]. This vascularization method not only supported the development and maturation of the organoids but also influenced their spatial organization, leading to a more complex and functional tissue architecture [36].

Generation of perfusable and functional vascularized organoids using organ-on-chip technology

Several pre-vascularized organoids discussed earlier in this review have shown enhanced maturation, forming perfused vessels after *in vivo* transplantation [3,6,13,22,25–28]. Advancements in OoC platforms are now offering an alternative by enabling the integration of organoids into dynamic cellular microenvironments. These systems support microscale fluid flow, enabling vascular network perfusion and precise control of the microenvironmental cues essential for organoid function and maturation. For example, Homan et al. demonstrated that perfusion under continuous flow enhanced vascularization, lumen formation and maturation in hiPSC-derived kidney organoids without the need for external vasculature [37]. However, this model did not support vascular network perfusion within the organoid. Taking a different approach, Kroll et al. integrated kidney organoids in a flow channel positioned next to an EC-covered channel, representing a macrovessel, within a hydrogel matrix [38]. Exposure to fluid flow stimulated the development of an extensive endogenous vascular network within the organoids, with angiogenic sprouting towards the macrovessel channel in a response to a VEGF gradient. This resulted in the formation of an interconnected vascular network between the kidney organoid and the macrovessel via anastomosis [38].

Another approach used involves creating an external vascular network surrounding organoids embedded in a hydrogel. Within a few days, ECs surrounding the organoids self-organize into a perfusable vascular network, accessible through medium channels (Figure 1). Promoting anastomosis between the vasculature within the organoids and the external vessel network can result in a fully functional vasculature on-chip. This method has been described in several recent vascularized organoid models [39—42]. For example, our group demonstrated bidirectional anastomosis between a pre-vascularized hiPSC-derived cardiac microtissue with a developing external network, which was further enhanced by continuous perfusion of fluid through the external network [39]. Cio et al. also found that embedding pre-vascularized cardiac spheroids into the hydrogel at the start of external vasculature formation led to successful anastomosis, while embedding at later time-point limited vessel invasion into the organoids [41]. In another study, Bender et al. developed vascularized human pancreatic islets integrated into self-assembled microvascular network that responded to glucose stimulation and allowed immune cell perfusion [*42]. Quintard et al. also reported flow-induced anastomosis between a developing external vessel network and vascularized mesenchymal spheroids or BVOs [*40]. Notably, connection to the external vascular network was essential for achieving perfusable and mature BVOs [*40]. Lastly, when non-vascularized cerebral organoids were integrated into a pre-formed external vessel network, they showed limited vascularization, mostly at periphery [43]. This could be enhanced with the addition of HDGF and CYR61 [43].

Incorporating controlled fluid flow into OoC systems is crucial for replicating the biomechanical factors and establishing long-term vascular perfusion. Some recent studies employed hydrostatic pressure difference or bidirectional rocking platforms [39,41,43], which often resulted in irregular flow. More advanced approaches, such as connecting microfluidic systems to pressure- or syringe-driven pumps, have been shown to enhance both the function and maturity of organoids [37,38,40,44]. For instance, Quintard et al. demonstrated controlled perfusion of BVOs over 2 weeks at physiological flow rates comparable to human capillaries, which supported organoid growth and maturation, closely resembling *in vivo* transplanted BVOs [*40]. Additionally, pre-vascularized pancreatic islet spheroids in this model showed improved insulin secretion in response to glucose under flow. Similarly, in a multicellular myocardium model, King et al. showed that continuous perfusion enhanced cardiomyocyte function by increasing Ca^{2+} transient amplitude and decreasing time-to-peak [44].

Challenges and perspectives

Despite multidisciplinary approaches tested to incorporate vasculature into organoids, key obstacles persist. One major issue is developing culture medium that can simultaneously support both organ-specific tissues and vascular cells. Alternative strategies, like co-differentiating hPSC-ECs with growth factors or ETV2 and co-assembling hPSC-ECs can help but often result in a naïve EC network, lacking the necessary supporting cells to form fully lumenized vascular structures. Co-assembly with BVO offers the advantage of integrating pre-formed vascular networks but faces variability in protocols and lack the control of differentiating ECs through inducible ETV2 expression or 3D bioprinting. Additionally, these approaches often miss the early interactions between multiple germ layers, which are crucial for the proper differentiation of progenitor cells and vascular specificity.

OoC technology holds promise by addressing limitations of *in vivo* transplantation, including the ethical concerns and immune compatibility. However, several obstacles still limit the standardization and scalability of OoC systems. These include: 1) appropriate culture medium to support both vascular network formation and organoid development; 2) the reliance on primary vascular cells instead of hPSC-derived cells, which impacts batch-to-batch variability, reproducibility and repeated availability of cells from the same genetic source; and 3) variability in microfluidic chip fabrication techniques, materials and ECM composition. Controlling continuous perfusion of microvasculature in organoids is also challenging due to the complex architecture of the vascular networks, which affects flow dynamics. Lastly, incorporating immune components into organoids and OoC systems remains a key challenge, since immune cells are a key underlying trigger of many disease states and tumor microenvironment.

Overcoming these challenges not only requires expertise in hPSC biology but also collaboration across materials science and bioengineering to achieve controlled and reproducible vascular network formation and perfusion in organoids. Nonetheless, recent advances that combine hPSC organoid vascularization with OoC technology show great promise and will likely pave the way towards broader integration of vascularized organoids in both clinical and basic research and eventually lead to regulatory acceptance of these models.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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