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Engineering vascularised organoid-on-a-chip: strategies, advances and future perspectives

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ABSTRACT

In recent years, advances in microfabrication technology and tissue engineering have propelled the development of a novel drug screening and disease modelling platform known as organoid-on-a-chip. This platform integrates organoids and organ-on-a-chip technologies, emerging as a promising approach for in vitro modelling of human organ physiology. Organoid-on-a-chip devices leverage microfluidic systems to simulate the physiological microenvironment of specific organs, offering a more dynamic and flexible setting that can mimic a more comprehensive human biological context. However, the lack of functional vasculature has remained a significant challenge in this technology. Vascularisation is crucial for the long-term culture and in vitro modelling of organoids, holding important implications for drug development and personalised medical approaches. This review provides an overview of research progress in developing vascularised organoid-on-a-chip models, addressing methods for in vitro vascularisation and advancements in vascularised organoids. The aim is to serve as a reference for future endeavors in constructing fully functional vascularised organoid-on-a-chip platforms.

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Introduction

Traditional tools for studying human biology include two-dimensional monolayer cell culture models and animal models. Although the two-dimensional cell models are simple and convenient, the cells grown in dishes fail to replicate most intricate cell interactions. Simultaneously, the cell morphology also differs significantly from that observed *in vivo*.[1-3] In contrast, animal models are employed to investigate the overall functionality of three-dimensional (3D) organs. However, metabolic levels, immune functions, and tissue morphology in animal models differ from those in humans.[4] Moreover, ethical concerns are inherent in the utilization of animal models.[5]

With the advancement of microfabrication technology and tissue engineering, a more advanced platform, organoid-on-a-chip, emerged in recent years. Combining two cutting-edge technologies, organoids and organ-on-a-chip, this platform has become a more promising method for constructing *in vitro* models, surpassing the limitations of both two-dimensional cell models and animal models. Organoid-on-a-chip involves microfluidic devices that simulate the specific physiological environment of organs. These devices can mimic the human body's physical, chemical, and mechanical cues more accurately, thus providing a more faithful reproduction of human biology.[6] Compared to traditional well plates and culture dishes, organoid-on-a-chip offers a more dynamic and flexible microenvironment, allowing for the simulation of more comprehensive human biology.

Various organoid models, including lungs,[7] kidneys,[8] hearts,[9] digestive systems,[10] livers,[11] vascular networks,[12] and neuronal networks,[13] have been developed using microfluidic

technology. These models find widespread applications in studying organ development, drug discovery, and various diseases.[14] For instance, Wang et al.[15] utilised intestinal organoids derived from human intestinal stem cells to mimic *in vivo* crypt structures and model the infection of Clostridium difficile. Nie et al.[16] induced the differentiation of induced pluripotent stem cells (iPSCs) into functional liver organoids capable of secreting albumin and urea, exhibiting partial hepatic functions and liver-specific characteristics. They employed these functional liver organoids to study hepatitis B. In addition to organoids derived from stem cells, organoids sourced from tumour tissues with different genetic backgrounds hold significant value in cancer biology research, predicting individual-level drug responses, drug screening, and precision therapies.[17-19] Furthermore, researchers have established serial or parallel multi-organoid models to capture the complexity of human organ physiology, investigating potential interactions between different organs, such as the liver and intestine.[20-22]

Despite the rapid progress of organoid models over the past decade, the lack of vasculature has been widely acknowledged as a significant obstacle hindering its effective recapitulation of *in vivo* biology.[23] Currently, most organoid models lack vascular structures, and the vascularisation of organoids is crucial for successful modelling. This is primarily because vascular networks can supply nutrients to organoids, supporting their long-term culture and promoting the maturation and functional enhancement of organoids.[24] Besides supporting the metabolism of organoids, in multi-organoid-on-a-chip models, vascular networks also facilitate the transport of signalling molecules, growth factors, and hormones, serving as a bridge for cell interactions between different organoids. Additionally, vascularisation is indispensable in organoid-on-a-chip models for drug screening and discovery applications. The presence of vasculature can replicate the authentic distribution of drugs, immune cells, antibodies, and immune factors to specific cells within organoids, mimicking the natural distribution of drugs in the human body. Therefore, it allows for a more accurate assessment of the efficacy and toxicity of drugs or new therapies, thereby improving drug development and personalised medical approaches.

Coincidentally, microfluidic chips have distinct advantages in the context of *in vitro* vascular construction. Microfluidic technology allows continuous perfusion of cell culture media to supply oxygen and nutrients while concurrently facilitating the removal of metabolic waste, which is essential for the long-term survival of vascular tissue.[25] Additionally, microfluidic chips can provide vascular cells with size-controllable fluid stimuli, which play a crucial role in the morphology, gene expression, proliferation, and migration of vascular cells.[26-29] For example, Homan et al.[30] employed a perfusable chip to culture kidney organoids derived from pluripotent stem cells (PSCs). They discovered that elevated fluid shear forces during kidney organoid development could stimulate the generation of vascular endothelial cells (ECs), thereby promoting the organoids' functional maturation and luminal structure formation. These findings suggest that shear force factors may activate endogenous vascular development pathways in organoids, providing a feasible avenue for the *in vitro* construction of vascularised organoids (vOrganoids).

To further understand the construction method of vOrganoid chips, the author searched PubMed and Web of Science and other databases; the search time limit is from the establishment of the database to March 2024. Finally, 73 articles were cited, and a literature review was conducted based on this. This review introduces the methods for constructing 3D vascularised models *in vitro* based on microfluidic technology. Subsequently, we discuss the strategies employed in the construction of vOrganoids. Finally, we discuss the potential solutions to the common issues of imperfect vascular function and difficulties in penetrating the interior of organoids with vascular networks. This review aims to provide insights for the future development of vascularised organoid-on-a-chip models with improved functionality.

The Methods for Constructing vascularised Models *In Vitro*

In the early attempts to construct vasculature *in vitro*, the common approach involved seeding ECs onto a flat surface, such as a culture dish[31] or a porous membrane,[32] to form a monolayer structure simulating the vascular wall. However, this method still adheres to traditional two-dimensional culture techniques and fails to replicate the physiological curvature of blood vascularisation. Microfluidic technology allows precise control of chemical gradients and mechanical stimuli during cell culture, making it widely employed in establishing *in vitro* 3D models.[33, 34] Additionally, microfluidic technology enables continuous perfusion of the cell culture medium to provide oxygen and nutrients while removing metabolic waste. This is necessary for the long-term survival of vascular tissues *in vitro*. Based on microfluidic technology, there are currently two main strategies for the *in vitro* construction of 3D vascularised models: top-down EC lining method, bottom-up self-assembly vascularisation method and hybrid method.

Top-down EC lining method

The EC lining method involves growing a monolayer of ECs on the inner wall of microchannels (**Figure 1A**). The size and shape of the formed micro-vasculature can be adjusted by manipulating the dimensions and configuration of the microchannels. Additionally, precise calculation and control of the shear stress experienced by ECs on the inner wall of microchannels can be achieved by varying the channel size and fluid flow velocity.

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Currently, microchannels are mainly constructed using polydimethylsiloxane (PDMS) or hydrogels. For microchannels based on PDMS, direct EC adhesion to its inner surface is impeded. Therefore, a layer of extracellular matrix (ECM) protein coating, such as laminin, fibronectin, or type IV collagen, is required to facilitate cell adhesion on the inner surface of PDMS microchannels.[35] Conversely, microchannels constructed with ECM proteins allow direct EC lining. Additionally, the modulus of PDMS is higher than that of the physiological vascular system, which may impact endothelial barrier function.[36] To address this issue, Qiu et al.[37] employed soft lithography to cast an agarose-gelatin interpenetrating network hydrogel on a master mould, creating a microvascular network with physiologically relevant stiffness. This model successfully reproduces the microvascular response to barrier-disrupting inflammatory mediators while studying the pathological processes of sickle cell disease and malaria, with microvasculature maintaining endothelial barrier function for up to 1 month. de Graaf et al.[38] developed scalable geometric microphysiological systems with a collagen cavity as a cell scaffold to simulate 3D *in vitro* culture of vascular cells (**Figure 1B**). This device offered a physiologically relevant platform for studying vascular biology and disease mechanisms. The formation of microvascular structures using the EC lining method is highly dependent on the microchannel structure. Most microchannels are prepared using microneedles as master moulds, resulting in straight microchannels.[39] Consequently, the EC lining such microchannels can only form straight microvasculature. Mandrycky et al.[40] overcame this limitation by embedding springs into uncured hydrogel. After the hydrogel solidified, they removed the springs by rotating them, creating a basis for developing a spiral vascular structure (**Figure 1C**). Compared to straight lumens, the fluid in helical lumens exhibits unique flow characteristics that promote the alignment of ECs in the flow direction and enhance cell proliferation. This model helps study cell developmental differences caused by various vascular structures. The soluble sacrificial layer method is another common technique within the EC lining methods. This method involves 3D printing a structurally complex framework, which is then immersed in an uncured hydrogel solution. After the hydrogel solidifies, the template material embedded in the cured gel is melted or dissolved to create microchannels with more intricate structures.[41] For instance, Miller et al.[42] utilised a 3D-printed carbohydrate glass framework as a template to prepare a multilayer microvascular network. After dissolving the carbohydrate glass with water, a hollow perfusable network structure was left in a fibrin gel. ECs were subsequently seeded in this hollow microstructure, forming a 3D vascular network.

It is important to note that the mechanism of vascular network formation by the top-down EC lining method differs from that *in vivo*. The top-down EC lining method is essentially engineering-oriented, where the size and branching of the resulting vascular network are determined by the artificially designed shapes and dimensions of microchannels. While this method allows precise control over the diameter and morphology of the vasculature, the vasculature diameters formed using this technique (> 100 μm) are typically larger significant than the capillary diameters found *in vivo* (< 20 μm), resulting in vascular densities that are also an order of magnitude lower than *in vivo*.[43]

Bottom-up self-assembly vascularisation method

The bottom-up self-assembly vascularisation method is a process that closely mimics the physiological processes in the human body. It utilises two *in vivo* vascular development mechanisms, vasculogenesis and angiogenesis, to construct vascular networks (**Figure 1A**).[25] Vasculogenesis is a physiological process involving the differentiation and organisation of endothelial precursor cells during embryonic development. The secreted proteins of fibroblasts, growth factors such as vascular endothelial growth factor and basic fibroblast growth factor, and the ECM (usually fibrinogen, collagen, or laminin), all have a certain effect on vasculogenesis. Whisler et al.[44] compared the vascular networks formed by individually culturing ECs and co-culturing ECs with fibroblasts. The results indicated that vascular networks formed by separately cultured ECs rapidly degrade, while co-culturing ECs with fibroblasts results in a stable vascular network. Moreover, an increase in the concentration of vascular endothelial growth factor in the medium leads to a reduction in the diameter of the formed vasculature. In addition to fibroblasts, co-culturing ECs with pericytes, smooth muscle cells, mesenchymal stem cells, and other supporting cells also promotes the formation and stability of vascular networks.[45-47] Angiogenesis, conversely, refers to forming new blood vasculature from existing ones. Angiogenesis involves processes such as the release of angiogenic signals, degradation of ECM by matrix metalloproteinases, migration and proliferation of ECs, formation and fusion of vascular sprouts, recruitment of support cells, vascular maturation, and termination of the process. Angiogenesis is regulated by many factors, including angiogenic factors, ECM, vascular endothelial growth factor, basic fibroblast growth factor, and other co-cultured cells.[48, 49] This complex process is crucial for blood Vascularisation growth, repair, and maintenance.

With the assistance of vascular endothelial growth factors, researchers can simulate the process of ECs degrading and invading hydrogels to form blood vascularisation *in vitro*. Yue et al.[50] developed a vertical double-layer microfluidic system that could generate large-scale perfused microvascular networks (**Figure 2A**). It offered high flexibility and scalability, with independently designed tissue chambers and media channels. The capillary break valves supported angiogenesis and anastomosis, and different combinations of tissue chambers and media channels were used to create perfused vascularised tissues. Fibroblasts play a crucial role in vascular formation. Nashimoto et al.[51] developed a perfusable vascular network model where fibroblast spheroids were co-cultured with human umbilical vein ECs (HUVECs), simulating the interaction between cardiovascular cells and fibroblasts under the angiogenesis mechanisms (**Figure 2B**). The results indicated that moderate adhesion, appropriate concentrations of growth factors, and a high frequency of culture medium exchange are indispensable for vascular generation on the chip. Phan et al.[52] injected a fibrin hydrogel containing human lung

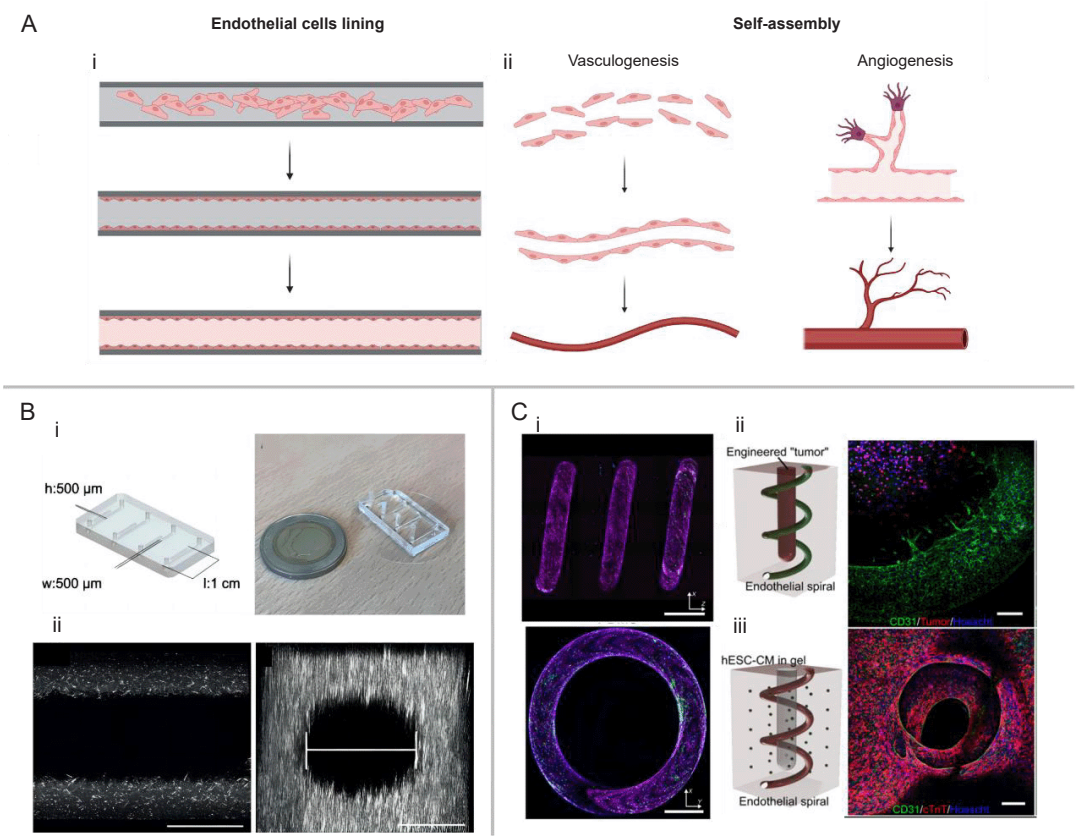

Figure 1. Schematic representation of the principles underlying two methods and top-down EC lining method for constructing vascularised models *in vitro*. (A) Schematic illustrating two methods for constructing vascularised models *in vitro*: (i) Principle of the endothelial cell lining method. (ii) Principle of the self-assembly method. Created with BioRender.com. (B) Microvascular system combined with viscous finger patterning technique: (i) Schematic of the microfluidic chip showing dimensions and layout of the microfluidic platform, four straight channels on a single chip with designed parameters 500 μm × 500 μm × 1 cm (w × h × l). (ii) 20× magnification confocal slice of a patterned lumen with 5 mg/mL collagen I. Scale bar: 200 μm. (iii) XZ reconstruction showing the flow field of the scaffold. Scale bar: 200 μm. Reprinted from de Graaf et al.[38] (C) Spiral microvascular system: (i) Cross-sectional fluorescence image of the spiral blood vessel. (ii) Spiral vascularised tumour model demonstrating vascular growth towards tumour cells. (iii) Spiral vascularised heart model. Scale bars: 200 μm. Reprinted from Mandrycky et al.[24] CM: cardiomyocyte; cTnT: cardiac troponin T; EC: endothelial cell; hESC: human embryonic stem cell.

fibroblasts and HUVECs into a microfluidic vascular chip. Tan et al.[53] designed a microfluidic vascular model as a non-contact cell culture device to study paracrine communication in co-culture (**Figure 2C**). In this device, the hydrogel channels for each cell type are separated by culture medium channels. The study found that multifunctional proteins, glycosaminoglycan, intercellular adhesion molecule 2, and tenascin-C can potentially promote the vascular network's maturity.

The bottom-up self-assembly method utilises angiogenesis and vasculogenesis mechanisms to form a vascular network. The geometric features (diameter and length), density, branching, stability, and permeability of the resulting vasculature depend on the complex interplay between biophysical signals provided to ECs (such as interstitial flow, intraluminal flow, and matrix stiffness) and biochemical signals (such as vascular endothelial growth factor).[54] These signals originate from the ECM and matrix cells. This approach has successfully achieved microvascular networks *in vitro* with dimensions close to capillaries, typically with diameters ranging from 15 to 50 μm. While this method closely mimics the natural vascular formation process in the human body, the size and shape of the formed vascular network are random and uncontrollable due to the complex interactions involved in this process.

Hybrid method

Furthermore, the top-down vascular construction methods and bottom-up vascular construction methods can be further combined. Wang et al.[54] prepared a vascularised chip that combines the EC lining method and the angiogenesis mechanism to construct an arterial-venous-capillary system. The vascular network formed based on the angiogenesis mechanism in the central rhomboid chamber matched the Vascularisation formed based on the EC lining method in the fluid channels on both sides (**Figure 3A**). By perfusing fluorescein isothiocyanate-dextran into the vascular chip, it was found that fluorescein isothiocyanate-dextran could be well confined within the vascular lumen, and no non-physiological leakage was observed. The results of this study suggest that the vascularised model constructed based on this method has a good barrier function. Park et al.[55] established a

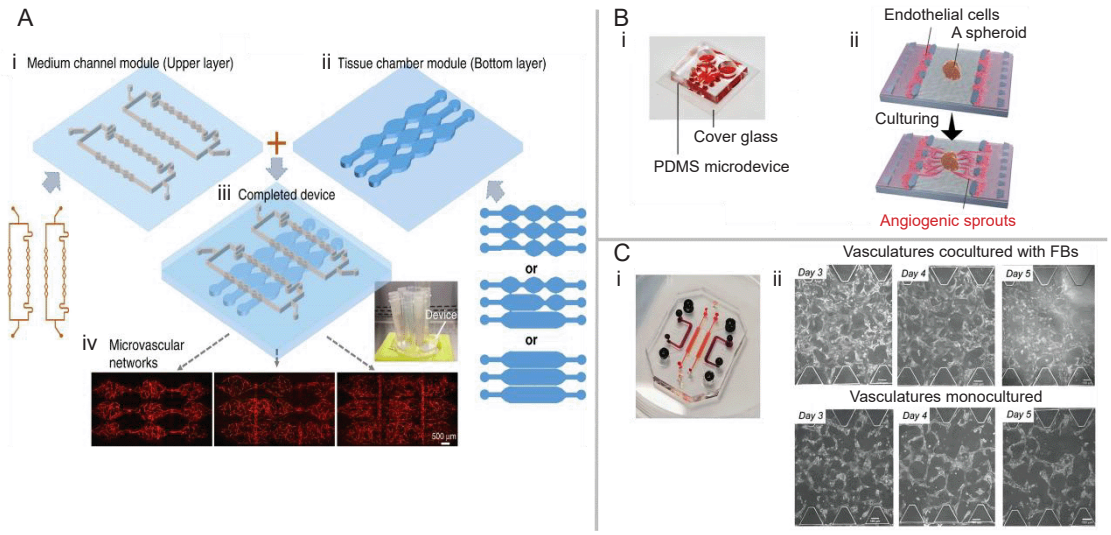

Figure 2. Bottom-up self-assembly vascularisation method for constructing vascularised models *in vitro*. (A) The modular microfluidic system combines two PDMS layers: (i) Medium channel module (upper layer). (ii) Tissue chamber module (bottom layer). (iii) A completed two-layered device shows the medium channels and tissue chambers in different layers. (iv) Large-scale perfused microvascular networks are generated using different configurations. Reprinted from Yue et al.[50] (B) Design diagram and overview of the microfluidic system with perfusion vascular network: (i) Photograph of a microfluidic device. (ii) Schematic diagram showing an overview of assays. Reprinted from Nashimoto et al. [51] Copyright 2017 The Royal Society of Chemistry. Reproduced with permission. (C) Design diagram and representative images of the microfluidic vascular model as a non-contact cell culture device: (i) Photograph of the microfluidic chip used in the study to generate a perfusable vascular network. (ii) Representative images show the comparison of cocultured ECs to monocultured ECs over 3 days. Scale bars: 100 μm. Reprinted from Tan et al.[53] EC: endothelial cell; FB: fibroblast: PDMS: polydimethylsiloxane.

microfluidic model simulating the invasion of extra-chorionic trophoblast cells during human embryo implantation (**Figure 3B**). This model reconstructed the 3D structure of the maternal-fetal interface on a chip, using primary human cells isolated from clinical specimens. The study demonstrates the directed migration of trophoblast cells towards microengineered maternal blood vascularisation and their necessary interaction with ECs for vascular remodelling. In addition, the study also reveals the unknown effects of pre-implantation maternal immune cells on trophoblast cell invasion. Debbi et al.[56] seeded ECs and support cells within a 3D porous polymer scaffold with hollow channels, constructing a perfusable multi-scale vascular network encompassing artificially patterned macro-vessels with a diameter of 600 μm and self-assembled capillaries with a diameter of 10 μm (**Figure 3C**). Redd et al.[57] demonstrated *in vitro* the vascular remodelling and anastomosis between pre-patterned vascular networks constructed with EC lining and self-assembled capillaries (**Figure 3D**). They observed that remodelled constructs with vascular anastomosis are associated with upregulated vascular and tissue development genes.

Hybrid methods offer greater flexibility, allowing for the construction of vasculature according to research needs. The self-assembly method can be employed when capillaries are required, while the EC lining method can be used to vascularize arterial or venous dimensions. Moreover, this method is frequently utilised to construct multi-scale vascular networks.

Table 1 compares different vascularisation construction methods. It can be seen that the ECs used in most methods are HUVECs. This is mainly because, on the one hand, these cells are easily obtainable. On the other hand, as cells derived from the fetal umbilical cord, they are compatible with organoid tissues with maturity close to that of a fetus or embryo. Meanwhile, **Table 1** also shows that the diameter of blood vessels obtained by the EC lining method is often an order of magnitude higher than that of the blood vessels obtained by the self-assembly method.

Strategies for Organoid Vascularisation

Organoid models demonstrate tremendous potential in simulating the complexity of human organs, providing powerful tools for drug discovery and disease modelling. However, a key challenge in organoid models is achieving vascularisation to mimic the *in vivo* microenvironment more accurately. Researchers have been actively exploring various strategies for constructing vOrganoid models, currently encompassing three main strategies: co-culture, co-differentiation, and organoid assembly (**Figure 4A**).

Co-culture strategy

The co-culture strategy involves co-culturing ECs with target organ progenitors or PSCs. For instance, Takebe et al.[58] successfully constructed vascularised liver buds by co-culturing iPSCs-derived hepatic endodermal cells, HUVECs, and umbilical cord-derived mesenchymal cells. In another approach, Shi et al.[59] initially co-cultured iPSCs with HUVECs to form embryoid bodies, which were subsequently cultured in neural induction medium, resulting in the development

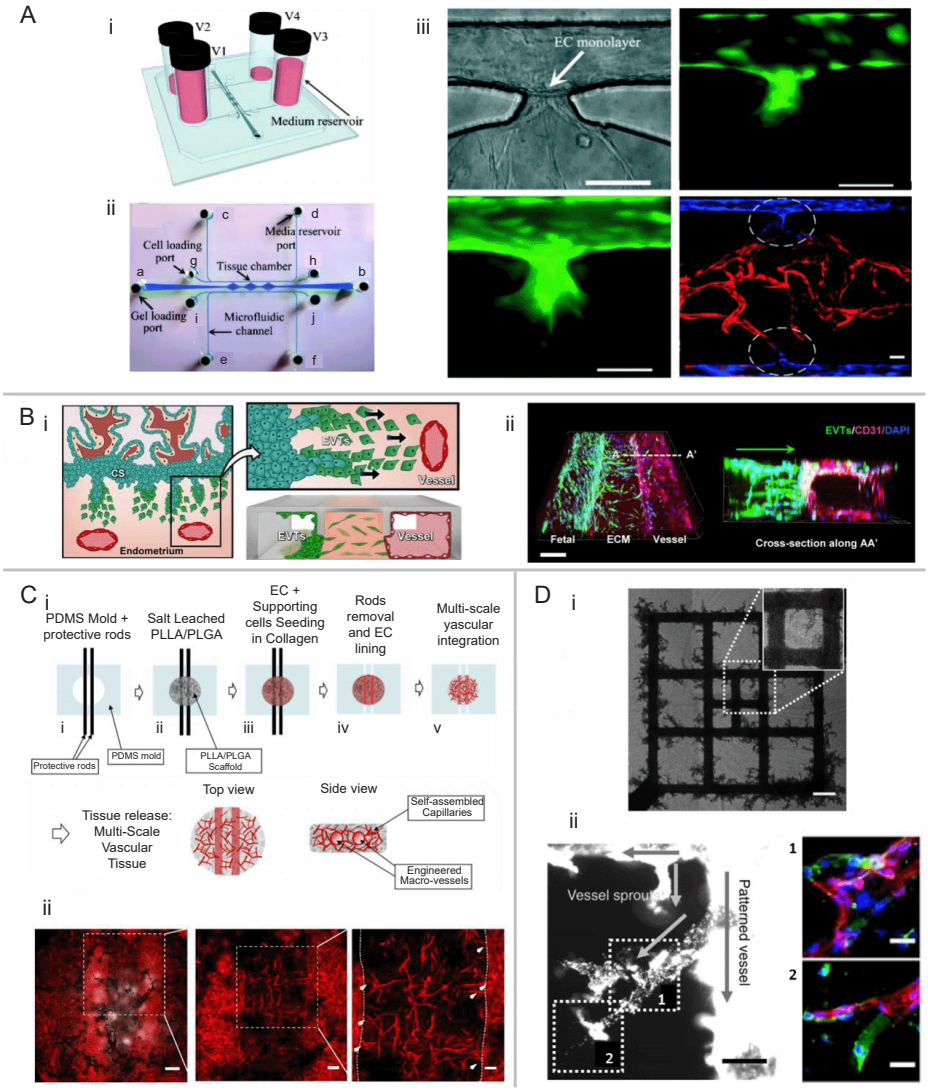

Figure 3. Hybrid method for constructing vascularised models *in vitro*. (A) Vascularised model construction combining vasculogenesis mechanism and EC lining method: (i) Schematic diagram of the microfluidic chip. The physiological level of mechanical stimulion vasculogenesis was established by filling the reservoirs to different culture medium heights (V1: 23 mm H_2O, V2: 8 mm H_2O, V3: 18 mm H_2O, V4: 3 mm H_2O (1 mm H_2O = 9.80665 Pa)). (ii) Microchannel design. The gel loading port (a, b), the media reservoir ports (c–f), cell loading port (g–j). (iii) Observation of angiogenesis phenomenon in the microfluidic chip. Scale bars: 100 μm. GFP (green)-labeled indicates the lined ECs at the cell–matrix interface invaded into the 3D fibrin gel and formed microvascular sprouts as early as 24 hours post-lining. From inside the tissue chamber, mCherry-expressing (red) ECs (ECs-mCherry) migrated outward to the laminin-coated microfluidic channel to connect with the lined BFP-labeled (blue) ECs (ECs-BFP) in the channels. The green image below represents more time of microvascular sprouts invasion than the green image above. Reprinted from Wang et al.[54] Copyright 2016 The Royal Society of Chemistry. (B) Diagram of the impact of chip implantation on maternal vascular remodelling: (i) Interactions between invading EVTs and maternal blood vessels were modeled in the implantation-on-a-chip. (ii) 3D projection images of EVTs (green) migrating across the ECM hydrogel towards maternal ECs. Scale bar: 200 μm. Reprinted from Park et al.[55] (C) MSVT construction: (i) Schematic of the MSVT fabrication process. (ii) Maximum intensity projection of the MSVT, with high magnifications. The middle image is an enlargement of the dashed box in the left image. The right image is an enlargement of the dashed box in the middle image. The white arrows of the right image indicate micro-vessels sprouts and the dashed lines of the right image indicate the macro-vessels borders. Scale bars: 500 μm (left), 100 μm (middle), 50 μm (right). Reprinted from Debbi et al.[56] (D) Fluorescent bead perfusion of engineered microvessels (μVs) and citrated whole-blood perfusion in μVs: (i) Brightfield stitched large image of red blood cell-filled pattern and sprouts with magnified view (inset, white dotted boundary) for hESC-ECs-seeded μV only constructs after 4 days of culture. Scale bar: 200 μm. (ii) μV + SA construct with perfused anastomotic connections. High magnification views of outlined regions 1 and 2 with corresponding in situ staining for mTm-hESC-ECs ($DsRed^+$, red) and GFP-hESC-ECs (GFP^+, green). Scale bars: 100 μm (left), 40 μm (right). Reprinted from Redd et al.[57] BFP: blue fluorescent protein; EC: endothelial cell; ECM: extracellular matrix; EVT: extravillous trophoblast; GFP: green fluorescent protein; hESC-EC: human embryonic stem cell-derived endothelial cell; MSVT: multi-scale vascular tissue; SA: self-assembled.

Table 1. Comparison of different *in vitro* vascularisation model construction methods

Study	Method	Cells used	Minimum diameter	Vascular network shape
Qiu et al.[37]	Top-down EC lining method	HUVECs, HDMVECs, HLMVECs	~20 μm	Cylindrical
de Graaf et al.[38]	Top-down EC lining method	iPSC-ECs, HBVPs	~300 μm	Cylindrical
Mandrycky et al.[40]	Top-down EC lining method	HUVECs	~200 μm	Spiral
Miller et al.[42]	Top-down EC lining method	HUVECs, 10T1/2 cells	~300 μm	Cylindrical
Yue et al.[50]	Bottom-up self-assembly vascularisation method	ECFC-ECs, NHLFs	~10 μm	Capillary
Nashimoto et al.[51]	Bottom-up self-assembly vascularisation method	HUVECs, NHLFs	~10 μm	Capillary
Phan et al.[52]	Bottom-up self-assembly vascularisation method	ECFC-ECs, NHLFs	~20 μm	Capillary
Tan et al.[53]	Bottom-up self-assembly vascularisation method	HUVECs, NHLFs	~20 μm	Capillary
Wang et al.[54]	Hybrid method	ECFC-ECs, NHLFs	~15 μm	Multi-scale vascular network
Park et al.[55]	Hybrid method	HEMVECs, HLMVECs, HBMVECs, NHLFs	~10 μm	Multi-scale vascular network
Debbi et al.[56]	Hybrid method	HAMEC, DPSCs	~20 μm	Multi-scale vascular network
Rebbi et al.[57]	Hybrid method	hESC-ECs, HUVECs	~20 μm	Multi-scale vascular network

Note: DPSCs: dental pulp stem cells; EC: endothelial cell; ECFC-ECs: human endothelial colony forming cell-derived endothelial cells; HBMVECs: human brain microvascular endothelial cells; HBVPs: human primary mural cells; HDMVECs: human dermal microvascular endothelial cells; HEMVECs: human endometrial microvascular endothelial cells; HLMVECs: human lung microvascular endothelial cells; HUVECs: human umbilical vein endothelial cells; iPSC-ECs: human-induced pluripotent stem cell-derived endothelial cells; NHLFs: normal human lung fibroblasts.

of vOrganoids resembling the human cerebral cortex with integrated vascular structures (**Figure 4B**). These vOrganoids exhibited neurogenesis and expressed genes associated with vascular development.[59]

However, due to their easy availability, HUVECs are also the most commonly used ECs type in co-culture strategies for vOrganoids. In constructing vOrganoids, organ-specific ECs derived from PSCs may offer advantages over HUVECs to closely resemble real organs. Salmon et al.[60] induced two batches of PSCs to differentiate into vascular and neural lineages, respectively. These cells were co-cultured in a 3D-printed microfluidic chip to construct vascularised brain organoids.[60] The results showed that the co-cultured ECs exhibited certain specific brain features. Significant developmental differences were observed between single and co-cultures, with accelerated differentiation. The generated vascular network penetrated the brain organoid.

Moreover, the immune system is closely related to the vasculature, relying on it to transport immune cells, immune factors, and antibodies to various body parts. Wan et al.[61] introduced a novel approach to enhance the vascularisation of tumour spheroids *in vitro*. This approach involves co-culturing pre-formed tumour spheroids with fibroblasts in a low-adhesion well plate, resulting in tumour spheroids surrounded by a high-density fibroblast periphery. This approach promotes the vascularisation of tumour spheroids, and the resulting vasculature is more easily perfused than vascularisation formed using traditional methods. Chimeric antigen receptor T cells exhibited enhanced recruitment and cytotoxicity levels within these vascularised tumour spheroids, primarily attributed to the increased level of vascularisation and improved perfusion around the tumour vasculature achieved by this method.

Co-differentiation strategy

This strategy involves adjusting the differentiation process of PSCs to obtain vOrganoids directly. Takasato et al.[63] manipulated the fate selection of stem cells by adjusting the exposure time to different growth factors, thereby increasing the proportion of mesenchymal cells forming the posterior nephron, actively inducing the formation of renal units. After 18 days in culture, they observed lumens formed by ECs within the kidney organoids. This approach is relatively simple, without involving exogenous cells or gene editing. However, the vascular structures obtained through this approach are limited to lumen formation, and further validation of vascular functionality is needed. Additionally, since blood vascularisation originates from the mesoderm, it is not feasible to simultaneously induce the formation of vascular tissues and organoids derived from other germ layers *in vitro* using the same batch of PSCs. Therefore, this approach applies to constructing vOrganoids originating from the same mesodermal layer.

To broaden the application scope of this strategy, Cakir et al.[62] employed genetic engineering techniques to overexpress the ETS variant 2 (ETV2) transcription factor in embryonic stem cells through lentiviral transduction. ETV2 is crucial in forming ECs and blood vascularisation during embryonic development.

They cultured embryonic stem cells in neural induction medium and subsequently activated ETV2 expression with puromycin. This method generated vascularised human cortical organoids with blood-brain barrier functionality (**Figure 4C**).

Organoid assembly strategy

The organoid assembly strategy refers to the assembly and fusion of vOrganoids and organoids requiring vascularisation. Both the vascular organoids and the organoids requiring vascularisation are derived from PSCs, making this strategy an extension of the co-differentiation strategy.

Song et al.[64] pioneered the use of this strategy in constructing vOrganoids. They innovatively fused ECs spheroids with cortical neural progenitor cell spheroids derived from iPSCs, introducing vasculature into cortical organoids. Because in this approach, ECs spheroids and cortical neural progenitor cell spheroids are separately differentiated from iPSCs before being assembled and fused, it circumvents the earlier issue regarding the distinct embryonic origins of blood vascularisation and the brain. However, it should be noted that although the resulting cortical organoids could express blood-brain barrier functional proteins, vascular structures were not observed. Sun et al.[65] took this strategy a step further. They separately constructed brain organoids and vascular organoids from iPSCs. In the later stages of culturing, they added neurotrophic factors to the vascular organoids to confer them with brain vascular functionality. Subsequently, when the two organoids reached the stages of vascular precursor cells and neural precursor cells, respectively, they were further fused. The vascularised brain organoids constructed using this method exhibited significantly increased maturity. Vascular structures were observed wrapping around and infiltrating the brain organoids, forming close connections with neural tissues (**Figure 4D**).

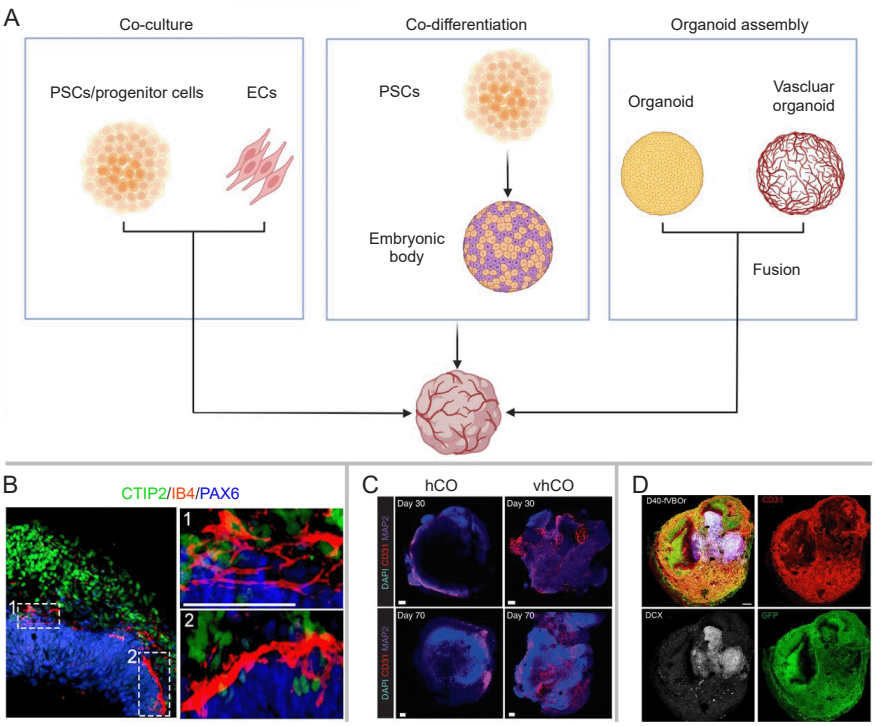

Figure 4. Strategies for organoid vascularisation. (A) Schematic representation of the principles underlying three strategies. Created with BioRender.com. (B) Representative immunofluorescence staining figure for CTIP2/IB4/PAX6 at day 65 to demonstrate that the vascular (IB4) structures would progressively extend into newborn neurons (CTIP2) with the development of vOrganoids. Scale bar: 50 μm. Reprinted from Shi et al.[59] (C) Immunostaining of whole-mount vhCOs and control hCOs at different time points (days 30 and 70) for DAPI/CD31/MAP2. It illustrates that vhCOs had significantly more vessel area and length than control hCOs. Scale bars: 100 μm. Reprinted from Cakir et al.[62] (D) Whole-mount staining results in the fused vasculature and brain organoids. D40-fVBOr represents the fused vasculature and brain organoids at day 40. Reprinted from Sun et al.[65] Scale bar: 200 μm. CTIP2: COUP TF1 interacting protein 2; DAPI: 4′,6-diamidino-2-phenylindole; DCX: doublecortin; EC: endothelial cell; GFP: green fluorescent protein; hCOs: human cortical organoids; IB4: isolectin B4; MAP2: microtubule-associated protein 2; PAX6: paired box 6; PSC: pluripotent stem cell; vhCOs: vascularised human cortical organoids; vOrganoids: vascularised organoids.

While several review articles have compared various strategies for the vOrganoids,[66-69] these discussions often remain superficial, merely comparing the cell sources and procedural complexities associated with different methods. Using single-cell RNA sequencing technology, Sato et al.[70] compared the cellular differentiation and transcriptomic characteristics among vascularised brain organoids derived from co-culture, co-differentiation, and organoid assembly strategies. Notably,

they observed ependymal-like cells in vascularised brain organoids generated through an ETV2 overexpression protocol within the co-differentiation strategy, a feature absent in organoids derived from the other two strategies. Furthermore, they reported that the method of vascularisation contributes to varying degrees of heterogeneity among the organoids. This finding underscores the influence of vascularisation techniques on vascluarized organoid characteristics, thereby highlighting the need for careful consideration when selecting an appropriate method for specific experimental objectives.

Discussion

In the previous part, we explored methods for constructing vascularised models *in vitro*, followed by an introduction to existing strategies for vascularizing organoids. However, in the various works on vOrganoids mentioned above, although vascular structures were observed, there has been limited practical verification of the complete functionality of the vasculature, such as perfusion. Except for vascularised tumour models,[71] most vOrganoids formed *in vitro* only possess vascular structures without achieving vascular functionality. To establish a fully perfusable vascular network, these organoids need to be transplanted into mice. This presents a challenge to the expected benefits associated with utilizing vOrganoids as *in vitro* models for drug screening and mechanistic studies, given that many applications of vOrganoids in drug screening hinge on perfusion functionality. In the envisaged scenario, the delivery of drugs to a vascularised organoid-on-a-chip relies on their transport through blood vessels, mimicking the *in vivo* context. Nevertheless, the realisation of this envisioned scenario becomes unattainable in the absence of perfusion functionality.

To address this challenge, we propose that combining the methods for constructing vascularised models with the strategies for vascularizing organoids might be a feasible solution. The idea is to construct a vascular network bed *in vitro* and implant pre-vOrganoids into this vascular network bed. Vasculatures from the network bed extend into the organoids, and the vascular structures within the pre-vOrganoids also grow outward. Through this mutual and interconnected growth, a fully perfusable vascular system is established inside and outside the organoids (**Figure 5**). Certainly, studies have demonstrated that under favorable conditions, the vasculature within pre-vOrganoids can embed into the surrounding ECM and grow. Homan et al.[30] mixed developing kidney organoids with endothelial progenitor cells and seeded them into a microfluidic chip containing a gelatin-fibrinogen mixture at the top of the ECM. Under the combined influence of fluid stimulation and growth factors, the interior of the kidney organoids not only formed a vascular network structure, but blood vascularisation also invaded the surrounding ECM. Therefore, theoretically, integrating vOrganoids into the ECM at the top of a vascular network bed and inducing the vascularisation within the organoids to infiltrate the surrounding ECM could establish a fully perfusable vascular system inside and outside the organoids in a concerted manner.

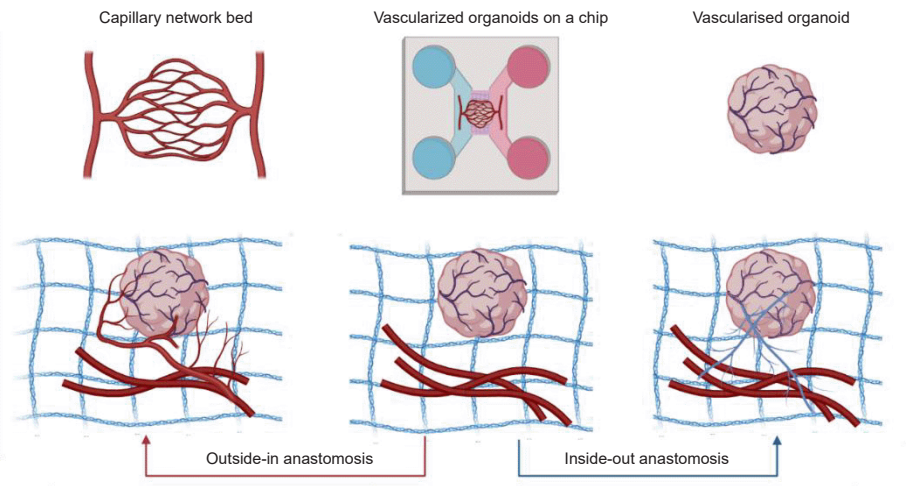

Figure 5. Bidirectional vascular anastomosis mechanism in organoid vascularisation. Created with BioRender.com.

Indeed, it is also possible to reverse the process by establishing vascular anastomosis from the outside to the inside. That is, it induces the invasion of blood vascularisation from the ECM into the organoid, connecting with the internal vasculature of the organoid. This concept has already been realised in tumour organoids. Nashimoto et al.[71] achieved a vascularised tumour organoid with bidirectional perfusable vasculature using a classic three-channel microfluidic chip. They seeded a mixed tissue spheroid of tumour cells, fibroblasts, and ECs in the central channel and ECs in the two side channels. On the second day of co-culture in the chip, neovascularisation was observed from both side channels, and by the seventh day, vascular buds from both sides were connected to the central spheroid. Fibroblasts and tumour cells played crucial roles in angiogenesis; they secreted angiogenic factors, and the combined action of these factors facilitated the invasion of vascularisation from the outside to the inside of the tumour, resembling the phenomenon of tumour angiogenesis *in vivo*. It

is worth noting that the effects of various angiogenic factors on vascularisation are not a simple linear accumulation but involve complex synergies. Although achieving a fully perfusable vascular system connecting the inside and outside has been demonstrated only in vascularised tumour organoids, recent research indicates that not only tumours but also organoids derived from PSCs can produce vascular endothelial growth factors.[72] In the next stage, harnessing this characteristic to achieve true vascularisation of all organoids will be a crucial focus for researchers.

Conclusion

Based on microfluidic technology, organoid-on-a-chip techniques have demonstrated immense potential in recapitulating the microphysiological environment of specific organs, including factors such as shear stress and nutrient gradients. During the preclinical stage of drug development, organoid-on-a-chip can be utilised to deeply investigate the mechanisms of drug action, potential toxicities, and can be compared with *in vivo* results to assess drug efficacy comprehensively. Organoid-on-a-chip also offers more possibilities for personalised therapies. By culturing cells and tissues from normal individuals or patients on the chip, specific populations or individuals can undergo personalised drug assessments, addressing to some extent the issue of disease heterogeneity, such as tumour heterogeneity. However, one of the significant challenges it faces is the lack of a functional vascular system. An available vascular system is crucial for the long-term culturing of organoids, promoting their maturation and overall functionality. This review introduces the construction methods of *in vitro* vascular models based on microfluidic technology. These methods can be categorized into three types: top-down EC lining, bottom-up self-assembly, and hybrid. Each method has its advantages and disadvantages. While the EC lining method can control the size and morphology of the generated vasculatures, it is limited to larger vasculature sizes. In contrast, although the self-assembly method cannot precisely control the size and shape of the resulting vasculatures, it mimics the natural vascular formation process in the human body, making it suitable for constructing capillary networks. Combining both methods allows for leveraging their respective strengths. Subsequently, the review discusses strategies for vascularizing organoids. Currently, co-culture, co-differentiation, and organoid assembly strategies have been developed, all successfully constructing vascularised organoid-on-a-chip models. Despite these advancements, the vasculature in current vOrganoids often exhibits only structural features, lacking complete functionality such as perfusion. This limitation somewhat undermines the anticipated advantages of using vascularised organoid-on-a-chip models in drug screening and mechanistic studies. A possible solution to this issue could be the implementation of a bidirectional vascular anastomosis mechanism. Future research efforts should focus on developing new strategies capable of creating vascularised organoid-on-a-chip models with improved functionality, thereby fully realising the potential of organ-on-a-chip technology in drug screening, disease modelling, and personalised medicine.

Author contributions

ZL, DY, XW designed the idea; ZL, DY, CZ, FW drafted the manuscript; ZL, DY, KL, YL collected references; ZL, DY, JX, LX, XW checked and polished the manuscript; ZL and DY contributed equally to this work. All authors perused and gave their consent to the concluding edition of the manuscript.

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Conflicts of interest statement

The authors have no conflicts to disclose.

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