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Engineering next generation vascularized organoids

Nicolas Werschler [a,b,*], Clement Quintard [a,c], Stephanie Nguyen [b], Josef Penninger [a,b,c,d,e,f]

[a] University of British Columbia, Life Sciences Institute, Vancouver, Canada
[b] University of British Columbia, School of Biomedical Engineering, Vancouver, Canada
[c] University of British Columbia, Medical Genetics, Vancouver, Canada
[d] Helmholtz Centre for Infection Research, Germany
[e] Eric Kandel Institute, Department of Laboratory Medicine, Medical University of Vienna, Austria
[f] IMBA Institute of Molecular Biotechnology, Vienna, Austria

A B S T R A C T

Organoids are self-organizing 3D cell culture models that are valuable for studying the mechanisms underlying both development and disease in multiple species, particularly, in humans. These 3D engineered tissues can mimic the structure and function of human organs *in vitro*. Methods to generate organoids have substantially improved to better resemble, in various ways, their *in vivo* counterpart. One of the major limitations in current organoid models is the lack of a functional vascular compartment. Here we discuss methodological approaches to generating perfusable blood vessel networks in organoid systems. Inclusion of perfused vascular compartments markedly enhances the physiological relevance of organoid systems and is a critical step in the establishment of next generation, higher-complexity *in vitro* systems for use in developmental, clinical, and drug-development settings.

1. Background and introduction

Pluripotent stem cells (PSCs) and induced pluripotent stem cells (iPSCs) are an *in vitro* analog of early embryonic cells and are characterized by their ability to self-renew and differentiate into all cell types of the body (Fig. 1A) [1]. Organoids can be generated from either pluripotent stem cells (PSCs), which encompass embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), or from adult stem cells (ASCs) obtained from specific tissues. ESCs are derived from the inner cell mass of a blastocyst-stage embryo. They are also considered the "gold standard" for pluripotency and downstream differentiation experimentation [2]. ESCs are not patient-specific and carry the risk of immune rejection after transplantation [3]. In contrast, iPSCs are derived from somatic cells (e.g., skin fibroblasts) by reprogramming using specific transcription factors [4]. They have similar pluripotent properties as ESCs but are patient-specific, avoiding ethical and immune rejection issues [2]. When working with iPSCs, researchers must be aware that genetic and epigenetic abnormalities may be introduced during the reprogramming process [3]. ASCs, however, are multipotent stem cells found in various adult tissues such as bone marrow and adipose tissue [5]. Due to their multipotency, they have a more limited differentiation potential compared to PSCs and are typically restricted to cell types of their tissue of origin. Although less controversial than ESCs, ASCs have limited proliferative capacity and plasticity in overall experimentation [5].

In the case of pluripotent stem cell-derived organoids, their potential *in vitro* can be leveraged via differentiation protocols that can give rise to relevant cells and engineered tissues for mechanistic studies or improved drug screening [6–8]. When aggregated into three-dimensional clusters and treated with growth factor combinations, PSCs/iPSCs undergo lineage specification and self-organization, giving rise to "organoids". Organoids are self-organizing 3-dimensional multicellular tissues generated *in vitro* that can replicate various structures and functions of their corresponding *in vivo* organ [7].

The concept of aggregation and self-organization of single cells into near-identical primary embryonic tissues from which they were derived is not new. Already in 1907, Wilson reported that sponge cells had the capability to self-assemble and regenerate a whole organism [9]. Reports spanning from 1952 to 1960 describe autonomous reconstruction from single cells derived from chick embryos *in vitro* [10,11]. Similarly, brain reaggregation and histogeneration of the fetal mouse hippocampus and isocortex was reported already in 1970 [12]. The advent of modern organoid models, however, is the directed differentiation and autonomous organization of *in vivo* like tissues not from differentiated tissues, but rather from iPSC and human embryonic stem cell (hESC) populations. While there exist many benefits in the use of PSCs for organoid generation such as unlimited patient-specific cell sourcing for disease modeling, cell therapy, and drug screening [13] PSC-derived

* Corresponding author. University of British Columbia, Life Sciences Institute, Vancouver, Canada.
E-mail address: nico.werschler@ubc.ca (N. Werschler).

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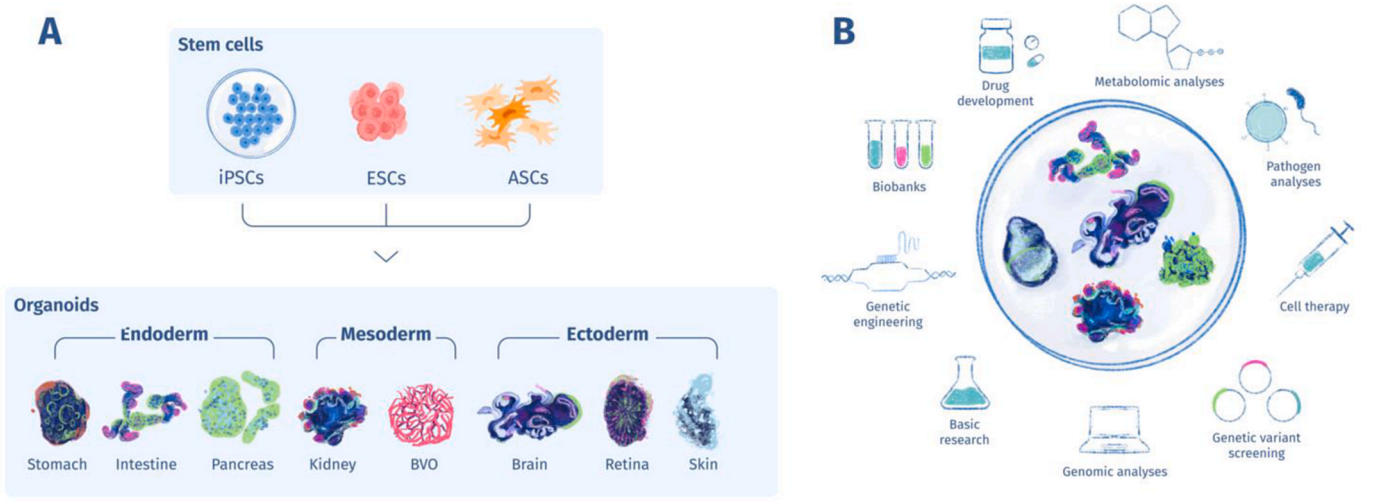

Fig. 1. Selected representation of the generation and application of organoids.
(A) Generation of organoids from iPSCs, ESCs, and ASCs. Brain, retina, skin, kidney, blood vessel (BVO), stomach, intestine, and pancreas represent a subset of multiple tissue organoid models that have been developed to date. (B) Application(s) of organoid technologies in both industry and academic settings.

organoid maturity and function *in vitro* often falls short of their organ counterpart *in vivo* [14,15].

Considering their underlying self-organization and maturation mechanisms closely mimic *in vivo* processes, organoids are frequently employed to investigate organogenesis or develop models for organ-specific diseases [16,17]. Additionally, the introduction of gene editing technology such as CRISPR/Cas9 allows for the generation of mutated or full disease-specific knockout stem cell lines or genetic modifications of already established organoids [18,19]. Organoids created from genetically modified, and disease specific cell lines offer higher resolution understandings of disease onset and progression in comparison to untailored cell lines.

During the last decade, the spectrum and application of organoid research has advanced significantly, now including brain, retina, tear duct, heart, lung, intestine, or kidney, among several other tissues – targeting usage in drug discovery, models of genetic disease, transplantation surgery, gene repairing, cancer, or toxicology [7]. From the promise of high throughput drug screening to regenerative and personalized medicine, organoids have moved to the forefront of many areas of clinical and academic research, with bioengineering and physiological sciences at the center. However, one omnipresent barrier to the application and scalable use of organoids *in vitro* is the absence of a perfusable vasculature, which hinders the growth and development, maturation, and physiological relevance of nearly every current engineered tissue and organoid model [20,21].

2. Importance of vascularization and perfusion in organoids

Endothelial cells (ECs) constitute a diverse cell population forming a monolayer on the inner surface of blood vessels, acting as a barrier between blood and interstitial fluid. Adult human ECs surface, spanning 1–7 m^2, consists of approximately 1–6 $\times$ 10^{13} cells [22]. These elongated cells are around 30 μm in length, 10 μm in width, and 0.3 μm in depth, also present with a negatively charged 10 nm deep glycosaminoglycan layer on their surface. While ECs share common features, regional diversity in morphological traits, molecular markers, and functional properties defines what we know as 'endothelial specialization' [23] - critical to proper vascular function and organogenesis.

Why does the lack of vascularization and/or a vascular compartment hamper the advancement and physiological relevance of organoid models? From a developmental perspective, the cardiovascular system (in combination with the haematological system) is, importantly, the first functioning organ system observed in all vertebrate embryogenesis [24] - supporting nutrient and oxygen transport, primitive circulatory network formation, initiation of hematopoiesis, and vascular networks that provide the foundations of organogenesis. Like ischemic complications *in vivo,* effective diffusion of oxygen and essential molecules from environments external to the developing organism is critical for cell growth and tissue morphogenesis. Although dependant on several factors (i.e., metabolic rate, bioavailability, static or dynamic environment), a general 150 mm O2 and nutrient diffusion limit has been established for *in vitro* tissue culture systems; values ranging from 70 to 200 μm have also been proposed [25]. Gated by principles of mass transport, the formation of vascular networks within and around organisms is crucial for the sustained delivery of oxygen and nutrients, which, in turn, support proper growth, differentiation, and maturation of developing tissues and organs. This effect translates across both *in vitro* and *in vivo* developmental platforms.

Apart from supplying oxygen, nutrients, and removing waste, there have been many recent reports outlining the role of blood vessels in directing non-vascular cell activity, suggesting further the importance of blood vessels in patterning developing organ structures in embryogenesis [26]. The direction and organization of non-vascular cells in angiogenesis and organogenesis is, to a remarkable degree, facilitated by paracrine signalling from endothelial cells [27,28]. Endothelial cells isolated from heart, lung, kidney, and liver at day 115 of embryonic development already present with organotypic markers and functions, suggesting early establishment of their role in directing organogenesis and maturation [29]. In essence, a reciprocal influence between organogenic cells and penetrating vasculature appears to exist: organs provide queues for vascular penetrance through angiogenic signalling, and the vascular endothelium signals back in a way that directs tissue morphogenesis and organogenesis [27].

Although general representation of *in vivo* cell types and structures can be achieved in organoids, lack of vascular networks (of any form) in and around the organoid tissue begs the accuracy of both developmental and disease modeling applications [21]. Generating vascular tissue in and around developing organoids will provide a next-generation, high-resolution perspective on the crosstalk between host tissue and vasculature in physiological, pathological, and drug-response settings [20]. Further, vascularization of organ tissues not only enhances physiological relevance, but also the speed and accuracy of which the developmental stages take place. For instance, vascularized cortical organoids more closely recapitulated fetal telencephalon development, showed further maturation, and did so in a more efficient manner [30]. Finally, the circulation of fluid within vessels and its subsequent flow

into the interstitial space play a pivotal role in regulating tissue function and providing vital cues in homeostatic maintenance. Thus, perfusing the vascular networks generated around and within otherwise avascular tissue constructs (i.e., organoids) serves as a promising means of overcoming size limitations that arise, in part, from the formation of necrotic cores within larger organoids [25].

The presence of a vascular network also enables the study of complex diseases, such as cancer and diabetes, which involve multifaceted interactions between the vasculature and other cell types. For example, the dysregulated formation of new blood vessels via angiogenesis is a hallmark of cancer [31] and the ability to study this process in the context of a 3D and time-resolved 4D organoid models could lead to the development of new cancer therapies.

3. Current approaches in the generation of vascularized organoids

Several approaches have been developed to generate vascularized organoids, reflecting the growing recognition of their importance in bioengineering and medical research. These methods aim to replicate the intricate vascular networks found in native organs, ensuring that organoids receive the necessary waste removal, nutrients, and oxygen for growth and function. This section delves into the predominant techniques and their underlying structural and molecular principles, offering insights into the current state of the art in the field.

3.1. Co-culture with endothelial cells

One approach to the generation of vascularized organoids is the use of a basic co-culture system, in which endothelial cells are co-cultured with organoid-forming cells. In vivo, endothelial cells, through mechanical [32,33] and chemical [34] processes, play a crucial role in the maturation and organization of human tissue through development, maturation, and sustained maintenance and function. Similarly, organoids exposed to endothelial cell co-cultures are subject to more physiologically relevant developmental patterns through higher-complexity cell signalling processes [35]. The developmental stage at which the organoid model is subject to endothelial cell co-culture vary based on experimental objectives, cell lines, and general differentiation timelines.

Endothelial cell-organoid co-culture systems stimulate the development of microvascular networks in and around the organoids, which exhibit enhanced functionality and anastomosis with host vasculature upon transplantation [36]. ECs can be obtained from various tissues such as the umbilical vein, aorta, and microvessels found in adipose tissue and foreskin [37]. Alternatively, ECs can be sourced through differentiation from hPSCs [38] and isolation from peripheral blood and bone marrow specimens [39]. Lim et al. effectively demonstrate that co-culture of hepatocellular carcinoma cell lines or patient-derived xenograft organoids with endothelial cells present increased levels of MCP-1, IL-8, and CXCL16, suggesting physiologically relevant aspects of angiocrine signalling. Co-culture models such as these serve as tools for studying and targeting interactions between angiogenic events and surrounding environments [40]. Furthermore, Ramachandran and colleagues spearheaded the creation of a 3D functional liver organoid incorporating three types of mature adult cells: hepatocytes, mesenchymal stem cells (MSCs), and endothelial cells (ECs). Their results demonstrated that the hepatocytes within these multi cell type liver organoids were genetically and functionally comparable to adult primary human hepatocytes and displayed architectural similarities to hepatic structures *in vivo* [41]. The combination of multiple cell types, including liver sinusoidal endothelial cells, played a crucial role in the maturation of the tissue construct. Achieving well-vascularized organoids *in vitro* simply through endothelial cell co-culture is challenging considering such a system omits the intricate coordination between endodermal epithelial, mesenchymal, and endothelial progenitors seen *in vivo*.

An alternative co-culture approach to achieving organoid vascularization is the incorporation of pre-formed vasculature or providing a scaffold for vascular growth into organoids. This can be achieved by embedding pre-formed microvessels or tissue-engineered blood vessels within the organoid, or by using a decellularized matrix from pre-vascularized tissue [35,42]. The decellularization process removes cellular components from the native tissue, leaving behind an extracellular matrix (ECM) scaffold [43,44]. Vascularized human cortical organoids were intracerebrally implanted into the S1 cortex of NOD-SCID mice by Shi and colleagues in which they identified both human ECs and mouse ECs within the blood vessels situated in the organoid graft areas. Two-photon fluorescence imaging revealed significant blood flow within the vascularized organoid grafts in mice, indicating the organoids' ability to recruit host ECs, reconstruct a functional vascular network, and support blood flow post-implantation [35]. In another study, Pham et al. employed a coating method combining iPSC-derived cortical cells and ECs. iPSCs were aggregated into organoids and subsequently differentiated into cortical cells [45]. Following a 34-day differentiation period, the organoids were coated with Matrigel and separately differentiated iPSC-derived ECs. Over three weeks of culture, the ECs spread and infiltrated the organoid, forming structures resembling blood vessels. Upon 2 weeks post-implantation, pre-vascularized organoids rapidly integrated with the host microvasculature, establishing a microvascular network within the organoid. Such approaches have shown promise in generating vascularized organoids but are limited by the availability of pre-formed vasculature and the complexity of the process. Methods to achieve decellularization involve the removal of cellular components from native tissues by perfusing vasculature with 0.1%–1% SDS detergent solution [46] or combinatorial treatment with detergents (e.g. SDS, Triton X-100), enzymatic agents (e.g. trypsin, endonucleases), and physical treatments such as freeze-thaw cycles [43,44], that yields a non-immunogenic, extracellular matrix (ECM) rich scaffold. Decellularized scaffolds (i.e., decellularized vascular scaffolds derived from blood vessels *in vivo*) (Fig. 2) are a promising means of delivering sustained perfusion to human blood vessel networks generated *in vitro*. In their original, free-standing form, decellularized scaffolds are amenable to cellular 're-seeding', or, 're-population' of the ECM-rich network with cells of the human blood vasculature to create a functional, intact vascular network on a predefined ECM-rich scaffold that can be present even before the aggregation and differentiation of 3-dimensional organoid constructs. This approach serves to provide a perfusable vascularized scaffolding around which organoid development, maturation, and tissue anastomosis can take place (Fig. 2).

3.2. 3D and 4D bioprinting

Another approach to address the challenge of perfusion is the use of bioprinting technologies. Bioprinting enables the precise placement of cells and ECM materials in space [26], as well as the printing of 3D vascular networks. Bioinks that encapsulate living cells are used in 3D bioprinting. Cell viability, or the ability of cells to survive and function properly, is a critical factor in the success of 3D bioprinting and is heavily influenced by the properties of the bioink formulation. For example, natural polymers like alginate, gelatin, and hyaluronic acid are commonly used as they provide a supportive 3D environment for cells. However, high concentrations can increase viscosity and shear stress, reducing cell viability [47]. Further, protein-based bioink hydrogels have been introduced that provide necessary peptides, as well as growth factors, to facilitate improved adhesion and cell growth [48]. Achieving high cell viability often comes at the cost of reduced printability. Current efforts in next-generation bioprinting aim to identify bioink formulations and printing parameters that strike a balance between maintaining structural integrity during printing and preserving cell viability and function [47–49].

With bioprinting, not only can the geometry and dimensions of the

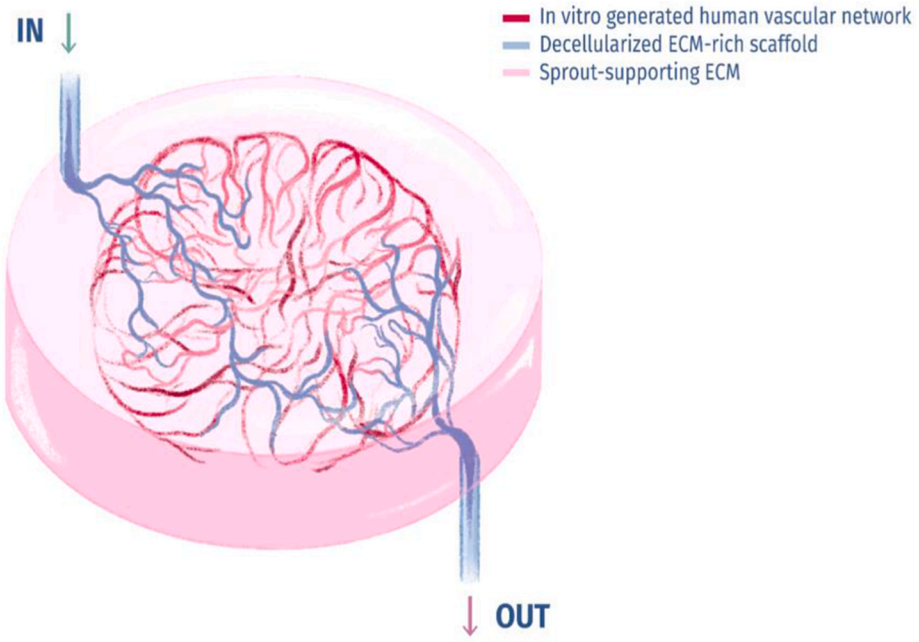

Fig. 2. Depiction of decellularized ECM-rich vascular scaffold (grey) anastomosing with human blood vessel networks (red) generated *in vitro*.
Human blood vessel networks are sprouting in response to VEGFA and FGF2 growing in sprout-supporting ECM matrix (pink). Self-directed organization and stochastic anastomosis between the human blood vessel networks and the decellularized ECM-rich scaffold facilitate perfusion of *in vitro* derived human blood vessels. A microfluidic system for active perfusion can be established by connecting one end of the scaffold to a pump for input and directing the medium flow through to the opposite end designated as the output.

tissue be consistently maintained and reproduced, but the flow parameters within perfusable bioprinted systems can also be monitored and controlled [26]. This approach has been successful in the generation of vascularized tissues, including cardiac constructs [50–52]. The use of bioprinting technology also offers the potential to create patient-specific vascularized organoids for personalized medicine applications. For example, determining disease processes and drug responses specific to the patient's genetic background to tailor treatment approaches. Other applications point towards utilizing patient-specific organoids for regeneration or repair of damaged tissues or organs, tailored to specific patient needs.

The emergence of 3D and 4D bioprinting may even address the complexity of the organoids, by printing vascular cells along with biomaterial to create an extensive network of microvessels [53]. Self-assembly techniques have been used to generate vascularized organoids, where organoid-forming cells are cultured in a 3D scaffold that promotes the formation of capillary-like structures [54]. These scaffolds can be made from a variety of materials such as collagen, hydrogels, alginate, along with natural, synthetic, and natural/synthetic hybrid polymers [55]. Bioactive synthetic hydrogels have gained attention as potential scaffolds due to their ability to offer customized biofunctions and modifiable mechanical characteristics [56]. Additionally, such hydrogels create a supportive, ECM-rich microenvironment conducive to cell growth and tissue development. The self-assembly of the scaffold and the formation of the vascular network can be influenced by various factors, including the composition of the scaffold and the presence of growth factors.

Although not yet widely used, 4D bioprinting is a promising method to more accurately mimic the formation of biological tissues through the incorporation of time. 4D bioprinting, while still fundamentally 3D, introduces the element of time as an additional dimension. In this context, 'time' refers not to the duration of the printing process itself, but to the ongoing evolution of the printed 3D biocompatible materials or living cellular constructs after printing and being exposed to different physical, biological, or chemical stimuli. The dynamic maturation process is facilitated using smart biomaterials which are designed to react to the aforementioned stimuli. Smart biomaterials, when combined with 4D bioprinting, offers printing capabilities of dynamic structures that can undergo shape changes, spatiotemporally controlled differentiation, and regulated release of bioactive agents. These factors are paramount to accurately recapitulate the dynamic behavior of living tissues [57]. Layer-by-layer bioprinting allows cells to be incorporated with hydrogels to form cylindrical, vasculature-like structures [58]. Maturation factors rapidly induce vascular cells to develop, leading to vascularization. Hong et al. demonstrated that multiple cell types, including MSCs, fibroblasts, and ECs, can be patterned in a hyaluronan-based hydrogel using a 4D printing system. Over a 16-day culture period, cell migration and aggregation occurred, forming vascular structures as indicated by endothelial-specific gene expression [59]. Bioprinted agarose rods have been utilized as filler material to create vascular tubes [58,60,61]. Norrotte et al. utilized human smooth muscle cells (SMCs) and fibroblasts, printing them within agarose molds where they fused and matured into vascular-like tubes with specific geometries, such as branching and double layers [58]. However, the scalability and complexity of these structures were limited by the removal of agarose fillers. Alternatively, vascular networks can be created using osmolarity gradients to form complex structures.

However, resolution, cell handling, and cross-technology integration (i.e. advanced imaging techniques and computational modeling) require further attention to improve the reproducibility and functional relevance of 3D printed vasculature [57,62,63]. The aforementioned 4D printing methods reduce operational difficulty for downstream handling and preparation for implantation and enhance the potential for creating larger vascular constructs, however they currently produce only single-layer structures, whereas native vascular networks consist of three layers [57]. Hence, consideration towards incorporating all these layers and cell types is crucial to create an integratable vascular structure.

While both 3D and 4D approaches have shown promise, there are still several challenges to be addressed to establish functional

vasculature in organoids via multidimensional bioprinting: development of reproducible methods for achieving vascularization, maintenance of vascular integrity, and regulation and quantification of blood flow and shear stress within organoids.

3.3. In vivo transplantation

Living organisms possess an inherent ability to stimulate vascularization. Previous studies have illustrated the feasibility and efficacy of organoid vascularization post transplantation *in vivo* [64]. Takebe et al. developed pre-vascularized liver buds by co-culturing iPSC derived hepatic endoderm cells with human bone marrow MSCs and human umbilical vein endothelial cells (HUVECs) [65]. Anastomosis of the liver organoid and host mouse vasculature was evident 48 h following the transplantation, as confirmed by dextran infusion, and the transplanted network grew over 44 % larger compared to the non-transplanted controls. Remarkably, the authors found that pre-vascularization of the organoids with HUVECs was crucial for successful engraftment. When control organoids without endothelial cells were transplanted, they failed to vascularize and engraft. The contact between host vessels and *in vitro* engineered vascular cells repeatedly presents as the most successful approach in transplantation-facilitated organoid anastomosis and vascularization [66].

Similarly, Takahashi et al. pre-vascularized pancreatic islets with HUVECs before transplantation under the renal capsule of immunodeficient mice and found that islets vascularization enhanced engraftment and early reperfusion, and thus therapeutic potential against diabetes [67]. Cakir et al. generated cortical organoids developing with vascular-like structures through transcription factor human ETS variant 2 (hETV2) induction [68]. When implanted subcutaneously into the hind limbs of immune-deficient mice, the vascularized cortical organoids functionally connected to the mouse vasculature, contrary to the control avascular organoids. Using a very different approach, Wang et al. engineered a 3D bio-printed blood vessel made of HUVECs in the lumen and human umbilical vein smooth muscle cells (HUVSMCs) on the outer surfaces, which anastomosed with the vena cava of the transplanted mice [69].). Importantly, the authors reported blood flow through the vena cava to the bio-printed vessel without leakage. Kawakami et al. have recently advanced the study of inflammation-induced thrombosis linked to SARS-CoV-2 by implanting human iPSC-derived vascular organoids beneath the cranial window in mice [70]. Remarkably, these organoids integrated with the host's vascular system and supported mouse blood perfusion within just three days.

Taken together, multiple studies show that engineered pre-vascularized organoids can functionally connect to the native vasculature of the host animal into which they are transplanted. Nevertheless, the necessity to transplant organoids into an animal for adequate vascularization and perfusion not only elevates the cost and duration of the process but is also strongly modulated by potential species-specific influences. Thus, having an *in vitro* tool enabling reproducible perfusion of functional vasculature would be highly valuable.

4. Next-generation approaches to create vascularized organoids

As the field of bioengineering evolves, so too do the methodologies employed in creating vascularized organoids. Besides the established techniques, researchers are now exploring cutting-edge strategies that prioritize enhanced efficiency, precision, and scalability. Moreover, a developing vasculature *in vivo* is subjected to a range of mechanical cues. Experienced primarily by endothelial cells, these cues span from the viscoelastic moduli of the surrounding ECM to the shear stress at the inner walls of vessels arising from the unidirectional flow of the bloodstream. Several engineered microfluidic platforms designed to study the impact of mechanical factors on angiogenesis, vasculogenesis, and 3D capillary morphogenesis *in vitro*, have underscored the critical role of these mechanical cues [43–46]. They highlight their importance in fostering a physiologically functional vasculature in future engineering of more complex organoids.

Table 1 highlights the listings of current perfusable and non-perfusable vascular models, general materials and equipment required, limitations, advantages, and application(s) for specific models of sprouting angiogenesis, organoid vascularization, and vascular perfusion.

Prefacing angiogenesis and vasculogenesis. Angiogenesis refers to the formation of new blood vessels from existing ones. Several key molecular players orchestrate this process [71]. Vascular endothelial growth factor (VEGF) stands out as a primary regulator, stimulating endothelial cell proliferation and migration [72]. Fibroblast growth factor (FGF) also plays a significant role by promoting angiogenesis through enhanced endothelial cell proliferation and migration [72]. Additionally, angiopoietins are crucial for regulating blood vessel maturation and stability. Platelet-derived growth factor (PDGF) aids in the recruitment of pericytes, contributing to vessel stabilization [72]. Finally, matrix metalloproteinases (MMPs) facilitate endothelial cell migration by remodeling the extracellular matrix [72]. In contrast, vasculogenesis, occurring primarily during embryonic development, is the de novo formation of blood vessels from endothelial progenitor cells [73]. Crucial molecular players include VEGFR-2, essential for progenitor cell differentiation and proliferation [74], Notch signalling guiding endothelial precursor cell differentiation [75,76], Ephrin-Eph receptor signalling directing precursor cells and aiding vessel assembly, and Sonic hedgehog (Shh) signalling contributing to embryonic vessel patterning and morphogenesis [76].

4.1. Co-culture with blood vessel organoids

Instead of relying on primary cell lines, like the frequently used HUVEC cells, for mimicking blood vessels, which have therefore changed their phenotype and exist only of endothelial cells, organoid technology now allows us to develop bona fide human blood vessels that resemble their initial state in a tissue. In the generation of human blood vessel organoids [77,78], following mesoderm induction via bone morphogenetic protein 4 (BMP4) and glycogen synthase kinase 3 (GSK-3) inhibitor CHIR99021 and, subsequently, vascular lineage priming using vascular endothelial growth factor A (VEFGA) and adenylyl cyclase activator Forskolin, endothelial cells are queued by both exogenous and endogenous signals to form angiogenic sprouts, primarily in response to proangiogenic factors such as VEGFA, fibroblast growth factor 2 (FGF2), or epidermal growth factor (EGF) [77–79]. Harnessing these processes *in vitro* has allowed the development of human blood vessel organoids (hBVOs) from hPSCs that consist of endothelial cells surrounding a central lumen, pericytes, as well as a basal membrane [77–79].

These hBVOs have been also applied *in vitro* and *in vivo* as a model of diabetic vasculopathy and towards identifying remedies for subsequent vascular defects. hBVOs have also been used to uncover mechanisms of SARS-CoV-2 transformation under the context of blood vessel maturity and function [80]. Importantly, these hBVOs present prototypical blood vessel cell types and form a perfused human vascular tree consisting of arterioles, capillaries, and venules following kidney capsule when transplantation in mice [77,78]. The primary application and progress of the hBVO technology towards disease modeling, drug identification, and vascular transplantation has opened multiple opportunities including the vascularization and perfusion of different hPSC/iPSC-derived organoids.

A noticeable example of this approach pertains to cerebral organoids. In 2013, the first brain or 'cerebral' organoids were reported [51]. Despite advancements in brain organoid culture and maintenance, conditions allowing growth and maturation up to and beyond 600 days, yielding organoids $\geq$6 mm in diameter, presenting hypoxia-driven and nutrient-deprived necrotic cores throws into question the physiological

Table 1
Presentation of current approaches to tissue engineered vascular models of organoid vascularization, perfusion, and co-maturation. Model type, technique/system, general equipment/materials, drawbacks, benefits, and primary use cases for *in vitro*, *in vivo*, in silico, and ex vivo conditions are discussed [140–189].

Type of study	Model Type	Model	Technique/System	Perfusable	Vascularizable	Equipment Needed	Benefits	Drawbacks	Best for Studying	References
in vitro	Static	2D Cell Culture	Pluripotent Stem Cells, Induced Pluripotent Stem Cells, Multipotent Stem Cells	Yes	Yes	Basic cell culture equipment (i.e., cell culture plates, ECM coating, incubator, media, BSC...etc)	Simple high quality culture maintenance, easy performance and reproducibility, long-term viability, low maintenance cost	Does not mimic in vivo tissues or tumours, limited physiological relevance (i.e. limited vessel tube formation)	Drug screening, gene therapy, hybridoma culture, endothelial cell sprouting assays	(140, 141)
in vitro	Static	Scaffold free 3D cell culture models	Tumor organoid models	Yes	Yes	Tumor cells, ECM, basic cell culture equipment	Recapitulates aspects of tumor microenvironment, allows study of tumor angiogenesis	Limited perfusion	Tumor angiogenesis, effect of drugs on tumor vasculature	(142, 144)
in vitro	Static	Scaffold free 3D cell culture models	Air•liquid interface culture	Yes	Yes	Collagen matrix, cultured tissue, basic cell culture equipment, cancer cell line, endothelial cells	Mucociliary differentiation, mucin secretion and tight junction formation, extracellular environment bears high resemblance to in vivo conditions	Lacks standardization and does not replicate or reflect recruitment of circulating immune cells into the tumor	Anastomosis, epithelial cell behaviours, response to pathogens and immune cell interactions	(145-149)
in vitro	Static	Scaffold free 3D cell culture models	3D Tumor spheroid culture	Yes	Yes	Method-dependant: ultra-low attachment cell culture plates, bioreactor, or hanging drop culture; basic cell culture equipment, cancer cell line, endothelial cells	High reproducibility, control of cellular shear stress and pressure, simple set up, continuous perfusion	Low throughput, optimization for each cell line is required, laborious	Tumor microenvironment, drug screening, biomarker discovery	(100,141,150–152)
in vitro	Static	Scaffold free 3D cell culture models	3D organoid culture	Yes	Yes	Traditional cell culture techniques and equipment, organoid(s) of interest	Robust division and differentiation programs, reflect features of endogenous tissue organization, can extract extensive quantitative data on various cell types and the condition of organ structures and functions	Optimization protocols are not standardized, lack of high-fidelity cell types, limited maturation, self-organizing construct anatomy	Disease and cancer modelling, organ development, drug screening, personalized medicine	(141,153,154)
in vitro	Dynamic	Scaffold free 3D cell culture models	Tumor spheroid culture	Yes	Yes	Same as for static cultures except now with the addition of a rocker or other perturbation device	Better mimicry of the physiological conditions; better diffusion of nutrients, signalling molecules, waste metabolites, and oxygen; no size constraint (from the cell wells); better preservation their transdifferentiation potential than in static culture	Introduction of shear stress may compromise cell viability and structure	Tumor microenvironment, drug screening, biomarker discovery	(138,155,156)
in vitro	Static	Scaffold Based 3D cell culture model	Hydrogel-based scaffolds	Yes	Yes	Hydrogel, basic cell culture equipment (i.e., cell culture plates, ECM coating, incubator, media, BSC... etc.), cancer cell line, endothelial cells	Can modulate scaffold durability and biodegradability	Volume-limited hydrogels, limited diffusion and mass transfer properties, variable cell viability based on hydrogel generation and composition	Drug delivery, wound healing, ophthalmic materials and tissue engineering (i.e. models of vascular development)	(141,157)
in vitro	Static	Scaffold Based 3D cell culture model	Solid-state scaffolds	Yes	Yes	Variety of hydrogel pre-polymers, desired cell types for seeding. Variable methods involving gas foaming, electrospinning, leaching, lyophilization equipment can be used	Enables concise positioning of cells in vitro in a controllable and reproducible manner	Challenging to control precise scaffold dimensions (i.e. pore size and shape)	Bread applications in cell and tissue engineering	(158,159)
in vitro	Dynamic or Static	3D microfluidic platform	Organ-on-a-chip/ 3D tumor vascular model	Yes	Yes	Tissue or cells, collagen matrix, microfluidic device, pumps	Precise control over microenvironment (i.e., flow velocity, and shear force), can model flow	Complex setup, technical expertise required, cost to produce microfluidic device may be costly	Sprouting angiogenesis, lumen formation, anastomosis, effect of flow, effect of drugs on ECs	(160,161)
in vitro	Dynamic	3D cell culture	Bioreactor/ rotating wall vessel/ microcarrier system culture	Yes	No	Microcarrier beads, basic cell culture equipment, bioreactor, spheroids or organoids	Promotes higher cell yield in small volumes of medium, integrable in bioprocessing systems, reduce cost, improves nutrient supply, provides dynamic environment for enhanced nutrient exchange, maintains a homogeneous oxygen gradient for stable growth, applies minimal shear force to cells	Cell growth is not well controlled, traditional microcarrier manufacturing methods cannot reproduce desired structure, too much shear stress may compromise cell viability and structure	Vaccine production, fermentation processes, mesenchymal stem cell production, measurement of oxygen content, bio-signal transduction, and vascular branching differentiation	(162,162–166)
in vitro	Static	4D cell culture	4D bioprinting	Yes	Yes	Smart biomaterials (ex: smart hydrogel systems that respond to thermal, magnetic, electrical, photo, pH, and water stimuli), 3D bioprinter, bioink such as polyethylene glycol (PEG), polylactic acid (PLA), and polycaprolactone (PCL), endothelial cells or other cell types of interest	Improve mechanical properties, has capability to respond to specific cellular signals	Non-homogeneous distribution can negatively affect cell migration and proliferation	Musculoskeletal, cardiovascular, nervous, and skin tissue repair	(167,168)
in vitro	Static	4D cell culture	Stem cell cultures	Yes	Yes	Smart biomaterials, stem cells, basic cell culture equipment	Mechanical response to stimuli, improve cell migration, addition or sequestration of growth factors to regulate stem cell differentiation	Complexity, high cost, technical expertise needed to work with 4D cultures	Stem cell based regeneration, disease progression, neural tissue engineering	(169,170)
in vitro	Static	Combination	Co-culture systems	No	Yes	Cell culture equipment, desired organoid and/or tissue constructs from desired cell sources, variable cell culture plates (i.e. ultra-low attachment vs cell culture treated)	Simplified approach to generating more complex organoid models through co-maturation. Influence of mat cell type presence and signalling increased construct complexity and maturation	Complexities in media composition for sustained viability of the co-cultured tissue constructs	Assembloids, organoid-organoid inflammasome dynamics, organoid vascularization and the influences of said vascularization on organoid maturation and development	(65,81)
in vitro	Dynamic	Combination	Organ-on-chip models	Yes	Yes	Organ-specific cells, microfluidic device	Allows study of organ-specific angiogenesis, effect of flow, and drug testing	Complex setup, limited throughput	Organ-specific angiogenesis, effect of flow, effect of drugs on ECs and mural cells	(171,172)
in vitro	Static	Embedded ex vivo tumor sections	---	No	---	Tumor tissue sections	Retains native tumor heterogeneity, combines the complexity of in vivo tumor microenvironments with the simplicity of in vitro models	No perfusion, limited study of angiogenesis	Characterization of tumor vasculature	(173,174)
in vitro	Static	Tumor Microvessel Model	Predefined scaffold	Yes	Yes	ECM, PDMS housing, template rod, endothelial cells, tumor cells	Well-defined vessel endothelium, shear stress is relatively easy to analyze	Vessel geometry has limited complexity and diameter (>50 μm)	Intravasation, extravasation, vessel paracrine signalling, transvascular migration	(161,174,175)
in vitro	Static	Tumor Microvessel Model	Self-Assembly	Yes	Yes	Endothelial cells on predefined ECM surfaces, tumor cells	Complex networks can be generated with varying sizes (5-50 um)	Randomized vessel network formation, unpredictable flow	Extravasation, drug toxicity screening, tumor dormancy	(174,176)
in vivo	Static	Zebrafish Model	---	Yes	Yes	Aquarium setup, microscopes	Transparent, genetic manipulability, live imaging	Specific to Zebrafish, may not fully replicate mammalian systems	Tumor angiogenesis, complex tissue regeneration, anastomosis	(177–180)
in vivo	Static	Preclinical Model	Chick Chorioallantoic Membrane (CAM) Assay	Yes	---	Incubators, imaging systems	Rapid, simple, cost-effective, accessible vasculature, allows high-throughput screening	Chick–specific, ethical considerations	Tumor angiogenesis, sprouting angiogenesis, acute toxicological studies for anti-cancer drugs	(181)
in vivo	Static	Mouse Retina Model	---	Yes	---	Animal facilities, microscopes	Mammalian system, high relevance to human physiology	Ethical considerations, cost, complexity of animal handling	Retinal angiogenesis, drug effects, effect of flow, retinal vasculature in developmental disorders	(182)
in vivo and *in vitro*	Static	Tumor Xenograft Model (Animal and Patient-derived)	---	Yes	---	Animal facilities, imaging systems, patient biopsy	Directly studies tumor angiogenesis, relevant to cancer research	Ethical considerations, cost, complex model	Tumor angiogenesis, drug effects on ECs and mural cells, effects of flow	(143,183)
ex vivo	Static	---	Aortic Ring Assay	No	---	Dissection tools, culture equipment	More complex tissue structure, intermediate between in vitro and in vivo	Limited to small vessel segments, no perfusion	Sprouting angiogenesis, matrix remodeling, lumen formation	(184)
in silico	Static or Dynamic	Computational Models	Simulations in silico can effectively replicate the angiogenic, vasculogenic, and vascularization processes observed in vivo and in vitro systems. These models can encompass various aspects, such as vascular perfusion, fluid shear stress, and flow parameters, in both healthy and pathological contexts.			Computer, simulation software, 3D CAD software, multiphysics modelling software	No ethical concerns, cost-effective, can model complex perfusable, microfluidic, and angiogenic systems under well-defined parameters	Model depends on parameter number, relevancy, and accuracy. Lacks biological complexity in contrast to in vitro or in vivo systems	Predictive modeling of angiogenesis, drug effects, theoretical studies of EC behavior, mural cell interactions, and fluid-shear-stress simulations	(185–189)

validity of such long-term experiments. An attempt to address these limitations was done by the fusion of hBVOs and brain organoids to obtain vascularized brain organoids [81]. As a result, the organoids exhibited a vascular network with blood-brain barrier structures. The microglia that were integrated responded actively to immune stimuli in the vascularized brain organoids and demonstrated their ability to engulf synapses. Further, inclusion of a vascular compartment in brain organoids resulted in the upregulation of the major glucose transporter GLUT-1, alongside preserved functional integrity of neuronal structures provided by the blood endothelium [81]. Kong et al. developed cortical-blood vessel assembloids by fusing cortical organoid with blood vessel organoid to provide vasculature to brain organoids [82]. Following individual maturation of blood vessel and cortical organoids, the fusion process can be achieved through a simple 4-day co-culture in ultra-low attachment 96 well plates [82]. The authors found increased expression of microglia and astrocytes in brain organoids when co-cultured with blood vessel organoids. Moreover, because ACE2 (the angiotensin-converting enzyme 2, a critical entry point for SARS-CoV-2 to infect human cells) [80] is highly expressed in microvascular pericytes, this vascularized cortical organoids model was used to provide SARS-CoV-2 viral receptors and thus recapitulate SARS-CoV-2 pathology. Similarly, Arslan et al. developed vascularized cardiac microtissues generated from the co-culture of blood vessel organoids and embryonic stem cell derived cardiomyocytes [83]. Thus, co-culture and co-maturation alongside human blood vessel organoids serves as an effective foundation in the generation of vascularized organoids (Fig. 3).

Kim et al. generated vascularized multilineage liver organoids through incorporation of hPSC-derived hepatic stellate cell-like cells and modulation of Notch signalling [84]. The vasculature formed within organoids was made of perfusable, hollow lumens, as evidenced by live perfusion of rhodamine conjugated Ulex Europaeus Agglutinin 1 and electron microscopy imaging [84]. Interestingly, the authors showed a reduced cell death and enhanced survival and growth of vascularized liver organoids compared to control avascular organoids. Similarly, Cui et al. engineered vascularized placenta-like organoids through a protocol inducing both trophoblast and vascular lineage [85]. Transmission Electron Microscopy (TEM) imaging revealed the presence of Weibel–Palade bodies, a known endothelial maturation marker, in the cytoplasm. Finally, in a very recent study, Khan et al. and Frenz et al. reported the generation of vascularized bone marrow organoids [86,87]. Following the general development principles of our blood vessel organoid protocol, the authors induced differentiation of vascular hemangioblasts through the addition of hematopoietic growth factors and cytokines [86].

A critical shortfall in the use of conventional well plate culture systems is the absence of controlled fluid flow perfusion, which is essential for harnessing the full potential across bioengineering and tissue engineering applications. In current lab settings, oxygen exchange mainly occurs at the medium's surface. However, this spans a considerable distance of several millimeters to a centimeter, which significantly deviates from the 10–30 μm diffusion distances commonly observed in mammalian tissues [88]. As previously mentioned, in these cultures, organoids without a functional vascular system rely solely on passive diffusion for nutrient, oxygen, and waste exchange. Typically, this diffusion efficiency does not extend beyond a few hundred micrometers, which in turn can result in the formation of a necrotic core [25]. Following successful perfusion of human blood vessel networks derived *in vitro*, a combination of co-maturation and perfusion culture systems should enable controllable delivery of cytokines, nutrients, and increased gas exchange to otherwise isolated regions of 3D organoid cultures [21]. This could be further achieved by generating vascularized organoids-on-a-chip as detailed in the next section (Fig. 4).

4.2. Vascularized organoids-on-a-chip

Organs-on-a-chip systems are miniaturized systems that employ microfluidic technology to create intricate three-dimensional structures, enabling the study of human cells in controlled environments [89]. In parallel, organoids have emerged as a distinct yet complementary technology, also aiming at a more accurate representation of human tissues [90–92]. While organoids stand out for their remarkable biological complexity, they are limited through the absence of biophysical cues and vasculature or immune cell compartments, which are crucial in the faithful recapitulation of human tissues. Organs-on-a-chip facilitate precision control of the environment and can incorporate functional vasculature, but often lack the biological complexity of human tissues. Merging these technologies into "organoids-on-a-chip" can potentially bridge this gap [21,93]. This nascent idea will allow us to leverage the cellular complexity of organoids while incorporating the dynamic biophysical environment provided by the microfluidic chips. The synergy

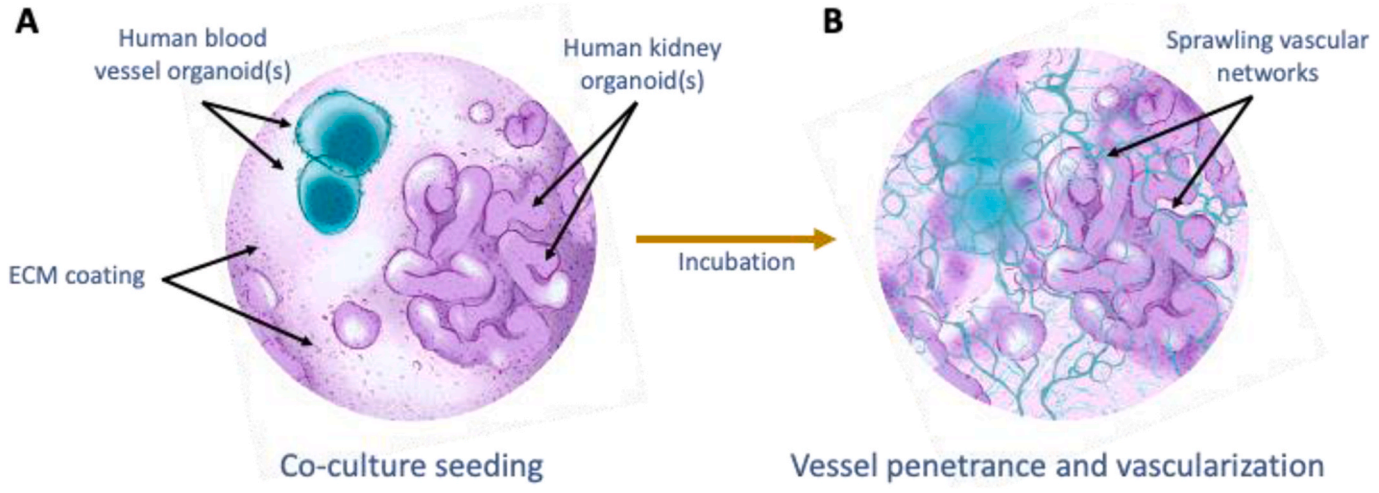

Fig. 3. Schematic representation of a static human blood vessel organoid and kidney organoid co-culture by which human vascular networks form within and around the kidney organoids.
(A) Seeding of human blood vessel organoids proximal to budding kidney organoids. A thin layer of ECM is applied to support the formation of new vascular networks within the co-culture environment. (B) Depiction of sprawling human vascular networks within and around the previously avascular kidney organoid following desired incubation period. NOTE: Not limited to kidney, this is a schematic representation of an approach that can be used to generate vascular networks within and around 2-dimensional organoid construct.

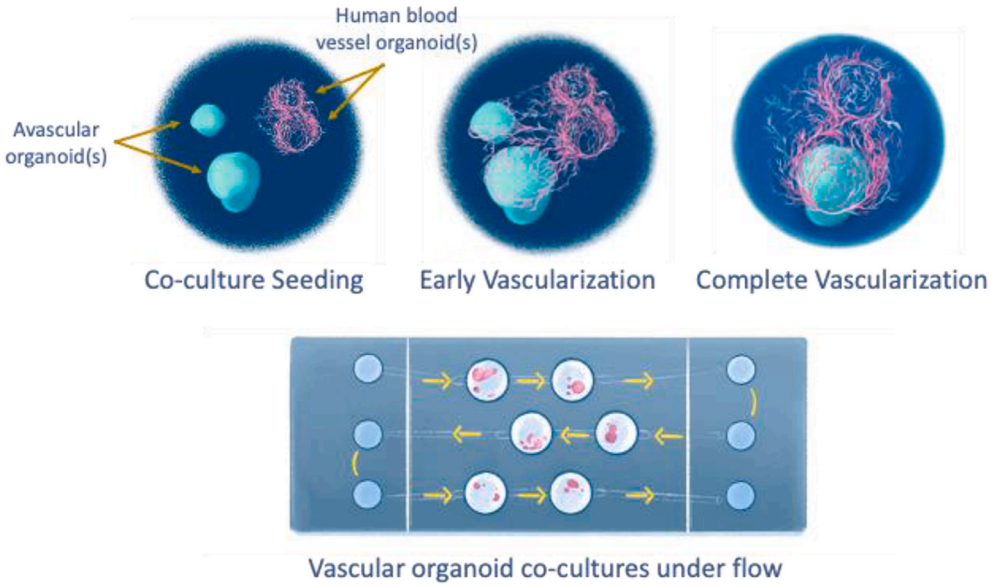

Fig. 4. Schematic representation of a dynamic human blood vessel organoid and target 3-dimensional organoid co-culture by which human vascular networks form within and around the target organoid while under perfusion culture conditions.
(Top, left to right) Depicts the seeding of human blood vessel organoids proximal to the target organoid inside the perfusion culture well(s). A thin layer of ECM can be applied to preserve organoid position and support the formation of new vascular networks while under flow conditions. During sprouting, vascular networks from the hBVO penetrate through the ECM to access and vascularize the nearby organoid. Progressing degrees of organoid vascularization are depicted by network density in early compared to late stages of vascularization. Late stages of vascularization present with penetrance of vascular networks within and around the target organoid, including potential visualization of flow through the newly formed vessels under correct perfusion conditions. (Bottom) Depiction of a microfluidic chip environment that supports the co-culture, co-maturation, and vascularization of avascular organoid models under flow conditions. NOTE: This is a schematic representation of an approach that can be used to generate vascular networks within and around 3-dimensional organoids under perfusion conditions.

between stem cell biology and microfluidics offers the possibility to provide mechanical stimuli in a controlled fashion to recapitulate *in vivo* physiology, functionality, and disease phenotypes, while preserving the scalability and ease of use required for *in vitro* applications [94] (Table 1).

The importance of administering flow and perfusion in vascularized organoids cannot be overstated. One of the major challenges in generating functional vascularized organoids is the lack of proper perfusion inside the lumens of the vessels. The presence of flow and its ample delivery of nutrients and oxygen is crucial for vascularization, which is dependent on flow [95]. This in turn is critical for maintaining the viability and functionality of the cells in the organoid. The lack of perfusion also affects the differentiation potential of cells under culture [96], further complicating the generation of functional tissues. Using microfluidic techniques, considerable effort has been directed towards addressing the challenge of organoids' vascularization through the generation of perfusable microvascular networks on-chip (MVNs), either as standalone [97–99], or by incorporation of other tissues to develop functional, vascularized organs-on-chips [100–104]. In this approach, endothelial cells and supportive cells are seeded into a central microfluidic chamber using hydrogels. These hydrogels provide structural support to the embedded cells facilitating self-organization into endothelial networks. The medium flows continuously into the lateral channels adjacent to the central microchamber, providing the nutrients and gas exchange required for long-term cell culture [105]. Numerous studies have presented the notable relationship between mechanical cues and the induction and maintenance of physiologically functionally vasculature [106–108]. The primary advantage of these systems is their ability to facilitate luminal flow within the formed vessels through microfluidic perfusion. Several research groups have therefore developed permeability assays to assess the leakiness of these vessels. Vascular permeability can be evaluated for molecules of various sizes, often using dextran with different molecular weights. In a study modeling the blood-brain barrier (BBB), the authors comprehensively characterized the permeability of the MVNs and found permeabilities comparable to those measured in animals. This suggests physiological nonspecific paracellular transport through endothelial cell junctions. Notably, they also showed that bevacizumab, a monoclonal antibody used in glioblastoma VEGF therapy, had a permeability similar to that of paclitaxel, a small molecule used in chemotherapy, indicating enhanced transcytosis across this BBB model [109]. To gain deeper insights into the morphological architecture of these MVNs, immunofluorescence staining can be used to evaluate endothelial cell junction expression and extracellular matrix deposition. Different studies have confirmed the presence of tight junction proteins such as ZO-1 and Claudin-5, adherens junction proteins like VE-cadherin, basement membrane proteins including laminin and collagen IV, as well as glycocalyx proteins such as heparan sulfate proteoglycan 2 and hyaluronic acid [109–111]. Despite the advances in microfluidic chip modeling of vascular development and function, considerably little effort has been put towards the *in vitro* development of fenestrated, or organotypic vasculature that is seen *in vivo* (e.g. kidney, liver) [112,113]. Next generation microfluidic organ-on-chip platforms are working to integrate flow and inflammation based microsensors, in combination with real time imaging, to capture, for example, drug toxicity, transport, and filtration in high-resolution [114].

Microfluidic devices can also be used to simulate disease conditions (i.e., modulation of shear stress, fluid composition, and pressure) allowing researchers to study the effects of diseases on the vascular system and test potential therapies [52].

To provide a vasculature to various biological tissues, one strategy is to utilize the HUVEC endothelial bed to connect spheroids or organoids. Spheroids, derived from immortalized cell lines, primary cells, or tissue fragments are formed by cell-cell adhesion and aggregation of cells into a spherical structure [115]. In contrast to that of organoids, spheroids take less time and money to generate but lack the multicellular complexity and organization of *in vivo* tissues due to their rapid generation and homogeneous cellular composition [115].

In a study by Nashimoto et al., a pre-vascularized spheroid composed of HUVECs, fibroblasts and breast cancer cells, was seeded in a central

microfluidic chamber [100]. HUVECs were introduced to the side walls of the central gel microchamber. Angiogenic factors, released by the co-cultured spheroid, prompted the ECs on the sidewalls to form angiogenic sprouts that penetrated the gel, moving towards the spheroid. By the fourth day, vessels from both the co-culture spheroid and sidewalls converged and anastomosed near the periphery of the spheroid [100]. These anastomoses resulted in a perfusable network throughout the central gel area, encompassing the spheroid. This was confirmed by the successful perfusion of a fluorescent dextran dye into the spheroid core. While this specific method has not been applied to PSC-derived organoids yet, it offers an encouraging blueprint for realizing a functional *in vitro* vasculature spanning an organoid and its surrounding endothelial bed [100].

Recently our group developed a microfluidic platform in which BVOs were rendered accessible through a neighboring endothelial HUVEC network, to organize a whole perfusable vascular tree [116]. When cultured under flow conditions, the vascularized BVOs displayed a physiological hierarchical organization with larger HUVEC vessels upstream and downstream of the BVO. The regions upstream and downstream with arteriole-venule sized vessels exhibited strong expression of the smooth muscle cell SM22 marker, while the narrow capillaries in the BVOs present physiological hollow lumen structures surrounded by pericytes. The BVOs' vessels were perfusable, as demonstrated by live microbead perfusion, and microbeads were also observed deep inside the BVOs' vasculature, suggesting successful organoid perfusion. Importantly, the combined presence of vascularization and flow enhances the growth, function, and maturation of organoids. To accomplish this, BVOs can be first embedded or seeded within a supportive matrix like hydrogels or decellularized scaffolds [21,117]. This pre-vascularized matrix containing the BVO-derived vascular plexus is then used as a niche to seed in organoid-derived cells or organoids. As the organoids develop within this niche, they integrate with the pre-formed BVO vasculature, leading to vascularization [21,117].

This platform also offers the flexibility to vascularize other types of organoids, spheroids, tumoroids, or human tissue explants. For instance, our results demonstrated that recreating a favorable pancreatic islet niche within the microfluidic platform significantly improved islets functionality [118]. However, it should be noted that these neighboring endothelial beds still rely on an HUVEC endothelial network, which lack the capacity for differentiation, do not represent tissue-specific characteristics, and have a limited potential of anastomosis with a native vasculature. Utilizing iPSCs instead of HUVECs provides greater flexibility and relevance for vascular studies, making it an additional approach for the development of personalized therapeutics and advancing disease modeling. Indeed, traditional *in vitro* studies have primarily utilized human HUVECs and other EC model systems, overlooking tissue-specific differences in phenotype and function. Recent transcriptomic advancements highlight the diversity of organ-specific EC expression patterns, questioning the suitability of HUVECs for organ-specific vascularized models [119,120]. The use of primary ECs in these models faces challenges due to variable tissue availability and patient variability, while advancements in induced pluripotent stem cell (iPSC) technologies offer a renewable, reproducible source for generating ECs with specific vessel and organ properties.

A new strategy emerged in Valeria Orlova's group, consisting in growing a fully hiPSC-derived vasculature on-chip [121]. Using protocols to derive from hiPSC lines, they seeded hiPSC-ECs alongside hiPSC-VSMCs in a fibrin microchamber. These combined cells subsequently self-organized into perfusable microvascular networks-on-chip, allowing for hereditary hemorrhagic telangiectasia modeling, a genetic disease characterized by the development of abnormally fragile blood vessels and caused by mutations in the *ENDOGLIN* gene [122]. In a very recent study, the authors took a step further by incorporating a cardiac organoid to their vascular bed on-chip [83]. The authors showed that cardiac tissue vasculature connected to the surrounding vascular network through anastomosis, and that vascularization enhanced endothelial cell - cardiomyocyte communication as well as inflammatory responses. While this approach offers several advantages compared to traditional HUVEC beds, it still lacks the genuine self-differentiation and organization traits inherent to organoids. Additionally, much like HUVEC vessels, the vessels produced using this method tend to be larger and don't accurately mirror the structure and function of the capillary-level vessel beds observed *in vivo* (see Fig. 4).

In 2019, Homan et al. reported a new 'millifluidic' chip for culturing kidney organoids under flow conditions [123]. The organoids were partially embedded in a hydrogel and continuously exposed to a superficial flow, leading to the development of vascular networks structures both within and sprouting out from the organoids. Compared to organoids cultured under static or low flow conditions, organoids subjected to high fluid shear stresses exhibited enhanced glomerular vascularization and foot process maturation [123]. While their device facilitated flow via superfusion (flow overtop the organoids), it did not readily support intravascular perfusion. However, through precise timing of flow administration and differentiation, self-organizing

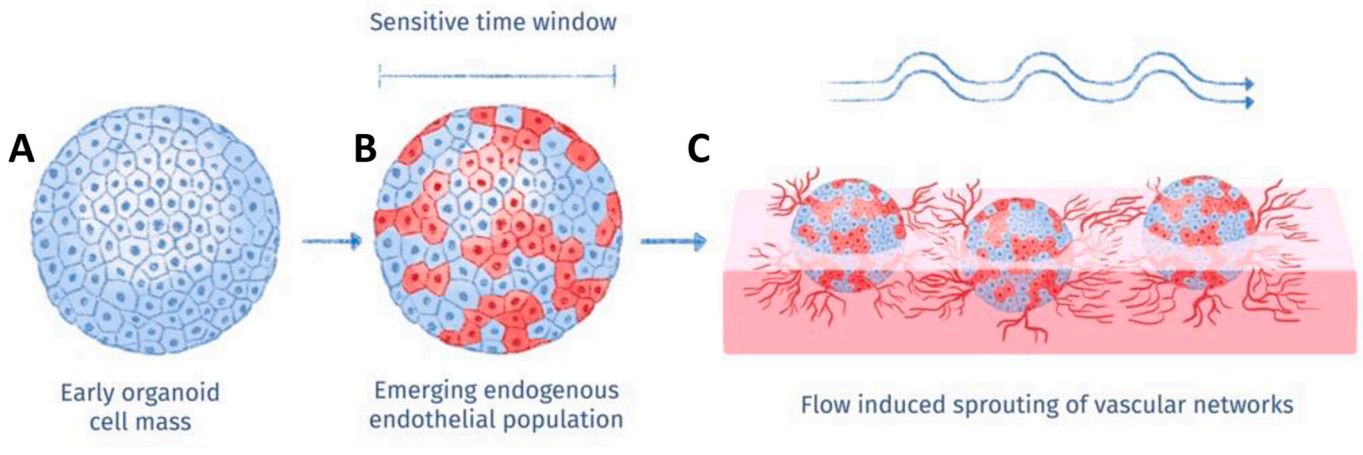

Fig. 5. Leveraging mechanical responsivity of endogenous endothelial cell populations to achieve vascular network formation in and around organoids under controlled flow conditions.
(A) Depiction of early organoid cell mass comprised of seemingly homogenous, undifferentiated cell population. (B) Progression through mesodermal lineage in organoid differentiation prompts the emergence of a sensitive time window in which endogenous endothelial cell populations emerge.(C) Schematic representation using top flow (superfusion) to induce vessel sprouting and vascularization within and around organoids presenting endogenous endothelial cell populations (as in Fig. 5B).

vascular network with mural cells emerged. Nonetheless, the process of sprouting endothelial and vascular progenitor populations endogenous to the target organoid tissue via mechanical and chemical cues is increasing in popularity as a means of generating vasculature within organoid cultures *in vitro* (Fig. 5).

4.3. Transition towards disease modeling

A critically overlooked component of the blood vasculature that can be effectively studied using microfluidic systems is the glycocalyx. Since its discovery some 40 years ago, the endothelial glycocalyx, acting as both a flow regulator and force transmitter, has been increasingly appreciated as an important factor in vascular function in both health and disease [124–126]. The primary structural components of the glycocalyx are glycoproteins and proteoglycans. While both contribute to structural support, proteoglycans, particularly due to their association with glycosaminoglycans, are considered the main support structures within the endothelial glycocalyx. The soluble apical layer of the endothelial glycocalyx shares a dynamic relationship with fluid shear stress and blood flow dynamics. A delicate balancing of glycocalyx thickness, composition, and restructuring proficiencies make geometrical characterization of the glycocalyx quite challenging [127]. Components of the glycocalyx anchor to the cell membrane via transmembrane domains (covalent links to molecules in the outer leaflet of the plasmalemma), or indirectly through receptor molecules like integrins or CD44. Various proteoglycans are present, but the most prominent in the endothelial glycocalyx are syndecans and glypicans, showcasing the intricate nature of this structure in vascular function Importantly, damage to the endothelial glycocalyx, accompanied by the structural degradation of its components, is an early sign of various pathologies including atherosclerosis and diabetes mellitus [128,129].

Considering the glycocalyx grows under the shear stress of blood flow, use of microchip and organoid-on-chip devices that recreate the fluid shear stress and better mimic natural flow conditions is a logical means of advancement in a rapidly expanding field of *in vitro* engineered vasculature. Our current understanding of the endothelial glycocalyx using on-chip systems extends to:

- Demonstrations of varying mean shear stresses, simulating arterial, capillary, and venous conditions, to form discrete and structurally unique glycocalyx structures along continuous endothelial monolayers [130].
- Validation of distinct glycocalyx structures by studying endothelial responses to inflammation (ICAM-1 expression, lymphocyte adhesion), primary hemostasis (VWF-expression, platelet adhesion), and cellular uptake of nanoparticles in on-chip platforms [130],
- Adhesion of tumor cells to the microvascular endothelium is mediated by the deposition of their glycocalyx in on-chip systems [111],
- Critical roles for tumor cell integrins, particularly integrin β1, in the extravasation process and the glycocalyx to enhance clustering of integrin binding sites on tumor cells with CD44 localization occurring on these clusters [111].

Many of the *in vitro* models of the endothelial glycocalyx focus primarily on visual representations of the glycocalyx, demonstrating changes in permeability, or studying specific interactions in static environments. Few models integrate all these aspects into one dynamic framework, illustrating the glycocalyx visually while assessing its barrier function in a flow-enriched environment [131]. These models primarily rely on HUVECs as their endothelial network foundation, omitting a potentially critical role of mural cell populations in glycocalyx structure and function. Further, relationships between shear stress, glycocalyx thickness, and disease pathologies must be key applications for perfused on-chip vasculature and vascularized organoid models.

In the case of atherogenesis, cumulating evidence suggests that preservation of glycocalyx structure serves as a protective barrier to vessel walls, whereas ill-structured or disrupted glycocalyx increased the vulnerability to atherogenesis [128]. In diabetes, increased shedding of the endothelial glycocalyx, marked by elevated circulating concentrations of hyaluronic acid, heparan sulfate, and syndecan, are correlated to elevated risk for diabetic vasculopathies. Further, diabetic patients present with elevated levels of hyaluronidase-1 (HYAL1) [132], a factor known to reduce glycocalyx thickness [129]. Thus, real time study of HYAL1 inhibitors using on-chip perfused vascular models can produce important data on the prevention of endothelial glycocalyx shedding in diabetes [129,132]. Importantly, employing methods to preserve glycocalyx structure and integrity for visualization and analysis *in vitro* are necessary, considering known challenges surrounding glycocalyx damage and degradation during tissue handling and fixation [133].

In oncology research, the integration of vascularization within organoid models has emerged as a significant focus area. In recent decades, the development of advanced 3D *in vitro* models like spheroids and organoids has revolutionized the simulation of the tumor microenvironment (TME), surpassing traditional 2D cultures in complexity [134]. Spheroids, despite their simplicity and ease of handling, offer limited structural complexity. Organoids more effectively replicate the TME and have already proven to be reliable indicators of chemotherapy responses in patients [16,135]. Ongoing research in oncology calls for novel *in vitro* models that reflect the dynamic and intricate interplay within tumors, encompassing diverse cell types, extracellular matrices, vasculatures, and spatial organizations—factors all pivotal in tumor growth, metastasis, and resistance to therapies. Microfluidics and organ-on-chip technologies show promise in developing next-generation 3D tumor models, offering controlled conditions, and the ability to incorporate mechanical stimuli. For instance, Fang et al. utilized microfluidics to mechanically stimulate intestinal muscles, enabling high-throughput culturing of human colon tumor organoids for nanomedicine screening [136]. When cultured under peristalsis-induced high interstitial fluid pressure condition, organoids showed an increase of stem cell markers (Lgr5) and proliferation markers (Ki67).

Complexity in chip designs varies, with the most advanced featuring perfusable MVNs –described in the section above – incorporating tumor spheroids. Recent research has further advanced this field by investigating the role of monocytes in cancer cell extravasation [137], and evaluating CAR-T cell recruitment, killing capacity, and inflammatory response [138]. These advancements allow exploration of key tumoral and therapeutic mechanisms. Yet, using HUVEC cells in these models presents challenges in faithfully reproducing the distinct characteristics of tumor vasculature. Looking ahead, iPSCs hold promise for progress due to their ability to be reprogrammed into different types of tissues. Lee et al. conducted pioneering work in that direction, by integrating iPSC-derived cardiac cells in a heart-breast cancer-on-chip system [139]. Moreover, iPSCs enable the creation of multi-organ platforms featuring an array of tissues all derived from a single donor.

5. Limitations in organoid vascularization and future directions

While significant progress has been made in generating vascularized organoids, there is still much work to be done to advance human tissue engineering. The importance of generating next-generation vascularized organoids stems from its transformative potential within the entire field of organoid technology and their breadth of biomedical application. The creation of perfusable, functionally vascularized organoid systems on microfluidic chips marks the emergence of a new era in biomedical and human tissue engineering. Such advanced models bridge the gap between traditional *in vitro* cell culture and *in vivo* studies, providing a more physiologically relevant platform for investigating disease

mechanisms, tissue development, or drug responses. Flow in microfluidic cultures is not only expected to enhance organoid growth, maturation and lifespan compared to conventional static approaches [123,190], but it is also a valuable tool for disease modeling. The development of new technologies, such as microfluidic devices and bioprinting, and the use of computational models and synthetic biology in cellular differentiation and organoid development [191,192]) may help to overcome these limitations and enable the creation of more accurate and complex vascularized organoids in the future.

Organoid-on-chip technology has garnered considerable interest for its potential to replicate the intricate complexity of human biology within miniature systems. However, as the biological relevance of these systems increases, so does their complexity, posing significant challenges in scalability and practical application. Such limitations hinder our ability to generate the extensive data required for accurate clinical predictions and deeper understandings of mechanistic processes that underpin human health and disease. To address these issues, researchers must transition away from time consuming traditional imaging techniques to automated imaging systems and adopt higher-throughput (i.e. not classic single-unit) devices [193].

The scalability of vascularized organoids is another critical area. While current approaches have enabled the generation of small, vascularized organoids, the ability to create larger and multiple organoids would enable more extensive studies and is essential for future clinical translation. One potential approach to scaling up vascularized organoid production is the use of bioreactor systems, which could provide the necessary nutrient and oxygen supply to large scale productions [194].). Bioreactor systems could also be used to study the effects of mechanical forces, such as fluid shear stress, on vascularized organoids [195–197]. Finally, the development of standardized protocols and quality control measures for vascularized organoid production is critical for advancing the field. Moreover, many current protocols rely on Matrigel, which not only elevates the cost of organoid cultures but also introduces batch-to-batch variability and the risk of immunogen transfer, thereby limiting potential clinical applications. Synthetic, chemically defined hydrogels present a promising alternative to Matrigel, potentially overcoming these limitations [92]. As the use of vascularized organoids becomes more widespread, it is important to ensure that the constructs are reproducible and consistent across different laboratories. Standardized protocols and quality control measures could also help to address issues of variability and reduce the potential for experimental artifacts. Further, the establishment of perfused vessels in organoids provides immense opportunities in the study of arteriovenous specification and the effect that this critical, yet largely unknown process, has on tissue development and organogenesis. Advances such as these are critical for the continued development and use of vascularized organoids as tools for use in drug screening, regenerative medicine, and disease modeling.

6. Conclusions

The inclusion of a vascular compartment in organoid models remains imperative in attempts to more effectively mimic the *in vivo* environment and enable more accurate studies of complex physiological processes. Various approaches have been developed to generate vascularized organoids, including the use of self-organizing systems, co-culture techniques, and bioprinting technologies. However, these approaches have limitations that need to be addressed to enable the generation of more accurate and complex vascular networks.

The importance of administering flow and perfusion inside the lumens of vascularized organoid constructs cannot be overstated. These mechanical forces are essential for maintaining the viability and functionality of the vascular network and mimicking the *in vivo* environment. Advances in microfluidic systems and flow perfusion techniques have enabled the study of the effects of mechanical forces on vascularized organoids and may lead to the development of more sophisticated models that faithfully represent human organs or cancer tissues (Table 1).

Overall, the field of vascularized organoids holds great promise for advancing our understanding of disease mechanisms, drug discovery, and regenerative medicine. Continued research and development in this area will be critical for realizing this potential and enabling the translation of vascularized organoids from the laboratory to the clinic (see graphical abstract).

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.The blood vessel organoid technology has been licensed to Stemcell Technologies. J.M.P. is a founder and shareholder of Angios Biotech that develops blood vessel organoids as vascular transplant therapy.

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