Development of an in vitro vascular network using zebrafish embryonic cells

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Chapter 2

Beyond organoids: *in vitro* vasculogenesis and angiogenesis using cells from mammals and zebrafish

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Abstract

The ability to culture complex organs is currently an important goal in biomedical research. It is possible to grow organoids *in vitro* and these small tissue masses can undergo a degree of histogenesis. However, a major limitation of organoids, and other 3D culture systems, is the lack of an *in vitro* vascular network. As a result, the growth and function of the organoid is constrained by the limited diffusion of nutrients, oxygen and growth factors. Protocols have been developed for establishing vascular networks *in vitro*, and they typically use human or rodent cells. The zebrafish is emerging as a complementary model to mammals in this regard. Its general advantages in developmental research and embryonic cell culture, and its specific advantages for studying vascular development (such as transgenic lines expressing green fluorescent in vascular cells) makes it of potential importance for developing *in vitro* vascular networks. The culture of functional (perfused) vascular networks is currently a major technical challenge. In this rapidly advancing field, some microfluidic devices are now getting close to the goal of an artificially perfused network in culture. One major hurdle is the need to sustain a microfluidic connection with a living vascular network that is growing rapidly in size, and therefore has an increasing demand for fluidic flow over time. In this review, we discuss the culture of endothelial cells and vascular networks from mammalian cells, and examine the prospects for using zebrafish cells for this objective. We also look into the future and consider how vascular networks *in vitro* might be successfully perfused using microfluidic technology.

Key words: Angiogenesis; *In vitro* vascular network; Microfluidics; Organ engineering; Vasculogenesis; Zebrafish

Introduction

In multicellular animals, nutrients and oxygen are carried by the cardiovascular system, and diffuse directly into the tissues [1]. Similarly, waste products are removed from the tissues by the same system. This allows the tissues to grow and develop into functional organs [1]. The cells in a living tissue are within 100-200 μm range of a blood capillary [2]. This is important for the survival of the cells as the oxygen and nutrients cannot diffuse through the tissue beyond this range [3]. A blood supply (vasculature connected to a pump) has therefore evolved to overcome the constraint on growth imposed by limited diffusion.

One area where blood vessel development is relevant is the tissue engineering for regenerative medicine and organ transplantation [4]. Currently, the lack of vascularization of tissues *in vitro* is a major hurdle in reaching this objective [5, 6]. This is unfortunate because cultured, vascularized tissues could not only have clinical applications [4], but could also be used as an alternative to whole animal models in research [7]. There are currently great efforts directed towards growing cells and tissues from a patient's own body (autologous transplantation), in order to overcome the potential danger of allogenic (from another individual) graft rejection, and graft-versus-host reactions [8, 9].

With current tissue culture techniques, tissues cannot be grown more than 100-200 μm in thickness, primarily because of the limited diffusion of nutrients and oxygen [10]. Tumor cells grown in non-adherent culture can develop into spherical masses (spheroids) up to 3mm in diameter, typically with a core of cells that are dead or dying due to diffusion limitation [11]. Similarly, masses of normal (non-malignant) cells grown *in vitro* are called organoids, and are currently the focus of great interest in biomedical research because they show some organization of tissues resembling *in vivo* organs [12]. We believe that the development of an *in vitro* vascular network could improve the culturing of spheroids and organoids, by allowing the tissues to grow and function in a way that is closer to the *in vivo* situation [13].

Other applications of vascular network culture could be fundamental studies of vascular development [14]; recapitulating disease conditions such as the retinal microvascular abnormalities seen in diabetes [15] or the abnormal angiogenesis in tumor development [16]; testing anti-angiogenic compounds in cancer research [17] or candidate drugs for their safe clinical application [18]; and studies in vascular regenerative medicine [19] (Figure 1).

It has long been known from the field of human and animal surgery, including transplant surgery, that tissue can become re-vascularized when grafted to a suitable site [20, 21]. Similarly, developmental studies have shown that embryonic tissues can also readily become re-vascularized, and continue to grow into functional organs, when transplanted to various locations in the embryo [22]. Furthermore, embryonic organ primordia can

become vascularized if transplanted not only to the embryo itself, but to the vascular network in the extra-embryonic membranes. A good example of this is the chicken embryo chorioallantoic membrane (CAM) system [23, 24]. In that model, organ primordia are placed onto the highly vascular CAM, the blood vessels first having been scratched to open them up. The organ primordia can then form a vascular connection with the CAM vessels, and undergo reasonable growth and morphogenesis. The CAM, however, is highly sensitive to environmental factors [25], therefore the development of the tissue graft is not perfect, possibly because it is not submerged in a supporting volume of fluid, but rather is exposed to the air. In a sense, therefore, the CAM and other developmental systems show that the growth of organs on vascular beds is a possibility. What is needed, however, is a vascular bed *ex vivo* that is perfused by some kind of microfluidic system.

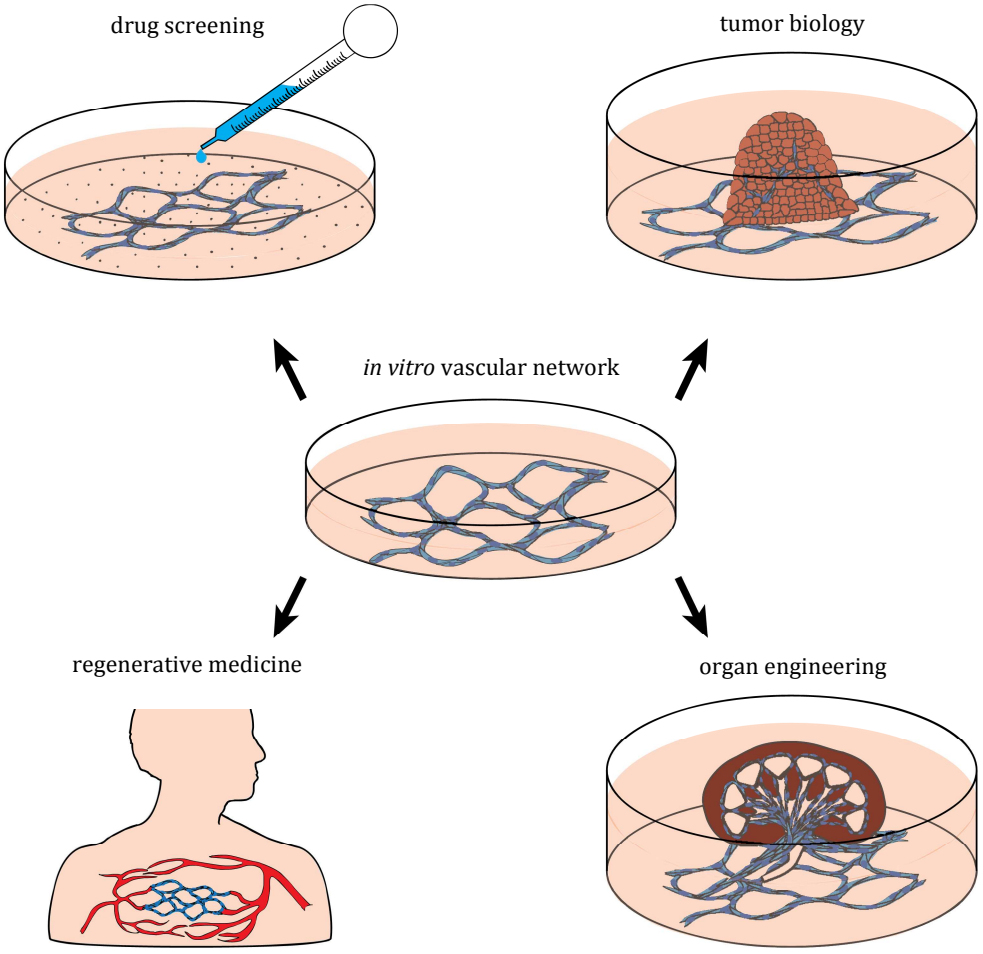

Figure 1: Potential applications of a vascular network culture.

Most of the current research describing vasculogenesis (de novo formation of blood vessels from progenitor cells) and angiogenesis (formation of blood vessels from existing blood vessels) uses mammalian models, mainly mice. However, these models are fairly expensive, time consuming and require ethical and other permissions [26]. Endothelial cell lines such as human umbilical vein endothelial cells (HUVECs) are commonly used for developing *in vitro* vascular networks [27]. Other sources for developing such cultures include embryonic or adult stem cells or tissue explants. The uses and limitations of these techniques are discussed in detail in the following sections.

Zebrafish can be an alternative to mammalian models [28]. The zebrafish produce a large number of fertilized eggs at low cost; the embryos are externally fertilized and therefore readily accessible for experiments [29]. In some jurisdictions, zebrafish embryos has fewer ethical restrictions. For example, in the European Union, the Directive 2010/63/EU on the protection of experimental animals allows zebrafish embryos to be used until 5 days post fertilization (dpf) without restriction [30]. Finally, the zebrafish genome has been sequenced and there is a high level of conservation between zebrafish and human protein coding genes [31]. This similarity supports the use of zebrafish to model various human diseases [32, 33]. Because of these advantages, the zebrafish is currently emerging as a model species to study vasculogenesis and angiogenesis *in vivo* [28]. Transgenic reporter lines are proving very useful in these studies [28].

In this review we give a general overview of vascular development *in vivo* and the role of various factors in the development of vasculature. Then, we review the current procedures used to culture vascular networks using mammalian endothelial cells and tissue explants. Then, we review the use of zebrafish to study various aspects of vasculogenesis and angiogenesis *in vivo*. Finally we look forward by summarizing the potential use of zebrafish model for *in vitro* studies of vascular development.

Development of vasculature *in vivo*

Formation of a vascular system is an essential process in embryonic development. Because multicellular tissues cannot survive without a blood supply, the cardiovascular system is one of the earliest systems formed during embryogenesis [34, 35]. The endothelial precursor cells (angioblasts) differentiate into endothelial cells and undergo the process of vasculogenesis in early embryos to form the primitive blood vessels [36]. Studies on zebrafish have shown that the angioblasts appear in the lateral mesoderm, migrate to the midline of the embryo and form the first blood vessels [37]. In adult mice and humans, endothelial progenitor cells reside in the bone marrow as multipotent adult progenitor cells, and contribute to the formation of new blood vessels [38].

Further development of blood vessels takes place by the extension of the pre-existing vascular network through the process of sprouting and non-sprouting angiogenesis [39].

During angiogenic sprouting, some endothelial cells within the existing blood vessel are selected as tip cells, and migrate in the direction of angiogenic stimuli [40]. The surrounding extracellular matrix is degraded by specific proteases released during the process [41]. Meanwhile, the stalk cells (endothelial cells following the tip cells) proliferate to extend the blood vessel [40]. Further in development the vascular network also extends through intussusceptive or non-sprouting angiogenesis [42]. The mature blood vessels attain arterial, venous and lymphatic differentiation types having different structures and functions [43]. Endothelial differentiation and blood vessel formation is a complex process which requires a number of growth factors, cell types and extracellular matrix (ECM) components, discussed in the following section.

Factors controlling vasculogenesis and angiogenesis *in vivo*

Exogenous protein factors influencing vascular development

The differentiation of endothelial cells and the formation of blood vessels is mainly controlled by several protein factors [44]. Some of these factors are released by the endothelial cells themselves, other factors are stabilizing signals released by other cell types [44]. The differentiation of angioblasts is induced mainly by fibroblast growth factor 2 (FGF2) and bone morphogenic protein 4 (BMP4) [43]. FGF2 induces the expression of vascular endothelial growth factor (VEGF) and other important chemokines required to control vascular morphogenesis [45]. The importance of FGF2 for vascular formation has been shown in studies on quail and zebrafish embryos [46, 47]. Similarly, BMP4 deficiency is associated with severe abnormalities in early mouse embryos, including the lack of a well-organized vasculature [48].

Among the endothelial growth factors, VEGFs play the predominant role in regulating the formation of blood vessels [49]. The VEGF family consists of several VEGF genes of which VEGF-A, which interacts with endothelial cells through VEGF receptor 2 (VEGFR2 also known as KDR or FLK1), is the main component responsible for the viability and proliferation of endothelial cells [50]. VEGFs also have important roles in the differentiation, migration and cell-cell adhesion of endothelial cells, as well as stimulating sprouting angiogenesis and the activation of tip cells [51]. Placental growth factor (PlGF), a member of VEGF family expressed in the placenta of early mammalian embryos, has a role in the activation of VEGFR2 and establishing interaction between VEGF-A and VEGFR2 [52]. PlGF has been demonstrated to increase the angiogenic potential of VEGF in ischemic myocardium in mouse [53]. PlGF expression is normally low in adult tissues, but high in pathological conditions, especially in cancer, where it promotes tumour angiogenesis [54].

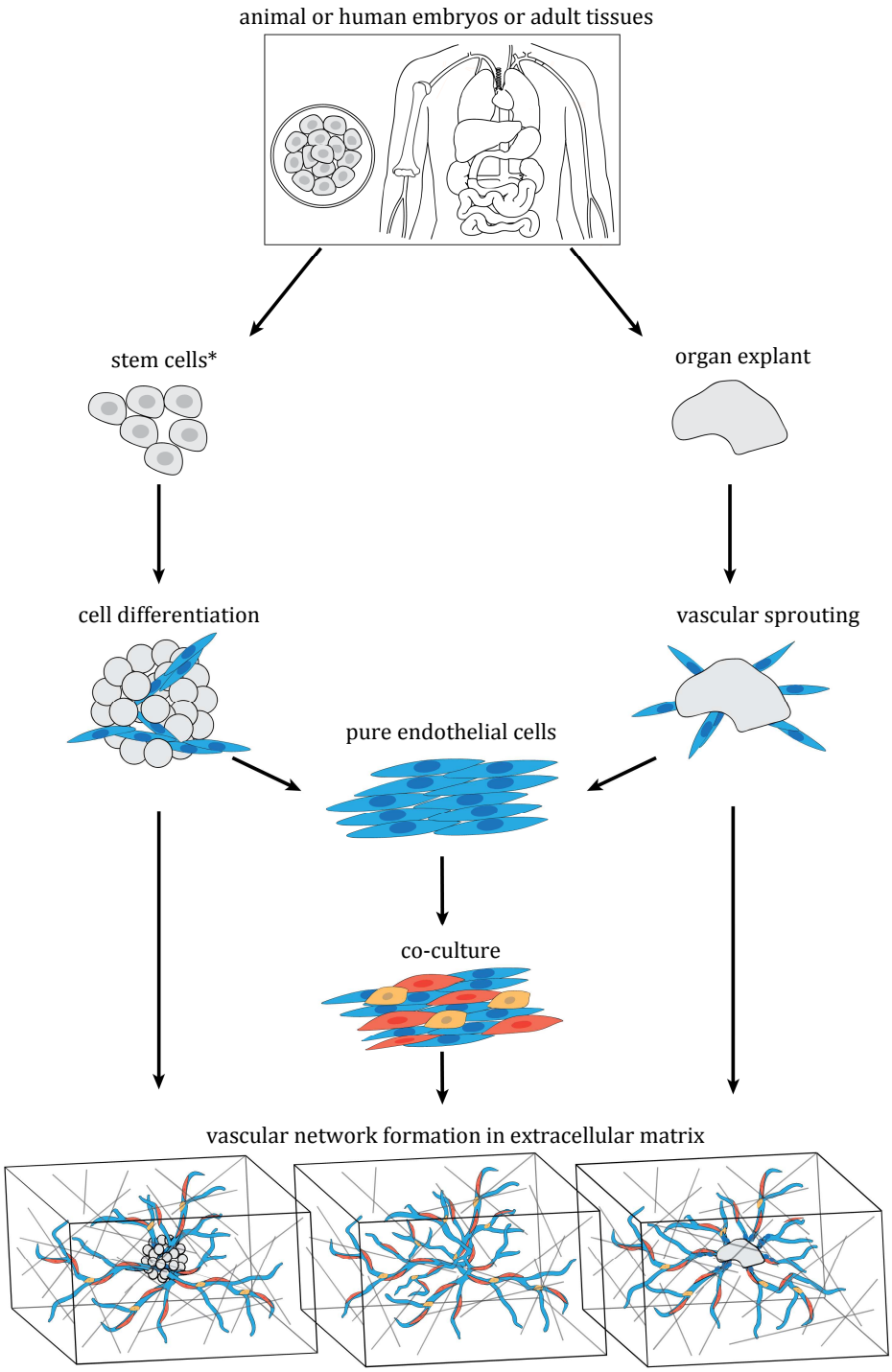

Figure 2. Schematic overview of three possible approaches to establishing cultures of vascular networks. *Stem cells, depending on their source, could be embryonic stem cells, mesenchymal stem cells, or induced pluripotent cells. Endothelial cells are in blue; diverse supporting cells are represented schematically by red and yellow.

Other growth factors involved in the spreading and maturation of blood vessels include angiopoietins (Ang-1 and Ang-2) [55], platelet-derived growth factor-B (PDGF-B) [56] and transforming growth factor β (TGF-β) [57], reviewed in Refs. [44, 49]. Many other transcription factors and signalling molecules have been identified to be involved in the differentiation of endothelial cells and the regulation of vascular development reviewed in Ref. [58]. In response to low oxygen levels in the tissues, Hypoxia inducible factors (HIFs) regulates the expression of a number of pro-angiogenic factors including VEGF, PlGF, Ang-1, Ang-2 and PDGF-B [59]. The HIFs are considered to be the principle mediators of *in vivo* vasculogenesis and angiogenesis at all developmental stages [59].

Role of membrane proteins and other cell types on vascular development

Membrane proteins on the surface of endothelial cells also play an important role in vascular morphogenesis. Examples of these membrane proteins include vascular endothelial cadherin (VE-cadherin) which functions to maintain endothelial cell-cell contact during VEGF-induced migration [60], epidermal growth factor like domain-7, which facilitates the formation of endothelial tubes [61], and delta like ligand-4 which specifies the tip cells for sprouting angiogenesis [62].

In addition to the soluble and bound protein factors, cell types other than endothelial cells also contribute to the formation of blood vessels. Pericytes and smooth muscle cells promote the proliferation and survival of endothelial cells and provide structural support to the blood vessels [63, 64]. Macrophages are reported to be involved in connecting two blood vessel sprouts in the process called anastomosis [65]. Under certain conditions (e.g. hypoxia), the parenchymal cells (neurons, hepatocytes, myocytes etc.) release angiogenic growth factors to initiate sprouting angiogenesis [66].

Role of extracellular matrix

Extracellular matrix (ECM) contributes to the formation and diversity of blood vessels in several ways including: (i) maintaining the histological structure and elasticity of the vessels, (ii) regulating the proliferation and differentiation of endothelial cells, and (iii) transporting, modifying or blocking the angiogenic growth factors [67]. The ECM is a complex network of macromolecules and its composition and properties are highly variable among different tissues, affecting the tissue specific differentiation of stem cells [68]. Research on the ECM of blood vessels have shown the presence of different ECM components at different stages of vascular development [69]. In the beginning of the process, the endothelial cells adhere to and migrate on a laminin-rich ECM which is later replaced by a collagen type-I rich ECM to support vascular tube formation [69].

Haemodynamic factors

Shear stress generated by blood flow on the luminal surface of endothelial cells is a mechanical factor that induces intracellular biochemical pathways resulting in gene expression changes and the modulation of the structure and function of blood vessels [70]. Heparin binding EGF-like growth factor is one such factor which is expressed in response to reduced blood flow and induces vessel narrowing [71]. Other molecular pathways involved in vascular remodelling are reported to be regulated by changes in shear stress leading to the expression of PlGF [72], Notch1 [73], and Smad6 (involved in TGB-β signalling) proteins [74].

Microfluidic culture of endothelial cells is currently an emerging technology which mimics the physiological shear stress on cultured cells to achieve the goal of culturing functional blood vessels for tissue engineering [13, 75]. A number of techniques for culturing vascular networks have been described in which endothelial growth factors, ECM components and microfluidics are combined; however, the development of fully functional blood vessels still remains a challenge [75].

Culture of vascular networks using endothelial cells

Pure endothelial cell populations can develop into vascular network-like structures in culture [27]. However, these networks are not sufficiently robust to be used for tissue engineering; they are mainly used to screen pro- and anti-angiogenic compounds for activity. Pure endothelial cell populations are derived from various sources including embryonic stem cells, induced pluripotent stem cells and adult tissues [76] (Figure 2). Human macro- and micro-vascular endothelial cells are commercially available (http://www.promocell.com/products-/human-primary-cells/) and have the ability to form vascular networks *in vitro*. The most commonly used endothelial cells in this regard are the HUVECs [27, 77-87], derived from the veins of the umbilical cord (Table 1). Other endothelial cell types such as bovine aortic endothelial cells [88] and rat aortic endothelial cells [89] have also been used to culture vascular networks.

By contrast, the culture of well-defined vascular networks with a lumen requires the co-culture of multiple cell types with endothelial cells [76]. The important supporting cell types, known to induce network formation by endothelial cells, include pericytes [90], mesenchymal stem cells [85], fibroblasts [79], hepatocytes [91], smooth muscle cells [92] and adipose-derived stem cells [78]. The importance of fibroblasts in enhancing angiogenesis has been shown in co-culture with HUVECs [81, 93]. The ECM components and angiogenic growth factors secreted by fibroblasts have been found to be critical for vascular tube formation from HUVECs [94].

Table 1. Endothelial cell cultures for vascular morphogenesis.

Interacting Cell types	Culture strategy	ECM Substrate	Medium additives	Main outcomes	Possible applications	Ref.
HUVECs hES-MCs	Static 3D	Col-1 + Fbn	DMEM/F12, VEGF, bFGF, HGF	hES-MC and HGF stabilize vascular network	Drug screening, regenerative medicine	[85]
HUVECs NHLF HPP	Flow 3D	Fibrin	EGM-2	Perfusable 3D vascular network	Drug screening; tissue engineering	[79]
HUVECs ASCs	Static 3D	Fibrin	EGM-2	Vasculogenesis is more stable in co-culture with ASC	Tissue engineering	[78]
HUVECs HDF	Static 2D		M199, ECGS, CS	HDF and bio-active silicate stimulate network formation	Tissue engineering	[81]
HUVECs	Flow 3D	Col-1	EGM-2	Directional neovessels growth in response to angiogenic signals	Drug screening	[80]
HUVECs HBMSCs	Static 3D	Col-1 + Laminin	EGM, DMEM	Laminin and HBMSCs promote vascular network formation	Tissue engineering	[84]
HUVECs MSCs	Static 3D	Col-1 + fibrin	EGM-2, DMEM	Increasing fibrin concentration increases vascular morphogenesis	Tissue engineering	[86]
HUVECs	Static 2D	BME	EGM-2	Vascular network formation	Drug screening	[27]
HUVECs HBVP	Flow 3D	Col-1	M199, ECGS, VEGF, bFGF	The vascular cells show an inflammatory response when stimulated	Modelling vascular diseases	[82]
HUVECs SF	Static 3D	Fibrin	EGM-2, VEGF, bFGF, Ang-1, TGF-β	vessel sprouting, lumen formation and vessel stability is regulated by different factors	Developmental studies	[83]
HUVECs HBMSCs OB	Flow 3D	Fibrin	EGM-2MV, VEGF, Ang1	Tumor cells introduced through the vessels escaped into the surrounding matrix	Drug screening, cancer research	[95]
HUVECs	Flow 3D	POMaC	EGM-2	The pre-formed vascular network allow the growth of tissues and show physiologically relevant responses	Drug screening, organ-on-a-chip technology	[87]
HMVECs	Flow 3D	Col-1	EGM-2MV, VEGF	Metalloproteinases modulate the length and diameter of vessels in 3D ECM	Developmental studies, tissue engineering	[96]
BAECs	Static 2D	Matrigel™	DMEM, VEGF, rGAL-8	GAL-8 promotes endothelial cell migration and capillary formation	Cancer research	[88]

Interacting Cell types	Culture strategy	ECM Substrate	Medium additives	Main outcomes	Possible applications	Ref.
RAECs	Static 2D	Matrigel™	DMEM, VEGF, roxarsone	Roxarsone promotes vascular formation *in vitro*	Cancer research	[89]

Abbreviations: Ang-1, angiopoietin-1; ASCs, adipose-derived stem cells; BAECs, bovine aortic endothelial cells; bFGF, basic fibroblast growth factor; BME, basement membrane extract (Trevigen); Col-1, collagen type-I; CS, calcium silicate; DMEM, Dulbecco's modified Eagle's medium; ECGS, endothelial cell growth supplement (Promocell); ECM, extracellular matrix; EGM, endothelial growth medium (Lonza or Promocell); EGM-MV, endothelial growth medium microvascular; F12, Ham's F-12 medium; Fbn, fibronectin; Flow, microfluidic flow-through culture; HBMSCs, human bone marrow-derived mesenchymal stem cells; HBVP, human brain vascular pericytes; HDF, human dermal fibroblasts; hES-MCs, human embryonic stem cell derived mesenchymal cells; HGF, hepatocyte growth factor; HMVECs, human dermal microvascular endothelial cells; HPP, human placental pericytes; HUVECs, human umbilical vein endothelial cells; M199, medium 199 (Lonza); MSCs, mesenchymal stem cells; NHLF, human normal lung fibroblasts; POMaC, poly(octamethylene maleate (anhydride) citrate); RAECs, rat aortic endothelial cells; rGAL8, recombinant galectin-8; SF, skin fibroblasts; Static, static replacement culture; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor.

The culture of vascular networks is usually established in synthetic matrices which mimic natural ECM; these matrices are particularly valuable for promoting successful vascular morphogenesis *in vitro* [97]. Examples of these matrices are Matrigel™ [98], Puramatix™ [99], collagen type-I [80], fibrin [78] and poly(ethylene glycol) hydrogel [100]. Combinations of these gel components can be used to mimic the complexity of natural ECM. The addition of laminin to collagen type-I scaffolds has been shown to increase network formation and VEGFR2 expression by HUVECs in culture [84]. Similarly, increased network formation from HUVECs was observed in composite collagen type-I/fibrin matrix compared to pure collagen [86].

Studies in which vascular networks are cultured from endothelial cells have revealed the important role of several cellular and molecular factors. In one study, adipose-derived stem cells (ASCs) were found to enhance vascular network formation from HUVECs in 3D fibrin gels [78]. That study showed that the expression of angiogenesis related genes (VE-cadherin, VEGFR2) and proteins (VEGF, FGF, Ang-1) was higher in ASCs/HUVECs co-culture compared to pure HUVECs culture [78]. The role of VEGF has been shown in specialization of the tip cells and directional migration of human micro vascular endothelial cells to form capillary-like structures [101]. Similarly, inducing the Notch signalling pathway upregulated VEGF-A, VEGF-B and VEGF-R1 expressions, and promoted vascular network formation in co-cultured mouse brain microvascular endothelial cells and ASCs in 3D collagen type-I gel [102].

Limitations of endothelial cell culture

Endothelial cell cultures are relatively easy to maintain. However, there are certain limitations which need to be considered while carrying out endothelial culture. A blood vascular network constitutes a number of vessel types, from large vessels to micro vessels,

and each vessel type has its own unique properties (including endothelial cell subtypes, as discussed below). Therefore it is challenging to attempt to recapitulate the formation of different vessel types using a homogeneous endothelial cell population [103]. Primary endothelial cell cultures are usually derived from terminally differentiated tissues; these cells have limited proliferative and regenerative capacity, and a short life span *in vitro* [104, 105].

Endothelial cells derived from different sites within the same tissue express different genes and respond differently to the same pro- or anti- angiogenic factors [106]. This supports the idea of functional subtypes among endothelial cells. Endothelial cells can be immortalised; however, this may change their behaviour and response to stimuli [107]. Immortalised endothelial cells may alter their gene expression and physiological properties with repeated passaging *in vitro*, resulting in loss of vasculogenesis efficiency [107]. The non-endothelial cell types that support *in vitro* vascular network formation from endothelial cells (e.g. fibroblasts), may represent an undesirable cell type if the resultant tissue is to be used for tissue engineering [76].

Use of stem cells for *in vitro* vasculogenesis

In recent years, stem cells have increasingly been used to develop vascular cultures; this is because stem cells have several advantages over terminally differentiated endothelial cells [19]. Stem cells are multipotent or pluripotent in nature, they show self-renewal, and their differentiation along various cell lineages can be manipulated by fine-tuning the culture conditions [19]. A few examples of the stem cells that can be used for endothelial, and ultimately vascular, differentiation are summarized in Table 2. Three main stem cell types used are: (i) embryonic stem cells (ESCs) [108], (ii) induced pluripotent stem cells (iPSCs) [109] and (iii) mesenchymal stem cells (MSCs) [110].

In addition, endothelial progenitor cells (EPCs), which originate in the bone marrow and contribute to the formation of new blood vessels in adults, are also useful in the study of *in vitro* vasculogenesis [105]. The differentiated endothelial cells arising from these stem cells directly undergo vasculogenesis because of the presence of other cell types that have also differentiated from the stem cells; alternatively, the endothelial cells can be isolated from the stem cell culture, without the unwanted additional cell types, and used for vascular morphogenesis (either in pure culture or co-culture with defined cell types) [111].

One of the advantages of using ESCs is that they can differentiate into multiple vascular cell lineages simultaneously in culture. In principle, these different lineages can contribute to the newly-formed vessels (neovessels) in a way that closely resembles the *in vivo* vasculogenesis in early embryos [19, 112]. Endothelial differentiation and vascular morphogenesis in ESCs is controlled by culture conditions (such as the presence of growth

factors in the medium and the use of feeder layers of stromal cells, or a substratum consisting of a synthetic hydrogel [112, 113]).

One approach to inducing the differentiation of ESCs in culture is to allow them to first aggregate into spherical cell masses, called embryoid bodies (EBs), in suspension culture [114]. The use of EBs as an intermediate step is common when ESCs are cultured for vascular differentiation (Table 2) [115]. In the absence of anti-differentiation factors (e.g. leukaemia inhibitory factor in mouse and feeder cell layer in human), ESCs differentiate into EBs consisting of mesodermal, ectodermal and endodermal lineages, similar to early embryogenesis [116]. In 2D (adherent) cultures the EB cells tend to proliferate and give rise to undesired cell types such as fibroblasts [114]. By contrast, in 3D culture (suspension or gels), the proliferation of EB cells is limited, allowing greater control of the differentiation of the desired cell type [114]. Significant effects of different factors, such as culture substrate (collagen type-IV or fibronectin), cell seeding density, concentration of VEGF and FGF in medium, and culture duration, have been observed on the endothelial differentiation in human, mouse and zebrafish ESC culture [117, 118]. Similarly, TGF-β has been identified to induce vascular differentiation in human ESCs [119].

Another important stem cell type, similar to ESCs in pluripotency and differentiation events, is the iPSCs [120]. An advantage of iPSCs is that they can be generated by genetic reprogramming of any adult somatic cell population, and therefore raise fewer ethical concerns compared to ESCs [19]. Endothelial differentiation in iPSCs can be induced by applying similar methods used for differentiation of ESCs [121]. Furthermore, gene expression in endothelial cells derived from ESCs and iPSCs is very similar [121]. MSCs are multipotent stem cells residing in adult tissues; they have limited differentiation potential compared to ESCs and iPSCs [19]. Endothelial differentiation in human amniotic fluid derived MSCs has been shown to be inducible by VEGF [110]. MSCs derived from various tissues (bone marrow, hair follicle, adipose tissue and muscles) have been used for vascular regeneration studies reviewed in Ref. [19]. In some studies the MSCs have been reported to promote and stabilize vascular network formation from HUVECs (Table 1).

In addition to the use of pluripotent and multipotent stem cells for endothelial differentiation and *in vitro* vasculogenesis, the unipotent EPCs also have the ability to differentiate into mature endothelial cells and form vascular tubes in culture [105]. The advantage of EPCs for culturing vascular networks is that these cells can be easily obtained from adult tissues such as peripheral blood [19]. *In vitro* studies have shown that the early EPCs do not directly undergo vascularization, but release factors to stimulate angiogenesis in distantly-cultured endothelial cells in a transwell [105]. Co-culture with MSCs has been proven to enhance vascular formation from EPCs both *in vitro* and after implantation *in vivo* [122, 123].

Table 2. The use of stem cell technology for endothelial differentiation and vascular development.

Stem cell type	Culture strategy	ECM Substrate	Medium additives	Main findings	Possible applications	Ref.
mESCs	EB static 3D	Col-1	IMDM, EPO, VEGF, bFGF,	RSK and TTK protein kinases modulate vascular formation	Drug screening, cancer research	[108]
mESCs	EB static 3D	Matrigel™	αMEM, VEGF	Reporter proteins in vascular cells allow track of vascular development	Developmental studies	[124]
mESCs	Static 3D	Col-1	IMDM, VEGF, bFGF	EB formation and angiogenic sprouting	Drug screening	[125]
mESCs	Static 2D	Gelatin	DMEM, VEGF	Endothelial differentiation, vascular network formation	Developmental studies, Drug screening	[126]
mESCs	EB static 2D	Col-1	DMEM, VEGF	VEGF receptors are involved in tip cell selection and sprouting	Developmental studies	[127]
mESCs	EB static 3D	Col-1	IMDM, EPO, VEGF, bFGF	Culture strategy, ECM substrate and growth factors effect vascular differentiation	Drug screening	[128]
hESCs	Static 2D	Matrigel™	EGM-2, BMP4	BMP4 increases vascular differentiation	Developmental studies, angiogenic therapy	[129]
hESCs	Static non-adherent		Knockout DMEM	Spontaneous endothelial differentiation and Vascular sprouting	Tissue engineering, regenerative medicine	[130]
miPSCs	EB static 3D	Col-1	DMEM, VEGF	TP73 gene regulate endothelial differentiation and vascular network formation	Cancer research	[131]
hiPSCs	Static 2D	Fbn	IMDM, VEGF, bFGF	Formation of vessel-like structures	Regenerative medicine, organ culture	[132]
hAFSCs	Flow 2D	Matrigel™	EGM-2	Shear stress promotes endothelial differentiation and vascular cord formation	Regenerative medicine	[133]
hAFSCs	Static 2D	Matrigel™	EGM-2, VEGF	EGM and VEGF promotes endothelial differentiation and vascular formation	Tissue engineering, angiogenic therapy	[110]
hTMSCs	Static 3D	Fibrin	EGM-2	TMSCs promote and stabilize vessel formation from endothelial cells	Regenerative medicine, tissue engineering	[134]

Abbreviations: αMEM, alpha-minimal essential medium (Cellgro); bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein-4; Col-1, collagen type-I; DMEM, Dulbecco's modified Eagle's medium; EB, embryoid body intermediate; ECM, extracellular matrix; EGM, endothelial growth medium (Cambrex or Clonetics); EPO, erythropoietin; Fbn, fibronectin; Flow, under flow of medium; hAFSCs, human amniotic fluid-derived stem cells; hESCs, human embryonic stem cells; IMDM, Iscove's modified Dulbecco's medium; mESCs, mouse embryonic stem cells; miPSCs, mouse induced pluripotent stem cells; RSK, ribosomal S6 kinase; Static, static replacement culture; TP73, tumor protein-73; TTK, threonine and tyrosine kinase; VEGF, vascular endothelial growth factor.

Issues and drawbacks with stem cell culture

Although stem cell technology has several advantages for vascular engineering and regenerative therapy, there are some limitations to its use [19]. Thus, while ESCs have been extensively studied in laboratory animals such as mouse and rats, and stable cell lines have been developed form these animals, the technique has been proven less successful for other species such as cattle, goat and dogs [120]. Furthermore, the very complexity of vascular differentiation from ESCs means that the growth factors necessary to support the generation and maintenance of multiple cell types need to be laboriously optimised [135]. Furthermore, the use of human ESCs for research raise ethical concerns [19].

ESCs and iPSCs are both pluripotent, and therefore it is challenging to direct the differentiation towards a specific lineage, and to obtain high quality pure cell cultures [120]. The iPSCs are developed by transfection of somatic cells with pluripotency genes; however, the efficiency of the process is very low (less than 1%) [136]. The iPSCs (in contrast to ESCs) are derived from adult differentiated cells by de-differentiation. Then, if re-differentiated into a specific cell type, they attain some of the characteristics of that cell type but are not identical to their normal counterparts [136]. Other issues with stem cells is that the isolation of MSCs from adult tissues requires invasive surgical procedures, and only yields small numbers of cells; the proliferation of these cells is also limited *in vitro* [137]. Furthermore, the MSCs isolated from different tissues or life stages are not the same, and therefore have different culture requirements and angiogenic potentials [138].

The isolation and culture methods for EPCs are only relatively recently developed (Asahara *et al.,* 1997 [139]). For this reason, there is no standard protocol among researchers. It should be noted that there are no specific markers for EPCs because many of the genes expressed by EPCs are also expressed in hematopoietic progenitors [140]. Similar to the iPSCs and MSCs, the number of EPCs found in isolated adult tissue cells is very low, and this greatly limits their study [19]. Finally, analysis of EPCs in long-term culture has shown that the late passage cells (45 days after the initiation of the primary culture) have changed morphology, reduced their proliferation rate, show high β-galactosidase expression and loss of vascular network formation ability, compared to the early passaged cells [141].

Use of tissue explants for *in vitro* angiogenesis

An important strategy for vascular morphogenesis *in vitro* is to stimulate the growth of the blood vessels existing in isolated sections or fragments of specific tissues [107]. The development of a well-defined blood vascular network requires the incorporation of multiple cell types both *in vivo* and *in vitro*, as discussed in the previous sections. The use of tissue explants is important in this context, because these explants already contain multiple cell types, and the angiogenesis stimulated in these cultures closely represents the corresponding process *in vivo* [107]. Furthermore, tissue explant experiments are relatively easy to perform and allow a large number of cultures to be derived from a single tissue sample [142].

Various tissue explants have been shown to have the ability to develop vascular sprouts *in vitro* (Table 3). Examples include cross sections of aorta called aortic rings [143]; metatarsal bones [144]; retina fragments [145]; choroid-sclera fragments [146]; and adipose tissue [147]. In most cases, the tissues for explant preparation are isolated from developing rodent embryos or neonates. Tissue explants from other species such as chick embryo aortic arch [148], rabbit aorta[149] and pig carotid artery [150] have also been adapted for sprouting angiogenesis. Furthermore, angiogenic sprouting has also been reported from human tissue explants e.g. adipose tissue [147], aortic explants from aborted embryos [151], placental explants [152, 153] and umbilical artery rings [154].

Explant cultures are usually established in a 3D gel matrix in the presence of angiogenic growth factors, and are examined for microvessel outgrowth (vascular sprouting)[107, 155]. The aortic ring model from various species is the most commonly used explant for studying *in vitro* angiogenesis (Table 3). The stimulatory effect of various factors, such as angiogenic growth factors (especially VEGF) and ECM components, on the growth of vascular sprouts from aortic ring have been extensively studied, reviewed in Ref. [156]. Recently developed explant cultures, using fetal metatarsals from mice, have shown advantages over the aortic ring model, in that they do not require a 3D matrix and exogenous growth factors for vascular sprouting [144]. In general, explant cultures can serve as an intermediate between the endothelial cell culture on the one hand, and *in vivo* models on the other. They are also thought to be more reliable for studying the mechanisms of angiogenesis and testing the role of regulatory factors [157].

Limitations of explant cultures

Besides the advantages of explant cultures, certain limitations need to be addressed before the technique can be fully accepted for research in tissue engineering and regenerative medicine. The mouse aortic ring model shows significant variability in microvessel sprouting from explants isolated from different age and strain of animals [158]. Variability in outcome has also been reported using explants isolated from different vessel types (artery or vein) of the same individual animal [159]. The vascular sprouts in

the aortic ring model regress over time in culture (with peak sprouting between days 6 and 7), and this limits the analysis time and increases variability in results with culture duration [143]. Furthermore, the aortic rings are derived from large vessels, and therefore do not truly represent *in vivo* angiogenesis, which is a microvascular process [107].

Table 3. Tissue explants used for sprouting angiogenesis *in vitro*.

Tissue explant	Culture strategy	ECM Substrate	Medium additives	Main findings	Possible applications	Ref.
mAR	Static 3D	Col-1	MCDB131, VEGF	Age of the mouse inversely affects vascular sprouting from explant	Drug screening	[160]
mAR	Static 3D	Col-1	ESFM	Endostatin inhibits vascular sprouting by modulating endothelial cell-ECM interaction	Cancer research	[161]
mAR	Static 3D	Col-1	Opti-MEM, VEGF	VEGF and collagen increase vessel sprouting	Drug screening	[143]
rAR	Static 3D	Matrigel™	EGM-200	Ascorbate inhibits sprouting angiogenesis	Cancer research	[162]
hUAR	Static 3D	BME	EGM-2	Capillary spouting upon VEGF stimulation	Drug screening, cancer research	[154]
cAA	Static 2D	Matrige™	bFGF, VEGF	Chemical compound releasing nitric oxide inhibits angiogenesis	Cancer research, drug screening	[163]
mAT	static 3D	Col-1	MCDB131, VEGF	Angiogenic sprouting	Drug screening	[164]
hAT	Static 2D	Matrigel™	EBM-2, EGM-2 MV	Angiogenic capacity reflects donor's physiology	Drug screening, angiogenic therapy	[147]
mRE	Static 2D	PTFE	DMEM, VEGF	VEGF stimulate vascular sprouting	Drug screening	[165]
mRE	Static 3D	Fibrin	DMEM, VEGF	VEGF stimulate sprouting angiogenesis	Drug screening	[166]
mMT	Static 2D	Gelatin OR Col-1	αMEM	Vascular sprouting occurs without additional growth factors	Developmental studies, Drug screening	[144]
mMT	Static 2D		αMEM, VEGF	VEGF recover the impaired vascular sprouting in endoglin deficient explants	Modelling vascular diseases, drug screening	[167]
mPE	Static 2D	Col-1	M199, VEGF, bFGF	Isoforms of VEGF differentially stimulate vascular sprouting	Developmental studies	[168]

Abbreviations: αMEM, alpha-minimal essential medium; bFGF, basic fibroblast growth factor; BME, basement membrane extract (BD Biosciences); cAA, chick aortic arch; Col-1, collagen type-I; DMEM, Dulbecco's modified Eagle's medium; EBM, endothelial basal medium (Lonza); ECM, extracellular matrix; EGM MV, endothelial growth medium microvascular; EGM-200, endothelial growth medium-200 (Cascade Biologics); ESFM, endothelial serum-free medium (Life Technologies); hAT, human adipose tissue explant; hUAR, human umbilical arterial ring; M199, medium 199 (Gibco); mAR, mouse aortic ring; mAT, mouse adipose tissue explant; MCDB131, basal medium (Invitrogen); mMT, mouse metatarsal explant; mPE, mouse proepicardium explant; mRE, mouse retinal explant; Opti-MEM, minimal essential reduced-serum medium (Gibco); PTFE, polytetrafluroethylene membrane; rAR, rat aortic ring; Static, static replacement culture; VEGF, vascular endothelial growth factor.

Tissues containing microvascular networks (e.g. adipose tissue and retina) can be used for explant preparation. However, these tissues are more difficult to isolate and, like the aortic explant, show variability between experiments [145]. The high levels of capillary sprouting observed in adipose tissue explant cultures are in many senses an advantage; however they do make it difficult to identify all the sprouts individually and interpret the results [147]. Similarly, angiogenic sprouts from fetal mouse metatarsal explants present microvascular features; however, their isolation and culture procedures also require advanced technical skills, which are key to the reproducibility of the research [144]. Finally, the metatarsal and chick aortic arch explants are isolated from developing embryos and have high proliferative capacity; therefore, angiogenesis in these models does not represent the *in vivo* situation in adults [107].

Zebrafish: a new model species for studying *in vivo* vasculogenesis and angiogenesis

The zebrafish is a freshwater teleost fish [169] that is emerging as a model of choice for studying vasculogenesis and angiogenesis [170]. The embryos and larvae are often used in these studies because of their external fertilization, optical transparency at early stages, and the ease of exposure to test substances (by simply adding the compound to the swimming water) [171]. Furthermore, the genome comparison study has revealed that there is at least one orthologue in zebrafish genome for more than 70% of human protein coding genes [31]. Vascular development and function in zebrafish are relatively conserved, compared to the same processes in other vertebrates [170].

The embryos develop a simple vascular system with circulating blood as early as 24 hours post fertilization (hpf) [37]. Vascular development can be directly observed non-invasively in the living, transparent embryo [28]. Enhanced visualization of vascular development can be achieved by injecting fluorescent micro particles into the blood stream, or by using transgenic lines such as *kdrl:GFP* and *fli:GFP* that express green fluorescent protein (GFP) in vascular cells [28].

For these and other reasons, vascular development in zebrafish — from early differentiation of angioblasts to the maturation of blood vessels — has been extensively

studied [32, 37, 172, 173]. Studies have shown similar angiogenic responses to the test substance irisin, in zebrafish embryos *in vivo*, and in HUVECs *in vitro* [174]. In another example, the genetic mutation (gridlock), which causes aortic malformations and congenital heart defects in humans, showed similar phenotypic effects in zebrafish [175]. Zebrafish have been successfully utilized to model several human vascular diseases reviewed in Ref. [32].

Similarly, a zebrafish *in vivo* xenograft model has been developed to study human carcinomas [176]. These studies have shown successful invasion, metastasis and extravasation of various human tumor cells in zebrafish embryos and adults [176]. It has been demonstrated that the transplantation of human WM-266-4 melanoma cells and breast adenocarcinoma cells in zebrafish embryos induced angiogenesis in the host vasculature; this led to the formation and infiltration of neovessels into the tumor masses [177, 178]. Other examples of human carcinomas studied in zebrafish include breast cancer bone metastasis [179], uveal melanoma [180] and retinoblastoma [181].

The zebrafish possesses remarkable regenerative capacity in several organs (including the caudal fin and heart [182, 183]) which makes it a useful model for studying regeneration [184]. The regeneration of organs also involves the regeneration of blood vessels, and therefore the regenerative capacity of zebrafish is also important for vascular regeneration studies [28].

Zebrafish transgenic reporter lines for vascular studies

Several transgenic lines have been developed for zebrafish which express fluorescent proteins under vascular cell specific promoters [28]. These transgenic lines allow the tracking of the differentiation, proliferation and migration of individual cells during vascular development *in vivo* and *in vitro* [28]. Moreover, different transgenes can be combined in the same embryo, as was done with a line (scl-PAC:GFP) expressing fluorescent proteins in both endothelial and blood cells; that line permitted the observation of the development of the vascular system and blood flow simultaneously [185].

The most important transgenic line that we are utilizing to study vascular development in zebrafish is the *kdrl:GFP* line (Figure 3A). This transgenic line is also known as *Tg(kdr:eGFP)* or *Tg(flk1:eGFP)* [186]. In this line, GFP is specifically expressed in endothelial cells under the control of the VEGFR2 or *kdr-like* gene [186]. The *kdrl:GFP* line allows high resolution analysis of single cell migration and vascular development in living embryos [186]. The utility of *kdrl:GFP* zebrafish embryos has been confirmed as a high-throughput toxicology screening model [187].

Other transgenic zebrafish lines are available for vascular studies, although they have some limitations. For example, In *Tg(Tie2:eGFP)* the GFP expression is relatively weak in the vascular cells [188]. Similarly, in *Tg(fli1:eGFP)* the GFP is expressed in certain non-

vascular cells which interferes with the results especially in the head region of the embryo [186]. Furthermore, studies on *Tg(fli1:eGFP)* have shown changes in the gene expression of a number of genes, compared to the wild type embryos, which may affect the results while using transgenic zebrafish for experiments [189].

In summary, zebrafish is a high-throughput, easily quantifiable, fast developing and relatively inexpensive *in vivo* model for vascular studies. However, there are some drawbacks associated with this model. The relevance of zebrafish embryo model to understand human angiogenesis is questioned, as there is a large evolutionary time difference between the two species [157]. Therefore, preclinical drug screening in zebrafish should always be followed by validation in mammalian models before going to clinical trials [28].

Future prospects for using zebrafish cells for *in vitro* vasculogenesis and angiogenesis

In principle, many of the techniques discussed in the previous sections for *in vitro* vasculogenesis and angiogenesis (using mammalian endothelial cells, ESCs and tissue explants), can also be adapted for use in the zebrafish model. With the availability of primary embryonic cells due to high fecundity of the species, and easy cell isolation procedures, the drawbacks associated with adapted cell lines can be avoided [190]. In addition to the above mentioned characteristics, zebrafish also possess specific desirable features for *in vitro* applications.

Cell culture techniques in zebrafish

The external fertilization and large number of fast developing embryos, allow easy harvesting of large numbers of cells and quantities of tissues from different developmental stages [191]. Zebrafish cells grow at a lower temperature (26-28 °C) than chick and mouse cells and do not usually require a CO_2-enriched atmosphere [191]. These properties allow zebrafish cells to be grown at room temperature, although the use of a simple incubator is recommended to help maintain sterile conditions [191]. The protective covering of the chorion, which is present until hatching at around 48 hpf, partly isolates the embryos from the environment [192]. This is important for *in vitro* studies because it maintains the embryos in an aseptic condition [193].

To harvest sterile cells or tissues from zebrafish embryos it is necessary to decontaminate the surface of the chorion. Using this approach, it is possible to isolate and culture sterile cells from blastula (3 hpf) or gastrula (24 hpf) stage embryos [191, 194]. In a recent study, we have shown that embryos with a chorion decontaminated at 24 hpf could be further cultured to 5 dpf under aseptic conditions [155]. The tissues and cells isolated from these embryos were successfully maintained free of contamination for eight days in culture.

Zebrafish embryonic stem cells

As is the case with mouse and human ESCs, it is possible to maintain zebrafish ESCs in a pluripotent state in long-term culture [195]. In zebrafish and medaka, the ESCs can be derived from the embryo before the blastocyst stage; these cells may possess a higher degree of pluripotency (and even totipotency), compared to mammalian ESCs derived from blastocysts [196]. When differentiation inhibition factors are depleted in the culture medium, the zebrafish ESCs have the ability to undergo differentiation into a range of specialized cell types [194]. The spontaneous differentiation of zebrafish ESCs into neuron-like cells, muscle-like cells, embryonic carcinoma-like cells and fibroblast-like cells has been demonstrated [191, 194, 197]. However, little is known about the condition needed to induce specific differentiation in ESCs from zebrafish.

The induction of myogenic differentiation in zebrafish primary ESCs has been shown by culturing these cells on a laminin substratum, in medium containing insulin [198], FGF [199] or sonic hedgehog protein [200]. In another study, the seeding density (between 1 and 2 × 10^4 cells/cm^2) of zebrafish primary ESCs, co-culture with a zebrafish fibroblast-like cell line (ZF4) and medium supplementation with insulin, were found to induce cardiomyocyte differentiation [201]. Similarly, an increase in the generation of cells resembling primordial germ cells was found in zebrafish blastocyst cell cultures after the addition of BMP4, EGF and retinoic acid to the medium [202]. Furthermore, the use of FGF and VEGF have been shown to increase differentiation towards the endothelial cell lineage in zebrafish blastocyst cells [203].

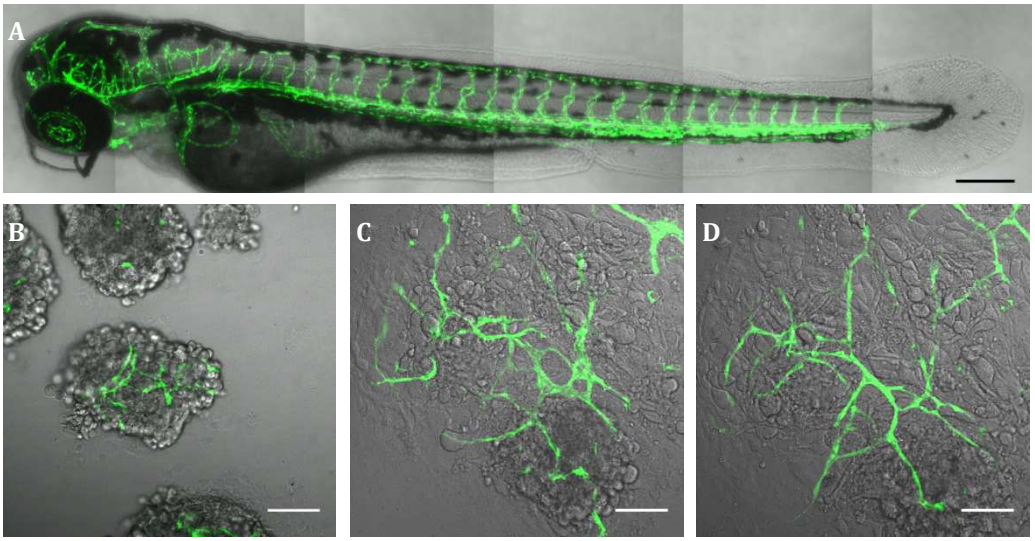

Figure 3: Confocal images of transgenic zebrafish *kdrl:GFP* whole embryo (A) and EB culture (B-D). (**A**) A 5 dpf *kdrl:GFP* embryo showing florescent endothelial cells forming blood vessels. Scale bar, 200 µm. (**B, C and D**) Developing embryoid body on subsequent days of culture (day 1, day 6 and day 8, respectively) on a mixture of collagen type-I, Geltrex™ and fibrin substratum, showing the development of vascular network-like structures from *kdrl:GFP*$^+$ cells. Scale bars, 100 µm.

Zebrafish blastocyst cells aggregate into EBs in culture. We have shown that the percentage of endothelial-like cells in EBs is increased by culturing them in suspension (hanging drop culture) rather than in adherent culture, and by adding endothelial growth supplements, including VEGF, to the medium [118]. We found that the endothelial cells in EB cultures form vascular network-like structures on hydrogel substrates (Figure 3B-D).

Cultures of specialised zebrafish cell types

Attempts have been made to develop cultures of specialised cell types using progenitor cells isolated from embryos. In one study, neural crest cells, isolated from dissociated 14 hpf (10-somite stage) zebrafish embryos, were maintained in culture [204]. These cells were able to proliferate, migrate and differentiate into neurons, chondrocytes and glial cells in the presence of specific factors [204]. Strategies have also been described for the culture of primary neurons from developing brain and spinal cord cells of zebrafish embryos [205]. We have found that the endothelial cells in dissociated hearts, isolated from 5 dpf zebrafish embryos, form colonies on a fibronectin substratum; however, these cultures could only be maintained for short periods, possibly because of low seeding-density [155].

These studies suggest that zebrafish embryonic cell culture can be an important model for studying endothelial differentiation and vascular morphogenesis *in vitro*. In principle, it could be possible to use zebrafish *kdrl:GFP* embryos for the isolation of endothelial cells using fluorescence activated cell sorting (FACS). The development of these cells into vascular networks in response to various signals could then be readily tracked in live cultures without the need to fix and stain them. Furthermore, tissues and organ explants from zebrafish embryos can be a promising model for sprouting angiogenesis, as we have shown using liver and heart explants from 5 dpf embryos [155].

In summary, the zebrafish allows easy access to large numbers of primary cells, and vascular development in cultures derived from these cells occurs in a complex environment of other cell types. In contrast, it is difficult to access primary cells and tissues in mammalian models, and the vascular culture using endothelial cell lines such as HUVECs does not reflect the complex process *in vivo*.

Disadvantages of zebrafish *in vitro* model

Zebrafish embryonic cell cultures usually combine cells or tissues from a large number of individuals due to the small size of embryos [198]. As a result, the cell population obtained is genetically heterogeneous, and may not be ideal for gene expression analysis [198]. Despite the simplicity of the required culture conditions (i.e. low incubation temperature and no requirement for a CO_2-enriched atmosphere), studies have shown that zebrafish primary embryonic cells require a complex medium containing FGF and fish embryo

extract for their growth [195]. The lower incubation temperature for zebrafish cells may not be ideal for human cells if a co-culture has to be established.

The zebrafish embryonic cells usually have to be cultured on a feeder layer of growth-arrested cells to maintain their pluripotency [193]. The development of vascular network in primary zebrafish embryonic cells and tissues in the presence of other supporting cell types may be considered as an advantages as it closely mimic the *in vivo* situation. However, this provides less control over the *in vitro* vascular development compared to endothelial cell lines. Because the zebrafish is a relatively new research model, the differentiation and culture conditions for its cells still needs to be optimised.

Beyond organoids: microfluidics and the development of a functional vascular network

In this final section we want to look at some future prospects in the field of *in vitro* vascular networks. These networks will always remain of limited value unless they support a functional blood flow. In a living animal, this flow is powered by the heart. But how can blood flow be initiated and maintained in a culture system? One obvious answer is to use microfluidic technologies to pump blood or some nutrient liquid through the vascular network.

Examples of microfluidic vascular culture systems

In one example of vascular culture in a microfluidic system, HUVECs and human lung fibroblasts were co-cultured in a perfused 3D fibrin gel [79]. The networks that developed allowed the transport of nutrients, molecules and cells, and also showed physiological responses to flow-induced shear stress [79]. In the same microfluidic device the authors showed that the presence of flow (regardless of flow direction) facilitates vasculogenesis by HUVECs, and that angiogenic sprouting occurs only in the direction opposite to the flow direction [206]. Similarly, angiogenic sprouting in 3D collagen type-I gel from HUVEC cultures in a microfluidic device showed morphological features resembling *in vivo* angiogenesis [80].

These studies employed a commonly-used microfluidic design containing a middle channel filled with ECM, and interconnected with two side channels which conduct the medium flow (Figure 4A). In this device the vascular network is connected to the media channels through the lumen openings [207]. This allows the vascular network to be perfused by chemical compounds or cells. The device allows rapid quantification of changes in vascular networks in response to test compounds [207]. Using the same design, Jeon et al. have shown the extravasation of cancer cells, introduced through the perfused vascular network, into the surrounding bone-mimicking matrix [95]. In a similar device, human colorectal or breast cancer cells co-cultured with endothelial cells in 3D

ECM in the middle channel have been shown to develop into vascularized tumor aggregates [208]. These tumor-like structures showed reduced growth, and sometimes even regression occurred, in response to standard vascular targeting therapies infused via the microvessels [208].

Another device (Figure 4B) mimics the tissue capillary interface by allowing the culture of endothelial cells on one side, and epithelial cells on the other, of a porous membrane inside a microfluidic channel [209]. An alternative approach is the pre-moulded hollow network (Figure 4C) in a 3D collagen type-I matrix with embedded supporting cells (in this case, human brain vascular pericytes [82]). The HUVECs are seeded into the cavities of cast. In this setup, the HUVECs first form a lining to the cast channels, and then form vascular sprouts into the surrounding matrix [82].

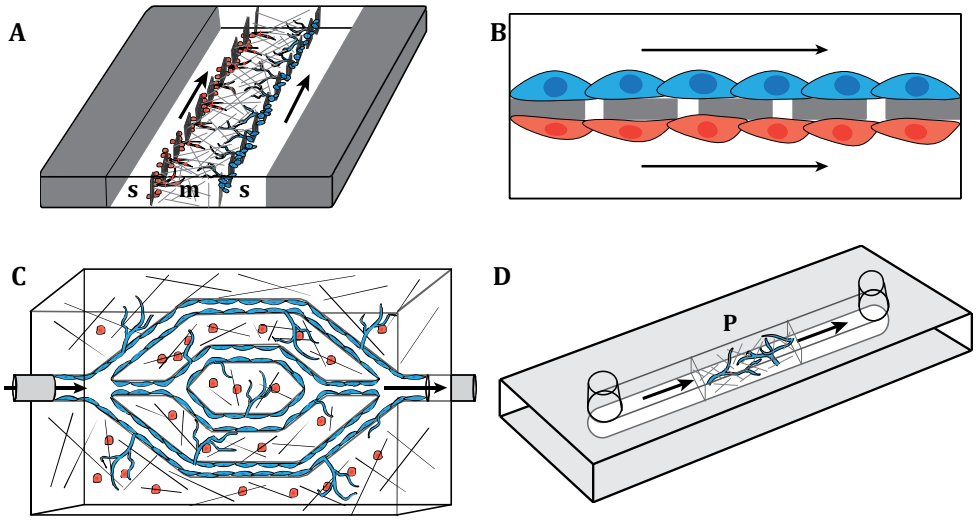

Figure 4. Microfluidic devices commonly used for culturing vascular-like networks. (**A**) Three channel microfluidic device [95, 206]. The middle channel (m) is filled with extracellular matrix with embedded cells. The two side channels (s) conduct the medium flow (arrows). (**B**) Microfluidic device mimicking capillary-tissue interface with endothelial cells on one side and epithelial cells on other side of a porous membrane [209]. The medium flow (arrows) can be established on the endothelial side only, or on both sides. (**C**) In this device, endothelial cells line the built-in channels cut into a hydrogel, with embedded supporting cells [82, 87]. (**D**) A microfluidic channel slide with a 3D matrix plug (P) in the middle of the channel [155]. The medium flows on either side of the plug (a small amount may penetrate the gel by diffusion). Note that (**C**) comes closest to a growing vascular bed connected to the microfluidic system. Arrows showing the direction of the medium flow. For further discussion of microfluidic devices in the field of vascular culture, see Refs. [13, 75, 210].

In a similar device, the luminized vasculature have been developed inside moulded channels in a biocompatible scaffold [87]. The network thus formed was able to vascularize cardiac and hepatic tissues cultured on the outside of the scaffold. The material used for the scaffold in that study was poly(octamethylene maleate (anhydride)

citrate) (POMaC; a biodegrade-able and biocompatible scaffold) [87]. In that setup, the micro pores incorporated into the scaffold allowed the uptake of nutrients, chemical compounds and cells from the vessels, and the release of metabolites into the vessels by the surrounding tissue [87].

In most cases, endothelial cell lines are used to culture vascular networks in a microfluidic system. The stem cells and explant cultures have been very little used in such studies. We have shown that zebrafish EBs embedded in 3D gel matrix, cultured in a microfluidic channel (Figure 4D) formed longer and wider vascular sprouts compared to the EBs cultured in conventional (static) 96-well plates [155].

Technical challenges for the future

As we have discussed above, scientists are beginning to realize the goal of a functional vascular network perfused by microfluidics *in vitro*. However, there are some severe technical challenges to be overcome. Presumably, some kind of synthetic interface or connector will be needed to connect the living vessels with microfluidic system. Another major challenge will be to maintain an increasing blood flow as the tissue explant, attached to the vascular network, grows in size. Thus, the growing tissue will require vessels of increasing diameter, and this in turn will require an expanding connection to the microfluidic system. Solutions to these problems will require intensive research.

Conclusions

In summary, the techniques developed for *in vitro* vascular network formation in mammals are producing rapid advances in our understanding. They can also be applied to zebrafish cells and tissues. For its benefits such as easy access to primary embryonic cells, availability of transgenic lines to visualize endothelial cells and conserved molecular pathways, the zebrafish can be a significant first-step model for studying *in vitro* vascular development. In recent years, microfluidic technology have shown great advances in developing *in vitro* vascular networks. Ultimately, the future of tissue culture and organ culture will be greatly extended if functional vascular networks with a fluidic flow can be grown *in vitro*. The major challenges in establishing such a system would be to recapitulate the key features of vascular system such as barrier function and vasoactivity, as wells as to compensate for the increasing demands of the growing tissue.

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