In vitro models for assessing therapeutic angiogenesis

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25–30-word teaser: A current understanding of *in vitro* models for assessing the angiogenic potential of cell therapy products (CTPs) or other angiogenic agents.

Abstract (100 words)

Arterial obstruction leading to ischemia causes a reduction of oxygen and nutrient supply to distal tissues. The physiological response to tissue ischemia triggers a cascade of events that results in the development of accessory vasculature to increase local tissue perfusion and to salvage tissue. However, this adaptive mechanism of repair is suboptimal in some patients. Therapeutic angiogenesis aims to stimulate new blood vessel formation via the local administration of pro-angiogenic agents or cell therapy products (CTPs). In this review, we provide a summary of the current understanding of *in vitro* models for assessing the angiogenic potential of a product.

Introduction

Vascular diseases, including peripheral artery disease, cerebrovascular disease and coronary artery disease, are commonly caused by progression of atherosclerotic plaques in the arteries, resulting in stenosis and a consequent reduction of distal tissue perfusion [1]. Artery occlusion activates two physiological mechanisms of neovascularization: angiogenesis and arteriogenesis [2, 3]. These processes differ from vasculogenesis, which is a type of vessel growth that occurs during embryogenesis (**Table 1**). Arteriogenesis is activated by fluid shear stress resulting from artery occlusion and involves the remodelling of pre-existing collateral arterioles into large conductance arteries that bypass the occlusion and restore blood flow. Angiogenesis is activated by hypoxia and involves the sprouting of endothelial tubes from pre-existing capillaries through the activation, proliferation, migration, and differentiation of endothelial cells (ECs) (**Figure 1**). Although angiogenesis and arteriogenesis are driven by different mechanisms, they share growth factors, chemokines and proteases that have a role in promoting and redefining these processes [4].

The endogenous process of neovascularization is usually enough to fully restore vasculature conductance [1]. However, this adaptive mechanism of repair is often impaired or insufficient in patients, because of underlying comorbidities and the disease microenvironment. In addition, bone marrow progenitor cell dysfunction can impair neovascularization capacity [5-8]. From a therapeutic point of view, activation of the endogenous neovascularization processes could offer a potential therapy for such patients. Thus, therapeutic angiogenesis aims to overcome the limitations of the natural angiogenic response by increasing the local concentrations of angiogenic growth factors.

Therapeutic angiogenesis for vascular diseases

Recently, new treatment modalities for therapeutic angiogenesis have been explored in the form of recombinant proteins or genes encoding angiogenic factors, and/or progenitor cells that synthesize therapeutic factors that participate in the process of blood vessel formation [3]. Therapeutic angiogenesis using these modalities has been widely examined in different human ischemic conditions, including coronary artery disease or peripheral artery disease [9-12], and also in processes such as wound healing [13, 14].

Protein and gene therapy using proangiogenic growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs), have been explored in both pre-clinical models and clinical trials [3]. Although results from these early studies were promising, larger randomized placebo-controlled studies showed little or no real clinical benefit [15, 16]. This failure might be

because single growth factor therapies are insufficient to stimulate neovascularization in humans. In addition, the poor stability and short half-life of the product *in vivo* and/or low up-take in ischemic target tissues might also contribute.

CTPs (i.e. stem and/or progenitor cells or secreted molecules) offer an alternative approach to therapeutic angiogenesis, that might have advantages including the ability to secrete multiple angiogenic factors. Cells secrete multiple molecules that participate in the angiogenic process at different steps, and also secrete factors, that influence important processes involved in physiological repair (e.g. inflammation or tissue repair). Different CTPs are currently being tested in both pre-clinical and early-phase human studies, including bone marrow-derived mononuclear cells (MNCs), endothelial progenitor cells (EPCs) and mesenchymal stem/stromal cells (MSCs) [3, 12, 17, 18]. These cells have shown pro-angiogenic properties both *in vitro* and i*n vivo*. Nevertheless, some concerns about the use of patient-derived MNCs and EPCs have arisen, especially for autologous transplantation approaches because these cells can have poor angiogenic potential because of disease-induced cell dysfunction [5, 8]. By contrast, MSCs might be a more suitable cell choice in not only allogeneic transplantation approaches because of hypoimmunogenic properties, but also autologous settings, where disease-induced cell dysfunction might be less prominent [19, 20]. The neovascularization capacity of MSCs has been mainly attributed to the secreted therapeutic factors, which have anti-inflammatory, immunomodulatory and pro-angiogenic effects [21, 22]. MSCs secrete a range of growth factors and cytokines, including VEGF, FGFs, angiopoietins (Ang), interleukin (IL)-8, monocyte chemoattractant protein 1 (MCP-1), urokinase-type plasminogen activator (uPA), platelet-derived growth factors (PDGFs), placental growth factor (PLGF), and matrix metalloproteinases (MMPs) [22-24], which are key participants in mechanisms of vascular repair, such as angiogenesis (**Figure 1**) and arteriogenesis [2].

Given that cell therapy can have effects mediated via paracrine mechanisms of action, it has been proposed that conditioned medium (i.e. secreted growth factors and cytokines) represents a cell-free therapeutic approach [22, 25, 26]. Interestingly, recent studies revealed that MSCs secrete other particles with therapeutic abilities, documented as exosomes, that is small membrane bound vesicles (30–100 nm) comprising of a variety of molecules, including lipids, proteins, messenger and mi-RNAs, which are important players in intercellular communication in both physiological and pathological processes. Exosomes have also been shown to have anti-apoptotic, anti-inflammatory, and angiogenic properties [27-30] and could represent a new promising cell-free therapeutic modality for angiogenesis.

Different therapeutic modalities are being tested for therapeutic angiogenesis in both preclinical studies and early-phase clinical trials, but no angiogenic treatment has yet gained market approval. Therapeutic agents are continually under investigation in both *in vitro* and *in vivo* angiogenic assays. In this review, we provide a comprehensive summary of the *in vitro* methods currently used for interrogating the angiogenic potential of CTPs and other therapeutic agents.

In vitro models for assessing the angiogenic potential of CTPs and other therapeutic agents

The angiogenic potential of CTPs or other therapeutic agents (e.g. drugs, growth factors or cytokines) can be assessed using both *in vitro* and *in vivo* assays. *In vivo* models might better reflect the neovascularization process and are vital for observing the efficacy of angiogenic products in living subjects. However, they require technical expertise and are highly complex, time consuming, and relatively expensive [31]. Moreover, neovascularization in the living organism is not an isolated process but involves multiple cell types and different mechanism of vascular repair, which ultimately complicate the understanding of the mechanism of action of a product. Nevertheless, *in vivo* models of angiogenesis are beyond the scope of this article, which focuses on *in vitro* assays.

In vitro angiogenesis assays provide a valuable tool for assessing the effect of angiogenic products [31-33]. They can be quickly and easily carried out using basic techniques and quantitative analysis. Furthermore, they are crucial for investigating the biochemical, cellular and molecular mechanism of action of a product, because they enable the analysis of not only isolated processes that contribute to angiogenesis such as EC migration, proliferation, sprouting, branching, differentiation and lumen formation, but also complex cell–cell and cell–extracellular matrix (ECM) interactions.

EC types for culture assays

ECs *in vivo* have different functions and characteristics depending on the vascular bed (macrovascular *vs.* microvascular), vessel type (artery *versus* vein), species of origin and organ from which they were isolated [33, 34]. The origin of these cells influences the EC phenotype, antigen and cytokine expression, lectin binding [33], MMP secretion [35], anchorage dependency and vascular cell adhesion molecules (VCAM) expression [36]. Therefore, when using *in vitro* angiogenesis assays, it is important to choose EC types that better resemble the *in vivo* condition under consideration.

Macrovascular EC types include human umbilical vein endothelial cells (HUVECs) and human arterial endothelial cells (HAECs). HUVECs are the most commonly used cells for *in vitro* angiogenesis assays. They are easily isolated, robust in culture, highly proliferative and prone to form capillaries. HAECs are isolated from big vessels (i.e. aorta) and can be used for studying pathological processes such as

thrombosis, atherosclerosis, and hypertension. Human microvascular endothelial cells (HMECs) are optimal for tumour neoangiogenesis studies because they are representative of ECs of the surrounding tumour tissues. HMECs should be isolated for the organ in question (e.g skin or lungs) because EC origin can influence their phenotype and functionality. Finally, continuous endothelial cell lines, such as HMECs (HMEC-1) can be used for culture assays; however, their use is not recommended because cell lines normally lose (very dependent on passage number) the native characteristics, phenotypic expression, cell surface markers, and response to an angiogenic stimulus [34].

Although angiogenesis only occurs in microvascular beds, ECs from macrovascular sources (e.g. HUVECs and HAECs) are regularly used in the laboratory because of their to availability and low maintenance costs. Thus, caution must be taken because differences between the lineages of these cells can lead to misinterpretation of results.

Methods for assessing EC migration

During the *in vivo* process of angiogenesis, ECs degrade basement membrane and migrate into the perivascular area in response to an angiogenic chemokine gradient. EC migration in response to this stimulus can be measured using different assays.

The scratch wound assay is a simple, quick (usually takes 8–18 h) and inexpensive method to quantify EC migration. It is based on the ability of EC to fill a cleared area that has been mechanically 'wounded' using a tip, needle, or cell scrapper, on a confluent monolayer of EC [37]. The rate and extent of EC migration towards the 'wounded' area can be monitored microscopically. The distance of each scratch and/or the area of the wound at time 0 are normally compared with that at the last time point, and can be quantified by computing software, such Image Pro-Plus (Media Cybernetics) or the free version Image J (http://rsb.info.nih.gov/ij/). Disadvantages include the difficulty in reproducing scratched areas of equal size, variability among wells and experiments because of the difficulty in maintaining identical conditions of initial cell confluence, mechanical damage of ECs when creating the wound, and difficulty in discriminating net migration events from cell spreading and proliferation [38]. Several modifications of this assay include the ring barrier-based assay [39] and the Teflon fence assay and its modifications [33]. These are based on the introduction of a fence or a barrier in the culture chamber. After cells reach confluence, the barrier is removed and migration into the denuded area is then monitored. These assays are highly reproducible, and overcome the problems of generating identical areas of EC damage. Finally, the electrical wound-healing assay (or electric fence assay) is an automated version of the labor intensive standard assay, which uses an

electrical means to both wound and follow the subsequent healing process. It also produces a highly reproducible wound with a diameter of 250 μm, which closes within a few hours, and enables the study of migration alone. However, it requires the use of the automated Electric Cell-substrate Impedance Sensing (ECIS) instrument (Applied Biophysics), which migth not be universally available [40, 41].

Transfilter assays, or Boyden chamber assays, are useful for studying EC migration towards a stimulus. These assays usually comprise two chambers divided by a polycarbonate filter of specific pore size [42]. This filter is often coated with ECM proteins, such as collagen, fibronectin, or Matrigel, to mimic the *in vivo* environment [43]. ECs are seeded on the upper chamber and culture medium with the angiogenic stimulus is placed in the lower chamber. EC migration through the chamber filter can be investigated using cell staining and manual or digital counting of cells attached to the filter. Migration of ECs can be studied after several hours of incubation up to 48 h later, depending on the cell type and growth rate, the thickness/type of the ECM used, loss of cell viability, or detachment of cells from the underside of the filter. This assay can be used to distinguish between chemotaxis (directional migration towards a stimulus) and chemokinesis (random motility) [33]. However, major disadvantages include technical difficulties in setting up the assay, time-consuming quantification, difficulty in maintaining gradients for prolonged periods of time, an inability to observe cell migratory processes in real time.

Regarding this last issue, several systems have been introduced that enable the real-time monitoring of EC migration. There are two instruments on the market that allows the automated monitoring of EC migration, adhesion, morphology, proliferation, motility, and cell density in real-time. These are the xCELLigence instrument (Roche) [44] and the ECIS Z instrument (Applied Biophysics) [41]. Briefly, cells are grown in special culture chambers covered with opposing, circular gold electrodes. A constant small alternating current is applied between electrodes and the potential across the electrodes is measured. Given that the cell membrane has insulating properties, it creates a resistance towards the electrical current flow, which results in an increased electrical potential between the electrodes. The impedance is calculated from the corresponding voltage change. Hence, this cellular impedance allows the automated monitoring of EC behavior under particular stimuli. Although the set-up of the experiment is simple and fully automatic, the underlying theory is complex and selection of the right settings and correct analysis and interpretation of the data are challenging.

Methods for assessing EC proliferation

The effects of angiogenic molecule(s) on EC proliferation (i.e. the number of cells dividing) can be assessed using different methods [33, 38]. It is often necessary to induce quiescence in ECs before starting an experiment. This can be achieved by serum-starving cells (reducing serum levels to 1%) followed by the re-introduction of full serum with the test substance.

The most simple and inexpensive means of assessing EC proliferation is using the common hemocytometer combined with a cell-viability dye, such as trypan blue. However, this is time-consuming and subject to operator errors. Mitotic cell divisions can be assessed by DNA synthesis quantification. This can be achieved by measuring the incorporation of [^{3}H] Thymidine into the DNA of dividing cells using a scintillation counter, where the amount of radioactivity is proportional to the synthesis to DNA. DNA binding dyes such as BrdU can be also used to quantify DNA synthesis as it incorporates into DNA during S-phase of the cell cycle. Other DNA-binding dyes, including PicoGreen, which detects double-stranded DNA can be also used. Finally, metabolic activity can be measured using colorimetric assays that measure the reduction in a specific compound by living cells as a means of assessing cells number. Nevertheless, in some cases, an increase in metabolic activity might not accurately reflect cell proliferation rates [45]. Therefore, a combination of two or more methods for assessing cell proliferation is strongly recommended for producing reliable results.

Methods for assessing morphogenesis and EC differentiation

2D angiogenesis model. In vitro assays that simulate the formation of capillary-like structures (CLS) can be used to investigate late stages of the angiogenic process. The 2D basement membrane tubule formation assay is one of the most widely used assays for investigating EC morphogenesis. It is a rapid (4–16 h for its completion) and quantitative assay that can be used as a first screen for testing stimulators or inhibitors of angiogenesis. Briefly, ECs are plated onto (or into) a layer of ECM compounds, such as collagen, laminin, fibrin, or most commonly Matrigel, which stimulate the attachment, migration and differentiation of ECs into CLS [46]. Assessment and quantification of CLS differ between laboratories. Usually, experiments require at least three replicates, taking random pictures at three or four sites. Whereas some laboratories measure the amount of tubes per field, others measure the tube length, the number of sprouts or branches, the number of ring structures, or combinations of these measurements [47]. Tubules structures and/or numbers be analyzed manually, or using image analysis programs. While using automated software can considerably speed up measurements, care has to be taken to ensure that a true assessment is being performed. Image analysis programs typically use thresholding to differentiate between tubules, single cells and

background. However, these processes cannot be fairly discriminated, leading to an increase and/or decrease in the true number of tubules [47]. Calcein AM dye can be added to enhance tubule visualization under fluorescence microscopy [46].

Generally, the use of a growth factor-reduced preparation of Matrigel is strongly recommended, because it avoids problems associated with the overstimulation of ECs that occurs in standard Matrigel [47, 48]. In addition, the amount of Matrigel and EC density and the EC type must be optimized because both can influence the formation of CLS [46]. Other non-EC (i.e. fibroblast and cancer cells) have the capability to form CLS on Matrigel, suggesting that tubule formation by ECs does not represent true differentiation [48]. Furthermore, there is some dispute as to whether tubules formed on Matrigel resemble capillaries with a lumen [47]. Nevertheless, the 2D matrigel tubule assay is the most widely used *in vitro* angiogenesis assay because of its simplicity and reliability.

3D angiogenesis sprouting models. Angiogenesis *in vivo* comprises migration of ECs from the confluent endothelium in pre-existing vessels towards a basement membrane, resulting in sprouting of capillaries. This process requires adequate cell–cell and cell–ECM interactions that cannot be mimicked in 2D assays. 3D assays have been developed for assessing sprouting angiogenesis, which closely mimic the *in vivo* situation. These 3D models can be fabricated in a variety of shapes<, including flat-shaped models, spherical-shaped models, or more sophisticated *in vitro* microvessel models.

The common 2D basement membrane assay can be converted into a 3-D assay by using a thicker basement membrane (i.e. collagen type I and fibrin) in which ECs can be mixed or also seeded at different layers positioned at the bottom, middle or top of the 3-D matrix [49]. ECs form CLS with lumen and also migration also occurs both horizontally and vertically [50]. Moreover, fibroblasts cultured under these conditions do not form networks, suggesting that is a specific behaviour of ECs [49].

The 3D scaffold-free cell sheet assay is becoming a powerful model for studying angiogenesis. In this assay, cells (e.g. fibroblasts or myoblasts) are cultured to confluence as monolayers, and then harvested without using proteolytic enzymes, for instance by using thermo-responsive polymer-grafted surface techniques. These polymers have the advantage of allowing easy detachment of cell sheets by changing temperature, in such a way that cell–cell connections and sheet structure is preserved [51, 52]. Cell sheets are then stacked to form a multi-layered sheet, where ECs can be mixed into or placed at different positions (e.g. bottom, middle, or top) as monolayers [53]. This

system allows not only EC migration in both vertical and horizontal planes, but also the formation of a network of mature CLS with lumen across the different planes.

These two systems are powerful tools to evaluate EC behavior such as migration and connection, as well as the formation of endothelial networks in a 3-D construct, and can be used to model *in vivo* angiogenesis after transplantation. Nevertheless, quantification of CLS can be difficult and often requires sectioning and histological assessment, or if possible, photography at multiple focal planes using confocal laser scanning microscopy. Also, care must be taken when designing these constructs, because the thickness of these matrices must be relatively thin to allow appropriate diffusion of oxygen and nutrients.

The microcarrier assay uses spherical scaffolds (made of collagen, fibrin, hyaluronic acid, or other materials) in which ECs are suspended and cultured for 2–4 days. Microcarriers containing ECs are then embedded in an ECM-type matrix. Stimulators or inhibitors of angiogenesis can be added, and basement membrane degradation, EC migration and sprouting can be monitored over time using bright field phase-contrast microscopy or also using immunocytochemistry combined with confocal laser scanning microscopy techniques [54-56]. Image software can be used for quantification of total network length and/or number of branches per spheroid over time. This system also allows real-time monitoring using a microscope adapted with an environmental chamber at 37 ºC and 5% CO_2 [55].

Alternatively, a scaffold-free spherical aggregate of cells, named microtissue, can be generated and embedded within 3D ECM matrix. An example of this is the 'radial invasion of matrix by aggregated cells' (RIMAC) assay, in which radial migration (invasion) of ECs from the microtissue into the collagen matrix occurs as a result of angiogenic stimuli, which can be then quantified using image software [57]. The 'responsive angiogenic implanted network' (RAIN)-Droplet model is based on the encapsulation of ECs in a spontaneously self-assembling, toroidal hydrogel droplet, which yields to preformed angiogenic networks that can be then embedded in 3-D matrices [58]. On embedding, radial growth of capillary-like sprouts and cell invasion is observed. Moreover, sprouts are not only formed as outgrowths from ECs on the surface of the droplet, but also, uniquely, from the pre-formed network structures within the droplet, which better mimic the *in vivo* angiogenesis process.

The advantage of these assays is that they recapitulate most of the stages seen in *in vivo* angiogenesis, including basement membrane degradation, sprouting, alignment, proliferation, tube formation and branching. They are also ideal for interrogating the pro-angiogenic or anti-angiogenic properties of therapeutic agents. However, they require advanced technologies for visualization and quantification

and can be difficult because of the thickness of the 3D matrices or the spherical shape of the constructs.

Although all the above-mentioned 3D *in vitro* models assess sprouting angiogenesis, none of them describe the morphogenetic steps of the angiogenic sprouting process *in vivo* (**Figure. 1**), including cell invasion, multicellular stalk formation, lumen formation, microvessel branching or anastomosis and neovessel perfusion, from native-like vessel structures with luminal flow. Currently, new and more sophisticated *in vitro* 3D models that resemble *in vivo* microvasculature are being developed and are available in a variety of platforms and fabrication methods [59]. These are mainly based on engineered perfusable endothelialized microtubes, which are embedded within ECM matrices. For instance, Nguyen et al. have engineered an elegant *in vitro* 3D biomimetic model of angiogenic invasion and sprouting that originates from preformed artificial vessels [60]. Herein, collagen type I is polymerized in a device that contains two 400 μm-diameter needles. After polymerization, the needles are removed, leaving two cylindrical channels in the matrix. ECs are seeded into one channel allowing the attachment and formation of a confluent monolayer on the interior wall. Proangiogenic factors are added to the opposite channel to establish a gradient across the collagen matrix to the endothelium and induce sprouting. EC sprouts formed here exhibit the typical morphological features of *in vivo* angiogenesis. Sprouts can be monitored in real time using bright field microscopy. Alternatively, immunochemistry and confocal laser scanning microscopy can be used for a more sophisticated analysis. Quantitative metrics include scoring number and length of sprouts and single cell migration using image software [60].

A similar approach was designed by Stroock's group, but using injection molding techniques to generate microchannels within a matrix of collagen [61]. These microchannels are then endothelialized within 1–2 days. The enclosed microstructure has an inlet and an outlet that enable perfusion of the vessels with medium during culture. ECs can be cultured alone or with perivascular cells within the bulk collagen. This system has been used for investigating the angiogenic remodeling process, the interactions between ECs and perivascular cells, and interactions between blood components and endothelium in both healthy and pathological scenarios [62]. This platform enables real-time fluorescence imaging of living engineered tissues, *in situ* confocal fluorescence of fixed cultures and transmission electron microscopy imaging of histological sections.

In vitro microvascular models provide new tools for fundamental and translational studies. These models can be used to understand not only the *in vivo* process of neovessel formation in response to

angiogenic factors [60, 63] but also the response to physical perturbations (e.g. shear stress or pressure) [62-64], the interactions of endothelium with immune cells (e.g. leukocyte adhesion) [65], perivascular cells [62, 65], or cancer cells [66]. Finally, *in vitro* microvessel models can also be used to study endothelial dysfunction and molecular mechanisms of diseases [61, 62].

Practical examples of potential CTPs applied in these *in vitro* models

In general, *in vitro* angiogenesis models can be used to investigate the direct and/or indirect effect of CTPs on EC functions (**Figure 2**). As previously discussed, MSCs appear to be an interesting CTP for therapeutic angiogenesis, and the angiogenic potential of this cell type has been interrogated to a significant extent using *in vitro* angiogenesis models. These *in vitro* assays, in particular, can be used for investigating various effects on EC function, as discussed below:

*The direct effect of stem/progenitor cells on EC function***.**

Duttenhoefer et al. investigated the effect of co-culturing MSCs with ECs in both, 2-D Matrigel assay and a 3-D construct [67]. Here, MSCs alone were unable to form luminal tubular structures. However, in a co-culture system with ECs, MSCs participated in the formation and stabilization of CLS, similar to pericyte-like cells. Interestingly, when EPCs were seeded alone, no pre-vascular network was observed in the 3D construct, indicating a major role of MSCs in directing EPCs towards a more mature EC phenotype able to form pre-vascular networks. In another study, Duffy et al. also showed an active role of MSCs in cellular processes involved in the formation, stabilization, and maturation of tubule networks [68]. Herein, MSCs increased EC proliferation and migration in a non-contact co-culture system. In addition, MSCs co-cultured with ECs in a 2D Matrigel assay stabilized and increased the persistence of pre-existing CLS [68].

The effect of conditioned medium on EC function.

As mentioned above, the paracrine effects of MSCs have been highlighted as important effector mechanisms in promoting blood vessel formation. Many studies have shown that conditioned medium from MSCs stimulate the proliferation, migration and tubule network formation by ECs. This observation has been investigated using some of the afore mentioned *in vitro* assays [22, 24, 25]. Recently, exosomes secreted by MSCs have become an interesting target for therapeutic angiogenesis, and the effect of exosomes on EC function has been investigated using *in vitro* angiogenesis models [30, 69, 70].

*Direct effects of stimuli have on EC function***.**

In vitro angiogenesis models have been used for investigating the process of neovessel formation in response to individual angiogenic factors [60, 63] or to combinations of angiogenic factors, which could have synergistic effects on EC function [56]. In addition, sophisticated 3D *in vitro* microvessel models have been used to investigate the effect of physical perturbations (e.g. shear stress or pressure) on ECs [62-64]. Another example includes the study by Zheng et al., in which a drug (phorbol-12-myristate-13-acetate; PMA) was used to stimulate *in vitro* engineered endothelium to elucidate the *in vivo* mechanisms of thrombosis [62].

Effects of stimuli on the secretome of stem/progenitor cells on EC functions.

Cell pre-conditioning (or cell priming) is a novel approach to enhance some properties of stem and/or stromal cells without genetic modification. In the literature, several priming conditions for pre-treating MSCs have been described. For instance, physiological preconditioning of MSCs via exposure to hypoxia has been shown to increase the expression of prosurvival and angiogenic factors [71] as well as enhancing EC proliferation, migration and the formation of EC tubular structures on a 2-D Matrigel matrix [72]. Preconditioning MSCs with inflammatory stimuli has resulted in enhanced secretion of angiogenic factors [73, 74]. However, these studies did not examine a correlation between secreted protein levels and related EC function using *in vitro* angiogenesis assays. Lopatina et al. showed that treatment of adipose-derived MSCs (ASC) with PDGF stimulated the secretion of extracellular vesicles (e.g. exosomes), changed their protein composition and enhanced their *in vitro* angiogenic potential [70].

Concluding remarks

In vitro angiogenic models provide a valuable tool for assessing the effects of CTPs or other agents on specific EC functions. The field of angiogenesis has matured sufficiently to enable the reconstruction of the complex morphogenetic changes that occur during *in vivo* neovascularization in simpler *in vitro* models. 3D *in vitro* models have many advantages over the 2D assays. Whereas 2D assays allow the study of independent steps in the process of angiogenesis, 3D assays enable the study of complex cell-cell and cell-ECM interactions that are essential for EC migration, proliferation, anastomosis, tube formation and neovessel perfusion. Moreover, sophisticated engineered *in vitro* microvessel models enable the study of the effect of physical perturbations (e.g. shear stress) on the endothelium, mimicking that which occurs during arteriogenesis, or can be used for modeling pathologies and studying molecular mechanisms of disease. Nevertheless, these assays are still

complex and technically challenging, and require advanced technology for fabrication and data analysis. Thus, 2D or simpler 3D *in vitro* assays are still the preferred choice of microvessel model.

To date, an optimal *in vitro* angiogenesis model, that can be easily and rapidly performed, reproducible with multiparameter assessment and easily quantified, has yet to be developed. Therefore, the limitations of *in vitro* assays should be understood, and results validated by using combinations of cell types and *in vitro* assays. Importantly, effects seen *in vitro* should correlate with results observed *in vivo*. Ideally, *in vitro* models should dominate preliminary screening studies, which could then provide a rationale for moving towards more sophisticated *in vivo* experiments, whose ultimate goal is to assess the therapeutic efficacy of specific products before clinical testing.

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References

1. Norgren L, et al. (2007) Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *Eur J Vasc Endovasc Surg*. pp S1–75
2. Buschmann I, Schaper W (1999) Arteriogenesis versus angiogenesis: Two mechanisms of vessel growth. *News Physiol Sci* 14:121–125.
3. Silvestre J-S, Smadja DM, Lévy BI (2013) Post-ischemic Revascularization: From Cellular and Molecular Mechanisms to Clinical Applications. *Physiological Reviews* 93:1743–1802.
4. van Royen N (2001) Stimulation of arteriogenesis; a new concept for the treatment of arterial occlusive disease. *Cardiovasc Res* 49:543–553.
5. Teraa M, et al. (2013) Bone marrow alterations and lower endothelial progenitor cell numbers in critical limb ischemia patients. *PLoS ONE* 8:e55592.
6. Tepper OM, et al. (2002) Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 106:2781–2786.
7. Li T-S, et al. (2009) Identification of risk factors related to poor angiogenic potency of bone marrow cells from different patients. *Circulation* 120:S255–61.
8. Heeschen C, et al. (2004) Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. *Circulation* 109:1615–1622.
9. Ouma GO, et al. (2013) Therapeutic angiogenesis in critical limb ischemia. *Angiology* 64:466–480.
10. Deveza L, et al. (2012) Therapeutic angiogenesis for treating cardiovascular diseases. *Theranostics* 2:801–

814.

11. Ko SH, Bandyk DF (2014) Therapeutic angiogenesis for critical limb ischemia. *Semin Vasc Surg* 27:23–31.

12. Lawall H, et al. (2010) Stem cell and progenitor cell therapy in peripheral artery disease. A critical appraisal. *Thromb Haemost* 103:696–709.

13. Maxson S, et al. (2012) Concise review: role of mesenchymal stem cells in wound repair. *Stem Cells Translational Medicine* 1:142–149.

14. Duscher D, et al. (2015) Stem Cells in Wound Healing: The Future of Regenerative Medicine? A Mini-Review. *Gerontology* 1-13.

15. Kalka C, Baumgartner I (2008) Gene and stem cell therapy in peripheral arterial occlusive disease. *Vascular Medicine* 13:157–172.

16. Belch J, et al. (2011) Effect of fibroblast growth factor NV1FGF on amputation and death: a randomised placebo-controlled trial of gene therapy in critical limb ischaemia. *The Lancet* 377:1929–1937.

17. Botti C, et al. (2012) Autologous bone marrow cell therapy for peripheral arterial disease. *Stem Cells Cloning* 5:5–14.

18. Liew A, O'Brien T (2012) Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia. *Stem Cell Research & Therapy* 3:28.

19. Gremmels H, et al. (2014) Neovascularization capacity of mesenchymal stromal cells from critical limb ischemia patients is equivalent to healthy controls. *Mol Ther* 22:1960–1970.

20. Sanz-Nogués C, O'Brien T (2014) MSCs Isolated From Patients With Ischemic Vascular Disease Have Normal Angiogenic Potential. *Mol Ther* 22:1888–1889.

21. Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. *J Cell Biochem* 98:1076–1084.

22. Kwon HM, et al. (2014) Multiple paracrine factors secreted by mesenchymal stem cells contribute to angiogenesis. *Vascular Pharmacology* 63:19–28.

23. Watt SM, et al. (2013) The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. *British Medical Bulletin* 108:25–53.

24. Burlacu A, et al. (2013) Factors secreted by mesenchymal stem cells and endothelial progenitor cells have complementary effects on angiogenesis in vitro. *Stem Cells Dev* 22:643–653.

25. Timmers L, et al. (2011) Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. *Stem Cell Res* 6:206–214.

26. Ranganath SH, et al. (2012) Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. *Cell Stem Cell 1*0:244–258.

27. Huang L, et al. (2015) Exosomes in mesenchymal stem cells, a new therapeutic strategy for cardiovascular diseases? *Int J Biol Sci* 11:238–245.

28. Lai RC, et al. (2015) Mesenchymal stem cell exosomes. *Semin Cell Dev Biol* 40:82–88.

29. Hu G-W, et al. (2015) Exosomes secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells attenuate limb ischemia by promoting angiogenesis in mice. *Stem Cell Research & Therapy* 6:10.

30. Zhang H-C, et al. (2012) Microvesicles derived from human umbilical cord mesenchymal stem cells stimulated by hypoxia promote angiogenesis both in vitro and in vivo. *Stem Cells Dev* 21:3289–3297.

31. Bahramsoltani M, et al. (2009) Quantitation of angiogenesis and antiangiogenesis in vivo, ex vivo and in vitro - an overview. *ALTEX* 26:95–107.

32. Ngo TX, et al. (2014) In Vitro Models for Angiogenesis Research: A Review. IJ *Tissue Reg* 5:3–45.

33. Bickell R (2006) Angiogenesis assays: a critical appraisal of current techniques.

34. Bouïs D, et al. (2001) Endothelium in vitro: a review of human vascular endothelial cell lines for blood vessel-related research. *Angiogenesis* 4:91–102.

35. Jackson CJ, Nguyen M (1997) Human microvascular endothelial cells differ from macrovascular endothelial cells in their expression of matrix metalloproteinases. *Int J Biochem Cell Biol* 29:1167–1177.

36. Müller AM, et al. (2002) Comparative study of adhesion molecule expression in cultured human macro- and microvascular endothelial cells. *Experimental and Molecular Pathology* 73:171–180.

37. Liang C-C, et al. (2007) In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols* 2:329–333.

38. Staton CA, et al. (2009) A critical analysis of current in vitro and in vivo angiogenesis assays. *International Journal of Experimental Pathology* 90:195–221.

39. Das AM, et al. (2015) A ring barrier–based migration assay to assess cell migration in vitro. *Nature Protocols* 10:904–915.

40. Keese CR, et al. (2004) Electrical wound-healing assay for cells in vitro. *Proceedings of the National Academy of Sciences* 101:1554–1559.

41. Szulcek R, et al. (2014) Electric Cell-substrate Impedance Sensing for the Quantification of Endothelial Proliferation, Barrier Function, and Motility. *Journal of Visualized Experiments*: JoVE.

42. Nicolson GL (2005) Transfilter cell invasion assays. *In: Cell Biology A Laboratory Handbook*., pp 359–362

43. Albini A, Benelli R (2007) The chemoinvasion assay: a method to assess tumor and endothelial cell invasion and its modulation. *Nature Protocol*s 2:504–511.

44. Rahim S, Üren A (2011) A Real-time Electrical Impedance Based Technique to Measure Invasion of Endothelial Cell Monolayer by Cancer Cells. *JoVE* e2792–e2792.

45. Quent VMC, et al. (2010) Discrepancies between metabolic activity and DNA content as tool to assess cell proliferation in cancer research. *J Cell Mol Med* 14:1003–1013.

46. Arnaoutova I, Kleinman HK (2010) In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract. *Nature Protocols* 5:628–635.

47. Smith EJ, Staton CA (2006) Tubule Formation Assays. In: Staton CA, Lewis C, Bicknell R (eds) *Angiogenesis Assays A Critical Appraisal of Current Techniques.* Angiogenesis Assays, pp 65–87

48. Donovan D, et al. (2001) Comparison of three in vitro human angiogenesis assays with capillaries formed in vivo. *Angiogenesis* 4:113–121.

49. Deroanne CF, et al. (1996) Modulation of Expression and Assembly of Vinculin duringin VitroFibrillar Collagen-Induced Angiogenesis and Its Reversal. *Exp Cell Res* 224:215–223.

50. Bayless KJ, Davis GE (2003) Sphingosine-1-phosphate markedly induces matrix metalloproteinase and integrin-dependent human endothelial cell invasion and lumen formation in three-dimensional collagen and fibrin matrices. *Biochem Biophys Res Commun* 312:903–913.

51. Nagamori E, et al. (2013) Network formation through active migration of human vascular endothelial cells in a multilayered skeletal myoblast sheet. *J Am Coll Cardiol* 34:662–668.

52. Haraguchi Y, et al. (2012) Scaffold-free tissue engineering using cell sheet technology. *RSC Adv* 2:2184–2190.

53. Asakawa N, Shimizu T, Tsuda Y, et al. (2010) Pre-vascularization of in vitro three-dimensional tissues created by cell sheet engineering. Biomaterials 31:3903–3909. doi: 10.1016/j.biomaterials.2010.01.105

54. Nakatsu MN, Hughes CCW (2008) An optimized three-dimensional in vitro model for the analysis of angiogenesis. *Meth Enzymol* 443:65–82.

55. Kniazeva E, Putnam AJ (2009) Endothelial cell traction and ECM density influence both capillary morphogenesis and maintenance in 3-D. *AJP: Cell Physiology* 297:C179–87.

56. Kim SK, et al. (2015) Combination of three angiogenic growth factors has synergistic effects on sprouting of endothelial cell/mesenchymal stem cell-based spheroids in a 3D matrix. *J Biomed Mater Res Part B Appl Biomater* 8:1-9

57. Vernon RB, Sage EH (1999) A novel, quantitative model for study of endothelial cell migration and sprout formation within three-dimensional collagen matrices. *Microvascular Research* 57:118–133.

58. Zeitlin BD, et al. (2012) RAIN-Droplet: a novel 3D in vitro angiogenesis model. *Lab Invest* 92:988–998.

59. Bogorad MI, et al. (2015) Review: in vitro microvessel models. *Lab Chip* 15:4242–4255.

60. Nguyen D-HT, et al. (2013) Biomimetic model to reconstitute angiogenic sprouting morphogenesis in vitro. *Proceedings of the National Academy of Sciences* 110:6712–6717.

61. Morgan JP, Delnero PF, Zheng Y, et al. (2013) Formation of microvascular networks in vitro. *Nature Protocols* 8:1820–1836.

62. Zheng Y, et al. (2012) In vitro microvessels for the study of angiogenesis and thrombosis. *Proceedings of the National Academy of Sciences* 109:9342–9347.

63. Song JW, Munn LL (2011) Fluid forces control endothelial sprouting. *Proc Natl Acad Sci USA* 108:15342–15347.

64. Li X, et al. (2015) In vitro recapitulation of functional microvessels for the study of endothelial shear response, nitric oxide and [Ca2+]i. *PLoS ON*E 10:e0126797.

65. Chrobak KM, et al. (2006) Formation of perfused, functional microvascular tubes in vitro. *Microvascular Research* 71:185–196.

66. Zhang Q, et al. (2012) A microfluidic-based device for study of transendothelial invasion of tumor aggregates in real-time. *Lab Chip* 12:2837.

67. Duttenhoefer F, et al. (2013) 3D scaffolds co-seeded with human endothelial progenitor and mesenchymal stem cells: evidence of prevascularisation within 7 days. *Eur Cell Mater* 26:49–64– discussion 64–5.

68. Duffy GP, et al. (2009) Bone marrow-derived mesenchymal stem cells promote angiogenic processes in a time- and dose-dependent manner in vitro. *Tissue engineering Part A* 15:2459–2470.

69. Anderson JD, et al. (2016) Comprehensive Proteomic Analysis of Mesenchymal Stem Cell Exosomes Reveals Modulation of Angiogenesis via Nuclear Factor-KappaB Signaling. *Stem Cells* 34:601–613.

70. Lopatina T, et al. (2014) Platelet-derived growth factor regulates the secretion of extracellular vesicles by adipose mesenchymal stem cells and enhances their angiogenic potential. *Cell Commun Signal* 12:26.

71. Chacko SM, et al. (2010) Hypoxic preconditioning induces the expression of prosurvival and proangiogenic markers in mesenchymal stem cells. *AJP: Cell Physiology* 299:C1562–70.

72. Chen L,et al. (2014) Conditioned medium from hypoxic bone marrow-derived mesenchymal stem cells enhances wound healing in mice. *PLoS ONE* 9:e96161.

73. Crisostomo PR, et al. (2008) Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. *American Journal of Physiology - Cell Physiology* 2015:C675–82.

74. Lee MJ, et al. (2010) Proteomic Analysis of Tumor Necrosis Factor-α-Induced Secretome of Human Adipose Tissue-Derived Mesenchymal Stem Cells. *J Proteome Res 9:*1754–1762.

FIGURES

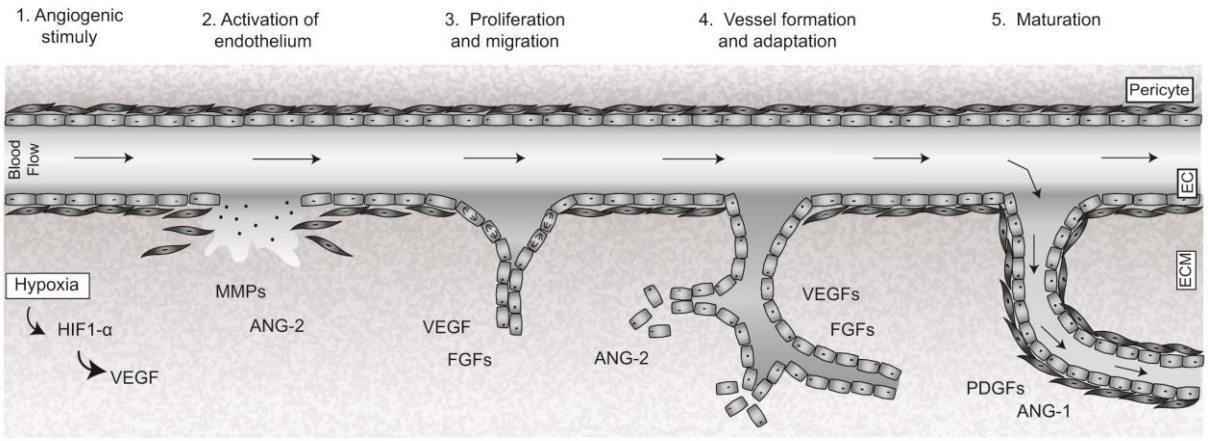

Figure 1. Cellular and molecular steps involved in the process of angiogenesis mediated by hypoxia. The physiological response to the development of profound muscle ischemia includes the upregulation of angiogenic growth factors and mobilization of circulating progenitor cells that enable development of an accessory vasculature. The main steps and factors involved in the process of *in vivo* capillary blood vessels formation are listed. EC–endothelial cells; ECM–extracellular matrix; VEGF–vascular endothelial growth factor; HIF1-α–hypoxia-inducible factor 1-alpha; ANG1 and 2–angiopoietin 1 and 2; FGF–fibroblast growth factor; MMPs–matrix metalloproteinase; PLGF–placental growth factor, PDGF – platelet-derived growth factor.

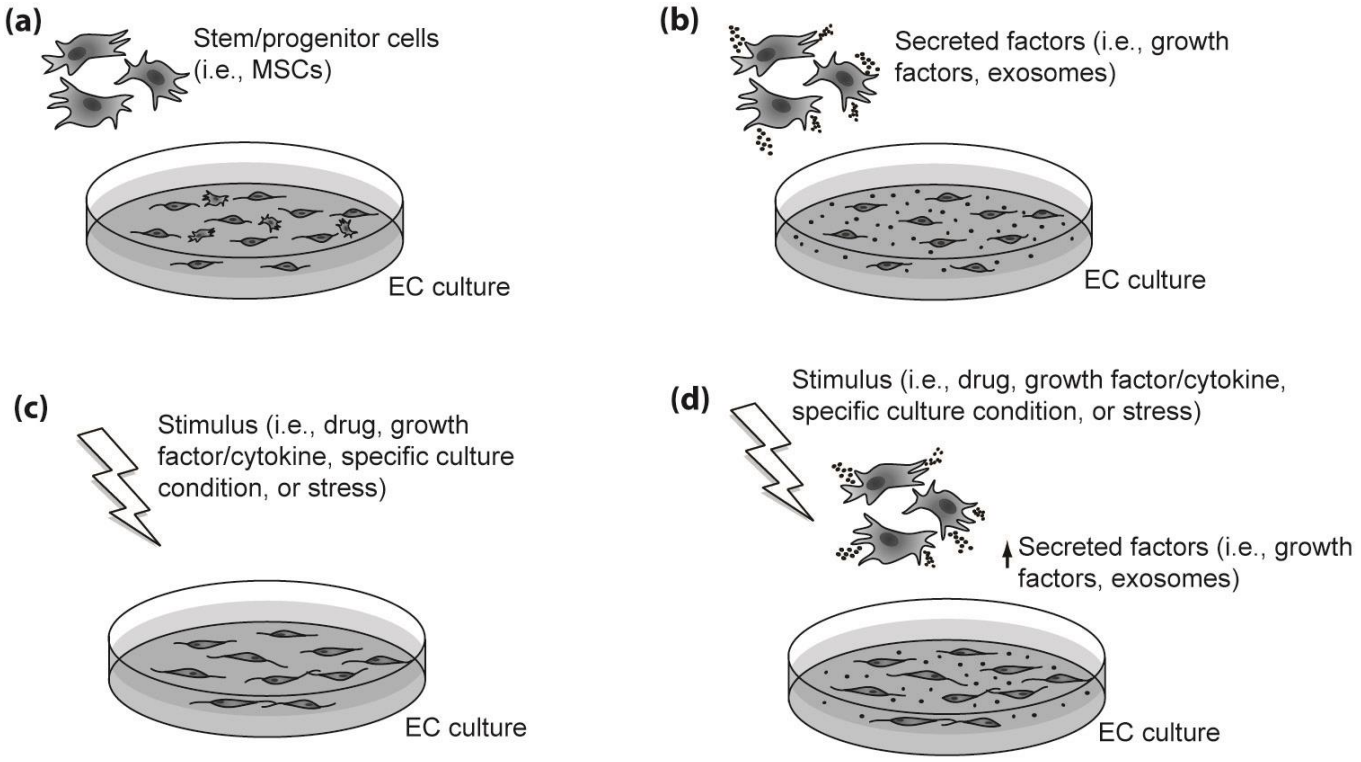

Figure 2. Schematic representation of *in vitro* models to study angiogenic potential of CTPs and other angiogenic agents. *In vitro* angiogenesis assays can be used to study: (A) the direct effect of stem/progenitor cells on endothelial (EC) function; (B) the effect of cell-secreted therapeutic factors on EC function; (C) the effect that a particular stimulus (i.e. drug, growth factor/cytokine, specific culture condition or stress) has on EC functions (direct effect) or D) the effect that a particular stimulus (i.e. drug, growth factor/cytokines, specific culture condition or stress) has on the secretome of stem/progenitor cells, and whether this enhances or decreases EC functions (indirect effect).

Table 1. Definition of the main terminology related to vascular growth.	
Vasculogenesis	*De novo* formation of blood vessels through differentiation of angioblasts (the precursors of EC) during embryo development.
Angiogenesis	Sprouting of small endothelial tubes from pre-existing capillaries through activation and proliferation of ECs. Hypoxia is the main driving force that triggers angiogenesis.
Arteriogenesis	Enlargement of pre-existing collateral arterioles into functional collateral arteries. Fluid shear stress, which occurs after vessel blockage is the primary stimulus.
Neovascularization	Development of new blood vessels, especially in tissues where circulation has been impaired by trauma or disease.
Revascularization	Restoration of blood perfusion to a body part or organ that has suffered ischemia. It is typically accomplished by surgical means. Vascular bypass and angioplasty are the two primary means of revascularization.
Therapeutic angiogenesis	Treating ischemic diseases through the activation of the endogenous process of neovascularization and physiological repair with increasing local concentrations of angiogenic growth factors either administering protein, genes, and stem and/or progenitor cells or secreted products.

Table 2. Summary table of *in vitro* models for assessing the angiogenic potential of cell therapeutic products (CTPs) or other angiogenic ag

Technique	Time	Step of angiogenesis process	Advantages [✓] & disadvantages [x]	Ref.
Scratch wound assays	Few hours - 18 h	EC migration towards a 'wounded' denuded area	√ Quick and inexpensive. × Difficulty in reproducing equal scratched areas and initial cell confluence. × Difficulty differentiating migration from proliferation and spreading. √ Modifications of assay includes the ring barrier-based, Teflon fence and electrical wound-healing assays, which overcome some of these problems.	[37, 39-41]
Transfilter or Boyden chamber assays	9-48 h	EC migration towards a stimulus	√ Distinguish between chemotaxis (directional migration towards a stimulus) and chemokinesis (random motility). × Technical difficulties in setting up assay, time-consuming quantification, difficulty in maintaining gradients for prolonged periods of time and inability to observe real time cell migration.	[42, 43]
Real-time electrical impedance-based techniques	Few hours - 24 h	Real time measurement of several aspects of EC behaviour	√ Easy and automated setting up. √ Allows analysis of different aspects of EC behaviour (e.g. migration, adhesion, proliferation, morphology, motility and cell density). × Complex theory behind the experiment and complex analysis and interpretation of results. × Requires exCELLigence or ECIS technology.	[44, 41]
Quantification of cells, DNA synthesis, or cell metabolic activity	Few hours	EC proliferation	√ Direct cell counting using haemocytometer is simple and inexpensive, × Time-consuming and subject to operator errors. × DNA-binding dyes usually require specific technology (i.e. radioactivity, flow cytometry). × Metabolic activity might not accurately reflect cellular proliferation or cell number. √ Combination of two or more assays is highly recommended.	[45]
2-D basement membrane tubule formation assay	6-18 h	EC morphogenesis	√ Quick, reliable, easy to set up, widely used across laboratories. √ Ideal for screening angiogenic stimulators and inhibitor. × Other non-EC (i.e. fibroblast and cancer cells) have capability to form CLS on Matrigel. × Discrepancies around whether tubes formed in Matrigel resemble capillaries with a lumen.	[46]
3-D flat-shape assay (3-D basement membrane and cell sheet assays)	3-10 d	EC migration, sprouting and differentiation into CLS	√ More closely mimics *in vivo* angiogenesis. √ Migration and tubule formation occurs in vertical and horizontal planes. × Difficulty to observe and quantify CLS requires histological techniques or confocal microscopy. × Thickness of matrix might result in difficulties in diffusion of oxygen and nutrients. × Difficult to handle because of fragility.	[49, 51, 53]
3-D spherical-shape assay (microcarrier or microtissue assay)	4-15 d	Basement membrane degradation and EC migration, sprouting tube formation and branching	√ ECs undergo identical morphological changes during tube formation to *in vivo* angiogenesis. √ Applicable for high-throughput screen of pro- or anti-angiogenic factors × Longer times of incubation × Require advanced imaging technologies and quantification can be difficult	[54-58]
***In vitro* microvessel assays**	Several weeks	EC migration, invasion, proliferation, lumen formation, anastomose and neovessel perfusion.	√ Allows study of endothelium structure and function, inflammation, changes in vessel permeability, response of endothelium to therapeutic factors, physical perturbation (shear stress or pressure), interaction with perivascular cells and cancer cells, and interaction with blood components. √ Allows study of molecular mechanisms of diseases. × Technically complex and challenging, requires advanced technology for its fabrication, time-consuming and difficult to image and quantify.	[60-63, 65]