The branching of life: human iPSC-based angiogenesis-on-chip

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Chapter 1

General Introduction to Angiogenesis

At the end of the 18th century, Dr. J. Hunter, a British surgeon, described in a treatise how the vasculature was found to be enlarged after surgery.[1]* Unknowingly, it is the first registry of the angiogenic process, without explicitly naming it "Angiogenesis". Philosophically, this process – the expansion of the vascular system – is in line with the Aristotelian law of change, in which form adapts, changes, and substances are temporary.[2]* Almost two hundred years after Hunter's observations, in 1971, Prof. J. Folkman, an American surgeon and biologist, transformed the cancer research field through his discoveries in tumor angiogenesis. These studies helped to understand how neoplasia are nourished from the blood supply by inducing neovascularization [1]. Due to his contributions to the development of antiangiogenic treatments in oncology, he is considered *"the Father of Angiogenesis*",[3]* from whom the modern history of angiogenesis began.

1.1. Angiogenesis: a combination of processes.

Throughout the early stages of embryonic life, endothelial cell precursors differentiate and develop *de novo* vasculature, a dynamic process called vasculogenesis [2,3]. The expansion of the vasculature from the primitive, or primary, preexisting blood vessels in response to biological demand, stimuli, or physical forces is termed angiogenesis [4].

During endothelial cell (EC) sprouting, the most studied and common in post-natal life, EC specializes into competent tip cells that are highly migratory and invasive, guiding the nascent vessels, which are followed by stalk cells. The stalk cells proliferate, lengthen, and stabilize the new vessel while creating a lumen [5,6]. The formation of the lumen is driven by the increased outer pressure that triggers the inverse membrane blebbing [7], or in a flow-independent manner by the coalescence of intracellular vacuoles [8]. Lumenized vasculature undergoes anastomosis with adjacent vessels while non-lumenized branches regress [9]. Lastly, *de novo* vasculature recruits mural and mesenchymal cells to stabilize and mature the vessel [10]. These cellular processes are tightly controlled and depend on

1* Hunter J. A treatise on the blood, inflammation and gunshot wounds. Palmer JF (Ed). p. 195, 1794; Philadelphia: Raswell, Barrington, and Haswell, 1840.
2* Barnes, J, 'Change', Aristotle: A Very Short Introduction, Very Short Introductions (Oxford, 2000; online edn, Oxford Academic, 26 Nov. 2015).
3* Cao, Y. and Langer, R. A review of Judah Folkman's remarkable achievements in biomedicine. Proc Natl Acad Sci U S A. 2008 Sep 9;105(36):13203-5. doi.org/10.1073/pnas.0806582105

the microvascular bed and angiogenic trigger, which shape the type of angiogenesis, as outlined in the following section.

1.2. Types of Angiogenesis

Angiogenesis occurs in prenatal and adult life during health and pathological situations in an organ-dependent and type-specific manner. In principle, there are two main types of angiogenesis, sprouting and nonsprouting angiogenesis. In addition, another form of angiogenesis termed tumor angiogenesis, receives special attention. It includes vessel co-option, vascular mimicry, and EC transdifferentiation (Fig. 1),[4*]. However, this falls in a separate category of vessel morphogenesis but is out of the scope of this thesis.

1.2.1. Nonsprouting angiogenesis.

Intussusceptive or splitting angiogenesis (IA) is the development of new vessels due to the growth of EC "columns" that expand and coalesce forming a lumen that is subsequently occupied by mural and mesenchymal cells leading to vessel division [11]. Although the molecular mechanisms of IA are not clearly defined, a zebrafish model revealed IA-like structures in the caudal vein plexus after sprouting angiogenesis occurs, and where hemodynamics is key in the regulation of IA. High-shear stress levels accelerate the development of middle posts, while low-shear stress areas are needed for merging the posts into columns [12]. This form of angiogenesis is considered fast and more efficient than sprouting, as it primarily comprises existing EC reorganization, without immediate requirement of EC proliferation or migration [13]. Recent insights on IA in skeletal muscle revealed that EphrinB2/EphB4 regulates vascular endothelial growth factor receptor 2 (VEGFR2) downstream phosphorylation of ERK1/2, which represents a therapeutic target for treating ischemic muscle tissue [14]. Also, IA in the context of inflamed colonic mucosa showed that the membrane-type 1 matrix metalloproteinase (MT1-MMP/MMP-14) regulates the cleavage of thrombospondin 1 and binds to αvβ3 integrity resulting in nitric oxide production and vasodilation leading to IA. These results propose the inhibition of IA for patients with colitis [13].

4* Domenico Ribatti, & Pezzella, F. Sprouting and nonsprouting angiogenesis in tumors. Elsevier EBooks, 2020; 1–13. https://doi.org/10.1016/b978-0-12-819494-2.00001-8

1.2.2. Sprouting angiogenesis.

Sprouting angiogenesis (SA) is a process that becomes active in compliance with the oxygen diffusion limit law, which established that cells must be located at a distance less than 100 μm from the capillary for optimal oxygenation [15,16]. Hypoxic environments activate the Hypoxia-Inducible Factor 1-alpha (HIF-1α) and its regulator, the microRNA-424 (miR-424), in ECs [17], as well as secretion of growth factors such as vascular endothelial growth factor (VEGF), angiopoietins, platelet-derived growth factor B (PDGFB) [18], basic fibroblast growth factor (bFGF), placental growth factor (PlGF) [19], transforming growth factor-beta 1 (TGF-β1), and phosphorylation of the suppressor of mothers against decapentaplegic 2/3 (SMAD 2/3) [20]. This results in the activation of pro-SA pathways. VEGF is the main driver of SA when binding to the VEGFR2, increasing EC survival, proliferation, migration, and permeability [21–24], required for the initiation, invasion, and elongation of the new sprouts. Initially, the sensing of the VEGF gradient by ECs in quiescence (phalanx cells) leads to the specialization of ECs that express high VEGR2 levels into "tip cells", which will lead the nascent sprouts. The activation of VEGFR2 upregulates the delta-like protein 4 (DLL4) that binds to the neurogenic locus notch homolog protein 1 (Notch 1) in the adjacent ECs inhibiting the tip cell phenotype in the neighboring ECs, a phenomenon termed lateral inhibition, and inducing the maintenance of "stalk cells" [25].

Tip cell metabolism is glycolysis dependent through PFKFB3, which enhances tip cell behavior and sprout directionality due to the formation of filopodia, and cytoskeleton rearrangement [26]. Also, during sprout development, spatial activation of autophagy regulates surface levels of VEGFR2/NOTCH to maintain tip/stalk phenotype as well as in the basement of the nascent vessel due to its high glycolytic and proliferative rates [27,28]. Neuropilin 1 (NRP1), a VEGF co-receptor with proangiogenic properties, especially in tumor angiogenesis [29], drives tip cell phenotype by suppressing ALK1/5 downstream effector SMAD 2/3. In contrast, stalk cells that displayed activated NOTCH cascades, downregulate NRP1, thus increasing ALK1/5 activity and NOTCH downstream targets *HEY1, HEY2,* and *HES* [30] (Fig. 1). Thus, the ALK1 pathway acts as a biological break of angiogenesis repressing the VEGF-mediated signaling upon Bone Morphogenetic Protein 9 (BMP9) stimulation [31]. In addition, tip cells secret Apelin (Apln), a protein that

contributes to the tip cell migration and filopodia morphology in an autocrine manner as a NOTCH downstream target and glycolytic activator [32]. This tightly orchestrated signaling determines the architecture of the newly formed sprout.

1.3. The challenges of mimicking angiogenesis.

The recapitulation of angiogenesis encompasses *ex vivo*, *in vivo*, and *in vitro* methods to create models with multilayered complexity and limitations. Whilst such assays provide specific insights into the molecular mechanisms of part of the process, all except in vivo models fail to mimic the complete angiogenic process [33]. Ex vivo systems like murine aortic ring explants received special attention as they emulate intrinsic mature tissue properties and can be subjected to different disrupting conditions and drugs cultured in different hydrogels. Critically, the specific origin of the ECs introduces another variable. For instance, employment of arterial ECs to study sprouting may not be ideal, because angiogenesis is a process often associated with the venous vasculature, which can create a mismatch between the cell source and the biological context under investigation [33–35]. In addition, *in vitro* models often only evaluate vessel morphogenesis (migration, EC proliferation). Whilst useful to investigate wound healing, chemotaxis, and invasion of ECs, these models do not provide insight into more tissue-specific cues, such as fluidic flow, hypoxia, angiokine gradients and pro-inflammatory microenvironments [33,36,37].

Matrix degradation and cell invasion are also key processes during angiogenesis. The EC tube formation assay allows investigation of network and lumen formation. This assay is difficult to control, especially because of sensitivity to seeding cell density. It frequently fails to reproduce vasculature in 3D [38,39]. To determine vessel morphogenesis in 3D which is often considered more physiological, EC fibrin-coated beads embedded in fibrin gel is simple method to reproduce sprouting although there is a lack of control over growth factor gradients, sprouting directionality, homogeneous bead distribution, mural cell interaction, and the assay is sensitive to external disturbances like pressure exerted by the media and weak hydrogel adhesion to the walls of the channel, which can produce gel rupture [40,41]. Another approach is the use of vessel organoids derived from hiPSCs to study early stages of development which are able to take into account individual genetic backgrounds of donors, and allow examination of EC metabolism and cell-to-cell-to-

matrix interplay [42]. Nonetheless, vascular organoids are immature, usually lack perfusion, are heterogenous in size, non-specific differentiation leading to differences in cell-type specific populations, and they may be costly and difficult to handle [43].

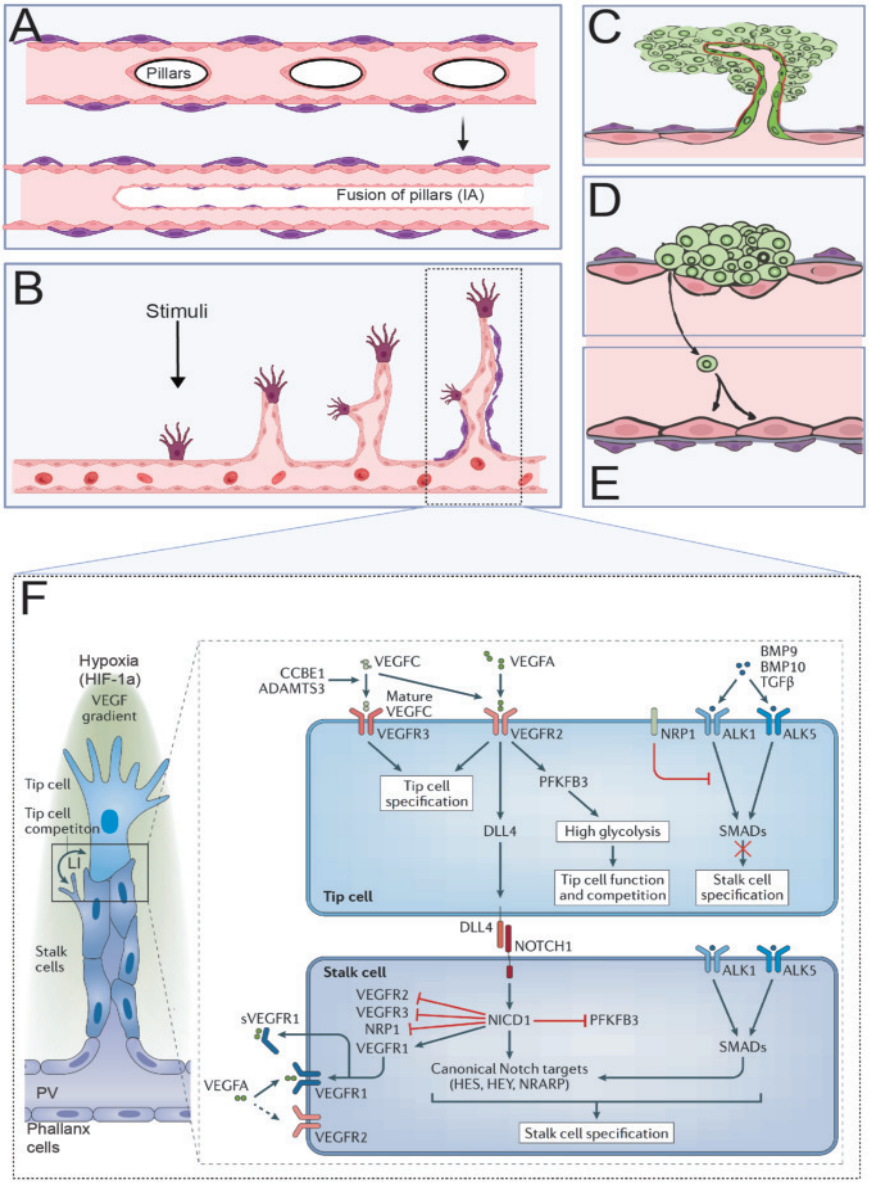

Figure 1. Schematic Angiogenesis Overview. Types of angiogenesis (A-E). Nonsprouting angiogenesis such as splitting angiogenesis (A), where transcapillary columns are created by ECs to fuse into a septum leading

to the generation of two new vessels. Other forms of angiogenesis comprise tumor angiogenesis where tumor-derived cells can develop a nonendothelial vasculature called vascular mimicry (C), hijack adjacent vessels to integrate them into the tumors in vessel cooption (D), or cancer cells can transdifferentiate into blood vessels in EC transdifferentiation (E). A-E figures taken and adapted from Eelen, *et al.* (2020) [44]. Sprouting angiogenesis (B) results from environmental stimuli such as hypoxia and VEGF release that induce tip/stalk formation from phalanx cells of the primary vessel. The EC subphenotype is controlled by a process termed lateral inhibition. The molecular mechanisms that regulate sprout formation such as the canonic VEGFR2-DLL4-NOTCH angiogenic pathways are depicted in (F), this figure was taken and adapted from Potente, *et al*. [45]. A-E panels Created with BioRender.com.

To fully model angiogenesis, it is important to induce the "angiogenic switch", a dynamic change in the pro-angiogenic factors leading to EC specialization into competent cells, that occurs *in vivo* [46]. Thus far, *in vivo* models have been able to recapitulate this process in zebrafish embryos, chicken chorioallantoic and yolk sac membranes, murine retina, gel plug assay and choroidal neovascularization [33,47]. However, major limitations restrict the use of these models, in particular, the lack of translation between data obtained from animals to humans [48]. Therefore, there is the need of not only more humane but also more human-relevant models to translate preclinical findings accurately. New approaches that incorporate the vascular components into microphysiological systems, called "angiogenesis-on-chip", is promising to bridge the gap between current models of angiogenesis and the gold-standard *in vivo* counterparts [49].

1.4. Advanced models of angiogenesis *in vitro*: Angiogenesis-on-Chip.

To date, just a few platforms and methods to examine angiogenic processes in 3D using organ-on-chip (OoC) have been published (Table 1 and Suppl. Fig. 1). Integrating a 2D compartmentalized EC monolayer capable of generating 3D sprouts within a separate compartment containing collagen as a matrix has demonstrated efficacy in controlling angiogenesis-related processes. Polydimethylsiloxane (PDMS)-made platforms allow for the control of aspects ranging from growth factor sensing and cellular signaling to vessel morphogenesis [50]. VEGF has been shown to induce sprouting angiogenesis in 3D at concentrations such as 50 ng/ml [50–54], 75 ng/ml [55], and 100 ng/ml [56]. Still, other angiokines also play important roles in sprouting induction.

Several PDMS-based OoC platforms that use type 1 collagen as a matrix have been developed. Engineered microvasculature models are useful because they include

microfluidic flow and allow the study of barrier function but fail to induce directional sprouting due to the angiokine gradient through the vessel lumen, rather than from the other side of the vessel [53]. This can be overcome when there is a parallel channel but as a screening platform, this is usually low throughput [55]. Angiokines such as Angiopoietin 1 (ANG-1), cooperate with VEGF to regulate tip/stalk cell assembly and sprout directionality [51]. Conversely, bead-based angiogenesis assays showed that siRNA knockdown of ALK1 and VEGFA reduce the number of tip cells, sprouting area and branching [41]. However, it has been demonstrated that a combination of specific growth factors such as VEGF, Sphingosine 1-phosphate (S1P), and phorbol 12-myristate 13-acetate (PMA) leads to multicellular and lumenized sprouts connected to primary vessels with little migration of ECs as single cells [55]. The sprout phenotype is not only shaped by the angiokine cocktail but also by the stiffness of the matrix and its degradability by metalloproteases [57].

Besides the chemotaxis induced by VEGF, interstitial flow influences sprout formation against the direction of flow and this is maintained over time. These observations were made in a “double process” model of vascular morphogenesis in which cells were first seeded in a compartment undergoing vasculogenesis in fibrin, followed by angiokine-mediated angiogenic stimuli in a side compartment, supported by the paracrine secretion of VEGF by fibroblasts [56,58]. Lung fibroblasts induce EC sprouting in a cell density-dependent manner, where 25 x10^6 cells/ml encapsulated in beads yields a VEGF release of 15 ng/ml. Under this co-culture condition, EC sprouted towards the encapsulated fibroblasts with subsequent formation of larger lumens [59]. Similarly, fibroblast- and pericyte-secreted angiokine gradients combined with a complex hydrogel, also induced a strong angiogenic response with functional barrier function and inflammatory response [60]. Tumor angiogenesis, a special form of angiogenesis, has been also studied using human fibrosarcoma-derived cells encapsulated in similar beads; this showed that VEGF secretion is two-fold higher than in the presence of fibroblasts [52]. Despite revealing the high angiogenic capacity of tumor cells, this approach has the drawback that thecell encapsulation methods are complex, especially in combination with microfluidic devices.

Table 1. Summary of angiogenesis-on-chip models.

Cell type (Final cell Density)	Matrix	[Final Conc.]	Co-culture (Final CD, cells/ml)	Angiokines [Conc.]	S (days)	HT	DT	Ref
hMVECs 2x10^6 cells/ml	Col1	2 mg/ml	-	VEGF and ANG1 50 ng/ml in EGM-2MV	6-8	-	-	[51]
hMVECs 0.5 to 5x10^6 cells/ml	Col1	2 mg/ml	-	VEGF 50 ng/ml In EGM-2MV	1-12	-	-	[50]
hMVECs 2x10^6 cells/ml	Col1	2.5 mg/ml	HDF 5x10^6	VEGF 50 ng/ml In EGM-2MV	7-9	-	-	[52]
hMVECs 2x10^6 cells/ml	Col1	2.5 mg/ml	IMR-90 in beads 6.25-25x10^6	EGM-2MV	5	-	-	[59]
HUVECs 5x10^6 cells/ml	Fn, Th	2.5 mg/ml, 50:1 ratio	Fibroblasts 7.5x10^6	VEGF 100 ng/ml S1P 1μM in EMG2	6	-	P	[56]
HUVECs/ hMVECs 10x10^6 cells/ml	Col1	2.5 mg/ml	-	VEGF, MCP and PMA -1 75 ng/ml and S1P 500 nM in EGM-2MV	4	-	P	[55, 57]
HUVECs 20x10^6 cells/ml	Fn, Th	2.5 mg/ml, 2 U/ml	NL-FBs 10x10^6	EGM-2	4-7	-	P	[58]
HUVECs 5x10^6 cells/ml	Fn, Th, Apro, Col1	2.5 mg/ml, 0.5 U/ml, 0.15 U/ml, 0.2 mg/ml	LFs 10x10^6 hPC-PL 5x10^5	EGM-2	3	-	-	[60]
HUVECs 10x10^6 cells/ml	pCol 1	2.4 mg/ml	-	VEGF 50 ng/ml in EC-CGM	10	-	P	[53]
HUVEC-VeraVec™ Or hiPSC-ECs 20x10^6 cells/ml	Col1	4 mg/ml	-	VEGF and bFGF 50ng/ml, S1P 500 nM and PMA 2 ng/ml in MV2	2-9	P	P	[54, 61,62]
HUVECs *	Fn or Col1	*	LFs *	EGM-2 FGM-2	6	P	P	[63]
HUVECs 6x10^6 cells/ml	Fn, Th	2.5 mg/ml, 0.25 U/ml	NL-FBs/ Cancer cells 3-6x10^6	EGM-2 FGM-2	7	P	P	[64]
HUVECs 3x10^6 cells/ml	Fn, Apro, Th	2.5 mg/ml, 0.15 U/ml, 0.5 U/ml	NL-FBs 7.5x10^6	EGM-2	3-4	P	P	[28]
HUVECs 1.5-5x10^6 cells/ml	Col1	2-6 mg/ml	-	S1P 0-500 nM and PMA 10-50 ng/ml in EGM-2	5	P	-	[65]
HUVECs 8x10^6 cells/ml	Col1	4 mg/ml	-	VEGF 50ng/ml, S1P 50 nM and PMA 2 ng/ml in EGM-2	3	P	P	[66]

Conc., concentration; S, stimuli duration; HT, high throughput; DT, drug testing; Ref., reference; CD, cell density; HUVECs, primary human umbilical vein endothelial cells; hMVECs, human (dermal) microvascular endothelial cells; IMR-90, human fetal lung cell line; HDF, Human dermal fibroblasts (fibrosarcoma); LF or NL-FBs, Normal human lung fibroblasts; hPC-PL, Human Pericytes from Placenta; Col1, Rat tail type 1 collagen; pCol1, porcine type 1 collagen; Fn, Fibrinogen; Th, Thrombin; Apro, aprotinin. HGF, hepatocyte growth factor; MCP-1, monocyte chemotactic protein-1; bFGF, basic fibroblast growth factor; S1P, sphingosine1-phosphate; PMA, phorbol 12-myristate 13-acetate; EC-CGM, endothelial complete growth medium; EGM-2, Endothelial Growth Media-2; FGM-2, Fibroblast Growth Medium-2; EGM-2MV or MV2, Microvascular Endothelial Cell Growth Medium-2; Not disclosed (*); not used/performed (-). P, Performed.

Building upon prior models, two platforms with increased reproducibility and throughput have been developed but the fundamental mechanisms for sprouting induction remain

similar. On the one hand, a 40-replicate platform containing a middle channel that separates the patterned collagen, and the Human umbilical vein endothelial cells (HUVEC) or Induced pluripotent stem cell-derived ECs (iPSC-ECs) by phaseguides, has shown successful results when sprouts are induced with VEGF, S1P, and PMA [54,61]. This platform also enabled antiangiogenic drug testing with optimal quality control metrics [62]. On the other hand, a more sophisticated platform with no structural division between compartments allowed angiogenic modeling combined with vascularized organoids or tumors. This is a 96-well plate format platform, well-suited to high-content screening [63]. Similarly, a 28-replicate microvascular device displays more control over drug testing and tumor angiogenesis [64]. Also, in this device, cell retrieval for single-cell RNA sequencing is possible, allowing insights into molecular mechanisms orchestrating the tip/stalk cell specification during sprout morphogenesis [28].

The use of high-content screening (HCS) platforms for angiogenesis makes the testing of multiple conditions possible to find optimal matrix concentration, cell density, and growth factor concentration. An 8-replicate platform has studied the balance between cell migration and proliferation to obtain multicellular sprouts connected to primary vessels. The study concluded that collagen concentration did not alter EC proliferation in the presence of PMA and S1P at specific concentrations [65]. Currently, it is also possible to test the efficacy and toxicity of more than 1500 antiangiogenic compounds on ECs in a 64-replicate device, although so far, only using primary ECs [66]. However, there are still limitations on the reproducibility and robustness of these platforms, since it seems to depend on the source of ECs used, impacting the generalizability of these results.

1.5. The frontier of modeling angiogenesis: the use of human hiPSC-derived ECs.

Modeling angiogenesis with the use of hiPSC-derived ECs represents an extra layer of complexity when employing in vitro microphysiological systems (Fig. 2). However, hiPSC-derived ECs are a sustainable source of vascular cells and they can be derived from relevant donors for advanced modeling of diseases using *in vitro* systems [67]. Several protocols to derive ECs from hiPSCs have been reported [68–72], increasing the availability of cells of different origins and molecular signatures. To date, the use of ECs from hiPSCs to mimic angiogenesis in high-content or high-throughput platforms is

limited as shown in Table 1. Current methods have largely used primary ECs and only one study has reported hiPSC-derived ECs being incorporated in a microphysiological device for antiangiogenic drug testing [61,62]. This comes as no surprise due to the challenges in obtaining and reprograming hiPSCs, differentiation into ECs, batch-to-batch quality control, organ-on-chip expertise, relative paucity of standards in the field, need for upscaling of platforms, and more importantly, appropriate technical expertise.

In contrast to the models of angiogenesis based on hiPSC-derived ECs, vasculogenesis models have, in general, shown superior performance [73–78], possibly due to the innate vasculogenic capacity of such cells arising from their immature identity [79]. Why angiogenic modeling results in less reproducible and variable outcomes is not clear but it could be because of the complex angiogenic cues that need to be mimicked *in vitro* [30,80,81] .

1.5. Fundamentals of angiogenesis in Hereditary Hemorrhagic Telangiectasia: a crosstalk defect amongst the BMP9- and VEGF-mediated signaling.

1.5.1. Genetics of neovasculature defects in HHT.

HHT is a genetic vascular disease associated with mutations in the genes encoding either endoglin (*ENG*) or activin receptor-like kinase 1 (*ACVRL1*), that lead to defects in the ALK1 pathway [82]. To date, approximately 1.4 million people worldwide are affected by HHT according to the International HHT Foundation (cureHHT).[5*][5]Dutch population suffering from HHT carries more than 188 mutations in *ENG* and *ACVRL1* genes [83]. In addition, *in silico* predictions indicate that HHT might be an underdiagnosed disease and that carriers of *ENG* or *ACVRL1* mutations might actually account for 2-to-12 higher prevalence [84]. The heterozygosity resulting in ALK1 or ENG haploinsufficiency, followed by the homozygosity derived from secondary allelic mutations, and local triggers leading to abnormal mechanical cues combined with an angiokine-enriched microenvironment are described as the triad for abnormal neovasculature in HHT [85].

5*International HHT Foundation. Consulted in 2025. www.curehht.org/understanding-hht/what-is-hht/

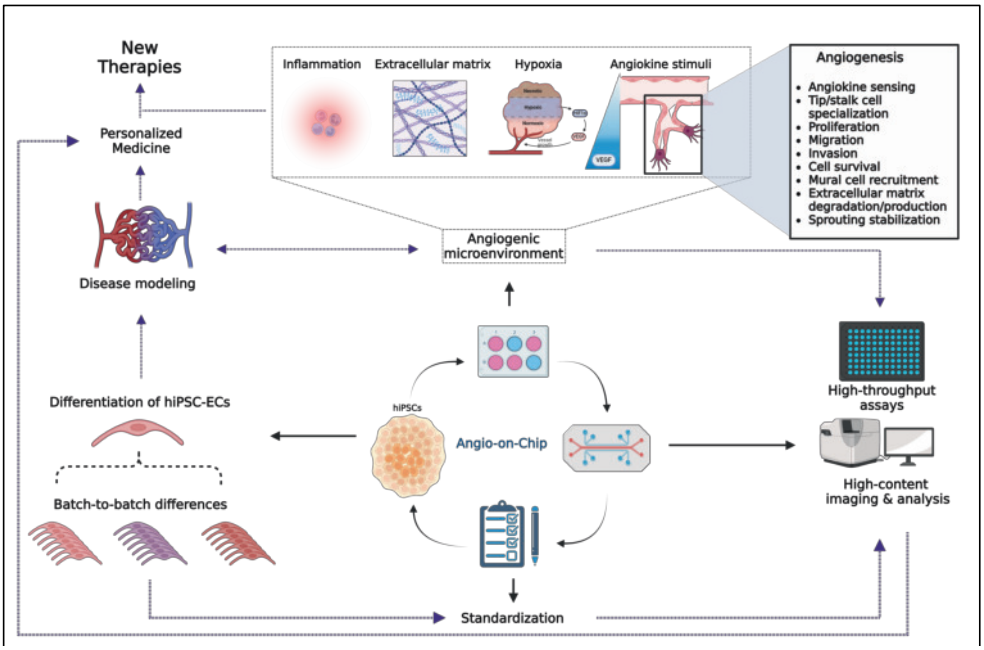

Figure 2. The complexity of modeling angiogenesis-on-chip. Several factors account for the constraints of these types of assays, from the recapitulation of the angiogenic stimuli in 3D microfluidic devices to the use of multiple donor-derived hiPSC-derived ECs for personalized medicine. Also, the incorporation of organ-on-chip technology for high-throughput screening and the lack of standards to be able to deconvolute the readouts. Created with BioRender.com.

In addition, the defects in mural cell recruitment also account for abnormal angiogenesis. Earliest data revealed that homozygous *ENG*$^{-/-}$ and *ACVRL1*$^{-/-}$ mice exhibit vessel dilation, aberrant Vascular Smooth Muscle Cells (VSMCs) formation and coverage [86–89]. This evidence suggests that the vessel defects are a consequence of the abnormal crosstalk between the mural and endothelial cells. Of note, *ACVRL1*$^{-/-}$ mice displayed higher mortality during embryonic life ~E10.5 and showed overexpression of proangiogenic molecules such as VEGF, Angiopoietin-2 (ANG-2), Urokinase-type plasminogen activator (uPA)/uPAr, and Plasminogen activator inhibitor-1 (PAI-1) [87]. In contrast, ENG-/- mice presented a disrupted vessel network and delayed growth at E9.5 but were viable, suggesting that ENG does not impair EC differentiation or vasculogenesis [86,90], contrary to ALK1, which is needed for embryonic viability [87]. Also, the retinas from heterozygous *ENG*$^{+/-}$ mice showed disproportionate angiogenesis characterized by increased branching points and low coverage of vascular smooth muscle cells (VSMCs). This phenotype is rescued upon Thalidomide treatment, which increases the PDGFB-

mediated pericyte and VSMC recruitment by the tip cells, leading to the inhibition of the sprouting [89].

Besides the mutations in the *ACVRL1* alleles, the enlargement of arterial vessels comprises a series of cooperative elements, such as mutations in *Gja5*, which encodes for the Connexin40 protein (Cx40). *ACVRL1;GJA5* double mutant mice induced generation of ROS resulting in flow changes and arterial enlargement, which confirms that ALK1 and Cx40 are associated with abnormal angiogenesis, increased proliferation of EC in the capillaries, and abnormal mural cell coverage in initial phases of the AVM development [85]. The onset of flow in *ACVRL1*$^{-/-}$ zebrafish showed that increased blood flow, secondary increase in EC population, and wall shear stress resulted in dilated vessels [91]. The migration of ECs intrinsically occurs in an against-flow manner. In the presence of *ACVRL1* mutations, flow impairs the negative regulatory effect of the BMP9-ENG-ALK1-SMAD1/5-SMAD4 signaling axis [92–95] and promotes the activation of the proangiogenic VEGFR2/PI3K/AKT/YAP/TAZ pathway [96–98]. The compromised interplay between these axes is possibly the key to the molecular understanding of HHT etiopathology.

1.5.2. Dysregulated canonical angiogenic pathways in HHT: *BMP9/10-ENG-ALK1-SMAD1/5* signaling: the regulatory switch of angiogenesis.

Bone Morphogenetic Protein (BMP)-9 and BMP10 bind to ALK1 inducing downstream SMAD1/5/8 phosphorylation, which is involved in the regulation of angiogenesis. Angiogenic regulatory mechanisms include the ALK1 ligands BMP9/10, which activates SMAD4 via SMAD1/5/8, resulting in a reduction of endothelial cell migration and proliferation. In addition, the agonist type III ENG receptor enhances the BMP9/10-mediated effects on ALK1 activity [99]. Hence, BMP9/10-mediated ENG/ALK1 activation co-regulates the downstream angiogenic mechanisms. Both BMP9 and BMP10 are critical during embryonic life, being BMP9 predominantly expressed in the liver and BMP10 in the heart. Despite the redundant binding capacity of BMP9 and BMP10 to ALK1, BMP10 activity controls vessel maintenance in mature vasculature. Zebrafish harboring double BMP10 and BMP10-like, but no BMP9, mutations develop AVMs in the liver and skin with hemorrhagic vasculature similar to the observed in *ACVRL1* mutants and HHT patients [94].

In mice, ALK1 depletion in liver sinusoidal endothelial cells (LSEC) reduced ENG levels, increased endomucin (EMCN), and loss of LSEC-specific LYVE-1 marker expression suggestive of vessel arterialization. Also, single-cell RNAseq data revealed that hepatic ECs developed an angiogenic signature characterized by a metabolic reduction, upregulation of tip cell markers such as Apelin, endothelial cell-specific molecule, and VE-Cadherin; and PI3K-AKT activation, suggesting the role of ALK1 in maintaining stalk cells and vessel quiescence [100]. Also, Wild-type BMP9-stimulated LSECs revealed activation of the BMP9/ALK1/ID1-3 axis that upon ALK1 inhibition overexpresses placental growth factor (PlGF)-2 and Prion-like protein doppel (PRND) [100], a modifier of the VEGFR2 activity [100,101]. In addition, BMP9-stimulated human arterial ECs showed an upregulation of mRNA levels of *ID1*, notch downstream effector *HEY2*, and significantly high levels of angiogenic regulator *GJA5* [85].

Similar to the lethal effect on ECs lacking BMP10 during embryonic life [94], Neuropilin-1 (NRP1) deletion is also lethal [102]. NRP1 regulates ENG and ALK1, and it is key for functional coverage of the neovessel by mural cells and the development of AVMs. Data from NRP1 knockout mice exhibit dilated vasculature, immune cell infiltration abnormal mural cell coverage, and reduced ALK1 downstream pSMAD1-5-8 effectors, leading to vessel instability and malformations [103]. Striking observations suggest that AVMs are more prone to develop in mosaic tissues where the ALK1 depletion is stochastic and heterogenous resulting from a combination of ALK1-preserved and ALK1-depleated ECs. Such malformations exhibit an increase of gene expression linked to angiogenesis and vascular remodeling; and a reduction of gene expression related to vessel homeostasis, stability, and recruitment of supporting cells with defective BMP9-ALK1 signaling. This raises a new hypothesis in which AVMs are formed in a cell non-autonomous mechanism resulting in an imbalance between vessel morphogenesis and remodeling [104].

1.5.3. Implications of the VEGFR2 hyperactivity on angiogenesis.

During sprouting angiogenesis, a tight tip/stalk cell specification takes place. The tip cell exhibits high migratory and invasive capacity in the tip cells primarily driven by VEGF-mediated signaling through VEGFR2/3, activating delta-like ligand 4 (DLL4), a canonical Neurogenic locus notch homolog protein (NOTCH). This results in NOTCH activation in

adjacent ECs, inducing a stalk phenotype by "lateral inhibition" that will extend and stabilize the sprout [45]. Besides VEGF-A as the main angiogenic driver [105], there are different pathways involved, resulting in a complex process to recapitulate *in-vitro.* Mutant *ACVRL1*$^{+/-}$ mice showed decreased mRNA expression of VEGFR1 but no changes in NOTCH or VEGFR2. However, VEGFR2 signaling is hyperactivated displaying higher VEGFR2 phosphorylation levels and increased vascular density in the same mouse model with a tracheal infection compared to the wild type. This phenotype was rescued upon selective VEGFR2-blocking treatment that converts capillaries into venule-like vessels, thus inhibiting the "proangiogenic phenotype". This data suggests that the imbalance between VEGFR1/VEGFR2 activity is crucial during pathological angiogenesis [106].

During sprout development, the activation of the DLL4-NOTCH pathway regulates the tip/stalk cell specification by ensuring specific phenotype development throughout the sprout morphology. It is widely established that indirect blocking of NOTCH using γ-secretase inhibitor DAPT orchestrates a tip cell specification, which results in sprouting enhancement [44,45,107]. The genetic signature of the tip/stalk ECs is shown to be spatially determined and regulated by autophagy. ECs in the primary vessel displayed a highly glycolytic and proliferative profile that supports the extension and formation of the sprouts. Conversely, the VEGF/NOTCH signaling and the LCB3, an autophagy marker, are characteristic in the sprout endpoints where tip cells are located. Furthermore, knockdown of autophagy reduces the sprouting suggesting that autophagy could regulate either the secretion, retrieval, or degradation of the VEGFR2 in tip/stalk cells [28]. In line with these observations, ENG variants leading to HHT1 accumulate in some cell types shifting from lysosomal to proteasomal degradation [108]. However, the autophagy-related markers are yet to be investigated using ENG and ALK1 knockouts or deficient ECs during angiogenic sprouting to evaluate the contribution of autophagy to AVM development in HHT1 and HHT2.

Several growth factors can independently regulate the VEGFR2 signaling switching on-off the EC sprouting. The Sphingosine-1-phosphate (S1P), which acts via G-protein coupled receptor activating Rac1 and MAPK pathways, amplifies the VEGFR2 signaling [109]. Also, S1P acts on DNA synthesis, chemotaxis, microtubule rearrangement, and sprout normalization mediated by the controlled balance between its receptors S1PR1, S1PR2,

S1PR3, and the VEGF-mediated signaling [110,111]. Another regulator of VEGFR2 is PRND, a coreceptor involved in VEGFR2 stabilization. Inhibition of this molecule leads to VEGFR2 internalization and therefore anti-angiogenic effects in ECs in the context of cancer [101] and blood-brain barrier sprouting angiogenesis [112]. Hepatic HHT revealed an upregulation of *PRND* as well as increased PlGF-2, and downregulated Wnt2, Wnt9, and Rspo3 in the ALK1 knockout mice [100]. Similarly, the PlGF-2 is involved in VEGFR2 degradation via the glycogen synthase kinase 3 (GSK-3) in the retina [113] but also increases VEGFA bioavailability to VEGFR2 by binding to VEGFR1 [19]. Therefore, these two molecules are considered to interact with the canonical VEGFR2 proangiogenic pathway. However, there is limited data in the context of HHT using humanized models.

VEGFR2 levels are also modulated by NRP1 via direct binding. However, the effects of NRP1 on angiogenesis do not depend on its binding affinity to VEGFR2 but rather on a decrease of the VEGFR2 activation as displayed in NRP1 mutated mice. This results in impaired vascular morphogenesis in the retina postnatally [114]. Moreover, sprouting angiogenesis is a metabolically active process that regulates EC migration. Evidence suggests that the glutamine-producing enzyme, glutamine synthetase (GS), controls the RHOA activation via the covalent addition of palmitic acid to RHOJ, causing a reduction of filopodia and cell migration in mouse retina and skin [115]. Similar to the GS effects over EC motility, the formation of tip cells is glycolysis dependent via the hypoxia-inducible PFKFB3 and upregulated by VEGFR2. Enhancement of PFKFB3 results in EC sprouting and its knockout leads to vascular abnormalities due to impaired cytoskeleton rearrangement and nonfunctional filopodia [26].

1.5.4. Organ-specific angiogenic defects in HHT linked to disrupted ENG/ALK1/VEGFR signaling.

Decreased ENG levels in different organs such as skin, lungs, brain, and intestine, particularly, in microvasculature and arteries of *ENG*$^{+/-}$ mice. Decreased ENG levels in humans have been found in the same organs as in the HHT1 mice. Interestingly, ENG haploinsufficiency seems to be required but insufficient to produce AVMs. However, VEGF-stimulated ENG-deficient hepatic and pulmonary ECs showed increased phosphorylation of VEGF-related downstream molecules, resulting in hyperactivity of such pathways.

Hence, this aberrant response is implicated in defects during angiogenic stimuli in organ-specific vascular beds in HHT1 [116].

Regarding HHT2, vascular malformations have been reported in several organs. The plasma from HHT2 patients showed low levels of sVEGR1 as well as low VEGR1 in skin biopsies [106]. Human skin biopsies revealed decreased Cx40 expression due to ALK1 haploinsufficiency [85]. In addition, dorsal ears from ACVRL1$^{+/-}$;GJA5$^{EGFP/+}$ mice showed dilation or arterial and pre-arteriolar vessels. Also, wounding of the ears revealed aberrant anastomosis and disturbed blood flow, leading to temporary small AVMs that turn into large AVMs due to sustained angiogenesis [85]. The retina, a highly vascularized structure, exhibits a hypersprouting phenotype in *ACVRL1*$^{+/-}$ mutant in contrast to the lesser-dense vessel network and reduced angiogenesis in ACVRL1$^{+/-}$;GJA5$^{EGFP/+}$ double mutant mice [85]. In addition, liver-specific BMP9 expression might account for the higher frequency of the hepatic AVMs observed in HHT2 patients [99]. A murine model of hepatic HHT revealed that liver-specific ALK1 knockdown results in hepatic AVM development, loss of sinusoidal compartmentalization, post-hepatic vessel enlargement, vessel arterialization, and activation of angiogenic signaling cascades without hindering the sinusoidal hepatic function [100]. Notably, NPR1 levels are reduced in mural cells of the liver, confirming the implications of the impaired smooth muscle cell coverage and the HHT pathogenesis [103].

Table 1. In vivo and in vitro models of angiogenesis in the context of HHT.

In vivo	Description	Findings	Ref.
ENG$^{-/-}$ mice	Homozygous endoglin knockout	Retarded animal growth. Defects on the yolk sac vascular network	[86]
ENG$^{+/-}$ mice	Heterozygous ENG model of HHT1	Abnormal retinal angiogenesis with increased vessel branching. Defects in mural cell coverage in retina and skin	[89]
ENG$^{+/-}$ mice	Heterozygous ENG model of HHT1	Microvascular bed-specific low ENG expression	[116]
ENG-iKO^{e} mice	Tamoxifen inducible ENG	AVM formation upon significant reduction in ENG levels, delayed vessel stabilization, abnormal EC proliferation	[117]
Acvrl1$^{+/-}$ mice	ALK1 heterozygous mutant	HHT2-like lesions, excessive sprouting, dense vasculature	[85]
Acvrl1$^{+/-}$;*Gja5*$^{EGFP/+}$ mice	ALK1/Cx40 heterozygous double mutant	Vasodilated vessels, reduced angiogenesis, ROS induction, and AVM shunts	[85]

Acvrl1-iHET;*Gja5*$^{EGFP/+}$ mice	Inducible ALK1 KO; Cx40 mutant mice	Severe angiogenic phenotype	[85]
Acvrl1$^{-/-}$ mice	Homozygous ALK1 knockout	Lethal during embryonic life	[87]
Acvrl1$^{-/-}$;*Tnnt2a* MO zebrafish	ALK1 homozygous + no heartbeat	No AVM development	[91]
Acvrl1$^{-/-}$;*GATA1a*$^{-/-}$ zebrafish	ALK1 homozygous + blood flow + no erythrocytes	Flow-dependent AVM development	[91]
Acvrl1^{HEC-KO} mice	LSEC-specific ALK1 knockout	HHT2 hepatic-specific lesions, arterialization, angiogenic cell phenotype	[100]
Acvrl1$^{+/-}$ mice	ALK1 heterozygous mutant/ *Mycoplasma pulmonis* infection	Excessive vessel density, branching points, migratory defects, and higher tip cell numbers in the retinal vascular network	[106]
Bmp mutant zebrafish	bmp9 mutant, bmp10 mutant bmp10; bmp10-like double mutant	Double bmp10;bmp10-like mutants displayed AVMs resembling HHT2 lesions	[94]
Mfsd2a, Esm1 and Bmx Cre^{ERT2} + ALK1$^{f/f}$ mice	Selective ALK1 deletion in venous, tip and arterial ECs	Impaired migratory capacity of ECs against the flow results in the development of AVMs	[98]
Nrp1^{SM22KO} mice	Selective NRP1 deletion in SMCs	AVM development, hemorrhagic lesions, decreased mural cells, dilated capillaries, reduced ENG and ALK	[103]
NRP1^{VEGF-} mice	NRP1 mutant with abrogation of the NR1P binding to VEGF	NRP1 modulates VEGF/VEGFR2 activity postnatal vessel formation	[114]
Vbg^{y6} zebrafish	ALK1 mutant vbg mutant allele	Aberrant vasculature due to increased EC number and edema.	[118]
In vitro	Description	Findings	Ref.
Acvrl1$^{+/-}$ mESC	ALK1 mutant embryoid bodies	No difference in EC number vs WT. Reduced VGFR1 levels in EC prompt tip cell specialization	[106]
si-Acvrl1 and IPTG-inducible sh- Acvrl1 HUVECs	ALK1 knockdown and knockout	Hyperdense network, AVM-like phenotype, HHT vascular phenotype, and TKI used for vascular normalization	[104]
ALK5$^{-/-}$ MEECs	ALK5 KO MEECS	Abrogation of ALK1 downstream targets pSMAD1/5	[119]
HHT1-hiPSC-ECs	ENG$^{c.1678C>T}$ mutant hiPSC-ECs	No differences in 2D assays. Morphological differences and abnormal EC-pericyte interaction of HHT1-hiPSC-ECs	[73]
HHT1-hiPSC-ECs	Prime edited ENG$^{c.360+1G>A}$ mutant hiPSC-ECs	Organoids with elevated proangiogenic phenotype	[120]

mESCs: mouse Embryonic stem cells, MEECs: mouse embryonic endothelial cells, ASO: antisense oligonucleotides, LSEC: Liver sinusoidal endothelial cells, ROS: reactive oxygen species, si; small interfering RNAs, IPTG: isopropyl ß-D-1-thiogalactopyranoside, sh: short hairpin RNAs, SMCs: smooth muscle cells, TKI: tyrosine kinase inhibitor, HHT1: ENG mutated cells, hiPSC-ECs: human induced pluripotent stem cell-derived endothelial cells.

1.6. Aims and Scope of the thesis

The overall aim of this thesis is to develop a hiPSC-EC-based model to mimic 3D sprouting angiogenesis for preclinical *in vitro* drug testing and disease modeling. However, as previously explained, current models that mimic angiogenesis encounter important challenges to overcome. Although there are platforms that enable the modeling of the angiogenic processes, the use of patient-specific hiPSC-derived EC for disease modeling and personalized medicine in the context of angiogenesis is still evolving.

In ***Chapter 2***, we describe a 3D culture method for sprouting angiogenesis based on hiPSC-derived ECs employing a platform for high-content screening. We determined the sprouting response of the cells to the combination of several angiokines as well as quality control metrics and exclusion criteria of microfluid channels prior to the analysis to evaluate the feasibility of the method for drug testing. Here we provide recommendations on the use of fibrin or collagen matrices. To assess the applications of the assay, we examined anti-angiogenic drugs that block different components of the VEGFR2 pathway in ***Chapter 3.*** This allowed measurement of the degree of inhibition of hiPSC-derived EC sprouting when VEGFR2 signaling is abrogated.

On the other hand, the basic aspects reported in the literature on the crosstalk defects between the BMP9- and VEGF-mediated signaling leading to abnormal angiogenesis in type 2 Hereditary Hemorrhagic Telangiectasia (HHT2) are summarized in the introduction**,** as a basis for selecting which model would be best suited to investigate disease mechanisms of HHT. Therefore, in ***Chapter 4,*** we report the generation and characterization of two patient hiPSC lines harboring heterozygous mutations leading to HHT2. Such hiPSC-derived ECs from patients with HHT2 will be further characterized in 2D and 3D by incorporating the HHT2 hiPSC-derived ECs in the organ-on-chip platform as a key direction to model angiogenic defects. Lastly, in **Chapter 5**, we discuss the research results in the context of current state-of-the-art, limitations, future perspectives, and the impact of our findings in the field of angiogenesis in health and disease.

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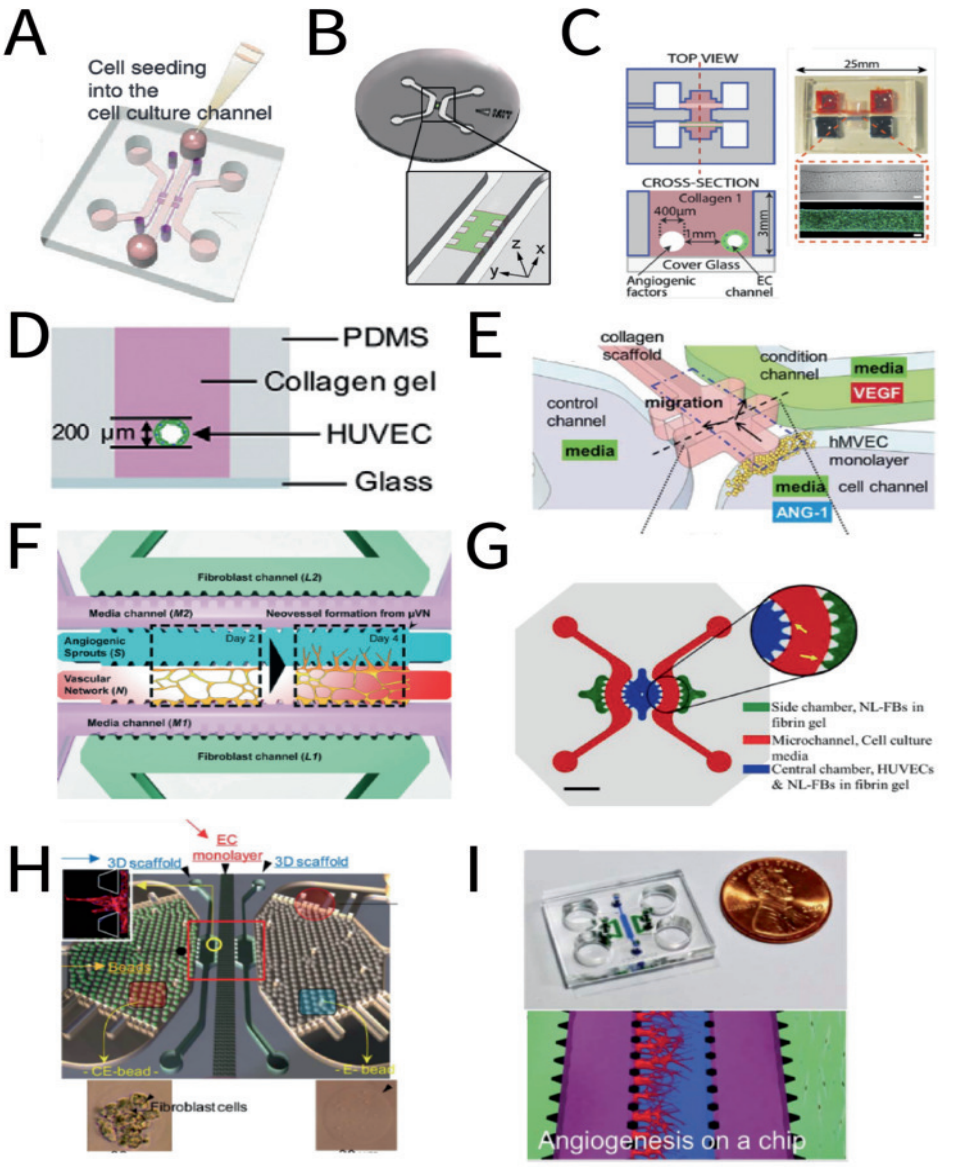

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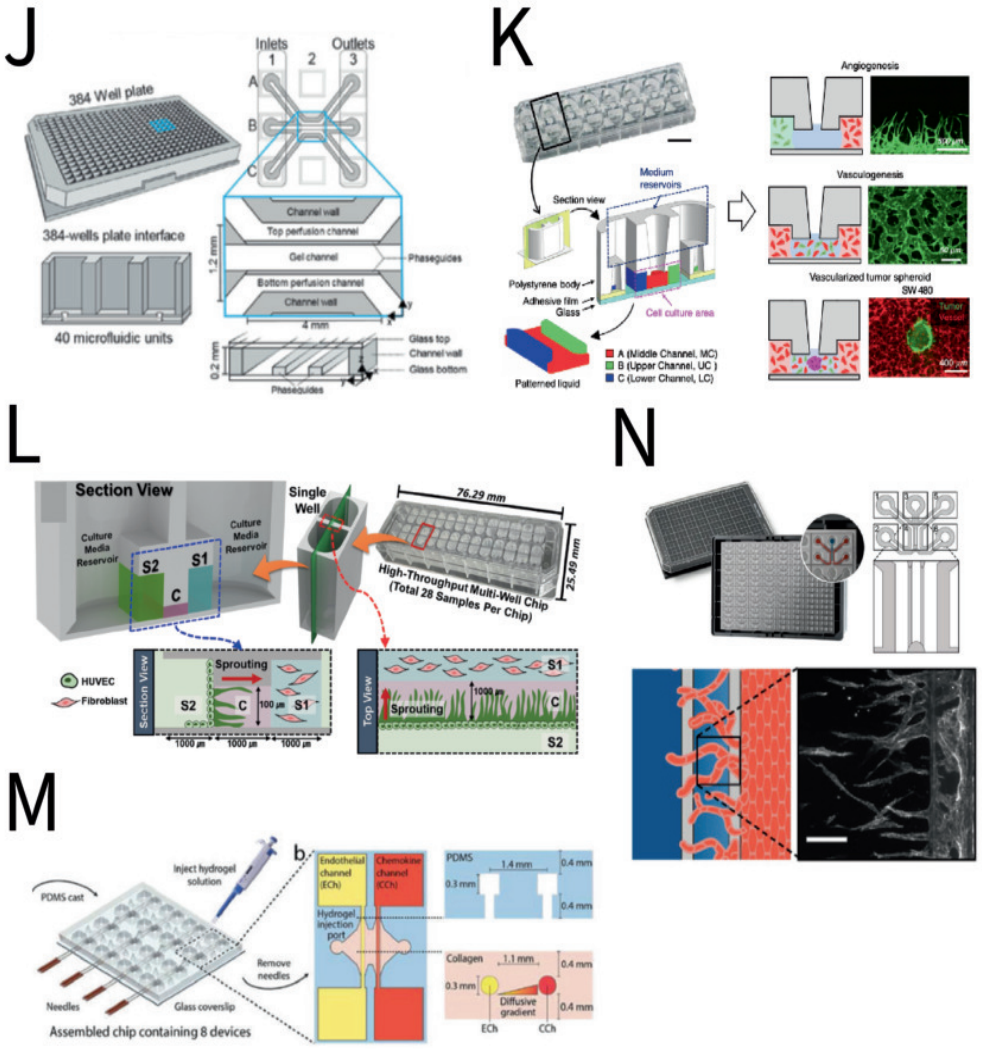

Supplementary Figure 1. Schematic overview of the organ-on-chip devices to model angiogenesis. Images taken from original articles A, Ref.[50]; B, Ref.[52]; C, Ref.[55]; D, Ref.[53]; E, Ref.[51]; F, Ref.[56]; G, Ref.[58]; H, Ref.[59]; I, Ref.[60]; J, Ref.[54]; K, Ref.[63]; L, Ref.[28,64]; M, Ref.[65]; N, Ref.[66]. F, dual vasculogenesis-angiogenesis model; A-I, low throughput platforms; J-M, high throughput platforms.