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

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Chapter 5

General Discussions and Future Perspectives

An assay from health to disease

Angiogenesis is important in multiple aspects of *in vitro* vascular disease modeling due to the crosstalk with microvascular bed-specific cells that support non-vascular cells and tissue function [1]. The outcomes of this thesis contribute to the growing field of angiogenesis in regenerative medicine and disease by developing a human iPSC-derived endothelial cell (hiPSC-EC)-based 3D *in vitro* system for sprouting angiogenesis (Ch. 2). To examine the functionality of the hiPSC-EC-based angiogenesis-on-chip (AoC) assay, selective VEGFR2 inhibitors were used to test the angiogenic inhibitory capacity by assessing vessel sprout inhibition through blocking the angiogenic canonical pathway (Ch.3). In an attempt to further elucidate the molecular mechanisms affected by mutations causing the vascular disease Hereditary Hemorrhagic Telangiectasia (HHT) and their impact on vessel morphogenesis, particularly sprouting angiogenesis, we successfully generated and genetically repaired type 2 HHT (HHT2) patient-derived hiPSCs (Ch. 4) for future angiogenic research on the underlying cellular mechanisms of HHT2. We considered the limitations of the angiogenic assay, and the use of hiPSC-ECs for the modeling of angiogenesis.

State-of-the-art angiogenesis-on-chip featuring hiPSC-ECs

Existing models of angiogenesis, often based on primary ECs in monolayer culture, have a number of issues that limit their use for disease modelling. The core hypothesis of this research is that hiPSC-EC-based grown in 3D culture could mimic sprouting angiogenesis for preclinical *in vitro* mechanistic studies and testing of therapeutics targeting vasculature. This work sought to overcome some of the challenges of current methodologies. Conventional 2D assays, such as the wound healing and tube formation, are simple methods to assess some of the central angiogenic processes, such as EC migration, invasion, and proliferation. Such assays are valuable and readily standardized, so they have contributed significantly to the field over several decades. However, their variability, high reagent usage, lack of control over the systems, inadequate perfusion, poor long-term cell survival, and the need to perform several different assays in parallel to provide informative data, limit their use when compared to 3D models [2–4]. Advanced 3D systems such as vascular spheroids in this context provide deeper insights into EC

specialization into tip and stalk cells and lumen formation, and enable cell collection for downstream functional assays but the absence of perfusion, controlled angiokine gradients, irregular cell distribution and spatial growth can limit reproducibility [5–8]. In addition, ECs are generally not exposed to the same mechanical fluidic flow or biological stimuli found *in vivo* during *in vitro* angiogenesis [3]. For instance, angiokines such as VEGF in the blood are in the picogram range (119-238 pg/ml) [9], while in vitro, they are only effective in nanogram concentrations (30-100 ng/ml) [10,11]. Also, lumen formation takes place by vacuole fusion, cytoskeleton rearrangement [12], and inverse membrane blebbing due to elevated hemodynamic pressure [13], often not mimicked *in vitro*. Fluidic flow rates in microvasculature *in vivo* are between 500-1500 µm/s and shear stress values range from 100-500 s^{-1} [14]. Most *in vivo* data have been obtained from animal models where all these events occur, but vessel size may differ from humans, or from primary (human or mouse) cells which may vary from donor to donor or model used. These models are nevertheless still widely used as the gold standard.

Microphysiological systems (MPS) have recently provided opportunities to recapitulate tissue-specific microenvironments to a certain extent by providing mechanical cues, structural scaffold, and connectivity for fit-for-purpose biologically relevant vasculature [15]. However, they still need further development. Very few human angiogenesis-on-chip models exhibit vascular sprouting characteristic of robust angiogenesis or its inhibition [11,16–19]. Furthermore, molecular insights from these models are rare, with only one platform to date showing evidence obtained from advanced molecular methods in which ECs had been retrieved from the MPS model for single-cell RNA sequencing. This data revealed that there is a spatial transcriptomic profiling throughout the sprout architecture, especially upregulation of genes associated with autophagy in the basal and tip cells which regulate NOTCH/VEGFR2 activation [20]. Noteworthy, only one model has reported the integration hiPSC-ECs and induction of sprouting [21]. We hypothesized that optimizing the microenvironment would enable hiPSC-ECs to develop quantifiable and reproducible sprouts in a 3D microfluidic platform. This hypothesis was partially confirmed. While achieving reproducible sprouting of hiPSC-ECs for parameters such as vessel density and vessel length, the assay's Z'-factor of $\geq 0 < 0.5$ (Ch. 2) suggests that it can be employed to investigate yes/no research questions but needs further optimization for phenotypical analyses or high-throughput studies.

Having established the 3D sprouting angiogenesis model based on hiPSC-ECs, however, we further hypothesized that it could be used for testing the efficacy of inhibitory angiogenic compounds. We applied the tyrosine kinase inhibitors pazopanib and nintedanib, which target the VEGFR2 pathway [22] and found that upon drug treatment, the angiogenic capacity of the hiPSC-ECs was completely abrogated, nintedanib being effective even at 10 nM, and pazopanib inducing partial inhibition at 100 nM, with full inhibition at 500 nM (Ch. 3). This successful pharmacological validation demonstrated that hiPSC-EC in this type of model may pave the road forward in individualized drug testing and phenotypic disease modeling [10,23–25]. This is important due to the capacity of hiPSC to yield large numbers of vascular derivatives over long periods for large and small blood vessel studies [23,26–28]. The approach builds upon several protocols that enable the generation of ECs from human pluripotent stem cells, with demonstrated utility when incorporated into advanced *in vitro* systems [4,29]. However, due to the limited data to date from the available phenotypic cell-based assays, it not yet clear whether such approaches will be amenable to large-scale applications or for comparing in meta-analyses , in particular for drug testing.

The frontiers and limitations of modeling angiogenesis in vitro

Our model recapitulated inducible angiogenic sprouting by employing a combination of angiokines containing a high concentration of VEGF, NOTCH inhibitor (DAPT), Sphingosine-1 phosphate (S1P), and basic fibroblast growth factor (bFGF) (Ch. 2). Similar to primary ECs, hiPSC-ECs are able to sense angiokines and adapt the angiogenic response [30,31]. The sprouting activation in our model is consistent with the use of VEGF-A signaling through VEGFR2, which is crucial to initiate the angiogenic switch [32], and for subsequent lumen formation [33]. Despite the limitation that we did not evaluate the lumen formation, key future research will be to determine if such sprouts develop lumens over an extended culture period. On the other hand, the evident enhanced tip cell formation in our assay was consistent with the DAPT effect, which increases tip cell specification via coregulation of NRP1, ALK1 and ALK5 [34]. Besides VEGF and DAPT, we observed that S1P and bFGF are needed to induce homogeneous sprouting throughout the gel channel of the AimBiotech chip used in the model we employed because S1P enhances

VEGF and cell survival [35], and bFGF stimulates the angiogenic switch and regulates EC behavior through serine- and arginine-rich proteins (SRSFs) and VEGFR1 splicing mechanisms [36]. To date, the two angiogenesis-on-chip models that used different angiokine stimuli are centered on the activation of the canonical VEGFR pathways. While one model stimulated EC sprouting in a monoculture setting by adding half of the VEGF concentration, we employed VEGF combined with other angiokines [19], another system induced sprouting angiogenesis by incorporating fibroblasts secreting VEGF to chemoattract the ECs towards the ECM [20]. Our approach demonstrated that high angiokine concentration is needed when a monoculture of hiPSC-ECs is employed.

Despite the use of an angiogenic cocktail able to activate the above pathway to some extent, our model lacks continuous flow. However, the application of flow might reduce throughput due to the technical challenges and variability in the readouts. As a result, important mechanobiological aspects known to be regulated by flow, such as mechanotransduction of KLF2, which induces the angiogenic switch via VEGF and Egfl7 in areas of low shear stress [37], was not taken into account in the system. This limitation is of interest for HHT research, where ALK1 deletions are the genetic cause of arteriovenous malformations (AVMs), possibly due to abnormal cell polarization and migration defects caused by the increased YAP/TAZ translocation and VEGFR2 signaling due to flow-induced defects [38]. In addition, blood flow activates NOTCH signaling, inducing against-the-flow migration as a safeguard of arterial identity [39], which was not investigated. In our model, we employed the mechanosensory regulator S1P, which is involved in vessel homeostasis through its receptor $S1P_1$. Our static assay did not address the effects of flow-dependent S1P signaling, which is critical to avoid flow perturbances resulting in junctional disarrangement and hypersprouting [40]. Notably, these aspects were not fully recapitulated as we employed temporal, gravity-driven flow and activated sprout induction using angiokines. It will be important in the future to further elucidate the incorporation of active and constant mechanostimuli versus the use of biological stimuli only.

Secondly, we evaluated the sprout responses to two different hydrogels, concluding that hiPSC-ECs perform significantly better in fibrin than in type I collagen (Ch. 2); fibrin was therefore selected for drug testing (Ch. 3). The choice of matrix is critical, as its

physicochemical properties provide essential "biosignals" that shape the angiogenic responses of the hiPSC-ECs [41]. Our finding that fibrin was superior is consistent with the literature showing that fibrin and collagen regulate angiogenesis through distinct mechanisms. Specifically, fibrin is known to synergistically promote the αVβ3 integrin signaling that is essential for robust sprout formation, an effect that is less pronounced in pure collagen hydrogels [42]. Furthermore, the angiogenic response is not just a passive reaction to the matrix but an active process of cell-mediated remodeling, and cells have been shown to remodel fibrin and collagen scaffolds differently, even when their initial stiffness is matched [43]. While our results establish the superiority of a fibrin hydrogel for this assay, other studies are exploring advanced collagen-fibrin blends to further optimize matrix malleability and stability [44]. Therefore, a key direction is testing the combination of such proteins to assess hiPSC-EC sprouting response using this model.

Although our results established pure fibrin as a robust natural matrix for our assay, the inherent batch-to-batch variability of such animal-derived proteins is a known limitation for standardization [41]. This is where alternative synthetic hydrogels represent a more stable matrix source to mimic angiogenesis. These engineered materials offer highly tunable and defined environments, which have been shown to improve invasiveness, stability, lumen formation, gel attachment and matrix degradation [45,46]. Therefore, transitioning our validated hiPSC-EC sprouting model from a natural fibrin matrix to a defined synthetic hydrogel is a critical future direction. This would likely enhance the reproducibility of the assay due to the characterization of the specific matrix-derived "biosignals" that govern angiogenesis.

The third constraint of this model is the lack of mural cells in the hiPSC-EC-based monoculture 3D assay. This absence meant that the EC interplay with mural cells like pericytes or smooth muscle cells, which is crucial for normal vessel morphogenesis [47], was absent. For instance, at a capillary level, pericytes display an Interleukin 6-mediated response important to shape angiogenesis [48]. This is particularly relevant for studying diseases like HHT, where mural cells have been shown to be an important component for rescuing disease phenotypes and preventing vascular leakage in *in vitro* assays [49,50]. It is important that HHT patient-derived hiPSC-ECs is combined with other hiPSC derivatives, especially hiPSC-pericytes, to support the capillary homeostasis or to study

vessel normalization [51,52]. Therefore, while our monoculture model was effective for inducing hiPSC-EC-based sprouting responses and drug testing, the observed phenotypes likely represent early, unstable phases of angiogenesis. The addition of mural cells, particularly pericytes, in future work would be essential to model the later stages of vessel maturation, stabilization, or normalization relevant to HHT.

The fourth limitation is the high coefficient of variation we observed in our hiPSC-EC angiogenic model. In what is sometimes regarded as the gold standard, primary human umbilical vein endothelial cells (HUVECs) are often used; here, batch-to-batch variation is typically low (<2%), and an acceptable criterion for angiogenic assay of ≤15 % coefficient of variation (CV) is attained [53], with a CV reported for tube formation assay of 12% [54]. In our experiments and those of others, but with a different platform, using hiPSC-ECs for angiogenesis models revealed an increase in the CV to 25-70%, which is parameter-dependent (vessel density, vessel length or number of ECs), the sprout/vessel length being the optimal parameter to evaluate such variation [10]. This is comparable to the CV obtained using hiPSC-ECs in our model based on AimBiotech chips (~28-43 % in type 1 collagen and ~32% in fibrin, Ch. 2). The angiogenic competence of hiPSC-ECs is regarded as higher than HUVECs due to their capacity to regulate VEGFR2 activity via epigenetic histone regulators, which prime the hiPSC-ECs to be in a "prone-to-sprout" state during regeneration [55]. To address such variation, it is important to implement quality control of the differentiation protocols, cell source, and cell phenotype, given that there will likely be donor-specific variability. In addition, basic endothelial characterization, along with phenotypic and functional assays such as induction of vessel morphogenesis, colony and tube formation, is employed as standard QA/QC in our group prior to using the cells in advanced models [56,57] such as AoCs.

Lastly, but most importantly, the experimental reproducibility constraints for high-content or high-throughput screening narrow the bottleneck. In our model, we report a Z'-Factor (fit to use to answer yes/no questions), acceptable SW, according to the criteria of Iversen, et al. 2006 [58]. Recently, important advances on standardizing multicellular 3D assays to improve robustness and reproducibility advise reporting specific quality metrics such as CV, Z'-Factor, signal-to-noise and signal-to-background ratios (S/N and S/B), intraclass correlation coefficient (ICC), and strictly standardized mean difference

(SSMD), which are undervalued and commonly not described. Such metrics are important to assess the heterogeneity of the results, especially donor-to-donor variability [59]. Our QC reporting is a critical contribution, as a single study has previously reported the QC metrics for angiogenesis-on-chip using hiPSC-ECs as yielding excellent Z-factor and Signal window (SW) [10], highlighting the gap that our work helps to address.

In our hands, we inferred that manual EC differentiation, non-automated hydrogel preparation, microfluidic device set-up, and subsequent platform handling are factors that account for the increased CV of *in vitro* models when employing hiPSC-ECs. It is important to highlight that these technical factors account for the fact that hiPSC-ECs intrinsically comprises differences not only based on the donor, but also in the differentiation protocol used. For instance, differentiation protocols based on growth factors have revealed important differences in the angiogenic-enriched pathways, like migration, and also displayed a more mature EC progenitor phenotype due to lower ECM remodeling [60]. Furthermore, the isolation of CD31+ cells during differentiation results in less batch-to-batch variability [61]. Therefore, our findings suggest that both manual handling and the known biological heterogeneity of hiPSC-ECs contribute to the variability observed in our experiments.

Altogether, state-of-the-art of advanced models of angiogenesis still have important challenges, especially when using hiPSC-ECs. As discussed in this section, several factors account for this in the type of assays used, from the recapitulation of the angiogenic stimuli in 3D microfluidic devices to the use of multiple donor-derived hiPSC-derived ECs for drug testing. A central challenge is balancing the increasing biological complexity of these models with the need for sufficient throughput and scalability for robust preclinical studies. This is exacerbated by the lack of standards that specify the parameters to quantify and the deconvolution of the readouts these models generate (Figure 1), making it difficult to determine true biological effects from cell- or model-specific artifacts, hindering cross-platform or cell type comparison of the readouts. Although the insufficient attempts to generate specific guidelines on angiogenesis [62], current efforts

are focused on first creating general benchmarks in the organ-on-chip field before embarking in depth into vasculature-on-chip models[6*].

Future directions on drug testing and disease modeling of HHT using in vitro systems

Our hypothesis was that our hiPSC-EC-based AoC model could serve as a functional assay for anti-angiogenic drug testing. To test this, we have implemented hiPSC-ECs in anti-angiogenesis drug testing targeting VEGFR2 inhibition in the first instance (Ch. 3). We found that VEGFR2 inhibitors robustly and dose-dependently reduced angiogenic sprouting. Few angiogenesis-on-chip models to date have reported drug testing even in a low-throughput manner, so that high throughput is more remote at the present time, despite the potential of hiPSC-ECs as an unlimited source of ECs [10,18–20]. The VEGF-mediated signaling through VEGFR2 is a canonical pathway of angiogenesis, which is highly relevant in cancer research where efforts were focused on targeting its inhibition to abrogate tumor growth and survival [22,63,64]. However, a more contemporary strategy is focused on vessel normalization to stabilize the vasculature and improve the hypoxic microenvironment, thus enhancing the delivery of chemotherapeutic drugs and access to the cancer site [65]. This is particularly important in the context of HHT2 because of its dysplastic vascular phenotype in which the regulatory mechanism of angiogenesis that includes the BMP9-ALK1 and VEGFR2 signaling are impaired [66], giving rise to the need for the normalization of abnormal vessels. Hence, targeting VEGFR2 could serve as a therapeutic approach based on the confirmed evidence showing the hyperactivity of the VEGFR2 pathway in HHT2 [67]. Taken together, the hiPSC-EC-based AoC model in which VEGR2 inhibition was demonstrated indicated that the model could be further developed as a tool for identifying angiogenic inhibitory compounds. This may be of interest in vascular diseases like HHT.

6* CEN/CENELEC Focus Group Organ-on-Chip (FGOoC). Organ-on-Chip Standardization Roadmap. 2024.

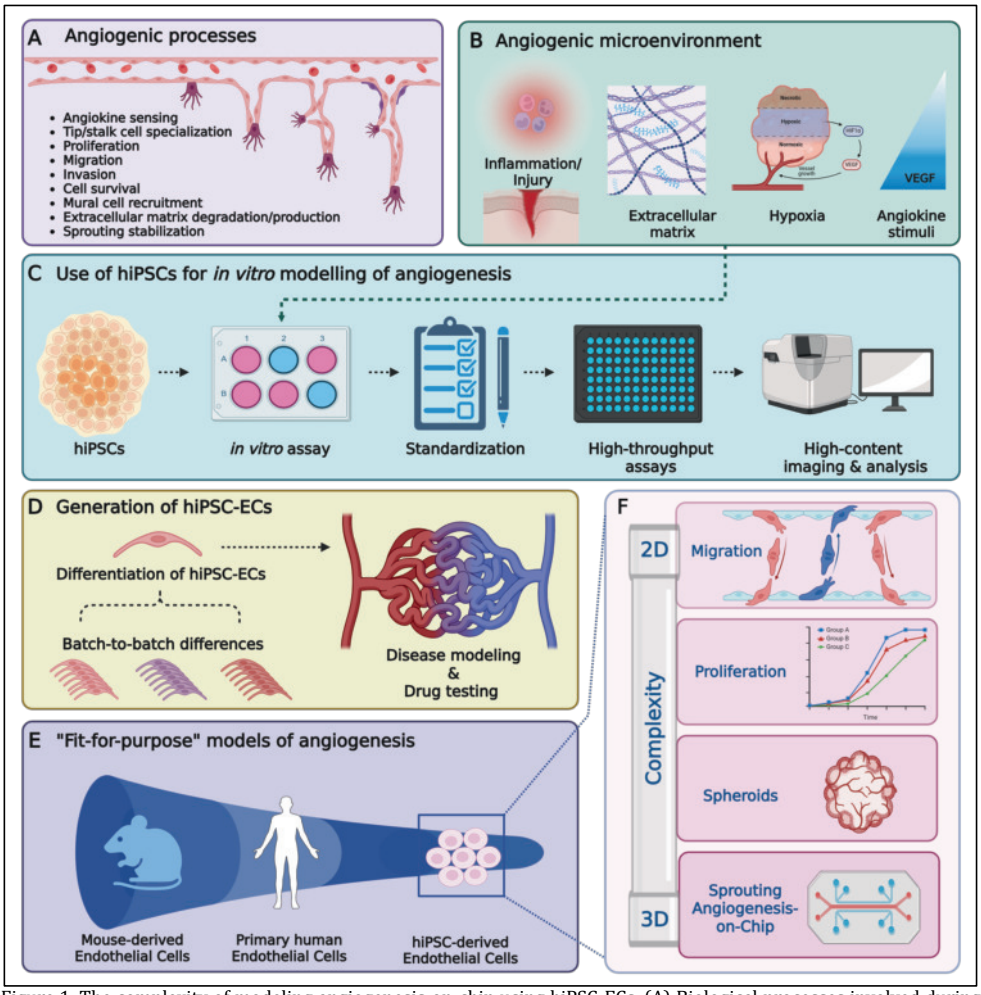

Figure 1. The complexity of modeling angiogenesis-on-chip using hiPSC-ECs. (A) Biological processes involved during sprouting angiogenesis. (B) Microenvironmental cues triggering angiogenesis. (C) Pipeline to the use of hiPSCs and Organ-on-Chip for modeling angiogenesis. (D) Optimization of hiPSC-EC generation for disease modeling and preclinical drug testing. (E) The use *in vivo* and *in vitro* systems to model angiogenesis. (E) The use of hiPSC derivatives employing gold-standard 2D methods towards animal-free 3D angiogenesis-on-chip. Created with BioRender.com.

Next, we hypothesized that hiPSC-ECs derived from HHT2 patients would recapitulate key disease phenotypes in our AoC model. To explore the molecular mechanisms leading to the angiogenic abnormalities found in patients with HHT2, we generated HHT2-hiPSCs from patients harboring mutations in the activin receptor-like kinase 1 (*ACVRL1*) (Ch. 4). Animal models to study HHT physiopathology, in particular the abnormal pro-angiogenic cues leading to AVM development have contributed to the current knowledge of the disease [68,69]. However, there is increasing interest in seeing the effectiveness of

potentially therapeutic compounds in human models. The use of the HHT-patient hiPSC as a source of ECs may become useful to model the vascular pathology of HHT, studying not only the underlying mechanisms but also confirming therapeutic options *in vitro* [27,70]. Having demonstrated that our platform recapitulates sprouting angiogenesis, key directions now include using this tool to dissect the underlying molecular mechanisms of HHT in the search for repurposed or novel therapeutic options.

Our platform provides a useful tool to address a significant gap in HHT research, as the application of hiPSCs to functionally study HHT remains limited. To date only a handful of studies have published the use of patient hiPSC to study the type 1 variant of HHT (HHT1) [27,71,72]; for HHT2, only the generation of hiPSC lines/hiPSC-EC has been published [73,74]. This leaves an important knowledge gap, as the current understanding of AVM development relies heavily on evidence from mouse models that proposes a multi-step process involving genetic and environmental triggers. This "three-event hypothesis" comprises a disease-causing heterozygous mutation [75], followed by a somatic secondary mutation known as the Knudson "2-hit" model [76], and an external vascular insult or trigger that results in abnormal angiogenic cues [77]. Although haploinsufficiency is the accepted pathogenic mechanism for AVM development, this might be an oversimplification, as different *ACVRL1* mutations could lead to distinct functional responses beyond the reduction in protein levels only. This can potentially explain the spectrum of phenotypes observed among patients that animal models cannot capture, limiting their generalizability. Therefore, the potential for patient-derived cells, together with isogenic gene-corrected hiPSC-ECs, could in the future accelerate the elucidation of the underlying mechanisms driving the AVMs development, which are not yet well understood.

The complexity of HHT physiopathology is further highlighted by compelling evidence indicating that a fourth mechanism is involved during AVM development. AVMs tend to arise in mosaic ECs, where ALK1 expression is unevenly lost due to random, heterogeneous depletion across the cell population. In these contexts, a mix of ALK1-intact and ALK1-deficient ECs contributes to the genesis of the AVMs. These vascular anomalies are characterized by elevated expression of genes inducing angiogenesis and vascular remodeling, coupled with decreased expression of those maintaining vessel

integrity, homeostasis, and recruitment of mural cells, which are hallmarks of disrupted BMP9-mediated signaling through ALK1. These findings support a revised model in which AVMs emerge through a cell non-autonomous process, driven by a dysregulated balance between vascular morphogenesis and remodeling [78,79]. This introduces a "fourth-event hypothesis", making HHT physiopathology even more complex to unravel. Therefore, our model offers an advantage because it can enable 3D co-culture of patient-derived mutant and gene-corrected isogenic hiPSC-ECs to investigate the cell non-autonomous mechanisms driving AVMs in a way that animal models cannot.

Despite the heterogeneous penetrance of the HHT-associated mutations, genotype-to-phenotype correlations are not fully predictable as it mainly depends on the mutated locus [80], together with the abovementioned hypothesis. This is precisely where hiPSCs could become valuable, in principle by enabling the creation of libraries of hiPSCs using CRISPR/Cas gene editing [81,82] harboring different mutations found in HHT instead of generating hiPSC lines directly from HHT patients or using primary cells (Figure. 2). Advanced methods allow the insertion of large DNA constructs, fluorescent tags, cassettes for inducible expression and knockouts [83–85]. Therefore, mimicking these non-sequential multi-step mechanisms *in vitro* using these powerful technologies is the current frontier in HHT modeling, moving from single-assay or single-patient observations towards a more comprehensive understanding of the disease.

To replace or not to replace the current gold-standard models of angiogenesis

The evidence on the outcomes of *in vitro* models of angiogenesis is currently insufficient to support arguments for the replacement of current gold-standard methods. To date, non-human systems, particularly animal models, are widely used due to their unique capacity to incorporate the biological systemic complexity, such as perfusion, immune responses, and the superior organ-to-organ interaction needed, which other advanced *in vitro* assays are yet unable to recapitulate. On the other hand, although missing physiologically relevant cues, widely scalable and used conventional 2D assays are still useful in the understanding of specific angiogenic processes like migration, proliferation, invasion and cell-matrix interactions [2,3]. 3D organ-on-chip models, including the one developed in this work, still suffer from significant limitations. As previously mentioned,

the absence of critical cues like continuous perfusion [37,39], EC interplay with mural cells [27,47], and the scarce data reporting QC metrics, limits their wide adoption in academia and industry and thus their translational potential. While these more humane and human-specific models can provide invaluable data, their failure to capture all the complex mechanobiological cues positions them for complementary use rather than a complete substitution of current gold standards for preclinical testing.

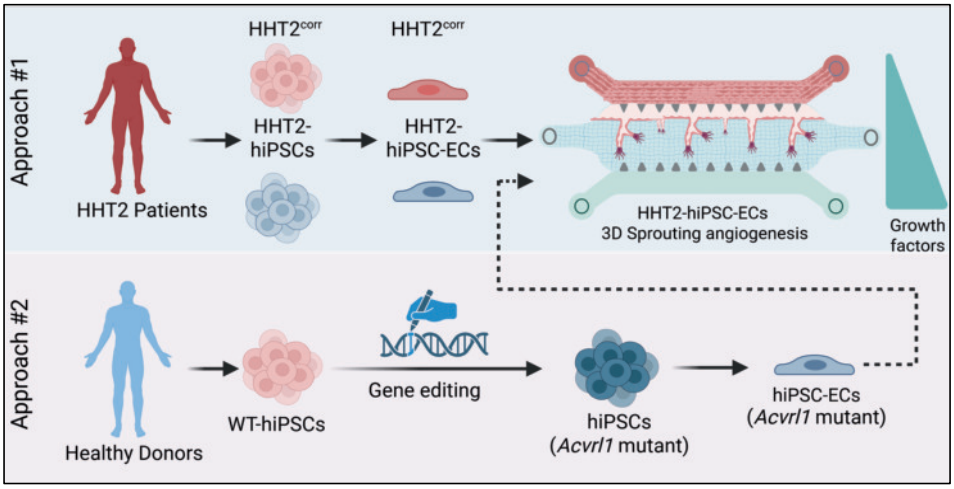

Figure 2. Future directions and strategies for the use of hiPSC-derived ECs and organ-on-chip technology for the purpose of HHT2 research. Created with BioRender.com.

Therefore, the usefulness of emergent assays lies in evaluating their application as fit-for-purpose tools to address in a more specific, faster, and cost-effective manner the research questions unfeasible to elucidate with conventional or animal models. The employment of hiPSC-based organ-on-chip models excels by allowing the study of patient-specific mutations, genetic manipulation, and drug testing [10,57,74,86]. Thus, hiPSCs represent a bridge between reductionist 2D cultures and animal-based studies, providing another layer of human relevance to generate predictive data, ethically sensitive but also translatable directly to human biology. In conclusion, our goal was not the replacement of any current method, but rather the smart integration of organ-on-chip models of angiogenesis to refine and reduce animal research in cases where human biology is paramount.

Final remarks

The field of angiogenesis has journeyed since the 20th century's foundational observations of angiogenesis during tumorigenesis by Dr. Folkman to the mechanistically complex models of today's microphysiological systems. Vascular derivatives of hiPSCs now enable the recapitulation of sprouting angiogenesis, though not without significant hurdles. These bottlenecks emphasize the urgent need for standardization across the field, particularly regarding biomaterials, cell source, stimuli, imaging, analysis, readout interpretation [3,62], especially the use of organ-on-chip technology [87][7*][8*], and the use of hiPSCs in advancing guidelines for drug testing.

This thesis represents a contribution to addressing these needs. Altogether, we have established conditions for modeling sprouting angiogenesis-on-chip employing hiPSC-ECs, functionally validated the model through inhibitory compounds, and ultimately generated hiPSCs from HHT2 patients to provide a platform for future research on the abnormal mechanisms of angiogenesis. Although in the opinion of the statistician George P. Box "*all models are wrong*" [9*], the scientific advancements in *in vitro* models suggest an addendum to this premise: "*all models are wrong only if used assuming they should be perfect replicas of reality but become powerful when employed in a fit-for-purpose manner, aware of their intrinsic limitations*".

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