Published in final edited form as:

Stem Cells. 2014 December ; 32(12): 3037–3045. doi:10.1002/stem.1797.

Concise Review: Tissue-Specific Microvascular Endothelial Cells Derived from Human Pluripotent Stem Cells

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Abstract

Accumulating evidence suggests that endothelial cells (ECs) display significant heterogeneity across tissue types, playing an important role in tissue regeneration and homeostasis. Recent work demonstrating the derivation of tissue-specific microvascular endothelial cells (TS-MVECs) from human pluripotent stem cells (hPSCs) has ignited the potential to generate tissue-specific models which may be applied to regenerative medicine and *in vitro* modeling applications. Here we review techniques by which hPSC-derived TS-MVECs have been made to date and discuss how current hPSC-EC differentiation protocols may be directed towards tissue-specific fates. We begin by discussing the nature of EC tissue specificity *in vivo* and review general hPSC-EC differentiation protocols generated over the last decade. Finally, we describe how specificity can be integrated into hPSC-EC protocols to generate hPSC-derived TS-MVECs *in vitro*, including EC and parenchymal cell co-culture, directed differentiation, and direct reprogramming strategies.

1. Introduction

Endothelial cells (ECs), which line the blood vessel lumen, are not simply passive barriers but instead function as active gatekeepers that dynamically respond to changes in the microenvironment. Positioned at the interface between the circulating blood components and the surrounding tissue, ECs play a critical role in regulating physiological and pathological processes, including control of microvascular permeability, angiogenesis, coagulation, and inflammation. To perform such diverse functions, ECs exhibit a high degree of heterogeneity across developmental stages [1], vascular classes (i.e., capillary, arteriole, or venule), and tissue types.

The microvasculature of various tissues differs on both a structural and functional level in order to meet the specific needs of that tissue. For example, ECs residing in the brain form a continuous, highly impermeable barrier, the blood-brain barrier (BBB), in order to maintain

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Author contributions: H.K.W: conception and design, manuscript writing and editing; S.G.C: conception and design, manuscript writing and editing; E.V.S.: conception and design, manuscript writing and editing; S.P.P.: conception and design, manuscript writing and editing

the delicate biochemical balance necessary for proper brain function. On the other hand, sinusoidal ECs in the liver are highly discontinuous to facilitate toxin clearance from the bloodstream, while glomerular ECs in the kidney help filter contents of the blood to remove waste products.

There exists much interest in generating tissue-specific microvascular endothelial cells (TS-MVECs) *in vitro* for use in both regenerative medicine and tissue modeling applications. Human pluripotent stem cells (hPSCs), specifically human embryonic and induced pluripotent stem cells (hESCs and hiPSCs, respectively), are an attractive source for generating TS-MVECs due to their capacity for extensive self-renewal and ability to differentiate into any somatic cell type. In particular, the ability to derive autologous cells and to study mechanisms of human tissue development *in vitro* makes hPSCs particularly appealing source of TS-MVECs.

Over the last decade, the development and refinement of protocols to differentiate hPSCs to ECs has advanced the understanding of the role that human ECs play in both physiological and pathological tissue states. Recently, several exciting advances have demonstrated hPSC differentiation into ECs that exhibit tissue-specific characteristics. The objective of this review is to summarize these advances and suggest promising directions that may expand the applications of TS-MVECs.

2. Characterizing EC tissue specificity *in vivo*

The ideal stem cell-derived TS-MVEC should match its *in vivo* counterpart as closely as possible in terms of gene and protein expression, structure, and functional characteristics. *In vivo* studies have identified major structural (reviewed in [2]) and functional (reviewed in [3]) differences in capillaries across tissues, suggesting a high degree of heterogeneity of which we are now beginning to identify the molecular basis. The need for *in vivo* TS-MVEC characterization is supported by the observation that in the absence of microenvironmental context *in vitro*, primary cultured ECs de-differentiate, losing expression of up to 50% of their tissue-specific genes [4].

A major function of ECs is the release of tissue-specific angiocrine factors that support tissue homeostasis and regeneration. For example, liver sinusoidal ECs (LSECs) influence liver regeneration via spatiotemporally regulated angiocrine signaling after partial hepatectomy [5, 6]). Ang-2 is initially downregulated [5], while Wnt2 and HGF are upregulated [6] in LSECs after hepatectomy, promoting hepatocyte growth and proliferation, which is followed by the gradual recovery of Ang-2 expression to promote angiogenesis [5]. In bone tissue, there are subtypes of capillary ECs, type H and type L ECs, which differ in their angiocrine responses [7, 8]. Type H ECs release niche signals that support the survival and proliferation of osteoprogenitor cells (including *Pdgfa* and *Pdgfb*), while Type L ECs do not [7]. Interestingly, increasing the number of type H ECs by enhancing HIF1 α levels increased the number of osteoprogenitors and enhanced bone formation [7].

Another major function of ECs is to regulate the permeability of ions, small molecules, proteins, and cells. As one example, the highly impermeable brain ECs comprising the BBB

are characterized by lack of fenestrae, diminished pinocytotic activity, expression of polarized efflux transporters, and high transendothelial electrical resistance (TEER) (reviewed in [9]). In fact, the TEER of brain capillaries has been measured up to $\sim 6000 \Omega \times \text{cm}^2$ depending on the age and species of the animal tested [10, 11], compared to below $20 \Omega \times \text{cm}^2$ in peripheral vessels [12]. Paracellular permeability is mediated by tight junction complexes that are heterogeneously expressed and differentially organized across tissues [13]. For instance, highly impermeable brain ECs express tight junction proteins occludin and claudin-5, whereas highly permeable LSECs do not express occludin and only heterogeneously express claudin-5 [14].

Gene microarrays have proven useful in identifying the molecular determinants of EC tissue specificity [15, 16]. Daneman *et al.* compared the transcriptomes of brain-, lung-, and liver-derived ECs in a Tie2-GFP mouse [15]. By focusing on sets of genes that have functional significance in producing the barrier phenotype of brain ECs, Daneman *et al.* described a number of brain EC-specific genes compared with lung and liver ECs, including tight junction proteins (occludin, Marvel2, Jam4) and transporters from the Slc, Slco, ATP, and ABC transporter families. Pathway analysis of BBB-enriched genes identified the canonical Wnt and retinoic X receptor (RXR) signaling pathways as upregulated in the brain vasculature. Interestingly, both canonical Wnt signaling [17-19] and retinoic acid signaling [20] have been implicated in the induction of brain-specific endothelial properties during development.

More recently, Nolan *et al.* isolated ECs from 9 different tissues in mice via intravital labeling and FACS purification [16]. They found that ECs from distinct tissues display significant differences in their transcriptomes, with the most dissimilar ECs (kidney and testis), only exhibiting an R^2 correlation in gene expression of 0.796, while the most similar ECs (heart and muscle) exhibited an R^2 of 0.976. Further, Nolan *et al.* identified sets of transcription factors, angiocrine factors, and surface markers that were differentially expressed between tissues. For example, the transcription factor SFPI1 was enriched in liver- and bone marrow-derived ECs, the angiocrine factor interleukin 33 was enriched in kidney ECs, and the surface marker CD133 was enriched in brain- and testes-derived ECs. However, clear examples of tissue-specific endothelial markers are rare. Collectively, these analyses indicate that tissue specificity should be defined by a unique combination of genes or proteins rather than a single factor.

3. Stem cell-derived endothelial cells

3.1 Human Stem Cell Sources

There are several distinct stem cell sources for deriving human ECs, including both pluripotent and adult stem cells. Adult stem cell populations, including bone marrow mononuclear cells [21], peripheral blood mononuclear cells [22-26], adipose-derived stem cells [27] and cardiac progenitors [28], have been shown capable of differentiating into ECs. However, adult stem cells are limited in their differentiation capabilities, often consist of heterogeneous populations [29], and in some instances lose proliferative and differentiation capacity with aging [30]. The derivation of hESCs from the inner cell mass of the blastocyst [31] and, later, the generation of hiPSCs from terminally differentiated somatic cells [32]

have overcome several of the limitations of adult stem cells. The ability to utilize hPSC-derived endothelial cells that represent a patient-specific phenotype [33] makes hPSCs a very powerful resource to further understand ECs in both their physiologic and pathophysiologic states and may play a critical role in cellular regeneration.

3.2 Characterization of Stem Cell-Derived Endothelial Cells

There is no single distinct marker of ECs; instead a combination of markers is beneficial in EC identification. The most definitive constitutively expressed endothelial markers include PECAM (CD31), vascular endothelial (VE)-cadherin (CD144), endothelial nitric oxide synthase (eNOS), von Willebrand Factor (vWF), vascular endothelial growth factor receptor-2 (VEGFR2), and Tie-2 [34]. A number of functional assays can be employed to characterize the phenotypes of hPSC-derived ECs. The release of nitric oxide by endothelial cells is critical in the regulation of blood flow and blood pressure *in vivo* [35] and can be measured *in vitro* [36]. The uptake of acetylated low-density lipoprotein is another characteristic of healthy ECs [37]. ECs are activated by inflammatory cytokines, such as TNF- α , inducing an up-regulation of adhesion molecules including ICAM-1, which captures circulating leukocytes and may be vital in paracrine signaling of endothelial progenitor-like cells to damaged vasculature [38]. *In vitro* angiogenic and vasculogenic assays that require endothelial cell proliferation and migration [39, 40] are conducted by seeding VEGF-treated ECs onto growth-factor reduced Matrigel and observing tube-like networks that contain lumens. Such assays can also be conducted *in vivo* by suspending cells into a Matrigel mixture with bFGF and subcutaneously injecting into an immune-deficient animal. The Matrigel plug can later be removed and capillaries identified via expression of endothelial- and species-specific markers.

3.3 Deriving Endothelial Cells from Human Pluripotent Stem Cells

The differentiation of hPSCs to ECs has significantly progressed over the last decade, and select advances are summarized in Table 1. First, primary cell co-culture has been employed in directing hPSCs towards an endothelial cell fate. Both hPSCs [41] and hiPSCs [42] were induced to differentiate into ECs in the presence of the mouse bone marrow stromal cell line OP9. The strong hematopoiesis-promoting activity of OP9 cells may be attributed to differences in Notch ligand expression, protein expression and unidentified paracrine factors, which may contribute to hESC differentiation to ECs. The authors of these studies hypothesized that secreted factors may contribute to directing hPSCs to an endothelial state, raising the possibility that paracrine signaling may regulate the derivation of tissue-specific endothelial cells from hPSCs.

An additional method often utilized to obtain hPSC-derived ECs employs embryoid body (EB) formation. A number of EB protocols utilize growth factors to first induce a CD31+CD34+ progenitor population that, once isolated and exposed to the proper environmental cues, can generate ECs. Levenberg *et al.* demonstrated that after 10 days of culture of hESC-derived EBs, 2% of cells expressed CD31 [43]. The addition of growth factors IGF and EGF in combination with VEGF and FGF2 yielded ~12% CD31+ cells [44]. Rufaihah *et al.* formed EBs from hiPSCs in the presence of BMP4 and VEGF [39]. Following EB formation and 10 days of cell expansion in the absence of BMP4,

approximately 15% of the population expressed CD31. In a recent study by James *et al.*, hESCs were aggregated into EBs and exposed sequentially to BMP4, Activin A and FGF2. On day 4-7, EBs were plated onto Matrigel and treated with VEGF-A. These conditions generated relatively sparse population of ECs as indicated by a VE-Cadherin-GFP reporter (~0.2%). However, inhibition of TGF β at day 7 increased endothelial differentiation efficiency by 10-fold. Subsequent TGF β inhibition of magnetic activated cell sorting (MACS)-purified CD31⁺ cells increased their expansion 36-fold [45]. These studies indicate that manipulation of specific signaling pathways following the initial differentiation to EC progenitors increases cell proliferation and conservation of the EC phenotype, approaches which could prove vital in the derivation of tissue-specific ECs. Pure populations of ECs can be readily obtained using EB differentiation protocols because of the ease of sorting and expanding cells expressing EC markers.

Monolayer-based 2D directed differentiation techniques utilizing small molecules, growth factors, and extracellular matrix proteins have also been designed to differentiate hPSCs to ECs. For example, Wang *et al.* cultured hESCs in a differentiation medium containing VEGF, bFGF, and BMP4 for 10 days, producing CD31⁺CD34⁺ angioblast-like cells that accounted for approximately 20% of the total cell population [46]. Following culture in endothelial growth medium supplemented with VEGF and bFGF, the majority of the cells formed adherens junctions, imported diI-acetylated low-density lipoprotein, and expressed VE-cadherin, CD31, vWF, Tie2 and VEGFR-2. In a similar study by Tatsumi *et al.*, hESCs were differentiated in the presence of a glycogen synthase kinase-3 β inhibitor and VEGF [37]. FACS analysis revealed that approximately 20% of the population expressed VE-cadherin, VEGFR-2, CD34, and CD31. Following VE-cadherin-based MACS purification, the ECs imported diI-acetylated low-density lipoprotein and formed capillary-like tubes *in vitro*. In another comprehensive study by White *et al.*, hiPSCs and hESCs cultured in BMP4, Activin A, VEGF, and bFGF yielded 6-70% VEGFR-2⁺ cells, indicating high variability between cell lines [40]. However, following VEGFR-2⁺ MACS isolation, the ECs could be expanded in EGM media containing EGF, IGF-1, bFGF, VEGF and RA, yielding 95% CD31⁺ cells. The CD31⁺ population imported diI-acetylated low-density lipoprotein, formed capillary-like tubes *in vitro* and *in vivo*, generated NO, and expressed a variety of endothelial markers. These 2D differentiation platforms permit finer chemical control over the microenvironment during differentiation than afforded by co-culture and EB methods, and thus may be more amenable to identifying and optimizing the presentation of specific cues that promote EC differentiation.

Although there has been extensive progress in the techniques and protocols utilized to develop stem cell-derived ECs, additional effort is necessary in understanding how to direct hPSCs to TS-MVECs.

4. Approaches to generate stem cell-derived tissue-specific endothelial cells

It is well accepted that tissue-specific characteristics of ECs are a consequence of the local microenvironment. The first studies demonstrating the notion of “organ imprinting” utilized chick-quail transplantations, which showed that the surrounding tissue was the source of

tissue-specific signals, rather than the invading vessels being driven by intrinsic tissue-specific programs [47]. Since then, a number of studies have demonstrated that microenvironmental cues such as soluble signaling factors [48-50] and extracellular matrix composition [51] contribute to tissue specificity of microvascular endothelial cells. Most recently, several studies have shown that TS-MVECs can be differentiated from hPSCs [49, 50, 52]. Here we review the successful strategies that have emerged to date (summarized in Table 2) and suggest approaches to drive derivation of hPSC-derived TS-MVECs in the future (summarized in Figure 1).

4.1 *In vivo* specification of stem cell-derived ECs

As researchers continue to understand and improve the differentiation of hPSCs to ECs, the question remains whether these ECs can be directed to acquire tissue specificity by providing proper inductive cues. Nolan *et al.* [16] demonstrated this was possible by stimulating mouse ESCs with BMP4, Activin-A, VEGF-A, and FGF2 as previously described by James *et al.* [45] followed by transduction with myrAkt1 [53]. 99.3% of cells in the purified population maintained expression of VE-cadherin and CD31 for four weeks and lacked markers predicted to be upregulated in certain tissues, such as CD133 (upregulated in brain ECs) or VCAM (upregulated in liver ECs) [16].

Next, ECs generated from mESCs in this manner were injected into mice that had undergone a 70% hepatectomy. Surprisingly, mESC-ECs engrafted in the kidney as well as the regenerating liver, suggesting that the surgical procedure created a permissive environment for engraftment in the kidney. Notably, the two mESC-EC populations showed divergent expression patterns, with high VCAM expression present in the liver mESC-ECs only, and elevated Tie2 and endoglin expression in the kidney mESC-ECs only, consistent with global gene expression patterns in primary liver and kidney ECs [16]. Importantly, this suggests that stem cell-derived ECs are responsive to tissue-specific microenvironmental cues. However, expression of only a few tissue-specific markers was assessed in the engrafted cells, and in the absence of further phenotypic and functional analyses these cells would not be considered bona fide liver or kidney ECs. Moving forward, perhaps TS-MVECs can be generated *in vitro* provided that the relevant microenvironmental cues are presented to populations of generic hPSC-ECs in the proper spatiotemporal fashion. While the identities of many of the microenvironment cues that can drive formation of TS-MVECs remain unknown, it is conceivable that once such factors are identified, they can be introduced *in vitro* during or after EC differentiation to induce tissue specificity.

4.2 Generating hPSC-derived TS-MVECs *in vitro*

4.2.1 Co-culture of ECs with parenchymal cells—Co-culture of primary ECs with neural cells, including neural progenitor cells [48] and astrocytes [54], has been widely adopted as a means to enhance BBB-specific properties of ECs *in vitro*. For example, Boyer-Di Ponio *et al.* found that astrocyte co-culture induced human cord blood-derived circulating endothelial progenitors to acquire BBB-specific markers and phenotypes [55]. In one of the few examples of functional hPSC-derived TS-MVECs to date, our group developed a strategy whereby hPSCs differentiate into a mixture of neural cells and ECs, resulting in a population of >60% brain-specific microvascular ECs (BMECs) [52]

(reviewed in [56]). These hPSC-derived BMECs recapitulate many key characteristics of the *in vivo* BBB including expression of tight junction proteins, elevated TEER, and polarized efflux transporter activity. We hypothesized that the co-differentiating neural cell population provides physiologically relevant developmental cues, including Wnt signaling [17-19], that instruct the nascent ECs to become brain-specific.

Although the neural and EC progenitor populations arise concurrently during hPSC-derived BMEC differentiation [52], BMEC barrier and transporter phenotypes are enhanced when hPSC-derived BMECs are co-cultured with cells from the neurovascular unit. Co-culture of hPSC-derived BMECs with primary rat astrocytes increased TEER from $221 \pm 51 \Omega \times \text{cm}^2$ in monoculture to $860 \pm 260 \Omega \times \text{cm}^2$ in co-culture, indicating tightening of the barrier [52]. In addition, a combination of retinoic acid treatment (as discussed in **Section 4.2.2**) and sequential co-culture of hPSC-derived BMECs with primary human pericytes followed by astrocytes and neurons differentiated from human neural progenitor cells achieved a TEER of approximately $5000 \Omega \times \text{cm}^2$ [50], approaching *in vivo* values [11].

While primary cell co-culture with hPSC-derived ECs has the potential to induce EC tissue specificity, the development of hPSC differentiation protocols for various parenchymal cell types, such as cardiomyocytes [57], hepatocytes [58], or renal cells [59], provides the opportunity to construct co-cultures of hPSC-derived ECs and hPSC-derived parenchymal cells. This strategy would permit the development of syngeneic models of ECs and parenchymal cells and represents an avenue to study mechanisms and dynamics of tissue-specification of EC progenitors *in vitro*.

Co-culture may also provide a critical first step in understanding the mechanisms of EC specification by parenchymal cells. For example, proteomic analysis has been used to quantify changes in protein expression in primary bovine brain ECs stimulated by astrocyte co-culture, highlighting astrocyte-induced changes in actin cytoskeleton [60] and the asymmetric dimethylarginine pathway [61]. In addition, genomic analysis of HUVECs co-cultured with fibroblasts identified 323 genes differentially expressed upon co-culture [62]; analysis of the promoters of these genes implicated a number of conserved C promoter binding factor (CBF)1/CSL elements, suggesting regulation of fibroblast/EC interactions via Notch signaling. Ideally, as these signals are identified they can be incorporated into the EC differentiation platform at the appropriate developmental stage to drive tissue specificity.

4.2.2 Directed differentiation of TS-MVECs via microenvironmental cues—

Directed differentiation strategies typically leverage existing endothelial differentiation protocols (described in **Section 3**) and incorporate elements of the *in vivo* microenvironment (i.e., growth factors, signaling proteins, tissue-specific matrices, and/or small molecules) to induce tissue specificity in the ECs. The role of extracellular matrices has been shown to be vital in regulating EC phenotype [63], and could play a critical role in deriving TS-MVECs. Small molecules and growth factors have already been successfully employed in deriving both brain-specific ECs [50] and cardiac-specific ECs [49] from hPSCs.

The first step in adopting a directed differentiation strategy to produce TS-MVECs is the identification of candidate specification cues. For example, retinoic acid (RA) was

hypothesized to enhance the BBB phenotype in the hPSC-derived BMECs for several reasons. First, retinol binding protein receptor *STRA6* has been detected in brain ECs but not peripheral ECs in adult mice, suggesting tissue specificity [64]. Moreover, addition of RA has been shown to upregulate certain BBB characteristics in a human immortalized BMEC cell line and has been suggested to be involved in BBB development *in vivo* [20]. Indeed, addition of RA during the later stages of hPSC-derived BMEC differentiation induced an earlier onset of VE-cadherin expression, enhanced tight junction fidelity, and increased TEER from an average of $228 \pm 57 \Omega \times \text{cm}^2$ to $2940 \pm 800 \Omega \times \text{cm}^2$ [50]. RA-treatment was synergistic with co-culture of human pericytes and human astrocytes and neurons differentiated from NPCs, increasing TEER to approximately $5000 \Omega \times \text{cm}^2$ [50].

In another example, Lui *et al.* [49] developed a method to direct hPSCs to a cardiac-specific EC fate. The authors identified VEGF-A as the most highly expressed growth factor in cardiac ECs and determined that its expression was specific to cardiac ECs. VEGF-A, either exogenously added or transfected into the progenitor population via chemically modified mRNA, drove hPSC-derived multipotent cardiovascular progenitors towards an endothelial fate (comprising 8-10% of the purified progenitor population), and further, these ECs expressed cardiac-specific markers.

Interestingly, while VEGF-A was identified to be highly expressed by human cardiac ECs by Lui *et al.* [49], it was not identified as highly expressed by mouse cardiac ECs compared to other tissues in the microarray database presented by Nolan *et al.* This discrepancy might be attributed to species differences. Additionally, the presence of VEGF-A in the differentiation medium could explain how the hPSC-derived ECs differentiated by Nolan *et al.* acquired expression of select cardiac-specific markers [16]. Also, VEGF-A was highly expressed in cardiac-specific ECs themselves, rather than cardiac parenchymal tissue, suggesting that autocrine signaling may contribute to EC tissue specification.

4.2.3 Tissue-specific endothelial reprogramming—During directed differentiation, microenvironmental cues stimulate a cascade of intracellular signaling that ultimately triggers a developmental program resulting in the expression of tissue-specific genes and acquisition of tissue-specific phenotypes. A more direct approach to generating TS-MVECs may be the forced expression of transcription factors (TFs) that regulate expression of tissue-specific endothelial genes.

One potential strategy toward reprogramming cells to TS-MVECs employs cell reprogramming via TFs that are common among all endothelial cells, followed by exposure of these reprogrammed ECs to microenvironments that impart tissue specificity. Indeed, ECs have been generated via transdifferentiation of human fibroblasts [65, 66]. Both of these studies utilized expression of pluripotency factors – either *OCT4*, *SOX2*, *KLF4*, and *C-MYC* [65] or *OCT4* and *KLF1* alone [66] – in combination with permissive culture conditions to obtain transdifferentiated ECs. In contrast, reprogramming of human amniotic cells to vascular ECs was accomplished via expression of 3 ETS family TFs (*ETV2*, *FLI1*, and *ERG1*), which were shown to induce vascular-specific genes while silencing non-vascular-specific genes [67]. In addition, reprogramming of mouse fibroblasts using 4 TFs implicated in the endothelial-to-hematopoietic transition and hematopoietic stem cell specification

(*GATA2*, *GFI1b*, *C-FOS*, and *ETV6*) induced an endothelial precursor cell phenotype [68]. While these studies have successfully produced reprogrammed ECs, it has not yet been shown that these cells can acquire tissue specificity.

A second potential strategy toward reprogramming TS-MVECs utilizes expression of endothelial tissue-specific TFs, which may be identified by mining genomics data for transcription factors that are heterogeneously expressed across vascular beds [16]. For example, the transcription factor *SFP11* is highly enriched in bone marrow and liver ECs [16], and may potentially regulate expression of bone marrow endothelial- and liver endothelial-specific genes. Ultimately, there may be different approaches to reprogram cells into tissue-specific ECs depending on both input cell type (i.e., EC vs. non-EC) and combinations of TFs (i.e., EC identity factors vs. tissue-specific TFs).

5. Conclusions and Future Challenges

The generation of TS-MVECs derived from hPSCs has the potential to reshape our understanding of endothelial function in health, disease, and development, create opportunities for therapeutic discovery, and offer an EC source that is well-suited for regenerative medicine. Defining the genes, proteins, and phenotypes that constitute endothelial tissue specificity is an active area of research, and the mechanisms directing this specificity are largely unknown. Given the substantial overlap in gene expression patterns between ECs from various tissues [16], researchers should be careful to avoid reducing complex EC heterogeneity to a few markers. Moving forward, the expansion of genomics and proteomics databases in conjunction with the investigation of tissue-specific EC phenotypes *in vivo* will be crucial for defining what constitutes hPSC-derived TS-MVEC behavior *in vitro*.

In this review, we have described various methods to generate TS-MVECs via hPSC differentiation and somatic cell reprogramming, including *in vivo*- and *in vitro*-guided approaches. Ultimately, the optimal approach depends on the desired application. *In vivo*-guided hPSC differentiation or EC maturation does not require *a priori* knowledge of specification mechanisms, which is of particular advantage as *in vitro*-guided processes aim to recapitulate *in vivo* signaling processes. However, ECs differentiated and matured *in vivo* face engraftment, viability, and isolation challenges [69] and are likely subject to the same de-differentiation processes as primary TS-MVECs should they be removed and expanded *ex vivo*. *In vitro* specification methods, on the other hand, could potentially generate the large quantities of purified TS-MVECs required for screening or therapeutic applications. Additionally, *in vitro* specification methods may be more controlled and better defined. However, the ability to derive fully mature, phenotypically relevant TS-MVECs has proven challenging and may be difficult to accomplish in an *in vitro* setting without a deeper understanding of specification mechanisms.

To date, researchers have generated hPSC-derived ECs populations displaying aspects of brain specificity [50, 52] and heart specificity [49]. These exciting breakthroughs provide evidence that hPSCs are indeed capable of differentiating into TS-MVECs that could be useful for scientific and pharmaceutical applications. While hPSC-derived TS-MVECs are

also a promising source of cells in regenerative medicine applications where vascularization is important, several key criteria must first be met. First, molecular and phenotypic hallmarks of tissue specificity in ECs must be identified. Also, epigenetic memory may influence hiPSC differentiation [70] and somatic cell reprogramming [71], thus cell sources for generating TS-MVECs must be carefully considered. TS-MVEC differentiation conditions would ideally be completely defined, and reprogramming methods should utilize non-integrating methods to minimize off-target effects. In addition, hPSC-derived TS-MVECs should be mature and fully differentiated. Finally, protocols to successfully transplant and engraft hPSC-derived TS-MVECs must be developed.

Acknowledgments

This work was funded in part by a National Science Foundation (NSF) Graduate Research Fellowship Program (HKW), NIH grants R01 NS083699 (EVS and SPP) and R21 NS085351 (SPP and EVS), and the Takeda Pharmaceuticals New Frontier Science Program (EVS and SPP).

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Abbreviations

BBB	Blood Brain Barrier
BMECs	Brain Microvascular Endothelial Cells
BMP4	Bone Morphogenetic Protein-4
EB	Embryoid Body
ECs	Endothelial Cells
eNOS	Endothelial Nitric Oxide Synthase
FACS	Fluorescent Activated Cell Sorting
FGF	Fibroblast Growth Factor
hESCs	Human Embryonic Stem Cells
hiPSCs	Human Induced Pluripotent Stem Cells
hPSCs	Human Pluripotent Stem Cells
LSECs	Liver Sinusoidal Endothelial Cells
MACS	Magnetic Activated Cell Sorting
mESCs	Mouse Embryonic Stem Cells
RA	Retinoic Acid
TEER	Trans-Endothelial Electrical Resistance
TFs	Transcription Factors
TS-MVECs	Tissue Specific Microvascular Endothelial Cells
VE	Vascular Endothelial
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor-2
vWF	von Willebrand Factor

Figure 1.

In vivo and *in vitro* approaches to generate hPSC-derived tissue-specific microvascular endothelial cells.

Table 1

Methods of differentiating hPSCs into endothelial cells.

Differentiation Technique	Source	Differentiation Factors	Purification	Expansion Factors	Differentiation Efficiency	Characterization	Reference
OP9 Co-Culture	hESC	N/A	MACs CD34 ⁺ VEGFR-2 ⁺ (~10% of total population)	ESFM media bFGF acidic FGF + heparin	~ 90 % CD144 ⁺ /vWF ⁺	Ac-LDL uptake <i>In vitro</i> vascular tube formation Inflammatory cytokine activation CD144 ⁺ , CD31 ⁺ , vWF ⁺ , eNOS ⁺ , VEGFR-2 ⁺	[41]
	hESC + hiPSC	N/A	MACs CD34 ⁺ CD31 ⁺ (3-6% of total population)	ESFM media bFGF acidic FGF + heparin	N/A	<i>In vitro</i> vascular tube formation CD144 ⁺ , CD31 ⁺ , vWF ⁺ , VEGFR-2 ⁺	[42]
Embryoid Body Formation	hESC	bFGF VEGF IGF EGF	FACs CD31 (12% of total population)	EGM-2 media	~93-97% CD31 ⁺ /CD144 ⁺ /vWF ⁺	Ac-LDL uptake <i>In vitro</i> and <i>in vivo</i> vascular tube formation CD144 ⁺ , CD31 ⁺ , vWF ⁺	[44]
	hiPSC	N/A	MACs CD144 ⁺ (18% of total population)	EC medium	~95% CD144 ⁺	Ac-LDL uptake <i>In vitro</i> vascular tube formation Inflammatory cytokine activation CD144 ⁺ , CD31 ⁺ , vWF ⁺ , eNOS ⁺ , VEGFR-2 ⁺	[72]
	hESC	N/A	FACs CD31 ⁺ (2% of total population)	EGM-2 media	~80% CD31 ⁺	Ac-LDL uptake <i>In vitro</i> and <i>in vivo</i> vascular tube formation CD144 ⁺ , CD31 ⁺ , vWF ⁺ , N-cadherin ⁺	[43]
	hESC	bFGF VEGF	FACs CD31 ⁺ /CD144 ⁺ (10-15% of total population)	EGM-2 media VEGF	~98% CD31 ⁺ /CD144 ⁺	Ac-LDL uptake <i>In vitro</i> and <i>in vivo</i> vascular tube formation CD144 ⁺ , CD31 ⁺ , vWF ⁺	[73]
	hESC	bFGF VEGF					

Differentiation Technique	Source	Differentiation Factors	Purification	Expansion Factors	Differentiation Efficiency	Characterization	Reference
2D Differentiation	hiPSC	BMP4 VEGF	FACs CD31 ⁺ (5-20% of total population)	EGM-2MV media	75-90% CD31 ⁺	Ac-LDL uptake <i>In vitro</i> and <i>in vivo</i> vascular tube formation CD144 ⁺ , CD31 ⁺ , vWF ⁺ , eNOS ⁺	[39] Wilson et al.
	hESC	BMP4 Activin A bFGF VEGF SB431542	MACs CD31 ⁺ (2% of total population)	Advanced DMEM/F12 SB431542	36-fold net expansion (CD144 ⁺ /VEGFR-2 ⁺)	<i>In vivo</i> vascular tube formation CD144 ⁺ , CD31 ⁺ , vWF ⁺ , eNOS ⁺	[45]
	hESC	BMP4 SCF Flt3 IL-3 IL-6 G-CSF VEGF	FACs CD144 ⁺ (10% of total population)	N/A	97% (CD31 ⁺)	<i>In vitro</i> vascular tube formation Inflammatory cytokine activation Endothelial Cell Migration Assay Wound-Healing Assay CD144 ⁺ , CD31 ⁺ , vWF ⁺	[74]
	hESC + hiPSC	bFGF Activin A BMP4 VEGF	MACs VEGFR-2 ⁺ (6-73% of total cell population)	ECM media EGF bFGF VEGF IGF-1 RA	93-95% (CD31 ⁺ /CD144 ⁺)	Ac-LDL uptake <i>In vitro</i> and <i>in vivo</i> vascular tube formation Endothelial Cell Migration Assay Nitric Oxide Measurement VE-cadherin ⁺ , CD31 ⁺ , vWF ⁺ , eNOS ⁺	[40]
	hESC	bFGF VEGF BMP4	MACs CD34 ⁺ (10% of total population)	EGM-2MV media bFGF VEGF	>85% (Adherent/ CD31 ⁺ /CD144 ⁺)	Ac-LDL uptake <i>In vitro</i> vascular tube formation CD144 ⁺ , CD31 ⁺ , vWF ⁺ , VEGFR-2 ⁺ , Tie-2 ⁺	[46]
	hESC	BIO VEGF	MACs CD144 ⁺ (20% of total population)	Stem Pro-SFM media VEGF	95% (CD144 ⁺) 100-fold net expansion	Ac-LDL uptake <i>In vitro</i> vascular tube formation CD144 ⁺ , CD31 ⁺ , VEGFR-2 ⁺	[37]
	hESC + hiPSC	BMP4, GSK3 β Inhibition, VEGF,	FACs CD 144 ⁺ , CD34 ⁺ , CD31 ⁺	EBM-2 Media FGF VEGF DAPT (Notch Inhibitor)	~80% (CD31 ⁺ /CD144 ⁺)	<i>In vitro</i> and <i>in vivo</i> vascular tube formation	[75]
							Page 17

Differentiation Technique	Source	Differentiation Factors	Purification (50% of total population) (50% of total population)	Expansion Factors	Differentiation Efficiency	Characterization	Reference
Direct Reprogramming	Human Fibroblasts	N/A	N/A	EGM-2 media	23-34% (CD144 ⁺ /CD31 ⁺)	CD144 ⁺ , CD31 ⁺ , vWF ⁺ <i>In vitro</i> and <i>in vivo</i> vascular tube formation CD144 ⁺ , CD31 ⁺ , VEGFR-2 ⁺ , eNOS ⁺ , vWF ⁺	Wilson et al. [65]
	Human Fibroblasts	EGM-2 Media bFGF VEGF BMP4	N/A	EGM-2 media SB431542	97% (CD31 ⁺)	Ac-LDL uptake <i>In vitro</i> and <i>in vivo</i> vascular tube formation CD31 ⁺ , CD144 ⁺ , vWF ⁺	[66]
	Human Amniotic Cells	EGM SB431542	FACS CD144 ⁺ , VEGFR-2 ⁺ , CD31 (~35% of total population)	EGM media	99% (CD144 ⁺ /CD31 ⁺)	Ac-LDL uptake <i>In vitro</i> and <i>in vivo</i> vascular tube formation CD31 ⁺ , CD144 ⁺ , VEGFR-2 ⁺	[67]

Table 2
Methods of differentiating hPSCs into TS-MVECs

Source	Type of Tissue Specificity	Factors Influencing Tissue Specificity	Differentiation Efficiency	Reference
hESC + hiPSC	Brain	Neural cell co-culture	>60%	[52]
hESC + hiPSC	Brain	Neural cell co-culture, retinoic acid	>60%	[50]
hESC	Heart	VEGF-A	8-10% of purified progenitor population	[49]