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Vascular Potential of Human Pluripotent Stem Cells

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Abstract

Cardiovascular disease is the number one cause of death and disability in the US. Understanding the biological activity of stem and progenitor cells, and their ability to contribute to the repair, regeneration and remodeling of the heart and blood vessels affected by pathologic processes is an essential part of the paradigm in enabling us to achieve a reduction in related deaths. Both human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are promising sources of cells for clinical cardiovascular therapies. Additional in vitro studies are needed, however, to understand their relative phenotypes and molecular regulation toward cardiovascular cell fates. Further studies in translational animal models are also needed to gain insights into the potential and function of both human ES- and iPS-derived cardiovascular cells, and enable translation from experimental and pre-clinical studies to human trials.

Clinical Need to Control Blood Vessel Formation

The vasculature is a ubiquitously distributed organ system that functions to provide oxygen and nutrients, as well as remove metabolic waste products, throughout the entire body. Disruption of blood vessel formation and/or function plays a central role in the progression of many prevalent disease processes. Thus, the clinical need for controlled blood vessel formation (promotion or suppression) plays a central role in many therapeutic approaches.

In the 1970s, Judah Folkman championed a vigorous campaign to suppress aberrant blood vessel formation for the treatment of angiogenesis-dependent diseases[1] (i.e. cancer, infantile hemangiomas[2], peptic ulcers[3], ocular neovascularization[4], rheumatoid arthritis[5] and atherosclerosis[6,7,8]). Since then, more than ten anti-angiogenic agents have entered clinical trials or have been approved for human use.

Equally challenging has been the effort to develop pro-angiogenic therapies for tissues suffering from chronic or acute ischemia, for tissues engineered ex vivo, and for the vascularization of implanted tissue grafts. Many groups have tried to stimulate endogenous neovascularization using various strategies including systemic administration of pro-

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angiogenic factors, mobilization of bone marrow-derived cells, and transplantation of putative vascular progenitor cells; all with promising, but still limited, results.

In conjunction with the need to optimize strategies to promote neovascularization in vivo, is the need to continuously identify, and evaluate the use of, human stem/progenitor cell types with potential to serve as vascular cell precursors. Since the discovery of human stem cells with pluripotent properties, including human embryonic stem (ES) cells[9] and human induced pluripotent stem (iPS) cells[10], there has been increasing interest in characterizing these cell types and controlling their differentiation towards specific cellular lineages. Herein, we will provide an overview of the vascular potential of human pluripotent stem cells, and endothelial cell differentiation there from.

Vasculogenesis

In situ differentiation of endothelial cells from multipotent progenitor cells, and the formation of endothelial tube networks, is referred to as vasculogenesis. The subsequent stages of blood vessel formation, including network remodeling and recruitment of surrounding mural cells (pericytes and smooth muscle cells), is referred to as angiogenesis. In mammals, extraembryonic vasculogenesis precedes intraembryonic vascular development and is initiated shortly after gastrulation, as cells from the epiblast migrate through the primitive streak and organize into the mesodermal germ layer[11,12]. While mesodermal cells are the most widely recognized source of embryonic endothelial precursors[13,14,15], neural progenitors have demonstrated the ability to differentiate into endothelial cells in certain conditions[16,17]. Nonetheless, the first endothelial cells are derived from mesodermal precursors in the extraembryonic yolk sac in structures referred to as blood islands, which consist of "primordial" (non-specialized) endothelial cells, as well as primitive erythroblasts[18,19,20]. Subsequent coalescence of blood islands contributes to the formation of a plexus of endothelial tubes that is then remodeled into a circulatory network that is invested by recruited mural cells.

Regulation of Endothelial Cell Development

Important insights into the regulation of endothelial cell differentiation have been gained using multiple development models including avian, zebrafish and mouse embryos. Studies, particularly in the mouse model system, have revealed soluble factors and transcriptional regulators involved in endothelial cell development in vivo, as discussed below and summarized in Table 1.

Signaling pathways

In the developing mouse, it is suggested that during yolk sac vasculogenesis, visceral endoderm-derived soluble factors, such as Indian Hedgehog (Ihh)[16,21], vascular endothelial growth factor (VEGF-A)[22], and basic fibroblast growth factor (bFGF)[23] promote endothelial cell development within the underlying mesoderm where their receptors, Ptc, VEGFR2/Flk1 and FGFR2, respectively, are localized[16,24–27]. Other signaling molecules proposed to be downstream targets of Ihh and VEGF-A signaling, such as bone morphogenic protein 4 (BMP4)[16,28], are similarly localized within the mesoderm. While all of these factors have been shown, individually, to be of importance in regulating murine blood vessel formation, the signaling hierarchy among them has not been clearly delineated. Whether similar signals, in a specific hierarchy, also regulate human endothelial cell development has only recently been investigated, and will be discussed in subsequent sections of this review.

Transcriptional regulators

Several transcription factor families have been implicated in endothelial cell development, and presumably function in conjunction with (upstream or downstream) the soluble effectors discussed above.

Ets Transcription Factors—There are at least 19 Ets factors known to be expressed in human endothelial cells[29]; the most widely studied are Ets-1, Elf-1, Fli-1, Tel, Erg and ER71. All characterized endothelial enhancers and promoters contain multiple essential ETS binding sites, and ETS motifs are strongly associated with endothelial genes throughout the human genome[30,31]. Likely due to redundancy among Ets factors in endothelial cell development, germline deletion or mutation of the majority of individual Ets genes in either mouse or zebrafish model systems has resulted in little or no vascular phenotype or has caused defects only in later vascular remodeling, while vasculogenesis remained largely intact[32–36]. One exception is Etv2 (ER71, Etsrp71) that appears to be essential for the development of endothelial and blood lineages in mouse. Expression of early vascular markers, such as VEGFR2/Flk-1, CD31/PECAM-1, and Tie-2 is almost completely abolished in the absence of Etv2[37–39]. Interestingly, while the majority of transcription factors have conserved functions among distinct species, Etv2 appears to be requried for myeloid lineage development in mice and zebrafish, but has no detectable effects on the expression of either myeloid or erythroid markers in Xenopus[40].

Forkhead proteins—Although no Forkhead proteins are specifically expressed in endothelial cells or their known progenitors, targeted disruption of several family members (i.e. FoxO1, FoxF1, FoxC1/2) results in severe vascular phenotypes and embryonic lethality[41–44]. FoxF1 is not expressed within endothelial cells of the differentiated embryonic vasculature, but is expressed earlier in the splanhnic mesoderm prior to endothelial cell specification and may regulate BMP signaling therein[45]. FoxC1 and C2 have important functions in arterial and lymphatic endothelial cell specialization and may be significant downstream effectors of Notch signaling in this process[46,47]

Kruppel-like factors (KLF)—Several KLF factors are known to be expressed in endothelial cells during vasculogenesis and early angiogenesis. KLF2 null mice die by embryonic day E14.5 due to hemorrhage resulting from lack of vessel stabilization and defective tunica media formation. In general, KLF family members appear to function within endothelial cells after initial specification and differentiation have occurred[48].

GATA Factors—GATA factors have long been known to play important roles in blood cell development. GATA2, specifically, has been shown to be important not only for hematopoiesis[49], but for endothelial cell development, as well[50]. In the mouse, GATA2 was shown to signal downstream of BMP4 within mesodermal progenitor cells to induce Scl/Tal-1 expression and differentiation into endothelial and hematopoietic cell types[50].

Scl/Tal-1—Scl/Tal-1 is able to induce paraxial and non-axial mesodermal cells to produce hematopoietic and endothelial cells at the expense of somite and pronephric precursors, indicating that it could act to respecify mesodermal progenitors cells[51]. As mentioned above, Scl/Tal-1 appears to be modulated by BMP4 signaling[52] and has been shown to regulate VEGFR2/Flk-1 and VE-cadherin expression in endothelial cells[53].

Vezf1—Vezf1 is a zinc finger protein predominantly expressed in endothelial cells during early embryonic development[54]. In mice deficient for Vezf1, endothelial cells do not develop appropriate cell-cell junctions or deposit a normal extracellular matrix resulting in hemorrhage and embryonic death[55].

Cell Systems to Study Endothelial Cell Differentiation

Although transcriptional regulators of endothelial cell differentiation have been implicated from embryonic studies, it is often difficult to delineate their precise, and potential interactive, role(s) in vivo due to functional redundancy with other family members or earlier functions within the embryo, including mesoderm formation. Many genes have also been deemed as essential for vascular development if their mutation or deletion resulted in embryonic lethality or caused a significant vascular phenotype in an animal model. However, every genetic manipulation affects not only the targeted gene but an array of functional pathways, whose coordinated function could be difficult to tease out in vivo. Thus, it is increasingly important that we take advantage of in vitro cell culture systems to delineate specific molecular regulators of endothelial cell differentiation from embryonic and adult progenitors, as well as determine the relevance of regulatory pathways identified in animal studies to modulating human endothelial cell formation for clinical therapeutic applications.

Various stem and progenitor cell types, with differing properties and potentials, have been shown to exhibit vascular potential. Although totipotent cells that can form cells of all lineages including the extraembryonic tissue (i.e. mammalian zygotes and early blastomeres[56]) can presumably form vascular cells, they are not typically used for vascular cell differentiation studies. In contrast, pluripotent cells such as ES[9] and iPS[57,58] cells, that have the ability to differentiate into all cell types of the body but not extraembryonic tissues (i.e. placenta), have been generated from human, as well as mouse, embryos and somatic cells, respectively. They propagate well in vitro, and have been adopted by many labs for the study of self-renewal and differentiation, including the differentiation of vascular cells. Multipotent cells, that are thought to be relatively lineage-restricted, such as those isolated from adult blood and blood vessels, also exhibit vascular potential; however, have proved less useful for molecular regulation studies due to heterogeneity and limited cell number. Thus, our review will specifically focus on the vascular potential of human pluripotent stem cells that serve as effective model systems for the study of early molecular events leading to endothelial cell development.

Human Pluripotent Stem Cells

Human ES cells

Since they were first isolated, there have been numerous reports demonstrating the differentiation potential of human ES cells into various derivatives of all three germ layers. Examples include ectodermal cells such as oligodendrocytes and neuroectoderm[59,60], mesodermal derivatives including neutrophils and cardiomyocytes[61,62] and endodermal cell types such as hepatocytes and pancreatic cells[63,64]. Along with these cell types, it has been well documented that human ES cells can generate both endothelial and hematopoietic cells[65–69]. It has been a particular challenge, however, to identify markers specific to vascular endothelium that are not overlapping with hematopoietic cells that develop in parallel. Thus, when studying the differentiation of endothelial cells in vitro, their phenotype is best defined by co-expression of multiple markers (i.e. VEGFR2/Flk-1, CD31/PECAM-1, VE-cadherin), lack of expression of blood cell markers (i.e. CD45), and demonstrated endothelial cell function (i.e. tube formation, eNOS production)[70–74].

Among the methods available for vascular differentiation from ES cells, the most widely used are the embryoid body (EB) formation method and co-culture on monolayers of OP9 cells, which are murine bone marrow stromal cells. EBs are formed by dissociating undifferentiated human ES cells and plating them onto nonadherent plates, then supplementing with cytokines to promote their vascular differentiation and/or proliferation[65,66,75]. The OP9 co-culture method was originally developed for hematopoietic differentiation of mouse ES cells[76] and later adapted for human ES cells[67]. The protocol allows dissociated undifferentiated human

ES cells to be plated directly on top of OP9 cells that have been growth arrested and serve as a feeder layer to promote endothelial cell differentiation. The OP9 co-culture method enables more robust (~5–10%) endothelial cell production[77] compared to the EB method (~1–2%), and requires less cytokine and growth factor supplementation.

A method to maintain human ES cells in an undifferentiated state on a feeder-free layer (i.e. Matrigel) with conditioned medium prepared from mouse embryonic fibroblast cultures was established in 2001[78], and adapted for use with an alternative conditioned media containing cloned zebrafish bFGF[79]. Endothelial cell generation from human ES cells maintained in a feeder-free culture system was achieved by growing the human ES cells on collagen IV-coated dishes with the addition of VEGF-A +/− pituitary extracts[66].

iPS cells

Recently, reprogramming of differentiated adult cells to a state of pluripotency has been achieved using both human and murine fibroblasts[57,58]. In groundbreaking work, two groups independently transduced genes encoding four transcriptional regulators (Nanog, Lin28, Oct4 and Sox2[57] or c-Myc, Klf4, Oct4 and Sox2[58]) in adult fibroblasts to induce a pluripotent phenotype. The resulting induced pluripotent stem (iPS) cells exhibited functional and genetic properties similar to that of human ES cells[80]. More recent reports indicate that induced pluripotency can be enhanced by small molecules such as methylation inhibitors, and can be achieved with as few as two reprogramming factors depending on the cell types used[81].

A shortcoming, with regard to clinical potential, of the initial reprogramming method was the use of retro- or lenti-viruses to transduce fibroblasts. These viruses are integrated into host chromosomes where they can cause insertional mutagenesis, interfere with gene transcription, and induce malignant transformation[82]. In the first report of germline competent mouse iPS cells, 20% of chimeric mice developed tumors that were attributable to the reactivation of the c-Myc proviral transgene that had integrated into the host cell genome[83]. Another group reported cancer-related deaths in 18 of 36 iPS chimeric mice[84]. Thus, several groups have sought alternatives to retroviral gene integration. Yamanaka's group has transfected plasmids without using viruses to generate mouse iPS cells[85]. Two recent papers have shown that mouse and human iPS cells can be produced by piggyBac transposition with four genes in a single plasmid, thus significantly improving induction efficiency[86,87]. Other methods employed Cre-recombinase-excisable viruses[88,89], non-integrating episomal vectors[90], insertion of transducing proteins[91], and replication-defective adenoviral vectors[92].

The process of deriving iPS cells is still not standardized, and there can be variation in the cell lines generated, even when using the same cells and induction protocol. For example, two cell lines derived from same donor using the same four factors show significant differences in hematopoietic differentiation potential, thought to be due to distinct viral integration sites in the clones[93]. Other groups[94], however, that generated iPS cells by transfection with Oct3/4, Sox2, Klf4 +/− c-Myc and compared four human iPS and three human ES cell lines found no obvious differences among the iPS cells lines, and no differences between iPS and ES cells, with regard to differentiation potential when subjected to the same endothelial-derivation protocol. Although the majority of the published studies, to date, emphasize the similarities between human ES and iPS cells, it is important to note that differences in potential do exist and likely reflect differences in regulatory pathways controlling endothelial cell development from these distinct types of human pluripotent stem cells.

Molecular Regulation of Endothelial Cell Differentiation from Pluripotent Stem Cells

Most of our current knowledge about the molecular regulation of endothelial cell differentiation, as summarized above, has come from animal models. Thus, the role of specific factors must be thoroughly investigated in human cell systems before applying such insights to clinical therapeutics. Importantly, there are significant differences between murine and human development that may limit the usefulness of the mouse as a model. Human embryos, for instance, have two phases of extraembryonic endoderm formation and limited reliance on the yolk sac circulation, while the mouse has one phase of extraembryonic endoderm generation and utilizes the yolk sac until birth[74]. There are also known regulatory differences; for example, in humans, TGFβ inhibits the expression of endodermal, endothelial and hematopoietic markers[95], which contrasts with findings in the mouse where exogenous TGFβ (TGFβ or overexpression of TGFβ receptor 2, Tgfβr2) reduces the level of endodermal markers but increases endothelial marker expression.

In addition, there are known differences between human and mouse ES cells. For example, human ES cells express a number of distinct cell surface antigens, exhibit leukemia inhibitory factor independency, and have a relatively long doubling time[95,96]. Therefore, it would not be surprising if there were differences in the molecular regulation of endothelial cell differentiation from mouse and human ES cells, as well as differences in the regulation of human ES and iPS cells. Herein, we summarize what is known about the regulation of endothelial cell differentiation from both types of human stem cells.

Human ES cells

In early studies of the role of VEGF-A in endothelial cell generation from human ES cells, it was determined that addition of this factor to cultures increased the production of endothelial cells[65,66]. Hence, it was suggested that VEGF-A promotes endothelial cell differentiation from human ES cells. However, the differentiation process in these studies was actually induced by other means (i.e. EB formation), and CD31-expressing cells were subsequently isolated from dissociated EB and expanded in response to exogenous VEGF-A. In more recent studies of the role of VEGF-A, as well as bFGF, in human ES cells, we determined that neither factor induces endothelial cell differentiation[77]. That is, when either or both are added to undifferentiated human ES cells, there is no increase in the expression of endothelial-specific genes or proteins. Although this does not exclude a role for either factor in later stages of endothelial cell development, such as survival or proliferation, neither factor appears to regulate the initial commitment to an endothelial cell lineage, which is consistent with studies in mouse ES cells, as previously reviewed[97].

In contrast, we found that other factors such as Ihh and BMP do play an inductive role in the process of endothelial cell differentiation from human ES cells[77]. Ihh is expressed in the murine yolk sac visceral endoderm as early as 6.5dpc[21,98]. Although the specific cellular role for Ihh in murine vascular development is not defined, Ihh-null mutants are embryonic lethal, exhibit impaired yolk sac vasculogenesis and vascular remodeling, and the yolk sacs have fewer endothelial cells[99]. In studies in human ES cells, we found that exogenous Ihh increases the expression of BMP4, VEGF-A, and VEGFR2/Flk1, as well as generation of differentiated endothelial cells; conversely, inhibition of hedgehog signaling suppresses formation of endothelial cells[66,68,100]. Furthermore, inhibitors of BMP signaling abolish the Ihh-mediated effects, indicating that BMP factors likely signal downstream of Ihh to modulate human endothelial cell development. Consistent with this idea, addition of rhBMP4 to human ES cells, in conjunction with hedgehog inhibition, rescues endothelial cell formation to control levels. Collectively, these studies reveal that Ihh signals via the BMP pathway to promote endothelial

cell differentiation from human ES cells[77]. Interestingly, a similar regulatory pathway has been defined for mouse ES cells, in which Ihh and BMP factors are needed to induce endothelial cell differentiation there from, and VEGF-A is necessary thereafter for endothelial cell expansion in culture[97]. Clearly, our understanding of the molecular regulation of endothelial cell differentiation from human ES cells is still very limited and more studies are needed to further dissect the regulatory pathways and factors involved.

iPS cells

The elements involved in the vascular differentiation of iPS cells have just started to be unraveled. The differentiation of human iPS cells toward an endothelial cell lineage can be induced using the same protocols used for the derivation of endothelial cells from human ES cells (i.e. EB and co-culture with OP9 cells), and the endothelial cell yield from human ES and iPS cells appears to be similar[94]. When VEGFR2/Flk-1- and VE-cadherin-expressing cells are sorted from iPS cell cultures and replated in the presence of exogenous VEGF-A, they form network-like structures in Matrigel and exhibit a cobblestone appearance on collagen IV-coated dishes[93]. Thus, there are similarities between human ES and iPS cell lines with regard to potential to differentiate towards an endothelial lineage, but whether the regulatory pathways that govern their differentiation are similar remains to be determined. Studies in both human ES and iPS cells, to date, suggest that factors implicated in endothelial cell differentiation are expressed in both cell systems, but in slightly different temporal patterns during the course of differentiation[65,67,68,101]. Thus, the hierarchy of signaling pathways throughout, and putative intermediate pre-endothelial phenotypes generated during, the differentiation process may differ even though the endothelial cell types ultimately generated from human ES and iPS cells exhibit similar characteristics.

Translation to Clinical Therapy

Cardiovascular disease is the number one cause of death and disability in the US. Understanding the biological activity of stem and progenitor cells, and their ability to contribute to repair, regeneration and remodeling of the heart and blood vessels injured by cardiovascular diseases, is an essential part of the paradigm in enabling us to achieve a reduction in related deaths. In addition, a deeper understanding of the signaling pathways that regulate vascular cell differentiation will enable optimized therapeutic strategies and interventions for many prevalent diseases associated with aberrant blood vessel formation.

Both human ES and iPS cells are promising sources of cells for clinical cardiovascular therapies, as they are able to undergo differentiation towards endothelial cells, vascular smooth muscle cells and cardiomyocytes in vitro[102]. There are increasing efforts to test these cells in cardiovascular injury models to gain further understanding of their potential and function in vivo. In a recent study tracking the fate of human ES cell-derived endothelial cells, survival of transplanted cells into infarcted murine myocardium was detected at 8 weeks post-infarct, and associated with functional improvement and increased vascular density[103]. Human ES cell-derived endothelial cells have been shown to integrate into the host circulation[69], and enhance revascularization in models of hindlimb ischemia and myocardial infarction[103,104]. Similarly, iPS cells have been shown to differentiate into cells of the cardiovascular lineages[105–107], and improve focal cerebral ischemia via subdural transplantation in rats[108].

Although these results are promising, continued studies are needed in both in vitro cell culture systems and in vivo translational animal models to gain needed insights into the potential and function of both human ES- and iPS-derived cardiovascular cells, and enable translation from experimental and pre-clinical studies to human clinical therapies.

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Table 1

Factors Implicated in Murine Endothelial Cell Development

	Soluble factors		**Transcription factors**
Ihh	- Expressed at E6.5 in visceral endoderm[21,109,110] - Required for vasculogenesis	Forkhead Family	- Expressed in the splanchnic mesoderm - Important in the arterial and lymphatic proliferation
BMP4	- Expressed prior to gastrulation - Required for mesoderm formation[28,111–113]	Klf Family	- Expressed at E7 - Required for vessel stabilization[116]
bFGF	- FGFR1 expressed on ectodermal cells at the egg cylinder stage and on migrating mesodermal cells[114] - Controls mesodermal differentiation.	Ets Family	- ER71 expressed at E7.5 - Required for induction of Flk1 expression[37, 117]
VEGFA/Flk-1	- Expressed at E4 in the primitive endoderm, and by E7 expression is restricted to the extraembryonic endoderm and mesoderm[115] - Required for migration and proliferation of endothelial cells	Scl/tal-1	- Expressed at E7.5 in mesodermal progenitors[118] - Regulates Flk-1 and VE-cadherin expression
		GATA 1/2	- Signals downstream of BMP4 to induce Scl expression and endothelial differentiation
		Vezf1	- Required for proper endothelial cell-cell junctions formation