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 pathological 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 preclinical studies to human trials. (Arterioscler Thromb Vase Biol. 2010;30:1110-1117.)

Key Words: angiogenesis ■ endothelium ■ vascular biology

Clinical Need to Control Blood Vessel Formation

The vasculature is an 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 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 (ie, cancer, infantile hemangiomas, peptic ulcers, ocular neovascularization, rheumatoid arthritis, and atherosclerosis). Since then, more than 10 antiangiogenic agents have entered clinical trials or have been approved for human use.

Equally challenging has been the effort to develop proangiogenic therapies for tissues experiencing 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 proangiogenic 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, there is the need to continuously identify and evaluate the use of human stem/progenitor cell types with the potential to serve as vascular cell precursors. Since the discovery of human stem cells with pluripotent properties, including human ES cells and human iPS cells, there has been increasing interest in characterizing these cell types and controlling their differentiation toward specific cellular lineages. Here, we provide an overview of the vascular potential of human pluripotent stem cells and endothelial cell differentiation from them.

Vasculogenesis

In situ differentiation of endothelial cells from multipotent progenitor cells and the formation of endothelial tube networks are 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), are 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. Although mesodermal cells are the most widely recognized source of embryonic endothelial precursors, neural progenitors have demonstrated the ability to differentiate into endothelial cells in certain conditions. 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” (nonspecialized) endothelial cells, as well as primitive erythroblasts. 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 the Table.

Signaling Pathways

In the developing mouse, it is suggested that during yolk sac vasculogenesis, visceral endoderm-derived soluble factors, such as Indian hedgehog (Ihh), BMP4, vascular endothelial growth factor (VEGF-A), basic fibroblast growth factor (bFGF), promote endothelial cell development within the underlying mesoderm, where their receptors, Ptc, VEGF receptor 2 (VEGFR2)/Flk1, and fibroblast growth factor receptor 2, respectively, are localized.

Other signaling molecules proposed to be downstream targets of Ihh and VEGF-A signaling, such as bone morphogenetic protein 4 (BMP4) and basic fibroblast growth factor (bFGF), are similarly localized within the mesoderm. Although 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 E26 transformation-specific (ETS) factors known to be expressed in human endothelial cells; 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. Likely because of 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, whereas vasculogenesis remained largely intact. One exception is Etv2 (ER71, Etsrp71), which appears to be essential for the development of endothelial and blood lineages in mice. Expression of early vascular markers, such as VEGFR2/Flik-1, CD31/platelet endothelial cell adhesion molecule-1, and Tie-2, is almost completely abolished in the absence of Etv2. Similarly, although the majority of transcription factors have conserved functions among distinct species, Etv2 appears to be required for myeloid lineage development in mice and zebrafish but has no detectable effects on the expression of either myeloid or erythroid markers in Xenopus.

Forkhead Proteins

Although no Forkhead proteins are specifically expressed in endothelial cells or their known progenitors, targeted disruption of several family members (ie, FoxO1, FoxF1, FoxC1/2) results in severe vascular phenotypes and embryonic lethality. FoxF1 is not expressed within endothelial cells or their known progenitors, targeted disruption of several family members (ie, FoxO1, FoxF1, FoxC1/2) results in severe vascular phenotypes and embryonic lethality. FoxF1 is not expressed within endothelial cells or their known progenitors, targeted disruption of several family members (ie, FoxO1, FoxF1, FoxC1/2) results in severe vascular phenotypes and embryonic lethality. FoxF1 is not expressed within endothelial cells or their known progenitors, targeted disruption of several family members (ie, FoxO1, FoxF1, FoxC1/2) results in severe vascular phenotypes and embryonic lethality.

Kruppel-Like Factors

Several Kruppel-like factors (KLF) are known to be expressed in endothelial cells during vasculogenesis and early angiogenesis. KLF2-null mice die by embryonic day (E) 14.5 because of 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.
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 but for endothelial cell development as well. 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.

Sel/Tal-1

Sel/Tal-1 is able to induce paraxial and nonaxial 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. As mentioned above, Sel/Tal-1 appears to be modulated by BMP4 signaling and has been shown to regulate VEGFR2/Flk-1 and VE-cadherin expression in endothelial cells.

Vezf1

Vezf1 is a zinc finger protein predominantly expressed in endothelial cells during early embryonic development. 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.

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 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 determining the relevance of regulatory pathways identified in animal studies to modulate 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 extraembryonic tissue (ie, mammalian zygotes and early blastomeres), can presumably form vascular cells, they are not typically used for vascular cell differentiation studies. In contrast, pluripotent cells, such as ES and iPS cells, which have the ability to differentiate into all cell types of the body, have been generated from human, as well as mouse, embryonic and somatic cells, respectively. They propagate well in vitro and have been adopted by many laboratories for the study of self-renewal and differentiation, including the differentiation of vascular cells. Multipotent cells, which are thought to be relatively lineage-restricted, such as those isolated from adult blood and blood vessels, also exhibit vascular potential; however, they may not be as useful for molecular regulation studies because of heterogeneity and limited cell number. Thus, our review specifically focuses 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 3 germ layers. Examples include ectodermal cells, such as oligodendrocytes and neuroectoderm; mesodermal derivatives, including neutrophils and cardiomyocytes; and endodermal cell types, such as hepatocytes and pancreatic cells. Along with these cell types, it has been well documented that human ES cells can generate both endothelial and hematopoietic cells. 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 coexpression of multiple markers (ie, Flk-1, CD31/PECAM-1, VE-cadherin), lack of expression of blood cell markers (ie, CD45), and demonstrated endothelial cell function (ie, tube formation, endothelial nitric oxide synthase production).

Among the methods available for vascular differentiation from ES cells, the most widely used are the embryoid body (EB) formation method and coculture on monolayers of OP9 cells, which are murine bone marrow stromal cells. EBs are formed by dissociating undifferentiated human ES cells, plating them onto nonadherent plates, and then supplementing with cytokines to promote their vascular differentiation or proliferation. The OP9 coculture method was originally developed for hematopoietic differentiation of mouse ES cells and later adapted for human ES cells. 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 coculture method enables more robust (~5% to 10%) endothelial cell production compared with the EB method (~1% to 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 (ie, Matrigel) with conditioned medium prepared from mouse embryonic fibroblast cultures was established in 2001 and adapted for use with an alternative conditioned media containing cloned zebrafish bFGF. 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.56

**iPS Cells**

In the last few years, reprogramming of differentiated adult cells to a state of pluripotency has been achieved using both human and murine fibroblasts.57,58 In groundbreaking work, 2 groups independently transduced genes encoding 4 transcriptional regulators (Nanog, Lin28, Oct4, and Sox259 or c-Myc, Klf4, Oct4, and Sox259) in adult fibroblasts to induce a pluripotent phenotype. The resulting iPS cells exhibited functional and genetic properties similar to those 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 2 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 retroviruses or lentiviruses 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 articles have shown that mouse and human iPS cells can be produced by piggyBac transposition with 4 genes in a single plasmid, thus significantly improving induction efficiency.86,87 Other methods used Cre-recombinase-excisable viruses,88,89 nonintegrating 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, 2 cell lines derived from same donor using the same 4 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, and Klf4±c-Myc and compared 4 human iPS and 3 human ES cell lines found no obvious differences among the iPS cell’s lines and no differences between iPS and ES cells with regard to differentiation potential when subjected to the same endothelial-differentiation 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 such insights are applied 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 2 phases of extraembryonic endoderm formation and limited reliance on the yolk sac circulation, whereas the mouse has 1 phase of extraembryonic endoderm generation and uses the yolk sac until birth.74 There are also known regulatory differences; for example, in humans, transforming growth factor β (TGFβ) inhibits the expression of endodermal, endothelial, and hematopoietic markers,95 which contrasts with findings in the mouse, where exogenous TGFβ 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 (ie, 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 6.5 days post-coitum.21,98 Although the specific cellular role for Ihh in murine vascular development is not defined, Ihh-null mutants are embryonic lethal and 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/Flik1, 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 recombinant human BMP4 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 inhibition, rescues endothelial cell formation to control levels. 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 (ie, EB and coculture with OP9 cells), and the endothelial cell yield from human ES and iPS cells appears to be similar.94 When VEGFR2/Flik-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 toward 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.55,67,68,101 Thus, the hierarchy of signaling pathways throughout the differentiation process, and putative intermediate pre-endothelial phenotypes generated during it, 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 1 cause of death and disability in the United States. 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 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 toward 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 postinfarct and was 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, additional 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 preclinical studies to human clinical therapies.

Disclosures
None.

References


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