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Endothelial Differentiation of Embryonic Stem Cells

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1. Introduction

Embryonic stem (ES) cells are a rich source of multiple different cell types of diverse lineages. Significant advances have recently been made in our understanding of the molecular mechanisms by which ES cells differentiate into endothelial cells. The differentiation of ES cells into endothelial cells can be enhanced by certain growth factors, environmental cues, cell-cell interactions, and extracellular matrix. A wide variety of signal transduction pathways and transcription factors have been shown to participate in this process. The use of ES cells for endothelial differentiation are not only of interest with respect to the molecular mechanisms but also for identifying sources of endothelial cells that can be used for a number of therapeutic purposes. The purpose of this chapter is to review recent advances in the molecular mechanisms underlying ES differentiation into endothelial cells and how endothelial cells derived from ES cells are being used therapeutically.

2. Culture conditions

ES cells are derived from the inner cell mass of a growing blastocyst [1]. As such, they have the capacity to differentiate into all the cell types of an organism [2]. To maintain their undifferentiated state ES cells are generally grown in the presence of feeder cells or leukemia inhibitory factor (LIF). In the absence of inhibitory factors ES cells can spontaneously differentiate into cells with characteristics of one of three germ layers; mesoderm, endoderm, or ectoderm. A variety of approaches have been used to differentiate human and mouse ES cells that include: (1) aggregating ES cells into three-dimensional cell masses called embryoid bodies (EBs); (2) by co-culturing the ES cells with other cell types; and (3) by culturing the ES cells on specific matrix surfaces together with defined media.
Murine ES cells have been used for several years to study differentiation along multiple lineages. Culturing ES cells into EBs has been the most frequently used model to study EC differentiation. The appearance of structures consisting of immature hematopoietic cells surrounded by endothelial cells and the formation of vascular-like channels suggests that the EBs produce factors necessary not only for endothelial differentiation but also for

vasculogenesis. These structures closely resemble the so-called blood islands of the yolk sac. One of the major advantages of this culture method is the highly reproducible timing of molecular events that occurs during the process of differentiation. The induction of both hematopoietic and endothelial markers at precise time points during the differentiation has greatly facilitated investigation of factors that promote or inhibit EC differentiation or hematopoiesis. Their close association has also led to commonly held belief that the hematopoietic and endothelial lineages stem from a common precursor known as the hemangioblast. The markers of hematopoiesis and endothelial differentiation also permit the isolation of specific populations of cells at different stages of differentiation. An example of one of the earliest markers of the endothelial lineage is VE-cadherin, and of the hematopoietic lineage is CD41. One of the potential disadvantages of this model is that only a fraction of the cells, on the order of 5-10%, ultimately become endothelial cells.

Human ES cells have also been used to generate EBs as a model of EC differentiation or hematopoiesis. endothelial cells can be isolated and propagated from human EBs at vary time points during differentiation. The EBs can also be used as a model of angiogenesis in addition to EC differentiation and vasculogenesis. 11-day old EBs are cultured within three dimensional type 1 collagen matrix gels that is supplemented with angiogenic growth factors including VEGF and FGF2 [3]. The sprouting of new vessels can be observed radiating out from the EBs into the surrounding matrix.

A second mouse model of ES cell differentiation is a two dimensional system that takes advantage of the calvaria stromal cell line OP9. ES cells are initially grown as EBs and then a subset of cells expressing VEGF-R2 are separated by flow cytometry. These VEGF-R2 cells are grown on the OP9 cells they gradually differentiate into endothelial cells expressing VE-cadherin and CD31 (PECAM-1). Interestingly, the endothelial cells exhibited two morphological characteristics. On the one hand endothelial cells grew as sheets of cells and on the other hand the cells coalesced into cord-like structures. It was determined that the sheet-like Endothelial cells were of lymphatic origin, whereas the other endothelial cells were more like blood endothelial cells. Two soluble factors made by the OP9 cells that contribute to the differentiation of these cells into lymphatic endothelial cells include Angiopoietin-1 and VEGF-C.

A third method of culturing the murine ES cells into endothelial cells consists of isolating a subpopulation of ES cells that express the VEGF receptor 2 (VEGF-R2) and allowing them to differentiate on an acellular matrix such as type IV collagen [4]. By adding growth factors to a serum free media it was determined that VEGF was a critical factor for differentiating VEGF-R2 expressing ES cells into endothelial cells.

3. Markers of the endothelial lineage

A critical determinant of endothelial differentiation is the identification of selective markers that separate the endothelial lineage from other lineages. Because of the close association between the hematopoietic and the endothelial lineage, the existence of a bipotent cell called the hemangioblast capable of differentiating into either the endothelial or hematopoietic lineages was suggested and identified [5,6]. A commonly used marker to define this cell type is the VEGF receptor 2 (VEGF-R2). Using an embryoid body (EB) model of ES the temporal expression pattern of several endothelial markers was evaluated by PCR and immunohistochemistry [7]. The expression of VEGF-R2 on day three of differentiation

preceded the expression of all of the endothelial markers. The expression of PECAM-1, Tie-1, Tie2, and VE-cadherin, followed shortly thereafter on days 4 and 5 of differentiation. Furthermore, the number of cells expressing these EC-specific markers represented a small fraction of the overall number of ES cells that appeared to coalesce into discreet regions of the EBs and by day 12 of differentiation when they formed primitive vascular structures. The number of cells expressing the endothelial marker PECAM-1 was enhanced by adding the growth factor VEGF on day 6 of ES cell differentiation [7]. We have similarly used the embryoid body model to identify a population of VEGF-R2 expressing mouse embryonic stem cells that also express the endothelial marker VE-cadherin and that are distinct from those that express the hematopoietic marker CD41 [8].

The close association between the hematopoietic and endothelial lineages that is seen during ES cell differentiation has also been observed in a variety of different organisms including the mouse, chicken, zebrafish and humans during embryonic development. In the mouse for example, cells of the hematopoietic and endothelial lineages are first observed in the yolk sac in regions called blood islands. In addition to the yolk sac, the generation of blood is first observed in the developing embryo in a region called the aorta-gonad-mesonephros (AGM), in the vitelline and umbilical arteries, and in the allantois/placenta region [9 10,11]. In the AGM region hematopoietic cells are found in close association with the endothelial wall of the aorta suggesting that these early hematopoietic cells are generated from an early intermediate called the hemogenic endothelium [9,12]. Additional support for the hemogenic endothelium has come from in vitro studies of mouse embryonic stem cell differentiation. Blast colony-forming cells (BL-CFCs) were identified that can give rise to cells of both the endothelial and hematopoietic lineages [5,6]. More recently, studies using time-lapse photography and FACS analysis of differentiating mouse embryonic stem cells, have demonstrated that the hemangioblast generates hematopoietic cells through the formation of a hemogenic endothelium intermediate [13]. In particular, $Tie2^{hi}c\text{-}Kit^{+}CD41^{-}$ expressing cells were identified as a population of cells that constitute the hemogenic endothelium.

Further differentiation of endothelial cells into cells of arterial, venous, and lymphatic endothelium has also been shown to occur in ES cells and do so in the developing embryo prior to the development of blood flow. The differentiation of embryonic stem cells into arterial endothelial cells is dependent on the presence and concentration of VEGF. When mouse ES cells are cultured in the presence of lower molecular weight isoforms of VEGF (120 and 164) they differentiate into endothelial cells expressing arterial markers [14]. The differentiation of ES cells into arterial versus venous endothelial cells is also dependent on the dose of VEGF. Whereas at low concentration of VEGF (2ng/ml) cultured VEGF-R2 expressing mouse ES cells differentiate into endothelial cells expressing venous EC markers such as neuropilin-2 (NRP2), at higher concentrations of VEGF (50 ng/ml) the same cells will differentiate into EC expressing the arterial markers Ephrin B2 and neuropiin-1 (NRP1) [15]. ES cells have also been used study their differentiation into lymphatic endothelial cells. For example, murine ES cells were aggregated to form EBs and then were cultured in a 3-dimensional collagen matrix for up to 18 days [16]. Treatment with a combination of growth factors including VEGF-C and VEGF-A enhanced the formation of lymphatic endothelial cells in the EBs [17]. An alternative approach of promoting the differentiating ES cell along the lymphatic lineage is to use VEGF-R2 expressing ES cells and to co-culture them with OP9 stromal cells [18]. The differentiation of the VEGF-R2 cells into lymphatic endothelial cells is dependent upon VEGF-C and angiopoietin-1.

4. Signal transduction pathways

A variety of signal transduction pathways have been implicated during the process of endothelial differentiation. At the top of the hierarchy of signal transduction molecules are the hedgehog (HH) family of signaling proteins, which play a number of different roles in determination of cell fate, embryonic patterning, and morphogenesis [19]. HH signaling activates the transcription factor GLI. Inhibition of Indian hedgehog (IHH) during the differentiation of mouse or human ES cells blocks their ability to differentiate along the endothelial and hematopoietic lineages [20] [21]. In addition to IHH, several studies have suggested a role for sonic hedgehog (SHH) in endothelial differentiation. In the absence of SHH, angioblasts fail to form into EC tubes or vascular networks [22]. Administration of SHH was able to promote the differentiation of human multipotent adult progenitor cells into arterial endothelial cells both in vivo and in vitro [23].

Bone morphogenic proteins (BMPs) belong to the TGF-beta family of proteins that are involved in regulating cell proliferation, survival, and differentiation during embryogenesis [24]. In particular, BMP-2 and BMP-4 are known mediators of endothelial function and differentiation during embryogenesis and ES cell differentiation [25,26]. BMP appears to function downstream of the HH proteins during the in vitro differentiation of ES cells along the EC lineage. Inhibition of EC differentiation by blocking the HH pathway can be rescued with BMP-4 [20]. Furthermore, when human ES cells are cultured in the presence of BMP-4, this augments their differentiation along the endothelial lineage [25].

BMPs can also activate a number of downstream signal transduction pathways. For example, the induction of angiogenesis by BMP-2 is dependent upon activation of the canonical and non-canonical WNT pathways [26]. BMPs can also activate the MAP kinase signaling pathways. For example, BMP-4 activation of EC sprouting is dependent upon p38 MAP kinase [27]. Inhibition of EC migration by BMPs is dependent upon the JNK and ERK pathways [28]. In contrast to BMP-2 and BMP-4, two BMPs, BMP-9 and BMP-10 inhibit the growth of endothelial cells and promote endothelial quiescence [29,30]. The role of BMP-9 and BMP-10 in endothelial differentiation has not been studied, however in contrast to other endothelial cells, BMP-9 promotes the proliferation of mouse embryonic-stem cell derived endothelial cells [31].

WNT signaling is involved in processes that determine cell fate, self-renewal of stem cells, polarity, and organogenesis. There are three classical WNT pathways: (1) the canonical or WNT/β-catenin pathway; (2) the planar cell polarity pathway; and (3) the WNT/Ca^{2+} pathway [32]. WNT5A is a mediator of EC proliferation, survival, and differentiation. WNT5A is expressed in the developing vasculature of several organs including the skin, retina, stomach, and liver [33]. WNT5A functions predominantly through the non-canonical WNT pathways. Signaling cascades activated by WNT5A include the protein kinase C (PKC) and c-Jun n-terminal kinase (JNK) pathways [34]. WNT5A can also inhibit the activity of the canonical WNT/β-catenin pathway [35]. WNT5A contributes to the regulation of the differentiation of ES cells along the endothelial lineage [36]. WNT5A deficient ES cells cannot differentiate into endothelial cells. In endothelial cells WNT5A can activate both the canonical and non-canonical WNT pathways. The WNT/Ca^{2+} pathway is the predominant WNT pathway activated in WNT5A deficient ES cells exposed to exogenous WNT5A. A role for WNT signaling has also been evaluated during the differentiation of the hemangioblast in human ES cells [37]. Administration of the WNT inhibitor dickkopf1 markedly inhibited the differentiation of ES cells towards the endothelial or hematopoietic

lineages. Likewise, when ES cells were cultured in the presence of WNT1, there was a marked increase in the number of hemangioblast like cells. In contrast, when ES cells were exposed to WNT5A, they did not have the same effect of increasing the number of hemangioblasts, suggesting that WNT5A principally acts at later stages of EC differentiation [38].

5. Transcription factors

A variety of transcription factors are known to play a critical role in cellular differentiation during embryonic development. In particular, selected transcription factors are known to regulate the differentiation of embryonic stem along the endothelial or hematopoietic lineages. As mentioned above, the hemangioblast is a bipotent cell capable of differentiating into either endothelial or hematopoietic cells. The basic helix-loop-helix (HLH) transcription factor SCL (Tal1) has been shown to be critical for blood and endothelial cell development [39]. SCL is expressed early during embryogenesis in hematopoietic and endothelial cells and its disruption in either mouse or zebrafish leads to severe defects in vasculogenesis [40-42]. SCL is also an early marker of the hemangioblast in embryonic stem cells during their differentiation towards the hematopoietic and endothelial lineages [43]. In this model of ES cells were cultured in serum free conditions and sequentially exposed to BMP4, activin A, bFGF, and VEGF. As the mesodermal marker brachyury gradually decreases there is an increase in the expression of SCL together with two other transcription factors Runx1 and Hhex. SCL deficient ES cells are unable to generate either primitive or definitive hematopoietic cells [44]. SCL also appears to be critical for the development of the hemogenic endothelium [13]. SCL deficient ES cells failed to differentiate into $Tie2^{hi}c\text{-}Kit^{+}CD41^{-}$ cells that constitute cells with the capacity to differentiate into hematopoietic and endothelial cells.

Members of the ETS family of transcription factors have also been shown to play a role in the regulation of EC-specific gene expression and EC differentiation. For example, ER71 has been shown to be critical for endothelial differentiation and vascular development in mice and zebrafish [45,46]. In ES cells ER71 is critical for the expression of VEGF-R2 [47]. ER71 appears to induce VEFGF-R2 downstream of BMP, Notch, and Wnt signaling. Another ETS factor that is expressed in the vasculature and regulates hematopoiesis is Fli-1. Fli-1 deficient mice die at embryonic day E12.5 of defective vasculogenesis leading to cerebral hemorrhage [48]. Fli-1 deficient ES cells also exhibit defective hematopoiesis with a marked reduction in the number of blast colony forming cells. In contrast to most other ETS factors we and other groups have shown that the ETS factor ERG exhibits an EC-restricted expression pattern [49-52]. Furthermore, it has also been shown that several EC-restricted genes including VE-cadherin, endoglin, and vWF, are regulated by ERG [53-55]. In addition to its role in regulating EC-restricted genes we have recently shown that ERG is critical effector of EC differentiation of ES cells that appears to be independent of hematopoiesis [56]. ERG was selectively expressed in $VEGF\text{-}R2^{+}VE\text{-}caherin^{+}$ cells and not in $VEGF\text{-}R2^{+}CD41^{+}$ cells. Suppression of ERG in ES cells by lentivirally delivery of shRNA resulted in a significant reduction in EC differentiation but not hematopoietic cells.

There are several transcription factors that facilitate the differentiation of ES cells into arterial, venous, and lymphatic endothelial cells. The critical transcription factor that promotes the differentiation of the hemangioblast or VEGF-R2 expressing cells into venous endothelial cells is COUP-TFII [15]. The differentiation of these cells was dependent upon the dose of VEGF. Whereas low doses of VEGF (2-10 ng/ml) induced the differentiation of VEGF-R2 cells into venous endothelial cells expressing high levels of COUP-TFII, high doses

of VEGF (50 ng/ml) repressed COUP-TFII levels and induced the differentiation of VEGF-R2 cells into endothelial cells expressing markers of arterial endothelial cells such as Ephrin B2. Transcription factors that are preferentially upregulated in arterial endothelial cells include the HLH factors Hey1 and Hey2. Notch signaling appears to be critical for promoting differentiation of ES cells into arterial endothelial cells. Inhibition of Notch signaling with a gamma secretase inhibitor preferentially leads to the expression of venous EC markers and a repression of arterial markers [15]. An environmental stimulus that promotes the differentiation of ES cells into arterial endothelial cells is hypoxia. Exposure of ES cells to hypoxia was associated with an increase in the expression of the Notch ligand Dll4 and the transcription factors Hey1 and Hey2 [57].

The prototypic transcription factor that regulates the differentiation of ES cells into lymphatic endothelial cells is Prox1. Prox1 expression can be induced by three-dimensional culture of murine ES cells into EBs in collagen matrices in the presence of VEGF-C and VEGF-A [16]. Similarly when VEGF-R2 cells were co-cultured with OP9 cells expression of Prox1 was observed in lymphatic endothelial cells on day 3 [18]. Expression of Prox1 in blood endothelial cell leads expression of lymphatic markers and transdifferentiation into lymphatic endothelial cells [58]. Another transcription factor that regulates the expression of the VEGF-R3, which binds to VEGF-C, is the T box transcription factor Tbx1 [59]. Although Tbx1 is not required for LEC differentiation it is required for the growth and maintenance of lymphatic vessels. A transcription factor that is involved in regulating later stages of lymphatic EC maturation is the forkhead transcription factor Foxc2 [60,61]. Foxc2 was shown to be important for the development of lymphatic valves and controlling interactions between lymphatic endothelial cells and mural cells.

6. Therapeutic implications

One of the ultimate goals of developing culture methods that promote the differentiation of stem cells into endothelial cell is to provide a source of cells for a variety of different therapeutic applications. One obvious application would be to generate new blood vessels or repair existing blood vessels in clinical settings where blood flow is compromised. As an initial proof of concept a population of VEGF-R2 expressing mouse ES cells were injected into chicken embryo hearts. These cells were shown to be able to integrate into the host vasculature and differentiate into two cellular components of the vasculature, endothelial cell and smooth muscle cells [62]. Based on these promising studies embryonic stem cell derived endothelial cell have been used in animal models of human disease to promote angiogenesis and ultimately improve blood flow. For example, murine ES cell derived endothelial cell were injected either intramuscularly or intra-arterially in a hindlimb model of ischemia, and were shown to engraft at the site of ischemia and improve tissue perfusion [63]. One of the concerns of using ES cells for therapeutic purposes is the potential formation of teratomas from undifferentiated ES cells. In the hindlimb ischemia study no teratomas formed when ES cell derived endothelial cell were used compared to their uniform development within two weeks when undifferentiated ES cells were used. A similarly promising study was done using human ES cell (hESC) derived endothelial cells [64]. The hESC derived endothelial cells were shown to engraft into blood vessels at sites of myocardial ischemia using a mouse myocardial infarction model. Together these exciting proof-of-concept studies provide evidence that ES cell derived endothelial cells can be used in a variety of settings to promote blood vessel growth at sites of ischemia. One of the major

challenges in using the currently available stem cells as a source of endothelial cells for therapeutic applications is the potential of significant immune responses to the engrafted cells. One potential mechanism of overcoming this hurdle more recently is the development of induced pluripotent stem (iPS) cells in which skin fibroblasts from any individual can be transformed into ES cells by introducing four transcription factors [65]. These iPS cells have subsequently been cultured to promote their differentiation into vascular cells that are very similar to those obtained from human ES cells [66].

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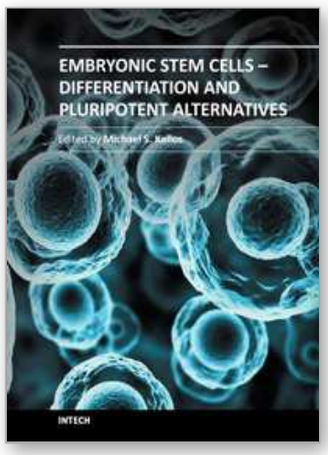

Embryonic Stem Cells - Differentiation and Pluripotent Alternatives
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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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