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Signalling pathways that regulate endothelial differentiation from stem cells

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1. ABSTRACT

Endothelial cells play a vital role in the human vascular system. Injury to this layer of cells can lead to devastating consequences and eventually mortality. As demonstrated by recent accumulating evidences, the injured endothelial layer can be rescued by endothelial cell-based therapy. However, the limited source of functional endothelial cells which can be used in clinical surgery, is hugely hampered. The discovery of pluripotent embryonic stem cells, nevertheless has raised hope for generating endothelial cells in the regenerative medicine field. It was demonstrated that the concerted and coordinated series of specific signaling pathways involving different molecules, guide the differentiation of these embryonic stem cells into functional endothelial cells. Moreover, it is believed that understanding the molecular mechanisms of endothelial development and signal pathways leading to endothelial differentiation from stem cells, will be essential for potential cell therapy for vascular disease. This review therefore, will summarize and discuss recent insights into endothelial development and the signaling pathways regulating embryonic stem cell differentiation towards the endothelial lineage.

2. INTRODUCTION

The human vascular system carries out vital transport of nutrients and oxygen to tissues as well as the removal of metabolic waste products. Endothelial cells (ECs) lining the endothelium of vessels are building blocks of blood vessels and act as a vital interface between the circulating blood stream and the body tissues. ECs play various major physiological and pathological roles, such as subendothelial matrix proteins synthesis, homeostasis, thrombolysis, vasomotor properties, antigen presentation and synthesis of growth factors such as platelet derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1) and fibroblast growth factor (FGF) which promote cell growth (1-3). ECs also play some crucial roles in cardiovascular diseases.

Cardiovascular diseases such as coronary heart disease (heart attack) and cerebrovascular disease (stroke) are the number one cause of global morbidity and mortality. Around 95% of cardiovascular diseases are caused by atherosclerosis that is the most common form of arteriosclerosis (stiffening of medium or large arteries) and a chronic disorder. The damage or dysfunction of the

endothelium leads to the initiation of atherosclerotic-plaque development. When the endothelium is injured by risk factors, adhesion molecules such as VCAM-1 (vascular cell adhesion molecule-1) are expressed on the surface of ECs allowing peripheral circulating monocytes to adhere and infiltrate the intima of the vessel (4). These monocytes subsequently mature and differentiate to form macrophages under the exposure of physiologically active substances such as platelet-derived growth factor and macrophage colony stimulating factor (MCSF), which are released in response to the injured vessel wall (5). The macrophages take up oxidized low-density lipoprotein (LDL) (6, 7) via their scavenger receptors and thereby resulting in the formation of foam cells and the accumulation of cholesterol esters. Formation of foam cells is a classical hallmark of early atherosclerosis.

Clinical atherosclerosis treatments such as balloon angioplasty, stenting and vein bypass graft result in post-operation complications such as angioplasty-induced restenosis, transplant arteriosclerosis and vein bypass graft atherosclerosis. These post-treatment complications are due to the loss and dysfunction of endothelium, with consecutive proliferation of vascular smooth muscle cells (SMC), hence leading to the narrowing of lumen. However, increasing evidence over the past recent years has shown the capability of endothelial progenitor cells in repair of vessel wall in the process of arteriosclerosis (8, 9). Our group has established a model of allograft vessel and we found that circulating progenitor cells have the ability to contribute to the endothelial replacement in arteriosclerotic lesions of allografts. Hu *et al.* also discovered that 30% of these circulating progenitor cells were bone marrow derived (10). Furthermore, our group also demonstrated that ApoE deficiency in our *in vivo* mouse model resulted in hyperlipidemia and subsequently a decrease in the number of the circulating endothelial progenitors (11). Since it was observed that these circulating progenitor cells regenerate the endothelium of vein graft atherosclerosis, the ApoE-deficient mice had enhanced lesions caused by aggravated hyperlipidemia.

Moreover, SMC proliferation, migration and subsequent vascular luminal narrowing after vascular injury could be inhibited with reendothelialisation with VEGFR-2+ or Tie-2+ cells which were capable of differentiating to mature ECs (12). As demonstrated by Xiao *et al*, large quantities of functional and mature ECs could be generated from embryonic stem cells to regenerate the endothelium of denuded vessels in mice and reduced neointimal lesion formation after arterial injury (13). These findings may have implications for a cell-based approach in vascular diseases and hence the next section will discuss the application of embryonic stem cell in vascular therapy.

Currently, cell transplantation therapies to treat vascular diseases are hampered by the lack of donor cells and the ability to acquire a vast number of harvested cells. Moreover, fully matured ECs isolated from patient's blood vessels have limited proliferation and expansion capabilities. Hence alternatives such as the use of embryonic stem cells (ESCs), which have an unlimited self-renewal capability, are increasingly sought after as the new cellular therapy for regenerating damaged areas. Understanding the mechanisms of endothelial development and signal pathways leading to endothelial cell differentiation will be essential for potential cell therapy for vascular disease.

3. ENDOTHELIAL DEVELOPMENT IN EMBRYOS

ESCs have an indefinite lifespan and are pluripotent, which refers to their ability to differentiate into the three embryonic germ layers – the ectoderm, mesoderm and endoderm (14). In addition, their capability in prolonged self-renewal has sparked enormous interest for their therapeutic applications. These applications include the formation of blood vessels, repair of damaged vessels and cell transplantation for the replacement of ischemic tissues. Recent advances have also enabled the possibility of engineering artificial vessels (15). Another beneficial area in using human embryonic stem cells (hESCs), is the creation of a valuable source of human disease model for drug discovery and toxicology *in-vitro* screening studies. Moreover, the availability of hESCs enables the study of the earliest events in human embryogenesis, early vascular development and developmental signalling pathways that regulate endothelial development, thus dismissing the need for using ethically complicated early stage human embryos for *in-vivo* research (15-18). On the other hand, studies have also strongly suggested that *in-vitro* differentiation could be a powerful and reliable model for recapitulating *in-vivo* embryonic development (19-21). Thus, established ESC models could provide valuable insights of complex signalling interactions at the cellular and molecular levels.

Over the past years, numerous reports of utilising ESCs to derive mature vascular ECs have been successfully achieved (22-26). The process of differentiating ESCs into vascular ECs has enabled the study of mechanisms and signalling pathways that govern this crucial differentiation route. In order to fulfil the potential of ESCs as a platform for treating cardiovascular diseases, the understanding of their roles and functional biology in vasculogenesis is pivotal. As such, the following sections will discuss current vasculogenesis studies focusing ESCs as a model system.

3.1. Formation of the hemangioblast

Both vasculogenesis and angiogenesis take place in the early developing embryo, enabling the establishment of the vascular system. In fact, the cardiovascular system is the first organ formed during early embryogenesis and during early stages of embryogenesis, the endothelium is the first tissue to differentiate. Formation of a functional *de novo* vascular network from embryonic mesoderm via the process of vasculogenesis is crucial for embryonic survival and organogenesis (27).

Vasculogenesis is driven by the invagination of epiblastic cells through the primitive streak to form the mesoderm during gastrulation. *De novo* mesodermal cells gradually organize into different segments such as the axial (notochord), paraxial (somites), intermediate mesoderm (kidney), lateral and extra-embryonic mesoderm (28). After

the formation of the coelome, the lateral mesoderm divides into two layers attributing to a dorsal sheet (somatopleural mesoderm) and a ventral sheet (splanchnopleural mesoderm). The extraembryonic mesoderm is situated at the posterior part of the mesoderm. Both lateral and posterior mesoderm which comprises the posterior two thirds of the embryo, were shown by Murray in 1932 to give rise to endothelial as well as hematopoietic cells (29). Formation of endothelial and hematopoietic cells occurs after migration of the newly formed lateral and posterior mesodermal cells toward the yolk sac where the cells differentiate. Florence Sabin was the first who observed under light microscope that migration leads to the appearance of mesdoderm cell clusters or known as hemangioblastic aggregates during the incubation period in living chick blastoderms (30). Cells present at the periphery of these aggregates flatten and differentiate into ECs, while cells in the core of the cluster differentiate into hematopoietic cells. Embryonic ECs aggregate and coalescence to form *de novo* vascular networks via the process of vasculogenesis. Ultimately, both the intra and extra embryonic vessels will form a vascular plexus.

Since both ECs and hematopoietic cells originate in close proximity during embryonic development, the hypothesis of a common vascular precursor or a hemangioblast was postulated (29, 30). However only recently, was the concept of hemangioblast, the putative ancestor for endothelial and hematopoietic lineages was supported experimentally. The hemangioblast concept was proven initially from studies in differentiating mouse embryonic stem cells (mESCs) (31-33) and later in early developing mouse embryos (34). In mESCs, embryoid bodies were spontaneously differentiated and found to give rise to blast colony-forming cell (BL-CFC) which generates both hematopoietic and adherent ECs. Based on kinetic analysis, Choi *et al* showed that the BL-CFC develops early during embryoid body development but is transient and disappears quickly (31). Early mESCs findings have also provided strong evidence that the BL-CFC represents the bipotent mesodermal common precursor of the hematopoietic and endothelial lineages, the hemangioblast (31-33). The identification of the BL-CFC in mESCs differentiation studies was also achieved in gastrulating mouse embryos (34). Similar findings for the presence of the hemangioblast have also been proven in hESCs studies. For instance, Zambidis *et al* derived semi-adherent mesodermal hematoendothelial (MHE) cluster colonies from hESCs, which contained both adherent and nonadherent cells which are capable of giving rise to endothelial and hematopoietic cells respectively (35).

As the hemangioblast differentiates, studies have shown certain similar and different traits between the hematopoietic and endothelial lineages. Genetic and molecular studies found that both lineages share a common set of gene expression such as Fetal Liver Kinase-1 (Flk-1; alternatively known as Vascular endothelial growth factor receptor 2 (VEGFR2) or Kinase-inserted domain containing receptor (Kdr)) and mesoderm-derived transcription factors Brachyury or bHLH transcription factor Tal1 (Scl/Tal1) (36, 37). Moreover an *in-vivo* study involving the mutation of VEGFR2 leads to complete absence of hematopoietic and ECs development (38). Although VEGFR2 is expressed in both early and mature endothelial cells, its expression presence in early hematopoietic cells does not persist in mature hematopoietic lineages. This indicates that hemangioblasts can be determined by the coexpression of VEGFR2 with mesoderm-derived transcription factors Brachyury or Scl/Tal1 (39). But as the EC matures, there is a gradual lost of brachyury expression followed by Scl/Tal1 but VEGFR2 expression remains (40).

3.2. Functional endothelial markers of embryonic stem cell derived endothelial cells

The first *in-vivo* observation of differentiating ESCs into ECs and subsequent vascular morphogenesis by Doetschman *et al* (41), has led to a series of EC differentiation studies carried out extensively in murine embryogenesis, including maturation steps, molecular events, and growth factor involvement(23, 25, 42, 43). *In-vitro* derivation of ECs from ESCs is either achieved by growing embryoid bodies, which closely recapitulates that of early *in-vivo* vascular development or by simply culturing ESCs in tightly controlled chemical conditions over a certain defined period of time. During differentiation, the appearance of specific hemangioblasts or EC markers aids in tracking the transition from the early stages of stem cell differentiation to the mature vessel, as well as distinguishing them from other vascular lineages, such as smooth muscle cells.

The combinations of multiple functional endothelial specific markers and functional assays such as Dil-labeled acetylated low density lipoprotein (Dil-Ac-LDL) uptake and vasculogenesis by matrigel assays are classic hallmarks in identifying and characterizing ESC-derived ECs. Observations under the microscope determined that ESC-derived ECs also acquire cobblestone morphology (44). Many of the EC markers are functionally important and, as well as for endothelial formation, maintenance and remodelling. Expression of PECAM, VEGFR2, VE-cad, endothelial nitric oxide synthase (eNOS), vWF and Tie1 were detected in a time-dependent manner from the early stages of ESCs differentiation to mature vessels via immunofluorescence staining and gene expression analysis in mouse EBs (25). Vittel *et al*, reported that VEGFR2 mRNA occurred at day 3 of differentiation whereas later stage markers such as VE-cad and Tie1 mRNAs were detected at day 5. VEGFR2 has been regarded as one of the earliest markers to appear during the endothelial linage development and has also been reported by other groups to appear around day 3-4 of differentiation (42). Also, because VEGFR2 is expressed by angioblasts as well as mature ECs, it cannot be used solely to distinguish between different stages of EC differentiation. On the other hand, expression of VE-cad and vWF are known as late or mature EC markers. The employment of functional EC markers has greatly contributed to the derivation of EC from ESCs. Henceforth, this further aids in the discovery of crucial mechanisms and signalling pathways, which influence ESC differentiation towards the EC lineage (Figure 1).

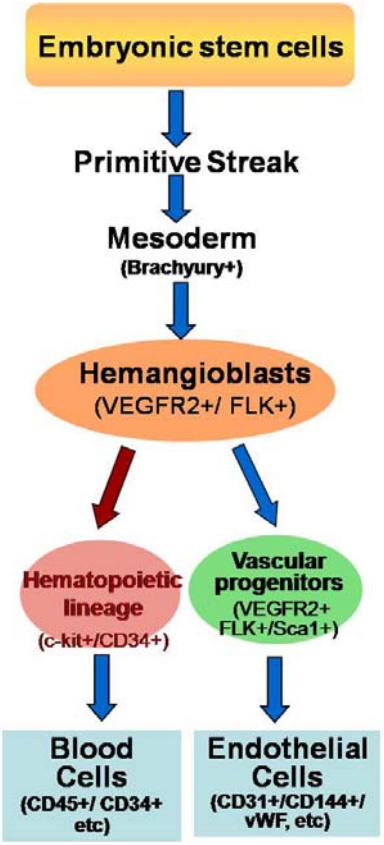

Figure 1. A diagram depicting the process of differentiating embryonic stem cells towards the endothelial lineage. The primitive streak and mesoderm layer can be induced from embryonic stem cells under appropriate differentiating conditions. Sorted VEGFR2+ cells can be differentiated into either vascular progenitors or hematopoietic cells. Eventually, vascular progenitor cells can be induced under growth factor differentiating conditions to derive ECs.

4. SIGNALLING PATHWAYS THAT REGULATE STEM CELL DIFFERENTIATION TOWARDS THE ENDOTHELIAL LINEAGE

In the early events of endothelial differentiation, different signalling cues play a role in the initiation of the development cascade. The release of signaling protein molecules such as VEGF, Wnts or Bone Morphogenic Proteins (BMPs) from a distinct cell population enables the trigger of one or more series of intracellular signaling cascades from their distant neighboring cells. A good grasp of knowledge in understanding the mechanisms that control the signaling events pertaining to early and late developmental progress of ECs in differentiating ESCs studies, can also offer insight as to how one might influence or stimulate these signals for therapeutic advantages.

4.1. Vascular Endothelial Growth Factor (VEGF)

Initiation of vascular development also requires VEGF-A, a secreted protein with high specificity for the endothelium and the most critical driver of early vascular formation. Its vital regulatory role in adult vasculogenesis and angiogenesis has been well-described (38, 45, 46). VEGF-A induces differentiative, proliferative and chemotactic responses from ECs, as well as promoting the coalescence of ECs into primitive vascular structures (47, 48). *In-vivo* models of mice lacking a single VEGF-A allele exhibit severe defects in vascular development and die early at mid-gestation (48). There are 5 members of the VEGF family, VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PLGF). In this context, VEGF-A is the most important member and it interacts with both specific tyrosine kinase receptors, VEGFR1, or known as Flt-1 (Fms-like tyrosine kinase-1) and VEGFR2. Several human alternatively spliced VEGF-A isoforms have been discovered. These are namely, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$ which range in length from 121 to 206 amino acid residues (49-54).

VEGF-A also binds to the semaphorin co-receptor, neuropilin-1 (NP1), with high affinity in a complex which promotes VEGF binding to VEGFR2 (55, 56). Both neuropilin coreceptors (NP1 and NP2) are transmembrane non-protein tyrosine kinase co-receptors which are reportedly required for yolk sac developmental and angiogenesis (57).

4.1.1. VEGFR2-phospholipase C-gamma and Ras signaling

VEGFR2 is the major receptor in ECs for VEGF-induced responses (45, 58, 59). The first expression of VEGFR2 is detected at embryonic day 7 (E7) in murine mesodermal yolk sac blood island progenitors. Its expression subsequently confers during later stages of endothelial development, in progenitors and developing cells (45, 46). VEGFR2 plays a pivotal role in early endothelial development as highlighted by a study using VEGFR2-/- mouse embryos, which die at E8.5-9.5 due to early defect in the development of hematopoietic and ECs (38). Moreover, VEGFR2 -/- mESCs were not capable of differentiating into ECs, suggesting that VEGF effects on ECs are mediated through VEGFR2 (60). VEGFR2 is also a marker for the hemangioblast, a common progenitor cell that can differentiate into hematopoietic and endothelial lineages (31, 61).

VEGF-A being a mitogen for ECs, interacts with VEGFR2 which, like most receptor tyrosine kinase receptors, induces cell proliferation via phosphorylation of the classical Ras-independent activation of extracellular signal-regulated kinases (Erk) pathway via a protein kinase C (PKC) -dependent pathway involving activation of phospholipase C-gamma (PLC-gamma) (62). The significance of PLC-gamma in vasculogenesis was highlighted in PLC-gamma1 deficient mouse embryos which die at approximately E9.0 with significantly diminished vasculogenesis and erythropoiesis (63). Furthermore, Sase *et al.* demonstrated that PLCgamma1 interacts with Y1175 (Figure 2) which is one of the major sites of phosphorylation of VEGFR2 (64). They also demonstrated that this VEGFR2-PLCgamma1 signalling

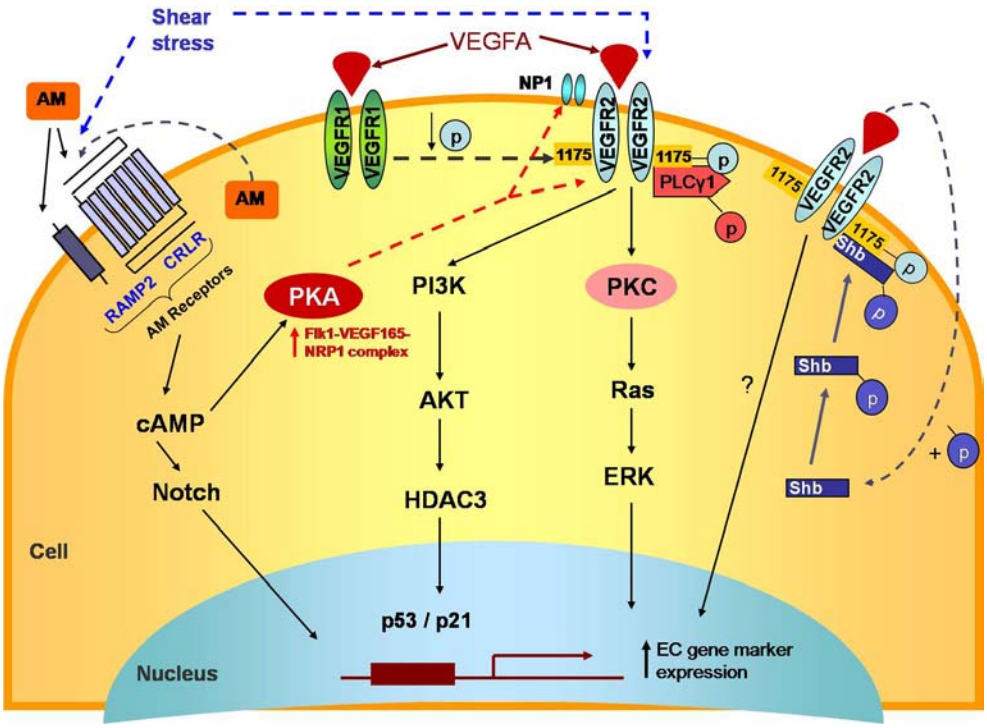

Figure 2. VEGF and its related signaling pathways that influence the differentiation of embryonic stem cells into ECs. VEGFR2-PLC gamma1 signaling relay via VEGFR2 Y1175, a major phosphorylation site in VEGFR2 and the downstream Ras signaling pathways to promote the expression of endothelial markers in differentiating stem cells. Whereas, Shb influences VEGFR2 signaling mainly via the binding of Shb SH2 domain to the phosphorylation site Y1175 in the activated VEGFR2. However, negative modulation by VEGFR1 results in the reduction of tyrosine phosphorylation of VEGFR2, striking a balance between phosphorylation and non-phosphorlyation of VEGFR2. Also, presence of adrenomedullin (AM) stimulates cAMP signaling which subsequently activated Notch signaling and enhanced VEGF-induced EC differentiation from VEGFR2+ vascular progenitor cells. The resultant increased levels in cAMP activate PKA, which induced the formation of the VEGFR2-VEGF165-NP1 complex. The VEGFR2-VEGF165-NP1 complex boosted VEGF signalling to efficiently induce EC differentiation and vascular formation. Shear stress upregulates HDAC3 via Flk-1-PI3K-Akt signal pathways and mediated p53 deacetylation and p21 activation which are crucial for shear- and VEGF-induced EC differentiation.

acts via VEGFR2 Y1175, and is essential for endothelial lineage specification from mESCs-derived VEGF2+ vascular progenitor cells. After activation of VEGFR2-PLCgamma1 signalling, it subsequently induces a downstream Ras activation pathway as shown by the abolishment of endothelial specification induced by PLCgamma1 signalling using a H-Ras pharmacological inhibitor (64) (Figure 2). Additionally, as shown in a separate study, the inhibition of H-Ras function using pharmacological inhibitor and knockdown techniques results in selective suppression of VEGF-A-dependent endothelial specification of mESCs derived VEGFR2+ vascular progenitor cells (65). Henceforth, the VEGFR2-PLC gamma1 signalling, and subsequent activation of VEGFR2 Y1175 and the downstream Ras signalling pathways play important cues in directing the EC lineage specification from embryonic stem cells derived vascular progenitor cells.

4.1.2. Shb mediated signalling via VEGFR2

Shb is a ubiquitously expressed adaptor protein with proline-rich motifs in its N terminus, a central phosphotyrosine binding domain and several tyrosine phosphorylation sites (66, 67). It also has a Src homology 2 (SH2) domain in the C-terminus. Stimulation of Shb with PDGF, FGF-2 and VEGF results in the tyrosine phosphorylation of Shb and its subsequent downstream binding to receptors such as PDGFR (68), FGFR-1 (68, 69) and VEGFR2 (70) via its SH2 domain.

Shb has been reported to promote blood vessel formation in embryoid bodies derived from mESCs by amplifying VEGFR2 and PDGFR-BB signalling (71). In this study, Rolny *et al.* overexpressed wild-type Shb adapter protein in mESCs, which promoted the outgrowth of vascular structures in differentiating EBs. The observed blood vessel formation partly resulted from the upregulated expression of VEGFR2 in Shb EBs. However, it remains uncertain how Shb promotes the expression of VEGFR2 in these cells. Shb influences VEGFR2 signalling mainly via the binding of Shb SH2 domain to the phosphorylation site Y1175 in the activated VEGFR2 (70) (Figure 2). It also appears that other than VEGFR2, Shb also promoted PDGFR-BB signalling in Ebs, which further aid in the maturation of endothelial precursor cells towards the EC lineage (71).

4.1.3. Interplay between VEGFR1 and VEGFR2

Apart from cell proliferation, other cellular responses mediated from VEGF signalling through VEGFR2 include EC migration and cell survival (58, 59). However, unlike VEGFR2, VEGF-A binding to VEGFR1 is not required for EC mitogenesis but essential for the organization of embryonic vasculature (72, 73). This is evident in VEGFR1-/- mice whereby the mutation of VEGFR1 in these transgenic mice resulted in death at mid-gestation with disorganization and vascular overgrowth (72). Moreover, studies involving embryos and differentiating mESC cultures implied that VEGFR1 also regulates vascular blood vessel formation by down-modulating the rate of EC division/proliferation (74). This negative modulation by VEGFR1 was shown to affect the VEGFR2-mediated signalling pathway by reducing its level of tyrosine phosphorylation and hence EC proliferation (75). Nevertheless, whether this negative modulation by VEGFR1 takes place during early (mesoderm formation) or late (vascular progenitor cells) differentiation is still unknown.

4.1.4. Protein Kinase A

Protein kinase A (PKA) or otherwise known as cAMP-dependent protein kinase, is a family of enzymes whose activity is dependent on the level of cellular cyclic adenosine monophosphate (cAMP). Activation of PKA and cAMP signalling in ECs can be induced by factors such as adrenomedullin and mechanical shear stress (76, 77). Yurugi-Kobayashi *et al.* discovered that adrenomedullin, an endogenous ligand-elevating cAMP, enhanced VEGF-induced EC differentiation from VEGFR2+ vascular progenitor cells (78). The stimulation of cAMP signalling subsequently activated Notch signalling in these differentiating ECs (Figure 2). Moreover, shear stress, a mechanical force generated by fluid flow, can also induce ESC differentiation towards the EC lineage. It was reported that fluid shear stress promotes EC differentiation from VEGFR2+ vascular progenitor cells derived from mESCs by up-regulating VEGFR2, VEGFR1 (Figure 2), vascular endothelial cadherin, and PECAM-1 expression (79). These studies indicated that PKA signalling might play a role in the differentiation of vascular progenitor cells towards the EC lineage.

Indeed a study demonstrated by Yamamizu, *et al.* has revealed that PKA activation significantly promoted the differentiation of mESCs derived VEGFR2+ vascular progenitor cells towards the EC lineage as well as vascular formation (80). The activation of PKA increased both VEGFR2 and NP1 expression (Figure 2) in these VEGFR2+ cells and induced the formation of the VEGFR2-$VEGF_{165}$-NP1 complex. This subsequently enhanced the "sensitivity" of the vascular progenitors to $VEGF_{165}$, which boosted VEGF signalling to efficiently induce EC differentiation and vascular formation.

4.2. Histone Deacetylases (HDACs)

Both histone acetyltransferases (HATs) and histone deacetylases (HDACs) are important regulators of chromatin structure and function. Since differentiating ESCs undergo complex gene-specific and chromatin structure remodelling, reports have pointed out the participation of some HDACs in ESC differentiation. Also, the inhibition of HDACs prevents the differentiation of ESCs (81).

Our group has demonstrated that HDAC3, a member of the HDACS is essential for differentiating stem cell antigen-1 positive (Sca1+) vascular progenitor cells derived from mESCs towards the EC lineage and in the presence of shear stress and VEGF (82). Luminal shear stress is generated by blood flow and has been associated with endothelial lineage determination from vascular progenitor cells. We found that luminal shear stress could increase differentiation of ESCs or Sca1+ cells toward ECs, which were fully capable to form vascular tube-like structures on Matrigel compared with static control. Furthermore, we also noticed that HDAC3 was up-regulated by shear stress, causing enhanced EC differentiation from Sca1+ cells. During differentiation, shear-induced HDAC3 also mediated p53 deacetylation and p21 activation as these observations were abolished when HDAC inhibitor TSA, was applied. It was also revealed that shear activated HDAC3 by posttranslational stabilization through Flk-1-PI3K-Akt signal pathways (Figure 2). Moreover, HDAC3 was also important in VEGF induced EC differentiation. Therefore, we proposed that HDAC3-mediated p53 deacetylation and p21 activation are crucial for shear- and VEGF-induced EC differentiation (82).

In a separate study, it was found that Sca1+ vascular progenitor cells could differentiate into functional ECs via activation of HDAC3 (13). These mature ECs were later infected with adenovirus-LacZ and injected into denuded femoral artery of mice. It was revealed that these cells accelerated re-endothelialization of injured arteries and a 73% reduction in neointimal area was observed 2 weeks after injury (13). From these findings, it was concluded that HDAC3 plays an important role in signal transduction for deriving functional ECs from vascular progenitor cells.

4.3. Wnt

Wnt is a large family of cysteine-rich secreted proteins involved in an array of diverse processes such as embryonic growth, migration, and differentiation (83, 84). Among the many different pathways that Wnts are known to activate, the Wnt canonical pathway is the most well described. It involves Wnt binding to Frizzled and LDL receptor related protein (LRP) 5/6 co-receptors on the plasma membrane, which induces beta-Catenin protein stabilization and entry into the nucleus (Figure 3) where it subsequently activates the transcription of target genes such as *Msx1, Msx2, fibronectin, cyclinD1, cyclinD2* and *Myc* (23, 85, 86). Non-canonical Wnt signalling is, however primarily mediated through the Frizzled receptors, inducing c-Jun N-terminal kinase (JNK) and PKC pathways (87, 88).

In vivo studies have shown that canonical Wnt signaling is required for primitive streak formation (89). Beta-catenin or Wnt co-receptors Lrp5/6 deficient mice fail to develop a primitive streak and hence the mesoderm layer

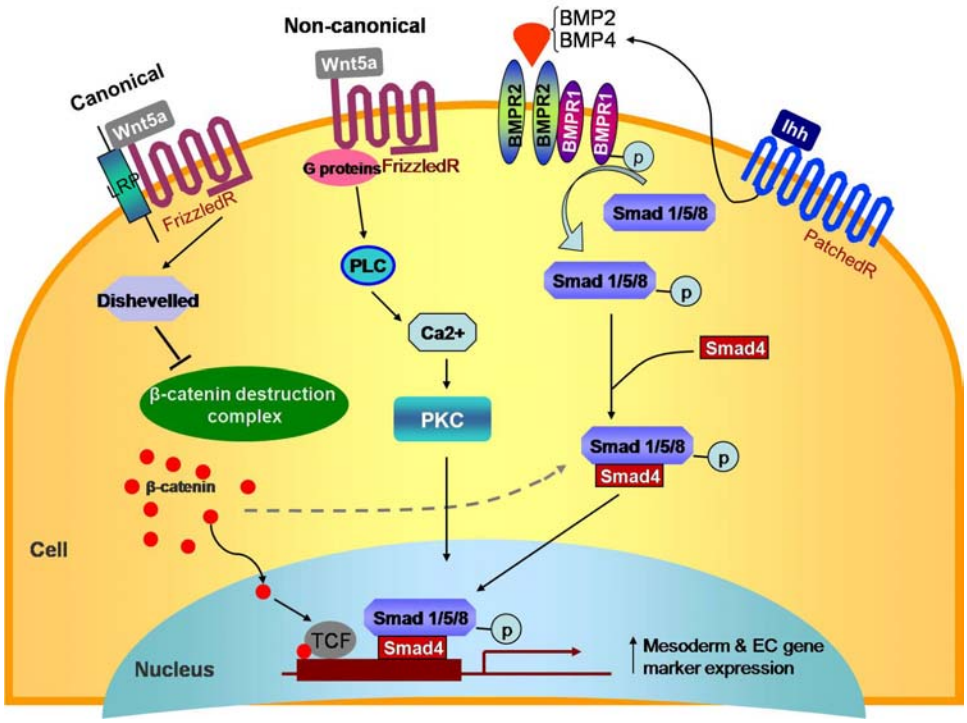

Figure 3. An overview of the involvement of Wnt and TGF-beta signaling in the differentiation of ESCs towards the EC lineage. In canonical Wnt signaling, the Wnt ligand, Frizzled receptor (FrizzledR) and LRP form complexes to activate a cytosolic protein called Dishevelled. Activated Dishevelled inhibits the beta-catenin destruction complex and thus increases the stabilization of beta-catenin by escaping destruction via proteasomes and then accumulates in the cytosol and nucleus. In the nucleus, beta-catenin forms a complex with T-cell factor (TCF) proteins that activates the transcription of specific target genes which drives mesoderm and EC gene expression. Canonical Wnt signaling may simultaneously interact with other signalling pathways such as BMP during the development of the mesoderm layer. Wnt5a has been reportedly able to function through both canonical and noncanonical pathways via beta-catenin and Protein Kinase C (PKC) respectively in the differentiation of VEGFR2 cells towards the EC lineage. Non-canonical Wnt signaling is primarily mediated through FrizzledR. In TGF-beta signaling, presence of Indian hedgehog (Ihh) binds to its patched receptor (PatchedR) to activate BMP4 signaling. During activation, BMP receptor type 2 (BMPR2) phosphorylates type I receptors (BMPR1) to form a complex. This complex phosphorylates the receptor Smads 1, 5 and 8, to form heteromeric complexes with Smad4. The complex subsequently translocates into the nucleus and increase the expression of mesoderm and EC genes.

(90, 91). In mESCs-derived CD4-Foxa2+GFP-Bry+ cell population, Wnt and TGF-beta/nodal/activin pathways were shown to simultaneously induce the formation of the primitive streak (92)**.** Further evidence demonstrated a requirement for canonical Wnt signalling during the initial steps of mesendodermal differentiation of mESCs (93). Lindsley *et al.* found that Wnt signalling is required for the expression of primitive streak and gastrulation associated genes such as brachyury, *Mixl1* and *Evx1* as well as the expression of epithelial to mesenchymal transition (EMT) and mesoderm-associated genes (93). Moreover, the stabilized form of beta-catenin alone is not sufficient to induce primitive streak-associated gene expression. Rather, canonical Wnt signaling may simultaneously interact with other signalling pathways such as BMP (Figure 3) and Nodal and their transcriptional targets during the development of mESC-derived mesoderm layer (93).

It seems that after the induction and formation of the mesoderm germ layer, active canonical Wnt signalling is still required for the generation of hematoendothelial progenitors (94). Human embryonic stem cells (hESCs) were differentiated using a coculture system with stromal cells engineered to express Wnt proteins to provide stable and active Wnt activity. The derived $CD34^{bright}CD31^{+}VEGFR2^{+}$ hematogenic endothelium cell population from the coculture system, were able to differentiate into both hematopoietic and ECs, further demonstrating the implication of Wnt signalling in ESC-derived ECs (94). Furthermore, components of Wnt signalling such as *Wnt2* and *Wnt5a* as well as Wnt target genes including *Msx1, Msx2, fibronectin, cyclin D1, cyclin D2,* and *Myc* were specifically upregulated in a gene expression profile analysis of *in vitro* ESC-derived VEGFR2+ cells. This denotes the critical role for Wnt signalling, which is necessary for EC differentiation and vascular formation (95).

Nevertheless, reports have also shown the participation of both canonical and non-canonical Wnt pathways in the differentiation of VEGFR2+ cells towards the EC lineage (Figure 3). Reverse transcription-

polymerase chain reaction (RT-PCR) analysis identified a significantly higher expression of *Wnt5a* and *Wnt11* genes in VEGFR2+ cells compared to VEGFR2-cells derived from mESCs. In addition, a higher level of non-canonical *Frizzled2*, *Frizzled5* and *Frizzled7* genes were also expressed in these cells (83). Wnt5a has been reportedly able to function through both canonical and noncanonical pathways via beta-catenin and PKC respectively (96-98). By establishing *Wnt5a*-/- mESCs, Yang *et al.* were able to investigate the roles of Wnt5a during differentiation towards the EC lineage. *Wnt5a*-/- mESCs could not differentiate into ECs but this phenomenon was rescued in the addition or treatment with Wnt5a (99). Further investigations also indicated that both beta-catenin and PKCalpha mediated signalling pathways were required for the differentiation of mESCs to EC induced by Wnt5a (Figure 3) as shown in the activation of both beta -catenin and PKCalpha in *Wnt5a*+/- but not in *Wnt5a*-/- mESCs. Moreover, transfection of *Wnt5a*-/- mESCs with either beta-catenin or PKCalpha alone did not induce the induction of Flk-1 nor Flt-1 mRNAs during the differentiation process. However, endothelial differentiation in *Wnt5a*-/- mESCs ensued when both beta-catenin and PKCalpha were cotransfected, hightlighting that canonical Wnt/ beta-catenin and noncanonical Wnt/PKC pathways function reciprocally in the process of endothelial differentiation by Wnt5a. Besides, an *in vivo* mouse model using *Wnt5a*-/- embryos revealed a defect in vascularisation that further demonstrated the crucial roles of Wnt5a in vascular development (99).

It appears that Wnt signalling, coupled with other signalling pathways such as BMP and Nodal, play an initial role in the formation of the primitive streak and then later in the formation of the mesoderm layer (89-93). Further differentiation of hESCs into hemangioblasts also demonstrated the involvement of Wnt signalling (94, 95). In particular, Wnt5a appears to act via both the canonical and non-canonical Wnt signalling pathways to aid in the differentiation of mESCs into the EC lineage (83, 99).

4.4. Transforming growth factor-beta and family members

Transforming growth factor-beta (TGF-beta) and their family members, including bone morphogenetic proteins (BMPs), Nodal and activins, have been implicated in the differentiation of ESCs. In a study by Poon *et al.* TGF-beta was applied for different time points after spontaneous differentiation using embryoid bodies (EBs) to assess its effect on differentiation in ESCs (100). They found that application of TGF-beta increased endothelial marker expression in mouse EBs and resulted in a reduced level of endodermal markers. However, this finding was not consistent in human EBs whereby TGF-beta application inhibited the expression of endodermal, endothelial and hematopoietic markers (100). The contrast in findings may reflect fundamental differences in early cell lineage development of the yolk sac between different species. Nevertheless, *in-vivo* mouse studies have revealed the importance of TGF-beta1 and its signalling components in early vascular development, particularly within the yolk sac where vessels appear *de novo* from yolk-sac mesoderm via vasculogenesis (101, 102).

TGF-beta family members have also been implicated in dynamic germ-layer specification process (103,104). Six members of the BMP family (BMP2 through BMP7) belong to the TGF-beta superfamily of proteins. *In vivo* mouse models showed that signal transduction through BMP 2 and 4 results in the mobilization of Smad proteins which aid in modulating early vascular development (105,106). Moreover, expression analyses and genetic studies have demonstrated that the coordinated activation and regional inhibition of BMP-4 and Nodal together with Wnt signaling pathways are necessary for germ-layer specification (104). Park *et al.* found that the initial formation of brachyury-positive and late VEGFR2+ mesoderm was developed after the addition of BMP4 (Figure 3) into differentiating ESC cultures in the absence of serum (107). Activin-activated Nodal signalling pathways were shown to induce a primitive streak population that expresses Foxa2 and Goosecoid, and the subsequent formation of mesoderm layer depending on varying concentrations of activin (104). Also demonstrated in a chick embryo study, after BMP induced derivation of the early ventral mesoderm, the presence of Notch signalling together with BMP, enhances lineage specification, resulting in the derivation of two progenitor cell populations, which are the SMCs progenitors and hemangioblasts (108).

Additionally, in mESC-derived ECs, BMP signalling has been found to induce the expression of VEGFR2 and Tie2 in these cells which promoted their proliferation and migration (109). Whereas the inhibition of TGF-beta and activin aids in the growth and integrity of ESC-derived ECs by hindering the expression of p21 and promoting the expression of claudin-5 which is a component of EC tight junctions (110). Using a hES-OP9 cell coculture system to derive ECs, Kelly *et al.* discovered that Indian Hedgehog (Ihh) a endoderm-derived soluble factor, work upstream of BMP4 signalling (Figure 3) to control EC differentiation form pluripotent hESCs (111). Using cyclopamine, which is a pan hedgehog inhibitor, they demonstrated that BMP2/4 rescued the effects of hedgehog inhibition on endothelial cell differentiation. In this experiment, it was suggested that the presence of Ihh binds to its patched receptor that is usually situated in the mesoderm, and activates BMP4 signalling (Figure 2), which drives endothelial differentiation (111).

The involvement of BMPs in EC differentiation was also recently highlighted by Moser *et al.* who reported bone morphogenetic protein–binding EC precursor-derived regulator (BMPER) which is a secreted protein that directly interacts with BMP2, BMP4 and BMP6 (112). Although BMPER seemed to be specifically expressed in VEGFR2+ ESCs, it also antagonises BMP4 dependent Smad5 activation and subsequent EC differentiation from ESCs. Despite requiring further elucidation of the precise role of BMPER in EC differentiation, Moser *et al.* hypothesized that VEGFR2+ positive cells express BMPER in order to modulate local BMP signalling and thereby inhibit the

recruitment of additional VEGFR2+ cells to prevent vascular overgrowth.

5. SUMMARY

The discovery of pluripotent embryonic stem cells has raised hope for generating endothelial cells for the treatment of vascular diseases or creating a disease model for drug discovery and toxicology *in-vitro* screening studies. Knowledge in understanding the mechanisms that control the signaling events pertaining to early and late developmental progress of ECs in differentiating ESCs studies, can offer insight as to how one might influence or stimulate these signals for regenerative and therapeutic advantages.

During embryonic stem cell differentiation towards the EC lineage, a complex compilation of signalling pathways act together in a coordinated fashion that surface at different differentiation time points. Generally, many proteins in a signalling cascade also perform in a balanced and regulated manner. From established reports, Wnt and BMP signalling are present throughout the EC differentiation process, from the formation of the primitive streak and mesoderm, through to the derivation of mature and functional ECs. Apart from Wnt and BMP, other signalling pathways including, TGF-beta, Nodal and activin were found to promote ESC differentiation towards the mesoderm germ layer specification. VEGFR2, the major receptor in ECs, has proven to be a crucial receptor for the numerous signalling pathways implicated in EC differentiation from VEGFR2+ vascular progenitor cells. Moreover, findings from our group have concluded that HDAC3-mediated p53 deacetylation and p21 activation are crucial for shear-and VEGF-induced functional EC differentiation from ESCs.

In summary, the discussed signalling pathways in this review demonstrate the involvement of various but specific molecules and their receptors, which subsequently trigger other signal transduction cascades to promote the differentiation of ESCs towards vascular ECs in an orderly and well-timed manner. Thus, the elucidation of signal pathways could provide basic information for manipulating cell differentiation towards an endothelial lineage, which is essential for stem cell therapy for cardiovascular diseases.

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Abbreviations: VEGF: vascular endothelial growth factor; ECs: endothelial cells; PDGF: Platelet derived growth factor; FGF: fibroblast growth factor; SMCs: smooth muscle cells; PECAM: platelet endothelial cell adhesion molecule-1; VE-cad: vascular endothelial-cadherin; vWF: von willebrand factor; PLC- gamma: phospholipase C-gamma; PKC: protein kinase C; HDACs: histone deacetylases; BMP: bone morphogenic protein

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