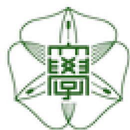
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Vascular formation : *in vitro* differentiation of vascular endothelial cells from pluripotent stem cells

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ABSTRACT : During early vascular development, vascular endothelial cells (ECs) differentiate, proliferate, migrate, and change morphologically, forming tube-like vascular structures. The ECs are then surrounded by vascular smooth muscle cells (SMCs), leading to the formation of stable vascular structures, or vascular maturation. The stages of cell or tissue development can be monitored and analyzed using in vitro differentiation systems of pluripotent stem cells. In this review, we present the molecular mechanisms that regulate the developmental processes of vasculature.

Key Words : vascular formation, vascular endothelial cells, VEGF, ES cells

1. The induction of vascular cell lineage differentiation using in vitro systems

Vascular or hematopoietic cell lineages can be generated from pluripotent stem cells using *in vitro* systems. The differentiated cells derived from stem cells can be purified using techniques such as fluorescence activated cell sorting (FACS) or magnetic-activated cell sorting (MACS), and subsequently used for research, such as cell lineage analysis[1, 2].

There are a number of *in vitro* systems available :

i) Embryoid body (EB) system :

Embryoid bodies are three-dimensional aggregates of stem cells that formed in suspension culture. Various cell lineages are induced from EBs. The initial stage of EB differentiation is considered to resemble an early embryo and the EB system provides an in vitro model for studying cell differentiation and development[3, 4].

ii) OP9 coculture system :

The OP9 stromal cell line is derived from osteopetrotic (op/op) mice lacking functional macrophage colony stimulating factor (M-CSF) due to a mutation of the M-CSF gene. This cell line is reported to support hematopoietic differentiation[5]. The embryonic stem (ES) cell and OP9 cell coculture system contains a low concentration of vascular endothelial growth factor (VEGF), primarily produced by the OP9 cells, and is used for induction of vascular or hematopoietic lineage differentiation[6].

iii) Collagen Type IV-coated culture plate system :

Embryonic stem cells have been reported to differentiate into vascular cell lineages when cultured on collagen Type IV-coated plates[7]. This culture system does not require feeder cells such as OP9 cells and is therefore suitable for analysis of signaling molecules involved in the regulation of cellular events.

2. Vascular cell lineages

VEGF is a potent angiogenic factor that stimulates various endothelial cell functions leading to vascular development. VEGF binds to and activates the tyrosine kinase receptor VEGF receptor-2 (VEGFR2/KDR/Flk1), and regulates EC behaviors such as survival, differentiation or proliferation[8, 9]. Lineage tracing in

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mouse ES cell in vitro systems demonstrates that VEGFR2^{+} mesodermal cells are vascular progenitor cells, which generate both VE-cadherin^{+} CD31^{+} ECs and alpha-smooth muscle actin (αSMA)$^{+}$ desmin^{+} SMCs[7, 10] (Fig. 1). Endothelial cells derived from VEGFR2^{+} vascular progenitor cells construct vessel-like structures, which are supported by SMCs.

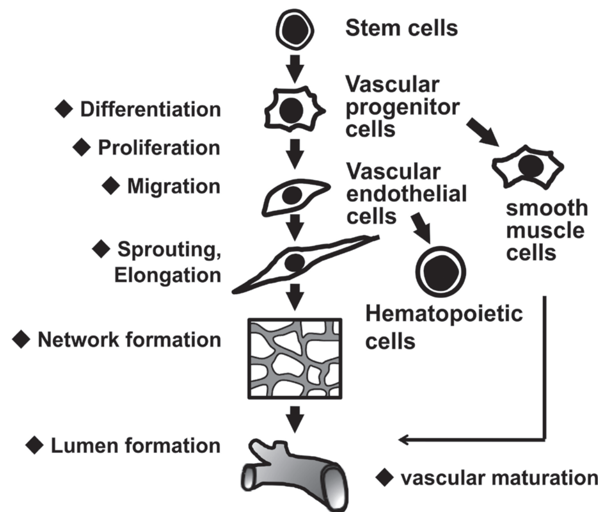

Fig. 1 Vascular cell lineages

Angiogenic functions are known to be regulated by numerous factors in addition to VEGF, including fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF), and transforming growth factor-β (TGF-β)[11, 12]. FGF or HGF stimulate many functions in ECs, such as proliferation and migration, and induce blood vessel formation[8, 13]. PDGF plays an important role in the recruitment of SMCs into ECs, preserving vascular integrity, and the differentiation of SMCs derived from ES cells has been reported to be induced by the presence of PDGF B-chain homodimers (PDGF-BB)[7]. The inhibition of TGF-β signals promotes the expansion of ECs derived from human ES cells depending on Id1, a protein that modulates cellular differentiation and cell fate determination[14, 15]. Additionally, TGF-β regulates the differentiation of vascular progenitor cells into SMCs, and the recruitment of SMCs[16].

Blood cell lineages, including leukocytes, erythrocytes or megakaryocytes, originate from hematopoietic stem cells[17]. Hematopoietic cells are considered to develop from hemogenic ECs, a subpopulation of ECs, via the process of endothelial-to-hematopoietic transition[18]. Hemogenic ECs derived from mouse ES cells are bipotential progenitors, and have the capacity to generate both hematopoietic and endothelial cells. Previously, we showed through analysis of cell lineages using a mouse ES cell-OP9 feeder coculture system, that bipotential hemogenic ECs arise from the VE-cadherin^{+} CD41^{+} fraction of ECs, and express chemokine (C-X-C motif) receptor 4 (CXCR4), also known as SDF1[19]. Chemokine (C-X-C motif) ligand 12 (CXCL12) binds to CXCR4, which is expressed on the surface of ECs. The CXCL12/CXCR4 signaling suppresses endothelial cell potential, leading to negative regulation of the bipotential state in hemogenic ECs.

3. Vascular endothelial cell specific transcriptional enhancer

The differentiation of vascular cells has been reported to be cooperatively regulated by the combination of Forkhead box (Fox) and Ets transcription factors[20]. Myocyte enhancer factor 2c (MEF2C), a member of the MEF2 family of transcription factors, also plays a key role in the development of vascular structures[21]. De Val *et al*. identified a 44-bp region of the MEF2C gene as an endothelial cell-specific enhancer (F10-44 enhancer)[20, 22], which contains two types of transcription factor binding sites : a Fox binding site and an Ets binding site (FOX : ETS motif). Similar FOX : ETS sequence motifs are present in multiple endothelial cell-specific genes, including VEGFR2, Tie2, Tal1, Notch4, and VE-cadherin. These observations suggest that activation of the FOX : ETS motif is associated with endothelial cell-specific gene expression and endothelial cell differentiation. In our previous report, lineage tracing in a mouse ES cell differentiation system showed that the F10-44 enhancer was activated in VEGFR2^{+} vascular progenitor cells[10]. The activation of the F10-44 enhancer continued in differentiated VE-cadherin^{+} ECs derived from the vascular progenitor cells, but was not observed in αSMA^{+} SMCs, suggesting that F10-44 enhancer activation is specific to endothelial cell lineages. However, which transcriptional factors mediate each stage of vascular cell differentiation remains to be elucidated. To this end, the mechanism of cell fate determination needs to be investigated further.

4. Regulation of vascular endothelial cell morphological change

New blood vessels sprout out and elongate from ECs derived from progenitor cells (defined as vasculogenesis),

or from a pre-existing vascular bed (defined as angiogenesis), and form a multicellular EC cord-like structure. This is followed by lumen formation, and finally, the vascular tube structure is formed. In the next section, we discuss the mechanisms that regulate the process of endothelial cell elongation and lumen formation.

Forkhead box O1 (Foxo1)-deficient mice show impaired vascular development around embryonic day (E) 9.5, and embryonic lethal phenotype around E11[23]. In our reports, in vitro differentiation of ES cells cultured on OP9 cells demonstrated that wild-type ES cells and Foxo1-deficient ES cells generated VE-cadherin$^+$ ECs, which formed around colonies on OP9 cell layers[10, 24]. When exposed to high doses of VEGF, wild-type ECs show a remarkable morphological change from a polygonal to an elongated shape, and form thread-like colonies. In contrast, Foxo1-deficient ECs remain polygonal-shaped and do not form such colonies[10, 23-25]. Wild-type and Foxo1-deficient ES cells produced αSMA$^+$ SMCs. These SMCs are morphologically indistinguishable. These observations suggest that Foxo1 is crucial to the regulation of endothelial cell morphological changes.

Formation of vessel-like structures can be induced using 3D culture systems. VEGFR2$^+$ vascular progenitor cells derived from ES cells are aggregated on a spheroid plate with a low-attachment surface, and then the aggregated cells are cultured in Type 1 collagen gel in the presence of a high concentration of VEGF. In 3D culture, both ECs and SMCs are derived from vascular progenitor cell aggregation. Endothelial cells sprout from the aggregation and form long, thin, vessel-like structures, then are covered with SMCs[7]. We demonstrated that Foxo1-deficient vascular progenitor cells did generate ECs in 3D culture; however they formed abnormally thick, short sprouts[10, 26]. Wild-type ECs had an arrangement of prominent actin filaments along the edge of the cells, and organization of long filamentous microtubules. In contrast, vascular sprouts of Foxo1-deficient ECs had abnormal cytoskeletal structures : dot-like accumulation of actin and disorganization of microtubules with small spikes at the tips of sprouts. Wild-type endothelial cell elongation and the formation of vessel-like structures was not affected by the inhibition of Rho kinase (ROCK) signaling[10]. However, ROCK inhibition caused extreme disorganization of actin and microtubules in Foxo1-deficient ECs, resulting in failure to maintain even short sprout form. Moreover, Foxo1-deficient ECs were not surrounded by Foxo1-deficient SMCs. These findings suggest that Foxo1 is involved in the regulation of morphological change of ECs, and recruitment of SMCs to ECs.

During the sprouting process, ECs are categorized as two types of cells, tip and stalk cells[27]. Tip cells, which have high motility, lead and guide sprouts. Stalk cells, which have high proliferative activity, follow the tip cells and organize the sprout body. The activity of VEGFR2 is induced in tip cells, and suppressed in stalk cells. Vascular endothelial-phosphotyrosine phosphatase (VE-PTP), which is specifically expressed in ECs, dephosphorylates and inactivates VEGFR2. Analysis of mouse ES cell-derived vessel structures showed that the inactivation of VE-PTP causes excessive activation of VEGFR2 in stalk cells and the loss of cell polarity and lumen formation, suggesting that VE-PTP is one of the crucial molecules for the formation of functional vessels[28].

5. Regulation of vascular endothelial cell elongation depending on the levels of VEGF

The effects of VEGF on ECs are diverse, and the signaling pathways responsible for each angiogenic effect are intricate. Recently, we reported that endothelial cell elongation is modulated in response to the microenvironmental levels of VEGF via two different pathways : phosphatidylinositol 3-kinase (PI3K)-Akt-Foxo1 and mammalian target of rapamycin complex 1 (mTORC1)-mTORC2 pathways[24] (Fig. 2).

Low concentrations of VEGF are necessary for the survival and proliferation of ECs derived from mouse ES cells, but are not sufficient for endothelial cell elongation in OP9 feeder coculture systems[23, 25].

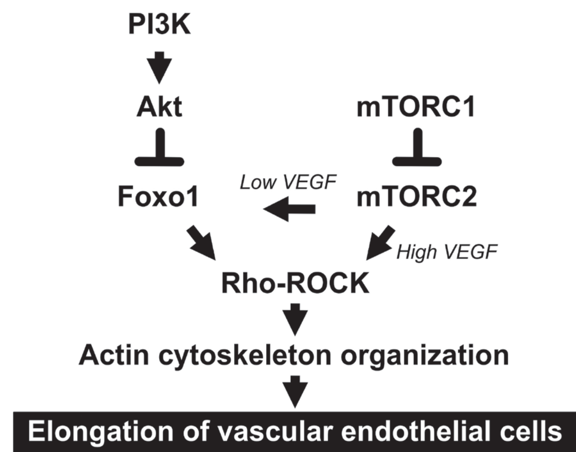

Fig. 2 Regulation of vascular endothelial cell elongation depending on the levels of VEGF

However, endothelial cell elongation can be induced when VEGF levels are low by the inhibition of PI3K-Akt or mTORC1 signaling. The PI3K-Akt signaling pathway mediates phosphorylation and nuclear exclusion of Foxo1, resulting in inhibition of Foxo1 transcription activity[29]. Therefore, inhibition of PI3K-Akt signaling is considered to link to the activation of Foxo1, promoting endothelial cell elongation. mTORC2 is a mediator that regulates remodeling or polymerization of the actin cytoskeleton[30, 31]. mTORC1 has been reported to induce phosphorylation of rapamycin-insensitive companion of mammalian target of rapamycin (Rictor), a subunit of mTORC2, through the activation of ribosomal protein S6 kinase 1, thereby inhibiting mTORC2 signaling[32]. Thus, inhibition of mTORC1 signaling likely activates mTORC2 and induces endothelial cell elongation. Moreover, the regulation of endothelial cell elongation by PI3K-Akt or mTORC1 inhibition depends on the organization of the actin cytoskeleton through the Rho-ROCK pathway. At low concentrations of VEGF, Foxo1-deficient ECs fail to elongate in the presence of PI3K-Akt or mTORC1 inhibitor, suggesting that both signaling pathways regulate EC morphology in a Foxo1-dependent manner.

High concentrations of VEGF strongly induce elongation of wild-type ECs and lead to a denser network of vessel-like structures, however, this has no influence on the morphology of Foxo1-deficient ECs. Inhibition of PI3K-Akt or mTORC1 signaling retained elongated morphological changes, but inhibited over-vascularization of wild-type ECs at high concentrations of VEGF. Interestingly, the elongation of Foxo1-deficient ECs was restored by the inhibition of mTORC1 signaling, but not by the inhibition of PI3K-Akt signaling. This induced elongation is also dependant on the Rho-ROCK pathway. Accordingly, the mTORC1-mTORC2 signaling pathway can regulate endothelial cell elongation in a Foxo1-independent manner at high concentrations of VEGF. These findings suggest that Foxo1 dependency changes in response to the microenvironmental levels of VEGF.

The establishment of proper vascularization is key to regeneration of tissue. VEGF has been used as a potent inducer of neovascularization in applications for tissue engineering[12, 33]. In contrast, transplanted myoblasts producing higher concentrations of VEGF have been shown to cause hemangiomas in host tissues[34]. Induction of vascular growth in preclinical therapeutic studies demonstrates that administration of recombinant VEGF or overexpression of VEGF promotes a vascular permeability reaction, leading to undesirable side effects such as tissue edema[35, 36]. Thus, the PI3K-Akt-Foxo1 or mTORC1-mTORC2 signaling pathways may be novel targets for induction of physiological angiogenesis, depending on the microenvironmental levels of VEGF.

Conclusion

Neovascularization is increasingly being recognized as an important component of tissue regeneration. Therapeutic studies of cardiac dysfunction using stem cells have shown that transplantation of cardiovascular cell sheets (consisting of cardiomyocytes, ECs, and SMCs derived from human induced pluripotent stem cells), improved cardiac function in infarcted hearts[37, 38]. In the engrafted area, neovascularization formed from host blood vessels, yet the molecular basis for this process is unclear. Further studies are needed to determine the underlying mechanism of vascular development. In vitro differentiation systems of pluripotent stem cells can contribute to the study of important cell biological processes during angiogenesis.

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