Roles of Cyclic Adenosine Monophosphate Signaling in Endothelial Cell Differentiation and Arterial-Venous Specification During Vascular Development

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Cyclic adenosine monophosphate (cAMP) is an important second messenger mediating physiological functions, including metabolism, gene expression, cell growth and differentiation. Recently, we demonstrated novel roles of cAMP pathway in endothelial cell (EC) differentiation and arterial–venous specification using an embryonic stem cell differentiation system. These studies offered a concept that vascular formation is accomplished by a 2-layered mechanism: (1) a basal mechanism for common EC differentiation, whereby vascular endothelial growth factor (VEGF) signaling plays a central role in the basal mechanism, and (2) a vascular diversification mechanism working on the basis of common EC differentiation. Vascular diversification, such as artery and vein formation, can be only achieved by enacting specific machineries in the presence of the basal EC machinery. cAMP/protein kinase A signaling contributes to common EC differentiation through upregulation of the VEGF-A receptors, Flk1 and neuropilin1. On the other hand, cAMP can activate phosphatidylinositol-3-kinase, which induces an arterial fate in vascular progenitors via dual activation of Notch and β-catenin signaling as an arterial-specific machinery. cAMP signaling thus plays a pivotal role in both the basal and diversification machinery during vascular development. (Circ J 2011; 75: 253–260)

Key Words: Cyclic AMP; Embryonic stem cells; Endothelial cells; Vascular progenitor cells

Cyclic adenosine monophosphate (cAMP) discovered in the late 1950s marked the birth of the second messenger theory and sparked signal transduction research.1 Adenylate cyclase generates cAMP from ATP in essentially all tissues in the body. This enzyme is embedded in the plasma membrane and is activated by transmembrane receptors that are coupled to trimeric G-proteins.2,3 The effects of cAMP are mediated by various downstream targets, such as protein kinase A (PKA), and exchange protein directly activated by cAMP (Epac).4 PKA and Epac contain an evolutionarily conserved cAMP-binding domain that acts as a molecular switch for sensing intracellular second messenger cAMP levels to control diverse biological functions.4 At the cellular level, cAMP plays an important role in almost every known physiological action, such as metabolism, gene expression, cell division and growth, cell differentiation and apoptosis, as well as secretion and neurotransmission.

In cardiovascular biology, cAMP is a critical second messenger in the modulation of vasodilation, cardiac chronotropic and inotropic responses, cellular growth, and hypertrophy.5 For example, cAMP/PKA signaling potently inhibits smooth muscle cell (SMC) proliferation and migration. Increased levels of cAMP markedly inhibit SMC proliferation by arresting the cells primarily in the G1 and G2/M phases of the cell cycle.6 Furthermore, cAMP-elevating G protein-coupled receptor (GPCR) agonists, including adrenomedullin (AM), prostacyclin, prostaglandin E2 (PGE2), and β-adrenergic agonists, reduce endothelial permeability.7,8 cAMP/Epac signaling enhances the vascular barrier property that stabilizes the VE-cadherin-mediated cell–cell adhesion and inhibits permeability.9 Although cAMP-mediated signaling pathways regulate a multitude of important vascular functions under physiological conditions, the role of cAMP in vascular development is still unclear.

Until now, most studies of vascular development have consisted of gene knockout and gene inhibitory studies using mice and zebrafish. Although these studies led to the discoveries of essential factors in vascular development, they could not sufficiently identify the conditions required for vascular formation. To clarify the “constructive” mechanisms underlying vascular development, we have developed a novel embryonic stem (ES) cell differentiation system, which exhibits early vascular development using VEGFR2 (Flk1) -positive cells as common progenitors for vascular cells (Figure 1).10,11 Using this system, we can systematically induce vascular cells in vitro and dissect their differentiating processes in detail. We
recently demonstrated that cAMP signaling plays a critical role in the reconstitution of endothelial cells (ECs) and arterial specification in vascular development. In this review, we focus on the molecular mechanisms of vascular development/differentiation and diversification from vascular progenitors.

**Molecular Mechanisms of EC Differentiation**

**Roles of VEGF Signaling in Vascular Development**

Numerous vascular formation factors, such as vascular endothelial growth factors (VEGF), neuropilin (NRP), angiopoietins, transforming growth factor-β, platelet-derived growth factor, fibroblast growth factor, ephrin, and Notch, have been identified within the past few decades. Among these factors, VEGF/Flik1 signaling plays the most important role in the production of vascular progenitors and EC differentiation. Lateral plate mesoderm expresses Flik1, and migrates into the extra-embryonic yolk sac to form a vascular capillary plexus, leading to the development of a functional circulatory system. Flik1 null mice die at E8.5–E9.5, without organized blood vessels. Heterozygous VEGF-A null mice die in early gestation due to failure in vascular system formation. On the other hand, two- to threefold overexpression of VEGF-A from its endogenous locus results in aberrant heart development and lethality at E12.5–E14, indicating that strictly balanced VEGF function is important in normal embryogenesis.

NRP1 acts as another VEGF-A receptor in blood vessels and endocardial cells of the heart. NRP1 is also expressed in particular classes of developing neurons, and functions as a receptor for the class 3 semaphorins that mediate semaphorin-elicited inhibitory axon guidance signals to neurons. NRP1, together with Flik1, forms a specific receptor for VEGF-A165, an isoform of VEGF, and the Flik1-VEGF-A165–NRP1 complex potently enhances Flik1 signaling. NRP1 null mice die midway through gestation at E10.5–E12.5 and exhibit defects of the heart, vasculature, and nervous system, indicating that the relationship between VEGF165 and NRP1 is critical in vascular development.

Growth factors and hypoxia are known to induce VEGF-A gene expression. Hypoxia-inducible factor (HIF) markedly produces VEGF and contributes to formation of the vascu-
Figure 2. Cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway plays a critical role in vascular development. (A) Experimental system for PKA activation. An endothelial cell (EC) cell line expressing the constitutive active (CA) form of PKA by tetracyclin-inducible expression system (Tet-Off) was established. Doxycycline (Dox) was added during the first 4.5 days of ES cell differentiation to Flk1+ cells. Flk1+ cells were sorted by MACS and subjected to 2-D culture on collagen-coated dishes or 3-D culture in collagen gel, and were cultured in the presence or absence of Dox (1 μg/ml). (B, C) 2-D culture with DM, at Flk-d3. (B) Double immunostaining for CD31 (purple) and αSMA (brown). Left panel, Dox (1 μg/ml) treatment. Right panel, Dox free. Culture with DM alone. Scale bar: 100 μm. (C) Flowcytometry for EC markers, CD31 and VE-cadherin. Percentages of CD31+/VE-cadherin+ ECs in total Flk1+ cell-derived cells are indicated. (D) Phase contrast images after 5 days’ culture in 3-D culture. Left panel, Dox (1 μg/ml) treatment. Right panel, Dox free. Scale bars: 100 μm. (E) In-gel double immunostaining for CD31 (purple) and αSMA (brown) in Dox-free conditions. Left panel, gross appearance of vascular structure. Right panel, higher magnification view. αSMA+ cells attached to CD31+ EC tube structure are observed (arrows). Scale bars: 100 μm. (F) Confocal microscopic analysis of vascular structure. Double fluorescent staining for CD31 and αSMA in Dox-free conditions. Left panel, CD31 (green). Middle panel, αSMA (red). Right panel, Merged image. αSMA+ cell attached to CD31+ EC tube structure is observed (arrow). CD31+ cells formed a true lumen (green) with attached mural cells (red) shown in xz image. Dashed line indicates sliced position. Scale bars: 100 μm. (G) Quantitative RT-PCR showing mRNA expression of Flk1 and neuropilin (NRP) 1 at Flk-d3 and d3 in the presence or absence of Dox. mRNA expression at Flk-d1 with Dox was set as 1.0. (H) Flowcytometry for CD31 expression in the presence (Dox+) (1 μg/ml) or absence of Dox (Dox–). X-axis: CD31, Y-axis: SSC. Flk1+ cells were incubated with various concentrations of vascular endothelial growth factor (VEGF)165 in the serum-free medium, SF03. Percentages of CD31+ ECs in the total Flk1+ cell-derived cells are indicated. (I) PKA activation increased both Flk1 and NRP1 expression in vascular progenitors and markedly enhanced the “sensitivity” of the progenitors to VEGF165 by inducing Flk1–VEGF165–NRP1 complex formation, and markedly enhancing EC differentiation.
lunar tube in embryogenesis as well as in adults. Null mice for HIF1a, HIF2a, and HIF-related genes have vascular defects and die at E9.5–E10.5,25 indicating that HIF-related VEGF production regulates vascular development. However, the mechanisms that regulate the expression of the VEGF receptors, Flk1 and NRP1, in vascular development are not fully elucidated.

**Roles of cAMP/PKA Signaling in Vascular Development**

We have previously shown that cAMP signaling enhances EC differentiation, using an ES cell differentiation system.26 We further investigated the molecular mechanisms of cAMP in EC differentiation, and we recently showed that activation of cAMP/PKA signaling increased the VEGF receptors, Flk1 and NRP1, but not the VEGF ligand. Furthermore, the cAMP/PKA pathway markedly formed the protein complex of Flk1–VEGF-A165–NRP1, and enhanced the sensitivity of the progenitors to VEGF165 by more than 10-fold (Figure 2).12 These results indicate that PKA regulates “progenitor sensitivity” in EC differentiation by changing the characters of the vascular progenitors, not by producing growth factors. NRP1 was largely co-expressed with Flk1 in vascular progenitors derived from human and mouse ES cells.27 These 2 functional markers for vascular progenitors might be commonly regulated by PKA to efficiently enhance their progenitor potential of responding to VEGF signaling.

Various factors, such as AM,7 PGI, PGE2,8 adiponectin,28 ghrelin,29 klotho, and mechanical stress, especially fluid shear stress,30 have been reported to activate the cAMP/PKA pathway in ECs. Our previous studies showed that AM enhanced the VEGF-induced EC differentiation from Flk1+ vascular progenitors.26 AM knockout mice demonstrated defective vascular formation and did not survive beyond mid gestation,31 indicating that AM is a one of the key factors regulating the cAMP/PKA pathway in vascular development. However, the main role of cAMP production in vascular development is not yet clearly understood.

**Molecular Machinery of Arterial-Venous Specification**

**Mechanisms of Artery Formation**

Molecular differences between arterial and venous ECs become apparent before circulation begins.32–34 The first genes for arterial–venous specification to be identified were ephrinB2 and EphB4. These members of the Eph–ephrin family were discovered to be differentially expressed in the endothelium of arteries and veins, even before the onset of blood flow and heart beat.32,33 Since then, various other arterial–venous markers have been identified. Arterial ECs are known to express Notch pathway molecules,35,36 such as Notch1, Notch4, Dll4, and Jagged1, NRP1,37 the chemokine receptor CXCR4,26,38,39 the gap junction protein connexin-37 and connexin-40, CD44, Alk1, and Bmx. Venous ECs are known to express EphB4, NRP2, COUP-TFI1, and the Apj receptor.

**Roles of Notch Signaling in Artery Formation**

A variety of evidence from mammals has highlighted the importance of Notch signaling in the proper formation of the vasculature. In ECs, Notch (Notch1, 4) activation can be induced by various Notch ligands, including Dll1, Dll4, and Jagged2, expressed in arterial ECs, and Jagged1 expressed in ECs and mural cells.35,36 All of this Notch signaling is considered to be mediated by the Notch intracellular domain (NICD) and RBP-J transcription factor (also called CSL, CBF-1 in mammals, Suppressor of Hairless [Sul(H)] in Drosophila and LAG-1 in Caenorhabditis elegans). Genetic animal studies of the Notch signal related-genes have shown that Notch1 and 4, Dll4, RBP-J, and Hey1/Hey2 are essential for arterial formation in the developing vasculature.41–45 However, EC-specific NICD transgenic mice partially induce arterial EC formation.46 Moreover, Notch activation, together with VEGF stimulation
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Roles of Convergence Signaling of NOTCH and β-Catenin in Artery Formation

Wnt/β-catenin signaling plays a key role in vascular biology. Mice deficient for Wnt2 displayed vascular abnormalities, including defective placental vasculature. Wnt receptor gene, Frizzled-5 knockout mice died in utero owing to defects in yolk sac angiogenesis. Defects of the β-catenin gene in ECs caused a defective vascular pattern and increased vascular fragility. We previously revealed that simultaneous activation of VEGF and cAMP in Flk1+ vascular progenitors leads to the induction of arterial ECs in vitro. We recently demonstrated a novel arterial specification machinery regulated by Notch and β-catenin signaling. Both Notch and GSK3β-mediated β-catenin signaling were activated downstream of cAMP through phosphatidylinositol-3 kinase. Forced activation of Notch and β-catenin synergistically enhanced expression of the arterial markers, ephrinB2 and CXCR4. Interestingly, a protein complex with RBP-J, NICD, and β-catenin was formed on RBP-J binding sites of arterial genes in arterial, but not venous, ECs. Thus, the formation of the protein complex on arterial genes induced by cAMP activation could be the central machinery for arterial EC specification. These findings lead to an integrated and more comprehensive understanding of vascular signaling (Figure 4). Furthermore, the Notch–β-catenin–RBP-J complex suppresses differentiation of neural precursor cells, indicating that the protein complex that directly converges Notch and β-catenin signaling may play a critical role in cell fate determination in various organs.

Beta-catenin signaling in ECs can be activated through Wnt ligands as well as by VE-cadherin. Thus, Wnt ligands such as Wnt2, 5a, and 10b, expressed in fetal blood vessels, are involved in EC differentiation. VE-cadherin is heavily tyrosine phosphorylated and is linked to β-catenin. When adherens junctions mature, the tyrosine residues in VE-cadherin tend to be dephosphorylated and β-catenin is partially released from the complex, allowing nuclear translocation of β-catenin and activation of downstream signaling cascades. As VE-cadherin and β-catenin are broadly expressed in ECs, and mice with EC-specific disruption of β-catenin show broad vascular phenotypes, β-catenin should have both a com-

Figure 4. Molecular mechanisms of arterial endothelial cell (EC) specification. cAMP signaling, which can be induced by adrenomedullin, shear stress, etc activates Notch and β-catenin signaling through PI3K (and GSK3β) in vascular progenitors (as well as in differentiating ECs). Notch and β-catenin signaling subsequently converges into a single protein complex with RBP-J, NICD and β-catenin (arterial complex) on arterial genes. Notch signaling from Notch ligand binding and β-catenin signaling from wnt and VE-cadherin would also participate in forming the complex. The arterial complex should play a central role in the specification of arterial cell fate in ECs.
mon role in ECs and a specific role in arterial ECs. A recent study showing that EC-specific β-catenin transgenic mice had enhanced Notch signaling and induced artery formation, also indicated that β-catenin signaling plays an important role in arterial EC specification.

Mechanisms of Vein Formation
Venous ECs are known to express EphB4,32,55 NRP2,36 COUP-TFII,56 and the Apj receptor57 (Figure 3). The vein was considered to be the default character in vascular development. However, You et al reported that the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) acts as an inducer of venous EC specification.56 COUP-TFII establishes venous identity by suppressing arterial fate through downregulating Notch signaling. Other mechanisms involved in venous specification are still largely unknown.

Environmental Factors and Plasticity of Arterial-Venous Specification
Although the molecular pathways for arterial–venous specification is now becoming clear, we cannot ignore the involvement of physical factors such as flow shear stress. Chick embryo studies have shown that hemodynamic forces can alter EC identity after the onset of circulation. Ligation of specific arteries in the avian embryo can result in a reversible switch from arterial-specific markers, such as ephrinB2 and NRP1, to venous markers such as EphB4.58 Furthermore, vascular progenitors or early ECs determined the arterial–venous specification by environmental conditions in a quail chick model. That is, the dorsal aorta, carotid artery and the cardinal and jugular veins were isolated with the vessel wall from quail embryos and grafted into the coelom of chick hosts. Early ECs, arterial ECs and venous ECs, can populate both artery and vein in host embryos and assume the appropriate molecular identity in their new locales,59 indicating that even after the acquisition of arterial or venous identity the endothelium during vascular development remains plastic for arterial–venous differentiation. We also revealed that ECs derived from ES cells and at the early differentiation stage possess plasticity for arterial–venous specification.13 Activation of Notch and β-catenin signaling in venous ECs derived from ES cells induced ephrinB2-positive arterial ECs. When activation of Notch and β-catenin signaling ceased in arterial ECs, ephrinB2 expression was attenuated and disappeared. Early arterial or venous ECs thus possess plasticity between the arterial and venous identities by the on/off of Notch and β-catenin signaling.13 This evidence suggests that ECs in the early developmental stage are still plastic, and acquire their diverse identities from physical and environmental factors, as well as genetic factors, which leads to the formation of an elaborate and complex vascular network in the body.

Figure 5. Endothelial cell (EC) differentiation and vascular diversification in vascular development. Vascular formation in embryogenesis is considered to have 2 main mechanisms: (1) a basal mechanism for common EC differentiation, whereby vascular endothelial growth factor (VEGF) signaling plays a central role, and (2) a vascular diversification mechanism working on the basis of common EC differentiation. Vascular diversification, such as artery and vein formation, can be achieved only by activating specific machinery in the presence of the basal EC machinery. cAMP/protein kinase A (PKA) signaling contributes to common EC differentiation through upregulation of VEGF-A receptors, Flk1 and neuropilin1. On the other hand, cAMP can activate phosphatidylinositol-3 kinase (PI3K), which leads to an arterial fate for vascular progenitors through the dual activation of Notch and β-catenin signaling to induce arterial gene expression.
Conclusion

Blood vessels are involved in the formation of most organs during ontogenesis, and they maintain homeostasis in the body. They have an abundance of diversification and play a specific role in respective organs. Our 2 recent studies of cAMP signaling in EC differentiation and other previous works present the concept that vascular formation in embryogenesis has 2 main mechanisms: (1) basal EC differentiation, and (2) vascular diversification in the context of ECs. cAMP signaling has the pivotal abilities of enhancing common EC differentiation through upregulation of Flk1 and NRPI, and determining the fate of arterial EC through dual activation of Notch and β-catenin signaling (Figure 5). These findings, however, only partially explain the vascular developmental processes in the body. How is vascular branching determined in vascular development? How are specific blood vessels formed, such as the blood–brain barrier and the blood–placenta barrier? A more detailed investigation into the local environment of ECs, such as interaction between SMCs, pericytes, bloods, and nerves, may lead to a more profound understanding of vascular diversity. Elucidation of all the cellular and molecular machinery of vascular formation will lead to a better understanding of various pathophysiology, as well as ontogenesis/regeneration, and provide novel strategies for new drugs and future medical therapies.

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