Adrenomedullin/Cyclic AMP Pathway Induces Notch Activation and Differentiation of Arterial Endothelial Cells From Vascular Progenitors

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Objective—The acquisition of arterial or venous identity is highlighted in vascular development. Previously, we have reported an embryonic stem (ES) cell differentiation system that exhibits early vascular development using vascular endothelial growth factor (VEGF) receptor-2 (VEGFR2)-positive cells as common vascular progenitors. In this study, we constructively induced differentiation of arterial and venous endothelial cells (ECs) in vitro to elucidate molecular mechanisms of arterial-venous specification.

Methods and Results—ECs were induced from VEGFR2$^+$ progenitor cells with various conditions. VEGF was essential to induce ECs. Addition of 8bromo-cAMP or adrenomedullin (AM), an endogenous ligand-elevating cAMP, enhanced VEGF-induced EC differentiation. Whereas VEGF alone mainly induced venous ECs, 8bromo-cAMP (or AM) with VEGF supported substantial induction of arterial ECs. Stimulation of cAMP pathway induced Notch signal activation in ECs. The arterializing effect of VEGF and cAMP was abolished in recombination recognition sequence binding protein at the Jκ site deficient ES cells lacking Notch signal activation or in ES cells treated with γ-secretase inhibitor. Nevertheless, forced Notch activation by the constitutively active Notch1 alone did not induce arterial ECs.

Conclusions—Adrenomedullin/cAMP is a novel signaling pathway to activate Notch signaling in differentiating ECs. Coordinated signaling of VEGF, Notch, and cAMP is required to induce arterial ECs from vascular progenitors. (***Arterioscler Thromb Vasc Biol.* 2006;26:1977-1984.)**

Key Words: angiogenesis ■ developmental biology ■ embryonic stem cells ■ endothelium ■ vascular biology

Vascular formation is a complicated but well-organized process that involves sprouting, branching, and differential growth of vessels from the primary plexus or existing vessels into a functioning circulation system.[1] During the process, vascular cell specification proceeds in an inseparably coordinated manner.[2] A transmembrane ligand, ephrinB2, and its receptor, the tyrosine kinase EphB4, are reported as molecular markers for arterial and venous endothelial cells (ECs), respectively.[3,4] Recently, various molecular markers specific for arterial ECs have been documented such as Delta-like 4 (Dll4), Bmx, Notch1, Activin receptor-like kinase 1 (Alk1), and others.[5,6] These findings enable the investigation of endothelial specification processes at the cellular and molecular levels being independent of the context of vessel location within the body plan.

The Notch pathway has been highlighted in arterial-venous specification.[7,8] Notch target genes, Hairy and Enhancer-of-split-related basic helix-loop-helix transcription factors, such as grl (gridlock) in zebrafish, or Hey1 and 2 in mammals, are required for arterial vascular development.[9,10] Arterial-venous specification mechanisms in zebrafish were further demonstrated to be a regulatory signaling cascade of sonic hedgehog-vascular endothelial growth factor (VEGF)-Notch-ephrinB2.[5] The molecular machinery for arterial-venous specification in mammals, however, is still undergoing investigation.

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cAMP is a ubiquitous second messenger produced in cells and is involved in various biological phenomena including cell growth and differentiation.[11] Nevertheless, little has been reported for the role of cAMP signaling in vascular development. Adrenomedullin (AM) is a multifunctional polypeptide that was originally isolated from human pheochromocytoma.[12] AM exerts its function by increasing the levels of

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intracellular cAMP through the binding to its receptor complex, calcitonin receptor-like receptor (CRLR), and receptor activity modifying proteins (RAMP)-2 or RAMP-3.[13] Targeted null mutation of the AM gene shows embryonic lethality[14] with aberrant vascular formation and hemorrhage,[15] or extreme hydrops fetalis and cardiovascular abnormalities, including underdeveloped arterial walls,[16] inferring the significance of AM/cAMP signaling in vascular development.

Pluripotent embryonic stem (ES) cells are potent materials for both regenerative therapeutic approaches and developmental research. We have developed a novel ES cell differentiation system devoid of embryoid body formation or feeder cells that exhibits early vascular development using VEGF receptor-2 (VEGFR2)-positive cells as common progenitors for vascular cells.[17,18] We demonstrated that ES cell-derived VEGFR2$^+$ cells can differentiate into both ECs and mural cells (MCs) (pericytes and vascular smooth muscle cells) and form mature vascular-like structures in vitro.[18] Moreover, transplantation of induced vascular cells can augment the blood flow in tumor angiogenesis.[19] Our ES-derived VEGFR2$^+$ cell differentiation system can recapitulate the vascular development processes and dissect the cellular and molecular mechanisms of each developmental step including endothelial differentiation and specification.

In this study, we aimed to specifically induce arterial and venous ECs and elucidate the mechanisms of arterial-venous specification using our ES cell differentiation system. We successfully induced arterial and venous ECs and demonstrated that the AM/cAMP pathway is another indispensable signaling pathway in EC differentiation and arterial specification in conjunction with VEGF and Notch by reconstructing the arterial EC differentiation process in vitro. Our constructive approach using this ES cell system provides a novel understanding of the cellular and molecular mechanisms of vascular developmental processes.

Methods

Antibodies

Monoclonal antibodies for murine E-cadherin (ECCD2), murine VEGFR2 (AVAS12), and murine VE-cadherin (VECD1) were described previously.[18] Monoclonal antibodies for murine CD31 and CXCR4 were purchased from Pharmingen (San Diego, Calif). MoAb for murine alpha smooth muscle actin (SMA) 1A4 and human estrogen receptor-α (ERα) (F-10) antibody were from Sigma (St Louis, Mo) and Santa Cruz Biotechnology (Santa Cruz, Calif), respectively. Cleaved Notch1 antibody was from Cell Signaling Technology (Beverly, Mass).

Cell Culture

Induction of differentiation of an ES cell line, CCE (gift from Dr Evans), were performed using differentiation medium (alpha minimal essential medium; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Equitech-Bio, Kerrville, Tex) and 5×10^{-5} mol/L 2-mercaptoethanol (Gibco) and VEGF165 (R&D System, Minneapolis, Minn) as previously described.[17,18] Other chemicals, rat AM (Peptide Institute. Inc, Osaka, Japan), 8-bromoadenosine-3′:5′-cyclic monophosphate sodium salt (8bromo-cAMP) (Nacalai Tesque, Kyoto, Japan), 8-bromoguanosine-3′:5′-cyclic monophosphate sodium salt (8bromo-cGMP) (Nacalai Tesque), 3-isobutyl-1-methyl-xanthine (IBMX) (Nacalai Tesque), or γ-secretase inhibitor IX, DAPT (Calbiochem, San Diego, Calif), and iloprost (Cayman Chemical, Ann Arbor, Mich) were occasionally added to VEGFR2$^+$ cell culture.

The recombination recognition sequence binding protein at the Jκ site (RBP-J$^{+/+}$), RBP-J$^{+/-}$ and RBP-J$^{-/-}$ D3 ES cell lines have been described previously.[20] The ES cell line NERT$^{\Delta O}$-7[21] was generated by stable introduction of CAG promoter-driven cDNA encoding a fusion protein of a constitutively active part of the intracellular domain of mouse Notch1 and a tamoxifen-sensitive mutant of the hormone binding domain of the human estrogen receptor α (NERT)[22] into EB5 ES cells (gift from Dr Niwa). To induce Notch activation, 4-hydroxytamoxifen (OHT) (50 to 500 nmol/L) (Sigma) was added to NERT$^{\Delta O}$-7 cell-derived VEGFR2$^+$ cells 12 hours after the plating. NERT$^{\Delta O}$-7/Hes-green fluorescent protein (GFP) cells were generated by stable introduction of Hes promoter-driven enhanced GFP (EGFP) gene[23] (gift from Dr Kageyama) into NERT$^{\Delta O}$-7 cells.

Flowcytometry and Cell Sorting

Fluorescence-activated cell sorting (FACS) of ES cells was performed as previously described.[17,18]

Immunocytochemistry

Immunostaining for cultured cells was performed as described.[18,24] Double immunofluorescent staining for CD31 and ERα was performed using anti-ERα antibody (1:50) and anti-CD31 antibody (1:300) as first antibodies, followed by second antibodies, Alexa Fluor 546-conjugated goat anti-rat IgG (1:500) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500) (Molecular Probes, Eugene, Ore). For double staining for ephrinB2 and CD31, the fixed culture slides were incubated with EphB4-human immunoglobulin Fc portion chimeric protein (EphB4-Fc) (1:50; R&D system), followed by peroxidase-conjugated goat IgG fraction to human IgG Fc (1:500; ICN Biomedicals, Inc, Aurora, Ohio). TSA Biotin system (Tyramid signal amplification; PerkinElmer Life Science, Boston, Mass) was used for amplification of the signal for EphB4-Fc staining. EphrinB2$^+$ cells were visualized by using streptavidin-Alexa Fluor488-conjugate (Molecular Probes). Phycoerythrin-conjugated anti-CD31 antibody (Pharmingen) and DAPI (Molecular Probes) were added together with streptavidin-conjugated alexa 488. Cleaved intracellular domain of Notch (NICD) staining was performed using TSA Biotin System (PerkinElmer) with cleaved Notch1 antibody (1:300), followed by peroxidase-labeled anti-rabbit IgH (1:250; Vector Laboratories, Burlingame, Calif).

Single-Cell Analysis

Single-cell sorting of VEGFR2$^+$ cells using 96-well dishes was performed as previously described.[18] Colonies were stained for ephrinB2 using EphB4-Fc by TSA kit with streptavidin-conjugated horseradish peroxidase, followed by addition of phycoerythrin-conjugated anti-CD31 antibody and DAPI. Numbers of colonies including CD31$^+$ cells (EC-including), colonies including ephrinB2$^+$ cells (arterial EC-including), and ephrinB2$^+$ arterial EC numbers in each arterial EC-including colonies, as well as the total number of colonies that appeared were counted. 1692 VEGFR2$^+$ cells were cultured with VEGF alone, and 1128 cells were cultured with VEGF and 8bromo-cAMP. Total colony numbers in every 100 sequential wells, EC-including or arterial EC-including colony numbers in every 10 sequential colonies that appeared, and the arterial EC number in each arterial EC-including colony were statistically evaluated.

Measurement of Intracellular cAMP

After 3 days culture of VEGFR2$^+$ cells (2 to 10×10^5 cells), cells were harvested and counted. Intracellular cAMP concentration in total harvested cells was evaluated using cAMP Biotrak Enzyme Immunoassay system kit (Amersham Bioscience). Concentration was normalized by cell number.

In Situ Hybridization

In situ hybridization for CXCR4 was performed as previously described.[25]

Reverse-Transcription Polymerase Chain Reaction Amplification

Total RNA was isolated from sorted VE-cadherin$^+$ ECs induced by VEGF alone, or 8bromo-cAMP and VEGF treatment, using ISOGEN (Nippon Gene, Toyama, Japan). The reverse-transcription polymerase chain reaction was performed as described[24] using indicated primers (supplemental Table I, available online at http://atvb.ahajournals.org).

Statistical Analysis

Statistical analysis of the data was performed using Student *t* test. $P<0.05$ was considered significant.

Results

We first examined the effects of AM and cAMP on EC differentiation from ES cell-derived VEGFR2$^+$ progenitor cells. VEGFR2$^+$ cells were sorted by FACS and re-cultured for 3 days on type IV collagen-coated dishes in differentiation medium (see Methods) with VEGF (50 ng/mL) and other factors. Double immunostaining of induced cells with an EC marker, CD31, and a MC marker, SMA, revealed that VEGF treatment selectively induced both CD31$^+$ ECs and SMA$^+$ MCs from VEGFR2$^+$ cells as previously reported[18] (Figure 1A). Simultaneous stimulation of cAMP signaling in the presence of VEGF substantially enhanced EC induction from VEGFR2$^+$ cells (Figure 1B to 1D). VEGF together with 0.5 mmol/L 8bromo-cAMP resulted in substantial induction of ECs (Figure 1D), whereas 8bromo-cAMP treatment alone exerted almost no effect (data not shown). Another cyclic monophosphate analog, 8bromo-cGMP, showed no effect on VEGF-induced EC induction (data not shown). Addition of 10^{-6}mol/L AM also enhanced VEGF-stimulated EC induction, but to a lesser extent than 8bromo-cAMP (Figure 1B). Enhancement of the effect of AM by the simultaneous administration of a phosphodiesterase inhibitor, IBMX, revealed comparable EC induction with 8bromo-cAMP (Figure 1C). We quantitatively evaluated the EC-inducing effects of AM and 8bromo-cAMP using flow cytometry. VEGF treatment induced ECs to ≈30% of total cells. AM increased VEGF-induced ECs up to ≈50%. AM with IBMX or 8bromo-cAMP showed efficient induction of ECs to ≈70% of total cells (Figure 1E). Intracellular concentration of cAMP in the differentiating cells was significantly increased by AM with VEGF (667.6 fmol±215.1/10^6 cells; n=6; $P<0.01$ versus VEGF alone), or AM and IBMX with VEGF (1142 fmol±270.1/10^6 cells; n=6; $P<0.001$ versus VEGF alone) than that with VEGF alone (372.2 fmol±58.5/10^6 cells; n=6), and was comparable or lower level with those observed in previous reports using human umbilical vein ECs.[26] These results indicated that the AM/cAMP pathway specifically and synergistically enhances the effect of VEGF on EC differentiation from VEGFR2$^+$ progenitor cells.

Next, we investigated the features of induced ECs with AM/cAMP treatment with regard to arterial-venous diversity. Arterial ECs were evaluated by ephrinB2 expression, an arterial EC marker, detected by the binding of EphB4-Fc.[27]

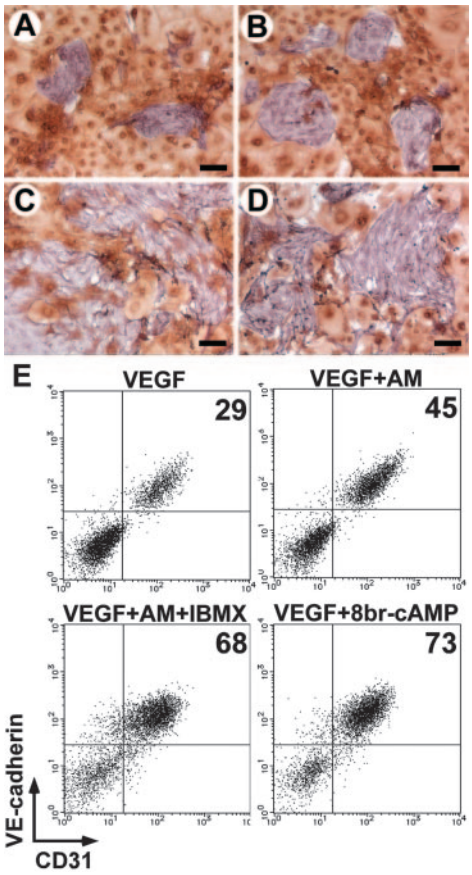

Figure 1. The effect of AM and cAMP on EC induction from VEGFR2$^+$ cells. A to D, Double immunostaining of induced ECs and MCs with an EC marker CD31 (purple) and MC marker SMA (brown) after 3 days of culture of VEGFR2$^+$ cells on type IV collagen-coated dishes in various conditions. A, VEGF treatment alone (50 ng/mL). CD31$^+$ EC sheets and SMA$^+$ MCs appear. B, VEGF with 10^{-6} mol/L AM. A slight increase of ECs is observed. C, VEGF with 10^{-6} mol/L AM and 10^{-4} mol/L IBMX. D, VEGF with 0.5 mmol/L 8bromo-cAMP. Remarkable EC induction occurs. Scale bars: 100 μm. E, Flow cytometry of induced cells from VEGFR2$^+$ cells with endothelial markers VE-cadhein and CD31. Left upper panel, VEGF treatment alone (50 ng/mL). Right upper panel, VEGF with 10^{-6} mol/L AM. Left lower panel, VEGF with 10^{-6} mol/L AM and 10^{-4} mol/L IBMX. Right lower panel, VEGF with 0.5 mmol/L 8bromo-cAMP. Percentages of VE-cadherin$^+$/CD31$^+$ ECs of total VEGFR2$^+$ cell-derived cells are indicated.

We double-immunostained ECs using anti-CD31 antibody and EphB4-Fc (Figure 2A to 2D). With VEGF treatment alone, very few ephrinB2$^+$ arterial ECs were observed among the ECs that appeared, indicating that venous ECs were mainly induced in this condition (Figure 2A). Surprisingly, remarkable appearance of ephrinB2$^+$ ECs was clearly observed by the stimulation of cAMP pathway. That is, addition of AM induced ephrinB2$^+$ EC appearance (Figure 2B). AM with IBMX, or 8bromo-cAMP together with VEGF, showed substantial induction of ephrinB2$^+$ ECs (Figure 2C and 2D). Messenger RNA expression of arterial EC markers, ephrinB2, Dll4, Notch1, Notch4, Alk1, and neuropilin1 (NRP1) were increased in 8bromo-cAMP and VEGF-treated ECs (Figure 2E). In contrast, venous EC markers, COUP-TFII transcription factor[28] and NRP2[29] mRNA were decreased by

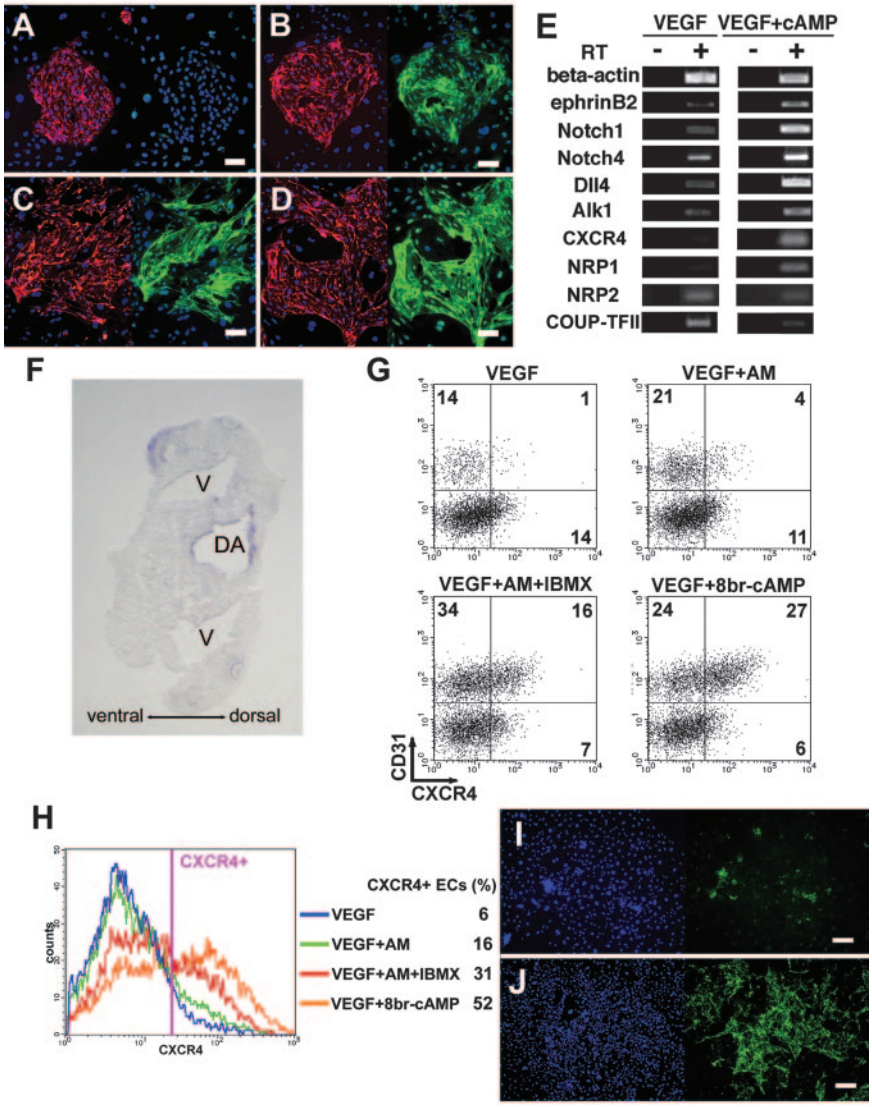

Figure 2. The effect of AM and cAMP on arterial EC induction from VEGFR2$^+$ cells. A to D, Double fluorescent staining for CD31 and ephrinB2 after 3 days of culture of VEGFR2$^+$ cells. Left panels, CD31 (pan-ECs, red) and DAPI (blue). Right panels, EphB4-Fc (ephrinB2$^+$ arterial ECs, green) and DAPI (blue). A, VEGF treatment alone (50 ng/mL). B, VEGF with 10^{-6} mol/L AM. C, VEGF with 10^{-6} mol/L AM and 10^{-4} mol/L IBMX. D, VEGF with 0.5 mmol/L 8bromo-cAMP. Scale bars: 100 μm. E, Reverse-transcription polymerase chain reaction showing mRNA expression of arterial markers (ephrinB2, Notch1, Notch4, Dll4, Alk1, CXCR4, and NRP1) and venous marker (NRP2 and COUP-TFII) in purified ECs induced by VEGF treatment alone or VEGF and 8bromo-cAMP treatment. F, Aortic EC-specific expression of CXCR4 (purple) by in situ hybridization of the isolated aorta-gonad-mesonephros (AGM) region in E11.5 mouse embryo. DA indicates dorsal aorta; V, Cardinal veins. G, Flow cytometry for CD31 and CXCR4 expression. Left upper panel, VEGF treatment alone (50 ng/mL). Right upper panel, VEGF with 10^{-6} mol/L AM. Left lower panel, VEGF with 10^{-6} mol/L AM and 10^{-4} mol/L IBMX. Right lower panel, VEGF with 0.5 mmol/L 8bromo-cAMP. H, Expression profile of CXCR4 in CD31$^+$ ECs by flowcytometry. VEGF treatment alone (blue line), VEGF with 10^{-6} mol/L AM (green line), VEGF with 10^{-6} mol/L AM and 10^{-4} mol/L IBMX (red line), and VEGF with 0.5mmol/L 8bromo-cAMP (orange line) are shown. Percentages of CXCR4$^+$ arterial ECs in total ECs are indicated. I and J, Gross appearance of ephrinB2$^+$ arterial EC induction from VEGFR2$^+$ cells (plated at 2×10^4 cells/cm^2). Left panels, DAPI (blue). Right panels, EphB4-Fc (ephrinB2$^+$ arterial ECs, green). I, VEGF treatment alone (50 ng/mL). J, VEGF with 0.5 mmol/L 8bromo-cAMP. Increase in cell number (DAPI) and substantial arterial EC induction were observed. Scale bars: 400 μm.

8bromo-cAMP and VEGF treatment (Figure 2E). These results indicated that stimulation of cAMP pathway induces arterial ECs.

We further attempted to quantitatively evaluate arterial EC induction at the cellular level. CXCR4, a 7-transmembrane G-protein-coupled receptor, is the receptor of CXCL12 (also known as stromal cell-derived factor-1). Recently, CXCR4 has been reported to be expressed in ECs in the superior mesenteric artery, but not in the superior mesenteric vein, and involved in the formation of arteries in the gastrointestinal tract.[25,30] We examined CXCR4 expression in the mouse embryo by in situ hybridization and found that CXCR4 was detected in ECs of the dorsal aorta but not of cardinal veins in aorta-gonado-mesonephros (AGM) region of E11.5 embryos (Figure 2F). In addition, mRNA expression of CXCR4 was increased in 8bromo-cAMP and VEGF-treated ECs together with other arterial EC markers (Figure 2E), indicating that CXCR4 is another arterial EC marker. FACS analysis using an anti-CXCR4 antibody successfully quantified arterial EC induction by AM or 8bromo-cAMP treatment. Most of ECs induced by VEGF treatment alone (>90% to 95%) were negative for CXCR4. CXCR4$^+$/CD31$^+$ arterial ECs were induced in the presence of AM together with VEGF. Addition of AM with IBMX, or 8bromo-cAMP further increased CXCR4$^+$/CD31$^+$ arterial EC appearance (Figure 2G). Overall, 8bromo-cAMP and VEGF treatment induced ≈5- to 10-fold more CXCR4$^+$ arterial ECs compared with VEGF treatment alone. AM with VEGF treatment showed slight effect on the arterial EC induction. Simultaneous administration of AM and IBMX with VEGF enhanced the arterializing effect of AM (Figure 2H). These results indicated that cAMP signaling mainly contributes to the arterial EC induction. The maximum percentage of arterial ECs within total ECs was increased to ≈60% by 8bromo-cAMP and VEGF (Figure 3F). Addition of 8bromo-cAMP with VEGF led to an increase in total cell number, total EC number, and arterial EC percentage, resulting in ≈70-times increment of induced arterial EC number than those by VEGF alone (Figure 2I and 2J). Higher doses of VEGF (100 to 200 ng/mL) alone or 8bromo-cGMP (0.5 mmol/L) with VEGF

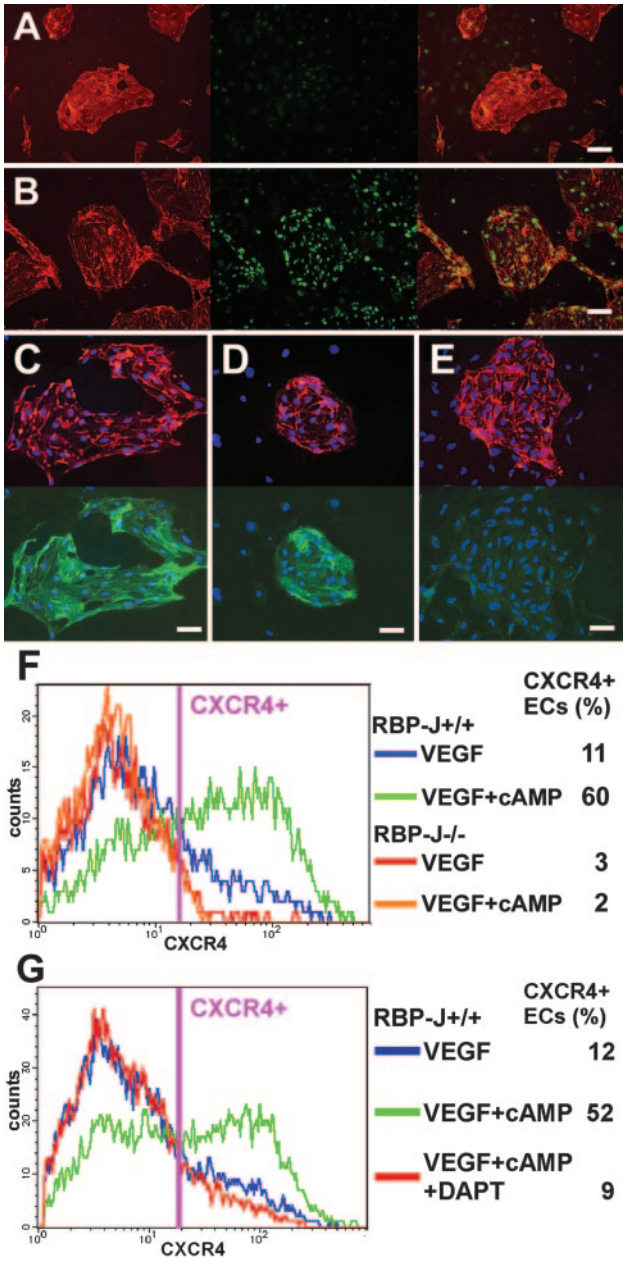

Figure 3. Essential role of Notch signaling in arterial EC induction. A and B, Double fluorescent staining of cleaved Notch intracellular domain (NICD) and CD31 for induced ECs. Left panels, CD31 (pan-ECs, red). Middle panels, Cleaved NICD (green). Right panels, Merged image. A, VEGF treatment alone (50 ng/mL). B, VEGF with 0.5 mmol/L 8bromo-cAMP. Scale Bars: 200 μm. C to E, Double-fluorescent staining of CD31 and ephrinB2 for ECs induced by VEGF with 8bromo-cAMP using RBP-J–deficient ES cells. Upper panels, CD31 (pan-ECs, red) and DAPI (blue). Lower panels, EphB4-Fc (ephrinB2$^+$ arterial ECs, green) and DAPI (blue). C, RBP-J$^{+/+}$ ES cells. D, RBP-J$^{+/-}$ ES cells. E, RBP-J$^{-/-}$ ES cells. Scale bars: 100 μm. F and G, Expression profile of CXCR4 in CD31$^+$ ECs. F, Blue and green lines: RBP-J$^{+/+}$ cells. VEGF treatment alone (blue line), VEGF with 0.5 mmol/L 8bromo-cAMP (green line). Red and orange lines: RBP-J$^{-/-}$ cells. VEGF alone (red line), VEGF with 0.5 mmol/L 8bromo-cAMP (orange line). Percentages of CXCR4$^+$ arterial ECs in total ECs are indicated. G, RBP-J$^{+/+}$ cells. VEGF treatment alone (blue line), VEGF with 0.5 mmol/L 8bromo-cAMP (green line). VEGF with 8bromo-cAMP and 2.5 μmol/L DAPT (red line).

Single-Cell Analysis of VEGFR2$^+$ Cell Culture

	VEGF Alone	VEGF With 8bromo-cAMP
Total colony, n (per every 100 sequential wells)	5.62±1.74 (n=16)	16.0±6.06 (n=11)*
EC-including colony, n (per every 10 sequential colonies)	3.40±2.20 (n=15)	7.00±1.70 (n=19)*
AEC-including colony, n (per every 10 sequential colonies)	1.27±1.10 (n=15)	3.63±1.30 (n=19)*
AEC number (per each AEC-including colony)	1.69±0.87 (n=16)	4.51±2.77 (n=76)*

*$P<0.01$ vs VEGF alone.
AEC indicates arterial endothelial cell; EC, endothelial cell.

treatment did not show arterial EC induction. Administration of iloprost (10^{-7} to 10^{-5} mol/L), an analogue of prostaglandin-I2 that elevates intracellular cAMP in mature ECs, showed almost no arterial inducing effect even with VEGF treatment (data not shown). These results indicated that AM/cAMP signaling is a novel potent and specific inducer of arterial ECs from vascular progenitor cells.

To further evaluate the mechanism of AM/cAMP-stimulated arterial EC induction, we performed single-cell culture of VEGFR2$^+$ cells. Colonies obtained from single VEGFR2$^+$ cells were counted and evaluated by staining for CD31, ephrinB2, and DAPI (Table). VEGF and 8bromo-cAMP treatment significantly increased the total number of colonies that appeared, number of EC-including colonies, and arterial EC-including colonies in appeared colonies, and arterial EC numbers in each arterial EC-including colony than VEGF alone. These results suggest that cAMP increased survival of VEGFR2$^+$ progenitor cells, differentiation of ECs and arterial ECs from progenitor cells that survived, and proliferation of arterial ECs. cAMP, thus, should be involved in multi steps of arterial EC differentiation processes.

We then examined the role of Notch signaling in arterial EC induction in this system. Activation of Notch on ligand binding is accompanied by proteolytic processing that releases intracellular domain of Notch (NICD) from the membrane. The NICD then translocates into the nucleus and associates with RBP-J, a DNA-binding protein, to form a transcriptional activator, which turns on transcription of a set of target genes.[31] First, we examined Notch activation by cAMP treatment with immunostaining of cleaved NICD. Whereas Notch signal was not activated in most of ECs induced by VEGF alone (Figure 3A), administration of 8bromo-cAMP together with VEGF clearly induced nuclear localization of cleaved NICD in ECs, indicating that stimulation of cAMP pathway can activate Notch signaling in differentiating ECs (Figure 3B). cAMP is, thus, found to be a novel signaling pathway that interacts with and activates Notch signaling in EC lineages. Then, we performed a loss-of-function study using RBP-J–deficient ES cells that lack Notch signaling activation.[20] VEGFR2$^+$ cells derived

from RBP-J$^{+/+}$, RBP-J$^{+/-}$, or RBP-J$^{-/-}$ ES cells were sorted and re-cultured with VEGF in the presence of 8bromo-cAMP. Arterial EC induction observed in RBP-J$^{+/+}$ (Figure 3C) or RBP-J$^{+/-}$ ES cells (Figure 3D) was completely abolished in RBP-J$^{-/-}$ ES cells (Figure 3E). FACS analysis using CXCR4 further demonstrated that induction of CXCR4^{+} arterial ECs observed in RBP-J$^{+/+}$ was completely abolished in RBP-J$^{-/-}$ ES cells (Figure 3F). Similarly, administration of γ-secretase inhibitor, DAPT (2.5μmol/L), which inhibits proteolytic processing of Notch to activate its signaling, to VEGFR2^{+} cell culture also completely blocked the arterial EC induction (Figure 3G). These results indicate that Notch signaling is essential for arterial EC induction in this ES cell system, and correlates with previous reports in zebrafish[32,33] and mouse[34,35] genetic animal models.

Next, we examined the effect of a gain-of-function of Notch in arterial EC induction. We used an ES cell line NERT$^{\Delta OP}$-7,[21] in which signaling of the activated intracellular domain of murine Notch1 can be regulated using an OHT-inducible system.[22] NERT$^{\Delta OP}$-7 ES cell-derived VEGFR2^{+} cells were sorted and re-cultured with VEGF in the presence or absence of OHT. In the absence of OHT, NERT protein was located mainly in the cytoplasm of induced CD31^{+} ECs and other cell types (supplemental Figure IA, available online at http://atvb.ahajournals.org). After addition of OHT, NERT protein translocated to the nucleus (supplemental Figure IB). Notch signal activation in VEGF-induced ECs was evaluated by FACS using NERT$^{\Delta OP}$-7/Hes-GFP cells carrying HES promoter-driven GFP gene (supplemental Figure IC). Addition of 8bromo-cAMP induced endogenous Notch activation in ECs, correlating with our previous results shown in Figure 3A and 3B. OHT treatment showed stronger Notch signal activation through NERT protein than 8bromo-cAMP treatment. Simultaneous stimulation by 8bromo-cAMP and OHT additionally enhanced Notch activation in induced ECs. These results indicate that NERT$^{\Delta OP}$-7 cell system can successfully induce Notch signal activation in differentiating ES cells. NERT$^{\Delta OP}$-7 cell-derived ECs induced by VEGF alone were negative for ephrinB2 (Figure 4A). Unexpectedly, hardly any arterial ECs appeared after Notch activation with OHT, even when co-stimulated with VEGF (Figure 4B). Although ephrin-B2^{+} arterial ECs were successfully induced by VEGF with 8bromo-cAMP (Figure 4C), no apparent effect of OHT was observed on the cAMP-stimulated arterial EC induction with ephrinB2 staining (Figure 4D). FACS analysis further demonstrated that activation of Notch signaling by OHT failed to induce CXCR4^{+} arterial ECs and, moreover, activation of Notch signaling with OHT did not affect, or often reduced, cAMP-induced CXCR4^{+} arterial EC induction (Figure 4E). These results indicate that Notch signal is not sufficient or at least aberrant activation of Notch is not beneficial, for arterial EC induction. This is compatible with the previous in vivo study using activated Notch4-transgenic mice in that activation of Notch signaling in embryonic endothelium led to disorganized vascular networks but did not document arterial induction.[36]

Taken together, VEGF appears essential for EC differentiation from VEGFR2^{+} cells, and venous ECs can be induced by VEGF alone. For arterial EC induction, however, VEGF and Notch signaling is essential but not sufficient. AM/cAMP pathway can activate Notch signaling, and is another important signaling to induce arterial ECs. Coordinated signaling of VEGF, Notch, and cAMP is the combination that composes a sufficient condition to constructively induce arterial ECs from vascular progenitor cells.

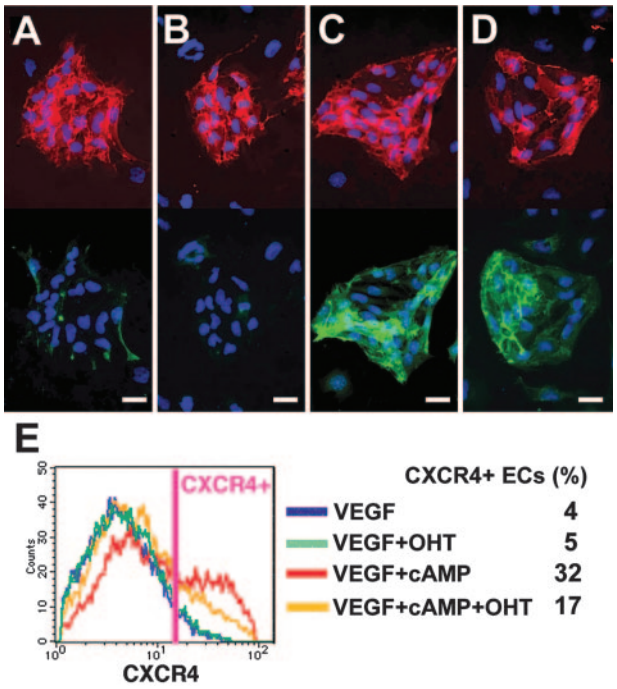

Figure 4. Effects of activated Notch on arterial EC induction from VEGFR2^{+} cells. A-D, Double-fluorescent staining of CD31 and ephrinB2 for induced ECs using NERT$^{\Delta OP}$-7 ES cells. Upper panels, CD31 (pan-ECs, red) and DAPI (blue). Lower panels, EphB4-Fc (ephrinB2^{+} arterial ECs, green) and DAPI (blue). A, VEGF treatment alone (50 ng/mL). B, VEGF and 150 nmol/L OHT. C, VEGF and 0.5 mmol/L 8bromo-cAMP. D, VEGF, 0.5mmol/L 8bromo-cAMP, and 150 nmol/L OHT. Scale bars: 100 μm. E, Expression profile of CXCR4 in CD31^{+} ECs. VEGF alone (blue line), VEGF and OHT (green line), VEGF and 8bromo-cAMP (red line), and VEGF, 8bromo-cAMP, and OHT (orange line) are shown. Percentages of CXCR4^{+} arterial ECs in total ECs are indicated.

Discussion

Our findings provide the first demonstration to our knowledge of arterial and venous EC induction from ES cells by constructively reproducing endothelial differentiation processes in vitro. Here we showed that cAMP and AM play specific roles in EC differentiation, especially for arterial EC induction, from VEGFR2^{+} vascular progenitors. We have shown that AM enhances proliferation and migration of cultured ECs and can promote angiogenesis in gel plug assays in vivo.[37] Recently, AM was reported to enhance angiogenic potency of bone marrow cell transplantation.[38] AM should be a novel potent candidate for an endogenous ligand for EC differentiation as well as arterial EC induction.

Our results showed that stimulation of cAMP pathway can activate Notch signaling in EC lineage. To date, little evidence of Notch activation by cAMP pathway has been reported. In neuronal cells, cAMP-response element-binding protein increased expression of presenilin-1, a component of

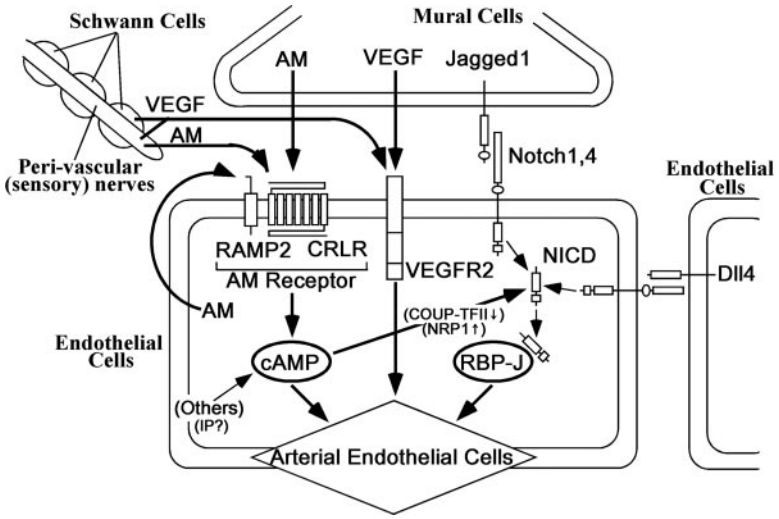

Figure 5. Cellular and molecular mechanisms of arterial EC induction. Putative autocrine/paracrine system for arterial induction in vascular wall. Signals for arterial induction, VEGF, Notch, and AM/cAMP components exist in the vascular wall. VEGFR2, AM receptor complex, RAMP2 and CRLR, and Notch1 and 4 are expressed in ECs. However, their ligands, VEGF, AM, and Jagged1 are expressed in mural cells (MCs). Moreover, AM is expressed in ECs and perivascular nerves. VEGF is produced from peripheral sensory nerves and Schwann cells. Notch ligands, Dll4, and Jagged1 are expressed in arterial ECs. These autocrine/paracrine signals among ECs, MCs, and other perivascular tissues should coordinately regulate the arterial induction and maintenance.

γ-secretase, through transcriptional activation.[39] A similar mechanism may contribute in EC and EC progenitors to induce Notch activation. Recently, COUP-TFII has been reported to repress Notch signaling through suppressing NRP1 expression to maintain vein identity.[28] Administration of 8bromo-cAMP did not increase mRNA expression of Notch ligands (ie, jagged1, 2, Delata-like1, 3, 4) in surrounding mural cells (data not shown), but suppressed COUP-TFII and increased NRP1 expression in ECs. These results suggest that cAMP pathway may activate Notch signaling through the suppression of COUP-TFII expression. cAMP pathway, thus, may regulate the determination of cell fates between arterial and venous ECs. Although Dll4 and Notch signaling were reported to be growth-suppressive on mature ECs through downregulation of VEGFR2 and NRP1 expression,[40] forced Notch activation with OHT did not affect on VEGFR2 and NRP1 mRNA expression in differentiating ECs (data not shown). Notch signaling may possess differentiation stage-specific roles in EC differentiation and proliferation. Precise molecular interactions among these pathways should be further investigated to figure out the whole scheme of arterial-venous specification.

In the vascular wall, VEGFR2, Notch1 and 4, and AM receptor complex, CRLR, RAMP-2 and -3, are expressed in ECs.[5,6] On the other hand, their ligands, VEGF, Jagged1, and AM, are expressed in MCs.[8,41,42] Dll4 and AM are also expressed in ECs. We confirmed AM mRNA expression in ES cell-derived ECs and MCs, and RAMP-2 and CRLR mRNA in ECs by reverse-transcription polymerase chain reaction analysis. Low-level expression of prostaglandin-I2 receptor mRNA was also observed in ECs (data not shown). Moreover, peripheral sensory nerve and Schwann cell-derived VEGF are reported to be involved in arterial EC induction.[43] AM is demonstrated to be expressed in perivascular nerves in the rat mesenteric artery.[44] The autocrine/paracrine cross-talk of VEGF, Notch, and AM/cAMP signaling between ECs and MCs, and signals from other perivascular tissues, should coordinately regulate vascular development including the induction and maintenance of the arterial structures (Figure 5). Combinatory signaling of VEGF, Notch, and cAMP may mimic these arterial-inducing machineries in vivo to achieve constructive induction of arterial ECs from vascular progenitor cells in vitro.

Our constructive approach has successfully provided a novel understanding for the mechanisms of arterial EC differentiation. This study, thus, would provide a potent novel strategy as constructive developmental biology to dissect cell differentiation processes and contribute to regenerative medicine.

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Disclosures

None.

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Arteriosclerosis, Thrombosis, and Vascular Biology

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Adrenomedullin/Cyclic AMP Pathway Induces Notch Activation and Differentiation of Arterial Endothelial Cells From Vascular Progenitors

Takami Yurugi-Kobayashi, Hiroshi Itoh, Timm Schroeder, Akiko Nakano, Genta Narazaki, Fumiyo Kita, Kentoku Yanagi, Mina Hiraoka-Kanie, Emi Inoue, Toshiaki Ara, Takashi Nagasawa, Ursula Just, Kazuwa Nakao, Shin-Ichi Nishikawa and Jun K. Yamashita

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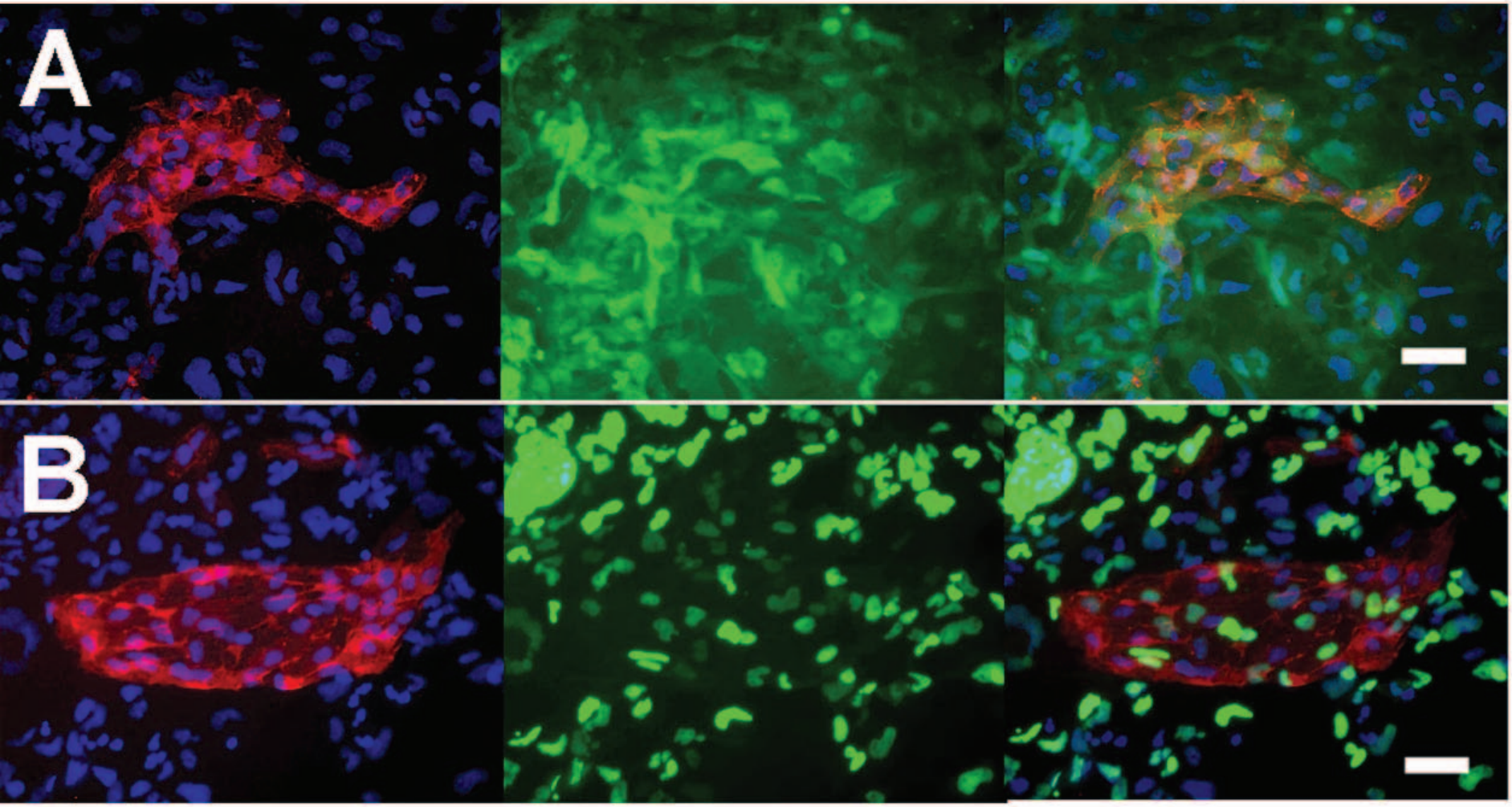
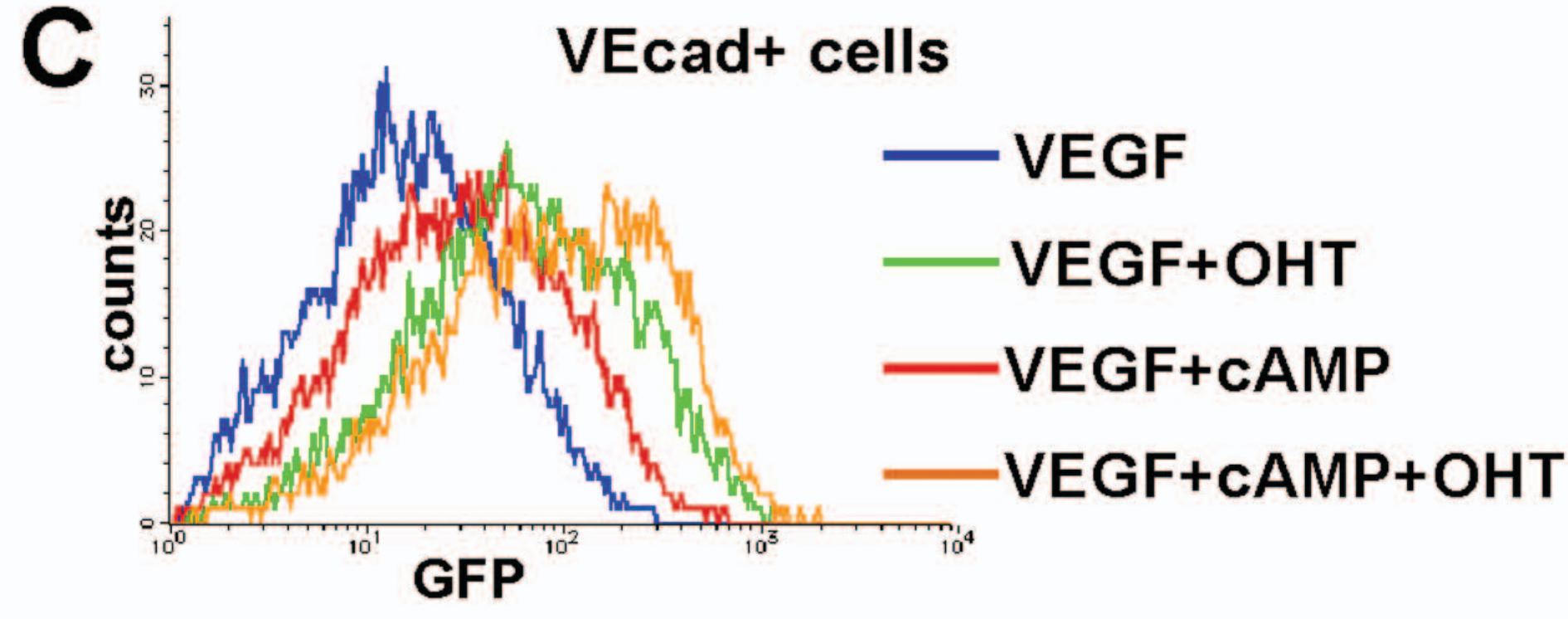
Online supplement Table I: Primer list for RT-PCR

Gene	Sense sequence (5' to 3') Antisense sequence (5' to 3')	Expected size of PCR product
ephrinB2	TCTGGGGTCTAGAATTTCAG AGTAAATGTTGGCAGGACTC	630 bp
Notch1	GACATCACGGATCACATGGA TGCCATTGGTACTCAGCACT	610 bp
Notch4	ACCCTGCTCCAATGGAGGAT AGAACCTCCGATTCACACTCC	1562 bp
Dll4	TGCTGTGGGTAAGATTTGGCGAACA GGTGAGTCCGCACAGGTCAAGGTAC	663 bp
Alk1	CTTGGGGAGCTTCAGAAGGGG GGTGGCCTCCAGCATCAGAGA	310 bp
CXCR4	TAGGATCTTCCTGCCCACCAT TGACCAGGATCACCAATCCA	77 bp
Neuropilin1	GGTTTTAACTGCGAGTTTG ATCATCCACAGCAATTCCACC	452 bp

Neuropilin2	GTACACAGTACACTCCCATG	377 bp
	GGTCATGTCTGAGTGCACTG	
COUP-TFII	GACTCCGCCGAGTATAGCTG	560 bp
	GCCCAACACAGGAGTTGTTT	
β-actin	GCTCGTCGTCGACAAGGGCTC	353 bp
	CAAACATGATCTGGGTCATCTTCTC	

Online supplement Figure I





Legend for online supplement figure

Online supplement Figure I: Notch signal activation in NERT$^{\Delta OP}$-7 ES cells

A and B, Double fluorescent immunostaining of CD31 and ERα (NERT protein) for induced ECs using NERT$^{\Delta OP}$-7 ES cells. Left panels: CD31 (pan-ECs, red) and DAPI (blue), middle panels: ERα (NERT protein, green), right panels: merged image. A, VEGF treatment alone (50ng/mL). B, VEGF and 150nmol/L OHT. Apparent nuclear translocation of NERT protein was observed. C, Flowcytometry for Hes promoter-driven GFP expression in induced EC fractions (VE-cadherin^{+}) using NERT$^{\Delta OP}$-7/Hes-GFP ES cells. VEGF treatment alone (blue line), VEGF with 150nmol/L OHT (green line), VEGF with 0.5mmol/L 8bromo-cAMP (red line), and VEGF with 8bromo-cAMP and OHT (orange line). Induction of Notch signal activation was observed in OHT and/or 8bromo-cAMP-treated ECs.