at Flk-d3 in venous ECs induced by VEGF alone, ephrinB2 expression could still be induced (Fig. S5), suggesting that ECs at the early differentiation stage still possess a plasticity for arterial–venous specification. The existence of the arterial protein complex in the adult vessels (Fig. 6) and synergistic effect of dual activation of Notch and β-catenin signaling on arterial gene expression during in vivo angiogenesis (Fig. 7) provide further supporting evidence that the complex should be involved in the induction as well as the maintenance of arterial phenotypes. Recently, Shin et al. (2009) reported that activation of Notch or β-catenin signaling during early mesoderm differentiation in the chick embryo induces separation of smooth muscle and blood/endothelial progenitor lineages. Notch and β-catenin signaling should play distinct roles in cell fate determination depending on each differentiation stage.

Interaction of Notch and β-catenin signaling has been reported in various contexts. Though Notch and β-catenin signaling pathways were reported to produce synergistic effects on maintenance of hematopoietic stem cells (Duncan et al., 2005), hair follicles (Estrach et al., 2006), and intestinal stem cells (Radtke and Clevers, 2005; van Es et al., 2005), direct molecular interaction of Notch and β-catenin was not clear from these reports. Direct interaction of Notch and β-catenin has been reported in *Drosophila*, in which the Notch C-terminal region to the cdc10/ankyrin repeats is associated with Armadillo/β-catenin and negatively regulates β-catenin/TCF transcription (Hayward et al., 2005). In our study, TCF4 was not involved in arterial specification, and β-catenin exerted its arterializing effect through a TCF-independent manner. Recently, Shimizu et al. (2008) showed that the Notch/β-catenin/RBP-J complex suppresses differentiation of neural precursor cells independent of TCF. The protein complex that directly converges Notch and β-catenin signaling may play a critical role in cell fate determination in various organs.

Until now, individual roles of VEGF, Flk1, PI3K, Notch, and β-catenin have been suggested in various studies, namely, VEGF and Flk1 in EC differentiation (Sakurai et al., 2005), PI3K and β-catenin in vascular formation (Monkley et al., 1996; Dayanir et al., 2001; Ishikawa et al., 2001; Goodwin and D'Amore, 2002; Shiojima and Walsh, 2002; Cattelino et al., 2003), and Notch in arterial specification (Xue et al., 1999; Lawson et al., 2001; Villa et al., 2001; Zhong et al., 2001; Duarte et al., 2004; Krebs et al., 2004; Sörensen et al., 2009). We constructively reproduced the cellular processes of arterial EC induction in vitro, which can lead to integrate these signaling pathways and offer comprehensive understanding of how these molecules interact during EC differentiation and arterial specification. The novel insights into the molecular machinery of cell differentiation and diversification would provide clues for clinical strategies with vascular-specific manipulation and drug discovery targeting ischemic disease and cancer.

Materials and methods

Generation of stable cell lines with inducible cDNAs

cDNA for CA-β-catenin (ΔN90-β-catenin; a gift from Dr. Y. Gotoh, University of Tokyo, Tokyo, Japan; Hirabayashi et al., 2004), CA-GSK3β (S9A-GSK; a gift from Dr. M.J. Birnbaum, University of Pennsylvania School of Medicine, Philadelphia, PA; Summers et al., 1999), DN-GSK3β (a gift from Dr. J.R. Woodgett, Samuel Lunenfeld Research Institute, Toronto, Canada; Rommel et al., 2001), and DN-TCF4 (a gift from Dr. H. Clevers, University Medical Center, Utrecht, Netherlands; van de Wetering et al., 2002) were introduced into the downstream region of tetracycline responsive element (TRE)–regulatable CMV promoter of Exchange vector (Yamamizu et al., 2009). We previously generated ES cells carrying recombination sites of loxP and a mutant loxP, loxP511, in ROSA locus and tetracycline-transactivator gene (ESiTA-ROSA; Yamamizu et al., 2009). Stable ES cell lines that express the desired cDNA under the control of the TRE-regulatable CMV promoter were generated by introducing Exchange vectors and pBS185 (Cre expression vector) to ESiTA-ROSA cells using mouse ES cell Nucleofector kit (Lonza). Hygromycin- and ganciclovir-resistant colonies were selected and subjected to further studies (cDNA ESiTA cells; Yamamizu et al., 2009). For DN-TCF4–expressing ES cells, total hygromycin- and ganciclovir-resistant colonies were collected and used. Tet-inducible expression and/or function of these cDNAs were confirmed by Western blotting and Wnt/β-catenin signaling reporter assays using TOP-FLASH TCF reporter plasmid (Millipore; Fig. S3, A–D).

An ES cell line carrying both tet-regulatable CA-β-catenin gene and OHT-regulated Notch activation system with $NERT^{\Delta OP}$, a fusion protein of N1ICD and ER (Yurugi-Kobayashi et al., 2006), was generated by introducing $NERT^{\Delta OP}$ plasmid carrying puromycin-resistant gene to CA-β-catenin ESiTA cells using mouse ES cell Nucleofector kit (Lonza). Cells were then plated on 10-cm dishes containing 1 μg/ml doxycycline (Dox^+). After 1 d, the medium was changed to Dox^+ medium containing 200 μg/ml hygromycin and 1 μg/ml puromycin. Hygromycin- and puromycin-resistant colonies were selected and subjected to further studies (CA-β-catenin/$NERT^{\Delta OP}$ ESiTA cells).

Cell culture

ES cell lines, D3, ESiTA-ROSA, and various ESiTA derivatives were maintained as described previously (Yurugi-Kobayashi et al., 2006; Yamamizu et al., 2009). Differentiation was induced in these ES cell lines using differentiation medium (DM) (α-minimal essential medium, MEM; Invitrogen) supplemented with 10% fetal calf serum (FCS; Japan Bioserum) and 5×10^{-5} M 2-mercaptoethanol (2-ME; Invitrogen) as described previously (Yamashita et al., 2000; Yurugi-Kobayashi et al., 2006; Yamamizu et al., 2009). In brief, undifferentiated ES cells were cultured on type IV collagen-coated dishes without leukemia inhibitory factor at a cell density of $0.75–10^3$ cells/cm^2 for 96–108 h. Cultured cells were harvested and subjected to magnetic cell sorting (MACS) purification. Purified $Flk1^+$ cells were then plated onto type IV collagen-coated dishes (BD) at a density of $0.75–10^4$ cells/cm^2 in DM. After 3 d of $Flk1^+$ cell differentiation (Flk-d3), induced ECs were examined by immunohistochemistry and flow cytometric analyses. Various reagents were occasionally added to the $Flk1^+$ cell culture, including human $VEGF_{165}$ (R&D Systems), 8-bromoadenosine-3′, 5′-cyclic monophosphate sodium salt (8-bromo-cAMP′ Nacalai Tesque), γ-secretase inhibitor, DAPT, PI3K inhibitor, LY294002, GSK3β inhibitor, Bio, Akt inhibitor, TAT-Akt-inh, PKA inhibitor, PKI or H89, p38 inhibitor, SB202190, MEK inhibitor, PD98059, PKCαβ inhibitor, PKCη inhibitor, and PKCζ inhibitor (EMD). Dox was added to ES cells during the first 4.5 d of ES cell differentiation culture for the tetracycline-regulated cDNA expression experiments. Subsequently, purified $Flk1^+$ cells were plated on type IV collagen-coated dishes and cultured in the presence or absence of 1 μg/ml Dox. 1 μg/ml Dox did not affect EC differentiation, proliferation, or arterial–venous specification in control ES cells (Yamamizu et al., 2009). For OHT-induced Notch activation, 150 ng/ml of OHT was added 24 h after $Flk1^+$ cell plating. OHT did not affect arterial–venous specification in control ES cells.

Immunocytochemistry

Immunostaining of cultured cells was conducted as described previously (Yurugi-Kobayashi et al., 2006, Yamamizu et al., 2009). ECs were fixed with 5% dimethyl sulfoxide/methanol. For double staining of ephrinB2 and CD31, fixed culture slides were incubated with EphB4-human immunoglobulin (Ig) Fc portion chimeric protein (EphB4-Fc; 1:50; R&D Systems) followed by human IgG Fc peroxidase-conjugated goat IgG fraction (1:500; ICN Biomedicals, Inc.). TSA biotin system (PerkinElmer) was used to amplify the signal for EphB4-Fc staining. $EphrinB2^{P+P}$ cells were visualized with streptavidin/Alexa Fluor 488 conjugate (Invitrogen; Yurugi-Kobayashi et al., 2006). $CD31^+$ cells were stained with phycoerythrin (PE)-conjugated monoclonal antibody (mAb) for CD31 (Mec13.3; 1:300; BD). Cleaved Notch intracellular domain (NICD) staining was performed with TSA Biotin System using anti-cleaved Notch1 antibody (1:300; Cell Signaling Technology), followed by peroxidase-labeled anti-rabbit IgH antibody (1:500; Vector Laboratories; Yurugi-Kobayashi et al., 2006).

Stained cells were photographed with an inverted fluorescent microscope (Eclipse TE2000-U; Nikon) with 10× and 20× objectives, Plan Fluor (Nikon), a digital camera system, AxioCam HRc (Carl Zeiss, Inc.), and AxioVision software (Carl Zeiss, Inc.). All images were taken at room temperature.

PI3K activity assay

Flk1$^+$ cells (10^4 cells/ml) were incubated on 96-well plates and stimulated with vehicle, VEGF (50 ng/ml), 8-bromo-cAMP (0.5 mM), VEGF with 8-bromo-cAMP, or VEGF with 8-bromo-cAMP and LY294002 (7.5 µM). After a 3-d culture of Flk1$^+$ cells, PI3K activity was evaluated using FACE PI3 Kinase p85 ELISA kit (Active Motif) according to the manufacturer's instructions. The concentration was normalized by cell numbers stained with crystal violet. PI3K activity was measured with a microplate reader (ARVO MX; PerkinElmer).

Cell purification and FACS analysis

After the induction of Flk1$^+$ cells, they were harvested and stained with allophycocyanin (APC)-conjugated AVAS12 antibody (Yamashita et al., 2000). Flk1$^+$ cells were sorted with auto MACS (Miltenyi Biotec) using anti-APC MicroBeads (Miltenyi Biotec) and recultured for EC differentiation. For the arterial and venous EC FACS analysis, cultured cells were harvested at Flk-d3 and stained with a combination of PE-conjugated anti-CD31 mAb (Mec13.3; BD) and biotin-conjugated anti-CXCR4 mAb (BD) followed by streptavidin-conjugated APC (BD), and then subjected to analysis using FACSVantage or FACSAria (BD). Arterial and venous ECs in vivo were isolated from embryos at E11.5. Embryos were diced, digested with dispase II (Roche), and hemolyzed. Arterial and venous ECs negative for CD45 were obtained using FACSVantage or FACSAria.

RT-PCR

Total RNA was isolated from arterial and venous ECs with RNeasy (QIAGEN) according to the manufacturer's instructions and reverse transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen). RT-PCR was performed as described previously (Yamashita et al., 2005) using the indicated primers (Table S1).

Subcellular proteome extraction and Western blotting

Sorted arterial and venous ECs were subjected to protein extraction according to their subcelluar localization using the ProteoExtract Subcelluar Proteome Extraction kit (EMD). Nuclear extraction or total lysates were subjected to SDS–PAGE using gradient gels (Atto Co.) followed by electrophoretic transfer onto nitrocellulose membranes. The blots were incubated for 1 h in Blocking One blocking agents (Nacalai Tesque). Then, the membranes were incubated overnight with the respective first antibodies (1:1,000) for β-catenin (BD), NICD (Cell Signaling Technology), histone H3 (Abcam), Notch1 (Abcam), Notch4 (Santa Cruz Biotechnology, Inc.), ephrinB2 (R&D Systems), neuropilin1 (R&D Systems), and β-actin (Sigma-Aldrich). Horseradish peroxidase (HRP)–conjugated anti–mouse Ig antibody (Invitrogen), HRP-conjugated anti–goat Ig antibody (Invitrogen), or HRP-conjugated anti–rabbit Ig antibody (Vector Laboratories) were used as secondary antibodies (1:10,000). The Can Get Signal Immunoreaction Enhancer Solution kit (Toyobo) was used to enhance the signal. Immunoreactivity was detected with the Chemi-Lumi One Enhanced Chemiluminescence kit (Nacalai Tesque). Signal intensity was calculated with Scion Image software (Scion Corp.).

Immunoprecipitation and chromatin immunoprecipitation assays

Arterial and venous EC cell lysates were subjected to immunoprecipitation using Protein G HP SpinTrap (GE Healthcare) and anti-NICD antibody, and then immunoblotted with antibody specific to β-catenin (see previous section).

Arterial and venous ECs were subjected to cross-linking with 3.7% formaldehyde. The aorta and vena cava were isolated from adult mice (8–10 wk old) and were also subjected to cross-linking with 3.7% formaldehyde, followed by the ChIP assay using a ChIP assay kit (Millipore). Chromatin was sheared to an average length of 0.4–1.0 kb. Antibodies to RBP-J (K0043; Tokusyu-meneki Laboratory, Tokyo, Japan), NICD (Cell Signaling Technology), and β-catenin (BD) were used for immunoprecipitation. PCR amplifications were performed in 20 µl with primers specific for the promoter analysis, as shown below. The sensitivity of the PCR amplifications was evaluated with serial dilutions of total DNA collected after sonication (input fraction). Amplified DNA was separated on 2% agarose gels and visualized with ethidium bromide. Sets of primers were used to amplify DNA sequences containing the conserved RBP-J binding sites (GTGGGAA) of the mouse ephrinB2 and Hes1 gene according to Grego-Bessa et al. (2007) and Shimizu et al. (2008), respectively (Table S2). Moreover, we performed in silico investigations of the cis-acting elements within various reported arterial-specific genes using VISTA browser (Mayor et al., 2000) and ClustalW (Thompson et al., 1994). We analyzed the RBP-J binding sites conserved between the mouse and human genomes in ±10 kb of each gene. We found conserved RBP-J binding sites in the *neuropilin1*, *dll4*, and *cxcr4* genes (Fig. S4 and Table S2). PCR amplification was conducted with a variable number of cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s).

Luciferase reporter assay

FACS-purified Flk1$^+$ cells were transfected with a Notch signaling reporter, Hes1-Luciferase plasmid carrying the firefly luciferase gene under the control of the Hes1 promoter region (−467 to +46; a gift from Drs. R. Kageyama and T. Ohtsuka, Kyoto University, Kyoto, Japan; Ohtsuka et al., 1999) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. After 24 h, luciferase activity was assayed using a luminometer (Lumat LB 9507; Berthold Technologies). The luciferase activity was normalized with that of control *Renilla* luciferase.

Construction and preparation of adenovirus vectors

HA-tagged N1ICD (generated by deleting the ER sequence from the NERT$^{\Delta OP}$ plasmid; Yurugi-Kobayashi et al., 2006) or CA-β-catenin was introduced into pENTR (Invitrogen), and LR recombination reactions were performed between the pAd/CMV/V5-DEST (Invitrogen) and pENTR with HA-tagged NICD or CA-β-catenin to generate full-length adenoviral vectors.

Plasmid DNAs encoding the full-length adenoviral vectors were linearized and transfected into 293 cells using Lipofectamine 2000 (Invitrogen). The resultant adenovirus vectors were propagated by serial passages on 293 cells and purified by $CsCl_2$ density gradient ultracentrifugation, as described previously (Ng and Graham, 2002).

In vivo Matrigel plug assay and cell recovery

In vivo induction of arterial ECs was evaluated with the Matrigel plug assay (Kim et al., 2002). VEGF (final concentration, 100 ng/ml; R&D Systems) and heparin (final concentration, 10 units/ml; Nacalai Tesque) were dissolved in BD Matrigels Matrix High Concentration Solution. Adenoviral vector solutions for vehicle, NICD, and/or CA-β-catenin were mixed with the Matrigel solution on ice (total 8×10^8 plaque-forming units [p.f.u.] of virus [20 µl]/500 µl Matrigel; control: 8×10^8 p.f.u. of vehicle; NICD: 4×10^8 p.f.u. of NICD and 4×10^8 p.f.u. of vehicle; β-catenin: 4×10^8 p.f.u. of CA-β-catenin and 4×10^8 p.f.u. of vehicle; NICD + β-catenin: 4×10^8 p.f.u. of NICD and 4×10^8 p.f.u. of CA-β-catenin). Matrigel solution (500 µl) containing growth factors and adenovirus was injected subcutaneously near the mid-abdomen of anesthetized nude mice, and the mice were sacrificed 7 d after injection. All animal experiments were performed in accordance with the guidelines for Animal Experiments of Kyoto University, which conforms to the Guide for the Care and Use of Laboratory Animals in Japan. Gel plugs were excised and fixed in 4% paraformaldehyde, subjected to an ethanol dehydration series, and embedded in paraffin. Serial sections were deparaffinized and stained with hematoxylin and eosin. Stained sections were photographed using a microscope (model BX51; Olympus) with 4× objectives, UPlanFLN (Olympus), and DP2-BSW software (Olympus). All images were taken at room temperature. For cell recovery, the excised Matrigel plugs were minced with a sterile scalpel, passed 10 times through an 18-gauge needle, treated with BD Cell Recovery Solution for 1 h at 4°C, centrifuged, and subjected to Western blotting.

Statistical analysis

Comparisons among values for all groups were performed with an analysis of variance (ANOVA). At least three independent experiments were performed. A p-value less than 0.05 was considered significant. Values are reported as means ± SDs.

Online supplemental material

Fig. S1 shows pharmacological studies for cAMP downstream targets on arterial EC induction. Fig. S2 shows activation Notch signaling by cAMP together with VEGF. Fig. S3 shows generation of stable ES cell lines with inducible CA-β-catenin, CA-GSK3β, or DN-TCF4. Fig. S4 shows in silico investigations of the cis-acting elements within arterial-specific genes. Fig. S5 shows plasticity for arterial–venous phenotypes in early ECs. Table S1 shows the primer list for RT-PCR. Table S2 shows the primer list for ChIP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200904114/DC1.

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心不全に対するiPS細胞の臨床応用

Clinical application of iPS cells to heart failure

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◎人工多能性幹細胞(iPS 細胞)は，線維芽細胞などの分化した細胞から誘導された ES 細胞様の新しい幹細胞である．ヒト iPS 細胞は，ヒト ES 細胞において認められた倫理的問題を回避できる画期的発明であるが，奇形腫形成や iPS 細胞特有の“遺伝子導入による細胞変異・癌化の問題”など今後地道に解決すべき課題は多い．マウス ES 細胞とマウス iPS 細胞，ヒト ES 細胞とヒト iPS 細胞はそれぞれ維持培養法，分化誘導法などにおいてほとんど同等の特性を有しており，ES 細胞で培われた技術を導入することにより心血管系細胞を分化誘導することが可能であった．iPS 細胞研究の心不全治療応用としては，心筋細胞シートや心筋ボールなどの移植技術を用いた細胞移植治療がおもなターゲットと考えられるが，それ以外にも患者特異的モデル細胞の構築などにより，病態解明や創薬治療応用などさまざまな形で臨床へ貢献することが期待される．

Key word：ES細胞，iPS細胞，再生，分化

iPS細胞登場の背景と意義

胚性幹細胞(embryonic stem cells：ES 細胞)は，マウスやヒトの早期胚(胚盤胞；blastocyst)の段階において，将来胎仔を形成する内細胞塊とよばれる部位を取り出して樹立した細胞株であり，体中すべての種類の細胞に分化することのできる，いわゆる万能の幹細胞と考えられている．マウス ES 細胞の樹立は 1981 年，ヒト ES 細胞は 1998 年に樹立され，再生医療への応用に期待が寄せられた．しかし，ヒト ES 細胞においては，①技術・安全面の問題，とくに未分化 ES 細胞があやまって移植されると奇形腫を形成する可能性がある，②倫理面の問題，すなわち，ⅰ)ヒト ES 細胞の樹立の際にヒト受精卵を壊す必要がある，ⅱ)免疫による拒絶を受けない ES 細胞を樹立するためにヒト体細胞クローン胚(成体細胞の核を除核未受精卵に移植したクローン胚)をつくる必要が考えられる，ということが実際の応用への大きな障壁になっていた．こうした状況を背景に生まれてきた新しい幹細胞が人工多能性幹細胞(induced pluripotent stem cells：iPS 細胞)である．

iPS 細胞は，線維芽細胞など分化した細胞に特定の遺伝子群(*Oct3/4*，*Sox2*，*Klf4*，*c-myc* など)を導入することにより，ES 細胞様の万能の幹細胞の性質をもたせることに成功した細胞である．最初の iPS 細胞は 2006 年に京都大学の山中らによって報告された[1]．2007 年には山中らおよびトムソンら[2,3]，その後さらに多くの他のグループによりヒト iPS 細胞の樹立が報告された．ヒト iPS 細胞は，ヒト ES 細胞において認められた倫理的問題，上記の②-ⅰ)，ⅱ)を一気に回避できる画期的発明である．しかし，iPS 細胞には依然として上記①の奇形腫の問題は存在しているし，iPS 細胞特有の“遺伝子導入による細胞変異・癌化の問題”などあらたな問題もあり，今後地道に解決すべき課題は多い．

マウスiPS細胞の心血管細胞分化

著者らはこれまで，マウスおよびヒト ES 細胞を用いて心血管細胞の分化再生研究を行ってきた．すなわち，マウス未分化 ES 細胞から血管内皮増殖因子(vascular endothelial growth factor：

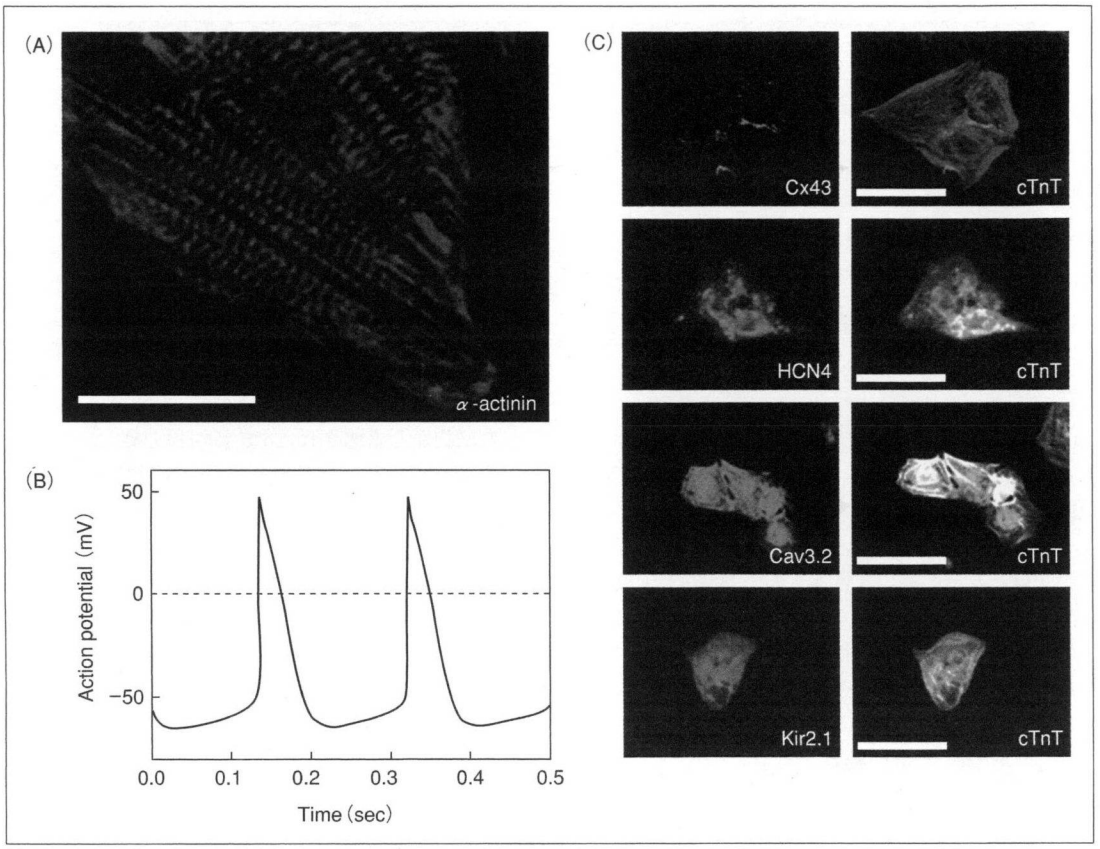

図 1　マウスiPS細胞からの心筋細胞分化[8]

Flk1 陽性細胞を OP9 ストローマ細胞上で培養することにより拍動心筋細胞が出現する．
A：拍動細胞は sarcomere 構造を示す(赤；アクチニン染色)．スケールバー：25 μm．
B：ペースメーカー細胞様の活動電位を示す．
C：誘導された心筋細胞(右：赤；cTnT 陽性)は Cx43, HCN4, Cav3.2, Kir2.1 など(左；緑)の種々の心筋細胞マーカーの発現を認めた．スケールバー：100 μm．

VEGF)の受容体のひとつであり，血管内皮・血球の前駆細胞や中胚葉細胞のマーカーでもある Flk1(2 型 VEGF 受容体；VEGF receptor-2)を発現する細胞を誘導し，Flk1 陽性細胞を共通の前駆細胞として，血管内皮細胞，血管壁細胞，血球細胞，心筋細胞といった循環器系細胞を系統的に分化誘導することに成功している[4,5]．この新しい分化誘導システムを用いて，ES 細胞由来の心筋前駆細胞の同定[5]や動静脈リンパ管内皮細胞をそれぞれ ES 細胞から誘導すること[6,7]にも成功している．ヒト ES 細胞からの血管細胞の誘導や虚血モデルへの移植実験などにも関与している(京都大学内分泌代謝内科との共同研究)．

このマウス ES 細胞の系統的心血管細胞分化システムをマウス iPS 細胞に導入し，著者らはいち早く iPS 細胞からのこれら心血管細胞の分化誘導に成功した[8]．すなわち，未分化マウス iPS 細胞を LIF(leukemia inhibitory factor)非存在下に培養することにより Flk1 陽性細胞が誘導された．Flk1 陽性細胞を VEGF および血清存在下に培養することにより，おもに静脈を中心とする内皮細胞および壁細胞が，VEGF に加えて cAMP シグナルを刺激することにより動脈内皮細胞が，OP9 ストローマ細胞上で培養することにより血球，リンパ管内皮細胞，心筋細胞が，それぞれ誘導された．誘導された心筋細胞は，種々の心筋細胞マーカー発現や sarcomere 構造，心筋様活動電位などの心筋細胞としての特性を示した．またペースメーカー細胞特異的イオンチャネル HCN4，T 型 Ca チャネル(Cav3.2)や心室筋特異的チャネル Kir2.1

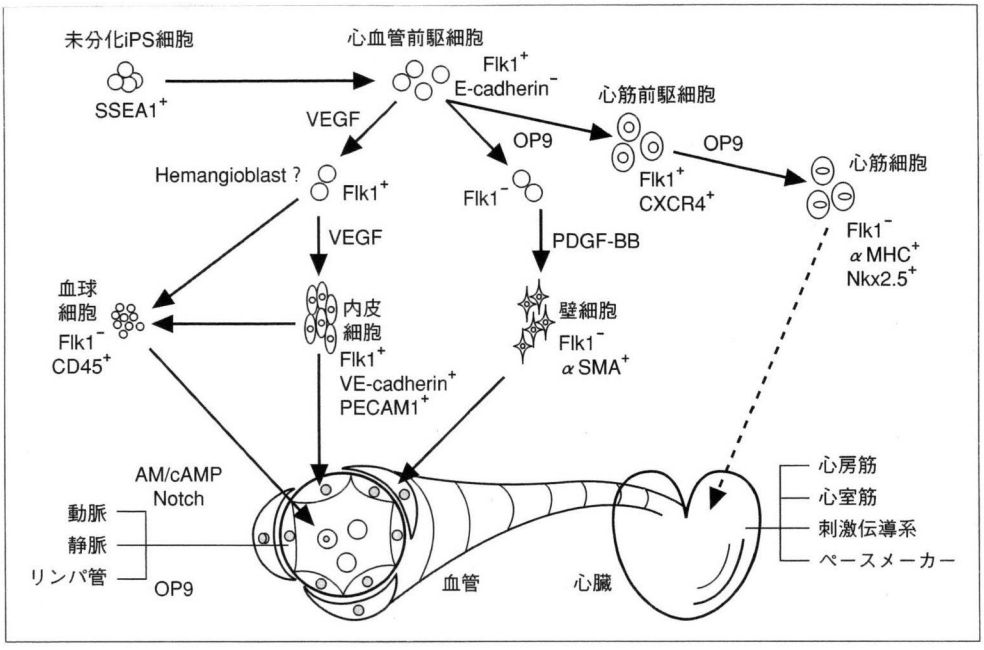

図 2　マウスiPS細胞からの系統的心血管細胞分化[8]

マウス iPS 細胞から誘導した Flk1 陽性細胞を共通の前駆細胞として，心血管系の構成細胞である血管内皮，壁細胞，心筋細胞，さらには動静脈リンパ管内皮細胞や種々の心筋細胞を系統的に分化誘導することができる．

を発現する細胞などが存在し，種々の心筋細胞が混在して誘導されていると考えられた(図 1)．マウス iPS 細胞からの Flk1 陽性細胞，(動静脈リンパ管)内皮細胞，壁細胞の分化様式，分化効率などはほとんどマウス ES 細胞と変わりがなかった．このように，マウス ES 細胞とマウス iPS 細胞はほぼ完全に同等な心血管分化能と分化動態を示し，マウス ES 細胞と同様に系統的に心血管細胞を分化誘導することが可能であった(図 2)．ほかにマウス iPS 細胞からの心血管細胞分化に関しては，心筋細胞を誘導したとの報告[9]，および著者らに類似した分化システムを用いた心血管細胞分化の報告[10]などがある．

ヒトiPS細胞の心血管細胞分化

著者らはヒト iPS 細胞の心血管細胞分化にも取り組んでいる．ヒト ES 細胞の維持培養条件に準じた環境で誘導・樹立されたヒト iPS 細胞は，その維持および分化誘導においてもヒト ES 細胞に類似した動態を示した．著者らは，ヒト ES 細胞において報告されている心筋分化誘導法[11]に準じて培養することにより，自己拍動する心筋細胞コロニーの誘導にすでに成功している．心筋に特徴的な sarcomere の形成や自己拍動に同調した Ca^{2+} の取込みなど形態的機能的特性も確認している(未発表)．また胚様体(embryoid body)法を用いてヒト iPS 細胞から心筋を誘導した報告が 2009 年 2 月に最初になされ[12]，その後日本などから胚様体法を用いて薬剤に反応する心筋細胞を誘導したことが報告されている[13-15]．マウス ES 細胞とマウス iPS 細胞，ヒト ES 細胞とヒト iPS 細胞はそれぞれ維持培養法，分化誘導法などにおいてほとんど同等の特性を有していると考えられる．今後の iPS 細胞研究においては，マウスおよびヒト ES 細胞研究がその土台となり，またつねに比較対象のスタンダードとなると考えられる．iPS 細胞の出現によって，ES 細胞研究は衰退するどころかさらにその重要性を増していると考えられる．

iPS細胞研究の臨床への貢献

ES 細胞，iPS 細胞研究の循環器領域における意義は，やはり心血管再生治療への応用が中心的に

期待されると考えられるが，それ以外にも患者特異的モデル細胞の構築という新しいアプローチができることにより，病態解明や創薬治療応用などさまざまな形での臨床面への貢献が可能である．

1. 誘導細胞の細胞移植応用

ヒト iPS 細胞は，ヒト ES 細胞に存在した倫理面の問題，および患者特異的 iPS 細胞を樹立できることにより移植免疫の問題も回避できるため，細胞移植による再生医療応用が期待されている．循環器領域においても，心筋再生による心筋梗塞や心筋症その他の心不全治療，血管再生による虚血性疾患の治療，生物学的ペースメーカーによる洞不全症候群などの治療などが細胞治療ターゲットとして想定される．しかし，ヒト iPS 細胞を用いてこれら細胞治療の実現に至るまでには数多くの乗り越えるべきハードルが残っている．

① **効率的心血管分化誘導法および純化法の開発**……ヒトの心筋梗塞においては 10^9個オーダーに至る心筋細胞が死ぬともいわれている．そのレベルの細胞数を用意することが可能な効率的誘導法を開発する必要がある．現在までででもっとも効率がよいと考えられるヒト ES 細胞からの心筋分化誘導法において，ヒト ES 細胞 1 個から心筋細胞 3 個と報告されている[16]．また，ヒト iPS 細胞はヒト ES 細胞と同様に奇形腫を形成するので，未分化ヒト iPS 細胞を厳密に除去できる細胞純化法が必要である．

② **移植用細胞の開発**……最終的にヒトに対して細胞を移植するためには，単に細胞を誘導して純化するということだけでは不十分で，GMP 基準の医薬品と同様な品質管理の元に移植用細胞を用意できるようにする必要がある．元になる iPS 細胞から血清やフィーダー細胞などを極力排除して，分化誘導・純化が行えるようにする必要があり，①から②の間には実は大きな隔たりがある．

③ **細胞移植法の開発**……①，②を経て用意された細胞をヒトに移植する際に，いかなる細胞群をどのような方法をもって移植すれば，有効かつ安全であるかを評価していく必要がある．最近 ES 細胞から誘導された心筋細胞の移植に関しては，単純に細胞懸濁液を作製して心臓に注入するだけでは，細胞が失われたり死滅したりして生着効率が非常に悪いということがコンセンサスとなりつつある．ある一定以上の細胞を mass として移植する必要があると考えられ，現在おもに 2 つの方法，i）東京女子医大で開発された細胞シート作製技術を用いた心筋細胞シートの移植（心臓に貼り付ける），および，ii）心筋細胞の浮遊培養により得られる心筋細胞塊（cardiac ball）の移植，が試みられている．いずれの方法でも生着心筋効率は改善していると思われる．今後機能的回復が十分なレベルまで技術が発展することが期待される．最近，ヒト iPS 細胞を未分化のままで 20 万個をマウス心筋梗塞モデルに移植すると，奇形水腫の出現は認めずに心筋，血管に分化して心機能が回復したという報告がなされている[17]．一方，マウス iPS 細胞由来神経細胞移植の実験では 0.05% 以下の未分化細胞の混入（100 個以下/合計）でも奇形水腫を形成したとの報告もある[18]．種差や臓器の違いがあるとはいえ，こうした大きな落差をもった報告がなされることは，細胞移植法の評価の大きな問題点である．厳密な評価法が確立されることが望まれる．

2. 患者特異的モデル細胞

患者自身から細胞を採取し患者特異的な iPS 細胞を樹立できるという iPS 細胞にしかない特性は，移植免疫を回避した細胞治療ということだけでなく，まったく新しい形で病態の解明や創薬への応用を可能にする[19,20]．

① **病態解明**……心筋症，QT 延長症候群，洞不全症候群など心臓を構成する細胞そのものに起因すると考えられる疾患が中心となると思われるが，患者自身の細胞から iPS 細胞を樹立し，そこから該当する細胞を分化誘導し種々のモデル細胞を構築できることは，病態解明にまったく新しい手段を提供する．すなわち，これまではごく少量の生検サンプルの解析に限局されていたものが，個々の症例から生きた細胞を潤沢に得られることにより，標的細胞の遺伝子解析，機能解析や薬剤の効果判定などを実際の症例に関して繰り返し行うことが可能となる．原因遺伝子不明の症例においてもモデル細胞が構築できるので，モデル細胞を用いた原因遺伝子探索も可能となる．このように病態解明に向けたアプローチの方法は飛躍的に

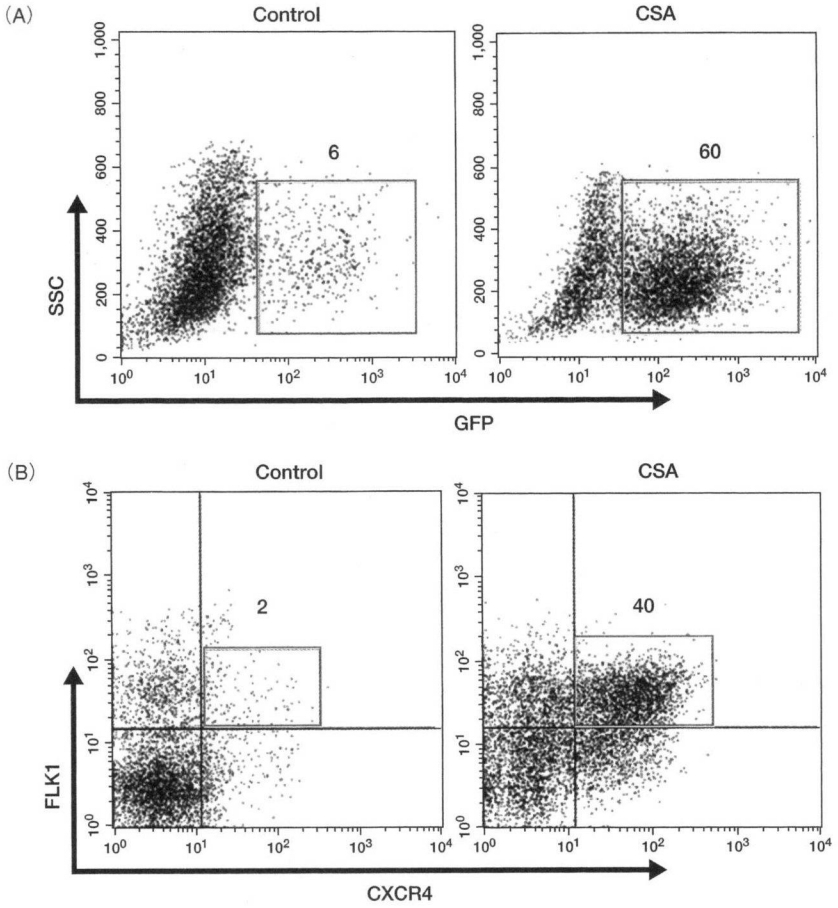

図 3　シクロスポリンA(CSA)による心筋前駆細胞および心筋細胞分化誘導効果：FACS解析

Flk1 陽性細胞を OP9 細胞上で培養する際に CSA を添加すると，心筋細胞および心筋前駆細胞が著しく増加する．

A：心筋特異的 GFP(αMHC プロモーター/GFP)発現．誘導された細胞の約 60%がαMHC/GFP 陽性の心筋細胞になる．

B：心筋前駆細胞．Flk1 陽性/CXCR4 陽性の心筋前駆細胞分画[5]が約 20 倍増加する．

増大すると考えられる．

② **創薬応用**……iPS 細胞の創薬応用には大きく，新規薬剤の探索と薬剤安全性試験への応用の 2 つが考えられる．疾患モデル細胞を用いて，同細胞の異常を改善する新規薬剤や疾患特異的に作用する薬剤などの探索が可能となる．著者らは最近，著者らの ES 細胞心筋分化系を用いて免疫抑制剤シクロスポリン A が中胚葉段階に特異的に作用し，強力な心筋前駆細胞および心筋細胞分化誘導作用を有することを見出した(図 3，4)[21]．こうした培養下における分化モデルを用いることにより，心筋分化促進物質などのあらたな生理活性物質の探索も可能となる．さらに同様のシステムを患者特異的 iPS 細胞を用いて構築することにより，疾患特異的作用物質の探索などにも展開可能と考えられる．

受精卵を用意することが必要であるヒト ES 細胞と比べて，iPS 細胞は数多くの細胞株を樹立しやすく iPS 細胞バンクが構築しやすい．そこから細胞を誘導して array(アレイ)化することにより，種々のヒトモデル細胞パネルのようなものを構築することができる．こうしたヒトモデル細胞パネルは，薬剤の安全性試験に応用可能と考えられる．たとえば，1,000 人分や 10,000 人分などの心筋細

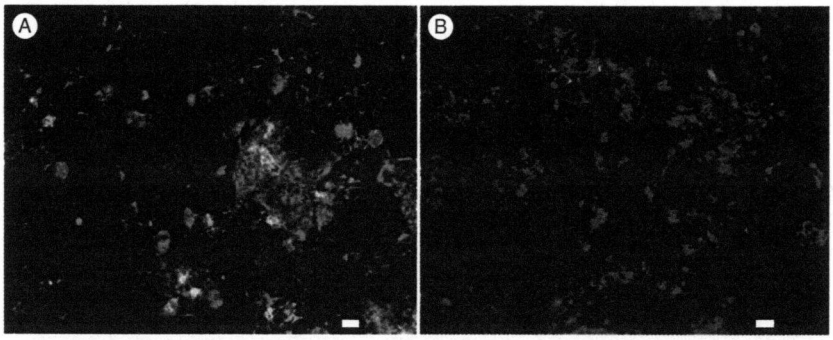

図 4　シクロスポリンA(CSA)による心筋前駆細胞および心筋細胞分化誘導効果：免疫染色
A：control，B：CSA.
CD31(赤；内皮細胞)/cTnT(緑；心筋細胞)二重染色. CSA により内皮細胞の出現が抑制され，対照的に心筋細胞が著しく増加する．スケールバー：400 μm.

胞や肝細胞を並べたパネルを用いて薬剤の細胞毒性をスクリーニングすることにより，まれに発生する心毒性や肝障害などを事前に検出できるかもしれない．さらには障害を起こす細胞を解析し原因を明らかにすることにより，副作用を起こす症例を事前に特定し投薬を避ける“テーラーメイド医療”に貢献しうる可能性もある．

3. その他動物モデルへの応用

循環器病関係のモデル動物には，マウスモデルばかりでなくマウス以外の動物種のものが数多くある(高血圧自然発症ラット，糖尿病モデルラット，心筋症ハムスターなど)．これらモデル動物からの iPS 細胞の樹立が可能となれば，モデル動物と同動物由来細胞を用いて，動物モデルと細胞実験を相互対応させながら新しい病態の解析を行うことなどが可能となると考えられる．

おわりに

哺乳類の成体の細胞がリプログラミングされて未分化なものに戻りうることは，クローンヒツジドリーによってすでに示されていたが，その後 10 年を経て報告されたヒト iPS 細胞樹立がそれを上まわる反響をもって迎えられたのは，iPS 細胞のもつ応用範囲の広さによるであろう．「世界中どこでも施行可能な簡単な方法で成人由来の分化細胞から未分化幹細胞を誘導できる」ということが将来的に科学や社会に及ぼす影響は計り知れない．そこには当然，功罪両面が生まれてくることになる．それはすべて科学者と社会が自ら責任を負うものである．極端な熱狂や批判に走ることなく，冷静にかつ良識と叡知をもって iPS 細胞の今後に対応していくことが必要と考えられる．

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Enhancement of vascular progenitor potential by protein kinase A through dual induction of Flk-1 and Neuropilin-1

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Fine tuning of vascular endothelial growth factor (VEGF) signaling is critical in endothelial cell (EC) differentiation and vascular development. Nevertheless, the system for regulating the sensitivity of VEGF signaling has remained unclear. Previously, we established an embryonic stem cell culture reproducing early vascular development using Flk1 (VEGF receptor-2)$^+$ cells as common progenitors, and demonstrated that cyclic adenosine monophosphate (cAMP) enhanced VEGF-induced EC differentiation. Here we show that protein kinase A (PKA) regulates sensitivity of Flk1$^+$ vascular progenitors to VEGF signaling for efficient EC differentiation. Blockade of PKA perturbed EC differentiation and vascular formation in vitro and ex vivo. Overexpression of constitutive active form of PKA (CA-PKA) potently induced EC differentiation and vascular formation. Expression of Flk1 and Neuropilin-1 (NRP1), which form a selective and sensitive receptor for $VEGF_{165}$, was increased only in CA-PKA–expressing progenitors, enhancing the sensitivity of the progenitors to $VEGF_{165}$ by more than 10 times. PKA activation induced the formation of a $VEGF_{165}$, Flk1, and NRP1 protein complex in vascular progenitors. These data indicate that PKA regulates differentiation potential of vascular progenitors to be endothelial competent via the dual induction of Flk1 and NRP1. This new-mode mechanism regulating "progenitor sensitivity" would provide a novel understanding in vascular development and regeneration. (Blood. 2009;114:3707-3716)

Introduction

Vascular endothelial growth factor (VEGF) signaling is a key regulator of vascular development during embryogenesis as well as neovascularization in the adult.[1-3] Intensity of VEGF signaling is strictly controlled during vascular development through ligand-receptor interaction.[4,5] Flk1 (also designated as VEGF receptor-2) is tyrosine-phosphorylated much more efficiently than Flt1 (VEGF receptor-1) upon VEGF binding and is thought to be the major receptor in endothelial cells (ECs) for VEGF-induced responses.[6-8] Whereas Flk1-null mice die at embryonic day 8.5 (E8.5) to E9.5 with no organized blood vessels,[9] Flt1-null mice die at midgestation with vascular overgrowth and disorganization.[10,11] Flt1 tyrosine kinase–deficient homozygous mice, in which VEGF can bind to the cell-surface domain of Flt1 but cannot conduct kinase signaling, developed normal vessels and survived,[12] indicating that VEGF signal intensity on Flk1 is regulated by absorption of VEGF to the higher affinity receptor, Flt1. VEGF-A heterozygotes die early in gestation due to failure in vascular system formation.[13] On the other hand, 2- to 3-fold overexpression of VEGF-A from its endogenous locus results in aberrant heart development and lethality at E12.5 to E14,[14] indicating that strictly balanced VEGF function is important in normal embryogenesis.

Neuropilin-1 (NRP1) is a type 1 membrane protein, which is expressed in particular classes of developing neurons[15,16] and functions as a receptor for the class 3 semaphorins mediating semaphorin-elicited inhibitory axon guidance signals to neurons.[17,18] NRP1 is also expressed in ECs of blood vessels and endocardial cells of the heart.[15,16,19] NRP1, together with Flk1, forms a specific receptor for $VEGF_{165}$, an isoform of VEGF, and the Flk1-$VEGF_{165}$-NRP1 complex potently enhances Flk1 signaling.[20] Coexpression of NRP1 with Flk1 in cultured ECs enhanced $VEGF_{165}$ binding to Flk1 and VEGF-elicited mitogenic and chemotactic activities.[20] Overexpression of NRP1 in mouse embryos resulted in an excess production of blood vessels and malformed hearts.[15] NRP1-null mice die midway through gestation at E10.5 to E12.5 and exhibit defects in the heart, vasculature, and nervous system.[16] These findings indicate that NRP1 plays an important role in regulating vascular development, and Flk1/NRP1 system would be important for controlling VEGF signal intensity. However, the regulatory mechanisms of Flk1/NRP1 expression in vascular development are not fully elucidated.

In the early embryo and in differentiating embryonic stem (ES) cells, Flk1 expression marks a common progenitor for both blood and endothelium.[21-24] To elucidate the mechanisms underlying vascular development, we have developed a novel ES cell differentiation system that exhibits early vascular development using Flk1$^+$ cells as common progenitors for vascular cells.[25] ES cell–derived Flk1$^+$ cells can differentiate into both ECs and mural cells (MCs: vascular smooth muscle cells and pericytes) and form mature vascular-like structures in vitro. We recently reported that adrenomedullin/cyclic adenosine monophosphate (cAMP) pathway enhanced EC differentiation and induced arterial EC appearance from Flk1$^+$ progenitors.[26] In the present study, to further elucidate

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the mechanisms of EC differentiation from vascular progenitor cells, we examined roles of cAMP pathways in EC differentiation. Here we report that protein kinase A (PKA) activation remarkably enhanced EC differentiation and vascular formation from Flk1$^+$ vascular progenitors. PKA markedly increased the sensitivity of vascular progenitors to VEGF through dual up-regulation of Flk1 and NRP1 and played a pivotal role in EC differentiation. This new-mode molecular system regulating "progenitor sensitivity" would offer novel insights for vascular development as well as molecular targets for vascular regeneration strategies.

Methods

Generation of ES cells carrying an inducible expression unit in ROSA locus

Murine ES cell line (EStTA5-4), expressing tetracycline-transactivator protein and containing the puromycin resistance gene,[27] was a kind gift from Dr T. Era (Kumamoto University, Kumamoto, Japan). We generated an ES cell line (EStTA-ROSA) by inserting a knockin vector carrying loxP and mutant loxP, loxP511, recombination sites flanking neomycin-resistant and herpes simplex virus thymidine kinase (HSV-TK) genes (a kind gift from Dr K. Tanimoto [University of Tsukuba, Tsukuba, Japan] and Dr P. Soriano [Mt Sinai School of Medicine, New York, NY]) into ROSA locus[28] of EStTA5-4 (supplemental Figure 1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Neomycin (200 μg/mL)–resistant colonies were selected and homogenous insertion of the loxP sites into ROSA locus was confirmed by Southern blotting using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics; supplemental Figure 1B-C). The probes were generated by polymerase chain reaction (PCR) amplification using the primer pair, 5′ probe: 5′-TTCAACAGGGATATCGCAAGG and 5′-AGCCTGGTAG-CAGGAAGATC, and Neo probe: 5′-CTCGACGTTGTCACTGAA and 5′-AAGAACTCGTCAAGAAGGCG.

Generation of ES cells for CA-PKA expression

cDNA for constitutive active form (CA)–PKA (a kind gift from Dr G. S. McKnight, University of Washington, Seattle, WA)[29] was introduced into the downstream region of tetracycline responsive element–regulatable cytomegalovirus promoter of Exchange vector (supplemental Figure 1A).

Stable ES cells that express the CA-PKA under the control of the tetracycline responsive element–regulatable cytomegalovirus promoter were generated by introduction of Exchange vectors and pBS185 (Cre expression vector) to EStTA-ROSA cells using mouse ES cells Nucleofector Kit (Amaxa Biosystems). Cells were then plated on 10-cm dishes containing 1 μg/mL doxycycline (Dox$^+$). After 1 day, the medium was changed to Dox$^+$ medium with 200 μg/mL hygromycin. After 10 days, the medium was changed to Dox$^+$ medium with 200 μg/mL hygromycin and 1 μg/mL ganciclovir. Total hygromycin- and ganciclovir-resistant colonies were collected and subjected to further studies.

Antibodies

Monoclonal antibodies for murine Flk1 (AVAS12) and murine vascular endothelial (VE)–cadherin (VECD1, for fluorescence-activated cell sorting [FACS]) were described previously.[24] Monoclonal antibodies for murine CD31 (1:500), VE-cadherin (for immunostaining, 1:200), and endothelial nitric oxide synthase (eNOS; 1:200) were purchased from BD Pharmingen. Monoclonal antibodies for murine α-smooth muscle actin (SMA; 1:1000) were from Sigma-Aldrich. Antibodies for SM22α (1:400) and calponin (1:500) were from Abcam. Polyclonal antibodies for murine Claudin-5 (1:100) were from Invitrogen. Polyclonal antibodies for murine VEGF and rat Neuropilin1 were from R&D Systems.

Cell culture

ES cell lines, D3, EStTA-ROSA, and CA-PKA–introduced EStTA-ROSA were maintained as described.[26] Induction of differentiation of these ES cell lines was performed using differentiation medium (DM; alpha minimal essential medium [MEM; Gibco] supplemented with 10% fetal calf serum [Japan Bioserum Co Ltd] and 5×10^{-5} M 2-mercaptoethanol [Gibco]) as previously described.[25,26] In brief, undifferentiated ES cells were cultured in the absence of leukemia inhibitory factor on collagen type IV–coated dishes (Becton Dickinson) at cell density 0.75 to 1×10^3 cells/cm^2 for 96 to 108 hours. Cultured cells were harvested and subjected to magnetic cell sorting (MACS) purification. Purified Flk1$^+$ cells were then plated onto type IV–coated dishes at cell density 0.75 to 1×10^4 cells/cm^2 in DM. After 3 days of Flk1$^+$ cell differentiation (Flk-d3), induced ECs were then examined by immunohistochemistry and flow cytometric analysis. Various reagents, human $VEGF_{165}$, $VEGF_{121}$ (R&D Systems), 8-bromoadenosine-3′:5′-cyclic monophosphate sodium salt (8bromo-cAMP; Nacalai Tesque), γ-secretase inhibitor, DAPT, PI3K inhibitor, LY294002, GSK3β inhibitor, Bio, Akt inhibitor, TAT-Akt-in, PKA inhibitor, PKI, H89, p38 inhibitor, SB202190, MEK inhibitor, PD98059, PKCαβ inhibitor, PKCη inhibitor, PKCζ inhibitor, H-Ras inhibitor, FTI-277 (Calbiochem), and phospholipase C (PLC) inhibitor, U73122 (Tocris Cookson Inc) were occasionally added to the Flk1$^+$ cell culture. (DAPT, LY294002, Bio, TAT-Akt-in, SB202190, PD98059, PKCαβ inhibitor, PKCη inhibitor, and PKCζ inhibitor did not inhibit cAMP effect.) Human $VEGF_{165}$ was used as the representative of VEGF isoforms unless stated otherwise. In serum-free culture, a defined medium, SFO3 (Sanko Junyaku; including insulin, transferrin, sodium selenite, and ethanolamine), was used instead of DM.[25]

Three-dimensional culture

Three-dimensional culture was performed as described previously.[25] Briefly, Flk1$^+$ cells (4×10^5 cells/mL) were incubated in DM with VEGF on uncoated Petri dishes for 16 hours to induce aggregation. Aggregates were resuspended in 2 × DM and mixed with an isovolume of collagen I-A gel (3 mg/mL; Nitta Gelatin). We plated 250 to 300 μL of this mixture onto a lucent insert disk, Cell disk (Sumitomo Bakelite), in 24-well dishes. After 30 minutes at 37°C to allow polymerization, we added 500 μL DM. To monitor vascular formation, collagen-embedded Flk1$^+$ cell aggregates were cultured in a temperature- and gas-controlled chamber (37°C, 5% CO_2), and phase-contrast images were acquired every 10 minutes with Metamorph software (Molecular Devices) for up to 5 days.

Cell sorting and flow cytometric analysis

After induction of Flk1$^+$ cells, cultured cells were harvested and stained with allophycocyanin (APC)–conjugated anti-Flk1 antibody (AVAS12).[24] Flk1$^+$ cells were sorted by auto MACS (Miltenyi Biotec) using anti-APC MicroBeads (Miltenyi Biotec). At Flk-d3, cultured cells were harvested and stained with monoclonal antibodies for phycoerythrin-conjugated CD31 (Mec13.3; BD Pharmingen) together with APC-conjugated VECD1 or biotin-conjugated CXCR4 (BD Pharmingen) followed by streptavidin-conjugated APC (BD Pharmingen) or AVAS12, then subjected to analysis by FACSVantage or FACSAria (Becton Dickinson).

Immunocytochemistry

Immunostaining for cultured cells was carried out as described.[25,26] Briefly, 4% paraformaldehyde-fixed cells were blocked by 1% skim milk (BD Biosciences) and incubated overnight with primary antibodies at 4°C. For immunohistochemistry, anti–rat immunoglobulin G (IgG) conjugated with alkaline phosphatase and anti–mouse IgG horseradish peroxidase (Invitrogen) were used as secondary antibodies. For immunofluorescent staining, anti–mouse, –rat, –rabbit, or –goat IgG antibodies conjugated with Alexa488 or Alexa546 (Invitrogen) were used for secondary antibodies. Nuclei were visualized with DAPI (4,6 diamidino-2-phenylindole; Invitrogen). Double staining for NRP1 and CD31 was performed using anti-NRP1 antibody (1:100; R&D Systems) as first antibody, followed by secondary antibody, Alexa Fluor 488–conjugated donkey anti–goat IgG (1:500; Molecular Probes). CD31$^+$ cells were visualized using phycoerythrin-

conjugated anti-CD31 antibody (1:300; BD Pharmingen). Stained cells were photographed with inverted fluorescent microscopy, Eclipse TE2000-U (Nikon) and digital camera system AxioCam HRc with the use of AxioVision Software (Carl Zeiss), or confocal fluorescent microscopy (TCS SP2; Leica).

Immunostaining for three-dimensional culture

Immunostaining for vascular structures in type I collagen gel was performed after the whole-mount immunostaining procedure as described.[25] In brief, gels were fixed with 4% paraformaldehyde and blocked by 1% skim milk/0.1% Triton X/phosphate-buffered saline solution and incubated with anti-CD31 (BD Pharmingen) and anti–α smooth muscle actin (αSMA; Sigma-Aldrich), or anti-NRP1 (R&D Systems) and anti-Flk1 antibodies (1:200). Alexa Fluor 488–conjugated anti–mouse or anti–goat IgG and Alexa Fluor 567–conjugated anti–rat IgG (1:500; Zymed) were used as secondary antibodies. Alternatively, anti–rat IgG conjugated with alkaline phosphatase and anti–mouse IgG conjugated with horseradish peroxidase (Zymed) were used as secondary antibodies for enzymatic color development.

Cross-section of three-dimensional culture and immunostaining

Gel clots including vascular structure were fixed in 3.7% formaldehyde for 24 hours. Paraffin-embedded gel clots were sectioned at 3-μm thickness. The sections were mounted on glass slides coated with 2% 3-aminopropyl triethoxy silane (Tokyo Kasei). After deparaffinization and washing in distilled water, hematoxylin-eosin or immunohistochemical staining was performed.[30] For double immunostaining for CD31 and SMA, CD31 was first stained with whole-mount in-gel staining using anti-CD31 antibody and anti–rat IgG antibody conjugated with horseradish peroxidase. Subsequently, gel clots were subjected to paraffin embedding. Anti-SMA antibody (1:100; DAKO) was applied to sectioned slides overnight at 4°C. They were incubated with biotinylated horse anti–mouse serum diluted to 1:300 followed by streptavidin-alkaline phosphatase.

RNA isolation, RT-PCR, and quantitative RT-PCR

Total RNA was isolated from cells in Dox treatment or Dox-free condition at Flk-d3 using RNeasy (QIAGEN), according to the manufacturer's instructions. Reverse-transcription was performed with the SuperScript III first-strand synthesis system (Invitrogen). Reverse-transcription (RT)–PCR was carried out as described[26] using indicated primers (supplemental Table 1). Quantitative RT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and StepOnePlus system (Applied Biosystems). The amount of target RNA was determined from the appropriate standard curve and normalized relative to the amount of *Gapdh* mRNA. Primer sequences are shown in supplemental Table 2.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed according to the report by Pan et al.[31] In brief, Flk1$^+$ cells were incubated with vehicle, anti-VEGF (5 μg/mL), or anti-NRP1 antibodies (5 μg/mL; R&D Systems) in serum-free medium, SFO3 (Sanko Junyaku),[25] for 30 minutes at 37°C. Cells were then cooled on ice for 15 minutes, and VEGF isoforms were added, followed by 30-minute incubation at 4°C. Cells were stimulated for 7 minutes at 37°C and then washed with ice-cold phosphate-buffered saline and lysed in lysis buffer. Cell lysates were subjected to immunoprecipitation using Protein G HP SpinTrap (GE Healthcare) and anti-Flk1 antibody, and immunoblotted with antibodies specific for NRP1 (R&D Systems). Samples were run on sodium dodecyl sulfate/polyacrylamide gel electrophoresis using gradient gel (Atto Co) followed by electrophoretic transfer onto nitrocellulose membranes. After the blots were incubated for 1 hour in blocking agents Blocking One (Nacalai Tesque), they were incubated overnight with the respective NRP1 antibodies (0.5 μg/mL; R&D Systems). Horseradish peroxidase–conjugated anti–goat antibody (Zymed Laboratories) was used as secondary antibody (1:1000). Can Get Signal Immunoreaction Enhancer solution kit (Toyobo) was used for signal enhancement. Immunoreactivity was detected with the enhanced chemiluminescence kit Chemi-Lumi One (Nacalai Tesque).

Ex vivo whole-embryo culture

Embryos were dissected out of the deciduum and placed in 500 μL dimethyl ether containing 50% rat IC serum (Charles River Laboratories), 5 mM nonessential amino acids, 50 mM sodium pyruvate, and 27.5 mM 2-mercaptoethanol, pre-equilibrated at 37°C with 5% CO_2. Embryos were cultured at 37°C with 5% CO_2 and analyzed. H89 (dissolved in dimethyl sulfoxide) was used at 30 μM. The concentration of dimethyl sulfoxide was set at 0.3% in all cultures.[32] Whole-mount staining of embryos and yolk sacs was performed as described previously,[33] and microscopy was performed using a microscope (MZ6; Leica) with 5× objectives (Leica 10411589). Images were imported using Adobe Photoshop software, and quantification of whole yolk sacs and CD31-stained areas was performed using ImageJ software (National Institutes of Health). Results of quantification were expressed as ratio of CD31$^+$/whole yolk sac area, which provides an estimate of the proportion of the yolk sacs that were occupied by CD31-stained vascular structures. Animal experiments were done under the approval of the Animal Research Committee of Kyoto University in accordance with the guidelines for animal experiments in the Guide for the Care and Use of Laboratory Animals in Japan.

Statistical analysis

At least 3 independent experiments were performed. Statistical analysis of the data was performed with the Student *t* test or analysis of variance. *P* values less than .05 was considered significant. Values are reported as means plus or minus SD.

Results

cAMP/PKA pathway plays a critical role in vascular development

In our ES cell differentiation system, first we induced Flk1$^+$ progenitor cells from undifferentiated ES cells. Flk1$^+$ cells that appeared after 96 to 108 hours of differentiation of undifferentiated ES cells were negative for EC markers, such as CD31 and VE-cadherin.[24-26] Then, purified Flk1$^+$ progenitor cells were cultured for further differentiation to vascular cells. As previously reported, whereas no CD31$^+$ ECs appeared when Flk1$^+$ cells were cultured for 3 days with DM ("Methods") alone, addition of VEGF to the Flk1$^+$ cell culture induced selective appearance of CD31$^+$ ECs and SMA$^+$ MCs (Figure 1A).[25,26] Almost all of CD31$^+$ cells were also positive for other EC markers, VE-cadherin, claudin-5, and eNOS (Figure 1A-B).[25,26] SMA$^+$ MCs, which were reciprocally negative for EC markers (Figure 1B), expressed other smooth muscle cell markers, SM22α and calponin (Figure 1A). In this culture condition, only these 2 cell types (ie, ECs and MCs), and no blood cells such as CD45$^+$ cells, were specifically induced from Flk1$^+$ cells (Figure 1C).[26]

Stimulation of cAMP signaling by addition of 8bromo-cAMP, an analog of cAMP, together with VEGF substantially enhanced CD31$^+$ EC induction from Flk1$^+$ cells (Figure 2A-B). Similar to ECs induced by VEGF alone (Figure 1), CD31$^+$ ECs that appeared by treatment of 8bromo-cAMP and VEGF were also positive for other EC markers, VE-cadherin, eNOS, and claudin5, but not CD45 (supplemental Figure 2). Compared with VEGF alone, VEGF with 8bromo-cAMP induced approximately 2-fold increase in EC population (CD31$^+$ cells: 26.5% ± 2.3% in VEGF alone vs 52.3% ± 2.7% in VEGF with 8bromo-cAMP, n = 16; $P < .001$; Figure 2C). Total EC numbers induced from the same number of Flk1$^+$ cells were similarly increased approximately 2.3 times by 8bromo-cAMP treatment (CD31$^+$ cells: 9.4 ± 0.8 [× 10^4] cells in VEGF alone vs 21.8 ± 0.9 [× 10^4] cells in VEGF with 8bromo-cAMP; n = 4; $P < .001$; Figure 2D). PKA inhibitors, PKI and

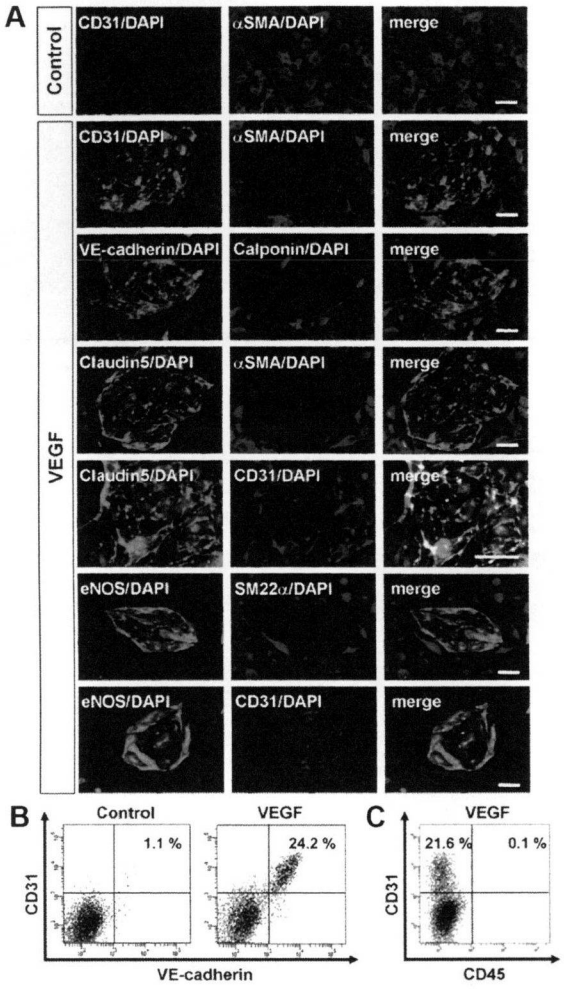

Figure 1. Vascular endothelial growth factor induces endothelial cells from vascular progenitors. (A-C) Cells after three-dimensional culture of Flk1$^+$ cells (Flk-d3). (A-B) Exclusive induction of endothelial cells (ECs) and mural cells (MCs) from Flk1$^+$ cells. (A) Expression of EC and MC markers. (Top panels) Double immunostaining of CD31 (green) and αSMA (red) cultured with differentiation medium (DM) alone (control). Note that no CD31$^+$ cells appeared. (Other panels) Vascular endothelial growth factor (VEGF) treatment (50 ng/mL). EC sheets appeared. Double staining with pan-EC markers (CD31, VE-cadherin, Claudin5, or eNOS; green) and MC markers (αSMA, SM22α, or Calponin; red). (Bottom panels) Double staining with eNOS (green) and CD31 (red). ECs and MCs were exclusively induced. Nuclei are stained with DAPI (blue). Scale bar represents 50 μm. (B) Flow cytometry. x-axis: VE-cadherin; y-axis: CD31. Percentages of CD31$^+$/VE-cadherin$^+$ ECs in total Flk1$^+$ cell–derived cells are indicated. (C) Flow cytometry. x-axis: CD45; y-axis: CD31. Percentages of CD31$^+$/CD45$^-$ ECs and CD31$^+$/CD45$^+$ blood cells in total Flk1$^+$ cell–derived cells are indicated. Note that almost no CD45$^+$ cells were induced in this culture.

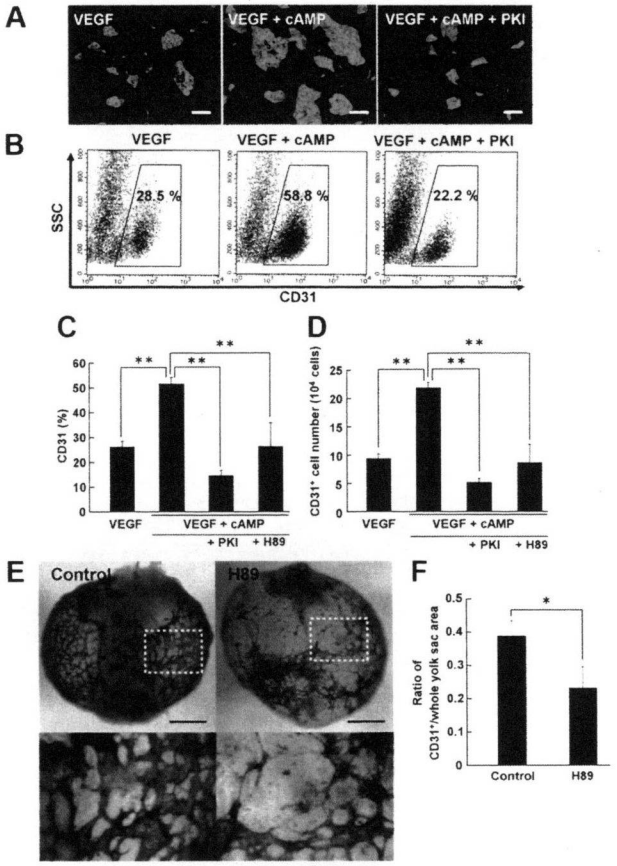

Figure 2. Cyclic adenosine monophosphate/protein kinase A pathway plays a critical role in vascular development. (A-D) Enhancement of EC induction by cyclic adenosine monophosphate (cAMP) through protein kinase A (PKA) at Flk-d3. (A) Fluorescent staining for CD31 (green). (Left panel) VEGF treatment alone (50 ng/mL). (Middle panel) VEGF with 8bromo-cAMP (0.5 mM). (Right panel) VEGF with 8bromo-cAMP and PKA inhibitor, PKI (10 μM). Nuclei are stained with DAPI (blue). Scale bars represent 250 μm. (B) Flow cytometry. x-axis: CD31; y-axis: side scatter (SSC). Percentages of CD31$^+$ ECs in total Flk1$^+$ cell–derived cells are indicated. (C-D) Quantitative evaluation of the effect of PKA inhibitors on CD31$^+$ EC induction from Flk1$^+$ cells by FACS. (C) Percentages of CD31$^+$ cell population in total Flk1$^+$ cell–derived cells. VEGF (50 ng/mL; n = 16); VEGF and 8bromo-cAMP (0.5 mM; n = 16); VEGF, cAMP, and PKI (10 μM; n = 7); and VEGF, cAMP, and H89 (10 μM; n = 6) treatment are shown (**$P < .01$ vs VEGF and 8bromo-cAMP). (D) CD31$^+$ cell number that appeared from 1.5 × 10^5 Flk1$^+$ cells. VEGF (50 ng/mL; n = 4); VEGF and 8bromo-cAMP (0.5 mM; n = 4); VEGF, cAMP, and PKI (10 μM; n = 4); and VEGF, cAMP, and H89 (10 μM; n = 4) treatment are shown (**$P < .01$ vs VEGF and 8bromo-cAMP). (E-F) Role of PKA in vascular formation in the embryo. (E) Representative results of ex vivo culture of mouse embryo. Isolated E6.75 concepti were cultured in the absence (control, left panels) or presence (right panels) of H89 (30 μM) for 3 days. Vasculature in yolk sacs of concepti was immunostained for CD31 (purple). Bottom panels correspond to boxed regions in control and H89, respectively. Apparent reduction of CD31$^+$ vascular formation was induced by H89 treatment. Scale bar represents 1 mm. (F) Quantitative evaluation of CD31$^+$ vasculature formation in yolk sacs of concepti. The ratio of CD31$^+$/whole yolk sac area was evaluated (n = 3; *$P < .05$ vs control).

H89, but not many other kinase inhibitors ("Methods"), specifically inhibited the cAMP effects on EC induction (Figure 2A-D). These results indicated that the cAMP/PKA pathway specifically enhances the effect of VEGF on EC differentiation from Flk1$^+$ progenitor cells.

We further examined the role of PKA in vascular development with ex vivo whole-embryo culture assay. Embryonic day 6.75 concepti were picked out from the uteri of pregnant mice and cultured for 3 days, during which CD31$^+$ blood vessels were formed in the yolk sac. Using this system, we could examine early phase of EC differentiation ex vivo. In the presence of H89 during ex vivo culture, formation of blood vessels, which were evaluated by CD31 staining, in yolk sac was markedly disturbed, showing malformation of vascular networks with decrease in the caliber size and CD31$^+$ areas (Figure 2E). Indeed, CD31$^+$ area within whole yolk sac area was significantly decreased in H89-treated embryo to approximately 59% of that in control (n = 3; $P = .025$; Figure 2F). These results indicate that PKA also plays an important role in early vascular development in vivo.

CA-PKA enhanced EC differentiation and vascular formation from Flk1$^+$ vascular progenitors

To dissect PKA function in EC differentiation, we generated ES cells expressing CA-PKA by tetracycline-regulatable system (Tet-Off; supplemental Figure 1). Although negative effects of high-dose Dox (~ 25 μg/mL) on EC differentiation, proliferation, and survival were

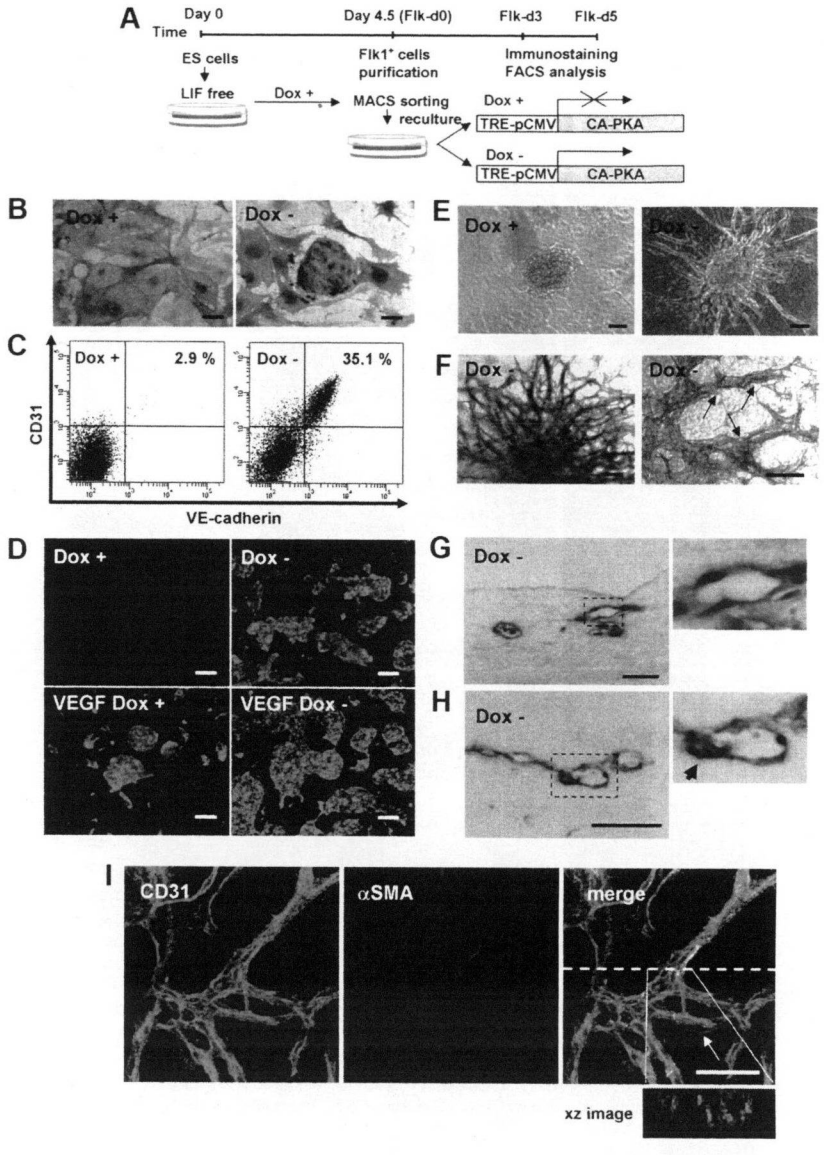

Figure 3. CA-PKA enhances EC differentiation from Flk1$^+$ vascular progenitors. (A) Experimental system for PKA activation. An embryonic stem (ES) cell line expressing constitutive active (CA) form of PKA by tetracycline-inducible expression system (Tet-Off) was established. Doxycycline (Dox) was added during the first 4.5-day culture of ES cell differentiation to Flk1$^+$ cells. Flk1$^+$ cells were sorted by magnetic cell sorting (MACS) and subjected to two-dimensional culture on collagen-coated dishes or three-dimensional culture in collagen gel, and were cultured in the presence or absence of Dox (1 μg/mL). (B-D) Two-dimensional 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 represents 100 μm. (C) Flow cytometry for EC markers, CD31 and VE-cadherin. Percentages of CD31$^+$/VE-cadherin$^+$ ECs in total Flk1$^+$ cell–derived cells are indicated. (D) Fluorescent staining for CD31 (green) and DAPI (blue). (Left panels) Dox (1 μg/mL) treatment. (Right panels) Dox-free. Flk1$^+$ cells stimulated with vehicle (top panels) or VEGF (50 ng/mL; bottom panels). Scale bars represent 250 μm. (E-I) Three-dimensional culture of Flk1$^+$ cell aggregates in type I collagen gel with DM alone. (E) Phase-contrast images after 5-day culture. (Left panel) Dox (1 μg/mL) treatment. (Right panel) Dox-free. Scale bars represent 100 μm. (F) In-gel double immunostaining for CD31 (purple) and αSMA (brown) in Dox-free condition. (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 represent 100 μm. (G-H) Cross-section of three-dimensional culture in Dox-free condition. (G) Hematoxylin-eosin staining. (H) Double immunostaining for CD31 (brown) and αSMA (red). Right panels correspond to boxed regions. Scale bars represent 250 μm. αSMA$^+$ cell attached to CD31$^+$ EC tube structure is observed (arrow). (I) Confocal microscopic analysis of vascular structure. Double fluorescent staining for CD31 and αSMA in Dox-free condition. (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 true lumen (green) with attached mural cells (red) shown in xz image. Dashed line indicates sliced position. Scale bars represent 100 μm.

reported,[34] lower concentration of Dox (1 μg/mL) did not affect EC appearance in control ES cells (ESrtTA-ROSA; supplemental Figure 3). We induced differentiation of ES cells in the presence of Dox for 4.5 days, and purified Flk1$^+$ cells were recultured on type IV collagen–coated dishes with DM alone in the presence or absence of Dox (Figure 3A). In the presence of Dox (Dox$^+$), only SMA$^+$ mural cells, but not ECs were induced (Figure 3B-C), compatible with our previous results[25] (Figure 1A-B). Surprisingly, considerable amounts of CD31$^+$ ECs were generated even in the absence of VEGF when CA-PKA expression was induced by the depletion of Dox (Dox$^-$; Figure 3B; supplemental Videos 1-2). Almost all of CA-PKA–induced CD31$^+$ cells on 2-dimensional culture condition were also positive for VE-cadherin, eNOS, and claudin5 (Figure 2C, supplemental Figure 4). CD31$^-$/VE-cadherin$^-$ cells observed in CA-PKA–activated condition were positive for SMA, SM22α, and calponin (supplemental Figure 4). When we tested the effects of CA-PKA with VEGF, EC appearance with VEGF in Dox$^+$ condition was further enhanced by expression of CA-PKA (Figure 3D). These results indicate that PKA should enhance EC differentiation from vascular progenitors.

We further examined vascular formation from Flk1$^+$ cells in three-dimensional culture[25] to investigate PKA function in vascular development. When aggregates of Flk1$^+$ cells were cultured in type I collagen gel with DM alone, no sprouting of vessels was observed in Dox$^+$ condition. In contrast, CA-PKA expression (Dox$^-$) induced vascular-like structure formation even in the absence of VEGF (Figure 3E; supplemental Video 3). In-gel immunostaining showed the vascular-like structure consisted of CD31$^+$ ECs with surrounding SMA$^+$ mural cells (Figure 3F). Cross-sections revealed true lumens with CD31$^+$ ECs and attached SMA$^+$ MCs (Figure 3G-H). Confocal microscopic study further showed vascular-like structure formation with EC tube and mural cell attachment (Figure 3I). In addition, PKA activation induced CD45$^+$ blood cells within the vascular lumen (supplemental Figure 5A). Occasionally, beating cardiomyocytes, which were positive for cardiac troponin T, were observed along with vascular structures (supplemental Figure 5B; supplemental Video 4). PKA, thus, should play an important role in vascular development through enhancement of EC differentiation.

Dual up-regulation of Flk1 and NRP1 was induced by PKA

Next, we investigated the molecular mechanism of the PKA effects in EC differentiation and vascular development. First we examined PKA activity in vascular progenitor cells. Whereas PKA activity was significantly increased by addition of 8bromo-cAMP, VEGF treatment did not induce PKA activation (supplemental Figure 6),

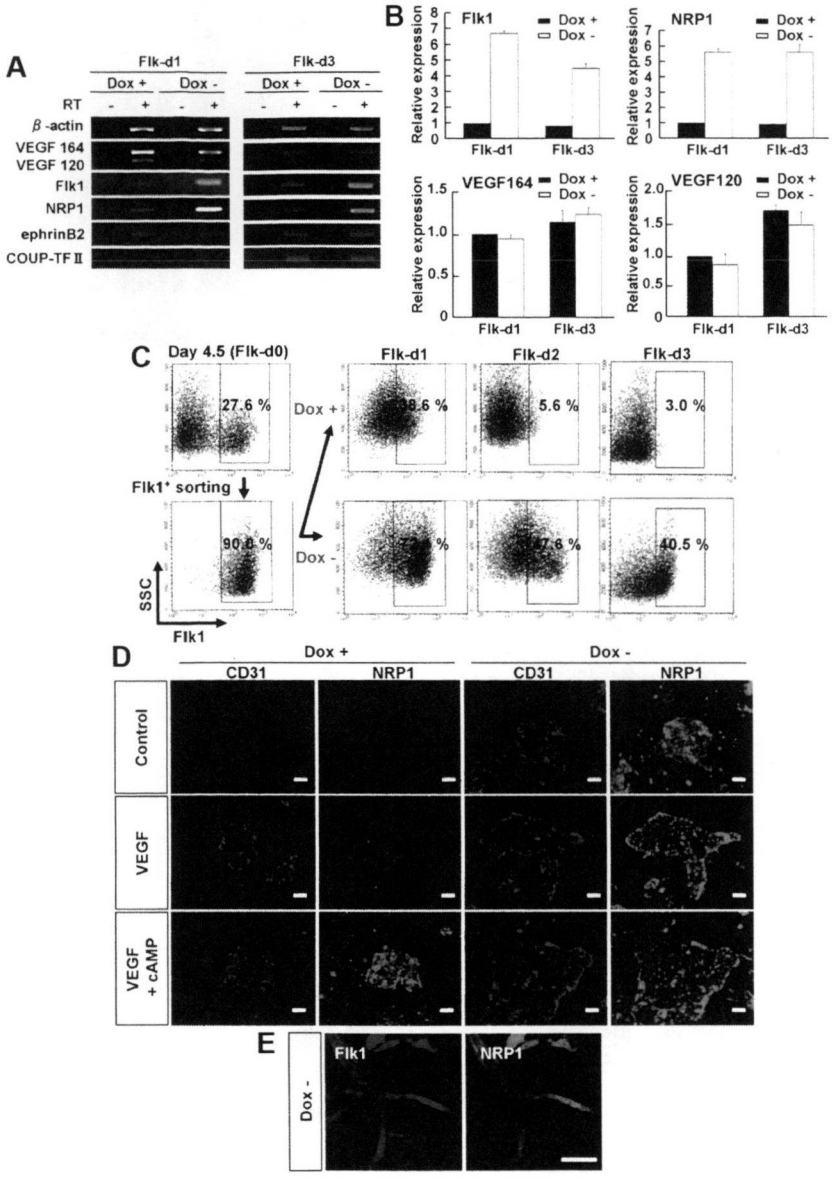

Figure 4. Dual up-regulation of Flk1 and NRP1 by PKA activation. (A-D) Two-dimensional culture of Flk1$^+$ cells. (A) RT-PCR showing mRNA expression of $VEGF_{164}$, $VEGF_{120}$, Flk1, NRP1, ephrinB2 (arterial marker), and COUP-TF II (venous marker) after 1 (Flk-d1) and 3 (Flk-d3) days of culture of Flk1$^+$ cells with DM alone in the presence or absence of Dox (1 μg/mL). (B) Quantitative RT-PCR showing mRNA expression of Flk1, NRP1, $VEGF_{164}$, and $VEGF_{120}$ at Flk-d1 and Flk-d3 in the presence or absence of Dox. mRNA expression at Flk-d1 with Dox was set as 1.0. (C) Time course of Flk1$^+$ cell appearance evaluated by FACS. Flk1$^+$ cell appearance was examined on differentiation day 4.5 before and after sorting, and at Flk-d1, Flk-d2, and Flk-d3 cultured with DM alone in the presence or absence of Dox (1 μg/mL). (Top panels) Dox treatment. (Bottom panels) Dox-free. Percentages of Flk1$^+$ cells are indicated. (D) Double fluorescent staining for CD31 and NRP1 at Flk-d3. (Left 6 panels) Dox treatment. (Right 6 panels) Dox-free. Flk1$^+$ cells stimulated with vehicle (top panels), VEGF (50 ng/mL; middle panels), or VEGF and 8bromo-cAMP (0.5 mM; bottom panels). Scale bars represent 100 μm. (E) Vascular formation from Flk1$^+$ cell aggregates in three-dimensional culture in Dox-free condition at Flk-d5. In-gel double fluorescent staining for Flk1 and NRP1. (Left panel) Flk1 (red). (Right panel) NRP1 (green). Scale bars represent 100 μm.

indicating that PKA pathway did not work downstream of VEGF signaling in vascular progenitor cells. In addition, activation of PKA by induction of CA-PKA in Flk1$^+$ cells did not increase VEGF mRNA expression (Figure 4A-B). These results indicate that PKA signaling did not enhance VEGF signaling through the formation of a positive feedback loop between VEGF and PKA. In contrast, overexpression of CA-PKA up-regulated Flk1 and NRP1 mRNA expression from the early stage of Flk1$^+$ cell culture (ie, at days 1 and 3 after Flk1 sorting; Figure 4A). Arterial EC marker, ephrinB2,[35,36] and venous EC marker, COUP-TF II,[37] were not affected by CA-PKA expression (Figure 4A). Quantitative RT-PCR analysis revealed that PKA activation induced approximately 5- to 7-fold increase in Flk1 and NRP1 mRNA expression in total cells at Flk-d1 and Flk-d3 (Figure 4B). Similar significant up-regulation of Flk1 and NRP1 mRNA expression was observed in 8bromo-cAMP–treated Flk1$^+$ cells (supplemental Figure 7A). We further confirmed the time course of Flk1 protein expression in the early stage of Flk1$^+$ cell culture by FACS. When purified Flk1$^+$ progenitor cells were cultured in the absence of VEGF, Flk1 expression was rapidly decreased and lost within 3 days, compatible with our previous results[25] (Figure 4C top panels). On the other hand, when CA-PKA was induced in purified Flk1$^+$ cells, Flk1 expression was essentially maintained, even in the absence of VEGF. At Flk-d1, almost all Flk1$^+$ cells were still negative for EC markers, CD31 and VE-cadherin (supplemental Figure 8), indicating that PKA should work at the progenitor stage to enhance EC appearance from Flk1$^+$ progenitor cells. As for NRP1 protein expression, whereas clear up-regulation of NRP1 expression was observed only in VEGF with 8bromo-cAMP treatment in Dox$^+$ condition, CA-PKA activation (Dox$^-$) induced NRP1 expression even in the absence of VEGF at Flk-d3 (Figure 4D). Furthermore, Flk1 and NRP1 were also coexpressed in vascular structure in vitro induced with CA-PKA expression (Figure 4E). These results suggest that PKA pathway should enhance EC differentiation and vascular formation through dual induction of Flk1 and NRP1.

Sensitivity of VEGF signaling is markedly enhanced by PKA

To precisely define the biologic function of up-regulated Flk1 and NRP1 by PKA activation, we tested dose-response effects of

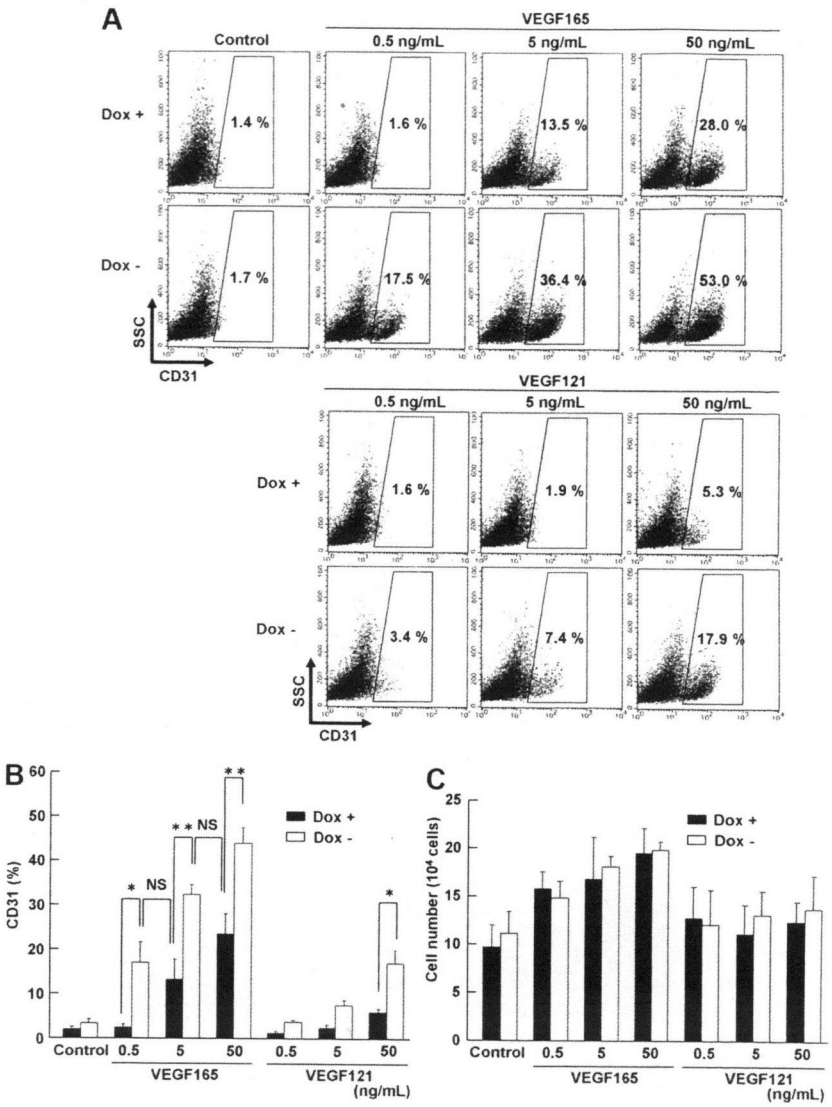

Figure 5. Sensitivity of VEGF signaling is enhanced by PKA. Serum-free culture of Flk1$^+$ cells on two-dimensional condition, at Flk-d3. (A-B) Flow cytometry for CD31 expression in the presence (Dox$^+$; 1 μg/mL) or absence (Dox$^-$) of Dox. x-axis: CD31; y-axis: SSC. Flk1$^+$ cells were incubated with various concentrations of $VEGF_{165}$ or $VEGF_{121}$ in serum-free medium, SFO3. Percentages of CD31$^+$ ECs in total Flk1$^+$ cell–derived cells are indicated. (B) Quantitative evaluation of effects of PKA activation on EC differentiation. Percentages of CD31$^+$ EC population in total Flk1$^+$ cell–derived cells are evaluated (n = 3; $^*P < .05$, $^{**}P < .01$ vs corresponding values; NS indicates not significant). (C) Quantitative evaluation of the number of induced ECs. Total cell number that appeared from 12.5×10^4 of plated Flk1$^+$ cells at Flk-d3 is shown.

$VEGF_{165}$ and $VEGF_{121}$ on Flk1$^+$ cells using serum-free culture with a defined medium, SFO3 (including insulin, transferrin, sodium selenite, and ethanolamine).[25] In the serum-free condition, CD31$^+$ ECs were not induced from Flk1$^+$ progenitors in the absence of $VEGF_{165}$. In the control condition (Dox$^+$), 5 to 50 ng/mL $VEGF_{165}$ induced CD31$^+$ ECs. Surprisingly, although almost no ECs were induced in the absence of $VEGF_{165}$ even with CA-PKA activation (Dox$^-$), CA-PKA expression induced distinct EC appearance in much lower concentration of $VEGF_{165}$ (ie, 0.5-5 ng/mL; Figure 5A-B). Similar or higher amounts of ECs were induced by 10 times lower concentration of $VEGF_{165}$ in Dox$^-$ condition compared with those in Dox$^+$ condition (CD31$^+$ cells: 18.1% ± 5.1% [Dox$^-$, 0.5 ng/mL $VEGF_{165}$] vs 14.2% ± 4.8% [Dox$^+$, 5 ng/mL $VEGF_{165}$]; 34.6% ± 2.4% [Dox$^-$, 5 ng/mL $VEGF_{165}$] vs 25.2% ± 4.8% [Dox$^+$, 50 ng/mL $VEGF_{165}$]; Figure 5B). There was no difference observed in the total cell number that appeared from Flk1$^+$ cells between Dox$^+$ and Dox$^-$ treatment (Figure 5C), suggesting that PKA activation should enhance EC differentiation but not proliferation. Furthermore, the potent enhancement of EC differentiation was observed specifically by $VEGF_{165}$ treatment, and not by $VEGF_{121}$, which does not bind to NRP1 (Figure 5A).[20] Significant increase in EC appearance with 50 ng/mL $VEGF_{121}$ (Figure 5B) should be induced by binding of $VEGF_{121}$ to up-regulated Flk1. Similarly, addition of 8bromo-cAMP in Dox$^+$ condition also enhanced response of Flk1$^+$ progenitor differentiation to VEGF (supplemental Figure 9). These results indicate that dual activation of Flk1 and NRP1 by PKA activation markedly enhanced sensitivity of Flk1$^+$ progenitors to $VEGF_{165}$.

PKA activation induces Flk1-VEGF-NRP1 complex formation

Finally, we confirmed the formation and function of Flk1-$VEGF_{165}$-NRP1 complex by PKA activation. One day after Flk1$^+$ cell culture in serum-free conditions (Flk-d1), cells were collected and protein interaction of Flk1, NRP1, and VEGF was examined by immunoprecipitation assay.[31] Western blot analysis for NRP1 using total cell lysates clearly revealed increase in NRP1 protein by CA-PKA expression (Dox$^-$) at Flk-d1 (Figure 6Aii). Total NRP1 expression was increased approximately 4.3-fold by PKA activation (n = 6; $P < .001$). In various conditions that we tested, only when added together with CA-PKA expression (Dox$^-$), $VEGF_{165}$ formed a distinct protein complex with Flk1 and NRP1 (Figure 6Ai lane 7). The protein complex was not formed in the control conditions (Dox$^+$) or Dox$^-$ conditions with the addition of $VEGF_{121}$. Similarly,

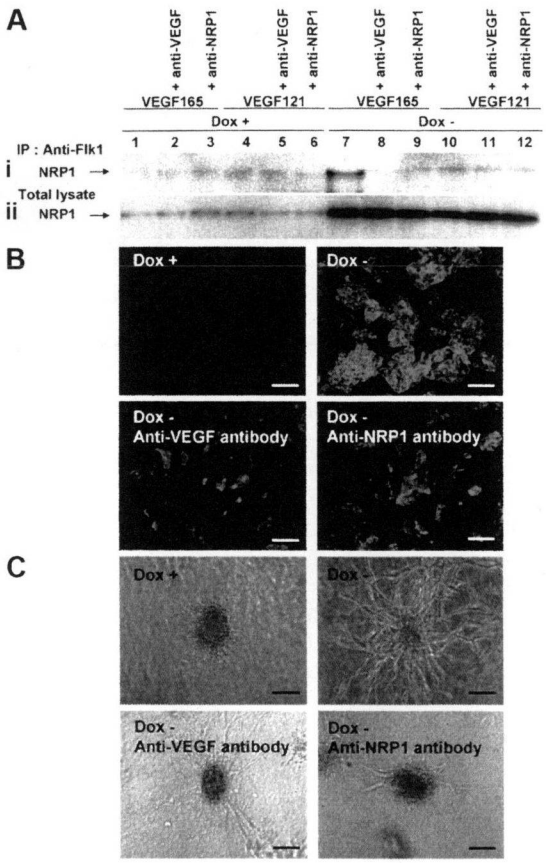

Figure 6. PKA enhanced to form Flk1-VEGF-NRP1 complexes. (A) Immunoprecipitation assay. Formation of Flk1-VEGF-NRP1 complex was examined at Flk-d1 cultured with serum-free medium, SFO3. Immunoblot with anti-NRP1 antibody for cell lysates immunoprecipitated with anti-Flk1 antibody (Ai) and total cell lysates (Aii). (Ai) Note that a distinct band was observed only when $VEGF_{165}$ was added to PKA-activated (Dox^-) condition (lane 7), which was inhibited by addition of anti-VEGF or anti-NRP1 antibodies. (Aii) Total NRP1 expression was markedly increased in PKA-activated condition. (B) Two-dimensional culture of $Flk1^+$ cells with DM, at Flk-d3. Fluorescent staining for CD31 (green). Nuclei are stained with DAPI (blue). (Top left panel) Dox treatment. (Other panels) Dox-free. (Bottom left panel) Dox-free with neutralizing antibody for VEGF. (Bottom right panel) Dox-free with neutralizing antibody for NRP1. Scale bars represent 250 μm. (C) Vascular formation from $Flk1^+$ cell aggregates in three-dimensional culture. (Top left panel) Dox treatment. (Other panels) Dox-free. (Bottom left panel) Dox-free with neutralizing antibody for VEGF. (Bottom right panel) Dox-free with neutralizing antibody for NRP1. Scale bars represent 100 μm.

8bromo-cAMP treatment also induced formation of a protein complex with Flk1, NRP1, and $VEGF_{165}$ (supplemental Figure 7B). These results clearly indicated that PKA activation induced both Flk1 and NRP1 expression in vascular progenitors, and $VEGF_{165}$ in turn specifically induced the protein complex formation of Flk1-$VEGF_{165}$-NRP1. The formation of Flk1-$VEGF_{165}$-NRP1 complex was completely blocked by the addition of anti-VEGF or anti-NRP1 neutralizing antibodies (Figure 6Ai lanes 8-9). Parallel to the Flk1-$VEGF_{165}$-NRP1 complex formation, the CA-PKA–induced EC differentiation as well as vascular formation in three-dimensional culture were drastically inhibited by the addition of anti-VEGF or NRP1 neutralizing antibodies, suggesting a functional significance of the Flk1-$VEGF_{165}$-NRP1 complex (Figure 6B-C). These results indicate that PKA regulates sensitivity of vascular progenitors to VEGF by dual induction of Flk1 and NRP1, which forms the Flk1-$VEGF_{165}$-NRP1 complex enhancing VEGF signaling to efficiently induce EC differentiation and vascular formation.

Discussion

Here, we showed a novel regulatory mechanism of EC differentiation and vascular formation through the modulation of progenitor properties to be endothelial competent. PKA activation increased both Flk1 and NRP1 expression in vascular progenitors and markedly enhanced the "sensitivity" of the progenitors to $VEGF_{165}$ by inducing Flk1-$VEGF_{165}$-NRP1 complex formation. This new-mode regulatory system would provide insights in vascular development and offer options for various therapeutic strategies with vascular manipulation.

Vascular formation is regulated by appropriate intensity, space, and timing of VEGF signaling. This study showed that PKA is involved in vascular formation process through its novel function regulating VEGF signal intensity. Various factors, such as adrenomedullin,[38] prostaglandins,[39] adiponectin,[40] ghrelin,[41] klotho,[42] and mechanical stress, especially fluid shear stress,[43] have been reported to activate PKA in ECs. We previously demonstrated that adrenomedullin could enhance EC differentiation from $Flk1^+$ cells through cAMP signaling.[26] Fluid shear stress was reported to enhance EC differentiation from $Flk1^+$ cells by up-regulating VEGF receptors,[44] and to induce Flk1 gene expression in EC cell lines.[45] These multiple PKA-activating signals should be involved in vascular development to modulate the progenitor sensitivity in vivo.

We previously reported that adrenomedullin/cAMP pathway induced differentiation of arterial ECs.[26] We also examined involvement of PKA in arterial-venous specification. Whereas addition of PKI, PKA inhibitor, to $Flk1^+$ cell culture with VEGF and 8bromo-cAMP significantly decreased total $CD31^+$ EC appearance (Figure 2A-D), PKI did not inhibit ephrinB2- or CXCR4-positive arterial EC differentiation (supplemental Figure 10). Moreover, expression of CA-PKA with VEGF did not induce arterial ECs from $Flk1^+$ vascular progenitors (supplemental Figure 11). These results indicated that PKA is not involved in arterial-venous specification. Activation of PKA in $Flk1^+$ cells did not induce prox1- or podoplanin-positive lymphatic ECs (supplemental Figure 11), further suggesting that PKA pathway is involved in common EC differentiation but not in EC specification processes.

Some studies have reported the roles of downstream molecules of Flk1 signaling in EC proliferation and differentiation. Tyrosine residue 1173 of Flk1 (Y1173, corresponding to Y1175 in human Flk1, KDR) is essential for Flk1 function in vasculogenesis.[46] Y1175 of KDR is a binding site of PLCγ and is important for VEGF-dependent EC proliferation.[47] Furthermore, Ras signaling acting downstream of Flk1 signaling plays a critical role in EC differentiation.[32] Indeed, PLC inhibitor, U73122, or H-Ras inhibitor, FTI-277, showed an inhibitory effect on EC differentiation from $Flk1^+$ cells (supplemental Figure 12), indicating that PLC and Ras pathway are both downstream molecules of Flk1 signaling. Enhanced VEGF signaling in vascular progenitors by PKA should be mediated by these molecules to induce basal EC differentiation.

Molecular mechanisms of PKA to induce and/or maintain Flk1 and NRP1 expression in vascular progenitors are largely unknown. NRP1 expression was reported to be up-regulated by cAMP/PKA pathway in olfactory neuron guidance.[48] Some other reports have shown that PKA pathway enhances differentiation of neuronal progenitor cells,[49] hippocampal progenitor cells,[50] and oligodendrocyte progenitor cells.[51] Recently, evidence is accumulating to suggest that blood vessels and nerves share a similar molecular machinery to form their networks.[52] Blood vessels and nerves may use PKA as common regulatory cues for their differentiation and

development. Further elucidation of molecular interaction among PKA, Flk1, and NRP1 should provide a novel molecular framework for tissue development.

Very recently, Cimato et al reported that NRP1 was largely coexpressed with Flk1 to identify endothelial precursors in human and mouse ES cells.[53] We confirmed that low-level expression of NRP1 was observed in Flk1$^+$ progenitors and was increased in ECs (supplemental Figure 13). These 2 functional markers for EC progenitors, Flk1 and NRP1, were, thus, commonly regulated by PKA to efficiently enhance their progenitor potentials responding to VEGF signaling.

We have succeeded in uncovering novel roles of PKA in EC differentiation and vascular development using our unique ES cell differentiation system. Elucidation of the new-mode cell fate determination mechanisms by modulation of progenitor potentials would provide novel insights in developmental biology, stem cell biology, and regenerative medicine.

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Authorship

Contribution: K.Y. performed all experiments and wrote the paper; K.K. and T.W. performed ex vivo whole-embryo experiments; S.K. helped with immunostaining experiments; and J.K.Y. supervised all experiments and wrote the paper.

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