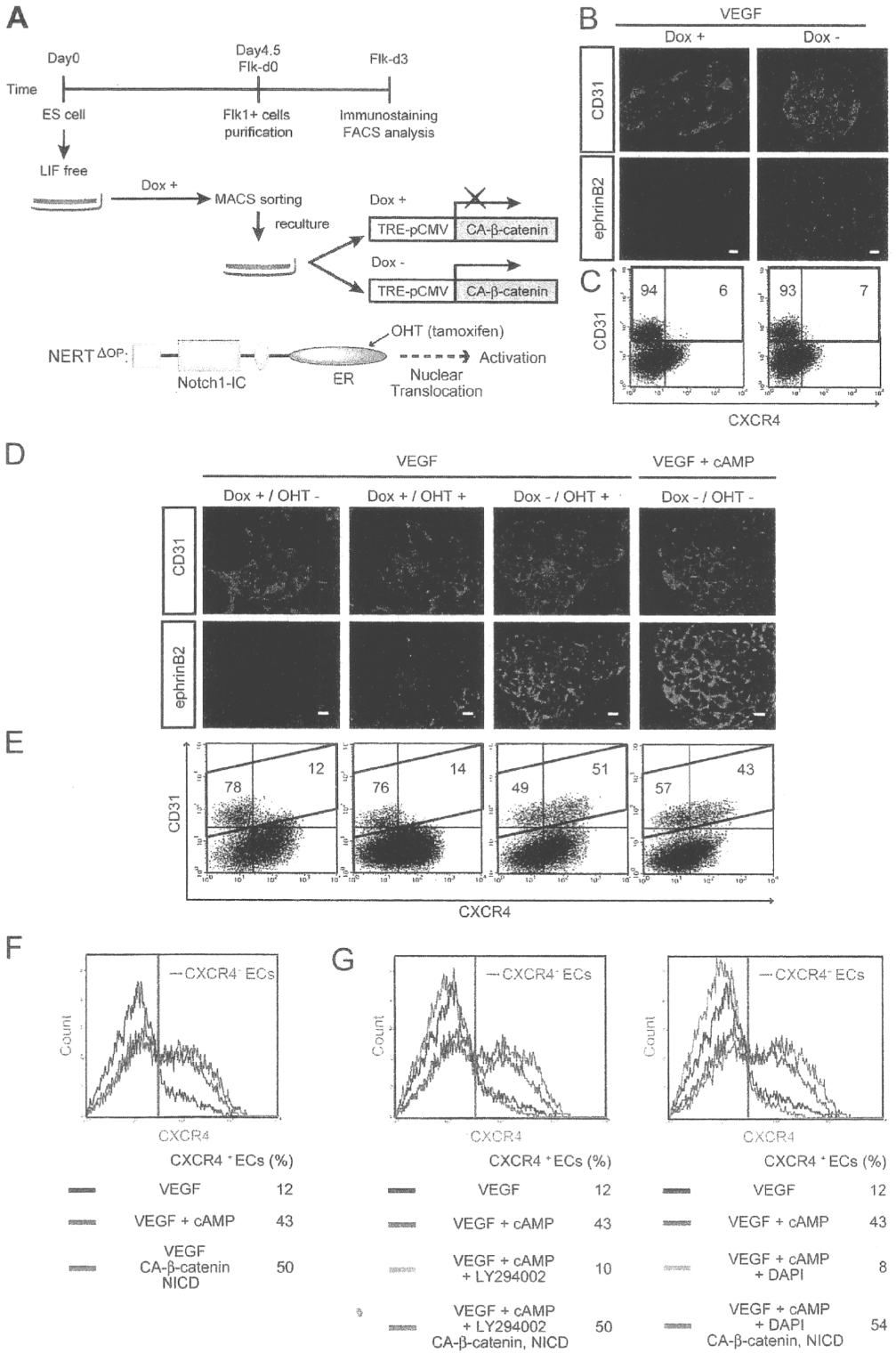

Figure 3. **Arterial EC induction by dual activation of β-catenin and Notch signaling.** (A) Experimental system for dual activation of Notch and β-catenin signaling. ES cell lines carrying CA-β-catenin regulated by Tet-Off system, and a fusion protein of N1ICD and estrogen receptor (ER), $NERT^{\Delta OP}$, were established. CA-β-catenin was induced by depletion of Dox, and Notch activation was induced by nuclear translocation of $NERT^{\Delta OP}$ with addition of 4-hydrotamoxifen (OHT). 1 µg/ml Dox was added during the first 4.5 d of culture of ES cell differentiation to $Flk1^+$ cells. After $Flk1^+$ cells were sorted by MACS and plated on type IV collagen-coated dishes, cells were treated with or without Dox and/or OHT (150 ng/ml). (B and C) Activation of β-catenin together with VEGF. (B) Double-fluorescent staining for CD31 and ephrinB2 at Flk-d3. Left panels, Dox treatment. Right panels, Dox free (expression of CA-β-catenin). (C) Flow cytometry for CD31 and CXCR4 expression at Flk-d3. Percentages of $CXCR4^+/CD31^+$ arterial ECs and $CXCR4^-/CD31^+$ venous ECs in total ECs ($CD31^+$ cells) are indicated. (D–F) Dual activation of Notch and β-catenin signaling. (D) Double-fluorescent staining for CD31 and ephrinB2

(Fig. 1 A and Fig. S1). We further quantitatively evaluated arterial EC induction at the cellular level with flow cytometry. We used a chemokine receptor, CXCR4, as an arterial EC marker (Tachibana et al., 1998; Ara et al., 2005; Yurugi-Kobayashi et al., 2006). $CXCR4^-/CD31^+$ venous ECs were mainly induced by VEGF treatment alone. $CXCR4^+/CD31^+$ arterial ECs were induced in the presence of 8bromo-cAMP together with VEGF. Addition of LY294002 almost completely inhibited $CXCR4^+$ arterial EC induction, but not total $CD31^+$ cell appearance (Fig. 1 B).

PI3K is known as one of the downstream molecules of VEGF signaling in adult ECs (Dayanir et al., 2001; Shiojima and Walsh, 2002). Although VEGF treatment alone induced no significant activation of PI3K in $Flk1^+$ cells, treatments with 8bromo-cAMP significantly activated PI3K (Fig. 1 C). These results indicated that cAMP signaling, but not VEGF, can activate PI3K in vascular progenitors or differentiating ECs and contributes to arterial EC induction.

Notch signaling is known to have important functions during arterial–venous specification (Xue et al., 1999; Lawson et al., 2001; Villa et al., 2001; Duarte et al., 2004; Krebs et al., 2004; Sörensen et al., 2009). Previously we demonstrated that addition of 8bromo-cAMP together with VEGF induced Notch activation in differentiating ECs (Fig. 1 D and Fig. S2; Yurugi-Kobayashi et al., 2006). Addition of LY294002 virtually abolished cAMP-induced Notch activation (Fig. 1 D), indicating that PI3K acts downstream of cAMP to activate Notch signaling in differentiating ECs.

GSK3β is negatively involved in arterial EC induction

We previously reported in our ES cell system that Notch signaling is essential but not sufficient for arterial EC induction (Yurugi-Kobayashi et al., 2006). We next investigated other downstream targets of the cAMP and PI3K pathways involved in arterial EC induction. When activation of glycogen synthase kinase (GSK) 3β, one of the downstream targets of PI3K (Cross et al., 1995), was blocked by addition of a GSK3β inhibitor, Bio, the inhibitory effects of LY294002 on $ephrinB2^+$ arterial EC induction was partially restored (Fig. 2 A). The inhibitory effect of LY294002 on $CXCR4^+/CD31^+$ arterial EC appearance was also partially reversed by the addition of Bio (Fig. 2 B). We generated ES cell lines expressing constitutively active (CA) or dominant-negative (DN) mutants of GSK3β using a tetracycline-regulatable system (Tet-Off; Fig. S3; Summers et al., 1999; Rommel et al., 2001; Yamamizu et al., 2009). We then induced expression of the CA- or DN-GSK3β in $Flk1^+$ cell cultures by depleting doxycycline (Dox), a tetracycline analogue (Fig. 2 C). CA-GSK3β expression in $Flk1^+$ progenitor cells inhibited arterial EC induction by VEGF and cAMP treatment (Fig. 2, D and E). On the other hand, DN-GSK3β expression in $Flk1^+$ progenitor cells weakly induced arterial ECs with VEGF treatment alone (Fig. 2 F and G), indicating that GSK3β negatively regulates arterial EC induction downstream of PI3K.

Activation of β-catenin and Notch signaling induces arterial ECs

Next, we investigated whether β-catenin, a negatively regulated downstream target of GSK3β (Nusse, 2005), is involved in arterial EC induction. We generated an ES cell line expressing CA-β-catenin regulated by the Tet-Off system (Fig. S3; Hirabayashi et al., 2004; Yamamizu et al., 2009). $Flk1^+$ cells were sorted and recultured with VEGF in the presence or absence of 1 μg/ml Dox (Dox^+ or Dox^-; Fig. 3 A). CA-β-catenin expression (Dox^-) together with VEGF stimulation showed almost no induction of $ephrinB2^+$ arterial ECs (Fig. 3 B) as well as $CXCR4^+/CD31^+$ arterial ECs (Fig. 3 C), indicating that β-catenin signaling alone is not sufficient for arterial EC induction.

Previously we established an inducible Notch activation system in ES cells using a fusion protein ($NERT^{\Delta OP}$) of the intracellular domain of murine Notch1 (N1ICD) and the estrogen receptor (ER), which allows regulated nuclear translocation of N1ICD with an ER ligand, 4-hydroxytamoxifen (OHT; Fig. 3 A; Schroeder et al., 2006; Yurugi-Kobayashi et al., 2006). We subsequently generated an ES cell line expressing both the $NERT^{\Delta OP}$ fusion protein and tetracycline-regulated CA-β-catenin. Activation of Notch by addition of 150 ng/ml OHT together with VEGF induced only a faint arterial EC induction, compatible with our previous results (Yurugi-Kobayashi et al., 2006). However, remarkable appearance of $ephrinB2^+$ ECs was clearly observed after dual activation of Notch and β-catenin signaling by the addition of OHT and depletion of Dox, respectively, even in the absence of cAMP (Fig. 3 D). FACS analysis further demonstrated that the dual activation of Notch and β-catenin signaling completely reproduced the effects of cAMP on arterial EC induction (Fig. 3, E and F). Moreover, though LY294002 or a γ-secretase inhibitor, DAPT, almost completely abolished arterial EC induction by cAMP, the dual activation of Notch and β-catenin signaling completely reversed their inhibitory effects (Fig. 3 G). These results indicate that the dual activation of Notch and β-catenin is sufficient to reconstitute the roles of cAMP in arterial EC induction.

Cytoplasmic β-catenin translocates into the nucleus where it forms a complex with transcription factors of the TCF/LEF family and activates target molecules (Orsulic and Peifer, 1996; Nusse, 2005). To investigate whether the TCF transcription factor

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at Flk-d3. Top panels, CD31 (pan-ECs, red) and DAPI (blue). Bottom panels, EphB4-Fc (ephrinB2$^+$ arterial ECs, green) and DAPI (blue). $Flk1^+$ cells were treated with VEGF alone (50 ng/ml), together with Dox^+ (control), Dox^+/OHT^+ (Notch activated), or Dox^-/OHT^+ (dual activated) condition. VEGF and 8bromo-cAMP (0.5 mM) treatment in Dox^+ condition is shown as positive control. Bars: 100 μm. (E) Flow cytometry for CD31 and CXCR4 expression. Percentages of $CXCR4^+/CD31^+$ arterial ECs and $CXCR4^-/CD31^+$ venous ECs in total ECs ($CD31^+$ cells) are indicated. (F and G) Expression profile of CXCR4 in $CD31^+$ ECs by flow cytometry. Percentages of $CXCR4^+$ arterial ECs in total ECs are indicated. (F) VEGF treatment alone (blue line), VEGF and 8bromo-cAMP (red line), and VEGF together with dual activation of β-catenin and Notch activation (Dox^-, OHT^+; green line) are shown. (G) VEGF treatment alone (blue line), VEGF and 8bromo-cAMP (red line), VEGF, 8bromo-cAMP, and LY294002 (7.5 μM; left panel), DAPT (2.5 μM; right panel, orange line), VEGF, 8bromo-cAMP, and LY294002 (left panel), or DAPT (right panel) together with dual activation of β-catenin and Notch activation (Dox^-, OHT^+; green line) are shown.

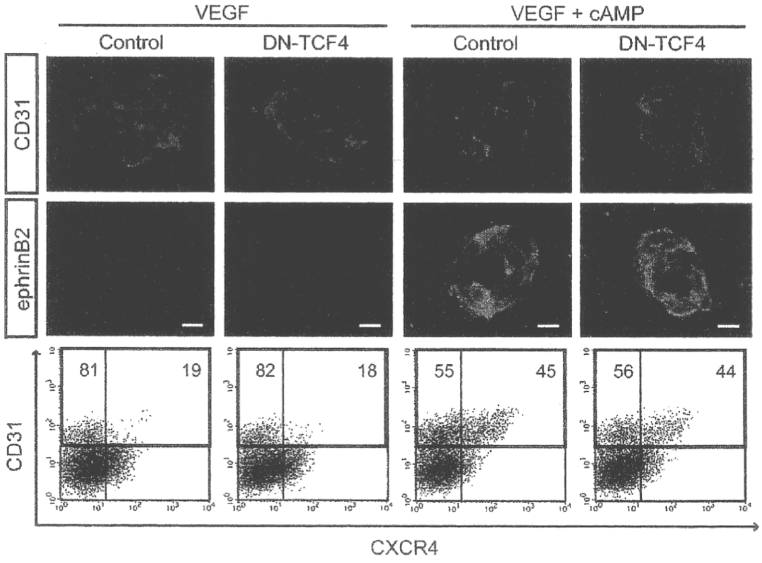

Figure 4. **Effects of DN-TCF4 on arterial EC induction from Flk1$^+$ cells.** (Top) Double-fluorescent staining for CD31 and ephrinB2 at Flk-d3. CD31 panels, CD31 (pan-ECs, red) and DAPI (blue). ephrinB2 panels, EphB4-Fc (ephrinB2$^+$ arterial ECs, green) and DAPI (blue). Flk1$^+$ cells induced from DN-TCF4 ES cell line were cultured with VEGF alone (50 ng/ml) or VEGF and 8bromo-cAMP (0.5 mM), in the presence or absence of 1 µg/ml Dox. Bars: 100 µm. (Bottom) Flow cytometry for CD31 and CXCR4 expression at Flk-d3. Percentages of CXCR4$^+$/CD31$^+$ arterial ECs and CXCR4$^-$/CD31$^+$ venous ECs in total ECs (CD31$^+$ cells) are indicated.

is involved in arterial EC induction, we generated an ES cell line expressing DN-TCF4 regulated by the Tet-Off system (Fig. S3; van de Wetering et al., 2002; Yamamizu et al., 2009). Even when we expressed DN-TCF4 in Flk1$^+$ vascular progenitors, cAMP-elicited arterial EC induction was not affected (Fig. 4, A and B). Thus, TCF did not appear to be involved in the arterial specification process.

A protein complex of RBP-J, NICD, and β-catenin is formed specifically in arterial ECs

We further investigated how Notch and β-catenin signaling pathways interact to induce arterial ECs. We first attempted to examine the expression and molecular interaction of these two molecules in purified arterial and venous ECs induced from ES cells (Fig. 5 A; Yurugi-Kobayashi et al., 2006). Purified arterial and venous ECs showed a distinct mRNA expression pattern of arterial EC markers (ephrinB2, Notch1, Dll4, Hes1, Alk1, CXCR4, and NRP1) and venous EC markers (NRP2 and COUP-TFII), respectively (Fig. 5 B). Western blot analysis revealed that Notch1 and 4 protein expression was specifically detected in arterial ECs but not in venous ECs (Fig. 5 C), suggesting that cAMP regulates both Notch induction and activation. Furthermore, the NICD was present almost specifically in the nuclear fraction of arterial ECs but not in that of venous ECs. Although β-catenin was observed in the nuclear fraction of both arterial and venous ECs, arterial ECs showed a higher nuclear β-catenin expression than venous ECs (Fig. 5 D). Immunoprecipitation (IP) assays revealed that the arterial-expressed NICD formed a protein complex with β-catenin (Fig. 5 E). We further confirmed the formation of a NICD/β-catenin protein complex on several arterial genes by chromatin-IP (ChIP) assays. Recently, RBP-J binding sites in mouse *ephrinb2* or *hes1* genes were reported to regulate their expression in response to Notch activation (Grego-Bessa et al., 2007; Shimizu et al., 2008). We further performed in silico investigations of RBP-J binding sites within other arterial-specific genes, and found conserved RBP-J binding sites in both the mouse and human genome in *neuropilin1*, *dll4*, and *cxcr4* genes (Fig. S4). ChIP assays demonstrated that although the RBP-J protein was present on these RBP-J binding sites in both arterial and venous ECs, NICD and β-catenin formed a protein complex with RBP-J only in arterial ECs (Fig. 5 F). We further confirmed the functional relevance of the dual activation of NICD and β-catenin on target gene expression in Flk1$^+$ cells. Compared with NICD alone, dual expression of NICD and β-catenin synergistically increased Hes1 gene promoter activity by approximately threefold ($n = 3$; $P < 0.002$; Fig. 5 G). Taken together, these results suggest that Notch and β-catenin signaling converge into a single protein complex with RBP-J, NICD, and β-catenin (arterial protein complex) on RBP-J binding sites specifically in arterial ECs, and that this heterotrimeric arterial protein complex synergistically activates target gene expressions in Flk1$^+$ vascular progenitors and induces arterial EC specification.

The arterial protein complex is also formed in embryonic and adult vessels

We purified arterial (CXCR4$^+$/CD31$^+$/CD45$^-$) and venous (CXCR4$^-$/CD31$^+$/CD45$^-$) ECs from embryonic day (E) 11.5 mouse embryos (Fig. 6 A) and performed ChIP assays for the RBP-J binding sites in *ephrinb2*, *neuropilin1*, *dll4*, *hes1*, and *cxcr4* genes. Purified arterial and venous ECs showed distinct mRNA expression patterns of arterial and venous EC markers, respectively (Fig. 6 B). Similar to ECs derived from ES cells, the arterial protein complex (NICD/β-catenin/RBP-J protein complex) was formed on RBP-J binding sites of arterial marker genes specifically in arterial ECs, but not in venous ECs in the embryo (Fig. 6 C). Moreover, we investigated whether the arterial protein complex is also formed in adult mice using isolated aorta and vena cava. Notch1 and 4 proteins were specifically detected in the aorta (Fig. 6 D). IP assays revealed that NICD and β-catenin formed a protein complex only in the aorta (Fig. 6 E). ChIP assays further demonstrated that the arterial protein complex was evidently identified in the aorta rather than

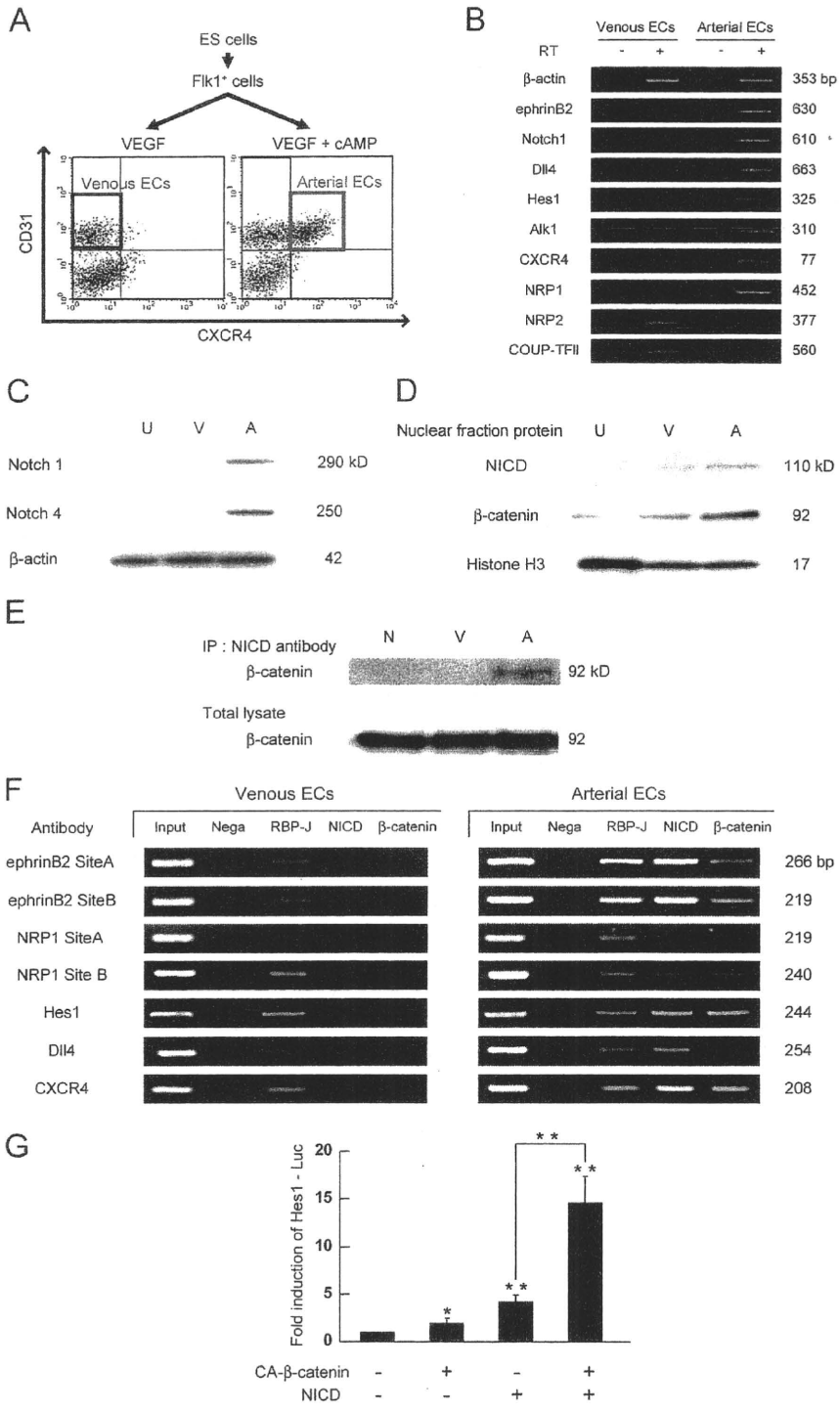

Figure 5. **Arterial-specific formation of protein complex with RBP-J, NICD, and β-catenin.** (A) Purification of arterial and venous ECs from ES cells. CXCR4$^+$/CD31$^+$ cells at Flk-d3 induced by VEGF (50 ng/ml) with 8bromo-cAMP (0.5 mM) and CXCR4$^-$/CD31$^+$ cells induced by VEGF alone were purified as arterial and venous ECs, respectively. (B) RT-PCR for mRNA expression of arterial and venous EC markers in purified arterial and venous ECs induced from ES cells as indicated in panel A. (C) Western blot for protein expression of Notch1 and Notch4 in purified arterial and venous ECs. U, undifferentiated ES cells; V, venous ECs; A, arterial ECs. (D) Nuclear localization of NICD and β-catenin. A representative result of Western blot analysis for NICD and β-catenin using nuclear fraction of purified arterial and venous ECs. Anti-histone H3: nuclear fraction control. (E) Immunoprecipitation assay. Immunoblot with anti–β-catenin antibody for total cell lysates or cell lysates immunoprecipitated with anti-NICD antibody. N: negative control, immunoprecipitated with normal rabbit-IgG antibody. (F) ChIP assays for RBP-J, NICD, and β-catenin on RBP-J binding sites of arterial markers in ECs from ES cells. Input: PCR products generated using DNA from nonimmunoprecipitated chromatin as a template. Negative control: immunoprecipitated with normal rabbit-IgG antibody. RBP-J, NICD, β-catenin: immunoprecipitated chromatin with antibodies for corresponding proteins. (G) Hes1 Luciferase reporter assay. A Notch signaling reporter, Hes1-Lucifearse plasmid was transiently transfected to MACS-purified Flk1$^+$ cells together with CA-β-catenin and/or NERT$^{\Delta OP}$ activation. After 24 h, the luciferase activities were measured (n = 3; *, $P < 0.05$; **, $P < 0.01$ vs. control or between corresponding values).

the vena cava (Fig. 6 F). Together, these results indicate the existence of the same molecular interaction in both embryos and adults.

Dual activation of Notch and β-catenin signaling enhances arterial gene expression during in vivo angiogenesis

Finally, to investigate whether Notch and β-catenin signaling is involved in arterial specification during angiogenesis in adults, we examined in vivo angiogenesis using a gel plug assay (Kim et al., 2002). Adenoviral vectors for NICD and/or CA-β-catenin were mixed into Matrigel plugs with VEGF (100 ng/ml) and heparin (10 units/ml). Transgene products were successfully induced in gel plugs at 1 wk after transplantation (Fig. 7 A). Formation of vascular structures and the presence of blood cells within the vascular lumen occurred in gel plugs to a similar extent under all experimental conditions tested (Fig. 7 B). Although expression of a pan-EC

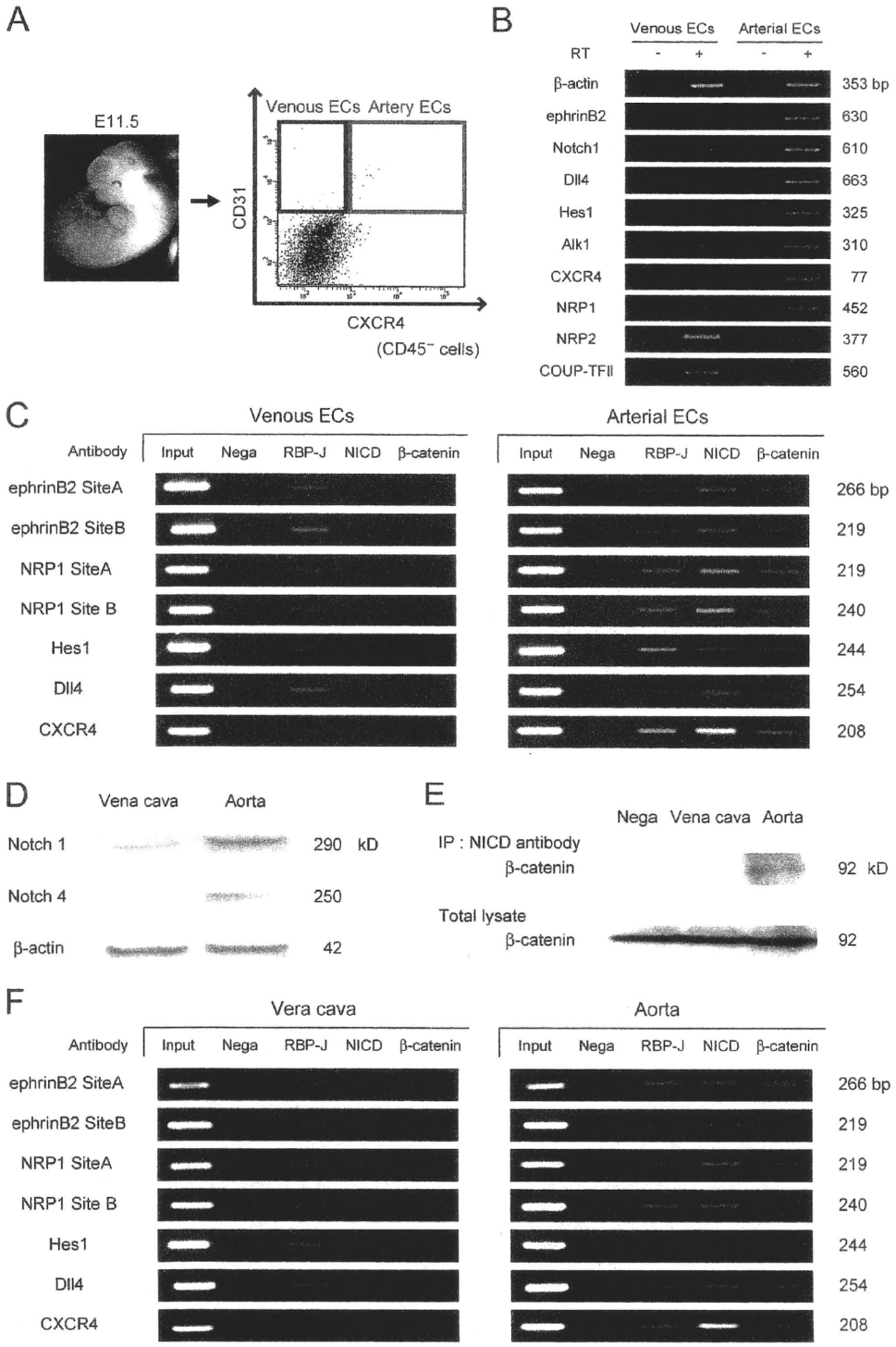

Figure 6. **Formation of the arterial protein complex in the embryo and adult vessels.** (A) Purification of arterial and venous ECs from the mouse embryo. Arterial ECs ($CXCR4^+/CD31^+/CD45^-$) and venous ECs ($CXCR4^-/CD31^+/CD45^-$) were isolated from E11.5 embryos. (B) RT-PCR for mRNA expression of arterial and venous EC markers in purified arterial and venous ECs from E11.5 mouse embryo. (C) ChIP assays for RBP-J, NICD, and β-catenin on RBP-J binding sites of arterial markers in ECs from embryos. (D) Western blot for Notch1 and Notch4 in isolated aorta and vena cava. (E) Immunoprecipitation assay for isolated aorta and vena cava. Immunoblot with anti–β-catenin antibody for total cell lysates or cell lysates immunoprecipitated with anti-NICD antibody. (F) ChIP assays for RBP-J, NICD, and β-catenin on RBP-J binding sites of arterial markers in the aorta and vena cava.

marker, VE-cadherin, was not altered, expression of the arterial markers ephrinB2 and Neuropilin1 was significantly increased in gel plugs only with the dual induction of NICD and CA-β-catenin (Fig. 7, C and D). These results indicate that dual activation of Notch and β-catenin signaling also functions in arterial EC induction during in vivo angiogenesis in adults.

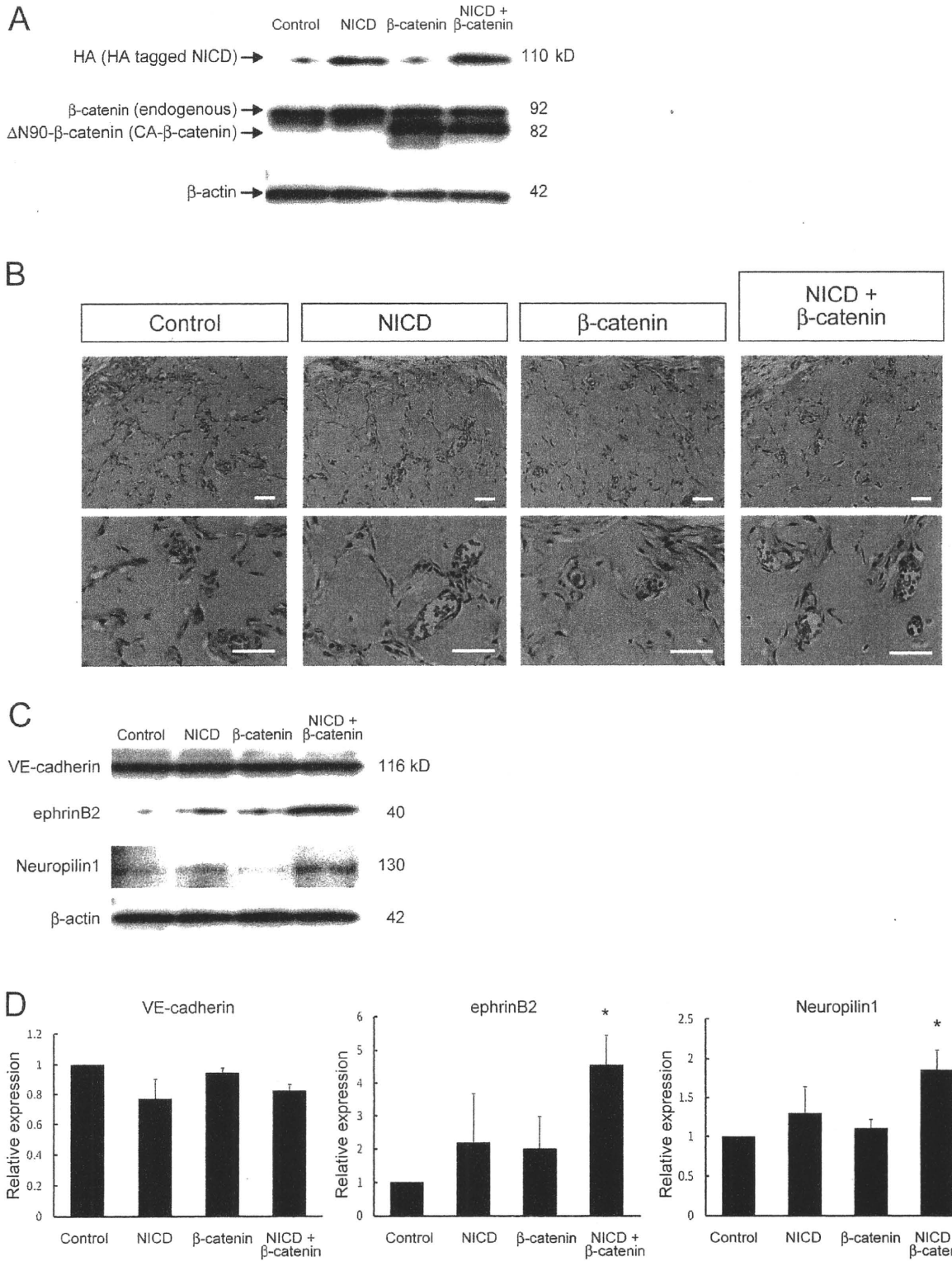

Figure 7. **Enhancement of arterial gene expression through dual activation of Notch and β-catenin during in vivo angiogenesis.** Matrigel containing VEGF (100 ng/ml), heparin (10 units/ml), and adenoviral vectors (vehicle [control], HA-tagged N1ICD [NICD], and/or CA-β-catenin) were injected subcutaneously in mice. After 7 d, the mice were sacrificed and plugs were excised. (A) Western blot for HA-tagged N1ICD and CA-β-catenin in recovered cells from Matrigel plugs. (B) Hematoxylin and eosin staining of Matrigel sections. Overall appearances were not different. Invasion of blood vessels with vascular lumen and blood cells were observed. Bars: 200 μm. (C) Representative result of Western blot for VE-cadherin, ephrinB2, and Neuropilin1 in recovered cells from Matrigel plugs. (D) Quantitative evaluation of VE-cadherin (left graph), ephrinB2 (middle graph), and Neuropilin1 (right graph) protein expression in Matrigels. Relative expression normalized with β-actin expression is shown. ($n = 3$; *, $P < 0.05$ vs. control).

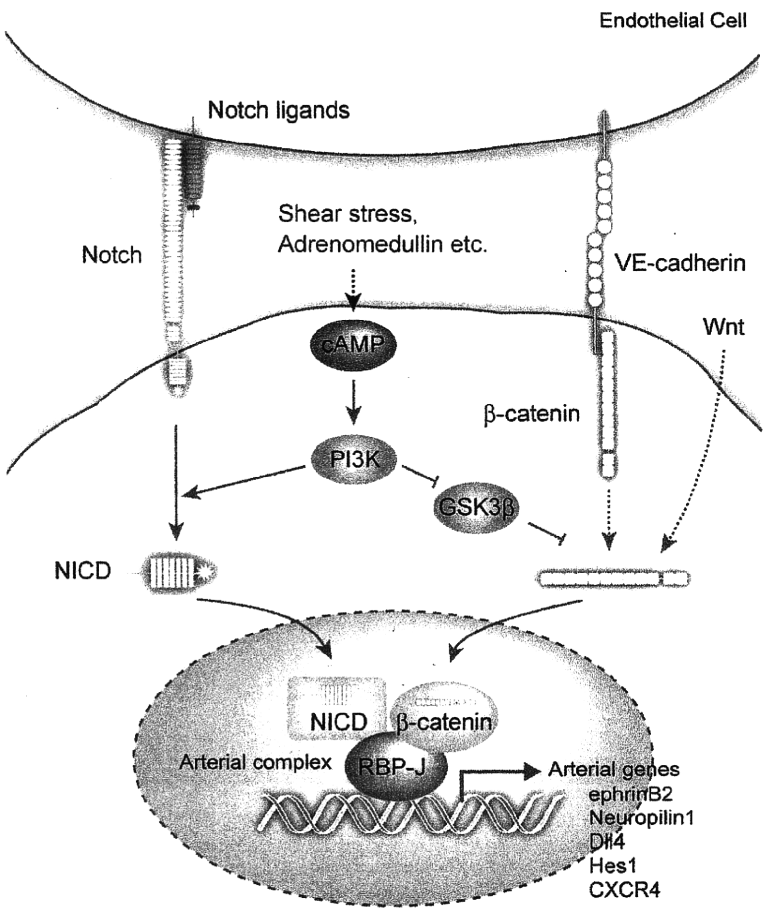

Figure 8. **Molecular mechanisms of arterial EC specification.** cAMP signaling, which could be induced by adrenomedullin, shear stress, and so on, activates Notch and β-catenin signaling through PI3K (and GSK3β) in vascular progenitors (as well as differentiating ECs). Notch and β-catenin signaling subsequently converges into a single protein complex with RBP-J, NICD, and β-catenin (arterial complex) on arterial genes. Notch signaling from Notch ligand binding and β-catenin signaling from Wnt and VE-cadherin should also participate in forming the complex. The arterial complex should play a central role in the specification of arterial cell fate in ECs.

Discussion

Here, we demonstrated that simultaneous activation of Notch and β-catenin signaling can constructively reproduce the induction processes of arterial ECs from Flk1$^+$ vascular progenitors through the formation of an arterial-specific protein complex. cAMP, PI3K, Notch, and β-catenin signaling interact and converge during EC differentiation to specify arterial cell fate. These findings provide novel insights into vascular signaling necessary for cell differentiation and diversification as well as into molecular mechanisms of cell fate determination.

In ECs, Notch (Notch1, 4) activation can be induced by various Notch ligands, including Dll1, Dll4, and Jagged2, which are expressed by arterial ECs, and Jagged1, which is expressed in ECs and mural cells (Villa et al., 2001; Sörensen et al., 2009). All of this Notch signaling is considered to be mediated by the NICD and RBP-J transcription factor. On the other hand, β-catenin signaling in ECs can be activated through Wnt ligands as well as VE-cadherin. Thus, Wnt ligands such as Wnt2, 5a, and 10b, expressed in fetal blood vessels, are involved in EC differentiation (Monkley et al., 1996; Goodwin and D'Amore, 2002). VE-cadherin is heavily tyrosine phosphorylated and is linked to β-catenin. When adherens junctions mature, tyrosine residues in VE-cadherin tend to be dephosphorylated and β-catenin is partially released from the complex (Dejana et al., 1999), allowing nuclear translocation of β-catenin and activation of downstream signaling cascades. As VE-cadherin and β-catenin are broadly expressed in ECs and mice with EC-specific disruption of β-catenin show broad vascular phenotypes (Cattelino et al., 2003), β-catenin should possess both common roles in ECs and specific roles in arterial ECs. Adrenomedullin, which is mainly secreted from vascular smooth muscle cells, activates the cAMP pathway and induces arterial ECs in the ES cell system (Yurugi-Kobayashi et al., 2006). Many other factors, such as fluid shear stress, should be involved in cAMP activation in ECs. All of this signaling in blood vessels should finally converge into the single arterial protein complex (NICD/β-catenin/RBP-J protein complex) and contribute to induce arterial ECs (Fig. 8).

In addition to induction of arterial ECs, Notch and β-catenin could also be potentially involved in maintenance of the arterial EC phenotype. When activation of Notch and β-catenin signaling was ceased in arterial ECs from Flk-d3, ephrinB2 expression was attenuated and disappeared until Flk-d12 (Fig. S5). On the other hand, When Notch and β-catenin signaling was activated

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 (ESrTA-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 ESrTA-ROSA cells using mouse ES cell Nucleofector kit (Lonza). Hygromycin- and ganciclovir-resistant colonies were selected and subjected to further studies (cDNA ESrTA 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 ESrTA 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/$NERT^{\Delta OP}$ ESrTA cells).

Cell culture

ES cell lines, D3, ESrTA-ROSA, and various ESrTA 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. $EphrinB2P^{+P}$ 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：

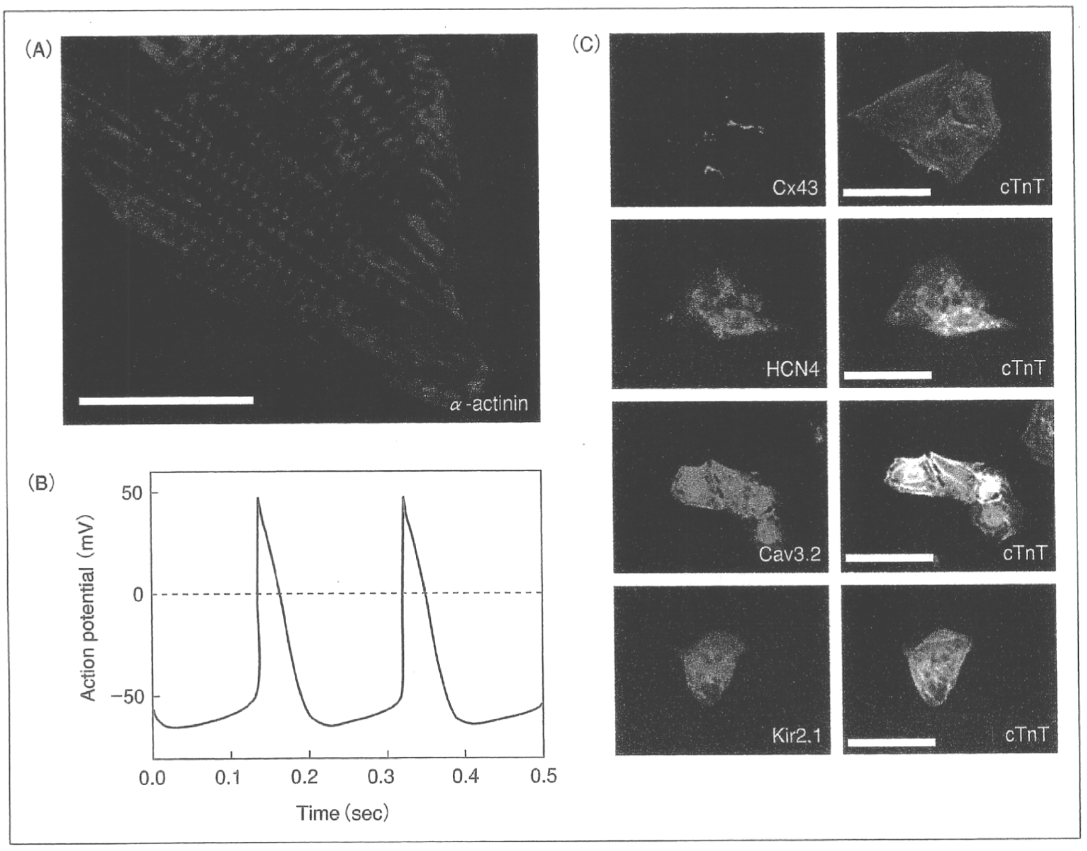

図 1　マウスiPS細胞からの心筋細胞分化[8]

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

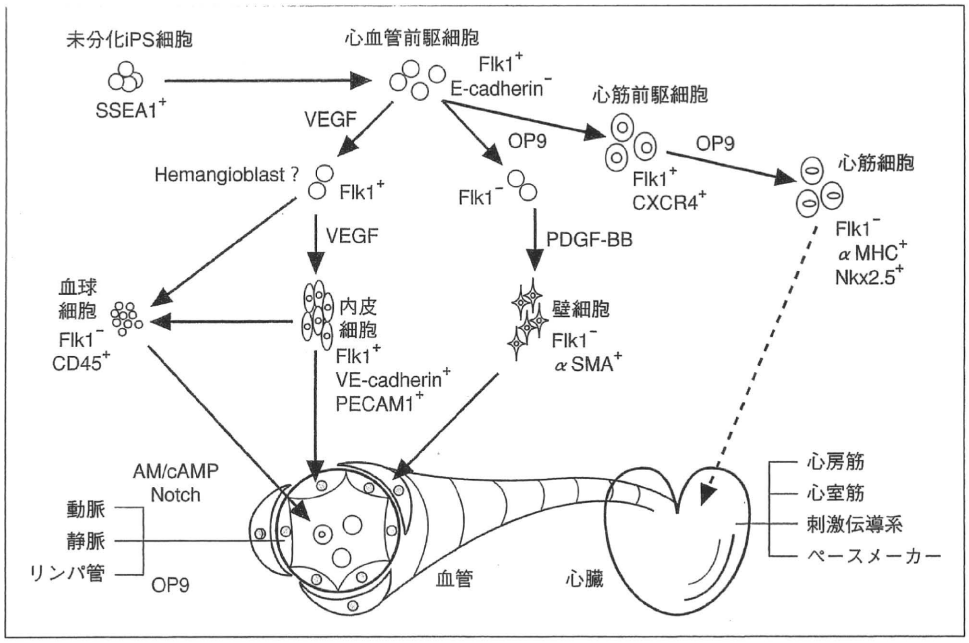

図 2　マウスiPS細胞からの系統的心血管細胞分化[8)]

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

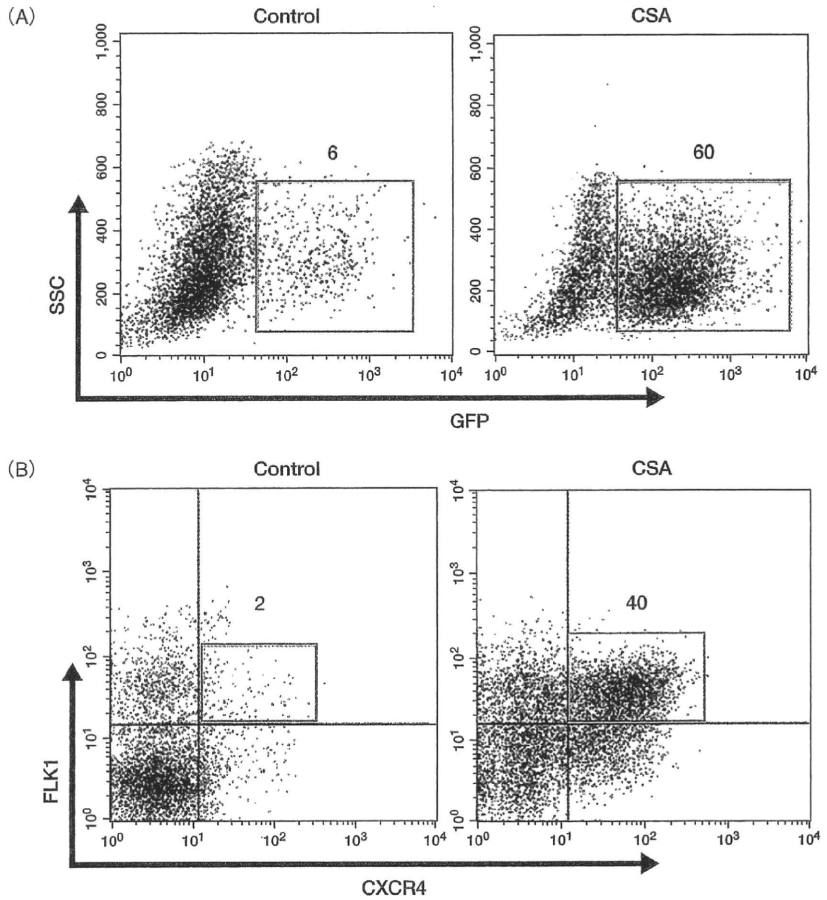

図 3　シクロスポリンA(CSA)による心筋前駆細胞および心筋細胞分化誘導効果：FACS解析

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

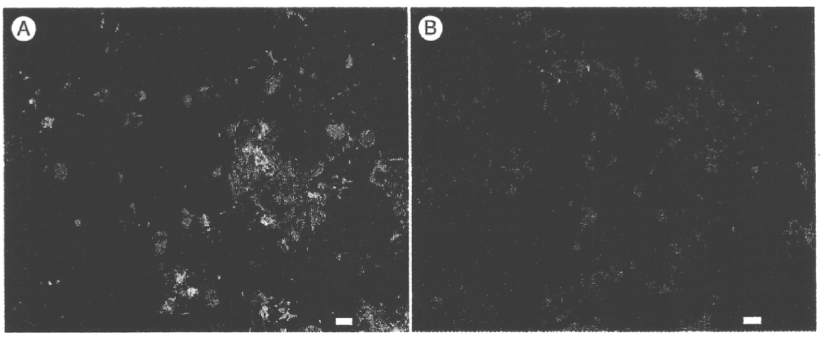

図 4　シクロスポリンA(CSA)による心筋前駆細胞および心筋細胞分化誘導効果：免疫染色
A：control，B：CSA.
CD31(赤；内皮細胞)/cTnT(緑；心筋細胞)二重染色．CSA により内皮細胞の出現が抑制され，対照的に心筋細胞が著しく増加する．スケールバー：400 μm.

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′-AGCCTGGTAGCAGGAAGATC, 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-