3　ES細胞による血管再生

筆者らはES細胞由来Flk1陽性細胞が生体内においても血管細胞に分化し、血管再生に寄与しうるかを検討するため、純化Flk1陽性細胞のニワトリ胎仔への移植実験を行った。心腔内注入により経血管的に移植されたFlk1陽性細胞は、内皮細胞および壁細胞に分化するとともにニワトリ胎仔発生にともなって形成された新生血管に寄与した[4]。筆者らはさらに、ES細胞由来細胞の血管再生治療応用における可能性を検討するため、ES細胞由来血管細胞の成体に対する移植効果を検討した[11]。すなわち、ES細胞由来血管細胞をヌードマウスに移植した腫瘍周囲に注入し、移植細胞の新生血管への寄与を検討したところ、ES細胞由来Flk1陽性細胞は、内皮細胞および壁細胞として新生血管へ寄与した。次に、成体への移植に適切な細胞の分化段階を検討するため、分化段階の異なる血管細胞、すなわち、ソート直後のFlk1陽性血管前駆細胞と、Flk1陽性細胞をさらに3日間培養して初期内皮細胞に分化した細胞（VE-カドヘリン陽性)の移植を比較した。Flk1陽性細胞を移植した群では、血管内皮細胞として寄与しているものの他に、それ以外の細胞として組織内に存在するものが多数(約60%)認められた。一方、初期内皮を移植した群では、ほとんど全ての細胞(95%以上)が内皮細胞として血管に寄与していた。また、Flk1陽性細胞移植群では、細胞移植した腫瘍における血流増加は認められなかったが、分化させた血管細胞を移植した群では、有意な血流増加が認められた。これらの結果より、ES細胞由来血管細胞の移植により、血管新生促進効果が認められるが、成体における血管新生をターゲットとした細胞移植においては、血管前駆細胞のレベルの細胞よりも、やや血管に分化した初期内皮細胞のステージがより有効かつ特異的であると考えられた。このように、ES細胞由来細胞の移植においては、むやみに未分化細胞を移植すればよいわけではなく、ドナー細胞の分化段階とレシピエント側の状況を対応させた至適な分化段階の細胞、—おそらくは標的細胞への分化が運命づけられた直近の前駆細胞—、を選択する必要があると考えられた。また同時に、移植をされる側においても標的細胞の分化を効率的に促進できる微小環境ができるだけ再現されていることが、有効な再生の実現には重要であると考えられる。

4　ヒトES細胞からの血管分化再生

ヒトES細胞を用いた血管細胞分化としては、胚様体を用いてCD31やVE-カドヘリン陽性内皮細胞の誘導と、フローサイトメトリーを用いての純化・再培養、培養下および免疫不全マウスに移植したゲル内における血管構造の形成が報告されている。京都大学のグループは、マウスES細胞と同様にサルES細胞においても2型VEGF受容体陽性細胞からの内皮細胞・壁細胞の分化[12]、培養下における血管構造形成に成功している。さらに同グループは、2002

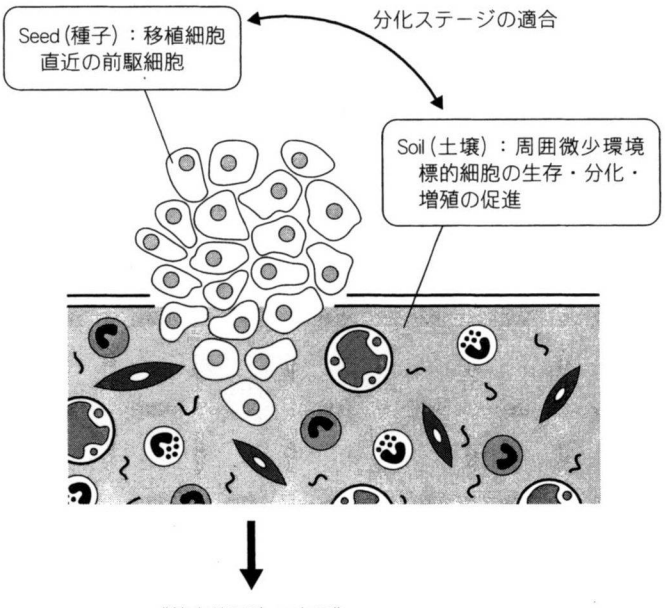
年より日本最初のヒトES細胞分化研究を輸入ヒトES細胞を用いて開始し、ヒトES細胞においても血管構成細胞の分化誘導と *in vitro* における管腔構造形成、さらにはマウス血管新生モデルにおける新生血管への移植細胞の寄与と血流改善効果を認めることを明らかにした[13),14)]。ヒトES細胞由来血管細胞の移植においては、純化した内皮細胞だけの移植よりも、血管壁細胞と混合した細胞群の方が血管新生作用が強かった[14)]。類似の現象は、心筋や神経に関する細胞移植においても報告されている。すなわち、純粋な心筋細胞や神経細胞のみならず、心臓間質細胞やグリア細胞などが共存する形の移植の方が心筋や神経再生効果が高い可能性が示唆されており、純粋に必要とされる細胞だけを移植するのではなく、移植細胞と周囲環境との相互作用、効果的に標的組織の再生を促進する局所微小環境を考慮した細胞移植戦略が新たな再生治療法の開発に有効であると考えられる。

5 iPS細胞からの血管・リンパ管分化

iPS細胞は、線維芽細胞等の成体由来分化細胞にOct4、Sox2、Klf4、c-mycの4因子(または3因子)を導入することにより誘導される新しい多能性幹細胞である[1)~3)]。筆者らは、マウスおよびヒトiPS細胞を用いた心血管分化研究にもいち早く取り組んでいる。[1]項に述べたマウスES細胞の血管分化誘導法をマウスiPS細胞に適用することにより、マウスES細胞と同様に、iPS細胞からの血管内皮細胞、壁細胞、動静脈リンパ管内皮細胞の分化誘導に成功した。内皮細胞および壁細胞からなる血管構造の3次元的形成にも成功した[15)]。また、担がんヌードマウスへの細胞移植実験により、内皮および壁細胞として生体内血管新生に寄与し得ることも確認した。マウスiPS細胞3クローンを用いて検討したが、クローン間で多少の分化能、増殖能に差異を認めたが、ES細胞においても認められるクローン間の差異と同程度かそれ以下のものであり、マウスiPS細胞はマウスES細胞とほぼ同様の分化特性を有していると考えられた。ただし、1～2ヵ月以上の長期分化誘導培養中にc-mycをはじめとするiPS細胞誘導時の導入遺伝子群の再発現を認める例があり、iPS細胞における特性の一つとして注意する必要があると考えられる。現在ヒトiPS細胞の心血管系への分化誘導も行っているが、マウスiPS細胞はマウスES細胞と、ヒトiPS細胞はヒトES細胞とほぼ同様の性質を持っていると考えられる。iPS細胞は、さまざまな病態モデル動物やヒト症例から比較的簡便に多能性幹細胞が誘導できるため、薬剤の安全性試験や新たなドラッグスクリーニングなど、直接的な細胞移植以外にも種々の応用が可能である。実際筆者らは、マウスiPS細胞からの3次元的血管形成モデルを用いて海洋生物由来HDAC阻害物質Ageladineの血管新生抑制作用を示すことに成功している[16)]。このようにiPS細胞を用いることにより、病態や疾患と幹細胞およびケミカルバイオロジーを結びつけた新しい再生医学や創薬研究が可能になると考えられる。

6　おわりに

このように、血管の発生・分化・再生機構に関してさまざまな知見が蓄積されてきているが、いまだ血管再生治療が明らかに有用な形で臨床応用されたといえるレベルには至っていない。臓器を構成する細胞を誘導して移植するあるいは前駆細胞を移植するというだけで臓器の再生が進むというほど単純ではないことがようやく学習されてきたというのが実情に近いであろう。今後は、細胞そのものの分化メカニズムの解析―細胞外シグナルから細胞内環境の変化と安定化の過程をエピジェネティックな視点も含めて解明する―に加えて、細胞間および細胞―細胞外マトリックス相互作用や臓器・組織間相互作用等臓器としての機能を果たし得る機能ユニットを形成するために必要な要素全てに関して理解を深め、それらを生体内でできるだけ再構成することが重要であろう。[3][4]の項でも述べたように、有効に分化し得る幹・前駆細胞(Seed：種子)と、分化と機能発現を可能にする周囲環境(Soil：土壌)の双方を整えた治療(Seed & Soil Therapy)(**図3**)を目指すことにより、再生医療はより実効性が期待されるものに近づ



細胞移植による臓器再生を行うためには、移植細胞(Seed：種子)とレシピエント側の微少環境(Soil：土壌)の分化ステージを適合させるとともに、双方の要因を整える必要がある。Seedとしては、標的細胞の直近の前駆細胞が好ましいと考えられる。Soilは、移植細胞の生着・生存、分化、増殖を促進できる微少環境が存在することが必要である。これらSeed & Soilを最適化することにより、効率的臓器再生が実現されると考えられる。

図3　Seed & Soil therapy

くと考えられる。

iPS細胞の樹立によって、成体組織からES細胞様の多能性幹細胞を得ることができるようになったことにより、ヒトES細胞樹立における倫理的問題や移植における免疫の問題とそれに絡むヒトクローン胚の問題も回避され、これらの細胞の再生医療応用におけるハードルは一気に低くなると考えられる。しかし、未分化細胞による奇形腫形成という従来からES細胞に認められている問題は残っていることに加え、iPS細胞誘導時の導入遺伝子の再発現やゲノムへの遺伝子導入等による腫瘍形成の可能性等iPS細胞特有の新しい問題もあり、iPS細胞そのものの改良や分化誘導・純化法等さまざまな技術開発が今後必要である。iPS細胞の出現は、これまでのES細胞研究に数多くの新たな可能性を与えた。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持ってiPS細胞の今後に対応していくことが必要と考えられる。iPS細胞研究が健全に成長することにより、分化再生機構の基礎研究から、再生治療法の開発や創薬とその産業化に至るまで、ES細胞研究が持っていたポテンシャルがさまざまな形で臨床応用へ向けて花開き、人々の幸福に貢献していくことを期待する。

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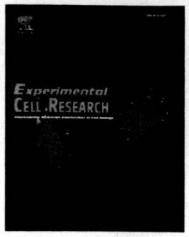

Review Article

ES and iPS cell research for cardiovascular regeneration

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ABSTRACT

Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, which are ES-like stem cells induced from adult tissues, are twin stem cells with currently (with the exception of fertilized eggs) the broadest differentiation potentials. These two stem cells show various similarities in appearance, maintenance methods, growth and differentiation potentials, i.e. theoretically, those cells can give rise to all kinds of cells including germ-line cells. Generation of human ES and iPS cells is further facilitating the researches towards the realization of regenerative medicine. The following three issues are important purposes of ES and iPS cell researches for regenerative medicine: (1) dissection of differentiation mechanisms, (2) application to cell transplantation, and (3) drug discovery. In this review, the current status of cardiovascular regenerative trials using ES and iPS cells is briefly discussed.

Contents

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Introduction

Cardiovascular diseases are the major causes of death in industrialized countries. Cardiovascular cells are, therefore, one of the most important targets in regenerative medicine. The regeneration of cardiomyocytes, which principally cannot proliferate and regenerate in the adult, is particularly expected to bring new hopes to cure cardiac diseases. Many cell types, such as endothelial progenitor cells from blood or bone marrow, cardiac progenitor cells from the heart, mesenchymal cells from bone marrow or other tissues, and ES and iPS cells, are currently being examined as cell sources for cardiovascular regenerative cell therapy [1]. Previously, the authors established a novel ES and iPS cell differentiation system, which can reproduce the early cardiovascular development processes in vitro [2–4]. Using and expanding this system as a tool for differentiation studies, cell transplantation and drug discovery, we are trying to explore novel cardiovascular regenerative strategies.

Differentiation from ES and iPS cells

Differentiation strategies – embryoid bodies vs. stepwise methods

To induce ES (or iPS) cell differentiation, embryoid bodies (EBs) which form as aggregates of ES cells are often used. Spontaneous differentiation of ES cells occurs in EBs by the interaction of cells within EBs, locally mimicking the body plan in the embryo. EBs contain various cell types including cardiomyocytes, endothelial cells (ECs) and mural cells (MCs; pericytes in capillary vessels and vascular smooth muscle cells in arteries and veins) and often form blood vessel-like structures [5]. Though the EB method is convenient for inducing differentiation and should be suitable for a large-scale preparation of cell sources, the method possesses several weak-points in dissecting cellular and molecular mechanisms during differentiation such as: (i) difficulty to dissect the differentiation mechanisms by highlighting cells and signals of interest in EBs, (ii) difficulty to directly observe differentiating cells at the cellular level by microscopy, and (iii) difficulty to conduct single cell analysis of differentiation.

To overcome these disadvantages in EB cultures, the authors developed a 2-dimentional culture-based, stepwise cardiovascular differentiation system (Fig. 1) [2,3,6]. In this system, Flk1$^+$ mesoderm cells are first induced from undifferentiated ES cells in a monolayer culture of ES cells in the absence of LIF (leukemia inhibitory factor). Flk1$^+$ cells, presumptive lateral plate mesoderm, are purified by FACS (flow cytometry-assisted cell sorting) using anti-Flk1 antibody. Various cardiovascular cells are then induced by re-culture of purified Flk1$^+$ cells as common precursor cells. Though this system requires purification and re-culture processes, it is amenable for easy monitoring and analysis of differentiating cells at the cellular level. To dissect molecular and cellular mechanisms of cell differentiation or to apply ES/iPS cell differentiation system to screen small molecules, such stepwise and systematic methods are very powerful (see below).

Differentiation of vascular cells – vasculodiversity and a constructive approach

Blood vessels consist of two cell types, ECs and MCs. The majority of vascular cells are considered to be derived from the mesoderm. Expression of Flk1 (also designated as vascular endothelial growth factor receptor-2 (VEGF-R2)) is an indicator of the lateral plate mesoderm [7]. Flk1 is also the earliest functional differentiation marker for blood and ECs [8]. The Flk1 ligand, VEGF, is a key factor for

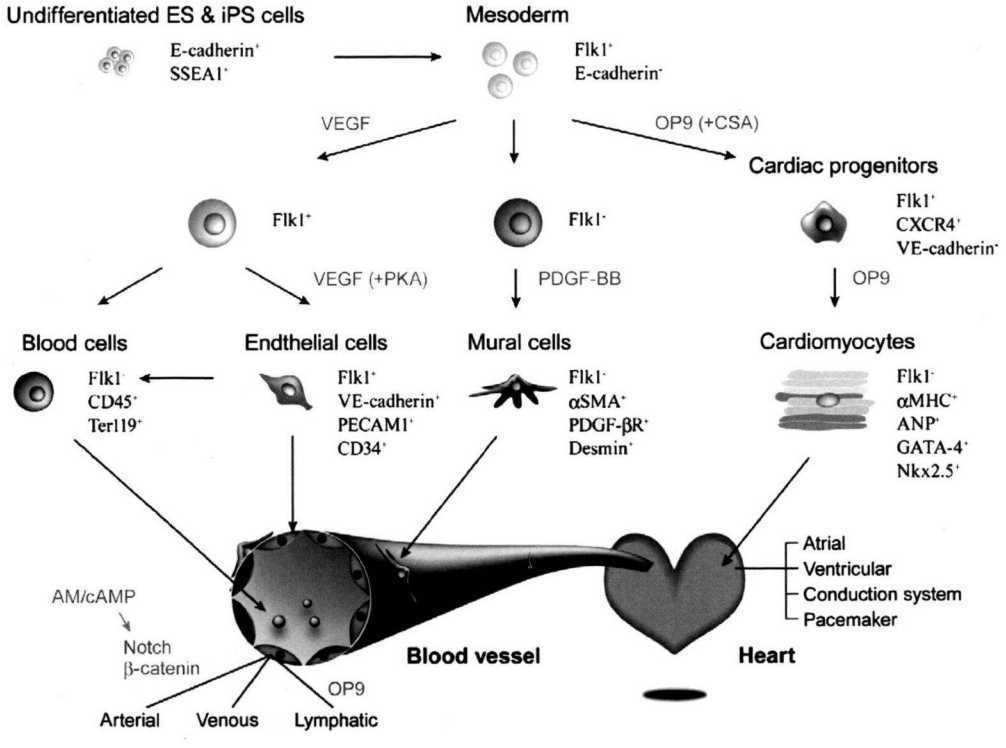

Fig. 1 – Systemic and stepwise cardiovascular cell differentiation system with mouse ES/iPS cells.

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EC differentiation. VEGF-A heterozygotes die early in gestation due to failure in vascular system formation, indicating that strict regulation of VEGF function is critical in normal vascular formation [9]. Recent reports for various molecular markers and functional molecules for arterial, venous, and lymphatic ECs [10] provide a novel research field as "vasculodiversity." A transmembrane ligand ephrinB2 and its receptor tyrosine kinase EphB4 are the first reported markers for arterial and venous ECs, respectively. The ephrinB2-EphB4 system is essential to establish mature blood vessel system with arterial-venous identity. Notch (Notch 1, 4) and its cell-surface ligands (Delta like-1 [Dll1], Dll4, and Jagged1 and 2) are expressed in arteries but not in veins. Genetic studies of Notch signaling components have shown that Notch signaling is essential for proper formation of the developing vasculature and arterial specification. On the other hand, insufficient Notch activation during angioblast differentiation to ECs leads ECs to venous fate. COUP-TFII, an orphan nuclear receptor transcription factor, was reported to repress Notch signaling through suppressing neuropilin1 expression to maintain vein identity. Lymphatic ECs originate from venous ECs. A subset of venous ECs expressing LYVE1, which are competent to lymphatic differentiation, are committed into lymphatic ECs with the expression of Prox1 homeobox transcription factor. Prox1 is considered as the most specific and functional lymphatic EC marker.

The author's group succeeded in inducing a variety of vascular cells from mouse ES cells using the stepwise ES cell differentiation system [2,5,11]. ECs and MCs are specifically induced from ES cell-derived $Flk1^+$ cells when they are cultured with VEGF and serum. In this condition, induced ECs mainly show venous phenotype. When cyclic AMP (cAMP) signaling is simultaneously stimulated with VEGF, arterial ECs are induced. A multifunctional polypeptide, adrenomedullin (AM), which exerts its function by increasing the levels of intracellular cAMP, is a candidate endogenous ligand that activates cAMP [12]. Prox1-positive lymphatic ECs are induced [13] when $Flk1^+$ cells are cultured on OP9 cells [14], which are stroma cells established from bone marrow of op/op (monocyte colony stimulating factor deficient) mice. Lymphatic ECs are also induced with EB methods. The authors group succeeded in inducing all three EC phenotypes, arterial, venous, and lymphatic ECs from ES cells [10].

Recently, novel roles of cAMP signaling in EC differentiation and arterial specification were demonstrated with the stepwise method. That is, a cAMP downstream gene, protein kinase A (PKA), specifically upregulates selective and sensitive receptors for $VEGF_{165}$, Flk1 and Neuropilin1, in vascular progenitors, and enhances the "sensitivity of the progenitors" to $VEGF_{165}$ by more than 10 times [15]. PKA activation increased the total EC number that appeared from $Flk1^+$ cells, but had no effect on arterial-venous specification. Arterial specification was caused by another pathway activated by cAMP, that is, Notch and GSK3β-mediated β-catenin signaling [16]. Notch and β-catenin signaling, both of which are activated through phosphatidylinositol-3 kinase downstream of cAMP, converges into single protein complex on arterial genes. The effect of cAMP in arterial specification was completely reproduced with neither Notch nor β-catenin, but with simultaneous activation. Thus, two distinct roles of cAMP pathways, common EC differentiation and arterial EC specification, were demonstrated though a constructive approach by building up each molecular functions to reproduce cell differentiation process in vitro [5,10]. The stepwise and constructive reproduction of vascular developmental processes with ES cell differentiation system can provide novel understanding in cellular and molecular mechanisms of vascular development from a new point of view.

Differentiation of cardiomyocytes, progenitors or stem cells

Cardiomyocytes are principally mesoderm derivatives. Mesodermal cells give rise to two cardiac progenitor populations that exist in so-called, primary heart field and secondary heart field [1,17,18]. Primary heart field is derived from the anterior splanchnic mesoderm and form cardiac crescent. Primary heart field is positive for Nkx2.5, Tbx5, and Hand1, and gives rise to mainly the left ventricle. Secondary heart field originates from the pharyngeal mesoderm and is situated medially to the primary heart field. Secondary heart field is positive for isl1, Tbx1, FGF8 and 10, and gives rise to mainly the right ventricle, outflow tract and inflow region. In addition to these two populations, proepicardial cells and neural crest cells also contribute to the heart structure [1].

Cardiomyocytes are one of the first cell types induced from ES cells. Appearance of self-beating cells in EBs was reported four years after the derivation of mouse ES cells [19]. The stepwise method showed that ES cell-derived $Flk1^+$ cells can give rise to vascular cells as well as cardiomyocytes [3]. When $Flk1^+$ cells were cultured on OP9 stroma cells, self-beating cardiomyocytes appeared in 4 days. ES cell-derived cardiac progenitors at a single cell level were first reported as $Flk1^+$/$CXCR4^+$/vascular endothelial cadherin$^-$ (FCV) cells [3]. Flk1, Nkx2.5, and/or islet1 were reported to mark multipotent cardiac progenitor population [20]. Though these markers mainly represent lateral plate mesoderm, primary heart field, and secondary heart field, respectively, these marker expressions overlap each other [21]. For example, islet1 is also expressed in $Flk1^+$ mesoderm, and Nkx2.5 is expressed in both heart fields. These progenitor populations should therefore be partially overlapping, and the relationship among them should be further clarified. In human ES cells, KDR (human Flk1)$^+$ cells and $Isl1^+$ cells were independently reported to be multipotent cardiovascular progenitors [22,23]. As for cardiac stem cells, some reports show clonal potential of tissue-derived cardiac stem cells, such as c-kit^+ cells or cardiac side population cells [24]. Though expansion of ES cell-derived progenitor cells was reported with a sphere [25] or feeder cell methods [23], the establishment of ES cell-derived cardiac stem cells has not been completely demonstrated.

Cardiomyocyte induction from mouse iPS cells was first reported in 2008 [4,26,27]. Cardiomyocytes could be induced from mouse iPS cells with similar methods from mouse ES cells with EBs or stepwise methods. Various cardiovascular cells, cardiomyocytes, arterial, venous, and lymphatic ECs, and blood cells, were systematically induced from $Flk1^+$ progenitor cells [4]. Comparable levels of cardiovascular cells could be induced from iPS cells and ES cells. As for human iPS cells, cardiomyocyte induction using EB methods was reported for the first time in 2009 [28]. Though functional analyses of induced cardiomyocytes suggest that human cardiac cell models could be established from human iPS cells, induction efficiency and stability are still not sufficient, especially for cell therapy purpose. Further improvements for more robust induction methods are still required.

Application to cell transplantation

As establishment of human iPS cells from human tissues can avoid the legal and ethical controversy over human ES cells, iPS cells are now one of the most promising cell sources for cardiac regenerative cell therapy. Nevertheless, many hurdles have yet to be overcome before the realization of cardiac regeneration by iPS cells.

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There are various cardiovascular lineage cells. What cells are suitable for cardiac regeneration? Cardiomyocytes? Cardiac progenitors? More specific cardiac cells such as pacemaker cells? Vascular cells? Mesenchymal cells? Alternatively, a mixture of different cell types? Many studies are now ongoing all over the world. A large-scale preparation and injection of human ES cell-derived cardiomyocytes (10^7 cells) are reported to be able to ameliorate cardiac function [29]. FCV cardiac progenitor cells from mouse ES cells were shown to efficiently generate cardiomyocytes after cell transplantation [21]. Recently, importance of non-cardiomyocytes in cardiac regeneration is highlighted as a source of various humoral factors that help cardiac regeneration in a paracrine fashion [30]. Transplantation of cardiomyocyte/non-cardiomyocyte mixtures may be a good strategy for efficient regeneration. Novel transplantation technologies such as cardiosphere (cardiac cell aggregates) [31] and cardiac cell sheets using temperature-responsive culture surface [32], would support mixture transplantation strategies. Cardiovascular progenitors, which can efficiently give rise to cardiomyocytes as well as endothelial cells and mural cells, should be a good cell source for mixture strategies. In addition to such technical hurdles, elimination of undifferentiated ES/iPS cells to avoid teratoma formation is, by far the most critical issue for the safety of ES/iPS cell therapy. Recently, an iPS cell-specific feature in the teratoma-forming propensity was reported, where some iPS cell lines showed a "differentiation-resistant" phenotype [33]. In a particular iPS cell line, which may be incompletely reprogrammed, undifferentiated cells persist even after induction of differentiation resulting in teratoma formation after transplantation. Establishment of standard for safe iPS cells would be critical to develop iPS cell therapy.

Drug discovery

iPS cell technology, which enables the establishment of patient-specific pluripotent stem cells and patient cell models, brings two new hopes in drug discovery. One is in vitro screening for adverse or toxic effects of drugs. The other is discovering new drugs for currently incurable diseases.

Safety test

Establishment of cardiac cell models from human iPS cells offers novel tools for drug safety test. QT elongation is a critical adverse effect caused by inhibition of human ERG (HERG) ion channel. Currently, the so-called HERG test, in which inhibitory effects of chemical substances are evaluated with HERG-overexpressed cell lines (such as HEK293), is mainly used for safety screening of QT elongation. HERG test often shows false negative results, that is, though HERG test is negative, QT elongation occurs in patients (ex. dl-sotarol). When inhibitory effects of substances on HERG current were evaluated using human cardiomyocytes prepared from human ES/iPS cells in vitro, the in vitro results precisely reflected in vivo QT elongation [34]. Human cell models, thus, are potent tool for drug safety test which may drastically simplify and facilitate drug development.

Cardiac regenerative drugs

It would be ideal if cardiac regeneration could be achieved with drugs. Some trials to discover small molecules which promote cardiomyocyte differentiation are being performed using ES cell differentiation systems. Cardiogenol, ascorbic acid, isoxazolyl-serines, sulfonyl hydrazones, and so on are reported to enhance cardiomyocyte differentiation from ES cells using EB methods [35]. Nevertheless, as these substances were added to EBs (or P19 carcinoma cell lines) from the initial step of differentiation, target cells or processes to which these substances act on are unclear. For example, cardiogenol and sulfonyl hydrazones should act on mesoderm induction stage. Differentiation-stage specific screening and evaluation using stepwise differentiation methods would be more powerful and suitable to discover cardiac regenerative drugs. Indeed, the authors recently demonstrated that an immunosuppressant, cyclosporin-A (CSA), showed a novel effect specifically acting on mesoderm cells to drastically increase cardiac progenitors (FCV cells) as well as cardiomyocytes by 10–20 times [21]. CSA had an effect on specific induction of cardiac lineage from mesoderm, that is, on cardiac commitment process. Drugs acting on the late stages of cardiac differentiation, such as cardiac commitment, differentiation, and cardiomyocyte proliferation, should be promising targets as cardiac regenerative drugs.

Novel perspective – epigenetic memory and iCM

Currently, iPS cells can be established from various cell types. Recent studies suggest that differentiation properties of iPS cells should be affected by their origin (personal communication). iPS cells established from blood cells have a tendency to well differentiate to blood cells but not to other lineages. This phenomenon suggests that epigenetic information as blood cells is still persistent in some degree as a kind of cell memory even after iPS cell derivation process, though the molecular identity of the epigenetic memory is still unknown. If iPS cells could be induced from cardiomyocytes, those iPS cells may be ideal for the efficient preparation of cardiomyocytes.

Recently, direct conversion of fibroblasts to functional neurons (iN cells) was reported by transduction of three defined transcription factors [36]. This result indicates that critical combination of transcription factors can induce and reproduce any kind of distinct cell types. Establishment of iN cells instantly prompts direct induction of cardiomyocytes from fibroblasts (iCM). iCM would offer another important option to research, cell therapy, and drug discovery toward cardiovascular regeneration. iPS cell technologies are still expanding, and will continue to bring various new hopes in cardiovascular regeneration.

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Convergence of Notch and β-catenin signaling induces arterial fate in vascular progenitors

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Molecular mechanisms controlling arterial–venous specification have not been fully elucidated. Previously, we established an embryonic stem cell differentiation system and demonstrated that activation of cAMP signaling together with VEGF induces arterial endothelial cells (ECs) from Flk1$^+$ vascular progenitor cells. Here, we show novel arterial specification machinery regulated by Notch and β-catenin signaling. Notch and GSK3β-mediated β-catenin signaling were activated downstream of cAMP through phosphatidylinositol-3 kinase. Forced activation of Notch and β-catenin with VEGF completely reconstituted cAMP-elicited arterial EC induction, and synergistically enhanced target gene promoter activity in vitro and arterial gene expression during in vivo angiogenesis. A protein complex with RBP-J, the intracellular domain of Notch, and β-catenin was formed on RBP-J binding sites of arterial genes in arterial, but not venous ECs. This molecular machinery for arterial specification leads to an integrated and more comprehensive understanding of vascular signaling.

Introduction

One of the earliest occurrences in organogenesis is the development of the vascular system. The vascular system is first formed as a primitive vascular network by differentiation and assembly of vascular progenitor cells. Molecular differences between arterial and venous endothelial cells (ECs) become apparent at this stage before circulation begins (Wang et al.,. 1998; Adams et al., 1999; Zhong et al., 2001). Although acquisition of arterial and venous EC identities from progenitors is a crucial step for establishing the complete circulation system, cellular and molecular processes that regulate arterial–venous specification are not fully elucidated.

Notch is a single-pass transmembrane receptor known for its function in controlling cell fate decisions and creating boundaries through cell–cell communication (Lai, 2004). Ligand binding to Notch leads to cleavage and release of the Notch intracellular domain (NICD), and NICD translocates to the nucleus and associates with the transcription factor RBP-J (also called CSL, CBF-1 in mammals, Suppressor of Hairless [Su(H)] in *Drosophila*, and LAG-1 in *Caenorhabditis elegans*; Christensen et al., 1996; Kidd et al., 1998; Artavanis-Tsakonas et al., 1999). Notch (Notch 1, 4) and its cell surface ligands (Delta like-1 [Dll1], Dll4, and Jagged1 and 2) are expressed in arteries but not in veins (Villa et al., 2001; Sörensen et al., 2009). Genetic studies of Notch signaling components have shown that these arterial EC markers are essential for proper formation of the developing vasculature (Xue et al., 1999; Lawson et al., 2001; Duarte et al., 2004; Krebs et al., 2004). Thus, Notch signaling plays an important role in arterial specification.

Wnt/β-catenin signaling regulates embryogenesis and is involved in the pathogenesis of a variety of diseases (Nusse, 2005; Clevers, 2006; Grigoryan et al., 2008). Recent studies suggested that Wnt/β-catenin signaling also plays a key role in vascular biology (Goodwin and D'Amore, 2002). Mice deficient for Wnt2 displayed vascular abnormalities including

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Abbreviations used in this paper: CA, constitutive active; ChIP, chromatin immunoprecipitation; CXCR4, CXC chemokine receptor 4; Dll, Delta like; DN, dominant negative; Dox, doxycycline; EC, endothelial cell; ER, estrogen receptor; ES, embryonic stem; Flk1, fetal liver kinase 1; GSK3, glycogen synthase kinase 3; NICD, Notch intracellular domain; NRP, neuropilin; OHT, 4-hydroxytamoxifen; PI3K, phosphatidylinositol-3-kinase; RBP-J, recombination signal sequence binding protein J; TCF4, transcription factor 4; Tet-Off, tetracycline-regulatable system.

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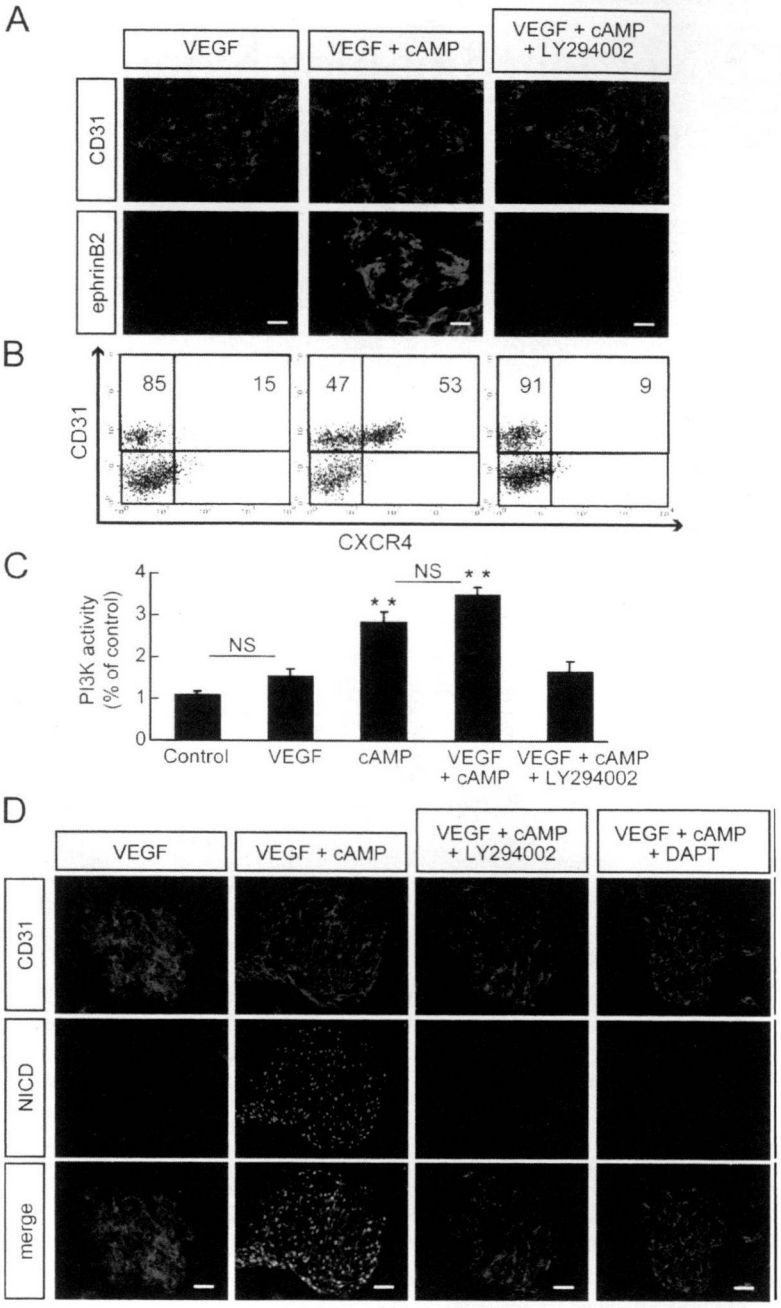

Figure 1. **Inhibitory effect of PI3K inhibitor LY294002 on arterial EC induction from Flk1$^+$ cells.** (A) Double-fluorescent staining for CD31 and ephrinB2 after a 3-d culture of Flk1$^+$ cells (Flk-d3). Top panels: CD31 (pan-ECs, red) and DAPI (blue). Bottom panels: EphB4-Fc (ephrinB2$^+$ arterial ECs, green) and DAPI (blue). Flk1$^+$ cells stimulated with VEGF alone (50 ng/ml; left panels), VEGF and 8bromo-cAMP (0.5 mM; middle panels), or VEGF, 8bromo-cAMP, and a PI3K inhibitor, LY294002 (7.5 µM; right panels). Bars: 100 µm. (B) 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. (C) PI3K activity at Flk-d3. Flk1$^+$ cells stimulated with vehicle, VEGF, 8bromo-cAMP, VEGF and 8bromo-cAMP, or VEGF, 8bromo-cAMP, and LY294002 (7.5 µM; $n = 3$; **, $P < 0.01$ vs. vehicle; NS: not significant). (D) Double-fluorescent immunostaining for cleaved Notch intracellular domain (NICD) and CD31 at Flk-d3. Left panels, CD31 (pan-ECs, red). Middle panels, cleaved NICD (green). Right panels, merged image. Flk1$^+$ cells stimulated with VEGF alone, VEGF and 8bromo-cAMP, VEGF, 8bromo-cAMP and LY294002, or VEGF, 8bromo-cAMP, and a γ-secretase inhibitor, DAPT (2.5 µM). Bars: 100 µm.

defective placental vasculature (Monkley et al., 1996). Knockout mice for the Wnt receptor gene, Frizzled-5, died in utero due to defects in yolk sac angiogenesis (Ishikawa et al., 2001). Defects of the β-catenin gene in ECs caused aberrant vascular patterning and increased vascular fragility (Cattelino et al., 2003). Nevertheless, the role of Wnt/β-catenin signaling in arterial–venous development is unknown.

We previously demonstrated that Flk1$^+$ (also designated as VEGF receptor 2) cells derived from embryonic stem (ES) cells serve as vascular progenitors and can constructively reproduce the early vascular organization processes including differentiation of both endothelial and mural cells (MCs; vascular smooth muscle cells and pericytes) and vascular structure formation (Yamashita et al., 2000; Yamamizu et al., 2009). We also reported that activation of the adrenomedullin/cAMP pathway induced differentiation of arterial ECs from Flk1$^+$ cells. Activation of the cAMP pathway induced Notch activation in differentiating ECs. Although inhibition of Notch signaling abolished arterial EC induction, activation of Notch using a NICD–estrogen receptor fusion protein together with VEGF treatment did not induce arterial ECs (Yurugi-Kobayashi et al., 2006). These results indicated that Notch signaling is essential but not sufficient for arterial EC induction, suggesting that other factors are involved in this process.

In this study, we investigated signal transduction events downstream of the cAMP pathway with the use of our ES cell differentiation system and found novel molecular machinery for

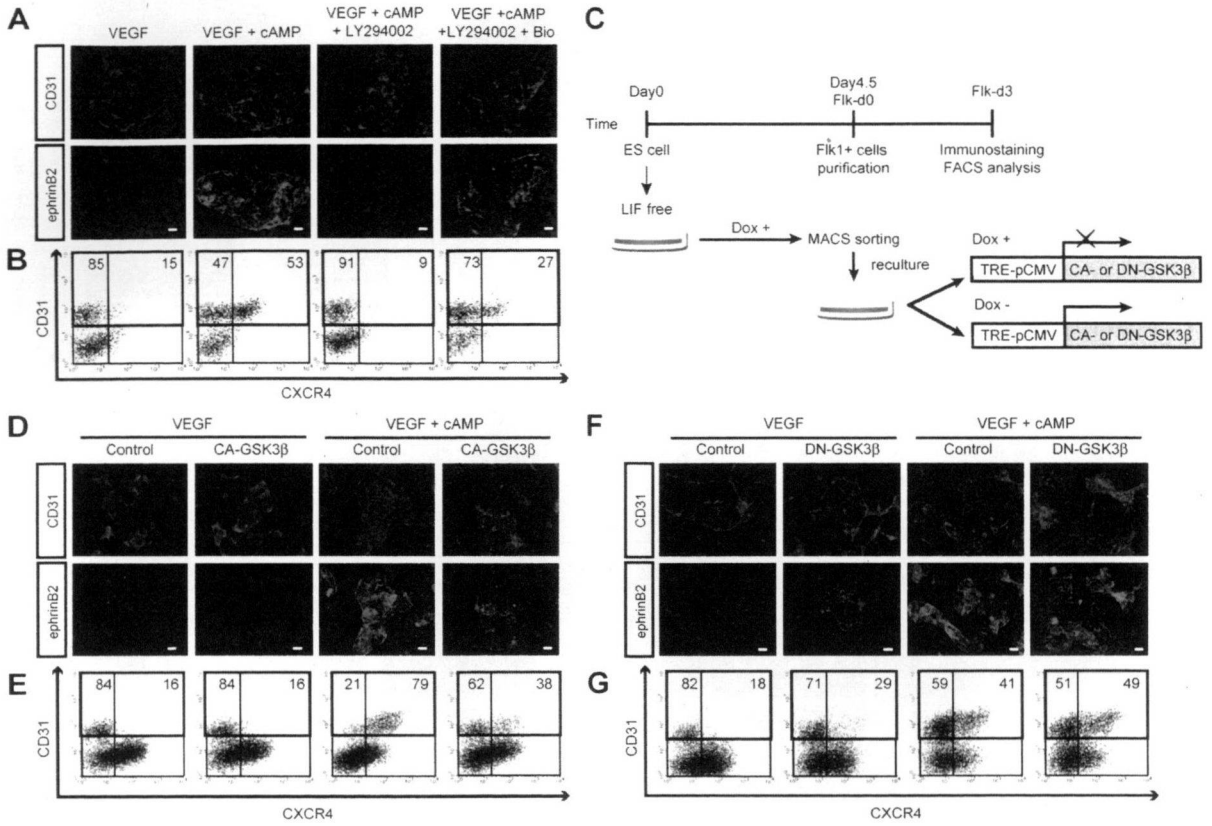

Figure 2. **Inhibitory effect of GSK3β on arterial EC differentiation.** (A) Double-fluorescent staining for CD31 and ephrinB2 at Flk-d3. Top panels, CD31 (pan-ECs, red) and DAPI (blue). Bottom panels, EphB4-Fc (ephrinB2$^+$ arterial ECs, green) and DAPI (blue). Flk1$^+$ cells stimulated with VEGF alone (50 ng/ml), VEGF and 8bromo-cAMP (0.5 mM), VEGF, 8bromo-cAMP, and LY294002 (7.5 μM), or VEGF, 8bromo-cAMP, LY294002, and a GSK3β inhibitor, Bio (100 nM). Bars: 100 μm. (B) 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. (C) Experimental system for GSK3β expression. ES cell line expressing constitutive active (CA) form or dominant-negative (DN) form of GSK3β by tetracycline-inducible expression system (Tet-Off) were established. Doxycycline (Dox) was added during the first 4.5 d of culture of ES cell differentiation to Flk1$^+$ cells. Subsequently, Flk1$^+$ cells were sorted by MACS and plated on type IV collagen-coated dishes, and cells were cultured in the presence or absence of 1 μg/ml Dox. (D and E) Induction of CA-GSK3β. (F and G) Induction of DN-GSK3β. (D and F) Double-fluorescent staining for CD31 and ephrinB2 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 cultured with VEGF alone, or VEGF and 8bromo-cAMP, in the presence or absence of Dox. Bars: 200 μm. (E and G) 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.

arterial EC induction. That is, not single, but dual activation of Notch and β-catenin signaling together with VEGF successfully reconstituted arterial EC induction from vascular progenitors. RBP-J, NICD, and β-catenin formed a protein complex specifically in arterial but not venous ECs both from ES cells and in vivo. Moreover, dual induction of NICD and β-catenin enhanced promoter activity of target genes in vitro and arterial gene expression during in vivo angiogenesis in adults. Thus, Notch and β-catenin signaling converge via formation of a single protein complex which should form a core molecular machinery that induces arterial fate in ECs.

Results

PI3K is involved in arterial EC induction downstream of cAMP

Previously, we reported that two vascular cell types, ECs (positive for CD31/vascular endothelial [VE]–cadherin/endothelial nitric oxide synthase [eNOS]/Claudin5) and MCs (positive for smooth muscle actin [SMA]/Calponin/SM22α), were selectively induced (Yamashita et al., 2000; Yamamizu et al. 2009) when purified ES cell–derived Flk1$^+$ cells were cultured with VEGF and serum. Under these culture conditions, only these two cell types (i.e., ECs and MCs), but not blood cells such as CD45$^+$ cells, were specifically induced (Yamamizu et al., 2009). Most of the CD31$^+$ ECs induced with VEGF and serum had a venous phenotype, which did not express ephrin B2, an arterial EC marker. Simultaneous stimulation of VEGF and cAMP signaling by addition of a cAMP analogue, 8bromo-cAMP, successfully induced ephrinB2$^+$ arterial ECs, indicating that the cAMP pathway regulates arterial EC induction (Fig. 1 A; Yurugi-Kobayashi et al., 2006).

We then investigated the downstream targets of cAMP. First we examined various kinase inhibitors (Fig. S1). Among them, LY294002, a phosphatidylinositol-3 kinase (PI3K) inhibitor, potently and specifically inhibited the cAMP-elicited induction of ephrinB2$^+$ arterial ECs (ephrinB2$^+$/CD31$^+$), but not total EC (CD31$^+$) appearance from Flk1$^+$ progenitor cells

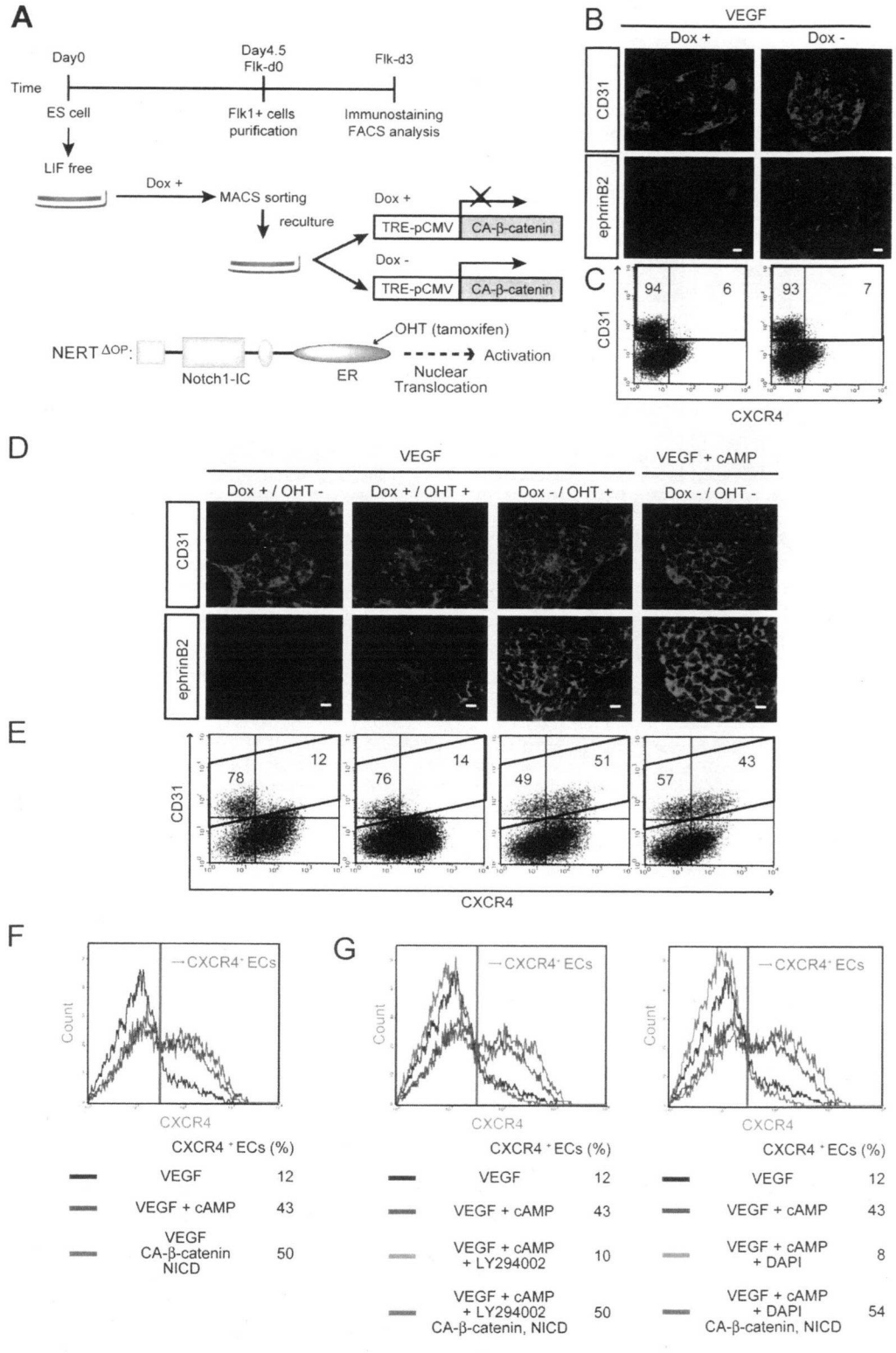

Figure 3. **Arterial EC induction by dual activation of β-catenin and Notch signaling.** (A) Experimental system for dual activation of Notch and β-catenin signaling. ES cell lines carrying CA-β-catenin regulated by Tet-Off system, and a fusion protein of N1ICD and estrogen receptor (ER), $NERT^{\Delta OP}$, 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.

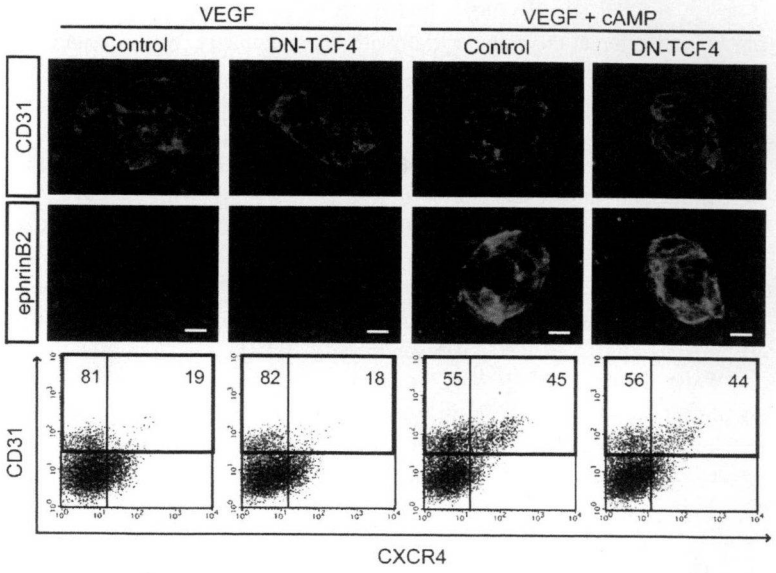

Figure 4. **Effects of DN-TCF4 on arterial EC induction from Flk1$^+$ cells.** (Top) Double-fluorescent staining for CD31 and ephrinB2 at Flk-d3. CD31 panels, CD31 (pan-ECs, red) and DAPI (blue). ephrinB2 panels, EphB4-Fc (ephrinB2$^+$ arterial ECs, green) and DAPI (blue). Flk1$^+$ cells induced from 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

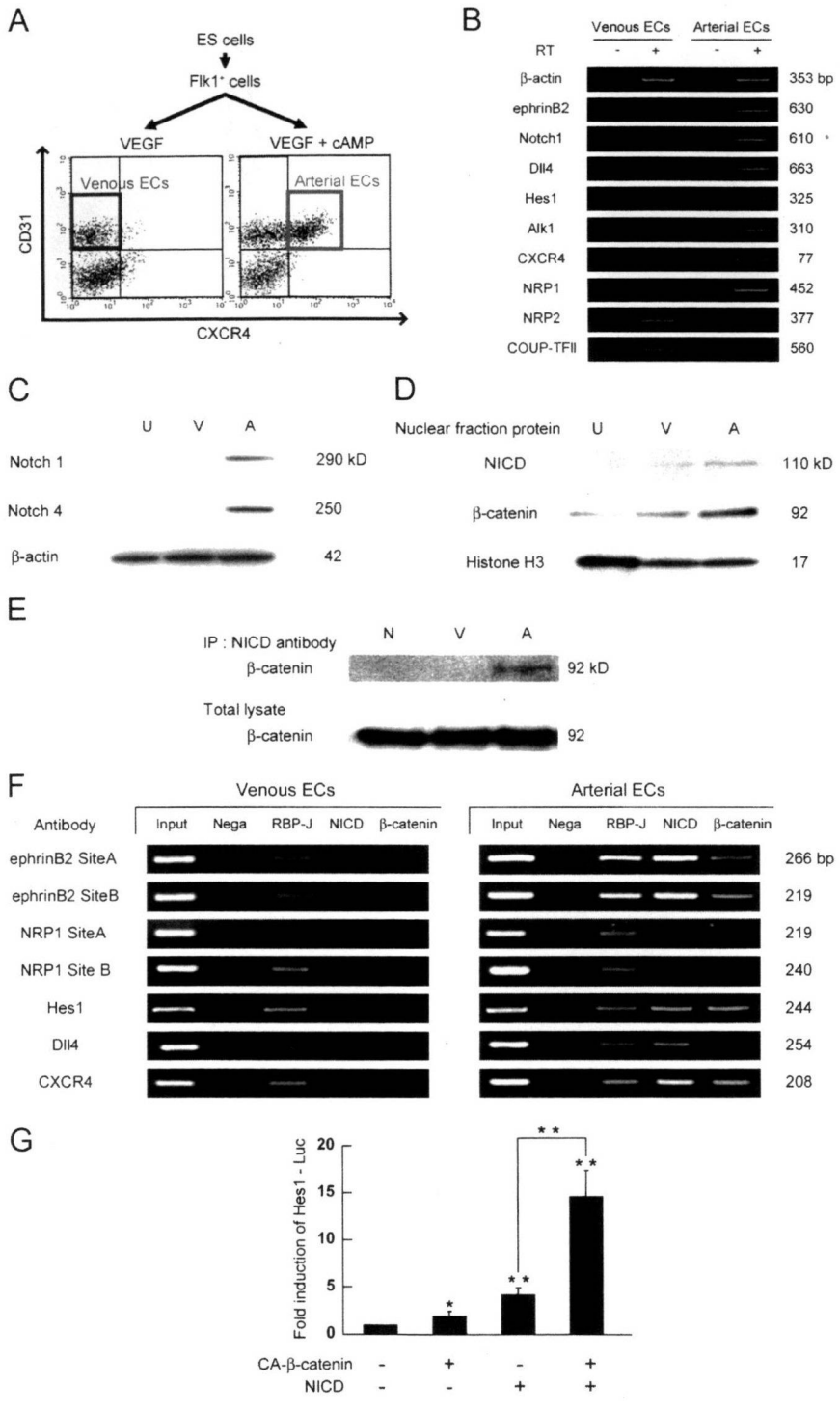

Figure 5. **Arterial-specific formation of protein complex with RBP-J, NICD, and β-catenin.** (A) Purification of arterial and venous ECs from ES cells. CXCR4$^+$/CD31$^+$ cells at Flk-d3 induced by VEGF (50 ng/ml) with 8bromo-cAMP (0.5 mM) and CXCR4$^-$/CD31$^+$ cells induced by VEGF alone were purified 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

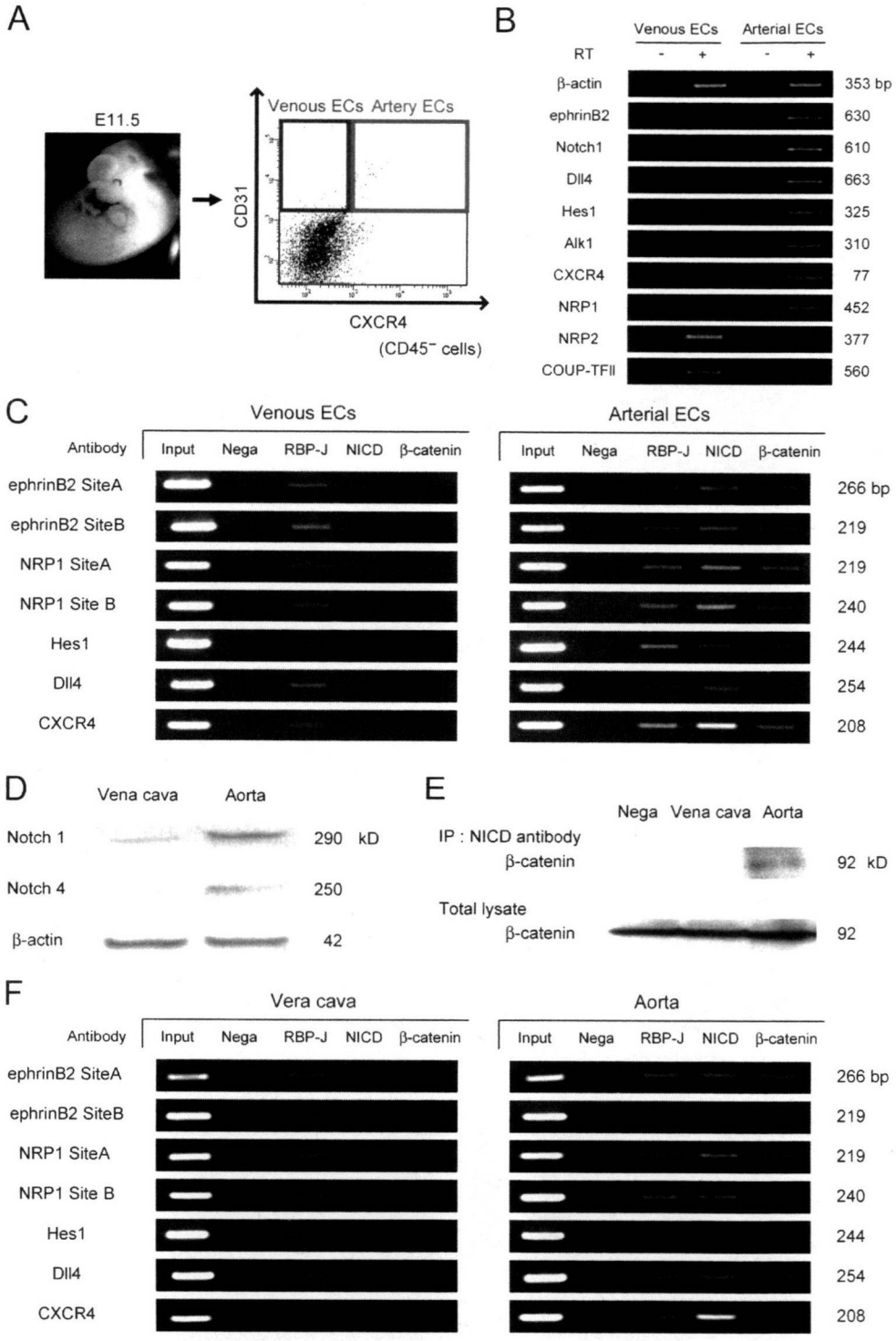

Figure 6. **Formation of the arterial protein complex in the embryo and adult vessels.** (A) Purification of arterial and venous ECs from the mouse embryo. Arterial ECs ($CXCR4^+/CD31^+/CD45^-$) and venous ECs ($CXCR4^-/CD31^+/CD45^-$) were isolated from E11.5 embryos. (B) RT-PCR for mRNA expression 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.

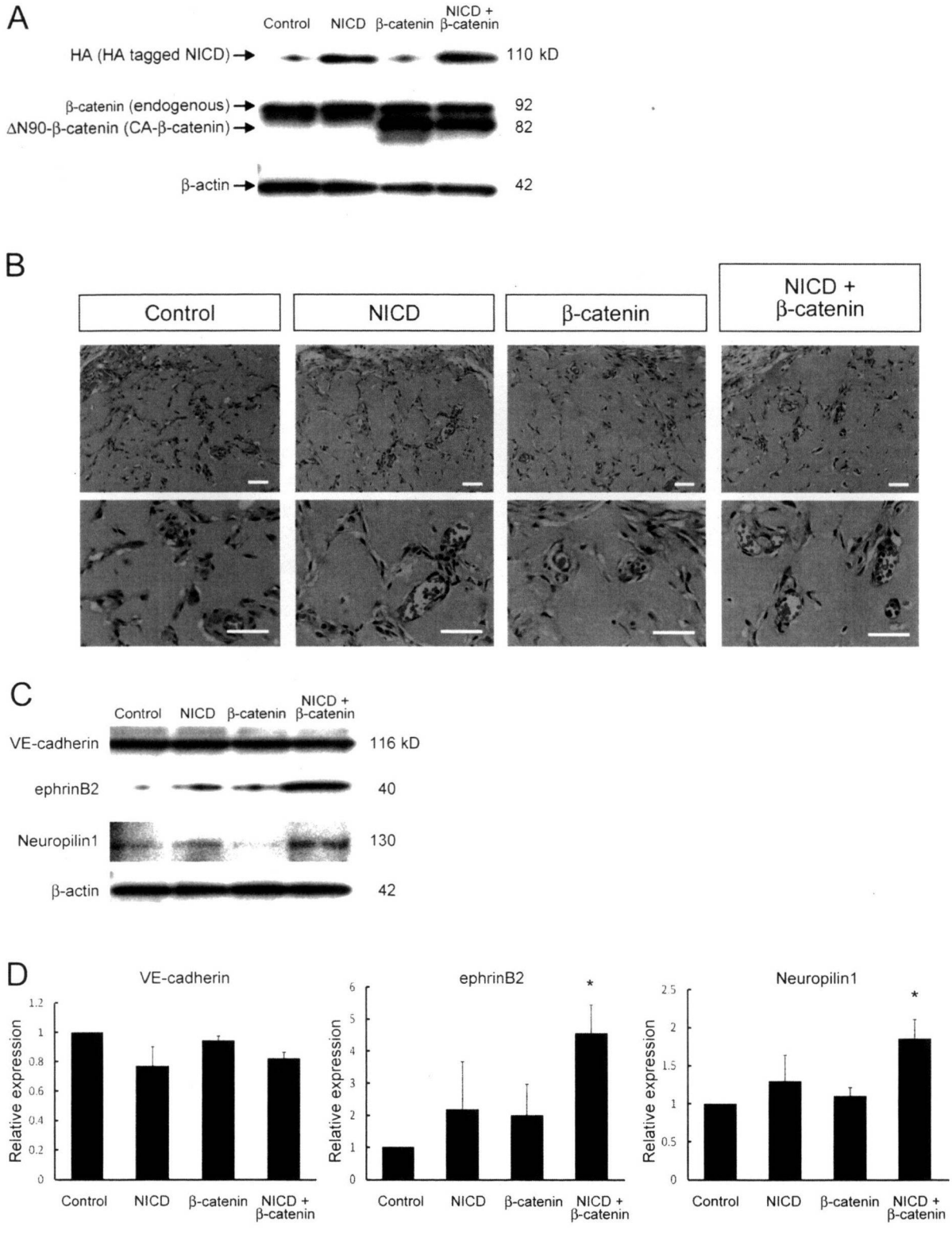

Figure 7. **Enhancement of arterial gene expression through dual activation of Notch and β-catenin during in vivo angiogenesis.** Matrigel containing VEGF (100 ng/ml), heparin (10 units/ml), and adenoviral vectors (vehicle [control], HA-tagged N1ICD [NICD], and/or CA-β-catenin) were injected subcutaneously 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).

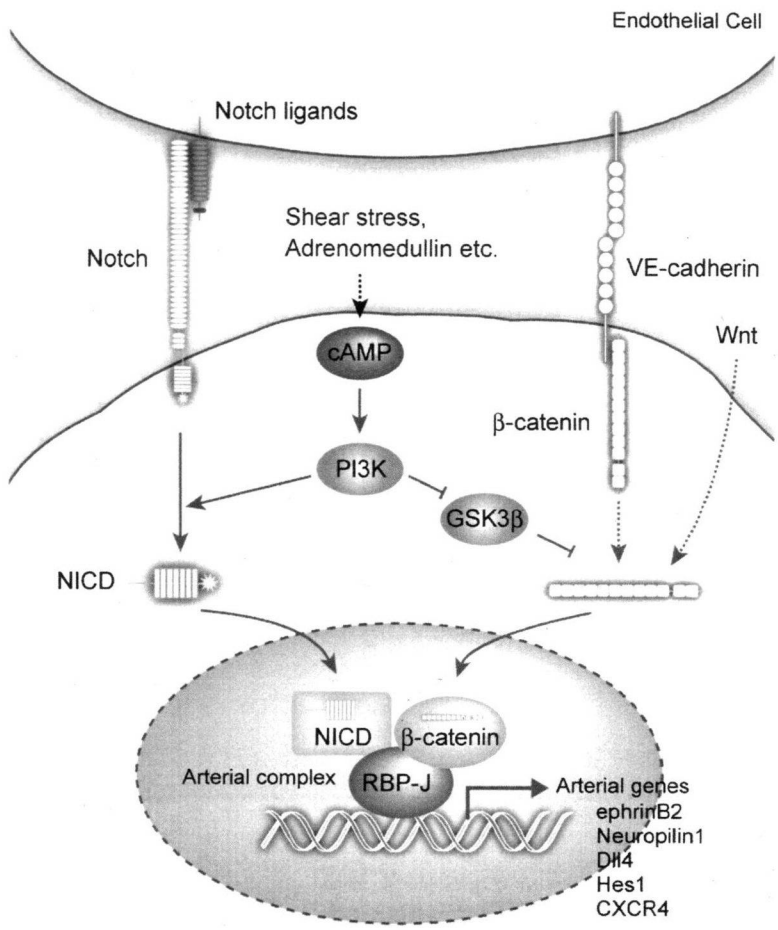

Figure 8. **Molecular mechanisms of arterial EC specification.** cAMP signaling, which could be induced by adrenomedullin, shear stress, and so on, activates Notch and β-catenin signaling through PI3K (and GSK3β) in vascular progenitors (as well as differentiating ECs). Notch and β-catenin signaling 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