基礎研究から臨床応用に至る幅広い知見が蓄積されてきた。なかでも血管は，骨髄細胞や末梢血細胞の虚血組織への移植，という細胞移植治療がすでに臨床の場にも応用され成果をあげており，近年の再生医療の発展において先駆的役割を果たしている。本稿はその中で万能の幹細胞として期待されるES細胞および最近樹立された新しい多能性幹細胞iPS細胞の血管分化とその応用に関する知見を中心に概説する。

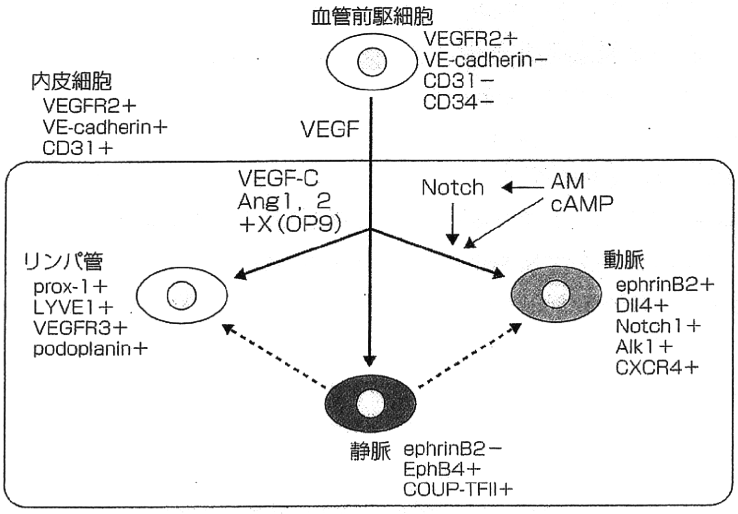
ES細胞由来血管前駆細胞とその分化

筆者らはES細胞由来VEGFR2（2型血管内皮増殖因子受容体；Flk1）陽性細胞が，血管を構成する細胞である血管内皮細胞と血管壁細胞（血管平滑筋細胞およびペリサイト）の共通の前駆細胞であり，VEGFR2陽性細胞から内皮細胞および壁細胞の双方が分化誘導でき，毛細血管様の高次構造を培養下に形成できることを示した[1]。VEGFR2陽性の血管前駆細胞は，VEGF（血管内皮増殖因子）の刺激により内皮細胞に，主にPDGF-BB（血小板由来増殖因子）により壁細胞に分化すると考えられる。また，血流による物理的刺激であるshearストレスや拍動性進展刺激がVEGFR2陽性細胞からの内皮細胞分化や壁細胞分化を誘導することも明らかにされている。

最近，動脈，静脈，リンパ管それぞれの内皮細胞特異的に発現している分子が数々報告され，内皮細胞の多様性に分子的根拠が与えられるようになってきた[2]。筆者らは最近，VEGFR2陽性細胞からの血管分化系を用い，ephrinB2陽性（動脈）内皮，ephrinB2陰性（静脈）内皮，およびprox-1陽性（リンパ管）内皮細胞と考えられる細胞の誘導と純化にそれぞれ成功した[3][4]。すなわち，VEGFR2陽性細胞をVEGFおよび血清存在下に内皮細胞に誘導するとほとんど（＞90～95％）の内皮細胞がephrinB2陰性の静脈内皮細胞となる。VEGFに加えて，cAMPアナログである8bromo-cAMPまたは細胞内cAMPを上昇させる液性因子の1つであるアドレノメデュリン（AM）を加えcAMP経路を活性化することにより，内皮細胞においてNotchシグナルの活性化が誘導され，ephrinB2陽性の動脈内皮細胞が誘導される。また一方，VEGFR2陽性細胞をOP9ストローマ細胞上で培養して内皮細胞を誘導したところ，prox-1陽性リンパ管内皮細胞が出現した。このOP9によるリンパ管誘導作用は，VEGF-Cおよびangiopoietinの作用をブロックすることによりほぼ完全に阻害された。これらの結果により，ES細胞を用いて，動脈，静脈，リンパ管内皮細胞のすべてを系統的に分化誘導することが可能になるとともにその新たな分化メカニズムが明らかになった[5]（図1）。こうした新たなアプローチで血管分化多様化機構を解析することにより，動脈特異的血管新生やリンパ管特異的新生抑制による抗癌治療などの開発も期待される。さらに詳細に臓器特異的な血管の多様性を解析し理解することは，血管を介した臓器機能や病態の理解とそれに基づくさまざまな新しい治療戦略の開拓に結びつくと考えられる。

ES細胞による血管再生

筆者らは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[6]。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VEGFR2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VEGFR2陽性血管前駆細胞と，VEGFR2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。VEGFR2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，内皮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，ほとんどすべての細胞（95％以上）が内皮細胞として血管に寄与していた。また，VEGFR2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



VEGFR2(Flk1)陽性血管前駆細胞は，主にVEGFのシグナルによりVE-カドヘリン陽性内皮細胞に分化する。静脈内皮細胞と考えられる細胞はVEGFおよび血清のみで誘導されるが，動脈内皮分化にはそれに加えてNotchおよびcAMPシグナルが，リンパ管内皮にはVEGF-C，angiopoietinとOP9細胞由来因子がそれぞれ必要である。動脈内皮およびリンパ管内皮は，静脈内皮(または厳密にはどれにも当てはまらないプロトタイプ内皮細胞？)からそれぞれ分化する可能性もある。

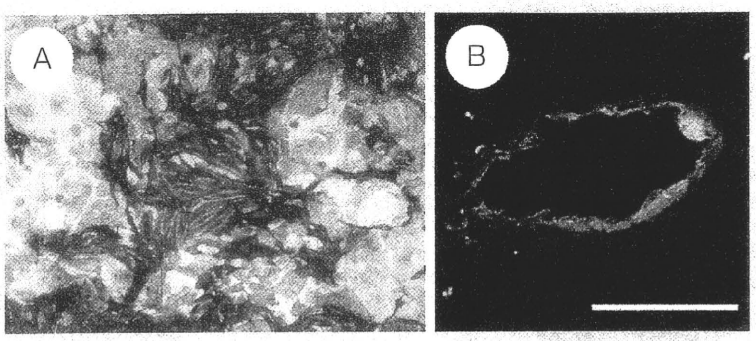
図1 VEGFR2陽性細胞からの動静脈リンパ管内皮分化

められた。これらの結果より，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合わ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。

血管再生治療においては，倫理面・安全面・技術面でハードルが低い骨髄細胞や末梢血，G-CSFなどの薬剤を用いた血管新生治療が先行して行われ，優れた効果をあげている。心筋や神経と異なり，既存の組織からの新生が可能な血管においては，細胞による純粋な再生は必ずしも必要ではなく，ES細胞治療がこれらの治療を凌駕して有用であるという知見は今のところない。しかし将来にわたっては，直接的な細胞移植治療のみならずさまざまな血管再生治療のターゲットとなる新たなシーズを生み出し，血管特異的血管新生なども含めさらに治療法を精緻に改善向上させていく上で，ES細胞の血管再生研究における意義は大きいと考えられる。

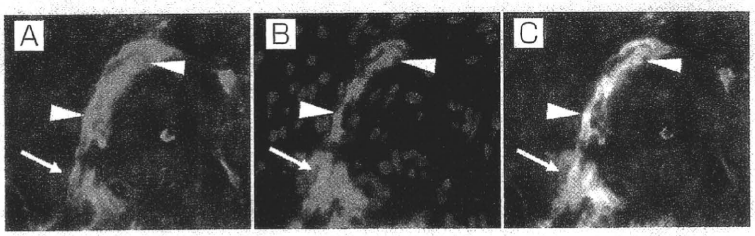
ヒトES細胞からの血管分化再生

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VEGFR2陽性細胞からの内皮細胞・壁細胞の分化[7]，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(図2)[8][9]。また細胞移植の際に，純化した誘導内皮細胞のみを移植するよりも同時に誘導される壁細胞と混在した形で移植するほうが血管再生効果が高いことを見い出している。同様の現象はES細胞由来心筋細胞移植の動物実験においても，純化



A：ヒトES細胞から誘導されたVEGFR2陽性細胞をFACSにより単離した後，Ⅳ型コラーゲン上で，VEGFおよび血清存在下に培養すると，CD31陽性内皮細胞（紫）と平滑筋αアクチン陽性血管壁細胞（茶）が分化誘導される。
B：ヌードマウス虚血肢に移植されたヒトES細胞由来内皮細胞は成体内血管再生に寄与する。全内皮細胞（isolectin染色：緑）。ヒトES細胞由来内皮細胞（DiI染色：赤）。Bar=50μm

図2　ヒトES細胞からの血管細胞分化（➔巻頭Color Gravure参照）



ヌードマウスの腫瘍周囲に移植されたマウスiPS細胞由来血管細胞は成体内再生に寄与する。A：CD31陽性内皮細胞（緑），B：iPS細胞由来細胞（赤），C：合成画像。核（青）。iPS細胞由来CD31陽性内皮細胞（矢頭）およびそれに隣接してiPS細胞由来CD31陰性壁細胞（矢印）が認められる。

図3　マウスiPS細胞由来血管細胞による生体内血管新生（➔巻頭Color Gravure参照）

心筋のみではなく，非心筋細胞と混在させたほうが心筋再生効率が良いことが報告されている。標的細胞に分化する前駆細胞のみならず，その分化や増殖，生存などを支える周囲の間質細胞の意義についても十分に考慮に入れる必要性が示唆される。このようにヒト細胞において新たな血管分化再生機構が明らかになることにより，より治療応用に結びついた新たな知見が生まれることが期待される。

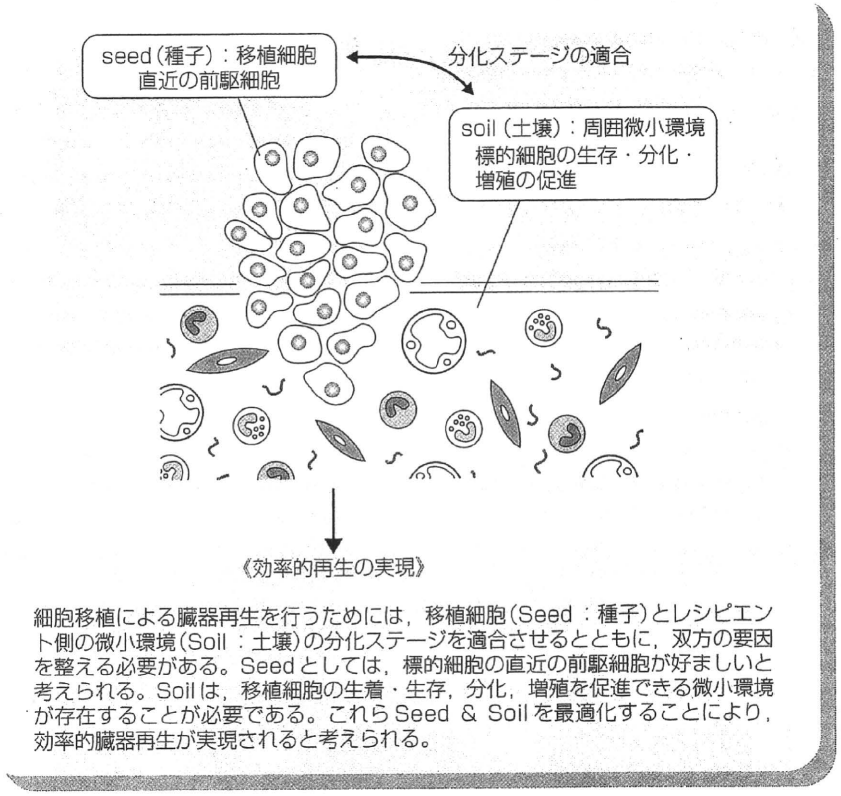
iPS細胞からの血管分化再生

iPS細胞は，線維芽細胞など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[10)11)]。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。前述したマウス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三次元的形成にも成功した[12)]。また，担癌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うることも確認した（**図3**）。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1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も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細胞からの三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[13]。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。

今後の可能性

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は発展途上にあると考えられる。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・組織間相互作用など臓器としての機能を果たしうる機能ユニットを形成するために必要な要素すべ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。有効に分化しうる幹・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4）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くと考えられる。



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小環境（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小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4　Seed & Soil therapy

また，iPS細胞の出現は，これまでのES細胞をはじめとする再生医学研究に数多くの新たな可能性を与えた。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細胞研究がもっていたポテンシャルがさまざまなかたち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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Induction and Enhancement of Cardiac Cell Differentiation from Mouse and Human Induced Pluripotent Stem Cells with Cyclosporin-A

Masataka Fujiwara[1,2], Peishi Yan[1,3¤], Tomomi G. Otsuji[4,5], Genta Narazaki[1,6], Hideki Uosaki[1,6], Hiroyuki Fukushima[1,6], Koichiro Kuwahara[2], Masaki Harada[2], Hiroyuki Matsuda[7], Satoshi Matsuoka[7], Keisuke Okita[8], Kazutoshi Takahashi[8], Masato Nakagawa[8], Tadashi Ikeda[3], Ryuzo Sakata[3], Christine L. Mummery[9], Norio Nakatsuji[10,11], Shinya Yamanaka[8,12], Kazuwa Nakao[2], Jun K. Yamashita[1,6]*

1 Laboratory of Stem Cell Differentiation, Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan, **2** Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto, Japan, **3** Department of Cardiovascular Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan, **4** Stem Cell and Drug Discovery Institute, Kyoto Research Park, Kyoto, Japan, **5** Laboratory of Embryonic Stem Cell Research, Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan, **6** Department of Cell Growth and Differentiation, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan, **7** Department of Physiology and Biophysics, Kyoto University Graduate School of Medicine, Kyoto, Japan, **8** Department of Reprogramming Science, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan, **9** Department of Anatomy and Embryology, Leiden University Medical Centre, Leiden, the Netherlands, **10** Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan, **11** Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Kyoto, Japan, **12** Gladstone Institute of Cardiovascular Disease, San Francisco, California, United States of America

Abstract

Induced pluripotent stem cells (iPSCs) are novel stem cells derived from adult mouse and human tissues by reprogramming. Elucidation of mechanisms and exploration of efficient methods for their differentiation to functional cardiomyocytes are essential for developing cardiac cell models and future regenerative therapies. We previously established a novel mouse embryonic stem cell (ESC) and iPSC differentiation system in which cardiovascular cells can be systematically induced from Flk1$^+$ common progenitor cells, and identified highly cardiogenic progenitors as Flk1$^+$/CXCR4$^+$/VE-cadherin$^-$ (FCV) cells. We have also reported that cyclosporin-A (CSA) drastically increases FCV progenitor and cardiomyocyte induction from mouse ESCs. Here, we combined these technologies and extended them to mouse and human iPSCs. Co-culture of purified mouse iPSC-derived Flk1$^+$ cells with OP9 stroma cells induced cardiomyocyte differentiation whilst addition of CSA to Flk1$^+$ cells dramatically increased both cardiomyocyte and FCV progenitor cell differentiation. Spontaneously beating colonies were obtained from human iPSCs by co-culture with END-2 visceral endoderm-like cells. Appearance of beating colonies from human iPSCs was increased approximately 4.3 times by addition of CSA at mesoderm stage. CSA-expanded human iPSC-derived cardiomyocytes showed various cardiac marker expressions, synchronized calcium transients, cardiomyocyte-like action potentials, pharmacological reactions, and ultra-structural features as cardiomyocytes. These results provide a technological basis to obtain functional cardiomyocytes from iPSCs.

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* E-mail: juny@frontier.kyoto-u.ac.jp

¤ Current address: Cardiovascular Department, Dalian Municipal Central Hospital, Dalian, China

Introduction

Induced pluripotent stem cells (iPSCs) are novel pluripotent stem cells generated from adult tissues by reprogramming originally with transduction of a few defined transcription factors, such as Oct4, Sox2, Klf4, and c-myc [1], [2]. Establishment of iPSC lines from adult human tissue is facilitating development of cell transplantation-based regenerative strategies and establishment of patient-derived cells as disease models. Efficient differentiation and dissecting the differentiation mechanisms of target cells would significantly contribute to elucidate the pathophysiology of diseases and provide a platform for developing new therapeutic strategies for specific diseases through such as drug discovery [3], [4].

Cardiomyocytes are a major target of regenerative medicine. Although cardiomyocyte differentiation has been reported from various progenitor and adult cell sources (e.g. bone marrow, cardiac biopsies, adipose tissue, umbilical cord, mesenchymal cells, etc), overall, the efficiencies of functional cardiomyocyte appearance have been still variable (<1–5%) [5]. Pluripotent cells, embryonic stem cells (ESCs) and iPSCs have thus emerged as among the most promising stem cell sources for inducing

functional cardiomyocytes in vitro. Several induction and purification methods have been reported, starting with either mouse or human ESCs. These include stem cell aggregation in suspension and growth as embryoid bodies (EBs), co-culture with stroma cells, serum-free culture in differentiation medium, or hypoxic culture [6], [7], [8], [9], [10], [11]. Overall, the efficiency of cardiomycyte differentiation in human ESCs [6] should be still lower than in mouse ESCs [8], [11]. In view of the similarities between iPSCs and ESCs, most cardiomyocyte induction methods from iPSCs are based on those tried and tested in ESCs. Several groups have thus reported cardiomyocyte formation from mouse iPSCs using either EBs or stroma cell co-culture [12], [13], [14]. Recently, several reports on cardiomyocyte induction from human iPSCs appeared with based on EB formation though the efficiencies are still varied [15], [16], [17], [18], [19]. Other new methods robust in human iPSCs remain to be explored and maybe of particular value for preparation of transplantation cell sources as well as dissecting the differentiation mechanisms and drug discovery.

Previously, we developed a novel ESC differentiation system that recapitulates early cardiovascular development in vivo [8], [20], [21]. Flk1 (also known as vascular endothelial growth factor (VEGF) receptor-2) is the earliest differentiation marker for endothelial cells (ECs) and blood cells, and is a marker of lateral plate mesoderm [21], [22]. We induced $Flk1^+$ cells from ESCs, purified them by fluorescence-activated cell sorting (FACS), and re-cultured the purified cells. We succeeded in inducing the major cardiovascular cell types from the common $Flk1^+$ progenitor cells: vascular ECs, mural cells (pericytes and vascular smooth muscle cells) [20] and cardiomyocytes [8]. When purified $Flk1^+$ cells were cultured on mouse bone marrow-derived stromal cells, OP9 cells, spontaneously beating cardiomyocytes as well as ECs can be induced within 3–4 days (Flk-d3-4) even from a single cell. We, thus, demonstrated that ESC-derived $Flk1^+$ cells serve as cardiovascular progenitors [8], [20], [23], which was further supported with following several mouse and human studies [9], [24], [25], [26]. We also identified a $Flk1^+$/$CXCR4^+$/vascular endothelial $cadherin^-$ (FCV) population as highly cardiogenic progenitor cells among the progeny of $Flk1^+$ mesoderm cells at the single cell level [8]. That is, in an intermediate stage of ESC differentiation between $Flk1^+$ mesoderm cells and cardiomyocytes (Flk-d2), purified FCV population could efficiently give rise to cardiomyocytes from a single cell. The cardiogenic potential of FCV cells was 15–20 times higher than that of other cell populations among the $Flk1^+$ cell progeny. We further confirmed FCV cells can differentiate into cardiomyocytes in vivo through cell transplantation experiments [11]. FCV cells, which are detected just 1–2 days before the cardiomyocyte appearance, are so far the nearest upstream cardiac progenitors to cardiomyocytes. This system proved amenable to induce various cardiovascular cells systematically from ESCs, explore novel differentiation methods, and dissect the differentiation processes [23], [27], [28]. Indeed, we recently succeeded in demonstrating that an immunosuppressant, cyclosporin-A (CSA) showed a novel potent effect specifically on $Flk1^+$ mesoderm cells to induce a dramatic increase in FCV cardiac progenitor cells and cardiomyocytes with the use of this ESC differentiation system [11]. That is, when CSA was added to $Flk1^+$ cells co-cultured on OP9 cells, appearance of FCV progenitor cells and cardiomyocytes were increased by 10–20 times.

Recently, we were able to systematically induce cardiovascular cells from mouse iPSCs in a way almost identical to that using mouse ESCs [12]. Here, we combined our technologies in ESCs and iPSCs and showed that FCV cardiac progenitors and cardiomyocytes were efficiently expanded from mouse iPSCs by CSA treatment. Moreover, we extended the CSA method to human iPSCs and showed that CSA also successfully worked in human iPSC differentiation and efficiently enhanced the appearance of spontaneously beating cells. Human iPSC-derived cardiomyocytes showed expected molecular, structural and functional features of human cardiomyocytes. We, thus, succeeded in inducing and enhancing cardiac cell differentiation from both mouse and human iPSCs.

Methods

Antibodies

Monoclonal antibodies (MoAbs) for murine E-cadhein (ECCD2), murine Flk1 (AVAS12) were prepared and labeled in our laboratory as described previously [8], [21], [29]. MoAb for cardiac troponin-T (cTnT) (1:2000) was purchased from Neo-Markers (Fremont, CA). For staining human ESCs and iPSCs, another MoAb for cTnT (1:100) was from Santa Cruz Biotechnology (Santa Cruz, CA). MoAbs for murine and human α-actinin (1:800) was from Sigma (St Louis, Mo). MoAb of phycoerithrin (PE)-conjugated AVAS12 was purchased from eBioscience (San Diego, CA). MoAbs for biotinylated-CXCR4 was purchased from BD Pharmingen (San Diego, CA). Anti-HCN4 (1:200) and anti-Cav3.2 (1:200) antibodies were from Chemicon (Temecula, CA). Anti-Kir2.1 (1:200) and anti-connexin 43 (1:200) antibodies were from Alomone (Israel) and Invitrogen (Carlsbad, CA), respectively.

Reagents

Cyclosporin-A (a gift from Novartis Pharma) was dissolved in Dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto Japan) at 30 mg/mL. Dilution of 1–3 μg/mL were made in differentiation medium at the time of use. PKH67 fluorescent dye was purchased from Sigma (St. Louis, MO).

Mouse iPSC culture

A germline-competent mouse iPSC line, 20D-17, carrying Nanog promoter-driven GFP/IRES/puromycin resistant gene (Nanog-iPS cells), was maintained as previously described [30]. Briefly, iPSCs were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 15% FCS, non-essential amino acids, 1 mmol/L sodium pyruvate, 5.5 mmol/L 2-mercaptoethanol, 50 units/mL penicillin and 50 mg/mL streptomycin on feeder layers of mitomycin-C-treated mouse embryonic fibroblast (MEF) cells carrying stably incorporated puromycin-resistance gene. OP9 stroma cells were maintained as described [21].

Induction of mouse cardiomyocyte differentiation

Induction of $Flk1^+$ cells and sorting for $Flk1^+$ cells were performed as previously described [8], [12], [20]. Briefly, mouse iPSCs were first plated on to gelatin-coated dishes and cultured for 30 min to eliminate attached feeder cells, then, non-adherent cells were collected and induced to differentiation. Mouse iPSCs were cultured at a density of $1–2.5\times10^3$ cells/cm^2 in differentiation medium (DM)(alpha minimum essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum) on type IV collagen-coated dishes (Biocoat, Beckton Dickinson) or mitomycin C-treated confluent OP9 cell sheets (MMC-OP9) for 96–108 h. Cells were collected and selected by FACS to purify $Flk1^+$ cells. $Flk1^+$ cells were then plated on to MMC-OP9 at a density of $1–10\times10^3$ cells/cm^2 and cultured in differentiation medium to induce cardiac differentiation. CSA (1–3 μg/mL) was added to $Flk1^+$ cells on OP9 cells. Medium was replaced every 2 days.

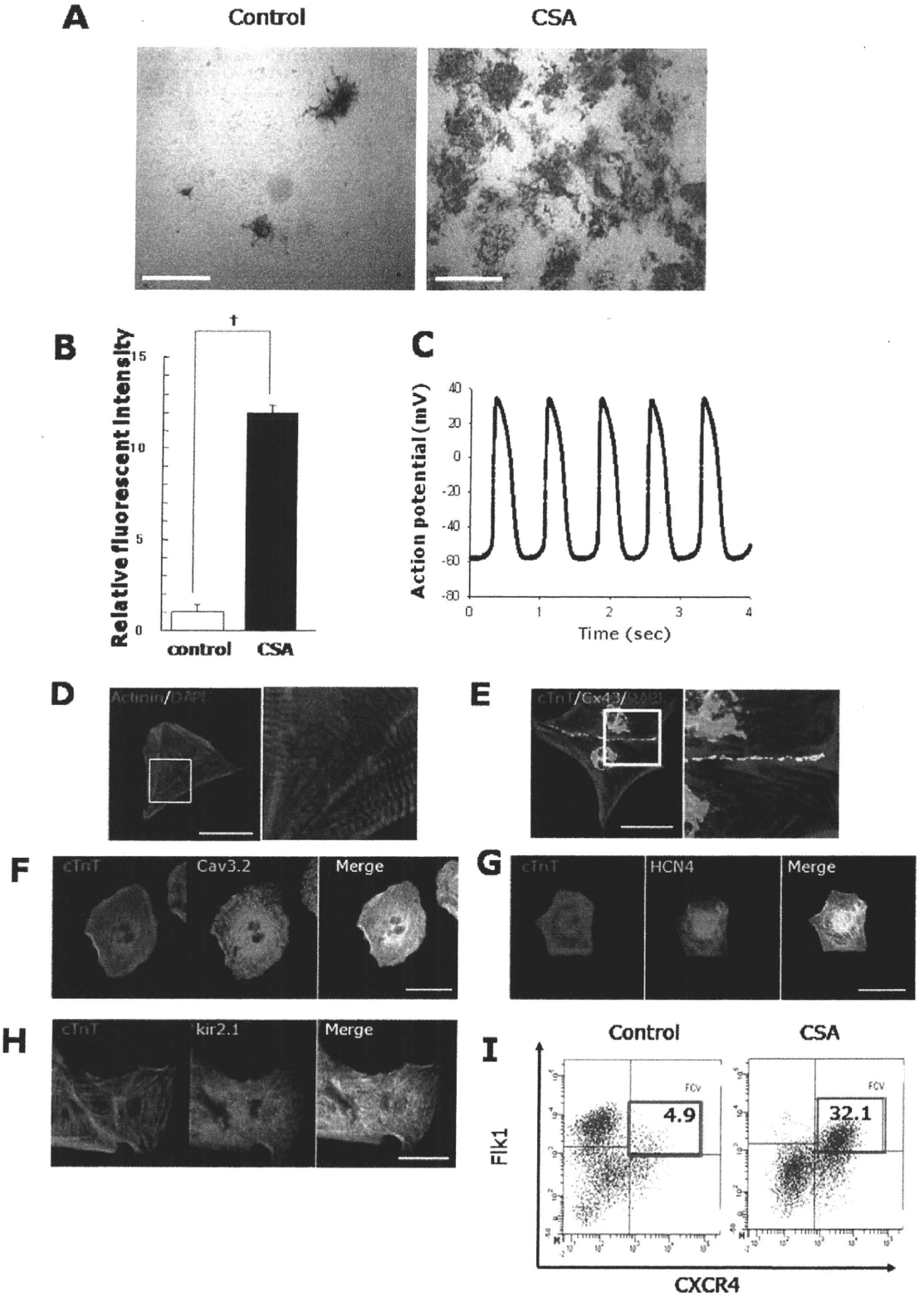

Figure 1. Cardiac cell expansion from mouse iPSC-derived Flk1$^+$ mesoderm by CSA. A. Gross appearance of cardiomyocyte induction by CSA. Six days after the Flk1$^+$ cell culture on OP9 cells (Flk-d6). cTnT staining (brown). Left panel: control. Right panel: CSA treatment. Scale bars = 400 μm. **B.** Quantitative evaluation of cardiomyocyte induction by fluorescent intensity of cTnT staining. Relative fluorescent intensity is

indicated (n = 4, †, $p<0.001$ vs control). **C.** Representative action potential of iPSC-derived spontaneously beating cardiomyocytes. **D.** Sarcomeric organization in TMRM-purified cardiomyocytes at Flk-d8. Immunostaining with anti-sarcomeric α-actinin antibody (red) and DAPI (blue). Right panel shows higher magnification of boxed area. Scale bar = 25 μm. **E–H.** Double immunostaining of TMRM-purified cardiomyocytes at Flk-d8 for connexin43 (Cx43) (green) and cTnT (orange) (E), Cav3.2 (green) and cTnT (orange) (F), HCN4 (green) and cTnT (orange) (G), Kir2.1 (green) and cTnT (orange) (H). Nuclei are visualized with DAPI. Scale bars = 25 μm. I. FACS analysis for cardiac progenitor induction from mouse iPSCs by CSA. X axis: CXCR4. Y axis: Flk1. Percentages of FCV cardiac progenitor cells (double positive population; red boxes) in total $Flk1^+$ cell progenies are indicated.
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Flowcytometry and cell sorting

FACS for differentiating mouse iPSCs was performed as described previously [8], [12], [20]. After 96–108 h of iPSC differentiation, cultured cells were harvested and stained with allophycocyanin (APC)-conjugated AVAS12 and FITC-conjugated ECCD2. Viable $Flk1^+$/E-cadherin$^-$ cells, excluding propidium iodide (Sigma), were sorted by FACS AriaII (Becton Dickinson). For FACS for FCV progenitor cells, after 2 days differentiation of purified $Flk1^+$ cell on PKH67-stained OP9 cells (Flk-d2), cultured cells were harvested and stained with a combination of MoAbs of PE-conjugated AVAS12 and biotinylated CXCR4 followed by addition of streptoavidin-conjugated APC, and subjected to FACS analysis. PKH-negative populations were analyzed and sorted as iPSC-derived cells. The $Flk1^+$/$CXCR4^+$ population (which was vascular endothelial cadherin-negative) [8] was designated "FCV cells". For FACS for cardiomyocytes, cells were harvested after 6–8 days culture of $Flk1^+$ cells on OP9 cells (Flk-d6-8). Induced cardiomyocytes were selected using tetramethyl rhodamine methyl ester (TMRM) (Invitrogen) [12], a fluorescent probe to monitor the membrane potential of mitochondria. In brief, cells were dissociated with 0.25% tryprin/EDTA, then incubated in DM with 50 nmol/L TMRM at 37°C for 15 minutes. Stained cells were washed twice and selected by FACS. TMRM-high population was considered as purified cardiomyocytes in iPSCs.

Human iPSC culture

END-2 cells were cultured as described previously [31]. Human iPS cell lines induced with transduction of four transcription factors (Oct4, Sox2, Klf4, and c-myc), 201B6 and 201B7, and Myc-negative human iPSC lines, 253G1 and 253G4 were maintained as previously described [1], [32]. 253G1 was used as the human iPS cell representative in all experiments unless stated otherwise. Induction of cardiomyocyte differentiation from human iPSCs was performed by co-culturing clumps of undifferentiated human iPSCs on END-2 cells, essentially as described previously [31]. To study the effect of CSA on cardiomyocyte differentiation, 3 μg/mL CSA was added to the culture medium on day 0 (END2-d0) or 8 (END2-d8) after start of co-culture. The number of beating colonies on END2-d12 was scored by microscopic examination. For intracellular Ca^{++} measurement and immunostaining for cTnT and actinin, beating colonies were mechanically excised, then gently dissociated by trypsin-EDTA treatment (at 37°C, 10 min), and replated on to gelatin-coated dishes. For electrophysiological analysis, beating colonies were mechanically excised and then dissociated by trypsin-EDTA with DNAse I (at 37°C, 10–15 min), and replated on to gelatin-coated dishes.

Immunohistochemistry

Immunostaining of murine cardiomyocytes was performed as described [8], [11], [12]. Briefly, 4% paraformaldehyde (PFA)-fixed cells were blocked by 2% skimmed milk (BD, bioscience) and incubated with 1st Abs. For immunohistochemistry, anti-mouse IgG –horse radish peroxidase (HRP) (Invitrogen) was used as 2nd Abs. For immunofluorescent staining, anti-mouse, rat and rabbit immunoglobulin conjugated with Alexa 488 or 546 were used for 2nd Abs. Nuclei were visualized with DAPI (Invitrogen). Cardiomyocyte differentiation was quantified as the fluorescent intensity of cTnT staining as described [8]. Immunostaining for human cardiomyocytes, 4% paraformaldehyde (PFA)-fixed cells were processed with 0.2% Triton X100 and 1% BSA (Sigma), and incubated with 1st Abs. Stained cells were photographed with inverted fluorescent microscopy, Eclipse TE2000-U (Nikon, Tokyo, Japan), digital camera system, AxioCam HRc (Carl Zeiss, Germany), or BIOREVO BZ-9000 (Keyence, Osaka, Japan).

Electrophysiology

Membrane potentials of single cells within a beating colony were measured using whole-cell patch clamp electrophysiology in the current-clamp mode (Axopatch200B, Axon Instruments/Molecular Devices Corp., Union City, CA). All recordings were carried out at room temperature [8].

Buffer compositions. Bath solution contained (in mmol/L) 140 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.45 $MgCl_2$, 1.8 $CaCl_2$, and 5 HEPES (pH = 7.4 with NaOH). Pipette solution contained (in mmol/L) 110 L-Aspartic acid, 30 KCl, 5 MgATP, 0.1 NaGTP, 5 K_2Creatine phosphate, 2 EGTA, 10 HEPES, and 10 NaOH (pH = 7.2 with KOH).

Field potential (FP) recordings of the beating colonies were performed using The MED64 multi-electrode array (MEA) system (Alpha MED Scientific Inc., Osaka, Japan) at a sampling rate of 20 kHz with low path filter of 500 Hz or high path filter of 1 Hz. All MEA measurements were performed at 37°C with heated perfusion system. The signals were recorded and processed with the Mobius software (WitXerx, US). The medium were perfused 1.7 ml/min as 37°C, and then the FPs were recorded for 5 min. Subsequently, E-4031(Calbiochem, US), isoproterenol (Proternol-L®, Kowa Pharmaceutical Company, Tokyo, Japan), or propranprol (Inderal®, AstraZeneca, Japan) was added to medium (discrete colony samples were used for each drug). Then, the FPs were measured for about 10 min.

Intracellular Ca measurement

Human iPSCs were loaded with 4 μM Quest Fluo-8 (ABD Bioquest, Inc. Sunnyvale, CA) for 30 min. Fluo-8 fluorescence (excitation at 495±10 nm and emission at 535±20 nm) of beating colony was measured every 16 msec with a back-thinned electron multiplier CCD camera (ImagEM; Hamamatsu Photonics, Hamamatsu, Japan). Four consecutive images were averaged. Ratio (F1/F0) to an image at minimum fluorescence intensity (F0) was calculated after background subtraction. The measurements were carried out at room temperature.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from various kinds of cell populations with the use of RNeasy Mini Kit (QIAGEN, Valencia, CA). cDNA was synthesized by the SuperScript III First-strand Synthesis System (Invitrogen). Polymerase chain reaction was performed with the use of KOD Plus (Toyobo, Tokyo, Japan) as described [33]. Primer sequences [34] are shown in Table S1.

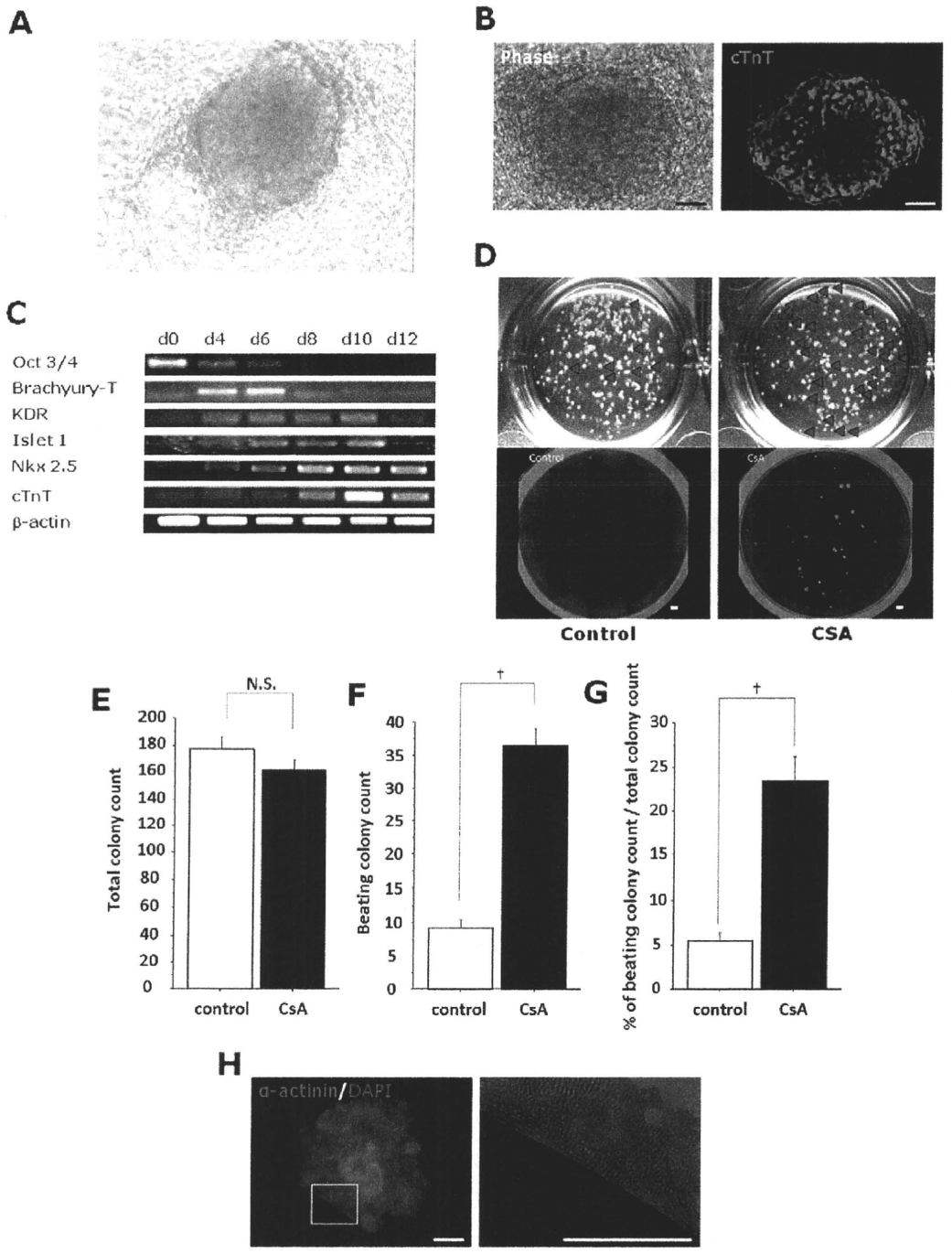

Figure 2. Induction and expansion of cardiomyocytes from human iPSCs. Human iPSCs were co-cultured with END-2 cells to differentiate cardiomyocytes. **A.** Gross morphology of a beating colony from human iPSCs (captured photo from Movie S1). **B.** cTnT staining of a beating colony on END-2 cells. Left panel: phase contrast image. Right panel: human cTnT staining (green). Scale bar = 50 μm. **C.** RT-PCR analysis for differentiation markers during cardiomyocyte differentiation of human iPSCs (from END2-d0 to d12). Oct3/4: Undifferentiated cell marker, Brachyury-T: mesendoderm marker, KDR (human Flk1): mesoderm marker, Islet1: mesoderm and cardiac progenitor marker, Nkx2.5: cardiac progenitor and cardiomyocyte marker, cTnT: cardiomyocyte marker. **D.** Representative gross appearance of human iPSC-derived beating colonies at END2-d12 in 12-well dishes. Left panels: control. Right panels: CSA treatment from END2-d8. Upper panels: phase contrast images. Beating colonies are shown by red arrows. Lower panels: cTnT staining (green). **E–G** Quantitative evaluation of beating colony appearance. **E.** Total colony count (control; 177±9.7/well (12-well dishes)(n = 8), CSA; 162±8.0/well (n = 9); N.S., p = 0.237), **F.** Beating colony count (control; 9.1±1.2/well (12-well dishes)(n = 8), CSA; 36.4±2.5/well (n = 9); †, $p<0.0001$), and **G.** Percentages of beating colonies (control; 5.4±0.9% (n = 8), CSA; 23.5±2.8% (n = 9); †, $p<0.0001$) in total colonies that appeared at END2-d12. **H.** Immunostaining of actinin (red) and DAPI (blue) in dissociated cardiomyocyte colonies. The same colony is shown in Movie S2. Right panel shows higher magnification of boxed area. Sarcomere structures are evident. Scale bar = 50 μm.
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Electron microscopic study

Human iPSC-derived beating colony was replated on multi-well chamber slide (NUNC Rochester, NewYork), fixed with 2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 30–60 min, washed and immersed with phosphate buffered saline for overnight at 4°C, and fixed in 1% buffered osmium tetroxide. The specimens were then dehydrated through graded ethanol and embedded in epoxy resin. Ultrathin sections (90 nm), double-stained with uranyl acetate and lead citrate, were examined under electron microscopy (H-7650; Hitachi, Tokyo, Japan).

Statistical Analysis

All data were obtained from at least three independent experiments. Statistical analysis of the data was performed using Student's t-test or ANOVA. $p<0.05$ was considered significant. All data are shown as mean ± S.D.

Results

Cardiomyocyte and cardiac progenitor expansion from mouse iPSCs by CSA

Recently, we reported that functional cardiomyocytes were induced from mouse iPSCs with our differentiation method in mouse ES cells [12]. In brief, undifferentiated mouse iPSC colonies maintained on MEFs were morphologically similar to mouse ESCs. We induced mesoderm differentiation from mouse iPSCs by culturing on type IV collagen-coated dish with DM (see Methods). $Flk1^+$ mesoderm cells that appeared were selected by FACS at 4.5 days of differentiation (iPS-d4.5) and then underwent a cardiomyocyte induction protocol involving co-culture on OP9 stroma cells; spontaneously beating cardiomyocytes began to appear after 3 to 4 days of culture (Flk-d3-4). Beating cells that appeared were positive for multiple cardiomyocyte markers and had electrophysiological features assessed by whole-cell patch clamp as previously reported [8], [12].

In the present study, we first tried to expand cardiomyocytes and cardiac progenitors from mouse iPS cells by CSA. When CSA was added to purified $Flk1^+$ cells, the appearance of $cTnT^+$ cardiomyocytes was increased 12-fold compared to controls (Fig. 1A, B), which was comparable with the increase observed in mouse ESCs [11]. CSA-expanded cardiomyocytes spontaneously beat and showed cardiomyocyte-like action potential (average interval: 0.74 sec, maximum diastolic potential: −58.6 mV and overshoot: 34.3 mV (n = 6)) (Fig. 1C). These cardiomyocytes also showed distinct sarcomere formation (Fig. 1D), expression of cTnT (Fig. 1E-H) and connexin 43 located at cellular boundaries (Fig. 1E). T-type calcium channel Cav3.2 (Fig. 1F), a pacemaker ion channel, HCN4 (Fig. 1G), and a ventricular ion channel, kir2.1 (Fig. 1H) were also detected in $cTnT^+$ cells. We also examined the effect of CSA on the induction of FCV cardiac progenitor cells in mouse iPSCs. Addition of CSA to $Flk1^+$ cells specifically increased the FCV population in mouse iPSCs to approximately 6.5 times of control. The maximum percentage of FCV cells within total $Flk1^+$ cell-derived cells was more than 30% by CSA (Fig. 1I), comparable with that observed in mouse ESCs, previously [11]. CSA can thus efficiently enhance the differentiation of functional cardiomyocytes and cardiac progenitors from mouse iPSCs.

Differentiation of cardiomyocytes from human iPSCs

We next examined cardiomyocyte differentiation from human iPSCs. We employed a human ESC differentiation method for cardiomyocytes using END-2 visceral endodermal stroma cells [31]. When human iPSCs were cultured on END-2 cells, spontaneously beating cardiomyocytes were successfully induced (Fig. 2A, Movie S1). Beating colonies were first detected after END2-d10 and became maximally evident after END2-d12.

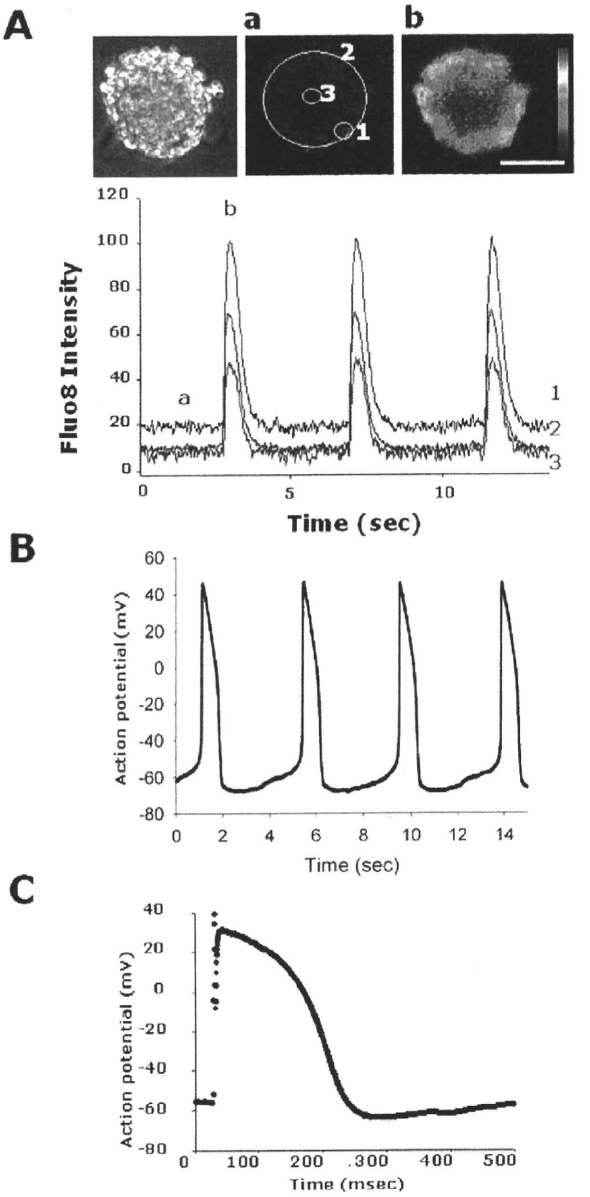

Figure 3. Functional analysis of expanded human cardiomyocytes. A. Ca^{++} transient in dissociated beating colonies. Cytoplasmic Ca^{++} change was monitored with fluo-8. Left panel: a transmission image of fluo-8 loaded iPSC colony. Middle and right panels: Fluo-8 images at the end (a) and the peak (b) of the fluorescence change. Scale bar = 50 μm. Lower panel: Time course of fluo-8 intensity change. The intensity was measured at the periphery (1), the entire colony (2) and the center (3) (ROIs shown in middle panel). Ratios (F1/F0) of the intensity to the one at the beginning of recording (F0) are indicated. Note that Ca transient is well synchronized within the colony. Real time video is shown in Movie S3. **B.** Representative action potential recorded from a cell in a beating colony. **C.** Representative single whole cell patch-cramp recording of a non-self beating human iPSC-derived cardiomyocyte after electrical stimulation.
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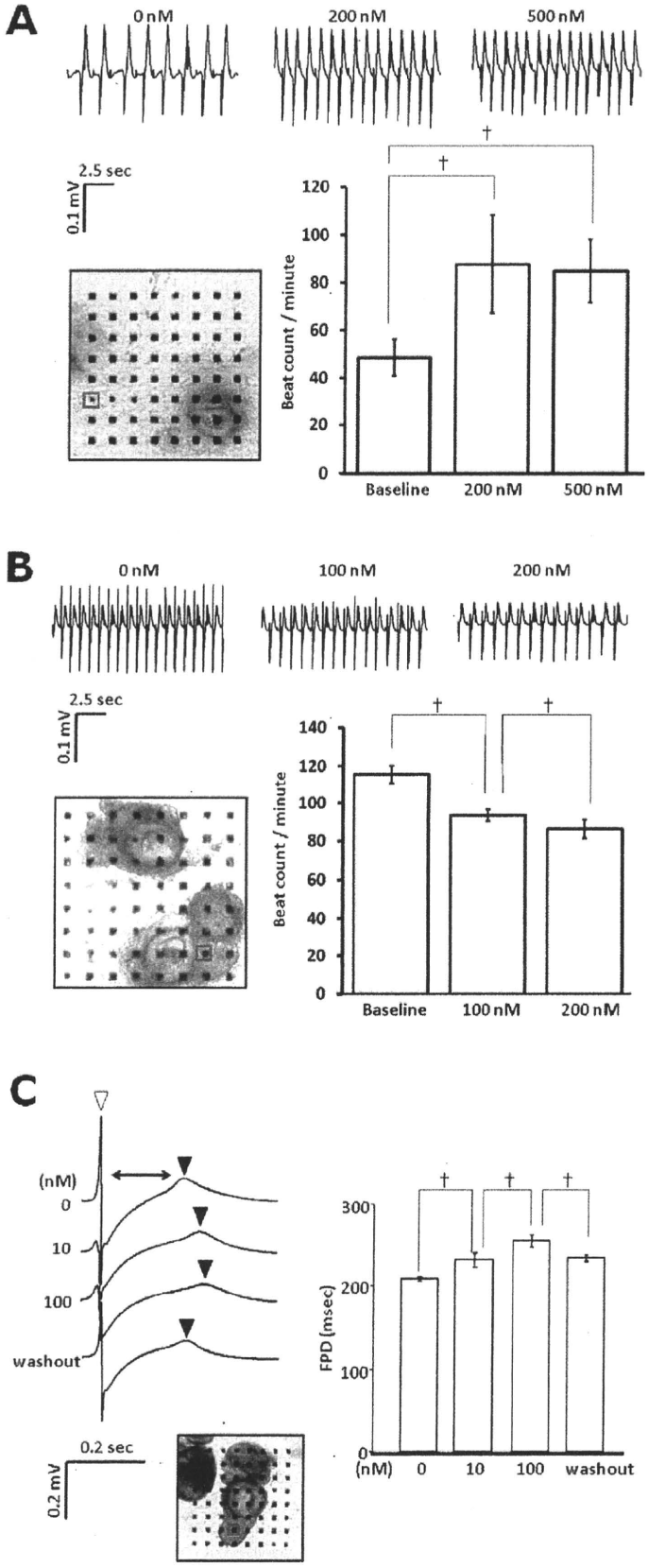
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Figure 4. Pharmacological responses of human iPSC-derived cardiomyocytes. Field potential recordings of replated beating colonies after stimulation with isoproterenol (**A**), propranorol (**B**), and E-4031 (**C**). Photos; array of multi-electrode and replated colonies. Data recorded at electrodes in red squares are shown. **A, B.** Beating frequency (beating/minite). **C.** QT elongation. The time period from the first negative peak (open triangle) to the first positive peaks (closed triangles) reflects QT time in electrocardiogram. n = 3, †, $p<0.001$.
doi:10.1371/journal.pone.0016734.g004

These beating colonies were positive for cTnT (Fig. 2B). During the differentiation of human iPSCs on END2 cells, sequential expression of various marker genes expected for cardiomyogenesis was observed (ex: Oct3/4; undifferentiated iPSCs, Brachyury; mesendoderm, KDR; mesoderm, islet1; mesoderm and cardiac progenitors, nkx2.5; cardiac progenitors and cardiomyocytes, cTnT; cardiomyocytes) (Fig. 2C). In our another previous study on human ESC differentiation, a Flk1 (in human, VEGF receptor-2)$^{+}$/TRA1-60^{-} mesoderm population appeared in culture approximately 8 days after induction of differentiation [35]. When CSA was added to differentiating human iPSCs at the mesoderm stage (i.e. on END2-d8), the appearance of beating colonies was increased (Fig. 2D) although no effect was observed with the CSA treatment on undifferentiated human iPSCs (i.e. from END2-d0) (data not shown). Whereas the total number of iPSC-derived colonies that appeared was not changed (Fig. 2E), the number and percentage of beating colonies that appeared at END2-d12 were significantly increased approximately 4.0 and 4.3 times by CSA treatment, respectively (Fig. 2F, G). Approximately 23±2.7% of total colonies was beating in average, and in an optimized condition, 39% of total colonies included beating cardiomyocytes. CSA-expanded colonies maintained self-beating after a mechanical isolation and re-plating, and were positive for α-actinin with distinct sarcomere formation (Fig. 2H, Movie S2). Thus, cardiomyocyte induction from human iPSCs could be similarly enhanced by CSA. The mesoderm stage-specific effect of CSA in human iPSCs suggests the similar machinery in mouse ES/iPSCs are robustly working in human iPSC differentiation to cardiomyocytes.

Functional features of expanded human iPSC-derived cardiomyocytes

We next evaluated functional features of CSA-expanded human iPSC-derived cardiomyocytes. Fluo-8 imaging revealed synchronized increases in intracellular Ca^{++} in beating colonies with contraction (Fig. 3A, Movie S3). Action potentials recorded by patch clamp electrophysiology identified cells with pacemaker potential (average of the interval: 4.26 sec, maximum diastolic potential: −67.6 mV overshoot: 46.6 mV (n = 6))(Fig. 3B). Replated colonies continued beating spontaneously for more than 10 months. Some isolated single cells obtained from beating colonies at 3 months culture period lost automaticity and showed some features of human ventricular cells such as action potential with rapid depolarization and prolonged plateau after electrical stimulation (Fig. 3C). These results indicate that various functional human cardiomyocytes could be induced in this system.

We further examined pharmacological reactions of CSA-expanded human cardiomyocytes to show the relevance as cardiac cell models. We recorded field potential of re-plated beating colonies with multi-electrode array under simulation of a β-stimulant, isoproterenol, a β-blocker, propranorol, and a HERG channel inhibitor, E-4031. Addition of isoproterenol significantly increased the beating frequency (Fig. 4A), on the other hand, propranorol significantly decreased the beating frequency (Fig. 4B). E-4031 dose-dependently prolonged the length of time from the first negative peak to first positive peak, which is corresponding to QT time in electrocardiogram (Fig. 4C). These results indicate that CSA-expanded human iPSC-derived cardiomyocytes can suffice multiple functional features as human cardiomyocyte cell models.

Ultra structural features of expanded human iPSC-derived cardiomyocytes

We finally confirmed features of CSA-expanded human iPSC-derived cardiomyocytes at the ultrastructural level using electron microscopy. Beating colonies induced from human iPSCs resembled native cardiomyocytes, showing myofibrillar bundles with transverse Z-bands and enriched mitochondria (Fig. 5A-D). Other cardiomyocyte-specific structures, such as intercalated disks with desmosomes (Fig. 5D), atrial secretory granule-like structures (Fig. 5E), and glycogen granules (Fig. 5F) were also observed.

Together, these results indicate that *bona fide* human cardiomyocytes can be successfully induced and expanded from human iPSCs with this method.

Discussion

Here we demonstrated the induction and expansion of cardiac progenitors and functional cardiomyocytes from iPSCs using potent and specific effect of CSA. Human cardiomyocytes with multiple expected structural and functional features could be induced with this method. This method provides a critical technological basis to obtain cardiac cells from human iPSCs.

We have demonstrated previously that CSA treatment is most effective in inducing FCV cardiac progenitor cells, the nearest upstream of cardiomyocytes in mouse ESCs [11]. Here we showed that CSA effects on FCV cardiac progenitor and cardiomyocyte induction were also completely reproduced in mouse iPSCs. Moreover, CSA also showed significant enhancing effects of cardiomyocyte differentiation from human iPSCs in the END-2 system. This is the first report to show the effect of CSA in human stem cells. In this study, we examined four human iPSC clones, 201B6, B7 (induced with four factors) [1], 253G1 and G4 (induced without c-myc) [32]. Though the basal efficiency of cardiomyocyte differentiation from 201B6, B7 and 253G4 were lower than that from 253G1, CSA treatment significantly enhanced cardiomyocyte appearance similarly in all these human iPSC clones (Figure S1). Thus, CSA robustly induced cardiogenic differentiation in mouse ESCs, iPSCs and human iPSCs regardless their species and derivation methods.

The molecular mechanisms conducting this potent CSA effect on cardiac lineage is important, but still it is unknown. Though we examined another calcineurin inhibitor, FK506, and a NF-AT inhibitor, 11R-VIVIT, both of them did not reproduce the effect of CSA [11], indicating that the cardiogenic CSA effect is mediated by other molecular target than immunosuppressing effect of CSA. Further elucidation of molecular mechanisms of CSA in cardiomyocyte differentiation would be critical for the exploration of cardiomyocyte differentiation and regeneration strategies.

CSA-expanded cardiomyocytes from human iPSCs exhibited many features sufficing as functional cardiomyocytes. Cardiomyocytes with pacemaker-like or ventricular-like action potentials were successfully induced. Nevertheless, they were still immature compared with mature adult cardiomyocytes [36], [37] and they

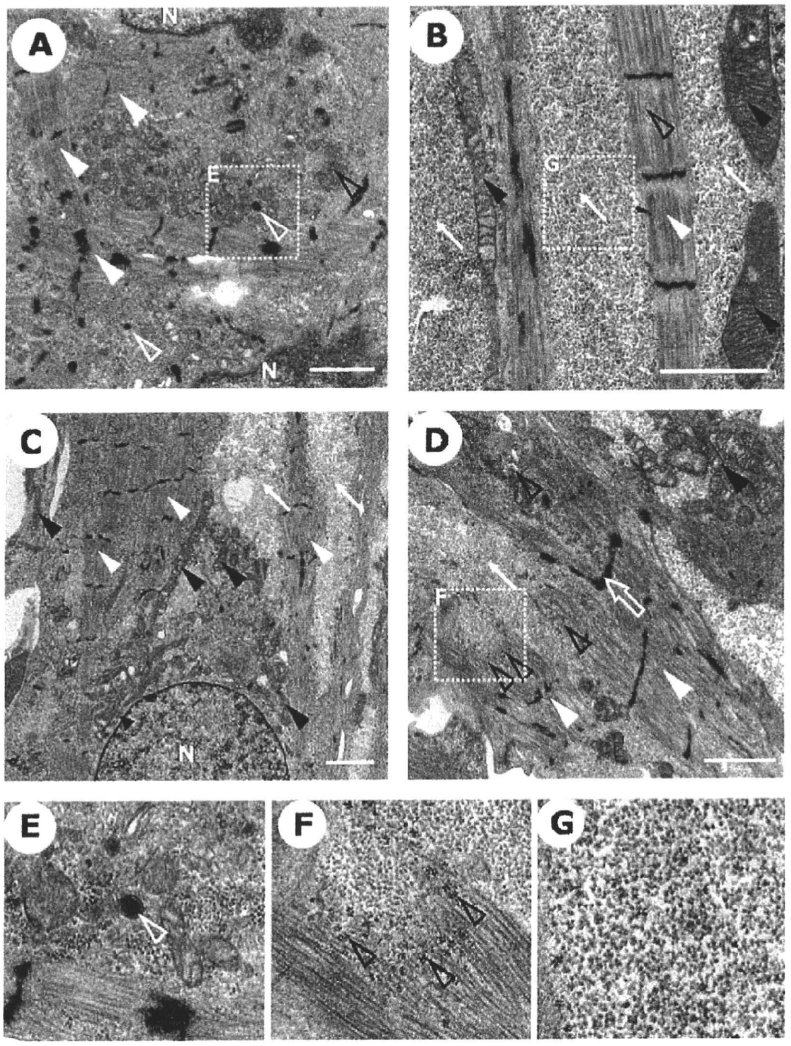

Figure 5. Ultrastructural analysis of human iPSC-derived cardiomyocytes. Transmission electron microscopic images of beating colonies. Myofibrils with Z-bands (white closed arrowheads in **A–D.**), mitochondria (black closed arrowheads in **B–D.**), intercalated disk-like structure with desmosome (white open arrow in **D.**), atrial sercetory granules (electron-dense granules surrounded by double membranes. White open arrowheads in **A.** and **E.** (magnified image of **A.**)), glycogen granules (electron-dense small granules. Black open arrowheads in **D.** and **F.** (magnified image of **D.**)), ribosomal granules (electron-lucent small granules. White arrows in **B–D.** and **G.** (magnified image of **B.**)). N: nucleus. Scale bar = 2 μm, direct magnify, ×3000 (A), ×7000 (B), ×4000 (C), ×5000 (D).
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also displayed some structural features of fetal cardiomyocytes, such as relatively low global electron density, sparse myofibrils, and abundant ribosome granules (Fig. 5). Methods for further maturation as well as specific induction and purification of the various cardiac cell types (pacemaker, atrial, ventricular, conduction system cells etc.) should be explored in future study.

Interestingly, a recent clinical report showed that CSA prevented cardiac reperfusion injury by protecting cardiomyocytes from apoptosis [38]. Cardiogenic effects of CSA in later stages of differentiation of human iPSCs imply that CSA may positively affect on endogenous cardiac progenitors to induce cardiac regeneration in patients. Though it is still unknown whether endogenous cardiac regeneration can be induced by CSA administration, our study may offer a scientific basis to support a clinical opportunity for CSA as a cardiac regenerative drug.

This novel cardiac cell differentiation method for iPSCs would thus broadly contribute to cardiac regenerative medicine by providing various options for cell preparation, transplantation strategies, and drug discovery.

Supporting Information

Figure S1 Quantitative evaluation of beating colony appearance in iPSC clones. 201B6 cells: Total colony count (control; 203±6.4/well (12-well dishes)(n = 3), CSA; 193±4.0/well (n = 3); N.S., p = 0.0915), beating colony count (control; 4.0±1.0/well (n = 3), CSA; 13.7±3.5/well (n = 3); *, p<0.05), percentages of beating colonies (control; 2.0±0.5% (n = 3), CSA; 7.1±1.7% (n = 3); **, p<0.01) in total colonies that appeared at END2-d12. 201B7 cells: Total colony count (control; 204±8.3/well (n = 3),

CSA; 200±2.0/well (n = 3); N.S., p = 0.43), beating colony count (control; 5.0±1.0/well (n = 3), CSA; 18.3±3.1/well (n = 3); **, p<0.01), percentages of beating colonies (control; 2.5±0.6% (n = 3), CSA; 9.2±1.5% (n = 3); **, p<0.01). 253G4 cells: Total colony count (control; 201±4.0/well (n = 3), CSA; 201±3.8/well (n = 3); N.S., p = 0.9216), beating colony count (control; 4.7±0.6/well (n = 3), CSA; 15.0±1.0/well (n = 3); **, p<0.05), percentages of beating colonies (control; 2.3±0.3% (n = 3), CSA; 7.5±0.6% (n = 3); †, p<0.001)
(TIF)

Movie S1 A beating colony induced from human iPS cells at END2-d12 (Fig. 2A).
(MOV)

Movie S2 A dissociated beating colony induced from human iPS cells on END-2 cells (Fig. 2H).
(MOV)

Movie S3 Real time monitoring of Ca^{++} transient by Fluo-8 in dissociated beating colony induced from human iPS cells (Fig. 3A). Clear and synchronized Ca^{++} transient is observed.
(MOV)

Table S1 Primers for PCR.
(RTF)

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Author Contributions

Conceived and designed the experiments: MF PY JKY. Performed the experiments: MF PY TGO GN HU HF HM SM. Analyzed the data: MF TI CLM JKY. Contributed reagents/materials/analysis tools: KO KT MN CLM. Wrote the paper: MF RS CLM NN KN SY JKY.

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REVIEW

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

Kohei Yamamizu, PhD; Jun K. Yamashita, MD, PhD

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

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

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

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

Until now, most studies of vascular development have consisted of gene knockout and gene inhibitory studies using mice and zebrafish. Although these studies led to the discoveries of essential factors in vascular development, they could not sufficiently identify the conditions required for vascular formation. To clarify the "constructive" mechanisms underlying vascular development, we have developed a novel embryonic stem (ES) cell differentiation system, which exhibits early vascular development using VEGFR2 (Flk1) -positive cells as common progenitors for vascular cells (**Figure 1**).[10,11] Using this system, we can systematically induce vascular cells in vitro and dissect their differentiating processes in detail. We

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Laboratory of Stem Cell Differentiation, Stem Cell Research Center, Institute for Frontier Medical Sciences; Department of Cell Growth and Differentiation, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan
Mailing address: Kohei Yamamizu, PhD, Laboratory of Stem Cell Differentiation, Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: yamamizu@frontier.kyoto-u.ac.jp
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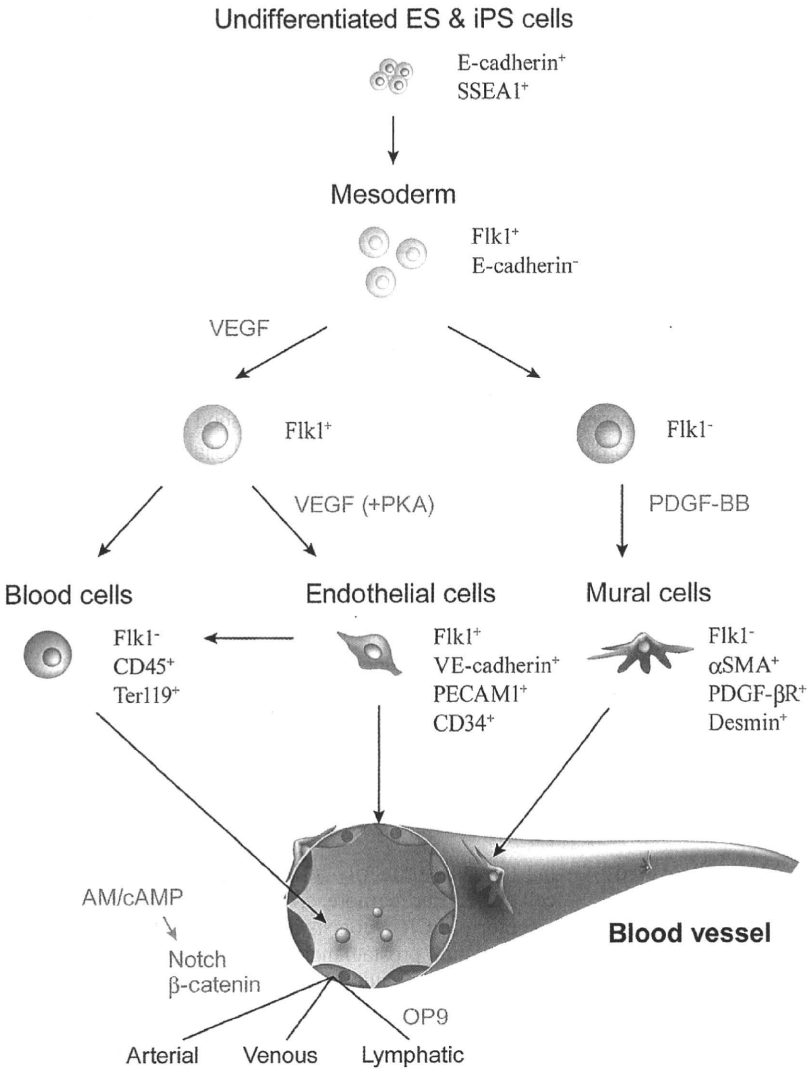

Figure 1. Vascular differentiation system using embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. Flk1 (VEGFR2) positive cells derived from ES/iPS cells are vascular progenitors that can differentiate into endothelial cells (ECs), mural cells, and blood cells. ECs have specific characters of arterial–venous-lymphatic ECs.

recently demonstrated that cAMP signaling plays a critical role in the reconstitution of endothelial cells (ECs) and arterial specification in vascular development.[12,13] In this review, we focus on the molecular mechanisms of vascular development/differentiation and diversification from vascular progenitors.

Molecular Mechanisms of EC Differentiation

Roles of VEGF Signaling in Vascular Development

Numerous vascular formation factors, such as vascular endothelial growth factors (VEGF), neuropilin (NRP), angiopoietins, transforming growth factor-β, platelet-derived growth factor, fibroblast growth factor, ephrin, and Notch, have been identified within the past few decades. Among these factors, VEGF/Flk1 signaling plays the most important role in the production of vascular progenitors and EC differentiation.[14] Lateral plate mesoderm expresses Flk1, and migrates into the extra-embryonic yolk sac to form a vascular capillary plexus, leading to the development of a functional circulatory system.[15] Flk1 null mice die at E8.5–E9.5, without organized blood vessels.[16] Heterozygous VEGF-A null mice die in early gestation due to failure in vascular system formation.[17] On the other hand, two- to threefold overexpression of VEGF-A from its endogenous locus results in aberrant heart development and lethality at E12.5–E14,[18] indicating that strictly balanced VEGF function is important in normal embryogenesis.

NRP1 acts as another VEGF-A receptor in blood vessels and endocardial cells of the heart.[19,20] NRP1 is also expressed in particular classes of developing neurons,[21,22] and functions as a receptor for the class 3 semaphorins that mediate semaphorin-elicited inhibitory axon guidance signals to neurons.[23,24] NRP1, together with Flk1, forms a specific receptor for VEGF-A_{165}, an isoform of VEGF, and the Flk1-VEGF-A_{165}–NRP1 complex potently enhances Flk1 signaling.[20] NRP1 null mice die midway through gestation at E10.5–E12.5 and exhibit defects of the heart, vasculature, and nervous system,[22] indicating that the relationship between $VEGF_{165}$ and NRP1 is critical in vascular development.

Growth factors and hypoxia are known to induce VEGF-A gene expression. Hypoxia-inducible factor (HIF) markedly produces VEGF and contributes to formation of the vascu-

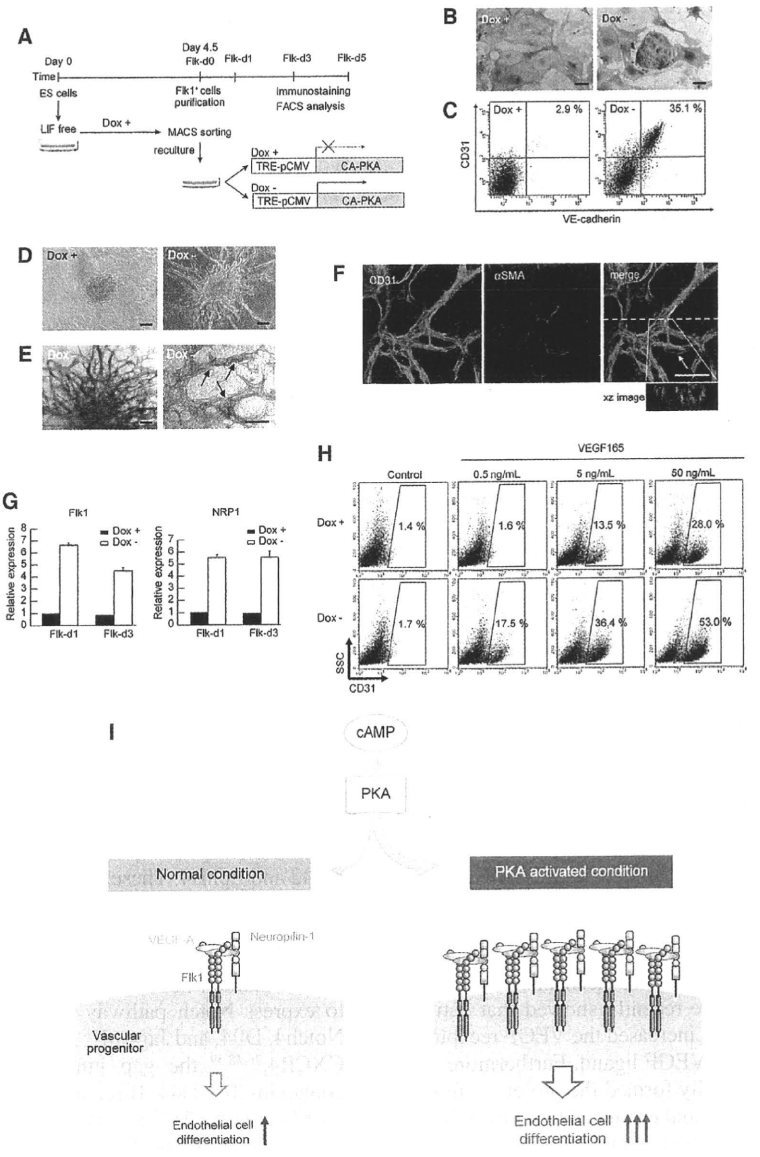

Figure 2. Cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway plays a critical role in vascular development. (**A**) Experimental system for PKA activation. An endothelial cell (EC) cell line expressing the constitutive active (CA) form of PKA by tetracyclin-inducible expression system (Tet-Off) was established. Doxycycline (Dox) was added during the first 4.5 days of ES cell differentiation to Flk1$^+$ cells. Flk1$^+$ cells were sorted by MACS and subjected to 2-D culture on collagen-coated dishes or 3-D culture in collagen gel, and were cultured in the presence or absence of Dox (1 μg/ml). (**B**,**C**) 2-D culture with DM, at Flk-d3. (**B**) Double immunostaining for CD31 (purple) and αSMA (brown). **Left panel**, Dox (1 μg/ml) treatment. **Right panel**, Dox free. Culture with DM alone. Scale bar: 100 μm. (**C**) Flowcytometry for EC markers, CD31 and VE-cadherin. Percentages of CD31$^+$/VE-cadherin$^+$ ECs in total Flk1$^+$ cell-derived cells are indicated. (**D**) Phase contrast images after 5 days' culture in 3-D culture. **Left panel**, Dox (1 μg/ml) treatment. **Right panel**, Dox free. Scale bars: 100 μm. (**E**) In-gel double immunostaining for CD31 (purple) and αSMA (brown) in Dox-free conditions. **Left panel**, gross appearance of vascular structure. **Right panel**, higher magnification view. αSMA$^+$ cells attached to CD31$^+$ EC tube structure are observed (arrows). Scale bars: 100 μm. (**F**) Confocal microscopic analysis of vascular structure. Double fluorescent staining for CD31 and αSMA in Dox-free conditions. **Left panel**, CD31 (green). **Middle panel**, αSMA (red). **Right panel**, Merged image. αSMA$^+$ cell attached to CD31$^+$ EC tube structure is observed (arrow). CD31$^+$ cells formed a true lumen (green) with attached mural cells (red) shown in xz image. Dashed line indicates sliced position. Scale bars: 100 μm. (**G**) Quantitative RT-PCR showing mRNA expression of Flk1 and neuropilin (NRP) 1 at Flk-d1 and d3 in the presence or absence of Dox. mRNA expression at Flk-d1 with Dox was set as 1.0. (**H**) Flowcytometry for CD31 expression in the presence (Dox$^+$) (1 μg/ml) or absence of Dox (Dox$^-$). X-axis: CD31, Y-axis: SSC. Flk1$^+$ cells were incubated with various concentrations of vascular endothelial growth factor (VEGF)$_{165}$ in the serum-free medium, SFO3. Percentages of CD31$^+$ ECs in the total Flk1$^+$ cell-derived cells are indicated. (**I**) PKA activation increased both Flk1 and NRP1 expression in vascular progenitors and markedly enhanced the "sensitivity" of the progenitors to VEGF$_{165}$ by inducing Flk1–VEGF$_{165}$–NRP1 complex formation, and markedly enhancing EC differentiation.

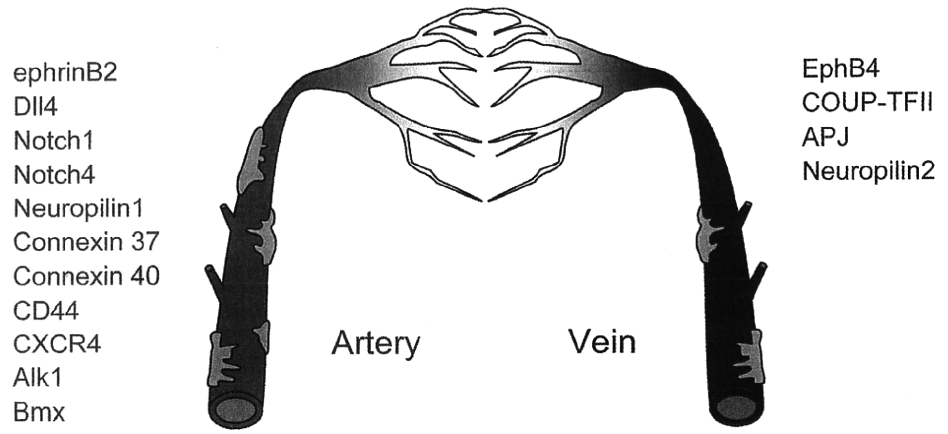

Figure 3. Arterial–venous specification markers in vascular formation. Arterial endothelial cells (ECs) are known to express ephrinB2, and Notch pathway molecules, such as Notch1, Notch4, Dll4, and Jagged1, neuropilin (NRP) 1, the chemokine receptor CXCR4, the gap junction protein connexin-37, connexin-40, CD44, Alk1, and Bmx. Venous ECs are known to express EphB4, NRP2, COUP-TFII, and the Apj receptor.

lar tube in embryogenesis as well as in adults. Null mice for HIF1a, HIF2a, and HIF-related genes have vascular defects and die at E9.5–E10.5,[25] indicating that HIF-related VEGF production regulates vascular development. However, the mechanisms that regulate the expression of the VEGF receptors, Flk1 and NRP1, in vascular development are not fully elucidated.

Roles of cAMP/PKA Signaling in Vascular Development

We have previously shown that cAMP signaling enhances EC differentiation, using an ES cell differentiation system.[26] We further investigated the molecular mechanisms of cAMP in EC differentiation, and we recently showed that activation of cAMP/PKA signaling increased the VEGF receptors, Flk1 and NRP1, but not the VEGF ligand. Furthermore, the cAMP/PKA pathway markedly formed the protein complex of Flk1–VEGF-A_{165}–NRP1, and enhanced the sensitivity of the progenitors to $VEGF_{165}$ by more than 10-fold (**Figure 2**).[12] These results indicate that PKA regulates "progenitor sensitivity" in EC differentiation by changing the characters of the vascular progenitors, not by producing growth factors. NRP1 was largely co-expressed with Flk1 in vascular progenitors derived from human and mouse ES cells.[27] These 2 functional markers for vascular progenitors might be commonly regulated by PKA to efficiently enhance their progenitor potential of responding to VEGF signaling.

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

Molecular Machinery of Arterial-Venous Specification

Mechanisms of Artery Formation

Molecular differences between arterial and venous ECs become apparent before circulation begins.[32–34] The first genes for arterial–venous specification to be identified were ephrinB2 and EphB4. These members of the Eph–ephrin family were discovered to be differentially expressed in the endothelium of arteries and veins, even before the onset of blood flow and heart beat.[32,33] Since then, various other arterial–venous markers have been identified. Arterial ECs are known to express Notch pathway molecules,[35,36] such as Notch1, Notch4, Dll4, and Jagged1, NRP1,[37] the chemokine receptor CXCR4,[26,38,39] the gap junction protein connexin-37 and connexin-40, CD44, Bmx, and activin receptor-like kinase 1 (Alk1) (**Figure 3**).[37] Recent accumulated evidence concerning the roles of the Eph–ephrin, Unc–Netrin, and NRP–plexin–semaphorin families suggests that blood vessels and nerves share a similar molecular machinery to form their networks, and that these molecules are associated with arterial–venous specification and intersomitic vessel guidance.[40]

Roles of Notch Signaling in Artery Formation

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

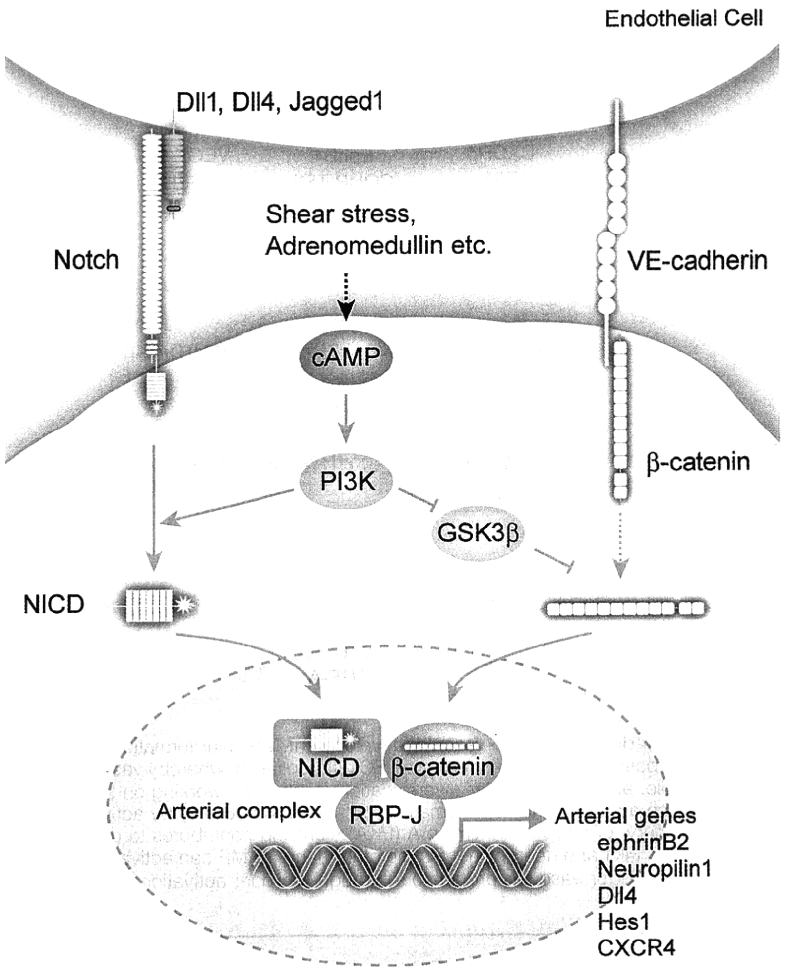

Figure 4. Molecular mechanisms of arterial endothelial cell (EC) specification. cAMP signaling, which can be induced by adrenomedullin, shear stress, etc activates Notch and β-catenin signaling through PI3K (and GSK3β) in vascular progenitors (as well as in differentiating ECs). Notch and β-catenin signaling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

in Flk1$^+$ cells, fails to induce arterial ECs in vitro,[26] indicating that Notch signaling is essential, but not sufficient, for arterial EC induction, suggesting that other factors are involved in this process.

Roles of Convergence Signaling of NOTCH and β-Catenin in Artery Formation

Wnt/β-catenin signaling plays a key role in vascular biology.[47] Mice deficient for Wnt2 displayed vascular abnormalities, including defective placental vasculature.[48] Wnt receptor gene, Frizzled-5 knockout mice died in utero owing to defects in yolk sac angiogenesis.[49] Defects of the β-catenin gene in ECs caused a defective vascular pattern and increased vascular fragility.[50] We previously revealed that simultaneous activation of VEGF and cAMP in Flk1$^+$ vascular progenitors leads to the induction of arterial ECs in vitro. We recently demonstrated a novel arterial specification machinery regulated by Notch and β-catenin signaling.[13] Both Notch and GSK3β-mediated β-catenin signaling were activated downstream of cAMP through phosphatidylinositol-3 kinase. Forced activation of Notch and β-catenin synergistically enhanced expression of the arterial markers, ephrinB2 and CXCR4. Interestingly, a protein complex with RBP-J, NICD, and β-catenin was formed on RBP-J binding sites of arterial genes in arterial, but not venous, ECs. Thus, the formation of the protein complex on arterial genes induced by cAMP activation could be the central machinery for arterial EC specification. These findings lead to an integrated and more comprehensive understanding of vascular signaling (**Figure 4**).[13] Furthermore, the Notch–β-catenin–RBP-J complex suppresses differentiation of neural precursor cells,[51] indicating that the protein complex that directly converges Notch and β-catenin signaling may play a critical role in cell fate determination in various organs.

Beta-catenin signaling in ECs can be activated through Wnt ligands as well as by VE-cadherin. Thus, Wnt ligands such as Wnt2, 5a, and 10b, expressed in fetal blood vessels, are involved in EC differentiation.[47,48] VE-cadherin is heavily tyrosine phosphorylated and is linked to β-catenin.[52] When adherens junctions mature, the tyrosine residues in VE-cadherin tend to be dephosphorylated and β-catenin is partially released from the complex,[53] 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,[50] β-catenin should have both a com-