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.
doi:10.1371/journal.pone.0016734.g001

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.

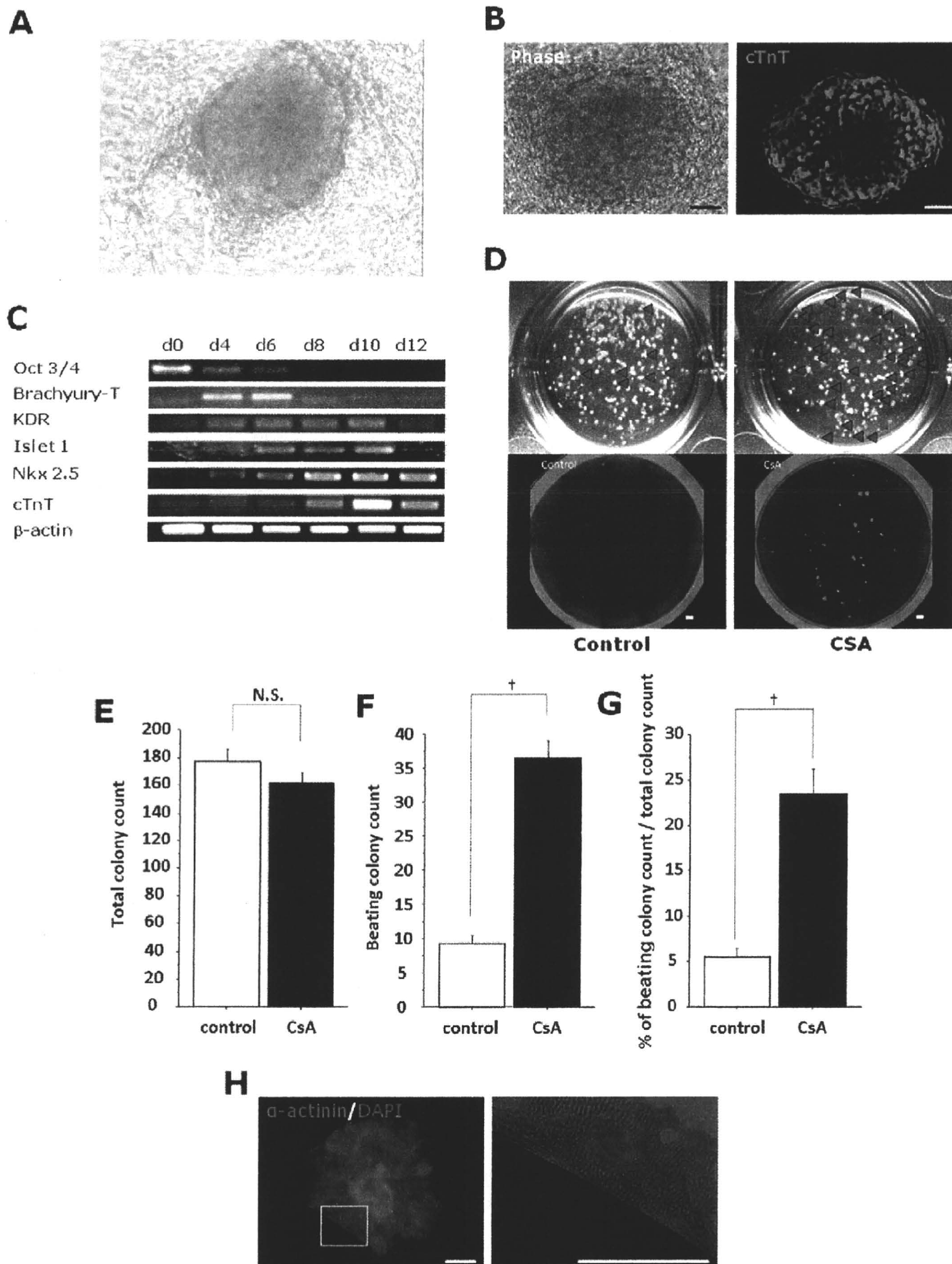

Figure 2. Induction and expansion of cardiomyocytes from human iPSCs. Human iPSCs were co-cultured with END-2 cells to differentiate cardiomyocytes. **A.** Gross morphology of a beating colony from human iPSCs (captured photo from Movie S1). **B.** cTnT staining of a beating colony on END-2 cells. Left 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.

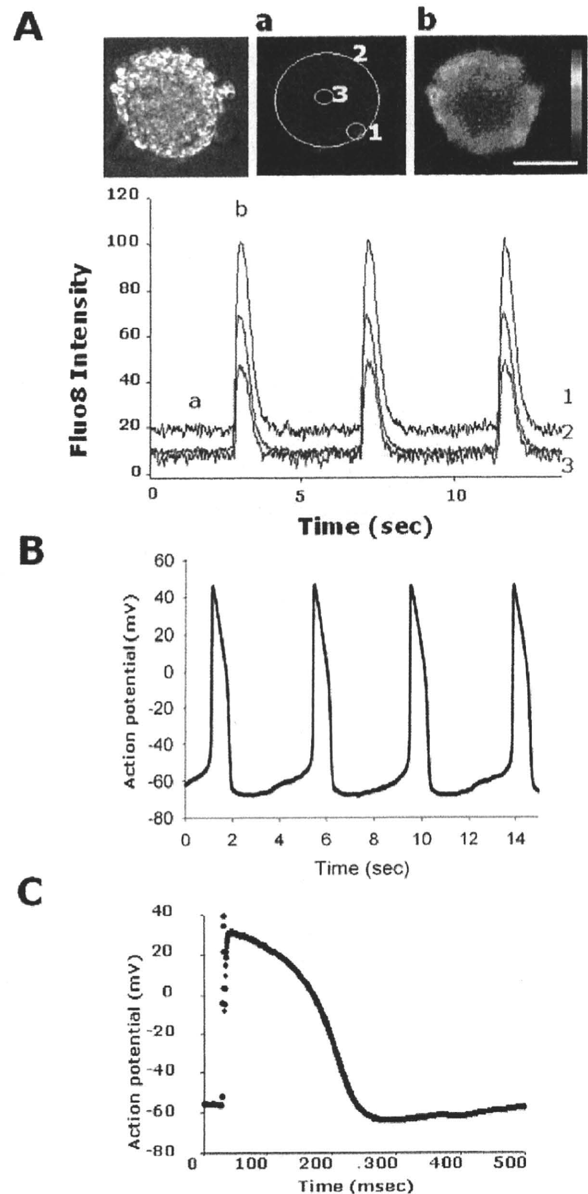

Figure 3. Functional analysis of expanded human cardiomyocytes. A. Ca^{++} transient in dissociated beating colonies. Cytoplasmic Ca^{++} change was monitored with fluo-8. Left panel: a transmission image of fluo-8 loaded iPSC colony. Middle and right panels: Fluo-8 images at the end (a) and the peak (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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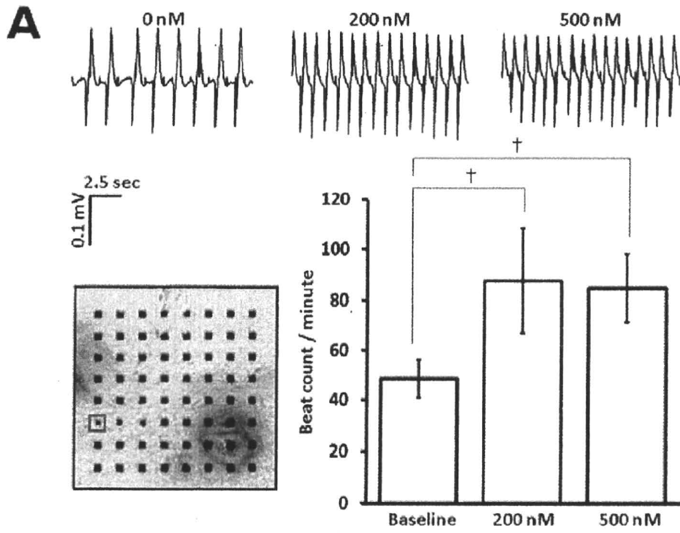
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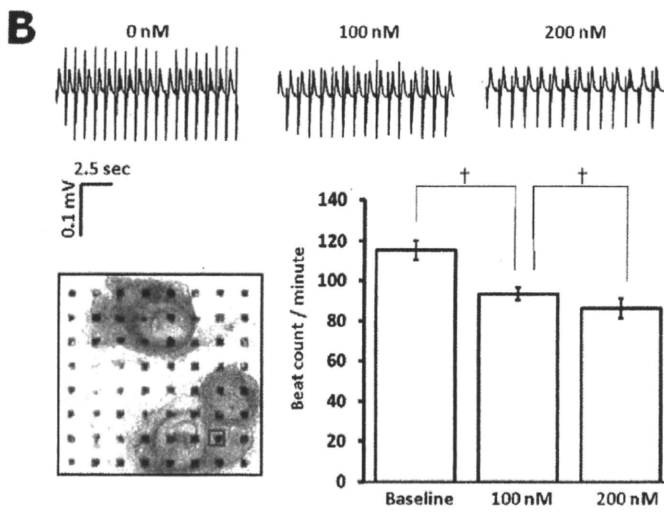
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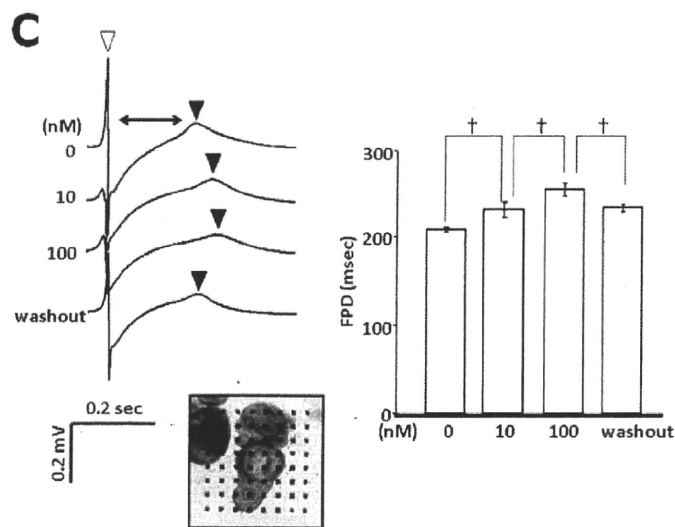
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Figure 4. Pharmacological responses of human iPSC-derived cardiomyocytes. Field potential recordings of replated beating colonies after stimulation with isoproterenol (**A**), propranorol (**B**), and E-4031 (**C**). Photos; array of multi-electrode 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

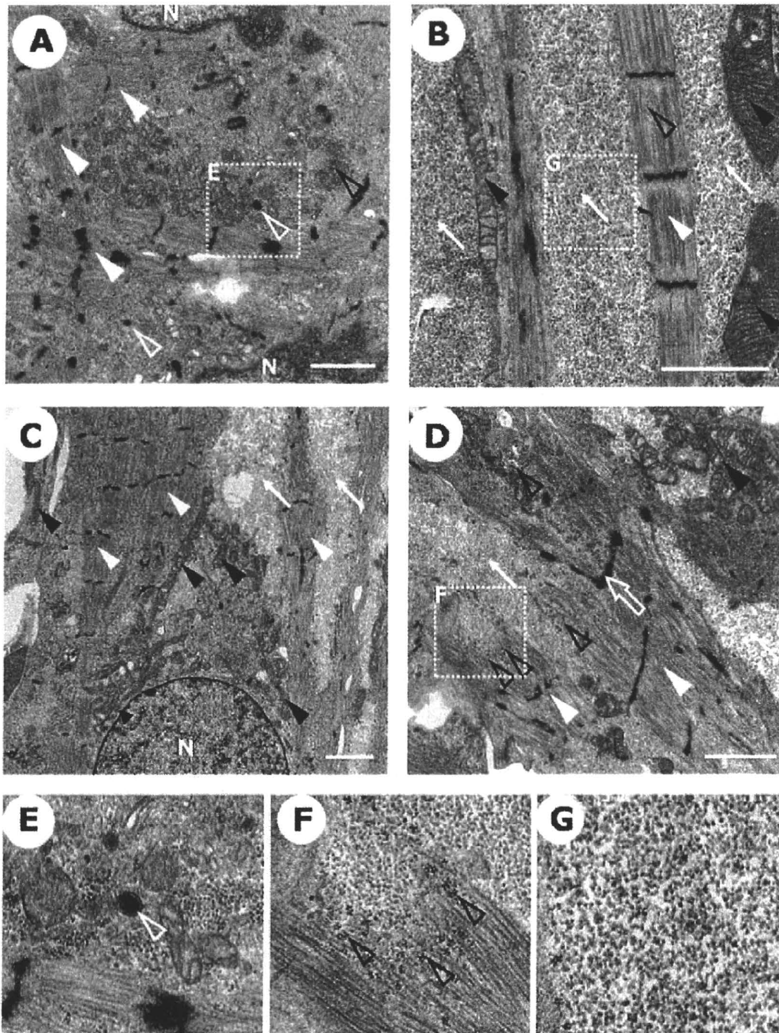

Figure 5. Ultrastructural analysis of human iPSC-derived cardiomyocytes. Transmission electron microscopic images of beating colonies. Myofibrils with Z-bands (white closed arrowheads in **A–D.**), mitochondria (black closed arrowheads in **B–D.**), intercalated disk-like structure with desmosome (white 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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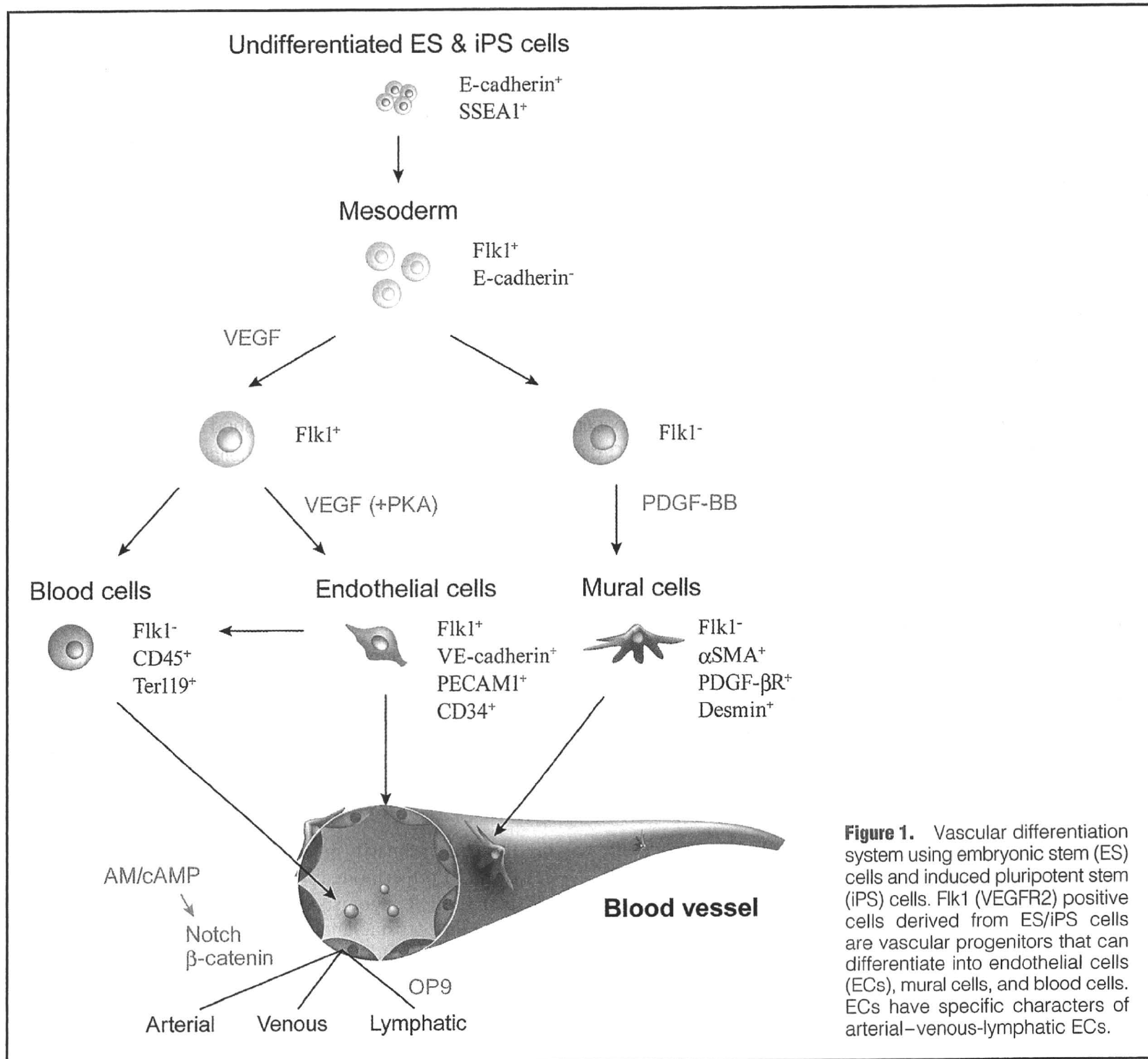

Figure 1. Vascular differentiation system using embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. Flk1 (VEGFR2) positive cells derived from ES/iPS cells are vascular progenitors that can differentiate into endothelial cells (ECs), mural cells, and blood cells. ECs have specific characters 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-A165, an isoform of VEGF, and the Flk1-VEGF-A165–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 VEGF165 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-

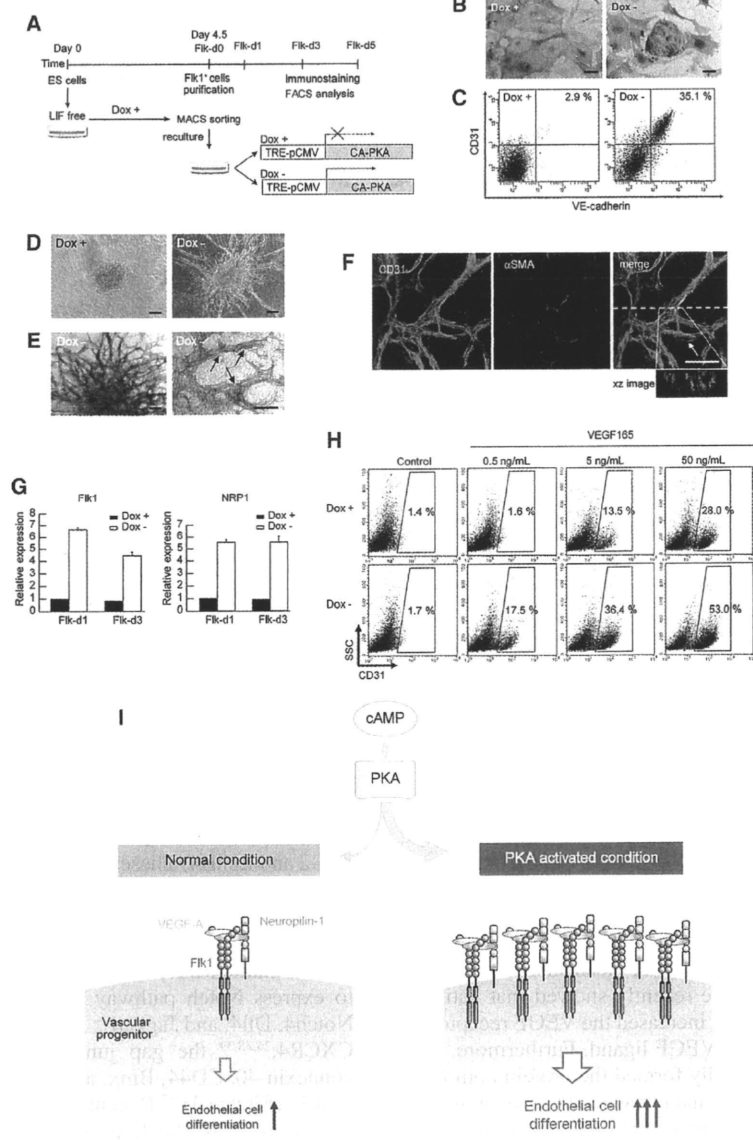

Figure 2. Cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway plays a critical role in vascular development. (**A**) Experimental system for PKA activation. An endothelial cell (EC) cell line expressing the constitutive active (CA) form of PKA by tetracyclin-inducible expression system (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.

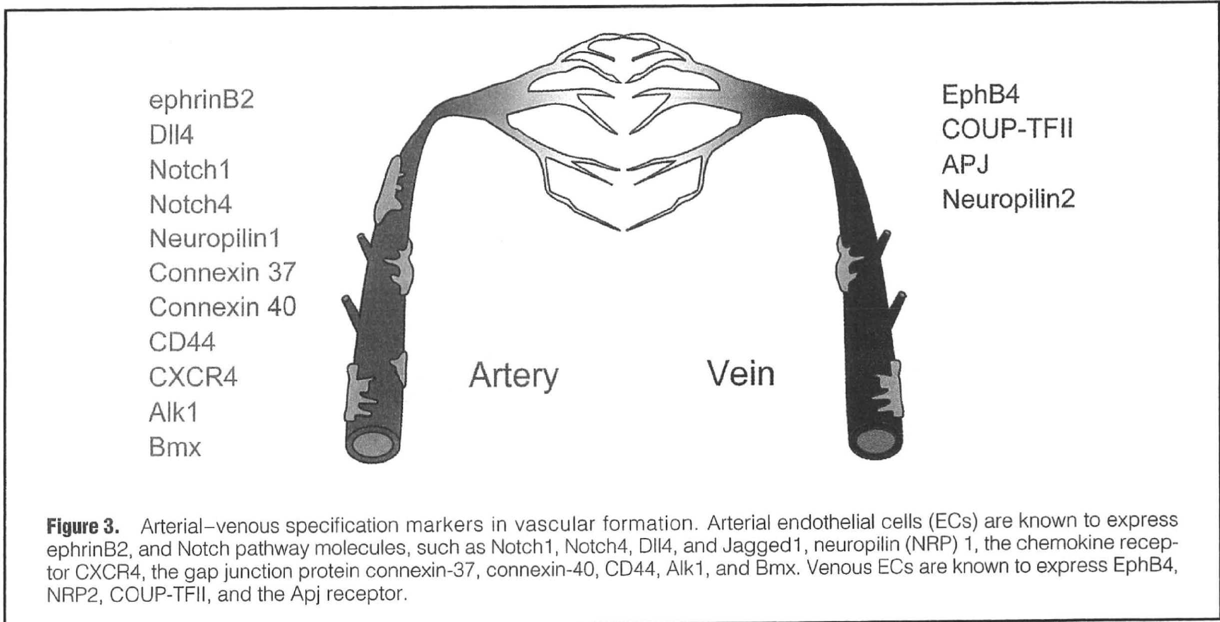

Figure 3. Arterial–venous specification markers in vascular formation. Arterial endothelial cells (ECs) are known to express ephrinB2, and Notch pathway molecules, such as Notch1, Notch4, Dll4, and Jagged1, neuropilin (NRP) 1, the chemokine receptor CXCR4, the gap junction protein connexin-37, connexin-40,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

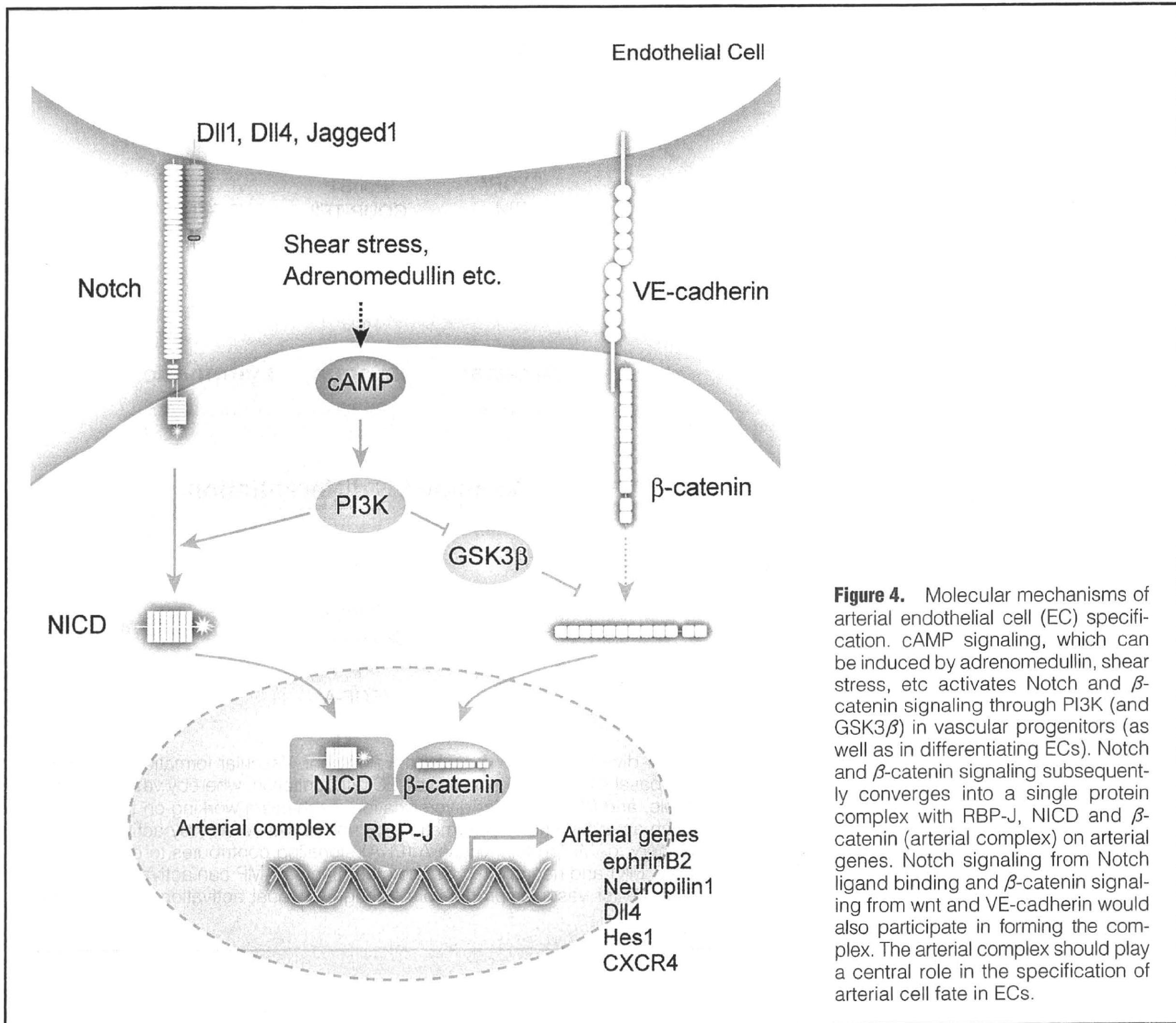

Figure 4. Molecular mechanisms of arterial endothelial cell (EC) specification. cAMP signaling, which can be induced by adrenomedullin, shear stress, etc activates Notch and β-catenin signaling through PI3K (and GSK3β) in vascular progenitors (as well as in differentiating ECs). Notch and β-catenin signaling 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-

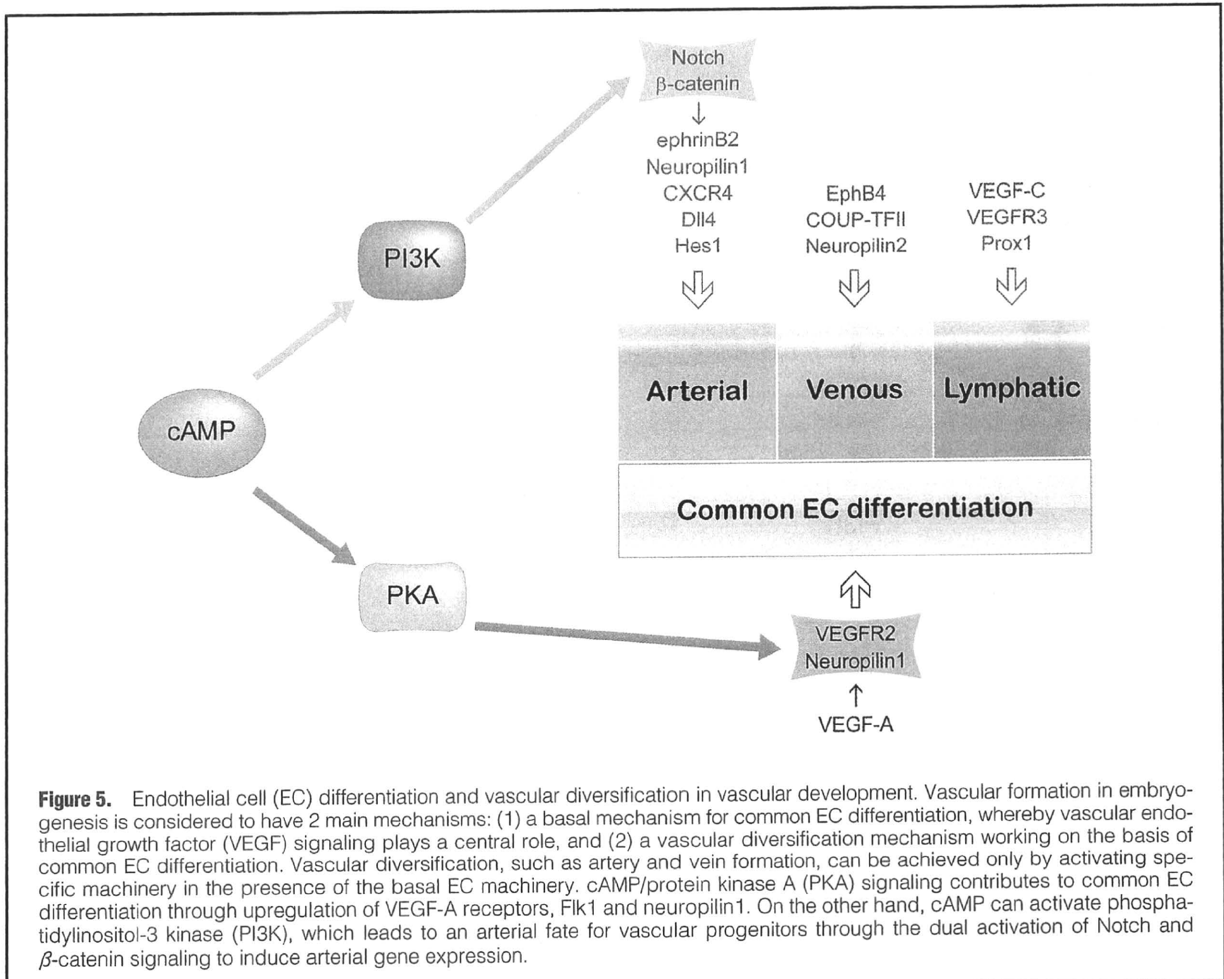

Figure 5. Endothelial cell (EC) differentiation and vascular diversification in vascular development. Vascular formation in embryogenesis is considered to have 2 main mechanisms: (1) a basal mechanism for common EC differentiation, whereby vascular endothelial growth factor (VEGF) signaling plays a central role, and (2) a vascular diversification mechanism working on the basis of common EC differentiation. Vascular diversification, such as artery and vein formation, can be achieved only by activating specific machinery in the presence of the basal EC machinery. cAMP/protein kinase A (PKA) signaling contributes to common EC differentiation through upregulation of VEGF-A receptors, Flk1 and neuropilin1. On the other hand, cAMP can activate phosphatidylinositol-3 kinase (PI3K), which leads to an arterial fate for vascular progenitors through the dual activation of Notch and β-catenin signaling to induce arterial gene expression.

mon role in ECs and a specific role in arterial ECs. A recent study showing that EC-specific β-catenin transgenic mice had enhanced Notch signaling and induced artery formation, also indicated that β-catenin signaling plays an important role in arterial EC specification.[54]

Mechanisms of Vein Formation

Venous ECs are known to express EphB4,[32,55] NRP2,[37] COUP-TFII,[56] and the Apj receptor[57] (**Figure 3**). The vein was considered to be the default character in vascular development. However, You et al reported that the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) acts as an inducer of venous EC specification.[56] COUP-TFII establishes venous identity by suppressing arterial fate through downregulating Notch signaling. Other mechanisms involved in venous specification are still largely unknown.

Environmental Factors and Plasticity of Arterial-Venous Specification

Although the molecular pathways for arterial–venous specification is now becoming clear, we cannot ignore the involvement of physical factors such as flow shear stress. Chick embryo studies have shown that hemodynamic forces can alter EC identity after the onset of circulation. Ligation of specific arteries in the avian embryo can result in a reversible switch from arterial-specific markers, such as ephrinB2 and NRP1, to venous markers such as EphB4.[58] Furthermore, vascular progenitors or early ECs determined the arterial–venous specification by environmental conditions in a quail chick model. That is, the dorsal aorta, carotid artery and the cardinal and jugular veins were isolated with the vessel wall from quail embryos and grafted into the coelom of chick hosts. Early ECs, arterial ECs and venous ECs, can populate both artery and vein in host embryos and assume the appropriate molecular identity in their new locales,[59] indicating that even after the acquisition of arterial or venous identity the endothelium during vascular development remains plastic for arterial–venous differentiation. We also revealed that ECs derived from ES cells and at the early differentiation stage possess plasticity for arterial–venous specification.[13] Activation of Notch and β-catenin signaling in venous ECs derived from ES cells induced ephrinB2-positive arterial ECs. When activation of Notch and β-catenin signaling ceased in arterial ECs, ephrinB2 expression was attenuated and disappeared. Early arterial or venous ECs thus possess plasticity between the arterial and venous identities by the on/off of Notch and β-catenin signaling.[13] This evidence suggests that ECs in the early developmental stage are still plastic, and acquire their diverse identities from physical and environmental factors, as well as genetic factors, which leads to the formation of an elaborate and complex vascular network in the body.

Conclusion

Blood vessels are involved in the formation of most organs during ontogenesis, and they maintain homeostasis in the body. They have an abundance of diversification and play a specific role in respective organs. Our 2 recent studies of cAMP signaling in EC differentiation and other previous works present the concept that vascular formation in embryogenesis has 2 main mechanisms: (1) basal EC differentiation, and (2) vascular diversification in the context of ECs. cAMP signaling has the pivotal abilities of enhancing common EC differentiation through upregulation of Flk1 and NRP1, and determining the fate of arterial EC through dual activation of Notch and β-catenin signaling (**Figure 5**). These findings, however, only partially explain the vascular developmental processes in the body. How is vascular branching determined in vascular development? How are specific blood vessels formed, such as the blood–brain barrier and the blood–placenta barrier? A more detailed investigation into the local environment of ECs, such as interaction between SMCs, pericytes, bloods, and nerves, may lead to a more profound understanding of vascular diversity. Elucidation of all the cellular and molecular machinery of vascular formation will lead to a better understanding of various pathophysiologies, as well as ontogenesis/regeneration, and provide novel strategies for new drugs and future medical therapies.

Acknowledgments

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HEART's Selection

iPS細胞の循環器疾患への応用

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人工多能性幹細胞（induced pluripotent stem cells；iPS細胞）は，線維芽細胞など分化した細胞に特定の遺伝子群を導入することにより，ES細胞様の万能の幹細胞の性質を持たせることに成功した細胞である．最初のiPS細胞は，2006年，京都大学の山中伸弥らによって報告された．2007年には，山中らおよびトムソンらによりヒトiPS細胞の樹立が報告された．

ヒトiPS細胞は，ヒトES細胞において認められた倫理的問題，すなわち，①ヒトES細胞の樹立の際にヒト受精卵を壊す必要がある，②免疫による拒絶を受けないES細胞を樹立するためにヒト体細胞クローン胚（成体細胞の核を除核未受精卵に移植したクローン胚）を作る必要が考えられる，を一気に回避できる画期的発明である．しかし，iPS細胞には依然として技術・安全面の問題，特に未分化細胞が誤って移植されると奇形腫を形成する可能性がある，という問題は存在しているし，iPS細胞特有の「遺伝子導入による細胞変異・がん化の問題」など新たな問題もあり，今後地道に解決すべき課題は多い．

iPS細胞研究の循環器疾患への応用としては，細胞移植治療が主たるターゲットと考えられるが，それ以外にも患者特異的モデル細胞の構築などにより，病態解明や創薬治療応用などさまざまな形で臨床へ貢献することが期待される．本特集は，iPS細胞が循環器医療全体に，実際にはどのようなことをもたらし得るのか，またそれにはどんなハードルがあるのかについて，できるだけ多角的視点から迫ることを目標とした．そこで，①細胞治療を中心としたiPS細胞の心筋再生への応用の試みと，②疾患特異的細胞モデルを用いた研究，さらに③それら細胞の商業レベルでの応用の可能性と，④再生医療実現のために必要とされる制度面の問題，をそれぞれのフロントランナーの方々に解説いただいた．iPS細胞研究の現状と未来について，多次元的位置づけと包括的理解に貢献できれば幸いである．

HEART's
Selection 1

iPS細胞の循環器疾患への応用

iPS細胞を用いた心筋再生

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マウスES/iPS細胞の心血管細胞分化研究

筆者らは，これまでマウスおよびヒトES細胞を用いて心血管細胞の分化再生研究を行ってきた．すなわち，マウス未分化ES(embryonic stem)細胞から血管内皮増殖因子(vascular endothelial growth factor；VEGF)の受容体の1つであり，血管内皮・血球の前駆細胞や中胚葉細胞のマーカーでもあるFlk1(2型VEGF受容体：VEGF receptor-2)を発現する細胞を誘導し，Flk1陽性細胞を共通の前駆細胞として，血管内皮細胞，血管壁細胞，血球細胞，心筋細胞といった循環器系細胞を系統的に分化誘導することに成功している[1)2)]．この新しい分化誘導システムを用いて，ES細胞由来の心筋前駆細胞の同定[2)]や，動静脈リンパ管内皮細胞をそれぞれES細胞から誘導すること[3)4)]にも成功している．ヒトES細胞からの血管細胞の誘導や虚血モデルへの移植実験などにも関与している(京都大学内分泌代謝内科との共同研究)．このマウスES細胞の系統的心血管細胞分化システムをマウスiPS細胞に導入し，筆者らは，いち早くiPS細胞からのこれら心血管細胞の分化誘導に成功した[5)]．すなわち，未分化マウスiPS細胞をLIF(leukemia inhibitory factor)非存在下に培養することによりFlk1陽性細胞が誘導された．Flk1陽性細胞をVEGFおよび血清存在下に培養することにより，主に静脈を中心とする内皮細胞および壁細胞が，VEGFに加えてcAMPシグナルを刺激することにより動脈内皮細胞が，OP9ストローマ細胞上で培養することにより血球，リンパ管内皮細胞，心筋細胞が，それぞれ誘導された．誘導された心筋細胞は，種々の心筋細胞マーカー発現やsarcomere構造，心筋様活動電位などの心筋細胞としての特性を示した．また，ペースメーカー細胞特異的イオンチャネルHCN4，T型Caチャネル(Cav3.2)や心室筋特異的チャネルKir2.1を発現する細胞など[6)]が存在し，種々の心筋細胞が混在して誘導されていると考えられた．マウスiPS細胞からのFlk1陽性細胞，(動静脈リンパ管)内皮細胞，壁細胞の分化様式，分化効率などはほとんどマウスES細胞と変わりがなかった．このように，マウスES細胞とマウスiPS細胞はほぼ完全に同等な心血管分化能と分化動態を示し，マウスES細胞と同様に系統的に心血管細胞を分化誘導することが可能であった(**図1**)．ほかにマウスiPS細胞からの心血管細胞分化に関しては，心筋細胞を誘導したとの報告[7)]および筆者らに類似した分化システムを用いた心血管細胞分化の報告[8)]などがある．

ヒトES/iPS細胞の心筋細胞分化

ヒトES細胞は，1998年米国James Thomsonらによって樹立されたが，マウスES細胞とは，①維持培養に必要とされるシグナルが異なる，②単一細胞からコロニーを形成しにくい，③フィーダー細胞なしでの培養が難しい，④相同組み換えなどの遺伝子導入が困難である，⑤導入遺伝子のサイレンシングがしばしば起こる，⑥分化誘導にも時間がかかる，⑦単一細胞からの分化も困難である，等々性質上のさまざまな相違点があり，そのいずれもがヒトES細胞を用いた研究開発をより困難なものとしている．ヒトiPS細胞は，ヒトES細胞とその維持方法，分化などの性質はほぼ同等と考えられる．ヒトiPS細胞の応用には，こうした基本的な技術的問題が解決されていく必要がある．

ヒトES細胞からの心筋分化誘導は，胚様体を用い

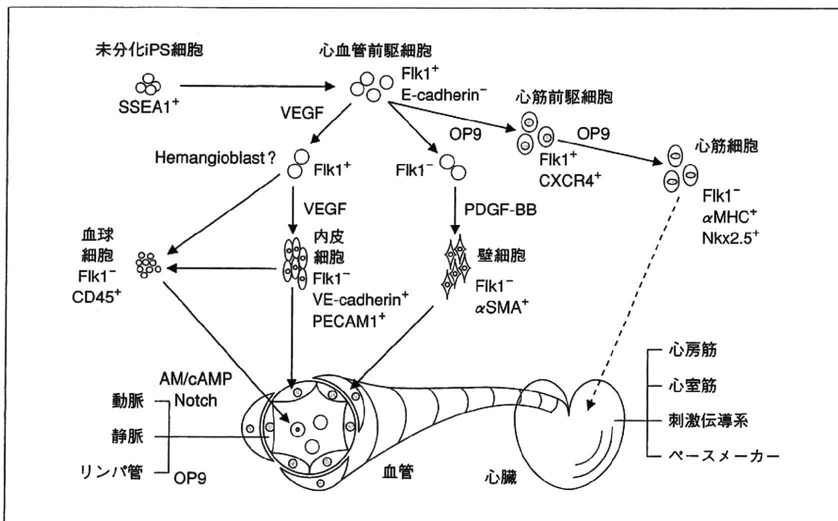

図1
マウスiPS細胞からの系統的心血管細胞分化
マウスiPS細胞から誘導したFlk1陽性細胞を共通の前駆細胞として，心血管系の構成細胞である血管内皮，壁細胞，心筋細胞，さらには動静脈リンパ管内皮細胞や種々の心筋細胞を系統的に分化誘導することができる．
（文献5より改変引用）

た方法やストローマ細胞との共培養（END2細胞）などにより報告されている[9]．その後，胎生期の中胚葉誘導シグナルや内皮細胞・心筋細胞誘導シグナルをさまざまに組み合わせ分化誘導法の改善が試みられている[10)11]．マウスES細胞に準じた形でヒトFlk1（KDR）陽性細胞[12]やislet1陽性細胞[13]が心血管系の共通の前駆細胞として機能することも示されている．現在中胚葉系列への特異的誘導と心筋分化シグナルを組み合わせて70～80％の心筋分化誘導効率というのがヒトES細胞で報告されている（Keller，国際幹細胞学会，2010，San Francisco）．筆者らは，END2細胞を用いたヒトES細胞心筋分化誘導法に準じて培養することにより，自己拍動する心筋細胞コロニーの誘導にすでに成功している．心筋に特徴的なsarcomereの形成や自己拍動に同調したCa^{2+}の取り込みなど形態的機能的特性も確認している（未発表）．また胚様体（embryoid body）法を用いてヒトiPS細胞から心筋を誘導した報告が，2009年2月に最初になされ[14]，その後，日本などから胚様体法を用いて薬剤に反応する心筋細胞を誘導したことが報告されている[15)16]．ヒトES細胞で開発された手法を導入し，ヒトiPS細胞からの心筋細胞誘導はさらに改善されていくものと考えられる．

iPS細胞研究の臨床への貢献

ES細胞，iPS細胞研究の循環器領域における意義は，やはり心血管再生治療への応用が中心的に期待されると考えられるが，それ以外にも患者特異的モデル細胞の構築という新しいアプローチができることにより，病態解明や創薬治療応用など，さまざまな形での臨床面への貢献が可能である．

1．誘導細胞の細胞移植応用

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

①効率的心血管分化誘導法および純化法の開発

ヒトの心筋梗塞においては10^9個オーダーにいたる心筋細胞が死ぬともいわれている．その10分の１量の細胞の補充を行うとしても，10^7から10^8オーダーの細胞数を用意することが可能な効率的誘導法を開発する必要がある．また，未分化ヒトiPS細胞が移植されると，ヒトES細胞と同様に奇形腫を形成するので，未分化ヒトiPS細胞を厳密に除去できる細胞純化法が必要である．心筋細胞に関しては，ミトコンドリアをラベルする蛍光色素により，iPS細胞由来心筋が純化できることが示されている[17]．

②移植用細胞の開発

最終的にヒトに対して細胞を移植するためには，単に細胞を誘導して純化するということだけでは不十分で，技術的にはGMP基準の医薬品と同様な品質管理のもとに移植用細胞を用意できることを目指す必要がある．もとになるiPS細胞から血清やフィーダー細胞などを極力排除して，分化誘導・純化が行えるようにする必要があり，①から②の間には実は大きな隔たりがある．またES/iPS細胞由来細胞の移植に関する法や手続き上の体制整備を行うこともこうした細胞治療の実現には不可欠である(Selection 4参照)．

③細胞移植法の開発

①，②を経て用意された細胞をヒトに移植する際に，いかなる細胞群をどのような方法をもって移植すれば，有効かつ安全であるかを評価していく必要がある．最近，ES細胞から誘導された心筋細胞の移植に関しては，単純に細胞懸濁液を作製して心臓に注入するだけでは，細胞が失われたり死滅したりして生着効率が非常に悪いということがコンセンサスとなりつつある．ある一定以上の細胞を集団として移植する必要があると考えられ，現在，主に２つの方法，①東京女子医科大学で開発された細胞シート作製技術を用いた心筋細胞シートの移植(心臓に貼り付ける)[18]，および②心筋細胞の浮遊培養により得られる心筋細胞塊(cardiosphere)[19]の移植が試みられている．いずれの方法でも生着心筋効率は改善していると思われる．機能的回復が十分なレベルまで技術が発展することが期待される．現在筆者らはマウスES細胞から誘導した心筋細胞を用いた心筋組織シートの開発を行っており，心筋梗塞モデルへの移植により明らかに心機能の改善が認められる予備的結果を得ている．同モデルをヒトiPS細胞へ拡張していくことが望まれる．

2．患者特異的モデル細胞

患者自身から細胞を採取し，患者特異的なiPS細胞を樹立できるというiPS細胞にしかない特性は，移植免疫を回避した細胞治療ということだけでなく，全く新しい形で病態の解明や創薬への応用が可能である[20][21] (Selection 2, 3参照)．

①病態解明

患者自身の細胞からiPS細胞を樹立し，そこから該当する細胞を分化誘導し種々のモデル細胞を構築できることは，病態解明に全く新しい手段を提供する．しかし実際には，モデル細胞を樹立するために多数のiPS細胞を樹立・解析する必要があること，そのようにして構築したモデル細胞が遺伝子異常や病態を反映した振る舞いを示すかどうかは不明であること，など未知数の部分も多い．

②創薬応用

iPS細胞の創薬応用には大きく，①新規薬剤の探索と，②薬剤安全性試験への応用の２つが考えられる．

① 疾患モデル細胞を用いて，同細胞の異常を改善する新規薬剤や疾患特異的に作用する薬剤などの探索が可能となる．筆者らは最近，筆者らのES細胞心筋分化系を用いて免疫抑制薬サイクロスポリンＡが中胚葉段階に特異的に作用し強力な心筋前駆細胞および心筋細胞分化誘導作用を有することを見出した(**図２**)[22]．こうした培養下における分化モデルを用いることにより，心筋分化促進物質などの新たな生理活性物質の探索も可能となる．実際，筆者らは，種々の化合物ライブラリーのスクリーニングを行い，心筋分化促進作用を有する化合物や心筋の分裂増殖を促進する物質など，種々の化合物の同定に成

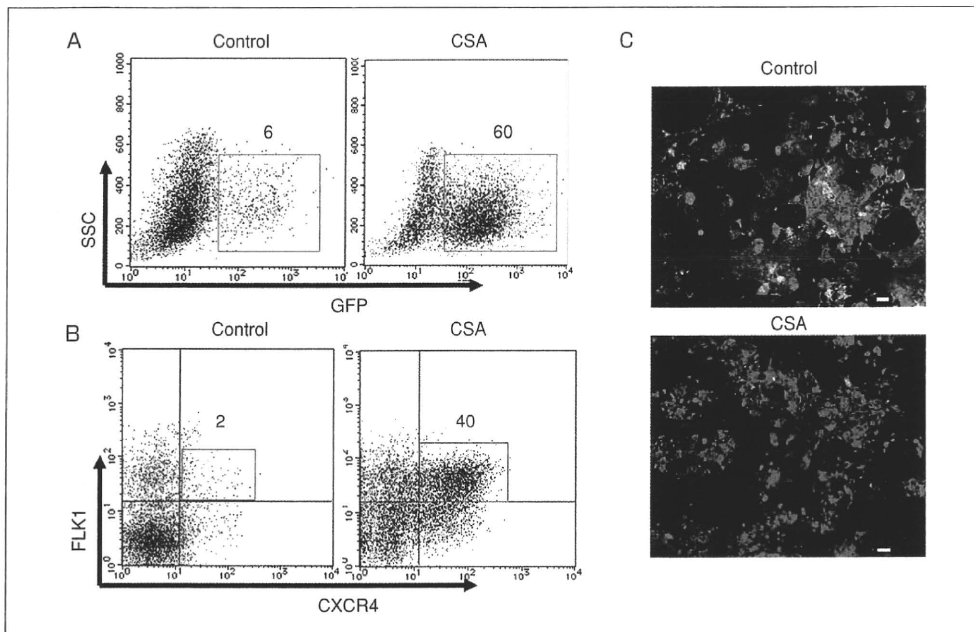

図2 サイクロスポリンA(CSA)による心筋前駆細胞および心筋細胞分化誘導効果

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

A，B：FACS解析．A；心筋特異的GFP(αMHCプロモーター/GFP)発現．誘導された細胞の約60%がαMHC/GFP陽性の心筋細胞になる．B；心筋前駆細胞．Flk1陽性/CXCR4陽性の心筋前駆細胞分画(文献2)が約20倍増加する．

C：免疫染色．CD31(赤；内皮細胞)/cTnT(緑；心筋細胞)2重染色．CSAにより内皮細胞の出現が抑制され，対照的に心筋細胞が著しく増加する．

(文献22より改変引用)

功している(未発表)．これらの中から生体においても効果を発揮するものがみつかれば，新しい心臓再生薬の誕生につながるかもしれない．

② 受精卵を用意することが必要であるヒトES細胞と比べて，iPS細胞は数多くの細胞株を樹立しやすくiPS細胞バンクが構築しやすい．そこから細胞を誘導して，種々のヒトモデル細胞パネルのようなものを構築することにより，薬剤の安全性試験に応用可能と考えられる．さらには，障害を起こす細胞を解析し原因を明らかにすることにより，副作用を起こす症例を事前に特定し投薬を避ける「テーラーメード医療」に貢献し得る可能性もある．

おわりに

iPS細胞が樹立されてから，マウスでは4年，ヒトでは3年が経ち，樹立，分化，移植などにおけるさまざまなiPS細胞に関する研究や，「分化した細胞を別の細胞にリモデリングする」というiPS細胞樹立の技術を応用した新しい研究が急速に進みつつある．例えば，樹立されたiPS細胞の株により奇形腫の形成性は異なり，分化誘導を行っても分化せずに未分化状態にとどまろうとする「分化抵抗性」のiPS細胞は，移植後に奇形腫を形成する頻度が高いことが報告された[23]．またiPS細胞は，樹立に使われたもとの細胞の性質をある程度保持しており，分化誘導させた時