Biochemical and Biophysical Research Communications 379 (2009) 115–120

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cyclosporin-A potently induces highly cardiogenic progenitors from embryonic stem cells

Peishi Yan [a,b], Atsushi Nagasawa [b], Hideki Uosaki [a], Akihiro Sugimoto [b], Kohei Yamamizu [a], Mizue Teranishi [a], Hiroyuki Matsuda [c], Satoshi Matsuoka [c], Tadashi Ikeda [b], Masashi Komeda [d], Ryuzo Sakata [b], Jun K. Yamashita [a,e,*]

[a] 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
[b] Department of Cardiovascular Surgery, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
[c] Department of Physiology and Biophysics, Kyoto University Graduate School of Medicine, Yoshida-Konoe, Sakyo-ku, Kyoto 606-8501, Japan
[d] Nagoya Heart Center, Japan
[e] Center for iPS Cell Research and Application, Institute for Integrated Cell-Material Sciences, Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

ARTICLE INFO

Article history:
Received 23 November 2008
Available online 16 December 2008

Keywords:
Cyclosporin-A
Cardiac progenitor
Cardiomyocytes
Differentiation
Embryonic stem cells

ABSTRACT

Though cardiac progenitor cells should be a suitable material for cardiac regeneration, efficient ways to induce cardiac progenitors from embryonic stem (ES) cells have not been established. Extending our systematic cardiovascular differentiation method of ES cells, here we show efficient and specific expansion of cardiomyocytes and highly cardiogenic progenitors from ES cells. An immunosuppressant, cyclosporin-A (CSA), showed a novel effect specifically acting on mesoderm cells to drastically increase cardiac progenitors as well as cardiomyocytes by 10–20 times. Approximately 200 cardiomyocytes could be induced from one mouse ES cell using this method. Expanded progenitors successfully integrated into scar tissue of infracted heart as cardiomyocytes after cell transplantation to rat myocardial infarction model. CSA elicited specific induction of cardiac lineage from mesoderm in a novel mesoderm-specific, NFAT independent fashion. This simple but efficient differentiation technology would be extended to induce pluripotent stem (iPS) cells and broadly contribute to cardiac regeneration.

Embryonic stem (ES) cell research has been providing various possibilities and strategies for regenerative medicine. Establishment of adult tissue derived, ES cell-like, stem cells, induced pluripotent stem (iPS) cells, is now further facilitating research for clinical application of stem cells [1–4]. Various ES cell studies showed induction of cardiomyocytes from ES cells and their contribution to cardiac tissues after transplantation [5]. Nevertheless, cell transplantation strategies of purified cardiomyocytes have not been fully established, mainly due to the limited proliferative potential and engraftment efficacy of differentiated cardiomyocytes [6,7]. Cardiac progenitors which can proliferate and give rise to cardiomyocytes should be suitable material to achieve efficient cardiac regeneration [8,9]. Methods to efficiently and specifically expand and purify cardiac progenitor cells, however, have not been reported.

Previously, we established a novel ES cell differentiation system that can reproduce the early process of cardiovascular development in vitro [8,10]. Endothelial cells (ECs), pericytes and vascular smooth muscle cells [10], and cardiomyocytes [8] are systematically induced from common mesodermal precursor, Flk1 (also designated as vascular endothelial growth factor receptor-2 (VEGFR2))-expressing cells. We also succeeded in identifying a cardiac progenitor population, FCV cells (Flk1$^+$/CXCR4$^+$/vascular endothelial cadherin$^-$ cell population) among the progeny of Flk1$^+$ mesoderm cells [8]. Though FCV cells, which are only a small subset of Flk1$^+$ cell progeny, showed highly cardiac specific progenitor activity, efficient expanding method of FCV cells remain unknown.

To explore a novel cardiac regenerative therapy, we investigated methods to efficiently induce cardiac progenitor cells and cardiomyocytes with the use of our ES cell differentiation system. Here we show that addition of an immunosuppressant, cyclosporine-A (CSA), to Flk1$^+$ mesoderm cells potently and specifically expand FCV progenitor population as well as cardiomyocytes. Expanded FCV cardiac progenitors showed differentiation potentials to cardiomyocytes in vivo after cell transplantation to rat myocardial infarction model. Discovery of the novel role of CSA

* Corresponding author. Address: 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. Fax: +81 75 751 4824.
E-mail address: juny@frontier.kyoto-u.ac.jp (J.K. Yamashita).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.12.019

with mesoderm-specific cardiogenic activity would provide a clue to explore cardiac regeneration strategies.

Materials and methods

Antibodies. Monoclonal antibodies (MoAbs) for murine E-cadherin (ECCD2), murine Flk1 (AVAS12) were prepared and labeled in our laboratory as described [8,10]. MoAb for cardiac troponin-T (cTnT) was purchased from NeoMarkers (Fremont, CA). Murine α-actinin was from Sigma (St. Louis, Mo). Phycoerythrin (PE)-conjugated AVAS12 was from eBioscience (San Diego, CA). MoAbs for mouse CD31 and biotinylated-CXCR4 were purchased from BD Pharmingen (San Diego, CA). Polyclonal rabbit antibody to GFP was from MBL (Nagoya, Japan).

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 (see below) at the time of use. FK506 (a gift from Astellas Pharma) was dissolved in DMSO at 10 mg/mL, dilution of 10 ng–1 μg/mL were made in differentiation medium. 11R-VIVIT was from Calbiochem (Darmstadt, Germany). PKH67 fluorescent dye was from Sigma.

Mouse ES cell culture. EMG7 mouse ES cells that carry mouse α-myosin heavy chain (MHC) promoter-driven EGFP gene were used for this study [8]. OP9 stroma cells were maintained as described [11].

Induction of cardiomyocyte differentiation. Induction and sorting of Flk1$^+$ cells were performed as previously described [8,10]. Briefly, mouse ES cells were cultured in differentiation medium (alpha minimum essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum) on type IV collagen-coated dishes (BIOCOAT™, Becton–Dickinson) or mitomycin C-treated confluent OP9 cell sheets (MMC-OP9) for 96–108 h. FACS-purified Flk1$^+$ cells were then plated onto MMC-OP9 and cultured in differentiation medium to induce cardiac differentiation. Medium was replaced every 2 days. Induction of FCV cells was performed as described [8]. Purified FCV progenitor cells were again plated onto MMC-OP9 cells. MMC-OP9 cells were pre-stained with PKH67 fluorescent dye (Sigma) before plating.

Flowcytometry and cell sorting. FACS for differentiating ES cells was performed as described [8,10]. After 96–108 h of ES cell 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 Vantage (Becton–Dickinson). For FACS analysis of FCV progenitor cells, after 2 days differentiation of purified Flk1$^+$ cells on OP9 cells (Flk-d2), cells were harvested and stained with a combination of MoAbs of PE-conjugated AVAS12 and biotinylated CXCR4 followed by addition of streptavidin-conjugated APC. PKH-negative populations were analyzed as ES cell-derived cells. For cardiomyocytes, cells were harvested at Flk-d6. GFP$^+$ population was evaluated and sorted as differentiated cardiomyocytes.

Immunohistochemistry. Immunostaining for ECs and cardiomyocytes was performed as described [8]. Anti-mouse IgG antibody conjugated with horse radish peroxidase (HRP) (Invitrogen, Carlsbad, California) or anti-mouse, rat and Rabbit Ig Abs conjugated with Alexa 488 or 546 (Molecular Probes, Eugene, OR) were used for 2nd Abs. Nuclei were visualized with DAPI (Invitrogen). Preparation of rat heart sections was performed as previously described [12]. Double staining for cTnT and GFP was performed with mixture of anti-cTnT Ab labeled with Zenon Alexa Fluor 546 labeling kit (Molecular Probes) and anti-GFP Ab, followed by Alexa Fluor488-conjugated anti-Rabbit Ig Ab (Molecular Probes).

Quantification of cardiomyocyte differentiation. Cardiomyocyte differentiation was quantitatively evaluated by fluorescent intensity of cTnT staining using Alexa Fluor 546 as described [8].

Reverse-transcription polymerase chain reation (RT-PCR). RT-PCR was performed as previously described [8]. Primers that were used are indicated in supplemental Table I online.

Annexin-V FITC apoptosis assay. Cells were harvested at Flk-d2 and stained for Flk1 and CXCR4. After addition of Annexin-V FITC (Invitrogen), cells were incubated for 15 min at room temperature in the dark and subjected to FACS analysis.

EdU cell proliferation assay. EdU solution (Invitrogen) (10 μM) was added to the culture medium 2 h before sorting FCV cells by FACS. The sorted FCV cells were plated onto glass slides by Cytospin (Thermo Shandon) (Waltham, MA), fixed by 4% PFA and EdU was detected by incubating with Click-iT™ reaction cocktail (Invitrogen) according to the manufacturer's instructions. The numbers of EdU positive DAPI positive FCV cells were counted in 10 randomly selected fields.

Analysis of induced endothelial and blood cells. At Flk-d6, floating cells and attached cells were collected and stained with biotin-conjugated anti-CD45 Ab or anti-CD31 Ab (BD Pharmingen) followed by addition of streptavidin-APC.

Model of heart failure. Ligation of rat left coronary artery was performed as described [12] in accordance with the guidelines for Animal Experiments of Kyoto University, which conforms to the law of "Guide for the Care and Use of Laboratory Animals" in Japan.

ES cell transplantation. Four weeks after ligation, nude rats with moderate-sized myocardial infarction (MI) (infarct size: 20–40%) were used for cell transplantation experiment. Through left thoracotomy, after putting mattress sutures with 6.0 polypropylene thread at injection points to prevent leakage, 100 μL culture medium containing 4×10^5 cells were subepicardially injected into the center of the scar using a 27-gauge needle. Bulging over the MI area was confirmed after injection [12].

Electrophysiological studies. Action potentials of FACS-purified GFP positive cardiomyocytes were recorded as previously described [13]. All experiments were performed at 36–37 °C.

Statistical analysis. Values are reported as means ± SD. Comparisons among values were performed by ANOVA. At least three independent experiments were performed. $p < 0.05$ was considered significant.

Results

In our differentiation system, cells representing four different differentiation stages are sequentially induced, that is, undifferentiated ES cells, Flk1$^+$ mesoderm cells, FCV cardiac progenitor cells, and cardiomyocytes. When purified Flk1$^+$ cells are cultured on OP9 stroma cells, self-beating cardiomyocytes appear after 4 days of culture (Flk-d4) [8]. When we were performing transplantation experiments in which purified cardiomyocytes were injected into immune-competent mice treated with CSA to suppress immune rejection, we examined the direct effects of CSA on cardiomyocyte differentiation and survival in vitro in a control experiment. Surprisingly, addition of CSA (1–3 μg/mL) to Flk1$^+$ cells showed a striking effect to increase beating cells at Flk-d6 (experiment 1 in supplemental figure 1 online) (Fig. 1A, and supplemental video 1 and 2 online). Addition of CSA induced approximately 13 times increase in cardiac troponin-T (cTnT)-positive cardiomyocyte appearance than control (Fig. 1B and C). CSA-induced cardiomyocytes showed distinct expression of cTnT (Fig. 1D), and sarcomere formation (Fig. 1E). Action potential in purified cardiomyocytes showed existence of cells with pacemaker potential (Fig. 1F), as well as ventricular type cells lacking pacemaker potential and

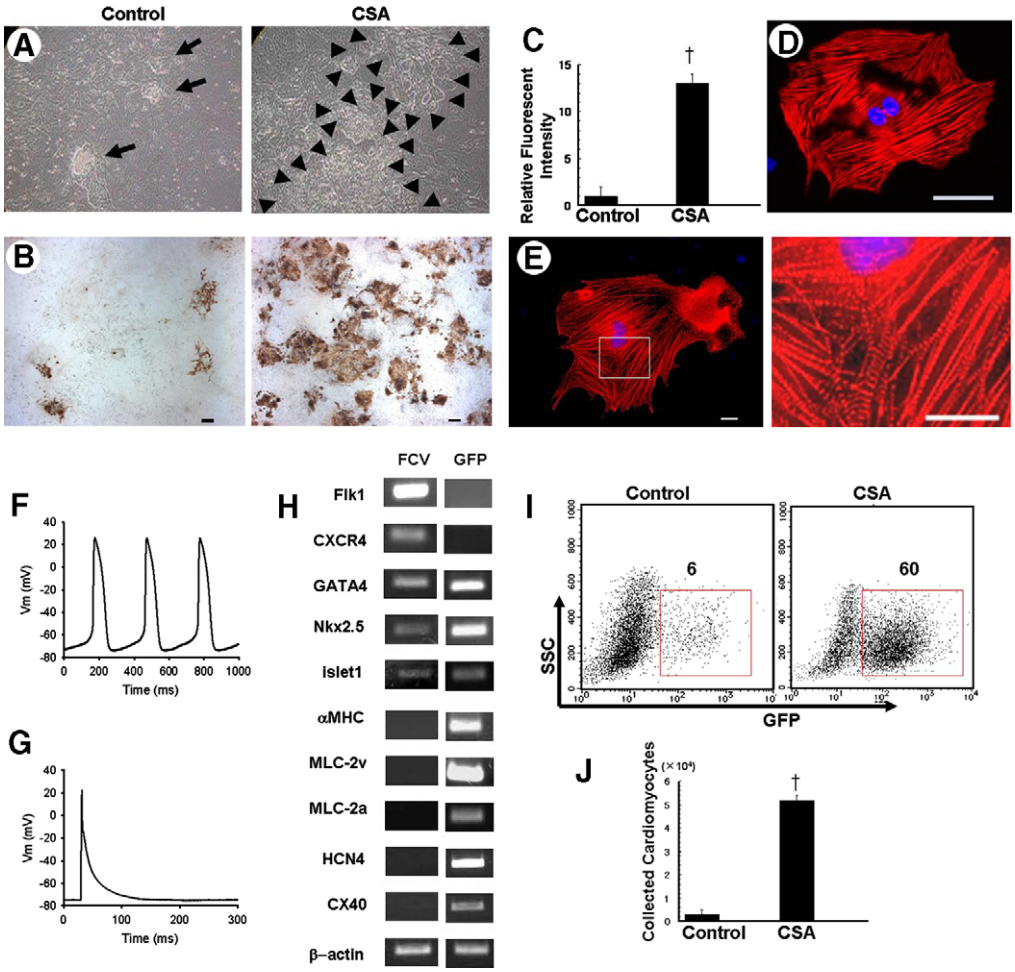

Fig. 1. Cardiomyocyte induction from Flk1$^+$ mesoderm by CSA (experiment 1). (A,B) Gross appearance of cardiomyocyte induction by CSA. Left panels: control. Right panels: CSA treatment. Scale bars = 100 μm. (A) Appearance of beating colony. Arrows indicate beating colonies. Arrow heads surround large beating area (see supplemental video 1 and 2 online). (B) Cardiomyocyte stained with cTnT (brown). (C) Quantitative evaluation of cardiomyocyte induction by fluorescent intensity of cTnT staining. Relative fluorescent intensity is indicated ($n = 9$, $^{\dagger}p < 0.001$ vs control). (D,E) Appearance of isolated cardiomyocytes. (D) cTnT (red) and DAPI (blue) staining. Scale bar = 50 μm. (E) Actinin (red) staining. Right panel shows higher magnification of boxed area. Apparent sarcomere structures are observed. Scale bar = 25 μm. (F,G) Action potentials of induced cardiomyocytes. (F) Cell with pacemaker potential and spontaneous beating. (G) Quiescent ventricular type cell. (H) mRNA expressions in purified FCV progenitor cells and MHC promoter-driven GFP$^+$ cardiomyocytes induced by CSA. (I) FACS analysis for GFP$^+$ cardiomyocyte induction by CSA. Left panel: control. Right panel: CSA treatment. *X* axis: GFP. *Y* axis: side scatter. All Flk1$^+$ cell-derived population was analyzed. Percentages of GFP$^+$ cardiomyocytes are indicated. (J) Yield of purified cardiomyocytes. Cell number of obtained GFP$^+$ cardiomyocytes from 10^4 Flk1$^+$ cells ($n = 12$, $^{\dagger}p < 0.001$ vs control).

self-beating (Fig. 1G).Purified CSA-induced cardiomyocytes showed expression of various cardiac marker mRNAs, such as GATA4, Nkx2.5, islet1, alpha-myosin heavy chain (MHC), myosin light chain (MLC) 2v, and 2a, a pacemaker marker, HCN4, and conduction system marker, Cx40 (Fig. 1H). These results indicate that functional cardiomyocytes were successfully induced and expanded by CSA treatment. At optimal conditions, approximately 60% of Flk1$^+$ cell-derived cells became cardiomyocytes, positive for MHC promoter-driven GFP (GFP$^+$) (Fig. 1I). The CSA treatment resulted in approximately 17-fold increase in the yield of FACS-purified cardiomyocytes starting from the same number of Flk1$^+$ cells (CSA-treatment: $5.0 \pm 0.35 \times 10^4$ cells/10^4 Flk1$^+$ cells vs. control: $0.3 \pm 0.023 \times 10^4$ cells/10^4 Flk1$^+$ cells, $n = 12$, $^{\dagger}p < 0.001$) (Fig. 1J). As a result, approximately 200 cardiomyocytes could be obtained from one ES cell (Supplemental figure 2 online).

We further evaluated differentiation stage-specific effects of CSA. CSA did not have any influence on Flk1$^+$ mesoderm cell appearance from undifferentiated ES cells (Fig. 2A, experiment 2). No apparent difference in endoderm and ectoderm marker expression was induced by CSA treatment (Fig. 2B). Surprisingly, addition of CSA to Flk1$^+$ cells specifically increased FCV population to approximately 10–20 times more than control (experiment 3) (Fig. 2C). The maximum percentage of FCV cells within total Flk1$^+$ cell-derived cells was increased up to 40% by CSA. The yield of purified FCV progenitor cells was increased approximately 22 times by CSA treatment (CSA-treatment: $4.3 \pm 0.23 \times 10^3$ cells/10^4 Flk1$^+$ cells vs. control: $0.2 \pm 0.001 \times 10^3$ cells/10^4 Flk1$^+$ cells, $n = 12$, $^{\dagger}p < 0.001$) (Fig. 2D). Purified CSA-induced FCV cells showed expression of various cardiac progenitor marker mRNAs, such as Flk1, CXCR4, GATA4, Nkx2.5, islet1, but not differentiated cardiomyocyte markers, such as MHC, MLC-2v and 2a (Fig. 1H). Comparable number of cardiomyocytes was induced from the same number of control FCV cells and CSA-expanded FCV cells (data not shown). These results indicate that CSA-expanded FCV cells retained their high cardiogenic potentials. When CSA was added to purified FCV cells (experiment 4), slight increase (approximately 2.6 times) in cardiomyocytes was observed (Fig. 2E and F). These results indicate that the novel potent cardiomyocyte inducing activity of CSA is restricted to the period after mesoderm formation, and acts principally between the mesoderm and cardiac pro-

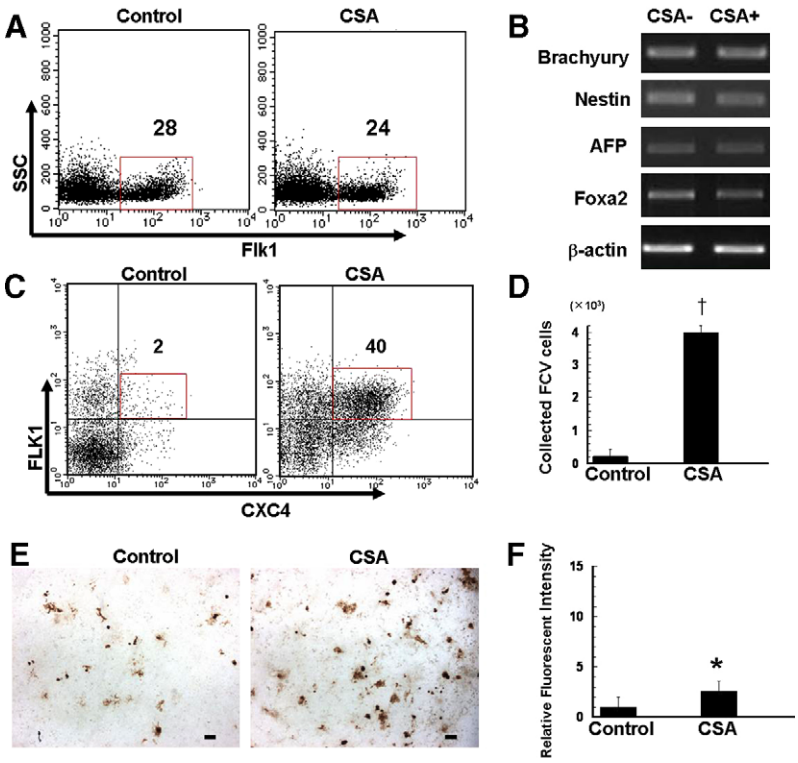

Fig. 2. Differentiation stage-specific effect of CSA. (A,B) Effects of CSA on early differentiation from undifferentiated ES cells (experiment 2). Undifferentiated ES cells were cultured in the absence of LIF for 4 days (ES-d4). (A) Induction of Flk1$^+$ mesoderm cells. FACS analysis for Flk1 and side scatter (SSC) in the absence (control) or presence (CSA) of CSA. (B) mRNA expressions for mesoendoderm marker, brachyury, ectoderm marker, Nestin, and endoderm markers, AFP and Foxa2 at ES-d4. (C,D) Induction of FCV progenitor cells from Flk1$^+$ mesoderm (experiment 3). (C) FACS analysis for cardiac progenitor induction by CSA. *X* axis: Flk1. *Y* axis: CXCR4. Percentages of FCV cells (double positive population) are indicated. (D) Yield of purified cardiac progenitor cells. Cell number of obtained FCV progenitor cells from 10^4 Flk1$^+$ cells ($n = 12$, $^{\dagger}p < 0.001$ vs control). (E,F) Cardiomyocyte induction from purified FCV cells by CSA (experiment 4). Purified FCV cells were recultured on OP9 cells for 4 days. (E) Gross appearance of cardiomyocytes stained with cTnT (brown). Left panel: control. Right panel: CSA treatment. Scale bars = 400 μm. (F) Quantitative evaluation of cardiomyocyte induction by fluorescent intensity of cTnT staining. Relative fluorescent intensity is indicated ($n = 3$, $p < 0.05$ vs control).

genitor stages to induce specific and efficient expansion of the cardiac progenitor cells.

We further examined cellular and molecular mechanisms of CSA. EdU incorporation and annexin V expression in FCV cells were not affected by CSA treatment (Fig. 3A–C), indicating that the increase in FCV cells by CSA was not due to proliferation and/or survival of FCV cells. CSA treatment on Flk1$^+$ cells (Flk-d0-6: experiment 1) induced drastic increase in cardiomyocytes with reciprocal decrease in ECs or blood cells from Flk1$^+$ mesoderm cells (Fig. 3D and E). Even when CSA was added only in mesoderm stage (Flk-d0-2: experiment 5), similar effects were observed (Fig. 3F), suggesting that CSA should act on Flk1$^+$ cells and may shift the cell fate from ECs or blood cells to cardiomyocytes. Calcineurin inhibitors, CSA and FK506 exert their immunosuppressing effect through inhibition of nuclear factor of activated T-cells (NFAT) signaling [14]. Nevertheless, FK506 and a NFAT inhibitor, 11R-VIVIT, showed no significant effect on cardiomyocyte induction in our system (Fig. 3G), suggesting that the main cardiogenic effect of CSA should be NFAT independent. These results indicate that the potent cardiomyocyte inducing activity of CSA should be evoked through novel mechanisms to induce specific and efficient expansion of the cardiac progenitor cells with mesoderm-specific, NFAT-independent fashion. Future studies on precise mechanisms of CSA action should provide novel molecular understanding of cardiomyocyte differentiation and regeneration.

This efficient expansion of the rare FCV progenitors allowed us to confirm their cardiogenic potential in vivo. Finally, we examined in vivo cardiogenic potential of expanded FCV cells. We performed transplantation of CSA-expanded FCV cells to chronic myocardial infarction model of rat. At 2 weeks after the injection, transplanted FCV cells were successfully differentiated into cardiomyocytes and integrated in the infarct heart to form GFP$^+$/cTnT$^+$ donor cell-derived cardiomyocyte bundle in the scar tissue (Fig. 4A and B). This result indicates that CSA-expanded FCV cells can show highly cardiogenic potentials also in vivo after cell transplantation.

Discussion

Here, we show a novel technology for the specific and efficient expansion of highly cardiogenic progenitors as well as cardiomyocytes from ES cells with a newly discovered mesoderm-specific effect of CSA. The immunosuppressive action of the calcineurin inhibitor CSA stems from the inhibition of NFAT signaling in T cells [14]. Through calcineurin-NFAT signaling, CSA is also involved in various cellular processes, such as cardiac valve formation [15], cardiac hypertrophy [16], and hair growth [17]. Though a weak inductive effect of CSA on cardiomyocytes in embryoid bodies was reported [18], the effect on cardiac progenitors and its molecular mechanism were unclear. Our novel sequential cardiomyocyte differentiation system should succeed in distinctively digging out the potent mesoderm-specific and novel NFAT-independent effect of CSA, which has been buried in the cell mixture of embryoid bodies. This novel culture system would be amenable to screen and discover novel cardiac regenerative drugs from small molecules using chemical biology strategies.

FCV cells, which are detected at 6–6.5 days after the differentiation of mouse ES cells and at 1–2 days before cardiomyocyte

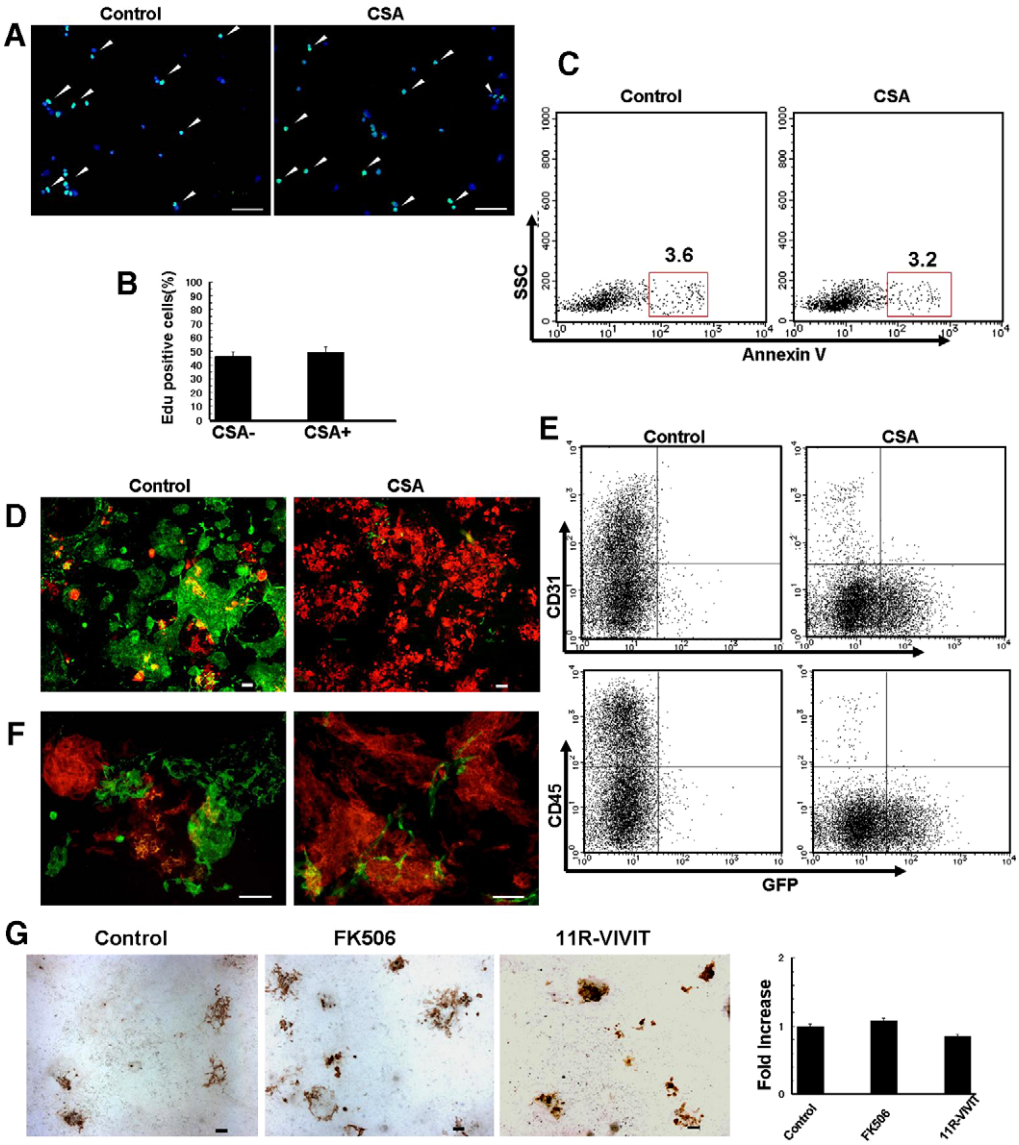

Fig. 3. Cellular and molecular mechanisms of CSA. (A,B) EdU incorporation. EdU-treated FCV cells were plated onto glass slides by cytocentrifugation. (A) Double staining for EdU (green) and DAPI (blue). Left panel: control. Right panel: CSA treatment. Double positive nuclei are indicated by arrowheads. Scale bar = 100 μm. (B) Quantitative evaluation of EdU positive FCV cells. Percentages of EdU positive cells in total cells are indicated (200 nuclei each, $n = 3$). (C) FACS analysis for annexin V expression. Gated FCV cell populations are shown. Percentages of apoptotic cells (annexin V^+, red box) are indicated. (D) Reciprocal appearance of ECs and cardiomyocytes by CSA treatment (experiment 1). Double immunostaining for CD31 (pan-ECs; green) and cTnT (red). Left panel: control. Right panel: CSA treatment. Scale bars = 400 μm. (E) Reciprocal appearance of ECs (upper panels) or blood cells (lower panels) with cardiomyocytes by CSA treatment. FACS analysis at Flk-d6 (experiment 1). Left panel: control. Right panel: CSA treatment. *X* axis: GFP (cardiomyocytes). *Y* axis: CD31 (pan-ECs), CD45 (pan-white blood cells). All $Flk1^+$ cell-derived population was analyzed. (F) Mesoderm-specific treatment of CSA (Flk-d0-2: experiment 5). Double immunostaining for CD31 (pan-ECs; green) and cTnT (red). Scale bars = 400 μm. (G) Effects of FK506 and NFAT inhibitor (11R-VIVIT) on cardiomyocyte differentiation (experiment 1). Left panels: gross appearance of cardiomyocytes stained with cTnT (brown), treated with vehicle (control), FK506, or 11R-VIVIT. Scale bars = 100 μm. Right panel: quantitative evaluation of cardiomyocyte induction by fluorescent intensity of cTnT staining. Relative fluorescent intensity is indicated ($n = 3$).

appearance, are, to our knowledge, the first identified distinct cardiac progenitor population [8] and so far the nearest upstream cardiac progenitors to cardiomyocytes. Recently, several kinds of multipotent cardiac progenitor populations were reported [19–21]. Cardiac progenitors reported by Kattman et al. were identified at an earlier stage of differentiation (i.e. at 4.25 days after the differentiation) than FCV cells [19]. Other $Nkx2.5^+$ or $islet1^+$ cardiac progenitors were reported at 4–6 days of ES cell differentiation [20,21]. In our FCV population, approximately 42% of cells were $Islet1^+$ (42 ± 18%, 1000 cells each, $n = 3$), 24% were $Nkx2.5^+$ (24 ± 5%), and 14% were double positive for Islet1 and Nkx2.5 (14 ± 1%). FCV cells, thus, should be an overlapped population with $Nkx2.5^+$ and/or $Islet1^+$ cardiac progenitors.

Recently, novel ES cell-like pluripotent stem cells, iPS cells, were generated from adult somatic cells by transduction of defined transcription factors [1–4]. We have just succeeded in establishing an iPS cell differentiation system for various cardiovascular cells similar to the ES cell system [22]. This CSA-mediated cardiac cell induction method would be extended and applied to iPS cells, and that would broadly contribute to exploring cardiac regeneration strategies.

Competing interests statement

The authors declare that they have no competing financial interests.

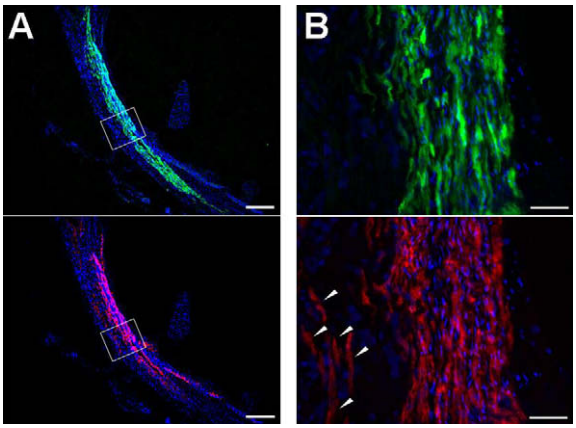

Fig. 4. Cardiagenic potential of expanded FCV cells in vivo. (A,B) Representative data of FCV cell transplantation (4×10^5 cells) to rat myocardial infarction model. Double immunostaining for GFP and cTnT. Upper panels: GFP (donor cell-derived cardiomyocytes, green) and DAPI (blue). Lower panels: cTnT (pan-cardiomyocytes, red) and DAPI. (A) Gross appearance of transplanted cell contribution to infarct area. Scale bars = 400 μm. (B) Higher magnification views of boxed area. Arrowheads show $cTnT^+/GFP^-$ endogenous cardiomyocytes. Scale bars = 50 μm.

Acknowledgments

We thank to Novartis Pharma for cyclosporin, Astellas Pharma for FK506, Dr. M. Takahashi for critical reading of the manuscript. This study was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Ministry of Health, Labour and Welfare, the New Energy and Industrial Development Organization (NEDO) of Japan, the Project for Realization of Regenerative Medicine.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.019.

References

[1] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (2006) 663–676.
[2] K. Okita, T. Ichisaka, S. Yamanaka, Generation of germline-competent induced pluripotent stem cells, Nature 448 (2007) 313–317.
[3] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (2007) 861–872.
[4] J. Yu, M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, I.I. Slukvin, J.A. Thomson, Induced pluripotent stem cell lines derived from human somatic cells, Science 318 (2007) 1917–1920.
[5] M.A. Laflamme, C.E. Murry, Regenerating the heart, Nat. Biotechnol. 23 (2005) 845–856.
[6] A. Lyon, S. Harding, The potential of cardiac stem cell therapy for heart failure, Curr. Opin. Pharmacol. 7 (2007) 164–170.
[7] E. Kolossov, T. Bostani, W. Roell, M. Breitbach, F. Pillekamp, J. Nygren, P. Sasse, O. Rubenchik, J. Fries, D. Wenzel, C. Geisen, Y. Xia, Z. Lu, Y. Duan, R. Kettenhofen, S. Jovinge, W. Bloch, H. Bohlen, A. Welz, J. Hescheler, S.E. Jacobsen, B.K. Fleischmann, Engraftment of engineered ES cell-derived cardiomyocytes but not BM cells restores contractile function to the infarcted myocardium, J. Exp. Med. 203 (2006) 2315–2327.
[8] J.K. Yamashita, M. Takano, M. Hiraoka-Kanie, C. Shimazu, P. Yan, K. Yanagi, A. Nakano, E. Inoue, F. Kita, S.I. Nishikawa, Prospective identification of cardiac progenitors by a novel single cell-based cardiomyocyte induction, FASEB J. 19 (2005) 1534–1536.
[9] D.J. Garry, E.N. Olson, A common progenitor at the heart of development, Cell 127 (2006) 1101–1104.
[10] J. Yamashita, H. Itoh, M. Hirashima, M. Ogawa, S. Nishikawa, T. Yurugi, M. Naito, K. Nakao, S.I. Nishikawa, Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors, Nature 408 (2000) 92–96.
[11] H. Kodama, M. Nose, S. Niida, S. Nishikawa, S.I. Nishikawa, Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells, Exp. Hematol. 22 (1994) 979–984.
[12] K. Tambara, Y. Sakakibara, G. Sakaguchi, F. Lu, G.U. Premaratne, X. Lin, K. Nishimura, M. Komeda, Transplanted skeletal myoblasts can fully replace the infarcted myocardium when they survive in the host in large numbers, Circulation 108 (Suppl. II) (2003) 259–263.
[13] K. Yanagi, M. Takano, G. Narazaki, H. Uosaki, T. Hoshino, T. Ishii, T. Misaki, J.K. Yamashita, Hyperpolarization-activated cyclic nucleotide-gated channels and T-type calcium channels confer automaticity of embryonic stem cell-derived cardiomyocytes, Stem Cells 25 (2007) 2712–2719.
[14] G.R. Crabtree, E.N. Olson, NFAT signaling: choreographing the social lives of cells, Cell 109 (Suppl) (2002) S67–S79.
[15] A.M. Ranger, M.J. Grusby, M.R. Hodge, E.M. Gravallese, F.C. de la Brousse, T. Hoey, Mickanin, H.S. Baldwin, L.H. Glimcher, The transcription factor NF-ATc is essential for cardiac valve formation, Nature 392 (1998) 186–190.
[16] J.L. de la Pompa, L.A. Timmerman, H. Takimoto, H. Yoshida, A.J. Elia, E. Samper, J. Potter, A. Wakeham, L. Marengere, B.L. Langille, G.R. Crabtree, T.W. Mak, Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum, Nature 392 (1998) 182–186.
[17] V. Horsley, A.O. Aliprantis, L. Polak, L.H. Glimcher, E. Fuchs, NFATc1 balances quiescence and proliferation of skin stem cells, Cell 132 (2008) 299–310.
[18] A. Sachinidis, S. Schwengberg, R. Hippler-Altenburg, D. Mariappan, N. Kamisetti, B. Seelig, A. Berkessel, J. Hescheler, Identification of small signalling molecules promoting cardiac-specific differentiation of mouse embryonic stem cells, Cell. Physiol. Biochem. 18 (2006) 303–314.
[19] S.J. Kattman, T.L. Huber, G.M. Keller, Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte Endothelial, and vascular smooth muscle lineages, Dev. Cell 11 (2006) 723–732.
[20] S.M. Wu, Y. Fujiwara, S.M. Cibulsky, D.E. Clapham, C.L. Lien, T.M. Schultheiss, S.H. Orkin, Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart, Cell 127 (2006) 1137–1150.
[21] A. Moretti, L. Caron, A. Nakano, J.T. Lam, A. Bernshausen, Y. Chen, Y. Qyang, L. Bu, M. Sasaki, S. Martin-Puig, Y. Sun, S.M. Evans, K.L. Laugwitz, K.R. Chien, Multipotent embryonic isl1+ progenitor cells lead to cardiac smooth muscle, and endothelial cell diversification, Cell 127 (2006) 1151–1165.
[22] G. Narazaki, H. Uosaki, M. Teranishi, K. Okita, B. Kim, S. Matsuoka, S. Yamanaka, J.K. Yamashita, Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells, Circulation 118 (2008) 498–506.