Flk1⁺ cardiac stem/progenitor cells derived from embryonic stem cells improve cardiac function in a dilated cardiomyopathy mouse model

Shiro Baba⁎, Toshio Heikea, Momoko Yoshimotoa, Katsutsugu Umedaa, Hiraku Doia, Toru Iwasaa, Xue Linb, Satoshi Matsuokac, Masashi Komeda, Tatsutoshi Nakahataa

a Department of Pediatrics, Graduate School of Medicine, Kyoto University, Kyoto, Japan, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
b Department of Cardiovascular Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan
c Department of Physiology and Biophysics, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Received 5 October 2006; received in revised form 2 May 2007; accepted 11 May 2007
Available online 17 May 2007
Time for primary review 18 days

Abstract

Objectives: Flk1⁺ cells derived from embryonic stem (ES) cells are known to differentiate into mesodermal lineages such as hematopoietic and endothelial cells. Here we demonstrate that they can develop into cardiomyocytes that support functional recovery in a dilated cardiomyopathy (DCM) C57/BL6 mouse model.

Methods: Flk1⁺ and Flk1⁻ cells were sorted at day 4 of differentiation, and cardiomyogenesis was assessed in vitro. Next, we transplanted these cells into the hearts of cardiomyopathy mice to assess improvement in cardiac function.

Results: Flk1⁺ cells, but not Flk1⁻ cells, isolated on day 4 after differentiation were efficiently converted into contractile cardiomyocytes. RT-PCR analysis and immunohistological assays demonstrated that contractile cells derived from Flk1⁺ cells in vitro expressed mature cardiac markers on day 10 after differentiation. Transplantation of sorted Flk1⁺ cells into DCM model mouse hearts improved cardiac function, as determined by echocardiography and cardiac catheterization. The in vivo differentiated Flk1⁺ cells expressed cardiac markers and had gap junctions, as demonstrated by immunohistochemistry. Furthermore, these cells generated ventricular type action potentials similar to those of adult ventricle.

Conclusion: These results indicate that Flk1 is a good marker for sorting cardiac stem/progenitor cells which can differentiate into mature cardiomyocytes both in vitro and in vivo.

© 2007 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Cell culture/isolation; Cell differentiation; Cell therapy; Cardiomyopathy

1. Introduction

At present, heart transplantation is the most frequently used and, indeed, the only effective therapy for end stage heart failure patients [1]. However, too few hearts are available for transplantation to meet demand.

To overcome this problem, work in recent years has attempted to develop cell transplantation protocols using various cell types. Although fetal cardiomyocytes [2] and skeletal muscle [3] are known candidates, it is not feasible to harvest fetal cardiomyocytes from human fetuses and skeletal muscle derived cells may promote life threatening arrhythmias because of a lack of gap junction formation between the transplanted cells and host cardiomyocytes [4,5]. Bone marrow is another candidate cell type [6]. However, while these cells can differentiate into vascular cells or induce angiogenesis, they very rarely differentiate into cardiomyocytes [7]. Thus, these previously considered cell types do not seem to be able to differentiate into cardiomyocytes efficiently.

Recently, embryonic stem (ES) cells and adult cardiac stem (CS) cells have been proposed as transplantable cell candidates that would avoid the problems described above [8–10]. With regard to CS cells, cardiomyocytes derived from adult CS cells in vivo have contractile capacity and bear gap junctions on their cell surface [10]. Although adult CS cells are good
candidates for heart cell transplantation, it is hard to harvest these cells abundantly from the patient’s own heart. With regard to ES cells, cardiomyogenesis from ES cells has been well studied in vitro [8,9]. However, procedure by which the ES cell progeny can be transplanted into the heart have not yet been established. Although Min et al. [11] did demonstrate improved cardiac function in post-infarcted rats upon transplantation with various cells, including mature cardiomyocytes derived from ES cells, it was difficult to purify the cardiomyocytes from this population by the hanging drop culture method. For heart cell transplantation, it is better to purify the transplanted cells as stem/progenitor cells that have the capacity to differentiate into cardiomyocytes. Therefore, a better transplantable cell candidate needs to be identified.

In this report, we identify cardiac stem/progenitor cells expressing Flk1, which is a lateral mesodermal marker, and demonstrate that these cells effectively differentiate into cardiomyocytes both in vitro and in vivo. Transplantation of these cells into the hearts of dilated cardiomyopathy (DCM) model mice significantly improves their cardiac function.

2. Materials and methods

2.1. Cell lines, culture and differentiation

For these experiments, we used Gact4 mouse ES cells and which were maintained in the presence of 1% FCS, 10% Knockout-SR (GIBCO) and 5000 units/ml leukemia inhibitory factor (LIF) on 0.1% gelatin coated culture dishes as described [12]. The Gact4 cell line, which was produced by stable transfection with the enhanced GFP gene driven by the CAG promter (a kind gift from Dr. Ogawa). Gact4 cells were differentiated as described previously [13]. Briefly, single cell dispersions of undifferentiated Gact4 cells were suspended in MEM Alpha medium (GIBCO) containing 10% FCS (EQUI TECH BIO Inc.) and 5 × 10^-5 M 2-mercaptoethanol in the absence of LIF (differentiation medium) at an initial concentration of 1.2 × 10^5 cells on 100 mm type IV collagen-coated culture dishes (Becton Dickinson).

2.2. FACS and comparison of Flk1+ and Flk1− cell differentiation into cardiomyocytes, endothelial colonies, and blood clusters in vitro

After 4 days of differentiation, Gact4 ES cells were dissociated into single cells by using Cell Dissociation Buffer (GIBCO), and separated Gact4 cells were labeled with an APC-conjugated AVAS12 antibody (anti-Flk1) [13] and sorted into Flk1+ and Flk1− cells using a FACS Vantage SE (BD Biosciences). Each cell type was co-cultured in differentiation medium without cytokines at 500 cells per well in 24-well plates (FALCON) on OP-9 stromal cells, which were kindly provided by Dr. Kodama and maintained as described previously [14]. These 4 days-differentiated Gact4 cells were also characterized using a PE-conjugated anti-mouse c-kit antibody (BD Biosciences). The number of contractile colonies in each 24-well plate was counted. Endothelial colonies were characterized by a sheet-like structure that stained with Dil-acetylated low-density lipoprotein (DiL-Ac-LDL) (Molecular Probes) [15] and were also counted. Hematoipoietic clusters were characterized by their cobblestone appearance [16]. Sorted cells were incubated in differentiation medium on OP-9 stromal cells as described above, and, after 24 h, the medium was replaced with fresh semisolid medium containing 10% fetal calf serum (EQUITECH BIO Inc.) and 1.2% methylcellulose (Shinetsu Chemical) without cytokines. The number of hematopoietic clusters on the 24-well plates were then counted as described previously [16].

2.3. Immunohistochemistry

Cells and 7 μm tissue slices were fixed with 4% paraformaldehyde and incubated with antibodies specific for the following markers: myosin light chain 2v (MLC2v) (Alexis Biochemicals, Inc.), atrial natriuretic peptide (ANP) (Protos Biotech Corporation), sarcomeric protein MF20 (MF20) (Hybridoma bank), sarcomeric protein CH1 (CH1) (Sigma), cardiac troponin-I (cTn-I) (Santa Cruz Biotechnology), connexin 43 (Cx43) (Zymed Laboratories, Inc.), CD45 (Becton Dickinson), CD31 (Becton Dickinson), skeletal myosin heavy chain (MHC) (Zymed Laboratories, Inc.), mouse Ki-67 (Dako Cytomation) and GFP (Nacalai tesque). Peroxidase- or Cy3-conjugated donkey anti-mouse IgG, Alkaline phosphatase (ALP)- or Cy3-conjugated donkey anti-rabbit IgG, ALP- or peroxidase-conjugated goat anti-mouse IgG, and peroxidase- or Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc.) served as secondary antibodies. ALP- or peroxidase-conjugated cells were visualized by using the Alkaline Phosphatase Substrate Kit III or the DAB Substrate Kit (Vector Laboratories, Inc.). All heart sections were incubated with these antibodies using the M.O.M. kit (Vector Laboratories, Inc.) to prevent non-specific reactions. Many heart slices were also stained with hematoxylin-eosin (HE) to detect abnormal cell growth due to transplanted Flk1+ or Flk1− cells.

2.4. RT-PCR analysis

PCR amplifications used the primers described in Table 1. The PCR conditions for all primers were DNA denaturation at 94 °C for 5 min, 25–30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, followed by a final extension step at 72 °C for 7 min.

2.5. Electrophysiological examination in vitro

Day 4-sorted Flk1+ Gact4 ES cells were co-cultured with OP-9 stromal cells on MED-probe dishes (ALPHA MED SCIENCES) and the electrical potentials of contractile colonies derived from these cells were recorded by using...
the MED64 System (Panasonic multi-electrode system; ALPHA MED SCIENCES).

2.6. Transplantation of ES cells to doxorubicin-induced DCM model mice

To produce DCM model mice, we used 8 wk-old male C57/BL6 mice. The MHC types of this mouse and Gact4 cells are very similar. The MHC H2 haplotypes of Gact4 and C57/BL6 are both type b. All animal handling procedures followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the guidelines of the Animal Research Committee of the Graduate School of Medicine, Kyoto University. We injected a 3 mg/kg dose of doxorubicin intraperitoneally (i.p.) 6 times over 2 weeks. Four weeks after the last i.p. injection, mice that had a left ventricular diastolic diameter (LVDd) larger than 21390A, PHILIPS). LVDd and left ventricular systolic diameter (LVDs) were measured and the left ventricular ejection fraction (LVEF) was calculated as described [6]. Cardiac catheterization was performed four weeks after cell transplantation, as described [17], using a 1.4-Fr micro-menometer-tipped catheter (Miller Instruments Inc.), a pressure transducer (model TCB-500, MILLER INSTRUMENT INC.) and a chart-strip recorder (Thermal Array Recorder, model RTA-1100M) (NIHON KOHDEN). Left ventricular maximum systolic velocity (+ dP/dt) and minimum diastolic velocity (−dP/dt) were calculated from the left ventricular pressure curve. Limb-lead electrocardiogram was recorded by a PowerLab System (PowerLab 4/25 ML845 and BIO Amp CF ML132) (ADInstruments). All measurements were performed under anesthesia with mask inhalation of 0.5–1.0% Sevoflurane and a heart rate of approximately 450/min.

2.8. FISH analysis

FISH analysis was performed on 20 μm tissue sections fixed with 4% paraformaldehyde according to the method described previously [18]. We used a DIG-labeled GFP probe [18] and a Biotin-labeled mouse chromosome Y specific probe (Cambio Ltd). The DIG-labeled GFP probe was visualized with rabbit anti-DIG/HRPDIG (Dako Cytomation) and the TSA Fluorescein System (PerkinElmer Life Sciences), and the Biotin-labeled mouse chromosome Y probe was visualized with Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc.) on a fluorescence microscope (Leica, LEICA DM 6000B). Finally, nuclei were counterstained with Hoechst33342. The figures were subjected to computer graphics analysis. The nuclei in the sample were dissected from top to bottom with a thickness of 1 μm. We confirmed that a complete observation of the nucleus had been achieved.

2.9. Isolation of single cells and action potential measurements

Ventricular myocytes were dissociated from mouse hearts by perfusing collagenase (Worthington Biochemical Corporation), protease (Sigma–Aldrich), and trypsin (Sigma–Aldrich) in essentially the same method as described previously [19]. GFP+ cardiomyocytes were identified on a fluorescence microscope and the membrane potential was recorded by the current-clamp and perforated patch techniques (0.3 mmol/L amphotericin B) using a patch clamp amplifier (Axopatch 200B, Axon Instruments, Inc.) in a control Tyrode solution (140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.5 mM MgCl2, 0.3 mM NaH2PO4, 5.5 mM glucose and 5.0 mM HEPES, pH=7.4 with NaOH) at 36–37 °C.

2.10. Ectopic transplantation

A freshly harvested 10 μl Flk1+ cell suspension (3 × 10^5/10 μl) was injected subcutaneously into the backs of five
mice and kidney capsules of five mice at 14 weeks of age. Tissues from these areas were removed four and ten weeks after transplantation.

2.11. Statistical analysis

Data were statistically analyzed using a two-tailed unpaired \( t \)-test within Microsoft Excel. Statistical significance was defined as \( p < 0.05 \).

3. Results

3.1. Flk1+ cells differentiate into cardiomyocytes, endothelial cells and hematopoietic cells more frequently than Flk1− cells

We sorted differentiated ES cells into Flk1+ and Flk1− cells after 4 days of culture in differentiation medium (Fig. 1A), and evaluated their differentiation potentials at days 5–10 after differentiation. On day 4 after differentiation,
the large population of freshly sorted Flk1+ cells expressed Sca-1 and c-kit (Fig. 1B), which are cardiac stem/progenitor cell markers [20]. In addition, the expression of Nanog and Oct4, which are undifferentiated ES cell markers, was significantly lower than in day 4 sorted Flk1− cells (Fig. 1C). By day 10 after differentiation, 12,000 Flk1+ and Flk1− cells yielded 640±353 and 46±15 contractile colonies, respectively (p<0.01) (Fig. 1D). We used RT-PCR to assess whether these Flk1+ cell derivatives expressed various cardiac markers. The day 4 sorted Flk1+ cells expressed GATA-4 and Nkx2.5, which are cardiac markers that are detected from an early stage in the developing heart, and did not express the mature ventricular cell marker MLC2v, the atrial cell marker MLC2a, or the gap junction markerCx43 (Fig. 2A). In contrast, day 4 sorted Flk1− cells did not express any of these markers (Fig. 2A). The day 10 Flk1+ cell derivatives express MLC2a, MLC2v and Cx43 at significantly greater levels than the day 10 Flk1− cell derivatives. Immunohistochemical analysis also demonstrated that the day 10 contractile colonies expressed MLC2v, the developing cardiac cell and atrial cell marker ANP, sarcomeric protein MF20 and the cardiac troponin-I (cTn-I) (Fig. 2B–F). We next measured the electrical potentials of contractile colonies derived from Flk1+ cells (left panel) were stained with an anti-GFP antibody (right panel, brown) to confirm GFP expression. Bar, 50 μm.

DCM mice were transplanted with Flk1+ cells [the FT(DCM) group], 19 DCM mice were injected with medium alone [the MT(DCM) group], 14 DCM mice were untreated [the NT (DCM) group], and 14 normal mice were used as controls (normal control). To assess the severity of the dilated cardiomyopathy in the DCM mice, LVDd and LVEF were assessed by echocardiography at −6 wks, −4 wks, day 0 and 4 wks. At −6 wks, −4 wks and day 0, all three DCM groups showed equivalent LVDd values which were significantly larger than the values for the normal control group (Fig. 3B, C). At 4 wks post-transplantation, the FT (DCM) group had significantly smaller LVDd values than the MT(DCM) and NT(DCM) groups (3.82±0.18 versus 4.22±0.22 and 4.0±0.19 mm, respectively) (Fig. 3D). The three DCM groups showed equivalent declining LVEF values until 4 wks post-transplantation, at which point the FT (DCM) group recovered significantly higher LVEF values than observed in the MT(DCM) and NT(DCM) groups (78.4±7.3 versus 70.1±5.0 and 70.9±5.6%, respectively) (Fig. 3E). However, the change in LVDd and LVEF values in the FT(DCM) group at 4 wks did not reach statistical significance when compared to their day 0 LVDd and LVEF values.

Cardiac catheterization was used to evaluate the +dP/dt and −dP/dt parameters and left ventricular end-diastolic pressure (LVEDp) at 4 wks post-transplantation. The FT (DCM) group showed significant improvements in their +dP/dt, −dP/dt and LVEDp values when compared to the values of the MT(DCM) and NT(DCM) groups (Fig. 3F–H). Thus, FT (DCM) group had significantly faster +dP/dt values than the MT(DCM) group and NT(DCM) groups (8858.3±2197.3 versus 6967.5±1765.7 and 6986.7±1645.3 mm Hg/s, respectively) (Fig. 3F), slower −dP/dt values (−8008.3±1706.9 versus −6673.9±1899.0 and −628.9±1711.6 mm Hg/s, respectively) (Fig. 3G), and lower LVEDp values (1.54±1.42 versus 3.50±2.74 and 3.61±2.37 mm Hg, respectively) (Fig. 3H). These results indicate that Flk1+ cell transplantation improved the systolic and diastolic cardiac function of DCM mice. Notably, when nine DCM mice were transplanted with Flk1− cells, their cardiac function did not improve at 4 wks post-transplantation as judged by echocardiography and cardiac catheterization (LVDd: 4.30±0.32 mm; LVEF: 71.5±8008.3±1706.9 mm Hg/s, respectively) (Fig. 3I, slower −dP/dt values (−8008.3±1706.9 versus −6673.9±1899.0 and −628.9±1711.6 mm Hg/s, respectively) (Fig. 3G), and lower LVEDp values (1.54±1.42 versus 3.50±2.74 and 3.61±2.37 mm Hg, respectively) (Fig. 3H).

Furthermore, we evaluated the cardiac function of each group at 10 wks after cell transplantation. A slight improvement in the cardiac function of the FT(DCM) group (n=3) was still observed compared to that of the MT(DCM) (n=3) or NT(DCM) (n=3) groups at this time point [LVDd: 3.8±

Fig. 3. Transplantation of Flk1+ cells into DCM mice significantly improves cardiac function. (A) Timeline of DCM mouse model generation and transplantation experiments. (B–G) Cross-sections of the left ventricle at the level of the papillary muscle of a normal 14 wk-old mouse (B) and a DCM mouse just prior to Flk1+ cell transplantation (C). (D–H) The FT(DCM) group generally showed significant improvements in their LVDd (D), LVEF (E), +dP/dt (F), −dP/dt (G), and LVEDp values (H) compared to the MT(DCM) and NT(DCM) groups. * p<0.01; † p<0.05. (I) Fluorescence detection of groups of engrafted GFP+ cells. The arrowheads in (J) indicates engrafted GFP+ cells along the scar area resulting from cell injection. (K) Engrafted GFP+ cells were also clearly detected in FT(DCM) mouse hearts 10 wks after cell transplantation. (L) GFP+ cells (left panel) were stained with an anti-GFP antibody (right panel, brown) to confirm GFP expression. Bar, 50 μm.
0.08 mm FT(DCM) versus 4.20±0.14 mm MT(DCM), respectively), [LVEF: 77.2±3.1% FT(DCM) versus 73.4±0.5% MT(DCM), respectively].

3.3. Cardiomyocytes that are derived from transplanted Flk1+ cells express cardiac specific markers

Using immunohistochemistry, we evaluated the differentiation of the transplanted GFP-expressing Flk1+ cells in the hearts of FT(DCM) mice. We detected groups of GFP+ engrafted cells in DCM hearts within or adjacent to the scar tissue resulting from the injection 4 wks after cell transplantation (Fig. 3I, J) and 10 wks after cell transplantation (Fig. 3K). We confirmed the presence of GFP by immunohistochemistry with an anti-GFP antibody (Fig. 3L). A part of the GFP+ cells expressed MLC2v, cTn-I, and the sarcomeric antibody. Although the nuclei of almost all undifferentiated Gact4 ES cells and just sorted Flk1+ cells were stained abundantly with the anti-Ki-67 antibody (Supplementary Fig. 2), the nuclei of the engrafted GFP+ cells that had differentiated in vivo were not stained with this antibody (Fig. 5A). Thus, the engrafted GFP+ cells that have differentiated from Flk1+ cells in vivo appear to be terminally differentiated.

Unlike Flk1+ cells, Flk1− cells transplanted into the hearts of DCM mice did not differentiate into cardiomyocytes, and calcification or adipose degeneration were detected in the Flk1− cell-transplanted hearts (data not shown). This may explain why the transplantation of these cells failed to improve cardiac function. In addition, we detected abnormal cell growth in the hearts of the three of nine Flk1− cell-transplanted mice (Fig. 5B). Furthermore, we investigated whether transplanted Flk1+ cells fused with host cardiomyocytes. We identified the mouse Y chromosome in Gact4 ES cells via PCR analysis (data not shown) and transplanted these cells into male mice. Although, assessed by FISH analysis, small number of GFP stained nuclei contained two Y chromosomes (n=9 in total 158 GFP+ nuclei; 5.7%), most of GFP stained nuclei contained no more than two Y chromosomes (n=149 in total 158 GFP+ nuclei; 94.3%) (Fig. 5H, I). These results indicated that there was only a small rate of fusion between Flk1+ cells and host cardiomyocytes.

3.4. Cardiomyocytes derived from Flk1+ cells have action potentials

Since we have found that transplanted Flk1+ cells effectively differentiate into ventricular type cells with gap junctions, we next assessed whether these cells have electrical activity. We isolated GFP+ cardiomyocytes from the Flk1+ cell-transplanted hearts and recorded their action potentials (n=5). The shape of the action potentials recorded from these engrafted GFP+ cells indicates that they are no embryonic or neonatal ventricular type cells, rather, they are adult ventricular type cells [21,22] (Fig. 5C–E), and the resting membrane potentials measured from these engrafted GFP+ cells (−70.3±3.9 mV) were equal to those from normal mouse ventricular cells (−71.1±4.4 mV). Moreover, the mean action potential duration at 90% repolarization (APD90) [23] from engrafted GFP+ cells (116.8±6.6 ms) was not significantly different from normal ventricular cells (113.4±26.5 ms). As expected, day 4-sorted Flk1+ cells had no action potential following electrical stimulation. Furthermore, we recorded electrocardiogram from FT(DCM) mice to investigate whether these differentiated cardiomyocytes produce arrhythmia. We could not detect arrhythmia not only during echocardiography and cardiac catheterization, but also during electrocardiography (Fig. 5J).

3.5. Flk1+ cell-transplantation induced angiogenesis

To assess another mechanism of cardiac improvement, we counted the number of vessels around the Flk1+ cell-injected
or medium-injected sites. Although we could not detect GFP⁺ CD31⁺ tubulo-vesicular structures, the number of vessels was significantly higher in the FT(DCM) group than in the MT(DCM) group (Fig. 5F, G). These data indicate that Flk1⁺ cell-transplantation has a favorable effect on angiogenesis.

Fig. 4. Engrafted GFP⁺ cells in the hearts of DCM mice express cardiac-specific markers. (A–G) Engrafted GFP⁺ cells shown in the left panels (green) were stained for MLC2v (A), cTn-I (B), CH1 (C), Cx43 (D), ANP (E), CD31 (F), and CD45 (G), shown in the middle panels (red). The merged images are shown in the right panels. The white arrowheads in the middle panel of (A) indicate the GFP⁺ cells surrounded by the host ventricular cells (VC) and the strong but non-specific staining of scar tissue is indicated by the blue arrows. The arrowheads in the middle panel of (D) indicate gap junctions formed between the engrafted cardiomyocytes and endogenous cardiomyocytes. The arrowhead in (G) indicates a CD45⁺ GFP⁺ cell. Bar, 50 μm. Nuclei were counterstained with Hoechst 33342 (blue).
4. Discussion

In this report, we demonstrate that Flk1+ cells have a greater potential than Flk1− cells to differentiate into cardiomyocytes. In addition, Flk1+ cells efficiently differentiated into adult ventricular type cardiomyocytes upon transplantation into DCM model mouse hearts, as shown by immunohistochemistry and electrophysiological examinations. These observations suggest that transplanted Flk1+ cells derived from ES cells may effect a functional improvement in injured hearts.

Fig. 5. Engrafted GFP+ cells derived from Flk1+ cells are terminally differentiated and have action potentials, but engrafted cells derived from Flk1− cells generate abnormal growth. The nuclei of engrafted GFP+ cells derived from Flk1− cells (green; left panel) do not express Ki-67 (red; middle panel). Right panel: merged images. The nuclei were counterstained with Hoechst 33342 (blue). Bar, 50 μm. (B) Hearts of DCM mice transplanted with Flk1− cells showed abnormal cell growth. (C-E) Analysis of the action potentials of GFP+ cardiomyocytes in hearts transplanted with Flk1+ cells. The engrafted cardiomyocytes were isolated and identified by fluorescence microscopy (C, panels a and b). The tip of a glass electrode (white arrowhead) was then pressed onto the membrane of a GFP+ cardiomyocyte and its action potential was measured (C, panel c). The GFP+ cardiomyocytes generate ventricular type action potentials following electrical stimulation (D). (E) The action potential of normal mouse cardiomyocytes. (F) The number of CD31+ endothelial cells (brown) stained in the scar area of the FT (DCM) group (left panel a) was significantly greater than those of the MT (DCM) group (right panel b). These endothelial cells were not GFP+ cells. Bar, 10 μm. (G) The number of vessels in scar area of FT (DCM) or MT (DCM) groups was counted. (H) Most of nucleus (blue) containing GFP (green) had only one Y chromosome (red) and did not have more than two. (I) Small number of nucleus (blue) containing GFP (green) had two Y chromosome (red). (J) No arrhythmia was detected from FT (DCM) mice.
Flk1 is a marker of early mesodermal cells, and Flk1+ cells have the potential to differentiate into hematopoietic and endothelial lineages [13,15]. Although cardiomyocytes are also derivatives of mesodermal cells, only a few reports have suggested that expression of Flk1 indicates a commitment to a cardiomyocyte lineage [12,24]. Moreover, while these reports suggest that Flk1+ cells could be cardiac stem/progenitor cells, their functions, differentiation and ability to induce cardiac regeneration in vivo have not been reported previously.

In this report, we found that Flk1+ cells generated regular contractile colonies that expressed cardiac-specific markers more frequently than Flk1- cells. Thus, Flk1 is an effective marker for not only hematopoietic and endothelial stem/progenitor cells but also for cardiac stem/progenitor cells expressing Sca-1 and c-kit, which have been reported to be one of the cardiac stem/progenitor cell markers [20]. Although, Nanog and Oct4 were expressed in the population of day 4-sorted Flk1+ cells in our experiments, Yamashita et al. previously reported that day 4-sorted Flk1+ cells lacked the expression of Oct4 [25]. According to this result, the very weak Oct4 and Nanog expressions of day 4-sorted Flk1+ cells in our experiments would be result from the contamination of Flk1- cells during cell-sorting process. Notably, a previous study by Kouskoff et al. found that the Flk1- Brachyury+ cell population has a strong potential to differentiate into cardiomyocytes, which seems to contradict our observations. However, this anomaly may be due to the fact that the Flk1- Brachyury+ cells were harvested on day 3.25 after differentiation in their experiments [26]. In our experiments, we found that some of Flk1- cells isolated on day 3 converted into Flk1+ cells on day 4 of differentiation. Kattman SJ et al. also reported the difference of cardiac differentiation capacity between day 3.25 differentiated ES cells and day 4.25 differentiated ES cells [27]. Moreover, Kouskoff et al. employed different methods for cell differentiation, which may be another reason for why there are differences between their findings and ours.

Heart cell transplantation using cardiomyopathy models has been the subject of many studies. Candidate cells for heart cell transplantation include fetal cardiomyocytes, skeletal muscle cells, and bone marrow cells. However, routine use of these cell types in the clinic is hampered either by ethical or practical limitations or by the failure of these cells to differentiate in vivo into mature cardiomyocytes. Other, more suitable, candidate cells are adult CS and ES cells, whose transplantation also improves cardiac function in models of cardiomyopathy [10,11]. However, morphological evaluation of the type of cardiomyocytes generated by these cells has not been performed.

We speculated that the best transplantable cell candidate would be one that is as close to the cardiac stem/progenitor level as possible, as these would not only have the capacity to differentiate in vivo into all cardiac lineages, particularly ventricular cells, they would also proliferate well in vitro making it easier to obtain cell numbers sufficient for transplantation. We show here that Flk1+ cells satisfy these requirements, as they have a good potential to proliferate and differentiate into cardiomyocytes upon cell transplantation. Moreover, we found by immunohistochemistry that the engrafted Flk1+ cell derived cardiomyocytes were close to ventricular type cardiomyocytes, as they expressed MLC2v, cTn-I, and CH1, but not ANP. In addition, the electrophysiological response of single engrafted cardiomyocytes, indicated that these cells possessed adult ventricular cell type action potentials, rather than embryonic or neonatal ventricular type potentials [21,22]. Additionally, most of these GFP+ cardiomyocytes arose as a result of regeneration in host hearts, assessed by FISH analysis. It is not yet clear why these cells spontaneously differentiate into ventricular type cardiomyocytes in vivo, but factors in the damaged ventricular environment may promote their differentiation. Moreover, we found that the engrafted cardiomyocytes have gap junctions on their surfaces. Although we have failed to show the actual direct functional coupling between the GFP+ cells and endogenous cardiomyocytes as previously reported [28], our results demonstrated that the transplanted cells could not induce any arrhythmias from FT(DCM) mice, which suggest the possibility of functional coupling. Thus, Flk1+ cells transplanted into cardiomyopathic hearts effectively generate into cardiomyocytes that have the potential to communicate with host cardiomyocytes and contribute to contractile function. Although an increased number of vessels in the scar area of the FT(DCM) treatment group was observed more frequently than in the MT(DCM) group, we could not detect GFP+ CD31+ cells forming vascular structures. This may be due to the fact that we did not use an ischemic animal model in which angiogenesis from Flk1+ cells would be strongly induced.

A major concern about using ES cells in clinical settings is that they may generate teratomas [29]. However, in this study, we did not find abnormal cell growth in the hearts of any of the FT(DCM) mice. We also conducted a longer term study of this problem by transplanting Flk1+ cells into an additional five mice and sacrificing them ten weeks post-transplantation. No abnormal cell growths were observed. Moreover, we could not detect Ki-67 in the nuclei of engrafted cells at 4 wks post-transplantation, which suggests that these cells have terminally differentiated. In contrast, the transplantation of Flk1- cells led to tumors in the hearts of three of nine mice. Thus, unlike the heterogeneous and undifferentiated Flk1- cells, which expressed high levels of Nanog and Oct4, Flk1+ cells sorted on day 4 of ES cell differentiation lack the potential to induce abnormal cell growth and are therefore likely to be relatively safer cell transplantation candidates than Flk1- cells.

Flk1+ cell-transplanted DCM mice were examined by echocardiography and cardiac catheterization to measure the effect of the transplantation on cardiac function. The cardiac function values determined by these methods revealed that the transplanted Flk1+ cells favorably influenced the systolic and diastolic function of the left ventricle. Although we cannot declare the engrafted GFP+ cTn-I+ cell number is...
sufficient for the recovery of cardiac function, this recovery in the transplanted DCM mice is likely to be partly due to the cooperative contractile function of the cardiomyocytes derived from the Flk1+ cells and angiogenesis induced by Flk1+ cell-transplantation. However, the improved cardiac function could be the result of other mechanisms that arise from the Flk1+ cell transplantation. These possibilities will be investigated in future experiments.

In conclusion, by using a simple cell sorting method, we were able to collect numerous cardiac stem/progenitor cells that have the potential to differentiate into cardiomyocytes both in vitro and in vivo from differentiating ES cells. Furthermore, the cardiomyocytes generated by these stem/progenitor cells improve cardiac function in a cardiomyopathy mouse model. However, we have not yet been able to isolate committed cardiac stem/progenitor cells that exclusively differentiate into cardiomyocytes. The search for these cells will be the subject of future studies.

Acknowledgements

We thank Dr. Ogawa for kindly providing the Gact4 ES cell line, Dr. Kodama for generously providing the OP-9 stromal cell line, and Dr. Shioya for valuable advice about cell isolation method.

This study was supported by the Program for Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation (NIBIO) (03-2) and Research of Japan, and by a Grant-in-Aid for Creative Scientific Research (13GS0009).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2007.05.013.

References


