Cardiomyocytes fuse with surrounding noncardiomyocytes and reenter the cell cycle

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The concept of the plasticity or transdifferentiation of adult stem cells has been challenged by the phenomenon of cell fusion. In this work, we examined whether neonatal cardiomyocytes fuse with various somatic cells including endothelial cells, cardiac fibroblasts, bone marrow cells, and endothelial progenitor cells spontaneously in vitro. When cardiomyocytes were cocultured with endothelial cells or cardiac fibroblasts, they fused and showed phenotypes of cardiomyocytes. Furthermore, cardiomyocytes reentered the G2-M phase in the cell cycle after fusing with proliferative noncardiomyocytes. Transplanted endothelial cells or skeletal muscle–derived cells fused with adult cardiomyocytes in vivo. In the cryo-injured heart, there were Ki67-positive cells that expressed both cardiac and endothelial lineage marker proteins. These results suggest that cardiomyocytes fuse with other cells and enter the cell cycle by maintaining their phenotypes.

Introduction

Many reports have indicated that adult stem cells have "plasticity" and transdifferentiate into various types of cells including cardiomyocytes (Jackson et al., 2001; Orlic et al., 2001a; Badorff et al., 2003). Bone marrow cells have been incorporated into the damaged myocardium and have expressed cardiac-specific proteins (Jackson et al., 2001; Orlic et al., 2001a; Mangi et al., 2003). Besides undifferentiated stem cells, differentiated somatic cells such as endothelial cells and skeletal muscle–derived cells have been also reported to transdifferentiate into cardiomyocytes when cocultured with cardiomyocytes (Condorelli et al., 2001; Iijima et al., 2003). However, the concept of plasticity has been challenged by the new findings that embryonic stem cells adopt the phenotype of bone marrow cells or central nervous stem cells by cell fusion (Terada et al., 2002; Ying et al., 2002). Bone marrow cells have been reported to fuse with hepatocytes in the severely injured liver and proliferate extensively, resulting in millions of highly aneuploid new hepatocytes (Vassilopoulos et al., 2003; Wang et al., 2003). In the brain, bone marrow cells form stable heterokaryons with Purkinje neurons in the absence of selective pressure. In this intracellular milieu, bone marrow cell–derived nuclei are reprogrammed to activate the Purkinje-specific gene, resulting in the phenotype of the Purkinje cells becoming dominant over time (Weimann et al., 2003). These results suggest that cell fusion might be one of the physiological mechanisms through which the cells change their lineage and the tissues are rejuvenated or regenerated.

Adult cardiomyocytes have been thought to be terminally differentiated and unable to divide, thus myocyte growth under pathologic conditions as well as physiologic conditions is believed to be accomplished only by cellular hypertrophy (Morgan and Baker, 1991; Chien, 1995). Cytoplasmic extracts of adult cardiomyocytes have been reported to reduce the expression of proliferating cell nuclear antigens in proliferating noncardiomyocytes (Engel et al., 2003), suggesting that some inhibitory molecules of the cell cycle might exist in the cytoplasm of adult cardiomyocytes. However, recent reports have indicated that adult cardiomyocytes can divide after myocardial infarction and at end-stage heart failure (Kajstura et al., 1998; Beltrami et al., 2001). The precise mechanism of how cardiomyocytes acquire proliferative ability is still elusive, but there is a possibility that mobilized bone marrow–derived stem cells or cardiac progenitor cells start to proliferate in response to some environmental cues (Orlic et al., 2001b; Beltrami et al.,

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Abbreviations used in this paper: ANF, atrial natriuretic factor; β-gal, β-galactosidase; CAT, chloramphenicol acetyltransferase; cFB, cardiac fibroblasts; cTnT, cardiac troponin T; EPC, endothelial progenitor cell; HUVEC, human umbilical vein endothelial cells; PH3, phosphohistone H3; RFP, red fluorescent protein; UEA-1, ulex europaeus agglutinin-1; vWF, von Willebrand factor.
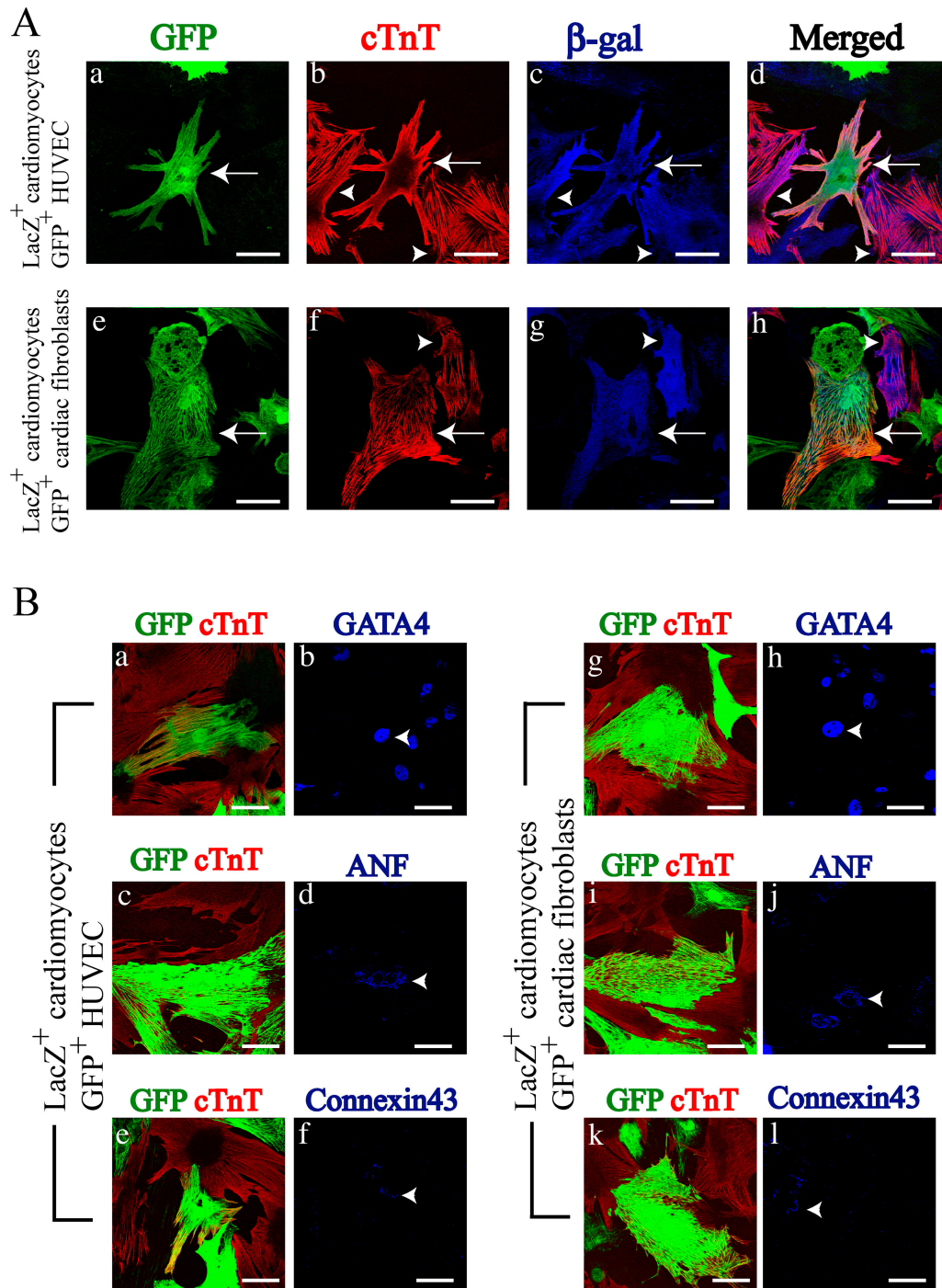
Figure 1. **HUVEC and cFB acquired the cardiac phenotype through cell fusion with cardiomyocytes.** (A) *LacZ*-expressing cardiomyocytes of neonatal rats were cocultured with GFP[+] HUVEC (a–d) or GFP[+] cFB (e–h) and stained with mouse monoclonal anti-cTnT (red) and rabbit polyclonal anti-β-gal antibodies (blue). Merged images were obtained from the same confocal plane. GFP[+] HUVEC and GFP[+] cFB (a and e, arrow) expressed cTnT (b and f, arrow) and β-gal (c and g, arrow) in the same cell (merged on d and h). Arrowheads indicate the nonfused cardiomyocytes. Bars, 50 μm. (B) *LacZ*-expressing cardio-myocytes of neonatal rats were cocultured with GFP[+] HUVEC (a–f) or GFP[+] cFB (g–l) and stained with mouse monoclonal anti-cTnT (red) and goat poly-clonal anti-GATA4 or rabbit polyclonal anti-ANF or anti-connexin43 antibodies (blue). cTnT-expressing GFP[+] HUVEC (a, c, and e) and cTnT-expressing GFP[+] cFB (g, i, and k) expressed GATA4 (b and h, arrowhead), ANF (d and j, arrowhead), and connexin43 (f and l, arrowhead). Bars, 50 μm.

2003; Matsuura et al., 2004). Recently, Oh et al. (2003) have reported that transplanted cardiac progenitor cells in the adult murine heart not only differentiate into cardiomyocytes, but also fuse with preexisting cardiomyocytes in the ischemia model. This finding indicates that there is another possible ex-

planation, in which the ability to proliferate might be conferred on cardiomyocytes by surrounding proliferative noncardio-myocytes by means of cell fusion in the diseased heart. To date, two studies have been reported regarding the cell fusion between cardiomyocytes and noncardiomyocytes. Evans et al.
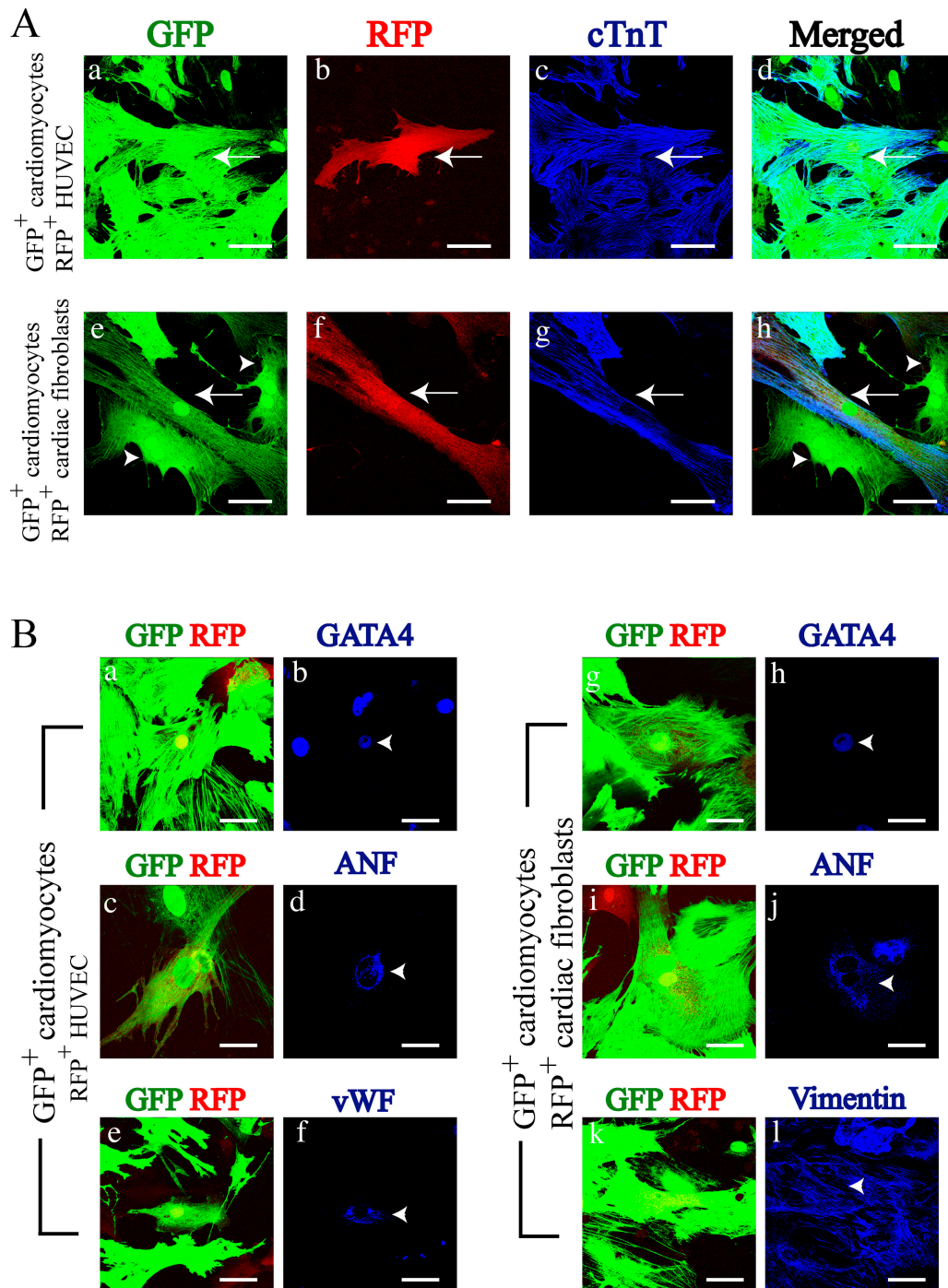
Figure 2. HUVEC and cFB acquired the cardiac phenotype through cell fusion with cardiomyocytes. (A) Cardiomyocytes of GFP transgenic neonatal rats were cocultured with RFP⁺ HUVEC (a–d) and RFP⁺ cFB (e–h) and were stained with mouse monoclonal anti-cTnT (blue). Merged images were obtained from the same confocal plane. RFP⁺ HUVEC and RFP⁺ cFB (b and f, arrow) expressed cardiomyocyte-derived GFP (a and e, arrow) and cTnT (c and g, arrow) in the same cell (merged on d and h). Arrowheads indicate GFP⁺ noncardiomyocyte. Bars, 50 μm. (B) Cardiomyocytes of GFP transgenic neonatal rats were cocultured with RFP⁺ HUVEC (a–f) and RFP1 cFB (g–l) and were stained with goat polyclonal anti-GATA4 or rabbit polyclonal anti-ANF, anti-vWF, or mouse monoclonal anti-vimentin antibodies (blue). GFP⁺/RFP⁺ cardiomyocyte-HUVEC fused cells (a and c) and GFP⁺/RFP⁺ cardiomyocyte-cFB fused cells (g and i) expressed GATA4 (b and h, arrowhead) and ANF (d and j, arrowhead). GFP⁺/RFP⁺ cardiomyocyte-HUVEC fused cells (e) express vWF (f, arrowhead) and GFP⁺/RFP⁺ cardiomyocyte-cFB fused cells (k) express vimentin (l, arrowhead). Bars, 50 μm.

(1994) have reported that neonatal cardiomyocytes lose their cardiac phenotypes when forced to fuse with embryonic fibroblasts by using polyethylene glycol. Alvarez-Dolado et al. (2003) have demonstrated that transplanted bone marrow cells fuse with cardiac myocytes in the heart and express cardiac contractile proteins. Currently, it is still unknown which of the mechanisms, transdifferentiation or fusion, plays a major role in phenotypic change of the cells in the heart. Therefore, it is important to examine the fusiogenic ability of cardiomyocytes with various types of cells in vivo and in vitro and to know

Figure 3. **Cardiac phenotype is dominated in fused cells.** (A and B) Each bar represents the number of GFP$^+$/RFP$^+$ fused cells in 10^5 RFP$^+$ HUVEC cocultured with GFP$^+$ neonatal rat cardiomyocytes at different time in culture. The number of GFP$^+$/RFP$^+$ fused cells was increased with the time-dependent manner. The percentage of cTnT-positive cells and the percentage of vWF-positive cells in GFP$^+$/RFP$^+$ fused cells are presented by line graphs. The percentage of cTnT-positive cells in GFP$^+$/RFP$^+$ fused cells (A) increased and that of vWF-positive cells (B) decreased with the time-dependent manner. Data are mean ± SD of three independent experiments. *, P < 0.01 vs. 1 d; †, P < 0.01 vs. 4 d; ‡, P < 0.05 vs. 1 d. (C and D) Each bar represents the number of GFP$^+$/RFP$^+$ fused cells in 10^5 RFP$^+$ cFB cocultured with GFP$^+$ neonatal rat cardiomyocytes at different time in culture. The number of GFP$^+$/RFP$^+$ fused cells was increased with the time-dependent manner. The percentage of cTnT-positive cells and the percentage of vimentin-positive cells in GFP$^+$/RFP$^+$ fused cells are presented by line graphs. The percentage of cTnT-positive cells in GFP$^+$/RFP$^+$ fused cells (C) increased and that of vimentin-positive cells (D) decreased with the time-dependent manner. Data are mean ± SD of three independent experiments. *, P < 0.01 vs. 1 d; †, P < 0.01 vs. 4 d; ‡, P < 0.05 vs. 1 d.
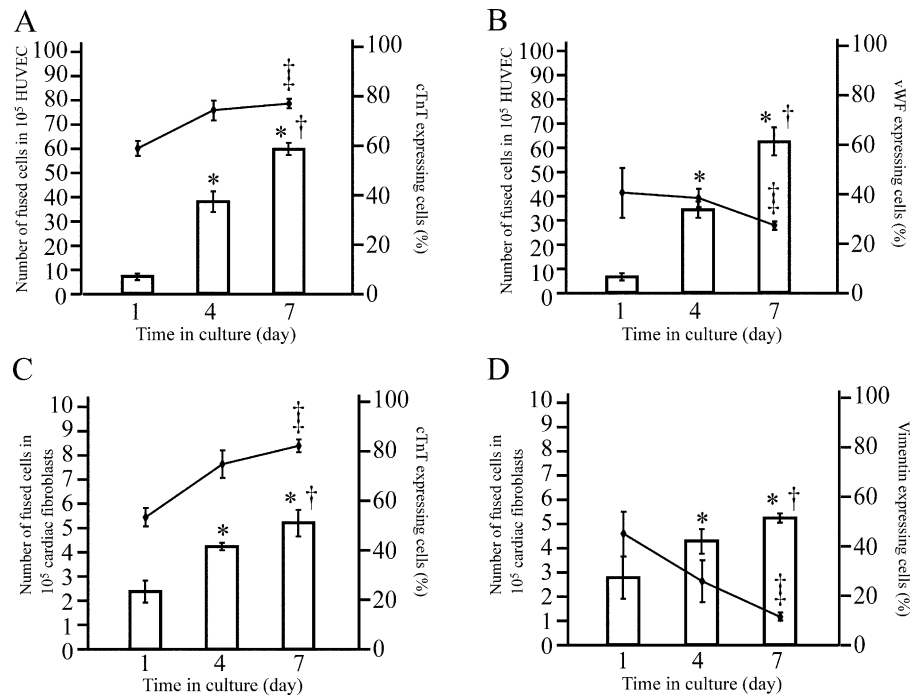
whether cardiomyocytes can obtain proliferative ability after fusion without losing cardiac phenotypes.

Here, we demonstrate that neonatal cardiomyocytes fuse with various kinds of somatic cells including human umbilical vein endothelial cells (HUVEC), cardiac fibroblasts (cFB), bone marrow cells, and endothelial progenitor cells (EPCs) spontaneously in vitro. When cardiomyocytes fused with HUVEC or cFB both phenotypes were observed at first, but cardiac phenotypes became dominant over time. Furthermore, terminally differentiated cardiomyocytes reentered the G2-M phase in the cell cycle after cell fusion with proliferative noncardiomyocytes. Cardiomyocytes spontaneously fused with transplanted HUVEC and skeletal muscle–derived cells in vivo and maintained the phenotypes of cardiomyocytes. Finally, we demonstrated that some cells in the cryoinjured heart expressed both cardiac and endothelial lineage marker proteins along with Ki67.

Results

HUVEC and cFB acquired the cardiac phenotype through cell fusion with cardiomyocytes

When GFP-expressing (GFP$^+$) HUVEC or GFP$^+$ cFB were cocultured with neonatal rat cardiomyocytes that were infected with the adenoviral vector carrying the *LacZ* reporter gene, some of GFP$^+$ HUVEC and GFP$^+$ cFB coexpressed both cardiac troponin T (cTnT) and β-galactosidase (β-gal) (Fig. 1 A, a–h, arrows). These GFP- and cTnT-expressing cells also expressed GATA4 (Fig. 1 B, a, b, g, and h, arrowheads), atrial natriuretic factor (ANF) (Fig. 1 B, c, d, i and j, arrowheads), and connexin43 (Fig. 1 B, e, f, k, and l, arrowheads). The expres-

sion of the cardiac proteins in GFP$^+$ cells was observed only in the coculture condition and all of cTnT-expressing HUVEC and cFB were positive for β-gal, suggesting that HUVEC and cFB acquired the cardiac phenotype through cell fusion with cardiomyocytes. The cTnT-positive GFP-expressing cells were found in 0.019% of GFP$^+$ HUVEC and 0.004% of GFP$^+$ cFB after 4 d of coculture. To rule out the possibility that noncardiomyocytes were infected with the adenoviral vector carrying the *LacZ* reporter gene during coculture, we examined the cell fusion by using neonatal cardiomyocytes prepared from GFP transgenic rats and red fluorescent protein-expressing (RFP$^+$) HUVEC or RFP$^+$ cFB. When RFP$^+$ HUVEC or RFP$^+$ cFB were cocultured with GFP$^+$ cardiomyocytes, some of RFP$^+$ HUVEC and RFP$^+$ cFB coexpressed cardiomyocyte-derived GFP and cTnT (Fig. 2 A, a–h, arrows). Some of both GFP- and RFP-expressing (GFP$^+$/RFP$^+$) fused cells expressed GATA4 (Fig. 2 B, a, b, g, and h, arrowheads), ANF (Fig. 2 B, c, d, i, and j, arrowheads), and connexin43 (unpublished data). Live imaging showed that GFP$^+$ cardiomyocytes fused with RFP$^+$ HUVEC beat spontaneously (see Fig. S1 and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200312111/DC1). They beat regularly and the beating rate was ∼80 beats/min, which was similar to that of cocultured cardiomyocytes, suggesting that cardiomyocyte function was maintained even after fusing with other cells.

Cardiac phenotypes became predominant in fused cells

The von Willebrand factor (vWF) and vimentin are phenotype-specific markers of endothelial cells and fibroblasts, respectively, and are never expressed in cardiomyocytes. When GFP$^+$ cardiomyocytes were cocultured with RFP$^+$ HUVEC or RFP$^+$

Table I. **The phenotypic analysis of fused cells between cardiomyocytes and HUVEC**

	Number of cells in 10^5 RFP$^+$ HUVEC (% of total fused cells)		
	1 d	4 d	7 d
GFP$^+$/RFP$^+$/TnT$^+$	3.86 ± 0.64 (58.6%)	27.2 ± 4.0 (73.7%)	44.2 ± 0.7 (76.2%)[c]
GFP$^+$/RFP$^+$/TnT$^-$	2.72 ± 0.26 (41.4%)	9.7 ± 0.9 (26.3%)	13.8 ± 1.2 (23.8%)[c]
GFP$^+$/RFP$^+$	6.59 ± 0.89	36.9 ± 3.2[a]	58.0 ± 1.9[a,b]
GFP$^+$/RFP$^+$/VW$^+$	2.79 ± 0.93 (41.3%)	13.5 ± 1.8 (39.2%)	17.4 ± 1.6 (27.7%)[c]
GFP$^+$/RFP$^+$/VW$^-$	3.71 ± 0.32 (58.7%)	20.7 ± 0.6 (60.8%)	45.2 ± 4.2 (72.3%)[c]
GFP$^+$/RFP$^+$	6.49 ± 1.17	34.2 ± 3.6[a]	62.6 ± 5.9[a,b]

Times indicate period after starting coculture. Fused cells were detected as GFP$^+$/RFP$^+$ cells. Each number represents the number of cells in 10^5 RFP$^+$ HUVEC.
Data are mean ± SD of three independent experiments.
[a]P < 0.01 vs. 1 d.
[b]P < 0.01 vs. 4 d.
[c]P < 0.05 vs. 1 d.

cFB, some of GFP$^+$/RFP$^+$ fused cells expressed vWF (Fig. 2 B, e and f) and vimentin (Fig. 2 B, k and l), respectively. To elucidate the phenotype in fused cells, we quantified the percentage of cTnT-, vWF-, or vimentin-expressing cells in the GFP$^+$/RFP$^+$ fused cells. When GFP$^+$ cardiomyocytes were cocultured with RFP$^+$ HUVEC, the number of GFP$^+$/RFP$^+$ fused cells was increased with the time-dependent manner (Fig. 3, A and B, bar graphs; Table I). The percentage of cTnT-expressing cells in the total fused cells was also increased with the time-dependent manner (d 1, 58.6 ± 2.9%; d 4, 73.7 ± 3.9%; d 7, 76.2 ± 1.9%); on the other hand, the percentage of vWF-expressing cells in fused cells was decreased with the time-dependent manner (d 1, 41.3 ± 10.3%; d 4, 39.2 ± 3.7%; d 7, 27.7 ± 1.6%), suggesting that the cardiac phenotype becomes predominant in the cells formed by fusion between cardiomyocytes and endothelial cells (Fig. 3, A and B, line graphs; Table I). When GFP$^+$ cardiomyocytes were cocultured with RFP$^+$ cFB, the number of GFP$^+$/RFP$^+$ fused cells was gradually increased (Fig. 3, C and D, bar graphs; Table II). The percentage of cTnT-expressing cells in fused cells was also increased with the time-dependent manner (d 1, 54.2 ± 3.8%; d 4, 75.8 ± 5.6%; d 7, 83.5 ± 2.6%); on the other hand, the percentage of vimentin-expressing cells (Fig. 2 B, k and l) in fused cells was decreased progressively (d 1, 45.3 ± 9.3%; d 4, 25.9 ± 8.8%; d 7, 11.5 ± 1.6%), suggesting that cardiac phenotypes also dominate in fused cells between cardiomyocytes and cFB (Fig. 3, C and D, line graphs; Table II).

Cardiomyocytes reenter the cell cycle by cell fusion with adult somatic cells in vitro

To determine whether cardiomyocytes reenter the cell cycle after fusion, we examined the expression of the cell cycle marker proteins such as Ki67, phosphohistone H3 (PH3), and cyclinB1 in fused cells. First, we examined whether monocultured HUVEC, cFB, and neonatal cardiomyocytes expressed Ki67, PH3, and cyclinB1 (Fig. 4). Many of HUVEC and cFB in the growth medium expressed Ki67, PH3, and cyclinB1 in their nuclei. Some neonatal rat cardiomyocytes expressed Ki67, but not PH3 and cyclinB1, suggesting that some neonatal cardiomyocytes are in the G1-S stage, but not in the G2-M stage of the cell cycle. However, when fused with HUVEC and cFB some cardiomyocytes expressed PH3 (Fig. 5 b for HUVEC and Fig. 5 d for cFB) and cyclinB1 (Fig. 5 f for HUVEC and Fig. 5 h for cFB). Among cardiomyocytes fused with HUVEC, ~9% of the cells expressed PH3 (Fig. 6 A). When treated for 6 h with nocodazole, an inhibitor of microtubule dynamics and cell cycle progression (Blajeski et al., 2002; Tamamori-Adachi et al., 2003), the number of PH3-expressing fused cells was increased up to ~21% in fused cells. At 3 h after the withdrawal of nocodazole, this number was decreased to ~15%. In cardiomyocytes fused with cFB (Fig. 6 B), ~14% of the cells expressed PH3, and at 24 h after the nocodazole treatment the number of PH3-expressing fused cells was increased up to ~29%. This number was decreased to ~20% at 3 h after the withdrawal of

Table II. **The phenotypic analysis of fused cells between cardiomyocytes and cardiac fibroblasts**

	Number of cells in 10^5 RFP$^+$ cardiac fibroblasts (% of total fused cells)		
	1 d	4 d	7 d
GFP$^+$/RFP$^+$/TnT$^+$	1.29 ± 0.19 (54.2%)	3.19 ± 0.20 (75.8%)	4.31 ± 0.33 (83.5%)[c]
GFP$^+$/RFP$^+$/TnT$^-$	1.08 ± 0.17 (45.8%)	1.02 ± 0.16 (24.2%)	0.86 ± 0.23 (16.5%)[c]
GFP$^+$/RFP$^+$	2.37 ± 0.31	4.21 ± 0.11[a]	5.17 ± 0.54[a,b]
GFP$^+$/RFP$^+$/Vimentin$^+$	1.28 ± 0.49 (45.3%)	1.12 ± 0.35 (25.9%)	0.59 ± 0.06 (11.5%)[c]
GFP$^+$/RFP$^+$/Vimentin$^-$	1.46 ± 0.25 (54.7%)	3.13 ± 0.20 (74.1%)	4.59 ± 0.15 (88.5%)[c]
GFP$^+$/RFP$^+$	2.74 ± 0.60	4.25 ± 0.37[a]	5.18 ± 0.14[a,b]

Times indicate period after starting coculture. Fused cells were detected as GFP$^+$/RFP$^+$ cells. Each number represents the number of cells in 10^5 RFP$^+$ cardiac fibroblasts.
Data are mean ± SD of three independent experiments.
[a]P < 0.01 vs. 1 d.
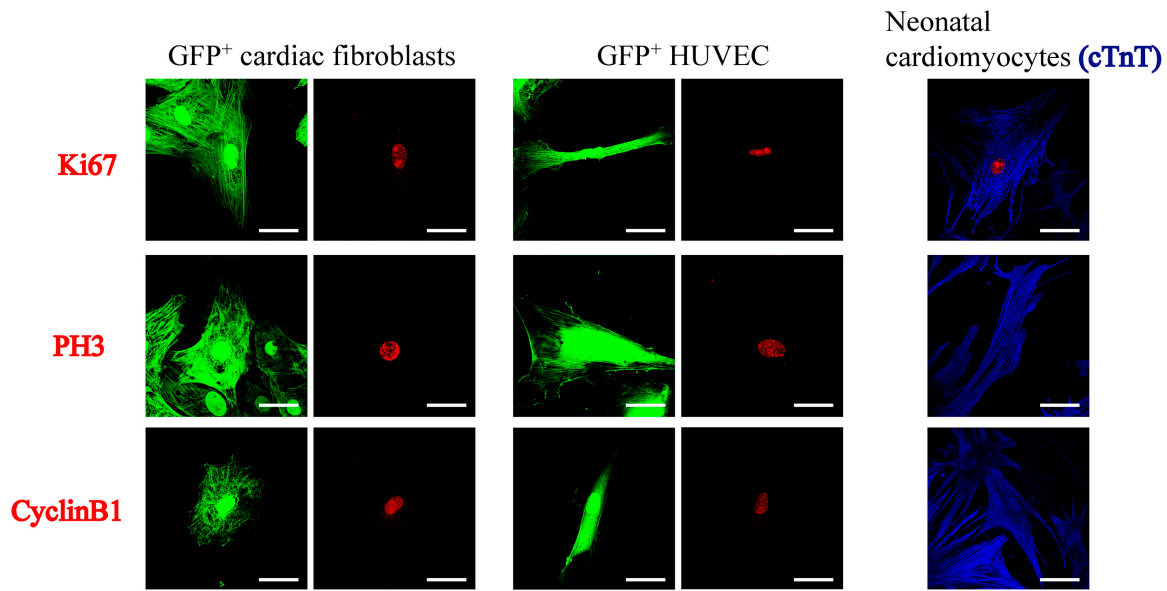[b]P < 0.01 vs. 4 d.
[c]P < 0.05 vs. 1 d.

Figure 4. **Localization of Ki67, PH3, and cyclinB1 in cFB, HUVEC, and neonatal cardiomyocytes.** GFP⁺ cFB, GFP⁺ HUVEC, and neonatal rat cardiomyocytes were stained with mouse monoclonal anti-Ki67 (top row, red), rabbit polyclonal anti-PH3 (middle row, red), and mouse monoclonal anti-cyclinB1 (bottom row, red). Cardiomyocytes were identified with anti-cTnT antibodies (blue). HUVEC and cFB expressed Ki67, PH3, and cyclinB1. Some of neonatal cardiomyocytes expressed Ki67, but not PH3 or cyclinB1. Bars, 50 μm.

nocodazole. These results suggest that some cardiomyocytes reenter the stage of mitosis after fusion with HUVEC and cFB. Mitosis of fused cardiomyocytes was confirmed by the existence of cells that showed visible chromosomes characteristic of the several distinct phases of mitosis (Fig. 6 C). In Fig. 6, GFP⁺ cardiomyocytes fused with RFP⁺ HUVEC (b and j) or RFP⁺ cFB (f) show prophase chromosomes (c), metaphase chromosomes (g), and anaphase chromosomes (k).

Cardiomyocytes spontaneously fuse with surrounding somatic cells in vivo

Next, we examined whether cardiomyocytes fuse with other mature somatic cells in vivo as well as in vitro. RFP⁺ HUVEC

or RFP⁺ skeletal muscle–derived cells isolated from neonatal Sprague-Dawley rats were first injected into the hearts of adult GFP transgenic Sprague-Dawley rats. At 7 d after injection, we observed GFP and RFP double-positive cells, which also expressed cTnT, in the heart (Fig. 7 A). The expression of two different kinds of dyes in the single cell suggests that spontaneous cell fusion could also occur in vivo in the heart. Furthermore, we examined the cell fusion in vivo by using the Cre/lox recombination assay. HUVEC infected with adenovirus containing the nuclear-localized Cre recombinase gene (Kanegae et al., 1995) were transplanted directly to the heart of mice that carry the loxP-flanked chloramphenicol acetyltransferase (CAT) gene located between the CAG promoter and the *LacZ*
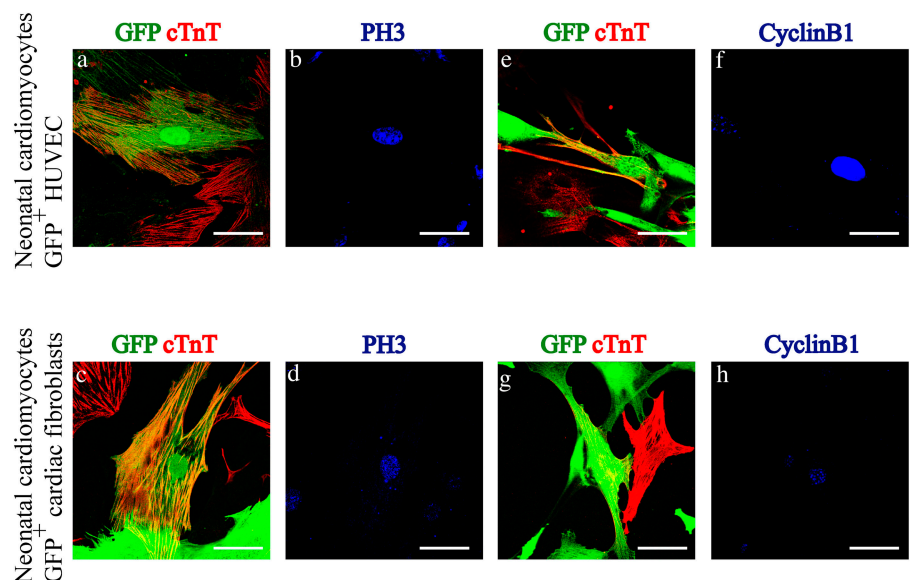

Figure 5. **Neonatal rat cardiomyocytes expressed PH3 and cyclinB1 after cell fusion with HUVEC or cFB.** Neonatal rat cardiomyocytes were cocultured with GFP⁺ HUVEC or GFP⁺ cFB and double stained with anti-cTnT (red) and anti-PH3 (blue) or anti-cyclinB1 (blue) antibodies. Fused cells between cardiomyocytes and HUVEC or cFB coexpressed both GFP and cTnT (a, c, e, and g, yellow in merged images), and expressed PH3 (b and d, blue) and cyclinB1 (f and h, blue) in their nuclei. Bars, 50 μm.
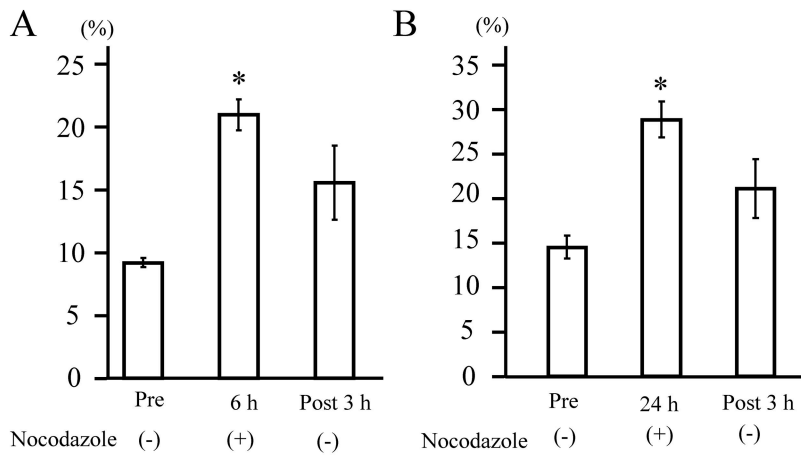
Figure 6. **Cardiomyocytes of neonatal rats reentered the cell cycle through cell fusion with HUVEC or cFB in vitro.** (A and B) Quantitative analysis of the percentage of PH3-expressing cardiomyocytes fused with HUVEC or cFB. The percentage of PH3-expressing cardiomyocytes in all fused cells with HUVEC (A) and cFB (B) was significantly increased with the treatment with nocodazole, and was decreased after the withdrawal of nocodazole. Data are mean ± SD of four independent experiments. *, P < 0.05 vs. pretreatment. (C) Fluorescent images of GFP[+] cardiomyocytes fused with RFP[+] HUVEC (top and bottom rows, a–d and i–l) or RFP[+] cFB (middle row, e–h) stained with Hoechst 33258 (blue). Typical prophase chromosome (c), metaphase chromosome (g), and anaphase chromosome (k) were visualized by Hoechst staining. Merged fluorescent images indicate that fused cells show mitotic figures. Bars, 50 μm.
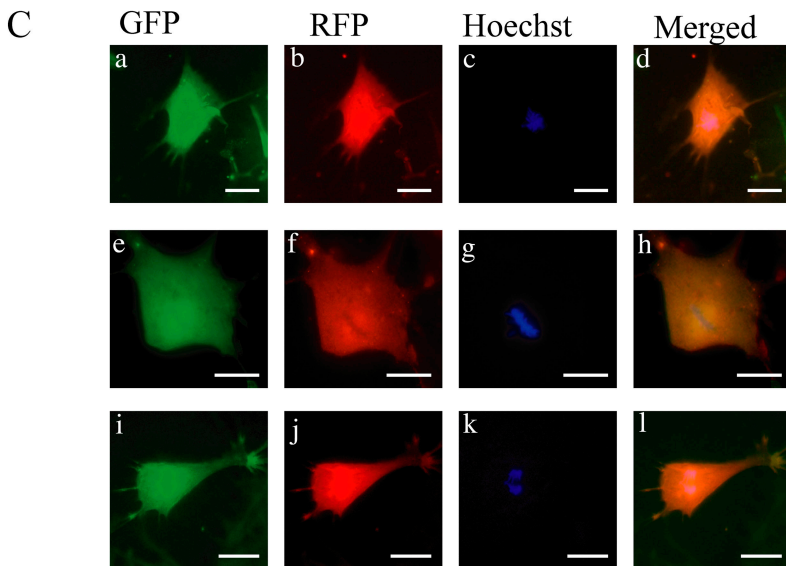
gene (Fig. 8 A, CAG-CAT-*LacZ*). At 4 d after transplantation, some β-gal^{+} cells were observed in the myocardium, and the same cells in the adjacent sections showed the expression of Cre and cTnT with the fine striated pattern. A typical image was presented in Fig. 8 B. Skeletal muscle–derived cells isolated from CAG-CAT-*LacZ* mice were transplanted to the heart of MerCreMer mice (Sohal et al., 2001) in which the expression of Cre was restricted to the cardiomyocytes under the control of the αMHC promoter after treatment with tamoxifen (Fig. 8 C). At 4 d after transplantation, we observed some β-gal^{+} cells that coexpressed Cre and cTnT in the myocardium (Fig. 8 D). These genetic results strongly suggest that HUVEC and skeletal muscle–derived cells could fuse spontaneously with cardiomyocytes in vivo. Next, we examined whether cardiomyocytes spontaneously fuse with endogenous surrounding cells in injured heart tissue. When adult rat hearts were cryoinjured there were cells expressing both cTnT and vWF at the border zone, but not at the normal and injured areas (Fig. 7 B). Staining of adjacent sections revealed that cells that expressed both cTnT and vWF also expressed desmin and Ki67 (Fig. 7 B), whereas there were no Ki67-expressing cardiomyocytes in the normal adult heart. These findings suggest that cardiomyo-

cytes fuse with surrounding endothelial lineage cells and reenter the cell cycle also in vivo.

Bone marrow–derived cells and EPCs can fuse with cardiomyocytes in vitro

Our in vitro and in vivo results suggest that cells expressing both cTnT and vWF in the damaged heart are fusion products of cardiomyocytes and endothelial cells. However, it has been reported that bone marrow–derived cells and EPCs differentiate into vascular cells and cardiomyocytes (Jackson et al., 2001; Badorff et al., 2003), leading us to examine whether bone marrow–derived or peripheral blood–derived EPCs may fuse with cardiomyocytes and express cTnT and vWF. Hematopoietic cells and mesenchymal cells from bone marrow of GFP transgenic mouse and human-derived EPCs were cocultured with neonatal cardiomyocytes that were infected with the adenoviral vector carrying the *LacZ* reporter gene. When GFP[+] bone marrow–derived mesenchymal cells were cocultured with *LacZ*[+] neonatal rat cardiomyocytes, ∼0.01% of GFP^{+} cells expressed cTnT and β-gal, suggesting that bone marrow–derived mesenchymal cells express cardiac-specific protein through cell fusion with cardiomyocytes (Fig. 9 A, a–d, arrow). In con-
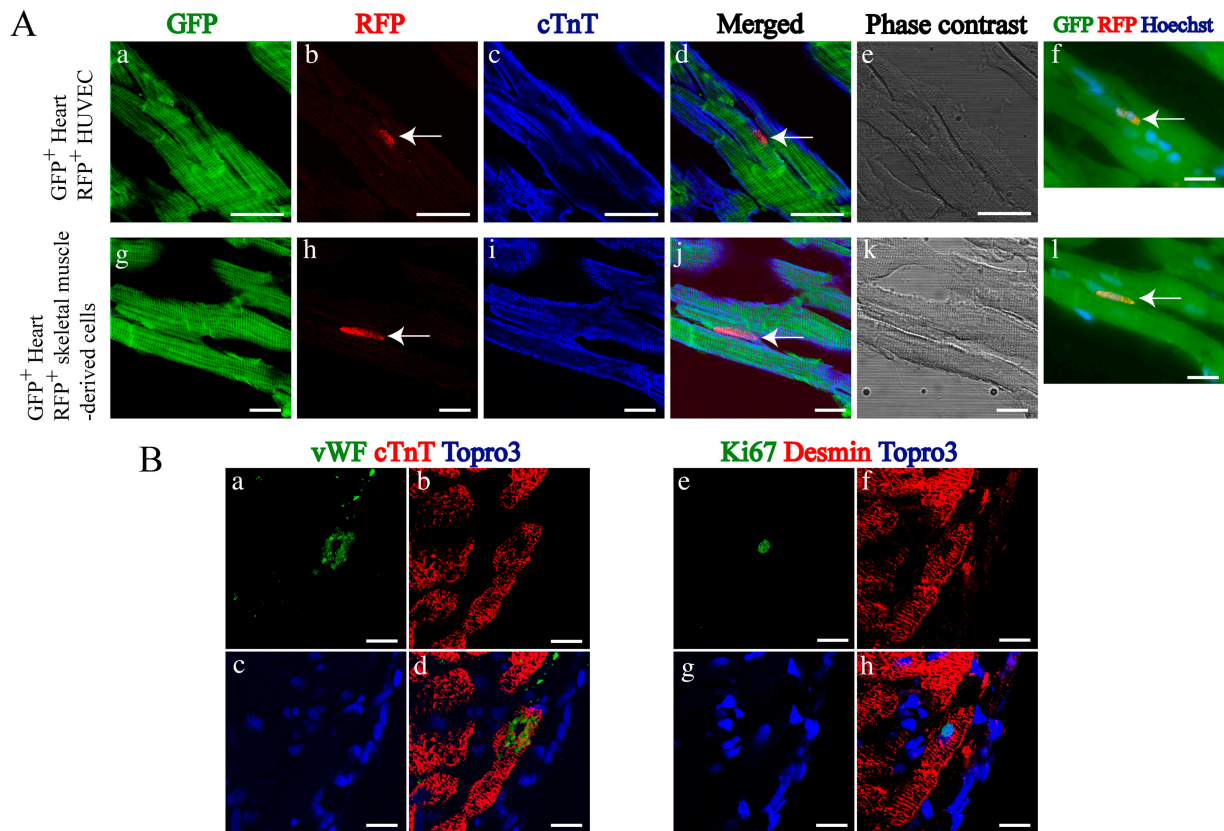
Figure 7. **Adult cardiomyocytes fused with somatic cells in vivo.** (A) RFP+ HUVEC or RFP+ skeletal muscle–derived cells were injected to the heart of GFP transgenic rats, and 6-μm sections were stained with rabbit polyclonal anti-RFP and mouse monoclonal anti-cTnT antibodies. Confocal microscopic images demonstrate that GFP+ cardiomyocytes (a and g, green) coexpress RFP, marker proteins of HUVEC (b, red) and skeletal muscle–derived cells (h, red), and cTnT (c and i, blue). d and j represent merged images; e and k represent phase-contrast images. The merged images of the same view were taken by fluorescent microscope (f and l). Nuclei were stained with Hoechst 33258 (f and l, blue). Arrows indicate the fused cells. Bars, 100 μm. (B) Two adjacent sections of 6 μm were cut from cryoinjured adult rat hearts. One section (a–d) was triple stained with mouse monoclonal anti-cTnT (red), rabbit polyclonal anti-vWF (green) antibodies, and Topro3 (blue). Another section (e–h) was triple stained with rabbit polyclonal anti-desmin (red), mouse monoclonal anti-Ki67 (green) antibodies, and Topro3 (blue). Confocal microscopic images in the border zone of cryoinjured hearts show a cell expressing vWF (a) and cTnT (b) with nucleus stained by Topro3 (c). Right panel shows a same fine-striated cell that expresses desmin (f) and Ki67 (e) with nucleus stained by Topro3 (g). d and h represent merged images. Bars, 100 μm.

trast, GFP+ hematopoietic cells expressed neither cTnT nor β-gal when cocultured with *LacZ*+ neonatal rat cardiomyocytes. Next, we isolated human-derived EPCs from the healthy volunteers and cultured them as described previously (Kalka et al., 2000). At 7 d after starting culture, spindle-shaped cells were stained with DiI-AcLDL and FITC-labeled ulex europaeus agglutinin-1 (UEA-1) lectin (Fig. 9 B, a and b), suggesting that these adherent cells were endothelial lineage cells. Immunocytochemical analysis revealed that ∼30% of these cells expressed vWF at this time (Fig. 9 B, c). When human-derived EPCs were cocultured with neonatal mouse cardiomyocytes that were infected with the adenoviral vector carrying the *LacZ* reporter gene, ∼0.1% of vWF-expressing cells expressed cTnT and β-gal (Fig. 9 C, a–d). Hoechst nuclear staining (Blau et al., 1983) revealed that these cells contained both a human-derived nucleus (smoothly appearance) and a mouse-derived nucleus (punctate appearance) (Fig. 9 C, e–h), suggesting that human-derived EPCs express cardiac-specific proteins through cell fusion with cardiomyocytes. These results suggest that bone marrow–derived mesenchymal cells and circulating EPCs, but not hematopoietic cells, fuse with cardiomyocytes.

Discussion

In this paper, we show that cardiomyocytes fuse with various types of cells including endothelial cells, cFB, bone marrow–derived mesenchymal cells, and EPCs in vitro. In the cells generated by fusion between cardiomyocytes and HUVEC or cFB, the cardiac phenotype became dominant until at least 7 d after starting coculture, and some fused cells reentered the cell cycle maintaining the cardiac phenotype. Moreover, cardiomyocytes fused with transplanted cells and surrounding cells in vivo as well as in vitro.

Heterokaryons have been used to determine whether specific traits of either parental cells are maintained or extinguished (Baron, 1993; Blau and Blakely, 1999). In this work, we identified fused cells retrovirally induced by two different fluorescent dyes. Analysis of lineage-specific marker proteins revealed that the phenotype of cardiomyocytes became more dominant than that of HUVEC and cFB as time passed. Besides the contractile proteins, a cardiac-specific secreted protein (ANF) and a cardiac-selective transcription factor (GATA4) were also expressed in the fused cells over 7 d. Moreover,
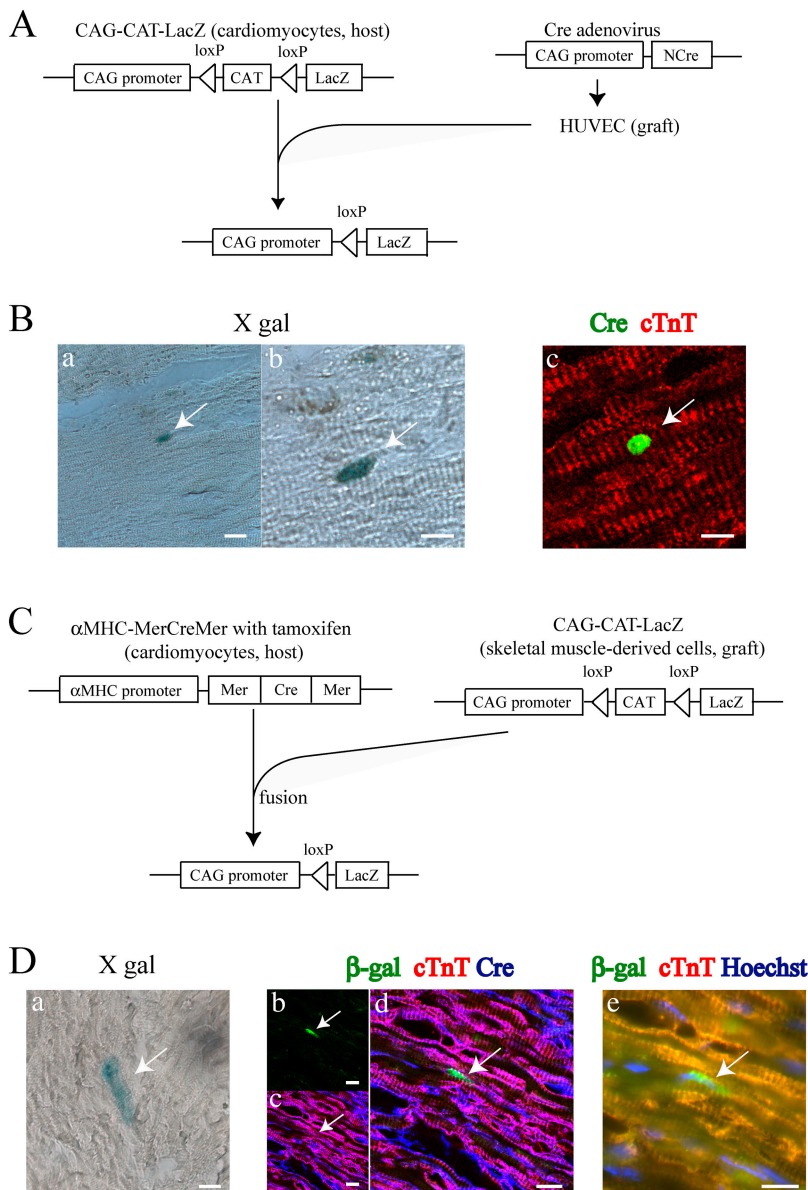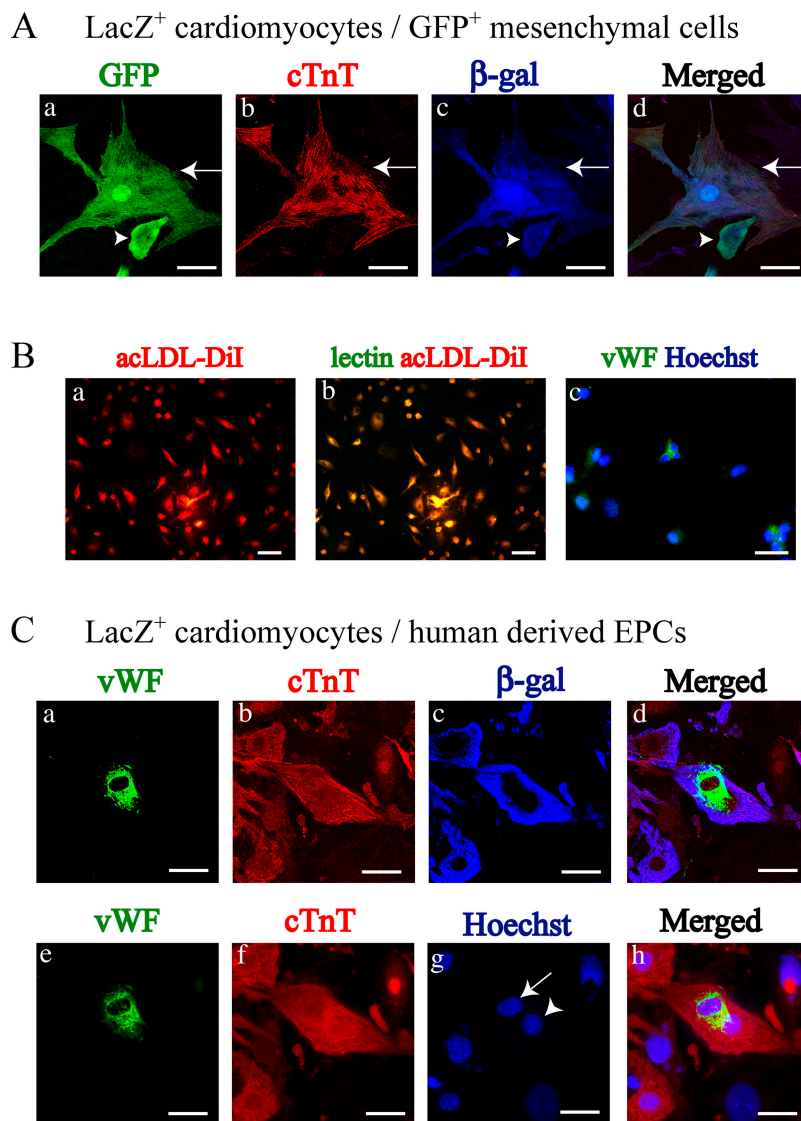
A CAG-CAT-LacZ (cardiomyocytes, host)

Cre adenovirus

HUVEC (graft)

B X gal

Cre cTnT

C αMHC-MerCreMer with tamoxifen (cardiomyocytes, host)

CAG-CAT-LacZ (skeletal muscle-derived cells, graft)

fusion

D X gal

β-gal cTnT Cre

β-gal cTnT Hoechst

Figure 8. **Cre/lox recombination assay for detection of cell fusion in vivo.** (A and C) Schematic representation of the transgenes expressed by the mouse line and the adenovirus used in HUVEC transplantation model (A) and in skeletal muscle–derived cell transplantation model (C). (B) Cre-expressing HUVEC were transplanted to the heart of CAG-CAT-LacZ mice. Two adjacent sections of 6 μm were prepared. One section treated with X-gal staining demonstrated a β-gal+ cell in the myocardium (a and b, arrow). The adjacent section, which was double stained with mouse monoclonal anti-Cre and goat polyclonal anti-cTnT antibodies, showed the expression of Cre (c, green, arrow) and cTnT (c, red, arrow) with the fine-striated pattern in the same cell in a and b. Bars: a, 50 μm; b and c, 100 μm. (D) Skeletal muscle–derived cells isolated from CAG-CAT-LacZ mice were transplanted to the heart of MerCreMer mice treated with tamoxifen. Sections were analyzed by X-gal staining or by triple staining with rabbit polyclonal anti-β-gal, goat polyclonal anti-cTnT, and mouse monoclonal anti-Cre antibodies. X-gal staining revealed a β-gal+ cell in the myocardium (a). The immunofluorescent confocal images demonstrated that β-gal+ (b and d, green) cells also expressed cTnT (c and d, red) and Cre (c and d, blue). The merged images of the same view of d were taken by fluorescent microscope (e). Nuclei were stained with Hoechst 33258 (e, blue). Arrows indicate the fused cells. Bars, 100 μm.

fused cells not only expressed cardiac-specific proteins, but also showed the function of cardiomyocytes (i.e., spontaneous beating). Evans et al. (1994) have reported that neonatal cardiomyocyte-fibroblast heterokaryons lose the expression of myosin light chain 2 gene, ANF, and muscle enhancer factor 2 until 6 d, suggesting that the cardiac phenotype is recessive. The discrepancy between our and Evans's results may be explained by the difference in used cells and the method of cell fusion. Evans et al. (1994) used embryonic fibroblasts for coculture and forced the inducing of cell fusion by using polyethylene glycol, whereas we used primary isolated cFB and examined the spontaneous fusion. It remains to be determined whether the nuclei of noncardiomyocytes are reprogrammed to express cardiac proteins by a dominantly acting cardiac factor.

An increase in cardiac mass during fetal period is accomplished predominantly by a cardiomyocyte proliferation, but soon after birth there is a transition from hyperplastic to hypertrophic growth (Morgan and Baker, 1991; Chien, 1995). Many

studies have been made to elucidate the mechanism by which cell cycle is arrested in postnatal cardiomyocytes (Agah et al., 1997; Tamamori-Adachi et al., 2003). The adenoviral delivery of cyclinD1 or E2F-1 has been reported to induce cardiomyocytes to reenter the G2/M stage of the cell cycle. In the present work, cardiomyocytes fused with noncardiomyocytes that have proliferative ability expressed G2-M stage cell cycle proteins. After the treatment with nocodazole, PH3-expressing fused cells were significantly enriched, and after the withdrawal of nocodazole, the number was decreased. Moreover, there were fused cells showing the mitotic figures, suggesting that the cell cycle was actively progressing toward the M stage in the fused cells. Engel et al. (2003) have reported that p21 in the cytoplasm of adult cardiomyocytes down-regulates the proliferating cell nuclear antigen protein level in S phase nuclei. The inhibitory effect of the adult cardiomyocyte extract was abolished when an excess volume of S phase cytoplasmic extract from noncardiomyocytes was present. In a similar way, the cytoplasmic factors

Figure 9. Cardiomyocytes fused with adult immature somatic cells in vitro. (A) *LacZ*-expressing cardiomyocytes of neonatal rats were cocultured with GFP⁺ bone marrow mesenchymal cells. After 4 d of coculture, cells were stained with mouse monoclonal anti-cTnT (red) and rabbit polyclonal anti-β-gal antibodies (blue). Merged image was obtained from the same confocal plane. GFP⁺ mesenchymal cells (a, arrow) expressed cTnT (b, arrow) and β-gal (c, arrow) in the same cell (merged on d). Arrowheads indicate bone marrow cells fused with noncardiomyocyte. Bars, 50 μm. (B) Fluorescent microscopic images of EPCs cultured 7 d after isolation from peripheral blood. EPCs were identified as double-positive cells of DiI-labeled AcLDL uptake (a, red) and UEA-1 lectin reactivity (b, yellow in merged images). Some adherent cells expressed vWF (c, green). Nuclei were stained with Hoechst 33258 (c, blue). Bars, 50 μm. (C) Human-derived EPCs were cocultured with neonatal mouse cardiomyocytes infected with *LacZ* adenovirus. After 4 d of coculture, cells were stained with rabbit polyclonal anti-vWF (green), goat polyclonal anti-cTnT (red), and mouse monoclonal anti-β-gal antibodies (blue in top row) and Hoechst 33258 (bottom row, blue). The fluorescent confocal microscopic images (a–d, top row) demonstrate that vWF-expressing cells (a) expressed cTnT (b) and β-gal (c) in the same cell. Note that Cy5-conjugated secondary antibodies were used to visualize β-gal. The images of the same cell were taken by fluorescent microscope (e–h, bottom row). Hoechst staining of the nuclei revealed that homogenously stained nuclei (arrow) were of human cell origin and that mouse nuclei showed a punctate appearance (arrowhead). d and h represent merged images. Bars, 50 μm.

A — LacZ⁺ cardiomyocytes / GFP⁺ mesenchymal cells

B

C — LacZ⁺ cardiomyocytes / human derived EPCs

of the proliferative cells in the G2-M stage may overcome the unknown endogenous cell cycle inhibitors in the heterokaryons.

We examined two kinds of cells for the in vivo transplantation model. Endothelial cells are a component of the cardiac interstitium, and it is possible that cardiomyocytes fuse with surrounding endothelial cells. Skeletal muscle cells do not exist in the myocardium, but myoblasts have the nature to fuse to form myotubes (Tajbakhsh, 2003), and clinical trials of autologous skeletal myoblast transplantation into the failed heart are currently underway (Menasche et al., 2003; Pagani et al., 2003). Consistent with our in vitro results, cardiomyocytes fused with transplanted HUVEC and skeletal muscle–derived cells. Reinecke et al. (2002) have reported that skeletal myoblasts differentiate into mature skeletal muscle and do not express cardiac-specific genes after been grafted into the heart. In their paper, rat satellite cells were tagged in vitro with BrdU, and the grafted cells were examined by double staining with the BrdU tag and cardiac-specific markers. However, this approach would have disadvantage of potential signal dilution if there is significant donor cell proliferation after transplantation (Dow-

ell et al., 2003). We used genetically modified animals and cells that carry ubiquitously expressed fluorescent proteins or that carry the Cre recombinase gene and the loxP-flanked CAT gene located between CAG promoter and the *LacZ* gene for monitoring donor cell fate after transplantation. These methods possibly enabled us to find rare fused cells in the heart tissue.

In the cryoinjured heart model, some cells in the border zone expressed both cardiomyocyte-specific and endothelial cell–specific proteins. The images were taken with optical sections through an appropriate confocal aperture, so that two different lineage markers were exactly recognized in the same cells. When we cocultured HUVEC with cardiomyocytes, some cells showed transient coexpression of vWF with cardiac sarcomeric proteins (unpublished data). Condorelli et al. (2001) have reported the same findings as a phenomenon that demonstrates the transition from one differentiated state (endothelium) to another (cardiac muscle). Our findings that all cTnT-expressing HUVEC coexpressed cardiomyocyte-derived β-gal and that transplanted HUVEC and cardiomyocytes formed the hybrid cells in the myocardium suggest that the cell fusion of cardiomyocytes with

surrounding endothelial cells occurs in the damaged heart. However, we cannot exclude the possibility of the transdifferentiation of endothelial cells into cardiomyocytes at present. Besides endothelial cells, EPCs have been reported to transdifferentiate into cardiomyocytes in an in vitro coculture model (Badorff et al., 2003). Bone marrow cells have been reported to contain stem cells, which transdifferentiate into various types of cells including vascular cells and cardiomyocytes (Jackson et al., 2001; Orlic et al., 2001a; Jiang et al., 2002). In our work, EPCs and bone marrow–derived mesenchymal cells expressed cardiac-specific proteins through cell fusion with cocultured cardiomyocytes. Therefore, it is possible that circulating EPCs or mesenchymal cells may differentiate into endothelial cells and then fuse with cardiomyocytes. Indeed, recent reports have suggested that transplanted bone marrow–derived cells fuse with preexisting hepatocytes and cardiomyocytes (Alvarez-Dolado et al., 2003; Vassilopoulos et al., 2003; Wang et al., 2003). Recently, cardiac stem cells have been reported to exist in the adult heart (Beltrami et al., 2003; Oh et al., 2003; Matsuura et al., 2004). Sca-1– or c-kit–positive cells from the heart differentiate into cardiomyocytes and other cells including endothelial cells in vitro. Oh et al. (2003) have shown that intravenously infused Sca-1–positive cardiac cells acquire the cardiac phenotypes by both transdifferentiation and fusion, suggesting that cardiac stem cells may differentiate into endothelial cells and then fuse with cardiomyocytes.

In the border zone of rat cryoinjured myocardium, some cardiomyocytes that coexpressed both cTnT and vWF were positively stained with anti-Ki67 antibodies. Ki67 is expressed in all phases of the cell cycle except G0, becomes particularly evident in the late S phase, and is increased further in the G2-M phase. Although Ki67 is not a specific marker for the G2-M stage, Beltrami et al. (2001) have concluded that cardiac myocytes divide in the pathological condition by the evidence of the Ki67 labeling of myocyte nuclei with the mitotic index. We could not detect mitotic figures in the cells that were positively stained with cTnT and vWF, but the expression of Ki67 was observed only in the heterotypic fused cells. Because there were no Ki67-positive nonfused cardiomyocytes, these results suggest that the fused cells enter the cell cycle in vivo as well as in vitro. Wagers and Weissman (2004) have proposed that cell fusion–mediated regeneration might be considered a physiological mechanism of repair. Our results suggest that augmented cell fusion in the diseased heart may contribute to the maintenance and replenishment of cardiomyocytes.

In conclusion, the present work demonstrates that cardiomyocytes have the fusiogenic activity with many different types of cells and obtain proliferative ability after fusion with somatic cells without losing their phenotypes in vitro and in vivo. Our future effort should be toward the understanding of the molecular mechanisms of phenotypic determination and cell cycle activation after fusion. During preparation of this manuscript, Reinecke et al. (2004) have reported that skeletal muscle cell grafting gives rise to skeletal–cardiac hybrid cells with unknown phenotypes. Our findings from the thorough examination of the fused cells are relevant to today's controversy concerning cell plasticity and provide further insights into the understanding of the consequences of cell therapy.

Materials and methods

Animals and reagents

Neonatal (0–1 d old) and adult Wistar rats (8 wk old) were purchased from Takasugi Experimental Animals Supply, Co., Ltd. Adult GFP transgenic mice (Okabe et al., 1997) were gifts from Dr. Okabe (Osaka University, Osaka, Japan). Neonatal and adult GFP transgenic rats (Ito et al., 2001) were purchased from Japan SLC. All protocols were approved by the Institutional Animal Care and Use Committee of Chiba University. The following antibodies were used for immunostaining: mouse monoclonal anti-cTnT (RV-C2, DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), goat polyclonal anti-cTnT, goat polyclonal anti-GATA4 (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-ANF (Peninsula Laboratories), rabbit polyclonal anti-connexin43 (Zymed Laboratories), rabbit polyclonal anti-desmin, rabbit polyclonal anti-vWF, mouse monoclonal anti–rat Ki67, mouse monoclonal anti–human Ki67 (Dako Cytomation), mouse monoclonal anti-β-gal, rabbit polyclonal anti-β-gal (CHEMICON International, Inc.), mouse monoclonal anti-vimentin, mouse monoclonal anti-Cre (Sigma-Aldrich), rabbit polyclonal anti-PH3 (Upstate Biotechnology), mouse monoclonal anti-cyclinB1 (Neomarkers), and rabbit polyclonal anti-RFP (MBL International Corporation). Fluorescent secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. pLEGFP-N1 and pDsRed2-N1 were purchased from CLONTECH Laboratories. Other reagents not specified were obtained from Sigma-Aldrich.

Cell culture

Neonatal rat cardiomyocytes and neonatal mouse cardiomyocytes were cultured as described previously (Komuro et al., 1990), basically according to the methods of Simpson and Savion (1982). Cardiomyocytes were plated at a field density of 10^5 cells/cm^2 on 35-mm culture dishes containing cover glasses coated by 1% gelatin, and cultured in DME with 10% FBS. cFB were obtained from primary culture described above by preplating technique. Fibroblasts on culture dishes were diluted fourfold, and infected with GFP- or RFP-expressing retroviral vector. Identification and characterization of GFP$^+$ or RFP$^+$ cFB was accomplished by immunocytochemistry and there were not vWF- and cTnT-expressing cells in GFP$^+$ or RFP$^+$ cFB. cFB from passages 3–5 were used. HUVEC were cultured on 0.1% gelatin-coated 100-mm dishes with EGM-2 (Cambrex Bio Science).

Bone marrow mononuclear cells were isolated from 10-wk-old GFP mouse by density gradient centrifugation with Histopaque-1083 as described previously (Zou et al., 2003). Primary culture of the bone marrow cells was performed according to Dexter's method with a few modifications (Dexter et al., 1977). Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FBS at 33°C in humid air with 5% CO$_2$. After 4 d in culture, nonadherent cells were collected as hematopoietic cells and were used to coculture with cardiomyocytes. Adherent cells were cultured though 14 d and were used to coculture with cardiomyocytes.

Human peripheral mononuclear cells were isolated from blood of human healthy volunteers by density gradient centrifugation with Histopaque-1077. Cells were plated on culture dishes coated with fibronectin in 0.5% gelatin solution and maintained in EGM-2. After 4 d in culture, nonadherent cells were removed by washing with PBS, and the culture was maintained though 7 d. After 7 d in culture, EPCs, recognized as attaching spindle-shaped cells, were assayed by costaining with DiI-labeled AcLDL (Biomedical Technologies) and FITC-labeled UEA-1 lectin. Cells were first incubated with 10 mg/ml DiI-labeled AcLDL at 37°C for 1 h and later fixed with 2% PFA for 10 min. After washes, the cells were reacted with 10 mg/ml FITC-labeled UEA-1 for 1 h. At 7 d in culture, ~30% of cells expressed vWF. Adherent cells at 7 d in culture were used to coculture with cardiomyocytes.

Skeletal muscle–derived cells were isolated from hind limbs of neonatal Sprague-Dawley rats or adult mice as described previously (Iijima et al., 2003). In brief, muscle tissues were minced smaller than 1 mm^3 and digested for a total of 45–60 min of three successive treatments with 0.05% trypsin-EDTA. The cells were collected in the supernatant after each treatment and resuspended in Ham's F10 medium in the presence of 20% horse serum, 0.5% chicken embryo extract, and 2.5 ng/ml bFGF. The cells were grown for 4 d in the same medium on the 2% gelatin-coated dishes. Then the medium was replaced by fresh medium supplemented with 20% FBS and cultured for 2 d.

Labeling of cells

DsRed2 sites of pDsRed2-N1 were subcloned in frame into Xho1 and Not1 sites of pLEGFP-N1 vector. Retroviral stocks were generated as described previously (Minamino et al., 2001). HUVEC and cFB were infected with the GFP- or RFP-expressing retroviral vector. Infected cells were selected for growth in the presence of 500 μg/ml neomycin for 2 wk. The

efficiency of transfection of GFP and RFP was over 95%. Skeletal muscle–derived cells at 4 d after isolation were infected with RFP-expressing retroviral vector. Infected cells were not selected and used for transplantation. The efficiency of transfection of RFP was ~50%.

Neonatal rat cardiomyocytes and neonatal mouse cardiomyocytes were tagged with recombinant adenovirus containing the *Escherichia coli* LacZ gene at a multiplicity of infection of 20 units for 24 h before coculture. After X-gal staining (Minamino et al., 2002), ~100% of cardiomyocytes were recognized to express β-gal.

The adenovirus AxCANCre (RIKEN BRC DNA Bank no. 1748) contains the Cre gene with a nuclear localized signal (NCre) (Kanegae et al., 1995) driven by the CAG promoter (Niwa et al., 1991). HUVEC were infected with AxCANCre at a multiplicity of infection of 50 units for 24 h before the transplantation. After immunostaining, ~100% of HUVEC were recognized to express Cre.

Coculture of neonatal cardiomyocytes with noncardiomyocytes
Neonatal cardiomyocytes were cultured through 4 d and then fluorescence-labeled HUVEC, cFB, bone marrow cells, and nonlabeled EPCs were cultured with cardiomyocytes at a 1:4 ratio. Coculture was maintained in adequate medium for each noncardiomyocyte. Cells were fixed with 4% PFA for 15 min at RT and were subjected to immunostaining at various time points after starting coculture.

Cell transplantation
GFP transgenic adult male rats were anesthetized with ketamine (50 mg/kg, i.p.) and xyladine (10 mg/kg, i.p.). A normal heart was injected with a standard dose of 10^6 RFP-expressing HUVEC or skeletal muscle–derived cells (Reinecke and Murry, 2003). In the HUVEC transplantation model, the immunosupressor FK506 (Fujisawa Pharmaceutical) was administered i.p. at 2.0 mg/kg body weight on the day of injection and maintained until the animals were killed. The hearts were fixed according to the periodate–lysine–PFA fixative methods and were snap-frozen in nitrogen and stored for subsequent immunohistochemical analysis.

MerCreMer mice express MerCreMer fusion protein driven by the αMHC promoter (Sohal et al., 2001). CAG-CAT-LacZ transgenic mice direct expression of the *E. coli* LacZ gene upon Cre-mediated excision of the loxP-flanked CAT gene located between the CAG promoter and the LacZ gene (Sakai and Miyazaki, 1997; Dr. Miyazaki, Osaka University, Osaka, Japan). A dose of 10^6 Cre-expressing HUVEC was transplanted to the heart of CAG-CAT-LacZ transgenic mice with the i.p. administration of FK506 at 2.0 mg/kg body weight on the day of injection and maintained until the animals were killed. A dose of 10^6 skeletal muscle–derived cells isolated from CAG-CAT-LacZ transgenic mice were transplanted to the heart of MerCreMer mice. MerCreMer mice were treated with tamoxifen (20 mg/kg/day, i.p.) 7 d before transplantation and the treatment was maintained until 2 d before the transplantation. At 4 d after transplantation, mice were killed and the hearts were perfused with 2% PFA and were snap-frozen in nitrogen. A couple of adjacent sections as a mirror image were prepared and fixed with 0.25% glutaraldehyde or 2% PFA for 15 min and were analyzed by X-gal staining or immunohistochemistry.

Cryoinjury
Male Wistar rats were anesthetized with ketamine (50 mg/kg, i.p.) and xyladine (10 mg/kg, i.p.) and a 6-mm aluminum rod, cooled to −190°C by immersion in liquid nitrogen applied to the left ventricular free wall to produce cryoinjury. The rats were killed at 4 d after cryoinjury. The hearts were snap-frozen in nitrogen. A couple of adjacent sections as a mirror image were prepared and fixed with 4% PFA and were subjected to immunostaining.

Immunohistochemistry
Fixed cells were preblocked with PBS containing 2% donkey serum, 2% BSA, and 0.2% NP-40 for 30 min. Primary antibodies were diluted with PBS containing 2% donkey serum, 2% BSA, and 0.1% NP-40 and applied over night at 4°C. FITC-, Cy3-, or Cy5-conjugated secondary antibodies were applied to visualize expression of specific proteins. Before mounting, nuclei were stained with Hoechst 33258 (1 μg/ml) or Topro3 (Molecular Probes, Inc.). Images of samples were taken by laser confocal microscopy (Radiance 2000; Bio-Rad Laboratories) or with a fluorescent microscope (Carl Zeiss MicroImaging, Inc.) equipped with a CCD camera (Axiocam; Carl Zeiss MicroImaging, Inc.).

Nocodazole treatment and cell cycle analysis
Neonatal rat cardiomyocytes fused with GFP+ HUVEC or GFP+ cFB were treated with 50 ng/ml nocodazole for 6–24 h and at each time cells were fixed and stained with anti-cTnT and anti-PH3 antibodies. Some of the cells treated with nocodazole for 6 h cocultured with HUVEC and for 24 h cocultured with cFB were released from nocodazole and cultured further for 3 h and fixed.

Statistical analysis
Values are presented as mean ± SD. The significance of differences among mean values was determined by one-factor ANOVA, chi-square independent test, and Kruskal-Wallis test. Probability (P) values were corrected for multiple comparisons by the Bonferroni correction. The accepted level of significance was P < 0.05.

Online supplemental material
Live images of beating cells were obtained with an inverted microscope (Carl Zeiss MicroImaging, Inc.) equipped with a chilled CCD camera (Hamamatsu Corporation) using I-O DATA Videorecorder software. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200312111/DC1.

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