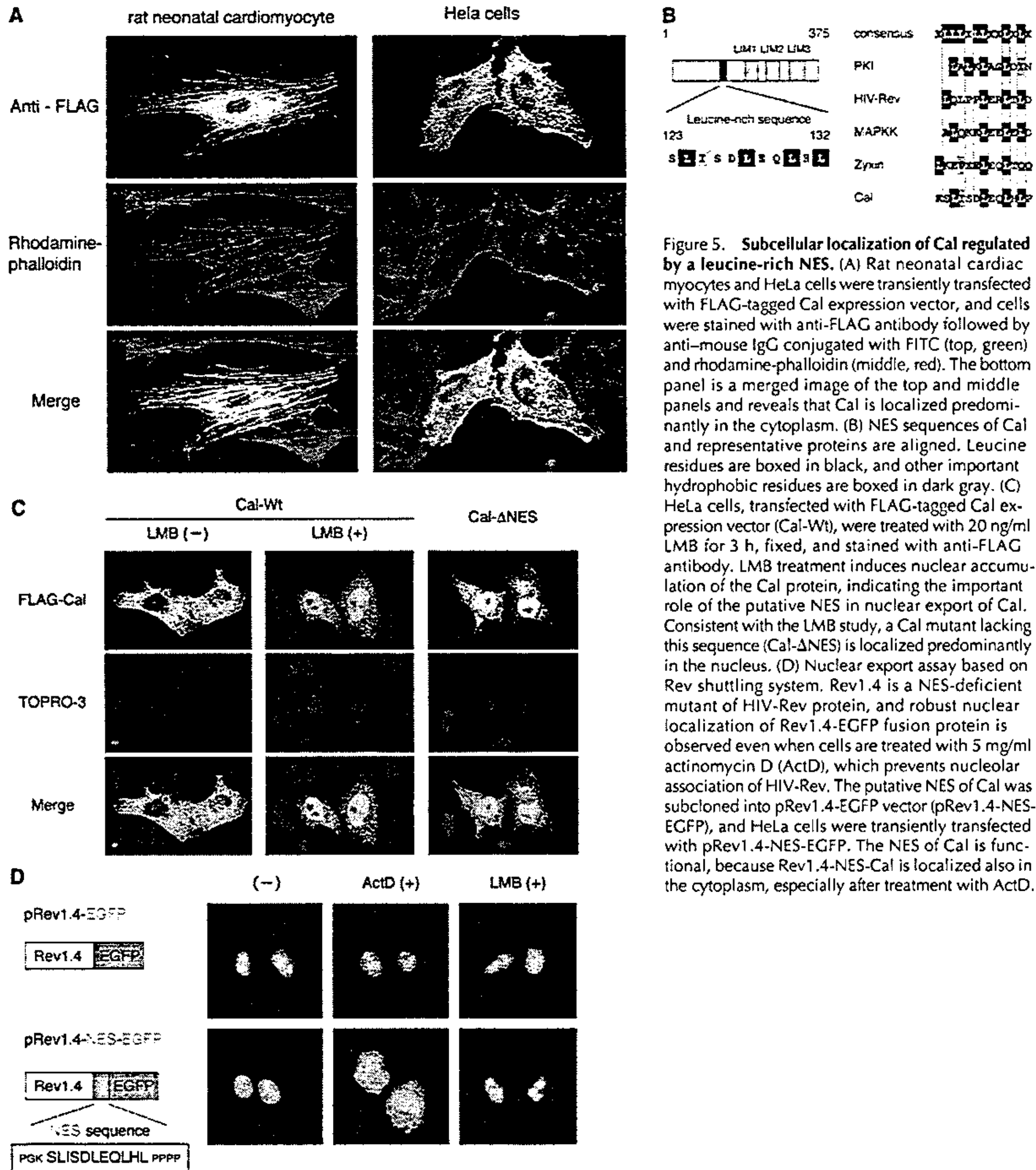


Figure 5. **Subcellular localization of Cal regulated by a leucine-rich NES.** (A) Rat neonatal cardiac myocytes and HeLa cells were transiently transfected with FLAG-tagged Cal expression vector, and cells were stained with anti-FLAG antibody followed by anti–mouse IgG conjugated with FITC (top, green) and rhodamine-phalloidin (middle, red). The bottom panel is a merged image of the top and middle panels and reveals that Cal is localized predominantly in the cytoplasm. (B) NES sequences of Cal and representative proteins are aligned. Leucine residues are boxed in black, and other important hydrophobic residues are boxed in dark gray. (C) HeLa cells, transfected with FLAG-tagged Cal expression vector (Cal-Wt), were treated with 20 ng/ml LMB for 3 h, fixed, and stained with anti-FLAG antibody. LMB treatment induces nuclear accumulation of the Cal protein, indicating the important role of the putative NES in nuclear export of Cal. Consistent with the LMB study, a Cal mutant lacking this sequence (Cal-ΔNES) is localized predominantly in the nucleus. (D) Nuclear export assay based on Rev shuttling system. Rev1.4 is a NES-deficient mutant of HIV-Rev protein, and robust nuclear localization of Rev1.4-EGFP fusion protein is observed even when cells are treated with 5 mg/ml actinomycin D (ActD), which prevents nucleolar association of HIV-Rev. The putative NES of Cal was subcloned into pRev1.4-EGFP vector (pRev1.4-NES-EGFP), and HeLa cells were transiently transfected with pRev1.4-NES-EGFP. The NES of Cal is functional, because Rev1.4-NES-Cal is localized also in the cytoplasm, especially after treatment with ActD.

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within the LIM2 and LIM3 domains, whereas LIM1 may function as a repressor domain.

### Cal is predominantly localized in the cytoplasm and shuttles between the cytoplasm and the nucleus

We examined the subcellular localization of Cal protein in cultured cells. Cultured cardiac myocytes of neonatal rats were transiently transfected with FLAG-tagged Cal expression vector, and immunofluorescence analysis was performed using anti-FLAG antibody. Cal protein was predominantly localized in the cytoplasm of cardiac myocytes at steady state (Fig. 5 A). Similar pattern of immunofluorescence was obtained in other cell lines such as HeLa (Fig. 5 A), COS7, and NIH3T3 cells (not depicted).

Within the amino acid sequence of Cal, there was a leucine-rich sequence that matched the consensus sequence of NES (Fig. 5 B). During a nuclear export cycle, an exportin molecule CRM1 recognizes the NES and forms a complex

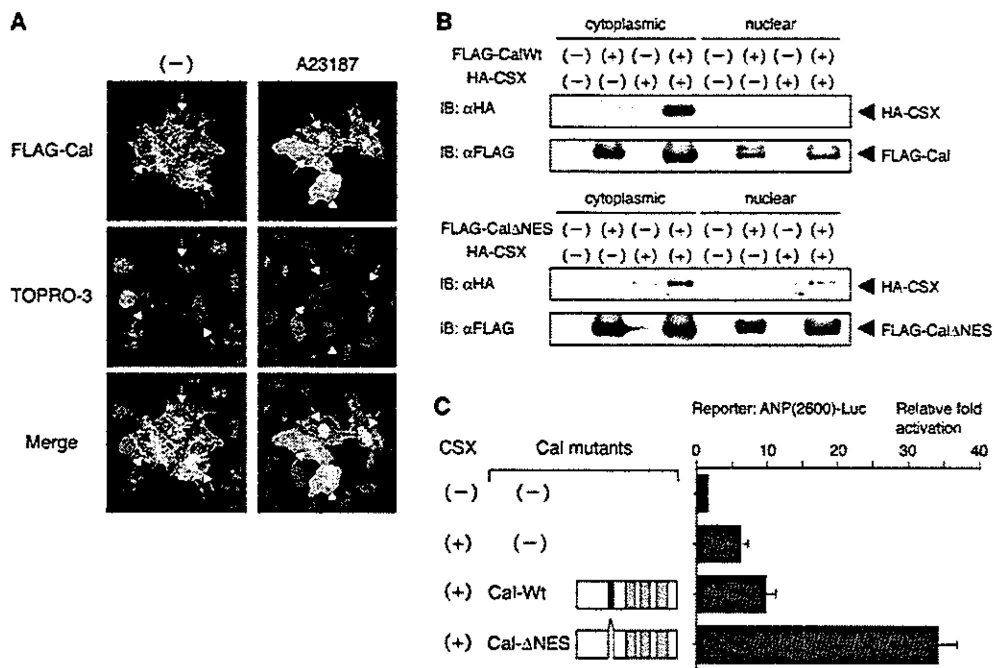


Figure 6. **Nuclear transport of Cal in response to calcium ionophore and implications of nuclear accumulation of Cal in transcriptional cooperativity with CSX/NKX2-5.** (A) HeLa cells, transfected with FLAG-tagged Cal expression vector, were treated with vehicle or calcium ionophore A23187 (2 μM) for 15 min, fixed, and stained with anti-FLAG antibody. Nuclear accumulation of Cal is observed in significant portions of transfected cells after treatment with A23187. The arrows indicate the nuclei of the transfected cells. (B) Coimmunoprecipitation of CSX/NKX2-5 and Cal (Cal-Wt) or nuclear form of Cal (Cal-ΔNES) in preparations of cytoplasmic or nuclear fractions of transfected COS7 cells. Cal-ΔNES showed significantly stronger interaction with CSX/NKX2-5 in the nucleus than Cal-Wt. (C) The luciferase reporter containing the *ANP* promoter was cotransfected in COS7 cells with the expression vectors of CSX/NKX2-5 and Cal-Wt or Cal-ΔNES. Cal-ΔNES showed much stronger synergistic activation with CSX/NKX2-5 than Cal-Wt. The results are expressed as the mean ± SEM.

with RanGTP, and mediates transport to the cytoplasm (Fornerod et al., 1997; Mattaj and Englmeier, 1998; Ohno et al., 1998; Kuersten et al., 2001). NES-dependent nuclear export is inhibited by leptomycin B (LMB) that interferes with the binding of CRM1 to NES (Kudo et al., 1998). Inhibition of CRM1-dependent nuclear export using LMB resulted in rapid nuclear accumulation of Cal protein in HeLa cells (Fig. 5 C). Although immunofluorescence studies indicated that the main compartment where Cal is localized at steady state was the cytoplasm, the accumulation of CAL after treatment with LMB suggested that Cal can shuttle between the cytoplasm and the nucleus.

To confirm that the putative NES contributes to nuclear export of Cal, we deleted the NES sequence (residues 123–132) in the FLAG-tagged Cal expression vector (Cal-ΔNES) and examined the subcellular localization of Cal-ΔNES mutant. Cal-ΔNES was predominantly localized in the nucleus (Fig. 5 C), suggesting that this sequence mediates the CRM1-dependent nuclear export of Cal. To test this sequence of Cal functions as an NES, we introduced this sequence into the export-deficient form of Rev-EGFP, and tested its nuclear export activity in HeLa cells. The putative NES of Cal displayed the export activity, especially in the presence of actinomycin D, which prevents nucleolar association of Rev protein (Fig. 5 D). These results indicate that this 123-132–amino acid sequence of Cal really functions as an NES.

## Cal shuttles into the nucleus in response to $Ca^{2+}$ signal

We explored a specific signal capable of targeting Cal protein to the nucleus. When intracellular $Ca^{2+}$ levels were increased by $Ca^{2+}$ ionophore A23187, Cal protein was transported to the nucleus (Fig. 6 A). Nuclear accumulation of Cal was detected at 10 min after addition of A23187. No other cellular signals possessed ability to transport Cal into the nucleus. For example, treatment with cytochalasin D, an actin filament disrupting reagent, tetradecanoylphorbol 13-acetate, PKC activator, forskolin, an adenylate cyclase activator, anisomycin, Jun-$NH_2$–terminal kinase agonist, okadaic acid, a serine/threonine phosphatase inhibitor did not induce nuclear translocation of Cal protein.

Next, we examined whether nucleocytoplasmic shuttling of Cal protein had important implications for modifying the transcriptional activity of CSX/NKX2-5. As indicated by coimmunoprecipitation experiments by using cytoplasmic and nuclear fractions of transfected cells, interaction between CSX/NKX2-5 and wild-type of Cal (Cal-Wt) was detectable predominantly in the cytoplasm and slightly in the nucleus (Fig. 6 B). When Cal-ΔNES, which lacks the NES and is predominantly localized in the nucleus, was cotransfected, the level of coprecipitating CSX/NKX2-5 in the nuclear fraction increased significantly (Fig. 6 B). Furthermore, Cal-ΔNES showed much stronger synergistic transactivation of the *ANP* promoter than Cal-Wt, when cotransfected with CSX/



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NKX2-5 (Fig. 6 C). These results suggest that nuclear translocation of Cal enhances CSX/NKX2-5–induced promoter activation by promoting mutual interaction in the nucleus.

### Nuclear accumulation of Cal induces cardiac differentiation of P19CL6 cells

To determine whether synergistic transactivation by Cal has a significant effect on cardiomyocyte differentiation, we isolated P19CL6 clones, which stably overexpress wild-type Cal (P19CL6-CAL-Wt) or Cal mutant lacking the NES (P19CL6-Cal-ΔNES). When cultured in the medium containing 1% DMSO, P19CL6 cells differentiated into cardiomyocytes, which exhibit spontaneous beating and express cardiac-specific genes (Monzen et al., 1999). The expression of cardiac-specific genes was examined in P19CL6 cells, P19CL6-Cal-Wt, and P19CL6-Cal-ΔNES during differentiation (Fig. 7 A). Northern blot analysis revealed that expression levels of a cardiac transcription factor *GATA-4* and *sarcoplasmic reticulum $Ca^{2+}$-ATPase 2* (*SERCA2*) as well as *connexin 43* and *calreticulin*, known as downstream targets for CSX/NKX2-5, were increased in P19CL6-Cal-ΔNES cells. RT-PCR analysis revealed that expression of *ANP* gene was also up-regulated in P19CL6-Cal-ΔNES cells, which was consistent with the results that Cal augmented *ANP* promoter activation induced by CSX/NKX2-5. Immunocytochemical analysis revealed that in P19CL6-Cal-ΔNES, a larger number of cells were stained positive with anticardiac troponin T antibody than the parental P19CL6 cells (Fig. 7 B), suggesting that nuclear accumulation of Cal strongly promotes cardiac differentiation.

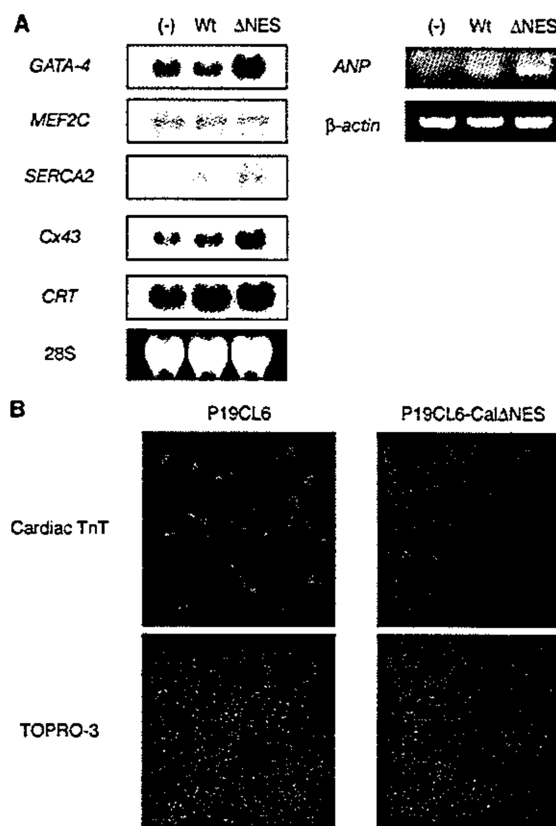


Figure 7. **Promotion of cardiac differentiation in P19CL6 cells by nuclear accumulation of Cal.** (A) Expression of cardiac genes was examined on differentiation day nine of P19CL6 cells, P19CL6 cells stably expressing Cal-Wt and Cal-ΔNES. Northern blot analysis was performed with *GATA-4, MEF2C, SERCA2, Connexin43* (*Cx43*), and *calreticulin* (*CRT*) cDNAs and RT-PCR was performed using specific primers for *ANP*. Notably, expression levels of target genes for CSX/NKX2-5 such as *Cx43, CRT,* and *ANP* were increased in P19CL6-Cal-ΔNES. (B) Cardiac differentiation in P19CL6 cells on differentiation day 14 was determined by immunofluorescence with anticardiac troponin T (TnT) antibody. Much larger number of cells were stained positive for cardiac TnT in P19CL6-Cal-ΔNES.

## Discussion

### Cal is a novel LIM domain–containing protein

We identified a novel protein Cal, which associates with the cardiac homeobox transcription factor CSX/NKX2-5. *Cal* is a member of Zyxin family, that commonly have a proline-rich region at the $NH_2$ terminus, a leucine-rich sequence, and three tandem LIM domains located at the COOH terminus. The proline-rich regions of Zyxin serve as interface to bind to SH3 domain of Vav (Hobert et al., 1996) and EVH1 domain of Ena/VASP family proteins (Renfranz and Beckerle, 2002) that are implicated in control of actin organization (Gertler et al., 1996). LPP also contains proline-rich motifs that are required for the interaction with the EVH1 domain (Prehoda et al., 1999). This proline-rich region of LPP directly interacts with VASP in vitro, and LPP is colocalized with VASP in the focal adhesion. The proline-rich regions of Ajuba interact with Grb2 (Goyal et al., 1999). Expression of Ajuba enhances MAPK activity in fibroblasts and promotes meiotic maturation of *Xenopus* oocytes through activation of MAPK in Grb2- and Ras-dependent manner (Goyal et al., 1999). The $NH_2$-terminal portion of *Cal* also contains stretches of proline-rich sequences. Especially, two proline-rich sequences (LPPPPPPP 98-105 and LPPPPPPPPP 133-142) of Cal lead us to speculate that Cal might associate with profilin and be involved in the organization of cytoskeletal actin in the cytoplasm because the sequence of consecutive prolines flanked by leucine has been thought to be a ligand motif for profilin (Mahoney et al., 1997). Identification of proteins binding to the proline-rich region of Cal would provide further insights into its cellular function.

### Cal interacts with CSX/NKX-2.5 both in vitro and in vivo

GST pull-down assays and coimmunoprecipitation experiments indicated the association of Cal with CSX/NKX2-5 both in vitro and in vivo. Analyses using mutants of both proteins revealed that the mutual binding was mediated through the homeodomain of CSX/NKX2-5 and the LIM domains of Cal. Besides binding to DNA, the homeodomain of CSX/NKX2-5 acts as a module for the interaction with its binding partner such as GATA-4 (Durocher et al., 1997; Lee et al., 1998; Shiojima et al., 1999), SRF (Chen and Schwartz, 1996), and Tbx-5 (Hiroi et al., 2001). The LIM domains of Cal have a cysteine-histidine rich, double zinc-finger motif that functions as a protein–protein in-

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teraction module (Dawid et al., 1998; Bach, 2000). The LIM domains of Zyxin interact with members of CRP family (Sadler et al., 1992) and serine/threonine kinase h-warts/LATS1 (Hirota et al., 2000). During mitosis, phosphorylation of Zyxin by Cdc2 promotes the binding between Zyxin and h-warts/LATS1, and the complex is targeted to the mitotic apparatus. The possibility that interaction between CSX/NKX2-5 and Cal is modulated by specific protein modification remains to be determined.

Abundant expression of *Cal* was detected in the heart during embryogenesis and maintained in the atrial and ventricular chambers through the adulthood. *Cal* was also expressed in a variety of tissues such as the aorta, lung, and intestine, but little expression was detected in the brain and liver. Although the functional roles of Cal in tissues other than the heart remain unknown at present, Cal may associate with other NK homeobox transcription factors, because the amino acid sequences of homeodomains, which are responsible for binding to Cal, are highly conserved among this class of homeoproteins. Interestingly, Ajuba has been reported to associate with thyroid transcription factor-1/Nkx2-1, a member of NK homeobox transcription factors, in mammalian cells, although the physiological function of their interaction remains unknown (Missero et al., 2001). It is possible that there are more combinatorial patterns of physical interaction between Zyxin family LIM proteins and NK homeoproteins.

### Cal shuttles between the cytoplasm and the nucleus

The leucine-rich sequence of Cal is thought to function as an NES, based on the following results: (a) the leucine-rich sequence of Cal matches the consensus of the NES; (b) predominant nuclear distribution was observed when treated with LMB, that is a specific inhibitor of CRM1-dependent nuclear export (Kudo et al., 1998); (c) the Cal mutant lacking the leucine-rich sequence was localized predominantly in the nucleus; and (d) fusion of leucine-rich sequence of Cal to Rev1.4-EGFP transported the Rev1.4-EGFP from the nucleus to the cytoplasm (Henderson, 2000). Functional leucine-rich NESs have been identified in other Zyxin family members such as *Zyxin* (Nix and Beckerle, 1997), *trip6* (Wang and Gilmore, 2001), *LPP* (Petit et al., 2000), and *Ajuba* (Kanungo et al., 2000). Although the role of Zyxin family members in the nucleus has not been fully defined, the interaction between Zyxin and h-wartz/LATS1 on the mitotic apparatus implicates the specific role of Zyxin in the regulation of cell cycle (Hirota et al., 2000).

### Cal augments CSX/NKX2-5–induced promoter activation

The interaction between CSX/NKX2-5 and Cal implicates a certain role of transcriptional regulation of cardiac-specific genes. CSX/NKX2-5 and Cal synergistically activated both the *ANP* promoter and the artificial promoter containing multimerized CSX/NKX2-5–binding sites. Furthermore, Cal enhanced cooperative promoter activation of *ANP* gene between CSX/NKX2-5 and GATA-4 or Tbx-5. These results suggest that transcriptional regulation by cardiac transcription factors may be fulfilled harmoniously by multiprotein complex.

The GAL4-based reporter assay revealed that Cal itself possesses transcriptional activity. LIM2 and LIM3 domains were endowed with the capacity to activate transcription, whereas the LIM1 domain suppressed the transcriptional activity. On the other hand, the ΔLIM1 mutant failed to augment CSX/NKX2-5–induced transactivation of the *ANP* reporter (Fig. 3 B). GST pull-down assays revealed that the LIM domains are required for binding to CSX/NKX2-5 and that deletion of LIM1 reduced the mutual binding (Fig. 2 C), suggesting that deletion of LIM1 may also decrease the binding affinity for CSX/NKX2-5. In addition, there is a possibility that the LIM1 interferes the GAL4-DNA binding but not inhibits the transcription. It has been reported that Trip6 and LPP have transcriptional activity, and the transactivation domains were attributed to the LIM domains and a region containing the NES of *trip6* (Wang and Gilmore, 2001) and to the LIM domains and the proline-rich region of *LPP* (Kanungo et al., 2000). Based on the fact that transactivation domains reside in modules for protein–protein interaction, it is likely that the interaction with components of transcriptional initiation complex is involved in transcriptional activation.

Cooperative transactivation of the *ANP* promoter by CSX/NKX2-5 and Cal was enhanced when Cal protein was targeted into the nucleus by deleting its NES. We found that treatment with $Ca^{2+}$ ionophore A23187 induced nuclear transport of Cal. Pathophysiological significance of $Ca^{2+}$ signaling in cardiac development has not been fully defined. However, $Ca^{2+}$ signals are induced by various conditions including G-protein–coupled receptors (Clapham, 1995) and receptor tyrosine kinases (Schlessinger, 2000). It is possible to assume that Cal might modulate the transcriptional activity of CSX/NKX2-5 in response to $Ca^{2+}$ signals triggered by G-protein–coupled receptors or receptor tyrosine kinases during cardiogenesis. Exploration of physiological ligands that activate $Ca^{2+}$ signals and subsequent nuclear import of Cal will undermine the molecular framework of cardiac development.

$Ca^{2+}$ signaling plays an important role in generation of cardiac hypertrophy (Frey et al., 2000). Nuclear import of NF-AT transcription factors is induced by $Ca^{2+}$-activated phosphatase calcineurin and that transgenic mice expressing nuclear form of NF-AT3 in the heart exhibited cardiac hypertrophy (Molkentin et al., 1998). CSX/NKX2-5 is expressed in the adult heart (Komuro and Izumo, 1993), and it has been proposed that CSX/NKX2-5 is involved in generation of cardiac hypertrophy (Akazawa and Komuro, 2003) on the basis of in vivo findings that expression levels of CSX/NKX2-5 were increased in response to hypertrophic stimuli including pressure overload (Thompson et al., 1998) and phenylephrine or isoproterenol (Saadane et al., 1999). Therefore, Cal may be another $Ca^{2+}$-sensitive effector that translocates into the nucleus like NF-AT transcription factors and it is possible to speculate that Cal may play a certain role in generation of cardiac hypertrophy by modulating transcriptional activity of CSX/NKX2-5.

### Cal may function as a signal mediator that links cytoplasmic signals and gene expression

Cal was localized in the cytoplasm at steady state and translocated into the nucleus in response to calcium, and Cal functioned as a transcriptional activator in the nucleus by cooper-

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ating with the cardiac transcription factor CSX/NKX2-5. These results indicate a novel function of LIM proteins that link cytoplasmic signals and nuclear gene expression.

Recently, some proteins associated with cell junctions have been reported to be involved in transcriptional regulation. A membrane-associated guanylate kinase, CASK/LIN-2, interacts with a T-box transcription factor, Tbr-1, and stimulates the transcriptional activity of Tbr-1 in the nucleus of mammalian cells (Hsueh et al., 2000). Jun activation domain-binding protein 1, colocalizing with integrin LFA-1, translocates into the nucleus in response to LFA-1 stimulation and acts as a coactivator for AP-1 complex (Bianchi et al., 2000). β-Catenin, linking cadherins to actin cytoskeleton at adherens junctions, interacts with T cell factor to form a transcriptional activator complex in response to Wnt signaling (Barth et al., 1997). Although CRP3/MLP binds to Zyxin and α actinin in the cytoplasm (Louis et al., 1997), forced expression of CRP3/MLP in the nucleus by fusing it to nuclear localization signal led to a cooperative enhancement of the transcriptional activity of MyoD (Kong et al., 1997). Trip6 also acts as a coactivator for v-Rel transcription factor (Zhao et al., 1999). However, it remains unclear how subcellular localization of CRP3/MLP and trip6 is regulated. We first clarify the molecular mechanism of how the cytoplasmic LIM protein is translocated into the nucleus and functions as a transcriptional activator.

### Cal promotes cardiac differentiation in P19CL6 cells

Mouse P19CL6 cells, derived from P19 embryonal carcinoma cells, are used as a good in vitro system for molecular analysis of cardiac differentiation. In the presence of 1% DMSO, mouse P19CL6 cell efficiently differentiate into spontaneously beating cardiac myocytes that exhibit the biological features recapturing embryonic cardiogenesis in vivo (Monzen et al., 1999, 2001). P19CL6 cells that overexpress nuclear form of Cal (P19CL6-Cal-ΔNES) differentiated into cardiac myocytes more efficiently than the parental P19CL6 cells. In P19CL6-Cal-ΔNES cells, expression levels of *SERCA2*, *calreticulin*, *connexin43*, *ANP*, and *cardiac troponin T* were up-regulated, which convey properties characteristic of cardiomyocytes. Expression levels of cardiac transcription factor *MEF2C* did not change, whereas expression levels of *GATA-4* were increased. Although there has been no evidence indicating that *GATA-4* is a downstream target for CSX/NKX2-5, it is possible that expression of *GATA-4* is up-regulated through undefined functions of Cal. Up-regulation of *GATA-4* might have an influence on myocardial cell differentiation in P19CL6-Cal-ΔNES. These results leave an open question whether the nuclear target for Cal is solely CSX/NKX2-5. However, based on the up-regulated expression of the target genes for CSX/NKX2-5, it is reasonable to assume that cooperation of CSX/NKX2-5 and Cal promoted cardiac differentiation in P19CL6 cells. Our present studies elucidate a novel role of LIM proteins in cardiac development as a transcriptional activator, and suggest that fine-tuned gene expression during cardiogenesis is orchestrated by multiprotein complex including LIM proteins as well as transcription factors.

## Materials and methods

### Molecular cloning of Cal

We performed a yeast two-hybrid screening using the MATCHMAKER Two-Hybrid System (CLONTECH Laboratories, Inc.) as described previously (Hiroi et al., 2001). The plasmid pGBT9-CSX, which encodes the GAL4 DNA-binding domain fused to the human CSX/NKX2-5, was used as a bait in screening of a human heart MATCHMAKER cDNA Library (CLONTECH Laboratories, Inc.). One clone containing a fragment of CAL cDNA was scored positive, and the full-length mouse Cal cDNA was obtained by screening a mouse heart cDNA library (Stratagene).

### Northern blot, RT-PCR, and in situ hybridization analysis

For Northern blot analysis, total RNA was hybridized with cDNA corresponding to 3′-UTR of *Cal*. Probes for *GATA-4*, *MEF2C*, *connexin 43*, and *SERCA2* were described previously (Hiroi et al., 2001). A probe for *calreticulin* was a gift from M. Michalak (University of Alberta, Alberta, Canada). RT-PCR analysis for *ANP* expression was performed as described previously (Hiroi et al., 2001). Digoxygenin labeled riboprobes were synthesized by using the 1.5-kb *Cal* cDNA, and RNA in situ hybridization was performed as described previously (Akazawa et al., 2000).

### Plasmids construction

The following plasmids were described previously: the expression vectors of CSX/NKX2-5 (pEFSA-HA-CSX), GATA-4 (pSSRa-hGATA4), and Tbx-5 (pcDNA3-Tbx5); the luciferase reporters containing the *ANP* promoter (ANP[600]-Luc and ANP[2600]-Luc); and multimerized CSX-binding sites (4×TTF-Luc; Shiojima et al., 1999; Hiroi et al., 2001). FLAG-tagged Cal was subcloned into pCAGGS vector (pCAGGS-FLAG-Cal; Niwa et al., 1991; Aoki et al., 2000). pCAGGS vector was provided by J. Miyazaki (Osaka University Graduate School of Medicine, Suita, Japan) and T. Kobayashi and O. Hino (The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan). Cal derivatives were subcloned into pcDNA3.1 (Invitrogen) and pBIND (Promega) for in vitro transcription and translation and expression of GAL4-fusion protein, respectively. For deletion analyses, the following Cal derivatives were subcloned into the corresponding vectors: Cal-ΔLIM1 (1-184, 221-375), Cal-ΔLIM2 (1-244, 279-375), Cal-ΔLIM3 (1-307, 345-375), Cal-ΔLIM123 (1-184), Cal-LIM123 (185-375), Cal-LIM23 (245-375), and Cal-ΔNES (1-121, 135-375).

### Cell culture, transfection, and reporter gene assay

Primary cultures of cardiac myocytes were prepared from ventricles of 1-d-old Wistar rats as described previously (Kudoh et al., 1997). Transient transfections were performed by standard calcium phosphate methods. For reporter gene assays, pRL-SV40 (Promega) was cotransfected as an internal control. Luciferase activities were measured as described previously (Shiojima et al., 1999). P19CL6 cells were cultured as described previously (Monzen et al., 1999). To isolate the permanent cell lines, P19CL6 cells were transfected with pcDNA3.1-Cal and pcDNA3.1-Cal-ΔNES by the lipofection method (TfxTM reagents; Promega). Stable transformants were selected with 400 μg/ml of neomycin (G418; Sigma-Aldrich).

### Coimmunoprecipitation experiment

We performed a coimmunoprecipitation experiment as described previously (Shiojima et al., 1999). COS-7 cells were transiently transfected with expression plasmids of pEFSA-HA-CSX and pCAGGS-FLAG-Cal or pCAGGS-FLAG-CalΔNES. For preparation of the cytoplasmic fraction, transfected cells were lysed in digitonin buffer (20 mM Hepes/KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 50 μg/ml digitonin) on ice for 10 min. The lysates were centrifuged at 1,000 *g* and the supernatant was collected as the cytoplasmic fraction. The pellets were resuspended Triton buffer (20 mM Hepes/KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 10 mg/ml Triton X-100) and the lysates were used as the nuclear fraction. Protein samples were subjected to immunoprecipitation with the anti-FLAG mAb M2 (KODAK), fractionated by 10% SDS-PAGE, and immunoblotted with the rabbit polyclonal anti-HA antibody (Santa Cruz Biotechnology, Inc.). HRP-conjugated anti-rabbit IgG antibody was used as the secondary antibody and immune complex was detected by the ECL detection kit (Amersham Biosciences).

### GST pull-down assay

We performed GST pull-down assays as described previously (Shiojima et al., 1999). GST fusion protein of CSX/NKX2-5 has been described previously. cDNA fragment corresponding to the full length of Cal was subcloned in frame into the EcoRI site of pGEX-3X (Amersham Biosciences). CSX/NKX2-5 derivatives (Shiojima et al., 1999) and Cal derivatives, subcloned

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into pcDNA3.1 vector (Invitrogen), were labeled with [$^{35}$S]methionine by the TNT Quick Coupled Transcription/Translation Systems (Promega). GST and GST fusion proteins immobilized on glutathione-Sepharose 4B beads were mixed with in vitro–translated proteins. Bound proteins were fractionated by SDS-PAGE and visualized by autoradiography.

### Immunostaining

Rat neonatal cardiac myocytes or HeLa cells were transfected with the expression vector of Cal and Cal mutants. Cells were stained with the anti-FLAG mAb M2 (KODAK), and visualized with FITC-labeled anti-mouse IgG (CAPPEL). Calcium ionophore A23187 was purchased from Sigma-Aldrich. Differentiated P19CL6 cells were stained with anticardiac troponin T mAb (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and visualized with Cy3-labeled anti-mouse IgG (CHEMICON International, Inc.). The cells were double stained using rhodamine-phalloidin (Molecular Probes) or TO-PRO-3 (Molecular Probes).

### Nuclear export assays

Nuclear export assays were performed as described previously (Henderson, 2000). pRev(1.4)-NES-EGFP plasmid was constructed by subcloning the NES of Cal between BamHI and AgeI sites of pRev(1.4)-EGFP plasmid (provided by B.R. Henderson, Westmead Institute for Cancer Research, Sydney, Australia). The NES of Cal was amplified by PCR using specific primers (5'-AGGGAAGCCCCCACCCCCGCCTC-3', and 5'-GGTGCGGGCTTCCCTG-GTAAGACA-3'). Actinomycin D (Sigma-Aldrich) was added at 5 mg/ml to prevent nucleolar association of Rev protein. LMB was provided by M. Yoshida (The University of Tokyo, Tokyo, Japan).

### Acquisition and processing of images

For light microscopic analysis (Fig. 1 C), images were acquired by a stereomicroscope (MZ12; objective lens, Plan 1.0×; Leica) and captured by DC100 program (Leica), or by a light microscope (Axioskop 2 plus; objective lens, Plan-Neofluar 2.5×/0.075; Carl Zeiss MicroImaging, Inc.) and captured by Axio Cam CCD camera and Axio Vision 3.0 imaging system (Carl Zeiss MicroImaging, Inc.). For immunofluorescence microscopic analysis, images were acquired by a laser-scanning microscope (model Eclipse E600; Nikon) using Plan-Fluor 10×/0.30 (Fig. 7 B), Plan-Fluor 40×/0.75 (Fig. 6 A), and Plan-Apo 60×A/1.40 oil (Fig. 5). Radiance 2000 confocal scanning system (Bio-Rad Laboratories) was used.

### Accession no.

The deduced amino acid sequence of mouse Cal was deposited in GenBank/EMBL/DDBJ accession no. AF513359.

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THE JOURNAL OF CELL BIOLOGY

# 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 cryoinjured 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.

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## 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.

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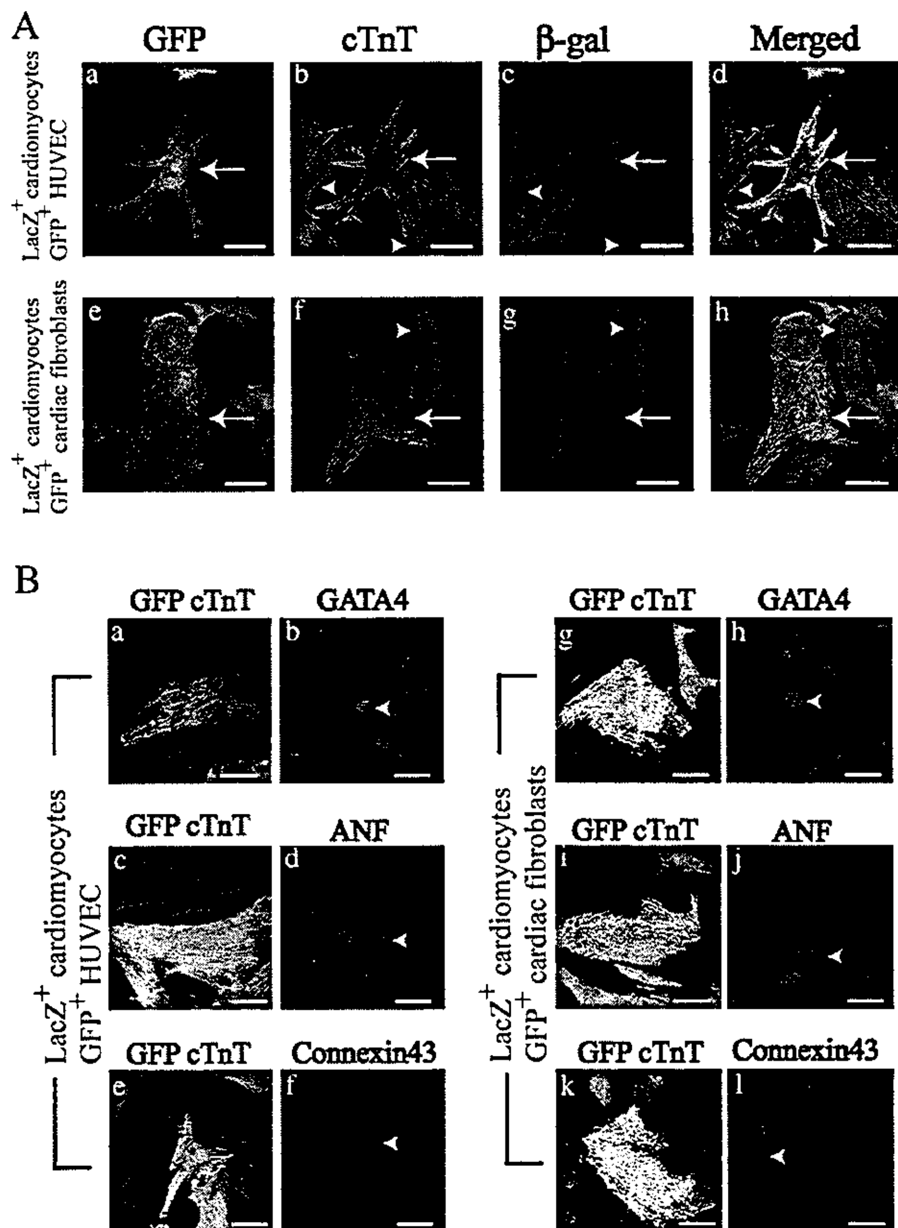


Figure 1. **HUVEC and cFB acquired the cardiac phenotype through cell fusion with cardiomyocytes.** (A) *LacZ*-expressing cardiomyocytes of neonatal rats were cocultured with GFP+ HUVEC (a–d) or GFP+ cFB (e–h) and stained with mouse monoclonal anti-cTnT (red) and rabbit polyclonal anti-β-gal antibodies (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 cardiomyocytes 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 polyclonal 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 explanation, in which the ability to proliferate might be conferred on cardiomyocytes by surrounding proliferative noncardiomyocytes 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.

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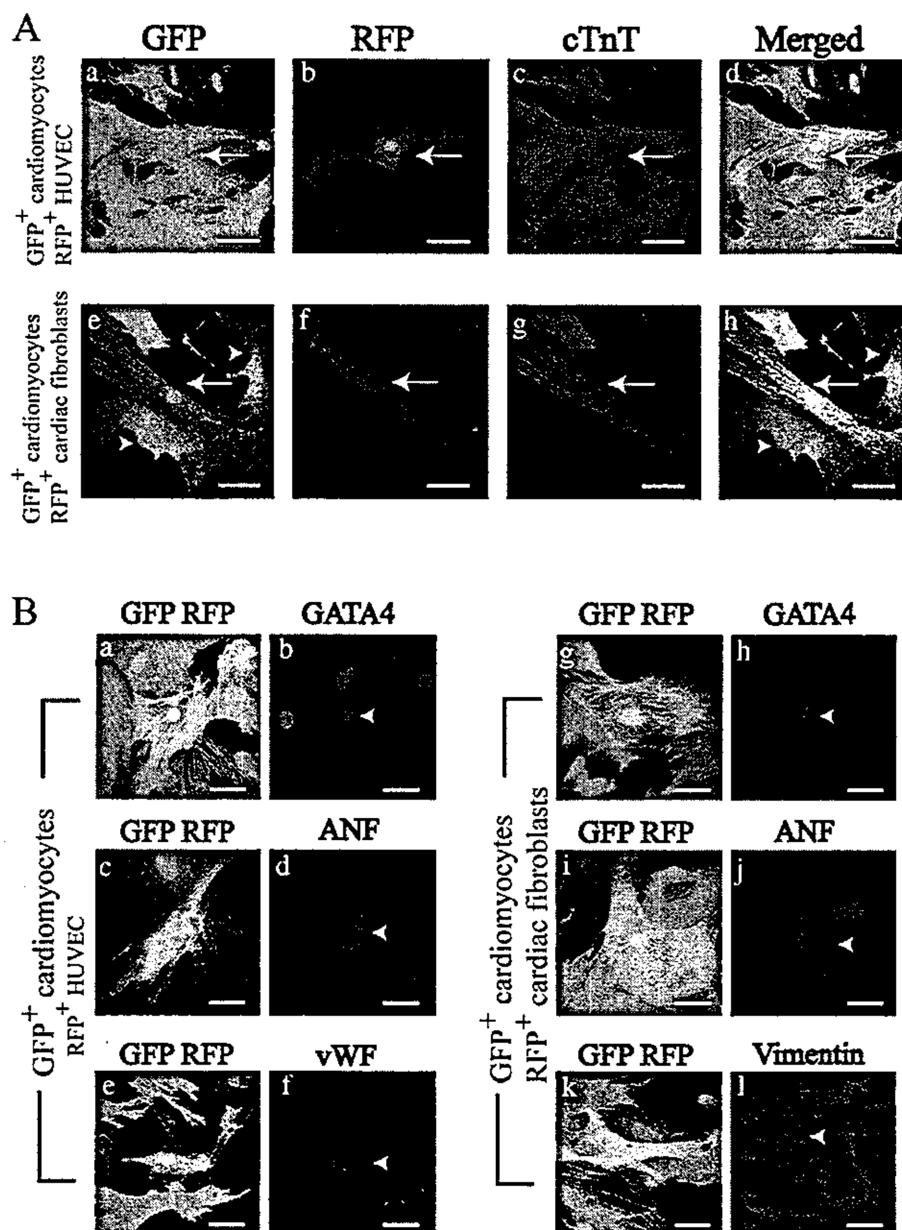


Figure 2. **HUVEC and cFB acquired the cardiac phenotype through cell fusion with cardiomyocytes.** (A) Cardiomyocytes of GFP transgenic neonatal rats were cocultured with RFP+ HUVEC (a–d) and RFP+ cFB (e–h) and were stained with mouse monoclonal anti-cTnT (blue). Merged images were obtained from the 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 RFP+ 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



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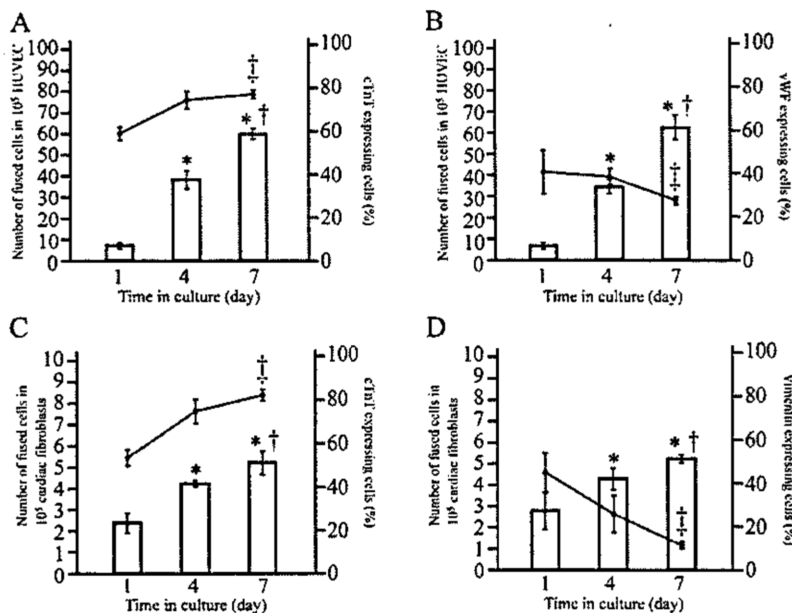


Figure 3. **Cardiac phenotype is dominated in fused cells.** (A and B) Each bar represents the number of GFP$^+$/RFP$^+$ fused cells in $10^5$ RFP$^+$ HUVEC cocultured with GFP$^+$ neonatal rat cardiomyocytes at different time in culture. The number of GFP$^+$/RFP$^+$ fused cells was increased with the 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 expression 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$^+$

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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

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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.

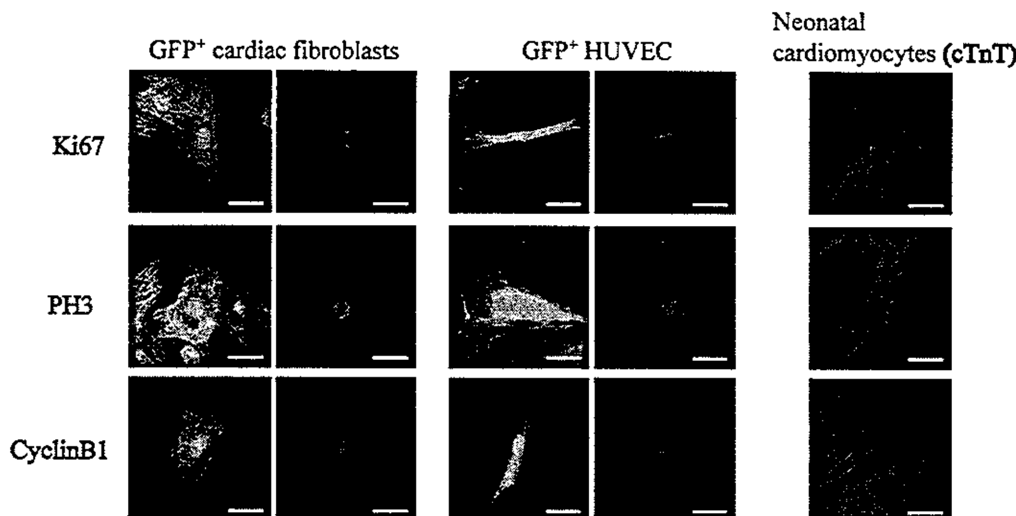


Figure 4. **Localization of Ki67, PH3, and cyclinB1 in cFB, HUVEC, and neonatal cardiomyocytes.** GFP+ cFB, GFP+ HUVEC, and neonatal rat cardiomyocytes were stained with mouse monoclonal anti-Ki67 (top row, red), rabbit polyclonal anti-PH3 (middle row, red), and mouse monoclonal anti-cyclinB1 (bottom 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.

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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*

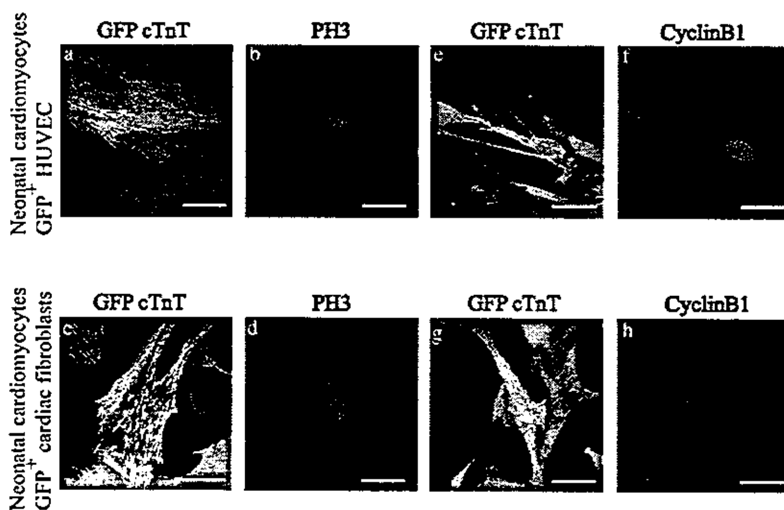



Figure 5. **Neonatal rat cardiomyocytes expressed PH3 and cyclinB1 after cell fusion with HUVEC or cFB.** Neonatal rat cardiomyocytes were cocultured with GFP+ HUVEC or GFP+ cFB and double stained with anti-cTnT (red) and anti-PH3 (blue) or anti-cyclinB1 (blue) antibodies. Fused cells between cardiomyocytes 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.

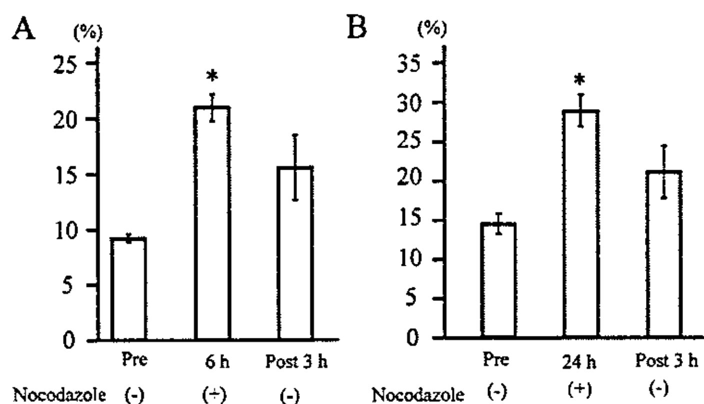
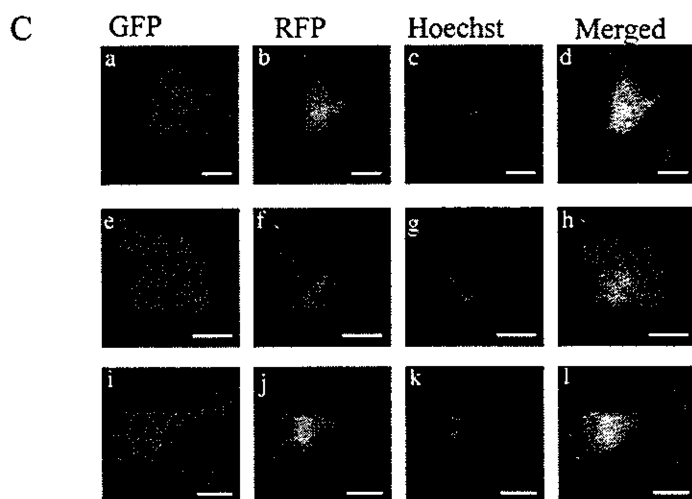



Figure 6. **Cardiomyocytes of neonatal rats reentered the cell cycle through cell fusion with HUVEC or cFB in vitro.** (A and B) Quantitative analysis of the percentage of PH3-expressing cardiomyocytes fused with HUVEC or cFB. The percentage of PH3-expressing cardiomyocytes in all fused cells with HUVEC (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.

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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 cardiomyocytes 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-

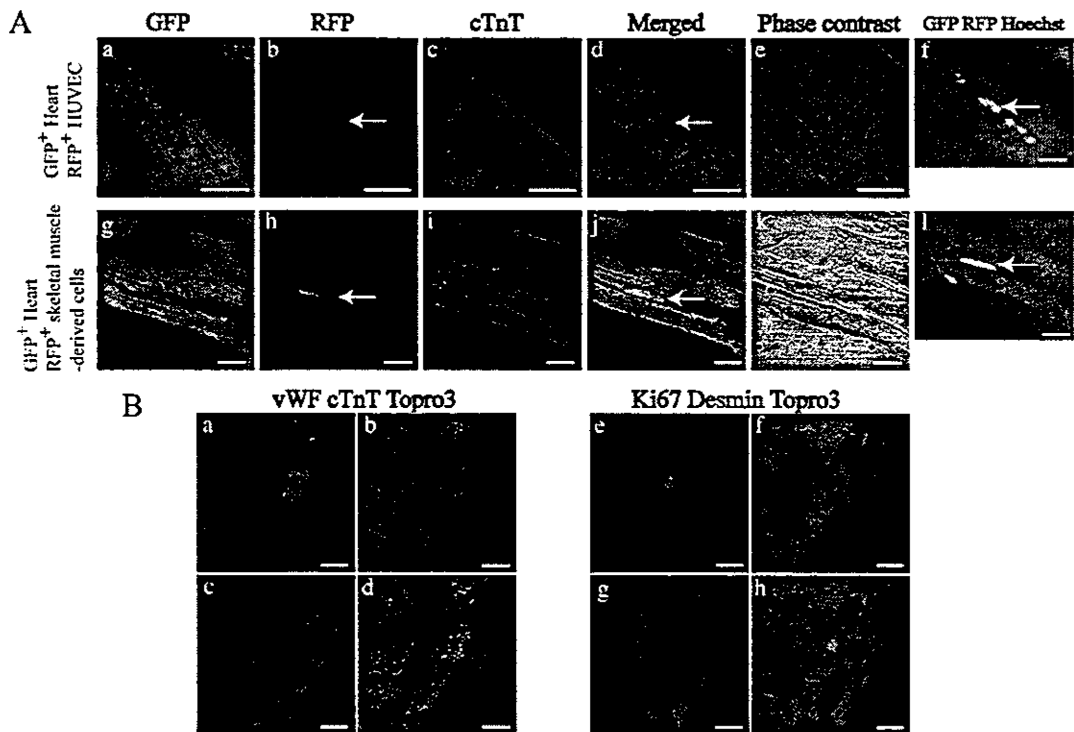


Figure 7. **Adult cardiomyocytes fused with somatic cells in vivo.** (A) RFP+ HUVEC or RFP+ skeletal muscle–derived cells were injected to the heart of GFP transgenic rats, and 6-μm sections were stained with rabbit polyclonal anti-RFP and mouse monoclonal anti-cTnT antibodies. Confocal microscopic images 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.

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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,

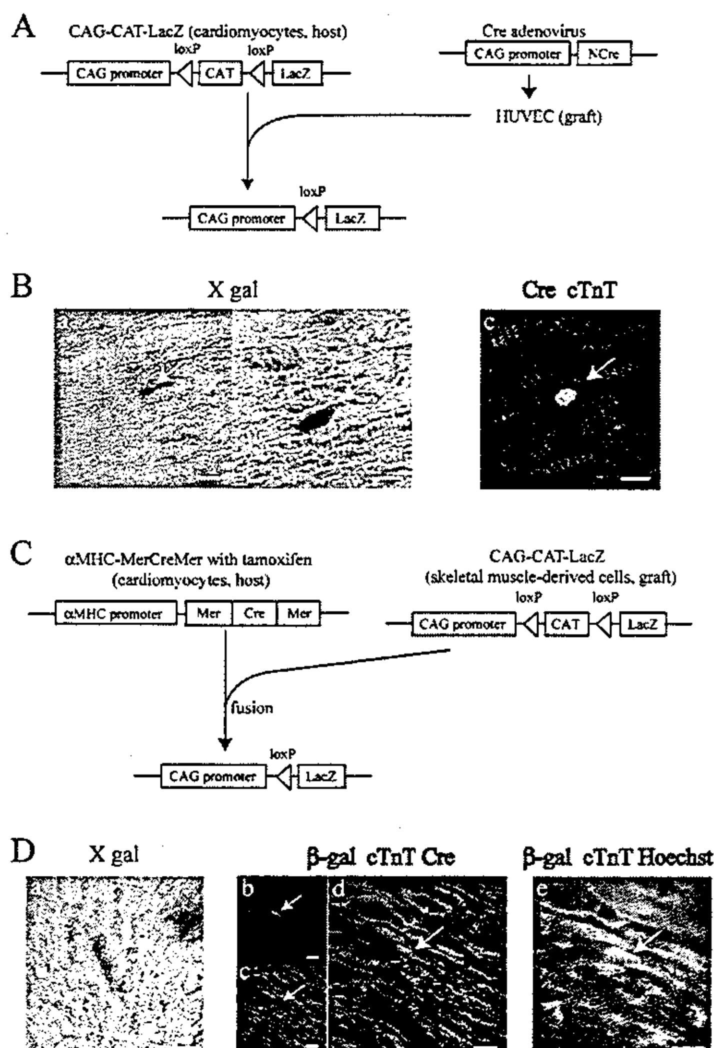


Figure 8. **Cre/lox recombination assay for detection of cell fusion in vivo.** (A and C) Schematic representation of the transgenes expressed by the mouse line and the adenovirus used in HUVEC transplantation model (A) and in skeletal muscle–derived cell transplantation model (C). (B) Cre-expressing 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.

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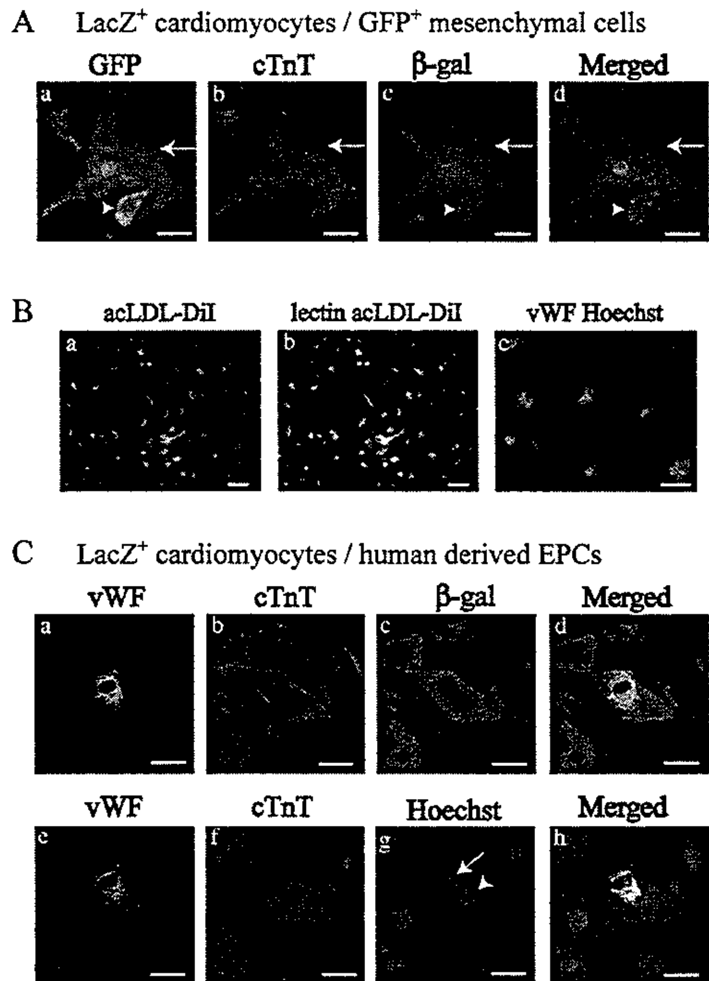
fused cells not only expressed cardiac-specific proteins, but also showed the function of cardiomyocytes (i.e., spontaneous beating). Evans et al. (1994) have reported that neonatal cardiomyocyte-fibroblast heterokaryons lose the expression of myosin light chain 2 gene, ANF, and muscle enhancer factor 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.

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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 (Dowell 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

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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 were 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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