

Fig. 5. Histological analysis of the transplanted CMG cells. (a–c) Confocal microscopy of the recipient heart transplanted with the sorted cardiomyocytes at 4 weeks. The transplanted cells could be clearly identified by EGFP signals. a and b show the transverse section of the transplanted cardiomyocytes, and c shows the longitudinal section. Bars indicate 50  $\mu\text{m}$ . (d) The emission profile of the green signal in GFP<sup>+</sup> cells was investigated by CLSM. The emission peak existed at 510–530 nm, and the profile was ascertained to be that of GFP, and not arising from nonspecific background. The inset shows the GFP<sup>+</sup> transplanted cells, and the cross indicates the site of the emission profile. (e–m) The bone-marrow-derived cardiomyocytes were sorted and marked with adenovirus-mediated LacZ gene. e is a photograph of the whole heart, and f shows an enlarged photograph of the injected site. The transplanted LacZ-positive cells were identified from the surface. g and h show the microscopy of the injected site of the left ventricle. The samples were stained with LacZ and hematoxylin–eosin. i–k show higher magnification of the same fields: i shows the transverse section, and j,k show the longitudinal section. (l, m) Transplanted CMG cells were stained with anticonnexin43 antibody and DAPI. Connexin43 was expressed at the both ends of the transplanted EGFP(+) CMG cells. Green: EGFP, Blue: DAPI, Red: connexin43.

not due to nonspecific background fluorescence, but due to the EGFP itself, using absorbance frequency analysis on a LSM510 Meta spectrometer (Fig. 5d).

Fig. 5e shows the entire murine heart stained with LacZ at 4 weeks after transplantation, and Fig. 5f showed an enlarged photograph of the site of injection. Cells were transplanted into the anterior free wall of the left ventricle and were observed to be rectangular in shape and located at the surface of the heart. Fig. 5g,h shows the site of injection

in a transverse section of the left ventricle stained with LacZ and hematoxylin–eosin 4 weeks after transplantation. The scar of the injection needle is shown in Fig. 5h. Granulomatous tissue was also observed around the site of injection. The LacZ-stained transplanted cells were clearly visible, and were located throughout the site of injection. Fig. 5i–k shows transverse and longitudinal sections of the transplanted cardiomyocytes at higher magnifications. This figure clearly shows the arrangement of the transplanted

cells parallel to the cardiomyocytes of the recipient heart. Fig. 5I,m shows expression of connexin43 at the longitudinal border between transplanted EGFP(+) CMG cells and adjacent cardiomyocytes of the recipient heart.

Transplanted cardiomyocytes survived in the recipient heart for more than 3 months and the estimated percentage of cells surviving transplantation was  $6.5 \pm 3.2\%$ . Table 1 shows the diameter of transplanted cardiomyocytes in transverse section. The diameter increased to almost the same size as the cardiomyocytes in the recipient heart over 4 weeks after which time no further increase was observed.

### 3.7. ECG recording and survival curve

Of the 35 mice that had undergone cell transplantation, 5 died within 24 h. This is most likely a result of the surgical procedure. The remaining 30 mice survived the duration of the observation period. ECGs recordings in 5 mice at 2 and 4 weeks, respectively, showed no evidence of arrhythmia (ventricular premature beats, ventricular tachycardia) during the recording period (data not shown). This finding suggests that survival of recipients in this model is not affected by arrhythmia.

## 4. Discussion

Since our report that cardiomyocytes can be regenerated from bone marrow stem cells [6,7], several studies have shown that transplantation of bone-marrow mononuclear cells or bone marrow stem cell fractions into the heart can improve cardiac function. Although the direct transplantation of these cells omits prior differentiation or purification, and thereby shortens the therapeutic period, it remains undetermined whether transplanted cells differentiate into the desired cardiomyocytes or endothelial cells, and not into other cell types including osteoblasts, chondroblasts, or adipocytes. The establishment of a reliable method to repair injured myocardium using cardiomyocyte transplantation requires the preparation of a sufficient number of well-characterized, purified regenerated cardiomyocytes, and an estimation of the survival rate of the transplanted cells.

Tomita [18] reported that the transplantation of 5-azacytidine-treated primary cultured marrow-stromal cells improved the function of the infarcted myocardium. Since the population of mesenchymal stem cells in primary cultured mice marrow stromal cells is less than 0.01%, it

is likely that most of the cells transplanted do not differentiate into cardiomyocytes, and that the observed improvement in cardiac function is caused by an improvement in ventricular remodeling or stimulation of angiogenesis.

Jackson transplanted adult stem cells [CD34(-)/low, c-Kit(+), Sca-1(+)] into lethally irradiated mice subsequently rendered ischemic by coronary artery occlusion followed by reperfusion, and reported that the engrafted cells migrated into ischemic cardiac muscle and blood vessels, differentiated to cardiomyocytes and endothelial cells, and contributed to the formation of functional tissue [19]. They found that the donor-derived endothelial cells were present at around 3.3%, primarily in small vessels adjacent to the infarct, and that donor-derived cardiomyocytes were present at around 0.02% and were found primarily in the peri-infarct region. Taken together, these findings show that differentiation from marrow stromal cells to cardiomyocytes *in vivo* is possible, but that their prevalence is less than other cell types. Condrelli [20] reported that neural stem cells differentiated into heart muscle cells when mixed with heart muscle cells from newborn rats in a process known as transdifferentiation. The mechanism of *in vitro* transdifferentiation is based on the idea that the developmental limitations of tissue specific stem cells are dictated by the environment, and that new signals that relax these limitations may be provided by cells from a different tissue [20].

It is most likely that the direct transplantation of stem cells into the heart does not facilitate their differentiation into cardiomyocytes, but merely results in their fusion with residual cardiomyocytes. We propose a more rigorous method to achieve repair of damaged tissue by first differentiating adult stem cells cardiomyocytes *in vitro*, and then transplanting a sufficient number of differentiated cardiomyocytes into the damaged heart tissue. To avoid possible adverse effects, we emphasize the importance of thoroughly investigating the molecular and electrophysiological characteristics of the stem cell-derived regenerated cardiomyocytes prior to transplantation.

In the present study, we used an EGFP reporter gene under the control of the MLC-2v promoter to tag isolated cardiomyocytes. Following FACS analysis, 99% of the isolated cardiomyocytes expressed EGFP, and when transplanted into the recipient heart they survived for at least 4 weeks. We observed no other cell types in the transplanted area, but this may have been because we only used a strongly expressing EGFP(+) fraction.

Table 1  
Diameter of the transplanted bone-marrow-derived cardiomyocyte

Time after transplantation (weeks)	2	4	8	12	Recipient cardiomyocytes
Diameter ( $\mu\text{m}$ )	$10.5 \pm 3.6^*$	$19.0 \pm 4.8\$$	$19.1 \pm 5.0\$$	$19.1 \pm 4.9\$$	$19.5 \pm 5.1$

The diameter of the transplanted cardiomyocytes was measured by the transverse section of the recipient hearts. Each data was obtained by measuring 200 cells. Mean  $\pm$  SD. \$: not significant vs. recipient cardiomyocytes.

\*  $p < 0.01$  vs. 4 weeks and recipient cardiomyocytes.

A plasmid encoding reporter genes and cardiac specific gene promoters was used in a previous study to isolate cardiomyocytes from ES cells or embryonic carcinoma cells (EC cell) [21]. Klug et al. [8] transfected a fusion gene containing the  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter and aminoglycoside phosphotransferase (NeoR) into pluripotent ES cells, then differentiated these cells in vitro prior to G418 selection. They reported high purification (>99%) and a survival period in the recipient heart of at least 7 weeks following transplantation. Zweigerdt et al. [22] and Zandstra et al. [23] reported a lab-scale protocol to generate cultures of highly enriched cardiomyocyte from ES cells transfected with a  $\alpha$ -MHC-NeoR containing plasmid, and suggest its application to a larger-scale process for the supply of stem cell based cardiomyocytes. Muller et al. [9] isolated a subpopulation of ventricular-like cardiomyocytes from ES cells by transfecting the EGFP gene under the control of the MLC-2v promoter and cytomegalovirus enhancer. Moore et al. [24] reported that EC cell (P19Cl6)-derived cardiomyocytes could be isolated using an EGFP reporter under the control of 250 bp of the MLC-2v promoter. They enzymatically digested embryoid bodies, then isolated a population of cardiomyocytes (97% pure) using Percoll gradient centrifugation and FACS analysis. Kolossov et al. [25] reported the use of EGFP under the control of the cardiac  $\alpha$ -actin promoter to isolate ES cell-derived cardiomyocytes. The present study confirmed the efficiency of this strategy for the isolation and purification of cardiomyocytes from bone-marrow-derived stem cells.

Reinecke and Murry [26] and Zhang et al. [27] highlighted the importance of a quantitative analysis of grafted cardiomyocytes, since a large number of fetal or neonatal cardiomyocytes often display apoptosis within several days of transplantation. They reported that only a small percentage of cardiomyocytes survive in the cryoinjured recipient heart, and that heat shock or adenoviral transfer of constitutive active Akt genes could increase their survival. In comparison, the present study reports a slightly higher survival rate for bone marrow-derived cardiomyocytes. One possible reason is the difference in the experimental models as the present study used a mouse uninjured model and not a rat cryoinjured heart model. Another reason is the small size of our not fully differentiated transplanted cells compared with fetal or neonatal cardiomyocytes. A small size may allow transplanted cells to go deep into the recipient heart without mechanical injury.

Recently, Takeda et al. [28] reported that the life span of human bone marrow mesenchymal stem cells could be prolonged by infecting the cells with the retrovirus encoding oncogene *bmi-1*, human papilloma virus E6 and E7, and human telomerase reverse transcriptase over 150 population doublings, and that these cells could be induced to differentiate into cardiomyocyte using 5-azacytidine and coculture with the rat cardiomyocytes. Although this procedure is not suitable for clinical application at the present stage, the findings provide valuable information on the use

of human bone marrow stem cells for the regeneration of cardiomyocytes.

In summary, the present study provides a new model for tissue engineering. Further studies are required to improve cardiomyocyte differentiation and to increase the efficiency of the transplantation procedure.

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# Regenerative Medicine for Cardiomyocytes

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**Abstract:** Heart transplantation is the ultimate treatment option for severe cardiac failure, but is available only for a very small fraction of cases due to a serious donor shortage. Increasing attention has become focused upon a novel therapeutic approach, regenerative medicine, to break the present impasse. Attempts to regenerate cardiomyocytes have been made by using pluripotent embryonic stem cells or marrow-derived mesenchymal stem cells (adult stem cells). Cardiomyocytes can be regenerated from embryonic stem cells as well as from adult stem cells, but the regenerated cells differ in characteristics depending on the source stem cells. These two groups of stem cells differ with respect to proliferative potency, pluripotency, method of induction of differentiation into cardiomyocytes, rejection reactions, and tumorigenic potential. Further studies to ascertain which type of stem cell will be more useful and safer for this purpose remain to be carried out. Studies in laboratory animals have reportedly demonstrated improvement of cardiac function through regenerated cardiomyocyte transplantation into the heart, encouraging the hope that a new treatment modality has been found for severe heart failure.

**Key words:** Embryonic stem cell; Adult stem cell; Cardiomyocyte; Regenerative medicine

## Introduction

In Japan, cases of heart disease have consistently been increasing with the aging of the population and the Westernization of the diet. A wide variety of pharmacotherapies has been developed for the treatment of intractable severe heart disease, with proven efficacy, however, heart transplantation is the sole radical

treatment option. There is still no increase in brain-dead donors and heart transplantation is a treatment available only for a very few cases.

To break the present impasse, therefore, a method to treat intractable cardiac failure by regenerating and transplanting cardiomyocytes is being sought. Studies of heart muscle cell regeneration have been making a steady progress, though at the level of laboratory ani-

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Table 1 Comparison of ES Cells and Adult Stem Cells as Materials for Regenerative Cardiomyocytes

	ES Cells	Adult Stem Cells
Origin	Post-fertilization early-stage embryo (inner cell mass of blastocyst)	Marrow stromal cell
Cell isolation technique, etc.	Method of cell establishment is already established, and is relatively easily performed.	Sparse among bone marrow cells, and method of cell establishment is yet to be established.
Proliferative potency	At present, cells are considered to infinitely proliferate.	Proliferate to some extent but the number of divisions is unknown.
Pluripotency	Differentiate into any type of cell <i>in vivo</i> . The cells differentiate into early developmental stage cells <i>in vitro</i> , but it is thought to be difficult for them to differentiate into cells that appear late in the fetal stage.	Recognized to be able to differentiate into mesodermal cells such as osteoblasts, chondroblasts and adipocytes, but reportedly undergo differentiation into nerve cells (ectoblast-derived) and cells of the liver (entoblast-derived) as well.
Differentiation into cardiomyocytes	Differentiate relatively easily, but a method to have ES cells specifically differentiate into cardiomyocytes has not been established.	Demonstrated to differentiate into cardiomyocytes, but a method to have the cells specifically differentiate into cardiomyocytes has not been established.
Rejection reactions	Occur	No rejection reactions if the cells are autologous.
Tumorigenic potential	There is potential risk of teratomas after transplantation if undifferentiated cells remain.	No

mal experiments, and will lead to development of research with human cells and their clinical application.

Stem cells currently used for regenerative medical therapy for the myocardium are broadly divided into two groups: embryonic stem cells (ES cells) obtained from early stage embryos post *in vitro* fertilization and marrow adult stem cells obtained from the bone marrow of adults. Whether one type is superior to the other for cardiomyocyte regeneration remains to be seen. Characteristics of the two types of stem cells are summarized in Table 1. This article outlines the current status and future prospects of regenerative medicine for the myocardium.

### Differentiation from ES Cells to Cardiomyocytes

Figure 1 illustrates the outline of cardiomyocyte regeneration using ES cells. ES cells are those cells constituting the inner cell mass, destined to form the fetus, from an early

embryo having reached the stage of blastocyst. These cells are known to differentiate into any type of cell *in vivo* and have been shown to differentiate *in vitro* into a variety of organic cells such as cardiomyocytes, skeletal muscle cells, vascular endothelial cells, smooth muscle cells, neurons, and hepatocytes. However, most types of cells that appear late in the fetal stage have not been demonstrated from ES cells *in vitro*.

It is generally recognized that various cell growth factors, cytokines, and cell adhesion factors are required for ES cell differentiation into those various cells. Recent studies have clarified a cascade operating for selective differentiation of ES cells into motor neurons, however, the cascade for ES cell differentiation into cardiomyocytes has not been fully elucidated to date.

A method to have ES cells form a cell mass (embryoid) has been introduced as a general means of inducing ES cell differentiation into cells capable of differentiation. The frequency with which differentiation from the embryoid

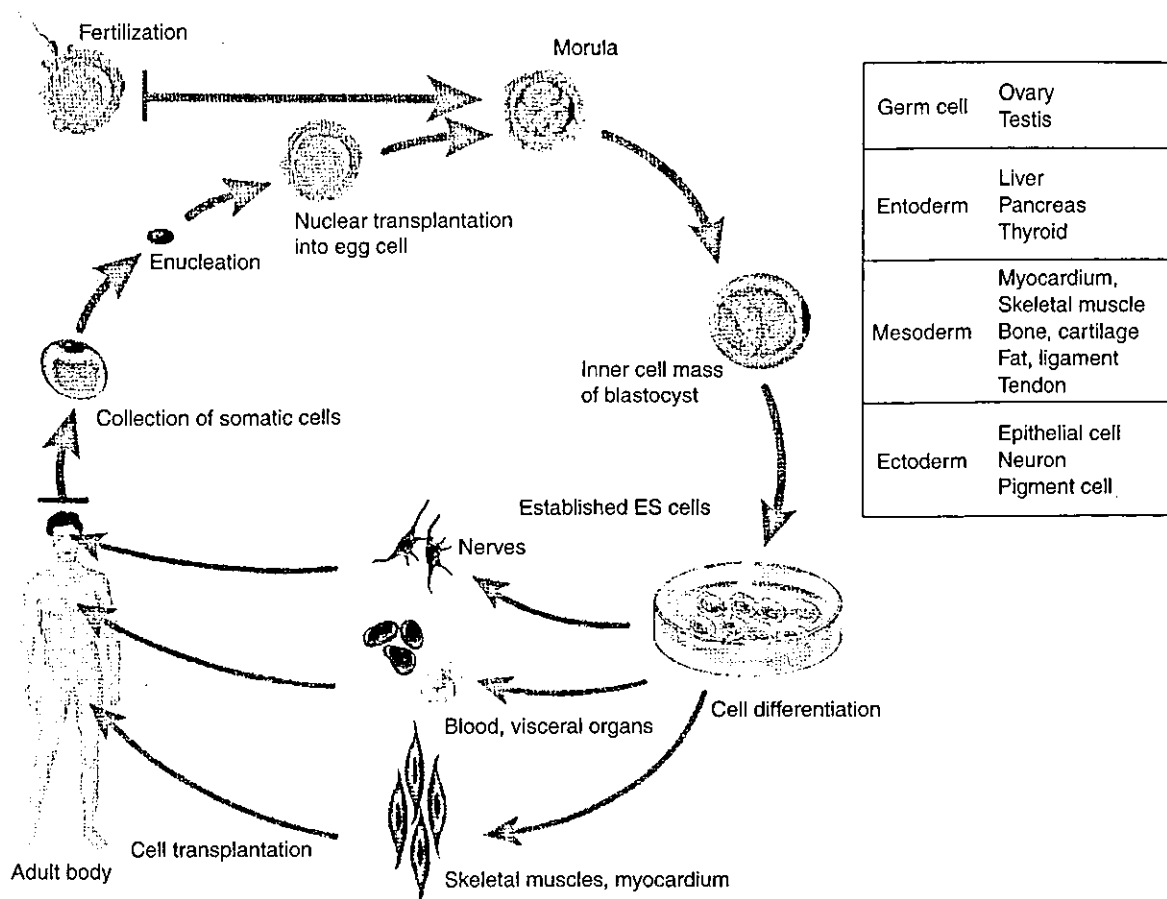


Fig. 1 Myocardial regeneration from ES cells

to cardiomyocytes is regarded as being about 7–8% at best. Humoral factors known to induce ES cell differentiation specifically into cardiomyocytes include bone morphogenetic protein-2 (BMP-2) and Wnt 11, which facilitate the differentiation, and Wnt 3 and Wnt 8, which act as inhibitors. Possible involvement of various other factors is presumed; the process seems to be quite intricate. Selective differentiation of ES cells into cardiomyocytes may become feasible from analysis of the pathways of the differentiation process.

### Adult Stem Cell Differentiation into Cardiomyocytes

Bone marrow has been universally recognized to be the site of hematopoiesis with the

predominance of hematopoietic stem cells. In fact, more than 99% of marrow cells take part in the production and development of blood cells. It was discovered recently that, among bone marrow cells, there are cells termed stromal cells that essentially do not represent blood cells but secrete cytokines and growth factors to support cells of the hematopoietic system. The presence of pluripotent stem cells capable of differentiating into various types of cells among the marrow stromal cells has become recognized. These marrow stromal cells with pluripotent capacity are referred to as mesenchymal stem cells on account of their ability to differentiate into such mesenchymal cells as osteoblasts, chondroblasts, and adipocytes.

In view of the mesenchymal stem cells being

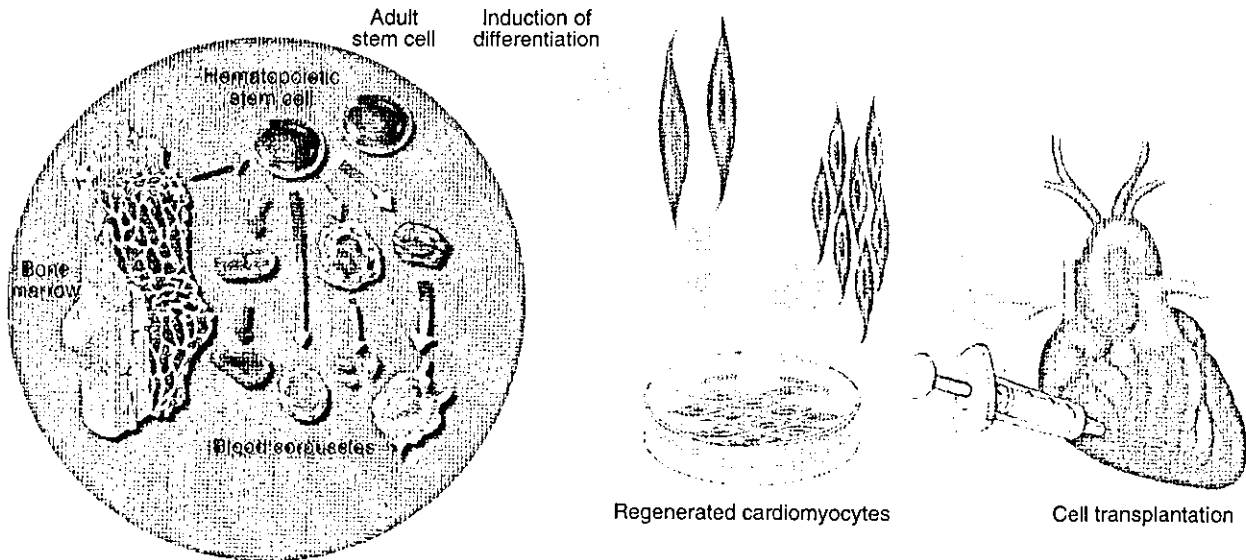


Fig. 2 Myocardial regeneration from adult stem cells and cell transplantation

able to differentiate into mesoblast-derived organs, we wondered if they could differentiate to become cardiomyocytes as well, which are also of mesodermal origin. Our studies demonstrated that cardiomyocytes that beat regularly by themselves can be obtained from mesenchymal stem cells. Figure 2 shows an outline of the process. Mesenchymal stem cells have recently been reported to undergo differentiation into nerve cells (ectoblast-derived) and cells of the liver (entoblast-derived) as well, and are now termed adult stem cells.

### Characteristics of Regenerated Cardiomyocytes

Cardiomyocytes derived from bone marrow show expression of fetal ventricular muscle type genes soon after their differentiation from adult stem cells, and thereafter gradually express adult type genes.<sup>1)</sup> Expression of genes for atrial natriuretic polypeptide and cerebral natriuretic polypeptide that are considered cardiomyocyte-specific have also been demonstrated. The cells proved to self-beat, and those differentiated from murine adult stem cells showed 120–250 beats/min. The cardiomyo-

cytes exhibited the sinus node pattern of action potentials early after their differentiation from adult stem cells, and the pattern gradually changed to the ventricular muscle cell type.

Catecholamin  $\alpha_1$  receptor (cardiac hypertrophic effect) and  $\beta_1$  and  $\beta_2$  receptors (positive chronotropic and positive inotropic effects) play important roles in the muscle cells of the heart. In regenerated cardiomyocytes of bone marrow origin,  $\alpha_1$  receptor switched to myocardial type (mainly  $\beta_{1A}$  and  $\beta_{1B}$  receptors) as the differentiation proceeded into heart muscle cells, and simultaneously,  $\beta_1$  and  $\beta_2$  receptors that had not existed early in the course of differentiation became expressed.<sup>2)</sup> Stimulation of these catecholamine receptors led to activation of subreceptor signaling to produce cardiac hypertrophy, increases in heart rate and increases in myocardial contractile force.

The above findings indicate that the regenerated cardiomyocytes are endowed with practically normal characteristics of cardiomyocytes.<sup>3)</sup>

### Treatment of Cardiac Failure by Cell Transplantation

Cardiomyocyte transplantation has been



extensively investigated since the mid-1990s at the level of animal experiments. Cells harvested in primary cultures of heart muscle cells obtained from the fetus or the neonate were transplanted into the heart of sexually mature animals, and the transplantation was shown to improve post-infarction cardiac function. Clinical experience with the transplantation of fetal midbrain obtained through artificial termination of pregnancy into patients with Parkinson's disease has yielded some gratifying therapeutic results. The amount of cell transplants required is apparently greater in the case of cardiomyocyte transplantation, so that it is not practical to use aborted fetuses as the source.

Regenerated cardiomyocytes derived from ES cells, when transplanted, reportedly proved to electrically bound to recipient myocardium and contract synchronously with surrounding cells, thus fueling hopes for the use of regenerated cardiomyocytes. Our experience with regenerated cardiomyocyte transplantation into the hearts of adult patients showed long-term engraftment with gratifying outcomes. However, there have been reports demonstrating that the number of cardiomyocytes taken as compared to that of cardiomyocytes transplanted diminished due to cellular necrosis during the course of engraftment. Further study is needed, including assessments of transplantation methods.

### Problems Associated with Myocardial Regeneration and Future Prospects

To bring regenerative cardiomyocyte trans-

plantation to realization requires securing regenerated cardiomyocytes and supplying those cells safely and at moderate expense. When adult stem cells and ES cells are compared, this author's view is that the latter will come into use earlier in the future. Supply of regenerated cardiomyocytes derived from ES cells will become a reality within several years. Major problems are rejection reactions and method of transplantation. To avoid rejection, it is essential to transplant the nucleus of a somatic cell into an egg cell, as shown in Fig. 1. The nuclear transplantation eventually has a close bearing upon the matter of human cloning, therefore it is important to hold nationwide discussion of this ethical problem.

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# Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction

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Bone marrow (BM) cells are reported to contribute to the process of regeneration following myocardial infarction. However, the responsible BM cells have not been fully identified. Here, we used 2 independent clonal studies to determine the origin of bone marrow (BM)-derived cardiomyocytes. First, we transplanted single CD34<sup>-</sup> c-kit<sup>+</sup> Sca-1<sup>+</sup> lineage<sup>-</sup> side population (CD34<sup>-</sup> KSL-SP) cells or whole BM cells from mice ubiquitously expressing enhanced green fluorescent protein (EGFP) into lethally irradiated mice, induced myocardial infarction (MI), and

treated the animals with granulocyte colony-stimulating factor (G-CSF) to mobilize stem cells to the damaged myocardium. At 8 weeks after MI, from 100 specimens we counted only 3 EGFP<sup>+</sup> actinin<sup>+</sup> cells in myocardium of CD34<sup>-</sup> KSL-SP cells in mice that received transplants, but more than 5000 EGFP<sup>+</sup> actinin<sup>+</sup> cells in whole BM cell in mice that received transplants, suggesting that most of EGFP<sup>+</sup> actinin<sup>+</sup> cells were derived from nonhematopoietic BM cells. Next, clonally purified nonhematopoietic mesenchymal stem cells (MSCs), cardiomyogenic (CMG)

cells, that expressed EGFP in the cardiomyocyte-specific manner were transplanted directly into BM of lethally irradiated mice, MI was induced, and they were treated with G-CSF. EGFP<sup>+</sup> actinin<sup>+</sup> cells were observed in the ischemic myocardium, indicating that CMG cells had been mobilized and differentiated into cardiomyocytes. Together, these results suggest that the origin of the vast majority of BM-derived cardiomyocytes is MSCs. (Blood. 2004;104:3581-3587)

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## Introduction

Recent studies have suggested that bone marrow (BM) cells can contribute to regeneration processes in various tissues.<sup>1,2</sup> Cardiomyocytes derived from BM cells have been observed after myocardial infarction (MI),<sup>3,4</sup> and BM-derived cells mobilized by cytokines were capable of regenerating the myocardial tissue, leading to an improvement in survival and cardiac function after MI.<sup>5</sup> BM contains both hematopoietic and nonhematopoietic cells, and the origin of the BM cells with the ability to repair damaged myocardial tissue remains unknown. The identification of specific cell types involved in myocardial repair is a crucial step toward the development of effective stem cell-based therapies for MI.

The most likely candidates for the BM-derived stem cells with the ability to regenerate myocardial tissue are hematopoietic stem cells (HSCs)<sup>3-5</sup> and nonhematopoietic mesenchymal stem cells (MSCs).<sup>6-10</sup> Alvarez-Dolado et al recently demonstrated that BM-derived cardiomyocytes sporadically detected in noninfarcted mice were exclusively generated by fusion with donor CD45<sup>+</sup> cells, possibly hematopoietic cells,<sup>11</sup> suggesting that the so-called phenomenon of HSC "plasticity" might result from the fusion of HSCs with cells residing in the target tissue. Two recent studies reported that

HSCs are unable to differentiate into cardiomyocytes in vivo even after MI.<sup>12,13</sup> MSCs also are candidates for the regeneration of cardiomyocytes in vivo; we previously reported that BM-derived MSCs could differentiate into spontaneously beating cardiomyocytes in vitro,<sup>6-8</sup> and other groups have repaired the myocardium using MSC transplantation in vivo.<sup>9,10</sup>

The aim of this study is to determine the precise origin of the BM cells mobilized by cytokines to repair infarcted myocardium. We transplanted genetically marked single HSCs or clonal MSCs into lethally irradiated recipient mice, induced MI, treated the mice with granulocyte colony-stimulating factor (G-CSF), and analyzed the cardiac tissues. Our results suggest that MSCs are the predominant source of regenerated cardiomyocytes.

## Materials and methods

### Mice

C57BL/6 (B6) mice and C3H/He (C3H) mice were purchased at 6 to 8 weeks of age from Japan CLEA (Tokyo, Japan). B6 transgenic mice that ubiquitously expressed enhanced green fluorescent protein (EGFP) under

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the control of the CAG promoter<sup>14</sup> were used as the BM donor in the transplantation studies using B6 mice. All the transgenic EGFP mice used in this study were heterozygous for the transgene.

#### Isolation of CD34<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> lineage<sup>-</sup> SP cells (CD34<sup>-</sup>KSL-SP cells)

The isolation of the CD34<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> lineage<sup>-</sup> SP cells was previously described.<sup>15</sup> Briefly, BM cells suspended at  $1 \times 10^6$  cells/mL in HBSS+ (calcium- and magnesium-free Hanks balanced salt solution supplemented with 2% fetal calf serum [FCS], 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 100 U/mL penicillin, and 100  $\mu$ M streptomycin) were incubated with 5 mg/mL Hoechst 33342 (Sigma Aldrich, St Louis, MO) for 60 minutes at 37°C. After washing, the cells were resuspended in ice cold HBSS+ at a cell density of  $10^7$  cells/mL and then stained for 30 minutes on ice with various monoclonal antibodies, including biotinylated CD34, allophycocyanin (APC)-conjugated c-kit, phycoerythrin (PE)-conjugated Sca-1, and PE-Cy5-conjugated lineage markers (Gr-1, Mac-1, B220, CD3, TER119). The biotinylated antibodies were visualized using PharRed (APC-Cy7)-conjugated streptavidin. All of these reagents were purchased from BD Pharmingen (San Diego, CA). Cell sorting was performed using a triple laser MoFlo (Cytomation, CO) using Summit software. Hoechst 33342 was excited at 350 nm, and the fluorescence emission was detected using 405/BP30 and 570/BP20 optical filters against Hoechst blue and Hoechst red, respectively, and a 555-nm long-pass dichroic mirror (Omega Optical, Brattleboro, VT) to separate the emission wavelengths. Both Hoechst blue and red fluorescence were shown on a linear scale. After collecting  $10^5$  events, the side population (SP) was defined as described previously, and additional gates were defined as positive for Sca-1 and c-kit and negative for CD34 and lineage markers according to the isotype control fluorescence intensity. Populations of CD34<sup>-</sup>KSL-SP cells with 99% purity were routinely prepared using this method. Single CD34<sup>-</sup>KSL-SP cells derived from transgenic EGFP mice were sorted directly into separate wells of a 96-well plate containing 100  $\mu$ L HBSS+ using a CyClone automated cell deposition unit.

#### HSC transplantation

Single CD34<sup>-</sup>KSL-SP cells or  $5 \times 10^6$  whole BM cells from transgenic EGFP mice were injected intravenously into the retro-orbital plexus of etherized recipient B6 mice that had been lethally irradiated with a dose of

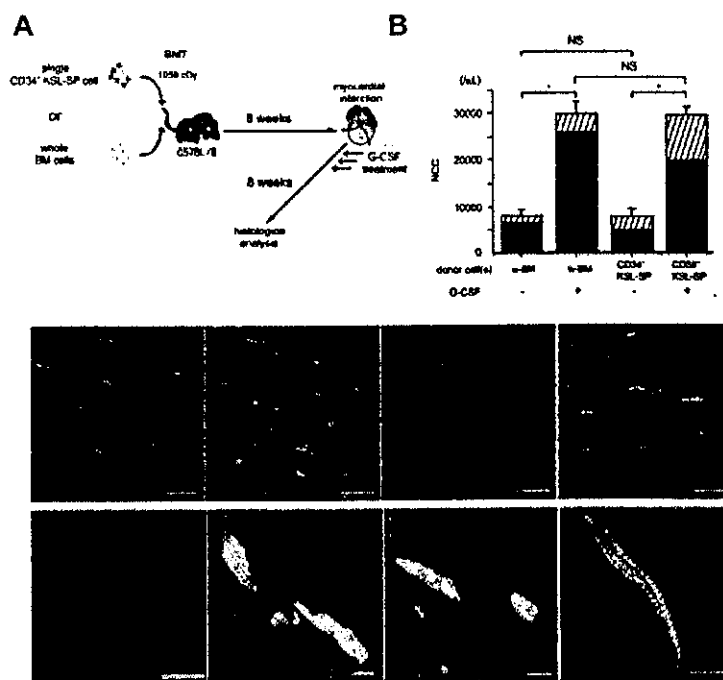
10.5 Gy (Figure 1A). For radioprotection, the single CD34<sup>-</sup>KSL-SP cells were transplanted along with  $2 \times 10^5$  whole BM cells as radioprotective cells from B6 mice that did not carry the CAG-EGFP transgene. Three months after BM transplantation, peripheral blood samples were collected from the recipient mice, and the erythrocytes in the samples were depleted using Ficoll-Paque. Dual-laser fluorescence-activated cell-sorter scanner (FACS) analysis was performed using a FACS Calibur (Becton Dickinson, San Jose, CA). Donor BM-derived cells were determined by the fluorescent intensity of the EGFP, compared with wild-type cells, and mice engrafted with more than 50% of the EGFP<sup>+</sup> cells were used for the experiments.

#### Myocardial infarction and mobilization of BM cells

The recipient mice were intubated and anesthetized with 0.5% isoflurane gas. After left thoracotomy, the left ventricle was exposed and the left coronary artery was ligated. Twenty-four hours later, the mice were subcutaneously injected with 300  $\mu$ g/kg of recombinant human G-CSF dissolved in saline solution once a day for 10 consecutive days. Control mice received a saline solution without G-CSF.

#### Immunohistological studies

Hearts were perfused from the apex with phosphate-buffered saline (PBS) and perfusion fixed with 4% paraformaldehyde/PBS. They were then dissected, immersion fixed overnight at 4°C in 4% paraformaldehyde, embedded in Optimal Cutting Temperature (OCT) Compound (TED PELLA, Redding, CA), and quickly frozen in liquid nitrogen. Cryostat sections (6  $\mu$ m thick) were stained overnight at 4°C using specific antibodies. Anti-GFP (MBL, Nagoya, Japan), anti- $\alpha$ -actinin (Sigma Aldrich), anti-sarcomeric myosin (MF20), anti-cardiac troponin I (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GATA4 (Santa Cruz), and anti-CD45 (BD Pharmingen, San Diego, CA) were used to identify EGFP<sup>+</sup> cells, cardiomyocytes, and hematopoietic cells. The sections were incubated with secondary antibodies conjugated with Alexa 488 or 594 (Molecular Probes, Eugene, OR). Nuclei were stained with TOTO-3 (Molecular Probes). Slides were observed under a confocal laser scanning microscope (LSM 510 META; Carl Zeiss, Jena, Germany). In some experiments, the liver, kidney, brain, skeletal muscle, and spleen of recipient mice also were analyzed by immunofluorescence photography. To observe the bone marrow cavity, bone tissue was fixed with 10% formaldehyde and decalcified in K-CX solution (Falma, Tokyo, Japan) at



**Figure 1.** G-CSF-induced mobilization of BM cells in mice receiving transplants of single CD34<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> lineage<sup>-</sup> side population (CD34<sup>-</sup>KSL-SP) cells or whole BM cells following MI. (A) Experimental protocol. Single EGFP<sup>+</sup> CD34<sup>-</sup>KSL-SP cells and radioprotective EGFP<sup>-</sup> BM cells were transplanted into the recipient mice. (B) The nucleated cell counts (NCCs) for peripheral blood samples obtained 24 hours after the last injection of G-CSF ( $n = 10$ ) or saline ( $n = 10$ ) are shown. ■ indicates EGFP<sup>+</sup> cells; □, EGFP<sup>-</sup> cells. NS indicates not significant; \* $P < .0001$ . Bars indicate the standard error. (C-F) Panels show representative results for immunofluorescent analysis using anti-GFP antibody and TOTO-3 dye in the hearts of mice receiving transplants of w-BM cells or single CD34<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> lineage<sup>-</sup> side population (CD34<sup>-</sup>KSL-SP) cells separated from the BM of transgenic EGFP mice. The green and blue signals indicate EGFP and nuclei, respectively. Saline-treated mice (G-CSF[-] mice) (C) and G-CSF-treated mice (G-CSF[+] mice) (D) in the w-BM group. G-CSF(-) mice (E) and G-CSF(+) mice (F) in the CD34<sup>-</sup>KSL-SP group. Bars indicate 200  $\mu$ m. (G) The control experiment using C57BL/6J mice as donors revealed that no green fluorescence was detected in the whole heart. Bar indicates 50  $\mu$ m. (H-J) Coimmunostaining with anti-GFP and anti-actinin antibodies in the infarcted myocardium of G-CSF(+) mice transplanted with w-BM (H, I) or single CD34<sup>-</sup>KSL-SP cells (J). The green, red, and blue signals indicate EGFP, actinin, and nuclei, respectively. (H, I) In the w-BM group, some of the EGFP<sup>+</sup> cells in the infarcted area were positive also for actinin and showed striation, indicating that they had differentiated into cardiomyocytes. (J) In contrast, very few EGFP<sup>+</sup> actinin<sup>+</sup> cells were seen in the infarcted area in the CD34<sup>-</sup>KSL-SP group. Bars indicate 50  $\mu$ m (H, I) and 20  $\mu$ m (J).

4°C for 2 days. Sections (3 μm) were incubated with anti-GFP antibody overnight at 4°C. A specific signal was visualized as a brown reaction product of peroxidase substrate 3,3'-diaminobenzidine (Sigma). Samples were counterstained with hematoxylin and eosin.

**Adherent cell culture and immunocytochemistry**

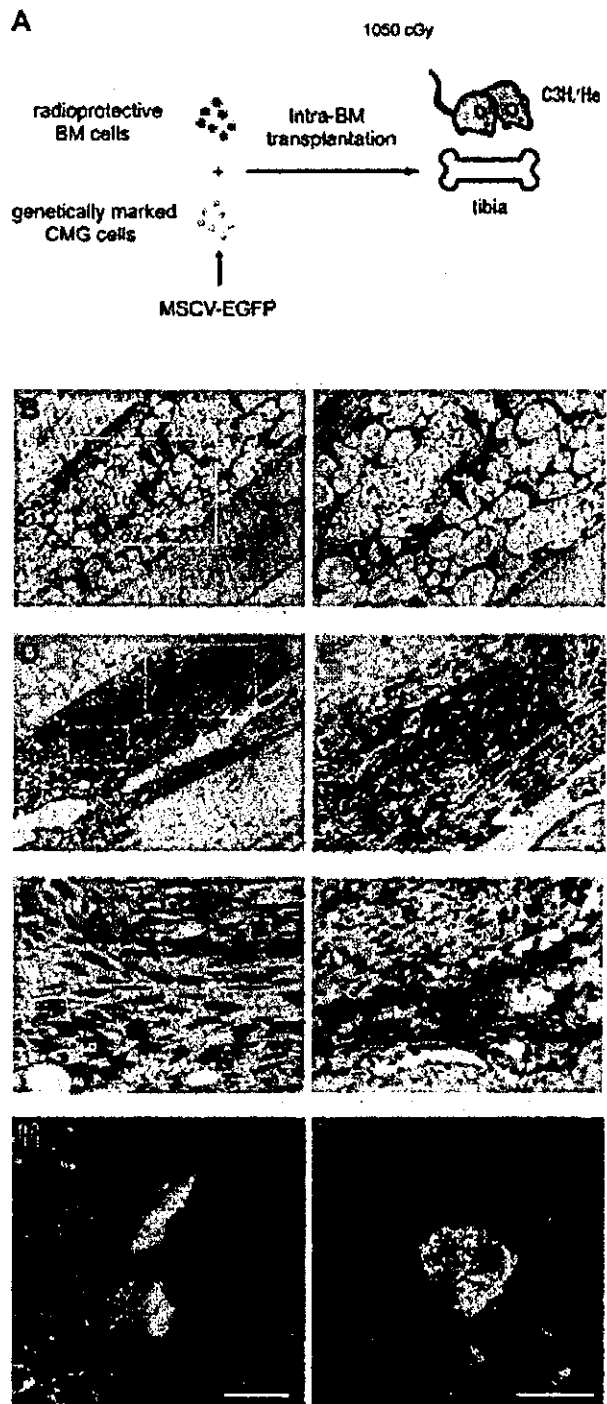
Twelve weeks after the transplantation of the single CD34<sup>+</sup>-KSL-SP cells or w-BM cells from transgenic mice that ubiquitously express EGFP,<sup>14</sup> peripheral blood samples were collected from the retro-orbital plexus of the recipients. If more than 80% of the donor-derived EGFP<sup>+</sup> cells were found in the peripheral blood, the mice were killed and the BM cells were flushed from the femurs and tibiae to prepare single-cell suspensions. After most of the erythrocytes were removed with Ficoll-Paque, 3 × 10<sup>5</sup> BM mononuclear cells were plated on the fibronectin-coated wells of 96-well plates (Costar, Cambridge, United Kingdom) and incubated in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a fully humidified atmosphere of 5% CO<sub>2</sub>. The medium was exchanged every 2 to 3 days, and nonadherent cells were removed. The cultures were terminated 2 weeks after seeding by removal of the culture medium. The samples were flushed 3 times with PBS and fixed with 4% paraformaldehyde, then stained with rabbit anti-GFP antibody (1:1000 dilution, Molecular Probes) and visualized using a secondary antibody of Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000 dilution, Molecular Probes). The specimens were also stained with PE-conjugated rat anti-mouse CD45 (1:1000 dilution; eBioscience, San Diego, CA) and Hoechst 33342 (Sigma Aldrich). The adherent cells in each plate were counted under a fluorescent microscope.

**Differentiation cultures for osteoblasts and adipocytes**

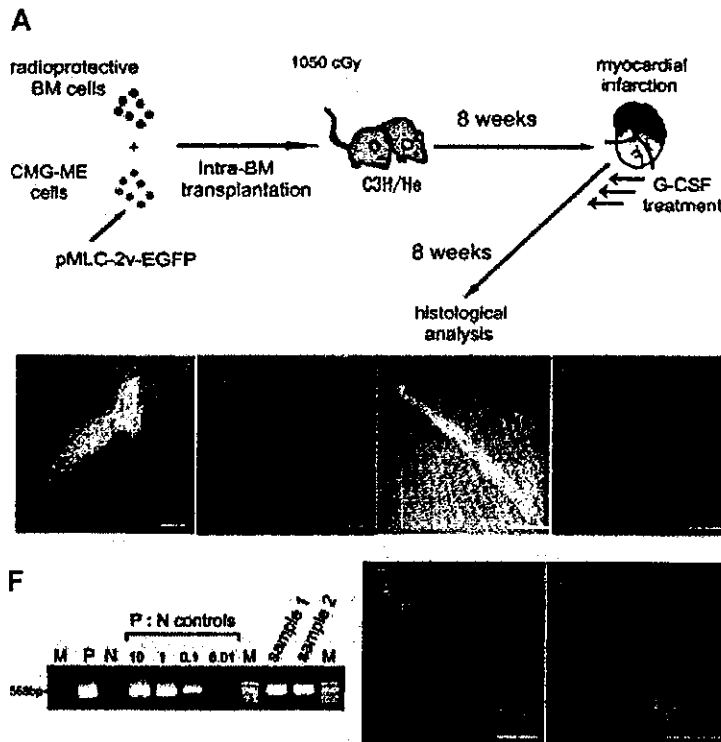
To induce osteocyte differentiation, the adherent cells were cultured on slides in DMEM with 10 mM β-glycerophosphate, 10<sup>-7</sup> M dexamethasone, and 0.2 mM ascorbic acid (all from Sigma Aldrich); the media was changed every 3 to 4 days. After 14 days, osteoblast differentiation was confirmed using alkaline phosphatase enzymatic staining. For the alkaline phosphatase staining, cells were fixed with methanol at -20°C for 2 minutes and then washed in 100 mM Tris [tris(hydroxymethyl)aminomethane]-HCl (pH 9.5), 100 mM NaCl, and 10 mM MgCl<sub>2</sub> buffer (all from Sigma Aldrich) for 10 minutes. The slides were then stained with fast 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium alkaline phosphatase substrate (both from Sigma Aldrich) for 10 minutes and rinsed in distilled water. To induce adipocyte differentiation, the adherent cells were cultured with 10% horse serum or 100 ng/mL insulin and were maintained for 14 days on the slides, with medium exchanges every 3 to 4 days. After 14 days, we confirmed the differentiation of the cells to lipid-laden adipocytes by staining with oil-red. For the oil-red staining, the cells were fixed with methanol at -20°C for 2 minutes and then rinsed in 50% alcohol. Slides were stained in oil-red-O (Sigma Aldrich) for 10 minutes and rinsed in 50% alcohol. After rinsing in distilled water, slides were counterstained with Mayer hematoxylin (Sigma Aldrich) for 1 minute.

**Transduction of CMG cells**

CMG cells derived from C3H mice were cultured in Iscoves modified Dulbecco medium supplemented with 20% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 85 μg/mL amphotericin B on fibronectin-coated dishes at 33°C in 5% CO<sub>2</sub>, as previously described.<sup>6</sup> In the experiments shown in Figure 2A, the CMG cells were transduced with the recombinant ecotropic retrovirus murine stem cell virus (MSCV)-EGFP<sup>16</sup> at multiplicity of infection (MOI) 10, and the EGFP<sup>+</sup> cells were sorted using a FACS Vantage (Becton Dickinson, Cockeysville, MD), as previously described.<sup>17</sup> In the experiments shown in Figure 3A, an expression vector, pMLC2v-EGFP, was constructed by cloning a 2.7-kb *HindIII-EcoRI* fragment of the rat MLC-2v promoter region<sup>18,19</sup> into the *HindIII-EcoRI* site of pEGFP-1 (Clontech, Palo Alto, CA) so that EGFP would be expressed under the control of the MLC-2v promoter. The MLC2v-EGFP plasmid was then transfected into the CMG cells by liposomal transfection. After culturing with 1000 μg/mL of G418 for 4 weeks, permanently transfected single



**Figure 2. Engraftment and multilineage differentiation of clonal mesenchymal stem cells (CMG cells).** (A) The experimental protocol for the intra-BM transplantation of CMG cells transfected with MSCV-EGFP is shown. (B-G) Transplanted CMG cells were observed in the recipients' BM. CMG cells were recognized as brown cells by immunohistochemistry. (B) CMG cells differentiated into adipocytes (arrows) (magnification, × 100). The rectangle in panel B is shown at a higher magnification (× 200) in panel C. (D) CMG cells also differentiated into osteocytes (arrows). Undifferentiated CMG cells exhibiting spindle-like shapes were also observed (arrowheads) (magnification, × 100). The rectangles in panel D are shown at higher magnifications in panels E (× 200) and F (× 400). (G) Undifferentiated CMG cells in other part (× 200). (H, I) CMG cells also were found in the liver (H) and kidney (I) by immunofluorescence photography. Bars indicate 10 μm. The green and blue signals indicate EGFP and nuclei, respectively.



**Figure 3.** Mobilization and differentiation of mesenchymal stem cells (CMG cells) into cardiomyocytes *in vitro* and *in vivo*. (A) The experimental protocol is shown. The CMG-ME cells are CMG cells that have been permanently transfected with a plasmid-encoding EGFP driven by the myosin light chain promoter (pMLC2v-EGFP). (B,C) Cultured CMG-ME cells were treated with 5-azacytidine and immunostained with anti-GATA4 (red) antibody on the seventh day of culture. GATA4 was expressed in the CMG-ME cells. Some of the CMG-ME cells became EGFP<sup>+</sup> (green). (D,E) At 3 weeks, CMG-ME cells that were positive for both EGFP and actinin were observed, indicating that the cells had differentiated into cardiomyocytes. (F) The engraftment of CMG-ME cells into the recipients' BM was confirmed by PCR. Representative results using BM samples collected from recipient mice (samples 1 and 2) are shown. The transgene was clearly detected in the 2 samples. P indicates positive control (CMG-ME cells); N, negative control (CMG cells); M, marker. P:N controls are shown as the percentage with respect to the positive control. (G,H) The myocardium of infarcted mice that received transplants of CMG-ME cells was analyzed using immunofluorescent microscopy. The green, red, and blue signals indicate EGFP, actinin, and nuclei, respectively. EGFP<sup>+</sup> actinin<sup>+</sup> CMG-ME cells were observed in the myocardium, indicating that the CMG-ME cells had been mobilized into the ischemic myocardium. Actinin and EGFP expression, driven by the MLC-2v promoter, was detected, indicating that the cells had differentiated into cardiomyocytes. Bars indicate 10  $\mu$ m.

cell-derived colonies were cloned and pooled (CMG-ME cells). For *in vitro* differentiation, CMG-ME cells were plated onto fibronectin-coated dishes and treated with 3  $\mu$ mol/L of 5-azacytidine (Sigma Aldrich) for 24 hours.

#### Intra-BM transplantation of CMG cells

One-hundred thousand CMG cells transduced with MSCV-GFP or CMG-ME cells were transplanted into the right tibia of lethally irradiated C3H mice by intra-BM injection using a previously described method.<sup>20</sup> Two-hundred thousand whole BM cells separated from C3H mice also were injected into the recipient mice to serve as radioprotective cells. When the mice were killed, the engraftment of the CMG-ME cells and the CMG cells that were transduced with MSCV-GFP was confirmed by polymerase chain reaction (PCR) and enzyme immunohistochemistry, respectively.

#### PCR analysis

The presence of the transgene was detected by PCR. The following primers were designed against the EGFP cDNA (5' primer: 5'-CCAGTTCAGCGTGTCCGGCG-3'; 3' primer: 5'-GGGGTCTTTGCTCAGGGCGG-3'). The PCR conditions have been previously described.<sup>17</sup>

#### Statistics

All values are presented as the mean  $\pm$  SEM. Statistical significance was evaluated using unpaired Student *t* tests for comparisons between 2 mean values. Multiple comparisons between more than 3 groups were performed using an ANOVA. A value of *P* less than .05 was considered significant.

## Results

### Single HSC progeny are not a major source of new cardiomyocytes after cytokine-enhanced post-MI repair

In a previous report, we demonstrated that a single-cell transplantation analysis using cells that had the strongest dye-efflux activity ("Tip"-SP cells) with a phenotype of CD34<sup>+</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup>Lin<sup>-</sup>

(CD34<sup>+</sup>KSL<sup>+</sup>SP) exhibited nearly complete (~96%) level of hematopoietic engraftment activity.<sup>15</sup> To first examine the contribution of BM-derived HSCs to the regeneration of cardiomyocytes after MI, we transplanted either whole BM cells (w-BM) or single CD34<sup>+</sup>KSL<sup>+</sup>SP cells from transgenic EGFP mice into irradiated mice, induced MI, treated the mice with G-CSF daily for 10 days to mobilize the BM cells to the heart, and then examined the hearts using histologic methods (Figure 1A). Eight weeks after transplantation, the mean percentages of EGFP<sup>+</sup> cells among the peripheral blood nucleated cells of the mice transplanted with w-BM and that of the mice transplanted with single CD34<sup>+</sup>KSL<sup>+</sup>SP cells were 87%  $\pm$  3% (*n* = 10) and 63%  $\pm$  10% (*n* = 10), respectively. The peripheral blood nucleated cell counts (NCCs), measured 24 hours after the last injection of G-CSF, were significantly higher in the G-CSF-treated (G-CSF[+]) mice than in saline-injected (G-CSF[-]) mice (Figure 1B). No significant difference in the NCC of G-CSF(+) mice was observed between the w-BM group and the CD34<sup>+</sup>KSL<sup>+</sup>SP group.

A histologic analysis performed 8 weeks after the MI showed a markedly higher degree of EGFP<sup>+</sup> cell infiltration into the infarcted area in G-CSF(+) mice than in G-CSF(-) mice (Figure 1C-F; Table 1). Among the G-CSF(+) mice, a greater degree of infiltration was seen in the w-BM group than in the CD34<sup>+</sup>KSL<sup>+</sup>SP group. Nonspecific autofluorescence was distinguished as reported previously.<sup>21</sup> As a control, the same experiment was performed using wild C57BL/6 mice-derived BM cells as donor cells for BM transplantation. Figure 1G shows that no green fluorescence was observed in the infarct area of control mice, thus, G-CSF treatment-induced augmentation of green fluorescence was not due to nonspecific fluorescence. The presence of the EGFP gene in the infarcted area also was confirmed by PCR analysis (data not shown).

In the w-BM group, some of the EGFP<sup>+</sup> cells in the ischemic area expressed actinin and showed striations, suggesting that they

**Table 1. Quantitative analysis of EGFP<sup>+</sup> cells in infarcted area**

Mice	EGFP <sup>+</sup> cells	
	Total*	Actinin <sup>+</sup>
w-BM G-CSF (-) (n = 5)	8841 (1478, 1757, 1515, 2348, 1743)	65 (18, 15, 9, 9, 14)
w-BM G-CSF (+) (n = 5)	119802 (25006, 19950, 28303, 17633, 28910)	5403 (1016, 982, 1328, 921, 1156)
CD34 <sup>-</sup> KSL-SP G-CSF (-) (n = 5)	1224 (254, 183, 210, 300, 277)	0 (0, 0, 0, 0, 0)
CD34 <sup>-</sup> KSL-SP G-CSF (+) (n = 5)	41779 (8654, 7005, 9217, 9034, 7869)	3 (0, 0, 1, 0, 2)

Whole bone marrow cells (w-BM) or single CD34<sup>-</sup> c-kit<sup>+</sup> Sca-1<sup>+</sup> lineage<sup>-</sup> side population (CD34<sup>-</sup> KSL-SP) cells from the BM of EGFP mice were transplanted into lethally irradiated C57BL/6 mice. Myocardial infarction (MI) was induced 8 weeks later. Commencing 24 hours after induction of MI, a saline solution (G-CSF (-)) or G-CSF (G-CSF (+)) was injected into the mice for 10 consecutive days. Eight weeks after induction of MI, the mice were sacrificed, and the hearts were subjected to immunohistological analysis. The number of EGFP<sup>+</sup> and EGFP<sup>+</sup> actinin<sup>+</sup> cells in the infarcted area was scored using 100 specimens per group of mice (20 samples from the mid-left ventricle per mouse).

\*Total numbers of EGFP<sup>+</sup> cells in the infarcted area.

were cardiomyocytes (Figure 1H-I; Table 1). Some EGFP<sup>+</sup> cells also expressed sarcomeric myosin or cardiac troponin I (data not shown). Very few EGFP<sup>+</sup> actinin<sup>+</sup> cells were seen in the 2 CD34<sup>-</sup> KSL-SP groups (Figure 1J; Table 1), which was consistent with previous reports.<sup>12,13</sup> The marked increase in EGFP<sup>+</sup> actinin<sup>+</sup> cells in the w-BM groups cannot be explained simply by the superiority of hematopoietic chimerism in the w-BM group (87% ± 3% vs 63% ± 10%) if regenerated cardiomyocytes were derived from HSCs. Collectively, these results suggest that the major population of cells mobilized from the BM and active in the regeneration of cardiomyocytes was nonhematopoietic in origin.

#### HSCs have little potency to differentiate into a mesenchymal lineage

Next, we compared the nonhematopoietic BM cell populations of mice transplanted with single CD34<sup>-</sup> KSL-SP cells or w-BM cells from transgenic EGFP mice. We cultured the BM cells from both groups of mice (n = 5 per group) with a high chimerism (> 80%) of EGFP<sup>+</sup> donor cells using fibronectin-coated, 96-well plates. After 14 days of culture, the adherent cells were stained with anti-GFP and anti-CD45, and the frequency of donor-derived nonhematopoietic cells was determined using 3 specimens from each mouse BM. A significant proportion of the adherent cells from the BM of the w-BM group was positive for EGFP (EGFP<sup>+</sup>CD45<sup>-</sup> mononuclear cells = 22.38%).

On the other hand, most of the adherent cells from the BM of the CD34<sup>-</sup> KSL-SP group were negative for EGFP (EGFP<sup>+</sup>CD45<sup>-</sup> mononuclear cells = 0.8%, *P* < .001). These adherent cells were further cultured in either adipocyte- or osteocyte-inducing conditions for another 14 days; 200 adipocytes and 200 osteocytes among the adherent cells of the CD34<sup>-</sup> KSL-SP group were counted, but no EGFP<sup>+</sup> cells were found (data not shown). These results suggest that purified HSCs have very little potency to differentiate into a mesenchymal lineage, while MSCs are feasible candidates for the source of regenerated cardiomyocytes among nonhematopoietic BM cells.<sup>6-10</sup> Based on these results, we focused on the contribution of MSCs in w-BM in the regeneration of cardiomyocytes after MI.

#### Clonal MSC progeny contribute to the regeneration of cardiomyocytes after cytokine-enhanced post-MI repair

Unlike HSCs, MSCs are difficult to purify prospectively according to surface phenotypes. Therefore, to assess the contribution of BM-derived MSCs to the regeneration of

cardiomyocytes after MI, we designed a series of experiments using cardiomyogenic (CMG) cells,<sup>6</sup> a clonally isolated cell line of MSCs (Figures 2A and 3A). CMG cells can differentiate into spontaneously beating cardiomyocytes in vitro after exposure to 5-azacytidine. Since cultured MSCs are known to lose their BM homing ability,<sup>22</sup> we first confirmed that the CMG cells, tagged by transfection with the MSCV-EGFP retrovirus vector,<sup>16</sup> could be engrafted into the recipients' BM (n = 5) by intra-BM injection,<sup>20</sup> rather than intravenous injection, and differentiate into multiple lineages in a manner similar to that of authentic MSCs (Figure 2A). We detected EGFP<sup>+</sup> cells in the BM, most of which had differentiated into adipocytes and osteocytes (Figure 2B-F), and we also found a small number of cells residing among the stromal cells (Figure 2D, F, G). We detected EGFP<sup>+</sup> cells in several other organs, although in very low numbers (4, 5, 0, 0, 0, and 0 EGFP<sup>+</sup> cells per 100 specimens [20 specimens per mouse] in the liver, kidney, heart, brain, skeletal muscle, and spleen, respectively) (Figure 2H-I).

To detect cardiomyocytes derived from CMG cells with a higher sensitivity and specificity, cells transfected with a pMLC2v-EGFP plasmid encoding EGFP driven by the myosin light chain promoter (CMG-ME cells) were prepared by selection with G418 and then treated with 5-azacytidine. Ten days after treatment, 6% of the cells were positive for EGFP and negative for actinin but expressed GATA4 (Figure 3B-C). After 3 weeks, most of the EGFP<sup>+</sup> cells became actinin positive and developed a rod-like appearance (Figure 3D-E); at 4 weeks, they exhibited spontaneous beating. These findings demonstrate that the CMG-ME cells that were committed to cardiomyocytes could be detected by the expression of EGFP in this system.

Uncommitted CMG-ME cells then were transplanted directly into the BM of lethally irradiated C3H/He (C3H) mice, MI was induced, and the mice were treated with G-CSF (Figure 3A). Eight weeks after induction of MI, the mice (n = 5) were killed, and PCR analysis confirmed the engraftment of CMG-ME cells in the BM of all recipients (Figure 3F). The myocardium of the 5 mice was analyzed using immunofluorescence, and a total of 1034 EGFP<sup>+</sup> actinin<sup>+</sup> cells in 100 samples (20 samples per mouse) were observed. The number of EGFP<sup>+</sup> actinin<sup>+</sup> cells from each group of 20 samples from each mouse was 253, 92, 135, 327, and 227. This result indicated that CMG-ME cells mobilized from the BM contributed to the regeneration of the myocardium after MI (Figure 3G-H). The presence of the EGFP gene in the infarcted area also was confirmed by PCR analysis (data not shown). Although the number of detected cells was

lower than that observed in mice transplanted with whole BM, CMG-derived cardiomyocytes were clearly visible in the ischemic myocardium. We did not detect EGFP<sup>+</sup> cells in any other organs such as liver, kidney, skeletal muscle, and BM (data not shown). These results suggested that the myosin light chain promoter restricted EGFP expression to cardiac tissue.

## Discussion

Although BM-derived cells were reported to differentiate into cardiomyocytes after MI,<sup>3-5</sup> the identity of specific cell types involved has not been determined. There are 2 possibilities: first, BM-derived HSCs trans-differentiated into cardiomyocytes. Second, other cell types, such as MSCs, regenerated cardiomyocytes. To address this issue, we performed 2 independent clonal analyses to test which cell types contribute to cardiomyocyte regeneration. The results suggest that MSCs, but not HSCs, in BM play a major role in the regeneration of myocardial tissue after MI.

Purified HSCs and their progeny rarely contributed to the regeneration of cardiomyocytes, even with G-CSF treatment after single-cell transplantation following MI induction. On the other hand, w-BM transplantation resulted in marked increase of the donor-derived cardiomyocytes after MI and subsequent treatment with G-CSF. The results suggest that the repopulation of cardiomyocytes was achieved by nonhematopoietic cells in BM rather than HSCs and their progeny. In addition, we did not detect any EGFP<sup>+</sup> MSCs in the CD34<sup>+</sup>KSL-SP group, while a precise analysis indicated that around 90% of the MSCs, assayed as fibroblastic colony-forming units (CFU-Fs), were derived from donor mice in the w-BM transplantation model when the recipient mice received more than 7 Gy of total body irradiation.<sup>22</sup> These results led us to focus on the nonhematopoietic population of MSCs in BM as a candidate for the donor-derived cardiomyocytes.

While HSCs can be purified prospectively according to their surface phenotypes, such purification is difficult to achieve for MSCs. Therefore, we used a cell line of MSCs to clonally identify the origin of BM-derived cardiomyocytes after MI. CMG cells, previously established from MSCs in the BM of C3H mice, can differentiate into spontaneously beating cells with a fetal ventricular cardiomyocyte phenotype *in vitro* after exposure to 5-azacytidine.<sup>6</sup> Since only a small fraction of these cells differentiate into cardiomyocytes and CMG cells were sporadically found in other organs after intra-BM injection (Figure 2H-I), we labeled the CMG cells to improve specific detection. Although the number of detected cells was smaller than that observed in mice that received transplants of whole BM, CMG-derived cardiomyocytes were clearly detected in the ischemic myocardium; these results are the first evidence that CMG cells can differentiate into cardiomyocytes in the cardiac niche without 5-azacytidine treatment. Most of the CMG cells that were transplanted directly into the BM differenti-

ated into bone tissue (Figure 2D-E). This is most likely because insertion of the needle into the BM cavity stimulated osteogenesis<sup>23</sup>; such activity may account for the small number of CMG-derived cardiomyocytes. Systemic intravenous delivery of CMG may obviate this loss of CMG cells to osteogenesis and improve cardiomyocyte differentiation.<sup>24</sup> However, the intravenous delivery of cultured MSCs to BM is limited by either a defect in the homing ability of the cells<sup>22</sup> or the entrapment of the cells in the lungs.<sup>25</sup> A limitation of these experiments is that the CMG cell population isolated *in vitro* might not be a natural cell lineage. An important future experiment is to determine whether authentic MSCs purified from the BM can regenerate cardiomyocytes using the protocol outlined in Figure 3A.

Human MSCs were reported to mobilize into the systemic circulation as a result of G-CSF treatment.<sup>26</sup> The present study also shows that G-CSF treatment induces mobilization of MSCs from the BM into the systemic circulation leading to engraftment at peripheral organ sites. Matrix metalloproteinases and elastase, released mainly from granulocytes, are thought to be the major mediators of HSC mobilization induced by G-CSF.<sup>27</sup> The role of these factors in mediating the mobilization of MSC is unknown and provides an important avenue for future research. As G-CSF treatment following MI is reported to improve cardiac function and survival,<sup>5</sup> MSC mobilization may provide a new strategy for regenerative treatment. Furthermore, the data presented here support the concept that one function of MSC in BM is to contribute to the repair of injured tissue by being mobilizing to the site of injury and differentiating according to the niche.<sup>25,26,28,29</sup>

While this study focused on cardiomyocytes derived from BM, the reported improvement of infarcted tissue by G-CSF cannot be solely attributed to these cells. Most of the donor-derived cells in the infarcted area were actinin-negative fibroblastic cells and endothelial cells derived from HSCs. It is possible that these cells are *f*-macrophages<sup>30</sup> or are generated by fusion.<sup>11</sup> The role of these cells in remodeling is now under investigation. Neovascularization by endothelial progenitor cells (EPCs) mobilized from BM by G-CSF is also reported to contribute to an improved outcome.<sup>27</sup> In addition, EPC are reported to transdifferentiate into cardiomyocytes.<sup>31</sup> Adult cardiac stem cells in the heart<sup>32,33</sup> and multipotent adult progenitor cells<sup>34</sup> also may be affected by G-CSF. Therefore, the clinical significance of cardiomyocyte regeneration by BM-derived cells must take into account the role of different populations of cells in the regeneration process. The outcome of this research should be the development of novel cytokine and cell-based therapies for MI.

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## Down-regulation of p27<sup>Kip1</sup> Promotes Cell Proliferation of Rat Neonatal Cardiomyocytes Induced by Nuclear Expression of Cyclin D1 and CDK4

EVIDENCE FOR IMPAIRED Skp2-DEPENDENT DEGRADATION OF p27 IN TERMINAL DIFFERENTIATION\*

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Mammalian cardiomyocytes lose their capacity to proliferate during terminal differentiation. We have previously reported that the expression of nuclear localization signal-tagged cyclin D1 (D1NLS) and its partner cyclin-dependent kinase 4 (CDK4) induces proliferation of rat neonatal cardiomyocytes. Here we show that the D1NLS/CDK4 cells, after their entry into the cell cycle, accumulated cyclin-dependent kinase inhibitor p27 in the nuclei and decreased the cyclin-dependent kinase 2 (CDK2) activity, leading to early cell cycle arrest. Biochemical analysis demonstrated that Skp2-dependent p27 ubiquitylation was remarkably suppressed in cardiomyocytes, whereas Skp2, a component of Skp1-Cullin-F-box protein ubiquitin ligase, was more actively ubiquitylated compared with proliferating rat fibroblasts. Specific degradation of p27 by co-expressing Skp2 or p27 small interfering RNA caused an increase of CDK2 activity and overrode the limited cell cycle. These data altogether indicate that the impaired Skp2-dependent p27 degradation is causally related to the loss of proliferation in cardiomyocytes. This provides a novel insight in understanding the molecular mechanism by which mammalian cardiomyocytes cease to proliferate during terminal differentiation.

Terminal differentiation of highly specialized cells such as neural cells, cardiomyocytes, and pancreatic  $\beta$  cells is an important biological process that ensures their proper mass and function in higher organism. This process is accompanied with cell cycle arrest that is a hallmark of terminally differentiated

cells. However, it is poorly understood how these cells cease to proliferate or more specifically whether their cell cycle can be reactivated. Mammalian cardiomyocytes irreversibly withdraw from the cell cycle soon after birth and lose the cell proliferative activity (1–4), although it has been shown that the adult heart contains a small population of cardiac progenitors that retain the proliferation capacity (5, 6). It is recently demonstrated that cardiac progenitor cells from embryonic or bone marrow stem cells are differentiated into functional cardiomyocytes when grafted in the damaged cardiac tissue (5). In addition to these, several approaches have been made to reactivate the cell cycle of terminally differentiated cardiomyocytes. For instance, adenoviral delivery of E2F allowed these cells to enter the cell cycle. However, this could not support cell proliferation but caused apoptotic cell death (7–9). This failure of cell proliferation may be largely because of our limited knowledge of the molecular events of cell cycle arrest underlying terminal differentiation of cardiomyocytes.

We have recently reported that the nuclear expression of cyclin D1 (D1NLS)<sup>1</sup>/cyclin-dependent kinase (CDK) 4 promoted the proliferation of rat neonatal cardiomyocytes in culture as well as adult rat heart *in situ* (10). Furthermore, there observed no induction of apoptotic cell death. This argues that the nuclear import of cyclin D1 is impaired in adult cardiac cells and these cells could be allowed to enter the cell cycle once this step is bypassed. This may illustrate one of the molecular features of adult cardiomyocytes that cease to grow after terminal differentiation. However, our knowledge is still limited regarding the molecular mechanism by which the proliferation of adult cardiomyocytes is suppressed or how it is reactivated. It is also unknown whether there is a cell cycle barrier other than the nuclear import of cyclin D1.

Progression of the mammalian cell cycle is regulated with a combination of positive and negative regulators (11). It is activated by a family of cyclins and CDKs. During the G<sub>1</sub> phase, cyclin D1 and other D-type cyclins accumulate in nuclei and assemble with their catalytic partners, CDK4 and CDK6. For example, the cyclin D1-CDK4 complex phosphorylates and in-

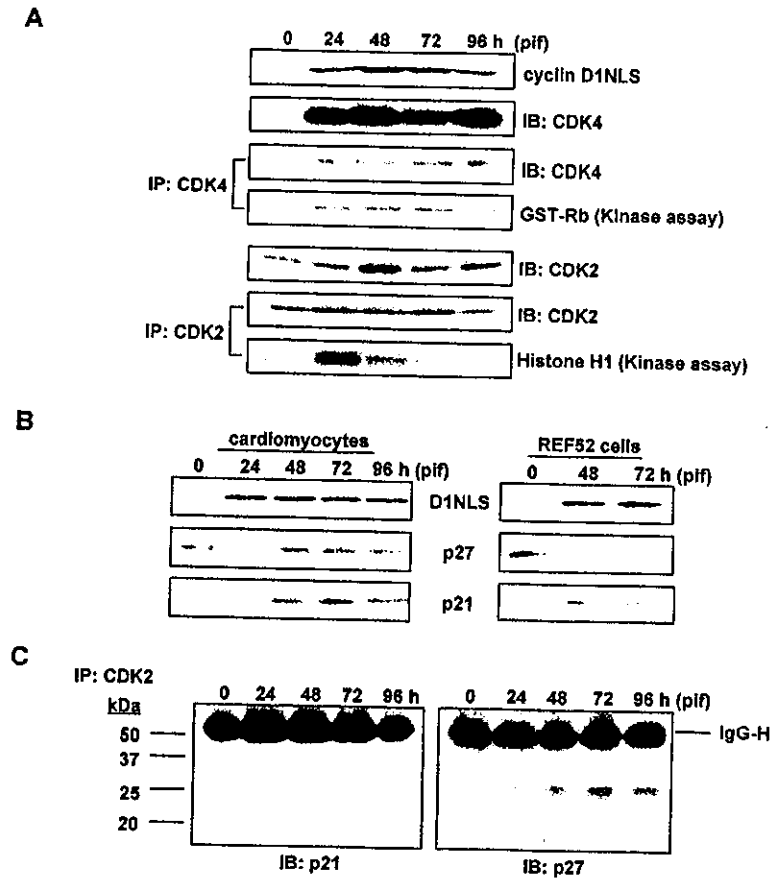
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<sup>1</sup> The abbreviations used are: D1NLS, nuclear localization signal-tagged cyclin D1; CDK, cyclin-dependent kinase; SCF, Skp1/Cul1/F-box; siRNA, small interfering RNA; APC/Cdh1, anaphase-promoting complex/cyclosome and its activator Cdh1.

**FIG. 1.** CDK2 activity was suppressed and p27 accumulated in cardiomyocytes after cell cycle entry by D1NLS/CDK4. Rat neonatal cardiomyocytes were infected with a combination of AdD1NLS and AdCDK4 as described under "Experimental Procedures." **A**, at each time indicated, cell extracts were immunoprecipitated using anti-CDK4 and anti-CDK2 antibodies, and assayed for CDK4 and CDK2 activity, respectively. In **B**, the cell extract from the D1NLS/CDK4-infected cardiomyocytes or REF52 cells was subjected to Western blot and assayed for CDK inhibitors, p27 and p21. **C**, extracts of the D1NLS/CDK4 cardiomyocytes were immunoprecipitated with anti-CDK2 antibody and analyzed for p21 and p27 protein by Western blot analysis using anti-IgG heavy chain-specific antibody as the secondary antibody. **IB**, immunoblot.



activates the retinoblastoma protein (Rb) and sequesters CDK inhibitors, thereby promoting cell cycle progression through the  $G_1$  to S phase. On the other hand, the CDK inhibitors, such as CIP/KIP family proteins, negatively regulate progression of the cell cycle by inhibiting the activity of cyclin-CDK complexes. The CIP/KIP family proteins, including p21 and p27, suppress the activities of cyclin A/CDK2 and cyclin E/CDK2, respectively, and mediate the exit from the cell cycle.

The level of p27 increases in quiescent non-proliferating cells and decreases on entry into the cell cycle and is controlled by the rate of degradation in both cytoplasm and nuclei. The degradation of p27 in cytoplasm is associated with the transition of cells from the  $G_0$  to  $G_1$  phase, whereas nuclear degradation occurs during S and  $G_2$  phases. In the latter, p27 is phosphorylated at threonine 187 and recruited to the F-box protein Skp2 of the SCF<sup>Skp2</sup> complex. This is followed by multiple ubiquitylation and degradation through the 26 S proteasome. Among this cascade reaction, the recognition of p27 by Skp2 is specific and plays a central role in p27 degradation. Therefore, p27 degradation is essential for cells to undergo cell cycle progression (12–19). In the developing heart, p27 is reported to accumulate as it loses the cell growth capacity in the late stage of embryo and after birth (20). Furthermore, mice deficient for p27 sustained cell growth capacity after birth (21). These findings may suggest a role of p27 in the terminal differentiation of cardiomyocytes.

In the present study, we examined the cell cycle progression of rat neonatal cardiomyocytes that were forced to enter the cell cycle by nuclear expression of D1NLS and its partner CDK4 (10). It was shown that D1NLS/CDK4 cardiac cells ceased to proliferate after only one or two cell cycles and accumulated the CDK inhibitor p27 in the nuclei. The *in vitro* p27 ubiquitylation was remarkably suppressed in cardiomyocytes, whereas that of

Skp2 was up-regulated. We further showed that knockdown of p27 or overexpression of Skp2 specifically degraded p27 and accelerated proliferation of D1NLS/CDK4 cardiomyocytes.

#### EXPERIMENTAL PROCEDURES

**Reagents**—Antibodies used in this study were as follow: mouse monoclonal anti-cyclin D1 (Ab-3, Oncogene Science), anti-sarcomeric actin (M0874, DAKO), anti-proliferating cell nuclear antigen (sc-056), and rabbit polyclonal anti-p21 (sc-6246), anti-p27 (sc-528), anti-Skp2 p45 (sc-7164), anti-CDK4 (sc-260), and anti-CDK2 (sc-163) from Santa Cruz Biotechnology, Santa Cruz, CA. Other biochemicals used were reagent grade.

**Cell Culture**—Rat neonatal cardiomyocytes were isolated and cultured as previously described (10, 22). Heart ventricles were isolated from 3-day-old postnatal Sprague-Dawley rats, trisected, and then digested 4 times with 1 mg/ml collagenase type II (Worthington) in Ads buffer (20 mM HEPES-KOH, pH 7.35, 116 mM NaCl, 5.4 mM KCl, 1 mM  $\text{NaH}_2\text{PO}_4$ , 0.8 mM  $\text{MgSO}_4$ , and 5 mM glucose) at 37 °C for 20 min. The dispersed cells were washed once by Ads buffer and then purified by centrifugation through a discontinuous Percoll gradient of 1.050, 1.060, and 1.082 g/ml, respectively. The cardiac cells at the interface between 1.060 and 1.082 g/ml were collected and plated on a 60-mm dish ( $2-3 \times 10^6$  cells) or 25-mm collagen-coated coverslips ( $2-3 \times 10^5$  cells) in minimum essential medium supplemented with 5% calf serum, 100 units/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Cardiomyocytes were cultured at 37 °C for 24 h in humidified air with 5% carbon dioxide, after which the medium was changed to serum-free minimal essential medium and further incubated for another 24 h. The purity of cardiomyocytes was determined by immunostaining with anti-sarcomeric actin antibody and over 95% of cells were positive for sarcomeric actin. Rat fibroblast REF52 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal calf serum as reported (10). Use of neonatal rats in this study was approved by the Institutional Animal Care and Use Committee of our university.

**Adenoviruses and Their Gene Transfer**—Adenoviruses for expressing nuclear localization signal-tagged cyclin D1 (Ad-D1NLS), CDK4 (Ad-CDK4), and Skp2 (Ad-Skp2) were as described (10, 23). Viruses were propagated in 293 cells, and the virus stocks were prepared as described (10, 22). Virus titer was determined by an indirect immunofluorescent

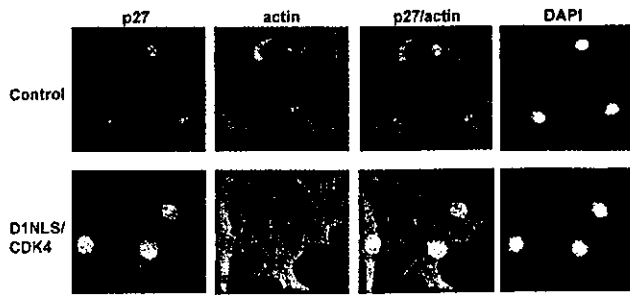


FIG. 2. p27 accumulated in the nuclei of cardiomyocytes after cell cycle entry by D1NLS/CDK4. Control untreated and D1NLS/CDK4 cells at 48 h post-infection were fixed and stained for p27 (green) and sarcomeric actin (red) with anti-p27 and anti-sarcomeric actin antibodies, respectively, as described under "Experimental Procedures." Cell nuclei (white) were also stained with 4',6-diamidino-2-phenylindole (DAPI).

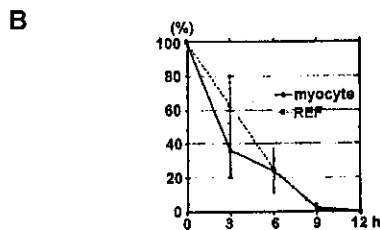
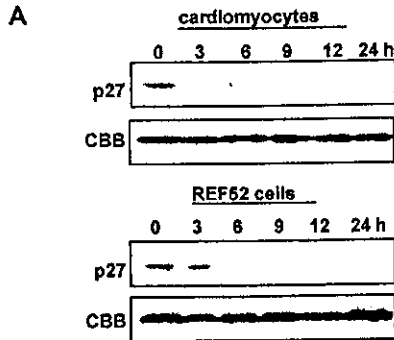


FIG. 3. Serum-induced degradation of p27 in cardiomyocytes was not different from REF52 cells. A, cardiomyocytes and REF52 cells were serum-starved for 48 h and then treated with serum (10% fetal bovine serum). At each time after adding the serum, cell extract (20  $\mu$ g) was assayed for p27 by Western blot analysis. Lower panels show the Coomassie Brilliant Blue (CBB) staining as the control for protein loading. In B, the amount of p27 protein was quantitated by densitometric measurement and expressed as percent of that at time 0. Results represent the mean  $\pm$  S.E. three independent experiments.

assay using anti-72K antibody. Cardiomyocytes were infected at the indicated multiplicity of infection in serum-free minimal essential medium for 60 min with brief agitation every 15 min, and then the medium was replaced with culture medium.

**Construction of Adenovirus Vector Encoding p27 siRNA**—Oligonucleotides for expressing stem-loop RNAs that target three different regions of rat p27 were ligated with human U6 promoter and the resulting transcription unit of the U6 promoter was subcloned into the SwaI site of the E1-deleted region of cassette cosmid vector pAxcw (Adenovirus Expression Vector Kit, TaKaRa BIO). Adenoviral vector was then prepared as in the protocol from Takara. Oligonucleotide sequences used were: 5'-CACCGGTAGGAGGTTCTTCTCAACGTGTGCTGTCCGTTGAAGAAGAATCTTCTGCCTTTTT-3' and 5'-GCATAAAAGGCAGAAAGATTCTTCTTCAACGGACAGCACACGTTGAAGAA-GAACCTCCTACC-3' (#1) for the p27 amino acid region from 830 to 848, 5'-CACCAAGTGAAGTGGAGTTTCGAACGTTGCTGTCCGTTTCGAAATCCACTTGGCGTTTTTT-3' and 5'-GCATAAAAAAGCGCAA-GTGGAAATTCGAACGGACAGCACACGTTGAAACTCCACTTACA-CT-3' (#4) from 532 to 550, or 5'-CACCGTGGGAGTGTTAATGGGA-

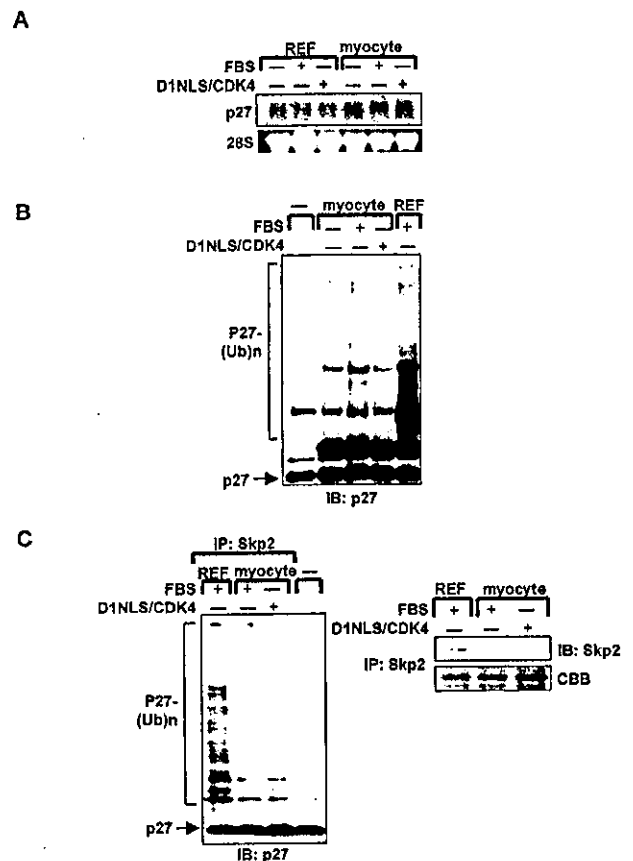


FIG. 4. Cardiomyocytes showed the impaired Skp2-dependent degradation of p27. A, total RNA (10  $\mu$ g) from the control, serum, or D1NLS/CDK4-treated cardiomyocytes and REF52 cells was analyzed for p27 mRNA by Northern blot analysis as described under "Experimental Procedures." B, equal amounts of cell extract (50  $\mu$ g of protein) treated as in A were assayed for the *in vitro* p27 ubiquitylation activity as described under "Experimental Procedures." C, cell extracts (50  $\mu$ g of protein) from cells treated as in B were immunoprecipitated with anti-Skp2 antibody and the resulting complexes were assayed for the Skp2 protein (right panel) and the *in vitro* p27 ubiquitylation activity (left panel), respectively. CBB, Coomassie Brilliant Blue; FBS, fetal bovine serum.

ACGTGTGCTGTCCGTTCCCGTTAGACTCTCACTTTTT-3' and 5'-GCATAAAAAAGTGAGAGTGTCTAACGGGAACCGACAGCACACGTTCCCATTAACACTCCCAC-3' (#6) from 372 to 390, respectively. Adenovirus vector encoding siRNA for hepatitis C viral protein (siRNA331) was as described (24) and employed as control.

**Whole Cell Extract Preparation and Western Blot Analysis**—Cardiomyocytes ( $2 \times 10^6$  cells) treated as indicated were washed in phosphate-buffered saline, resuspended in 50  $\mu$ l of lysis buffer (50 mM Hepes-KOH, pH 7.6, 150 mM NaCl, 1% Tween 20, 10 mM glycerophosphate, 2.5 mM EGTA, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml each leupeptin and aprotinin, 0.1 mM sodium vanadate, 1 mM NaF, and 10% glycerol), and incubated on ice for 30 min. After centrifugation at 12,000 rpm for 10 min, whole cell extract was obtained. The amount of protein was measured with the Lowry method using bovine serum albumin as standard. Whole cell extracts (usually 20  $\mu$ g of protein) were separated by SDS-PAGE, transferred onto an Immobilon-P membrane (Millipore), and subjected to Western blot using the protocol of the ECL kit (Amersham Biosciences) as described (20).

**Immunoprecipitation and Kinase Assays**—Whole cell extracts (50  $\mu$ g of protein) were immunoprecipitated with anti-CDK4 (0.4  $\mu$ g) or anti-CDK2 antibody (0.4  $\mu$ g) followed by Western blot with the indicated antibody, and bands were detected using monoclonal anti-rabbit IgG ( $\gamma$ -chain specific) peroxidase-conjugated secondary antibody (Sigma). To assay CDK4 and CDK2 kinase activity, the immunoprecipitated complexes were incubated with glutathione S-transferase-Rb and histone H1, respectively, as substrates as described (10, 22).

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated by the acid-guanidium method using Isogen (Nippon Gene, Japan),

fractionated on a formaldehyde/agarose gel, transferred to a Hybond-N membrane, and hybridized to random-primed cDNA probes for the mouse *p27* and *skp2* genes as described (22). The membrane was exposed and analyzed with a Bas 2500 Bioimage analyzer (Fujifilm Co., Tokyo, Japan). DNA fragments (100 ng) for these cDNAs were radiolabeled with 50  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]dCTP (6000 Ci/mmol, Amersham Biosciences) using a random primer labeling kit from Takara and used as probe for RNA preparation and Northern blot analysis.

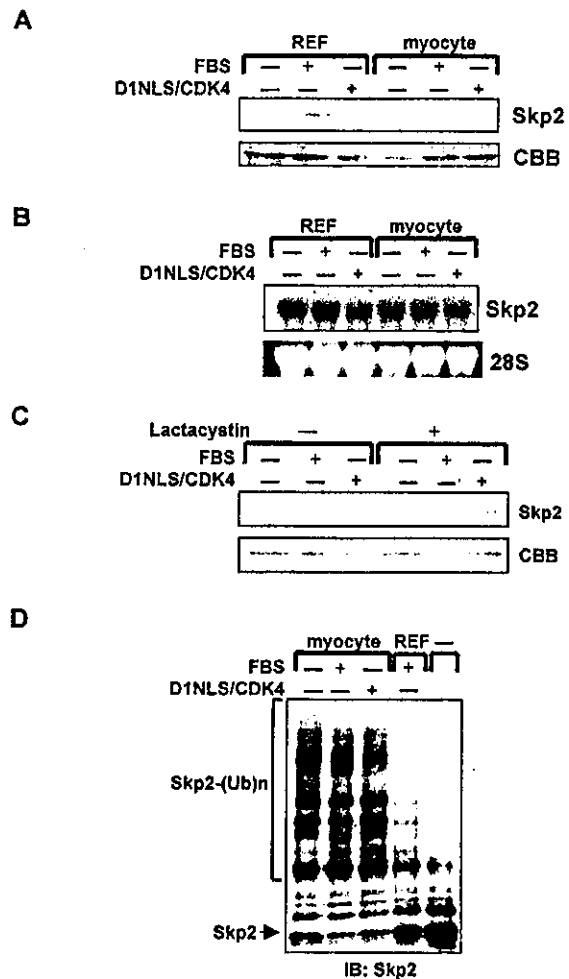
**In Vitro Ubiquitylation Assay**—Cells were treated in lysis buffer (20 mM Hepes-KOH, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml each leupeptin and aprotinin, and 25% glycerol) on ice for 30 min, and cell debris was removed by centrifugation at 15,000 rpm for 20 min. Cell extract (50  $\mu$ g of protein) was incubated with substrates in 15  $\mu$ l of buffer of 40 mM Hepes-NaOH (pH 7.9), 60 mM potassium acetate, 2 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 10% glycerol, and 1.5 mM ATP containing 50 ng of Uba1, 100 ng of UbcH5A, 3  $\mu$ g of glutathione *S*-transferase-ubiquitin. After incubating at 26 °C for 30 min, reaction products were separated by SDS-PAGE and analyzed by Western blot with the indicated antibody. As substrates, FLAG-p27 or -Skp2 were prepared using 1  $\mu$ g of pcDNA3Flag-p27 or pcDNA3Flag-skp2 (23) and 25  $\mu$ l of rabbit reticulocyte lysate (TnT kit from Promega) according to its protocol. The *in vitro* p27 ubiquitylation activity was also assayed after immunoprecipitation of cell extracts with 0.4  $\mu$ g of anti-Skp2 antibody.

**Immunocytological Study and Cell Cycle Analysis**—To examine the expression of p27 in cardiomyocytes, cells grown on glass coverslips were fixed in 70% ethanol, double-stained with anti-p27 and anti-sarcomeric actin antibodies, and visualized using fluorescent tyramide reagent according to the manufacturer's protocols (TSA-direct NEL-701, PerkinElmer Life Sciences, Inc.). Confocal images were obtained using the laser-scanning confocal image system (LSM510, Zeiss). For cell cycle analysis, cells were stained with anti-sarcomeric actin and anti-mouse antibodies conjugated with fluorescein isothiocyanate (23799, Polysciences), followed by treatment with propidium iodide (50  $\mu$ g/ml) and RNase A (500  $\mu$ g/ml). The DNA content of cells positive for sarcomeric actin was analyzed using a laser scanning cytometer (LSC 101, Olympus).

**Statistical Analysis**—Quantitative data were expressed as mean  $\pm$  S.E. Statistical analysis was performed with the Student's *t* test.

## RESULTS

**CDK2 Activity Was Suppressed and the Cell Cycle Inhibitor p27 Accumulated in the Nuclei of Cardiomyocytes after Cell Cycle Entry by D1NLS/CDK4**—We have previously shown that D1NLS/CDK4 expression allowed the cardiomyocytes to enter the cell cycle (10). However, the proliferation of these cells was limited to approximately one or two cycles. To understand the mechanism by which the cells cease to proliferate, we first assayed the activity of G<sub>1</sub> cyclin kinase, CDK4 and CDK2. As shown in Fig. 1A, the CDK4 activity was up-regulated at 24 h post-infection of D1NLS/CDK4, and remained active for at least 96 h. The CDK2 activity was also activated at 24 h, but suppressed again at 48 h, whereas the amount of CDK2 was not significantly affected. We, therefore, examined the expression of CDK2 inhibitors, p27 and p21. Fig 1B showed that p27 was accumulated in the serum-starved non-proliferating cardiomyocytes. When these cells were allowed to enter the cell cycle by D1NLS/CDK4, the p27 protein was rapidly degraded within 24 h. However, it accumulated again at 48 h, at which time CDK2 was inhibited (Fig. 1A). In REF52 fibroblasts, p27 was also rapidly degraded after entering the cell cycle by D1NLS/CDK4, but no accumulation was observed for at least 72 h. The expression of p21 was significantly suppressed in serum-starved cardiomyocytes and REF52 cells, but up-regulated at 48 h after the cell cycle entry. In Fig. 1C, immunoprecipitation of the cardiomyocyte extract was performed using anti-CDK2 antibody. Results showed that p27 was little associated with CDK2 in the serum-starved cells, but the higher amount of p27 was detected in the CDK2 complex at 48–96 h. In contrast, appreciable amounts of p21 were not detected in the CDK2 complex. It was further noted that the expression of p57 and p53, other cell cycle inhibitors, was not significantly altered in D1NLS/CDK4 cells of this study (data not shown).



**FIG. 5. Down-regulation of Skp2 was inversely correlated to the *in vitro* Skp2 ubiquitylation activity in cardiomyocytes.** A, cardiomyocytes and REF52 cells were treated with serum (10% FBS) or D1NLS/CDK4 for 48 h, and their cell extracts (20  $\mu$ g of protein) were assayed for Skp2 protein by Western blot analysis. B, cardiomyocytes and REF52 cells were treated as in A, and their total RNA (10  $\mu$ g) was analyzed for Skp2 mRNA by Northern blot. C, cells were treated as in A in the presence or absence of 20  $\mu$ M lactacystin, and their cell extracts were assayed for Skp2 protein by Western blot analysis. Lower panels show the Coomassie Brilliant Blue (CBB) staining as control for protein loading. D, cell extracts (50  $\mu$ g of protein) were assayed for the *in vitro* Skp2 ubiquitylation activity as described under "Experimental Procedures." IB, immunoblot.

Next, the immunocytological study of p27 expression was performed. As shown in Fig. 2, p27 accumulated significantly in the nuclei of D1NLS/CDK4 cells, whereas its expression was little detected in control cells. These data altogether indicated that the p27 protein was degraded after initiation of cell cycle, but was accumulated again in the nuclei during cell cycle progression of cardiomyocytes.

**Serum-induced Degradation of p27 in Cardiomyocytes Was Not Different from REF52 Cells**—The protein level of p27 is predominantly controlled by the rate of degradation through the proteasome-dependent pathway. This degradation is mediated by the Skp2-independent ubiquitin ligase in the cytoplasm at G<sub>0</sub> to G<sub>1</sub> transition and by the Skp2-dependent ligase in the nuclei at S to G<sub>2</sub> phase, respectively (12, 17–19). In Fig. 3A, p27 accumulated when the cardiomyocytes and REF52 cells were serum-starved. We stimulated these cells with serum and the degradation of p27 was determined by Western blot analysis. Results showed that p27 was degraded in both cell types with