planted cell numbers appear to have been relatively low in the ASTAMI trial. Indeed, cell number did not significantly affect LV function in our study (Fig 4). Importantly, our study results also suggests that PBMNCs, which were even not culture-expanded, show great capability as a comparable cell source to BMCs.

In view of BMCs homing into the heart, the microenvironment (eg, niche) within the infarct tissue and the timing of cell delivery may be important for the incorporation of BMCs. Recent observations indicate that after intracoronary transfer only 1.3–2.6% of ^{18}F-FDG-labelled unselected BMMNCs were detected in the infarcted heart, whereas most cells homed into the liver and spleen within ≈1 h after intracoronary delivery[32] Therefore, the findings do not support the likelihood that progenitor cells home into jeopardized myocardium and transdifferentiate into cardiac myocytes capable of generating active force development in scar tissue, but rather suggest other potential mechanisms through angiogenesis and reduced apoptosis. Indeed, recent articles have shed light on the potential of BMCs to differentiate into hematopoietic and endothelial lineages able to secrete proangiogenic factors[33] rather than transdifferentiation into other cell lineages such as cardiac myocytes[34,35] These subsets of mature hematopoietic cells, either derived from bone marrow or peripheral blood, may cooperate with transplanted or resident cardiac and endothelial stem/progenitor cells to enhance their capacity for tissue repair through angiogenesis, anti-apoptosis, and myocyte proliferation after ischemic injury[36,37]

The most potential advantage of our method is its feasibility and safety in collecting PBMNCs from patients with depressed cardiac performance and bleeding tendency, by administration of enough antiplatelet agents, such as aspirin and ticlopidine. Although previous studies emphasize the feasibility and safety of BMCs aspiration, this maneuver is always accompanied by the risk of s serious bleeding accident from bone in patients receiving antiplatelet therapy. There are also a few other reports indicating a benefit of intracoronary infusion of granulocyte colony-stimulating factor (G-CSF) mobilized-PBMNCs for AMI[38–40] However, this procedure still involves several possible adverse effects of G-CSF, including serious thrombosis, bone pain, fever, and aggravation of in-stent restenosis[38–40] In contrast to those previous studies, we could easily collect ≈5×10^9 cells PBMNCs, avoiding contamination with neutrophils, within 2 h without any hemodynamic or bleeding problems. We could concentrate the collected PBMNCs to 10 ml by density gradient centrifugation aseptically through bag to bag, instead of by Ficoll gradient sedimentation methods. Our present data, therefore, show for the first time that intracoronary infusion of non-expanded PBMNCs alone can promote improvement of LV function without any bleeding accident or G-CSF-related serious adverse effects. Because we can easily obtain levels of ≈6×10^6 CD34$^+$ cells, which is higher than in either the BOOST or REPAIR-AMI trials, we never need another laboratory to expand the PBMNCs, giving substantial merit that this protocol can be easily accepted in any hospital worldwide.

A major limitation of our study is that evaluation of the present regeneration therapy was not randomized, double-blind, and controlled. Moreover, cardiac function was not assessed with state-of-the-art imaging modalities, such as magnetic resonance imaging (MRI), and LV angiography was used exclusively for the serial assessment of LV function. Although angiography is well suited to delineate regional contractile function for AMI by LAD occlusion, the use of MRI to assess global LV function would have more precisely depicted changes in the distorted geometry of the infarcted hearts. Although we choose contemporary controls, the control group does not reproduce the exact conditions of the cell therapy group to which the cells were transferred, including PBMNC collection and a placebo intracoronary injection. Therefore, the true benefit of cell transfer can not be fully appreciated and further research is needed to address these issues.

In conclusion, intracoronary infusion of PBMNCs in patients with AMI is associated with improved global LV contractile function; cell therapy preferentially improves LV function in patients with early reperfusion, but relatively depressed contractility after AMI, prevents end-diastolic and end-systolic LV volume expansion, and has not increased any adverse clinical events so far. Transplantation of PBMNCs might be an effective and novel therapeutic option for AMI, if cell transfer occurs expeditiously and in appropriate subjects. This less invasive and more feasible approach to collecting EPCs may be a novel therapeutic option for improving cardiac function after AMI.

Acknowledgments

This study was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology and from the Ministry of Health, Labor and Welfare in Japan.

References

1. Cleaver O, Melton DA. Endothelial signaling during development. *Nat Med* 2003; **9:** 661–668.
2. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275:** 964–967.
3. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* 1999; **18:** 3964–3972.
4. Asahara T, Kawamoto A. Endothelial progenitor cells for postnatal vasculogenesis. *Am J Physiol Cell Physiol* 2004; **287:** C572–C579.
5. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation* 2001; **104:**1046–1052.
6. Qin SL, Li TS, Takahashi M, Hamano K. In vitro assessment of the effect of interleukin-1beta on angiogenic potential of bone marrow cells. *Circ J* 2006; **70:** 1195–1199.
7. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, et al; Therapeutic Angiogenesis using Cell Transplantation (TACT) Study Investigators. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: A pilot study and a randomised controlled trial. *Lancet* 2002; **360:** 427–435.
8. Goodell MA, Jackson KA, Majka SM, Mi T, Wang H, Pocius J, et al. Stem cell plasticity in muscle and bone marrow. *Ann NY Acad Sci* 2001; **938:** 208–218.
9. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, et al. Chimerism of the transplanted heart. *N Engl J Med* 2002; **346:** 5–15.
10. Fukuda K. Progress in myocardial regeneration and cell transplantation. *Circ J* 2005; **69:** 1431–1446.
11. Leor J, Patterson M, Quinones MJ, Kedes LH, Kloner RA. Transplantation of fetal myocardial tissue into the infarcted myocardium of rat: A potential method for repair of infarcted myocardium? *Circulation* 1996; **94:** II-332–II-336.
12. Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. Skeletal myoblast transplantation for repair of myocardial necrosis. *J Clin Invest* 1996; **98:** 2512–2523.
13. Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, et al. Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. *Nat Med* 1998; **4:** 929–933.

14. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. *Nature* 2001; **410:** 701–705.
15. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci USA* 2001; **98:** 10344–10349.
16. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet* 2003; **361:** 45–46.
17. Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, Lau CP. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet* 2003; **361:** 47–49.
18. Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* 2002; **106:** 1913–1918.
19. Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation* 2002; **106:** 3009–3017.
20. Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, et al. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: The BOOST randomised controlled clinical trial. *Lancet* 2004; **364:** 141–148.
21. Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, et al; REPAIR-AMI Investigators. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med* 2006; **355:** 1210–1221.
22. Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Egeland T, et al. Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N Engl J Med* 2006; **355:** 1199–1209.
23. Janssens S, Dubois C, Bogaert J, Theunissen K, Deroose C, Desmet W, et al. Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: Double-blind, randomised controlled trial. *Lancet* 2006; **367:** 113–121.
24. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Amano K, Iba O, et al. Improvement of collateral perfusion and regional function by implantation of peripheral blood mononuclear cells into ischemic hibernating myocardium. *Arterioscler Thromb Vasc Biol* 2002; **22:** 1804–1810.
25. Iba O, Matsubara H, Nozawa Y, Fujiyama S, Amano K, Mori Y, et al. Angiogenesis by implantation of peripheral blood mononuclear cells and platelets into ischemic limbs. *Circulation* 2002; **106:** 2019–2025.
26. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 2001; **103:** 2776–2779.
27. Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet* 2003; **362:** 697–703.
28. Halkin A, Stone GW, Grines CL, Cox DA, Rutherford BD, Esente P, et al. Prognostic implications of creatine kinase elevation after primary percutaneous coronary intervention for acute myocardial infarction. *J Am Coll Cardiol* 2006; **47:** 951–961.
29. Katayama T, Nakashima H, Takagi C, Honda Y, Suzuki S, Iwasaki Y, et al. Prognostic significance of time-delay to peak creatine kinase after direct percutaneous coronary intervention in acute myocardial infarction patients. *Int Heart J* 2005; **46:** 607–618.
30. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 2005; **353:** 999–1007.
31. Yeh ET, Zhang S, Wu HD, Korbling M, Willerson JT, Estrov Z. Transdifferentiation of human peripheral blood CD34$^+$-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells in vivo. *Circulation* 2003; **108:** 2070–2073.
32. Hofmann M, Wollert KC, Meyer GP, Menke A, Arseniev L, Hertenstein B, et al. Monitoring of bone marrow cell homing into the infarcted human myocardium. *Circulation* 2005; **111:** 2198–2202.
33. Heil M, Ziegelhoeffer T, Mees B, Schaper W. A different outlook on the role of bone marrow stem cells in vascular growth: Bone marrow delivers software not hardware. *Circ Res* 2004; **94:** 573–574.
34. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 2004; **428:** 664–668.
35. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* 2004; **428:** 668–673.
36. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 2001; **7:** 430–436.
37. Wollert KC, Drexler H. Clinical applications of stem cells for the heart. *Circ Res* 2005; **96:** 151–163.
38. Kang HJ, Kim HS, Zhang SY, Park KW, Cho HJ, Koo BK, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: The MAGIC cell randomised clinical trial. *Lancet* 2004; **363:** 751–756.
39. Kang HJ, Lee HY, Na SH, Chang SA, Park KW, Kim HK, et al. Differential effect of intracoronary infusion of mobilized peripheral blood stem cells by granulocyte colony-stimulating factor on left ventricular function and remodeling in patients with acute myocardial infarction versus old myocardial infarction: The MAGIC Cell-3-DES randomized, controlled trial. *Circulation* 2006; **114:** I-145–I-151.
40. Li ZQ, Zhang M, Jing YZ, Zhang WW, Liu Y, Cui LJ, et al. The clinical study of autologous peripheral blood stem cell transplantation by intracoronary infusion in patients with acute myocardial infarction (AMI). *Int J Cardiol* 2007; **115:** 52–56.

Available online at www.sciencedirect.com

ScienceDirect

Biochemical and Biophysical Research Communications 352 (2007) 668–674

BBRC

www.elsevier.com/locate/ybbrc

Skeletal myosphere-derived progenitor cell transplantation promotes neovascularization in δ-sarcoglycan knockdown cardiomyopathy

Tetsuya Nomura [a,b], Eishi Ashihara [a], Kento Tateishi [a,b], Satoshi Asada [a,b], Tomomi Ueyama [a], Tomosaburo Takahashi [a,b], Hiroaki Matsubara [a,b], Hidemasa Oh [a,*]

[a] *Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto 606-8507, Japan*
[b] *Department of Cardiovascular Medicine, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan*

Received 10 November 2006
Available online 27 November 2006

Abstract

Bone marrow cells have been shown to contribute to neovascularization in ischemic hearts, whereas their impaired maturation to restore the δ-sarcoglycan (δ-SG) expression responsible for focal myocardial degeneration limits their utility to treat the pathogenesis of cardiomyopathy. Here, we report the isolation of multipotent progenitor cells from adult skeletal muscle, based on their ability to generate floating-myospheres. Myosphere-derived progenitor cells (MDPCs) are distinguishable from myogenic C2C12 cells and differentiate into vascular smooth muscle cells and mesenchymal progeny. The mutation in the δ-SG has been shown to develop vascular spasm to affect sarcolemma structure causing cardiomyopathy. We originally generated δ-SD knockdown (KD) mice and transplanted MDPCs into the hearts. MDPCs enhanced neoangiogenesis and restored δ-SG expression in impaired vasculatures through trans-differentiation, leading to improvement of cardiac function associated with paracrine effectors secretion. We propose that MDPCs may be the promising progenitor cells in skeletal muscle to treat δ-sarcoglycan complex mutant cardiomyopathy.

Keywords: Stem cells; Skeletal muscle; Angiogenesis; δ-Sarcoglycan; Mesenchymal cell

Satellite cells reside beneath the basal lamina of adult skeletal muscle and mediate the postnatal growth and regeneration of muscle [1]. However, a growing number of studies are reporting the isolation of stem cells from adult skeletal muscle tissue, distinct from or descendant from satellite cells [2,3]. Multipotent skeletal muscle-derived stem cells (MDSCs) were demonstrated to be composed of a subset of a Sca-1^+/$CD34^+$/$CD45^-$ cell population [4]. These cells exhibited greater neoangiogenesis as well as regeneration of cardiomyocytes when transplanted into myocardial infarction [5] or dystrophin-deficient mdx mice [6]. Myogenic and endothelial cell progenitors were also identified in the interstitial space of adult skeletal muscle. They were defined as a $CD34^+$/$CD45^-$ fraction, and differentiated into vascular endothelial cells and skeletal muscle fibers after transplantation into intact skeletal muscle [7].

To take advantage of potential therapeutic opportunities, as an easily accessible tissue source for autologous transplantation, we isolated the cells from adult skeletal muscle, based on the characteristics of adult stem cells having a distinct proliferative potential to form floating-spheres, termed myospheres [8]. Myosphere-derived progenitor cells (MDPCs) expressed phenotypic characteristics resembling microvascular pericytes [9] or mesenchymal stem cells (MSCs) [10]. When introduced into ischemic hearts, MSCs were shown to prevent deleterious remodeling and to improve cardiac function [11].

Cardiomyopathy is a multifactorial disease that includes both inherited and acquired forms and is one of the most

* Corresponding author. Fax: +81 75 751 4741.
E-mail address: hidemasa@kuhp.kyoto-u.ac.jp (H. Oh).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.11.097

common causes of chronic heart failure. A mutation in the δ-sarcoglycan (δ-SG) gene was demonstrated to lead to sarcoglycan complex disruption and dystrophic changes [12]. The absence of δ-SG specifically in vascular smooth muscle produced microinfarcts in the heart that resulted in cardiomyopathy characterized by irregularities of the coronary vasculature and focal degeneration [13]. In this study, we originally generated cardiomyopathy model by targeting δ-SG transcripts with efficient knockdown (KD) vector pDECAP-δ-SG [14]. δ-SG KD mice showed both less vascular density and reduced δ-SG expression in the hearts, resulted in cardiac dysfunction.

Bone marrow-derived side population (BM-SP) transplantation has been shown to engrafted into δ-SG-deficient hearts in the absence of restoration of δ-SG expression in cardiac muscle [15]. Therefore, the present study was designed to address the efficacy of cell therapy using MDPCs for the treatment of δ-SG KD-induced cardiac dysfunction. Our results showed that the implanted MDPCs not only regenerated new vessels but also promoted the secretion of paracrine effectors, thereby improving cardiac function.

Materials and methods

MDPC isolation. The primary hind limb muscle cells were isolated from 8-week-old C57BL/6J mice (Shimizu Laboratories Supplies) and green fluorescent protein (GFP) transgenic mice (generously donated by M. Okabe, Osaka University) using 470 U/ml collagenase type II (Worthington) for digestion. Cells were suspended in DMEM/F12 (Invitrogen) supplemented with B27, 20 ng/ml epidermal growth factor (EGF) (Sigma), and 40 ng/ml recombinant basic fibroblast growth factor (bFGF) (Promega). Cell suspensions were then cultured onto a non-coated dish at 20 cells/μl density over 7 days. Individual GFP^- spheres were transferred onto a 24-well fibronectin-coated plate in the growth medium composed of DMEM/F12, 2% fetal bovine serum (FBS), 20 ng/ml EGF, 10 ng/ml bFGF, and 10 ng/ml leukemia inhibitory factor (LIF) (Chemicon).

MDPC differentiation. Culture medium was replaced by specific medium composed of DMEM, 10% FBS, 0.5 mM isobutyl-methylxanthine, and 1 μM dexamethasone for adipogenic differentiation. Osteogenic differentiation was induced by treating cells with 250 ng/ml recombinant bone morphogenetic protein 2 (Sigma). Differentiation medium containing DMEM/F12 and 10% FBS supplemented with 10 ng/ml vascular endothelial growth factor or 50 ng/ml platelet-derived growth factor (R&D Systems) was used to induce endothelial or smooth muscle cell differentiation, respectively.

Generation of δ-SG KD mice. The plasmid to express first 498-bp coding region of δ-SG RNA was cloned by PCR using the full-length of δ-SG cDNA (generously donated by M. Imamura, National Institute of Neuroscience, Tokyo, Japan) [16]. A plasmid expressing the 498-bp of double-stranded δ-SG RNA was constructed into the KD vector, pDECAP (generously donated by S. Ishii, RIKEN Tsukuba Institute, Japan) [14], as an inverted repeat with a 12-bp spacer (CTCTCTGGTACC). The 2.2-kbp *Bgl*II–*Bam*HI fragment of pDECAP-δ-SG was released and injected into fertilized mouse oocytes.

RNA extraction and gene expression analysis. Total RNA was extracted using TRIzol reagent and first-strand cDNA was synthesized by SuperScript III kit (Invitrogen). Primers used were Sca-1-f: CTCTGAGGATG GACACTTCT, Sca-1-r: GGTCTGCAGGAGGACTGAGC; CD34-f: TTGACTTCTGCAACCACGGA, CD34-r: TAGATGGCAGGCTGG ACTTC; Pax7-f: GAAAGCCAAACACAGCATCGA, Pax7-r: ACCCTG ATGCATGGTTGATGG; MyoD-f: ACATAGACTTGACAGGCCC CGA, MyoD-r: AGACCTTCGATGTAGCGGATGG; Myogenin-f: TAC GTCCATCGTGGACAGCAT, Myogenin-r: TCAGCTAAATTCCCTC GCTGG; β-actin-f: GCTCGTCGTCGACAACGGCTC, β-actin-r: CAAACATGATCTGGGTCATCTTCTC; δ-SG-f: CCATGACCATC TGGATTCTCAAGG, δ-SG-r: GATGGCTTCCATATTGCCAGCTTC; and smooth muscle myosin heavy chain (Sm-MHC)-f: AGGAACTCC AAGCAAGTTGCAGG, Sm-MHC-r: CTGGAAGGAACAAATGAA GCCTCG. To evaluate hepatocyte growth factor (HGF) and stromal-cell-derived factor 1 (SDF-1) expression, cDNA was analyzed by kinetic real-time RT-PCR using the ABI Prism 7700 Sequence Detector system (Applied Biosystems) with Assay-on-Demand™ primer-probes sets. mRNA levels were expressed relative to an endogenous control (18S RNA).

Fluorescence activated cell sorting (FACS) analysis. Cells were stained with the following antibodies; FITC-conjugated antibodies against Sca-1, CD29, CD31, CD44, CD45, CD106, and CD117, PE-conjugated antibodies against CD34 and CD90 (BD Biosciences), and rat monoclonal anti-CD105 (Southern Biotechnology) followed by APC-labeled goat anti-rat IgG (BD Biosciences). Non-viable cells were stained with propidium iodide and 30,000 events were collected per sample by FACS Calibur flow cytometer (BD Biosciences). Gates were established by non-specific-Ig binding in each experiment.

Immunofluorescence. Specimens were fixed in 4% paraformaldehyde and stained with rat monoclonal anti-CD31 (BD Biosciences); rabbit polyclonal anti-type I collagen (LSL); mouse monoclonal antibodies against vimentin, Sm-MHC (DAKO), and δ-SG (Novocastra). Secondary antibodies were conjugated with Alexa 488 or Alexa 555, and nuclei were visualized using 4′,6-diamino-2-phenylindole (DAPI) (Invitrogen). Mouse monoclonal antibody against α-smooth muscle actin (α-SMA, Sigma) was conjugated with Cy3. M.O.M. Kit (Vector). Cells were labeled with 10 μM 5-bromo-2′-deoxyuridine (BrdU) solution for 1 h in culture and BrdU detection kit (Roche) was used according to the manufacturer's instruction. Images were captured with BZ-8000 (Keyence).

Oil red O and Alizarin red staining. Formalin-fixed cells were stained with 0.3% oil red O (Sigma) in 60% isopropanol for 30 min at room temperature. To stain calcium deposits, cells were covered with 2% alizarin red S solution (pH 4.2, Sigma) for 3 min.

Masson's trichrome and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) staining. Hearts from 28-week-old δ-SG KD mice were fixed with 10% formalin. Paraffin-embedded hearts were sectioned and stained with Masson's trichrome. Cell-implanted hearts were fixed by perfusion with 4% paraformaldehyde, and stained with the solution composed of 1 mg/ml X-gal (Invitrogen), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% NP-40 for overnight.

Retroviral transduction. GP2-293 cells were co-transfected with the envelope vector pVSV-G and pMSCV-puro vectors using FuGENE6 (Roche). The medium supernatant was collected and centrifuged to concentrate viral stocks according to the manufacturer's instruction. MDPCs were infected with the retrovirus for 24 h, and the infected cells were selected with 2.5 μg/ml puromycin.

Surgical procedure. Anesthetized 28-week-old δ-SG KD mice ($n = 15$) were intubated and positive-pressure ventilation was maintained. A half million MDPCs diluted in 20 μl of phosphate-buffered saline (PBS) were directly transplanted into three distinct sites of myocardium. All experimental procedures and protocols using animals were approved by the Animal Care and Use Committee of Kyoto University.

Cardiac function. Echocardiograms were performed using SONOS 5500 and 15 MHz probe (PHILIPS). M-mode measurements of left ventricular end diastolic diameters (LVDd) were measured and used for the calculation of fractional shortening (FS) of the left ventricle (LV). As an index of LV diastolic function, transmitral early filling/atrial contraction ratio (E/A) values were determined from five independent measurements by using spectral Doppler traces.

Statistical analysis. All experiments were performed at least three times. Data were expressed as means ± standard error and analyzed by one-way ANOVA with post hoc analysis. A value of $p < 0.05$ was considered significant.

Results

Isolation and expansion of MDPCs

We isolated myospheres from adult skeletal muscle, based on the characteristics of adult stem cells having a distinct proliferative potential to form floating-spheres, by co-culturing the single cells from GFP transgenic and wild-type (WT) mice to exclude cell aggregation as confirmed by green mosaic fluorescence [17]. By day-7 in culture, spherical colonies composed of entirely GFP-positive or -negative cells had been formed ($0.11 \pm 0.03\%$ of initial cells, Fig. 1A). RT-PCR demonstrated that a single myosphere was positive for Sca-1 and CD34 but lacked essential myogenic transcription factors, including Pax7, MyoD, and Myogenin, which are typically present in myogenic C2C12 cells (Fig. 1B).

For MDPC expansion, individual GFP^- myospheres were transferred onto fibronectin-coated 24-well plates in the growth medium and the myospheres were allowed to attach on culture plates. Many cells migrated from the colony and were mitotically active cells as confirmed by BrdU incorporation (Fig. 1C). MDPCs continued to proliferate in the growth medium (Fig. 1D), and reached more than 120 population doublings as confirmed in three individual cell lines (Fig. 1E).

MDPCs have mesenchymal cell-like phenotype and differentiate into endothelial and vascular smooth muscle cells

FACS analysis showed that MDPCs expressed CD29, CD44, CD90, CD105, and CD106, a typical profile for mesenchymal cells. The lack of CD31, CD45, and CD117 indicated that the cells did not include endothelial or hematopoietic progenitors. Of note, Sca-1 and CD34 were highly expressed in MDPCs (Fig. 2A). To further address their mesenchymal-cell phenotype, undifferentiated MDPCs were stained for vimentin ($90.2 \pm 3.3\%$, Fig. 2B) and type I collagen ($87.4 \pm 6.9\%$, Fig. 2C). Induction of adipogenic- and osteogenic-lineage differentiation was examined in vitro. Accumulation of lipid vacuoles was clearly visualized by oil red O staining (Fig. 2D), and Alizarin red staining detected calcium deposits in osteogenic culture (Fig. 2E), indicating that MDPCs have a mesenchymal cell-like phenotype.

To determine the angiogenic potential of MDPCs, cells were cultured under specific inductions. Immunofluorescence analysis showed that MDPCs differentiated into $CD31^+$ vasculature (Fig. 2F) and Sm-MHC^+ smooth muscle cells in vitro (Fig. 2G).

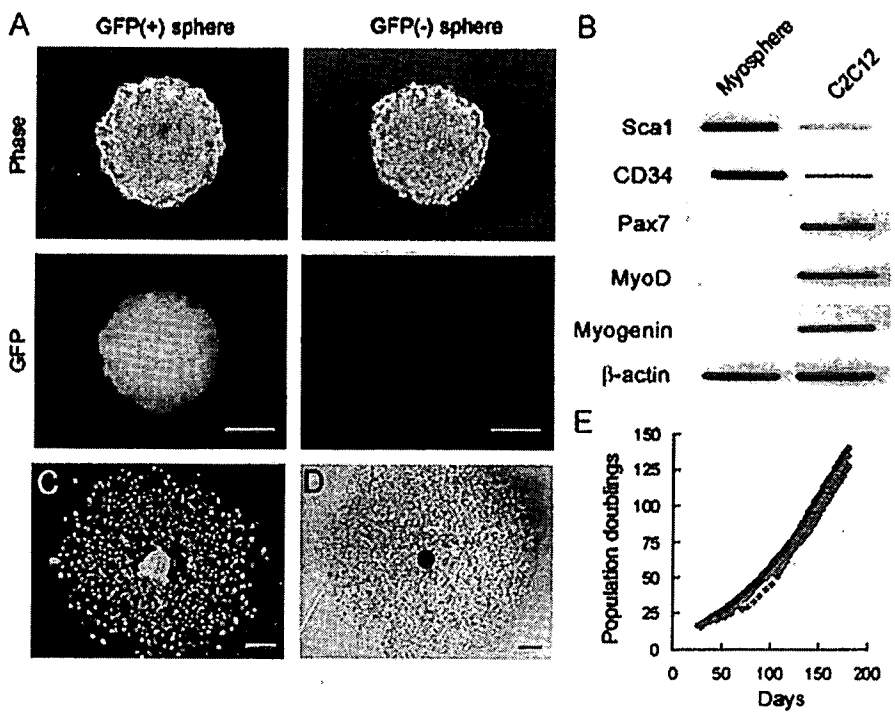

Fig. 1. Isolation and expansion of MDPCs. (A) Representative phase contrast and fluorescent images of myospheres generated from the mixed-cultures of single cells isolated from GFP transgenic (left panels) and WT mice (right panels). (B) RT-PCR analysis of Sca-1, CD34, and myogenic transcription factors. (C) MDPCs that migrated from single myosphere actively incorporated BrdU (green). DAPI (blue). (D) MDPCs propagated in the growth medium. (E) Growth kinetics of 3 independent cell lines in long-term culture. Scale bars represent 50 μm in (C) and (D), and 20 μm in (A).

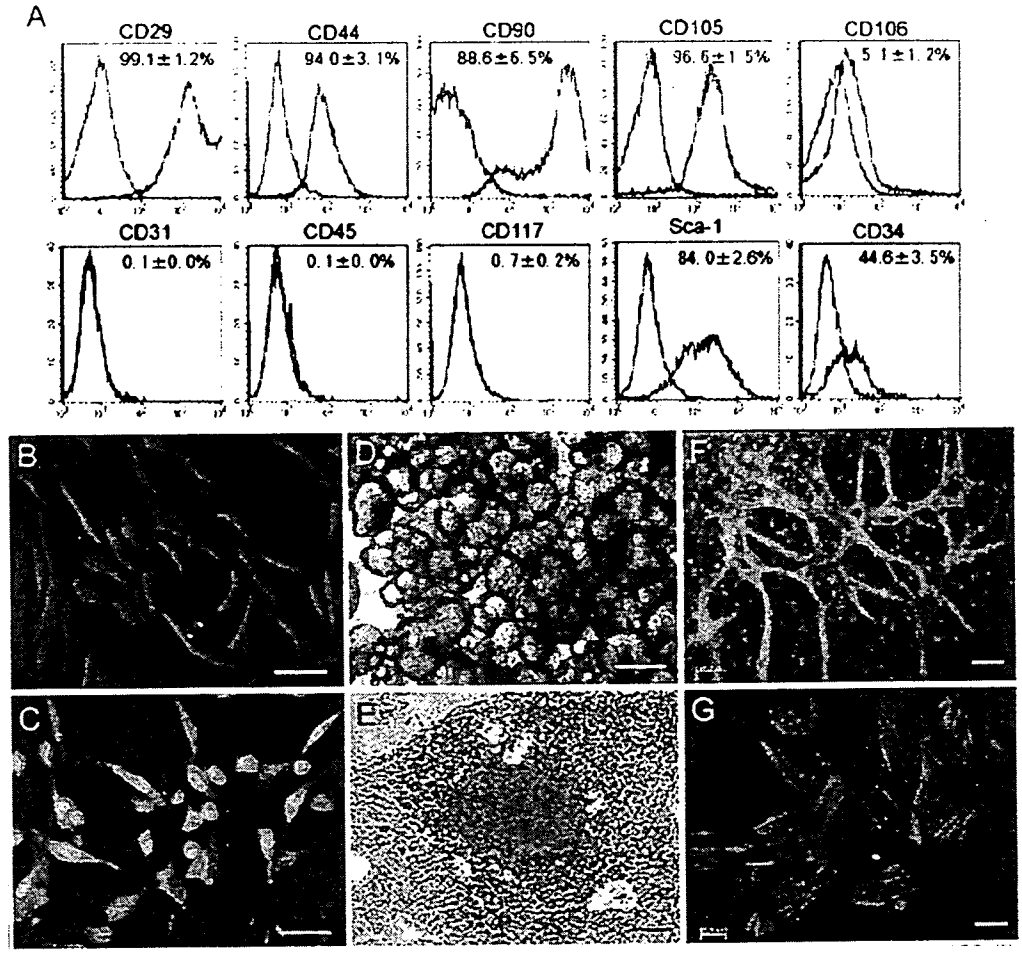

Fig. 2. MDPCs have mesenchymal cell-like phenotype and differentiate into endothelial and smooth muscle cells. (A) FACS analysis of MDPCs. Isotype controls were overlaid (blue lines) on each antigen tested (red lines). Immunostaining of mesenchymal markers on undifferentiated MDPCs. Vimentin (B, red) and type I collagen (C, green) are shown. DAPI (blue). Adipogenic- and osteogenic-inductions were verified by Oil red O (D, red) and Alizarin red (E, red), respectively. MDPCs were also induced into $CD31^+$ endothelial (F, green) and Sm-MHC^+ smooth muscle cells (G, red) by specific medium. DAPI (blue) Scale bars represent 50 μm in (E) and 20 μm in (B–D, F, and G).

MDPCs regenerate vascular smooth muscle cells with the restoration of δ-SG expression in vivo

We next generated δ-SG KD mice as a cardiomyopathy model by targeting δ-SG transcripts with an efficient KD vector, pDECAP-δ-SG [14]. Compared with non-transgenic littermates (NTG), the δ-SG expression on the membrane of cardiac muscle was disrupted in 28-week-old δ-SG KD mice (Fig. 3A left panels). The δ-SG expression along the vessels was also decreased, resulting in narrow vascular lumens with constrictive morphology (Fig. 3A middle panels). Masson's trichrome staining demonstrated extensive fibrosis surrounding the vessels (Fig. 3A right panels).

To determine whether MDPC transplantation can restore the δ-SG expression as well as regenerate the degenerated vessels in δ-SG KD hearts, a half million MDPCs transduced with a LacZ reporter gene were directly injected into three individual sites of myocardium. All transplanted hearts showed substantial $LacZ^+$ cell engraftment 4 weeks after implantation. $LacZ^+$ vascular smooth muscle cells could be readily detectable (Fig. 3B), and those were co-localized with δ-SG expression to regenerate new vessels (Fig. 3C arrows).

Transplantation of MDPCs improves cardiac function partially through the paracrine effectors production

We next asked whether MDPCs might restore δ-SG expression during differentiation process and found that MDPCs expressed δ-SG transcripts through smooth muscle cell lineage induction in vitro (Fig. 4A). A significant neoangiogenesis in the MDPC-injected area was observed in the MDPC-transplanted group compared with that in PBS-treated hearts (Fig. 4B). Cardiac function at baseline of δ-SG KD and NTG littermates was analyzed by echocardiography and showed a significant increase in LVDd and impaired systolic and diastolic functions in δ-SG KD

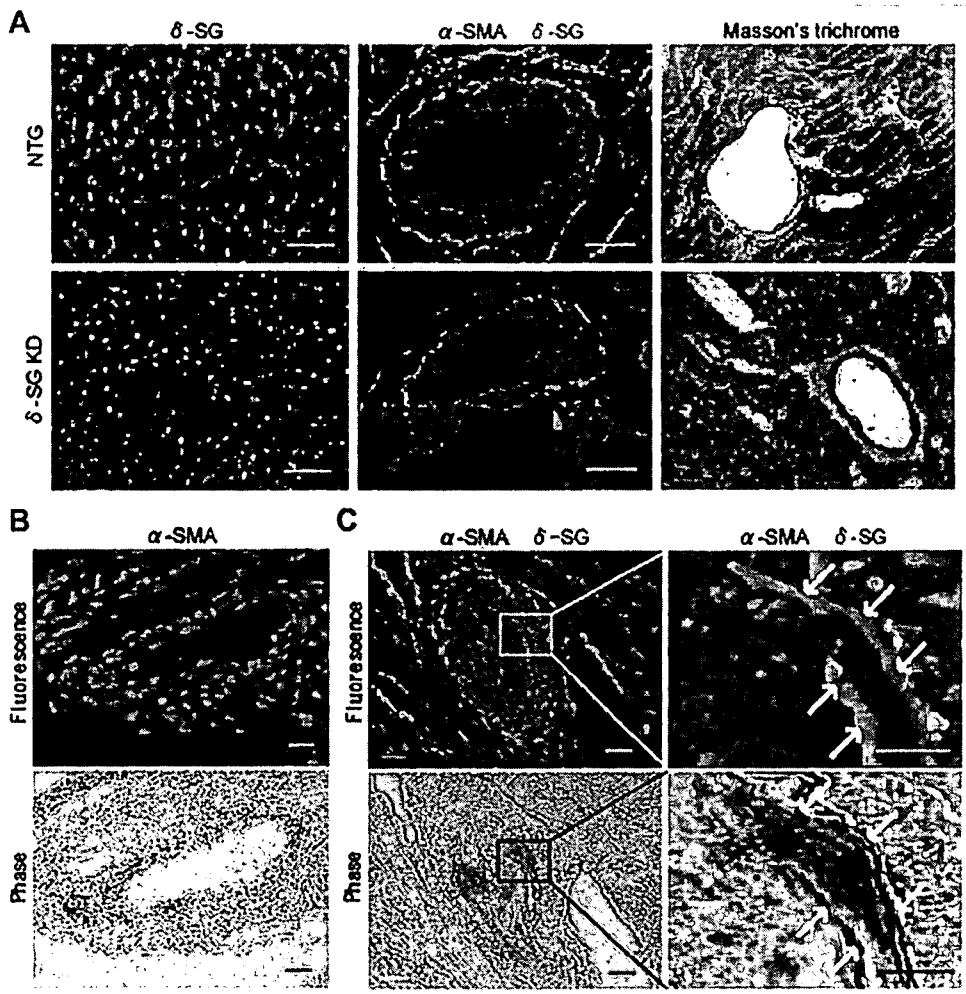

Fig. 3. Vascular regeneration in δ-SG KD hearts with the restoration of δ-SG expression. (A, left panels) Decreased δ-SG (red) expression in cardiac muscle was significantly observed in δ-SG KD hearts compared with NTG littermates. DAPI (blue). (A, middle panels) Vascular lumens were more constrictive and narrower with irregular distribution of perivascular δ-SG expression (green) in δ-SG KD hearts. α-SMA (red). DAPI (blue). (A, right panels) Masson's trichrome staining showed perivascular fibrosis in δ-SG KD hearts. (B) Transplanted LacZ$^+$ MDPCs differentiated into smooth muscle cells in δ-SG KD hearts. α-SMA (red DAPI (blue). (C) δ-SG expression (green) was restored in newly formed vessels (arrows). The right panels are magnified images of the rectangle areas in the left panels. α-SMA (red) DAPI (blue). Scale bars represent 50 μm in the left and right panels of (A), (B), and the left panels of (C), and 20 μm in the middle panels of (A) and the right panels of (C).

hearts (Fig. 4C). Transplantation of MDPCs did not result in any significant reduction in cardiac enlargement compared with that in PBS-treated hearts, but did significantly improve LV performance 4 weeks after cell implantation (Fig. 4C). To elucidate the mechanisms of functional recovery in the MDPC-transplanted hearts, relative gene expression of paracrine mediators was measured by real-time RT-PCR. Gene expression for HGF and SDF-1 significantly increased in the MDPC-implanted hearts compared with that in the control hearts 2 weeks after cell transplantation (Fig. 4D).

Discussion

Autologous transplantation is the ideal system of cell therapy. From this practical point of view, skeletal muscle is one of the most easily accessible tissue sources. There are accumulating reports of multipotent progenitors in skeletal muscle, but the differentiation potential of these cells remains controversial [2]. A recent report demonstrated the isolation of myospheres from the adult skeletal muscle [8]. As opposed to the MDPCs we described here, these cells expressed Pax7 at baseline and tended to differentiate into a myogenic lineage, suggesting that these cells were originated from satellite cells. In this study, we demonstrated Pax7$^-$ MDPCs regenerated endothelial and vascular smooth muscle cells in vitro and in vivo. These MDPCs displayed prolonged self-renewal capacity, mesenchymal cell-like phenotype, and expressed part of the embryonic stem cell markers such as Nanog, Oct-4 and Sox2 (data not shown), indicative of thier marked plasticity.

Although few reports to date have described the origin of skeletal muscle containing stem cell-like population, the characteristics of MDPCs shown here indicated that

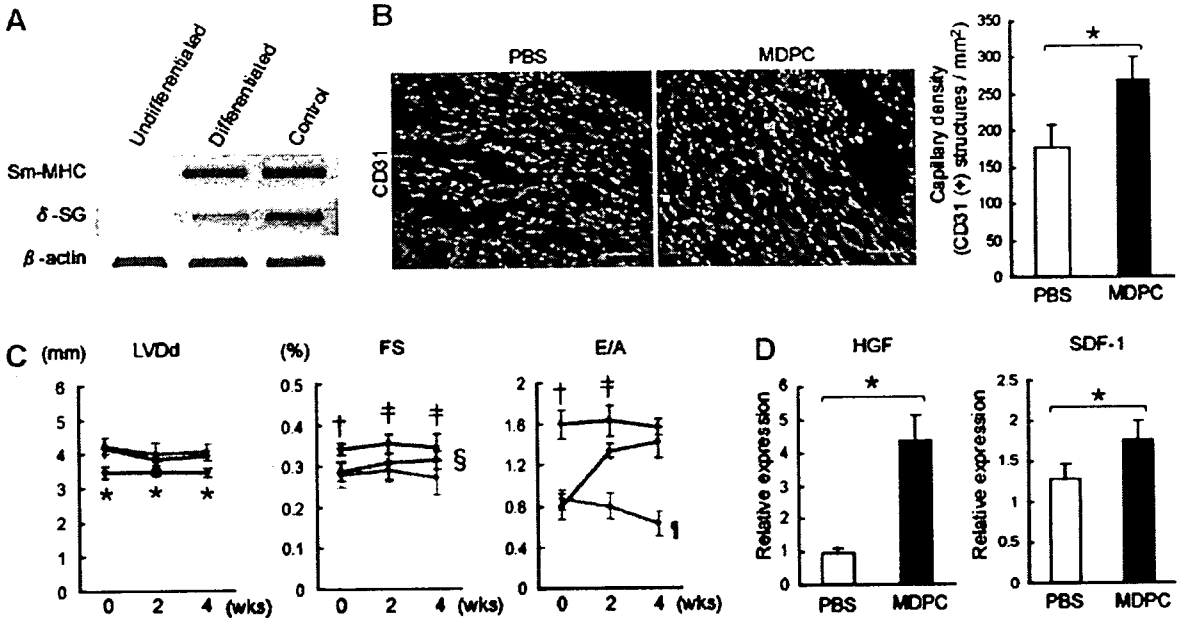

Fig. 4. MDPC transplantation improves cardiac function partially through paracrine effectors production (A) δ-SG expression was observed in differentiated MDPCs in vitro. (B) Comparison of the capillary density between PBS-treated and MDPC-transplanted δ-SG KD hearts. CD31 (red). DAPI (blue). $^{*}p < 0.01$. (C) The effect of MDPC transplantation in δ-SG KD hearts shown by echocardiograms. Black lines: NTG mice. Blue lines: PBS-treated group. Red lines: MDPC-transplanted group. $^{*}p < 0.05$; $^{\dagger}p < 0.01$ vs. PBS- and MDPC-treated mice. $^{\ddagger}p < 0.01$ vs. PBS-injected mice and $p < 0.05$ vs. MDPC-transplanted mice. $^{\S}p < 0.05$ vs. PBS-injected mice. $^{\P}p < 0.01$ vs. NTG and MDPC-treated mice compared with the same time point. (D) Relative gene expression for HGF and SDF-1 was measured by real-time RT-PCR. $^{*}p < 0.01$. Scale bars represent 50 μm in (B).

they might be reminiscent of mesenchymal cells derived from perivascular cells (PVCs) [18] or mesoangioblasts that are putative ancestors of PVCs [19], which can be classified as pericytes in capillaries and are essential for the development of functional vessel walls. Because PVCs are thought to have the potential to regenerate mesenchymal cells, MDPCs may reflect in some aspects of the phenotype of MSCs originally isolated from bone marrow stroma.

Previous report demonstrated that BM-SP cells could be engrafted in δ-SG null hearts, but failed to restore the δ-SG expression [15]. The absence of δ-SG expression after transplantation suggested that cellular fusion, as opposed to de novo differentiation, occurred with transplanted BM-SP cells which led to impaired maturation of implanted cells. In contrast, we observed that transplanted MDPCs did differentiate into mature vascular cells with the restoration of δ-SG expression, indicating autonomous vascular-differentiation might occur after MDPC transplantation.

It is important to determine whether local intramuscular injection of MDPCs into δ-SG KD heart is sufficient to deliver the cells into focally degenerated lesions and contributes to functional recovery. We observed extensive angiogenesis induced by MDPC transplantation to achieve a better preservation of cardiac function. However, the lack of improvement in diastolic dimension did not favor a scaffolding effect of the grafted MDPCs in δ-SG KD hearts similar to the previous report [20]. In our study, engrafted MDPCs were incorporated mostly into vascular cells, but muscular regeneration was rarely observed. One of the reasons is that δ-SG KD mice showed a predominantly lower expression of δ-SG along vascular smooth muscle cells, as previously reported [21], leading to scarce muscular artery and particularly extensive fibrosis surrounding the vessels. This focal defect in the δ-SG KD heart might be one of the causes for transplanted MDPCs to differentiate into vascular cells more efficiently than into cardiac or skeletal muscle fibers.

Our results also suggested that transplanted MDPCs could induce the secretion of HGF and SDF-1, that is consistent with the recent reports demonstrating that HGF could promote stem cell activation and reduced cardiomyocyte apoptosis in the myocardium of δ-SG-null hamsters [22], and that SDF-1 was sufficient to induce therapeutic stem cell homing to injured myocardium [23]. Taken together, the beneficial effects of MDPC transplantation might be due to increased blood supply produced by angiogenesis and promoted secretion of specific growth factors, leading to modulation of adverse LV remodeling and improvement of cardiac function.

In conclusion, transplantation of MDPCs induced substantial angiogenesis and increased secretion of paracrine mediators, resulting in the improvement of cardiac function in δ-SG KD mice. Our findings indicate that MDPCs may be the promising progenitor cells in adult skeletal muscle for cell therapy to treat δ-sarcoglycan complex mutant cardiomyopathy.

Acknowledgments

We thank the investigators cited for generously donating plasmids: S. Ishii, and M. Imamura; Y. Yoshida, A. Kosugi, and M. Nishikawa for technical assistance. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Grants-in-Aid from the Ministry of Health, Labor, and Welfare of Japan.

References

[1] C.A. Collins, I. Olsen, P.S. Zammit, L. Heslop, A. Petrie, T.A. Partridge, J.E. Morgan, Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche, Cell 122 (2005) 289–301.

[2] I.W. McKinnell, G. Parise, M.A. Rudnicki, Muscle stem cells and regenerative myogenesis, Curr. Top Dev. Biol. 71 (2005) 113–130.

[3] N. Hashimoto, T. Murase, S. Kondo, A. Okuda, M. Inagawa-Ogashiwa, Muscle reconstitution by muscle satellite cell descendants with stem cell-like properties, Development 131 (2004) 5481–5490.

[4] Z. Qu-Petersen, B. Deasy, R. Jankowski, M. Ikezawa, J. Cummins, R. Pruchnic, J. Mytinger, B. Cao, C. Gates, A. Wernig, J. Huard, Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration, J. Cell Biol. 157 (2002) 851–864.

[5] H. Oshima, T.R. Payne, K.L. Urish, T. Sakai, Y. Ling, B. Gharaibeh, K. Tobita, B.B. Keller, J.H. Cummins, J. Huard, Differential myocardial infarct repair with muscle stem cells compared to myoblasts, Mol. Ther. 12 (2005) 1130–1141.

[6] T.R. Payne, H. Oshima, T. Sakai, Y. Ling, B. Gharaibeh, J. Cummins, J. Huard, Regeneration of dystrophin-expressing myocytes in the mdx heart by skeletal muscle stem cells, Gene Ther. 12 (2005) 1264–1274.

[7] T. Tamaki, A. Akatsuka, K. Ando, Y. Nakamura, H. Matsuzawa, T. Hotta, R.R. Roy, V.R. Edgerton, Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle, J. Cell Biol. 157 (2002) 571–577.

[8] R. Sarig, Z. Baruchi, O. Fuchs, U. Nudel, D. Yaffe, Regeneration and transdifferentiation potential of muscle-derived stem cells propagated as myospheres, Stem Cells 24 (2006) 1769–1778.

[9] H. Gerhardt, C. Betsholtz, Endothelial-pericyte interactions in angiogenesis, Cell Tissue Res. 314 (2003) 15–23.

[10] L. da Silva Meirelles, P.C. Chagastelles, N.B. Nardi, Mesenchymal stem cells reside in virtually all post-natal organs and tissues, J. Cell Sci. 119 (2006) 2204–2213.

[11] M.F. Pittenger, B.J. Martin, Mesenchymal stem cells and their potential as cardiac therapeutics, Circ. Res. 95 (2004) 9–20.

[12] A. Sakamoto, K. Ono, M. Abe, G. Jasmin, T. Eki, Y. Murakami, T. Masaki, T. Toyo-oka, F. Hanaoka, Both hypertrophic and dilated cardiomyopathies are caused by mutation of the same gene, delta-sarcoglycan, in hamster: an animal model of disrupted dystrophin-associated glycoprotein complex, Proc. Natl. Acad. Sci. USA 94 (1997) 13873–13878.

[13] R. Coral-Vazquez, R.D. Cohn, S.A. Moore, J.A. Hill, R.M. Weiss, R.L. Davisson, V. Straub, R. Barresi, D. Bansal, R.F. Hrstka, R. Williamson, K.P. Campbell, Disruption of the sarcoglycan-sarcospan complex in vascular smooth muscle: a novel mechanism for cardiomyopathy and muscular dystrophy, Cell 98 (1999) 465–474.

[14] T. Shinagawa, S. Ishii, Generation of Ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter, Genes Dev. 17 (2003) 1340–1345.

[15] K.A. Lapidos, Y.E. Chen, J.U. Earley, A. Heydemann, J.M. Huber, M. Chien, A. Ma, E.M. McNally, Transplanted hematopoietic stem cells demonstrate impaired sarcoglycan expression after engraftment into cardiac and skeletal muscle, J. Clin. Invest. 114 (2004) 1577–1585.

[16] S. Noguchi, E. Wakabayashi, M. Imamura, M. Yoshida, E. Ozawa, Developmental expression of sarcoglycan gene products in cultured myocytes, Biochem. Biophys. Res. Commun. 262 (1999) 88–93.

[17] P. Vourc'h, M. Romero-Ramos, O. Chivatakarn, H.E. Young, P.A. Lucas, M. El-Kalay, M.F. Chesselet, Isolation and characterization of cells with neurogenic potential from adult skeletal muscle, Biochem. Biophys. Res. Commun. 317 (2004) 893–901.

[18] B. Brachvogel, H. Moch, F. Pausch, U. Schlotzer-Schrehardt, C. Hofmann, R. Hallmann, K. von der Mark, T. Winkler, E. Poschl, Perivascular cells expressing annexin A5 define a novel mesenchymal stem cell-like population with the capacity to differentiate into multiple mesenchymal lineages, Development 132 (2005) 2657–2668.

[19] G. Cossu, P. Bianco, Mesoangioblasts—vascular progenitors for extravascular mesodermal tissues, Curr. Opin. Genet. Dev. 13 (2003) 537–542.

[20] J. Pouly, A.A. Hagege, J.T. Vilquin, A. Bissery, A. Rouche, P. Bruneval, D. Duboc, M. Desnos, M. Fiszman, Y. Fromes, P. Menasche, Does the functional efficacy of skeletal myoblast transplantation extend to nonischemic cardiomyopathy? Circulation 110 (2004) 1626–1631.

[21] M.T. Wheeler, M.J. Allikian, A. Heydemann, M. Hadhazy, S. Zarnegar, E.M. McNally, Smooth muscle cell-extrinsic vascular spasm arises from cardiomyocyte degeneration in sarcoglycan-deficient cardiomyopathy, J. Clin. Invest. 113 (2004) 668–675.

[22] R. Fiaccavento, F. Carotenuto, M. Minieri, C. Fantini, G. Forte, A. Carbone, L. Carosella, R. Bei, L. Masuelli, C. Palumbo, A. Modesti, M. Prat, P. Di Nardo, Stem cell activation sustains hereditary hypertrophy in hamster cardiomyopathy, J. Pathol. 205 (2005) 397–407.

[23] A.T. Askari, S. Unzek, Z.B. Popovic, C.K. Goldman, F. Forudi, M. Kiedrowski, A. Rovner, S.G. Ellis, J.D. Thomas, P.E. DiCorleto, E.J. Topol, M.S. Penn, Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy, Lancet 362 (2003) 697–703.

Clonally amplified cardiac stem cells are regulated by Sca-1 signaling for efficient cardiovascular regeneration

Kento Tateishi[1,2], Eishi Ashihara[1], Naofumi Takehara[1], Tetsuya Nomura[1,2], Shoken Honsho[2], Takuo Nakagami[2,3], Shigehiro Morikawa[4], Tomosaburo Takahashi[1,2], Tomomi Ueyama[1], Hiroaki Matsubara[1,2,*] and Hidemasa Oh[1,*]

[1]Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto 606-8507, Japan
[2]Department of Cardiovascular Medicine, [3]Departments of Pathology and Cell Regulation, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan
[4]Molecular Neuroscience Research Center, Shiga University of Medical Science, Shiga 520-2192, Japan
*Authors for correspondence (e-mail: matsubah@koto.kpu-m.ac.jp; hidemasa@kuhp.kyoto-u.ac.jp)

Accepted 25 March 2007
Journal of Cell Science 120, 1791-1800 Published by The Company of Biologists 2007
doi:10.1242/jcs.006122

Summary

Recent studies have shown that cardiac stem cells (CSCs) from the adult mammalian heart can give rise to functional cardiomyocytes; however, the definite surface markers to identify a definitive single entity of CSCs and the molecular mechanisms regulating their growth are so far unknown. Here, we demonstrate a single-cell deposition analysis to isolate individually selected CSCs from adult murine hearts and investigate the signals required for their proliferation and survival. Clonally proliferated CSCs express stem cell antigen-1 (Sca-1) with embryonic stem (ES) cell-like and mesenchymal cell-like characteristics and are associated with telomerase reverse transcriptase (TERT). Using a transgene that expresses a GFP reporter under the control of the TERT promoter, we demonstrated that $TERT^{GFP}$-positive fractions from the heart were enriched for cells expressing Sca-1. Knockdown of Sca-1 transcripts in CSCs led to retarded ex vivo expansion and apoptosis through Akt inactivation. We also show that ongoing CSC proliferation and survival after direct cell-grafting into ischemic myocardium require Sca-1 to upregulate the secreted paracrine effectors that augment neoangiogenesis and limit cardiac apoptosis. Thus, Sca-1 might be an essential component to promote CSC proliferation and survival to directly facilitate early engraftment, and might indirectly exert the effects on late cardiovascular differentiation after CSC transplantation.

Key words: Cardiac stem cells, Proliferation, Regeneration, Stem cell antigen-1, Survival, Telomerase

Introduction

The adult mammalian heart harbors a population of mitotically competent cardiac stem cells (CSCs) that can be isolated by using FACS to recognize the cells expressing surface antigens KIT and stem cell antigen-1 (Sca-1) or by targeting a reporter gene driven by the promoter for islet-1, a LIM-homeodomain transcription factor (Beltrami et al., 2003; Laugwitz et al., 2005; Matsuura et al., 2004; Moretti et al., 2006; Oh et al., 2003; Pfister et al., 2005). These cells express essential cardiac transcriptional factors but do not express more mature markers of structural genes; however, the exact contribution of cell fusion in the process of adopting cardiac muscle phenotype after cell transfer into ischemic myocardium remains controversial (Beltrami et al., 2003; Oh et al., 2003). Within the adult heart, CSCs often reside in cardiac niches with supporting cells that provide a specialized environment to replenish and maintain a balance of survival, proliferation and self-renewal of CSCs through symmetric or asymmetric division in order to replace the mature cells that are lost during injury or turnover (Urbanek et al., 2006).

The general lack of definitive molecular markers to identify cardiac stem cells raises the fundamental question of whether these cardiac stem cells are derived from a single entity. CSCs in the mammalian heart share several cell-surface markers with hematopoietic and endothelial progenitor cells (Linke et al., 2005; Messina et al., 2004; Urbanek et al., 2003). Although the hierarchies of hematopoietic stem cells have been well characterized, evidence supporting the role of bone marrow-derived *Lin*$^{-}$*-Kit*$^{+}$ cells in cardiac-lineage induction has been controversial (Kawada et al., 2004; Murry et al., 2004; Orlic et al., 2001). Recent reports have demonstrated that genetic disruption of *Kit* in mice mainly affects marrow-derived hematopoietic and endothelial cell development for cardiac repair, that could be rescued by bone marrow replacement with wild-type cells, through the failure of progenitor-cell mobilization from marrow and reduced release of cytokines and chemokines that may participate in the cardioprotective paracrine signaling (Ayach et al., 2006; Fazel et al., 2006). These studies do not exclude the possible functional role of KIT in resident CSCs as the principal mediator in the regenerating process during cardiac injury, but suggest that defining CSCs using specific cell-surface markers may not be optimal to address the identity of these cells, as indicated by their partially overlapping expression in human hearts (Urbanek et al., 2005b).

Decline of CSC function may be a major cause of the decrease in regenerative capacity in aging and disease (Rota et al., 2006). Although some of the growth factors involved in *Kit*$^+$ CSC proliferation and survival have been identified, factors regulating *Kit*$^-$ CSCs have yet to be defined (Gude et al., 2006; Limana et al., 2005; Urbanek et al., 2005a). In this study, we sought to identify single proliferative cells from the adult heart without progenitor selection using particular surface markers. Using this unbiased approach, we have established clonal CSC lines and demonstrated that the majority of the telomerase-active progenitor-cell colonies expressed Sca-1 and showed mesenchymal-cell-like character. We also show that targeting the Sca-1 transcripts in CSCs used for cell grafting leads to failure of their ability to prevent cardiac remodeling after myocardial infarction. The antiapoptotic and angiogenetic paracrine activities of intrinsic Sca-1 signaling in CSCs promote direct CSC proliferation and survival, and contribute to neovascularization in the host myocardium for efficient cardiovascular regeneration.

Results

Clonal isolation of cardiac stem cells in the adult heart

To identify the single entity of CSCs in the adult heart, we employed an unbiased approach using a single-cell clonogenic isolation technique to isolate a proliferative cell population. Singly dissociated GFP-labelled transgenic cells derived from the hearts of GFP transgenic mice were plated at a density of one cell per well in serum-free medium (Fig. 1A,B). Altogether, 11,520 single cells were deposited, and from 9541 single cells determined by inspection on day 1 to be present as one individual cell per well, a total of 11 clones arose within 7 days. Eight out 11 clones failed to grow in serum-free medium after 7 days in culture, and 3 clones (~0.03%) could proliferate to form spherical clusters and were continuously expanded after 14 days (Fig. 1C). The three independent colonies were re-dissociated and re-plated in low-serum for expansion, and individual CSC colonies were used for the following experiments to characterize clonal CSCs.

Characterization of clonally amplified CSCs

Immunophenotyping revealed that the clonal CSCs strongly expressed Sca-1, which is used as a marker to identify cardiac progenitor cells from the adult heart (Matsuura et al., 2004; Oh et al., 2003), whereas KIT-positive cells were rarely detected (Fazel et al., 2006; Gude et al., 2006; Pfister et al., 2005) (Fig. 1D). Notably, CSCs did not express the hematopoietic and endothelial progenitor-cell-specific surface antigens CD45, CD34 and CD31, but did express the typical mesenchymal stem-cell surface antigens CD90, CD105, CD29, CD44, CD106, CD73 and CD13 (Pittenger and Martin, 2004). The three individual CSCs exhibited an identical immunophenotyping for the surface marker analysis. The cell membrane antigens Sca-1, KIT, CD45, and CD34 were not destroyed by collagenase treatment as tested in bone marrow (data not shown).

Gene expression was then examined in CSC clones using reverse transcriptase (RT)-PCR (Fig. 1E). Three individual colonies were analyzed and most of the clones expressed Bcrp1, polycomb group protein Bmi1 and also telomerase reverse transcriptase (TERT), which has been reported to be absent in cardiac fibroblasts (Leri et al., 2001). Although Nanog was detectable in all of the colonies examined, none of the colonies – unlike embryonic stem (ES) cells – were positive for OCT4 or UTF1. Some but not all of the colonies expressed HNF3β, brachyury and SOX2, which are endodermal, mesodermal and ectodermal precursor markers, respectively. These results distinguished clonal CSCs from mouse fibroblasts, which are negative for all of the ES cell markers described above (Takahashi and Yamanaka, 2006). In addition, all of the colonies analyzed expressed nestin, a marker of immature neural progenitor cells (Joannides et al., 2004).

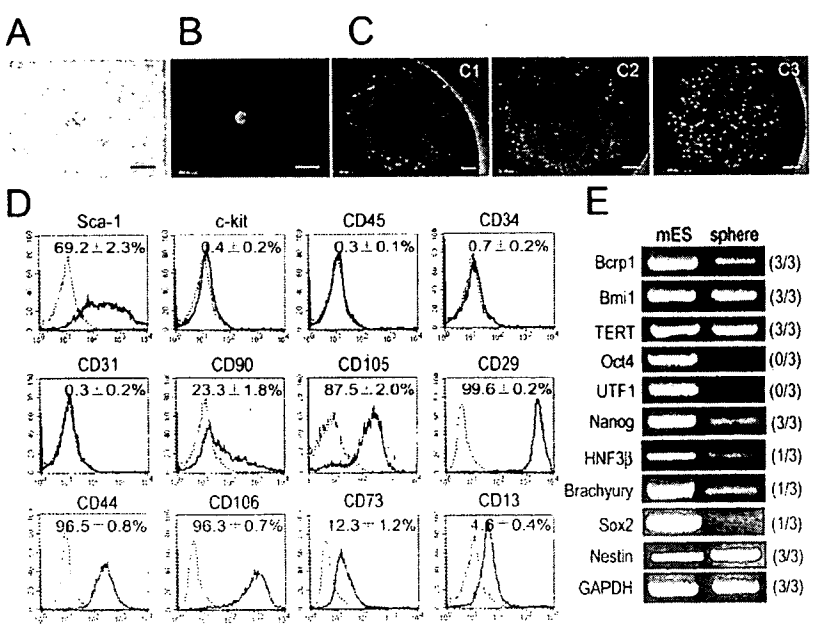

Fig. 1. Clonal isolation and characterization of stem cells in the adult heart. (A,B) Single-cell deposition analysis was performed by the limiting dilution technique. At day 1 of the culture period, wells were inspected for the presence of single cells by phase contrast (A) and GFP fluorescence (B). (C) Colony formation from single cells in 96-well plates at 14 days of culture in serum-free medium. Three-independent colonies derived from single cells are shown. (D) FACS analysis of CSCs. Black line, control IgG; red line, corresponding antibody. Data are representative of three independent clonal CSCs. (E) RT-PCR for CSC clones. The numbers on the right indicate the number of individual colonies that expressed the corresponding genes out of the colonies examined. mES, mouse ES cells used as positive control. Data are representative of three independent clonal CSCs. Bars, 20 μm in A,B; 500 μm in C.

TERT-expressing cells in postnatal heart are associated with Sca-1 expression

TERT has been identified as a key factor controlling telomerase activity, telomere length, and cell growth (Blackburn, 2001). We measured the telomerase activity in clonal CSCs. The three individual CSCs displayed significantly elevated telomerase activity (Fig. 2A). To directly characterize TERT-expressing cells in the adult heart, we engineered transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of the mouse *Tert* promoter (Fig. 2B). We identified two transgenic founders by genomic DNA screening and established two independent lines (Fig. 2C). In order to examine the efficacy of mouse *Tert* promoter in the heart in vivo, cells isolated from transgenic hearts were sorted by EGFP signal and the EGFP-positive cells were found to be TERT-expressing cells, which were not detectable in EGFP$^-$ populations (Fig. 2D). As expected from high telomerase activities of clonal CSCs shown in Fig. 2A, three individual CSC clones showed TERT expression (Fig. 2D). To further characterize the TERT-positive cells in the heart, FACS analysis was performed. FACS of the cells prepared from the heart of adult mice expressing TERT-EGFP indicated that TERT^{GFP}-positive cells constitute a population that is positive for Sca-1 but rarely expressing detectable levels of c-kit, CD45, CD34, or CD31 (Fig. 2E).

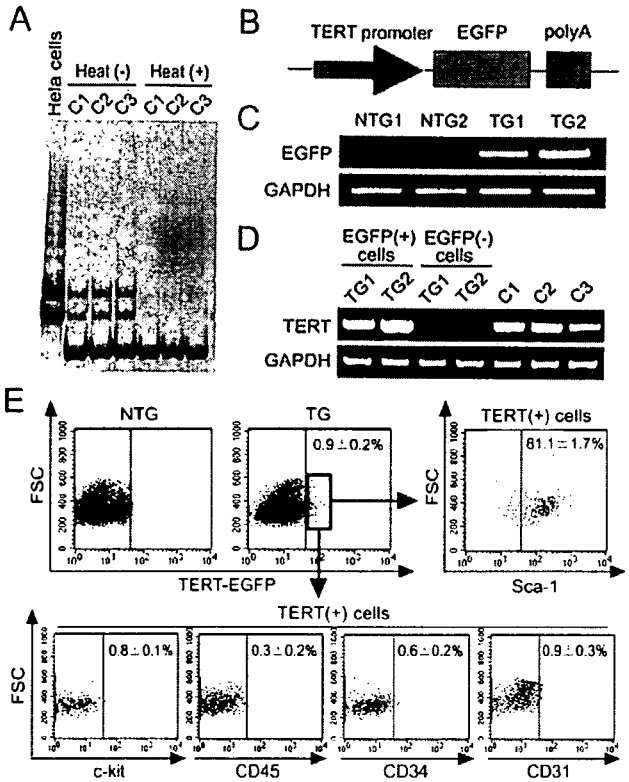

Fig. 2. TERT-expressing cells in adult heart are associated with Sca-1 expression. (A) Telomerase activity was measured by the TRAP assay in three independent clonal-CSCs. The cells were treated with or without heat and used as templates. Hela cells were used as positive controls. (B) Construction of EGFP transgene under the control of the TERT promoter. (C) PCR of genomic DNA from 2 independent TERT-EGFP transgenic lines and respective NTG littermate controls. (D) The expression of TERT on EGFP-positive and EGFP-negative cells sorted from TERT-EGFP transgenic hearts is shown by RT-PCR. The TERT expression was detectable in all three independent clonal CSCs shown in Fig. 1C. (E) FACS analysis of the primary EGFP-positive cells isolated from TERT-EGFP mice (TG). Expression of Sca-1, KIT, CD45, CD34, and CD31 in EGFP-positive cells was examined. Cells from NTG littermates were used as negative control. Data are representative of six independent experiments.

Generation of Sca-1 knockdown (KD) mice

To functionally characterize clonal CSCs, majority of which could be marked by Sca-1 expression, we generated Sca-1 KD mice in which double-stranded (ds)-Sca-1 RNA was expressed under the control of an RNA polymerase II promoter (Fig. 3A) (Shinagawa and Ishii, 2003). The vector pDECAP-Sca-1 expressing ds-Sca-1 RNA with a small loop for transcript pausing and full-length Sca-1 were co-transfected into HEK 293 cells at various concentrations, and the reduction in Sca-1 expression was examined by both RT-PCR and FACS (Fig. 3B,C). Two lines of Sca-1 KD mice were obtained, in which endogenous Sca-1 protein levels (Fig. 3D) in the heart were apparently reduced.

Targeting Sca-1 transcripts affects proliferation and survival but not differentiation of CSCs

To test the function of Sca-1 in CSC development, we examined the ability to clonally proliferate in vitro of single cells from the adult heart of Sca-1 KD and non-transgenic (NTG) littermate mice using a single-cell deposition analysis. This revealed that the percentage of colony-forming cells from Sca-1 KD hearts was significantly lower than that from NTG hearts (Sca-1 KD ~0.007% vs NTG ~0.03%, Fig. 3E). We isolated 11 clones from NTG hearts and four clones from Sca-1 KD hearts, of which eight NTG- and two Sca-1 KD-clones exhibited features of mesenchymal phenotype (data not shown), showed Nanog and Bcrp1 expression by RT-PCR, and could proliferate for more than 14 days (Fig. 3F). Of the clones obtained, four clones expressed brachyury, which is a primitive streak marker (Gadue et al., 2006). Sca-1 expression in CSCs isolated from Sca-1 KD mice was markedly inhibited compared with NTG controls (Fig. 3G). Therefore, we investigated whether Sca-1 expression affects the replicative growth of clonal CSCs in independent cell-culture (Fig. 3H). As shown in Fig. 3I, Sca-1 KD CSCs showed significantly impaired growth kinetics compared with those of NTG CSCs. We determined the molecular mechanisms by which Sca-1 KD mice showed retarded CSC growth. As shown in Fig. 3J, BrdU incorporation and phosphorylation of histone H3 were clearly reduced in the Sca-1 KD CSCs compared with NTG controls, whereas p53 expression levels were significantly increased in the Sca-1 KD CSCs. Telomerase activities were also significantly impaired in the Sca-1 KD CSCs (Fig. 3K).

Of the CSC clones isolated, clones 2, 3 and 6 from NTG and clone 1 from Sca-1 KD mice, all of which expressed brachyury, were chosen for subsequent series of experiments. We asked whether the decrease in clonal CSC growth mediated by Sca-1 KD is associated with an increase in apoptosis. CSCs were isolated from the hearts of NTG and Sca-1 KD mice and were incubated with 100 and 200 μM H_2O_2 for 18 hours, and the surviving cells were analyzed by TUNEL staining. As shown

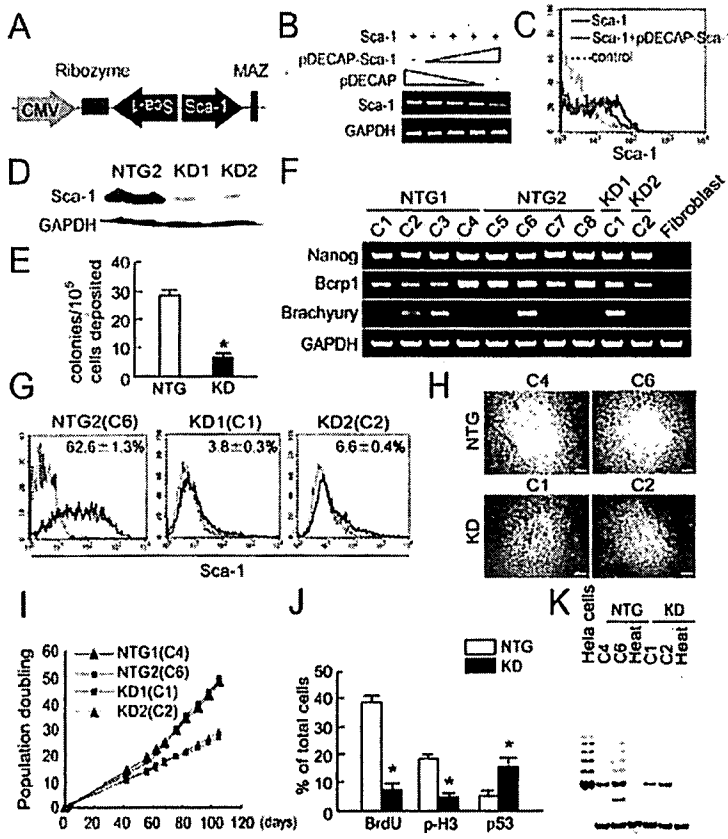

Fig. 3. Generation of Sca-1 KD mice. (A) Construction of the pDECAP-Sca-1 vector. (B,C) Decrease in exogenous Sca-1 expression induced by pDECAP-Sca-1 as shown by RT-PCR (B) and FACS (C) in HEK 293 cells. The total amount (8 μg) of plasmids co-transfected was the same in each experiment (n=3). (D) Decrease in Sca-1 protein levels in the hearts from two independent Sca-1 KD lines. (E) The frequency of CSC colonies from NTG- and Sca-1 KD hearts is shown. Data are expressed as the mean number of colonies formed per 10^5 single cells deposited ± s.e. (n=4). *P<0.01 vs NTG. (F) RT-PCR for embryonic and mesodermal precursor markers. Cardiac fibroblasts were used as negative control. (G) Decrease in Sca-1 expression from two independent Sca-1 KD CSCs clones. Black line, control IgG; red line, Sca-1. (H) Phase-contrast images of respective CSC clones at 14 days of culture in serum-free medium. Bars, 500 μm. (I) Growth kinetics of two independent clonal CSCs isolated from NTG (black lines, C4 and C6) and Sca-1 KD (red lines, C1 and C2) mice. (J) BrdU incorporation, phosphorylated histone-H3 (p-H3) and p53 expression from five independent experiments are shown. *P<0.01 vs NTG. (K) Loss of telomerase activity in the clonal CSCs (C1 and C2) isolated from two independent lines of Sca-1 KD mice.

in Fig. 4A, H_2O_2 induced apoptosis in a dose-dependent manner, and the extent of apoptosis was significantly higher in CSCs isolated from Sca-1 KD hearts than that in NTG-CSCs.

Activation of EGF and bFGF signaling in endothelial cells leads to the phosphorylation of a number of downstream effectors, including Akt and MAPKs (Sulpice et al., 2002). To test the role of these kinases in Sca-1-mediated CSC growth, the activation of Akt and MAPKs in response to EGF and bFGF was examined (Fig. 4B). Incubation of CSCs with EGF and bFGF resulted in a rapid enhancement of Akt, ERK1/2, and JNK1/2, but not in phosphorylation of p38. Although activation of Akt could be abolished by inhibition of Sca-1 transcripts, phosphorylation of three MAPKs was unaffected. These results raise the issue of whether Sca-1-mediated signaling regulates CSC differentiation in vitro. The potential of CSCs to give rise to cardiovascular lineages was not affected by targeting Sca-1 transcripts – as shown by immunostaining (Fig. 5A) and by RT-PCR to assess gene profiles typical of cardiac muscle, smooth muscle and endothelial cell differentiation after specific inductions for 14 days (Fig. 5B),

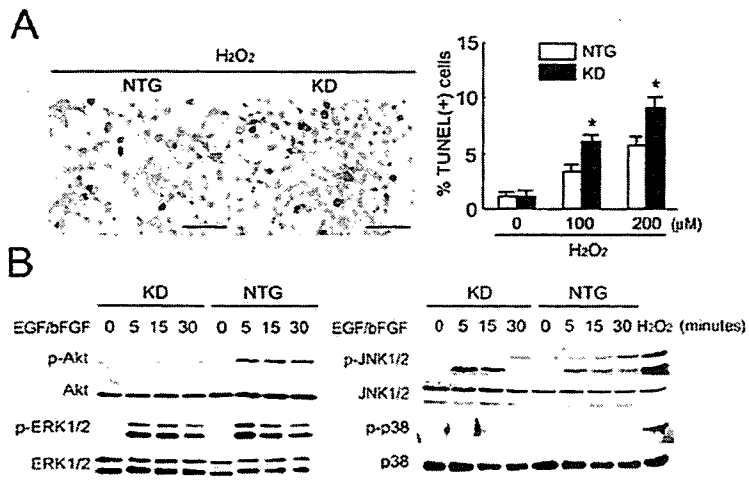

Fig. 4. Sca-1-mediated signaling activates Akt to support CSC proliferation and survival.
(A) Representative photographs of TUNEL assay obtained from NTG- or Sca-1 KD-derived CSCs treated with 200 μM H_2O_2 for 18 hours. The numbers of apoptotic cells (brown nuclei) in NTG (C6)- or Sca-1 KD-CSCs (C1) are shown (n=8). *P<0.01 versus NTG. (B) Loss of Sca-1 diminished EGF and bFGF-induced Akt activation in CSCs. CSCs treated with 200 μm H_2O_2 for 15 minutes were used as positive controls. Bars, 50 μm in A.

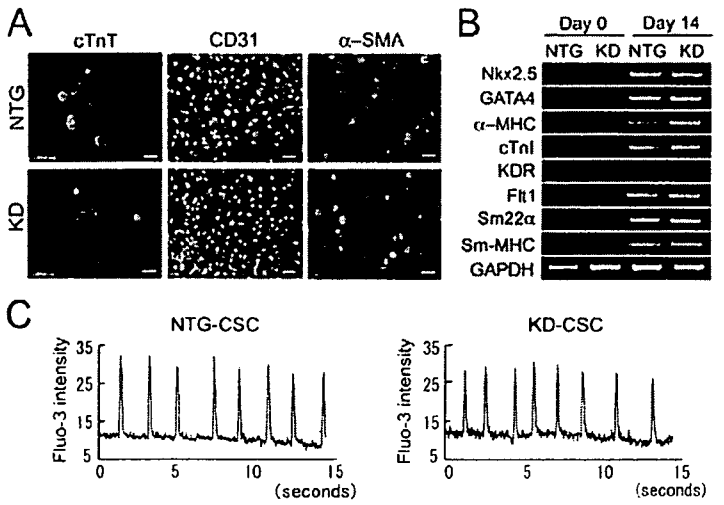

Fig. 5. Loss of Sca-1 transcripts does not affect the differentiation potential of clonal CSCs.
(A) Differentiation of NTG-derived or Sca-1 KD-derived clonal CSCs. Cardiac muscle cell (cardiac troponin-T), endothelial cell (CD31) and vascular smooth muscle cell (α-SMA) differentiation were 1.24±0.3%, 12.4±1.8% and 31.9±2.5%, respectively, for NTG-CSCs (C2, C3 and C6, respectively), and 1.23±0.3%, 12.1±2.1% and 32.2±4.7%, respectively, for Sca-1 KD-CSCs (C1). Nuclei are stained by DAPI (blue). Bars, 20 μm in A. (B) RT-PCR showed that the differentiation potential into the three different lineages were similar for both types of CSCs (n=3).
(C) Representative Ca^{2+} transient in beating cardiomyocytes. Clone 2, 3 and 6 from NTG and clone 1 from Sca-1 KD mice, all expressed brachyury at baseline, were used for analysis. Intensities were corrected by background amplitude and expressed as arbitrary units (n=3).

and a study investigating the Ca^{2+} transient in beating cardiomyocytes (Fig. 5C).

Loss of Sca-1 transcripts in CSCs fails to improve cardiac function due to diminished donor-cell proliferation, survival and engraftment after cell transplantation

The data described above support the hypothesis that loss of Sca-1 results in a retarded regenerative capacity of CSCs in vivo. To further examine this possibility, we performed cell transfer experiments into ischemic myocardium. 5×10^5 CSCs that had been clonally isolated and expanded from the hearts of Sca-1 KD (C1) and NTG (C6) mice were transplanted into wild-type (WT) mice 1 hour after myocardial infarction. Cardiac MRI was performed 4 weeks after cell grafting and showed that transplantation of Sca-1-KD CSCs resulted in significantly larger left ventricular volume and an increased infarct rate as compared with NTG-CSC implantation (Fig. 6A,B).

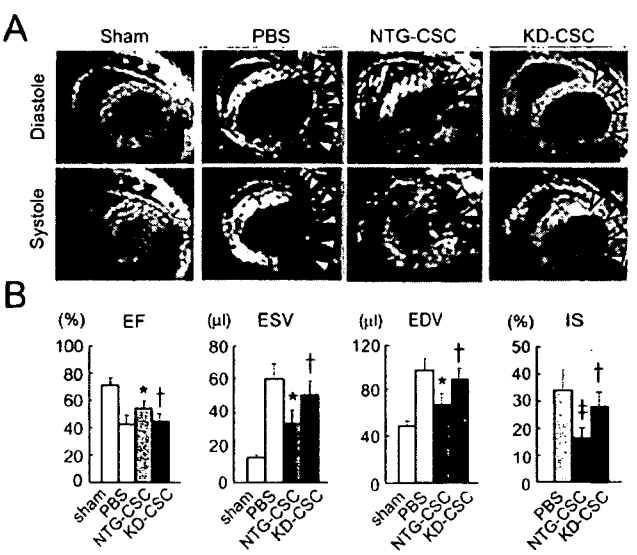

Fig. 6. Sca-1 KD CSC transplantation fails to prevent cardiac remodeling after myocardial infarction. (A,B) WT mice received transplantation of either NTG- or Sca-1 KD mice-derived CSCs 1 hour after infarction. Cardiac MRI was performed 4 weeks after CSC transplantation (n=8). Arrowheads indicate akinetic regions. White bars, sham-operated. Myocardial infarction with PBS injection (light gray bars), NTG-CSC (C6, dark gray bars) or Sca-1 KD-CSC (C1, black bars) transplantation are shown. *P<0.05 vs PBS; †P<0.05 versus NTG-CSC; ‡P<0.01 versus PBS injection. EF, ejection fraction; ESV, end-systolic volume; EDV, end-diastolic volume; IS, infarcted size.

We examined the in-vivo effects of Sca-1-mediated CSC regulation we observed in vitro. At day 3 after CSC transplantation into ischemic myocardium, Sca-1 KD CSCs showed significantly fewer engraftments than NTG-CSCs, as verified by measurement of *lacZ* activity (Fig. 7A). This observation was confirmed by the lower Ki67 expression in Sca-1 KD CSCs, indicating that the proliferative potential was significantly impaired in Sca-1 KD CSCs (Fig. 7A). To assess whether Sca-1-mediated control of CSC survival may also be applied to the process of donor-cell engraftment, we analyzed the viability of grafted CSCs, labeled by β-galactosidase (β-gal) staining, on day 3 after the cell grafting. As shown in Fig. 7B, grafted Sca-1 KD CSCs in the ischemic myocardium resulted in more apoptotic cells than NTG-CSCs, suggesting that the transplanted Sca-1 KD CSCs were also susceptible to cell death in vivo.

To further test whether these effects of Sca-1 during the early phase of CSC transplantation may contribute to the early CSC-engraftment and late regeneration process of cardiovascular-lineage cells, we investigated the presence of *lacZ*$^+$ donor cells at day 7 and characterized their individual phenotypes 4 weeks after transplantation. As shown in Fig. 8A, the frequency of *lacZ*$^+$ cells observed 7 days post cell transfer was significantly lower in Sca-1 KD CSC grafts compared with NTG-CSC transplantation, resulting in substantially insufficient cardiovascular regeneration within the ischemic regions 4 weeks after CSC transplantation (Fig. 8B-D).

Sca-1 KD CSC transplantation fails to prevent myocardial apoptosis and limits angiogenesis, partially due to the failure of paracrine effector secretion

Last, we assessed whether the loss of Sca-1 in transplanted

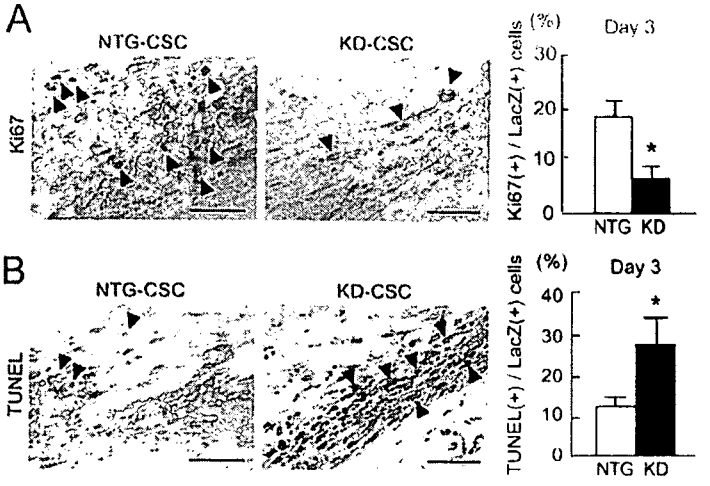

Fig. 7. Sca-1 transcripts are required for CSC proliferation and survival in vivo. (A) Immunohistochemistry of transplanted *lacZ*$^+$ cells 3 days after infarction. Transplanted *lacZ*$^+$ cells entering cell cycle were detected as Ki67-positive cells (arrowheads) (n=6). Myocardial infarction transplanted with NTG-CSCs (C6) and Sca-1 KD CSCs (C1) are shown. *P<0.01 versus NTG. (B) Apoptotic features (arrowheads, brown nuclei) of *lacZ*$^+$ engrafted cells are shown at day 3 after NTG- or Sca-1 KD-CSC transplantation. *P<0.01 versus NTG-CSC transplantation (n=6). Bars, 50 μm in A,B.

CSCs affects myocardial apoptosis and angiogenesis. At day 3, transplantation of Sca-1 KD CSCs resulted in a high level of myocardial apoptosis in the ungrafted area of the infarcted border zone, whereas fewer TUNEL-positive cells were observed in NTG-CSC-injected hearts (Fig. 9A). Furthermore, transplantation of Sca-1 KD CSCs failed to improve capillary density 2 weeks after infarction in the ischemic region as compared with NTG-CSC injection (Fig. 9B). To explore the molecular mechanisms of Sca-1-mediated myocardial apoptosis and neoangiogenesis, we then oxygen-starved CSCs for 8 hours and measured the levels of mRNA for secreted paracrine factors by RT-PCR. As shown in Fig. 9C, downregulation of hepatocyte growth factor (HGF) in Sca-1 KD CSCs was evident at baseline normoxia. After hypoxia, a greater increase in the expression of VEGF and HGF was observed in NTG-CSCs compared with that in Sca-1 KD CSCs. The expression pattern of insulin-like growth factor-1 (IGF1) under normoxic and hypoxic conditions was comparable in CSCs from NTG and Sca-1 KD hearts.

Discussion

Recent reports have shown that clonogenic CSCs reside in

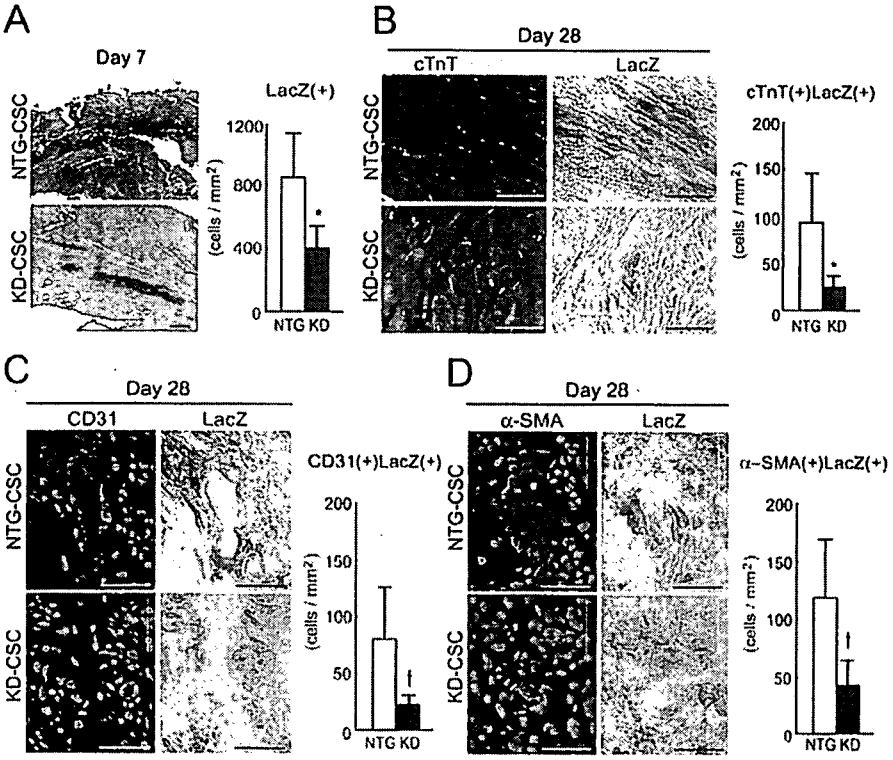

Fig. 8. Loss of Sca-1 transcripts in clonal CSC transplantation shows less donor-cell engraftment, resulting in the decrease in late cardiovascular regeneration. (A) Engrafted *lacZ*$^+$ cells in NTG (C6)- and Sca-1 KD-CSC (C1)-transplanted hearts at day 7 after infarction. Sections were counterstained using H&E. (B-D) The representative images and frequencies of cardiomyocytes (cardiac troponin-T, red), and endothelial (CD31) and smooth muscle cells (α-SMA) in *lacZ*$^+$ cells at day 28 are shown (n=6). Note that differentiated *lacZ*$^+$ cardiomyocytes co-express connexin-43 (yellow). Bars, 100 μm in A; 20 μm in B; 50 μm in C,D.

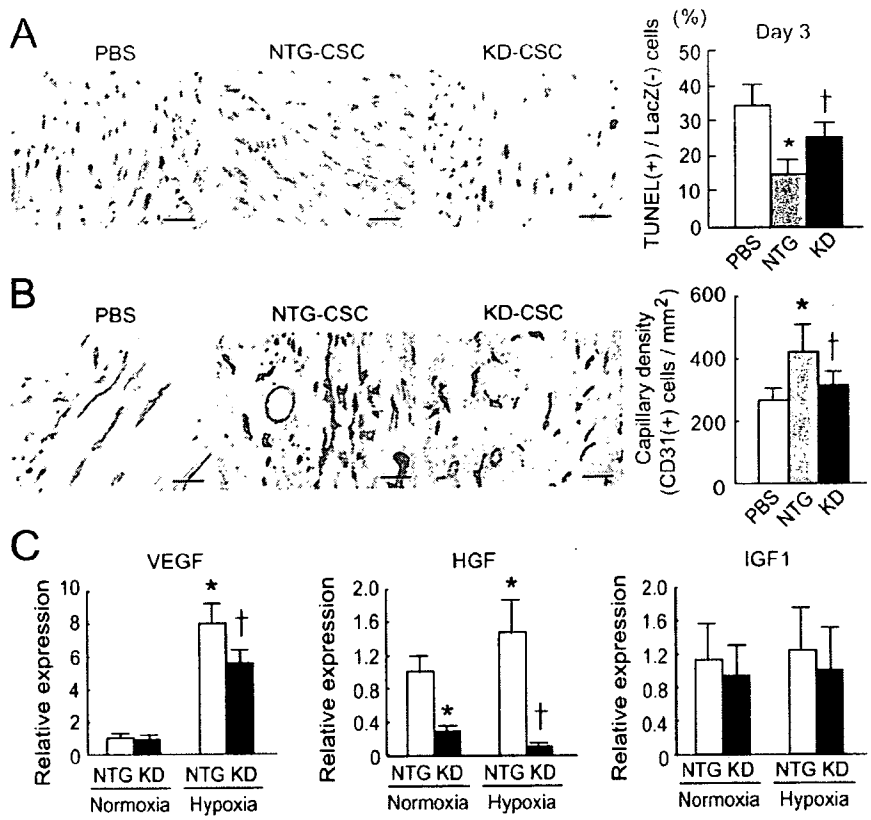

Fig. 9. Transplantation of Sca-1 KD CSCs fails to prevent myocardial apoptosis and limits neoangiogenesis after myocardial infarction partially due to the failure of paracrine effector secretion. (A) TUNEL staining revealed the apoptotic cardiomyocytes in the border zone of PBS-treated, NTG (C6)- and Sca-1 KD-CSC (C1)-transplanted hearts at day 3; (n=6). *P<0.01 vs PBS. †P<0.01 vs NTG. (B) Capillary density of infarcted border zone of transplanted hearts at day 14 after myocardial infarction. Capillary density was measured by staining of CD31 (brown) and corrected by the area analyzed. (n=8). *P<0.01 vs PBS-treated mice; †P<0.01 vs NTG-CSC-transplanted mice. Bars, 50 μm in A,B. (C) Relative mRNA expression levels of VEGF, HGF, and IGF1 normalized by 18S expression in NTG- and Sca-1 KD-CSCs under normoxic and hypoxic conditions (n=7). *P<0.01 vs NTG-CSCs under normoxia; †P<0.01 vs NTG-CSCs under hypoxia.

mammalian hearts, judging on the basis of specific cell-surface markers which are also expressed by hematopoietic and endothelial progenitor cells (Beltrami et al., 2003; Linke et al., 2005). Using an unbiased approach, our study has demonstrated that clonally proliferated CSCs express Sca-1 with ES cell-like and mesenchymal-cell-like characteristics, and are associated with TERT expression.

Our results, showing low expression of KIT in clonal *Kit*$^+$ CSCs, differ from the findings of some previous studies (Linke et al., 2005; Messina et al., 2004) but are consistent with those of recent reports about the adult heart (Fazel et al., 2006; Gude et al., 2006; Matsuura et al., 2004; Oh et al., 2003; Pfister et al., 2005; Tateishi et al., 2007). The reasons for this discrepancy are unclear. However, retrospective analysis data that directly sorted TERT-expressing cells from TERT-EGFP hearts (that were genetically isolated without the modification by cell culture) have shown that the majority of heart-resident TERT-positive cells could be identified via the expression of Sca-1. This implies that our findings were neither the result of intra-clonal variability nor due to contamination by cardiac fibroblasts during cell expansion. Recent report demonstrated that the expression levels of Sca-1 and KIT appear to be changed during myocardial maturation in ES cells (Wu et al., 2006).

Mesenchymal stem cells have been isolated from many tissues, including human heart (da Silva Meirelles et al., 2006; Tateishi et al., 2007). It is notable that CSCs expressed general characteristics of mesenchymal stem cells according to the analysis of cell-surface markers and partially showed the embryonic factors, as previously reported in clonal amniotic fluid-derived mesenchymal stem cells (Tsai et al., 2006). These observations indicated that the epithelial-mesenchymal transition (EMT) may occur in adult CSCs to produce proliferative precursors, which may undergo a reversible commitment into the directions of either mesenchymal or cardiac lineage, depending on the inductive conditions (Wessels and Perez-Pomares, 2004). Several reports suggest that the source of CSCs may include the neural crest (Tomita et al., 2005), primitive epicardial cells (Hay, 2005) or perivascular cells (da Silva Meirelles et al., 2006) through the EMT.

Regulating stem cell self-renewal is an essential feature of the niche where stem cells must be exposed to sufficient intrinsic-factors to maintain the proper stem cell number for the demands of tissue repair. We focused on Sca-1-mediated regulation in CSCs for the first time and found that normal Sca-1 function is associated with CSC proliferation and survival, contributing to early donor-cell engraftment and late cardiovascular differentiation, which is consistent with the prevailing view of the role of Sca-1 in the ability of hematopoietic stem cells and bone-marrow-derived mesenchymal stem cells to self-replicate (Bonyadi et al., 2003; Ito et al., 2003). Although the function of Sca-1 in skeletal muscle progenitors was not consistent with our observations in CSCs, the cell fate decision might be cell-type specific and/or age dependent (Mitchell et al., 2005). The mode of action of *Lin*$^-$*-Kit*$^+$ cells in the heart or bone marrow has been intensively investigated in gain- (Dawn et al., 2006; Urbanek et al., 2005a)

and loss-of-function experiments, and the function of these cells was validated by bone marrow reconstitution studies to completely rescue the defective cardiac repair in c-kit mutant mice after infarction (Ayach et al., 2006; Fazel et al., 2006), consistent with the lack of cardiac decompensation after pressure-overload in c-kit mutant mice (Hara et al., 2002) and our present observation indicating the rarity of c-kit$^+$ cells in TERT-expressing CSCs.

Sca-1 was originally identified as an antigen upregulated in activated lymphocytes, and was shown to be linked to the lipid bilayer as a glycosyl phosphatidylinositol (PtdIns)-anchored protein that activates cell signaling via mediators such as Akt (Reiser et al., 1986). The proliferation of CSCs appears to be dependent on the capacity of the cells to undergo cell cycle progression through the phosphorylation of Akt in response to EGF and bFGF stimulation, as observed in neural stem cells (Groszer et al., 2006). Our observations are supported by two independent gain-of-function studies demonstrating that the nuclear-targeting of Akt leads to the rapid expansion of comparatively rare *Kit*$^+$ CSCs in the postnatal heart (Gude et al., 2006), and ex-vivo transduction of Akt to bone marrow-derived MSCs can functionally repair the ischemic myocardium through the upregulation of secreted paracrine effectors (Gnecchi et al., 2006; Jiang et al., 2006). Consistent with these studies, our present study also demonstrated that the functional improvement of damaged myocardium after CSCs transplantation was attenuated by Sca-1 KD, in which new vessel formation and inhibition of myocardial apoptosis by release of angiogenic growth factors and myocyte regeneration by grafted CSCs were severely impaired.

Taken together, our results suggest that Sca-1 is expressed in the majority of intrinsic CSCs in the adult heart, which have characteristics of ES-like and mesenchymal-like cells, and implicate the role of Sca-1 in CSC maintenance and function. Sca-1-mediated signaling is important in CSC development in normal circumstances and its beneficial effect might be involved the responses to hypoxic and ischemic conditions. The cardioprotective effect of CSC transplantation that we have shown here indicates that Sca-1-mediated ligand responses may participate in the production of angiogenic and antiapoptotic paracrine effectors, consistent with recent observations demonstrating that induction of VEGF and HGF activates bone marrow-derived mesenchymal stem cells through PI 3-kinase–Akt pathway (Forte et al., 2006; Okuyama et al., 2006). It will be of interest to assess the gene expression profile in CSCs by targeting Sca-1 transcripts to identify the factors responsible for optimizing CSC therapy in heart failure.

Materials and Methods

Clonal isolation and culture of CSCs

Hearts from 6-week-old to-12-week-old GFP transgenic mice (provided by M. Okabe, Osaka University Medical School) (Okabe et al., 1997), Sca-1 KD mice or NTG mice were excised and were perfused with cold PBS to remove the blood cells. The tissues were washed twice, and aortic and pulmonary vessels were removed from the hearts. The dissected hearts were minced, and digested twice for 20 minutes at 37°C with 0.2% type II collagenase and 0.01% DNAse I (Worthington Biochemical Corp, NJ). The cells were passed through a 40-μm filter to remove the debris and were plated into 25-cm^2 dishes in DMEM (Invitrogen) for 30 minutes to allow fibroblasts to adhere. The non-adherent cells were collected and size-fractionated with a 30-70% Percoll gradient to obtain single-cell suspensions by removal of mature cardiomyocytes. For clonal analysis, the resulting cell suspensions were plated in 96-well plates at 1 cell per 100 μl by the limiting dilution technique (Yoon et al., 2005) with serum-free growth medium: DMEM/F12 containing B27 supplement, 20 ng/ml EGF (Sigma), and 40 ng/ml bFGF (Promega). Wells were visually inspected 24 hours after plating to exclude those containing more than one cell per well; then, clones derived from a single cell were further cultivated. On day 14, clonally expanded CSCs from single cells were cultured in low-serum medium consisting of growth medium supplemented with 1×B27 supplement, 2% FBS, and 10 ng/ml leukemia inhibitory factor (CHEMICON). At 60-70% confluence, cells from individual clones were serially reseeded in six-well plates, 25-cm^2, 75-cm^2 and 175-cm^2 flasks for further expansion. Hypoxic conditions were created by incubating cells at 37°C in a CO_2 multi-gas incubator (ASTEC) with an atmosphere of 5% CO_2 and 95% N_2 for 8 hours.

CSC differentiation

For cardiac differentiation analysis, single-cell-derived CSCs were cultured in differentiation medium containing 10% FBS, insulin-transferrin-selenium, and 10 nM dexamethasone (Sigma) for 14 days. Differentiation medium consisting of DMEM/F12 supplemented with 10 ng/ml VEGF or 50 ng/ml PDGF-BB (both from R&D Systems) and 10% FBS was used to induce endothelial and smooth muscle cell differentiation for 14 days, respectively.

Construction of targeting vector and generation of transgenic mice

Full-length Sca-1 cDNA was cloned using the following primers: forward: 5′-CTCTGAGGATGGACACTTCT-3′, reverse: 5′-GGTCTGCAGGAGGACTGA-GC-3′. The 404-bp ds-RNA fragment targeting the N-terminus of Sca-1 was selectively amplified and subcloned into the pDECAP vector (Shinagawa and Ishii, 2003). The plasmid encoding EGFP driven by the mouse *Tert* promoter was provided by N. Hole (University of Durham) (Armstrong et al., 2000) and subcloned into the human growth hormone polyadenylation sequence. Each expression cassette was released and microinjected into the pronuclei of fertilized C57BL/6 oocytes. PCR analysis of tail DNA was used to identify founder transgenic mice.

Retroviral transduction of CSCs

To track cells after injection into the infarcted myocardium, CSCs were engineered to express the bacterial *lacZ* reporter gene. This was done by retroviral infection with a vector (pMSCV-LacZ) encoding the *lacZ* gene and a puromycin resistance gene. After selection with puromycin, the transduction efficiency was evaluated by X-gal staining.

FACS analysis and cell sorting

Single-cell suspensions were stained with the following antibodies: phycoerythrin (PE)-conjugated antibodies against Sca-1, KIT, CD45, CD44, CD90, CD31, CD73, CD106, CD34, CD13, CD29, and isotype control IgG (all from BD Biosciences). Allophycocyanin (APC)-conjugated goat anti-rat IgG was used to detect rat anti-mouse CD105 (Southern Biotech). Dead cells were eliminated using propidium iodide (Sigma) and 10,000 to 50,000 events were collected per sample using a FACS Calibur flow cytometer (BD Biosciences). Bone marrow cells were flushed from the tibiae and femurs of 6-week-old to 12-week-old C57BL/6 mice and compared (with or without collagenase and filtration) (Oh et al., 2003). Single-cell suspensions were harvested from TERT-EGFP transgenic and NTG hearts as the method for CSC preparation, and the EGFP-positive cells were analyzed and sorted on BD FACSAria (Becton Dickinson).

RT-PCR and telomerase activity

Total RNA was prepared from cultured cells using TRIzol (Invitrogen) and cDNA was generated with the SuperScript III First-Strand Synthesis System (Invitrogen). PCR reactions were performed with gene-specific primers. Primer sequences are available on request. To evaluate VEGF, HGF, and IGF1 expression, cDNA was subjected to 40 rounds of amplification (ABI PRISM 7700, Applied Biosystems) with Assay-on-Demand™ primer-probes sets (Applied Biosystems). The mRNA levels were expressed relative to an endogenous control (18S RNA) and the fold-increase in the respective groups versus normoxia NTG-CSCs was calculated. Telomerase activity of single-cell-derived CSCs was measured using a TRAP assay kit, TRAPEZE (CHEMICON), as previously described (Oh et al., 2001).

Calcium transient

Cells were washed three times with 1 mM Ca^{2+} Tyrode's solution as previously described (Kaneko et al., 2000), additional 15 minutes incubation with 1 mM Ca^{2+} Tyrode's containing 1 mM probenecid at 37°C was performed to allow hydrolysis of acetoxymethyl esters within the cells. Fluorescence imaging was performed at 24°C using a fixed-stage microscope (BX50WI, Olympus, Japan) equipped with a multi-pinhole-type confocal scanning system (CSU-21, Yokogawa, Japan). Digitized fluorescence signals were analyzed with Image J software.

Western blotting

Whole protein lysates were extracted with lysis buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF and protease inhibitor (PIERCE). For phosphorylation of Akt, ERK1/2, JNK1/2 and p38, 1 mM Na_3VO_4 and 1 mM NaF were added. Transferred

membranes were incubated with rat anti-mouse Sca-1 monoclonal antibody (clone D7, BD Biosciences), antibodies against phosphorylated Akt (S473), Akt, phosphorylated ERK1/2 (T202/Y204), ERK1/2, phosphorylated SAPK/JNK (Thr 183/Tyr185), SAPK/JNK, phosphorylated p38 MAPK (Thr180/Tyr182), p38 MAPK (all from Cell Signaling), or mouse monoclonal anti-GAPDH (Chemicon). Horseradish peroxidase (HRP)-conjugated goat anti-rat IgG, HRP-conjugated sheep anti-mouse IgG and HRP-conjugated donkey anti-rabbit IgG (Amersham Biosciences) were used as secondary antibodies.

Sample fixation and X-gal staining

Hearts were fixed in 1% paraformaldehyde, 0.2% glutaraldehyde, and 0.2% Nonidet P-40. X-gal staining was performed with the following reagents: 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.2% Nonidet P-40, and 1 mg/ml X-gal (Invitrogen). After staining, samples were post-fixed with 4% paraformadehyde and embedded in frozen OCT compound or paraffin.

Histology and immunofluorescence

Fixed cells and frozen sections were stained using the following primary antibodies: mouse anti-cardiac troponin-T (Ab1, Neo Markers), rat anti-mouse CD31 (BD Biosciences), Cy3-conjugated anti-α-SMA (Sigma), rabbit anti-p53 (FL-393, Santa Cruz) and rabbit anti-phosphorylated histone H3 (Ser10, Upstate). Secondary antibodies were conjugated to Alexa Fluor 555 or Alexa Fluor 568, and nuclei were visualized using DAPI (Molecular Probes). BrdU incorporation was examined by incubation with 10 μM BrdU for 1 hour using a detection kit (Roche). For Ki67 immunohistochemistry, we used a Vectastain ABC Elite kit (Vector Laboratories). After antigen retrieval using citrate buffer (pH 6.0) and blockage of endogenous peroxidase activity using 0.3% hydrogen peroxide, the sections were incubated with rat anti-mouse Ki67 antibody (DAKO) for 1 hour at room temperature. Then, the sections were treated with biotinylated secondary antibody followed by incubation with avidin horseradish peroxidase complex. Finally, the sections were counterstained with hematoxylin or H&E staining. Capillary density was estimated by CD31 immunostaining with a Vectastain ABC Elite kit. Apoptotic CSCs or cardiomyocytes were evaluated by the TUNEL assay in fixed cells and paraffin-embedded sections with an ApopTag kit (Chemicon). H_2O_2 was purchased from Wako. Images were captured with a BZ-8000 (Keyence, Japan) and IX 71 (Olympus Corporation, Japan).

Myocardial infarction and cell grafting

Ligation of the left anterior descending (LAD) coronary artery was performed in 12-week-old to 24-week-old C57BL/6 mice (Shimizu Laboratory Supplies, Japan) in accordance with the animal care and use guidelines at Kyoto University Hospital. One hour after the LAD ligation, 5×10^5 cells were suspended in 20 μl of PBS and injected into two sites of the infarcted border zone. In the control group, mice were sham-operated by receiving a left thoracotomy without coronary artery ligation.

Cardiac function and infarct size

Cardiac MRI studies were performed using a 7 T MR scanner, Unity Inova (Varian Inc., Palo Alto, CA) with a 25-mm home-built solenoid-type volume coil. Analysis of end-systolic and end-diastolic LV volumes and LV mass was done using an operator-interactive threshold technique, and stroke volume and cardiac output were calculated. All measurements were performed and analyzed by an individual blinded to the animal group. For in vivo determination of infarct size, end-diastolic epicardial and endocardial contours were traced on the MRI short-axis slices; only akinetic and dyskinetic segments were considered to be infarcted areas (Yang et al., 2004).

Statistics

Data are expressed as the mean ± s.e. Two-tailed Student's *t* test was used to compare the clonality of Sca-1 KD- and NTG-CSCs. Comparison of groups in remaining experiments was unpaired analyses using two-tailed Student's *t* test. Significance level was set at $P<0.05$ (StatView).

We thank the following investigators for their kind gifts of mice or plasmids: M. Okabe, N. Hole, S. Ishii and Y. Yoshida, A. Kosugi. We also thank M. Nishikawa for technical assistance. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, and by Grants-in-Aid from the Ministry of Health, Labor and Welfare.

References

Armstrong, L., Lako, M., Lincoln, J., Cairns, P. M. and Hole, N. (2000). mTert expression correlates with telomerase activity during the differentiation of murine embryonic stem cells. *Mech. Dev.* **97**, 109-116.

Ayach, B. B., Yoshimitsu, M., Dawood, F., Sun, M., Arab, S., Chen, M., Higuchi, K., Siatskas, C., Lee, P., Lim, H. et al. (2006). Stem cell factor receptor induces progenitor and natural killer cell-mediated cardiac survival and repair after myocardial infarction. *Proc. Natl. Acad. Sci. USA* **103**, 2304-2309.

Beltrami, A. P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., Urbanek, K. et al. (2003). Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114**, 763-776.

Blackburn, E. H. (2001). Switching and signaling at the telomere. *Cell* **106**, 661-673.

Bonyadi, M., Waldman, S. D., Liu, D., Aubin, J. E., Grynpas, M. D. and Stanford, W. L. (2003). Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. *Proc. Natl. Acad. Sci. USA* **100**, 5840-5845.

da Silva Meirelles, L., Chagastelles, P. C. and Nardi, N. B. (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J. Cell Sci.* **119**, 2204-2213.

Dawn, B., Guo, Y., Rezazadeh, A., Huang, Y., Stein, A. B., Hunt, G., Tiwari, S., Varma, J., Gu, Y., Prabhu, S. D. et al. (2006). Postinfarct cytokine therapy regenerates cardiac tissue and improves left ventricular function. *Circ. Res.* **98**, 1098-1105.

Fazel, S., Cimini, M., Chen, L., Li, S., Angoulvant, D., Fedak, P., Verma, S., Weisel, R. D., Keating, A. and Li, R. K. (2006). Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. *J. Clin. Invest.* **116**, 1865-1877.

Forte, G., Minieri, M., Cossa, P., Antenucci, D., Sala, M., Gnocchi, V., Fiaccavento, R., Carotenuto, F., De Vito, P., Baldini, P. M. et al. (2006). Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation. *Stem Cells* **24**, 23-33.

Gadue, P., Huber, T. L., Paddison, P. J. and Keller, G. M. (2006). Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **103**, 16806-16811.

Gnecchi, M., He, H., Noiseux, N., Liang, O. D., Zhang, L., Morello, F., Mu, H., Melo, L. G., Pratt, R. E., Ingwall, J. S. et al. (2006). Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J.* **20**, 661-669.

Groszer, M., Erickson, R., Scripture-Adams, D. D., Dougherty, J. D., Le Belle, J., Zack, J. A., Geschwind, D. H., Liu, X., Kornblum, H. I. and Wu, H. (2006). PTEN negatively regulates neural stem cell self-renewal by modulating G0-G1 cell cycle entry. *Proc. Natl. Acad. Sci. USA* **103**, 111-116.

Gude, N., Muraski, J., Rubio, M., Kajstura, J., Schaefer, E., Anversa, P. and Sussman, M. A. (2006). Akt promotes increased cardiomyocyte cycling and expansion of the cardiac progenitor cell population. *Circ. Res.* **99**, 381-388.

Hara, M., Ono, K., Hwang, M. W., Iwasaki, A., Okada, M., Nakatani, K., Sasayama, S. and Matsumori, A. (2002). Evidence for a role of mast cells in the evolution to congestive heart failure. *J. Exp. Med.* **195**, 375-381.

Hay, E. D. (2005). The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. *Dev. Dyn.* **233**, 706-720.

Ito, C. Y., Li, C. Y., Bernstein, A., Dick, J. E. and Stanford, W. L. (2003). Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. *Blood* **101**, 517-523.

Jiang, S., Haider, H., Idris, N. M., Salim, A. and Ashraf, M. (2006). Supportive interaction between cell survival signaling and angiocompetent factors enhances donor cell survival and promotes angiomyogenesis for cardiac repair. *Circ. Res.* **99**, 776-784.

Joannides, A., Gaughwin, P., Schwiening, C., Majed, H., Sterling, J., Compston, A. and Chandran, S. (2004). Efficient generation of neural precursors from adult human skin: astrocytes promote neurogenesis from skin-derived stem cells. *Lancet* **364**, 172-178.

Kaneko, T., Tanaka, H., Oyamada, M., Kawata, S. and Takamatsu, T. (2000). Three distinct types of Ca(2+) waves in Langendorff-perfused rat heart revealed by real-time confocal microscopy. *Circ. Res.* **86**, 1093-1099.

Kawada, H., Fujita, J., Kinjo, K., Matsuzaki, Y., Tsuma, M., Miyatake, H., Muguruma, Y., Tsuboi, K., Itabashi, Y., Ikeda, Y. et al. (2004). Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. *Blood* **104**, 3581-3587.

Laugwitz, K. L., Moretti, A., Lam, J., Gruber, P., Chen, Y., Woodard, S., Lin, L. Z., Cai, C. L., Lu, M. M., Reth, M. et al. (2005). Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433**, 647-653.

Leri, A., Barlucchi, L., Limana, F., Deptala, A., Darzynkiewicz, Z., Hintze, T. H., Kajstura, J., Nadal-Ginard, B. and Anversa, P. (2001). Telomerase expression and activity are coupled with myocyte proliferation and preservation of telomeric length in the failing heart. *Proc. Natl. Acad. Sci. USA* **98**, 8626-8631.

Limana, F., Germani, A., Zacheo, A., Kajstura, J., Di Carlo, A., Borsellino, G., Leoni, O., Palumbo, R., Battistini, L., Rastaldo, R. et al. (2005). Exogenous high-mobility group box 1 protein induces myocardial regeneration after infarction via enhanced cardiac C-kit+ cell proliferation and differentiation. *Circ. Res.* **97**, e73-e83.

Linke, A., Muller, P., Nurzynska, D., Casarsa, C., Torella, D., Nascimbene, A., Castaldo, C., Cascapera, S., Bohm, M., Quaini, F. et al. (2005). Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. *Proc. Natl. Acad. Sci. USA* **102**, 8966-8971.

Matsuura, K., Nagai, T., Nishigaki, N., Oyama, T., Nishi, J., Wada, H., Sano, M., Toko, H., Akazawa, H., Sato, T. et al. (2004). Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. *J. Biol. Chem.* **279**, 11384-11391.

Messina, E., De Angelis, L., Frati, G., Morrone, S., Chimenti, S., Fiordaliso, F., Salio, M., Battaglia, M., Latronico, M. V., Coletta, M. et al. (2004). Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ. Res.* **95**, 911-921.

Mitchell, P. O., Mills, T., O'Connor, R. S., Graubert, T., Dzierzak, E. and Pavlath, G. K. (2005). Sca-1 negatively regulates proliferation and differentiation of muscle cells. *Dev. Biol.* **283**, 240-252.

Moretti, A., Caron, L., Nakano, A., Lam, J. T., Bernshausen, A., Chen, Y., Qyang, Y., Bu, L., Sasaki, M., Martin-Puig, S. et al. (2006). Multipotent embryonic Isl1(+)

progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell* **127**, 1151-1165.

Murry, C. E., Soonpaa, M. H., Reinecke, H., Nakajima, H., Nakajima, H. O., Rubart, M., Pasumarthi, K. B., Virag, J. I., Bartelmez, S. H., Poppa, V. et al. (2004). Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* **428**, 664-668.

Oh, H., Taffet, G. E., Youker, K. A., Entman, M. L., Overbeek, P. A., Michael, L. H. and Schneider, M. D. (2001). Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival. *Proc. Natl. Acad. Sci. USA* **98**, 10308-10313.

Oh, H., Bradfute, S. B., Gallardo, T. D., Nakamura, T., Gaussin, V., Mishina, Y., Pocius, J., Michael, L. H., Behringer, R. R., Garry, D. J. et al. (2003). Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc. Natl. Acad. Sci. USA* **100**, 12313-12318.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. (1997). 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407**, 313-319.

Okuyama, H., Krishnamachary, B., Zhou, Y. F., Nagasawa, H., Bosch-Marce, M. and Semenza, G. L. (2006). Expression of vascular endothelial growth factor receptor 1 in bone marrow-derived mesenchymal cells is dependent on hypoxia-inducible factor 1. *J. Biol. Chem.* **281**, 15554-15563.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M. et al. (2001). Bone marrow cells regenerate infarcted myocardium. *Nature* **410**, 701-705.

Pfister, O., Mouquet, F., Jain, M., Summer, R., Helmes, M., Fine, A., Colucci, W. S. and Liao, R. (2005). CD31– but not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. *Circ. Res.* **97**, 52-61.

Pittenger, M. F. and Martin, B. J. (2004). Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ. Res.* **95**, 9-20.

Reiser, H., Oettgen, H., Yeh, E. T., Terhorst, C., Low, M. G., Benacerraf, B. and Rock, K. L. (1986). Structural characterization of the TAP molecule: a phosphatidylinositol-linked glycoprotein distinct from the T cell receptor/T3 complex and Thy-1. *Cell* **47**, 365-370.

Rota, M., LeCapitaine, N., Hosoda, T., Boni, A., De Angelis, A., Padin-Iruegas, M. E., Esposito, G., Vitale, S., Urbanek, K., Casarsa, C. et al. (2006). Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the p66shc gene. *Circ. Res.* **99**, 42-52.

Shinagawa, T. and Ishii, S. (2003). Generation of Ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter. *Genes Dev.* **17**, 1340-1345.

Sulpice, E., Bryckaert, M., Lacour, J., Contreres, J. O. and Tobelem, G. (2002). Platelet factor 4 inhibits FGF2-induced endothelial cell proliferation via the extracellular signal-regulated kinase pathway but not by the phosphatidylinositol 3-kinase pathway. *Blood* **100**, 3087-3094.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676.

Tateishi, K., Ashihara, E., Honsho, S., Takehara, N., Nomura, T., Takahashi, T., Ueyama, T., Yamagishi, M., Yaku, H., Matsubara, H. et al. (2007). Human cardiac stem cells exhibit mesenchymal features and are maintained through Akt/GSK-3beta signaling. *Biochem. Biophys. Res. Commun.* **352**, 635-641.

Tomita, Y., Matsumura, K., Wakamatsu, Y., Matsuzaki, Y., Shibuya, I., Kawaguchi, H., Ieda, M., Kanakubo, S., Shimazaki, T., Ogawa, S. et al. (2005). Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. *J. Cell Biol.* **170**, 1135-1146.

Tsai, M. S., Hwang, S. M., Tsai, Y. L., Cheng, F. C., Lee, J. L. and Chang, Y. J. (2006). Clonal amniotic fluid-derived stem cells express characteristics of both mesenchymal and neural stem cells. *Biol. Reprod.* **74**, 545-551.

Urbanek, K., Quaini, F., Tasca, G., Torella, D., Castaldo, C., Nadal-Ginard, B., Leri, A., Kajstura, J., Quaini, E. and Anversa, P. (2003). Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. *Proc. Natl. Acad. Sci. USA* **100**, 10440-10445.

Urbanek, K., Rota, M., Cascapera, S., Bearzi, C., Nascimbene, A., De Angelis, A., Hosoda, T., Chimenti, S., Baker, M., Limana, F. et al. (2005a). Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. *Circ. Res.* **97**, 663-673.

Urbanek, K., Torella, D., Sheikh, F., De Angelis, A., Nurzynska, D., Silvestri, F., Beltrami, C. A., Bussani, R., Beltrami, A. P., Quaini, F. et al. (2005b). Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. *Proc. Natl. Acad. Sci. USA* **102**, 8692-8697.

Urbanek, K., Cesselli, D., Rota, M., Nascimbene, A., De Angelis, A., Hosoda, T., Bearzi, C., Boni, A., Bolli, R., Kajstura, J. et al. (2006). Stem cell niches in the adult mouse heart. *Proc. Natl. Acad. Sci. USA* **103**, 9226-9231.

Wessels, A. and Perez-Pomares, J. M. (2004). The epicardium and epicardially derived cells (EPDCs) as cardiac stem cells. *Anat. Rec. A Discov. Mol. Cell. Evol. Biol.* **276**, 43-57.

Wu, S. M., Fujiwara, Y., Cibulsky, S. M., Clapham, D. E., Lien, C. L., Schultheiss, T. M. and Orkin, S. H. (2006). Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the Mammalian heart. *Cell* **127**, 1137-1150.

Yang, Z., Berr, S. S., Gilson, W. D., Toufektsian, M. C. and French, B. A. (2004). Simultaneous evaluation of infarct size and cardiac function in intact mice by contrast-enhanced cardiac magnetic resonance imaging reveals contractile dysfunction in noninfarcted regions early after myocardial infarction. *Circulation* **109**, 1161-1167.

Yoon, Y. S., Wecker, A., Heyd, L., Park, J. S., Tkebuchava, T., Kusano, K., Hanley, A., Scadova, H., Qin, G., Cha, D. H. et al. (2005). Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. *J. Clin. Invest.* **115**, 326-338.

■新しい治療■

急性冠症候群への再生医療
—細胞治療と造血性サイトカイン治療—

松永晋作　五十殿弘二　浅田　聡
辰巳哲也　松原弘明

心筋梗塞への再生医療として，末梢血単核球あるいは骨髄単核球を利用した血管新生治療が臨床応用されている。心筋梗塞はST上昇型と非ST上昇型に二分されるが，再生医療の主たる対象は前者のST上昇型である。急性心筋梗塞（AMI）の経皮的冠動脈形成術（PCI）治療後に骨髄単核球を冠動脈から注入する血管新生治療が，欧米で2001年ごろからスタートした。初期のオープンラベル臨床試験では半年後の心機能が10%前後と改善し世界中の注目を浴びたが，最近の二重盲検試験では有意な改善がみられないとの報告もあり，適応症例の選択が必要になった。造血性サイトカイン（G-CSF）をAMI後に投与して，心機能を改善させる臨床試験も実施されている。一方，陳旧性心筋梗塞（OMI）への骨髄単核球の直接心筋移植は，有効例が多く報告され，開胸・カテーテルを利用した再生医療が期待されている。ヒト心筋からの多能性幹細胞も分離され低心機能の重症心筋梗塞への移植もまもなくである。心筋梗塞への再生医療の最新の臨床試験の成績を中心に述べ，将来展望についてもふれてみたい。

● 骨髄細胞による血管新生と心筋再生

骨髄細胞中には造血系や間葉系幹細胞が含まれる。血管内皮細胞は造血系・間葉系幹細胞の両細胞群から分化可能とされる。造血系・間葉系幹細胞を含む骨髄単核球移植は虚血下肢や心筋において血管新生を誘導するが，新生血管のすべてが移植骨髄細胞から派生した（vasculogenesis）ものではなく，移植細胞から分泌されるVEGF，bFGFなどの血管内皮増殖因子が血管新生（angiogenesis）に大きな役割を演じている。虚血下肢への骨髄単核球移植による血管新生細胞治療の有効性は国際的に承認され，わが国だけでなく世界中において実施されている[1]。

骨髄造血系幹細胞からの心筋細胞分化は現在では否定されている，まれに観察されたとしても既存心筋細胞との融合現象であろう。骨髄間葉系幹細胞には“MAPC”とよばれる多能性幹細胞群が存在し心筋細胞に分化可能とされる。骨髄中に存在するとされる間葉系多能性幹細胞（MAPC）の存在数の低さを考えると，OMIやAMIへの骨髄単核球移植による心臓ポンプ機能の改善効果は心筋再生によるものとは考えにくく，移植細胞からの血管新生誘導因子や心筋保護因子の分泌などの関与と考えるのが正しいであろう。

● 急性心筋梗塞に対する骨髄単核球を利用した血管新生治療

急性心筋梗塞の際には急性期4〜7日目をピークとして骨髄から末梢血に血管内皮前駆細胞が動員されることや，幹細胞のhoming factorであるstromal cell-derived factor 1（SDF-1）が心筋に発現し，SDF-1を導入した線維芽細胞を移植しておくと梗塞心に骨髄幹細胞のhomingが促進され，血管新生効果と心機能改善効果が増強されることが示されている[2,3]。

最近，ST上昇型AMIのPCI再灌流成功後に骨髄単核球細胞または末梢血内皮前駆細胞を採取し，さらに梗塞責任冠動脈より低圧バルーン拡張カテーテ

まつなが しんさく，いそどの こうじ，あさだ さとし，たつみ てつや：京都府立医科大学大学院医学研究科 循環器内科学
まつばら ひろあき：京都府立医科大学大学院医学研究科 循環器内科学，京都大学医学部探索医療センター「重症心不全への細胞移植プロジェクト」