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).

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Human cardiac stem cells exhibit mesenchymal features and are maintained through Akt/GSK-3β signaling

Kento Tateishi [a,b], Eishi Ashihara [a], Shoken Honsho [a,b], Naofumi Takehara [a], Tetsuya Nomura [a,b], Tomosaburo Takahashi [b], Tomomi Ueyama [a], Masaaki Yamagishi [c], Hitoshi Yaku [c], 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 School of Medicine, Kyoto 602-8566, Japan*
[c] *Department of Cardiovascular Surgery, Kyoto Prefectural University School of Medicine, Kyoto 602-8566, Japan*

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Abstract

Recent evidence suggested that human cardiac stem cells (hCSCs) may have the clinical application for cardiac repair; however, their characteristics and the regulatory mechanisms of their growth have not been fully investigated. Here, we show the novel property of hCSCs with respect to their origin and tissue distribution in human heart, and demonstrate the signaling pathway that regulates their growth and survival. Telomerase-active hCSCs were predominantly present in the right atrium and outflow tract of the heart (infant > adult) and had a mesenchymal cell-like phenotype. These hCSCs expressed the embryonic stem cell markers and differentiated into cardiomyocytes to support cardiac function when transplanted them into ischemic myocardium. Inhibition of Akt pathway impaired the hCSC proliferation and induced apoptosis, whereas inhibition of glycogen synthase kinase-3 (GSK-3) enhanced their growth and survival. We conclude that hCSCs exhibit mesenchymal features and that Akt/GSK-3β may be crucial modulators for hCSC maintenance in human heart.

Keywords: Cardiac stem cells; Mesenchymal cells; Proliferation; Survival; Akt/GSK-3β

The postmitotic heart was shown to exhibit a previously unappreciated self-renewing phenotype, in which primitive cells proliferated and differentiated into specific progeny under acute or chronic workloads [1,2]. Recent studies have challenged this paradigm and shown the existence of intrinsic cardiac stem or progenitor cells in the mammalian heart [3–5]. CSCs expressing c-kit were clonogenic and multipotent [4,6], and were also able to be isolated from human heart in the floating culture system [7]. Furthermore, hCSCs were reported to be activated in response to myocardial ischemia and increased workload [8,9]. These cells have a significant impact on future clinical application to treat patients with heart failure. However, it is necessary to further examine the property and regulatory mechanism of hCSC growth to obtain a sufficient number of stem cells from a small amount of tissue samples to achieve an efficient regenerative-therapy.

Recent reports have suggested that bone marrow-derived mesenchymal stem cells (MSCs) enhanced with Akt, a serine/threonine protein kinase, can repair infarcted myocardium, prevent remodeling, and normalize cardiac performance through the prevention of apoptosis as well as a paracrine effect on resident cells [10,11]. Recently, insulin-like growth factor-1 (IGF1) has been shown to maintain murine CSC (mCSC) viability and growth through activation of Akt [12,13]; however, the downstream signals of

* Corresponding authors. Fax: +81 75 751 4741.
E-mail addresses: matsubah@koto.kpu-m.ac.jp (H. Matsubara), hidemasa@kuhp.kyoto-u.ac.jp (H. Oh).

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Akt pathway in hCSC growth remain to be investigated. In the present study, we characterized the property of hCSCs and clarified the role of Akt/GSK-3β signaling pathway in hCSC growth and survival. These results suggest that pharmacological inhibition of GSK-3β may have practical application in hCSC transplantation therapy in human heart failure.

Materials and methods

Tissue samples. The heart samples were obtained from 18 patients undergone cardiac surgery (9 males and 9 females aged from 9 days to 77 years old) in confirmation with the guidelines of the Kyoto University Hospital and Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Isolation of hCSCs. The heart samples were excised, minced, and digested with 0.4% type II collagenase and 0.01% DNAse. Obtained cells were then plated at 20 cells/μl in ultra-low culture dishes to generate cardiospheres with growth medium containing DMEM/F12, 5% FBS, 20 ng/ml EGF (Sigma), and 40 ng/ml bFGF (Promega). For the analyses described below, generated cardiospheres were dissected into single cells to obtain hCSCs by exposure to a 0.05% Trypsin/EDTA solution.

hCSC differentiation. For cardiac differentiation, hCSCs were cultured in differentiation medium containing 10% FBS, insulin-transferrin-selenium, and 10 nM dexamethasone. Differentiation medium containing DMEM/F12 supplemented with 10 ng/ml VEGF or 50 ng/ml PDGF-BB (R&D Systems) and 10% FBS was used to induce endothelial or smooth muscle cell differentiation, respectively. For the assay of cell proliferation and survival, specific inhibitors for Akt and GSK-3 (BIO) were purchased from Calbiochem.

FACS analysis. hCSCs were labeled with the following antibodies; phycoerythrin-conjugated antibodies against c-kit, CD45, CD34, CD31, CD90, CD29, CD73, CD71 (BD Biosciences), CD105 (Ancell Corp), and Stro-1 (R&D Systems). Cell events were collected by FACS Calibur flow cytometer and data were analyzed by Cell Quest (BD Biosciences).

RT-PCR and telomerase activity. Total RNA was extracted from cells using TRIzol and RT-PCR was performed with a SuperScript III First-Strand Synthesis System. The primer sequences are available upon request. Telomerase activity was measured with a TRAP assay kit, TRAPEZE (Chemicon).

Immunochemistry. Fixed cells and sections were stained with primary antibodies against cardiac troponin-I (Scripps), CD31, Ki67 (DAKO), α-SMA, connexin 43 (Sigma), collagen typeI (LSL), vimentin, and human nuclei (Chemicon). Secondary antibodies were conjugated to Alexa 488 and Alexa 555, and nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI). Apoptotic hCSCs were evaluated by TUNEL assay with ApopTag kit (Chemicon). Images were captured with a BZ-8000 (Keyence) and IX71 (Olympus Corporation).

Myocardial infarction (MI) and cell grafting. MI was created in 12- to 24-week-old NOD/*scid* mice (Jackson Laboratories) in accordance with the animal care and use guidelines at Kyoto University Hospital. MI was induced by ligation of the left anterior descending coronary artery. One hour after MI, 3×10^5 hCSCs were injected into two sites of the infarcted border zone. In the control group, mice were sham-operated on receiving a thoracotomy but no ligation of coronary artery.

Echocardiography. Two-dimensional and M-mode recordings (Sonos 5500, PHILIPS) were obtained from the short-axis view at the midpapillary muscle level.

Western blotting. Cell lysates were extracted with lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 1× protease inhibitor, 1 mM Na_3VO_4, and 1 mM NaF. Transferred membranes were incubated with primary antibodies against GSK-3β (BD Biosciences), phospho-GSK-3β (Ser9), phospho-Akt (S473), and Akt (Cell Signaling). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG were used as secondary antibodies.

Statistics. Data are means ± SE, and were analyzed by ANOVA and Scheffe's test, using a significance level of $p < 0.05$ (StatView).

Results

Identification and distribution of hCSCs in human heart

To characterize the hCSCs in human heart, primary heart-derived cells from patients were cultured at low density with low serum condition in a floating culture system using a modification of the method previously reported [7]. At day-14, spherical colonies were generated at a frequency of 63.1 ± 16.5 spheres per 200,000 viable cells (Fig. 1A). The initial yield of digested cells was proportional to the number of spheres, and the number of isolated cells was significantly increased in heart tissues from the right atrium (RA) and outflow tract (OFT) than in tissue from the left ventricle (LV) (Fig. 1B). Moreover, the isolated cells were 5-fold greater and had higher telomerase activity in the infant heart than the adult heart (Fig. 1C and D).

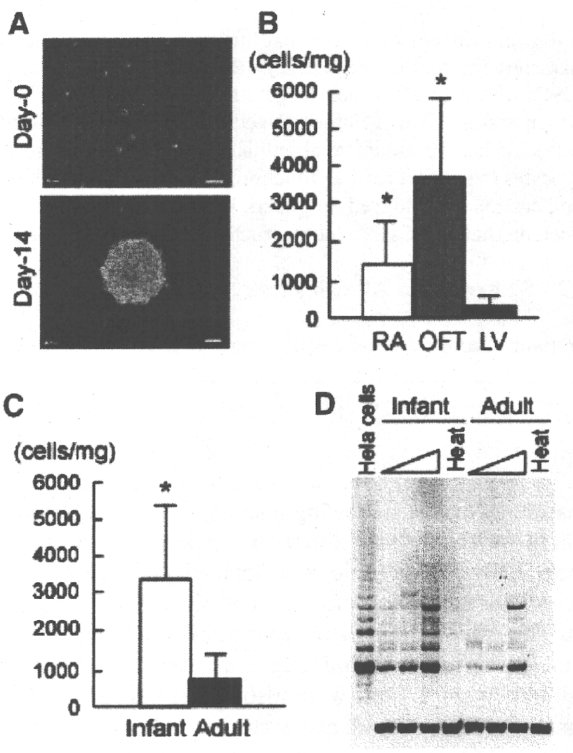

Fig. 1. Isolation and distribution of hCSCs. (A) Generation of cardiosphere from human heart. Bars, 20 μm. (B, C) The initial progenitor cell number harvested by primary isolation as indicated. Total yield was corrected by tissue weight (mg). Distribution of hCSCs corresponding to the parts of the heart (B) or the patients' age (C). $^*p < 0.05$ versus LV in (B); $^*p < 0.01$ versus adult in (C). (D) Telomerase activity in hCSCs. Threefold serial dilutions of hCSCs isolated from infant and adult hearts were treated with or without heat and used as templates. HeLa cells were used as a positive control ($n = 3$).

hCSCs exhibit mesenchymal features

Immunophenotyping revealed that hCSCs rarely expressed c-kit and did not express the hematopoietic and endothelial progenitor cell-specific surface antigens: CD45, CD34, and CD31, while they were positive for typical MSC surface antigens: CD105, CD90, CD29, CD73, CD71, and Stro-1 (Fig. 2A) [14,15]. Human cardiospheres also expressed both vimentin and collagen type 1 (Fig. 2B), and had a spindle shaped morphology in attached cell-culture experiments (Fig. 2C). RT-PCR showed that hCSCs expressed ATP-binding cassette transporter subfamily G member 2 (ABCG2), which was associated with Hoechst's efflux properties prerequisite for the side population cells [16]. Human cardiospheres also expressed Rex1, Nanog, and Sox2, although Oct4 was not detectable (Fig. 2D), suggesting that hCSCs express the embryonic stem cell markers and contain the mesenchymal cell-like population.

hCSCs give rise to cardiovascular lineages in vitro and in vivo

To determine the differentiation potential of hCSCs *in vitro*, hCSCs were cultured in differentiation medium. Immunostaining showed that hCSCs gave rise to smooth muscle cells, endothelial cells, and cardiomyocytes co-expressing connexin-43 (Fig. 3A). Furthermore, cardiac-specific transcriptional factors such as Nkx2.5 and GATA4, ANP, and structural genes, including α-cardiac-actin, cardiac troponin-T, MLC2a, MLC2v, α-MHC, and β-MHC, were detected in the differentiated cardiomyocytes by RT-PCR (Fig. 3B).

To investigate the regenerative potential of hCSCs *in vivo*, we performed cell transplantation into MI using NOD/*scid* mice. The injected cells formed a successful engraftment within the border and infarcted regions. The differentiation of hCSCs into the cardiovascular-lineage cells was verified by the presence of smooth muscle cells, endothelial cells, and cardiomyocytes, colocalized with human nuclei (Fig. 3C). Capillary density was also increased in the implanted hearts compared with the PBS-treated hearts (Fig. 3D).

After the transplantation of hCSCs, cardiac function was analyzed by echocardiography (Fig. 3E). In PBS-treated mice, the ejection fraction (EF) and fractional shortening (FS) were significantly decreased (EF: $81.5 \pm 2.0\%$ to $46 \pm 2.0\%$, $p < 0.01$; FS: $43.7 \pm 2.0\%$ to $20.2 \pm 1.0\%$, $p < 0.01$), and LV diastolic dimension (Dd) was expanded (35.2 ± 2.0 to 47.0 ± 3.0 mm, $p < 0.01$) at day-14 after MI compared with baseline. In contrast, the implantation of hCSCs effectively ameliorated the cardiac dysfunction (EF: $46 \pm 2.0\%$ vs $58 \pm 2.0\%$, $p < 0.01$; FS: $20.2 \pm 1.0\%$ vs $26.2 \pm 2.0\%$, $p < 0.01$) and reduced LV dilatation (Dd: $47.0 \pm 3.0\%$ vs $40.7 \pm 2.0\%$, $p < 0.01$) compared with PBS-injected mice. These parameters showed that the

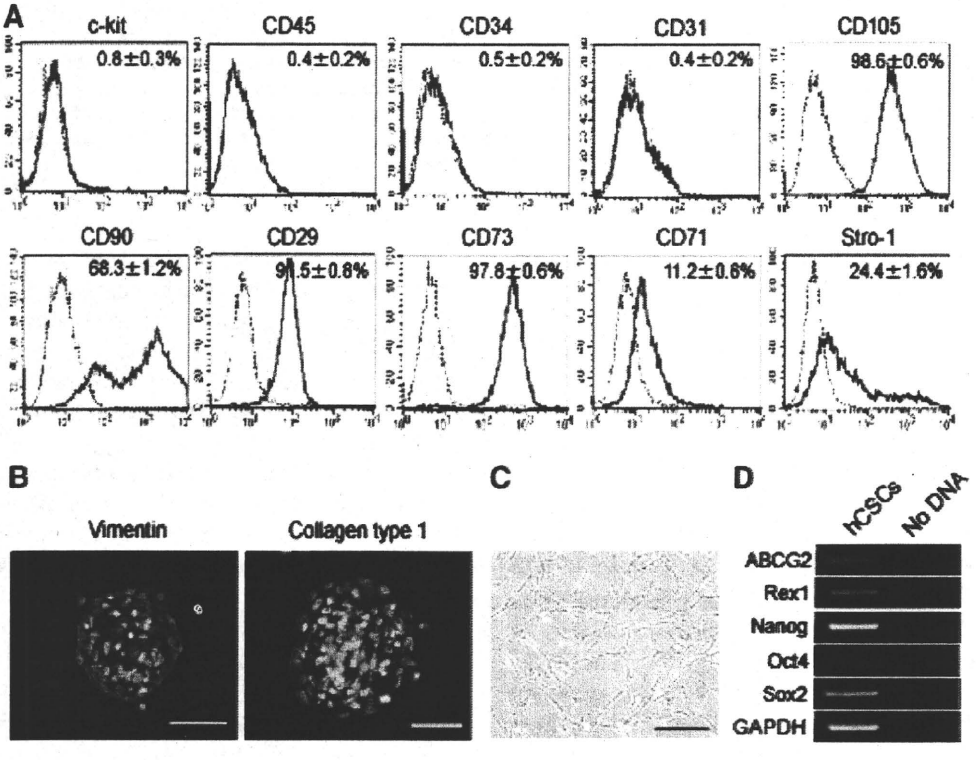

Fig. 2. Characterization of hCSCs. (A) FACS analysis of hCSCs. Black line, control IgG; red line, corresponding antibody ($n = 3$). (B) Immunostaining of human cardiospheres. Red signals show the expression of vimentin (left) and collagen type1 (right). Scale bars, 50 μm. (C) Phase contrast image of hCSCs in attached cell-culture. Scale bars, 100 μm. (D) Gene expression profile by RT-PCR examined in hCSCs. No DNA template was used as a negative control ($n = 6$).

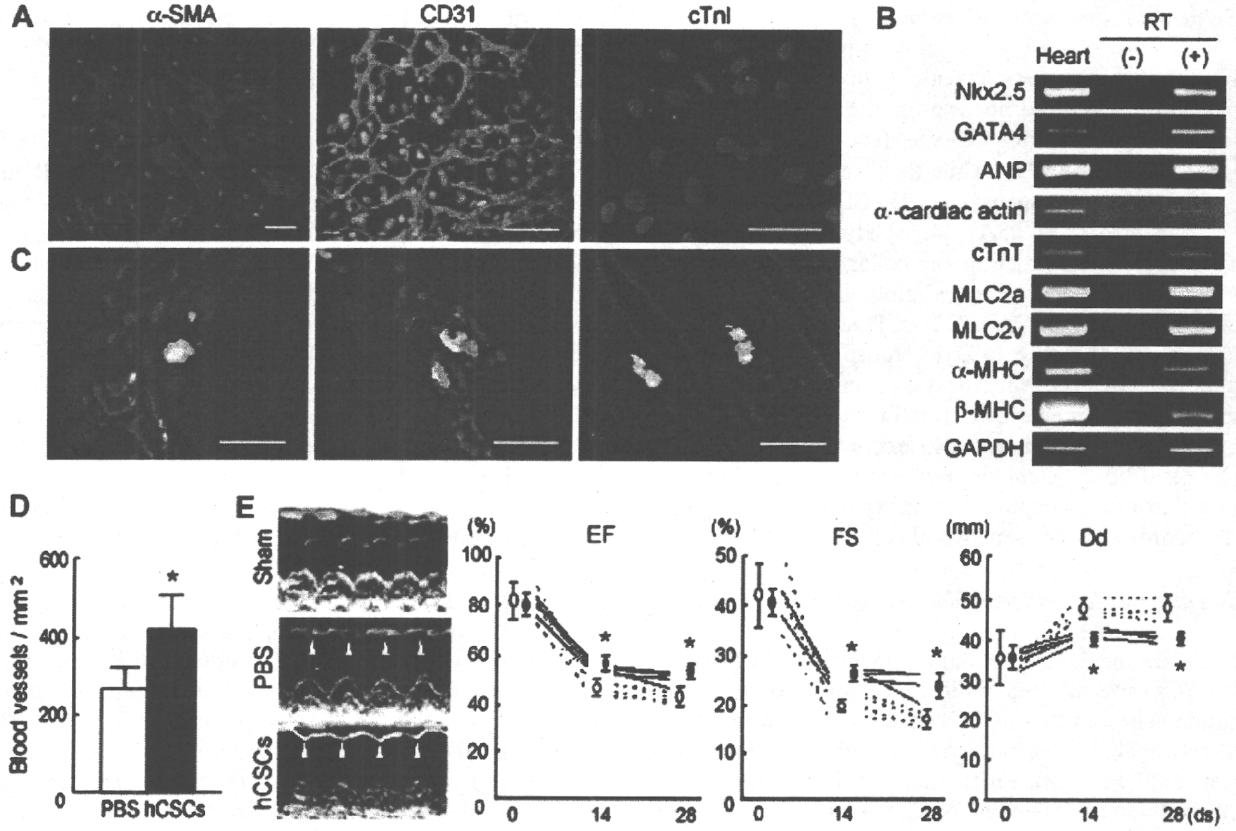

Fig. 3. Functional differentiation of hCSCs *in vitro* and *in vivo*. (A) *In vitro* differentiation of hCSCs into smooth muscle cells (left: α-SMA, red), endothelial cells (middle: CD31, green), and cardiomyocytes (right: cardiac troponin-I, red; connexin-43, yelow). DAPI, blue. (B) RT-PCR shows cardiac differentiation of hCSCs. Heart tissue was used as positive control ($n = 3$). (C) *In vivo* differentiation of hCSCs. Smooth muscle cells (left: α-SMA, red), endothelial cells (middle: CD31, red), and cardiomyocytes (right: cardiac troponin-I, red), counterstained with human nuclei (green) are shown. DAPI, blue ($n = 4$). (D) Capillary density was assessed by CD31 immunohistochemistry in the border zone. $^*p < 0.01$ versus PBS treated mice. (E) Serial assessment of cardiac function by echocardiography. Representative M-mode images of sham-operated, PBS-injected, and hCSC-transplantated hearts at 28 days after MI. Closed circles, hCSC transplanted hearts; open circles, PBS-injected hearts ($n = 8$). Arrowheads indicate significantly improved anterior wall movement on stem cell implantation. $^*p < 0.01$ versus PBS-treated mice. Scale bars, 50 μm in (A); 20 μm in (C).

significant recovery was observed 2 and 4 weeks after hCSC implantation.

The proliferation and survival of hCSCs depend on Akt/GSK-3β pathway

Akt pathway plays a crucial role to mediate the proliferation activity in mCSCs [13]. To verify whether Akt pathway was involved in hCSC proliferation, we examined the activation of Akt in hCSCs and found that EGF/bFGF treatment of hCSCs caused a rapid activation of Akt (Fig. 4A) and also augmented sustained phosphorylation of GSK-3β, which is one of the downstream targets of Akt, to inactivate GSK-3β function (Fig. 4B). The EGF/bFGF-induced activation of Akt in hCSCs was inhibited by Akt inhibitor, Akt-I, in a dose-dependent manner (Fig. 4C). In contrast, the levels of phosphorylated GSK-3β (inactive form of GSK-3β) could be enhanced by the treatment of 10 nM GSK-3-inhibitor, BIO (Fig. 4D), as previously reported in renal epithelial cells [17].

If Akt mediates hCSC proliferation through the inhibition of GSK-3β, the pharmacological inhibition of Akt/GSK-3β signaling pathways may affect the growth of hCSCs. To test this hypothesis, the diameter of cardiospheres was measured in the presence or absence of 10 μM Akt-I or 10 nM BIO, the minimal doses needed to achieve an effect shown above (Fig. 4C and D). Our results demonstrated that Akt-I significantly decreased the diameter of EGF/bFGF-expanded cardiospheres (Fig. 4E), whereas addition of BIO significantly increased their growth at the range of sphere size more than 100 μm (Fig. 4F).

We next determined the underlying mechanisms by which Akt/GSK-3β pathway modulated sphere formation and growth of hCSCs. $TUNEL^+$ cells were significantly increased in cardiospheres treated with Akt-I compared with control, whereas BIO apparently reduced the number of $TUNEL^+$ cells (Fig. 4G). In contrast, Ki67-positive cells were apparently decreased in cardiospheres treated with Akt-I compared with control, whereas a significant

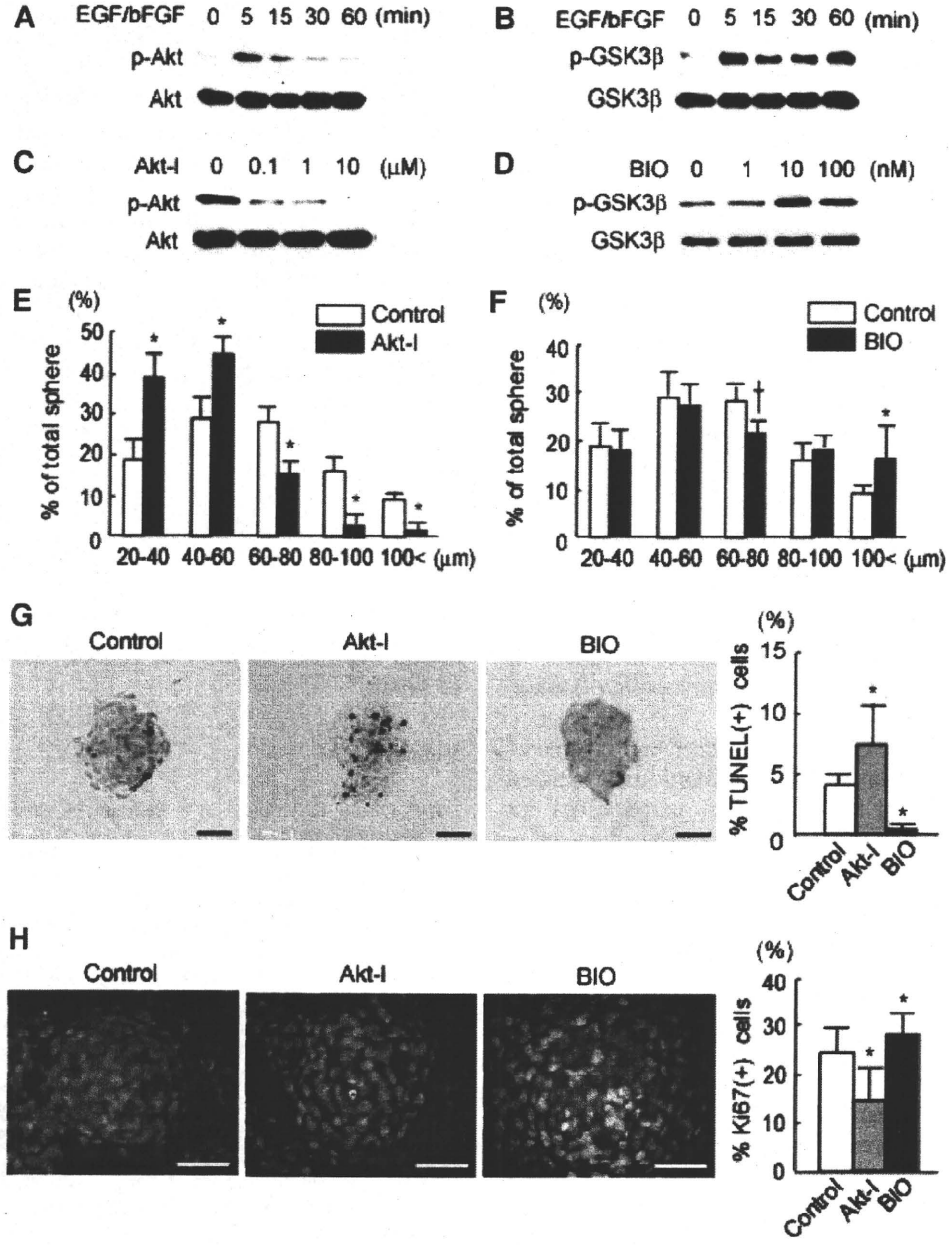

Fig. 4. Akt/GSK-3β signaling regulates the proliferation and survival of hCSCs. (A,B) Phosphorylation of Akt (A) and GSK-3β (B) induced by EGF/bFGF in hCSCs. After serum starvation for 2 h, hCSCs were treated with EGF/bFGF for the period of time indicated. (C) Activation of Akt induced by EGF/bFGF treatment for 5 min was abolished by the pretreatment of Akt-I for 4 h in a dose-dependent manner. (D) Phosphorylation of GSK-3β (inactive) induced by EGF/bFGF treatment for 5 min was enhanced by the pretreatment of 10 nM BIO for 4 h. (E,F) Size distribution of cardiospheres cultured in EGF/bFGF-containing medium in the presence of either 10 μM Akt-I (E), or 10 nM BIO (F) for 6 days ($n = 7$). $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ versus DMSO control. (G,H) TUNEL assay (G) and Ki67 staining (H) of cardiospheres exposed to 10 μM Akt-I and 10 nM BIO. Ki67, red. DAPI, blue. $^{*}p < 0.01$ versus DMSO control ($n = 3$). Scale bars, 50 μm.

increase in the number of Ki67-positive cells was observed in cardiospheres exposed to BIO (Fig. 4H).

Discussion

Our present study provides the novel evidence that hCSCs exhibit a mesenchymal cell-like property and Akt/GSK-3β signaling is involved in their proliferation and survival. Furthermore, our study shows that hCSCs are predominantly present in the right atrium and outflow tract of the heart (more expressed in infant heart rather than adult heart).

A recent report has suggested that cellular aging induces a functional impairment of mCSC growth that may result from the reduction in Akt phosphorylation and telomerase inactivation [18]. Consistent with these data, we showed

here less telomerase activity in hCSCs from the adult heart than that from the infant heart. The abundance of hCSCs isolated from RA and OFT may reflect their specific distribution in the human heart. Although there is a possibility of bias caused by the patients' background, including disease, age, and sex, our results are consistent with a recent report showing that the stem cell niches are predominantly present in the atrium in the murine heart [19].

Messina et al. [7] have demonstrated that hCSCs can be isolated from human heart using floating culture system. We employed the essentially similar method to isolate hCSCs. However, in contrast to the previous report, we found that c-kit expression was extremely low in the isolated cells from both infant and adult hearts. Several reports demonstrated that c-kit expression was diminished on the lineage-committed cardiac progeny as observed in murine cardiac progenitor cells and cardioblasts [3,5,19]. It is possible that cardiospheres contain a mixed population of cells that, as in the niche, can promote the viability of c-kit progenitors and contribute to their proliferation [7]. Our observation suggest that mixed progenitor populations may exist during the process of lineage-commitment of hCSCs in the human heart as during hematopoietic homeostasis [20].

It is notable that hCSCs have a mesenchymal-like character. Mesenchymal stem cells were conventionally isolated from bone marrow and the presence in many tissues but not heart has recently been reported [21]. In the developing heart, the neural crest cells are known to migrate into the cardiac outflow tract to supply the cells from the primitive epicardial epithelium through a process of epithelial-to-mesenchymal transition [22]. These epicardially derived cells have a mesenchymal phenotype and stem cell property in human adult hearts [23]. Thus, it may be conceivable that hCSCs isolated from the human heart might be originated from the primitive epicardial epithelium.

The mechanism to regulate the proliferation and survival of stem cells has been examined. Akt is a nodal signaling kinase linked to both the proliferation and survival of somatic stem or progenitor cells in neural tissue and blood [24,25]. Our studies demonstrate that the proliferation of hCSCs appears to be dependent on the activation of Akt in response to EGF/bFGF stimulation. Furthermore, we have documented that inhibition of Akt pathway impairs cell growth and survival. Our observations are consistent with two independent studies demonstrating that *ex vivo* transduction of Akt prevents bone marrow-derived MSCs from the oxidative stress-induced apoptosis [10] and that the nuclear-targeting of Akt leads to an acceleration of mCSC expansion [13].

The novel finding we showed here that GSK-3β is also associated with the proliferation and survival of hCSCs may provide the new prospect for stem cell therapy. GSK-3β is one of the substrates of Akt and participates in regulating the cell cycle in various cell types [26]. We found that BIO stimulated the growth kinetics of hCSCs consistent with the observation seen in BIO-mediated proliferation of differentiated cardiomyocytes [27]. Thus, our findings suggest that Akt/GSK-3β pathway is crucial in hCSC growth and survival as well as mCSCs.

In conclusion, the present study demonstrates that the resident CSCs in human hearts have mesenchymal characteristics and proliferate through Akt/GSK-3β pathway. Understanding whether pharmacological inhibition of GSK3β by BIO may act through direct activation of the Wnt signaling pathway for stem cell maintenance [28] will provide a new insight into the signaling pathways required for hCSC expansion and engraftment *in vivo*. These novel findings may enable practical applications for establishing hCSC lines and provide an advanced cell therapy for patients with heart failure.

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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*

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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).

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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).

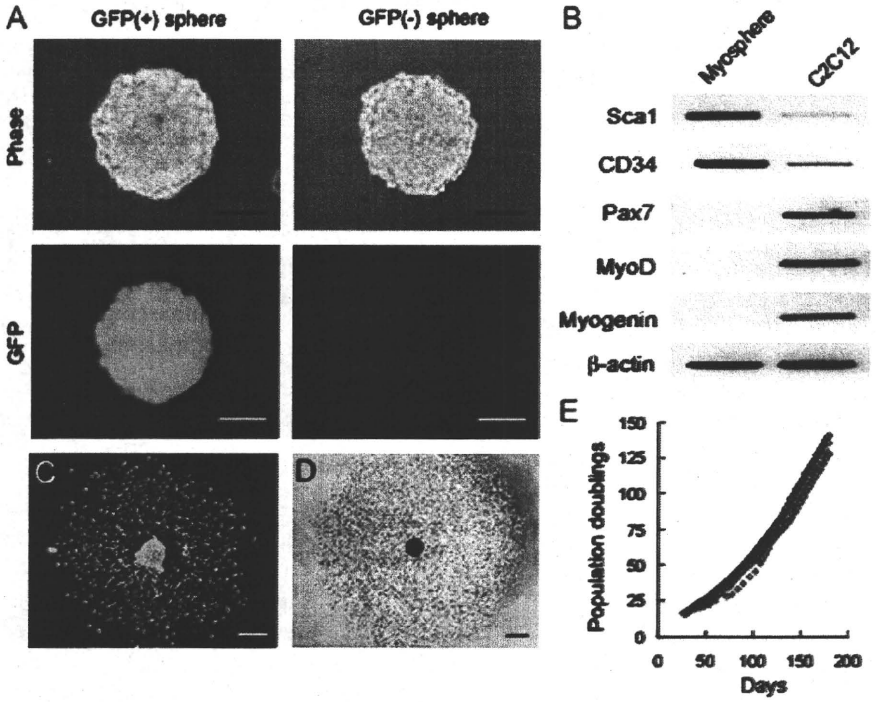

Fig. 1. Isolation and expansion of MDPCs. (A) Representative phase contrast and fluorescent images of myospheres generated from the mixed-cultures of single cells isolated from GFP transgenic (left panels) and WT mice (right panels). (B) RT-PCR analysis of Sca-1, CD34, and myogenic transcription factors. (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).

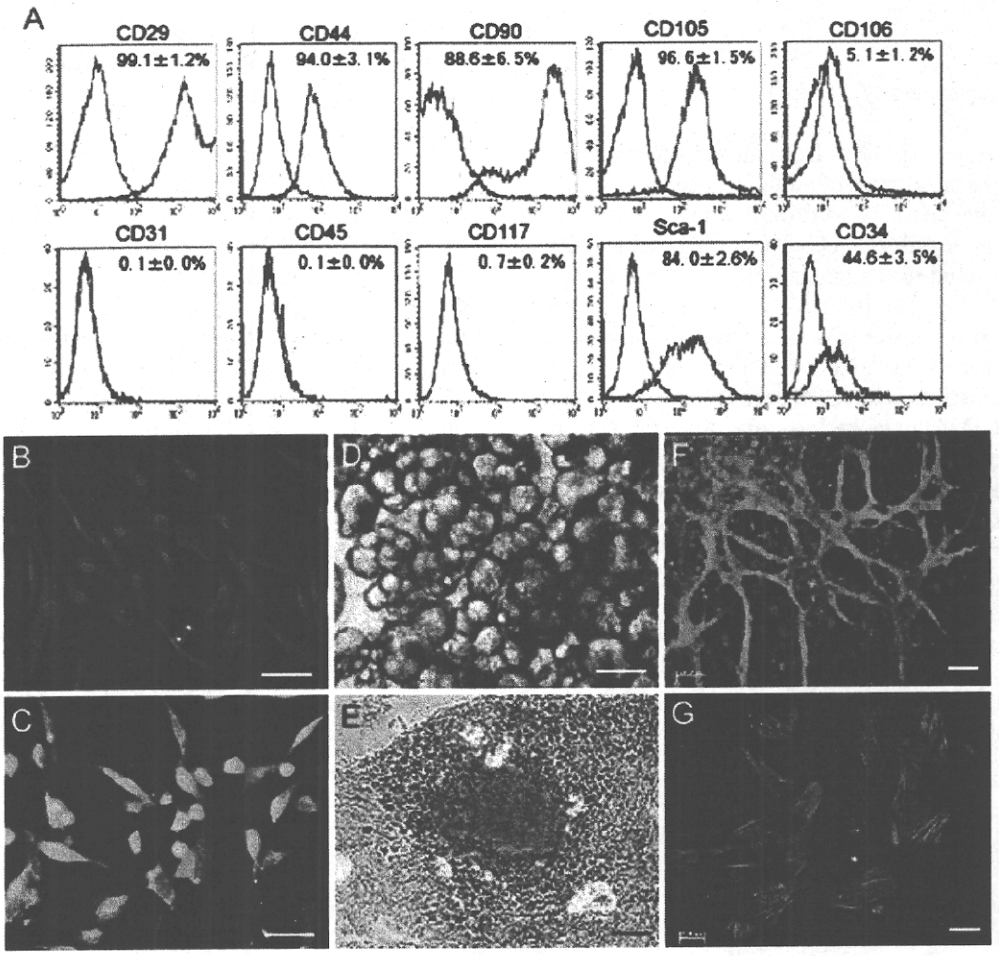

Fig. 2. MDPCs have mesenchymal cell-like phenotype and differentiate into endothelial and smooth muscle cells. (A) FACS analysis of MDPCs. Isotype controls were overlaid (blue lines) on each antigen tested (red lines). Immunostaining of mesenchymal markers on undifferentiated MDPCs. Vimentin (B, red) 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 $Sm\text{-}MHC^+$ 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

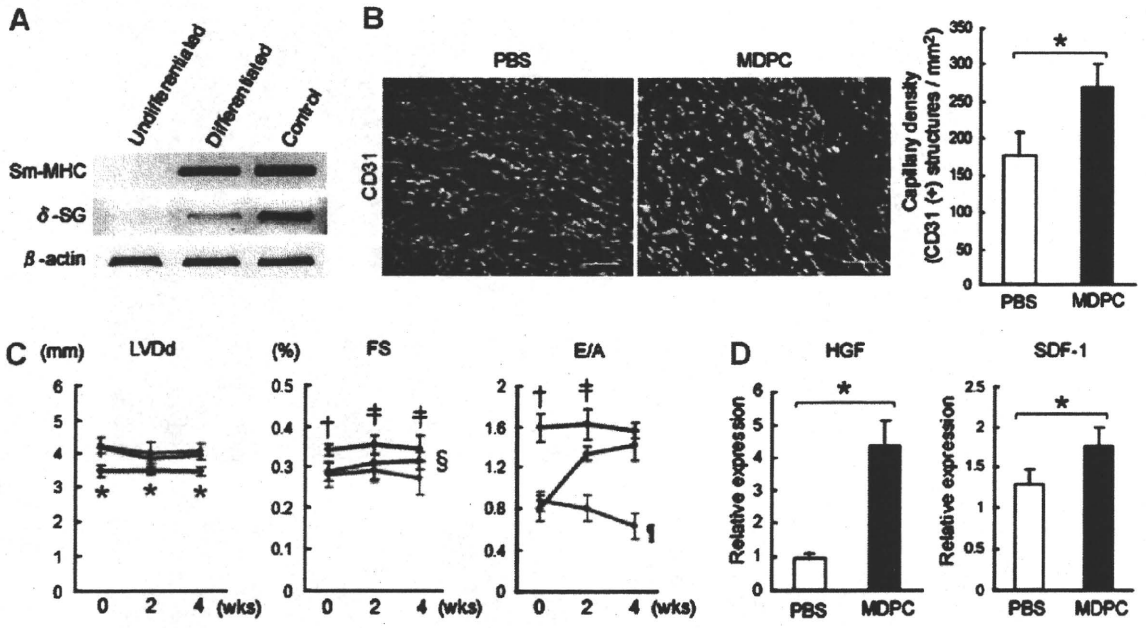

Fig. 4. MDPC transplantation improves cardiac function partially through paracrine effectors production (A) δ-SG expression was observed in differentiated MDPCs in vitro. (B) Comparison of the capillary density between PBS-treated and MDPC-transplanted δ-SG KD hearts. CD31 (red). DAPI (blue). $^{*}p < 0.01$. (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.

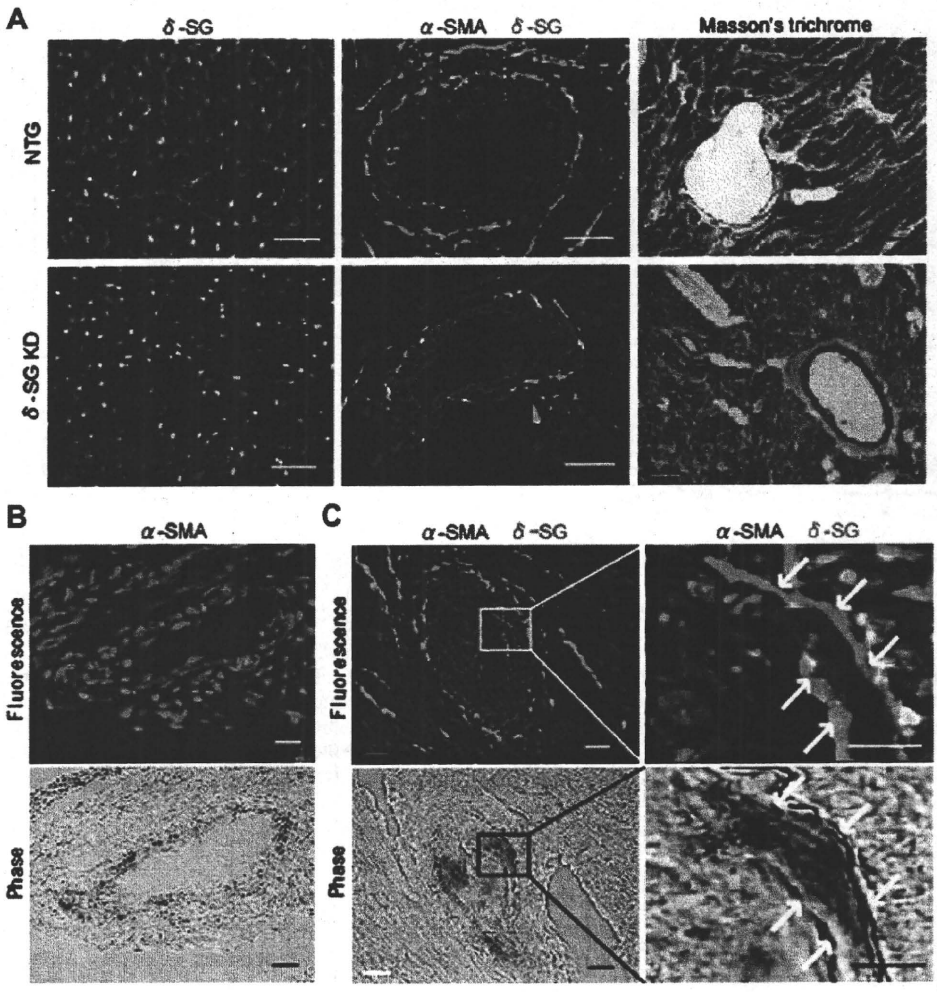

Fig. 3. Vascular regeneration in δ-SG KD hearts with the restoration of δ-SG expression. (A, left panels) Decreased δ-SG (red) expression in cardiac muscle was significantly observed in δ-SG KD hearts compared with NTG littermates. DAPI (blue). (A, middle panels) Vascular lumens were more constrictive 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

Acknowledgments

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