厚生労働科学研究費補助金（基礎研究成果の臨床応用推進研究事業）
（分担）研究報告書

心筋幹細胞移植実現化へ向けての最適な血管新生療法探索研究に関する研究

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研究要旨

重症心不全への心臓移植事業がドナー不足のため充分には機能していない現状を打開することを目的とする末期的心不全への幹細胞移植医療実現化へ向けての研究基盤形成研究の中で、私は細胞移植による心筋・血管再生医療が実現化されるために最も適した血管を誘導できる新生方法の確立、血管新生因子の探索と同定に関する研究を行う。

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A. 研究目的

重症心不全への心臓移植事業がドナー不足のため充分には機能していない現状を打開することを目的とする末期的心不全への幹細胞移植医療実現化へ向けての研究基盤形成研究の中で、私は細胞移植による心筋・血管再生医療が実現化されるために最も適した血管を誘導できる新生方法の確立、血管新生因子の探索と同定に関する研究を行う。

B. 研究方法

アンジオポエチン様血管新生因子ファミリー(Angptl)、特にAGF/Angpotl6の血管新生因子としての機能解析を、臨床応用に向けてマウス病態モデルを用い血管新生への貢献の検討を行った。また組み替えタンパクを作成しin vitroでのAGF/Angpotl6の血管内皮細胞への作用に関して分子機構の解明を行った。さらにAngptl2の血管新生因子としての機能解析を、遺伝子改変マウスを作製し検討を行った。。さらに生理的な血管新生を誘導するための条件を検討するために、種々の眼内血管新生病をモデルに病的血管新生の発症分子機構を解析した。

（倫理面への配慮）

動物操作にあたっては各施設の動物実験指針に従って行った。基礎的研究においては、遺伝子改変マウス、プラスミドDNAを用いる場合は仕様に際しては、遺伝子組み換え生物な

どの使用等の規則による生物の多様性の確保に関する法律に基づき研究を実施する。今後の臨床研究に関しては、学内の倫理委員会承認のもとで進め、患者、ボランティアに対するインフォームド・コンセントは書面で行う。

C.研究結果

AGF/Angptl6は生理的な血管新生促進用のみならず、上皮細胞への増殖作用も有し、注入される細胞にとっての足場形成には適した分子であること、重症新心不全の原因となる重篤な虚血性心疾患の基盤病態と言えるメタボリックシンドロームに対しても治療効果を有していることを明らかにし、単に心筋幹細胞移植時に最適な血管新生療法を行える分子としてのみならず、その基盤となる病態改善にも寄与できる可能性を明らかにしてきた。本年度の研究では、マウス下肢虚血モデルを用いてAGF/Angptl6の血管新生療法への可能性を検討する研究を行った。AGF/Angptl6投与群では血管新生（angiogenesisとarteriogenesis）により血流の回復が著明に見られ下肢切断のイベントが有意に減少された。以上の結果よりAGF/Angptl6シグナルの調節が血管新生療法への標的になることが示唆された。また、その分子機構としてはNOの活性化が重要であることを明らかにした(論文準備中)。一方、ファミリー分子であるAngptl2は、持続的に発現させると血管新生は促進されるが、白血球の接着等が亢進するような病的血管新生を誘導することが明らかとなり、血管新生療法への標的としては適さないことが示唆された(論文準備中)。病的血管新生の発症分子機構としてRAS（レニンーアンジオテンシン系）の活性化、STAT3の活性化が関わっていることを明らかにし、生理的な血管新生を誘導するためにはこれら病的血管新生誘導シグナルの活性化の抑制の考慮が示唆された。

D.考察

欠損した組織で注入された細胞が増殖し組織を再生誘導するためには、適切な細胞増殖因子と細胞周囲の環境が重要である。また、重症心不全の原因となる重篤な虚血性心疾患の基盤病態と言えるメタボリックシンドロームへの治療は細胞移植治療後の再発の危険度を下げる意味で重要である。血管新生作用と足場形成作用を有し、抗肥満、抗インスリン抵抗性作用を介してメタボリックシンドロームを改善する作用を有するAGFが実際にマウス下肢虚血病態モデルにおいて血流を回復させ下肢の切断イベントを有意に減少させる分子であることを明らかにしたことは、心筋幹細胞移植による心不全への細胞移植事業実現化へ向けて最も適した血管を誘導できる方法を探索し検証する、細胞移植治療後の再発の危険度を下げる治療標的を探索し検証するという点において多大に貢献したものと考えられる。

E.結論

心筋幹細胞移植による心不全への細胞移植事業実現化へ向けて最も適した血管新生療

法の開発にアンジオポエチン様因子のAGF/Angptl6が貢献できる可能性を見出した。

F. 健康危険情報

G. 研究発表

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1. 特許取得

特になし

2. 実用新案登録

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3. その他

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研究成果の刊行に関する一覧

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

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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

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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 type1 (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).

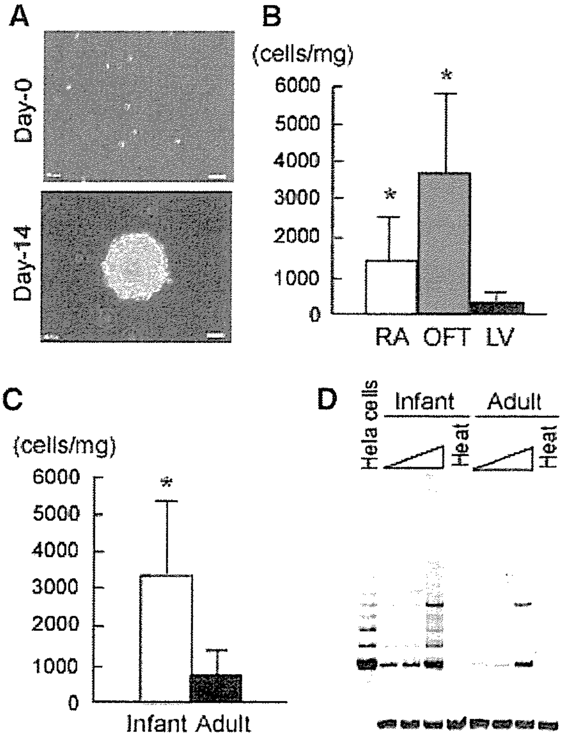

Fig. 1. Isolation and distribution of hCSCs. (A) Generation of cardiosphere from human heart. Bars, 20 μm. (B, C) The initial progenitor cell number harvested by primary isolation as indicated. Total yield was corrected by tissue weight (mg). Distribution of hCSCs corresponding to the parts of the heart (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 ± 2.0% to 46 ± 2.0%, $p < 0.01$; FS: 43.7 ± 2.0% to 20.2 ± 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 ± 2.0% vs 58 ± 2.0%, $p < 0.01$; FS: 20.2 ± 1.0% vs 26.2 ± 2.0%, $p < 0.01$) and reduced LV dilatation (Dd: 47.0 ± 3.0% vs 40.7 ± 2.0%, $p < 0.01$) compared with PBS-injected mice. These parameters showed that the

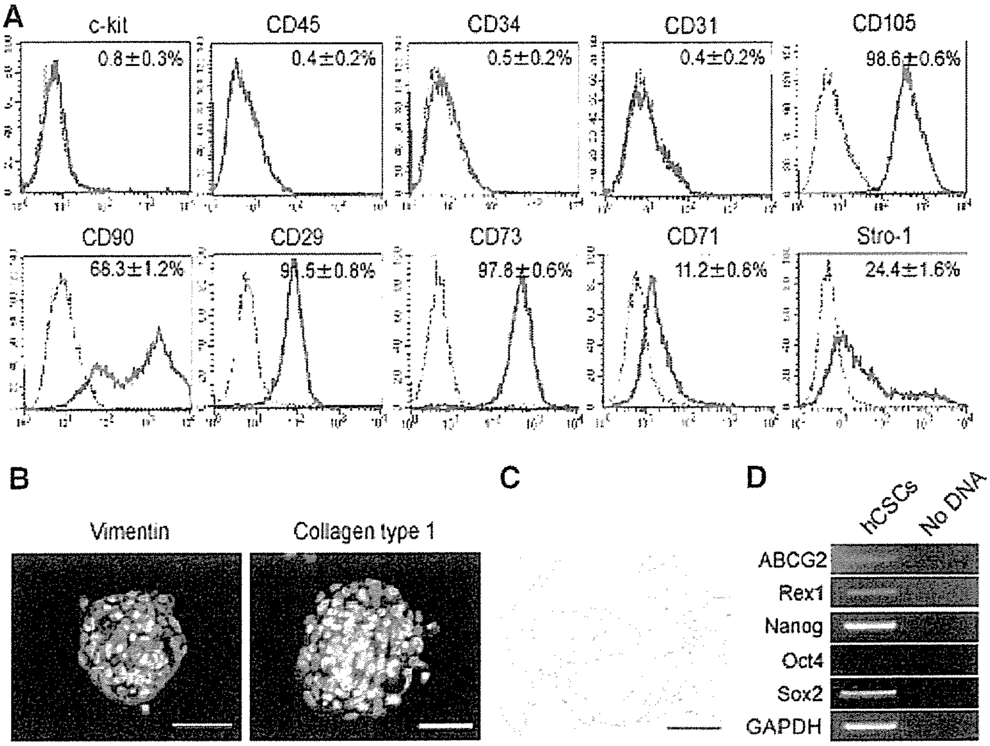

Fig. 2. Characterization of hCSCs. (A) FACS analysis of hCSCs. Black line, control IgG; red line, corresponding antibody ($n = 3$). (B) Immunostaining of human cardiospheres. Red signals show the expression of vimentin (left) and collagen type1 (right). Scale bars, 50 μm. (C) Phase contrast image of 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 ($n = 6$).

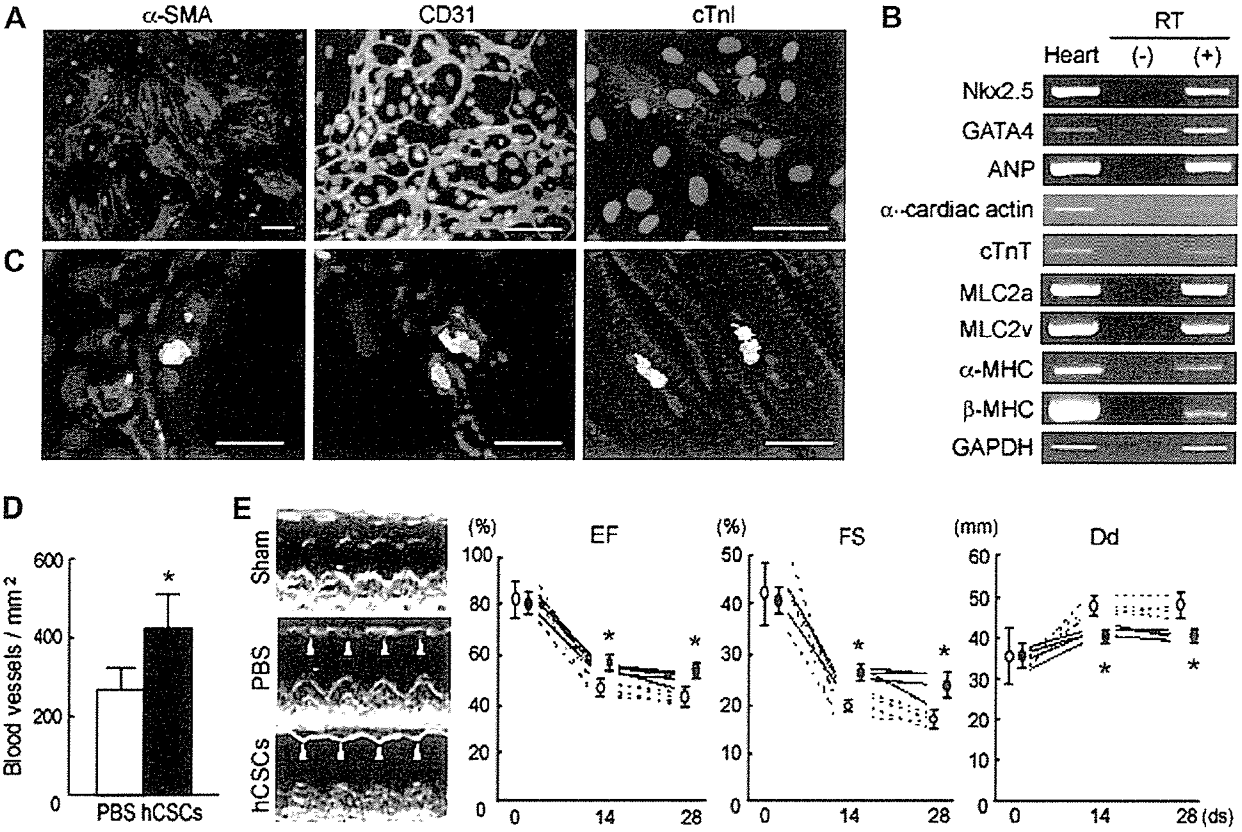

Fig. 3. Functional differentiation of hCSCs *in vitro* and *in vivo*. (A) *In vitro* differentiation of hCSCs into smooth muscle cells (left: α-SMA, red), endothelial cells (middle: CD31, green), and cardiomyocytes (right: cardiac troponin-I, red; connexin-43, yelow). DAPI, blue. (B) RT-PCR shows cardiac 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

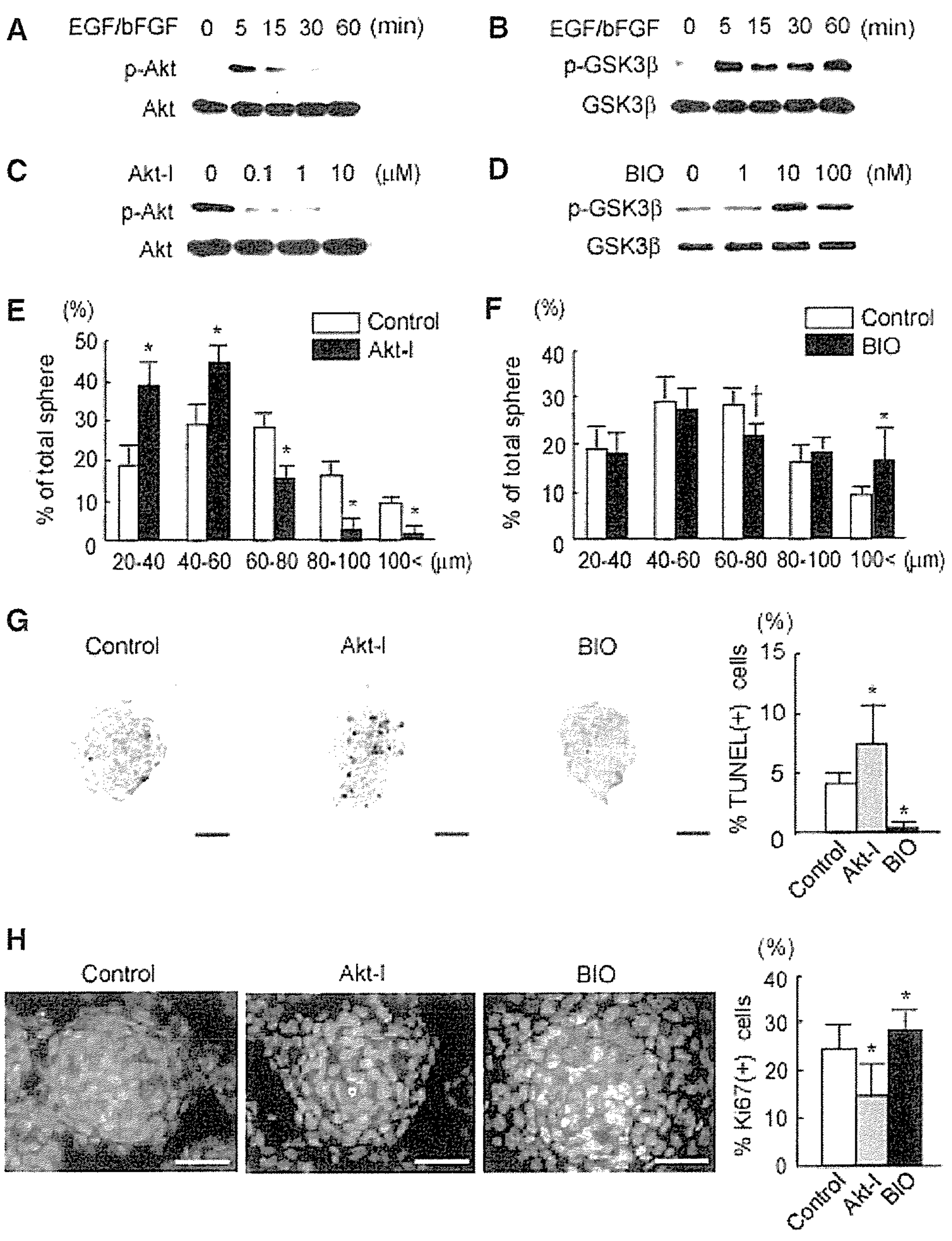

Fig. 4. Akt/GSK-3β signaling regulates the proliferation and survival of hCSCs. (A,B) Phosphorylation of Akt (A) and GSK-3β (B) induced by EGF/bFGF in hCSCs. After serum starvation for 2 h, hCSCs were treated with EGF/bFGF for the period of time indicated. (C) Activation of Akt induced by EGF/bFGF treatment 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.

Acknowledgments

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

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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

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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: CTCTGAGGATGGACACTTCT, Sca-1-r: GGTCTGCAGGAGGACTGAGC; CD34-f: TTGACTTCTGCAACCACGGA, CD34-r: TAGATGGCAGGCTGGACTTC; Pax7-f: GAAAGCCAAACACAGCATCGA, Pax7-r: ACCCTGATGCATGGTTGATGG; MyoD-f: ACATAGACTTGACAGGCCCCGA, MyoD-r: AGACCTTCGATGTAGCGGATGG; Myogenin-f: TACGTCCATCGTGGACAGCAT, Myogenin-r: TCAGCTAAATTCCCTCGCTGG; β-actin-f: GCTCGTCGTCGACAACGGCTC, β-actin-r: CAAACATGATCTGGGTCATCTTCTC; δ-SG-f: CCATGACCATCTGGATTCTCAAGG, δ-SG-r: GATGGCTTCCATATTGCCAGCTTC; and smooth muscle myosin heavy chain (Sm-MHC)-f: AGGAACTCCAAGCAAGTTGCAGG, Sm-MHC-r: CTGGAAGGAACAAATGAAGCCTCG. 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 ± 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 ± 3.3%, Fig. 2B) and type I collagen (87.4 ± 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).

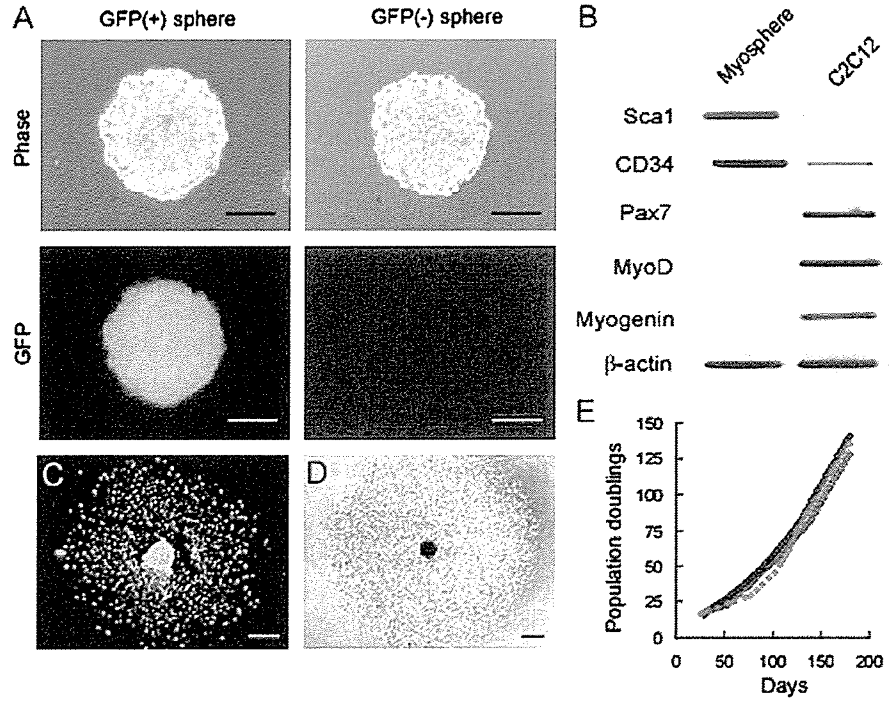

Fig. 1. Isolation and expansion of MDPCs. (A) Representative phase contrast and fluorescent images of myospheres generated from the mixed-cultures of single cells isolated from GFP transgenic (left panels) and WT mice (right panels). (B) RT-PCR analysis of Sca-1, CD34, and myogenic transcription factors.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

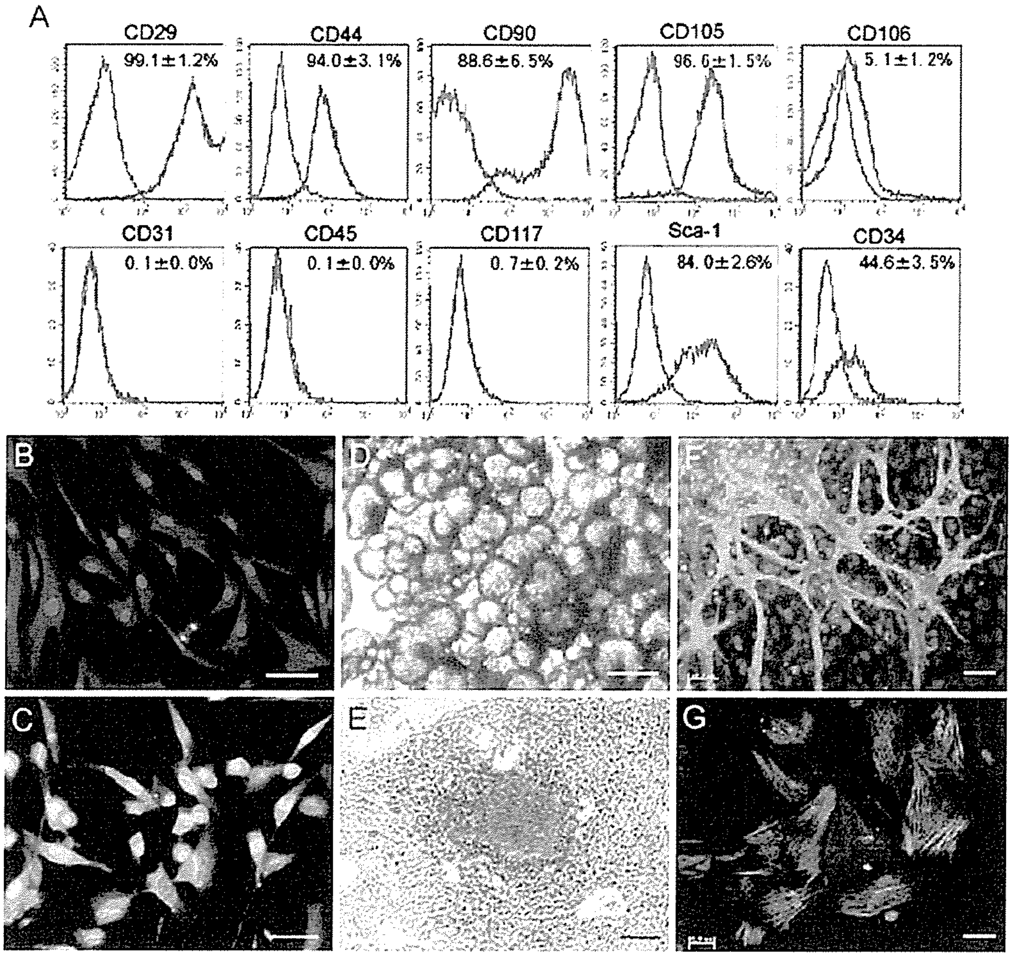

Fig. 2. MDPCs have mesenchymal cell-like phenotype and differentiate into endothelial and smooth muscle cells. (A) FACS analysis of MDPCs. Isotype controls were overlaid (blue lines) on each antigen tested (red lines). Immunostaining of mesenchymal markers on undifferentiated MDPCs. Vimentin (B, red)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

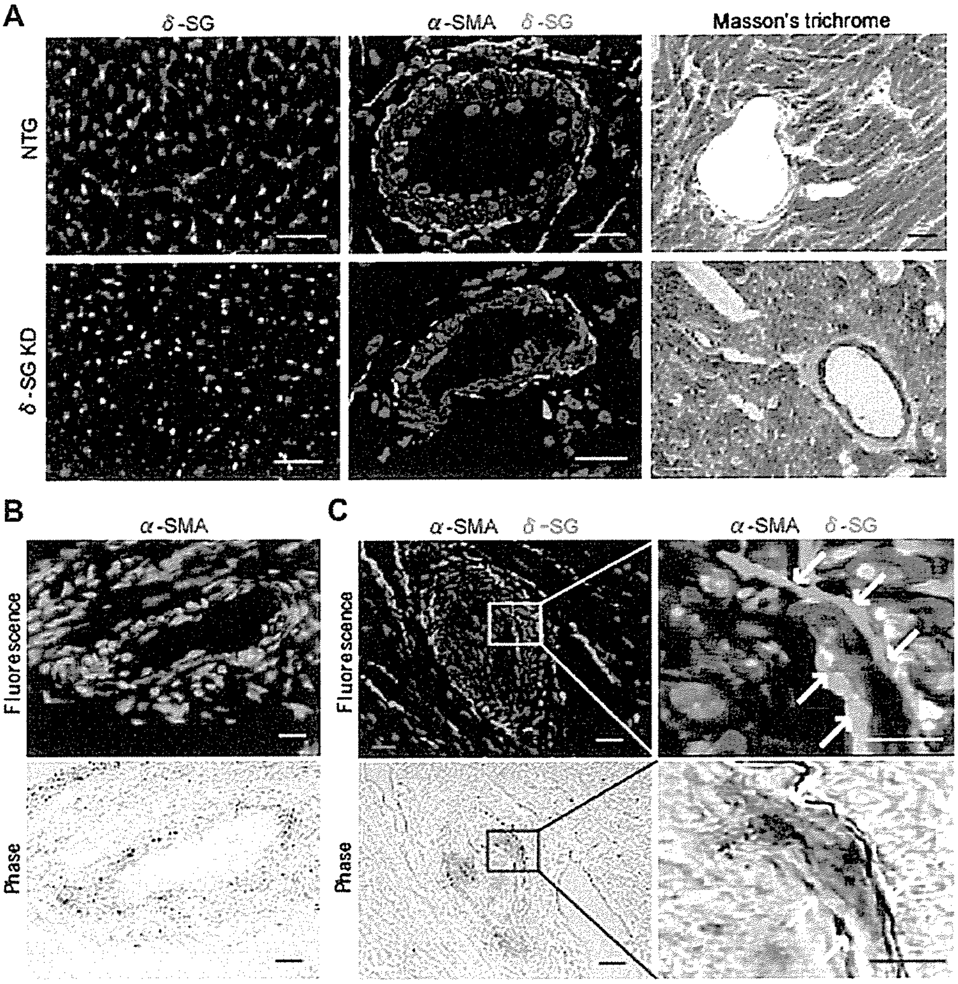

Fig. 3. Vascular regeneration in δ-SG KD hearts with the restoration of δ-SG expression. (A, left panels) Decreased δ-SG (red) expression in cardiac muscle was significantly observed in δ-SG KD hearts compared with NTG littermates. DAPI (blue). (A, middle panels) Vascular lumens were more constrictive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