Biochemical and Biophysical Research Communications 425 (2012) 859–865

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Intracoronary artery transplantation of cardiomyoblast-like cells from human adipose tissue-derived multi-lineage progenitor cells improve left ventricular dysfunction and survival in a swine model of chronic myocardial infarction

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ARTICLE INFO

Article history:
Received 12 July 2012
Available online 7 August 2012

Keywords:
Cellular cardiomyoplasty
ADMPCs
Myocardial infarction
Transplantation

ABSTRACT

Transplantation of human cardiomyoblast-like cells (hCLCs) from human adipose tissue-derived multi-lineage progenitor cells improved left ventricular function and survival of rats with myocardial infarction. Here we examined the effect of intracoronary artery transplantation of human CLCs in a swine model of chronic heart failure. Twenty-four pigs underwent balloon-occlusion of the first diagonal branch followed by reperfusion, with a second balloon-occlusion of the left ascending coronary artery 1 week later followed by reperfusion. Four weeks after the second occlusion/reperfusion, 17 of the 18 surviving animals with severe chronic MI (ejection fraction <35% by echocardiography) were immunosuppressed then randomly assigned to receive either intracoronary artery transplantation of hCLCs hADMPCs or placebo lactic Ringer's solution with heparin. Intracoronary artery transplantation was followed by the distribution of DiI-stained hCLCs into the scarred myocardial milieu. Echocardiography at post-transplant days 4 and 8 weeks showed rescue and maintenance of cardiac function in the hCLCs transplanted group, but not in the control animals, indicating myocardial functional recovery by hCLCs intracoronary transplantation. At 8 week post-transplantation, 7 of 8 hCLCs transplanted animals were still alive compared with only 1 of the 5 control ($p = 0.0147$). Histological studies at week 12 post-transplantation demonstrated engraftment of the pre DiI-stained hCLCs into the scarred myocardium and their expression of human specific alpha-cardiac actin. Human alpha cardiac actin-positive cells also expressed cardiac nuclear factors; nkx2.5 and GATA-4. Our results suggest that intracoronary artery transplantation of hCLCs is a potentially effective therapeutic strategy for future cardiac tissue regeneration.

1. Introduction

End-stage heart failure remains a major cause of death worldwide, mainly due to myocardial ischemia. Cardiac transplantation and mechanical support using implantation of the left ventricular assist system (LVAS) were established as the ultimate means of support for these patients [1,2]. However, these treatment entities have certain limitations including donor shortage, rejection, and LVAS durability, and alternative strategies are needed in such circumstances.

Cellular cardiomyoplasty was developed as a new approach to restore normal heart function, [3,4] using a variety of cell types [3–5]. Mesenchymal stem cells (MSC) seem particularly advantageous for cellular therapy in general because they are multipotent, potentially immune privileged [6]. MSC also proliferate rapidly and differentiate into cardiomyogenic cells [7–10]. MSC can be isolated from human adipose tissue, which can be resected easily and safely in most patients [11,12]. In fact, we have reported that adipose tissue-derived multilineage progenitor cells (ADMPCs), which met the criteria as mesenchymal stem cells [13], can differentiate into hepatocytes both *in vitro* and *in vivo* [14,15]. Recently, we demonstrated that human cardiomyoblast-like cells (hCLCs) from human adipose tissue-derived multi-lineage progenitor cells transplanted into rats with chronic myocardial infarction reversed wall thinning

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http://dx.doi.org/10.1016/j.bbrc.2012.08.004

in the scarred area with the engrafted cells forming a thick stratum, and that the hCLCs reversed left ventricular dysfunction in the long term and survival of rats with experimentally-induced myocardial infarction [16].

The present study is an extension to the above study and was designed to accelerate the clinical application of hCLCs. Specifically, we examined in pre-/non-clinical studies the effects of hCLCs transplantation on cardiac dysfunction and on long-term survival with swine chronic myocardial infarction model. We also documented the histological regeneration of damaged myocardium after transplantation of hCLCs *in vivo*.

2. Materials and methods

2.1. Adipose tissue

Adipose tissue samples were resected from five human subjects during plastic surgery (all females, age, 20–60 years) as excess discards. Ten to 50 g of subcutaneous adipose tissue were collected from each subject after obtaining of informed consent. The protocol was approved by the Review Board for Human Research of Kobe University Graduate School of Medicine, Osaka University Graduate School of Medicine and Foundation for Biomedical Research and Innovation.

2.2. Isolation of hADMPCs and preparation of hCLCs

Human adipose tissue-derived multi-lineage progenitor cells (hADMPCs) were prepared as described previously [13–17]. After passaging 5 to 6 times, the hADMPCs were replated and treated with 0.1% dimethyl sulfoxide (DMSO) (Cryoserve, GE Healthcare Biosciences, Uppsala, Sweden) for 48 h.

2.3. Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from hADMPCs and cardiomyoblasts using an RNAeasy kit (Qiagen, Hilden, Germany). After treatment with DNase, cDNA was synthesized from 500 ng total RNA using Superscript III reverse transcriptase RNase H minus (Invitrogen, Carlsbad, CA). Real-time PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). 20X Assays-on-Demand™ Gene Expression Assay Mix for *nkx2.5* (Hs00231763_m1), *islet-1* (Hs00158126_m1), *GATA-4* (Hs00171403_m1), *alpha-cardiac actin* (Hs01109515_m), *cardiac troponin I* (Hs00165957_m1), *myosin light chain* (*MLC*) (Hs00166405_m1), *myosin heavy chain* (*MHC*) (Hs00411908_m1) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (Hs99999905_m1) were obtained from Applied Biosystems. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2X), was also purchased from Applied Biosystems. Reactions were performed in quadruplicate and the mRNA levels were normalized relative to human GAPDH expression. Then the fold-inductions of hCLCs were compared to hADMPCs.

2.4. Animal model of myocardial infarction and cell transplantation

Five weeks before transplantation, the first diagonal branch (D1; #9) of the coronary arteries of 24 pigs (8-week-old female, 30.5 ± 0.7 kg, mean ± standard error of the mean) was balloon-occluded for 60 min followed by reperfusion, (Fig. 1A). One week later, the left ascending coronary artery of the same animals was balloon-occluded just proximal of the first septal branch divergence (#6), followed by reperfusion (Fig. 1A). To rescue the better baseline survivals and to obtain severe old myocardial infarction swine model, two separate reperfused infarcts one week apart were performed. From 5 days before cell transplantation to the end of the experiment, the swine received tacrolimus as an immunosuppressant (0.1 mg/kg/day intramuscularly) (Fig. 1B) as previously reported [18] with modification. Four weeks after the second occlusion/reperfusion (day 0), we examined 17 animals with chronic severe MI (ejection fraction <35% by echocardiography) of only 18 survivors. The tacrolimus-immunosuppressed chronic MI swine were randomly assigned to receive intracoronary transplantation of hCLCs (3×10^5 cells/mL concentration of cell

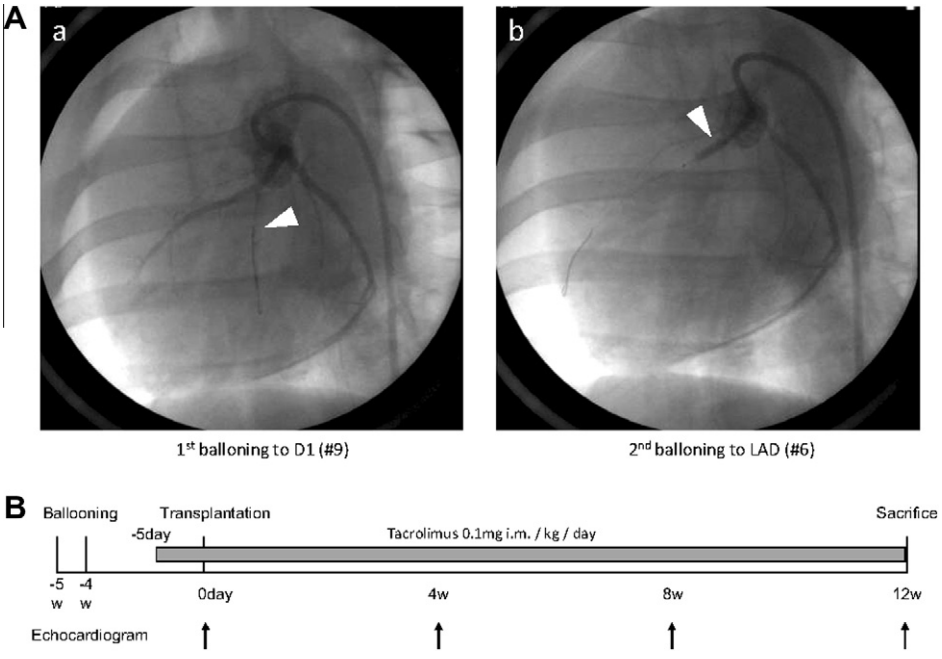

Fig. 1. Study protocol and angiographic demonstration of transient coronary artery occlusion. (A) Five weeks before transplantation, the first diagonal branch (D1; #9) of the coronary arteries was balloon-occluded followed by reperfusion (a, arrowhead). One week later, the left ascending coronary artery of the same animals was balloon-occluded just proximal of the first septal branch divergence (#6), followed by reperfusion (b, arrowhead). (B) From 5 days before cell transplantation to the end of the experiment, the swine received tacrolimus as an immunosuppressant. At day 0, 17 animals with chronic severe MI were applied for the experiment.

Table 1
Cardiocytic induction of hCLCs.

	Fold induction	
	Mean	SE
nkx2.5	2.49	1.02
islet-1	1.32	0.36
GATA-4	6.84	1.47
Alpha-Cardiac actin	1.46	0.22
Cardiac troponin I	2.36	0.47
Myosin light chain	1.89	0.49
Myosin heavy chain	109.89	6.13

suspension, 1 mL/kg cell suspension was transplanted.) ($n = 8$), hADMPCs (3×10^5 cells/mL concentration of cell suspension, 1 mL/kg cell suspension was transplanted.) ($n = 4$), or placebo lactic Ringer's solution with heparin ($n = 5$), at 4 weeks after the second occlusion/reperfusion. Transplantation procedure was performed as following, the transarterial catheter was placed in the left coronary artery, and then the cell-suspensions or placebo control solutions were transplanted into LAD (#6). The Osaka University Graduate School of Medicine Standing Committee on Animals approved all experimental protocols.

2.5. Assessment of swine cardiac function and histological analysis

Cardiac ultrasound studies were performed before cell-transplantation and at 4, 8 and 12 weeks after transplantation using a VIVID 7 system (GE Healthcare Biosciences, Uppsala, Sweden) and the data at the day transplantation, 4- and 8-week-after transplantation were applied for the statistical analysis. The studies were shown as M-mode with short axis view observed from left fifth intracostal space.

For histological analysis, the swine hearts were dissected out at the end of the experiment and immediately fixed overnight in 4% paraformaldehyde and processed for embedding in paraffin wax. Sections were cut at 3-μm thickness, deparaffinized and then rehydrated through a graded ethanol series into distilled water. The sections were then immersed in Target Retrieval Solution (Dako, Glostrup, Denmark) and boiled, followed by cooling at room temperature for 20 min. Sections were incubated overnight with 10% blocking solution (Nacalai tesque) in TBS-T, and then in a humidity chamber for 16 h at 4 °C with mouse monoclonal antibodies to human alpha-cardiac actin (American Research Products., Belmont, MA), human myosin heavy chain (MHC) (mouse monoclonal anti-human myosin heavy chain cardiac antibody, Cat: 05-833., Upstate, NY) and CD34 (ab81289 [EP373Y], Abcom) diluted in blocking solution, followed by Alexa Fluor 488-labeled anti- IgG (Molecular Probes, Eugene, OR) with counter DAPI-staining. Hematoxylin and eosin stain, Masson trichrome stain and Sirius red stain were also performed. The stained all slides were viewed on a BioZero laser scanning microscope (Keyence, Osaka, Japan). The scarred area percentages of the middle portion and apex side of LV were calculated by area stained blue with Masson's trichrome staining/total of 10 each independent sections using software Dynamic Cell Count (Keyence, Osaka, Japan).

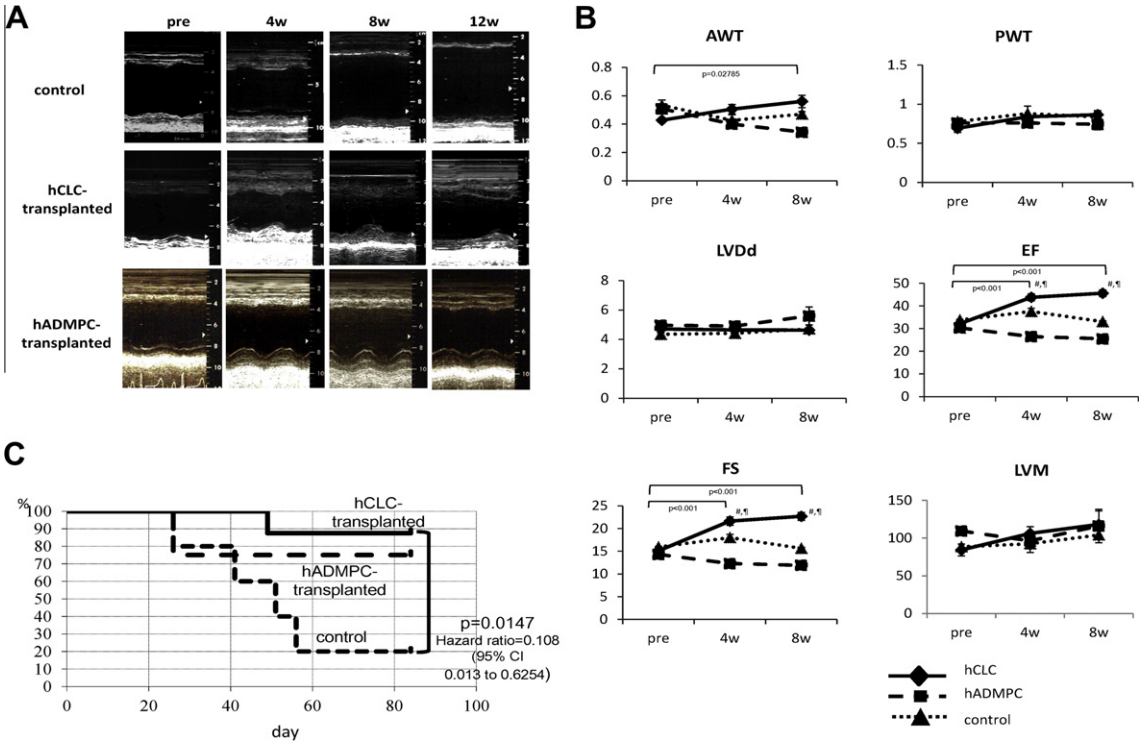

Fig. 2. Effects of hCLCs transplantation on cardiac function and survival rate. (A) In the hCLCs transplanted group, M-mode echocardiography showed improved wall motion within 4 weeks of transplantation. In contrast, worsening of the wall motion was noted in the mock-transplanted control swine. (B) Anterior wall thickness (AWT), ventricular ejection fraction (EF) and fractional shortening (FS) improved significantly in the hCLCs transplanted group, as confirmed by echocardiography. In the hCLCs transplanted swine, cardiac functions were recovered from transplantation to the end of the study. In the hADMPCs transplanted swine, cardiac functions were maintained from transplantation to the end of the study. In contrast, worsening of these cardiac function parameters was noted after mock-transplantation. The left ventricular diastolic dimension (LVDd) was maintained during the course of the experiment in hCLCs transplanted swine, but increased in the control groups. Posterior wall thickness (PWT) and left ventricular mass (LVM) showed no significant difference in the groups. Solid lines and squares indicated the transplanted group and the dashed lines and open squares indicated the control group. The symbol # indicated $p < 0.01$ hCLCs -transplanted vs control and indicated $p < 0.01$ hADMPC-transplanted vs control, respectively. Bars indicated mean ± standard error of the mean (SEM). (C) Effect of hCLCs transplantation ($n = 8$), hADMPCs transplantation ($n = 4$) and lactic Ringer's solution injection ($n = 5$) on long-term survival rates of swine. Kaplan–Meier survival curve analysis demonstrated significant difference in the survival rates between the hCLCs group and the lactic Ringer's solution group.

2.6. Statistical analysis

Longitudinal changes between groups were tested with the use of mixed-model repeated-measures analysis of variance, with adjustment for baseline values. When the overall *P* value for the main effect of group or time, or interaction between group and time was less than 0.05, the post hoc multiple comparisons with the use of the single-step adjustment method as implemented by Hothorn et al. were performed [19]. Survival curves were constructed by the Kaplan–Meier method and survival among groups was compared using the Log-Rank test (StatMate III for Windows, Atoms, Tokyo).

3. Results

3.1. Cardiocytic commitment of hADMPCs into hCLCs

The potential for hADMPCs to commit into CLCs was evaluated from the mRNA expression of several cardiocytic markers by quantitative reverse transcriptase-PCR before and after DMSO induction, as follows: *islet-1* is a cardiac stem cell marker; *nkx2.5* and *GATA-4* are transcription factors required for subsequent cardiac differentiation; and *alpha-cardiac actin*, *myosin light chain* (*MLC*), and *myosin heavy chain* (*MHC*) are markers of cardiocytic commitment (Table 1). After induction, hADMPCs expressed all markers with increment, indicating that hADMPCs could be successfully committed into cells of the cardiac lineage, hCLCs.

3.2. Effects of hCLCs transplantation on cardiac function and survival rate

Cardiac function was assessed by echocardiography. Four weeks after intracoronay transplantation of hCLCs, wall motion was improved but not in the placebo group (Fig. 2A). The wall motion of control swine worsened at 12 weeks after transplantation, while the improved motion was maintained after the hCLCs transplant (Fig. 2A). In the early post-transplantation period, there was no significant difference in left ventricular diastolic dimension (LVDd) between hCLCs -transplanted swine and the control. During the course of the study, LVDd exacerbated gradually in the control swine while it did not change significantly in the transplant swine (Fig. 2B). Likewise, the left ventricular ejection fraction (EF) and fractional shortening (FS) improved in the implanted group, but not in control swine (Fig. 2B). After hCLCs transplantation via left anterior descending (#6), the anterior wall thickness improved in the implanted group, but not in control swine. These results indicate that intracoronary transplantation of hCLCs resulted in recovery of cardiac function.

The Kaplan–Meier survival curves showed higher long-term survival rates for the hCLCs transplanted group than the control (Fig. 2C). Notably, only 1 of 8 swine died after transplantation of hCLCs. Survival at 12 weeks after transplantation was significantly higher in the hCLCs group (87.5%) than the control group (20%, 1 of 5) (Log-rank test: $p = 0.0147$. Hazard ratio = 0.108: 95% CI 0.013 to 0.625). These results suggest that transplantation of hCLCs

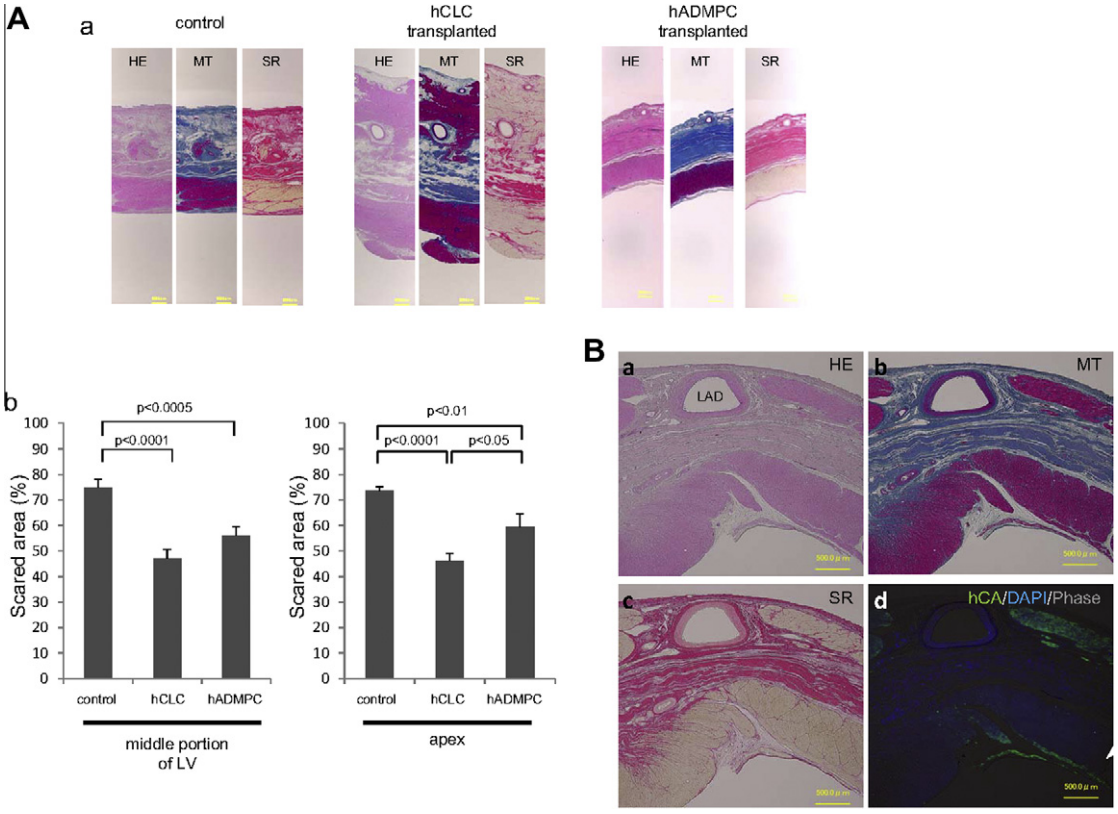

Fig. 3. Effects of hCLCs transplanted via coronary artery on cardiac structure. (A) (a) Photomicrographs of representative myocardial sections of the scarred area stained with hematoxylin/eosin (HE), Masson trichrome (MT) and Sirius red (SR) in the hCLCs-, hADMPC-transplantation and mock-transplanted control groups. Transplantation of hCLCs improved myocardial wall thickness in the infarcted myocardium and resulted in the development of new cardiac muscles on the surface. Bars = 500 μm. HE; hematoxylin and eosin staining, MT; Masson trichrome staining, and SR; Sirius red staining. (b) The scarred area percentages of the middle portion and apex side of LV. The scarred area percentages of hCLCs-, hADMPC-transplantation and control groups were calculated by area stained blue with Masson's trichrome staining /total of 10 independent sections. The error bar indicated SEM. (B) Photomicrographs of representative myocardial sections of apical side of the anterior wall stained with HE (a), MT (b), SR (c) or phase contrast merge image of neighboring sections stained with anti-human alpha-cardiac actin (hCA; green), and DAPI as counter staining (d). In the HE-, MT-, and SR-stained sections, cardiac muscles were distributed on the scarred areas, and some parts of these muscles expressed human alpha-cardiac actin (green). Bars = 500 μm. LAD; left anterior descending.

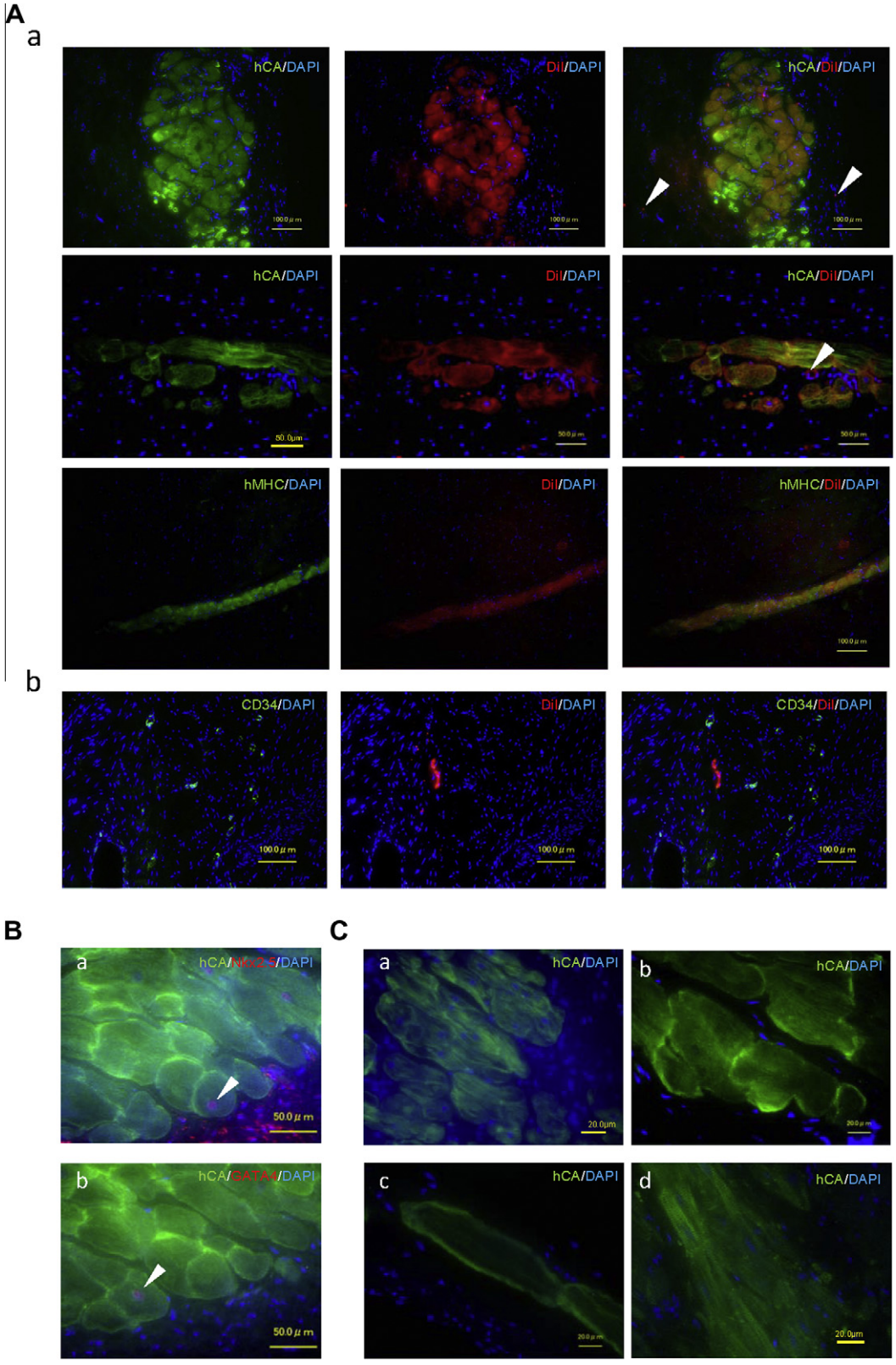

Fig. 4. hCLCs survive *in situ*. (A) (a) *In situ* survivals of the fluorescent DiI-prestained hCLCs into cardiomyocytes at 12-week- after transplantation. Note the presence of human alpha-CA positive cardiac muscle bundles or cells and that almost all cells exhibit DiI-fluorescence. Only minor part of DiI-positive cells did not express human alpha-CA (arrowheads). DiI-prestained cells were also positive for human myosin heavy chain (lower panel). (b) Survivals of hCLCs outside of vessel capillaries. The vessel capillaries were stained with anti-CD34 antibody and localization of DiI-positive cells were examined using fluoromicroscpy. DiI-positive cells exist outside of vessel capillaries which were stained with anti-CD34 antibody (B) Co-expression of human alpha-CA (green) and Nkx2.5 (purple) (a) or GATA-4 (purple) (b) in the nuclei of human alpha-CA positive cells. (C) Typical expression patterns of human alpha-CA on the cells. Human alpha-CA exhibited a brush pattern in oval cells (a), a spot pattern in cell-to-cell contact areas (b), as a sarcomeric structure beneath around the cell surface (c), and in a pattern resembling cardiomyocytes (d). Bars = 20 μm.

improves long-term survival rate of swine with heart failure induced by chronic myocardial infarction.

3.3. Effects of hCLCs transplantation on cardiac structure

Twelve weeks after transplantation, the treated swine were sacrificed and cardiac tissues prepared for histological examination for further analysis of cardiac structure and delineate the difference between hCLCs transplanted animals and controls (Fig. 3). Hematoxylin/eosin, Masson's trichrome and Sirius red staining showed the presence of a thin layer of cardiac muscles and massive fibrosis in the scarred anterior left ventricular wall of the control and hADMPCs transplanted swine (Fig. 3Aa). In contrast, the same staining techniques in hCLCs -transplanted swine showed significant thickening of the infarcted myocardium and layers of cardiomyocytes on the anterior ventricular wall (Fig. 3Aa). Next, to confirm the hCLCs could rescue from the fibrosis on cardiac structure, the scarred area percentages of the middle portion and apex side of LV were calculated. As shown in Fig. 3Ab, the percentage of scarred area of hCLCs -transplantation heart reduced compared to the control swine heart and hADMPC-transplanted one in both middle portion of LV and apex side.

3.4. hCLCs integrated in situ with the cardiac milieu

The *in situ* differentiation capacity of the implanted hCLCs into cardiomyocytes after grafting onto the scarred myocardium was assessed by immunohistochemical staining for human alpha-CA (Fig. 3B). Thin layers of cardiomyocytes were noted on the scarred myocardium by hematoxylin and eosin staining and Masson trichrome staining. Furthermore, clusters of human alpha-CA-positive cells were identified on the scarred myocardium (Fig. 3B; Green, arrowhead), indicating that hCLCs might integrate *in situ* with the cardiac milieu.

To confirm that the transplanted hCLCs survived *in situ*, we chased the fluorescent DiI-prestained hCLCs *in situ* 12 weeks after transplantation using histochemical technique. The top panel of Fig. 4Aa shows human alpha-CA positive cardiac muscle bundle and almost all cells of the bundle were DiI-fluorescent. The middle panel shows that all human alpha-CA-expressing cells were prestained DiI-fluorescent. DiI-prestained cells were also positive for human myosin heavy chain (Fig. 4Aa lower panel). On the other hand, DiI-positive cells exist outside of vessel capillaries which were stained with anti-CD34 antibody (Fig. 4Ab). Since cardiomyocytes are known to express the nuclear transcriptional factors; Nkx2.5 and GATA-4, we examined the expression of these molecules on human alpha-CA positive cells. The nuclei of human alpha-CA positive cells (green) expressed Nkx2.5 (purple) (Fig. 4Ba) and those of human alpha-CA positive cells (green) expressed GATA-4 (purple) (Fig. 4Bb), adding further confirmation that hCLCs might differentiate into cardiac marker positive cells.

The expression patterns of human alpha-CA on the cells were presented in Fig. 4C. The first pattern of human alpha-CA expression was the brushed pattern in oval-shaped cells (Fig. 4Ca). Alpha-CA also showed a spot pattern in the cell-to-cell contact areas (Fig. 4Cb). Resident alpha-CA-like immunoreactivity also appeared as sarcomeric structure beneath and around the cell surface (Fig. 4Cc). The fourth pattern of alpha-CA was cardiomyocyte structure-like pattern (Fig. 4Cd). These results indicate that hCLCs survive *in situ* and integrate into the cardiac milieu.

4. Discussion

There are several advantages to intracoronay transplantation of hCLCs for regeneration therapy. First, the source of adipose-derived cells is easily and safely accessible and large quantities of the cells can be obtained without serious ethical issues. Second, hCLCs can survive *in vivo* within the myocardial milieu. Finally, the reconstruction of a thick myocardial wall rescued cardiac dysfunction after chronic myocardial infarction and improved long-term survival in our swine model.

The choice of cell source is critical for realizing success in cellular therapy [19,20]. The adipose tissue is easily and safely accessible without serious ethical issues, and the cells can be obtained in large quantities since liposuction surgeries yield from 100 ml to >3 L of lipoaspirate tissue [21]. In the literature, isolation of cells from adipose tissue was first described by Bjorntorp et al. [22]. This procedure was then modified for the isolation of cells from human adipose tissue specimens [23–25]. In this context, Zuk et al. [11] reported the presence of cells with properties resembling those of mesenchymal stem cells resident in adipose tissue and they renamed the cell populations as adipose tissue-derived stromal/stem cells (ADSC). Recently, we have reported hADMPC as a novel cell population in human adipose tissue and indicated that these cells have stem cell features resembling mesenchymal stem cells including their ability to differentiate into cardiomyocytes in rat infracted cardiac milieu, into hepatocytes in rabbit hepatic milieu *in situ*, and into clusters of islet-like cells and hepatocytes *in vitro* [13–16]. Based on the above advantages, hADMPCs represent a potentially promising source of cells for cellular therapy, including patients with severe heart failure.

While the differentiation of ADSCs *in vitro* has been reported [26], only a few studies reported their differentiation into cardiomyocytes *in vivo* [27–29]. In one study, rat ADSCs were isolated and grown in intact monolayer sheets using temperature-responsive culture dishes. Placement of the rat ADSC sheets onto scarred myocardium in rats reduced the scarring and enhanced cardiac structure and function. Histological analysis demonstrated that the engrafted rat ADSC sheets grew to form a thickened layer that included newly formed vessels and few cardiomyocytes. In this context, Gimble et al. [20] suggested that hADSCs might secrete angiogenic factors. In our previous study, hCLCs survived within the rat myocardial milieu *in vivo*, as indicated by immunohistological results, suggesting that the newly developed myocardium could augment cardiac function.

As indicated in this study, transplantation of the hCLCs via the coronary artery resulted in the development of a new thick myocardial tissue, rescued cardiac dysfunction after MI in the swine model, and improved long-term survival rate compared to the control. Our findings suggest that hCLCs can be engrafted and survive within the myocardial infarct milieu, acquire phenotypic markers consistent with cardiomyocytic lineages, and have a positive impact on structural and functional endpoints. These are desirable outcomes for cardiac function and survival. Despite these encouraging results, much progress is needed to realize the hope of cell therapies for myocardial damage. First, delivery of the cell number to patients should be optimized for each given disease. Second, the risk–benefit based approach should be considered in the infracted or affected tissues after transplantation. Finally, the value and impact of hCLCs -transplantation should be confirmed in Investigational New Drug approval before embarking on clinical trials and applications.

In conclusion, we showed that the hCLCs were successfully engrafted into the scarred myocardium. The hCLCs -transplantation via the coronary artery also resulted in recovery of cardiac function and improved survival rate. Thus, transplantation of hCLCs in heart patients is a potentially effective therapeutic strategy for cardiac tissue regeneration within a few years.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Akifumi Matsuyama from the Ministry of Health, Labor and Welfare

(MHLW) (http://www.mhlw.go.jp/ Grant No: H21-Nanchi-Ippan-219) and by a Grant-in Aid for Akifumi Matsuyama by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) (http://www.nibio.go.jp/ Grant No: 09-19).

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