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A novel approach for myocardial regeneration with educated cord blood cells cocultured with cells from brown adipose tissue

Yoshihiro Yamada [a,*], Shin-ichiro Yokoyama [b], Noboru Fukuda [b], Hiroyasu Kidoya [a], Xiao-Yong Huang [a], Hisamichi Naitoh [a], Naoyuki Satoh [a], Nobuyuki Takakura [a,*]

[a] *Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita-shi, 565-0871, Japan*
[b] *Second Department of Internal Medicine, Nihon University School of Medicine, Ooyaguchi-kami 30-1, Itabashi-ku, Tokyo 173-8610, Japan*

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Abstract

Umbilical cord blood (CB) is a promising source for regeneration therapy in humans. Recently, it was shown that CB was a source of mesenchymal stem cells as well as hematopoietic stem cells, and further that the mesenchymal stem cells could differentiate into a number of cells types of mesenchymal lineage, such as cardiomyocytes (CMs), osteocytes, chondrocytes, and fat cells. Previously, we reported that brown adipose tissue derived cells (BATDCs) differentiated into CMs and these CMs could adapt functionally to repair regions of myocardial infarction. In this study, we examined whether CB mononuclear cells (CBMNCs) could effectively differentiate into CMs by coculturing them with BATDCs and determined which population among CBMNCs differentiated into CMs. The results show that BATDCs effectively induced CBMNCs that were non-hematopoietic stem cells (HSCs) (educated CB cells: e-CBCs) into CMs in vitro. E-CBCs reconstituted infarcted myocardium more effectively than non-educated CBMNCs or CD34-positive HSCs. Moreover, we found that e-CBCs after 3 days coculturing with BATDCs induced the most effective regeneration for impaired CMs. This suggests that e-CBCs have a high potential to differentiate into CMs and that adequate timing of transplantation supports a high efficiency for CM regeneration. This strategy might be a promising therapy for human cardiac disease.

Keywords: Stem cell; Cord blood; Adipose tissue; Myocardial regeneration; Cell fusion

Myocardial regeneration is currently a popular topic in cardiac medicine, and research in regenerative medicine has advanced in an explosive manner. Many cell types such as bone marrow mesenchymal stem cells (BM-MSCs) [1–3], embryonic stem (ES) cells [4,5], and cardiac tissue stem cells [6,7] have been found to undergo myocardial differentiation and can be used as a source for cardiomyocytes (CMs). Additional cell types may also prove to have cardiac differentiation ability. With regard to human therapy, umbilical cord blood (CB) is a promising source because transplantation of CB has already been established for patients with blood diseases. Moreover, usage of CB overcomes considerable problems encountered with other sources of CMs, such as allergenic, ethical, and tumorigenic issues. Furthermore, CB contains both hematopoietic stem cells (HSCs) [8] and MSCs [9]. Also, stem cells are more abundant in CB than in adult human peripheral blood or bone marrow (BM) and stem cells in CB have a higher proliferative potential associated with an extended life span and longer telomeres [10–12].

Indeed, $CD34^+$ cells derived from human CB homed to infarcted hearts and reduced the size of the infarcted area; this was not through direct differentiation into CMs, but through enhancing neovascularization [13,14]. These studies showed no evidence of myocytes of human origin in the infracted myocardium; however, it was reported that unrestricted somatic stem cells (USSCs) from human CB could differentiate into CMs in vitro and in vivo [15]. Such

* Corresponding authors. Fax: +81 6 6879 8314.
E-mail addresses: yamaday@biken.osaka-u.ac.jp (Y. Yamada), ntakaku@biken.osaka-u.ac.jp (N. Takakura).

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USSCs are fibroblastic in appearance and negative for hematopoietic cell markers, such as c-kit, CD34, and CD45. USSCs injected into immunosuppressed pig model of myocardial infarction (MI) improved perfusion and wall motion, reduced infarct scar size, and enhanced cardiac function. USSCs seem to be a useful source for myocardial regeneration; however, they are a rare population, therefore, expansion of USSCs is required for application to clinical therapy. In spite of these challenges for the repair of CM, to date, no clinical studies of CB have been reported. Previously, we reported that brown adipose tissue derived cells (BATDCs) included cardiac progenitor cells and they effectively differentiated into CMs in vitro and in vivo [16]. This indicated that our culture system of BATDCs contained differentiation molecular cues for CMs. When BATDCs were injected into MI rats, they differentiated into CMs as well as endothelial cells (ECs) and smooth muscle cells (SMCs), supported the growth of resident cells and vascular cells, and restored cardiac function. This suggested a potential therapeutic use for BATDCs in human ischemic heart disease. However, in humans, BAT exists only in the embryonic stage and infants, therefore, it is difficult to obtain BATDCs to treat adult cardiac disease. In this study, we examined whether mononuclear cells from CB can differentiate into CMs upon coculturing with BATDCs. Moreover, usefulness of CB cells that were exposed to BATDCs for MI repair was evaluated.

Materials and methods

Cell preparation and flow cytometry. Brown adipose tissue (BAT) was dissected from postnatal day (P1) to P7 neonates of C57BL/6 mice. BAT was dissociated by DispaseII (Roche, Mannheim, Germany), drawn through a 23G needle and prepared as single cell suspension as previously reported [16]. Human CB mononuclear cells (CBMNCs) were purchased from Cambrex (Baltimore, MD). The cell-staining procedure for the flow cytometry was also as previously described [17]. The monoclonal antibodies (mAbs) used in immunofluorescence staining were anti-human CD45, -34, and -HLA–ABC mAbs (Pharmingen, SanDiego, CA). All mAbs were purified and conjugated with fluorescein isothiocyanate (FITC), PE (phycoerythrin), biotin or allophycocyanin (APC). Biotinylated antibodies were visualized with PE-conjugated streptavidin (Pharmingen) or APC-conjugated streptavidin (Pharmingen). Cells were incubated for 5 min on ice with CD16/32 (FcγIII/II Receptor) (1:100) (Fcblock™, Pharmingen) prior to staining with primary antibody. Cells were incubated in 5% fetal calf serum/phosphate-buffered saline (FCS/PBS; washing buffer) with primary antibody for 30 min on ice, and washed twice with washing buffer. Secondary antibody was added and the cells were incubated for 30 min on ice. After incubation, cells were washed twice with, and suspended in, the washing buffer for fluorescence-activated cell sorter (FACS) analysis. The stained cells were analyzed and sorted by EPICS Flowcytometer (BECKMAN COULTER, San Jose, CA). The sorted cells were added to 24-well dishes (Nunc, Roskilde, Denmark), precoated with 0.1% gelatin (Sigma, St. Louis, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma), supplemented with 10% FCS and 10^{-5}M 2-mercaptoethanol (2-ME), at 37 °C in a 5% CO_2 incubator.

Cell coculture. BATDCs were prepared as described above. When BATDCs and human (h) CBMNCs were cocultured in contact conditions, 1×10^5 BATDCs were plated per well of a 24-well plate and cultured for 7 days, and then, 1×10^5 CBMNCs, or 1×10^4 $CD34^+$ HSCs were cultured with BATDCs for 10 days. Staining was performed with anti-cardiac troponinT (Santa Cruz), -MEF-2C (Cell signaling) and -HLA ABC antibodies (Pharmingen).

Supplemental information reveals the Materials and methods for RT-PCR analysis, Immunohistochemistry, FISH staining, and procedure for mouse myocardial infarction (MI) model and echocardiography.

Results

Differentiaion of CBMNCs into CMs by coculturing with BATDCs in vitro

Previously, we reported that BATDCs differentiate into CMs spontaneously; this suggests that BATDCs produce molecules that induce self differentiation into CMs by an autocrine loop. Moreover, CBMNCs contained cells with a potential to differentiate into various cell types, such as osteoblasts, chondrocytes, and CMs. Therefore, to determine whether molecules produced from BATDCs induce CBMNCs to differentiate into CMs effectively, we cultured CBMNCs with BATDCs and observed the differentiation of CBMNCs into CMs. At first, dissociated BATDCs from P1 to P7 neonatal mice were cultured on 0.1% gelatin-coated dishes. After 1 week, human CBMNCs were added and cocultured with BATDCs. After coculturing CBMNCs with BATDCs for 14 days, among HLA-positive CBMNCs, nuclear located MEF2C positive and cardiac troponinT-positive cells (Fig. 1A and B), or cardiac troponinI-positive cells (Fig. 1C) were effectively produced. In contrast, sorted $CD34^+38^-$HSC population from CBMNCs, which was previously reported to differentiate into CMs [18], was differentiated into cardiac troponinT-positive CMs (Fig. 1E); however, the frequency of CM differentiation from HSC population was lower than that from total CBMNCs (Fig. 1I). CBMNCs alone did not differentiate into cardiac troponinT-positive or MEF2C-positive cells spontaneously under the same culture medium without coculturing with BATDCs (Fig. 1G, H, and I).

The expression of CM-specific genes in CBMNCs educated by culturing with BATDCs

Next, we evaluated the length of time required for CBMNCs to become committed CM lineage cells when cocultured with BATDCs. For this purpose, we attempted to coculture CBMNCs with BATDCs for 1 to 7 days and $HLA^+CD45^-CD34^-$ non-hematopoietic cells [we termed them educated CB cells (e-CBCs)] were then sorted and mRNA was extracted from the cells as indicated in Fig. 2A. Because mature hematopoietic cells from cocultured CBMNCs did not differentiate into CMs (data not shown) and HSC population barely differentiated into CMs (Fig. 1), we deduced that cardiac stem/progenitor cells were more abundant in non-hematopoietic cells and were therefore $CD45^-CD34^-$. We analyzed the expression of CM-specific genes on days 0, 3, and 7 as indicated in Fig. 2B and confirmed that cardiac actin, myosin light chain 2v, and specific transcriptional factor, such as

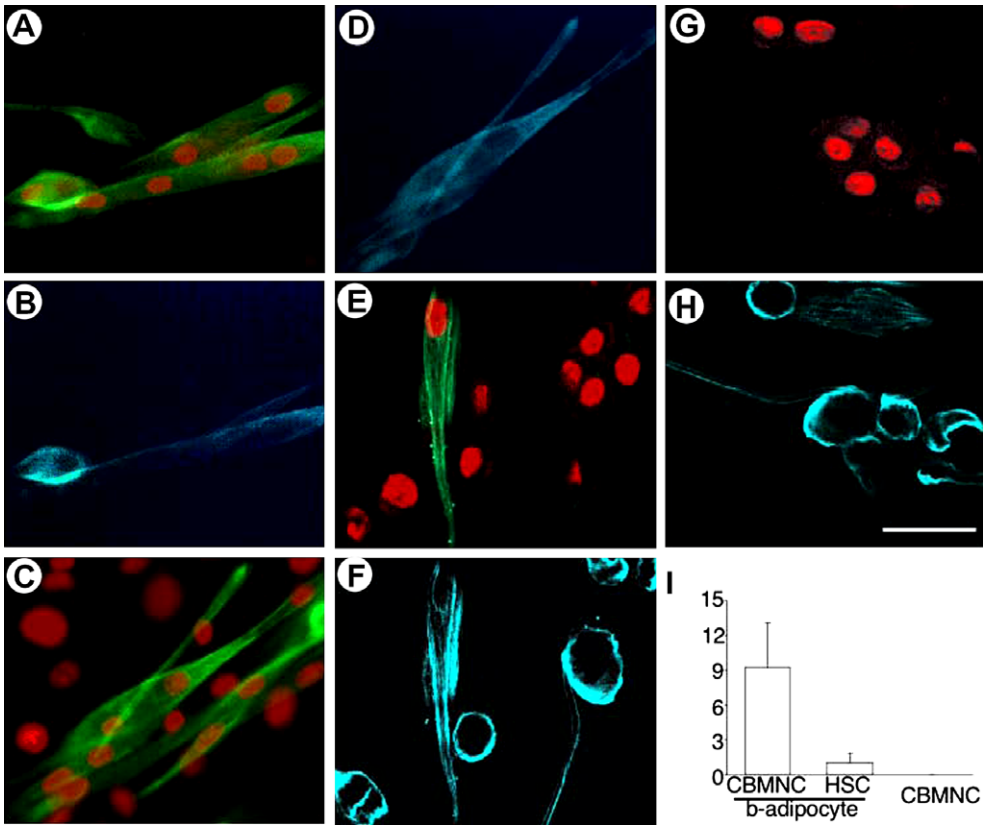

Fig. 1. CBMNCs can differentiate into CMs upon coculturing with BATDCs. Immunocytochemical analysis of human CBMNCs (A–D), and $CD34^{+}CD38^{-}$HSCs (E, F) cocultured with BATDCs from wild type mice, or CBMNCs (G, H) cultured without BATDCs for 14 days. (A) Expression of cardiac troponinT (green) and MEF2C (red). (B) Human HLA expression (blue) in the same field as (A). (C, E, and G) Expression of cardiac troponinI (green) and nuclear staining with PI (red). (D, F, and H) Human HLA expression (blue) in the same fields as (C, E, and G), respectively. Scale bar in (H) indicates 5 μm. (I) Quantitative evaluation of differentiated cardiac troponinT and MEF2C-positive CMs among adhering HLA-positive CB-derived cells. Data for CBMNC, and $CD34^{+}CD38^{-}$HSCs cocultured with BATDCs (b-adipocyte) and CBMNCs cultured without BATDCs are displayed. Results represent means ± SD of five independent experiments.

GATA-4 and Nkx2.5 were expressed on day 3 of coculture (Fig. 2B). MHC alpha and beta mRNAs were not expressed on day 3; however, they started to be expressed around day 7.

Educated CBMNCs in non-hematopoietic lineage contributed to myocardial regeneration

As indicated in Fig. 2, 3 days of coculturing with BATDCs was enough for commitment of CBMNCs into CM lineage. Next, in order to determine whether e-CBCs could effectively contribute to the regeneration of the heart, we injected the e-CBCs into the hearts of nude rats after the induction of an acute MI as indicated in Fig. 3A. At first, we cocultured CBMNCs with BATDCs for 3 days, purified $HLA^{+}CD45^{-}CD34^{-}$ cells (e-CBCs) by FACS and injected the cells into the hearts of experimental MI nude rats at each of five sites at the border of the infarcted tissue. As a control, infarcted hearts were injected with either equal volumes and numbers of CBMNCs that were not exposed to BATDCs (non-e-CBCs), or $CD34^{+}38^{-}$HSCs directly sorted from freshly isolated CBMNCs. Upon injection of e-CBCs, donor-derived human HLA- and SA-positive cells were detected abundantly in the infarct border zone (Fig. 3B, a, b, and c; 23.4 ± 3.1% of total cardiomyocytes in one field), but the contribution to CMs by the injection with non-e-CBCs and $CD34^{+}38^{-}$HSCs in the MI was 20- (Fig. 3B, d, e, and f; 1.1 ± 0.3%) and 15-fold (Fig. 3B, g, h, and i; 1.5 ± 0.3%), respectively, less than that of e-CBCs. e-CBC-derived SA-positive CMs also expressed connexin 43 (Fig. 3Bc), indicating that transplanted e-CBC-derived CMs formed gap junctions with host CMs. Moreover, the assessment of cardiac function by echocardiography revealed that the hearts injected with e-CBCs showed improved contractions of movement of the infarcted anterior walls and reduced left ventricular remodeling compared with the hearts injected with non-e-CBCs or $CD34^{+}38^{-}$HSCs (Fig. 4).

To clarify the origin of CMs in recipient tissue, donor-derived human chromosomes and host-derived rat chromosomes were simultaneously detected by using species-specific chromosome probes using fluorescent in situ hybridization (FISH) analysis. In this analysis, we used centromere probes, because 5 μm thick slices may not always include sex chromosomes in the nuclei as previously described [18]. Result showed that

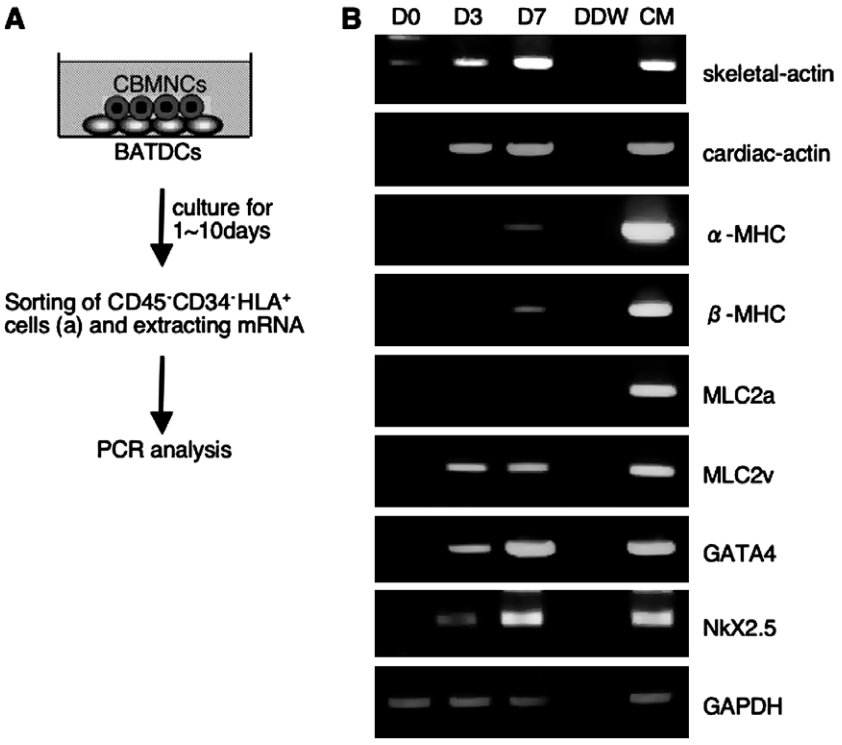

Fig. 2. Expression of CM-specific genes in e-CBCs. (A) Design of experiment for the isolation of human e-CBCs. CBMNCs were cocultured with BATDCs for 1 to 10 days, and then $CD45^{-}CD34^{-}$ human HLA^{+} cells were sorted and total RNA was extracted. (B) PCR analysis was performed with CM-specific primers in e-CBCs after coculturing with BATDCs for 0 day (D0; freshly isolated CBCs without exposure to BATDCs), 3 days (D3) and 7 days (D7). Distilled water (DDW) and CMs from embryos (CM) were used as a negative and a positive control, respectively. GAPDH was used as an internal control.

implanted e-CBCs of human origin transferred into female nude rats formed CMs that stained only with probes specific for human chromosomes (Fig. 3C, a and b). We also checked the serial confocal imaging to exclude the possibility that they arose from cell overlay as previously reported [18], but could not observe any superimposed cells (data not shown). This indicated that in vivo cardiac differentiation of e-CBCs was not induced by the fusion mechanism. In contrast, when $CD34^{+}CD38^{-}$HSCs were implanted into MI induced nude rats, human HLA-positive CMs were stained with both human and rat chromosome probes (data not shown). This indicated that generation of CM-derived HSCs was due to the fusion mechanism between donor-derived cells and host CMs as previously reported [18,19].

Discussion

So far, various kinds of sources for CMs, such as adult BM HSCs [19,20], MSCs [1–3], and ES cells [4,5] have been reported; however, there is some controversy regarding the efficiency of cardiomyoplasty. In terms of the myocardial regeneration therapy for human, human CB cells seem to be a safe and useful source compared to other sources, because these cells have already been utilized in CB transplantation for managing patients with blood disease. However, no clinical trials using CB cells to treat heart disease have been reported.

In this study, we raised two important points. The first is that e-CBCs of non-hematopoietic origin were more effectively differentiated into CMs compared with $CD34^{+}CD38^{-}$HSCs in vitro. Moreover, we showed that e-CBCs differentiation into CMs was not through the cell fusion mechanism. On the other hand, CMs derived from $CD34^{+}CD38^{-}$HSCs were generated through cell fusion with host CMs in vivo. Previously, it was reported that MSCs but not HSCs from BM could migrate into the heart and differentiate into CMs in mouse MI model [21]. This suggested that MSCs were the predominant source for myocardial regeneration. Our report is the first to show that non-hematopoietic cells can be used as CM source and how these compare with HSCs in human cord blood cells.

The second is the new strategy for myocardial regeneration using CB cells and BATDCs. Using the coculturing method described here, CB cells were effectively induced to differentiate into CMs in vitro. Moreover, e-CBCs were effectively differentiated into CMs in immunodeficient rat MI model and improved the cardiac function. Furthermore, we found that an adequate duration of coculturing of CB cells with BATDCs was critical for CM regeneration. In the present method, three days of coculturing was the most effective to produce CMs from e-CBCs and improve the CM function and this timing was consistent

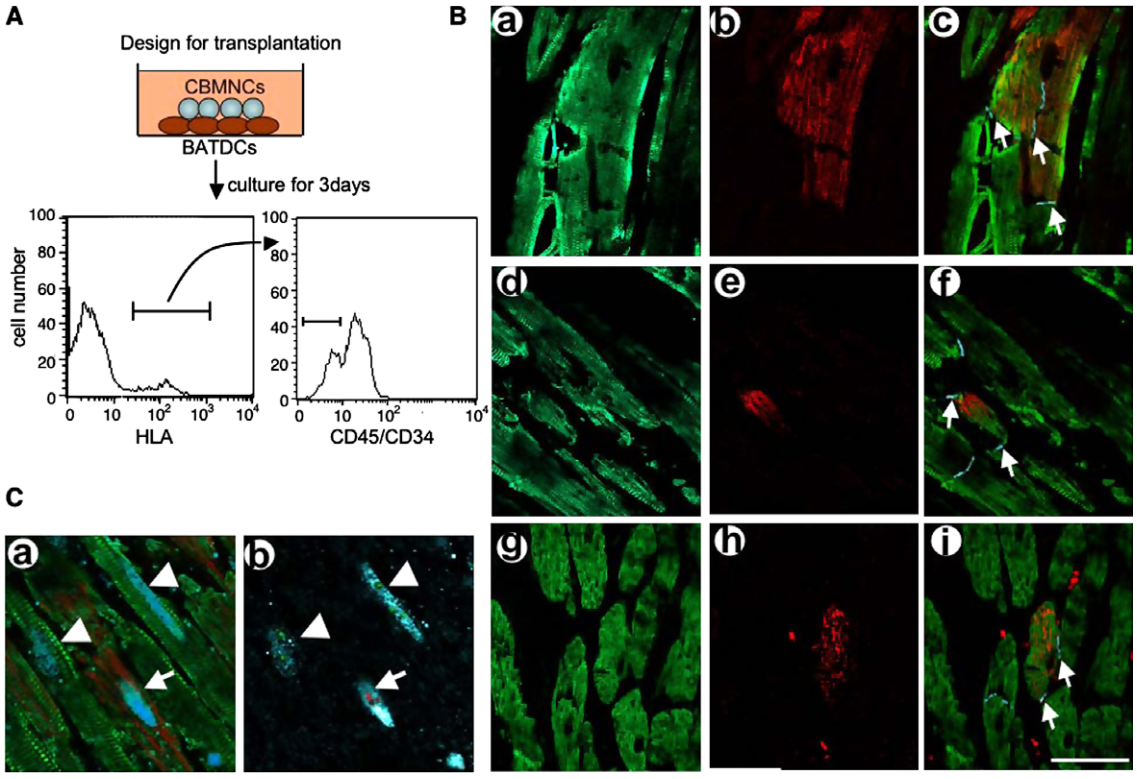

Fig. 3. CBMNCs cocultured with BATDCs contributed to cardiac regeneration. (A) Strategy for transplantation. After human CBMNCs were cocultured for 3 days with BATDCs from mice, human $HLA^{+}CD45^{-}CD34^{-}$ cells (e-CBCs) were sorted by FACS and injected into the border zone of ischemia induced nude rats. As a control, CBMNCs that were not cocltured (non-e-CBCs) and freshly isolated $CD34^{+}CD38^{-}$HSCs were used. (B) CM development from injected e-CBCs (a–c), non-e-CBCs (d–f), and $CD34^{+}CD38^{-}$HSCs in MI induced heart. (a, d, and g) Expression of SA (green). (b, e, and h) human HLA (red). (c, f, and i) are merged image of (a and b), (d and e), and (g and h), respectively, and stained with anti-connexin 43 antibody (blue). Arrows in (c, f, and i) indicate the regions in which human CB-derived connexin positive CMs make tight junction with resident host CMs. Scale bar in (i) indicates 20 μm. (C) (a) Expression of human HLA (red) and SA (green) in the site of implantation of e-CBCs in MI induced heart. Nuclei were counter stained with TOPRO3 (blue). (b) FISH staining in a serial section of (a). Green colors indicate rat chromosome, and red color indicates human chromosome. Nuclear staining was performed with TOPRO3 (blue). Cells expressing human chromosome (red) in the nuclei indicate that these cells were derived from e-CBCs and did not fuse with host CM expressing only rat chromosome (green). Arrowheads indicate nuclei from host CMs and arrow indicates human nuclei in e-CBCs (a, b). Scale bar indicates 5 μm.

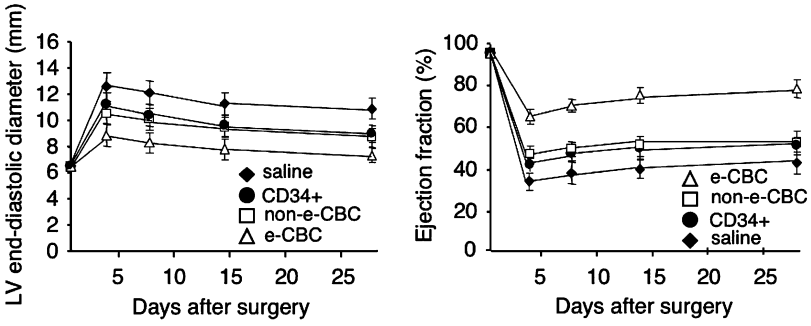

Fig. 4. e-CBCs transplantation improved cardiac function. LV diameter (LV end-diastolic diameter) and function (Ejection fraction) were assessed by echocardiography at 0, 3, 7, 14, and 28 days following myocardial infarction and injection of saline, e-CBCs, non-e-CBCs, and HSCs (CD34+). Note that in case of injection of e-CBCs, enlargement of LV diameter was reduced and LV function was significantly improved compared with injection of saline, non-e-CBCs, and HSCs. Each data point is the mean of five determinations; bars denote ±SD.

with the expression of Nkx2.5 and GATA-4 on CBMNCs (Fig. 2). In order to determine how e-CBCs differentiated into CMs, we tested a number of growth and survival factors and found that Akt activation seemed to play a role in the differentiation of CBMNCs into CMs (Supplemental data 1). Akt, a serine threonine kinase, transduces powerful survival signals in many systems [22,23]. Recently, it was reported that overexpression of Akt1 in MSCs increased the post-transplantation viability of these cells and enhanced their therapeutic efficiency [24]. In fact, intramyocardial injection of MSCs that had been transfected with a retroviral vector containing the Akt gene resulted in the

differentiation of MSCs into CMs and led to the prevention of ventricular remodeling and to the restoration of cardiac function after MI. In order to examine the survival signals in e-CBCs in each culture day as indicated, we checked the phosphorylation level of Akt (p-Akt) in e-CBCs on days 0, 3, and 7. Before extraction of cell lysate, e-CBCs were exposed to hypoxic condition for 24 h. The p-Akt level of e-CBCs on day 3 was 10-fold higher than that on day 7 and it was higher (1.7×) than that on day 0 before coculturing with BATDCs (Supplement data 1). The resistance of cell to apoptosis induced by hypoxic stimuli was proportional to the level of p-Akt in e-CBCs, i.e., it was higher on day 3 compared to day 0 and day 7. This anti-apoptotic effect might contribute to the high incidence of CMs derived from e-CBCs and to prevention of cardiac remodeling caused by conditions such as hypoxia, inflammation, and mechanical stress, and many endogenous factors such as angiotensin II, endothelin-1, and norepinephrine [25] in MI model.

We have as yet not clarified the precise mechanism whereby CB cells expressed high levels of p-Akt in hypoxic conditions after short-term coculturing with BATDCs. BATDCs were derived from adipose tissue, which possessed many beneficial factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), angiopoietin-1, and so on [16]. Therefore, these factors might support the high level of p-Akt in e-CBCs. Identification of such factors may enable effective myocardial regeneration. Use of CBCs together with such factors for protection against cell apoptosis may enable the application of the strategy described here to the clinic for managing patients of ischemic disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.017.

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