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Original Article

Cardiosphere-derived cell sheet primed with hypoxia improves left ventricular function of chronically infarcted heart

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Abstract: Cardiosphere-derived cells (CDCs) isolated from postnatal heart tissue are a convenient and efficientresource for the treatment of myocardial infarction. However, poor retention of CDCs in infarcted hearts often causes less than ideal therapeutic outcomes. Cell sheet technology has been developed as a means of permitting longer retention of graft cells, and this therapeutic strategy has opened new avenues of cell-based therapy for severe ischemic diseases. However, there is still scope for improvement before this treatment can be routinely applied in clinical settings. In this study, we investigated whether hypoxic preconditioning enhances the therapeutic efficacy of CDC monolayer sheets. To induce hypoxia priming, CDC monolayer sheets were placed in an incubator adjusted to 2% oxygen for 24 hours, and then preconditioned mouse CDC sheets were implanted into the infarcted heart of old myocardial infarction mouse models. Hypoxic preconditioning of CDC sheets remarkably increased the expression of vascular endothelial growth factor through the PI3-kinase/Akt signaling pathway. Implantation of preconditioned CDC sheets improved left ventricular function inchronically infarcted hearts and reduced fibrosis. The therapeutic efficacy of preconditioned CDC sheets was higher than the CDC sheets that were cultured under normaxia condition. These results suggest that hypoxic preconditioning augments the therapeutic angiogenic and anti-fibrotic activity of CDC sheets. A combination of cell sheets and hypoxic preconditioning offers an attractive therapeutic protocol for CDC transplantation into chronically infarcted hearts.

Keywords: Old myocardial infarction, cell-based therapy, cardiosphere-derived cells, cell sheet, hypoxic preconditioning

Introduction

Myocardial infarction (MI) is one of the leading causes of death in the western world. MI leads to the loss of functional cardiomyocytes followed by scarring [1]. Heart transplantation is the gold-standard therapeutic approach for end-stage heart failure, including MI; however, there is a critical shortage of donor hearts worldwide. Hence, an alternative to heart transplantation is needed for the treatment of end-stage heart failure, including MI. Cell-based therapy is thought to be a good alternative to organ transplantation. Among a wide variety of graft cell types, cardiosphere-derived cells (CDCs) from postnatal heart tissue are one of the most potentially useful for cell-based MI therapy due to their considerable regenerative potential in infarcted hearts [2]. CDCs are composed of a heterogeneous cell population, including cardiac progenitor cells (CPCs), and can give rise to cardiomyocytes, smooth muscle cells, and endothelial cells. Direct injection of CDCs into an infarcted heart has been shown to result in improvements in heart function [3, 4]. Following initial, successful animal studies, a Phase I clinical trial (CADUCEUS) conducted from 2009 to 2010 established the safety of

CDCs for therapeutic use in human subjects [5]. A common issue with these cells, however, is that the retention of grafted cells in the infarcted heart is extremely poor following implantation. More than 90% of grafted cells are lost due to washing out from injected area and cell death from ischemia, anoikis, and inflammation [3, 6-9]. Conversely, it is almost-certain that longer retention of grafted cells in infarcted hearts would result in an improvement of cardiac function in the clinical setting.

Biological scaffolds are potentially powerful delivery methods, allowing grafted cells to be retained in the infarcted heart for longer. Indeed, the use of a hyaluronan-gelatin hydrogel has been shown to enable longer retention of grafted CDCs in infarcted hearts [3]. However, the risk of bacterial contamination during the manufacturing process of biological scaffolds cannot be prevented fully. Recently, scaffold-free cell sheets, which maintain cell-cell contact and matrix components in the desired region, were developed froma wide variety of cells such as mesenchymal stem cells, skeletal myoblasts, and cardiomyocytes [10-12]. Implantation of cell sheets into infarcted hearts has been shown to induce neovascularization and angiogenesis through increased secretion of angiogenic factors from the sheet, resulting in improved cardiac function, observed as changes in the left ventricular ejection fraction (LVEF). In addition, CPC sheets have been shown to regenerate cardiac muscle in infarcted hearts through cardiomyogenesis of sheet-derived progenitors, there by resulting in the replacement of damaged tissues with healthy heart muscle and the recovery of cardiac function [13]. In addition, a recent study demonstrated that overexpression of vascular endothelial growth factor (VEGF) in adipose-derived stem cell sheets, induced by viral vector infection, enhanced therapeutic efficacy in rabbit infarcted hearts [14], suggesting that modification of the cell sheet is not just necessary to enable longer retention of grafted cells, but also to enhance therapeutic efficacy in infarcted hearts. However, genetic modification of graft cells should be avoided as much as possible in the clinical setting, and a safer protocol-should be developed to augment the therapeutic features of cell sheets.

Hypoxic preconditioning, which involved a brief incubation in hypoxic conditions before cell transplantation, has been investigated as a means of reinforcing the multiple cellular functions of graft cells, such as angiogenic activity and oxidative stress resistance, resulting in therapeutic angiogenesis in ischemic tissue after transplantation [15-18]. The simplicity and safety of this protocol led us to consider the possibility of hypoxic preconditioning of CDC monolayer sheets as a realistic "booster shot" in a clinical setting. Therefore, in the present study we examined whether hypoxic preconditioning augments the therapeutic features of CDC sheets, and whether preconditioned CDC sheets could become effective therapeutic tools in cases of chronic MI.

Materials and methods

Ethical approval

Animal experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University Graduate School of Medicine (no. 31-089). The protocol for isolating human cells was approved by the Ethics Review Board for Clinical Research at Yamaguchi University (no. 2010025), and the study was conducted in accordance with the Declaration of Helsinki. Informed written consent for participation in the study was obtained from all patients.

Preparation of cardiosphere-derived cells

Briefly, right atrial biopsy samples (~100 mg) were collected from patients scheduled for open-heart surgery and digested with 0.5% trypsin for 5 min. The biopsy samples, termed "explants", were then placed onto fibronectin-coated cell culture dishes and incubated in Iscove's Modified Dulbecco's medium (IMDM; Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS; Life Technologies), 2 mM L-glutamine (Life Technologies), 2 nM β-mercaptoethanol, and 1% penicillin/streptomycin (Life Technologies) at 37°C [19, 20]. Cells that migrated from the explants were collected and placed in cardiosphere medium (Supplementary Table 1) [20]. The culture flask was pre-coated with poly-2-hydroxymethyl methacryate (Sigma-Aldrich, St. Louis, MO) to prevent the attachment of cells to the surface [21]. After 4 days, cardiospheres were collected and plated onto fibronectin-coated cell culture dishes to isolate CDCs propagated from spheres.

Mouse CDCs were isolated from male C57BL/6 mice (8-10 weeks old) using the same protocol. In all experiments, CDCs obtained after the second passage were used.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (Wako, Osaka, Japan) or ice-cold methanol (Wako). After rinsing with Phosphate-buffered Saline (PBS), cells were permeabilized with 0.1% Triton-X/Protein blocking solution (Dako-Japan, Tokyo, Japan), and then incubated with primary antibodies (Supplementary Table 2). After incubation with primary antibodies, cells were incubated with secondary antibodies conjugated with Alexa Fluor 594 or 488 (Life Technologies). Cell nuclei were visualized with DAPI after incubation with secondary antibodies. Immunofluorescence images were acquired using a BIOREVO microscopy system (BZ-9000 Generation II system; Keyence, Osaka, Japan).

Fluorescence-activated cell sorting

Cardiospheres were gently dissociated with TrypLE® (Life Technologies) and then incubated with antibodies against CD90, CD105, and c-Kit conjugated with PE or FITC. After incubation with antibodies, cells were applied to a flow cytometer (FACSCalibur, BD Biosciences, SanJose, CA) to analyze cell surface proteins.

Preparation and hypoxic preconditioning of cardiosphere-derived cell sheets

Temperature-responsive cell culture dishes (UpCell®; CellSeed, Tokyo, Japan) were used to prepare both human and mouse CDC sheets [22-24]. CDCs from one well of 6-well culture dishes were divided between three wells of 24-well UpCell® dishes and cultured in 10% FBS/IMDM until fully confluent. To prepare fluorescently labeled CDC sheets, PKH26 (Sigma-Aldrich) was added to the cell suspension before re-plating onto the temperature-responsive dish. To prepare hypoxically preconditioned cell sheets, CDCs on the UpCell dishes were placed in an incubator adjusted to a 2% oxygen level and 33°C and cultivated for 24 hours.

Enzyme-linked immunosorbent assay

To assess the production of growth factors and matrix metalloproteinases (MMPs), conditioned medium was collected from the human CDC sheet cultures, and enzyme-linked immunosorbent assay (ELISA) was performed, targeted at hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), VEGF, insulin-like growth factor-I (IGF-I), MMP2, MMP3, and endoglin/CD105 (Quantikine ELISA kit; R&D systems, Minneapolis, MN) according to the manufacturer's protocol. Endogenous MMP2 activity in the conditioned medium was also analyzed using an ELISA kit (MMP2 Activity Assay System; GE Health care, Buckinghamshire, UK).

Western blotting

Human CDC sheets were dissolved in RIPA buffer containing protease/phosphatase inhibitor cocktails, and 30-μg of proteins was applied into polyacrylamide gels. Information concerning primary antibodies used is displayed in Supplementary Table 2. Proteins were visualized with HRP substrate (ECL Prime Western Blotting Detection System; GE Healthcare). Band intensities were quantified using the Image J software package.

Cell proliferation assay

Mouse intestinal myofibroblasts (SmcMF) [25] were seeded onto 24-well plates (3.5 x 10^4 cells/well) and cultured in 10% FBS/IMDM. Twenty-four hours later, culture medium was replaced with the conditioned medium from human CDC sheets (Hypo or Normo), and cells were cultured for a further 24 hours. To identify proliferating cells, immunocytochemistry for phosphorylated histone H3 (pHH3) was performed, and the ratio of $pHH3^+$ cells to total counted cells were compared between Normo and Hypo-derived conditioned medium.

Tube formation assay

Human umbilical endothelial cells (HUVECs; 3 x 10^5 cells) maintained in EGM2 medium (Lonza, Bazel, Switzerland) were seeded onto Matrigel-coated 96-well plates and further cultured in 10% FBS/DMEM for 24 hours. Then, culture medium was replaced to the conditioned medium, which was prepared from normoxically- or hypoxically-cultured human CDC sheet, and cultivated for 12 hours. To verify contribution of VEGF for tube formation, neutralizing antibody (R&D systems) was added into the conditioned medium from preconditioned CDC sheet (10

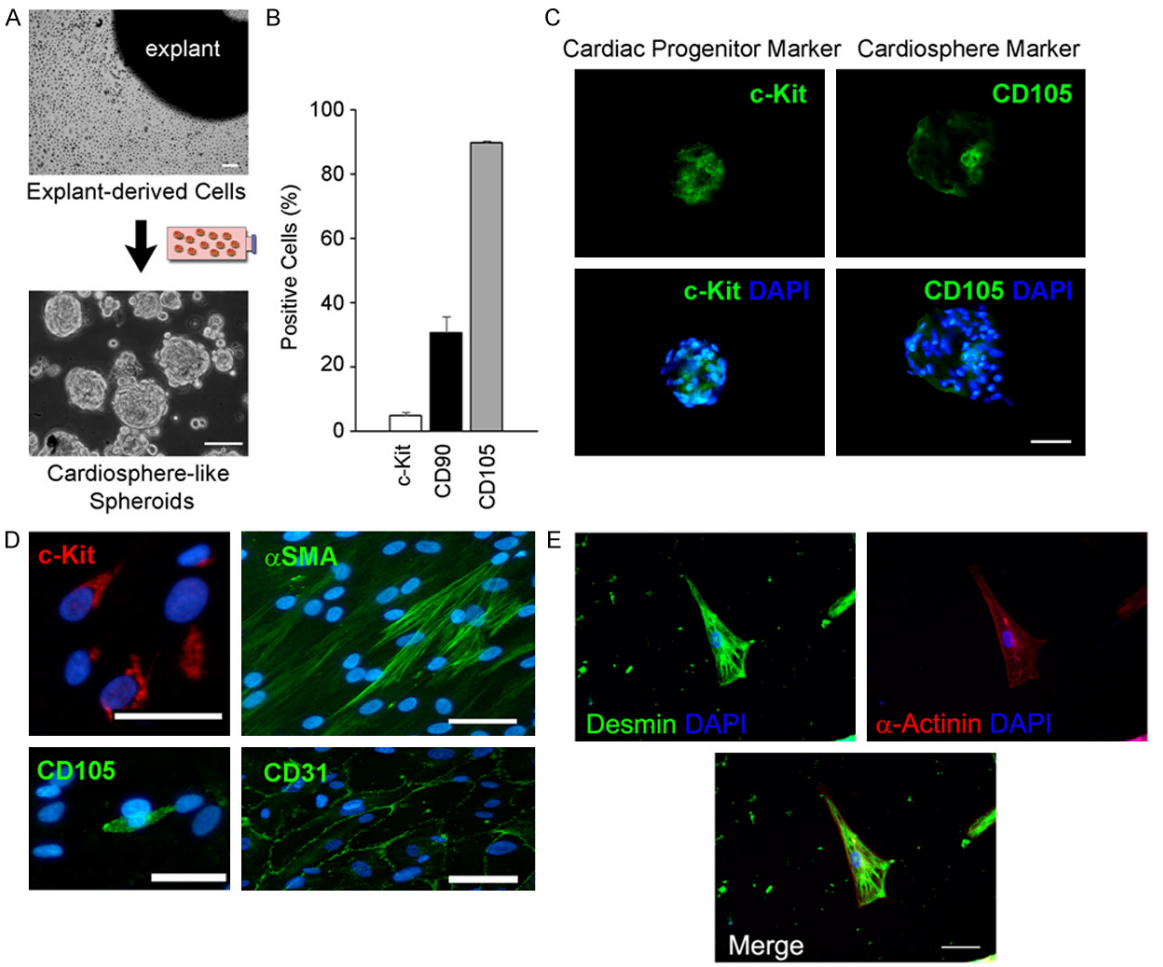

Figure 1. Isolation of cardiospheres and cardiosphere-derived cells (CDCs) from human hearts. A. Cardiosphere-like spheroids were developed by direct free-floating culture of explant-derived cells. B. Fluorescence-activated cell sorting of cardiosphere-like spheroids revealed expression of the cardiosphere markers CD90, CD105, and the cardiac progenitor marker c-Kit (n = 3). C. Immunocytochemistry for c-Kit and CD105 in cardiosphere-like the spheroids. D. CDCs spontaneously gave rise to vascular endothelial cells (CD31) and smooth muscle cells (a-smooth muscle actin), whereas cardiac progenitor (c-Kit) and mesenchymal (CD105) cells were maintained. E. CDCs differentiated into cardiomyocytes (Desmin$^+$/α-Actinin$^+$) under cardiomyogenic differentiation conditions. Scale bar = 50 μm.

μg/ml) and then HUVECs were cultured for 12 hours. Mouse IgG_2 was added into the medium from the Normo and Hypo groups as an isotype control. Total tube numbers in 4 different wells for each groups were counted (4 x microscopic field).

Old MI mouse model and sheet implantation

Briefly, mice were placed under general anesthesia with isoflurane, and tracheal intubation was performed with a 20-gauge intravenous catheter. Mice were artificially ventilated with room air at 80 breaths per minutes. A left thoracotomy was performed through the fourth intercostal space, and the left anterior descending artery (LAD) was completely ligated with an 8-0 polypropylene suture under microscopic guidance [4]. The ligated mice were caged for 1 month to establish the old myocardial infarction (o-MI) condition. At 1 month after surgery, mice were subjected to echocardiography to choose the most appropriate o-MI mice for the implantation experiments.

Mouse CDC sheets were placed on Seprafilm® (Sanofi US, Bridgewater, NJ), which is used to prevent adhesion after surgery, and then carefully implanted into the infarcted hearts of o-MI mice. Seprafilm® without the CDC sheet was

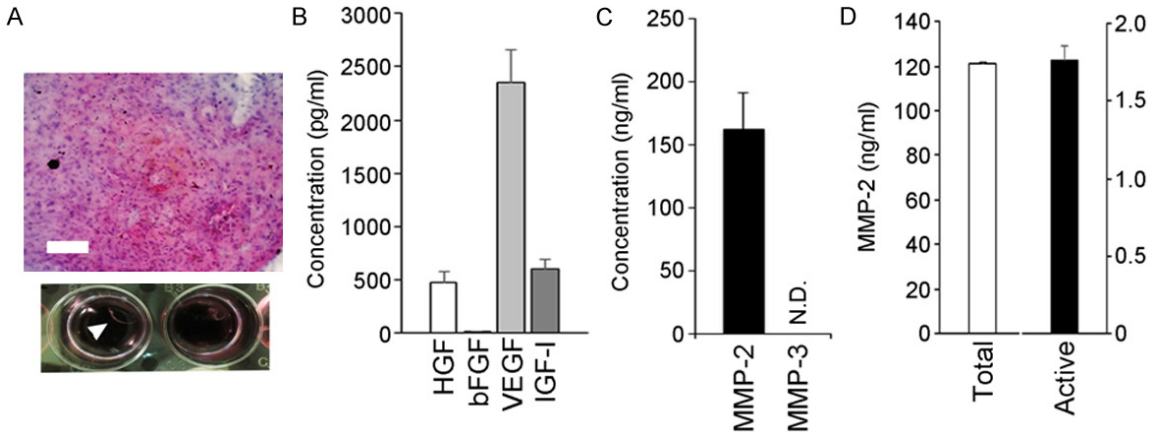

Figure 2. CDC sheets secrete angiogenic factors and MMPs. A. Representative images of a human CDC sheet (upper: hematoxylin and eosin staining). CDC sheets were detached from the temperature-responsive culture dish (lower: arrowhead). Scale bar = 100 μm. B. Human CDC sheets secrete angiogenic growth factors (n = 3). C. Human CDC sheets secrete MMP2 but not MMP3. The concentration of MMP2 and MMP3 in conditioned medium from human CDC sheet cultures was measured by ELISA (n = 3). N.D.: Not detected. D. Human CDC sheets secrete biologically active MMP2 (n = 3).

implanted into the MI heart as the o-MI control group. Mice were euthanized 4 weeks after the implantation of CDC sheets.

Echocardiography

Echocardiograms were performed before implantation, at 2 weeks, and at 4 weeks following implantation of the mouse CDC sheets using the VeVo770 high-resolution imaging system (Visual Sonics, Toronto, Canada). LVEF, left ventricular fractional shortening (LVFS), systolic-inter ventricular septum, and left ventricular internal dimension were measured from the echocardiograms.

Histology

After echocardiogram analysis, the mice were euthanized and the hearts were collected. Hearts were immersed in 10% formalin overnight at 4°C, and 5-μm thick tissue sections were prepared for histological analysis. Infarcted heart sections were stained with Masson's trichrome stain to visualize the fibrotic area, and images were taken using a BIOREVO microscopy system. The degenerated area was calculated using a BZ-II analyzer (Keyence). The fluorescent signal for PKH26 in the infarcted hearts was directly detected using the same microscope.

To analyze new vessel formation, infarcted heart sections were stained with DyeLight 488-conjugated Tomato-Lectin and cardiac Troponin T (cTnT) to visualize capillaries and cardiomyocytes. Judging from immunofluorescent images, we decided that cTnT-negative area in the heart is as an infarcted area. Percentage of capillary area in the border zone, which was chosen 200 x 200 μm area partly including infarcted area, was calculated and compared between the control (no-sheet delivery), Normo-sheet, and Hypo-sheet groups.

Statistical analysis

All values are expressed as mean ± SD. A two-tailed Student *t*-test was used to compare left ventricular functions (LVEF and LVFS) of the CDC sheet-implanted group with those of the control group. One-way analysis of variance with a post-hoc Tukey's test was used to analyze the differences between multiple groups. Statistical analysis was performed using IBM SPSS Statistics 20 (IBM-Japan, Tokyo, Japan). *P*-values less than 0.05 or 0.01 were considered statistically significant.

Results

Cardiosphere and cardiosphere-derived cell isolation from mouse and human hearts

To isolate CDCs, we used a slightly modified protocol implementing a combination of tissue explant and free-floating techniques, instead of the colony formation procedure with poly-D-

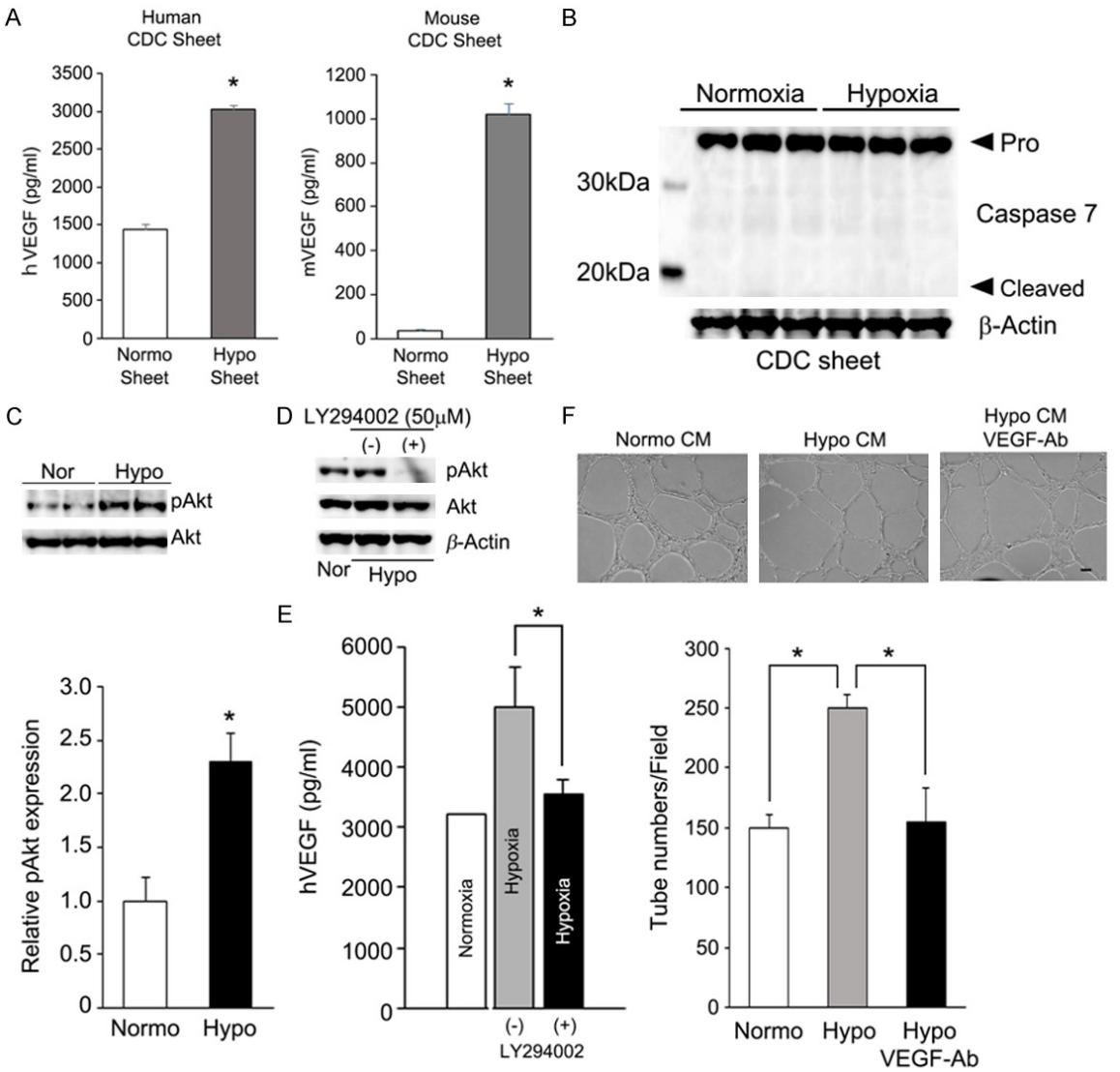

Figure 3. Hypoxic pretreatment accelerates VEGF expression in CDC sheet through the PI3-kinase/Akt signaling pathway. A. Hypoxic pretreatment up-regulated VEGF expression in both human and mouse CDC sheets. Both human and mouse CDC sheets were exposed to hypoxic culture conditions (2% oxygen) for 24 hours. B. Hypoxic pretreatment does not induce apoptotic response of CDC sheet. Cleaved Caspase 7 was not detected in both normoxically and hypoxically cultured mouse CDC sheet. C. Hypoxic pretreatment accelerates Akt-associated signaling pathway in CDC sheet. D, E. The PI3-kinase inhibitor LY294002 blocked phosphorylation of Akt and further acceleration of VEGF production in CDC sheets. Human CDC sheets were incubated in hypoxic conditions with or without LY294002 (50 μM) for 24 hours, then the VEGF level in the medium was calculated by ELISA. F. Hypoxic preconditioning enhanced angiogenic activity of CDC sheet. Human umbilical vascular endothelial cells (HUVECs) were cultured in the conditioned medium form CDC sheet with/without Hypoxia for 12 hours. To verify contribution of VEGF, neutralizing antibody was added into the conditioned medium from Hypoxia. Total tube numbers were counted in the field of 4 x microscopic magnification (n = 3). *p<0.05. Normo or Nor; Normoxia. Hypo; Hypoxia. Normo CM; Conditioned medium from Normo Sheet. Hypo CM; Conditioned medium from Hypo Sheet. VEGF-Ab; neutralizing antibody for human VEGF. Scale bar = 50 μm.

lysineas described in the original protocol [20]. Sphere-forming cells in EDCs rapidly aggregated, expanded, and developed cardiosphere-like cells in the free-floating culture system (**Figure 1A**).

Fluorescence-activated cell sorting was performed following our protocol to characterize the cardiosphere-like cells using general cardiosphere markers (CD90, CD105, and c-Kit) [20, 26]. The majority of human cardiosphere-

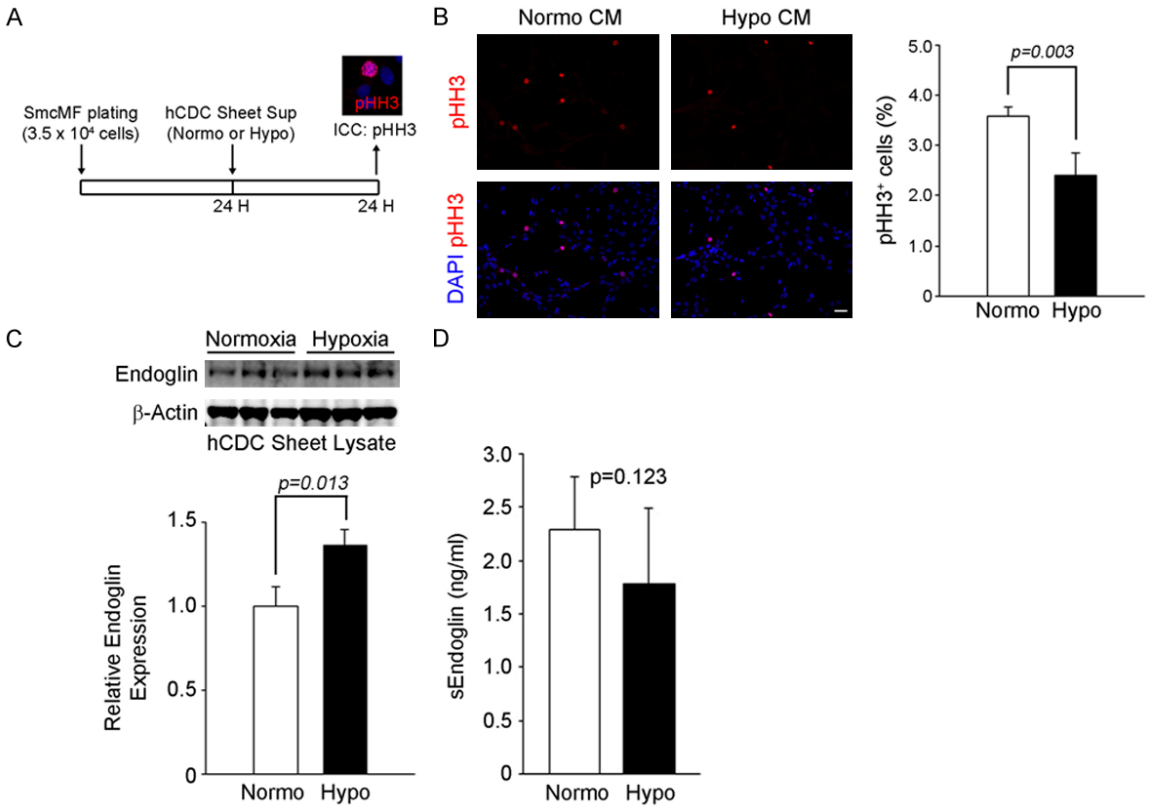

Figure 4. Hypoxically preconditioned CDC sheets inhibit myofibroblast proliferation. A. Experimental design of the proliferation assay. Conditioned medium from human CDC sheets was added to the culture of mouse intestinal myofibroblast cell line (SmcMF). B. Proliferation of intestinal myofibroblasts (phosphorylated-Histone $H3^{+}$) was inhibited by the conditioned medium from hypoxically pretreated CDC sheets. Counted total cell numbers were 2,667 (Normoxia) and 2,721 (Hypoxia), respectively. C. Hypoxic pretreatment up-regulated endoglin, a TGF-β receptor antagonist, in CDC sheets. Human CDC sheets were incubated in 2% (Hypoxia) or 20% (Normoxia) oxygen for 24 hours, and then CDC sheet lysates were applied to Western blot analysis targeted at endoglin. n = 3. D. Soluble endoglin level was not increased by hypoxic treatment. The conditioned medium were collected from CDC sheets cultured in normoxic or hypoxic condition. Endoglin levels in the conditioned medium was analyzed by ELISA (n = 8).

like cells expressed CD105 (89.7% ± 0.5%), and one-third of cells were CD90-positive (30.8% ± 4.6%). Only a small number of cells expressed c-Kit (4.6% ± 1.0%) (**Figure 1B**). Both CD105-and c-Kit-positive cells were also confirmed by immunostaining (**Figure 1C**). Taken together with the expression profiles for cardiosphere markers, we determined that our protocol generated cardiospheres from heart tissues to a level consistent with the original protocols. However, the number of c-Kit-positive cells (~5%) in our protocol was lower than that observed in previous studies (17%~30%) [13, 26], indicating that our cardiospheres included a lower number of CPCs.

Human cardiospheres were plated onto fibronectin-coated cell culture dishes to isolate CDCs. Consistent with the results of previous investigation [20], CDCs that migrated from the cardiospheres expressed c-Kit, α-smooth muscle actin, CD105, and CD31 (**Figure 1D**). In addition, human CDCs can give rise to cardiomyocytes under specific culture conditions [27] (**Figure 1E**). Similar protein expression was confirmed in mouse CDCs isolated from adult heart (Supplementary Figure 1).

Cardiosphere-derived cell sheets secrete multiple growth factors and matrix metalloproteinase-2

Human CDCs were plated onto temperature-responsive cell culture dishes to form CDC sheets (**Figure 2A**). It is known that CDC-derived growth factors are potential repair factors for MI after cell transplantation [28, 29], so we assessed whether the CDC sheets secrete-

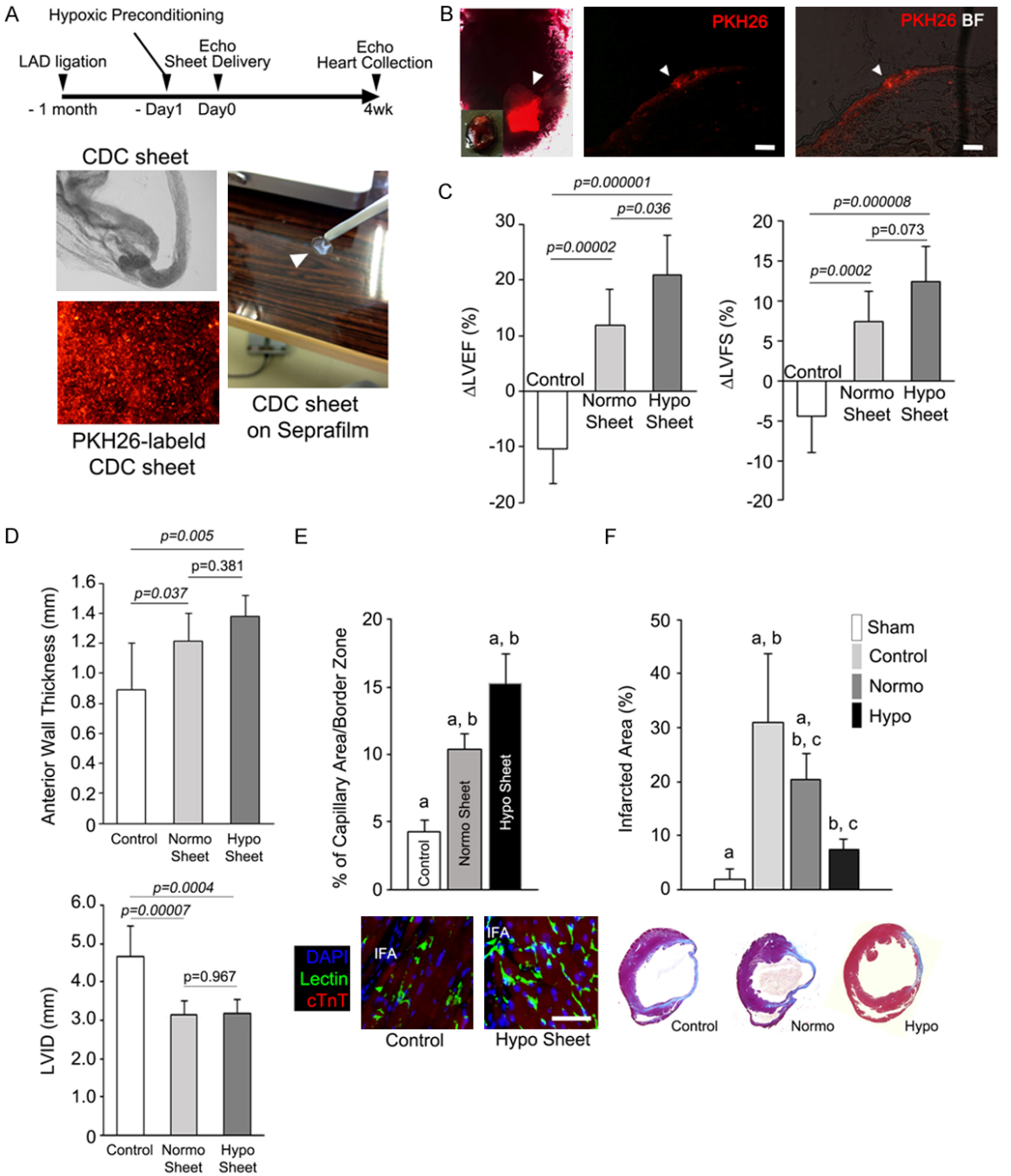

Figure 5. A combination of hypoxic preconditioning and CDC sheets improves left ventricular function and reduces fibrosis. A. Schematic depiction of hypoxic preconditioning and cell sheet delivery. Mouse CDC sheets were transiently labeled with PKH26 fluorescent dye and placed onto Seprafilm® for delivery into the hearts of o-MI mice (arrowhead). B. CDC sheets were maintained in the infarcted heart for at least 4 weeks following implantation. Macroscopic (x 4 magnification) and microscopic images of a PKH26-labeled CDC sheet (arrowhead) in an o-MI heart. Insert in upper left indicates an image of the whole heart. BF: Bright field. C. Changes of LVEF and LVFS at 4 weeks after cell sheet implantation. Preconditioned CDC sheets significantly recovered left ventricular function (n = 5, Control; and n = 10, Normo sheet; n = 6, Hypo sheet). D. Anterior wall thickness was significantly increased by CDC sheet implantation. In addition, LVID was significantly increased in sheet-delivered infarcted hearts. The IVS-s at 4 weeks after sheet implantation was measured from echocardiograms (n = 6, Control; and n = 10, Normo sheet; n = 6, Hypo sheet). E. Preconditioned CDC sheet accelerated angiogenesis in infarcted heart. CDC sheets were delivered onto infarcted heart, and then Lectin$^+$ capillaries in the border zone were counted at 4 weeks after sheet delivery (n = 4). Scale bar = 50 μm. cTnT: Cardiac Troponin T. IFA: Infarcted area. F. Fibrotic area in the infarcted

heart was significantly reduced by preconditioned CDC sheet implantation (n = 5, Sham; n = 4, Control; and n = 10, Normo sheet; n = 7, Hypo sheet). Matching letters indicate a statistically significant difference ($p<0.05$). Images represent Masson's trichrome-stained heart tissue.

growth factors into the culture medium. Consistent with individual CDCs, the human CDC sheets were shown to secrete growth factors including HGF, IGF-I, VEGF, and bFGF (**Figure 2B**). Importantly, the increased secretion of VEGF indicates the potential angiogenic activity of the CDC sheets after delivery to an infarcted heart.

It has been reported that CDCs express MMPs, indicating the possibility that the grafted CDCs themselves may provide a suitable environment for survival in an ischemic area after implantation [26]. In fact, human CDC sheets secreted MMP2 but not MMP3. The sheet-derived MMP2 was also shown to have enzymatic activity (**Figure 2C**, **2D**), indicating that the CDC sheet produces not only pro-MMP2 but also active-MMP2.

Hypoxic pretreatment accelerates vascular endothelial growth factor expression in cardiosphere-derived cell sheets through the PI3-kinase/Akt signaling pathway

In our previous studies, hypoxic stimulation was shown to accelerate the expression of VEGF in blood-derived cells resulting in neovascularization in ischemic tissue after cell transplantation [17, 18]. We therefore tested whether hypoxic pretreatment of CDC sheets up-regulates angiogenesis-associated factors in a similar way to individual cells. As expected, hypoxic preconditioning accelerated VEGF production in both human and mouse CDC sheets (**Figure 3A**). In addition, we also found in the analysis of Caspase cleavage that this hypoxia condition does not induce apoptosis of CDC sheet (**Figure 3B**). These results suggest that hypoxic pretreatment augments the angiogenic activity of CDC sheets without apoptotic response.

Next, we attempted to clarify the regulatory mechanism responsible for accelerating VEGF expression under hypoxic conditions. Cell lysates from normoxically- and hypoxically-cultured human CDC sheets (Normo and Hypo, respectively) were subjected to western blot analysis for pAkt which is one of major signaling transducers responding to hypoxia [30]. As a result, hypoxic preconditioning induced phosphorylation of Akt in CDC sheet (**Figure 3C**). Moreover, administration of LY294002, which is a PI3-kinase (PI3K) inhibitor, blocked both the up-regulation of VEGF in the Hypo sheet culture and the dephosphorylation of Akt (**Figure 3D**). Because VEGF gene expression under hypoxic conditions is regulated by the PI3K/Akt signaling pathway [31], these results suggest that hypoxic preconditioning augments the angiogenic activity of CDC sheets through this pathway. To examine whether hypoxic preconditioning enhanced angiogenic activity of CDC sheet, we performed *in vitro* angiogenesis assay using HUVECs. As a result, tube formation of HUVECs was significantly increased in the culture with the conditioned media from hypoxically-preconditioned CDC sheet (**Figure 3E**). In addition, neutralizing antibody for human VEGF inhibited an increased tube formation of HUVECs in the culture with the media from preconditioned CDC sheet, indicating that increased VEGF in the Hypo sheet is one of possible factors to enhance angiogenesis (**Figure 3F**).

Preconditioned cardiosphere-derived cell sheet inhibits myofibroblast proliferation

Myofibroblasts were cultured in the conditioned medium from Normo sheets or Hypo sheets (**Figure 4A**). Proliferating myofibroblasts were decreased when cells were cultured in the conditioned medium from Hypo sheets (**Figure 4B**). A recent study demonstrated that cardiospheres reverse fibrosis in infarcted hearts through the production of endoglin, which inhibits TGF-β signaling [32]. Therefore, we speculated whether hypoxic preconditioning could augment the anti-fibrotic action of CDC sheets in addition to accelerating angiogenic activity. The significant increase of endoglin in the Hypo sheet suggested that hypoxic preconditioning enhanced the anti-fibrotic action of the CDC sheets (**Figure 4C**). However, soluble endoglin level was not increased in the conditioned medium from Hypo sheets (**Figure 4D**).

Hypoxically preconditioned cardiosphere-derived cell sheet improves vascularization and fibrosis in a chronically infarcted heart

Finally, we investigated whether hypoxically preconditioned CDC sheets could improve LV

function in chronically infarcted hearts after implantation. One day before sheet implantation, mouse CDC sheets were reinforced by hypoxic preconditioning. And then, preconditioned CDC sheet was delivered onto infarcted heart of o-MI mouse model (**Figure 5A**). In this mouse model, cardiac function was deteriorated as a result of the LAD ligation for 4 weeks (Supplementary Figure 2). After implantation of the mouse CDC sheets, both LVEF and LVFS were measured by echocardiography to check for functional improvement in the infarcted heart. Mouse CDC sheets labeled with PKH26 fluorescence dye were mounted on Seprafilm®, and then delivered to the infarcted heart (**Figure 5A**). Macroscopic analysis indicated that the CDC sheet remained on the infarcted heart for 4 weeks post-implantation (**Figure 5B**). The rate of change of LVEF and LVFS were calculated at 4 weeks after implantation, and compared between the Normo, Hypo, and control groups. Hypo sheets improved LV function with greater therapeutic recovery in LVEF than Normo sheets (**Figure 5C**). In addition, the anterior wall in both treatment groups was thicker than that in the control group and left ventricular internal dimension (LVID) was significantly decreased in treatment groups, although there were no significant differences between the two treatment groups (**Figure 5D**).

Histological changes in the infarcted hearts induced by CDC sheets were evaluated after implantation. At 4 weeks after sheet implantation, we assessed the effects of the CDC sheets on new vessel formation and heart fibrosis. In the border zone, capillary numbers were significantly increased in sheet-delivered groups. In particularly, the preconditioned sheet showed higher angiogenic activity in infarcted heart in consistent with *in vitro* study (**Figure 5E**). In addition, the extent of fibrosis was assessed using Masson's trichrome staining, and the percentage of overall fibrotic area in the infarcted heart was calculated from the tissue-cross section. At the time of euthanasia, the non-treated infarcted hearts presented heavier fibrosis than that of the normal heart group (1.8 ± 1.9%). In contrast, extensive fibrosis in the infarcted heart was reduced in the CDC sheet-implanted groups. Importantly, hypoxically preconditioned CDC sheets significantly reduced fibrotic area in infarcted hearts more than the Normo sheet group (Normo: 21% ± 5%; Hypo: 7.29 ± 2%; $p<0.05$, **Figure 5F**). These results suggest that delivery of preconditioned CDC sheets effectively reverses cardiac remodeling in infarcted hearts.

Discussion

CDCs are one of the most promising cell types for clinical applications, and autologous CDCs have already been evaluated in a clinical trial for patients with heart failure (CADUCEUS, NCT00893360 at clinicaltrials.gov). In this previous trial, however, the impact of CDC delivery on LV function was insignificant in spite of a marked reduction in scarring [5]. Poor retention of CDCs in the recipient heart is one of the primary reasons why the effects of CDCs on the functional recovery of the LV were unsatisfactory in humans [3]. Based on this, we propose that a combinational strategy using cell sheet technology would enable transplanted CDCs to recover LV function in human patients as well as in animal models. Cell sheets allow implanted cells to be retained longer in the infarcted heart and also induce cardiac regeneration, as previously shown in experimental animal studies [33-35]. In addition, the availability of cell sheets for clinical use has been demonstrated in patients with dilated cardiomyopathy that was treated with implanted autologous myoblast sheets [36]. Thus, these studies demonstrating the positive correlation between longer cell retention and efficacy further support our motivation to use this technology for CDC transplantation. In our study, CDC sheets remained intact for at least 4 weeks within the infarcted mouse heart, as shown under macroscopic analysis, indicating that cell sheet technology can retain CDCs in infarcted hearts for a longer period than direct CDC injection [3]. These findings suggest that cell sheet technology may enable human CDCs to survive longer in human infarcted hearts, resulting in functional improvement of the LV and morphological alterations. More number of detailed studies is needed in the future to demonstrate that longer retention of CDCs correlates to functional improvement, with direct comparisons between sheet-mediated and direct delivery of CDCs.

One potential therapeutic pathway by which implanted CDC sheets act is via a paracrine effect to accelerate angiogenesis in an infarcted heart, since CDC sheets secrete angiogenic

factors including VEGF, IGF-I, and HGF. These growth factors are released from directly injected CDCs, and are known to contribute to neovascularization *in vivo* [28]. Therefore, CDC sheets, which are retained longer within an infarcted heart, may synergistically affect therapeutic angiogenesis through the secretion of multiple growth factors after implantation, and accelerated angiogenesis stimulated by CDC sheets may induce vascularization and cardiac regeneration in the infarcted heart.

The migration of CPCs from CDC sheets presents another potential therapeutic pathway. Isolated CDCs have been shown to give rise to cardiomyocytes *in vitro,* and to contribute to cardiac regeneration *in vivo* when CDCs are directly engrafted into the myocardium of an infarcted heart [20, 37]. Furthermore, hypoxic preconditioning up-regulates CXCR4 expression of CDCs, resulting in efficient migration of CPCs into infarcted heart and cardiac regeneration after intravenous injection [38]. It is essential to determine whether CPCs in the CDC sheets can migrate to the myocardium through the epimyocardium layer, and to contribute to cardiac regeneration in a manner similar to that observed by direct cellular injection into the myocardium. In our study, however, $PKH26^+$ cardiomyocytes were not identified in sheet-implanted infarcted hearts (data not shown). This observation suggests that the therapeutic effects of CDC sheet implantation were restricted with respect to therapeutic angiogenesis, but not to cardiomyocyte replacement, a finding that is consistent with previous results for myoblast sheet implantation [34].

Hypoxic preconditioning is a simple but powerful protocol to enhance the therapeutic efficacy of cell transplantation, and this strategy acts upon multiple cellular aspects such as oxidative stress resistance and angiogenic activity [17]. In this study, we demonstrated the therapeutic efficacy of a combined strategy of hypoxic preconditioning and cell sheet technology for severe MI. As expected, hypoxic pretreatment dramatically increased VEGF expression in both human and mouse CDC sheets, mediated by activation of the PI3K/Akt signaling pathway. As suggested by the results of our inhibitory experiment using LY294002, Akt is one of the major signal transducers in hypoxic preconditioning, leading to accelerated VEGF expression in CDC sheets. In addition, hypoxic preconditioning enhanced HGF expression, another angiogenic factor, in CDC sheets (Supplementary Figure 3). Recent work has demonstrated that simultaneous overexpression of IGF-I, HGF, and VEGF in myoblasts activated each signaling pathway and enhanced angiogenic potential after implantation into infarcted hearts [39]. Therefore, simultaneous enhancement of growth factor expression by hypoxic preconditioning likely augments the angiogenic activity of CDC sheets.

Hypoxic pretreatment also reinforced the anti-fibrotic capacity of CDC sheets. In fact, the fibrotic area in infarcted hearts was significantly reduced after implantation of preconditioned CDC sheets. Because of the hypoxia response element (HRE) in the promoter region of endoglin, hypoxic preconditioning may directly up-regulate endoglin expression in CDC sheet through the HRE [40]. In addition, more recent study demonstrated that CDC-derived soluble endoglin can inhibit myofibroblast proliferation [32]. Taken together, we speculate that increased-levels of endoglin in the preconditioned CDC sheet can account for inhibition of myofibloblast proliferation and fibrosis in the infarcted heart. However, soluble endoglin level was not increased in the conditioned media from preconditioned CDC sheets, suggesting another possibility that other cytokines responded to hypoxic preconditioning are potential soluble factors from the preconditioned sheet to inhibit fibrosis. In fact, we also found that HGF, which possess anti-fibrotic activity, was up-regulated in the conditioned media from the preconditioned sheet [41] (Supplementary Figure 3). We need to additional detail studies to clarify what factors inhibits myofibroblast behaviors in response to hypoxic preconditioning. In addition, soluble cytokine-mediated inhibition of myofibroblast proliferation does not completely explain how preconditioned CDC sheets reduced pre-existing scarring in chronically infarcted hearts. Although there are no clear answers to this question so far, we speculate that MMP2 in CDC sheets might be a key factor in reducing pre-existing scarring after sheet implantation. Degradation and remodeling of the extra cellular matrix are important events for cardiac growth, and this process is partially mediated by MMPs. Thus, expression of MMP2 in the sheet might result in remodeling of

infarcted hearts and improvements in heart function after implantation, along with an inhibitory effect on myofibroblasts.

Our results show that cell sheet technology maintains CDCs in chronically infarcted hearts. After implantation, CDC sheets improve cardiac contractile function. Importantly, the therapeutic efficacy of CDC sheets was dramatically reinforced by hypoxic preconditioning, resulting in higher functional improvement of the LV after implantation than normally cultured sheets. Although the precise therapeutic mechanisms of preconditioned CDC sheets are not fully understood, enhanced therapeutic angiogenic and anti-fibrotic capacities might synergistically affect cardiac regeneration in chronically infarcted hearts. Thus, scaffold-free cell sheet technology may enhance the therapeutic effects of CDCs, and a combined strategy with hypoxic preconditioning should therefore be considered for future clinical trials using autologous CDCs.

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Disclosure of conflict of interest

All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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References

[1] http://www.who.int/mediacentre/factsheets/fs310/en/index.html. 2014.

[2] Marban E. Breakthroughs in cell therapy for heart disease: focus on cardiosphere-derived cells. Mayo Clin Proc 2014; 89: 850-858.

[3] Cheng K, Blusztajn A, Shen D, Li TS, Sun B, Galang G, Zarembinski TI, Prestwich GD, Marban E, Smith RR and Marban L. Functional performance of human cardiosphere-derived cells delivered in an in situ polymerizable hyaluronan-gelatin hydrogel. Biomaterials 2012; 33: 5317-5324.

[4] Li TS, Hayashi M, Ito H, Furutani A, Murata T, Matsuzaki M and Hamano K. Regeneration of infarcted myocardium by intramyocardial implantation of ex vivo transforming growth factor-beta-preprogrammed bone marrow stem cells. Circulation 2005; 111: 2438-2445.

[5] Makkar RR, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, Czer LS, Marban L, Mendizabal A, Johnston PV, Russell SD, Schuleri KH, Lardo AC, Gerstenblith G and Marban E. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. Lancet 2012; 379: 895-904.

[6] Al Kindi A, Ge Y, Shum-Tim D and Chiu RC. Cellular cardiomyoplasty: routes of cell delivery and retention. Front Biosci 2008; 13: 2421-2434.

[7] Freyman T, Polin G, Osman H, Crary J, Lu M, Cheng L, Palasis M and Wilensky RL. A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction. Eur Heart J 2006; 27: 1114-1122.

[8] Robey TE, Saiget MK, Reinecke H and Murry CE. Systems approaches to preventing transplanted cell death in cardiac repair. J Mol Cell Cardiol 2008; 45: 567-581.

[9] Zhang M, Methot D, Poppa V, Fujio Y, Walsh K and Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. J Mol Cell Cardiol 2001; 33: 907-921.

[10] Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M and Okano T. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res 2002; 90: e40.

[11] Memon IA, Sawa Y, Fukushima N, Matsumiya G, Miyagawa S, Taketani S, Sakakida SK, Kondoh H, Aleshin AN, Shimizu T, Okano T and Matsuda H. Repair of impaired myocardium by means of implantation of engineered autologous myoblast sheets. J Thorac Cardiovasc Surg 2005; 130: 1333-1341.

[12] Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, Ishino K, Ishida H, Shimizu

T, Kangawa K, Sano S, Okano T, Kitamura S and Mori H. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med 2006; 12: 459-465.
[13] Zakharova L, Mastroeni D, Mutlu N, Molina M, Goldman S, Diethrich E and Gaballa MA. Transplantation of cardiac progenitor cell sheet onto infarcted heart promotes cardiogenesis and improves function. Cardiovasc Res 2010; 87: 40-49.
[14] Yeh TS, Fang YH, Lu CH, Chiu SC, Yeh CL, Yen TC, Parfyonova Y and Hu YC. Baculovirus-transduced, VEGF-expressing adipose-derived stem cell sheet for the treatment of myocardium infarction. Biomaterials 2014; 35: 174-184.
[15] Akita T, Murohara T, Ikeda H, Sasaki K, Shimada T, Egami K and Imaizumi T. Hypoxic preconditioning augments efficacy of human endothelial progenitor cells for therapeutic neovascularization. Lab Invest 2003; 83: 65-73.
[16] Kubo M, Li TS, Suzuki R, Shirasawa B, Morikage N, Ohshima M, Qin SL and Hamano K. Hypoxic preconditioning increases survival and angiogenic potency of peripheral blood mononuclear cells via oxidative stress resistance. Am J Physiol Heart Circ Physiol 2008; 294: H590-595.
[17] Kudo T, Hosoyama T, Samura M, Katsura S, Nishimoto A, Kugimiya N, Fujii Y, Li TS and Hamano K. Hypoxic preconditioning reinforces cellular functions of autologous peripheral blood-derived cells in rabbit hindlimb ischemia model. Biochem Biophys Res Commun 2014; 444: 370-375.
[18] Kudo T, Kubo M, Katsura S, Nishimoto A, Ueno K, Samura M, Fujii Y, Hosoyama T and Hamano K. Hypoxically preconditioned human peripheral blood mononuclear cells improve blood flow in hindlimb ischemia xenograft model. Am J Transl Res 2014; 6: 570-579.
[19] Davis DR, Zhang Y, Smith RR, Cheng K, Terrovitis J, Malliaras K, Li TS, White A, Makkar R and Marban E. Validation of the cardiosphere method to culture cardiac progenitor cells from myocardial tissue. PLoS One 2009; 4: e7195.
[20] Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR and Marban E. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 2007; 115: 896-908.
[21] Hosoyama T, McGivern JV, Van Dyke JM, Ebert AD and Suzuki M. Derivation of myogenic progenitors directly from human pluripotent stem cells using a sphere-based culture. Stem Cells Transl Med 2014; 3: 564-574.
[22] Kwon OH, Kikuchi A, Yamato M, Sakurai Y and Okano T. Rapid cell sheet detachment from poly (N-isopropylacrylamide)-grafted porous cell culture membranes. J Biomed Mater Res 2000; 50: 82-89.
[23] Okano T, Yamada N, Sakai H and Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly (N-isopropylacrylamide). J Biomed Mater Res 1993; 27: 1243-1251.
[24] Sekiya S, Shimizu T, Yamato M, Kikuchi A and Okano T. Bioengineered cardiac cell sheet grafts have intrinsic angiogenic potential. Biochem Biophys Res Commun 2006; 341: 573-582.
[25] Kawasaki H, Ohama T, Hori M and Sato K. Establishment of mouse intestinal myofibroblast cell lines. World J Gastroenterol 2013; 19: 2629-2637.
[26] Li TS, Cheng K, Lee ST, Matsushita S, Davis D, Malliaras K, Zhang Y, Matsushita N, Smith RR and Marban E. Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell-matrix interactions, rationalizing their enhanced functional potency for myocardial repair. Stem Cells 2010; 28: 2088-2098.
[27] Gago-Lopez N, Awaji O, Zhang Y, Ko C, Nsair A, Liem D, Stempien-Otero A and MacLellan WR. THY-1 Receptor Expression Differentiates Cardiosphere-Derived Cells with Divergent Cardiogenic Differentiation Potential. Stem Cell Reports 2014; 2: 576-591.
[28] Chimenti I, Smith RR, Li TS, Gerstenblith G, Messina E, Giacomello A and Marban E. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. Circ Res 2010; 106: 971-980.
[29] Li TS, Cheng K, Malliaras K, Smith RR, Zhang Y, Sun B, Matsushita N, Blusztajn A, Terrovitis J, Kusuoka H, Marban L and Marban E. Direct comparison of different stem cell types and subpopulations reveals superior paracrine potency and myocardial repair efficacy with cardiosphere-derived cells. J Am Coll Cardiol 2012; 59: 942-953.
[30] Beitner-Johnson D, Rust RT, Hsieh TC and Millhorn DE. Hypoxia activates Akt and induces phosphorylation of GSK-3 in PC12 cells. Cell Signal 2001; 13: 23-27.
[31] Laughner E, Taghavi P, Chiles K, Mahon PC and Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 2001; 21: 3995-4004.
[32] Tseliou E, Reich H, de Couto G, Terrovitis J, Sun B, Liu W and Marban E. Cardiospheres reverse

adverse remodeling in chronic rat myocardial infarction: roles of soluble endoglin and Tgf-beta signaling. Basic Res Cardiol 2014; 109: 443.

[33] Masumoto H, Matsuo T, Yamamizu K, Uosaki H, Narazaki G, Katayama S, Marui A, Shimizu T, Ikeda T, Okano T, Sakata R and Yamashita JK. Pluripotent stem cell-engineered cell sheets reassembled with defined cardiovascular populations ameliorate reduction in infarct heart function through cardiomyocyte-mediated neovascularization. Stem Cells 2012; 30: 1196-1205.

[34] Saito S, Miyagawa S, Sakaguchi T, Imanishi Y, Iseoka H, Nishi H, Yoshikawa Y, Fukushima S, Saito A, Shimizu T, Okano T and Sawa Y. Myoblast sheet can prevent the impairment of cardiac diastolic function and late remodeling after left ventricular restoration in ischemic cardiomyopathy. Transplantation 2012; 93: 1108-1115.

[35] Suzuki R, Hattori F, Itabashi Y, Yoshioka M, Yuasa S, Manabe-Kawaguchi H, Murata M, Makino S, Kokaji K, Yozu R and Fukuda K. Omentopexy enhances graft function in myocardial cell sheet transplantation. Biochem Biophys Res Commun 2009; 387: 353-359.

[36] Sawa Y, Miyagawa S, Sakaguchi T, Fujita T, Matsuyama A, Saito A, Shimizu T and Okano T. Tissue engineered myoblast sheets improved cardiac function sufficiently to discontinue LVAS in a patient with DCM: report of a case. Surg Today 2012; 42: 181-184.

[37] Malliaras K, Li TS, Luthringer D, Terrovitis J, Cheng K, Chakravarty T, Galang G, Zhang Y, Schoenhoff F, Van Eyk J, Marban L and Marban E. Safety and efficacy of allogeneic cell therapy in infarcted rats transplanted with mismatched cardiosphere-derived cells. Circulation 2012; 125: 100-112.

[38] Tang YL, Zhu W, Cheng M, Chen L, Zhang J, Sun T, Kishore R, Phillips MI, Losordo DW and Qin G. Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression. Circ Res 2009; 104: 1209-1216.

[39] Konoplyannikov M, Haider KH, Lai VK, Ahmed RP, Jiang S and Ashraf M. Activation of diverse signaling pathways by ex-vivo delivery of multiple cytokines for myocardial repair. Stem Cells Dev 2013; 22: 204-215.

[40] Sanchez-Elsner T, Botella LM, Velasco B, Langa C and Bernabeu C. Endoglin expression is regulated by transcriptional cooperation between the hypoxia and transforming growth factor-beta pathways. J Biol Chem 2002; 277: 43799-43808.

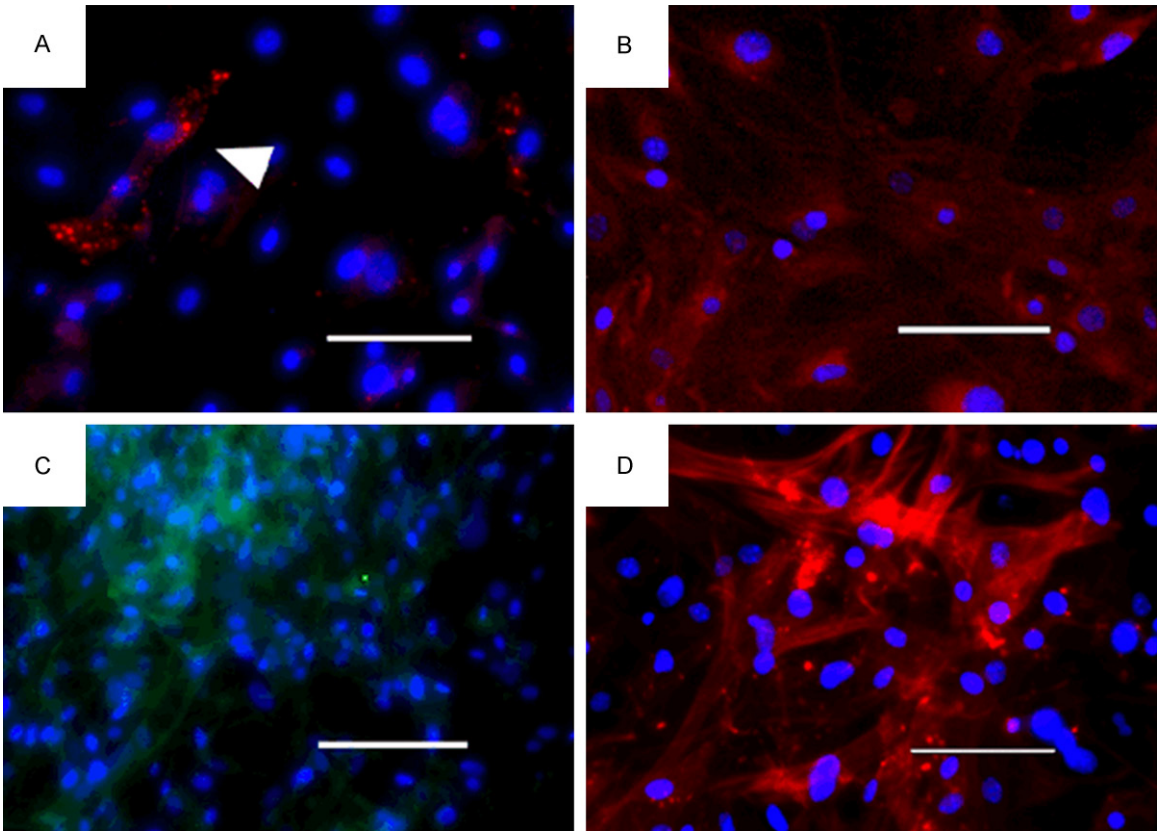
[41] Wang Y, Ahmad N, Wani MA and Ashraf M. Hepatocyte growth factor prevents ventricular remodeling and dysfunction in mice via Akt pathway and angiogenesis. J Mol Cell Cardiol 2004; 37: 1041-1052.

Supplementary Table 1. Cardiosphere Medium Recipe

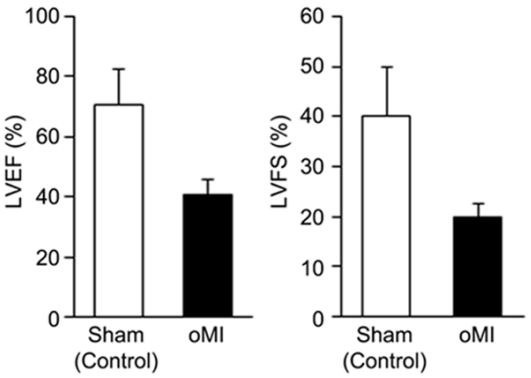
Materials	Final Conc.	Vendor/Cat #
IMDM	35%	Life Technologies/12440-053
DMEM/F12	65%	Life Technologies/11320-033
FBS	3.5%	Life Technologies/26140-079
L-Glutamine	1 mM	Sigma-Aldrich/G7513
β-mercaptoethanol	0.1 mM	Wako/139-07525
Thrombin	1 unit/ml	Sigma-Aldrich/T6884
B-27	1%	Life Technologies/17504-044
bFGF	80 ng/ml	Sigma-Aldrich/F0291
EGF	25 ng/ml	Sigma-Aldrich/E9644
Cardiotrophin-1	4 ng/ml	Sigma-Aldrich/SRP4011
Heparin	5 ng/ml	Sigma-Aldrich/H3149
Penicillin/Streptomycin	1%	Life Technologies/15140

Supplementary Table 2. Primary Antibodies

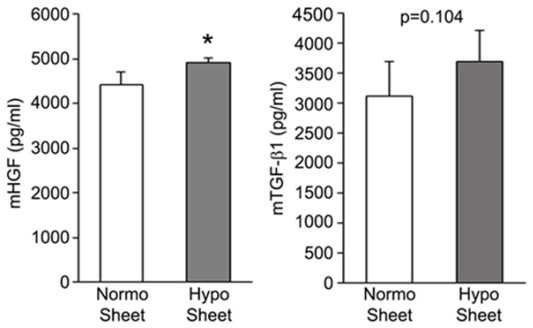
Antigen	Vendor/Cat #	Use	Dilution
c-Kit	Santa Cruz Biotechnology/sc-168	Immunofluorescence	1:50
CD105	Diagnostic Biosystems/Mob364	Immunofluorescence	1:50
α-Smooth Muscle Actin	Enzo Life Science/ENZ-C34933	Immunofluorescence	1:50
CD31	Abcam/ab28364	Immunofluorescence	1:50
Desmin	Millipore/MAB1698	Immunofluorescence	1:100
α-Actinin	Cell Signaling Technology/#6487	Immunofluorescence	1:50
Phospho-Histone H3	Cell Signaling Technology/#9701	Immunofluorescence	1:1000
CD90-PE conjugated	eBioscience/12-0909	FACS	1:1000
CD105-PE conjugated	eBioscience/12-1057	FACS	1:1000
c-Kit-FITC conjugated	eBioscience/12-1178	FACS	1:1000
Akt	Cell Signaling Technology/#9272	Western Blot	1:1000
pAkt	Cell Signaling Technology/#9271	Western Blot	1:1000
Endoglin	Santa Cruz Biotechnology/sc-20632	Western Blot	1:200
β-Actin-HRP conjugated	Novus Biologicals/NB600-532H	Western Blot	1:5000
DyeLight 488 conjugated Tomato Lectin	Vector Laboratories/DL-1174	Immunofluorescence	1:200
Cardiac Troponin T	Abcam/ab10214	Immunofluorescence	1:100



Supplementary Figure 1. Characterization of mouse CDCs. Mouse CDCs were isolated from adult mouse heart, and then stained with anti-c-Kit (A), CD90 (B), CD105 (C), and αSMA (D) antibodies. Scale bar = 100 μm.



Supplementary Figure 2. Permanent ligation of left anterior descending artery (LAD) diminishes left ventricular function of the mouse heart. At 4 weeks after LAD ligation, both LVEF and LVFS were measured and compared between non-operated mice and LAD-ligated mice (oMI). In both parameters, o-MI mice showed diminished left ventricular function (n = 4).



Supplementary Figure 3. Hypoxic preconditioning increased HGF expression in mouse CDC sheets but did not affect TGF-β1 expression. Both HGF and TGF-β1 level in supernatant were measured and compared between normoxically cultured (Normo Sheet) and hypoxically cultured (Hypo Sheet) mouse CDC sheets by ELISA. HGF expression was significantly increased in Hypo Sheets (left graph), whereas TGF-β1 expression was not altered by hypoxic preconditioning (right graph) (n = 6, respectively. *p < 0.05).