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Regenerative Medicine

Generation of Induced Cardiospheres via Reprogramming of Skin Fibroblasts fo Myocardial Regeneration

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ABSTRACT

Recent pre-clinical and clinical studies have suggested that endogenous cardiospheres (eCS) are potentially safe and effective for cardiac regeneration following myocardial infarction (MI). Nevertheless the preparation of autologous eCS requires invasive myocardial biopsy with limited yield. We describe a novel approach to generate induced cardiospheres (iCS) from adult skin fibroblasts via somatic reprogramming. After infectio with Sox2, Klf4, and Oct4, iCS were generated from mouse adult skin fibroblasts treated with Gsk3β inhibitor-(2′Z,3′E)- 6-Bromoindirubin-3′-oxime and Oncostatin M. They resembled eCS, but contained a higher percentage of cells expressing Mesp1, Isl1, and Nkx2.5. They were differentiated into functional cardiomyocytes in vitro with similar electrophysiological properties, calcium transient and contractile function to eCS and mouse embryonic stem cell-derived cardiomyocytes. Transplantation of iCS (1×10^6 cells into mouse myocardium following MI had similar effects to transplantation of eCS but significantly better than saline or fibroblast in improving left ventricular ejection fraction, increasing anterior/septal ventricular wall thickness and capillary density in the infarcted region 4 weeks after transplantation. No tumor formation was observed. iCS generated from adult skin fibroblasts by somatic reprogramming and a cocktail of Gsk3β inhibitor-6-Bromoindirubin-3′-oxime and Oncostatin M may represent a novel source for cell

This study provides the first proof-of-principle results that demonstrate the feasibility of generating donor-specific induced cardiospheres via a somatic reprogramming process, providing a new source of cell therapy for treatment of myocardial infarction

INTRODUCTION

Cardiovascular diseases (CVDs), especially myocardial infarction (MI), are the leading cause c death worldwide and contribute to over 30% of global mortality 1, 2. Despite recent advance in the treatment of CVDs, a significant proportion of patients still die of progressive heart failure. Therapy remains palliative and does not address the fundamental pathogenic proce of CVDs, that is, loss of functional cardiomyocytes. As a result, cell-based therapies have bee proposed as a promising strategy to treat CVDs via replenishment of the damaged cells. Different types of stem cells, including bone marrow stem cells, mesenchymal stem cells, skeletal myoblasts, endothelial progenitor cells and cardiac progenitor cells, have been investigated as sources for cell therapy in myocardial regeneration 3-5. Although improved heart function has been observed in preclinical studies after transplantation of non-heart origin stem cells into the injured myocardium, there is very limited evidence of myocardial regeneration via trans-differentiation into functional cardiomyocytes in vivo. Most studies h shown that the improvement in cardiac function, especially with bone marrow-derived cells, due to paracrine effects 6, 7. On the contrary, it has been demonstrated that various types c cardiac progenitor cells, including endogenous cardiospheres (eCS), can differentiate into functional cardiomyocytes in vivo, and thus may represent a more effective cell-based thera 8, 9.

Cardiac progenitor cells, residing in the heart with the ability to differentiate into three majo cardiac cell types (cardiomyocyte, endothelial cell, and smooth muscle cell), have been defin and isolated using different approaches, such as different marker-positive cell population (cKit+ 10, Flk+ 11, Wt1+ 12, Mesp1+ 13, Isl1+14, and Nkx2.5+15), side population 16 as well a the eCS 8, 9. These cardiac progenitor cells have been consistently shown to improve heart function in different pre-clinical small and large animal models of MI 17, 18. Among them, e has been one of the most commonly used cell sources for cardiac regeneration in animal

approach 21. Previous reports have demonstrated that mouse embryonic fibroblasts can be directly converted into cardiomyocytes through a direct somatic reprogramming strategy; a this process passed through a cardiac progenitor stage 22, 23. To evaluate the feasibility of isolating these cardiac progenitor cells via the formation of cardiospheres during the somat reprogramming process, different panels of cardiotrophic growth factors or chemicals were screened.

Our results demonstrated that the combination of Gsk3β inhibitor-(2′Z,3′E)- 6-Bromoindirub 3′-oxime (BIO), and Oncostatin M (OSM) promoted the generation of spheres from both embryonic fibroblasts and adult skin fibroblasts during somatic reprogramming. These spheres, which resemble eCS, contained Mesp1, Isl1, and Nkx2.5 positive cardiac progenitor cells but not pluripotent stem cells and possessed the differentiation ability of cardiomyocyt thus they were termed induced cardiospheres (iCS). These iCS had therapeutic potential comparable with eCS in a mouse model of MI. Therefore, we provide the first proof-of-principle results to show the feasibility of generating iCS for cardiac regeneration by somatic reprogramming of mouse fibroblasts without passing through a pluripotent stem cell stage using a panel of pluripotent transcription factors and cardiotrophic growth factors.

Materials and Methods

Cell Isolation and Maintenance

Adult skin fibroblasts were isolated from 4-week-old mice. Neonatal cardiomyocytes were isolated from 1 to 2 day old neonatal mice. Adult cardiomyocytes were isolated from 4 to 6 weeks old mice using the Langendorff retrograde perfusion method 24. Endogenous cardiospheres were isolated as described before 8, 9. In brief, mouse eCS were isolated fror weeks old mice. The mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and their hearts were removed quickly and incubated with cold Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) (Invitrogen, Grand Island, NY, USA, https://www.thermofisher.com/hk/en/home/brands/invitrogen.html). The heart was washed with PBS via aorta perfusion, and then minced in the digestion buffer (4 mg/ml collagenase and 4 mg/ml dispase (Roche Life Science, Penzberg, Germany, https://lifescience.roche.com/ in DMEM/High Glucose) and digested for 30 minutes at 37°C. The isolated single cells and tissue fragments were washed twice with PBS and plated on a culture dish with culture medium (DMEM/High Glucose, 10% FBS (fetal bovine serum), 100 U/ml penicillin, 100 µg/ml

complete IMDM/65% DMEM–Ham F-12 mix containing 2% B27, 0.1 mM 2-mercaptoethanol, ng/ml epidermal growth factor (EGF), 20 ng/ml basic fibroblast growth factor (bFGF), 40 nM cardiotrophin-1, 40 nM thrombin, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine). Three days after plating, the eCS were formed and used for subsequent experiments.

Mouse embryonic stem cells (mESC, cell line R1) were maintained on 0.1% gelatin coated plates with culture medium (DMEM/High Glucose, 15% FBS (HyClone, South Logan, Utah, US http://www.gelifesciences.com/), 2 mM GlutaMAX, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol (Gibco, Grand Island, NY, USA, https://www.thermofisher.com/), 1% penicillin-streptomycin, and 1,000 U/ml leukemia inhibitory factor (LIF, Millipore, Darmstadt, Germany, http://www.emdmillipore.com/). The medium was refreshed every day. Human dermal fibroblasts (Thermo Fisher, Grand Island, NY, USA, https://www.thermofisher.com/) were maintained according to manufacturer's instructions.

iCS Generation and Differentiation

Retrovirus was produced by introducing pMXs-Oct4 (Addgene 13366, Cambridge, MA, USA, https://www.addgene.org/), pMXs-Sox2 (Addgene 13367), and pMXs-Klf4 (Addgene 13370) 2 and packaging plasmid pCL-Eco (Addgene 12371) 26 into 293T cells with lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, the viral supernatant was collected and filtered using a 0.45 μm PVDF-based filter for somatic cell infection. Mouse adult skin fibroblasts were plated on a 0.1% gelatin coated plate at a cell density of 2×10^4 cells per we The fibroblasts were infected overnight in the presence of 8 μg/ml polybrene (Sigma). Then medium was changed to basal medium: DMEM/High Glucose, 10% Knockout Serum Replacement, 2 mM GlutaMAX, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol, 0. N2 (Gibco) and 4 ng/ml bFGF (Peprotech, Rocky Hill, NJ, USA, https://www.peprotech.com/) f induced cardiosphere generation. Growth factors or chemicals were added at a final concentration of 20 ng/ml [EGF, bFGF, bone morphogenetic protein 4 (BMP4), Interleukin 6 from Peprotech], 50 ng/ml [vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and Actinin A from Peprotech; and OSM from Sigma], 100 ng/ml [Wnt3a, Cardiotrophin 1 (CT-1) from Peprotech], 20 mM Lithium chloride (Sigma) and 2.5 μM BIO (Sigma).

For knock down experiments, shRNA targeting to β-catenin (shRNA target

time as Oct 4, Sox2, and Klf4 infection.

For spontaneous differentiation, newly formed iCS were plated onto Matrigel (1:40, BD Biosciences, San Jose, CA, USA, http://www.bdbiosciences.com/) coated plates with differentiation medium (DMEM/High Glucose, 15% FBS (HyClone), 2 mM GlutaMAX, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol (Gibco), 50 μg/ml Ascorbic acid (Sigma for 15 days. Beating area appeared at day 12 after differentiation and the number increased till day 15. There was no obvious further increasing of beating area after 15 days of differentiation. This differentiation process is consistent in all experiments performed. Beati areas were manually dissected and digested with 1 mg/ml collagenase B for subsequent experiments. The other differentiated cells were used for endothelial and smooth muscle ce marker staining.

Similarly, spontaneous differentiation of eCS was performed as described before 8, 9. The e were plated onto 0.1% gelatin coated plates with cardiomyocytes differentiation medium [DMEM/High Glucose, 15% FBS (HyClone), 2 mM GlutaMAX, 1% nonessential amino acids, 0. mM 2-mercaptoethanol (Gibco), 50 μg/ml Ascorbic acid (Sigma)]. The beating clusters from d 15 of differentiation were manually picked out and digested with 1 mg/ml collagenase B for hours at 37°C, followed by 5 mM EDTA treatment for 5 minutes. The single cardiomyocytes were plated on 0.1% gelatin coated coverslips with cardiomyocyte differentiation medium fo days and then used for subsequent experiments, including flow cytometry, action potential, calcium transient, and contractility analysis.

mESC Differentiation

Cardiomyocyte differentiation of mESC was performed as described before 27. In brief, 1 × cells were suspended in 10 ml differentiation medium [DMEM/High Glucose, 15% FBS (HyClone), 2 mM GlutaMAX, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol (Gibc 50 μg/ml Ascorbic acid (Sigma)] for 7 days to allow embryoid body (EB) formation. On day 7, the EBs were plated onto 0.1% gelatin coated bacterial dishes for further differentiation. On day 9, beating areas were observed in some EBs. The beating cluster from day 15 of differentiation was manually picked out and digested with 1 mg/ml collagenase B for 2 hour at 37°C, followed by 5 mM EDTA treatment for 5 minutes. The single cardiomyocytes were plated on 0.1% gelatin coated coverslips with cardiomyocyte differentiation medium for 3 da and then used for subsequent experiments.

MA, USA, https://www.neb.com/) and random primers (Invitrogen). Real-time PCR was performed with SYBR Green Master Mix (Clontech, Mountain View, CA, USA, https://www.clontech.com/) on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems Grand Island, NY, USA, http://www.thermofisher.com/hk/en/home/brands/applied-biosystems.html) according to the manufacturer's instructions. Primers used for real-time P are listed in Supporting Information Table 1. qPCR determination of gene level was calculate using the ΔΔCt method with treatment group compared with baseline and β-actin serving a internal control.

Immunofluorescence

Cells were plated onto 0.1% gelatin coated coverslips for 48 hours, fixed with 4% paraformaldehyde (Sigma) for 10 minutes at room temperature, washed twice with PBS, permeablized with 0.2% Triton-X 100 in PBS with 10% FBS for 30 minutes, and incubated wit primary antibodies (Mesp1 (Abcam, 1:100, Cambridge, UK, http://www.abcam.com/), Isl1 (Abcam, 1:100), Nkx2.5 (Santa Cruz, 1:100, Dallas, Texas, USA, http://www.scbt.com/), α-Actir (Sigma, 1:1,000), myosin light chain 2 atrial (Mlc2a, Abcam, 1:100), myosin light chain 2 ventricular (Mlc2v, AXXORA, 1:10, Farmingdale, NY, USA, http://www.axxora.com/), von Willebrand Factor (vWF) (Millipore, 1:100), α-smooth muscle actin (R&D Systems, 1:100, Minneapolis, MN, USA, https://www.rndsystems.com/), or Nanog (Fisher Scientific, 1:100, Grand Island, NY, USA, https://www.fishersci.com/)) in blocking buffer (0.1% Triton-X 100 in F with 10% FBS) overnight at 4°C. Cells were washed five times with PBST (0.1% Triton-X 100 ir PBS), five minutes each time, and then incubated with secondary antibody Alexa Fluor 594 conjugate for 1 hour. The cells were further counterstained and mounted with SlowFade Go antifade reagent with DAPI (Invitrogen). For counting Mesp1, Isl1, and Nkx2.5 positive cells, four views per coverslip were counted and three coverslips were used for each round of experiment. The experiment was repeated three times.

Flow Cytometry and Cell Purification

Spheres and differentiated cardiomyocytes were dissociated with 1 mg/ml collagenase B (Roche Life Science) for 30 minutes at 37°C, followed by 2 mM EDTA for 5 minutes. Cells wer centrifuged and re-suspended in PBS with 5% FBS. For cell surface marker staining, cells wer ready for use. For cardiomyocyte marker staining, cells were fixed with 2% paraformaldehyc for 15 minutes, then permeablized with 0.5% Tween 20 for 15 minutes. Cells were stained w

Fluor 488 conjugate for 1 hour. Cells were analyzed with the FC500 (Beckman Coulter, Inc., Indianapolis IN, USA, https://www.beckmancoulter.com/) and data analyzed with FlowJo software. cKit, Flk1, and CXCR4 positive cells were purified by BD FACSAria SORP cell sorter (Biosciences).

Patch Clamping

The whole-cell patch clamp technique was used 28. Borosilicate glass electrodes (1.2 mm outside diameter) were pulled with a Brown-Flaming puller (model P-97; Sutter Instrument (Novato, CA, USA, http://www.sutter.com/) with tip resistances of 2–3 MΩ when filled with pipette solution (20 mM KCl, 110 mM K-aspartate, 1.0 mM MgCl2, 10 mM HEPES, 0.05 mM EGTA, 0.1 mM GTP, 5.0 mM Na2-phosphocreatine, and 5.0 mM Mg2-ATP; the pH was adjuste to 7.2 with KOH.). Cells were perfused with Tyrode solution (136 mM NaCl, 5.4 mM KCl, 1.0 n MgCl2, 1.8 mM CaCl2, 0.33 mM NaH2PO4, 10 mM glucose, and 10 mM HEPES; the pH was adjusted to 7.4 with NaOH.). The cellular electrophysiological parameters were measured in spontaneous beating cardiomyocytes for differentiated cardiomyocytes and neonatal cardiomyocytes, and during pacing with 1 Hz for adult cardiomyocytes. The nodal, atrial, and ventricular like cardiomyocytes were defined by maximum diastolic membrane potential, action potential amplitude (APA) and the ratio of action potential duration at 90% to 50% repolarization (APD90/50) 27. Both atrial and ventricular cardiomyocytes have lower maximu diastolic membrane potential (< −50 mV) while nodal is higher than −50 mV. Both atrial and ventricular cardiomyocytes have higher APA (>90 mV) while nodal has lower than 90 mV. Atr cardiomyocytes have a ratio of APD90/APD50 more than 2.0 without plateau phase while ventricular cardiomyocytes have a ratio less than 1.9 with plateau phase.

Calcium Transient

Calcium transient studies were conducted as in our previous studies 29-32. In brief, Fluo-4 A (Invitrogen) was diluted in culture medium and loaded into the cells with a final concentratio of 10 μM for 30 minutes at room temperature in the dark. Cells were washed with Tyrode (1 mM $CaCl_2$) solution and ready for imaging 30 minutes later. The coverslip was transferred in the glass bottom dish for confocal imaging (Corning Incorporated, Corning, NY, USA, https://www.corning.com/). A LSM510 (Zeiss, Oberkochen, Germany, http://www.zeiss.com/) confocal microscope equipped with a 20× objective was used for calcium transient recordin The Fluo-4 was excited by a 488 nm line of an Argon laser and emission signals above 505 n

Cell contractility was recorded by MyoCam-S (ION-OPTIX, Westwood, MA, USA, http://www.ionoptix.com/) and IonWizard software (ION-OPTIX) 33. The coverslip was transferred into a perfusion chamber containing Tyrode buffer (1.8 mM $CaCl_2$) and maintain at 37°C. The beating cardiomyocyte cell edge was identified by the left and right cursor and located with IonWizard software under the microscope. The cell edge movement was recorc at a magnification of 40× with the capturing frequency at 250 Hz. Contractility was expresse as a percentage of cell length shortening, calculated with IonWizard software.

Western Blot

Cells were lysed using RIPA buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.1 SDS, 140 mM NaCl) with proteinase inhibitor cocktail (Sigma). The lysate was separated with 8% SDS-PAGE. Membrane was incubated overnight at 4°C with rabbit anti-β-catenin (Abcam 1:1,000), Mesp1 (Abcam, 1:1,000), Isl1 (Abcam, 1:1,000), Nkx2.5 (Santa Cruz, 1:1,000), β-actin (Abcam, 1:4,000). Goat anti-Rabbit IgG (H + L)/HRP (horseradish peroxidase) (Dako, 1:2000, Glostrup, Denmark, http://www.dako.com/) was used as a secondary antibody and the signa was detected with Clarity ECL Western Blotting Substrate (Bio-Rad, Hercules, CA, USA, http://www.bio-rad.com/). Band density was measured by Image J (National Institutes of Health, USA, http://www.ncbi.nlm.nih.gov/).

Whole Genome Arrays and Data Analysis

Gene expression profile was determined on the GeneChip Mouse Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA, http://www.affymetrix.com/) at the Genome Center of the University of Hong Kong. Three microgram of total RNA were used for cDNA synthesis, fragmentation and hybridization (Affymetrix). Arrays were scanned with a GeneArray scanne 7G (Affymetrix). Data were background-subtracted and normalized with the Robust Multichi Average method using the software GeneSpring (Agilent Technologies, Santa Clara, CA, USA, https://www.agilent.com/). Expression profiles were established on the basis of duplicate samples collected from independent experiments. Statistical significance was determined w one-way ANOVA test and multiple testing corrections were performed using Benjamini-Hochberg test. $p < 0.05$ was considered significant. All samples were compared against the group “Fibroblast” and differentially expressed genes were identified with a fold change cut-of >2.0. Hierarchical clustering was performed with Cluster 3.0 software 34.

upside of the infarcted region, three sites in total, 5 µl for each site) and the infarcted region (one site in the middle of the infarcted region, 5 µl).

Echocardiography

Four weeks after transplantation, animals were anesthetized with ketamine 100 mg/kg. The heart was monitored in 2D mode with Vivid q system (GE Healthcare, Little Chalfont, UK, http://www.gehealthcare.com/) with a 11.5-MHz transducer GE 10-RS (GE Healthcare). The image was recorded in 2D mode in the parasternal long axis view. The M-mode line passed through the basal segment of the left ventricle between the mitral valve and the papillary muscle. M-mode line was adjusted to be perpendicular to the walls of the left ventricle. Left ventricular ejection fraction was derived from the measurement of LV end-diastolic diamete and LV end-systolic diameter in standard M-mode.

Hemodynamic Assessment

Hemodynamic assessment was performed as described before 35, 36. At 4 weeks after transplantation, the animals were anesthetized with ketamine 100 mg/kg and xylazine 10 mg/kg. The right carotid artery was gently exposed and isolated. A 1.2-F, 3.5 mm electrode spacing rodent pressure-volume catheter (Transonic, Ithaca, NY, USA, http://www.transonic.com/) connected to ADVantage Small Animal PV Foundation System (Iworx, Dover, NH, USA, https://www.iworx.com/) was inserted into the artery. Hemodynami parameters including left ventricular end systolic pressure, maximum increase in left ventricular pressure (dP/dt) and the slope of end systolic pressure-volume relationship were recorded and analyzed with LabScribe2 (Iworx).

Histological Assessment

At 4 weeks after transplantation, the animals were sacrificed and the hearts were fixed with 10% neutral buffered formalin (Sigma), embedded in paraffin. The heart was sectioned (5 µr in thickness) serially from the apex to the base. Every tenth serial section was chosen for further analysis. Sections were used for trichrome staining (ScyTek, Logan, UT, USA, http://www.scytek.com/) or immunohistochemistry [α-Actinin 1:1,000 (Sigma), green florescence protein (GFP) 1:200 (Santa Cruz), vWF 1:200 (Millipore)]. The infarcted wall thickness was measured by the ratio of anterior to septal wall thickness and the percentage infarction determined by the ratio of blue positive area to whole left ventricle. The

Cardiospheres were dissociated into single cells by 1 mg/ml collagenase B, followed by 2 mM EDTA treatment. Single cells were labeled with DiR (Life Technologies, Waltham, MA USA, https://www.thermofisher.com/) for 15 minutes and then washed three times with PBS. Labeled cells were transplanted into MI mice. At different time points following transplantation, the mouse was sacrificed and the heart was dissected out for signal detecti using the IVIS Spectrum Preclinical In Vivo Imaging System (PerkinElmer, Waltham , MA, USA http://www.perkinelmer.com/). The quantity of DiR labelled cells in the heart was analyzed using IVIS Spectrum software.

Statistical Analysis

Data were analyzed using SPSS software for Windows (IBM, Armonk, NY, USA, http://www.ibm.com/) and are shown as mean ± SEM (standard error of the mean). Student *t*-test was used for two group comparison and 1-way ANOVA for multiple group comparison with normal data distribution, parametric test, and Turkey Post Hoc tests. A level of $p < 0.05$ was considered statistically significant.

RESULTS

GSK3β Inhibitor-BIO and Oncostatin M Promote Generation of iCS from Mouse Fibroblasts with Somatic Reprogramming

Mouse embryonic fibroblast cells were infected with retrovirus expressing three transcriptic factors (Oct4, Sox2, and Klf4). Following overnight infection, the medium was refreshed with Knockout Serum Replacement-based medium that has been proven to be a more efficient system for somatic reprogramming 37. Quantitative PCR analysis showed that the mRNA lev of cardiac progenitor markers Isl1 and Nkx2.5 was significantly upregulated during somatic reprogramming, starting from day 16 (for Isl1) and day 18 (for Nkx2.5) compared with the control green fluorescence protein (GFP) vector at day 6 post retrovirus infection. The mRNA level of Isl1 and Nkx2.5 in cells infected with control GFP remained unchanged at day 22 compared with day 6 (Fig. 1A). The mRNA level of mesoderm marker Bry, another cardiac marker Gata4 and early cardiac progenitor marker Mesp1 were also upregulated compared with the control GFP (Fig. 1B). Nonetheless Mesp1 declined at day 20 compared with day 16 (Fig. 1B). As a result, day 18 post infection was chosen as the experimental time point for the next screening step, when both early (Mesp1) and late (Nkx2.5) cardiac progenitor markers

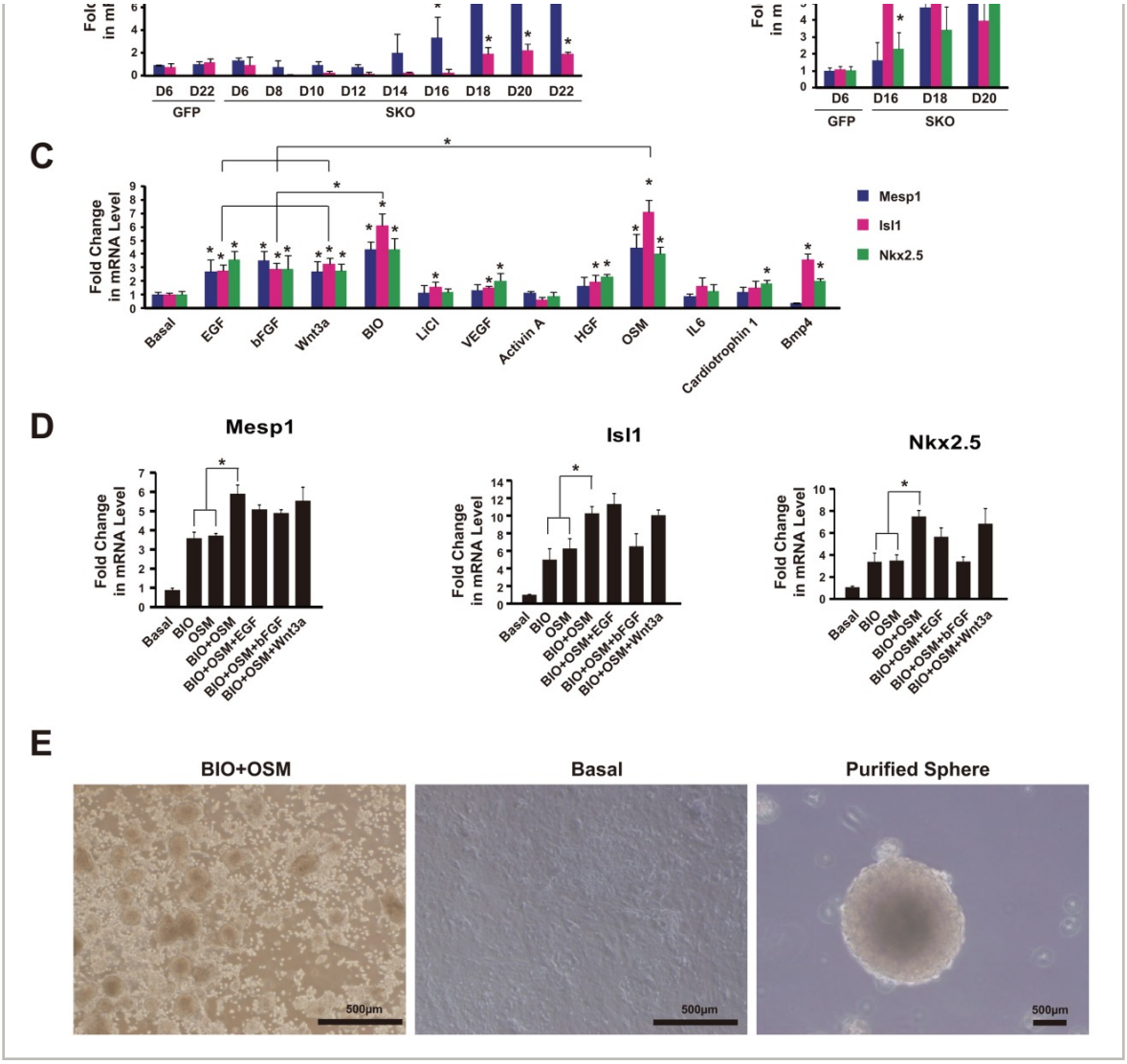

Figure 1

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BIO and OSM elevated the mRNA level of cardiac progenitor marker Mesp1, Isl1, and Nkx2.5 and promoted sphere formation in embryonic fibroblasts. (**A**): qPCR analysis of Isl1 and Nkx2.5 showed that their mRNA level in reproprogramming fibroblasts was overexpressed with SKO (Sox2, Klf4, and Oct4) when the cells were cultured in basal medium compared with GFP from day 6 (D6) to day 22 (D22) during somatic reprogramming ($n = 3$, biological replicates; *$p < .05$; day time point of interest vs. D6 GFP group); (**B**) qPCR analysis of mesodermal marker Bry, early cardiac progenitor marker Mesp1

CT-1, and BMP4. As BIO and OSM enhanced the mRNA level of Isl1 more than other factors (Fig. 1C), we further determined whether a combination of these two factors could provide a additive effect. Indeed, combining these two factors significantly increased the mRNA level c Mesp1, Isl1, and Nkx2.5 (Fig. 1D). Nonetheless adding a further growth factor into the mediu with BIO and OSM did not further increase the same mRNA levels (Fig. 1D).

After plating the cells onto poly-D-lysine coated plates, which were used for cardiosphere formation and isolation 9, cells treated with BIO and OSM, not those untreated, began to aggregate as spheres (Fig. 1E). These spheres, formed during somatic reprogramming with E and OSM treatment, contained Mesp1, Isl1, and Nkx2.5 positive cells (Supporting Informatio Fig. 1A). They had the capability to form beating cluster of cardiomyocytes after 12 days of differentiation; and the number of beating cluster reached a maximum at 15 days. Those beating cardiomyocytes expressed different cardiomyocytes markers including α-Actinin, myosin light chain 2 atrial (atrial specific marker), and myosin light chain 2 ventricular (ventricular specific marker) (Supporting Information Fig. 1B and Movie 1). Furthermore, the differentiated cells also contained vWF and α-smooth muscle actin positive cells, indicating their endothelium and smooth muscle differentiation capability (Supporting Information Fig 1C, 1D).

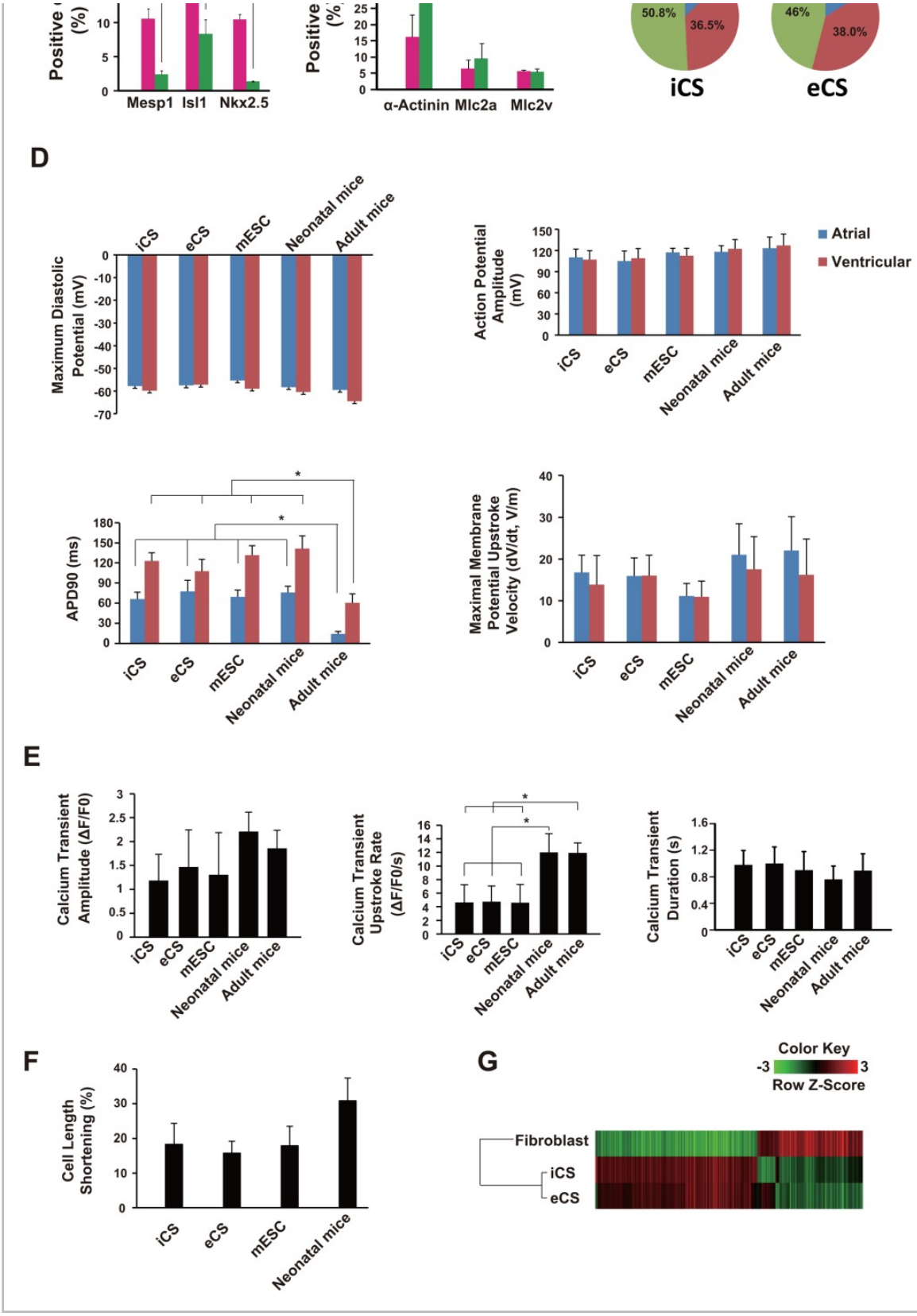
Cell surface marker analysis for cardiospheres revealed that both spheres and no-sphere forming cells derived from reprogramming contained similar high proportion of Sca1 positiv and $CD105^{high}CD45^{negative}$ cells as eCS (Supporting Information Fig. 1E). Conversely, spheres forming cells and eCS contained significantly higher population of cKit, Flk1, and CXCR4 positive cells comparing to non-sphere forming cells (Supporting Information Fig. 1E). Moreover, those spheres forming cells were further enriched with cells expressing cKit, Flk1 and CXCR4 on further passage (Supporting Information Fig. 1F). Purified cKit, Flk1, or CXCR4 positive cells from those spheres forming cells had the capability to differentiate into α-Actin positive cardiomyocytes but not those negative populations (Supporting Information Fig. 1G Furthermore, qPCR analysis revealed that these spheres did not express pluripotent stem ce marker Oct, Sox2, Nanog, or Rex1, confirming that they did not contain pluripotent stem cel These findings were further confirmed by immune-staining of Nanog in the dissociated spheres (Supporting Information Fig. 1H, 1I).

The reprogramming approach using BIO and OSM treatment of mouse embryonic fibroblas was also applied to adult skin fibroblasts. Similarly, the spheres generated from adult skin

termed iCS. In general, beating clusters of cardiomyocytes were observed at 32–35 days of culturing from fibroblasts: 18 days for reprogramming and factor induction, 2 days for sphe formation, and 12–15 days for cardiomyocytes differentiation. This reprogramming and differentiation approaches are highly consistent and effective for both mouse embryonic (repeated 13 times) and adult skin fibroblast (repeated 45 times) cells for generation of iCS. subsequent experiments, iCS generated from adult skin fibroblasts were used for further functional assessment.

Comparison of iCS with eCS and Native Cardiomyocytes

Compared with eCS isolated from the mouse heart, those iCS derived from adult skin fibroblasts contained a higher percentage of Mesp1, Isl1, and Nkx2.5 positive cells (Fig. 2A a Supporting Information Fig. 2). Nevertheless, both types of cardiospheres showed similar efficacy for in vitro cardiac differentiation when the percentage of α-Actinin, myosin light cha 2 atrial, and myosin light chain 2 ventricular positive cells were analyzed (Fig. 2B). Using the patch clamp technique, the action potential was recorded from a single isolated cardiomyoc to characterize the phenotype of these cells. As defined by the ratio of action potential duration (APD) at 90% to 50% repolarization 27, both types of cardiosphere showed a simila level of differentiated nodal-like, atrial-like, and ventricular-like cardiomyocytes (Fig. 2C; Supporting Information Fig. 3).


Positive (%)
Mesp1 Isl1 Nkx2.5
α-Actinin Mlc2a Mlc2v
50.8%
36.5%
iCS
46%
38.0%
eCS
D
Maximum Diastolic Potential (mV)
iCS eCS mESC Neonatal mice Adult mice
Action Potential Amplitude (mV)
Atrial
Ventricular
APD90 (ms)
Maximal Membrane Potential Upstroke Velocity (dV/dt, V/m)
E
Calcium Transient Amplitude (ΔF/F0)
Calcium Transient Upstroke Rate (ΔF/F0/s)
Calcium Transient Duration (s)
F
Cell Length Shortening (%)
G
Color Key
-3 3
Row Z-Score
Fibroblast
iCS
eCS

upstroke velocity. Nonetheless the adult cardiomyocytes had a significantly shorter APD at 90% repolarization compared with other cardiomyocytes (Fig. 2D).

Calcium transient analysis showed that the iCS, eCS, and mESC derived cardiomyocytes had similar level of calcium amplitude and duration of calcium transient. But these differentiated cardiomyocytes had a significantly lower calcium signal upstroke velocity than native cardiomyocytes (Fig. 2E). Assessment of cell contractile function showed that cardiomyocyte from different sources had a similar percentage of cell shortening (Fig. 2F). Our results demonstrated that the in vitro functional parameters of cardiomyocytes derived from eCSs, iCSs, or mESCs were similar; but were less mature compared with native cardiomyocytes. Whole genome expression profile analysis showed that the iCS was clustered with eCS but t fibroblast cells (Fig. 2G), further proving the similarity of iCS and eCS.

Transplantation of iCS Improves Cardiac Function Following Myocardial Infarction

We investigated the therapeutic effects of transplanting iCS versus eCS into an animal mode of acute MI in immunodeficient mice (NOD/SCID). Compared with phosphate-buffered salin or the iCS parental fibroblast cell injection, both iCS and eCS transplantation significantly improved left ventricular ejection fraction at week 4 after cell transplantation as determined serial echocardiogram (Fig. 3A, 3B). Invasive hemodynamic assessment at week 4 also confirmed that compared with saline or fibroblast injection, both iCS and eCS transplantatio improved left ventricular end systolic pressure, maximum increase in left ventricular pressu and the slope of end systolic pressure-volume relationship (Fig. 3C, 3D).

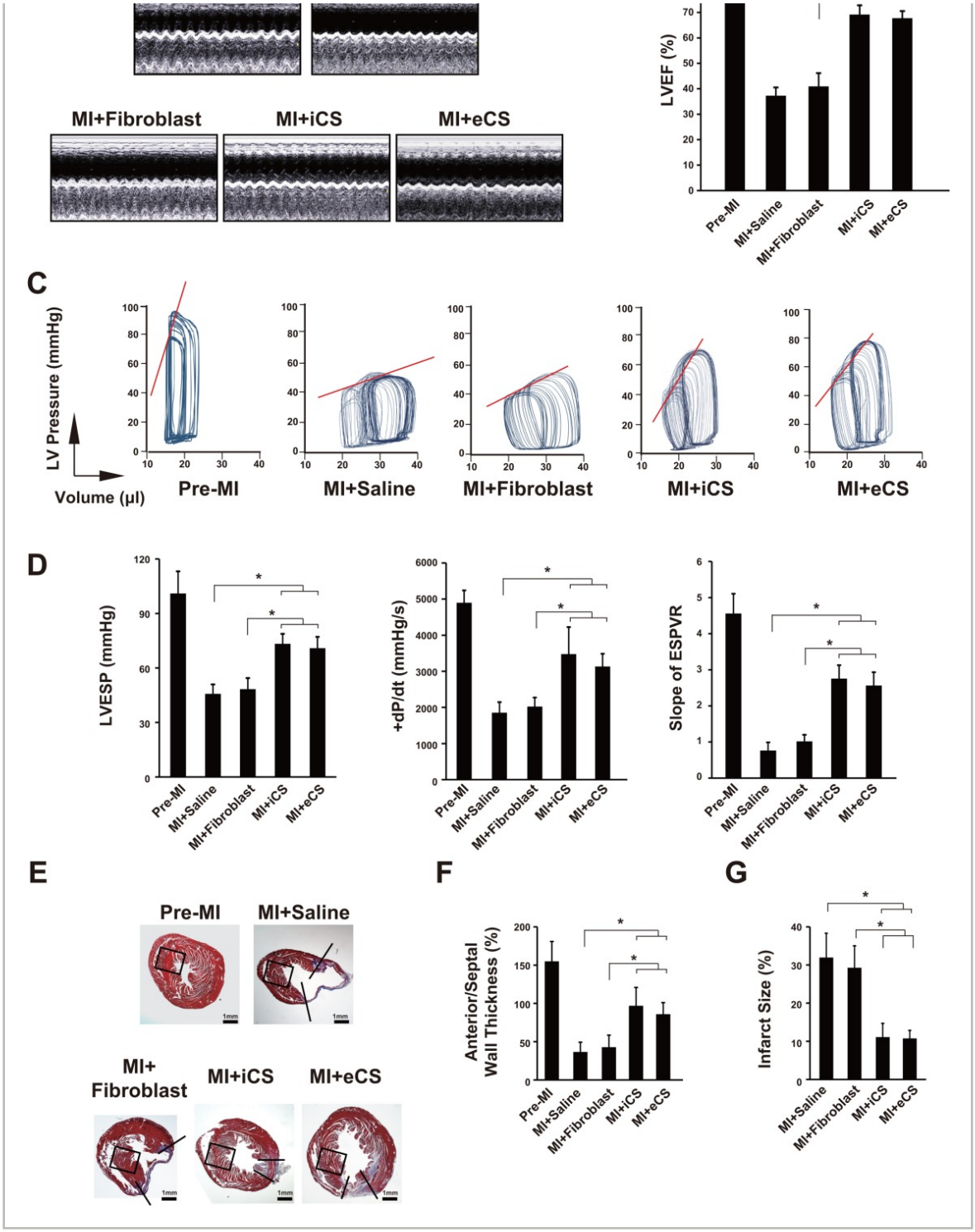


Figure 3

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Transplantation of iCS improves cardiac function as eCS following MI. (**A**):

12 weeks after transplantation. Nevertheless, the average radiant efficiency of DiR was significantly reduced at week 4 compared with 5 minutes after cell transplantation (week 0) and this was further reduced at week 12 (Fig. 4A, 4B).

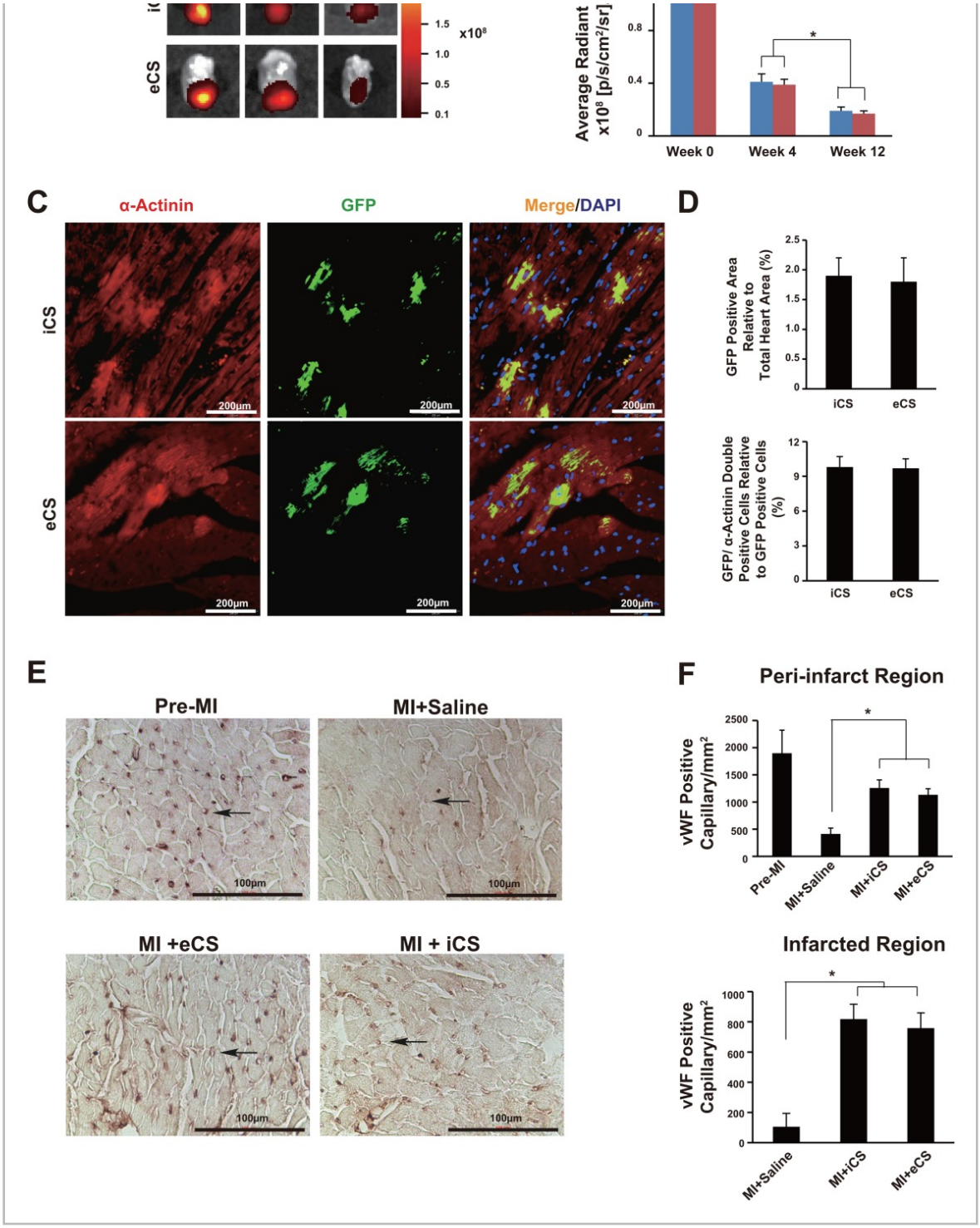

Figure 4

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Transplanted iCS participate in cardiac regeneration in vivo and promote

weeks after cell transplantation (Fig. 4C, 4D).

In addition, transplantation of both iCS and eCS significantly increased the capillary density a determined by vWF staining of the peri-infarct region and infarcted region compared with saline injection (Fig. 4E, 4F). In addition, double staining of GFP and vWF revealed potential endothelial cell differentiation of transplanted iCS or eCS (Supporting Information Fig. 4). In summary, iCS have similar therapeutic effects to eCS and improve cardiac function following MI. Of note, no tumor formation was observed 12 weeks following transplantation.

Induction of Cardiosphere Formation from Fibroblasts by BIO and OSM via β-Catenin

β-catenin is stabilized by the Gsk3β inhibitor-BIO and is critical for the maintenance of cardi progenitor cells 38, 39. Accordingly, we hypothesized that BIO and OSM induced cardiosphe formation via upregulation of β-catenin. Indeed, both BIO and OSM alone or in combination increased the protein level of β-catenin as well as the expression of Mesp1, Isl1, and Nkx2.5 when compared with basal medium without BIO and OSM (Fig. 5A). Knocking down β-cateni through shRNA decreased the protein level of Mesp1, Isl1, and Nkx2.5 compared with cells infected with scramble shRNA (Fig. 5B), and reduced cardiosphere formation (Fig. 5C). Nonetheless overexpressing β-catenin upregulated the protein level of Mesp1, Isl1, and Nkx compared with GFP (Fig. 5D), and promoted the formation of cardiospheres in the absence BIO and OSM (Fig. 5E). These findings confirm the critical role of β-catenin in the induction o cardiosphere formation from fibroblasts.

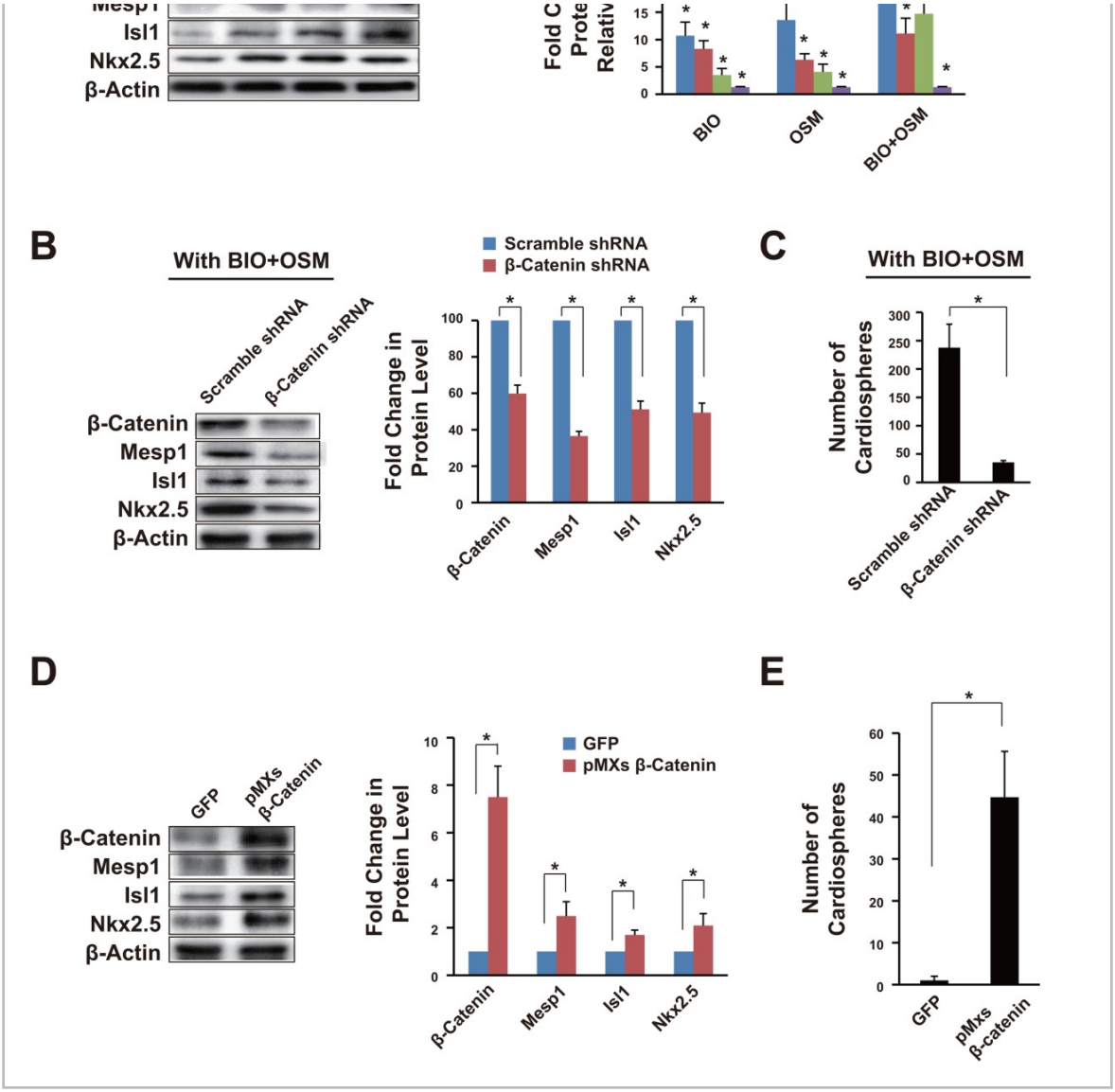

Figure 5

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The BIO and OSM induced cardiosphere (iCS) generation through β-catenin pathway in adult skin fibroblasts infected with SKO (Sox2, Klf4, and Oct4) treated with BIO or OSM or BIO and OSM. (**A**): Western blot of β-catenin, Mesp1, Isl1, and Nkx2.5 at day 15 after iCS formation. Representative immunoblot (left panel) and band density measurement (right panel) showed the β-catenin level was elevated by BIO and OSM stimulation, similar to Mesp1, Isl1, and Nkx2.5 ($n = 3$; $*p < 0.05$; groups with growth factors vs. basal group): Basal: medium without BIO and OSM. (**B**): Representative immunoblot

cardiac progenitor cell markers Mesp1, Isl1, and Nkx2.5 (Supporting Information Fig. 5B). Wh the differentiated cardiomyocytes were positive for α-Actinin, Mlc-2a and Mlc-2v (Supporting Information Fig. 5C), no spontaneously beating could be observed as previous reported in human eCS 8, 9. Nevertheless, those differentiated cardiomyocytes had functional calcium transient activity and responded to caffeine stimulation (Supporting Information Fig. 5D).

Discussion

Cardiac progenitor cells have been proposed as a novel therapy for the treatment of MI. Cardiospheres are a type of cardiac progenitor cell that is potentially safe and effective for cardiac regeneration following MI in both animal 40 and human 20 studies. Nevertheless, th widespread clinical application of autologous eCS is limited by the need for invasive harvesting, and their number, proliferation and differentiation ability decline during the agir process 17, 19, 40, 41. Direct reprogramming of fibroblasts into cardiomyocytes is another promising approach to achieve cardiac regeneration but is limited by the low conversion rat and the potential arrhythmia risk associated with cardiomyocyte transplantation. We preser new strategy to generate iCS from adult mouse skin fibroblasts using a cocktail approach wi expression of pluripotent stem cell factors and combination of Gsk3β inhibitor-BIO and OSM Furthermore, human iCS can be generated using similar approach. Our results suggest that these cardiotrophic growth factors induce cardiosphere formation via upregulation of β-catenin. These iCS are capable of differentiation into functional cardiomyocytes in vitro, fr the perspective of action potential and calcium transient, and incorporate into the myocardium in a MI model as eCS with subsequent cardiac function improvement. This stuc provides the first proof-of-principle results that demonstrate the feasibility of generating donor-specific iCS via a somatic reprogramming process, providing a new source of cell therapy for treatment of MI.

First, we used the expression of a panel of cardiac transcription factors including Mesp1, Isl1 and Nkx2.5 to screen for the optimal cocktail of cardiotrophic growth factors for iCS formati after reprogramming with pluripotent stem cell factors. These transcription factors have bee shown via lineage tracing studies to be important for heart development and maintenance c cardiac progenitor cells 13-15. Quantitative real-time PCR analysis showed that all these markers were upregulated during somatic reprogramming and were further enhanced after adding two more cardiotrophic factors, Gsk3β inhibitor-BIO and OSM. Sphere formation cou be observed after seeding the cells onto poly-D-lysine coated plates as described in the

accordance with previous reports 9, 19, 40.

The cardiomyocytes derived from the iCS generated from adult skin fibroblasts were comparable with those derived from eCS and mESC in terms of cellular electrophysiology, calcium handling, and contractile function. Moreover, transplantation of the iCS following M provided similar improvement to that of eCS in terms of cardiac function, reduction in infarc size and increased capillary density, consistent with previous findings in eCS 7.

Our results also provide novel insight into the mechanism of cardiosphere formation. β-catenin is an important regulator of cardiac progenitor cell fate maintenance, proliferatior and cardiomyocyte differentiation 42. It directly regulates isl1 expression 43 and loss of β-catenin decreases the cardiac progenitor population and leads to failure in heart development 44. It is well known that Gsk3β inhibitor-BIO is a stabilizer of β-catenin that is critical for heart development 38, 39. While the action of OSM on β-catenin is unknown, prio studies have shown that it promotes cardiomyocyte dedifferentiation and cardiac progenito cell proliferation in both MI and heart failure 45. Here, we demonstrated the pivotal role of β-catenin in the formation of iCS. Both BIO and OSM significantly increased the protein level β-catenin. Manipulating the expression of β-catenin during the formation of iCS significantly affected the efficiency of generation of iCS.

This study had several limitations. First, the use of retrovirus vector may limit the clinical application of this approach. Whether the use of other nonintegrated vectors or small molecules for reprogramming can provide similar efficacy remains unclear. Second, the iCS contains a mixed progenitor cell population as in the eCS. Therefore, the contribution of different types of progenitor cells in iCS to cardiac differentiation and regeneration needs to be further investigated. Third, the long-term safety and efficacy of iCS transplantation are unknown. Although we did not observe any tumor formation after 3 months, the proarrhythmic risk of iCS transplantation was not addressed in this study and their long-terr therapeutic efficacy is unclear. Finally, the low retention of transplanted cells hinders the potential applications, which is one of the most important issues should be addressed in the cell therapy field for MI.

Conclusion

iCS generated from adult skin fibroblasts by somatic reprogramming and a cocktail of Gsk3β inhibitor-BIO and OSM may represent a novel source for cell therapy in MI.

Author Contributions

J.-Y.X.: conception and design, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing; Y.-K.L.: manuscript writing; X.R.: administrative support, provision of study material; S.-Y.L.: data analysis and interpretation; J.Y.: administrative support; K.-W.A.: administrative support, provision of study material; W.-H.L.: administrative support, provision of study material; M.A.E.: manuscript writing; H.-F.T.: conception and design, data analysis and interpretation, manuscript writing, final approval c manuscript.

Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

Supporting Information

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