Spheroid Formation and Enhanced Cardiomyogenic Potential of Adipose-Derived Stem Cells Grown on Chitosan

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Abstract

Mesenchymal stem cells may differentiate into cardiomyocytes and participate in local tissue repair after heart injury. In the current study, rat adipose-derived adult stem cells (ASCs) grown on chitosan membranes were observed to form cell spheroids after 3 days. The cell seeding density and surface modification of chitosan with Arg-Gly-Asp–containing peptide had an influence on the sizes of ASC spheroids. In the absence of induction, these spheroids showed an increased level of cardiac marker gene expression (Gata4, Nkx2-5, Myh6, and Tnnt2) more than 20-fold versus cells on the tissue culture polystyrene (TCPS) dish. Induction by 5-azacytidine or p38 MAP kinase inhibitor (SB202190) did not further increase the cardiac marker gene expression of these spheroids. Moreover, the enhanced cardiomyogenic potential of the spheroids was highly associated with the chitosan substrates. When ASC spheroids were plated onto TCPS with either basal or cardiac induction medium for 9 days, the spheroids spread into a monolayer and the positive effect on cardiomyogenic marker gene expression disappeared. The possible role of calcium ion and the up-regulation of adhesion molecule P-selectin and chemokine receptor Cxcr4 were demonstrated in ASC spheroids. Applying these spheroids to the chronic myocardial infarction animal model showed better functional recovery versus single cells after 12 weeks. Taken together, this study suggested that the ASC spheroids on chitosan may form as a result of calcium ion signaling, and the transplantation of these spheroids may offer a simple method to enhance the efficiency of stem cell–based therapy in myocardial infarction.

Key words: biomaterials; cardiology; regeneration; stem cells; tissue engineering

Introduction

MYOCARDIAL INFARCTION (MI) IS ONE OF THE primary cardiovascular diseases which lead to cardiomyocyte ischemia, ventricular hypertrophy, heart failure, and death. Once MI results in cardiomyocyte ischemia, it is difficult to recover the damaged cardiomyocytes by self-repair. Several studies have successfully induced embryonic stem cells (ESCs) to differentiate into cardiomyocytes that express cardiac-specific genes, proteins, and ion channels and exhibit spontaneous contractile activity with the pacemaker-like action potential.[1] Cardiomyogenesis from ESCs is generally based on the classical embryonic body formation or co-culture with a mouse endodermal cell line (e.g., END-2).[2,3] Combining the p38 MAP kinase inhibitor may further enhance the cardiomyogenesis of human ESCs up to 2.5-fold higher than that of control.[4] However, the pluripotency of the undifferentiated portion of ESC-derived cells may lead to formation of teratomas.[5] Concerns regarding the homogeneity of ESC-derived cells remain to be resolved prior to their therapeutic applications in human.

Mesenchymal stem cells (MSCs) are multipotent cells with self-renewal property. They have multilineage differentiation capacity and may differentiate into osteocytes, chondrocytes, adipocytes, and myocytes.[6] Adipose-derived adult stem cells (ASCs) are considered to be more abundant and easier to

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harvest than the other types of adult MSCs. ASCs exhibit regular doubling time and low levels of senescence after culture for several months *in vitro* and can be derived into various types of cells in response to lineage-specific induction factors.[7] Treatment with 5-azacytidine (5-aza) may trigger ASCs to differentiate into cardiomyocytes by random demethylation of genomic DNA.[8] Furthermore, transplantation of ASCs was reported to regenerate different types of tissues under various conditions, such as liver after partial hepatectomy, brain after ischemia, as well as neoangiogenesis in hindlimb ischemia.[9–11]

Chitosan is primarily obtained from shellfish by deacetylation of chitin, which is the second most abundant natural polysaccharide next to cellulose.[12] Chitosan has been demonstrated as a biomimetic material and is extensively used in tissue engineering.[13] However, the use of chitosan is limited by its cell adhesion properties.[14] Some surface modifications have been demonstrated to improve cell seeding efficiency, such as modification by type I or II collagens.[15] Arg-Gly-Asp (RGD) is an adhesive recognition sequence that is present in the extracellular matrix.[16] RGD peptide conjugated with alginate has been shown to improve the proliferation and adhesion of human umbilical vein endothelial cells and promote the differentiation of MSCs for various applications.[17] A genetically engineered RGD-chimeric protein that contains the cellulose-binding domain (CBD-RGD) can promote the cell adhesion and proliferation when coated on various synthetic polymers without special cross-linking.[18]

In this study, chitosan membranes and those modified by CBD-RGD were prepared. The cardiomyogenic potential of rat ASCs was first evaluated *in vitro*. Specifically, we observed that ASCs in basal medium formed spheroids on these membranes after 3 days. These spheroids showed enhanced cardiomyogenesis either with or without the cardiac induction medium. The therapeutic potential of these spheroids was further demonstrated in the MI animal model.

Materials and Methods

Isolation and culture of ASCs

Fresh subcutaneous fat was obtained from the hind legs and side abdominal regions of Sprague-Dawley rats (body weight from 350 to 500 g). The adipose tissues were cut into several pieces and treated with 1 mg/mL type I collagenase (Sigma-Aldrich) in Hank's buffered salt solution at 37°C for 1 h with gentle agitation. Digested cells were collected by centrifugation at 500 g for 10 min. The cellular pellet was washed by phosphate-buffered saline (PBS), centrifuged at 500 g for 10 min and resuspended in PBS. The debris was removed through a 40-μm mesh filter (Falcon®, BD Biosciences). Cells were collected by centrifugation at 500 g for 10 min and then plated in T150 tissue culture flasks (Falcon, BD Biosciences) at approximately 3500 cells/cm^2 with low-glucose Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM-LG/F-12; 1:1; Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 20 mM HEPES (Gibco), 50 mg/mL bovine serum albumin fraction V (Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco) at 37°C with 5% CO$_2$. Once the adherent cells were more than 70% confluent, they were detached with 0.25% trypsin-EDTA and replated at a 1:3 dilution under the same culture conditions. All experiments were performed with cells of the fifth passage.

Flow cytometry

The surface markers of ASCs were examined by the flow cytometry. Cells were harvested by treatment with 0.25% trypsin-EDTA for 5 min at 37°C and washed twice with PBS. Cells were incubated with the relevant test antibodies or isotype controls at 4°C for 30 min. The following test antibodies were used. Fluorescein isothiocyanate (FITC)–conjugated CD29 (CD29-FITC), phycoerythrin (PE)-conjugated CD31 (CD31-PE), CD44-FITC, and CD90-PE were used for direct staining; mouse monoclonal anti-CD34 (Santa Cruz Biotechnology), mouse monoclonal anti-CD45 (Santa Cruz Biotechnology), mouse monoclonal anti-CD73 (BD Biosciences) and mouse monoclonal anti-CD105 (BD Biosciences) were used for indirect staining. The corresponding isotypic antibodies were mIgG1-FITC and mIgG1-PE (Serotec) for direct staining. Secondary antibodies used in this study were FITC-conjugated goat-anti-mouse antibody (Chemicon). The final concentration was the same for the isotype controls and test antibodies. A flow cytometer (BD FACSCalibur™ system) was used to assess the fluorescence intensities. Analysis was conducted using the WinMDI 2.9 software (The Scripps Research Institute, San Diego, CA, USA).

Preparation of chitosan membranes

Two kinds of chitosan were used in this study. One has a molecular weight of 416 kDa with a lower degree of deacetylation of 73% (CS1; Fluka); the other has a molecular weight of 170 kDa with a higher degree of deacetylation of 97% (CS2; Kiotec). The contact angle of CS1 and CS2 was measured by a static contact angle meter (First Ten Angstrom). The surface zeta potential was determined by electrophoretic light scattering using the Delsa Nano C Analyzer (Beckman Coulter) with a flat solid cell. The wet modulus was obtained by a dynamic mechanical analyzer (Q800, TA Instruments). The degree of deacetylation was confirmed by NMR spectroscopy (Bruker AVIII-500MHz FT-NMR).

Chitosan powder was dissolved and stirred at room temperature for 24 h in 1% aqueous acetic acid solution to obtain a 1% chitosan solution. Chitosan membranes were made from casting 1.5 mL of chitosan solution on 3-cm microscope coverslip glass (Assistant, Glaswarenfabrik Karl Hecht KG) and air-dried. The membranes were treated with 0.1 N NaOH for 10 min, washed extensively, and antiseptically rinsed with 70% ethanol before use. The CBD-RGD–modified chitosan membranes were prepared by coating 16 μg of CBD-RGD (in 200 μL of PBS) on each of the sterilized chitosan membranes before use.

Cell culture on chitosan membranes and cardiomyogenic induction

Chitosan membranes and those modified by CBD-RGD were placed in each well of a six-well tissue culture plate. To study the density effect on spheroid formation, ASCs were seeded in three different seeding densities (1.4×10^4, 2.8×10^4, or 5.6×10^4 cells/cm^2) and cultured in the basal medium. After seeding, the morphology of ASCs on the membranes was continually examined by an inverted microscope (Leica, DMIRB). To induce cardiac differentiation, cells were cultured on the membranes in the basal medium for 3 days and the medium was then replaced by the induction

medium, which contained $10\,\mu M$ of 5-aza or SB202190 (SB; Sigma-Aldrich) in the basal medium. After treatment with the induction medium for 3 days, the medium was replenished by the basal medium at 3-day intervals (twice). The total culture period was 3 days (basal medium) + 9 days (induction, basal, and basal medium). The expression of cardiomyogenic marker genes (Gata4, Nkx2-5, Tnnt2, and Myh6) was further analyzed by quantitative real-time PCR. To confirm the cell spheroid formation was a favorable stage during cardiomyogenesis, ASCs were cultured on chitosan membranes with the basal medium for the first 3 days and the spheroidal ASCs were detached from the well by shaking and then transferred onto tissue culture polystyrene (TCPS) dish with the basal or induction medium for another 9 days. The gene expression of Gata4, Nkx2-5, Tnnt2, and Myh6 was determined by quantitative real-time PCR.

Ion adsorption on the surface of chitosan membranes

Chitosan membranes were placed in each well of a 24-well tissue culture plate where 1 mL of DMEM-LG was added. After incubation at 37°C for 24 h, the medium was collected for later analysis of the free calcium remained in the bulk solution. The membrane surface was gently rinsed with PBS. One milliliter of EDTA solution (5 mM) was then added into each well and incubated at 37°C to elute the surface bound calcium. The EDTA solution was again collected after 24 h. A blank well (TCPS) was used as the control. The concentration of calcium in each of the collected solutions was measured by atomic absorption spectrometry (iCE 3300; Thermo Scientific). In the other groups, the chitosan membranes prior to or after EDTA treatment were examined by a scanning electron microscope (SEM, JEOL 6700) and the elements were analyzed by the energy dispersive spectrometer (EDS; INCAx-act LN2-free Analytical Silicon Drift Detector) equipped with the SEM.

Analysis of intracellular calcium and calcium-dependent cell surface molecules

ASCs (2.8×10^4 cells/cm^2) were seeded on TCPS or chitosan membranes (CS1 or CS2) in basal medium. After 24 h, the gene expression of two calcium-dependent cell surface molecules (Selp and Cxcr4) was analyzed by quantitative real-time PCR. The intracellular calcium in the ASCs was specifically stained by Calcium Green™-1, AM (Invitrogen) and washed with PBS. Then, the nuclear was stained by 4′,6-diamidino-2-phenylindole (Invitrogen) and rinsed with PBS. Visualization of the stained sections was achieved through a confocal microscope (BD Pathway 435). The monolayer and spheroids sections were analyzed at same exposure times. To eliminate the autofluorescence effect of ASC spheroids, these cells were trypsinized and washed by PBS twice (800 g, 5 min). The suspensions of single cells or ASC spheroids were then sonicated for 30 min and homogenized in PBS. The quantification of intracellular calcium fluorescence was achieved by a microreader (SpectraMax M5, Molecular Derives) at 506/531 nm and normalized by the respective total protein.

RNA extraction and quantitative real-time PCR analysis

Total RNA of cells at the end of the culture period was extracted by the Trizol® Reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesis and amplification via quantitative real-time PCR were performed using the RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas). Paired forward and reverse primers were designed from UniSTS database in National Center for Biotechnology Information. One hundred nanograms of cDNA was used for quantitative real-time PCR using the SYBR Green Master Mix (Finnzymes) with 150 nM targeted gene oligonucleotide primers: GATA binding protein 4 (Gata4), F: CAT GCT TGC AGT TGT GCT AG, R: ATT CTC TGC TAC GGC CAG TA; NK2 transcription factor related, locus 5 (Nkx2-5), F: ATC GCG GTG CCG GTG TT, R: GCC CGA ATT GCC CTG TG; cardiac troponin T type 2 (Tnnt2), F: CAA GGA ACA GAG CTT TGT CGA A, R: CAC AAC CTA GAG GCC GAG AAG T; cardiac α-myosin heavy chain 6 (Myh6), F: TAG TGA CCA GAG CTC GCT GAA C, R: CTG AGG CCA GCG ATA TAC ACA G; chemokine (C-X-C motif) receptor 4 (Cxcr4), F: TGA AAT CAA TCC ACC CTT GAA A, R: GAA CTG AAC GCT CCA GAA TGT G; P-selectin (Selp), F: GTG GAC AGG CGA CAT ACA ACA T, R: CAT CTG ACT GCA GAC TTT CCG T; β-actin, F: TCC TGC GTC TGG ACC TGG, R: CCA TCT CTT GCT CGA AGT. Forty cycles of PCR consisting of denaturing at 95°C for 10 sec (7 min in the first cycle) and annealing and extension for 30 sec at 62°C were performed by a Chrom4 Thermal Cycler System (MJ Research). Amplification of specific transcripts was further confirmed by the melting curve profile analysis. Primer efficiency was calculated[19] using the formula $[10^{(-1/slope)}]-1$. Threshold cycle (Ct) values were obtained and relative gene expression ratio was calculated[19] using the formula $(1 + \text{efficiency of target gene})^{\Delta \text{Ct of target } (\beta\text{-actin}-\text{target gene})} / (1 + \text{efficiency of } \beta\text{-actin})^{\Delta \text{Ct of } \beta\text{-actin } (\beta\text{-actin}-\text{target gene})}$. The value of each sample was normalized using the expression of the β-actin housekeeping gene in the same sample.

Cell transplantation and the heart functional assay

ASCs before transplantation were rinsed twice with PBS and stained by PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich) based on the manufacturer's protocol. The labeled ASCs were rinsed twice again with PBS. All surgical procedures and animal care followed ethical guidelines and were approved by the Animal Care and Use Committee of National Taiwan University. Male Sprague-Dawley rats (body weight, 300–450 g) were euthanized after functional measurements in deep anesthesia with chloral hydrate (intraperitonal), in compliance with the above guidelines. Rats were divided into four groups ($n=6$ for each group). In group I (sham-operated), rats received surgical procedures without ligation of the left anterior descending artery (LAD) or ASC treatment. In group II (PBS), rats received injection of PBS as the blank control. In group III (LAD ligation/spheroids), rats received injection of CS1-derived ASC spheroids (40–60 μm diameter) into the area of myocardial ischemia. In group IV (LAD ligation/single cells), rats received injection of ASCs (single cells) into the area of myocardial ischemia. By left lateral thoracotomy and pericardiectomy, the LAD was identified and ligated with silk suture while the heart was still beating. After LAD ligation for 2 weeks, rats were injected with PBS or PKH67-labeled spheroids (1×10^7 cells) or single cells (1×10^7 cells) on the area of myocardial ischemia. There were multiple

injection sites in the scar region and the total volume of injection was 200 μL.

During pre-LAD ligation, post-LAD ligation (2 weeks), and the end point (14 weeks) of the experiment, echocardiography was performed to evaluate the left ventricular internal end diastolic dimension (LVIDd), left ventricular internal end systolic dimension (LVIDs), ejection fraction (EF), and fractional shortening (FS) values. The iE33 xMATRIX Echocardiography System (PHILIPS) equipped with a 2–7 MHz transducer was employed and MI rats were analyzed by the short axis view approach.

To locate transplanted ASCs, frozen hearts were embedded in optimum cutting temperature compound and sectioned at 10-μm thickness. The heart slices were then fixed in 10% (v/v) buffered formalin and stained with hematoxylin and eosin (HE).

Statistical analysis

Numerical values were expressed as the mean ± standard deviation (SD). Differences between two groups were assessed by unpaired two tailed t-tests. Results involving more than two groups were assessed by an analysis of variance procedure. Duncan's new multiple-range test was used to evaluate differences among means (SAS Institute, Inc.). A significant difference was indicated at $p \leq 0.05$. In all studies, independent experiments were performed for each type of experiments.

Results

Characteristics of rat ASCs

The expression profile of cell surface proteins analyzed by flow cytometry is shown in Figure 1. The surface proteins were positive for CD29 (95%), CD44 (93%), CD73 (96%), CD90 (94%), and CD105 (91%) and were negative for CD31 (7%), CD34 (3%), and CD45 (8%).
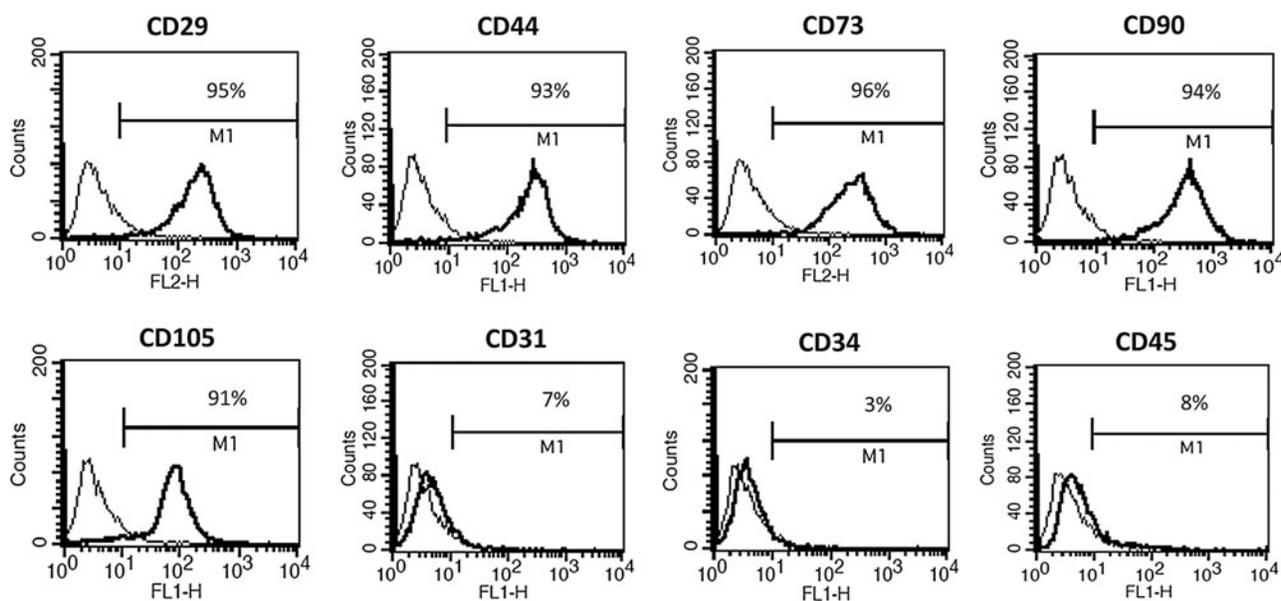
Properties of chitosan

The contact angle of CS1 and CS2 was in the range of $\sim 80°$–$90°$ for both. The surface zeta potential was close to neutral (both ~ 1 mV). The wet modulus was ~ 4 MPa for both types of chitosan. Thus, no statistically significant difference in these properties was observed between CS1 and CS2. They were very distinct differences in the degree of deacetylation (73% for CS1 vs. 97% for CS2), which was confirmed by NMR spectroscopy. TCPS had a contact angle of $\sim 65°$, surface zeta potential of approximately -20 mV (rather negative), and modulus of ~ 3–4 GPa (much stiffer).

The effect of cell seeding density on spheroid formation

The effect of seeding density on the spheroid formation of ASCs is shown in Figure 2. Cells grown on chitosan membranes gradually formed aggregates at day 1. The phenomenon of aggregation was more evident as the seeding density increased. No aggregation was found at the low seeding density of 1.4×10^4 cells/cm^2, as shown in Figure 2A. At day 2, the aggregates started to form spheroids gradually. Most spheroids remained attached on the membranes rather than suspended (Fig. 2B, 2E). After 2 days, the sizes of the spheroids on CS1 and CS2 membranes under the medium seeding density (2.8×10^4 cells/cm^2) were 52.3 ± 10.0 μm and 65.7 ± 13.2 μm, respectively. At the same time, the sizes were significantly greater in the case of high seeding density (5.6×10^4 cells/cm^2) and were 87.0 ± 21.7 μm and 106.2 ± 39.3 μm, respectively. After 5 days, spheroids became a little smaller in general with sizes 54.9 ± 12.4 μm (CS1) and 57.2 ± 19.0 μm (CS2) at the medium seeding density, and 64.8 ± 20.1 μm (CS1) and 88.6 ± 29.8 μm (CS2) at the high seeding density (Fig. 2C, 2E). No spheroid formation was observed when ASCs were seeded at the low density (1.4×10^4 cells/cm^2) even after long (12 days) of culture on either CS1 or CS2 (Fig. 2A–D). The sizes of spheroids became steady after 12 days and were 48.9 ± 11.3 μm (CS1) and 54.1 ± 14.9 μm (CS2) at the medium seeding density and

FIG. 1. Flow cytometric analysis of various surface markers in rat adipose-derived adult stem cells (ASCs). The results showed that cells were negative for CD31, CD34, and CD45 and positive for CD29, CD44, CD73, CD90, and CD105.

FIG. 2. The effect of cell seeding density on spheroid formation. ASCs at three different seeding densities (1.4×10^4 [14K], 2.8×10^4 [28K], and 5.6×10^4 [56K] cells/cm^2) were seeded on chitosan membranes (CS1 [Fluka] and CS2 [Kiotec]) and cultured in the basal medium. The phenomenon of cell aggregations was observed after 1 day **(A)**, 2 days **(B)**, 5 days **(C)**, and 12 days **(D)**. The scale bar represents 100 μm. The average size of spheroidal rat ASCs was calculated and is shown in **(E)**. The results were the means from five independent experiments with ASCs isolated from five different rats ($n = 5$). [abc]Means without a common letter differ significantly ($p \leq 0.05$).
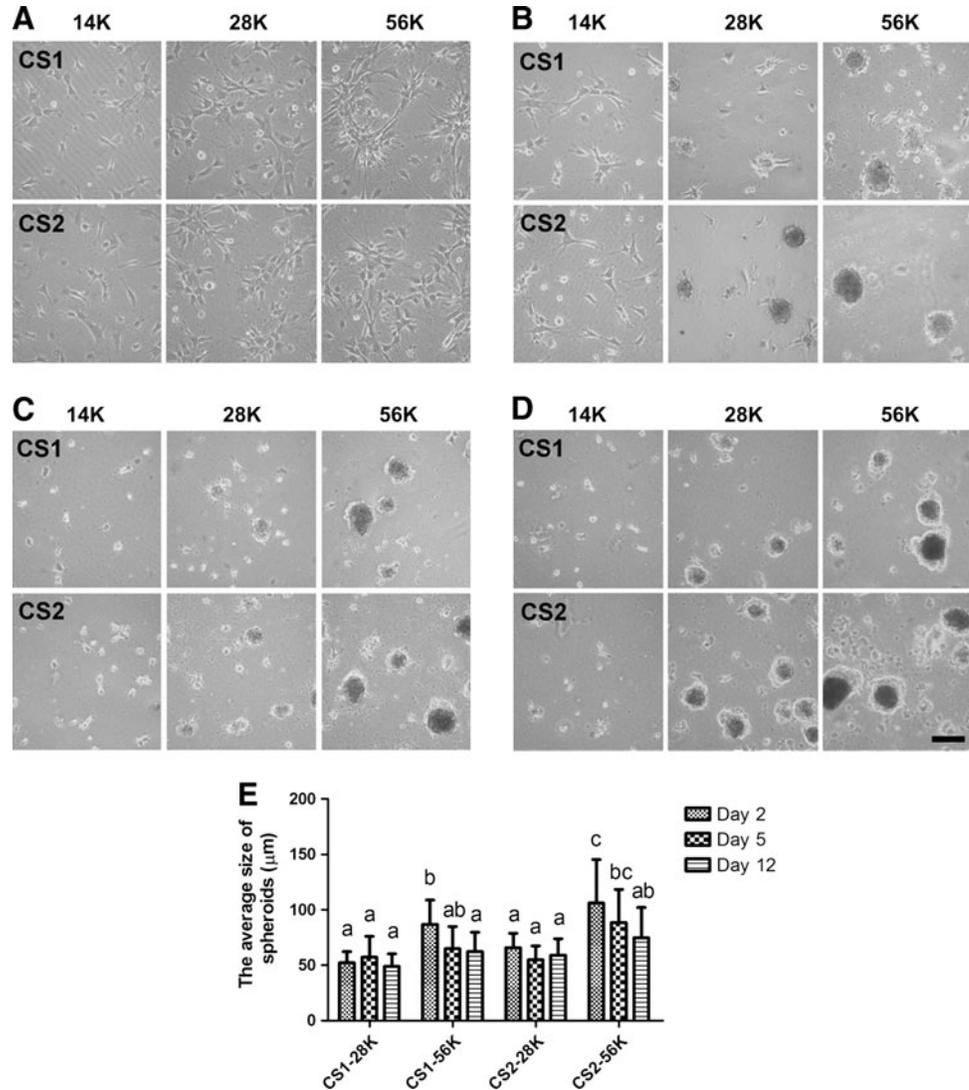
62.3 ± 17.4 μm (CS1) and 74.7 ± 27.4 μm (CS2) at the high seeding density (Fig. 2D, 2E). Statistical analysis revealed that CS2 had significantly larger spheroids than CS1 only at the beginning of the culture period (2 days) in the high density group. These findings indicated that the size of the spheroids was mostly dependent on the density of ASCs seeded on chitosan membranes.

The morphology of ASCs cultured on chitosan membranes

The morphology of ASCs (2.8×10^4 cells/cm^2) grown on TCPS, chitosan membranes, and CBD-RGD–modified chitosan membranes is shown in Figure 3. ASCs attached on TCPS had a fibroblast-like morphology at 24 h and became confluent after 3 days. ASCs cultured on chitosan membranes were poorly spread and did not show the fibroblast-like morphology at 24 h. As mentioned, they became aggregated and formed spheroids (50–100 μm). On the other hand, ASCs attached and spread on CBD-RGD–modified chitosan membranes and displayed fibroblast-like morphology at 24 h. They formed spheroids with a larger average size (175 ± 90 μm) than those on bare chitosan membranes after 3 days. Even after 12 days (either with or without 5-aza induction), the spheroids on CBD-RGD–modified chitosan mem-

branes remained larger. During the whole period, spheroid formation was not observed for cells on TCPS. These results indicated that chitosan membranes either with or without CBD-RGD modification could have prompted ASCs to form spheroids that remained to adhere on the membranes. Although the spheroids on chitosan could be detached by shaking the dish at 3 days when the pH of the culture medium became lower, the spheroids on CBD-RGD–modified chitosan membranes attached to the surface rather firmly.

Cardiac marker gene expression for ASC spheroids

The cardiomyogenic potential of ASC spheroids on chitosan membranes was evaluated by cardiac marker gene expression and the results are illustrated in Figure 4. After cell seeding for 3 days, ASCs were treated with (or without) 10 μM 5-aza or SB202190 to induce cardiomyogenesis. When cultured on TCPS, which is a traditional two-dimensional environment, the gene expression of cardiac transcript factors Gata4 (Fig. 4A) and Nkx2-5 (Fig. 4B) as well as that of cardiac structure proteins Tnnt2 (Fig. 4C) and Myh6 (Fig. 4D) was pretty low. Treatment with the induction medium (5-aza or SB202190) on TCPS increased the cardiac gene expression.
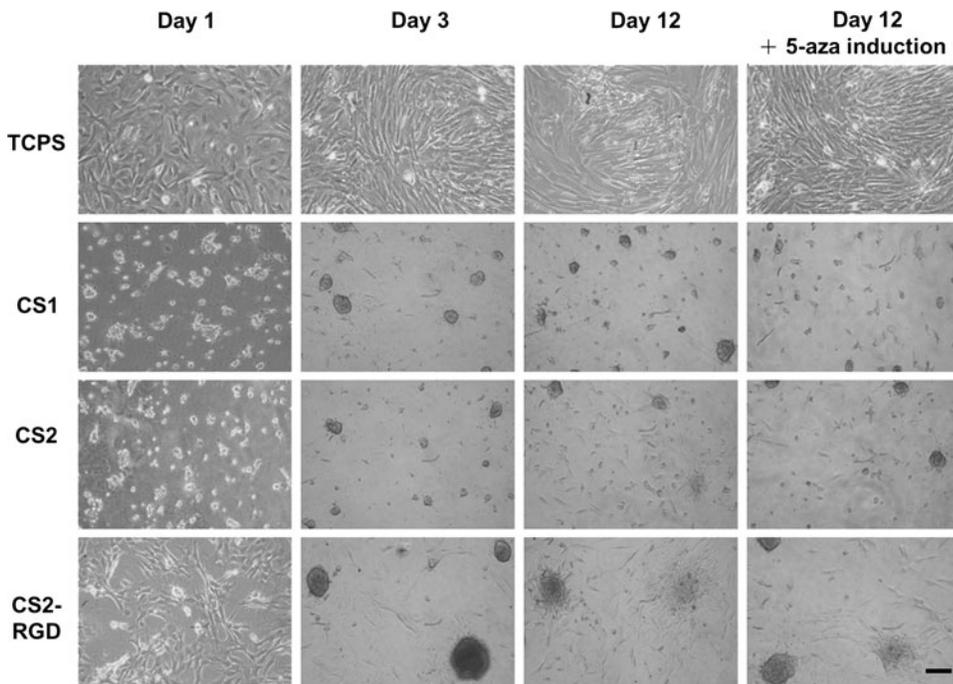
FIG. 3. The morphology of ASCs (2.8×10^4 cells/cm^2) cultured on chitosan membranes (CS1 and CS2), CBD-RGD–modified chitosan membranes (CS2-RGD), or tissue culture polystyrene (TCPS). Cells were cultured on the membranes in the basal medium for 3 days and the medium was replaced by the induction medium, which contained 10 μM 5-azacytidine (5-aza) in the basal medium to induce cardiac differentiation. After treatment with the induction medium for 3 days, the medium was replenished by basal medium at 3-day intervals (twice). The scale bar represents 100 μm. CBD, cellulose-binding domain; RGD, Arg-Gly-Asp.
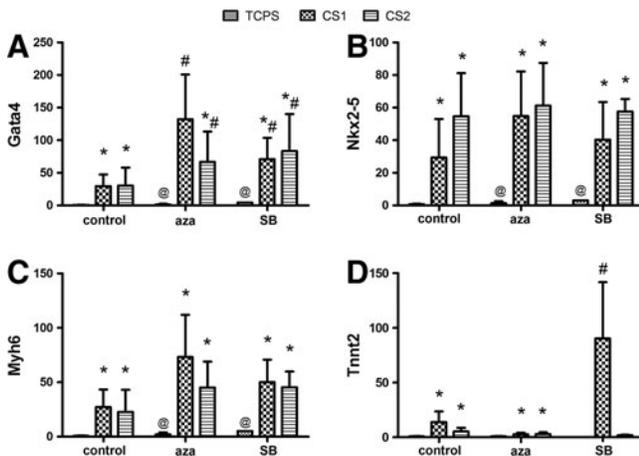

FIG. 4. Cardiac marker gene expression for ASC spheroids. ASCs (2.8×10^4 cells/cm^2) were seeded on chitosan (CS1 and CS2) membranes or TCPS. After the cells were cultured in the basal medium for 3 days, the medium was replaced by the induction medium, which contained 10 μM 5-aza or SB202190 (SB) in the basal medium to induce cardiac differentiation. After treatment with the induction medium for 3 days, the medium was replenished by basal medium at 3-day intervals (twice). After culture, the cellular mRNA concentration of Gata4 **(A)**, Nkx2-5 **(B)**, Myh6 **(C)**, and Tnnt2 **(D)** was analyzed by the real-time PCR and normalized to the β-actin mRNA concentration in the same sample. The term "control" indicates that cells were cultured in basal medium during the whole (12-day) period. "aza" and "SB" each indicate that cells were induced by 5-aza and SB for 3 days. The data were expressed as means±SD from five independent replicates ($n = 5$). Means without a common symbol differ significantly ($p \le 0.05$).

Even without the induction, culturing cells on both chitosan membranes could increase the cardiac gene expression more than 20-fold (Fig. 4A–D, control groups). Treatment with 5-aza or SB202190 did not further increase the cardiac gene expression of ASC spheroids cultured on chitosan membranes. These results suggested that chitosan may promote the differentiation of ASCs toward the myocardial lineage in the absence of the traditional cardiac induction. Since the spheroid formation of ASCs was the most distinct feature between the chitosan and TCPS groups, we speculated that three-dimensional (3D) cellular growth on chitosan membranes might be a key factor during the cardiomyogenesis of ASCs and conducted the following experiments.

The necessity of spheroid formation for enhanced cardiomyogenesis of ASCs on chitosan membranes

The spheroidal ASCs collected and replated were observed to re-attach onto the TCPS dish and spread out with the fibroblast-like morphology after 3 days (data not shown). However, the spheroidal ASCs without the support of chitosan membranes could not maintain the expression of cardiac genes (such as Gata4, Fig. 5A; Nkx2-5, Fig. 5B; Myh6, Fig. 5C; and Tnnt2, Fig. 5D) even with the supply of cardiac induction medium (SB9d groups). Compared to the original membranes, CBD-RGD modification did not further increase the expression of cardiac genes (Fig. 5). This result suggested that the CBD-RGD may not be a critical factor to influence the cardiomyogenic potential of ASCs. The spheroid formation was a necessary step for chitosan membranes to enhance the cardiomyogenesis of ASCs.

Surface bound calcium on chitosan, the intracellular calcium and up-regulation of Ca^{2+}-dependent molecules

The calcium deposition on chitosan after incubation with the medium was examined by SEM, and the respective EDS
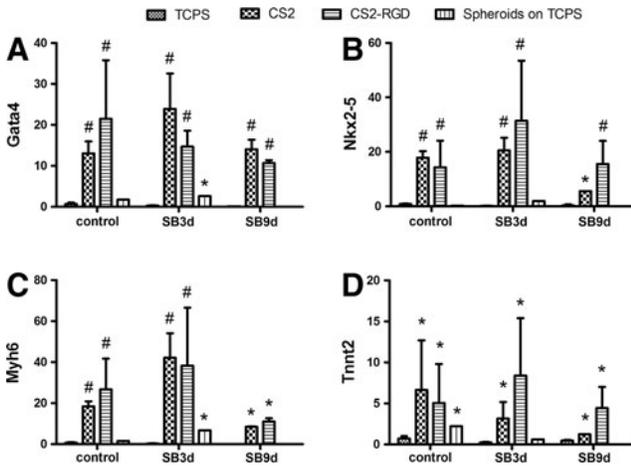
FIG. 5. The necessity of spheroid formation for cardiomyogenesis of ASCs on chitosan membranes. ASCs (2.8×10^4 cells/cm^2) were seeded on chitosan membranes (CS2), CBD-RGD–modified chitosan membranes (CS2-RGD), or TCPS. After the cells were cultured in the basal medium for 3 days, the medium was replaced by the induction medium, which contained 10 μM SB in the basal medium to induce cardiac differentiation. To confirm that spheroid formation of ASCs was a favorable stage for cardiomyogenesis, ASCs cultured on chitosan membranes with basal medium for the first 3 days were transferred onto TCPS dish with SB-induction medium for 3 days (followed by basal for 6 days) or with SB-induction medium for 9 days. The medium was refreshed at 3-day intervals. After culture, the cellular mRNA concentration of Gata4 **(A)**, Nkx2-5 **(B)**, Myh6 **(C)**, and Tnnt2 **(D)** was analyzed by real-time PCR and normalized to the β-actin mRNA concentration in the same sample. The term "control" indicates that cells were cultured in basal medium during the whole (12-day) period. "SB3d" and "SB9d" each indicate that cells were induced by SB for 3 days and 9 days. The data were expressed as means ± SD from five independent replicates ($n = 5$). Means without a common symbol differ significantly ($p \leq 0.05$).

data are shown in Figure 6A. Both CS1 and CS2 membranes had calcium deposited on the surface after incubation with the medium, of which the amount detected by SEM/EDS was 4.82% and 1.42%, respectively, after incubation with the medium. After EDTA treatment, no surface-bound calcium was detected by SEM/EDS. The concentration of the free (unbound) calcium remained in the bulk solution and that of the surface bound calcium in the EDTA eluted solution are shown in Figure 6B. Both data indicated that chitosan bound significantly more calcium than TCPS. Besides, there was no statistically significant difference in the calcium concentration (either free or bound) between CS1 and CS2. After EDTA elution for 24 h, some calcium may remain bound with chitosan (not fully eluted), judging from the larger sum of unbound and bound calcium for TCPS. Since no calcium on chitosan surface was detected by SEM/EDS after EDTA elution, calcium may be absorbed inside the membranes. The resistance to EDTA elution suggested the tightness of binding between calcium and chitosan. The influences of chitosan on the expression of Ca^{2+}-dependent cell adhesion molecule or surface receptor are further shown in Figure 6C. The gene expression level of P-selectin (Fig. 6C, Selp) was significantly higher for ASCs on chitosan versus

on TCPS. The gene expression of a chemokine receptor (Cxcr4) was also significantly up-regulated. This applied to cells grown on both types of chitosan. Because the homeostasis of calcium by chitosan may have been modified, we went further to determine the amount of intracellular calcium. With fluorescent calcium staining, the intracellular calcium of CS1- and CS2-derived ASC spheroids could be visualized by the confocal microscope as shown in Figure 6D. The ASC spheroids grown on chitosan membranes expressed stronger green fluorescence intensities than cells on TCPS. The quantitative data of intracellular calcium revealed that there was a significant increase in the intracellular calcium level for ASCs grown on chitosan membranes.

The effect of ASC spheroids evaluated by the MI rat model

A rat model of MI was performed to assess the cardiomyogenic potential of CS1-derived ASC spheroids (40- to 60-μm diameter, 1×10^7 cells) *in vivo*. The schematic diagram of ASC transplantation and the heart functional assay procedure are described in Figure 7A. Results from echocardiography showed that at 2 weeks after LAD ligation the rats exhibited significantly larger LVIDs and smaller EF and FS values (Fig. 7B–E), compared with the sham-operated group. At this time (2 weeks), PBS, regular ASCs, or CS1-derived ASC spheroids were injected in the ischemic area of the heart. At 12 weeks post transplantation (14 weeks after LAD ligation), the heart function was measured again. As shown in Figure 7B–E, the transplanted CS1-derived ASC spheroids, but not the regular ASCs (single cells), significantly improved LVIDd, LVIDs, EF, and FS values. Animals treated with regular ASCs showed equal or higher LVIDd and LVIDs values, as well as equal or lower EF and FS values at 14 weeks, compared with the PBS-injected group or the sham control. Transplantation of CS1-derived ASC spheroids led to better recovery from cardiac dysfunction than that of regular ASCs.

The engrafting of transplanted ASCs in MI heart was visualized by the merged HE-stained and fluorescent images shown in yellow in Figure 8. In the regular ASC single cells group (L/SC), the transplanted ASCs were mainly located in the periphery of blood vessels. The transplanted CS1-derived ASC spheroids (L/S) were widely distributed between cardiac muscle and pericardium. This observation suggested that the higher engrafting efficiency of CS1-derived ASC spheroids versus single cells in MI hearts may account for the better cardiac function recovery of the group receiving ASC spheroids. The transplanted single cells and spheroids both expressed high levels of tight junction protein Zo-1 (Supplementary Data). However, only the CS1-derived ASC spheroids appeared as clusters in MI heart.

Discussion

To prolong the life of MI patients, it is important to increase the regeneration rate of cardiomyocytes so the heart function can be improved. Human ESCs have been reported for their pluripotency to differentiate into early cardiomyocytes.[3] However, the plasticity of ESCs makes the condition difficult to control, and inefficient differentiation has been shown in most studies. Although a few animal studies were promising, the inadequate efficiency of cardiomyogenesis from ESCs still posed limitation to their clinical applications. Most studies employed a common strategy (i.e., formation of embryoid bodies in the
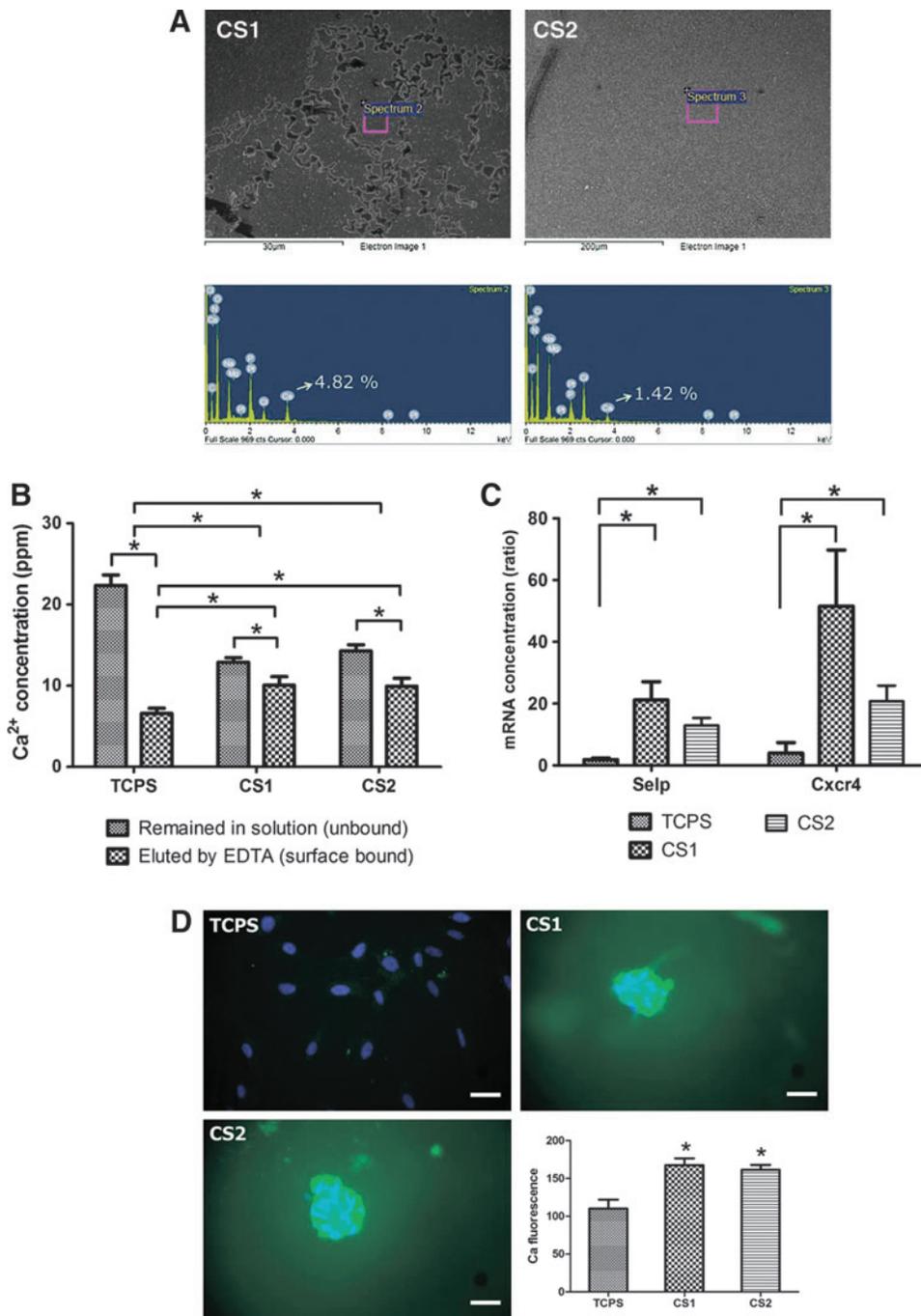
FIG. 6. The surface bound and intracellular calcium associated with the up-regulation of Ca^{2+}-dependent molecules. Scanning electron microscope/energy dispersive spectrometer (SEM/EDS) data **(A)** showed the presence of calcium on the surface of chitosan membranes after soaking in the medium for 24 h (*left:* CS1; *right:* CS2). Quantitative measurements **(B)** of the free (unbound) calcium remained in the bulk solution and the surface bound calcium in the EDTA eluted solution demonstrated the ability of chitosan to bind more calcium (vs. TCPS). The up-regulation of mRNA expression for Ca^{2+}-dependent adhesion molecule P-selectin (Selp) as well as the chemokine receptor Cxcr4 after 24 h was analyzed by the real-time PCR and normalized to that of the β-actin mRNA in the same sample **(C)**. The increase in the intracellular calcium was visible and quantified by green fluorescence **(D)**. The data were expressed as means $\pm$ SD from five independent replicates ($n = 5$). The scale bar represents 20 μm. *Significant difference, $p \leq 0.05$.

suspension culture) to differentiate human ESCs into the cardiac lineage, where they were able to cluster into 3D aggregates and differentiate into spontaneously contracting cardiomyocytes. A recent study indicated that the size of embryoid bodies may play an important role during cardiomyogenesis.[20] The addition of exogenous growth factors, such as Activin A and BMP4, further enhanced the embryoid body formation and differentiation of human ESCs into spontaneously beating cells.[21] On the other hand, reprogrammed somatic cells (induced pluripotent stem cells) also formed the embryoid bodies that differentiated into cardiomyocytes.[22,23] These results suggested the process of embryoid body formation may be an important process for cardiomyogenesis of human ESCs.

In the current study, chitosan membranes were found to induce the spheroid formation of rat ASCs, which mimicked the embryoid body formation in morphology. Recent research has indicated that ASC spheroids grown on chitosan may enhance the multilineage differentiation capacities including chondrogenesis, osteogenesis, neurogenesis, and hepatogenesis.[24,25] We thus hypothesized that chitosan might have the potential to induce cardiomyogenesis in rat ASCs. Indeed, we showed that the chitosan membranes could increase the cardiac marker gene expression in rat ASCs. Furthermore, the effect of cardiomyogenesis was strongly owing to the chitosan membranes. When the spheroidal ASCs were plated onto TCPS dish with basal or cardiac induction medium, the
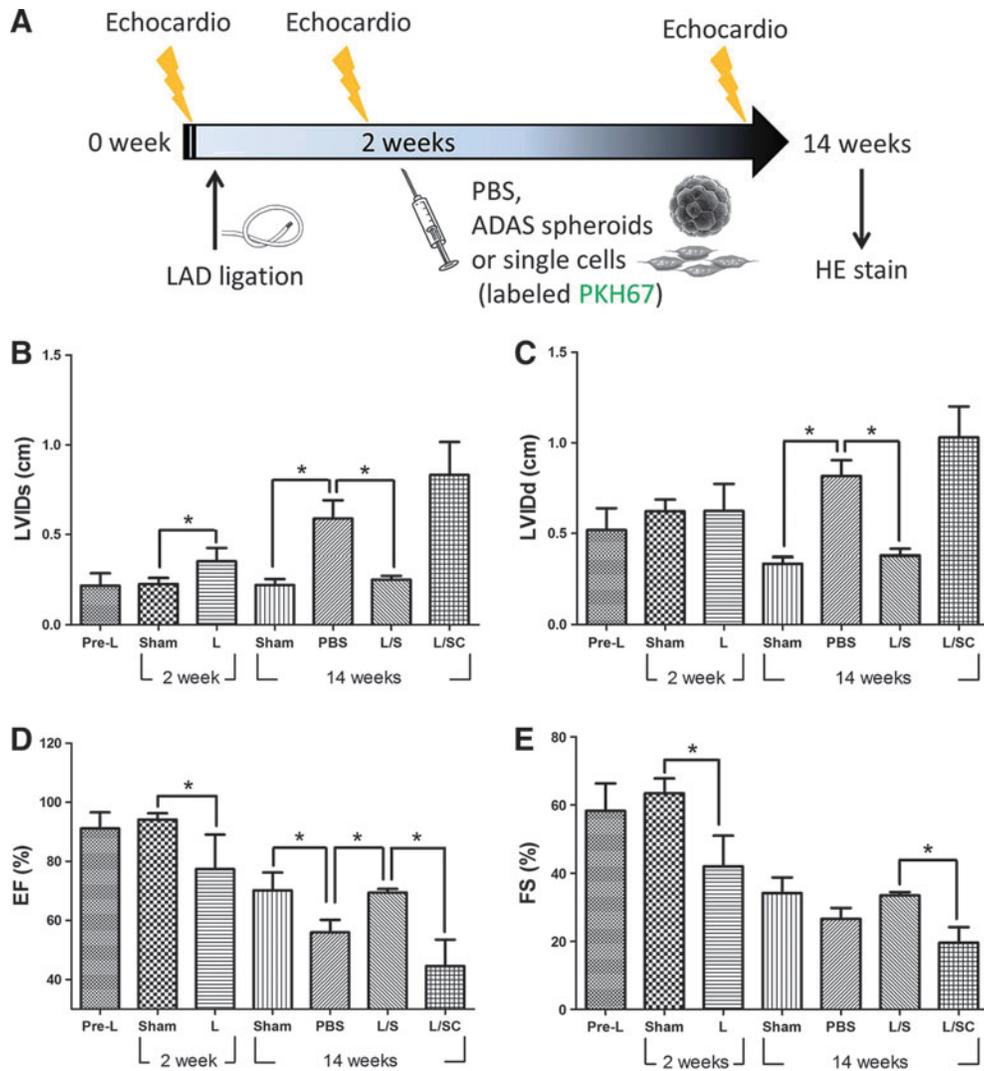
FIG. 7. The improvement of heart functions after transplantation of ASC spheroids. Schematic diagram of ASC transplantation and the heart functional assay procedures **(A)**. The LVIDs **(B)**, LVIDd **(C)**, EF **(D)**, and FS **(E)** values were assayed by echocardiography to evaluate the heart functions of rats in chronic myocardial infarction model during pre-LAD ligation, post-LAD ligation "2 weeks," and the end point "14 weeks." Rats were injected with PBS, CS1-derived ASC spheroids, or ASCs at 2 weeks post-LAD ligation. The data were expressed as means ± SD from four independent replicates ($n = 4$). *Significant difference, $p \leq 0.05$. LVIDs, LV internal dimension in systole; LVIDd, LV internal dimension-diastole; EF, ejection fraction; FS, fractional shortening; Pre-L, pre-LAD ligation; L, LAD ligation; L/S, LAD ligation/CS1-derived ASC spheroids; L/SC, LAD ligation/ASC single cells; HE, hematoxylin and eosin.

effect disappeared. These findings encouraged us to optimize the culture condition for ASCs during cardiomyogenesis. We found that the seeding density and surface modification with CBD-RGD peptide may affect the sizes of spheroidal ASCs. In addition, the spheroids on the CBD-RGD–modified surface attached more tightly, while the spheroids on chitosan may be detached and collected at 3 days possibly due to a pH decrease in medium that increased the hydration (nonadherence) of the chitosan. Nevertheless, the spheroid size (CS1, 52.3 ± 10.0 μm; CS2, 65.7 ± 13.2 μm after 2 days) did not show critical influence on the expression of cardiac genes. Induction by 5-aza or p38 MAP kinase inhibitor (SB202190) did not further stimulate the cardiomyogenesis of rat ASCs. These results suggested that the differentiation of rat ASCs into the cardiac lineage may require progression through a se-

ries of cardiomyogenic stages that remained largely unclear to us. We speculated that chitosan might promote ASCs entering a similar state as the embryoid body, which awaits an appropriate signal to take the next step into cardiomyogenesis.

Random demethylation of rat MSC by the treatment of 5-aza can trigger cardiomyogenesis *in vitro*, but the detailed mechanism is still unknown.[26] Recent studies suggested that 5-aza may induce demethylation of CpG islands in the promoter of the glycogen synthase kinase-3 and lead to the increased cardiomyogenesis of rat MSC via phosphorylation of β-catenin.[27,28] We found that 5-aza could increase the cardiac gene expression of rat ASCs cultured on TCPS dish in our study. However, 5-aza had no positive effect when the cardiomyogenesis of MSC was induced by co-culture with
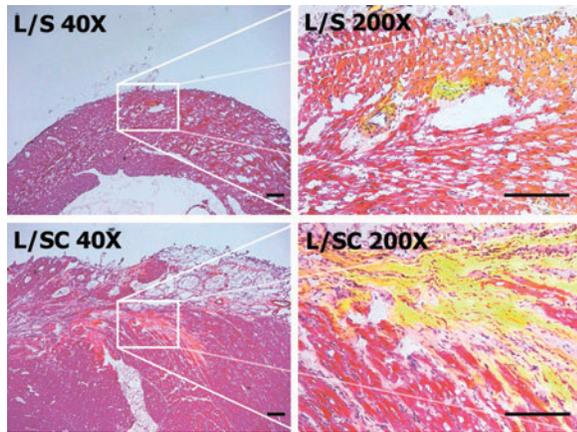
FIG. 8. Tracking of transplanted ASCs. Frozen hearts were sectioned and stained by HE. The merged images of cardiac cytoplasm (red color) and transplantable ASCs (PKH67 prelabeled cells, green fluorescence) were visualized in yellow. Magnifications (40×, 200×) are indicated. Scale bars represent 200 μm.

cardiomyocytes.[29] This was somewhat parallel to our observation that 5-aza had no further effect for rat ASCs cultured on chitosan membranes. It also indicated that 5-aza were not always effective for inducing the cardiomyogenesis of rat ASCs *in vitro*. p38 MAP kinase inhibitors were reported to enhance the cardiac mesoderm formation in human ESC.[30] In this study, we also showed that the cardiac gene expression of rat ASCs cultured on TCPS dish was increased by a p38 MAP kinase inhibitor, SB202190. Similar to the effect of 5-aza, the p38 MAP kinase inhibitor increased the phosphorylation of β-catenin through Wnt signaling pathway in HEK293 cells.[31] It was possible that Wnt/β-catenin signaling pathway might play an important role during the cardiomyogenesis of rat ASCs. However, the Wnt signaling pathway is one of the primary pathways that are directly involved in embryonic development. Both β-catenin–dependent and independent Wnt signaling pathways existed in cardiac and vascular development.[32] More work is required to verify the possible role of Wnt signaling in the cardiomyogenesis of rat ASCs.

Several extracellular matrix (ECM) molecules and their analogs have been shown to play an important role in manipulating cell differentiation and maturation. For example, mouse ESCs cultured on cardiac fibroblasts produced ECM that improved the cardiomyogenesis of ESCs into the functional beating cardiomyocytes.[33] A previous study has indicated that the injection of chitosan hydrogel into the infarcted rat heart improved the myocardial performance without the help from ESCs.[34] Our *in vitro* data further demonstrated that chitosan may be a suitable ECM for the cardiomyogenesis of rat ASCs to repair myocardial infarction in the future. Using RGD-modified ECMs to deliver MSC has been reported to improve heart function in MI rats.[35] In this study, we demonstrated that ASCs may form larger and more adhesive spheroids on RGD-modified chitosan membranes (Fig. 3). However, ASC spheroids of larger size did not further enhance the cardiomyogenic marker gene expression (Fig. 5). On the other hand, RGD may improve heart

function indirectly through angiogenesis. ASCs were also reported to increase the angiogenesis in 3D scaffolds.[36] Therefore, the *in vivo* efficacy of the combination of RGD, chitosan, and ASC spheroids is still worth future investigations.

Both types of chitosan as substrata promoted the formation of ASC spheroids in this study. The two types of chitosan differ in the degree of deacetylation, which actually did not lead to different results in cellular studies. Therefore, spheroid formation seemed to be favored by the common surface properties of the two chitosans such as the relatively neutral zeta potential and intermediate mechanical modulus (~MPa instead of GPa or kPa). Other than physical properties such as substrata stiffness, additional environmental factors brought by chitosan may come into play. There is not yet sufficient evidence to explain why chitosan could induce spheroid formation from individual cells of ASC. We speculated that the changes of ionic environment for ASCs by the presence of chitosan may play an important role in spheroid formation. Indeed, we showed that chitosan membranes may adsorb a few different ions, including Na^+, K^+, Cl^-, Ca^{2+}, and Mg^{2+}. In particular, the relative greater amounts of adsorption of calcium ion on chitosan versus TCPS as well as release by EDTA buffer were confirmed by atomic absorption spectrometry. A recent study showed that calcium ion bound the amino groups of chitosan and formed chitosan/calcium ions complexes ($CS-NH_2...Ca^{2+}$).[37] In addition, we found that the intracellular calcium was increased in ASC spheroids cultured on chitosan membranes. Hence, chitosan may trigger the expression of adhesion molecules in ASCs via a Ca^{2+}-dependent pathway and offer a 3D niche through formation of ASC spheroids. Selectins, a family of Ca^{2+}-dependent carbohydrate binding proteins, are the key adhesion molecules that mediate leukocyte trafficking, hemostasis, and stem cell homing.[38] In addition to endothelial cells and leukocytes, human cardiac fibroblasts were reported to express selectins (E-selectin) in response to proinflammatory cytokines.[39] The expression of coronary P-selectin was upregulated in a monkey heart ischemia reperfusion model.[40] These findings suggest that selectins may play a part in the therapeutic effect of ASCs. Other than selectins, the chemokine receptor CXCR4 is the key receptor responsible for stem cell homing. The expression of CXCR4 was shown to be restored in MSC aggregates formed by hanging drops and to regulate MSC adhesion to endothelial cells.[41] Calcium ion was a positive regulator of CXCR4 expression that promoted stem cell mobilization, homing, and therapy.[42] In this study, we found that the expression of P-selectin and Cxcr4 in rat ASCs was significantly up-regulated by chitosan membranes. The increased cell–cell adhesion and cell mobilization may account for the spheroid formation on chitosan. The preferential adsorption of Ca^{2+} on chitosan and the increase in the intracellular calcium also suggested that chitosan may promote the cardiomyogenic differentiation of rat ASCs by the regulation of adhesion molecules and Cxcr4 via Ca^{2+}-dependent pathways.

The mobilization of extra- and intracellular calcium has been recognized to play a critical role in regeneration after MI.[43,44] Transplantation of 3D aggregates of human amniotic-fluid stem cells formed by culture on microwelled hydrogel was reported to improve heart functions in immune-suppressed rats.[45] Our *in vitro* and *in vivo* data indicated that CS1-derived ASC spheroids dramatically

increased the intracellular calcium and recovered the heart functions in MI rats. Both ASC single cells and spheroids expressed high levels of Zo-1 protein in MI rats. The histology also suggested that ASC spheroids may recruit more MSC and differentiate into fibroblast-like cells to repair the MI heart. The low heart function in the sham control group may be caused by the possible synechia of thoracic cavity after two surgical procedures. Although 5-aza or SB202190 did not further induce cardiomyogenesis, the heart microenvironment may support CS1-derived ASC spheroids to direct a better regeneration effect than single cells. Nevertheless, the long recovery period for heart injury made it difficult to fully track the transplanted cells or spheroids. Our current study has proven the benefit of using CS1-derived ASC spheroids to treat MI rats, but the role of these spheroids in MI therapy remains unclear. Further investigations are needed to clarify the associated mechanisms.

Taken together, findings from this study suggest that the cardiomyogenic potential of ASCs may be enhanced by spheroid formation on chitosan membranes. The cell seeding density and surface modification of chitosan membranes with RGD influenced the sizes of ASC spheroids. Combination with the traditional induction medium, such as 5-aza or the p38 MAP kinase inhibitor, did not further increase the cardiomyogenic potential. The spheroids may form as a result of increased cell–cell adhesion via Ca^{2+} signaling on chitosan membranes, which further provides a 3D environment to support their differentiation toward the cardiac lineage. Injection of these spheroids in MI rats significantly improved the heart functions, suggesting the advantage of using spheroids formed on chitosan over single cells in repairing the MI heart.

Acknowledgment

Author Disclosure Statement

No competing financial interests exist.

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