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Porous nanofibrous poly(l-lactic acid) scaffolds supporting cardiovascular progenitor cells for cardiac tissue engineering

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Abstract

Myocardial infarction (MI) is the irreversible necrosis of heart with approximately 1.5 million cases every year in the United States. Tissue engineering offers a promising strategy for cardiac repair after MI. However, the optimal cell source for heart tissue regeneration and the ideal scaffolds to support cell survival, differentiation, and integration, remain to be developed. To address these issues, we developed the technology to induce cardiovascular progenitor cells (CPCs) derived from mouse embryonic stem cells (ESCs) towards desired cardiomyocytes as well as smooth muscle cells and endothelial cells. We fabricated extracellular matrix (ECM)-mimicking nanofibrous poly(l-lactic acid) (PLLA) scaffolds with porous structure of high interconnection for cardiac tissue formation. The CPCs were seeded into the scaffolds to engineer cardiac constructs in vitro. Fluorescence staining and RT-PCR assay showed that the scaffolds facilitated cell attachment, extension, and differentiation. Subcutaneous implantation of the cell/scaffold constructs in a nude mouse model showed that the scaffolds favorably supported survival of the grafted cells and their commitment to the three desired lineages in vivo. Thus, our study suggested that the porous nanofibrous PLLA scaffolds support cardiac tissue formation from

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Disclosure

There are no conflicts of interest.

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CPCs. The integration of CPCs with the nanofibrous PLLA scaffolds represents a promising tissue engineering strategy for cardiac repair.

Graphical abstract

Keywords

Embryonic stem cell; Cardiovascular progenitor cell; Cardiac differentiation; Extracellular matrix; Porous nanofibrous scaffold; Cardiac tissue engineering

1. Introduction

Myocardial infarction (MI) frequently leads to irreversible cell loss and scar formation, and ultimately to heart failure due to the inability of the myocardium to heal itself [1]. In patients with severe MI, heart transplantation is the only efficacious therapy [2]. However, there is a chronic shortage of human donor tissues, and there are rejection complications in recipients. Therefore, new technologies for heart tissue regeneration, for example, cell-based therapies, such as intracoronary and intramyocardial cell injections, have been explored to repopulate the damaged cardiac tissue with therapeutic cells [3]. Unfortunately, these approaches were largely hampered by the poor retention and rapid death of the administered cells after engraftment. Thus, there is an urgent need to develop novel strategies to improve the retention of therapeutic cells and promote their cardiac differentiation for regeneration. One such solution is to integrate cardiac stem cells and biodegradable scaffolds to generate functional heart tissue [4, 5].

As mature heart tissue is mainly composed of cardiomyocytes, smooth muscle cells, endothelial cells, and pacemaker cells [6], it is desired for the seeded cells to be multipotent towards these lineages for cardiac repair. Embryonic cardiovascular progenitor cells (CPCs), expressing *Mesp1*, *ISL1*, or *Nkx2-5*, have been identified during cardiogenesis [7–9]. These cells are multipotent and can differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells. The CPCs derived from embryonic stem cells (ESCs) have a similar capacity to differentiate into the three major lineages in vitro. The protocol to direct ESCs towards CPCs facilitates their subsequent differentiation for cardiac tissue regeneration and

ensures to a certain degree the safety to use these cells, preventing uncontrolled differentiation leading to teratoma formation [10]. It remains a challenge to differentiate CPCs down the main desired lineages to form integrated and functional cardiac tissue.

Biomaterials can assist cell survival, integration and communication with a proper microenvironment that closely mimics the native tissue architecture [11, 12], and therefore, might promote the proper differentiation and maturation of CPCs for cardiac tissue regeneration. Various types of scaffolds, including natural [13–18] and synthetic [19–22] biomaterials, have been explored to accommodate heart tissue cells. Natural biomaterials show good biocompatibility, but may lack ideal physical properties, degradation profiles, or consistency during production [23]. In contrast, synthetic scaffolds are advantageous in their pure chemical composition, good mechanical performance, controllable degradation rate, and can be tailored with significantly lower batch-to-batch variation [24]. However, these synthetic scaffold materials need to mimic certain advanced features of the natural extracellular matrix (ECM) to improve biological performance [25]. Nanoscale design can tailor biomaterials to closely mimic the ECM [26]. Porous scaffolds with nanotopographical features to imitate ECM in native tissues may benefit the interactions between seeded cells and scaffolds, new ECM formation, as well as the intercellular connections. The fibrous structure of tissue matrix is known to be critical to cellular functions [27–29]. Thus, our lab recently developed a phase-separation technique to fabricate nanofibrous (NF) poly(l-lactic acid) (PLLA) matrix, which enhanced aortic smooth muscle cell differentiation [30]. Furthermore, the NF PLLA scaffolds with improved macroporous structure were found to be beneficial for vascular tissue engineering [30–32]. Based on these findings, we hypothesized that the porous NF PLLA scaffolds would also facilitate the growth and cardiac differentiation of CPCs under three-dimensional (3D) conditions and promote cardiac regeneration.

In this study, we used the porous NF PLLA scaffolds to support CPC growth and differentiation in 3D for cardiac tissue formation. We derived CPCs from mouse ESCs, which can differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells. The scaffolds seeded with CPCs were evaluated to determine if they can facilitate cell attachment, extension, and differentiation in vitro. The cell/scaffold constructs were further assessed in a mouse subcutaneous implantation model to determine if the scaffolds favorably support survival of the grafted cells and their commitment to the three desired lineages in vivo.

2. Materials and Methods

2.1 Culture of mouse ESCs and cellular differentiation towards cardiomyocytes as well as smooth muscle cells and endothelial cells

Mouse ESCs were isolated from Strain 129 mouse (Charles River Laboratories, Wilmington, MA) embryos on day E3.5. The ESCs were cultured under feeder-free conditions with DMEM (Gibco, Life Technologies Corporation, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS, Gibco), 2 mM Glutamax-1 (Gibco), 0.1 mM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 450 μ M 1-thioglycerol (Sigma-Aldrich, St. Louis, MO), 0.5 μ M mitogen/extracellular signal-regulated kinase (MEK) inhibitor

PD0325901 (Stemgent, Cambridge, MA), 3 μ M glycogen synthase kinase (GSK) 3 inhibitor CHIR99021 (R&D Systems, Minneapolis, MN), and leukemia inhibitory factor (LIF). After expansion in vitro, the modified stage-specific differentiation protocol (Fig. 1) was followed to induce differentiation of mouse ESCs into CPCs, cardiac progenitor cells, and then further into cardiomyocytes associated with smooth muscle cells and endothelial cells [33]. In brief, mouse ESCs were dissociated and transferred into petri dishes in suspension at 75,000 cells/mL to induce embryoid body (EB) formation with the differentiation medium (IMDM:Ham's F12 (3:1, Gibco), supplemented with 0.05% BSA (Gibco), 2 mM Glutamax-1 (Gibco), 0.5 $\times$ B27 (Gibco), 0.5 $\times$ N2 (without retinoic acid, Gibco), 450 μ M 1-thioglycerol (Sigma-Aldrich), and 0.1 mM ascorbic acid (Sigma-Aldrich)). Two days later, the EBs were dissociated and the cells were cultured in suspension at 100,000 cells/mL in differentiation medium supplemented with 5 ng/mL VEGF (R&D Systems), 10 ng/mL Activin A (R&D Systems) and 0.8 ng/mL BMP4 (R&D Systems). One and a half days later, the newly formed aggregates were dissociated into single cells (CPCs) again to further generate cardiomyocytes associated with smooth muscle cells and endothelial cells in culture plates or in scaffolds.

To generate the desired cells in culture plates, CPCs were seeded into 6-well plates (pre-coated with 0.1% gelatin, Sigma-Aldrich) at 470,000/cm² and cultured with differentiation medium supplemented with 5 ng/mL VEGF (R&D Systems). After 1.5 and 7 days, the cells were harvested for the following analyses.

2.2 Immunofluorescence staining for the cell culture in plates

After 1.5 and 7 days, the cells cultured in 6-well plates were fixed with ice-cold 4% paraformaldehyde for 15 min, washed twice with PBS, and then permeabilized with 0.25% Triton X-100 (Sigma-Aldrich) for 10 min. After blocked with 3% BSA (Sigma-Aldrich) for 30 min, monoclonal mouse anti-ISL1 (1:200, DSHB, Iowa City, IA) and α -SMA (1:200, Abcam, Cambridge, MA), polyclonal rabbit anti-GATA4 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), SM-22 α (1:200, Abcam), and CD31 (1:100, Abcam), and polyclonal goat anti-cardiac troponin T (cTnT, 1:200, Santa Cruz Biotechnology) primary antibodies were applied at 4°C overnight, and the signals were visualized with the secondary antibodies of donkey anti-mouse IgG conjugated with Alexa Fluor 488 and 594 (Life Technologies Corporation), donkey anti-rabbit IgG conjugated with Alexa Fluor 488 and 594 (Life Technologies Corporation), and donkey anti-goat IgG conjugated with Alexa Fluor 488 (Life Technologies Corporation). Cellular nuclei were counter-stained with 1 μ g/mL Hoechst 33258 (Life Technologies Corporation). The fluorescence images were taken under an Olympus BX53 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

2.3 Flow cytometry assay

The two-dimensional (2D) culture in plates was dissociated into single cells with 0.25% trypsin, fixed with 4% paraformaldehyde at room temperature for 30 min, and washed twice with PBS. After permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 30 min and blocked with 0.4% BSA (Blocking solution, Sigma-Aldrich) for 30 min, the cells were mounted with primary antibodies of polyclonal goat anti-cTnT (1:200, Santa Cruz Biotechnology), and polyclonal rabbit anti-SMMHC (1:100, Abcam) and CD31 (1:100,

Abcam) in blocking solution at 4°C overnight. Then the cells were washed twice with PBS and incubated with blocking solution carrying FITC-conjugated donkey anti-rabbit and goat IgG secondary antibodies (Life Technologies Corporation) at room temperature for 1 h. After washed twice, the cells were resuspended in PBS and analyzed by flow cytometry (MoFlo Astrios, Beckman Coulter, Brea, CA). Cells treated the same way without antibody mounting were set as negative control.

2.4 Fabrication of porous NF PLLA scaffolds

The porous NF PLLA scaffolds were fabricated according to our previous report [34]. In brief, the PLLA (Boehringer Ingelheim, Ingelheim, Germany) with an inherent viscosity of approximately 1.6 dL/g was dissolved in tetrahydrofuran (10% w/v) at 60°C and cast into an assembled sugar template (composed of bound D-fructose spheres, 125–250 µm in diameter) under mild vacuum. The PLLA/D-fructose composites were phase-separated at –80°C overnight and then immersed into cyclohexane to exchange tetrahydrofuran for 2 days. The resulting composites were freeze-dried and the D-fructose spheres were leached out in distilled water to form the network with interconnected spherical pores. After freeze-drying again, the highly porous scaffolds were acquired and cut into circular discs with the dimension of 4 mm in diameter and 2 mm in thickness by a Uni-Punch Seamless Disposable Biopsy Punch (Premier Medical Products Company, Plymouth Meeting, PA). The macro-images of the scaffolds were taken under a Leica M165 FC stereo microscope (Leica Microsystems Inc., Buffalo Grove, IL).

2.5 Cell culture in porous NF PLLA scaffolds

For 3D construction, the porous NF PLLA scaffolds were pre-wetted in 70% ethanol for 30 min, and then the internal air bubbles were exhausted under mild vacuum. Each scaffold was seeded with 2×10^6 CPCs after being washed 3 times with PBS and 2 times with culture medium. In brief, we suspended 2×10^6 CPCs by 30 µL culture medium, and 15 µL cell suspension was dropped slowly into the scaffold from one side. The whole scaffold was confirmed to be saturated by the 15 µL cell suspension while seeing color change from white to red (culture medium contains phenol red). Then the scaffold was moved into 24-well culture plate and placed in tissue culture incubator at 37°C. One hour later, the scaffold was turned over and another 15 µL cell suspension was dropped slowly into the scaffold from the other side. After another 1 hour, 1 mL culture medium was gently added into the well to culture the construct. The cell/scaffold constructs were cultured with differentiation medium supplemented with 5 ng/mL VEGF (R&D Systems), which was changed twice a week.

2.6 Scanning electron microscopy (SEM) observation

The blank PLLA scaffolds were first observed under a stereo microscope as described above, and then sputter-coated with gold before SEM observation. After 1.5 and 7 days of culture, the cell/scaffold constructs were fixed with 2.5% phosphate-buffered glutaraldehyde (Sigma-Aldrich) overnight at 4°C, and post-fixed with 1% osmium tetroxide (Sigma-Aldrich) for 1 h. These samples were then rinsed 3 times with PBS, dehydrated through a graded series of ethanol and dried using hexamethyldisilazane (HMDS, Sigma-Aldrich) as described previously [35]. Processed cell/scaffold constructs were cut open from the middle. Samples were sputter-coated with gold and imaged at 10 kV with a Philips XL30 FEG

scanning electron microscope (Philips, The Netherlands) to detect the cell adhesion, spreading, and ECM formation inside the scaffolds.

2.7 Hematoxylin and Eosin (H&E) and immunofluorescence staining for the cell culture in scaffolds

The cell/scaffold constructs cultured in vitro were fixed with ice-cold 4% paraformaldehyde for 10 min and then placed in 5% sucrose-PBS for 1 h and in 15% sucrose-PBS for another 1 h. Specimens were embedded in CRYO-OCT Compound (Andwin Scientific, Tryon, NC) and frozen at -80°C . The 7- μm sections were acquired and immunofluorescence was stained as described above. Cellular F-actin was stained with Alexa Fluor 555-conjugated phalloidin (Life Technologies Corporation). To detect connexin 43 expression, the primary antibody of polyclonal rabbit anti-connexin 43 (1:200, Santa Cruz Biotechnology) was applied, which was further detected by a secondary antibody of donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (Life Technologies Corporation). Cell distribution and growth inside the scaffolds were also detected by H&E staining.

2.8 Gene expression analysis by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from cells (2D culture) or cell/scaffold constructs (3D culture) was extracted using Trizol reagent (Life Technologies Corporation). We used disposable plastic pestles (Fisher Scientific, Pittsburg, PA) to disrupt and smash the cell/scaffold constructs in Trizol for total RNA extraction. The concentration of RNA was determined from the optical absorbance at 260 nm of the extract. Complementary DNA (cDNA) was synthesized using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instruction. Polymerase chain reaction (PCR) was performed with Platinum Taq DNA Polymerase (Life Technologies Corporation) to evaluate the following genes: Mesp1, ISL1, cTnT, SMMHC and CD31. The β -actin was chosen as an internal control to normalize the signal from genes of interest. Sequences of primers, individual annealing temperature, and amplicon lengths were shown in Table 1. The PCR was performed in a T100 Thermal Cycler (Bio-Rad). The amplified products were separated in a 2.0% agarose gel and visualized with ethidium bromide (Sigma-Aldrich). Densitometry for the target bands normalized by β -actin was performed with the software ImageJ 1.48v (NIH).

2.9 Subcutaneous implantation

Seven days after culture in vitro, the subcutaneous implantation was performed in 6–8-week-old male athymic nude mice (Charles River Laboratories, Wilmington, MA) according to the protocol that was approved by the Use and Care of Laboratory Animals Committee at the University of Michigan. The surgery was performed under general inhalation anesthesia with isoflurane. One midsagittal incision was made on the dorsa and a subcutaneous pocket was created on each side of the incision via blunt dissection. The cell/scaffold constructs were implanted subcutaneously into each pocket. Two samples were implanted for each mouse. After implantation, the incisions were closed with surgery staples. The implantation of Cells Only and Scaffold Only was also performed as controls. Seven days post-surgery, the mice were euthanized with CO_2 asphyxiation followed by cervical dislocation, and the

implants were harvested for the following histological and immunohistochemical detection. Fig. 6 schematically showed the process from cell seeding to subcutaneous implantation.

2.10 Histological assays and tracking of the seeded cells after subcutaneous implantation

Seven days post-surgery, the implants were harvested and cryo-sectioned. Cell distribution and ECM formation were examined using H&E staining. For immunohistochemical staining, the polyclonal goat anti-cTnT (1:200, Santa Cruz Biotechnology), and polyclonal rabbit anti-SMMHC (1:100, Abcam) and CD31 (1:100, Abcam) primary antibodies were mounted that were further detected by the Anti-Goat and Rabbit HRP-AEC Cell & Tissue Staining Kits (R&D Systems). The slides mounted with goat or rabbit IgG Isotype Control (Abcam) instead of primary antibody were observed as controls.

To appraise how the seeded cells survived after in vivo implantation, the cells were labeled with green fluorescence using a PKH67 Fluorescent Cell Linker Kit (Sigma-Aldrich) before seeding them into the scaffolds. Seven days post-surgery, the implants were harvested and cryo-sectioned. The green fluorescence of the samples was observed under an Olympus BX53 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

3. Results

3.1 In vitro cardiac differentiation of CPCs derived from ESCs

Since embryonic CPCs can give rise to both cardiac muscle tissue and blood vessel system, we decided to integrate CPCs with our scaffolds for cardiac tissue engineering. First, we performed the 2D differentiation of CPCs in vitro as a reference for the 3D study in the scaffolds. The CPCs were acquired from mouse ESCs following a typical differentiation protocol (Fig. 1) [33]. Immunofluorescence staining showed that, under 2D culture in plates, after 1.5 days of induction supplemented with VEGF, the seeded CPCs positively expressed cardiac progenitor markers, such as ISL1 and GATA4 (Fig. 2A). Similar results were obtained from RT-PCR assay (Fig. 2B). The CPCs initially expressed *Mesp1*, an early CPC marker, which quickly diminished during cardiac differentiation (Fig. 2B). These cells also expressed ISL1, which peaked at day 1.5, and diminished rapidly after 7 days (Fig. 2B). At day 7 of cardiac induction, most of the cells differentiated into committed lineages with strong positive expression of cTnT (cardiomyocytes), SM-22 α and α -SMA (smooth muscle cells), and rather modest expression of CD31 (endothelial cells) (Fig. 2A). Consistent with fluorescence staining, RT-PCR (Fig. 2B) showed an increased expression of cTnT (cardiomyocyte marker) and SMMHC (smooth muscle cell marker), but very weak CD31 (endothelial cell marker) expression after 7 days of differentiation. We next performed flow cytometry to quantify the percentage of desired cell types (Fig. 2C). After 7 days of induction, approximately 21.0% of CPCs differentiated into cardiomyocytes, 48.0% into smooth muscle cells, and 2.69% into endothelial cells.

3.2 Nanotopographical and interconnected porous features of the NF PLLA scaffolds to support cell attachment and growth

To facilitate cell seeding, differentiation, and cardiac tissue formation, the supporting scaffolds need to have a homogeneous and interconnected porous structure with an optimal

range of pore diameters. Therefore, we fabricated the PLLA scaffolds according to a method developed in our laboratory (see methods for details). The resulted PLLA scaffolds possess a uniform porous structure and a high level of interconnectivity among the pores (Fig. 3). The walls of the pores consist of nanofibers with the average diameter between 100 and 200 nm, mimicking NF feature of natural collagen fibers in ECM [36]. These features supported CPC retention and intercellular connections 1.5 days after seeding, with the cells spreading and adhering onto the NF walls inside the scaffolds. Seven days later, the seeded cells looked more extended and produced more ECM along the NF walls inside the scaffolds (Fig. 3).

3.3 Cell growth and desired differentiation in the scaffolds

First, we determined if the scaffolds support the cell growth and intercellular connections. As shown in Fig. 4 (F-actin staining), 1.5 days after seeding, the cells displayed a spherical morphology inside the scaffolds. Seven days later, most of the cells stretched out and extended along the NF matrix walls. The cells also produced significantly more connexin 43, a gap junction protein in differentiated cardiomyocytes [37], at day 7 than day 1.5. As gap junctions are essential for many physiological processes, increased expression of connexin 43 with increasing 3D culture time indicated that these cells on the NF matrix were robust enough to communicate with one another for tissue construction [38, 39].

Then, we investigated the differentiation of the CPCs in the scaffolds. We detected a dramatic transition of the seeded cells in the scaffolds from cardiac progenitors to the three desired cell types (Fig. 5A). At day 1.5, the cells showed strong expression of CPC marker ISL1 as indicated by intense green fluorescence, which was almost undetectable after 7 days of induction. After 7 days of culture, the seeded cells were found growing evenly within the scaffolds (Fig. 5B), positively expressing cardiomyocyte marker (cTnT) and smooth muscle cell markers (SM-22 α and α -SMA). However, endothelial cell marker (CD31) expression was relatively weaker. The differentiation profile in 3D scaffolds was consistent with that in 2D culture plates. Moreover, RT-PCR assay showed that the differentiation trend in 3D scaffolds was similar to that in 2D culture plates (Fig. 5C). When the induction proceeded further, the seeded cells lost their progenitor features and differentiated into specific lineages. The semi-quantification data as shown in Fig. 5D clearly indicated the robust differentiation under 3D culture. Taken together, the above data indicated that the porous NF PLLA scaffolds favorably supported the cell extension, growth, and differentiation towards desired lineages in vitro.

3.4 Scaffolds supporting differentiated cells in vivo

To investigate whether the scaffolds could facilitate the survival and the desired commitment of the seeded cells in vivo, after 7 days of differentiation in vitro, we implanted the cells/scaffold constructs subcutaneously in a nude mouse model (Fig. 6). Immunohistochemistry (Fig. 7) showed that, after 7 days of subcutaneous implantation, the differentiated cells inside the constructs positively displayed markers of both cardiomyocytes (cTnT) and smooth muscle cells (SMMHC). Interestingly, in contrast to in vitro differentiation, more extensive CD31 expression was detected inside the scaffolds (indicated by black arrows) in the subcutaneous implantation model.

To further track the grafted cells and determine the possible invasion of host cells into the cell/scaffold constructs, we labeled the CPCs with green fluorescence before seeding them into the porous scaffolds. The green fluorescent dye (PKH67) has long aliphatic tails penetrating into lipid regions of the cell membrane. Due to the long aliphatic carbon tails, PKH67 exhibits reduced cell-cell transfer. Seven days after subcutaneous implantation, H&E staining (Fig. 8A) showed that the cells were distributed evenly inside the scaffolds. Many cells within the pores showed strong green fluorescence (Fig. 8B) indicating that they were derived from the seeded CPCs. Non-fluorescent cells were derived from host tissue. Based on these results, we expect a robust integration of the engineered constructs with host tissue when transplanted into injured hearts. Collectively, our study revealed that the porous NF scaffolds supported the survival and cardiac commitment of the grafted cells, as well as their integration with the host tissue in vivo.

4. Discussion

MI leads to large amount of cardiac cell death. Sufficient cells of cardiac phenotype are needed to restore the damaged tissue. Lack of appropriate cell source impedes cardiac tissue regeneration. ESC-derived CPCs, instead of cardiomyocytes or mixed terminal cardiac lineage cells, were used here for cardiac tissue formation. Since heart tissue contains cardiomyocytes, smooth muscle cells, and endothelial cells [40, 41], it would be advantageous to use one type of stem cells that can derive all of the three desired lineages. As has been reported, the ISL1-positive cardiac progenitor cells are multipotent, capable of forming cardiomyocytes, smooth muscle cells, and endothelial cells, which are needed for cardiac tissue regeneration [42]. Since CPCs are an earlier cell type than cardiac progenitor cells, we aimed to develop the technology that facilitates CPC differentiation towards the three desired lineages in this study. Under 2D culture with the optimized multi-induction protocol, we observed positive differentiation of the seeded CPCs towards both cardiomyocytes (21.0%) and smooth muscle cells (48.0%). We noticed that the differentiation of these CPCs into endothelial cells was relatively poor in vitro (2.69%). We hypothesized that the in vivo environment might facilitate the angiogenesis in the cell/scaffold constructs possibly through host tissue invasion as well as the interactions between the grafted cells and the host cells. The results of our in vivo study appeared to support this hypothesis.

The other challenge in the construction of cardiac tissue is to develop suitable scaffold to support cardiac cell attachment, differentiation, and maturation. As has been reported, the natural nano-structured ECM play important roles in regulating essential cell and tissue structure and function [43]. Successful engineering of bone [44], cartilage [43, 45], bladder tissue [46], and blood vessels [32] manifested the significance of emulating natural ECM to recapitulate a unique microenvironment that facilitates tissue regeneration using nano-structured scaffolds. While decellularized rat hearts preserving the underlying ECM was reported to support cardiac tissue regeneration [47], such matrix may raise concerns about immunological rejection and disease transmission. Therefore, in this study, we aimed to fabricate ECM-mimicking scaffolds with nanotopographical feature for cardiac repair. Since collagen is one of the key structural components in cardiac ECM [48, 49], we fabricated PLLA scaffolds composed of nanofibers with the average diameter between 100 and 200 nm

to mimic NF feature of natural collagen fibers in ECM [36]. Besides mechanical support, it was reported that the ECM-mimicking structure might also provide instructive cues to the developing tissue [50]. As confirmed by our SEM assay (Fig. 3), the NF network favorably supported cell attachment at early time point and integrated well with natural ECM generated by seeded cells at later time points. The abundant ECM deposition on the scaffolds may facilitate further cell differentiation and tissue regeneration [35].

Another determinant of the NF PLLA scaffolds for cardiac tissue engineering is the porous structure. Electrospinning technique has been explored using natural and synthesized materials to prepare scaffolds of nano-structure for cardiac tissue regeneration [51–54]. However, it's hard to fabricate 3D scaffolds with well-defined pore structure using the electrospinning technique. We developed the technique to fabricate scaffolds of an open porous structure with highly interconnected pore networks that would facilitate nutrient and oxygen diffusion, waste removal, cell proliferation and migration, cell communication and reorganization, as well as host tissue invasion [55]. During 3D culture with cardiac induction *in vitro*, we found much connexin 43 expression in the scaffolds (Fig. 4), illustrating improved intercellular coupling and communications, as well as initiating maturation of cardiac differentiation.

In addition, the mouse subcutaneous implantation model showed the desired commitment of the grafted cells as well as the interactions between the cell/scaffold constructs and the host tissue *in vivo*. Increased angiogenesis in the constructs was detected after 7 days of subcutaneous implantation. Our study also revealed that some of the grafted cells in the Cells Only group expressed endothelial cell marker *in vivo*, and the host tissue invaded the blank scaffold in the Scaffold Only group but didn't show clear angiogenesis in the scaffold (Fig. 7). Thus, the host tissue invasion and the interactions between the grafted cells and the host cells likely facilitated the angiogenesis in the constructs *in vivo*, probably via mechanisms involving paracrine secretion of growth factors and cytokines [56]. Further studies are needed to confirm and elucidate the underlying mechanisms. Moreover, our *in vivo* study showed that the porous scaffolds protected the seeded cells well. A large number of the fluorescence-labeled cells survived after the subcutaneous implantation and appeared to integrate and communicate with one another inside the pores for tissue formation. In addition, the open porous structure of the scaffolds should have facilitated the nutrient supply from host tissue to support the constructs *in vivo*. Thus, the porous NF PLLA scaffolds associated with CPCs warrant a promising prospect to engineer cardiac tissue *in vitro* and a favorable integration with the surrounding host tissue *in vivo*.

While our short-term study showed advantages of the porous NF PLLA scaffolds to facilitate engineering of the cardiac constructs with CPCs *in vitro* and support the desired differentiation and commitment of the seeded CPCs as well as their interactions with the host tissue *in vivo*, further long-term implantation studies are needed to confirm whether longer-term engineering *in vitro* may lead to any better construction of functional cardiac tissue. Furthermore, optimization of the degradation rate of the scaffolds may facilitate the intercellular connections to promote the organization of tissue construction. We are adjusting the molecular size of the PLLA to have its degradation rate match the regeneration of cardiac tissue with the seeded cells and the integration of the constructs with the host

tissue. Moreover, electrical [56, 57] and mechanical [58, 59] stimuli have been reported to facilitate cardiac differentiation and cardiac tissue organization. Thus, both rhythmic electrical and mechanical stimuli will be utilized in the construction of cardiac tissue in our future studies.

5. Conclusions

We developed the porous NF PLLA scaffolds to mimic the NF feature of natural ECM beneficial for the construction of cardiac tissue with CPCs. The scaffolds facilitated cell attachment, extension, and differentiation in vitro, and favorably supported large number of living cells expressing the key cardiomyocyte, smooth muscle cell, and endothelial cell marker proteins in a mouse subcutaneous implantation model. Our study made a significant advance in cardiac regeneration, and indicated a promising future to use the porous NF scaffolds and CPCs derived from patient iPSCs for patient-specific cardiac repair.

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References

1. Shah RV, Holmes D, Anderson M, Wang TY, Kontos MC, Wiviott SD, et al. Risk of heart failure complication during hospitalization for acute myocardial infarction in a contemporary population: insights from the National Cardiovascular Data ACTION Registry. *Circulation Heart failure*. 2012; 5:693–702. [PubMed: 23051950]
2. Takeuchi R, Kuruma Y, Sekine H, Dobashi I, Yamato M, Umezumi M, et al. In vivo vascularization of cell sheets provided better long-term tissue survival than injection of cell suspension. *Journal of tissue engineering and regenerative medicine*. 2014
3. Shevach M, Soffer-Tsur N, Fleischer S, Shapira A, Dvir T. Fabrication of omentum-based matrix for engineering vascularized cardiac tissues. *Biofabrication*. 2014; 6:024101. [PubMed: 24464690]
4. Shevach M, Fleischer S, Shapira A, Dvir T. Gold nanoparticle-decellularized matrix hybrids for cardiac tissue engineering. *Nano letters*. 2014; 14:5792–6. [PubMed: 25176294]
5. Wang H, Shi J, Wang Y, Yin Y, Wang L, Liu J, et al. Promotion of cardiac differentiation of brown adipose derived stem cells by chitosan hydrogel for repair after myocardial infarction. *Biomaterials*. 2014; 35:3986–98. [PubMed: 24508080]
6. Martin-Puig S, Wang Z, Chien KR. Lives of a heart cell: tracing the origins of cardiac progenitors. *Cell stem cell*. 2008; 2:320–31. [PubMed: 18397752]
7. Bondué A, Blanpain C. Mesp1: a key regulator of cardiovascular lineage commitment. *Circulation research*. 2010; 107:1414–27. [PubMed: 21148448]
8. Bondué A, Lapouge G, Paulissen C, Semeraro C, Iacovino M, Kyba M, et al. Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell stem cell*. 2008; 3:69–84. [PubMed: 18593560]
9. Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, et al. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell*. 2006; 127:1151–65. [PubMed: 17123592]
10. Cao F, van der Bogt KE, Sadrzadeh A, Xie X, Sheikh AY, Wang H, et al. Spatial and temporal kinetics of teratoma formation from murine embryonic stem cell transplantation. *Stem cells and development*. 2007; 16:883–91. [PubMed: 17896868]

11. Kuraitis D, Giordano C, Ruel M, Musaro A, Suuronen EJ. Exploiting extracellular matrix-stem cell interactions: a review of natural materials for therapeutic muscle regeneration. *Biomaterials*. 2012; 33:428–43. [PubMed: 22014942]
12. Liu J, Zhang Z, Liu Y, Guo C, Gong Y, Yang S, et al. Generation, characterization, and potential therapeutic applications of cardiomyocytes from various stem cells. *Stem cells and development*. 2012; 21:2095–110. [PubMed: 22428725]
13. Christman KL, Fok HH, Sievers RE, Fang Q, Lee RJ. Fibrin glue alone and skeletal myoblasts in a fibrin scaffold preserve cardiac function after myocardial infarction. *Tissue engineering*. 2004; 10:403–9. [PubMed: 15165457]
14. Serpooshan V, Zhao M, Metzler SA, Wei K, Shah PB, Wang A, et al. The effect of bioengineered acellular collagen patch on cardiac remodeling and ventricular function post myocardial infarction. *Biomaterials*. 2013; 34:9048–55. [PubMed: 23992980]
15. Miyagi Y, Chiu LL, Cimini M, Weisel RD, Radisic M, Li RK. Biodegradable collagen patch with covalently immobilized VEGF for myocardial repair. *Biomaterials*. 2011; 32:1280–90. [PubMed: 21035179]
16. Kouris NA, Squirrell JM, Jung JP, Pehlke CA, Hacker T, Eliceiri KW, et al. A nondenatured, noncrosslinked collagen matrix to deliver stem cells to the heart. *Regenerative medicine*. 2011; 6:569–82. [PubMed: 21916593]
17. Liu Z, Wang H, Wang Y, Lin Q, Yao A, Cao F, et al. The influence of chitosan hydrogel on stem cell engraftment, survival and homing in the ischemic myocardial microenvironment. *Biomaterials*. 2012; 33:3093–106. [PubMed: 22265788]
18. Rajabi-Zeleti S, Jalili-Firoozinezhad S, Azarnia M, Khayyat F, Vahdat S, Nikeghbalian S, et al. The behavior of cardiac progenitor cells on macroporous pericardium-derived scaffolds. *Biomaterials*. 2014; 35:970–82. [PubMed: 24183165]
19. Chen QZ, Ishii H, Thouas GA, Lyon AR, Wright JS, Blaker JJ, et al. An elastomeric patch derived from poly(glycerol sebacate) for delivery of embryonic stem cells to the heart. *Biomaterials*. 2010; 31:3885–93. [PubMed: 20153041]
20. Wickham AM, Islam MM, Mondal D, Phopase J, Sadhu V, Tamas E, et al. Polycaprolactone-thiophene-conjugated carbon nanotube meshes as scaffolds for cardiac progenitor cells. *Journal of biomedical materials research Part B, Applied biomaterials*. 2014; 102:1553–61.
21. Qazi TH, Rai R, Dippold D, Roether JE, Schubert DW, Rosellini E, et al. Development and characterization of novel electrically conductive PANI-PGS composites for cardiac tissue engineering applications. *Acta biomaterialia*. 2014; 10:2434–45. [PubMed: 24561709]
22. Marsano A, Maidhof R, Luo J, Fujikara K, Konofagou EE, Banfi A, et al. The effect of controlled expression of VEGF by transduced myoblasts in a cardiac patch on vascularization in a mouse model of myocardial infarction. *Biomaterials*. 2013; 34:393–401. [PubMed: 23083931]
23. Karam JP, Muscari C, Montero-Menei CN. Combining adult stem cells and polymeric devices for tissue engineering in infarcted myocardium. *Biomaterials*. 2012; 33:5683–95. [PubMed: 22594970]
24. Castells-Sala C, Semino CE. Biomaterials for stem cell culture and seeding for the generation and delivery of cardiac myocytes. *Current opinion in organ transplantation*. 2012; 17:681–7. [PubMed: 23080064]
25. Ma PX. Biomimetic materials for tissue engineering. *Advanced drug delivery reviews*. 2008; 60:184–98. [PubMed: 18045729]
26. Ban K, Park HJ, Kim S, Andukuri A, Cho KW, Hwang JW, et al. Cell Therapy with Embryonic Stem Cell-Derived Cardiomyocytes Encapsulated in Injectable Nanomatrix Gel Enhances Cell Engraftment and Promotes Cardiac Repair. *ACS nano*. 2014
27. Eldsle T, Bard J. Collagen substrata for studies on cell behavior. *J Cell Biol*. 1972; 54:626–37. [PubMed: 4339818]
28. Grinnell F, Bennett MH. Ultrastructural studies of cell-collagen interactions. *Methods Enzymol*. 1982; 82(Pt A):535–44. [PubMed: 7078448]
29. Strom SC, Michalopoulos G. Collagen as a substrate for cell growth and differentiation. *Methods Enzymol*. 1982; 82(Pt A):544–55. [PubMed: 7043209]

30. Hu J, Sun X, Ma H, Xie C, Chen YE, Ma PX. Porous nanofibrous PLLA scaffolds for vascular tissue engineering. *Biomaterials*. 2010; 31:7971–7. [PubMed: 20673997]
31. Hu J, Xie C, Ma H, Yang B, Ma PX, Chen YE. Construction of vascular tissues with macro-porous nano-fibrous scaffolds and smooth muscle cells enriched from differentiated embryonic stem cells. *PloS one*. 2012; 7:e35580. [PubMed: 22545119]
32. Wang Y, Hu J, Jiao J, Liu Z, Zhou Z, Zhao C, et al. Engineering vascular tissue with functional smooth muscle cells derived from human iPS cells and nanofibrous scaffolds. *Biomaterials*. 2014; 35:8960–9. [PubMed: 25085858]
33. Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, et al. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell stem cell*. 2011; 8:228–40. [PubMed: 21295278]
34. Wei G, Ma PX. Macroporous and nanofibrous polymer scaffolds and polymer/bone-like apatite composite scaffolds generated by sugar spheres. *J Biomed Mater Res A*. 2006; 78:306–15. [PubMed: 16637043]
35. Liu Q, Cen L, Yin S, Chen L, Liu G, Chang J, et al. A comparative study of proliferation and osteogenic differentiation of adipose-derived stem cells on akermanite and beta-TCP ceramics. *Biomaterials*. 2008; 29:4792–9. [PubMed: 18823660]
36. Feng G, Zhang Z, Jin X, Hu J, Gupte MJ, Holzwarth JM, et al. Regenerating nucleus pulposus of the intervertebral disc using biodegradable nanofibrous polymer scaffolds. *Tissue engineering Part A*. 2012; 18:2231–8. [PubMed: 22690837]
37. You JO, Rafat M, Ye GJ, Auguste DT. Nanoengineering the heart: conductive scaffolds enhance connexin 43 expression. *Nano letters*. 2011; 11:3643–8. [PubMed: 21800912]
38. Plotkin LI. Connexin 43 hemichannels and intracellular signaling in bone cells. *Frontiers in physiology*. 2014; 5:131. [PubMed: 24772090]
39. Garcia-Dorado D, Rodriguez-Sinovas A, Ruiz-Meana M. Gap junction-mediated spread of cell injury and death during myocardial ischemia-reperfusion. *Cardiovascular research*. 2004; 61:386–401. [PubMed: 14962471]
40. Stevens KR, Kreutziger KL, Dupras SK, Korte FS, Regnier M, Muskheli V, et al. Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:16568–73. [PubMed: 19805339]
41. Sekine H, Shimizu T, Hobo K, Sekiya S, Yang J, Yamato M, et al. Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. *Circulation*. 2008; 118:S145–52. [PubMed: 18824746]
42. Bu L, Jiang X, Martin-Puig S, Caron L, Zhu S, Shao Y, et al. Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. *Nature*. 2009; 460:113–7. [PubMed: 19571884]
43. Liu X, Jin X, Ma PX. Nanofibrous hollow microspheres self-assembled from star-shaped polymers as injectable cell carriers for knee repair. *Nature materials*. 2011; 10:398–406. [PubMed: 21499313]
44. McMahon RE, Wang L, Skoracki R, Mathur AB. Development of nanomaterials for bone repair and regeneration. *Journal of biomedical materials research Part B, Applied biomaterials*. 2013; 101:387–97.
45. Kon E, Delcogliano M, Filardo G, Fini M, Giavaresi G, Francioli S, et al. Orderly osteochondral regeneration in a sheep model using a novel nano-composite multilayered biomaterial. *Journal of orthopaedic research: official publication of the Orthopaedic Research Society*. 2010; 28:116–24. [PubMed: 19623663]
46. Tian H, Bharadwaj S, Liu Y, Ma H, Ma PX, Atala A, et al. Myogenic differentiation of human bone marrow mesenchymal stem cells on a 3D nano fibrous scaffold for bladder tissue engineering. *Biomaterials*. 2010; 31:870–7. [PubMed: 19853294]
47. Ott HC, Matthiesen TS, Goh SK, Black LD, Kren SM, Netoff TI, et al. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nature medicine*. 2008; 14:213–21.

48. Mishra PK, Givvimani S, Chavali V, Tyagi SC. Cardiac matrix: a clue for future therapy. *Biochimica et biophysica acta*. 2013; 1832:2271–6. [PubMed: 24055000]
49. Chachques JC, Pradas MM, Bayes-Genis A, Semino C. Creating the bioartificial myocardium for cardiac repair: challenges and clinical targets. *Expert review of cardiovascular therapy*. 2013; 11:1701–11. [PubMed: 24215199]
50. Dvir T, Timko BP, Kohane DS, Langer R. Nanotechnological strategies for engineering complex tissues. *Nature nanotechnology*. 2011; 6:13–22.
51. Heydarkhan-Hagvall S, Gluck JM, Delman C, Jung M, Ehsani N, Full S, et al. The effect of vitronectin on the differentiation of embryonic stem cells in a 3D culture system. *Biomaterials*. 2012; 33:2032–40. [PubMed: 22169822]
52. Kumar A, Patel A, Duvalsaint L, Desai M, Marks ED. Thymosin beta4 coated nanofiber scaffolds for the repair of damaged cardiac tissue. *Journal of nanobiotechnology*. 2014; 12:10. [PubMed: 24661328]
53. Ji Y, Liang K, Shen X, Bowlin GL. Electrospinning and characterization of chitin nanofibril/polycaprolactone nanocomposite fiber mats. *Carbohydr Polym*. 2014; 101:68–74. [PubMed: 24299750]
54. Wang F, Guan J. Cellular cardiomyoplasty and cardiac tissue engineering for myocardial therapy. *Advanced drug delivery reviews*. 2010; 62:784–97. [PubMed: 20214939]
55. Loh QL, Choong C. Three-dimensional scaffolds for tissue engineering applications: role of porosity and pore size. *Tissue engineering Part B, Reviews*. 2013; 19:485–502. [PubMed: 23672709]
56. Haneef K, Lila N, Benadda S, Legrand F, Carpentier A, Chachques JC. Development of bioartificial myocardium by electrostimulation of 3D collagen scaffolds seeded with stem cells. *Heart international*. 2012; 7:e14. [PubMed: 23185681]
57. Tandon N, Taubman A, Cimetta E, Saccenti L, Vunjak-Novakovic G. Portable bioreactor for perfusion and electrical stimulation of engineered cardiac tissue. *Conference proceedings: Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Annual Conference*. 2013; 2013:6219–23.
58. Huang Y, Zheng L, Gong X, Jia X, Song W, Liu M, et al. Effect of cyclic strain on cardiomyogenic differentiation of rat bone marrow derived mesenchymal stem cells. *PloS one*. 2012; 7:e34960. [PubMed: 22496879]
59. Bovendeerd PH. Modeling of cardiac growth and remodeling of myofiber orientation. *Journal of biomechanics*. 2012; 45:872–81. [PubMed: 22169149]

Fig. 1.

A diagram to show differentiation of mouse embryonic stem cells (ESCs) into cardiovascular progenitor cells (CPCs) and the desired lineages for cardiac repair. Mouse ESCs were cultured and passaged under feeder-free conditions. The modified stage-specific differentiation protocol was followed to induce differentiation of mouse ESCs into CPCs, and terminally differentiated cardiomyocytes associated with smooth muscle cells and endothelial cells. After 1.5 days of cardiovascular induction for the embryoid body (EB), we harvested CPCs for the subsequent two-dimensional (2D) and three-dimensional (3D) studies. This time point was set as day 0 for the following cardiac induction.

Fig. 2.

Differentiation potential of mouse CPCs into desired lineages for cardiac repair under 2D culture. Mouse CPCs were cultured in plates following a modified stage-specific differentiation protocol. At days 1.5 and 7 of cardiac induction in vitro, immunofluorescence staining was performed to detect cardiac progenitor (ISL1 and GATA4), and cardiomyocyte (cTnT), smooth muscle cell (SM-22α and α-SMA), and endothelial cell (CD31) marker expression, respectively (A, top panel). The cellular nuclei were counter-stained with Hoechst 33258 (A, bottom panel). Bar scale=100 μm. RT-PCR was performed to detect the transition of gene profile from CPCs to specific lineages (B). At day 7 of cardiac induction in vitro, differentiation rate for the desired lineages (cardiomyocyte, smooth muscle cell, and endothelial cell) was appraised by flow cytometry (C).

Fig. 3.

Gross and SEM view of porous nanofibrous (NF) PLLA scaffolds as well as cell attachment and extension in the scaffolds. Macro and SEM observation showed that the PLLA scaffolds were fabricated with a uniform porous structure of high interconnectivity, and the walls of the pores consist of nanofibers (top panel). The cells attached (Day 1.5) and extended (Day 7) well in the scaffolds (bottom panel).

Fig. 4. Scaffolds supporting cell growth and intercellular connections under 3D culture. Mouse CPCs were seeded into porous NF PLLA scaffolds. After 1.5 and 7 days of induction, fluorescence staining was performed to detect F-actin and connexin 43 expression. The cellular nuclei were counter-stained with Hoechst 33258. Bar scale=100 µm.

Fig. 5.

Transition from CPCs to the desired lineages for cardiac repair under 3D culture. Mouse CPCs were seeded into porous NF PLLA scaffolds. After 1.5 and 7 days of induction, immunofluorescence staining was performed to detect ISL1 expression (A). At day 7, H&E and immunofluorescence staining was performed to detect cell growth and differentiation (B). Bar scale=100 μ m. RT-PCR showed the transition of gene profile from CPCs to specific lineages (C). Densitometry of the target bands for RT-PCR was performed to show the reverse courses during cardiac differentiation (D).

Fig. 6.
Schematic view of cell/scaffold construction in vitro and subcutaneous implantation surgery in a nude mouse model.

Fig. 7.

Scaffolds supporting cardiac cell/scaffold constructs in vivo. The cell/scaffold constructs engineered in vitro were subcutaneously implanted in a nude mouse model. The implantation of Cells Only and Scaffold Only was performed as controls. Seven days later, the desired features were appraised by immunohistochemistry to detect cTnT, SMMHC, and CD31 expression. The slides stained with goat or rabbit IgG isotype control instead of primary antibodies were observed as controls. Bar scale=100 μ m.

Fig. 8.

Tracking of seeded cells in the scaffolds after subcutaneous implantation. The CPCs were labeled with green fluorescent dye (PKH67) before seeding into the porous scaffolds. After construction in vitro, the cardiac constructs were subcutaneously implanted in a nude mouse model. Seven days later, the constructs were harvested and H&E staining was performed to observe cell distribution and growth inside the constructs (A). Fluorescence detection revealed that some of the cells within the scaffolds showed strong green signals to identify their origin (B). Bar scale=100 μ m.

Table 1

Sequences of Primers and RT-PCR Conditions

Gene	Primers (F=forward; R=reverse)	Amplicon size (bp)	Annealing temperature (°C)
Mesp1	F : 5'-GCTCGGTCCCCGTTTAAGC-3' R : 5'-ACGATGGGTCCCACGATTCT-3'	104	58
ISL1	F : 5'-ATGATGGTGGTTTACAGGCTAAC-3' R : 5'-TCGATGCTACTTCACTGCCAG-3'	174	58
cTnT	F : 5'-CAGAGGAGGCCAACGTAGAAG-3' R : 5'-CTCCATCGGGGATCTTGGGT-3'	138	58
SMMHC	F : 5'-AAGCTGCGGCTAGAGGTCA-3' R : 5'-CCCTCCCTTTGATGGCTGAG-3'	238	58
CD31	F : 5'-GTGAAGGTGCATGGCGTATC-3' R : 5'-CACAAAGTTCTCGTTGGAGGT-3'	192	58
β-actin	F : 5'-GGCTGTATTCCCCTCCATCG-3' R : 5'-CCAGTTGGTAACAATGCCATGT-3'	154	60