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Influence of decellularized pericardium matrix on the behavior of cardiac progenitors

Sasan Jalili-Firoozinezhad,[1,2*] Sareh Rajabi-Zeleti,[1*] Anna Marsano,[2] Nasser Aghdami,[1] Hossein Baharvand[1]

[1]Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

[2]Departments of Surgery and Biomedicine, University Hospital Basel, University of Basel, Basel CH-4031, Switzerland

*Co-First Authors: Sasan Jalili-Firoozinezhad and Sareh Rajabi-Zeleti

Correspondence to: H. Baharvand (E-mail: Baharvand@Royaninstitute.org) or N. Aghdami (E-mail: Nasser.Aghdami@Royaninstitute.org)

ABSTRACT: Following myocardial infarction, heart muscle has a limited capacity of self-healing. Biological platforms providing the natural biochemical and biophysical cues of the native myocardium might be crucial to address current therapeutic shortcomings. The aim of this study was to assess the effect of decellularized human pericardium (DPc), as a bioactive platform, on viability, attachment, proliferation and differentiation of human cardiac progenitors (CPs), and evaluate the possibility of using DPc as a substitute of tissue culture polystyrene (TCPS) substrate for culturing CPs *in vitro*. The decellularization process removed more than 99% of the cellular components from Pc, yet well preserved its macro-/micro-structure and extracellular collagen and glycosaminoglycan content. DPc supported the viability, attachment, metabolism and proliferation of CPs, and enhanced their differentiation into mature cardiomyocytes compared to TCPS. Decellularized pericardium appeared thus to have a high potential for cardiac cell culture and could be applied as a superior alternative to common TCPS. DPc could be then utilized for further tissue engineering applications.
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INTRODUCTION

Heart failure, the leading health problem in industrialized nations, accounts for approximately 40% of all human mortality.[1] Current therapeutic strategies to treat damaged cardiac tissue are limited and cell therapies have not gained significant outcomes.[2] Massive cardiomyocytes loss and noncontractible scar tissue formation occurs following myocardial infarction. Lacking of an appropriate biomaterial to keep the cells in the defect site and prevent their apoptosis after injection has blurred cell therapy success.[3] Therefore, biomaterials technology has emerged and shown great promise in providing better environment for culturing the cells, improving cell therapy efficiency and eventually promoting the treatment of myocardial infarction.[4] Synthetic materials, such as tissue culture polystyrene (TCPS) which is used in daily routine cardiac cell culture for expansion of cardiac progenitors and/or harvesting mature cardiomyocytes, do not have bioactive cues and thus, limit the regeneration capacity of the cells. Natural biomaterials such as fibrin, collagen, and chitosan which even contain some native signaling traits have not shown promising outcomes when employed in the damaged myocardium.[5–7] Interestingly, the idea of fabricating complex substrates through combining different proteins and factors, and so, mimicking advanced composition of native extracellular matrices (ECMs), have shown to enhance the proliferation and differentiation of cells considerably.[8] Thus, native ECM platforms are required to support cellular attachment, survival, proliferation, and maturation, and eventually proper regeneration of the intended tissue.

As an ideal bio-complex matrix, decellularized tissues prepared through physical, chemical, and enzymatic methods have been used for several tissue engineering and regenerative medicine applications including myocardium engineering.[9,10] Naturally

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derived matrices containing glycosaminoglycan (GAG) and different proteins provide more physiologically relevant surface with a closer mimic to the *in vivo* environment.[11] Different types of decellularized tissues have been studied for ischemic myocardium treatment, including small intestinal submucosa and pericardium tissue.[12–14] However, a serious limitation shared by the majority of these tissues is their xenogeneic or allogeneic basis which is unfavorable in sense of disease transferring and immunogenicity.[15] Among them, pericardium can be recruited from patient's own tissue with great resemblance to myocardium biochemical traits. The application of pericardium as a biomaterial is a promising approach in myocardial tissue engineering.[16] Pericardium, readily available collagen-rich biological tissue, can be surgically resected without adverse consequences.[17,18] Moreover, preparing this native extracellular matrix (ECM) scaffold is cost-effective, and the final sample can easily be free of donor-derived pathogens and provide a potential source for an autologous scaffold in tissue engineering strategies.[19,20] Thus, native pericardium is a good candidate to be considered as a biological platform for cardiac cell culture and myocardial engineering applications because of its similarity to heart ECM and biomechanical properties.[21]

So far, different strategies have been implemented to use human pericardium (Pc) membrane in the form of an injectable gel and macroporous three-dimensional (3D) sponge in combination with cardiac cells for cardiac tissue engineering applications.[16,22] Although the biochemical cues of Pc were maintained during fabrication of the gel and sponge, the 3D biophysical nature of the tissue was eliminated in these processes. So, in order to study the simultaneous effect of biochemical and biophysical traits of Pc on behavior of cardiac cells, the microstructure of Pc should be maintained while preserving the biochemical elements. Moreover, for evaluating the suitability of Pc application *per se*, such as heart valve replacements and covering blood vessels,[23–25] and eventually assessing its potential as a bioactive substrate for cardiac cell culture studies, the response of cardiac cells should be investigated on intact but decellularized human pericardium (DPc) membrane. For this reason, in the current study human cardiac progenitors (CPs) were selected for *in vitro* evaluations. The heart appears to contain self-renewal, clonogenic, and multipotent CPs population, capable to differentiate into cardiomyocyte, endothelial, and smooth muscle cells.[26–29]

The objective of the present study was to evaluate the attachment, survival, metabolism, and proliferation of CPs on DPc *per se*. The first signs of DPc impact on directing the differentiation/maturation of CPs were also investigated. The results of cell monolayer culture on DPc group were compared to the common standard control group, TCPS. We aimed at introducing DPc as an alternative to TCPS, to support expansion of CPs *in vitro* and enhance the probability of harvesting mature cardiomyocytes, which could then find utilization for cellular therapies and regenerative medicine prospects.

EXPERIMENTAL

CPs Harvesting and Culture

Heart biopsies were obtained during routine surgeries in Royan Cell Therapy Center from congenital heart patients (2–10 year-old children, six donors) in accordance with Iran legislation, guidelines established by Royan Institute Cell Bank Services and approval of Royan Institute Ethical Committee. One cm^3 of right ventricle tissues from each patient were transferred to our laboratory in a medium containing cold IMDM (Sigma, 13390), 10% fetal bovine serum (FBS, Gibco, 10270-106) and 10% penicillin/streptomycin (Gibco, 15070-063). Transferred samples were washed twice with cold phosphate-buffered saline (PBS). Minced samples were incubated with an enzyme cocktail containing collagenase I (2 mg/mL, Sigma, C0130), collagenase II (2 mg/mL, Gibco, 17101-015), collagenase IV (1 mg/mL, Gibco, 17104-019) and trypsin (2 mg/mL, Gibco, 27250-018) at 37°C. The cell suspensions from the subsequent 4–6 digestions were centrifuged (1500 rpm, 5 min) and resuspended in culture medium [IMDM, 10% FBS, 10% penicillin/streptomycin and L-glutamine (Gibco, 25030-024)], counted and cultured on tissue culture flasks. Cells were pre-plated for 65 min to enrich for CPs. After an overnight culture, the medium was transferred to another tissue culture flask and allowed to reach confluency. To promote cell growth, 5 ng/mL EGF (Invitrogen, PHG 0315), 5 ng/mL IGF-1 (Sigma, 13769) and 10 ng/mL bFGF (Royan) were supplemented to the culture medium. Trypsin/EDTA (0.025%, Gibco, 15400) was used to passage the cells, and CPs at passage 2–3 were recruited for further experiments.

CPs Characterization

To determine the distribution of cell surface markers on isolated cells, flow cytometry analysis was used. The surface marker expression of CPs was measured by staining with fluorochrome-conjugated antibodies against CD90 (Dako F7274), CD117 (Miltenyi Biotec, 130-091-734), CD105 (R&D Systems FAB10971P), CD34 (BD, 550619), CD45 (Dako F0861), Sca-1 (eBioscience, 12-5981-81), and CD31 (BD Pharmingen, 555445) on a 2-laser FACSCalibur flow cytometer (BD, Becton Dickinson, USA). For each marker 10^5 cells were treated in the conditions mentioned in the antibody data sheet. In brief, cells were washed with PBS and resuspended in PBS containing 1% FBS. Cells were then incubated in primary antibody at 4°C, washed with flow cytometry washing buffer and stained with secondary antibody at 4°C in the dark. Following a washing step, cells were tested by flow cytometry device and obtained data were analyzed using WinMDI 2.9 software.

Decellularization of Human Pericardium

Human pericardia from cadaveric donors were supplied by the National Tissue Services with appropriate informed consent for research use and Ethical Committee approval. Tissues were decellularized as previously described with slight modifications.[16] In brief, following removal of fat tissues, the pericardial membrane was rinsed in double distilled water (D.D.W.) for 2 h, treated with 1% sodium dodecyl sulfate (SDS, 1% w/v in PBS) for 24 h, washed in RNase/DNase nuclease solution, and finally treated with acetic acid (0.2*M*). Samples were then incubated in PBS for three 60-min. periods with agitation. After completion of the decellularization process, samples were freeze-dried and stored at 4°C.

Histological Studies

In order to confirm removal of cells and preservation of biochemical structures of decellularized tissues, a piece of Pc and DPc were cut for histological analyses. Samples were first fixed in 10% neutral buffered formalin solution in PBS (pH 7.4) overnight at 25°C.

They were then rinsed in D.D.W, dehydrated in graded alcohol series, embedded in paraffin and sectioned at 5 μm. Tissue slides were subsequently stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome (MT) and observed under an optical microscope.

DNA Quantification

DNA content of native ECM and DPc was measured. Samples were thoroughly solubilized in 1 mL lysis buffer (50 m*M* tris-HCl, 50 m*M* EDTA, 1% SDS, 10 m*M* NaCl, pH 8.0). Samples were subsequently digested overnight in the presence of proteinase K in a water bath at 65°C, followed by a phenol/chloroform extraction. Using 100% ethanol, DNA was precipitated from the aqueous phase. The pellet was dissolved in RNase-free water and the concentration of DNA obtained with a spectrophotometer at 280 nm. The amount of DNA was expressed as μg per mg dry weight of the samples.

Collagen Content

The amount of collagen in Pc and DPc were measured using the Sircol^{TM} Kit (Biocolor, UK). Samples were completely homogenized prior to solubilizing the collagen in a 0.1*M* HCl-pepsin solution. Collagen content of the samples was obtained after adding Sirius red dye and measuring the absorbance at 555 nm. Serial dilution of bovine collagen was plotted as standard curve.

GAG Quantification

The sulfated GAG contents of samples were analyzed with the Blyscan GAG assay (Biocolor, UK). In brief, 30 mg of each sample was digested in 1 mL of papain solution (Sigma, P3125) for 5 h at 65°C. The sample aliquots were centrifuged and incubated with 1, 9-dimethyl-methylene blue dye. Absolute GAG levels were obtained at 656 nm by extrapolating values from the standard curve of bovine tracheal chondroitin-4-sulfate (Sigma, 27042).

Cell Seeding onto Scaffolds

In order to sterilize the DPc, the sample was washed thoroughly with 70% ethanol and sterile PBS. Afterwards, the scaffold was incubated with IMDM/10% FBS for 24 h. After transferring into a tissue culture plate, DPc was loaded with CPs (5×10^5 cells/cm^2) and incubated at 37°C and 5% CO_2. Culture medium was exchanged daily. In order to induce CPs differentiation into cardiomyocytes, the cells on TCPS and DPc were cultured according to the protocol by Smits *et al.*[30] In brief, the cells were treated with 5 μM 5-azacytidin (Santa Cruz, Sc-221003) for 72 h in differentiation medium, 1 : 1 IMDM/Hams F12 (PAA Laboratories) supplemented with L-glutamine, 2% horse serum (Gibco, 2605), nonessential amino acids, insulin–transferrin–selenium (Gibco, 41400-045) supplement and $10^{-4}M$ ascorbic acid (Sigma, A4403). The differentiation process was followed by 1 ng/mL TGF-ß1 (Invitrogen, PHG9209).

Scanning Electron Microscopy (SEM)

In order to investigate cell morphology and attachment on scaffolds, after three days samples were processed for SEM examinations. DPc was removed from the culture plate and gently rinsed with PBS. Then, the specimen was fixed with 2.5% glutaraldehyde in PBS overnight, followed by washing with PBS and sequentially dehydrated in a 30%, 50%, 70%, 80%, 90%, and 100% ethanol series; thereafter, the sample was observed by SEM device (VEGA\TESCAN, Czech Republic).

Immunofluorescence Staining

Three days after CPs culturing on TCPS and DPc, the cells were fixed with 4% formaldehyde for 20 min, permeabilized with 0.5% Triton-X 100 for 30 min, washed with PBS, blocked with 2% bovine serum albumin (BSA) for 15 min, and then incubated with antibodies against Ki67 (Abcam, ab66155) at 4°C overnight. After rinsing with PBS, the cells were treated with secondary antibody for 2 h at 25°C. Cells were all counterstained for nuclei detection with DAPI. Secondary antibody incubation alone was used as a negative control. Immunostaining was visualized and analyzed by at least three blind investigators using a light microscope (Olympus, BX51) and imaged with an Olympus DP72 digital camera that was mounted on the microscope. The percentage of Ki67 positive cells was calculated as the number of green cells divided by total number of cells (blue).

Cell Metabolic Activity

Metabolic activity of the CPs on the TCPS and DPc was measured by the 3-(4,5-dimethylthiazoyl-2-yl)−2,5-diphenyltetrazolium bromide (MTT) reduction assay. Cells were seeded on TCPS and DPc at a density of 10×10^4 cells/cm^2 in 24-well plates and incubated under standard conditions for 1, 3, and 5 days. Then, 40 μL of 5 mg/mL MTT solution was added to each well, and the plates were incubated at 37°C for 4 h. The medium was removed and the formazan crystals were dissolved in DMSO. The formazan solution transferred to a 96-well plate and absorbance at 570 nm was measured using an ELISA plate reader (Thermo Scientific Multiscan Spectrum).

Quantitative Real-Time PCR

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed to assess the mRNA expression patterns of different genes involved in early and late heart development. Total RNA was extracted by TRIzol reagent (Ambion, 15596018) and chloroform based on the manufacturer's instructions. DNA contamination was removed using a DNase1 kit (Fermentaz, en0521). First strand cDNA synthesis and PCR reaction were performed with TaKaRa, PrimeScript 1st Strand cDNA Synthesis (TaKaRa, 6110A) and Ex Taq kits (TaKaRa, RR001A) according to their product data sheets. Quantitative PCR reactions were performed in a Rotor-Gene^{TM} 6000 Real-Time PCR System (Corbett Life Science) using the following program: stage 1: 95°C for 10 min; stage 2: 95°C for 10 s, 60°C for 20 s and 72°C for 20 s, for 40 cycles. Reaction mixtures contained 10 μL of SYBR® Premix Ex Taq^{TM} II (RR081Q, Takara Bio), 6 μL D.D.W., 1 μL forward or reverse primers (5 pmol/μL) and 2 μL of 1:4 cDNA. The final concentration for each reaction was 25 ng of cDNA template. cDNA was synthesized 1 μg per sample and diluted four times then. The threshold cycle (Ct) of each target gene obtained was normalized by GAPDH as internal standard gene. Primer sequences are listed in Table I. The RNA integrity was also evaluated by performing a denaturating agarose gel electrophoresis using a 1% agarose gel and TAE buffer.

Table I. Real-Time PCR Primers

Target gene	Primer sequence	Accession number	Annealing temp.(°C)	Product size
GATA4	FOR: 5′ CCTGTCATCTCACTACGG 3′ REV: 5′ GCTGTTCCAAGAGTCCTG 3′	NM_002052.3	60	180bp
MEF2C[a]	FOR: 5′ TCCGAGTTCTTATTCCACC 3′ REV: 5′ ATCCTCCCATTCCTTGTC 3′	NM_002397.3	60	168bp
cTnT[b]	FOR: 5′ ATGATGCATTTTGGGGGTTA 3′ REV: 5′ CAGCACCTTCCTCCTCTCAG 3′	NM_00364.2	60	108bp
α-MHC[c]	FOR: 5′ ATTGCTGAAACCGAGAATGG 3′ REV: 5′ CGCTCCTTGAGGTTGAAAAG 3′	NM_002471.3	60	146bp
Cx43[d]	FOR: 5′ GCTATGACAAGTCTTTCCCA 3′ REV: 5′ CAGTTTCTCTTCCTTTCGCA 3′	NM_00165.3	60	124bp
GAPDH[e]	FOR: 5′ GTTCTTCATTCACTAAGGAAGG 3′ REV: 5′ CAAGAGCATCATTGAACTTCAC 3′	NM_002046.5	60	122bp

[a] Myocyte enhancer factor 2C.
[b] Cardiac Troponin I.
[c] Alpha-myosin heavy chain.
[d] Connexin 43.
[e] Glyceraldehyde 3-phosphate dehydrogenase.

Statistical Analysis

All data are presented as mean ± standard deviation. All assays were performed at least in triplicate. Statistical analysis was accomplished using *t*-test with SPSS 16.0 software. P values of less than 0.05 were considered significant.

RESULTS

DPc Preparation and Characterization

Decellularization protocol illustrated in Figure 1(A) was applied to obtain DPc scaffold from native tissue. As an indication of a successful decellularization process, the color of native ECM turned white at the end of decellularization run [Figure 1(B)]. Histological study was performed to evaluate the ECM structure after decellularization. The results from H&E and MT staining showed that the majority of the cell nuclei were washed out from Pc whilst the natural microstructure of the ECM was still preserved following decellularization [Figure 1(B)]. As shown in Figure 1(C), DNA content analysis confirmed the almost complete (approximately 99%) removal of DNA from Pc (2.15 ± 0.23 μg/mg) compared with DPc (0.02 ± 0.001 μg/mg). Quantification of collagen content showed higher amounts in Pc (106.32 ± 4.57 μg/mg of tissue) compared to DPc (84.79 ± 3.26 μg/mg of tissue). GAG content of Pc and DPc was determined to be 10.94 ± 2.13 μg and 8.34 ± 1.78 μg GAG per mg of dry ECM, respectively [Figure 1(D)].

CPs Characterization

Primary cultures of CPs were successfully established and passages obtained from ventricle biopsies. The cells had fibroblast-like morphology and they were potential to form colonies [Figure 2(A)]. In order to evaluate the cell surface markers, flow cytometry analysis was performed. Three different surface marker groups—mesenchymal stromal cells (MSCs) CD90 and CD105, cardiac stem cells Sca-1 and CD117, and hematopoietic and endothelial cells CD34, CD45 and CD31 were analyzed. As shown in Figure 2(B), isolated cells were positive for MSCs markers but they did not express hematopoietic markers. Cells also expressed CD31 and Sca-1. Only a small percentage of isolated cells (2.26 ± 0.12%) were positive for CD117.

Morphology of CPs and DPc

In order to evaluate the surface structure of DPc and morphology of seeded cells, SEM analysis was performed. Our results showed that the surface microstructure of DPc was normal and without pores and disruptions. As depicted in Figure 3(A), CPs adhered on top surface of DPc after five days of culture. CPs spread to the substrate and showed an elongated morphology resembling the cell shape during culture on TCPS. H&E and SEM images of DPc following decellularization and cell seeding revealed the placement of cells within the construct and presence of dense layers in DPc (Supporting Information Figure S1).

Proliferation and Metabolic Activity Evaluations

In order to compare cell proliferation on DPc with TCPS, immunofluorescence staining for Ki67 was performed. Based on the obtained results, there was no significant difference between the percentage of Ki67 positive cells on TCPS (10.83 ± 2.47%) and DPc (7.48 ± 1.59%), Figure 3(B,C). To evaluate the viability of CPs cultured on DPc samples, MTT assay was performed and quantified at specified time intervals. As shown in Figure 3(D), the metabolic activity of CPs cultured on the TCPS and DPc was identical and there did not appear to be a significant difference between these two groups after 3 and 5 days of culture.

Real-Time PCR Analysis

In order to evaluate the effect of DPc on CPs cardiogenic differentiation, quantitative real-time PCR was performed for the following genes: *GATA4*, *MEF 2c*, α-myosin heavy chain (*α-MHC*), *Connexin 43*, and cardiac troponin T (*cTNT*). RNA integrity was also confirmed by developing a nondenaturing agarose gel electrophoresis, where the 28s and 18s rRNA bands were clearly observed, and no DNA contamination was detected (Supporting Information Figure S2). As illustrated in Figure 4, although

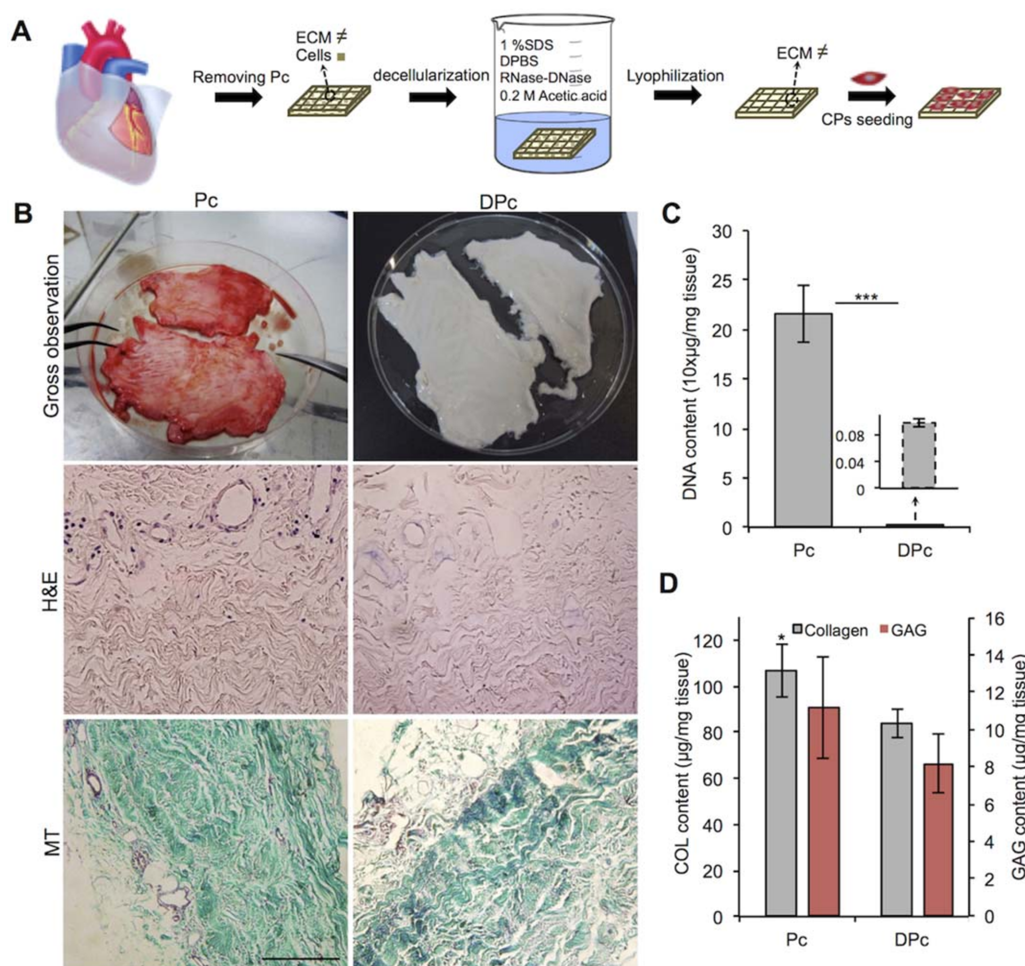

Figure 1. Characterization of DPc. (A) Schematic presentation of decellularization process and subsequent cell seeding. (B) Gross image of Pc before and after decellularization. H&E and MT stainings show removal of cellular components and remaining of histological structure of Pc after decellularization. Scale bar: 100 μm. (C) DNA quantification shows complete removal of DNA following decellularization treatments, $n = 3$ (*t*-test statistical analysis, $^{***}P < 0.001$). (D) GAG and collagen quantification of Pc and DPc, $n = 3$, (*t*-test statistical analysis, $^{*}P < 0.05$). H&E: Hematoxylin and eosin, GAG: Glycosaminoglycan, Pc: Human pericardium, DPc: Decellularized human pericardium. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

there were not considerable differences between TCPS and DPc in the context of expression of the some cardiac markers such as *MEF 2c* (1.10 ± 0.15 fold), *cTNT* (1.21 ± 0.18 fold) and *connexin 43* (0.95 ± 0.21 fold), the early cardiac marker gene *GATA4* increased significantly by 1.50 ± 0.23 fold 25 days after seeding on DPc. Moreover, gene expression analysis of α-*MHC* revealed a significant increase (3.40 ± 0.54 fold) compared to TCPS.

DISCUSSION

A central goal of myocardial engineering has been to design and fabricate templates to provide an optimum mimicry of the heart's biological environment.[31] Among synthetic and natural biomaterials, substrates with native ECM components encompass a unique significance.[32–34] So in an effort to employ these native ECMs, we decellularized human pericardium membrane and evaluated cardiac progenitors behavior *in vitro*. Our results demonstrated the removal of cellular contents through the decellularization protocol. The SDS treatment procedure did not cause any change in the structure of the native ECM as previously reported.[22] The efficiency of DNA removal was higher than that reported with other decellularization techniques.[35] Although some diminution in collagen and GAGs contents was evident after decellularization and washing steps, about 75% of the biochemical components of the native tissue were thoroughly preserved. As the native ECM enhances the cell–matrix interaction,[32] the maintained biochemical structure of the Pc could address superior cellular behavior such as attachment, proliferation, and differentiation on DPc. These bioactive components not only have major roles in both structure and function of ECM, but they are also able to bind and modulate various proteins.[36]

We investigated the ability of CPs to adhere and proliferate on DPc compared to TCPS. This study reveals that DPc can support adhesion, metabolism, and proliferation of CPs similar to TCPS. Our findings from cell viability demonstrated that the Pc after decellularization with SDS had no cytotoxic effects on cells after a five-day culture period according to previous

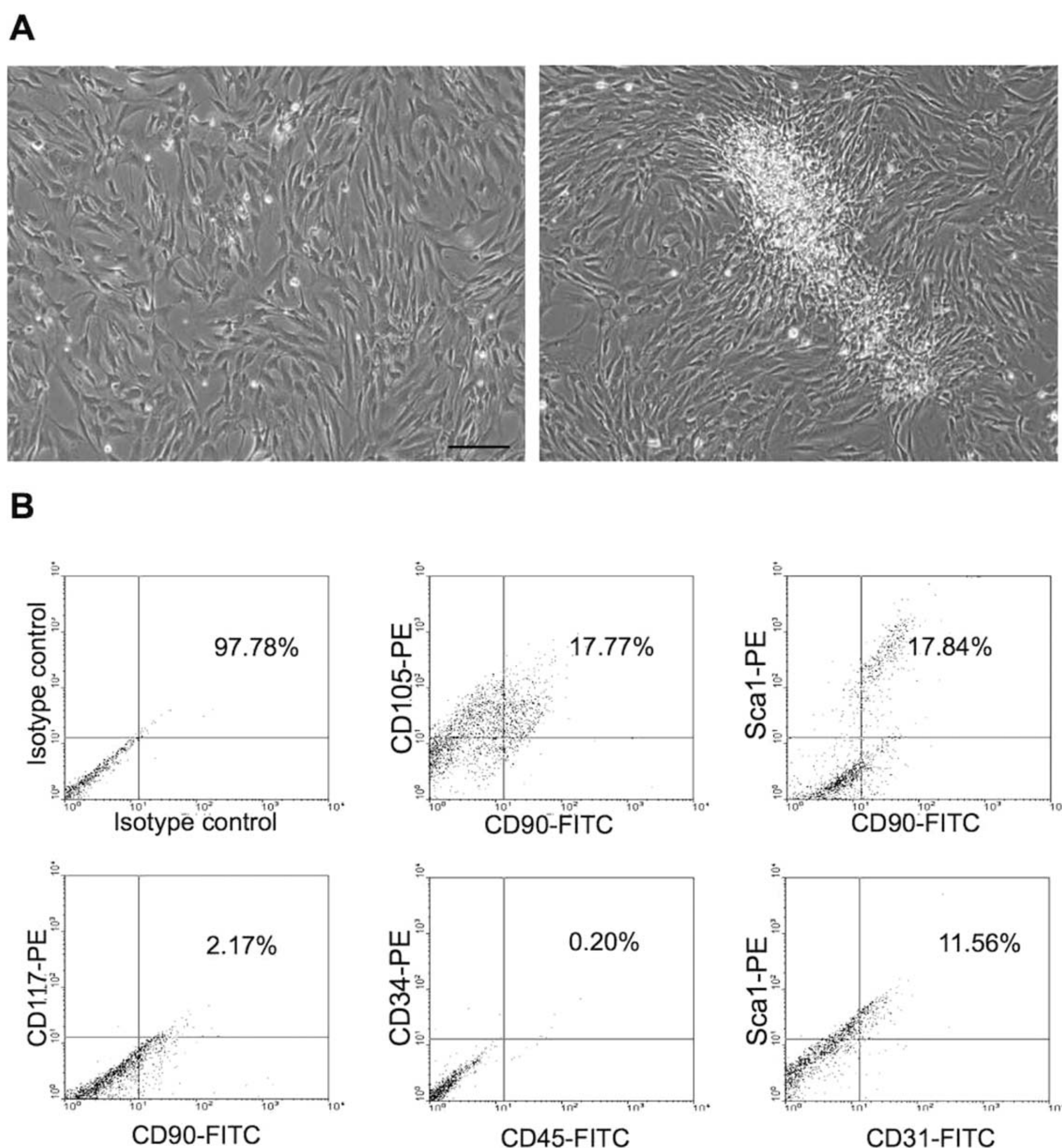

Figure 2. CPs characterization. (A) Fibroblast-like morphology of the cultured CPs (left panel) in their clonogenic form (right panel). (B) Flow cytometry analysis of CPs stained for CD90, CD117, CD105, CD34, CD45, Sca-1, and CD31 antibodies. The percentage of double-positive cells is shown in the plots. $n = 6$. Scale bars: 100 μm. CPs: Cardiac Progenitors.

studies.[16,35,37] Since in cellular therapies most of cells are lost or diffuse away from the injection site,[38,39] the great adhesion of CPs on DPc might be important for cell transplantation procedures. Moreover, considerable proliferation has been shown to be advantageous in the sense of increasing the chance of tissue repair.[40]

Native ECMs that composed of complex and interconnected network of proteoglycans, proteins, and GAGs have a profound effect on differentiation direction and maturation of stem cells.[41,42] They play an important role in regulation of various cellular activities and tissue development.[43] To repair the infarcted myocardium, new cardiomyocytes should be generated.[44] While CPs have the potential to differentiate into all cardiac cell types,[27] our study demonstrated that expressions of early (*GATA4*) and mature (*α-MHC*) cardiomyocyte genes within DPc were increased significantly after a 25-day treatment period compared to the TCPS control group. Because of greater level of α-MHC expression in DPc, it is possible that CPs might present better regeneration capacity on this native substrate. Moreover, it has been shown that *α-MHC* is involved in heart development following *GATA4* expression that its increased expression improved regenerative capacity.[26]

Although biochemical properties of pericardium tissue appear to be slightly different among various human donors, the application of DPc is not limited to only autologous tissue repair. So, pericardium tissue could be used as an off-the-shelf substrate for valve replacement, mitral valve extension and wound healing applications; and considered as a great candidate for cardiac repair.[45,46]

While previous studies were mainly focused on evaluating allogeneic and xenogeneic pericardium tissues, limited studies have been done so far to compare native human pericardium membrane with standard TCPS. Moreover, previous reports aimed mainly at studying the behavior of mesenchymal stromal cells on chemically modified, e.g., glutaraldehyde cross-linked, pericardium membrane rather than cardiac-specific progenitors/stem cells on intact tissue.[24,46,47] Although we tried to completely maintain biochemical and biophysical traits of pericardium tissue, previous reports on engineering pericardium in form of injectable hydrogels and 3D sponges might provide better solution for some specific cardiac conditions, where pericardium tissue cannot be implanted in its intact form.[16,22] Pericardium membrane with interesting biochemical traits can improve retention, survival, integration, and maturation of seeded cells, and is of considerable clinical interest.[48]

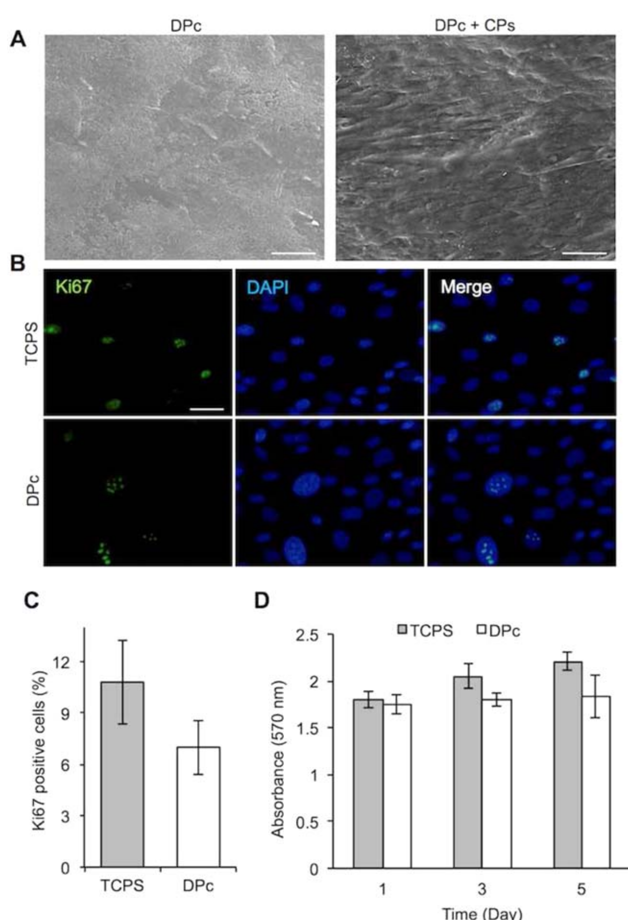

Figure 3. Viability and proliferation assessments. (A) SEM images of surface topography of DPc (scale bar: 5 μm) and CPs attachment and colonization on the surface of DPc scaffold (scale bar: 25 μm). (B) Cellular proliferation according to immunostaining results for Ki67 on TCPS and DPc at day 5. Scale bar: 100 μm. (C) Quantitative analysis of Ki67-positive cells (*t*-test statistical analysis, ns). (D) MTT assay of CPs at different time points postseeding on TCPS and DPc surface, $n = 4$ (*t*-test statistical analysis, ns). SEM: Scanning electron microscopy, CPs: Cardiac progenitors, DPc: Decellularized human pericardium, TCPS: Tissue culture polystyrene, MTT: 3-(4,5-dimethylthiazoyl-2-yl)−2,5-diphenyltetrazolium bromide, ns: Not significant. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

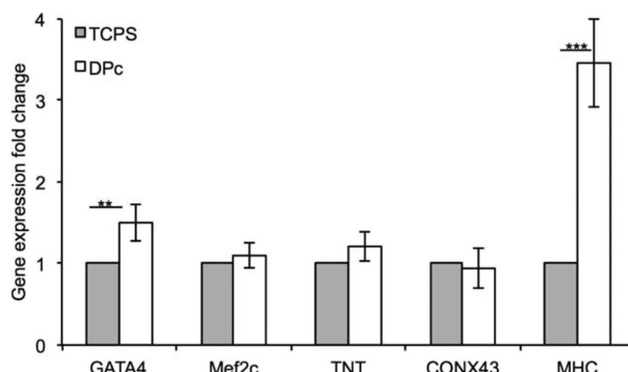

Figure 4. Gene expression analysis for cardiac markers on DPc after 25 days of culture. The results are normalized to GAPDH and expressed as fold change for DPc over TCPS ($\Delta\Delta$Ct), $n = 6$ (*t*-test statistical analysis, $^{**}P < 0.01$ and $^{***}P < 0.001$). DPc: decellularized human pericardium, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, α-MHC: Alpha-myosin heavy chain, cTnT: Cardiac Troponin T, Cnx 43: Connexin 43, MEF2C: Myocyte enhancer factor 2C.

However, to get better vision about the functionality of this tissue, performing long-term *in vivo* studies is beneficial.

CONCLUSIONS

We successfully decellularized human pericardium and completely removed cellular nuclei while preserved the extracellular matrix components and structure. We have also demonstrated that DPc with preserved extracellular matrix cues supports adhesion and proliferation of CPs, and enhance the commitment of these cells to a cardiac lineage compared to standard control TCPS. This study suggested that this decellularized human pericardium tissue could find utilization in cardiac engineering strategies; however, further studies improving 3D structure of the DPc and evaluating this scaffold in cardiac defects are beneficial.

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