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Tailoring Material Properties of Cardiac Matrix Hydrogels To Induce Endothelial Differentiation of Human Mesenchymal Stem Cells

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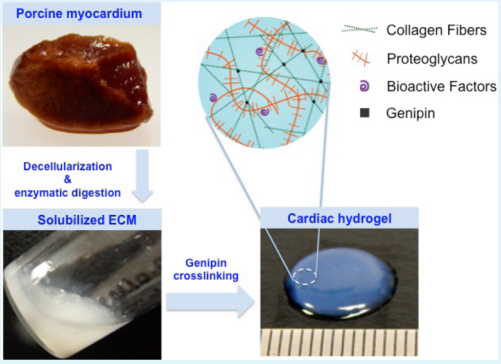
ABSTRACT: Cardiac matrix hydrogel has shown great promise as an injectable biomaterial due to the possession of cardiac-specific extracellular matrix composition. A cardiac matrix hydrogel facilitating neovascularization will further improve its therapeutic outcomes in cardiac repair. In this study, we explored the feasibility of tailoring material properties of cardiac matrix hydrogels using a natural compound, genipin, to promote endothelial differentiation of stem cells. Our results demonstrated that the genipin cross-linking could increase the mechanical properties of the cardiac matrix hydrogel to a stiffness range promoting endothelial differentiation of human mesenchymal stem cells (hMSCs). It also decreased the swelling ratio and prolonged degradation without altering gelation time. Human mesenchymal stem cells cultured on the genipin cross-linked cardiac matrix hydrogels showed great viability. After 1 day culture, hMSCs demonstrated down-regulation of early endothelial marker expression and up-regulation of mature endothelial marker expression. Especially for 1 mM genipin cross-linked cardiac matrix hydrogels, hMSCs showed particularly significant expression of mature endothelial cell marker vWF. These attractive results indicate the potential of using genipin cross-linked cardiac matrix hydrogels to promote rapid vascularization for cardiac infarction treatment through minimally invasive therapy.



KEYWORDS: *cardiac extracellular matrix, injectable hydrogel, genipin, human mesenchymal stem cells, stem cell differentiation*

1. INTRODUCTION

Myocardial infarction (MI) and heart failure following MI are the major contributors to cardiovascular related deaths. In the United States, approximately 800,000 people will experience a new MI every year and an additional 470,000 will have a recurrent attack.[1] Additionally, 5.7 million people suffer heart failure and 670,000 new cases are reported annually.[1] Current clinical treatments for MI mainly focus on slowing the negative progression toward heart failure. Heart transplantation is the only effective option for long-term cardiac function restoration, but it is limited by organ shortage and complications associated with transplant rejection. Thus, there is a critical need for the development of alternative therapeutics to treat MI and prevent heart failure.

Cardiac stem cell therapy has shown great potential to repair the injured heart and improve cardiac function after MI.[2−4] The capability to differentiate into multiple lineages makes stem cells the most attractive therapeutics in regenerative medicine. Over the past decade, there has been tremendous enthusiasm in the quest for a stem cell capable of regenerating infarcted myocardium and restoring cardiac function. Mesenchymal stem cells (MSCs) have emerged as one of the leading candidates because of their unique properties (e.g., easy isolation from bone marrow, fast growth rate, immunological tolerance, and multilineage potential, etc.).[5,6] Results from large-animal preclinical studies and current clinical trials demonstrated the ability of MSCs to improve left ventricular (LV) function, reduce scar size, and increase myocardial tissue perfusion in post-MI heart regardless of delivery methods.[7−9] However, the efficiency of MSC engraftment and differentiation in the heart is extremely low although robust functional recovery is observed after cell transplantation.[10,11] The improvement of cell retention and cardiovascular differentiation after transplantation could significantly enhance the efficacy of cardiac stem cell therapy.

Recently, solubilized cardiac extracellular matrix (ECM) has gained increasing attention as an injectable biomaterial for cardiac tissue engineering. Decellularized cardiac tissue contains tissue-specific biochemical composition and structure.[12−15] When further processed into a liquid, the solubilized ECM preserves the unique combination of chemical and biological cues mimicking the native microenvironment. It can also assemble into three-dimensional (3D) hydrogels at physiological pH and temperature. It has been shown that solubilized porcine myocardial matrix could be delivered using a catheter that is used clinically for endocardial injection, indicating the potential for minimally invasive therapy.[16] In addition, the safety and efficacy of using solubilized cardiac ECM as an

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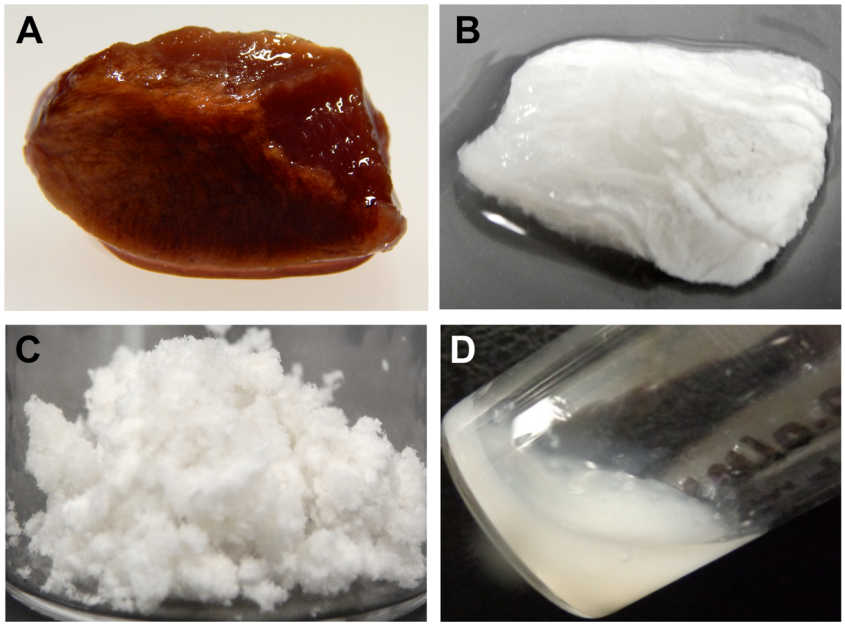

Figure 1. Decellularization and digestion of porcine cardiac ECM tissue. The porcine cardiac tissue was sliced into sections (A) and then decellularized (B). The decellularized tissue was further lyophilized and ground into powder (C), and then enzymatically digested into a liquid at room temperature (D).

injectable hydrogel for treating MI have been validated in both small- and large-animal studies.[14,16] However, the therapeutic mechanisms have been mostly related to the increased survival of resident cardiomyocytes and recruitment of endogenous cardiac stem cells through the released bioactive factors during hydrogel degradation.[16] Surprisingly, we notice that angiogenesis induced by cardiac matrix hydrogel has not been as significant as we observed with other natural hydrogels such as fibrin gels.[17] Revascularization is the key to improve the cell viability and engraftment after transplantation. Enhancing the angiogenic effects of cardiac matrix hydrogels will greatly improve the therapeutic outcomes.

In this study, we aimed to explore the feasibility of tuning the material properties of a cardiac matrix hydrogel to induce endothelial differentiation of stem cells. Our long-term goal is to use our developed genipin cross-linked cardiac matrix hydrogel to facilitate neovascularization and therefore boost its therapeutic efficiency in cardiac repair after MI. In this study, solubilized cardiac ECM from porcine tissue was cross-linked using various amounts of genipin. The influence of genipin on the gelation, mechanical and degradation properties of cardiac matrix hydrogel were investigated. Human mesenchymal stem cells (hMSCs) were cultured on the genipin cross-linked cardiac matrix hydrogel for cell viability evaluation. Vascular differentiation of hMSCs was demonstrated by expression of endothelial and smooth muscle cell markers.

2. MATERIALS AND METHODS

2.1. Materials. Sodium dodecyl sulfate (SDS), pepsin, genipin, phenol/chloroform/isoamyl alcohol, chloramine T/oxidation buffer, collagenase, calcium chloride ($CaCl_2$), bovine serum albumin (BSA), and glycine were purchased from Sigma and used as received. Collagen and Hoechst 33342 were obtained from Invitrogen.

2.2. Decellularization and Solubilization of Porcine Cardiac Tissue. The porcine cardiac tissue was decellularized using a detergent SDS and then solubilized using enzyme pepsin as previously described.[14,18] Briefly, fresh hearts were harvested from adult pigs (weighing 80−100 kg) in a local slaughterhouse. The cardiac tissue was cut into slices (2 mm thick; Figure 1A) and rinsed in deionized (DI) water to completely remove blood. The cardiac slices were then decellularized in 1% (w/v) SDS/phosphate buffered saline (PBS) solution for 3−4 days, until the tissue became white (Figure 1B). The obtained decellularized cardiac ECM was immersed in DI water to remove SDS residual. Finally, the decellularized cardiac ECM was lyophilized and ground into powder (Figure 1C). The powder was added in pepsin/0.01 M hydrochloric acid (HCl) solution at a pepsin:matrix ratio of 1:10 for 48 h with stirring. Then the solubilized cardiac ECM (Figure 1D) was neutralized using 0.1 M sodium hydroxide (NaOH) solution and 10× PBS, and then diluted into pregel solutions with desirable ECM concentrations using 1× PBS.

2.3. Genipin Cross-Linking. The solubilized cardiac ECM was cross-linked using genipin at various concentrations during self-assembly. Different concentrations of genipin solutions (0, 1, 2, and 5 mM) were prepared by dissolving the required amount of genipin in PBS (Corning; pH = 7.4). The mixture of solubilized cardiac matrix (final concentration, 6 mg/mL) and genipin solution was then incubated at 37 °C for 24 h. The formed cardiac matrix hydrogel was thoroughly rinsed in cell culture medium to remove residual genipin for further measurement and cell studies.

2.4. Characterization of Decellularized and Solubilized Cardiac Matrix. Haematoxylin and eosin (H&E) staining was used to evaluate the cellular content and general structure of the porcine cardiac tissue before and after decellularization. The tissue samples were fixed in 10% formalin, dehydrated with ethanol, embedded in paraffin, cut into 5 μm sections using a microtome (Leica Microsystems), and mounted on glass slides. These slides were stained with H&E (Leica) and imaged using an Axiovert A1 (Carl Zeiss, Inc.) microscope equipped with an AxioCam MRc camera (Carl Zeiss, Inc.).

Solubilized cardiac matrix was analyzed by SDS-PAGE and compared to collagen type I. The solutions were run on a Mini-PROTEAN TGX, 4−15% polyacrylamide gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in Tris/glycine/SDS buffer (Bio-Rad). Gel electrophoresis was performed in a Mini-PROTEAN Tetra Cell (Bio-Rad) at 200 V and then stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

DNA content of decellularized cardiac matrix was measured using PicoGreen DNA assay (Life Technologies, Inc.). Briefly, the solution of solubilized cardiac matrix was centrifuged at 2980*g* for 10 min for

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Table 1. Real Time PCR Primers

name	forward 5′−3′	reverse 5′−3′
18s rRNA	CGGGGAGGTAGTGACGAAAAATA	CGGCTGCTGGCACCAG
GAPDH	ACGCACATCGCTCAGACA	GCCCAATACGACCAAATCC
CD31	TGATGCCGTGGAAAGCAGAT	GCATCTGGCCTTGCTGTCTA
VECadherin	TGGTCACCCATGCATCTTCC	CCATGACGAAGGGTGAGCTT
GATA-6	TCTCCATGTGCATTGGGGAC	AAGGAAATCGCCCTGTTCGT
alpha SMA	AATACTCTGTCTGGATCGGTGGCT	ACGAGTCAGAGCTTTGGCTAGGAA
vWF	AGCCACCCCTCAGTGAAATG	ATGTCTGCTTCAGGACCACG

protein precipitation. The collected supernatants were then buffered in phenol/chloroform/isoamyl alcohol (25:24:1; Sigma) and centrifuged at 10000*g* for 30 min. The DNA was obtained by precipitating in 3 M sodium acetate/ethanol solution (v/v = 1:20) at −20 °C overnight, followed by air-drying at room temperature. The isolated DNA was quantified using PicoGreen DNA assay. Samples were run in triplicate.

Total collagen content analysis of the decellularized cardiac matrix was performed using a hydroxyproline assay (Sigma) with a hydroxyproline/collagen conversion factor of 14.3%.[19,20] The samples were hydrolyzed with 6 M HCl and then reacted with chloramine T/oxidation buffer mixed solution for 5 min, followed by addition of dimethylaminobenzaldehyde solution and incubation at 60 °C for 90 min. The results were read at 560 nm using an Infinite M200 plate reader (Tecan, Reading, U.K.). GAG content in the decellularized cardiac matrix was quantified using the Blyscan sulfated glycosaminoglycan assay following the manufacturer's instruction (Biocolor, Carrickfergus, U.K.). Samples were run in triplicate.

2.5. Turbidimetric Gelation Kinetics. The gelation kinetics of the cardiac matrix hydrogels with various genipin concentrations was evaluated turbidimetrically.[21] The pregel solutions were prepared and stored on ice. For each concentration, 100 μL/well was added in triplicate into a 96 well plate and read spectrophotometrically in a Synergy H1 Hybrid microplate reader (BioTek) preheated to 37 °C. Absorbance at 405 nm was recorded every 2 min for 60 min. The absorbance readings were scaled from 0 (at time 0) to 100% (at maximum absorbance) according to eq 1, where NA is normalized absorbance, A is the absorbance at a given time, A_0 is the initial absorbance, and A_{max} is the maximum absorbance.

$$\mathrm{NA} = (A - A_0)/(A_{max} - A_0) \quad (1)$$

The kinetic parameters were defined as follows: time to half-gelation, time to reach 50% absorbance; the gelation rate, slope of the linear region of the gelation curve; and the lag time, the intercept of the linear region with 0% absorbance.

2.6. Swelling Ratio. The solubilized cardiac matrix solution (0.5 mL, 6 mg/mL) was added into a 1 cm (i.d.) metal cylinder mold. The mold was incubated at 37 °C for 1 day to obtain the cylinder hydrogel, and then it was immersed in PBS for 24 h at 37 °C to measure its swelling ratio. The hydrogel was weighed immediately to obtain the swollen weight (W_s) after excess water was gently removed with a Kimwipe. The weighted swollen hydrogels were then lyophilized and weighed to obtain its dry weight (W_d). The swelling ratio (%S) was determined according to eq 2.

$$\%S = (W_s - W_d)/W_d \times 100 \quad (2)$$

2.7. Rheological Measurement. Prehydrogel solutions (0.5 mL, 6 mg/mL) were added into a metal cylinder mold (i.d. = 1 cm) and allowed to gel at 37 °C overnight. Mechanical properties were measured using parallel plate rheometry (Ares RFS III). A 0.8 mm gap between plates was maintained for all hydrogels. Three consecutive frequency sweeps were conducted on each hydrogel within the linear viscoelastic strain region of 1.5% (determined experimentally), throughout the range of 0.1−5.0 rad/s. The complex modulus and phase angle were reported at 2.5 rad/s.

2.8. In Vitro Degradation. The solubilized cardiac matrix with varying genipin concentrations (0, 1, and 2 mM) was exposed to collagenase type I to evaluate the degradation as previously described.[22−24] Briefly, the pregel solutions (0.5 mL, 6 mg/mL) were added in preweighed 1.5 mL microcentrifuge tubes and incubated at 37 °C for 24 h to form solid hydrogels. The hydrogels were then exposed to 0.5 mL of collagenase type I (125 U/mL) in PBS with 0.9 mM $CaCl_2$ for 5 or 24 h; the control gels were exposed to PBS alone instead of collagenase. At each time point, the collagenase type I solution was then completely removed from the tubes, and the tube was immediately weighed to obtain the hydrogel wet weight (W_1). The control hydrogel in PBS was weighed (W_2) as described above. The percent weight remaining was calculated as $W_1/W_2 \times 100\%$; $n = 3$ for each condition at each time point.

2.9. Human Mesenchymal Stem Cell Culture. hMSCs (Lonza, Walkersville, MD, USA) were cultured with Poietics MSCGM BulletKit (Lonza) containing mesenchymal stem cell growth supplement, L-glutamine, and GA-1000. Culture was maintained in a humidified 37 °C cell incubator and 5% CO_2. Media was replaced every 2−3 days, and cells were split prior to reaching 80% confluence. Cells at passages 3−7 were used for all studies.

2.10. Cell Viability. Cylinder hydrogels (1 cm diameter) were incubated with hMSC culture media for 24 h. hMSCs (2×10^4) were were then seeded on the hydrogel surface and cultured for 24 h. The samples were then stained with calcein AM, ethidium homodimer-1 (2 mM; Molecular Probes), and Hoechst 33342 (1:1000) at 37 °C for 30 min. After washing with PBS, the samples were imaged with an Olympus Fluoview FV1000 confocal microscope. Three representative areas from each hydrogel were imaged serially through the thickness of the hydrogel. The images were stacked using FV1000 software to show all of the cells through the imaged hydrogel, and the cells were counted using ImageJ software (National Institute of Health, Bethesda, MD, USA). Cell viability (live cells/total cell number) was determined by taking the average cell numbers of the three images per sample.

2.11. Gene Expression. Real-time, quantitative, reverse transcription-polymerase chain reaction (RT-PCR) was performed to elucidate the effects of genipin cross-linking. RNA was extracted from hMSCs after 24 h of culture on the cardiac matrix hydrogels with or without genipin cross-linking. The hydrogels (6 mg/mL) were formed in wells of a 6 well plate with 2.5×10^5 hMSCs seeded on top. Cells on tissue culture plastic (TCP) without cardiac matrix hydrogel served as a control. RNA was extracted by digesting the hydrogel with collagenase type II (4 mg/mL) and then isolated using an Absolutely RNA Microprep Kit (Stratagene). The total amount and purity of isolated RNA was measured using a Synergy H1 Hybrid Reader nanodrop and 1% agarose gel electrophoresis. Total RNA was reverse transcribed into cDNA with a Verso cDNA kit (Thermo Scientific) per manufacturer's instructions. cDNA and reverse transcription controls with no enzyme were evaluated via RT-PCR using SYBR GreenER qPCR SuperMix (Invitrogen). Relative gene expression was analyzed with the comparative C_t method. The results were normalized to 18s rRNA and GAPDH and expressed as a fold change for cardiac matrix relative to TCP control ($\Delta\Delta C_t$). Primer sequences are listed in Table 1.

2.12. Immunocytochemistry. Hydrogels (1 cm diameter, 6 mg/mL) were incubated with media for 24 h and then seeded with 2×10^4 hMSCs. The cells were cultured for 48 h and then fixed with 1 mL 4% paraformaldehyde (PFA) (USB Corp.) for 30 min. After rinsing with fresh PBS, the samples were frozen with OCT and sectioned into 20 μm thick slices using cryostat (Leica CM1860 UV). The slides were postfixed with 2% PFA for 10 min and rinsed with PBS containing 0.01% Triton X-100. The slices were then incubated for 10 min with

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PBS containing 0.20% Triton X-100 to improve penetration of the antibodies. The sections were blocked with 5% horse serum for 30 min, followed by incubation with vWF antibody (Santa Cruz Biotechnology, Inc.) at 4 °C overnight. After PBS rinse containing 0.01% Triton X-100, the secondary antibody, donkey antigoat IgG, was added and incubated for 45 min at room temperature. The slides were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Inc.) to stain the cell nuclei. Images were taken with a Carl Zeiss microscope.

2.13. Statistical Analyses. All results presented as mean ± standard deviation. Statistical analyses were performed using Minitab 17 statistical software. Statistical comparisons were made by performing single factor analysis of variance (ANOVA) with Tukey's post-hoc test and a student's *t* test for ECM components (DNA, collagen, and GAG). *p* values less than 0.05 were considered statistically significant.

3. RESULTS

3.1. Characterization of Decellularized and Solubilized Cardiac Matrix. The solubilized cardiac matrix was derived through decellularization and enzymatic digestion of porcine cardiac tissue (Figure 1). To examine the preserved ECM components in solubilized cardiac matrix, we revealed the protein components using gel electrophoresis (Figure 2A).

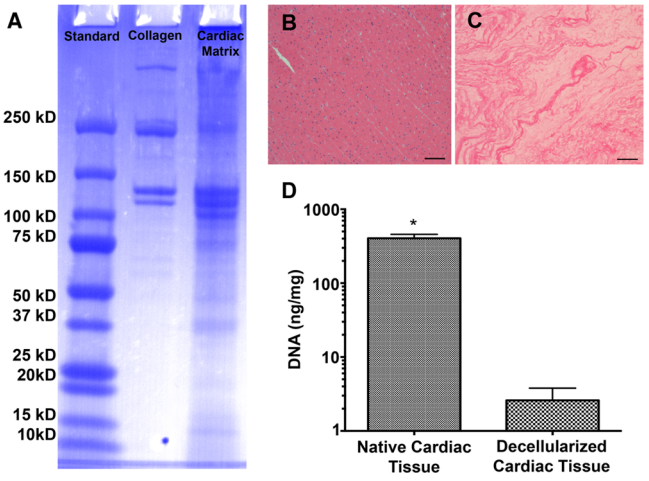

Figure 2. Characterization of decellularized cardiac ECM. (A) SDS-PAGE analysis of the solubilized cardiac ECM compared to rat-tail type I collagen. H&E staining of native cardiac tissue (B) and decellularized cardiac ECM (C). Scale bars represent 100 μm. (D) DNA contents of native tissue and decellularized cardiac ECM. $n = 3$. (Asterisk (*) marks $p < 0.01$.)

Compared to collagen type I, the solubilized cardiac matrix contained a more complex mixture of ECM components, indicated by several lower molecular weight bands. H&E staining of the sectioned cardiac matrix after decellularization confirmed the complete removal of cellular nuclei (Figure 2B,C). The absence of cell nuclei and significantly decreased DNA content compared with native cardiac tissue (Figure 2D) demonstrated sufficient decellularization of porcine cardiac tissue following our protocol. In addition, we quantitatively assessed the collagen and GAG content before and after decellularization and digestion, and our results were generally consistent with the values reported in previous studies.[20,21,25] The collagen content of the solubilized cardiac matrix was determined to be 39.6 ± 1.5% dry weight, which was greater than the 17.6 ± 4.6% found for the native cardiac tissue. Conversely, the total sulfated GAG content of the solubilized matrix was reduced to 0.5 ± 0.1 μg/(mg of dry ECM), compared with 2.1 ± 0.5 μg/(mg dry native tissue).

3.2. Gelation and Turbidimetric Gelation Kinetics. The cardiac matrix hydrogels were obtained by cross-linking from 6 mg/mL solubilized cardiac matrix solution with 1 and 2 mM genipin for 24 h. The normally opaque cardiac matrix hydrogels turned blue with increasing intensity as genipin concentration increased (Figure 3A). The turbidimetric gelation kinetics for

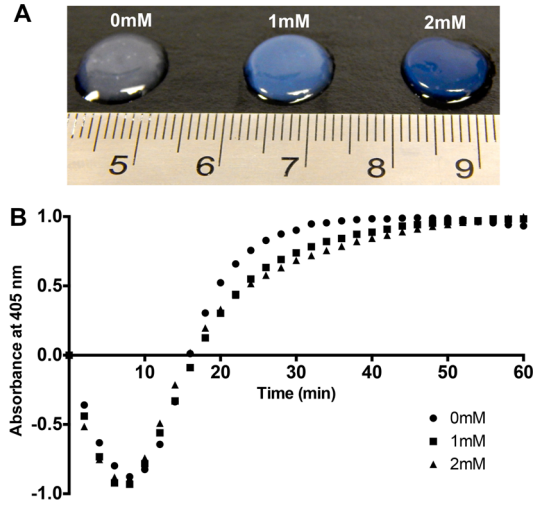

Figure 3. Genipin cross-linking and turbidimetric gelation kinetics of cardiac matrix hydrogels. (A) The opaque cardiac matrix hydrogels turned blue with increasing intensity as genipin concentration increased. (B) The gelation kinetic curves of cardiac matrix hydrogels with and without genipin cross-linking.

all of the cross-linking conditions showed a sigmoidal shape (Figure 3B) with gelation occurring after a lag period (t_{lag}). Cross-linking with genipin for 24 h did not have an effect on gelation kinetics. The times to 50% gelation ($t_{1/2}$) for 0, 1, and 2 mM genipin cross-linking were 14.9 ± 4.9, 15.3 ± 6.5, and 15.6 ± 3.9 min, respectively, without significant difference.

3.3. Swelling Ratio. The swelling ratio of cross-linked cardiac matrix hydrogels with 1 and 2 mM genipin was calculated and compared to un-cross-linked hydrogels (Figure 4). The swelling ratio of the cardiac matrix hydrogel without cross-linking was 7067 ± 192%. After cross-linking with genipin, the swelling ratios were markedly reduced ($p < 0.05$). The hydrogel cross-linked with 1 mM genipin had a swelling ratio of 4961 ± 327%, which was significantly higher

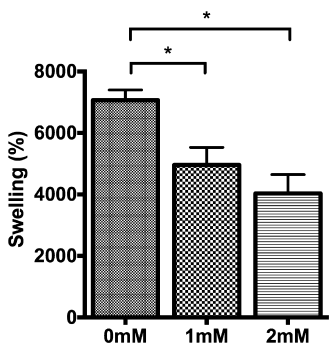

Figure 4. Swelling ratios of cardiac matrix hydrogels cross-linked using 0, 1, and 2 mM genipin.

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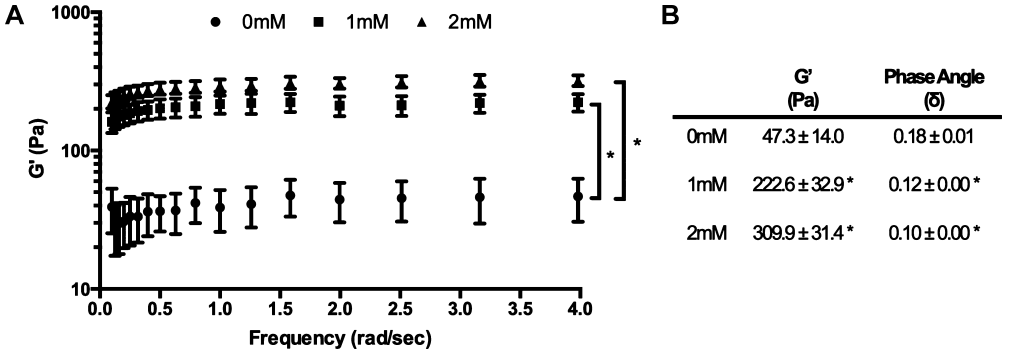

Figure 5. Rheological effects of genipin cross-linking. (A) Plot of rheological data and (B) list of storage modulus (G') and phase angle (δ) of cardiac matrix hydrogels cross-linked using 0, 1, and 2 mM genipin.

than that of the hydrogel cross-linked with 2 mM genipin (4034 ± 353%; $p < 0.05$).

3.4. Rheometry of Genipin Cross-Linked Cardiac Matrix Hydrogels. Parallel plate rheometry was performed on cardiac matrix hydrogels to quantify the stiffness of genipin cross-linked hydrogels. A frequency sweep at a constant strain of 1.5% was conducted and shown over the range of 0.1−5 rad/s (Figure 5A). At 1.5 rad/s the un-cross-linked cardiac matrix hydrogel had a storage modulus (G') value of 47.3 ± 14.0 Pa. The genipin cross-linking was shown to significantly increase the stiffness of the cardiac matrix hydrogels. The G's of the hydrogels cross-linked with 1 and 2 mM genipin were 222.6 ± 32.9 and 309.9 ± 31.4 Pa, respectively, which were significantly higher than that of the un-cross-linked hydrogel (Figure 5B). The phase angle (δ), which indicates the relative elasticity or viscosity of a material, was significantly lower when the cardiac matrix was cross-linked with genipin, indicating that cross-linking is able to bring the material closer to an ideal elastic material ($\delta = 0$; Figure 5B).

3.5. Degradation of Cardiac Matrix Hydrogels. The effect of genipin cross-linking on the rate of degradation of cardiac matrix hydrogels was evaluated using 125 U/mL type I collagenase (Figure 6). After 5 h, the percent weight remaining

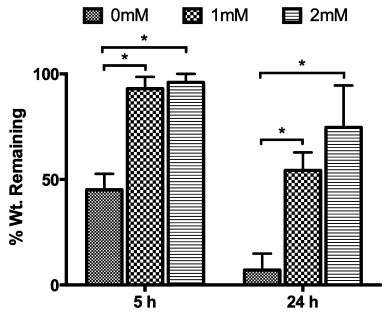

Figure 6. Degradation of un-cross-linked and genipin cross-linked cardiac matrix hydrogels after 5 and 24 h incubation in bacterial collagenase I solution.

of the un-cross-linked hydrogel was significantly lower (45.3 ± 4.4%) than those of the genipin cross-linked hydrogels (93.0 ± 3.2% for 1 mM genipin cross-linked and 96.3 ± 2.3% for 2 mM genipin cross-linked). After 24 h, the un-cross-linked cardiac matrix hydrogel was nearly fully degraded with a percent weight of 7.0 ± 4.5, which was significantly lower than the genipin cross-linked hydrogels. The 1 and 2 mM genipin cross-linked hydrogels remained intact and degraded to 54.3 ± 4.9% and 74.7 ± 11.5%, respectively.

3.6. Cell Viability. The cytotoxic effect of genipin was evaluated by culturing hMSCs for 24 h on the genipin cross-linked cardiac matrix hydrogel. hMSCs attached on all of the genipin cross-linked cardiac matrix hydrogels 1−2 h after cell seeding. The result of live/dead staining indicated that genipin at 1 and 2 mM did not cause significant cell death (Figure 7A). Cell viability was comparable between cardiac matrix hydrogels with or without genipin cross-linking (Figure 7B).

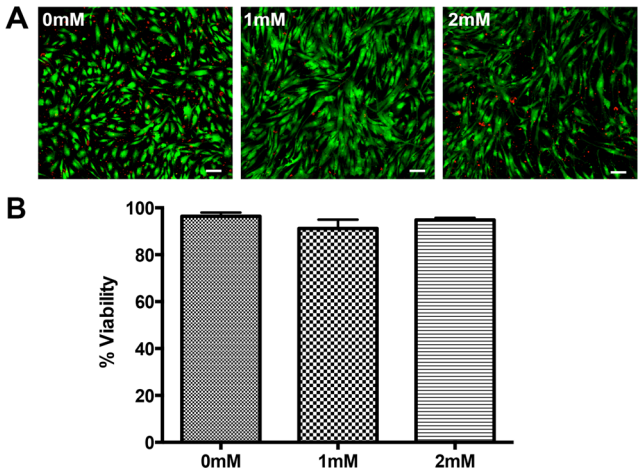

Figure 7. Effect of genipin (0, 1, and 2 mM) on the viability of hMSCs cultured on cardiac matrix hydrogels at day 1. (A) Representative live/dead confocal microscopy images of hMSCs (live cells in green, dead cell in red) and (B) quantitative measure of hMSCs viability using ImageJ analysis.

3.7. Cardiovascular Differentiation of hMSCs. After 24 h of hMSCs culture on genipin cross-linked cardiac matrix hydrogels, we analyzed the cardiovascular gene expression of hMSCs via RT-PCR. Various cardiovascular genes were assessed including: endothelial genes (CD 31, VE cadherin, vWF), smooth muscle gene (αSMA), and cardiac gene (GATA-6). As shown in Figure 8, the culture of hMSCs on cardiac matrix hydrogels (with and without genipin cross-linking) induced the expression of all of the assessed cardiovascular genes. Genipin cross-linked cardiac matrix hydrogels significantly up-regulated the expression of early cardiac marker, GATA-6, and smooth muscle cell marker αSMA. Interestingly, the expression of early endothelial cell markers (CD31 and VE cadherin) was significantly decreased when hMSCs were

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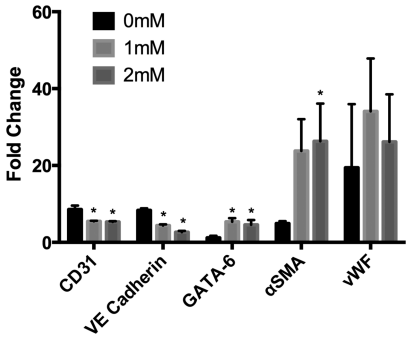

Figure 8. qPCR analysis of vascular marker gene expression in hMSCs after 1 day culture on un-cross-linked or genipin cross-linked cardiac matrix hydrogels. The results were normalized to 18s rRNA and expressed as a fold change for hydrogels over TCP ($\Delta\Delta C_t$).

cultured on cardiac matrix hydrogels with genipin cross-linking compared with hydrogels without genipin cross-linking ($p < 0.05$). However, there was a trend for increased expression of mature endothelial cell marker vWF of cells cultured on genipin cross-linked cardiac matrix hydrogels. Immunofluorescence staining also confirmed the vWF expression of cells cultured on all genipin cross-linked cardiac matrix hydrogels on day 1. Robust vWF expression was only shown on cells cultured 3 days on 1 mM genipin cross-linked cardiac matrix hydrogels (Figure 9).

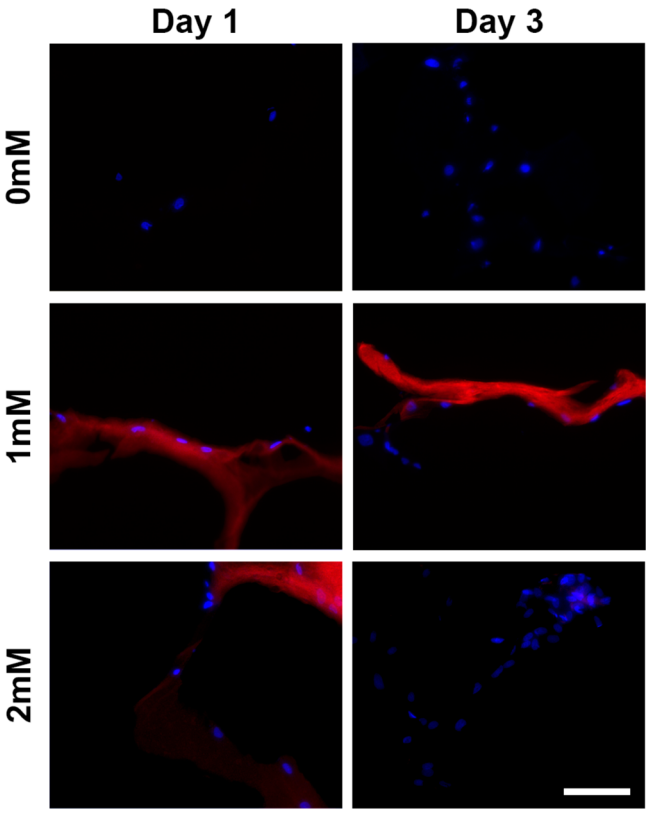

Figure 9. Immunofluorescence staining of *von Willebrand factor* (vWF) expression in hMSCs after 1 day and 3 day culture on un-cross-linked (0 mM) and genipin cross-linked cardiac matrix hydrogels (1 and 2 mM): red, vWF expression; blue, cell nuclei. Scale bar = 50 μm.

4. DISCUSSION

Cardiac matrix hydrogel has many superior advantages as a biologically derived material for tissue engineering applications. Derived from solubilized acellular cardiac tissue, it retains the organ-specific biochemical composition mimicking the native biochemical microenvironment of cells. Biochemical cues from the cardiac matrix have been shown to enhance cardiac differentiation of human embryonic stem cells, c-kit $^+$ cardiac progenitor cells, and MSCs in vitro.[17,26,27] Additionally, the appropriate gelation time of cardiac matrix hydrogel enables its minimally invasive delivery. Recent large-animal studies have demonstrated that solubilized cardiac matrix could gel in situ after catheter delivery and proved the safety and efficacy of using cardiac matrix hydrogel for treating MI.[28] Furthermore, it is biodegradable and has the potential to be used as a platform for prolonged retention and delivery of bioactive factors.[29,30] Despite mounting evidence suggesting the promise of a cardiac matrix hydrogel as a biomimetic scaffold, further tuning of the materials properties needs to be conducted to fully exploit their advantages.

Generally, weak mechanical properties and rapid enzymatic degradation are the two limiting factors restraining the potentials of cardiac matrix hydrogels.[31] Cross-linking strategies have been explored to overcome these limitations. Chemical cross-linking agent, glutaraldehyde (GA), has been used to increase the stiffness and slow the degradation rate of cardiac matrix hydrogels.[32] But the undesirable cytotoxic effects of chemical cross-linking agents prevent them from being used in cell-based applications especially in a clinical setting. Another strategy that has been explored is to create hybrid hydrogels by combining cardiac matrix hydrogel with other synthetic or natural hydrogels such as poly(ethylene glycol), collagen and fibrin.[31,33,34] Although the incorporated component in the hybrid hydrogels has dramatically enhanced the tunability, the alteration of the organ-specific biocomposition within the cardiac matrix hydrogel could affect their interactions with cells.

Genipin, on the other hand, is an attractive cross-linking agent. It is a natural compound extracted from the fruit of *Gardenia jasminoides* Ellis, which has been used over hundreds of years in traditional Chinese medicine for the treatment of inflammation, jaundice, headache, edema, fever, hepatic disorders, and hypertension.[35] The therapeutic effects of genipin have been mostly related to anti-inflammation and protecting cells from stress-induced cytotoxicity.[36,37] In addition, genipin is a remarkable water-soluble cross-linking agent. The utilization of genipin to fabricate stable and biocompatible cross-linked biomaterials has been explored in several studies. Genipin cross-linked collagen, chitosan, and fibrin all demonstrated improved mechanical properties and high biocompatibility.[38,39] The results of an in vitro cytotoxicity evaluation using 3T3 fibroblasts have proved that genipin was about 10000 times less cytotoxic than glutaraldehyde for biological tissue fixation.[40] The excellent cross-linking ability and superior biocompatibility have enabled genipin, as a natural cross-linking agent, to overcome the disadvantages of chemical cross-linking.

In this study we explored the feasibility of fine-tuning the material properties of a cardiac matrix hydrogel using genipin to induce endothelial differentiation of hMSCs. Several strategies have been used to mediate the material properties of decellularized cardiac matrix hydrogels but with the focus of inducing cardiac differentiation.[17,33,34] To the best of our

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knowledge, this is the first study on tuning material properties of cardiac matrix hydrogels specifically toward promoting endothelial differentiation of hMSCs. Mechanical cues play a critical role in directing cell response. Our group has previously demonstrated that the PEGylated fibrin gel with the stiffness range of 100−200 Pa would dramatically induce the vascular differentiation of hMSCs without the addition of chemical inducers.[41,42] The stiffness of the cardiac hydrogel without genipin cross-linking is much lower than this value, while the hydrogel stiffness (200−300 Pa) is comparable to this range after genipin cross-linking (Figure 5). It might be a major contribution to improve angiogenic effects of cardiac matrix hydrogels.

The genipin involvement has great effects on hydrogel properties (Figures 3−6). The increase of genipin concentration increased the cross-linking degree of the hydrogel, which resulted in higher stiffness while lowering the swelling ratio and slowing degradation. In the pilot study, it was found that cell viability was significantly lower in the cardiac matrix hydrogel cross-linked using 5 mM genipin (data not shown). Thus, a concentration limit of 2 mM was set for genipin use in this study. Interestingly, we found that genipin cross-linking would not significantly alter the gelation kinetics of the cardiac matrix hydrogel. This suggests that the cross-linked hydrogel could still be delivered through a catheter for minimally invasive therapy. The major component of the ECM is collagen, which majorly contributes to the gelation of ECM hydrogel without genipin cross-linking.[21] Thus, the gelation of this genipin cross-linked ECM hydrogel includes genipin cross-linking and fibril formation of the ECM protein components. It was reported that the low concentration of genipin has no significant effect on the gelation of collagen hydrogel during a beginning period of approximately 1−2 h, and it took 24 h to complete the cross-linking reaction.[43] The ECM gelation without genipin showed a gelation time at ∼30 min at 37 °C. Thus, the genipin addition has no obvious effect on the gelation time during our observation period. Additionally, the genipin cross-linking reaction still continued although the plateau at 30 min of the gelation curve was observed. Hence, we kept the samples for 24 h to allow the completed cross-linking reaction prior to the mechanical testing and biological evaluation. The degradation rate of the cardiac matrix hydrogel has shown to be significantly decreased after genipin cross-linking, which will lead to prolonged release of bioactive factors and may boost their efficacy in facilitating cardiac repair. The ultimate effects of prolonged degradation will need to be examined in the future.

With regard to vascular differentiation, the endothelial markers (CD31, VE Cadherin, and vWF) were expressed by hMSC cultured on both genipin cross-linked and un-cross-linked cardiac matrix hydrogels. However, cells on genipin cross-linked hydrogels showed down-regulation of early endothelial cell markers (CD31) and up-regulation of mature endothelial cell marker (vWF) compared with cells on un-cross-linked hydrogels. This indicates that genipin cross-linking of the hydrogel may accelerate the process of endothelial differentiation and maturation of hMSCs. Chemical and mechanical cues regulate stem cell differentiation. Decellularized matrix derived from highly vascularized cardiac tissue may contain vascular-specific chemical cues to potently promote endothelial differentiation of stem cells. Genipin cross-linking can tune the degradation and mechanical properties of cardiac matrix hydrogel, which may synergistically integrate the chemical and mechanical cues to facilitate the endothelial differentiation process. Further investigation will be performed in future studies to elucidate the mechanisms behind the accelerated endothelial differentiation induced by genipin cross-linked hydrogels. In addition, the increased mechanical properties also promote the expressions of vascular marker (αSMA) and early cardiac marker (GATA-6). It should be noted that all of the above observations on vascular differentiation are based on relatively short time interactions between cells and hydrogels (1 day and 3 days). In future studies we will be looking to further explore the induction of vascular differentiation on genipin cross-linked cardiac matrix hydrogels during a longer time frame (e.g., 7 and 14 days). One limitation of the current study is derived from the 2D culture of cells, although it did provide the information enabling us to test our hypothesis in a simple and straightforward way. It is suggested to further investigate the effects of genipin cross-linking on cardiac matrix hydrogels using 3D cell culture and in a rat MI model.

5. CONCLUSIONS

A natural cross-linker, genipin, was used to tune the material properties of porcine myocardium-derived hydrogels. The genipin cross-linking increased the mechanical properties of the cardiac matrix hydrogel to a stiffness range promoting vascular differentiation of hMSCs. It also decreased the swelling ratio and prolonged degradation without altering gelation time. hMSCs cultured on the genipin cross-linked cardiac matrix hydrogels showed great viability. After 1 day culture, hMSCs demonstrated down-regulation of early endothelial marker expression and up-regulation of mature endothelial marker expression. Especially for 1 mM genipin cross-linked cardiac matrix hydrogels, hMSCs showed particularly significant expression of mature endothelial cell marker vWF after 3 day culture. These attractive results indicate the potential of using our developed genipin cross-linked cardiac matrix hydrogels to promote rapid vascularization for cardiac infarction treatment through minimally invasive therapy.

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Notes

The authors declare no competing financial interest.

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