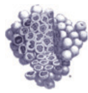
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Effects of Matrix Metalloproteinases on the Performance of Platelet Fibrin Gel Spiked With Cardiac Stem Cells in Heart Repair

DELIANG SHEN,[a,*] JUNNAN TANG,[a,b,c,*] MICHAEL TAYLOR HENSLEY,[b,c] TAOSHENG LI,[d] THOMAS GEORGE CARANASOS,[e] TIANXIA ZHANG,[b] JINYING ZHANG,[a] KE CHENG[b,c,f]

Key Words. Cardiac stem cells • Platelet fibrin gel • Matrix metalloproteinases • Heart repair

ABSTRACT

Stem cells and biomaterials have been studied for therapeutic cardiac repair. Previous studies have shown the beneficial effects of platelet fibrin gel and cardiac stem cells when cotransplanted into rodent hearts with myocardial infarction (MI). We hypothesized that matrix metalloproteinases (MMPs) play an important role in such protection. Thus, the present study is designed to elucidate the effects of MMP inhibition on the therapeutic benefits of intramyocardial injection of platelet fibrin gel spiked with cardiac stem cells (cell-gel) in a rat model of acute MI. In vitro, broad-spectrum MMP inhibitor GM6001 undermines cell spreading and cardiomyocyte contraction. In a syngeneic rat model of myocardial infarction, MMP inhibition blunted the recruitment of endogenous cardiovascular cells into the injected biomaterials, therefore hindering de novo angiogenesis and cardiomyogenesis. Echocardiography and histology 3 weeks after treatment revealed that metalloproteinase inhibition diminished the functional and structural benefits of cell-gel in treating MI. Reduction of host angiogenesis, cardiomyocyte cycling, and MMP-2 activities was evident in animals treated with GM6001. Our findings suggest that MMPs play a critical role in the therapeutic benefits of platelet fibrin gel spiked with cardiac stem cells for treating MI. STEM CELLS TRANSLATIONAL MEDICINE *2016;5:793–803*

SIGNIFICANCE

In this study, the effects of matrix metalloproteinase inhibition on the performance of platelet gel spiked with cardiac stem cells (cell-gel) for heart regeneration are explored. The results demonstrate that matrix metalloproteinases are required for cell-gel to exert its benefits in cardiac repair. Inhibition of matrix metalloproteinases reduces cell engraftment, host angiogenesis, and recruitment of endogenous cardiovascular cells in rats with heart attack.

[a]Department of Cardiology, First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, People's Republic of China; [b]Department of Molecular Biomedical Sciences and Comparative Medicine Institute, North Carolina State University, Raleigh, North Carolina, USA; [c]Department of Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, North Carolina, USA; [d]Department of Stem Cell Biology, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan; [e]Division of Cardiothoracic Surgery, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA; [f]Cyrus Tang Hematology Center, Soochow University, Suzhou, Jiangshu, People's Republic of China

[*]Contributed equally.

Correspondence: Ke Cheng, Ph.D., North Carolina State University, 1060 William Moore Drive, Raleigh, North Carolina 27607, USA. Telephone: 919-513-6157; E-Mail: ke_cheng@ncsu.edu or ke_cheng@unc.edu; or Jinying Zhang, Ph.D., M.D., First Affiliated Hospital of Zhengzhou University, No. 1 East of Jianshe Road, Zhengzhou 450052, People's Republic of China. Telephone: 86-13503830283; E-Mail: jyzhang@zzu.edu.cn

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INTRODUCTION

Heart disease remains the number one cause of death in developed countries, generating substantial socioeconomic costs [1]. A typical myocardial infarction (MI) causes the death of billions of cardiomyocytes and can further develop into heart failure. Once failure occurs, only palliative and sympathetic treatment options are available to patients.

Stem cells and biomaterials are promising alternatives to conventional pharmacologic therapies for therapeutic cardiac repair [2, 3]. Because of their minimally invasive nature, injectable biomaterials are appealing choices to be administered, alone or blended with stem cells [4, 5]. Numerous injectable biomaterials have been studied for cardiac repair, including fibrin gel [6], collagen [7], self-assembling peptides [8], hydrogel [9], polypropylene [10], and decellularized myocardium matrix [11]. Among them, platelet fibrin gel (also known as platelet fibrin scaffold) is an appealing choice for therapeutic development because it can be easily produced from blood as either an allogeneic or autologous product. Cardiac stem cells (CSCs) have been proven to be safe and effective in early phases of clinical trials for treating myocardial infarction [12, 13]. Previous studies from our group showed that injection of platelet gel alone attenuated adverse left ventricular remodeling and preserved cardiac function in rats with acute MI [14], whereas injection of platelet gel spiked with CSCs had even better therapeutic benefits [15]. It is unclear whether the extra benefits were merely from the addition of cardiac stem cells or complex interactions between the cells and the biomaterial.

Matrix metalloproteinases (MMPs) are a family of structurally related, zinc-containing enzymes that contribute to the degradation of

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extracellular matrix proteins and connective tissue proteins [16]. MMPs have vital roles in the development, physiology, and pathology of the cardiovascular system by acting on cardiovascular remodeling, atherosclerotic plaque formation and plaque instability, vascular smooth muscle cell migration, and restenosis [17]. MMPs also play key roles in extracellular matrix catabolism and activation and inactivation of cytokines, chemokines, growth factors, and other proteinases at the cell surface and within the extracellular matrix. Their activities are tightly regulated in a number of ways, such as transcriptional regulation, proteolytic activation, and interaction with tissue inhibitors of metalloproteinases [18]. In the wound healing response, MMP inhibition has a negative effect on cell phenotype and dynamics [19–21]. In the CSC/platelet fibrin gel (cell-gel) system that we used for cardiac repair, MMPs are likely to play an important role in promoting CSCs to migrate outside of the gel and, conversely, recruiting endogenous cardiomyocytes and endothelial cells into the gel. Thus, we hypothesized that MMPs play an essential role in the therapeutic benefits of cell-gel in cardiac repair. In the present study, we aimed to explore the therapeutic benefits of intramyocardial injection of cell-gel (platelet fibrin gel spiked with CSCs) in a rat model of acute MI with or without the broad-spectrum MMP inhibitor GM6001.

Materials and Methods

Derivation of Platelet Fibrin Gel

Platelet fibrin gel was derived from the vein blood of Wistar-Kyoto (WKY) rats [22]. Under deep anesthesia (by inhalation of isoflurane), an incision was created at the rat's abdominal skin, and the vena cava was located. Venous blood (3–5 ml) was drawn from the vena cava. To stop the coagulation cascade, the blood was immediately citrated with 10% (vol/vol) 10 mM sodium citrate (Sigma-Aldrich, St Louis, MO, http://www.sigmaaldrich.com), followed by centrifugation at 1,000 *g* for 10 minutes and collection of the supernatant (platelet-containing plasma). Whole blood samples were sealed and left at room temperature for a period of 2 hours and placed overnight at 4°C to allow blood cells and blood plasma to fractionate. Samples were then centrifuged at 1,000 *g* for 10 minutes, and the supernatant was collected. Supernatants were centrifuged for a second time at 1,000 *g* for 10 minutes to remove any residual blood cells, and blood plasma was pooled and frozen at −20°C. For gel formation, the prewarmed platelet-containing plasma was mixed with prewarmed Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com) at a ratio of 1:1 (vol/vol) and returned to 37°C for ~3–5 minutes (Fig. 1). The calcium in DMEM reinitiates the coagulation process, which leads to the formation of a stable gel.

Derivation of Rat CSCs

CSCs were derived from the hearts of WKY rats using the reported cardiosphere method as previously described [23–27]. Myocardial specimens harvested from WKY rats were cut into fragments of <2 mm^3, washed with phosphate-buffered saline, and partially digested with collagenase (Sigma-Aldrich). The tissue fragments were cultured as cardiac explants on a 0.5-mg/ml fibronectin solution–coated surface in Iscove's modified Dulbecco's medium (IMDM; Thermo Fisher Scientific Life Sciences) containing 20% fetal bovine serum. A layer of stromal-like cells emerged from the cardiac explant with phase-bright cells over them. The explant-derived cells were harvested using TryPEL Select (under direct visualization of no more than 5 minutes) (Thermo Fisher Scientific Life Sciences). Harvested cells were seeded at a density of 2×10^4 cells/ml in UltraLow Attachment flasks (Corning, Corning, NY, http://www.corning.com) for cardiosphere formation. In ~3–7 days, explant-derived cells spontaneously aggregated into cardiospheres. The cardiospheres were collected and plated onto fibronectin-coated surfaces to generate cardiosphere-derived CSCs. CSCs were embedded in the scaffold during gel formation to become cell-gel (Fig. 1). The culture was maintained in IMDM (Thermo Fisher Scientific Life Sciences) containing 10% fetal bovine serum.

Cell proliferation, viability, and morphology in the gel were characterized and compared with the control cells cultured on tissue culture plates (TCPs). For cell proliferation, 1×10^4 rat CSCs were cultured in 1 ml platelet fibrin gel and on TCPs for 7 days. Representative cell cultures were then stained with Live/Dead Viability/Cytotoxicity Kit (Thermo Fisher Scientific Life Sciences) after 12 hours and 3 and 7 days. The number of live cells in three randomized microscopic fields was counted. Cell numbers were normalized to the numbers at 12 hours to generate a cell growth curve. Similarly, for the viability assay, 1×10^4 rat CSCs were cultured in platelet fibrin gel and on TCPs for 7 days and then stained with the same Live/Dead Viability/Cytotoxicity Kit, based on green fluorescent calcein-AM (live) and red fluorescent ethidium homodimer-1 (dead). Cell morphology was characterized from the same images using ImagePro software.

Culture of Neonatal Rat Cardiomyocytes or Mononuclear Cells in Cell-Gel With or Without GM6001

To examine the impact of MMP inhibitor GM6001 on cardiomyocytes or bone marrow mononuclear cells (MNCs) cultured in cell-gel, we cultured neonatal rat cardiomyocytes (NRCMs) or MNCs in the cell-gel with GM6001 (EMD Millipore, Darmstadt, Germany, http://www.emdmillipore.com/US/en) or a scrambled control (GM6001 Negative Control [NC]) from the same vendor at a concentration of 20 μM. These NRCMs and MNCs were derived from the same strain of WKY rats from which the CSCs were derived (Fig. 1), as previously described [28, 29]. The cells were incubated at 37°C and 5% CO_2. To distinguish between the NRCMs or MNCs and CSCs in the gel, CSCs were prelabeled with green fluorescent DiO and NRCMs or MNCs were stained with red fluorescent CM-DiI (Thermo Fisher Scientific Life Sciences). To evaluate cell-gel degradation under physiological conditions, we measured the thickness of the gel/cell composite over time by determining the *z* distance of cell distribution with a confocal microscope.

Release of In Vitro Growth Factors

To study sustained release of growth factors, we collected conditioned media at various time points (days 2, 5, 9, and 14), and fresh media was added back into the well to be conditioned for future collection. The concentrations of various growth factors were determined with enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, http://www.rndsystems.com).

Animal Models

All animal work is compliant with the Institutional Animal Care and Usage Committee (IACUC, North Carolina State University).

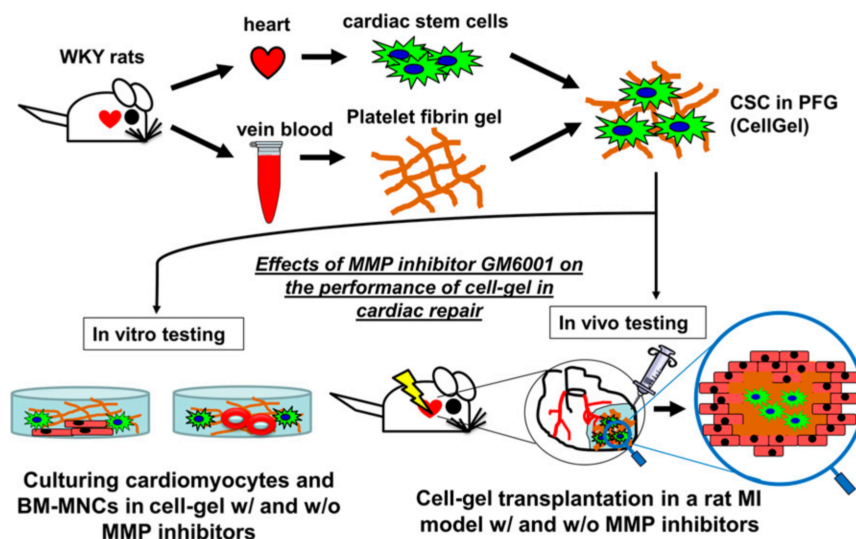

Figure 1. Study design. CSCs and PFG were harvested from WKY rat hearts and venous blood, respectively. CSCs were embedded in the PFG to form cell-gel. For in vitro studies, neonatal rat cardiomyocytes and BM-MNCs were cultured in cell-gel with or without MMP inhibitor GM6001. In vivo studies involved testing the treatment effects of cell-gel with or without GM6001 in a rat model of myocardial infarction. Abbreviations: BM-MNC, bone marrow mononuclear cell; CSC, cardiac stem cell; MMP, matrix metalloproteinase; PFG, platelet fibrin gel.

We used an acute MI model as previously described [30]. Female WKY rats (8–10 weeks old; *n* = 55) underwent left thoracotomy under general anesthesia, and MI was produced by permanent ligation of the left anterior descending coronary artery. We derived platelet fibrin gel and CSCs from WKY rats and intramyocardially injected them into the infarcted hearts of syngeneic animals. In general, an apparent infarct area with pale gray color was visible after ligation. The animals were immediately subjected to intramyocardial injections with a 29-gauge needle at four sites in the peri-infarct area, according to one of four randomly assigned groups: (a) MI + control: injection of 150 μl vehicle (DMEM); (b) MI + cell-gel: injection of 150 μl platelet fibrin gel containing 1 million CSCs (75 μl host plasma mixed with 75 μl prewarmed DMEM containing 1 million CSCs); (c) MI + cell-gel + GM6001: injection of 150 μl platelet fibrin gel containing 1 million CSCs plus daily intraperitoneal injections of 50 mg/kg GM6001; and (d) MI + cell-gel + GM6001-NC: injection of 150 μl platelet fibrin gel containing 1 million CSCs plus daily intraperitoneal injections of 50 mg/kg GM6001-NC (Fig. 1).

Heart Morphometric Analysis

Animals were euthanized 3 weeks after various treatments, and the hearts were harvested and prepared for cryosections. Sections every 100 μm (10-μm thickness) were prepared and stained with Masson trichrome (six sections per heart, collected at 400-μm intervals) [23]. Images were acquired with a PathScan Enabler IV slide scanner (Advanced Imaging Concepts, Princeton, NJ, http://www.aic-imagecentral.com). From the stained images, morphometric parameters including infarct size, infarct wall thickness, and viable tissue in the risk area were measured using ImageJ software (NIH, Bethesda, MD, http://imagej.nih.gov/ij). Six measurements were averaged for each heart. Respectively, viable (normal) tissue and scar (infarct) tissue could be distinguished with red and blue under Masson trichrome staining. To evaluate infarct thickness, measurements of wall thickness were taken at the thinnest part of the infarction and then averaged (six sections per heart, collected at 400-μm intervals). Viable tissue, expressed as a percentage of risk region, was calculated by the sum of red area in the risk region (defined with previously published methods [23]). Infarct size was calculated by the sum of blue area and expressed as a percentage of total left ventricular area.

Histology

For immunohistochemistry, heart cryosections were fixed with 4% paraformaldehyde, permeabilized, and blocked with Protein Block Solution (Dako, Carpinteria, CA, http://www.dako.com) containing 1% saponin (Sigma-Aldrich) and then incubated with the following antibodies overnight at 4°C: rabbit anti–von Willebrand factor (Abcam, Cambridge, MA, http://www.abcam.com), mouse anti–α sarcomeric actin (Sigma-Aldrich), rabbit anti-Ki67 (Abcam), or fluorescein isothiocyanate (FITC)-conjugated isolectin B4 (Vector Laboratories, Burlingame, CA, http://vectorlabs.com). FITC- or Texas red secondary antibodies were used for detection. Images were taken with a confocal microscopy system.

Cardiac Function Assessment

To access cardiac function, blinded echocardiography analysis was performed by a single observer using a Vevo Imaging System (VisualSonics, Toronto, ON, Canada, http://www.visualsonics.com). Light general anesthesia was induced with isoflurane. Two-dimensional long-axis images were recorded from the left caudal (apical) view. left ventricular end diastolic volume (LVEDV) and left ventricular end systolic volume (LVESV) were measured/calculated from two-dimensional long-axis views taken through the infarcted area. Left ventricular ejection fraction (LVEF) was calculated as (LVEDV – LVESV/LVEDV) $\times$ 100%.

Tissue Western Blot Analysis

Myocardial samples from the peri-infarct area were collected, lysed, and homogenized. Homogenates were centrifuged at 10,000 relative centrifugal force for 10 minutes on ice,

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supernatants were collected, and protein content was quantified with a BCA Protein Assay (Thermo Fisher Scientific Life Sciences). The equivalent of 15 μg total protein per lane was loaded onto 12% Precise Protein gels (Bio-Rad, Hercules, CA, http://www.bio-rad.com) and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat milk and incubated overnight with primary antibodies against β-actin (Cell Signaling Technologies, Danvers, MA, http://www.cellsignal.com), MMP-2 (Abcam), and MMP-3 (Abcam). Subsequently, the appropriate horseradish peroxidase-conjugated secondary antibodies were used, and the blots were visualized with SuperSignal West Femto substrate and exposed to the Gel Doc XR System (Bio-Rad). Quantitative analysis was performed with ImageJ software, and expressions were first normalized to β-actin and then to the MI + control group.

Statistical Analysis

All results are presented as mean $\pm$ SD. Comparisons between any two groups were performed with two-tailed unpaired Student's *t* test, except that statistical significance between baseline and 3-week LVEFs was determined using two-tailed paired Student's *t* test. Comparisons among more than two groups were performed with one-way analysis of variance followed by post hoc Bonferroni correction. Differences were considerd statistically significant when $p < .05$.

RESULTS

Characterization of CSCs in Platelet Fibrin Gel

CSCs cultured in platelet fibrin gel exhibited distinct elongated morphology compared with cells cultured on TCPs (Fig. 2A). The proliferation rates of CSCs cultured in the platelet fibrin gel were comparable to those cultured on TCPs (Fig. 2B). The Live/Dead assay showed that CSCs cultured in platelet fibrin gel had cell viability comparable to those cultured on TCPs (Fig. 2C), whereas MMP inhibitor GM6001 reduced CSC viability in the gel (supplemental online Fig. 1) ($p < .05$ compared with CSCs treated with GM6001-NC).

Effects of MMP Inhibition on Cardiomyocytes and MNCs Cultured in Cell-Gel

The elongated cell morphology of CSCs in the platelet gel indicates their ability to degrade and soften the surrounding matrix. Meanwhile, we have found that the presence of CSCs in platelet fibrin gel promotes cardiomyocyte spreading and contracting. Here we characterized the morphology and function of NRCMs and MNCs cultured in cell-gel (Fig. 3A, 3B) under MMP inhibition. Less spreading of NRCMs or MNCs was confirmed in cell-gel with MMP inhibitor GM6001 (Fig. 3E, 3F) than those in cell-gel with GM6001-NC (Fig. 3C, 3D). Quantitative cell morphology analysis revealed that GM6001 decreased cell body elongation (Fig. 3G) and cardiomyocyte contraction (Fig. 3H) ($p < .05$). To evaluate the effects of MMP on cell-gel degradation, we measured the thickness of cell-gel over time by confocal microscopy. MMP inhibition slowed matrix degradation: larger thicknesses were found in cell-gel treated with MMP inhibitor GM6001 at days 7 and 14 (Fig. 3I) ($p < .05$). Meanwhile, we detected that the release of growth factors (namely vascular endothelial growth factor [VEGF] and insulin-like growth factor 1 [IGF-1]) was persistently blocked by MMP inhibitor GM6001 (Fig. 3J, 3K) (VEGF and IGF-1, $p < .01$ on days 2 and 5 and $p < .05$ on days 9 and 14).

Effects of MMP Inhibition on Recruitment of Endogenous Cells In Vivo

To confirm our findings in vivo, we examined the impact of MMP inhibitor GM6001 on the integration of cardiomyocytes and endothelial cells into the injected cell-gel in the post-MI heart (Fig. 4A). To enable histologic detection, CSCs were prelabeled with CM-DiI (Thermo Fisher Scientific Life Sciences). cell-gel was intramyocardially injected into WKY rat hearts immediately after MI, and animals were sacrificed 7 days later for histologic examination. More cardiomyocytes (Fig. 4B, 4C, green) and endothelial cells (Fig. 4G, 4H, green) could be detected residing in cell-gel with GM6001-NC (Fig. 4B, 4G, red) than in cell-gel with GM6001 (Fig. 4C, 4H, red). Quantitative analysis confirmed those findings (Fig. 4D, 4I). Higher magnification revealed integration of cardiomyocytes (Fig. 4E, 4F) and endothelial cells (Fig. 4J, 4K) into the cell-gel with the presence of GM6001-NC or GM6001. MMP inhibitor GM6001 diminished such integration between the injected cell-gel and endogenous cells. Quantitative analysis revealed decreased numbers of infiltrating cardiomyocytes (supplemental online Fig. 2A) and endothelial cells (supplemental online Fig. 2B) in the cell-gel with MMP inhibitor GM6001, whereas GM6001-NC had no effects on cell integration ($p < .05$).

Heart Morphology and Cardiac Function

Adverse ventricular remodeling, indicated as dilated chamber and thinning of infarcted wall, can lead to heart failure. Therefore, prevention of adverse ventricular remodeling after a myocardial infarction is a key strategy to attenuate the progress of heart failure. Masson trichrome staining permitted simultaneous detection of scar (blue) and healthy myocardial (red) tissues (Fig. 5A; supplemental online Fig. 3). Snapshots of the infarcted area indicated the amount of viable tissue in the risk area (Fig. 5B; supplemental online Fig. 3). Consistent with our previous findings, hearts injected with cell-gel exhibited less left ventricular chamber dilatation and infarct wall thinning than those that received control (vehicle) injection. MMP inhibitor GM6001 blunted the therapeutic benefits of cell-gel: compared with hearts receiving cell-gel with GM6001, those that received cell-gel alone or cell-gel with GM6001-NC showed smaller infarct size (Fig. 5C), thicker infarcted walls (Fig. 5D), and more viable tissue (Fig. 5E). As one of the most valuable indicators of therapeutic benefits clinically, LVEF can evaluate the improvement or preservation of cardiac function. There was no significant difference in LVEF among the four treatment groups at baseline (4 hours after MI), suggesting a comparable degree of initial injury (Fig. 5F). Over the next 3 weeks, LVEF declined progressively in the control group, whereas injection of cell-gel or cell-gel with GM6001-NC improved cardiac functions. MMP inhibition (cell-gel plus GM6001) diminished the functional benefits of cell-gel (Fig. 5G, blue bar). To facilitate comparisons, we calculated the treatment effect, which reflects the changes in LVEF at 3 weeks relative to baseline. Control treatment had a negative effect, as LVEF decreased over time; cell-gel + GM 6001 showed some degree of LVEF preservation. In contrast, MI hearts treated with cell-gel or cell-gel with GM6001-NC exhibited sizable LVEF improvement (Fig. 5H). These results suggest that MMP inhibition blunted the structural and functional benefits of cell-gel injection.

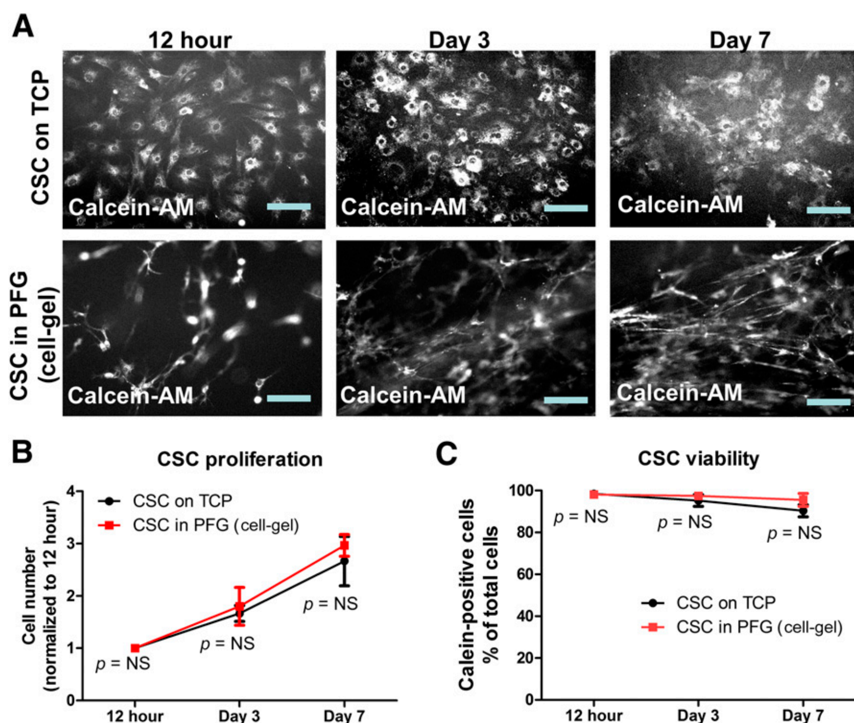

Figure 2. Characterization of CSCs in platelet fibrin gel. **(A):** CSCs cultured in platelet fibrin gel exhibited distinct elongated morphology compared with control cells cultured on TCPs at days 0.5, 3, and 7. **(B):** Proliferation of CSCs grown in platelet fibrin gel (red line) or on TCPs (black line) ($n = 3$). **(C):** Live/Dead assay showed that CSCs cultured in platelet fibrin gel (red line) at day 7 had cell viability (more calcein AM–positive cells) comparable to those cultured on TCPs (black line) ($n = 3$). Scale bar = 50 μm. p = NS indicates $p > .05$ compared with one another. Abbreviations: CSC, cardiac stem cell; NS, not significant; PFG, platelet fibrin gel; TCP, tissue culture plate.

Effects of MMP Inhibition on Mechanisms Underlying Cell-Gel-Mediated Cardiac Repair and Protection

We have shown in our previous work that CSCs exert the majority of their therapeutic benefits through indirect regenerative mechanisms (e.g., paracrine effects) [31, 32]. Two important indirect regenerative mechanisms of cell-gel are its ability to promote angiogenesis and cardiomyocyte proliferation [15]. Thus, we explored whether MMP inhibitor GM6001 altered the regenerative mechanisms of cell-gel in those two aspects. Three weeks after MI, transplanted CSCs were identified with CM-DiI fluorescence. Isolectin $B4^+$ capillaries (Fig. 6A, 6C) and cycling cardiomyocytes ($ki67^+/\alpha\text{-SA}^+$) (Fig. 6D) were consistently detected. The capillary density (percentages of Lectin $B4^+$ capillaries) and number of cycling cardiomyocytes ($Ki67^+/\alpha\text{-SA}^+$) in the peri-infarct zone from the cell-gel or cell-gel + GM6001-NC group were significantly higher than those from the control or cell-gel + GM6001 group (Fig. 6B, 6E), suggesting that MMP inhibition suppressed the proangiogenic and procardiomyogenic effects of cell-gel. $Ki67^+$ nuclei were detected in DiI^+ cells, suggesting that a portion of injected CSCs were proliferating (supplemental online Fig. 4). Western blot analysis confirmed that GM6001 treatment decreased expression of MMP-2 and MMP-3 in the heart ($p < .05$) (Fig. 7).

Discussion

CSCs have been developed for treating MI and heart failure over the last decade. Animal studies and a proof-of-concept human study support the notion that CSCs may benefit patients with ischemic cardiomyopathy [26]. Platelet fibrin gel represents an appealing choice of injectable biomaterial for heart repair because it is biodegradable, nontoxic, and nonimmunoreactive and provides mechanical and biological support to the injured heart. It has been previously reported in the same rat model of MI studied here that CSCs transplanted in fibrin gel had a favorable engraftment rate compared with those transplanted in saline [33]. Our group has also reported in a mouse model of MI that CSCs transplanted in another type of hydrogel had a 24-hour engraftment rate of ~40% versus ~5% transplanted in saline [34]. In addition, we reported the reparative and protective effects of platelet fibrin gel injection for the treatment of acute MI [14]. We further showed that transplantation of platelet fibrin gel spiked with CSCs could boost structural and functional benefits compared with bare gel injection [15]. MMPs are zinc-containing calcium-dependent endopeptidases that are released as inactive zymogens in a latent form [35]. MMPs and their tissue inhibitors regulate matrix degradation, which further affects cardiac fibrosis and myocardial performance [35]. It has been reported that CSCs secrete proteins and microRNAs to inhibit cardiac fibrosis and apoptosis and enhance contractility and angiogenesis [36]. All these regenerative pathways could be mediated by matrix modulation.

GM-6001 (ilomastat or gelardin), a broad-spectrum MMP inhibitor, has been extensively used in various experimental models of disease. The present study, which assessed the impact of MMP inhibitor on the therapeutic performance of cell-gel in a rat model of acute MI, has several important implications. First, we demonstrated that MMP inhibition could not only alter cell morphology but also blunt the proliferation and viability of CSCs cultured in platelet fibrin gel (supplemental online Fig. 1), suggesting that

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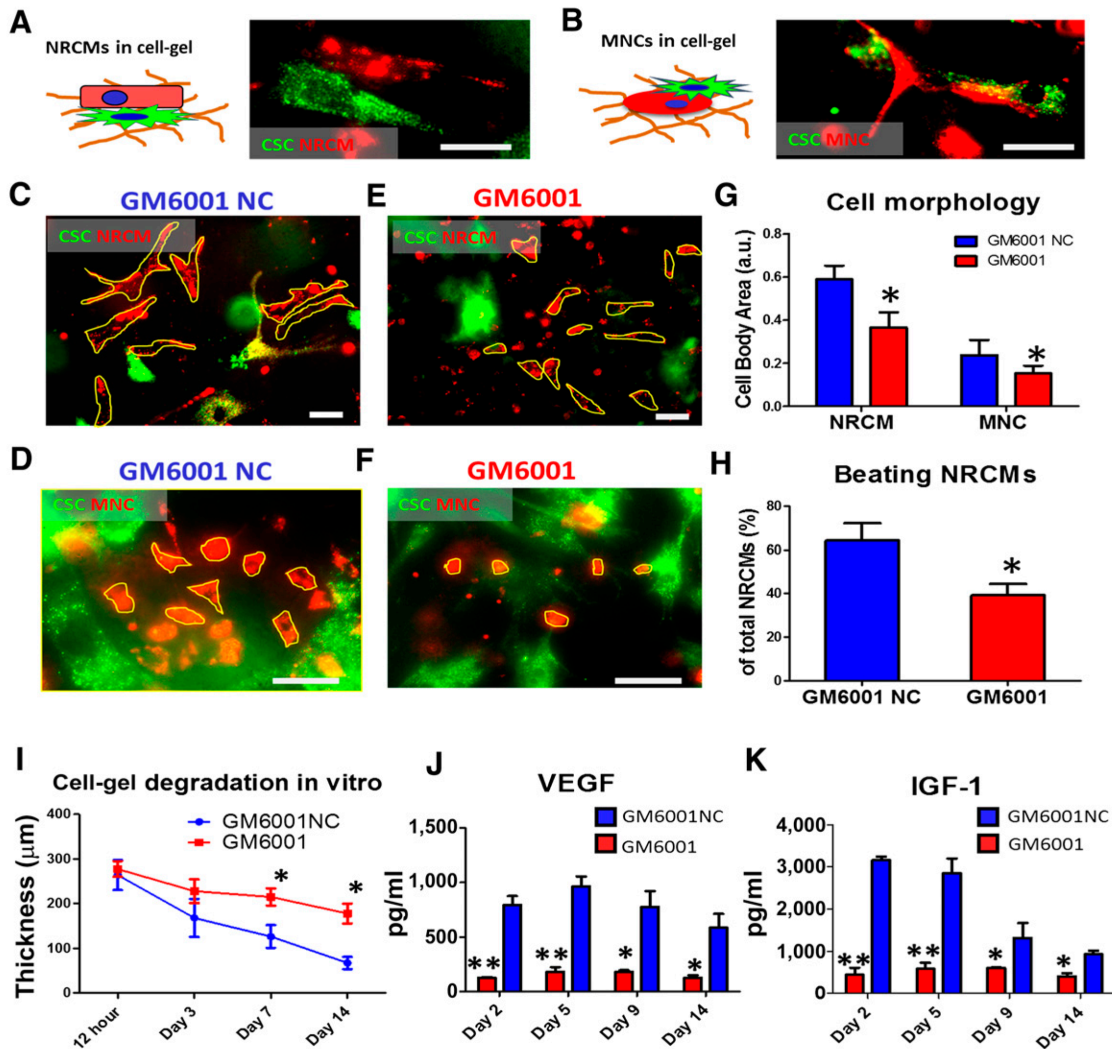

Figure 3. Effects of MMP inhibition on cardiomyocytes and MNCs cultured in cell-gel. **(A, B):** Schematic and fluorescent micrograph showing neonatal rat cardiomyocytes (NRCMs, red [**A**] or MNCs, red [**B**]) stained with red fluorescent CM-DiI cultured in cell-gel (CSCs labeled with green fluorescent DiO). **(C, E):** Less spreading of NRCMs (red, yellow outline) were confirmed in cell-gel (CSCs, green) containing MMP inhibitor GM6001 **(E)** than those in cell-gel with GM6001 scrambled control **(C)**. **(D, F):** Less spreading of MNCs (red, yellow outline) were confirmed in cell-gel (CSCs, green) containing MMP inhibitor GM6001 **(F)** than those in cell-gel with GM6001 scrambled control **(D)**. **(G):** Quantitative cell morphology analysis showing cell body area of NRCMs cultured in cell-gel with MMP inhibitor GM6001 (red bar) or GM6001-NC (blue bar) ($n = 4$). **(H):** Percentage of beating NRCMs in cell-gel containing GM6001 (red bar) or scrambled control (blue bar) ($n = 3$). **(I)** Thickness of cell-gel with MMP inhibitor GM6001 (red line) or GM6001-NC (blue line) decreased over time in culture ($n = 3$ per time point). **(J, K)** Concentrations of VEGF and IGF-1 in conditioned media of cell-gel with MMP inhibitor GM6001 (red bar) or GM6001-NC (blue bar) at various time points ($n = 3$ per time point) determined by enzyme-linked immunosorbent assay. Scale bar = 10 μm. *, $p < .05$, **, $p < .01$ compared with the GM6001-NC group. Abbreviations: a.u., arbitrary unit; CSC, cardiac stem cell; IGF-1, insulin-like growth factor 1; NC, negative control; MMP, matrix metalloproteinase; MNC, bone marrow mononuclear cell; NRCM, neonatal rat cardiomyocyte; VEGF, vascular endothelial growth factor.

MMPs are important for cells to maintain a healthy function in matrix. Second, our in vitro results showed that MMP inhibitor GM6001 slowed cell-gel degradation and decreased the secretion of growth factors (e.g., VEGF and IGF-1) (Fig. 3). Consistently, previous studies have shown that MMPs can mobilize growth factors such as VEGF through modulating the extracellular matrix [37, 38]. Third, we assessed the role of MMPs in cell-gel-induced angiogenesis, cardiac function protection, and adverse remodeling prevention after MI. Our results indicated that MMP inhibitor GM6001 diminished the integration of cardiomyocytes and endothelial cell into the cell-gel (Fig. 4 and supplemental online Fig. 2) and the therapeutic benefits of cell-gel (Fig. 5 and supplemental online Fig. 3).

Meanwhile, our study revealed that CSCs in gel (cell-gel) promoted angiogenesis and myocyte cycling, but MMP inhibition suppressed the proangiogenic and procardiomyogenic effects of cell-gel (Fig. 6). Previous studies have shown that MMPs can facilitate stem cell differentiation through remodeling of extracellular matrix [39], as well as promote epithelial cell ingression through MMP-mediated breakdown of the basement membrane [40, 41]. Similarly, we postulated that MMP inhibitor may block CSCs' "matrix softener" function, which is essential for their migration and the integration of local cardiomyocytes and endothelial cells into the injected gel. MMP inhibition blunted cell–cell interaction between injected CSCs and endogenous cardiomyocytes and endothelial cells, which has been proven to be essential

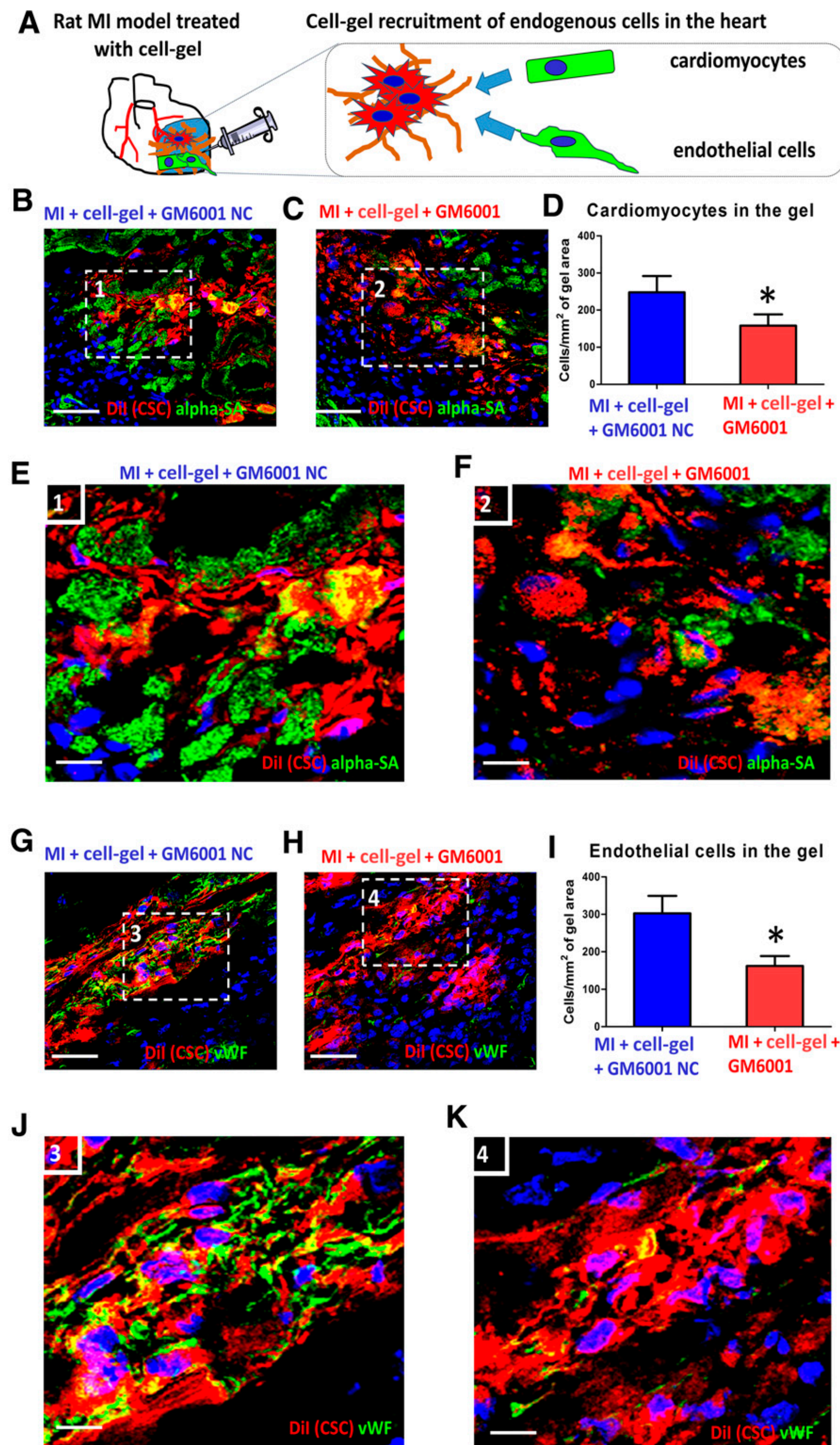

Figure 4. Effects of MMP inhibition on recruitment of endogenous cells to cell-gel. **(A):** Schematic showing endogenous cell integration with injected cell-gel. **(B)** and **(C):** Cardiomyocytes stained with α-sarcomeric actin (green) populating the injected cell-gel, treated with GM6001-NC (**[B]**, red) or cell-gel with GM6001 (**[C]**, red) 7 days after injection. **(D):** Quantitation of cardiomyocytes infiltrating into the cell-gel (*n* = 3 animals per group). **(E, F):** High-magnification images of the white box area in **B** and **C**. **(G, H):** Endothelial cells stained with vWF (green) populating the injected cell-gel, treated with GM6001-NC (**[E]**, red) or cell-gel with GM6001 (**[F]**, red) 7 days after injection. **(I):** Quantitation of endothelial cells infiltrating into injected cell-gel (*n* = 3 animals per group). **(J, K):** High-magnification images of the white box area in **(G)** and **(H)**. Scale bar = 100 μm. $*$, $p < .05$ compared with MI + cell-gel + GM6001-NC group. Abbreviations: alpha-SA, α-sarcomeric actin; CSC, cardiac stem cell; MI, myocardial infarction; MMP, matrix metalloproteinase; NC, negative control; vWF, von Willebrand factor.

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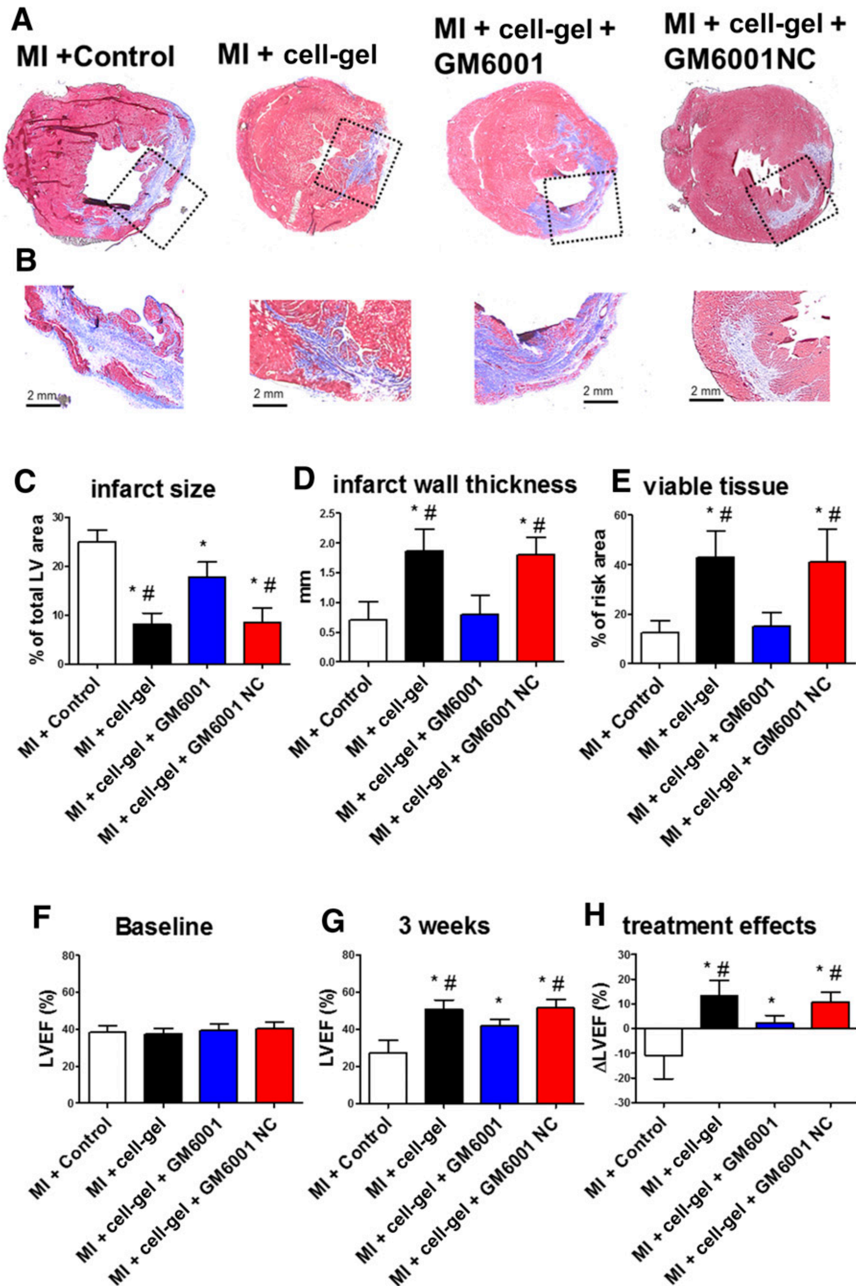

Figure 5. Heart morphometry and cardiac function. **(A):** Representative Masson trichrome–stained myocardial sections 3 weeks after treatment with control (vehicle), cell-gel alone, cell-gel with GM6001, or cell-gel with GM6001-NC. Scar tissue and viable myocardium are identified by blue and red, respectively. **(B):** Snapshots of the infarcted area. **(C–E):** Quantitative analysis of infarct size, infarct wall thickness, and viable tissue percentages from Masson trichrome–stained images (n = 5 animals per group). **(F, G):** LVEF was measured by echocardiography at baseline (4 hours after MI) (**F**) and 3 weeks later **(G)** (n = 6–7 animals per group). **(G):** Treatment effects (change in LVEF at 3 weeks relative to baseline, in each group). Scale bar = 2 mm. ∗, $p < .05$ compared with MI + control group; #, $p < .05$ compared with MI + cell-gel + GM6001 group. Abbreviations: LV, left ventricular; LVEF, left ventricular ejection fraction; MI, myocardial infarction; NC, negative control.

for CSC-mediated cardiomyogenesis and angiogenesis [42]. In addition, GM6001 is a broad-spectrum MMP inhibitor, targeting MMP-1, -2, -3, -7, and many others. We have previously reported that MMP-2 and MMP-3 were secreted by CSCs and likely to play positive roles in post-MI heart regeneration [43]. Here, we confirmed the decreased expressions of MMP-2 and MMP-3 in animals treated with GM6001 (Fig. 7).

Nevertheless, our study has several limitations. First, we did not elucidate the roles of individual MMP subtypes (e.g., MMP-2, MMP-3) in cell-gel-mediated heart repair. In addition, we used echocardiographic results obtained from 4 hours post-MI as the postinjury baseline. An echocardiography measurement before MI surgery would rule out any congenital differences in cardiac functions in different animals. Moreover, to obtain an accurate cell survival/engraftment rate, quantitative methods such as polymerase chain reaction (e.g., on *SRY* in the case of male-to-female transplantation) are needed. Nevertheless, our previous report in the same rat model of MI showed that CSCs

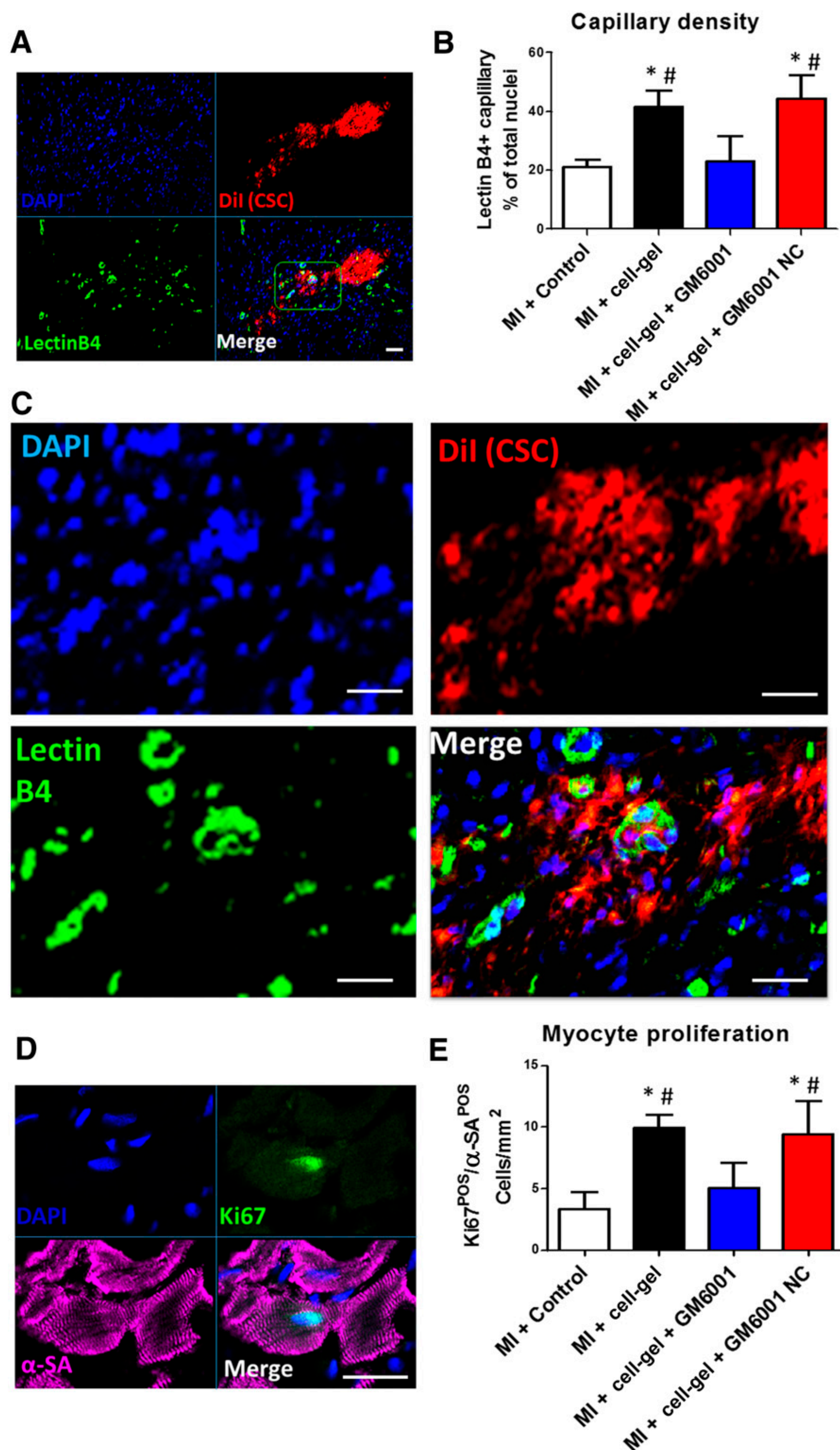

Figure 6. Effects of matrix metalloproteinasesinhibition on cell-gel-mediated cardiac repair mechanisms. **(A):** Injected cell-gel (CSCs labeled with DiI, red) were surrounded by capillaries (stained with FITC-lectinB4, green) in the post-MI heart. **(B):** Quantification of capillary density (percentages of Lectin $B4^+$ capillaries) in hearts treated with control (vehicle), cell-gel alone, cell-gel + GM6001, and cell-gel + GM6001-NC (n = 5 animals per group). **(C):** High-magnification images of the white box area in **(A)**. **(D):** $Ki67^+$ cardiomyocytes were detected in hearts treated with cell-gel. **(E):** Quantification of cycling cardiomyocytes ($Ki67^+/\alpha\text{-SA}^+$) in hearts treated with control (vehicle), cell-gel alone, cell-gel + GM6001, and cell-gel + GM6001-NC (n = 5 animals per group). Scale bar = 50 μm. $*$, $p < .05$ compared with MI + control group; #, $p < .05$ compared with MI + cell-gel + GM6001 group. Abbreviations: α-SA, α-sarcomeric actin; CSC, cardiac stem cell; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; MI, myocardial infarction; NC, negative control.

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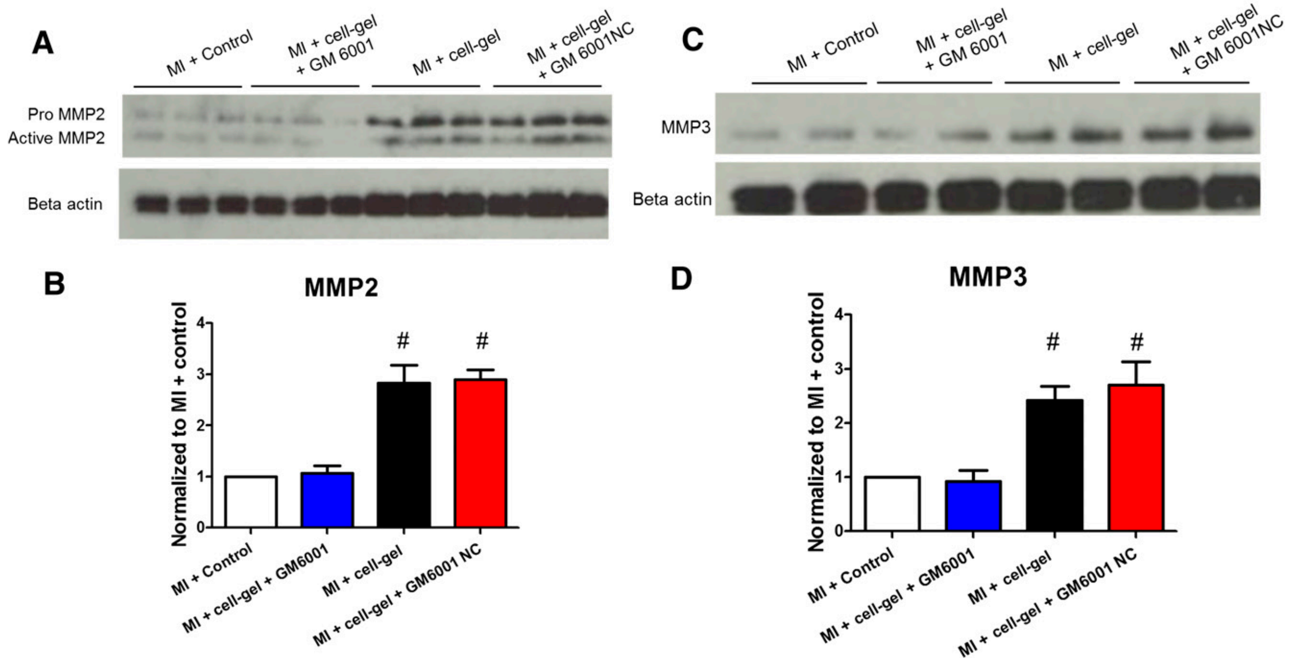

Figure 7. MMP-2 or -3 activities in the post-MI heart. **(A, C):** Immunoblotting was performed to examine the relative abundance of MMP-2 **(A)** and MMP-3 **(C)** in myocardial tissue obtained from the peri-infarct zone 7 days after MI with various treatments. **(B, D):** Quantitation of MMP-2 **(B)** and MMP-3 **(D)** levels in the myocardium ($n = 3$ animals per group). #, $p < .05$ compared with MI + cell-gel + GM6001 group. Abbreviations: MI, myocardial infarction; MMP, matrix metalloproteinase; NC, negative control.

transplanted in fibrin gel had a favorable engraftment rate compared with those transplanted in saline [33], and the scrambled drug GM6001-NC had no effect on the performance of cell-gel compared with cell-gel alone (supplemental online Fig. 2; cell-gel alone group reproduced from Cheng et al. [15]). In addition, the toxic effects of the scrambled control on cardiomyocyte viability and function in vitro need to be explored further.

Conclusion

Our study demonstrates that MMP plays a critical role in the therapeutic benefits of platelet fibrin gel spiked with cardiac stem cells for treating myocardial infarction. Our findings provide impetus for further investigation of the synergistic effects of cell-gel and MMPs in treating myocardial infarction.

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Author Contributions

D.S.: conception and design, collection and/or assembly of data; J.T.: data analysis and interpretation, manuscript writing; M.T.H., T.L., T.G.C., and T.Z.: collection and/or assembly of data; J.Z.: financial support; K.C.: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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