Bone marrow-derived mesenchymal stem cells in fibrin augment angiogenesis in the chronically infarcted myocardium

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Abstract

Aims—Current efforts to treat myocardial infarction include the delivery of cells and matrix scaffolds. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are multipotent stem cells that secrete angiogenic growth factors, and fibrin has been shown to be a biomaterial that provides structural support to cells and tissues. The objective of this study was to characterize the attachment and viability of BM-MSCs in fibrin *in vitro*, and then to assess the efficacy of treatment with BM-MSCs in fibrin for promoting neovascularization in the chronically infarcted myocardium.

Materials & methods—BM-MSCs were cultured in fibrin and assessed for cell attachment and viability by using immunofluorescence staining for actin filaments and Live/Dead® viability assays, respectively. To determine the efficacy of BM-MSCs in fibrin *in vivo*, chronically infarcted rat hearts were treated with either cells, cells in fibrin, fibrin or saline (n = 9). After 5 weeks, the infarct scar tissues were assessed for neovascularization.

Results—BM-MSCs exhibited robust cell attachment and viability when cultured in fibrin *in vitro*. Furthermore, when injected together into the infarcted tissue, BM-MSCs in fibrin could enhance neovasculature formation by increasing capillary density, in comparison to treatment by cells or fibrin separately. Concomitant to significant improvement in capillary density was an increase in the levels of VEGF in the infarct scar.

Conclusion—This study demonstrates the angiogenic potential of the combined delivery of BM-MSCs and fibrin, and highlights the advantage of stem cell-matrix approaches for myocardial repair.

Keywords

angiogenesis; cardiac tissue engineering; extracellular matrix; fibrin; mesenchymal stem cell; myocardial infarction

Despite advancements in treating myocardial infarction (MI), therapies that improve the structural and functional capacities of the myocardium remain lacking. To replace cells in damaged myocardium, numerous cell types have been studied for therapeutic transplantation, including fetal cardiomyocytes [1], skeletal myoblasts [2], embryonic stem cells [3] and bone marrow-derived mesenchymal stem cells (BM-MSCs) [4]. In particular, BM-MSCs are multipotent stem cells that have shown tremendous therapeutic potential. It has been demonstrated that BM-MSC transplantation after MI can improve cardiac performance, regenerate the infarcted myocardium and promote angiogenesis in the ischemic infarct area [4-6]. When applied to asynchronously beating cardiomyocytes, BM-MSCs could restore synchronization, most likely by connexin-43 coupling [7]. Recent data show that BM-MSCs secrete numerous angiogenic growth factors that implicate a paracrine mechanism of involvement in therapeutic repair *in vivo* [8]. Among the growth factors expressed by BM-MSCs, VEGF is known to promote endothelial cell proliferation and migration and can enhance postischemic neovascularization [9,10].

Concomitant with cell delivery, biomaterials are another emerging area of research in cardiac repair. When used alone or in combination with cells, biomaterials have been shown to prevent the negative remodeling associated with the MI [3,11-13]. For example, Zimmermann *et al*. demonstrated that the grafting of tissue constructs containing neonatal heart cells, collagen I and Matrigel™ to the infarcted heart could improve cardiac function and wall thickening [13]. Biomaterials can serve as delivery vehicles that mediate cell alignment, cell migration and growth factor release [14-17]. One potential biomaterial for myocardial repair is fibrin, a US FDA-approved biomaterial that is commercially available as a sealant and adhesive. Fibrin is bioresorbable and serves as a provisional matrix for cellular ingrowth during wound repair.

Previous studies have characterized the *in vitro* effect of fibrin on BM-MSC survival [18] and differentiation towards vascular or osteogenic lineages [18,19]. In addition, their therapeutic effect has been assessed for repair in the setting of acute MI [20], which is shortly after the induction of MI, during which the infarct zone actively undergoes a remodeling process characterized by the infiltration of inflammatory cells and elaboration of cytokines [21]. Compared with acute MI, a more clinically relevant setting is the model of chronic MI, which occurs several weeks following the induction of MI when the pathological remodeling process is complete and myocardial pumping capacity is compromised, predisposing the heart to congestive heart failure [22,23]. However, the angiogenic effect of BM-MSCs in fibrin for treatment of chronic MI remains to be elucidated.

In this study, we characterized the attachment and viability of human BM-MSCs in fibrin *in vitro*, and then assessed the efficacy of treatment with BM-MSCs in fibrin for promoting neovascularization in the chronically infarcted myocardium.

Materials & methods

Fibrin characterization

To characterize the porous structure of fibrin (Tisseel, Baxter Healthcare Corp., CA, USA), the fibrinogen and thrombin components were prepared according to our previous studies [12,14]. In brief, fibrinogen and thrombin were briefly mixed at a 1:1 ratio to form 100 μl fibrin matrices. For scanning electron microscopy (SEM), the fibrin samples were sputter-coated with gold:palladium (40:60) particles to a thickness of 10-15 nm. Fibrin pore sizes were visualized using a Hitachi S-5000 cold field emission scanning electron microscope [24]. For histological analysis of pore size, fibrin was embedded in OCT compound (Sakura Finetek, CA, USA) and cryosectioned for routine hematoxylin and eosin (H&E) staining [25]. Pore sizes were quantified by measurement of the pore diameter of H&E-stained fibrin cross-

sections using Image J software (NIH, MD, USA) (n = 3). At least 40 pores were assessed in each sample.

Cell morphology & survival in fibrin *in vitro*

Human BM-MSCs (Lonza, MD, USA) were cultured in MSC growth medium (MSCGM, Lonza). These cells were tested by the manufacturer for positive expression of BM-MSC phenotypic markers including CD105, CD166 and CD44. To characterize cell morphology and survival in 3D fibrin gels *in vitro*, the BM-MSCs were resuspended in 100 μl thrombin, transferred to tissue-culture dishes and mixed with 100 μl fibrinogen. After solidification of fibrin, the gels were incubated with MSCGM. As a control, BM-MSCs were cultured on tissue-culture dishes. After 2 days, cell viability in fibrin was assessed by incubation with 2 μM calcein-AM and 4 μM ethidium homodimer (Live/Dead® viability assay; Molecular Probes, OR, USA) for 1 h before visualization. To track cell migration in 3D fibrin, a sprouting assay was carried out in which BM-MSC aggregates were formed by hanging drops for 24 h, and then embedded within 200 μl fibrin. After 1 h, the gels were immersed in MSCGM. As a control, cell aggregates was cultured on tissue-culture dishes in MSCGM. After 2 days, the aggregates were evaluated for cell migration by bright field microscopy and then fluorescently stained with Alexafluor-488-conjugated phalloidin (Molecular Probes). The samples were visualized with a Zeiss Axioskop 2 fluorescent microscope.

MI model & injections

This study was performed in accordance with the Committee for Animal Research of the University of California, San Francisco. Female nude (*rnu* homozygous) rats aged 8-12 weeks underwent occlusion of the left anterior descending (LAD) coronary artery for 17 min, followed by reperfusion, according to our previous studies [26]. A midline thoracotomy was performed 5 weeks post-MI [14,25] and the infarct scar was injected intramuscularly with 50 μl of one of four treatments, namely 0.5% bovine serum albumin (BSA) in phosphate-buffered saline, 2×10^6 cells in 0.5% BSA, fibrin or 2×10^6 cells in fibrin (n = 9 per group).

For treatment of fibrin, the fibrinogen and thrombin components were maintained in separate syringes in liquid form until injecting simultaneously using the supplied Duploject applicator into the infarct area, where the two components solidified into a 3D fibrin matrix. For treatment of BM-MSCs in fibrin, the cells were briefly suspended in thrombin before joining with fibrinogen using the Duploject applicator and then solidifying within the infarct. The BM-MSCs used in this study were cultured in MSCGM prior to *in vivo* studies and used within eight passages. At 2 days or 5 weeks after treatment, the animals were euthanized by pentobarbital overdose (200 mg/kg) and the hearts were freshly frozen in OCT compound for histology and immunohistochemical analysis (n = 9 per group at each time point).

Histology & immunohistochemistry

The hearts were cryosectioned into 10-μm transverse slices. Representative cryosections spanning the infarct were processed for routine H&E staining [26] for quantification of infarct size. The infarct size was measured as the (infarct scar area)/(total left ventricular area), according to our previous studies [26]. Neovascularization was evaluated by immunohistochemical staining of anti-CD31 (BD Biosciences, CA, USA) for the identification of endothelial cells and anti-smooth muscle α-actin (SMA, Sigma-Aldrich, MO, USA) for smooth muscle cells, as previously described [14]. Capillary and arteriole densities were analyzed according to our previous studies [14]. Briefly, for measurement of capillary density, five fields within the infarct of each tissue section were imaged under 40× objectives, and capillaries with less than 10 μm diameters were counted. The capillary density was quantified as the (total CD31-positive microvessels)/mm². Arteriole density was quantified as the (total

SMA-positive microvessels)/mm^2 within the infarct area. Vascular densities were averaged out of at least four tissue sections spanning the infarct region for each animal.

In addition, the level of infarct scar VEGF expression was semiquantified by immunohistochemical staining of VEGF using a human- and rat-reactive polyclonal antibody (Santa Cruz Biotechnology, CA, USA) and the Vectastain ABC kit (Vector Labs, CA, USA). For semiquantification of VEGF expression, five fields within the infarct area of each tissue section were imaged under 40× objectives, and the area of positive VEGF expression was measured by Image J software and expressed as a percentage of the total infarct area [27]. The expression for each animal was averaged out of at least four tissue sections spanning the infarct region.

BM-MSC retention & differentiation in infarct scar

Bone marrow-derived mesenchymal stem cells were identified in cryosections of the infarcted myocardium by immunofluorescence staining of nuclear matrix antigen (NuMA, Calbiochem, CA, USA) and vimentin (Dako, CA, USA) using human-specific antibodies that did not cross-react with rat tissue. Gap junctional proteins were assessed by anti-connexin-43 (Sigma) antibody. Differentiation towards cardiovascular lineages was assessed by the expression of SMA for SMC phenotype and Nkx2.5 (Santa Cruz Biotechnology) for cardiomyocyte lineage. Fluorescein or tetramethyl rhodamine isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch, PA, USA) were then applied. Cell nuclei were dyed with Hoechst 33342 (Molecular Probes) before visualizing with a Zeiss Axioskop 2 microscope or Leica TCS SL confocal microscope. Human and rat samples were used as positive and negative control tissues, respectively, for the human-specific antibodies. For quantification of BM-MSC survival in the infarct scar at 2 days or 5 weeks after cell delivery using human-specific NuMA antibody, at least four fields within the infarct area of each tissue section were imaged using 40× objectives. The number of NuMA-expressing nuclei in each section was counted and averaged out of at least four tissue sections for each animal.

Statistical analysis

Results are shown as mean ± standard error of the mean. Vascular densities among multiple groups followed normal distributions and were compared by analysis of variance (ANOVA) with Holm's adjustment. Statistical analysis of cell retention between delivery in saline or fibrin was carried out by an unpaired t-test. Significance was accepted at $p < 0.05$.

Results

In vitro characterization of cell viability on fibrin

Scanning electron microscopy images showed that fibrin was a porous 3D matrix (FIGURE 1A). From H&E-stained cross-sections, the average pore size was 84 ± 4 μm in diameter (FIGURE 1B). After 2 days in culture, Live/Dead viability assay demonstrated that the BM-MSCs in fibrin gels were comparable to those on the control substrate (FIGURES 1C & 1D), based on the abundance of viable cells (green) and dead cells (red). In fibrin gels, the BM-MSCs retained an elongated morphology. To assay their ability to migrate within fibrin, we performed a sprouting assay in which aggregates of BM-MSCs were embedded in fibrin or cultured on control tissue-culture substrates. After 2 days, sprouting BM-MSCs could be observed migrating in the radial direction from the aggregates on the control substrate (FIGURE 1E) as well as within fibrin (FIGURE 1F). The outgrowths from both substrates had organized F-actin assembly in the radial direction (FIGURES 1G & 1H). Taken together, these data demonstrated that the fibrin formulation was porous and did not interfere with cell attachment, migration and viability *in vitro*.

Cell engraftment *in vivo*

The therapeutic effects of delivering BM-MSCs in fibrin were then tested *in vivo* for repair of chronic MI. To assess the retention of BM-MSCs in the infarct area, we utilized human-specific antibodies against NuMA and vimentin to detect the cells. Figures 2A & 2B show the nuclear localization of NuMA and cytoskeletal assembly of vimentin *in vitro* as positive sample controls for these antibodies. The BM-MSCs were also shown to express connexin-43 gap junctions robustly. To verify successful intramuscular injections of fibrin and cells 2 days after injection, H&E-stained cross-sections showed the presence of fibrin in ischemic tissue (Figure 2C), and BM-MSCs could be visualized in an adjacent tissue section by immunofluorescence staining for NuMA (Figure 2D). Quantification of NuMA-expressing cells in the infarct after 2 days revealed an average of 46 ± 8 BM-MSCs per high power field when delivered in BSA, which was not significantly different from 47 ± 6 cells when delivered in fibrin. The BM-MSC survival was estimated to be less than 1% 5 weeks after injection, based on the average of 33 ± 23 NuMA-expressing cells found in each 10 μm thick section within the infarct, regardless of delivery in BSA or fibrin. The surviving BM-MSCs in the infarct showed elongated NuMA-positive nuclei and vimentin-positive filaments (Figures 2E & 2F). In addition, they expressed connexin-43 gap junctions that were often within the vicinity of the gap junctions of the host myocardium, as demonstrated by double staining of connexin-43 with NuMA (Figure 2E) or with vimentin (Figure 2F). However, double immunofluorescence staining for NuMA with cardiovascular differentiation markers Nkx2.5 and SMA demonstrated no co-localization of staining among the cells assessed, suggesting that the BM-MSCs did not differentiate into cardiovascular cell types after 5 weeks of any treatment. In addition, the BM-MSCs did not appear to differentiate into osteogenic, adipogenic or chondrogenic lineages based on histological analysis of H&E-stained transverse sections of the infarcted tissue (data not shown).

Immunohistochemical analysis of neovascularization

After demonstrating BM-MSC survival in the infarct in the absence of differentiation at 5 weeks after delivery, we assessed whether BM-MSCs had a paracrine effect by enhancing neovasculature formation, and whether the effect could be augmented by fibrin. Indeed, quantification of capillaries revealed that the BM-MSCs in fibrin group had significantly higher capillary density (273 ± 38 microvessels/mm^2), in comparison to the BM-MSC (159 ± 28 microvessels/mm^2) (p = 0.015), fibrin (118 ± 14 microvessels/mm^2) (p < 0.0001) and BSA (123 ± 22 microvessels/mm^2) groups (p < 0.0001) (Figures 3A-3E). This finding indicated that the combined delivery of BM-MSCs in fibrin could augment capillary density, when compared with delivery of each component separately.

To understand the mechanism governing the enhanced capillary formation due to the combined delivery of BM-MSCs in fibrin, we examined whether VEGF, an angiogenic cytokine known to be secreted by BM-MSCs, was differentially expressed by BM-MSCs between treatment groups [8]. Indeed, semiquantification of the infarct scar VEGF expression (Figures 4A-4E), as assessed by a human-reactive VEGF antibody, demonstrated that the VEGF staining occupied $15 \pm 1\%$ of the infarct area in the BM-MSCs in fibrin group, which was significantly higher than that of the BSA group ($7 \pm 1\%$; p < 0.0001), BM-MSCs group ($11 \pm 2\%$; p = 0.029) and fibrin group ($8 \pm 1\%$; p < 0.0001). VEGF staining was more pronounced in regions near vasculature. These data suggested that the augmented VEGF expression level may be involved in the enhanced capillary formation in the BM-MSCs in fibrin group.

Since arterioles are mature vessels that can contribute to blood perfusion, SMA staining for arterioles was carried out and quantified. Arteriole density analysis revealed that the BM-MSCs in fibrin group had significantly higher arteriole density (13 ± 2 arterioles/mm^2), when compared with both the BSA (8 ± 1 arterioles/mm^2; p = 0.025) and fibrin (8 ± 1 arterioles/

mm^2; p = 0.016) groups, but not when compared with the BM-MSCs group (12 ± 2 arterioles/mm^2) (FIGURES 5A-5E). Based on these data, the combined treatment of BM-MSCs and fibrin could enhance capillary formation and VEGF levels, when compared with all other treatment groups, as well as improve arteriole formation when compared with the fibrin or BSA groups.

Morphometric analysis of infarct size at 5 weeks after therapeutic injections revealed nonsignificant comparisons between treatment groups. The infarct size of the BSA group was $26 \pm 9\%$, which was not significantly different from other treatment groups. These data suggested that treatment of BM-MSCs in fibrin could enhance angiogenesis but could not reduce the infarct size.

Discussion

In the present study, the feasibility of augmenting the angiogenic effect of BM-MSCs using fibrin was demonstrated. We showed that BM-MSCs had robust cell attachment, migration and viability when cultured in fibrin *in vitro*, a finding in concurrence with published literature [18,19,28]. When delivered together as an injectable therapy to the infarct scar, the BM-MSCs in fibrin treatment could enhance capillary formation. A significant improvement in capillary density was associated with increased levels of VEGF expressed in the infarct scar. Although the present method used for VEGF assessment can only be semiquantified, this technique provided spatial information regarding VEGF localization within the infarct tissue.

The enhancement of VEGF expression after treatment of BM-MSCs in fibrin can provide cardioprotective effects to cardiomyocytes. This finding is supported by *in vivo* studies in which treatment of BM-MSCs overexpressing VEGF improved cardiac function [29]. In related studies, Markel *et al*. demonstrated that knockdown of VEGF in BM-MSCs by siRNA impaired VEGF production and diminished stem cell-mediated postischemic myocardial recovery [30].

Although the BM-MSCs demonstrated low cell survival and no evidence of cardiovascular differentiation at 5 weeks after delivery - a finding supported by other reports [31,32] - the transient retention of BM-MSCs may be sufficient to promote the paracrine release of angiogenic cytokines such as VEGF. Moreover, our findings suggest that fibrin could modulate the angiogenic effect of BM-MSCs by promoting significantly increased neovascularization, when compared with the treatment by each individual component. However, the inability of BM-MSCs and fibrin to reduce the infarct size in the present study suggests that other additional treatment factors may be required, such as prosurvival cues or cell-cell interaction [33,34].

The ability of fibrin to regulate BM-MSC behavior has been demonstrated in previous studies [18,28], in which fibrin stimulated BM-MSC proliferation and migration and could modulate BM-MSC phenotype towards cardiovascular lineages. The effect of fibrin on BM-MSCs could be due to factors such as matrix elasticity [35] or specific integrin binding [36]. These potential factors are interesting and warrant further investigation.

An advantage of this approach of delivering BM-MSCs in fibrin is the ability to enhance VEGF levels in the infarct without relying on viral approaches to induce VEGF overexpression [30, 37]. Furthermore, the treatment of BM-MSCs in fibrin can potentially become an off-the-shelf therapy. BM-MSCs can be easily isolated from numerous sources and expanded *in vitro* one million-fold while sustaining multilineage potential [38-40]. Also, BM-MSCs lack MHC II antigens that are responsible for immune rejection, making them a favorable candidate for allogeneic cell transplantation [41]. In addition, fibrin is already an FDA-approved material. Therefore, the delivery of BM-MSCs and fibrin could provide a treatment strategy for patients nearing congestive heart failure who require immediate therapeutic intervention.

In contrast to previous studies that demonstrated angiogenic effects of fibrin treatment alone [14,26], the current study showed no enhancement in neovascularization in the fibrin group. This finding is supported by others and by our previous work showing that fibrin alone does not affect capillary formation or microvascular density [26,42]. The difference in results may also be related to the type of animal model used. Earlier reports suggest that fibrin's ability to modulate angiogenesis may, in part, be related to its ability to recruit inflammatory cells that secrete angiogenic factors [43]. However, since the animals used in this study were immunocompromised, they may have had a limited ability to respond to the treatment of fibrin. Instead, our results suggest that it may be necessary to deliver both BM-MSCs and fibrin in order to augment capillary formation and VEGF expression for repair of chronic MI in the immunocompromised setting.

Earlier reports on the combined delivery of cells with fibrin for myocardial repair remain limited. Previous studies of delivering myoblasts in fibrin in an acute MI model also demonstrated significant increases in neovascularization and cardiac functional improvement [12,26], but myoblasts lack the ability to form electrical connections with cardiomyocytes and therefore pose the risk of arrhythmia [44-46]. Transplantation of endothelial cells with fibrin into the ischemic myocardium has been shown to improve ejection fraction and neovascularization [47], but endothelial cells also have limited ability to transdifferentiate into cardiomyocytes. The therapeutic effect of delivering bone marrow mononuclear cells alone or in conjunction with fibrin resulted in significantly higher microvascular density and improvement in cardiac function [42,48,49]. In addition, Zhang *et al*. demonstrated that controlled release of stromal cell-derived factor-1α conjugated to a poly(ethylene glycol) fibrin patch could improve recruitment of c-kit-expressing cells and improve cardiac function [50]. With respect to BM-MSCs and fibrin delivery for myocardial repair, Liu *et al*. transplanted patches containing BM-MSCs in fibrin onto acutely infarcted porcine hearts and reported enhanced angiogenesis when compared with acellular fibrin constructs [20]. However, the patch approach relied on migration of the BM-MSCs from the patch into the myocardium, a process reported by the authors as only 10% efficient. By contrast, the current study utilizes an injectable delivery system that efficiently delivers the cells locally into the myocardium. To date, this is the only study that has investigated the injectable delivery of BM-MSCs in fibrin using a chronic model of MI.

Conclusion

In summary, we have demonstrated that BM-MSCs have robust cell attachment and viability when cultured in fibrin *in vitro*. When injected into the chronically infarcted myocardium, the BM-MSCs and fibrin treatment could enhance neovasculature formation by the formation of a greater number of capillaries when compared with delivery of cells or fibrin alone. Concomitant to significant improvement in neovascularization was an increase in VEGF expression in the infarct scar, in comparison to delivery by cells or fibrin alone. This study highlights the role of stem cell-matrix approaches for myocardial repair.

Executive summary

Introduction

☐ Bone marrow-derived mesenchymal stem cells (BM-MSCs) are multipotent stem cells that secrete angiogenic factors.

☐ Fibrin is a biomaterial that provides structural support to cells and tissues.

☐ The objective of this study was to characterize the attachment and viability of BM-MSCs in fibrin *in vitro*, and then to assess the efficacy of treatment with BM-MSCs

in fibrin for promoting neovascularization in the chronically infarcted myocardium.

Materials & methods

□ BM-MSCs were cultured in fibrin and assessed for cell attachment and viability by immunofluorescence staining for F-actin and Live/Dead® viability assays, respectively.

□ Chronically infarcted rat hearts were treated with either cells, cells in fibrin, fibrin or saline. After 5 weeks, the infarct scar tissues were assessed for neovascularization.

Results

□ BM-MSCs exhibited robust cell attachment and viability when cultured in fibrin *in vitro*.

□ BM-MSCs in fibrin could enhance neovasculature formation by increasing capillary density, in comparison to treatments by cells or fibrin separately.

□ BM-MSCs in fibrin could enhance infarct VEGF expression, in comparison to treatments by cells or fibrin separately.

Conclusion

□ This study demonstrates the angiogenic potential of combined delivery of BM-MSCs and fibrin, and highlights the advantage of stem cell-matrix approaches for myocardial repair.

Acknowledgments

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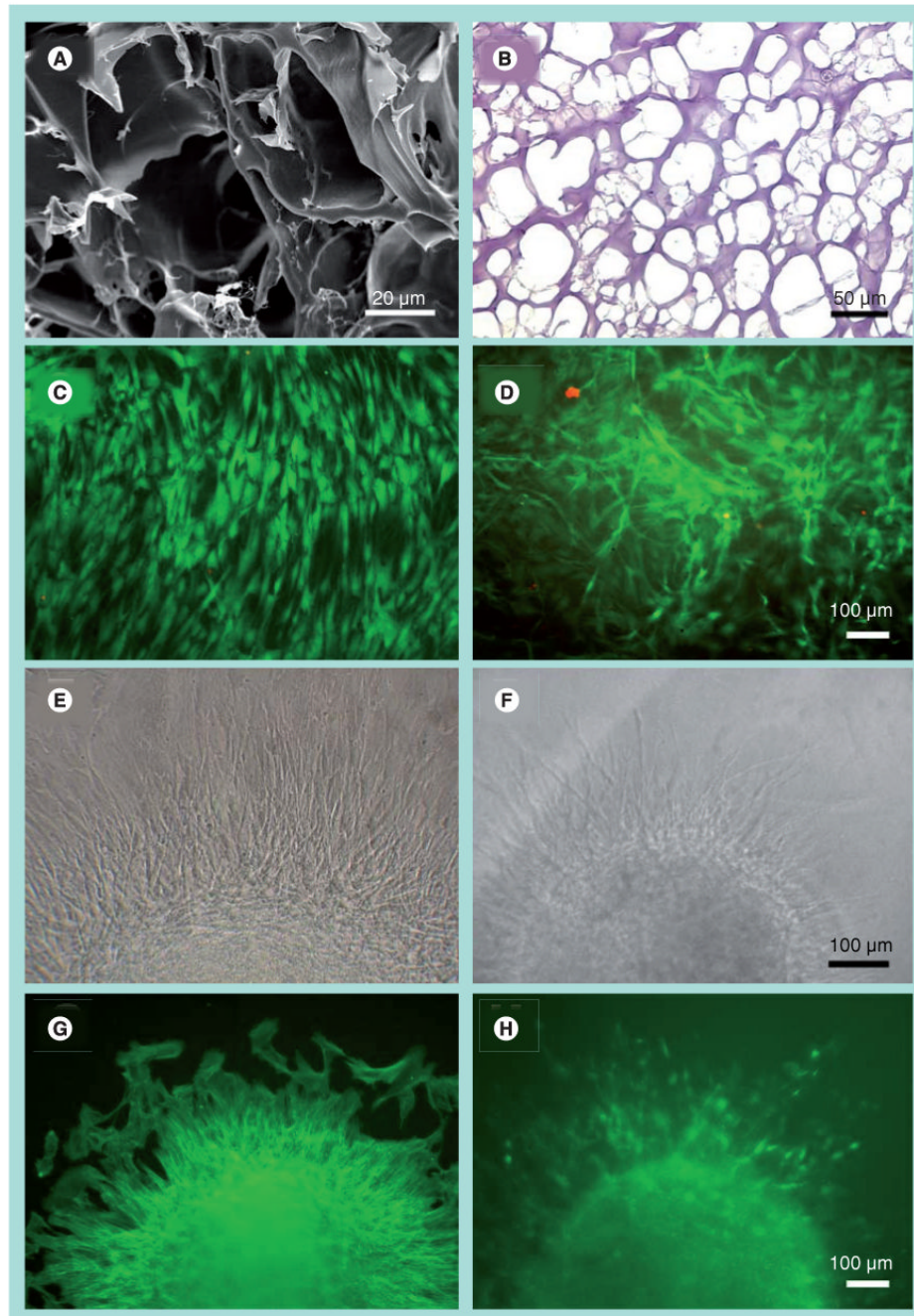
Figure 1. *In vitro* **characterization of bone marrow-derived mesenchymal stem cell morphology and survival in 3D fibrin after 2 days**

Visualization of porous fibrin structure by **(A)** scanning electron microscopy and **(B)** hematoxylin and eosin of a cross-sectional slice. Live/Dead® viability assay depicts viable (green) and dead (red) cells on **(C)** control tissue culture dishes or in **(D)** fibrin gels. Migration of bone marrow-derived mesenchymal stem cells from aggregates on **(E & G)** control or **(F & H)** fibrin gels as shown by bright field **(E & F)** or by immunofluorescence staining of F-actin **(G & H)**.
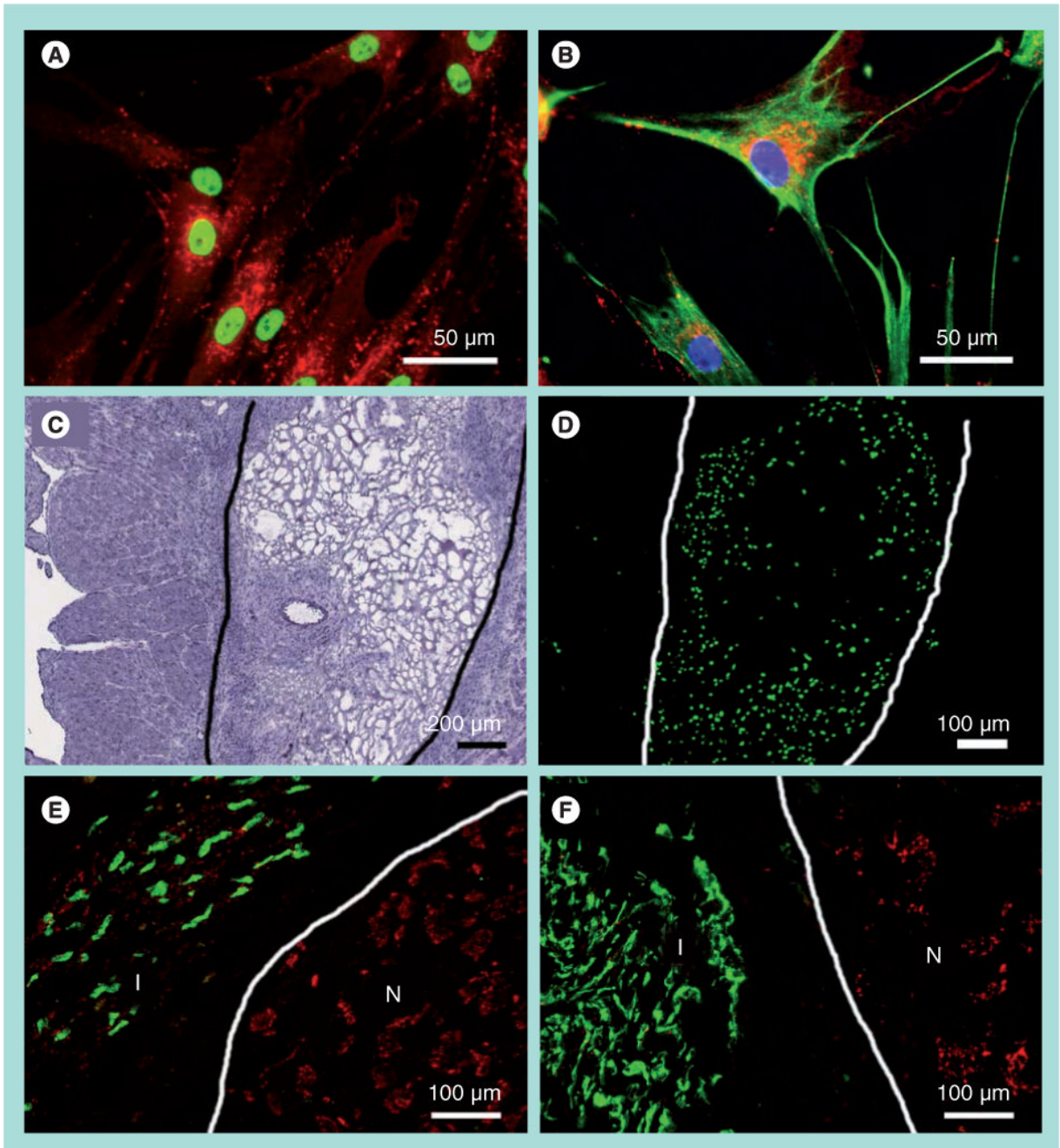
Figure 2. *In vivo* **delivery of bone marrow-derived mesenchymal stem cells in fibrin into the infarcted myocardium**
Immunofluorescence staining of **(A)** human-specific nuclear matrix antigen (NuMA, green) and connexin-43 (red) and **(B)** human-specific vimentin (green), connexin-43 (red) and Hoechst nuclear dye (blue). At 2 days postinjection, fibrin was visible by **(C)** hematoxylin and eosin staining, and bone marrow-derived mesenchymal stem cells (BM-MSCs) were visualized by **(D)** NuMA-positive BM-MSCs in an adjacent tissue section. The boundary of the fibrin matrix is marked for clarity. BM-MSCs persisted in the infarct after 5 weeks of injection, as shown by immunofluorescence staining of **(E)** NuMA (green) and connexin-43 (red), and

(F) vimentin (green) and connexin-43 (red). The boundaries between infarct and normal myocardium are marked for clarity.
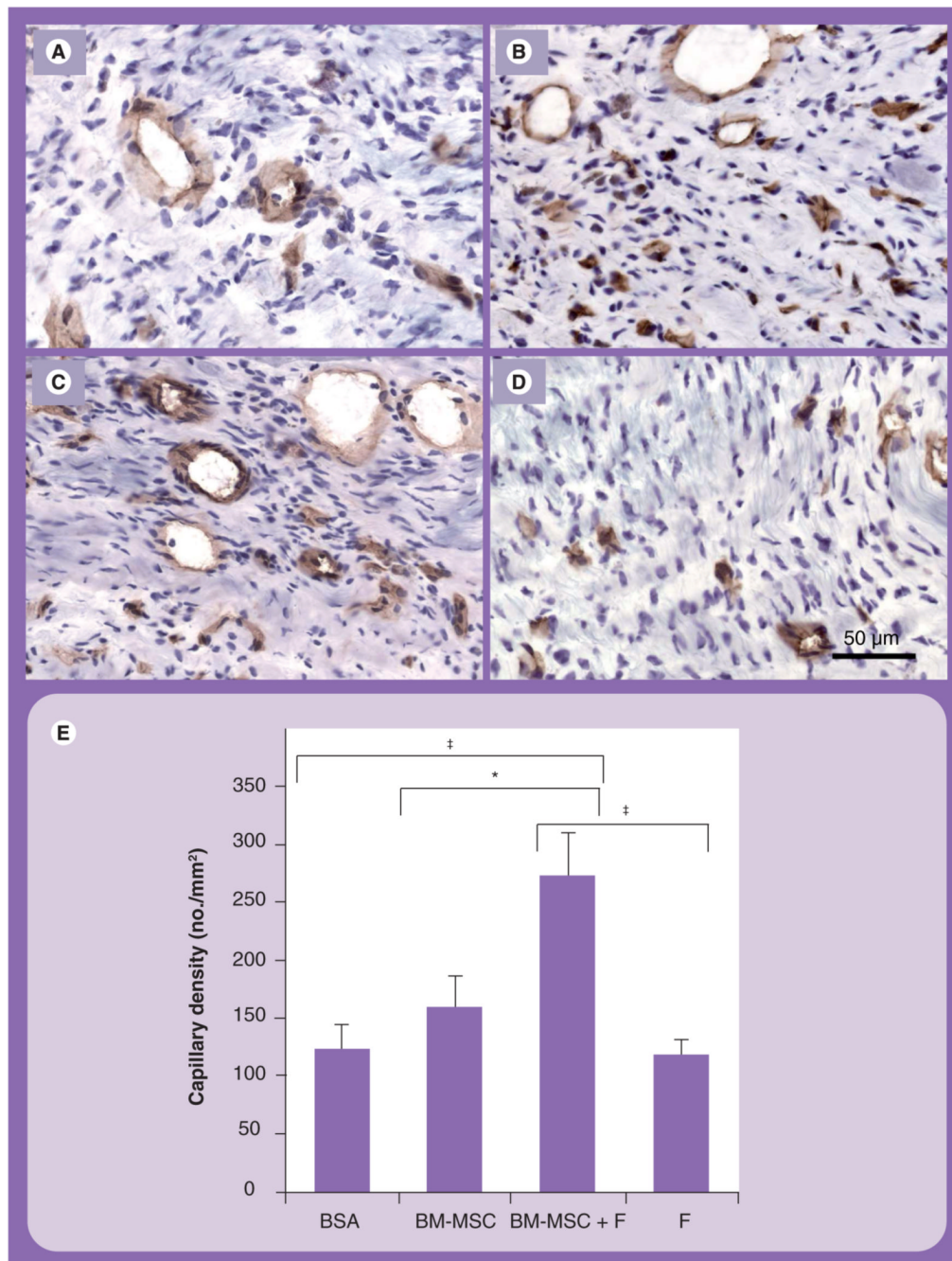
I: Infarct; N: Normal.

Figure 3. Capillary density assessment in the infarct area
Immunohistochemical staining of CD31 for endothelial cells in **(A)** BSA, **(B)** BM-MSC, **(C)** BM-MSC in fibrin and **(D)** fibrin groups. Samples were counterstained with hematoxylin. **(E)** Quantification of capillary density.
*p = 0.015; ‡p < 0.0001.
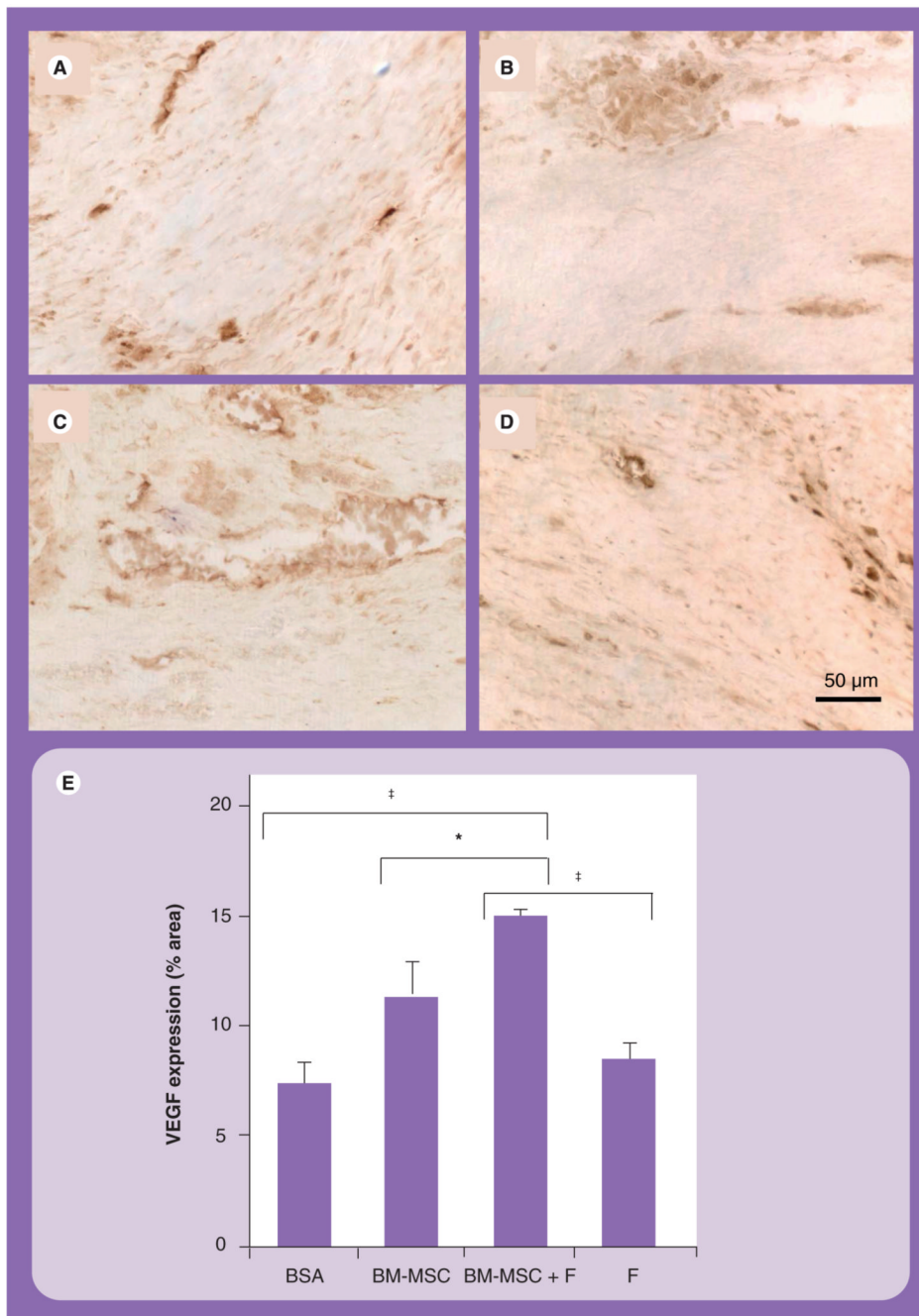BM-MSC: Bone marrow-derived mesenchymal stem cell; BSA: Bovine serum albumin; F: Fibrin.

Figure 4. VEGF expression in the infarct area
Immunohistochemical staining of VEGF in **(A)** BSA, **(B)** BM-MSC, **(C)** BM-MSC in fibrin
and **(D)** fibrin groups. **(E)** Semiquantification of VEGF expression area.
*p = 0.029; ‡p < 0.0001.
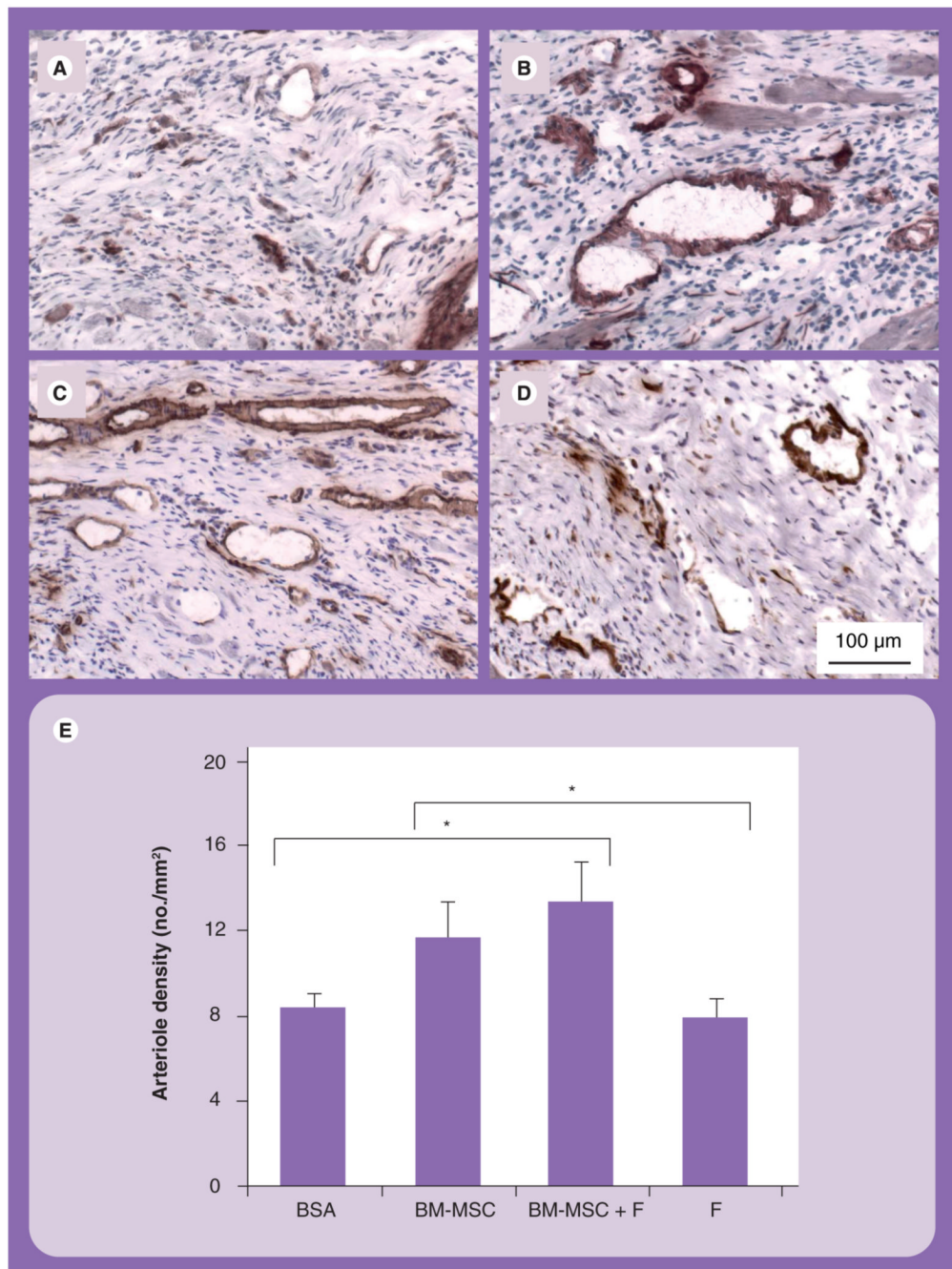BM-MSC: Bone marrow-derived mesenchymal stem cell; BSA: Bovine serum albumin; F:
Fibrin.

Figure 5. Arteriogenesis in the infarct area
Immunohistochemical staining of smooth muscle α-actin for smooth muscle cells in **(A)** BSA, **(B)** BM-MSC, **(C)** BM-MSC in fibrin and **(D)** fibrin groups. Samples were counterstained with hematoxylin. **(E)** Quantification of arteriole density.
*p = 0.025, BSA versus BM-MSCs in fibrin; p = 0.016, fibrin versus BM-MSCs in fibrin.
BM-MSC: Bone marrow-derived mesenchymal stem cell; BSA: Bovine serum albumin; F: Fibrin.