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Original Article

Bone Marrow–Derived Mesenchymal Stem Cells Promote Angiogenic Processes in a Time- and Dose-Dependent Manner *In Vitro*

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Bone marrow–derived mesenchymal stem cells (MSCs) have received much attention as a potential treatment for myocardial infarction because of their potential to integrate into the host myocardium and repair the injured heart. The mode of action of stem cell–mediated cardiac repair is still somewhat unclear, including the potential role of MSCs in neovascularization. The objective of this study was to determine the *in vitro* effect of MSCs on angiogenesis-related endothelial cell (EC) behavior, including migration, monolayer permeability, and vessel formation and stabilization. In a noncontact coculture system, we found that MSCs increase EC proliferation and migration, promoting early events of angiogenesis, while also decreasing EC monolayer permeability. Further, in a time- and dose-dependent manner, MSCs in direct coculture with ECs on Matrigel™ can increase the persistence of preexisting vessels by greater than threefold, with complex vessels remaining stable for more than 10 days. The results demonstrate that MSCs play an active role in the cellular processes involved in the formation, stabilization, and maturation of newly formed vessels. Further, these outcomes are not governed solely by either paracrine or direct contact effects and are both time and dose dependent.

Introduction

Cardiovascular disease is still the leading cause of morbidity and mortality in the developed world.[1] The degenerative mechanism underlying myocardial infarction (MI) involves a decline in the number of viable cardiomyocytes after a loss in myocardial perfusion due to an obstruction of flow in the coronary vasculature. Over time, the infarcted region expands and the heart compensates by ventricular dilation and cellular hypertrophy to sustain output requirements.[2] Simplistically, reparative mechanisms are overwhelmed, and progress to left ventricular wall thinning and congestive heart failure continues.

Stem cells have received much attention as a treatment for MI because of their potential to integrate into the host myocardium, differentiate to replace lost cells, and repair the injured heart.[3] The optimal stem cell source still remains unclear. Bone marrow–derived mesenchymal stem cells (MSCs), an endogenous population of cells found in the bone marrow, have the potential to differentiate into a number of cell types, including fat, bone, and cartilage.[4,5] A number of studies have reported cardiovascular differentiation[6–8] leading to the MSC population being widely studied in preclinical models of MI.[9–12] Further, these initial studies have been translated to a clinical setting with several human trials showing MSCs to be advantageous as a therapy in early and late cardiovascular disease.[13–15]

The mode of action MSCs have in cardiac repair is still somewhat unclear. A number of mechanisms have been reported, including cellular differentiation,[16] release of paracrine factors,[17] and the recruitment of endogenous repair cells.[18] One other mechanism of particular interest is the ability of MSCs to promote neovascularization in and surrounding the infarct zone after cell delivery. This may restore partial levels of perfusion to the ischemic myocardium, reduce scar growth, and slow the continued degenerative process to heart failure. MSCs have been shown to release proangiogenic factors that promote vascular cell function and new blood vessel formation,[19] and an increase in vascular density has been reported in a number of preclinical studies of MI after cell delivery.[20,21] The use of MSCs as a proangiogenic therapy also has implication in the treatment of peripheral vascular disease as well as wound healing. A recent study comparing MSCs to bone marrow mononuclear cells has shown that MSCs surpass mononuclear cells in their efficacy in improving vascular density in a model of

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hind limb ischemia.[22] Further, in the setting of wound healing, MSCs were shown to promote accelerated wound healing and closure through angiogenesis and differentiation.[23]

The mechanism by which vascularity is increased due to MSC therapies in post-MI cardiac tissue as well as peripheral tissue is unknown. It still remains unclear whether MSCs integrate into the new vessels formed promoting vessel maturity or promote neovascularization indirectly by the release of paracrine cues. Understanding the role MSCs play on the different steps of angiogenesis and vasculogenesis will allow not only optimization for cardiac repair (e.g., time and dose of implantation), but also translation to perfusion of other ischemic native and engineered tissues. Due to the limited assessments possible in clinical studies, research in *in vitro* and preclinical studies is necessary to determine the mechanisms by which MSC-based therapies promote cardiac repair. A recent study has shown that MSCs in coculture could have a beneficial effect on the formation of long-lasting vessels *in vivo*;[24] however, the effect of MSCs on EC processes and behavior has yet to be characterized. Thus, the objective of this study was to determine the *in vitro* effect of MSCs on angiogenesis-related EC behavior, including migration, monolayer permeability, and vessel formation and stabilization. We found that MSCs promote cell processes involved during the initial stages of new vessel formation, as well as vessel maturation.

Materials and Methods

Mesenchymal stem and EC source and culture

Bone marrow aspirates were obtained from the iliac crest of normal human donors; all procedures were performed with informed consent and approved by the Clinical Research Ethical Committee at University College Hospital, Galway. MSCs were isolated by direct plating, expanded in culture, and tested for osteogenic and adipogenic potential as described previously.[25] All experiments were carried out with three individual MSC isolations. Human aortic ECs, smooth muscle cells (SMCs), and appropriate growth medium were obtained from Cambrex (East Rutherford, NJ). MSCs and ECs were each culture-expanded to passage five, and SMCs were expanded to passage three.

Adipogenic and osteogenic differentiation assays

MSCs were seeded into six-well plates at 20×10^4 cells/well and cultured until they became confluent in complete MSC growth medium. Cells were then placed in adipogenic induction medium (DMEM–high glucose, 10% FBS [Hyclone, Logan, Utah], 5% rabbit serum [RS], 1 μM dexamethasone, 200 μM indomethacin, insulin 10 μg/mL, and 0.5 mM isobutylmethylxanthine) for 3 days and transferred to adipogenic maintenance medium (DMEM–high glucose, 10% FBS, 5% RS, and insulin 10 μg/mL) for 3 days.[26] After three cycles of induction and maintenance cell cultures were fixed in 10% neutral buffered formalin. Fixative was removed, and the wells rinsed in distilled water and were covered in Oil red O staining solution. Cells were counterstained with hematoxylin for 1 min and rinsed with warm water. Oil red O–stained lipid droplets were visualized with an inverted Olympus IX71 microscope. For Osteogenesis, MSCs were seeded into 24-well plates at 1.3×10^4 cells/well and cultured for 24 h. Cells were placed in osteogenic induction medium (α-MEM, 10% FBS, 10 mM β-glycerophosphate, 10 mM ascorbic acid 2-phosphate, 10 mM dexamethasone, and 1% antibiotic–antimycotic) for 15 days, with medium changes every 3 days. Cells in complete MSC growth media acted as controls. Calcium depositions were visualized after von Kossa staining as above.

EC migration

EC migration due to noncontact coculture with MSCs was assessed using a modified Boyden chamber as described previously with modifications.[27] Briefly, ECs were serum starved in OptiMEM medium (Invitrogen, Carlsbad, CA) for 2 h and seeded onto 8-μm-pore transwell inserts (12 well; BD Biosciences, Bedford, MA) at 2×10^4 cells/well. MSCs (5×10^4 cells/cm^2) were grown in the bottom chamber in expansion medium for 24 h and equilibrated in OptiMEM medium for 5 h before the placement of the EC-seeded upper chamber. As a control, EC-seeded upper chambers were also cultured over bottom chambers filled with only OptiMEM medium. After either 10 or 18 h of coculture, transwells were fixed in 3% formaldehyde (Polysciences, Warrington, PA). The bottom side of the transwells was imaged to visualize the cells that had migrated through the porous membrane. Migration was quantified by counting cell number in five random fields using a 20× magnification.

EC growth

EC cell number changes due to noncontact coculture with MSCs were assessed using a Coulter Counter (Beckman Coulter, Fullerton, CA). ECs were seeded at 7×10^3 cells/well above MSCs seeded at 5.7×10^3 cells/cm^2 in the bottom chamber of a 4-μm-pore insert system (six multiwell; BD Biosciences). Cells were trypsinized off inserts and EC number quantified on days 2 and 4 in EC growth medium.

EC permeability

Permeability was assessed by quantitating the diffusion of horseradish peroxidase (HRP) through a confluent EC monolayer.[28] ECs were plated at 6×10^4 cells/cm^2 on fibronectin-coated 4-μm-pore inserts (24 multiwell; BD Biosciences). Inserts were cultured for 4 days with medium changes every other day above plate wells with either EC growth medium (control) or MSCs seeded at 5.7×10^3 cells/cm^2. To test permeability of the control and experimental EC samples, HRP (1.5 mg/mL; Sigma-Aldrich, St. Louis, MO) was added to the top well for 30 min. The amount of HRP that diffused through the cell monolayer was determined colorimetrically using 3,3′,5,5-tetramethylbenzidine (Sigma-Aldrich) and absorbance readings at 490 nm. In separate parallel samples, permeability was challenged with vascular endothelial growth factor (VEGF; Invitrogen) at a concentration of 50 ng/mL for 30 min before the HRP test.

Cell–cell interactions in ECs

ECs were assayed for cadherin-related proteins using both immunocytochemistry and flow cytometry to establish how coculture with MSCs affected adherence molecule expression between ECs.[29] Endothelial monolayers, similarly prepared to those for permeability testing, were assessed for

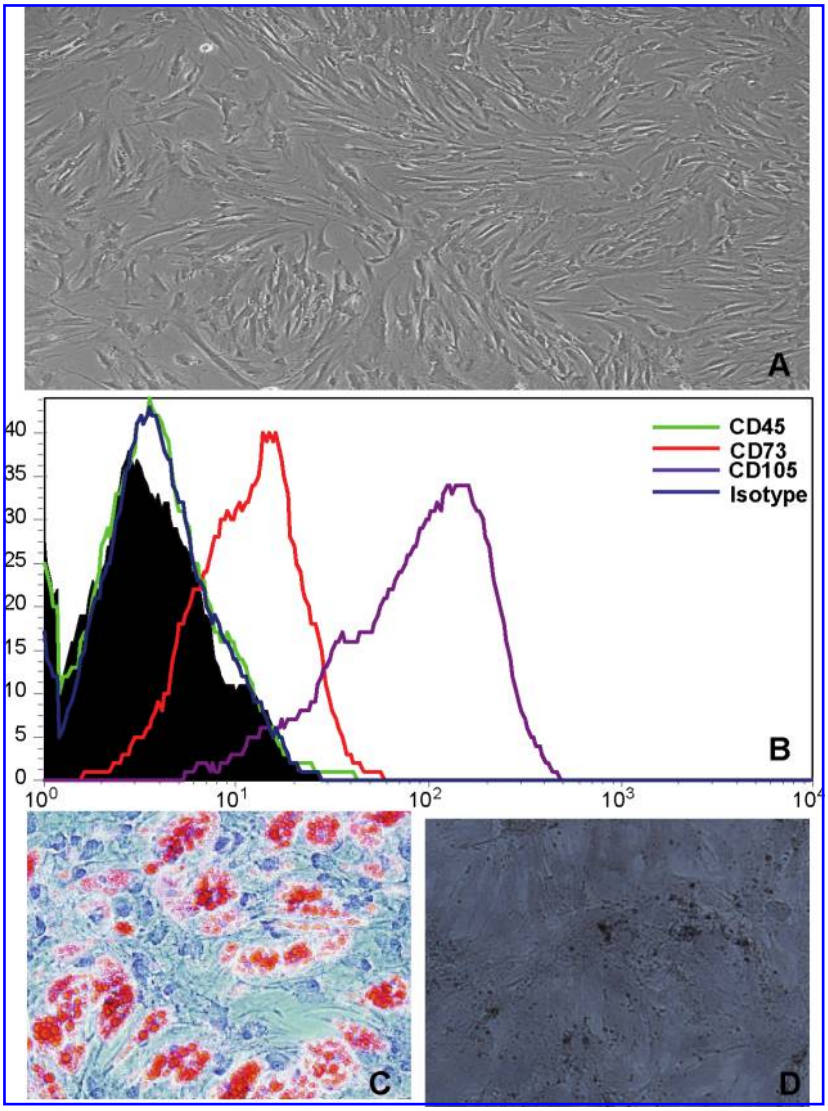


FIG. 1. MSC characterization and differentiation potential. (**A**) Phase contrast micrograph showing the fibroblastic morphology of MSCs in culture at passage 5. (**B**) Flow cytometry histograms indicate the fluorescent intensity of unstained cells (solid), or cells labeled with anti-CD45, anti-CD73, anti-CD105 antibodies, or anti-mouse IgG 1 isotype control (colored). MSCs cultured in adipogenic and osteogenic differentiation conditions for 15 days stained positively with Oil red O for lipid droplets in the cytoplasm (**C**) and von Kossa for calcium deposits in the well (**D**).

VE-cadherin (extracellular), β-catenin (intracellular subunit of cadherin complex), or general cadherin expression (pan-cadherin). For visualization, membranes were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich), blocked for nonspecific binding with 4% donkey serum (Sigma-Aldrich), stained with goat anti-human VE-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-human β-catenin (Abcam, Cambridge, MA) primary antibodies, and indirectly fluorescently labeled with Alexa Fluor 488–conjugated anti-goat or anti-rabbit antibodies (Invitrogen). The actin cytoskeleton and nuclei were also fluorescently labeled using Phalloidin-AF 546 (Molecular Probes–Invitrogen) and Hoechst 33258 (Invitrogen), respectively. Fluorescent images were taken on a confocal microscope (LSM 510; Zeiss, Maple Grove, MN). Separate samples were prepared for flow cytometry: cells were trypsinized from membranes, stained as above with a goat anti-human pan-cadherin antibody (Abcam), and analyzed on a digital cytometer (BD LSR; BD Biosciences).

Vessel structure formation

EC-vessel-like structure formation *in vitro* was carried out as described previously, with modifications.[30] Briefly, ECs (3×10^4/well) were plated on growth-factor-reduced Matrigel™ (120 μL/well; BD Biosciences) in 48-well TC-treated culture plates. MSCs at varying ratios (1:1 to 1:10) were added at time points ranging from 0 to 24 h after EC plating. For some samples, SMCs were added at a 1:1 ratio at 24 h to compare MSCs to another mesenchymal cell type that is known to stabilize vessels.[31] Images were taken over 72 h and then at 10 days. For fluorescent images, ECs were labeled with 250 μM PK2 (green fluorescence; Sigma-Aldrich) and MSCs/SMCs were labeled with 250 μM PKH26 (red fluorescence; Sigma-Aldrich), respectively, before plating on Matrigel™. Image analysis was carried out using Olympus Cell R imaging software. Tubule length, junction area, and cross section diameter were measured on 10× images ($n=5$).

Statistical analysis

All values are presented as mean ± standard deviation of the mean. Data were analyzed using the Student's *t*-test or a general linear model two-way ANOVA with a *post hoc* Tukey test to compare between groups as appropriate. Value of $p<0.05$ was considered statistically significant.

Results

MSCs were isolated from the bone marrow and expressed characteristic cell surface markers

Cell isolation, expansion, and differentiation of MSCs using direct plating were established according to other reports.[25] Isolated MSCs adhered to culture plastic had a fibroblastic morphology (Fig. 1A) and proliferated up to passage five with a doubling time of approximately 2 days. The isolated cells were negative for CD45, a known hematopoietic marker, but positive for MSC characteristic markers CD73 and CD105 (Fig. 1B). MSCs also differentiated along the adipogenic and osteogenic pathways as evidenced by the accumulation of lipid droplets in the cytoplasm and deposition of calcium in the well (Fig. 1C, D).

MSCs increase EC migration and cell growth

In a noncontact coculture model, the effect of MSCs on endothelial cellular processes was determined for migration and cell growth (Fig. 2). Under serum-free conditions, ECs were cultured in the top well with just medium or MSCs in the bottom well of a modified Boyden Chamber. Images of ECs that had migrated to the bottom of the transwell (Fig. 2A) indicate that the total number of migrated cells increased from 10 to 18 h, with further increases in the presence of MSCs. Quantification revealed that the presence of MSCs increased the migration of ECs significantly at both 10 and 18 h after plating ($p < 0.001$; Fig. 2E). In this same configuration, EC cell growth was assessed at days 2 and 4. In the subconfluent EC layer there was an increase in cell number between 2 and 4 days for both media and MSC groups (Fig. 2F). However, coculture with MSCs resulted in a significant increase ($p < 0.05$) in the number of ECs at day 4, compared to cells cultured with just medium in the bottom well. These results show that through indirect communication MSCs promote EC migration and growth.

MSCs decrease EC monolayer permeability

The effect of MSC coculture on EC permeability was assessed via diffusion using HRP and cell–cell communication focused on cadherin-related proteins (Fig. 3). After 4 days of culture, confluent EC layers had a statistically significant reduction ($p < 0.01$; Fig. 3A) in HRP diffusion when cocultured in the presence of MSCs compared to medium alone. The presence of MSCs also significantly ($p < 0.05$) mitigated the increase in diffusion when challenged with VEGF, known to increase EC permeability. The observed changes in permeability, however, were independent of cell density as there was no significant difference in cell number at the time of assessment ($p = 0.223$; $n = 4$).

Permeability of endothelial vessel lining is dependent on cell adhesion, cell–cell communication, and vessel maturity,[32] which are often associated with cadherin-related proteins. Immunostaining indicates the presence of VE-cadherin and β-catenin in all samples, with an apparent upregulation of expression at EC cell junctions for those samples cocultured with MSCs (Fig. 3B–E). Further, when cells were trypsinized and stained with a pan-cadherin antibody, flow cytometry results showed a marked increase in the overall expression of cadherins in those ECs that were cocultured with MSCs compared to media alone (Fig. 3F). Taken together, these results indicate that noncontact coculture with MSCs may increase the functionality of an EC lining by decreasing permeability and upregulating adherin junction proteins.

MSCs promote vessel-like structure stabilization in a time-dependent manner

ECs seeded on Matrigel™ *in vitro* form a lattice of vessel-like structures within 12 h, which is most extensive at ~24 h, but then proceeds to degrade with only structure fragments present at 72 h (Fig. 4A, top row). At time points varying from 0 to 24 h after initial EC seeding, MSCs (at MSC:EC ratio of 1:10) were added to the Matrigel™ surface. The addition of MSCs at 0 h inhibited lattice formation, though addition at either 0 or 12 h promoted cellular bunching (Fig. 4, arrows) by 48 h. However, when MSCs were added after an extensive lattice structure already existed, 24 h after initial EC seeding, structures stabilized to persist for over 72 h. This observation was confirmed by tubule length quantification at 72 h, which was significantly higher in the ECs cocultured with MSCs than ECs cultured alone ($p < 0.05$, Fig. 5Ei). Inspection at higher magnification at 48 h (Fig. 4B) showed that, on Matrigel™, ECs form lattices through extension (Fig. 4B, left), MSCs form cell clusters (Fig. 4B, right), but coculture interaction results in cellular processes that can be seen extending away from cell bunches and tubules (Fig. 4B, middle images). Thus, this sequence of images shows that MSCs affect EC vessel formation and stabilization in a time-dependent manner, possibly through direct cellular contact.

MSC addition increases vessel-like structure persistence, as well as vessel complexity in a dose-dependent manner

MSCs were added at increasing doses (1:10 to 1:1 MSC:EC ratio) 24 h after initial EC seeding onto Matrigel™ and visualized at 48 h. The addition of MSCs seemed to increase vessel-like structure thickness and junction size in a dose-dependent manner (Fig. 5A). Image analysis demonstrated a significant increase in tubule cross-sectional diameter and junction area in ECs cocultured with MSCs at a 10:1 and 1:1 ratio versus ECs alone ($p < 0.05$, Fig. 5Eii, iii). Independent fluorescent staining of ECs (green) and MSCs (red) was used to visualize cell interaction in a 1:1 MSC:EC coculture dose (Fig. 5B–D). Despite the same initial seeding density of ECs, in the coculture system there was an increase in the number of ECs in both the vessel structures (Fig. 3Bi) and junctions (Fig. 5Bii) compared to EC-only cultures. The two-dimensional confocal images indicate that the MSCs form the outer layer for both vessel structures and junctions. To determine the relative location of MSCs to ECs in three-dimensional space, a series of confocal images were taken and reconstructed into a *z*-stack (Fig. 5C). Cross sections of the projected image show that MSCs are intimately wrapped around ECs (Fig. 5C, side panels) in vessels. Overall, in coculture systems, vessel-like structures and junctions were each composed of both MSCs and ECs, in closely associated but spatially distinct locations.

Vessel-like form is substantially different in coculture systems compared to EC-only cultures. At 48 h, MSC:EC (1:1) cocultures generate vessels with cross-sectional diameters of

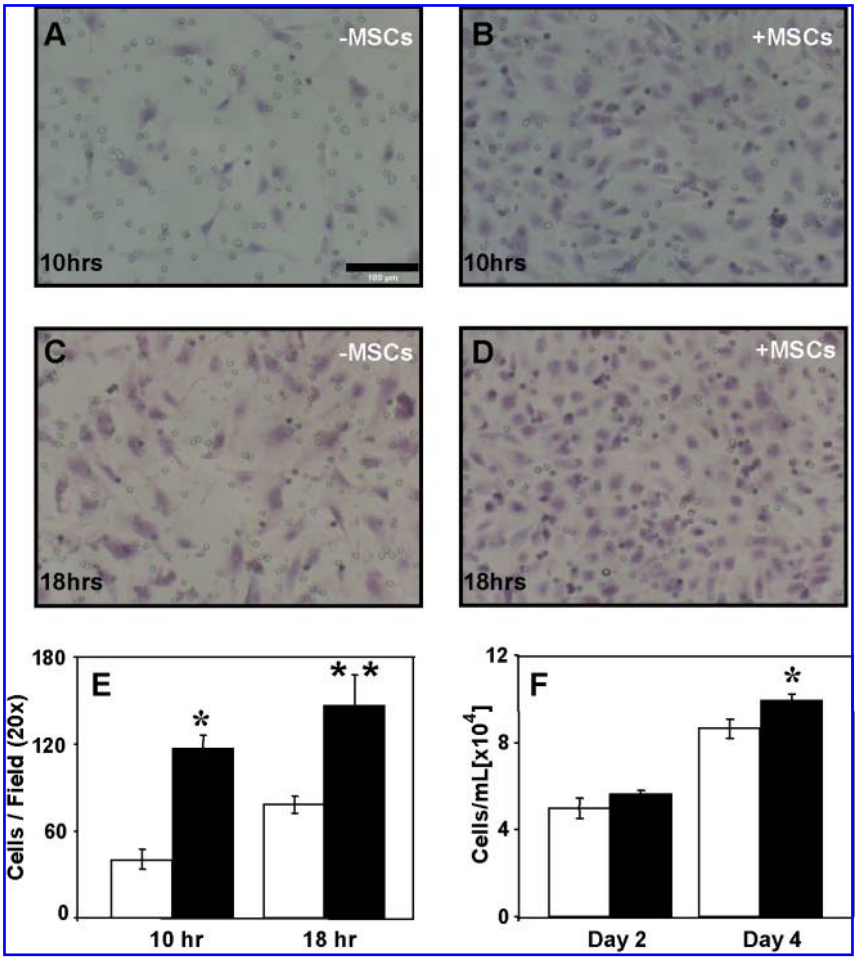


FIG. 2. Effect of MSC coculture on EC migration and growth. Assessment of the migration of ECs to the bottom of transwell membranes in a modified Boyden chamber. (**A**–**D**) Representative images of hematoxylin-stained membranes indicate EC migration after 10 h (**A, B**) and 18 h (**C, D**) of coculture with MSCs (**B, D**) or medium alone (**A, C**). Scale bar indicates 100 μm. (**E**) Quantification of migrated ECs after coculture with MSCs (black bars) or medium alone (white bars). Data are presented as the mean ± SD ($n = 4$). Asterisks indicate $p < 0.001$ (*) or $p < 0.001$ (**) for MSC versus medium samples at 10 and 18 h, respectively. (**F**) Quantification of EC cell number after 2 or 4 days of coculture with MSCs (black bars) or medium alone (white bars). Data are presented as mean ± SD. Asterisk (*) indicates $p < 0.05$ for MSC versus medium samples.

500 μm at some points, in comparison to 50 μm diameters in EC-only cultures (Fig. 5Eii, iii), and lengths that approximate 2 mm. Further, there is evidence of occasional "branch points" where two cords come together to continue as a larger vessel (Fig. 5D). These large complex vessel-like structures formed in an MSC-EC coculture (Figs. 5D, 4B, and 6Aiii) are vastly different from the long, thin processes formed in EC-only cultures (Fig. 6Ai) and more similar to the types of vessels formed in an SMC-EC coculture system (Fig. 6Aiii). Also, the MSCs interact with ECs in a similar manner to SMCs (Fig. 6Aiii) by attaching and forming sheaths around the EC tubules. In addition, these hybrid MSC-EC elongated vessel structures have a stability that allows persistence beyond 10 days (Fig. 6Biii), at which time EC-only or MSC-only cultures have already regressed to either no remaining lattice structure or only cell bunches (Fig. 6Bi, iii). Thus, the addition of MSCs after EC vessel formation on Matrigel™ may be used to increase vessel size and stability *in vitro*. To try and understand whether MSC dose was an important requirement for independent tubule formation, MSCs were added to Matrigel at increasing densities up to 600,000 cells/well. No vessel structures formed, and characteristic cell bunches became apparent after 24 h in culture (Fig. 6Ci, ii). Finally, to understand whether the sequence of cell addition is important for vessel stabilization, MSCs were added to the Matrigel at T0 at varying ratios, ECs were added at 24 h after MSC seeding, and characteristic cell bunches formed with no demonstratable lattice structure formation (Fig. 6Ci).

Discussion

Angiogenesis is often considered in the context of myocardial tissue repair.[33] In most cases, chronic ischemia persists after MI, causing negative ventricular remodeling that can lead to heart failure and death.[2] The importance of restoring a vascular supply to the ischemic myocardium is therefore of high clinical relevance. The development of MSC implantation as a cellular therapy for post-MI patients is ongoing, with safety and efficacy shown in a number of clinical trials.[13–15] In preclinical models of MI, MSC delivery has been shown to augment a proangiogenic effect in ischemic myocardium, as evidenced by an increase in vascular density and reperfusion.[20,21,34] Further, increases in global cardiac function, seen in initial clinical trials, have also been attributed to increased perfusion in the ischemic myocardium.[35–38] Early discussion on whether MSCs incorporate directly into these new vessel structures[19] has been replaced with a more recent focus on the indirect interaction between MSCs and ECs through the release of paracrine factors.[39–42]

Moreover, the use of MSCs to promote angiogenesis has implications in the reperfusion of other pathological ischemia, as well as for engineered tissues. For instance,

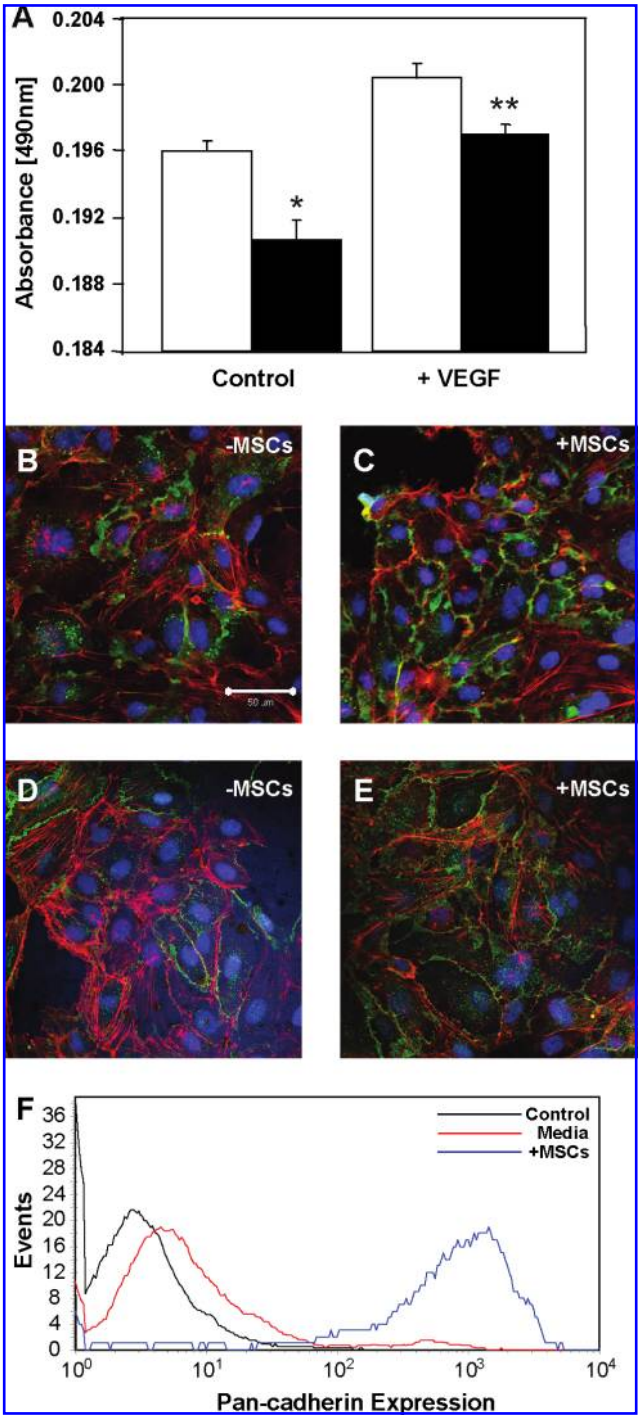


FIG. 3. Effect of MSC coculture on EC permeability. (**A**) Quantification of HRP diffusion through EC monolayers cocultured with MSCs (black bars) or medium alone (white bars), without (control) or with VEGF challenges to permeability. Data are presented as mean ± SD ($n = 4$). Asterisks indicate $p < 0.01$ (*) or $p < 0.05$ (**) for MSC versus medium samples. Immunofluorescent staining for VE-cadherin (green; **B, C**) or β-catenin (green; **D, E**) in ECs cocultured with MSCs (**C, E**) or medium alone (**B, D**). All samples were also stained for F-actin cytoskeleton (red) and nuclei (blue). (**F**) Flow cytometry histograms of unstained cells, or ECs cocultured with MSCs or medium alone and stained using a pan-cadherin antibody to indicate general cadherin expression.

peripheral arterial disease is strongly associated with a high risk of cardiovascular morbidity and mortality.[22] The ability to promote vascularization in these patients would have great clinical consequence in reducing the associated morbidity. Interestingly, MSCs have recently been shown to improve vascular supply in affected limbs in the setting of peripheral arterial disease through the promotion of angiogenesis and differentiation.[43] Also, as biomaterial and bioreactor technologies advance, there continues to be an increase in the complexity and size of tissues/organs generated *in vitro*.[44] Maintaining the cells viable within these constructs will likely require vascularization, perhaps before or after implantation, involving graft or host cells. Recent efforts in using stem cells for tissue engineering—particularly, MSCs in bone formation—further elevate interest in understanding the role of MSCs in angiogenesis.

These present studies investigated the effect of MSCs on EC processes involved in angiogenesis. In a noncontact coculture system, we found that MSCs increase EC proliferation and migration, promoting early events of angiogenesis. Further, in a time- and dose-dependent manner, MSCs in direct coculture can increase the persistence of preexisting vessels by greater than threefold, with complex vessels stable more than 10 days later. While there was no evidence of lumens in these vessel-like structures formed on Matrigel™, assays on EC monolayers showed that the presence of MSCs decreased permeability and increased expression of cadherin-related (e.g., cell–cell contact) proteins. Thus, MSCs can play a role at various stages of new vessel formation, from releasing paracrine factors to promote early angiogenic events to assuming functions of vascular SMCs in vessel stabilization and functionalization.

Early steps of angiogenesis and vasculogenesis depend on the potential for EC migration and proliferation. In these *in vitro* studies, MSCs increased overall EC migration within 10 h, which is consistent with the cell bunching seen when MSCs were added to ECs at early time points during vessel formation. While these studies did not investigate the soluble factors in the coculture medium, it has been shown that MSCs release a number of proangiogenic cytokines during culture, including VEGF, bFGF, and PDGF.[19] VEGF, in particular, is a major component involved in EC migration during vessel formation.[45] Less cell bunching occurred when MSCs were added after an extensive EC lattice had formed. Once in fully formed structures, it is consistent with vessel maturity that ECs would no longer be as responsive to a paracrine migratory stimulus. We also assessed cell growth in response to MSC noncontact coculture and found that EC cell number was increased after 4 days of culture. Further, direct coculture, in an MSC dose-dependent manner, seemed to increase EC numbers found in elongated vessels and junctions, perhaps contributing to the initial stages of vessel fictionalization. Thus, MSCs may contribute to the early phases of angiogenesis *in vivo* by promoting EC migration and proliferation, potentially through paracrine and/or direct contact effects.

Latter stages of angiogenesis require mural cells for vessel stabilization and functionalization. We found that the addition of MSCs to an established EC lattice stabilized vessel-like structures for greater than 10 days, resulting in more than a threefold increase in vessel persistence. In these vessels, MSCs were positioned as a sheath around the ECs,

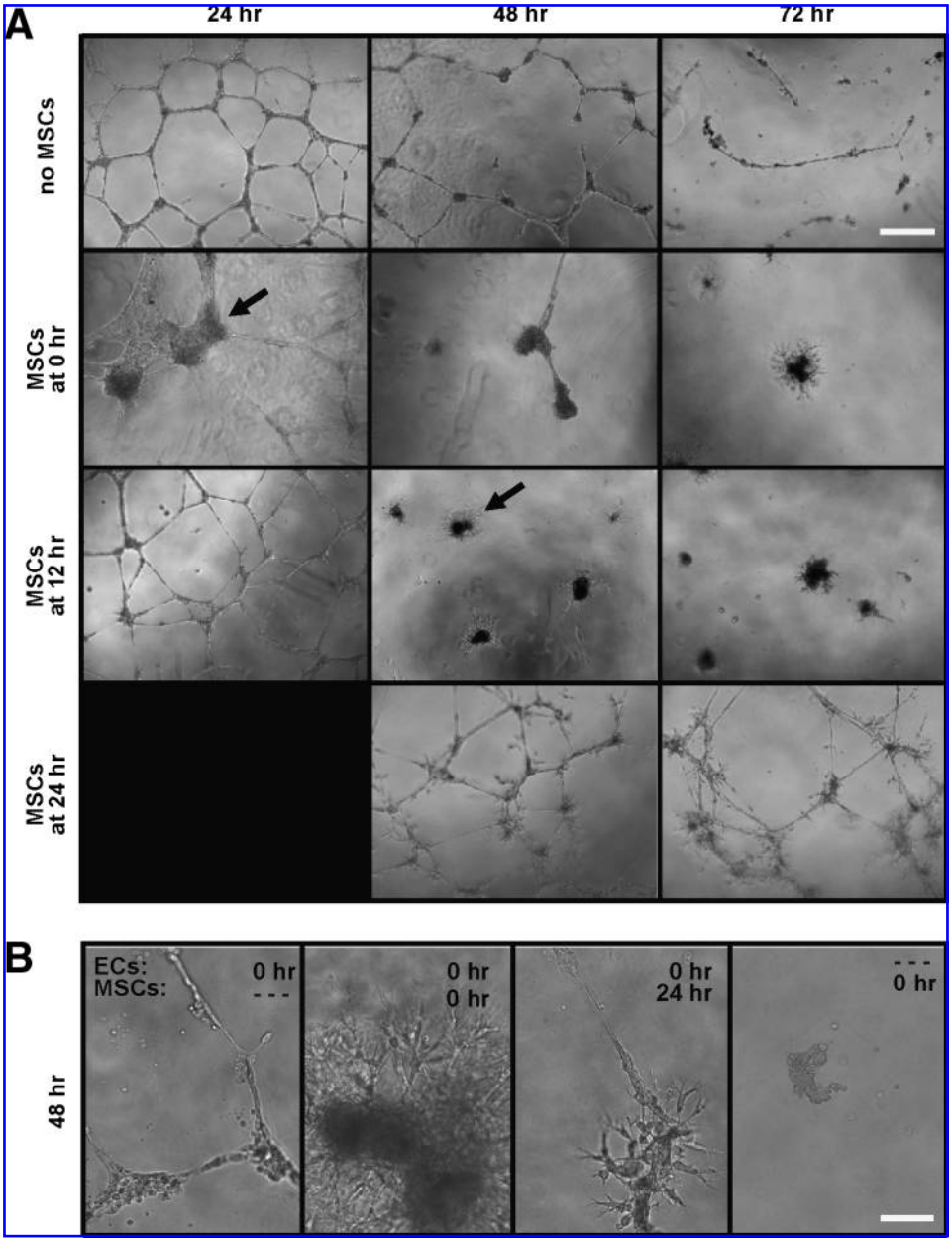
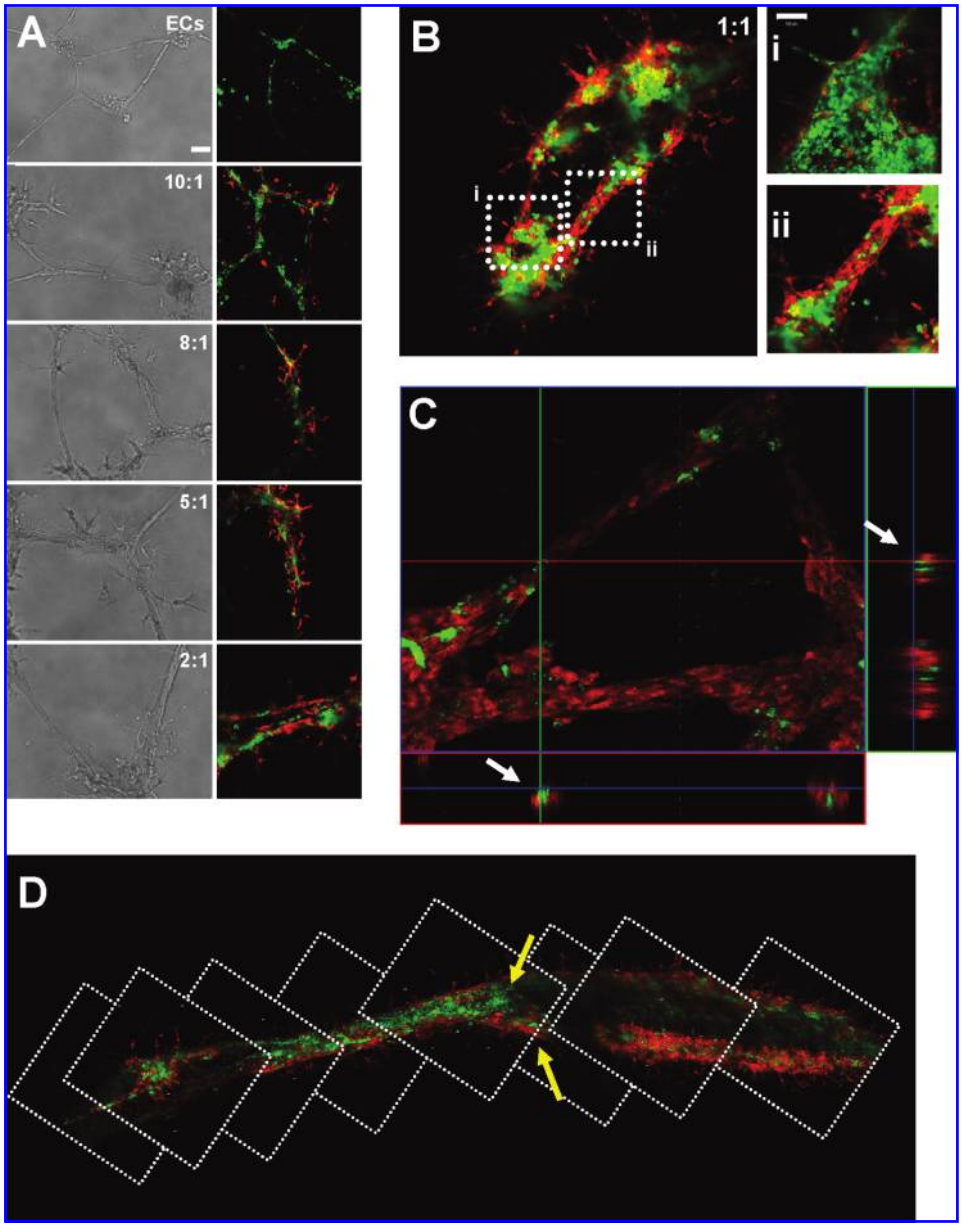


FIG. 4. Time-dependent effect of MSCs on vessel formation and stabilization. (**A**) Representative phase micrographs of vessel formation on Matrigel over 72 h. ECs form a lattice structure within 12 h, which is most extensive at 24 h, and degrades by 72 h. MSCs were added at a ratio of 1:10 (MSC:EC) at 0, 12, and 24 h. The addition of MSCs at 0 and 12 h resulted in cellular bunching (arrows), whereas addition at 24 h promoted structure stability beyond 72 h. (**B**) High-magnification images at 48 h of ECs alone, of ECs with MSCs added at 0 or 24 h, or of MSCs alone. Scale bar indicates 400 μm (**A**) or 100 μm (**B**).

similar to the observed interaction of pericytes and ECs.[46] Direct comparison of MSCs and SMCs in coculture with ECs reveals that both cell types form similar multi-cellular thick vessel-like structures. Further, other studies have shown that MSCs in culture release factors involved in adhesion molecule expression[47] and maturity[19] in vessels. It remains to be determined whether MSCs could provide a functioning vessel wall, including a properly organized matrix and the capacity for vasoactivity. Nonetheless, these studies indicate that MSCs may be capable of replicating some of the functions of pericytes and vascular SMCs during angiogenesis. A recent study by Au *et al.* demonstrated that MSCs efficiently stabilized nascent blood vessels *in vivo* by functioning as perivascular precursor cells.[24] When MSCs were added with venous arterial cells in collagen gels *in vivo*, they organized themselves into vessels that remained stable for 130 days. This is in line with the results seen in our study, where MSCs associated with the outer layers of ECs acting like pericytes and stabilized the vessel-like structures for up to 10 days *in vitro*.

During normal vessel stabilization, ECs adopt a quiescent phenotype and become less permeable.[45] In our studies, the direct coculture of MSCs and ECs did not yield structures with lumen, likely due to the absence of shear stress and hypoxia that serve as physiological cues during normal vessel formation.[32] In noncontact coculture, however, the presence of MSCs seems to promote EC-EC interactions, as evidenced by a decrease in endothelial monolayer permeability and an increase in cadherin-related protein expression. VE-cadherin and its intracellular subunit β-catenin were found to be upregulated in ECs cocultured with MSCs, and a large increase was seen in pan-cadherin expression in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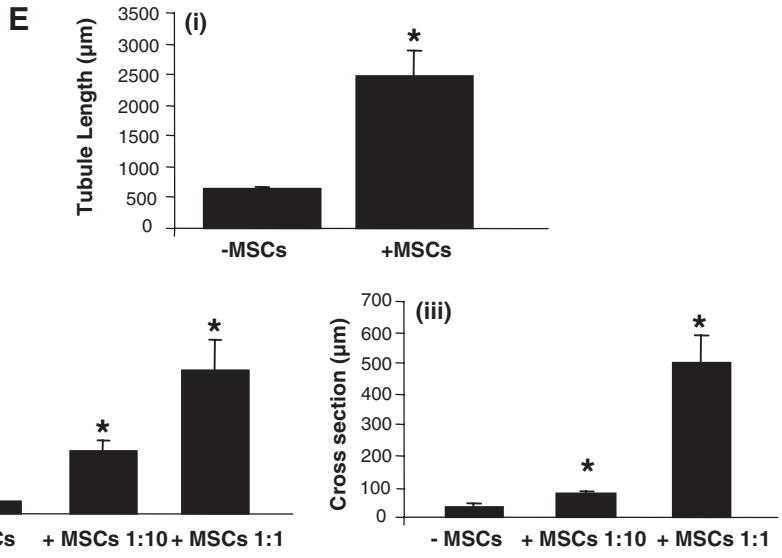
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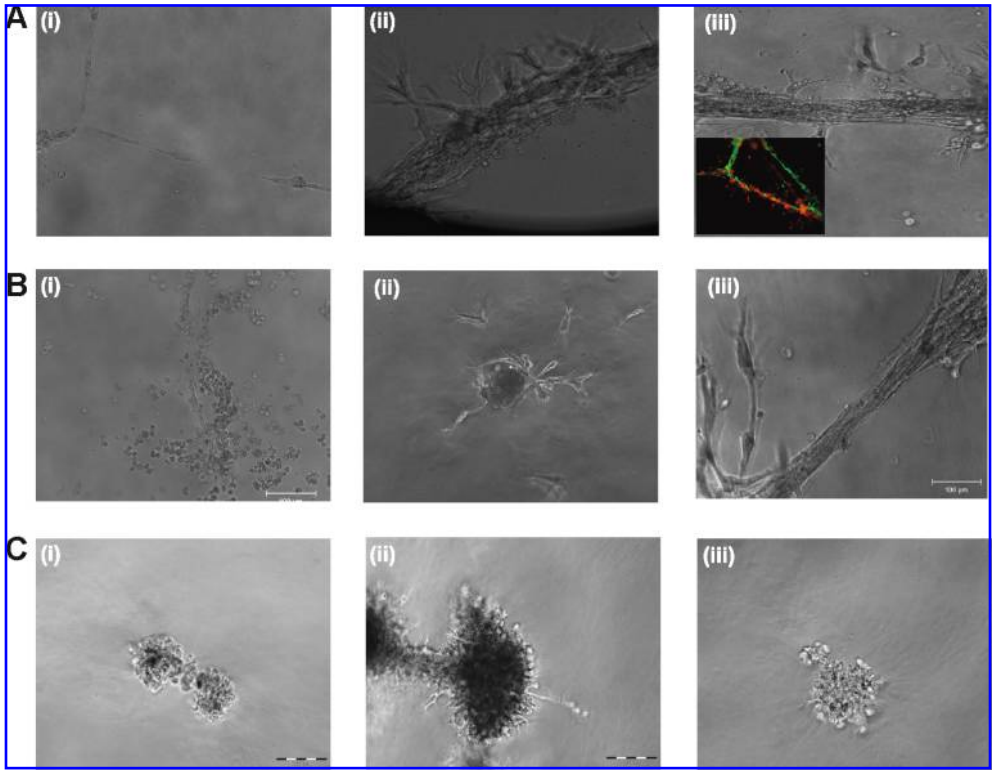

FIG. 6. MSC-EC vessel form and persistence. (**A, i–iii**) Phase micrographs of cells seeded on Matrigel™ for 2 days (scale bar indicates 100 μm). After 2 days, ECs cultured alone (**i**) form long, thin processes, while MSCs (**ii**) or SMCs (**iii**) added at a 1:1 ratio at 24 h resulted in complex, thick vessels. Inset (**iii**) shows SMCs (red) interacting with the outside of ECs (green) in a similar manner to MSCs. (**B, i–iii**) Phase micrographs after extended culture for 10 days, ECs (**i**) or MSCs (**ii**) cultured alone have either no remaining lattice structure or only cell bunches, respectively. Conversely, when MSCs were added in a 1:1 ratio at 24 h after initial EC seeding, complex and thick vessels persisted for 10 days (**iii**). (**C, i, ii**) Representative phase micrographs of MSCs added at increased ratios (60,000 and 600,000, respectively). There were no tubules formed with increased cell dose. (**C, iii**) Representative phase image of MSCs/ECs cocultures where ECs were added 24 h after MSC seeding; no vessel-like structures were present.

cocultured ECs in our assessment. Indeed, studies have shown that the cytokines Ang-1[47] and TIMP-1,[48] both known to be released by MSCs, promote the functionality of endothelium, by decreasing EC proliferation and monolayer permeability. We found that MSCs release these cytokines along with VEGF, PDGF, and TIMP 2 in culture (data not shown), which may account for the marked increase in pan-cadherin seen in our studies and the decrease in EC permeability. Thus, through the release of paracrine factors, MSCs may aid in the maturation of an endothelial lining, promoting the patency and anti-thrombogenic properties of new vessels.

The effect of MSC coculture on vessel-like structure stability and form was found to be time and dose dependent. Application of MSCs after an EC lattice was established resulted in extended vessel persistence, while equally high doses of MSCs and ECs supported the formation of large spatially organized complex vessel structures. We found that the sequence of cell addition is also an important requirement for vessel-like structure formation, where MSCs added before EC addition resulted in cell bunching and loss of lattice structure formation. The time and dose appropriate for *in vivo* applications, however, still need to be empirically determined. After an MI, there is a large angiogenic stimulus

FIG. 5. Dose-dependent effect and complexity of MSC-EC vessel formation. (**A**) Phase images (**A**, left panel) at 48 h of MSCs added in increasing ratios (1:10 to 1:2 of MSC:EC) 24 h after initial EC seeding on Matrigel™. Scale bar indicates 200 μm. Fluorescent micrographs (**A** [right panel], **B, C, D**) show MSCs and ECs as red and green, respectively. Increasing the concentration of MSCs resulted in increased tubule thickness, along with an increase in the EC cell number associated with tubules and junctions. At an MSC:EC ratio of 1:1 (**B, C, D**), (**B**) two-dimensional fluorescent images show that MSCs form an outer layer over ECs in both elongated structures (**i**; scale bar indicates 50 μm) and junctions (**ii**). (**C**) A reconstructed three-dimensional *z*-stack of confocal images taken through an elongated structure show that MSCs are intimately associated with and wrap around EC vessels (white arrows). Side panels are cross-sectional images of the 3D projection at the indicated intersection. (**D**) Composite representation of fluorescent images captured along a large vessel shows the joining of two cord structures (yellow arrows) to form a greater complex structure. (**E**) Graphical representation of tubule length (**i**), tubule junction area (**ii**), and tubule cross section (**iii**). Data are presented as mean ± SD ($n = 5$). Asterisks indicate $p < 0.05$ (*) for EC-MSC versus EC alone.

released by the injured heart in response to an obstruction of flow in the coronary vasculature. Three to 7 days after MI, concentrations of VEGF and SDF-1, factors involved in angiogenesis and cell recruitment, are the highest, and there is also an increase in circulating progenitor cells.[49] Application of MSCs as a cardiac therapy during this time may leverage the naturally available angiogenic factors while avoiding the initial inflammatory response. In fact, restoration of cardiac function in rats was maximal when MSCs were implanted 1 week after MI.[50] The form of MSC therapy also needs to be further defined: conditioned media from MSCs under a number of different culture conditions, including hypoxia,[51] mechanical loading,[52] and 3D cluster culture,[39] has also been reported to promote endothelial function and tubule formation. However, we found that conditioned media from MSCs did not increase EC vessel stabilization in a similar fashion to direct cell coculture (data not shown). Clinical studies are necessary, therefore, to determine the time, dose, and delivery method of the MSC-based therapy that best helps form, stabilize, and mature newly formed vessels to positively impact post-MI cardiac repair.

MSCs can play an active role in the formation, stabilization, and maturation of newly formed vessel-like structures. We found that these outcomes are not governed solely by either paracrine or direct contact effects and are both time and dose dependent. The multiple aspects by which MSCs affect angiogenesis complicate the interpretation of clinical improvement in cardiac function due to MSC implantation. Thus, further *in vitro* and preclinical studies are necessary to better devise MSC-based therapies for reperfusion of tissues *in vivo*.

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Disclosure Statement

No competing financial interests exist.

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