Cell-based gene therapy modifies matrix remodeling after a myocardial infarction in tissue inhibitor of matrix metalloproteinase-3–deficient mice

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Objective: Cell-based gene therapy can enhance the effects of cell transplantation by temporally and spatially regulating the release of the gene product. The purpose of this study was to evaluate transient matrix metalloproteinase inhibition by implanting cells genetically modified to overexpress a natural tissue inhibitor of matrix metalloproteinases (tissue inhibitor of matrix metalloproteinase-3) into the hearts of mutant (tissue inhibitor of matrix metalloproteinase-3–deficient) mice that exhibit an exaggerated response to myocardial infarction. Following a myocardial infarction, tissue inhibitor of matrix metalloproteinase-3–deficient mice undergo accelerated cardiac dilatation and matrix disruption due to uninhibited matrix metalloproteinase activity. This preliminary proof of concept study assessed the potential for cell-based gene therapy to reduce matrix remodeling in the remote myocardium and facilitate functional recovery.

Methods: Anesthetized tissue inhibitor of matrix metalloproteinase-3–deficient mice were subjected to coronary ligation followed by intramyocardial injection of vector-transfected bone marrow stromal cells, bone marrow stromal cells overexpressing tissue inhibitor of matrix metalloproteinase-3, or medium. Functional, morphologic, histologic, and biochemical studies were performed 0, 3, 7, and 28 days later.

Results: Bone marrow stromal cells and bone marrow stromal cells overexpressing tissue inhibitor of matrix metalloproteinase-3 significantly decreased scar expansion and ventricular dilatation 28 days after coronary ligation and increased regional capillary density to day 7. Only bone marrow stromal cells overexpressing tissue inhibitor of matrix metalloproteinase-3 reduced early matrix metalloproteinase activities and tumor necrosis factor α levels relative to medium injection. Bone marrow stromal cells overexpressing tissue inhibitor of matrix metalloproteinase-3 were also more effective than bone marrow stromal cells in preventing progressive cardiac dysfunction, preserving remote myocardial collagen content and structure, and reducing border zone apoptosis for at least 28 days after implantation.

Conclusions: Tissue inhibitor of matrix metalloproteinase-3 overexpression enhanced the effects of bone marrow stromal cells transplanted early after a myocardial infarction in tissue inhibitor of matrix metalloproteinase-3–deficient mice by contributing regulated matrix metalloproteinase inhibition to preserve matrix collagen and improve functional recovery.

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Supplemental material is available online.

We recently demonstrated that the paracrine benefits of cell transplantation[1] can be enhanced by transfecting the implanted cells with genes to increase their cytokine production[2-4]; these ''cell-based gene therapies'' permit the temporally and spatially regulated release of the gene product. This study assessed the potential for this technique to reduce post–myocardial infarction (MI) matrix remodeling in the remote myocardium and facilitate functional recovery. Cell-based gene therapy could represent a clinically relevant method to prevent congestive heart failure.

After an MI, diminished elasticity and increased wall stress cause the infarcted region to thin and expand while the remote myocardium undergoes extensive remodeling. An increase in the activity of matrix metalloproteinases (MMPs) relative to that of their natural tissue inhibitors (TIMPs) contributes to ventricular dilatation and dysfunction[5] through remodeling of the extracellular matrix (ECM), a dynamic structure that provides a scaffold for cardiomyocytes, facilitates contraction, and supports homeostasis.[6] Although the importance of modifying post-MI matrix remodeling using MMP inhibitors has been established,[7] clinically relevant strategies have been difficult to

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Abbreviations List:

ADAM = a disintegrin and a metalloproteinase domain
ANOVA = analysis of variance
BMSC = bone marrow stromal cell
ECM = extracellular matrix
ELISA = enzyme-linked immunosorbent assay
FAC = fractional area change
LV = left ventricular
MI = myocardial infarction
MMP = matrix metalloproteinase
TIMP = tissue inhibitor of matrix metalloproteinase
timp3$^{-/-}$ = TIMP-3-deficient mice
TNF-α = tumor necrosis factor α
TUNEL = terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling
VEGF = vascular endothelial growth factor

implement[8]: Chronic administration of MMP inhibitors[9,10] and the initial clinical trials[11] were not beneficial, and gene therapies[12] are nonspecific.[10] Implanting cells genetically modified to overexpress TIMP-3 into the heart after an MI may regulate MMP inhibition in the infarcted and remote regions,[10] establishing the proof of concept for cell-based gene therapy as an effective, clinically relevant approach to matrix modulation.

We evaluated cell-based gene therapy in TIMP-3 deficient (*timp-3*$^{-/-}$) mice, a model in which we previously reported spontaneous heart failure after age 12 months due to unopposed ECM degradation[13,14] and premature heart failure in response to coronary ligation.[15]

MATERIALS AND METHODS

Animals

All experimental procedures performed on animals were approved by the Animal Care Committee of the University Health Network, according to the ''Guide for the Care and Use of Laboratory Animals'' (National Academy Press, 1996). Eight- to 10-week-old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine). *Timp-3*$^{-/-}$ C57BL/6 mice were generated as previously described.[13]

In Vitro Studies

Bone marrow stromal cells: Isolation, transfection, and characterization. Procedures to generate the bone marrow stromal cells (BMSCs) employed in these studies are described in detail in Appendix E1 (supplementary methods). Briefly, adherent cells harvested from C57BL/6 mice were transfected by liposomal lipofectamine 2000 (Invitrogen) with a purified plasmid consisting of cDNA for murine TIMP-3 cloned into pcDNA3.1^{+} (Invitrogen), as previously described.[16] Cellular TIMP-3 mRNA levels were assessed by semiquantitative reverse-transcriptase polymerase chain reaction, using specific primers, as previously described.[17] Protein levels of synthesized (BMSC lysate) and secreted (supernatant) TIMP-3 were evaluated with an immunoblotting procedure. MMP-2 protein levels in the cultured cells and culture medium were quantified by Western blot. MMP-2 activities were evaluated using gelatin zymography, as previously described.[14]

In Vivo Studies

Surgical procedure. In the *timp-3*$^{-/-}$ mouse model, the left coronary artery was permanently ligated proximally under the left atrial appendage through a thoracotomy. Ischemia was confirmed by the appearance of hypokinesis and pallor distal to the occlusion, and by ST elevation (electrocardiographic monitoring). The animals were randomly separated into 3 groups. At 5 minutes after ligation, BMSCs transfected 3 days earlier with TIMP-3 plasmid (BMSC-T3 group) or vector (BMSC group) were delivered by 3 direct, intramyocardial injections (1×10^5 cells in 5 μL of Iscove's modified Dulbecco's medium per injection) into 3 separate points within the infarcted area (identified visually as a dark brown area). Control animals (medium control group) received 3 injections of culture medium (5 μL per injection). Early mortality for the surgical procedure was 26%. Assessments were performed before coronary ligation and cell or medium implantation (0 days), early after coronary ligation and implantation (3 and 7 days), and late after ligation/implantation (28 days). Measurements were taken in a blinded fashion so that operators performing echocardiographic, histologic, and biochemical analyses were not aware of the groups to which the animals were randomized.

Functional assessment. Left ventricular (LV) dilatation and systolic function were evaluated by echocardiography (Sequoia C256 System, Siemens Medical, Malvern, Penn.); 15-MHz linear array transducer) prior to and 3, 7, and 28 days after MI and cell or medium implantation (n = 6 per group). Detailed procedures are described in Appendix E1 (supplementary methods). Fractional area change (FAC) was calculated from 2-dimensional images.

Morphometric assessment. At 28 days after implantation (n = 6 each for medium, BMSC, BMSC-T3 groups; n = 3 for normal myocardium), hearts were fixed and sliced transversely (1-mm-thick slices), then photographed for the measurement of infarct area (as a percentage of LV area) and body weight–indexed LV chamber volume. Detailed procedures are described in Appendix E1 (supplementary methods).

Remote myocardial collagen content and structure. Twenty-eight days after implantation, LV slices from the perfusion-fixed hearts (n = 6 each for medium, BMSC, BMSC-T3 groups; n = 4 for normal myocardium) were paraffin embedded, cut into 15-μm-thick sections, and stained with 0.1% picrosirius red as previously described.[18] Sections were imaged using a laser scanning confocal microscope (MRC 1024, Bio-Rad, Hercules, Calif). Five fields (n = 5 per heart) were randomly selected from the remote myocardium (viable tissue located in the lateral wall of the LV), as previously described.[6] For each image, total collagen content (% pixel^2) and average perimysial collagen fibril length and diameter (in micrometers after image scaling) were measured.

Apoptosis. Frozen sections from hearts harvested on days 3, 7, and 28 following implantation (n = 5 per group per time point) were subjected to terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (in situ cell death detection kit AP, Roche, Basel, Switzerland), as previously described.[19] For each sample, apoptotic nuclei counts from 5 random microscope fields from the infarct border zone (identified using hematoxylin and eosin staining for basic histologic structures on the preceding serial section) were averaged, and apoptosis was expressed as the mean percentage of apoptotic nuclei/total nuclei per field.

Capillary density. Frozen sections from hearts harvested on days 3 and 7 following implantation (n = 5 per group per time point) were immunohistochemically stained with immunoglobulin G rat-anti-mouse CD31 antibody. For each sample, capillary density per millimeter squared was calculated from the mean number of capillaries (large, CD31^{+} structures) counted in 5 random, high-powered microscope fields (0.05 mm^2) from

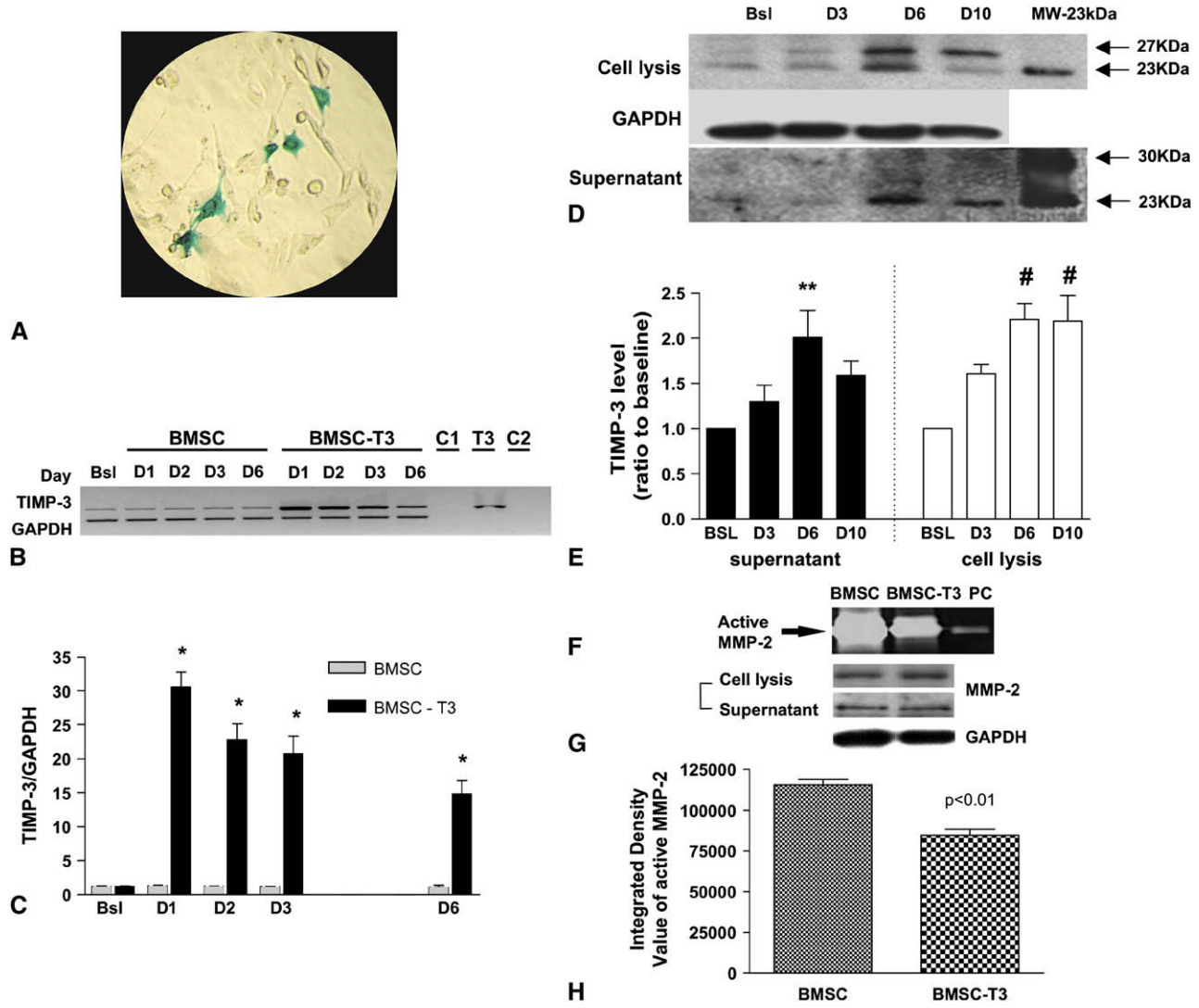
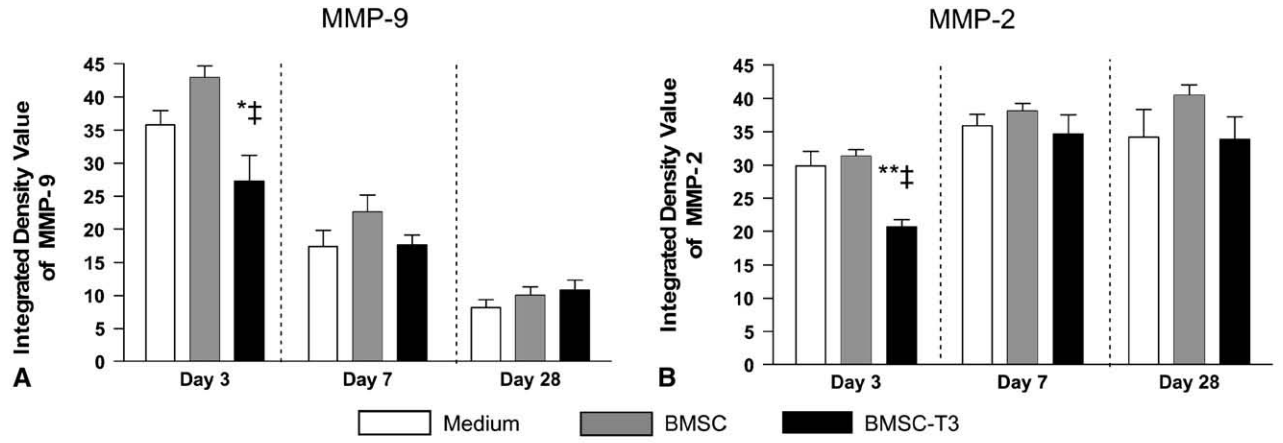


FIGURE 1. In vitro analysis of tissue inhibitor of matrix metalloproteinase-3 *(TIMP-3)* and matrix metalloproteinase-2 *(MMP-2)*. A, Transfection efficiency. β-Galactosidase control vector expression (in *blue*) 3 days after transfection into bone marrow stromal cells (*BMSCs;* ×400). B, C, Semiquantitative reverse-transcriptase polymerase chain reaction (B) and densitometry (TIMP-3 mRNA levels normalized relative to glyceraldehyde-3-phosphate dehydrogenase *[GAPDH]* expression: TIMP-3/GAPDH). (C), Analyses showing TIMP-3 mRNA levels and GAPDH expression at days *(D)* 1, 2, 3, and 6 after cultured BMSCs were transfected with *timp-3* (BMSC-T3) or vector (BMSC). *Bsl,* baseline; *C1,* control sample without reverse transcription; *T3,* TIMP-3 plasmid; *C2,* solution control; n = 5 gels in (C). *P < .05 compared with Bsl. D, E, Time course analysis of TIMP-3 protein levels by immunoblot in cell lysis and supernatant at Bsl and days *(D)* 3, 6, and 10 after *timp-3* transfection. *Bsl,* Nontransfected cells (cell lysis) or culture medium (supernatant); *MW,* molecular weight; n = 6 gels in E. **P < .01 compared with Bsl; #P < .001 compared with Bsl. F–H, Gelatin zymography (F) with immunoblot (G) and densitometry analysis (H), showing a significant reduction (∼30%; P < .01) in MMP-2 activity in the supernatant, with no difference in MMP-2 expression in the cell lysis or supernatant or in internal standard GAPDH expression, 3 days after transfection in the BMSC-T3 group relative to the BMSC group. *PC,* Positive control; n = 6 samples per group in H, equal amounts of protein loaded. Data are mean ± standard error of the mean.

the infarct border zone (identified using hematoxylin and eosin staining on the preceding serial section).

Myocardial MMP expression and activity. Myocardial samples from animals sacrificed on days 3, 7, and 28 after implantation (n = 6 mice per group per time point) were snap-frozen. Half of each heart homogenate was treated at 4°C for 1 hour with an extraction buffer for determination of MMP-2 and -9 activities. The other half was treated at 4°C for 1 hour with an extraction buffer containing 0.15 NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1% Triton X-100, 10 mmol/L Tris-Cl, and small amounts of proteinase inhibitor cocktail for measurement of MMP-2 and -9 protein levels. Protein levels and activities were evaluated as described in Appendix E1 (supplementary methods), with integrated density values of active MMP-9 and -2 used as markers of MMP-9 and -2 activation.

Myocardial tumor necrosis factor α levels. Mice were sacrificed on days 3 and 7 after implantation (n = 6 per group). Hearts from normal mice (n = 4) were collected as normal controls. The myocardial tissue


MMP-9
MMP-2
Integrated Density Value of MMP-9
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*‡
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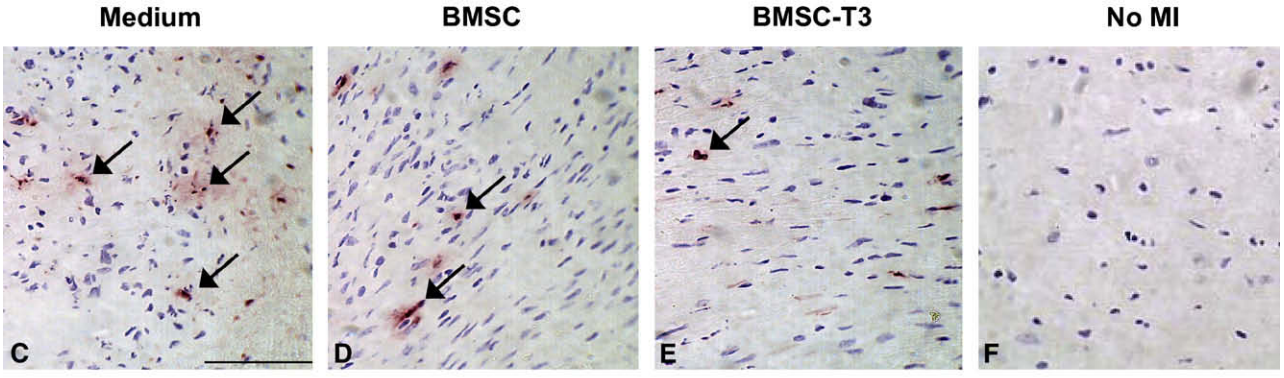
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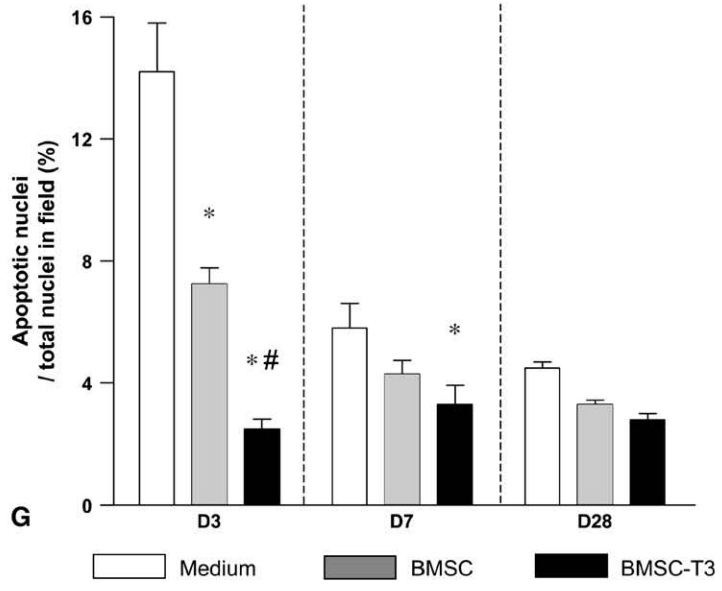
Apoptotic nuclei / total nuclei in field (%)
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D3
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Medium
BMSC
BMSC-T3

CSP

was homogenized and supernatants were collected. Tumor necrosis factor α (TNF-α) levels were quantified using enzyme-linked immunosorbent assay (ELISA) with commercial Murine TNF-α ELISA Kit (Endogen, Rockford, Ill.) as per manufacturer's instructions. Each sample at each time point was performed in triplicate, then averaged.

Statistical Analyses

Results are expressed as mean ± standard error of the mean unless otherwise specified. Data recorded at several time points were analyzed using 2-way, repeated measures analysis of variance (ANOVA; to detect time and group effects) followed (when significant) by Bonferoni's post hoc multiple comparison test, or using 1-way ANOVA followed by Tukey multiple comparison test. Unless otherwise specified, data recorded at a single time point were analyzed using 1-way ANOVA followed by Newman–Keuls multiple comparison test. Differences were considered statistically significant at $P < .05$.

RESULTS

In Vitro Studies

Appendix E1 (supplementary results) describes the phenotype of cultured BMSCs at passage 20 that were employed for the in vivo studies. The efficiency of gene transfection was 15% ± 2%. As shown in Figure 1, TIMP-3 protein levels in the BMSC lysate (synthesized TIMP-3) and supernatant (secreted TIMP-3) were elevated for at least 6 days after *timp-3* transfection ($P < .01$), and MMP-2 activity was decreased in medium cultured with BMSC-T3 ($P < .05$ vs BMSC), confirming that the TIMP-3 expressed by the BMSC-T3 cells was functional (Figure 1).

In Vivo Studies

TIMP-3 overexpression reduced myocardial MMP activity. After MI, cardiac MMP-9 and MMP-2 activities increased. Gelatin zymography provided an estimate of protease activity (after removal of any inhibition by natural TIMPs). By this measure, BMSC-T3 implantation significantly reduced the activities of both MMP-9 ($P < .01$ vs BMSC, $P < .05$ vs medium) and MMP-2 ($P < .01$ vs BMSC and medium) at 3 days after cell transplantation (Figure 2, *A* and *B*), despite the fact that neither MMP-9 nor -2 protein levels (assessed by immunoblotting, relative to internal standard glyceraldehyde-3-phosphate dehydrogenase levels) differed significantly among groups at this time point (data not shown). No significant differences in MMP-9 and -2 expressions or activities were observed among groups beyond 3 days after transplantation. Implantation of vector-transfected BMSCs did not significantly change MMP activities compared to medium implantation at any time point.

TIMP-3 overexpression reduced myocardial TNF-α levels. MI significantly increased myocardial TNF-α levels (from 0.21 ± 0.01 pg/μg of TNF-α in the normal myocardium), producing a spike during the acute phase (1.67 ± 0.2 pg/μg), followed by a reduction on day 7 (to 1.22 ± 0.04 pg/μg) in the medium recipients. BMSC-T3 implantation decreased TNF-α levels during the acute phase (to 1.01 ± 0.06 pg/μg; $P < .05$ vs BMSC and medium), but BMSC implantation alone did not (1.49 ± 0.2 pg/μg). The decrease in TNF-α levels persisted at day 7 in the BMSC-T3 group, at which time levels were statistically similar among the groups.

TIMP-3 overexpression enhanced the early reduction of border zone apoptosis by BMSCs. Three days after implantation, TUNEL staining revealed a significant reduction in apoptosis in the border zone of BMSC-implanted hearts relative to medium controls ($P < .05$). Implantation of BMSC-T3 cells significantly enhanced the early antiapoptotic effect of BMSC implantation ($P < .05$ vs BMSC), which persisted in the BMSC-T3 group at day 7 ($P < .05$ vs medium, Figure 2, *C* to *G*). No significant differences were observed among groups at day 28.

TIMP-3 overexpression enhanced the preservation of remote myocardial collagen by BMSCs. MI resulted in significant fragmentation and thinning of the matrix fibrils (Figure 3, *A* to *D*). Compared with medium injection, BMSC and BMSC-T3 transplantation significantly reduced collagen degradation in the remote myocardium at day 28 after cell implantation ($P < .05$ for all groups; Figure 3E). Compared with the medium control group, both length and diameter of the collagen fibrils were preserved by BMSC implantation ($P < .05$ for both measures). These effects were significantly enhanced with BMSC-T3 implantation ($P < .05$ for both measures vs BMSC and medium control group; Figure 3, *F* and *G*).

TIMP-3 overexpression delayed early blood vessel formation in the border zone. Following MI, medium-injected hearts exhibited low capillary densities in the border

FIGURE 2. In vivo analysis of matrix metalloproteinase *(MMP)* activity and apoptosis. A, B, Densitometry analyses estimating MMP activity in myocardial extracts at days 3, 7, and 28 after implantation of culture medium, bone marrow stromal cells *(BMSCs)*, or *timp-3*-transfected BMSCs *(BMSC-T3)*. The pooled results demonstrate that MMP-9 (A) and MMP-2 (B) gelatinolytic activities were reduced at day 3 after implantation of BMSC-T3 (n = 6 per group per time point). Gelatin zymography does not measure what activity is inactivated by natural inhibitors in vivo. C–F, Representative light micrographs (×400) illustrating terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining of apoptotic cells *(arrows)* in the border zone of mouse hearts 3 days after implantation of *medium* (C), *BMSCs* (D), or *BMSC-T3* (E). Normal myocardium (*No MI*, F) is presented as a positive control. *Scale bar* = 50 μm in (C–F). G, Apoptotic nuclei (expressed as a % of total cells per field on days *[D]* 3, 7, and 28 after implantation; n = 5 per group per time point) were significantly reduced at D3 with BMSC implantation, and further reduced with BMSC-T3 implantation. The depression in apoptotic cell numbers persisted at D7 in the BMSC-T3 compared with the medium group. No significant differences were observed between groups at D28. Data are mean ± standard error of the mean. *$P < .05$ versus medium, **$P < .01$ versus medium; #$P < .05$ versus BMSCs; ‡$P < .01$ versus BMSCs.

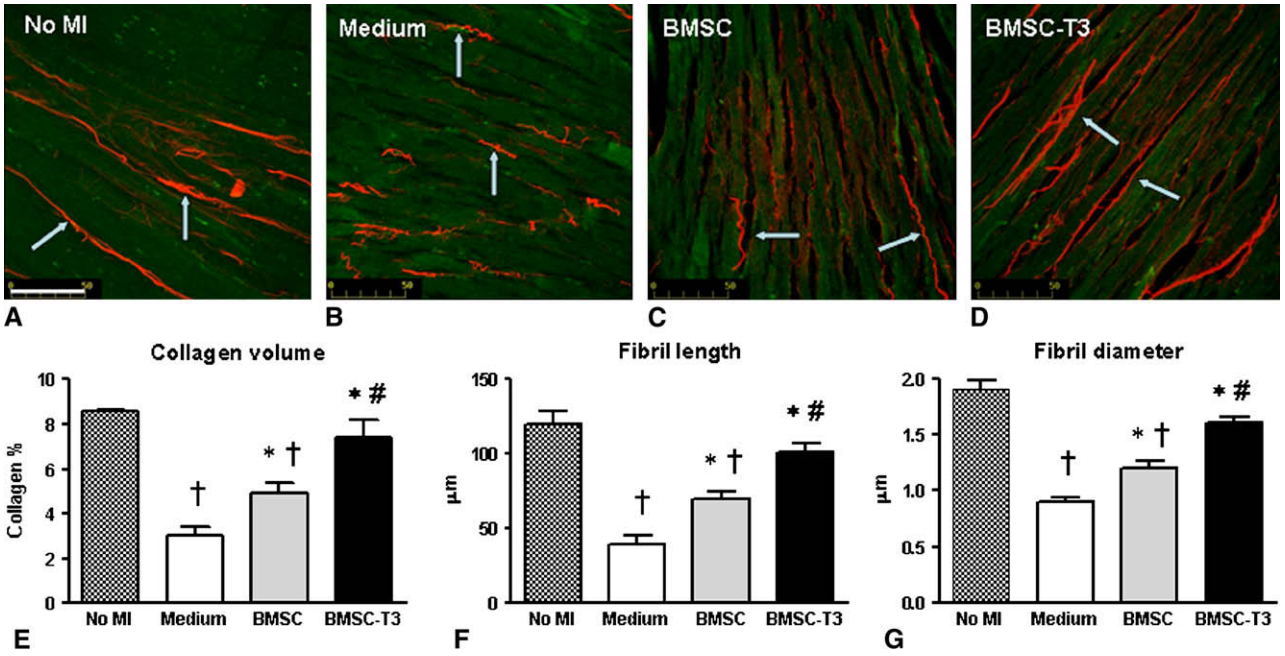


FIGURE 3. Collagen content and structure in the remote myocardium. A–D, Representative confocal micrographs (left ventricular sections) showing the fibrillar collagen network (stained with picrosirius red, *arrows*) in normal myocardium (*No MI,* A), and in the remote (noninfarcted) myocardium of infarcted animals, 28 days after implantation of medium (B), bone marrow stromal cells (*BMSC,* C) or *timp-3*-transfected BMSCs (*BMSC-T3,* D). Cardiomyocytes appear green due to autofluorescence. Scale bar = 50 μm in (A–D). Collagen fibers and connectivity were clearly preserved in the BMSC group and further preserved in the BMSC-T3 group. E–G, Collagen content (expressed as % of pixel^2, E), fibril length (F), and fibril diameter (G), determined for all 4 groups (n = 6 per group for *Medium, BMSC, BMSC-T3;* n = 4 for *No MI*) by computer image analysis of confocal stacks, were all increased to *No MI* levels by BMSC-T3 implantation. Data are mean ± standard error of the mean. *P < 0.05 versus medium, #P < .05 versus BMSC, †P < .05 versus No MI.

zone (Figure 4, *A* to *G*). BMSC implantation significantly increased capillary densities in this region early (by day 3, P < .05 vs BMSC-T3 and medium). Interestingly, TIMP-3 overexpression temporarily reduced the effect of the implanted cells on capillary formation early after implantation (at day 3) but not later, as capillary densities were similarly elevated (P < .05) relative to medium controls in both BMSC and BMSC-T3 groups at day 7 after implantation (Figure 4, *H*).

TIMP-3 overexpression enhanced the preservation of systolic function by BMSCs. Echocardiographic measurements of LV end diastolic dimension showed significant LV dilatation in the medium-implanted group as early as 3 days after MI, worsening until the end of the experiments at 28 days (Figure 5, *A* to *C*). BMSC and BMSC-T3 implantation significantly reduced LV dilatation (relative to controls) at 7 and 28 days following MI (P < .05 group and time effects by 2-way ANOVA), with beneficial effects evident earlier (by day 3) in the BMSC-T3 group (Figure 5, *C*).

Systolic function decreased progressively after MI in the medium control group. BMSC implantation significantly improved systolic function (FAC) compared with the medium control group at days 7 (P < .05) and 28 (P < .05) following treatment. TIMP-3 overexpression further increased the functional benefits of cell implantation (P < .05 for BMSC-T3 vs BMSC at days 3 and 28), and the effects were evident earlier, by 3 days following cell treatment (Figure 5, *D*).

Cell transplantation preserved LV morphometry. Twenty-eight days after cell implantation, morphologic analysis demonstrated a significantly smaller (P < 0.01) scar area in the cell-implanted groups than in the medium control group (Figure 5, *E*). In agreement with the echocardiographic data, cell transplantation also reduced LV dilatation to a level similar to that seen in noninfarcted myocardium (Figure 5, *F*).

DISCUSSION

Cell transplantation improves ventricular function after an MI,[4,20-23] primarily due to paracrine cytokine activation that induces angiogenesis, marrow progenitor cell recruitment, and matrix modulation[1]; however, functional recovery may be enhanced by cell-based gene therapy. Here, BMSCs transplanted immediately after MI preserved matrix structure in the remote (viable) myocardium of *timp-3*$^{-/-}$ mice, and overexpressing TIMP-3 in the implanted BMSCs significantly enhanced these effects. The *timp-3*$^{-/-}$ mice exhibit exaggerated ECM degradation and ventricular dilatation following coronary ligation,[13-15] which may

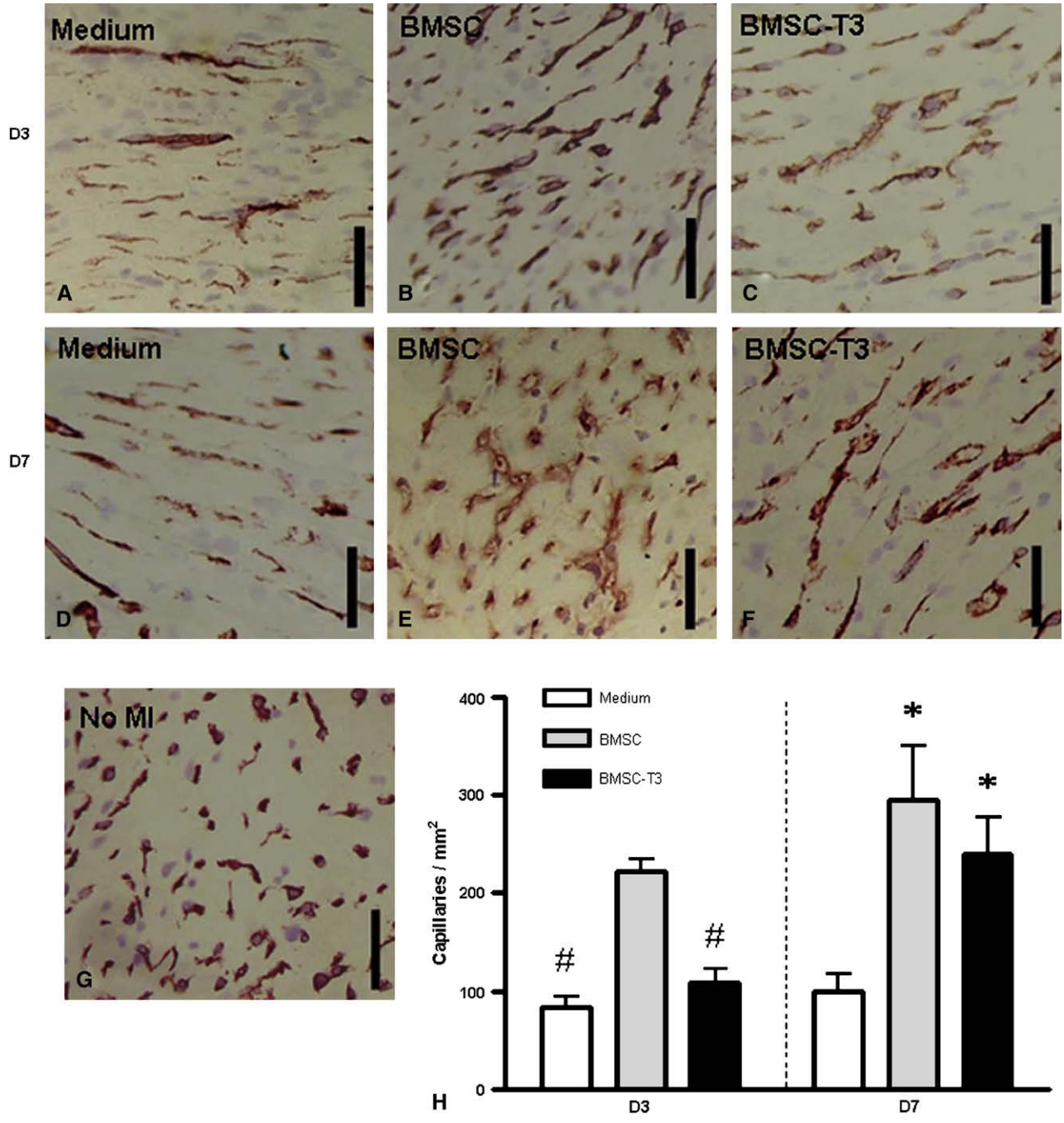
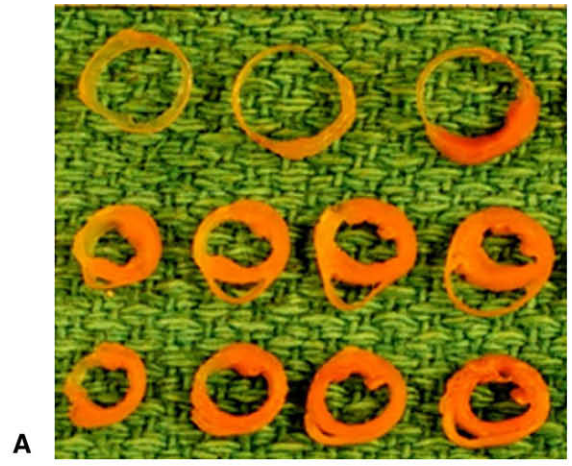


FIGURE 4. Capillary density. A–G, Representative light micrographs (×250) showing CD31 staining (vascular structure; brown in color) in the infarct border zone (A–F) of hearts at days *(D)* 3 and 7 after implantation of medium (A, D), bone marrow stromal cells (*BMSC,* B, E) or *timp-3*-transfected BMSCs (*BMSC-T3,* C, F). Normal myocardium (*No MI,* G) was a positive control. Scale bar = 25 μm in A–G. H, Blood vessel density (expressed as number of capillaries per mm^2; n = 5 per group per time point) was significantly greater at D3 with BMSC implantation compared with BMSC-T3. By D7, vessel density was equally increased in both cell transplant groups. Data are mean ± standard error of the mean. *P < .05 versus medium, #P < .05 versus BMSC.

be similar to that seen in patients who progress rapidly to heart failure after an MI (perhaps due to an inadequate TIMP injury response).[6,18] This preliminary study establishes the proof of concept that cell-based gene therapy could modify matrix modulation following an MI. Studies in wild-type rodents and large animals will be required to determine whether the approach is clinically applicable. If it is, then this technique might provide an opportunity to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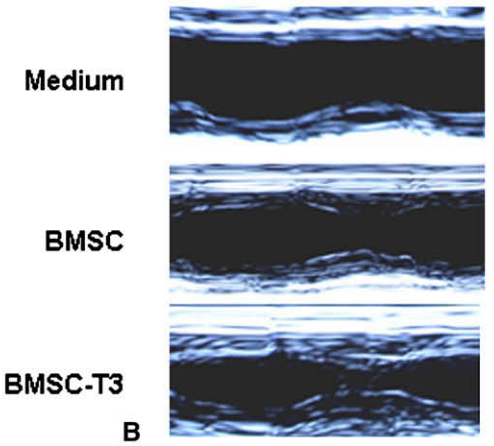
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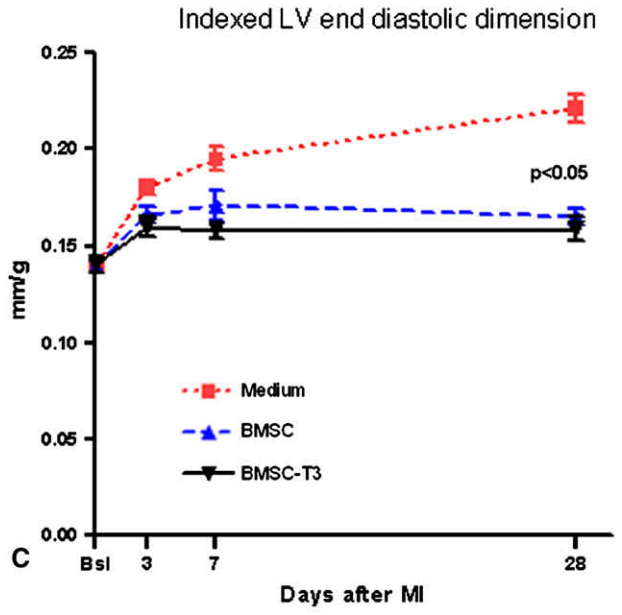
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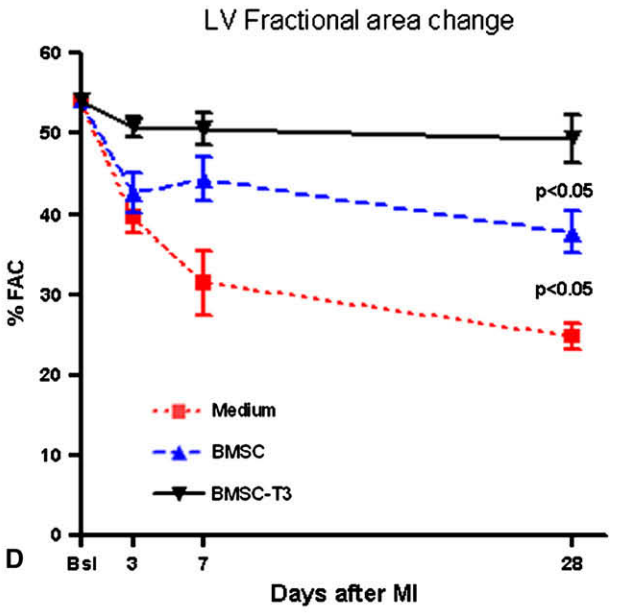
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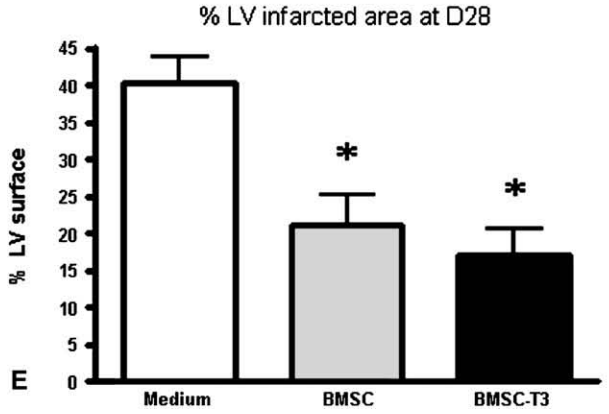
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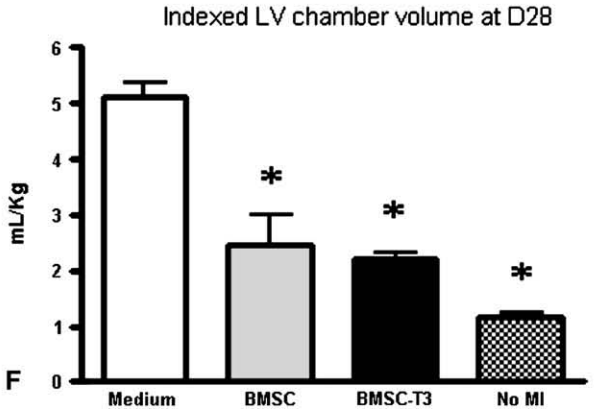
Indexed LV chamber volume at D28
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CSP

rejuvenate stem cells in elderly patients whose natural regenerative capacity is diminished.

Cell-based gene therapy may boost the paracrine effects of cell transplantation. Ventricular function was enhanced by transfecting implanted cells with the genes for stem cell factor, vascular endothelial growth factor (VEGF), insulin-like growth factor, and/or Akt.[20-24] This is the first study to show that TIMP-3 overexpression in the implanted cells enhanced the preservation of the matrix structure after an MI. The early reduction in capillary density by BMSC-T3s may have coincided with the early inhibition of MMPs, as matrix degradation is required for endothelial cell mobilization and the branching of new blood vessels. The early effects on capillary density could also be attributed to alternate (but slower) angiogenic pathways. Alternatively, TIMP-3 overexpression may have inhibited VEGF-mediated angiogenesis and induced other secondary effects.[25]

We have previously suggested that cell transplantation after an MI may modify matrix remodeling in the remote myocardium as well as in the infarct area, but this effect has been difficult to demonstrate due to the multiple events associated with coronary ligation.[6,13] In a hamster model of progressive cardiomyopathy, we showed that cell implantation preserved the collagen network and the regional MMP/TIMP balance in both the implanted area and the remote myocardium.[4] In rats following an MI, we found that cells implanted into the infarct area reduced matrix remodeling in that area.[26] The current study demonstrated that BMSCs implanted into the infarct area of *timp-3*$^{-/-}$ mice promoted matrix preservation not only in that area but also in the remote myocardium. This finding provides further support for the theory that matrix homeostasis in the remote myocardium is a critical determinant for the improvement of cardiac function by implanted cells.

In addition to affecting matrix modulation, TIMP-3 overexpression may also have contributed to the reduced cardiac production of TNF-α, which induces adverse cardiac remodeling and ventricular dysfunction.[27] TIMP-3 is a potent, endogenous inhibitor of ADAMs (a disintegrin and a metalloproteinase domain) and TNF-α converting enzyme,[28,29] which are increased in the hearts of *timp-3*$^{-/-}$ mice.[13] In the current study, implanting cells overexpressing TIMP-3 significantly modified the early pattern of post-MI TNF-α levels relative to that observed with vector-transfected BMSCs. TNF-α also increases apoptosis and MMP gene expression,[27] which may explain further reductions in cell loss and matrix structural remodeling in mice implanted with the gene-enhanced cells.

Cell-based gene therapy may permit the regulated, transient MMP inhibition that has not been achieved by current approaches.[10] Jayasankar and colleagues[12] overexpressed TIMP-1 by intramyocardial gene transfer in the border zone after coronary ligation in rats. They observed transient inhibition of MMP-1 activity as well as limited improvements in systolic function and geometric remodeling. Gene-enhanced cells influence not only the infarct (implanted) area but also the border zone and the noninfarcted myocardium.[21,23] We previously reported that the transient release of angiogenic gene products following cell-based gene therapy produced prolonged (6-month) improvements in capillary density and cardiac function.[2] In the current study, we observed a transient (~6-day) elevation of TIMP-3 mRNA in the transfected cells. We believe that short-term, regulated amplification of the TIMP response with this clinically relevant approach may confer long-term benefits for matrix modulation in patients. However, we did not determine the number of cells engrafting in the heart, and we did not compare our results to those with gene therapy alone or gene therapy using inert cells.

Paracrine mediators likely act through multiple, diverse, and interrelated mechanisms to produce synergistic effects on the remodeling process. However, although future studies will be required to determine whether TIMP-3-enhanced cell transplantation will modify post-MI matrix remodeling in wild-type mice or humans, the current study suggests that cell-based gene therapy is a potentially effective method to generate transient MMP inhibition and achieve functional recovery following an MI.

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FIGURE 5. Left ventricular *(LV)* morphometry and function. A, Representative macrographs of heart slices at 28 days after myocardial infarction *(MI)* and implantation of medium, bone marrow stromal cells *(BMSC)*, or *timp-3*-transfected-BMSCs *(BMSC-T3)*. Increased infarct size and LV dilatation is apparent in medium control hearts compared with those from BMSC and BMSC-T3 groups. B, These observations are confirmed by echocardiographic parasternal small-axis M mode images of the hearts for morphologic study. C, By echocardiography (n = 6 per group per time point), LV dilatation (body weight-indexed LV end diastolic dimension in mm/g) over 28 days following MI was significantly reduced (relative to medium controls) after implantation of BMSC (at days 7 and 28; $P < .05$) and BMSC-T3 (at days 3, 7, 28; $P < .01$). D, LV systolic function (fractional area change in %) over 28 days following MI was significantly preserved after implantation of BMSC (compared with medium controls) and BMSC-T3 (compared with BMSC and medium groups). E, F, By morphometric assessment (n = 6 per group), LV infarcted areas (expressed as % of LV surface, E), and body weight–indexed LV chamber volumes (normal heart [*No MI*, n = 3] as control, F) were significantly smaller (compared with medium controls) at 28 days *(D)* after BMSC or BMSC-T3 implantation. No significant differences were observed between BMSC and BMSC-T3 groups. Data are mean ± standard error of the mean. *$P < .05$ versus medium.

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APPENDIX E1

Supplementary Methods

The bone marrow stromal cells (BMSCs) employed in these studies have been previously employed to improve ventricular function after a myocardial infarction and to act as a carrier for cell-based gene therapy. These cells are particularly appropriate for this purpose because of their ability not only to induce angiogenesis but also to transdifferentiate into a variety of cell types required for infarct healing: endothelial, smooth muscle cells, and myofibroblasts. However, BMSCs are not easily identified by surface markers and therefore are not easily separated by common cell-sorting techniques. Instead, BMSCs are identified by their ability to adhere to plastic surfaces and to rapidly expand in response to cytokines.

In Vitro Studies

BMSCs: Isolation, expansion, and characterization. BMSCs were isolated from C57BL/6 mice and cultured as previously described.[E1] Whole bone marrow was plated in Iscove's modified Dulbecco's medium (Gibco, Rockville, Md) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 U/mL). Cells were cultured at 37°C with 5% CO_2 in humid atmosphere. Nonadherent cells were removed by aspiration 48 hours later. Adherent cells were harvested in 0.05% Trypsin solution with 1 mmol ethylenediaminetetraacetic acid and subcultured. Cultured cell phenotype was assessed by fluorescence-activated cell sorting (FACS), using fluorescein isothiocyanate–conjugated or phycoerythrin-conjugated monoclonal antibodies recognizing Sca-1, CD81, CD34, CD45, CD31, and c-kit (Becton Dickinson, Franklin Lakes, NJ). Analysis was with an EPICS XL-MCL flow cytometer (Coulter, Beckman, Fullerton, Calif) equipped with Expo32 ACD Xa software.

Generation of BMSCs overexpressing murine tissue inhibitor of matrix metalloproteinase-3. cDNA for murine tissue inhibitor of matrix metalloproteinase-3 (TIMP-3) was cloned into pcDNA3.1+(Invitrogen, Carlsbad, Calif) as previously described.[E2] The purified plasmid was transfected into BMSCs (BMSC-T3) by liposomal transfection using lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. Cotransfection with pSV-β-galactosidase control vector (Promega, Madison, Wis) was performed to assess transfection efficiency. β-galactosidase expression was assessed after staining of BMSCs as per manufacturer's instructions (Promega). Blue-stained β-galactosidase positive cells were counted on 10 random fields and expressed as percent of total cells per field. BMSCs transfected with unmodified pcDNA3.1+ were stained as negative controls.

Cellular TIMP-3 expression. Cellular TIMP-3 mRNA levels at 0, 1, 2, 3, and 6 days after transfection (n = 5 per group per time point) were assessed by semiquantitative reverse-transcriptase polymerase chain reaction, using specific primers, as previously described.[E3]

Protein levels of synthesized (BMSC lysate) and secreted (supernatant) TIMP-3 were evaluated with an immunoblotting procedure using a polyclonal rabbit anti-human antibody for TIMP-3 (1:500, Triple Point Biologics, Forest Groves, Orel) at 0, 3, 6, and 10 days after gene transfection (n = 6 per group per time point).

Cellular matrix metalloproteinase-2 expression and activity. Protein levels of matrix metalloproteinase-2 (MMP-2) in the cultured cells and culture medium 3 days (n = 6 per group) after *timp-3* transfection were quantified by Western blot, using a rabbit anti-human antibody against MMP-2 (1:1000, Chemicon International, Billerica, Mass.), as previously described.[E4]

Activities of MMP-2 were evaluated at 3 days after gene transfection using gelatin zymography, as previously described.[E4] Equal amounts (10 μg) of protein (n = 6 per group) were fractionated by 10% zymography gels impregnated with 0.1% gelatin (Novex, Invitrogen). Gels were renatured with 2 × 2.5% Triton X-100 washes for 30 minutes at room temperature. The gels were then equilibrated with developing buffer (49.1 mmol/L Tris, 4.81 mmol/L $CaCl_2$, 0.002% NaN_3) at room temperature for 15 minutes with agitation. Finally, gels were incubated with developing buffer 18 to 20 hours at 37°C. Coomassie blue R-250 (Sigma Chemical Co, St Louis, Mo) staining was used to detect enzymatic activity; following staining, areas of gelatinolytic activity appeared as clear bands against the dark blue background. Bands obtained from immunoblot and gelatin zymography were quantified using densitometric analysis. The latent (pro) and active forms of MMP-2 (72 and 68 kDa, respectively) were detected. Molecular weights of the bands were estimated using recombinant MMP-2 protein. Integrated density values (band area X relative intensity) of active MMP-2 were used as markers of MMP-2 activation.

In Vivo Studies

Functional assessment. Left ventricular (LV) dilatation and systolic function were evaluated by echocardiography (Sequoia C256 System, Siemens Medical; 15-MHz linear array transducer) prior to and 3, 7, and 28 days after MI and cell or medium implantation (n = 6 per group). M-mode and 2-dimensional images were obtained in the parasternal short axis at the level of the papillary muscles. For each measurement, 3 consecutive cardiac cycles were recorded and averaged by a single blinded examiner. LV end diastolic dimension was determined in M-mode imaging and indexed to the animal's body weight recorded at the time of echocardiography. In 2-dimensional imaging, the LV end diastolic area (LVEdA) was determined as the largest cavity

size and the LV end systolic area (LVEsA) as the smallest. Fractional area change (FAC) was calculated as follows: FAC = ((LVEdA − LVEsA)/LVEdA) ×100. Afterward, hearts from each group were fixed for morphometric and collagen analyses.

Morphometric assessment. At 28 days after implantation (n = 6 each for medium, BMSC, BMSCs transfected 3 days earlier with TIMP-3 plasmid [BMSC-T3] groups; n = 3 for normal myocardium), following perfusion fixation with buffered 10% formalin at controlled ventricular pressure, left ventricles were cut into 1-mm-thick transverse slices from apex to base. Heart slices were photographed with a digital camera, and unmagnified images were analyzed by a blinded observer using Image J software. The border between infarcted and noninfarcted myocardium was clearly identifiable in each image (the infarcted myocardium appeared visibly thinner and paler in color than the noninfarcted myocardium). Infarct size was measured as described by Takagawa and colleagues,[E5] with modifications. Briefly, epicardial infarct length and epicardial circumference of LV myocardium were manually traced and automatically measured in serial digital images. To calculate infarct and LV areas for each slice, infarct length or LV circumference were multiplied by the 1-mm thickness. To calculate infarct size (expressed as a percentage), the sum of infarct areas from all slices was divided by the sum of LV areas from all slices (including those without infarct scar) and multiplied by 100. LV chamber volume was measured in the same manner and indexed by body weight (mL/kg).

SUPPLEMENTARY RESULTS

In Vitro Studies

Characterization of cultured BMSCs. The phenotypes of cultured BMSCs at passages 10, 20, and 40 were evaluated by FACS analysis. The cell population was homogenous, and more than 99% of cells stably expressed Sca-1$^+$, CD81$^+$, CD34$^-$, CD45$^-$, CD31$^-$, c-kit$^-$, without contamination by hematopoietic stem cells or endothelial progenitor cells. BMSCs from passage 20 were used in this study to improve cardiac function and as carriers of the *timp-3* gene.

Cellular TIMP-3 expression. Gene transfection efficiency, evaluated by staining for β-galactosidase expression (Figure 1, *A*), was 15% ± 2% at 3 days after transfection. TIMP-3 mRNA levels in the gene-transfected cells were significantly greater ($P < .01$) than those in the vector-transfected cells for the 6-day duration of the study (Figure 1, *B* and *C*). TIMP-3 protein levels in the BMSC lysate (synthesized TIMP-3) and supernatant (secreted TIMP-3) were greatly elevated from baseline for at least 6 days after *timp-3* transfection ($P < .01$ for all groups, Figure 1, *D* and *E*).

Cellular MMP-2 expression and activity. Gelatin zymography showed a significant reduction in MMP-2 activity in the medium cultured with BMSC-T3 compared with that cultured with BMSC at 3 days after transfection ($P < .01$; Figure 1, *F*), though immunoblot analysis showed no difference in MMP-2 expression between the 2 groups (Figure 1, *G*). These data confirm that BMSC-T3 cells expressed functional TIMP-3 following gene transfection.

Supplementary References

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