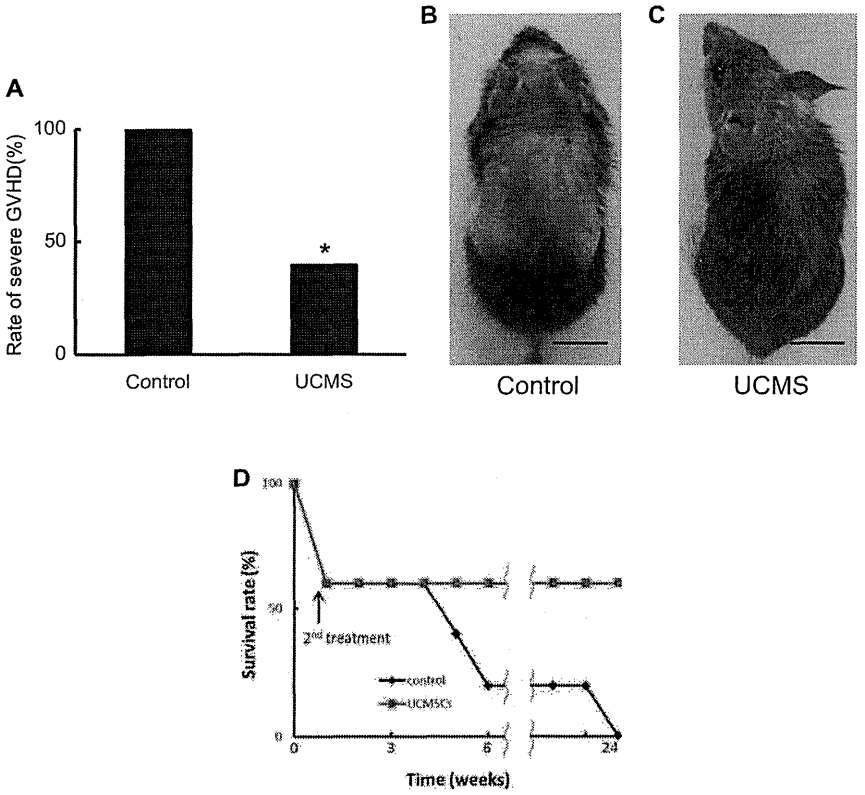

Figure 5. Suppression of GvHD by UCMS cells transplantation. Although all mice in the control group died or showed severe GvHD at 6 weeks (score 6; 1 mouse, dead; 4 mice), treatment with non-expanded UCMS cells significantly reduced the severity of GvHD (score 3; 1 mouse, score 4; 2 mice, dead; 2 mice) (A). Representative pictures of animals from the control group (B) and non-expanded UCMS cell-treated group (C) are shown. (D) Survival curve after radiation and allogeneic hematopoietic stem cell transplantation ($n = 5$, in each group). All of the mice in the control group were dead by 25 weeks after allogeneic hematopoietic stem cell transplantation. In contrast, no mice in the UCMS group were dead after the second infusion of non-expanded UCMS cells. $^*P < 0.05$ versus control. Scale bar, 1 cm (B, C).

Removal of the umbilical artery results in the loss of Wharton's jelly, which contains significant numbers of MSC. Preservation of the blood vessels before digestion with collagenase and hyaluronidase also significantly reduced contamination by endothelial cells. This finding may be attributed to removal of intact vascular structures from the cell suspension before digestion with trypsin, as vasculature was not considerably degraded in the presence of collagenase and hyaluronidase according to our protocol. Furthermore, our method has a significant advantage in that no *in vitro* cell culture is required for isolation of MSC from umbilical cord. In contrast, recently published methods described by Capelli *et al.* (11) and Lu *et al.* (10) involve cell culture over several days for isolation of MSC. The advantage of protocols using *in vitro* expansion is that non-adhesive cells can be removed, resulting in an enriched adhesive cell population. However, our results indicate that freshly isolated and non-selected umbilical cord-derived cells obtained by our method suppressed GvHD in an animal model. *In vitro* expansion of MSC carries a risk of tumorigenesis (42) and clinical use of the expanded cells requires strict monitoring to ensure safety. Our modified method has significant advantages in the reduced time required for the isolation procedure and its safety, both of which are important for cell banking. For the studies reported here, we used only the umbilical cords obtained at full-term Cesarean sections. It is likely that the properties and number of MSC in umbilical cord are similar comparing Cesarean section and vaginal delivery. Although caution should be taken regarding cleanliness/sterility after vaginal delivery, we expect that the same procedure can be used for umbilical cords obtained at full-term vaginal delivery.

In addition to offering a cell source for regenerative medicine, MSC have the potential to suppress GvHD after hematopoietic stem cell transplantation. Although the mechanisms and actual cell fraction underlying suppression of GvHD by MSC transplantation remains contentious (43), our results indicate

that human umbilical cord-derived non-expanded MSC suppressed GvHD in a murine model. The optimal type of MSC for suppression of GvHD, in terms of source, level of maturity and HLA-matching to the donor, is unclear. However, our results indicate that non-expanded UCMS cells represent a potential candidate cell source, particularly when co-banked with cord blood-derived hematopoietic cells (provided there is also strict control for contamination with infectious agents with HLA-typing). Furthermore, co-transplantation of HLA-matched hematopoietic stem cells and MSC may be advantageous to reduce clearance of transplanted MSC via immunologic recognition of HLA-matched hematopoietic cells.

In conclusion, our results indicate that umbilical cord-derived non-expanded MSC represent a potential cell source for cell banking and subsequent therapeutic use. Our results indicate that the combination of banking UCMS cells with identical cord blood-derived hematopoietic stem cells could be an important source for cell-based therapies in a range of settings.

Acknowledgments

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Allogeneic Transplantation of Fetal Membrane–Derived Mesenchymal Stem Cell Sheets Increases Neovascularization and Improves Cardiac Function after Myocardial Infarction in Rats

Shin Ishikane,[1,2] Hiroshi Hosoda,[1,7] Kenichi Yamahara,[1] Yoshiharu Akitake,[1,2] Jung Kyoungsook,[1] Kenichi Mishima,[3] Katsunori Iwasaki,[3] Michihiro Fujiwara,[3] Mikiya Miyazato,[2] Kenji Kangawa,[4] and Tomoaki Ikeda[1,5,6]

Background. Mesenchymal stem cell (MSC) transplantation has been pursued as a new method to repair damaged myocardium. We focused on the fetal membrane (FM) as an alternative source to bone marrow (BM)–derived MSCs. In this study, we investigated whether transplantation of allogeneic FM-MSC sheets could attenuate myocardial dysfunction in a rat chronic myocardial infarction (MI) model.

Methods. Sheets of allogeneic FM-MSC or autologous BM-MSC were transplanted into the scarred myocardium 4 weeks after coronary ligation.

Results. Four weeks after transplantation, both allogeneic FM-MSC and autologous BM-MSC sheets had significantly improved cardiac function and reduced myocardial fibrosis compared with the untreated MI group. In both MSC sheet-transplanted groups, the peri-infarct regional capillary density was increased. Some engrafted MSCs formed vascular structures and were positive for lectin I and α-smooth muscle actin. The numbers of engrafted cells and differentiated cells were very low after both types of MSC sheet transplantation. $CD3^+$ T cells did not increase in the transplantation site, but $CD163^+$ M2 macrophages increased in the groups transplanted with allogeneic FM-MSC and autologous BM-MSC.

Conclusions. Transplantation of allogeneic FM-MSC or autologous BM-MSC sheets attenuated myocardial dysfunction in a rat MI model to a similar degree. The engraftment rate of transplanted cells and immune cell infiltration into the transplanted area did not differ between the two types of MSC transplants. M2 macrophage induction has possible involvement in the therapeutic effects of MSC transplantation. Allogeneic FM-MSC sheet transplantation might be a new therapeutic strategy after MI.

Keywords: Fetal membrane, Mesenchymal stem cells, Cell sheet, Myocardial infarction, Allogeneic transplantation.
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Myocardial infarction (MI) causes loss of cardiac tissue and impairment of left ventricular function. Recent reports suggest that mesenchymal stem cells (MSCs) are a

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The authors declare no conflicts of interest.
[1] Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan.
[2] Department of Biochemistry, National Cerebral and Cardiovascular Center Research Institute.
[3] Department of Neuropharmacology, Faculty of Pharmaceutical Science, Fukuoka University, Fukuoka, Japan.
[4] National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan.
[5] Department of Perinatology, National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan.
[6] Department of Obstetrics and Gynecology, Mie University School of Medicine, Mie, Japan.
[7] Address correspondence to: Hiroshi Hosoda, M.D., Ph.D., Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan.
E-mail: hosodah@ri.ncvc.go.jp

S.I. participated in research design, performance of the research, data analysis, and writing of the article. H.H. and T.I. participated in research design, performance of the research, and writing of the article. K.Y., K.M., K.I., M.F., M.M., and K.K. participated in the performance of the research and contributed new reagents or analytic tools. Y.A. and J.K. participated in the performance of the research and data analysis.
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valuable cell source for cell therapy after MI and that bone marrow (BM) represents a major source of MSCs. Several clinical trials of autologous BM-MSC transplantation for MI have reported therapeutic success (*1–3*).

BM harvest is a surgical procedure that requires general anesthesia or sedation, and both the proliferative potential and the differentiation capacity of MSCs seem to decrease in older donors (*4, 5*). In addition, BM procurement procedures in humans may yield low numbers of MSCs after cell processing. To address this issue, we focused on the fetal membrane (FM) of the placenta, which is generally discarded as medical waste after delivery, as an alternative source of autologous MSCs. Several studies have reported that the human FM contains multipotent cells similar to BM-MSCs and that these cells are easy to expand (*6, 7*). We demonstrated previously that the allogeneic transplantation of FM-MSCs did not elicit any lymphocyte proliferative response despite their allogeneic origin and induced therapeutic effects in a rat model of hind-limb ischemia and acute myocarditis (*8, 9*).

In some types of MSC transplantation, dissociated MSCs are injected into the myocardium to induce cardiac regeneration. However, it is difficult to reconstruct sufficient cardiac mass in the thinned scar area after MI. Imanishi et al. (*10*) reported that approximately 90% of cells injected into the myocardium are lost within 1 day. Okano et al. recently developed cell sheets using temperature-responsive culture dishes (*11–14*). These cell sheets allow for cell-to-cell connections and maintenance of adhesion proteins. In a rat MI model, the engraftment rate of transplanted cells was higher after transplantation of cell sheets compared with intramyocardial transplantation of dissociated cells (*15, 16*). These results suggest that transplantation of allogeneic FM-MSC sheets may be a new strategy for the treatment of heart failure.

In this study, we designed a set of experiments with the following aims: (i) to compare the therapeutic effects of transplantation of allogeneic FM-MSC sheets and autologous BM-MSC sheets in a rat chronic MI model, (ii) to investigate the engraftment and differentiation of transplanted MSCs, and (iii) to investigate whether transplanted allogeneic FM-MSC sheets evade immune rejection.

RESULTS

Preparation and Transplantation of Two-Layered MSC Sheets

FM-MSCs derived from green fluorescent protein (GFP)-transgenic Sprague–Dawley rats (3.3×10^6 cells) or BM-MSCs derived from GFP-transgenic Lewis rats (3.3×10^6 cells) were cultured in temperature-responsive 35-mm dishes for 1 day. When the culture temperature was decreased from 37°C to 20°C, both types of MSC sheets detached spontaneously and floated into the culture medium as a monolayer MSC sheet that could be stacked into two-layer constructs (Fig. 1A, C, and D). We transplanted two-layered FM-MSC sheets or BM-MSC sheets over the anterior wall of the heart, including the infarcted area, and then attached them to the heart surface (Fig. 1B).

Engraftment of Transplanted Allogeneic FM-MSC and Autologous BM-MSC Sheets in Infarcted Hearts

One day and 1 and 2 weeks after transplantation, GFP-positive allogeneic FM-MSCs and autologous BM-MSCs were present as sheets on the infarcted area of the anterior wall (n=3 in each group). GFP-positive allogeneic FM-MSCs and autologous BM-MSCs were observed in the anterior infarcted area 3 and 4 weeks after transplantation (n=4 in each group) (Fig. 1E). However, semiquantitative analysis demonstrated that the engraftment rate decreased with time in both MSC sheet-transplanted groups (Fig. 1F). The engraftment rate did not differ significantly between the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC.

Improvement in Cardiac Function after Transplantation of Allogeneic FM-MSC and Autologous BM-MSC Sheets

Heart failure developed 4 weeks after coronary ligation, as indicated by deterioration of left ventricular function and thinning of the infarct wall. The ejection fraction, fractional shortening, anterior wall thickness, posterior wall thickness, left ventricular diastolic dimension, and left ventricular systolic dimension measurements at baseline did not differ significantly between the three MI groups. One of the 10 rats in the untreated MI group died on day 53 after coronary ligation; no rats died in the MI groups transplanted with allogeneic FM-MSC or autologous BM-MSC.

Hemodynamic analysis revealed significant improvements in the MI group transplanted with allogeneic FM-MSC compared with the untreated MI group for the left ventricular systolic pressure, maximum dP/dt, and minimum dP/dt ($P<0.05$; n=10 in each group) (Fig. 2B,C; see **Table S1, SDC,** http://links.lww.com/TP/A849).

Echocardiographic analysis revealed significant improvements in ejection fraction, fractional shortening, and left ventricular systolic dimension ($P<0.05$ for each) in the MI group transplanted with allogeneic FM-MSC compared with the untreated MI group. Anterior wall thickness was also significantly greater in the MI group transplanted with allogeneic FM-MSC than in the untreated MI group (Fig. 2D,E; see **Table S2, SDC,** http://links.lww.com/TP/A849).

The hemodynamic and echocardiographic parameters did not differ significantly between the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC.

Reduction in Myocardial Fibrosis after Transplantation of Allogeneic FM-MSC and Autologous BM-MSC Sheets

Eight weeks after coronary ligation, Masson's trichrome staining of the myocardium from the untreated MI group demonstrated prominent and diffuse interstitial fibrosis in the anterior scar area. This was attenuated markedly in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC (Fig. 3A). Quantitative assessment of myocardial fibrosis of the left heart showed that the fraction of Masson's trichrome-stained collagen volume was significantly smaller in the MI groups transplanted with allogeneic FM-MSC or autologous BM-MSC than in the untreated MI group ($P<0.05$; n=10 in each group) (Fig. 3B).

Angiogenesis and Differentiation of Transplanted Allogeneic FM-MSC and Autologous BM-MSC Sheets in Infarcted Hearts

Four weeks after transplantation, vascularization was assessed by lectin I staining and was observed in the allogeneic FM-MSC–transplanted MI group and the autologous

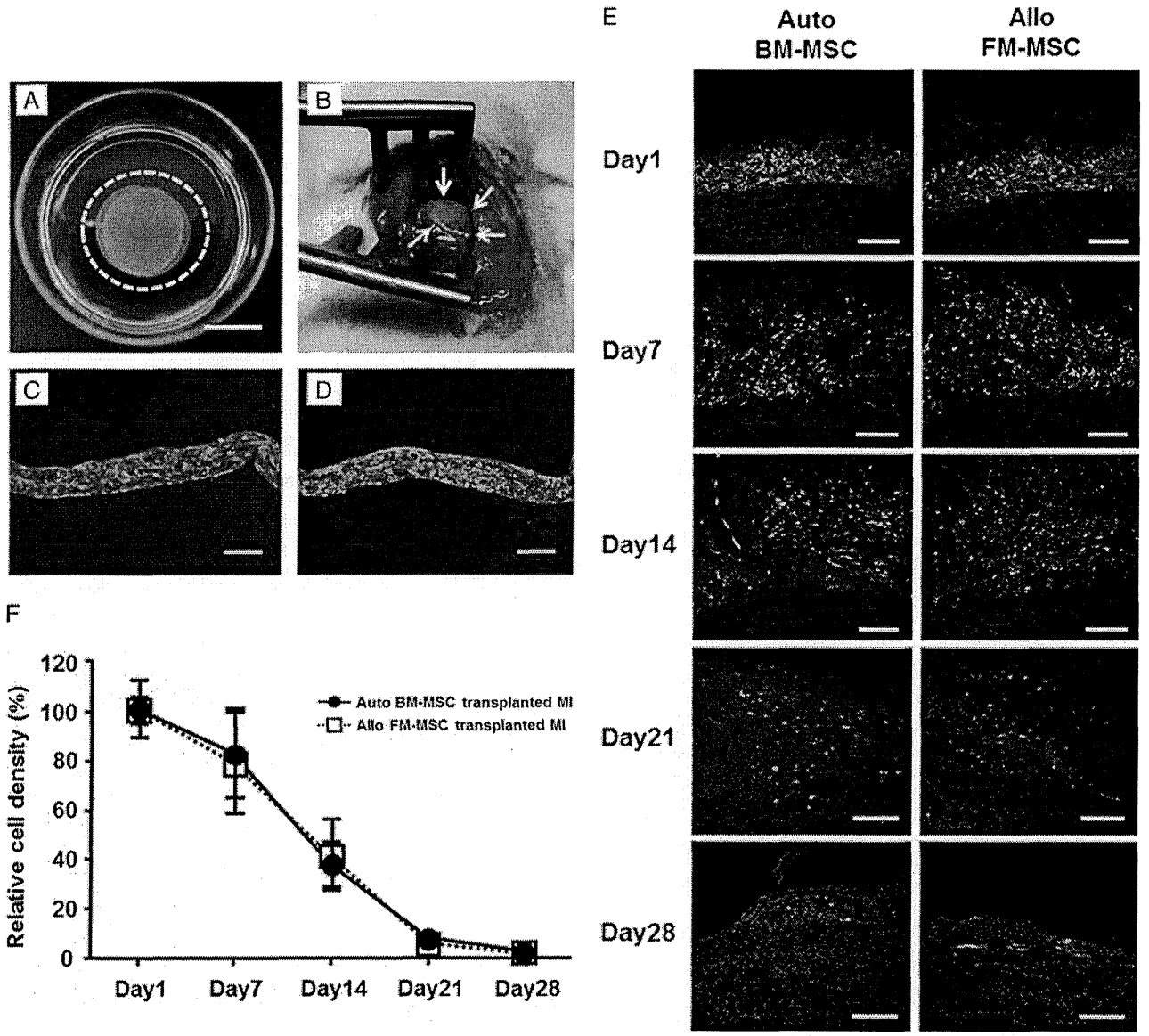

FIGURE 1. Stacked MSC sheets and their transplantation into infarcted hearts. A, two MSC sheets harvested from temperature-responsive culture surfaces were stacked successfully, producing a two-layer construct. Scale bar, 10 mm. B, two-layered MSC sheets were transplanted over the anterior wall of the infarcted heart and formed a stable attachment to the heart surface (arrows). C, cross-sectional staining of a GFP-expressing two-layered FM-MSC sheet. Scale bar, 100 μm. D, cross-sectional staining of a GFP-expressing two-layered BM-MSC sheet. Scale bar, 100 μm. E, allogeneic FM-MSCs and autologous BM-MSCs were present over the area surrounding the scar on days 1, 7, 14, 21, and 28. Scale bar, 100 μm. F, semiquantitative analysis showed that the engraftment rate of cells decreased with time in both groups transplanted with MSC sheets (days 1, 7, and 14, n=3 in each group; days 21 and 28, n=4 in each group). Data are expressed as mean±SE.

BM-MSC–transplanted MI group (Fig. 4B). Quantitative analysis showed increased capillary density in the infarcted area in both MSC-transplanted groups compared with the untreated MI group (n=6 in each group) (Fig. 4C). The capillary density in the peri-infarct area was similar in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC; both values were significantly higher than in the untreated MI group ($P<0.05$; n=6 in each group) (Fig. 4D).

GFP-positive FM-MSCs and BM-MSCs were observed in the peri-infarct area of the anterior wall, but GFP–lectin I/α-smooth muscle actin (αSMA) double-positive cells were not observed 1 day or 1 week after transplantation (data not shown). Two weeks after transplantation, engrafted GFP-positive FM-MSCs and BM-MSCs formed vascular structures and were positive for lectin I and αSMA (Fig. 4E,F). The GFP–lectin I/αSMA double-positive cells comprised less than 1% of the engrafted cells.

Immune Responses to Transplanted Allogeneic FM-MSCs and Autologous BM-MSCs in Infarcted Hearts

To compare the host immune responses to transplanted allogeneic FM-MSCs and autologous BM-MSCs, we performed immunohistochemical staining for CD3 (T cells) and CD68

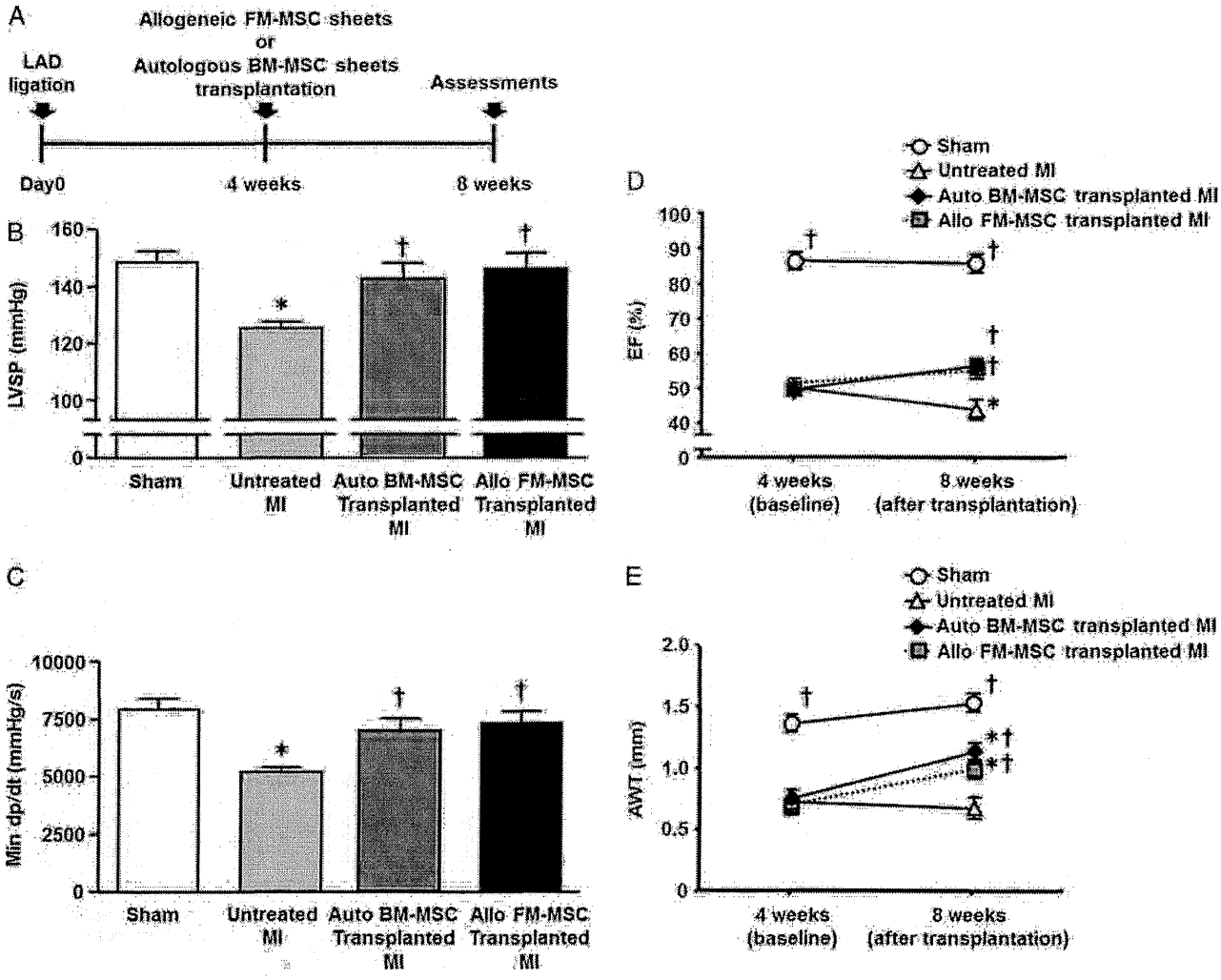

FIGURE 2. Effects of transplantation of allogeneic FM-MSC and autologous BM-MSC sheets on hemodynamic and echocardiographic parameters after MI. A, study flowchart. B and C, four weeks after transplantation, left ventricular systolic pressure and minimum d*P*/d*t* had improved significantly in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC compared with the untreated MI group (n=10 in each group). Data are expressed as mean±SE. **P*<0.05 vs. sham group; †*P*<0.05 vs. untreated MI group. D and E, four weeks after transplantation, the ejection fraction and anterior wall thickness in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC had improved significantly compared with the untreated MI group (n=10 in each group). Data are expressed as mean±SE. **P*<0.05 vs. baseline; †*P*<0.05 vs. time-matched untreated MI group. AWT, anterior wall thickness; EF, ejection fraction; LVSP, left ventricular systolic pressure.

(monocytes and macrophages) in sections of MSC-transplanted infarcted hearts 4 weeks after transplantation. Compared with the sham group, the numbers of $CD3^+$ and $CD68^+$ cells in the infarcted and peri-infarct areas were increased in the untreated MI group and in the MI groups transplanted with allogeneic FM-MSC or autologous BM-MSC (Fig. 5A,D). Quantitative analysis demonstrated no significant differences in $CD3^+$ cell infiltration between the MI groups that were untreated or transplanted with allogeneic FM-MSC and autologous BM-MSC (n=8 in each group) (Fig. 5C).

In the infarcted areas, there were no differences in the number of $CD68^+$ cells between the three MI groups, untreated or transplanted with allogeneic FM-MSC or autologous BM-MSC (n=8 in each group). By contrast, the number of $CD68^+$ cells in the peri-infarct area was significantly higher in both MSC-transplanted MI groups than in the untreated MI group (*P*<0.05 vs. untreated MI group; n=8 in each group) (Fig. 5E,F). The intensity of CD3 and CD68 staining did not differ between the two MSC-transplanted groups. $CD163^+$ cells were observed in the serial sections of the sites infiltrated by $CD68^+$ cells from all three MI groups (see **Figure S2b, SDC,** http://links.lww.com/TP/A849).

DISCUSSION

In the present study, we have demonstrated five points. First, transplantation of allogeneic FM-MSC sheets and autologous BM-MSC sheets improved cardiac function and prevented ventricular remodeling in a rat model of MI to a similar degree. Second, massive angiogenesis was observed in the areas transplanted with allogeneic FM-MSC sheets and autologous BM-MSC sheets but was not observed in the area

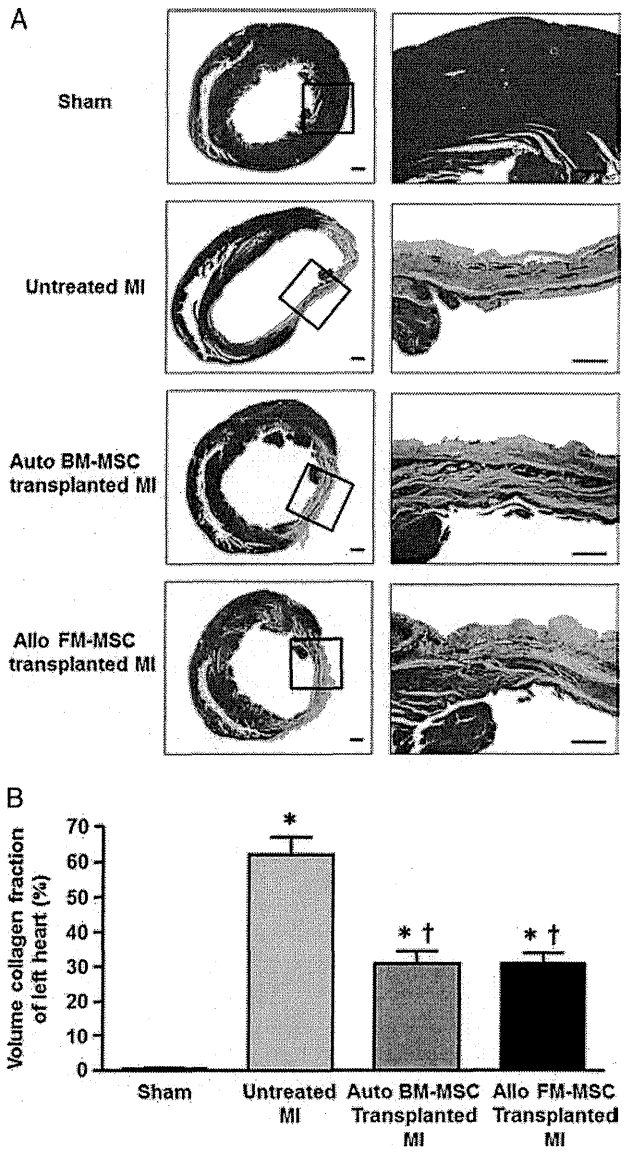

FIGURE 3. Masson's trichrome staining of heart cross-sections at the left ventricular papillary muscle level from MI rats transplanted with allogeneic FM-MSC and autologous BM-MSC sheets. A, four weeks after transplantation, the myocardial fibrosis area was smaller in the MI groups transplanted with FM-MSC and autologous BM-MSC than in the untreated MI group. Right row shows a higher resolution of the image in the black box in the respective left row. Scale bar, 1 mm (left row) and 500 μm (right row). B, quantitative analysis demonstrated that the fibrosis area was significantly smaller in the MI groups transplanted with FM-MSC and autologous BM-MSC compared with the untreated MI group (n=10 in each group). Data are expressed as mean±SE. *P<0.05 vs. sham group; †P<0.05 vs. untreated MI group.

of the infarcted myocardium. Third, transplanted allogeneic FM-MSCs engrafted in the infarcted myocardium from 1 day to 4 weeks after transplantation, but the number of engrafted cells decreased markedly with time. Fourth, some of the engrafted FM-MSCs were positive for lectin I or αSMA, but these cells comprised less than 1% of the engrafted cells. Fifth, the engraftment rate and host immune cell responses did not differ between groups transplanted with allogeneic FM-MSC and autologous BM-MSC.

Several studies have reported that transplantation of autologous BM-MSC improves cardiac function in ischemic heart disease (*17–20*). However, there are several limitations when using autologous BM-MSCs for clinical applications, including the invasiveness of the harvesting procedure, inadequate cell numbers, and donor site morbidity (*21*). We have reported that allogeneic FM-MSCs are an alternative to autologous BM-MSCs (*8, 9*). Although allogeneic, transplanted FM-MSCs exerted therapeutic effects in experimental rat models of hind-limb ischemia and acute myocarditis and did not elicit alloreactive lymphocyte proliferation. In this study, we showed a significant improvement in cardiac function and a reduction in myocardial fibrosis in rats with chronic MI that were transplanted with allogeneic FM-MSC sheets or with autologous BM-MSC sheets. The FM contains large quantities of MSCs, and their use is considered to present few ethical concerns; thus, FM-MSCs can provide a cell source for regenerative medicine (*22, 23*).

The mechanisms underlying the effectiveness of MSC therapy in treating ischemic heart failure may involve both the differentiation of transplanted MSCs into vascular cells and cardiomyocytes and the secretion of several growth factors by transplanted cells (paracrine effects). Two to 4 weeks after transplantation, some of the engrafted FM-MSCs and BM-MSCs stained positively for lectin I and participated in vessel formation. Staining for αSMA revealed that both types of MSCs differentiated into vascular smooth muscle cells, which play an important role in vessel maturation. A few engrafted MSCs may transdifferentiate in the vessel, but the number of such cells would be insufficient to be the main mechanism responsible for the therapeutic gain. We did not find desmin-positive or troponin T–positive engrafted allogeneic FM-MSCs or autologous BM-MSCs (data not shown). Earlier studies reported that transplanted MSCs differentiated into cardiomyocytes, vascular endothelial cells, and smooth muscle cells (*24–26*), but more recent studies have reported that transplanted MSCs appear to differentiate into these cells at a very low frequency (*27–30*).

After the discovery of the paracrine effect of MSCs, many studies have confirmed that the success of stem cell therapy for heart failure depends on this mechanism mainly by the promotion of angiogenesis, myocardial protection, and immune regulation (*31, 32*). In our previous study, transplanted FM-MSCs and BM-MSCs secreted angiogenic and cardioprotective cytokines, including vascular endothelial growth factor (VEGF) and hepatocyte growth factor, in the ischemic tissues (*8, 33*). These growth factors secreted from engrafted MSCs may help prevent ventricular remodeling. The response of the MSC sheets was similar, with large amounts of VEGF secreted into the culture media by FM-MSCs and BM-MSCs (see **Figure S1a, SDC,** http://links.lww.com/TP/A849). In both MI groups transplanted with MSC sheets, VEGF expression was up-regulated in the peri-infarct areas (see **Figure S1b, SDC,** http://links.lww.com/TP/A849). These results suggest that the therapeutic effects observed in our study may be attributable to the paracrine effects of transplanted FM-MSCs rather than to their differentiation into vascular endothelial cells and cardiomyocytes.

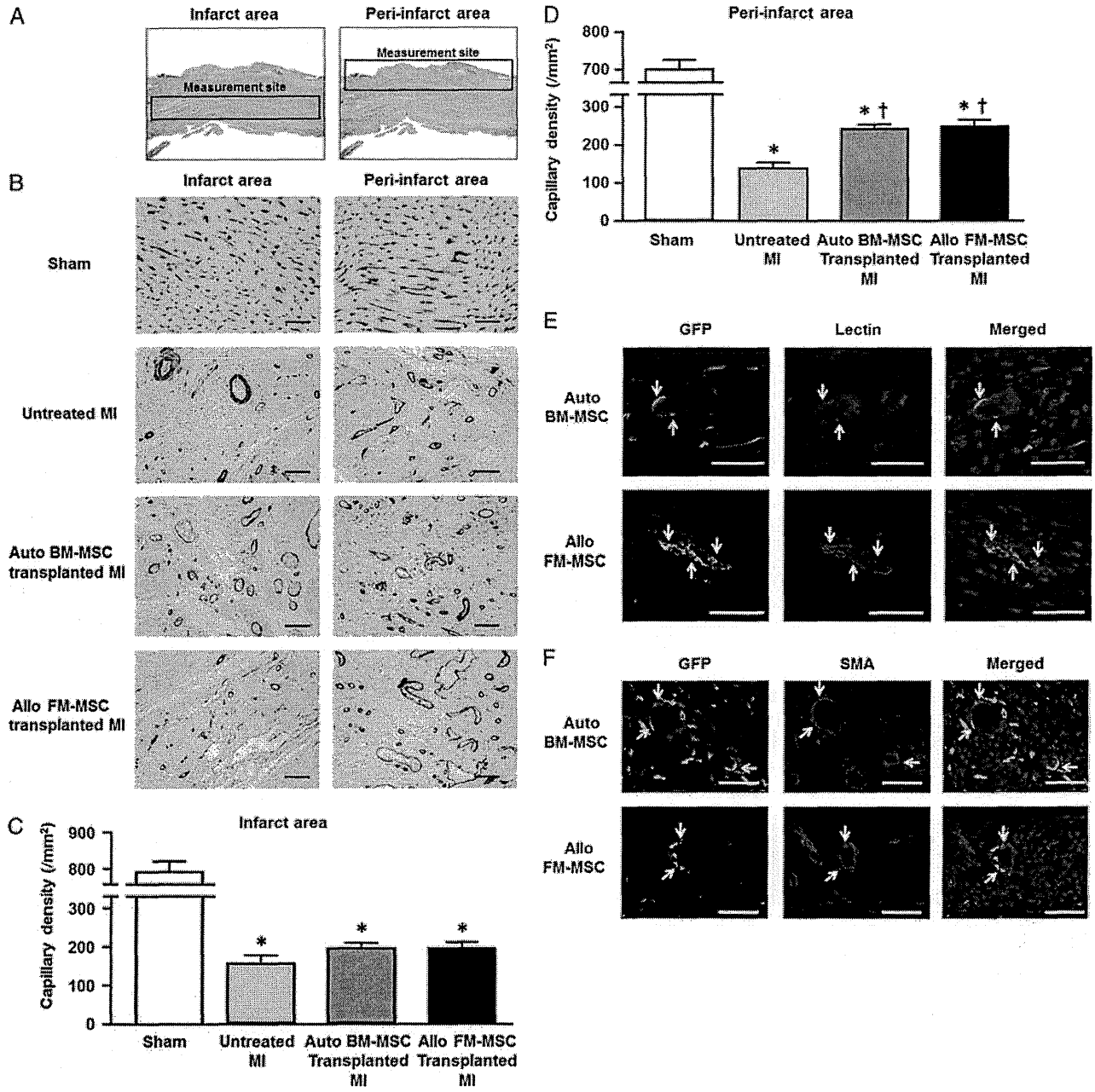

FIGURE 4. Vascularization and differentiation into vascular endothelial cells in the myocardial tissue grafted with allogeneic FM-MSC and autologous BM-MSC sheets. A, representative measurement section sites. B, four weeks after transplantation, the numbers of lectin I–positive capillaries were greater in the infarcted and peri-infarct areas in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC than in the untreated MI group. Quantitative analysis demonstrated that the capillary densities in the transplanted area were significantly higher in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC compared with the untreated MI group (infarcted area [C] and peri-infarct area [D]; n=10 in each group). E, two weeks after transplantation, GFP-expressing FM-MSCs and BM-MSCs were identified in a thick stratum on the epicardial side of the myocardium. Some allogeneic FM-MSCs and autologous BM-MSCs (green; white arrows) were positive for lectin I (red). F, some allogeneic FM-MSCs and autologous BM-MSCs (green; white arrows) were positive for αSMA (red). Nuclei are stained with TOPRO3 (blue). Scale bar, 50 μm. Data are expressed as mean±SE. *P<0.05 vs. sham group; †P<0.05 vs. untreated MI group.

MSCs are positive for major histocompatibility complex (MHC) I and negative for MHC II and costimulatory factors such as CD40, CD80, and CD86, so are considered to be nonimmunogenic (*34, 35*). We reported previously that FM-MSCs did not express MHC class II and did not induce alloreactive T lymphocyte proliferation (*8*). In this study, immunohistochemical staining showed few infiltrating $CD3^+$ T cells in the areas transplanted with allogeneic

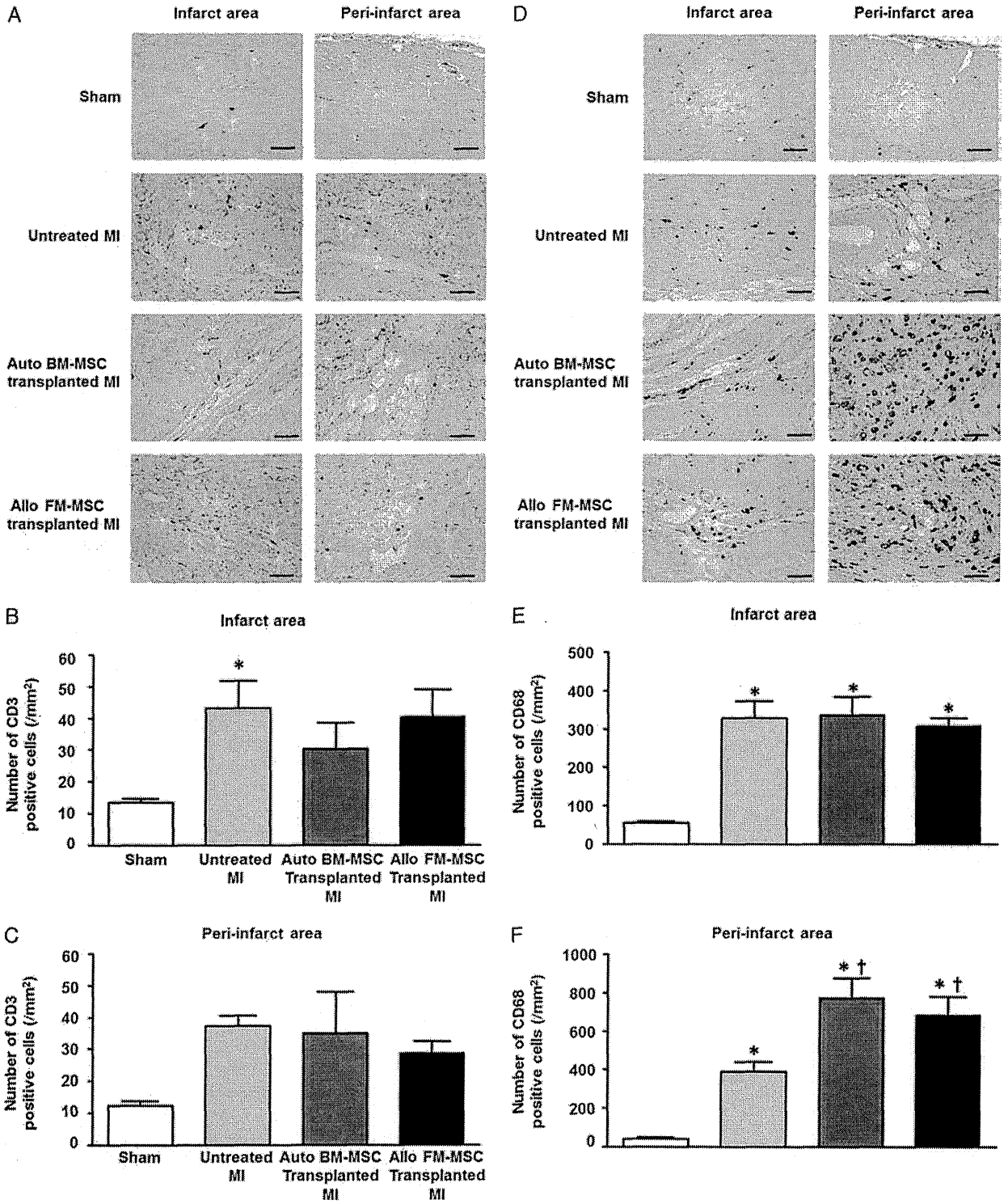

FIGURE 5. Immune responses in the myocardium transplanted with MSC sheets. A, in the infarcted and peri-infarct areas, the numbers of infiltrating $CD3^+$ cells did not differ between the untreated MI group and MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC (yellow arrows: $CD3^+$ cells). B and C, quantitative analysis of $CD3^+$ cells in the infarcted and peri-infarct areas showed no significant differences between the three MI groups (n=8 in each group). D, in the infarcted area, the number of infiltrating $CD68^+$ cells did not differ between the three MI groups, but marked $CD68^+$ cell infiltration was found in the peri-infarct area in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC. E and F, quantitative analysis of $CD68^+$ cells in the infarcted area showed no significant difference between the three MI groups. In the peri-infarct area, significantly more $CD68^+$ cells were found in the MI groups transplanted with allogeneic FM-MSC and autologous BM-MSC than in the untreated MI group (n=8 in each group). Scale bar, 50 μm. Data are expressed as mean±SE. *P<0.05 vs. sham group; †P<0.05 vs. untreated MI group.

FM-MSCs and the infarcted areas at 4 weeks after transplantation, and the infiltrating T cells were almost all $CD8^+$ T cells (see **Figure S2a, SDC,** http://links.lww.com/TP/A849). However, there were no differences between the infarcted hearts transplanted with allogeneic FM-MSC sheets and untreated infarcted hearts in the number of infiltrating $CD3^+$ and $CD8^+$ T cells and the degree of $CD3^+$ T-cell infiltration, and the engraftment rate did not differ between allogeneic FM-MSC transplantation and autologous BM-MSC transplantation. Thus, this limited T-cell infiltration may have been caused by chronic myocardial inflammation. In addition, there were few infiltrating $CD45RA^+$ B cells in the allogeneic FM-MSC-transplanted areas (see **Figure S3, SDC,** http://links.lww.com/TP/A849). These results suggest that allogeneic FM-MSCs are unlikely to activate host immune responses. In contrast, massive $CD68^+$ macrophage/monocyte infiltration was observed in the areas transplanted with either type of MSCs. There are two conceivable reasons for this macrophage infiltration. First, the infiltrating macrophages may have phagocytosed apoptotic cells, because terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling–positive cells were observed in areas transplanted with both types of MSCs (see **Figure S4, SDC,** http://links.lww.com/TP/A849). Second, there is a possibility that the macrophage infiltration was induced by the MSCs. It was reported that MSCs increased macrophage infiltration via a paracrine mechanism during wound healing after MI (*36*). There are two types of macrophages/monocytes: the classically activated proinflammatory M1 type and the alternatively activated anti-inflammatory M2 type (*37*). In our present study, many of the infiltrating macrophages were $CD163^+$ M2 macrophages (see **Figure S2b, SDC,** http://links.lww.com/TP/A849). M2 macrophages secrete several angiogenic factors promoting neovascularization (*38, 39*). Freytes et al. (*40*) reported that M2 macrophages modulated the viability of MSCs, and MSCs were reported to mediate a switch of macrophages to an anti-inflammatory activation state, which may be associated with the enhancement of cardiac function (*36, 41, 42*). Although further studies are needed, the induction of M2 macrophages may be one of the therapeutic mechanisms of MSC transplantation in MI.

The mechanisms responsible for the therapeutic effects of transplantation of allogeneic FM-MSC sheets in chronic MI are still unclear, and poor long-term survival and low differentiation rates of both types of transplanted MSC sheets are limitations of our study. In our previous study using the MI model, monolayer adipose tissue–derived MSC sheets gradually grew and developed into a thick stratum (*12*). The different results obtained in this study may have been caused by the difference in the cell sources. Several studies indicate differences between adipose tissue–derived MSCs and BM-MSCs (*43, 44*). Some studies tried to increase the therapeutic effects of cell transplantation by, for example, gene transduction or using a combination of drugs (*45–48*). Xu et al. (*49*) reported that lovastatin protected BM-MSCs from hypoxia-induced apoptosis, and Yang et al. (*50*) demonstrated that simvastatin improved the therapeutic efficacy of BM-MSC transplantation in an acute MI model by promoting cell survival and cardiovascular differentiation. These drug treatments may improve cell viability and increase the therapeutic effects of transplantation of MSC sheets in heart failure.

In conclusion, transplantation of allogeneic FM-MSC sheets improved cardiac function in a rat model of MI possibly by inducing angiogenesis and inhibiting myocardial fibrosis. The therapeutic effects were similar to those of transplanting autologous BM-MSC sheets and might be caused by the paracrine effects and the M2 macrophage induction. FM-MSC could be considered a new cell source, allowing wider clinical applications of MSC transplantation therapy. Although further experiments are needed to apply the current results to human cardiomyoplasty, transplantation of allogeneic FM-MSC sheets may provide a new therapeutic strategy for the treatment of MI.

MATERIALS AND METHODS

Animals

Male 8-week-old Lewis rats (Japan SLC, Hamamatsu, Japan) were used in this MI model. Male GFP-transgenic Lewis rats (Institute of Laboratory Animals, Kyoto University, Japan) and female GFP-transgenic Sprague–Dawley rats (Japan SLC) were also used for the harvest of transplanted cells. The experimental protocols were approved by the Animal Care Committee of the National Cerebral and Cardiovascular Center Research Institute (Osaka, Japan).

Expansion of FM-MSCs and BM-MSCs

The isolation and expansion of FM-MSCs and BM-MSCs were performed as described previously (see details in the **Materials and Methods, SDC,** http://links.lww.com/TP/A849) (*8*). In all experiments, FM-MSCs and BM-MSCs were used at passages 4 to 8.

Preparation of MSC Sheets

To prepare MSC sheets, we used 35-mm temperature-responsive dish (UpCell, CellSeed, Tokyo, Japan). Preparation of MSC sheets was performed as described previously (see details in the **Materials and Methods, SDC,** http://links.lww.com/TP/A849) (*12*).

Model of MI

To create an MI model, male Lewis rats (220–250 g) were anesthetized, and the left coronary artery was ligated, as described previously (see details in the **Materials and Methods, SDC,** http://links.lww.com/TP/A849) (*12*). The sham group underwent thoracotomy and cardiac exposure without coronary ligation.

We randomly assigned the rats to four groups: (a) rats with chronic heart failure that underwent transplantation with allogeneic FM-MSC sheets (allo-FM-MSC–transplanted MI group; n=10), (b) rats with chronic heart failure that underwent transplantation with autologous BM-MSC sheets (auto-BM-MSC–transplanted MI group; n=10), (c) rats with chronic heart failure without transplantation (untreated MI group; n=10), and (d) sham-operated rats without transplantation (sham group; n=10). Four weeks after coronary ligation, the allo-FM-MSC–transplanted MI group and auto-BM-MSC–transplanted MI group underwent transplantation with the respective two-layered cell sheets. The sheets were placed on the anterior wall, including the scar area, and then covered with oxidized regenerated cellulose (INTERCEED [TC7], Johnson & Johnson Medical, Tokyo, Japan). The other two groups underwent the same operative procedures without transplantation.

Hemodynamic Studies

Hemodynamic studies were performed 8 weeks after coronary ligation (4 weeks after transplantation (see details in the **Materials and Methods, SDC,** http://links.lww.com/TP/A849).

Echocardiographic Studies

Echocardiography was performed 4 weeks (before transplantation) and 8 weeks (4 weeks after transplantation) after coronary ligation (see details in the **Materials and Methods, SDC,** http://links.lww.com/TP/A849).

Immunohistochemical Studies

Immunohistochemical details (see **Materials and Methods, SDC,** http://links.lww.com/TP/A849).

Statistical Analysis

Data are expressed as mean±SE. Analysis of variance was used to compare each variable between groups, and the post hoc Tukey's test was used to locate significant differences. Differences were considered significant at $P<0.05$.

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Transplantation of allogenic fetal membrane-derived mesenchymal stem cells protect against ischemia-reperfusion-induced acute kidney injury

Hidetoshi Tsuda[1,2], Kenichi Yamahara[2], Kentaro Otani[2], Masayoshi Okumi[3], Koji Yazawa[3], Jun-ya Kaimori[1, 4], Akihiko Taguchi[2, 5], Kenji Kangawa[6], Tomoaki Ikeda[2,7], Shiro Takahara[1], Yoshitaka Isaka[4]

[1] Department of Advanced Technology for Transplantation, Osaka University Graduate School of Medicine, Osaka, Japan
[2] Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan
[3] Department of Urology, Osaka University Graduate School of Medicine, Suita, Japan
[4] Department of Geriatric and Nephrology, Osaka University Graduate School of Medicine, Suita, Japan
[5] Department of Regenerative Medicine Research, Institute of Biomedical Research and Innovation, Kobe, Japan
[6] Department of Biochemistry, National Cerebral and Cardiovascular Center, Osaka, Japan
[7] Department of Obstetrics and Gynecology, Mie University Graduate School of Medicine, Tsu, Japan
Corresponding Author: Yoshitaka Isaka, M.D., Ph.D., Department of Geriatric and Nephrology, Osaka University Graduate School of Medicine, Suita, Japan
isaka@kid.med.osaka-u.ac.jp
Running head; Renoprotective effect of FM-MSCs on IR injury

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ABSTRACT

Mesenchymal stem cells (MSCs) are an attractive therapeutic cell source for treating renal diseases. MSCs administration has been shown to improve renal function, although underlying mechanisms are incompletely understood. We recently showed that allogenic fetal membrane-derived MSCs (FM-MSCs), which are available non-invasively in large amounts, had renoprotective effect in an experimental glomerulonephritis model. Here, we investigated whether allogenic FM-MSCs administration could protect kidneys from ischemia/reperfusion (I/R) injury.

Lewis rats were subjected to right nephrectomy and left renal I/R injury by clamping left renal artery as acute kidney injury (AKI) model. After declamping FM-MSCs (5×10^5 cells) obtained from major histocompatibility complex (MHC) mismatched-ACI rats were intravenously administered.

I/R injured rats exhibited increased serum creatinine and BUN, whereas FM-MSCs administration significantly ameliorated renal function. Histological analysis revealed that FM-MSCs administration significantly suppressed tubular apoptosis and infiltration of macrophages and T cells. Administration of FM-MSCs mainly homed into lung, but increased serum IL-10 levels. Of interest is that renoprotective effects of FM-MSCs were abolished by using anti-IL-10 neutralization antibody, suggesting that IL-10 would be one of the candidate factors to protect rat kidney from I/R injury in this model. We concluded that allogenic FM-MSCs transplantation is a potent therapeutic strategy for the treatment of AKI.

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Keywords: fetal membrane; cell therapy; acute kidney injury

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INTRODUCTION

Renal ischemia-reperfusion (I/R) injury is a major cause of acute kidney injury (AKI), which is associated with prolonged hospitalization and excess morbidity and mortality (38,42). As its prevalence has risen, it is increasingly recognized as a significant cause of end-stage renal diseases (6). Despite decades of laboratory and clinical investigations and the advent of renal replacement therapy, no significantly effective new therapy has been introduced into clinical practice in decades (13,44). Therefore, due to the clinical importance of renal I/R injury, effective therapy for such injury should be considered.

Mesenchymal stem cells (MSCs) are multipotent stem cell present in bone marrow (BM), adipose tissue, and many other organs, and capable of differentiating into cell of different tissue lineages including adipocyte, osteocyte, and chondrocyte (9,10,34). Previous studies have shown that BM-MSCs have renoprotective effects and can ameliorate the renal damage due to AKI induced by ischemia-reperfusion (25,41). Although BM-MSCs are promising in renoprotective effects, BM aspiration may be invasive and sometimes yields low numbers of MSCs after processing. Therefore, alternative sources of MSCs would be beneficial for both research and therapeutic purposes. Fetal membrane (FM), which is generally discarded as medical waste after delivery, has been shown to be a rich, and easily expandable source of MSCs (21,35). Recently, we established a protocol to isolate MSC from FM, and reported that administration of allogenic FM-derived MSCs (FM-MSCs) (5×10^5cells/rat) as well as

autologous BM-MSCs (5×10^5cells/rat) induced therapeutic angiogenesis in a rat hindlimb ischemia model (22). Moreover, we reported that same number of FM-MSCs administration (5×10^5cells/rat)have a therapeutic effect in anti-Thy1 nephritic rats (43). These results suggest that this dose is an adequate to improve tissue damage and allogeneic FM-MSCs proposes an attractive alternative to autologous BM-MSCs as a source of regenerative therapy.

We previously reported the behavior of transplanted FM-MSCs; a large number of FM-MSCs were observed in lung, and a small number of FM-MSCs were detected in liver, spleen and kidney 24 hrs after transplantation, but FM-MSCs were rarely found in the lung, liver or spleen at 7 days after transplantation and no FM-MSCs were detected at 28 days (23,43). Similar observation was also reported using adipose-derived mesenchymal stem cells (16). However, therapeutic effects of FM-MSCs administration were shown on day 7 or 14 after transplantation. These results suggested that FM-MSCs contributed to tissue repair by producing humoral factors rather than by differentiating into inured cell types. Moreover, we showed that FM-MSCs-secreted prostaglandin E2 (PGE2) were involved renal repair in anti-Thy1 nephritic rats (43). It was reported that PGE2- induced interleukin-10 (IL-10), in macrophages(29,39) and T cells(11), could possess both anti-inflammatory and immunosuppressive properties. As our preliminary experiments revealed that the FM-MSCs transplanted rats exhibited increased IL-10 production, we hypothesized that intravenously injected FM-MSCs could stimulate IL-10 secretion via PGE2, and investigated whether allogenic FM-MSCs administration can be protective against

renal structural and functional injury in I/R injury as a model of AKI. Furthermore, we also examined that the role of FM-MSCs-induced IL-10 by using anti-IL-10 neutralizing antibody.

MATERIALS AND METHODS

Animals

All experiments had received prior approval by the Animal Care Committee of the National Cerebral and Cardiovascular Center Research Institute. Different strains of rats were used according to their major histocompatibility complex (MHC) antigen disparity: Lewis (MHC haplotype: RT-1l) and ACI (MHC haplotype: RT-1a) rats (Japan SLC, Hamamatsu, Japan). Green fluorescent protein (GFP)-transgenic Lewis rats (Institute of Laboratory Animals, Kyoto University, Japan) were used to study the distribution of transplanted FM-MSCs.

Isolation and expansion of FM-MSCs

Isolation and expansion of FM-MSCs were performed as previously described (22). In brief, FM was obtained from pregnant rats on day 15 postconception. Minced FM was digested with type II collagenase solution (300 U/ml; Worthington Biochemical, Lakewood, NJ) for 1 hour at 37°C. After filtration and centrifugation, FM-derived cells were suspended in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Thermo Scientific, Woburn, MA, USA) and penicillin/streptomycin (Invitrogen) and cultured in standard plastic dishes. The adherent MSCs populations appeared by days 5-7 and these FM-MSCs were used for the experiments at passage 3-6.