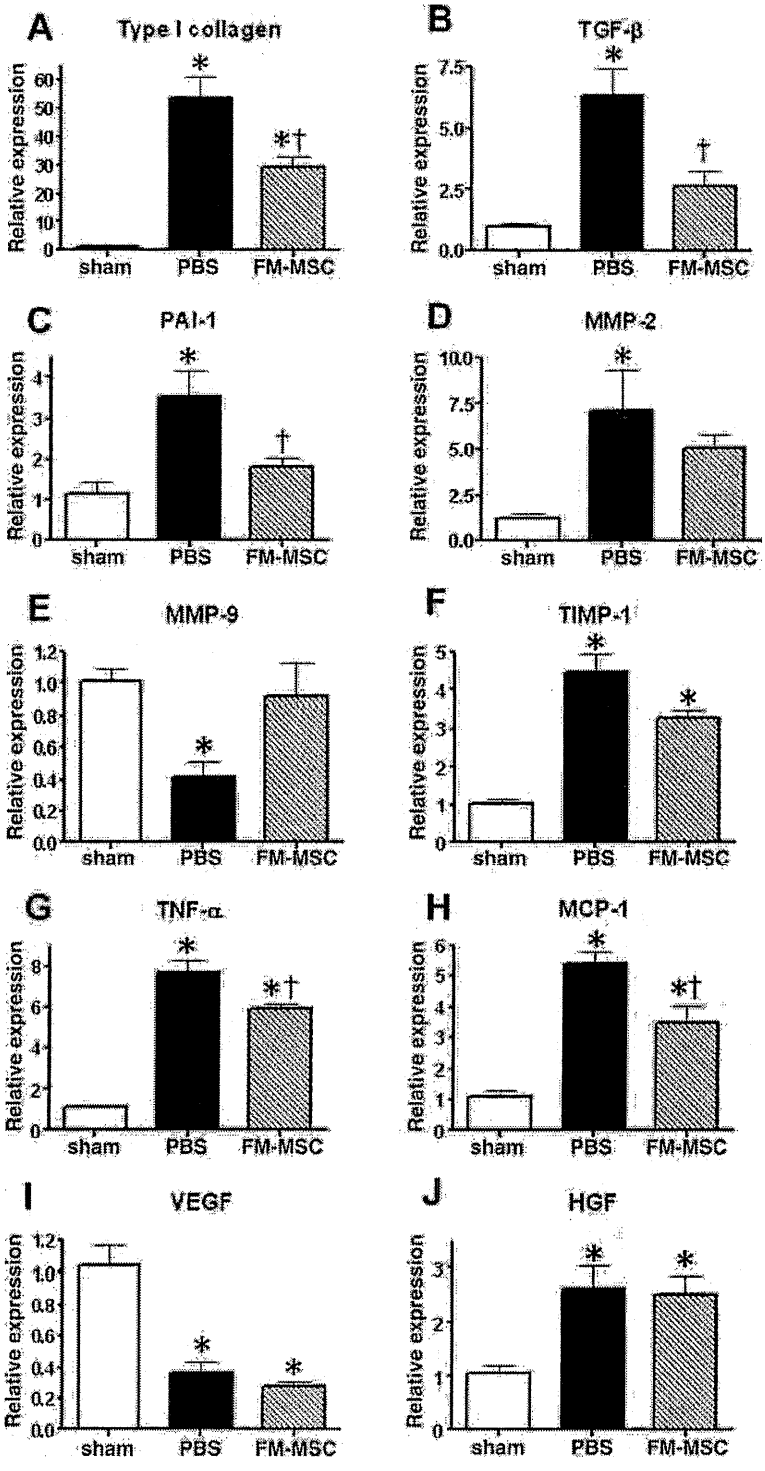

Fig. 3. Quantitative RT-PCR analysis of extracellular matrix, inflammatory-, and angiogenesis-related genes in the kidney of rats with anti-Thy1 nephritis and FM-MSC administration. Expression of mRNA for type I collagen (*A*), transforming growth factor (TGF)-β (*B*), type 1 plasminogen activator inhibitor (PAI-1; *C*), TNF-α (*G*), and monocyte chemoattractant protein (MCP-1; *H*) in glomeruli was markedly increased in anti-Thy1 nephritic rats on *day 7*, which was significantly attenuated in FM-MSC-transplanted rats. No significant difference in mRNA expression of membrane-type matrix metalloproteinase 2 (MMP-2; *D*), MMP-9 (*E*), tissue inhibitor of MMP-1 (TIMP-1; *F*), VEGF (*I*), and HGF (*J*) was seen between the PBS and FM-MSC groups. (n = 8/group). *$P < 0.05$ vs. Sham group. †$P < 0.05$ vs. PBS group.

Engraftment of intravenously injected FM-MSC in rats with anti-Thy1 nephritis. To investigate the behavior of intravenously administered FM-MSC in anti-Thy1 nephritic rats, FM-MSC derived from GFP transgenic Lewis rats were intravenously administered into allogenic ACI rats on *day 2* after mAb injection (n = 4). Twenty-four hours after FM-MSC transplantation, several GFP-positive cells were detected in the kidney sections (12.7 ± 0.3 cells/cm^2) including glomeruli (Fig. 5*A*), proximal tubule (Fig. 5*B*), and interstitial area (Fig. 5*C*). We also detected GFP-positive FM-MSC in sections of lung (Fig. 5*D*), liver (Fig. 5*E*), and spleen (Fig. 5*F*). A significant number of GFP-positive FM-MSC were seen in the

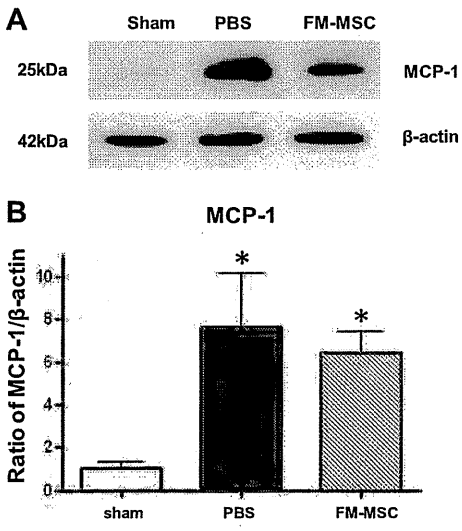

Fig. 4. Decreased MCP-1 protein expression in the renal tissue of nephritic rats after FM-MSC administration. *A*: representative Western blot analysis of MCP-1 in the Sham, PBS, and FM-MSC groups. *B*: quantitative analysis of immunoreactive bands for MCP-1 demonstrated that MCP-1 protein expression in the PBS group was significantly upregulated compared with the Sham group ($P < 0.05$, $n = 8$), and FM-MSC administration showed a tendency toward decreased expression.

lung (119.2 ± 5.1 cells/cm^2), and a similar distribution of GFP-positive cells were observed in the liver and spleen (38.4 ± 1.5 and 32.1 ± 2.9 cells/cm^2, respectively). Although no GFP-positive cell was found in the kidney at 7 days after systemic injection of GFP-positive FM-MSC, several GFP-expressing cells were detected in sections of lung, liver, and spleen (37.6 ± 3.1, 26.1 ± 2.7, and 4.8 ± 0.3 cells/cm^2, respectively).

Anti-inflammatory effect of FM-MSC-conditioned medium on cultured MC. Next, we examined whether FM-MSC possess direct anti-inflammatory effects on MC. When MC were cultured in standard medium, gene expression of TNF-α showed a peak at 8 h and then decreased ($n = 3$–12, Table 2). FM-MSC-conditioned medium induced a significant (>50%) decrease in TNF-α expression after incubation for 8 h ($P <$ 0.05 vs. standard medium) (Table 2). MCP-1 expression in cultured MC showed a peak at 4 h and then decreased (Table 2). Between FM-MSC-conditioned and standard medium, a significant reduction in MCP-1 expression was seen at 4 (>20%) and 8 (>70%) h ($P < 0.05$ vs. standard medium).

Because recent reports have shown that PGE_2 is one of the key modulators for the MSC-induced anti-inflammatory response, PGE_2-depleted conditioned medium of FM-MSC was prepared by treatment with NS-398, a selective inhibitor of COX2 activity (38). ELISA revealed that a significant amount of PGE_2 was detected in FM-MSC-conditioned medium (888.1 ± 123.3 pg/ml), and NS-398 treatment significantly suppressed its production (23.2 ± 2.4 pg/ml, $P < 0.01$). After incubation for 8 h, conditioned medium of NS-389-treated FM-MSC markedly abolished the decreased expression of TNF-α and MCP-1 in rat MC (1.19 ± 0.12- and 0.82 ± 0.06-fold, respectively) (Fig. 6).

DISCUSSION

In this study, we demonstrated that *1*) intravenous injection of allogenic FM-MSC improved disease manifestations in rats with anti-Thy1 glomerulonephritis; *2*) allogenic FM-MSC administration suppressed MC proliferation, glomerular monocyte/macrophage infiltration, mesangial matrix accumulation, and the glomerular expression of inflammatory and extracellular matrix-related molecules in anti-Thy1 nephritis; and *3*) FM-MSC-conditioned medium attenuated the expression of these inflammatory cytokines/chemokines in cultured MC through a PGE_2-dependent mechanism. Therefore, our data indicate that allogenic FM-MSC transplantation would be a potent therapeutic strategy for the treatment of acute glomerulonephritis.

MSC are considered to be an attractive cell source for application in regenerative medicine because of their excellent capacities in proliferation and differentiation (8, 33, 35, 62). MSC are present in various tissues, but the most characterized population is BM-MSC (9, 11, 42). Therefore, the potential of MSC for renal repair has been investigated using BM-MSC (17, 30–32, 54, 56). To consider the clinical setting, donor BM is a suitable source of MSC, because MSC are relatively easy to obtain from BM aspirates and autologous donor MSC are unlikely to be immunologically rejected. However, autologous MSC transplantation is difficult to attempt on acute glomerulonephritis patients, because of a cell-preparatory period and cell transplantation timing. Therefore, allogenic MSC transplantation has more practical therapeutic value in clinical medicine. We have previously characterized a population of MSC from FM tissue, which possesses great advantages due to its abundance, easy accessibility, and angiogenic activity (20). In this study, we demonstrated that intravenous injection of allogenic FM-MSC, similar to reported autologous BM-MSC (31, 58), provided significant improvement in rats with anti-Thy1 nephritis, indicating that allogenic FM-MSC have potential as a source for regenerative-based therapy for glomerulonephritis.

In this study, we demonstrated that allogenic ACI-derived FM-MSC have a therapeutic effect in MHC-mismatched Lewis rats with anti-Thy1 nephritis. FM is known to play a role in preventing rejection of the fetus and is thought to have low immunogenicity (2, 3, 59). MSC have been reported to fail to trigger allogenic T cell proliferation and induce immune tolerance (1, 6). Indeed, we previously demonstrated that FM-MSC expressed surface antigens similar to those of BM-MSC. For example, both types of MSC are negative for MHC II (19). We also confirmed that FM-MSC did not provoke alloreactive lymphocyte proliferation in mixed lymphocyte culture (20). A recent report described that intravenous injection of BM-MSC induced recovery from anti-Thy1 nephritis in outbred allogenic as well as inbred autologous settings (31). These results suggest that FM-MSC as well as BM-MSC could evade T lymphocyte alloreactivity and would be successfully transplantable across MHC barriers.

Several studies have shown beneficial effects of BM-MSC transplantation in renal diseases (17, 30–32, 54, 56). However, the mechanisms underlying the benefit of MSC transplantation remain controversial. One possible mechanism is the differentiation into renal cells of injected MSC. Intravenously administered MSC have been shown to con-

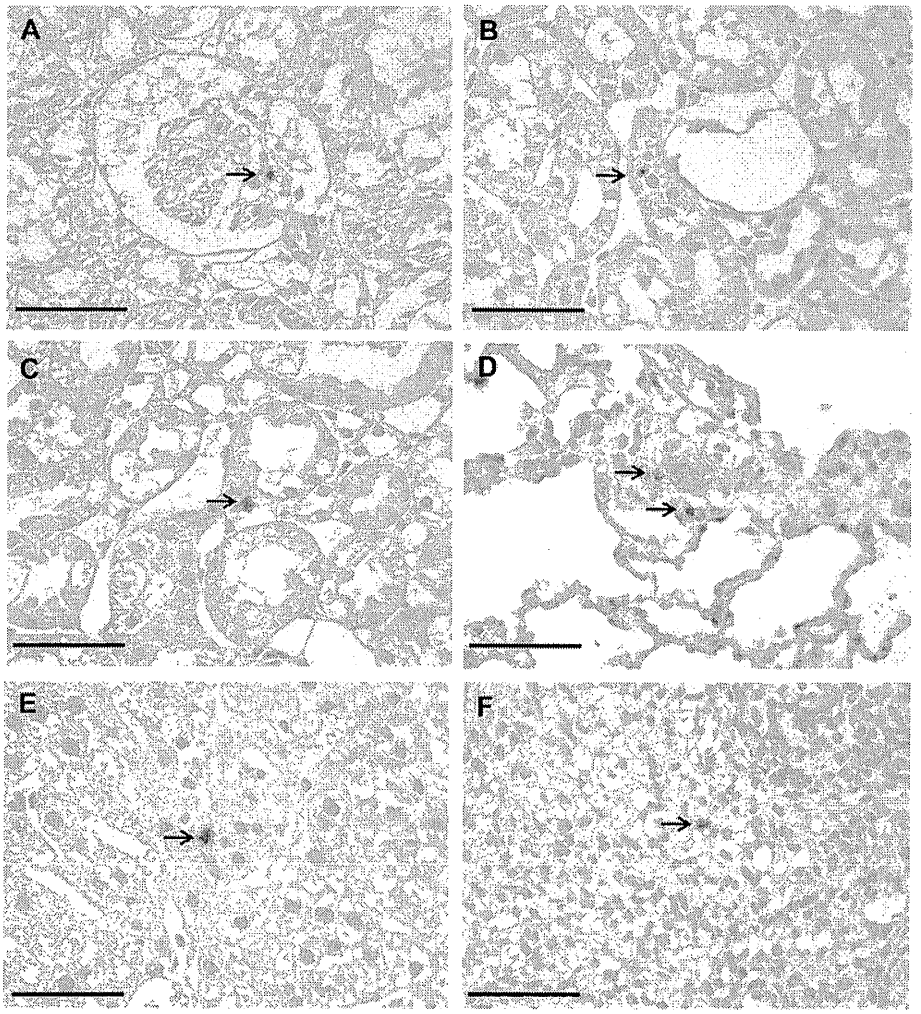

Fig. 5. Engraftment of intravenously injected FM-MSC in rats with anti-Thy1 nephritis. The presence of intravenously injected green fluorescent protein (GFP)-positive FM-MSC was observed 24 h after cell injection (n = 4). *A–F*: representative GFP immunohistochemical staining shows GFP-positive transplanted FM-MSC were found in glomeruli (*A*), proximal tubule (*B*), and renal interstitial tissue (*C*) as well as the lung (*D*), liver (*E*), and spleen (*F*; black arrows). Scale bars = 50 μm.

tribute, via differentiation and engraftment, to the cells of many organs, including the kidney (17, 23, 45, 47). In this study, however, we confirmed that these engraftments were low-frequency events that cannot explain the prompt regenerative responses MSC elicit in damaged kidneys. Using the same anti-Thy1 nephritis model, Kunter et al. (31) reported that they failed to detect any evidence of transdifferentiation of MSC into renal cells. This evidence suggests that the direct contribution of transplanted MSC to tissue regeneration is minimal.

Another possibility explaining how transplanted MSC mediate the protective and regenerative effects in damaged kidney tissue is paracrine action (31, 56, 58). Our previous studies found that FM- and BM-MSC secreted VEGF and HGF, which are well-known potent angiogenic and anti-apoptotic factors that elicited angiogenesis in a hindlimb ischemia model (20). In experimental ischemic acute kidney injury or glomerulonephritis, VEGF or HGF secreted from MSC exerted beneficial effects (31, 46, 56, 58). Based on these results, we examined the glomerular expression of these regenerative factors in anti-Thy1 nephritic rats. Contrary to our expectation, however, no significant induction of VEGF or HGF expression in the kidney was seen after FM-MSC transplantation. Therefore, contribution of these FM-MSC-derived growth factors might be minimal in the repair process of anti-Thy1 nephritis.

Table 2. *Time course of TNF-α and MCP-1 mRNA levels in MC after incubation with standard or FM-MSC-conditioned medium*

	TNF-α			MCP-1		
	4 h	8 h	12 h	4 h	8 h	12 h
Standard medium	100.0 ± 22.4%	155.5 ± 28.5%	154.3 ± 23.2%	100.0 ± 14.7%	20.2 ± 1.7%	26.9 ± 2.9%
FM-MSC-conditioned medium	64.6 ± 19.7%*	83.4 ± 10.6%*	154.4 ± 61.6%	19.9 ± 11.5%*	14.4 ± 1.9%*	31.9 ± 6.7%

MC, mesangial cells; FM-MSC, fetal membrane-derived mesangial stem cells. *$P < 0.05$ vs. standard medium.

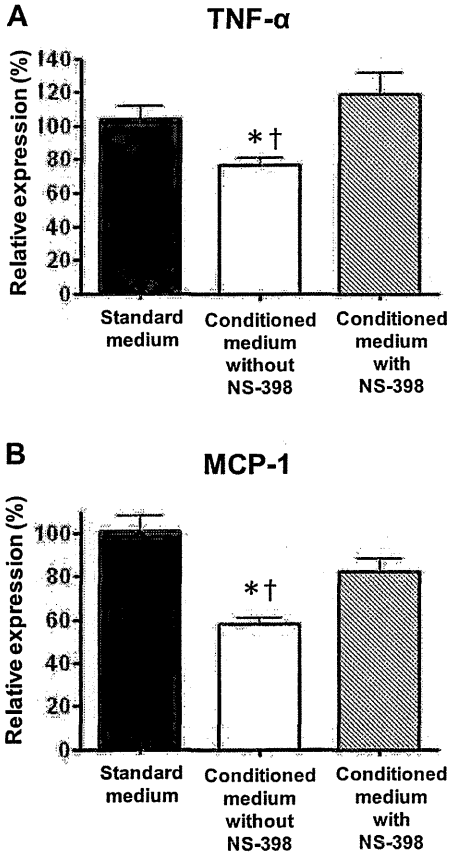

Fig. 6. Effect of FM-MSC-conditioned medium on TNF-α and MCP-1 gene expression in cultured MC. Quantitative RT-PCR analysis in MC after 8 h in culture revealed that TNF-α (*A*) and MCP-1 (*B*) expression was significantly reduced when MC were cultured in FM-MSC-conditioned medium, and this decrease was significantly abolished by the treatment with NS-398 (0.1 μM; $n = 6$–12). *$P < 0.05$ vs. standard medium.

Recently, MSC have been demonstrated to possess immunomodulatory properties (12–14, 22, 27, 28, 37, 49, 51, 52). They are able to modulate the function of lymphocytes (12–14, 22, 28), dendritic cells (37, 49), natural killers (27, 51, 52), and macrophages (26, 38). Several in vivo studies have shown that MSC administration could downregulate the systemic and local inflammatory responses and prevent tissue damage in inflammatory models (4, 15, 63). Therefore, FM-MSC injection might act by modulating the inflammatory responses after the induction of anti-Thy1 nephritis. We previously demonstrated that MSC transplantation attenuated myocardial inflammation in an acute myocarditis model associated with an increase in MCP-1 as well as infiltration of macrophages (40). In the present study, we confirmed that glomerular expression of TNF-α and MCP-1 and macrophage infiltration was significantly suppressed by the administration of FM-MSC. In glomerulonephritis, these inflammatory cytokines/chemokines participate in the activation and accumulation of macrophages, and their infiltration is strongly associated with proteinuria and declining renal function (10, 44, 48, 60).

Recent reports demonstrate that MSC-mediated immunosuppression is mediated by direct contact with immunomodulatory cells including T cells, NK cells, and macrophages, followed by paracrine action of secreted PGE_2 and TGF-β (13, 27, 38). Because intravenously administered FM-MSC survived in kidney, lung, and reticuloendothelial organs including the spleen and liver, these transplanted FM-MSC might change the activity of immunomodulatory cells in nephritic rats by direct contact and paracrine action, which would reduce the inflammatory state of anti-Thy1 nephritis. Previous studies demonstrate that PGE_2 is one of the leading candidates for MSC-induced immune suppression (38). In this study, we confirmed that FM-MSC-conditioned medium contained a significant amount of PGE_2, which was completely depleted by treatment with the COX2 inhibitor NS-398. Gene expression of MCP-1 and TNF-α in MC was decreased by FM-MSC-conditioned media, and this decrease was significantly restored by the treatment with NS-398. A previous report demonstrated that PGE_2 suppressed cytokine/chemokine expression including TNF-α and MCP-1 in MC, which would relate to its anti-inflammatory activity (50). Therefore, PGE_2 would be one of the candidate factors to cause the downregulation of TNF-α and MCP-1 in FM-MSC-treated rats with anti-Thy1 nephritis. Together, previous studies including our own support the hypothesis that paracrine/endocrine actions are of major importance in mediating the protective and regenerative effect of administered MSC after tissue damage.

We have recently reported that MSC transplantation improved cardiac function through an antifibrotic effect in a rat model of dilated cardiomyopathy and acute myocarditis (36, 40) and also demonstrated that the highly expressed genes in cultured MSC included a number of molecules involved in the biogenesis of extracellular matrix (39). These results suggest that transplanted MSC inhibit the fibrogenic process through paracrine actions. In this study, we confirmed that FM-MSC transplantation in anti-Thy1 nephritic rats resulted in reduced mesangial matrix accumulation. In addition, the glomerular expression of several genes involved in fibrogenesis including type I collagen, TGF-β, and PAI-1 was significantly decreased in the FM-MSC group compared with the PBS group. These results support our hypothesis that transplanted MSC possesses antifibrotic activity. However, because the expression of type I collagen, TGF-β, and PAI-1 is associated with renal disease severity (5, 7, 57), decreased expression of these fibrogenic genes in the FM-MSC group might only reflect the degree of renal damage; the precise mechanism by which transplanted FM-MSC prevent renal fibrosis in anti-Thy1 nephritis remains to be elucidated.

In conclusion, our observation that FM-MSC transplantation helped recovery from anti-Thy1 nephritis demonstrates the renoprotective effect of FM-MSC. Because FM-MSC is available non-invasively in large amounts, we suggest that cultured, banked FM-MSC could provide a new therapeutic strategy for the treatment of kidney injury.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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Original article

Allogeneic administration of fetal membrane-derived mesenchymal stem cells attenuates acute myocarditis in rats

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ABSTRACT

We reported previously that the autologous administration of bone marrow-derived mesenchymal stem cells (BM-MSC) significantly attenuated myocardial dysfunction and injury in a rat model of acute myocarditis by stimulating angiogenesis and reducing inflammation. Because BM aspiration procedures are invasive and can yield low numbers of MSC after processing, we focused on fetal membranes (FMs) as an alternative source of MSC to provide a large number of cells. We investigated whether the allogeneic administration of FM-derived MSC (FM-MSC) attenuates myocardial injury and dysfunction in a rat myocarditis model. Experimental autoimmune myocarditis (EAM) was induced in male Lewis rats by injecting porcine cardiac myosin. Allogeneic FM-MSC obtained from major histocompatibility complex-mismatched ACI rats (5×10^5 cells/animal) were injected intravenously into Lewis rats one week after myosin administration. At day 21, severe cardiac inflammation and deterioration of cardiac function were observed. The allogeneic administration of FM-MSC significantly attenuated inflammatory cell infiltration and monocyte chemoattractant protein 1 expression in the myocardium and improved cardiac function. In a T-lymphocyte proliferation assay, the proliferative response of splenic T lymphocytes was significantly lower in cells obtained from FM-MSC-treated EAM rats that reacted to myosin than in cells obtained from vehicle-treated rats with EAM. T-lymphocyte activation was significantly reduced by coculture with FM-MSC. The allogeneic administration of FM-MSC attenuated myocardial dysfunction and inflammation, and the host cell-mediated immune response was attenuated in a rat model of acute myocarditis. These results suggest that allogeneic administration of FM-MSC might provide a new therapeutic strategy for the treatment of acute myocarditis.

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1. Introduction

Acute myocarditis is a nonischemic heart disease characterized by myocardial inflammation and edema. This disease is associated with rapidly progressing heart failure, arrhythmia, and sudden death [1,2]. Although the early evidence showing the efficacy of immunoglobulin and interferon therapies appears promising, these results have yet to be demonstrated in randomized controlled clinical trials. The current options are restricted to supportive care for patients with heart failure and arrhythmia. The lack of a specific treatment and the potential severity of the illness emphasize the importance of new effective therapeutic strategies for myocarditis.

Mesenchymal stem cells (MSC) are multipotent stem cells present in the bone marrow (BM), adipose tissue, and many other tissues, and these cells can differentiate into a variety of cells, including

Abbreviations: MSC, mesenchymal stem cells; BM, bone marrow; BM-MSC, bone marrow-derived mesenchymal stem cells; FMs, fetal membranes; FM-MSC, fetal membrane-derived mesenchymal stem cells; MHC, major histocompatibility complex; ACI, August–Copenhagen–Irish; GFP, green fluorescent protein; EAM, experimental autoimmune myocarditis; PBS, phosphate-buffered saline; α-MEM, α-minimal essential medium; FBS, fetal bovine serum; TGF-β3, transforming growth factor-β3; FITC, fluorescein isothiocyanate; LVSP, left ventricular systolic pressure; LVDs, left ventricular systolic dimension; LVDd, left ventricular diastolic dimension; H&E, hematoxylin and eosin; MCP1, monocyte chemoattractant protein 1; HRP, horseradish peroxidase.

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adipocytes, osteocytes, chondrocytes, endothelial cells, and myocytes [3–5]. MSC are a promising cell source for regenerative therapies. We have reported that the autologous administration of BM- or adipose tissue-derived MSC improves cardiac function in rat models of dilated cardiomyopathy and myocardial infarction [6–8]. We also recently demonstrated that the administration of autologous BM-derived MSC (BM-MSC) attenuates myocardial injury and dysfunction in rats with acute myocarditis [9].

However, there are limitations to the application of autologous BM in clinical situations. BM procurement procedures in humans may be painful and may yield low numbers of MSC after processing. An alternative source of MSC that could provide large quantities of cells would be advantageous. To address this issue, we focused on fetal membranes (FMs), which are generally discarded as medical waste after delivery, as an alternative source of autologous MSC. Several studies have reported that human FMs contain multipotent cells similar to BM-MSC, which are easy to expand [10,11]. We demonstrated recently that the allogeneic transplantation of FM-derived MSC (FM-MSC) and BM-MSC induces therapeutic angiogenesis in a rat hind-limb ischemia model [12]. MSC have been reported to induce immune tolerance [13,14], and we confirmed that the transplantation of FM-MSC did not elicit any lymphocyte proliferative response despite their allogeneic origin.

In this study, we investigated whether the intravenous allogeneic administration of FM-MSC improves cardiac function and decreases myocardial inflammation in rats with myosin-induced myocarditis, and the mechanisms underlying the changes induced by allogeneic FM-MSC administration.

2. Materials and methods

2.1. Animals

Different strains of rats were used, based on their major histocompatibility complex (MHC) antigen disparities: Lewis rats (MHC haplotype: RT-1A^{l}; Japan SLC, Hamamatsu, Japan), and August–Copenhagen–Irish (ACI) rats (MHC haplotype: RT-1A^{a}; Japan SLC). Green fluorescent protein (GFP)-transgenic Lewis rats (Institute of Laboratory Animals, Kyoto University, Japan) were also used to investigate the distribution of the transplanted FM-MSC. Adult rats, aged 8–12 weeks, were used for the induction of experimental autoimmune myocarditis (EAM) and were maintained in our animal facility. The experimental protocols were approved by the Animal Care Committee of the National Cardiovascular Center Research Institute.

2.2. Preparation of FM-MSC

The isolation and expansion of FM-MSC were performed as described previously [12]. In brief, pregnant ACI rats (15 days postconception) were sacrificed, and their uteri were harvested and placed in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA). We chose 15 days postconception as the day of FM retrieval because that point was the best in terms of cell isolation and reproducibility in rats. After separation from the placenta, the FMs were minced with scissors and digested with type II collagenase solution (300 U/mL; Worthington Biochemicals, Lakewood, NJ, USA) for 1 h at 37 °C in a shaking water bath. Enzyme activity was neutralized with α-minimal essential medium (α-MEM; Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen). After filtration through a mesh filter (100 μm; BD Biosciences, Bedford, MA, USA) and centrifugation at 300×*g* for 5 min, the dissociated FM cells were suspended in α-MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen), plated onto 100-mm uncoated culture dishes, and incubated at 37 °C in 5% CO_2. The population of adherent, spindle-shaped MSC was expanded. Almost all of non-adherent cells were hematocytes in the morphological observation. The isolation of FM-MSC was repeated three times to evaluate its reproducibility. In all experiments, the FM-MSC were used at passages 5–7.

2.3. Differentiation of FM- and BM-MSC into adipocytes, osteocytes, and chondrocytes

The multipotency of FM-MSC was assessed as described previously [12]. FM-MSC were seeded into six-well plates, and the differentiation into adipocytes and osteocytes was induced at 40–50% confluence. To induce differentiation into adipocytes, MSC were cultured with adipocyte differentiation medium: 0.5 mM 3-isobutyl-1-methylxanthine (Wako Pure Chemical Industries, Osaka, Japan), 1 μM dexamethasone (Wako Pure Chemical Industries), 50 μM indomethacin (Wako Pure Chemical Industries), and 10 μg/mL insulin (Sigma-Aldrich, St. Louis, MO) in α-MEM. After two weeks of differentiation, adipocytes were identified by the existence of lipid vesicles stained with oil red O (Sigma-Aldrich).

To induce differentiation into osteocytes, MSC were cultured in α-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma, Osaka, Japan), according to the manufacturer's instructions. After two weeks of differentiation, osteocytes were identified by the existence of mineral nodule deposition stained with alizarin red S (Sigma-Aldrich).

To induce differentiation into chondrocytes in three-dimensional culture, the pellet culture method was used. MSC were centrifuged at 150×*g* for 5 min and resuspended at a density of 1×10^6 cells/mL in an hMSC Differentiation BulletKit-Chondrogenic (Cambrex Bio Science, Walkersville, MD) supplemented with transforming growth factor-β3 (TGF-β3; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, 5×10^5 cells were placed in a 15-mL polypropylene tube and centrifuged at 150×*g* for 5 min. Fresh medium was added every third day. After three weeks of differentiation, cell pellets were fixed with 4% paraformaldehyde and embedded in paraffin. Differentiation into chondrocytes was identified by the existence of proteoglycan deposition stained with Safranin O (Sigma-Aldrich). Differentiation of FM-MSC was repeated three times to evaluate reproducibility.

2.4. Flow cytometry

Cultured FM-MSC were analyzed by flow cytometry (FACSCalibur, BD Biosciences) as described previously [12]. Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against rat CD34 (clone ICO-115, Santa Cruz Biotechnology, Santa Cruz, CA), CD45 (clone OX-1, BD Biosciences), CD73 (clone 5F/B9, BD Biosciences), CD90 (clone OX-7, BD Biosciences), RT1A^{a,b,l} (clone B5, BD Biosciences), and RT1B (clone OX-6, BD Biosciences) were used. Isotype-identical antibodies served as controls. Flow cytometric analysis of FM-MSC was repeated three times to evaluate reproducibility.

2.5. Acute myocarditis model

Purified cardiac myosin was prepared from the ventricular muscles of pig hearts according to a previously described procedure [15]. The antigen was dissolved at a concentration of 20 mg/mL in PBS containing 0.3 M KCl and mixed with an equal volume of complete Freund's adjuvant containing 11 mg/mL *Mycobacterium tuberculosis* (Difco Laboratories, Sparks, MD, USA). The rats were anesthetized with an intraperitoneal injection of 20 mg/kg sodium pentobarbital, and 0.1 mL of the antigen–adjuvant emulsion was injected into each footpad.

Forty-five Lewis rats were assigned randomly into the following three groups and treated with: 1) 0.2 mL of PBS only (Sham group,

$n=15$); 2) 0.2 mL of cardiac myosin only (MyoC group, $n=15$); and 3) 0.2 mL of cardiac myosin and FM-MSC (MyoC + FM-MSC group, $n=15$). One week after the myosin injection, allogeneic FM-MSC (5×10^5 cells/animal) or vehicle (PBS) was administered intravenously via the tail vein. We chose an intravenous route for the administration of FM-MSC because of its clinical applicability. We chose the time for the cell injection of one week after myosin injection on the basis of our previous report showing that this time was the most effective compared with other schedules [9]. In this study, we used the 5×10^5 cell administration because this cell number did not elicit the significant and persistent rise in pulmonary arterial pressure.

To assess the distribution of the injected cells, FM-MSC (5×10^5 cells/animal) derived from GFP-transgenic Lewis rats were administered intravenously via the tail vein seven days after the myosin injection. One day, one week or four weeks after the injection of the cells, the rats were sacrificed, and sections of tissues were obtained from the heart, lung, spleen, and liver, and embedded in paraffin ($n=4$ for each tissue).

2.6. Hemodynamic studies

Hemodynamic studies were performed on day 21 after the myosin injection. Anesthesia was maintained with isoflurane (1.5–2.0 vol.% in air), and a polyethylene catheter (model PE-50, BD Biosciences) was placed into the left ventricle through the right carotid artery. The heart rate was monitored by electrocardiography. Heart rate, mean arterial pressure, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure, maximum dP/dt, and minimum dP/dt were used as the hemodynamic indices and were recorded simultaneously during ventilation after a minimum equilibration period of 20 min. The hemodynamic studies were performed with the investigators blinded to the treatment group and the analyses were performed offline.

2.7. Echocardiographic studies

Echocardiography was performed on day 21 after the myosin injection. The rats were anesthetized with isoflurane (1.5–2.0 vol.% in air). A 12-MHz probe was placed at the left fourth intercostal space for M-mode imaging using two-dimensional echocardiography (Sonos 5500, Philips, Bothell, WA, USA). The left ventricular systolic dimension (LVDs), left ventricular diastolic dimension (LVDd), anterior wall thickness, posterior wall thickness, and ejection fraction were measured and recorded as the average for three beats. Fractional shortening (%) was calculated as $([LVDd-LVDs]/LVDd)\times100$. The echocardiography studies were performed with the investigators blinded to the treatment group and the analyses were performed offline.

2.8. Histopathological studies

The hearts were excised above the origin of the great vessels on day 21 after the myosin injection, and the heart and body weight were recorded. The heart, spleen, pancreas, kidney, and liver were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned to 4-μm thickness, and stained with hematoxylin and eosin (H&E) or Masson's trichrome. H&E-stained sections were evaluated by a cardiovascular pathologist (H.I.-U.) with no knowledge of the experimental groups, who characterized the myocardial injury and inflammation using the following scale: 0, absent or questionable presence; 1, limited focal distribution; 2–3, intermediate severity; and 4, coalescent and extensive foci throughout the entire transversely sectioned ventricular tissue. To evaluate fibrosis, the collagen volume fraction was analyzed with image-processing software (Win ROOF, Mitani Co. Ltd., Tokyo, Japan).

2.9. Immunohistochemical studies

Deparaffinized sections were incubated with Protein Block (DakoCytomation, Glostrup, Denmark) and then with mouse anti-rat CD68 (clone ED-1; Millipore, Bedford, MA, USA), CD3 (BD Biosciences), monocyte chemoattractant protein 1 (MCP1; BD Biosciences), or rabbit anti-GFP antibody (Invitrogen) in diluent for 40 min, followed by incubation with horseradish peroxidase (HRP)-linked rabbit anti-mouse IgG or DakoCytomation Envision+ System-HRP Labeled Polymer (DakoCytomation) for 30 min. The sections were visualized with 0.5% diaminobenzidine (DakoCytomation) and 0.03% hydrogen peroxide, and counterstained with hematoxylin. Five random fields from each rat were photographed (Biorevo BZ-9000; Keyence, Osaka, Japan). The numbers of CD68- and CD3-positive cells and the MCP1-positive areas were analyzed with the image-processing software. The number of GFP-positive cells was counted in 20 randomly selected fields per section.

2.10. T-lymphocyte proliferation assay

T lymphocytes were isolated from the spleens of rats with myocarditis on day 21 using a previously described procedure [12]. The responder T lymphocytes were isolated from untreated control Lewis rats (sham-TL), myosin-treated Lewis rats with myocarditis (MyoC-TL), or allogeneic FM-MSC-administered myosin-treated Lewis rats with myocarditis (MyoC + FM-MSC-TL). The responder T lymphocytes (1×10^5 cells/well) were cultured in a 96-well culture plate with 50 μg/mL of purified porcine heart myosin (Sigma-Aldrich) as the stimulator, with or without the modulator cells. The modulator cells were allogeneic FM-MSC obtained from MHC-mismatched ACI rats (1×10^5 cells/well). When no allogeneic FM-MSC were added, T lymphocytes isolated from normal Lewis rats were added to adjust the cell number (1×10^5 cells/well). The modulator cells were irradiated at 30 Gy before they were cultured. A total of 2×10^5 cells were cocultured in 0.2 mL of tissue culture medium (RPMI 1640; Invitrogen) supplemented with 20% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in 96-well flat-bottomed plates for three days. The proliferation of the responding cells was assessed using the Cell Proliferation Biotrak ELISA System (GE Healthcare, Piscataway, NJ, USA), according to the manufacturer's instructions. T-lymphocyte proliferation is presented as the percentage of the relative proliferation response, as follows: % change in proliferative response = $(\text{absorbance}_{\text{each group}}/\text{absorbance}_{\text{sham-TL without modulator}})\times100$.

2.11. Statistical analysis

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

3. Results

3.1. Distribution of intravenously administered FM-MSC in rats with myocarditis

We obtained FM-MSC from ACI rats (MHC haplotype: RT-1A^a) to examine whether the allogeneic administration of FM-MSC could attenuate myocarditis in Lewis rats (MHC haplotype: RT-1A^l). FM-MSC differentiated into adipocytes, osteocytes, and chondrocytes ($n=3$, each) (Figs. 1(B)–(D)). Flow cytometric analysis of cultured FM-MSC at passage 5 ($n=3$) demonstrated that FM-MSC were positive for CD73, CD90, and MHC class I (i.e., RT1A) but were negative for CD34, CD45, and MHC class II (i.e., RT1B) (Fig. 1(E)).

To investigate the distribution of the intravenously injected FM-MSC in the rats with myocarditis, we intravenously administered

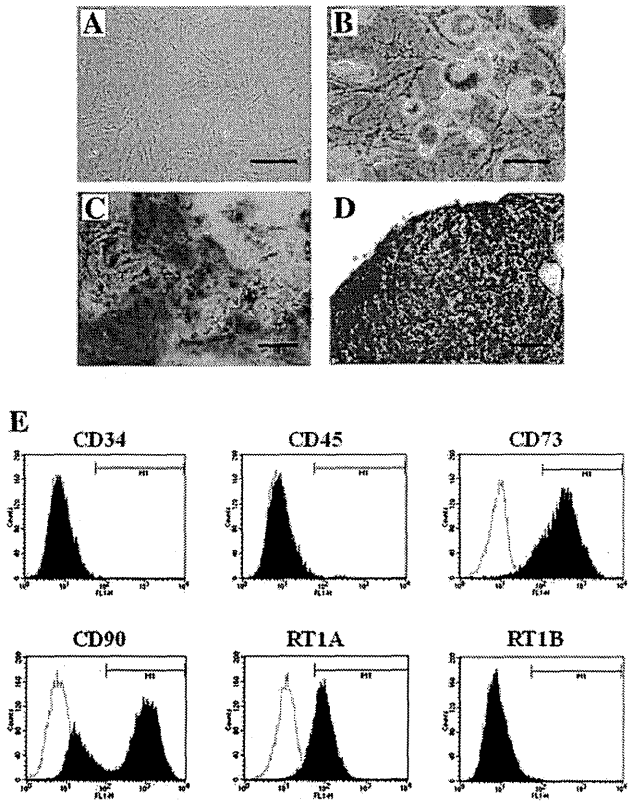

Fig. 1. Characterization of FM-MSC. (A) Morphology of FM-MSC. Scale bar = 100 μm. (B–D) Multipotency of FM-MSC. FM-MSC differentiated into adipocytes (B), osteocytes (C), and chondrocytes (D) ($n=3$ each). Scale bars = 50 μm. (E) Flow cytometric analysis of FM-MSC ($n=3$ each).

GFP-expressing FM-MSC obtained from GFP-transgenic Lewis rats one week after the myosin injection. GFP immunostaining demonstrated that GFP-positive transplanted FM-MSC were present in the heart, lungs, spleen, and liver at both one day and one week after FM-MSC injection (Fig. 2(A)). Semiquantitative analysis demonstrated that a significant number of GFP-positive cells were observed in the lung (day 1, 4.0 ± 0.4 cells/mm^2; and day 7, 2.4 ± 0.4 cells/mm^2), whereas only a few GFP-expressing engrafted cells were observed in the other organs at day 1 (heart, 1.1 ± 0.5 cells/mm^2; spleen, 0.3 ± 0.2 cells/mm^2; and liver, 0.2 ± 0.1 cells/mm^2) and one week (heart, 0.7 ± 0.3 cells/mm^2; spleen, 0.8 ± 0.1 cells/mm^2; and liver, 1.0 ± 0.5 cells/mm^2) after the FM-MSC injection ($n=4$ for each) (Fig. 2(B)). We found no GFP-positive cells four weeks after the FM-MSC injection.

3.2. Improvement in cardiac function by allogeneic administration of FM-MSC

All rats with myocarditis survived the 21-day observation period. On day 21, the heart weight/body weight ratio was significantly lower in the MyoC + FM-MSC group than in the MyoC group (3.7 ± 0.1 vs 4.3 ± 0.1, $P<0.05$). Hemodynamic analysis revealed significant improvements in the MyoC + FM-MSC group compared with the MyoC group in LVSP (119.1 ± 3.4 vs 102.0 ± 4.3 mmHg), mean arterial pressure (84.3 ± 3.2 vs 69.0 ± 3.1 mmHg), and maximum dP/dt (8202 ± 516 vs 6445 ± 373 mmHg/s) ($P<0.05$ for all; $n=15$ in each group) (Figs. 3(A) and (B), and Table 1).

Echocardiographic analysis revealed significant improvements in the MyoC + FM-MSC group compared with the MyoC group in LVDs (3.8 ± 0.2 vs 4.7 ± 0.1 mm), fractional shortening (45.4 ± 1.8 vs $36.8\pm1.2\%$), and ejection fraction (83.2 ± 1.6 vs $74.2\pm1.4\%$) ($P<0.05$ for all). Wall thickness was also significantly thinner in the MyoC + FM-MSC group than in the MyoC group (anterior wall thickness diastole, 2.1 ± 0.2 vs 2.5 ± 0.2 mm; and posterior wall thickness diastole, 2.2 ± 0.1 vs 2.5 ± 0.1 mm) ($P<0.05$ for all; $n=15$ in each group) (Figs. 3(C)–(E) and Table 2).

3.3. Attenuation of myocardial inflammation after allogeneic administration of FM-MSC

Histological analysis on day 21 after the induction of experimental myocarditis showed severe myocardial inflammatory changes. The semiquantitative grading of H&E-stained heart sections by a pathologist (H.I.-U.) using a blinded method showed significantly lower in the MyoC + FM-MSC group than in the MyoC group for tissue granulation (2.7 ± 0.2 vs 3.1 ± 0.1), eosinophil infiltration (1.8 ± 0.1

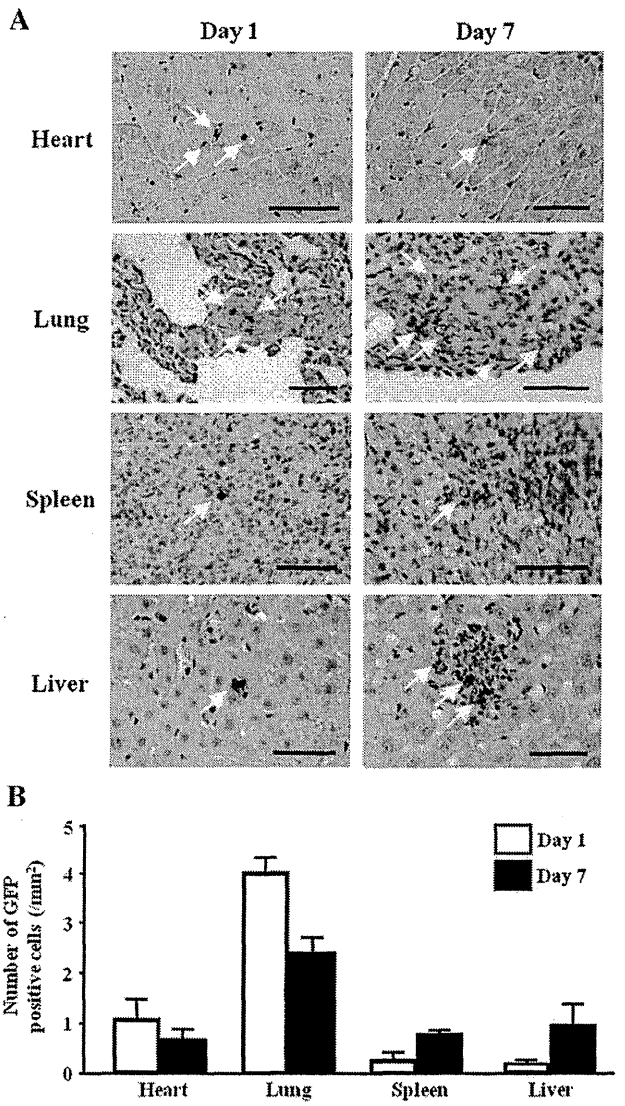

Fig. 2. Distribution of intravenous-administered FM-MSC in acute myocarditis. (A) GFP-positive-administered FM-MSC were present in the heart, lung, spleen, and liver one day and one week after cell administration (brown stain; yellow arrows). Scale bars = 50 μm. (B) Semiquantitative analysis demonstrated that a significant number of GFP-positive cells were observed in the lung, whereas only a few GFP-expressing engrafted cells were observed in the other organs at one day and one week after the FM-MSC injection ($n=4$ for each organ).

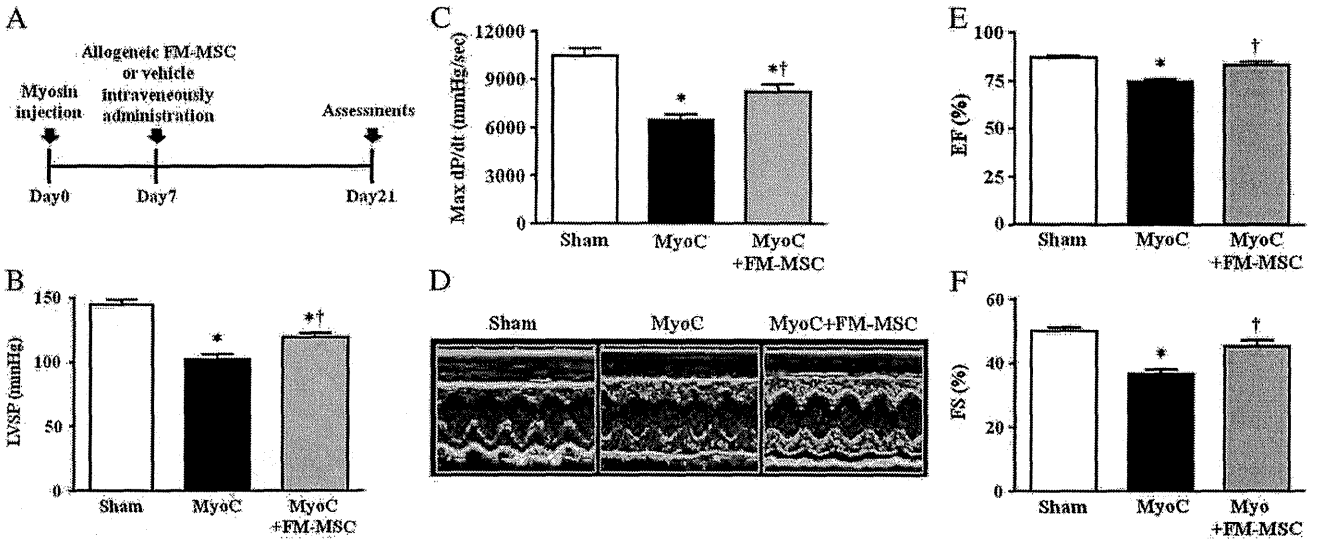

Fig. 3. Effects of administration of allogeneic FM-MSC on hemodynamic and echocardiographic parameters in acute myocarditis. (A) The study flowchart. (B) Left ventricular systolic pressure (LVSP) and (C) maximum dP/dt (max dP/dt) were measured in sham-treated rats given vehicle (Sham group), myosin-treated rats given vehicle (MyoC group), and myosin-treated rats given FM-SC (MyoC + FM-MSC group; $n = 15$ in each group). (D) Representative echocardiographic images showing wall thickening and poor movement in the MyoC group, and improved cardiac contractility in the MyoC + FM-MSC group. (E, F) Allogeneic FM-MSC administration significantly improved the ejection fraction (EF) and fractional shortening (FS; $n = 15$ for each group). Data are expressed as mean ± SEM. *$P<0.05$ vs the Sham group; †$P<0.05$ vs the MyoC group.

vs 2.7 ± 0.1), and edema (2.4 ± 0.1 vs 3.1 ± 0.1) ($P<0.05$ for all; $n = 15$ in each group) (Figs. 4(A) and (B)).

Masson's trichrome staining of the myocardium derived from the MyoC group on day 21 demonstrated prominent and diffuse interstitial fibrosis, which was attenuated dramatically in the MyoC + FM-MSC group (Fig. 4(C)). Quantitative assessment of myocardial fibrosis showed that the Masson's trichrome-stained collagen volume fraction was significantly smaller in the MyoC + FM-MSC group than in the MyoC group (3.1 ± 1.4 vs 7.7 ± 1.9%) ($P<0.05$; $n = 15$ in each group) (Fig. 4(D)).

Immunohistochemical analysis of the myocardial tissue on day 21 showed significantly attenuated infiltration of CD68-positive monocytes/macrophages in the MyoC + FM-MSC group compared with the MyoC group (3713 ± 426 vs 6528 ± 590 cells/mm^2) ($P<0.05$; $n = 15$ in each group) (Figs. 5(A) and (B)).

Immunohistochemical staining of the myocardial tissue for MCP1 on day 21 showed a few positive cells in the myocardial interstitium of the normal heart (Fig. 5(C)). In the rats with myocarditis, increased MCP1 expression was observed in the myocardial interstitium and in the vascular wall of the heart tissue. The hearts of the MyoC + FM-MSC group showed a partial reduction in MCP1 expression. Quantitative analysis demonstrated less MCP1 expression in the MyoC + FM-MSC group than in the MyoC group (0.85 ± 0.1 vs 1.46 ± 0.2%) ($P<0.05$; $n = 15$ in each group) (Fig. 5(D)).

Table 1
Physiological parameters in the three experimental groups.

	Sham	MyoC	MyoC + FM-MSC
HW/BW (g/kg)	2.5 ± 0	4.3 ± 0.1*	3.7 ± 0.1*,**
HR (bpm)	371.9 ± 7.0	337.9 ± 7.6*	364.3 ± 8.5**
MAP (mmHg)	100.1 ± 2.7	69.0 ± 3.1*	84.3 ± 3.2*,**
LVEDP (mmHg)	4.5 ± 1.0	5.7 ± 0.8	6.5 ± 1.0
Min dP/dt (mmHg/s)	−8258.9 ± 422.2	−4980.5 ± 278.6*	−6135.6 ± 375.9*

Sham, sham-operated rats given vehicle; MyoC, myosin-treated rats given vehicle; MyoC + FM-MSC, myosin-treated rats given FM-MSC (5×10^5 cells/animal); FM-MSC, fetal membrane-derived mesenchymal stem cells; HW/BW, heart weight-to-body weight ratio; HR, heart rate; MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; Min dP/dt, minimum dP/dt.

* $P<0.05$ vs the Sham group.

** $P<0.05$ vs the MyoC group.

3.4. Attenuation of T-cell infiltration by allogeneic administration of FM-MSC

Marked T-cell infiltration was demonstrated by CD3 immunostaining of the cardiac tissues of the MyoC group on day 21. By contrast, T-cell infiltration was attenuated significantly in the MyoC + MSC group (2014 ± 196 vs 3068 ± 455 cells/mm^2) ($P<0.05$ vs the MyoC group; $n = 15$ in each group) (Figs. 5(E) and (F)).

3.5. Suppression of T-lymphocyte activation by allogeneic treatment with FM-MSC demonstrated in a T-lymphocyte proliferation assay

To examine whether allogeneic FM-MSC suppress T-lymphocyte activation, we performed a T-lymphocyte proliferation assay [12]. T lymphocytes collected on day 21 from the MyoC group with myocarditis were cocultured with porcine heart myosin and with irradiated T lymphocytes derived from normal Lewis rats. The proliferative response was significantly higher in the MyoC group with myocarditis than in the sham group T lymphocytes (183.2 ± 2.5 vs 100.0 ± 2.6%, $P<0.05$; $n = 8$ in each group). However, on day 21, the proliferative response was significantly lower in the T lymphocytes derived from the MyoC + FM-MSC group with myocarditis (164.2 ± 0%) than in the MyoC group ($P<0.05$, $n = 8$). T-cell activation was significantly reduced when T lymphocytes from the MyoC group or MyoC + FM-MSC group were cocultured with allogeneic ACI-derived

Table 2
Echocardiographic findings in the three experimental groups.

	Sham	MyoC	MyoC + FM-MSC
LVDs (mm)	3.6 ± 0.1	4.5 ± 0.1*	3.8 ± 0.2**
LVDd (mm)	7.1 ± 0.1	7.2 ± 0.1	7.0 ± 0.2
AWT diastole (mm)	1.4 ± 0	2.5 ± 0.2*	2.1 ± 0.2*,**
PWT diastole (mm)	1.7 ± 0	2.5 ± 0.1*	2.2 ± 0.1*,**

Sham, sham-operated rats given vehicle; MyoC, myosin-treated rats given vehicle; MyoC + FM-MSC, myosin-treated rats given FM-MSC (5×10^5 cells/animal); FM-MSC, fetal membrane-derived mesenchymal stem cells; LVDs, left ventricular systolic dimension; LVDd, left ventricular diastolic dimension; AWT, anterior wall thickness; PWT, posterior wall thickness.

* $P<0.05$ vs the Sham group.

** $P<0.05$ vs the MyoC group.

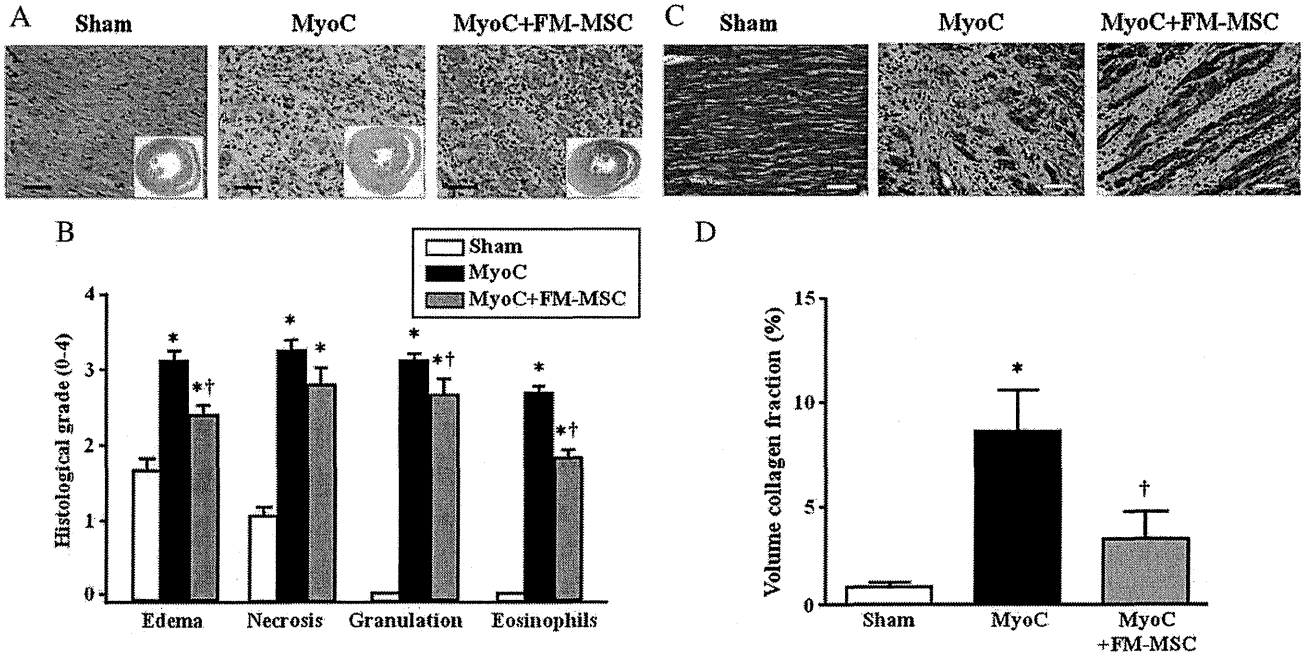

Fig. 4. Histopathological changes of acute myocarditis induced by administration of allogeneic FM-MSC. (A) Myocardial sections show markedly less inflammation in the rats given allogeneic FM-MSC than in the MyoC group. Insets are transverse sections of the myocardium. (B) The semiquantitative histological grading of edema and eosinophil infiltration were markedly lower in the MyoC + FM-MSC group than in the MyoC group ($n = 15$ in each group). (C) Myocardial fibrosis was markedly lower in the allogeneic FM-MSC group than in the MyoC group. (D) The semiquantitative area of fibrosis was smaller in the MyoC + FM-MSC group than in the MyoC group ($n =$ for 15 each group). Scale bars = 50 μm. Data are expressed as mean ± SEM. *$P<0.05$ vs the Sham group; †$P<0.05$ vs the MyoC group.

FM-MSC instead of autologous normal Lewis T cells; the values were 145.1 ± 4.6% for MyoC-TL with allogeneic FM-MSC vs 183.2 ± 2.5% for MyoC-TL without allogeneic FM-MSC and 136.2 ± 3.6% for MyoC + MSC-TL with allogeneic FM-MSC vs 164.2 ± 3.7% for MyoC + MSC-TL without allogeneic FM-MSC ($P<0.05$, $n = 8$ in each group) (Fig. 6). However, in the sham-TL group, proliferation did not differ in the presence or absence of allogeneic FM-MSC. These results show that allogeneic FM-MSC had both an acute suppressive effect on T-lymphocyte proliferation in vitro and a chronic suppressive effect on T-lymphocyte proliferation in the pathological studies.

4. Discussion

In this study, we investigated the therapeutic potential of allogeneic FM-MSC in the acute phase of myocarditis. In our rat model of acute myocarditis, intravenous allogeneic administration of FM-MSC one week after the myosin injection significantly improved cardiac function and the pathological findings in the heart three weeks after the myosin injection. The pathological findings included the attenuated expression of the proinflammatory factor MCP1 and a reduction in the infiltration of T cells and macrophages into the hearts treated with FM-MSC. The T-lymphocyte proliferation assay demonstrated that splenic lymphocytes from rats with myocarditis treated with FM-MSC reacted to myosin less strongly than did the splenic lymphocytes from untreated rats with myocarditis.

Experimental autoimmune myocarditis (EAM) is induced by immunization with cardiac myosin in Lewis rats and is characterized by severe myocardial dysfunction and the appearance of multinucleated giant cells. EAM has been used as an animal model of human giant cell myocarditis [15,16]. This myocarditis model is triphasic and comprises an antigen-priming phase on days 0–13, an autoimmune response phase on days 14–21, and a reparative phase thereafter, which is associated with a chronically dilated cardiomyopathy phenotype. Although the pathogenesis of EAM has not been clarified fully, severe inflammatory cell infiltration is a characteristic of the disease [15,16].

In a recent study, we tried to determine whether MSC improve the function and pathological findings in the affected heart in EAM. The intravenous administration of autologous BM-MSC one week after myosin injection significantly attenuated the myocardial dysfunction in rats with acute myocarditis [9]. Moreover, the intramyocardial transplantation of autologous BM-MSC five weeks after the myosin injection, corresponding to the reparative phase, improved cardiac function in a model of chronic dilated cardiomyopathy [7]. These findings suggest that autologous BM-MSC are an attractive source of cells for transplantation. However, there are several limitations in using an autologous cell source, including their invasiveness, inadequate cell numbers, and donor-site morbidity [17]. In addition, a waiting period is required for transplantation of autologous BM-MSC to prepare an adequate number of cells. Because acute myocarditis is often associated with rapidly progressive heart failure, time-consuming transplantation of autologous BM-MSC is not suitable. Human FMs, which are generally discarded as medical waste after delivery, have been shown recently to be rich sources of MSC [18,19]. Previous reports have suggested that FM-MSC might have regenerative potential for cell therapy. Using a rat hind-limb ischemia model, we demonstrated that allogeneic FM-MSC and autologous BM-MSC are suitable cell sources for tissue regeneration [12]. We infer that, as an alternative source to autologous BM-MSC, allogeneic FM-MSC might be a promising candidate for the cell therapy-based treatment of acute myocarditis.

In this study, intravenous allogeneic administration of FM-MSC significantly improved cardiac function and the pathological findings in the hearts exhibiting EAM. The extent of the improvement was in the range of 30–60% in the indices of the dysfunction level, which are equivalent to those observed in our previous study of administration of autologous BM-MSC [9]. Our previous report focused on the angiogenesis effects and the paracrine action of MSC in EAM, and we focused on the immunomodulatory properties of MSC in this study.

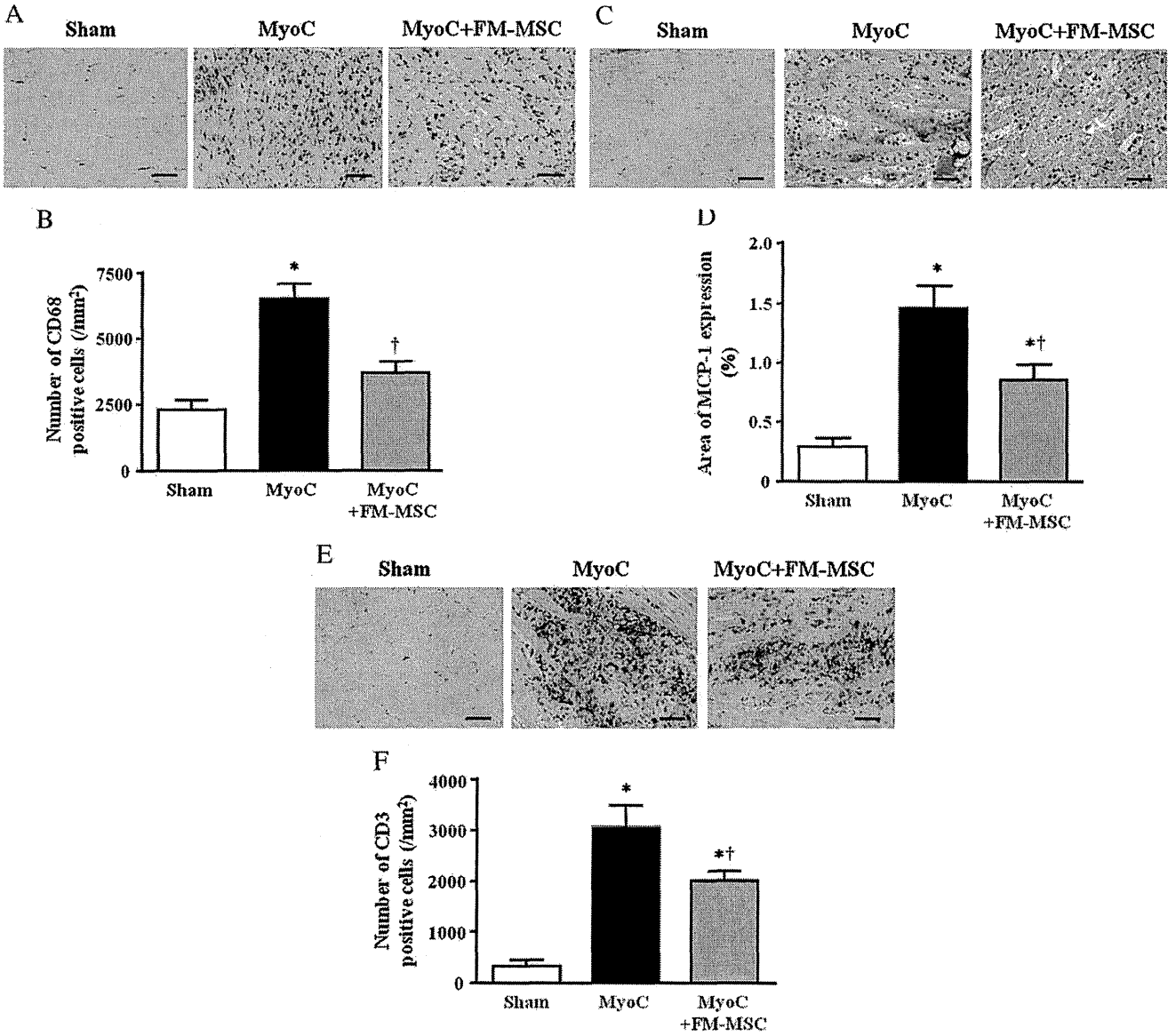

Fig. 5. Effects of administration of allogeneic FM-MSC on myocardial infiltration of inflammatory cells and MCP1 expression in acute myocarditis. (A, B) CD68-positive macrophage/monocyte infiltration was markedly lower in the MyoC + FM-MSC group than in the MyoC group. (C, D) The area of MCP1 expression was significantly smaller in the MyoC + FM-MSC group than in the MyoC group. (E, F) Significantly fewer infiltrating CD3-positive T cells were observed in the allogeneic FM-MSC group than in the MyoC group. Scale bars = 50 μm, $n = 15$ for each group. Data are expressed as mean ± SEM. $^{*}P<0.05$ vs the Sham group; $^{\dagger}P<0.05$ vs the MyoC group.

Because, the migration of activated T cells into the myocardium is considered the initial process of EAM [20]. Subsequently, large numbers of inflammatory cells, including macrophages and T cells, infiltrate the myocardium where they induce severe inflammation.

In this study, GFP-positive cells were detected in the heart tissue one day and one week after the intravenous administration of GFP-expressing FM-MSC, although only a few engrafted cells were found, even one day after injection, which is consistent with a previous work [6]. The low percentage of cells migrating to the heart is in agreement with other reports [21–23]. In several clinical applications, MSC are administered preferentially by an intravenous route [24,25]. However, limited data are available regarding the fate of systemically infused MSC. Studies in rodents suggest that a broad distribution of transplanted MSC is observed initially, followed by a limited capacity for sustained engraftment [22,26]. In addition, the number of von Willebrand factor-positive capillaries in the heart did not increase in the EAM rats given FM-MSC compared with the untreated EAM group two weeks after cell administration (data not shown), and we found no GFP-positive cells and cardiomyocyte-differentiated cells four weeks after cell administration. A recent study reported that engraftment of MSC is very low and that transplanted MSC appear to differentiate into cardiomyocytes at a very low frequency [27]. These results suggest that the angiogenic effect and the cardiomyocyte differentiation of FM-MSC in the heart are not the main effects in EAM therapy.

We have demonstrated that some of the administered GFP-positive cells were found in the lung, spleen, and liver. It is interesting that the number of GFP-positive cells in the spleen, the hub of immunoreactions of T cells, was greater at one week after administration than at one day after administration. One may speculate that the systemically engrafted allogeneic FM-MSC can exert a therapeutic effect in the treatment of myocarditis.

Recent studies have highlighted the potential immunomodulatory or anti-inflammatory effects of MSC [28,29]. MSC can suppress T-cell

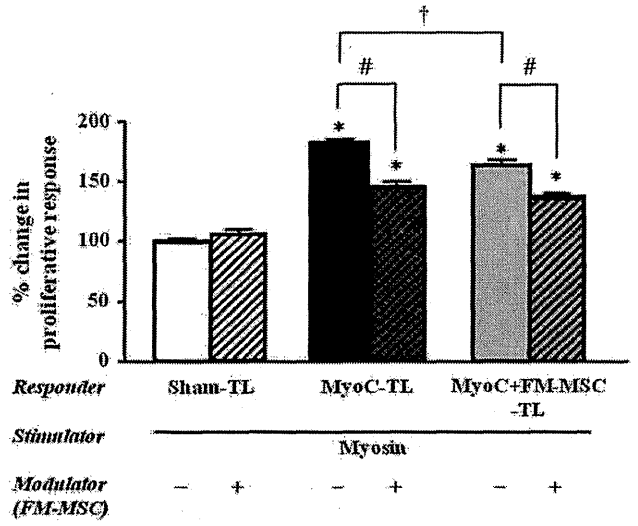

Fig. 6. Suppressive effects of T-lymphocyte activation by allogeneic FM-MSC. The T-lymphocyte proliferative response was significantly lower in the MyoC + FM-MSC group than in the MyoC group. The T-lymphocyte-activated proliferation was markedly attenuated by allogeneic FM-MSC ($n=8$ each). Data are expressed as mean ± SEM. *$P<0.05$ vs Sham-TL; †$P<0.05$; #$P<0.05$.

activation and proliferation both in vitro and in vivo [30,31]. Recent clinical studies found that the intravenous injection of BM-MSC ameliorates acute graft-versus-host disease [13,14,32]. Interestingly, the suppression of T-cell proliferation by MSC causes no immunological restriction, insofar as similar suppressive effects were observed with cells that were either autologous or allogeneic to the responder cells [33,34]. Several independent reports have suggested multiple mechanisms by which MSC inhibit T-cell responses. Prostaglandin E2, nitric oxide, indoleamine 2,3-dioxygenase, and galectin are among the molecules postulated to be involved in the inhibition of T-cell proliferation by MSC [35–39]. Our present study demonstrated that allogeneic administration of FM-MSC significantly attenuates the infiltration of macrophages and T cells into EAM hearts. In the T-lymphocyte proliferation assay, splenic T lymphocytes derived from rats with myocarditis given allogeneic FM-MSC had a reduced activated proliferative response compared with the response of splenic T lymphocytes from untreated myocarditis rats, even two weeks after the injection of FM-MSC. In addition, activated T-lymphocyte proliferation was also suppressed by coculture with allogeneic FM-MSC in vitro. These results suggest that allogeneic FM-MSC reduce the severity of EAM by inhibiting T-cell activation and proliferation through both a direct effect and a systematic effect, and that these combined effects lead to the amelioration of impaired cardiac function.

However, GFP-positive cells could not be detected in the spleen, lung, and liver as with the heart at four weeks after administration. In this study, we administered a low number of FM-MSC to avoid pulmonary embolism. More effective results might be obtained with administration of more cells or multiple administrations of FM-MSC in EAM. One study reported that transplantation of allogeneic BM-MSC into a rat model of myocardial infarction improved cardiac functions at four weeks after transplantation, although this benefit was transient [40]. Currently, we are extending our observation up to three months in EAM. Although the results are an inadequate number to be presented, improvement of cardiac function appears to be maintained in the intravenous allogeneic FM-MSC administration group compared with the untreated EAM group.

In conclusion, this study showed that the intravenous allogeneic administration of FM-MSC ameliorated cardiac dysfunction in a rat model of acute myocarditis. These beneficial effects may be mainly attributable to the suppression of T-lymphocyte activation rather than to angiogenesis and cardiomyocyte differentiation of the administered allogeneic FM-MSC. Although further experiments are needed to apply the current results to human cardiomyoplasty, the allogeneic administration of FM-MSC may provide a new therapeutic strategy for the treatment of severe acute myocarditis.

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ORIGINAL ARTICLE
Regenerative Medicine

Gene and Protein Expression Analysis of Mesenchymal Stem Cells Derived From Rat Adipose Tissue and Bone Marrow

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Background: Mesenchymal stem cells (MSC) are multipotent and reside in bone marrow (BM), adipose tissue and many other tissues. However, the molecular foundations underlying the differences in proliferation, differentiation potential and paracrine effects between adipose tissue-derived MSC (ASC) and BM-derived MSC (BM-MSC) are not well-known. Therefore, we investigated differences in the gene and secretory protein expressions of the 2 types of MSC.

Methods and Results: ASC and BM-MSC were obtained from subcutaneous adipose tissue and BM of adult Lewis rats. ASC proliferated as rapidly as BM-MSC, and had expanded 200-fold in approximately 2 weeks. On microarray analysis of 31,099 genes, 571 (1.8%) were more highly (>3-fold) expressed in ASC, and a number of these genes were associated with mitosis and immune response. On the other hand, 571 genes (1.8%) were more highly expressed in BM-MSC, and some of these genes were associated with organ development and morphogenesis. In secretory protein analysis, ASC secreted significantly larger amounts of growth factor and inflammatory cytokines, such as vascular endothelial growth factor, hepatocyte growth factor and interleukin 6, whereas BM-MSC secreted significantly larger amounts of stromal-derived factor-1α.

Conclusions: There are significant differences between ASC and BM-MSC in the cytokine secretome, which may provide clues to the molecule mechanisms associated with tissue regeneration and alternative cell sources.
(*Circ J* 2011; **75:** 2260–2268)

Key Words: Cell therapy; Mesenchymal stem cells; Microarray; Secretory protein

Mesenchymal stem cells (MSC) are multipotent cells that reside within various tissues, including bone marrow (BM), adipose tissue and many other tissues,[1,2] and can differentiate into a variety of cell types of mesodermal lineage.[1,3] MSC can be expanded in vitro over the short term, and they are thought to be an attractive tool for cell therapy. It has been demonstrated in animal and human studies of cardiovascular disease that transplanted BM-MSC induce neovascularization and differentiate into functional cells.[4–8] In addition, recent studies suggest that MSC exert tissue regeneration, secreting various kinds of angiogenic and cytoprotective factors.[6,9,10]

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Subcutaneous adipose tissue can be harvested more safely and noninvasively than BM, and ASC have emerged as a possible alternative cell source to BM-MSC.[9,11] We and others have demonstrated that ASC transplantation induces neovascularization in animal models of myocardial infarction and hindlimb ischemia.[12,13] ASC are similar to BM-MSC in terms of morphology and surface marker expression.[14] However, few data exist regarding their differences in biological activity, such as proliferative activity, differentiation potential and productive ability. Using microarray and enzyme-linked immunosorbent

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assay (ELISA), we have performed a comprehensive analysis to evaluate both the differences between ASC and BM-MSC, and their usage as an effective transplanted cell source from the point of view of the gene and protein expression profile of the 2 MSC sources.

Methods

Isolation and Culture of ASC and BM-MSC

All protocols were performed in accordance with the guidelines of the Animal Care Committee of the National Cardiovascular Center Research Institute and Kanazawa University. MSC isolation and culture were performed according to previously described methods.[15] In brief, we harvested BM from male Lewis rats (Japan SLC, Hamamatsu, Japan) weighing 200–250 g by flushing their femoral cavities with phosphate-buffered saline. Subcutaneous adipose tissue was harvested from the inguinal region and minced with scissors, then digested with 0.1% type I collagenase (300 U/ml; Worthington Biochemical, Lakewood, NJ, USA) for 1 h at 37°C in a water-bath shaker. After filtration with 100-μm filter mesh (Cell Strainer; Becton Dickinson, Bedford, MA, USA) and centrifugation at 1,240 g for 5 min, MSC were cultured in complete culture medium: α-minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). A small number of cells developed visible symmetric colonies by days 5–7. Nonadherent hematopoietic cells were removed, and the medium was replaced. The adherent, spindle-shaped MSC population expanded to >5×10^7 cells within 3–5 passages after the cells were first plated.

Cell Proliferation

We compared the proliferative activity of ASC and BM-MSC in cell culture, as reported previously.[16] In brief, cells (3×10^5 cells/dish) at passage 1 were cultured in a 10-cm dish with complete culture medium, and harvested at 70–90% confluency at each passage. Cell number was counted with a hemocytometer (n=5).

Differentiation of ASC and BM-MSC Into Adipocytes and Osteoblasts

MSC (1×10^5 cells/well) were seeded onto 12-well plates, and differentiation into adipocytes and osteocytes was induced when MSC were 70–80% confluent. MSC were cultured in α-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma, Osaka, Japan) according to the manufacturer's instructions. After 14–17 days of differentiation, cells were fixed and stained with Alizarin Red S (Sigma-Aldrich, St Louis, MO, USA). To induce differentiation into adipocytes, MSC were cultured with adipocyte differentiation medium: 0.5 mmol/L 3-isobutyl-1-methylxanthine (Wako Pure Chemical Industries, Osaka, Japan), 1 μmol/L dexamethasone (Wako Pure Chemical Industries), 50 μmol/L indomethacin (Wako Pure Chemical Industries), and 10 μg/ml insulin (Sigma-Aldrich) in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 10% FBS. After 21 days of differentiation, adipocytes were stained with Oil Red O (Sigma-Aldrich). In order to measure lipid accumulation, isopropyl alcohol was added to the stained culture plate, the extracted dye was immediately collected, and the absorbance was measured spectrophotometrically at 490 nm (Bio-Rad, Hercules, CA, USA).

Microarray Analysis of ASC and BM-MSC

To compare the gene expression of ASC and BM-MSC, microarray analysis was performed according to previously reported methods.[17] Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified by spectrometry, and its quality was confirmed by gel electrophoresis. Double-stranded cDNA was synthesized from 10 μg of total RNA, and in-vitro transcription was performed to produce biotin-labeled cRNA using GeneChip One-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. After fragmentation, 10 μg of cRNA was hybridized with a GeneChip Rat Genome 230 2.0 Array (Affymetrix) containing 31,099 genes. The GeneChips were then scanned in a GeneChip Scanner 3000 (Affymetrix). Normalization, filtering and Gene Ontology analysis of the data were performed with GeneSpring GX 7.3.1 software (Agilent Technologies, Palo Alto, CA, USA). The raw data from each array were normalized as follows: each CEL file was preprocessed with RMA, and each measurement for each gene was divided by the 80th percentile of all measurements. Genes showing at least a 3-fold change were then selected.

Quantitative Real-Time Reverse-Transcription–Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cultured BM-MSC and ASC as described, and 5 μg of total RNA was reverse-transcribed into cDNA using a QuantiTect reverse-transcription kit (Qiagen) according to the manufacturer's instructions. PCR amplification was performed in 50 μl containing 1 μl of cDNA and 25 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, amplified from the same samples, served as an internal control. After an initial denaturation at 95°C for 10 min, a 2-step cycle procedure was used (denaturation at 95°C for 15s, annealing and extension at 60°C for 1 min) for 40 cycles in a 7700 sequence detector (Applied Biosystems). Gene expression levels were normalized according to that of GAPDH.

ELISA

To investigate differences in protein secretion between ASC and BM-MSC, we measured the levels of various bioactive proteins, including proliferative and anti-apoptotic factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and adrenomedullin (AM); chemokines such as stem cell-derived factor-1α (SDF-1α); inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6); and adipokines such as leptin and plasminogen activator inhibitor-1 (PAI-1). Protein levels were measured in conditioned medium 24 h after medium replacement. MSC (1×10^6 cells/dish) were plated in 10-cm dishes and cultured in complete culture medium. After 24 h, conditioned medium (n=6) was collected and centrifuged at 2,000 g for 10 min, and the supernatant was filtered through a 0.22-μm filtration unit (Millipore, Bedford, MA, USA). Angiogenic and growth factors were measured by ELISA according to each of the manufacturer's instructions (VEGF, TNF-α: R&D Systems, Minneapolis, MN, USA; HGF: Institute of Immunology, Tokyo, Japan; AM: Phoenix Pharmaceuticals, Burlingame, CA, USA; IL-6: Pierce, Rockford, IL, USA; adiponectin: AdipoGen, Seoul, Korea; PAI-1, Oxford Biomedica Reseach, Oxford, CT, USA).

Statistical Analysis

Data are expressed as mean±standard error of the mean. Comparisons of parameters among groups were made by 1-way ANOVA, followed by Newman-Keuls' test. Differences were

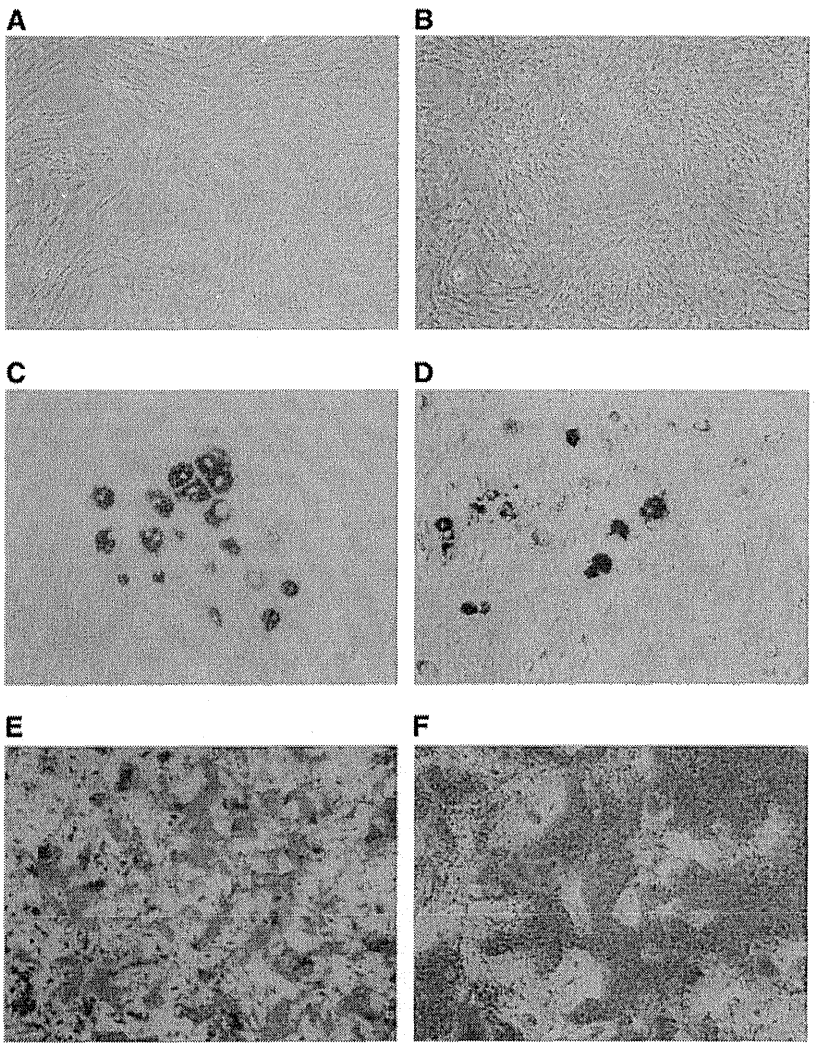

Figure 1. Morphology and differentiation of adipose tissue-derived mesenchymal stem cells (ASC) and bone marrow-derived mesenchymal stem cells (BM-MSC). Cultured ASC (**A**) and BM-MSC (**B**) at passage 2 (×100). Their typical morphology is a similar spindle shape, but ASC are slightly longer than BM-MSC. ASC (**C**) and BM-MSC (**D**) differentiated into adipocytes (×100; Oil Red O). ASC (**E**) and BM-MSC (**F**) differentiated into osteoblasts (×100; Alizarin Red S).

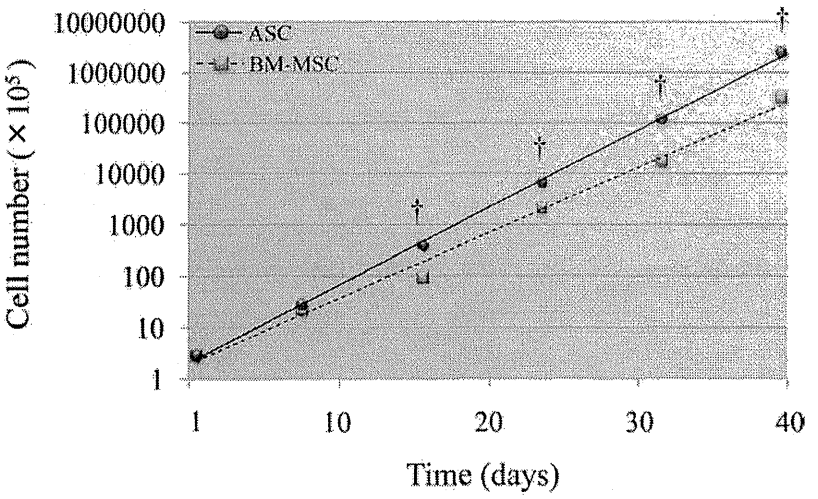

Figure 2. Proliferation of adipose tissue-derived mesenchymal stem cells (ASC) and bone marrow-derived mesenchymal stem cells (BM-MSC) shown as a growth chart. Vertical axis, cell number; horizontal axis, day after first passage. Day 1 is the first day at passage 1. Values are mean±SEM. *P<0.05, †P<0.01 vs. BM-MSC.

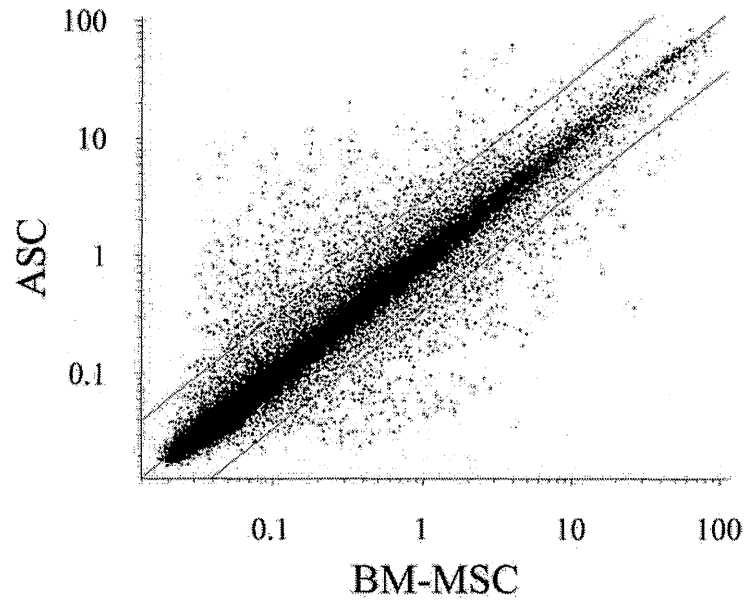

Figure 3. Gene expression profiles of adipose tissue-derived mesenchymal stem cells (ASC, vertical axis) and bone marrow-derived mesenchymal stem cells (BM-MSC, horizontal axis) by microarray. The scatter plot shows normalized microarray datasets of ASC and BM-MSC. All 31,099 gene probes are represented in these plots. The outer lines indicate a 3-fold difference; the central line represents equality.

Table 1. Genes Upregulated in ASC in Comparison With BM-MSC (>10-Fold Upregulation)

Gene name	GenBank Acc. no.	Fold change
Interleukin 1α (Il1a)	NM017019	38.1
Interleukin 1 receptor, type II (Il1r2)	NM053953	21.7
Chemokine (C-X-C motif) ligand 1 (Cxcl1)	NM030845	21.6
Lipocalin 2 (Lcn2)	NM130741	21.5
Fast myosin alkali light chain (Rgd:620885)	NM020104	20.6
Interleukin 6 (Il6)	NM012589	20.5
Chemokine (C-C motif) ligand 20 (Ccl20)	AF053312	17.6
Twist homolog 2 (Twist2)	NM021691	17.5
RAS, dexamethasone-induced 1 (Rasd1)	AF239157	17.1
Complement component 3 (C3)	NM016994	16.9
NADPH oxidase 1 (Nox1)	NM053683	16.3
Matrix metallopeptidase 9 (Mmp9)	NM031055	15.2
Colony-stimulating factor 3 (Csf3)	NM017104	14.5
Prostaglandin E synthase (Ptges)	AB048730	12.8
Adenosine A2B receptor (Adora2b)	NM017161	12.5
Oxidized low-density lipoprotein receptor 1 (Oldlr1)	NM133306	12.4
Uterine sensitization-associated gene 1 protein (Sostdc1)	AA892798	12.1
Chemokine (C-X-C motif) ligand 5 (Cxcl5)	NM022214	11.9
Neuregulin 1 (Nrg1)	U02315	11.8
CD24 antigen (Cd24)	BI285141	11.6
Cathepsin c (Ctsc)	AA858815	11.2
Lymphocyte antigen 68 (C1qr1)	BI282932	11.2
Interleukin 1 receptor antagonist (Il1rn)	NM022194	11.1
Chemokine (C-C motif) ligand 2 (Ccl2)	NM031530	10.8

ASC, adipose tissue-derived mesenchymal stem cells; BM-MSC, bone marrow-derived mesenchymal stem cells.

considered significant at $P<0.05$.

Results

Proliferation and Differentiation of ASC and BM-MSC

Both ASC and BM-MSC could be expanded on a plastic dish, and they exhibited a similar fibroblast-like morphology (**Figures 1A,B**). To examine the potential of ASC and BM-MSC to differentiate into adipocytes, the cells were cultured in adipogenesis medium for 21 days (**Figures 1C,D**). Although lipid droplets were not observed in undifferentiated ASC or BM-MSC, ASC and BM-MSC cultured in adipogenesis

Table 2. Genes Upregulated in BM-MSC in Comparison With ASC (>10-Fold Upregulation)

Gene name	GenBank Acc. no.	Fold change
WNT1 inducible signaling pathway protein 2 (Wisp2)	NM031590	202.5
Complement component factor H (Cfh)	NM130409	81.9
Osteomodulin (Omd)	NM031817	67.4
Solute carrier organic anion transporter family, member 2a1 (Slco2a1)	AI407489	65.8
Dynein, cytoplasmic, intermediate chain 1 (Dncic1)	NM019234	64.8
3-α-hydroxysteroid dehydrogenase (RGD:708361)	BF545626	37.7
Preproenkephalin, related sequence (Penk-rs)	NM017139	29.3
Fc receptor, IgG, low affinity lib (Fcgr2b)	X73371	29.3
Actin, γ 2 (Actg2)	NM012893	25.9
α–2-macroglobulin (A2m)	NM012488	23.2
Lysozyme (Lyz)	L12458	22.2
Jagged 1 (Jag1)	NM019147	19.3
Phospholamban (Pln)	BI290034	17.6
Procollagen, type XI, α 1 (Col11a1)	BM388456	16.2
Gamma sarcoglycan (RGD:1359577)	AA850867	15.3
Pleiomorphic adenoma gene-like 1 (Plagl1)	NM012760	15.0
Matrix metallopeptidase 12 (Mmp12)	NM053963	14.7
Cyclin D2 (Ccnd2)	L09752	14.4
Transforming growth factor, β 2 (Tgfb2)	NM031131	14.3
Solute carrier family 29, member 1 (Slc29a1)	NM031684	14.1
Tissue inhibitor of metalloproteinase 3 (Timp3)	AA893169	13.2
Procollagen, type XI, α 1 (Col11a1)	BM389291	13.1
Down syndrome critical region gene 1-like 1 (Dscr1l1)	AI138048	12.8
Bone morphogenetic protein 4 (Bmp4)	NM012827	12.7
Matrix metallopeptidase 13 (Mmp13)	M60616	11.8
Macrophage galactose N-acetyl-galactosamine specific lectin 1 (Mgl1)	NM022393	11.2
Glycoprotein nmb (Gpnmb)	NM133298	10.7
Aquaporin 1 (Aqp1)	AA891661	10.6
Cadherin 13 (Cdh13)	NM138889	10.5
Selenoprotein P, plasma, 1 (Sepp1)	AA799627	10.5
Secreted frizzled-related protein 4 (Sfrp4)	AF140346	10.4
Cellular retinoic acid binding protein 2 (Crabp2)	U23407	10.2

ASC, adipose tissue-derived mesenchymal stem cells; BM-MSC, bone marrow-derived mesenchymal stem cells.

medium stained positively with Oil Red O in 3 weeks. To quantify lipid accumulation, the absorbance of the extracted cells was measured; however, there was no difference in the absorbance between differentiated ASC and BM-MSC. In addition, both ASC and BM-MSC differentiated identically into osteocytes (**Figures 1E,F**). ASC proliferated more rapidly than BM-MSC; the number of ASC was approximately 10-fold higher than that of BM-MSC at the 40th day (**Figure 2**). In approximately 2 weeks, ASC had expanded almost 200-fold, whereas BM-MSC had expanded nearly 30-fold.

Differences in the Gene Expression of ASC and BM-MSC

Of 31,099 genes analyzed, 571 (1.8%) were more highly (>3-fold) expressed in ASC, whereas 571 genes (1.8%) were more highly (>3-fold) expressed in BM-MSC (**Figure 3**). The genes showing the most enriched expression (>10-fold) in ASC and BM-MSC are listed in **Table 1**. Of note, the genes that were highly expressed in ASC included various types of molecules involved in inflammation, such as IL-1α and IL-6, and chemotaxis, such as chemokine (C-C motif) ligand 20 and chemokine (C-X-C motif) ligand 5 (**Table 1**). The genes that were highly expressed in BM-MSC included differentiation-associated genes, such as WNT1-inducible signaling pathway protein 2 (Wisp2), osteomodulin and jagged1 (**Table 2**). Furthermore, the differential expression patterns of 5 representative genes in ASC and BM-MSC obtained by microarray were confirmed by qRT-PCR, which gave the relative expression of IL-1α as 438.2±560.9 (ratio ASC/BM-MSC, n=5), IL-6 as 54.0±26.6, MMP9 as 3.9±2.2, VEGF 1.8±0.4, and Wisp2 as 7.0±2.2.

To evaluate the genes upregulated in ASC, 571 genes that were more highly expressed in ASC were classified by functional annotation using gene ontology terms (**Table 3**). The 31 terms listed had a P-value <0.00001, and included mitosis (eg, pituitary tumor-transforming 1, cyclin B1, cyclin-dependent kinase 2), immune response (eg, chemokine (C-C motif) ligand 20, cathepsin C and IL-1α) and response to stress (glutathione peroxidase 2, superoxide dismutase 2 and metallothionein). In BM-MSC, 22 terms were listed for the 571 enriched genes, and included regulation of organ development (eg, Wisp2, osteomodulin and bone morphogenetic protein 4), morphogenesis (cadherin 13, elastin and Neuropillin 2) and cell migration (chemokine (C-X3-C motif) ligand 1 and chemokine (C-X-C motif) receptor 4) (**Table 4**).

Differences Between ASC and BM-MSC in Secretory Proteins Determined by ELISA

In previous reports, MSC evoked a cell protective effect and induced angiogenesis via secretion of various cytokines, includ-