Mol. Cells, Vol. 19, No. 3, pp. 402-407

Molecules and Cells

Transfection of Mesenchymal Stem Cells with the FGF-2 Gene Improves Their Survival under Hypoxic Conditions

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(Received January 28, 2005; Accepted March 18, 2005)

Bone marrow mesenchymal stem cells (MSCs) have shown potential for cardiac repair following myocardial injury, but this approach is limited by their poor viability after transplantation. To reduce cell loss after transplantation, we introduced the fibroblast growth factor-2 (FGF-2) gene *ex vivo* before transplantation. The isolated MSCs produced colonies with a fibroblast-like morphology in 2 weeks; over 95% expressed CD71, and 28% expressed the cardiomyocyte-specific transcription factor, Nkx2.5, as well as α-skeletal actin, Nkx2.5, and GATA4. In hypoxic culture, the FGF-2-transfected MSCs (FGF-2-MSCs) secreted increased levels of FGF-2 and displayed a threefold increase in viability, as well as increased expression of the anti-apoptotic gene, *Bcl2*, and reduced DNA laddering. They had functional adrenergic receptors, like cardiomyocytes, and exposure to norepinephrine led to phosphorylation of ERK1/2. Viable cells persisted 4 weeks after implantation of 5.0×10^5 FGF-2-MSCs into infarcted myocardia. Expression of cardiac troponin T (CTn T) and a voltage-gated Ca^{2+} channel (CaV2.1) increased, and new blood vessels formed. These data suggest that genetic modification of MSCs before transplantation could be useful for treating myocardial infarction and end-stage cardiac failure.

Keywords: Fibroblast Growth Factor-2; Mesenchymal Stem Cell; Myocardial Infarction.

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Introduction

Myocardial infarction leads to irreversible loss of tissue and deficits in cardiac performance (Ho *et al.*, 1993). The remaining myocytes in infarcted tissues undergo progressive replacement by fibroblasts, forming scar tissue (Makino *et al.*, 1999). Recent attempts to repair infarcted hearts revealed that skeletal myoblasts or cardiomyocytes from fetal or neonatal mice can populate cardiac tissue after injury (Reinecke *et al.*, 1999; Taylor *et al.*, 1998). It is now clear that bone marrow stem cells (MSCs) are also able, when appropriately stimulated, to differentiate into cardiac myocytes, endothelial cells, and vascular smooth muscle cells (Orlic *et al.*, 2001a; 2001b; 2002). They can be passaged several times and have an attached, well-spread and undifferentiated appearance (Pittenger *et al.*, 1999; Wang *et al.*, 2000). In clinical application to cellular cardiomyoplasty, MSCs have shown potential in terms of the number of cells needed to improve cardiac function. Although MSCs represent a suitable source of autologous cells in such cell therapy, MSC therapy is limited by poor cell viability after transplantation.

Recently, there have been attempts, by *in vitro* expansion (Bianchi *et al.*, 2003) and *in vivo* genetic engineering (Mangi *et al.*, 2003), to improve the viability of MSCs. FGF-2 has mitogenic activity for various cells of mesenchymal, neuronal, and epithelial origin. It also regulates events in normal embryonic development, angiogenesis, wound repair, and neoplasia (Houchen *et al.*, 1999; Kim

Abbreviations: FGF-2, fibroblast growth factor-2; MSCs, mesenchymal stem cells.

et al., 2003). Extracellular FGF-2 binds to cell surface receptors and heparan sulfate proteoglycans, and activates a number of intracellular signaling pathways leading to cell proliferation, while intracellular FGF-2 directly enhances mitogenic activity within the nucleus (Nugent and Iozzo, 2000).

In this study, we tested whether transfection with the FGF-2 gene could improve the survival of MSCs after transplantation into infarcted myocardium. We present evidence that such FGF-2-MSCs survive better in hypoxic conditions and show significant retention in infarcted regions of the myocardium. They also expressed cardiac-specific markers and improved neovascularization. Our results suggest that genetic modification of MSCs with FGF-2 may improve treatment of myocardial infarction and end-stage cardiac failure.

Materials and Methods

Isolation and culture of MSCs MSCs were isolated from the femoral and tibial bones of rats (Wang *et al.*, 2000). We collected bone marrow-derived mesenchymal stem cells from aspirates of the femurs and tibias of 4-week-old male Sprague-Dawley rats (about 100 g) with 10 ml of MSC medium consisting of Dulbecco's modified Eagle's medium (DMEM)-low glucose, supplemented with 10% fetal bovine serum and 1% antibiotic-penicillin and streptomycin solution. Mononuclear cells recovered from the interface after centrifugation in Percoll were washed twice, resuspended in 10% FBS-DMEM, and plated in flasks at 1×10^6 cells/100 cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 or 72 h, nonadherent cells were discarded, and the adherent cells were thoroughly washed twice with PBS. Fresh complete medium was added and replaced every 3 or 4 days for about 10 d. To further purify the MSCs, we used the Isolex magnetic cell selection system (Nexell Therapeutics Inc., USA). Briefly, cells were incubated with Dynabeads® M-450 coated with anti-CD34 monoclonal antibody. A magnetic field was applied to the chamber, and the CD34+ cell-bead complexes were separated magnetically from the rest of the cell suspension. The remaining CD34-negative fraction was cultured further. The cells were harvested after incubation with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C, replated in 100 cm^2 plates, and again grown for about 10 d. To estimate their proliferation rate, cell numbers were measured with the non-radioactive colorimetric assay WST-1 (Boehringer Mannheim), based on the cleavage of a tetrazolium salt. To generate hypoxic conditions, the cells were incubated anaerobically for 24 h in deoxygenated DMEM plus 0.5% FBS in an anaerobic chamber (Thermo Forma Anaerobic System Model 1025, USA). To measure activation of ERKs by norepinephrine via adrenergic receptors, neonatal rat cardiomyocytes, MSCs and FGF-2-MSCs were pretreated with prazosin (PRA) (100 nM) or propranolol (PRO) (2 μM) for 30 min, and exposed to norepinephrine (NE) (1 μM) for 10 min.

MSC labeling After transfection, MSCs were labeled with green fluorescent protein (GFP) to detect expression of FGF-2. To generate GFP-FGF2, an FGF-2 DNA fragment produced by *Eco*RI was cloned into pEGFP-N3 (Clontech, USA). Intracellular GFP expression was detected by fluorescence and light microscopy (BX 51TR, Olympus Korea) using a GFP filter set (excitation maximum 488 nm; emission maximum 507 nm) and photographed, or captured digitally with Image Analysis Software using a CCD color video camera (U-CMAP3, Olympus Korea). To label viable MSCs with DAPI, sterile DAPI solution was added to the culture medium on the day of implantation at a final concentration of 50 μg/ml for 30 min. The cells were rinsed 6 times in PBS to remove unbound DAPI, detached with 0.25% (w/v) trypsin and suspended in serum-free medium for grafting.

Flow cytometry The MSCs were harvested by standard trypsinization, washed once in PBS and fixed in 70% ethanol at 4°C for 30 min, with agitation. They were then washed twice in PBS, resuspended at 2×10^6 cells/ml in blocking buffer (1% BSA, 0.1% FBS) containing anti-Nkx2.5 rabbit antibody (Santa Cruz Biotechnology, Inc., USA) diluted 1:200, and agitated for 20 min at room temperature. They were washed twice more and labeled with FITC conjugated anti rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., USA) diluted 1:400 for 20 min at room temperature in the dark. After two more washes, flow cytometric analysis was performed on a FACSCalibur system (Becton Dickinson, USA) using CellQuest™ software with 10,000 events recorded for each sample. Data was acquired in single parameter histograms with appropriate gating for particle size and light scattering.

Transfection Transfection of pEGFP fused to *FGF-2* was performed with LIPOFECTAMINE PLUS™ reagent (Invitrogen, USA) according to the manufacturer's instructions. DNA fragmentation was measured with a TACS™ Apoptotic DNA Laddering kit (R&D Systems, Minneapolis, MN).

Production of myocardial scars, and cell transplantation 8-week-old male Sprague-Dawley rats (about 250 g) were intubated under general anesthesia, and positive-pressure ventilation (180 ml/min) was maintained with room air supplemented with oxygen (2 L/min) using a Harvard ventilator. The rat hearts were exposed through a 2-cm left lateral thoracotomy. Cryoinjury was produced with a metal probe (8 × 10 mm in diameter) cooled to −190°C by immersion in liquid nitrogen and applied to the left ventricular free wall for 15 s. This procedure was repeated 5 times, and then for a total of 10 times each for 1 min. The muscle layer and skin incision were closed with 3-0 silk sutures. For cell transplantation, MSCs (5.0×10^5 cells) were suspended in 30 μl serum-free medium and injected from the injured region to the border with a Hamilton syringe and a 30-gauge needle. The rats were divided into 3 groups of 6 rats each: MSCs, MSCs transfected with *GFP*, and MSCs transfected with *FGF-2*.

Immunoblot analysis Proteins were separated by SDS-PAGE

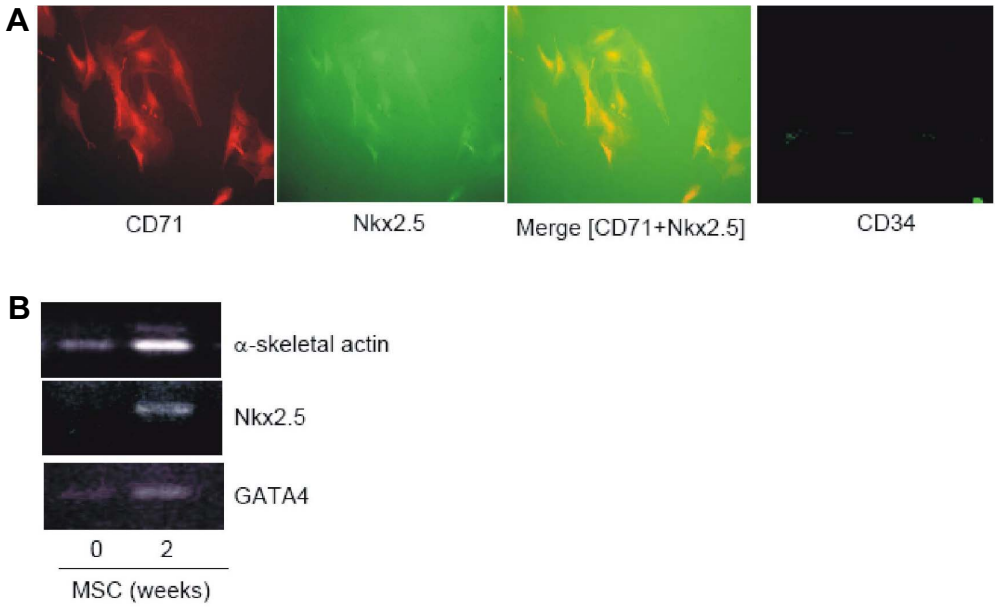

Fig. 1. A. Immunocytochemical characterization of MSCs. Cells cultured from bone marrow after density fractionation are shown 10 days after plating. At 14 days, the cells were 95 to 99% homogeneous and were negative for antigen CD34, which is found on hematopoietic cells. Merged image of double staining of cells for the transferrin receptor, CD71 (red) and for the transcription factor, Nkx2.5 (green) demonstrates colocalization of the surface marker with the cardiac specific transcription factor. **B.** RT-PCR analysis of cardiac-specific genes, α-skeletal actin, Nkx2.5, and GATA4, in MSCs.

using 10–12% polyacrylamide gels and electrotransferred to methanol-treated polyvinylidene difluoride membranes. The blotted membranes were blocked by incubation with 5% nonfat dried milk in PBS buffer. After one hour at room temperature, the membranes were probed overnight at 4°C with mouse polyclonal antibodies against ERK1/2 and bcl2 followed by rabbit anti-mouse IgG-peroxidase. The blots were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech.).

Histology and immunohistochemistry Rats with transplants were killed at intervals after implantation and their hearts excised. The hearts were perfusion-fixed with 10% (v/v) neutral buffered formaldehyde for 24 h, sectioned transversely into four comparably thick sections, and embedded in paraffin by routine methods. Sections 2 μm in thickness were mounted on gelatin-coated glass slides to ensure that different stains could be used on successive sections through the implantation areas. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin. Other sections were analyzed with mouse anti-FGF-2 (Upstate Biotechnology, USA), rabbit anti-CaV2.1 (alamone labs, Israel), goat anti-CTn T (Santa Cruz Biotechnology Inc., USA), and rabbit anti-Nkx2.5 (Santa Cruz Biotechnology Inc., USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Lab., USA) and Texas red-conjugated goat anti-mouse IgG or mouse anti-goat IgG (Jackson ImmunoResearch Lab., USA) were used as secondary antibodies. All images were made with an excitation filter and a reflected light fluorescence microscope, and transferred to a computer equipped with MetaMorph software

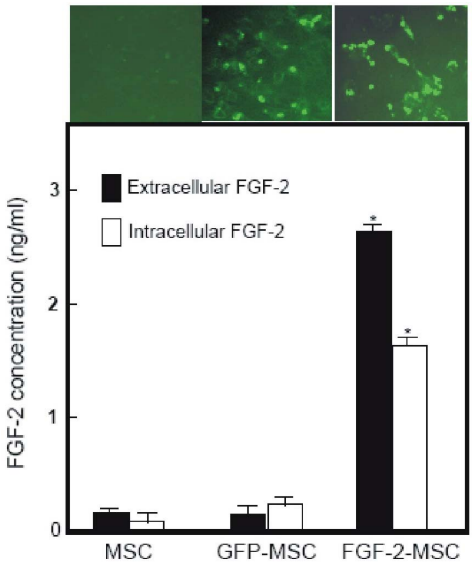

Fig. 2. Expression of FGF-2 in transfected MSCs. MSCs were transfected with a CMV-based pEGFP vector encoding native FGF-2. GFP expression in transfected cells was detected by fluorescence microscopy with a GFP filter set (excitation 488 nm; emission 507 nm). After 24 h exposure to hypoxia, intracellular and secreted FGF-2 was detected with an FGF-2 ELISA kit.

ver. 4.6 (Universal Imaging Corp.).

Statistical analysis All data are expressed as means ± SE. *P*

values less than 0.05 were considered statistically significant (ANOVA and Student's *t*-test).

Results

Isolation and characteristics of MSCs MSCs were isolated from mixed cultures of hematopoietic cells as described in **Materials and Methods**. They retained a fibroblastic morphology through repeated passages, and their identity was confirmed by immunocytochemistry: over 95% of the MSCs expressed CD71, 28% expressed the cardiac transcription factor Nkx2.5, and the hematopoietic marker CD34 (Fig. 1A) was not expressed. RT-PCR analysis showed that they also expressed a-skeletal actin, Nkx2.5 and GATA 4 (Fig. 1B).

Transfection of *FGF-2* into MSCs improves cell survival and function GFP-FGF-2 was introduced into the MSCs using Lipofectamine Plus™ with over 70% efficiency. The expressed FGF-2 totaled 4200 ± 52 pg/ml; secreted and intracellular FGF-2 were 2604 ± 29 pg/ml and 1596 ± 23 pg/ml, respectively, during twenty-four hours' exposure to hypoxia (85% N_2, 10% CO_2, and 5% H_2) and serum starvation (0.5% FBS) (Fig. 2). Overexpression of FGF-2 led to a threefold increase in viable cells under hypoxic conditions, and was associated with increased expression of the antiapoptotic gene *Bcl2* and a reduction in DNA laddering (Fig. 3). To evaluate the extent of differentiation of FGF-2-MSCs into cardiomyocyte-like cells, we measured Nkx2.5 expression by flow cytometry. Nkx2.5 expression was significantly higher than in control MSCs, 60 ± 3% compared to 28 ± 6% ($p < 0.001$) (Fig. 4). Interestingly, MSCs directly exposed to FGF-2 also expressed higher levels of Nkx2.5 than control MSCs. To further evaluate the phenotype of FGF-2-MSCs as cardiomyocyte-like cells, we stimulated them with norepinephrine and measured phosphorylation of ERK1/2 to estimate their adrenergic response (Fig. 5). In neonatal cardiomyocytes, norepinephrine-induced phosphorylation is completely inhibited by prazosin and partially inhibited by propranolol. Control MSCs hardly gave rise to any phosphorylation in response to norepinephrine, whereas phosphorylation took place in the FGF-2-MSCs and was inhibited by prazosin and propranolol, as in neonatal cardiomyocytes. These results indicate that the FGF-2-MSCs have functional adrenergic receptors.

Behavior of implanted FGF-2-MSCs and neovascularization H&E staining of implants in the infarcted heart showed alignment of the FGF-2-MSCs with non-labeled cells (host cardiomyocytes), in DAPI-stained regions, indicating that DAPI-labeled donor cells were incorporated into the host myocardium (Fig. 6). Expression persisted in the DAPI-labeled cells, but the regions of expression of FGF-2 did not match with the DAPI staining, indicating that some of the FGF-2 was secreted, as it is *in vitro*. To confirm that the implanted cells had formed cardiac myocytes, we showed by immunohistochemistry that the cardiac specific markers, CTn T and Cav2.1, were detectible in the regions that were DAPI stained and contained FGF-2. To evaluate the extent of development of collateral vessels in the regions overexpressing FGF-2, we investigated the expression of alpha smooth muscle

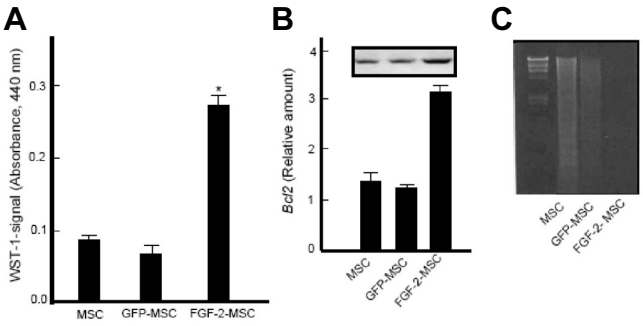

Fig. 3. Effect of transfection of MSCs with *FGF-2*. **A.** Assay of MSC proliferation. MSCs (1.5×10^4) were seeded in 96-well culture plates and cultured for 24 h. After 24 h exposure to hypoxia and serum starvation (0.5% FBS), 10 µl of WST-1 reagent was added to each well for 30 min at 37°C, and cell numbers were assessed spectrophotometrically (λ=440 nm). The data shown are means ± SEM (n = 4). * $P \leq 0.05$. **B.** Representative Western blot and relative amounts of the apoptotic gene *Bcl2* in three experiments. **C.** DNA fragmentation in MSCs. Genomic DNA (10 µg) from *FGF-2*-transfected and control MSCs was resolved on a 1% agarose gel.

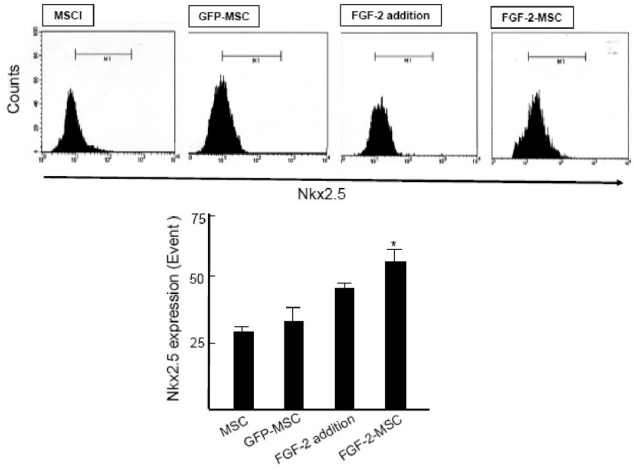

Fig. 4. Flow cytometric analysis of Nkx2.5 expression in *FGF-2*-transfected MSCs. MSCs, GFP-MSCs, FGF-2-MSCs and MSCs treated with FGF-2 (20 ng/ml) were labeled with FITC-conjugated antibody against Nkx2.5, and prepared for FACS analysis as described in **Materials and Methods**. The results are presented as fluorescence intensity histograms.

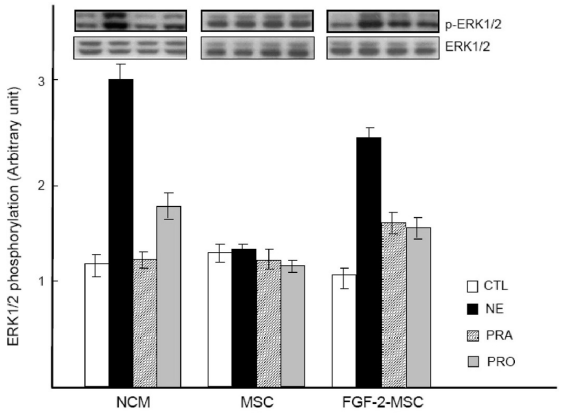

Fig. 5. Activation of ERKs by norepinephrine via adrenergic receptors in *FGF-2*-transfected MSCs. After 30 min pretreatment with prazosin (PRA) (100 nM) or propranolol (PRO) (2 μM), neonatal rat cardiomyocytes (NCM), MSCs and FGF-2-MSCs were stimulated with norepinephrine (NE) (1 μM) for 10 min. Signals were quantified by scanning densitometry and are shown relative to the maximal level of ERK activity in the control (CTL). Results are means of three independent experiments.

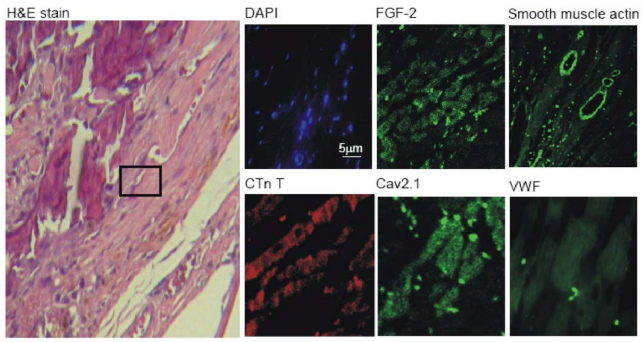

Fig. 6. Analysis of myocardial repair 4 weeks after implantation of MSCs into cryo-injured hearts. H&E staining shows infiltration of viable, mature cardiac myocytes from the border zone into the scar area. Fluorescent images are of DAPI staining, and immunohistochemical analysis for FGF-2, cardiac-specific voltage-gated Ca^{2+} channel (Cav2.1), cardiac troponin T (CTn T), smooth muscle actin and vonWillebrand Factor (VWF).

actin and von Willebrand factor (VWF) by immunohistochemistry. New vessel formation was much greater in the FGF-2-MSC group than in the control MSCs as judged by the levels of alpha smooth muscle actin and VWF, suggesting that the FGF-2 promoted angiogenesis.

Discussion

In this study, we established that FGF-2-transfected MSCs delivered into the myocardium after cryoinjury survive better and form more cells with cardiomyocyte characteristics than control MSCs. Moreover, norepinephrine induced phosphorylation of ERK1/2 in these cells, and this phosphorylation was regulated by prazosin and propranolol, as in neonatal cardiomyocytes.

Therapeutic trials of cells for the treatment of ischemic myocardium can be divided into two types: differentiation and therapeutic implantation. Despite several differentiation studies, the mechanism by which MSCs differentiate into cardiac myocyte-like cells remains controversial, even though several *in vitro* investigations have shown that such differentiation does occur (Makino *et al.*, 1999; Pittenger *et al.*, 1999; Prockop, 1997). After treatment with 5-azacytidine, murine bone marrow MSCs generated a cardiomyogenic cell line (CMG) that formed myotubes connected by intercalated discs, and beat synchronously (Makino *et al.*, 1999). Several cell types, including skeletal myoblasts (Murry *et al.*, 1996; Reinecke *et al.*, 2000; Taylor *et al.*, 1998), cardiac myocytes (adult, fetal or neonatal myocytes) (Li *et al.*, 1997; Watanabe *et al.*, 1998), and embryonic stem cell-derived cardiac myocytes (Min *et al.*, 2002), have been investigated in cell implantation studies for cardiac infarction, but each cell type had clinical limitations. We also have shown above that MSCs have the potential to form cardiomyocyte-like cells, based on the expression of the cardiac-specific transcription factors, Nkx2.5 and GATA4, and the presence of transcripts of the cardiomyocyte-specific protein, α-skeletal actin (Figs. 1A and 1B).

Autologous MSCs have advantages for generating functional cardiac myocytes in the infarcted myocardium because of the ease with which they can be prepared from adult patients, and their immunologic safety. However, the frequency of MSC engraftment was extremely low, despite implanting large numbers of cells (Wang *et al.*, 2000), and the exact mechanism of therapeutic cardiac repair by MSCs was ill-defined. Recently, in a new approach to enhancing the viability of MSCs, they were exposed to the survival signal, Akt, in the early post-transplant period (Mangi *et al.*, 2003).

Our attempt to enhance the survival of MSCs and hence to increase neovascularization involved introduction of the FGF-2 gene into the implanted MSCs. FGF-2 activates several signaling components related to cell survival, including mitogen-activated protein kinase (MAPK) (Tokuda *et al.*, 2000), src, and protein kinase C (PKC) (Nugent and Iozzo, 2000). It also has a role in stimulating the growth and development of new blood vessels (Debiais *et al.*, 2004). When FGF-2 is added to the culture medium of MSCs, it may act via receptor-mediated mechanisms. But after transfection of the FGF-2 gene, it can act both intracellularly and extracellularly, and modulates several signals (Fig. 2). Transfection with *FGF-2* increased the viability of the MSCs and promoted the formation and survival of myocytes in the border regions of the infarcted myocardium. In addition, flow cytometric

analysis showed that Nkx2.5 expression was significantly higher in the FGF-2-MSCs, than in control MSCs.

The activation of ERKs plays an important role in gene regulation in cardiac hypertrophy, and is a sensitive and quantitative marker for the hypertrophic response of cardiac myocytes (Lee *et al.*, 2003). Stimulation of protein synthesis by α_1-AR as well as β-AR agonists is a symptom of hypertrophy, and the pathways by which norepinephrine induces cardiomyocyte hypertrophy are well defined. Interestingly, we found that adrenergic signal transduction in FGF-2-MSCs is similar to that in neonatal cardiomyocytes, indicating that FGF-2-MSCs have functionally active adrenergic receptors, unlike control MSCs.

We have shown here that FGF-2-transfected MSCs survived better in hypoxic conditions and were significantly retained in infarcted regions of myocardium. They also expressed cardiac-specific markers and improved neovascularization. Our results suggest that genetic modification of MSCs with FGF-2 before transplantation offers a novel therapeutic approach to the treatment of myocardial infarction and end-stage cardiac failure.

Acknowledgments This study was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ10-PG8-EC01-0016) and was supported (in part) by Yonsei University Research Fund of 1999 year.

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