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MINI-FOCUS: CELL-BASED THERAPY

Controlled Delivery of Basic Fibroblast Growth Factor Promotes Human Cardiosphere-Derived Cell Engraftment to Enhance Cardiac Repair for Chronic Myocardial Infarction

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Objectives This study was designed to determine whether controlled release of basic fibroblast growth factor (bFGF) might improve human cardiosphere-derived cell (hCDC) therapy in a pig model of chronic myocardial infarction.

Background Current cell therapies for cardiac repair are limited by loss of the transplanted cells and poor differentiation.

Methods We conducted 2 randomized, placebo-controlled studies in immunosuppressed pigs with anterior myocardial infarctions. Four weeks after coronary reperfusion, 14 pigs were randomly assigned to receive an intramyocardial injection of placebo medium with or without bFGF-incorporating hydrogel implantation. As a second study, 26 pigs were randomized to receive controlled release of bFGF combined with or without hCDCs or bone marrow–derived mesenchymal stem cell transplantation 4 weeks after reperfusion.

Results Controlled release of bFGF in ischemic myocardium significantly augmented the formation of microvascular networks to enhance myocardial perfusion and contractile function. When combined with cell transplantation, the additive effects of bFGF were confined to hCDC-injected animals, but were not observed in animals receiving human bone marrow–derived mesenchymal stem cell transplantation. This was shown by increased donor-cell engraftment and enhanced cardiomyocyte differentiation in the transplanted hearts, resulting in synergistically improved ventricular function and regional wall motion and reduced infarct size.

Conclusions Controlled delivery of bFGF modulates the post-ischemic microenvironment to enhance hCDC engraftment and differentiation. This novel strategy demonstrates significant functional improvements after myocardial infarction and may potentially represent a therapeutic approach to be studied in a clinical trial in human heart failure. (J Am Coll Cardiol 2008;52:1858–65) © 2008 by the American College of Cardiology Foundation

Stem cell therapies offer tremendous possibilities for curative approaches toward restoring lost myocardium and cardiac function; however, recent studies have indicated that effective cardiac muscle regeneration might be hindered by poor cell engraftment and inefficient cardiomyocyte differentiation of the transplanted cells in the absence of integration with the host myocardial environment after infarction (1). Although prior studies (2–5)

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including our report have suggested human cardiac stem/progenitor cells as an attractive cell source for cardiac repair, the beneficial effects of these cells in large animal models have yet to be investigated.

The basic fibroblast growth factor (bFGF) is a pluripotent mitogen and possesses properties to promote stem cell differentiation, proliferation, and survival (3,6,7). Biodegradable gelatin is a useful delivery modality to circumvent the short half-life of recombinant bFGF in vivo. We have designed a controlled-release system for bFGF composed of acidic gelatin, which forms a poly-ion complex with bFGF (8). Biodegradable hydrogels display excellent biocompatibility demonstrated by the absence of rejection and inflammation and achieve a controlled release of bFGF in vivo as a result of hydrogel degradation within 3 weeks (9). Controlled release of bFGF has been shown to effectively enhance neoangiogenesis in human ischemic limbs (10).

This study was conducted to test whether the cell engraftment, survival, and differentiation potential of human cardiosphere-derived cells (hCDCs) could be promoted by controlled delivery of bFGF-incorporating hydrogel in response to experimental myocardial infarction, ultimately leading to improved performance in cardiovascular regeneration.

Methods

Isolation and expansion of hCDCs from human heart samples. Human samples were obtained from 10 male patients undergoing cardiac surgery, in conformity with the guidelines of the Kyoto University Hospital and Ministry of Education, Culture, Sports, Science, and Technology, Japan. Samples were excised, minced, and digested with 0.2% type II collagenase and 0.01% DNase I (Worthington Biochemical Corp., Lakewood, New Jersey) to obtain single cell suspensions to generate cardiospheres as described previously (3). Cardiospheres were mechanically selected from the cultures and expanded in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium containing 10% fetal bovine serum, 2% penicillin and streptomycin, and 40 ng/ml human recombinant bFGF (Promega Corp., Madison, Wisconsin). Six independent human bone marrow–derived mesenchymal stem cells (hBMCs) were purchased from the RIKEN Cell Bank (RIKEN Bioresource Center, Ibaraki, Japan) (11). The hBMCs were plated in DMEM containing 10% fetal bovine serum, 2% penicillin, streptomycin, and 4 ng/ml bFGF. Cells were harvested at passage 2, frozen at −80°C, and were thawed to process the third rounds of passage 3 weeks before the transplantation. The expanded hBMCs were characterized by a fluorescence-activated cell sorter using CD29, CD105, CD71, and CD90.

Generation of gelatin hydrogel sheet. The gelatin was isolated by an alkaline process from bovine bone with an isoelectric point of 5.0 as previously described (10). The water content of gelatin hydrogel was prepared to 94% by chemical cross-linking at 140°C for 72 h. The gelatin was reinforced by polytetrafluoroethylene (W. L. Gore and Associates, Inc., Flagstaff, Arizona) pericardial sheet (12) to provide strength against the beating heart. Human recombinant bFGF (Kaken Pharmaceutical Co., Tokyo, Japan) was incorporated into the gelatin hydrogel by impregnation for 3 h before implantation.

Abbreviations and Acronyms

bFGF = basic fibroblast growth factor
DMEM = Dulbecco's Modified Eagle Medium
FISH = fluorescent in situ hybridization
hBMC = human bone marrow–derived mesenchymal stem cell
hCDC = human cardiosphere-derived cell
LV = left ventricle/ventricular
LVEF = left ventricular ejection fraction
MRI = magnetic resonance imaging
SPIO = superparamagnetic iron oxide
SRS = systolic radial strain

Animal models and study protocol. Based on computer-generated random allocation, we performed 2 randomized studies of chronically instrumented animals (Fig. 1). Myocardial infarction was created in 60 female Yorkshire pigs by inflating the balloon at the left ascending coronary artery for 90 min, followed by reperfusion. Thirteen of the study pigs died in the early postoperative period. We excluded 7 pigs with an ejection fraction <35% or >45% determined by transthoracic echocardiography using the Teichholz method before randomization. Animals were assigned for randomization 1 week after the creation of myocardial infarction, and then cells were grown in culture for 3 weeks to prepare for transplantation. Four study pigs died within 1 week after randomization but before the treatment due to heart failure.

In study 1, the eligible pigs (n = 14) were randomized to receive DMEM intramyocardial injection with or without 200 μg bFGF-incorporating gelatin hydrogel implantation 4 weeks after reperfusion. In study 2, we randomly assigned eligible pigs (n = 26) to receive bFGF hydrogel sheet implantation with intramyocardial injections of either DMEM, 2.0×10^7 hBMCs, or 2.0×10^7 hCDCs. All animals in both studies were immunosuppressed with cyclosporine A (Novartis Pharmaceuticals, East Hanover, New Jersey) 5 mg/kg daily from 5 days before transplantation until the time for sacrifice (13). Transplantation was performed by a 3-ml injection at 30 different sites along the border zone and in the center of the scar area.

Fluorescence-activated cell sorter analysis. Single cell suspensions were stained with the following antibodies: phycoerythrin-conjugated antibodies against CD29, and fluorescein isothiocyanate–conjugated antibodies against CD45 (all from BD Biosciences, San Jose, California) or CD105 (Ancell, Bayport, Minnesota). Antibody against mouse anti-human CD90 was detected by phycoerythrin-conjugated gout anti-mouse immunoglobin G (BD Biosciences). Samples were analyzed by FACSCalibur flow cytometer (BD Biosciences).

Generation of retroviral vectors. The *LacZ* reporter gene was subcloned into a human cardiac troponin I promoter

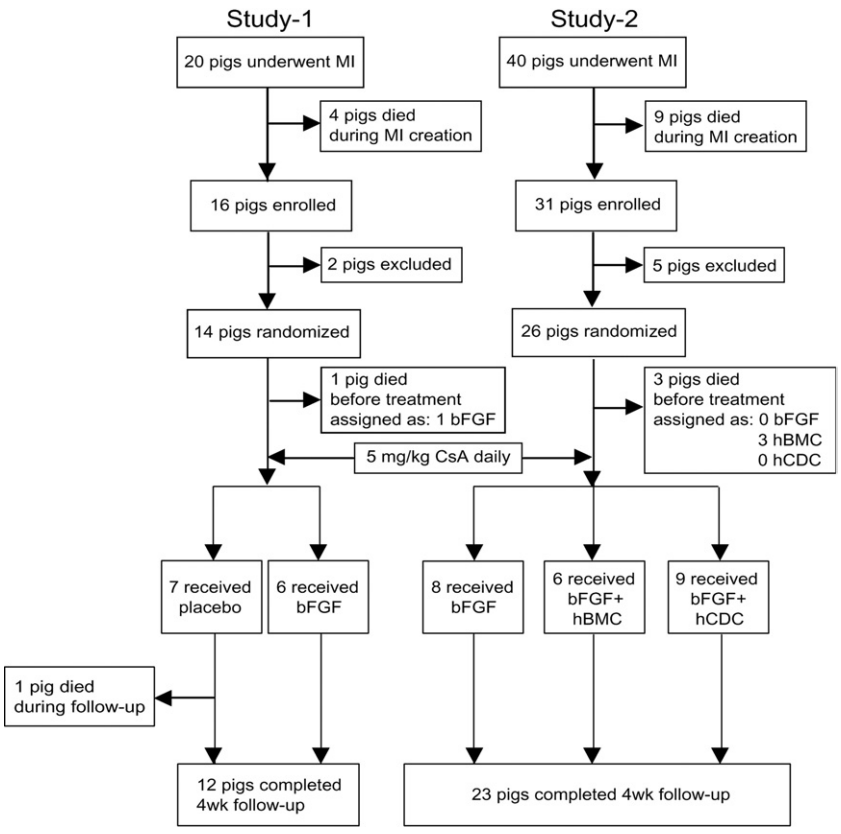

Figure 1 Study Design

bFGF = basic fibroblast growth factor; CsA = cyclosporine A; hBMC = human bone marrow-derived mesenchymal stem cell; hCDC = human cardiosphere-derived cell; MI = myocardial infarction.

containing plasmid (14). The hCDCs were infected by retrovirus containing pDsRed2-1 (Clontech Laboratories, Inc., Mountain View, California) and were co-cultured with neonatal rat ventricular myocytes for 4 to 5 days.

Calcium transients and electrophysiological studies. Cells were loaded with fluo-4/AM (0.625 mg/ml) and incubated with fluo-4-free Tyrode's solution at 37°C as previously described (15). Confocal images of fluo-4 and DsRed fluorescence intensities were obtained under excitation with an argon-laser and a krypton laser, respectively. The signals of action potentials from the electrodes were digitized and displayed on a digital oscilloscope (Model 310, Nicolet Instrument Technologies, Madison, Wisconsin) and stored onto a computer for offline analysis.

Immunostaining and fluorescence in situ hybridization (FISH) analysis. Paraffin-fixed sections were stained using the following primary antibodies: Cy3-conjugated mouse anti–alpha-smooth muscle actin, mouse anti-sarcomeric alpha-actin, rabbit anti-connexin 43 (all from Sigma-Aldrich, St. Louis, Missouri), mouse anti-myosin heavy chain, or rabbit anti-beta-galactosidase (all from Abcam Inc., Cambridge, Massachusetts). Secondary antibodies were conjugated to Alexa fluorochromes and nuclei were visualized using 4,6-diamino-2-phenylindole (Invitrogen Corp. [Molecular Probe], Carlsbad, California). Arteriolar density was evaluated morphometrically by histological examination of 10 randomly selected fields recognized as anti–alpha-smooth muscle actin positive structures corrected by the total area of tissue sections measured. FISH was performed as previously described (16). Deoxyribonucleic acid probes for Cy3-conjugated human Y-chromosome (classical satellite) and Cy5-conjugated porcine-specific genome were from Masahisa Tsuji (Chromosome Science Laboratory, Hiroshima, Japan). Images were captured with a BZ-8000 (Keyence, Osaka, Japan) and confocal microscope (Leica Microsystems, Wetzlar, Germany).

Reverse transcriptase polymerase chain reaction and Western blotting. Total ribonucleic acid was extracted with Trizol reagent (Invitrogen) and complementary deoxyribonucleic acid was generated using a SuperScript III complementary deoxyribonucleic acid synthesis kit (Invitrogen). Polymerase chain reactions were performed with human-specific primers as shown in the Supplemental Table. Transferred membranes were incubated with primary antibodies against phospho-Akt (S473), Akt, phospho–extracellular signal-related kinase 1/2, extracellular signal-

related kinase 1/2, phosho-p38, p38, phospho–Jun N-terminal kinase 1/2, or Jun N-terminal kinase 1/2 (all from Cell Signaling Technology Inc., Danvers, Massachusetts) as described previously (7). Horseradish peroxidase–conjugated anti-rabbit immunoglobin G was used as a secondary antibody.

Cardiac magnetic resonance imaging (MRI). MRI images were obtained on a 1.5-T MR scanner (GE Medical Systems, Milwaukee, Wisconsin) using electrocardiography-gating. Global left ventricular (LV) function was assessed using a steady-state precession pulse sequence from 8 contiguous short-axis slices (17). Infarct size was analyzed by the late-enhancement MRI technique using contrast agent (Omniscan, Daiichi-Sankyo, Japan). The LV mass was analyzed using MASS software (Medis, Leiden, the Netherlands) and infarct size was calculated as enhanced LV mass (g)/total LV mass (g).

Iron-oxide labeling. The hCDCs were magnetically labeled before injection by using a superparamagnetic iron oxide (SPIO) (Nihon Schering, Osaka, Japan) with hemagglutinating virus of Japan envelope (GenomONE Neo) (Ishihara Sangyo Kaisha Ltd., Osaka, Japan). Magnetically labeled hCDCs were assessed from 14 to 18 contiguous short-axis images by using pulse parameters for cardiac gated, fast gradient-recoiled echocardiography. The SPIO-labeled areas were measured and corrected by the values of SPIO densities of interest (18).

Echocardiography. Echocardiography was performed using a Vivid 7 Echocardiography System (GE Vingmed Ultrasound, Horten, Norway) with a M3S transducer. Real-time myocardial contrast echocardiography was performed by intravenous infusion of Levovist (Nihon, Schering) as previously described (19). Equal-sized transmural regions of interest were placed in the ischemic border area and nonischemic posterolateral wall. Signal intensities in each region of interest were assessed 3 times and the ratios were calculated.

Two-dimensional strain echocardiography was performed using the EchoPAC program (GE Vingmed). The ischemic region, border area, and nonischemic posterolateral area of the LV were analyzed at medial and apical levels as described previously (20). The LV wall motion index was defined as the ratio of peak systolic radial strain (SRS); [(medial level ischemic area SRS/medial level control area SRS) + (medial level border area SRS/medial level control area SRS) + (apical level ischemic area SRS/apical level control area SRS) + (apical level border area SRS/apical level control area SRS)]/4.

Statistics. Changes in variables from baseline to 4 weeks after treatment were analyzed with the paired *t* test. Differences between any 2 groups from baseline to 4 weeks were assessed with the Student *t* test using JMP software (SAS Institute, Cary, North Carolina). A value of $p < 0.05$ was regarded as statistically significant.

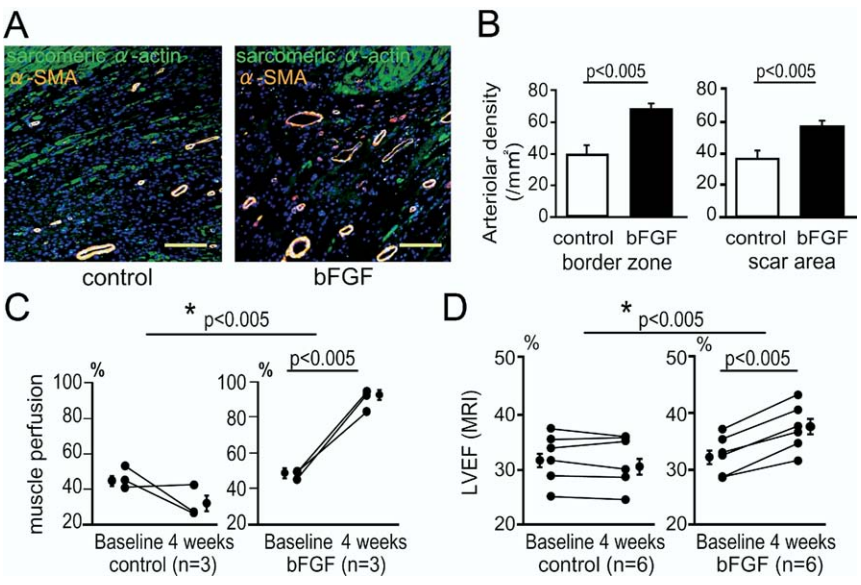

Figure 2 **Controlled Delivery of bFGF Promotes Neoangiogenesis and Restores Cardiac Function in Ischemic Myocardium**

(A and B) Arteriolar density was measured by alpha-SMA staining in control and bFGF-treated hearts (n = 6). **Bars** = 50 μm **(A)**. **(C)** Myocardial perfusion was measured by myocardial contrast echocardiography. Signal intensities in the ischemic zone corrected by control area are shown. **(D)** Functional recovery with bFGF treatment MI analyzed by magnetic resonance imaging. **Asterisks** indicate the comparisons of absolute change in respective measurement from baseline to 4 weeks follow-up in control and bFGF-treated groups. SMA = smooth muscle actin; other abbreviations as in Figure 1.

Results

Randomized, placebo-controlled trial 1: controlled delivery of bFGF enhances myocardial perfusion and restores cardiac function in ischemic myocardium. Immunosuppressed pigs were randomized to receive intramyocardial injection of culture medium with or without bFGF-incorporating gelatin hydrogel implantation 4 weeks after myocardial infarction. A significant increase in the formation of arterial vessels was found in both infarct border zones (66.5 ± 5.8/mm^2 vs. 38.6 ± 5.8/mm^2; $p < 0.005$) and necrotic areas (56.9 ± 4.2/mm^2 vs. 36.9 ± 4.3/mm^2; $p < 0.005$) as compared with control hearts (Figs. 2A and 2B). Myocardial contrast echocardiography showed that bFGF-treated animals exhibited remarkably enhanced myocardial perfusion when compared with myocardial perfusion of the control group at 4 weeks (89.7 ± 5.9% vs. 26.3 ± 0.6%, $p < 0.005$) (Fig. 2C). In addition, bFGF significantly improved left ventricular ejection fraction (LVEF) at 4 weeks (LVEF: 37.1 ± 4.2% vs. 31.8 ± 4.7%; $p < 0.005$). The absolute change in LVEF from baseline to 4 weeks was significantly greater in the pigs treated by bFGF compared with the change in the LVEF of the control group (+4.9 ± 0.5% vs. −0.4 ± 0.4%; $p < 0.005$) (Fig. 2D).

Placebo-controlled trial: bFGF increases hCDC engraftment. As a preliminary experiment to confirm the beneficial effects of bFGF on hCDCs, we conducted a placebo-controlled study to compare engraftment efficiency of hCDC transplantation with or without controlled release of bFGF using SPIO nanoparticles to track hCDCs in vivo. The morphological, surface marker, stem cell transcription factor expression, calcium flux, and single cell electrical data indicate that the hCDCs that we cultured were the same cells described in previous studies (Online Fig. 1) (3,4). At 4 weeks after implantation, there was a significant retention of transplanted hCDCs in the ischemic myocardium when combined with bFGF treatment compared with hCDC injection alone (Figs. 3A and 3B). Absolute changes in LVEF and infarct volume were both synergistically improved in hCDC transplantation with bFGF than in hCDC injection alone at 4 weeks after implantation (Figs. 3C and 3D).

Randomized, placebo-controlled trial 2: epicardial delivery of bFGF with cell therapy improves cardiac function. We next performed a second, randomized study to determine whether the combination of bFGF with stem cell therapy could improve cardiac function further after chronic

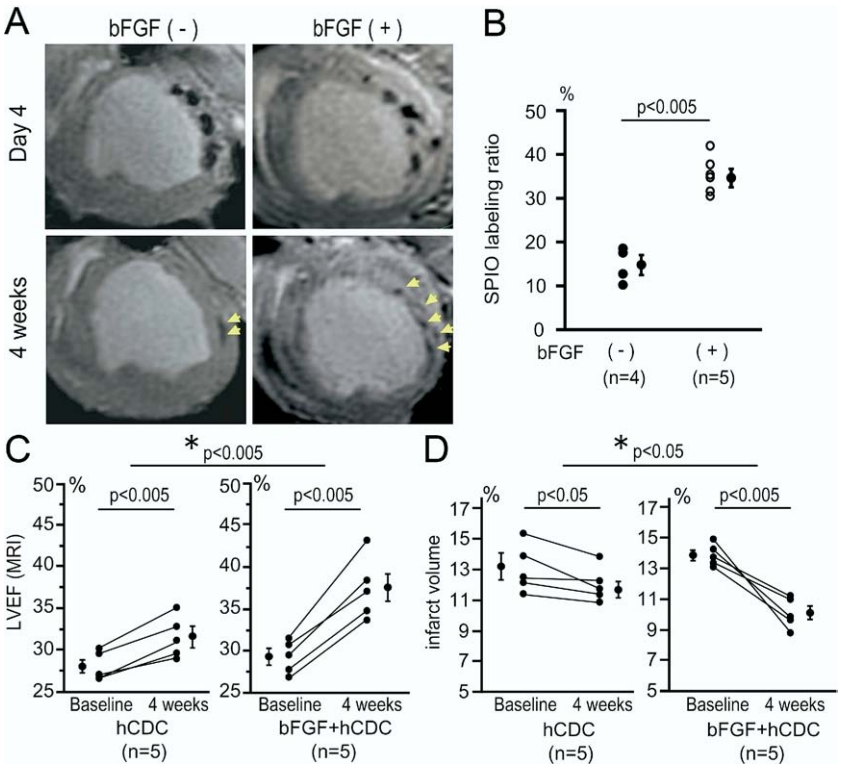

Figure 3 The Impact of bFGF on hCDCs In Vivo

(A) Paired MRIs were examined at day 4 and 4 weeks after hCDC transplantation with or without bFGF treatment. The low intensity of SPIO-labeled area is shown by **yellow arrowheads**. **(B)** Cell engraftment was estimated by the retention of SPIO-labeled cells at 4 weeks corrected by that at day 4. **(C)** Functional recovery of hCDC transplantation with or without bFGF analyzed by MRI. **(D)** Infarct size was evaluated and compared before and after treatment. **Asterisks** indicate the comparisons of absolute change in respective measurement from baseline to 4 weeks' follow-up in control and bFGF-treated groups. LVEF = left ventricular ejection fraction; MRI = magnetic resonance imaging; SPIO = superparamagnetic iron oxide; other abbreviations as in Figure 1.

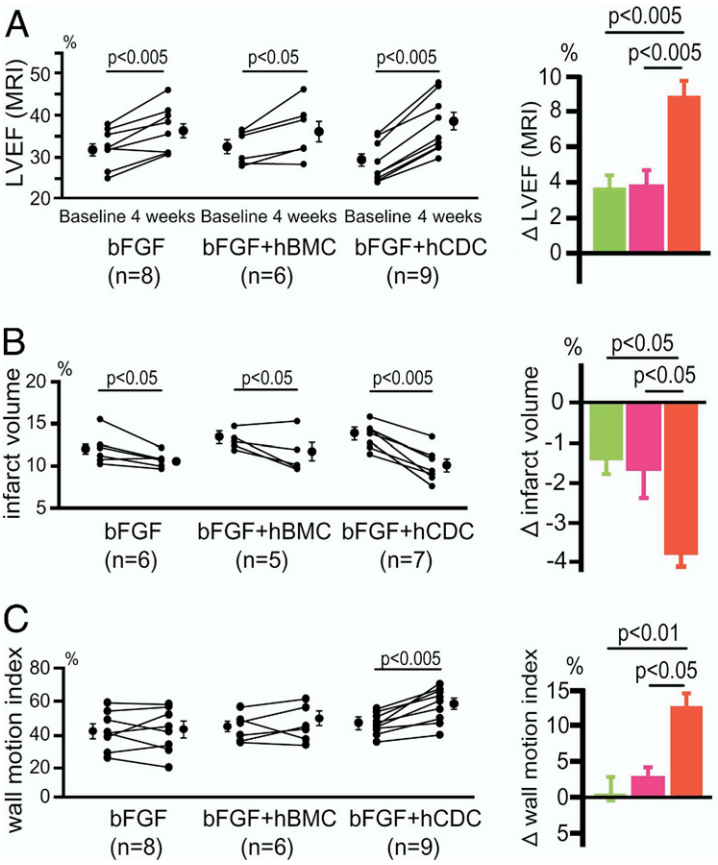

Figure 4 **Controlled Delivery of bFGF With Cell Therapy Improves Cardiac Function**

(A and B) The LVEF and infarct size at baseline and 4 weeks after treatment was measured by MRI. **(C)** The wall motion index was assessed by 2-dimensional strain echocardiography. Absolute change in each measurement was summarized and is shown on the **right. Green bars** = bFGF; **pink bars** = bFGF+hBMC; **red bars** = bFGF+hCDC. Abbreviations as in Figures 1 and 3.

myocardial infarction. Although bFGF alone significantly improved LVEF and reduced infarct size compared with the baseline, transplantation of hBMCs with bFGF had no additive effects on cardiac function (Figs. 4A and 4B). In contrast to the negligible effects of hBMCs, ischemic hearts implanted with hCDCs and bFGF-incorporating hydrogels, both LVEF (38.4 ± 6.2% vs. 30.1 ± 4.3%, $p < 0.005$) and regional wall motion (58.5 ± 11.7% vs. 46.2 ± 7.2%, $p < 0.005$) showed significant improvements; infarct volume was also remarkably reduced compared with the baseline (Figs. 4A to 4C). The magnitude of improvement after transplantation of hCDCs with bFGF in cardiac function and infarct size are significantly evident compared with cardiac function and infarct size of the control group by a comprehensive analysis of both studies 1 and 2 (Online Fig. 2).

Effects of controlled release of bFGF on cardiac regeneration after cell transplantation. To assess if cardiac function was restored, at least in part, by cardiomyocyte differentiation from the transplanted cells, we engineered 3 independent hCDCs to express a *LacZ* reporter gene under the control of human cardiac troponin-I promoter (cTnI-LacZ) by retrovirus infection (14). Immunofluorescence analyses in hCDC transplants showed that beta-gal-expressing cells were co-localized with myosin heavy chain and sarcomeric alpha-actin and were functionally coupled in the ischemic border zone (Figs. 5A to 5D).

We performed FISH experiments to identify the transplanted human male donor cells in female recipients (Fig. 5E). The functional improvement observed in hCDC transplantation with bFGF was confirmed by a greater magnitude of myocyte conversion compared with the injection of hBMCs combined with bFGF (Figs. 5F to 5H). Although myocyte regeneration was almost exclusively (>90%) through cell fusion in hBMC transplants with bFGF treatment, approximately 33% of the differentiated human cardiomyocytes were independent from cell fusion in hCDC injection with bFGF (Fig. 5H).

Discussion

Successful cell engraftment is a critical component to achieve a significant improvement in LVEF for long-term

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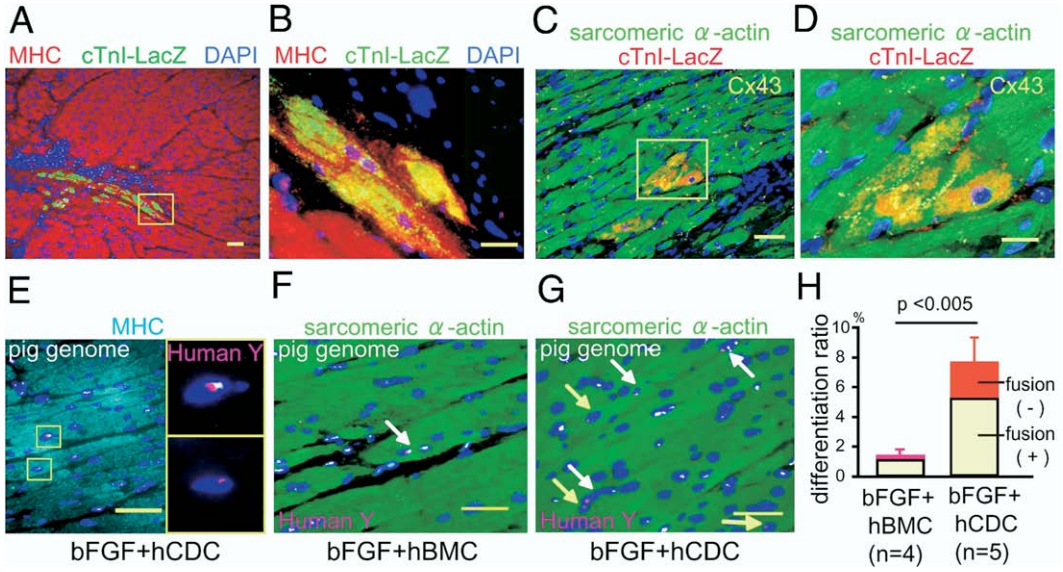

Figure 5 **Myocardial Repair by Combination of bFGF and Cell Transplantation**

(A) Differentiated cardiomyocytes expressing beta-gal **(green)** were counterstained with MHC **(red)**. **(B)** Magnified image is shown. **(C and D)** Differentiated human cardiomyocytes expressed connexin 43 **(yellow)** and sarcomeric alpha-actin **(green)**. **(E)** The FISH analysis. In hCDC transplantation, hCDCs **(red)** differentiated into human cardiomyocytes **(light blue)** with pig genome sequences **(white)** suggesting cell fusion **(upper inset)**. Autonomous differentiation is shown by hCDC-derived human cardiomyocytes lacking pig genome sequences **(lower inset)**. **(F and G)** Myocyte regeneration with **(white arrows)** or without **(yellow arrows)** cell fusion events is shown. **(H)** Quantitative analysis of cardiomyocyte regeneration and fusion. Human Y chromosome and sarcomeric alpha-actin–positive cells were counted and corrected by the total number of myocytes analyzed (>15,000 cells). The DAPI stain is shown as **blue. Bars:** 100 μm **(A and C)**; 50 μm **(E to G)**; 20 μm **(B and D)**. DAPI = 4,6-diamino-2-phyenylindole; MHC = myosin heavy chain; other abbreviations as in Figure 1.

results. Our findings demonstrated that transplantation of hCDCs with bFGF treatment combined the advantages of cardiomyocyte repopulation and stable vascular network formation, resulting in synergistically improved cell therapy efficiency.

Local delivery of combination of growth and survival factor(s), such as insulin-like growth factor 1, on scaffold in cardiac muscles has been shown to improve donor cell survival and enhance tissue repair (21,22). Resident hCDCs may also require joint effects of multiple factors that modulate cell proliferation and differentiation in response to injury. Clinical trials of single protein infusion (23) in human heart disease have shown that the efficacy was not significant in the long term. It is possible that the recombinant protein infused could be rapidly diffused in situ or a combinatorial protein release may be required to achieve the desired degree of angiogenesis in the absence of cell transfer (24). Previous mouse experiments also supported the beneficial effect of bFGF-releasing hydrogel transplantation in the current study, demonstrating that bFGF could be slowly released over time to promote neovascularization initially in situ, and its biological effect was completely terminated as a consequence of hydrogel biodegradation in vivo within 3 weeks after implantation (9).

The less synergistic improvement in animals receiving hBMC injection combined with bFGF than those in hCDC transplants with bFGF was unexpected. Experimental studies in animals suggested that transplantation of BMCs in myocardial infarction could prevent LV remodeling and attenuate the infarct size, mainly through Akt-mediated paracrine effects, despite minimal myocyte regeneration (25). Our results suggested that controlled release of bFGF itself might stimulate these paracrine effectors on host myocardium and showed no additive effects when hBMCs were transplanted.

The cell tracking system using iron-oxide labeling has been reported to be useful to serially monitor the transplanted cells in vivo. However, iron-positive cells may not directly reflect surviving cells, but they are likely to detect the dead cells colocalized with phagocytic macrophages in the host myocardium (26). At least, our results suggested that direct cell injection alone might not be sufficient to achieve efficient myocyte regeneration.

Although our results are limited in the experimental setting of human-pig chimera transplantation, the host animals were immunosuppressed with a dose of cyclosporine A as reported previously (13). Our findings demonstrate a previously undescribed therapeutic efficacy that a combination of bFGF with hCDCs can induce functionally stable microvascular networks to support efficient donor cell engraftment and differentiation. This novel strategy may potentially enhance the cell therapy practically needed to treat patients with heart failure.

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Key Words: cell therapy ■ bFGF ■ gelatin hydrogel ■ heart failure ■ myocardial infarction.

APPENDIX

For a discussion of the isolation and characterization of hCDCs, Supplemental Figures 1 and 2, and a Supplemental Table, please see the online version of this article.