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Effects of Myocardial Transplantation of Marrow Mesenchymal Stem Cells Transfected with Vascular Endothelial Growth Factor for the Improvement of Heart Function and Angiogenesis after Myocardial Infarction

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Key Words
Mesenchymal stem cell · Bone marrow · Cell therapy · Gene therapy · Vascular endothelial growth factor · Ischemic heart disease

Abstract
Objective: To establish the transfection method of vascular endothelial growth factor (VEGF) gene into mesenchymal stem cells (MSCs), to investigate the effect of this gene-transfected MSCs for heart function restoration and angiogenesis after myocardial infarction, and to compare the therapeutic differences among cell therapy, gene therapy, and combined therapy. ***Methods:*** Ischemic heart models were constructed in inbred Wistar rats by ligation of the left anterior descending coronary artery. MSCs of Wistar rats were isolated by density gradient centrifugation and purified on the basis of their ability to adhere to plastic, and identified by checking the surface markers and their differentiation capacity, and then followed by transfection of $pcDNA_{3.1}$-$hVEGF_{165}$ using the liposome-mediated method. The expression of $hVEGF_{165}$ in the transfected cells was detected by Enzyme-Linked Immunosorbent Assay, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western Blot Analysis. The ligated animals were randomly divided into four groups (12 in each) and, after 2 weeks, were injected at the heart infarct zone with $hVEGF_{165}$-transfected MSCs (Combo group), MSCs (Cell group), liposome-hVEGF gene plasmid (Gene group), or medium (Control group). And other six ligated rats (without any injection) were used as Model-assessment group for the baseline heart infarcted size evaluation, and other 12 non-ligated rats (Non-ischemic group) were used as the normal control. Four weeks after the injection, the rats' cardiac function was measured by the Buxco system. Brdu and Troponin-T double labeling and factor VIII were identified by immunohistochemical staining to demonstrate the survival and differentiation of engrafted cells or to evaluate the angiogenesis in the injured heart area; heart infarcted size was calculated by Evan's blue staining. VEGF expression was evaluated by RT-PCR. ***Results:*** MSCs can be successfully isolated and cultured by density gradient centrifugation followed by adherence-separation. The cultured MSCs were CD34–, CD45–, CD44+ and SH+. They can differentiate into osteoblasts and adipocytes successfully. The expression of $hVEGF_{165}$ in the transfected MSCs was demonstrated with Enzyme-Linked Immunosorbent Assay, RT-PCR and Western Blot Assay. Four weeks after the cells were transplanted, among all groups but the Non-ischemic group, the Combo group had the smallest heart infarcted size and the best heart function. The capillary density of the Combo

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group was significantly greater than those of both Cell and Control groups. The heart infarcted size, heart function and capillary density of both Cell and Gene groups were similar with each other and smaller, better and greater than those of the Control group, respectively. Brdu and Troponin-T double staining detected a varied increase in the number of survived cardiomyoctyes at the heart infarcted area, some of which were double stain positive. RT-PCR showed that the $hVEGF_{165}$ gene was expressed in the Combo and Gene groups, and that the former was higher than the latter. ***Conclusions:*** Eukaryotic expression vector $pcDNA_{3.1}$-$hVEGF_{165}$ can effectively be expressed in MSCs. Transplantation of VEGF gene-transfected MSCs can bring better improvement in myocardial perfusion and in restoration of heart function than either cellular or gene therapy alone.

Introduction

After acute myocardial infarction occurs, the basic pathology is characterized by irreversible loss of the massive cardiomyocytes, which are eventually replaced by fibrous non-contractile cells [1]. Although myocytes in the surviving myocardium undergo hypertrophy and cell division occurs in the border area of the dead tissue [2], it is not sufficient to alleviate the progressive deterioration of congestive heart failure. Present therapeutic approaches, including pharmacological and interventional therapies and cardiovascular surgery, cannot result in satisfying outcome in some special patients [3]. Recently, both cellular cardiomyoplasty using bone marrow mesenchymal stem cells (MSCs) [4], myoblasts [5] and embryonic stem cells [6] and therapeutic angiogenesis using vascular endothelial growth factor (VEGF) [7] and basic fibroblast growth factor were reported by some researchers. Yau et al. [8] had evaluated the angiogenic effect of heart cells transfected with VEGF and transplanted into a myocardial scar. However, to our knowledge, reports related to the evaluation of the effects of MSCs transfected with VEGF gene for treating ischemic heart disease are rare.

MSCs, early cells of the mesoderm [9], are present in adult bone marrow tissue. These cells can 'self-renew' and have the plasticity to differentiate into multiple tissues. Some advantages of MSCs are that they can be isolated easily from a variety of sources, have genetic stability, and do not carry immunological or ethical concerns. Hence, they were considered very suitable candidate donor cells for stem cell therapy and target cells for gene transfer [10, 11]. After becoming transfected with an exogenic gene by various vectors, these cells can retain genetic stability and their capacity to differentiate, resulting in long-term and stable gene expression with better performance of incorporation into surrounding tissue, contributing to better cellular transplant effects and higher differentiation efficiencies [12]. We put forward the hypothesis that the combination of MSC transplantation and VEGF gene transfer would be superior to either strategy alone for treatment of chronic myocardial ischemia.

Materials and Methods

Experimental Animals

All animals received humane care in compliance with the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the 'Guide to the Care and Use of Experimental Animals' by the Chinese Council on Animal Care.

The model of chronic myocardial ischemia was constructed as previously described [13] in male Wistar rats (250–320 g, Shanghai Laboratory Animal Center, Chinese Academy of Science). Briefly, after a small thoracotomy was done through the left fourth intercostal space, the rat's left anterior descending coronary artery was ligated by a 6-0 surgical suture. Seventy-eight rats were used for construction of the ischemic model, and 15 for the non-ischemic model (without ligation). Six of 54 rats, selected from the 68 surviving ligated animals, were randomly re-selected as the Model-assessment group for the heart infarcted size baseline evaluation; then, the other 48 rats were randomly and meanly divided into four groups (Combo, Cell, Gene and Control group, 12 rats each). Twelve rats, selected from non-ligated animals, were set as the normal control (Non-ischemic group).

MSC Isolation, Culture and Identification, VEGF Gene Transfer Procedures

MSCs of Wistar rats were isolated by density gradient centrifugation and purified on the basis of their ability to adhere to plastic. In short, the cells were isolated from the bone marrow of limb bones and separated by gradient centrifugation with 1.073 g/ml Percoll solution (Promega, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, USA) containing 15% fetal bovine serum, 1 ng/ml basic fibroblast growth factor and 200 mmol/l glutamine at 37°C in a humidified atmosphere containing 5% carbon dioxide (Forma, USA). When the cells reached about 80% confluence, 0.5 $\mu l/cm^2$ lipofectamine 2000 (Qiagen, Germany) and 1 $\mu g/cm^2$ $pcDNA_{3.1}$-$hVEGF_{165}$ (National Genetic Laboratory, China) were added into the medium. After 6 h of incubation, the medium containing liposome and plasmid was replaced. With full medium replacement after 24 h of culturing, about 10% of the cells were discarded (because they were dead) and the remaining cells were kept in culture until cell density within colonies reached about 80% confluence.

After MSCs were cultured in vitro for 4 weeks, Flow cytoMeter (FCM) (FACSort, B-D Co., USA) was used to analyze the superficial markers such as CD44, SH3, CD34 and CD45 of MSCs. In brief, MSCs were gathered and diluted by PBS at a concentration of 10^6 cells/ml. After incubated with fluorescence-labeled anti-

bodies for 15 min at room temperature, cells were washed twice with PBS and dispersed to make single cell suspension. Fluorescent intensity was detected using exciting light of 488 nm and emission light of 515 nm. Osteoblast differentiation from MSCs was induced by dexamethasone, β-glycerol phosphate and ascorbic acid, and shown by alizarin S on day 14. Adipocyte differentiation from MSCs was induced by insulin and shown by Sudan IV on day 21.

VEGF Gene Expression Analysis in vitro

Enzyme-Linked Immunosorbent Assay

The culture medium (triplicated for each group) was harvested at 2, 4, 6, 8, 11, 14, 17, 21, 23, 25, 27, and 29 days after liposome transfection. hVEGF protein levels in the medium were quantified by an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Jingmei biotech, China) following the protocols listed in the instructions manual.

Reverse Transcription-Polymerase Chain Reaction

MSCs were collected separately from the $pcDNA_{3.1}$-$hVEGF_{165}$ transfected group, the $pcDNA_{3.1}$ group, and the non-transfected group when the cell densities reached about 90% confluence. Then total RNA was extracted by using the Trizol (Gibco BRL, USA) reagent, and cDNA was composed by a Reverse Transcription System Kit (Promega) according to protocols listed in the instructions manual.

Sequences of the human specific primers for $VEGF_{165}$ were designed by using Primer 3 software based on cDNA sequences from Genebank. The forward primer was 5′-CCT TGC TGC TCA CCT CCA C-3′; the reverse primer was 5′-ATC TGC ATG GTA GAT GTT GGA-3′. The length of the product was 280 bp. Two microliters of cDNA product, 10× PCR buffer 2 μl, 10 mM dNTPs 0.4 μl, and 1 U Taq enzyme were mixed in a 20 μl volume in the presence of 50 ng each of 5′ and 3′ primer for PCR. The conditions for PCR amplification were: denaturation at 94°C, annealing at 60°C for 30 s and extension at 72°C for 30 s. After 30 cycles, PCR products were separated by electrophoresis in 2% agarose gel.

Western Blot Assay

The medium of $pcDNA_{3.1}$-$hVEGF_{165}$ transfected and non-transfected MSCs were collected separately and loaded on different 15% SDS polyacrylamide gels for electrophoresis. One type of gel used for both mediums was stained by Coomassie brilliant blue (Sigma, USA). Another gel's protein was transferred to PVDF membranes at 120 V for about 2 h. Subsequently, the membrane's mark path was cut for Coomassie brill blue staining, while the rest was blocked by a blocking buffer at 4°C overnight, followed by incubation with rabbit anti-human VEGF antibody (diluted, 1:200, Sigma) at room temperature for 1 h. Next, the membrane was washed three times with PBS containing 0.1% Triton (Gibco BRL), incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (diluted, 1:1,000) at room temperature for 1 h, and washed again. Finally, the membrane was visualized with an ECL detection system.

Cell Transplantation and Gene Delivery

Two weeks after the ligation and 1 day before transplantation, BrDu solution (Sigma) was added to the culture medium at a final concentration of 10 μg/ml. The animals' chests were reopened and, using a 30-gauge needle, the border of the infarcted zone was injected intramyocardially with the suspension of gene-modified MSCs (3×10^6 cells) for the Combo group, unmodified MSCs (3×10^6 cells) for the Cell group, gene-liposome compound containing 5 μg $pcDNA_{3.1}$-$hVEGF_{165}$ for the Gene group, and 300 μl medium for the Control group. The Non-ischemic group's hearts were penetrated without any injections.

Evaluation of Heart Function

Six weeks after ligation (4 weeks after the injection) was set as the point in time to evaluate the therapeutic effects. After evaluation of heart function, the rat hearts were harvested and divided randomly and evenly into two groups. One was fixed into 4% formaldehydum polymerisatum for immunohistochemistry, the other one was protected with liquid nitrogen for Reverse Transcription Polymerase Chain Reaction (RT-PCR) after the heart scar size calculation.

Just before they were killed, the rats were anesthetized with pentobarbital. A microtip pressure transducer catheter connected to an electrostatic chart recorder (Buxco Electronics, USA) was penetrated into the left ventricular chamber. The left ventricular systolic pressure (LVSP), the left ventricular end-diastolic pressure (LVEDP), and the peak rates of pressure rise and fall (+dp/dt_{max} and −dp/dt_{max}) were recorded and evaluated.

Calculation of Heart Infarcted Size

Two weeks after the ligation (just before the injection) was set as the baseline evaluation, the infarcted size of all the 6 rats in Model-assessment group were measured at this time. The final infarction area evaluation time point was set at 6 weeks after the ligation. Two rats from the Control group were found dead during the 5th week and were included in the final assessment of heart infarcted size. After the hemodynamic measurements, the hearts of animals in all groups were arrested in diastole with 1 ml potassium chloride (100 mmol/l) injected into the ventricular chambers, then removed from the chests after 2 ml 5% Evan's blue (Sigma) was injected through the catheter. The hearts were perfused with 0.9% NS. The left ventricles (including septum) were dissected and weighed, and the remainders of the hearts were discarded. Transverse sections for every 2 mm of the left ventricle were sectioned perpendicular to its longitudinal axis, following a bath with PBS containing 0.1%. N-BT (Sigma) at 37°C for 30 min (pH 7.4). The heart infarcted zone was unstained while the live tissue was stained blue. Next, the specimens were washed again and the previously unstained tissues were cut and weighed. The weight percent of ischemic myocardium in the left ventricle was calculated by the following formula: heart infracted size (%) = (net weight of unstained area/net weight of left ventricle) × 100%. Afterward, these specimen were protected with liquid nitrogen for RT-PCR.

Immunohistochemical Stain

The fixed myocardial specimens were embedded in paraffin and cut perpendicular to the long axis of myocardial fibers in order to yield 5-μm-thick sections for every 1 mm along that axis.

Capillary vessels were processed through two-step reactions with rabbit anti-factor VIII antibodies and stained with DAB (Maixin bio, China) as described in the manufacturer's specifications. After becoming restained with hematine, the samples were coverslipped and photographed. The cytoplasm of the endothelial cells was stained red. To measure the capillary density, the

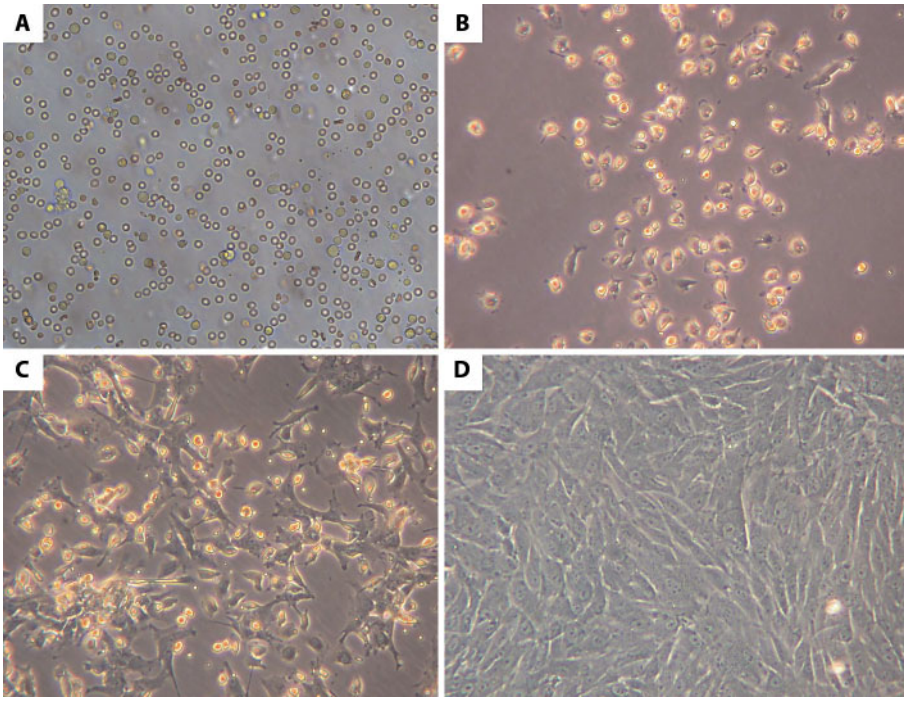

Fig. 1. Morphology of MSCs, newly planted bone marrow cells contained different kinds of other cells (**A**) 24 h later, the cells had short-spindle or short-stick shapes (**B**). 5–8 days later, the cells proliferated into spindle shapes (**C**) and new daughter MSCs grew in the same spindles (**D**). Magnified 100×.

number of capillary vessels in the scar tissue of all groups was counted using a fluorescent microscope (Leica, Germany) at a ×200 magnification. Five high-power fields in each scar were randomly selected, and the number of capillaries in each was averaged as the capillary density. Criteria for being counted consisted of having diameters less than 50 μm and including single or tiny vascular endothelial cells.

BrDu and myocardial Troponin-T double-staining samples were first incubated with monoclonal antibodies against BrDu (Sigma) at 1:80, and then stained with BCIP/NBT (Zymed, USA), which turned the nucleus dark blue. Thereafter, the samples were incubated with monoclonal antibodies against myocardial Troponin T (NeoMarkers, USA) at 1:1,200, then stained with ACE (Maixin bio), which turned the cytoplasm red. Finally samples were incubated with general biotin connecting second antibodies.

VEGF Gene Expression Analysis in vivo

Total RNA was extracted from the frozen infarction tissue using the Trizol reagent, and cDNA was composed by using the Reverse Transcription System Kit according to the company's instructions manual. Two pairs of $VEGF_{165}$ primers were used separately for the PCR amplification. One was the human $VEGF_{165}$ specific primers (primer A) whose sequences were the same with the primer used in vitro mentioned above; another (primer B), whose forward primer was 5′-TAC CTC CAC CAT GCC AAG T-3′ and reverse primer was 5′-GTT GTG CTG TAG GAA GCT CA-3′ and the length of the product was 330 bp, was the primer for both human and rat. And the PCR reaction system and reaction conditions were performed as also the same with those in vitro mentioned above. β-Actin of the rats was selected as references. The primer sequences of β-actin were: forward 5′-ATG CCA TCC TGC GTC TGG ACC TGG C-3′; reverse 5′-CTG CTC CAC CTT GGG CTT GCG ACC CAC-3′. PCR products were separated by size in 2% agarose gel electrophoresis. Then photographs of the gels were taken and the OD values of objective bands were quantified by densitometry. The relative OD ratio of each RNA transcript was calculated with reference to the β-actin.

Statistical Analysis

Data were expressed as mean ± SE. Statistical Product and Service Solutions 11.0 software (SPSS Institute) was used for all analyses. Independent-sample t test and 1-way ANOVA were performed for analysis. The critical level for these analyses was set at $p < 0.05$.

Results

MSC Isolation and Culture

MSCs were isolated from bone marrow, which contains different kinds of cells (fig. 1A), by Percoll gradient centrifugation, and then purified through adherence-separation culturing. MSCs were adhered to the culture bottles within 24 h with short-spindle or short-stick shapes (fig. 1B), and 2–3 days after plantation these cells' shapes and attachments became much more pronounced. Five–eight days after plantation (fig. 1C), MSCs were in spindle shapes proliferating in colonies irradiating from single points or arranged in a vortex. MSCs proliferated into a layer after about 9–14 days. After passage, the new

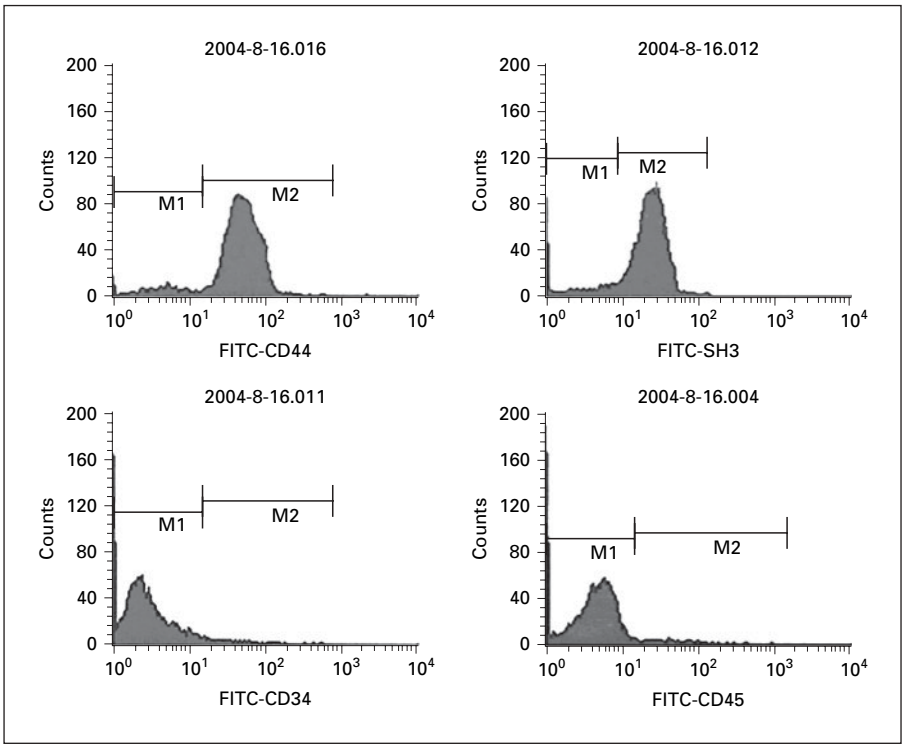

Fig. 2. Cell marker expression CD44 (92.6%), SH3 (99.2%), CD34 (2.93%), CD45 (1.85%).

daughter MSCs grew in spindle shapes with the same morphology (fig. 1D).

Identification of the Surface Markers of MSCs

Immunophenotypic analysis by FACS of cells after four-cell passages showed that cells expressed high level of CD44 (92.6%) and SH3 (99.2%). Cells did not express CD34 (2.93%) and CD45 (1.85%) (fig. 2).

Differentiation of MSCs

Osteoblasts Differentiation from MSCs

After 14 days, a mass of calcium salinity accumulated (fig. 3A), which was red when stained by alizarin S (fig. 3B).

Adipocyte Differentiation from MSCs

The shape of cells changed after day 14 of induced culturing and few fat drop appeared (fig. 4A). After 21 days, more than 80% of cells differentiated into lipid-laden cells which were stained by Sudan IV (fig. 4B).

VEGF Gene Expression in Transfected Cells

ELISA shows the VEGF concentration of the $pcDNA_{3.1}$-$VEGF_{165}$-transferred group peaked 5–6 days after transplantation and no positive results were found in the $pcDNA_{3.1}$-transferred group or in non-transferred group (fig. 5A). After RT-PCR, a new 280-bp band was found in the $pcDNA_{3.1}$-$VEGF_{165}$-transferred group (fig. 5B) and a new 23-kDa band was found in the $pcDNA_{3.1}$-$VEGF_{165}$-transferred group by Western blot assay (fig. 5C).

Ischemic Model

Two rats from the Control group died during 5 weeks after the ligation and were included in the final assessment for heart infarcted size.

The Non-ischemic group heart specimen were well ordered and evenly developed, without thinning of the myocardium (fig. 6A1). The three layers of the myocardium were well defined. There was no evidence of cardiac fibrosis, and breakage of myocytes (fig. 6A2). A significant loss of cardiac contractility after the ligation was observed in specimens from the other groups. The color of the myocardium gradually darkened from red to black. Six weeks after ligation, the infarcted size of the Control group hearts increased continually (fig. 6B). However, the infarcted size of the Combo group hearts increased to a limited extent only. The myocardium of the infarcted areas in Combo group specimens was thicker than that in Control group specimens (fig. 6C).

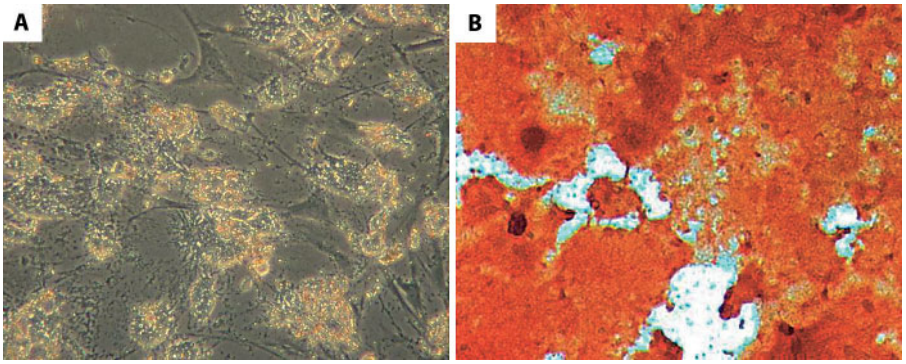

Fig. 3. Calcium salinity accumulated after 14 days of induced culturing (**A**), calcium salts were red when stained by Alizarin S (**B**) (100×).

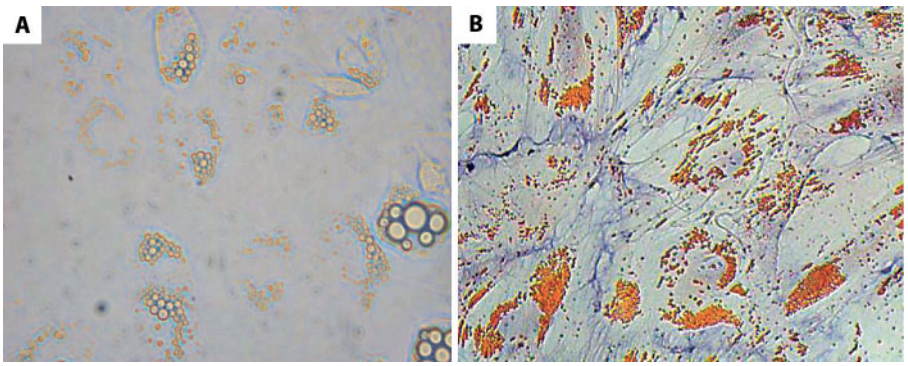

Fig. 4. Fat drops present after day 14 (**A**); fat drops were red when stained with Sudan IV and nuclei were blue when stained with hematoxylin (**B**) (200×).

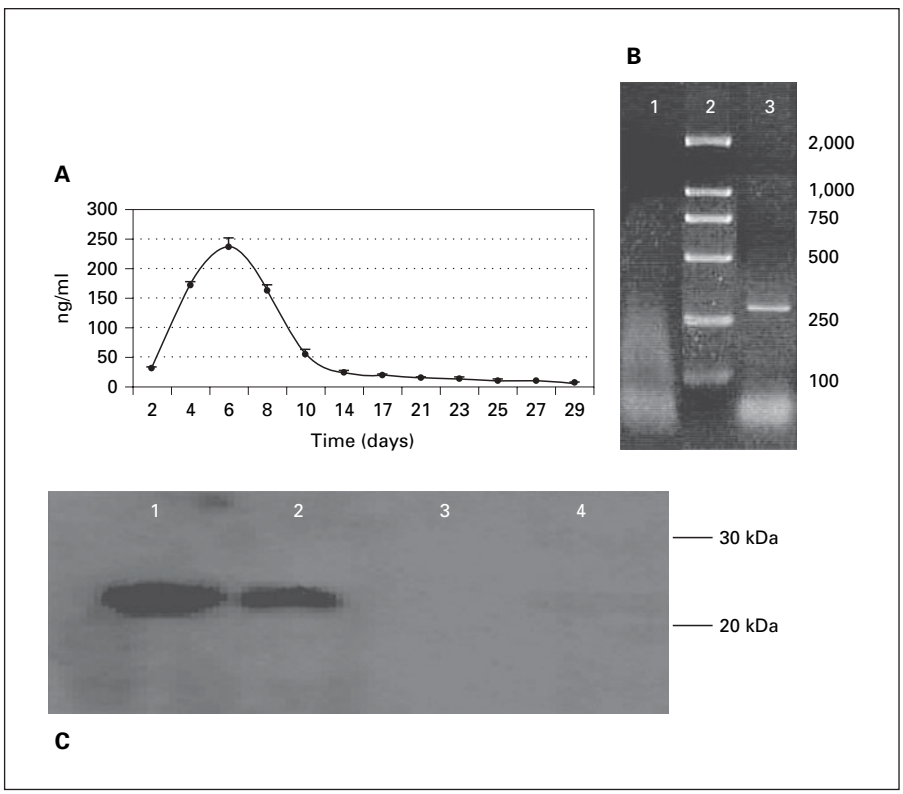

Fig. 5. **A** The VEGF concentration of the pcDNA$_{3.1}$-VEGF$_{165}$-transferred group. **B** The RT-PCR analysis: (1) non-transferred MSCs, (2) marker, (3) MSCs transferred with pcDNA$_{3.1}$-VEGF$_{165}$. **C** The Western blot analyses: (1) and (2) MSCs transferred with pcDNA$_{3.1}$-VEGF$_{165}$, (3) and (4) non-transferred MSCs.

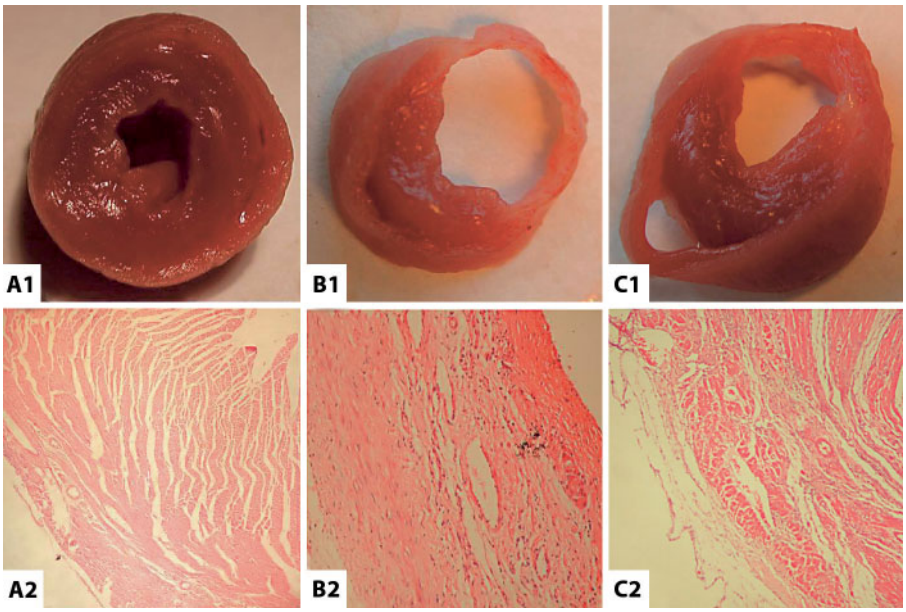

Fig. 6. Pictures of hearts' transverse sections and hematoxylin and eosin staining (50×), the detailed description was in the article above.

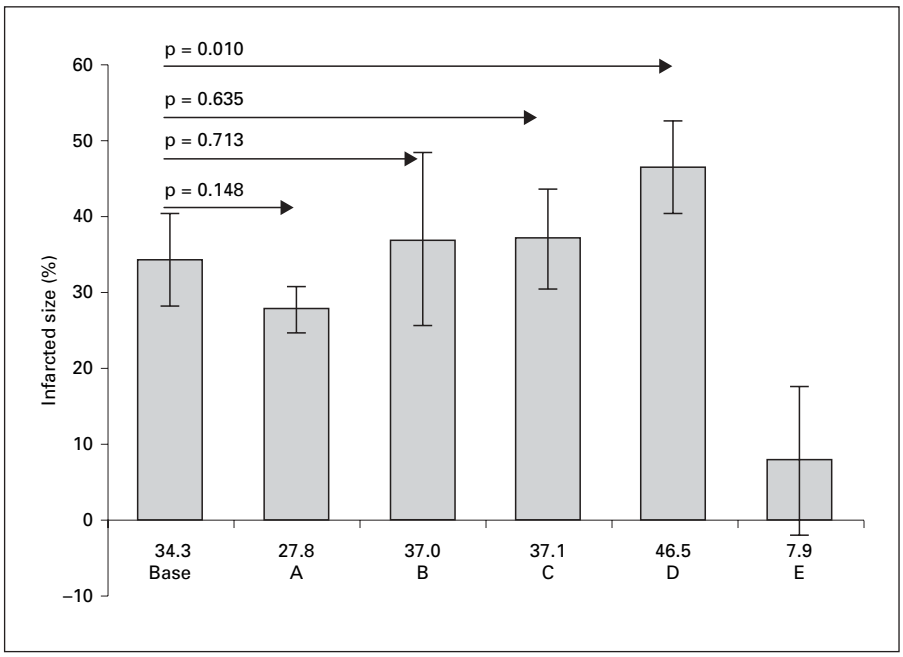

Fig. 7. Compared with the baseline, the heart infarcted size of Combo group (A) was not enlarged, neither did those of Cell (B) and Gene (C) group, the infarction expansion of Control group (D) was continued.

Heart Scar Size

Compared to the Baseline, the Infarction Expansion of All the Treated Groups Was Limited

The heart infarcted size of baseline was gained from the 6 rats of Model-assessment group at the time point of 2 weeks after the coronary artery ligation. Figure 7 shows that 4 weeks after the injection, the infarction expansion of Combo, Cell and Gene group was stopped, while the infarcted size on the Control group's heart was still enlarged.

Four Weeks after the Injection, the Infarcted Size of the Combo Group Was Smaller than Those of Cell, Gene and Control Group

The heart infarcted size, 4 weeks after the injection, is shown in figure 8. Compared to the others but the Non-

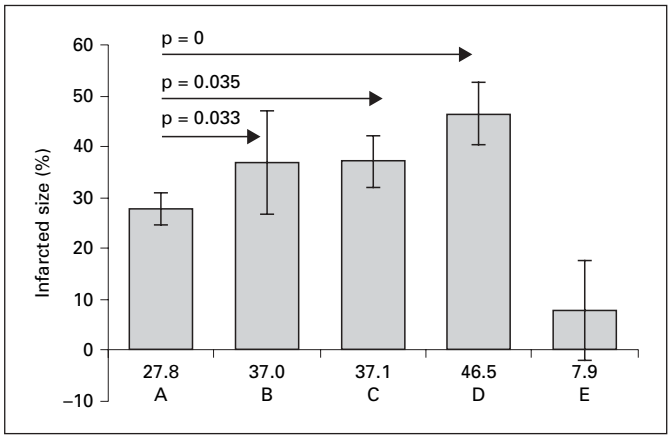

Fig. 8. The heart infarcted sizes of all groups at 4 weeks after the injection. Combo group (A) had smaller sizes than those in the Cell (B) and Gene (C) group, which were smaller than those in the Control group (D). Non-ischemic group (E) had the smallest infarcted sizes.

ischemic group, the Combo group had the smallest heart scar size (p = 0.035 vs. Cell group, 0.033 vs. Gene group, 0.000 vs. Control group). Sizes in the Cell and Gene groups were similar (p = 0.875) and both were smaller than the sizes in the Control group (p = 0.044, 0.032).

Measurement of Heart Function

Four Weeks after the Injection, Combo Group Heart Function Mostly Improved

Except for the Non-ischemic group, the Combo group had the highest values of LVSP and $+dp/dt_{max}$. Next highest were the Cell and Gene groups, followed by the Control group, with the lowest values of LVEDP and $-dp/dt_{max}$, followed consequent was the same of above. The higher values are for LVSP and $+dp/dt_{max}$ and the lower the values are for LVEDP and $-dp/dt_{max}$ indicates the better heart function. All of these indexes in the Combo

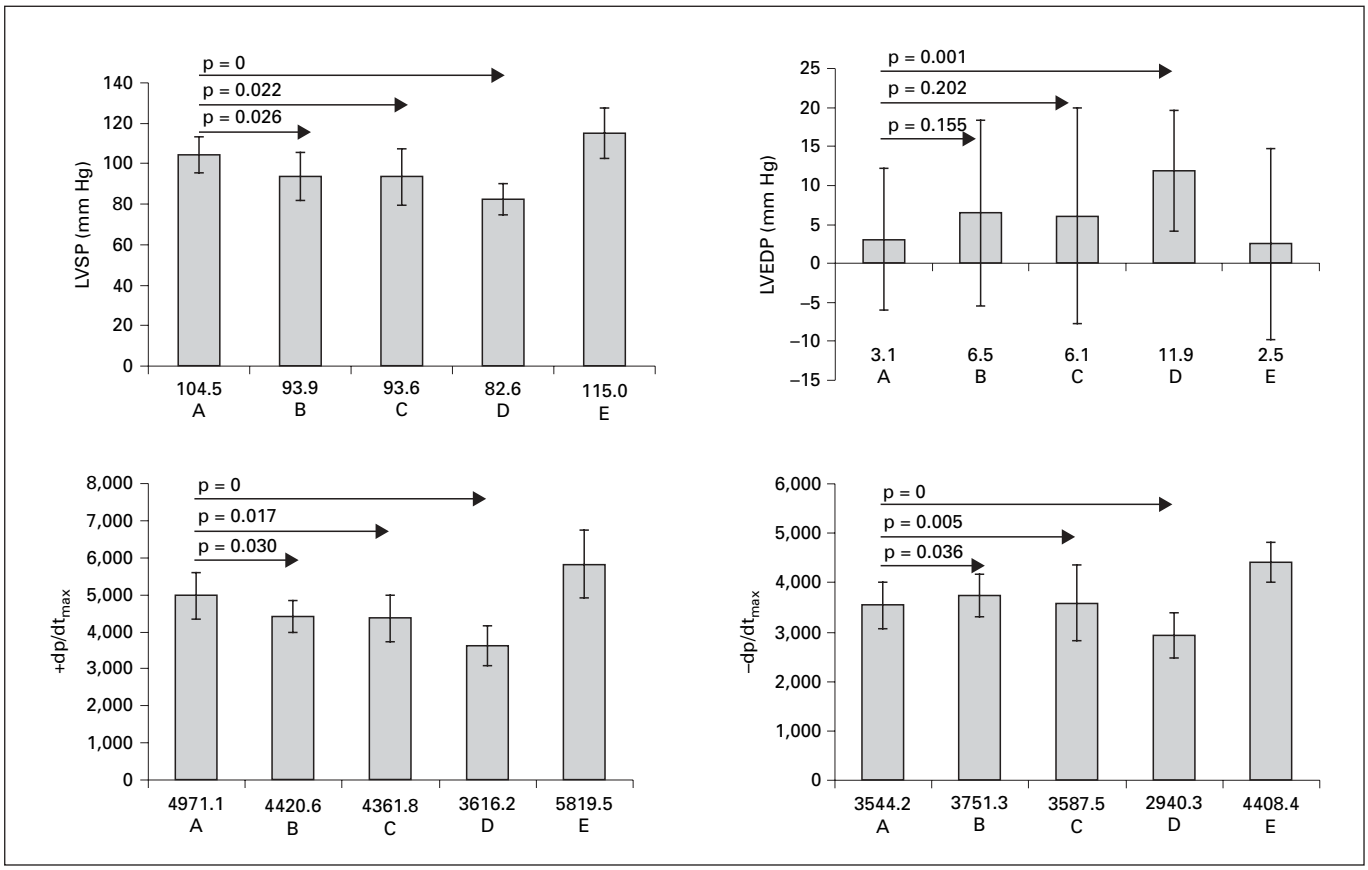

Fig. 9. Heart function comparative figure. LVSP and $+dp/dt_{max}$ of the Combo group (A) were significantly higher than those of the Cell (B) and Gene groups (C), $-dp/dt_{max}$ of the Combo group was significantly lower than those of the Cell and Gene groups. LVEDP of the Combo group had a trend of lower LVEDP and $-dp/dt_{max}$ than those of the Cell and Gene groups. Control group (D) had the worst heart function; non-ischemic group (E) had the best heart function.

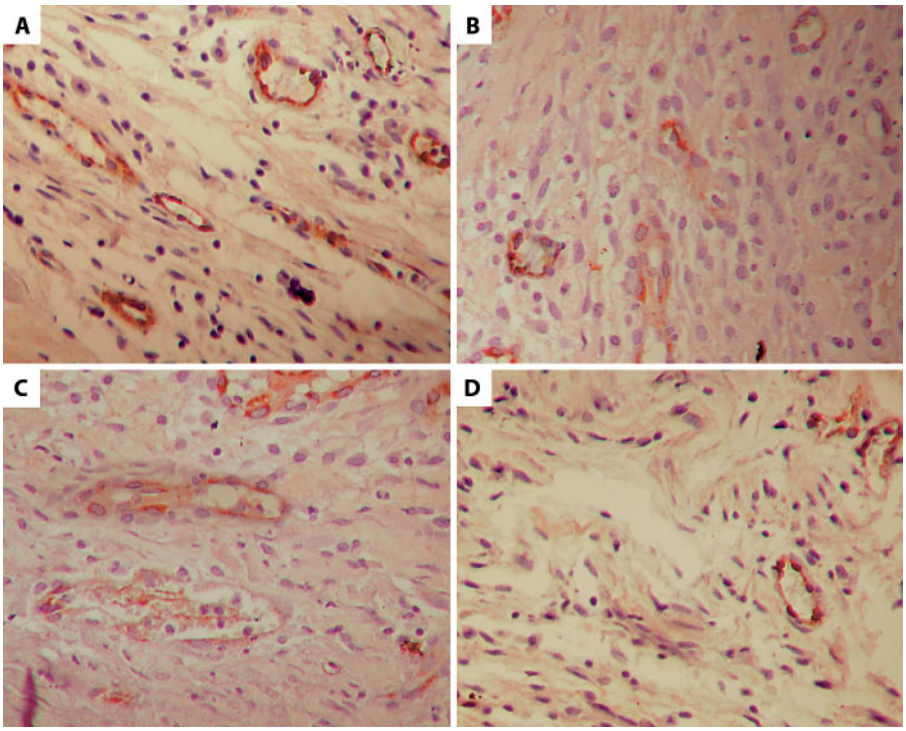

Fig. 10. Factor VIII-related antigen immunohistochemical staining; the positive stain was identified by DAB which displayed endothelial cells as brown-red. A varying increase in the number of vessels was shown in the heart infarcted zone of the Combo (**A**), Cell (**B**) and Gene (**C**) groups, while less growth was observed in the Control (**D**) group (200×).

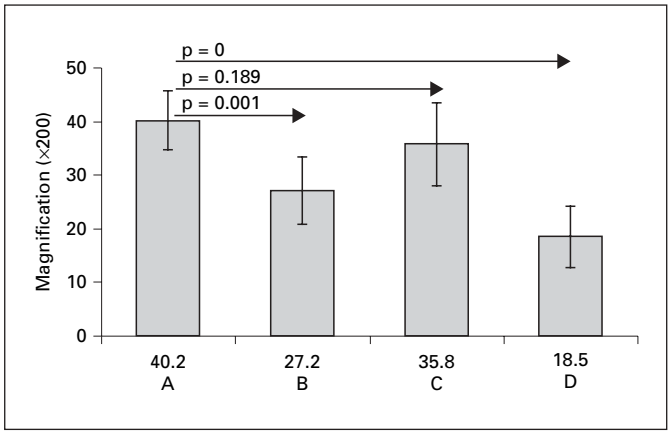

Fig. 11. The Combo group (A) had the greatest capillary density among all the groups, significantly greater than that of the Control (D) and Cell (B) groups, and with a trend higher than that of the Gene group (C).

group were superior to those in the Control group. In addition, the Combo group's indexes for LVSP and +dp/dt_{max} were superior to those in the Cell and Gene groups, which in turn were superior to those in the Control group again (all $p < 0.05$) (fig. 9).

Immunohistochemical Assay

Factor VIII-Related Antigen Staining

Endothelial cells of capillary vessels were shown through the immunohistochemistry reaction with rabbit anti-factor VIII antibody and DAB staining (fig. 10).

A varying increase of capillaries was observed in the heart infarcted zone of the Combo, Cell and Gene groups. Both the Combo and the Gene groups had greater capillary densities than the Cell group ($p = 0.001$, 0.029). The densities in the Cell group were significantly greater than those in the Control group ($p = 0.029$) (fig. 11).

BrDu and Troponin-T Double Staining

In BrDu and myocardial Troponin-T double staining, the former showed cell nuclei as dark blue or purple spots, stained by BCIP/NBT, and the latter turned the cytoplasm red, stained by ACE (fig. 12). Figure 12A showed there was only Troponin-T double staining-positive cells, which were thought to be host cardiomyocytes, located at the heart non-ischemic area. While at the heart infarcted zone of the Control group, almost no myocytes were observed (fig. 12B). And at the infarcted area of both the Combo (fig. 12C) and the Cell groups (fig. 12D), a varying increase of surviving myocytes were observed, furthermore, some of them were BrDu and Troponin-T double staining-positive cells.

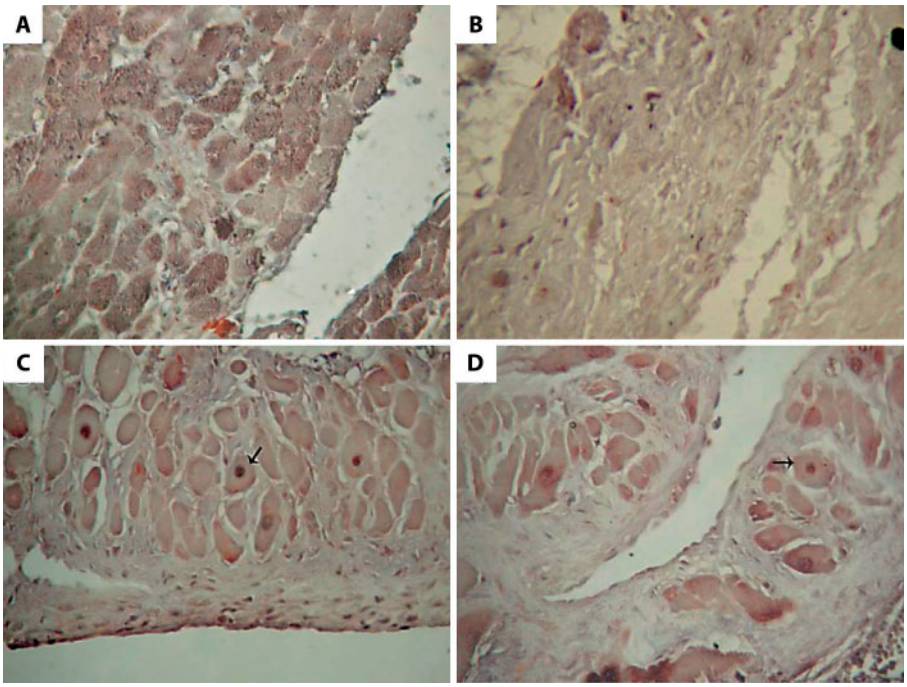

Fig. 12. Immunohistochemical characterization of BrDu and Troponin-T double staining at 4 weeks after the transplantation.

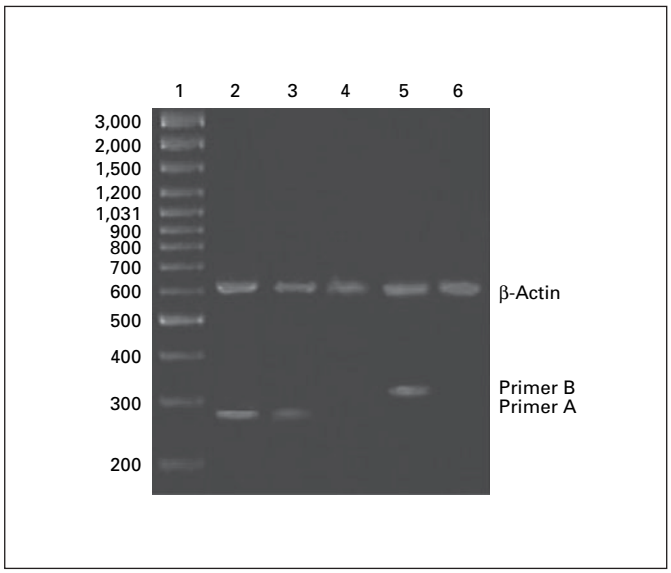

Fig. 13. hVEGF mRNA expression in rat hearts. The 330-bp band was the amplification of primer B, the 280-bp was primer A and the 607-bp band was the β-actin. The first lane was a DNA marker. Positive expression (280 bp) was only observed in the Combo (2) and Gene (3) groups, not in the Cell (4) and Control (6) groups. The Combo group had a greater hVEGF expression than that of Gene group. The 5th lane was Cell group amplified by the primer B.

VEGF Gene Expression in vivo

Human VEGF-specific primer amplified mRNA was observed only in the Combo and Gene groups. The relative concentration of hVEGF mRNA of the former was higher than that of the latter (0.182 ± 0.043 vs. 0.095 ± 0.031, p = 0.007) (fig. 13).

Discussion

MSCs were firstly cultured by utilizing the adhesion to plastic by Friedenstein et al. [9]. Due to the complexity of marrow cells, the MSCs isolated by adhesion way was quite a mixture. Although with the increase times of passage and exchange of fluid other cells would be eliminated slowly, the homogeneity of BMMSC growth and properties would be disturbed. In this study, 1.073 g/ml Percoll-mediated density-gradient centrifugation made the adhesive cells grow to a satisfied configuration. Some scholars isolated the MSCs by immunomagnetic microspheres, with the surface marker on it and got more purified MSCs, but this was not suitable and widely used either in clinical or experimental researches because of its high expenditure, no specific surface marker on MSC and the necessity for further purification by adhesion.

The plasticity of differentiating to multi-tissue also gives MSCs the feature of lacking special surface markers,

which bring great difficulty to its identity work. Inspecting the surface antigens with flow cytometry combined with differentiation tests provides good practicability. Analyzing more than 70 antibodies of MSCs selected from 500 different individual samples, Pittenger et al. [14] did not find even one specific marker for the exclusion. We examined four markers, and the cells shown in the result were SH3+, CD34–, CD45– and CD44+, which are consistent with the quality of MSCs. We successfully induced MSCs into both osteoblasts and adipocytes within the same sample and got the similar results on multiple specimens. Added with the inspection of morphologic and surface markers, we can safely draw the conclusion that we have set up a steady culture system for MSCs in vitro.

Two days after the transfection of $pcDNA_{3.1}$-$VEGF_{165}$, evaluated by RT-PCR, the gene-transfected MSCs could express $VEGF_{165}$ mRNA. We can infer $VEGF_{165}$ protein could be secreted by gene-transfected MSCs by specific positive cross-fertilize band of Western. ELISA assays showed that quite amount of $VEGF_{165}$ in the supernatant fluid of culture medium 24 h after transfection and reached a climax at the 6th day. Then there goes a predominant descending phase, but maintains an expression level that could be detected for 4 weeks after transfection. Above all, we can infer that if the target cells could be transplanted into ischemic myocardium in 3 days, the secretion climax of $VEGF_{165}$ would definitely establish in vivo.

The use of stem cells as the gene transfer vehicle in vitro has several advantages. This strategy can be easily implemented, and it keeps the host cell from coming into direct contact with the liposome. Thus, the potential for liposome toxicity is low [15]. In this study, MSCs were used as cellular vehicles for $hVEGF_{165}$ gene transfer, and the ELISA demonstrated that the $hVGEF_{165}$ expression can last about 30 days, which meets the demands of vascular growth and can avoid the side-effects caused by prolonged gene expression. At the same time, MSCs may undergo transdifferentiation and regenerate cardiomyocytes.

Two weeks after the LAD ligation, when the local edema has almost disappeared and a tight fibrotic scan has not yet been formed, it is the optimal time for injection of the transplanted cells, as this is most advantageous for the survival of the cells. Four weeks after the injection, all treated groups had their heart infarction expansion limited when compared with the baseline and the Combo group had the smallest heart infarcted size when compared to the Cell and Gene groups, which in turn had smaller heart infarcted sizes than the Control group. Consistent with the infarcted size, the Buxco system revealed that the cardiac function improvements had occurred in the Combo, Cell and Gene groups, but most dramatically in the Combo group. So, our data indicate that the combination of cellular and gene therapy may be superior to either strategy alone for limiting the ventricular remodeling process, preventing the infarct from thinning and dilatating.

The initial aim of cellular transplantation, based on the assumption that the engrafted cells can undergo milieu-dependent differentiation into cardiomyocytes, was to replace the necrotic cardiomyocytes with new contractile cells. But recently, whether those exogenous engrafted cells could transdifferentiate into cardiomyocytes, and if any, whether they could form membrane potential couples with the host cardiomyocytes has been under hot debate. Four weeks after the intramyocardial injection of the border of the infarcted zone, BrDu and Troponin double stain-positive cells located in the surviving myocardium of the Combo and Cell groups were observed. Without high-power laser-scanning confocal microscopy, it is very difficult to determine whether those double stain-positive cells are newly differentiated endogenous cells or the product of cell infusion [16]. But the results of heart function improvement and more surviving myocytes in the infarcted zone can support the suggestion raised by Zhang et al. [17] that both transdifferentiation and cell infusion are two approaches of human stem cells to differentiate into cardiomyocytes. Consequently, we think that the differentiation of engrafted MSCs, mobilization of endogenous stem cells, and the decrease of myocyte apoptosis and necrosis [18] caused by angiogenesis all contributed to more surviving myocytes in the infarcted zone of Combo, Cell and Gene groups, while few myocytes survived in the Control group.

MSCs may differentiate into vascular endothelium and incorporate into growing capillaries or promote angiogenesis, mitigating the adverse effects of ventricular remodeling through paracrining a series of angiogenic cytokines, including VEGF [19]. And these cytokines contribute to the rescue of hibernating cardiomyocytes in the infarcted area and mobilization of endogenous stem cells to home the injured site that results in the inhibiting of the further expansion of infarct and fibrosis of the myocardium after myocardial infarction [20]. The increased vessel permeability is in favor of MSCs to optimize the arrangement of extracellular matrix associating with better support functions [21, 22], and then, better global heart performance. Neovascularization in the in-

farcted myocardium facilitates the survival and transdifferentiation of implanted stem cells. Above all, MSCs may explain why the combination of cellular and gene therapy could yield better effects than cellular use alone. Additionally, pri-genetic modification can enhance the transplanted stem cell's survival ability in the scar fibrosis environment [23]. Mangi et al. [12] demonstrated that transplanted Akt (pro-survival) modified MSCs could prevent remodeling and restore performance of infarcted hearts.

VEGF, a highly specific mitogen for vascular endothelial cells, induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and plays a pivotal role in the regulation of vasculogenesis [24]. And the expression of VEGF gene is restrictedly controlled by a hypoxia-inducible factor [15].

Using specific primer of human VEGF, mRNA proliferation was observed by RT-PCR in hearts of Combo and Gene groups, and the former was higher than the latter, which indicates that the strategy of using stem cells as delivery vehicles may generate a more stable gene expression than direct gene injection does. The expression of VEGF strongly relates to formation of a new vessel. Factor VIII-related antigen, existing on the normal endothelial cells of vessel and cardiac tissues, demonstrated by an immunohistochemical stain showed that the capillary density was greater in treated groups than that in the Control group, which coincides with angiogenic effect of VEGF. Compared with the Gene group, the Combo group had a higher VEGF gene expression but did not have a significantly greater capillary density. Regarding the greater heart function improvement and smaller infarcted size of the Combo group, we add that it is precisely a microenvironmental VEGF concentration, not total dose that determines a threshold between normal and aberrant angiogenesis [25]. Our data suggest, while favorably affecting stable gene expression, the stem cells contained in the combined regimen may also contribute to precise control and physiological free-back calibration of VEGF concentration though paracrine multiple cytokines [26], resulting in optional control of angiogenesis and not only strengthening the newly formed vessel for effective transport function but also preventing the abnormal angiogenesis from associating with angioma.

For chronic ischemic heart disease, there are fewer cardiomyocytes that survive, although VEGF obviously stimulates the formation of new vessels, without replenishing contractile cells, the further cardiac function improvements of injection of liposome-VEGF compound is limited because there are no effective regeneration of cardiomyocytes. Regardless, some host cells located at the border of infarcted zones can be rescued.

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