The therapeutic effects of MSCs-based IGF-1/EGFP dual gene by sorting system in rat myocardial infarction model

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The therapeutic effects of MSCs-based IGF-1/EGFP dual gene by sorting system in rat myocardial infarction model

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ABSTRACT

The therapeutic effects of MSCs-based IGF-1/EGFP dual gene by sorting system in rat myocardial infarction model

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Myocardial infarction (MI) is the most important disease of the cardiovascular diseases because it causes cardiomyocyte death, scar formation, wall thinning and collagen degradation via blockage of the coronary arteries. Mesenchymal stem cells (MSCs)-based gene therapy has attracted attention due to their unique therapeutic properties such as capacity of multipotent differentiation into variety of cell types, immunomodulation, and paracrine effects. Furthermore, Insulin-like growth factor-1 (IGF-1) has substantially beneficial effects on cardiomyocyte function including cell proliferation and survival. In the present study, a simplified method have been established the MSCs-based IGF-1/EGFP gene therapy using the selection plasmid vector with sorting system.

To improve the transfection efficiency of MSCs using non-viral carriers, the selection vector was constructed by inserting IGF-1 coding sequences as a target gene into

pEF1/His C vector, and sequentially cloned enhanced green fluorescent protein (EGFP) as a selection marker gene with its CMV promoter. By using the selection vector (pEF1/His C::IGF-1::EGFP), both IGF-1 and EGFP genes were expressed in MSCs *in vitro*. Dual-genes expressing MSCs were collected solely via fluorescence activated cell sorter (FACS) system and the purity was approximately 95.1%.

To investigate the therapeutic effects by sorted pEF1/His C::IGF-1::EGFP-MSCs, rat MI models were divided into 4 groups; PBS, control MSCs, unsorting MSCs and sorting MSCs. After 2 weeks after MSCs transplantation, the MI hearts were harvested. The green fluorescence as transfected MSCs was visualized in myocardium of ischemic left ventricle and observed in both unsorting and sorting groups. Interestingly, the IGF-1 transfected MSCs were significantly localized in sorting groups compared to unsorting groups. Also, the expression of IGF-1 was increased in sorting groups.

The TTC staining and the Masson's trichrome staining were proceeded to evaluate the therapeutic effects of dual gene system with FACS through histological assessment. The infarct size by TTC staining was significantly decreased in sorting groups compared to other groups (sorting 5.74% vs. PBS 31.50%, Intact MSCs 12.61%, unsorting 10.42%). The fibrosis area of myocardium layer relatively decreased in sorting group compared to other groups.

The present study demonstrated that the expression of IGF-1 can attenuate the fibrosis and reduce the infarct size in rat MI models. Also, the selection plasmid vector with sorting system efficiently enhanced therapeutic effects in MSCs-based gene therapy. Furthermore, this selection plasmid vector together with sorting system could be a practical application in a wide range of diseases. Also, it could be used in cell-based gene therapy as a treatment tool, by simply replacing the gene of interest or based cell source.

Key words: insulin-like growth factor (IGF-1), selection plasmid vector, dual promoters, sorting system, transduced gene effect, myocardial infarction (MI)

I. INTRODUCTION

Cardiovascular diseases (CVDs) are the heart or blood vessel diseases such as myocardial infarction, hypertensive heart disease, cardiomyopathy, atrial fibrillation, peripheral artery disease and venous thrombosis. Furthermore, CVDs are the leading cause of death throughout the world [1,2,3]. One of the important CVDs is myocardial infarction (MI) because it causes cardiomyocyte death, scar formation, wall thinning and collagen degradation via blockage of the coronary arteries [4,5]. Therefore, therapeutic strategies are essential to repair the damaged cardiac function and prevent the destructive ventricular remodeling after MI.

Many clinicians and researchers have been trying to treat MI such as balloon angiography and stent, bypass surgery, heart transplantation, and artificial heart surgeries [6,7,8,9]. However, the current treatments for MI still remain the problems to be solved as follows; myocardial salvage is incomplete due to restenosis and a very high cost. Furthermore, heart transplantations are interrupted by shortage of donor organs [8,9]. Recently, the stem cell-based therapies have been suggested as a promising approach for people suffering a MI [8].

For restoration of damaged cardiac after MI, prior investigators have attempted cells transplantation into the injured myocardium to repair of cardiac function. A variety of cell types was used to restore damage heart as follow; cardiomyocyte progenitor cells, hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), cardiac stem cells (CSCs), embryonic stem cells (ESCs), skeletal myoblast, fetal and umbilical cord blood cells [8,10].

MSCs are free of ethical concerns and can be easily obtained from the patient [11]. That is why MSCs are the best cell type as a source for transplantation into MI heart.

Moreover, MSCs have many advantages such as ease of isolation, capacity of multipotent differentiation into variety of cell types, immunomodulation, and paracrine effects [9,11,12]. However, MSCs-based cell therapy has limitation as a new treatment for patients with various diseases because of their low transplantation and poor cell viability [12]. Hence, many studies have been tried to overcome the limitation and enhance the therapeutic effects of MSCs-based cell therapy, including gene transfection [13].

MSCs-based gene therapy has developed the strategies of cell treatment via viral carrier or non-viral carrier. However, the therapeutic effects of transfected MSCs are reported to be low. The reason is that MSCs have low transfection efficiency using non-viral carrier [13,14]. Originally, gene delivery systems can be divided into two strategies: viral carrier and non-viral carrier [14]. The viral carrier systems including retrovirus, lentivirus, and adenovirus are high efficient in gene transfection (also called infection). However, the viral carrier systems still remain disadvantages such as insertional mutagenesis and immunogenicity, and pathogenic risk for clinical trials. On the other hand, non-viral carriers are efficient delivery systems due to their safety, stability, easy manufacturing, and low immunogenicity**.** But, the disadvantage of non-viral carrier systems is relatively low transfection efficiency compared to viral infection, approximately 20~25% [14,15,16]. Therefore, the low transfection efficiency of non-viral carrier systems is the most critical for clinical trials.

Insulin-like growth factor-1 (IGF-1) is similar to structure of insulin hormone and also called somatomedin C [17]. The IGF-1 is secreted by almost all tissues and plays an important role in cell growth, differentiation, and transformation [18,19]. In the heart, IGF-1 has substantial beneficial effects on cardiomyocyte function including proliferation and survival [18]. According to *Davis et. al.*, IGF-1 deficiency in IGF-1 knockout mice leads to an impaired cardiac remodeling and increases apoptosis in myocardial infarction. While overexpression of IGF-1 prevents cell death of myocardium, attenuates ventricular dilatation and wall stress in infarcted heart [18]. Researchers have noticed that IGF-1 administration would be promising method to prevent ischemia-reperfusion injury.

Methods of IGF-1 administration are classified into two categories; gene and protein delivery. Injection of recombinant protein is a simple method, but it has limitations involving expensive price or short half-lives of the protein to delivery an adequate amount of the protein. Whereas gene transfer therapy, especially using non-viral gene transfer, is inexpensive and has low cytotoxicity compared to viral gene transfer method [20]. Also, the angiogenic factors including IGF-1, vascular endothelial growth factor (VEGF), and transforming growth factor-beta (TGF-beta) were significantly increased after IGF-1 treatment in MI animal models. Furthermore, previous investigators demonstrated that IGF-1 induced the activation of resident cardiac stem cells and increased engraftment and survival of several cell types including MSCs, embryonic stem cells and smooth muscle cells [18].

To improve the therapeutic effect of MI, selection vector system containing dual gene was constructed by enhanced green fluorescent protein (EGFP) as a selective marker and IGF-1 as a gene of interest. The dual promoter vectors were used for blocking of fusion proteins. The aim of this study is the verification of therapeutic effects via MSCs-based gene (IGF-1) therapy using FACS sorting system in rat MI models.

II. MATERIALS AND METHODS

2.1. Animals

Spraque-Dawley (SD) rats from ORIENT BIO (Seongnam, Korea) were cared for according to the Association for Assessment and Accreditation of Laboratory Animal Care International system. All animal experiments confirmed to the International Guide for the Care and Use of Laboratory Animals. All experimental procedures were examined and approved by the Animal Research Committee of Yonsei University College of Medicine.

2.2. Construction of selection vector - pEF1/His C::IGF-1 & pEF1/His C::IGF-1::EGFP

pEF1/His C::IGF-1::EGFP selection vector was constructed by inserting rat IGF-1 cDNA and EGFP DNA into pEF1/His C vector. pEF1/His C vector (invitrogen) are 6.2kb size designed for overexpression of recombinant proteins in mammalian cell lines. This construction of vector was divided into two steps; first step was rat IGF-1 cDNA was inserted into pEF1/His C vector (pEF1/His C::IGF-1 vector). And second step was EGFP DNA inserted into pEF1/His C::IGF-1 vector (pEF1/His C::IGF-1::EGFP vector).

Rat IGF-1 cDNA was amplified by PCR with DNA polymerase (TaKaRa) using MSCs cDNA as a template. The forward and reverse primers for IGF-1 were tagged EcoRI sequence and XbaI sequence, respectively. (Table 1). The PCR cycling conditions were 95℃ for 5min, 32 cycles of 95℃ for 1min, 65℃ for 1min 30sec, 72℃ for 40sec and 72℃ for 10min for final extension.

The amplified IGF-1 PCR products were loaded in 0.8% agarose gel and run. Then, IGF-1 fragments were eluted with gel extraction kit (LaboPass), and the IGF-1 elution and

pEF1/His C vector was digested with restriction enzymes EcoRI (1830) and XbaI (1869), respectively. After enzyme cleavage, IGF-1 and vector fragments were purified and concentrated by using clean-up spin column (LaboPass). To fusion with IGF-1 and pEF1/His C vector fragments, DNA ligation reaction was conducted at 16℃ overnight incubation using T4 ligase (NEW ENGLAND BioLabs). Ligation products were transformed into DH5α competent cells. Transformed colony was screened by colony PCR to confirm whether they have an insert of interest. And this chosen cloning vector was confirmed by digesting PstI sites and verified through sequencing via Cosmogenetech. pEF1/His C::IGF-1 vector was constructed by this step.

Next step was sub-cloning by inserting EGFP DNA into pEF1/His C::IGF-1 vector. It was too large to insert EGFP DNA (1.6kb) into pEF1/His C::IGF-1 vector (6.6kb), so pEF1/His C::IGF-1 vector was reduced by cutting in restriction enzyme PvuII sites (2152, 3248, 4317). EGFP DNA was isolated from pEGFP-C1 vector by cutting the site of restriction enzyme AseI (7) and MluI (1641). And then, Pfu DNA polymerase (ELPIS) has been used to make sticky ends into blunt ends with filling in step at 72℃ for 30min. Shortly after DNA precipitation step was processed. DNA ligation was conducted at 16℃ overnight incubation. This final cloning IGF-1/EGFP vector was confirmed by digesting EcoRI and NdeI restriction enzyme sites and examined through sequencing.

2.3. MSCs isolation, culture, and characterization

MSCs were isolated from four-week-old male Sprague Dawley rats (100±5g) by flushing the femurs and tibias with low glucose-Dulbecco's modified Eagle's medium (DMEM) (Welgene) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin solution (Gibco), using a 19-gauge needle. Flushing media were centrifuged at 1,600rpm for 5 minutes and suspended in complete medium. And then, suspension cells media were loaded onto a Ficoll-Paque (GE Healthcare Life Sciences). After being centrifuged at 1,600rapm for 30min, the following three layers (top, middle, and bottom) were visible. Transferred middle layer (mononuclear bone marrow cells) in

new 15ml conical tube and washed twice with PBS. This mononuclear bone marrow cells were seeded onto 100mm culture dishes. After 72hrs incubation, non-adherent cells were discarded, and the adherent cells were thoroughly washed twice with PBS and changed fresh MSCs media. This washing step was repeated every 2 days for 1 week.

Characterization of MSCs was identified by presence of cell surface marker through Flow cytometry. Suspended single cells in FACS buffer ($1X10^6$ cells/100ul) were stained at 4℃ for 1hr with surface marker antibodies such as CD29-FITC (Biolegend), CD54-PE (Biolegend), CD90-FITC (Biolegend), CD45-PE (Biolegend), CD49d-FITC (Biolegend) according to the manufacturer's instructions. After 1hr, cells were washed twice with PBS and resuspended in 300ul PBS. Fluorescent intensities of MSCs stained by antibodies were detected by BD FACSVerse™ flow cytometry. Data of each sample were detected by FITC (488nm) and PE (561nm) fluorescent parameters. Also, compensation step was proceeded to prevent for overlapping signals between two fluorescence dyes. Data analysis was performed by using BD FACSuite™ software.

2.4. *in vivo* transfection using non-viral carrier and pEF1/His C::IGF-1::EGFP vector in MSCs (complexes of pEF1/His C::IGF-1::EGFP + PAM-ABP)

To confirm optimal time and concentration of transfection, the MSCs were plated at a density of $2x10^5$ cells in 60mm culture dishes for 48hrs. MSCs were transfected with non-viral carrier PAM-ABP and selection vector complexes at serial amount of pEF1/His C::IGF-1::EGFP ranging from 0 μg to 16 μg (0 μg, 0.5 μg, 1 μg, 2 μg, 4 μg, 8 μg and 16 μg). Complexes of plasmid DNA and PAM-ABP were mixed by weight ratio of 1:5, and conjugated in 100μl PBS at room temperature (RT) for 30 minutes. Also, MSCs medium was exchanged with serum free DMEM for starvation during complex conjugation time. And then, PAM-ABP and selection vector complexes were treated in the MSCs. After 4hrs of transfection, MSCs were washed with PBS and replaced with fresh complete medium. Continuously, transfected MSCs were incubated in 37℃ incubator for 24hrs or 48hrs. After that time, the transfected cells were confirmed by fluorescent microscope for

detection of enhanced green fluorescent protein (EGFP). The conditioned medium was collected at 24hrs or 48hrs after transfection and measured the level of IGF-1 protein secreted by transfected MSCs using an ELISA kit (R&D Systems). Also, transfected MSCs were harvested using RNeasy mini kit (QIAGEN) for extraction of RNA.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

pEF1/His C::IGF-1 or pEF1/His C::IGF-1::EGFP transfected cells were harvested 24hrs or 48hrs after transfection and total RNA was extracted by using RNeasy mini kit (QIAGEN). cDNA was synthesized by using Promega RT reagents (Promega) according to manufacturer's protocol. Briefly, RNA with oligo(dt) primer and Nuclease-free water was preheated for 5min at 70℃, and immediately chilled in 4℃ for at least 5min. Reverse transcription reaction mix (nuclease-free water, ImProm-II™ 5X Reaction Buffer, MgCl2, dNTP Mix, Recombinant RNasin® Ribonuclease Inhibitor , ImProm-II™ Reverse Transcriptase) was added to RNA and primer mix for final reaction volume of 20μl per tube. This mix was annealed at 25℃ for 5min, extended at 42℃ for 1hr, inactivated reverse transcriptase at 70℃ for 15min.

RT-PCR was processed with amfiEco kit (GenDEPOT). Primer sequences of rat IGF-1 and rat GAPDH were listed on Table 1. The PCR cycling conditions of rat IGF-1 were 95℃ for 5min, 28 cycles of 95℃ for 1min, 65℃ for 1min 30sec, 72℃ for 40sec and 72℃ for 10min for final extension. And GAPDH PCR conditions were 95℃ for 5min, 28 cycles of 95℃ for 1min, 60℃ for 1min, 72℃ for 40sec and 72℃ for 10min for final extension. PCR products were loaded in 1.2% agarose gel containing red-safe (Intron).

Table 1. Sequences of primers for RT-PCR

	Forward	Reverse
IGF-1	5'-TTT GAA TTC ATG GGG AAA ATC AGC AGT CT-3' * GAATTC : EcoRI sequence	5'-TTT CTA GAC TGC ACT TCC TCT ACT TGT GTT C-3' * TCTAGA : XbaI sequence
EGFP	5'-GTA GGT GTC ATT CTA TTC TGG GG-3'	5'-AAC CGT ATT ACC GCC TTT GA-3'
GAPDH	5'-AAT GCA TCC TGC ACC ACC AAC TGC-3'	5'-GGA GGC CAT GTA GGC CAT GAG GTC-3'

IGF-1: Insulin-like growth factor-1; EGFP: Enhanced green fluorescent protein;
GAPDH: Glyceraldehyde 3-phophate dehydrogenase

2.6. Enzyme-linked immunosorbent assay (ELISA)

The conditioned medium of transfected MSCs was collected by after 24hrs or 48hrs after incubation. Total 3ml of conditioned medium was harvested and was centrifuged at 1600rpm for 3min. Supernatant was transferred to new 1.5ml eppendorf tube and the medium was measured by using rat IGF-1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) following the manufacturer's instruction. Serum-free low glucose DMEM was used as a negative control. All controls and samples were assayed in triplicate and optical density was measured at 450nm.

2.7. Fluorescence-activated cell sorting (FACS)

Fluorescence-activated cell sorting (FACS) was processed to selection of only transfected MSCs using BD FACSAria^{TM} III (BD Biosciences). 24hrs after transfection, transfected MSCs were harvested and washed with FACS buffer once. And then, MSCs were suspended in 1ml FACS buffer, entrusted FACS specialist with samples. Transfected MSCs with pEF1/His C::IGF-1::EGFP was visualized as green fluorescence and selected by FITC filter (488nm). Data were analyzed by BD FACSDiva^{TM} software (BD Biosciences).

2.8. Preparation of rat myocardial infarction (MI) model

Experimental myocardial infarction was induced at an age of 8 weeks to 9 weeks male Sprague-Dawley rats (270±10 g) as described previously. Rats were anesthetized by intraperitoneal injection of Zoletil (30mg/kg) and Rompun (10mg/kg). The anesthetized rats were ventilated with positive pressure (180ml/min) using Ventilator (Harvard Apparatus). And then, the rat heart was exposed through 2cm incision at the left lateral costal rib. The left anterior decending (LAD) was ligated with 6-0 prolene (ETHICON) beneath the left atrium for 30 minutes. After occlusion, the ligation was considered successful when color of left ventricle region became pale. After that time, followed by reperfusion and immediately proceeded with intramyocardial injection of cells or PBS.

100 μl of PBS or control MSC ($1X10^6$ cells in 100 μl PBS) or unsorting_pEF1/His C::IGF-1::EGFP-MSCs ($1X10^6$ cells in 100 μl PBS) or sorting_pEF1/His C::IGF-1::EGFP- MSCs ($1X10^6$ cells in 100 μl PBS) were injected into 3-4 different sites of border zone using 31-gauge insulin needle (Becton, Dickinson and Company).

Animal groups were divided into four groups: PBS injection (LAD ligation and reperfusion, n=9), control MSCs (LAD ligation and reperfusion, $1.0X10^6$ cells, n=8), unsorting_pEF1/His C::IGF-1::EGFP-MSCs (LAD ligation and reperfusion, $1.0X10^6$ cells, n=8), sorting_pEF1/His C::IGF-1::EGFP- MSCs (LAD ligation and reperfusion, $1.0X10^6$ cells, n=8). 2 weeks after transplantation, the animals were re-anesthetized and sacrificed for MSCs localization and histological study, respectively.

2.9. MSCs localization in injection sites

To analyze MSCs engraftment (or localization) within infarcted myocardium following experimentally induced rat myocardial infarction, MSCs treated animals were sacrificed 2 weeks after transplantation. The heart of rat was perfused with PBS and fixed in 10% formalin solution overnight. And next day, heart tissue was embedded in OCT compound (SAKURA) and frozen with dry ice, and sectioned transversally with cryostat. Cryosections were mounted with mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) (Santa cruz Biotechnology, Inc) and covered with cover slide. Mounted sections were examined under confocal fluorescent microscope, and DAPI and EGFP were detected in heart sections.

2.10. Immunofluorescence (IF)

To confirm that IGF-1 expression from MSCs transfected with pEF1/His C::IGF-1::EGFP in ischemic heart. MSCs treated animals were sacrificed 2 weeks after transplantation. The heart of rat was perfused with PBS and fixed in 10% formalin solution overnight. And next day, heart tissue was embedded in OCT compound (SAKURA) and frozen with dry ice, and sectioned transversally with cryostat.

Cryosections were washed PBS containing Triton X-100 (Sigma). And sections were permeabilized with mix of 100% methanol and 30% H_2O_2. Then, washed 3 times with PBS and incubated with 3% BSA (blocking step). Next, cryosections were incubated with primary antibody (IGF-1 antibody, Santa cruz Biotechnology, Inc, 1:50) in blocking solution at RT for 2hr. and then, the sections were washed with PBS three times and incubated with secondary antibody (Cy5.5 Donkey anti-goat antibody, Biolegend, 1:200) at RT for 1hr. Sections were washed with PBS, mounted with mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) (Santa cruz Biotechnology, Inc) and covered with cover slide. Sections were examined under Olympus fluorescence microscope, and DAPI and Cy5.5 were detected in heart sections.

2.11. 2,3,5-Triphenyltetrazolium chloride (TTC) staining

Myocardial infarct size was measured by using TTC (Sigma-Aldrich) staining. PBS or MSCs (control or unsorting or sorting) treated animals were sacrificed 2 weeks after implantation. Heart was perfused with PBS and incubated in 1% TTC for 15 minutes at 37℃ water bath. And then, heart tissue were fixed in 10% formalin at 4℃ for overnight. This heart was sectioned transversally and photographed with a digital camera. The infarct size was measured by calculating the ratio of cumulative infarcted area to the entire left ventricle. Analysis of the infarcted area was performed using Image J software program.

2.12. Trichrome staining

Heart was perfused with PBS and fixed in 10% formalin solution. Heart sections were stained with Trichrome stains (Masson) kit (Sigma-Aldrich) according to the manufacturer's recommendations. In briefly, the cryosection slides were stained with Bouin's solution at 56℃ for 15min and washed in running tap water until yellow color from sections was disappeared. And then, the slides were stained with Weigert's iron hematoxylin solution for 15min and washed under tap water for 5min. And the sections

were stained with Blebrich scarlet-acid fucshin solution for 5min and rinsed in deionized water. Next, the sections were placed in working Phosphotungstic/Phosphomolybdic acid solution for 5min and Anilline blue solution for 5min. the sections were treated with 1% acetic acid for 2min and rinsed deionized water. The sections were dehydrated through from 70% alcohol to 100% alcohol, clear in xylene. And then, the sections were mounted using permount (Fisher Scientific).

2.13. Statistical analysis

Data are presented as mean ± standard deviation. The data was analyzed using T-test followed by Mann-Whitney test. A p-value less than 0.05 were considered to indicate statistical significance. All statistical analyses were performed using GraphPad Prism 5 for Windows (version 5.01, GraphPad Software).

III. RESULTS

3.1. Construction of selection plasmid vector expressing dual gene; IGF-1 and EGFP

pEF1/His C::IGF-1::EGFP selection vector was constructed by inserting rat IGF-1 cDNA and EGFP DNA into pEF1/His C vector. The construction was divided into pEF1/His C::IGF-1 vector and pEF1/His C::IGF-1::EGFP vector, respectively (Figure 1, 2). In the first step of cloning, pEF1/His C::IGF-1 was intermediated products of cloning selection vector that made by inserting rat IGF-1 cDNA into pEF1/His C vector (Figure 1A). Enzyme cutting with PstI site and sequencing were proceeded to confirm whether construction of cloned vector is correct. Figure 1B and 1C indicated that correct size of constructed vector after enzyme cutting, and the cloned vector was identified by a 100% in sequencing data (Figure 1). Size of final cloned vector pEF1/His C::IGF-1 was 6645bp (Figure 3A). To confirm the gene expression of constructed IGF-1 by human EF1 alpha promoter, pEF1/His C::IGF-1 was transfected in H9c2 cells which is a subclone of the original clonal cell line derived from embryonic BD1X rat heart tissue, and the IGF-1 expression was identified by RT-PCR. Levels of IGF-1 expression were increased in dose-dependent manner (0 μg to 4 μg) (Figure 1D).

Finally, EGFP gene was inserted into pEF1/His C::IGF-1 vector. To confirm the cloned vector, enzyme cutting using EcoRI and NdeI, and sequencing were processed (Figure 2). pEF1/His C::IGF-1::EGFP vector was successfully constructed and the size of final cloned vector pEF1/His C::IGF-1::EGFP was 6121bp (Figure 3B).

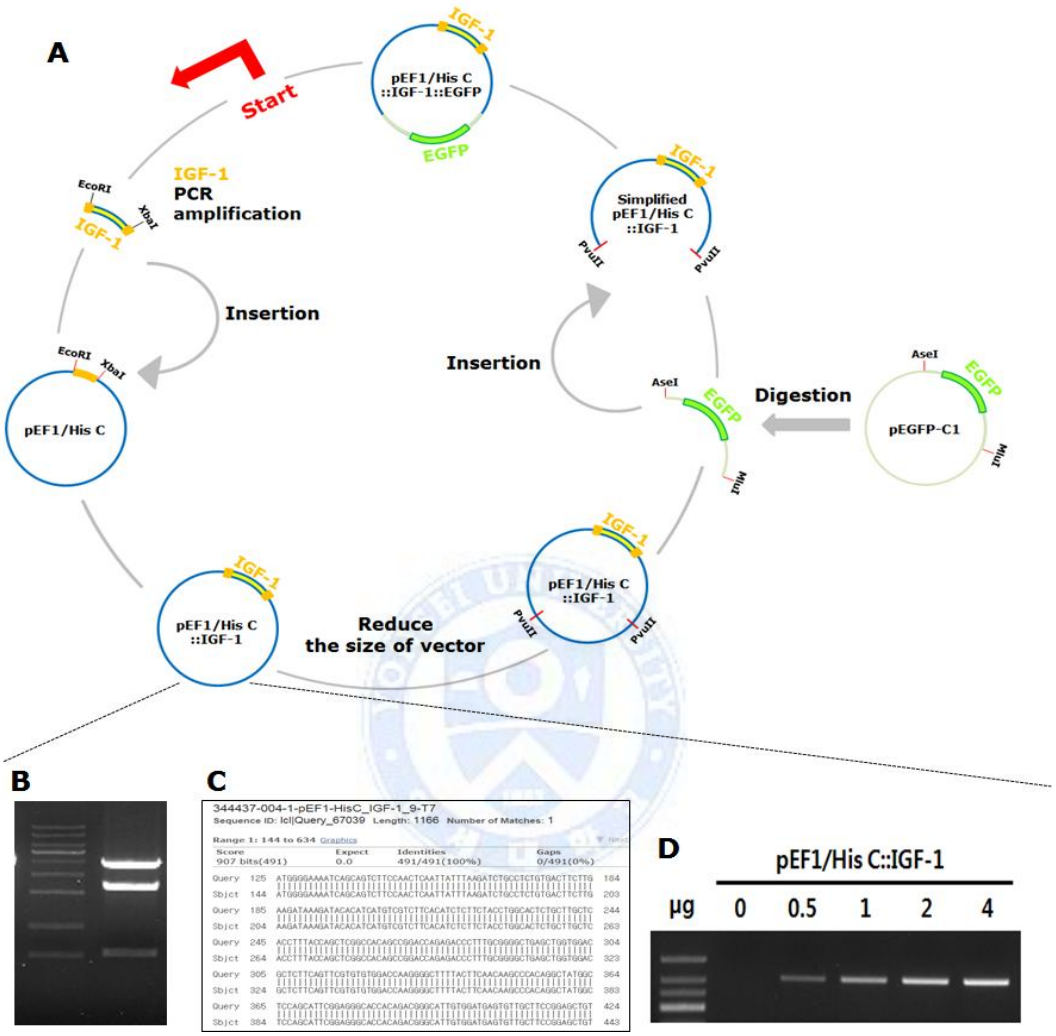

Figure 1. Construction of pEF1/His C::IGF-1 and identification of cloned vector. (A) First step in construction of cloning vector; pEF1/His C::IGF-1. Enzyme cutting and sequencing analysis were processed to confirm whether construction of pEF1/His C::IGF-1 vector is correct. (B) Agarose gel electrophoresis of digested products with PstI restriction enzyme. (C) Result of sequencing. (D) Agarose gel electrophoresis of RT-PCR for IGF-1. The band intensities were increased in dose-dependent manner at 0 μg to 4 μg.

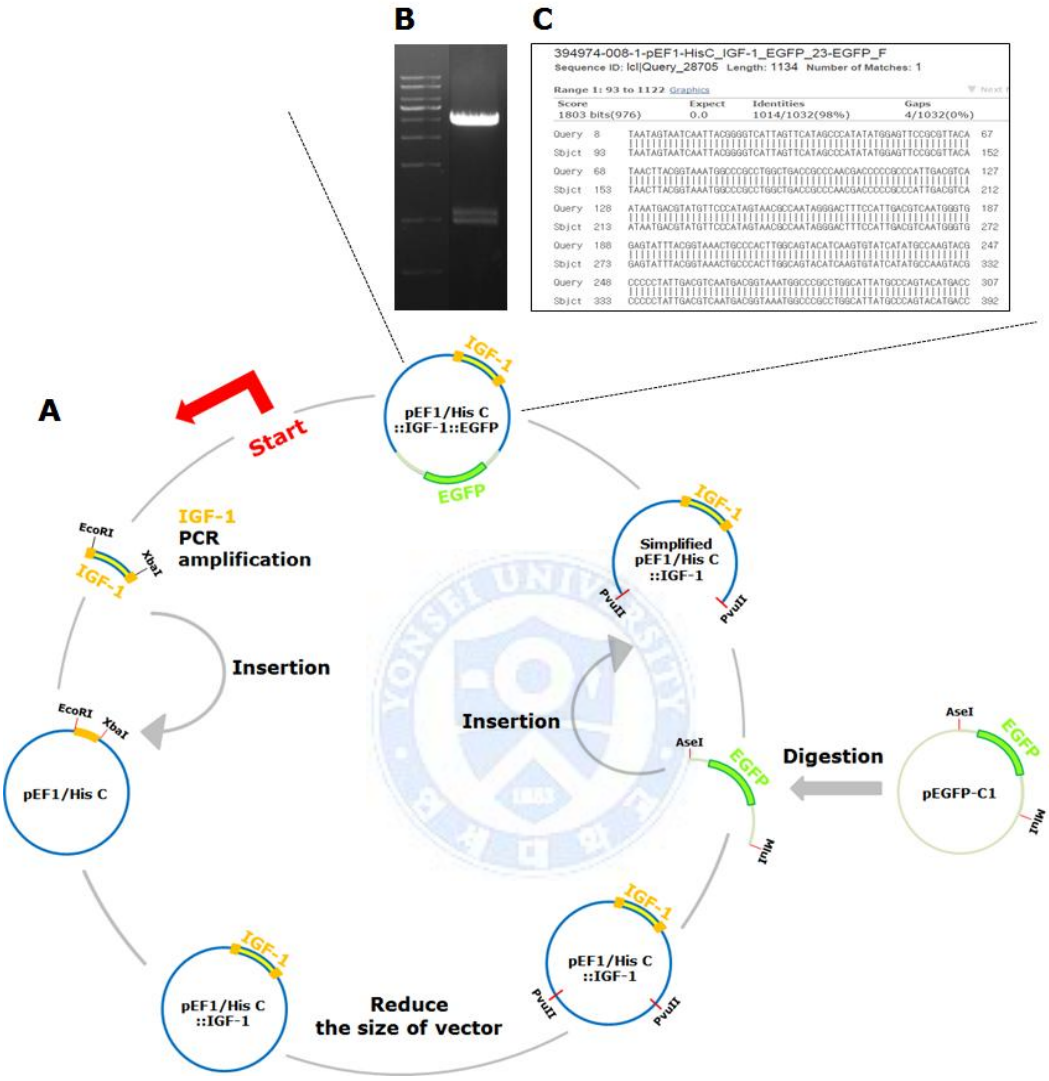

Figure 2. Construction of pEF1/His C::IGF-1::EGFP and identification of cloned vector. (A) Second step in construction of cloning vector; pEF1/His C::IGF-1::EGFP. Enzyme cutting and sequencing analysis were processed to confirm whether construction of pEF1/His C::IGF-1::EGFP vector is correct. (B) Agarose gel electrophoresis of digested products with EcoRI and NdeI restriction enzyme. (C) Result of sequencing.

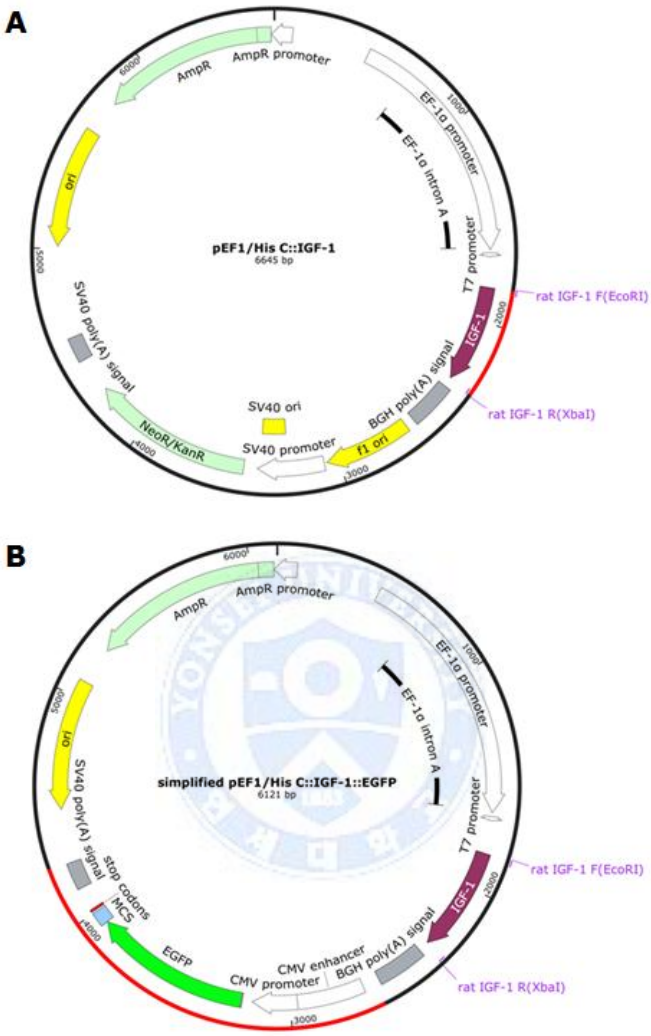

Figure 3. pEF1/His C::IGF-1 and pEF1/His C::IGF-1::EGFP selection vector maps. Construction of pEF1/His C::IGF-1 and pEF1/His C::IGF-1::EGFP vector. (A) pEF1/His C::IGF-1 vector. Rat IGF-1 cDNA was inserted into pEF1/His C vector. (B) pEF1/His C::IGF-1::EGFP selection vector. CMV promoter and EGFP in pEGFP-C1 vector were inserted into pEF1/His C::IGF-1 vector. The constructed vector contains dual promoters and dual genes; human EF1 alpha promoter and IGF-1 gene, CMV promoter and EGFP gene. Further information was described in materials and methods.

3.2. Characterization of bone marrow-derived mesenchymal stem cells (BM-MSCs)

Rat BM-MSCs were isolated by using the Ficoll-Plaque gradient centrifugation. The morphology of MSCs was typically observed such as spindle-shaped (Figure 4A). To characterize the phenotype of primary BM-MSCs, the cultured cells were stained with positive and negative cell surface markers, such as CD29-FITC, CD54-PE, CD90-FITC, CD45-PE, and CD49d-FITC; CD29, CD54 and CD90 were positive marker, CD45 and CD49d were negative marker of MSCs. The BM-MSCs were positive for MSCs markers such as CD29 (99.8%), CD54 (96.3%), and CD90 (99.7%). Also, MSCs were negative for CD45 (0.34%) and CD49d (0.42%) (Figure 4B).

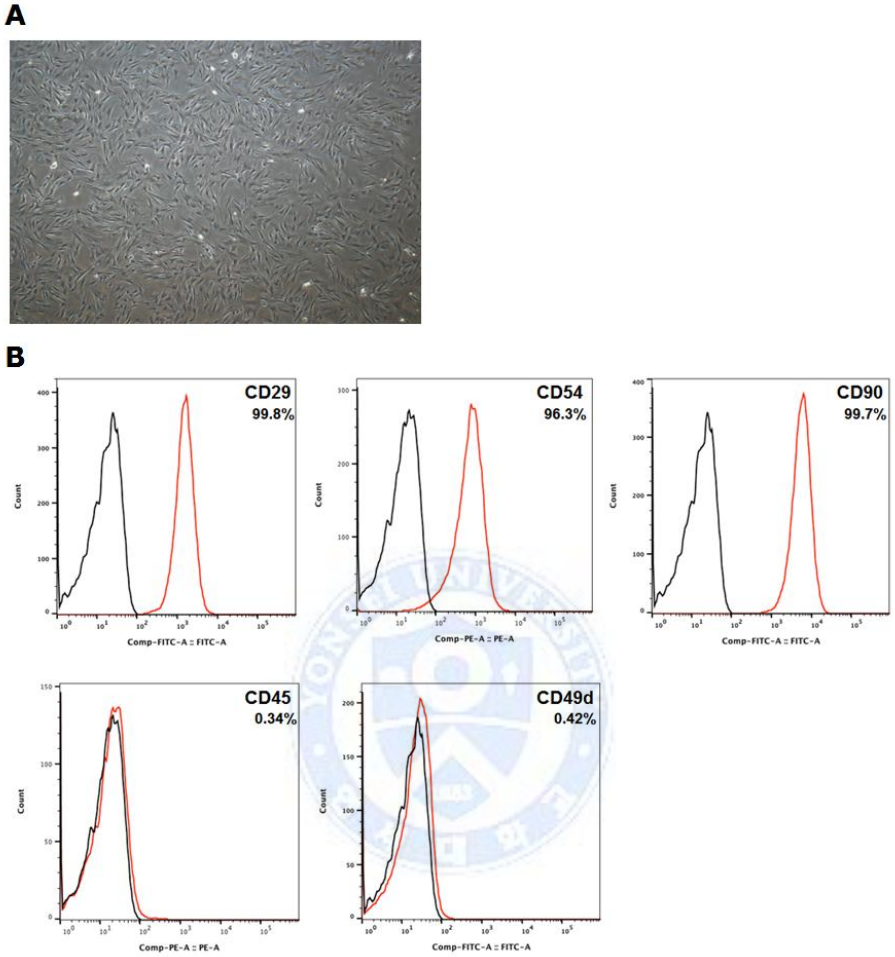

Figure 4. Characterization of MSCs derived from rat bone marrow. (A) Morphology of primary cultured BM-MSCs showed spindle-shaped appearance. And cell adherence to cell culture plate. Magnification 40X. (B) Expression of surface markers in isolated MSCs. Histogram showed the relative staining intensity of MSCs by flow cytometry for CD29-FITC, CD54-PE, CD90-FITC, CD45-PE, and CD49d-FITC. The red histograms represent staining with respective surface marker antibody, whereas the black histograms mean staining with isotype controls.

3.3. The transfection efficiency of pEF1/His C::IGF-1::EGFP in rat BM-MSCs

To determine the transfection dose of pEF1/His C::IGF-1::EGFP in rat BM-MSCs, final selection vector was transfected in a dose-dependent (from 0 μg to 16 μg) manner. RT-PCR, ELISA and fluorescence microscopy were performed in time-dependent (24hr and 48hr) after transfection. The EGFP expressions by fluorescence microscope were dose-dependently visualized from 1 μg and maximized at 8 μg dose in both 24hr and 48hr (Figure 5). Also, the IGF-1 mRNA expressions were dose-dependently increased and maximized at 8 μg dose in RT-PCR (Figure 6A, B, C).

To determine the levels of IGF-1 protein, ELISA was performed by the conditioned culture medium of transfected MSCs. Conditioned medium of pEF1/His C::IGF-1::EGFP transfected MSCs showed a significantly higher level of IGF-1 compared to negative control (0 μg) (Figure 6D). Levels of IGF-1 secretion were enhanced up to 8 μg, and then began to decline both 24hr and 48hr after transfection (Figure 6D). Furthermore, amount of IGF-1 secretion from transfected MSCs at 48hr post transfection is increased when compared to 24hr after transfection. Therefore, transfection efficiency of pEF1/His C::IGF-1::EGFP was achieved maximum at 8 μg dose.

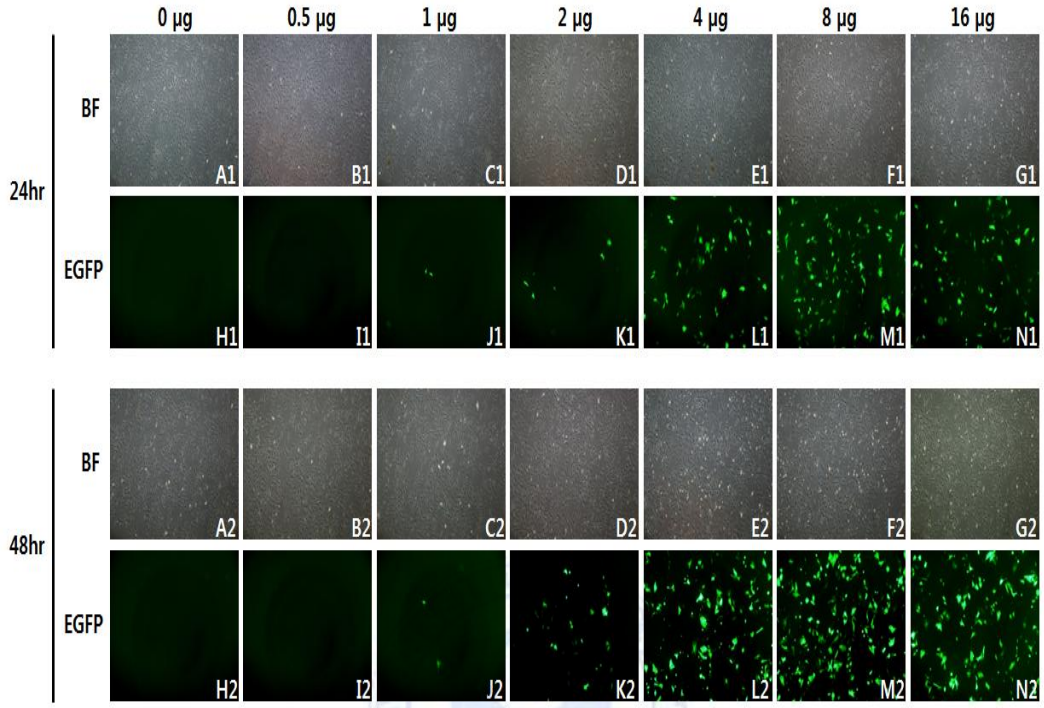

Figure 5. EGFP expression in MSCs serial-transfected with pEF1/His C::IGF-1::EGFP using fluorescence microscopy. MSCs were successfully transfected with pEF1/His C::IGF-1::EGFP and non-viral carrier, as shown by green fluorescence. The expression of EGFP was analyzed by using phase contrast and fluorescence microscopy at serial amount of pEF1/His C::IGF-1::EGFP. EGFP expression was peaked at 8ug. Magnification 40X. A1 to G1: Bright field (24hr), A2 to G2: Bright field (48hr), H1 to N1: Fluorescence microscopy (24hr), H2 to N2: Fluorescence microscopy (48hr).

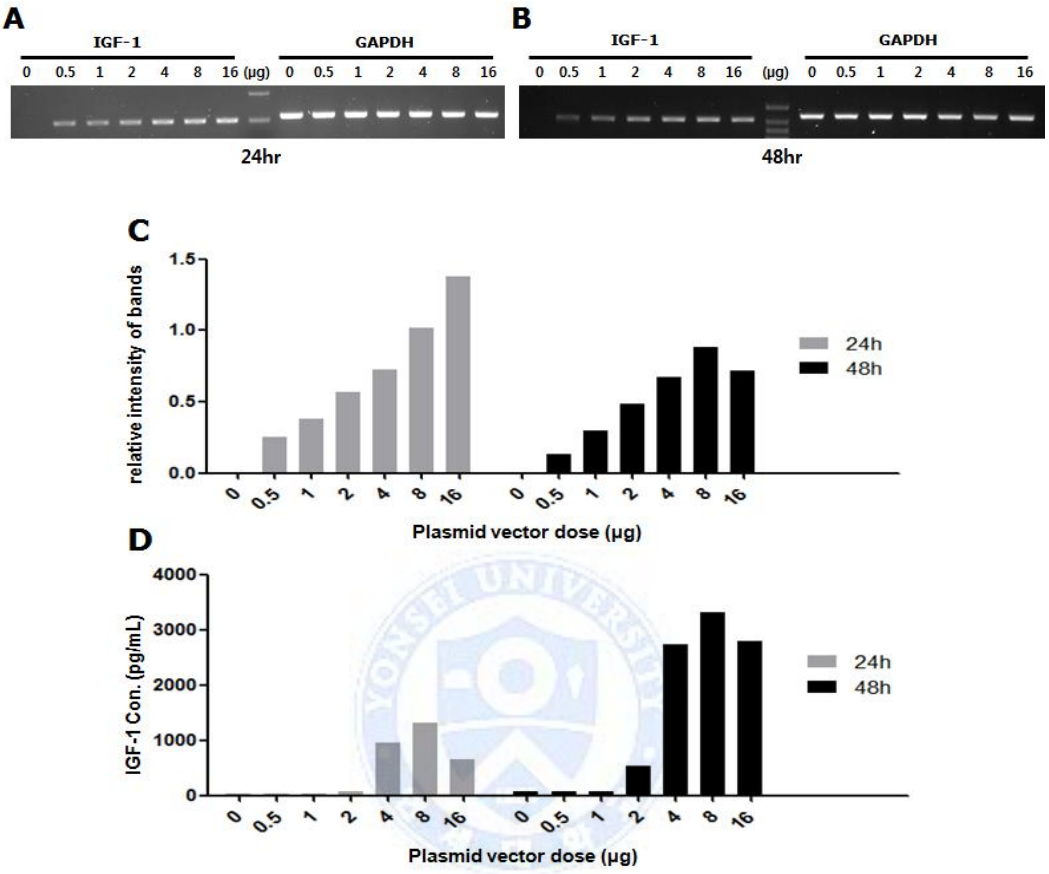

Figure 6. Analysis of IGF-1 expression in mRNA and protein levels. pEF1/His C::IGF-1::EGFP and non-viral carrier were transfected into MSCs. After 24hr and 48hr, MSCs culture supernatant and cells were harvested for analysis of ELISA and RT-PCR, respectively. Agarose gel electrophoresis of RT-PCR for IGF-1 and GAPDH at 24hr (A) and 48hr (B).The band intensity (C) was quantified image J analysis. (D) ELISA. 24hr and 48hr after transfection, IGF-1 protein secreted by the transfected cells were measured by conditioned medium using ELISA. It was showed that IGF-1 expression was increased gradually in dose dependent manner and reached a maximum at 8ug. For the comparison between the two times (24hr and 48hr), levels of IGF-1 expression in 48hr were 2.6 fold higher than IGF-1 expression in 24hr.

3.4. Transfection efficiency evaluation by FACS in BM-MSCs

Transfection efficiency of MSCs with non-viral carrier PAM-ABP was evaluated by FACS. Transfection efficiency was estimated on the basis of the percentage of MSCs expressing EGFP via flow cytometric analysis. The transfection efficiency of pEF1/His C::IGF-1::EGFP and non-viral carrier PAM-ABP in MSCs was approximately 20% (Figure 7A). After sorting, the transfection efficiency was increased up to 95.1% by EGFP selection (Figure 7B). The purity of transfected MSCs was increased approximately 4.8 fold after sorting process.

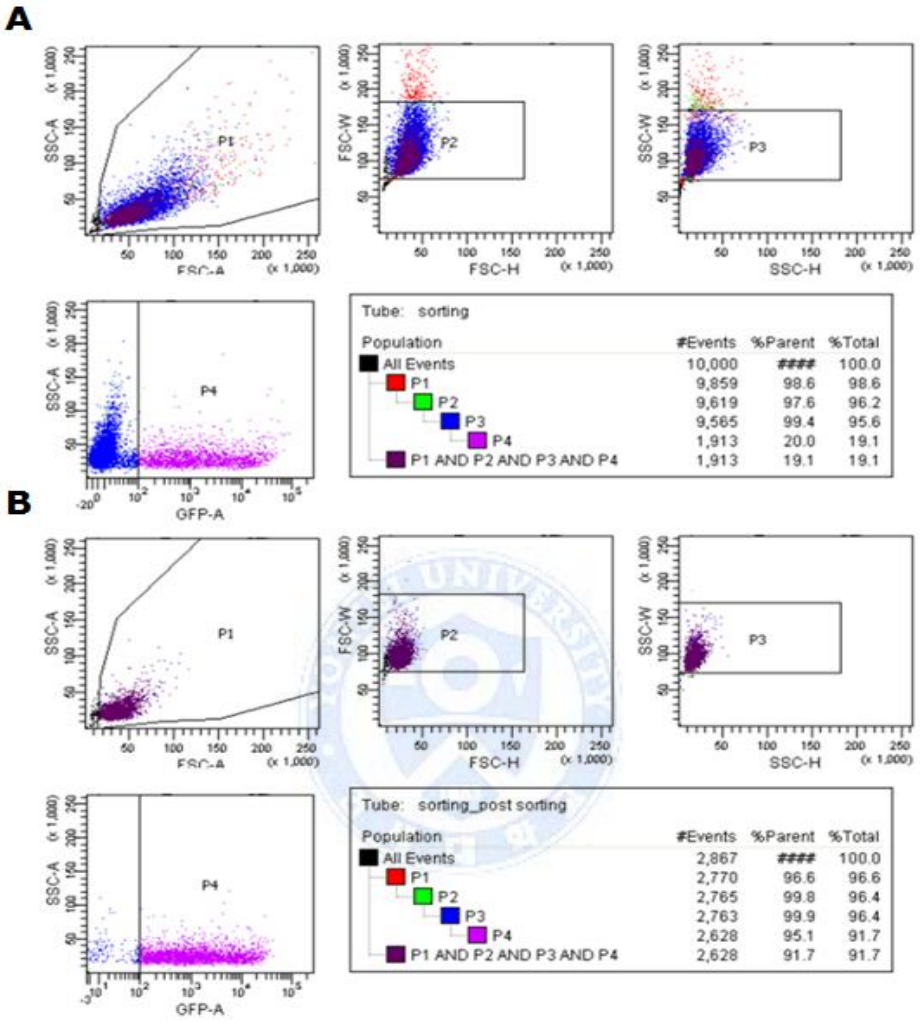

Figure 7. Transfection efficiency of MSCs with pEF1/His C::IGF-1::EGFP and purity following FACS selection. A. Prior to FACS cell sorting. Transfection efficiency of MSCs with pEF1/His C::IGF-1::EGFP and non-viral carrier PAM-ABP was approximately 20% (P4). B. After FACS cell sorting. Transfected MSCs were collected following FACS and its purity was increased up to 95.1%. That is meaning of that almost all MSCs shows a green fluorescent signal (EGFP) and IGF-1.

3.5. Engraftment of pEF1/His C::IGF-1::EGFP transfected MSCs in ischemic heart

The MI rat models were divided four groups; MI-PBS, MI-control MSCs, MI-unsorting MSCs and MI-sorting MSCs (Figure 8). Experimental design and schematic flow diagram were described in Figure 8.

The green fluorescence as pEF1/His C::IGF-1::EGFP transfected MSCs was visualized in both unsorting and sorting groups. The transfected MSCs were observed much more in MI-sorting groups compared to MI-unsorting groups (Figure 9). Furthermore, the pEF1/His C::IGF-1::EGFP transfected MSCs were localized in myocardium of ischemic left ventricle (Figure 9C, F).

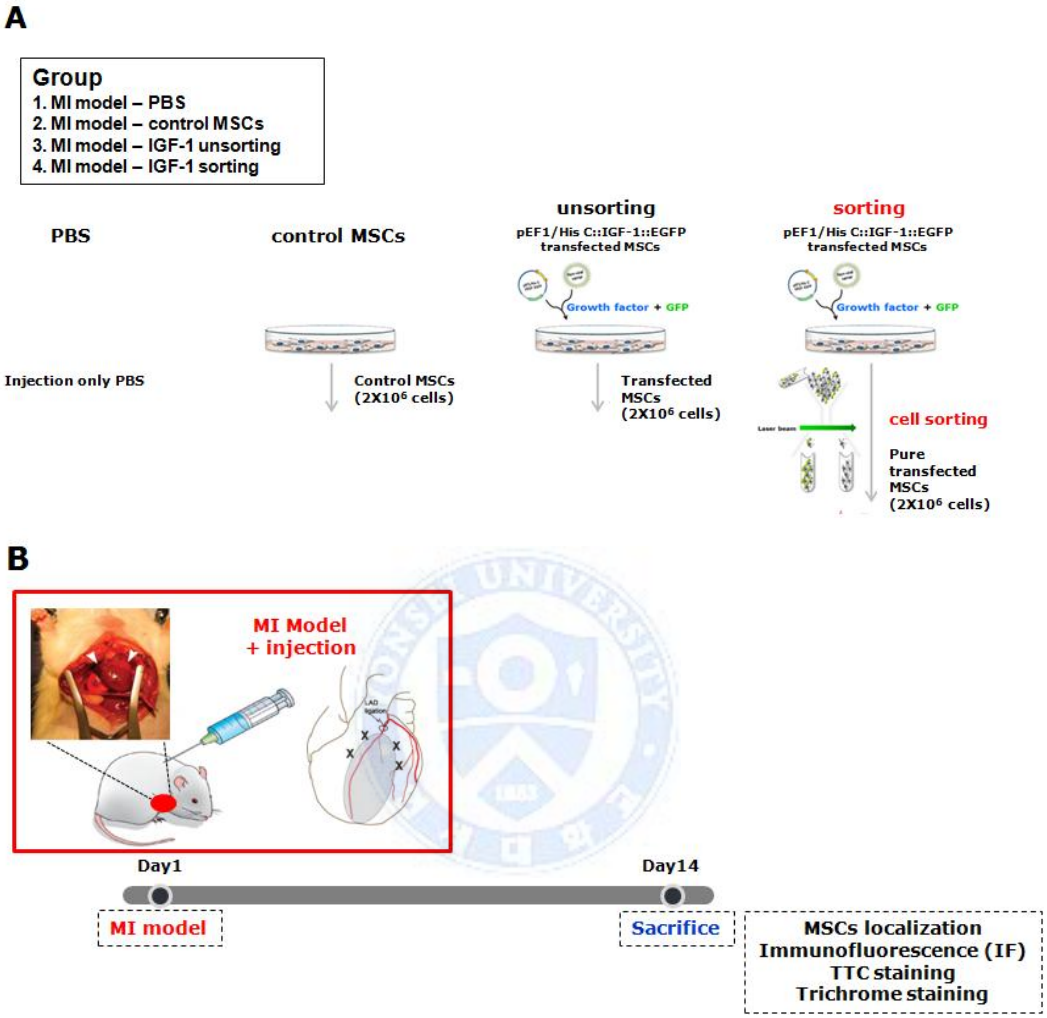

Figure 8. Experimental design and schematic flow diagram of in vivo application. (A) The systematic flow diagrams of cell sorting for in vivo application. Myocardial infarction models were divided into 4 groups; PBS, control MSCs, unsorting MSCs, and sorting MSCs. MSCs injected groups; control MSCs, unsorting, and sorting group were received same amount of cells ($1X10^6$ cells). (B) Experimental design for in vivo. Animals were sacrificed at 2 weeks after transplantation. And then MSC localization, immunofluorescence, TTC staining, and trichrome staining were processed.

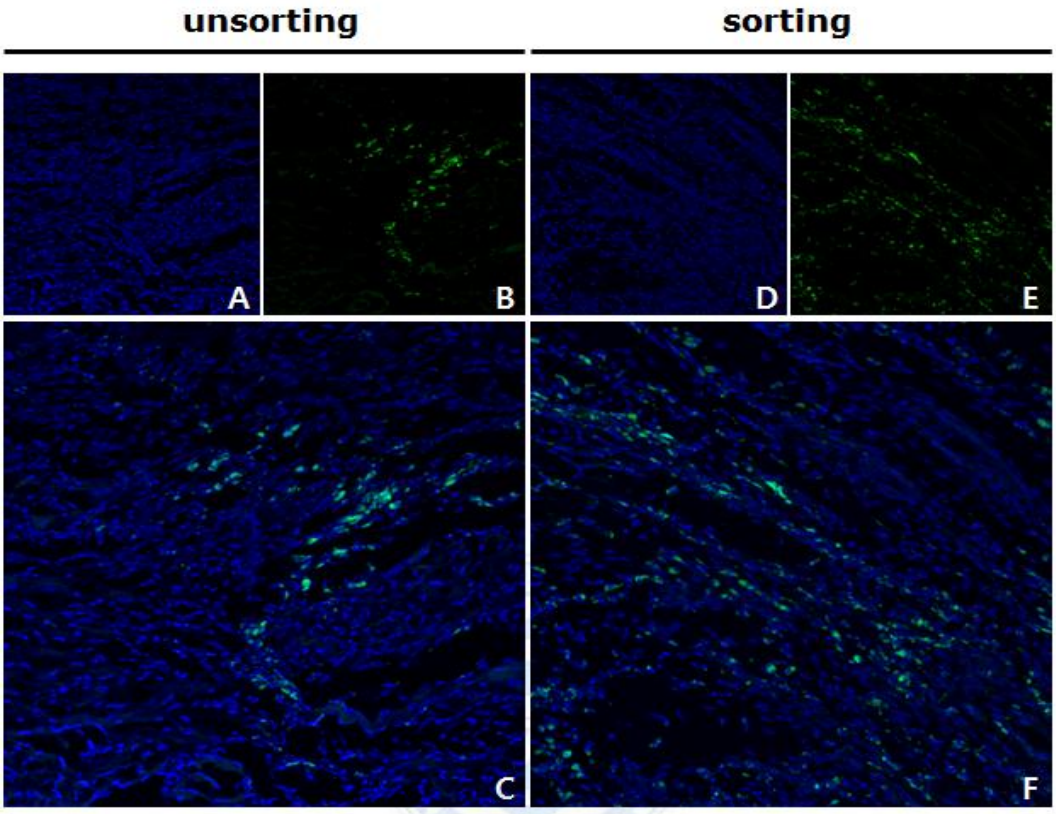

Figure 9. The localization of MSCs transfected with pEF1/His C::IGF-1::EGFP and non-viral carrier PAM-ABP in ischemic heart. The green fluorescence indicated EGFP distribution in ischemic left ventricle. Fluorescence imaging showed more green fluorescence in MI-sorting group compared to unsorting group. (A to C) unsorting group in ischemic heart; (D to F) sorting group in ischemic heart. Magnification 100X.

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3.6. Expression of IGF-1 in ischemic heart after transplantation of MSCs with pEF1/His C::IGF-1::EGFP

To investigate IGF-1 expression in MSCs transfected with pEF1/His C::IGF-1::EGFP, the red fluorescence as IGF-1 expression was detected in myocardium layer of both unsorting and sorting groups (Figure 10). The expression of IGF-1 was significantly increased in MI-sorting group compared with MI-unsorting group.

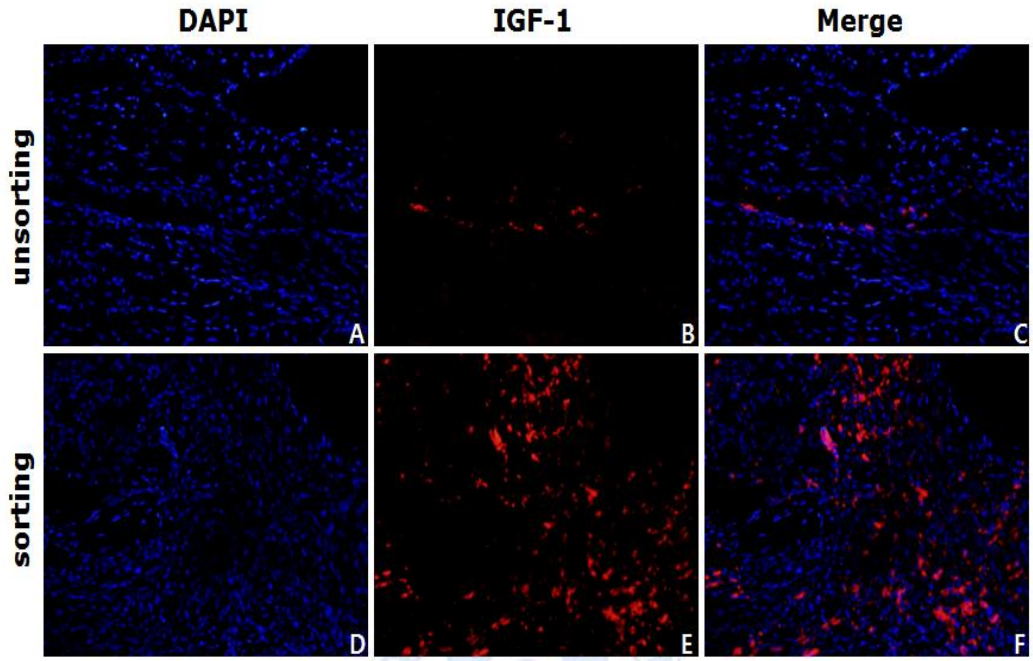

Figure 10. Image for expression of IGF-1 in MSCs transfected with pEF1/His C::IGF-1::EGFP and non-viral carrier PAM-ABP in ischemic heart.

The expression of IGF-1 in BM-MSCs was observed in immunofluorescence staining. IGF-1 was significantly expressed in MI-sorting group compared to MI-unsorting group. IGF-1 is stained in red (Cy 5.5), Magnification 100X. (A to C) unsorting group in ischemic heart; (D to F) sorting group in ischemic heart.

3.7. Improved myocardial repair in ischemic myocardium with pEF1/His C::IGF-1::EGFP

To investigate the therapeutic effects by sorting system of IGF-1 transfected MSCs in myocardial infarction, TTC and Masson's trichrome staining were performed. The infarct size was measured using Image J software program and the results are as follows: PBS 31.50%, control MSCs 12.61%, unsorting MSCs 10.42% and sorting MSCs 5.74% (Figure 11B). The infarct size was significantly decreased in sorting group compared to PBS group (Figure 11A, B). Furthermore, the infarct size of sorting group was protected 2 fold compared to unsorting group (sorting 5.74% vs. unsorting 10.42%, Figure 11).
Masson's trichrome staining is used to distinguish collagen from muscle in heart. The density of stained myocardium layer in sorting group was lower than other groups (Figure 11C).

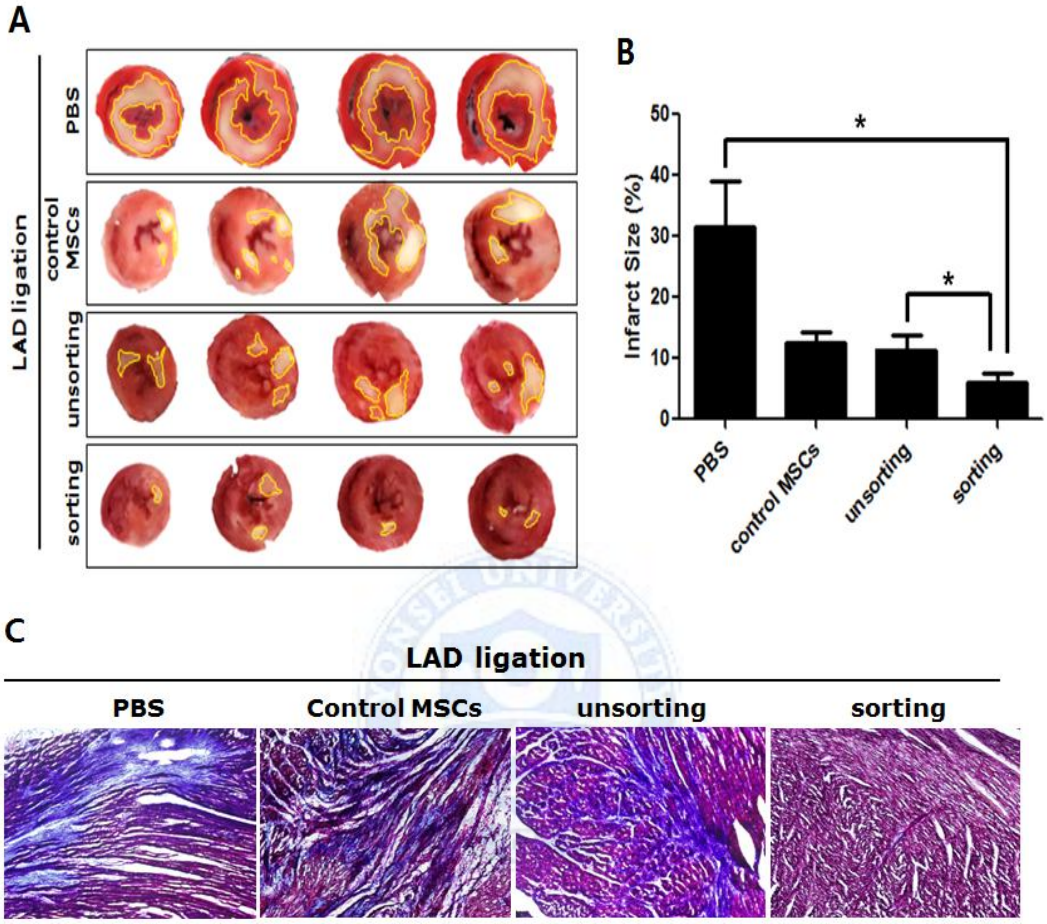

Figure 11. Analysis of histological evaluation. The infarcted hearts were stained by TTC staining (A) and Masson's trichrome staining (C). The infarct area was quantified by image J analysis (B). *$P < 0.05$. The infarct size was significantly decreased in sorting group compared to other groups. In Masson's trichrome staining, the fibrosis was lower than other groups. The hearts muscle were stained a red, whereas the collagen was stained blue. Magnification 200X.

IV. DISCUSSION

The heart constantly beats to supply with oxygen and nutrients, like any muscle in the body. The cardiac ischemia or MI was commonly known as a heart attack. If one of arteries or branches in heart becomes blocked suddenly, a portion of the heart is starved of oxygen, a condition called cardiac ischemia [4,21]. The MI causes the death of a patient resulting in necrosis of cardiac cells. For that reason, MI was known a major cause of death worldwide [4,22]. The infarcted heart causes cardiomyocyte death, scar formation, wall thinning and collagen degradation [4,5]. Therefore, prior investigators have tried to repair the damaged cardiac function and prevent the destructive ventricular remodeling. Recently, new strategies have been suggested to overcome the limitation of current treatment such as stem cell and gene therapy.

This study suggested the verification of therapeutic effects via MSCs-based IGF-1 gene therapy using FACS sorting system in myocardial infarction animal models. Especially, the therapeutic effects of BM-MSCs transplantation were demonstrated that cardiac function, capillary density and ventricular remodeling are significantly increased in MSCs treatment models compared with controls [12,23]. However, some problems such as poor cell transplantation and low viability still remain. Many studies have been researched to enhance therapeutic effects of MSCs cell therapy or MSCs-based gene therapy [12,13].

The viral vector system was known as high transfection efficiency compared to non-viral carrier. However, the viral vector systems do not recommend for clinical trials because of safety aspects [14,15,16]. Normally, primary culture cells including BM-MSCs have poor transfection efficiency when using non-viral carrier. The disadvantage of non-viral carrier methods is relatively low transfection efficiency, approximately 5 to

25% [13,14,24]. Nevertheless, the non-viral carrier is proposed an efficient delivery system due to their characteristics as follow; safety, stability, easy manufacturing and low immunogenicity [15,16].

To overcome the low transfection efficiency of non-viral carrier in MSCs-based gene therapy, new method was developed to maximize therapeutic effects of transfected gene via FACS analysis. In this experiment, the pEF1/His C::IGF-1::EGFP selection vector was constructed by inserting IGF-1 as a gene of interest and EGFP as a selective marker. This selection vector expressing dual gene (IGF-1 and EGFP) simultaneously was constructed by two steps (Figure 1, 2). First step is rat IGF-1 cDNA into pEF1/His C vector (pEF1/His C::IGF-1) and second step is EGFP and CMV promoter in pEGFP-C1 into pEF1/His C::IGF-1 (pEF1/His C::IGF-1::EGFP). The pEF1/His C plasmid vector was selected for selection vector expressing dual gene because human EF1 alpha promoter of pEF1/His C plasmid vector has a high expression activity of target gene in stem cell line [25,26]. GFP is a convenient reporter gene for observation of transfection efficiency in mammalian cells because of easy detection in fluorescence microscopy and emits a strong green fluorescence when cells are exposed by blue light or ultraviolet (UV) [27,28]. *Zhang et al*. constructed the enhanced GFP (EGFP) vector, which makes the protein 35 times brighter than GFP. So, EGFP was used to visualize transfected MSCs for cell sorting system [28].

Furthermore, we manipulated dual genes with dual promoters in one vector to avoid make a fusion protein. In other words, we cloned that each gene has own promoters in one plasmid vector; EGFP gene with CMV promoter, IGF-1 gene with human EF1 alpha promoter. To confirm the regulation of rat IGF-1 gene expression by human EF1 alpha promoter and EGFP gene expression by CMV promoter, the pEF1/His C::IGF-1::EGFP vector and the non-viral vector PAM-ABP were transfected into BM-MSCs. PAM-ABP (ABP-conjugated PAMAM dendrimer) is a bio-reducible dendrimer-type polymer as a non-viral transfection reagent. It is an efficient gene delivery carrier due to their high transfection efficiency and low cytotoxicity compared to other reducible polymers [29].

According to *Won et al*, PAM-ABP at weight ratio 5 for *in vivo* study provided the suitable balance in high level of transfection efficiency and minimal toxicity [30]. So, we applied weight ratio 5 of plasmid and non-viral carrier PAM-ABP for transfection of MSCs.

Expression of EGFP is evaluated through fluorescence microscopy. In these results, the pEF1/His C::IGF-1::EGFP was dose-dependently visualized in green fluorescence and maximized at 8 μg dose in fluorescence microscopy (Figure 5). In RT-PCR and ELISA, the expression of IGF-1 was maximized at 8 μg dose (Figure 6). Therefore, the concentration of transfection by pEF1/His C::IGF-1::EGFP was determined at 8 μg dose in BM-MSCs.

Before sorting of transfected MSCs, the transfection efficiency was approximately 20%. Therefore, IGF-1 expressed MSCs was one-fifth of the total in transfected MSCs by pEF1/His C::IGF-1::EGFP (Figure 7). After sorting, the purity of transfected-MSCs was approximately 95.1% through FACS selection. As a result, the purity of MSC expressed IGF-1 and EGFP is increased from 20% to 95.1%. (Figure 7) It implies that almost all MSCs express EGFP and IGF-1.

To investigate the therapeutic effects by sorted pEF1/His C::IGF-1::EGFP-MSCs, rat MI groups were divided into 4 groups; PBS, control MSCs, unsorting MSCs and sorting MSCs. The green fluorescence as transfected MSCs was visualized in myocardium of ischemic left ventricle and observed in both unsorting and sorting groups. Interestingly, the IGF-1 transfected MSCs were significantly localized in sorting group compared to unsorting group (Figure 9). Also, the IGF-1 expression was increased in sorting groups (Figure 10). Therefore, the new therapeutic strategy by sorting system significantly improves the efficiency of MSCs-based gene therapy.

The TTC staining and the trichrome staining were proceeded to evaluate the therapeutic effects of dual gene system with FACS through histological assessment. As a result, infarct size and fibrosis were relatively decreased in sorting group compared to other groups (Figure 11). The increased IGF-1 expression by sorting system probably

induces the therapeutic effects as protection of fibrosis.

According to prior reports, IGF-1 is secreted by almost all tissues and plays an important role in cell growth, differentiation, and transformation [18,19]. Particularly, IGF-1 in the heart has substantially beneficial effects on cardiomyocyte function including proliferation and survival [18]. *Raffay et al*. demonstrated that exogenous administration of IGF-1 results in cardioprotective effects mediated through Akt pathway, pro-survival signaling [31]. Furthermore, survival and engraftment of MSCs are increased, and attenuated cardiac dysfunction, decreased myocardium apoptosis after transplantation of MSCs treated IGF-1 [32].

The present study demonstrated that the IGF-1 expression can protect the fibrosis of myocardium and induce decreased infarct size in MI animal models. Furthermore, the new therapeutic strategy by sorting system significantly improves the efficiency of MSCs-based gene therapy.

V. CONCLUSION

The present study demonstrated the therapeutic effects of MSCs-based IGF-1/EGFP dual gene by sorting system in myocardial infarction. The IGF-1 is probably an effective treatment for MI. Furthermore, the MSCs-based gene therapy by sorting system would be a new strategy for cardiovascular disease. Promising further study will prove our hypothesis and reveal biological role for diverse diseases.

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ABSTRACT (In Korean)

인슐린 유사 성장인자와 녹색 형광 단백질로 유전자 변형된 중간엽 줄기세포와 선별 시스템을 이용한 랫드 심근 경색 모델의 치료효과 확인

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정수빈

심근경색은 대표적인 심혈관 질환으로 관상동맥 폐쇄가 일정시간 동안 지속되어 심근세포가 괴사되는 상태로, 가장 전형적인 증상은 흉통이며, 호흡곤란, 발한, 구역, 피로감, 실신 같은 비전형적인 증상이 나타난다. 손상된 심장의 재생을 위하여 다양한 줄기세포 치료법이 제시되고 있으나, 임상적 안전성

측면에서 중간엽 줄기세포가 가장 대표적인 세포치료법으로 제안되고 있다. 최근, 중간엽 줄기세포의 치료 효과를 증진시키기 위하여 질환 특이적 유전자를 도입한 세포치료법이 활발히 연구되고 있으나, 낮은 유전자 도입 효율 등의 한계가 해결 되어야 할 문제점으로 알려져 있다.

본 연구에서는 기존의 세포 기반 유전자 치료의 효과를 향상시키기 위하여 두 가지 유전자를 동시에 발현하는 유전자 벡터와 선별 시스템을 이용하는 단순하면서도 효율적인 방법을 이용하였고, 랫드 심근경색 동물모델에서 그 치료 효과를 확인하였다.

pEF1/His C 벡터에 질환 특이적 유전자인 인슐린 유사 성장인자 (Insulin-like growth factor, IGF-1)를 삽입하였고, 선별 마커로써 녹색 형광 단백질 (enhanced green fluorescent protein, EGFP) 유전자를 복제하여 유전자 벡터를 제작하였다. 유전자 벡터 (pEF1/His C::IGF-1::EGFP)를 중간엽 줄기세포에 유전자 도입하였을 때, 두 가지 유전자 모두 균일하게 발현하는 것을 확인하였다. 유전자 도입 효율을 극대화 시키기 위해 형광이용세포분류기 (FACS)를 이용하여 타겟 유전자를 발현하는 중간엽 줄기세포를 선별하였고 그 효율은 95.1%로 확인되었다.

심근경색 특이적 유전자 발현 중간엽 줄기세포의 치료효과를 검증하기 위하여 랫드 심근경색 동물모델을 대조군 (PBS), 줄기세포군 (control MSCs), 비선별 인슐린 유전자 도입 줄기세포군 (unsorting MSCs), 선별 인슐린 유전자 도

입 줄기세포군 (sorting MSCs)으로 분류하였다. 세포 주입 2주 후 실험동물을 희생시켜 심장조직을 면역 형광 염색, TTC 염색, Masson's trichrome 염색을 통하여 치료효과를 확인하였다.

선별 인슐린 유전자 줄기세포 군에서 녹색 형광 발현 세포와 인슐린 발현 세포가 현저히 많이 관찰되었다. TTC 염색의 경우에도 선별 인슐린 유전자 줄기세포 군에서 심근경색의 크기가 감소하였고 (PBS 31.50%, control MSCs 12.61%, unsorting 10.42%, sorting 5.74%), 심근섬유화가 현저히 감소된 것을 확인하였다.

그러므로, 인슐린 유사 성장인자는 허혈성 심장의 경색과 섬유화를 억제하는 것을 확인할 수 있었다. 또한, 두 가지 유전자를 발현하는 유전자 벡터와 선별 시스템은 유전자 도입 효율을 높임으로써 인슐린 유사 성장인자의 심근경색의 치료효과를 극대화하였다. 더 나아가, 질환 특이적 유전자 도입 세포 선별방식은 심근경색뿐만 아니라 다양한 질병들에 적용할 수 있을 것으로 기대된다.

핵심 되는 말: 인슐린 유사 성장인자, 특이적 플라스미드 벡터, 선별 시스템, 듀얼 프로모터, 도입된 유전자 효과, 심근 경색