수의학박사학위논문

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2020년 2월

서울대학교 대학원

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이 논문을 수의학박사학위논문으로 제출함

2019년 11월

서울대학교 대학원
수의학과 수의생명과학 전공 (수의생화학)
조 현 민

조현민의 수의학박사 학위논문을 인준함
2019년 12월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

# Therapeutic Effects of Genome-edited Mesenchymal Stem Cells Expressing VEGF and LEF1 on Myocardial Infarction

**Under the supervision of Professor Je-Yoel Cho**

**DISSERTATION**

Presented in Partial Fulfillment of the Requirement for the Degree of DOCTOR OF PHILOSOPHY

**By**

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February 2020

# ABSTRACT

# Therapeutic Effects of Genome-edited Mesenchymal Stem Cells Expressing VEGF and LEF1 on Myocardial Infarction

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Cardiovascular disease is one of the leading causes of death worldwide, and according to the 2016 World Health Organization (WHO) statistics, more than 9 million patients die each year from different forms of this disease. Among these, myocardial infarction is a serious disease in which cardiomyocytes that do not receive blood due to coronary artery obstruction die, and the number of patients is increasing the fastest among cardiovascular diseases. The major therapy for myocardial infarction is stent implantation, which expands the endoscopic diameter by inserting the stent into the blocked vessel. Although, it is a fundamental

treatment for the survival of myocardial tissues, side effects such as reperfusion injury may occur, and since cardiomyocytes have very low regenerative capacity, additional treatment strategies are needed to regenerate damaged myocardial tissues after surgery.

In this regard, human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) have received special attention. These cells have fast self-renewal and differentiation into cardiomyocytes, and secrete various cytokines such as HGF, bFGF, VEGF-D, and PDGF to help repair damaged areas in the heart and form new blood vessels. However, despite these advantages, hUCB-MSCs are limited in that less than 3% of cells survive after transplantation and they secrete very little VEGF-A, which has the most important effect on angiogenesis even after successful transplantation. These limitations raise the need for the induction of VEGF-A and the enhancement of cell proliferation in therapies using hUCB-MSCs.

In this study, we applied VEGF-A and LEF1 genes to the AAVS1 locus, a safe harbor site in stem cells, using the TALEN and CRISPR / Cas9 systems. The effect of restoring heart function was confirmed, thus suggesting a new strategy for treating myocardial infarction of stem cells into which therapeutic genes have been introduced through target genomic engineering.

In CHAPTER I, the vascular endothelial growth factor (VEGF-A) was used to improve the angiogenic capacity of hUCB-MSCs. After coronary artery occlusion, neovascularization is essential to replenish heart tissue and slow the progression of

myocardial necrosis. To this end, mesenchymal stem cells (VEGF / hUCB-MSCs) secreting therapeutic levels of VEGF-A were produced using the TALEN system, a second-generation gene editing technology, and the therapeutic effect of these cells was confirmed in the myocardial infarction model.

VEGF-A can induce angiogenesis and form new blood vessels for therapeutic purposes, but excessive amounts of VEGF-A can cause abnormal blood vessel formation and hemangioma growth. Therefore, in order to utilize this for therapeutic purposes, a device capable of controlling its expression was required. For this purpose, the vector was designed to only produce VEGF-A protein in the presence of doxycycline. In addition, to solve the problems of low gene transfer efficiency, stability, and specific expression, which are limitations of conventional gene therapy, the TALEN gene editing technology was used to introduce the VEGF expression vector cassette in the AAVS1 locus.

Thus, the VEGF/hUCB-MSCs produced secreted VEGF-A only upon doxycycline treatment, and consequently increased the expression of genes associated with angiogenesis and improved angiogenesis in animal models. Finally, when VEGF/hUCB-MSCs were implanted into the rat myocardial infarction model and doxycycline was supplied via negative water, cardiac function was restored, MI size and fibrosis decreased, and the recovery effect of the heart damaged by myocardial infarction could be confirmed.

In CHAPTER II, hUCB-MSCs incorporating the LEF1 gene were studied for their proliferation and survival effects. It has previously been noted that there is potential for therapeutic applications, but hUCB-MSCs have certain limitations in their application to the treatment of myocardial infarction because of their low post-transplantation survival rate. Accordingly, there is demand for a treatment strategy using stem cells into which a therapeutic gene has been introduced. In this section, hUCB-MSCs (LEF1/hUCB-MSCs) incorporating the LEF1 gene were constructed using the CRISPR/Cas9 system and applied to the myocardial infarction model.

Through In silico literature surveys, LEF1 was identified as a therapeutic gene, and it was overexpressed in hUCB-MSCs to confirm cell proliferation and survival. After fully confirming the possibility of applying the treatment to the actual disease model, a rat myocardial infarction model was prepared by introducing stem cells (LEF1/hUCB-MSCs) into which the LEF1 gene had been incorporated into the AAVS1 locus via the CRISPR/Cas9 system. Upon subsequent transplantation of LEF1/hUCB-MSCs survival after myocardial infarction improved, and myocardial protective effects such as recovery of cardiac function, fibrosis and reduction of infarct area was later noted.

In conclusion, human mesenchymal stem cells expressing VEGF-A and LEF1 by targeted genome editing (VEGF/hUCB-MSCs and LEF1/hUCB-MSCs) were generated in the present study. Each of these cells showed enhanced angiogenesis

and cell proliferation. Furthermore, when implanted into the myocardial infarction model, the cardiovascular recovery was improved. Through this, we proposed a new cell therapy strategy that overcomes the limitations of existing cell therapies.

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**Keywords:** Myocardial infarction, Mesenchymal stem cells, Targeted genome engineering, Vascular endothelial growth factor, Lymphocyte enhancer-binding factor 1, Recovery of cardiac function

**Student Number:** 2012-21532

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CHAPTER 1

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CHAPTER 2

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## CHAPTER 1

# CHAPTER 2

# ABBREVIATIONS

| | |
|---|---|
| bFGF | Basic fibroblast growth factor |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CAD | Coronary artery disease |
| DSBs | Double-stranded breaks |
| EF | Ejection fraction |
| FS | Fractional shortening |
| HGF | Hematocyte growth factor |
| HDR | Homology-directed repair |
| IL | Interleukin |
| IHD | Ischemic heart disease |
| LEF1 | Lymphoid enhancer-binding factor 1 |
| MSCs | Mesenchymal stem cells |
| MI | Myocardial infarction |
| TALEN | Transcription activator-like effector nuclease |
| VEGF | Vascular endothelial growth factor |
| ZFN | Zinc finger nuclease |

# LITERATURE REVIEW

## 1. Myocardial infarction

Ischemic heart disease (IHD) is one of the leading causes of death. According to the World Health Organization (WHO) statistics in 2016, more than 9 million patients die from this disease each year (Nowbar et al., 2019) (Figure B-1). The main cause of this disease is the blockage of the coronary artery due to an atherosclerotic plaque, which causes disruptions in blood flow and oxygen supply. Severe or prolonged ischemia causes death of surrounding tissues (Mills et al., 2009, Avery et al., 2010).

Myocardial infarction (MI) is a type of ischemic heart disease (Alpert, 2009), and it refers to the irreversible death of the cardiac tissue (Figure B-2). The main therapeutic strategy for MI is reperfusion therapy through primary percutaneous coronary intervention (PPCI) (Frohlich et al., 2013). This treatment is a non-surgical procedure that reopens the blocked coronary artery by inserting a catheter into the blood vessel. Although this therapeutic process is a pre-requisite for the survival of myocardial tissue, it can lead to paradoxical phenomena termed ‘myocardial reperfusion injuries,’ including additional intravascular and myocardial damage and death of remaining cardiomyocytes (Carden and Granger, 2000).

In order to overcome this problem, combined therapies using anti-platelet drugs such as prasugrel, abciximab and anti-thrombotic agents such as bivalirudin have been tried (Showkathali and Natarajan, 2012, Metharom et al., 2015). However, it has proven insufficient in restoring myocardial reperfusion injury, and since cardiomyocytes have a very low regenerative capacity, there is a need for additional measures to regenerate damaged myocardium after the intervention (Kikuchi and Poss, 2012).

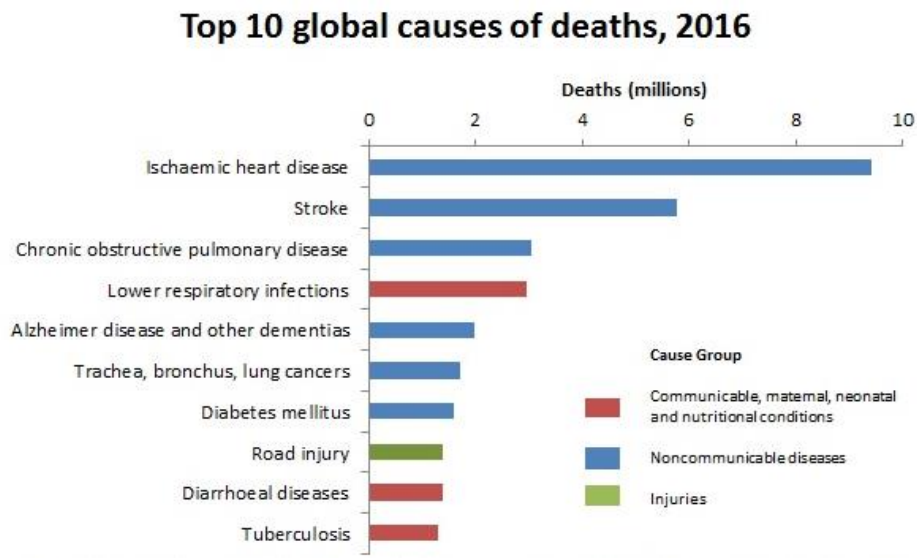


**Figure B-1. Top 10 global causes of deaths, 2016.**

Of the 56.9 million deaths, more than half (54%) were due to the top 10 causes. Cardiovascular diseases are the world's biggest killers, accounting for a combined 9.3 million deaths in 2016. These diseases have remained the leading cause of death globally in the last 10 years.

Adapted from Geneva, World Health Organization et al., 2018

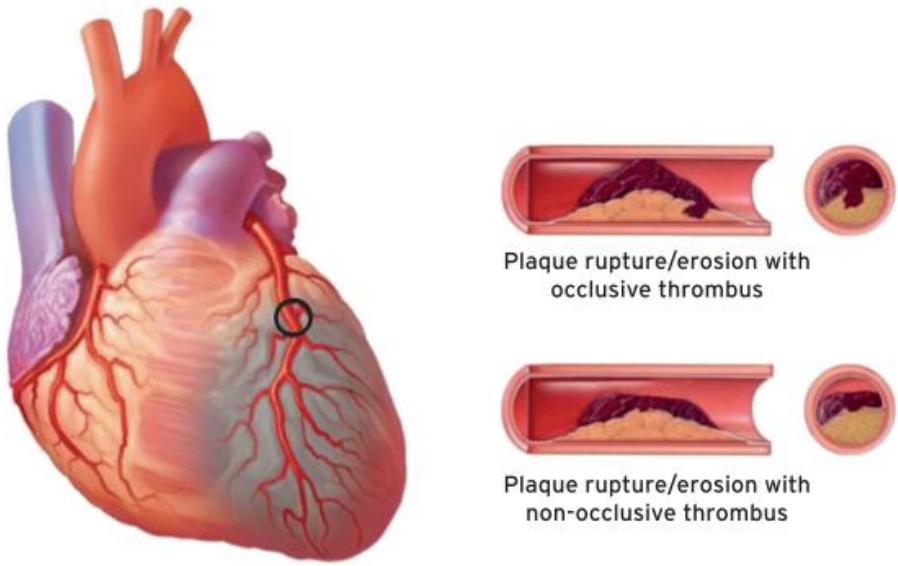


**Figure B-2. Myocardial infarction.**

Myocardial infarction (MI) occurred by atherothrombotic coronary artery disease (CAD) and usually precipitated by plaque disruptions (rupture or erosion). Prolonged atherosclerosis causes thrombosis, and dynamic thrombus components eventually lead to necrosis of cardiomyocytes.

Adapted from Thygesen K et al., 2018

## 2. STEM CELL THERAPY IN MI

After myocardial infarction, more than 1 billion cardiomyocytes die. The lost cells are replaced by fibrotic connective tissue through the process called ‘cardiac fibrosis’. This process causes scarring and stiffness in the cardiac muscle, and ultimately leads to thinning of the heart wall and weakness of heart function (Talman and Ruskoaho, 2016). Since cardiomyocytes are terminally differentiated cells and their regenerative capacity is so limited, additional strategies based on stem cells are required (Foglia and Poss, 2016). Stem cells have the potential to proliferate and differentiate into various types of cells, including cardiomyocytes (Slack, 2007, Slack, 2013). In this respect, the therapeutic strategies utilizing stem cells have sufficient potential to be applied to MI. Different types of stem cells in various administration methods have been investigated, and promising results have been shown in many preclinical studies. Particularly Mesenchymal stem cells (MSCs), with their proliferative capacity and ability to be differentiated into various lineage cells, are promising candidates for MI treatment (Kubo et al., 2009, Shafei et al., 2017) (Table B-1).

MSCs were first discovered in 1970. According to the international society for cellular therapy, MSCs are defined as self-renewing cells that express CD90 and CD73 but do not express CD45, CD34, CD14, CD11b, CD79a, or CD19 cell surface markers. MSCs can be derived from a variety of sources, including bone marrow, adipose tissue, umbilical cord blood, dental pulp, etc (Kern et al., 2006). Each type of MSC has a different isolation protocol and culture conditions, and due

to their various sources, each has its own unique properties.

Recently, human umbilical cord blood derived mesenchymal stem cells (hUCB-MSCs) have received special attention in regenerative medicine because they form a connection between embryonic and adult stem cells, and have the advantages of both (Han et al., 2019). Compared to adult stem cells, hUCB-MSCs have a higher proliferative and differentiative capacity, enabling better clinical applications. On the other hand, when compared to embryonic stem cells, hUCB-MSCs have a lower potential for teratoma formation, thus enabling safer treatment. Especially regarding cardiovascular diseases, hUCB-MSCs have shown positive results in various preclinical studies due to their various advantages such as fast self-renewal, immuno-modulation, or capacity to differentiate to various lineages. Additionally, hUCB-MSCs could encourage neovascularization and anti-fibrosis (Lee, 2018, Bagno et al., 2018) (Figure B-3).

The major function of hUCB-MSCs involves their paracrine effects in MI. Transplanted cells secrete various cytokines that could restore cardiac metabolism. These are cell chemotactic factors that assist cell recruitment to the ischemic region, thereby reducing the scar formation. They also promote angiogenesis, alleviate the symptoms caused by limb ischemia, and restore blood supply that has been interrupted. hUCB-MSCs produce high levels of angiogenic growth factors such as hematocyte growth factor (HGF), basic fibroblast growth factor (bFGF), VEGF-D, and platelet-derived growth factor (PDGF) (Majka et al., 2017) (Figure B-4). However, the interesting thing is that they produce very little VEGF-A (Akimoto et al., 2013). These facts indicate that the cells can induce angiogenesis independently

of VEGF-A, and thus there is no need for induction of VEGF-A in cell therapy using hUCB-MSCs. In addition to neovascularization, the immunomodulatory properties of hUCB-MSCs targeted to inflammation are thought to be responsible for additional paracrine effects. HUCB-MSCs produce interleukin (IL)-6, which promotes the acquisition of tolerogenic phenotypes, and prostaglandin E2 (PGE2), which suppresses cytotoxicity of NK cells and proliferation of CD4+/CD8+ T-cells (Cortes-Araya et al., 2018). In addition, hUCB-MSCs secrete anti-inflammation cytokines, such as interferon-a, identifying these cells as the major regulators of early inflammation. Transplantation of hUCB-MSCs has a positive effect on the innate immune system, as well as on the adaptive immune system. Transplanted cells produce IL-6 and HGF-rich microenvironments, which primes the monocytes to produce IL-10 (Wang et al., 2009).

With regards to cardiac regeneration, the introduction of hUCB-MSCs for cell therapy showed various effects such as anti-fibrotic, anti-inflammatory and pro-angiogenic effects. This has been demonstrated in many preclinical studies and offers ample potential for clinical studies (Singh et al., 2016). Nevertheless, there remains many limitations to overcome for application in clinical treatment, including the low accuracy of delivered cells to the damaged areas and low survival rates of transplanted hUCB-MSCs due to harsh conditions of infarcted regions (Yu et al., 2008). To overcome these limitations, a variety of additional strategies have been attempted, including combinations with other cell types (e.g., CSCs or macrophages), additional pharmaceutical approaches, and enhancing specific cellular functions through gene overexpression. However, the therapeutic

effects are short-lived or incomplete. Therefore, continuous and diverse efforts should be encouraged to develop the best treatment strategy using hUCB-MSCs.

Table 1: Clinical trials with mesenchymal stem cells (https://clinicaltrials.gov/).

| | Study | Year (country) | Study status | Age | Number treated | Phase | Study ID |
|---|---|---|---|---|---|---|---|
| 1 | Mesenchymal Stem Cells and Myocardial Ischemia | 2010–2014 (France) | Completed | 18 years and older | 10 | Phase 1 Phase 2 | NCT01076920 |
| 2 | Administration of Mesenchymal Stem Cells in Patients with Chronic Ischemic Cardiomyopathy (MESAMI2) | 2015–2016 (France) | Ongoing | 18 years to 75 years | 90 | Phase 2 | NCT02462330 |
| 3 | Stem Cell Therapy for Vasculogenesis in Patients with Severe Myocardial Ischemia | 2009–2013 (Denmark) | Completed | 30 years to 80 years | 31 | Phase 1 Phase 2 | NCT00260338 |
| 4 | Human Umbilical-Cord-Derived Mesenchymal Stem Cell Therapy in Ischemic Cardiomyopathy | 2015–2018 (China) | Ongoing | 18 years to 80 years | 40 | Phase 1 Phase 2 | NCT02439541 |
| 5 | MesenchYmal STROMAL CELL Therapy in Patients with Chronic Myocardial Ischemia (MyStromalCell Trial) | 2010–2014 (Denmark) | Completed | 30 years to 80 years | 60 | Phase 2 | NCT01449032 |
| 6 | Safety and Exploratory Efficacy Study of UCMSCs in Patients With Ischemic Heart Disease (SEESUPIHD) | 2016–2017 (China) | Ongoing | 18 years to 70 years | 64 | Phase 1 Phase 2 | NCT02666391 |
| 7 | Intracoronary Autologous Mesenchymal Stem Cells Implantation in Patients with Ischemic Dilated Cardiomyopathy | 2012–2015 (Malaysia) | Ongoing | 35 years to 75 years | 80 | Phase 2 | NCT01720888 |
| 8 | Therapy of Preconditioned Autologous BMMSCs for Patients With Ischemic Heart Disease | 2015–2017 (China) | Ongoing | up to 75 years | 200 | Phase 1 Phase 2 | NCT02504437 |
| 9 | The TRansendocardial Stem Cell Injection Delivery Effects on Neomyogenesis STudy (The TRIDENT Study) | 2013–2017 (USA) | Ongoing | 21 years to 90 years | 30 | Phase 2 | NCT02013674 |
| 10 | Mesenchymal Stem Cell Administration in the Treatment of Coronary Graft Disease in Heart Transplant Patients | 2014–2017 (France) | Ongoing | 18 years to 80 years | 14 | Phase 1 Phase 2 | NCT02472002 |
| 11 | Safety and Efficacy of Intracoronary Adult Human Mesenchymal Stem Cells after Acute Myocardial Infarction | 2007–2011 (Korea) | Completed | 18 years to 70 years | 80 | Phase 2 Phase 3 | NCT01392105 |
| 12 | Human Umbilical Cord Strona MSC in Myocardial Infarction | 2014–2017 (Turky) | Ongoing | 30 years to 80 years | 79 | Phase 1 Phase 2 | NCT02323477 |
| 13 | Stem Cell Injection to Treat Heart Damage during Open Heart Surgery | 2012–2020 (USA) | Ongoing | 18 years to 85 years | 60 | Phase 1 | NCT01557543 |
| 14 | Safety Study of Adult Mesenchymal Stem Cells (MSC) to Treat Acute Myocardial Infarction | 2005–2014 (Australia) | Completed | 21 years to 85 years | 53 | Phase 1 | NCT00114452 |
| 15 | RELIEF (A Randomized, Open labEled, mul.ticenter Trial for Safety and Efficacy of Intracoronary Adult Human Mesenchymal stEm Cells Acute Myocardial inFarction) | 2012–2016 (Korea) | Ongoing | 20 years to 70 years | 135 | Phase 3 | NCT01652209 |
| 16 | Intracoronary Human Wharton's Jelly-Derived Mesenchymal Stem Cells (WJ-MSCs) Transfer in Patients with Acute Myocardial Infarction (AMI) | 2011–2015 (China) | Completed | 18 years and older | 160 | Phase 2 | NCT01291329 |
| 17 | Ex Vivo Cultured Bone Marrow Derived Allogenic MSCs in AMI | 2009–2013 (India) | Completed | 20 years to 70 years | 20 | Phase 1 Phase 2 | NCT00883727 |
| 18 | "ESTIMATION Study" for Endocardial Mesenchymal Stem Cells Implantation in Patients after Acute Myocardial Infarction | 2011–2016 (Russia) | Ongoing | 30 years to 75 years | 50 | Phase 3 | NCT01394432 |
| 19 | Prochymal® (Human Adult Stem Cells) Intravenous Infusion following Acute Myocardial Infarction (AMI) | 2009–2016 (Australia) | Ongoing | 21 years to 85 years | 220 | Phase 2 | NCT00877903 |
| 20 | Plasmonic Nanophotothermic Therapy of Atherosclerosis | 2007–2015 (Russia) | Completed *Has results* | 45 years to 65 years | 180 | Phase 1 Phase 2 | NCT01270139 |
| 21 | The Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis Pilot Study (The POSEIDON-Pilot Study) | 2010–2015 (USA) | Completed *Has results* | 21 years to 90 years | 31 | Phase 1 Phase 2 | NCT01087996 |
| 22 | The Transendocardial Autologous Cells (hMSC or hBMC) in Ischemic Heart Failure Trial (TAC-HFT) | 2008–2015 (USA) | Completed *Has results* | 21 years to 90 years | 65 | Phase 1 Phase 2 | NCT00768066 |
| 23 | Safety and Efficacy Study of Stem Cell Transplantation to Treat Dilated Cardiomyopathy | 2013–2015 (Slovenia, USA) | Completed | 18 years to 80 years | 110 | Phase 2 | NCT00629018 |

**Table B-1. 23 Clinical trials using MSCs in relation to MI.**

Clinical Trials related to MSCs in myocardial infarction.

Adapted from Seung Taek Ji et al., 2017

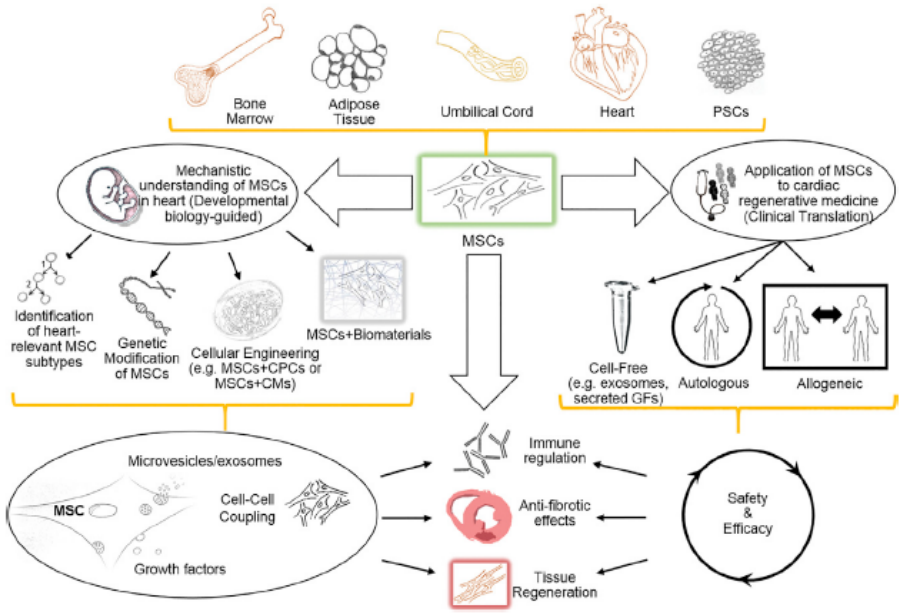


**Figure B-3. MSCs are an attractive cellular platform for cardiac regenerative medicine.**

MSCs can be isolated from various tissues, including bone, adipose tissue, umbilical cord, or they can be originated from pluripotent stem cells. Ongoing research, examining the relationship of MSCs to heart regeneration, will lead to the development of more innovative strategies, such as isolating cardiac MSC lineages, generating genetically engineered MSCs with various growth factors and exosome secretomes, and optimizing combination strategies of MSCs with biomaterials similar to heart tissue.

Adapted from Luiza Bagno et al., 2018

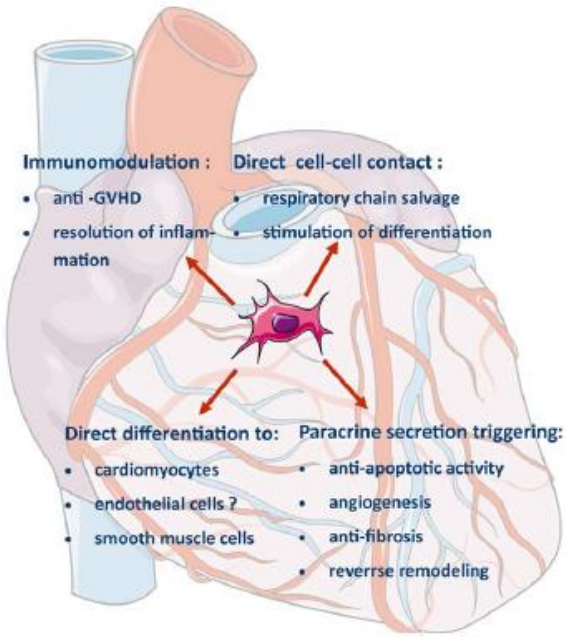


**Figure B-4. Therapeutic effect of mesenchymal stem cells in myocardial infarction.**

Mesenchymal stem cells mechanisms of action in myocardial infarction. Abbreviation: GVHD, graft-versus-host disease.

Adapted from Marcin Majka et al., 2017

## 3. VEGF AND LEF1

Vascular endothelial growth factor (VEGF) is a special mitogen that induces vascular endothelial cells to begin mitosis. It is one of the most actively studied angiogenic growth factors, and is known to play major roles in vascular formation during embryonic development, injury, bone formation and even cancers (Shibuya, 2011).

In 1989, Henzel and Ferrara first purified and cloned the mitogen from endothelial cells, and began to name this factor VEGF. The factor can be divided into VEGF-A, B, C, E and F depending on the structure, and each interacts with different receptors. Each member has a variety of roles, such as angiogenesis, embryonic angiogenesis and development of lymphatic vasculature, depending on the interacting receptors (Ferrara, 2016).

Among them, VEGF-A is a protein which has two 23kDa homodimer subunits. The gene encoding this protein in humans is located on p21.1 of chromosome 6 and its size is 16,304 bases. VEGF-A is closely associated with angiogenesis of most organs during vertebrate development, and is associated with angiogenesis during organ remodeling in adults (Figure B-5). Endothelial cell differentiation is highly dependent on the expression of this gene, so deletion of this gene during development could be embryonic lethal. VEGF-A has various isoforms due to alternative splicing. Five isoforms are produced in humans (VEGF-A 121, 145, 165, 183, 189, and 206), and the most expressed isoform is VEGF-A 165 (Holmes and Zachary, 2005) (Figure B-6).

Lymphoid enhancer-binding factor 1 (LEF1) is a protein in the nucleus of pre-B and T cells, and has a molecular weight of 48kDa (Milatovich et al., 1991). In humans, it is translated from the LEF1 gene at position q25 of chromosome 4. It is known that this growth factor attaches to T cell receptor-alpha enhancers and induces maximum enhancer activity and is also expressed at the early stages of differentiation in B-cells thus playing a key role in cell survival and division. In addition, it is considered to be an important mediator of the Wnt/β-Catenin signaling pathway, one of the factors of the T-cell factor (TCF)/LEF1 transcription factor family (Cadigan and Waterman, 2012). Currently, seven LEF1 isoforms, including LEF1A, LEF1B and LEF1DN, have been identified through alternative promoter usage and alternative splicing. In addition, through computational mapping, three more potential isoforms are also suspected to exist.

LEF1 has received much attention in recent cardiac regeneration research. In 2009, it was confirmed that LEF1 is necessary for the development of major blood vessels and heart tissue through xenopus tropicalis models (Roel et al., 2009). In 2019, LEF1, along with other TCF families, was reported to induce cardiac maturation through temporal and spatial control in cardiac development through Wnt/β-Catenin signaling (Ye et al., 2019). LEF1 was mainly expressed in the valvular region. Furthermore, stem cell research continues to report that LEF1 promotes self-renewal. According to a 2010 study by Huang C, LEF1 increased the activity of the Oct4 promoter and physiologically interacted with Nanog to help maintain mouse embryonic stem cells (Huang and Qin, 2010b). In addition, it has been reported that the proliferation of adult hippocampal precursor cells as well as

Pro-B cells is also promoted by LEF1.

Considering these studies and results, we can expect that LEF1 plays a major role in heart development and promoting cell proliferation. However, studies related to cell proliferation and survival of LEF1 in hUCB-MSCs have not been reported yet. Experimental confirmation of these functions and application of LEF1 overexpressed MSCs to the MI to confirm its therapeutic applicability seems to be necessary.

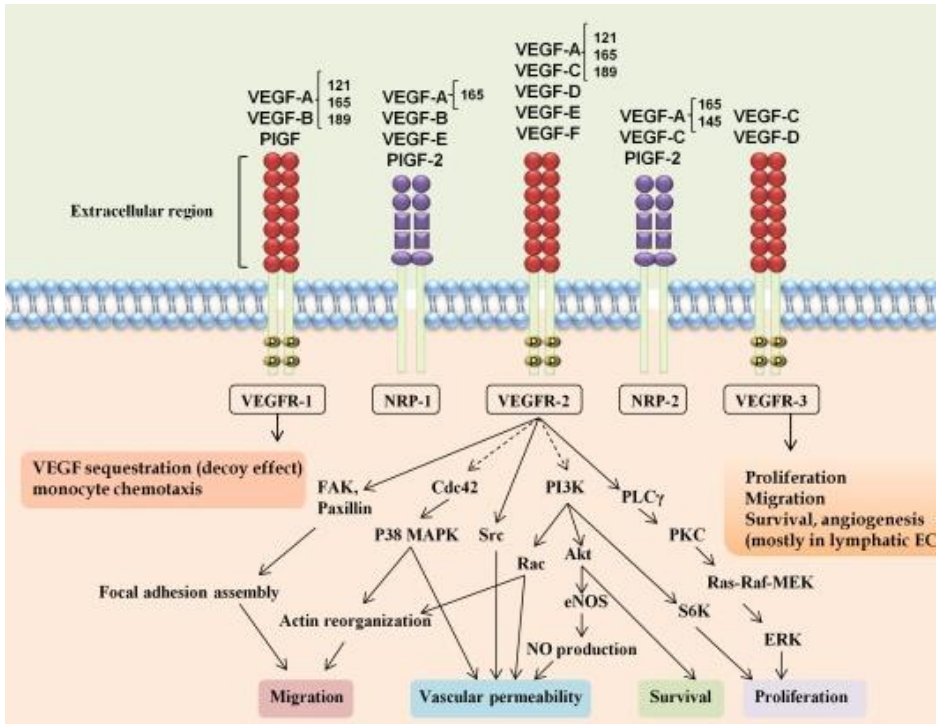


**Figure B-5. Growth factors of the VEGF family.**

The three signaling receptors of the VEGF family (VEGFR-1, VEGFR-2, and VEGFR-3) and the accessory isoform specific receptors neuropilin-1, neuropilin-2 are displayed with structural features.

Adapted from Masoumi Moghaddam S et al., 2012

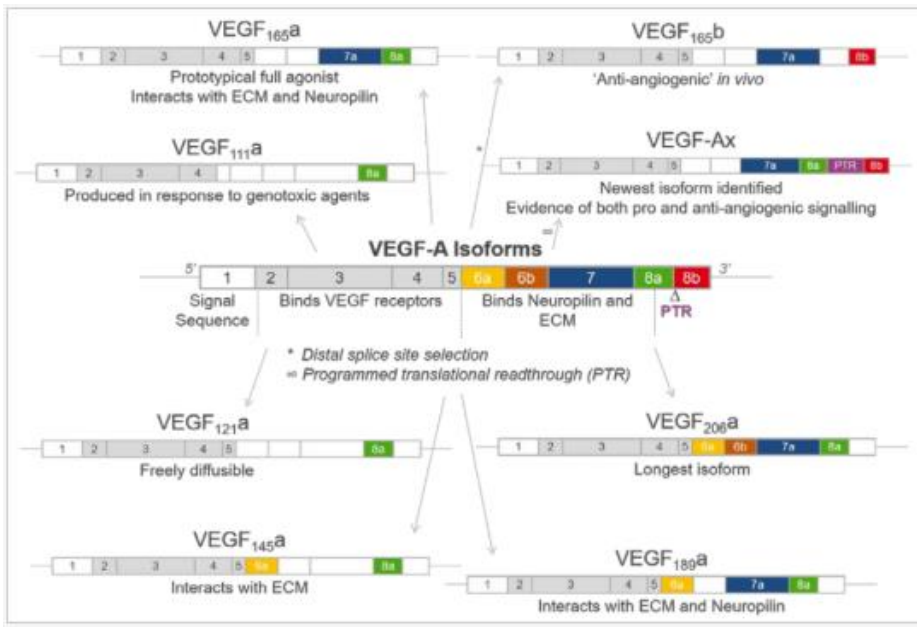


**Figure B-6. Various isoforms of the VEGF.**

Vascular endothelial growth factor (VEGF) mRNA generates four different isoforms, VEGF121, VEGF165, VEGF189, or VEGF206 after alternative splicing. Of these, VEGF121 is a nonheparin-binding acidic protein, which is freely diffusible.

Adapted from Chloe J. Peach et al., 2018

# 4. TALEN AND CRISPR/CAS9

Genome editing technology is an emerging therapeutic strategy that manipulates eukaryotic genomes using specific nucleases. These nucleases are composed of restriction enzymes and DNA binding domains engineered to bind to specific sequences and produce double-stranded breaks (DSBs). There are many known genome-editing nucleases, but they share the same platforms. After the nucleases cut a specific sequence on the genome, the DSB can be repaired by the following two endogenous DNA repair systems: non-homologous end joining (NHEJ) and homology-directed repair (HDR) with Donor DNA (Cox et al., 2015, Lieber et al., 2003).

NHEJ is efficient, but often causes errors during the process. Thus, the repeated break and repair process at the same genomic locus results in deletions or small insertions (indels) in the desired site. These indels may result in mRNA degradation or production of nonfunctional proteins. Therefore, programmed NHEJ can be used to permanently disrupt target genes (Hilton and Gersbach, 2015). In contrast, HDR requires donor DNA to be exogenously delivered. The donor DNA contains homologous sequences with DSBs and foreign sequences need to be inserted between the homologous sequences. After a DSB, the donor DNA makes an accurate modification at the locus, and in the process the external sequence can be integrated into the endogenous locus. Thus, HDR-based genome editing is used to knock in target genes at specific locus to induce expression patterns of the genes (Lee et al., 2018) (Figure B-7). There are three major platforms for genome-editing

nucleases to induce DSBs in the genome: zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9).

ZFNs and TALENs generate site recognition specificity via protein–DNA interactions. TALEN consists of a transcription activator-like effector (TALE) domain that binds to DNA and a FOK Ⅰ nuclease, which is a cleavage domain. Although additional molecular cloning is still required, TALEN has high specificity as well as potency since it can be designed quickly. Unlike the two conventional nucleases, the CRISPR/Cas9 system consists of a guide RNA molecule, which forms a base pair with the target sequence, and a CAS nuclease (Jiang and Doudna, 2017). The system requires a simple manufacturing process because it can assign target sequences on various genomes, by simply changing the guide RNA sequences (Figure B-8). For this reason, the recent application of this system to gene therapy has had a tremendous impact.

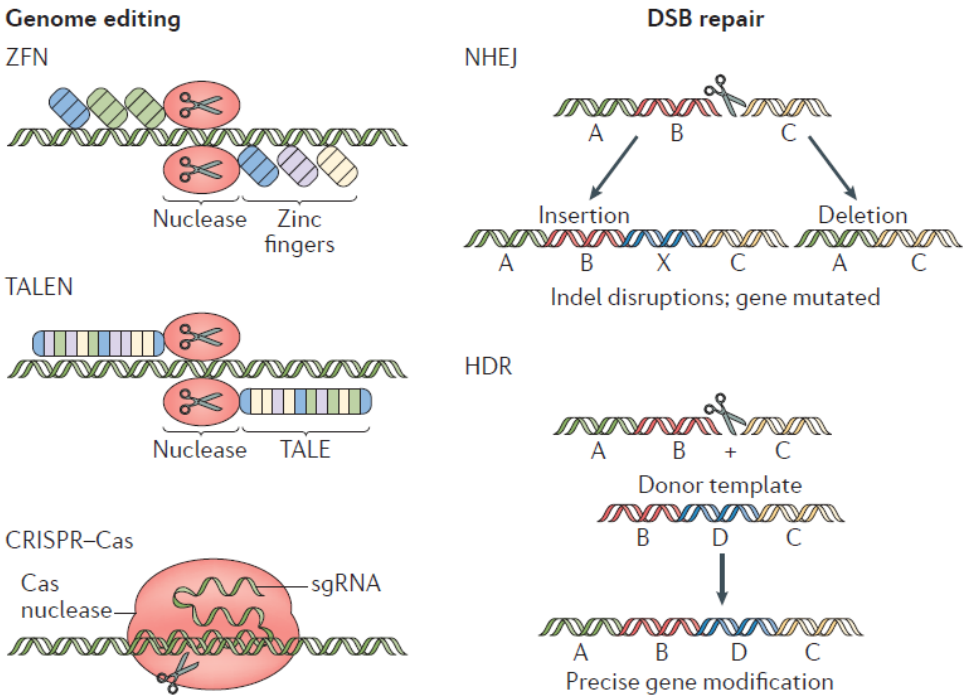


**Figure B-7. The mechanisms of genome editing.**

Zinc-finger nuclease (ZFN), transcription activator-like effector (TALE) nuclease (TALEN) and CRISPR–Cas systems induce double-strand breaks (DSBs) in DNA. There are two mechanisms repair the DSB for genome editing: non-homologous end joining (NHEJ) and homology-directed repair (HDR). Among these, NHEJ disrupts the target genome region through insertions or deletions, whereas HDR inserts donor DNA template into the target genomic region to install insertions, deletions or alterations of genomic sequences.

Adapted from Hao Yin et al., 2017

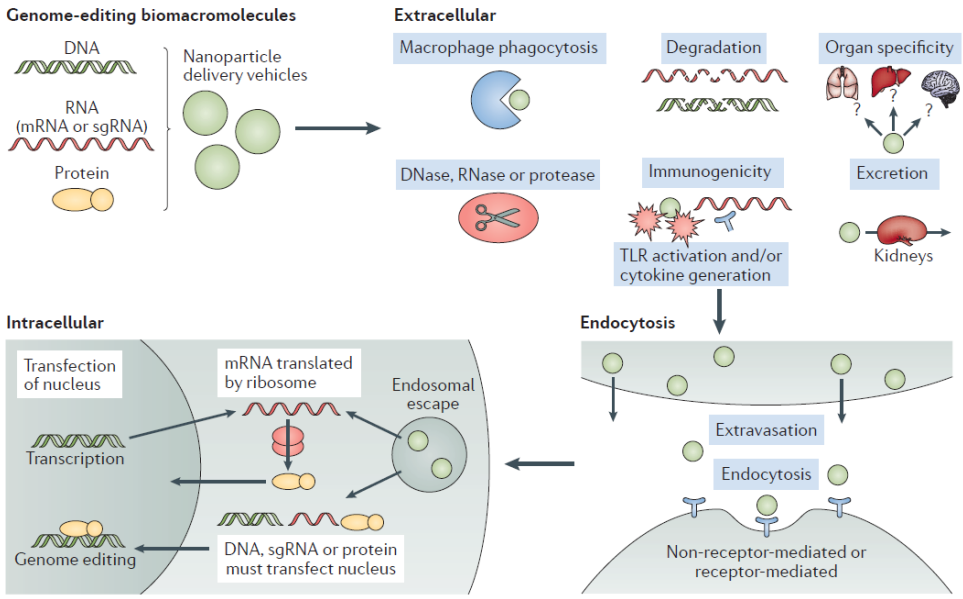


**Figure B-8. Barriers to delivery of genome-editing components.**

Genome-editing bio macromolecules (DNA, RNA, protein) or nanoparticles containing these bio macromolecules should avoid extracellular barriers. These barriers include phagocytosis, degradation through enzymatic means, and the induction of an immune response and the cytokines.

Adapted from Hao Yin et al., 2017

# CHAPTER Ⅰ

# Targeted Genome Engineering to Control VEGF Expression in Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells: Potential Implications for the Treatment of Myocardial Infarction

# INTRODUCTION

Myocardial infarction (MI) is a serious health and economic problem and a primary cause of mortality in developed countries. A primary therapy for this disease is cardiac regeneration, which represents a difficult and immense challenge. During the process of MI, the coronary artery is occluded after plaque rupture. After a few weeks, the ischemic region is replaced with a thin fibrotic area that finally causes cardiac dysfunction (Vallee et al., 2012). The cardiomyocytes in the ischemic site have a limited capacity for self-regeneration, and their repopulation is almost impossible (Segers and Lee, 2008). Thus, other sources of cells and factors are required to regenerate the infarcted region of the heart.

Stem cell-based therapeutic approaches have attracted interest for heart regeneration and cardiac repair after MI due to their regenerative potency. Various trials have treated the injured myocardium using diverse mesenchymal stem cell populations (Kang et al., 2014, Karam et al., 2012, Shi and Li, 2008). Some of these studies showed restoration of damaged cardiomyocytes, enhancement of cardiac function and a reduced infarct size to certain degrees. Cell therapy using human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) has also shown positive effects in pre-clinical trials (Kang et al., 2014). However, stem

cell therapy has encountered problems, such as low survival and proliferation rates because the MI region is localized in a hostile microenvironment and the oxygen supply is low. Therefore, to acquire efficient and rapid therapeutic efficacy of stem cells, providing an intrinsic vascularization stimulus by growth factors are demanded.

Vascular endothelial growth factor (VEGF) is an important angiogenic factor that promotes the survival of endothelial cells (EC) and prevents EC apoptosis (Ferrara et al., 2003). VEGF can be used as a therapeutic reagent in ischemic injuries. Rat mesenchymal stem cells expressing VEGF transiently by adenovirus induced angiogenesis and protected the remaining cardiomyocytes in MI animal models (Matsumoto et al., 2005). VEGF protein with HGF has also been demonstrated to have a cardio-protective effect by increasing the tolerance of cardiomyocytes to ischemia and reducing cardiomyocyte apoptosis (Deuse et al., 2009). However, transient gene expression and protein injection are limited for long-term therapeutic effect accomplishment in myocardial infarction. Thus, for long-term gene expression, retrovirus-mediated gene delivery has been attempted. However, gene delivery through a retroviral vector for cell transduction also carries a risk of stem cell neoplastic transformation and oncogene activation by the LTR promoter (Baum et al., 2003). Therefore, this retrovirus system has a limit for therapeutic application and may cause tumors and insertional mutagenesis via the incorporation of the growth factor gene into a critical region of the host chromosome. Moreover, high and uncontrollable level of VEGF itself also has the potential to cause abnormal blood vessel formation and hemangiomas (Ozawa et al.,

2004).

One way to overcome these issues is to use a controllable system for the transgene and to ensure integration into a safe harbor site in the chromosome. The tetracycline (Tet)-controlled system is commonly used for gene regulation in mammalian cells (Gossen and Bujard, 1992). In this system, rtTA binds to the Tet-On promoter in the presence of doxycycline (Dox) and activates Tet-On/CMV-driven gene transcription (Watanabe et al., 2007). For safe gene insertion into a safe harbor site of human chromosome, genome editing technologies have been used; zinc-finger nucleases (ZFN), Transcription activator-like effector nucleases (TALEN) and recent clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas9 (Gaj et al., 2013). TALENs are targetable nucleases composed of a *Fok*I nuclease domain and customizable DNA binding domain (Bogdanove and Voytas, 2011). The DNA-binding domain has conserved repeats that originate from transcription activator-like effectors (TALEs) found in Xanthomonas plant pathogens. TALENs have rapidly emerged as a genome editing technology with high efficiency and low toxicity that allow safe, targeted non-viral gene delivery to a specific chromosome locus (Joung and Sander, 2013). It is known that the human chromosome 19 AAVS1 integration site is a 'safe harbor site' for the targeted insertion of foreign genes by producing DNA double-strand breaks and subsequent repair via homologous recombination (HR) (Mussolino et al., 2014).

In this study, we developed inducible VEGF-secreting hUCB-MSCs by TALEN-mediated *VEGF* gene integration into a safe harbor site of the chromosome. These engineered stem cells were capable of secreting VEGF at

physiological concentration upon induction by Dox treatment. *VEGF*/hUCB-MSCs were implanted in a myocardial infarction rat model to assess whether they could enhance angiogenesis and provide a cardioprotective effect.

# MATERIAL AND METHODS

***Isolation and culture of human UCB-MSCs***

Human UCB-MSCs were isolated as previously described (Seo et al., 2011). Human UCB-MSC isolation was performed according to the procedure approved by the Borame Hospital Institutional Review Board and Seoul National University (IRB No. 0603/001-002-07C1). The medium was changed at 48 hr intervals and the cells were subcultured after they reached 90% confluence, unless described.

***Designing new TALEN vectors and Inducible VEGF donor vectors***

The left and right TALEN plasmids were newly constructed as follows. The target sequences of the chromosome 19 AAVS1-targeting TALENs were: 5′-TGGAGCCATCTCTCTCCTT-3′ (Left) – gccagaacctctaa (spacer) - 5′-GGTTTGCTTACGATGGA-3′ (Right). The plasmids encoding the TALENs targeting this sequence were prepared as previously described (Kim et al., 2013). To prepare the efficient targeting donor DNA, we also designed new 800 bp homology arms that were approximately 50 bp apart and flanked both sides of the TALEN target site. The homology arms were PCR-amplified from human genomic DNA and cloned into the pGEM T-Easy vector. The left and right homology arms

were isolated using pairs of restriction enzymes (KpnI/AgeI/NotI for the left and NotI/EcoRI/SphI for the right homology arms) and cloned into the KpnI/SphI site of the pUC19 vector.

The inducible donor vectors were constructed using three DNA fragments. The Tet-on mini-CMV promoter (Addgene®, Cambridge, MA, USA), VEGF cDNA (synthesized by Bioneer Co. Ltd) and hEF1a-rtTA-pA (Addgene®, http://www.addgene.org) were amplified by PCR using specific primers containing the flanking sequences. Each amplified DNA fragment was cloned into the pZDonor-AAVS1-puromycin DNA vector (Sigma–Aldrich) digested with the AgeI and EcoRI restriction enzymes (New England Biolabs® ) using the In-Fusion® HD Cloning Kit (Clonetech) (Figure 1). Then, the whole insert TetO-CMV-VEGF-hEF1a-rtTA was transferred into the pUC19-AAVS1 donor vector that contained newly designed HA-L and HA-R sequences to target to the chromosome 19 AAVS1 site.

***TALEN-mediated homologous recombination***

For the genetic modification of hUCB-MSCs to introduce the inducible *VEGF* DNA construct into the safe harbor site, 8 x $10^5$ cells seeded on a 60 mm dish were transfected with TALEN left (L), right (R) (1.5 ug) and the pUC19-TetOn-CMV-VEGF-hEF1a-rtTA donor vector (3 ug). At 5 days post-transfection, the medium was changed; then, the cells were sub-cultured two times at 4.5 day intervals. Genomic DNA was isolated from the hUCB-MSCs by adding 1 mL of the SNET extraction buffer per $10^6$ cells (20 mM Tris-HCl [pH 8], 5 mM EDTA [pH 8], 400

mM NaCl, and 1% SDS) containing proteinase K (100 mg/mL; Sigma-Aldrich). The DNA samples were incubated at 55℃ for 2 hrs. Proteinase K was inactivated by incubating the DNA samples at 98℃ for 10 min; then, RNase (1 mg/mL) was added and the cells were incubated at 37℃ for 30 min. The DNA was precipitated and its concentration was determined by spectrophotometry. TALEN-mediated HR was detected using PCR genotyping. PCR amplification of the genomic DNA was performed using the following parameters: an initial denaturation step at 94℃ for 5 min, followed by 35 cycles at 94℃ for 15 s, 65℃ for 45 s and 72℃ for 150 s with a final extension step at 72℃ for 10 min. The amplified products were analyzed on a 1% agarose gel.

***Subcutaneous transplantation of VEGF/hUCB-MSCs on cell sheets***

*VEGF/*hUCB-MSC cell sheets were subcutaneously transplanted on the mouse dorsal skin as previously described (Obokata et al., 2011). Briefly, *VEGF/*hUCB-MSCs were cultured on the UpCell surface at a density of $1x10^6$ cells per 35 mm dish. Six female mice were anesthetized and the dorsal skin was lifted and cut vertically. The cell sheet was transferred onto a PVDF membrane and the PVDF membrane was slowly removed. Doxycycline was added to the drinking water at a concentration of 1 μg/mL. Eight days after transplantation, the animals were sacrificed for analysis.

***Cell-Matrigel transplantation into SCID mice***

The animal protocol was approved by the Seoul National University Institutional Animal Care and Use Committee (IACUC) prior to the experiment. Vascular formation was evaluated in 7- to 8-week-old female SCID mice (Orient Bio, Seongnam, Korea). Matrigel was loaded with a final concentration of $1x10^6$ cells/100 μl. The injection sites were cleaned with ethanol twice prior to injection of Cell-Matrigel into each site using 1 mL syringes and 27 G needles. Eight days after the injections, the animals were sacrificed for analysis.

***Myocardial infarction (MI) rat model and cell transplantation***

The myocardial infarction rat models were surgically induced as previously described (Kang et al., 2014). Briefly, Male Sprague-Dawley rats weighting 260-300 g (Orient Bio, Seongnam, Korea) were used for the MI model. All animal procedures were performed in accordance with the guidelines of the SNU IACUC. After MI induction, the rats were divided into four treatment groups: rats that received *VEGF*/hUCB-MSCs (n=12, later divided into 2 groups), hUCB-MSCs (n=6), or a sham operation (Control, n=6).

Immediately after the MI induction surgery, the UpCell sheets layered with *VEGF*/hUCB-MSC or hUCB-MSC cells on PIPAAm-grafted surfaces were detached from the plates by incubating at 20℃ for 20 min. Then, the cell sheets were attached to polypropylene supporting membranes (Thermo Scientific) and transplanted onto the injured epicardial anterolateral area. Seven minutes after transplantation, the supporting membrane was removed and the cell sheets showed

stable attachment. The same procedures were repeated to make a bilayer cell sheet composed of 2x $10^6$ cells. To prevent the rat's immune rejection of the human cells, all rats received cyclosporine as previously described (Kang et al., 2014).

***Functional assessment of the infarcted myocardium***

The cardiac functional assessment was performed as previously reported by our group (Kang et al., 2014). Briefly, cardiac functions were assessed by transthoracic echocardiography prior to MI surgery (normal baseline) and 1 week and 4 weeks after MI for each cell transplantation group.

**Statistical Analysis**

All statistical analyses were performed with SPSS version 20.0 (SPSS, Chicago, IL, USA). A Kruskal-Wallis test was used to assess differences among the groups. The Mann-Whitney U test was performed as the post hoc test. A p value less than 0.05 was considered to be statistically significant.

# RESULTS

***Genetic engineering of an inducible VEGF-expressing DNA cassette into the hUCB-MSC chromosome by TALEN-mediated genome editing***

Figure 1-1A shows the TALEN domains that consisted of specific binding sequences targeting the *AAVS1* locus (safe harbor site) and the FokI non-specific endonuclease domains. As illustrated in Fig 1-1B, TALEN-mediated AAVS1 site integration of an inducible VEGF-secreting cassette was accomplished in two steps. First, the TALEN left (L) and right (R) arms bind to their target sequences in the host chromosome and the FOKI endonuclease induces a DNA double-strand break. Then, the DSB is repaired by the HR machinery using a pUC19-TetO-CMV-VEGF-hEF1a-rtTA donor vector. To confirm the TALEN-mediated transgene insertion in hUCB-MSCs, the cells were co-transfected with TALEN-L/R and the pUC19-TetO-CMV-VEGF-hEF1a-rtTA donor vector. At 5 days post-transfection, the medium was changed and the cells were sub-cultured 2 times at 4.5 day intervals. After 2 weeks, the genomic DNA was extracted and PCR genotyping was performed. The amplification of 2160 bp and 2007 bp PCR fragments indicated the site-specific integration of the inducible VEGF-secreting donor cassette into the AAVS1 locus (Fig 1-2A).

Next, to confirm whether the inducible VEGF expression system from the *VEGF*/hUCB-MSCs is properly induced by treatment with Dox, western blotting was performed on *VEGF*/hUCB-MSC samples. VEGF expression and secretion were detected in the cell lysates (Fig 1-2B) and secreted media (Fig 1-2C) after Dox treatment for 48 hrs. In the transient episomal plasmid transfection experiment, we observed the induction of the VEGF secretion into the media after Dox treatment by ELISA (Fig 1-2D). Similar VEGF levels were measured in the *VEGF*/hUCB-MSC cells with the inducible system integrated into the chromosome by TALEN-mediated genome editing (Fig 1-2E). Too high a continuous level of VEGF is known to result in angioma formation, which is one of the main obstacles for VEGF therapy. Thus, the VEGF concentration is the main consideration for the treatment of ischemic disease with VEGF. In our study, the VEGF concentration secreted into the media following induction was 50.74 ng/$10^6$ cells/day, which was within the physiological concentration range (32.3-70 ng/$10^6$ cells/day). From these results, we confirmed that *VEGF*/hUCB-MSCs harboring an inducible *VEGF* gene in a safe harbor site secreted VEGF into the cell culture media in the physiological therapeutic concentration range.

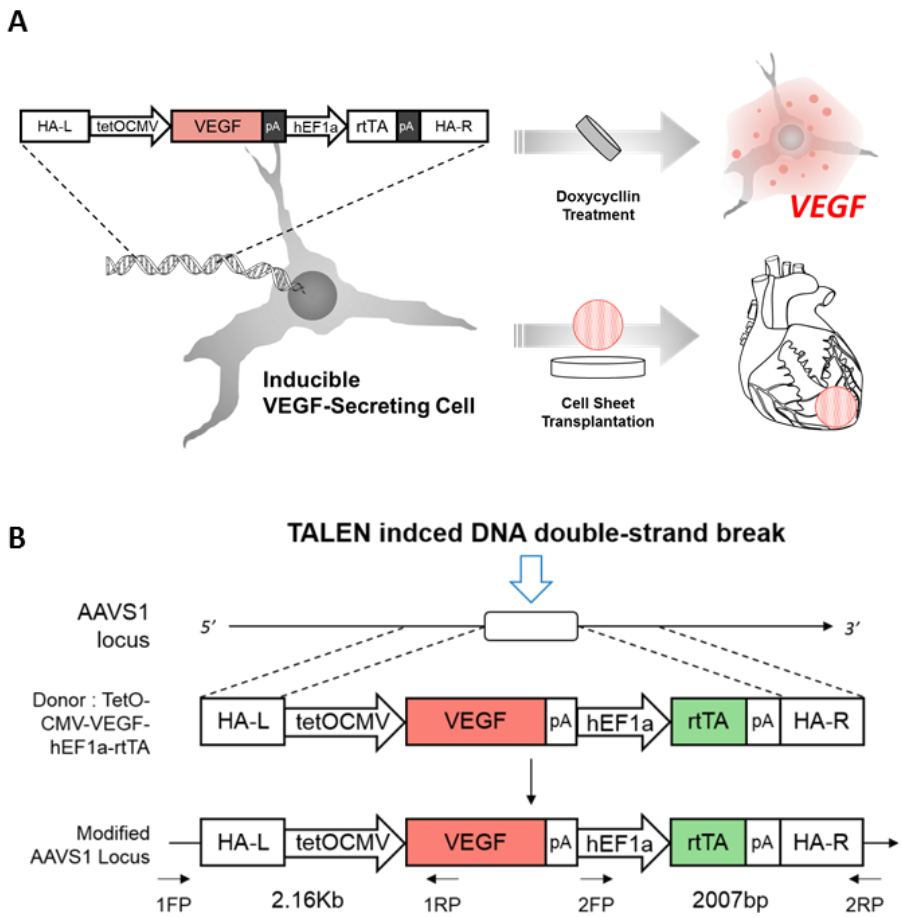


**Figure 1-1. Generation of VEGF/hUCB-MSCs.**

(A) A schematic diagram of the TALEN binding domains in AAVS1 locus (FokI denotes the FokI endonuclease). The colored DNA sequences indicate the TALEN-binding sites (red letters and blue letters). (B) The integration of the inducible VEGF secretion DNA cassette into the AAVS1 site following the TALEN-mediated DNA double-strand break. HR (L) and HR (R), the left and right arms for

homologous recombination. FP and RP, forward primer and reverse primer binding sites for the PCR primers.

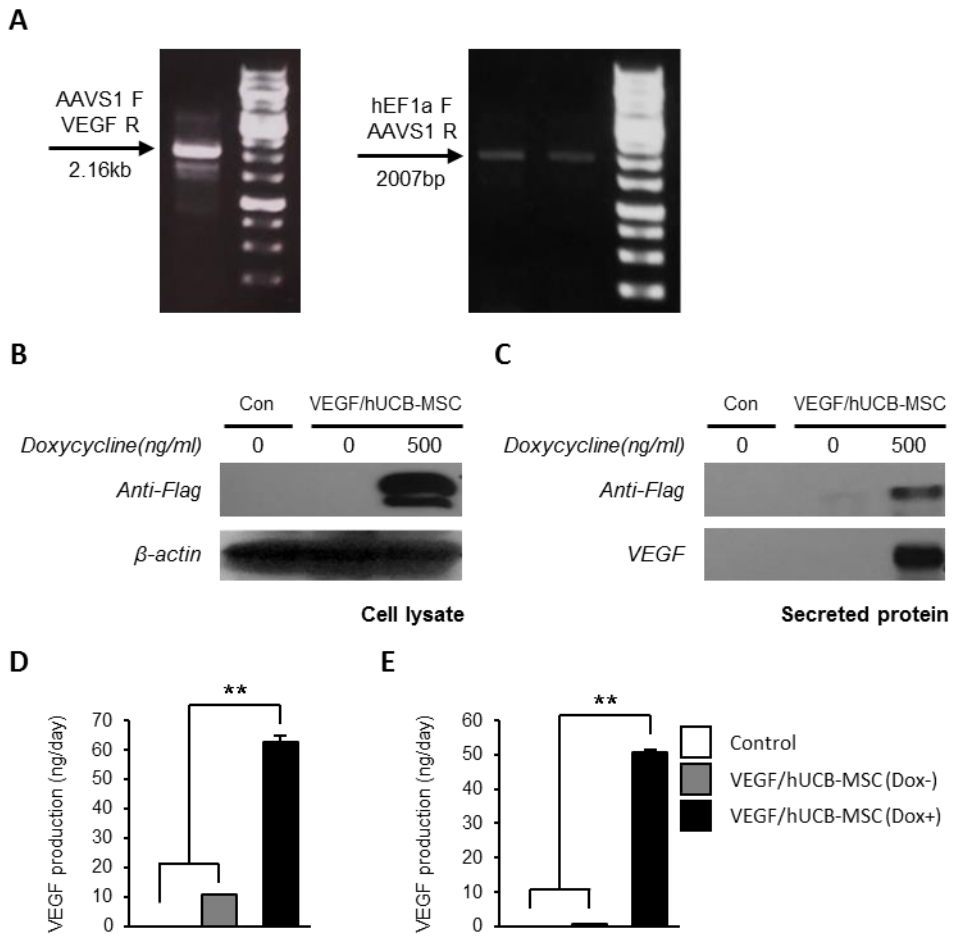


**Figure 1-2. Confirmation of conditional VEGF secretion.**

(A) Junction PCR analysis for the confirmation of the integrated inducible VEGF secretion cassette with two different PCR primer sets. For validation of the gene integration into the UCB-MSC genome, two sets of primers were used to detect the specific sites; a 2.16 Kb fragment with the AAVS1 (forward) and VEGF (reverse) region primers and a 2007 bp fragment with the hEF1a (forward) and AAVS1 (reverse) region primers. (B) Western blotting analysis detected inducible VEGF expression in VEGF/hUCB-MSC cell lysates and (C) inducible VEGF secretion in

the VEGF/hUCB-MSC culture media. The doxycycline treatment was maintained for 48 hrs. (D) Quantification of the secreted VEGF in the VEGF/hUCB-MSC (Dox+) culture media 2 days after transient transfection and (E) 2 weeks after stable transduction. The amount of VEGF production is indicated as ng/2x$10^6$ cells/day on the Y axis. The error bars indicate the SEM of three replicate measurements per group. (*: $p < 0.05$, **: $P < 0.01$).

***Increased angiogenic markers and decreased cell cycle inhibitor expression by VEGF induction***

To examine the biological effects of VEGF secreted from *VEGF/*hUCB-MSCs, we compared the expression levels of angiogenic markers and cell cycle-related genes in each group by real-time RT-PCR. At 48 hrs after the induction of VEGF secretion by Dox, the *VEGF/*hUCB-MSCs showed a significant increase in the expression of the angiogenic markers FLK1, NRP1 and ANGPT1, and a reduction in the expression of the cell cycle arrest- and senescence-related genes p21 and p16 (Fig 1-3). These results indicate that the *VEGF/*hUCB-MSCs exhibit endothelial cell-like marker characteristics through VEGF autocrine effects.

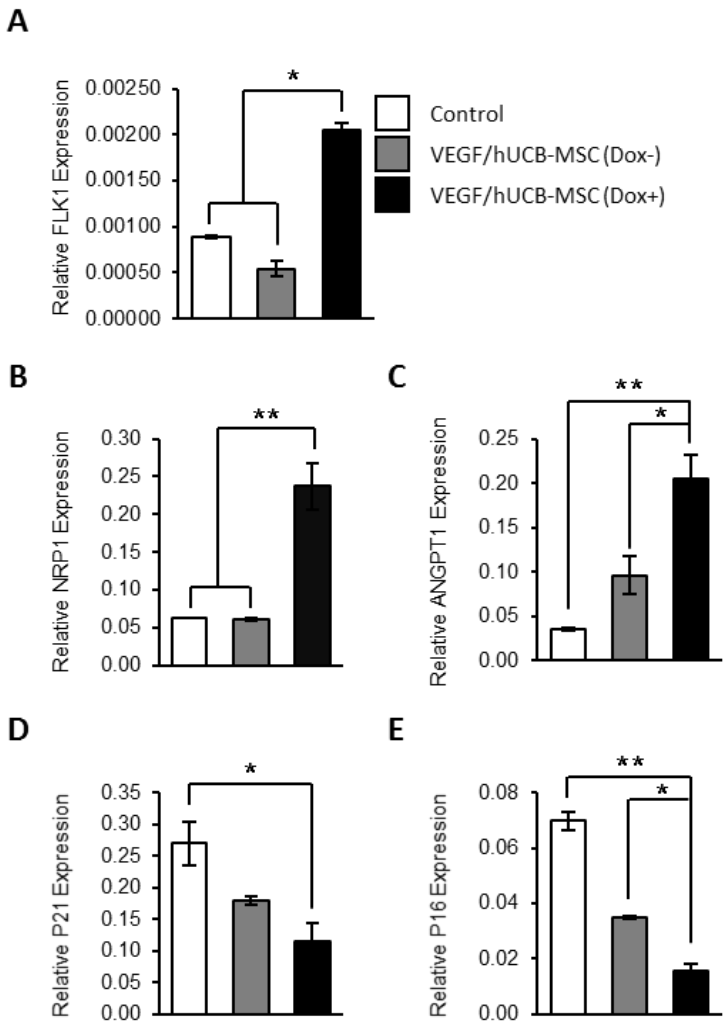


**Figure 1-3. Effect of VEGF secretion from VEGF/hUCB-MSCs on the gene expression of neo-angiogenesis markers and cell cycle regulators.**

(A-C) Induced VEGF enhanced the expression of angiogenesis-related markers. The mRNA levels of Flk-1, NRP1, and ANGPT1 in VEGF/hUCB-MSCs (Dox+) were evaluated by comparing to control hUCB-MSCs (Con) and VEGF/hUCB-MSCs (Dox-) using real-time RT-PCR. (D-E) Cell cycle regulation in VEGF/hUCB-MSCs in response to secreted VEGF. Secreted VEGF from

VEGF/hUCB-MSCs (Dox+) decreased cell cycle inhibitors (p21 and p16), suggesting that VEGF had a positive effect on hUCB-MSC survival. The experiments were performed in triprlicates, and the data were presented as the means±SE. (*: $P < 0.05$, **: $P < 0.01$, n=3).

***Functional assessment of transplanted VEGF/hUCB-MSCs* in a mouse model**

Prior to the application of *VEGF/*hUCB-MSCs to the MI animal model, we first tested: 1) whether VEGF was secreted properly by the *VEGF/*hUCB-MSCs upon Dox treatment *in vivo* and 2) whether VEGF secreted from *VEGF/*hUCB-MSCs could enhance neo-angiogenesis.

To confirm VEGF secretion by the implanted *VEGF/*hUCB-MSCs, we divided the mice into 3 groups: the hUCB-MSC cell only group, the *VEGF/*hUCB-MSC without Dox group and the *VEGF/*hUCB-MSC with Dox group. Using an UpCell transplantation system, hUCB-MSC or *VEGF/*hUCB-MSC cell sheets were transplanted onto the skin on the backs of the mice. For the *VEGF/*hUCB-MSC (Dox+) group, the mice were freely administered Dox in their drinking water (1 ug/mL) for 4 days starting one day after UpCell transplantation of *VEGF/*hUCB-MSCs (Fig 1-4A). VEGF expression was detected in the transplanted subcutaneous tissues in the Dox(+) group mice by Western blot analysis using anti-Flag and anti-VEGF antibodies (Fig 1-4B). The amount of secreted VEGF from the extracted subcutaneous tissue was quantified by ELISA. Significantly higher levels of VEGF were detected in the *VEGF/*hUCB-MSC (Dox+) group mice (15.4 ng/mg) compared to the Dox- and cell only groups (Fig 1-4C).

To test the angiogenetic function of the *VEGF/*hUCB-MSCs, we subcutaneously injected the mice with Matrigel mixed with *VEGF/*hUCB-MSCs (Fig 1-4D). The injected Matrigel plugs were removed 8 days after implantation. The plugs of the Dox-fed mice showed a more reddish color compared with the group not provided Dox, indicating greatly enhanced angiogenesis (Fig 1-4E). Immunostaining of the

Matrigel with an anti-VEGF antibody (Fig 1-4F) and an anti-vWF antibody (Fig 1-4G) showed high levels of VEGF secretion and higher levels of blood vessel formation in the doxycycline-treated group. These results demonstrated that the inducible VEGF-secreting hUCB-MSC cells secreted VEGF within normal physiological ranges and induced neo-vascularization *in vivo* following doxycycline treatment.

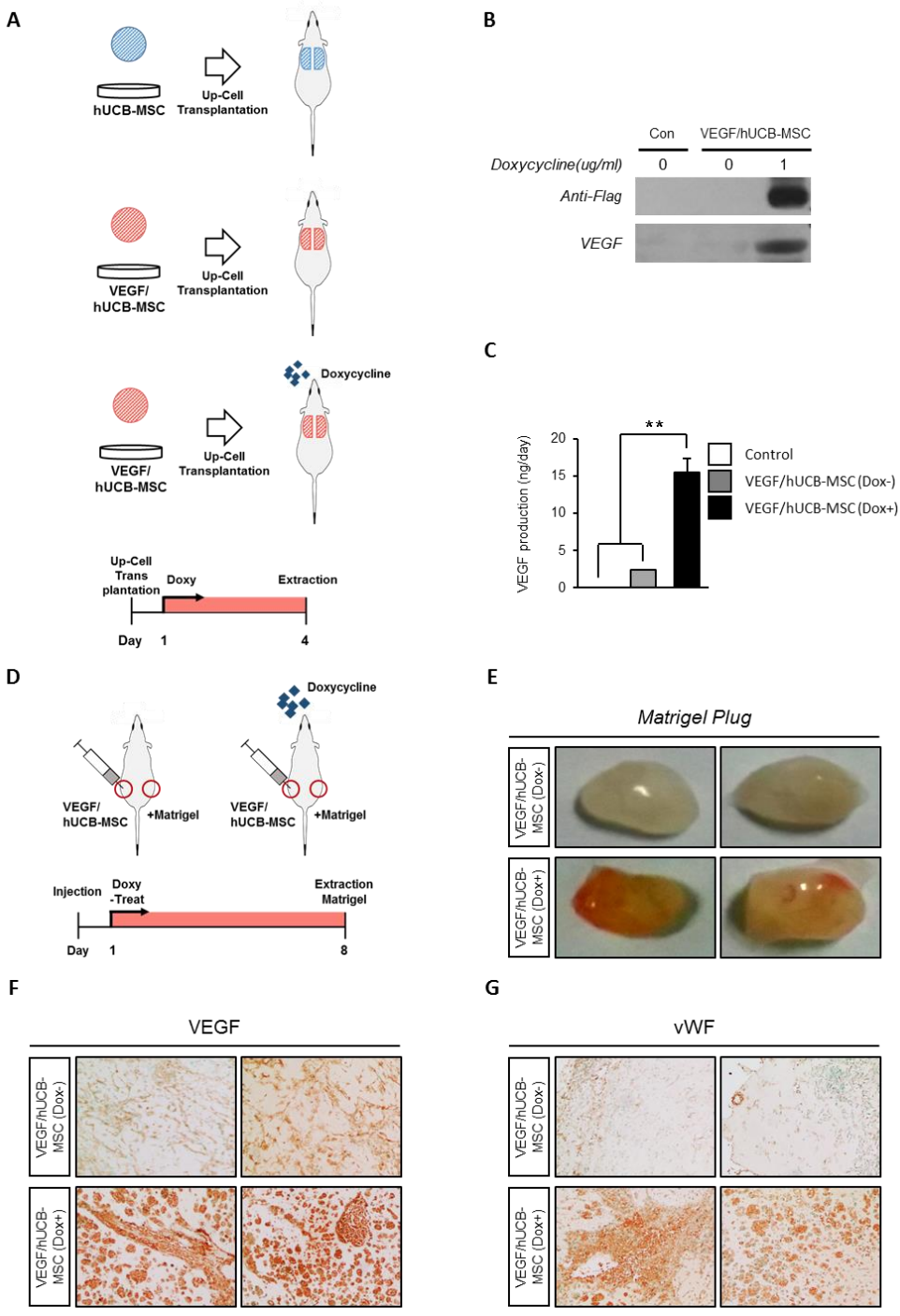
A
hUCB-MSC
Up-Cell Transplantation
VEGF/ hUCB-MSC
Up-Cell Transplantation
Doxycycline
VEGF/ hUCB-MSC
Up-Cell Transplantation
Up-Cell Trans plantation
Doxy
Extraction
Day 1 4
B
Con
VEGF/hUCB-MSC
Doxycycline(ug/ml)
0 0 1
Anti-Flag
VEGF
C
VEGF production (ng/day)
20
15
10
5
0
**
Control
VEGF/hUCB-MSC (Dox-)
VEGF/hUCB-MSC (Dox+)
D
Doxycycline
VEGF/ hUCB-MSC
+Matrigel
VEGF/ hUCB-MSC
+Matrigel
Injection
Doxy -Treat
Extraction Matrigel
Day 1 8
E
Matrigel Plug
VEGF/hUCB-MSC (Dox-)
VEGF/hUCB-MSC (Dox+)
F
VEGF
VEGF/hUCB-MSC (Dox-)
VEGF/hUCB-MSC (Dox+)
G
vWF
VEGF/hUCB-MSC (Dox-)
VEGF/hUCB-MSC (Dox+)

**Figure 1-4. The induction of VEGF expression by VEGF/hUCB-MSCs transplanted on mouse skin and neo-angiogenesis by secreted VEGF in Matrigel plugs.**

(A) In vivo test design for the transplantation of the UpCell seeded with hUCB-MSCs and VEGF/hUCB-MSCs. (B-C) Western blot and ELISA analysis for VEGF secreted at the UpCell transplantation site. Error bars indicate the SEM of three replicate measurements per clone or population. (**: $P < 0.01$, n=3). (D) Design of the blood vessel induction test in Matrigels with VEGF/hUCB-MSCs. (E) Blood vessels formed in the Matrigel containing VEGF/hUCB-MSCs without doxycycline (Dox-) and with doxycycline (Dox+). (F-G) Immunohistochemical staining for VEGF and newly formed vessels by vWF in Matrigel with VEGF/hUCB-MSCs (Dox- or Dox+). (Scale bar: 100 μm).

***Enhanced cardiac function by VEGF secretion from VEGF/hUCB-MSCs in the rat MI model***

Next, we tested whether the *VEGF/*hUCB-MSCs could enhance cardiac function upon VEGF induction in the rat myocardial infarction model. After MI generation in 6 rats each group total 24 rats including sham control, hUCB-MSCs or *VEGF/*hUCB-MSCs grown on the UpCell cell sheets were transplanted into the infarcted region of the rat hearts; a Sham operation without MI generation was also performed as a control. Echocardiography was performed to evaluate the therapeutic efficacy of VEGF secretion in combination with *VEGF/*hUCB-MSC cells in the rat MI-induced heart. Healthy heart function indicators (i.e., ejection fraction (EF), fractional shortening (FS), left ventricle inner diameter at diastole (LVIDd) and left ventricle inner diameter at systole (LVIDs)) were measured 1 week and 3 weeks after the coronary artery ligation and implantation (Fig 1-5A & B). One week after transplantation of *VEGF/*hUCB-MSCs (Dox+), the values of LVIDd, LVIDs, EF and FS showed improved heart function compared with the hUCB-MSC alone and *VEGF/*hUCB-MSC (Dox-) groups. Further improved cardiac functions were observed 3 weeks after the treatment. LV dilation and dysfunction was reduced in the *VEGF/*hUCB-MSC (Dox+) group compared to the control and other groups (Fig 1-6). Three weeks after implantation, the ejection fraction in the *VEGF/*hUCB-MSC (Dox+) group (65.39 ± 3.5%) was much higher than the ejection fraction in the control (35.31 ± 4.10%), hUCB-MSC (49.95 ± 4.19%), and *VEGF/*hUCB-MSC (Dox-) (49.24 ± 2.92%) groups (Fig 1-7A). Similarly, fraction shortening in the *VEGF/*hUCB-MSC (Dox+) group (31.87 ±

1.94%) was significantly higher compared to the control (14.61 ± 1.88%), hUCB-MSC (22 ± 1.87%), and *VEGF*/hUCB-MSC (Dox-) (21.80 ± 2.28%) groups (Fig 1-7B). Although the LVIDd values in the hUCB-MSC alone (8.71 ± 0.32 mm) and *VEGF*/hUCB-MSC (Dox-) (8.43 ± 0.87 mm) groups were slightly reduced compared to the control group (9.83 ± 0.61 mm), the *VEGF*/hUCB-MSC (Dox+) group (6.42 ± 0.26 mm) showed a significant reduction ($p<0.01$) (Fig 1-7C & D).

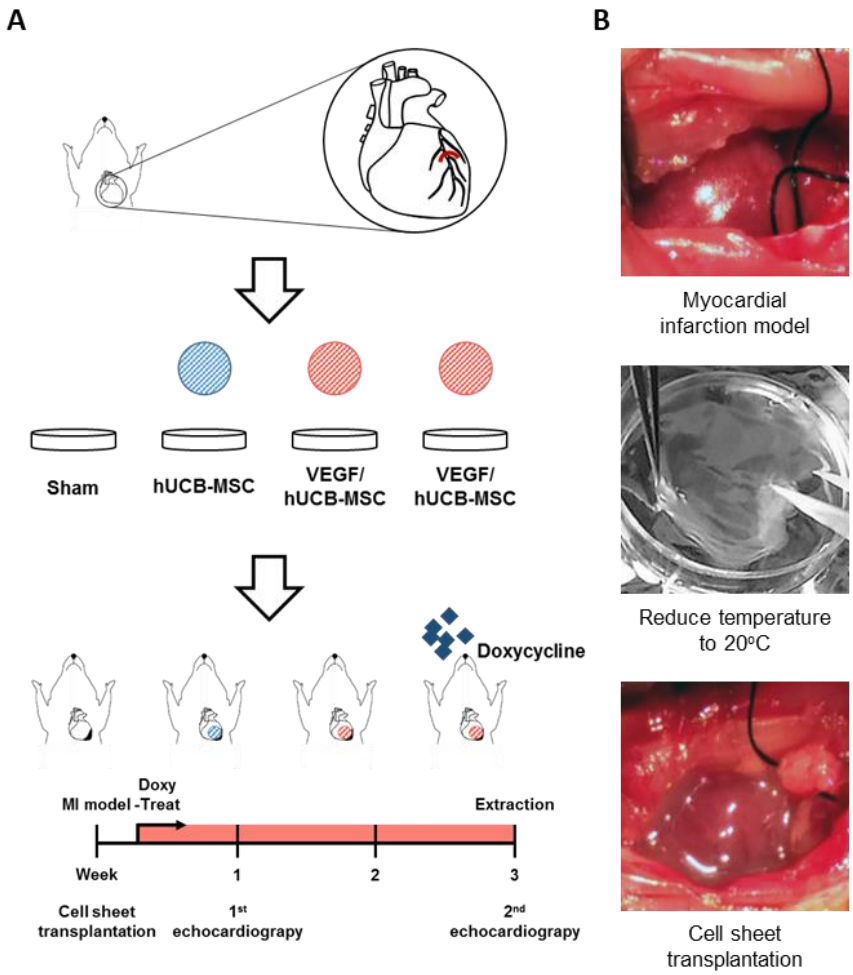


**Figure 1-5. Engraftment of VEGF/hUCB-MSCs.**

(A) Schematic illustration of treatment of MI by VEGF/hUCB-MSCs. Echocardiography measurements performed one week and 3 weeks after MI induction and cell transplantation. (B) The image of an MI and the transplantation process of the UpCell system.

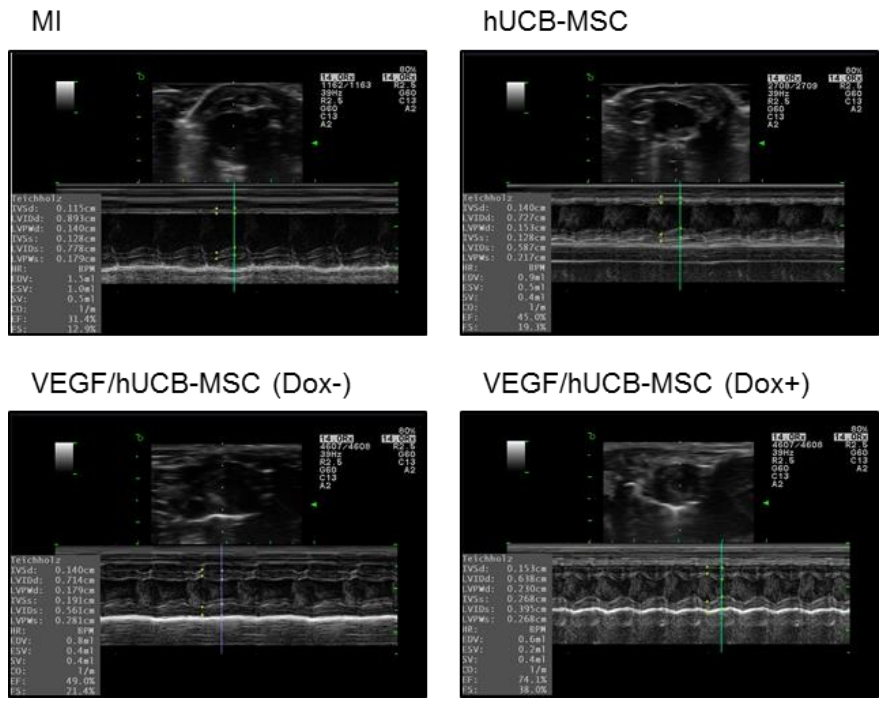


**Figure 1-6. Representative echocardiogram images.**

Representative echocardiogram images in the MI site after 3 weeks. The infarcted rat heart served as the control for transplantation with hUCB-MSCs, VEGF/hCUB-MSC (Dox-), and VEGF/hUCB-MSC (Dox+). In the quantitative analysis, treatment of VEGF/hUCB-MSCs with doxycycline significantly enhanced the cardiac function in one week and 3 weeks. The VEGF/hUCB-MSC (Dox+) group showed.

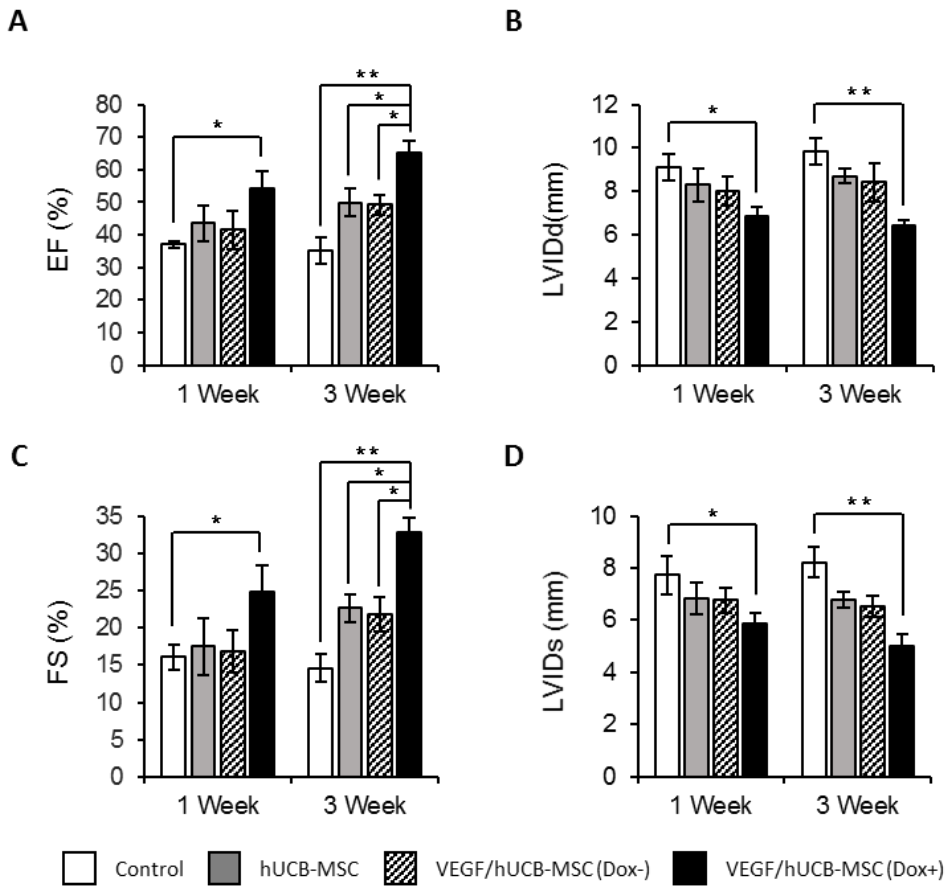


**Figure 1-7. Recovery of cardiac function.**

(A) an increase in the ejection fraction (EF) and (B) fractional shortening (FS) and (C) a decrease in the left ventricle inner diameter at diastole (LVIDd) and (D) left ventricle inner diameter at systole (LVIDs). (*: $P < 0.05$, **: $P < 0.01$, n=6 each group). All values represent the mean ± SEM.

***Reduced MI size and fibrosis but thicker left ventricle due to VEGF secretion by VEGF/hUCB-MSCs in the rat MI model***

We conducted histological and histomorphometric analyses to better understand the improvement of cardiac function by Masson's trichrome staining of the rat hearts at 3 weeks after implantation. Fibrosis due to MI appears blue and the preserved myocardium appears red in Masson's trichrome staining. Dramatic left ventricle (LV) wall thinning was detected in the MI model in the hUCB-MSC alone group. The *VEGF/*hUCB-MSC (Dox-) group showed a minor protective effect. However, a large anti-fibrosis effect was observed in the LV wall in the rats treated with *VEGF/*hUCB-MSCs (Dox+) (Fig 1-8). The MI size was greatly reduced in the *VEGF/*hUCB-MSC (Dox+) group compared to the MI control, hUCB-MSC alone, and *VEGF/*hUCB-MSC (Dox-) groups (Fig 1-9). The ventricular fibrosis rate was significantly reduced in the *VEGF/*hUCB-MSC (Dox+)-treated rats compared with the other groups (Fig 1-9). However, the LV wall thickness of the *VEGF/*hUCB-MSC (Dox+) group was significantly thicker than the MI, hUCB-MSC alone, and *VEGF/*hUCB-MSC (Dox-) groups (Fig 1-9).

Immunohistochemical staining was performed to investigate Dox-induced VEGF secretion in the infarcted area following treatment with *VEGF/*hUCB-MSC + Dox. Heart tissues were stained with a VEGF antibody. The infarcted area in the *VEGF/*hUCB-MSC (Dox+) group showed high expression of VEGF, which was not observed in the control, hUCB-MSC, and *VEGF/*hUCB-MSC (Dox-) groups (Fig 1-10A). These results suggested that VEGF was secreted properly by the *VEGF/*hUCB-MSC cells upon doxycycline treatment. Furthermore, anti-von

Willibrand factor (vWF) staining showed that the *VEGF/*hUCB-MSC (Dox+) group had significantly increased vessel density in the infarcted area, whereas the hUCB-MSC alone and *VEGF/*hUCB-MSC (Dox-) groups showed small increases in vessel density compared to the control group (Fig 1-10B). These results suggest that VEGF secreted from *VEGF/*hUCB-MSCs greatly enhanced neo-angiogenesis compared to stem cell therapy alone by directly and indirectly stimulating angiogenic factors or EC cell-like factors.

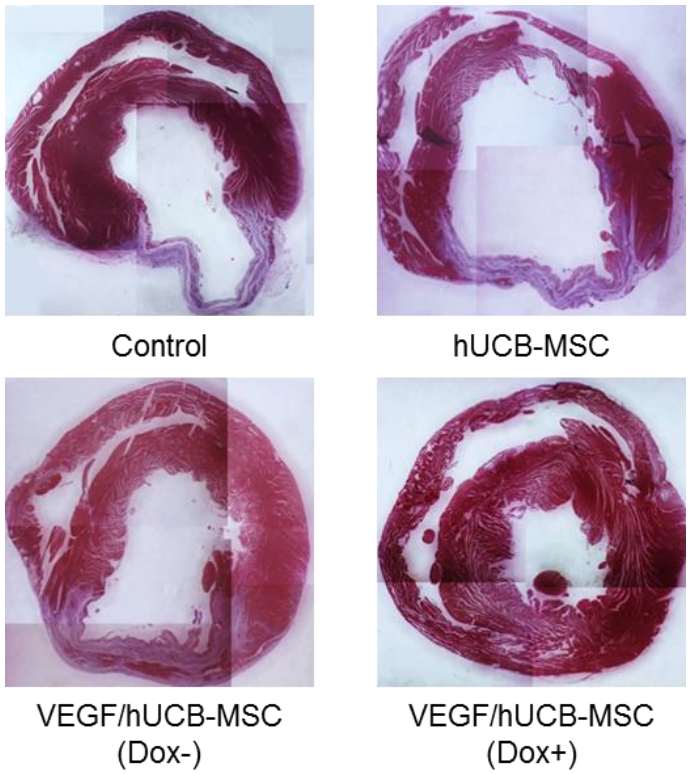


**Figure 1-8. Representative images of heart sections.**

Representative images of heart sections stained with Masson's trichrome show fibrosis and wall thinning at the infarcted area. Fibrotic areas are colored in blue and the viable myocardium is colored in red.

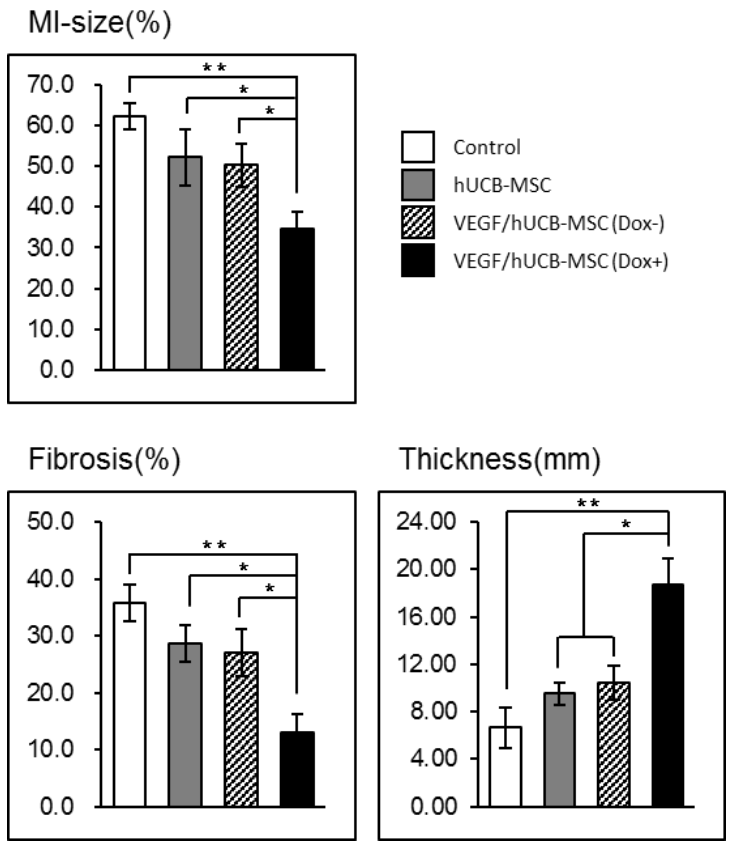


**Figure 1-9. Evaluation of MI size, LV fibrosis and wall thickness after the transplantation of VEGF/hUCB-MSCs.**

Infarct size, percentage of LV fibrosis and LV wall thickness were compared among the different groups. (*: $P < 0.05$, **: $P < 0.01$). All values represent the mean ± SEM.

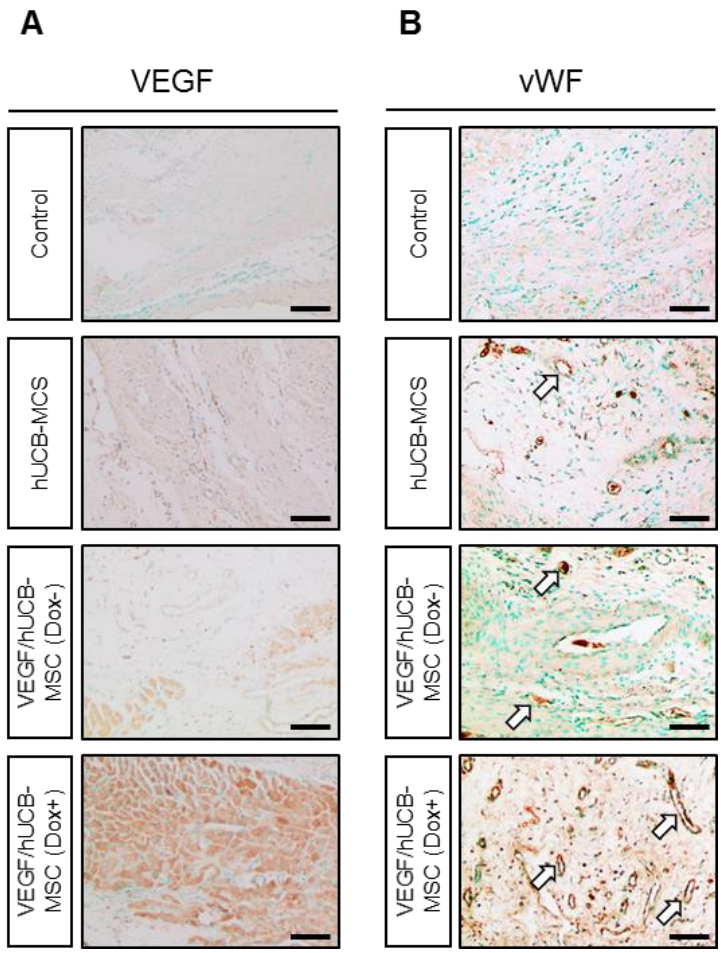


**Figure 1-10. Immunohistochemical staining of VEGF, blood vessel formation by VEGF/hUCB-MSCs.**

MI-induced rats were divided in to 4 group. Control (MI alone), treated with hUCB-MSCs, VEGF/hUCB-MSCs (Dox-), and VEGF/hUCB-MSCs (Dox+). (A) VEGF staining were performed at the infarcted area. High levels of VEGF were detected only in the VEGF/HUCB-MSC (Dox+) group. (B) Generation of blood vessels in the MI regions. More blood vessels were observed in VEGF/hUCB-MSC (Dox+) group than other groups.

***Prolonged cell survival of transplanted human VEGF/hUCB-MSCs in the infarcted region***

To determine the effects of VEGF secreted from *VEGF/*hUCB-MSCs (Dox+) on the survival of transplanted human stem cells, we detected transplanted *VEGF/*hUCB-MSC in rat hearts via immunohistochemical staining. An anti-lamin A+C antibody was used to detect human cells in the infarcted region. Lamin A+C-positive cells were not detected in the MI control group (Fig 1-11). Significant numbers of transplanted human cells were detected above the myocardium of the hUCB-MSC alone and *VEGF/*hUCB-MSC (Dox-)-transplanted groups. However, in the *VEGF/*hUCB-MSC (Dox+) group, a large number of Lamin A+C-positive cells were detected above the myocardium (Fig 1-11). These results indicate that secreted VEGF also has a positive effect on stem cell survival and thus leads to the induction of enhanced paracrine effects and improved protection from cardiac damage.

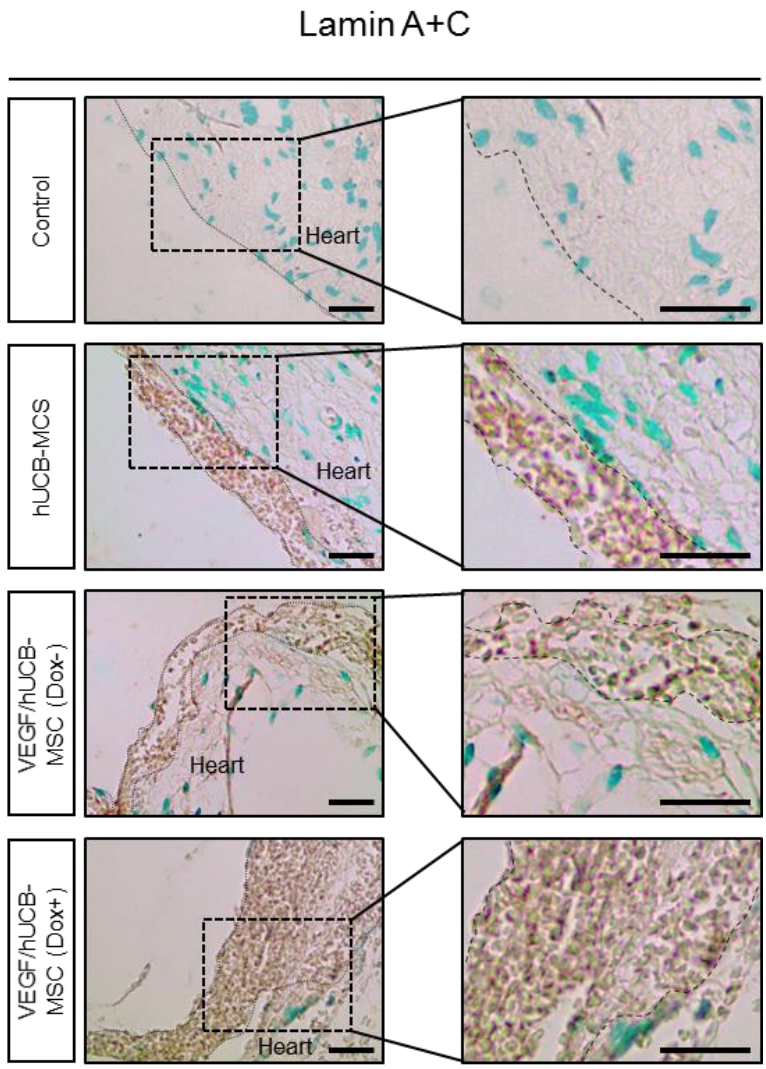


**Figure 1-11. Prolonged cell survival of transplanted human VEGF/hUCB-MSCs.**

An anti-human Lamin A+C antibody was used to visualize the transplanted human stem cells. More human Lamin A+C positive cells were observed in rat hearts transplanted with VEGF/hUCB-MSC (Dox+). (Scale bar = 100 μm).

# Discussion

Stem cell-based therapy has been widely investigated for the treatment of myocardial infarction. In recent studies, stem cell therapy using MSCs offered a positive result for the treatment of myocardial infarction due to paracrine effects and revascularization (Williams and Hare, 2011). However, the application of cell therapy faces many obstacles due to the low survival of the implanted cells and insufficient oxygen and nutrients. Moreover, cardiomyocytes have a limited regeneration capacity (Mangi et al., 2003). To overcome these obstacles with ischemic disease, combined therapy using vascular stimulating growth factor and MSCs is required. In our previous study, VEGF protein delivery with stem cells showed a therapeutic effect on ischemic disease (Kim et al., 2014). However, the protein therapy highlighted the limitation of the application periods due to the short half-life of the VEGF proteins (Henry et al., 2003). To overcome this limitation, several studies reported genetic modifications using retroviruses for the integration of transgenes into the host chromosome to induce their long-term expression (Misteli et al., 2010, Chen et al., 2005). However, retroviruses can produce oncogene activation or tumorigenesis due to the random and unpredictable integration of the transgene, which is a major side effect. Furthermore,

uncontrolled VEGF expression also causes side effects such as hemangioma and nonstandard blood vessel formation.

To address these issues, we generated an inducible VEGF-secreting stem cell using TALEN-mediated transgene integration into a specific safe harbor site by AAVS1 locus-directed homologous recombination. With this TALEN-mediated safe harbor gene delivery strategy, the human cord blood-derived mesenchymal stem cells were able to secrete VEGF upon doxycycline treatment, protect the host from cardiac damage and improve cardiac function in a rat myocardial infarction model.

In this study, we used TALENs technology to introduce an inducible VEGF-165a gene cassette into the *AAVS1* safe harbor site in human umbilical cord blood mesenchymal stem cells. The initial targeting vector with the left and right homology arm (HA-L & HA-R) cassette containing the inducible *VEGF* gene did not produce a sufficient number of cells integrated with the gene cassette. Therefore, we redesigned several TALEN-L/R targeting vectors along with several HA-Ls and HA-Rs in donor vectors and tested their efficiency. Finally, we could generate a quite efficient combination of TALEN-L/R targeting vector and HA-L and HA-R donor vector cassette for the AAVS1 locus by giving a 50 bp space apart between the homology arms and the TALEN target sites. With these newly designed vectors, we could generate efficient integration of the *VEGF* gene cassette that resulted in the production of a physiologically relevant concentration of VEGF.

Successful stem cell-based cell transplantation combined with vascular growth factors such as VEGF would induce vascularization to treat ischemic or vascular

disease (Vunjak-Novakovic et al., 2011). However, excessive amounts of VEGF can induce unwanted effects. Thus, the regulation of VEGF expression is important. The proper VEGF concentration for therapeutic angiogenesis was reported to be below 100 ng per $10^6$ cells per day *in vitro* (Ozawa et al., 2004). Our *VEGF/*hUCB-MSCs secreted VEGF at a concentration of approximately 50 ng/$10^6$ cells/day when exposed to doxycycline (Fig 1-2E), thereby providing a safe therapeutic ranges of VEGF against vascular diseases.

In addition to the long-term expression of VEGF, the secreted VEGF has to be biologically functional and capable of binding to the VEGF receptor (Flk-1) to induce angiogenic gene expression and prevent cell cycle arrest (Yla-Herttuala and Alitalo, 2003). Our results showed that VEGF secreted from *VEGF/*hUCB-MSCs by Dox induction increased the expression of angiogenesis markers and down-regulated cell cycle arrest factors (Fig 1-3). These results indicate that the VEGF secreted by hUCB-MSCs gave endothelial cell-like characteristics to these cells by autocrine stimulation. This approach may assist in the neovascularization of vascular disease. We also demonstrated that VEGF secreted by hUCB-MSCs significantly enhanced new vessel formation in *in vivo* mouse models using both the UpCell system and Matrigel transplantation (Fig 1-4).

In this study, our goal was to improve cardiac function and repair the infarcted heart using the combination of the *VEGF/*hUCB-MSCs and the VEGF secreted form the stem cells. The *VEGF/*hUCB-MSCs patch was implanted on the infarcted area and VEGF secretion was induced by Dox in the rat animal model. The echocardiography results showed that rats treated with *VEGF/*hUCB-MSCs (Dox+)

for 1 and 3 weeks showed improved cardiac function and attenuated LV remodeling. These results suggest that combination therapy of *VEGF*/hUCB-MSCs with secreted VEGF further improved significantly heart function and cardiomyocyte restoration compared to *VEGF*/hUCB-MSCs or parental stem cells alone. The apparent improvement observed for this combination therapy can be explained as follows. First, the impaired cardiac tissue may have been regenerated better by the transplanted stem cells. According to our previous results and this result, transplanted MSCs could regenerate the impaired cardiac tissue and promote the proliferation of cardiac progenitor cells near the transplanted area (Kang et al., 2014). But, the cardiac regenerative effect was not clearly observed in our MSC alone group, and other researchers also reported that the frequency of the occurrence of these regenerative processes by MSC was also very low (Cashman et al., 2013). However, our combinatorial approaches of induced VEGF and *VEGF*/hUCB-MSCs greatly contributed to the prevention of LV remodeling and the protection of the remaining cardiomyocytes as well as cardiac regeneration. The second explanation for these positive results is the paracrine effects of the combination therapy. Previous studies also suggested that the main therapeutic effect of transplanted MSCs occurred due to paracrine effects (Fedak, 2008, Segers and Lee, 2008). The transplanted cells secrete many cytokines, and these cytokines activate cell survival, alleviate the fibrosis, and contribute to cardiomyocyte preservation. Furthermore, tuning the cytokine secretion toward angiogenic factors will further help the therapeutic effect of stem cells in myocardial infarction. Indeed, our data showed that the induced VEGF could enhance the release of

cytokines related to the angiogenic paracrine such as cytokine Angiopoietin1 (Fig 1-3), and finally prevent cardiac remodeling by improving implanted cell survival in the harshly infarcted region. These VEGF stimulating paracrine effects were also demonstrated in myoblast and endothelial cells in mouse model (Cucina et al., 2003, Marsano et al., 2013). Third, *VEGF/*hUCB-MSCs and secreted VEGF promoted neovascularization in the implanted area and this neovascularization might maintain *VEGF/*hUCB-MSC survival after cell sheet integration and the cells' therapeutic functions in the MI microenvironment. Our data showed that more neovascularization occurred in the infarcted area when the engineered cells were boosted with secreted VEGF (Fig 1-10). Other studies also reported that the therapeutic concentration of VEGF was able to induce stable capillary formation and protect the remaining cardiomyocytes in MI model (Marsano et al., 2013, Misteli et al., 2010). Therefore, the newly formed vessels also increased the cell survival of the implanted hUCB/MSCs, thereby contributing to the formation of a compact cell sheet in the myocardial surface of the infarcted area as demonstrated by anti-human Lamin A+C staining (Fig 1-11). Furthermore, the increased vessel formation may contribute to protection against negative LV remodeling, the deterioration of heart tissues and myocardial functional enhancement through an improved nutrient and oxygen supply.

TALENs have been successfully applied to edit targeted genomes in various cell types (Carroll, 2011, Joung and Sander, 2013). However, the limitations associated with engineered nucleases remain. The expression of TALEN pairs could cause additional DNA cleavage in human cells, and this unexpected DNA

cleavage at off-target sites could reduce cell survival and cell cycle arrest (Bogdanove and Voytas, 2011). To predict off-target cleavage activities, high-throughput sequencing assessment is required. In previous reports, the off-target cleavage activities of AAVS1-specific TALENs were investigated and revealed to be approximately 0.13% (Niu et al., 2014, Mussolino et al., 2014). Although we did not profile TALEN off-target activity using whole genome sequencing in a *VEGF*/hUCB-MSC cell population in this study, similar activities were expected. When we tested the 3 sites with the highest homology to AAVS1 safe harbor site by PCR, the results showed no off-target integration.

# CHAPTER Ⅱ

# Transplantation of human mesenchymal stem cells genome-edited with LEF1 improves cardio protective effects in myocardial infarction

# INTRODUCTION

Myocardial infarction (MI) is the most common coronary heart disease, which in turn is the leading cause of morbidity and mortality worldwide (Collaborators, 2017, Jessup and Brozena, 2003). The main cause of the disease is an obstruction of the coronary artery that leads to a massive loss of cardiomyocytes, resulting in myocardial dysfunction and heart failure (Neri et al., 2017). The primary therapy should be the restoration of lost cardiomyocytes, but there is a clear limitation to the regenerative capacity of the adult mammalian heart, meaning that additional therapeutic approaches are mandatory.

Stem cell therapy has received constant attention as a potential strategy for the regeneration of infarcted hearts, and human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) are regarded as a promising candidate for this therapy due to its unique properties, such as multiple lineage potential, lack of teratoma formation, easy expansion and low immunogenicity (Nagamura-Inoue and He, 2014). Various trials have treated the injured myocardium using diverse MSC populations, including hUCB-MSCs. Some of these studies showed restoration of damaged cardiomyocytes, enhancement of cardiac function and, to certain degrees, reduced infarct size (Amado et al., 2005, Tang et al., 2005, Cai et

al., 2016). Although many studies have attempted to utilize hUCB-MSCs and provided positive outcomes in preclinical trials, there are still some hurdles to be surmounted, such as low graft and survival rates in the hostile microenvironment of the infarcted region with its insufficient supply of oxygen and nutrients. Therefore, to enhance hUCB-MSCs' functionality, additional strategies such as cell patch and genome editing of the hUCB-MSCs are required to improve cell survival rate and paracrine effects. There have been a few trials with different target genes that obtained the expected outcomes and addressed these issues. Our recent study reported that hUCB-MSCs overexpressing vascular endothelial growth factor (VEGF) gene controlled by doxycycline (Dox) induction successfully improved cardiac function via the increase of angiogenesis in MI (Cho et al., 2017). VEGF is an angiogenic factor that can promote endothelial cell survival and can be suggested as a therapeutic reagent (Ferrara et al., 2003). Although, methods were varied, introduction of ISL and HGF also enhanced therapeutic effects of stem cells (Xiang et al., 2018, Zhao et al., 2016). However, there is still room to improve the functionality of hUCB-MSCs via not only endocrine and paracrine growth factors but also autocrine effects. It is ultimately necessary to survey as many therapeutic target genes as possible.

We indeed focused on the activation of canonical wingless/integrase-1 (Wnt) signaling pathways that have been known to contribute to mouse ESC self-renewal and help maintain the undifferentiated status of ESCs through modulation of Oct4 and Nanog (Li et al., 2012, Huang and Qin, 2010a, Kim et al., 2011). Lymphoid enhancer-binding factor-1 (LEF1), a 48-kD nuclear protein, is a crucial

transcription factor for proliferation and survival of B- and T-cells (Okamura et al., 1998, Staal et al., 2008). The involvement of LEF1 in canonical Wnt signaling pathways has been studied and activation of the pathways via the overexpression of LEF1 has been shown to contribute to mouse ESC self-renewal (Huang and Qin, 2010a). Furthermore, it has also reported that the gene regulate the follicle morphogenesis and proliferation of neural progenitor cells (Armenteros et al., 2018, Zhang et al., 2013).

Recent studies have focused on the functions of LEF1 related to stem cells and cardiogenesis. High expression of LEF1 between the mesoderm and cardiac progenitor cell stage has been identified in various studies and it has been suggested that the gene plays an important roles between these stages (Li et al., 2015, Liu et al., 2017). Moreover, direct and temporal contribution of LEF1 in mouse heart maturation has also been reported. It has been demonstrated that the gene is mainly expressed in MSCs in the valvular region during the murine heart development (Ye et al., 2019), but its function in mouse and human mesenchymal stem cells needs additional study. Despite this positive potential in cell proliferation, survival and cardiac differentiation, the cardio-protective effects from MI in stem cell therapy has not been demonstrated yet.

In parallel, diverse gene introduction systems such as viruses, zinc- finger nucleases, transcription activator- like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeat (CRISPR) and CRISPR-associated 9 (Cas9) have been developed (Gaj et al., 2013, Carroll, 2011, Joung and Sander, 2013, Cong et al., 2013). Since 2012 when the CRISPR system was initially

demonstrated, it has been improved to reduce off-target mutations (Cong et al., 2013) and has been widely adopted as the simplest and most efficient gene integration systems in various cells and organisms, including human, rat and mouse (Hsu et al., 2014, Sander and Joung, 2014, de la Fuente-Nunez and Lu, 2017).

Taken together, the objective of this study is to examine the therapeutic efficacy of the hUCB-MSCs stably expressing the LEF1 by CRISPR/CAS9-mediated gene integration (LEF1/hUCB-MSCs) in MI. We first investigated autocrine effects of overexpressing LEF1 in hUCB-MSCs on cell proliferation and survival of hUCB-MSCs in both normal and oxidative stress conditions and then survival promoting effects of the cells in the MI region *in vivo*. Moreover, paracrine effects via stimulation of growth factor and cytokine secretion by LEF1 were also analyzed to explain the recovery of cardiac function in the MI animal model by LEF1.

# MATERIAL AND METHODS

### *Isolation and culture of hUCB-MSCs*

hUCB-MSCs were isolated as previously described (Kang et al., 2014) and by following the procedure approved by the Borame Hospital Institutional Review Board and Seoul National University (IRB No. 0603/001-002-07C1). In brief, UCB samples were harvested from term and preterm deliveries at the time of birth with the mother's informed consent (Seoul City Borame Hospital Cord Blood Bank). Separation of the mesenchymal stem cells from UCB was performed using Ficoll-Paque TM PLUS (Amersham Bioscience, Uppsala, Sweden). The cells were suspended in DMEM media (Gibco, Grand Island, NY, USA) with 20% fetal bovine serum (FBS; Gibco), 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. After 24 hours, the cells were washed twice in PBS and cultured in DMEM media with 10% FBS.

### *Transfection and cell proliferation assays*

To introduce the expression vector, transfection was performed using Lipofectamine 3000 by following the manufacturer’s manual (Thermo Fisher Scientific, Waltham, MA). The hUCB-MSCs and LEF1 introduced hUCB-MSCs

were subjected to a cell proliferation assay. In brief, 150,000 cells were seeded in 60-mm plates on day 0 and counted after 24, 48, and 72 hours by an automated cell counter (Arthur, NanoEnTek, Seoul, Korea). Experiments were run in triplicate.

***RNA isolation and cDNA synthesis***

The total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, USA) and the quality was measured by the ratio of 260/280 using the Nanodrop Epoch Microplate Spectrophotometer (BioTek Instruments, VT, USA). Reverse transcription was performed on 2ug of RNA using the Omniscript RT kit, following the manufacturer`s guideline (Qiagen) TRIzol reagent (Invitrogen, Carlsbad, USA)

***Conventional and Real-time PCR analyses***

Conventional PCR was performed on 1 μl of cDNA using GoTaq polymerase in the following conditions: denaturation at 95℃ for 2min, 35 cycles of 95℃ for 30s, 60℃ for 30s and 72℃ for 30s followed by final extension at 72℃ for 5 min. Real-time PCR was performed in the same reaction conditions except for the introduction of SYBR Green (Bio-rad CFX Manager, CA, USA) and the relative expressions were calculated by the delta-delta CT method. GAPDH served as an internal control.

***Western blot analysis***

After treatment with RIPA buffer for 30 minutes, approximately 20 to 30 μg of

cell lysates were used for Western blot analysis as previously reported (Lee et al., 2010). Proteins were detected with the following antibodies; Anti-LEF1 (1: 1,000, Cell Signaling Technology; C12A5), Anti-c-MYC (1:1,000, Cell Signaling Technology; D84C12), Anti-cyclin D1 (1: 1,000, Cell Signaling Technology; 92G2), Anti-Bcl-2 (1:1,000, Abcam; ab692), Anti-Bax (1:1,000, Santa Cruz Biotechnology; SC-493) and Anti- β-Actin antibody (1:2,000, Santa Cruz Biotechnology; SC-32233).

***Cell cycle analysis***

The cells in each group were collected after 48 h of transfection. After washing with cold PBS three times, the cell pellets were re-suspended in PBS with the cell density of $1x10^5$ cells/ml. After treatment with 400 μl of Propidium iodide (PI, Sigma-Aldrich Chemical Company, St Louis MO, USA), the samples were incubated for 30 min in the darkness at 4 ℃. The cell cycle was detected using red fluorescence with an automated fluorescence cell counter (Arthur, NanoEnTek, Seoul, Korea).

***Flow cytometry***

After cell transfection, the cells in each group were digested with ethylene diamine tetraacetic acid (EDTA), and then collected in a flow tube. After 3 PBS washes, each sample was subjected to the Annexin-V fluorescein isothiocyanate (FITC) cell apoptosis kit (Sigma-Aldrich Chemical Company, St Louis MO, USA), following the kit instructions. The Annexin-V-FITC/PI dye was added to the $1x10^6$

cell suspension. After a 15min incubation at room temperature, the 525 nm and 620 nm band pass filter was used for FITC and PI fluorescence detection and the excitation wavelength of 488 nm was used for the detection of cell apoptosis.

***Donor construct design and CRISPR/Cas9 mediated gene editing***

The transgene insertion system using CRISPR/Cas9 in to the AAVS1 locus was derived from Origene Technologies (https://www.origene.com/products/gene-expression/crispr-cas9), and consists of pCas-Guide-AAVS1 (#GE 100023) and pAAVS1-EF1a-Puro-DNR (#GE 100046). The donor vector contains 550 bp left, right homology arms matched to the AAVS1 locus in each side followed by the EF1a promoter for the constitutive expression of the target gene.

To insert the LEF1 gene in the donor vector, LEF1 plasmid (#RC 208663) was purchased from Origene (Rockville, MD, USA). The donor vector and LEF1 plasmid were digested with SgfI/MluI (NEB, Ipswich, MA, USA). This fragment was synthesized *de novo* and ligated into donor vector to generate pAAVS1-EF1a-LEF1-DNR. To generate the LEF1/hUCB-MSCs, hUCB-MSCs were co-transfected with pCas-Guide-AAVS1 and pAAVS1-EF1a-LEF1-DNR through electroporation with the Neon™ Transfection System (Thermo Fisher Scientific).

***Myocardial infarction (MI) rat model and cell sheet transplantation***

The myocardial infarction rat models were surgically induced as previously described (Cho et al., 2017). Briefly, male Sprague-Dawley rats weighing 260-300 g (Orient Bio, Seongnam, Korea) were used as the MI model. All animal

experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC; SNU-190307-2) (Seoul National University, Korea). The rats were divided into four groups: rats that received *LEF1/*hUCB-MSCs with MI, hUCB-MSCs with MI, non-treated with MI and sham (no-MI) and each group consisted of at least 5 rats.

For the transplantation of stem cells, the Upcell system was employed (Thermo Fisher Scientific, Waltham, MA). Cell sheets of *LEF1/*hUCB-MSCs or hUCB-MSCs were detached from the plates by incubating at 20℃ for 20 min. After transplantation, the supporting membrane was removed and the cell sheets showed stable attachment. To prevent the rat's immune rejection of the human cells, all rats received cyclosporine as previously described (Kang et al., 2014).

***Masson`s trichrome staining and histological analysis***

After extraction of all mice hearts, they were washed with PBS, fixed, embedded and sectioned into 5 μm sections. Masson`s Trichrome staining was performed according to instructions of Trichrome stain kit (Sigma-Aldrich Chemical Company, St Louis MO, USA). The infarct size, fibrosis and scar thickness were quantified with the Image J software (NIH, Bethesda, MD, USA).

***Immunohistochemical staining***

Immunohistochemical staining was performed using a standard protocol. The heart sections were incubated overnight with the following primary antibodies: Anti-lamin A+C (1:200, abcam; ab108922), Anti-LEF1 (1:200, Cell Signaling

Technology; C12A5) and Anti-VEGF (1:200, abcam; ab69479). And then they were subsequently exposed to biotinylated secondary antibody and streptavidin peroxidase complex using the Histostain-Plus kit (Sigma-Aldrich Chemical Company, St Louis MO, USA). Then, the sections were stained with, 3'-diaminobenzidine tetra hydrochloride (Liquid DAB substrate kit; Abcam).

***Functional assessment of the infarcted myocardium***

The measurement of cardiac function was performed as previously reported (Cho et al., 2017). Briefly, it was assessed by transthoracic echocardiography prior to MI surgery (normal baseline) and both 1 and 4 weeks after MI for every experimental group.

***Statistical Analysis***

Data are expressed as the mean ± SEM. For statistical analysis of multiple groups, 1-way ANOVA was performed, followed by a Bonferroni post hoc test. For verification of the therapeutic effects in MI, 2-way ANOVA was carried out followed by a Bonferroni post hoc test. A p-value less than 0.05 was considered statistically significant.

# RESULTS

### *LEF1 promotes hUCB-MSC proliferation*

To find the target gene for hUCB-MSCs therapy, we examined multiple transcriptome analyses from 4 different studies that were associated with cardiomyocyte differentiation from pluripotent cells. Since the genes that exhibit cell specific expression from mesoderm to cardiac progenitor cell stage promote self-renewal to maintain the stage or differentiation to cardiac progenitor cells (Tateno et al., 2017, Lian et al., 2012, Witman and Sahara, 2018). We focused on genes that are enriched in these stages. From 8 different analyses, we first examined the 100 most strongly up-regulated genes in these stages in comparison to human pluripotent stem cells and then listed them individually (S1 Table). From these gene lists, the 11 genes that were commonly enriched in 5 or more analyses were selected (Fig S1A). Among these, 36% (4 out of 11) of the genes are known to function in cardiac differentiation, e.g., *PLXNA2*, *TBX3*, *BMP4*, and in MSC proliferation, e.g., *LIX1*, and were excluded from further target selection, finally leaving only 7 target genes.

The 7 target genes selected from the *in silico* literature surveys were subjected to conventional PCR (Fig S1B) and qRT-PCR, to examine the gene expression in hUCB-MSCs and expression patterns during the cardiomyocyte differentiation.

Various patterns were observed (Fig S2), but the aim of this study was to enhance the protection efficacy of hUCB-MSCs by insertion of target genes, so we focused on the genes that maintained a constant low expression (*LEF1*, *ZEB2*). However, the effects of LEF1 on cell proliferation, as well as the direct and temporal contributions to heart development, have been reported (Ye et al., 2019, Reya et al., 2000, Huang and Qin, 2010a). Consequently, all additional studies proceeded with LEF1 (Fig 2-1A & B). Since low proliferative capacity is one of the limits in the use of differentiated stem cells (Segers and Lee, 2008), we first examined if introducing LEF1would enhance hUCB-MSC proliferation. The hUCB-MSCs transfected with LEF1 showed significantly increased numbers of cells 72 hours after incubation without any aberrant changes in morphology (Fig 2-1C & D). The activation of canonical Wnt/β-Catenin signaling by LEF1 transfection was confirmed by RT-PCR of β-catennin (CTNNB1) and c-Myc, as well as LEF1 itself. LEF1 significantly increased the expression of CTNNB1 and c-Myc. CyclinD1 expression was also dramatically increased, representing stimulated cell cycles by LEF1 (Fig 2-1E & F). This result was confirmed in protein levels, as Western blot and densitometry analysis clearly showed that Wnt/β-Catenin signaling and CyclinD1 was up-regulated by LEF1 (Fig 2-1G & H). We then presented the evidence for the proliferative fate of hUCB-MSCs enhanced in LEF1 transfection. Cell cycle analysis using PI staining combined with an automated fluorescence cell counter showed cell fates shifted up from G0/G1 to S phases in the LEF1-transfected hUCB-MSC population.

An approximately 40% cell cycle enhancement was observed within the hUCB-MSCs transfected with LEF1 (Fig 2-1I & J).

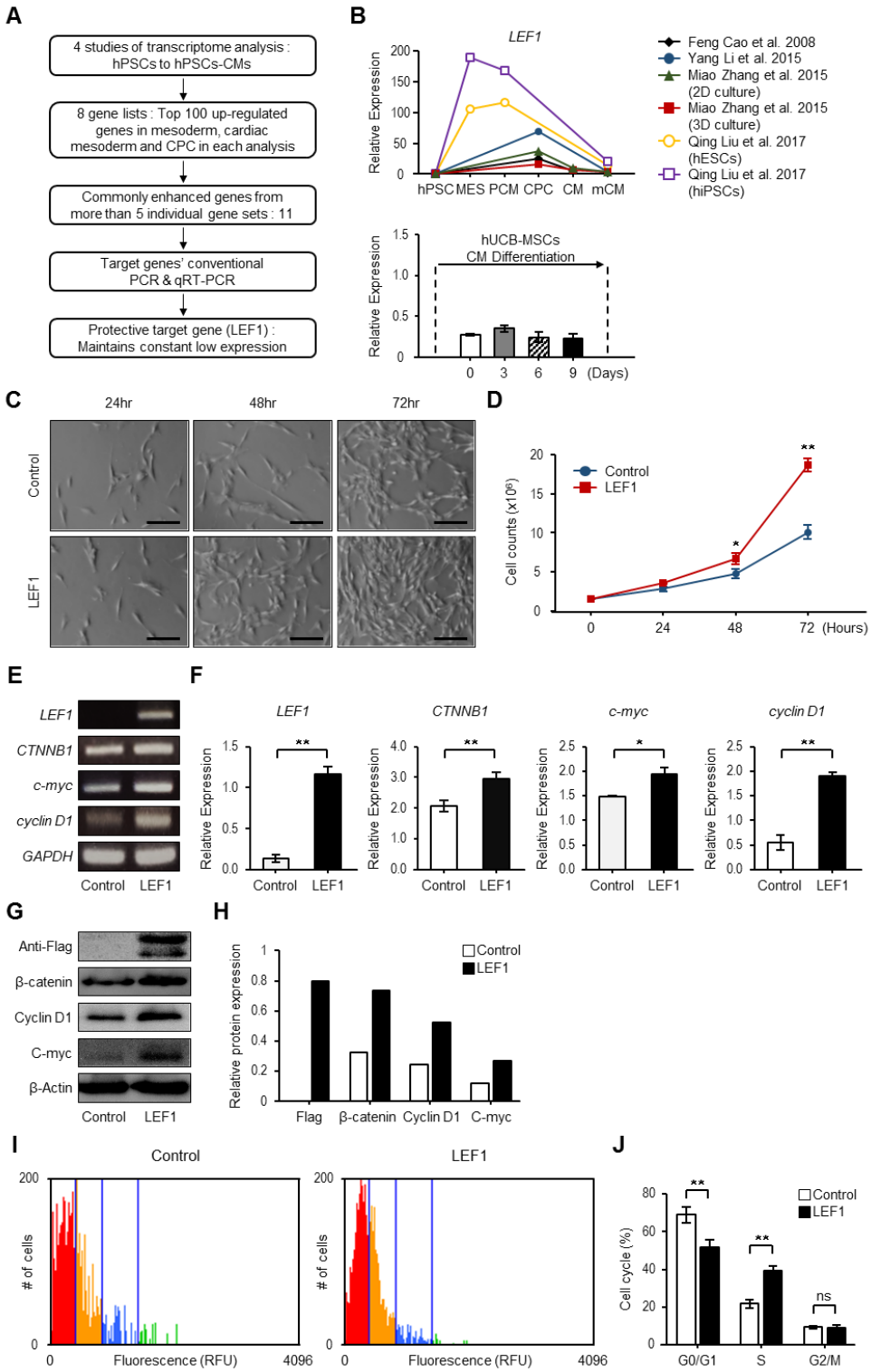
A
4 studies of transcriptome analysis : hPSCs to hPSCs-CMs
8 gene lists : Top 100 up-regulated genes in mesoderm, cardiac mesoderm and CPC in each analysis
Commonly enhanced genes from more than 5 individual gene sets : 11
Target genes' conventional PCR & qRT-PCR
Protective target gene (LEF1) : Maintains constant low expression
B
LEF1
Relative Expression
hPSC MES PCM CPC CM mCM
Feng Cao et al. 2008
Yang Li et al. 2015
Miao Zhang et al. 2015 (2D culture)
Miao Zhang et al. 2015 (3D culture)
Qing Liu et al. 2017 (hESCs)
Qing Liu et al. 2017 (hiPSCs)
hUCB-MSCs
CM Differentiation
0 3 6 9 (Days)
C
24hr
48hr
72hr
Control
LEF1
D
Control
LEF1
Cell counts (x10⁶)
0 24 48 72 (Hours)
E
LEF1
CTNNB1
c-myc
cyclin D1
GAPDH
Control LEF1
F
LEF1
CTNNB1
c-myc
cyclin D1
Relative Expression
Control LEF1
G
Anti-Flag
β-catenin
Cyclin D1
C-myc
β-Actin
Control LEF1
H
Control
LEF1
Relative protein expression
Flag β-catenin Cyclin D1 C-myc
I
Control
LEF1
# of cells
Fluorescence (RFU)
0 4096
J
Control
LEF1
Cell cycle (%)
G0/G1 S G2/M
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**Figure 2-1. Selection process of therapeutic target gene LEF1 and cell proliferation effects of LEF1 in hUCB-MSCs.**

(A) Schematic depiction of the overall workflow. (B) Comparison of LEF1 gene expression with in silico literature surveys and qRT-PCR data. (C) Representative images from phase-contrast microscopy at 24, 48 and 72 hrs after LEF1 transfection of hUCB-MSCs. Scale bars = 100 um. Ctrl: no DNA, LEF1: LEF1:pDC3.1. (D) hUCB-MSCs (1.5x105 cells) treated with no DNA or LEF1:pDC3.1 were seeded and the increased number of cells were counted at 24, 48 and 72 hr time points. * $p<0.05$ and ** $p<0.01$. (E) Conventional PCR for Wnt pathway and cell cycle related genes. (F) Significant difference in gene expression levels were confirmed by real-time PCR. Relative expression to GAPDH was calculated by the ΔΔ CT method. ** $p<0.01$ (G) Western blot analysis confirmed the increased expression in protein level under LEF1 overexpression. (H) Densitometry showed relative protein expression to β-Actin level. (I) Cell cycle analysis was performed by automated fluorescence cell counting in hUCB-MSCs differentially treated with no DNA or LEF1:pDC3.1. Colors indicate different stages, red: G0/G1 phase, yellow: S phase, blue: G2/M phase. (J)The histogram for the cell cycle distribution after transfection of LEF1 and scrambled control DNA. **$P<0.01$ compared to control.

***LEF1 prevents hUCB-MSCs from hydrogen peroxide-induced apoptosis***

A major issue in stem cell therapy for ischemic heart diseases, including MI, is the low survival of transplanted cells in the ischemic region. It is reported that most hMSCs implanted onto ischemic hearts died within 4 days after transplantation (Toma et al., 2002). Therefore, we examined the protective function of LEF1 from the hydrogen peroxide induced cell death of hUCB-MSCs *in vitro*. Apoptosis was induced by 500μM $H_2O_2$ treatment for 48hr in hUCB-MSCs, while hUCB-MSCs expressing LEF1 still survived at a significantly higher number of cells (Fig 2-2A). Slightly more cells were counted in LEF1 transfected hUCB-MSCs than in the control hUCB-MSCs under normal conditions. Yet, the number of cells remained drastically higher in the LEF1 expressing hUCB-MSC group (80% survival) than the control hUCB-MSC group (30% survival) under severe oxidative stress conditions induced by $H_2O_2$ (Fig 2-2B). Western blot analysis for Bax and Bcl-2 proteins revealed that LEF1 blocked pro-apoptotic Bax but induced anti-apoptotic Bcl-1 even without oxidative stress, thus presenting a reduced level of Bax but an increased level of Bcl-2 in both normal and severe oxidative stress conditions (Fig 2-2C-E). These results demonstrate that the cells had protective effect upon $H_2O_2$ treatment. We next demonstrated that LEF1 could protect hUCB-MSCs from apoptosis under severe oxidative stress using flow cytometry with Annexin V/ PI labeling. As shown in Fig 2-2F and G, a drastic increase in the apoptotic cells was observed in control hUCB-MSCs upon $H_2O_2$ treatment (~25%). However, a remarkable reduction in the apoptotic cell ratio was shown in LEF1 transfected hUCB-MSCs under both normal (~4%) and oxidative stress conditions (~10%).

Altogether, these results indicate that the LEF1 plays an important role in anti-apoptosis of hUCB-MSCs under oxidative stress.

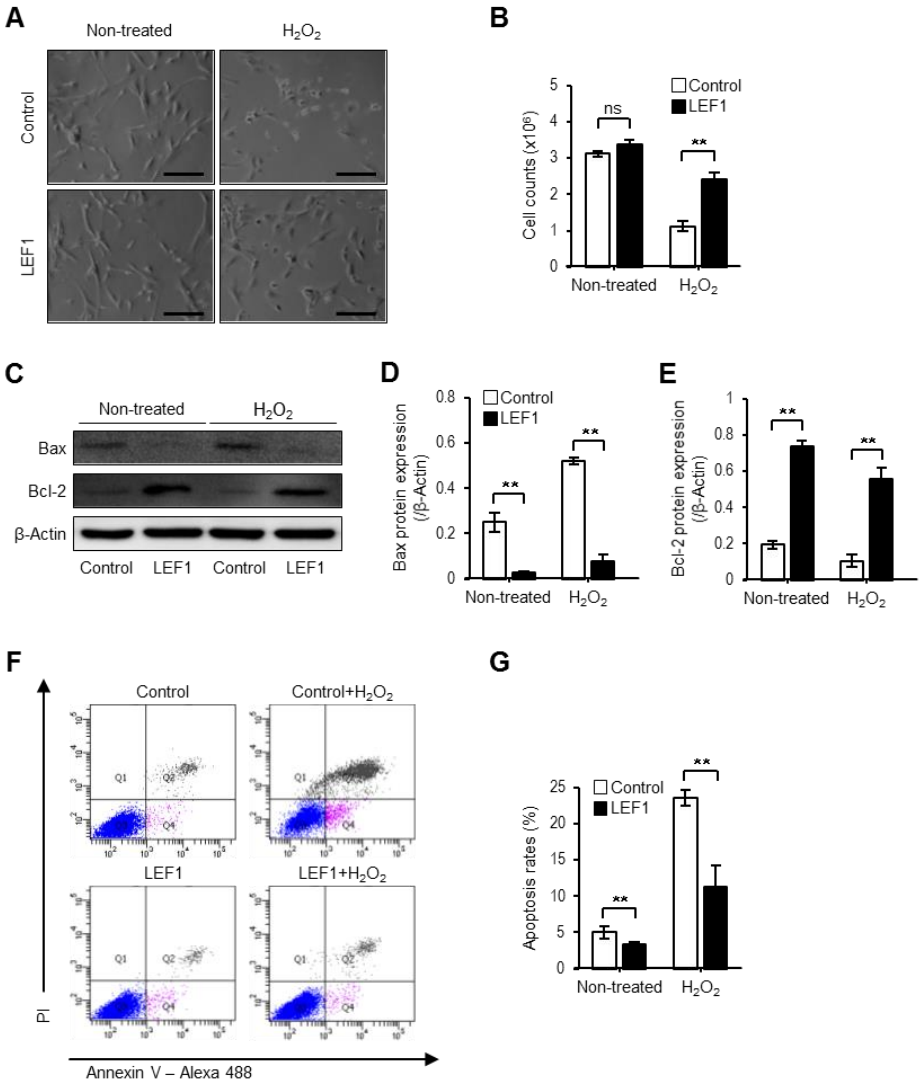


**Figure 2-2. LEF1 overexpression protects hUCB-MSCs from oxidative stress induced apoptosis.**

(A) Microscopy demonstrated that oxidative stress caused cell death was attenuated by LEF1 overexpression. Scale bars = 100 μm. (B) Quantification of reduced cell death in LEF1 overexpression under oxidative stress condition. ** indicates $p < 0.01$. (C) Drastic increase in Bcl-2 and decrease in Bax expression in LEF1

overexpressing hUCB-MSCs was observed by Western blot. (D - E) Image analysis confirmed the significant changes of Bax and Bcl-2 expression in LEF1 overexpressing hUCB-MSCs. (F) FACS analysis using PI and Annexin V successfully presented increased apoptotic cell populations (Q2) under $H_2O_2$ treatment in hUCB-MSCs (top right), but less cells were dead in LEF1 expressing hUCB-MSCs (bottom right). (G) Reduced apoptosis triggered by $H_2O_2$ in LEF1 transfection. ** indicates $p < 0.01$.

***LEF1/hUCB-MSC transplantation improves cardiac dysfunction after myocardial infarction***

A number of studies have demonstrated that stem cell therapy is effective in myocardial regeneration via enhancement of angiogenesis in the region of MI (Segers and Lee, 2008). Various types of stem cells, different treatments or gene editing, and transplantation methods have been developed and tested to improve therapeutic efficacy (Tompkins et al., 2017). In the present study, we first constructed LEF1/hUCB-MSCs that steadily express LEF1 using the CRISPR/Cas9 system, followed by homologous recombination on the AAVS1 genomic safe harbor site (Fig 2-3A). Successful integration of LEF1 on AAVS1 was confirmed by genomic PCR spanning from LEF1 to the AAVS1 site (Fig 2-3B & C). LEF1 protein was stably expressed by LEF1/hUCB-MSCs until 14 days after transfection (Fig 2-3D). To determine the cardioprotective potential of LEF1/hUCB-MSCs in ischemic heart disease, we conducted an experiment using transplantation of LEF1/hUCB-MSCs and control hUCB-MSCs in a post-MI rat model. This experiment included a group of sham rats (surgery without MI) and MI rats (non-treat with MI) as controls (Fig 2-3E). The cell transplantation of each groups was administered as a cell patch using the Upcell system (Fig 2-3F).

A total of 20 rats, 5 rats in each group, were initially designed and subjected to MI surgery. The rats were randomized to the following groups: operation + non-MI, MI + non-treat, MI + hUCB-MSCs, MI + LEF1/hUCB-MSCs. Each cell sheet was transplanted 30 minutes after visual inspection of the infarction. However, 6 out of 11 rats with MI died within 2 weeks after the operation (45.5% survival). Three out

of 8 (62.5% survival) died in the MI + hUCB-MSCs group, and 1 out of 6 in the MI + LEF1/hUCB-MSCs group (83.3% survival) (Fig 2-3G).

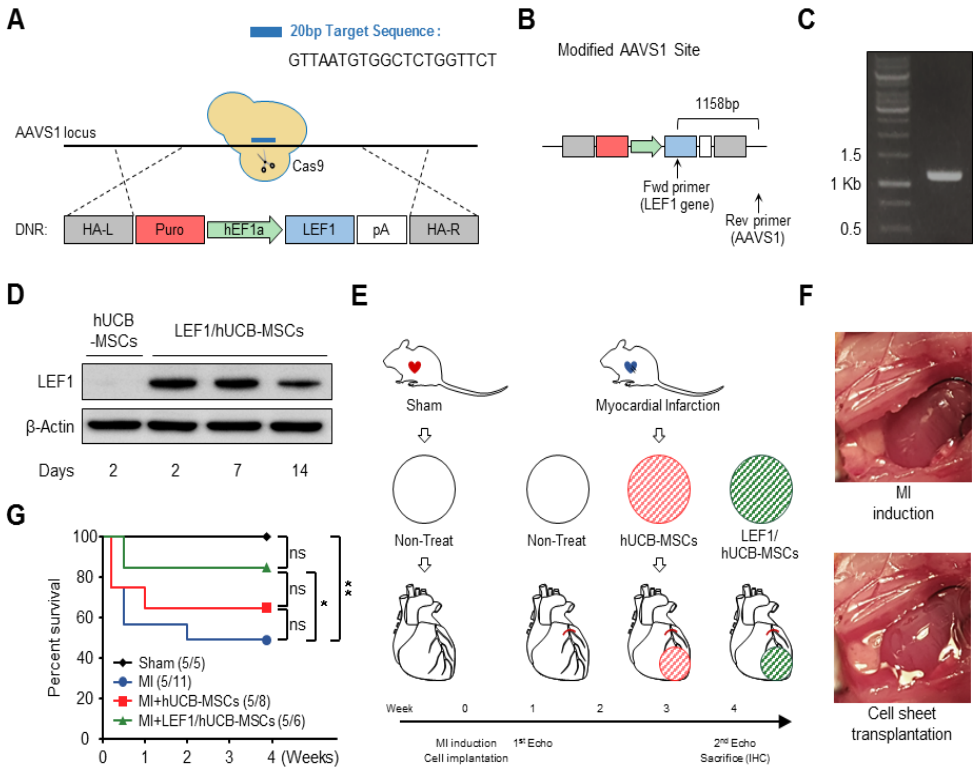


**Figure 2-3. Experimental strategy of therapeutic hUCB-MSC transplantation system.**

(A) Schematic diagram of the LEF1 gene integration procedure by CRISPR/Cas9-mediated knock-in to the AAVS1 site of hUCB-MSCs. Twenty nucleotide length sgRNA target sequence on AAVS1 locus, LEF1 introduction cassette flanked by homologous arm - Left (HR-L) and Right HR-R were indicated. (B) The architecture of donor DNA in the AAVS1 locus. Arrows: primers designed for the detection of successful homologous recombination. (C) Confirmation of the correct integration of LEF1 cassette into the AAVS1 locus using PCR (1,158 expected size). (D) Western blot analysis showing stable expression of LEF1 protein from

LEF1/hUCB-MSCs for 2 weeks. (E) Schematic illustration of the therapeutic procedure with cell sheet transplantation of hUCB-MSCs and LEF1/hUCB-MSCs in MI model. Of note, the cell sheet transplantation was performed with induction surgery of MI on day 0, and echocardiography measurements were performed prior to MI surgery, as well as 1 week and 4weeks after transplantation. Four groups (Sham: surgery without MI, MI: MI alone, MI+hUCB-MSCs: MI treated with hUCB-MSCs, and MI+LEF1/hUCB-MSCs: MI treat with LEF1/hUCB-MSCs). (F) Representative images depicting before and after MI induction and stem cell transplantation using the UpCell system. (G) Survival curves of the experimental groups. The survival rate was significantly enhanced in the LEF1/hUCB-MSCs group (n=5-11). *$p<0.05$, **$p<0.01$, ns=not significant.

To measure the combined therapy in myocardial infarction, we performed echocardiography at 1 week and 4 weeks after MI surgery (Fig 2-4A). A successful induction of MI in the rat model was confirmed by echocardiography showing drastic reduction of left ventricular ejection fraction (EF) (sham: 89.01 ± 2.56 % vs. MI: 36.33± 5.11 %), and fraction shortening (FS) (sham: 60.48 ± 3.82 % vs. MI: 16.24 ± 1.41 %) when comparing the sham and MI group at 1 week post-surgery (Fig 2-4B & C). Damage of the heart muscle was also measured by an increase of the left ventricle inner diameter at diastole (LVIDd) (sham: 4.33 ± 0.41 mm vs. MI: 8.77 ± 0.27 mm) and left ventricle inner diameter at systole (LVIDs) (sham: 2.26 ± 0.33 mm vs. MI: 7.60 ± 0.41 mm) (Fig 2-4D & E). To evaluate functional improvement of hUCB-MSCs and LEF1 overexpression in hUCB-MSCs on the MI, we also measured these 4 values at 4 weeks post-surgery (Fig 2-4B-E). The MI+hUCB-MSC group tended to have some protective effects when compared with MI, but there was no significant difference between the values taken 1 and 4 weeks post-surgery; EF (43.56 ± 3.62 %), FS (21.6 ± 1.88 %), LVIDd (8.68 ± 0.37 mm), LVIDs (7.64 ± 0.18 mm). Of note, in the comparison between the three MI groups (MI alone, MI+hUCB-MSCs, and MI+LEF1/hUCB-MSCs), LEF1 expressing hUCB-MSCs significantly improved in all 4 functional values in 4 weeks; EF (63.53 ± 4.34 %), FS (37.11 ± 2.78 %), LVIDd (6.29 ± 0.19 mm), LVIDs (4.72 ± 0.34 mm). These results demonstrate the protective effects of LEF1/hUCB-MSCs in myocardial infarction.

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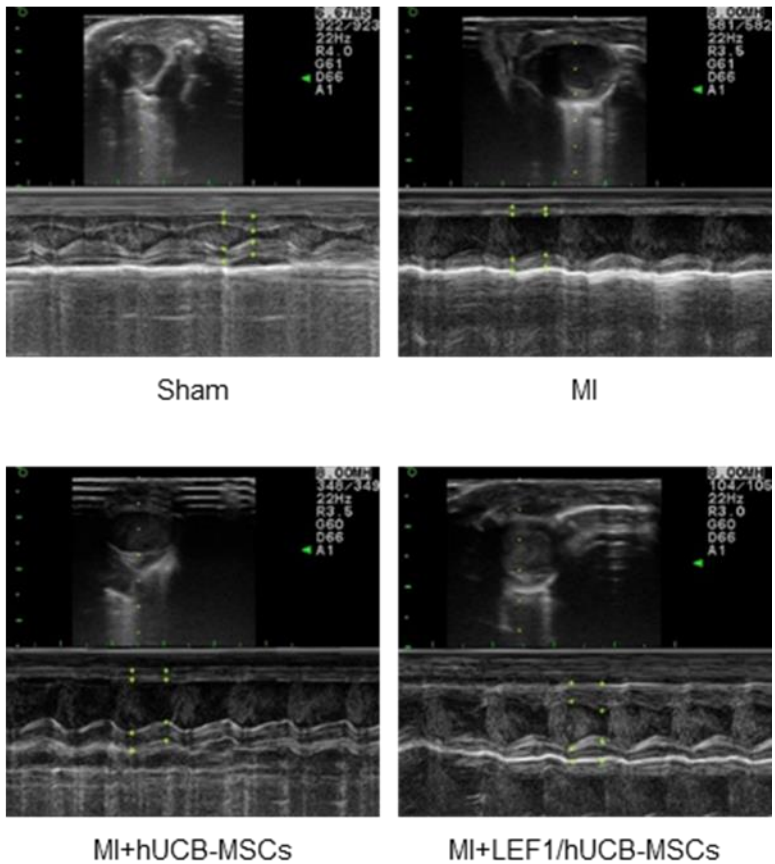


**Figure 2-4. Transplantation of LEF1/hUCB-MSCs recovered cardiac function in MI rat.**

(A) Representative images of echocardiography showed successively improved cardiac function in MI, MI+hUCB-MSCs and MI+LEF1/hUCB-MSCs at 4 weeks post-surgery.

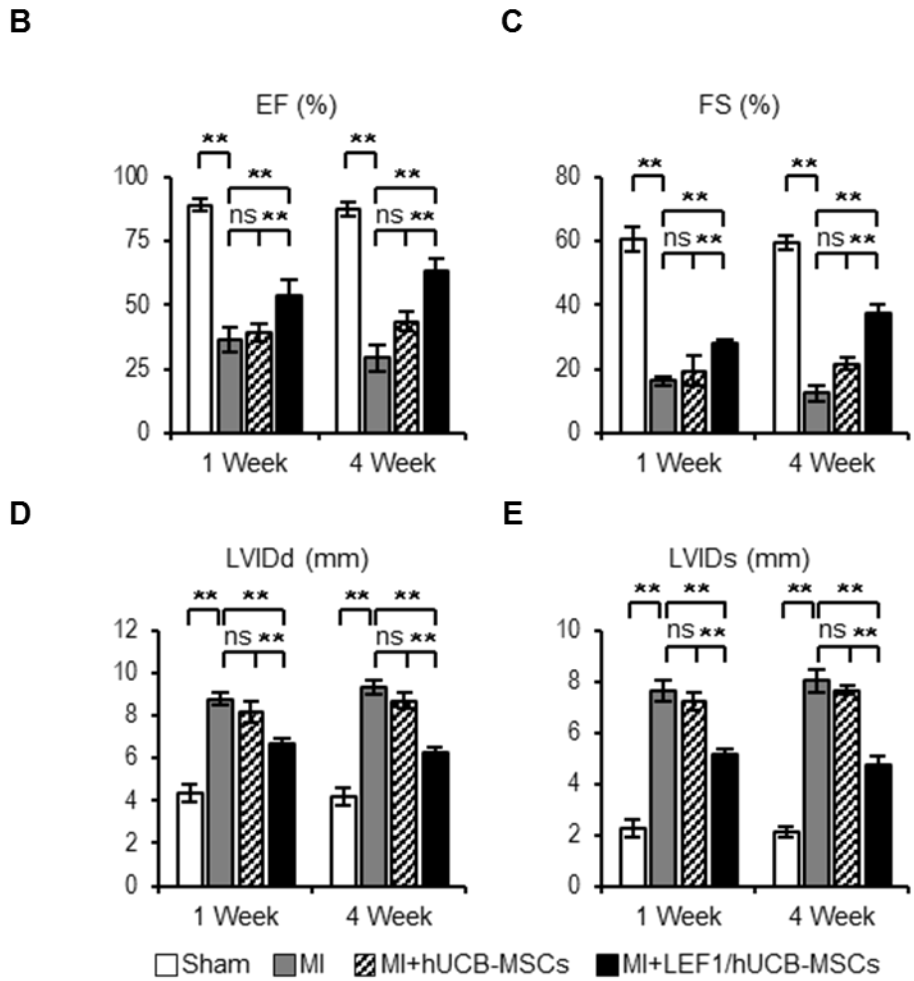


**Figure 2-4. Transplantation of LEF1/hUCB-MSCs recovered cardiac function in MI rat.**

(B - E) Four values representing cardiac function (EF, FS, LVIDd and LVIDs) were measured and compared in histograms. Significant improvements were detected in all MI+LEF1/hUCB-MSCs groups.*$p<0.05$, **$p<0.01$, ns=not significant. Error bar: standard error. White box: MI, gray: sham, pattern: MI+hUCB-MSCs and black: MI+LEF1/UCB-MSCs.

***LEF1/hUCB-MSCs show reduced MI size and Fibrosis, and protect the left ventricular wall from thinning***

Transplantation of LEF1/hUCB-MSCs reduced MI size and fibrosis and protected the left ventricular wall from thinning (Fig 2-5). The improvement of cardiac function was observed from a histological analysis. Masson's trichrome staining on the rat heart 4 weeks after sham surgery presented heart muscle in red (Sham in Fig 2-5A). The other three hearts with induced MI produced different ranges of regions stained with blue, which represents fibrosis in the left ventricle (LV). A very thin blue stained wall fiber was detected in the MI induced control model. No live heart muscle was stained in the MI region. Instead, a little thicker LV was noted in the hUCB- MSC treated MI group, but large fibrosis was still stained. Some live heart cells stained in red could be seen in the region of fibrosis. This may indicate that hUCB- MSC itself has a minor protective effect (MI+hUCB-MSCs in Fig 2-5A). In particular though, a clear improvement in anti- fibrosis effect was detected in the group treated with LEF1/hUCB- MSCs (MI+LEF/hUCB-MSCs in Fig 2-5A). The region of fibrosis stained blue was greatly reduced and the number of heart muscle cells remaining (stained with red) in the fibrotic region were more readily observed in the magnified image (Fig 2-5A). For the quantitation of efficacy in cardiac function, three factors, MI-size, fibrosis and wall thickness, were measured by analyzing the stained areas. MI-size and fibrosis were dramatically decreased in the LEF1/hUCB-MSC group when compared with both the MI and MI+ hUCB- MSC groups (Fig 2-5B & C). Furthermore, decreasing LV wall thickness resulting from MI was significantly

protected from in the LEF1/hUCB- MSC group, while the LV wall thickness tends to appear drastically reduced in the hUCB- MSC group which was not significantly different from the MI group (Fig 2-5D). These results suggest that LEF1 abundantly expressed in hUCB- MSCs not only has an autocrine effect (enhancing the survival of hUCB- MSCs) but also somehow has paracrine effects, possibly via growth factors and cytokines , which can protect and regenerate damaged rat heart cells in the region of MI.

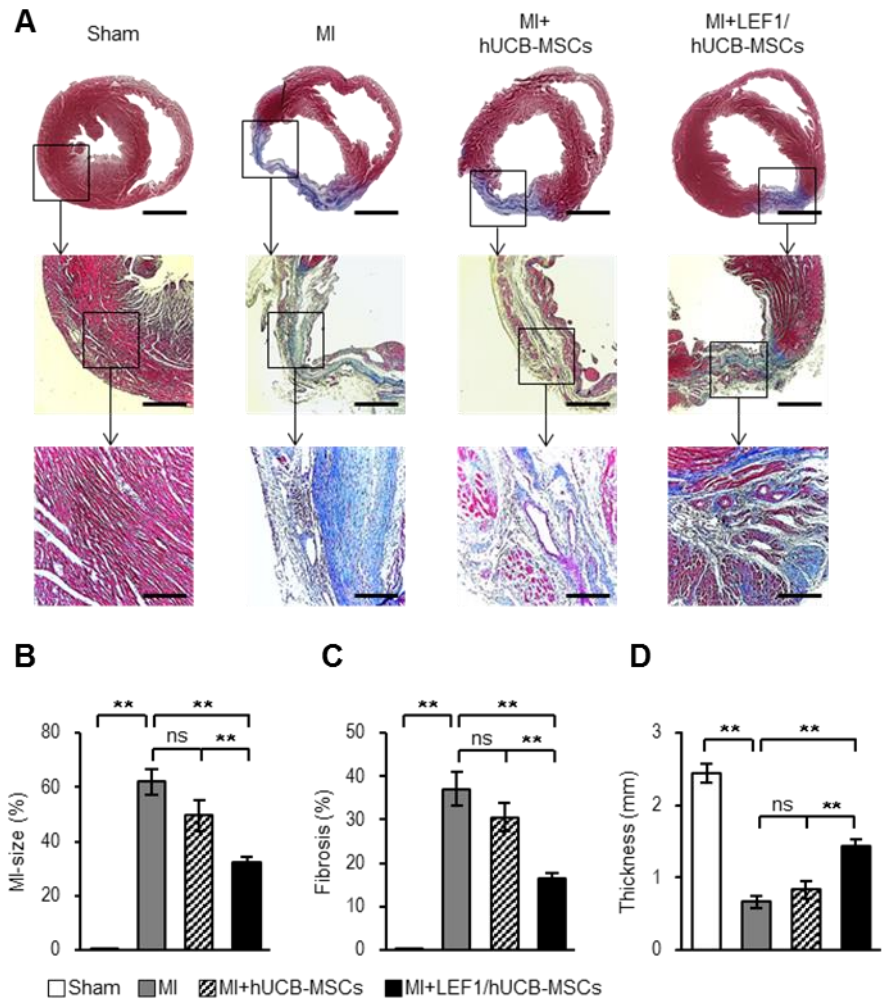


**Figure 2-5. The LEF1/hUCB-MSC transplantation greatly reduced MI size, fibrosis and restored the LV wall thickness.**

(A) Masson's trichrome staining showed MI regions at 4 weeks after surgery. Scale bars = (Top row, 1 mm; Middle row, 400 μm; Bottom row, 200 μm). The red stained region means the viable myocardium and the blue stained region means the fibrotic area. Large fibrous region stained in blue found in MI alone compared to

MI+hUCB-MSCs and MI+LEF1/hUCB-MSCs. Serial magnification distinctively presented damaged heart in MI and enhanced heart protective efficacy of MI+ LEF1/hUCB-MSCs (B - D) Quantification of infarct size, fibrosis region, and the wall thickness of LV in each group (n=5). *$p<0.05$, **$p<0.01$, ns=not significant.

***LEF1 expression in hUCB-MSCs helps to prolong survival of implanted stem cells in MI***

LEF1 expression was confirmed by immunohistochemical staining in MI induced rat heart tissues. Fibrous heart structure was found in the MI only group with few cells stained with methyl green (MI in Fig 2-6A). There were some cells that survived in the patch of hUCB- MSCs, but no LEF1 expression was detected in the MI+hUCB- MSCs group. LEF1 expression was stained only in the tissues from the cell patch of LEF1/hUCB- MSCs attached on the MI region (MI+ LEF1/hUCB- MSCs in Fig 2-6A). This suggests that there was no endogenous expression of LEF1 in the patched hUCB- MSCs. In addition, substantially more cells were counted in the LEF1/hUCB- MSCs group than the hUCB- MSCs group 4 weeks after surgery. This might contribute to the thicker LV in the MI region with LEF1/hUCB- MSCs. Quantification of the LEF1 immunohistochemistry (IHC) data successfully displayed prolonged survival of LEF1/hUCB- MSCs (Fig 2-6B). To distinguish implanted human stem cells from rat heart cells, IHC with anti- Lamin A+C antibody was performed in the MI region. We took a picture from the edge of the MI region to include rat heart cells as a control. No lamin A+C- positive cells were detected from rat heart cells in the MI group (Fig 2-6C). Only a thin layer of human cells were stained with Lamin A+C- positive cells in the hUCB-MSCs groups, whereas a large number of Lamin A+C- positive implanted human stem cells were detected in the MI+LEF1/hUCB- MSCs groups. Strikingly, rat heart myocardium still remained thicker at 4 weeks after MI induction following LEF1/hUCB- MSC treatment (Fig 2-6D). These results

suggest that LEF1 expression in hUCB- MSCs has positive effects not only on the survival of stem cells but also on protecting rat myocardial cells from MI via paracrine effects.

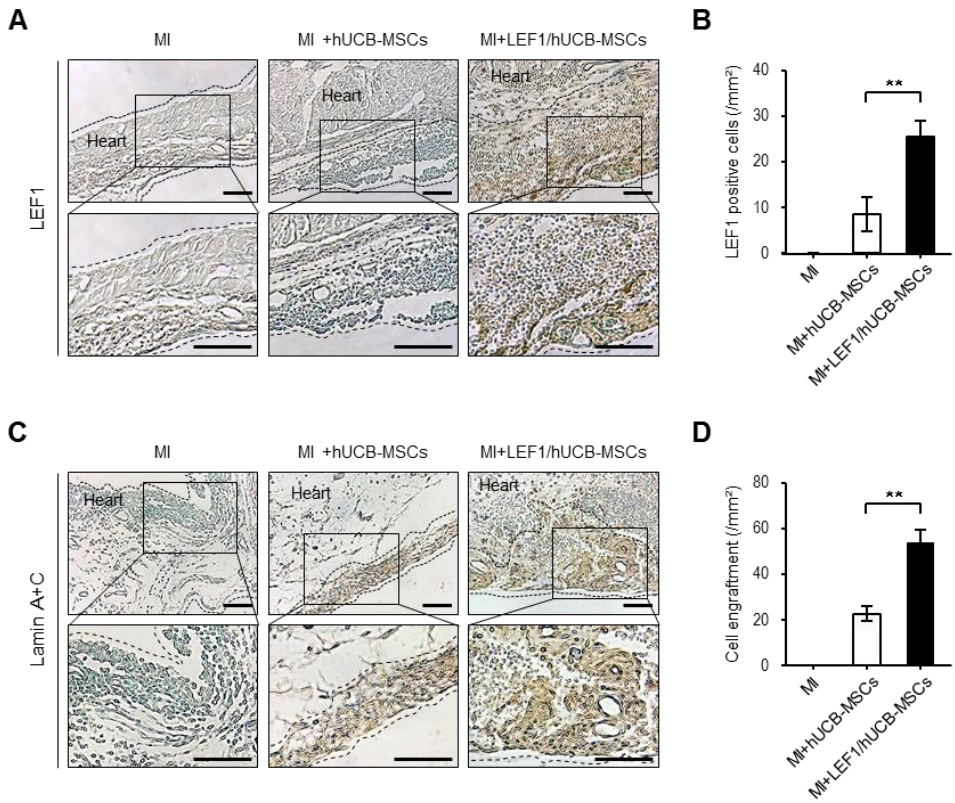


**Figure 2-6. Immunohistochemical staining confirmed the cells surviving and expressing LEF1 from the engrafted MI+ LEF1/hUCB-MSCs.**

(A) LEF1 was detected only from MI+LEF1/hUCB-MSCs. No rat heart cells and hUCB-MSCs expressed LEF1. Scale bar: 100 μm. (B) LEF1 positive cells in the heart tissue were quantitatively measured (n=5). (C) No Lamin signal was detected in the MI alone group. A thin layer was found in the group of MI+hUCB-MSCs and a thicker layer was stained from MI+ LEF1/hUCB-MSCs engrafted MI. Scale bar: 100 μm. (D) Human cell engraftment was quantitatively measured (n=5). **p<0.01.

***LEF1 expression triggers growth factor production in hUCB-MSCs that may protect heart from MI***

Growth factors and cytokines such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and hepatocyte growth factor (HGF), interleukin-1β (IL-1β) and IL-6 have been investigated as therapeutic targets in various cell types and diseases (Enoki et al., 2010, Cho et al., 2017, Chang et al., 2018, Pannitteri et al., 1997). Recently, these molecules secreted by MSCs have been examined in repair and regeneration therapy (Tompkins et al., 2017). We thus investigated the paracrine effect of LEF1/hUCB- MSCs via enhanced secretion of growth factors and cytokines. Expression levels of three growth factors (HGF, IGF and VEGF) and cytokine (IL-8) were compared between control hUCB- MSCs and LEF1/hUCB- MSCs. As expected, HGF, IGF, VEGF and IL-8 were increased in LEF1/hUCB- MSCs (Fig 2-7A). Quantitative RT-PCR showed an approximately two-fold increase in the expression of VEGF, IL-8 and IGF in LEF1/hUCB- MSCs (Fig 2-7B). VEGF protein expression was confirmed by IHC staining (Fig 2-7C). Rat heart at the edge of the MI region was faintly stained due to cross reactivity of VEGF antibody between human and rat. A strong VEGF signal was detected in the surrounding regions of LEF1/hUCB- MSCs (Fig 2-7D). These results determined that LEF1 integration in hUCB- MSCs triggers expression of diverse growth factors resulting in protective effects on myocardial cells in the rat MI model.

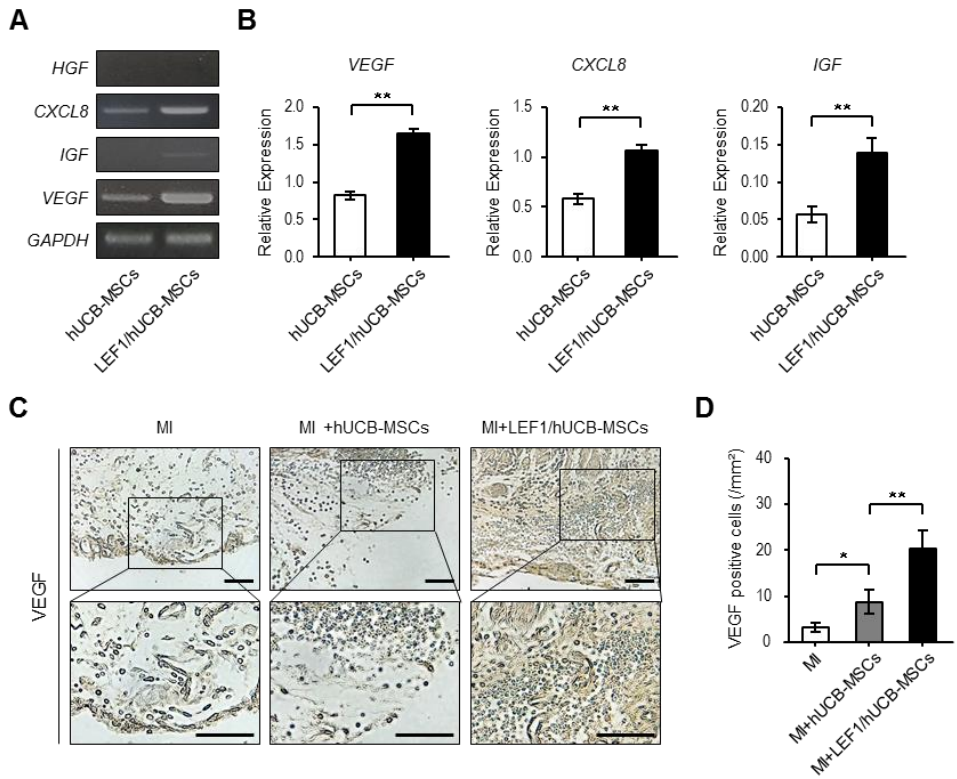


**Figure 2-7. LEF1 triggered growth factors (HGF, IGF, VEGF) and IL-8 expressions in MI+ LEF1/hUCB-MSCs.**

(A) Expression of growth factors HGF, CXCL8, IGF, and VEGF were measured by Conventional PCR. (B) Increased mRNA expression of growth factors in LEF1/hUCB-MSCs compared with hUCB-MSCs. Real-time PCR demonstrated that the relative gene expressions of both VEGF, CXCL8 and IGF to GAPDH were increased in LEF1/hUCB-MSCs. **$p<0.01$. (C) Immunohistochemical staining for VEGF in three MI groups; MI alone, MI+hUCB-MSCs and LEF1/MI+hUCB-MSCs. Anti-VEGF antibody faintly stained rat heart, but stronger signal was detected in LEF1/hUCB-MSCs. Scale bar: 100 μm. (D) VEGF protein expression was measured in infarcted heart among different groups (n=5). *$p<0.05$, **$p<0.01$.

# Discussion

Stem cell-based therapies have emerged as a promising treatment for heart regeneration after infarction (Segers and Lee, 2008). Among them, hUCB-MSCs have been recognized as good candidates due to trophic activities such as multiple lineage potential, lack of teratoma formation, easy expansion and low immunogenicity (Lee et al., 2016). In cardiovascular disease, MSC-based therapies have been attempted in a lot of pre-clinical research and has shown sufficient potential. However, the low cell survival rates and the engraftment failure of implanted cells in the infarcted region are the main obstacles in this field that still remain. Therefore, enhancement of stem cell proliferation and its survival under the harsh conditions of the ischemic area are necessary to utilize MSC's for clinical application.

In the current study, we thus focused on enhancing stem cell activities in cell proliferation and its survival in the harsh conditions of the ischemic region. LEF1 has been known to play a role in regulating cell proliferation via Wnt/β-Catenin signaling (Li et al., 2012, Reya et al., 2000, Aloysius et al., 2018). Huang et al. recently reported that LEF1 plays a crucial role in sustaining self-renewal in mouse embryonic stem cells as well (Huang and Qin, 2010a). Therefore, we investigated

the effects of LEF1 expression in hUCB-MSCs in normal and harsh conditions such as oxidative stress *in vitro*. Introduction of LEF1 to hUCB-MSCs resulted in the overexpression of LEF1 stimulated stem cells' cell cycle and proliferation via the canonical Wnt pathway in normal conditions (Fig 2-1). In addition, understanding of cell response to oxidative stress *in vitro* is important because the cells' micro-environment after implantation is hypoxic, which can lead to apoptosis (Denu and Hematti, 2016). This study clearly showed that LEF1 expression protected hUCB-MSCs from oxidative stress conditions by increasing Bcl-2 expression. This was confirmed through flow cytometry showing a reduced number of apoptotic cells induced by $H_2O_2$ (Fig 2-2).

We then generated therapeutic hUCB-MSCs that stably express LEF1 through CRISPR/Cas9-mediated genome editing (LEF1/hUCB-MSCs) in order to examine whether the induction of the LEF1 gene in hUCB-MSCs affects the cell engraftment, survival and tolerance in hypoxic conditions. The CRISPR/Cas9 gene integration system was employed on the AAVS1 locus to overcome side effects such as tumorigenesis, or unpredictable integration of the transgene, which could be induced by the viral approach (de la Fuente-Nunez and Lu, 2017). Steady expression of LEF1 was detected until 2 weeks in LEF1/hUCB-MSCs. As expected from the *in vitro* study, the LEF1/hUCB-MSC group showed strong positive effects in the *in vivo* MI model. Echocardiography and histological staining analysis clearly showed evidence that LEF1/hUCB-MSCs have a protective effect in the MI region. EF, FS, LVIDd and LVIDs, which represent left ventricular cardiac

functions, were greatly improved in LEF1/hUCB-MSCs compared with MI alone and hUCB-MSC treatment.

In addition, the protective effect of LEF1/hUCB-MSCs was measured by MI-size, fibrosis and wall thickness using Masson's trichrome staining. MI and fibrosis were formed approximately 52% less in LEF1/hUCB-MSCs compared with the MI control group. This is a huge improvement compared with the hUCB-MSC group showing only a 21% reduction. Furthermore, wall thickness loss in MI was suppressed by LEF1/hUCB-MSCs while it was not significantly different between MI alone and the MI+ hUCB-MSCs group. Most cardiac muscle cells were replaced by fibrosis in MI alone, but heart muscle structure was still retained in the MI region treated with LEF1/ hUCB-MSCs. This result may suggest two major mechanisms to explain these enhanced therapeutic effects. One is a paracrine effect and the other one is direct trans-differentiation. After transplantation, the engrafted MSCs could secrete the therapeutic factors that regenerate the damaged cardiac tissue and cause neovascularization via paracrine effects, and also prevent the cell loss of cardiomyocytes by direct trans-differentiation or inhibition of fibrosis. However, according to previous studies, there is little evidence that the induction of the LEF1 gene in stem cells directly affected the differentiation of cardiomyocytes or the decrease of fibrosis. In contrast, it has been reported that LEF1 expression secretes many therapeutic factors associated with cell cycling, proliferation, and survival (Kim et al., 2000, Reya et al., 2000, Huang and Qin, 2010a, Li et al., 2012). This mechanism that enhances positive effects in cell cycle, proliferation and cell survival under oxidative stress was confirmed by this *in vitro* study (Fig 2-1 &

2).We also tested paracrine effects of LEF1 expression in hUCB-MSCs. As we showed in Fig 2-7, increased VEGF and IL-8 expressions were detected in LEF1/hUCB-MSCs *in vitro* and was confirmed by IHC in a rat MI model with transplanted LEF1/hUCB-MSCs. These results indicated that LEF1/hUCB-MSCs enhanced the secretion of various growth factors associated with micro-environmental neovascularization, proliferation and immune responses, resulting in protective effects on hearts damaged by MI.

Although we presented several positive aspects of genome editing to lead LEF1 expression in hUCB-MSCs, there are also several studies regarding the aberration of LEF1 gene expression in various cases of tumorigenesis and cancer cell proliferation, migration, and invasion (Zhao et al., 2018, Lamb et al., 2013). Particularly, LEF1 expression has been reported in cancer cell types such as some leukemias, lymphoma, squamous cell carcinoma and colorectal cancer (Shtutman et al., 1999, Espada et al., 2009). Lack of teratoma formation has been known in MSCs, however, the tumorigenic effect of LEF1 in hUCB-MSCs needs be tested in regard to long-term expression and survival.

In conclusion, we provide the evidence that LEF1 promotes hUCB-MSCs proliferation and attenuates the apoptosis from oxidative stress. The hUCB-MSCs in which the LEF1 gene was integrated by CRISPR/Cas9 system, displayed enhanced cell survival and improved cardio-protective effects in an animal model of myocardial infarction. These results suggest that the introduction of LEF1 could be a novel strategy in stem cell therapy after myocardial infarction.

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## 국문초록

# 유전자 편집 기법으로 VEGF 와 LEF1 을 발현시킨 중간엽 줄기세포의 심근경색 치료효과

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지도교수 조 제 열

심혈관 질환은 전 세계적으로 주요한 사망 원인 중 하나로서, 2016년 세계보건기구 (World Health Organization; WHO) 통계자료에 따르면, 매년 9백만 이상의 환자들이 이 질환으로 인하여 사망한다. 이중 심근경색증은 관상동맥의 폐색으로 혈액을 공급받지 못한 심근세포들이 죽어가는 심각한 질환으로, 그 환자수가 심혈관 질환 중 가장 빠르게 증가하고 있다.

심근경색의 가장 주요한 치료법은 막힌 혈관에 스텐트를 삽입하여 혈관내경을 다시 넓혀주는 스텐트 삽입술이다. 이는 심근조직의 생존을 위한 근본적인 치료법 이지만, 재관류 손상의 부작용이 일어날 수 있으며, 또한 심근세포는 매우 낮은 재생능력을 지니고 있으므로, 수술 후 손상된 심근조직을 재생하기 위한 추가적인 치료전략이 필요한 상황이다.

이와 관련하여, 인간 제대혈 유래 중간엽 줄기세포 (hUCB-MSCs)는 특별한 주목을 받고 있다. 이 세포는 빠른 자가재생과, 심근세포로의 분화 능력을 가지고 있으며, HGF, bFGF, VEGF-D, PDGF 등의 다양한 사이토카인들을 분비하여, 심장 내 손상된 부위의 회복 및 새로운 혈관형성 등에 도움을 줄 수 있다. 그러나, 이러한 장점에도 불구하고, hUCB-MSCs 는 이식 후 3% 이하의 세포들 만이 생존하며, 성공적 이식 이후에도 혈관형성에서 가장 중요한 영향을 미치는 VEGF-A 를 매우 적게 분비한다는 한계점을 가지고 있다. 이와 같은 특징들은 hUCB-MSCs 를 활용한 치료요법에서 VEGF-A 의 유도 필요성과 세포증식능력을 증진시킬 필요성을 제기하고 있다.

이를 위하여 본 연구에서는 TALEN 및 CRISPR/Cas9 system 을 활용하여, 줄기세포 내 safe harbor site 인 AAVS1 locus 에 VEGF-A 와 LEF1 유전자를 유도하는 전략을 적용했고, 초음파 검사 및 조직학적 분석을 통해 이 세포들의 심장기능 회복효과를 확인하였다. 이는 표적 유전체 공학을 통해 치료적 유전자들이 도입된 줄기세포의 새로운 심근경색 치료 전략으로서의 가능성을 제시해 주고 있다.

CHAPTER Ⅰ 에서는 혈관내피성장인자 (VEGF-A) 를 활용하여, hUCB-MSCs 의 혈관형성 능력을 향상시켰으며, 심근경색모델에 이 세포를 이식해 주었을 때의 치료효과를 확인하였다. 관상동맥 폐쇄 이후, 심장조직에 혈액을 재 공급하고 심근 괴사의 진행을 늦추기 위해서는 신생혈관의 형성이 필수적이다. 이를 위해 2세대 유전자 편집 기술인 TALEN 시스템을 활용하여 치료적 수준의 VEGF-A 를 분비하는 중간엽 줄기세포 (VEGF/hUCB-MSCs) 를 제작했고, 심근경색 모델에서 이 세포의 치료효과를 확인하였다. VEGF-A 는 신생혈관형성을 유도하여 치료적 목적으로 새로운 혈관을 형성 할 수 있으나, 과도한 양의 VEGF-A 는 비정상적인 혈관의 형성 및 혈관종의 성장을 유발 할 수 있다. 따라서 이를 치료적 목적으로 활용하기 위해서는 그 발현을 조절해 줄 수 있는 제어장치가 필요하다. 이를 위하여 독시사이클린 (Doxycycline) 존재 시에만 VEGF-A 단백질이 발현되도록 벡터를 디자인 하였다. 또한 기존 유전자 요법의 한계점인 낮은 유전자 전달효율, 안정성 문제, 특이적 발현 등의 문제를 해결하기 위하여, TALEN 유전자 편집 기술을 활용해 세이프 하버 (Safe Harbor) 인 AAVS1 유전자좌에 VEGF 의 발현을 유도할 수 있는 유전자 카세트를 도입하였다.

이렇게 제작한 VEGF/hUCB-MSCs 는 독시사이클린 처리시에만 VEGF-A 를 분비 하였으며, 이는 혈관형성과 관련된 유전자들의 발현을 증가시카고 동물모델에서의 혈관형성을 향상시켰다. 최종적으로 랫드 심근경색 모델에 VEGF/hUCB-MSCs 이식을 진행하고 음수를 통해 독

시사이클린을 공급하였을 때, 심장기능이 회복되고, MI 크기 및 섬유증이 감소하여 심근경색으로 인해 손상된 심장의 회복효과를 확인할 수 있었다.

CHAPTER Ⅱ 에서는 LEF1 유전자가 도입된 hUCB-MSCs 에 대해 세포 증식 및 생존에서의 증진효과 및 심장보호효과에 대해 연구하였다. 이전부터 치료적인 응용에 대한 잠재성이 있다고 주목되어 왔으나, hUCB-MSCs 는 이식후의 낮은 생존율 때문에 심근경색 치료에 적용하기엔 분명한 한계가 있다. 이에 따라, 치료적 유전자가 도입된 줄기세포를 이용한 치료전략이 새롭게 요구되고 있는 실정이다. 이 단원에서는 CRISPR/Cas9 시스템을 활용하여 LEF1 유전자가 도입된 hUCB-MSCs (LEF1/hUCB-MSCs) 를 제작하고, 이를 심근경색 모델에 적용하여 심장보호 및 치료효과를 확인하고자 하였다.

4개의 문헌조사 (*in silico* literature surveys) 를 통해 LEF1 을 치료적 유전자로 선별할 수 있었고, hUCB-MSCs 에서, 이를 과발현 시켜 세포증식 및 생존효과의 증진을 확인 할 수 있었다. 그 결과 실제 질환 모델에 치료를 응용할 수 있는 가능성을 충분히 확인하여, 다음으로 CRISPR/Cas9 시스템을 통해 LEF1 유전자를 AAVS1 유전자 좌로 도입시킨 줄기세포 (LEF1/hUCB-MSCs) 를 제작하여 이를 랫드 심근경색 모델에 이식하는 과정을 수행했다. LEF1/hUCB-MSCs 의 이식은 심근경색 이후 생존율을 개선시켰으며, 이후 심장기능의 회복, 섬유증

및 경색 부위의 감소 등의 심근보호효과도 보여주었다.

결론적으로, 본 연구에서는 유전자 편집 기법을 통하여 VEGF-A 와 LEF1 을 발현하는 중간엽 줄기세포 (VEGF/hUCB-MSCs, LEF1/hUCB-MSCs) 를 제작하였다. 이들 세포들은 각각 증진된 혈관 형성 능력과 세포증식 능력을 보여 주었으며, 심근경색 모델에 이식 하였을 때 향상된 심혈관 기능회복 효과를 보여주었다. 이를 통해 기존 세포 치료법이 가지고 있는 한계점을 극복한 새로운 세포치료 전략을 제시할 수 있었다.

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**주요어:** 심근경색, 중간엽 줄기세포, 표적 유전체 공학,
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**학번:** 2012-21532