A Dissertation for the Degree of Doctor of Philosophy

Enhanced Angiogenesis and Reendothelialization by Mesenchymal Stem Cells Secreting Growth Factors Produced by Genome Editing

유전자 편집기술을 이용한 혈관 성장인자를 발현하는 중간엽줄기세포의 혈관신생 및 재내피화 촉진

August 2018

Graduate School of Veterinary Medicine

Seoul National University

Veterinary Medicine Major

Hyun-Kyung Chang

Enhanced Angiogenesis and Reendothelialization by Mesenchymal Stem Cells Secreting Growth Factors Produced by Genome Editing

By Hyun-Kyung Chang

Supervised by Je-Yoel Cho, D.V.M., Ph.D.

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

In

Department of Veterinary Medicine,

Graduate School of Seoul National University

We accept this thesis as confirming to the required standard.

August 2018

Chairman of Committee:	Hang, Lee	(Signature)
Vice chairman of Committee:	Je-Yoel, Cho	(Signature)
Committee member:	Kyung-Sun, Kang	(Signature)
Committee member:	Dong-Keun, Han	(Signature)
Committee member:	Pyung-Hwan, Kim	(Signature)

Enhanced Angiogenesis and Re-endothelialization by Mesenchymal Stem Cells Secreting Growth Factors Produced by Genome Editing

By Hyun-Kyung Chang

Supervised by Je-Yoel Cho, D.V.M., Ph.D.

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

In

Department of Veterinary Medicine,

Graduate School of Seoul National University

We accept this thesis as confirming to the required standard.

August 2018

Chairman of Committee: Hang, Lee (Signature)

Vice chairman of Committee: Je-Yoel, Cho (Signature)

Committee member: Kyung-Sun, Kang (Signature)

Committee member: Dong-Keun, Han (Signature)

Committee member: Pyung-Hwan, Kim (Signature)

Enhanced Angiogenesis and Reendothelialization by Mesenchymal Stem Cells Secreting Growth Factors Produced by Genome Editing

지도교수 조 제 열

이 논문을 수의학박사 학위논문으로 제출함

2018 년 8 월

서울대학교 대학원

수의학과 수의생화학 전공

장 현 경

장현경의 수의학박사 학위논문을 인준함

2018 년 8 월

위 원 장 이 항 (인)

부위원장 조 제 열 (인)

위 원 강 경 선 (인)

위 원 한 동 근 (인)

위 원 김 평 환 (인)

Enhanced Angiogenesis and Re-endothelialization by Mesenchymal Stem Cells Secreting Growth Factors Produced by Genome Editing

지도교수 조 제 열

이 논문을 수의학박사 학위논문으로 제출함

2018 년 8 월

서울대학교 대학원

수의학과 수의생화학 전공

장 현 경

장현경의 수의학박사 학위논문을 인준함

2018 년 8 월

위 원 장 이 항 (인)

부위원장 조 제 열 (인)

위 원 강 경 선 (인)

위 원 한 동 근 (인)

위 원 김 평 환 (인)

Abstract

Enhanced Angiogenesis and Reendothelialization by Mesenchymal Stem Cells Secreting Growth Factors Produced by Genome Editing

Hyun-Kyung Chang
Department of Veterinary Medicine
Graduate School of
Seoul National University

Vessel is an important network in body, and it has various types ranging from the aorta and the capillary. Diseases in these vessels are critical and difficult to cure fundamentally. To overcome this huddle, the fundamental way to treat these diseases has been established by regenerating blood vessels with the combination of stem cell therapy and gene therapy. Mesenchymal stem cells enhance the regeneration of blood vessels, but their survival rate and cytokine secretion in an injected site are limited. Therefore, another approaches to solve these problems are required. To address this, hepatocyte growth factor (HGF) gene that facilitates vascular regeneration was integrated into the safe-harbor site in the chromosome of mesenchymal stem cells (MSCs) using the TALEN system. The Tet-on system was also used to control the expression of HGF. The expression regulated by Doxycyclin was

successfully validated at protein level after the integration into the host chromosome. Inducible HGF expressing MSCs promoted migration in the short term, while prevented cell death and promoted angiogenesis in the long term. They were then encapsulated in RGD-alginate microgel, which includes a phenotype of a peripheral vascular disease, and applied to the limb ischemia model to actually evaluate the vessel regeneration ability of functional MSCs. As a result, the regeneration of blood vessels was improved by the injection of HGF secreting MSCs. It proved that the inducible HGF secreting MSCs are a valuable therapeutic tool for the treatment of vascular diseases that critically require angiogenesis.

Based on these therapeutic effects, it was also applied to the large animal model with aortic disease. Atherosclerosis is a fatal disease and is very difficult to treat. Although stents are used to treat these problems, there are still limitations such as restenosis and stent thrombosis. In order to solve the existing problems, the rapid reendothelialization should be induced after the stent transplantation. Thus, to induce reendothelialization, the stent was combined with stem cells that secrete HGF and vascular endothelial growth factor (VEGF), known as a strong vessel induction factor, in an inducible manner.

Angiogenic growth factor secreting MSCs were adhered to a stent coated with polydopamin, fibronectin and extracellular matrix. The expression of HGF and VEGF on the stent was confirmed.

Furthermore, the growth of cells on stent was confirmed while maintaining their original characteristics. When transplanted into pigs, VEGF reduced restenosis in the short term, but excessive restenosis was observed after 2 weeks−time point. In the case of HGF, the restenosis tended to decrease even after 2 weeks, and the micro CT showed a flattened endothelial layer. The mixed condition of HGF and VEGF expressing MSCs at the ratio of 5:1 highly reduced the restenosis after 4 weeks of transplantation. Reendothelialization was accelerated in the HGF and VEGF 5: 1 groups, confirmed by immunohistochemistry. We also found the human cells that were injected with the stent populated around a porcine cardiac vein. These results suggest that stem cells expressing HGF and VEGF in the stent promote reendothelialization and reduce restenosis in swine models. In conclusion, it is possible to promote vascular regeneration and highlighted the possibility of treatment using the stem cells with the regulated expression of angiogenic growth factors.

Keywords: Vessel regeneration, Reendothelialization, MSC, Angiogenic growth factor (HGF&VEGF), TALEN, Cell therapy

Student Number: 2012−23579

Contents

List of figures

General Introduction

1. The vascular disease

1.1 Vascular system and constituent

The vascular system is one of the most important network that delivers oxygen, nutrients and hormones by circulating our body. It includes the coronary artery from the heart and the peripheral artery leading to the distal end. Arteries, arterioles, capillaries, venules, and veins compose vascular system. Arteries and veins consist of tunica intima, tunica media, and tunica adventitia. Tunica intima, the innermost layer of an artery or vein, is composed of endothelium, subendothelial layer and elastic or fenestrated layer. Tunica media consists of smooth muscle cells and elastic tissue that lies between tunica intima and tunica adventitia. Tunica adventitia (or extern) is the outermost layer of vein that is mainly composed of collagen (Sternberg 1992).

1.2 The homeostasis function of endothelium

Endothelium exists as a semipermeable barrier between blood and the smooth muscle layer which is the main composition of tunica media. Endothelium makes direct contact with blood. In the past, endothelium was recognized only as a simple mechanical barrier, but it is a very active metabolic and endocrine organ. The endothelial cells are important to maintain homeostasis. The normal

endothelium inhibits the thrombus activation process as well as synthesizes and secretes substances that prevent platelet aggregation, including thrombomodulin, protein C and heparin sulfate proteoglycan (HSPG)(Munoz et al. 2004). In addition, fibrinolysis is regulated to maintain blood flow in the blood vessel by tissue plasminogen activator (tPA) and von Willebrand factor (vWF)(van Hinsbergh 2012). It also maintains the tension of the vessel by nitric oxide and endothelin (Deanfield et al. 2007). Endothelium secretes Toll-like receptors (TLRs) that monitor pathogen and external substances in the blood. Furthermore, endothelium restores damaged blood vessels from pulsatile pressure like sheer stress, cyclic strain and pulsatile pressure that stimulates the expression of nitric oxide synthase (eNOS)(Califano et al. 2010).

1.3 The mechanism of atherosclerosis

Arteries can become narrow and hard, reducing the overall blood flow of the body which is called atherosclerosis. The principal cause of atherosclerosis is due to the plaque that is made up of cholesterol and other substances in the blood. The plaque can clog vessels and block the blood flow to the heart or other organs. Consequently, the weakened blood vessels can rupture and cause internal bleeding (Ross 1993). Atherosclerosis is initiated by a decline of endothelial cells. The most principal mechanism that reduces endothelial cell function is the increase of oxidative stress.

In detail, oxidative radical is increased by active NADPH oxidase or uncoupling NOS. The increased oxidative radical stimulates ion channels, NF-kB, AP-1, tyrosine kinase and MMP, which leads to dysfunction of endothelial cells. Then, cholesterol, monocytes and macrophages infiltrate the blood vessel wall giving rise to foam cells and migrating smooth muscle cells. This results in the formation of a fibrous cap by synthesizing extracellular matrix proteins. A chronic inflammatory state persists to promote atherosclerosis (Fig. I.).

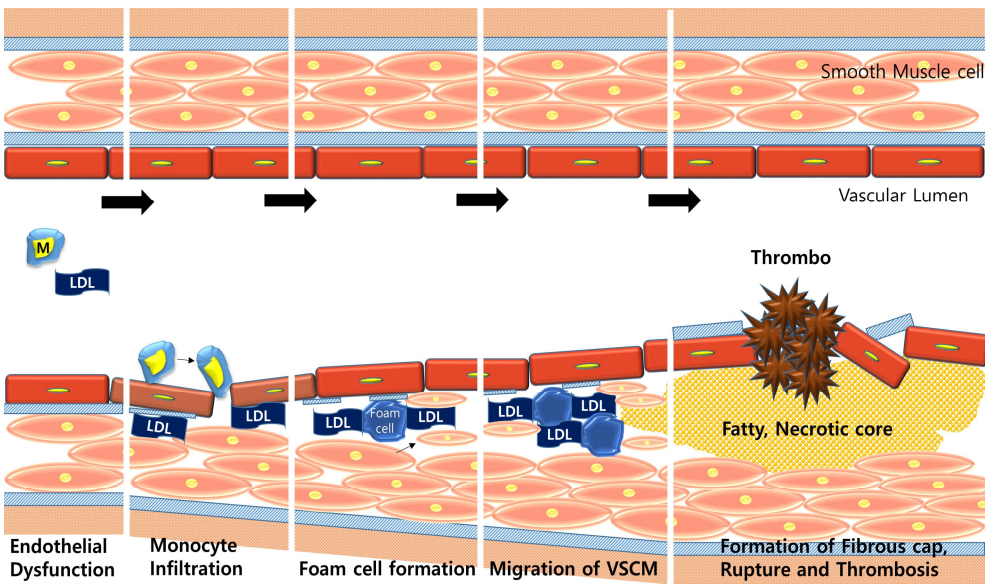


Figure I. The progression of atherosclerosis.

Therefore, the normal function of endothelium is significantly important to cure the atherosclerosis. I focused on 2 common atherosclerosis that are peripheral artery disease (PAD) and coronary artery disease (CAD).

1.4 Peripheral artery disease and coronary artery disease

PAD generally describes the damage of blood flow to the extremities as a result of atherosclerotic occlusive disease. It is a common artery disease hallmarked with narrowed arteries involving the aorta, iliac, and lower-extremity vessels such as limbs. It causes leg pain and limb amputation in severe case (Fowkes et al. 2013, Patel et al. 2015, Olin et al. 2016). CAD is another group of disease in atherosclerosis that includes angina, myocardial infarction and sudden cardiac death (Wong 2014). The main cause of these diseases is also due to the blocked blood flow by plague accumulation in coronary artery.

2. Stem cell therapy for the vascular disease

2.1 Endothelial progenitor cells (EPCs)

EPCs were founded in 1997 by Asahara team in circulating blood cells that can be differentiated into the mature blood vessel endothelial cells. It was the first case of EPC and adult neoangiogenesis. EPCs express the surface marker CD34 and kinase insert domain receptor (KDR) and promote vascular repair and the restoration of blood flow in animals with hindlimb ischemia (Asahara et al. 1997). EPCs observed the neoangiogenesis by replacing to the site of ischemia and differentiating into EC. In numerous trials, EPCs exhibited the effect for blood vessel repair and regeneration in a pre-clinical animal model (Critser et al. 2010, Tongers et al. 2010). The most of the trials were related to

myocardial diseases, ischemic cardiomyopathy and peripheral arterial disease (Devanesan et al. 2009).

2.1.1 Clinical trial

In clinical trial, 115 patients, 74 limbs PAD and 41 limbs thromboangiitis obliterans, were randomly injected with autologous bone marrow-mononuclear cells (BMNC) or placebo peripheral blood-mononucler cells into the gastrocnemius of the ischemic limb. At 4 weeks, the legs with BMNC had alleviated the symptoms of disease and improvements sustained even at 24 weeks (Masaki et al. 2002).

The measure of symptoms, including leg pain scale, ulcer size, pain free walking distance, were all improved significantly, but ankle-brachial pressure index and The leg pain scale, ulcer size and pain free walking distance were improved significantly, but ankle-brachial pressure index and transcutaneous oxygen pressure value did not change during the 2-year (Matoba et al. 2008). The circulating CD34+/KDR+ EPCs also improved cardiac vascular disease. Patients with arterial hypertension showed systolic blood pressure. This highlighted the negatively correlated with the number of circulating CD133+ and CD34+/KDR+ EPC. Impaired EPC activity in hypertensive patients incurred the acceleration of EPC senescence via angiotensin II (Touyz 2004).

2.1.2 Limitation

The limitation of EPC is to control characterization and quantification for clinical application. Even though EPCs present few

markers like CD34 and KDR, the unique marker for EPCs has not been reported for a human subject. This left the method to isolate the cells from blood difficult (Hirschi et al. 2008, Asahara et al. 2011). The source of an EPC also influences verifying markers. The number of markers are shared but few other markers are different depending on sources, such as cardiac muscle, adipose tissue, liver and intestinal tissues (Devanesan et al. 2009). A sufficient amount of cells is required to show the effect, but there is still a limitation for EPC to grow in *in vitro* system. Even the EPC with stemness, it still has limited proliferation and the differentiation evidence of progenitor cell lineage during culture is lacking. The fine control of cell quality is necessary.

2.2 Mesenchymal stem cells (MSCs)

Later in the 1970s, heterogeneous populations of undifferentiated, adherent cells from bone marrow was found to have an ability to differentiate into a various cell like osteoblasts, chondrocytes, myocytes and adipocytes [7, 8]. This is the discovery of mesenchymal stem cells (MSCs) that can be derived from bone-marrow, peripheral blood, adipose, umbilical cord blood and placenta. MSCs are multipotent cells and are determined by surface markers of CD73, CD105, CD39, CD44, CD90 and CD106 (Lv et al. 2014). MSCs also have a differentiation ability into endothelial and smooth muscle cells that were crucial for angiogenesis and neo-vasculogenesis(Lin et al. 2010, Ransohoff et al. 2012). Numerous

studies have demonstrated that MSCs can regenerate the vascular system of PAD and CAD (Caplan 2007, Wu et al. 2007, Garikipati et al. 2014, Gu et al. 2017). The therapeutic effect of MSCs was its ability to directly differentiate into injured cells and its paracrine ability from protein that secreted from cells (Tao et al. 2016).

Additionally, MSCs avoid allogeneic rejection by lacking the expression of MHC-II and costimulatory molecule expression. It also prevents T cell responses through the modification of dendritic cells and NK cell. These properties make MSCs a new frontier medicine for angiogenesis therapy.

2.2.1 Clinical trials

The use of MSCs in clinical trials of CVDs treatment was performed. The most of clinical trial used autologous BM-MSC. In phase I to III, autologous BM-MSC was used for myocardial infarction and heart dysfunc111111tion. MSCs repair and restore heart function by reducing fibrosis, neoangiogenesis and neomyogenensis. The damaged myocardium was repaired via paracrine signaling (Trachtenberg et al. 2011, Hare et al. 2012, Heldman et al. 2014, Lee et al. 2014). With the allogeneic BM-MSCs, the safety in patients and transdifferentiation of MSCs into cardiomyocytes was proved in phase I and II clinical trial (Hare et al. 2009, Chullikana et al. 2015). A number of trials have confirmed that MSCs injection is safe and has the capability to improve cardiac function.

2.2.2 Limitation

The growth factors secreted by MSCs are important because of their therapeutic efficacy (Tang et al. 2005, Gnecchi et al. 2008). However, the amount of growth factors from MSCs are lacking for a therapeutic treatment, and their expression is difficult to control. The secretion of growth factor was different base on the cell state and passage number (Raff 2003, Smith et al. 2007). Therefore, therapeutic doses and control of the cells are still left as challengeable part. These challenges could be overcome by gene editing system and controlled release system.

3. Protein and gene therapy application

3.1 Protein/gene therapy for PAD and CAD

Risk factors for both coronary and peripheral artery diseases are age, diabetes, hyperlipidemia that are associated with the deficiency or reduction of angiogenic growth factors. From the early 1990s, conceptual proof for the therapeutic angiogenesis were provided and supported in preclinical studies with vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF).

Vascular endothelial growth factor (VEGF) is one of the most effective proteins to enhance vascular regeneration (Ferrara et al. 2003, Hoeben et al. 2004, Murray et al. 2017). VEGF-A has been particularly studied in most in preclinical and clinical trials for therapeutic angiogenesis. VEGF-A has 4 isoforms that have been identified with 121, 165, 189 and 206 amino acids. VEGF 121 has no capacity to bind with heparin but VEGF 165 and VEGF 189 has

(Shibuya 2011). The heparin binding capacity provides close attachment to other cells and extracellular matrix. The receptor for VEGF-A is KDR or VEGF receptor-2 that transduce angiogenic signals (Enomoto et al. 2003).

Fibroblast growth factor (FGF) is also one of the most widely studied factor for therapeutic angiogenesis. Basic FGF (FGF2) and acidic FGF (FGF1) are endothelial cell mitogens that stimulate endothelial cell synthesis. FGF is essential for myocardial development and activate Hedgehog signaling to induce VEGF expression and form the coronary vasculature. FGF also control the other growth factors and chemokines such as PDGF, HGF and MCP-1 to mature vessels and arteries (Murakami et al. 2008).

Hepatocyte growth factor (HGF) is a pleiotropic factor that induces motogenesis, mitogenesis, survival and, in some cell types, morphogenesis (Nakamura et al. 1996). HGF is known to stimulate endothelial-cell specific growth via binding to the c-Met receptor on endothelial cells. Although it does not induce vascular smooth muscle-cell proliferation, it accelerates the process of reendothelialization (Nakamura et al. 1996, Hayashi et al. 2000).

3.2 Clinical trial of protein and gene therapy

More than 1000 patient studies published with the different isoforms of VEGF, FGF or HGF proteins or genes. However, the therapeutic effect was not significant. 337 patients participated in FGF initiating RevaScularization Trial (FIRST) and exhibited the

mean change in angina frequency at 90 days but not in 180 days. The exercise tolerances test time also did not change (Simons et al. 2002). In Angiogenic gene therapy trial (AGENT), results also did not exhibit significant improvement for test groups compared with the placebo (Grines et al. 2002). In case of VEGF, the VEGF in Ischemia for Vascular Angiogenesis (VIVA) trial showed no significant improvements at 60 days or 1 year (Henry et al. 2003). No adverse responses were reported in The Kuopio Angiogenesis Trial (KAT) trial of intracoronary infusion of VEGF165 cDNA with 109 patients (Hedman et al. 2003). The concept of therapeutic angiogenesis utilizing protein and gene unfortunately failed in clinical trials. The possible reason of these failures was the problem of target tissue with exogenous growth factors and cardiac muscle that provided pro-angiogenic factors (Masaki et al. 2002).

3.3 Limitation

Angiogenic growth factors have high potential to be a therapeutic reagent with their strong angiogenic effect. However, protein injection and transient expression of plasmid has limitation in sustaining the therapeutic effect. Both protein and gene are easily degraded, and their effects are not stable (Weatherall 1995, Kaufmann et al. 2013). Some proteins have a very transient half-life. In the case of HGF, the level of HGF is very low in normal condition, and restricted to cells of mesenchymal origin (Bottaro et al. 1991). It also has a very short half-life of <3~5 min *in vivo*

(Kawaida et al. 1994). As HGF and VEGF promote cell proliferation, the uncontrolled long-term expression can cause cancer. Therefore, a system that can strictly control the expression and persistent their effect for a long-term is required.

4. Stent application for CAD

4.1 Stent development

To restore blood flow, stents are widely used to maintain the arteries open in the clogged artery for CAD (Faxon et al. 2004, Aziz et al. 2005, Disease et al. 2016). In 1977 the first percutaneous coronary intervention (PCI) in a conscious human was performed by Andreas Gruntzig, using a rudimentary balloon angioplasty catheter. He treated a 38-year-old man who had a high-grade stenosis in the left anterior descending artery (Grüntzig 1978, Meier 2001). However, the balloon angioplasty resulted in the early abrupt vessel closure and resulted in increased rate of restenosis on the treated site. To overcome these limitations, a modified method using metallic stent was invented. In 1986, first human coronary stent implantation was performed. This resulted in a less acute thrombosis and decreased rate of restenosis (Sigwart et al. 1987). Even with the improvements of metallic stent, two significant limitations were recognized. There were late stent failure due to in-stent restenosis (ISR) and stent thrombosis still existed after the stent transplantation (Serruys et al. 1991, Kastrati et al. 1993). Due to the late neointimal hyperplasia, the

development of drug-eluting stent (DES) was promoted. DES decreased stent failure and expanded the opportunity of PCI, even to high-risk patients (Stefanini et al. 2014, Wiebe et al. 2014). However, even the DES was not able to overcome the limitation of late restenosis due to the incomplete endothelialization, along with immune response triggered by the metal (Figure 1). Therefore, variety of stents including the thinner stent and biocompatible polymer stent was developed.

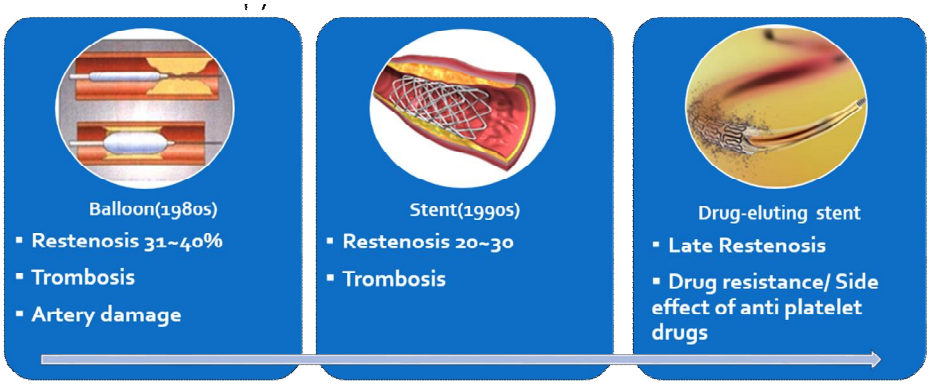


Figure II. The development and limitation of therapy for CAD.

4.2 Reendothelialization for CAD

Even DES stent has limitations including late restenosis and stent thrombosis after stent transplantation. To overcome these problems, rapid or complete endothelial regeneration is necessary. The endothelialization of coronary stents decreased the in-stent restenosis (Padfield et al. 2010, Reejhsinghani et al. 2015). The endothelialization after stent insertion was considered an important factor for preventing thrombosis, and for reducing the proliferation and migration of vascular smooth muscle cells (VSMCs) (Versari et

al. 2007, Tan et al. 2012, Bedair et al. 2017). Therefore, the capability of rapid surface endothelialization may have the potential to be used as the next-therapeutic reagent.

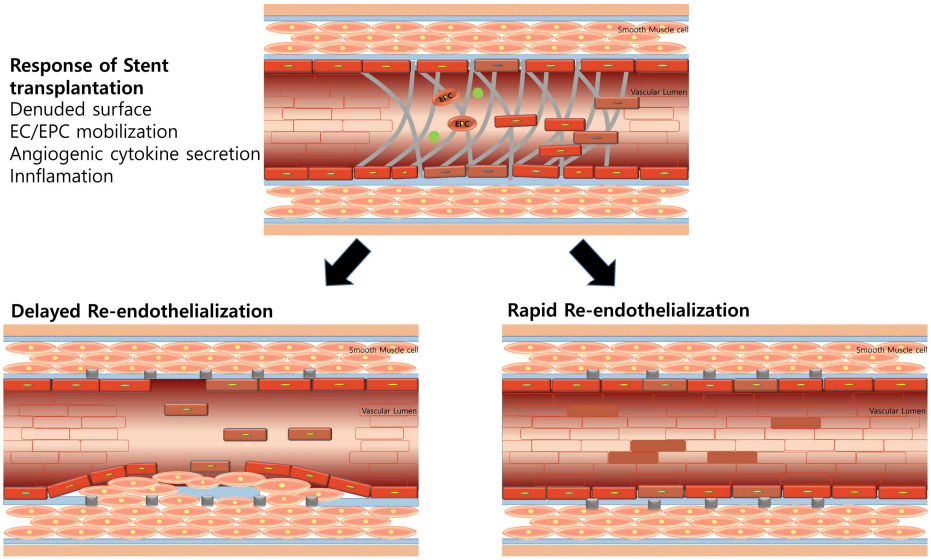


Figure III. The importance of rapid reendothelialization in Percutaneous Coronary Intervention. (Padfield et al. 2010)

5. New strategies and tools for vascular regeneration

To treat vessel disease(s), clinical researchers utilized a method using angiogenic factors or stem cells (Huang et al. 2008, Gu et al. 2017) to encourage collateral blood vessel development in both clinical trials and animal models. However, it was insufficient to examine the increased therapeutic efficacy. The effective strategies to the induce vascular structure that results in significant increase in capillary number and blood flow to ischemic site, are still required. To enhance the therapeutic effects for the application of

clinical trial, combination strategy is necessary. Here, the new tools for vascular regeneration are introduced and elaborated.

5.1 Gene editing tool

The new, stable and controllable, expression system is needed for the proper control of therapeutic genes in vascular regeneration. To resolve the problem of gene expression, genome editing technique was implemented. Genome editing was discovered by targeting DNA double strand breaks (DSBs). DSBs in the DNA are repaired through homology directed repair (HDR) or nonhomologous end joining (NHEJ) (Takata et al. 1998). A homologous sequence substitutes into the broken end site by HDR and subsequently the repair of the break occurs in a template-dependent manner. NHEJ functions repair DSBs without a template through direct re-ligation of the cleaved ends (Lieber et al. 2003). There are 3 major platforms for inducing site-specific DSBs; zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and discovered most recently the CRISPR/Cas system (Maeder et al. 2016).

The zinc finger nuclease (ZFN) technology combined the DNA-binding domain and the cleavage domain of the FokI restriction endonuclease (Li et al. 1992). The FokI DNA-binding domain can be modulated to generate chimeric nucleases with novel binding specificities (Bibikova et al. 2001). ZFN induces DSBs and modifies the genome through either NHEJ or HDR (Smith et al.

2000, Bibikova et al. 2001, Porteus et al. 2003). This first generation of engineering technology has successfully modified genes in human somatic and pluripotent stem cells (Urnov et al. 2005, Hockemeyer et al. 2009, Zou et al. 2009, Sebastiano et al. 2011).

For the next generation of gene editing tool, TALEN was discovered from a simple one-to-one code dictating the DNA-binding specificity of TALE proteins (Boch et al. 2009, Moscou et al. 2009). Unlike the 30 amino acid zinc finger that binds to three bases of DNA, TALENs require 34 amino acids to specify a single base pair. TALEN is an attractive platform with its unlimited targeting range and the ease of engineering new proteins. The limitation of TALEN is delivery of protein *in vivo* because of its large size and repetitive nature of TALE arrays (Zou et al. 2009).

Next, the most recent tool in gene editing part is the utilization of CRISPR-Cas, of which RNA-guided nucleases derived from bacteria's immune system against the malicious invading viruses and plasmids. They consist of CRISPR RNAs (crRNAs), trans-activating crRNAs (tracrRNAs), and CRISPR-associated (Cas) proteins that produce the specificity of DNA cleavage (Horvath et al. 2010, Fineran et al. 2012, Hsu et al. 2014). A protospacer-adjacent motif (PAM), located immediately 3' to the target site, is specific to the species of Cas9 (Kleinstiver et al. 2015). CRISPR/Cas nucleases do not need to engineer novel proteins for each DNA target site. This makes CRISPR/Cas system a highly

attractive tool to introduce a site-specific DSBs. This system can induce DSBs at several loci with multiple gRNAs due to un-direct coupling of Cas9 and gRNA. Therefore, multiple new CRISPR-based gene-editing technologies emerged, including RNA-guided endonuclease Cpf (Zetsche et al. 2015).

5.1 Safe harbor site

The majority of gene therapy applications are based on the addition of gene. To efficiently insert and express transgene can be performed using the viral vectors (Cartier et al. 2009, Papapetrou et al. 2016). However, there are possibility of external genes inserting into a random genomic positions and thus interacting with the host genome. Unexpected interaction of transgene and reciprocal gene may lead to attenuation or complete silencing. Genomic safe harbor sites have been used to minimize the risk of unpredicted interaction with the host genome (Papapetrou et al. 2011). There are 3 sites that have been mostly targeted for the purpose of transgene addition into human genome. (a) The adeno-associated virus site 1 (AAVS1) is a naturally occurring integration site of AAV virus on chromosome19. (b) The chemokine (C-C motif) receptor 5 (CCR5) gene is a HIV-1 co-receptor. (c) The human ortholog of the mouse Rosa 26 locus that is an extensively validated locus in the murine setting for the insertion of ubiquitously expressed transgenes (Kotin et al. 1992, Liu et al. 1996, Irion et al. 2007, Papapetrou et al. 2016). AAVS1 is the most popular site

because of the commercial availability and ability to express in multiple cell type (Emery 2011). These AAVS1, CCR5, and other intragenic regions possess a high possibility for research applications of gene therapy. The ability to manipulate any genomic sequence into safe harbor site has opened diverse opportunities in treating different diseases and disorders.

5.2 The Control System

The ability to control the transgene expression is critically important for the safety of gene therapy. This has resulted in development of several regulatory mechanisms, to ensure quantitative and temporal control of gene expression. We focused on the Tet-On and Tet-Off system that allowed the regulation of an interesting gene by administration or withdrawal of tetracyclines in eukaryotic cells (Baron et al. 2000, Fussenegger 2001). The prototype tetracycline (Tc) and its derivatives such as doxycycline (dox) have been used widely in humans as antibiotic. Tet-on/off system consists of Tet repressor protein (tTA or rtTA) domain, secreted proteins that bind to operator with Tc, and tet operator (tetO) domain that controls the transcription of interest gene. The Tet-On system activates the gene expression by the administration of Tc or Dox, but the Tet-Off system silences the gene expression by Tc or dox (Das et al. 2016). These Escherichia coli derived systems have been significantly improved and applied in multiple animal and human trials. It gives a good tool for controllable

angiogenesis in biological research and gene therapy applications.

5.3 Materials

Various biomaterials emerged to support vascular engineering and increase long-term patency of vascular function. Both the cell seeding scaffolds and bioactive polymers for vessel regeneration yielded promising results. An ECM is a biocompatible and cell-supporting substance that provides cells with mechanical and physiological support, increasing cell survival, adhesion, proliferation, and differentiation (Wozniak et al. 2004, Trappmann et al. 2012). It also traps and sustains growth factors and soluble molecules through proteoglycans, thus are major components of the ECM (Kim et al. 2011).

The synthetic bio-materials, poly (lactide-co-glycolide) (PLGA) and polyethylene glycol (PEG), offer high controllability, specificity to cell-adhesion and incorporation of angiogenic factor that is optimized for angiogenesis (Hern et al. 1998, Turturro et al. 2013). As similar to all materials, safe use of synthetic materials in clinical application still attracts controversy, but synthetic materials allow strict consistency between batches and has low immunogenicity.

These tools are now being utilized in various fields. Many of the methods that showed efficacy at preclinical stage, however showed limited therapeutic efficacy at the clinical stage. Therefore, through the collaboration of new methods, we can overcome the therapeutic hurdle. For example, using a control system and gene editing

methods, we made stable and controllable system that bypassed the limitation of gene / protein therapy. The combined tool can be used to maximize the therapeutic effect. A practical example of this new strategy for angiogenesis and reendothelialization will be explained in the future chapter.

My studies focused on investigating the therapeutic effects of MSCs secreting angiogenic growth factors in a controllable manner. This was achieved by inserting the genes in to a safe harbor site through genome-editing on endothelialization and angiogenesis. The objectives of these studies are to determine (a) the settlement of HGF secreting MSCs via TALEN and its effect of vessel regeneration and (b) the effect of reendothelialization with the HGF and VEGF combined MSCs on coronary stent in swine model.

Chapter I

Inducible HGF-secreting Human Umbilical Cord Blood-Derived MSCs Produced via TALEN-mediated Genome Editing Promoted Angiogenesis

Abstract

MSCs promote therapeutic angiogenesis to cure serious vascular disorders. However, their survival period and cytokine-secretory capacity are limited. Although, Hepatocyte Growth Factor (HGF) can accelerate the rate of angiogenesis, recombinant HGF is limited because of its very short half-life (< 3~5 min). Thus, continuous treatment with HGF is required to obtain an effective therapeutic response. To overcome these limitations, we produced genome-edited MSCs that secreted HGF upon drug-specific induction. The inducible HGF expression cassette was integrated into a safe harbor site in an MSC chromosome using the TALEN system, resulting in the production of TetOn-HGF/hUCB-MSCs. Functional assessment of the TetOn-HGF/hUCB-MSCs showed that they had enhanced mobility upon the induction of HGF expression. Moreover, long-term exposure by Dox treated TetOn-HGF/hUCB-MSCs enhanced the antiapoptotic responses of genome-edited MSCs subjected to oxidative stress and improved the tube formation ability. Furthermore, TetOn-HGF/hUCB-MSCs encapsulated by RGD-alginate microgel induced to express HGF improved *in vivo* angiogenesis in a mouse hindlimb ischemia model. This study showed that the inducible HGF expressing hUCB-MSCs are competent to continuously express and secrete HGF in a controlled manner. Thus, the MSCs that express HGF in an inducible manner are a useful therapeutic modality for the treatment of vascular diseases requiring angiogenesis.

1.1. Introduction

Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) can regenerate organs(Caplan 2007) and enhance angiogenesis(Wu et al. 2007). These cells can differentiate into endothelial and smooth muscle cells that participate in angiogenesis and neovasculogenesis(Lin et al. 2010). Additionally, these cells exhibit a low level of immunogenicity upon allogenic transplantation. These properties make hUCB-MSCs ideal for angiogenesis therapy. Generally, the therapeutic efficacy of these MSCs is due to the paracrine effects of the growth factors and cytokines that they secrete (Tang et al. 2005, Gnecchi et al. 2008). Therefore, growth factor secretion by MSCs is therapeutically important. However, the amounts of growth factors that these cells secrete are often insufficient for a therapeutic effect, and it is difficult to control their levels of expression/secretion to achieve physiologically adequate concentrations. The level of growth factor secretion varies depending on the state of the cells and their passage number (Raff 2003, Smith et al. 2007). Furthermore, the *in vitro* methodologies used to harvest, cultivate, and maintain MSCs so that therapeutic doses of the cells are obtained are challenges that require solution before these cells can be applied in the clinic. These challenges must be overcome and a better approach to stem cell therapies must be developed.

To control the amount of a secreted growth factor in a system, recombinant protein is widely applied. Depending on the specific

concentration of a recombinant growth factor, it has an effect similar to that produced by MSC treatment (Ferrara 2004). However, some growth factors have a short half-life and a very low level of therapeutic efficacy. Hepatocyte growth factor (HGF), which is also known as scatter factor (SF) and has been identified as a superb factor for therapeutic angiogenesis, has a very short half-life of only <3~5 min (Kawaida et al. 1994). Although recombinant HGF showed promise in *in vitro* assays, its *in vivo* utility is negligible due to its short half-life.

HGF, a growth factor that is secreted by MSCs, binds to the c-Met receptor on endothelial cells. HGF not only stimulates endothelial cell growth without inducing vascular smooth muscle cell proliferation but also accelerates reendothelialization while causing a low level of intimal hyperplasia (Nakamura et al. 1996, Hayashi et al. 2000). HGF also prevents the death of endothelial cells through its anti-apoptotic activities (Ponzetto et al. 1994, Morishita et al. 1997, Nakagami et al. 2001). Moreover, HGF is one of the major determinants of whether the epithelium remains in a quiescent state or switches to a proliferative state during development and tissue repair (Kopp 1998). However, the level of HGF in normal liver, kidney and spleen cells is very low, and HGF expression is restricted to cells of mesenchymal origin (Bottaro et al. 1991). Although the endogenous HGF level increases after injury, the level reached is not sufficient for repair due to a very short half –life of <3~5 min *in vivo* (Kawaida et al. 1994). Thus, more new stable and

controllable expression system is required for the improved angiogenic therapy. One of many strategies to resolve HGF problem in clinical field is genome editing technique capable of expressing therapeutic gene into the PPP1R12C site on human chromosome 19 (called the safe harbor site).

With respect to these, we integrated a doxycycline inducible HGF expression system into the safe harbor site of hUCB-MSCs via Transcription activator like effector nucleases (TALEN)-mediated genome editing to allow long term and controllable HGF secretion. Herein, we report that the HGF secreted by these hUCB-MSCs had long term and controllable therapeutic effects on endothelialization and angiogenesis. We also showed that treatment with the genome edited stem cells had an improved therapeutic effect on the mouse hindlimb ischemia model.

1.2. Materials and Methods

Cell cultures

Human umbilical cord blood-mesenchymal stem cells (hUCB-MSCs) isolated from human umbilical cord blood were collected as previously described (Kang et al. 2014). The human UCB-MSC isolation procedure was approved by the Borame Institutional Review Board and Seoul National University (IRB N. 0603/001-002-07C1). The hUCB-MSCs were maintained in Keratinocyte SFM supplemented with human recombinant EGF and bovine

pituitary extract (Gibco, Life Technologies, Grand Island, NY) at 37 ℃ in 5% CO_2. Adipose-derived stem cells (ADSCs) isolated from human adipose tissue were kindly provided by EHL Bio and were maintained in MesenPro medium containing the supplement kit reagents (Gibco, L.T.) under the same conditions. HEK293T cells, a human embryonic kidney cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, GE, South Logan, Utah) supplemented with 10% fetal bovine serum (FBS) (HyClone, GE) and penicillin/streptomycin (Gibco, L.T.).

Preparation of a plasmid containing human HGF cDNA and the Tet-On system

To clone the human HGF cDNA into a specific homologous recombination vector for inducible expression, primers that overlapped the end of each DNA fragment were designed, and PCR was performed. We first separately cloned TetOn/CMVmin promoter, HGF-pA and hEF1a-rtTA-pA constructs into a pGEM vector. Then, these 3 DNA fragments were PCR-amplified and were ligated into the basic plasmid pGEM using an In-fusion kit (Clontech Lab Inc., Central America, USA) (Fig 1.1. A). Then, the human HGF (hHGF) cDNA and Tet-On construct were transferred into the pUC19-AAVS1 vector, which contains two homologous recombination sites (HA-L and HA-R) for targeting AAVS1 on

human chromosome 19. In this plasmid, the expression of hrHGF cDNA is regulated by the Tet-on/minimal promoter, which is activated by the tetracycline-rtTA protein that is expressed via the EF1α promoter (Fig 1.1. A). Correct ligation into the vector was confirmed using restriction mapping (using NotI and AgeI) and colony PCR (Fig 1.2. A). The final plasmid was also evaluated by DNA sequencing and was found to be correct (Fig 1.2. B). The purified pUC19-TetOn-HGF plasmid was transfected into HEK 293T cells using Lipofectamine. The expression of HGF was confirmed by Western blotting using an antiHGF antibody (ab83760, Abcam, Cambridge, UK) after TCA based precipitation of the proteins in the medium conditioned by the transfected cells.

Western blotting and ELISA

After the HGF expression plasmid was transfected into cells using a Neon electroporation device (Invitrogen, Carlsbad CA, USA), $2x10^5$ transfected cells/well were seeded in a 6 well culture dish (Thermo-Nunc, Jiangsu, China). The cells were treated with doxycycline (at 5 μg/ml) for 2 days at 37 ℃. Then, the cells in each well were washed with PBS and were lysed using 100 μl of lysis buffer (RIPA buffer, Thermo Fisher) containing a protease inhibitor cocktail (PIC, Roche). The cells were collected using a scraper, and the cell lysates were incubated on ice for 20 min. The cell extracts were centrifuged for 10 min at 13,000 rpm to remove debris. The supernatants were collected, and the protein concentrations were

determined using a Bradford assay kit (Bio-Rad). To produce conditioned media, the cells were incubated in serum free media for 48 hrs under different conditions. Then, the proteins in the conditioned media were precipitated using TCA, as previously described. Western blotting assays were performed as previously described (Ahn et al. 2014). Briefly, 20 μg of each protein sample were separated in a 10% gel by SDS-PAGE. After blocking the blot for 1 hr using 5% skim milk, the blot was incubated with an anti-human HGF antibody for 16 hrs at 4 ℃ and then was incubated with an anti-rabbit secondary antibody for 1 hr at RT. Then, the labeled bands were detected using ECL+ reagents (Thermo Fisher). For the ELISA assay, the media were filtered using a 30 K cut-off filter (Millipore, MA, USA) and the proteins were assayed using an anti-human HGF ELISA kit (ab100534, Abcam), as previously described (Nishida et al. 2013).

Junction PCR

To confirm that the hHGF expression cassette was integrated into the AAVS1 on chromosome 19, a forward primer (5'-ACTAAGTAAGGATCCA GACATGATAAGA-3') was designed to detect the hHGF portion of the cassette and a reverse primer (5'-CCCACCCCAATGCTCCAGGC-3') was designed to detect part of the PPPR12C genomic locus. PCR was performed using LA taq polymerase (TaKaRa) according to the following protocol: one cycle of 92 ℃ for 3 min, then 35 cycles of 92 ℃ for 1 min 30 s,

60.7 ℃ for 3 min, and 72 ℃ for 2 min, and a final cycle of 72 ℃ for 3 min. The PCR product was evaluated using a 1% agarose gel. The PCR product was also sequenced using the primers described above.

Cell migration assay

For the wound healing/cell migration assay, TetOn-HGF/hUCB-MSCs (5 x 10^4 cells) were seeded in 24-well plates. The cells were cultured until reaching confluence and then were starved for 24 hrss. A linear wound was created in each monolayer using a pipette tip. Cell motility in terms of wound closure was evaluated by photographing three random fields 24 hrs after wounding the monolayer. For the trans-well migration assay, the bottom of the upper chamber of the trans-well was coated with 0.2% gelatin (#8422-8 ㎛, Corning). Each group of cells were starved for 16hrs, then same number of the cells (2.5×10^4) were resuspended in each conditioned medium, and then were seeded in the upper chamber. The plate was then incubated in 5% CO_2 at 37 ℃ for 24 hrs. Then, the cells on the membrane were stained with 0.1% crystal violet. The migration rate was determined by counting the number of migrated cells in three random fields under a light microscope.

Cell viability assay

TetOn-HGF/hUCB-MSC cells were seeded in 48 well plates at 1×10^4 cells/well. The MTT assay (Sigma) was used to determine the relative rate of cell growth at 1, 3, 5 and 7 days of cultivation.

MTT reagent (100 μl of a 0.2 mg/ml solution) was added to the media and the plates were incubated for 5 hrs at 37 ℃. After removing the culture media, the crystals remaining were dissolved in 500 μl of DMSO and the absorbance at 470 nm was measured.

Antiapoptosis and antioxidative stress assay

After TetOn-HGF/hUCB-MSC cells ($5x10^5$) had been grown under each condition (with Dox or without Dox), they were seeded in 60 ø culture plates (Thermo Fisher) and were treated with STC (30 nM) for 24 hrs or with H2O2 (150 mM) for 3 hrs. Then, the cell morphology was observed under a light microscope (Juli, NanoEndTech, Seoul, Korea) and the cell viability rate was measured using the MTT assay.

Tube formation

Human umbilical vein endothelial cells (HUVECs, 5×10^4 cells per well) were seeded on a layer of BD Matrigel (BD Biosciences, San Jose, CA) in 24 well plates. The cells were then exposed to VEGF at 50 ng/ml (R&D) and conditioned media that had been collected from hUCB-MSCs, TetOn-HGF/hUCB-MSCs that were grown with Dox and without Dox (2×10^6 cells per 100 ø dish) in high-glucose DMEM (Thermo Fisher) containing 5% FBS (Thermo) and antibiotics/antimycotics (Gibco) for 48 hrs. DMEM media containing 5% FBS was used as a control. The protein concentrations in the conditioned media were enriched 10-fold using a 30 K cut-off

filter, and the filtrates were added to HUVECs, after which the cells were incubated for 12 hrs to allow them to form tube-like structures. Tube formation was analyzed by counting the number of branches per high power field.

Cell microgel transplantation in the hindlimb Ischemia Model

RGD-alginate/cell microgels were prepared as described in our previous publication(Kim et al. 2014). To assess the level of HGF secretion by the cells in the microgels, media conditioned by different cells were collected after 48 hrs of incubation in high glucose DMEM containing 10% FBS. Then, the HGF levels in the conditioned media were determined using a Western blotting assay and an ELISA. The shapes of the cells in the microgels were examined using a JuLI microscope (NanoEnTek).

The hindlimb ischemia model was generated as described in our previous publication(Kim et al. 2014). One week after surgery, the mice were injected with either PBS, hUCB-MSCs only, human recombinant HGF (2 μg), a microgel containing hUCB-MSCs (2×10^7 cells in 1 mL of RGD-alginate per mouse), a microgel containing TetOn-HGF/hUCB-MSCs or a microgel containing TetOn-HGF/hUCB-MSCs treated with Dox. Mircogels containing cells were injected into three or four gracilis muscles at the medial thigh in the ischemic limbs. About 0.5 ml of mixture were injected into the gracilis muscles using insulin syringes. The blood perfusion of the hindlimb before occlusion and the complete lack of blood

perfusion immediately after occlusion were evaluated using a Laser-Doppler Flowmeter (Moor LDI, Moor Instruments Ltd., Devon, UK); the perfusion of both hindlimbs was then evaluated weekly for 4 weeks. The data were analyzed using Moor LDI™ PC software. All of the animal studies were performed according to the Seoul National University Animal Care Committee guidelines after acquiring permission from the committee (Protocol #SNU-1).

Immunohistochemistry

Tissues were harvested, fixed in 4% paraformaldehyde (Wako), embedded in paraffin, and cut into 5 μm sections (Leica, Buffalo Grove, IL). IHC was performed using an anti-vonWillebrand factor (vWF) antibody at a dilution of 1:100 (Merck Millipore, Billerica, MA) and the appropriate secondary antibody. Sections were also stained with hematoxylin and eosin (H&E). The number of blood vessels were counted as previously described(Ahn et al. 2014, Kang et al. 2014). The data presented are the mean values ± s.e. of three hindlimbs per group.

Statistical analysis

The data were expressed as the mean values ± standard error. The data were analyzed using one-way ANOVA and t-test (Prism5, CA, USA) to compare the data pertaining to the different experimental groups. Statistical significance is indicated in the figure legends.

1.3. Results

Generation of the inducible TetOn-HGF-expression construct and induction of HGF expression in hUCB-MSCs

For consistent delivery of HGF *in vivo*, we designed a human HGF cDNA construct for integration into human stem cells. However, because consistent HGF-Met signaling is known to trigger tumor growth (Gao et al. 2005), the level of HGF expression must be controlled. Thus, we created a construct in which HGF expression was under the control of a Tet-On inducible system. In this system, tetracycline/doxycycline (Dox) treatment activated the expression of the target HGF cDNA. After cloning the HGF cDNA, we tested whether HGF expression was controlled by Dox.

We first cloned the inducible HGF-expression construct into the interim pGEM vector, resulting in the production of pGEM-TetOn/CMVm-HGF-EF1a-rtTA, as explained in the Materials and Methods section (Fig 1.1. A). This plasmid was transiently transfected into 293T cells, and their expression of HGF via this vector was evaluated by Western blotting (Fig 1.1. B). HGF expression was strongly induced by Dox treatment. The expression level was similar to that of a positive control in which HGF expression was under the control of the CMV promoter in the pcDNA3.1 vector. The level of HGF expression was dependent on the Dox concentration at lower concentrations below 5 μg/ml (Fig 1.1. C). The level of expression was decreased when the Dox dosage was greater than 10 μg/ml (Fig 1.5. C), most likely due to

cellular damage caused by the toxic effect of Dox because we observed cell death above that concentration. Indeed, it has been reported that 10 μg/ml of Dox suppresses cell proliferation(Fife et al. 1997). Based on our results and the cited report, we decided that ideal Dox dose would be 5 μg/ml to maximize HGF expression and minimize the side effects of Dox treatment.

To safely integrate the inducible TetOn-HGF-expression construct into human stem cells, we then cloned this construct into a pUC19 vector that has two homologous recombination arms (HA-L & HA-R) for targeting the safe harbor PPPR12C site on chromosome 19. (Fig 1.1. D). The pTetOn-HGF plasmid was evaluated by restriction mapping, colony PCR and DNA sequencing (Fig 1.2. A & B). The induction of HGF expression via the pUC-TetOn-HGF vector and the secretion of HGF were confirmed by WB analysis of adipose-derived stem cells (ADSCs) and the medium conditioned by these ADSCs, respectively (Fig 1.1. E), and of transfected human umbilical cord blood-MSCs (hUCB-MSCs) (Fig 1.1. F) treated with Dox. Transfected hUCB-MSCs secreted more HGF than transfected ADSCs. When we tested the transfection efficiency of these two cell types using a GFP-expression plasmid, hUCB-MSCs were found to be transfected at greater than 50% efficiency, whereas ADSCs were transfected at approximately 10% efficiency (Fig 1.3. A & B). The high transfection efficiency of hUCB-MSCs will be beneficial for later genome editing. It is also known that hCB-MSCs have a low level of

immunogenicity. For these reasons, we mainly used hUCB-MSCs for the subsequent studies that we performed. Taken together, the results obtained at this stage showed that HGF expression and secretion could be controlled by Dox via the pUC19 Tet-On system in which rtTA expression was driven by the EF1α promoter.

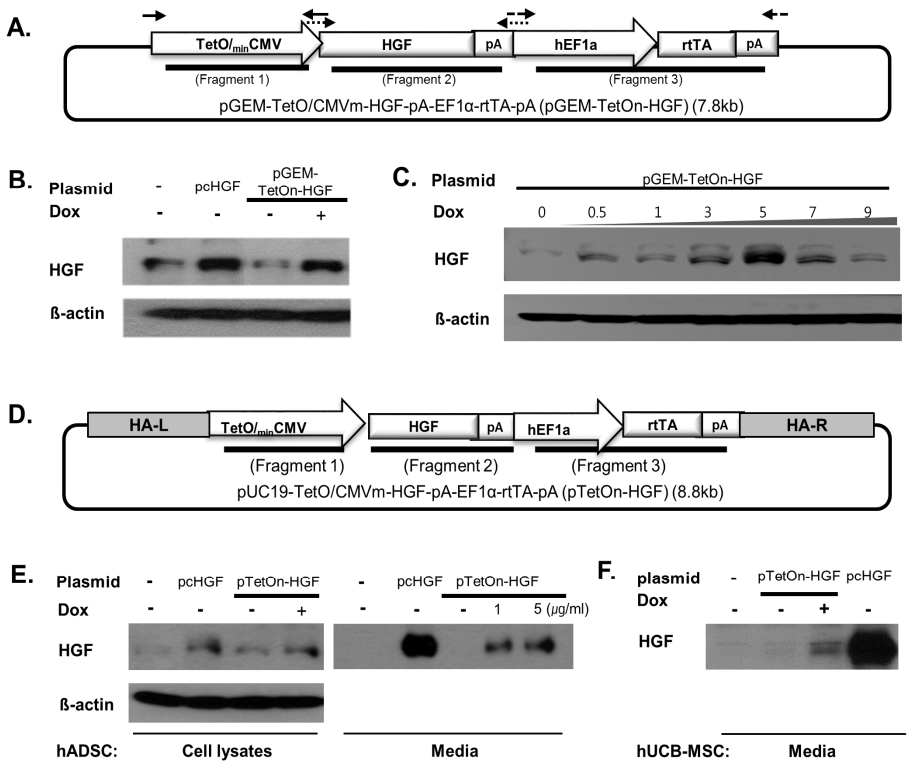


Figure 1.1. Transient expression of HGF was controlled by the Dox concentration. (A) Diagram of the inducible HGF-expression system. The In-fusion system was used to combine the Tet-On and HGF systems in the vector. (B) WB analysis of HGF expression by 293T cells at 48 hrs post-Dox treatment. (C) The level of HGF expression by 293T cells was dependent on the Dox concentration. (D) Diagram of the vector constructed for integration into the

PPPR12C site of chromosome 19 using the In-fusion system. **(E)** WB analysis of HGF expression via the integration vector in ADSCs cultured for 48 hrs and the level of HGF in the medium that they conditioned as well as that **(F)** in the hUCB-MSC conditioned medium.

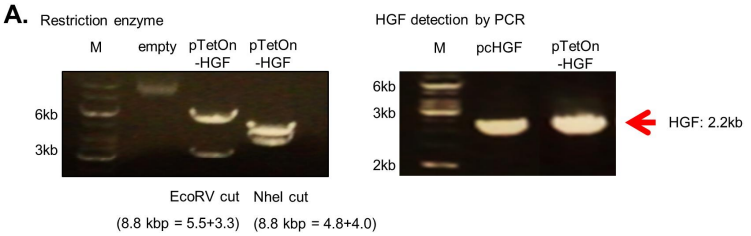


Figure 1.2. HGF vector construction. **(A)** The production of the final vector was confirmed by restriction mapping using EcoRV and NheI. Colony PCR was also performed using HGF primers, and the size of the product was 2.2 kb. **(B)** The results of sequencing the final vector.

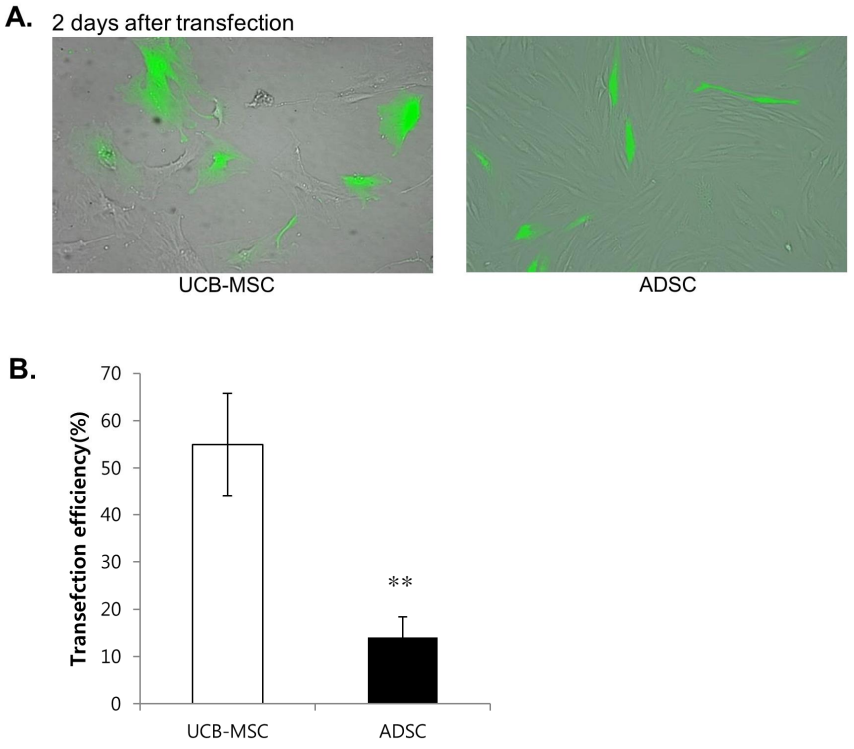


Figure 1.3. Comparison of the transfection efficiency of ADSCs and hUCB-MSCs. (A) Images of hUCB-MSCs and ADSCs at 2 days after transfection with a GFP-expression vector. (B) Transfection efficiencies (** P <0.01).

TALEN-mediated generation of hUCB-MSC MSCs with a safe-harbored inducible HGF expression system

For the consistent and safe expression of HGF, the inducible pUC19-TetOn-HGF expression cassette was integrated into the safe-harbor PPPR12C site on chromosome 19 via TALEN-mediated genome editing (Fig 1.4. A). The pUC19 vector has two arms that are coordinated with the TALEN-L/R sequences for homologous recombination. The original TALEN-L/R sequences and the commercially available HA-L/R sequences did not result in efficient gene integration. Thus, we designed several different TALEN-L/R and HA-L/R sequences and selected very effective (greater than 10 fold increase in efficiency compared with those of the original L/R sequences) sequences that had 50 bp spacers between the HA-L/R sequences of each TALEN-L/R sequences (Cho et al, manuscript submitted). With the newly designed TALEN system and new HA-L/R sequences in the pUC19 vector, we produced potent HGF-secreting hUCB-MSCs. Ten days after co-transfecting hUCB-MSCs with the TALEN and TetOn-HGF systems, they were treated with Dox for two days. Integration of the pTetOn-HGF-expression cassette into the safe harbor site was confirmed using a junction-PCR assay (Fig 1.4. B) and by sequencing the PCR product. The results showed the expression cassette and the rtTA construct had been integrated correctly into the PPP1R12C site (Fig 1.5.). HGF secretion was also demonstrated 12 days after co-transfection when the transiently

present genes had disappeared (Fig 1.4. C). ELISA analysis showed the concentration of secreted HGF was approximately 15 times higher in medium conditioned by Dox-treated HGF/hUCB-MSC cells than in that conditioned by non-Dox-treated controls (Fig 1.4 D). After generation of TetOn-HGF/hUCB-MSC, MSC was characterized with stem cell markers, to test it still has stemness property with or without dox (Fig 1.6.). Integration of the expression system in human ADSCs was also observed 12 days after their transfection (Fig 1.5. B). The level of HGF secretion by hADSCs was also optimal when they were treated with 5-7 μg/ml of Dox (Fig 1.5. C). The results showed that using the newly designed TALEN system and new HA-L/R sequences in the pUC19 vector, we produced potent HGF-secreting hUCB-MSCs.

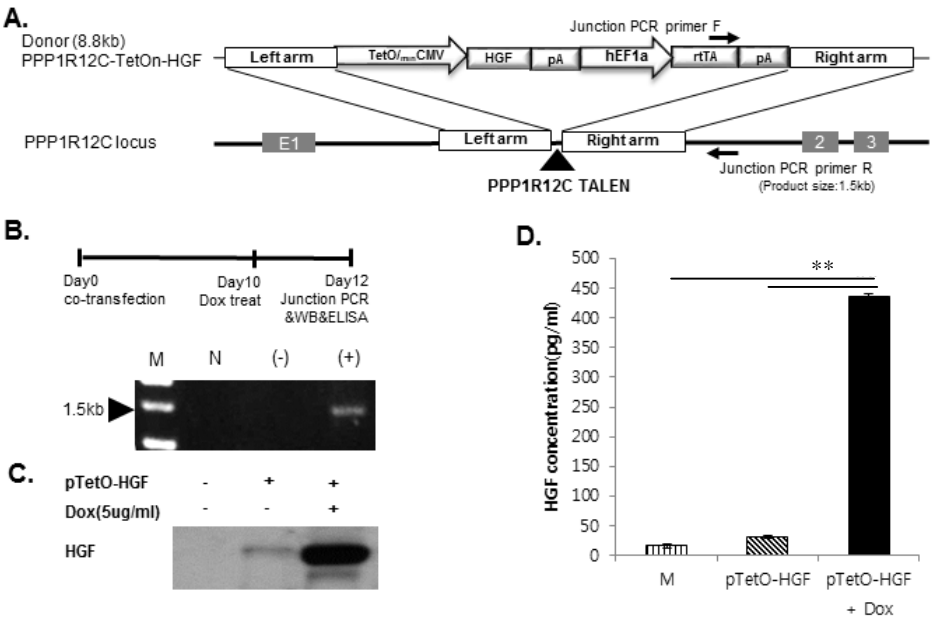


Figure 1.4. Integration of the inducible HGF-expression cassette into the safe harbor site of an MSC chromosome via a TALEN system. (A) Schematic representation of integration into the PPP1R12C site of chromosome 19 via the junction PCR primers. (B) Schematic showing junction-PCR sample preparation and the results obtained by PCR of hUCB-MSCs. M: marker, N: negative control, hUCB-MSC only genomic DNA, -: GFP-gene transfected hUCB-MSCs, (+): pTetOn-HGF vector-transfected hUCB-MSCs. (C) WB analysis of HGF expression at 12 days post-transfection of cells grown with and without Dox at 5 μg/ml. (D) Results of the ELISA-based analysis (**P <0.01).

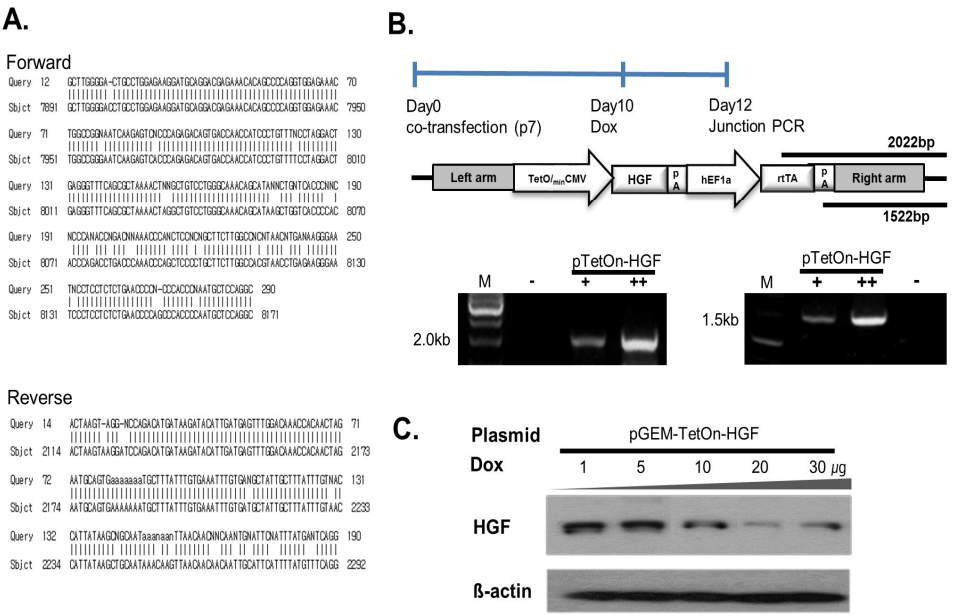


Figure 1.5. The integration of the HGF-expression system was confirmed by sequencing junction-PCR fragments and WB analysis of the expression and secretion of HGF by ADSCs. (A) Sequences of the junction-PCR fragments of the beginning and end of the integrated system. (B) Schematic showing junction-PCR sample preparation and the results obtained by PCR of ADSCs. M: marker, -: GFP vector-transfected ADSCs, (+): pTetOn-HGF vector-transfected ADSCs treated with 5 μg/ml of Dox, (++): pTetOn-HGF vector-transfected ADSCs treated with 10 μg/ml of Dox. The sizes of the PCR products were 2 kb and 1.5 kb. (C) WB analysis of the HGF level 10 days after transfection.

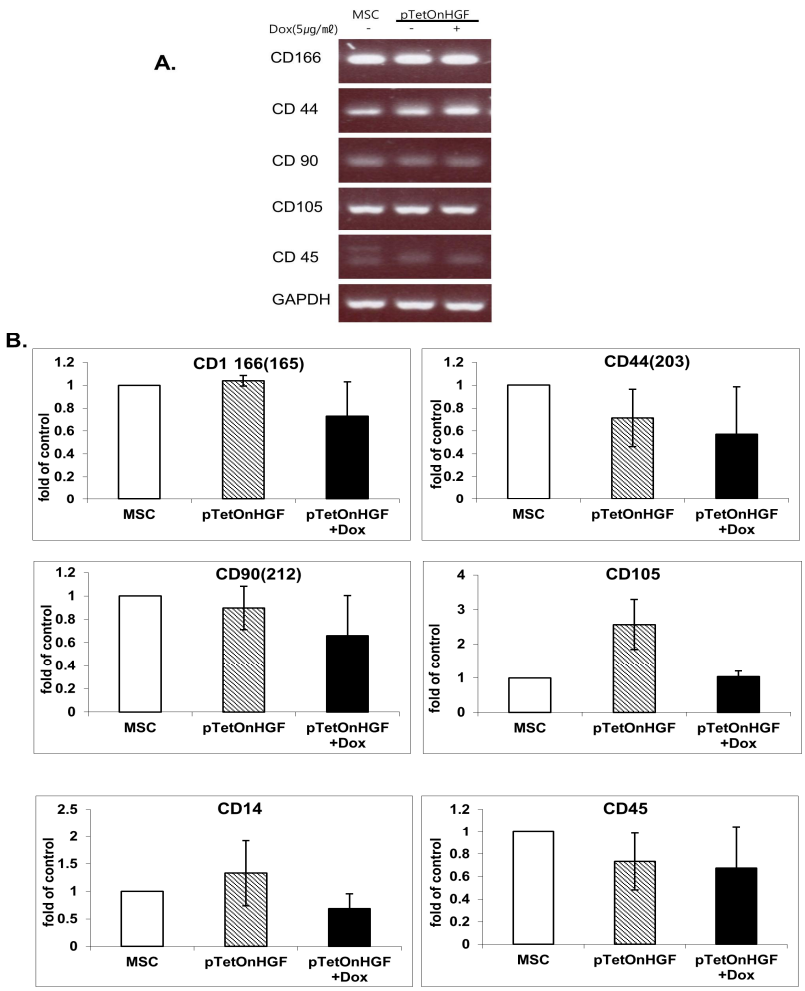


Figure 1.6. The expression of hUCB-MSC makers after transfection and doxycycline treatment. (A) Conventional RT-PCRs were done for the MSC positive markers, CD166, CD44, CD90 and CD105, and MSC negative marker CD45 on the RNAs from hUCB-MSC, pTetOn-HGF/hUCB-MSC and pTetOn-HGF/hUCB-MSC with Dox 5 μg/ml. (B) Quantitative real-time RT-PCR was done also for the MSC positive markers for the same samples. There were no significant differences among three cell groups, suggesting that genome-edited TetOn-HGF/hUCB-MSC maintained the stemness property after the transfection and doxycycline treatment.

HGF secreted by HGF/hUCB-MSCs promoted cell migration

The HGF/Met pathway plays an important role in vascular remodeling after tissue damage (Gallo et al. 2014, Gallo et al. 2015). We evaluated the migration-enhancing effect of HGF on HGF/hUCB-MSCs, which is one of its most noted effects (Naldini et al. 1991). Stem-cell migration is crucial for tissue regeneration (Nagai et al. 2005, Matsumoto et al. 2014). In the wound-healing assay, hUCB-MSCs induced to express HGF by Dox treatment migrated at a significantly higher rate than did untreated controls, as did hUCB-MSCs treated with recombinant human HGF (at 50 ng/ml) (Fig 1.7. A). The results of the trans-well cell-migration assay also showed that hUCB-MSCs induced to express HGF by Dox treatment migrated at a significantly higher rate than did their untreated counterparts (Fig 1.7. B). These results were expected because HGF, which is also known as scatter factor, causes cells to migrate. When we counted the cells one and two day after seeding, the cell number in TetOn-HGF/hUCB-MSCs (Dox+) group was not significantly different from the TetOn-HGF/hUCB-MSCs (Dox-) and hUCB-MSCs control groups (data not shown), suggesting that the cell migration was not due to cell proliferation in short-term culture. Thus, our results showed that HGF secreting hUCB-MSCs had an increased migration (scattering effect).

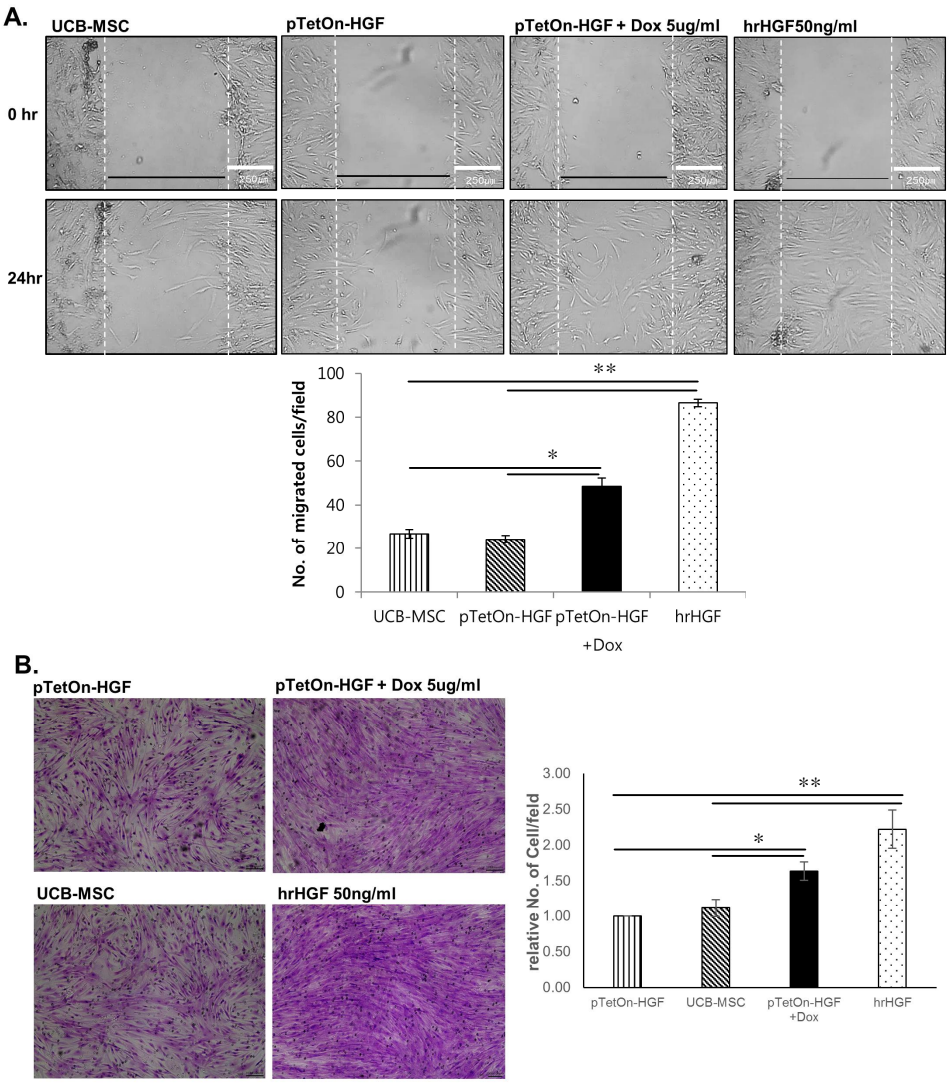


Figure 1.7. Functional effects of conditionally expressed HGF. (A) Wound healing at 24 hrs after scratching monolayers of hUCB-MSCs, pTetOn-HGF-transfected hUCB-MSCs grown with and without Dox or treated with human recombinant HGF at 50 ng/ml. The graph shows the number of cells within the area between the lines ($*P < 0.05$, $**P < 0.01$). (B) Trans-well migration analysis at 24 hrs after treatment with Dox or hrHGF. The graph shows the relative number of stained cells per field ($*P < 0.05$, $**P < 0.01$).

Long-term biological effects of HGF secreted by HGF/hUCB-MSCs

Although TetOn-HGF/hUCB-MSCs (Dox+) did not show changes in their short-term proliferation rate, long-term cultures of these cells showed a high rate of cell viability even at late passages (Fig 1.8. A & B). The viability rate of TetOn-HGF/hUCB-MSCs (Dox+) group was slightly higher than that of the hrHGF-treated group. Previous studies showed that HGF has anti-apoptotic and anti-oxidative activities(Gallo et al. 2014). To test whether TetOn-HGF/hUCB-MSCs (Dox+) were more resistant to apoptosis under pro-apoptotic conditions, TetOn-HGF/hUCB-MSCs were treated with staurosporine (STS), a pro-apoptosis reagent, or with H_2O_2, an oxidative reagent, at two-day intervals for 10 days. TetOn-HGF/hUCB-MSCs (Dox+) treated with STS had a better viability rate than that of STS-treated control cells (Fig 1.8. C & D), suggesting that HGF secreted by the former cells protected them against apoptosis. A similar protective effect was also observed in TetOn-HGF/hUCB-MSCs (Dox+) treated with 150 μM H_2O_2 (Fig 1.8. E & F), indicating that long-term HGF treatment had an anti-oxidative effect. This cell-protective effect was not affected by Dox itself because the morphology and viability rate of hUCB-MSCs treated or not treated with Dox did not differ (Fig 1.9. A & B). These data showed that the long-term secretion of HGF by TetOn-HGF/hUCB-MSCs (Dox+) enhanced their survival of pro-apoptotic and oxidative-stress conditions.

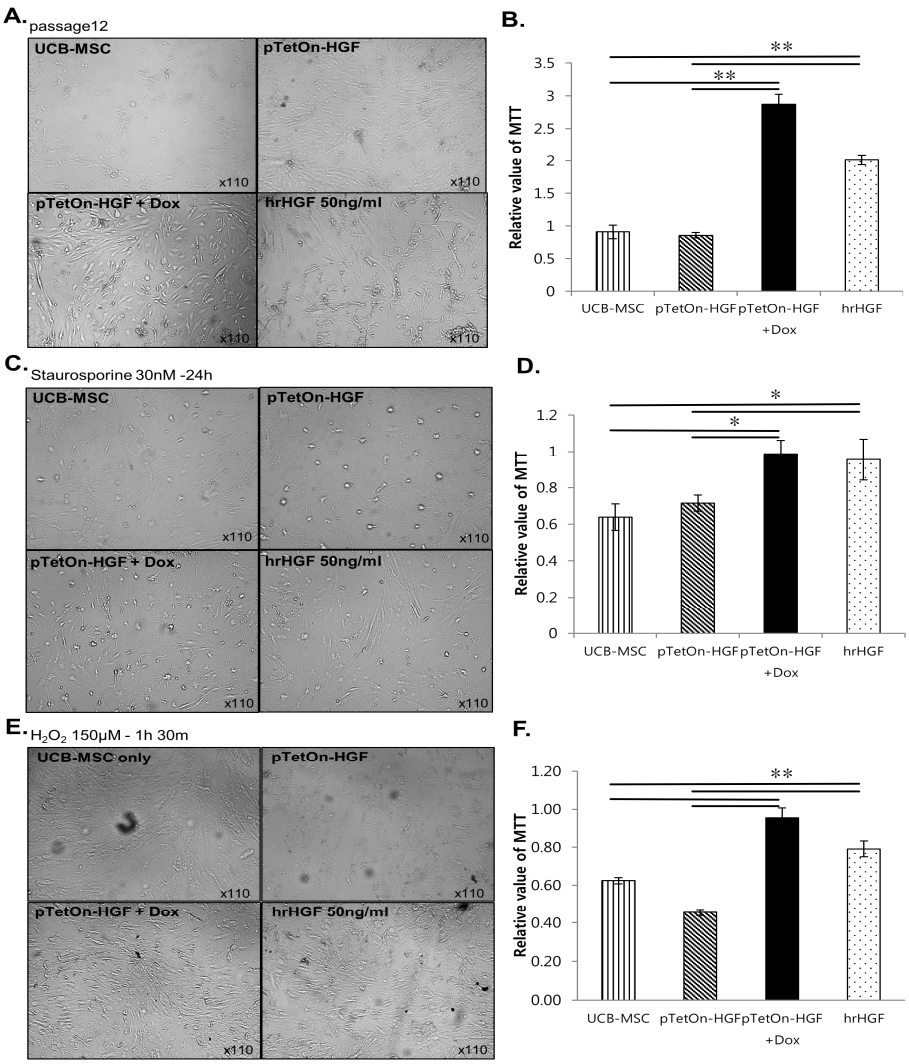


Figure 1.8. Biological effects of the long-term secretion of HGF by engineered MSCs. (A) Images demonstrating the long-term natural anti-senescence effect of the HGF secreted by passage-12 cells that were not exposed to Dox or were exposed to Dox at 5 μg/ml for approximately 10 days. (B) Viability analysis of the passage-12 cells. (C) Images of cells treated with STS at 30 nM for 24 hrs. (D) MT-based viability analysis of STS-treated cells. (E) Images of cells treated with H_2O_2 at 150 μM for 1hr 30min. (F) MT-based viability analysis of the H_2O_2-treated cells (* $P < 0.05$, ** $P < 0.01$).

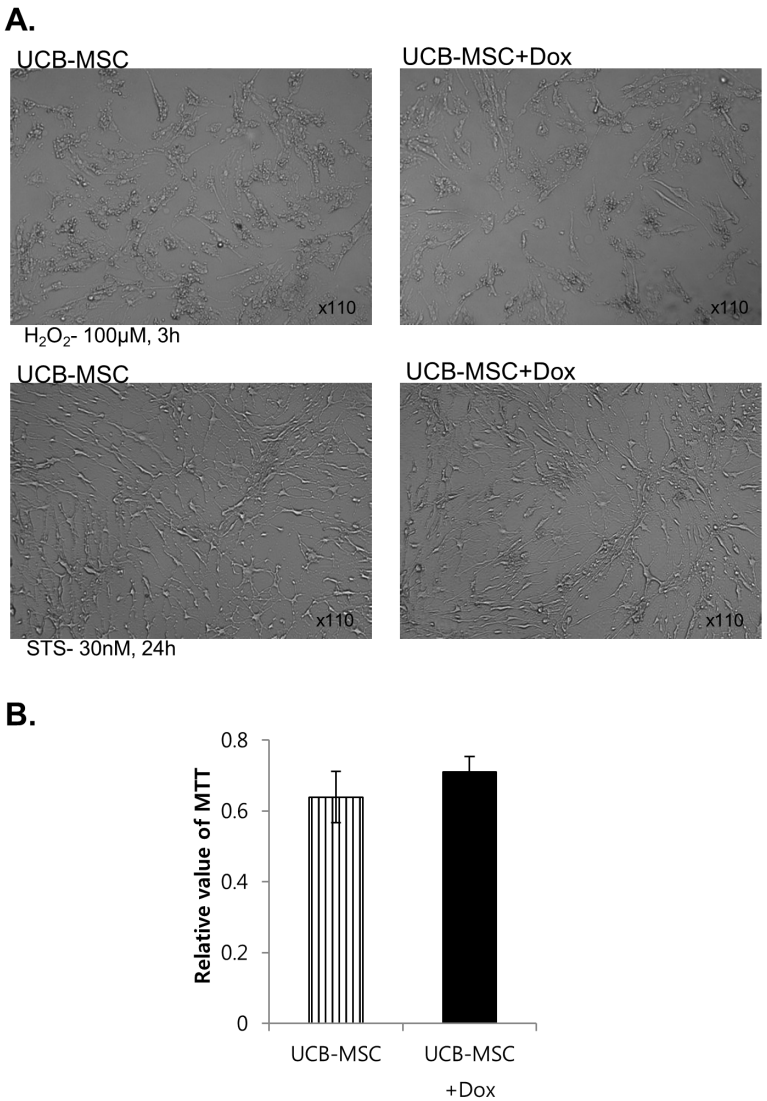


Figure 1.9. Doxycycline treatment did not affect cell proliferation. (A) Images of cells treated with Dox at 5 μg/ml for 48 hrs and then treated with H_2O_2 or STS. (B) MTT-based viability analysis of the STS treated cells.

Angiogenesis is enhanced by medium conditioned by TetOn-HGF/hUCB-MSCs (Dox+)

HGF is an essential factor for reendothelialization without vascular smooth-muscle cell hyperplasia(Hayashi et al. 2000). To evaluate the pro-angiogenesis effect of medium conditioned by TetOn-HGF/hUCB-MSCs (Dox-), a tube formation assay was performed. Media conditioned by cells grown under different conditions were collected and were used for a tube formation assay of HUVECs grown with a minimal concentration of VEGF in medium. Conditioned by hUCB-MSCs or by TetOn-HGF/hUCB-MSCs (Dox-) demonstrated a weak tube-formation capacity.

However, their tube formation was dramatically enhanced by medium conditioned by HGF-secreting TetOn-HGF/hUCB-MSCs (Dox+) and medium to which hrHGF was added (Fig 1.10. A & B). The effect of the former medium was comparable with that of medium containing 50 ng/ml of human recombinant HGF.

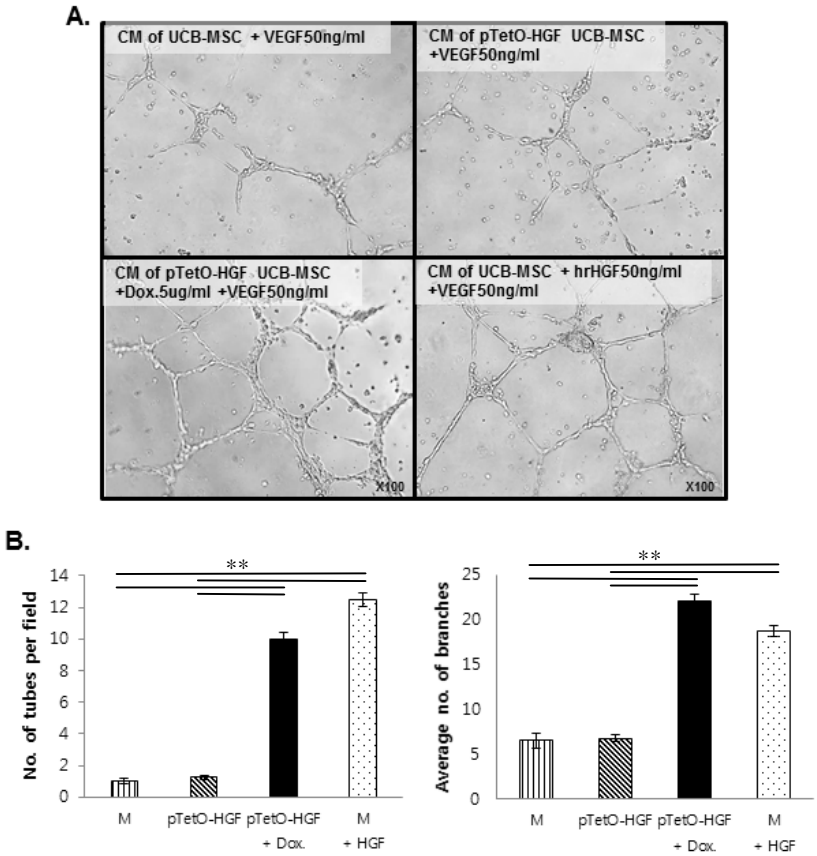


Figure 1.10. Secreted HGF enhanced angiogenesis. (A) Images of HUVECs subjected to the matrigel assay. HUVECs were seeded at $5x10^4$ cells per well and 200 μl of matrigel was used per well. After 48 h of cultivation in DMEM containing 50 ng/ml of VEGF and 5% FBS, the conditioned media were collected. The media were conditioned by only MSCs, by HGF−transfected MSCs or by $1x10^6$ HGF−transfected MSCs treated with Dox at 5 μg/ml. After the CMs were filtered using a 30 K filter, HUVEC were incubated in each CM for 12 hrs. **(B)** Analysis of endothelial tube formation according to the numbers of tubes and branches present (**$P < 0.01$).

TetOn-HGF/hUCB-MSCs (Dox+) enhanced blood flow in the mouse hindlimb ischemia model

HGF secreted by TetOn-HGF/hUCB-MSCs (Dox+) enhanced angiogenesis *in vitro*. To test the effect of TetOn-HGF/hUCB-MSCs (Dox+) *in vivo*, we used the mouse hindlimb ischemia model. One of the hurdles that must be overcome when applying stem cell treatments to *in vivo* animal models is the loss of the injected cells. Before modified stem cells can have an effect, they can be lost due to an immune response or a biological activity. For this reason, we encapsulated the engineered cells in a bio-degradable RGD-alginate material. In a previous study, encapsulating cells in RGD-alginate microgels not only decreased the rate of cell loss but also increased the survival rate of the cells and the period over which the growth factor was secreted into the tissues surrounding the injection site(Kim et al. 2014). Before and after microgel formation, the level of HGF secretion by TetOn-HGF/hUCB-MSCs (Dox+) was determined using a Western blotting assay and an ELISA (Fig 1.11. A, C & D). The concentration of HGF secreted by TetOn-HGF/hUCB-MSCs (Dox+) was slightly but not significantly changed after bead formation.

The blood flow in both hindlimbs of the model mice was measured before and after inducing ischemia in one hindlimb. One week after inducing hindlimb ischemia, the mice were treated with PBS, hUCB-MSC, hrHGF, an RGD-alginate microgel containing hUCB-MSCs, an RGD-alginate microgel containing TetOn-HGF/hUCB-MSCs

(Dox−) or an RGD−alginate microgel containing TetOn−HGF/hUCB−MSCs (Dox+). The blood perfusion rates were measured using a Doppler flowmeter weekly for 4 weeks. As shown in Fig 1.12. A, the blood flow to the affected hindlimb was greatly decreased after surgery had been performed. At the early post−surgery point, the non−microgel−treated groups (treated with MSCs or hrHGF) showed increased blood flow that gradually decreased by the later weeks post−surgery. In contrast, the microgel bead−treated groups showed a consistently increased level of blood flow in the ischemic limb. The TetOn−HGF/hUCB−MSCs (Dox+)−treated group showed a high level of continuous improvement in blood flow during the entire experimental period (Figs 1.12. A & B). As expected, despite treatment with a high concentration of hrHGF (2 μg), the blood flow in the ischemic limb was not restored in the hrHGF group and this limb was lost within one week post−surgery (Fig 1.12. A & B). The greatly improved effect of TetOn−HGF/hUCB−MSCs (Dox+) could be attributed not only to the proangiogenic paracrine effect of MSCs but also to their constant secretion of HGF.

Enhanced angiogenesis was also observed under the skin of the hindlimb ischemic models treated with TetOn−HGF/hUCB−MSCs (Dox+) encapsulated in microgels (Data not shown). Using H&E staining and IHC analysis of vWF expression, we demonstrated an increase in the number of capillaries and microvessels in the group treated with microgel−encapsulated TetOn−HGF/hUCB−MSCs

(Dox+) but not in the groups treated with non-encapsulated cells (Fig 1.12. C). The thickness of the endothelial cells in the former group was also increased. These data showed that TetOn-HGF/hUCB-MSCs (Dox+) encapsulated in an RGD-alginate microgel significantly improved the level of *in vivo* angiogenesis through the combined effects of the hUCB-MSCs and the HGF secreted by these cells.

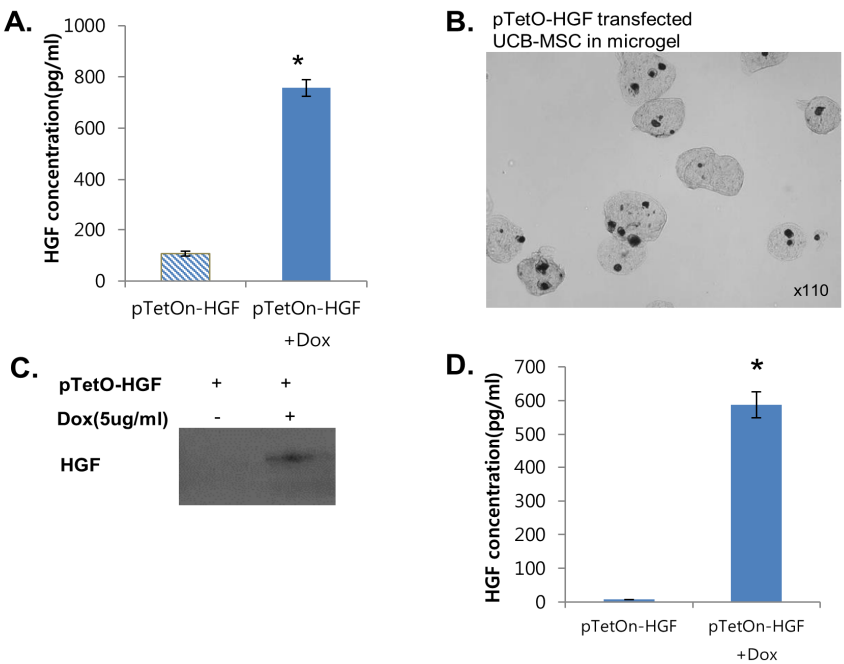
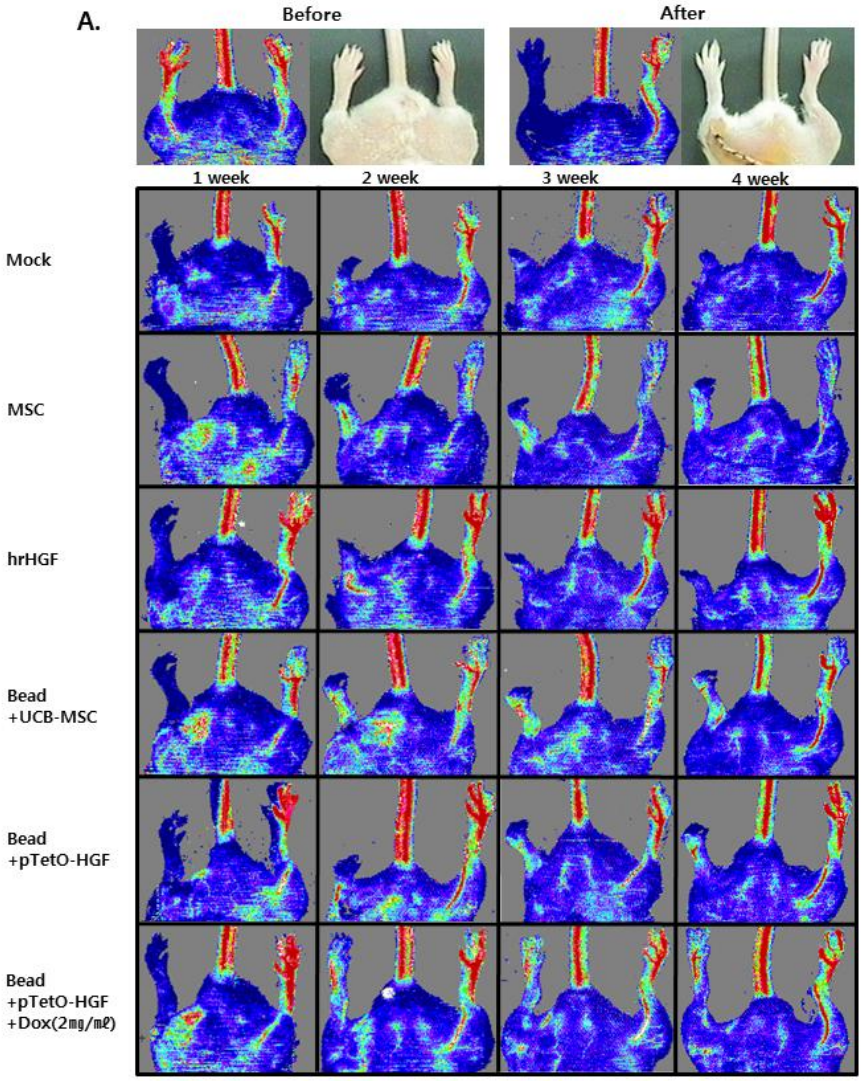
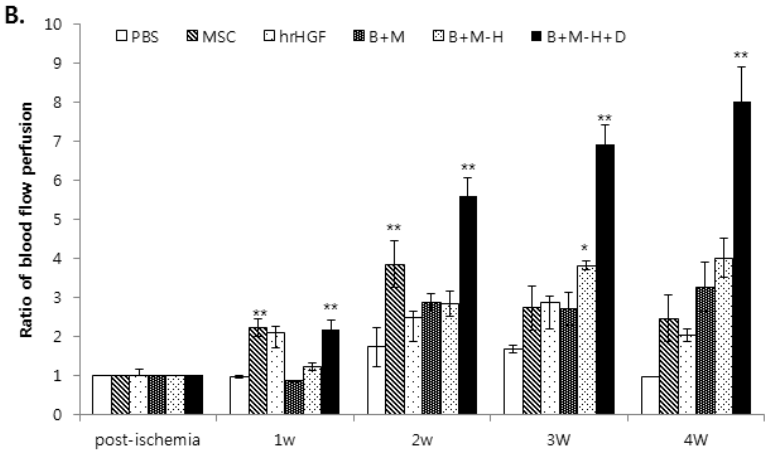


Figure 1.11. HGF expression by engineered hUCB-MSCs encapsulated in a microgel. **(A)** Before bead formation, an ELISA was performed to determine the level of HGF in media conditioned by engineered hUCB-MSCs treated or not treated with Dox. The concentrations of the conditioned media were enriched by approximately 10-fold. **(B)** Image of beads containing engineered hUCB-MSCs. **(C)** HGF levels in medium conditioned by the beads, as determined by WB analysis and **(D)** an ELISA (**$P < 0.01$).


A.
Before
After
1 week
2 week
3 week
4 week
Mock
MSC
hrHGF
Bead
+UCB-MSC
Bead
+pTetO-HGF
Bead
+pTetO-HGF
+Dox(2㎎/㎖)


B.
PBS
MSC
hrHGF
B+M
B+M-H
B+M-H+D
Ratio of blood flow perfusion
post-ischemia
1w
2w
3W
4W

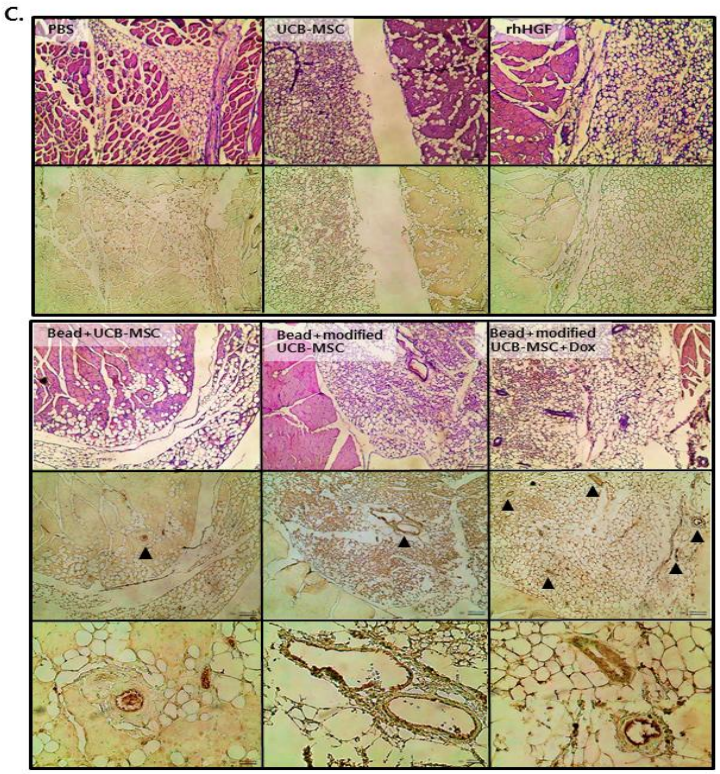


Figure 1.12. HGF enhanced angiogenesis in the mouse hindlimb ischemia model. A 1 week of induced hindlimb ischemia, mice were treated with PBS, hUCB-MSCs only, hrHGF only, an RGD-alginate microgel containing hUCB-MSCs, an RGD-alginate microgel containing HGF integrated hUCB-MSC, an RGD-alginate microgel containing HGF-secreting hUCB-MSCs treated with Dox. After treatment, the levels of blood perfusion of the hindlimbs were measured using a laser-Doppler flowmeter weekly for 4 weeks. **(A)** Images showing the blood flow in mice given each treatment. The ischemic limbs are on the left and the normal limbs are on the right. **(B)** Ratio of the blood flow perfusion rate of the ischemic limbs versus those of the normal limbs. **(C)** Image of H&E stained sections and of sections stained with an anti-vWF antibody (IHC).

1.4. Discussion

It is no doubt that stem cells are strong regenerative material and potent therapeutic source. The paracrine effects of cytokines or proteins secreted by MSCs greatly facilitate regeneration and therapeutic outcomes (Tang et al. 2005, Gnecchi et al. 2008). However, the therapeutic effects of cytokines secreted by MSCs are inadequate due to their short half-life and the rapid degradation of various types of MSCs. Therefore, new strategy to improve the therapeutic efficacy and paracrine effect of stem cell is required. For this, we developed the improved functional MSCs that secrete a growth factor using Transcription activator-like effector nucleases (TALEN)-mediated genomic editing. Genetically modified-MSCs are able to controllably express and induce HGF under special condition using a drug-responsive promoter cassette. TALENs are promising tools for editing genomes. A TALEN is an artificial restriction enzyme that is generated by fusing a TAL effector DNA-binding domain to a DNA-cleavage domain. TALENs exhibit strong and specific protein-to-nucleotide recognition (Bedell et al. 2012).

Although TALENs can target any site, few sites are safe for exogenous-gene integration and are in an open chromosomal state. The adeno-associated virus integration site 1 (AAVS1), which is in the PPP1R12C gene on human chromosome 19, is one of the safe harbor sites. When a gene is inserted into the AAVS1 on chromosome 19, it is efficiently expressed (Luo et al. 2014). The

engineered MSCs produced using our safe-harbor site system secreted HGF protein continuously in an inducible manner that could be controlled by Doxycycline. Thus, the TALEN-mediated integration of the HGF-expression system into a chromosome of the stem cells not only provided stem cells for therapy but also solved the problem of the short half-life of a therapeutic protein.

An HGF ELISA showed that the concentration of HGF secreted by the TetOn-HGF/hUCB-MSCs reached 0.5~0.8 ng/ml (Fig 1.4. D and Fig 1.11. A, D). Although this concentration is approximately 1% that of the human recombinant HGF used as a positive control, the effects of the HGF-secreting TetOn-HGF/hUCB-MSCs in most of the *in vitro* experiments were similar to those of the control (Figs 1.7., 1.8., 1.10.). These comparable effects might be due to the consistent release of HGF by the TetOn-HGF/hUCB-MSCs with Dox. We postulated that due to the short half-life of HGF (approximately 5 min), spike treatment of a high concentration of human recombinant HGF was not sufficient and only temporarily effective. Then, continuous exposure of HGF at physiological concentration produced by the engineered cells had effects comparable with those of a high concentration of hrHGF. Thus, our results showed the importance of the continuous secretion of a short half-life cytokine such as HGF.

One of major hurdle of stem cell-based cell therapy for clinical application is cell survival under harsh condition at treated site. Stem cells of superior ability are difficult to survive in the presence

of oxidative environment condition. However, HGF has the function to overcome anti-apoptotic and anti-oxidative stress mediated cellular damage. For that reason, the maintenance of proper and constant concentration of HGF is particularly important *in vivo*. The long-term therapeutic effect of a low concentration of HGF secreted by the engineered stem cells was proven in the *in vitro* cell-viability assay, which showed that even the viability rate of these cells was higher than that of the positive control group treated with a high dose of HGF (50 ng/ml) (Fig 1.8.). To evaluate whether the long-term secreted HGF from genetically engineered stem cells has more significant effect *in vivo*, immune competent mice were induced hindlimb ischemia. To improve the therapeutic efficacy and overcome low cell survival, engineered stem cells were encapsulated in injectable RGD-alginate microgel by electrospinning. In Fig 6, the hrHGF-treated group showed the least improvement in the ischemic hind-limb among all of the treated groups. The mice in the hrHGF-treated group lost their injured hindlimb within the first week following femoral-artery ligation and hrHGF treatment. The degree of limb deterioration was similar to that of the negative control group treated with PBS. On the other hand, HGF secreting MSCs in microgel significantly improved therapeutic effects among all groups. These results strongly indicate that the persistent secretion of HGF greatly enhanced angiogenesis.

Notably, we did not administer an immune suppressor to the mice

even though human stem cells were implanted in them. Reportedly, immune modulation can be accomplished not only by protecting stem cells by encapsulating them in a microgel but also through the effects of the MSCs and HGF. We speculated that due to the combined effect of RGF-alginate microgel-encapsulated hUCB-MSCs and the HGF that these cells secreted, our therapeutic system provided even better immune modulation so that treatment with an immune-system suppressor was not required for the therapeutic efficacy of this system(Okunishi et al. 2005, Abdi et al. 2008, Hoogduijn et al. 2010).

Although doxycycline-treated TetOn-HGF/hUCB-MSCs greatly enhanced the rates of tube formation and *in vivo* angiogenesis, further improvement is required before they can be used as therapeutic agents. Because the hUCB-MSCs are primary cells, their transfection efficiency rate and the rate of integration of the TetOn-HGF transgene differs depending on their status. Different rates of gene integration via TALEN may result in different concentrations of the secreted protein. Late passages of MSCs are an important concern. By the time that the expression system has become integrated into the hUCB-MSCs, they begin to reach senescence. Therefore, it is difficult to produce sufficient numbers of TetOn-HGF/hUCB-MSCs for use as therapeutic agents. Further studies are needed to standardize the procedure we utilized and to overcome the problem of senescence.

Safety is always an important issue for stem-cell therapy. A major

problem for stem cell-mediated gene therapy is controlling the level of gene expression. However, our system provided an example of how to control the side effects of stem-cell and gene therapies. Regarding the side effects of gene therapy, HGF transgene expression by our system can be controlled by a drug, doxycycline (tetracycline) because it is under the control of a tetracycline-on system, which was also integrated into the safe-harbor site using TALEN-mediated gene delivery. Regarding the side effects of cell therapy, hUCB-MSCs are tolerated by the immune system, and these cells are further protected from immune-system attack by being encapsulated in RGD-alginate. Taken all together, our inducible HGF secreting MSCs are an effective tool as a stem cell-based therapeutic agent.

In conclusion, HGF is a pleiotropic cytokine that has long been known to be involved in cell and tissue regeneration. However, the available tools that can produce its therapeutic effects are clinically insufficient. In this study, we generated inducible TetOn-HGF/hUCB-MSCs via TALEN-based genome editing. Notably, these cells in RGD-alginate microgel secreted HGF and enhanced cell mobility, protected cells against apoptosis, and improved the level of angiogenesis in a mouse hindlimb ischemia model. Our study clearly demonstrated that gene editing allowed HGF secretion by hUCB-MSCs and overcame the limitations of other HGF-based pro-angiogenesis therapies for vascular diseases such as ischemia.

Chapter II

Coronary Stents with Inducible VEGF/HGF-secreting hUCB-MSCs Reduced Restenosis and Increased Reendothelialization in Swine Model

Abstract

Atherosclerotic plaques within the vasculature can eventually lead to heart failure. Currently, cardiac stenting is the most effective and least invasive approach to treating the disease. However, in-stent restenosis is a complex chronic side-effect of the stenting treatment. In this study, to reduce stent restenosis and induce reendothelialization within the artery, we applied coronary stents coated with stem cells secreting angiogenic growth factors via an inducible genome-editing system. After confirming the characteristics of the cells and their adhesion properties on the stents, we transplanted the stents into a swine model to evaluate the restenosis and potential therapeutic use of the stents with stem cells. Restenosis was evaluated via optical coherence tomography, micro-computed tomography and angiography, and reendotheliali zation by immunostaining after cardiac stent treatment. Compared to a bare metal stent or a parental umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs)-coated stent, the stents that had stem cells capable of the controlled release of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) successfully reduced restenosis within the stent and induced natural reendothelialization. Furthermore, hUCB-MSCs exhibited the ability to differentiate into endothelial cells in Matrigel, and HGF and VEGF improved the differentiation. Our study indicates that the stents coated with hUCB-MSCs secreting VEGF/HGF reduced the restenosis side effects of cardiac stenting with improved

reendothelialization.

2.1. Introduction

Coronary artery disease is an angiocardiopathy that severely impairs health, and it is still the principal cause of mortality in the world. The goal of treatment is to restore the blood flow in the clogged artery to a near-normal rate (Faxon et al. 2004, Aziz et al. 2005, Mortality et al. 2015). Coronary stents are a widely used treatment strategy to keep the arteries open. However, the success of stent treatment has been limited by restenosis and stent thrombosis. Delayed or incomplete endothelial regeneration is believed to be a key factor responsible for these events.

In-stent restenosis was found to be decreased by the endothelialization of coronary stents (Padfield et al. 2010, Reejhsinghani et al. 2015). This was considered an important factor for preventing thrombosis and for reducing the proliferation and migration of vascular smooth muscle cells (VSMCs). Therefore, a coronary stent capable of rapid surface endothelialization has the potential to become the next-generation stent (Versari et al. 2007, Tan et al. 2012, Bedair et al. 2017). To improve reendothelialization, we used a very effective combination strategy combining gene therapy and cell therapy, in which genome edited stem cells release proangiogenic growth factors.

Vascular endothelial growth factor (VEGF) is one of the most effective signaling proteins that stimulates vasculogenesis (Hoeben et al. 2004). Hepatocyte growth factor (HGF) is a pleiotrophic

factor that induces motogenesis, mitogenesis, survival and, in some cell types, morphogenesis (Ellison et al. 2011, Chang et al. 2016). Thus, the integration of these genes into the genome of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) enhanced their ability to stimulate angiogenesis. To integrate these genes into stem cells, we used the TALEN genome editing system to introduce targeted DSBs into the chromosome 19 safe-harbor site. Using the Tet-on system, we controlled the gene expression with doxycycline. Our previous studies showed that the VEGF and HGF-secreting hUCB-MSC (VEGF/hUCB-MSC and HGF/hUCB-MSC) showed enhanced angiogenesis in both a rat myocardial infarction model and a mouse hind limb ischemia model (Chang et al. 2016, Cho et al. 2017). VEGF/hUCB-MSC and HGF/hUCB-MSC were proven to be a very effective and powerful cell therapy system to restore the blood vessels and blood flow.

The stents coated with polydopamine (pDA), fibronectin (FN) and extracellular matrix (ECM) have been used to enhance adhesion of stem cells including MSCs to the metallic stents (Park 2016). An ECM is a biocompatible and cell-supporting substance and provide the cells with mechanical and physiological support resulting in the increase of cell survival, adhesion, proliferation, and differentiation (Wozniak et al. 2004, Trappmann et al. 2012). It also traps and keeps some growth factors and soluble molecules through proteoglycans which are major components of the ECM (Kim et al. 2011). However, ECM still needs strong connector to be attached

on the metal surface. Therefore, pDA and FN are used as chemical connectors. FN can recognize and bind to ECM molecules by integrin. Its carboxyl termini covalently bind to the pDA. Thus, the FN-pDA layers serve as linkers to immobilize ECM molecules such as fibrin, collagen, heparin, and fibronectin on the surface (Prewitz et al. 2013). Dopamine is a strong adhesive molecule derived from the mussel (Waite 2008). It binds firmly to both of organic and inorganic surfaces by a catechol, which consists of a benzene ring with two hydroxyl groups. pDA also provides a functional group of amine for immobilizing the molecules on the surface. After coating with those 3 components on the stent, stem cells can be efficiently seeded on stents.

The aim of this study was to investigate the potential of the stents with angiogenic growth factor-secreting MSCs to enhance reendothelialization and the reduction of restenosis by rapid reendothelialization. We loaded the coronary stent with functional stem cells VEGF/hUCB-MSC and HGF/hUCB-MSC and assessed both the efficacy of in-stent stenosis reduction and coronary artery reendothelialization in a swine model.

2.2. Materials and methods

Cell culture and cell preparation

MSCs isolated from human umbilical cord blood (hUCB) were kindly provided by Kang's lab in Seoul National University. The cells were isolated from hUCB as previously described (Seo et al. 2011). The hUCB-MSC isolation procedure was approved by the Borame Institutional Review Board and Seoul National University (IRB No. 0603/001-002-07C1). The hUCB-MSCs were maintained in mesenchymal stem cell medium (KSB-3, Kangstem Biothech, South Korea) supplemented with KSB-3 supplements and 10% fetal bovine serum (Rocky Mountain Biologicals Inc., MT, USA) at 37 ℃ in 5% CO_2. After cell culture, cells were transfected by NEON with the TALEN system and HGF or VEGF secreting plasmids as previously described (Chang et al. 2016, Cho et al. 2017).

Viability assay

The live cells were taken by fluorescence microscopy after labeling with green fluorescent dye (PKH67, Sigma, USA) on pre-coated stent material. The metal materials were coated with polydopamin, fibronectin and ECM. Cell viability was testing via the crystal violet assay and cell counting. Cells (5×10^4) were seeded on stent material coated with fibronectin and an extracellular matrix layer, and then incubated for 7 days. After adding 50 μl of 0.5% crystal violet solution, an image of the stained cells was captured.

After taking the image, 200 μl of methanol was added to each well for 20 min at RT. Cell density was detected at 570 nm by a spectrophotometer (Epoch, BIoTek, VT, USA). Cells were also counted by a hemocytometer after trypan blue (Gibco, NY, USA) staining.

Western blot (WB)

To confirm growth factor expression, WB was performed as previously described (Chang et al. 2016, Cho et al. 2017). Transfected cells (5×10^5) were seeded on stent material. After 24 hrs, the cells were treated with Dox (at 5 μg/ml) for 2 days. Then, the conditioned medium was collected and precipitated by trichloroacetic acid (TCA, Sigma, USA). The pellet was dissolved in 200 μl of RIPA buffer (Thermo, IL, USA). SDS-PAGE was performed with a 1% acryl amide gel. The HGF (R&D Systems, MN, USA) and VEGF (Cell Signaling, MA, USA) antibodies were used at a dilution of 1:1,000.

Conventional PCR and real-time quantitative PCR

To assess MSC markers, conventional PCR and real-time PCR were performed 7 days after the cells were seeded on stent material. The sequences of primers for CD166, CD105, CD90, CD45, CD14, and GAPDH are listed in supplemental dataTable 1. PCR was performed using Go taq polymerase (Promega, MN, USA) according to the following protocol: one cycle of 95 ℃ for 5 min, 35 cycles of

95 ℃ for 30 sec, 60.1 ℃ for 30 sec, 72 ℃ for 30 sec, and a final cycle of 72 ℃ for 3 min. The PCR product was evaluated using a 1.5% agarose gel. Real-time PCR was performed with the same PCR conditions using a SYBR Green-based method.

Tube formation

Cells (5×10^4 cells/well) were seeded on a layer of BD Matrigel (BD Biosciences, CA, USA) in 24-well plates. The cells were then exposed to high-glucose DMEM (HyClone) containing 2% FBS for 48 hrs. MSC cells alone were used as a control. The cells were incubated for 12 hrs to allow them to form tube-like structures. Tube formation was analyzed by counting the number of branches per high-power field.

Preparation of stent and confirmation of cell adhesion

The stents were coated with fibronectin and extracellular matrix as previously described (Bedair et al. 2017). Cells (2.4×10^6) were seeded on stents and incubated for 12 hrs in a CO_2 incubator. Then, the cells on the stent were detected by fluorescence microscopy (July, Nanoentec, South Korea). To observe the cells remaining on the stent after transplantation, scanning electron microscopy (SEM) analysis was performed. The samples were rinsed in 2.5% glutaraldehyde in α-MEM without serum and fixed for 30 min at RT. The samples were then fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate pH 7.2 with 0.1 M sucrose for an additional 30 min at RT.

The samples were treated with 1% osmium tetroxide in distilled water for 1 hr, followed by dehydration through a graded series of ethanol solutions from 70, 80, 95 to 100 %. A freeze-dryer was used to dry samples. The samples were mounted on aluminum holders and coated with a 10-nm conducting layer of gold platinum. The samples were examined in the SEM (Jeol JSM7400F, Tokyo, Japan) using a voltage of 10 kV.

Transplantation into swine model

This animal study was approved by the Ethics Committee of Chonnam National University Medical School and Chonnam National University Hospital (CNU IACUC-H-2013-12), and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Yorkshire × Landrace F1 crossbred castrated male pigs (20-25 kg) were observed in the laboratory animal center of Chonnam National University Medical Institute for 5-10 days before the experiment.

On the day of the procedure, the pigs were anesthetized with zolazepam and tiletamine (2.5 mg/kg, Zoletil50®, Virvac, Caros, France), xylazine (3 mg/kg, Rompun®, Bayer AG, Leverkusen, Germany), and azaperone (6 mg/kg, Stresnil®, Janssen-Cilag, Neuss, Germany). An intravenous (IV) catheter was placed in the marginal ear vein for the administration of fluid and emergency drugs such as epinephrine and anti-arrhythmic agents (amiodarone

hydrochloride). IV fluid administration with 0.9% saline was continued throughout the experiment. After intubation, anesthesia was maintained with inhalation anesthetic consisting of sevoflurane (1%) in oxygen (100%). Pigs were mechanically ventilated. Tramadol HCl (5 mg/kg, Trodon®, Aju pharm, Korea) was administered IV pre- and post-operatively to reduce pain. The stent was then inserted into the coronary artery of an 8-week-old pig, and its placement was confirmed by angiography. We used LAD (Left anterior descending) and LCX (Left circumflex artery). Pigs were premedicated with 100 mg aspirin and 75 mg clopidogrel per day for 5 days before the procedure. We also treated cyclosporine (CIPOL.N, Chong Kun Dang, South Korea), immunosuppressant, for 3 days after stent transplantation. The pigs were also treated with immunosuppressant Cyclosporine (CIPOL.N, Chong Kun Dang, South Korea) for 3 days after stent transplantation.

Stenosis imaging: Angiography, OCT, and mCT

Imaging analysis for stenosis evaluation was performed as previously described (Lim et al. 2016). Briefly, a follow-up coronary angiogram was performed 4 weeks post-stent transplantation. At the end of the experiment, pigs were anesthetized and then sacrificed with an overdose of potassium chloride. The hearts were rapidly removed, extracted and grossly sectioned at 1 cm intervals. The myocardial sections were stained with 2,3,5-triphenyl tetrazolium chloride (TTC) solution (1% in

phosphate-buffered saline) for 30 min at 37 °C. After TTC staining, sectioned heart tissues were fixed in 10% neutral buffered formalin overnight and embedded in paraffin for histological analysis.

Angiography: A 7F coronary artery guiding catheter was placed within the opening of the coronary artery, and a baseline coronary angiogram was obtained using the nonionic contrast agent Omnihexol (Omnihexol 300, Korea United Pharm Co., Seoul, Korea) under fluoroscopic guidance with a mobile fluoroscopy system (BV Pulsera, Philips Medical Systems, Andover, MA, USA). Angiography was performed to confirm the obstruction of the mid-LAD. Finally, the guide wire, balloon catheter, and guiding catheter were removed and the left carotid artery was ligated.

Optical coherence tomography (OCT): After the carotid artery was excised, the neointima of pig blood vessels was measured using OCT (Model C7Xr OCT imaging system). A guidewire was connected to a water box dedicated for *in vitro* experiments, and the coronary artery was fixed to the guide wire. An imaging catheter (C7 Dragonfly) was inserted through the guide wire into the coronary artery. OCT images were obtained by connecting the imaging catheter and the Dragonfly Duo, and the neointimal vessels were measured using LightLab imaging (offline review workstation).

Micro-computed tomography (mCT): Tomograms of each sample were acquired using a micro-computed tomography scanner (SkyScan 1172, Bruker). The harvested coronary artery tissues were stored in 10% formalin solution and transferred to de-ionized

water prior to μ-CT analysis. The tissue was fixed vertically in a cylindrical plastic container and mounted on a specimen stub using soft clay. The plastic container was sealed with paraffin film to prevent drying of the sample. The scanning was operated at 10 W (100 kV/ 100 μA) X-ray generation power with an aluminum-copper filter. Images were recorded at 0.4° rotation for one step. The acquired images were reconstructed and visualized using the software (NRecon/CTan, Bruker). The in-stent restenosis area (ISR area) was calculated by subtracting the area of the lumen from the area within the stent strut.

Immunohistochemical (IHC) staining

Tissues were harvested, fixed in 4% paraformaldehyde (Wako), embedded in paraffin, and cut into 5 μm sections (Leica, Buffalo Grove, IL). Immunohistochemistry was performed using an anti-von Willebrand factor (vWF) (Abcam, USA, anti-Fibrin (Abcam, USA), and anti-Lamin A+C (Abcam, USA) antibodies at a 1:100 dilution and followed by the appropriate secondary antibodies. The tissue samples were also stained with hematoxylin and eosin (H&E) and Masson's trichrome. TTC, H&E, and Masson's trichrome stains were performed to evaluate the infarcted area of the ventricle. Histological evaluation of the infarcted myocardium was performed by an experienced cardiac pathologist.

Tumor formation

BALB/C Nude mice (Orientbio, South Korea) were used for tumor formation *in vivo*. hUCB-MSCs and HGF- or VEGF-secreting hUCB-MSCs ($5x10^6$) were injected with 50 μl of serum-free DMEM and 50 μl of Matrigel (Corning, USA). MDA-MB-231 breast cancer cells were used as a positive control. Mice were observed for 4 weeks, and the tumor volume was measured every week. After 4 weeks, mice were sacrificed with CO_2, and tumor formation and tumor weights were analyzed.

Statistical analysis

The data were expressed as the mean values ± standard error. The data were analyzed using Prism5 (GraphPad, CA, USA), and a t-test or ANOVA was used to compare the data pertaining to the different experimental groups. Statistical significance is indicated in the figure legends.

2.3. Results

Characterization of HGF/hUCB-MSCs and VEGF/hUCB-MSCs on stent material

The full experiment proceeded as describe in the schematic image (Fig 2.1. A). We first tested whether the engineered cells are successfully implanted on the stent. We confirmed that the stent material does not affect cell growth and the expression of hUCB-MSC markers. The characteristics of VEGF/hUCB-MSC and HGF/hUCB-MSC were evaluated after long-term interactions with the stent material. After seeding the cells on materials for more than 7 days, the proliferation of HGF- and VEGF-secreting hUCB-MSCs was increased by Doxycycline (Dox) (Fig 2.1. B~D). The secretion of HGF and VEGF by the Tet-On system was confirmed with immunoblot analysis (Fig 2.1. E). The MSC markers, positive markers of CD116, CD105, and CD90 and negative markers of CD45 and CD14- exhibited no change after HGF and VEGF secretion (Fig 2.1. F & Fig 2.2. A). Every time that cells were established on a stent, we confirmed the secretion of HGF and VEGF and the integration into the genome (Fig 2.1. B). There was a concern related to the effect of Dox on cell proliferation, but in this test, Dox did not affect MSC proliferation or morphology (Fig 2.3. A & B). We confirmed that the hUCB-MSCs maintained their properties and growth on the stent, and we showed that the secretion of HGF and VEGF from the engineered stem cells promoted cell proliferation.

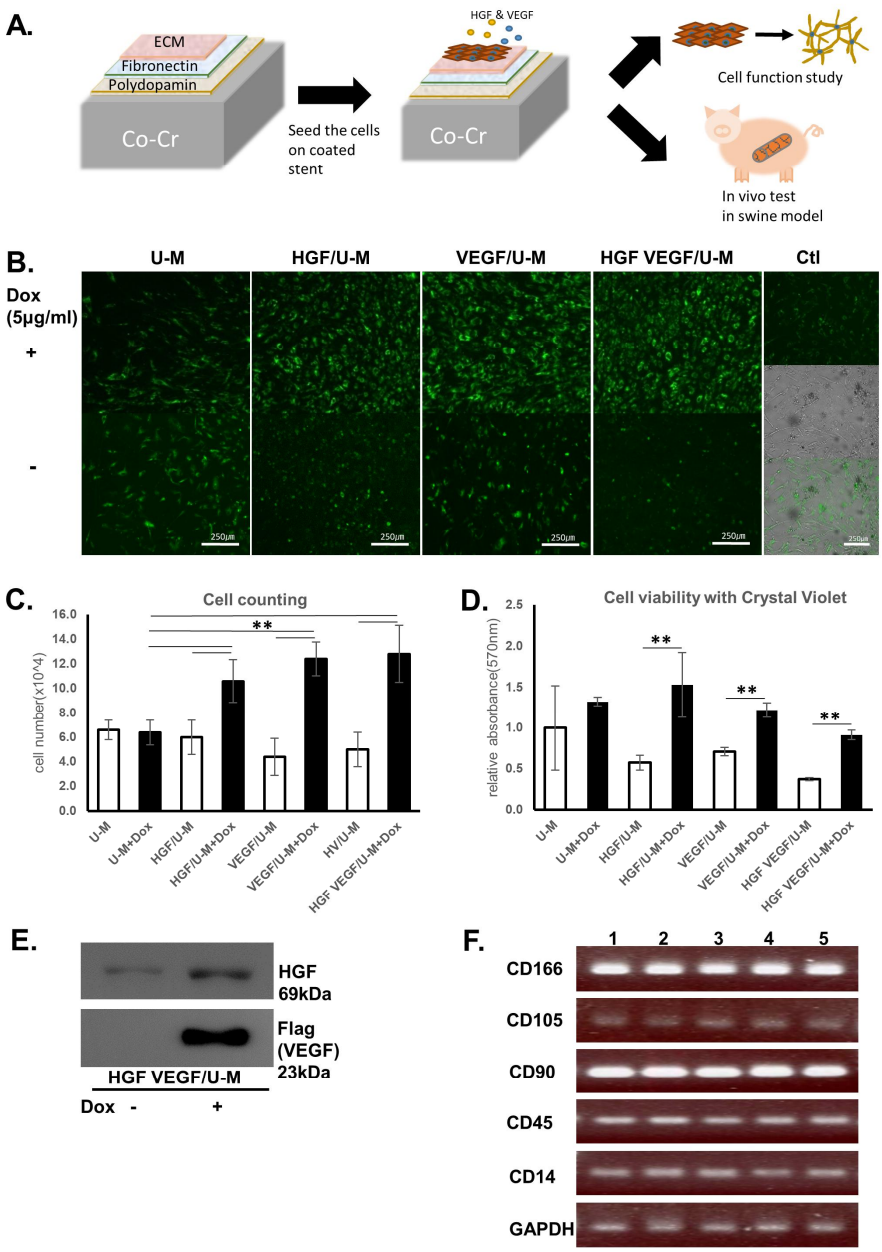


Fig. 2.1. The properties of HGF/hUCB-MSCs and VEGF/hUCB-MSCs are maintained on stent material. (A) Schematic illustration of the experiment using the stent with HGF/hUCB-MSCs and VEGF/hUCB-MSCs secreting the angiogenic factors in an inducible

manner. The stents were coated with polydopamine, fibronectin, and extracellular matrix (ECM), followed by *in vitro* and *in vivo* swine experiments. **(B)** The human UCB-MSCs secreting HGF and VEGF were seeded onto the pre-coated material sheets to confirm cell viability on the stent material. The cells stained with green fluorescent dye were detected by fluorescence microscopy. U-M: human UCB-MSCs, Ctl: control (n=3). **(C)** The cells were counted 7 days postseeding after detaching the cells from the stent material and staining the dying cells with trypan blue (** denotes a p <0.01). **(D)** Cell viability was analyzed by a crystal violet assay on the stent 7 days postseeding (** denotes a p <0.01). **(D)** **(E)** HGF and VEGF secretion were detected in conditioned media by Western blotting. HGF/hUCB-MSCs and VEGF/hUCB-MSCs were treated with 5 μg of doxycycline for two days in a 6-well plate. **(F)** MSC markers showed no change on the stent material even in the HGF- and VEGF-secreting cells. Lane 1: hUCB-MSCs, 2: hUCB-MSCs+Dox, 3: hUCB-MSCs+Dox on material, 4: HGF/hUCB-MSCs +Dox on material, 5: HGF+VEGF/hUCB-MSCs +Dox on material (n=3 experiments per group).

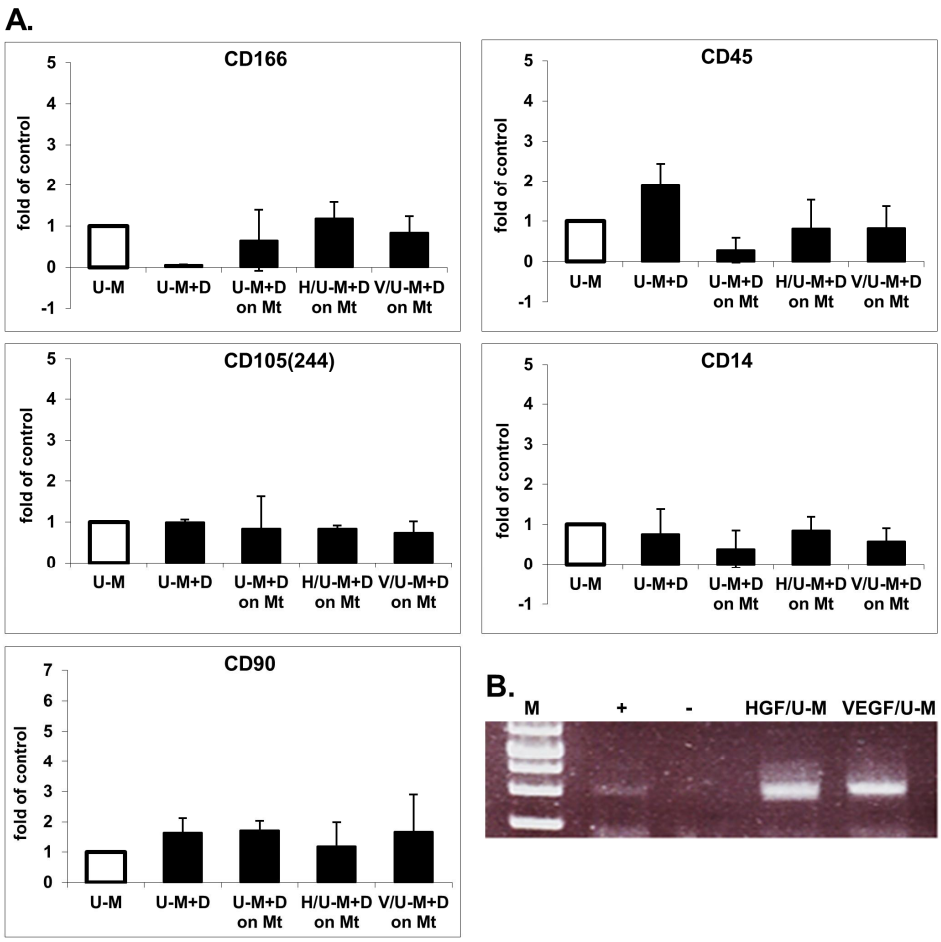


Fig 2.2. Characterization of HGF+VEGF/hUCB-MSCs on the stent material. (A) MSC markers were analyzed by quantitative real-time PCR, and no difference was observed after seeding the cells on stent material. (B) Junction PCR of HGF/hUCB-MSCs and ·VEGF/hUCB-MSCs confirmed the integration of the HGF and VEGF genes in the human genome.

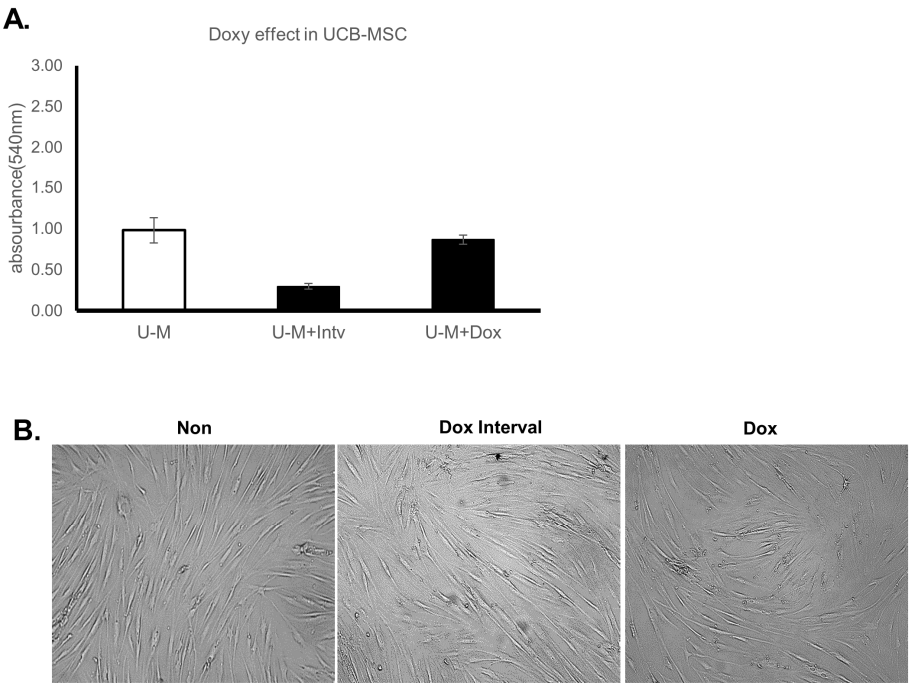


Fig 2.3. The characteristics of hUCB-MSCs are retained after Doxycycline treatment. (A) The MTT assay exhibited no difference in proliferation after doxycycline treatment of hUCB-MSCs. (B) Images of cell morphology showed no change after doxycycline treatment 7 days after treatment.

Cells on the stents after coronary stent transplantation

Since cell adhesion to the bare stent material is limited, we designed stents with biocompatible matrices. The poly-dopamine and fibronectin were sequentially conjugated onto the Co-Cr stent. Then, fibroblasts were seeded on the stent surfaces, which were then decellularized to provide extracellular matrices secreted by the fibroblasts (Fig 2.1. A). Then, the hUCB-MSCs with eGFP genes integrated to the Chr 19 safe harbor site by TALEN were attached on the stent surfaces with the extracellular matrix. After attaching the cells to the stent, the cell adhesion was confirmed by fluorescence microscopy (Fig 2.4. A). Due to a concern about the presence of the adhered cells on the stents after coronary artery transplantation, scanning electron microscopy (SEM) was performed to identify whether the cells remained after mimicking stent implantation in the swine model. Surgical procedures led to the detachment of some cells because of the strong physical friction caused by balloon dilatation. However, the cells still remained on the lateral side of stent after transplantation (Fig 2.4. B). These results implied that the stem cells implanted on the stent remained after surgery.

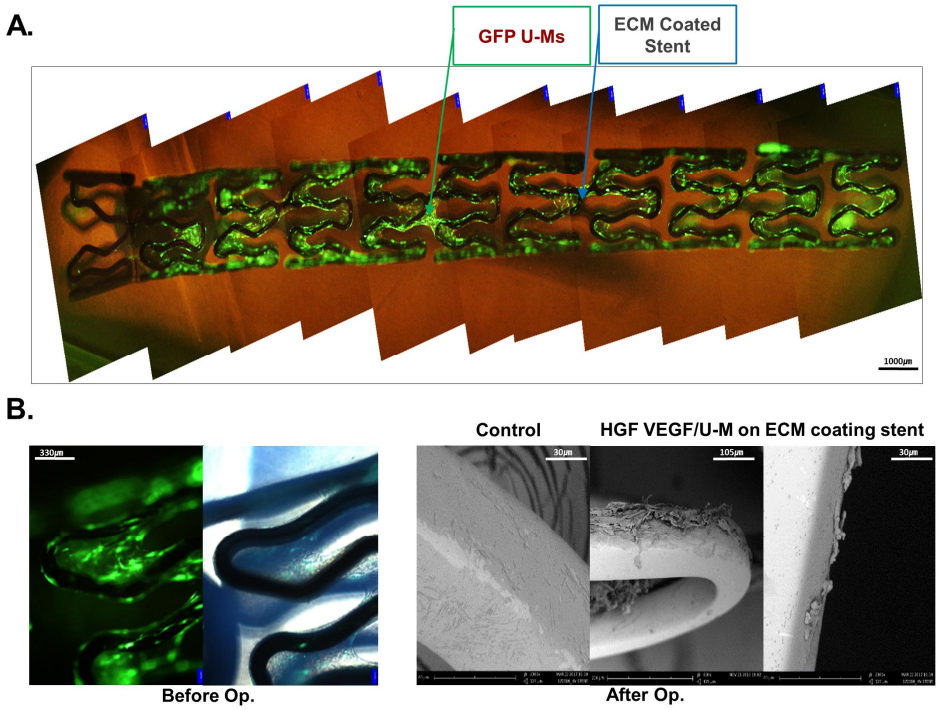


Fig. 2.4. The cells remained on the stent after transplantation. (A) Fluorescence images showing adhesion of the GFP-expressing hUCB-MSCs on the stents. (B) Fluorescence images before the operation and stent seeding in the swine model were captured by a microscope, and the SEM images after the end of the experiment were captured by scanning electron microscopy (n=3).

The effect of each HGF and VEGF application period on coronary restenosis

To evaluate the effects of HGF/hUCB-MSC and VEGF/hUCB-MSC seeded stents on the coronary artery, stents were applied to a swine model and observed for 2 weeks after stent implantation. Among the bare metal stent, stents with hUCB-MSCs, HGF/hUCB-MSCs and VEGF/hUCB-MSCs, the HGF/hUCB-MSCs group showed the lowest level of neointima formation (Fig 2.5. HGF group). OCT and mCT analysis displayed that the HGF/hUCB-MSCs group had not only the least stenosis formation but also a uniform pattern in the stenosis area (Fig 2.5. C). An even inner surface is known to be a crucial/essential criterion for stent reendothelialization. In contrast, excessive neointima formation was observed in the VEGF/hUCB-MSC group after 2 weeks (Fig 2.5. VEGF group). In a 3-day trial, the VEGF group exhibited the lowest level of neointima formation, but this was not observed at 2 weeks. The excessive amount of VEGF secreted from VEGF/hUCB-MSCs might stimulate vascular smooth muscles during longer term exposure to the cells. Other studies have shown that patients with an increase in VEGF after implantation had a restenosis rate of 26.2% compared to 2.4% in patients with basal levels of VEGF (Katsaros et al. 2014, Yang et al. 2014). Furthermore, restenosis was reduced in the HGF/hUCB-MSCs group, but the effect was not significant over the longer term. Therefore, it was necessary to optimize the application of the cell

population and the periodic conditioning of HGF/hUCB-MSCs and VEGF/hUCB-MSCs to promote reendothelialization and minimize the neointima side effect.

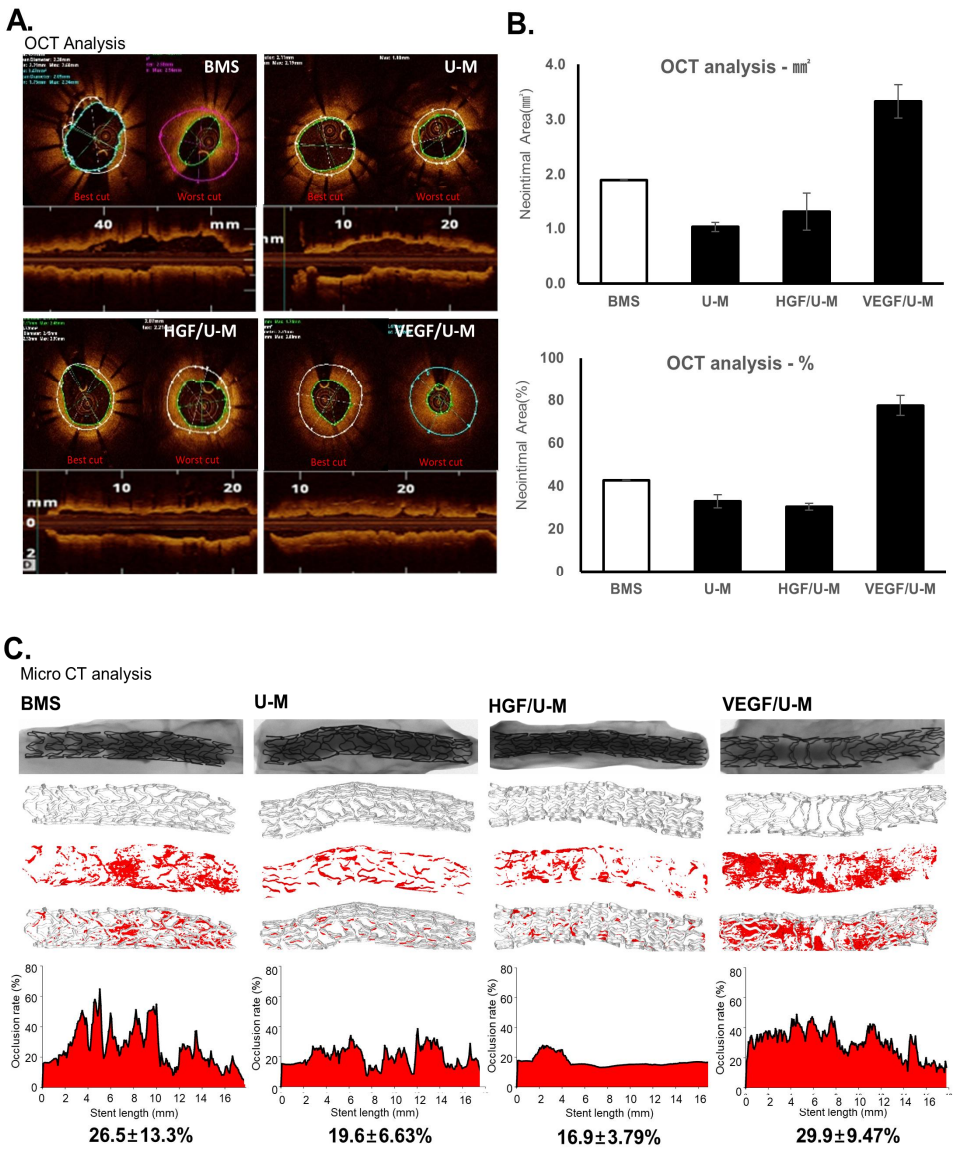


Fig. 2.5. The single conditions of HGF/hUCB-MSCs and VEGF/hUCB-MSCs alone showed opposite effects on the neointima levels 2 weeks after transplantation. (A) OCT images showing the neointimal degrees 2 weeks after stent transplantation. BMS: bare

material stent, U-M: human UCB-MSC coated stent, HGF/U-M: stent coated with HGF/hUCB-MSCs, VEGF/U-M: stent coated with VEGF/hUCB-MSCs. (B) OCT analysis showing the neointimal areas in mm2 and in % units. (C) The microCT results were analyzed at a 50 kV/200 μA spatial resolution with 17-μm units to show the surface smoothness and neointima areas (n=3).

Optimization of HGF and VEGF secreting stem cell and the enhanced tube formation with HGF/hUCB-MSCs and VEGF/hUCB-MSCs

The stents with VEGF-secreting stem cells for longer implants (2-week) caused even more severe restenosis (Fig 2.5.). This might be because VEGF is one of the strongest factors stimulating the fibrosis and proliferation of all cells in the vascular area (Yang et al. 2014, Park et al. 2015). However, cells secreting only HGF or VEGF were not able to efficiently reduce restenosis (Fig 2.5.). Furthermore, under angiogenic conditions, combining VEGF-A with HGF can promote neovascularization, especially reendothelialization, by enhancing intracellular signaling and allowing more finely regulated control of the signaling molecules involved in the regulation of the cytoskeleton and cellular migration and morphogenesis (Eric Sulplce 2009). Thus, the combined condition is necessary and the dose of HGF and VEGF secreting cells is also needed to optimize. From our previous studies, the amount of HGF and VEGF secreted from this system was analyzed. Fifty ng VEGF and 2.2 ng HGF were secreted from $2x10^6$ cells per a day (Chang et al. 2016, Cho et al. 2017). Based on these results, we maximized the HGF and minimized the VEGF because HGF is known to promote endothelial cells (EC) more specifically, whereas VEGF increases overall vascular cell activity (Hayashi et al. 2000). When the 5:1 ratio of HGF to VEGF-secreting cells was tested *in vitro*, the cell proliferation was similar to the control single condition (Fig 2.1, HGF VEGF/U-M group). This condition included a range

known to be suitable *in vivo* (Deindl et al. 2007).

A source of endothelial cells is needed to facilitate coronary in-stent reendothelization. Endothelial progenitor cells (EPCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been used as endothelial cell sources *in vitro*. More importantly, MSCs differentiate into endothelial cells (Oswald et al. 2004, Janeczek Portalska et al. 2012). We assumed that our functional HGF VEGF/hUCB-MSC might have the potential to differentiate into ECs. On Matrigel, the formation of tubes with HGF+VEGF/hUCB-MSC was observed, even in the culture condition excluding endothelial differentiation medium and bFGF, whereas hUCB-MSCs (U-M) themselves did not form the tube properly in the absence of Matrigel (Fig 2.6.). HGF+VEGF secretion from the cells in response to doxycycline induction (HGF+VEGF/U-M+Dox) enhanced the tube number and branch numbers (Fig 2.6. B & C). Furthermore, the tubes persisted longer in the HGF+VEGF/U-M+Dox group (Fig 2.6. A). Additionally, at early times (3-9 hrs), the 5:1 combination of HGF:VEGF/hUCB-MSC in response to Dox stimulation resulted in better tube formation and branch numbers (Fig 2.6. B & C). Based on these results, we concluded that HGF+VEGF secreting stem cells at a 5:1 ratio is an optimal condition for vascular reendothelialization.

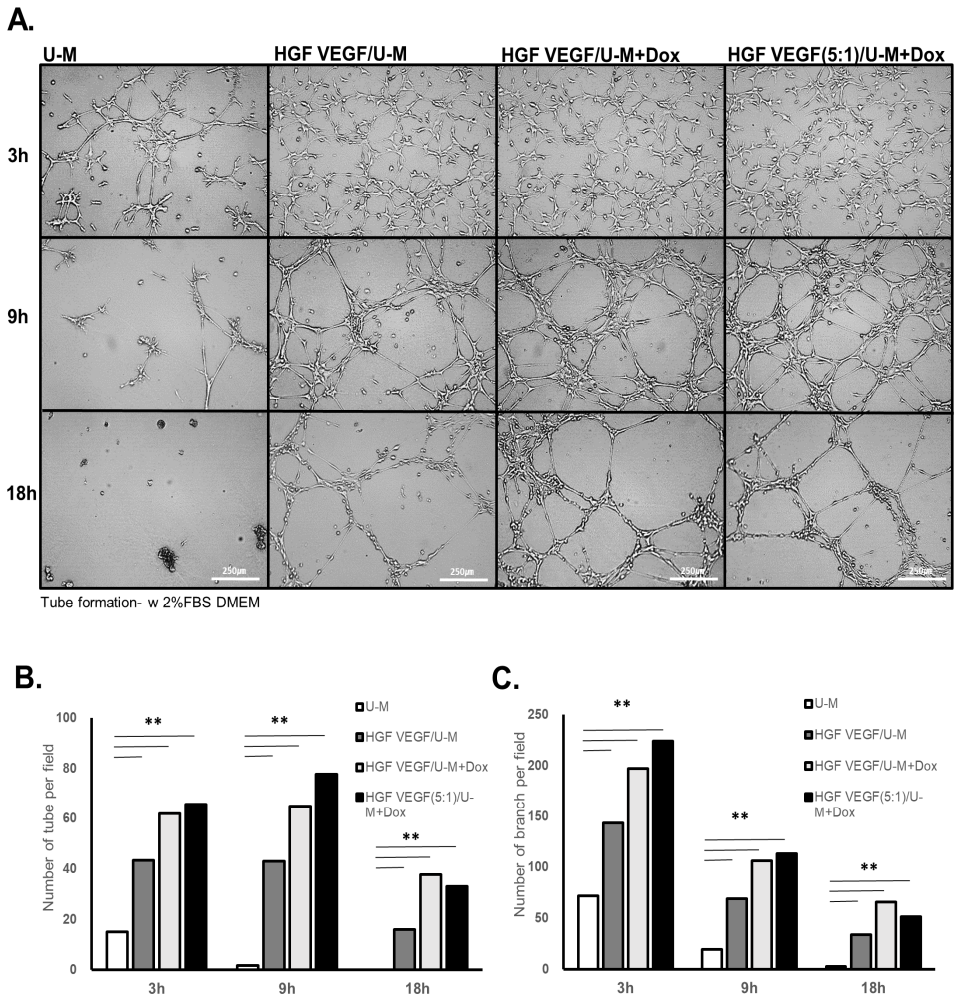
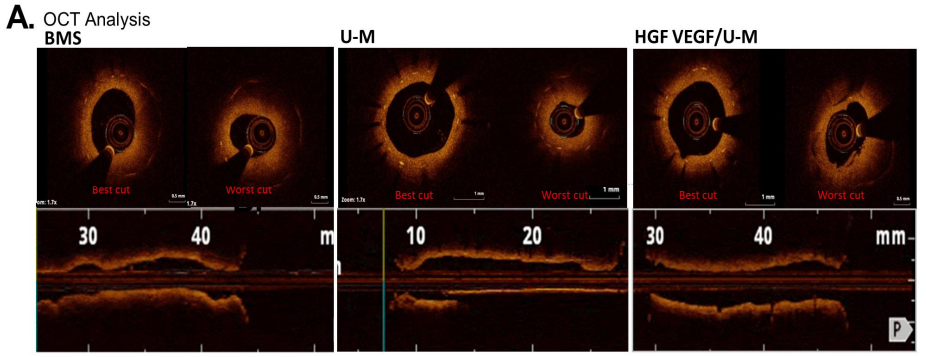
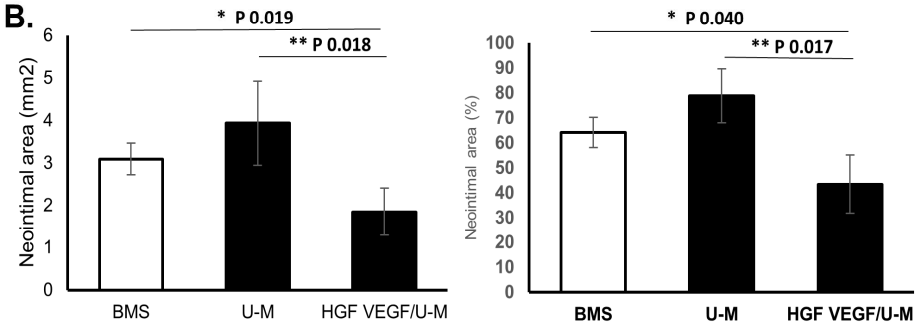


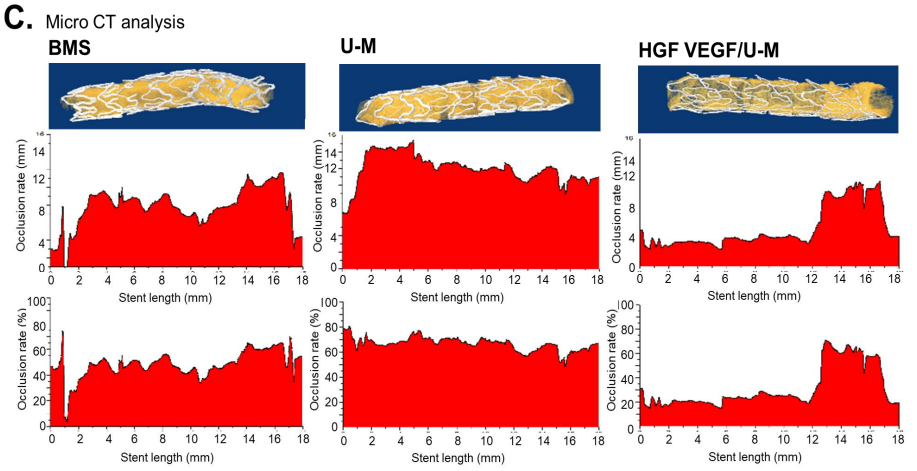
Fig. 2.6. HGF+VEGF/hUCB-MSCs enhanced tube formation upon HGF+VEGF induction. (A) Images of tube formation were acquired for the hUCB-MSCs, HGF+VEGF/hUCB-MSCs (1:1 ratio) without Doxycycline (Dox) and with Dox, and HGF+VEGF (5:1 ratio)/hUCB-MSCs with Dox. (B-C) Tube formation was analyzed based on the number of tubes (B) and the number of tube branches (C) (n=3).

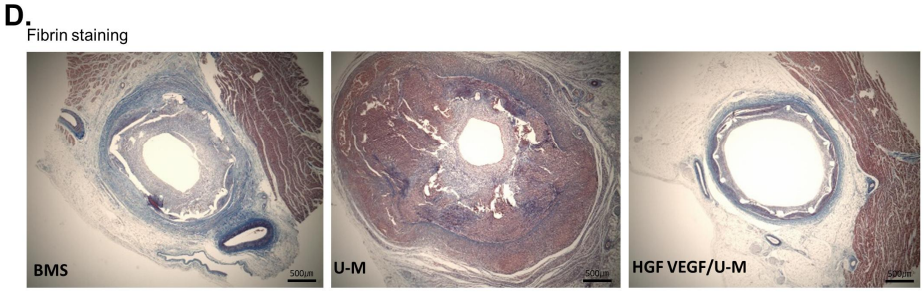
Restenosis reduction in the presence of HGF and VEGF (5:1)

The HGF+VEGF/hUCB-MSC combination stent was transplanted in the swine model to test the efficacy of the stents coated with combination of HGF+VEGF (5:1) secreting stem cells. The swine coronary arteries were observed 4 weeks after transplantation. Compared to the BMS and hUCB-MSC groups, the HGF/hUCB-MSC+VEGF/hUCB-MSC (+Dox) group exhibited the lowest restenosis (Fig 2.7.). The HGF/hUCB-MSC+VEGF/hUCB-MSC group showed the lowest neointimal area by OCT analysis (Fig 2.7. A & B) and mCT analysis (Fig 2.7. C). Fibrin staining also displayed the lowest fibrosis in the HGF/hUCB-MSC+VEGF/hUCB-MSC (+Dox) group (Fig 2.7 D & E). Live angiography analysis also demonstrated normal blood flow in the HGF/hUCB-MSC+VEGF/hUCB-MSC (+Dox) group (Fig 2.7. F). Taken together, these results indicated that the swine coronary stents with HGF/hUCB-MSC+VEGF/hUCB-MSC in a 5:1 combination significantly decreased the neointima and restenosis and thus provided the best blood flow.


A.
OCT Analysis
BMS
U-M
HGF VEGF/U-M
Best cut
Worst cut
30
40
10
20
mm


B.
* P 0.019
** P 0.018
Neointimal area (mm2)
BMS
U-M
HGF VEGF/U-M
* P 0.040
** P 0.017
Neointimal area (%)


C.
Micro CT analysis
BMS
U-M
HGF VEGF/U-M
Occlusion rate (mm)
Occlusion rate (%)
Stent length (mm)


D.
Fibrin staining
BMS
U-M
HGF VEGF/U-M

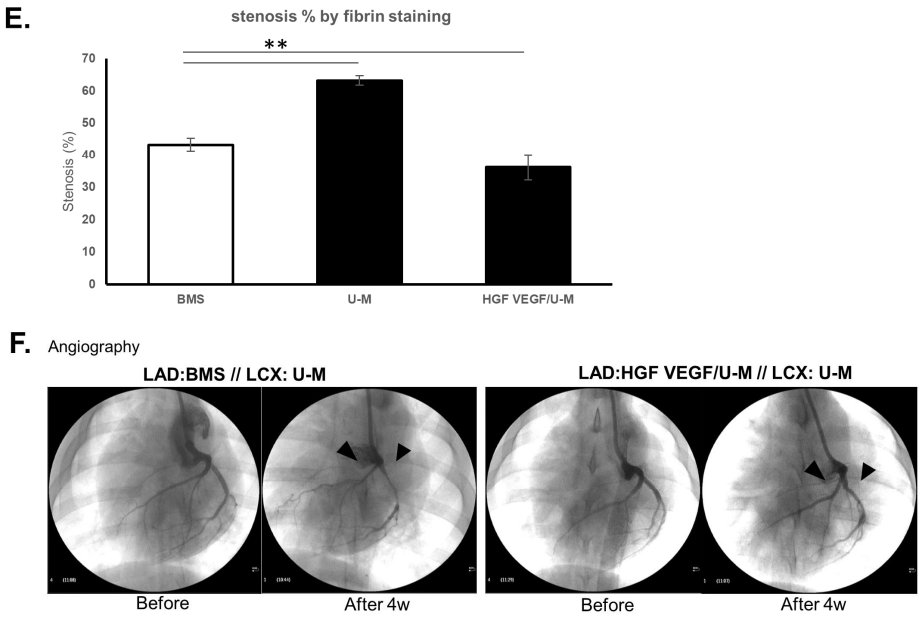
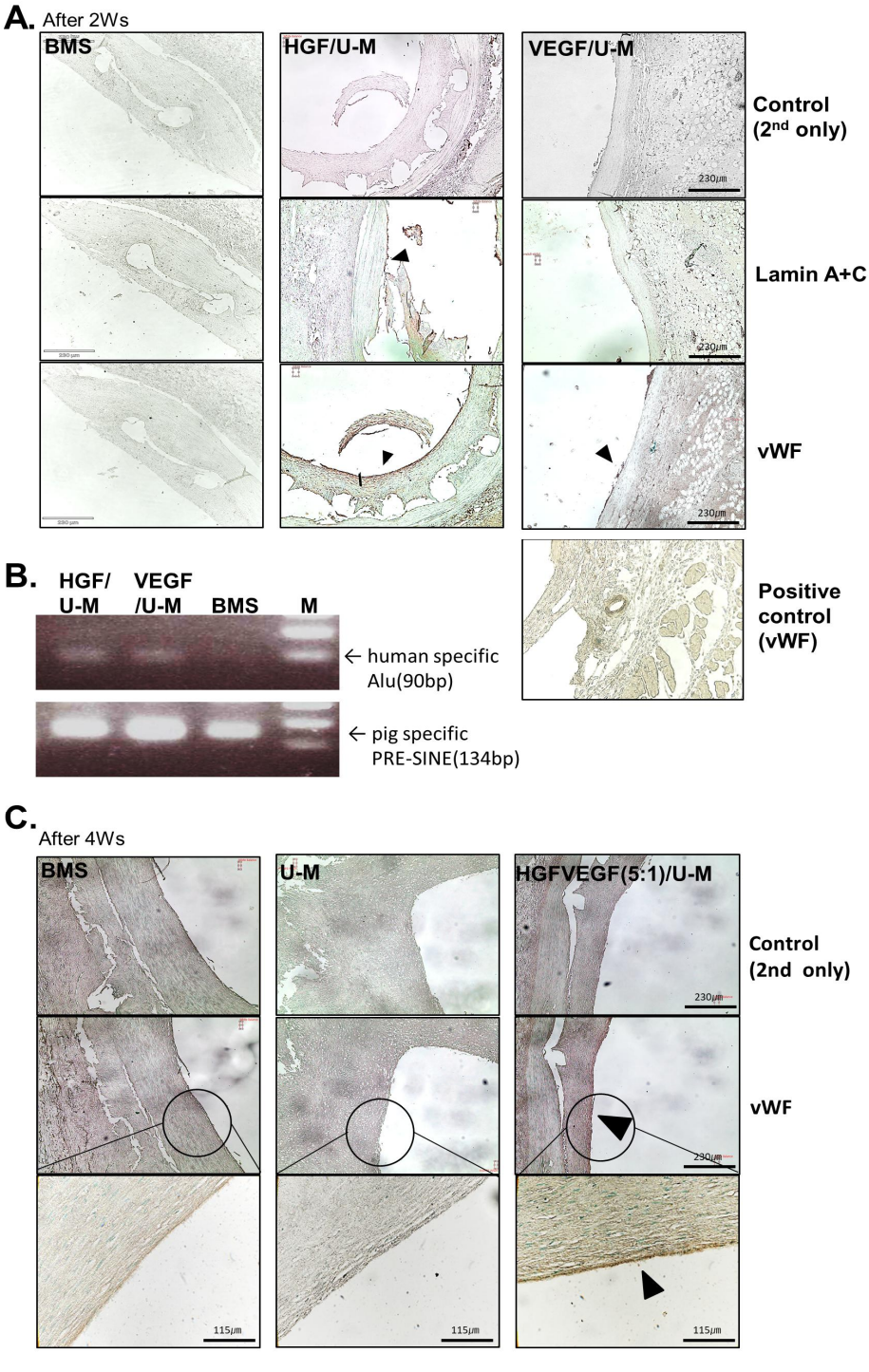


Fig. 2.7. Stents coated with HGF+VEGF/hUCB-MSCs reduced the neointimal area 4 weeks after transplantation. **(A)** OCT results showing the neointima degree in the lumens of the coronary arteries 4 weeks after transplantation. **(B)** The neointima degree was analyzed in mm2 and the % of the neointima area (* p of 0.019 and 0.040, ** p of 0.018 and 0.017). **(C)** Representative microCT images of the stents 4 weeks after transplantation. **(D)** Image of fibrin staining showing the fibrotic area with a cross-section of the transplanted stents. **(E)** The stenosis degrees were assessed by the % of fibrin staining (** p of 0.01). **(F)** Angiography showing the blood flow in the coronary artery with the transplanted stent (BMS; n=6, U-M; n=6, HV/U-M; n=5).

Enhanced reendothelialization in the stent with HGF/hUCB-MSC+VEGF/hUCB-MSC

To visualize the reendothelialization after stent transplantation, we stained the stent area of the coronary artery. Immunostaining for the endothelial cell marker vWF showed EC layers in the HGF/hUCB-MSC and VEGF/hUCB-MSC groups 2 weeks after transplantation (Fig 2.8. A). Four weeks after transplantation, the HGF/hUCB-MSC+VEGF/hUCB-MSC (5:1) group showed vWF staining, indicating reendothelialization, but vWF staining was not observed in the MSC only or BMS groups (Fig 2.8. C). Transplanted human MSCs were observed in the HGF/hUCB-MSC and VEGF/hUCB-MSC groups by genomic PCR with human-specific Alu (Fig 2.8. B). The human-specific Alu was detected in the HGF/hUCB-MSC and VEGF/hUCB-MSC stent groups but not in the BMS group. These results suggested that human cells coated the endothelium after HGF/hUCB-MSC or VEGF/hUCB-MSC transplantation in the stent.


A.
After 2Ws
BMS
HGF/U-M
VEGF/U-M
Control
(2nd only)
Lamin A+C
vWF
Positive
control
(vWF)
B.
HGF/
U-M
VEGF
/U-M
BMS
M
← human specific
Alu(90bp)
← pig specific
PRE-SINE(134bp)
C.
After 4Ws
BMS
U-M
HGFVEGF(5:1)/U-M
Control
(2nd only)
vWF

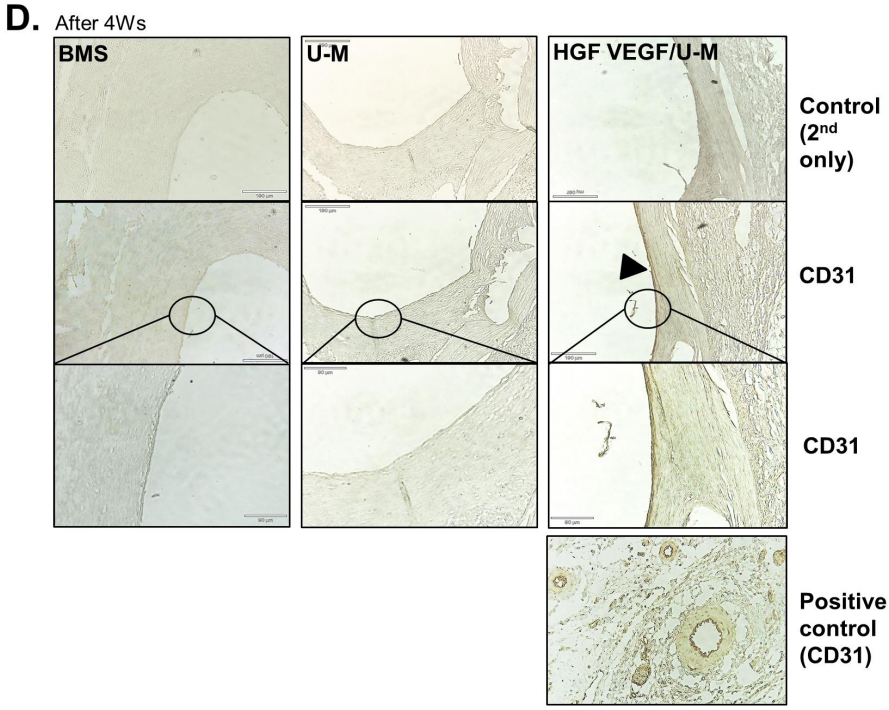


Fig. 2.8. Reendothelialization was observed in coronary arteries with transplanted stents coated with HGF+VEGF/hUCB-MSCs and human MSCs in the swine model. (A) The coronary arteries transplanted with the stem cell-loaded stent were sectioned and stained with anti-vWF (1:200) and anti-Lamin A+C (1:200) antibodies. The samples were harvested 2 weeks after transplantation. (B) Human-specific Alu was detected by RT-PCR in the coronary arteries 2 weeks after transplantation. (C) Anti-vWF immunostaining showing the reendothelialized portion of the coronary arteries 4 weeks after transplantation for the stents coated with the HGF+VEGF/hUCB-MSCs. (D) Anti-CD31 (1:50) immunostaining showing the reendothelialized region of the coronary arteries (n=3).

No tumor formation by HGF/hUCB-MSCs + VEGF/hUCB-MSCs

To confirm the safety of the functional stem cells, we performed the tumor formation assay *in vivo*. Since the UCB stem cells that we used secrete VEGF and HGF growth factors, these cells are speculated to have the potential to generate cancer cells. It is also known that MSCs have a dual potential, either pro- or anti-tumorigenic (Bergfeld et al. 2010). When the HGF/hUCB-MSC+VEGF/hUCB-MSC cells and hUCB-MSC cells were transplanted into nude mice, no tumors were formed in any mouse until 30 days, although the breast cancer cell line MDA-MB-231 formed a tumor mass (Fig 2.9. A & C)). The size of the mixture of HGF/hUCB-MSC+VEGF/hUCB-MSC and Matrigel decreased drastically and disappeared between 1 and 2 weeks (Fig 2.9. B).

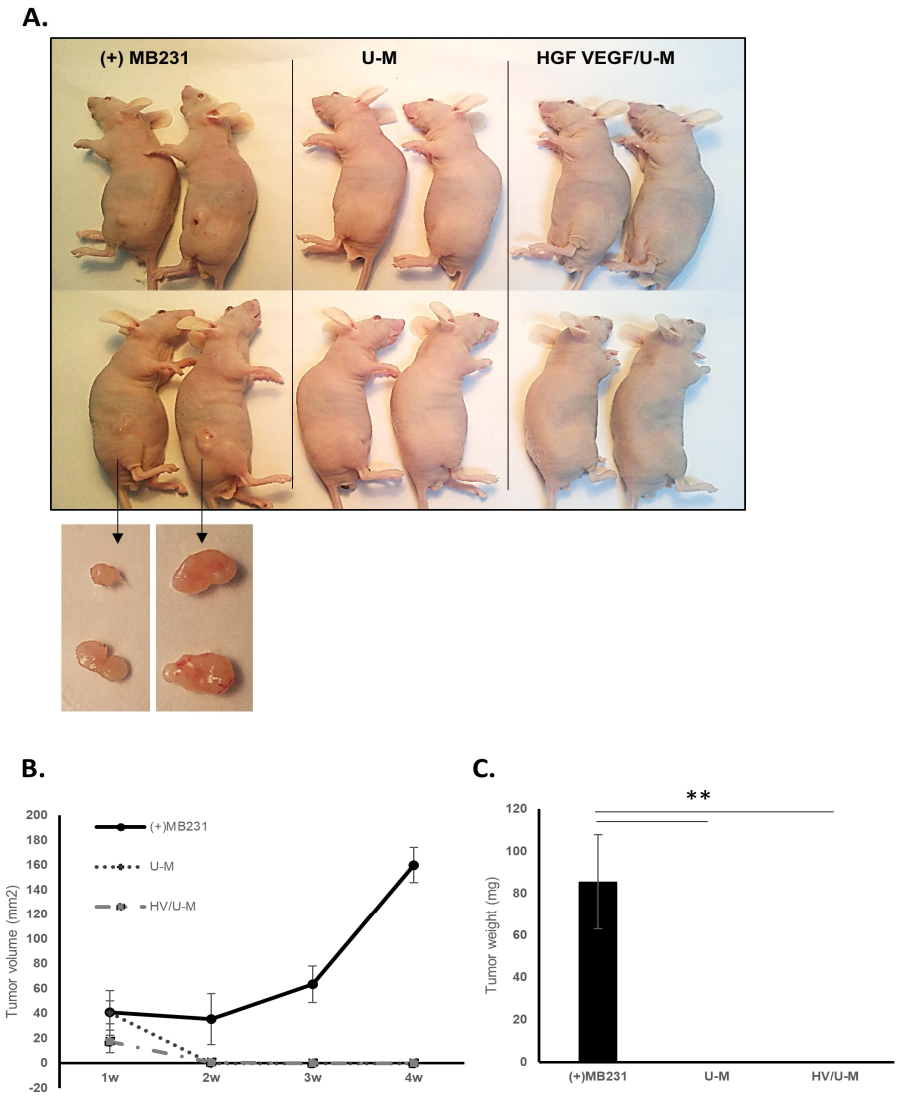


Fig. 2.9. No tumors were formed in nude mice transplanted with the HGF+VEGF/hUCB-MSCs. (A) Tumor formation was observed 4 weeks after cell transplantation in BALB/c nude mice. MDA-MB-231 cells were used as a positive control. Tumors were not formed in the hUCB-MSC and HGF+VEGF/hUCB-MSC groups. (B) The tumor volume was measured every week. (C) The mice were sacrificed and dissected 4 weeks after transplantation, and tumor formation was analyzed (n=4).

2.4. Discussion

Growth factor secreting stem cells play a pivotal role in stimulating reendothelialization. The cells serve as carriers of growth factors and sources of endothelial cells. As carriers, these cells naturally convey HGF and VEGF to other cells, such as endothelial cells. Furthermore, these cells could release HGF and VEGF in response to Doxycycline in a controlled manner. This inducible system can reduce the side effect of growth factor overproduction and help find an optimal condition in stent-mediated reendothelialization.

In this study, the HGF/hUCB-MSCs and VEGF/hUCB-MSCs on the coronary stents were used to prevent restenosis and for therapeutic purposes. We also used these HGF or VEGF releasing functional stem cells as a source of endothelial cells on the stent for reendothelialization. MSCs are known to have a high potential to become endothelial cells (Oswald et al. 2004, Janeczek Portalska et al. 2012). In this experiment, we have shown that HGF or VEGF releasing MSCs can differentiate to endothelial cells to induce tube formation without additional growth factors (Fig 2.6. A). Although angiogenesis and reendothelialization are distinct processes, these phenomena share the same regulatory molecular mechanism (2000, Bergfeld et al. 2010). By improving tube formation, we confirmed the reendothelialization of MSCs *in vitro*. We also showed that the cells transdifferentiated into an endothelial cell layer or at least provided a suitable micro-environment to form endothelial cells in the swine model (Fig 2.8.).

However, there are still some hurdles to overcome for these cell-based stent systems. Cell attachment to the stent needs to be improved. In this experiment, a significant number of cells were detached from the stent because of the physical force exerted on the inner and outer surfaces of the stent during transplantation. Cells remained on the lateral side of the stent, but only approximately one-third of the cells remained (Fig 2.4. B). More cells could enable greater restenosis reduction and reendothelialization. In future studies, better cell attachment technology will be applied because studies to improve cell attachment on stents are still ongoing (Zhou et al. 2009, Raina et al. 2014).

In this study, we found the optimal combination of HGF and VEGF secreting MSCs for the coronary stent to accomplish optimal restenosis reduction and endothelialization. We tried several different combinations of these two functional MSCs to find the best combination of HGF and VEGF releasing cells at a ratio of 5:1. VEGF showed a significant effect on neointima reduction in short-term trials (3 days) but not over 2-week or longer periods (Fig 2.5.). VEGF can promote fibrosis in endothelial cells (Yang et al. 2014, Park et al. 2015, Cho et al. 2017). Thus, longer exposure to a higher amount of VEGF might stimulate fibrosis instead of endothelialization. On the other hand, HGF led to a decrease in restenosis in 2 week trials, but this decrease was not enough compared to BMS group. Furthermore, seeding HGF secreting

MSCs alone on the stent could not reduce restenosis 4 weeks after implantation. Several reports indicated that HGF exclusively stimulates endothelial cell growth and reduces fibrosis without the replication of vascular smooth muscle cells (Xia , Van Belle et al. 1998, Hayashi et al. 2000, Ellison et al. 2011, Perin et al. 2011). However, the HGF/hUCB-MSCs could not survive more than 2 weeks *in vivo*. Therefore, a subsidiary support was necessary. To solve this problem, we combined HGF secreting cells with VEGF secreting cells. The cross-talk between HGF and VEGF improves stem cell survival and angio-architecture and increases cell proliferation and migration (Eric Sulplce 2009). Thus, in this study, we found the optimal combination of HGF and VEGF secreting cells to promote reendothelialization and minimize restenosis side effects.

Drug-eluting stents can result in a dramatic reduction in stenosis, and several commercial drug-eluting stents are currently in use (Htay et al. 2005, Taniwaki et al. 2014). However, these stents exhibit the fatal problem of arterial restenosis at later stages. This is in part due to the inability to form a barrier to protect the artery. Thus, reendothelialization is important to protect the coronary artery from forming restenosis (Douglas et al. 2013). Another drawback of drug-eluting stents is that the drug itself is anti-proliferative, with non-specific effects on surrounding cells. Thus, the drug prevents the regeneration of both VSMCs and ECs. There is currently no other way to promote EC regeneration with drug eluting stents without affecting VSMCs. To overcome this, the

functional HGF/VEGF secreting stem cells seeded on the stent can enhance EC regeneration and prevent VSMC growth.

In summary, coating stents with HGF- and VEGF-secreting mesenchymal stem cells reduced the side effects of coronary stents by promoting reendothelialization. HGF promoted natural endothelialization and led to an even lumen side of the vessel wall. VEGF, although it promoted fibrosis, activated cell survival through the controlled release of the appropriate dosage at the appropriate time, thus promoting angiogenesis. Our strategy of growth factor secreting MSCs has significant implications for clinical stent therapy.

General conclusion

In this study, we generated inducible TetOn-HGF/hUCB-MSCs via TALEN-based genome editing. Notably, these cells in RGD-alginate or ECM materials secreted angiogenic growth factors and enhanced therapeutic effect.

In part I, the easily degradable HGF is made persistent by TetOn-HGF/hUCB-MSCs. The cells can be inducible and regulated by simple antibiotics dox. The modified MSCs enhanced cell mobility, protected cells against apoptosis, and improved the level of angiogenesis in a mouse hind-limb ischemia model. By using this system, one of the major problem of gene therapy, controlling the gene expression, was resolved. This system also suggested effective method combine gene therapy and cell therapy.

In part II, we generated the coated stents with HGF- and VEGF-secreting mesenchymal stem cells. The angiogenic growth factors secreting MSCs reduced the restenosis, a side effect of coronary stents, by promoting reendothelialization. Individual HGF or VEGF was not enough to promote reendothelialization but combination of HGF and VEGF highlighted a synergy effect and thus, decreased restenosis. HGF promoted natural endothelialization and led an even vessel wall at the lumen side. VEGF, although known to promote fibrosis, activated cell survival through the controlled release of the

appropriate dosage at the appropriate time, thus promoting angiogenesis.

Our study clearly demonstrated that gene editing allowed hUCB-MSCs to secrete angiogenic growth factor and overcame the limitations of protein based pro-angiogenesis therapies for vascular diseases such as ischemia and stent restenosis. Our strategy of growth factor secreting MSCs has significant implications for clinical therapy.

Reference

Abdi, R., P. Fiorina, C. N. Adra, M. Atkinson and M. H. Sayegh (2008). "Immunomodulation by mesenchymal stem cells: a potential therapeutic strategy for type 1 diabetes." Diabetes 57(7): 1759-1767.

Ahn, J. M., H. J. Sung, Y. H. Yoon, B. G. Kim, W. S. Yang, C. Lee, H. M. Park, B. J. Kim, B. G. Kim, S. Y. Lee, H. J. An and J. Y. Cho (2014). "Integrated Glycoproteomics Demonstrates Fucosylated Serum Paraoxonase 1 Alterations in Small Cell Lung Cancer." Molecular & Cellular Proteomics 13(1): 30-48.

Asahara, T., A. Kawamoto and H. Masuda (2011). "Concise review: Circulating endothelial progenitor cells for vascular medicine." Stem Cells 29(11): 1650-1655.

Asahara, T., T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman and J. M. Isner (1997). "Isolation of putative progenitor endothelial cells for angiogenesis." Science 275(5302): 964-967.

Aziz, S. and D. R. Ramsdale (2005). "Chronic total occlusions--a stiff challenge requiring a major breakthrough: is there light at the end of the tunnel?" Heart 91 Suppl 3: iii42-48.

Baron, U. and H. Bujard (2000). "Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances." Methods Enzymol 327: 401-421.

Bedair, T. M., M. A. ElNaggar, Y. K. Joung and D. K. Han (2017). "Recent advances to accelerate re-endothelialization for vascular stents." J Tissue Eng 8: 2041731417731546.

Bedell, V. M., Y. Wang, J. M. Campbell, T. L. Poshusta, C. G. Starker, R. G. Krug, 2nd, W. Tan, S. G. Penheiter, A. C. Ma, A. Y. Leung, S. C. Fahrenkrug, D. F. Carlson, D. F. Voytas, K. J. Clark, J. J. Essner and S. C. Ekker (2012). "In vivo genome editing using a high-efficiency TALEN system." Nature 491(7422): 114-118.

Bergfeld, S. A. and Y. A. DeClerck (2010). "Bone marrow-derived mesenchymal stem cells and the tumor microenvironment." Cancer Metastasis Rev 29(2): 249-261.

Bibikova, M., D. Carroll, D. J. Segal, J. K. Trautman, J. Smith, Y. G. Kim and S. Chandrasegaran (2001). "Stimulation of homologous recombination through targeted cleavage by chimeric nucleases." Molecular and Cellular Biology 21(1): 289-297.

Boch, J., H. Scholze, S. Schornack, A. Landgraf, S. Hahn, S. Kay, T. Lahaye, A. Nickstadt and U. Bonas (2009). "Breaking the code of DNA binding specificity of TAL-type III effectors." Science 326(5959): 1509-1512.

Bottaro, D. P., J. S. Rubin, D. L. Faletto, A. M. L. Chan, T. E. Kmiecik, G. F. Vandewoude and S. A. Aaronson (1991). "IDENTIFICATION OF THE HEPATOCYTE GROWTH-FACTOR RECEPTOR AS THE C-MET PROTOONCOGENE PRODUCT." Science 251(4995): 802-804.

Califano, J. P. and C. A. Reinhart-King (2010). "Exogenous and endogenous force regulation of endothelial cell behavior." J Biomech 43(1): 79-86.

Caplan, A. I. (2007). "Adult mesenchymal stem cells for tissue engineering versus regenerative medicine." J Cell Physiol 213(2): 341-347.

Cartier, N., S. Hacein-Bey-Abina, C. C. Bartholomae, G. Veres, M.

Schmidt, I. Kutschera, M. Vidaud, U. Abel, L. Dal-Cortivo, L. Caccavelli, N. Mahlaoui, V. Kiermer, D. Mittelstaedt, C. Bellesme, N. Lahlou, F. Lefrere, S. Blanche, M. Audit, E. Payen, P. Leboulch, B. l'Homme, P. Bougneres, C. Von Kalle, A. Fischer, M. Cavazzana-Calvo and P. Aubourg (2009). "Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy." Science 326(5954): 818-823.

Chang, H. K., P. H. Kim, H. M. Cho, S. Y. Yum, Y. J. Choi, Y. Son, D. Lee, I. Kang, K. S. Kang, G. Jang and J. Y. Cho (2016). "Inducible HGF-secreting Human Umbilical Cord Blood-derived MSCs Produced via TALEN-mediated Genome Editing Promoted Angiogenesis." Mol Ther 24(9): 1644-1654.

Cho, H. M., P. H. Kim, H. K. Chang, Y. M. Shen, K. Bonsra, B. J. Kang, S. Y. Yum, J. H. Kim, S. Y. Lee, M. C. Choi, H. H. Kim, G. Jang and J. Y. Cho (2017). "Targeted Genome Engineering to Control VEGF Expression in Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells: Potential Implications for the Treatment of Myocardial Infarction." Stem Cells Transl Med 6(3): 1040-1051.

Chullikana, A., A. S. Majumdar, S. Gottipamula, S. Krishnamurthy, A. S. Kumar, V. S. Prakash and P. K. Gupta (2015). "Randomized, double-blind, phase I/II study of intravenous allogeneic mesenchymal stromal cells in acute myocardial infarction." Cytotherapy 17(3): 250-261.

Critser, P. J. and M. C. Yoder (2010). "Endothelial colony-forming cell role in neoangiogenesis and tissue repair." Curr Opin Organ Transplant 15(1): 68-72.

Das, A. T., L. Tenenbaum and B. Berkhout (2016). "Tet-On Systems For Doxycycline-inducible Gene Expression." Curr Gene Ther 16(3): 156-167.

Deanfield, J. E., J. P. Halcox and T. J. Rabelink (2007). "Endothelial function and dysfunction: testing and clinical relevance." Circulation 115(10): 1285-1295.

Deindl, E. and C. Kupatt (2007). Therapeutic Neovascularization - Quo vadis?, Springer Netherlands.

Devanesan, A. J., K. A. Laughlan, H. R. Girn and S. Homer-Vanniasinkam (2009). "Endothelial progenitor cells as a therapeutic option in peripheral arterial disease." Eur J Vasc Endovasc Surg 38(4): 475-481.

Disease, G. B. D., I. Injury and C. Prevalence (2016). "Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015." Lancet 388(10053): 1545-1602.

Douglas, G., E. Van Kampen, A. B. Hale, E. McNeill, J. Patel, M. J. Crabtree, Z. Ali, R. A. Hoerr, N. J. Alp and K. M. Channon (2013). "Endothelial cell repopulation after stenting determines in-stent neointima formation: effects of bare-metal vs. drug-eluting stents and genetic endothelial cell modification." Eur Heart J 34(43): 3378-3388.

Ellison, G. M., D. Torella, S. Dellegrottaglie, C. Perez-Martinez, A. Perez de Prado, C. Vicinanza, S. Purushothaman, V. Galuppo, C. Iaconetti, C. D. Waring, A. Smith, M. Torella, C. Cuellas Ramon, J. M. Gonzalo-Orden, V. Agosti, C. Indolfi, M. Galinanes, F. Fernandez-Vazquez and B. Nadal-Ginard (2011). "Endogenous cardiac stem cell activation by insulin-like growth factor-1/hepatocyte growth factor intracoronary injection fosters survival and regeneration of the infarcted pig heart." J Am Coll Cardiol 58(9): 977-986.

Emery, D. W. (2011). "The use of chromatin insulators to improve the

expression and safety of integrating gene transfer vectors." Hum Gene Ther 22(6): 761-774.

Enomoto, H., I. Inoki, K. Komiya, T. Shiomi, E. Ikeda, K. Obata, H. Matsumoto, Y. Toyama and Y. Okada (2003). "Vascular endothelial growth factor isoforms and their receptors are expressed in human osteoarthritic cartilage." American Journal of Pathology 162(1): 171-181.

Eric Sulplce, S. D. (2009). "Cross-talk between the VEGF-A and HGF signalling pathways in endothelial cells."

Faxon, D. P., M. A. Creager, S. C. Smith, Jr., R. C. Pasternak, J. W. Olin, M. A. Bettmann, M. H. Criqui, R. V. Milani, J. Loscalzo, J. A. Kaufman, D. W. Jones, W. H. Pearce and A. American Heart (2004). "Atherosclerotic Vascular Disease Conference: Executive summary: Atherosclerotic Vascular Disease Conference proceeding for healthcare professionals from a special writing group of the American Heart Association." Circulation 109(21): 2595-2604.

Ferrara, N. (2004). "Vascular endothelial growth factor as a target for anticancer therapy." Oncologist 9: 2-10.

Ferrara, N., H. P. Gerber and J. LeCouter (2003). "The biology of VEGF and its receptors." Nat Med 9(6): 669-676.

Fife, R., B. Fife, C. Rougraff, G. Proctor and Sledge (1997). "Inhibition of proliferation and induction of apoptosis by doxycycline in cultured human osteosarcoma cells." The Journal of laboratory and clinical medicine 130(5): 530-534.

Fineran, P. C. and E. Charpentier (2012). "Memory of viral infections by CRISPR-Cas adaptive immune systems: acquisition of new information."

Virology 434(2): 202-209.

Fowkes, F. G., D. Rudan, I. Rudan, V. Aboyans, J. O. Denenberg, M. M. McDermott, P. E. Norman, U. K. Sampson, L. J. Williams, G. A. Mensah and M. H. Criqui (2013). "Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis." Lancet 382(9901): 1329-1340.

Fussenegger, M. (2001). "The impact of mammalian gene regulation concepts on functional genomic research, metabolic engineering, and advanced gene therapies." Biotechnol Prog 17(1): 1-51.

Gallo, S., V. Sala, S. Gatti and T. Crepaldi (2014). "HGF/Met Axis in Heart Function and Cardioprotection." Biomedicines 2(4): 247-262.

Gallo, S., V. Sala, S. Gatti and T. Crepaldi (2015). "Cellular and molecular mechanisms of HGF/Met in the cardiovascular system." Clin Sci (Lond) 129(12): 1173-1193.

Gao, C., G. F. V. Gao and Woude (2005). "HGF/SF-Met signaling in tumor progression." Cell Research 15(1): 49-51.

Garikipati, V. N., S. Jadhav, L. Pal, P. Prakash, M. Dikshit and S. Nityanand (2014). "Mesenchymal stem cells from fetal heart attenuate myocardial injury after infarction: an in vivo serial pinhole gated SPECT-CT study in rats." PLoS One 9(6): e100982.

Gnecchi, M., Z. Zhang, A. Ni and V. Dzau (2008). "Paracrine mechanisms in adult stem cell signaling and therapy." Circulation research 103(11): 1204-1219.

Gnecchi, M., Z. P. Zhang, A. G. Ni and V. J. Dzau (2008). "Paracrine

Mechanisms in Adult Stem Cell Signaling and Therapy." Circulation Research 103(11): 1204-1219.

Grines, C. L., M. W. Watkins, G. Helmer, W. Penny, J. Brinker, J. D. Marmur, A. West, J. J. Rade, P. Marrott, H. K. Hammond and R. L. Engler (2002). "Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris." Circulation 105(11): 1291-1297.

Grüntzig, A. (1978). "Transluminal Dilatation of Coronary-Artery Stenosis." The Lancet 311(8058): 263.

Gu, W., X. Hong, C. Potter, A. Qu and Q. Xu (2017). "Mesenchymal stem cells and vascular regeneration." Microcirculation 24(1).

Hare, J. M., J. E. Fishman, G. Gerstenblith, D. L. DiFede Velazquez, J. P. Zambrano, V. Y. Suncion, M. Tracy, E. Ghersin, P. V. Johnston, J. A. Brinker, E. Breton, J. Davis-Sproul, I. H. Schulman, J. Byrnes, A. M. Mendizabal, M. H. Lowery, D. Rouy, P. Altman, C. Wong Po Foo, P. Ruiz, A. Amador, J. Da Silva, I. K. McNiece, A. W. Heldman, R. George and A. Lardo (2012). "Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial." JAMA 308(22): 2369-2379.

Hare, J. M., J. H. Traverse, T. D. Henry, N. Dib, R. K. Strumpf, S. P. Schulman, G. Gerstenblith, A. N. DeMaria, A. E. Denktas, R. S. Gammon, J. B. Hermiller, Jr., M. A. Reisman, G. L. Schaer and W. Sherman (2009). "A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction." J Am Coll Cardiol 54(24): 2277-2286.

Hayashi, K., S. Nakamura, R. Morishita, A. Moriguchi, M. Aoki, K.

Matsumoto, T. Nakamura, Y. Kaneda, N. Sakai and T. Ogihara (2000). "In vivo transfer of human hepatocyte growth factor gene accelerates re-endothelialization and inhibits neointimal formation after balloon injury in rat model." Gene Therapy 7(19): 1664-1671.

Hayashi, K., S. Nakamura, R. Morishita, A. Moriguchi, M. Aoki, K. Matsumoto, T. Nakamura, Y. Kaneda, N. Sakai and T. Ogihara (2000). "In vivo transfer of human hepatocyte growth factor gene accelerates re-endothelialization and inhibits neointimal formation after balloon injury in rat model." Gene Ther 7(19): 1664-1671.

Hedman, M., J. Hartikainen, M. Syvanne, J. Stjernvall, A. Hedman, A. Kivela, E. Vanninen, H. Mussalo, E. Kauppila, S. Simula, O. Narvanen, A. Rantala, K. Peuhkurinen, M. S. Nieminen, M. Laakso and S. Yla-Herttuala (2003). "Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT)." Circulation 107(21): 2677-2683.

Heldman, A. W., D. L. DiFede, J. E. Fishman, J. P. Zambrano, B. H. Trachtenberg, V. Karantalis, M. Mushtaq, A. R. Williams, V. Y. Suncion, I. K. McNiece, E. Ghersin, V. Soto, G. Lopera, R. Miki, H. Willens, R. Hendel, R. Mitrani, P. Pattany, G. Feigenbaum, B. Oskouei, J. Byrnes, M. H. Lowery, J. Sierra, M. V. Pujol, C. Delgado, P. J. Gonzalez, J. E. Rodriguez, L. L. Bagno, D. Rouy, P. Altman, C. W. Foo, J. da Silva, E. Anderson, R. Schwarz, A. Mendizabal and J. M. Hare (2014). "Transendocardial mesenchymal stem cells and mononuclear bone marrow cells for ischemic cardiomyopathy: the TAC-HFT randomized trial." JAMA 311(1): 62-73.

Henry, T. D., B. H. Annex, G. R. McKendall, M. A. Azrin, J. J. Lopez, F. J. Giordano, P. K. Shah, J. T. Willerson, R. L. Benza, D. S. Berman, C. M.

Gibson, A. Bajamonde, A. C. Rundle, J. Fine, E. R. McCluskey and V. Investigators (2003). "The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis." Circulation 107(10): 1359-1365.

Hern, D. L. and J. A. Hubbell (1998). "Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing." Journal of Biomedical Materials Research 39(2): 266-276.

Hirschi, K. K., D. A. Ingram and M. C. Yoder (2008). "Assessing identity, phenotype, and fate of endothelial progenitor cells." Arterioscler Thromb Vasc Biol 28(9): 1584-1595.

Hockemeyer, D., F. Soldner, C. Beard, Q. Gao, M. Mitalipova, R. C. DeKelver, G. E. Katibah, R. Amora, E. A. Boydston, B. Zeitler, X. D. Meng, J. C. Miller, L. Zhang, E. J. Rebar, P. D. Gregory, F. D. Urnov and R. Jaenisch (2009). "Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases." Nature Biotechnology 27(9): 851-U110.

Hoeben, A., B. Landuyt, M. S. Highley, H. Wildiers, A. T. Van Oosterom and E. A. De Bruijn (2004). "Vascular endothelial growth factor and angiogenesis." Pharmacol Rev 56(4): 549-580.

Hoogduijn, M. J., F. Popp, R. Verbeek, M. Masoodi, A. Nicolaou, C. Baan and M. H. Dahlke (2010). "The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy." Int Immunopharmacol 10(12): 1496-1500.

Horvath, P. and R. Barrangou (2010). "CRISPR/Cas, the immune system of bacteria and archaea." Science 327(5962): 167-170.

Hsu, P. D., E. S. Lander and F. Zhang (2014). "Development and

Applications of CRISPR-Cas9 for Genome Engineering." Cell 157(6): 1262-1278.

Htay, T. and M. W. Liu (2005). "Drug-eluting stent: a review and update." Vasc Health Risk Manag 1(4): 263-276.

Huang, N. F. and S. Li (2008). "Mesenchymal stem cells for vascular regeneration." Regen Med 3(6): 877-892.

Irion, S., H. Luche, P. Gadue, H. J. Fehling, M. Kennedy and G. Keller (2007). "Identification and targeting of the ROSA26 locus in human embryonic stem cells." Nat Biotechnol 25(12): 1477-1482.

Janeczek Portalska, K., A. Leferink, N. Groen, H. Fernandes, L. Moroni, C. van Blitterswijk and J. de Boer (2012). "Endothelial differentiation of mesenchymal stromal cells." PLoS One 7(10): e46842.

Kang, B. J., H. Kim, S. K. Lee, J. Kim, Y. Shen, S. Jung, K. S. Kang, S. G. Im, S. Y. Lee, M. Choi, N. S. Hwang and J. Y. Cho (2014). "Umbilical-cord-blood-derived mesenchymal stem cells seeded onto fibronectin-immobilized polycaprolactone nanofiber improve cardiac function." Acta Biomater 10(7): 3007-3017.

Kastrati, A., A. Schomig, R. Dietz, F. J. Neumann and G. Richardt (1993). "Time course of restenosis during the first year after emergency coronary stenting." Circulation 87(5): 1498-1505.

Katsaros, K. M., S. P. Kastl, K. A. Krychtiuk, R. Hutter, G. Zorn, G. Maurer, K. Huber, J. Wojta, G. Christ and W. S. Speidl (2014). "An increase of VEGF plasma levels is associated with restenosis of drug-eluting stents." Eurointervention 10(2): 224-230.

Kaufmann, K. B., H. Buning, A. Galy, A. Schambach and M. Grez (2013). "Gene therapy on the move." EMBO Mol Med 5(11): 1642-1661.

Kawaida, K., K. Matsumoto, H. Shimazu and T. Nakamura (1994). "Hepatocyte growth factor prevents acute renal failure and accelerates renal regeneration in mice." Proc Natl Acad Sci U S A 91(10): 4357-4361.

Kim, P. H., H. G. Yim, Y. J. Choi, B. J. Kang, J. Kim, S. M. Kwon, B. S. Kim, N. S. Hwang and J. Y. Cho (2014). "Injectable multifunctional microgel encapsulating outgrowth endothelial cells and growth factors for enhanced neovascularization." J Control Release 187: 1-13.

Kim, S. H., J. Turnbull and S. Guimond (2011). "Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor." J Endocrinol 209(2): 139-151.

Kleinstiver, B. P., M. S. Prew, S. Q. Tsai, V. V. Topkar, N. T. Nguyen, Z. Zheng, A. P. Gonzales, Z. Li, R. T. Peterson, J. R. Yeh, M. J. Aryee and J. K. Joung (2015). "Engineered CRISPR-Cas9 nucleases with altered PAM specificities." Nature 523(7561): 481-485.

Kopp, J. B. (1998). "Hepatocyte growth factor: Mesenchymal signal for epithelial homeostasis." Kidney International 54(4): 1392-1393.

Kotin, R. M., R. M. Linden and K. I. Berns (1992). "Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination." EMBO J 11(13): 5071-5078.

Lee, J. W., S. H. Lee, Y. J. Youn, M. S. Ahn, J. Y. Kim, B. S. Yoo, J. Yoon, W. Kwon, I. S. Hong, K. Lee, J. Kwan, K. S. Park, D. Choi, Y. S. Jang and M. K. Hong (2014). "A randomized, open-label, multicenter trial for the

safety and efficacy of adult mesenchymal stem cells after acute myocardial infarction." J Korean Med Sci 29(1): 23-31.

Li, L., L. P. Wu and S. Chandrasegaran (1992). "Functional domains in Fok I restriction endonuclease." Proc Natl Acad Sci U S A 89(10): 4275-4279.

Lieber, M. R., Y. Ma, U. Pannicke and K. Schwarz (2003). "Mechanism and regulation of human non-homologous DNA end-joining." Nat Rev Mol Cell Biol 4(9): 712-720.

Lim, K. S., J. K. Park, M. H. Jeong, I. H. Bae, J. W. Nah, D. S. Park, J. M. Kim, J. H. Kim, S. Y. Lee, E. J. Jang, S. Jang, H. K. Kim, D. S. Sim, K. H. Park, Y. J. Hong, Y. Ahn and J. C. Kang (2016). "Effect of stents coated with a combination of sirolimus and alpha-lipoic acid in a porcine coronary restenosis model." J Mater Sci Mater Med 27(4): 66.

Lin, C.-S., Z.-C. Xin, C.-H. Deng, H. Ning, G. Lin and T. Lue (2010). "Defining adipose tissue-derived stem cells in tissue and in culture." Histology and histopathology 25(6): 807-815.

Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup and N. R. Landau (1996). "Homozygous Defect in HIV-1 Coreceptor Accounts for Resistance of Some Multiply-Exposed Individuals to HIV-1 Infection." Cell 86(3): 367-377.

Luo, Y., C. Liu, T. Cerbini, H. San, Y. Lin, G. Chen, M. S. Rao and J. Zou (2014). "Stable enhanced green fluorescent protein expression after differentiation and transplantation of reporter human induced pluripotent stem cells generated by AAVS1 transcription activator-like effector nucleases." Stem Cells Transl Med 3(7): 821-835.

Lv, F. J., R. S. Tuan, K. M. Cheung and V. Y. Leung (2014). "Concise review: the surface markers and identity of human mesenchymal stem cells." Stem Cells 32(6): 1408-1419.

M., M. E. (2000). "Angiogenesis: From the molecular to integrative pharmacology. Proceedings of the 5th biannual meeting. Crete, Greece, July 1-7, 1999." Adv Exp Med Biol 476: 1-382.

Maeder, M. L. and C. A. Gersbach (2016). "Genome-editing Technologies for Gene and Cell Therapy." Mol Ther 24(3): 430-446.

Masaki, H., E. Tateishi-Yuyama, H. Matsubara, T. Murohra, S. Shintani, K. Amano, U. Ikeda, K. Shimada, H. Takahashi, T. Iwasaka and T. Imaizumi (2002). "Therapeutic angiogenesis for patients with critical limb ischemia using autologous bone marrow cell transplantation." Circulation 106(19): 474-474.

Matoba, S., T. Tatsumi, T. Murohara, T. Imaizumi, Y. Katsuda, M. Ito, Y. Saito, S. Uemura, H. Suzuki, S. Fukumoto, Y. Yamamoto, R. Onodera, S. Teramukai, M. Fukushima, H. Matsubara and T. F.-u. S. Investigators (2008). "Long-term clinical outcome after intramuscular implantation of bone marrow mononuclear cells (Therapeutic Angiogenesis by Cell Transplantation [TACT] trial) in patients with chronic limb ischemia." Am Heart J 156(5): 1010-1018.

Matsumoto, K., H. Funakoshi, H. Takahashi and K. Sakai (2014). "HGF-Met Pathway in Regeneration and Drug Discovery." Biomedicines 2(4): 275-300.

Meier, B. (2001). "The first patient to undergo coronary angioplasty--23-year follow-up." N Engl J Med 344(2): 144-145.

Morishita, R., S. Nakamura, Y. Nakamura, M. Aoki, A. Moriguchi, I. Kida, Y. Yo, K. Matsumoto, T. Nakamura, J. Higaki and T. Ogihara (1997).

"Potential role of an endothelium-specific growth factor, hepatocyte growth factor, on endothelial damage in diabetes." Diabetes 46(1): 138-142.

Mortality, G. B. D. and C. Causes of Death (2015). "Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013." Lancet 385(9963): 117-171.

Moscou, M. J. and A. J. Bogdanove (2009). "A simple cipher governs DNA recognition by TAL effectors." Science 326(5959): 1501.

Munoz, E. M. and R. J. Linhardt (2004). "Heparin-binding domains in vascular biology." Arterioscler Thromb Vasc Biol 24(9): 1549-1557.

Murakami, M. and M. Simons (2008). "Fibroblast growth factor regulation of neovascularization." Curr Opin Hematol 15(3): 215-220.

Murray, J. E., N. Laurieri and R. Delgoda (2017). "Proteins." 477-494.

Nagai, T., I. Shiojima, K. Matsuura and I. Komuro (2005). "Promotion of cardiac regeneration by cardiac stem cells." Circ Res 97(7): 615-617.

Nakagami, H., R. Morishita, K. Yamamoto, S. Yoshimura, Y. Taniyama, M. Aoki, H. Matsubara, S. Kim, Y. Kaneda and T. Ogihara (2001). "Phosphorylation of p38 mitogen-activated protein kinase downstream of bax-caspase-3 pathway leads to cell death induced by high D-glucose in human endothelial cells." Diabetes 50(6): 1472-1481.

Nakamura, Y., R. Morishita, J. Higaki, I. Kida, M. Aoki, A. Moriguchi, K. Yamada, S. Hayashi, Y. Yo, H. Nakano, K. Matsumoto, T. Nakamura and T. Ogihara (1996). "Hepatocyte growth factor is a novel member of the

endothelium-specific growth factors: Additive stimulatory effect of hepatocyte growth factor with basic fibroblast growth factor but not with vascular endothelial growth factor." Journal of Hypertension 14(9): 1067-1072.

Naldini, L., K. M. Weidner, E. Vigna, G. Gaudino, A. Bardelli, C. Ponzetto, R. P. Narsimhan, G. Hartmann, R. Zarnegar, G. K. Michalopoulos and et al. (1991). "Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor." EMBO J 10(10): 2867-2878.

Nishida, S., Y. Hirohashi, T. Torigoe, R. Inoue, H. Kitamura, T. Tanaka, A. Takahashi, H. Asanuma, N. Masumori, T. Tsukamoto and N. Sato (2013). "Prostate cancer stem-like cells/cancer-initiating cells have an autocrine system of hepatocyte growth factor." Cancer Sci 104(4): 431-436.

Okunishi, K., M. Dohi, K. Nakagome, R. Tanaka, S. Mizuno, K. Matsumoto, J. i. Miyazaki, T. Nakamura and K. Yamamoto (2005). "A Novel Role of Hepatocyte Growth Factor as an Immune Regulator through Suppressing Dendritic Cell Function." The Journal of Immunology 175(7): 4745-4753.

Olin, J. W., C. J. White, E. J. Armstrong, D. Kadian-Dodov and W. R. Hiatt (2016). "Peripheral Artery Disease: Evolving Role of Exercise, Medical Therapy, and Endovascular Options." J Am Coll Cardiol 67(11): 1338-1357.

Oswald, J., S. Boxberger, B. Jorgensen, S. Feldmann, G. Ehninger, M. Bornhauser and C. Werner (2004). "Mesenchymal stem cells can be differentiated into endothelial cells in vitro." Stem Cells 22(3): 377-384.

Padfield, G. J., D. E. Newby and N. L. Mills (2010). "Understanding the Role of Endothelial Progenitor Cells in Percutaneous Coronary Intervention." Journal of the American College of Cardiology 55(15): 1553-1565.

Papapetrou, E. P., G. Lee, N. Malani, M. Setty, I. Riviere, L. M. Tirunagari, K. Kadota, S. L. Roth, P. Giardina, A. Viale, C. Leslie, F. D. Bushman, L. Studer and M. Sadelain (2011). "Genomic safe harbors permit high beta-globin transgene expression in thalassemia induced pluripotent stem cells." Nat Biotechnol 29(1): 73-78.

Papapetrou, E. P. and A. Schambach (2016). "Gene Insertion Into Genomic Safe Harbors for Human Gene Therapy." Mol Ther 24(4): 678-684.

Park, C. (2016). "Study on Extracellular Matrix-Coated Cardiovascular Materials for Encompassment of Outgrowth Endothelial Cells." UNIVERSITY OF SCIENCE AND TECHNOLOGY.

Park, S., J. W. Kim, J. H. Kim, C. W. Lim and B. Kim (2015). "Differential Roles of Angiogenesis in the Induction of Fibrogenesis and the Resolution of Fibrosis in Liver." Biol Pharm Bull 38(7): 980-985.

Patel, M. R., M. S. Conte, D. E. Cutlip, N. Dib, P. Geraghty, W. Gray, W. R. Hiatt, M. Ho, K. Ikeda, F. Ikeno, M. R. Jaff, W. S. Jones, M. Kawahara, R. A. Lookstein, R. Mehran, S. Misra, L. Norgren, J. W. Olin, T. J. Povsic, K. Rosenfield, J. Rundback, F. Shamoun, J. Tcheng, T. T. Tsai, Y. Suzuki, P. Vranckx, B. N. Wiechmann, C. J. White, H. Yokoi and M. W. Krucoff (2015). "Evaluation and treatment of patients with lower extremity peripheral artery disease: consensus definitions from Peripheral Academic Research Consortium (PARC)." J Am Coll Cardiol 65(9): 931-941.

Perin, E. C., G. V. Silva, D. C. Vela, Y. Zheng, F. Baimbridge, A. Gahremanpour, X. Quan, W. Hahn, J. Kim, K. Wood and M. Kitamura (2011). "Human hepatocyte growth factor (VM202) gene therapy via transendocardial injection in a pig model of chronic myocardial ischemia." J Card Fail 17(7): 601-611.

Ponzetto, C., A. Bardelli, Z. Zhen, F. Maina, P. Dallazonca, S. Giordano, A. Graziani, G. Panayotou and P. M. Comoglio (1994). "A MULTIFUNCTIONAL DOCKING SITE MEDIATES SIGNALING AND TRANSFORMATION BY THE HEPATOCYTE GROWTH-FACTOR SCATTER FACTOR-RECEPTOR FAMILY." Cell 77(2): 261-271.

Porteus, M. H. and D. Baltimore (2003). "Chimeric nucleases stimulate gene targeting in human cells." Science 300(5620): 763.

Prewitz, M. C., F. P. Seib, M. von Bonin, J. Friedrichs, A. Stissel, C. Niehage, K. Muller, K. Anastassiadis, C. Waskow, B. Hoflack, M. Bornhauser and C. Werner (2013). "Tightly anchored tissue-mimetic matrices as instructive stem cell microenvironments." Nature Methods 10(8): 788-+.

Raff, M. (2003). "Adult stem cell plasticity: fact or artifact?" Annu Rev Cell Dev Biol 19: 1-22.

Raina, T., J. Iqbal, N. Arnold, H. Moore, B. Aflatoonian, J. Walsh, S. Whitehouse, K. Al-Lamee, S. Francis and J. Gunn (2014). "Coronary stents seeded with human trophoblastic endovascular progenitor cells show accelerated strut coverage without excessive neointimal proliferation in a porcine model." Eurointervention 10(6): 709-716.

Ransohoff, J. D. and J. C. Wu (2012). "Imaging stem cell therapy for the treatment of peripheral arterial disease." Curr Vasc Pharmacol 10(3): 361-373.

Reejhsinghani, R. and A. S. Lotfi (2015). "Prevention of stent thrombosis: challenges and solutions." Vasc Health Risk Manag 11: 93-106.

Ross, R. (1993). "The pathogenesis of atherosclerosis: a perspective for the 1990s." Nature 362(6423): 801-809.

Sebastiano, V., M. L. Maeder, J. F. Angstman, B. Haddad, C. Khayter, D. T. Yeo, M. J. Goodwin, J. S. Hawkins, C. L. Ramirez, L. F. Z. Batista, S. E. Artandi, M. Wernig and J. K. Joung (2011). "In Situ Genetic Correction of the Sickle Cell Anemia Mutation in Human Induced Pluripotent Stem Cells Using Engineered Zinc Finger Nucleases." Stem Cells 29(11): 1717-1726.

Seo, Y., S. R. Yang, M. K. Jee, E. K. Joo, K. H. Roh, M. S. Seo, T. H. Han, S. Y. Lee, P. D. Ryu, J. W. Jung, K. W. Seo, S. K. Kang and K. S. Kang (2011). "Human umbilical cord blood-derived mesenchymal stem cells protect against neuronal cell death and ameliorate motor deficits in Niemann Pick type C1 mice." Cell Transplant 20(7): 1033-1047.

Serruys, P. W., B. H. Strauss, K. J. Beatt, M. E. Bertrand, J. Puel, A. F. Rickards, B. Meier, J. J. Goy, P. Vogt, L. Kappenberger and et al. (1991). "Angiographic follow-up after placement of a self-expanding coronary-artery stent." N Engl J Med 324(1): 13-17.

Shibuya, M. (2011). "Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies." Genes Cancer 2(12): 1097-1105.

Sigwart, U., J. Puel, V. Mirkovitch, F. Joffre and L. Kappenberger (1987). "Intravascular stents to prevent occlusion and restenosis after transluminal angioplasty." N Engl J Med 316(12): 701-706.

Simons, M., B. H. Annex, R. J. Laham, N. Kleiman, T. Henry, H. Dauerman, J. E. Udelson, E. V. Gervino, M. Pike, M. J. Whitehouse, T. Moon and N. A. Chronos (2002). "Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized,

controlled clinical trial." Circulation 105(7): 788-793.

Smith, J., M. Bibikova, F. G. Whitby, A. R. Reddy, S. Chandrasegaran and D. Carroll (2000). "Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains." Nucleic Acids Res 28(17): 3361-3369.

Smith, S., W. Neaves and S. Teitelbaum (2007). "Adult versus embryonic stem cells: treatments." Science 316(5830): 1422-1423; author reply 1422-1423.

Stefanini, G. G., M. Taniwaki and S. Windecker (2014). "Coronary stents: novel developments." Heart 100(13): 1051-1061.

Sternberg, S. S. (1992). Histology for pathologists, Raven Press.

Takata, M., M. S. Sasaki, E. Sonoda, C. Morrison, M. Hashimoto, H. Utsumi, Y. Yamaguchi-Iwai, A. Shinohara and S. Takeda (1998). "Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells." Embo Journal 17(18): 5497-5508.

Tan, A., M. S. Alavijeh and A. M. Seifalian (2012). "Next generation stent coatings: convergence of biotechnology and nanotechnology." Trends Biotechnol 30(8): 406-409.

Tang, Y., Q. Tang, X. Zhao, L. Qin, L. Shen, J. Cheng, M. I. Ge and Phillips (2005). "Paracrine Action Enhances the Effects of Autologous Mesenchymal Stem Cell Transplantation on Vascular Regeneration in Rat Model of Myocardial Infarction." The annals of thoracic surgery 80(1): 229-237.

Taniwaki, M., G. G. Stefanini, S. Silber, G. Richardt, P. Vranckx, P. W. Serruys, P. E. Buszman, H. Kelbaek, S. Windecker and R. A.-C. Investigators (2014). "4-year clinical outcomes and predictors of repeat revascularization in patients treated with new-generation drug-eluting stents: a report from the RESOLUTE All-Comers trial (A Randomized Comparison of a Zotarolimus-Eluting Stent With an Everolimus-Eluting Stent for Percutaneous Coronary Intervention)." J Am Coll Cardiol 63(16): 1617-1625.

Tao, H. Y., Z. B. Han, Z. C. Han and Z. J. Li (2016). "Proangiogenic Features of Mesenchymal Stem Cells and Their Therapeutic Applications." Stem Cells International.

Tongers, J., J. G. Roncalli and D. W. Losordo (2010). "Role of endothelial progenitor cells during ischemia-induced vasculogenesis and collateral formation." Microvasc Res 79(3): 200-206.

Touyz, R. M. (2004). "Reactive oxygen species and angiotensin II signaling in vascular cells -- implications in cardiovascular disease." Braz J Med Biol Res 37(8): 1263-1273.

Trachtenberg, B., D. L. Velazquez, A. R. Williams, I. McNiece, J. Fishman, K. Nguyen, D. Rouy, P. Altman, R. Schwarz, A. Mendizabal, B. Oskouei, J. Byrnes, V. Soto, M. Tracy, J. P. Zambrano, A. W. Heldman and J. M. Hare (2011). "Rationale and design of the Transendocardial Injection of Autologous Human Cells (bone marrow or mesenchymal) in Chronic Ischemic Left Ventricular Dysfunction and Heart Failure Secondary to Myocardial Infarction (TAC-HFT) trial: A randomized, double-blind, placebo-controlled study of safety and efficacy." Am Heart J 161(3): 487-493.

Trappmann, B., J. E. Gautrot, J. T. Connelly, D. G. Strange, Y. Li, M. L.

Oyen, M. A. Cohen Stuart, H. Boehm, B. Li, V. Vogel, J. P. Spatz, F. M. Watt and W. T. Huck (2012). "Extracellular-matrix tethering regulates stem-cell fate." Nat Mater 11(7): 642-649.

Turturro, M. V., M. C. Christenson, J. C. Larson, D. A. Young, E. M. Brey and G. Papavasiliou (2013). "MMP-sensitive PEG diacrylate hydrogels with spatial variations in matrix properties stimulate directional vascular sprout formation." PLoS One 8(3): e58897.

Urnov, F. D., J. C. Miller, Y. L. Lee, C. M. Beausejour, J. M. Rock, S. Augustus, A. C. Jamieson, M. H. Porteus, P. D. Gregory and M. C. Holmes (2005). "Highly efficient endogenous human gene correction using designed zinc-finger nucleases." Nature 435(7042): 646-651.

Van Belle, E., B. Witzenbichler, D. Chen, M. Silver, L. Chang, R. Schwall and J. M. Isner (1998). "Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis." Circulation 97(4): 381-390.

van Hinsbergh, V. W. (2012). "Endothelium--role in regulation of coagulation and inflammation." Semin Immunopathol 34(1): 93-106.

Versari, D., L. O. Lerman and A. Lerman (2007). "The importance of reendothelialization after arterial injury." Curr Pharm Des 13(17): 1811-1824.

Waite, J. H. (2008). "Surface chemistry - Mussel power." Nature Materials 7(1): 8-9.

Weatherall, D. J. (1995). "Scope and limitations of gene therapy." Br Med Bull 51(1): 1-11.

Wiebe, J., H. M. Nef and C. W. Hamm (2014). "Current Status of Bioresorbable Scaffolds in the Treatment of Coronary Artery Disease." Journal of the American College of Cardiology 64(23): 2541-2551.

Wong, N. D. (2014). "Epidemiological studies of CHD and the evolution of preventive cardiology." Nat Rev Cardiol 11(5): 276-289.

Wozniak, M. A., K. Modzelewska, L. Kwong and P. J. Keely (2004). "Focal adhesion regulation of cell behavior." Biochim Biophys Acta 1692(2-3): 103-119.

Wu, Y., L. Wu, P. Chen, E. Scott and Tredget (2007). "Mesenchymal Stem Cells Enhance Wound Healing Through Differentiation and Angiogenesis." Stem cells 25(10): 2648-2659.

Xia, J.-L. "Hepatocyte growth factor attenuates liver fibrosis induced by gile duct ligation." Epithelial and Mesenchymal cell biology.

Yang, L., J. Kwon, Y. Popov, G. B. Gajdos, T. Ordog, R. A. Brekken, D. Mukhopadhyay, D. Schuppan, Y. Bi, D. Simonetto and V. H. Shah (2014). "Vascular endothelial growth factor promotes fibrosis resolution and repair in mice." Gastroenterology 146(5): 1339-1350 e1331.

Zetsche, B., J. S. Gootenberg, O. O. Abudayyeh, I. M. Slaymaker, K. S. Makarova, P. Essletzbichler, S. E. Volz, J. Joung, J. van der Oost, A. Regev, E. V. Koonin and F. Zhang (2015). "Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System." Cell 163(3): 759-771.

Zhou, Z., S. Shi, M. Song, H. Huang, K. Chen, J. Mi, L. Li, G. Chen, C. Hou, G. Huang and C. Zhu (2009). "Development of transgenic endothelial progenitor cell-seeded stents." J Biomed Mater Res A 91(2): 623-628.

Zou, J., M. L. Maeder, P. Mali, S. M. Pruett-Miller, S. Thibodeau-Beganny, B. K. Chou, G. Chen, Z. Ye, I. H. Park, G. Q. Daley, M. H. Porteus, J. K. Joung and L. Cheng (2009). "Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells." Cell Stem Cell **5**(1): 97-110.

초 록

혈관은 우리 몸을 이루는 중요한 네트워크로 대동맥부터 모세혈관까지 다양한 종류로 구성되어 있다. 이런 혈관에서 질병이 발생하면 치명적이고 근본적으로 치료하기 힘들다. 혈관을 재생시켜 근본적인 치료를 할 수 있는 방법을 찾고자 했고, 이를 위해 줄기세포 치료와 유전자 치료를 접목하였다. 무엇보다 중간엽 줄기세포는 혈관 재생을 도우나 그 생존율과 사이토카인 분비가 한정적이다. 그래서 혈관재생을 돕는 간성장인자 (Hepatocyte Growth Factor) 유전자를 TALEN system을 이용하여 중간엽 줄기세포의 chromosome 내의 safe-harbor site에 통합시켜 영구적으로 혈관성장인자가 발현되도록 하였다. 또한 Tet-on 시스템을 이용하여 성장인자의 발현을 조절하였다. HGF의 통합과 Doxycyclin에 의한 발현조절은 RNA와 단백질 수준에서 검증되었다. 이렇게 검증된 HGF 분비 세포는 단기적으로는 중간엽 줄기세포의 이동을 촉진시키고 장기적으로 세포사멸을 방지하고 혈관형성을 촉진하였다. 이를 RGD-alginate microgel에 캡슐화하여 말초혈관계 질병인 하지허혈 모델에 적용하였다. 그 결과, HGF가 조절 발현되는 줄기세포를 주입하였을 때, 혈관의 재생이 향상되어 하지허혈 증세가 치료됨을 확인하였다. 이를 통해 HGF 조절 발현되는 중간엽 줄기세포가 혈관 신생을 필요로 하는 혈관성 질환의 유용한 치료법이 될 수 있음을 확인했다.

이런 치료효과를 바탕으로, 대동맥 질환 모델에도 적용하였다. 관상동맥경화증 (Atherosclerosis)은 혈관계 질환 중 가장 치명적이며 치료가 어려운 질병이다. 이를 치료하기 위해 스텐트가 이용되고 있지만

재협착증, 협심증 등 부작용이 여전히 존재하며, 이를 해결하기 위해 빠른 재내피화가 스텐트 삽입 후에 일어나야 한다. 재내피화를 촉진시키는 스텐트를 만들기 위해 혈관성장인자인 Hepatocyte growth factor (HGF)와 Vascular endothelial growth factor (VEGF)가 조절 발현되는 줄기세포를 이용하였다. 스텐트 내 HGF와 VEGF가 조절 발현되는 줄기세포가 부착되고 각 성장인자가 발현되었다. 또한 스텐트 위에서도 세포가 성장하고 고유의 성격이 유지됨을 확인하였다. 이를 돼지에 이식하였을 때, 단기적으로는 VEGF가 재협착증을 줄였으나 2주 이상 지나면서 오히려 과도한 재협착증이 생겼다. HGF의 경우, 2주 후에도 재협착이 감소하는 경향이 보이며, 무엇보다 micro CT결과에서 신생된 내막층이 평평한 패턴을 보였다. HGF와 VEGF를 혼합시켰을 때 가장 큰 효과를 보였는데, 4주 후에도 HGF와 VEGF를 5:1 비율로 혼합한 군에 가장 적은 재협착증이 생김을 확인하였다. 이렇게 재협착증이 가장 적게 생긴 HGF와 VEGF 5:1 군에서 재내피화가 촉진되었음을 면역화학염색으로 확인하였다. 또한 스텐트가 삽입된 돼지 심장혈관에서 주입한 사람의 세포를 발견하였다. 이를 통해, 스텐트 내 HGF와 VEGF를 발현하는 줄기세포가 돼지 모델 내에서 재내피화 촉진하고 재협착증을 줄임을 확인하였다. 결론적으로, 성장인자가 조절 발현되는 줄기세포를 통해 혈관 재생을 촉진할 수 있었고, 이를 이용한 치료법의 가능성을 보였다.

주요어 : 혈관 재생, 재내피화, 중간엽 줄기세포, 혈관성장인자, TALEN, 세포치료

학 번 : 2012-23579

감사의 글

2012년 무더운 여름 시작했던 학위가 6년 뒤 또 다른 무더운 여름에 이렇게 끝을 맺을 수 있는 것은 많은 분들의 도움이 있었기에 가능하였습니다.

연구실에 처음 입학하여 부족한 저를 이끌어주시고 연구자로서 필요한 소양들을 지도해 주신 조제열 교수님께 진심으로 감사의 인사를 드립니다. 교수님의 가르침으로 여기까지 올 수 있었고 앞으로 이 배움을 바탕으로 새로운 시작을 할 수 있었습니다.

그리고 처음 프로젝트를 진행할 때부터 마무리까지 지켜봐 주시면 많은 도움과 가르침을 주신 건양대학교 김평환 교수님께 감사의 마음을 전합니다. 또한 실험 진행의 도움뿐만 아니라 논문 정리 및 학위 논문의 방향을 잡아주시고 점검해주신 서울대학교 이항교수님과 강경선 교수님 그리고 차의과학대학교 한동근 교수님께도 깊은 감사 드립니다.

대학원 생활 동안 항상 마음과 지식을 나눠주신 김동욱 박사님과 이강훈 박사님, 함께해 주며 갇힌 저에게 항상 새로운 시각을 제시해준 서나언니와 용인이, 봄이, 보리스, 다빈이에게 고마움을 전합니다. 그 외에도 삶에서 필요한 많은 것들을 알려주신 실험실 선배님들과 제게 많은 도움을 주고 같이 고생한 후배들에게 이 자리를 빌어 고마움을 전합니다.

끝으로 학위과정 동안 전심으로 응원해주고 아낌없이 지지해 주었던 부모님과 오빠에게 진심으로 감사 드리고 사랑한다고

전하고 싶습니다.

학위과정 동안 기쁜 일도 많았지만 지치고 좌절하던 일도 많았습니다. 그럼에도 학위를 마무리하고 새로운 시작을 할 수 있게 된 것은 언제나 함께 하시는 하나님의 사랑이 있기 때문이라 생각합니다.

학위 과정은 마쳤지만 이것이 또 다른 출발이라고 생각합니다. 지금껏 도와주셨던 모든 분들께 보답할 수 있도록 학위 과정 동안 배운 많은 배움을 바탕으로 항상 겸손하며 작은 일부터 최선을 다할 수 있는 사람이 되겠습니다. 다시 한 번 진심으로 감사 드립니다.

2018년 8월

장 현 경 올림