A DISSERTATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Therapeutic potential of hepatocyte growth factor overexpressing human adipose tissue derived mesenchymal stem cells and its mechanism study in an amyotrophic lateral sclerosis mouse model

간세포성장인자 유전자 도입 사람지방유래중간엽줄기세포를 이용한 근위축성측삭경화증 마우스 모델에서 치료효능 및 기전연구

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By

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Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder and seriously affects lower and upper motor neurons, resulting in progressive weakness and atrophy of skeletal muscles. Although several mechanisms have been proposed to likely contribute to

sporadic disease pathogenesis, the etiology of motor neuron death remains elusory and there exists no method for effective treatment of ALS. Recently, stem cell therapy has been a promising therapeutic approach for this devastating disorder and has raised great expectations.

In this study, hepatocyte growth factor overexpressing human adipose tissue derived mesenchymal stem cells (HGF-hATMSCs) were generated by liposomal transfection with pMEX expression vector and their therapeutic potential for ALS was assessed *in vitro*. To assess the bioactivity of HGF-hATMSCs engineered to overexpress HGF following pMEX expression vector-mediated transduction, expression of the human HGF was evaluated by RT-PCR and ELISA. The secretion of HGF protein in HGF-hATMSCs increased by 7.7-fold compared to the control. These results demonstrate that HGF-hATMSCs were successfully generated by liposomal transfection. Immunophenotypic characteristics of HGF-hATMSCs were confirmed by positivity to CD29, CD44, CD73, CD90, and CD105 markers and negativity to CD31, CD34, CD45, and HLA-DR using flow cytometry. There was no difference in expression of surface markers between HGF-hATMSCs and control (unmodified hATMSCs). Thus, the results of flow cytometry analysis indicate that expression of stem cell surface markers did not change due to HGF gene transfection. To investigate the effect of HGF-hATMSCs on motor neuron proliferation, NSC34 cells and HGF-hATMSCs

were co-cultured for 1, 2, and 3 days under transwell co-culture system. The WST-1 cell proliferation assay and trypan blue exclusion assay showed that proliferation of NSC34 cells was significantly promoted in a time-dependent manner in the HGF-hATMSCs groups. To assess the stimulatory effect of HGF-hATMSCs on motor neuron proliferation, cell cycle analysis was performed after culturing NSC34 cells and HGF-hATMSCs under indirect co-culture system. NSC34 cells were harvested for cell cycle analysis by flow cytometry. Cell cycle analysis showed that 73.27±4%, 10.68±2%, and 15.23±1.5% of control (NSC34 cells alone) were in G0/G1, S, and G2/M phases, respectively. For NSC34 cells after co-culturing with hATMSCs, G0/G1, S, and G2/M phases represented 63.1±2.1%, 15.1±1.83%, and 20.4±2% of the cell population, respectively. In the NSC34 cells co-cultured with HGF-hATMSCs, G0/G1, S, and G2/M phases represented 57.12±1.5%, 17.23±1.1%, and 24.1±1.3% of NSC34 cell population, respectively. The results of cell cycle analysis indicate that HGF-hATMSCs contribute to proliferation of NSC34 cells via the increase of S and G2/M phases during NSC34 cell cycle. Results from western blot analysis showed marked increases in the expression of cyclin D1 involved in S phase and mitosis, when NSC34 cells was co-cultured with HGF-hATMSCs or hATMSCs, and revealed that HGF produced from HGF-hATMSCs activates c-Met known as its receptor in NSC34 cells. These data show that HGF-hATMSCs can

contribute to enhancing motor neuron proliferation. To investigate whether HGF-hATMSCs have a stimulatory effect on survival of motor neurons after induction of endoplasmic reticulum (ER) stress, NSC34 cells pre-treated with thapsigargin were cultured with HGF-hATMSCs in an indirect co-culture system. Survival rate of NSC34 cells was assessed by the WST-1 cell proliferation assay. After co-culture with HGF-hATMSCs, NSC34 cells viability (76±2% at Day 1 and 68.4±3% at Day 2) was highly increased compare to that of control (NSC34 cells alone, 52.1±2% at Day 1 and 25.2±1% at Day 2) or that of co-culture with hATMSCs (65.1±3% at Day 1 and 48.3±2% at Day 2). The results indicate that HGF-hATMSCs can indirectly stimulate survival of NSC34 cells when they were received ER stress.

In addition, to determine inhibitory effect of HGF-hATMSCs on apoptosis of motor neurons, NSC34 cells, after co-culture with HGF-hATMSCs, were analyzed by Annexin V and Propidium Iodide (PI) staining and flow cytometry. Annexin V positive cells were prominently decreased in NSC34 cells co-cultured with HGF-hATMSCs (8.6±0.2%) compared with control (28.6±0.1%). NSC34 cells co-cultured with hATMSCs showed decrease of Annexin V positive cells (22.6±0.1%) than control. Consistent with inhibitory effect of HGF-hATMSCs on apoptosis of motor neurons, the results of western blot analysis demonstrated that activation of PARP and

caspase 3 was markedly lower in NSC34 cells co-cultured with HGF-hATMSCs than control. Taken together, these results show that HGF-hATMSCs can strongly inhibit apoptosis of NSC34 cells.

Therapeutic potential of HGF-hATMSCs for ALS was assessed *in vivo*. To investigate therapeutic effects of HGF-hATMSCs in a mouse model of ALS, the SOD1 G93A transgenic mice were divided into three groups including treatment with HGF-hATMSCs, hATMSCs, and saline as control, and were administrated by intra-spinal cord injection. After transplantation of HGF-hATMSCs, diseases onset point and rotarod failure were assessed to test progression of symptoms and the motor function. Treatment of HGF-hATMSCs significantly delayed symptom onset (111±2.7 days of age) in the SOD1 G93A transgenic mice compared to that of control (101±2.4 days of age). Symptom onset of the hATMSCs-treated group (108±1.6 days of age) began slower than that of control group. Average time of rotarod failure in HGF-hATMSCs-treated group, hATMSCs-treated group, and control group was 131.7±3.2, 128.1±3.1, and 120.8±2.9 days after birth, respectively. These results indicate that HGF-hATMSCs contribute to improvement of motor function and retardation of symptom onset in the SOD1 G93A transgenic mice. Furthermore, the lifespan of the mice was remarkably prolonged in HGF-hATMSCs-treated group (141.6±4.1 days) compare to the control group (127±4.1 days). The lifespan of hATMSCs-treated group

was longer (135.8±2.7 days) than that of control group.

In conclusion, these data show that HGF-hATMSCs have a clear therapeutic potential in slowing down the disease progression in mice model with ALS and their application present the possibility as a novel approach for ALS treatment.

A new and useful alternative gene based stem cell therapy that can enable motor neuron regeneration or cell transplantation with which the limitation of ALS therapy for future use in human clinical applications as well as veterinary medicine may be overcome.

Keywords: amyotrophic lateral sclerosis / apoptosis / hepatocyte growth factor / human adipose tissue derived mesenchymal stem cells / motor neuron proliferation

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transgenic mice

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ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
ATMSCs	Adipose tissue derived mesenchymal stem cells
bFGF	Basic fibroblast growth factor
CCR1	C-C chemokine receptor type 1
CO_2	Carbon dioxide
C9orf72	Chromosome 9 open reading frame 72
CREG	Cellular repressor of E1A-stimulated genes
CXCR4	CXC chemokine receptor 4
DMEM	Dulbecco's modified eagle's medium
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
FITC	Fluorescein isothiocyanate
FUS/TLS	RNA binding protein fused in sarcoma/translocated in sarcoma
GAPDH	Glyceraldehyde phosphate dehydrogenase
GDNF	Glial derived neurotrophic factor
GRPs	Glial restricted progenitor cells
hATMSCs	Human adipose tissue derived mesenchymal stem cells
HGF	Hepatocyte growth factor
iNOS	Inducible nitric oxide synthase
iPSCs	Induced pluripotent stem cells
IRB	Institutional review board

mRNA	Messenger RNA
MSCs	Mesenchymal stem cells
NSCs	Neural stem cells
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDF1	Stromal cell-derived factor 1
SF	Scatter factor
SFRP2	Secreted frizzled-related protein 2
SOD1	Cu/Zn superoxide dismutase 1
TDP43	TAR DNA binding protein 43
VEGF	Vascular endothelial growth factor

LITERATURE REVIEW

1. ALS

ALS, also known as Lou Gehrig's disease and motor neuron disease, is a specific disease that causes the death of neurons which control voluntary muscles. Some also use the term motor neuron disease for a group of conditions of which ALS is the most common. ALS is characterized by stiff muscles, muscle twitching, and gradually worsening weakness due to muscles decreasing in size. This results in difficulty in speaking, swallowing, and eventually breathing. ALS is a rapidly progressing neurodegenerative disease leading almost irrevocably to paralysis and death within 5 years after the first symptoms. The cause is not known in 90% to 95% of cases. About 5–10% of cases are inherited from a person's parents. The diagnosis is based on a person's signs and symptoms with tests to rule out other potential causes (Boillee *et al*. 2006; Cirulli *et al*. 2015).

The pathophysiological mechanism of the disease appears to be multifactorial and several mechanisms contribute to neurodegeneration. An increase of the neurotransmitter glutamate in the synaptic cleft (glutamate excitotoxicity), due to the impairment of its uptake by astrocytes, leads to an

increased influx of Ca^{2+} ions in the motoneurons. The increased levels of Ca^{2+} ions, which in physiological conditions could be removed by mitochondria (calcium homeostasis), remain high in the cytoplasm due to mitochondrial dysfunction and can cause neurodegeneration through activation of Ca^{2+}-dependent enzymatic pathways contributing to oxidative stress. Mutant misfolding proteins SOD1, C9orf72, TDP-43 and FUS form intercellular aggregates, contributing to an increase of oxidative stress and mitochondrial dysfunction, which could lead to the accumulation of neurofilaments and dysfunction of axonal transport. Moreover, activated astrocyte and microglia release inflammatory mediators and toxic factors, contributing to neurotoxicity (Roberta *et al*. 2017).

Despite similar final output of neuronal death, the underlying pathogenic causes are various and no common cause of neuronal damage has been identified to date. Inflammation-mediated neuronal injury is increasingly recognized as a major factor that promotes disease progression and amplifies the motor neuron death-inducing processes. The neuroimmune activation is not only a physiological reaction to cell-autonomous death but also an active component of nonautonomous cell death. Such injury-perpetuating phenomenon is now proved to be a common mechanism in many human disorders characterized by progressive neurodegeneration. Therefore, it represents an interesting therapeutic target.

To date, no single cell population has been proved to play a major role. Anti-inflammatory compounds that are currently undergoing preclinical study and novel suitable molecular targets are also mentioned (Crisafulli *et al*. 2017).

While the genetics of ALS are becoming more understood in familial cases, the mechanisms underlying disease pathology remain unclear and there are no effective treatment options. Without understanding what causes ALS it is difficult to design treatments. However, in recent years stem cell transplantation has emerged as a potential new therapy for ALS patients. While motor neuron replacement remains a focus of some studies trying to treat ALS with stem cells, there is more rationale for using stem cells as support cells for dying motor neurons as they are already connected to the muscle. This could be through reducing inflammation, releasing growth factors, and other potential less understood mechanisms. Prior to moving into patients, stringent pre-clinical studies are required that have at least some rationale and efficacy in animal models and good safety profiles. However, given our poor understanding of what causes ALS and whether stem cells may ameliorate symptoms, there should be a push to determine cell safety in pre-clinical models and then a quick translation to the clinic where patient trials will show if there is any efficacy (Thomsen GM *et al.* 2014).

A medication called riluzole may extend life by about two to three months. Non-invasive ventilation may result in both improved quality and length of life. Since the approval of riluzole, all other therapeutic trials have been negative, including many that followed hopeful preclinical and early clinical data. Therapeutic strategies for the fatal neurodegenerative ALS are actually minimally effective on patients' survival and quality of life.

Although stem cell therapy has raised great expectations, information on the involved molecular mechanisms is still limited. New approaches are needed to uncover effective treatments for this still-devastating disease (Gordon *et al*. 2013; Marconi *et al*. 2013).

2. Stem cell therapy using MSCs

Cell therapy based on stem cells for tissue repair and regeneration holds great therapeutic potential as a sub-classification of regenerative medicine. Stem cell therapy describes the process of introducing stem cells into tissue to treat a disease with or without the addition of gene therapy. ESCs and iPSCs can give rise to almost all cell lineages and are the most promising cells for regenerative medicine. However, they may seriously compromise their utility is their potential for teratoma formation as well as ethical concerns of ESCs (Han *et al*. 2014; Wei *et al*. 2013).

MSCs have therapeutic potential in a variety of diseases (Mimeault *et al*. 2008). MSCs exhibit a limited capacity for self-renewal and are able to differentiate into mesodermal cell types *in vitro* (Yamamoto *et al.* 2007). MSCs are isolated by their capacity to adhere to culture-dish plastic. The cells can be expanded in culture while maintaining their multipotency during standard cell culture and are immunologically characterized by a specific panel of markers (Baer *et al.* 2012). The International Society for Cellular Therapy proposed three minimal criteria for the definition of cultured MSCs; plastic adherence, expression of CD73, CD90, and CD105, and lack of CD11b or CD14, CD 19 or CD79α, CD45, and HLA-DR expression, and their trilineage differentiation potential into adipocytes, chondrocytes, and osteoblasts (Ankrum *et al.* 2010; Dominici *et al.* 2006; Han *et al*. 2014).

Furthermore, MSCs have reduced immunogenic properties and an immunosuppressive potential, which make them also attractive for allogenic stem cell therapy (Han *et al*. 2014; Le Blanc 2003; Mitchell *et al.* 2006). In addition, ideal MSCs for use in therapeutic approaches need to be isolated with minimal harm for the patient, must be available in high cell numbers, proliferate in culture, and differentiate into a broad spectrum of lineages (Fig. 1).

3. Stem cell therapy for ALS

There is much to be investigated about the specific characteristics of stem cells and about the efficacy and safety of the new drugs based on this type of cells, both embryonic as adult stem cells, for several therapeutic indications (cardiovascular and ischemic diseases, diabetes, hematopoietic diseases, and liver diseases). Along with recent progress in transference of nuclei from human somatic cells, as well as iPSC technology, has allowed availability of lineages of all three germ layers genetically identical to those of the donor patient, which permits safe transplantation of organ-tissue-specific adult stem cells with no immune rejection (Liras 2010). Transplanted allogeneic MSCs can be detected in recipients at extended time points, indicating a lack of immune recognition and clearance. As well, a role for bone marrow derived MSCs in reducing the incidence and severity of graft-versus-host disease during allogeneic transplantation has recently been reported (Aggarwal *et al*. 2005).

ALS pathogenesis seems to involve several pathological mechanisms (i.e., oxidative stress, inflammation, loss of the glial neurotrophic support, and glutamate toxicity) with different contributions from environmental and genetic factors. This multifaceted combination highlights the concept that an effective therapeutic approach should

simultaneously counteract different aspects; stem cell therapies are able to maintain or rescue motor neuron function and modulate toxicity in the central nervous system (CNS) at the same time, eventually representing the most comprehensive therapeutic approach for ALS. To achieve an effective cell-mediated therapy suitable for clinical applications, several issues must be addressed, including the identification of the most performing cell source, a feasible administration protocol, and the definition of therapeutic mechanisms. The method of cell delivery represents a major issue in developing cell-mediated approaches since the cells, to be effective, need to be spread across the CNS, targeting both lower and upper motor neurons (Faravelli *et al*. 2014)

MSCs exhibit neuroprotective properties when introduced into the degenerating central nervous system through different putative mechanisms including secretion of growth factors and transdifferentiation (Boucherie *et al*. 2009). Experimental therapeutics for degenerative and traumatic diseases of the nervous system have been recently enriched with the addition of NSCs as alternatives to fetal tissues for cell replacement (Xu *et al*. 2006).

Stem cell based therapies hold great promises in regenerative medicine because of their inherent biological properties of plasticity, self-renewal, and migration. Also, stem cell based therapies are novel therapeutic strategies that hold key for developing new treatments for

diseases and conditions with very few or no cures (Srivastava *et al*. 2014).

Current cell therapy strategies utilize various types of stem cells to study disease pathophysiology, support neurons or surrounding cells through gene therapy or release of neurotrophic factors, or directly replace cells (Gordon *et al*. 2013). Cell replacement therapy has been suggested as a promising therapeutic approach for multiple neurodegenerative diseases, including motor neuron disease (Ferrero *et al*. 2008). ALS is a progressive neurodegenerative disease characterized by motor neuron loss. Although the underlying cause of the disease remains unclear, a variety of pathogenic mechanisms have been proposed. Despite promising preclinical studies showing the modification of the disease progression, most trials have failed to demonstrate any significant improvement in outcome (Kim *et al*. 2010).

Therefore, MSCs are a good candidate for ALS cell therapy; they can survive and migrate after transplantation in the lumbar spinal cord, where they prevent astrogliosis and microglial activation and delay ALS-related decrease in the number of motoneurons, thus resulting in amelioration of the motor performance (Vercelli *et al*. 2008).

4. Genetic modification of MSCs

The ability to induce rapid proliferation of cells with desired

phenotypes is crucial to the success of stem cell therapy and tissue engineering approaches for repairing damaged tissues (Majd *et al.* 2009).

Despite of the benefits of stem cells for tissue regeneration, stem cell therapeutic application is still limited by poor survival and engraftment (Jung *et al.* 2013). In addition, *in vitro* culturing conditions can affect both MSC pluripotency and expression of homing receptors (Han *et al*. 2014; Wagner *et al.* 2007). Therefore, these issues need to be solved for the use of MSC in the clinical field.

Although the mechanisms of the therapeutic effect of MSC are not fully understood, MSC participate in tissue repair by direct transdifferentiating and reducing cell damage. To improve cell survival after injection, genetic modification strategies have been applied (Fig. 2). The overexpression of FGF-2 or Akt increased cell viability and survival rate in the transplanted organ (Gnecchi *et al.* 2005; Tang *et al*. 2005). In addition, SFRP2 was identified as one of the key paracrine factors released by Akt-MSCs and plays a critical role in the survival of ischemic cardiac myocytes (Deng *et al*. 2010; Fan *et al*. 2009; Han *et al*. 2014; Mirotsou *et al*. 2007; Zeng *et al*. 2008).

To enhance stem cell homing, chemokines that induce chemotaxis in nearby cells are considered (Chavakis *et al*. 2008). SDF1 and its receptor CXCR4 are important mediators of stem cell recruitment (Zaruba *et al*.

2009). In addition, CCR1 overexpression was also revealed to induce increase in MSC migration (Han *et al*. 2014; Huang *et al*. 2010).

Several antigenic factors have also been successfully employed for MSC genetic modification. HGF and VEGF increased the density of capillaries and promoted angiogenesis (Duan *et al*. 2003; Han *et al*. 2014; Yang *et al*. 2010).

Besides, genetically engineered MSCs have been used as a potential therapy for various disorders including neurological disease, cancer, bone formation and renal failure (Jung *et al.* 2013). Genetically modified MSCs and their applicability as therapeutic agents remain an emerging and developing field. Gene modification of MSC has attributed to MSC therapy by secreting effective proteins. A new generation of viral vectors or the use of RNA silencing could be used for the genetic modification of MSCs, and the combination with traditional gene modification protocols or alone, is currently under investigation (Han *et al*. 2014; Nixon *et al*. 2007). According to Hodgkinson *et al*. (2010), it was expected that as more becomes understood about MSC biology, the engineering of MSCs will become more refined and useful in the treatment of a greater number of diseases.

5. HGF and c-Met signaling

HGF, a pleiotropic cytokine of mesenchymal origin which promotes migration, proliferation and survival in a wide spectrum of cells, can also modulate various biological responses in stem cells (Forte *et al*. 2006). All biological effects of HGF are mediated by a single tyrosine kinase receptor, c-Met (Nakamura *et al*. 2010; Trusolino *et al*. 2010), and the role of the HGF/c-Met pathway in this process is still unclear (Ishikawa *et al*. 2012). c-Met activation by its ligand HGF induces c-Met kinase catalytic activity that triggers phosphorylation of the tyrosine kinase c-Met receptor (Fig. 3). Signaling initiated by the receptor promotes progression of cell growth through autocrine mechanisms activated by expression of the c-Met ligand, HGF (Li *et al*. 2011).

The HGF/c-Met signaling pathway plays an important role not only in embryogenesis and development but also in angiogenesis. This multifunctional signaling pathway induces mitogenesis, motogenesis, morphogenesis and angiogenesis (You *et al*. 2008). It has been reported that increased HGF supports tissue regeneration by promoting the viability of stem cells, but also by enhancing cell growth in stem cell based tissue engineering (Forte *et al*. 2006; Matsumoto *et al*. 2001).

6. HGF for ALS

HGF prevented induction of caspase-1 and iNOS in motoneurons and retained the levels of the glial-specific glutamate transporter (excitatory amino acid transporter 2/glutamate transporter 1) in reactive astrocytes. HGF is one of the most potent survival-promoting factors for motoneurons, comparable to GDNF *in vitro* (Sun *et al*. 2002).

HGF was initially identified and molecularly cloned as a potent mitogen for mature hepatocytes. Subsequent studies revealed that HGF exerts multiple biological effects, including mitogenic, motogenic, morphogenic, and anti-apoptotic activities in a wide variety of cells, including neurons, by binding to the c-Met receptor tyrosine kinase. HGF is one of the most potent *in vitro* and *in vivo* survival-promoting factors for neurons. For example, neurotrophic effects of HGF have been demonstrated in cultured hippocampal neurons and in cultured embryonic spinal motoneurons, and its anti-apoptotic activity in motoneurons is comparable to that of GDNF. Indeed, reflecting the *in vitro* neurotrophic activity of HGF on motoneurons and the expression of c-Met in motoneurons of G93A mice, the transgenic overexpression of rat HGF in the nervous system attenuates spinal motoneuronal death and axonal degeneration, delays onset of the disease and prolongs the lifespan of G93A mice (Kadoyama *et al*. 2007).

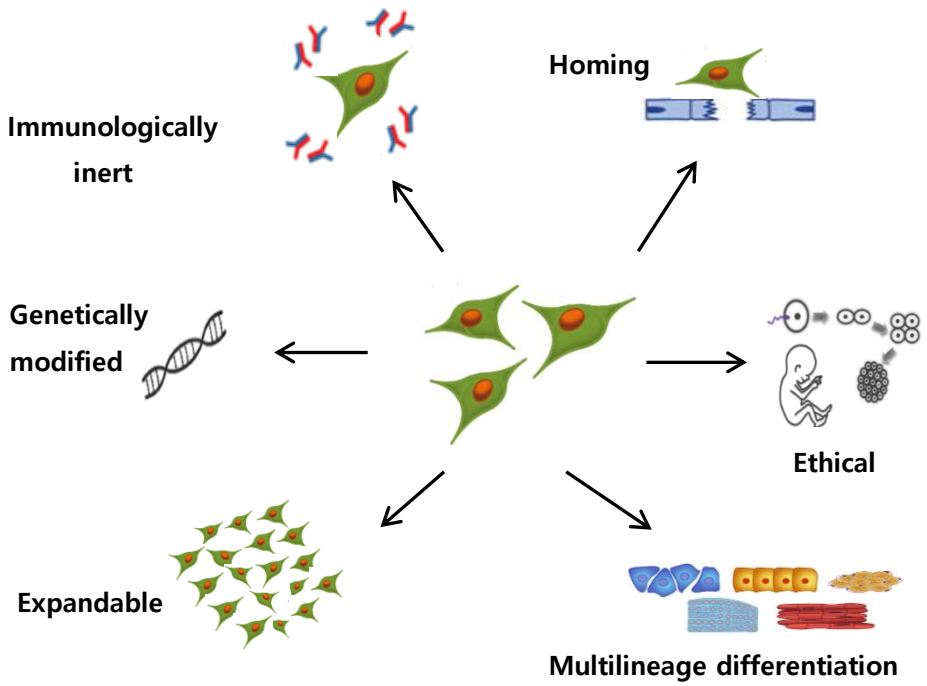

Fig. 1. Advantages of MSCs therapy. The cellular characteristics of MSCs are important for their therapeutic uses. These characteristics include being easily isolated and manipulated *ex vivo*, being immunologically inert, homing into the injury area, and having multilineage differentiation capacity. Genetic engineering of MSCs is aimed at improving their survival and engraftment as well as enhancing their repair mechanisms (adapted from Jung *et al.* 2013).

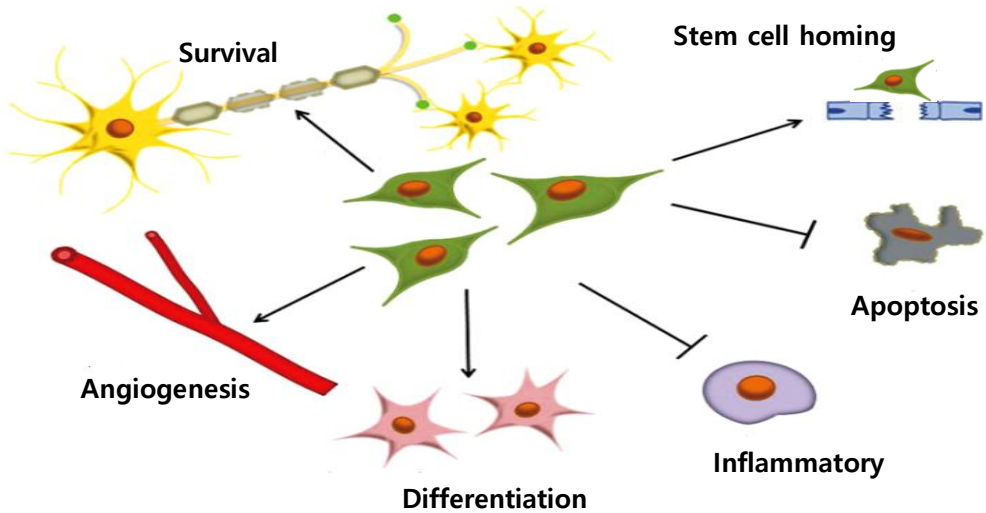

Fig. 2. Genetic modification of MSCs enhances their therapeutic potential. Genetic modification of MSCs is aimed toward enhancing different cellular process such as MSCs survival after transplantation, homing, differentiation, angiogenesis, anti-apoptosis, and anti-inflammatory effects (adapted from Jung *et al*. 2013).

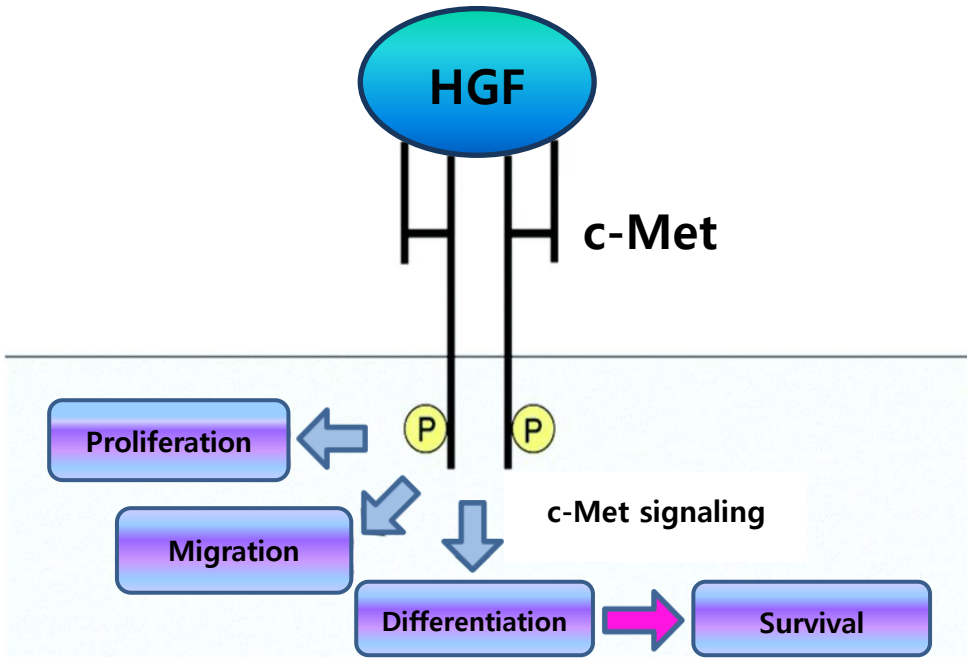

Fig. 3. The interaction of HGF and c-Met. c-Met activation by its ligand HGF induces c-Met kinase catalytic activity that triggers phosphorylation of the tyrosine kinase c-Met receptor mechanisms (adapted from Li *et al*. 2011).

CHAPTER I

Effects of HGF-hATMSCs on proliferation and apoptosis of motor neurons *in vitro*

1. Introduction

ALS, also known as Lou Gehrig's disease, is a fatal disease in which motor neurons die exclusively in the spinal cord and brain. It usually occurs between the ages of 45 and 60, and results in muscle weakness and atrophy. Due to progressive neuronal death, the vast majority of patients die from respiratory muscle paralysis within five years (Boillee *et al*. 2006). The pathogenesis of ALS is believed to be multifactorial. There exist two types of ALS disease; familial ALS and sporadic ALS. Familial ALS occupies only roughly 10% of the onset rate, including mutation of SOD1, TDP43, FUS/TLS, the C9orf72 gene, and the recently discovered TBK1 gene encoding a protein involved in two essential cellular pathways of emerging interest in ALS research; autophagy and inflammation (Cirulli *et al*. 2015). In the common forms of sporadic ALS, neurodegeneration might result from an intricate interaction among various cell types and several different mechanisms, including protein aggregation, glutamate-mediated excitotoxicity, mitochondrial dysfunction, oxidative stress, impaired axonal

transportation, altered glial cell function, and deficiency of neurotrophic factors (Boillee *et al*. 2006). All of these factors can eventually lead to the disruption of axonal transport processes via intracellular accumulation of neurofilaments (Kiernan *et al*. 2011; Robberecht *et al*. 2013). However, the exact cause of sporadic ALS is not known, and to date, there is no cure for this disease.

Stem cell therapy is a promising potential treatment option for ALS because stem cells have remarkable plasticity and ability to differentiate into multiple neuronal lineages (Gordon *et al*. 2013). When locally or systemically transplanted, stem cells are capable of migrating to disease-associated loci to exert the desired therapeutic effect (Srivastava *et al*. 2014). Several types of stem cells have been studied as possibilities for treating ALS, including NSCs, MSCs, GRPs, ESCs, and IPSCs (Traub *et al*. 2011). Their application may take advantage to modify disease pathophysiology (Karumbayaram *et al*. 2009), slow down or even halt the progression of disease, possibly by providing protective or proliferative factors to surrounding cells, inhibiting inflammation, modulating the host immune environment, or even replacing damaged cells (Boucherie *et al*. 2009; Corti *et al*. 2007; Corti *et al*. 2010; Kim *et al*. 2010; Ohnishi *et al*. 2009; Xu *et al*. 2006).

MSCs can be easily obtained from adult tissues, and their application does not raise substantial ethical issues (Liras 2010; Pflegerl *et al*. 2008), and since ALS does not influence MSCs expansion and differentiation (Ferrero *et al*. 2008), the cells can be extracted from patients themselves, thus avoiding immune rejection. Thus, MSCs are an attractive candidate for ALS cell therapy, and a number of studies have investigated their therapeutic potential by injecting cells either peripherally or directly into the spinal cord of animal models of ALS. The publication assessed the efficacy of the systemic administration of ATMSCs in SOD1-mutant mice and showed that the cells not only significantly delayed loss of motor function for 4–6 weeks and maintained the number of motor neurons but also up-regulated the levels of GDNF and bFGF in the spinal cord (Marconi *et al*. 2013). These findings indicated that ATMSCs may promote neuroprotection either directly or by modulating the response of local glial cells toward a neuroprotective phenotype (Marconi *et al*. 2013). In another study, MSCs were genetically modified to release GDNF or VEGF, and when injected into animals, they extended survival and reduced the loss of motor function (Krakora *et al*. 2013). Given the fact that MSCs can deliver various effective factors such as neurotrophic, anti-inflammatory, and immunomodulatory molecules (Aggarwal *et al*. 2005; Mahmood *et al*. 2003), application of these cells is a promising treatment approach for ALS.

The HGF has multiple biological functions required for generation, development and maturation of parenchymal organs. HGF is expressed mainly by mesenchymal cells, while c-Met is identified on the parenchyma during organogenesis (Ohmichi *et al*. 1998), suggesting that HGF-c-Met signals play an essential role in mammalian and non-mammalian developments. In the previous study, overexpression of HGF-gene prolongs the lifespan of mice during ALS via neurotrophic and anti-inflammatory mechanisms (Sun *et al*. 2002), and this effect was reproducible when recombinant human HGF was locally administered in a rat model of ALS. So, HGF seems to be one of the most potent survival-promoting factors for motor neurons and extensive evaluations of HGF are needed to be considered to improve ALS.

Although several mechanisms have been proposed to likely contribute to sporadic disease pathogenesis, the etiology of motor neuron death remains elusory and there exists no method for effective treatment of ALS. The important impact of this study lies in providing insight regarding improvement of ALS by a new approach using stem cell based HGF gene therapy.

2. Materials and Methods

1) Ethics statement

Subcutaneous abdominal adipose tissue from donor was obtained with the patient's informed, written consent for research use under a protocol approved by the Institutional Review Board of the Biostar RBio Co. Korea (IRB No. RBIO 2016-01-001). The cells were provided to the investigators with all identifying information removed except cell passage number.

2) Cell cultures

Human ATMSCs were maintained in MSC media (MSC proliferation media, Biostar RBio Inc, Seoul, Korea) at subconfluent levels to prevent spontaneous differentiation. The mouse motor neuron cell line (NSC34 cells) was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 U/mL penicillin (Gibco BRL), and 100 μg/mL streptomycin (Gibco BRL) at 37°C in a 5% CO_2-enriched atmosphere.

3) Generation of HGF-hATMSCs

The HGF-pMEX plasmid (Dualsystems Biotech, Zurich, Switzerland) was constructed by inserting the full-length cDNA of human HGF (2.2 kb) into the *Sal*I restriction sites. hATMSCs were plated in a 6-well plate at a density of 2×10^5 cells/well 24 hrs prior to gene transfection. The HGF-pMEX plasmid was transfected into MSCs using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Lipofectamine 2000 reagent (37 μL) and plasmid DNA (15 μg) were diluted in 1 mL of serum-free medium, followed by equilibration at room temperature for 5 min after mixing. Lipofectamine 2000 reagent-DNA complex was added to hATMSCs in 9 mL of hATMSC culture medium, and the cells were incubated for 6 hrs. The culture medium was changed after 24 hrs of transfection. Infected cells either at P4 or P5 were frozen to maintain a cryopreserved cell stock. hATMSCs labelled with green fluorescent nanoparticles (Cell Stallker CSF, CellTAGen, Daejeon, Korea) were used to analyze their engraftment in spinal cord of SOD1-mutant transgenic mice model.

4) Flow cytometry analysis of stem cell surface markers

HGF-hATMSCs (2×10^5 cells) were suspended in 100 μL of phosphate-buffered saline (PBS) containing 5% bovine serum

albumin(Gibco BRL, USA) and were stained with fluorescein isothiocyanate (FITC)-conjugated CD31, CD34, CD45, and HLA-DR (1:100; BD Biosciences, Franklin Lakes, NJ, USA), phycoerythrin (PE)-conjugated CD29, CD44, CD73, CD90 (BD Biosciences), and CD105/Endoglin (R&D Systems, Minneapolis, MN, USA) antibodies. The immunophenotype of HGF-hATMSCs was analyzed using a FACS calibur flow cytometer (BD Biosciences) using CELLQuest software (BD Biosciences).

5) RT-PCR analysis

Total RNA was isolated from each sample using Trizol (Invitrogen) following the manufacturer's protocol. Briefly, samples were transferred to a tube containing 1 mL RNA extraction solution. The homogenate was then subjected to chloroform extraction, isopropanol precipitation, ethanol washing, and resuspension in 30 μL distilled water. RNA concentration and purity were determined using a Nanophotometer (Implen, Munich, Germany) at 260 and 280 nm. Samples exhibited an absorbance ratio (260/280) of ≥ 1.8. First-strand cDNA was obtained by reverse transcription using 3 μg total RNA, M-MuLV RT, and an oligo (dT)-18 primer according to the manufacturer's instructions (Invitrogen). Primer sequences are shown in Table 1. PCR products were electrophoresed on 1.5% agarose gels to verify

DNA fragment sizes.

6) ELISA of human HGF

HGF-hATMSCs were incubated in hATMSC culture medium for 24 hrs and the supernatant was collected as HGF-hATMSC-conditioned media, centrifuged for 20 min to remove the cell debris, and stored at -80℃ until use. The human HGF concentration was determined by an enzyme-linked immunosorbent assay (ELISA) using an anti-hHGF monoclonal antibody (RayBiotech, Inc., Madison, WI, USA) according to the manufacturer's instructions.

7) Immunocytochemistry

To perform the immunocytochemistry, the cells with the cover-slip were rinsed with PBS and fixed by 4% PFA for 15 min in a dish. The cells were blocked using 5% BSA with 0.25% Triton x-100 in PBS overnight at 4℃. The following day, the dishes were stained using an anti-hHGF monoclonal antibody (1:500; Santa Cruz Biotechnology) for 2 hrs at 37℃. The samples were then rinsed three times with PBS. Sections were stained with each secondary antibody (1:500) and DAPI (1:200) for 1 hr at room temperature. Finally, the sections were dried and covered on the slide with

the fluorescence mounting medium.

8) Cell proliferation analysis

To evaluate the effect of HGF-hATMSCs on NSC34 cells proliferation, cell proliferation and viability were evaluated using a WST-1 cell proliferation assay and trypan blue exclusion assay. For the WST-1 cell proliferation assay, HGF-hATMSCs were seeded in transwell insert above NSC34 cells present in 12-well plates containing 100 μL of DMEM with 10% FBS at 2×10^4 cells/well and incubated for 1, 2, and 3 days. After separation of each insert containing HGF-hATMSCs, 50 μL of the WST-1 reagent (Roche, Mannhein, Germany) was added to each well containing NSC34 cells, and the reaction proceeded for 2 hrs at 37℃ with 5% CO_2. The absorbance of the samples at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). For trypan blue exclusion assay, NSC34 cells were collected from each well and stained with a 0.4% trypan blue solution. Enumeration of viable cells was carried out under a phase contrast microscope with a hemocytometer. Each experiment was performed in triplicate and repeated three times.

9) Cell cycle analysis

HGF-hATMSCs (1×10^5 cells/well) were plated in transwell insert above NSC34 cells (1.0×10^5 cells/well) present in 6-well plates and co-incubated after 24 hrs. After one day, NSC34 cells were collected by centrifugation, counted and fixed in 70% pre-chilled ethanol overnight. Next day, cells were incubated in PBS containing propidium iodide (PI; 50 μg/mL) and RNase A (50 μg/mL) for 1 hr at room temperature. The fluorescence of 10,000 cells per sample was measured using a FACS calibur flow cytometer (BD Biosciences) using CELLQuest software (BD Biosciences).

10) Survival assay

To determine anti-apoptotic effect of HGF-hATMSCs on survival of NSC34 cells treated with ER stress agent, HGF-hATMSCs (1×10^5 cells/well) were plated in transwell insert above NSC34 cells (1×10^5 cells/well) treated with 400 nM of thapsigargin for 4 hrs. Cells were co-cultured for 1 and 2 days, respectively. After separation of each insert containing HGF-hATMSCs, 50 μL of the WST-1 reagent (Roche, Mannhein, Germany) was added to each well containing NSC34 cells, and the reaction proceeded for 2 hrs at 37℃ with 5% CO_2. The absorbance of the samples at 450 nm was

measured using a microplate reader (Bio-Rad).

11) Annexin V-FITC assay

HGF-hATMSCs (2.0×10^5 cells/well) plated in transwell insert above NSC34 cells (1.0×10^5 cells/well) treated with 400 nM of thapsigargin for 4 hrs. After cells were co-cultured for 2 days, NSC34 cells were treated according to the manufacturer's instructions (BioVision, Mountain View, CA, USA; Annexin V-FITC Apoptosis Detection kit). Briefly, NSC34 cells were collected by centrifugation, resuspended in 500 μL of binding buffer and 5 μL of Annexin V-FITC added. After incubation for 5 min on ice in the dark, 1 μg of PI was added to the cell suspension. The samples were analysed by generating a plot showing FL1 (for Annexin V-FITC) using the flow cytometer.

12) Western blot analysis

Activated c-Met, cyclin D1, cleaved caspase-3, cleaved poly ADP-ribose polymerase (PARP), and β-actin expression levels were examined in NSC34 cells by western blot analysis. NSC34 cells and hATMSCs were indirectly co-cultured in transwell system for 24 hrs. Cells (5×10^6 cells)

were lysed in 200 μL of cold protein lysis buffer (Intron Co. Ltd., Seoul, Korea) for 1 hr and kept on ice to stop further biochemical activity. Protein content was quantified according to the Bradford method before adding sample buffer. Equal amounts of protein samples (30 μg/sample) were loaded on SDS-PAGE gels and transferred to PVDF membranes (Whatman inc, Sanford, ME, USA). The membranes were blocked with Tris-buffered saline-Tween 20 (TBST, 0.1% Tween 20/100 mM NaCl/10 mM Tris-HCl, pH 7.6) containing 5% non-fat dried milk for at least 1 hr and then the blots were probed with c-Met, cyclin D1, caspase-3, PARP, and β-actin antibodies (1:200, 1:500, 1:200, 1:500, and 1:1000 dilution, respectively) at 4℃ overnight. The membranes were washed three times and then incubated with horseradish peroxidase-conjugated anti-IgG (1:2000 dilution) in a blocking buffer for 2 hrs. Finally, the blots were developed with enhanced chemiluminescence (Gen Depot, TX, USA) and exposed to X-ray film (Eastman-Kodak, Rochester, NY, USA). Polyclonal anti-PARP (#9542; Danvers, MA, USA) was purchased from Cell Signaling Technology. Anti-c-Met, anti-cyclin D1 (H-295), anti-caspase-3 (H-277), and anti-β-actin (SC-47778) were obtained from the company (Santa Cruz, CA, USA).

13) Statistical Analysis

All experiments were performed at least twice. Values are expressed as Mean±standard deviation (SD). The probability associated with a Student's *t*-test was performed for comparison of the parameters between 2 groups. *P*-values less than 0.05 were considered statistically significant.

3. Results

1) Increase of HGF expression and secretion in hATMSCs modified with the HGF gene

To assess the bioactivity of HGF-hATMSCs engineered to overexpress HGF following pMEX expression vector-mediated transduction, expression of the human HGF was evaluated by RT-PCR and ELISA (Fig. 4). Expression of HGF mRNA was remarkably increased in HGF-hATMSCs that had been incubated for 24 hrs compared to the control (Fig. 4A). A significant amount of human HGF could be detected by ELISA in the culture supernatants of HGF-hATMSCs that had been incubated for 24 hrs (Fig. 4B). The secretion of HGF protein in HGF-hATMSCs increased by 7.7-fold (2,050±185 pg/mL, $P<0.05$) compared to the control (250±50

pg/mL). Immunofluorescence analysis using a HGF antibody also revealed the expression of HGF in HGF-hATMSCs (Fig. 4C). These results demonstrate that HGF-hATMSCs were successfully generated by liposomal transfection.

2) Immunophenotypic profile of HGF-hATMSCs

To evaluate the immunophenotypic characteristics of HGF-hATMSCs at passage 5 after gene transfection, the expression of stem cell surface markers was analyzed using flow cytometry (Table 2). Immunophenotypic characteristics of HGF-hATMSCs were confirmed by positivity (> 95%) to CD29, CD44, CD73, CD90, and CD105 markers and negativity (< 2%) to CD31, CD34, CD45, and HLA-DR. There was no difference in expression of surface markers between HGF-hATMSCs and control (unmodified hATMSCs). Thus, the results of flow cytometry analysis indicate that expression of stem cell surface markers did not change due to HGF gene transfection.

3) Promotion effect of HGF-hATMSCs on proliferation of motor neurons

To investigate the effect of HGF-hATMSCs on motor neuron

proliferation, NSC34 cells and HGF-hATMSCs were co-cultured for 1, 2, and 3 days under transwell co-culture system. The level of cell proliferation was quantified using the cell proliferation reagent WST-1 and trypan blue exclusion assay (Fig. 5). The WST-1 cell proliferation assay showed that proliferation of NSC34 cells was significantly promoted in a time-dependent manner in the HGF-hATMSCs groups (Fig. 5A). This result was further confirmed by counting the number of viable cells using the trypan blue exclusion assay (Fig. 5B).

4) Increase of S and G2/M phase distribution of motor neurons by contribution of HGF-hATMSCs

To assess the stimulatory effect of HGF-hATMSCs on motor neuron proliferation, cell cycle analysis was performed after culturing NSC34 cells and HGF-hATMSCs under indirect co-culture system for 24 hrs. NSC34 cells (5×10^5 cells) were harvested for cell cycle analysis by flow cytometry. Cell cycle analysis showed that 73.27±4%, 10.68±2%, and 15.23±1.5% of control (NSC34 cells alone) were in G0/G1, S, and G2/M phases, respectively (Fig. 6A). For NSC34 cells after co-culturing with hATMSCs, G0/G1, S, and G2/M phases represented 63.1±2.1%, 15.1±1.83%, and 20.4±2% of the cell population, respectively (Fig. 6A). In the NSC34 cells

co-cultured with HGF-hATMSCs, G0/G1, S, and G2/M phases represented 57.12±1.5%, 17.23±1.1%, and 24.1±1.3% of NSC34 cell population, respectively (Fig. 6A). The results of cell cycle analysis indicate that HGF-hATMSCs contribute to proliferation of NSC34 cells via the increase of S and G2/M phases during NSC34 cell cycle. Progression of cell proliferation during the cell cycle is closely regulated by cyclin D1, a protein that activates cyclin-dependent kinases. Results from western blot analysis showed marked increases in the expression of cyclin D1 involved in S phase and mitosis (Fig. 6B) when NSC34 cells were co-cultured with HGF-hATMSCs or hATMSCs, and revealed that HGF produced from HGF-hATMSCs activates c-Met known as its receptor in NSC34 cells (Fig. 6B). These data show that HGF-hATMSCs can contribute to enhancing motor neuron proliferation.

5) Inhibitory effect of HGF-hATMSCs on apoptosis of motor neurons

To investigate whether HGF-hATMSCs have a stimulatory effect on survival of motor neurons after induction of ER stress, NSC34 cells pre-treated with 400 nM of thapsigargin for 4 hrs were cultured with HGF-hATMSCs in an indirect co-culture system (Fig. 7A). After co-culture for 1 and 2 days, survival rate of NSC34 cells was assessed by the WST-1 cell

proliferation assay (Fig. 7A). After co-culture with HGF-hATMSCs, NSC34 cell viability (76±2% at Day 1 and 68.4±3% at Day 2) was highly increased compared to that of control (NSC34 cells alone, 52.1±2% at Day 1 and 25.2±1% at Day 2) or that of co-culture with hATMSCs (65.1±3% at Day 1 and 48.3±2% at Day 2). The results indicate that HGF-hATMSCs can indirectly stimulate survival of NSC34 cells when they were received ER stress.

In addition, to determine inhibitory effect of HGF-hATMSCs on apoptosis of motor neurons, NSC34 cells after co-culture with HGF-hATMSCs for 2 days were analyzed by Annexin V and Propidium Iodide (PI) staining and flow cytometry (Fig. 7B). As indicated in Fig. 7B, Annexin V positive cells were prominently decreased in NSC34 cells co-cultured with HGF-hATMSCs (8.6±0.2%) compared with control (28.6±0.1%). NSC34 cells co-cultured with hATMSCs showed decrease of Annexin V positive cells (22.6±0.1%) than control. Consistent with inhibitory effect of HGF-hATMSCs on apoptosis of motor neurons, the results of western blot analysis demonstrated that activation of PARP and caspase 3 was markedly lower in NSC34 cells co-cultured with HGF-hATMSCs than control (Fig. 7C). Taken together, these results show that HGF-hATMSCs can strongly inhibit apoptosis of NSC34 cells.

4. Discussion

The potency of stem cell therapy has been verified in experimental models with ALS by using human neuron-like cells (Garbuzova-Davis *et al*. 2006), human NSCs (Xu *et al*. 2006), rodent and human bone marrow derived MSCs (Corti *et al*. 2004; Vercelli *et al*. 2008), and human umbilical cord blood stem cells (Garbuzova-Davis *et al*. 2012; Knippenberg *et al*. 2012). However, the source and availability of stem cells raised an ethical issue for their clinical application and the invasiveness of isolation process may limit their application in humans. In this regard, ATMSCs have been received much attention recently because of the high frequency and expandability and the peculiar adhesion molecule profile which favors their migration into damaged CNS after systemic injection (Constantin *et al*. 2009). Regarding the reason, hATMSC was used as carrier for delivery of HGF gene with their potency for treatment of ALS. Thus, this study investigated whether hATMSCs based gene transfer of HGF, a pleiotrophic cytokine with a highly potent neurotrophic activity for motor neurons, specifically to motor neurons of ALS model mice may play a role in symptom retardation. HGF-hATMSCs were generated using the pMEX expression system, which is a non-viral vector system that avoids some of the disadvantages of viral vector systems, such as immunogenicity, virus-

mediated insertional mutagenesis, high cost, and manufacturing difficulties. Advantage of the pMEX expression vector is that it facilitates high-level, stable target protein expression in mammalian cells. After transfection of hATMSCs with the pMEX expression vector containing the HGF gene, HGF-hATMSCs showed significantly high levels of HGF expression *in vitro* (Fig. 4A, B, and C). In addition, genetic modification of hATMSCs containing the HGF gene did not alter MSC surface markers based on the results of immunophenotyping results obtained from flowcytometric analysis (Table 2). These results demonstrate that HGF-hATMSCs were successfully generated in this study. Although an exact therapeutic mechanism by HGF-hATMSCs on moto neuron-specific disease progression is not verified in this study, the possible events are revealed in the *in vitro* study. This study found that HGF-hATMSCs contribute to proliferation and survival of motor neurons *in vitro*. The results of WST-1 cell proliferation assay and trypan blue exclusion assay showed that motor neurons co-cultured with HGF-hATMSCs had significantly higher viability and proliferation rates than control cells (Fig. 5). These results were further evaluated in cell cycle progression by cell cycle analysis and western blot analysis of phosphorylated c-Met and cyclin D1 (Fig. 6). The cell cycle is a series of events that occurs in a cell leading to its division and replication, and is a critical process for determining cell proliferation and senescence

(Yoon *et al*. 2011). The G0/G1 phase is the non-proliferative or resting phase and the S phase involves DNA replication. In G2 phase, the cell is ready for division and mitosis occurs in the M phase. In cell cycle and western blot analysis, motor neurons co-cultured with HGF-hATMSCs result in an extended S phase and G2/M phase, and upregulation of cyclin D1 indicating their expression influences cell cycle progression and consequently, motor neuron proliferation. Thus, these observations appear to indicate a stronger effect of HGF-hATMSCs on motor neuron proliferation, at least in part, by accelerating the transition of cells from G1 to S phase. In addition, HGF-hATMSCs showed inhibitory effect on apoptosis of motor neurons by using Annexin V and PI staining and flow cytometry (Fig. 7). Western blot analysis demonstrated HGF-hATMSCs can induce anti-apoptotic effect on motor neurons through decrease of activated caspase-3 and PARP expression. Caspase-3 is an effector caspase that cleaves other protein substrates within the cell to trigger the apoptotic process (Alnemri *et al*. 1996; Danial *et al*. 2004), and PARP is a family of proteins involved in cellular processes involving DNA repair and programmed cell death (Dasari *et al*. 2010; Yu *et al*. 2006). PARP can also directly induce apoptosis; this mechanism appears to be caspase-independent.

HGF/SF is a multifunctional growth factor which stimulates the proliferation, motility, or branching morphogenesis of various cell types

(Jeffers *et al*. 1996; Rosen *et al*. 1994). HGF/SF was originally identified as a mitogen for hepatocytes (Nakamura *et al*. 1989), and a motogen (motility stimulator) for epithelial cells (Stoker *et al*. 1987). These diverse actions of HGF all appear to be mediated by the c-Met receptor tyrosine kinase (Bottaro *et al*. 1991; Weidner *et al*. 1993). Both HGF and c-Met are expressed in the nervous system, and possible roles in nervous system development have been suggested on the basis of the ability of HGF to promote neural induction (Stern *et al*. 1990; Streit *et al*. 1995), stimulate Schwann cell proliferation (Krasnoselsky *et al*. 1994), and increase c-Fos expression in septal neurons (Jung *et al*. 1994). Based on presented report, HGF produced by HGF-hATMSCs implicate as a survival factor for motor neurons. Since c-Met is currently the only known receptor for HGF and motor neurons express c-Met, the actions of HGF on motor neurons are mediated by c-Met and probably contribute to their proliferation and survival.

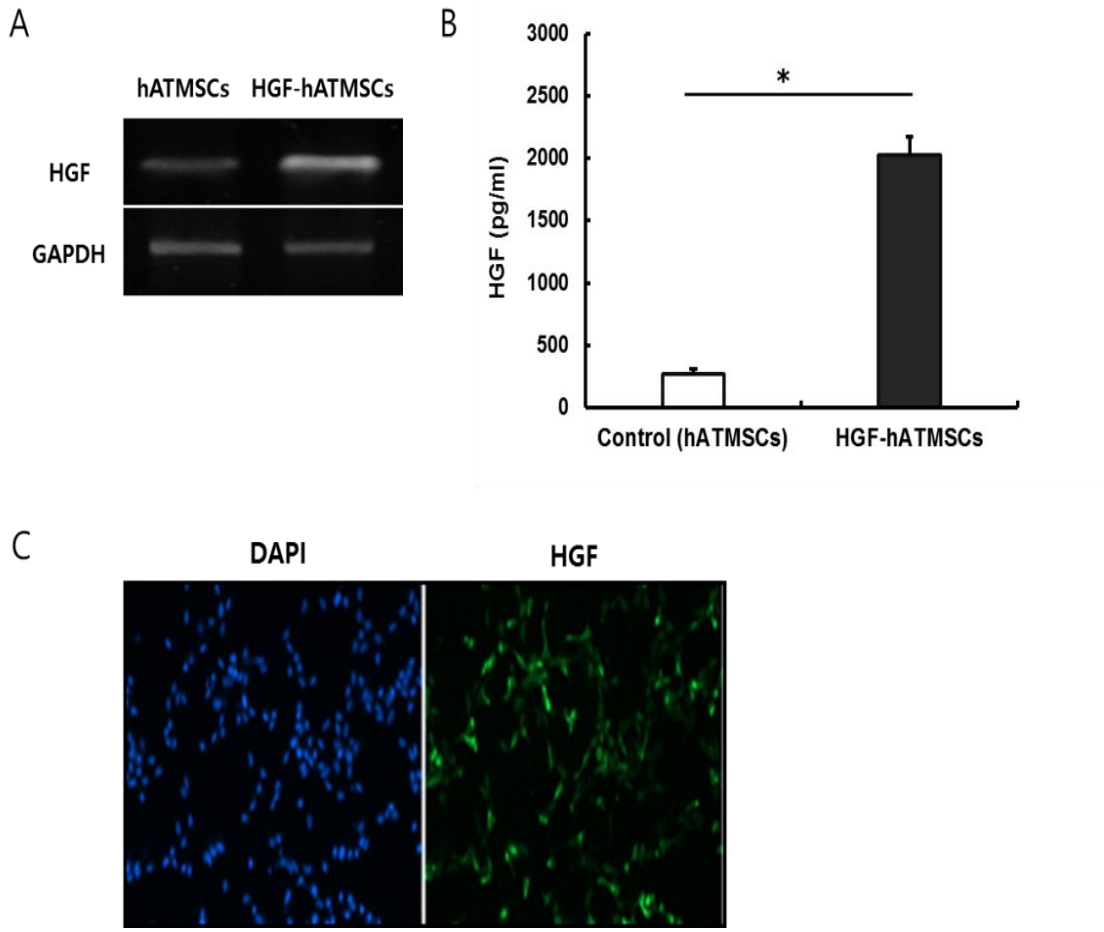

Fig. 4. Expression analysis of HGF in HGF-hATMSCs. HGF expression was analyzed in HGF-hATMSCs cultured for 24 hrs by RT-PCR (A), ELISA (B), and immunofluorescence (C) analysis. The results showed a significant increase in HGF expression at the mRNA (A) and protein levels (B) in HGF-hATMSCs compared with controls (hATMSCs). Immunofluoresence using HGF antibody revealed the expression of HGF in HGF-hATMSCs (C). Data are representative of three independent experiments with similar results. Data are expressed as the Mean±SD. **P*<0.05 compared with the corresponding control value as determined by the Student's *t*-test.

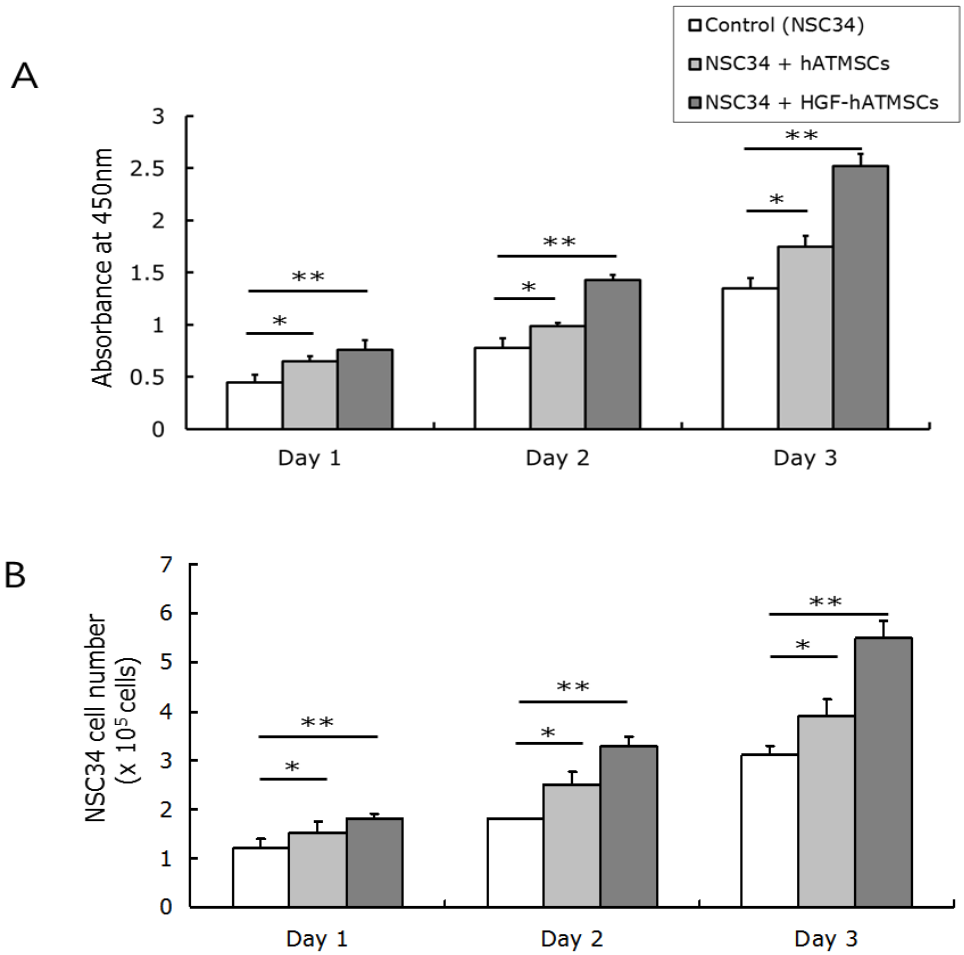

Fig. 5. Promotion effect of HGF-hATMSCs on motor neuron proliferation. NSC34 cells and HGF-hATMSCs were co-cultured for 1, 2, and 3 days under transwell co-culture system and proliferation of NSC34 cells was evaluated by the WST-1 cell proliferation assay (A) and trypan blue exclusion assay (B). The WST-1 cell proliferation assay and trypan blue exclusion assay showed that HGF-hATMSCs promote proliferation of NSC34 cells. Data are representative of three independent experiments with similar results. Data are expressed as the Mean±SD. **P*<0.05 and ***P*<0.01 compared with the corresponding control value as determined by the Student's *t*-test.

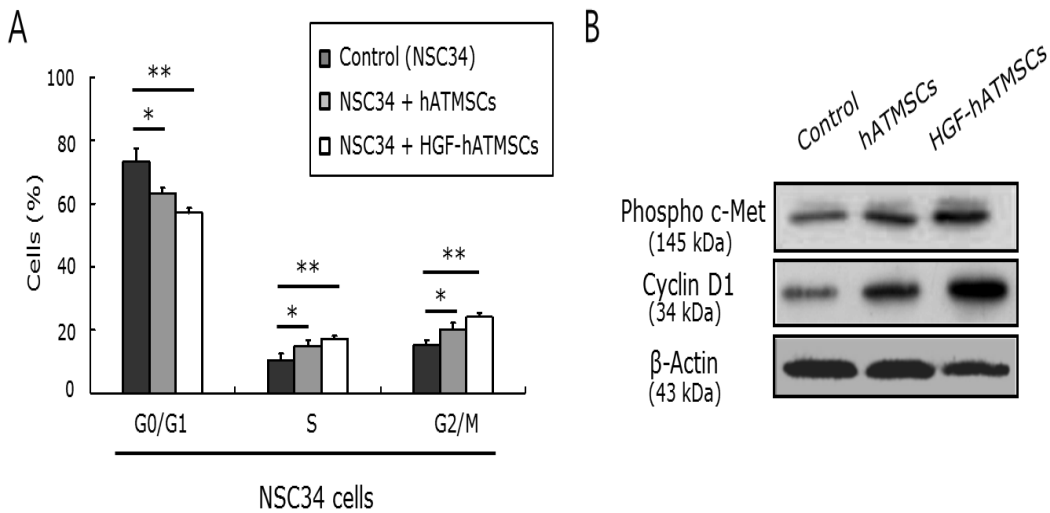

Fig. 6. Cell cycle analysis by flow cytometry and western blot analysis of c-Met and cyclin D1 in NSC34 cells after co-culture with HGF-hATMSCs. NSC34 cells and HGF-hATMSCs were co-cultured for 24 hrs under indirect co-culture system and cell cycle of NSC34 cells was analyzed by flow cytometry (A). Compared with the control (NSC34 cells alone), cell cycle analysis showed an increase in the proportion of NSC34 cells after co-culture with HGF-hATMSCs in S and G2/M phases during cell cycle. In addition, expressions of cyclin D1 and activated c-Met in NSC34 cells after co-culture with HGF-hATMSCs for 24 hrs were assessed by western blot analysis (B). The results revealed an increase of expression of cyclin D1 and activated c-Met in NSC34 cells after co-culture with HGF-hATMSCs compare to those of control. Data are representative of three independent experiments with similar results. Data are expressed as the Mean±SD. **P*<0.05 and ***P*<0.01 compared with the corresponding control value as determined by the Student′s *t*-test.

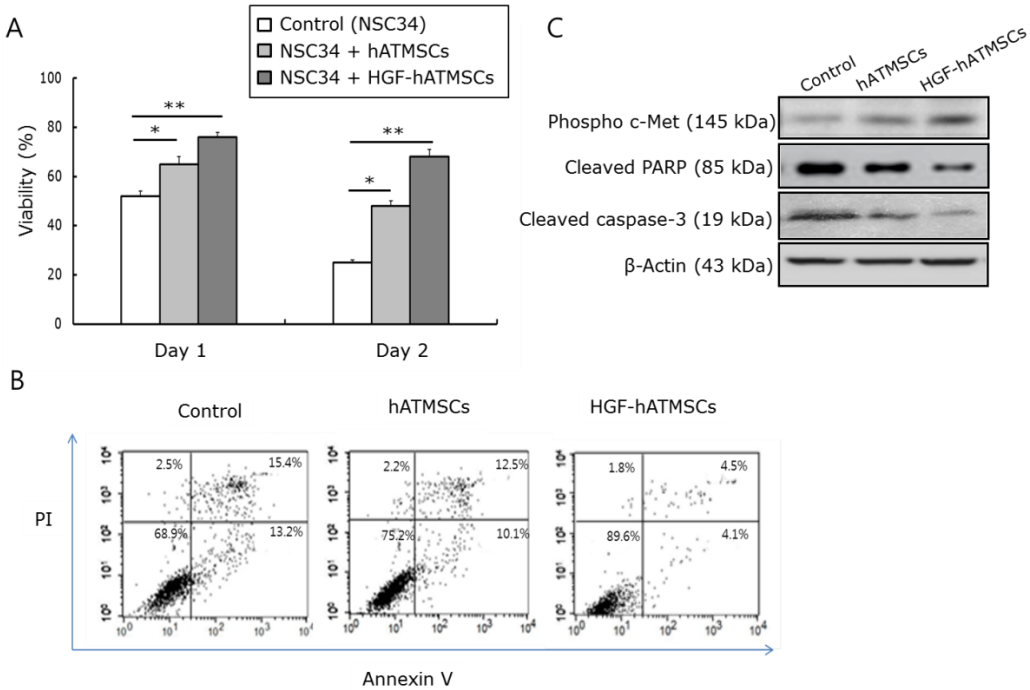

Fig. 7. Inhibitory effect of HGF-hATMSCs on motor neuron apoptosis. After co-culture of NSC34 cells (pre-treatment with thapsigargin for 4 hrs) and HGF-hATMSCs, survival assay (A), Annexin V/PI staining and flow cytometry analysis (B), and western blot analysis of c-Met, cleaved PARP, and caspase-3 (C) were performed in NSC34 cells. Survival assay showed stimulatory effect of HGF-hATMSCs on survival of NSC34 cells. In the Annexin V/PI staining and flow cytometry analysis, Annexin V positive cells in NSC34 cells co-cultured with HGF-hATMSCs were prominently decreased compare to those of control. The results of western blot analysis demonstrated that the levels of the activated c-Met, PARP, and caspase 3 proteins were remarkably declined in NSC34 cells co-cultured with HGF-hATMSCs compare to those of control. Data are representative of three independent experiments with similar results. Data are expressed as the Mean±SD. **P*<0.05 and ***P*<0.01 compared with the corresponding control value as determined by the Student's *t*-test.

Table 1. Primer sequences for RT-PCR analysis

Genes		Sequences of primers	Gen bank accession numbers	Product size (bp)
hHGF	Forward	5'-TGCAATTAAAACATGCGCTG-3'	EI 5445	827
	Reverse	5'-TCTCATCTCCTCTTCCGTGGACAT-3'		
GAPDH	Forward	5'-AAGTGGATATTGTTGCCATC-3'	NM 002046	445
	Reverse	5'-ACTGTGGTCATGAGTCCTTC-3'		

Table 2. Immunophenotyping of HGF-hATMSCs by flow cytometry

Surface markers		hATMSCs	HGF-hATMSCs
Positive	CD 29	99.30 ± 1.20	99.10 ± 1.10
	CD 44	99.25 ± 0.90	99.75 ± 1.30
	CD 73	96.57 ± 1.50	97.21 ± 0.80
	CD 90	99.45 ± 1.10	98.63 ± 0.90
	CD 105	95.49 ± 1.30	96.77 ± 1.50
Negative	CD 31	0.92 ± 0.03	0.99 ± 0.02
	CD 34	1.95 ± 0.07	1.91 ± 0.05
	CD 45	0.77 ± 0.02	0.84 ± 0.02
	HLA-DR	1.28 ± 0.10	1.52 ± 0.03

Data show the average values from three independent experiments and are expressed as the Mean±SD

CHAPTER II

Therapeutic effects of HGF-hATMSCs in a transgenic mouse model of ALS

1. Introduction

ALS is an adult-onset neurodegenerative disease characterized by progressive loss of both cortical and spinal motor neurons (Cleveland *et al*. 2001; Sejvar *et al*. 2005). The human SOD1 gene mutation G93A over-expressing transgenic mice (the SOD1 G93A transgenic mice) exhibit a phenotype that reproduces clinical symptoms and histopathological features of human ALS. Since sporadic ALS and familiar ALS do not differ in histopathology, SOD1-mutant mice provide a good model to investigate the pathogenesis of ALS and to test therapeutic approaches (Bendotti *et al*. 2004; Kato 2008; Turner *et al*. 2008). Several therapeutic strategies have been attempted in the SOD1 G93A transgenic mice models, but to date there is no treatment that can cure or significantly ameliorate the quality of life of ALS patients. However, there is now accumulating evidence that adult stem cell therapy may be a promising therapeutic approach for this devastating

disorder (Corti *et al*. 2010; Garbuzova-Davis *et al*. 2008; Gould *et al*. 2011).

In particular, applications of adult stem cells have been shown to delay symptom progression and prolong lifespan in the SOD1 G93A transgenic mice (Corti *et al*. 2010; Garbuzova-Davis *et al*. 2008). Such beneficial effects seem to be due to the ability of stem cells to release cytokines and growth factors, which have been demonstrated to directly support motor neuron survival, rather than their ability for neuronal replacement (Gould *et al*. 2011; Li *et al*. 2007; Park *et al*. 2009). In some study, an ability of stem cells has been shown to stimulate the migration and differentiation of endogenous neuronal and glial precursors as well as their ability modulate the host immune inflammatory response and support neuronal survival (Caplan *et al*. 2006; Huang *et al*. 2010). The majority of studies on stem cell therapeutic treatments have utilized MSCs derived from the bone marrow or the umbilical cord. Recently, much attention has been paid to ATMSCs, because ATMSCs can be obtained in large quantities and they display high proliferation kinetics and slow senescence ratio *in vitro* (Hipp *et al*. 2008). Furthermore, ATMSCs secrete a variety of growth factors and cytokines (Constantin *et al*. 2009), which may positively influent neural cell survival. Especially, HGF is one of the most potent survival-promoting factors for motor neurons *in vitro* (Ebens *et al*. 1996). The neurotrophic effects of HGF on embryonic spinal motor neurons during

development and on adult motor neurons after axotomy of the hypoglossal nerve have been shown *in vivo* (Novak *et al*. 2000; Okura *et al*. 1999). On this basis, this study explored the beneficial effects of HGF-hATMSCs in the SOD1 G93A transgenic mice.

2. Materials and Methods

1) Animal models and transplantation of HGF-hATMSCs

Transgenic mice originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) and expressing a high copy number of mutant human SOD1 with Gly93Ala substitution (SOD1 G93A) were bred and maintained on a B6/SJL mice strain. Tail samples were collected from the embryos for genotyping. All procedures were performed in accordance with the Institutional Animal Care and Use Committee (IAUCC) guidelines of Seoul National University for the care and use of laboratory animals (SNU-160223-2-1). This strain generally leads to death around 120 days after birth, the survival rate at that time point is 50%. SOD1 G93A ALS mice were divided into three groups; control, hATMSCs, and HGF-hATMSCs groups. Each group consisted of five mice. Mice were anesthetized and transplanted with HGF-hATMSCs using a glass micropipette attached to a stereotaxic

injector. The tip of the micropipette was inserted into the lumbar 2 region (L2) in the spinal cord, and 4 μL of saline containing HGF-hATMSCs (1×10^5 cells) was injected at a rate of 1 μL/min. For tissue processing, mice were harvested before death. They were transcardially perfused with PBS and 4% paraformaldehyde (PFA). The spinal cord and brain were removed and filled with 4% PFA in a conical tube at 4℃. The following day, the solution was replaced with 10% sucrose, 20% sucrose, and 30% sucrose in PBS. The tissues were placed in a disposable mold with OCT compound (Tissue-Tek, Alphen aan den Rijn, Netherlands) on dry-ice and kept at -80℃.

2) Behavioral test and disease onset

To test motor neuronal function, animals performed rotarod testing every other day, running on the machine 3 times for a maximum of 5 min each. The disease onset point of the animals was assessed using a neurological score developed by the Jackson Laboratory. One point onset indicates that the mouse did not overturn within 30 seconds. Two point onset indicates dragging the legs on the ground. Three point onset indicates when the tails pick-up and the legs are folded or trembling. Rotarod failure indicates that the mouse failed to run on the machine for 15 seconds. The end point was the day of death.

3) Statistical analysis

Statistical analysis was carried out using SPSS version 18.0 (SPSS Inc., IL, USA). The survival function data were analyzed by the Kaplan-Meier method. The other data were analyzed using a Student's *t*-test in Microsoft Excel version 2007. All data are expressed as the Mean±SEM, and findings were considered statistically significant at $P<0.05$.

3. Results

1) Therapeutic effects of HGF-hATMSCs transplantation in a mouse model of ALS

To investigate therapeutic effects of HGF-hATMSCs in a mouse model of ALS, the SOD1 G93A transgenic mice were divided into three groups including treatment with HGF-hATMSCs, hATMSCs, and saline as control, and were administrated by intra-spinal cord injection (Fig. 8A). For intra-spinal cord injection, laminectomy was performed at vertebral bodies Th12/L1 with sharp scissors to expose the spinal cord at level lumbar 1-4 (L1-L4) (Fig. 8A). A Hamilton syringe with an elongated glass capillary on top (50-80 μm diameter) was used for injection and $1x10^5$ cells (20,000 cells

per side in a volume of 1 μL) were administered bilaterally into L2 region of the spinal cord. The transplanted cells-labeled with green fluorescent nanoparticles were found to be well-engrafted onto spinal cord tissue of the SOD1 G93A transgenic mouse by fluorescence imaging analysis (Fig. 8B). After transplantation of HGF-hATMSCs, diseases onset point and rotarod failure were assessed to test progression of symptoms and the motor function (Fig. 9A). Treatment of HGF-hATMSCs significantly delayed symptom onset (111±2.7 days of age) in the SOD1 G93A transgenic mice compared to that of control (101±2.4 days of age). Symptom onset of the hATMSCs-treated group (108±1.6 days of age) began slower than that of control group. Average time of rotarod failure in HGF-hATMSCs-treated group, hATMSCs-treated group, and control group was 131.7±3.2, 128.1±3.1, and 120.8±2.9 days after birth, respectively. These results indicate that HGF-hATMSCs contribute to improvement of motor function and retardation of symptom onset in the SOD1 G93A transgenic mice. Furthermore, the lifespan of the mice was remarkably prolonged in HGF-hATMSCs-treated group (141.6±4.1 days) compared to the control group (127±4.1 days) (Fig. 8A and B). The lifespan of hATMSCs-treated group was longer (135.8±2.7 days) than that of control group.

4. Discussion

Given the complexity of ALS pathogenesis, to date, there is no effective treatment to cure or significantly ameliorate the quality of life of patients. Recently, several therapeutic approaches using stem cells have been presented to delay symptoms and improve the quality of life of ALS patients. Therapeutic approach using MSCs is of great interest given their ability to migrate to injury sites and promote tissue repair and regeneration. Several studies have provided evidence for the efficacy of MSCs *in vivo* models of ALS, demonstrating that their applications can delay the death of motor neurons, decrease the inflammatory response and prolong survival of the animals. Here, hATMSCs modified with HGF gene generated in this study and their therapeutic potential were evaluated in the SOD1 G93A transgenic mice.

In the *in vivo* study, treatment of HGF-hATMSCs demonstrated improved motor performance, delayed disease progression and extended the lifespan of the SOD1 G93A transgenic mice. These findings showed that the intra-spinal injection of HGF-hATMSCs in SOD1 G93A transgenic mice at the clinical onset significantly delayed the deterioration of motor performance as compared to control group. The analysis of distribution of

green fluorescence nanoparticles-labelled HGF-hATMSCs administrated by intra-spinal injection confirmed their engraftment in spinal cord (Fig. 8B), as seen in other experimental models (Marconi *et al*. 2012). These results showed that HGF-hATMSCs have a clear therapeutic potential in slowing down the disease progression in mice model with ALS.

Although the mechanism of motoneuron-specific disease progression is not exactly understood, regulation or accumulation of a series of molecules, including SOD1, neurofilament and apoptotic molecules, is thought to be involved in motoneuronal degeneration (Al-Chalabi *et al*. 2000; Vukosavic *et al*. 1999). From the viewpoint of effective ALS treatment, most of all, it is important to maintain a satisfying neurological functional status with complete appreciation on the neuropathological mechanism.

In this study, *in vivo* data provided the possible evidence that HGF produced by HGF-hATMSCs can attenuate degeneration of motor neuron death, resulting in improved motor performance, delayed disease progression, and extension of the lifespan of the SOD1 G93A transgenic mice. Furthermore, treatment of HGF-hATMSCs demonstrated the accelerating therapeutic effects in the SOD1 G93A transgenic mice than hATMSCs-treated group. In previous study, HGF could attenuate induction

of caspase-1 in spinal cord of the SOD1 G93A transgenic mice (Sun *et al*. 2002). In addition, the *in vitro* study exhibited that HGF produced by HGF-hATMSCs significantly inhibit caspase-3 expression of motor neurons after induction of the ER stress. These results suggest that inhibition of caspase-1 and -3 expressions in motor neurons may be responsible for HGF activity in preventing motor neuron death, and neurotrophic activity of HGF might play an important role associating improvement of the motor performance, prolongation of disease progression, and extension of the lifespan of the SOD1 G93A transgenic mice. Thus, the strategy using hATMSCs modified with HGF could represent a powerful and valuable stem cell based gene therapy for motor neuron degeneration.

In conclusion, these data show that HGF-hATMSCs have a clear therapeutic potential in slowing down the clinical course in the SOD1 G93A transgenic mice with ALS and could have important implication for their therapeutic use. Furthermore, the present results indicate the possibility as a novel tool for ALS treatment.

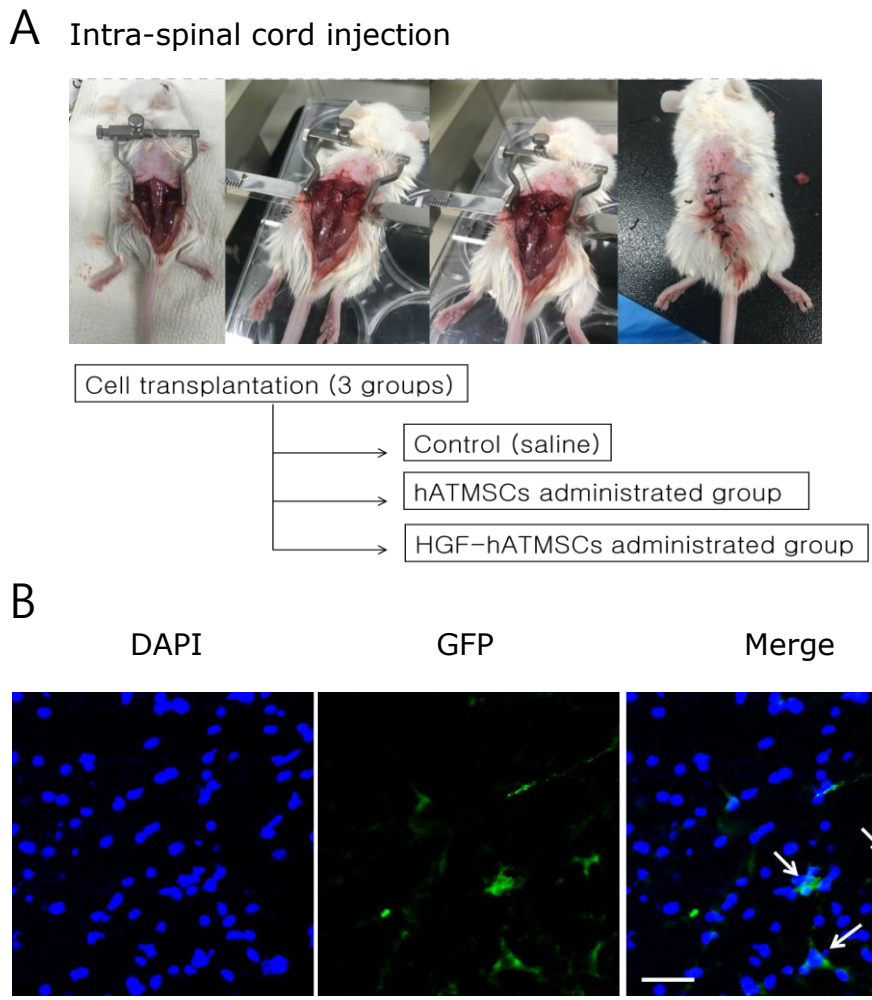

Fig. 8. Transplantation of HGF-hATMSCs by intra-spinal cord injection in the SOD1 G93A transgenic mice and engraftment of transplanted HGF-hATMSCs onto spinal cord tissue. (A) HGF-hATMSCs were administered bilaterally into L2 region of the spinal cord. (B) Engraftment of transplanted HGF-hATMSCs was observed in spinal cord tissue of the SOD1 G93A transgenic mouse by fluorescence imaging analysis. Scale bar, 100 μm.

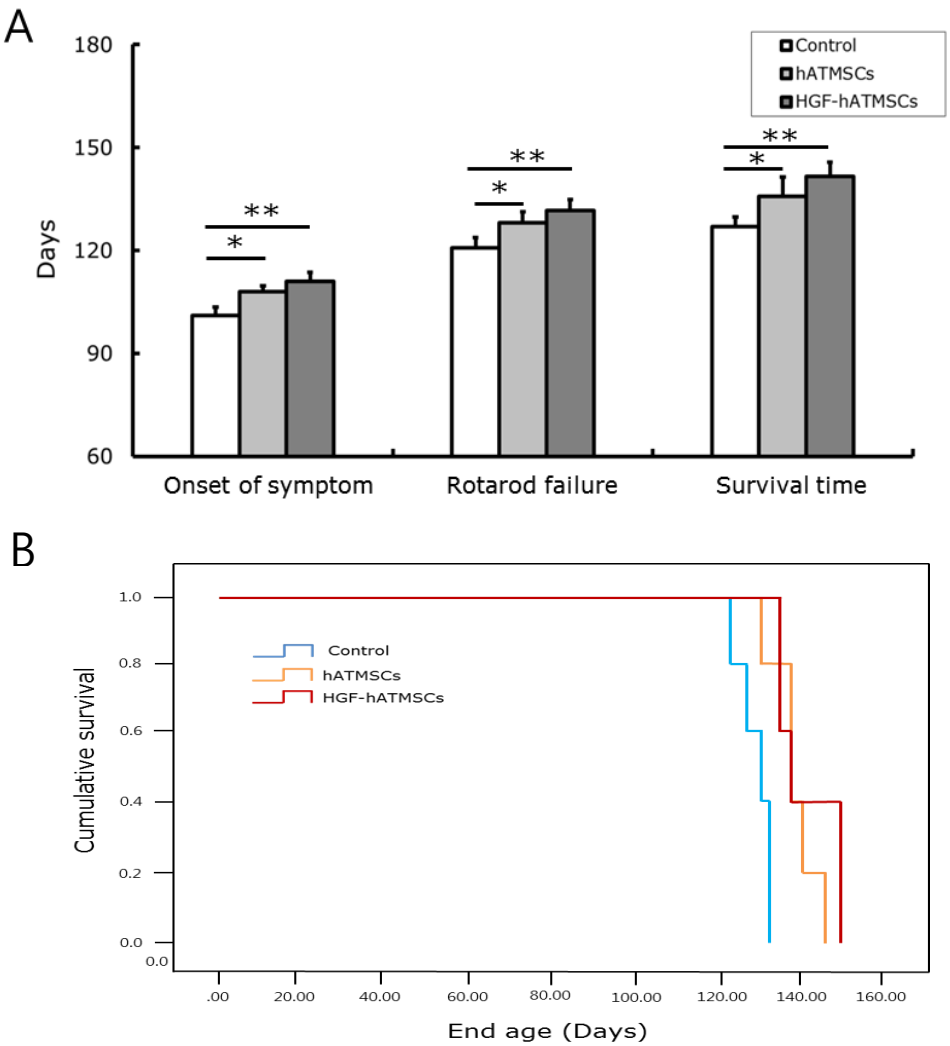

Fig. 9. Effects of HGF-hATMSCs transplantation on disease progression, motor function, and survival of the SOD1 G93A transgenic mice. (A) Transplantation of HGF-hATMSCs significantly delayed symptom onset and rotarod failure in the SOD1 G93A transgenic mice and remarkably prolonged their survival. Data are expressed as the Mean±SD. **P*<0.05 and ***P*<0.01 compared with the corresponding control value as determined by the Student's *t*-test. (B) Survival times of the SOD1 G93A transgenic mice were analyzed by Kaplan-Meier method.

CONCLUSIONS

This study was carried out to evaluate the therapeutic potential of HGF-hATMSCs.

The conclusions are as follows;

1) HGF-hATMSCs were successfully generated by liposomal transfection. Expression of the human HGF was evaluated by RT-PCR and ELISA. Immunofluorescence analysis using a HGF antibody also revealed the expression of HGF in HGF-hATMSCs.

2) There was no difference in expression of surface markers between HGF-hATMSCs and control (unmodified hATMSCs). Thus, the results indicate that expression of stem cell surface markers did not change due to HGF gene transfection.

3) The WST-1 cell proliferation assay and trypan blue exclusion assay showed that proliferation of NSC34 cells was significantly promoted in the HGF-hATMSCs groups.

4) The results of cell cycle analysis indicate that HGF-hATMSCs contribute to proliferation of NSC34 cells. Western blot analysis

(Cyclin D1 and phosphorylated c-Met) show that HGF-hATMSCs can contribute to enhancing motor neuron proliferation.

5) HGF-hATMSCs can strongly inhibit apoptosis of NSC34 cells (Cell cycle, PI positive cell, expression of activation of PARP, caspase-3, and phosphorylated c-Met).

6) HGF-hATMSCs contribute to improvement of motor function and retardation of symptom onset in the SOD1 G93A transgenic mice.

Altogether, HGF-hATMSCs showed neuronal capacity (proliferation and anti-apoptotic) *in vitro*, which results were interpreted at the time as the indication of *in vivo* multipotency and self-renewal properties. Thus, HGF-hATMSCs are a good candidate for ALS cell therapy, a new and useful alternative gene based cell therapy that can enable motor neuron regeneration or cell transplantation as a novel tool for ALS treatment. Genetically modified HGF-ATMSCs may offer better outcomes for patients with brain and spinal cord disease in human and veterinary medicine.

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국 문 초 록

간세포성장인자 유전자 도입 사람지방유래 중간엽줄기세포를 이용한 근위축성측삭경화증 마우스 모델에서 치료효능 및 기전연구

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근위축성측삭경화증은 루게릭병으로 잘 알려진 질병으로서 매우 파괴적인 신경퇴행성 질환이다. 골격근의 위약과 위축, 호흡근 마비 등의 증상이 동반되며, 상위 운동뉴런과 하위 운동뉴런 모두에 심각한 영향을 미치는 것으로 알려져 있다. 여러 기전들이 질병의 발병에 기여할 수 있다고 제시되어 왔으나, 운동뉴런 사멸의 원인과 기전은 아직 명확하게 밝혀지지 않았다.

그 동안 여러 가지 약제와 줄기세포 치료 관련 임상실험들이 진행되었으나, 치료제로서 효과가 입증된 사례는 없었다.

간세포성장인자의 여러 가지 기전이 신경세포의 기능을 도와주고, 신경 관련 질병의 진행 속도를 늦출 수 있음을 여러 논문과 연구 결과들을 통해서 제시되었다. 중간엽줄기세포는 생체 내에서 적응능력과 분화능력을 가지고 있기 때문에 국소적으로나 전신적으로 적용하였을 때 해당 질병부위에 작용하여, 치료효과를 기대할 수 있는 것으로 알려져 있다. 또한 중간엽줄기세포의 신경세포에 대한 치료적인 잠재력도 연구 결과를 통해서 확인되었다. 따라서 간세포성장인자와 중간엽줄기세포 각각의 치료적인 잠재력을 바탕으로 유전자(간세포성장인자) 도입 줄기세포 치료가 운동뉴런의 기능과 퇴행에 보다 높은 시너지 효과가 있을 것이라고 가정하고 본 연구를 계획하였다.

본 연구는 간세포성장인자가 과발현된 사람지방유래 중간엽줄기세포의 운동뉴런에 대한 증식과 세포자멸 억제효과에 관한 생체 외 실험과 근위축성측삭경화증 마우스 모델에 대한 치료적인 효과에 관한 생체 내 실험으로 나누어 진행하였다.

간세포성장인자가 과발현된 사람지방유래중간엽줄기세포는 pMEX발현벡터에 간세포성장인자의 형질주입을 통해서 만들어졌다. 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포가 성공적으로 만들어졌는지 확인하기 위해서 역전사중합효소 연쇄반응(RT-PCR), 효소면역측정법(ELISA), 그리고 간세포성장인자 항체를 이용한 면역형광분석을 시행하였다.

역전사중합효소연쇄반응분석 결과, 대조군에 비해서 현저하게 발현이 증가한 것을 확인하였으며, 효소면역측정법 결과, 대조군에 비해 7.7배 이상 과발현되었음을 확인하였다. 면역형광분석 결과 간세포성장인자의 발현을 직접 눈으로 확인할 수 있었다. 또한 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포의 유세포분석 결과, 줄기세포 양성표지 마커는 각각 99% 이상, 음성표지 마커는 2% 미만으로 확인되어 대조군과 큰 차이가 없는 것을 확인할 수 있었다. 이러한 결과는 유전자 도입을 통해 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포가 성공적으로 제작되었음을 의미한다. 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포가 운동뉴런 증식에 미치는 효과를 확인하기 위해서, 세 그룹(①NSC34세포(마우스운동뉴런세포), ②NSC34세포와 사람지방유래중간엽줄기세포, ③NSC34세포와 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포)으로 나누어 간접공생배양(indirect coculture)한 후, WST-1 세포증식측정법과 트립판블루세포배제측정법(trypan blue exclusion assay)을 진행한 결과, 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포와 공생배양한 그룹의 NSC34세포수가 현저하게 증가되었음을 확인할 수 있었다. 마찬가지로 각각 공생배양한 그룹에서 얻은 NSC34세포를 유세포분석 방법을 통하여 세포주기분석을 시행하였다. 대조군의 경우, 휴지기인 GO/G1 기, 유전자 합성기인 S 기, 유사분열 단계인 G2/M 기에 각각, 73.27±4%, 10.68±2%, 15.23±1.5%, 사람지방유래중간엽줄기세포 그룹은 각각, 63.1±2.1%, 15.1±1.83%, 20.4±2%, 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포 그룹은 각각,

57.12±1.5%, 17.23±1.1%, 24.1±1.3% 의 결과를 나타내었다. 세포주기분석 결과, G0/G1 기는 대조군에 비해 사람지방유래중간엽줄기세포 그룹에서, 사람지방유래중간엽줄기세포 그룹에 비해 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포 그룹에서, 상대적으로 세포수가 감소하였으며, S 기, G2/M 기에는 각각 세포수가 유의적으로 증가한 것을 확인할 수 있었다. 세포주기에서 G1 기에서 S 기로의 진행의 조절과 유전자 복제신호에 매우 중요한 역할을 하는 Cyclin D1의 과발현과 활성화 c-Met의 과발현은 NSC34세포의 증식이 활발히 진행되고 있음을 의미한다. 이러한 결과는 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포가 운동뉴런의 증식능력을 향상시키고 있음을 의미한다. 세포자멸이 진행되고 있는 운동뉴런에 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포의 세포자멸 억제효과를 확인하기 위해서, NSC34세포에 세포자멸을 유발한 후, 각각의 그룹과 간접공생배양하여, WST-1세포증식측정법을 진행하였다. 세포자멸을 유발한 NSC34세포의 생존 세포수가 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포와 공생배양한 그룹이 현저하게 높음을 확인할 수 있었다(간세포성장인자가 과발현된 사람지방유래중간엽줄기세포 그룹은 각각 76±2%(1일), 68.4±3%(2일), 사람지방유래중간엽줄기세포 그룹은 각각 65.1±3%(1일), 48.3±2%(2일), 대조군은 각각 52.1±2%(1일), 25.2±1%(2일)). Annexin V 와 PI 염색 후 유세포분석을 시행한 결과, Annexin V 양성 세포수가 대조군(28.6±0.1%)에 비해 사람지방유래중간엽줄기세포 그룹(22.6±0.1%)에서, 사람지방유래중간엽줄기세포 그룹에 비해 간세포성장인자가 과발현된

사람지방유래중간엽줄기세포 그룹(8.6±0.2%)에서, 유의적으로 감소되어 있는 것을 확인할 수 있었다. 웨스턴블럿분석 결과, 대조군에 비해 사람지방유래중간엽줄기세포 그룹에서, 사람지방유래중간엽줄기세포 그룹에 비해 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포 그룹에서, 활성화된 caspase-3와 PARP의 발현이 상대적으로 감소되어 있었으며, 활성화된 c-Met의 발현은 증가되었음을 확인할 수 있었다. 이러한 결과들은 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포가 NSC34세포의 세포자멸을 억제할 수 있음을 뒷받침한다.

근위축성측삭경화증 마우스 모델인 SOD1 G93A 유전자 변이 마우스에 대한 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포의 치료적인 효과를 확인하기 위해서, 세 그룹(①대조군(생리식염수), ②사람지방유래중간엽줄기세포, 그리고 ③간세포성장인자가 과발현된 사람지방유래 중간엽줄기세포)으로 나누어, 각각의 세포를 척수 내 주입하였다. 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포를 이식한 마우스 그룹이 사람지방유래중간엽줄기세포를 이식한 마우스 그룹과 대조군에 비해서 증상발현 시작시점(각각, 111±2.7일, 108±1.6일, 101±2.4일)이 늦어짐을 확인할 수 있었으며, 신경기능 저하시점(각각, 131.7±3.2일, 128.1±3.1일, 120.8±2.9일)도 늦어짐을 확인하였다. 마우스의 평균수명(각각, 141.6±4.1일, 135.8±2.7일, 127±4.1일) 또한 유의적으로 증가한 것을 확인할 수 있었다. 이러한 결과는 간세포성장인자가 과발현된 사람지방유래중간엽줄기세포가 SOD1 G93A 유전자 변이 마우스의 운동뉴런 기능을 증진시키고,

증상발현 시점을 늦춰주는데 기여할 수 있음을 보여준다.

결론적으로, 간세포성장인자가 과발현된 사람지방유래 중간엽줄기세포를 이용한 유전자 도입 세포치료가 운동뉴런의 증식 향상과 세포자멸 억제의 측면에서 강력한 신경 관련 잠재력을 가지고 있음을 보여주고 있으며, 난치성 질병인 근위축성측삭경화증에 대한 새로운 치료방법으로서 가능성을 제시하였다.

주요어: 간세포성장인자 / 근위축성측삭경화증 /
사람지방유래중간엽줄기세포 / 세포자멸 / 운동뉴런 증식

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