이학박사학위논문

신경 재생에서 간세포 성장인자의 역할과 이에 미치는 요인들

Roles of Hepatocyte Growth Factor in Nerve Regeneration and Factors Affecting its Activity

2021 년 2 월

서울대학교 대학원

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이나연의 박사학위논문을 인준함

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ABSTRACT

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Hepatocyte growth factor (HGF) is a neurotrophic factor and its role in adult peripheral nerves has been relatively unknown. In this study, biological functions of HGF and its receptor c-met have been investigated in the context of regeneration of damaged peripheral nerves. Axotomy of the peripheral branch of sensory neurons from embryonic dorsal root ganglion (DRG) resulted in the increased protein levels of HGF and phosphorylated c-met. When the neuronal cultures were treated with a pharmacological inhibitor of c-met, PHA665752, the length of axotomy-induced outgrowth of neurite was significantly reduced. On the other hand, the addition of recombinant HGF proteins to the neuronal culture facilitated axon outgrowth. In the nerve crush mouse model, the protein level of HGF was increased around the injury site by almost 5.5-fold at 24 hours post injury compared to control mice and was maintained at elevated levels for another 6 days. The amount of phosphorylated c-met receptor in sciatic nerve was also observed to be higher than control mice. When PHA665752 was locally applied to the injury site of sciatic nerve, axon outgrowth and injury mediated induction of cJun protein were effectively inhibited, indicating the functional involvement of HGF/c-met pathway in the nerve regeneration process. When extra HGF was

exogenously provided by intramuscular injection of plasmid DNA expressing HGF, axon outgrowth from damaged sciatic nerve and cJun expression level were enhanced.

I also investigated the role of HGF in the regeneration of injured peripheral nerves using cultured adult DRG neurons. When cells were treated with HGF protein, the length of the neurite was increased 1.4-fold compared to the untreated control group. HGF greatly increased the level of phosphorylated STAT3 at serine 727, thereby translocating the protein to the mitochondria. HGF treatment increased the activity of mitochondrial complex I. When DRGs were cultured in the presence of U0126, a pharmacological inhibitor of Erk, the HGF-mediated increase in neurite outgrowth and the level of pSTAT3 (Ser 727) were both suppressed.

In clinical studies involving HGF (in the form of plasmid DNA expressing HGF), HGF showed a peculiar bell-shaped dose response curve. Data from endothelial cell migration assays showed that the number of migrated cells was increased as HGF concentration increased but was significantly reduced at concentrations higher than a certain point. It was found that excessive doses of HGF increased the phosphorylation of tyrosine 1003 involved in the ubiquitination of c-met, and phosphorylated c-met was diverted toward the proteasomal degradation pathway. That is, the c-met receptor was degraded when unnecessarily large amounts of HGF were present. This result may explain why HGF showed a bell-shaped dose response in human studies.

In summary, HGF plays important roles in axon outgrowth by directly interacting with sensory neurons and HGF/c-met pathway is tightly regulated by a negative feedback loop through an ubiquitin-proteasomal degradation pathway.

Keywords: hepatocyte growth factor, mitochondria, sensory neuron, neurite outgrowth, axon regeneration, negative feedback

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ABBREVIATIONS

AAV	adeno-associated virus
ATF3	activating transcription factor 3
Cdc42	cell division control protein 42
CHX	cycloheximide
CSF1	colony stimulating factor 1
CREB	cAMP-response-element-binding protein
DLK	dual leucine zipper kinase
DRG	dorsal root ganglion
eDRG	embryonic DRG
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
Erk	extracellular signal-regulated kinases
ETC	electron transport chain
FACS	fluorescence-activated cell sorting
Gab1	Grb2-associated-binding protein 1
Gap43	growth associated protein 43
GDNF	glial cell-derived neurotrophic factor
GGA3	golgi-localized, gamma adaptin ear-containing, arf-binding 3
Grb2	growth factor receptor-bound protein 2

HGF	hepatocyte growth factor
HUVEC	human umbilical cord vein endothelial cell
IBA1	ionized calcium-binding adapter molecule 1
ICC	immunocytochemistry
IHC	immunohistochemistry
IL-6	interleukin-6
IRF	interferon regulatory factor
IM	intramuscular
JNK	cJun N-terminal kinase
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
NGF	nerve growth factor
PI3K	phosphoinositide 3-kinase
RAG	regeneration associated gene
Rac1	Ras-related C3 botulinum toxin substrate 1
RTK	receptor tyrosine kinase
Shp2	SH2 containing protein tyrosine phosphatase 2
STAT3	signal transducer and transcription 3

Chapter I

Introduction

1. Hepatocyte growth factor (HGF)

HGF was originally discovered as a mitogen for hepatocyte, promoting the DNA synthesis and proliferation of liver cells [1, 2]. However, HGF was later found to contain multiple biological activities such as angiogenesis, anti-inflammation, anti-apoptosis, and anti-fibrosis. HGF is also a neurotrophic factor that it promotes the development, differentiation, and proliferation of neuronal cells [3].

Structure of HGF:

HGF is secreted as a single chain, known as pro-form (Fig. I-1). Single chain HGF is cleaved by serine protease such as urokinase plasminogen activator and tissue-type plasminogen activator, into α-chain (containing four kringle domains) and β-chain (containing serine protease-like structure). These chains are linked by a disulfide bond to become a mature form. This mature HGF is a biologically active form and shows high affinity for c-met receptor [4, 5, 6].

Biological roles of HGF:

It has been reported that HGF/c-met pathway contributes to the development of nervous systems. In the developmental stage, HGF was shown to increase the length of neurite of cultured embryonic DRG neurons in NGF dependent manner. HGF also promotes the survival of DRG neurons. In addition, mice which have mutant form of c-met receptor

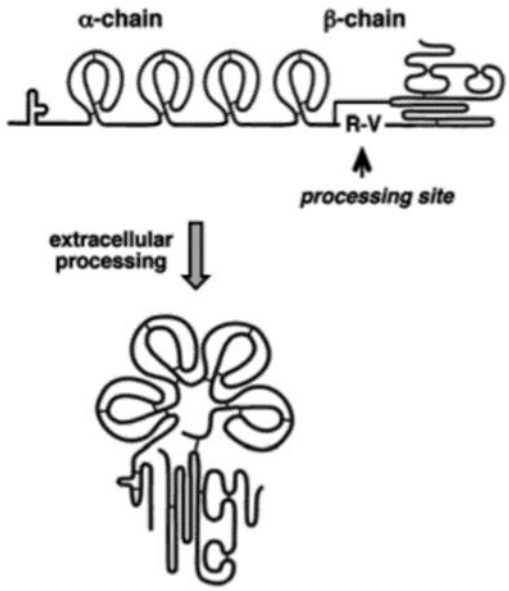

Figure I-1. The structure of HGF (Taken from Nakamura *et al.*, 2011)

HGF is produced as an inactive single chain. Then, HGF is cleaved into α-chain and β-chain and linked by a disulfide bond forming a mature form.

show reduced innervation of sensory neurons to skin. In embryonic motor neurons, HGF enhances the survival and the proper innervation of neurons to target tissue. HGF also facilitates the neurite outgrowth of embryonic sympathetic neurons [7, 8, 9].

In adult stages, HGF prevents neuronal death of cerebellar neurons and hippocampal neurons [10, 11]. It has been demonstrated that HGF/c-met pathway enhances the neurite outgrowth and survival of various types of neurons in developmental stages, however, the role of HGF/c-met pathway in adult stages is relatively unknown.

Peripheral nerve injury activates the HGF/c-met pathway in Schwann cells located in distal regions of injury site. HGF induces the proliferation and migration of cultured Schwann cells and upregulates the expression of GDNF and LIF by activating the Erk pathway. Finally, injury-induced HGF/c-met pathway enhances the re-myelination of Schwann cells, thereby promoting the process of peripheral nerve regeneration [12]. However, it has not been elucidated whether the HGF/c-met pathway directly interacts with sensory neurons to promote the axon regeneration process.

HGF and therapeutic potential:

HGF shows significant analgesic effects in mouse neuropathic pain model, chronic constriction injury. Intramuscular injection of HGF expressing plasmid DNA, pCK-HGF-X7, reduces the mechanical allodynia and thermal hyperalgesia. Expression levels of ATF3, α2δ1, and CSF-1 in DRGs were down-regulated by treatment of HGF. Also, injection of HGF expressing vector reduces the expression of cathepsin S, IRF8, IRF5, and IBA1 in spinal cord. Furthermore, HGF attenuated the activation of microglia and astrocyte in spinal cord [13]. It

has been reported that HGF regulates the various types of neurons as a neurotrophic factor in developmental stages. Also, HGF/c-met pathway increases the nerve regeneration by interacting with Schwann cell after peripheral nerve injury. In addition, HGF expressing plasmid DNA lowers the neuropathic pain by reducing the pain-related factors in sensory neurons. However, it has not been uncovered whether the HGF/c-met pathway induced the axon regeneration by directly interacting with sensory neurons.

Given known biological activities of HGF, it was thought to have significant therapeutic potentials. Initially, recombinant protein technology was explored which mostly failed due to a very short half-life. It was reported that the level of HGF protein in plasma decreased with a half-life of 3 to 5 minutes when the protein was intravenously delivered. To obtain therapeutically meaningful levels of HGF in the target tissue, a variety of delivery methods were used including plasmid DNA and adeno-associated virus (AAV) [14, 15].

2. c-met receptor

In 1980s, c-met receptor was identified as an oncogene. c-met is a representative member of RTKs (receptor tyrosine kinase), which is expressed in various tissues [16]. Produced as a single chain, c-met is cleaved at a furin site into α-chain and β-chain, and then linked by disulfide bond.

Upon binding to c-met, HGF induces dimerization and trans-phosphorylation of the c-met (Fig. I-2) [17]. Phosphorylation of tyrosine 1234/1235, catalytic tyrosine residue, occurs

followed by phosphorylation of tyrosine 1349/1356, docking tyrosine residue. Then, several molecules such as Gab1, Grb2, Shp2, and PI3K can be recruited to the c-met. Shp2 and Grb2 phosphorylates the Ras. Then, Ras phosphorylates Cdc42/Rac1 axis and/or Raf1/Mek/Erk axis. PI3K phosphorylates the Akt, which results in activation of mTOR. STAT3 can be recruited to the c-met without JAK [18].

Many RTKs and its ligands are regulated to a moderate level by a negative feedback loop. For example, the amounts of EGF and EGFR complex are regulated by endocytosis. At moderated amounts of EGF, the EGF and EGFR complex is internalized through clathrin-mediated endocytosis and preferentially recycled. However, at unnecessarily higher amounts of EGF, the EGF and EGFR complex is internalized via non-clathrin endocytosis and preferentially degraded [19]. HGF and c-met complex, one of the typical members of RTKs, could also be recycled to the plasma membrane or degraded by the lysosome or proteasome [20]. HGF and c-met complex could be recycled mediated by GGA3 adaptor proteins. Upon HGF stimulation, GGA3 is recruited to c-met within Rab4 endosome. Knock-down of GGA3 reduced the recycling of c-met, thereby decreasing the sustained Erk activation [21]. On the other hand, tyrosine 1003 phosphorylated c-met is destined to the lysosome or proteasome dependent degradation pathway. CBL, E3 ubiquitin protein ligase, is recruited to the tyrosine 1003 phosphorylated c-met and leads to the ubiquitination of c-met. Then, c-met undergoes proteasomal or lysosomal degradation [22]. However, it remains uncovered under what situations HGF and c-met complex is recycled or degraded.

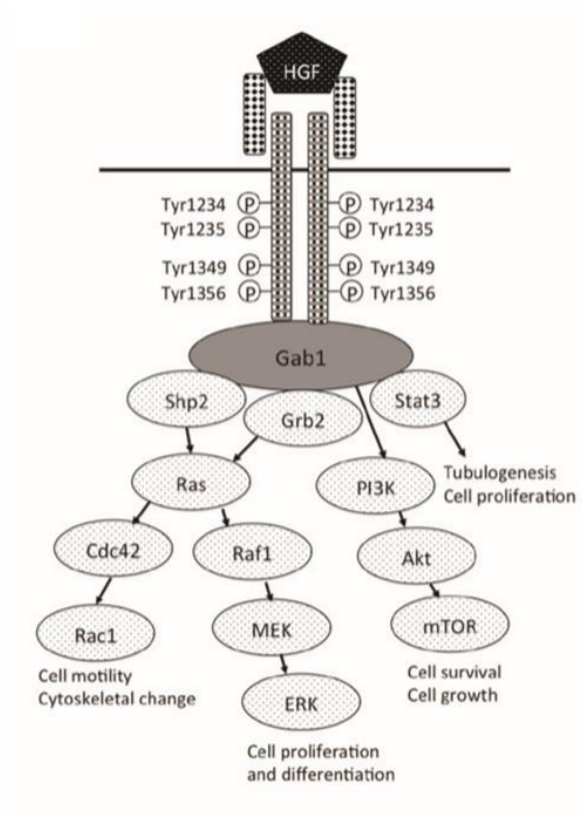

Figure I-2. The structure of c-met receptor (Taken from Kato, 2017)

Binding of HGF induces the trans-phosphorylation of c-met. Phosphorylation of tyrosine 1234/1235 occurs followed by phosphorylation of tyrosine 1349/1356. Then several molecules can be docked into the c-met, which results in activation of various signaling pathways.

3. Overview of thesis research

In this study, I investigated the role of HGF/c-met pathway in peripheral nerve regeneration using *in vitro* primary sensory neurons and *in vivo* nerve crush mouse model. Using primary embryonic DRG neurons, I investigated the level of activated HGF/c-met pathway upon axotomy in *in vitro*. Then, the neurite length of c-met inhibitor or HGF treated sensory neurons was measured. Nerve crush mouse model was used to mimic the peripheral nerve injury in *in vivo*. First, I measured the level of HGF expression and c-met phosphorylation in injured site. I analyzed the effect of c-met inhibitor and HGF expressing plasmid DNA on axon regeneration by using nerve pinch test, immunohistochemistry, and western blot. Next, I tested how HGF/c-met pathway induced neurite regrowth of cultured primary adult DRG neurons in *in vitro*. I analyzed the amounts of mitochondrial STAT3 and the activity of mitochondrial electron transport chain. Based on human studies involving HGF expressing plasmid DNA, I also tested the bell-shaped dose response of HGF/c-met pathway in primary endothelial cells in *in vitro*. I measured the number of migrated cells treated with various doses of HGF. The amount of c-met receptor was assessed and the mechanism how c-met receptor is regulated by HGF was studied.

In summary, the objective of this thesis was to investigate the role of HGF/c-met pathway in peripheral nerve regeneration process both *in vitro* and *in vivo* and to understand the underlying mechanisms at the molecular and cellular levels.

Chapter II

Materials and Methods

1. Animals

All experimental protocols were performed under the guidelines of the Seoul National University Institutional Animal Care and Use Committee (Permission No. SNU-170426-1-3). Eight-week-old male C57BL/6 mice were purchased from Orient Bio Inc. (Gyeonggi-do, Korea). Mice were housed at 22°C with a 12-hour light-dark cycle, given access to food, and provided water ad libitum. To induce peripheral nerve injury, sciatic nerve crush was performed. Animals were anesthetized with isoflurane and the sciatic nerve was exposed by making a small incision in the skin and muscle. The exposed nerve was then crushed for 15 seconds using fine hemostatic forceps (FST, British Columbia, Canada). Incisions were sutured and animals were monitored for their recovery.

For nerve pinch tests, mice were mildly anesthetized with low concentration of isoflurane. The sciatic nerve was exposed and pinched from distal to proximal direction until a reflex response of the hindlimb was observed. The distance between the pinched site where animals showed the reflex and injury site was measured.

After nerve crush injury, plasmid DNAs were injected to the bicep femoris muscle with 200 μg/head. For PHA665752 (Tocris, Bristol, United Kingdom) administration, surgifoam was soaked with DMSO solution containing 25 mg/kg of PHA665752 and delivered to the local injury site.

2. Primary embryonic DRG culture

The DRGs from E13.5 embryonic mice were collected, following trypsinization with 0.25% Trypsin/EDTA (Gibco, MA, United States) for 22 minutes. Each well coated with PDL/laminim (Thermofisher, MA, United States) was spotted with 5-7 DRGs and dried for 18 minutes. Cells were then cultured with 500 μL of Neurobasal media (Gibco) including 25 ng/mL of the recombinant NGF protein (R&D, MN, United States), 1:50 of B27 (Gibco), 1:100 of Penicillin/Streptomycin (Gibco), 1:100 of Glutamax (Gibco), and 1:200 of fluorodeoxyuridine (FdU) (Sigma, MO, United States). A week after spotting, axotomy was performed with a chisel (FST), followed by SCG10 staining after 12 to 40 hours to measure regenerated axons.

3. Primary adult DRG culture

The DRGs from 5-week-old male adult ICR were dissected and dissociated. Briefly, the DRGs from thoracic and lumbar were removed and the roots were trimmed, followed by incubation with 1 mg/mL of collagenase (Sigma) and 0.4 unit/mL of dispase I (Sigma) for 1 hour. Then, DRGs were trypsinized with 0.25% Trypsin/EDTA (Gibco) for 7 minutes. Dissociated cells were suspended in culture medium (10% FBS, 1% penicillin/streptomycin, and 2 mM glutamine in DMEM/F12). Then, triturated cells were centrifuged with 15% BSA (Sigma) at 900 rpm for 10 minutes, followed by resuspension with culture medium. Each well coated with poly-D-lysine (Sigma) was plated with 20000 cells/well.

4. Neurite outgrowth

Cultured cells were fixed with 4% formaldehyde for 15 minutes at room temperature and washed three times with PBS, followed by permeabilization with 0.3% Triton X-100 in PBS for 5 minutes. Cells were then incubated with a blocking solution (10% FBS and 0.2% Triton X-100 in PBS) at room temperature for 1 hour. Cells were incubated with primary antibody specific to βIII tubulin (BioLegend, CA, United States) in a blocking solution at 4°C for overnight. After three times of washing with PBS, cells were incubated with secondary antibody specific to mouse IgG (Invitrogen, MA, United States) at room temperature for 2 hours. Immunofluorescence was detected with the IN Cell Analyzer 2000 (GE healthcare, MA, United States). Quantification of the length of neurite was performed by measuring the total pixel area of the neurite with ImageJ software (NIH) and the WIMASIS website.

5. Immunocytochemistry (ICC)

Cultured cells were fixed in 10% formalin for 15 minutes at room temperature. Cells were then washed three times with PBS followed by incubation with the blocking solution (10% FBS with 0.2% Triton X-100 in PBS) at room temperature for 1 hour. Primary antibodies specific to SCG10 (Novus, CO, United States), phosphorylated c-met (Cell Signaling Technology, MA, United States), and βIII tubulin (BioLegend) were used at 4°C for overnight. After three times of PBS washing, cells were incubated with secondary antibodies specific to rabbit IgG (Invitrogen) and mouse IgG (Invitrogen) at room temperature for 2 hours. Immunofluorescence was measured using the IN Cell Analyzer 2000 (GE Healthcare).

6. Immunohistochemistry (IHC)

Sciatic nerves were fixed in 10% formalin at 4°C for overnight incubation. After washing with PBS, the tissues were sequentially immersed in 15% sucrose at 4°C for 8 hours and then 30% sucrose at 4°C for overnight incubation followed by cryopreservation in the OCT compound (Sakura Tissue Tek, CA, United States). Samples were then cryosectioned with the thickness of 12 μm. After an hour of incubation in blocking solution (2% BSA with 0.1% Triton X-100 in PBS), samples were treated with primary antibody specific to SCG10 (Novus), phosphorylated c-met (Cell Signaling Technology), and βIII tubulin (BioLegend) at 4°C for overnight incubation. After three times of washing with PBS, samples were then incubated with secondary antibodies specific to rabbit IgG (Invitrogen) or mouse IgG (Invitrogen) at room temperature for 2 hours. Immunofluorescence was measured with the IN Cell Analyzer 2000 (GE Healthcare) after mounting with DAPI (Vectashield, PA, United States).

7. Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed according to the manufacturer's protocol. Briefly, after 48 hours of axotomy, supernatants were harvested followed by Mouse/Rat HGF quantikine ELISA kit (R&D). After 1, 3, and 7 days of injury, sciatic nerves were lysed using a RIPA Buffer (Cell Signaling Technology) with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology). Equal amounts of protein lysate were loaded in a Mouse/Rat HGF quantikine ELISA kit (R&D).

8. Western blot

Electrophoresis was performed with the equal amounts of protein lysate loaded in a 4-12% Bis Tris gradient gel (Invitrogen). The gel was then transferred to PVDF membranes (GE Healthcare). Membranes were blocked with Blocker Casein in PBS (Thermofisher) at room temperature for 45 minutes. Membranes were incubated with primary antibodies specific to pc-met (Cell Signaling Technology), c-met (Sigma), cJun (Cell Signaling Technology), pSTAT3 (Cell Signaling Technology), STAT3 (Cell Signaling Technology), pErk (Cell Signaling Technology), Erk (Cell Signaling Technology), pAkt (Cell Signaling Technology), Akt (Cell Signaling Technology), complex IV (Abcam, Cambridge, United Kingdom), VDAC/porin (Abcam), βIII tubulin (Sigma), β-actin (Sigma), and GAPDH (Cell Signaling Technology) in a blocking solution at 4°C for overnight incubation. After three times of washing with TBST (Invitrogen), membranes were incubated with secondary antibody specific to rabbit IgG (Cell Signaling Technology) and mouse IgG (Cell Signaling Technology) in a blocking solution at room temperature for 1 hour and washed three times with TBST. Membranes were developed with Super Signal West Pico Chemiluminescent Substrate (Thermofisher) and Super Signal West Femto Maximum Sensitivity Substrate (Thermofisher) and signals were visualized with ImageQuant Las 4000 (GE Healthcare).

9. Isolation of mitochondrial fraction

Mitochondrial fraction was isolated using a Mitochondria Isolation Kit for Cultured cells (Abcam) according to the manufacturer's instructions. The concentration of obtained mitochondrial proteins was measured using a Pierce BCA Protein Assay Kit (Thermofisher).

10. Activity of mitochondrial electron transport chain

Activities of mitochondrial complex I and complex II were measured using a Complex I Enzyme Activity Microplate Assay Kit (Abcam) and Complex II Enzyme Activity Microplate Assay Kit (Abcam) according to the manufacturer's instructions. Equal amounts of protein lysate were loaded. Results were calculated as rate mOD450/minutes.

11. Migration assay

Transwell 24 well plates (pore size: 8 μm) were purchased from Corning (Corning, NY, United States). Plates were coated with a 1% gelatin solution for 30 minutes. HUVECs and C2C12 cells were plated in the upper chambers at 20000 cells/well and the recombinant human HGF protein was treated in the lower chambers in a dose-dependent manner. After a two-hour incubation, the cells were fixed with 3.7% form-aldehyde for 15 minutes and stained with crystal violet for 30 minutes. Migrated cells were counted using the ImageJ program.

12. Flow cytometry

Cells were washed with PBS and harvested using Trypsin-EDTA (Thermofisher). Cells were incubated with primary antibody at 4°C for 30 minutes, then washed with PBS. A secondary antibody was treated into cells and incubated at 4°C for 30 minutes. Then cells were washed twice with PBS, and resuspended for analysis with BD FACSCalibur (BD Bioscience, NJ, United States).

13. Statistical analysis

Data are presented as the mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Statistical significance was assessed using unpaired t-test or one-way ANOVA followed by Bonferroni's or Dunnett's multiple comparison testing. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Antigen	Host	Supplier	Cat. No.	Dilutions
pc-met (Y1234/1235)	Rabbit	CST	8218	1:500
pc-met (Y1003)	Rabbit	CST	3135	1:500
c-met	Rabbit	Sigma	SAB4300599	1:500
cJun	Rabbit	CST	9165	1:500
pSTAT3 (S727)	Rabbit	CST	94994	1:1000
pSTAT3 (Y705)	Rabbit	CST	9145	1:500
STAT3	Rabbit	CST	12640	1:1000
pErk (T202/Y204)	Rabbit	CST	4370	1:500
Erk	Rabbit	CST	4695	1:500
pAkt (S473)	Rabbit	CST	9271	1:500

Table 1. List of antibodies used for Western blot

Antigen	Host	Supplier	Cat. No.	Dilutions
Akt	Rabbit	CST	4691	1:500
Complex IV	Rabbit	Abcam	ab16056	1:500
VDAC/porin	Rabbit	Abcam	ab15895	1:500
βIII tubulin	Mouse	BioLegend	801202	1:1000
β-actin	Mouse	Sigma	A3854	1:2000
GAPDH	Rabbit	CST	3683	1:500

Table 1. List of antibodies used for Western blot analysis (continued)

Antigen	Host	Supplier	Cat. No.	Dilutions
SCG10	Rabbit	Novus	NBP1-49461	1:500
βIII tubulin	Mouse	BioLegend	801202	1:500
pc-met (Y1234/1235)	Rabbit	CST	8218	1:100

Table 2. List of antibodies used for immunostaining

Chapter III

Hepatocyte Growth Factor is Necessary for Efficient Outgrowth of Injured Peripheral Axons in *in vitro* Culture System and *in vivo* Nerve Crush Mouse Model

1. Background

The regeneration process of peripheral nerve is initiated immediately after nerve injury [23]. Initially, two types of signaling pathways are activated to induce the formation of the growth cone. Calcium rapidly enters the cell, and is then transported to the DRG, acting as an injury signal [24, 25]. Later, additional signaling pathways involving ERK, DLK, JNK, and STAT3 are relayed to the DRG to induce the expression of regeneration- associated genes (RAGs) including ATF3, cJun, STAT3, galanin, and GAP43 [26]. A variety of biological reactions and activities follow the expression of these genes, including the retro/anterograde transport of protein cargos, rearrangement of cytoskeletons, and translation of axonal mRNAs to promote the axon outgrowth process [27].

Hepatocyte growth factor (HGF) plays an important role in survival, proliferation and migration of various cell types by activating c-met receptor [3, 4]. In the context of the peripheral nerve, the HGF/c-met pathway is known to induce neurite outgrowth of sensory neuron during the developmental stage [7, 8]. HGF also activates c-met receptors present in Schwann cells to induce the re-myelination process of injured nerves [12, 28]. However, it has not been clear whether HGF also interacts directly with peripheral neurons to exert biological effects on axon outgrowth.

In this study, the role of HGF/c-met pathway was investigated in damaged peripheral neurons. In axotomized embryonic DRG cultures, the level of both HGF and phosphorylated c-met was highly increased. Similar observations were made in the nerve crush mouse model. The inhibition of c-met receptor by PHA665752 hindered the outgrowth

of axons and injury-mediated induction of cJun protein. When additional HGF was exogenously provided, axon outgrowth was facilitated as measured by nerve pinch test. Our results strongly suggest that HGF is an important part of the axon regeneration process in injured nerves by directly interacting with neurons.

2. Results

2.1. Axotomy induces the expression of HGF in primary embryonic DRG (eDRG)

It has been reported that HGF/c-met pathway plays important roles in the development of sensory neurons [7, 8], however, its role in injured peripheral nerve has largely been unknown. To investigate the involvement of HGF in damaged peripheral neurons, the level of HGF was first examined in cultured DRG. Primary DRG, which consists mostly of sensory neurons, was prepared from E13.5 mouse embryo and the axotomy procedure was performed to mimic the peripheral nerve injury [29]. Embryonic DRG produced approximately 160 pg/mL of HGF protein under culture conditions, and its level was increased by 2.2-fold when axotomized (Fig. III-1). When axotomized, the number of cells positive for phosphorylated c-met was highly increased compared to control group, indicating that c-met was activated in injured neurons (Fig. III-2). Taken together, these data indicated that nerve injury increased the expression level of HGF and activated c-met receptor.

To test if HGF/c-met pathway is involved in axotomy-induced axon outgrowth, axotomized primary eDRGs were treated with PHA665752, a pharmacological agent that

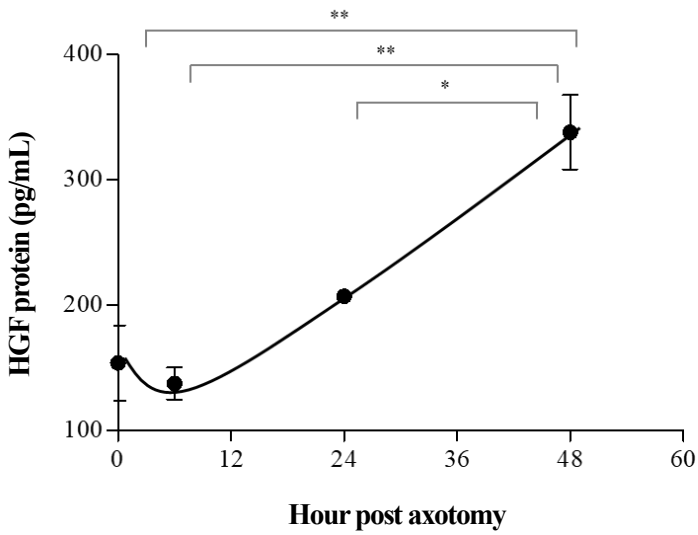

Figure III-1. Induction of HGF proteins by axotomy

Cultured eDRGs were axotomized. After 6, 24, and 48 hours, the protein level of HGF in the culture supernatants was measured using ELISA specific for murine HGF. For statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison testing was performed. Values are presented as mean ±SD. *$p<0.05$; **$p<0.01$.

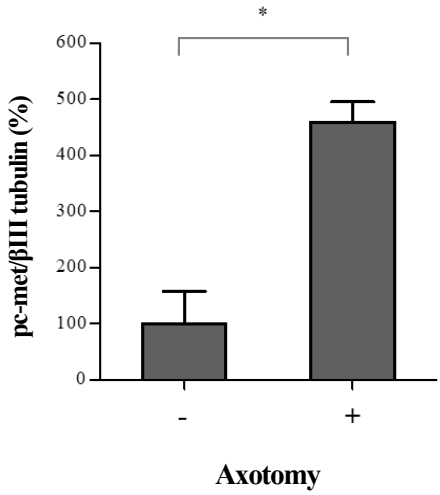

Figure III-2. Phosphorylation of c-met receptors by axotomy

Cultured eDRGs were axotomized. Cells were fixed and subjected to IHC using antibody specific to phosphorylated c-met. Data was analyzed by unpaired t-test. Values are presented as mean ±SD. *$p<0.05$.

specifically inhibits activation of c-met receptor. The level of axon outgrowth was assessed by staining the cells with an antibody to SCG10 protein, a marker specific for regenerating axons [29, 30]. One day after axotomy, the sprouting pattern of SCG10-positive axons was readily observed. When treated with PHA665752, the number of SCG10-positive axons was decreased by 50%, suggesting that the blockade of c-met receptor signaling hindered the axon outgrowth program (Fig. III-3).

The additional supply of HGF protein to the neuronal cultures was then examined for further possible enhancement of axon regeneration. As shown in Fig. III-4, the number of SCG10-positive axons increased in a dose-dependent manner by up to 48% at the 100 ng/mL dose. These results suggested that HGF/c-met pathway was indeed involved in the regeneration of injured DRG axons and that exogenously added HGF protein could enhance this process.

2.2. Peripheral nerve injury induces the expression of HGF and activation of c-met in the nerve crush mouse model

Involvement of HGF/c-met pathway in axon outgrowth was further tested in the nerve crush mouse model, which is widely used in the study of peripheral neuropathy-related axon regeneration program [31]. First, the expression kinetics of the HGF protein were examined. The level of HGF protein was highly increased at day 1 post injury, and then gradually decreased through day 7 in the injured sciatic nerves (Fig. III-5). The level of phosphorylated c-met was also measured in the sciatic nerve and DRG. The level of

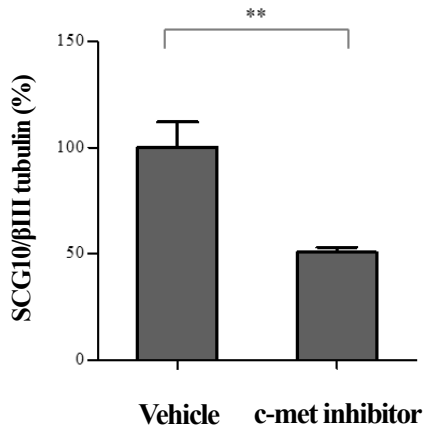

Figure III-3. Effect of c-met inhibitor, PHA665752, on neurite outgrowth

Cultured eDRGs were axotomized and treated with 1 μM of PHA665752. Cells were fixed and stained with SCG10 and βIII tubulin. The intensity of SCG10-positive axons in distal region was counted and summarized. Unpaired t-test was performed for statistical analysis. Values are presented as mean ±SD. **$p<0.01$.

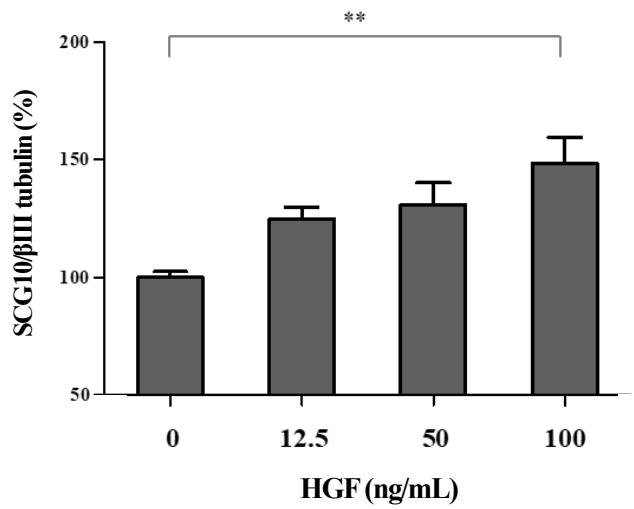

Figure III-4. Effects of addition of recombinant HGF protein on neurite outgrowth

Cultured eDRGs were axotomized and treated with various concentrations of HGF recombinant proteins. Cells were fixed and stained with SCG10 and βIII tubulin. Intensity of SCG10-positive axons in distal region was quantified. One-way ANOVA was performed, followed by Bonferroni's multiple comparison testing. Values are presented as mean ±SD. **$p<0.01$.

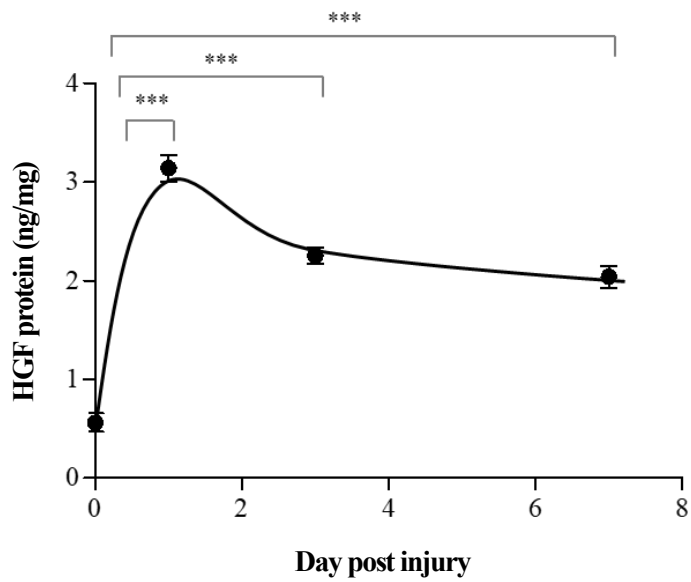

Figure III-5. Effect of peripheral nerve injury on HGF expression

Increased amount of HGF proteins by sciatic nerve crush injury. Nerves from proximal to distal region were collected 0, 1, 3, and 7 days after injury, and proteins were prepared followed by ELISA specific for murine HGF. Values are shown as mean ±SEM. For statistical analysis, data was analyzed by one-way ANOVA followed by Bonferroni's multiple comparison testing. ***$p<0.001$.

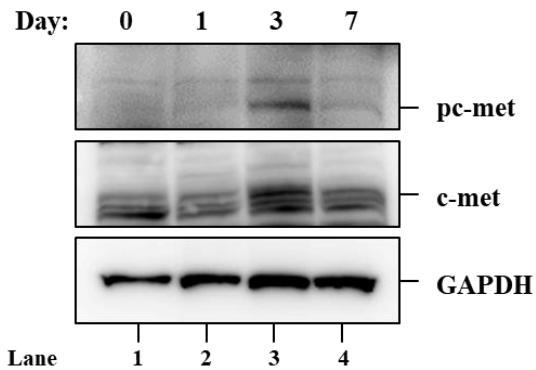

Figure III-6. Phosphorylation of c-met receptor on injured site by nerve crush injury

1, 3, and 7 days after injury, nerves from proximal to distal region were harvested. Proteins were prepared and subjected to Western blot analysis.

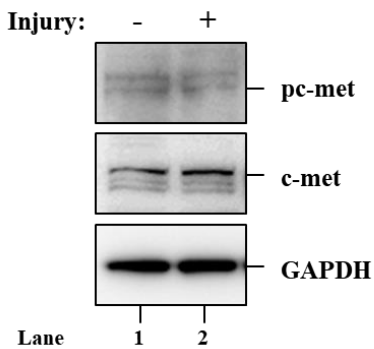

Figure III-7. Phosphorylation of c-met receptor on DRGs by nerve crush injury

DRGs were harvested 3 days after injury and total proteins were prepared followed by Western blot.

phosphorylated c-met was highly increased at day 3, and eventually reduced to the basal level by day 7 (Fig. III-6). In DRG, no significant change in c-met phosphorylation was observed, indicating that nerve injury-mediated c-met activation might be restricted to the sciatic nerve (Fig. III-7).

Activation of c-met receptors was also analyzed by immunohistochemistry. A large number of cells positive for phosphorylated c-met (green) were observed in the proximal, distal, and injury sites of the damaged nerve. Many of these cells were merged with those positive for βIII tubulin-positive axons in the proximal region of the sciatic nerve, indicating that c-met was activated in neurons (Fig. III-8). Taken together, these data suggested that nerve injury might induce the expression of HGF in the sciatic nerve, consequently leading to the activation of c-met receptor in proximal peripheral axons.

2.3. The HGF/c-met pathway is involved in the peripheral axon outgrowth in the nerve crush mouse model

The functional involvement of HGF/c-met pathway was tested in the nerve crush mouse model using PHA665752. After nerve crush injury, mice were treated with PHA665752 around the injury site using surgifoam for three days. The level of phosphorylated c-met was increased after nerve injury, but was reduced to background levels by local treatment of the injury site with PHA665752. This result shows that the procedure involving surgifoam with PHA665752 could effectively block c-met phosphorylation (Fig. III-9).

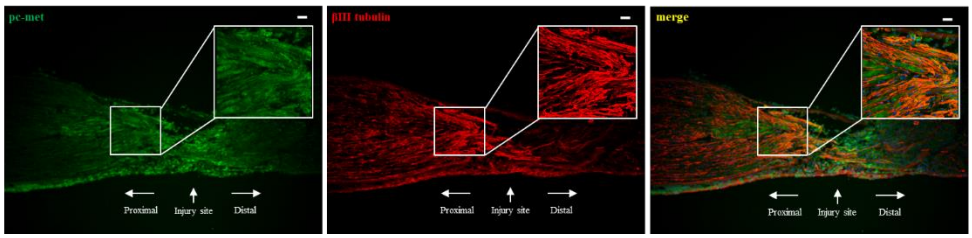

Figure III-8. Phosphorylated c-met receptors localized in βIII tubulin positive axons

3 days after injury, nerves were subjected to IHC using antibodies specific to βIII tubulin (in red) and phosphorylated c-met (in green). Scale, 100 μm.

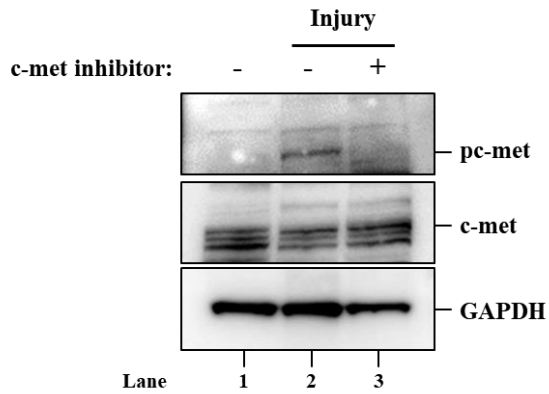

Figure III-9. Effect of PHA665752 on activated c-met

Sciatic nerves were crushed and treated with 25 mg/kg of PHA665752. Injured nerves from proximal to distal region were harvested by day 3, and proteins were prepared, followed by Western blot.

Next, the sciatic nerve pinch test was performed to assess the effect of HGF/c-met pathway on axon regeneration [32]. Mice were subjected to nerve crush, and 5 days later, the distance between the injury and distal sites where mouse show withdrawal reflex was measured. In control mice, the distance was 1.66 ± 0.31 mm. When c-met inhibitor was applied locally to the injury site, the length was reduced by 65% to 0.58 ± 0.27 mm (Fig. III-10). Histological analysis also confirmed the inhibition of axon regeneration by PHA665752. When the injured nerve was analyzed by staining SCG10 protein, regenerating axons were readily observed at the distal region of the sciatic nerve. When PHA665752 was applied to the injury site, however, SCG10-positive regenerating neurons were hardly seen in distal region (Fig. III-11). Taken together, these data suggested that HGF/c-met pathway played a role(s) in the regeneration process of injured peripheral nerves.

The transcription factor, cJun, has been proposed to be a key player in the nerve regeneration process, and is often used as a maker for regeneration [33]. To test if HGF was involved in the induction of cJun expression, sciatic nerve crush was conducted in the presence or absence of PHA665752, and 3 days later, the protein level of cJun was analyzed in the DRGs. The cJun level was low in normal mice (Fig. III-12, lane 1), and highly increased after nerve injury (lane 2). When PHA665752 was applied, its level was greatly reduced (lane 3). This result suggested that one of downstream targets of HGF/c-met pathway might be cJun during the peripheral nerve regeneration.

2.4. Effect of intramuscular injection of pCK-HGF-X7 on peripheral nerve regeneration

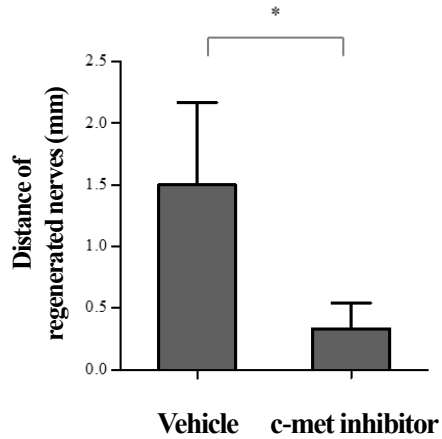

Figure III-10. Effect of PHA665752 in the nerve pinch test

After 5 days of injury, the distance between the crush and pinch-responsive site was measured. Values are presented as mean ±SEM. For statistical analysis, we performed unpaired t-test. *$p<0.05$.

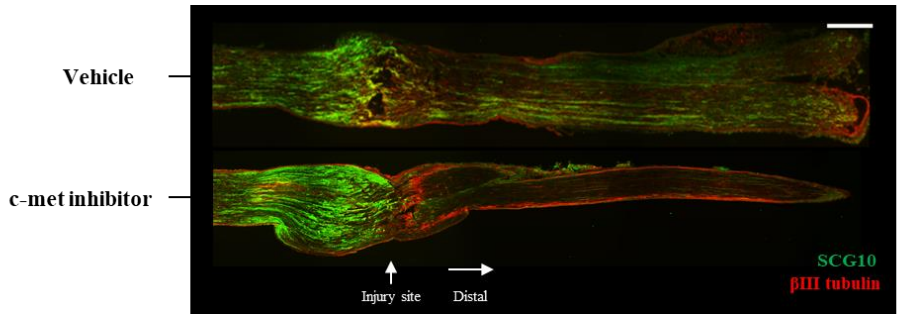

Figure III-11. Effect of PHA665752 on axon regrowth

Injured nerves from the injury site to distal region were isolated 3 days after injury and subjected to IHC using antibodies specific to SCG10 (in green) and βIII tubulin (in red). Scale, 100 μm.

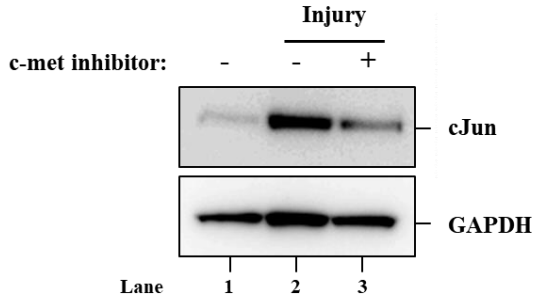

Figure III-12. Effect of PHA665752 on cJun expression in DRGs

DRGs were harvested 3 days after injury and proteins were prepared and then analyzed by Western blot.

The nerve crush mouse model was then used to investigate if an exogenous supply of HGF could facilitate axon outgrowth. The half-life of HGF protein is very short, less than 5 minutes, so the use of recombinant protein is not expected to be an efficient way of delivering HGF. In this study, we used a plasmid DNA designed to express human HGF, pCK-HGF-X7, which has been tested in a variety of clinical studies as well as animal experiments [12, 13, 34].

Nerve crush injury was introduced followed by intramuscular injection of plasmid DNAs, control, and HGF expression vectors to the thigh muscle. Animals were then subjected to the sciatic nerve pinch test. In mice injected with a pCK control vector lacking the HGF sequence, the length of regenerated axons was 1.25 ± 0.40 mm, but this length was highly increased by 3.3-fold, to 4.2 ± 0.41 mm, in mice injected with pCK-HGF-X7 (Fig. III-13). Consistent with this result, the increased length of SCG10-positive axon was also observed in mice injected with an HGF expression vector (Fig. III-14). The injury-mediated cJun induction in the DRGs was further enhanced in the group of mice injected with HGF-expressing plasmid DNA (Fig. III-15). Together, these data suggested that additional supply of HGF, in the form of plasmid DNA expression vector, enhanced the regeneration of damaged peripheral nerves by promoting axon outgrowth.

3. Discussion

This study investigated the role of HGF/c-met pathway in injured peripheral nerves. In *in vitro* primary eDRGs, axotomy increased the level of HGF expression and also activated

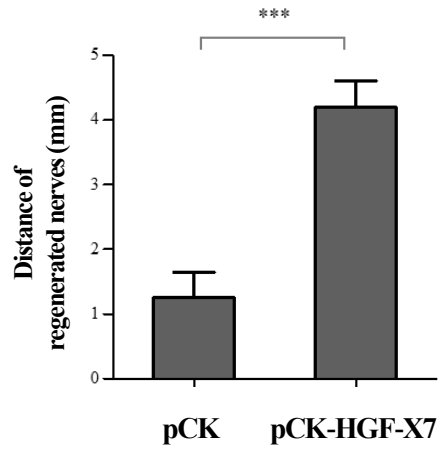

Figure III-13. Effect of HGF expressing plasmid DNA in the nerve pinch test

Sciatic nerves were crushed, and 200 μg of plasmid DNA was injected into the thigh muscle of mice. After 7 days, the distance between the crush and pinch-responsive sites was measured. Values are shown as mean ±SEM. Data was analyzed by unpaired t-test for statistical analysis. ***$p<0.001$.

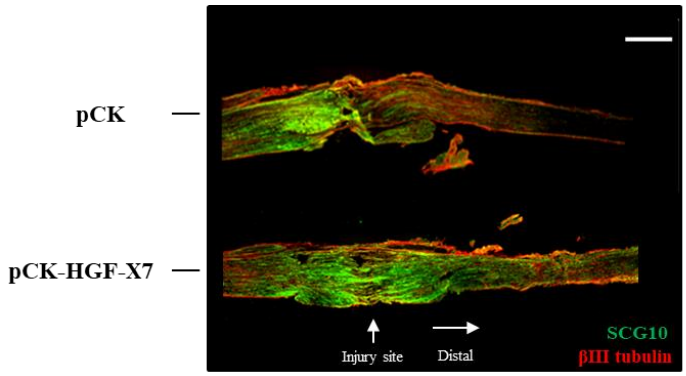

Figure III-14. Effect of HGF expressing plasmid DNA on axon regrowth

Three days after injury, injured nerves from the injury site to distal region were collected, followed by IHC using antibodies specific to SCG10 (in green) and βIII tubulin (in red). Scale, 100 μm.

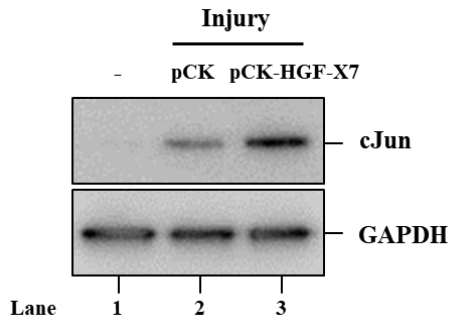

Figure III-15. Effect of HGF expressing plasmid DNA on cJun expression in DRGs

DRGs were harvested 7 days after injury and proteins were prepared followed by Western blot.

c-met. Treatment with PHA665752 hindered the neurite outgrowth, while addition of recombinant HGF proteins enhanced this process. In the *in vivo* nerve crush mouse model, nerve injury significantly increased the level of HGF expression, consequently leading to the activation of c-met receptor in peripheral axons. When PHA665752 was applied locally to the injured nerves, the axon outgrowth process was inhibited and the level of cJun expression in DRGs was also decreased. Finally, exogenously administered HGF by intramuscular injection of HGF-expressing plasmid DNA to mice further accelerated the axon outgrowth and increased the expression of cJun.

For axon regeneration to occur efficiently after injury, two components have to be involved, neuronal intrinsic and non-neuronal extrinsic systems [35]. HGF seems to work through both systems. In this study, data from eDRG cultures indicated that HGF could directly interact with sensory neurons. HGF appears to be one of early proteins upregulated by axon injury, and subsequently interacts with c-met receptors on cells around the injury site. It has been reported that HGF proteins are expressed by fibroblast upon nerve injury [28]. In addition, some serine proteases such as urokinase plasminogen activator and tissue type plasminogen activator are highly expressed by nerve crush injury [5, 6]. Thus, the peripheral nerve injury elicits the expression of HGF mature-form. It is interesting that activated c-met receptors in proximal regions were localized in axons. For example, it has been shown that activated molecules such as Erk, STAT3, DLK, and JNK move to the DRGs through microtubules and regulate the expression of RAGs including ATF3, CREB, galanin, and cJun in the cell body [36, 37, 38, 39, 40, 41, 42]. However, the upstream factors that regulate these signaling molecules in injury site are largely unknown. In this study, we observed that HGF/c-

met pathway was activated in the injured sciatic nerve, but not in the soma, while the expression of cJun in DRGs was regulated under the influence of this signaling. Therefore, the HGF/c-met pathway activated by nerve injury might induce some factor(s) in situ that is used to send a signal to the cell body.

HGF also plays roles through the extrinsic system. We have previously shown that HGF induced upon nerve injury interacts with Schwann cells located in distal region, and activated HGF/c-met pathway increased the proliferation and migration of Schwann cells, thereby accelerating the re-myelination process [12]. Therefore, HGF may be an important integral part of axon regeneration system when peripheral nerves are injured.

In summary, HGF appears to contain multiple bioactivities in the context of peripheral nerve regeneration. It interacts with sensory neurons as well as Schwann cells, two key cell types of the peripheral nervous systems. In addition, HGF showed strong anti-inflammatory activities and analgesic effects by controlling the expressions of inflammatory molecules such as CSF-1 and IL-6 and channel proteins like α2δ1 [13]. Given such a wide spectrum of biological effects and their potential to contribute to the regeneration of damaged nerves, HGF may be an excellent starting point for developing new therapeutics for various clinical conditions resulting from peripheral nerve damage.

Chapter IV

Hepatocyte Growth Factor Induces pErk and pSTAT3 to Promote Mitochondrial Activity and Neurite Outgrowth in Primary Dorsal Root Ganglion Cultures

1. Background

Axon regeneration requires the coordination of multiple cellular processes such as transport of injury signaling to soma, expression of regeneration associated genes, local translation of RNA in axons, reorganization of the cytoskeleton, and mitochondrial transport to injury sites [27, 43, 44, 45]. One of the most essential steps is to supply adequate energy to injured axons, because axon regrowth is a high energy consumption process. Indeed, Caenorhabditis elegans (C. elegans) manipulated for diminished mitochondrial activity showed a poor capacity for axon regeneration [46]. As such, ATP synthesis in mitochondria has been considered an indispensable part of the axon regeneration process [47, 48, 49].

STAT3 plays multiple important roles in peripheral nerve regeneration. Nerve injury induces the local translation of STAT3 RNAs in axons [50]. Thereafter, several MAPKKs phosphorylate STAT3 at tyrosine 705 and/or serine 727. pSTAT3 (Tyr 705) is transported to the nucleus and regulates the transcription of the RAGs. On the other hand, pSTAT3 (Ser 727) translocates to the mitochondria, activating the electron transport chain and increasing ATP synthesis. Both forms of STAT3, each located in the nucleus and mitochondria, are required for axon regeneration [51, 52, 53]. It remained unclear which factors regulate STAT3 phosphorylation in tyrosine and/or serine residues.

Hepatocyte growth factor (HGF) exhibits pleiotropic biological activities in diverse organs such as the liver, kidney, and nervous systems [54]. In the context of the peripheral nervous systems, HGF has been reported to play important roles in the survival and outgrowth of sensory neurons during the developmental stages [7, 8]. Recently, it was also revealed that

injury-induced activation of the HGF/c-met pathway promoted peripheral nerve regeneration in adult mice by interacting with Schwann cells, but its roles in sensory neurons was not clearly understood [12, 28].

To investigate the effect of HGF on sensory neurons, we used primary DRGs isolated from adult mice in this study. Treatment with recombinant HGF proteins activated Erk, increased the level of pSTAT3 (Ser 727), facilitated the translocation of STAT3 to the mitochondria, and enhanced the activity of mitochondrial complex I. Our results revealed that the HGF/Erk/STAT3 axis is involved in axon regeneration by controlling the activity of the mitochondrial electron transport chain.

2. Results

2.1. HGF/c-met pathway promotes neurite outgrowth of primary neurons

To investigate whether the HGF/c-met pathway is involved in injury-induced neurite outgrowth, primary DRGs from adult mice were prepared and cultured. This procedure mimics peripheral nerve injury [55]. First, the effect of HGF proteins on the level of phosphorylated c-met was tested in these injured nerves. Treatment with recombinant HGF proteins highly increased the level of phosphorylated c-met compared to the control cells (Fig. IV-1, compare lanes 1 and 2). Neurite outgrowth was also assessed by staining the cells with an antibody to βIII tubulin, a neuron-specific marker. As shown in Fig. IV-2, the length of the βIII tubulin-positive neurite was increased 1.4-fold compared to the untreated control cells.

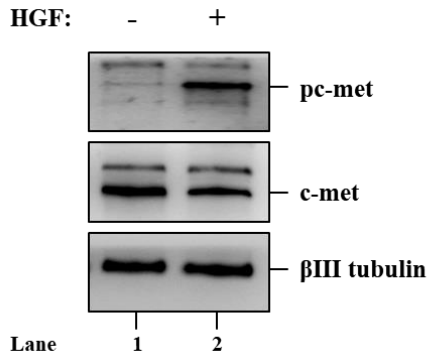

Figure IV-1. Effect of recombinant HGF protein on phosphorylation of c-met receptor

Cultured DRGs were treated with 50 ng/mL of recombinant HGF protein for 3 hours followed by Western blot using antibodies specific to phosphorylated c-met and total c-met. βIII tubulin was used as a loading control.

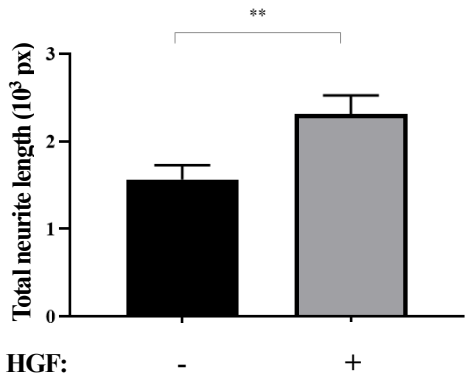

Figure IV-2. Effects of recombinant HGF protein on neurite outgrowth

Cells were fixed and stained with βIII tubulin. The lengths of βIII tubulin-positive neurites were measured. Values are presented as mean ±SEM. An unpaired t-test was performed for statistical analysis. **$p<0.01$.

These results suggest that the HGF/c-met pathway might facilitate the regeneration process in injured sensory neurons prepared from adult mice.

2.2. HGF induces translocation of STAT3 to mitochondria and enhances activity of the electron transport chain

To investigate which signaling pathways are affected by the HGF/c-met pathway, primary DRGs were treated with HGF proteins, followed by Western blot analysis. The two amino acid residues serine 727 and tyrosine 705 are important for STAT3 activities; the former is involved in the transportation of this protein to the mitochondria and the subsequent control of the electron transport chain's activity, while the latter is needed for STAT3 to reach the cell body and take action on downstream molecules [50, 51, 52, 53]. As shown in Fig. IV-3, the level of pSTAT3 (Ser 727) was increased by HGF treatment, while no significant change in that of pSTAT3 (Tyr 705) was observed.

We further investigated whether the translocation of STAT3 to the mitochondria indeed occurred by phosphorylation at serine 727 of STAT3. Primary DRGs were treated with HGF proteins, followed by isolation of the mitochondrial and cytosolic fractions. In the cytosolic fraction, HGF treatment did not change the level of pSTAT3 (Ser 727) (Fig. IV-4, compare lanes 3 with 4). However, in the mitochondrial fraction, HGF treatment substantially increased the amount of pSTAT3 (Ser 727) (Fig. IV-4, compare lanes 1 with 2). Taken together, these data suggest that HGF could activate STAT3 by phosphorylating serine 727 to translocate the protein to the mitochondria.

We also tested whether HGF could regulate the activity of the mitochondrial

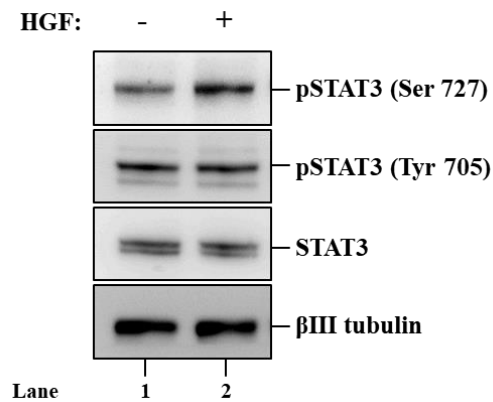

Figure IV-3. Effect of recombinant HGF protein on STAT3 phosphorylated at serine 727

Cultured DRGs were treated with 50 ng/mL of recombinant HGF protein for 3 hours followed by Western blot using antibodies specific to phosphorylated STAT3 at serine 727, phosphorylated STAT3 at tyrosine 705, and total STAT3.

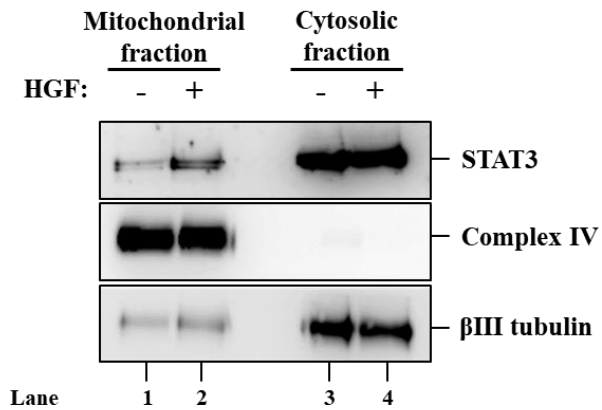

Figure IV-4. Effect of recombinant HGF protein on translocation of STAT3 to mitochondria

Cultured DRGs were treated with 50 ng/mL of recombinant HGF protein for 3 hours. Mitochondrial and cytosolic fractions were isolated followed by Western blot using an antibody specific to STAT3s. Complex IV was used as a marker for mitochondrial fraction, and βIII tubulin was used as a marker for cytosolic fraction.

electron transport chain. Cultured DRGs were treated with recombinant HGF proteins, after which the activity of the mitochondrial complex was measured. Treatment with HGF proteins enhanced the activity of mitochondrial complex I by 30% (Fig. IV-5), while its effect on that of mitochondrial complex II was marginal (Fig. IV-6).

2.3. HGF/ERK/STAT3 axis plays an important role in neurite outgrowth

Erk is known to play an important role in the phosphorylation of STAT3 at serine 727 [56, 57, 58, 59]. To test the involvement of Erk in the HGF-mediated mitochondrial transport of STAT3, U0126, a pharmacological inhibitor of Erk, was used in HGF-treated DRGs. When the culture was treated with HGF, the level of phosphorylated Erk was increased compared to the untreated control (Fig. IV-7, compare lanes 1 with 2), but was reduced in the presence of U0126 (Fig. IV-7, compare lanes 2 and 4).

To investigate whether the HGF-mediated activation of Erk indeed induces the localization of STAT3 to the mitochondria, cultured DRGs were treated with HGF proteins in the absence or presence of U1026, and the content of mitochondrial fraction was analyzed. As shown in Fig. IV-8, the level of STAT3 in the mitochondrial fraction was increased by HGF but was highly reduced by U0126 treatment.

The involvement of Erk in HGF-mediated neurite outgrowth was also tested. Primary DRGs were cultured and treated with HGF proteins in the presence or absence of U0126. Treatment with HGF proteins increased the length of the neurite 1.5-fold compared to that of the control group, which was reduced by 55% when U0126 was present (Fig. IV-9).

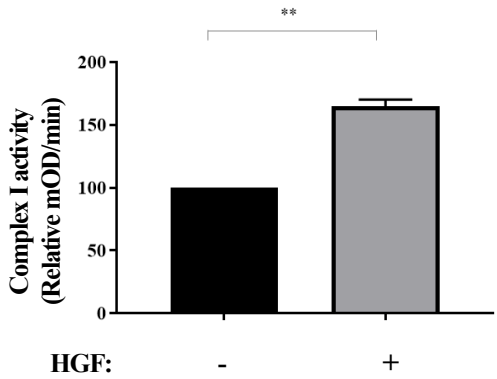

Figure IV-5. Effect of recombinant HGF protein on mitochondrial complex I activity

Cultured DRGs were treated with 50 ng/mL of recombinant HGF protein for 18 hours followed by the measurement of mitochondrial complex I activity. Values are presented as mean ±SEM. An unpaired t-test was performed for statistical analysis. **$p<0.01$.

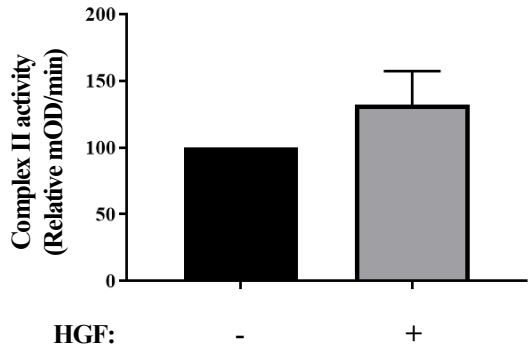

Figure IV-6. Effect of recombinant HGF protein on mitochondrial complex II activity

Effect on mitochondrial complex II activity. Cultured DRGs were treated with 50 ng/mL of recombinant HGF protein for 18 hours followed by the measurement of mitochondrial complex II activity. Values are presented as mean ±SEM. An unpaired t-test was performed for statistical analysis.

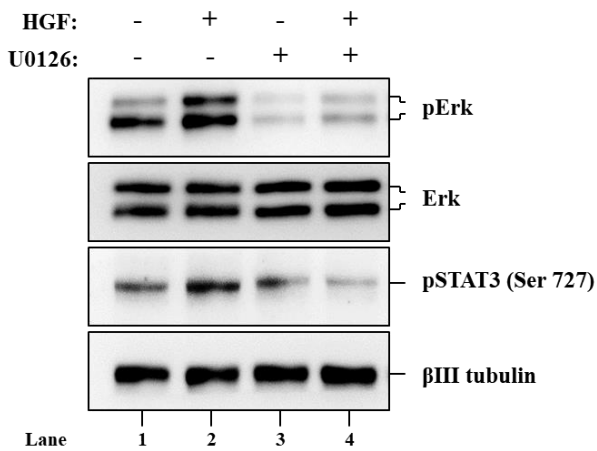

Figure IV-7. Effect of Erk inhibitor, U0126 on STAT3 phosphorylated at serine 727

Cultured DRGs were treated with 20 μM of U0126 for 1 hour, then treated with 50 ng/mL of recombinant HGF protein for 3 hours followed by Western blot using antibodies specific to phosphorylated Erk, Erk, and pSTAT3 (Serine 727).

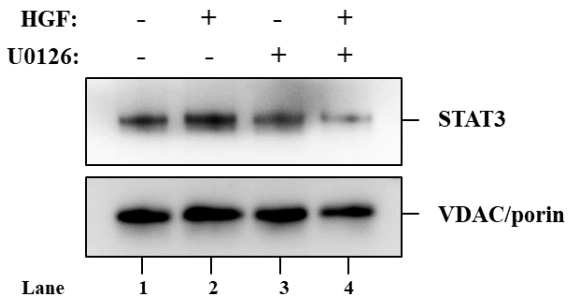

Figure IV-8. Effect of U0126 on translocation of STAT3 to mitochondria

Cultured DRGs were treated with 20 μM of U0126 for 1 hour, then treated with 50 ng/mL of recombinant HGF protein for 3 hours. Mitochondrial and cytosolic fractions were isolated followed by Western blot using an antibody specific to STAT3. VDAC/porin was used as a marker for mitochondrial fraction.

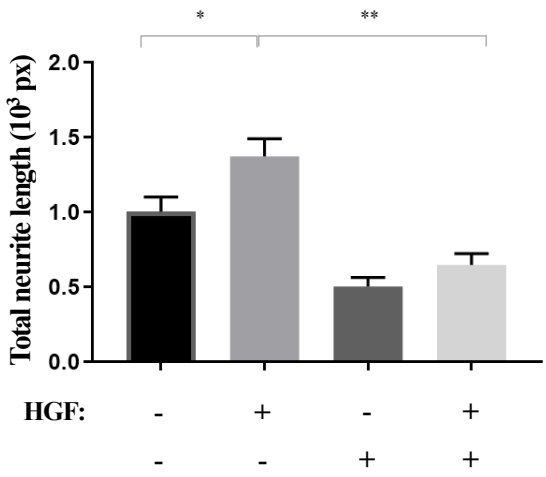

Figure IV-9. Effects of U0126 on neurite outgrowth

Cultured DRGs were treated with 20 μM of U0126 for 1 hour, then treated with 50 ng/mL of recombinant HGF protein for 24 hours. Cells were fixed and stained with βIII tubulin. The lengths of βIII tubulin-positive neurites were measured. Values are presented as mean ±SEM. One-way ANOVA followed by Dunnett's multiple comparison testing was performed for statistical analysis. *$p<0.05$, **$p<0.01$.

These data suggest that the effect of HGF on neurite outgrowth results from the activation of Erk and phosphorylation of STAT3 at serine 727.

3. Discussion

Using primary sensory neurons prepared from adult mice, this study found that the HGF/c-met pathway promotes axon regeneration by regulating mitochondrial activity. It appears that the HGF/c-met pathway achieves this by controlling the phosphorylation, first of Erk and then of STAT3 at serine 727, consequently translocating STAT3 to the mitochondria and elevating the activity of mitochondrial complex I to produce the ATP needed for neurite outgrowth. To prove HGF promotes the neurite outgrowth by controlling mitochondrial complex I activity, it should be tested whether the HGF-induced neurite outgrowth is inhibited by mitochondrial complex I blockers.

Peripheral nerve injury induces the transport of mitochondria to the injured axons. Because the formation of the growth cone is a high energy consumption process, supplying adequate energy to the damaged axon is an essential step. Thus, mitochondria are anterogradely transported to the damaged axons and provides ATP [47, 48, 49]. STAT3 plays dual roles in peripheral nerve regeneration. Nerve injury induces the phosphorylation of STAT3 at tyrosine 705 in injured axons. Then, pSTAT3 (Tyr 705) is retrogradely transported to the soma, acting as a transcription factor. On the other hand, nerve injury phosphorylated the STAT3 at serine 727 in injured axons. Then, pSTAT3 (Ser 727) translocates to the

mitochondria transported to the injured site, activating the mitochondrial complex I and increasing ATP synthesis [51, 52, 53]. Thus, injury-induced pSTAT3 (Tyr 705) functions as a transcription factor in DRGs, and pSTAT3 (Ser 727) functions in injured axons. In this study, we could not demonstrate where the activated HGF/c-met pathway plays role(s). We observed that HGF activates the mitochondrial complex I by phosphorylating the STAT3 at serine 727. HGF/c-met pathway could not elevate the phosphorylation of STAT3 at tyrosine 705. Moreover, in chapter III, it appears that injury activated HGF/c-met pathway in injured axons only. These results suggest that the region where the activated HGF/c-met pathway functions to promote neurite outgrowth is the injured axons. The precise mechanism remains to be determined.

In this study, HGF/c-met pathway induces the phosphorylation of STAT3 at serine 727, not tyrosine 705. It has been known that HGF phosphorylates the STAT3 at tyrosine 705, and then pSTAT3 (Tyr 705) is transported to nucleus. However, it's role in the phosphorylation of the STAT3 at serine 727 has been relatively unknown. CNTF, one of the well-known neurotrophic factors, phosphorylates the STAT3 both at tyrosine 705 and serine 727 to regenerate the damaged axons. CNTF regulates the phosphorylation of STAT3 at serine 727 in an Erk-dependent manner, while enhancing the phosphorylated STAT3 at tyrosine 705 in an Erk-independent manner. In this study, treatment with HGF proteins increases the phosphorylation of Erk. Also, HGF-induced pSTAT3 (Ser 727) was reduced by treatment with Erk inhibitor. These results presented here reveal that HGF/c-met pathway selectively promotes the phosphorylation of STAT3 at serine 727 in an Erk-dependent manner.

It has been reported that the nerve regeneration capacity diminishes with aging due to reduced mitochondrial activity [46]. In this study, HGF/c-met pathway promotes the neurite outgrowth by controlling the activity of mitochondrial electron transport chain. Thus, it is possible that HGF/c-met pathway might link the reduced nerve regeneration capacity and mitochondrial activity. We observed that peripheral nerve injury activated the HGF/c-met pathway in young mice, but nerve injury could not activate the HGF/c-met pathway in old mice (data not shown). Therefore, diminished nerve regeneration capacity with aging might be due to the reduced activation of HGF/c-met pathway. The exact mechanisms should be addressed in further studies.

Our previous reports revealed that HGF could effectively regenerate damaged peripheral nerves by directly interacting with Schwann cells to promote remyelination [12], and with sensory neurons to facilitate axon outgrowth. In sensory neurons, HGF seems to exert effects in damaged areas of both the axon and soma. HGF expression was highly increased around the nerve injury site. This injury-mediated induction of HGF expression led not only to the increased level of phosphorylated c-met in axons proximal and Schwann cells distal from the injury site, but also to significant changes in the expression of various genes in DRGs [13]. Therefore, HGF seems to be working at two levels: directly on the injury site and/or indirectly through the control of gene expression in the soma.

Together with the results described in this report, HGF appears to have pleiotropic effects on the nerve regeneration process by producing multiple biological activities in different cell types and different cellular compartments. Given the importance of understanding the nerve regeneration process in clinical as well as neurobiological contexts,

further investigations are warranted to break down the various bioactivities of HGF at anatomical, cellular, and molecular levels in different organs and tissues.

Chapter V

Disproportionately High Levels of HGF Induce the Degradation of the c-met Receptor through the Proteasomal Degradation Pathway

1. Background

Hepatocyte growth factor (HGF), also known as scatter factor, is one of the representative receptor tyrosine kinases (RTKs) and exerts pleiotropic effects [60]. HGF plays important roles in cell proliferation, survival, and motility [61]. During embryonic development, HGF-null mice have reduced liver size [62], show deficits in the migration of muscle progenitor cells [63], and fail to survive sensory neurons [7] and sympathetic neurons [8]. In the adult stage, HGF plays a decisive role in the regeneration of the liver and kidney [64, 65, 66].

RTKs regulate numerous cellular processes through physical interactions between specific ligands and respective cellular receptors. The binding of almost all ligands -except that of the insulin receptor family- induces dimerization of their receptors and autophosphorylation followed by recruitment of intracellular substrates [67]. After the binding, ligand and receptor complex is internalized by endocytosis [68] and sorted into recycling endosomes or late endosomes destined for degradation [69, 70].

In the case of EGF and EGFR, at rate-limiting concentrations of ligand, the EGF and EGFR complex is internalized through clathrin-mediated endocytosis and preferentially recycled. At unnecessarily higher concentrations of EGF, however, the ligand and receptor complex is internalized via non-clathrin endocytosis subjected to the degradation process [19]. It was reported that the internalized HGF and c-met complex could also be recycled to the plasma membrane or degraded by the lysosome or proteasome [20]. But it remains poorly

understood under what situations the internalized HGF and c-met complex is recycled or degraded.

In this study, we found that the HGF-induced migration of human umbilical vein endothelial cells (HUVECs) showed an interesting bell-shaped dose response; at lower concentrations, the number of migrated cells was increased as HGF concentration increased, but was significantly reduced at concentrations higher than a certain point. Data from experiments involving cycloheximide (CHX) indicated that the c-met receptor degraded rapidly at high concentrations. Treatment with MG132, an inhibitor of proteasomes, blocked such c-met degradation. Consistent with this result, the amount of c-met phosphorylation at Y1003 involved in the ubiquitination process was increased. Our results show that the c-met receptor underwent ubiquitin-proteasome associated degradation in the presence of disproportionately large amounts of HGF.

2. Results

2.1. HGF-mediated cell migration showed a bell-shaped dose response curve

While investigating the effect of HGF on the migration of HUVECs, we found that cells responded to different doses of HGF in a bell-shaped form. HUVECs were plated in the upper chambers, while the lower chambers contained recombinant human HGF proteins. Two hours after culture, cells that had migrated to the lower chambers were stained, visualized, and counted. The number of migrated cells was increased by a HGF treatment in a dose-dependent manner and peaked at 50 ng/mL, but then decreased at higher concentrations (Fig.

V-1). Treatment with 50 ng/mL of HGF induced cell migration by almost 2-fold compared to the non-treated group, while it dropped to 75% at 200 ng/mL of HGF, indicating that when HGF is present at high levels beyond a certain point, its biological activity is reduced.

This response was not restricted to a particular cell type. In another experiment involving a murine myoblast line, C2C12 cells whose migration has been reported to be induced by human HGF protein, again, a bell-shaped dose response curve was observed [71]. As summarized in Fig. V-2, the number of migrated cells was increased by HGF treatment, peaked at 50 ng/mL, and then decreased consistent with data from HUVECs. Similar observations were made for other cells like primary motor neurons (data not shown). We designated 50 ng/mL and 200 ng/mL of HGF as "optimal" and "excessive" doses, respectively, for further experiments.

HGF binds to its receptor c-met and activates a variety of downstream signaling pathways such as Erk and Akt to promote cell migration [72]. To test whether the level of activity of downstream proteins is somehow lowered at excessive concentrations, HUVECs were treated with optimal and excessive doses of HGF for 30 minutes, followed by Western blot analysis. Cells treated with optimal doses showed an increased amount of phosphorylated Erk and Akt (Fig. V-3, compare lanes 1 and 2), and this high level was more or less maintained at the excessive concentration (Fig. V-3, compare lanes 2 and 3). Taken together, these data indicate that HGF-mediated cell migration shows a bell-shaped dose response, but that the downstream signaling molecules might not be involved in this phenomenon.

2.2. Excessive doses of HGF induced c-met degradation

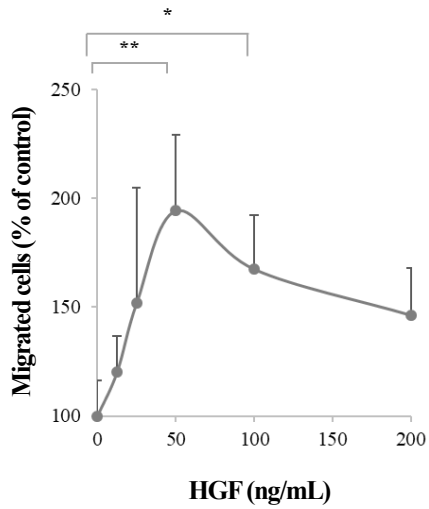

Figure V-1. Cell migration assay using HUVECs

Cells were plated in the upper chambers, while various concentrations of recombinant HGF protein were present in the lower chambers. Two hours later, cells that migrated through the membrane pore were stained and counted. one-way ANOVA followed by Bonferroni's multiple comparison testing. *$p<0.05$; **$p<0.01$.

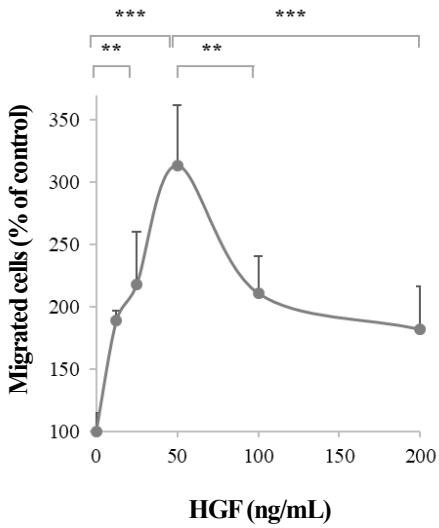

Figure V-2. Cell migration assay using C2C12 cells

Cells were plated in the upper chambers, while various concentrations of recombinant HGF protein were present in the lower chambers. Two hours later, cells that migrated through the membrane pore were stained and counted. one-way ANOVA followed by Bonferroni's multiple comparison testing. **p<0.01; ***p<0.001.

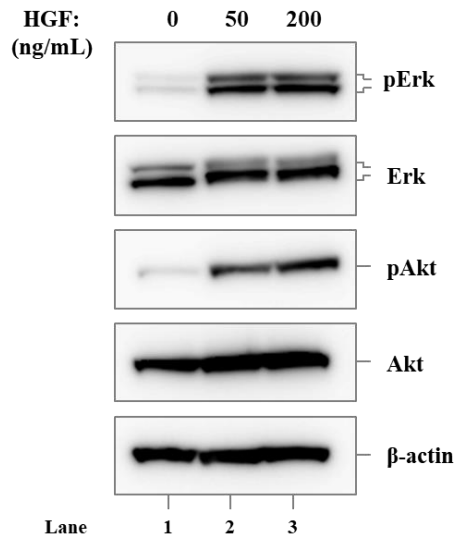

Figure V-3. Effects of different concentrations of HGF on signaling proteins

HUVECs were treated with 0, 50, 200 ng/mL of recombinant HGF protein. Thirty minutes later, cells were lysed and proteins were prepared followed by Western blot using antibodies specific to pErk, Erk, pAkt, and Akt. β-actin was used as a loading control.

To further investigate the underlying mechanism of the HGF-mediated bell-shaped response, it was hypothesized that as in the case of EGF and EGFR, cellular receptors of HGF might undergo degradation when unnecessarily large amounts of HGF are present [73]. HUVECs were treated with optimal and excessive doses of HGF in the presence of CHX for 2 hours to block the translation process followed by Western blot. When cells were treated with an optimal dose, the protein level of c-met was reduced by 21% compared to non-treated cells (Fig. V-4, compare lanes 1 and 2), but at the excessive dose, it was further lowered by 55% compared to non-treated cells (Fig. V-4, compare lanes 1 and 3), suggesting that the c-met receptor might be degraded at higher concentrations of HGF.

The surface level of c-met was also analyzed by FACS using a specific antibody. Under normal circumstances, 82% of HUVECs contained c-met, while treatment with an optimal dose reduced the level to 68 % due to the normal internalization process (Fig. V-5, compare graphs 1 and 2). When cells were treated with an excessive dose, however, the level of c-met protein present on the cell surface dropped even further to 41% (Fig. V-5, compare graphs 2 and 3). These data suggest that HGF could also downregulate the surface level of c-met at a higher dose, which might have led to decreased levels of HGF-mediated cell migration.

2.3. Excessive doses of HGF attenuated the sustained activation of HGF/c-met pathway

To examine whether an excessive amount of HGF affected downstream signaling, cells were treated with HGF at two steps. First, HUVECs were treated with an optimal or excessive dose of HGF in the presence of CHX for 2 hours. During this period, new protein

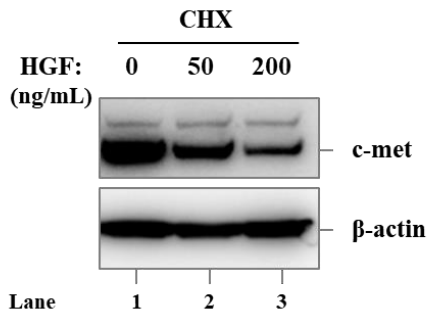

Figure V-4. Effects of large amounts of HGF on c-met degradation

HUVECs were treated with 0, 50, 200 ng/mL of recombinant HGF protein in the presence of cycloheximide. Two hours later, cells were lysed and proteins were prepared followed by Western blot, using an antibody specific to c-met. β-actin was used as a loading control.

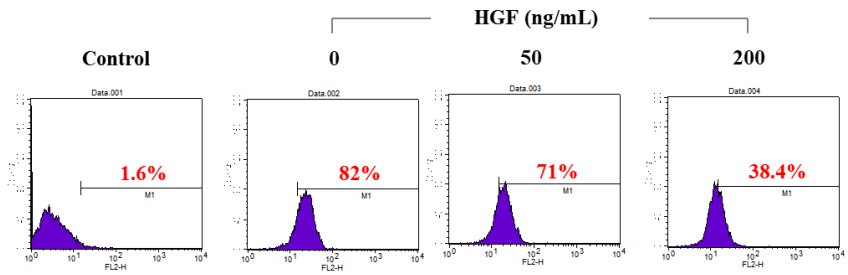

Figure V-5. Effects of excessive doses of HGF on the surface level of c-met

HUVECs were treated with 0, 50, 200 ng/mL of recombinant HGF protein. Thirty minutes later, cells were detached with trypsin and analyzed with FACS using an antibody specific to surface c-met.

synthesis was blocked, degrading the c-met protein to a very low level, and most signaling proteins under the influence of c-met would have returned to the basal state. At this point, cells were washed and treated again, this time with an optimum level of HGF for 30 minutes only. Since cells were in the basal state at this point, treatment with an optimal dose would again activate downstream signalings.

When proteins were isolated at the end of the first step, the level of c-met was significantly lower at 200 ng/mL of HGF (Fig. V-6, compare lanes 1 and 3), consistent with the above results. At this point, the amounts of phosphorylated Erk and Akt were all low when analyzed 2 hours after treatment with HGF as the signaling returned to the basal point (Fig. V-6, compare lanes 1 and 3). When cells were treated with 50 ng/mL of HGF during the second step, the level of phosphorylated Erk and Akt surged again in cells previously treated with 50 ng/mL of HGF (Fig. V-6, compare lanes 2 and 5). However, these levels did not increase at all in cells previously treated with an excessive dose during the first step (Fig. V-6, compare lanes 3 and 6). This data suggests that an excessive dose had placed the cells into an exhausted state rather than basal state, depleting related signaling molecules, and thus rendering cells inefficiently responsive to the second stimulation.

2.4. Excessive doses of HGF induced the proteasomal degradation of c-met

The degradation of cellular protein is mediated by lysosomal or proteasomal degradation pathways [74]. To test which pathway is involved in c-met protein degradation, we used chemical inhibitors widely used to block the proteasomal degradation pathways [75].

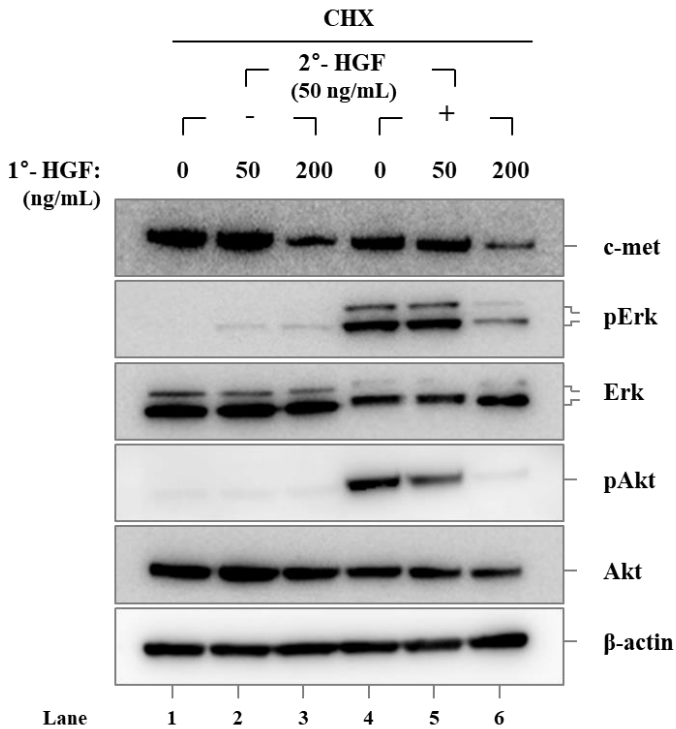

Figure V-6. Effects of excessive amounts of HGF on signaling proteins in HGF/c-met axis

HUVECs were treated with 0, 50, 200 ng/mL of recombinant HGF protein in the presence of cycloheximide. Two hours later, cells were washed with PBS and treated with 0, 50 ng/mL of recombinant HGF protein again. Thirty minutes later, cells were lysed and proteins were prepared followed by Western blot using antibodies specific to c-met, pErk, Erk, pAkt, and Akt. β-actin was used as a loading control. 1° and 2° each represent the first and second treatments of HGF, respectively.

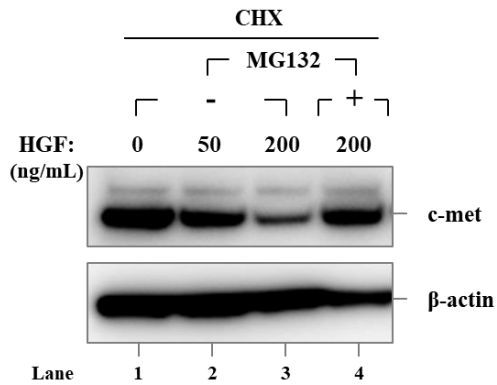

Figure V-7. Effects of proteasome inhibitor, MG132

HUVECs were treated with 0, 50, 200 ng/mL of recombinant HGF protein in the presence of cycloheximide. Additionally, 5 μM of MG132 was added to 200 ng/ml of HGF-treated cells. Two hours later, cells were lysed and proteins were prepared followed by Western blot using an antibody specific to c-met. β-actin was used as a loading control.

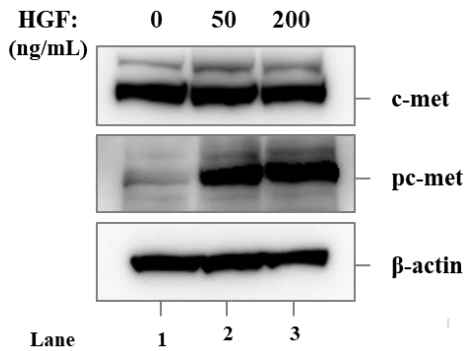

Figure V-8. Effects of excessive doses of HGF on tyrosine 1003 of c-met protein

HUVECs were treated with 0, 50, 200 ng/mL of recombinant HGF protein. Thirty minutes later, cells were lysed and proteins were prepared followed by Western blot using antibodies specific to c-met, pc-met. β-actin was used as a loading control.

HUVECs were treated with optimal or excessive doses of HGF in the presence of MG132, a specific inhibitor of proteasome. The degradation of c-met protein induced by excessive HGF treatment was suppressed by MG132 (Fig. V-7, compare lanes 3 and 4), suggesting that c-met degradation might be mediated by the proteasomal degradation pathway.

Tyrosine residue at the 1003 position (Y1003) of the c-met protein is phosphorylated by the ubiquitination complex, leading to the degradation of this protein [20]. To measure the level of Y1003-phosphorylated c-met, HUVECs were treated with an optimal or excessive dose of HGF for 30 minutes, and cell lysates were subjected to Western blot analysis. When cells were treated with an optimal dose of HGF, the level of c-met phosphorylated at Y1003 was comparable to that of untreated cells (Fig. V-8, compare lanes 1 and 2). Treatment with excessive HGF, however, increased the level of Y1003 phosphorylated 3-fold, indicating that c-met might indeed undergo an ubiquitination process to be degraded. Taken together, excessive doses of HGF appear to lead its cellular receptor to the proteasomal degradation pathway by inducing the phosphorylation of the Y1003 residue.

3. Discussion

In this study, we showed that the migration of HUVECs responded to HGF in a bell-shaped dose response manner, and that when unnecessarily large amounts of HGF were present, its cellular receptor, c-met, underwent degradation through the ubiquitination-proteasome pathway as evidenced by the blocking of this process by MG132 and the

increased level of Y1003 of c-met at an excessive dose. Our results are consistent with previous reports showing that RTK signaling is regulated to a moderate level [76].

It is well known that signaling pathways important during embryonic development and organ regeneration are often over-activated in cancer cells [77]. Although HGF plays major roles in organ development and regeneration, overexpression or overactivation of HGF and c-met pathway may increase the possibility of cancer onset and metastasis [78]. Indeed, chronic activation of HGF has been observed in glioblastoma, breast carcinoma, rhabdomyosarcoma, and osteosarcoma [79, 80, 81, 82]. Therefore, it is important to tightly control HGF-mediated bioactivity.

Negative feedback regulation of RTK signaling is achieved by various mechanisms -for example, ligand sequestration [83], inhibition of ligand and receptor binding [84], blocking of the autophosphorylation of receptors [85], expression of inhibitory proteins to downstream signaling proteins [86] and ligand-mediated receptor degradation [87]. It was reported that phosphorylation of Y1003 of the c-met protein could recruit CBL, E3 ubiquitin ligase, leading the protein to a proteasomal degradation pathway [20]. However, it has not been clear under what circumstance this function is used. In this study, we identify at least one condition leading to this pathway, that is, when HGF is present at a level higher than necessary. However, we showed the proteasomal degradation of c-met receptor in the presence of cycloheximide. Thus, to know the amount of c-met newly synthesized, and the rate of c-met recycling, further experiments are needed in the absence of cycloheximide.

Our data may explain an explanation why there was a bell-shaped dose response in human studies involving HGF. In phase I and II studies for painful diabetic peripheral

neuropathy employing the plasmid expressing HGF, VM202 (also called pCK-HGFX7), a peculiar dose response was observed. In the phase I study, 3 different doses were used; 8 mg plasmid DNA per leg per subject yielded a higher analgesic effect than 4 mg, while doubling the dose to 16 mg reduced pain to a lesser degree. This result was reproduced in a larger scale phase II study [88, 34]. Since then, such a bell or U shaped dose response curve, depending on what is being measured, has been observed in a variety of experimental settings performed in our laboratory, including behavior tests in neuropathic pain model (data not shown). In this model, about 100 ng/mg of HGF proteins was detected by administration of HGF expressing plasmid DNA. Although it is difficult to predict the exact optimum or excess amount of HGF proteins, it is likely that sufficient amounts of HGF proteins are expressed by plasmid DNA injection to exhibit bell-shaped dose response. In addition, the expression of HGF proteins was maintained about 14 days. Thus, to mimic the above situations in *in vitro* systems, repeated treatments with recombinant HGF proteins should be performed. Furthermore, to prove the exact mechanisms, it is essential to test the c-met degradation in *in vivo*.

Our results indicate that c-met might be tightly controlled depending on the concentrations of its ligand. It is possible that HGF may have evolved such that the over-activation of cell signalings by disproportionately large amounts of this protein is prevented by degrading its receptor. It would be interesting to test whether the same stands for other proteins with similar activities or functions. Given the importance of many growth factors in clinical settings, it is vital to understand the quantitative relationship between growth factors and their receptors in the context of the biological activities and therapeutic effects.

Chapter VI

Concluding Remarks

In this thesis, I investigated the role of HGF/c-met pathway in the peripheral nerve regeneration by using *in vitro* embryonic and adult DRG neurons and *in vivo* nerve crush mouse model. In *in vitro* primary eDRGs, axotomy increased the level of HGF expression and also activated c-met. Treatment with PHA665752, a specific c-met inhibitor, hindered the neurite outgrowth, while addition of recombinant HGF proteins enhanced this process. In the *in vivo* nerve crush mouse model, nerve injury significantly increased the level of HGF expression, consequently leading to the activation of c-met receptor in peripheral axons. When PHA665752 was applied locally to the injured nerves, the axon outgrowth process was inhibited and the level of cJun expression in DRGs was also decreased. Finally, exogenously administered HGF by intramuscular injection of HGF-expressing plasmid DNA to mice further accelerated the axon outgrowth and increased the expression of cJun. These results indicated that HGF interacts directly with sensory neurons as well as Schwann cells as previously reported by our group, two key cell types of the peripheral nervous systems.

Next, I also investigated the role of HGF using primary sensory neurons prepared from adult mice. I found that the HGF/c-met pathway promotes axon regeneration by regulating mitochondrial activity. It appears that the HGF/c-met pathway achieves this by controlling the phosphorylation, first of Erk and then of STAT3 at serine 727, consequently translocating STAT3 to the mitochondria and elevating the activity of mitochondrial complex I to produce the ATP needed for neurite outgrowth. Therefore, HGF appears to promote axon regeneration by controlling the activity of the mitochondrial electron transport chain via Erk and STAT3.

In phase I and II clinical studies for painful diabetic peripheral neuropathy

employing the plasmid expressing HGF, VM202 (also called pCK-HGFX7), a peculiar dose response was observed. In the phase I study, 3 different doses were used; 8 mg plasmid DNA per leg per subject yielded a higher analgesic effect than 4 mg, while doubling the dose to 16 mg reduced pain to a lesser degree. This result was reproduced in a larger scale phase II study. Since then, such a bell or U shaped dose response curve, depending on what is being measured, has been observed in a variety of experimental settings performed in our laboratory, including behavior tests in mice, cell migration assays, and the effects on axon outgrowth, among others. I tried to understand this phenomenon in the *in vitro* cell culture system. It was found that that the migration of HUVECs responded to HGF in a bell-shaped dose response manner, and that when unnecessarily large amounts of HGF were present, its cellular receptor, c-met, underwent degradation through the ubiquitination-proteasome pathway as evidenced by the blocking of this process by MG132 and the increased level of Y1003 of c-met at an excessive dose. Our results are consistent with previous reports showing that RTK signaling is regulated to a moderate level.

In summary, HGF plays important roles in axon outgrowth by directly interacting with sensory neurons and HGF/c-met pathway is tightly regulated by a negative feedback loop through an ubiquitin-proteasomal degradation pathway.

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국문초록

간세포 성장인자는 신경영양성 인자로 잘 알려져 있지만 손상된 말초 신경에 어떠한 영향을 미치는 지에 대해서는 밝혀진 바가 거의 없었다. 본 연구에서는 말초 신경의 재생 관점에서 간세포 성장인자와 그 수용체인 c-met 의 생물학적인 기능에 대하여 조사하였다. 태아의 후근 신경절 (dorsal root ganglion)에서 얻은 감각 신경을 배양한 후 axotomy 를 가하면 간세포 성장인자의 발현과 c-met 의 인산화가 증가하였다. Axotomy 를 가한 후 감각 신경에 c-met 저해제인 PHA665752 를 처리하면 다시 자라나는 신경 돌기의 길이가 상당히 감소하였다. 반면 간세포 성장인자를 처리하면 다시 자라나는 신경 돌기의 길이가 증가하였다. 동물 모델에서 말초 신경에 손상을 가하면 손상 부위에서 간세포 성장인자의 발현이 약 5.5 배 증가하였으며, 수용체인 c-met 의 인산화도 증가하였다. 신경 손상과 함께 PHA665752 를 신경 손상 부위에 처리한 경우 다시 자라나는 신경의 길이가 감소하였고, 후근 신경절에서 cJun 의 발현이 감소하였다. 이는 HGF/c-met 신호 전달 체계가 신경 재생 과정에 관여할 수 있음을 암시한다. 또한 간세포 성장인자를 플라스미드의 형태로 근육에 주사한 경우, 신경 재생 과정이 크게 촉진되었다.

다음으로, 성체 후근 신경절에서 얻은 감각 신경을 배양하여 신경 돌기가 뻗는 과정을 간세포 성장인자가 어떻게 조절하는지 조사하였다. 감각 신경 세포에 간세포 성장인자를 처리하면 신경 돌기의 길이가 약 1.4 배 증가하였다. 간세포 성장인자는 STAT3 의 serine 727 의 인산화를 증가시켜

STAT3 를 미토콘드리아로 이동시켰으며, 이를 통해 미토콘드리아의 전자전달계 complex I의 활성을 증가시켰다. 또한 Erk 저해제인 U0126 을 처리하면 이러한 간세포 성장인자의 효과가 억제되는 것을 관찰할 수 있었다.

간세포 성장인자를 이용한 임상시험에서 간세포 성장인자의 생물학적 활성이 독특한 종 모양 용량 반응 (bell-shaped dose response)을 보이는 것을 관찰하였다. 따라서 내피 세포 (endothelial cell)를 이용하여 간세포 성장인자의 종 모양 용량 반응에 대하여 조사해보았다. 처리한 간세포 성장인자의 용량이 증가할수록 내피 세포의 이동이 증가했지만 특정 용량 이상에서는 오히려 이동한 세포의 수가 감소하였다. 과다한 용량의 간세포 성장인자는 c-met 의 tyrosine 1003 의 인산화를 증가시켜 프로테아좀에 의해 분해되도록 하였다. 즉, 필요 이상의 고용량의 간세포 성장인자가 존재하는 경우, 수용체인 c-met 이 분해되었다. 이러한 결과는 간세포 성장인자가 임상시험에서 종 모양 용량 반응을 보인 이유에 대하여 설명해준다.

종합하면 간세포 성장인자는 감각 신경에 직접적으로 작용하여 미토콘드리아의 활성을 조절함으로써 신경 재생을 촉진시키며, 이러한 HGF/c-met 신호 전달 체계는 유비퀴틴-프로테아좀 경로를 통해 음성 피드백에 의해 조절된다.

핵심어: 간세포 성장인자, 미토콘드리아, 감각 신경, 신경 돌기, 신경 재생, 음성 피드백

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