이학박사학위논문

간세포 성장인자가 슈반세포를 통해 말초 신경 재생에 미치는 영향 연구

Promotion of Peripheral Nerve Regeneration by Hepatocyte Growth Factor through Activation of Schwann Cells

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고 경 량

Abstract

Promotion of Peripheral Nerve Regeneration by Hepatocyte Growth Factor through Activation of Schwann Cells

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During the peripheral nerve regeneration process, a variety of neurotrophic factors play roles in nerve repair by acting on neuronal or non-neuronal cells. In this thesis work, I investigated the role(s) of hepatocyte growth factor (HGF) and its receptor, c-met, in peripheral nerve regeneration. When mice were subjected to sciatic nerve injury, the HGF protein level was highly increased at the injured and distal sites. The levels of both total and phosphorylated c-met were also highly up-regulated, but almost exclusively in Schwann cells (SCs) distal from the injury site. When mice were treated with the c-met inhibitor PHA-665752, myelin thickness and axon regrowth were decreased, indicating that re-myelination was hindered. HGF promoted the migration and proliferation of cultured SCs, and also induced the expression of various genes such as GDNF and LIF, presumably by activating ERK pathways. Furthermore, the exogenous supply of HGF around the injury site via intramuscular

injection of a plasmid DNA expressing human HGF enhanced the myelin thickness and axon diameter in injured nerves.

During the above work, it was found that c-Fos is one of many cellular genes whose expressions are affected by the increased levels of HGF. c-Fos is an important component of the AP-1 heterodimer, but its role has not been clearly understood in the context of HGF and SCs. I investigated the relationship between HGF and c-Fos. First, it was confirmed that in the nerve crush model, both RNA and protein levels of c-Fos were increased, while this effect was abrogated by PHA-665752, an inhibitor of the c-met receptor. Increased expression of c-Fos was observed predominantly in distal SCs as measured by immunostaining. When primary SCs were treated with recombinant HGF protein, c-Fos expression was regulated in a typically quick and transient fashion at both RNA and proteins levels. HGF-mediated induction of c-Fos expression was highly suppressed by specific inhibitors of ERK and CREB, U0126 and CBPi, respectively. The knockdown of c-Fos expression by siRNA almost completely blocked various HGF-mediated effects in SCs—such as the induction of the gene expression of GDNF, LIF, and c-Myc, and the migration of SCs—indicating that c-Fos might play a key role in HGF effects. My thesis work revealed that HGF and c-met played important roles in Schwann cell-mediated nerve repair, that HGF gene transfer might provide a useful tool for treating peripheral neuropathy, and also that c-Fos played a key role in HGF-mediated effects on neuropathic gene and cell migration. These results show that HGF has great potential to be used as a platform for developing novel therapeutics for a variety of neurological diseases.

Key words: Hepatocyte growth factor, c-met, Nerve regeneration, Re-myelination, Schwann cells.

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Chapter 1. Introduction

1. Peripheral nervous system (PNS)

1.1 Overview of the peripheral nervous system

Members of the mammalian class, which includes humans and mice, typically have two nervous systems: the peripheral nervous system (PNS) and the central nervous system (CNS). These two systems differ in structure and function. The CNS is constituted of the brain and spinal cord, which process neuronal information. The PNS consists of 12 cranial nerves and 31 pairs of spinal nerves (excepting the brain and spinal cord) responsible for relaying and transmitting sensory or motor information (Figure 1).

The PNS is composed of neurons and supporting glial cells, specifically satellite cells and Schwann cells (SCs). Two types of peripheral neurons play a role in the PNS; sensory neurons and motor neurons. Sensory neurons normally carry information from peripheral sensory receptors to the CNS, whereas motor neurons transmit signals from the CNS to muscles or glands. Similar to oligodendrocytes in the CNS, SCs in the PNS form the myelin sheath of the peripheral neuron, which helps neurons to properly transmit electro potential signals and maintain their functions (Sherman and Brophy, 2005).

1.2. Development of Schwann cells (SCs) in the PNS

In the PNS, SCs are the most abundant cells, and sheathe axons in two different ways: myelinating SCs and non-myelinating SCs (Remak bundles). Together, these two types sheathe all the

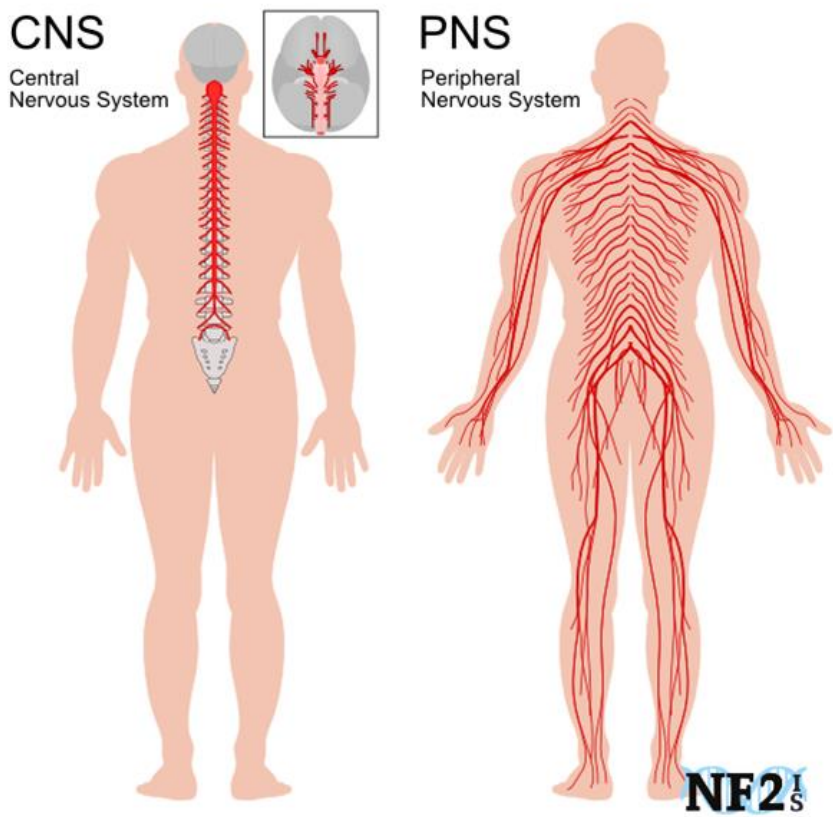


Figure 1. Peripheral nervous system and Central nervous system

axons of the peripheral neurons and provide electrical insulation. Among the peripheral neurons, large diameter axons (>1μm) acquire a 1:1 relationship with SCs and become myelinated, leaving unmyelinated gaps (nodes of Ranvier) between adjacent SCs, which facilitates neural conduction. The myelin structure comprises various lipids and proteins compacted into a multilayer structure. Non-myelinating SCs do not individually wrap the neurons, but a bundle of small diameter axons are grouped together and surrounded by unmyelinated SCs (Figure 2).

Both myelinating and non-myelinating SCs share the same cell lineage. SCs are derived from neural crest cells, which are multi-potent and can develop into a diverse cell lineage that includes melanocytes, cartilage, smooth muscle, peripheral neurons, and glial cells. During embryo development, some neural crest cells are developed into Schwann cell precursors (SCPs), the first transitional stage in the SC lineage. SCPs are then converted into immature SCs. During the late embryo and early post-natal stages, one or a few immature SCs together envelop groups of axons communally, forming irregular axon/SC columns, each of which is covered by nascent basal lamina and surrounded by an extracellular matrix. Radial sorting then starts, and a single large diameter axon (>1μm) is segregated from the axon/SC families and becomes individually sheathed by a single SC. The remaining axons differentiate into Remak bundles.

Unlike other types of cells like neurons, SCs can freely switch to differentiation/dedifferentiation states. As development progresses, SCs stably maintain their myelin structure and reach a quiescent state in which they no longer proliferate or migrate.

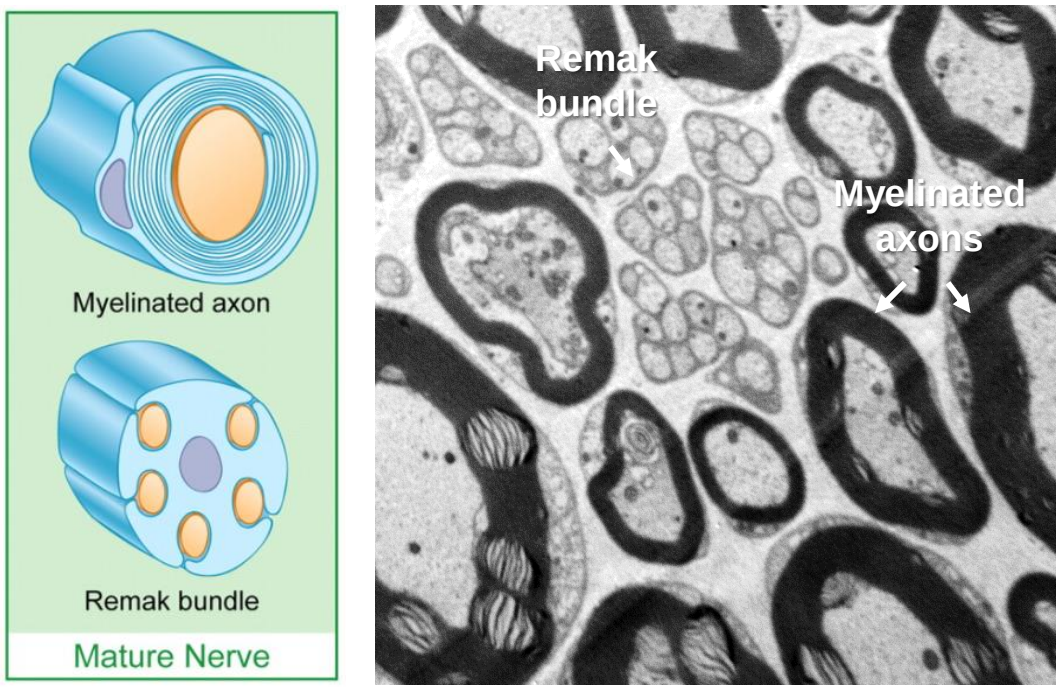


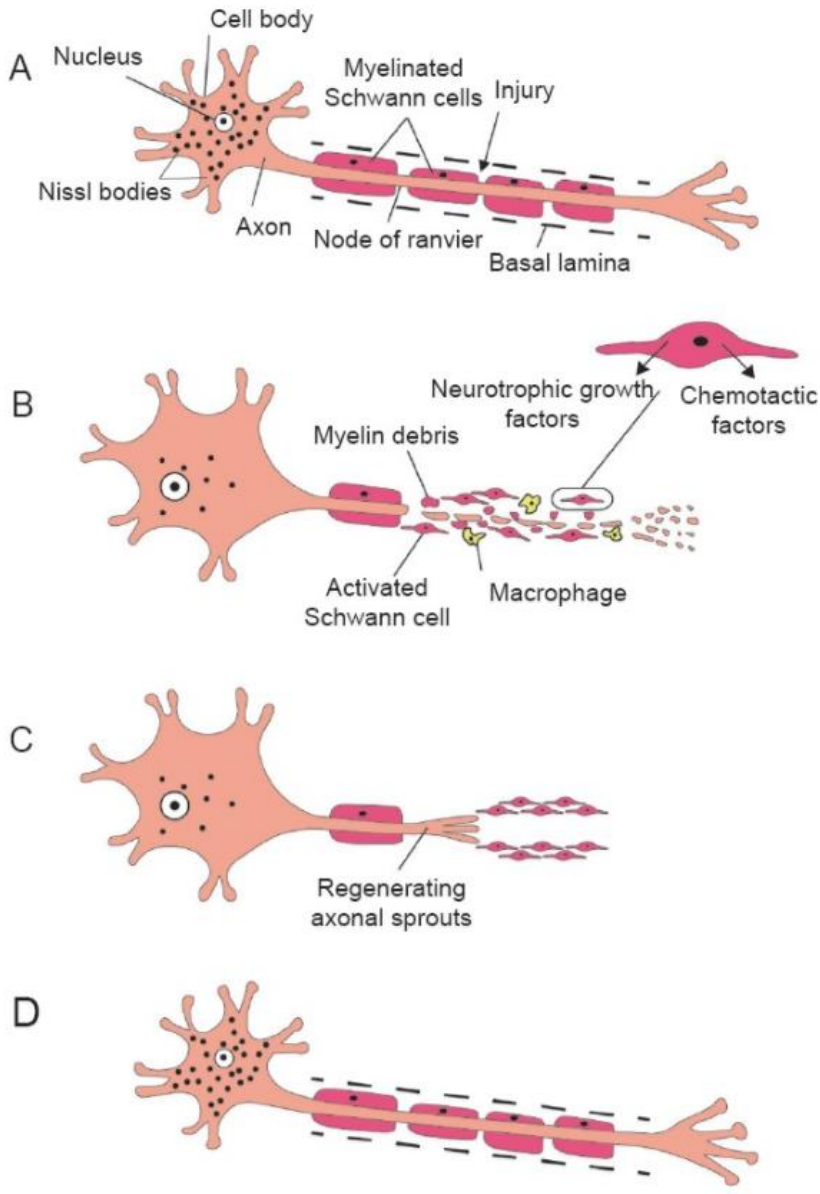
Figure 2. SCs derived structure in mature peripheral nerve.

In PNS, SCs sheathe the axons in two different way. The large diameter axons (>1μm) acquire 1:1 relationship with SCs and become myelinated axons, while a bundle of small diameter axons are grouped and surrounded by non-myelinated SCs (Remak bundle).

However, once SCs are damaged—such as through mechanical injury or metabolic stress—they are converted to the dedifferentiation state and change their physiological activity to promote nerve regeneration. After the nerve is repaired, SCs re-myelinate the neurons and return to the differentiation state.

1.3. The role of SCs in peripheral nerve regeneration.

When the peripheral nerve is wounded, SCs are dedifferentiated and initiate the regeneration process (Gaudet et al., 2011). Immediately after the myelin structure is destroyed, SCs are separated from axons and the expression of myelin-associated genes, such as Krox20 (Egr2), MBP, and MPZ, is decreased. The detached SCs are dedifferentiated and begin to produce not only cytokines or chemokines such as TNF-α, LIF, and CCL2/MCP-1, but also secrete various neurotrophic factors such as GDNF, NGF, and VEGF to promote axon elongation and activate other cells for nerve repair (Fontana et al., 2012; Frostick et al., 1998; Gaudet et al., 2011). After nerve injury, macrophages stimulated with LIF or MCP-1 are recruited to the injury site and involved in neuro-vascularization and the clearance of myelin or axon debris. In addition, SCs directly interact with the peripheral neurons to guide regenerating axons to the distal nerve. Once axons regrow, SCs in close proximity to newly regenerated neurons bind to axons and start the re-myelination process. As a result, regrown axons innervate their target tissues, and new myelin structures are formed (Burnett and Zager, 2004; Son and Thompson, 1995)



(Al-zer and Kalbouneh, Neural Regen Res, 2015)

Figure 3. The role of SCs in peripheral nerve regeneration.

When peripheral nerves get wounded, SCs started to de-differentiation and participated in nerve regeneration process.

2. Peripheral Neuropathy

Although the PNS has a high regenerative capacity compared to the CNS, continuous damage to peripheral nerves as a result of pathogenic conditions such as metabolic stress (e.g. long-term diabetes) or exposure to toxic substances (e.g. anti-cancer chemotherapy) can cause various side effects. For example, such peripheral neuropathy can result in sensory symptoms (numbness, tingling), autonomic symptoms, motor dysfunction, or neuropathic pain (firing, stabbing, electrical). Diabetic neuropathy is the most common cause of peripheral neuropathy in Western societies, and 30~66% of diabetes patients are known to suffer from sustained diabetic neuropathy pain.

Treatment methods of peripheral neuropathy depend largely on the cause of the nerve damage. For example, peripheral neuropathy caused by a vitamin deficiency can be treated with vitamin therapy and an improved diet. Peripheral neuropathy caused by toxic substances like alcohol or drugs can often be corrected by avoiding said substances. However, neuropathy diseases recruiting by genetic deficiencies or metabolic dysfunctions are difficult to treat because of their complex pathology. Extant medications serve primarily to relieve neuropathic symptoms, especially pain, rather than completely treat diseases. For example, aspirin, gabapentin, or cymbalta are frequently prescribed to relieve neuropathic pain, but the effects last only several hours, and often produce severe side effects such as drowsiness, loss of coordination, or dizziness. Various therapeutic strategies have been devised to provide more fundamental treatment methods, including delivering deficient genes by virus vector, treatment with neurotrophic growth factors, surgical tissue transplantation, or cell therapy.

Table 1. Current therapeutic strategies for peripheral neuropathy.

Methods	Candidates	Targets	Clinical Trial
Gene therapy	Adeno virus		X. Experimental
	Herpes simplex virus	DRG	X. Experimental
	AAV		X. Experimental
	Plasmid DNA		O
Growth factors	NGF	Sensory neurons and small axons	X. Experimental
	NT3	Sensory and motor neurons, Schwann cells	X. Experimental
	IGF-1	Inflammatory cells, sensory and motor neurons, Schwann cells	O
Cell therapy	Bonemarrow-derived mesenchymal cells		O
	Schwann cells		X. Experimental
	Adipose-derived mesenchymal cells		X. Experimental.

However, these are still in the early stages of development and effective medicines have yet to be made available for widespread use.

3. Neurotrophic growth factors

3.1 NGF family

Neurotrophins, including NGF, BDNF, NT-3, NT-4/5 and NT-6, belong to a family that shares highly related structures and functions (Figure). The biological functions of the neurotrophins are mediated by two classes of cell surface receptors: p75NTR and Trk receptor tyrosine kinases (TrkA, TrkB and TrkC). Because each neurotrophin has a different affinity to these receptors, neurotrophins show diverse effects on the nervous system. For example, p75NTR is a low affinity neurotrophic receptor to which all neurotrophins bind, while the Trk family only interacts with specific neurotrophins; for example, NGF to TrkA, BDNF and NT-4 to TrkB, and NT-3 to TrkC.

In the nervous system, neurotrophins play important roles in development, function, plasticity, and neuronal survival of the PNS and CNS (Chao, 2003; Sebben et al., 2011). In the PNS, both the SCs and neurons express neurotrophins and their receptors. For instance, mice that have completely lost neurotrophin genes die in the first few weeks following birth. In addition, the optimal level of NGF is crucial for the developmental process of the brain, especially in memory acquisition and maintenance. As neurotrophins are required for neurological development, they also have a great influence on peripheral nerve regeneration. For example, NGF and BDNF are highly secreted by neurons or SCs after nerve injury and enhance

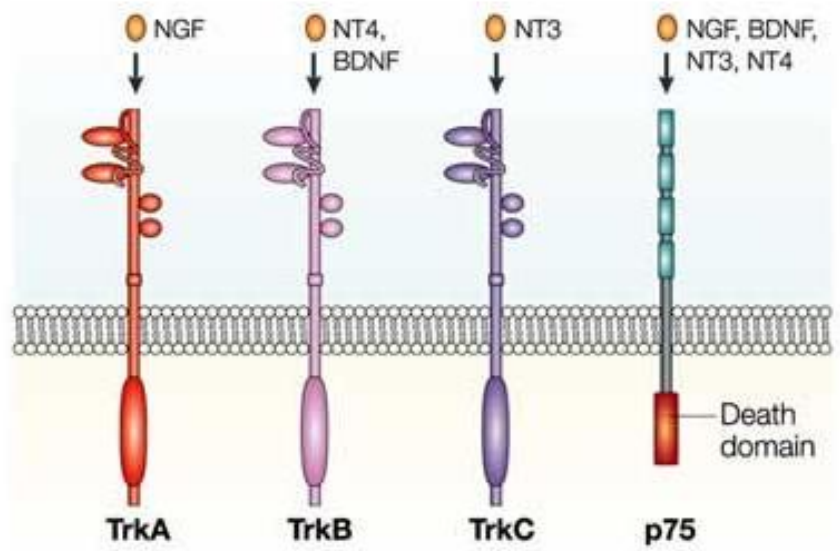


Figure 4. Neurotrophins and their receptors.

Neurotrophins, including NGF, BDNF, NT3, NT4/5, and NT6 have function on nervous system by binding to two classes of receptor; p75NTR and Trk receptor tyrosine kinases (TrkA, TrkB and TrkC).

axonal regeneration and neurite outgrowth during peripheral nerve repair. Because of their important roles in the nervous system, various neurotrophins have been tested for different neurodegenerative diseases (Table X).

3.2 VEGF family

Vascular endothelial growth factor (VEGF) is a well-known growth factor family that promotes vascularization and angiogenesis. In mammalians, the VEGF family comprises five members: VEGF-A, PGF, VEGF-B, VEGF-C, and VEGF-D. Each subtype has different functions. For example, VEGF-A usually promotes angiogenesis and vasodilation while VEGF-C induces lymphangiognesis. Members of the VEGF family are expressed by a high number of different cell types such as fibroblasts, macrophages, neurons, or cancer cells, and effect various physiological changes.

In addition to its angiogenic properties, VEGF also has various effects on neuronal systems. In both the PNS and CNS, VEGF has been found to play important roles in neurogenesis and glial cell development (Carmeliet and Storkebaum, 2002; Dumpich and Theiss, 2015; Pereira Lopes et al., 2011). VEGF has also been reported to be involved in nerve regeneration. For instance, hypoxia-induced VEGF after injury directly increases neuron survival rate and axon outgrowth. VEGF also promotes migration of SCs and vascularization of the injured nerve to reconstitute its microenvironment, facilitating the nerve repair process (Carmeliet and Storkebaum, 2002; Hobson et al., 2000; Rosenstein and Krum, 2004)

3.3 Hepatocyte growth factor (HGF)

Hepatocyte growth factor (HGF), first discovered by Toshikazu Nakamura's research group in the 1980s, is a multi-functional growth factor that generates a variety of biological reactions in many tissues. HGF is mainly secreted from mesenchymal lineage cells and binds to the c-met receptor, the only known cellular receptor for HGF (Nakamura and Mizuno, 2010).

HGF is a dimeric growth factor composed of a 69-kDa α-subunit and a 34-kDaβ-subunit linked by a disulfide bond. The α-chain contains four kringle domains while the β-chain contains a serine-protease-like structure. After HGF pre-proteins are synthesized, they secrete outward and are cleaved by serine proteases at Arg 494-Val 495. In mature HGF, Cys 487 in the α-chain and Cys 604 in the β-chain form an interchain bridge.

It has been reported that HGF not only triggers motility (migration), proliferation, and morphogenesis in a variety of epithelial cell types, but that it is also involved in angiogenesis, organ regeneration in the liver or kidney, and tumor invasions. HGF also plays important roles in both the CNS and PNS directly and indirectly.

In the CNS, HGF and c-met are expressed in the central neurons of the embryo and their expression is maintained into adulthood (Achim et al., 1997; Jung et al., 1994; Thewke and Seeds, 1996). During embryo development, the interaction between HGF and c-met promotes neuron survival and neurogenesis. Therefore, c-met KO mice are terminated during the early embryo stage (Uehara et al., 1995). C-met is expressed not only in neuron cells but also in

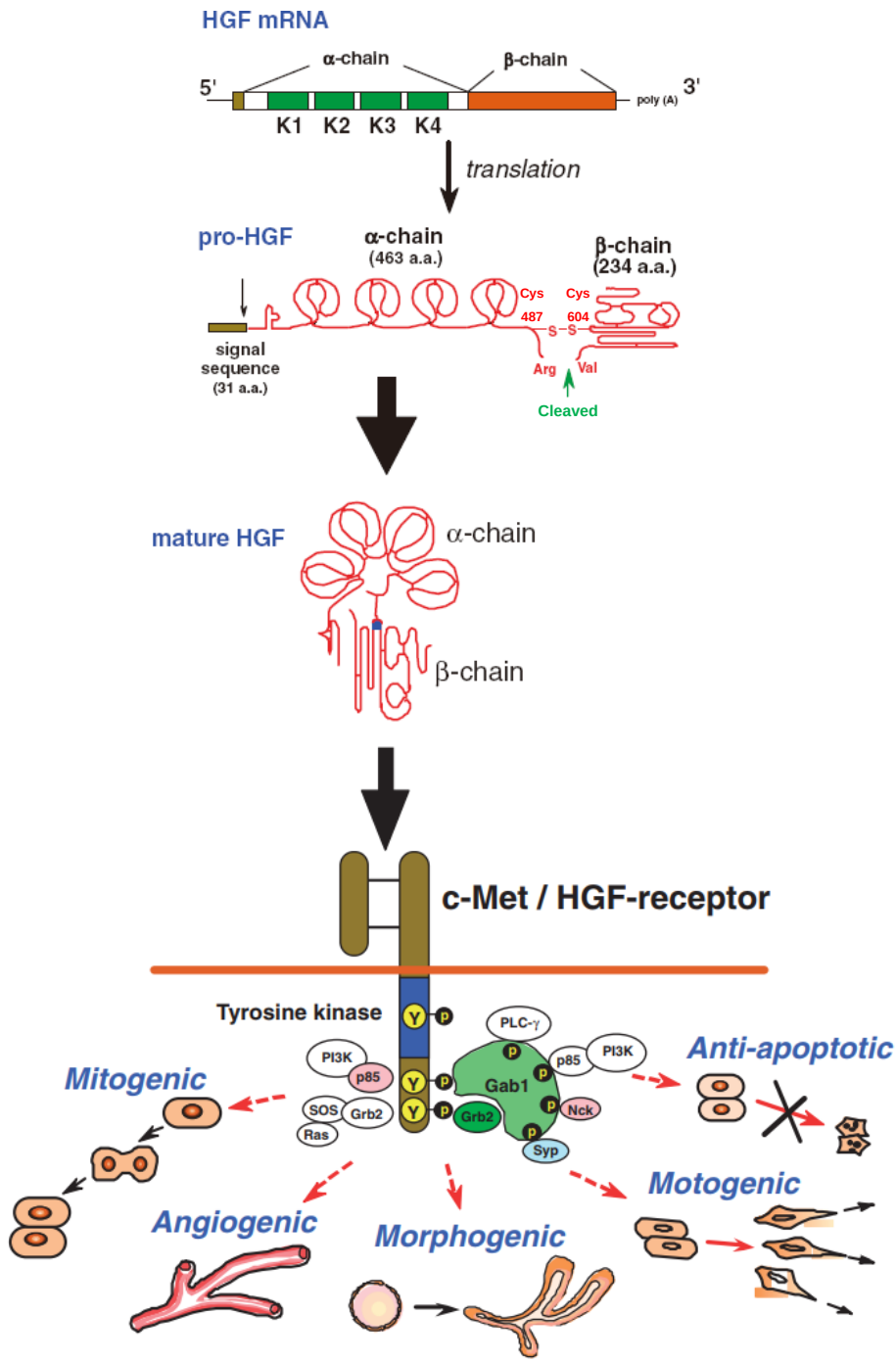
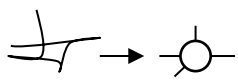
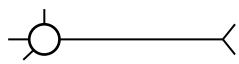
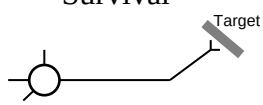


Figure 5. Hepatocyte growth factor and c-met receptor

Table 2. The role of HGF in PNS

	Motor neuron	**Sensory neuron**
Differentiation 		In vitro
Axonal outgrowth 	Defective in HGF/met mutants	In vitro Defective in met mutants
Chemoattraction 	In vitro	
Survival 	In vitro	In vitro Defective in met mutants

microglial cells and in SCs, for which HGF functions as a mitogen (Di Renzo et al., 1993; Krasnoselsky et al., 1994).

In the case of the PNS, HGF can enhance the survival and axon outgrowth of cultured motor neurons (Ebens et al., 1996; Novak et al., 2000; Wong et al., 1997; Yamamoto et al., 1997). It is also known to interact with NGF to exert neurotrophic effects on sensory neurons (Table 2, Maina et al., 1997). Recently, it was reported that HGF can activate the Gab2 protein to induce the migration and proliferation of SCs (Shin et al., 2017). However, the exact roles of HGF and c-met in peripheral nerve regeneration and the extent of HGF involvement in the nerve repair process have remained largely unknown.

4. AP-1 family

4.1 c-Jun

C-Jun, a member of the AP-1 (activator protein-1) family, is known to function by dimer with other AP-1 transcript factors. Those AP-1 family members are composed of basic region-leucine zippers (bZIP), which are responsible for dimerization with other bZIP proteins and determine DNA-binding sequences. Depending on the AP-1 dimer composition, the genes or promoter affinity to be bound is altered (Figure 6). For example, Jun and ATF2 proteins can form either a homodimer or heterodimer, while the Fos protein cannot form a homodimer but will form a heterodimer with other AP-1 family members to function. Jun/Jun, Jun/Fos and Jun/Fra dimer compositions bind to TREs sequences with high affinity, whereas Jun/ATF2 heterodimers bind predominantly to CRE sequences (Karin,

AP-1 Family Members			
FOS	*ATF*	*JUN*	*MAF*
c-FOS	ATF-2	c-JUN	c-MAF
FOSB	ATF-3	JUNB	MAFA
FRA-1	ATF-4	JUND	MAFB
FRA-2	ATF-5		MAFF
	ATF-6		MAFG
	ATF-6B		MAFK
	ATF-7		
	BATF		
	BATF-2		
	BATF-3		
	JDP2		

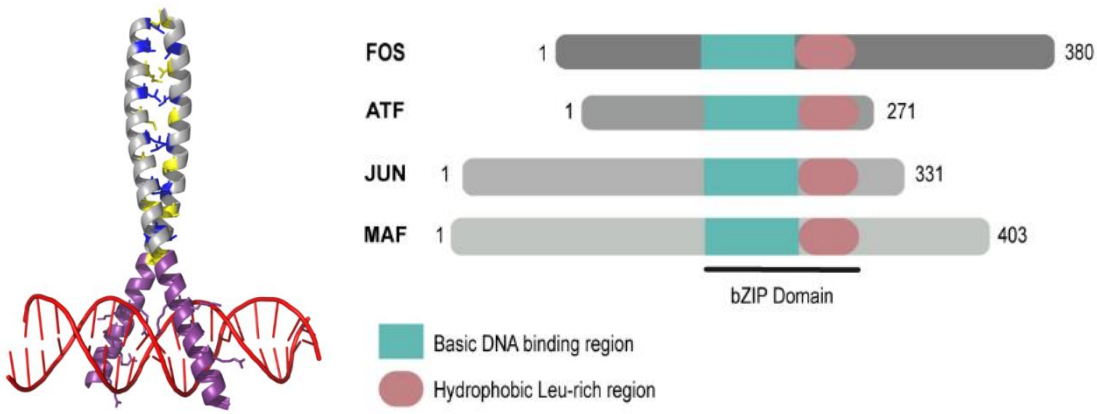


Figure 6. AP-1 family

1995; Shaulian and Karin, 2001, 2002; Whitmarsh and Davis, 1996; Wisdom, 1999).

Among SCs, c-Jun is considered the most important transcript factor that controls injury-induced SCs reprogramming; conversion to the dedifferentiated state. Following peripheral nerve damage, c-Jun expression is rapidly induced to high levels in SCs and promotes SCs to dedifferentiate and activate. When c-Jun is increased after injury, the expression of myelin-associated genes is suppressed, and important neurotrophic factors and cell surface proteins are increased, which support survival or axonal regrowth, including GDNF, BDNF, Artemin and p75NTR. Furthermore, c-Jun also promotes the myelinophagy necessary for myelin debris clearance and SCs' morphological change. In the absence of c-Jun, the formation of repair phenotype SCs is suppressed, and failure of regeneration and functional recovery is observed.

4.2 c-Fos

C-Fos is a transcription factor that also belongs to the AP-1 family, and is known to function by forming a heterodimer with other AP-1 family members like Jun (c-Jun, JunB, and JunD), Maf (c-Maf, MafB, MafA, MafG/F/K and NRL), or ATF (ATF2, ATF3, B-ATF, JDP1 and JDP2)(Ozanne et al., 2007; Shaulian and Karin, 2002). When a nerve is damaged, c-Fos expression is known to be up-regulated in distal Schwann cells (SCs) and involved in myelin clearance (Liu et al., 1995; Pyykönen and Koistinaho, 1991). Although c-Fos is an important component of the AP-1 heterodimer, which plays important roles in pain generation, re-myelination, and axon outgrowth, the exact role(s) of the c-Fos protein in SCs and how its gene expression is regulated after nerve injury remain poorly understood.

5. Purpose of this Study

Although many studies have reported that HGF has neurotrophic effects on peripheral neurons (Ebens et al., 1996; Shin et al., 2017; Wong et al., 1997), the in vivo roles of HGF in nerve regeneration after injury have been very poorly understood. In this thesis work, I investigated the exact role(s) of HGF and c-met in peripheral nerve regeneration, both in vivo and in vitro. My initial data indicated that HGF expression was highly induced after nerve crush at the injury site, while the levels of phosphorylated and total c-met were greatly increased in SCs distal from the injury site. Based on the observation, I investigated whether increased HGF expression is actually involved in peripheral nerve regeneration by blocking c-met activity or overexpressing the HGF protein. I found that HGF plays a very important role in re-myelination and axonal regrowth by increasing myelin thickness and axon diameter. The further underlying mechanisms of HGF were also studied using relevant cell lines and primary rat primary SCs . My investigation focused on the effects of HGF on the migration and proliferation of SCs, as well as the expression level of various neurotrophic factors and inflammatory cytokines. My thesis work was performed to understand the roles of HGF in the regeneration of damaged peripheral nerves at the molecular and cellular levels. Taken together, our data suggest that HGF has neurotrophic effects on peripheral regeneration, and might be a new candidate to target neurodegenerative diseases.

Chapter 2

Chapter2. Materials and Methods

1. Cell culture

1.1 Primary Schwann cell culture

Schwann cell cultures were performed as previously described in Kaewkhaw et al. (2012) (Kaewkhaw et al., 2012a). After isolation, primary Schwann cells were expanded in the culture medium [DMEM - d-valine (Welgene, Korea)+2 mM glutamine (Invitrogen)+10% FBS (Corning)+1% N2 Supplement (Invitrogen)+20 μg/ml bovine pituitary extract (Sigma)+5 μM forskolin (Sigma)+100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen)+0.25 μg/ml amphotericin B (Sigma)]. Primary SCs were used only until 3~4 passage. To study the HGF signaling pathway, primary Schwann cells were incubated in the DMEM medium containing 1% FBS+PSG for 4hrs for serum starvation. All reagents, including inhibitors and recombinant human HGF (R&D) were diluted in 1% FBS+PSG DMEM media prior to treatment.

1.2 siRNA Transfection

siRNA transfection was performed according to protocols provided by Lipofectamin RNAi MAX Reagent (Invitrogen, catalog number 13778). Primary SCs were seeded in 6 well plates (1.5 x 10^5 cells/well). The sequences of synthesized siRNA (Invitrogen) listed in table .

2. In vivo

2.1 Nerve crush model

Nerve crush was performed as previously described45. For nerve crush, C57BL/6 mice were anesthetized with isoflurane. All

Table 3. siRNA sequences

Gene	Catalog number
Rat c-Jun	Invitrogen Assay ID s127980
Rat c-Fos	Invitrogen Assay ID 261240

surgical protocols were approved by the Institutional Animal Care and Use Committee. For each mouse, the area above the right thigh was shaved and sterilized with Povidone and 70% EtOH. The right sciatic nerve was exposed by a small incision made to the skin and muscle. Two millimeter of the nerve was crushed for 15 seconds with fine hemostatic forceps (FST), which had been dipped in powdered carbon (Sigma) before use to mark the crush site (Bauder and Ferguson, 2012). Then the incision was sutured using a 5-0 black silk suture (ALEE). PHA665752, a c-met inhibitor, was intraperitoneally injected into each mouse in a dosage of 20mg/kg. Two hundred microgram of pCKor pCK-HGF-X7 were diluted in 100μl PBS and directly injected once to the thigh muscles around the sciatic nerves.

2.2 Sciatic nerve preparation

After mice were sacrificed at appropriate time points, ipsilateral sciatic nerves were isolated. Injured sciatic nerve was divided into three areas, proximal (the 2mm upper portion of injury site), injury (the site crushed by hemostatic forceps and labeled with powdered carbon) and distal (the 2mm lower portion of injury site) sites.

2.3 Von frey test

Animals were habituated to the testing environment for 3 days before behavioral studies. The development of mechanical allodynia was assessed by using von Frey filaments assay and pain symptoms were evaluated weekly. Von Frey measurements were made to evaluate the mechanical sensitivity of mice. First, animals were placed individually in the cylinder on top of the metal mesh floor for 3 hous for adaptation. Mechanical sensitivity of mice was assessed by stimulating the hind paw with von Frey filaments of different thicknesses. We also measured the frequency of hind paw

withdrawal by using a constant thickness (0.16 g) of the filaments.

2.4 Sciatic nerve explants culture

Sciatic nerve transaction model was performed as previously described (Shin et al., 2017). For Nerve injury, C57BL/6 mice were anesthetized with isoflurane. All surgical protocols were approved by the institutional Animal Care and Use Committee. After sciatic nerve transection was introduced, sciatic nerve was isolated at day 5 and then culture for 3 days in DMEM containing 10% FBS and 1mM of PHA-665752, a c-met inhibitor.

3. Molecular biology and biochemical techniques

3.1 Transmission electron microscopy

The animals were anesthetized with isoflurane. Mice were fixed by cardiovascular perfusion with 2% paraformaldehyde + 2% glutaraldehyde in 0.1M PBS. After fixation, the right ipsilateral sciatic nerve was prepared, and the tissue was immediately incubated in fixation solution for 4 hrs at 4°C, followed by treatment with 1% osmium tetroxide in 0.1M PBS for 2 hrs at 4°C for post-fixation. Fixed tissues were washed twice in distilled water, and en bloc staining was performed with 2% aqueous uranyl acetate (UA) overnight at 4°C in the dark. On the subsequent day, tissues were dehydrated by a serial passage in 30%, 50%, 70%, 80%, 90%, 100%, 100%, and 100% ethanol for 10 min each. Before resin embedding, the dehydrated tissues were suspended in propylene oxide twice for 10 min at 4°C and incubated in a series of propylene oxide plus Spurr resin mixtures (2:1, 1:1, 1:2) for 1 hr each. Tissues were then

Table 4. Antibodies

Antibody target	Catalog number
GAPDH	Abcam ab9485
c-Fos	Cell signaling #4384
c-Fos	Santa cruz sc-52
c-Jun	Cell signaling #9165
phospho c-met (Y1234/1235)	Cell signaling #3077
phospho c-met (Y1349)	Cell signaling #3121
c-met	Sigma SAB4300599
phospho AKT	Cell signaling #13038
AKT	Cell signaling #9272
phospho ERK1/2	Cell signaling #9106
ERK	Cell signaling #1695
S100β	Dako Z0311
GFAP	Dako M0761
phospho S6K	Cell signaling #9205
S6K	Cell signaling #2708
phospho mTOR	Cell signaling #2971
mTOR	Cell signaling #2983
HGF	Thermo scientific MA5-14160
anti-Rabbit IgG secondary antibody Alexa Flour 555	Thermo scientific A-31572
anti-mouse IgG secondary antibody Alexa Flour 555	Thermo scientific A-21206
anti-mouse IgG secondary antibody Alexa Flour 555	Thermo scientific A-31570
anti-mouse IgG secondary antibody Alexa Flour 488	Thermo scientific A-11006
anti-mouse IgG HRP	Sigma A0168
anti-rabbit IgG HRP	Sigma RABHRP1

embedded with 100% resin overnight. Samples were polymerized with Spurr resin embedded in molds and incubated in a 70°C dry oven overnight. Sample sectioning and visualizing images by Transmission Electron Microscopy were done by NICEM (Seoul National University, Korea). The lengths of axon diameter and myelin thickness and the number of regenerating axons were manually determined by Image J program (NIH).

3.2 Quantitative RT-PCR

Total RNAs were prepared from sciatic nerves and cultured cells using Trizol reagent (Invitrogen), and cDNAs were synthesized from 1 mg of respective RNA samples by using an oligo (dT) primer and AMV-RT enzyme (Roche, Indianapolis, IN). Real-time quantitative RT-PCR was performed with SYBR green (Takara Bio), using the Smart Cycler System's (Takara) with the following protocol: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The sequences of synthesized PCR primer sets (Bioneer Co. Ltd., Seoul, Korea) were listed in Table 4.

3.3 Western blot assay

For Western blot analysis, sciatic nerves and primary Schwann cells were prepared using RIPA buffer containing a protease inhibitor (Roche), phosphatase inhibitor cocktail (Roche) and 1 mM PMSF. Equal amounts of protein samples were subjected to SDS-PAGE on 10% polyacrylamide gels, and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 0.1% TBST containing 1% (w/v) BSA (Invitrogen, Carlsbad, CA), and incubated with primary antibodies diluted in a 3% BSA blocking solution overnight at 4 °C. Membranes were then incubated with HRP-conjugated anti-mouse or

Table 5. quantitative RT-PCR primer sequences.

Gene	Forward (5'→3')	Reverse (5'→3')
Rat 18s	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
Rat GDNF	CTGACCAGTGACTCCAATATGC	GCCTCTGCGACCTTTCCC
Rat LIF	TCAACTGGCTCAACTCAACG	AAAGTGGGAAATCCGTCAT
Rat Myc	CTGCTCTCCGTCCTATGTTG	TCTCCTCCAAGTAGCTCGGT
Rat c-Fos	TCCCAGAGGAGATGTCTGTG	GGCTCCAGCTCTGTGACCAT
Rat c-Jun	TGAAAGCGCAAAACTCCGA	TGTGCCACCTGTTCCCTGA
Rat Oct6	GGGCACCCTCTACGGTAATG	CACTTGTTGAGCAGCGGTTT
Rat shh	GCGGGCATCCACTGGTACT	TCGGACTTCAGCTGGACTTGA
Rat Krox20	TTTTCCATCTCCGTGCCA	GAACGGCTTTCGATCAGGG
Rat P0	CCCCAGTAGAACCAGCCTCA	TCCAGGCCCATCATGTTCTT
Rat MBP	CAATGGACCCGACAGGAAAC	TGGCATCTCCAGCGTGTTC
Rat Egr1	AGAAGGCGATGGGTGGAGACGA	TGCGGATGTGGGTGGTAAGGT
Rat JunB	CCGGATGTGCACGAAAATGGAACAG	ACCGTCCGCAAAGCCCTCCTG
Rat Sox10	GCTATCCAGGCTCACTACAAG	ACTGCAGCTCTGTCTTTGG
Rat Notch1	GCACCTGCATTGATGATGTC	CTCCTTGCATACCCCACTGT
Rat VEGFa	GAGTTAAACGAACGTACTTGCAGA	TCTAGTTCCCGAAACCCTGA
Rat NGF	TCAACAGGACTCACAGGAGCA	GGTCTTATCTCCAACCCACACAC
Rat TFNa	CTGACCAGTGACTCCAATATGC	TCATCACAGGGCTTGAGCTC
Rat MCP-1	GCAGGTCTCTGTCACGCTTCT	GCAGGTCTCTGTCACGCTTCT
Rat IL1b	ATATGTTCTCAGGAGATCTTGGAA	TGCATCATCGCGTTCATACA
Rat IL-6	AGGAAGGCAGTGTCACTCATTGT	CTTGGGTCCTCATCCTGGAA
Mouse GAPDH	TCTCGAGCAGGTTCGAATGG	AAGAACCGTCGCAAACTTTA
Mouse c-Fos	ACTTCTTGTTTCCGGC	AGCTTCAGGGTAGGTG
Mouse GDNF	TCTCGAGCAGGTTCGAATGG	AAGAACCGTCGCAAACTTTA
Mouse LIF	ACGCGTGTCAGCGACAAA	GGCAGCCAGTCCTGAGATGA

anti-rabbit IgG (1:100,000; Sigma) for 1hr, and protein bands were visualized with ECL (Millipore, Billerica, MA, USA) and X-Omat film (Kodak, Rochester, NY).

3.4 Immunoprecipitation assay

Immunoprecipitation assay was performed according to protocols provided by DynabeadsTM Protein A IP Kit and Magnet Starter Pack(Invitrogen). Total protein extracts were prepared by using 1% Triton-X100 in PBS solution containing a protease inhibitor (Roche) and phosphatase inhibitor cocktail (Roche).The antibodies used for western blot analysis and IP assay were listed in Table 2.

3.5 ELISA

Mouse HGF, and mouse VEGF ELISA kits were purchased from R&D Systems (R&D Inc.). All ELISAs were performed according to protocols provided by R&D Inc. When in vivo samples were prepared, sciatic nerves were isolated and homogenized in RIPA lysis buffer containing a protease inhibitor (Roche), phosphatase inhibitor cocktail (Roche) and PMSF (Sigma) by using polypropylene pestles (Bel-Art Scienceware). After preparation, samples were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were used to detect mouse HGF.

3.6 Immunohistochemistry assay

Immunohistochemical analyses were performed as previously described (Ahn et al., 2014). Briefly, mice were fixed in 4% paraformaldehyde and cryo-sectioned to 12uM thickness. Sections were washed in 0.1M PBS (pH7.4) twice, then blocked for 1 h with PBS containing 5% fetal bovine serum (Corning), 5% donkey serum (Jackson ImmunoResearch Laboratories), 2% BSA (Sigma) and 0.1%

Triton X-100. Samples were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Sections were washed four times in PBS and incubated for 1 hr at room temperature with secondary antibodies (Invitrogen) diluted in PBS. Immunostained samples were further washed 6 times and counterstained with Hoechst 33342 (Sigma) for nuclear staining. The fluorescence images were obtained using a Zeiss LSM 700 confocal microscope (Zeiss, Oberkochen, Germany).

3.7 Immunofluorescence assay

Immunofluorescence assays were performed as previously described (Ahn et al., 2014). Briefly, primary adult SCs were fixed using a 4% paraformaldehyde solution for 15 mins and washed by PBS three times. These were then permeabilized with 0.5% Triton X-100 in 0.1M PBS (pH7.4) for 15 minutes. The samples were washed with PBS three times and, followed by blocking with [10% FBS+2.5% donkey serum in PBS] for 1 hour. The samples were then incubated overnight at 4°C with primary antibodies diluted in the blocking solution. After 3 washes with PBS, the samples were incubated with fluorescence dye conjugated secondary antibodies for 1 hour at room temperature. Nuclear staining was done using 1ug/mL Hoeschst 33248 (Sigma).

3.8 WST-1 assay

WST-1 assay was performed according to protocols provided by CellVia cell viability assay kit (Abfrontier). Primary SCs were seeded in 96 well (0.5X10^4cells/well) and cultured in presence or absence of HGF for 24 hours.

3.9 Migration assay

Primary Schwann cells (2.5×10^4 cells/ well) in 1% FBS

media were seeded on Costar filter chambers (Bottom diameter: 6.5 mm, 8 μm pore size, Costar, Cambridge, MA) which had been coated with poly-L-lysine and laminin. Cells were allowed to migrate for 3hr 30mins. Migrated cells were fixed using a 4% paraformaldehyde solution for 15 mins and then stained with crystal Violet solution (0.2% crystal violet in 20% Methanol) for 30mins. The number of cells in each image was counted by using Image-J cell counting program.

3.10 Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 7.0. Statistical significance was estimated by one-way ANOVA with Tukey correction or Student's t-test. Sample sizes are as described in the figure legends. Data were considered statistically significant if the p-value is < 0.05.

4. Reagents

4.1 Human HGF overexpression plasmid constructs (pCK-HGF X7)

As a tool for overexpressing the HGF protein in mouse, a DNA plasmid (pCK-HGF-X7) that contains novel genomic-cDNA hybrid human hepatocyte growth factor (HGF) coding sequence (HGF-X7) was used. pCK-HGF-X7 has previously been shown to produce two isoforms of human HGF, HGF_{723} and HGF_{728}, and used in clinical studies as well as in a variety of animal models (Carlsson et al., 2008; Gu et al., 2011; Kessler et al., 2015; Pyun et al., 2010). The expression kinetics of human HGF from pCK-HGF-X7 in mice has been well established (Hahn et al., 2011; Nho et al.,

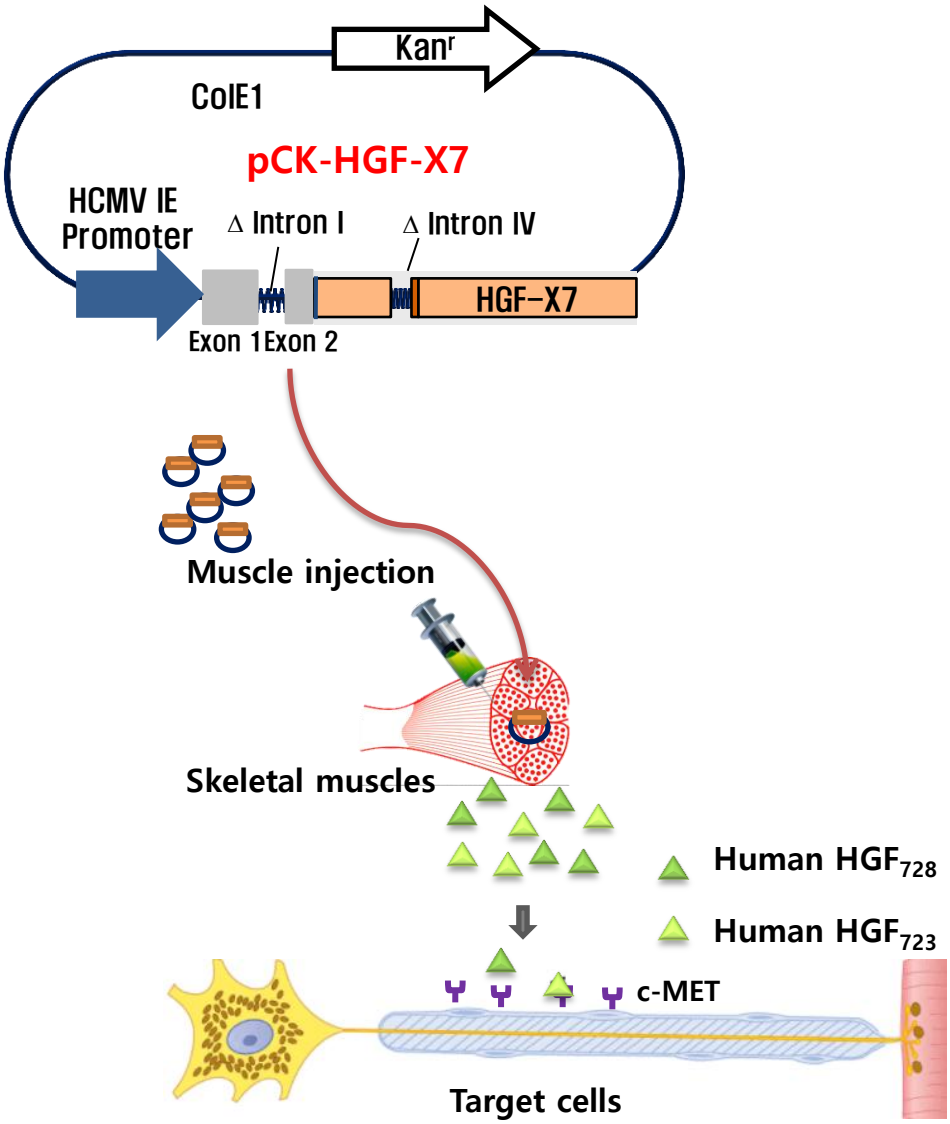


Figure 7. pCK-HGF-X7

2018).In this research, 200μg of pCK-HGF-X7diluted in 100μl PBS was intramuscularly injected into the thigh muscles around the sciatic nerve at the time of nerve crush surgery. pCK, a plasmid DNA lacking the HGF sequence, was used as a control.

4.2 PHA-665752

PHA-665752, a chemical c-met inhibitor, blocked c-met activity by inhibiting the phosphorylation of tyrosine receptor. PHA-665752 (Tocris, Catalog number 2693) was diluted in DMSO solution. 10mg/ml of PHA-665752 solution was daily intraperitoneally injected into mice by 20mg/kg.

4.3 Antibodies

The antibodies used for in this research were listed in Table 2.

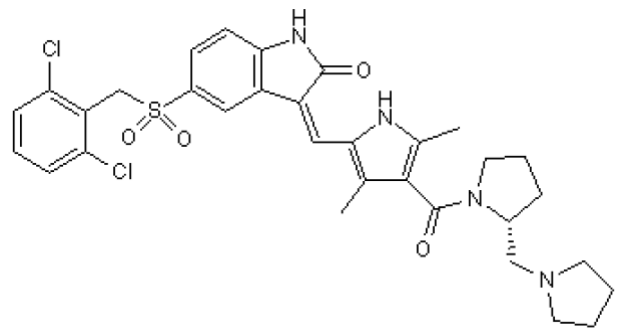


Figure 8. The structure of PHA-665752

Chapter 3

Chapter3. HGF promotes peripheral nerve regeneration by activating repair SCs

1. Background

A variety of neurotrophic factors have been studied for their involvement in nerve regeneration. The most extensively studied genes include GDNF, NGF and VEGF for their functions and actions in the peripheral nerve regeneration process (Fine et al., 2002; Lindsay, 1988; Terenghi, 1999; Zhang et al., 2000). These growth factors are highly expressed in injured nerve and activate neuronal and non-neuronal cells for repair of the injured nerves (Fu and Gordon, 1997). For instance, VEGF directly increases the neuron survival rate and axon outgrowth. VEGF also promotes the vascularization of the injured nerve to reconstitute its microenvironment, leading to the facilitation of the nerve repair process (Carmeliet and Storkebaum, 2002; Hobson et al., 2000; Rosenstein and Krum, 2004). Hepatocyte growth factor (HGF) is another angiogenic factor that has been reported to highly induced in injured nerve and might work on the nervous system (Maina and Klein, 1999), however, whether HGF and c-met receptor have any role in peripheral nerve injury is largely unknown.

2. Results

2.1 The spatiotemporal expression pattern of endogenous HGF in injured nerve

To investigate the role(s) of HGF in peripheral nerve regeneration, we first analyzed the temporal and spatial pattern of HGF expression in the injured nerve, employing the mouse nerve

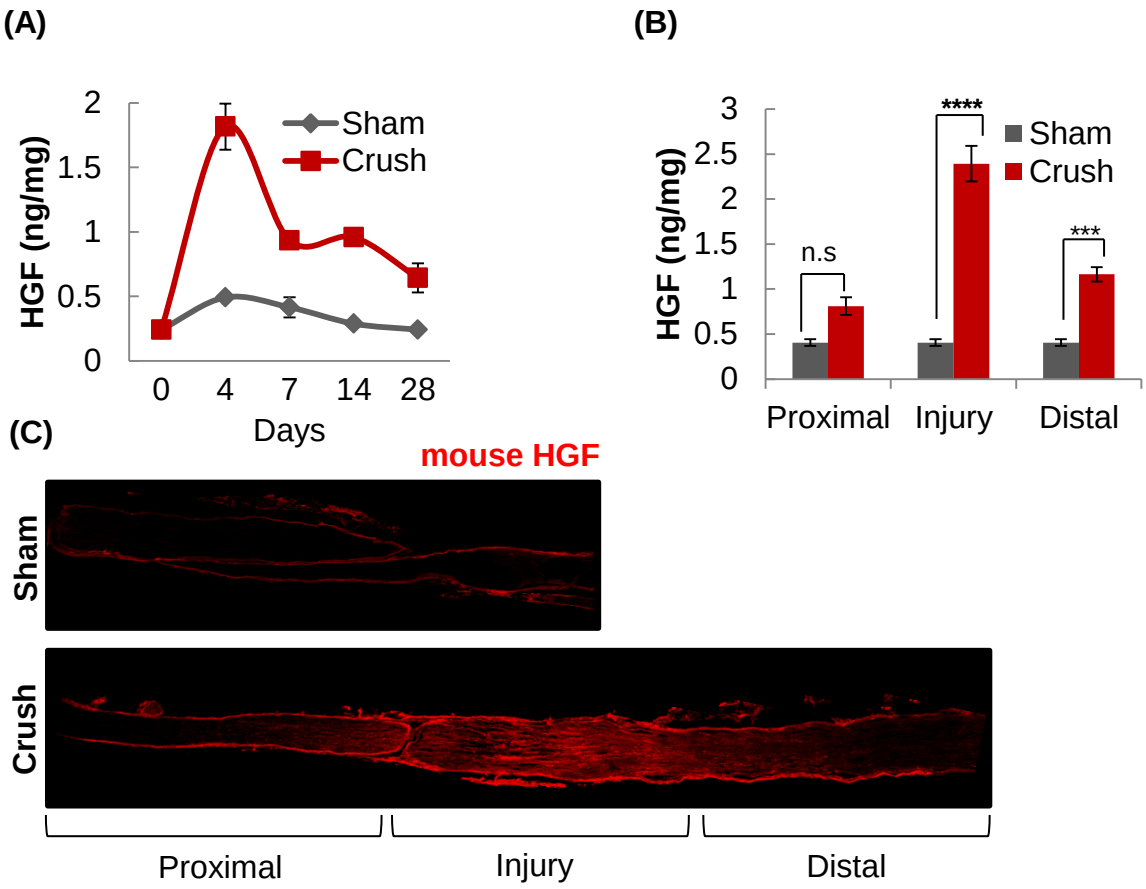


Figure 9. The spatiotemporal expression pattern of HGF in injured sciatic nerve. (A) Time kinetics of HGF expression in the sciatic nerve after nerve injury. Following nerve crush, total ipsilateral sciatic nerves were isolated at appropriate time points, and total proteins were analyzed by ELISA to measure the HGF protein level. (B) Spatial distribution of HGF expression in the injured sciatic nerve. The level of the HGF protein was measured in three different areas of the sciatic nerve (proximal, injury and distal sites) at 4 d.p.i by ELISA. (C) High level expression of HGF in the injured site of sciatic nerve. Ipsilateral nerves were isolated at 4 d.p.i and subjected to immunohistochemical assay using an antibody to HGF (red). Scale bar = 100μm. ***p<0.001, ****p<0.0001, n.s = not significant . Values represent the mean ± S.E.M.

crush model widely used in the study of peripheral nerve regeneration. After nerve crush was introduced, injured sciatic nerves were isolated at different time points, and the protein level of HGF was analyzed by ELISA. In a sham control, the sciatic nerve was exposed by muscle incision, but without nerve injury. As shown in Figure 9A, the protein level of HGF was highly increased from the basal point (approximately ~0.24 ng/mg), reaching a peak (1.82 ng/mg of total sciatic nerve protein) at 4 days post injury (d.p.i). Thereafter, its expression level was decreased to 0.93ng/mg at day 7. Even at day 28, however, the HGF level was higher by 2.6 fold than that of sham mice.

The spatial pattern of HGF expression after injury was also studied by measuring the level of HGF in three different areas of crushed nerves (proximal, injury and distal sites) by ELISA and immunohistochemistry (IHC) at 4 d.p.i. As shown in Figure 9B, the level of the HGF protein was significantly increased in both the injury and distal sites of damaged sciatic nerves, with induction level always highest at the injury site. In the proximal area, there was little difference between sham and crushed mice. This spatial pattern of HGF expression was also confirmed by IHC assay in which the strongest positive HGF signal was detected at the injured site followed by the distal site (Figure 9C)

2.2 The expression pattern of the HGF protein in other tissues

To determine whether HGF expression was also altered in other tissues as a result of nerve injury, total proteins were isolated at 0 and 4 d.p.i from the dorsal root ganglia (DRG) and thigh muscles around injured sciatic nerves followed by mouse HGF ELISA. At 4 d.p.i, the HGF level was slightly increased in both tissues compared to day 0, presumably due to the muscle incision

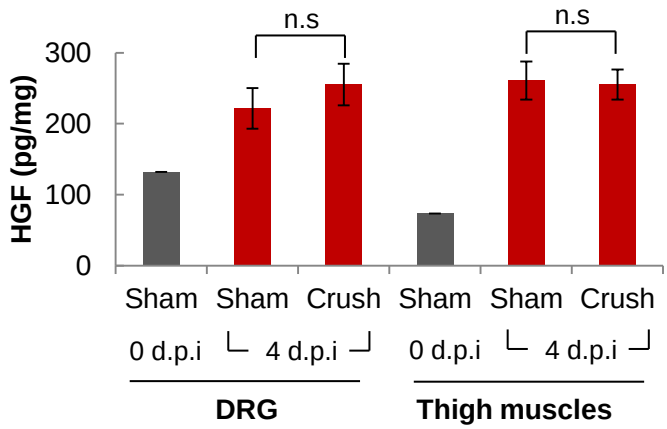


Figure 10. The expression of HGF in DRG and Thigh muscles

The protein level of HGF was measured in DRG and thigh muscles at 0, and 4 d.p.i by ELISA. n=3 for each group. n.s = not-significant. Values represent the mean ± S.E.M.

procedure, but there was no difference between sham and crushed animals (Figure 10). Taken together, these data indicated that nerve injury induced HGF expression mainly at the injury and distal areas of the sciatic nerve, but not in the DRG or thigh muscles.

2.3 The identification of HGF producing cells in injured nerves

Next, it was investigated which cell types in sciatic nerves produced the HGF protein after injury. As the HGF protein was observed mainly at the injured site, IHC was performed for cells around the injury area, where contains a variety of cell types such as fibroblasts, macrophages, neutrophils or SCs. To identify the HGF producing cell type(s) under the nerve injury situation, ipsilateral sciatic nerves were stained with antibodies to mouse HGF and cell markers (S100b for SCs and CD11b for macrophages). SCs and macrophage themselves did not seem to produce HGF during injury, as mouse HGF signal was not merged with SCs and macrophage markers (Figure 11A).

In addition, the level of HGF expression in primary was not changed when treated with a variety of stimulants including IL1β, $CoCl_2$-induced hypoxia and HGF itself. Although it was reported that macrophages might secret the HGF protein under hypoxic conditions, one of the most frequently used macrophage cell line, Raw264.7, did not produce any HGF protein when treated with $CoCl_2$ (Figure11B). Fibroblasts, which are well known to produce HGF in a variety of organs, are a good candidate for a major HGF producer cell type in our case, as they were previously reported that recruited to the injury site after nerve damage. In this thesis work, however, I was not able to stain fibroblast because there was no proper cell marker labeling the fibroblast only; vimentin or fibronectin, which are most commonly

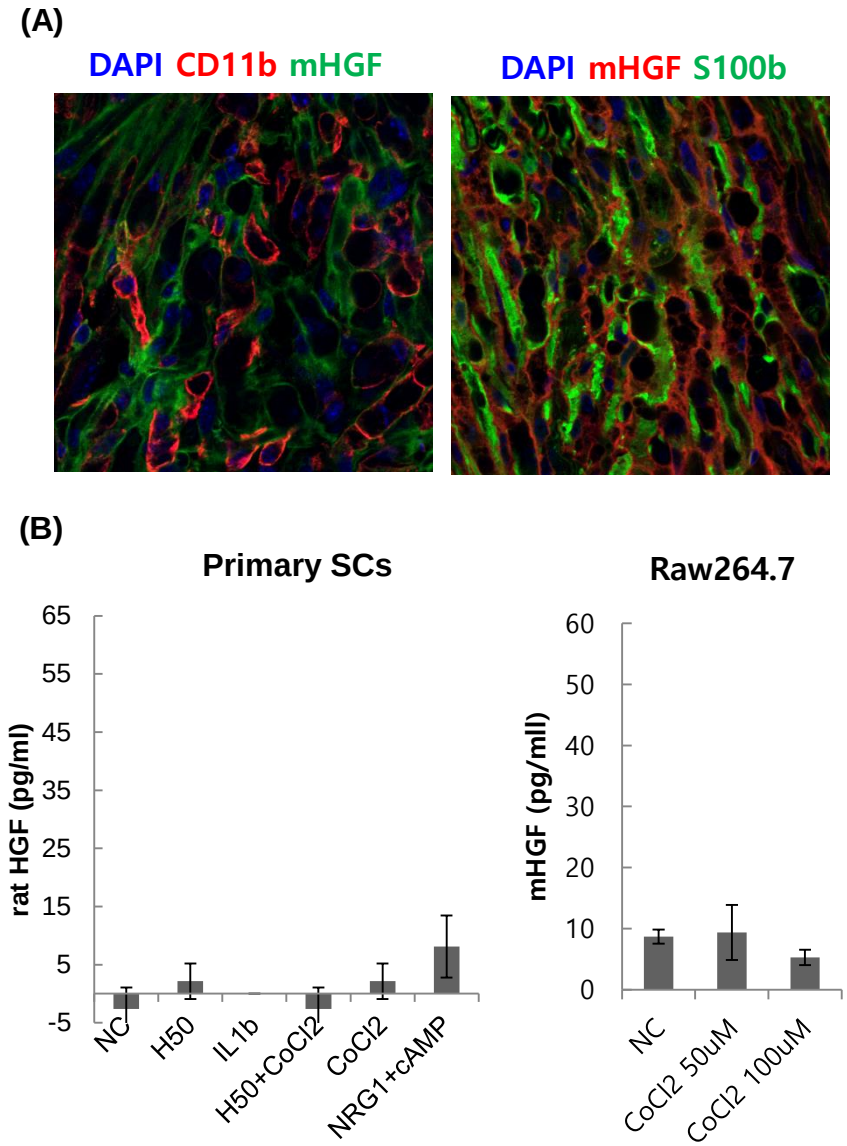


Figure 11. The identification of HGF producing cells in injured nerves

(A) To identify the HGF producing cell type(s), injured sciatic nerves were stained with antibodies to mouse HGF, S100b for SCs and CD11b for macrophages at 4 d. p.i. (B) To determine whether SCs or macrophages did not produce the HGF protei n, primary SCs and Raw264.7 cells were treated with various stimulates for 24hou rs and detected by rat HGF ELISA.

used for fibroblast staining could not be used because they are also abundant in SCs of sciatic nerves.

2.4 The spatiotemporal expression pattern of c-met in injured nerve

Since c-met is the only known receptor of HGF (Nakamura and Mizuno, 2010), the expression pattern of c-met around injured nerves was also analyzed. The three sites of the sciatic nerve (proximal, injury, and distal) were isolated at different time points followed by Western blot analysis, using antibodies to phosphorylated or total c-met. The change in the level of phosphorylated c-met was evident. In the distal region, its level was increased from day 2, reached a peak at day 4, and became barely detectable at day 7 (Figure 12A, compare lanes 1, 4, 7, and 10. In the proximal and injury areas, the level of phosphorylated c-met remained very low. The change in the level of total c-met was similar. In the distal region, it was increased from 2 d.p.i. and reached the maximum level at day 4 followed by a decrease at day 7 (Figure 12A, compare lanes 1, 4, 7, and 10). No change was seen in the proximal area at all three time points.

The pattern of c-met expression at 4 d.p.i. was also analyzed by IHC; a high level of total c-met was clearly visible, but the signal intensity was lower at the injury site and virtually undetectable at the proximal region (Figure 12B). Taken together, these data suggested that HGF produced from the nerve injury site might lead to the activation of c-met receptor present in distal region cells.

2.5 Identification of c-met expressing cells in injured nerve

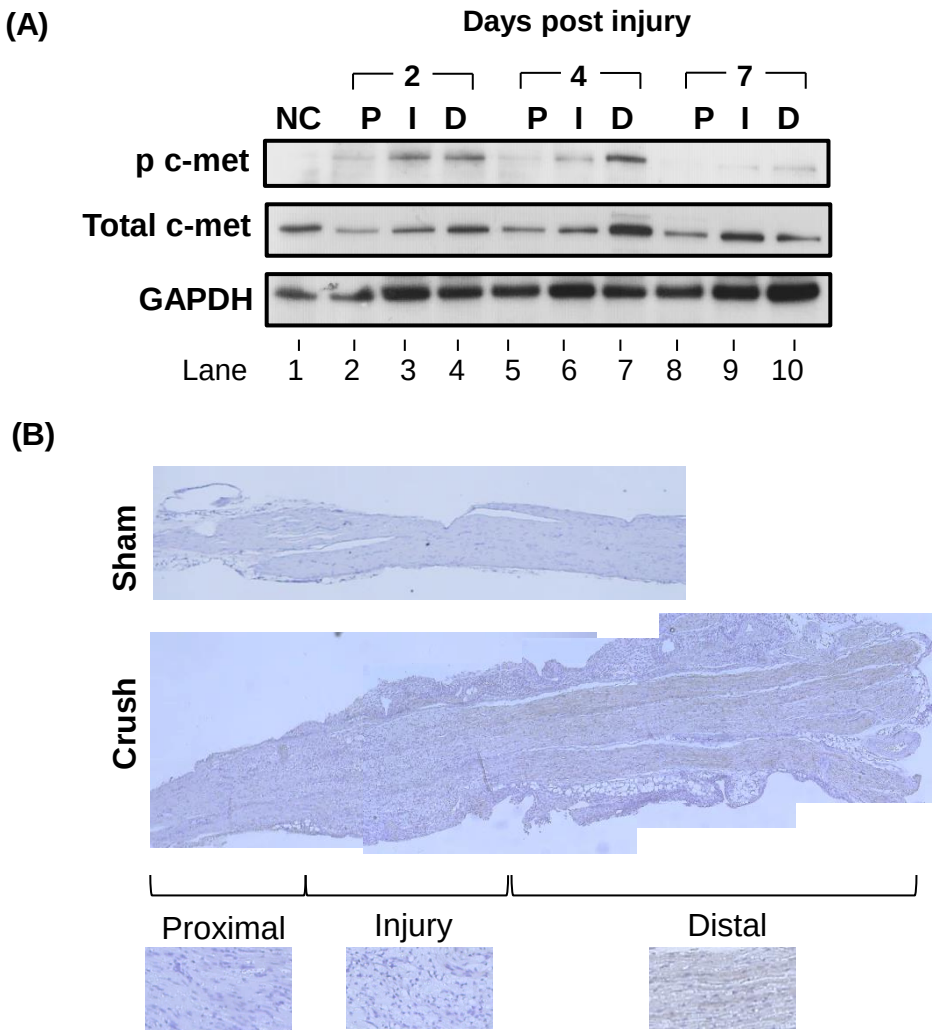


Figure 12. The spatiotemporal expression pattern of c-met in injured nerve.

. (A) c-met expression in different areas of the injured nerve. After nerve crush, pro ximal (P), injury (I), and distal (D) regions were isolated at different time points follo wed by Western blot using an antibody to phosphorylated or total c-met. GAPDH w as used as a loading control. (B) Immunohistochemical analysis of c-met receptor. Injured sciatic nerves were isolated at 4 d.p.i.

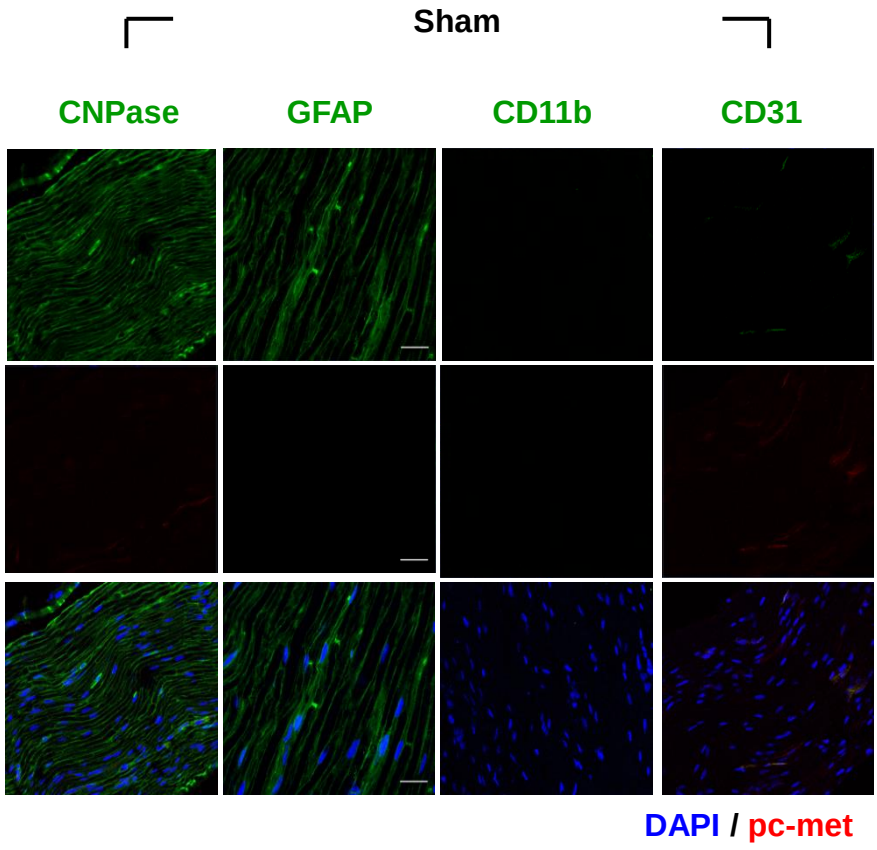


Figure 13. The staining pattern of phosphorylated c-met in naïve sciatic nerves. The naïve sciatic nerves were analyzed for various cell markers by immunohistochemistry assay using antibodies to CNPase and GFAP for SCs, CD11b for macrophages, CD31 for endothelial cells (all green), and phosphorylated c-met (red). Nuclei were counterstained with Hoechst (blue).n = 3 for each group. Scale bar = 20μm

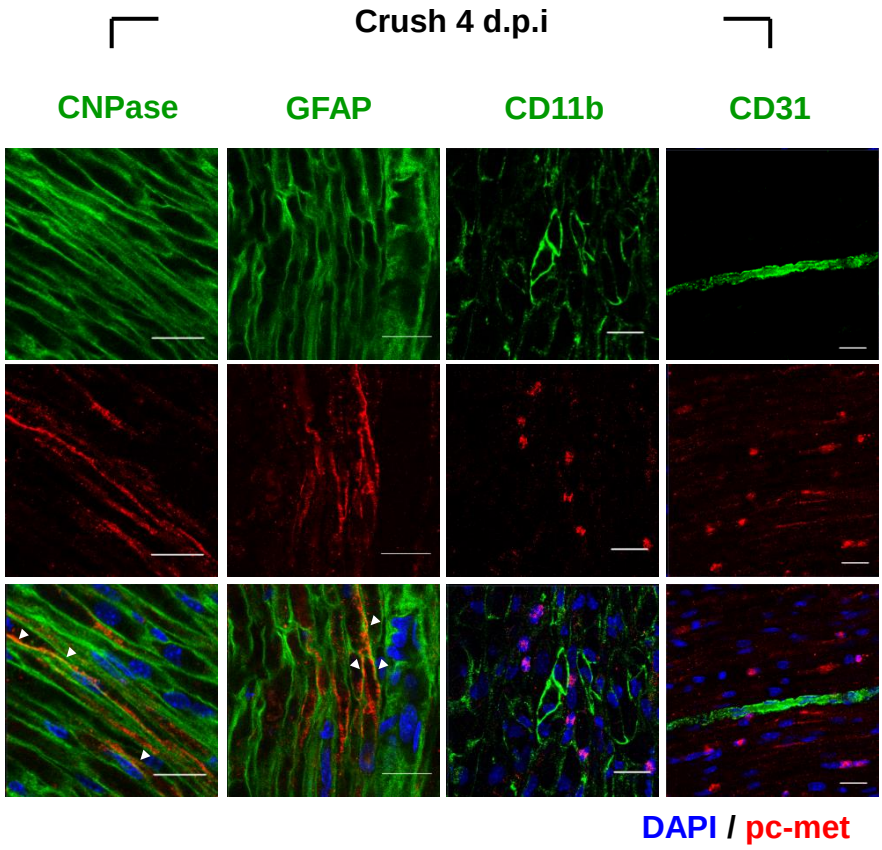


Figure 14. The staining pattern of phosphorylated c-met in injured sciatic nerves. The distal sites of injured sciatic nerves were analyzed for various cell markers by immunohistochemistry assay using antibodies to CNPase and GFAP for SCs, CD11b for macrophages, CD31 for endothelial cells (all green), and phosphorylated c-met (red).The phosphorylated c-met was mainly merged with SCs marker (white arrow). Injured sciatic nerves were prepared at crush 4d.p.i. Nuclei were counterstained with Hoechst (blue).n = 3 for each group. Scale bar = 20μm

A variety of different cell types are known to express c-met receptor and respond to HGF, including fibroblasts, endothelial cells, skeletal muscles, and neurons (Organ and Tsao, 2011). To identify the c-met expressing cell type(s) under the nerve injury situation, IHC was performed for cells around the distal sciatic nerve area by using antibodies to phosphorylated c-met, CNPase and GFAP for SCs, CD11b for macrophages, and CD31 for endothelial cells, respectively. As shown in Figure 14, activated c-met was detected mainly in distal SCs (Figure 14, white arrow for activated c-met).Endothelial cells also contained some positive signals for phosphorylated c-met, but they formed a small fraction among c-met positive cells. The c-met signal was not observed in macrophages. These data suggested that dedifferentiated SCs in the distal area were probably the major target of HGF produced from injured nerves.

2.6 A c-met inhibitor, PHA-665752 effectively blocked the c-met activity *in vivo*.

To study whether the increased level of HGF and c-met was indeed involved in nerve regeneration, a specific inhibitor of c-met, PHA-665752, was used. After sciatic nerve crush, mice were intraperitoneally injected with 20mg/kg of PHA-665752 on a daily basis until they were sacrificed. Again, the level of both total and phosphorylated c-met was increased in the distal region of sciatic nerves (Figure 15A lane 4). When mice were treated with PHA-665752, the level of phosphorylated c-met, but not that of total c-met, was highly reduced (Figure 15A lane 4 and 8). The protein level of HGF was not altered by PHA-665752 treatment in the injured mouse groups, indicating that it inhibited c-met activity

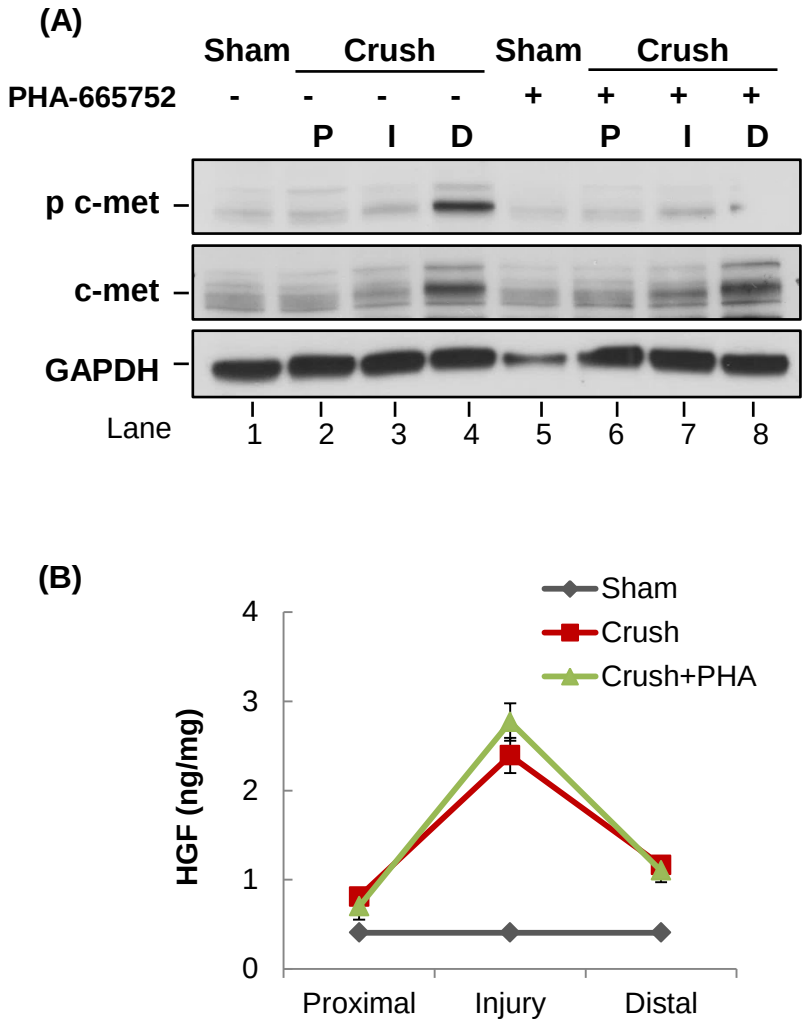


Figure 15. Effects of PHA-665752 on the level of phosphorylated and total c-met. After sciatic nerve crush, mice were intraperitoneally injected with 20mg/kg of PHA -665752 daily until sacrifice, followed by Western blot assay and ELISA. (A) Effects of PHA-665752 on c-met expression. Sciatic nerves were isolated at 4 d.p.i, and to tal proteins were prepared from three different areas followed by Western blot usin g specific antibodies to phosphorylated and total c-met. (n=4 for each group). (B) E ffects of PHA-665752 on HGF expression in the injured nerve. Injured nerves were isolated at 4 d.p.i and analyzed by ELISA. n=3 for each group.

without affecting the expression level of HGF in the sciatic nerve (Figure 15B).

2.7. Effects of PHA-665752 on pain

The effect of PHA-665752 treatment on pain was first measured by Von frey test, a well-known mechanical pain index. After nerve damage, Mice were daily injected with 20mg/kg of PHA-665752 for 4 weeks, and mechanical sensitivity was measured in frequency response method every week. In the nerve injured group, frequency response was gradually increased and sustained for 4 weeks compared to sham group (Figure 17). Interestingly, PHA-665752 injection induced mechanical allodynia in both sham and crush groups, indicating that c-met inhibition by PHA-665752 might further enhance the level of mechanical pain after injury.

2.8 Effects of PHA-665752 on re-myelination

Effects of c-met inhibition on nerve repair were observed by using a high-resolution transmission electron microscopy (TEM) at 14 d.p.i, which is the mid-point of the re-myelination in the nerve crush model (Grinsell and Keating, 2014). Treatment with PHA-665752 did not produce any visible effects in sham surgery animals (Figure 18). In the nerve crush group injected with PHA-665752 (Crush-PHA), however, c-met inhibition significantly disrupted the nerve regeneration, as evident by the reduction in axon diameter, myelin thickness, the number of re-myelinated neuron and myelin clearance (Figure 18, white arrow).

To further analyze the level of myelination in the injured nerve, g-ratio was measured. After nerve crush was introduced, g-ratio value was increased to 0.8413 ± 0.00313 (Crush-DMSO), as myelin thickness was decreased. When injured mice were treated

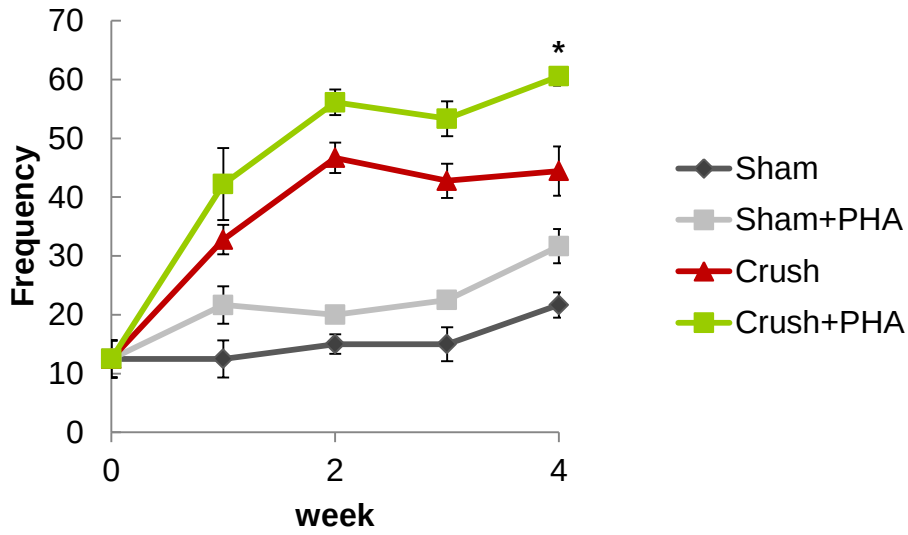


Figure 17. Effects of PHA-665752 on mechanical pain.

After sciatic nerve crush, mice were intraperitoneally injected with 20mg/kg of PHA -665752 daily. Mechanical sensitivity of mice was measured weekly by using von fr ey filaments test.

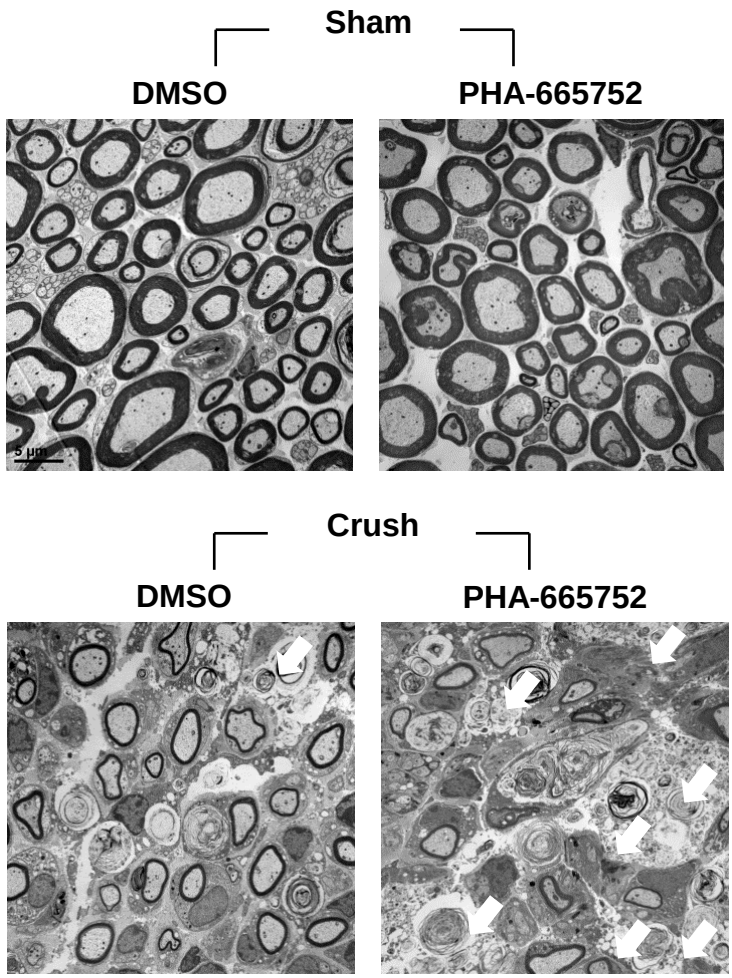


Figure 18. Effects of PHA-665752 on nerve regeneration.

After sciatic nerve crush, mice were intraperitoneally injected with 20mg/kg of PHA -665752 daily until sacrifice, followed by TEM. Electron micrographs of sciatic nerv es showed the cross section of sciatic nerve at 14 d.p.i, 4 mm from injury site. The white arrows indicate apoptotic cells. n=3 for each group. Scale bar, 5μm.

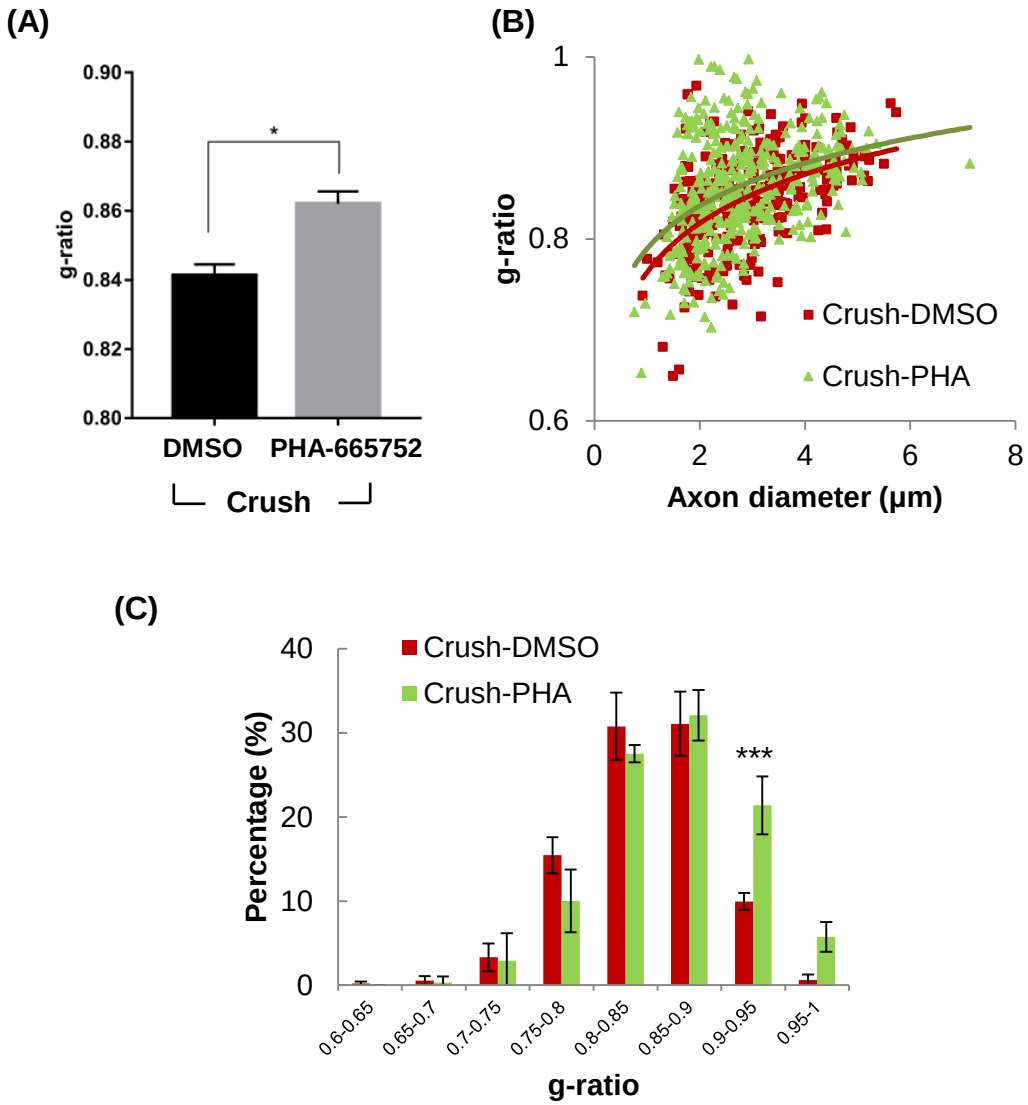


Figure 19. Effects of PHA-665752 on re-myelination.

Effects of PHA-665752 on re-myelination was measured by TEM. (A) The bar grap h shows the g-ratio vaule. (B)The scatter plot shows the distribution of myelin thick ness versus axon diameter in PHA-995752 treated and untreated groups. (C) The bar graphs show g-ratio value and the distribution of g-ratio. 250~300 axons, n=3 f or each group. *p<0.05, ***p<0.001.

with PHA-665752, the g-ratio value was further increased to 0.8621 ± 0.003495 (Figure 19A). Furthermore, the distribution graph of g-ratio showed that Crush-PHA group was shifted right compared to the Crush-DMSO group, indicating that neurons in Crush-PHA mice became hypo-myelinated compared to the Crush-DMSO group (Fig. 19C). These data indicated that c-met inhibition might block or delay the re-myelination process by acting on SCs.

2.9 Effects of PHA-665752 on axonal regeneration.

To investigate the effect of c-met inhibition on neuronal regeneration, axon diameter and the number of re-myelinated neuron were measured by using Image J program. The axon diameter distribution of the Crush-PHA group was shifted left compared to the Crush-DMSO group, indicating that neuron diameters were decreased in the PHA-665752 treated mice (Figure 20A). Furthermore, the number of re-myelinated neurons in injured nerves was also decreased by PHA-665752 treatment (Figure 20B), while the number of non-myelinated neuron (neuron diameter > 1μm) showed no difference between injured groups. These data showed the total number of neurons was reduced by PHA-665752 treatment.

To test whether PHA-665752 directly inhibits axon outgrowth during repair process, IHC analysis was performed by using antibody to SCG10, a regenerating axon maker (Shin et al., 2014; Shin et al., 2012). Immunostaining for axonal markers revealed that regenerating axons in Crush-PHA groups were stacked at proximal region, whereas regenerating axons were regrown and well penetrated into the distal nerve segments in Crush-DMSO group (Figure 21, * indicates the injured site). These data demonstrated that administration of c-met inhibitor, PHA-665752, could block or delay the neuron outgrowth.

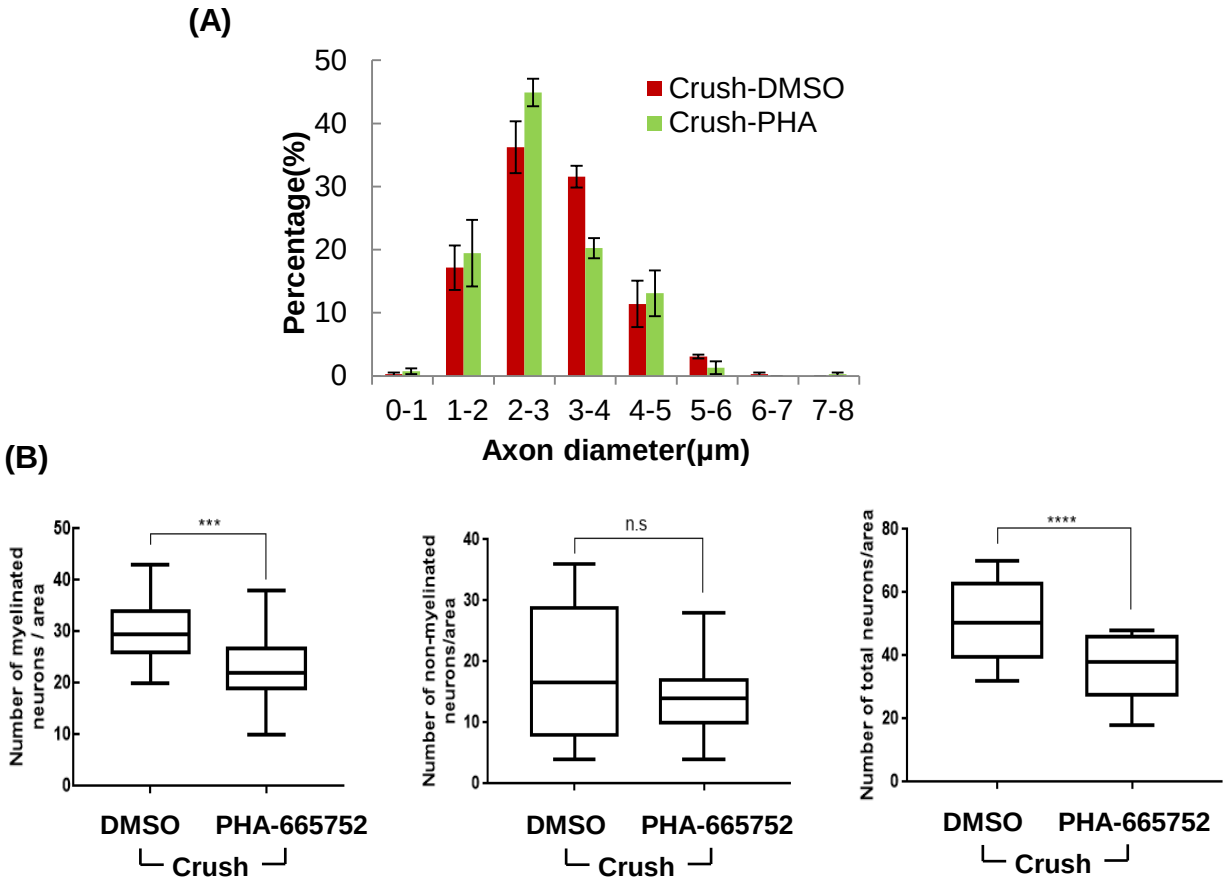


Figure 20. Effects of PHA-665752 on axonal regeneration.

Effects of PHA-665752 on axonal regeneration was measured by TEM. (A) Effects on axon diameter. The graph shows the distribution of axon diameter. (B) Effects o n the number of re-myelinated axons. The number of axons was calculated from T EM photos. 250~300 axons, n=3 for each group. ***p<0.001.In all these experimen ts, values represent the mean ± S.E.M

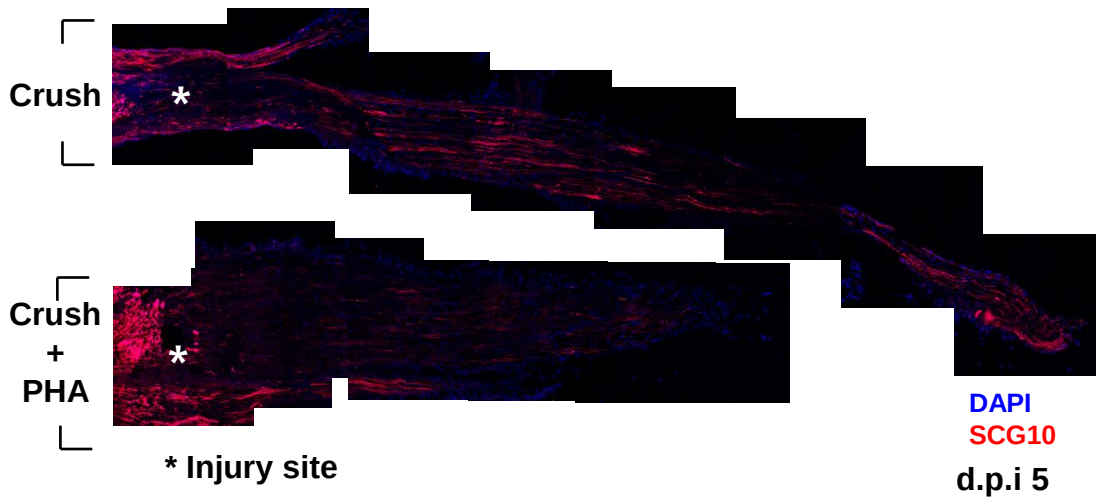


Figure 21. Effects of PHA-665752 on axon regrowth.

To test whether PHA-665752 directly inhibit axon outgrowth during nerve repair process, IHC analysis was performed at 5 d.p.i by using antibody SCG10, a regenerating axon maker. (* indicates injured site). Nuclei were counterstained with Hoechst (blue).n = 3 for each group.

2.10 Effects of HGF on gene expression in primary SCs

After nerve was damaged, the HGF protein and c-met expression were mostly increased at the first week, the period that SCs were in de-differentiation state. Re-myelination was started later, and the re-myelinated axon structure was able to observe least 2 weeks after injury. Due to this reason, we hypothesized that increased HGF and c-met during injury involved in nerve regeneration by promoting de-differentiated SCs phenotypes such as secreting cytokines and neurotrophic factors, migration and proliferation.

To prove our hypothesis, the primary SCs culture system was used. Our *in vivo* data suggested that HGF produced from the injury site might interact with the c-met receptor present on SCs. We tested the effect of recombinant human HGF protein binding to c-met on SCs, using primary SCs isolated from an adult rat. To be certain, the expression of c-met in these cells was first tested. The basal level of the total c-met protein was readily detectable as measured by Western blot and immunofluorescence assays, and was not changed by HGF treatment (Figure 22). Phosphorylated c-met was barely detectable without HGF treatment, but its level was increased when cells were treated with the HGF protein, indicating that under the normal situation, c-met is present in an un-phosphorylated form in SCs.

It is well known that during dedifferentiation of SCs, there are great changes in the activity and expression level of many cellular proteins. The effects of HGF in primary SCs were investigated, first on the RNA level of genes known to participate in

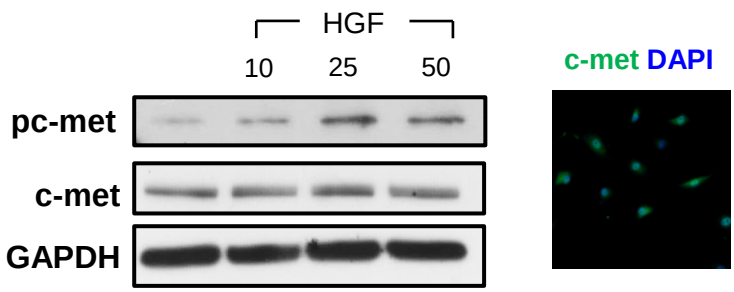


Figure 22. The activation and expression of c-met in primary SCs.
Primary Schwann cells isolated from rat sciatic nerves were treated with recombinant human HGF protein. Expression of c-met receptor in primary SCs was measured by western blot and IFA assay. Scale bar = 25μm. The samples derived from the same experiment and blots were processed in parallel.

or be affected by the nerve regeneration process. Primary SCs were treated with 25 ng/ml of hHGF for 1 hour, and its effects on cellular genes were analyzed by microarray and quantitative RT-PCR.

Interestingly, data from microarray analysis indicated that the expression of limited number of genes was controlled by HGF compared to other growth factors (Figure 23). 87 of genes up-regulated in SCs more than 2 folds compared to control, while 35 of genes were down-regulated by HGF -treatment. Among those genes, the expression of a lot of genes, which are known to be up-regulated in dedifferentiated SCs in injured nerves, including Egr1, c-Fos, JunB, LIF, Myc, MCP-1 and IL-6, were enhanced by HGF protein.

The effects of HGF on the RNA level of these genes were confirmed by quantitative RT-PCR in Table 6. Among various transcription factors, a change in the RNA level of Egr-1 was the most prominent. The role of Egr-1 in nerve regeneration has not yet been well characterized. The RNA level of c-Fos and JunB was also increased by HGF, more than 3-fold. These molecules could form AP-1, which is widely known to control the expression of various genes taking part in SC-mediated nerve repair. HGF did not alter the expression of c-Jun and Sox10. GDNF is the most affected growth factor of the proteins tested. Among 5 inflammatory proteins, the RNA level of TNF-α and LIF, which attracts macrophages to the injury site, was increased by more than 3-fold. Overall, data from microarray and quantitative RT-PCR assays suggested that HGF could induce the expression of selective cellular genes known to play roles in the promotion of SC dedifferentiation.

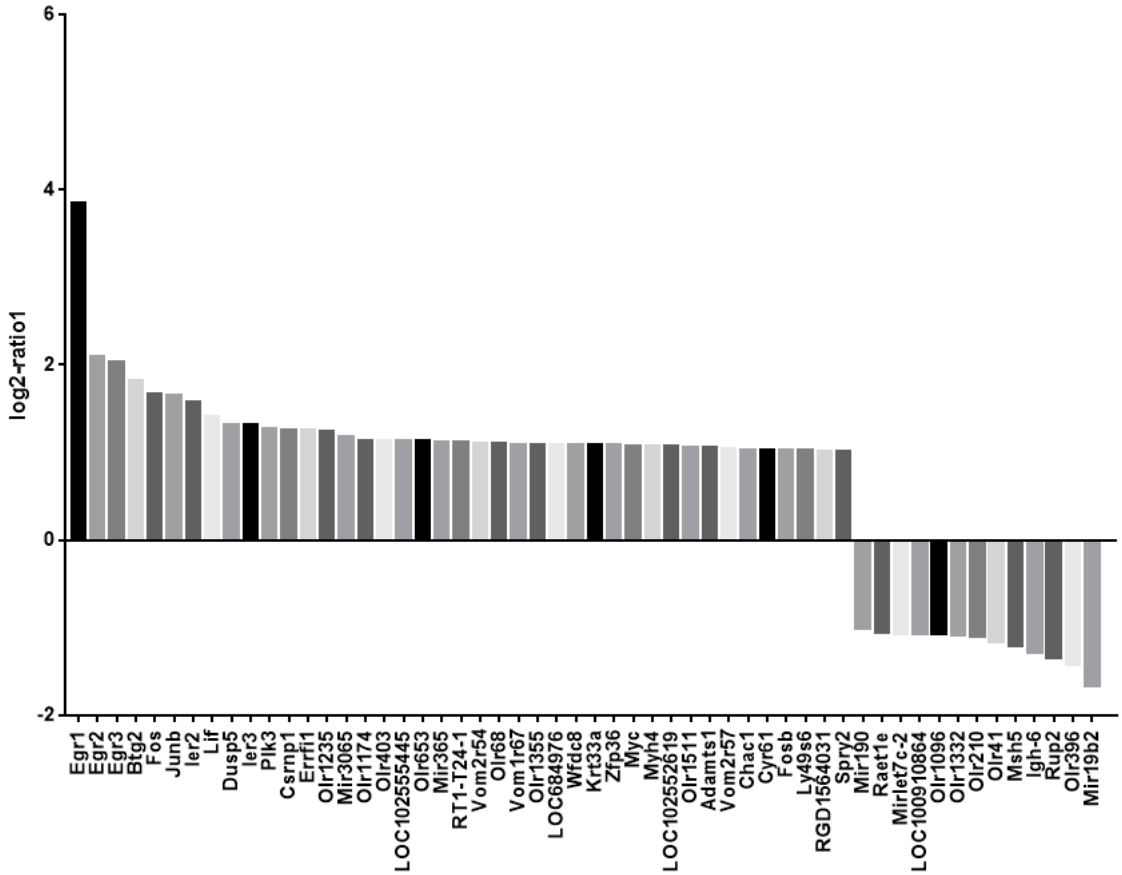


Figure 23. Effects of HGF on gene expression analyzed by microarray.

Primary SCs were treated with 25 ng/ml of HGF protein for 1hr. Total RNAs were isolated from SCs and analyzed by microarray.

Table 6

Functions		Fold increase*	STD	P-value
Transcription factors	Egr1	239.701	48.998	0.014
	c-Jun	0.872	0.068	1.000
	c-Fos	3.143	0.246	0.002
	JunB	3.827	0.244	0.001
	Sox10	1.091	0.048	0.600
	HIF1a	1.632	0.104	0.011
	Notch1	1.445	0.323	0.009
Growth factors	VEGFa	1.202	0.046	0.001
	NGF	1.055	0.191	0.724
	GDNF	4.118	0.077	0.001
Inflammatory proteins	TNFa	3.003	0.012	0.010
	LIF	7.662	0.066	0.004
	CCL2/MCP-1	1.266	0.230	0.031
	IL1b	1.682	0.099	0.001
	IL-6	2.041	0.368	0.063

Table 6. Effects of HGF on RNA levels of genes increased in repair SCs. Primary SCs were treated with 25 ng/ml of HGF protein for 1hr. Total RNAs were isolated from SCs and analyzed by quantitative RT-PCR. * Fold increase after HGF treatment relative to no treatment. Values represent the mean ± S.E.M. of three independent experiments.

2.11 Effects of HGF on cell signaling pathway in primary SCs

The effects of HGF on various signaling molecules are shown in Figure 39. The level of phosphorylated c-met was increased 5 min after HGF treatment, reaching the highest point at 15min and then decreased (Figure 24, lanes 2, 4, 6 and 8). The level of total c-met remained unchanged throughout the experimental period. The response of phosphorylated ERK1/2 (pERK1/2) was similar to c-met, but the change was more rapid and dramatic in quantity (Figure 24, lanes 2 and 4). RSK, one of the downstream targets of ERK signaling was changed in a similar way to that of ERK; the level of phosphorylated RSK was highest at 5 min, and undetectable at any other time point. The level of phosphorylated AKT was also highly increased at 5min, but to a lesser extent than that of pERK1/2. After the peak level, it was reduced slowly.

S6K is the downstream target of AKT /mTOR pathway. The phosphorylated level of S6K reached a peak at 5 min, and then was gradually reduced. The level of phosphorylated mTOR was not affected by HGF treatment, so the increase in the level of phosphorylated S6K is not due to mTOR pathway. In the same experiments, HGF did not have any effect on STAT3 and JNK, although they have been previously known to be activated by HGF in other cell types. Taken together, these data suggested that HGF might induce the expression of selective genes involved in dedifferentiation, probably through the activation of ERK or AKT signaling.

2.12 Effects of HGF on SCs migration in primary SCs

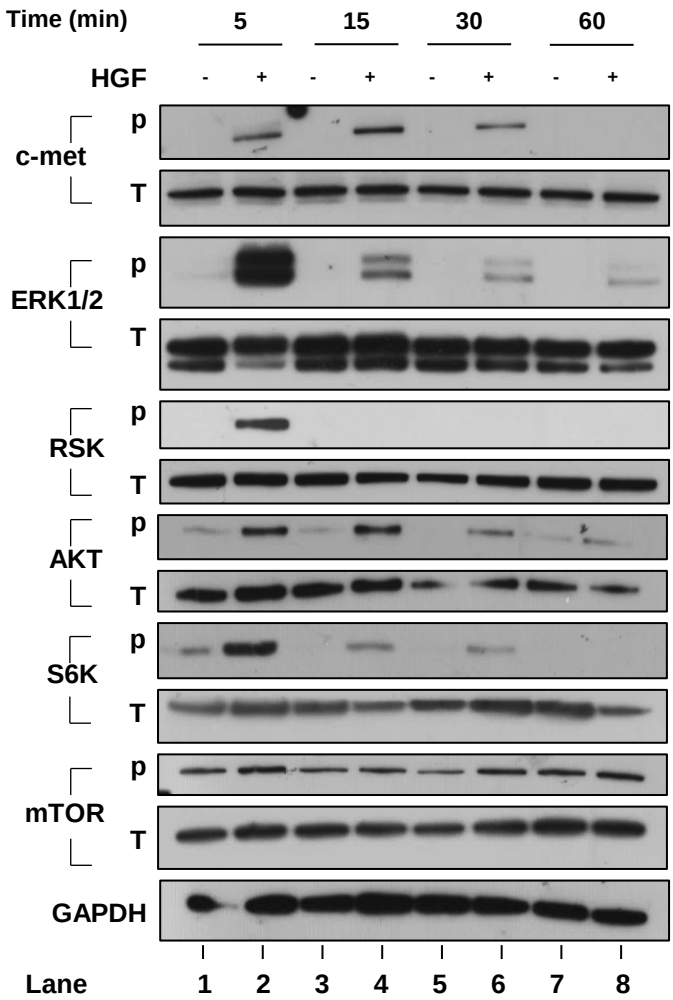


Figure 24. Effects of HGF on signaling pathway in primary SCs.

Signaling cascades activated by HGF treatment in SCs. Primary Schwann cells isolated from rat sciatic nerves were treated with recombinant human HGF protein. Total proteins were isolated from SCs treated with HGF at appropriate times and analyzed by Western blot using antibodies to respective proteins. The samples derived from the same experiment and blots were processed in parallel.

Because c-met expression is highly induced in distal SCs undergoing dedifferentiation, it was tested if HGF could affect migration of SCs, as it is key features of repair SCs. Primary SCs were treated with 10 and 25 ng/ml of recombinant human HGF protein and followed by Boyden chamber assay (Figure 25). When SCs were treated with 10 ng/ml of hHGF protein, SCs migration was induced about 70% compared to control group, however, as treated dose was increased, the number of migrated SCs was decreased to basal level. Taken together, these data indicated HGF might induce SCs migration in dose dependent manner.

2.13 Effects of HGF on SCs proliferation in primary SCs

To determine whether HGF alter the SCs proliferation, SCs were treated with diverse doses of HGF protein for 24 hours and analyzed by WST-1 assay and IFA staining. As shown in Figure 26A, HGF also increased the proliferation of SCs, although its effects were relatively mild compared to other well-known mitogenic growth factors such as neuregulin-1 (data not shown).Induction of proliferation by HGF was also confirmed by immunocytochemistry assay, using an antibody specific for Ki67 (Figure 26B). Taken together, these results suggested that HGF could directly interact with SCs to activate c-met and subsequently increase migration and proliferation of SCs.

2.14 The ERK pathway appears to play a major role(s) in HGF-mediated responses in primary SCs

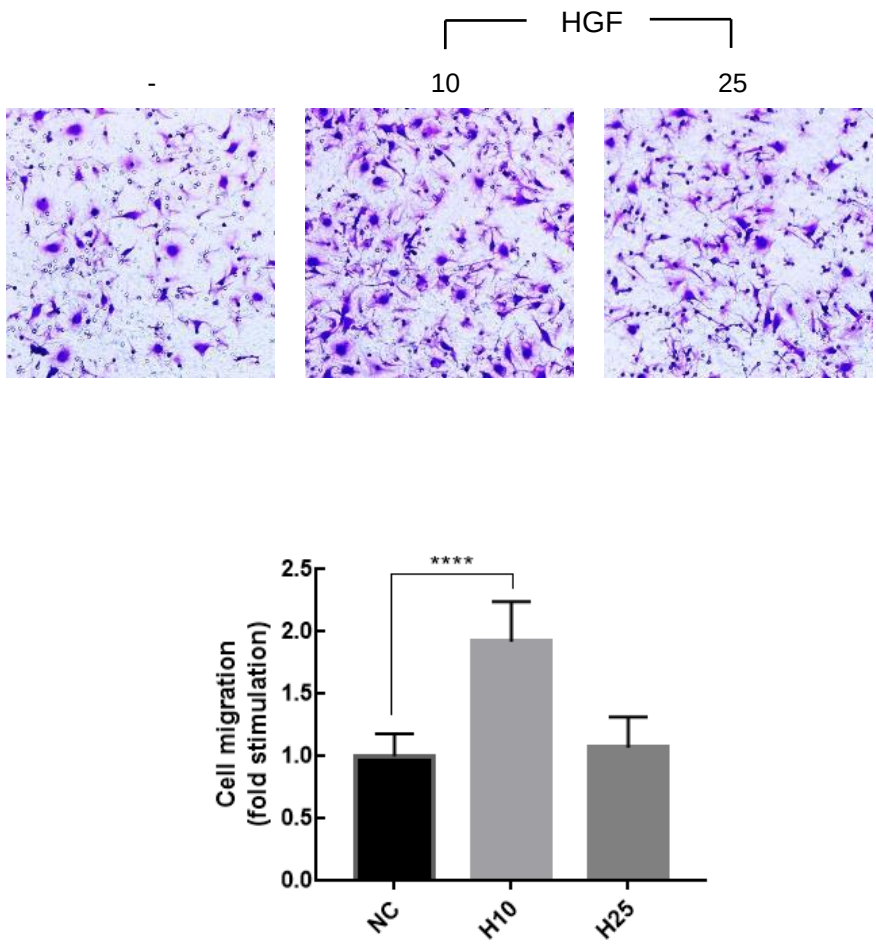


Figure 25. Effects of HGF on SCs migration.

Primary SCs were treated with 10 ng/ml and 25 ng/ml of recombinant HGF protein, and their migration was analyzed using Boyden chambers assay. ****p<0.0001. Values represent the mean ± S.E.M. of three independent experiments.

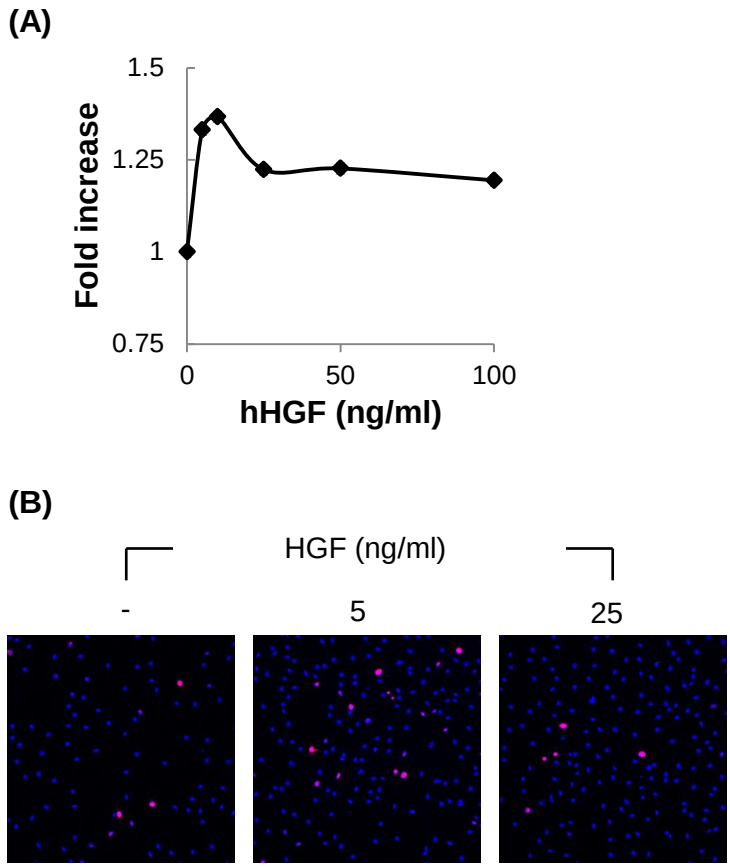


Figure 26. Effects of HGF on SCs proliferation.

(A) SCs were treated with 5, 10, 25, 50, and 100 ng/ml HGF and SC proliferations were analyzed by WST-1 assay. (B) SCs proliferation was analyzed by immunocytochemistry assay, using an antibody to Ki67 (Red). Scale bar = 50μm

When nerves get injury, both the ERK and AKT pathways have a critical role in SCs dedifferentiation. To test which of the two signaling pathways, ERK or AKT, played a more dominant role in HGF-induced phenotypes, chemical inhibitors for ERK or AKT were used. Primary SCs were treated with HGF in the presence or absence of U0126 or AKTi, an inhibitor of ERK or AKT respectively, followed by quantitative RT-PCR, Boyden chamber and WST-1 assays. As shown in Figure 27, an HGF-mediated increase in the RNA level of four genes (Egr1, c-Fos, GDNF, and LIF) involved in SC-mediated nerve regeneration was effectively suppressed by treatment with U0126, while the completely opposite result was obtained for AKTi, which further enhanced the expression level increased by HGF (Figure 28).

Similar results were obtained in cell migration assay. Treatment with U0126 also effectively inhibited HGF-induced migration of SCs, while AKTi had no effects (Figure 29 and 30). Interestingly, the effect on cell proliferation was different; an HGF-mediated increase in the cell number was inhibited by AKTi, but not by U1206 (Figure 31), indicating that AKT signaling might play a role(s) in cell proliferation rather than cell migration. These results suggested that the ERK pathway might be needed for HGF to induce migration of SCs as well as activate the expression of cellular proteins known to involve in nerve regeneration.

2.15 Exogenous administration of HGF by pCK-HGFX7 injection did not alter the expression of endogenous HGF protein expression.

Because the HGF/c-met pathway seemed to play a role in the

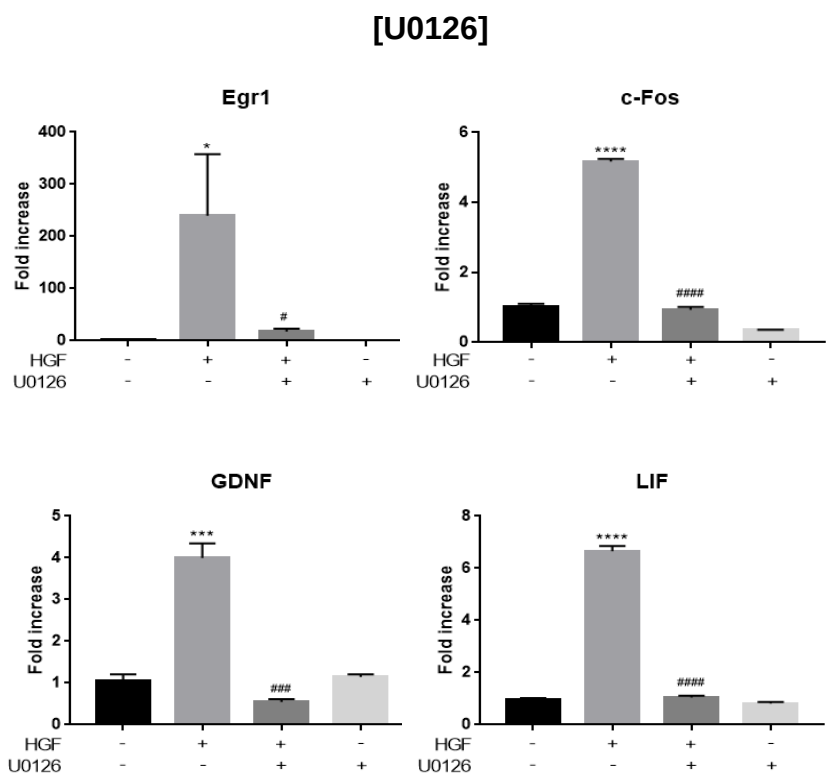


Figure 27. Effects of U0126 on HGF-induced gene expression.
Primary SCs were treated with 25 ng/ml of recombinant HGF protein in the presence of 10μM of U0126. Total RNAs were prepared at 1hr and subjected to quantitative RT-PCR. Effects of U0126 on the RNA level of Egr-1, c-Fos, GDNF and Lif. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. control. #p<0.05, ##p<0.01, ###p<0.001, #### p<0.0001 vs. HGF only treated group.

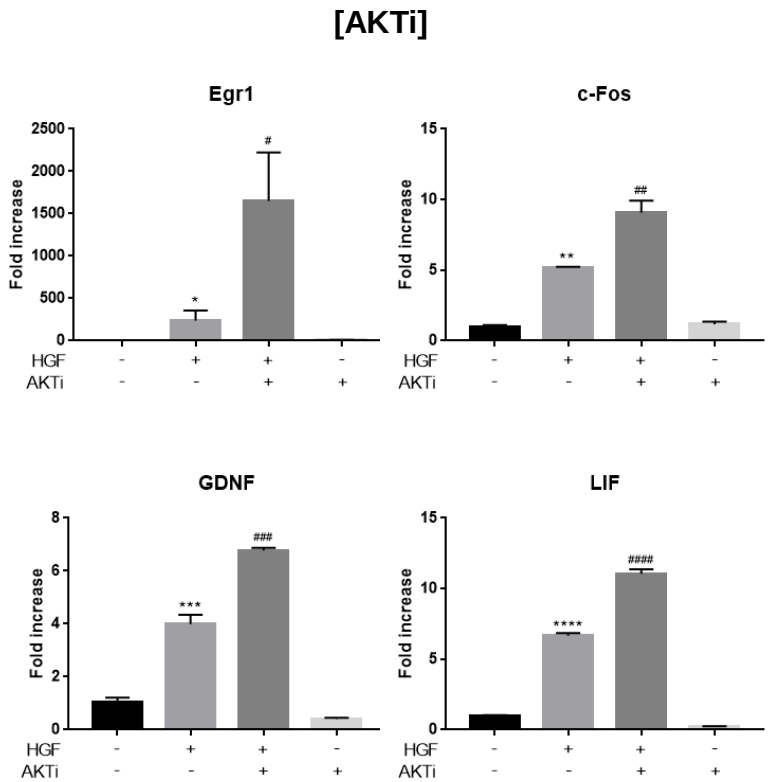


Figure 28. Effects of AKTi on HGF-induced gene expression.

Primary SCs were treated with 25 ng/ml of recombinant HGF protein in the presence of 10μM of AKTi. Total RNAs were prepared at 1hr and subjected to quantitative RT-PCR. Effects of AKTi on the RNA level of Egr-1, c-Fos, GDNF and Lif. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. control. #p<0.05, ##p<0.01, ###p<0.001, #### p<0.0001 vs. HGF only treated group.

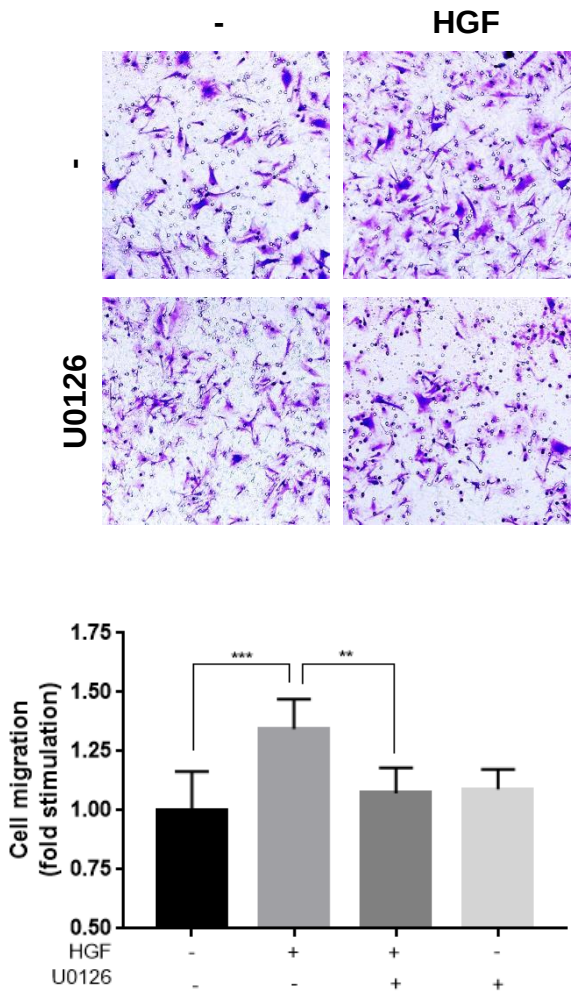


Figure 29. Effects of U0126 on HGF-induced SCs migration.

Primary SCs were treated with 25 ng/ml of recombinant HGF protein in the presence of 10μM of U0126. Effect of U0126 on SC migration. Was measured by Boyden Chamber assay. **p<0.01, ***p<0.001, n.s = not significant

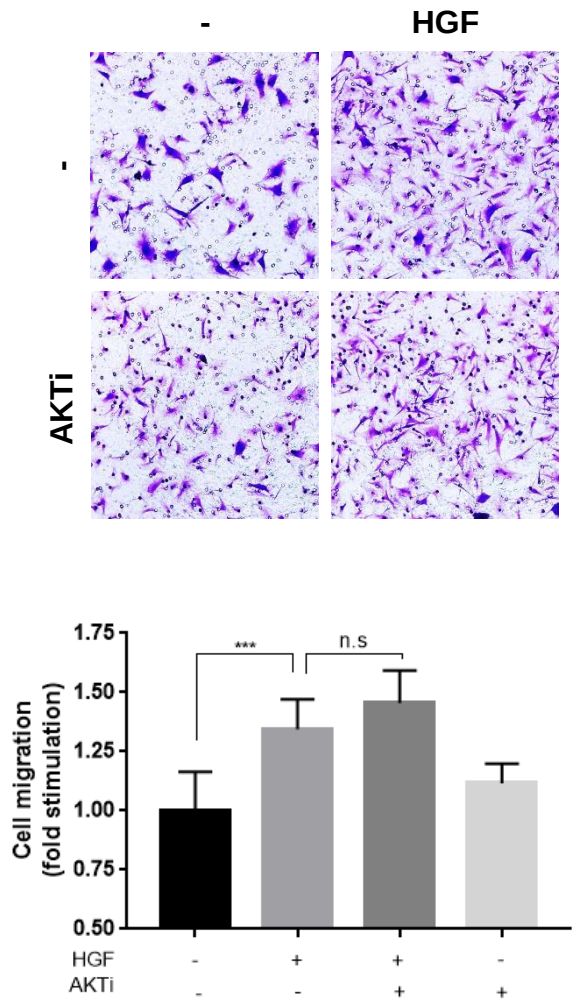


Figure 30. Effects of AKTi on HGF-induced SCs migration.

Primary SCs were treated with 25 ng/ml of recombinant HGF protein in the presence of 10μM of AKTi. Effect of AKTi on SC migration. Was measured by Boyden Chamber assay. ***p<0.001, n.s = not significant

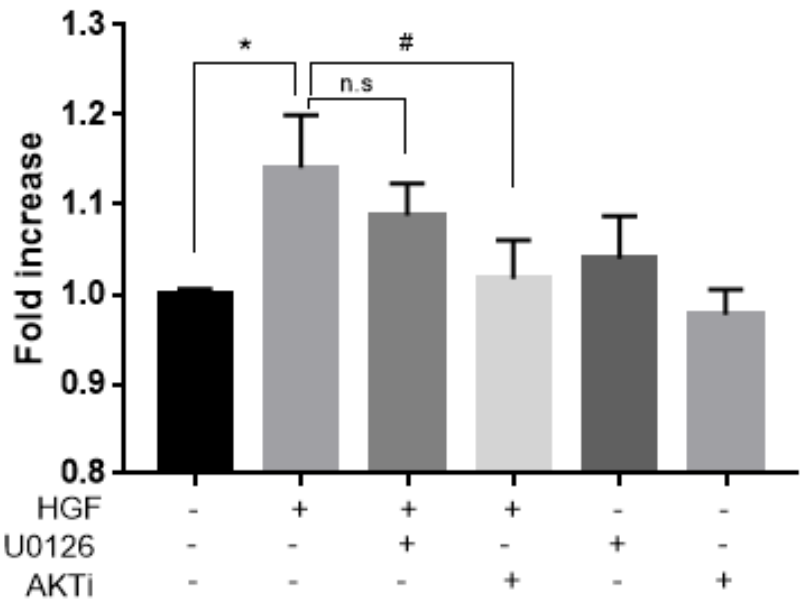


Figure 31. Effects of U0126 and AKTi on HGF-induced SCs proliferation.

Effect of U0126 and AKTi on SC proliferation was measured by WST-1 assay. *p <0.05 vs. control. #p<0.05 vs. HGF only treated group

nerve repair process such as re-myelination or axon regrowth, we tested whether exogenous administration of HGF could improve the injured nerve, as in the case of NT3, GDNF and VEGF (Chen et al., 2010; Guaiquil et al., 2014; Pereira Lopes et al., 2011; Wood et al., 2012).

In this thesis, we employed a gene transfer method to test whether HGF could have any positive results on nerve regeneration in the mice model. We did not use a recombinant protein, because of the short half of the HGF protein (Nakamura and Mizuno, 2010). A plasmid DNA expressing HGF, pCK-HGF-X7, was intramuscularly injected into the thigh muscles around the sciatic nerve at the time of nerve crush surgery. pCK, a plasmid DNA lacking the HGF sequence, was used as a control. The expression kinetics of human HGF from plasmid pCK-HGF-X7 in mice has been well established; the protein level of HGF peaked (a ~100ng/mg of total cellular protein scale in thigh muscles around the injection site and a ~300pg/mg range in sciatic nerves), and then decreased to become undetectable at 14 d.p.i (Nho et al., 2018)

To test whether human HGF expressed in pCK-HGF-X7injected thigh muscles have influence on the production of mouse HGF inipsilateral thigh muscles and sciatic nerves, total proteins were prepared at 7 d.p.i followed by mouse HGF ELISA (Figure 32). The protein level of mouse HGF was not altered by pCK-HGF-X7 in sciatic nerves and thigh muscles.

2.16 Effects of hHGF overexpression on pain

Because treatment with PHA-665752 induced the mechanical

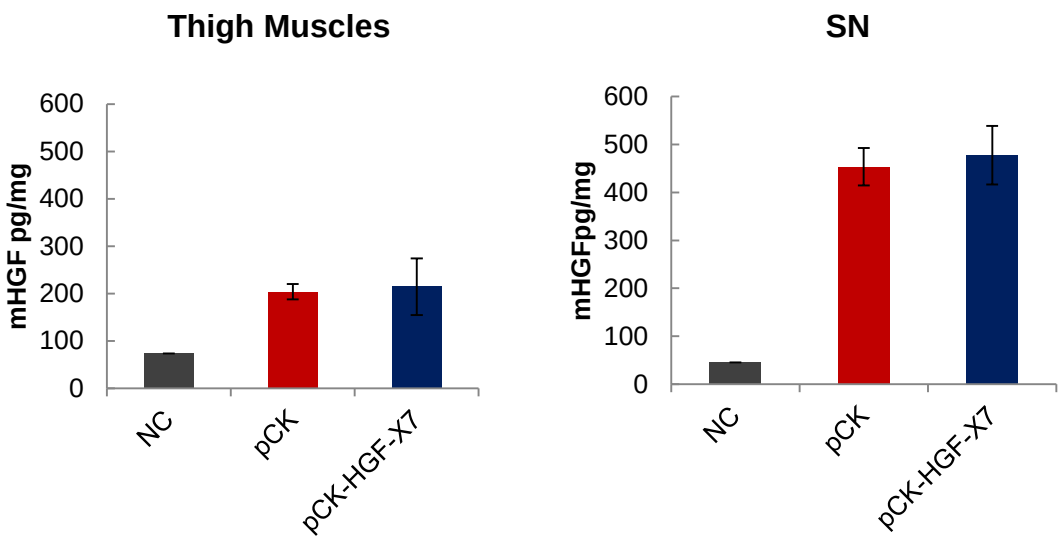


Figure 32. Exogenous administration of HGF by pCK-HGF-X7 injection did not alter the expression of endogenous HGF protein expression. The protein level of mouse HGF in both the thigh muscles and sciatic nerves was measured at 7 d.p.i by ELISA. n-=3 for each groups.

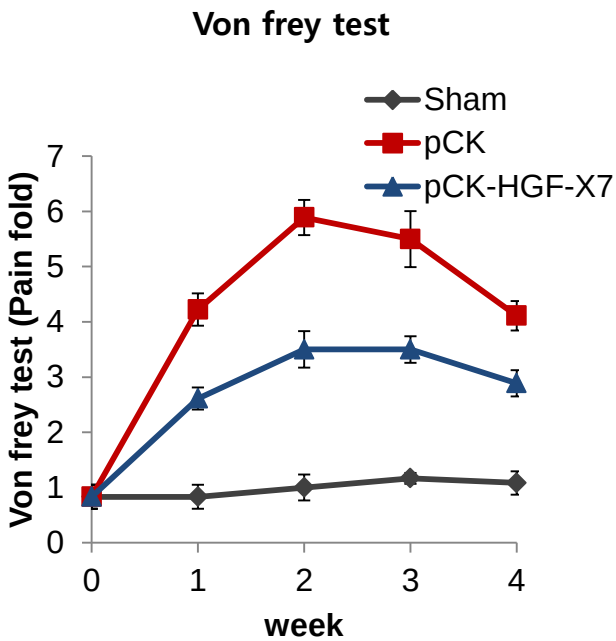


Figure 33. Effects of pCK-HGF-X7 on mechanical pain.

pCK-HGFX7 was i.m. injected to the thigh muscle around the injured sciatic neuron at the time of nerve crush surgery. Mechanical sensitivity of mice was mea sured weekly by using von frey filaments test. pCK lacking the HGF sequence was used as a control.

pain in mouse crush model (Figure 23), we tested whether overexpression of HGF could relieve the pain index. After nerve damage, pCK or pCK-HGFX7 was injected to thigh muscle at 0 d.p.i, and mechanical sensitivity was measured by Von frey method every week for 4 weeks (Figure 33). In nerve injury group, frequency response was gradually increased and then reached the stable level at 4 weeks compared to sham group. pCK-HGFX7 injection diminished the mechanical allodynia compared to control groups at least for 4 weeks, indicating that hHGF overexpression could block or decreased the pain induction.

2.17 Effects of hHGF overexpression on re-myelination

Because the HGF/c-met pathway seemed to involve in the nerve repair process, we tested whether exogenous administration of HGF could improve the injured nerve, as in the case of VEGF and GDNF (Chen et al., 2010; Guaiquil et al., 2014; Pereira Lopes et al., 2011; Wood et al., 2012). The effect of pCK-HGF-X7 on re-myelination was studied at 7, 14, and 28 d.p.i by analyzing the site 4mm distal from the injury site by TEM. Little difference was found between pCK-HGF-X7 and pCK groups at 7 d.p.i, while improvement in myelin thickness and neuron diameter was clearly visible at 14 and 28 d.p.i (Figure 34).

The scatter plots of the g-ratio of each group measured from the TEM image at 28 d.p.i, showed that the average value of myelin thickness was higher in the pCK-HGF-X7 group compared with the pCK control group (Figure 35A). As shown Figure 46B, g-ratio value was increased to 0.7553 ± 0.003052 in control group (Crush-pCK), whereas g-ratio value was reduced to 0.7312 ±

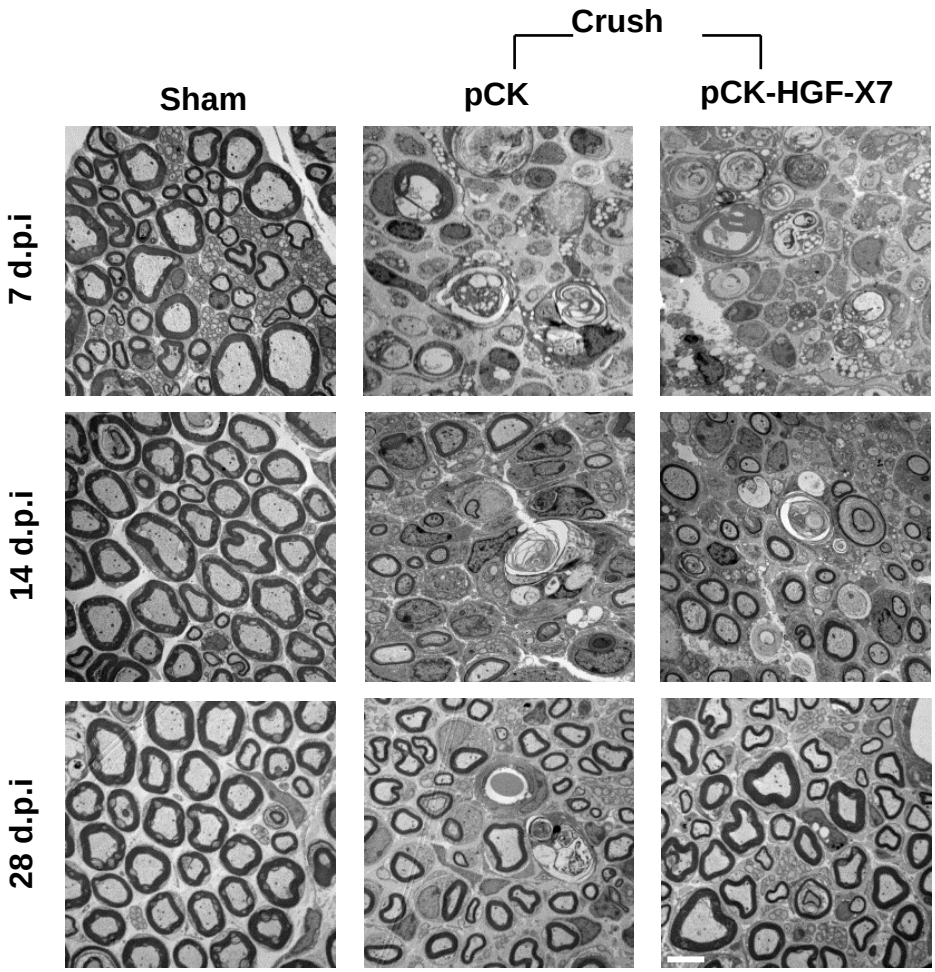


Figure 34. Effects of pCK-HGF-X7 on nerve regeneration.

pCK-HGFX7 was i.m. injected to the thigh muscle around the injured sciatic neuron at the time of nerve crush surgery. pCK lacking the HGF sequence was used as a control. Sciatic nerves were isolated at 7, 14, and 28 d.p.i (4 mm distal from injury site) and analyzed by TEM n=3 for each groups. Scale bar, 5μm.

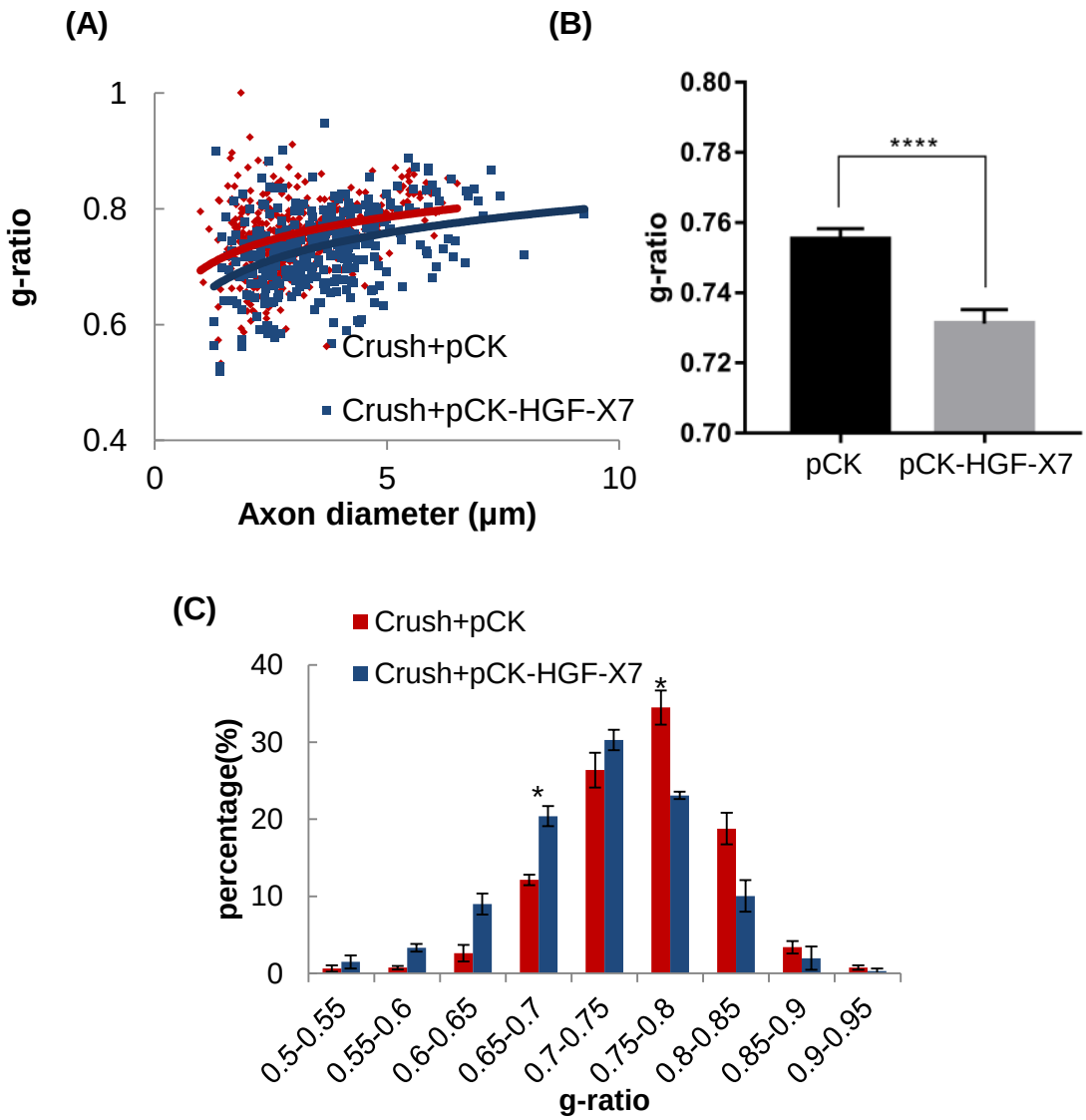


Figure 35. Effects of pCK-HGF-X7 on re-myelination.

The graph shows scatter plots of g-ratio in pCK-HGFX7 and pCK control groups at 28 d.p.i. The bar graphs show g-ratio value and the distribution of g-ratio. 300~400 axons, n=3 for each groups. *p<0.05, ****p<0.0001, Values represent the mean ± S.E.M.

Crush 14 d.p.i

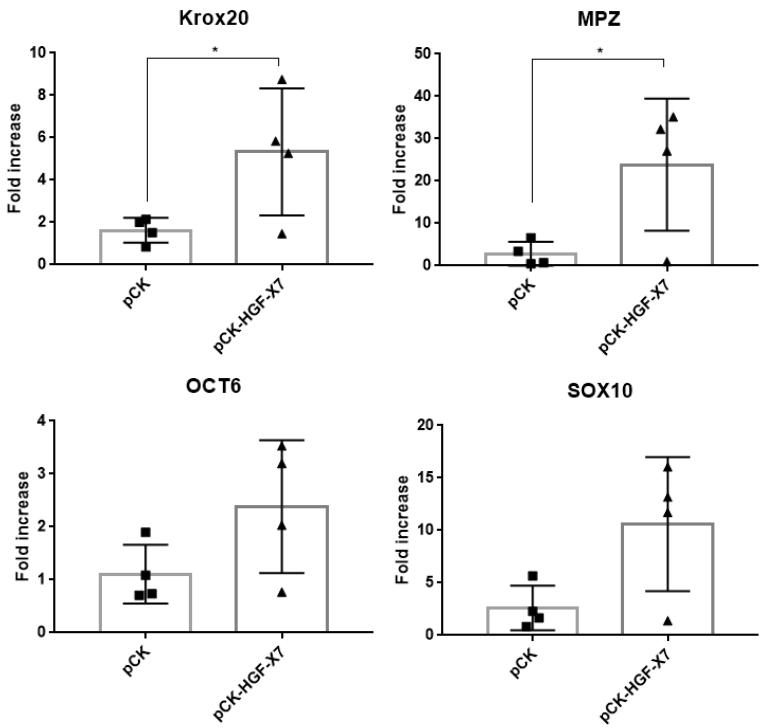


Figure 36. Effects of pCK-HGF-X7 on the expression of re-myelination associated genes. Injured sciatic nerves were isolated at 14 d.p.i .The expression of four myelin-associated genes was analyzed by quantitative RT-PCR. n=4 for each groups. One way Anova, Turkey's test, *p<0.05. Values represent the mean ± S.E. M

0.003953 in pCK-HGF-X7 injected group (Crush-pCK-HGFX7). In addition, the distribution graph of g-ratio showed that Crush-pCK-HGFX7 was shifted to left compared to the Crush-pCK group, indicating re-myelination was promoted by HGF overexpression (Figure 35C).

To test whether pCK-HGF-X7 have effects on re-myelination in molecular level, total RNAs of injured sciatic nerves were prepared at 14 d.p.i followed by quantitative RT-PCR. As shown in figure 47, the RNA level of four myelin-associated genes, including Krox20 (Egr2), Myelin protein Zero (MPZ), OCT6, and Sox10, was highly induced by pCK-HGF-X7 treatment (Figure 36). Taken together, these data indicated that exogenous administration of HGF could improve re-myelination after nerve injury.

2.18 Effects of hHGF overexpression on axonal regeneration

In addition, we also observed that axonal regeneration was also facilitated by pCK-HGF-X7 treatment. The distribution of myelinated axon size of the pCK-HGF-X7 injected mice shifted toward the right, indicating an increase in the neuron diameter of regenerating axons (Figure 37A). The number of myelinated neurons was not altered in both the pCK and pCK-HGF-X7 injected groups (Figure 37B).

To demonstrate whether HGF overexpression directly promote axon outgrowth during nerve repair process, IHC analysis was performed by using antibody SCG10 (Figure 38). Immunostaining of axonal markers revealed that regenerating axons in Crush-pCK-HGF-X7 groups began to grow and penetrate into the distal region from 1 d.p.i, whereas those in Crush-pCK control groups were still stacked at proximal portion. Taken together, our data

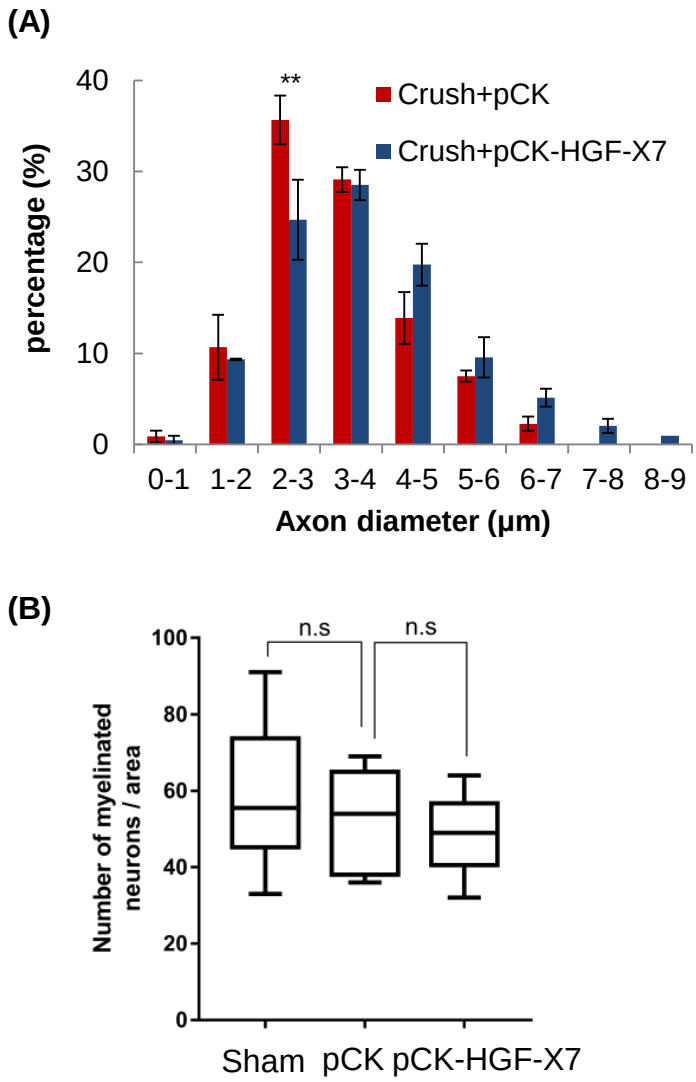


Figure 37. Effects of pCK-HGF-X7 on axonal regeneration.

The graph presents the distribution of axon diameter at 14 d.p.i (300~400 axons, n=3 for each group) . Effects of HGF on number of myelinatedaxons in injured nerves. n=3 for each group. n.s = notsignificant.,**p<0.01 .Values represent the mean ± S.E.M.

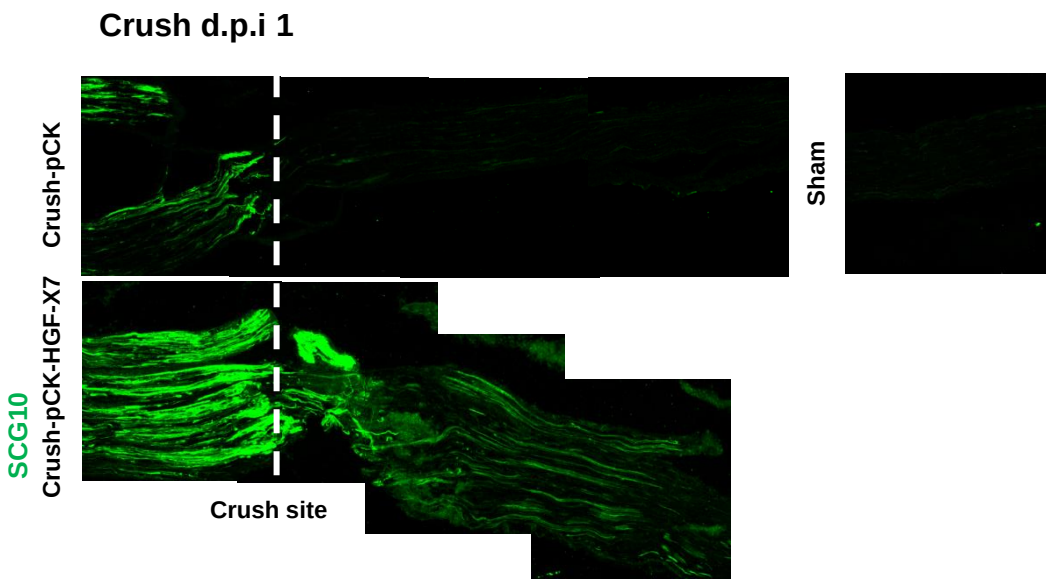


Figure 38. Effects of pCK-HGF-X7 on axon regeneration. IHC analysis was performed at 1 d.p.i by using antibody SCG10(green), a regenerating axon maker. (white line indicates injured site). n = 3 for each group.

demonstrated that exogenously added HGF, delivered in the form of plasmid DNA, could improve axon regeneration.

2.19 Effects of post-pCK-HGF-X7 injection on peripheral nerve regeneration

It was also tested whether i.m injection of pCK-HGF-X7 could have effects when introduced at 21 d.p.i, the time points that SCs were in re-myelination process. Twenty one days after sciatic nerve crush was introduced, 200μg of pCK or pCK-HGF-X7 were injected into ipsilateral thigh muscles, and injured nerves were analyzed by TEM at 42 d.p.i (Figure 39). The groups injected with pCK-HGF-X7 at 0 d.p.i used as control. As shown in Figure 40, the g-ratio value was decreased in both co-injected (HGF-X7/pCK) and post-injected (pCK/HGF-X7) groups compared to Crush-pCK group, indicating that HGF could improve myelin thickness in those groups.

However, the post injection of pCK-HGF-X7 at 21 d.p.i showed no improvement in axon diameters, while the distribution curve of axon diameter of co-injection group (HGF-X7/pCK) was shift right, indicating that injection of pCK-HGF-X7 could promotes axonal regeneration as well as re-myelination (Figure 41). Taken together, these results suggested that HGF might improve re-myelination whenever it was overexpressed, whereas effect on axonal regeneration was depending the injection time.

3. Discussion

In this thesis works, we demonstrated the involvement of HGF in Schwann cell-mediated regeneration of peripheral nerves in the nerve crush model. HGF expression was highly induced in the

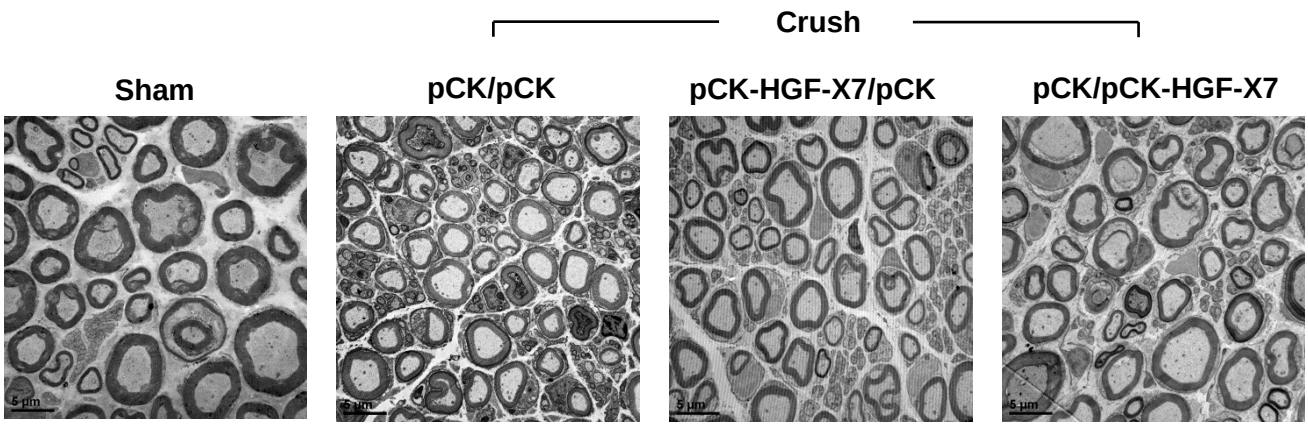


Figure 39. Effects of post pCK-HGF-X7 injection on nerve regeneration. pCK-HGFX7 was i.m. injected to the thigh muscle around the injured sciatic neuron at 21 d.p.i. pCK lacking the HGF sequence was used as a control. Sciatic nerves were isolated at 42 d.p.i (4 mm distal from injury site) and analyzed by TEM. n=3 for each groups. Scale bar, 5μm

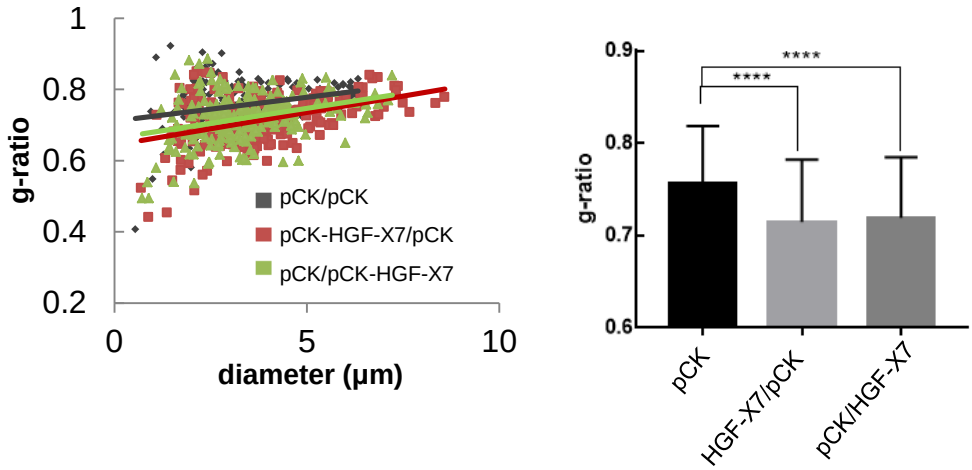


Figure 40. Effects of post pCK-HGF-X7 injection on re-myelination.

The graph shows scatter plots of g-ratio in pCK-HGF-X7 injected groups at 42 d.p.i. The bar graphs show g-ratio value and the distribution of g-ratio. 250~300 ax ons, n=3 for each groups. ****p<0.0001, Values represent the mean ± S.E.M.

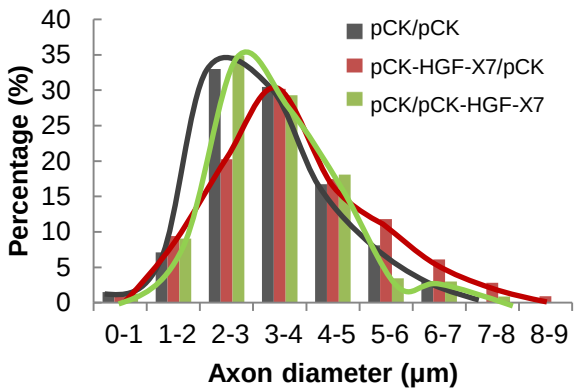


Figure 41. Effects of post pCK-HGF-X7 injection on axonal regeneration.

The graph presents the distribution of axon diameter at 42 d.p.i (250~300 axons, n=3 for each group) .

injured and distal areas while the expression of both total and phosphorylated c-met receptor was increased almost exclusively in SCs at distal sites. When mice were treated with a c-met inhibitor, PHA-665752, re-myelination of the injured nerve was suppressed as evidenced by a decrease in myelin thickness determined by TEM analysis, indicating that HGF might have a role(s) in nerve regeneration. Treatment of primary SCs with the HGF protein enhanced migration and proliferation of SCs, and increased the expression level of neurotrophic factors such as GDNF and LIF involved in nerve regeneration after injury. As evidenced by data from the experiments involving specific chemical inhibitors, ERK or AKT signaling pathways seem to play important roles. Finally, overexpression of HGF around the nerve injury site by i.m injection of HGF expression plasmid DNA, pCK-HGF-X7, increased the myelin thickness and axon diameter. Taken together, our data suggest that HGF and c-met play important roles in peripheral nerve regeneration by activating SCs.

The HGF protein was observed mainly around the injured site, but we have not yet identified the exact producer cell type(s). In naïve mice, the basal level of HGF was detected at 200 pg/mg of total protein in the sciatic nerve (Figure 9A and 9B). Data from IHC indicated that cells present in the epineurium might be one of the major sites of HGF production after nerve injury as well as in normal, undamaged nerves (Figure 9C). Fibroblasts, already known to produce HGF in a variety of organs, are a major candidate for a HGF producer cell type in our case, as they are known to present around the epineural membrane of the naïve nerve and are recruited to the injury site. Both the SCs and macrophages themselves do not seem to produce HGF during injury as the HGF signal was not

merged with their cell markers in IHC staining and the level of HGF expression was not changed when treated with a variety of stimulants. Despite HGF protein was basally expressed in both *in vivo* DRG tissue (100~200pg/mg) and *in vitro* DRG neuron (data not shown), sensory neurons were also not a candidate for HGF producing cell during nerve injury, because no induction or reduction of HGF protein was observed in DRG. The further studies to prove that fibroblast is the HGF producing cells in injured nerve has been on way.

One of the most interesting observations made in this study is that c-met expression is highly induced, but selectively in the distal SCs at the injury site. It is well established that most cells around the injury site undergo apoptosis after nerve crush, and the distal part of the nerve undergoes Wallerian degeneration. SCs present in the distal site become dedifferentiated, while a majority of those in the proximal region maintain their original differentiation status. Our data indicated that only distal SCs undergoing dedifferentiation expressed a large amount of the c-met protein.

High level induction of HGF on the injury site and of the c-met protein in distal SCs appear to be two independently occurring events as treatment with a c-met inhibitor reduced the amount of phosphorylated c-met in distal SCs, while having no effect on the total amount of c-met and mouse HGF protein. If HGF produced from the injury site had effects on the level of total c-met expression in distal SCs, c-met expression would have been decreased by this inhibitor interacting with the surface receptor. AP-1, Pax3, and NF-kB are the major candidates responsible for the induction of c-met expression in distal SCs. These transcription factors are well

known to be highly expressed in dedifferentiated SCs, and they act as transcription factors driving gene expression from the c-met promoter, together with Pax7, and Ets-1. Further studies are needed to pinpoint the main driving force(s) of c-met transcription in distal SCs.

Among the signaling molecules activated by HGF treatment, ERK pathway seems to play a major role in HGF-mediated nerve regeneration. The use of U0126, an ERK inhibitor, effectively suppressed HGF-mediated induction of Egr1, c-Fos, GDNF, and Lif as well as SC migration. The role(s) of AKT signaling is puzzling as treatment with AKTi increased the expression levels of those 4 genes involved in nerve regeneration, while inhibiting cell proliferation with no effect on cell migration. A bulk of literatures showed that expression of neurotrophic factors and cell migration are important in the nerve regeneration process, while SC proliferation plays no or minor role in nerve repair5,7,36-41. Taking several lines of data into consideration, it seems to be the ERK pathway that plays important roles in HGF-mediated nerve regeneration through SCs.

HGF overexpression around the injury site by i.m. injection of the HGF-expressing plasmid facilitated the re-myelination process, confirming HGF as an important player in Schwann-mediated nerve regeneration. Interestingly, we founded that HGF overexpression promoted the re-myelination and induced myelin thickness regardless the injection time point of pCK-HGF-X7.In the case of PNS injury or disease model, there are very few cases in which re-myelination was convincingly demonstrated by transfer of neurotrophic genes. To our knowledge, there are two cases showing the effects on both axonal regeneration and re-myelination; one was the case of using NT-3 by

AAV142,43 and the other was the case of using GDNF by adenovirus44.We showed that overexpression of HGF improved myelin thickness in the injured nerve, to an extent that the g-ratio of some nerves became similar to that of the control groups. On the other hand, results from phase I and II studies, involving the HGF-expressing plasmid used in this report, in patients with diabetic peripheral neuropathy, indicated that pCK-HGF-X7 was safe and furthermore effectively reduced the pain level with a sign of recovery of the sensory function as measured by monofilament testing. Taken together, HGF may be used as the platform or a starting material in developing therapeutics that can provide fundamental treatment methods for a variety of neurological diseases including peripheral neuropathy.

Appendix

Appendix

Data in this section indicated that HGF effects observed in this chapter may not be the result of the formation of blood vessel (neuro-vascularization) around the injury site or of the suppression of inflammation. Though interesting, these results are "negative" in that the possible involvement is excluded, requiring more rigorous proof procedure according to the tradition. Therefore, these data are shown as Appendix.

1. Effects of HGF protein on neuro-vascularization

It was well established that blood vessel is highly formed in injury site after nerve injury and that SCs migrate along the vessel to across the injury site. VEGF-α, an angiogenic growth factor secreted by macrophage around injury site, has been indentified to play major in such neuro-vascular formation (Cattin et al., 2015). Since HGF is also highly induced in injury site and one of the well-known angiogenic factors, we tested whether inhibition of c-met by PHA-665752 or HGF overexpression by pCK-HGF-X7 have effect on neuro-vascularization at injury site. IHC was performed for sciatic nerve at 5 d.p.i by using antibodies to CD31 for vessels and S100β for SCs. As shown Figure 42, blood vessels were highly induced in injury site, while treatment with PHA-665752 or pCK-HGF-X7 did not alter neuro-vascularization. These data indicated that HGF might not play important role in neuro-vascularization.

Effects of HGF protein on neuro-vascularlization

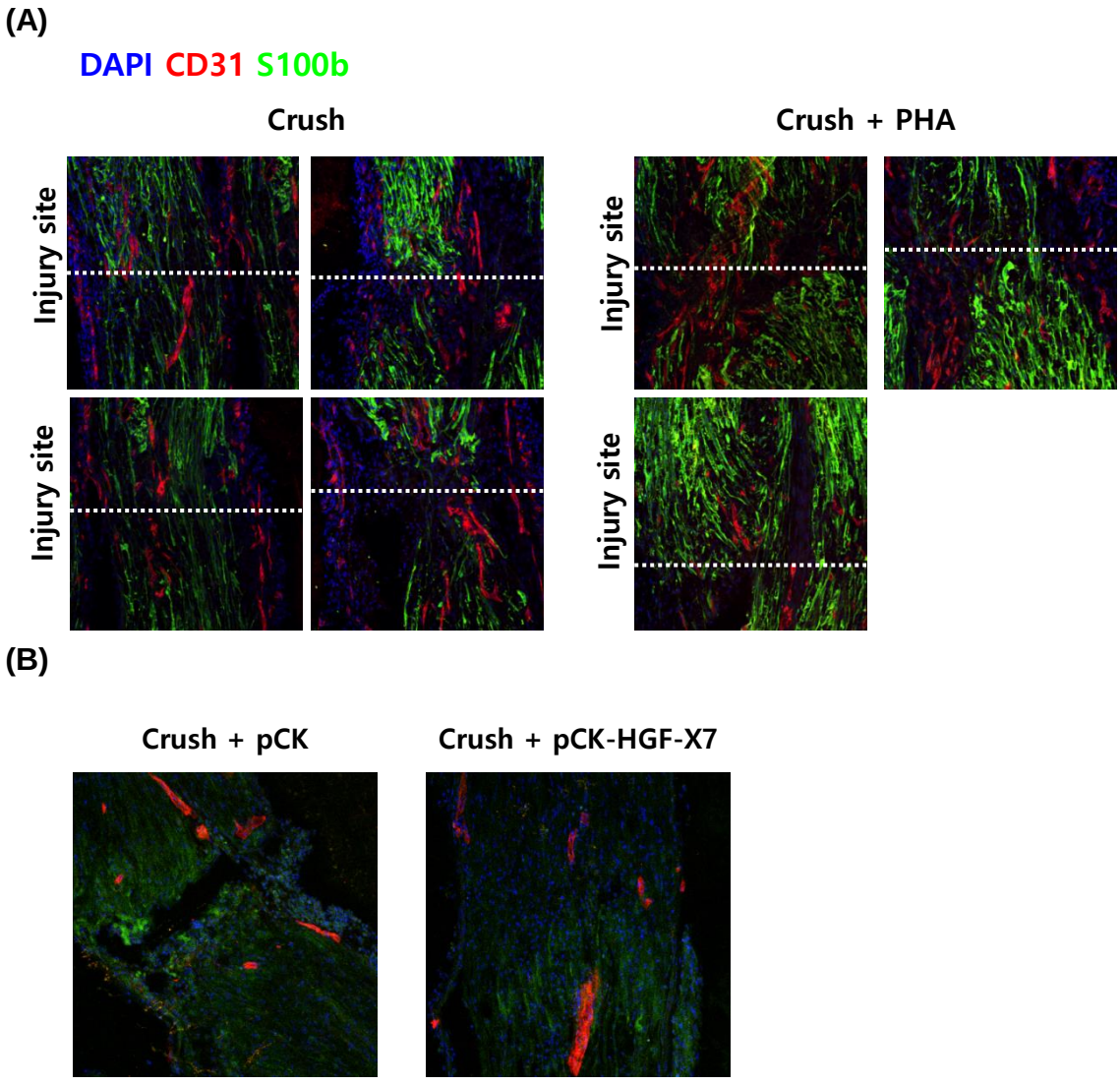


Figure 42. Effects of HGF on neuro-vascularization.

(A) Injured mice were intraperitoneally injected with 20mg/kg of PHA-665752 daily and prepared at 5 d.p.i followed by IHC staining. (B) Effects of pCK-HGF-X7 on neuro-vascularization. Injured mice were i.m injected with pCK or pCK-HGF-X7 and prepared at 5 d.p.i followed by IHC staining. The injury sites of sciatic nerves were stained with antibodies to S100b (SCs marker) and CD31 (Vessel marker). Nuclei were counterstained with Hoechst (blue). n = 3 for each group. Scale bar = 20μm

2. Effects of HGF protein on inflammation after nerve injury.

During the regeneration process, macrophages recruited to injured sciatic nerve play an important role in inflammation, myelin clearance, axonal regrowth or neuro vascularization. Some cytokines secreted by SCs are stimulate macrophages to move to injured nerve, while macrophages with c-met receptor, it was tested whether treatment with PHA-665752 could alter macrophage activation or infiltration by using qRT-PCR and IHC staining. As shown in figure 43A, PHA-665752 treatment decreased the induction of TNF-α, MCP-1 and LIF, which are known to be mainly produced by SCs during the very early stage of injury and facilitate macrophage recruitment to the injury site. However, the signal intensity of CD68, a marker for activated macrophage, showed no difference between crush-DMSO and crush-PHA groups (Figure 43B), indicating that macrophage activation and recruitment might not be affected by PHA-665752 treatment.

To test whether overexpression of HGF protein have effects on inflammatory responses, total RNA were prepared form injured sciatic nerves at 4 d.p.i followed by quantitative RT-PCR. The expression of inflammatory or anti-inflammatory factors, including IL-10, MMP9, TNF-α and CXCR4, were measured. As shown in Figure 33, the RNA level of these four genes was not changed by i.m injection of pCK-HGF-X7, indicating that HGF effects might not have resulted from the control of inflammation (Figure 44)

Effects of HGF protein on inflammation after nerve injury.

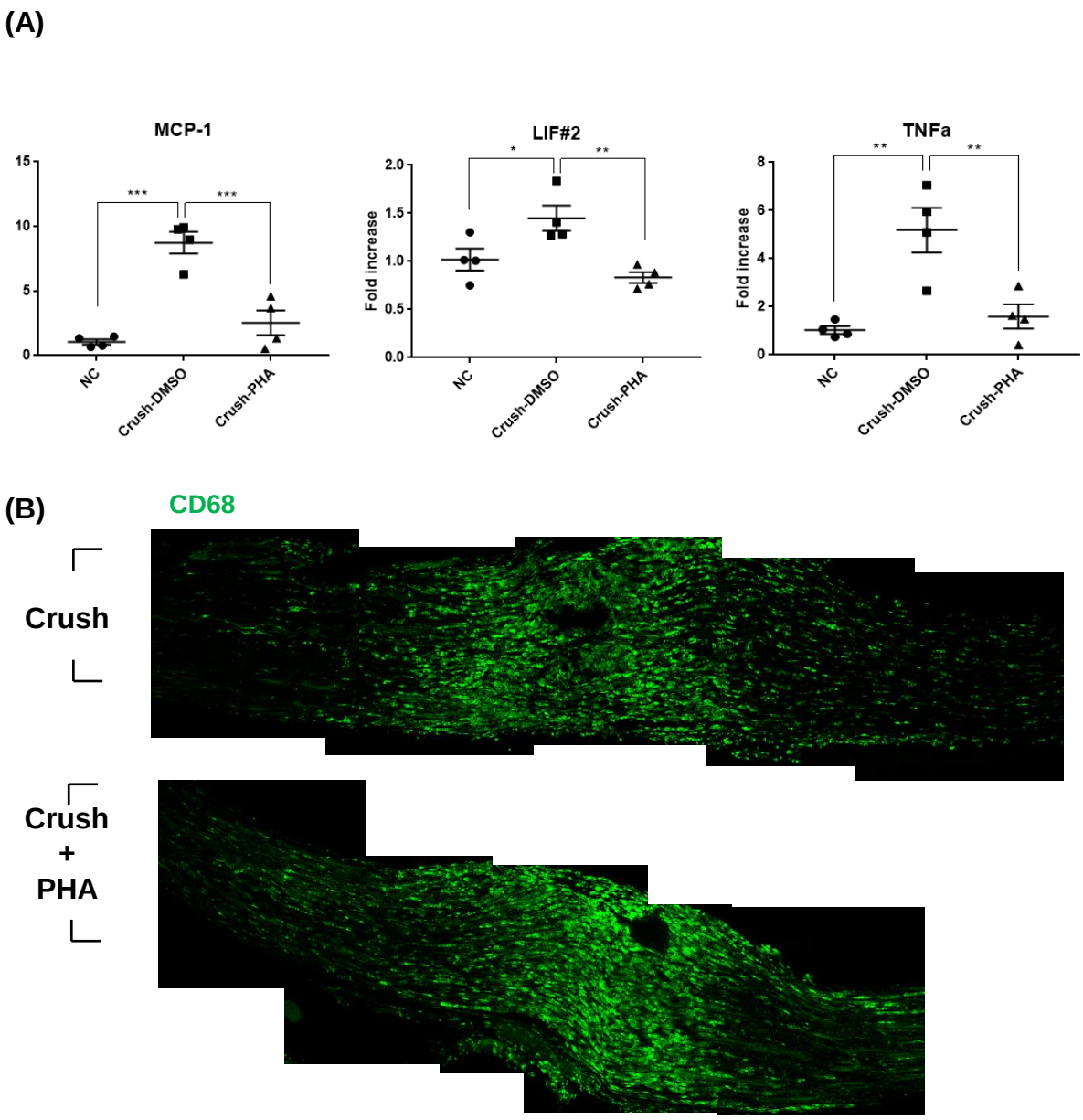


Figure 43. Effects of PHA-665752 on Inflammation

(A) Injured mice were intraperitoneally injected with 20mg/kg of PHA-665752 and t otal RNA were prepared at 1 d.p.i followed by quantitative RT-PCR. n = 4 for each group. *p<0.05, **p<0.01, ***p<0.001.In all these experiments, values represent th e mean ± S.E.M. (B) Effects of PHA-665752 on macrophage infiltration.

To test whether PHA-665752 block macrophage infiltration during nerve repair process, IHC analysis was performed at 5 d.p.i by using antibody CD68 (green), a marker for active macrophage. n = 3 for each group.

Effects of HGF protein on inflammation after nerve injury.

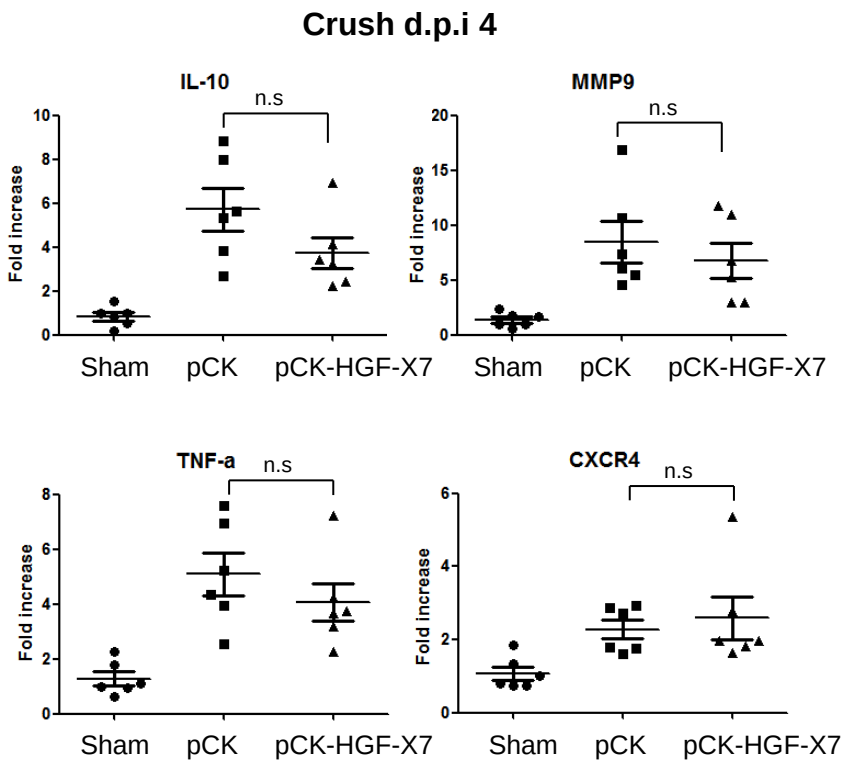


Figure 44. Effects of pCK-HGF-X7 on the expression of inflammatory genes. Injured mice were i.m injected with 200 μg of pCK-HGF-X7 and total RNA were prepared at 4 d.p.i followed by quantitative RT-PCR. n = 6 for each group. n.s = not-significant. In all these experiments, values represent the mean ± S.E.M.

Chapter 4

Chapter 4. HGF-induced responses in SCs were mediated by ERK/CREB/c-Fos pathway.

1. Background

c-Fos is a transcription factor (TF) that belongs to the AP-1 (activator protein-1) family, and is known to function by forming a heterodimer with another AP-1 sub-family member, such as Jun (c-Jun, JunB, and JunD), Maf (c-Maf, MafB, MafA, MafG/F/K and NRL), or ATF (ATF2, ATF3, B-ATF, JDP1 and JDP2)(Ozanne et al., 2007; Shaulian and Karin, 2002). It has been reported that when the nerve gets damaged, c-Fos expression is up-regulated in distal Schwann cells (SCs) and involved in myelin clearance (Liu et al., 1995; Pyykönen and Koistinaho, 1991). However, the exact role(s) of c-Fos protein in SCs and how its gene expression is regulated after nerve injury remain poorly understood.

AP-1 plays important roles in pain generation, re-myelination, and axon outgrowth, while c-Fos is one of two important molecules in forming AP-1. HGF has been shown to enhance the AP-1 activity by increasing the expression level and also phosphorylation status of various AP-1 family members in numerous cell types such as endothelial cells, skeletal muscle cells, and keratinocytes. We previously observed that HGF expression was highly induced after nerve injury and that it plays an important role in the re-myelination of SCs [1]. Although c-Fos is a significant component of AP-1 heterodimer, the role or importance of c-Fos in SCs has not been well understood.

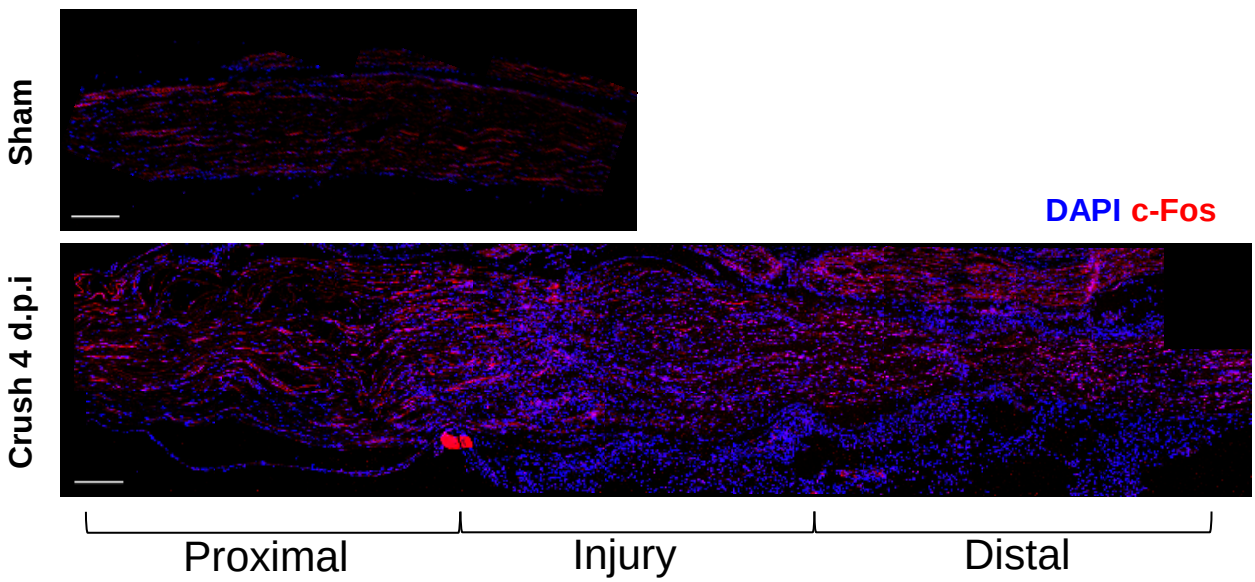


Figure 45. The c-Fos expression in injured sciatic nerves.

To define the expression pattern of c-Fos in injured sciatic nerves, IHC analysis was performed at 4 d.p.i by using antibody to c-Fos (red). Nuclei were counterstained with Hoechst (blue).n = 3 for each group.

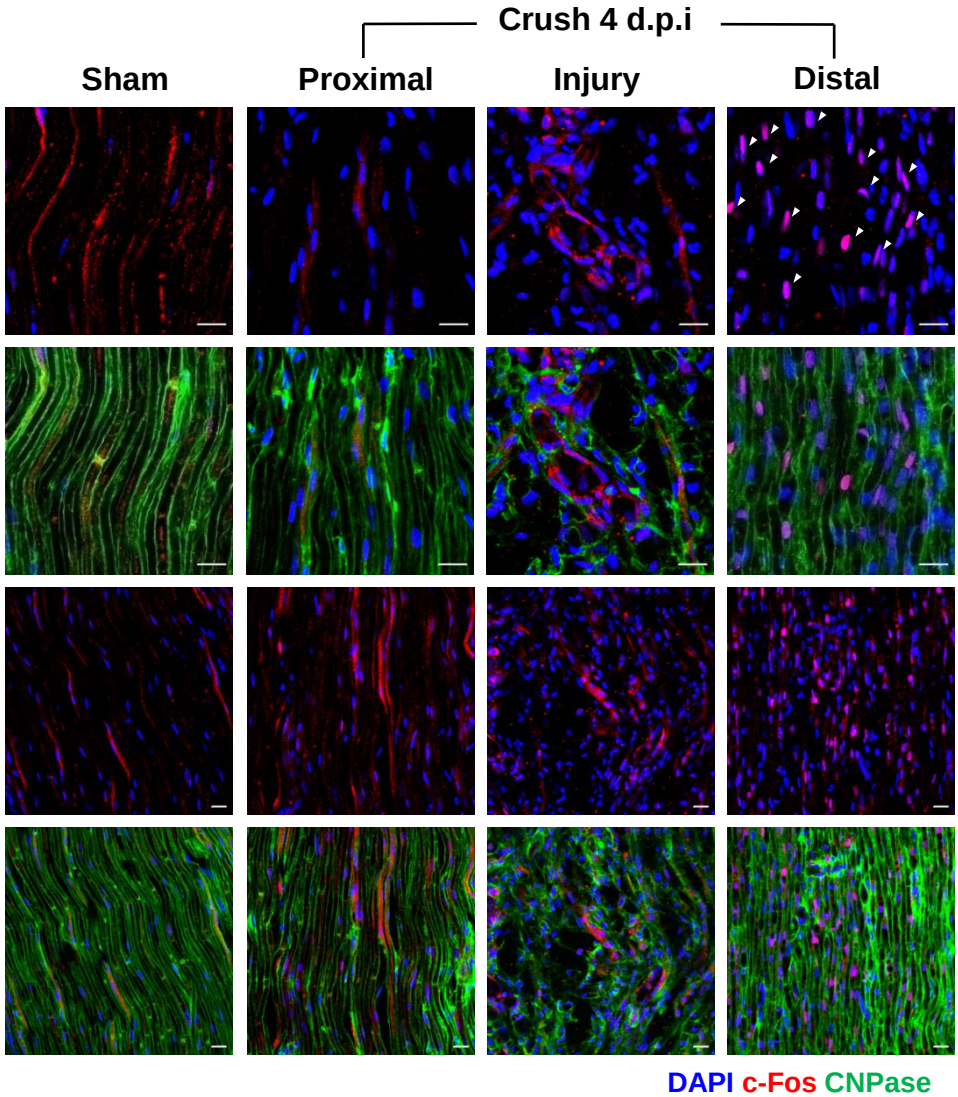


Figure 46. The c-Fos expression in three regions of injured sciatic nerves.

To define the expression pattern of c-Fos in injured sciatic nerves, IHC analysis was performed at 4 d.p.i by using antibodies to CNPase (green) for SCs and c-Fos (red). Nuclei were counterstained with Hoechst (blue). n = 3 for each group.

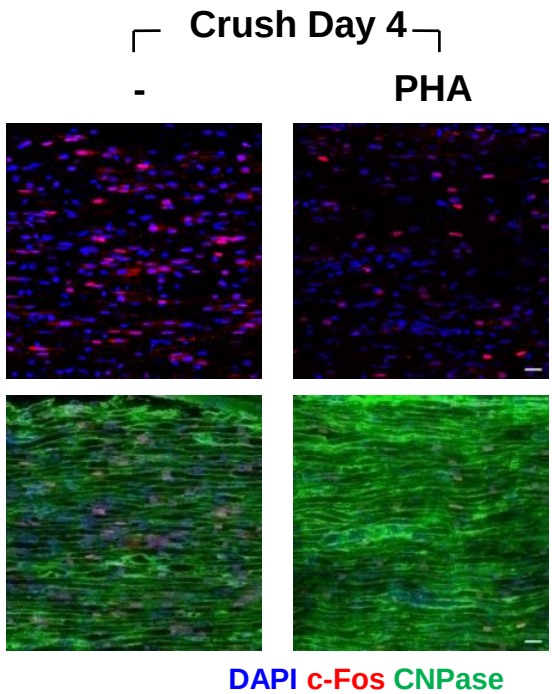


Figure 47. Effects of c-met inhibitor (PHA-665752) on injury-induced c-Fos expression.

To test whether HGF regulates c-Fos expression after nerve injury, mice were daily i.p injected with 20 mg/kg of PHA-665752 and analyzed by IHC analysis by using antibodies to CNPase (green) and c-Fos (red). Nuclei were counterstained with Hoechst (blue). n = 3 for each group.

2. Results

2.1 HGF regulates c-Fos expression in injured sciatic nerves.

We previously reported that HGF expression was highly induced after nerve injury and play important roles in the re-myelination of SCs, while we and others showed that c-Fos is one of many genes whose RNA levels are increased following nerve damage [1, 12, 13]. To confirm this observation, the protein level of c-Fos was measured after nerve injury. After crush injury was introduced to mice, the ipsilateral sciatic nerves were isolated at day 4 followed by IHC staining. As shown in Figure 45, the signal of c-Fos protein was highly increased in injured sciatic nerves. When antibodies specific for c-Fos protein and CNPase, a Schwann cell marker, were used, the trans-localization of c-Fos to nucleus was observed mainly in distal SCs, but not in the cells around the proximal and injury sites (Figure 46). When mice were intraperitoneally injected on a daily basis, with 20 mg/kg of PHA-665752, a specific inhibitor for c-met receptor, the intensity of c-Fos signal and the number of c-Fos positive nucleus in distal sites were highly reduced (Figure 47).

To confirm the involvement of HGF/c-met pathway in c-Fos expression, we used the sciatic nerve explant culture system which has been shown to faithfully mimic peripheral nerve injury (Figure 48). Following sciatic nerve transection, the sciatic nerve was isolated at day 5, and cultured for 3 days in DMEM containing 10% FBS and 1mM of PHA-665752. As shown in Figure 1D, PHA-665752 reduced the level of phosphorylated c-met (Figure 48, lanes 1 and 3). When the sciatic nerve was injured, the expression level of c-Fos was highly increased (Figure 48, lanes 1 and 3). However, inhibition of

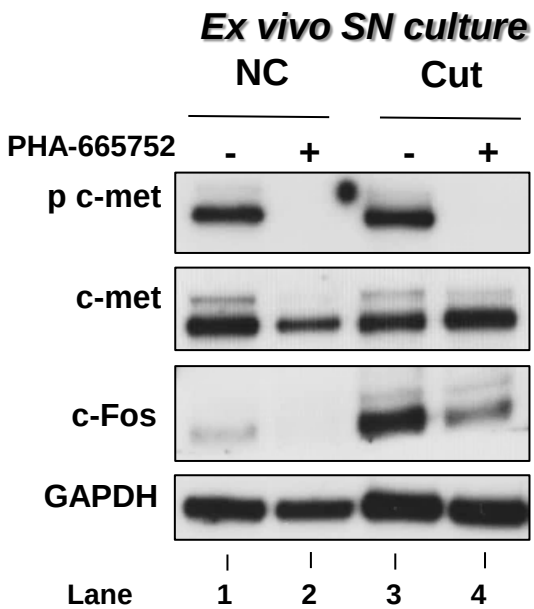


Figure 48. Effects of c-met inhibitor (PHA-665752) on transection-induced c-Fos expression.

Following the nerve transection, injured sciatic nerves were prepared at day 5 and culture in the DMEM medium in the presence of 1mM PHA-665752 for 3 days and analyzed by Western blot analysis. n = 3 for each group.

c-met by PHA-665752 decreased the protein level of c-Fos in both control and injured groups (Figure 48, compare lanes 1and 2; lanes 3 and 4), indicating that HGF/c-met indeed controls the expression of c-Fos and in both normal and injury situations. Taken together, these data indicated that c-Fos expression was up-regulated at both RNA and protein levels after nerve injury and that HGF/c-met axis is involved in the induction of c-Fos

2.2 HGF regulates c-Fos expression in SCs.

To understand the relationship between HGF and c-Fos at the molecular level, we used primary SCs isolated from adult rats as previously reported (Kaewkhaw et al., 2012b). First, the time kinetics of HGF-induced c-Fos expression was studied. Primary SCs were treated with 25ng/ml of recombinant human HGF (hHGF) protein, and total RNAs or proteins were prepared at appropriate times followed by quantitative RT-PCR or Western blot hybridization analyses, respectively. As shown in Figure 49A, the RNA level of c-Fos was highly increased by HGF treatment at the 3hr time point, and then decreased to the basal level at 24hr.The expression kinetics of the c-Fos protein was a bit faster, its level reaching the peak at 30 min and then returning to the basal point at 3hr (Figure 49B). Taken together, these data showed that HGF tightly regulated the expression of c-Fos at both RNA and protein levels.

To understand the relationship between HGF and c-Jun, usually formed heterodimer with c-Fos, primary SCs were treated with 25ng/ml of recombinant human HGF (hHGF) protein, and total RNAs or proteins were prepared at appropriate times followed by

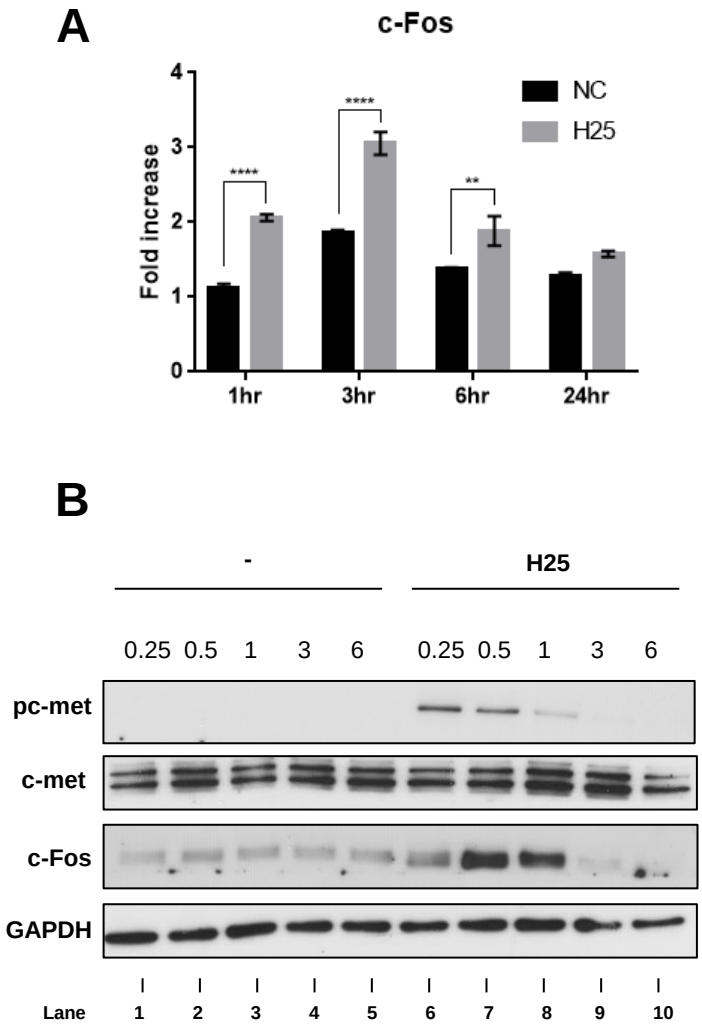


Figure 49. HGF treatment highly increased c-Fos expression in SCs. Primary SCs isolated from rat sciatic nerves were treated with 25 ng/ml of recombinant human HGF protein. Total RNA and protein were prepared at appropriate time points and subjected to quantitative RT-PCR and Western blot analysis. (A) Effects of HGF on the RNA level of c-Fos (B) Effects of HGF on the protein level of c-Fos. Three independent experiments were performed and results were highly reproducible. **p<0.01, ****p<0.0001. Values represent the mean ± SEM.

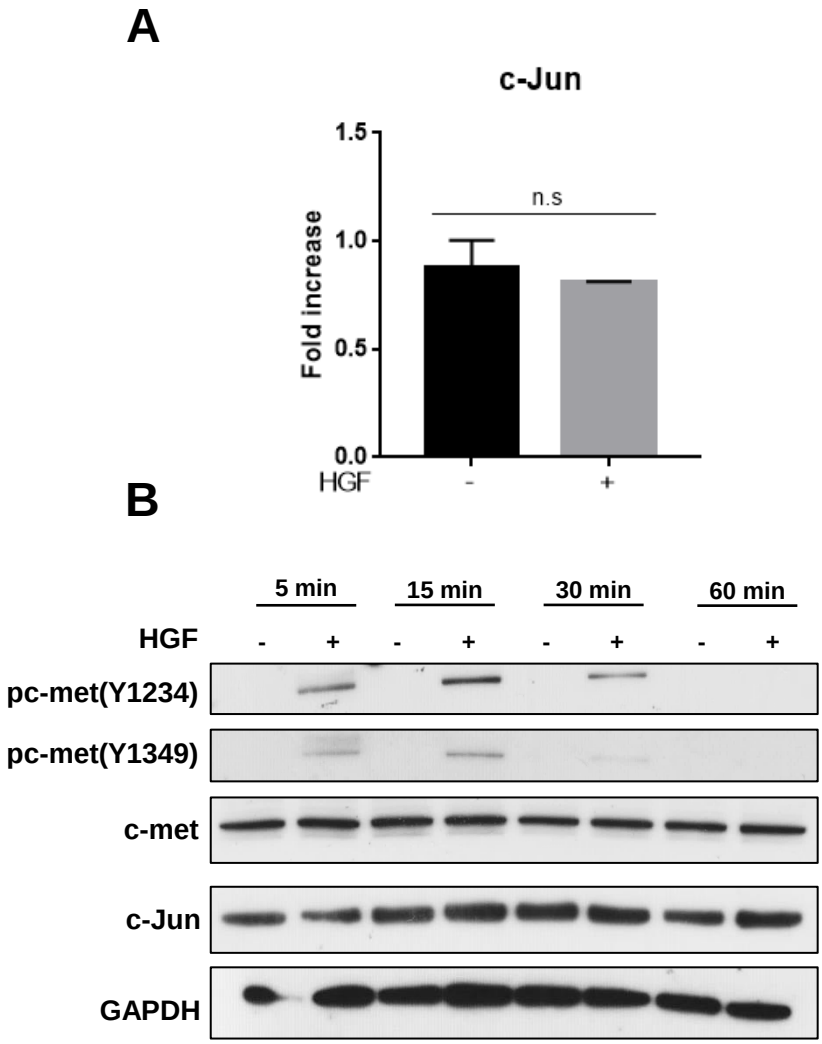


Figure 50. HGF treatment did not change c-Jun expression in SCs. Primary SCs isolated from rat sciatic nerves were treated with 25 ng/ml of recombinant human HGF protein. Total RNA and protein were prepared at appropriate time points and subjected to quantitative RT-PCR and Western blot analysis. (A) Effects of HGF on the RNA level of c-Jun (B) Effects of HGF on the protein level of c-Jun. Three independent experiments were performed and results were highly reproducible. n.s= not-significnat. Values represent the mean ± SEM.

quantitative RT-PCR or Western blot analyses. As shown in Figure 50A, the RNA level of c-Jun was not changed by HGF treatment. Consistent with RNA data, western blot data suggested that c-Jun expression was not altered by HGF protein (Figure 50B). Taken together, these data showed that HGF tightly regulated the expression of c-Fos, not c-Jun in SCs at both RNA and protein levels.

2.3 HGF induced c-Fos expression in SCs by ERK/CREB pathway

We previously reported that HGF promotes the expression of various neurotrophic genes and migration of SCs by using the ERK pathway (Ko et al., 2018). Transcription factor CREB is known to be needed for c-Fos expression and is activated through the ERK pathway. It was tested whether HGF affected c-Fos expression through the same pathway in SCs. Primary SCs were treated with hHGF and ERK inhibitor, U0126, for 5 and 60min, and then total proteins were analyzed by Western blot hybridization. As shown in Figure 3A, treatment with U0126 lowered the HGF-mediated increase of the c-Fos protein at 60 min (Figure 51A, lanes 6 and 7). It was also found that the level of phosphorylated CREB, working downstream from ERK, was highly increased by HGF treatment, which was then suppressed in the presence of U0126 (Figure 51B, lanes 2 and 3).

To test whether CREB was indeed directly involved in the HGF-mediated induction of c-Fos expression in SCs, CBPi, a specific inhibitor for CREB activity, was used. As shown in Figure 3C,

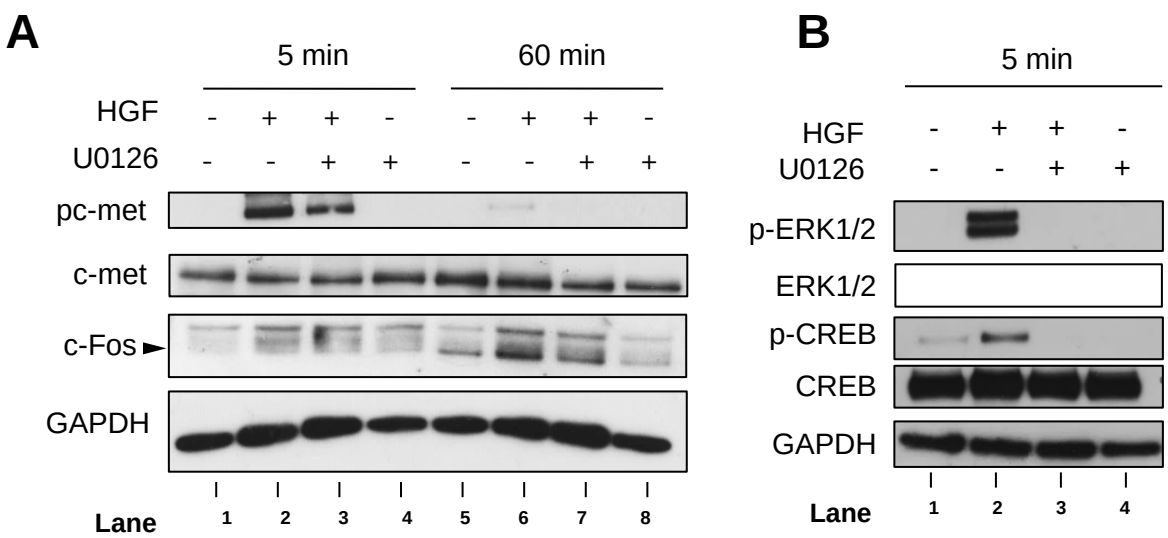


Figure 51. HGF induced c-Fos expression in SCs by ERK pathway. Primary SCs were treated with HGF (25 ng/ml) or an inhibitor of ERK (5 μM U0126). Total protein were prepared at appropriate time points and analyzed by Western blot analysis (A) Effects of U0126 on HGF-mediated induction of c-Fos expression were measured at 5 and 60 min (B) Effects of U0126 on HGF-mediated increase of phosphorylated CREB were detected at 5 min.

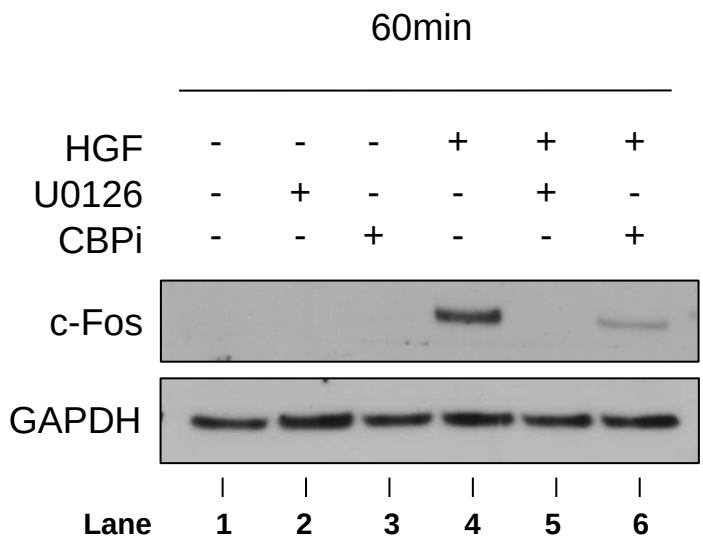


Figure 52. HGF induced c-Fos expression in SCs by ERK/CREB pathway. Primary SCs were treated with HGF (25 ng/ml) or an inhibitor of CREB (4 μM CBPi). Total protein were prepared at appropriate time points and analyzed by Western blot analysis. Effects of CBPi on HGF-mediated c-Fos expression were analyzed at 60 min.

treatment with CBPi decreased the HGF-mediated induction of c-Fos expression at 60 min, similar to the case of U0126 (Figure 52, compare lanes 4, 5 and 6).These results indicated that HGF control the c-Fos expression by ERK/CREB signaling pathway.

2.4 The knock down of c-Fos protein using specific siRNA inhibited various HGF-induced effects in SCs

To define whether c-Fos plays a key role in the HGF-mediated up-regulation of various gene expressions in SCs, siRNA specific for c-Fos was employed, while that for c-Jun was used as a control. siRNAs that effectively lowered the level of respective proteins were chosen for subsequent experiments (Figure 53). Primary SCs transfected with siRNAs for c-Fos and c-Jun were treated with 25ng/ml of hHGF protein, and total RNAs were isolated 1 hour later followed by quantitative RT-PCR for three genes, GDNF, LIF and c-Myc. As shown in Figure 54, knock-down of c-Fos protein by siRNA completely inhibited HGF-mediated induction of expression of all three genes (Figure 54). To our surprise, siRNA for c-Jun had little effects.

Since we previously observed that HGF could increase the SCs migration, the effects of c-Fos or c-Jun knock down on cell motility was also measured by Boyden chamber assay. There was no statistical difference between the control and c-Fos siRNA transfected groups, while knock down of c-Jun decreased the basal cell motility by about 80% compared to control, as previously reported (Jenkins, 2015). SCs migration was increased by about 50% by HGF treatment, compared to the control group, but this effect was almost completely

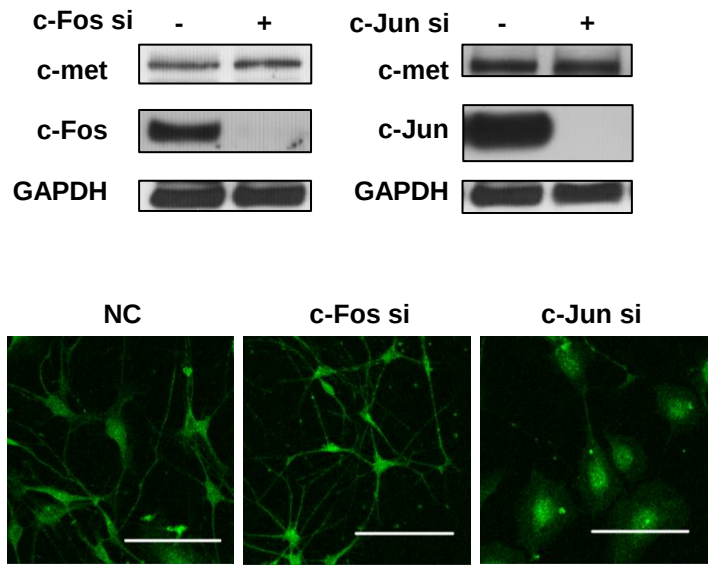


Figure 53. The knock down of c-Fos or c-Jun in primary SCs.

Knock down efficiency of c-Fos or c-Jun siRNA was measured by Western blot analysis and IFA analysis, using specific antibodies to c-Fos and c-Jun protein. Scale bar = 100μm

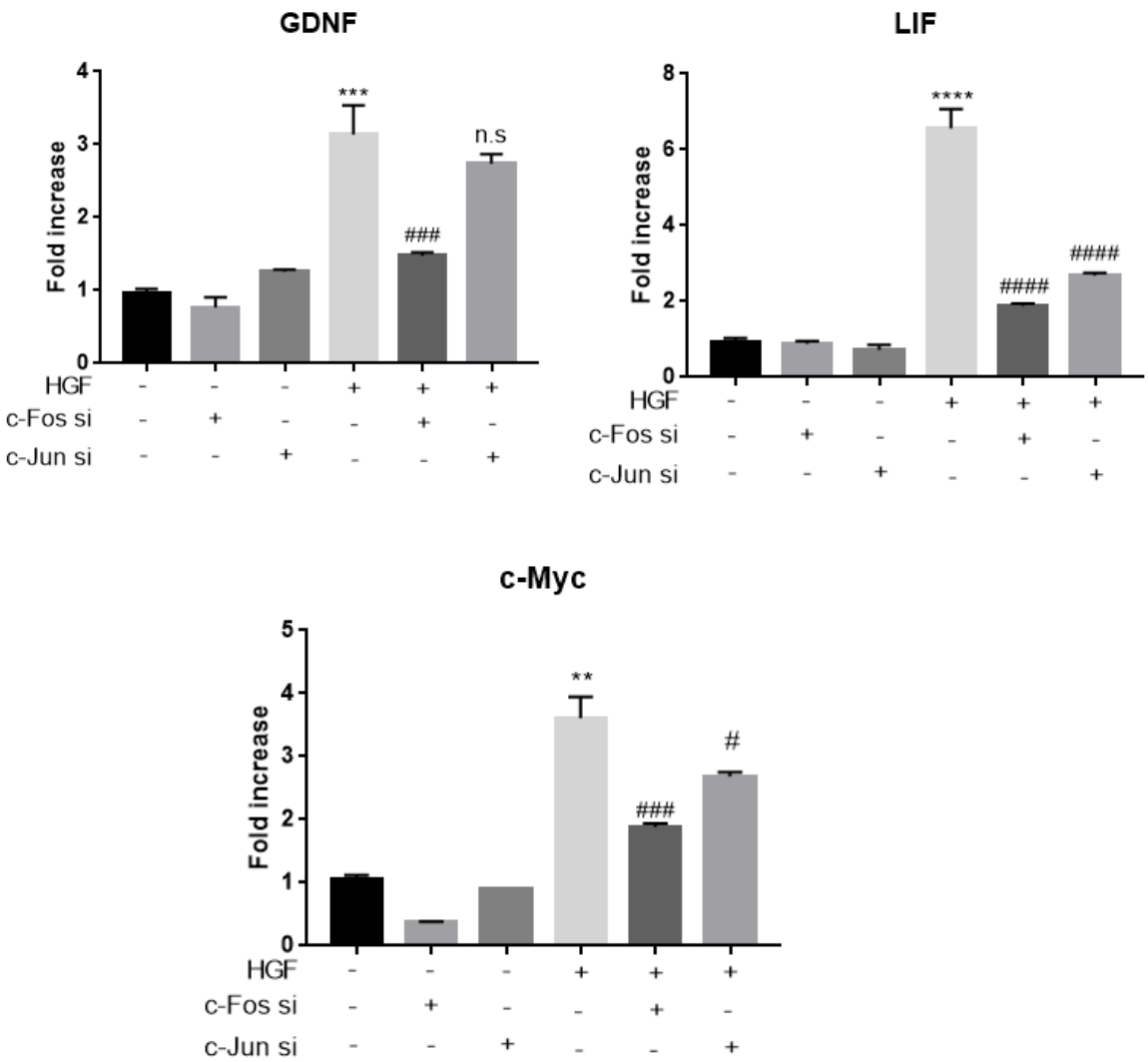


Figure 54. The knock down of c-Fos protein disrupted the HGF mediated responses in SCs. Primary SCs transfected with c-Fos or c-Jun siRNA were treated with 25ng/ml of hHGF protein. Effects of c-Fos or c-Jun knock down on gene expression were measured by quantitative RT-PCR. n.s = non-significant, *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001. Values represent the mean ± SEM.

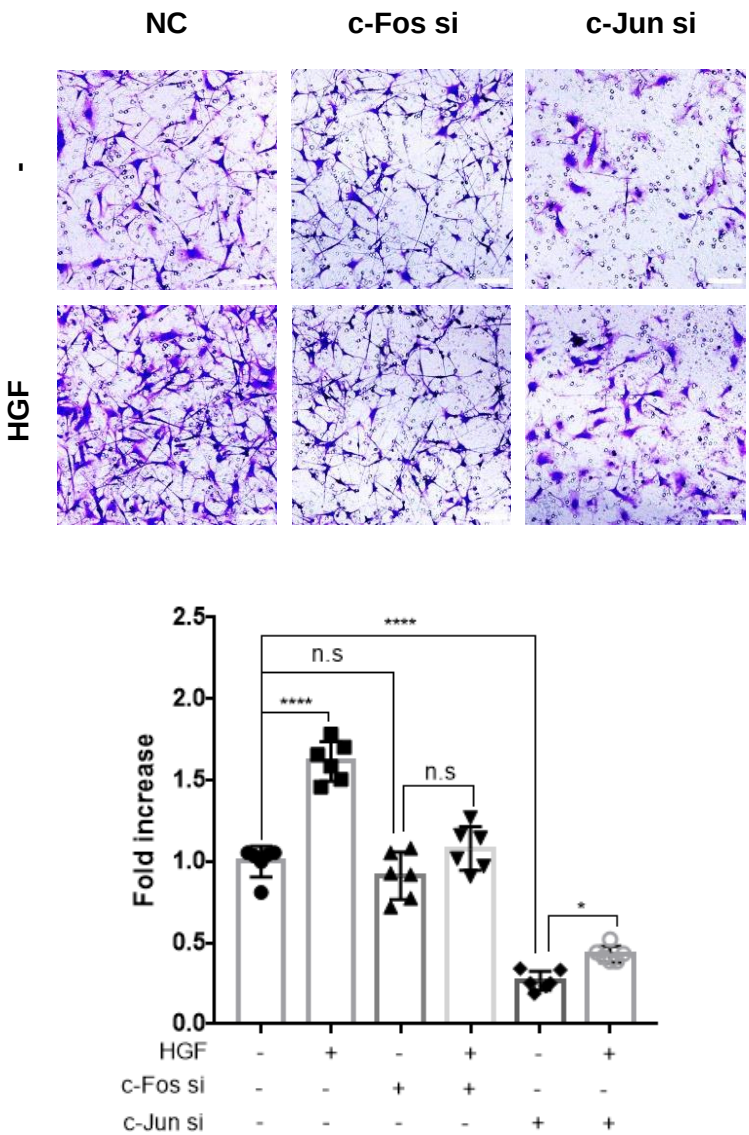


Figure 55. The knock down of c-Fos protein blocked the HGF mediated migration in SCs. Primary SCs transfected with c-Fos or c-Jun siRNA were treated with 25ng/ml of hHGF protein. Effects of c-Fos or c-Jun knock down on SCs migration were measured by boyden chamer assay. n.s = non-significant, *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001. Values represent the mean ± SEM.

blocked by c-Fos siRNA (Figure 55). Again, c-Jun siRNA did not affect cell migration induced by HGF treatment. Taken together, these results showed that c-Fos might act as a key molecule in various HGF-induced effects observed in SCs and that c-Jun might not be a counterpart of c-Fos in AP-1 heterodimer needed for HGF to function.

3. Discussion

In this study, we found that c-Fos protein acts as a key factor in various HGF-mediated effects in SCs. After nerve injury, the protein levels of c-Fos were highly increased, which was suppressed by the treatment with an inhibitor of c-met receptor, PHA-665752, indicating that HGF/c-Met signaling plays an important role. Treatment of primary SCs with recombinant HGF protein indeed highly increased the expression level of c-Fos, showing a quick and short transient induction kinetics, typical of this gene. HGF-mediated induction of c-Fos was blocked by chemical inhibitors of ERK or CREB. The importance of c-Fos in HGF-mediated effects was evident from the data showing that knock-down of c-Fos expression by specific siRNA resulted in almost complete suppression of HGF-mediated migration of SCs and an increase in the expression of GDNF, LIF and c-Myc genes.

Several interesting observations were made from our study. First, the c-Fos protein, whose level was highly increased after nerve crush in mice, was trans-localized to the nucleus of distal SCs only. We previously reported (1) that HGF, whose level was highly increased after nerve injury, was involved in re-myelination and axon

outgrowth, (2) that the high level activation of its cellular receptor c-met was seen almost exclusively in distal SCs, and (3) that HGF up-regulated the expression of GDNF, LIF, and MCP-1 in SCs which are all involved in regeneration (). Taken together, it appears that HGF may repair damaged nerves by using c-Fos as a key molecule.

Second, the role of c-Fos seems to be more important than previously thought. Unlike c-Fos, c-Jun has been analyzed in detail at various steps during nerve injury, ranging from inflammatory reactions to nerve regeneration involving re-myelination and axon outgrowth. c-Jun functions as a dimer to form AP-1, usually a heterodimer with c-Fos, pointing the obvious importance of the latter molecule. According to our literature search, however, c-Fos seems to have received very little attention, especially in the context of SCs (Liu et al., 1995; Pyykönen and Koistinaho, 1991). Our results indicated that c-Fos play an equally important role(s) during the repair of damaged nerves, at least in SCs.

Third, our results indicated that in the context of AP-1, the counter part of c-Fos may not be c-Jun, but a different member of the Jun family. In the same experiments, we used both siRNA for c-Fos and c-Jun, but it was only the former siRNA that suppressed HGF-mediated effects in SCs. Efforts to identify the counterpart of c-Fos are under way.

In summary, c-Fos appears to be an extremely important element needed for HGF to exert its effects in SCs, controlling the expression of various processes needed for nerve regeneration. Although c-Fos is one component of AP-1 heterodimer, its role has not been established as clearly as c-Jun. Given the high magnitude of induction after nerve injury and such clear effects of c-Fos knock

down in primary SCs, further studies are warranted to understand its roles in the biology of SC, especially in re-myelination and ultimately the nerve repair process.

Chapter 5

Summary and Concluding Remarks

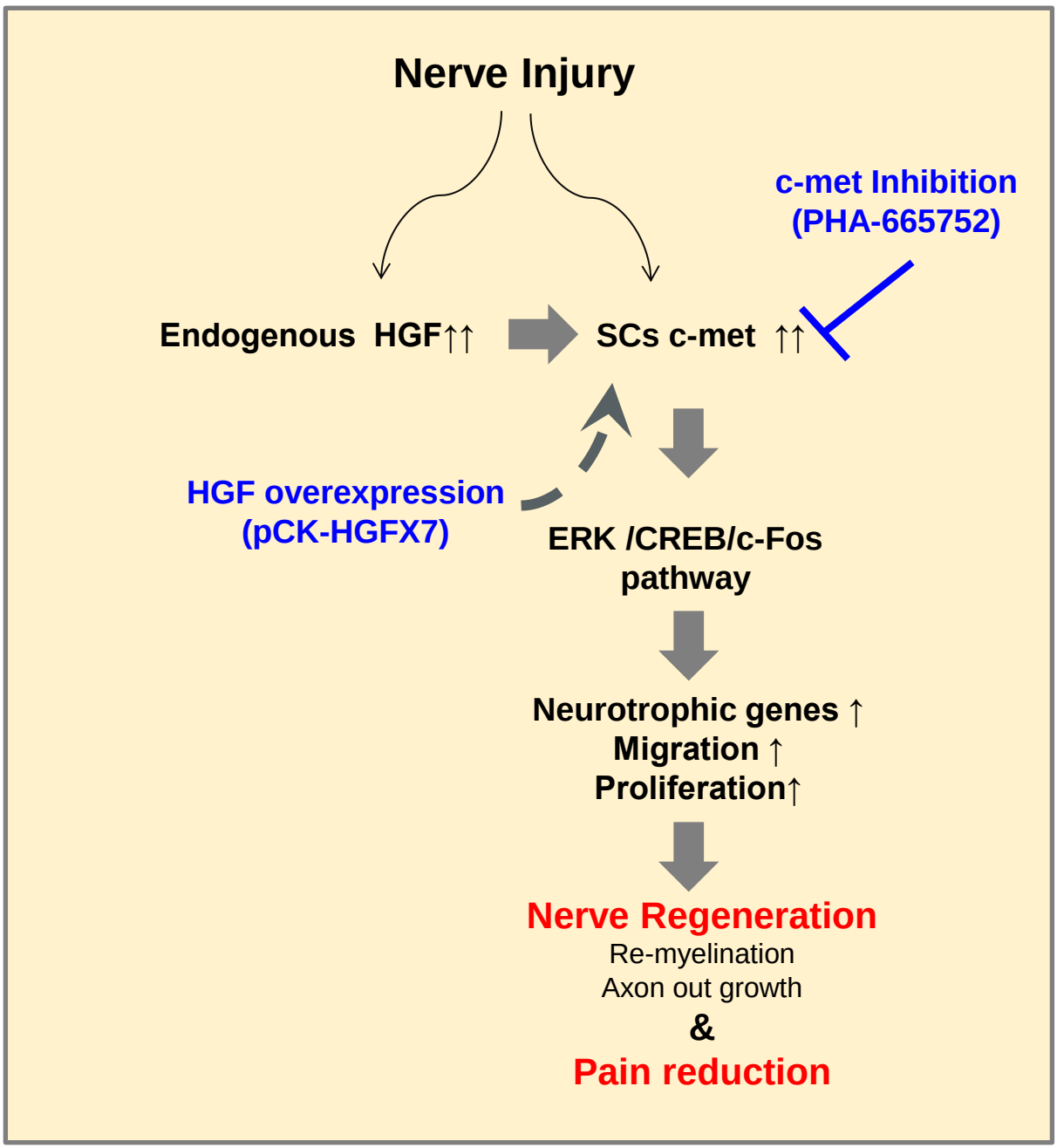


Figure 56. A schematic diagram of HGF effects in this thesis work

Discussion and Concluding Remarks

In my thesis work, I demonstrated that HGF was involved the in Schwann cell-mediated regeneration of peripheral nerves in the nerve crush model, and that the c-Fos protein acts as a key factor in various HGF-mediated effects in SCs. HGF expression was highly induced in the injured and distal areas while the expression of both total and phosphorylated c-met receptors was increased almost exclusively in SCs at distal sites. When mice were treated with the c-met inhibitor PHA-665752, re-myelination of the injured nerve was suppressed as evidenced by a decrease in myelin thickness determined by TEM analysis, indicating that HGF might play a role in nerve regeneration. Treatment of primary SCs with the HGF protein enhanced the migration and proliferation of SCs, and increased the expression level of neurotrophic factors such as GDNF and LIF involved in nerve regeneration after injury. As evidenced by data from experiments involving specific chemical inhibitors, ERK or AKT signaling pathways seem to play important roles. Finally, overexpression of HGF around the nerve injury site by i.m injection of the HGF expression plasmid DNA pCK-HGF-X7 increased the myelin thickness and axon diameter.

Another interesting finding from my thesis work was that c-Fos plays a role equally or possibly even more important than c-Jun. After nerve injury, the protein levels of c-Fos were highly increased, then suppressed by treatment with the c-met receptor inhibitor PHA-665752, indicating that HGF/c-Met signaling plays an important role. Treatment of primary SCs with recombinant HGF protein did indeed highly increase the expression level of c-Fos, showing a quick and short transient induction kinetics, typical of this

gene. The HGF-mediated induction of c-Fos was blocked by chemical inhibitors of ERK or CREB. The importance of c-Fos in HGF-mediated effects was evident from the data showing that the knockdown of c-Fos expression by specific siRNA resulted in the almost complete suppression of the HGF-mediated migration of SCs and an increase in the expression of GDNF, LIF, and c-Myc genes. Taken together, our data suggest that HGF and c-met play important roles in peripheral nerve regeneration by activating SCs.

One of the most interesting observations made in this study is that c-met expression is highly and selectively induced in the distal SCs at the injury site. It is well established that after nerve crush damage, most cells around the injury site undergo apoptosis, and the distal part of the nerve undergoes Wallerian degeneration. SCs present in the distal site become dedifferentiated, while a majority of those in the proximal region maintain their original differentiation status. These data indicated that only distal SCs undergoing dedifferentiation expressed a large amount of the c-met protein.

High-level induction of HGF at the injury site and of the c-met protein in distal SCs appear to be two independently occurring events, as treatment with a c-met inhibitor reduced the amount of phosphorylated c-met in distal SCs while having no effect on the total amount of c-met and mouse HGF protein. If HGF produced from the injury site affected the level of total c-met expression in distal SCs, c-met expression would have been decreased by this inhibitor interacting with the surface receptor. AP-1, Pax3, and NF-kB are the major candidates responsible for the induction of c-met expression in distal SCs. These transcription factors are well

known to be highly expressed in dedifferentiated SCs, and are key transcription factors driving gene expression from the c-met promoter together with Pax7 and Ets-1. Further studies are needed to pinpoint the main driving force(s) of c-met transcription in distal SCs.

The HGF protein was observed mainly around the injured site, but we have not yet identified the exact producer cell type(s). In naïve mice, the basal level of HGF was detected at 200 pg/mg of the total protein in the sciatic nerve. Data from IHC indicated that cells present in the epineurium might be one of the major sites of HGF production after nerve injury as well as in normal, undamaged nerves. Fibroblasts, already known to produce HGF in a variety of organs, are a candidate for a major HGF producer cell type in our case, as they are present around the epineural membrane of the control nerve and are recruited to the injury site. SCs themselves do not seem to produce HGF during injury as the level of HGF expression in primary SCs did not change when treated with a variety of stimulants including IL1β, hypoxia induced by CoCl2, and HGF itself. Efforts to identify the producer cell type(s) are currently underway.

Among the signaling molecules activated by HGF treatment, the ERK pathway seems to play a major role in HGF-mediated nerve regeneration. The use of U0126, an ERK inhibitor, effectively suppressed the HGF-mediated induction of Egr1, c-Fos, GDNF, and Lif, as well as SC migration. The role(s) of AKT signaling is puzzling, as treatment with AKTi increased the expression levels of those 4 genes involved in nerve regeneration, while inhibiting cell proliferation had no effect on cell migration. A bulk of literature shows that expression of neurotrophic factors and cell migration are

important in the nerve regeneration process while SC proliferation plays little to no role in nerve repair. Taking several lines of data into consideration, it seems to be the ERK pathway that plays an important role in HGF-mediated nerve regeneration through SCs.

My work on c-Fos yielded several interesting observations. First, the c-Fos protein, whose level was highly increased after nerve crush in mice, was trans-localized to the nuclei of distal SCs only. We previously reported that (1) HGF, whose level was highly increased after nerve injury, was involved in re-myelination and axon outgrowth, (2) the high level activation of its cellular receptor c-met was seen almost exclusively in distal SCs, and (3) HGF up-regulated the expression of GDNF, LIF, and MCP-1 in SCs that are all involved in regeneration (Chen et al., 2010; Fine et al., 2002; Wood et al., 2012). Taken together, it appears that HGF may repair damaged nerves by using c-Fos as a key molecule. Second, the role of c-Fos seems to be more important than previously thought. Unlike c-Fos, c-Jun has been analyzed in detail at various steps during nerve injury, ranging from inflammatory reactions to nerve regeneration involving re-myelination and axon outgrowth. C-Jun functions as a dimer to form AP-1, usually a heterodimer with c-Fos, pointing to the obvious importance of the latter molecule. According to our literature search, however, c-Fos seems to have received very little attention, especially in the context of SCs (Liu et al., 1995; Pyykönen and Koistinaho, 1991). Our results indicate that c-Fos plays an equally important role in the repair of damaged nerves, at least in SCs. Third, our results indicate that in the context of AP-1, the counterpart of c-Fos may not be c-Jun, but a different member of the Jun family. We used both siRNA for c-Fos and c-Jun in the same experiments, but it was only the former siRNA that suppressed

HGF-mediated effects in SCs. Efforts to identify the counterpart of c-Fos are underway. In summary, c-Fos appears to be an extremely important element needed for HGF to exert its effects in SCs, controlling the expression of various processes needed for nerve regeneration. Although c-Fos is one component of the AP-1 heterodimer, its role has not been established as clearly as c-Jun's. Given the high magnitude of induction after nerve injury and such clear effects of the c-Fos knockdown in primary SCs, further studies are warranted to understand its roles in the biology of SCs, especially in re-myelination and ultimately the nerve repair process.

Data from experiments involving the i.m injection of pCK-HGF-X7 have extremely important implications in the context of novel therapeutics for neuropathic pain and a variety of diseases associated with nerve damage. HGF overexpression around the injury site by i.m. injection of the HGF-expressing plasmid facilitated the re-myelination process, confirming HGF as an important player in Schwann-mediated nerve regeneration. In the case of PNS injury or disease models, there are very few cases in which re-myelination was convincingly demonstrated by the transfer of neurotrophic genes. To my knowledge, there are two cases showing the effects on both axonal regeneration and re-myelination; one was the case of using NT-3 by AAV1, and the other was the case of using GDNF by adenovirus. We showed that the overexpression of HGF improved myelin thickness in the injured nerve to the extent that the g-ratio of some nerves came close to that of the control groups. On the other hand, results from phase I and II studies involving the HGF-expressing plasmid used in this report indicated that pCK-HGF-X7 was safe for patients with diabetic peripheral neuropathy, and that it furthermore effectively reduced pain levels

with signs of recovery of sensory functions as measured by monofilament testing. In conclusion, my thesis work produced not only a scientifically compelling discoveries, but also clinically valuable insights. HGF may be used as the platform or a starting material in developing therapeutics that can provide fundamental treatment methods for a variety of neurological diseases, including peripheral neuropathy.

REFERENCES

Ahn, J., Jang, J., Choi, J., Lee, J., Oh, S.-H., Lee, J., Yoon, K., and Kim, S. (2014). GSK3β, but not GSK3α, inhibits the neuronal differentiation of neural progenitor cells as a downstream target of mammalian target of rapamycin complex1. Stem cells and development *23*, 1121-1133.

Bauder, A.R., and Ferguson, T.A. (2012). Reproducible mouse sciatic nerve crush and subsequent assessment of regeneration by whole mount muscle analysis. JoVE (Journal of Visualized Experiments), e3606-e3606.

Carlsson, M., Osman, N.F., Ursell, P.C., Martin, A.J., and Saeed, M. (2008). Quantitative MR measurements of regional and global left ventricular function and strain after intramyocardial transfer of VM202 into infarcted swine myocardium. American Journal of Physiology-Heart and Circulatory Physiology *295*, H522-H532.

Carmeliet, P., and Storkebaum, E. (2002). Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. Paper presented at: Seminars in cell & developmental biology (Elsevier).

Cattin, A.-L., Burden, J.J., Van Emmenis, L., Mackenzie, F.E., Hoving, J.J., Calavia, N.G., Guo, Y., McLaughlin, M., Rosenberg, L.H., and Quereda, V. (2015). Macrophage-induced blood vessels guide Schwann cell-mediated regeneration of peripheral nerves. Cell *162*, 1127-1139.

Chao, M.V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. Nature Reviews Neuroscience *4*, 299.

Chen, J., Chu, Y., Chen, J., and Li, B. (2010). Synergistic effects of NGF, CNTF and GDNF on functional recovery following sciatic nerve injury in rats. Advances in medical sciences *55*, 32-42.

Dumpich, M., and Theiss, C. (2015). VEGF in the nervous system: an important target for research in neurodevelopmental and regenerative medicine. Neural regeneration research *10*, 1725.

Ebens, A., Brose, K., Leonardo, E.D., Hanson Jr, M.G., Bladt, F., Birchmeier, C., Barres, B.A., and Tessier-Lavigne, M. (1996). Hepatocyte growth factor/scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. Neuron *17*, 1157-1172.

Fine, E.G., Decosterd, I., Papaloïzos, M., Zurn, A.D., and Aebischer, P. (2002). GDNF and NGF released by synthetic guidance channels support sciatic nerve regeneration across a long gap. European Journal of Neuroscience *15*, 589-601.

Fu, S.Y., and Gordon, T. (1997). The cellular and molecular basis of peripheral nerve regeneration. Molecular neurobiology *14*, 67-116.

Grinsell, D., and Keating, C. (2014). Peripheral nerve reconstruction after injury: a review of clinical and experimental therapies. BioMed research international *2014*.

Gu, Y., Zhang, J., Guo, L., Cui, S., Li, X., Ding, D., Kim, J.M., Ho, S.H., Hahn, W., and Kim, S. (2011). A phase I clinical study of naked DNA expressing two isoforms of hepatocyte growth factor to treat patients with critical limb ischemia. The journal of gene medicine *13*, 602-610.

Guaiquil, V.H., Pan, Z., Karagianni, N., Fukuoka, S., Alegre, G., and Rosenblatt, M.I. (2014). VEGF-B selectively regenerates injured peripheral neurons and restores sensory and trophic functions. Proceedings of the National Academy of Sciences *111*, 17272-17277.

Hahn, W., Pyun, W.B., Kim, D.S., Yoo, W.S., Lee, S.D., Won, J.H., Shin, G.J., Kim, J.M., and Kim, S. (2011). Enhanced cardioprotective effects by coexpression of two isoforms of hepatocyte growth factor

from naked plasmid DNA in a rat ischemic heart disease model. The journal of gene medicine *13*, 549-555.
Hobson, M.I., Green, C.J., and Terenghi, G. (2000). VEGF enhances intraneural angiogenesis and improves nerve regeneration after axotomy. Journal of anatomy *197*, 591-605.
Jenkins, B.E. (2015). The role of c-Jun in Schwann cell morphology and migration (UCL (University College London)).
Kaewkhaw, R., Scutt, A.M., and Haycock, J.W. (2012a). Integrated culture and purification of rat Schwann cells from freshly isolated adult tissue. Nature protocols *7*, 1996.
Kaewkhaw, R., Scutt, A.M., and Haycock, J.W. (2012b). Integrated culture and purification of rat Schwann cells from freshly isolated adult tissue. Nature protocols *7*, 1996-2004.
Karin, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. Journal of Biological Chemistry *270*, 16483-16486.
Kessler, J.A., Smith, A.G., Cha, B.S., Choi, S.H., Wymer, J., Shaibani, A., Ajroud Driss, S., and Vinik, A. (2015). Double blind, placebo controlled study of HGF gene therapy in diabetic neuropathy. Annals of clinical and translational neurology *2*, 465-478.
Ko, K.R., Lee, J., Lee, D., Nho, B., and Kim, S. (2018). Hepatocyte Growth Factor (HGF) Promotes Peripheral Nerve Regeneration by Activating Repair Schwann Cells. Scientific reports *8*, 8316.
Lindsay, R.M. (1988). Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. Journal of Neuroscience *8*, 2394-2405.
Liu, H.M., Yang, L.H., and Yang, Y.J. (1995). Schwann cell properties: 3. C-fos expression, bFGF production, phagocytosis and proliferation during Wallerian degeneration. Journal of Neuropathology &

Experimental Neurology *54*, 487-496.
Maina, F., and Klein, R. (1999). Hepatocyte growth factor, a versatile signal for developing neurons. Nature neuroscience *2*, 213-217.
Nakamura, T., and Mizuno, S. (2010). The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. Proceedings of the Japan Academy, Series B *86*, 588-610.
Nho, B., Lee, J., Lee, J., Ko, K.R., Lee, S.J., and Kim, S. (2018). Effective control of neuropathic pain by transient expression of hepatocyte growth factor in a mouse chronic constriction injury model. The FASEB Journal, fj. 201800476R.
Organ, S.L., and Tsao, M.-S. (2011). An overview of the c-MET signaling pathway. Therapeutic advances in medical oncology *3*, S7-S19.
Ozanne, B., Spence, H., McGarry, L., and Hennigan, R. (2007). Transcription factors control invasion: AP-1 the first among equals. Oncogene *26*, 1.
Pereira Lopes, F., Lisboa, B., Frattini, F., Almeida, F., Tomaz, M., Matsumoto, P., Langone, F., Lora, S., Melo, P., and Borojevic, R. (2011). Enhancement of sciatic nerve regeneration after vascular endothelial growth factor (VEGF) gene therapy. Neuropathology and applied neurobiology *37*, 600-612.
Pyun, W., Hahn, W., Kim, D., Yoo, W., Lee, S., Won, J., Rho, B., Park, Z., Kim, J., and Kim, S. (2010). Naked DNA expressing two isoforms of hepatocyte growth factor induces collateral artery augmentation in a rabbit model of limb ischemia. Gene therapy *17*, 1442-1452.
Pyykönen, I., and Koistinaho, J. (1991). c-fos protein like immunoreactivity in non-neuronal cells of rat peripheral nerve after

transection. Acta neuropathologica *82*, 66-71.
Rosenstein, J.M., and Krum, J.M. (2004). New roles for VEGF in nervous tissue—beyond blood vessels. Experimental neurology *187*, 246-253.
Sebben, A.D., Lichtenfels, M., and Silva, J.L.B.d. (2011). Peripheral nerve regeneration: cell therapy and neurotrophic factors. Revista brasileira de ortopedia *46*, 643-649.
Shaulian, E., and Karin, M. (2001). AP-1 in cell proliferation and survival. Oncogene *20*, 2390.
Shaulian, E., and Karin, M. (2002). AP-1 as a regulator of cell life and death. Nature cell biology *4*, E131.
Shin, J.E., Geisler, S., and DiAntonio, A. (2014). Dynamic regulation of SCG10 in regenerating axons after injury. Experimental neurology *252*, 1-11.
Shin, J.E., Miller, B.R., Babetto, E., Cho, Y., Sasaki, Y., Qayum, S., Russler, E.V., Cavalli, V., Milbrandt, J., and DiAntonio, A. (2012). SCG10 is a JNK target in the axonal degeneration pathway. Proceedings of the National Academy of Sciences *109*, E3696-E3705.
Shin, Y.K., Jang, S.Y., Yun, S.H., Choi, Y.Y., Yoon, B.A., Jo, Y.R., Park, S.Y., Pak, M.G., Park, J.I., and Park, H.T. (2017). Cooperative interaction of hepatocyte growth factor and neuregulin regulates Schwann cell migration and proliferation through Grb2 associated binder 2 in peripheral nerve repair. Glia.
Terenghi, G. (1999). Peripheral nerve regeneration and neurotrophic factors. Journal of anatomy *194*, 1-14.
Whitmarsh, A., and Davis, R. (1996). Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. Journal of molecular medicine *74*, 589-607.
Wisdom, R. (1999). AP-1: one switch for many signals. Experimental

cell research *253*, 180-185.
Wong, V., Glass, D.J., Arriaga, R., Yancopoulos, G.D., Lindsay, R.M., and Conn, G. (1997). Hepatocyte growth factor promotes motor neuron survival and synergizes with ciliary neurotrophic factor. Journal of Biological Chemistry *272*, 5187-5191.
Wood, M.D., Kim, H., Bilbily, A., Kemp, S.W., Lafontaine, C., Gordon, T., Shoichet, M.S., and Borschel, G.H. (2012). GDNF released from microspheres enhances nerve regeneration after delayed repair. Muscle & nerve *46*, 122-124.
Zhang, J.Y., Luo, X.G., Xian, C.J., Liu, Z.H., and Zhou, X.F. (2000). Endogenous BDNF is required for myelination and regeneration of injured sciatic nerve in rodents. European Journal of Neuroscience *12*, 4171-4180.

국문 초록

간세포 성장인자가 슈반세포를 통해 말초 신경 재생에 미치는 영향 연구

고 경 량

자연과학대학 생명과학부

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신경 손상 이후 말초 신경이 재생되는 과정에는 매우 다양한 신경 영양인자들이 주요하게 관여한다는 사실은 잘 알려져 있다. 본 박사 학위 과정 동안, 본 연구자는 간세포 성장인자와 그 수용체인 c-met 이 신경재생 과정에서 어떤 역할을 수행하는지를 주요하게 연구하였다. 생쥐의 좌골신경에 신경 손상을 가하였을 때, 간세포 성장인자의 발현이 신경 손상을 가한 부위 특이적으로 증가하는데 반해 그 수용체인 c-met 의 발현 및 활성은 손상 부위 아래쪽 원위부위에 위치해있는 슈반세포 특이적으로 증가하는 것을 관찰하였다. 이때 c-met의 활성을 저해한다고 알려져 있는 케미컬 억제제인 PHA-665752를 생쥐에 투여하였을 때, 신경 손상 이후 전반적인 회복과정-엑손 재성장 및 재수초화- 가 저해되는 현상을 확인하였다. 렛트로부터 분리한 프라이머리 슈반세포에 간세포성장인자를 처리하였을 때, 간세포 성장인자가 ERK 시그널링 경로를 통해 슈반세포에서 GDNF, LIF 와 같은 신경영양인자의 발현이 증가시키며, 슈반세포의 이동을 촉진시키는 현상을 관찰하였다. 또한 간세포 성장인자를 발현할 수 있는 플라스미드 벡터 시스템을 통해 생쥐에서 간세포성장인자를 과발현시킨 결과, 전반적인 엑손의 재생 및 재수초화 과정이 촉진되는 현상을 관찰하였다. 이러한 결과는 신경 손상이 발현이 증가한 간세포 성장인자 및 c-met 이 슈반세포를 통해 말초 신경 재생과정에 주요하게 관여할 수 있다는 것을 의미한다.

위와 같은 연구를 통해 본 연구자는 간세포 성장인자가 슈반세포에서 c-Fos의 발현을 유의미하게 올리는 것 관찰하였으며, 그 다음으로 간세포 성장인자와 c-Fos의 상관관계를 밝히는 연구를 수행하였다. 생쥐에 신경 손상을 가하면 원위부위에 존재하는 슈반세포 특이적으로 c-Fos의 발현이 증가하며, PHA-665752를 복강 주사하여 c-met의 활성을 저해하면 증가하였던 c-Fos 의 발현이 저해되는 현상을 관찰하였다. 또한 프라이머리 슈반세포를 이용하여 간세포 성장인자가 ERK/CREB 시그널링 경로를 통해 슈반세포에서 c-Fos의 발현을 증가시키며, 이때 siRNA를 이용하여 c-Fos의 발현을 저해하면 간세포 성장인자에 의해 일어나는 전반적인 변화 - 신경영양인자 발현 및 세포 이동- 이 저해되는 현상을 관찰하였다. 이러한 현상은 간세포성장인자가 c-Fos를 통해 슈반세포의 활성을 조절할 수 있다는 것을 의미한다.

본 연구자는 박사 학위 과정을 통해 간세포 성장인자가 말초 신경 재생과정에 주요하게 관여하며, 특히 ERK/CREB/c-Fos 시그널링 경로를 통해 슈반세포에서 분비되는 다양한 신경영양인자 (GDNF, LIF 등)의 발현을 증가시킬 뿐만 아니라 세포의 이동을 촉진함으로써 신경재생에 관여할 수 있다는 것을 증명하였다. 이러한 결과들은 간세포 성장인자가 신경 손상성 질환에 대한 새로운 치료제로써의 가능성을 제시하였다는 점에서 의의가 있다.

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