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Effective Control of Neuropathic Pain by Hepatocyte Growth Factor Transiently Expressed from Naked Plasmid DNA

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노 보 람

Abstract

Neuropathic pain is caused by pathological insults within the somatosensory nervous system, including peripheral fibers and central neurons. Neuropathic pain is associated with abnormal perception of pain including allodynia and hyperalgesia. Many people worldwide suffer from this devastating diseases, yet it still remains to be a great burden to the society and patients for its pathologic mechanisms are poorly understood. There are few current treatment options for neuropathic pain with limited efficacy and significant adverse effects. Therefore, there is a great need in the development of new investigational drug for the treatment of neuropathic pain.

In an effort to develop an effective treatment method for painful neuropathy, I have explored the possibility of using plasmid expression human hepatocyte growth factor (HGF). HGF is a multifunctional protein containing angiogenic and neurotrophic properties. In this study, I investigated the analgesic effects of HGF using a plasmid vector engineered to co-express two isoforms of human HGF, pCK-HGF-X7 (or VM202), in a chronic constriction nerve injury (CCI)-induced mouse neuropathic pain model. Intramuscular injection (*i.m.*) of pCK-HGF-X7 into proximal thigh muscle induced expression of HGF in the muscle, and sciatic nerve. This gene transfer procedure significantly attenuated mechanical allodynia and thermal hyperalgesia in mouse CCI model. A single *i.m.* injection of pCK-HGF-X7 was able to reduce neuropathic pain for up to 8 weeks. Furthermore, the injury-induced expression of activating transcription factor (ATF3), calcium channel subunit α2δ1, and serotonin reuptake transporter (5-HTT) was significantly suppressed. These results suggest that pCK-HGF-X7 effectively

attenuates CCI-induced neuropathic pain, and inhibits the expression of pain-related genes such as ATF3, α2δ1, and 5-HTT.

Complex neuroinflammatory responses within the peripheral and central nervous system is another key mechanism that are involved in the development and maintenance of neuropathic pain. Inflammatory mediators including cytokines and chemokines are released to promote undesirable sensitization of nociceptors generating pain symptoms. Here, the role of HGF in the regulation of inflammatory responses were investigated. It was shown that pCK-HGF-X7 successfully reduces the expression of CCI-induced IL-6 and CSF1 in the ipsilateral DRG neurons. In addition, the decreased CSF1 expression in the ipsilateral DRG neurons by pCK-HGF-X7 treatment was accompanied with a noticeable suppression of the nerve injury-induced glial cell activation in the spinal cord dorsal horn. Further investigation using primary sensory neurons and schwann cells revealed that HGF suppresses LPS-induced production of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α. Taken together, these results suggest that HGF downregulates the expression of pain-causing inflammatory cytokines, and thereby effectively ameliorating neuropathic pain.

In this thesis work, I have identified the anti-nociceptive effect of pCK-HGF-X7 in CCI-induced neuropathic pain model, and that it downregulates injury/pain-causing factors within the peripheral and central nervous system. This is the first study to clearly prove the possibility of using pCK-HGF-X7 as a therapeutic agent for neuropathic pain in animal models. The findings from this thesis may provide insights into the development of innovative therapeutic agent for neuropathic pain, and also the potential use of gene therapy as tool for treating certain diseases.

Key words: HGF, neuropathic pain, DRG, microglia

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Abbreviations

ATF3, activating transcription factor 3

α2δ1, calcium channel subunit α2δ1

AP-1, activating protein 1

CCI, chronic constriction injury;

CNS, central nervous system

c-Met, tyrosine-protein kinase Met or hepatocyte growth factor receptor

CREB, cAMP response element-binding protein

CSF1, colony stimulating factor 1

CSF1R, colony stimulating factor 1 receptor

CTSS, cathepsin S

CX3CR1, CX3C chemokine receptor 1

DAP12, DNAX activation protein of 12 kDa

DPN, diabetic peripheral neuropathy

DRG, dorsal root ganglion

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

ELISA, enzyme-linked immunosorbent assay

GAL, galanin

GFAP, glial fibrillary acidic protein

GSK3β, Glycogen synthase kinase 3 beta

HGF, hepatocyte growth factor

HGFR, hepatocyte growth factor receptor

5-HTT, serotonin transporter

IASP, international association for the study of pain

Iba1, ionized calcium-binding adapter molecule 1

IHC, immunohistochemistry

IL-1β, interleukin 1β

IL-6, interleukin 6

I.m, intramuscular

IRF5, interferon regulatory factor 5

IRF8, interferon regulatory factor 8

LPS, lipopolysaccharide

NF-κB, nuclear factor kappa B

NSAID, nonsteroidal anti-inflammatory drug

NPY, neuropeptide Y

PHA665752, c-Met inhibitor

qPCR, quantitative PCR

PBS, phosphate buffered saline

PDPN, painful diabetic peripheral neuropathy

PNS, peripheral nervous system

SC, schwann cell

SN, sciatic nerve

STZ, streptozotozin

TUJ1, neuron-specific class III beta-tubulin

TNF-α, tumor necrosis factor α

TRP, transient receptor potential

Chapter I

Introduction

1. Pain

1.1 Neuropathic Pain

The ability of sensory nervous system to respond to harmful stimuli have been conserved across human evolution due to its potentially beneficial effects on survival. Pain is an alert signal that comes to an attention after actual or potential tissue damage, and is a part of essential sensory function that protects our body from harmful situations. However, an abnormal functioning of these sensory neurons may lead to complex pathologies that results in chronic pain conditions. Pain syndromes can be divided into nociceptive, inflammatory, dysfunctional, and neuropathic pain, which all of them are considered a disease per se for it usually exerts deleterious impact on quality of life (**Fig. I-1**). Pain is the primary reason why 40% of US population seek medical care (1), and the annual cost spent on chronic pain medications are amounted approximately $150 billion USD (2) .

Nociceptive pain is sensing of the pain through high-threshold myelinated Aδ and unmyelinated C sensory neurons that transduces pain signaling pathway to the central nervous system (CNS). Sensory nerve fibers consist of large myelinated, small myelinated, and unmyelinated nerve fibers, and the unmyelinated nerve fiber is mostly known as “pain” fibers. They express nociceptors such as transient receptor potential (TRP) channels that are responsible for the detection of noxious stimuli that are transferred as nerve impulses which the brain interprets as “pain” (3). Unmyelinated “pain” fibers react to high intensity signals caused by thermal (hear or cold), mechanical (crushing or tearing), and chemical (iodine or cytokines) stimuli, where these types of intense pain only continue to persist in the presence of the stimuli

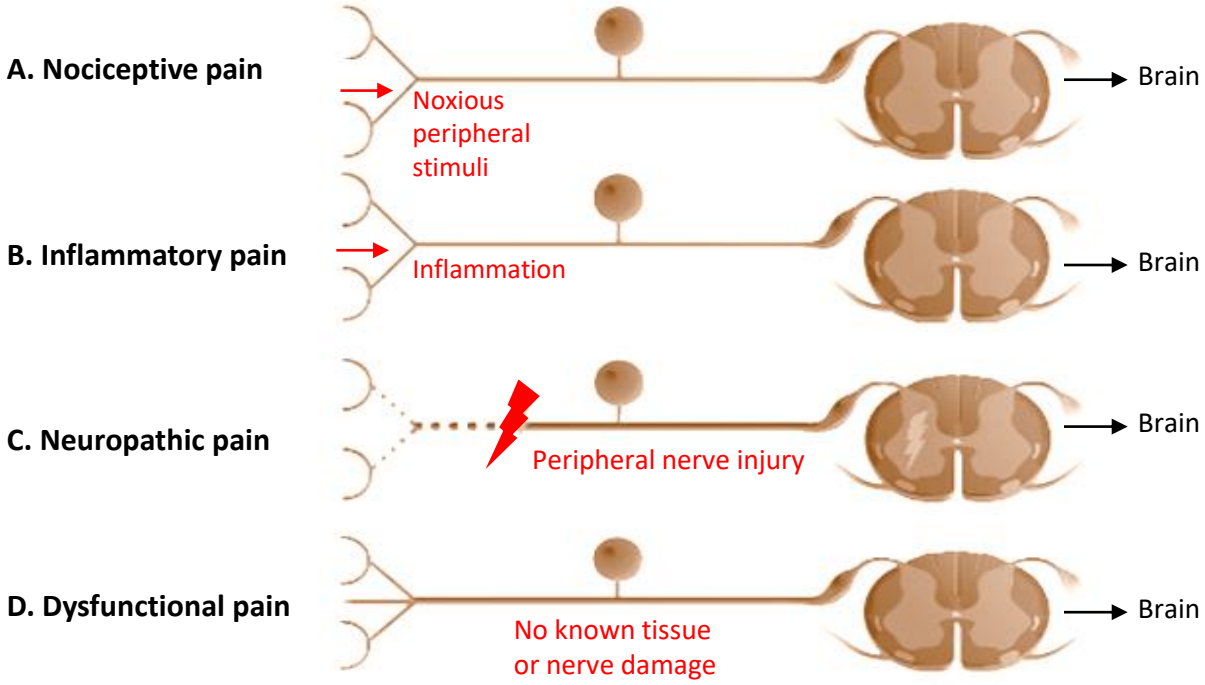


Figure I-1. Four types of pain syndromes. Four types of pain syndromes are simplified to compare the differences between causes of pain within the nervous system. (Adapted from: i) Woolf CJ. Ann interm Med 2004; 140:441-451, ii) Chong MS, Bagwa ZH. J Pain Symptom Manage. 2003;25: S4-S11, and iii) Dr. Mahmoud Abbas, Chronic pain as a disease state)

(**Fig. I-1A**). Physiological injuries or activation of pain receptors are the main cause of nociceptive pain syndrome.

Another type of pain includes inflammatory pain where it occurs in response to tissue damage. Inflammatory mediator such as arachidonic acid and prostaglandins triggers inflammation that results in peripheral nociceptive sensitization (4) (**Fig. I-1B**). The increased responsiveness is an important mechanism of protecting our body from additional injury. However, the change in the responsiveness leads to excessive amplification of nociceptive signals within the peripheral and central nervous system that are usually prolonged. Heightened sensitivity not only allows fast detection of noxious stimuli, but also is activated upon innocuous stimuli. Typical problem of inflammatory pain is that even if the injured tissues are healed, it may be developed into chronic pain disorder such as rheumatoid osteoarthritis or chronic back pain.

The most common form of pain syndrome is known to be neuropathic pain (**Fig. I-1C**). It is defined as "pain initiated or caused by a primary lesion or dysfunction in the peripheral or central nervous system" by IASP (International Association for the Study of Pain). The clinical symptoms of neuropathic pain include allodynia (pain sensation in response to non-noxious stimuli) and hyperalgesia (increased sensitivity to pain) (5, 6). Diverse pathological situations at different anatomical sites can lead to neuropathic pain. For example, central neuropathic pain arises from pathophysiological insults within the central nervous system (CNS) such as stroke, traumatic brain injury, multiple sclerosis, spinal cord injury, and HIV myelopathy (7). On the other hand, peripheral neuropathic pain results from lesions in the somatosensory system that are caused by viral infection, metabolic disturbance such as diabetes, cancer, and neurodegenerative

disorders. The persistence of neuropathic pain involves secondary changes in the entire nervous system, which remains to be a major clinical problem.

Lastly, dysfunctional pain is exaggerated and prolonged pain due to sensitized nociceptors in the absence of either inflammatory stimuli or neural lesions (6) (**Fig. I-1D**). It is unclear in most cases where the pain arises from, and how it is prolonged, but it usually accompanies exaggerated sensitivity of nociceptors in the CNS. The general features of dysfunctional pain are known to be similar to neuropathic pain, where it is characterized by reduced pain threshold and spatial diffuseness.

1.2 Nociceptive processing

Nociception is a process of brain perceiving pain provoked by noxious stimuli. The nociceptive processing is divided into four processes: i) transduction, ii) transmission, iii) modulation, and iv) perception (8) (**Fig. I-2**). Transduction is the first process that occurs in response to noxious stimuli. It is the process of converting noxious stimuli into electrical signals by peripheral nociceptors that are present on the afferent nerve endings. Transmission is the propagation of electrical signals by the first order neurons including myelinated Aδ fibers, and unmyelinated C fibers. Modulation is when primary afferent neurons make synaptic contact with secondary neurons in the dorsal horn spinal cord. Excitatory neurotransmitters are known to hypersensitize central transmission circuitry and amplify ascending signals of pain. Perception is the response of cerebral cortical neurons that are projected via tertiary neurons, which are responsible for emotional component of pain. Four phases of nociception are usually dismissed after the resolution of the initial tissue damage. However, in the case of persistent activation of

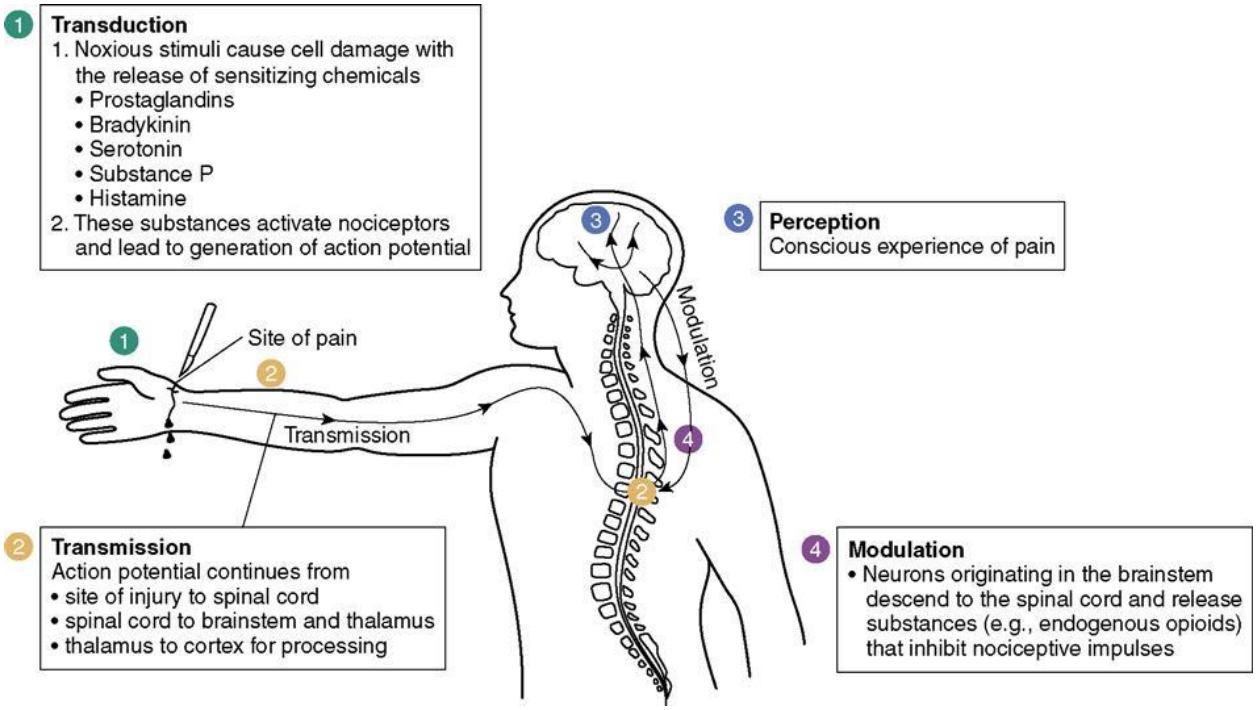


Figure I-2. Four phases of nociceptive processing. The process of nociception is divided into four phases: i) transduction, ii) transmission, iii) modulation, and iv) perception. Nociceptive transmission is usually dismissed once the initial source of peripheral insults is solved (from Lewis *et al*., 2011).

nociceptive signaling leads to intense peripheral and central sensitization that are known to be a major clinical challenge.

1.3 Peripheral sensitization

Nociceptors present on primary afferent neurons are activated upon tissue injury or inflammatory mediators that are released at the site of injury (9). Peripheral tissue damage promotes recruitment of immune cells such as mast cells, neutrophils, and platelets, which play crucial roles in the production of inflammatory mediators (10) (**Fig. I-3**). Primary afferent neurons are also involved in the production of "inflammatory soup", which include neuropeptides (bradykinin), lipids (prostaglandins), neurotransmitters (5-HT and ATP), cytokines (IL-1β and IL-6), and neurotrophins (NGF). These early-response inflammatory reactions provoke the release of vasoactive mediators including calcitonin gene-related peptide (CGRP), substance P, and nitric oxide that give rise to hyperaemia and swelling (11). The subsequent responses of inflammation enhance the invasion of circulating immune cells, where macrophages, T lymphocytes, and mast cells are recruited to the site of injury triggering Wallerian degeneration and thereby clearing of the debris generated by damaged tissues. Moreover, the chemical mediators including IL-1β, IL-6, TNF-α, LIF, and TGF-β1 facilitate the production of proteases and adhesion molecules that helps subsequent invasion of immune cells, and neurotrophic factors that orchestrates communication between neurons, peripheral glia, and immune cells (12). These chemical transducers are also responsible for amplifying sensitivity of nociceptors to noxious stimuli via interacting with transducer channels and voltage-gated ion channel on the primary sensory terminals, eventually leading to increased hyper-excited neurons with reduced activation threshold. Because of the complex mechanisms behind neuropathic pain

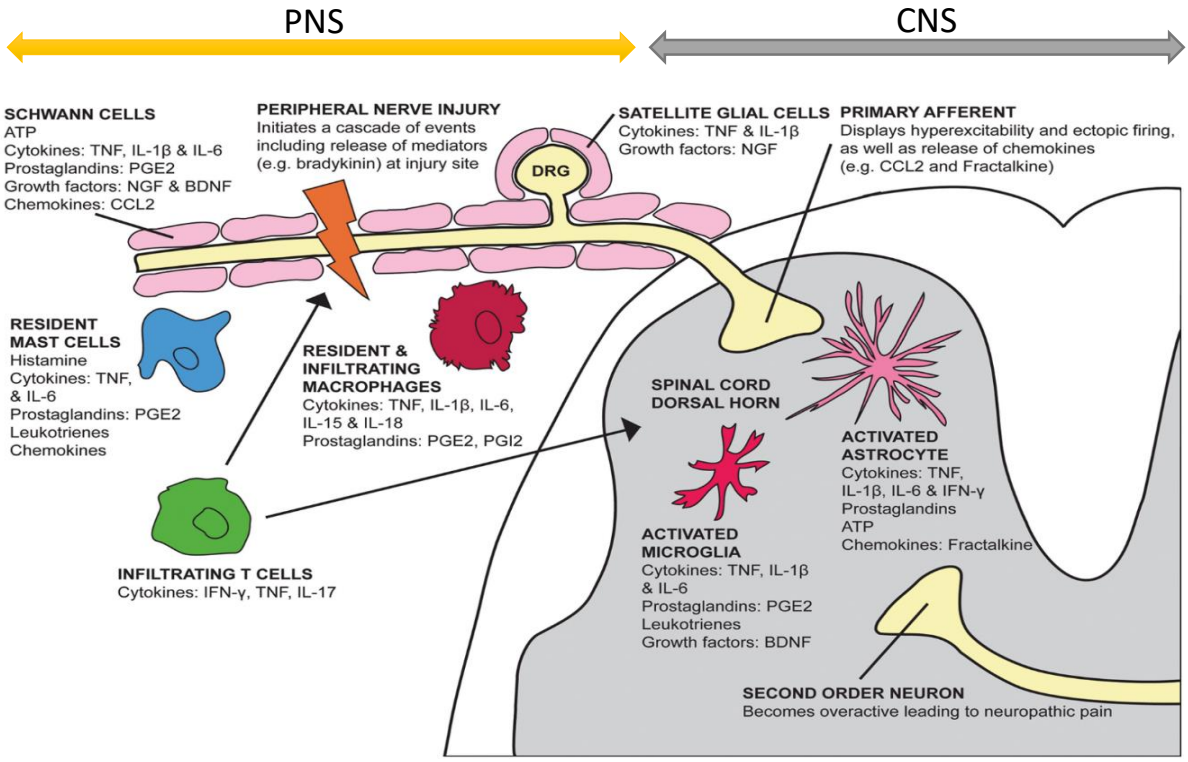


Figure I-3. Peripheral sensitization after somatosensory nerve injury. The initial tissue or nerve injury promotes inflammatory mediator release, recruiting different types of immune cells such as mast cells, T cells, and macrophages. The complex interaction between immune cells, neurons, and glial cells promote the hypersensitization of nociceptors present of primary afferent neurons. Continues nociceptor firing eventually leads to central sensitization via activating microglia and astrocytes, where prolonged activation of these cells result in chronic pain (from “the condition of neuropathic pain” section 2 neuropathic pain HJ87 – made by Cambridge.com)

involving various cell types participating in inflammation and peripheral sensitization, the development of pain therapeutics has always been extremely challenging.

Other than immune responses at the periphery, the somatosensory neurons also experience significant changes in the gene expression profile, particularly in the dorsal root ganglia (DRG), after pathological insults within the peripheral nerves or tissues. DRG is a collection of cell bodies of sensory neurons that convey sensory information from the periphery to the CNS. These neurons belong to the pseudo-unipolar neuron type, meaning that they contain an axon that splits into two branches, distal and proximal, which lies from the periphery to the spinal cord, respectively. Studies involving gene expression profiling and mass spectrometry suggest that neuropathic pain is associated with strong activation of certain neuronal genes, as well as immune cell responses involving both macrophages and satellite glial cells. Even though DRG is quite distant from the site of lesion, the resident macrophages and satellite glial cells in DRG react to nerve injury via inflammatory responses (13, 14). At the early stage of injury, macrophages and other immune cells alters the local environment of primary sensory neurons via increased synthesis and release of cytokines, and directly modulate neuronal activity and elicit spontaneous action potential sensitivity. Gene expression profiling in DRG after nerve injury showed changes in neuropeptide (Galanin, Sprr1A), ion channels (Ca^{2+} & Na^{+} channels), transcription factors (ATF3, CREB), and inflammatory genes (IL-6, CSF1), which all are critical in neuropathic pain development (15-19). In addition to rapid modulation of protein expression, post-translational modifications including phosphorylation of signaling molecules can alter excitability of sensory neurons leading to neuropathic pain. In the late stage of injury, T cells are recruited and activated through injury-induced chemokine expression such as CX3CL1 and CCL2, causing prominent increase in the density of macrophages (14). Monocytes are converted

into active phagocytes, which begins to clear up the debris within several weeks after initial nerve injury. It is reported that more than 50% of DRG neurons are lost after 2 months, and contribute to persistent induction of active macrophages and T-lymphocytes (20).

Conventional concepts of neuropathic pain were mostly focused on alteration of neurons, and that the neurons are responsible for pathophysiological conditions of peripheral nerve injury. However, it has been found that immune cells, glia, and neurons known as “neuropathic pain triad” are actively involved in the development and persistence of pain. Thus, the complex mechanisms of various cell types must be taken into account when developing new therapeutic approaches for neuropathic pain.

1.4 Central sensitization

Central sensitization is provoked by continuous nociceptor afferent input as a result of nerve or tissue damage. In most cases, it occurs within the dorsal horn of the spinal cord which is responsible for processing of the sensory information. Persistent nociceptor input originating from peripheral damage is transmitted through DRG neurons, and it triggers hypersensitization of the central nervous system, that accompanies a variety of physiological changes. The activation threshold of secondary neurons within the dorsal horn spinal cord is significantly reduced, and it contributes to the sensing of non-noxious stimuli as pain. Furthermore, the responsiveness of dorsal horn neurons is enhanced, and the neuronal receptive field properties are amplified that promotes chronic states of pain.

Increased responsiveness towards nociceptive input is due to changes in the CNS environment which is characterized by complex interaction between neurons and glial cells. Approximately 70% of CNS cells are composed of glial cells that may be divided into microglia,

astrocytes, oligodendrocytes and radial cells. Neurons and glial cells communicate through nerve impulses, neurotransmitters, and analgesic factors that contributes to pain sensation. Among different types of glial cells, microglial cells are the first to be activated and proliferated in the case of peripheral nerve injury. Microglia undergoes strong morphological changes, where "amoeba" like quiescent microglia are converted into activated shape with thicker processes (21-23). Activation of microglia involves many different pathways, but it was recently reported that CSF1 produced from the injured DRG neurons could be transported to the dorsal horn spinal cord and act on CSF1 receptor (CSF1R) present on microglia. Activation of CSF1R turns on DAP12-dependent signaling, that promotes the induction of several genes including IRF5/8, cx3cr1, TNFa, IL1b, and cathepsin S (15). The enhanced expression of these genes contributes to microgliosis, resulting in the development of neuropathic pain and central sensitization (**Fig. I-4**). Persistent induction of these analgesic factors give rise to the formation of pro-inflammatory environment within the CNS, eventually leading to hypersensitization and prolonged pain. Peripheral injury not only causes hyperactivation of somatosensory neurons but also promotes microglial cell activation within the CNS. The understanding of disease-causing mechanism in pain involving CNS will be important for the development of therapeutics for neuropathic pain.

1.5 Treatment options for neuropathic pain and its limitations

The heterogeneity of clinical pain conditions, together with the complexity and multiplicity of underlying pathophysiological mechanisms, has made it difficult to identify tractable targets for neuropathic pain. The IASP's recommendation as first line treatment for neuropathic pain includes gabapentinoids (gabapentin & pregabalin), which they had originally been developed as anti-epileptic drugs, but later have shown significant efficacy in the treatment

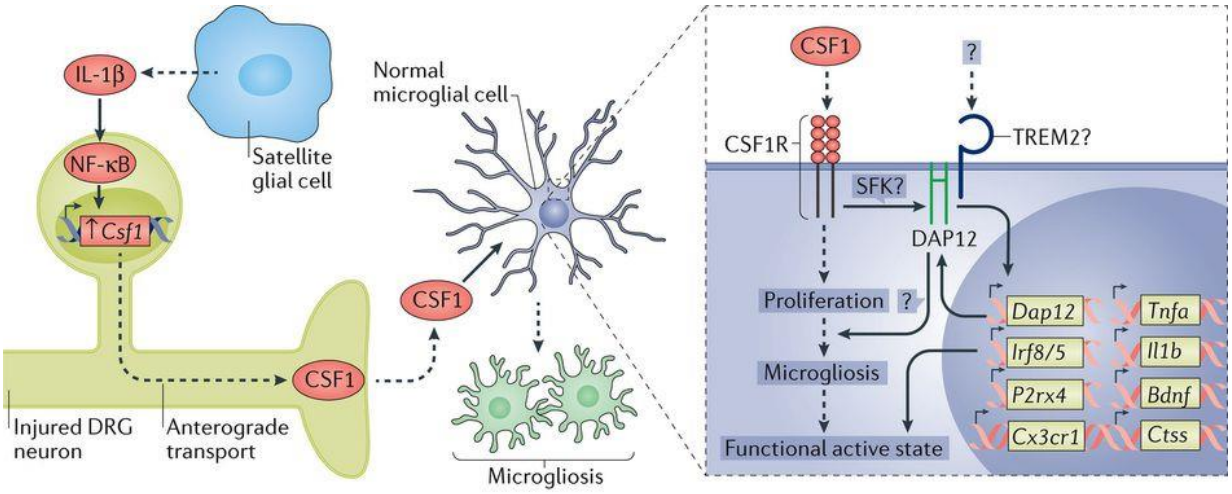


Figure I-4. Central sensitization via CSF1 induction in injured sensory neurons. Activation of microglial cells in spinal dorsal horn is promoted via CSF1 released in DRG after nerve injury. CSF1 is anterogradely transported to spinal dorsal horn and binds to CSF1 receptor present on microglia. Activation of DAP-12 dependent pathway triggers regulation of genes such as BDNF, CTSS, TNF-α, and IRF 5/8, which converts microglia into functionally active state giving rise to the development of pain (from Inoue *et al*., 2018).

of neuropathic pain (6, 24). Another frequently used drug for neuropathic pain is anti-depressants (Duloxetine), a selective serotonin reuptake inhibitor, that is known to treat painful diabetic peripheral neuropathic pain (25, 26). Other treatment options for neuropathic pain include opioids, topical anesthetics, cannabinoids, NSAIDS, and complementary medicines, each of which only provides limited efficacy that is palliative rather than curative, often causing severe side effects. Furthermore, growing misuse of prescription analgesics and consequent fatal overdose has become major problems in patients with neuropathic pain. Thus, the development of drugs without side effects has long been the holy grail of analgesia, but may now be potentially achievable through completely new therapeutic strategies.

2. Hepatocyte growth factor (HGF)

Hepatocyte growth factor (HGF) was first identified as mitogen for hepatocytes, however, it is now known as a multifunctional cytokine with mitogenic, motogenic, angiogenic, and anti-apoptotic activities. HGF is also known to exhibit neurological functions for it plays critical roles in the development of both central and peripheral nervous systems (27-29). The interaction between HGF and its receptor (c-Met) is known to be essential in the early developmental stages of adult brains, including cerebral cortex, hippocampus, sensory glia, and spinal cord. In neurons, HGF not only promotes the development and maintenance of nervous system, but also is associated with neuron survival and outgrowth (30, 31). These properties of HGF suggest that this growth factor may be used to understand and overcome certain diseases associated within the nervous system. Many studies have identified HGF as a powerful neurotrophic factor that can be applied neurodegenerative diseases, parkinson’s disease, and peripheral neuropathies (32-35).

2.1 Structure of HGF

Hepatocyte growth factor (HGF), also known as scatter factor (SF), was first discovered in 1989 by Nakamura group, and its first known function was its mitogenic activity within hepatocytes (36, 37). However, further research of HGF confirmed that it is rather a multifunctional protein with potent neurotrophic properties and extraneural functions, including angiogenesis, mitogenesis, and tissue regeneration. HGF is secreted by various cell types of mesenchymal origin, and acts on cells of mainly epithelial origin, or any other cells expressing c-Met.

HGF is synthesized as an inactive pro-HGF form and is converted into biologically active form when the bond between Arg494 and Val496 are cleaved by extracellular serine proteases (38) (**Fig. I-5A**). HGF is a dimeric molecule composed of α-subunit (69kDa) and β-subunit (34kDa) that are held by a disulfide bond between Cys487 of a α-subunit and Cys604 of a β-subunit (36, 39). HGF consists of six domains where the α-subunit contains an N-terminal hairpin loop (HL) followed by four kringle domains. The β-subunit contains serine protease homology (SPH) domain that lacks catalytic activity. Once the active HGF binds to c-Met, it activates a variety of signaling pathways to exert different cellular functions at appropriate situations (40).

2.2 Structure of c-Met

The receptor for HGF is called c-Met and it was identified as a new member of the tyrosine kinase family in 1992 by Bottaro group (**Fig. I-5B**). c-Met contains 50 kDa α-subunit and 145kDa β-subunit where α-subunit is exposed extracellularly and β-subunit is a transmembrane protein containing tyrosine kinase domain (41). The extracellular portion of c-

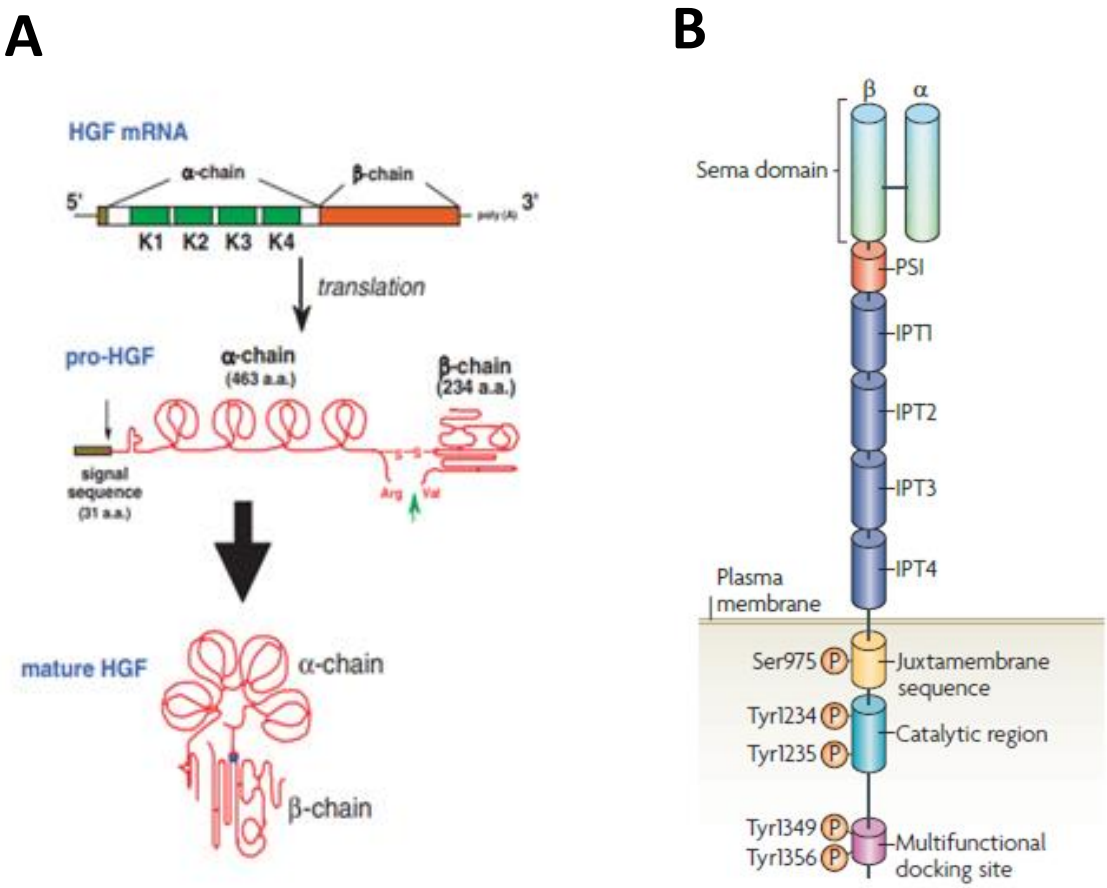


Figure I-5. Structure of hepatocyte growth factor and c-Met. (A) HGF is initially synthesized as pro-HGF inactive form, and the cleavage between Arg494 and Val496 by serine protease promotes the production of biologically active form of HGF. The mature form of HGF is composed of two subunits including α-subunit (69kDa) and β-subunit (34kDa) that are linked via disulfide bond (from Nakamura *et al*., 2010). **(B)** c-Met is composed of two subunits including 50 kDa α-subunit and the 145 kDa β-subunit. The intracellular portion of c-Met plays crucial roles in the HGF/c-Met signaling pathways for they are involved in recruitment of adaptors, and control of kinase activities (from Trusolino *et al.*, 2010).

Met is composed of three domain types: i) SEMA domain, ii) PSI domain, and iii) IPT domains. The SEMA domain (found in semaphoring and plexins) spans the first 500 residues of N-terminal domain that encompasses whole α-subunit and part of the β-subunit. The PSI (plexins, semaphorins and intergrins) domain consists of 50 residues with four disulphide bonds that are connected to the transmembrane helix via four immunoglobulin-plexin-transcption (IPT) domains. The intracellular portion of c-Met is composed of three segments: i) a juxtamembrane sequence that negatively regulates kinase activity via phosphorylation of Tyr1003, ii) the catalytic tyrosines Y1234 and Y1235, which positively controls kinase activity, and iii) the carboxyl-terminal multifunctional docking site (Y1349 and Try1356) that are involved in the recruitment of adaptors and transducers.

2.3 HGF/c-Met signaling

Met-dependent signals are organized in pathways that transmit biochemical information from the cell membrane to the nucleus. When HGF binds to c-Met, the receptors are homodimerized resulting in the phosphorylation of two tyrosine residues, Y1234 and Y1235, that are located in the catalytic loop of tyrosine kinase domain (**Fig. I-5B**). Consequently, it results in the phosphorylation of tyrosines in the carboxy-terminal tail leading to the recruitment of signaling effectors such as growth factor receptor bound protein 2 (GRB2), Src homology-2-containing (SHC), and GRB2-associated binding protein (GAB1) (42-44). These adaptor proteins and scaffold molecules lead to activation of major downstream signals (**Fig. 1-6**), including the mitogen-activated protein kinase (MAPK) cascades, PI3K/Akt axis, and the STAT3/JNK pathways (45, 46). The MAPK cascade involves Ras kinase that regulates terminal effectors such as extracellular signal-regulated kinases (ERKs). They are usually involved in the

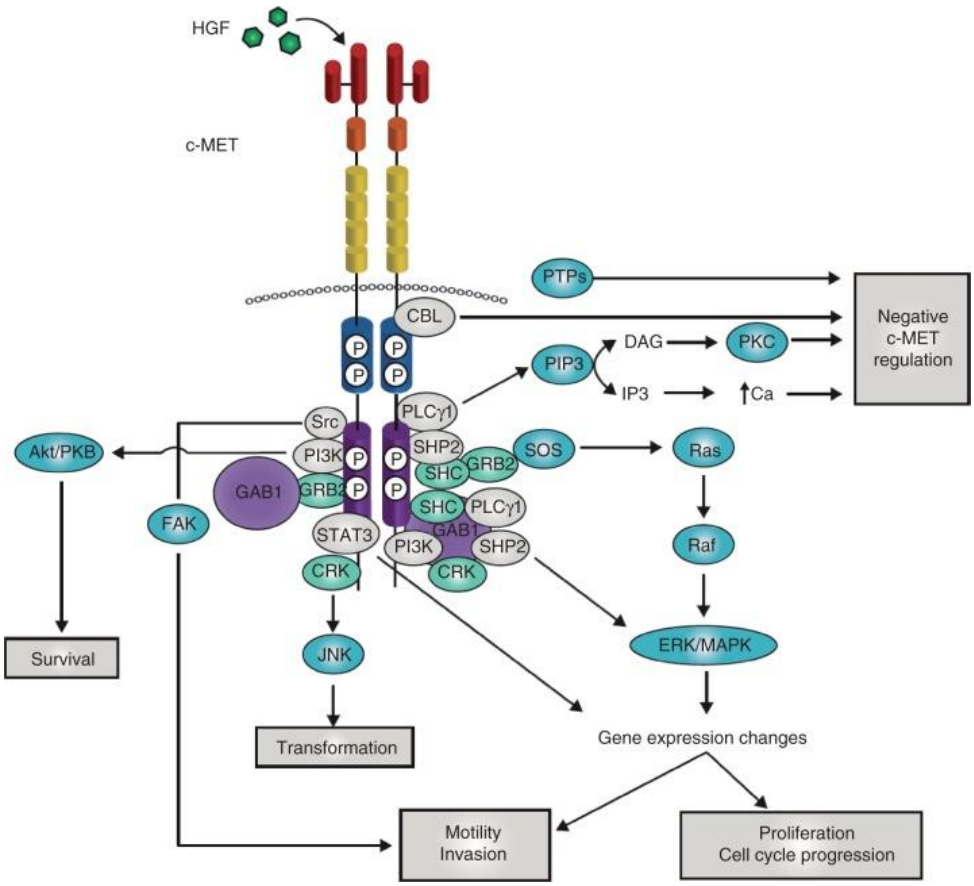


Figure I-6. Signaling pathways of HGF/c-Met. The binding of HGF onto c-Met leads to the phosphorylation of tyrosine residues, and activates the multiple docking adaptor molecules. Downstream signaling cascades lead to functional changes such as transformation, migration, and cellular proliferation (from Organ *et al*., 2011).

activity of various transcription factors associated with cell proliferation and transformation. Phosphoinositide 3-kinase (PI3K) is a lipid kinase that are associated with multifunctional docking site, and results in the formation of phosphotidylinoositol (3, 4, 5)–triphosphate which interacts with Ser/Thr kinase Akt. Akt signaling is involved in the suppression of apoptosis through inactivation of pro-apoptotic protein BCL-2, and also in the inactivation of glycogen synthase kinase 3β (GSK3β) resulting in the regulation of cell apoptosis and proliferation (47). Furthermore, c-Met activation leads to tyrosine phosphorylation of STAT3, which causes homo-dimerization and translocation to the nucleus. Eventually, STAT dimers act as transcription factors regulating the expression of various genes involved in cell differentiation and proliferation (45). These differentially regulated pathways are activated upon appropriate stimulations leading to the developmental morphogenesis, wound repair, angiogenesis, and nerve regeneration (40). Therefore, the controlled activation of HGF/c-Met signaling may open new avenues for developing innovative therapeutics of nerve regeneration, neuroprotection, and neuropathy-induced pain alleviation.

3. Gene therapy

Despite the great potential of HGF as a therapeutic reagent, there have been limitations in the delivery system of HGF into the body for it is very unstable in the blood circulation, where the half-life of HGF is known to be less than 15 minutes. In order to overcome unstable properties of HGF, there was a great need for the development of an effective delivery method, which is capable of expressing a therapeutically meaningful level of HGF for a relatively short period of time, say a week (48). Among the possible methods of delivering HGF, my group have developed a plasmid DNA expression vehicle designed to promote transient expression of HGF

when injected intramuscularly (49). Plasmid DNA is known to be relatively safe for it does not integrate into the chromosome and it only lasts in the body for a certain period of time. It has been reported that 99.99% of transfected plasmid DNA disappears within 90 days.

The plasmid used in this thesis, called pCK-HGF-X7 (or VM202), was designed to express two isoforms of HGF, namely HGF_{723} (or dHGF) and HGF_{728} (or cHGF). It is a 7377 base pair long plasmid DNA used in previous studies for ischemic diseases (**Fig. I-7**) (49). In addition to the usual replication origins (ColE1) and drug selectable marker (kanamycin resistance gene), it is composed of the 5' control region from the immediate early region of human cytomegalovirus (including the promoter, exon 1, deleted intron 1, and untranslated exon 2), and the genomic-complementary DNA hybrid sequence from the human HGF gene. This hybrid gene consists of 18 exons and 17 introns spread over 67.4 kb, and the alternative splicing between exons 4 and 5 results in two isoforms of HGF, HGF_{723} and HGF_{728}. The genomic-cDNA hybrid HGF sequence in pCK-HGF-X7 contains truncated intron 4, which was engineered to produce high levels of HGF_{723} and HGF_{728}. Co-expression of these two isoforms were previously shown to be biologically active in various studies involving a variety of different animal models (50, 51). This plasmid, pCK-HGF-X7, had been used throughout my thesis in order to investigate the role of HGF in alleviation of neuropathic pain.

4. Overview of thesis research

At the time when I began my thesis research, pCK-HGF-X7 had been tested in clinical studies as well as animal models for ischemic diseases, such as critical limb ischemia and coronary artery disease. In relation to my work, phase II study was just completed for painful diabetic peripheral neuropathy (PDPN) (34, 52). Data from this clinical trial clearly showed that

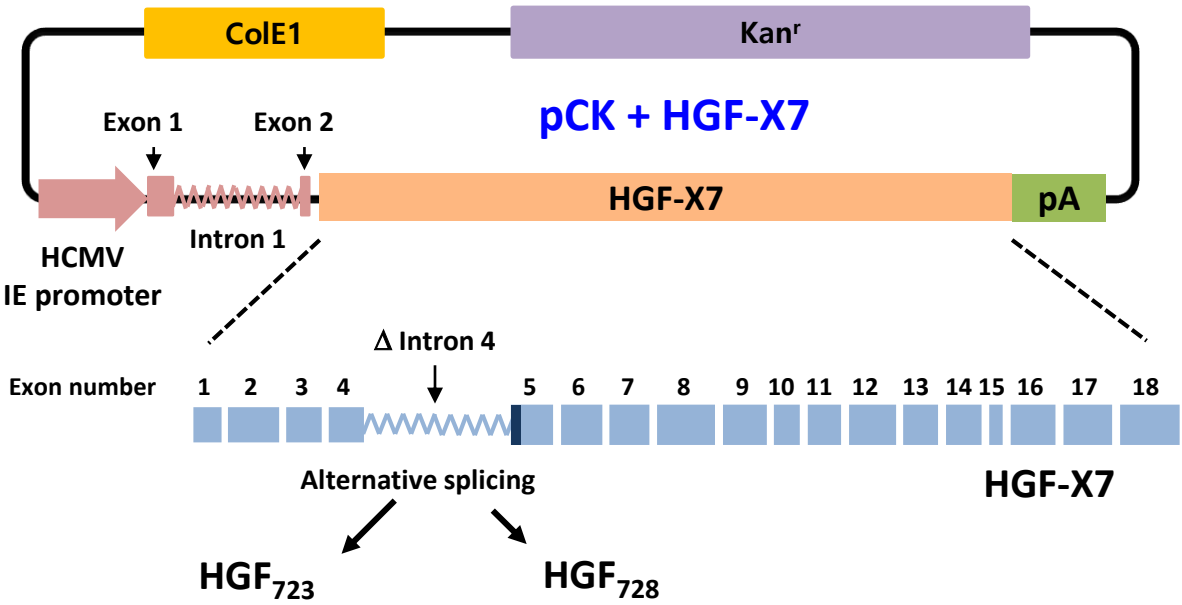


Figure I-7. Structure of pCK-HGF-X7. The genomic-cDNA hybrid sequence of HGF is present in the pCK backbone. Major immediate-early (IE) region of HCMV (pink arrow, boxes, and wavy lines); HGF-X7 (orange box), consisting of genomic-cDNA hybrid sequence of HGF including truncated form of intron 4 (blue boxes and wavy lines); polyadenylation signal of bovine hormone gene (green box); kanamycin resistant gene, phosphotransferase (purple box); ColE1, origin of replication, from E. Coli (yellow box) (from Lee *et al*., 2000).

pCK-HGF-X7 is capable of significantly improving pain measurement for a long period of time without any severe side effects. In addition, data from monofilament tests suggested that pCK-HGF-X7 may aid in recovery of sensory functions and it has a potential to become a disease-modifying drug. Based on this clinical trial results, I have tested the putative analgesic effects of pCK-HGF-X7 and its underlying mechanisms in the CCI-induced mouse neuropathic pain model. In this study, I found that *i.m.* injection of pCK-HGF-X7 effectively suppresses neuropathic pain for several weeks in mice after CCI, and also inhibited the expression of various genes involved in nerve injury and development of neuropathic pain. Of particular importance, pCK-HGF-X7 injection inhibited the nerve injury-induced colony stimulating factor-1 (CSF1) expression in the injured DRGs, and the subsequent microglia and astrocyte activation in the spinal cord. In addition, HGF showed anti-inflammatory effects in LPS-stimulated Schwann cells, which implies the possible role of HGF in neuropathic pain initiated via inflammatory responses. Taken together, my data suggests that *i.m.* injection of pCK-HGF-X7 could effectively reduce neuropathic pain by modulating the expression of injury- or pain-related factors and thus controlling the complex pain transmission circuitry. In this study, at least a part of the working mechanism(s) of pCK-HGF-X7 on the alleviation of neuropathic pain was shown. For these reasons above, pCK-HGF-X7 appears to have the potential to become a candidate for the next generation of therapeutic agents for neuropathic pain.

Chapter II

Methods and Materials

1. Animals and surgery

Male ICR mice (24 ~ 26g) were purchased from Orient Bio Inc (Seoul, Korea), and used for behavioral studies. All surgical and experimental procedures were approved by the Institutional Animal Care and Use committee at Seoul National University. Animals were housed in the animal facility for at least 7 days under a 12-hour light-dark cycle before they received any surgeries or treatments. Mice were given ad libitum access to food and water.

Mice were anesthetized with intraperitoneal administration of alfaxan (80mg/kg). Neuropathic pain was mimicked using CCI of the sciatic nerve (53), which consists of several processes that result in chronic nerve injury in the periphery. In order to perform CCI in mice, they were given about 1-cm long blunt dissections to expose the right sciatic nerve, which usually lies between the *gluteal* and *biceps femoris* muscles. Once the sciatic nerve proximal to the trifurcation site was exposed, it was given loose ligatures three times with 0.5 mm spacing using 6-0 silk (AILEE CO, Korea) sutures. The ligatures were slightly tightened until there was a noticeable twitch of the right hind limb. Then, 100uL of the control plasmid, pCK (2mg/ml), or pCK-HGF-X7 (2mg/ml) were intramuscularly injected in the area of *bicep femoris* and *quadriceps femoris* muscles. Sham-operated mice were given the same dissections in the right thigh, but did not receive ligatures in the sciatic nerve. As a control, sham mice were intramuscularly injected with 50uL of filtered PBS into the same muscles. After the surgery, the lesions were closed with sutures, and povidone-iodine was applied in order to prevent wound infection. Eye ointments were applied to animals to prevent corneal drying and damage from anesthetics, and they were also monitored under a warm pad during recovery.

2. Plasmid DNA preparation

The cloning of genomic-cDNA hybrids of HGF gene and construction of expression vector, pCK-HGF-X7, plasmid have been described in detail by Pyun et al. All expression vectors used in the experiments were purified using an EndoFree plasmid Maxi or Giga prep kit (Qiagen, Hilden, Germany), dissolved in 0.9% NaCl, diluted to 2 mg/ml and stored at -80℃ before use.

3. RNA analysis

For quantitative real-time RT-PCR (qRT-PCR), the ipsilateral L4–L6 DRGs, and L4 – 5 spinal cords were collected 4 days after CCI. Samples were mechanically homogenized using polypropylene pestles (Bel-Art Scienceware, NJ), and total RNAs were extracted in RNAiso plus (Takara, Japan). Quantification of RNA was done by using a nanodrop instrument. Equal amounts of RNA were used to synthesize cDNAs using Reverse Transcriptase XL (AMV) (Takara). Quantitative PCR was performed using SYBR Premix Ex Taq (Takara). Primers used for expression analysis are as follows: ATF3 gene: Forward 5'-GAGGATTTTGCTAACCTGACACC-3'; Reverse 5'-TTGACGGTAACTGACTCCAGC-3'; CACNA2D1 gene: Forward 5'-CTGCTGGCCTTGACTCTGAC-3'; Reverse 5'-CACTCCACTTGCTGTTTTTGC-3'; CSF1 gene: Forward 5'-TGCTAAGTGCTCTAGCCGAG-3'; Reverse 5'-CCCCCAACAGTCAGCAAGAC-3'; Cathepsin S: Forward 5'-CCATTGGGATCTCTGGAAGAAAA-3'; Reverse 5'TCATGCCCACTTGGTAGGTAT-3'; IRF8 gene: Forward 5'-GGGCAGCGTGGGAACC-3'; Reverse 5'-GCTTCCAGGGGATACGGAAC-3'; IRF5 gene: Forward 5'-TGGGGACAACACCATCTTCA-3'; Reverse 5'-CTGGAAGTCACGGCTTTTGT-3'; Iba1 gene: Forward 5'-ATCAACAAGCAATTCCTCGATGA-3'; Reverse 5'-

CAGCATTCGCTTCAAGGACATA-3'; HPRT gene: Forward 5'-TCAGTCAACGGGGGACATAAA-3'; Reverse 5'-GGGGCTGTACTGCTTAACCAG-3'; GAPDH gene: Forward 5'-AGGTCATCCCAGAGCTGAACG-3' Reverse 5'-CACCCTGTTGCTGTAGCCGTA-3'. Gene expression levels were normalized using housekeeping genes, HPRT and GAPDH, and relative expressions were compared between respective experimental controls.

4. Western blotting

For immunoblotting, animals were sacrificed 4 days after CCI and ipsilateral L4-6 DRG neurons and L5 spinal dorsal horns were collected, followed by lysis using RIPA buffer with protease and phosphatase inhibitor cocktail (Roche Diagnostic Ltd., IN). Equal amounts of protein were separated on 10% SDS-polyacrylamide gel, and transferred to a western membrane (PVDF). The membranes were blocked with 1% BSA in TBST (10mM Tris-HCl, pH 7.4, 0.9% NaCl, and 0.1% Tween 20) for 1 hour and probed with primary antibodies diluted in blocking solution at 4℃ overnight. The primary antibodies used included ATF3 (1:1000, Santa Cruz, sc-188), CACNA2D1 (1:1000, thermo, MA3-921), and β-actin (1:5000, sigma, A5411). After washing with TBST, membranes were incubated with HRP-conjugated goat anti-mouse or rabbit IgG secondary antibody (Sigma, MO, USA) at room temperature (RT) for 1 hour. The blots were then washed three times with TBST, and the protein bands were visualized with the enhanced chemiluminescence system (Millipore, MA, USA). Quantification of the bands was done by using Image J software (National Institutes of Health, MD). When needed, blots were stripped using stripping buffer with vigorous shaking for 30 minutes at RT followed by three washes in TBST.

5. ELISA

In vivo samples, including L4-5 spinal cords, L4-6 DRGs, sciatic nerves and muscles, were prepared after CCI, and they were homogenized in lysis buffer containing protease inhibitor (Sigma, 11697498001), phosphatase inhibitor cocktail (Sigma, 4906845001), and PMSF (Sigma, P-7626) using polypropylene pestles (Fisher scientific, 13-717-270). The samples were centrifuged at 12000 rpm for 15 minutes at 4℃, and the supernatants containing total protein were subjected to human HGF ELISA (R&D Systems, SHG00) and mouse CSF1 ELISA (R&D Systems, MMC00) as shown in the manufacturer's protocol. The level of HGF or CSF1 protein detected was normalized to the total amount of protein extracts from the tissue, as measured by BCA protein assay kit (Thermo, IL).

6. Immunofluorecence Assay (IFA)

Animals were deeply anesthetized and transcardial perfusion was performed using PBS and 4% paraformaldehyde. Then, the tissues of interest, including L4-6 DRGs and L4-5 spinal cords, were removed and postfixed with 4% para-formaldehyde (PFA) once again overnight at 4℃. The samples were washed with PBS once, followed by incubation in 30% sucrose phosphate buffer for 48 hours. DRGs and spinal cord samples were embedded in optimum cutting temperature (O. C. T) blocks (Tissue-Tek, ann den Rijn, Netherlands) and sections were prepared on gelatin-coated glass slides using cryocut microtome (Leica CM3050S, Germany). The sections were blocked with 5% normal goat serum (Jackson ImmunoResearch Laboratories, PA, USA), 2% BSA, and 0.1% triton X-100 (Sigma) dissolved in PBS for 1 hour at room temperature. Then, the samples were washed with PBS three times, and incubated overnight at 4℃ with the primary antibodies. The primary antibodies used in this study include Iba-1 (1:1000, Wako, 019-19741), and GFAP (1:5000, Dako, Z0334). After rinsing with PBS, the

sections were incubated in secondary antibodies for 1 hour at room temperature. The secondary antibodies used include donkey anti-mouse IgG Alexa Fluor® 488 conjugate (1:1000, Thermo, A21202), donkey anti-rabbit IgG Alexa Fluor® 488 conjugate (1:1000, Thermo, A21206), donkey anti-mouse IgG Alexa Fluor® 555 conjugate (1:1000, Thermo, A31570), and donkey anti-rabbit IgG Alexa Fluor® 555 conjugate (1:1000, Thermo, A31572). Thereafter, another three washes were done using PBS, and nuclear staining was performed using 1ug/mL Hoechst 33248 (Thermo, H1399). The sections were mounted using Vectashield mounting solution (Vector Laboratories, H100), and fluorescence images were obtained using a confocal microscope (LSM700, Carl Zeiss, Germany). Quantification of the fluorescent signal intensities was performed using Image J software (National Institutes of Health).

7. Behavioral studies

Animals were habituated to the testing environment for 3 days prior to the behavioral studies. The development of mechanical allodynia and thermal hyperalgesia was assessed using von Frey filaments assay and Hargreaves test, respectively, and pain symptoms were evaluated weekly. Von Frey measurements were made in order to evaluate the mechanical sensitivity of mice. First of all, animals were placed individually in the cylinder on top of the metal mesh floor for 3 hours for adaptation. The mechanical sensitivity of mice was assessed by stimulating the hind paw with von Frey filaments of different thicknesses. Also, the frequency of hind paw withdrawal was measured using constant thickness (0.16g) of the filaments. Hargreaves test was also performed in a similar manner to assess the thermal sensitivity of nerve-injured mice. Briefly, animals were placed on top of the heat-controlled glass for adaptation followed by stimulation of radiant heat generated to the hind paw. The latency time was measured until the hind paw was withdrawn upon heat stimulation.

8. Primary sensory neuron culture

DRG neurons were dissected from adult ICR mice (22g~26g) and digested with 1mg/mL of collagenase A (Sigma) and 0.8unit/mL of diapase I (Sigma) for 60 minutes at 37℃ 5% CO2 incubator. Then, the cells were enzyme-digested with trypsin-EDTA (0.05%) in the presence of HBSS buffer (20mM HEPES buffer included) for 7 minutes at 37℃. The samples were washed twice with DMEM/F12 (Welgene, Korea) containing 10% FBS, and seeded on to a 6-well plates. Plates were coated with 100ug/mL of Poly-D-Lysine (Sigma) 24 hours prior to cell seeding. After 2 days of culture, the cells were treated with 1uM Ara-C (Sigma) for 24 hours in order to inhibit proliferation of non-neuronal cells. Then, cells were given fresh media, and it was additionally grown for another day. On the fifth day of culture, cells were treated with LPS (Escherichia coli, 0111: B4), and rhHGF (R&D Systems) for 6 hours, and it was prepared for various analysis.

9. Sciatic nerve explants culture

Sciatic nerve was removed from 10 weeks old Sparague-Dawley rats. Sciatic nerve was cut into approximately 1 cm sections, and the perineurial sheath was removed. Then, each nerves were transferred to Poly-D-Lysine (Sigma) dish, and they were maintained in DMEM containing 10% FBS. After 2-3 days, they were treated with LPS (Escherichia coli, 0111: B4), and/or rhHGF (R&D Systems) to perform variety of experiments.

10. Primary Schwann cell culture

Primary Schwann cell cultures were prepared as described in Kaewkhaw *et al* (54). After the isolation of Schwann cells from rat sciatic nerves, they were expanded in appropriate culture medium: DMEM-d-valine (Welgene), 2mM glutamine (Invitrogen), 10% FBS (Corning), 1% N2 supplement (Invitrogen), 20ug/ml bovine pituitary extract (Sigma), 5uM forskolin (Sigma),

100U/ml penicillin (Invitrogen), 100ug/ml streptomycin (Invitrogen), and amphotericin B (Sigma). They were grown for 3 weeks, and subjected to various experiments.

11. Statistical analysis

All values are presented as mean ± standard error mean (SEM) from more than two independent experiments. Differences between values were determined by one-way or two-way ANOVA followed by Tukey's post-hoc test or Bonferroni's multiple comparison test, provided by the GraphPad Prism software.

Chapter III

Effective control of neuropathic pain by transient expression of hepatocyte growth factor in mouse CCI model

1. Background

Neuropathic pain is a pathological chronic condition caused by dysfunctions in the nervous system. The clinical symptoms of neuropathic pain include allodynia, pain sensation in response to non-noxious stimuli, and hyperalgesia, increased sensitivity to pain. Millions of people worldwide suffer from this devastating disease, yet it still remains to be a major clinical challenge because its pathologic mechanisms are poorly understood. Current treatment methods for neuropathic pain include anticonvulsants, antidepressants, and opioids, each of which only provides limited efficacy that is palliative rather than curative, often causing significant side effects. Therefore, there is a great need for the development of safer, and more effective therapeutic drugs.

In an effort to develop an effective treatment method for painful neuropathy, our group has been exploring the possibility of using plasmid expression human hepatocyte growth factor (HGF). HGF is a multifunctional protein with potent neurotrophic properties produced by various cell types of mesenchymal origin. In humans, two isoforms of HGF, namely HGF_{723} (or dHGF) and HGF_{728} (or cHGF), are produced from the HGF gene by alternative splicing (55, 56). Co-expression of these two isoforms has been reported to be necessary for optimum biological activities of HGF. Biological functions of HGF is mediated by its receptor called c-met, and they are expressed in sensory neurons, Schwann cells, and smooth muscle cells among others, which all play important roles in the functions of peripheral nerve after pathological insults. Indeed, it has been reported that HGF acts on sensory neurons to enhance their survivability and neurite outgrowth. Moreover, intramuscular injection (*i.m.*) of plasmid DNA expressing HGF has been shown to significantly reduce neuropathic pain symptoms in phase I and phase II clinical studies.

At the time when I began my research, the effects of HGF, when delivered in the form of plasmid DNA, on painful diabetic peripheral neuropathy (PDPN) had been tested in phase II study. The clinical results have shown that pCK-HGF-X7 effectively alleviates pain in patients with PDPN for maximally 12 months with strong safety profile. Additionally, pCK-HGF-X7 improved sensory functions in monofilaments test implying that HGF may be involved in the recovery of injured nerves. To understand the molecular mechanism(s) underlying such significant analgesic and potential regenerative effects of pCK-HGF-X7, I have adapted the used of CCI, initially to test whether I could reproduce data from clinical studies in the experimental animal model.

2. Results

2.1. Expression kinetics of HGF protein in skeletal muscles and sciatic nerve after *i.m.* injection of pCK-HGF-X7

First, the expression kinetics of HGF after intramuscular injection of pCK-HGF-X7 was studied. Two hundred micrograms of pCK-HGF-X7 was administered into the thigh muscles using mouse CCI model, and the expression pattern of hHGF within the muscles and peripheral nervous system was measured. To verify HGF protein expression derived from pCK-HGF-X7 around the injection site, total proteins were isolated from the thigh muscles, and the level of hHGF protein was measured by ELISA, which only detects hHGF and does not cross-react with endogenous murine HGF. The level of the hHGF protein was gradually increased, reaching its highest point (97 ng/mg of total protein) at day 7, and thereafter decreased to an undetectable level by day 14 (**Fig. III-1A**). Low, but readily detectable, levels of hHGF were also found in the sciatic nerve (267 pg/mg), 4 days after nerve injury (**Fig. III-1B**).

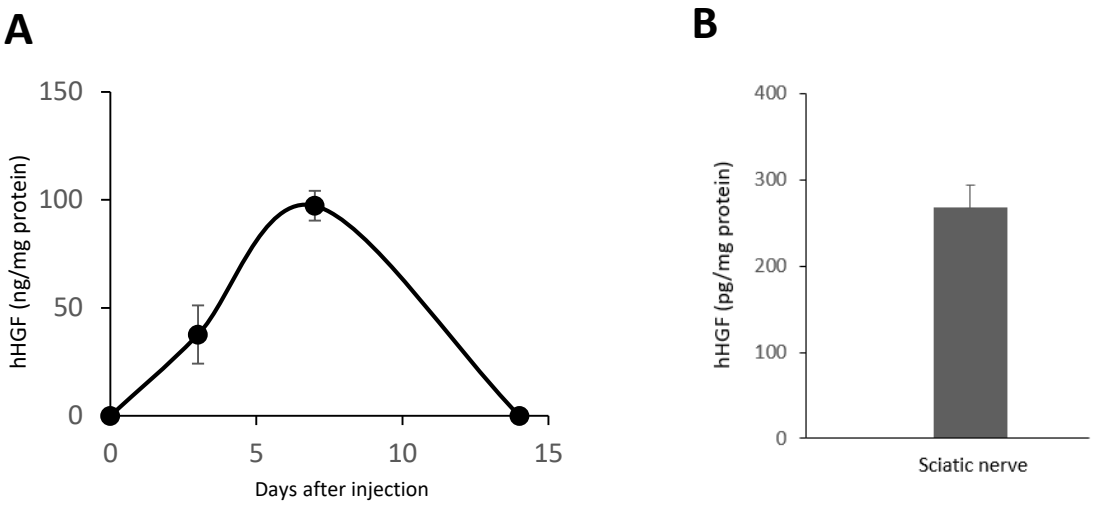


Figure III-1. Expression kinetics of HGF protein in skeletal muscles and sciatic nerve after *i.m.* injection of pCK-HGF-X7. (A) Skeletal muscles around the injection site were isolated, at appropriate time points, and total proteins were prepared followed by ELISA specific for hHGF (n = 4). **(B)** hHGF expression in the peripheral nervous system. Sciatic nerves were isolated on 4 days after CCI, and subjected to hHGF ELISA. *Note: In sham-operated, and control vector lacking the HGF sequence (pCK) injected groups, hHGF was not detected (n = 4).

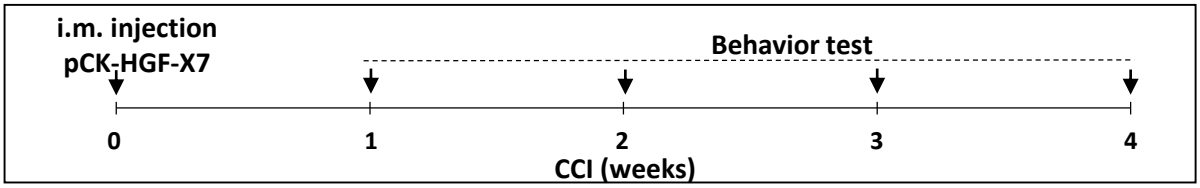
These results suggested that *i.m.* injection of pCK-HGF-X7 successfully produced HGF in the injected muscles, and it could be delivered to the peripheral nerve tissue such as sciatic nerve. HGF produced from pCK-HGF-X7 did not circulate in a systemic manner, presumably as it contains the heparan sulfate binding site in its N-terminal region (57, 58).

2.2. Effect of pCK-HGF-X7 injection on CCI-induced neuropathic pain in mouse

The effects of *i.m.* pCK-HGF-X7 injection on neuropathic pain development were studied by measuring mechanical allodynia and thermal hyperalgesia by von Frey filaments and Hargreaves tests, respectively. Sham-operated mice were not affected by either tests as expected **(Fig. III-2A, B, C, and D)**. In CCI-induced and control vector (pCK)-injected mice, the paw withdrawal threshold and frequency were changed in a way consistent with the increased level of mechanical allodynia **(Fig. III-2A and B)**, and the level of neuropathic pain was sustained for 4 weeks. The level of thermal hyperalgesia was also increased as evident by the reduction in thermal withdrawal latency **(Fig. III-2C)**. In animals injected with pCK-HGF-X7, however, the level of neuropathic pain was effectively suppressed as demonstrated by increase in the paw withdrawal thresholds and decrease in the paw withdrawal frequencies **(Fig. III-2A and B)**. Moreover, the withdrawal latencies measuring thermal hyperalgesia were significantly increased in the pCK-HGF-X7 group, which lasted for 4 weeks **(Fig. III-2 C)**. The pain-relieving effects lasted up to 4 weeks with a single injection of pCK-HGF-X7 in this particular experiment and up to 8 weeks in another longer term experiments **(Fig. III-2D)**.

2.3. Effect of different doses of pCK-HGF-X7 in mouse CCI model

Next, we investigated the effect of different doses of pCK-HGF-X7 on CCI-induced neuropathic pain. Five different concentrations of pCK-HGF-X7 were selected and they were



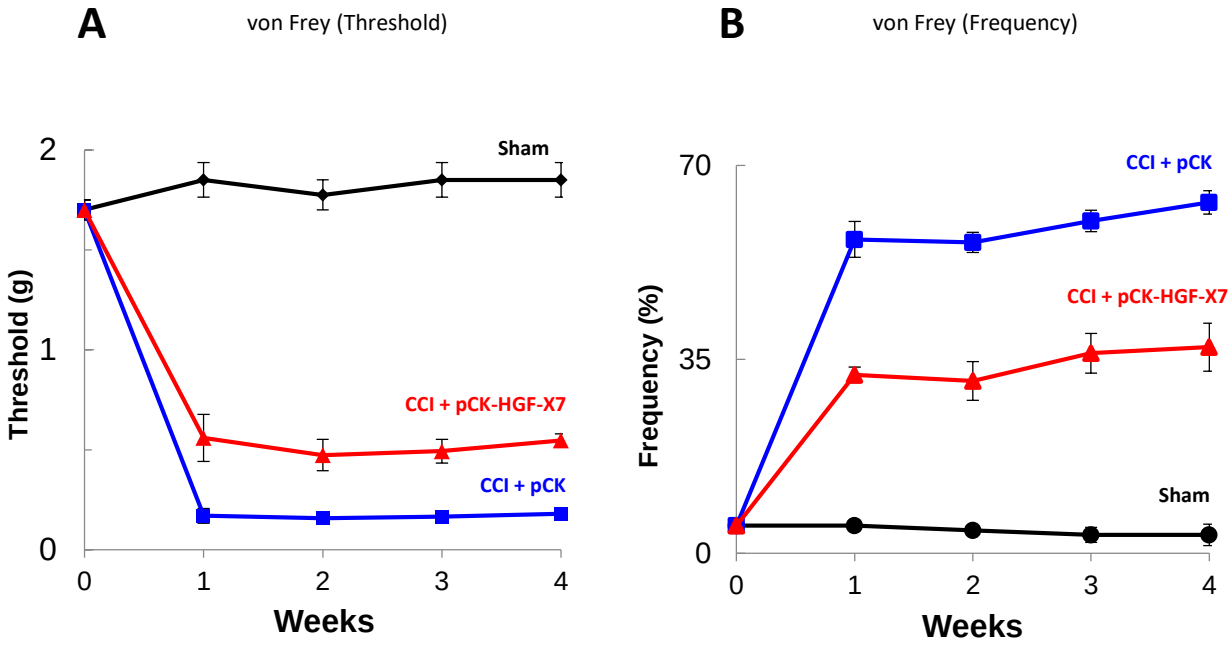


Figure III-2. Effect of pCK-HGF-X7 injection on CCI-induced neuropathic pain in mouse. pCK and pCK-HGF-X7 (200 µg) were intramuscularly injected on the day of CCI, and the pain sensitivity towards mechanical stimuli was measured at appropriate times by **v**on Frey filaments test **(A, B).** Each group consisted of 6 mice, and more than 3 independent experiments were performed (mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way ANOVA).

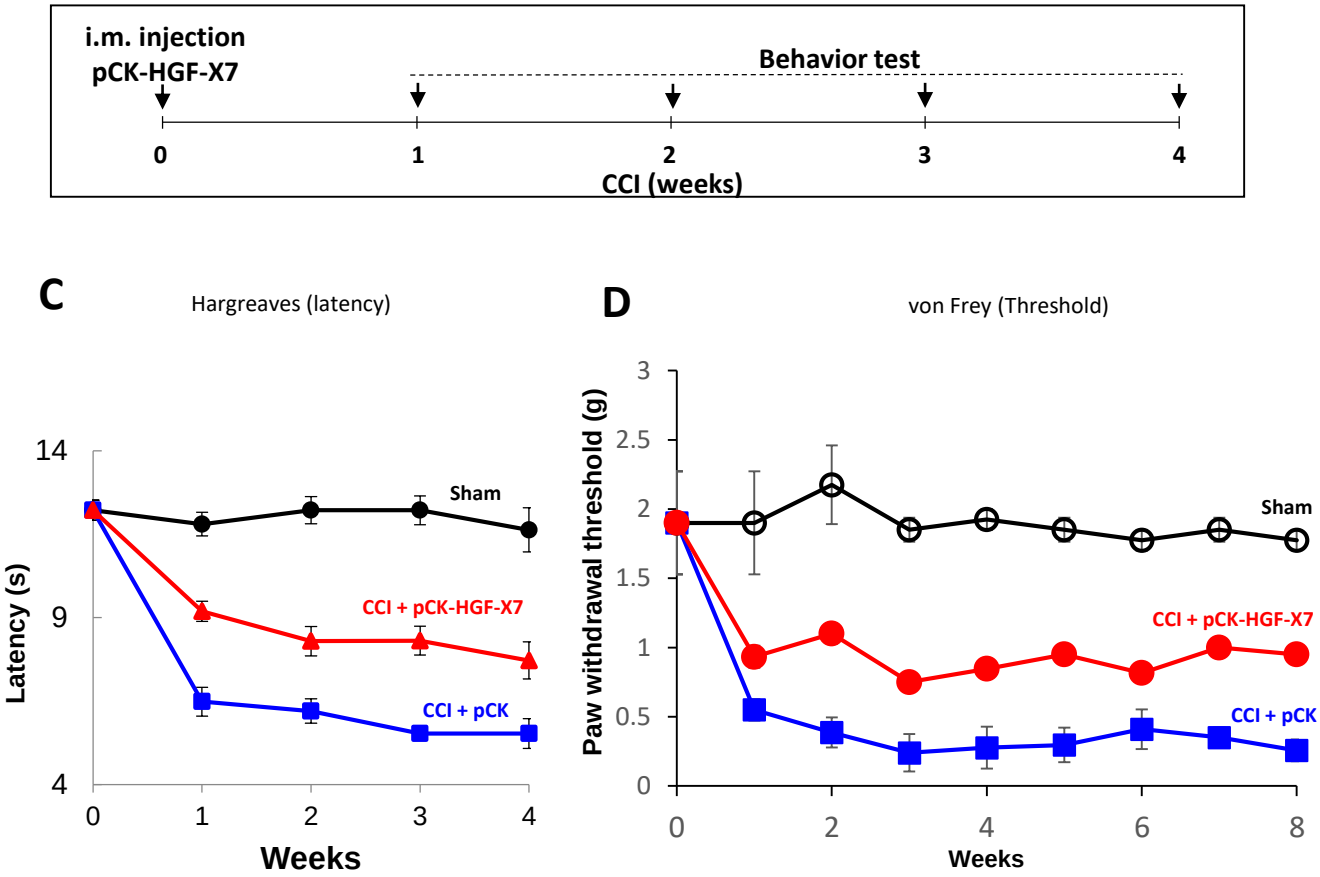


Figure III-2. Effect of pCK-HGF-X7 injection on CCI-induced neuropathic pain in mouse. pCK and pCK-HGF-X7 (200 µg) were intramuscularly injected on the day of CCI, and **(C)** thermal hyperalgesia was measured at appropriate times by Hargreaves test. **(D)** Long term pain-relieving effect of pCK-HGF-X7 was tested with con Frey filaments test for 8 weeks long. Each group consisted of 6 mice, and more than 3 independent experiments were performed (mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way ANOVA).

intramuscularly administered around the injury site on the day of ligation. The pain levels were measured at appropriate times by paw withdrawal frequencies and thermal withdrawal latencies. The behavioral test was conducted 2 weeks post injury, for it is the earliest time point that presents clear analgesic effect of pCK-HGF-X7. As shown in **Figure III-3A and B**, the pain relieving effect was increased in a dose dependent manner, reaching a peak at 200μg, and then somewhat declining thereafter in both measurements. Thus, the optimum dose that produced maximum pain relieving effect was reproducibly found to be 200μg of pCK-HGF-X7 in this particular model, and this concentration was used throughout the experiments in other behavioral studies and gene analysis.

2.4. Effect of i.m injection of pCK-HGF-X7 on baseline sensory function

To confirm the effect of pCK-HGF-X7 on baseline sensory function, behavioral studies in sham-operated mice was performed. Mice were given sham surgery by giving incision around the *gluteal* and *biceps femoris* muscles and injected with PBS, or pCK, or pCK-HGF-X7. As shown in **Fig. III-4A and B**, it was revealed that a single *i.m.* injection of pCK-HGF-X7 did not affect baseline sensory function as there were no significant changes in the withdrawal frequencies and latencies did not change in sham surgery mice. This experiment showed that pCK-HGF-X7 could reduce ongoing neuropathic pain for a long period of time, without affecting the baseline sensory function.

2.5. Effect of repeated administration of pCK-HGF-X7 on CCI-mediated neuropathic pain

To test whether repeated injection of pCK-HGF-X7 could further improve pain symptoms, mice were given CCI and pCK or pCK-HGF-X7 injection as like in the previous experiments, and an additional injection of pCK-HGF-X7 was applied after various time points.

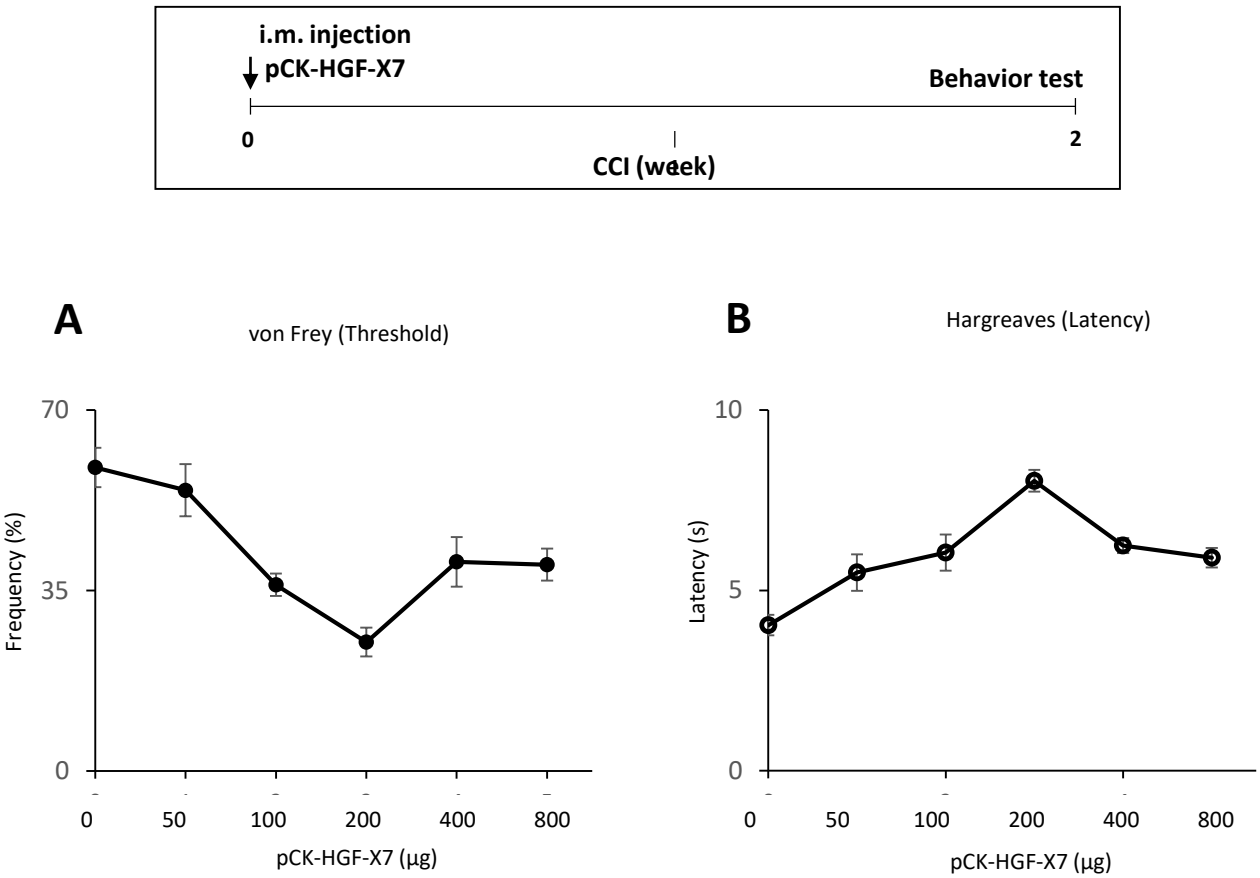


Figure III-3. Effect of different doses of pCK-HGF-X7 in mouse CCI model. Different doses of pCK-HGF-X7 were intramuscularly injected, and 2 weeks later, the pain sensitivity towards mechanical and thermal stimulation was measured by **(A)** von Frey filaments and **(B)** Hargreaves tests, respectively (n=6 for each group; mean + SEM).

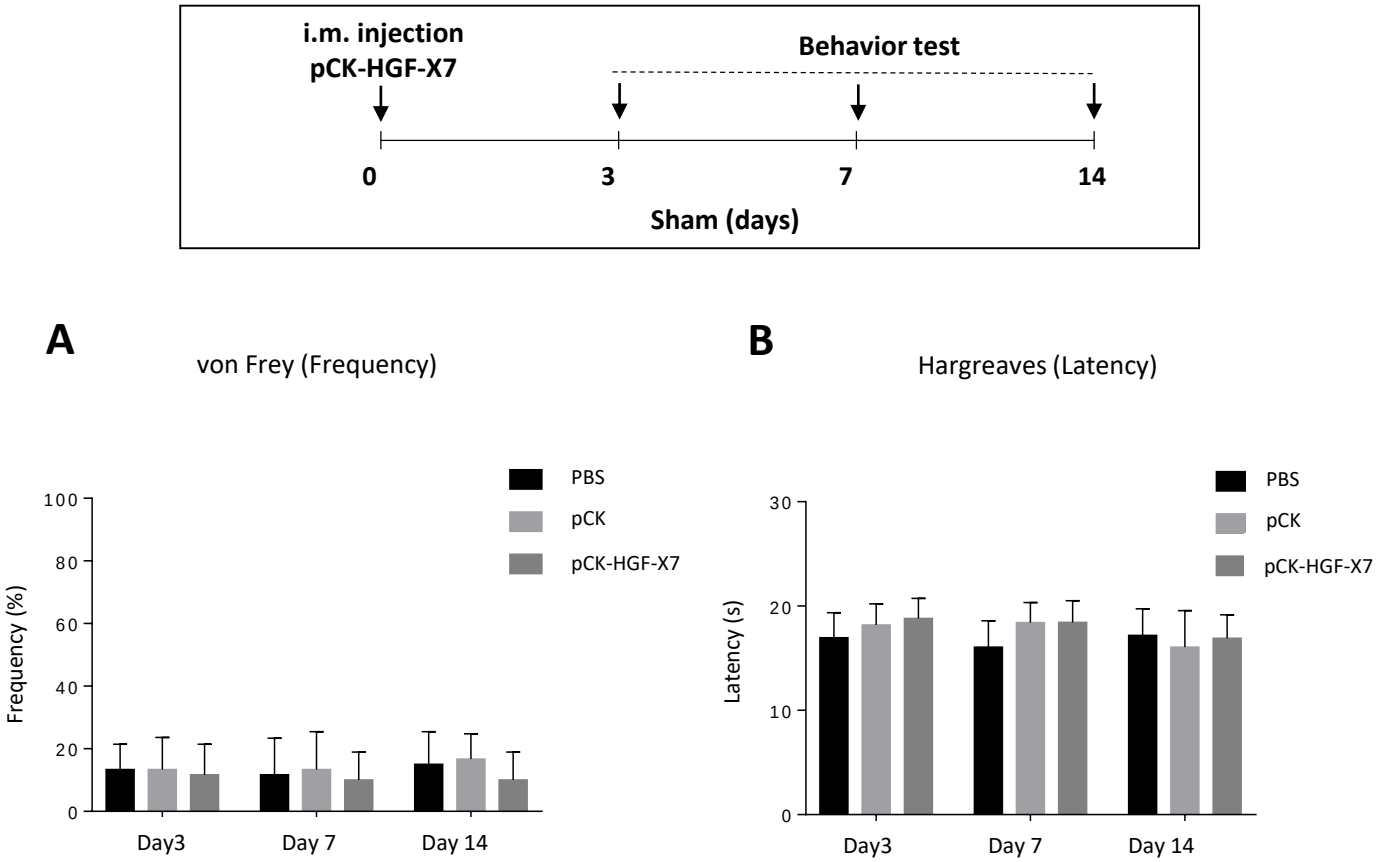
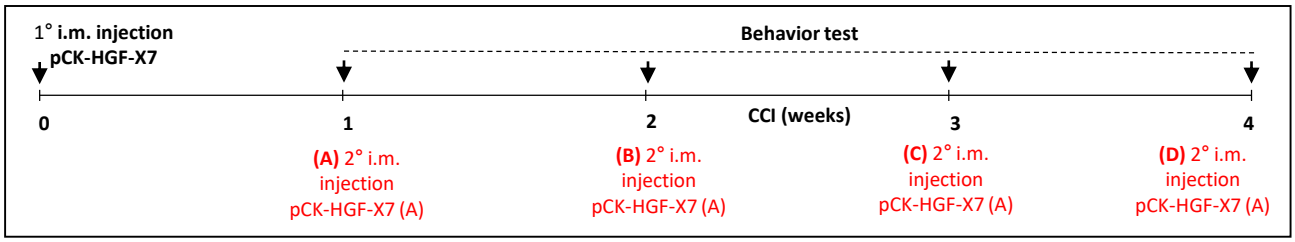


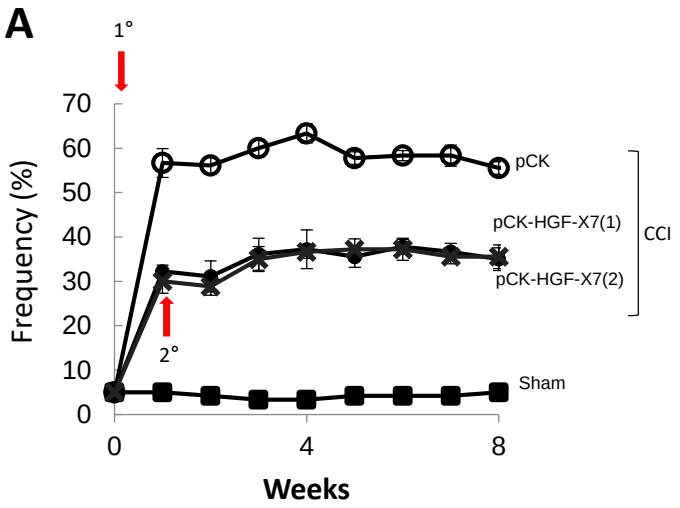
Figure III-4. Effect of pCK-HGF- X7 on baseline sensory function. PBS, pCK, or pCK-HGF-X7 (200 µg) were intramuscularly introduced to naïve mice, and the effect of plasmid injection on baseline pain function was measured by **(A)** von Frey filaments and **(B)** Hargreaves tests (n = 6 for each group; one-way ANOVA).

When the second injection of pCK-HGF-X7 was introduced at 1 or 2 weeks after the initial injection, there was no significant influence in the paw withdrawal frequencies **(Fig. III-5A, and B)** compared to the single pCK-HGF-X7-injected group. However, when the second pCK-HGF-X7 injection was introduced either 3 or 4 weeks after the initial injection, we observed further enhancement in the analgesic effect was observed with pCK-HGF-X7 (**Fig. III-5C and D)**. These data suggested that the additional injection of pCK-HGF-X7 at appropriate times could further improve the pain-relieving effect.

2.6. Effect of pCK-HGF-X7 injection 1~2 weeks after the induction of CCI (therapeutic model)

In prior experiments, pCK-HGF-X7 was injected at the same time when CCI was given. To mimic the clinical situation in this study, pCK-HGF-X7 was injected after the initial event of nerve injury. The purpose of this study was to confirm that pCK-HGF-X7 not only prevents pain production, but also shows effective amelioration of neuropathic pain in therapeutic model. The administration time after CCI varied, ranging from 1 to 4 weeks. When pCK-HGF-X7 was administered 1 week after CCI, the pain-reducing effect became noticeable one week after the injection and lasted up to 4 weeks (**Fig. III-6A and B)**. Similarly, when pCK-HGF-X7 was injected 2 weeks after CCI, the analgesic effect was clear one week after the initial injection, which also lasted up to 4 weeks. Taken together, pCK-HGF-X7 was effective in therapeutic model of neuropathic pain, where the plasmid is administered 1 or 2 weeks after peripheral nerve injury, and its anti-nociceptive effect becomes evident 1 week after the injection and lasts up to 1 month.





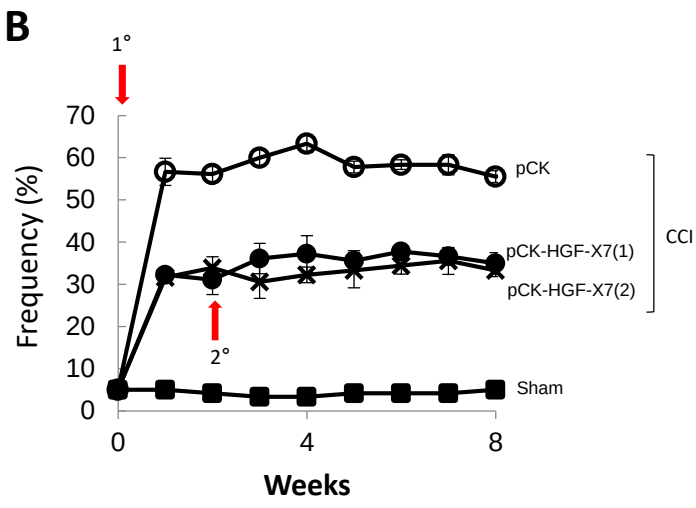
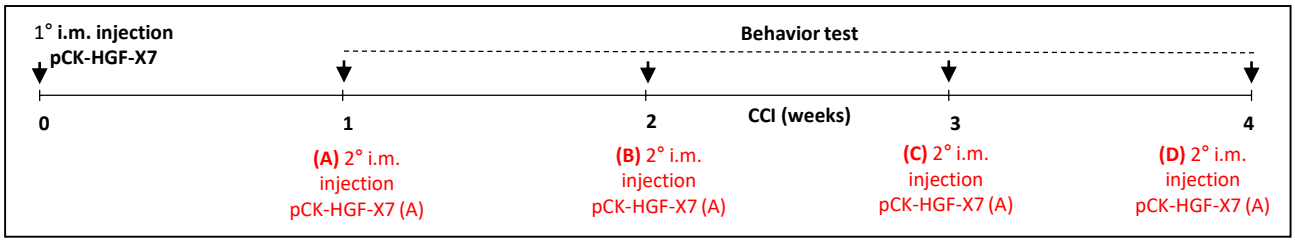
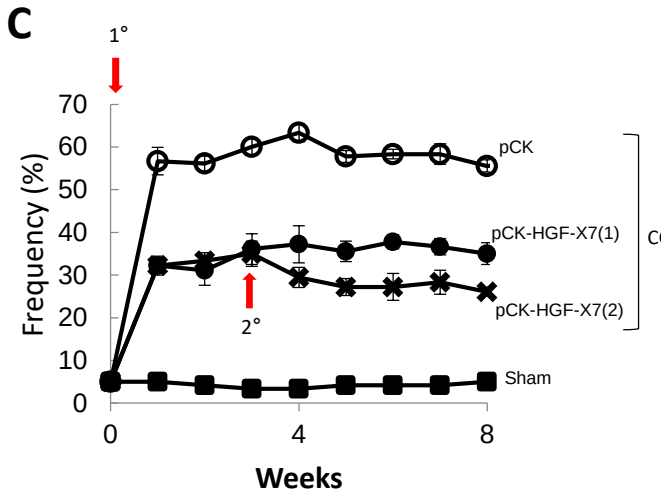


Figure III-5. Effect of repeated administration of pCK-HGF-X7 on CCI-mediated neuropathic pain. pCK and pCK-HGF-X7 (200 µg) were delivered via *i.m* injection on the day of CCI, and the second injection was introduced, as indicated by the red arrows, after **(A)**1 week and **(B)** 2 weeks**.** Pain sensitivity to a non-noxious mechanical stimuli was measured by von Frey filaments (n = 6 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way ANOVA). Sham-operated (■); CCI + pCK (○); CCI + pCK-HGF-X7(1) (●); CCI + pCK-HGF-X7(2) (**X**).





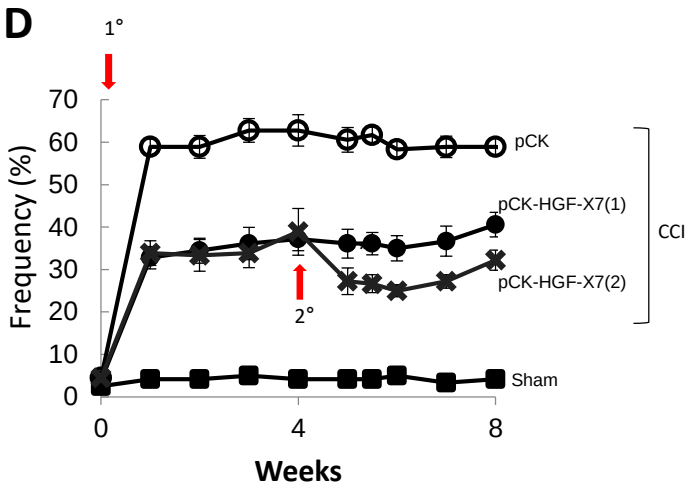
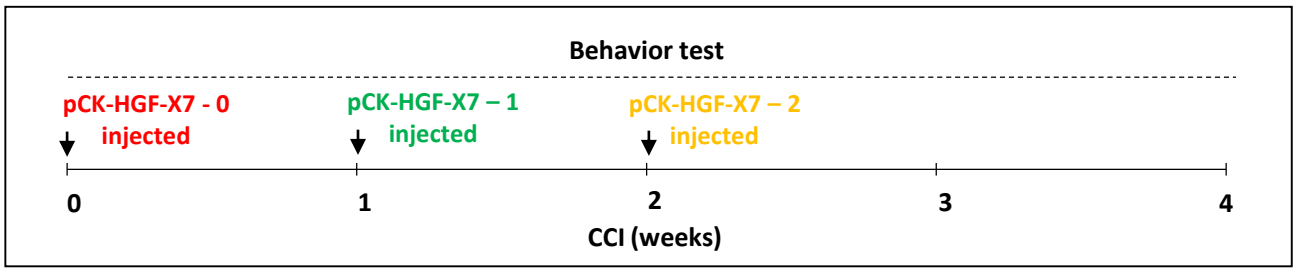
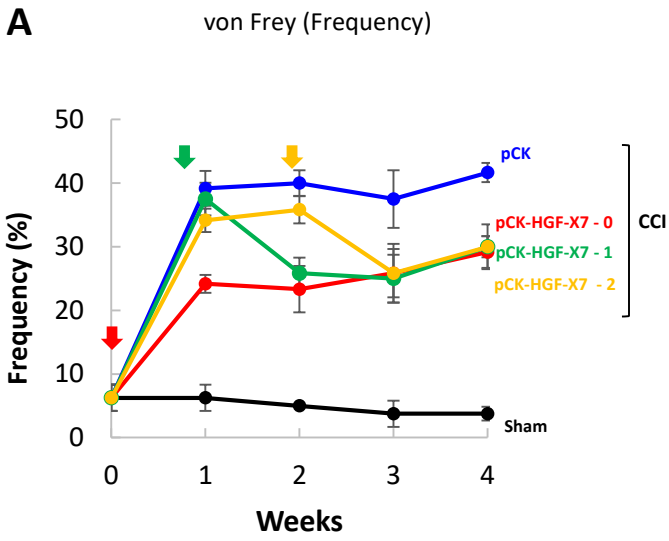


Figure III-5. Effect of repeated administration of pCK-HGF-X7 on CCI-mediated neuropathic pain. pCK and pCK-HGF-X7 (200 µg) were delivered via *i.m* injection on the day of CCI, and the second injection was introduced, as indicated by the red arrows, after **(C)** 3 weeks and **(D)** 4 weeks. Pain sensitivity to a non-noxious mechanical stimuli was measured by von Frey filaments (n = 6 for each group; mean + SEM; *, p<0.05; **, p<0.01; ***, p<0.001; two-way ANOVA). Sham-operated (■); CCI + pCK (○); CCI + pCK-HGF-X7(1) (●); CCI + pCK-HGF-X7(2) (**X**).





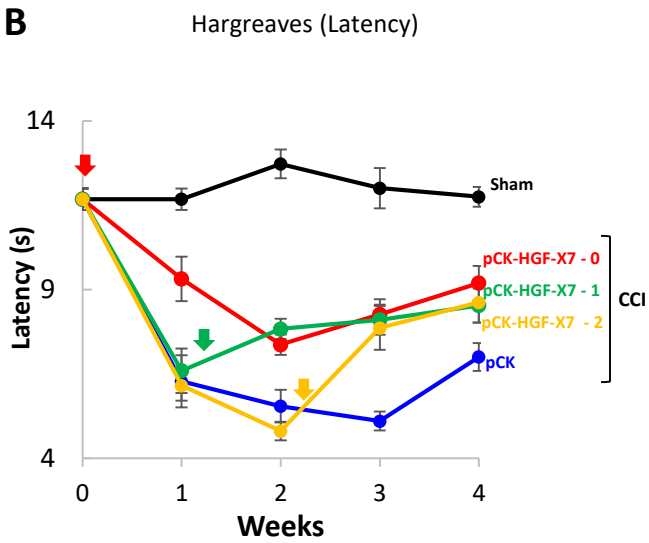


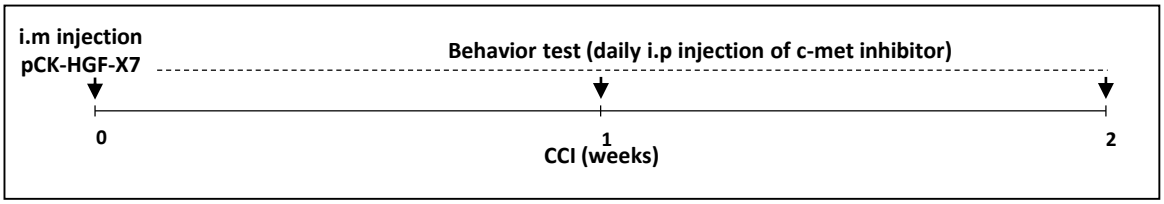
Figure III-6. Effect of pCK-HGF-X7 injection 1~2 weeks after the induction of CCI. 200ug of pCK or pCK-HGF-X7 was delivered intramuscularly 0-2 weeks after nerve injury, as indicated with colored graphs. Mechanical allodynia and thermal hyperalgesia was measured by von Frey filaments and Hargreaves tests filaments (n = 6 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way ANOVA). Sham-operated (●); CCI + pCK (●); CCI + pCK-HGF-X7 (0 week) (●); CCI + pCK-HGF-X7 (1 week) (●); CCI + pCK-HGF-X7 (2 week) (●)

2.6. Effect of pCK-HGF-X7 and c-met inhibitor (PHA665752) on CCI-induced neuropathic pain

To further validate the critical role of HGF in the suppression of nerve injury-induced neuropathic pain, I used c-Met inhibitor, PHA665752 was used in CCI model. The inhibitor was delivered via daily intraperitoneal (i.p) injection in the presence of pCK or pCK-HGF-X7. As expected, the mechanical withdrawal frequencies were increased upon CCI and pCK injection, and the application of PHA665752 in pCK-treated group had no effect on CCI-induced pain. However, the delivery of PHA665752 in pCK-HGF-X7 injected group was able to reverse pCK-HGF-X7-induced inhibition of allodynia at 1~2 weeks after CCI (**Fig. III-7A and B)**. These results suggested that HGF/c-Met signaling might be critical in the suppression of neuropathic pain, and that HGF is essential in producing anti-nociceptive effect in CCI-induced neuropathic pain.

2.7. Effect of pCK-HGF-X7 on streptozotocin-induced neuropathic pain

To investigate whether pCK-HGF-X7 shows efficacy in other neuropathy models, the effect of pCK-HGF-X7 was evaluated using streptozotocin model. Streptozotocin is a compound that has specific toxicity towards pancreatic β cells, and it is known to promote diabetic neuropathy and neuropathic pain. In this model, C57/BL6 strain mice were given streptozotocin intraperitonealy on a daily basis for 2 weeks to establish neuropathic pain, and pCK or pCK-HGF-X7 was administered intramuscularly to test mechanical allodynia using von Frey filaments. The pain-relieving effect of pCK-HGF-X7 in STZ-induced neuropathic pain became evident 6 weeks after injection, and lasted up to 12 weeks (**Fig. III-8)**. These results suggested that pCK-HGF-X7 could effectively control STZ-induced neuropathic pain, and might be used to treat a variety of neuropathif pan as well as diabetic peripheral neuropathy (DPN).



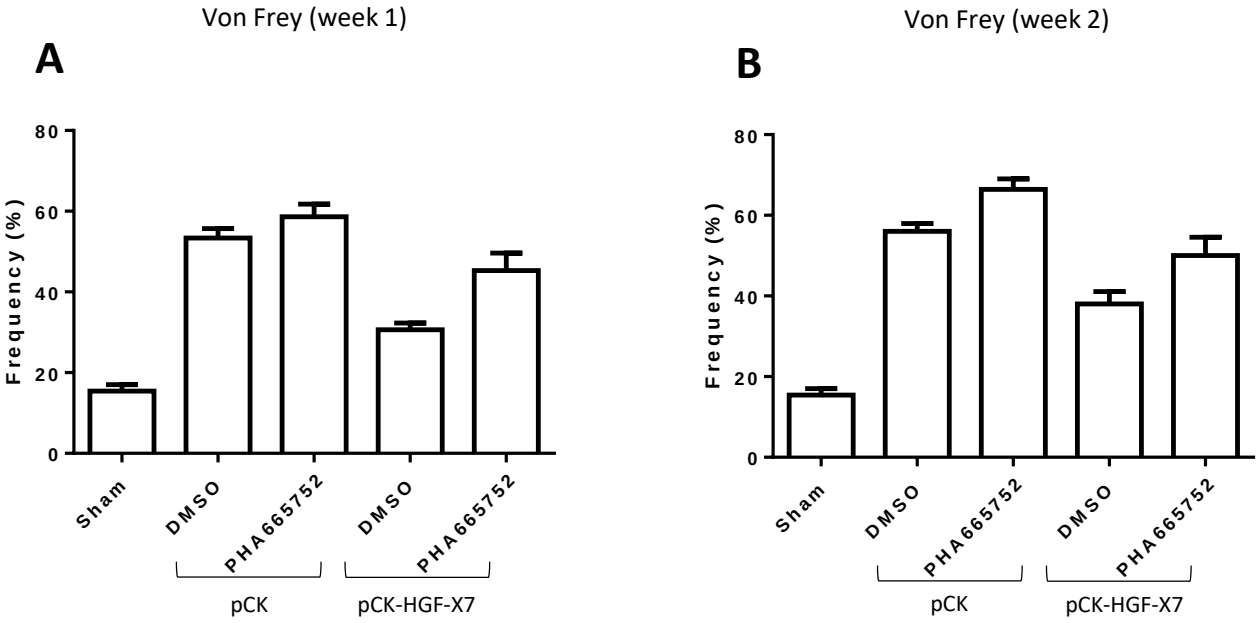
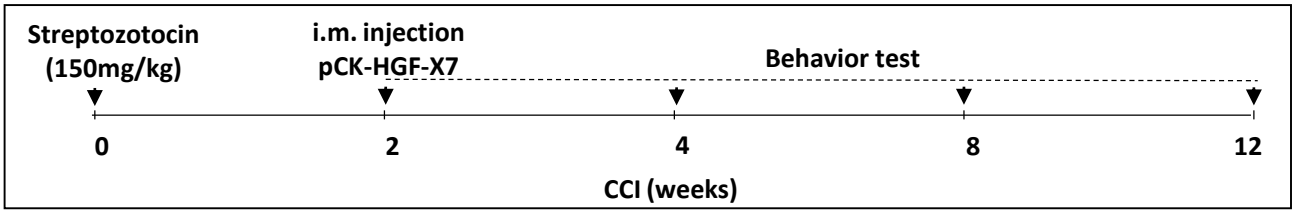


Figure III-7. Effect of pCK-HGF-X7 and c-Met inhibitor (PHA665752) on CCI-induced neuropathic pain. pCK or pCK-HGF-X7 was intramuscularly injected on the day of CCI, and c-Met inhibitor (PHA665752, 15mg/kg) was treated daily via intraperitoneal injection. Pain sensitivity towards mechanical stimuli was assessed using von Frey filaments test after (A) 1 week, and (B) 2 week.



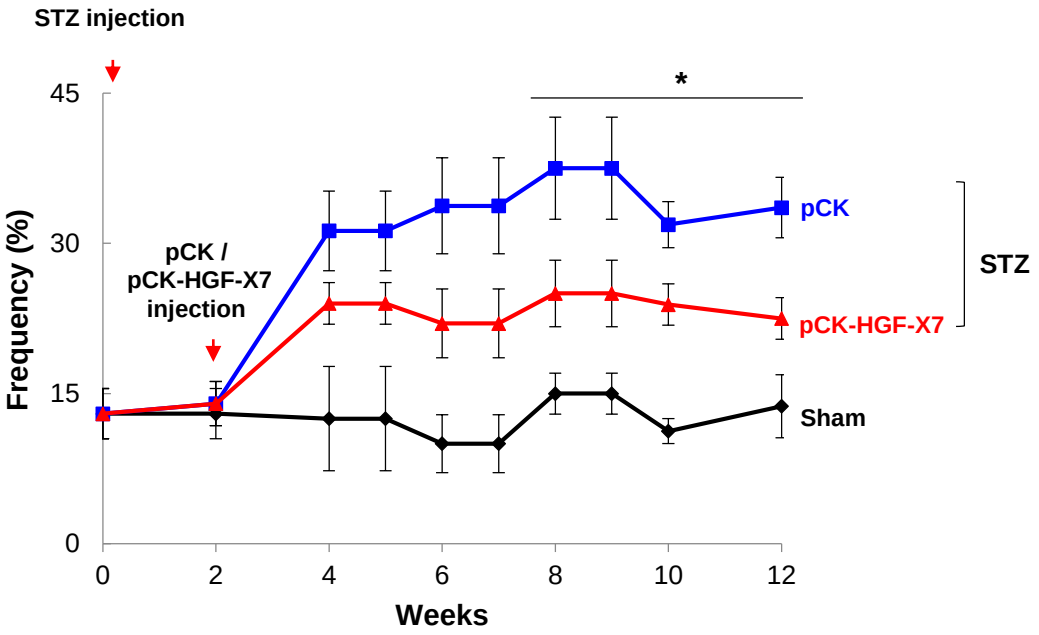


Figure III-8. Effect of pCK-HGF-X7 on streptozotocin-induced neuropathic pain. Adult mice were given CCI, and daily injection of STZ. After 2 weeks, pCK or pCK-HGF-X7 was intramuscularly injected, and pain sensitivity towards mechanical stimuli was assessed using von Frey filaments test for up to 12 weeks. (n = 6 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way ANOVA). Sham-operated (●); CCI + pCK (●); CCI + pCK-HGF-X7 (●).

3. Discussion

HGF is an ideal candidate for the development of therapeutic agents targeting the control of neuropathic pain and nerve regeneration, as it has been shown to possess potent angiogenic and neurotrophic properties. In this study, I have shown that the *i.m.* injection of plasmid DNA expressing both isoforms of HGF, pCK-HGF-X7, could effectively reduce neuropathic pain in a mouse CCI model. These results are consistent with data from clinical studies involving the same plasmid DNA.

Dose response of pCK-HGF-X7 showed an interesting bell- or U-shaped curve. The analgesic effect of pCK-HGF-X7 on neuropathic pain was increased only up to 200μg, and at higher concentrations of DNA, it was gradually diminished. This result is consistent with the data from the actual phase I and phase II clinical studies involving pCK-HGF-X7 (VM202) that were conducted in patients with painful diabetic peripheral neuropathy. In these clinical studies, 8 mg/leg of pCK-HGF-X7 produced a higher level of pain reduction than 4 mg/leg, but 16 mg/leg was less effective than 8 mg/leg. Although the reason for the bell- or U-shaped dose-response curve is not yet entirely clear, our recent publication suggested that the c-met receptor is degraded when the level of HGF reaches beyond a certain point (59), as in the case of EGF and EGFR.

Considering the fact that the pain reducing effect of pCK-HGF-X7 was observed as early as 4 days after CCI and lasted up to 8 weeks, the action points of this plasmid DNA might be divided into two. During the early stage, the HGF protein released from the transfected muscle cells may be directly involved in pain reduction by interacting with c-met receptor on sensory neurons to control pain- or injury-related genes by activating its downstream signaling

pathways. On the other hand, the long-term analgesic effect of pCK-HGF-X7 may be due to the repair of the damaged nerve or the fundamental change in the pain circulatory in the CNS, as the expression of HGF from the plasmid DNA is no longer available at this late stage. Investigation of the underlying mechanism(s) are under way. Whatever the case is, the pain-reducing effect observed after 2 weeks of pCK-HGF-X7 injection should have resulted from the consequence of biological reactions triggered by exogenous HGF.

Data from the repeated-injection experiments have shown that a second injection introduced 1 or 2 weeks after the initial injection does not provide any additional effect on pain reduction. When the repeated injection was performed 3 or 4 weeks later, however, the pain-relieving effect of pCK-HGF-X7 was further increased compared to that of the group which only received a single injection on the day of CCI. One possible explanation is that the HGF production from the initial injection ceases after 2 weeks, and therefore a second injection administered only after this time point might be effective.

Since pCK-HGF-X7 was also effective in STZ-induced neuropathic pain, it suggests that pCK-HGF-X7 not only suppresses pain, but also may have an effect on axon regeneration. STZ damages pancreatic β cells, which leads to hypoinsulinemia and hyperglycemia that give rise to peripheral nerve damage (60). The fact that pCK-HGF-X7 shows anti-allodynic effect in both models of CCI and STZ implies the involvement of pCK-HGF-X7 in the regeneration processes of injured nerves. Moreover, our group recently published a paper reporting that HGF plays a critical role in nerve regeneration via activating repair Schwann cells, supporting this notion (30).

In summary, the results from this chapter showed that an *i.m.* injection of plasmid DNA expressing HGF could produce sustained neuropathic pain relief in mice after CCI. Together with the data from phase I and phase II studies for PDPN involving the same plasmid DNA, pCK-HGF-X7 appears to have the potential to become a candidate for the next generation of therapeutic agents for neuropathic pain.

Chapter IV

Effect of hepatocyte growth factor on the expression of cellular genes involved in nerve injury and pain generation

1. Background

Peripheral nerve injury leads to extensive change in the gene expression profile within the peripheral and central nervous system. Nociceptors are rapidly activated due to inflammatory mediators that are released at the site of injury, which promotes the recruitment of various immune cells including macrophages, mast cells, and neutrophils. Furthermore, these chemical transducers in the periphery leads to transcriptional reprogramming of the injured DRG neurons. Out of many injury-induced genes that contributes to chronic pain, we have chosen a few key pain-related factors, such as ATF3, α2δ1, CSF1, and 5-HTT, to investigate the effect of pCK-HGF-X7 on the expression of these genes.

Activating transcription factor 3 (ATF3) is a well-known marker for injured sensory neurons (61), and its level is known to be dramatically increased in the nucleus of DRG neurons upon peripheral nerve injury. It is a member of ATF/cyclic AMP responsive element binding family of transcription factor and it is known as a classical responsive gene towards stressful stimuli. ATF3 expression are usually quite low in normal neurons and glia, but their expression is rapidly upregulated in response to peripheral nerve injury, where the expression increases as early as in 12 hours, and remains at high levels for almost up to 2 weeks. For these reasons above, ATF3 is considered as a standard marker for pathological insults in peripheral nerves and its expression level is closely related to the state of injury.

Another pain-related factor includes calcium channel subunit, α2δ1, in which numerous transgenic approaches have identify α2δ1 as a key modulator in mediating neuropathic pain. α2δ1 knockout mice have shown behavioral deficits towards mechanical and cold sensitivity via involving alterations in calcium influx within the nervous system. The gabapentinoid drugs,

gabapentin and pregabalin, are known to target α2δ1, and they are first-line treatment option for various neuropathic pain that reduces neuronal excitability and seizures.

The serotonin reuptake transporter, 5-HTT is also known to be involved in the transmission of neuropathic pain. 5-HTT knockout mice was shown to display a major deficit in sensitivity towards thermal hyperalgesia (62). One of the major anti-depressant drug, Cymbalta, targets 5-HTT, and has been used as another first-line treatment for diabetic peripheral neuropathic pain that is known to provide pain-relief. Serotonin reuptake transporters were reported as a key component that regulates the expression level of norepinephrine and serotonin in the synaptic cleft thereby controlling the pain transmission circuitry within the spinal cord.

Complex inflammatory response within the nervous system is another mechanism that initiates severe pain in the periphery. Inflammation is a response of an organism towards tissue injury that activates immune cells to release various mediators. Different causes of neuropathic pain are known to involve excessive inflammation in both peripheral and central nervous system. Inflammatory mediators including cytokines and chemokines are released to promote undesirable sensitization of nociceptors generating pain symptoms. Therefore, there have been many attempts to target cellular proteins involved in neuroinflammation in order to develop potential therapeutic agents for neuropathic pain

Major inflammatory cytokines that are known to be released in the case of nerve injury include IL-1β, IL-6, TNF-α, and CSF1. These cytokines are involved in the invasion of immune cells that interacts with neurons and glia to provide complex networking of these cells to provoke persistent pain. In particular, proinflammatory cytokines produced upon nerve injury are known to contribute to macrophage infiltration into the DRG, and they are involved in the development

of pain hypersensitivity. Similarly, microglia recruitment is also evident in the dorsal horn spinal cord after peripheral nerve injury. It was recently reported that CSF1 is the key factor that is upregulated in the ipsilateral DRG, and is transported to the spinal cord that leads to activation and proliferation of microglial cells (15). All of these inflammatory responses are major factors that leads to the genesis of neuropathic pain.

For the reasons above, the transcriptional changes of key pain mediators and inflammatory cytokines were investigated in the presence or absence of pCK-HGF-X7 after peripheral nerve injury. Microarray analysis was performed to identify genes with altered expression upon nerve injury and pCK-HGF-X7 injection. Based on the results from microarray analysis, several key factors were selected and investigated in detailed. In this chapter, it was described that the expression of ATF3, α2δ1, and 5-HTT is inhibited after the administration of pCK-HGF-X7. In addition, pCK-HGF-X7 treatment reduced neuroinflammation that actively occurs within the PNS and CNS after nerve injury. Taken together, these data suggested that *i.m.* injection of pCK-HGF-X7 could effectively reduce neuropathic pain, presumably by modulating the expression of injury- or pain-related factors and thus controlling the complex pain transmission circuitry.

2. Results

2.1. Microarray analysis

To get an overview of the overall gene expression profile in the context of nerve injury and pCK-HGF-X7, I performed preliminary microarray analysis using RNAs isolated from DRGs of mice with *i.m* injected plasmid DNAs at different time points. Male ICR mice were given chronic constriction injury via making loose ligatures in the sciatic nerve, and 200μg of pCK or pCK-HGF-X7 were intramuscularly injected in the area of *bicep femoris* and *quadriceps femoris* muscles. At 12h, 1, 4, 7 days after CCI, the injured L4-6 DRGs were collected, and analyzed by microarray experiment. When comparing injured DRG neurons with the control DRGs, approximately 200 of 40000 genes (approx. 0.5%) exhibited more than 2-fold change from the baseline. In addition, 45 of 200 genes were shown to be affected by the administration of pCK-HGF-X7. Out of these genes, nerve injury-related genes were selected, and further comparison studies were performed between different time points.

At the acute stage of nerve injury (12 h post-CCI), the injury-associated factors were slightly upregulated (~1.5-fold increase) within the injured DRG neurons. At 1 day after injury, the stress responsive transcription factors such as ATF3 and Jun were upregulated by approximately 3-fold. These transcription factors are known to induce pain-promoting factors, such as neuropeptide Y (NPY), galanin (gal) and sprr1A, involved in the pathogenesis of neuropathic pain (18, 63). As shown in Fig. IV-1B, the level of injury-induced ATF3 and Jun was reduced by pCK-HGF-X7, when pCK-HGF-X7 was injected, indicating that pCK-HGF-X7 might also inhibit NPY and Gal expression at later time points. As expected, at day 4, the expression level of pain-associated neuropeptides, NPY and Gal, was increased by nerve injury,

and they were lowered in the pCK-HGF-X7 group. These results imply that pCK-HGF-X7 may regulate the expression of early stress response transcription factors (ATF3), and thereby controlling the induction level of pain-mediating factors (NPY and gal). Furthermore, at this late time point, the regulation of channels involved in nociception was noticeable, especially 5-HTT (serotonin reuptake transporter) and cacnα2δ1 (calcium channel subunit α2δ1). These channels are well-known mediators of neuropathic pain, and indeed are the major targets of pain killers such as Pregabalin (Pfizer) and Cymbalta (Eli Lily), respectively. Later in this chapter, further experiments were performed in order to analyze in detail the role of ATF3, α2δ1, and 5-HTT in the development of pain and the effect of pCK-HGF-X7 on these factors.

The inflammatory response genes were also shown to be affected by nerve injury and pCK-HGF-X7. Especially, the expression level of IL-6 and CSF1 was noticeably altered on day 4 and 7. However, it was interesting to observe no significant change in the level of IL-1β and TNFα. In the case of IL-6 and CSF1, they were clearly induced by CCI and suppressed by the treatment of pCK-HGF-X7 at various time points. Based on the results from microarray analysis, in this chapter, I investigated the overall neuroinflammatory responses after CCI and/or pCK-HGF-X7 injection.

2.2. Effect of pCK-HGF-X7 on the expression of pain-related factors

<u>ATF3</u>

ATF3 is one of the most frequently used marker in pain research, which is known to be highly upregulated in DRG neurons upon peripheral nerve injury. In this chapter, the expression level of ATF3 in ipsilateral DRG neurons after CCI and pCK-HGF-X7 injection was investigated. CCI was performed as a model for nerve injury and 200μg of pCK-HGF-X7 was

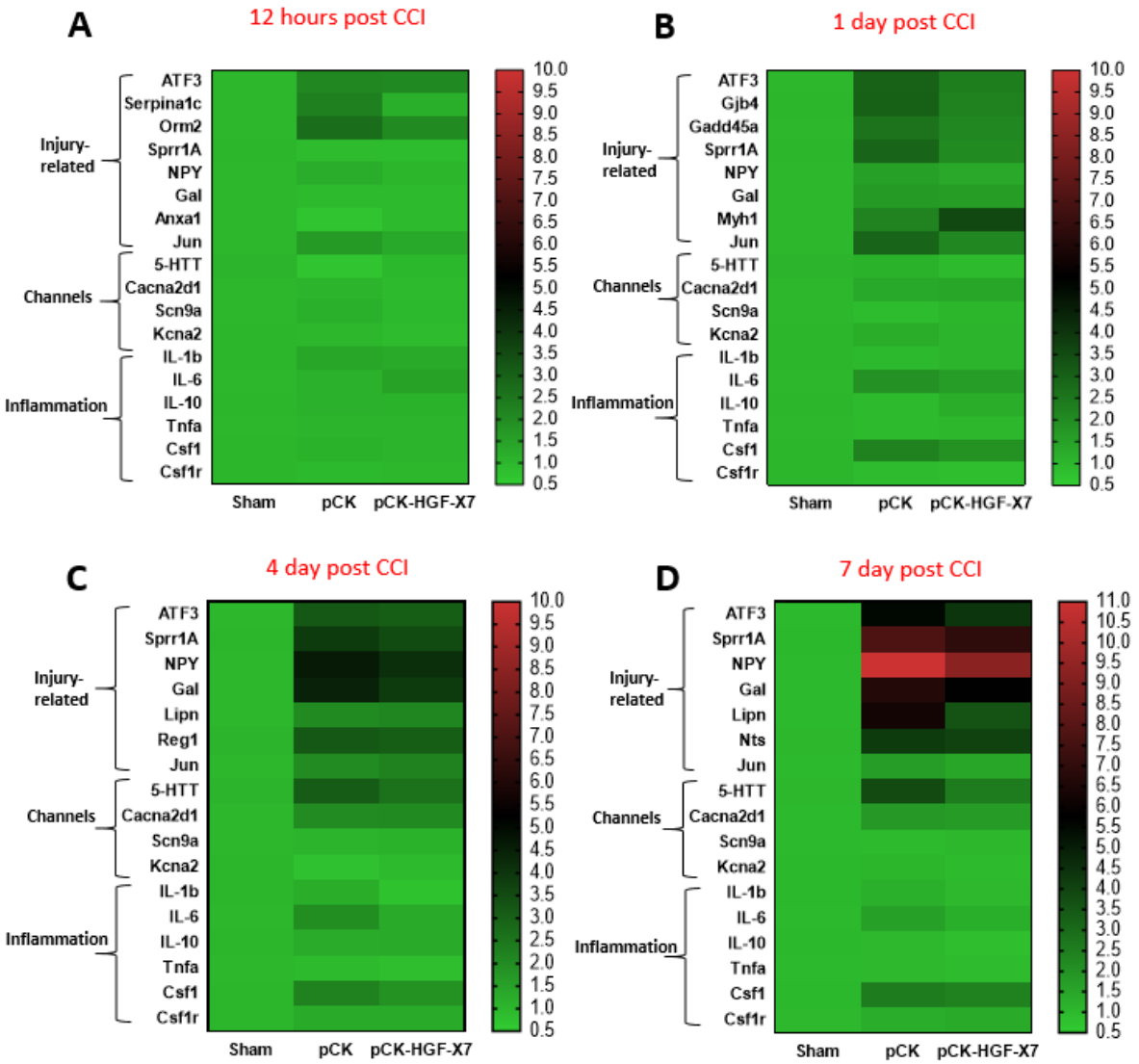


Figure IV-1. Microarray analysis of ipsilateral DRG neurons. *I.m* injection of pCK or pCK-HGF-X7 (200μg) and CCI was introduced, and the injured DRGs (L4-6) were isolated at appropriate time points, followed by microarray analysis, at **(A)** 12 hours **(B)** day 1, **(C)** day 4 and **(D)** day 7 post CCI. (n = 4 for each group, pooled)

administered into the thigh muscles. RNAs were isolated from L4-L6 DRGs and the RNA levels of ATF3 was measured at various time points including day 1, 4, 7, 14, and 28 after CCI (**Fig. IV-2A**). In sham animals, the RNA level was slightly increased on day 1, presumably due to muscle incision, but returned to its basal level. In CCI-induced mice injected with pCK control, the RNA level of ATF3 was upregulated by 22-fold, reaching a peak on day 4. When mice were administered with pCK-HGF-X7, the ATF3 level was reduced by 44% (day 4) and 63% (day 7) compared to pCK control at a given time. By day 28, the expression of ATF3 was close to its basal level in both pCK and pCK-HGF-X7 treated groups. To be certain, the protein level of ATF3 was also analyzed on day 4. The protein level of ATF3 was very low in the sham-operated animals (**Fig. IV-2B, lane 2**), but was significantly increased in the CCI-operated mice (**Fig. IV-2B, compare lanes 2 and 4**). The ATF3 expression level of the control plasmid pCK-injected, CCI-operated mice was comparable to that of the PBS-injected, CCI-operated mice, indicating that *i.m.* pCK injection per sei had little effect on ATF3 expression (**Fig. IV-2B, compare lanes 4 and 6**). When mice were injected with pCK-HGF-X7, however, the injury-induced increase in ATF3 expression was markedly inhibited (**Fig. IV-2B, compare lanes 6 and 8**). Similar trends were also observed when ATF3 western blot analysis was quantified using Image J (**Fig. IV-2C).** In immunostaining assay, ATF3 was scarcely detected in the sham surgery mice, but was highly increased in L4-L5 DRG neurons of CCI-induced and pCK-injected mice (**Fig. IV-2D)**. Treatment with pCK-HGF-X7 suppressed CCI-induced ATF3 expression 4 days after CCI (**Fig. IV-2D and E)**. Next, I have also confirmed the effect of pCK-HGF-X7 in a therapeutic model, where CCI was initially induced, and pCK-HGF-X7 is injected after 1 week of injury. pCK-HGF-X7 was also effective in downregulating the expression of ATF3 even if it was injected 1 week after nerve injury. This strongly supports the notion that pCK-HGF-X7 may be used in

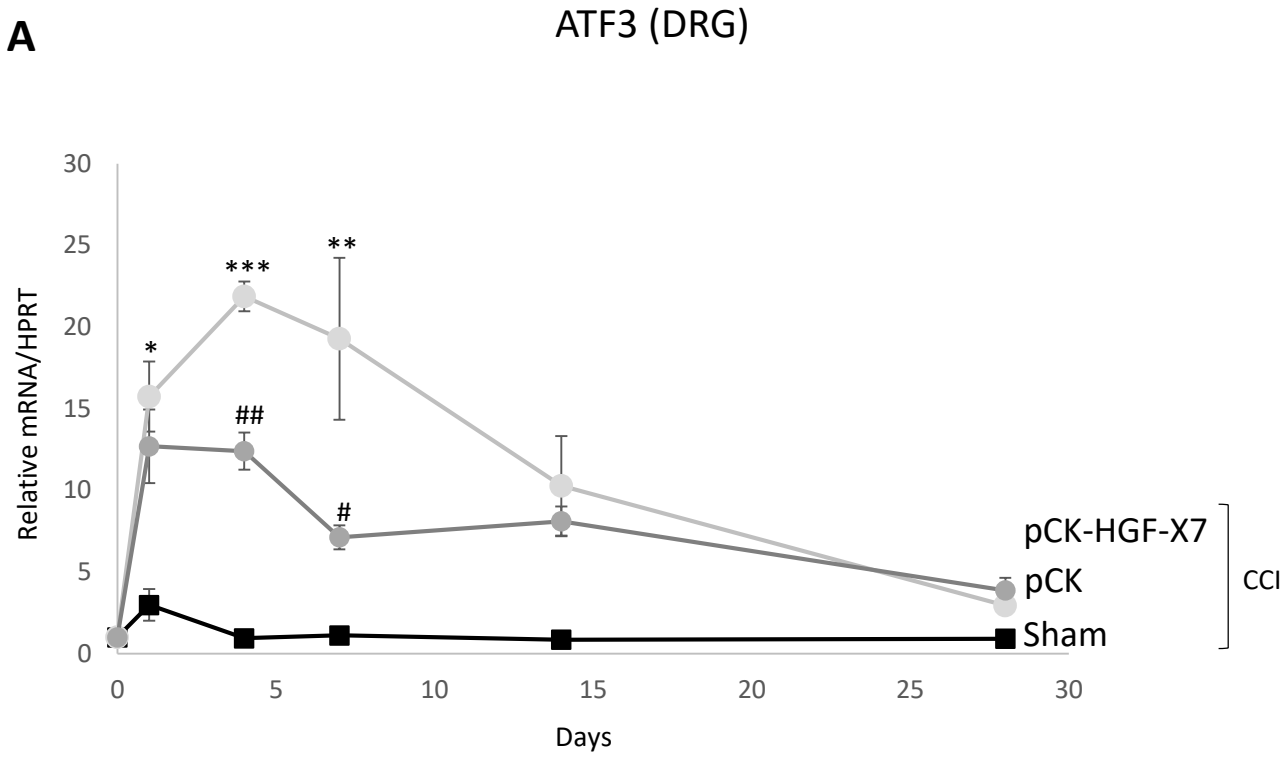


Figure IV-2. Effect of pCK-HGF-X7 on the expression of ATF3 in ipsilateral DRG neurons. *I.m.* injection of 200 μg of pCK or pCK-HGF-X7 and CCI were introduced, and the injured DRGs (L4-6) were isolated on 1, 4, 7, 14, and 28 days later. Total RNAs were prepared to perform **(A)** RT-qPCR. The level of mRNA was normalized to the expression of the contralateral DRG, and HPRT was used as housekeeping gene (n = 3 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with sham; one-way ANOVA; scale bar, 50 μm).

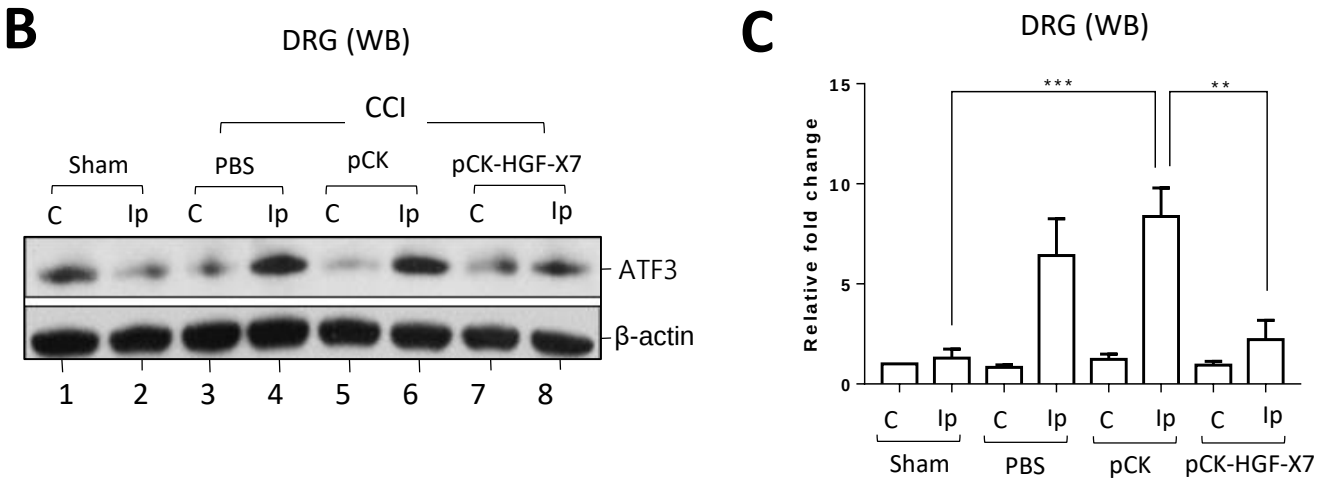
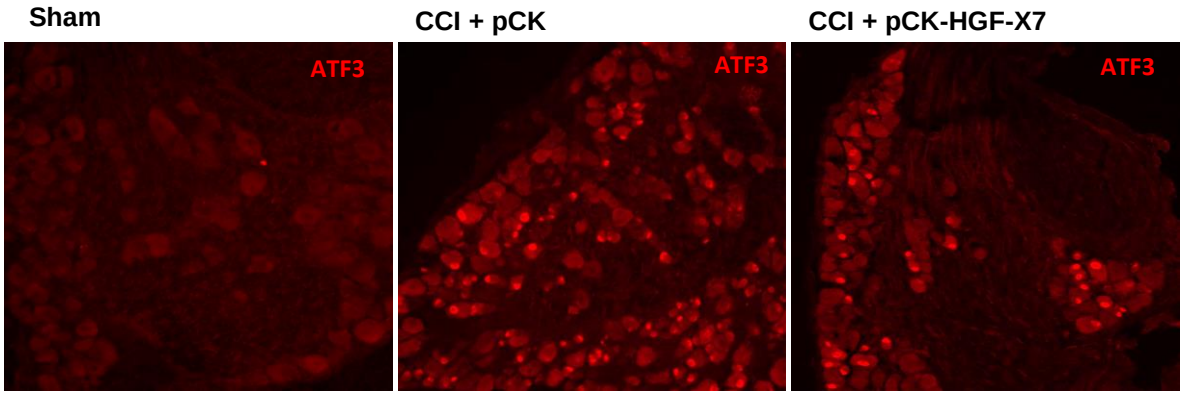


Figure IV-2. Effect of pCK-HGF-X7 on the expression of ATF3 in ipsilateral DRG neurons. *I.m.* injection of 200 μg of pCK or pCK-HGF-X7 and CCI were introduced, and the injured DRGs (L4-6) were isolated 4 days later. Total proteins were prepared to perform **(B, C)** Western blot using antibody specific to ATF3. (n = 3 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with sham; one-way ANOVA; scale bar, 50 μm). In Western blot analysis, β-actin was used as a loading control (C = contralateral; Ip = ipsilateral).

D



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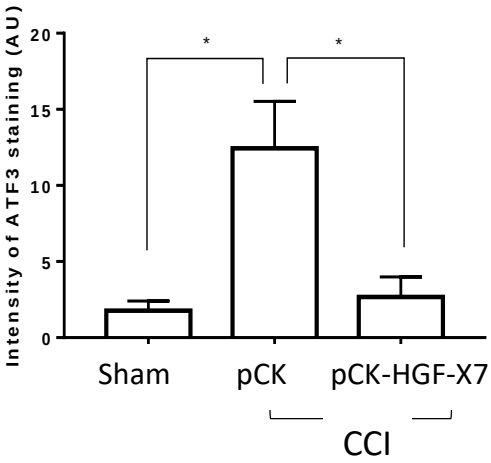

Figure IV-2. Effect of pCK-HGF-X7 on the expression of ATF3 in ipsilateral DRG neurons. *I.m.* injection of 200 μg of pCK or pCK-HGF-X7 and CCI were introduced, and the injured DRGs (L4-6) were isolated 4 days later. DRG sections were prepared to perform **(D)** immunostaining using antibody specific to ATF3. (E) Quantification of ATF3 positive staining. (n = 3 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with sham; one-way ANOVA; scale bar, 50 μm).

clinical cases of neuropathic pain. All of these results indicated that the *i.m.* injection of pCK-HGF-X7 could protect DRG neurons from severe nerve damage, which might have halted or reversed the neuropathic pain and disease progression as observed in the previous experiments.

α2δ1

Calcium channel subunit, α2δ1, is one of the major proteins that are known to conduct pain signals in nociceptors. They are also known as a cellular target of the most frequently prescribed drugs, Pregabalin, for painful diabetic peripheral neuropathy (PDPN). Therefore, the expression pattern of α2δ1 was analyzed using qRT-PCR and Western blot in the ipsilateral DRG neurons and sciatic nerves, 4 days after CCI. The RNA level of α2δ1 in the injury-induced mice was increased by 9-fold compared to the sham-operated mice (**Fig. IV-3A**). However, in the pCK-HGF-X7-treated mice, the level of α2δ1 was decreased by 40~50%. To confirm the decreased expression of α2δ1 at the protein level, Western blot analysis was performed. The level of the α2δ1 protein was increased in the injured DRG and sciatic nerve upon CCI (**Fig. IV-3B and D, compare lanes 2 with 4 or 6**). However, it was decreased in pCK-HGH-X7-injected mice (**Fig. IV-3D and E, lanes 6 and 8**). Taken together, these results suggested that HGF produced from pCK-HGF-X7 might downregulate α2δ1 expression and decrease synaptic transmission of pain circuitry, which may partly contribute to the analgesic effects of pCK-HGF-X7.

5-HTT

As mentioned earlier, 5-HTT is a serotonin reuptake transporter that is known to be involved in the generation of neuropathic pain. It is a cellular target protein of duloxetine, which is a frequently used pain killers for painful DPN. The level of 5-HTT was investigated after

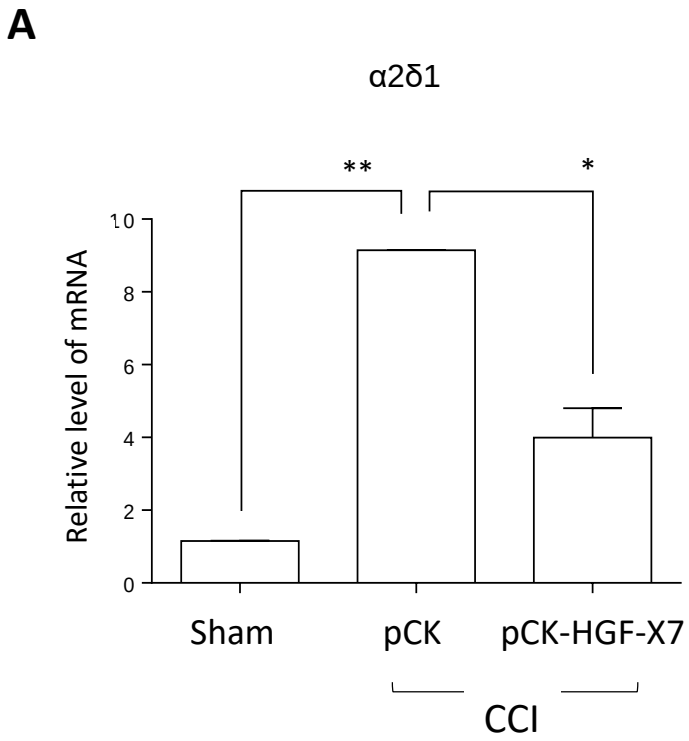

Figure IV-3. Effect of pCK-HGF-X7 on the expression of α2δ1 in ipsilateral DRG neurons. *I.m* injection of pCK or pCK-HGF-X7 (200 μg) and CCI were performed, and the affected DRGs were prepared 4 days later for **(A)** RT-qPCR The level of mRNA was normalized to the expression of the contralateral DRG, and HPRT was used as housekeeping gene (n = 3 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with sham; one-way ANOVA).

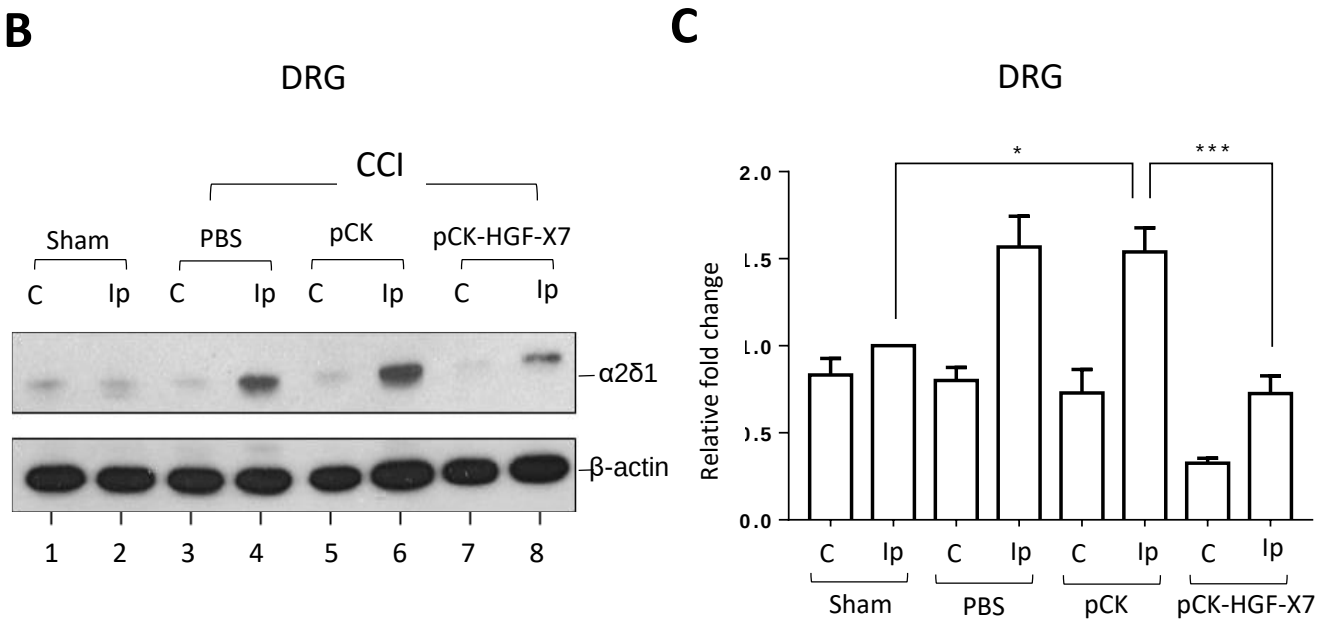

Figure IV-3. Effect of pCK-HGF-X7 on the expression of α2δ1 in ipsilateral DRG neurons.

I.m injection of pCK or pCK-HGF-X7 (200 μg) and CCI were performed, and the affected DRGs were prepared 4 days later for **(B, C)** Western blot analysis, respectively. (n = 3 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with sham; one-way ANOVA). In Western blot analysis, β-actin was used as a loading control (n = 3 for each group; C = contralateral; Ip = ipsilateral).

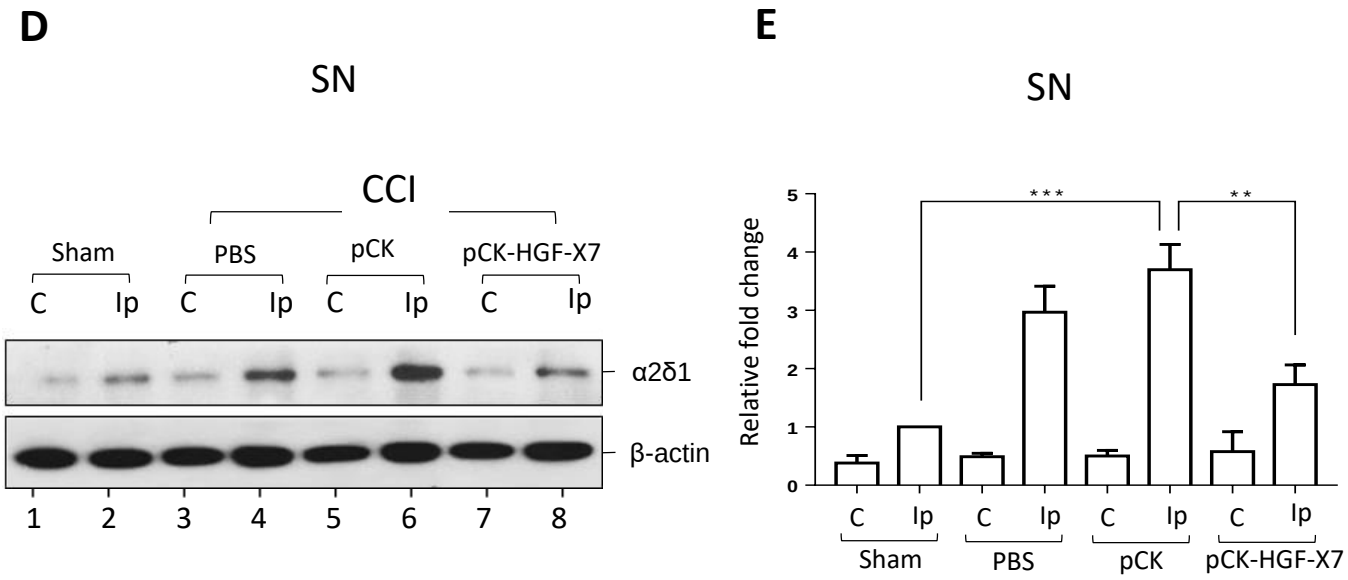

Figure IV-3. Effect of pCK-HGF-X7 on the expression of α2δ1 in ipsilateral DRG neurons.

I.m injection of pCK or pCK-HGF-X7 (200 μg) and CCI were performed, and the affected sciatic nerves were prepared 4 days later for **(D, E)** Western blot analysis. (n = 3 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with sham; one-way ANOVA). In Western blot analysis, β-actin was used as a loading control (n = 3 for each group; C = contralateral; Ip = ipsilateral).

nerve injury in ipsilateral DRG neurons. As shown in **Fig. IV-4A**, the level of 5-HTT was highly increased in injured DRG neurons upon CCI, but it was significantly reduced in the pCK-HGF-X7 treated group. The RNA level of 5-HTT was also tested in therapeutic model, where CCI is given, and plasmid DNA is injected 1 week after nerve injury (**Fig. IV-4B**). RNAs were prepared for RT-qPCR 4 days after the injection, which is a total of 11 days after the initial nerve injury. In this post-injection model, pCK or pCK-HGF-X7 injection again reduced injury-mediated induction of 5-HTT expression. Downregulation of 5-HTT by HGF produced from the plasmid may be another factor contributing to pain-relieving effect of pCK-HGF-X7.

2.3 Effect of pCK-HGF-X7 on inflammatory responses after CCI

After peripheral nerve injury, both immune cells and non-immune cells are involved in the production of pro-inflammatory and anti-inflammatory cytokines, which contributes to the development of neuropathic pain. Thus, the regulation of inflammatory responses after peripheral nerve injury is a crucial process in the understanding of the pathology of neuropathic pain. Here, the control of inflammatory reactions within the ipsilateral DRG neurons were investigated, and the role of pCK-HGF-X7 in the regulation of these pain-inducing inflammatory cytokines were studied.

<u>IL-6</u>

Data from microarray analysis indicated that the level of IL-6 was decreased when pCK-HGF-X7 was introduced to mice after CCI. To confirm this results, quantitative RT-PCR was performed. Total RNAs were isolated from L4-6 DRGs in the pCK or pCK-HGF-X7 injected group at days 1, 4, 7, and 14. In sham-operated animals, the level of IL-6 was maintained at the basal level. In CCI-operated and pCK-injected groups, however, the level of IL-6 was markedly

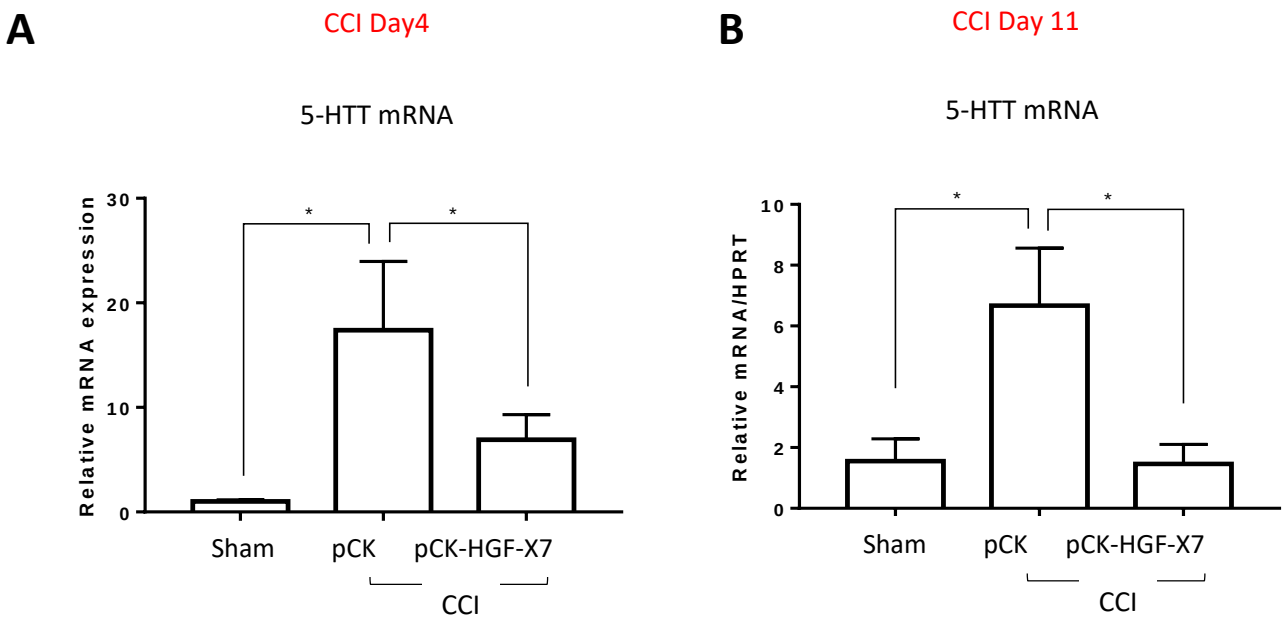

Figure IV-4. Effect of pCK-HGF-X7 on serotonin reuptake transporter (5-HTT) in ipsilateral DRG neurons. *I.m.* injection of 200 μg of pCK or pCK-HGF-X7 and CCI were introduced, and the injured DRGs (L4-6) were isolated at appropriate times. Total RNAs were extracted for RT-qPCR, and the expression of 5-HTT was analyzed. The level of mRNA was normalized to the expression of the contralateral DRG, and HPRT was used as housekeeping gene (n = 4 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with sham; #, $p<0.05$; ##, $p<0.01$ compared with CCI + pCK group; one-way ANOVA)

increased at days 1 and 4 in the ipsilateral DRG neurons after nerve injury, and gradually decreased through days 7 and 14. The expression levels of IL-6 was generally lower in the pCK-HGF-X7 treated mice up to day 7, suggesting that HGF produced from this plasmid suppressed the expression of IL-6 in the injured DRG neurons.

To test whether the reduced level of IL-6 indeed resulted from HGF expressed from pCK-HGF-X7, the activity of HGF was blocked by intraperitoneal injection of PHA665752, a selective inhibitor for its receptor c-met, for 4 days after nerve injury and plasmid injection. The expression level of IL-6 was increased by 8-fold on day 4 after CCI (**Fig. IV-5B**), and pCK-HGF-X7 administration reduced the induction level by approximately 45%. When mice were treated with PHA665752, the effect of pCK-HGF-X7 on IL-6 suppression was inhibited, to an extent that the IL-6 level became comparable between pCK-HGF-X7 and pCK group.

CSF1

CSF1 has recently been discovered as a key factor that contributes to neuropathic pain by promoting activation and proliferation of microglial cells in the spinal dorsal horn. The expression level of CSF1 in ipsilateral DRG was measured 4 days after CCI. Data from quantitative RT-PCR analysis indicated that CSF1 was highly upregulated in the injured sensory neurons, by approximately 15 fold (**Fig. IV-6**). *I.m.* injection of pCK-HGF-X7 inhibited the CCI-mediated upregulation of CSF1 by approximately 50%, and similar levels of inhibition were observed when the CSF1 protein levels were analyzed by ELISA (**Fig. IV-6A and B**). In addition, CSF1 was highly induced in the therapeutic model, and when pCK-HGF-X7 was injected 1 week after CCI, the injury-induced CSF1 expression was still suppressed in injured DRG neurons (**Fig. IV-6C)**. These results suggested that HGF produced from pCK-HGF-X7

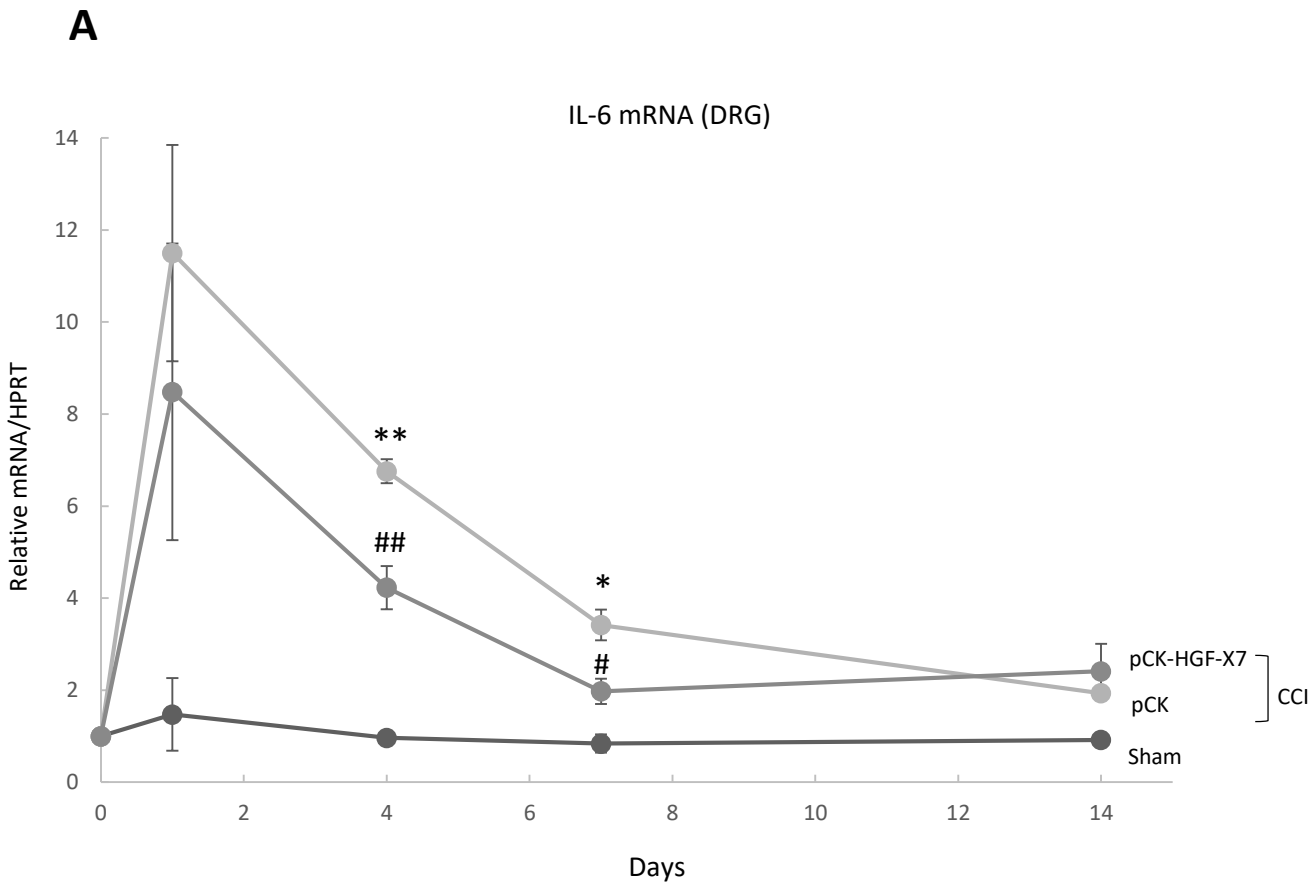

Figure IV-5 . Effect of pCK-HGF-X7 on IL-6 expression in ipsilateral DRG neurons. *I.m.* inj ection of 200 μg of pCK or pCK-HGF-X7 and CCI were introduced, and the injured DRGs (L4-6) were isolated 4 days later. Total RNAs were prepared to perform RT-qPCR, and (A) the level of I L-6 expression was measured. The level of mRNA was normalized to the expression of the contra lateral DRG, and HPRT was used as housekeeping gene (n = 4 for each group; mean + SEM; *, p <0.05; **, p<0.01; ***, p<0.001 compared with sham; #, p<0.05; ##, p<0.01 compared with CCI + pCK group; one-way ANOVA)

B

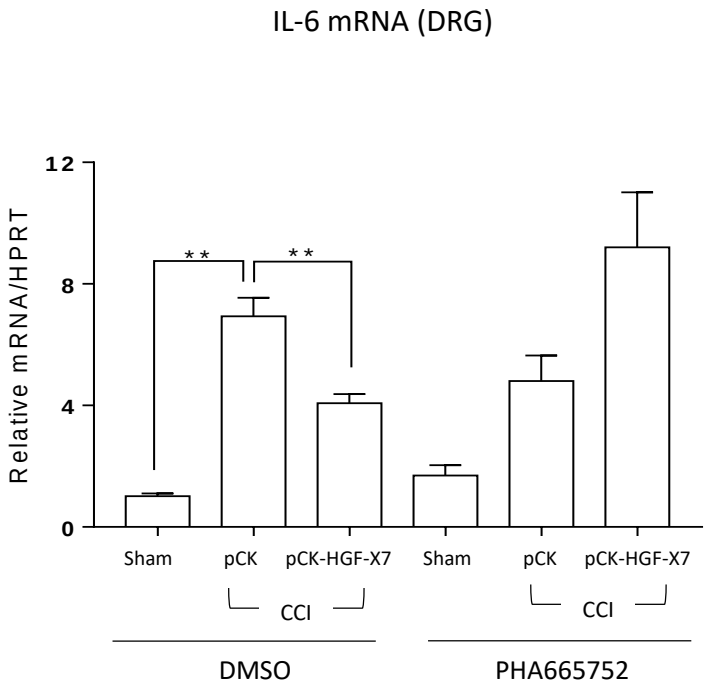

Figure IV-5. Effect of c-Met inhibitor on CCI-induced expression of IL-6 after pCK-HGF-X7 injection and CCI. *I.m.* injection of 200μg of pCK or pCK-HGF-X7 and CCI were introduced, and PHA665752 (15mg/kg) was intraperitoneally injected on a daily basis. The injured DRGs (L4-6) were isolated 4 days later, and total RNAs were extracted for RT-qPCR. The level of mRNA was normalized to the expression of the contralateral DRG, and HPRT was used as housekeeping gene (n = 4 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; one-way ANOVA)

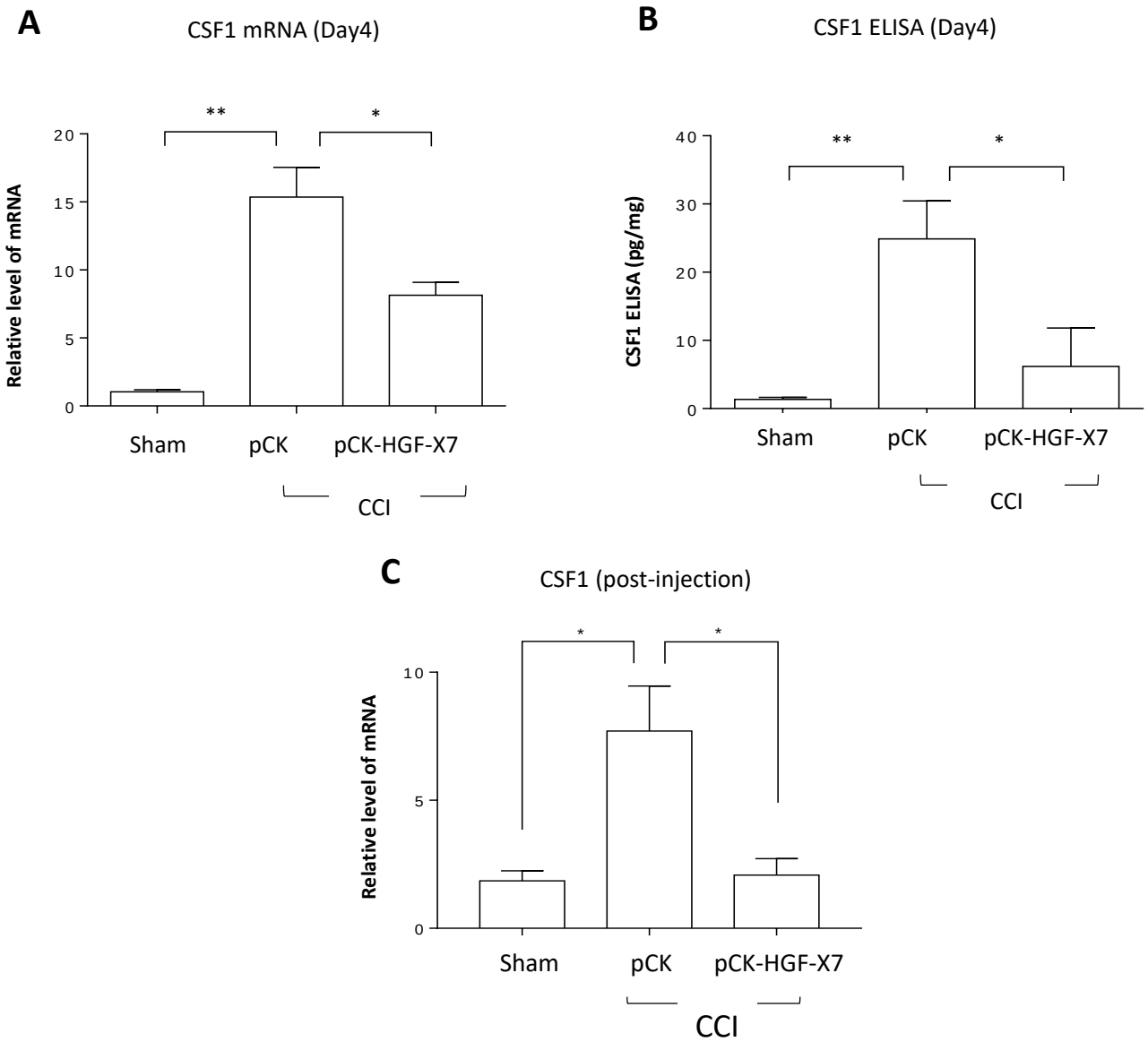

Figure IV-6. **Effect of pCK-HGF-X7 on CSF1 expression in injured DRG neurons**. CCI was introduced, and 200 μg of pCK/pCK-HGF-X7 were transferred via *i.m.* injection, simultaneously. The ipsilateral DRGs (L4-6) were isolated 4 days later. Total RNAs and proteins were prepared for **(A)** RT-qPCR and **(B)** ELISA specific for CSF1, respectively. The level of mRNA was normalized to the expression of the contralateral DRG, and HPRT was used as housekeeping gene (n = 3 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with sham; one-way ANOVA).

might exert its analgesic effects at least partly by downregulating CSF1 expression in the DRG.

2.4 Effect of pCK-HGF-X7 on distribution of inflammatory immune cells

Macrophage infiltration to DRG

Neuroinflammation is defined as immune responses that occur within the nervous tissues, and it is known to contribute to the development of neuropathic pain. It is usually associated with activation of glial cells and infiltration of immune cells. Here, I investigated the effect of pCK-HGF-X7 on infiltrating macrophages into the ipsilateral DRG neurons after nerve injury. In lumbar 4-5 DRG neurons of sham-operated mice, Iba-1 positive macrophages were barely detected, while its number being increased by 3-fold in the injured DRG neurons 4 days after CCI (**Fig. IV-7A)**. When CCI-induced mice were administered with pCK-HGF-X7, however, the level on injury-induced macrophage infiltrations became similar to that of sham (**Fig. IV-7A and B**). On the other hand, such suppression of macrophage infiltration by pCK-HGF-X7 injection was inhibited by the administration of PHA665752, implying the involvement of HGF/c-Met in immune cell infiltration (**Fig. IV-7C and D**). These data indicated that pCK-HGF-X7 delivered intramuscularly could abrogate neuroinflammatory responses such as macrophage infiltration.

Glial cell pattern in spinal cord

Several cell types that reside in the spinal cord, such as astrocytes and microglia, have been implicated in the development of central sensitization via complex modulation of pain transmission circuitry (64). In particular, it was recently reported that CSF1 produced from the injured DRG neurons could be transported to the dorsal horn spinal cord and induces microglial activation and proliferations, leading to the development of neuropathic pain and central

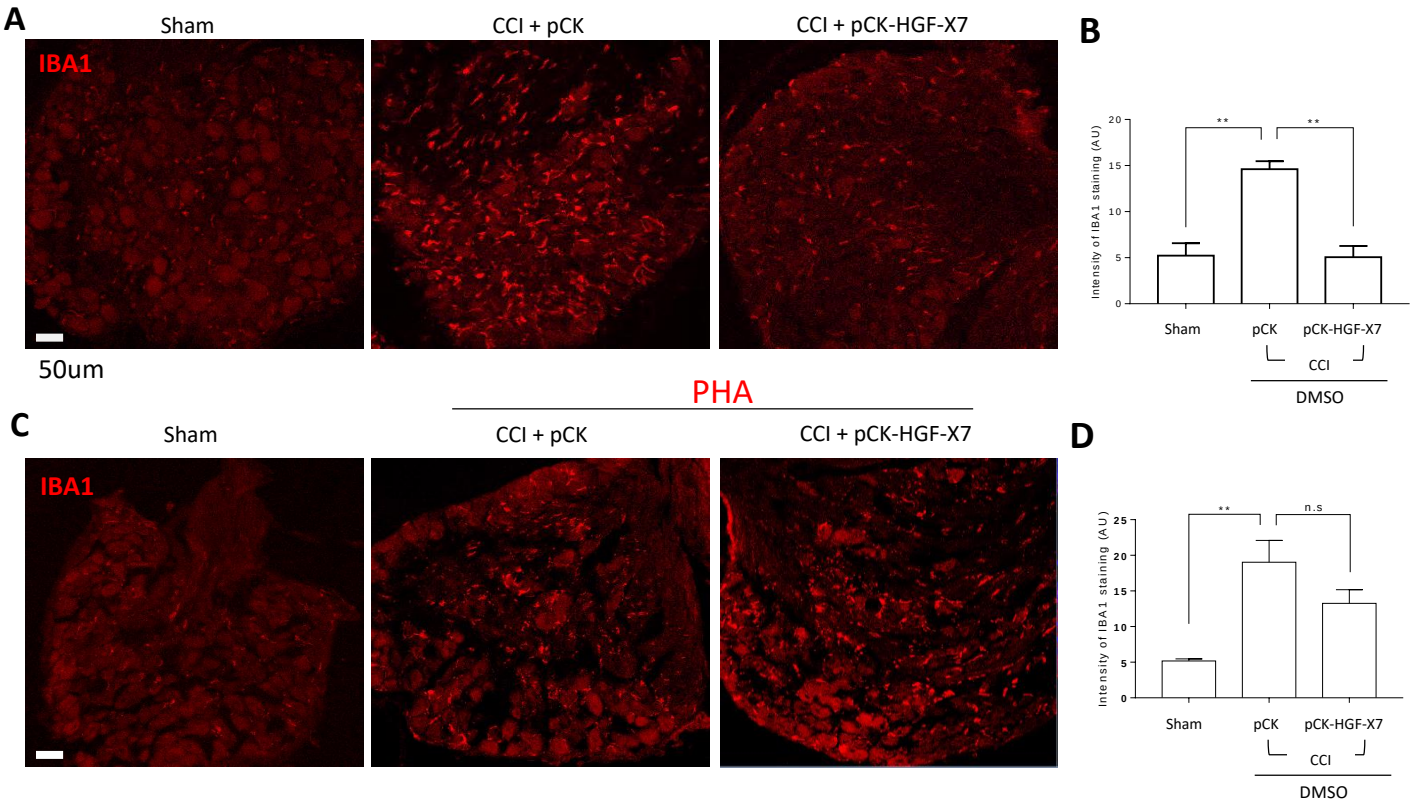

Figure IV-7. Effect of pCK-HGF-X7 on macrophage infiltration in ipsilateral DRGs. CCI and *i.m.* injection of 200 μg of pCK or pCK-HGF-X7 were performed. The affected DRG neurons were removed 4 days later, and cryocut sections were prepared. DRG sections (A) without PHA665752 treatment and (B) with PHA665752 treatment were subjected to IHC assay, using antibodies specific to Iba1 (in red; marker for macrophage) (n=4; scale bar, 50 μm).

sensitization (15, 65). To test if pCK-HGF-X7 plays any roles in this pathway, the microglia activation in the spinal cord dorsal horn was studied 4 days after CCI. As shown in **figure IV-8**, the density of microglia in sham-operated animals was comparable between contralateral and ipsilateral sides of the spinal cord. However, there was a substantial increase in the number of Iba1-positive microglia in the ipsilateral dorsal horn after CCI, consistent with other reports (15). Furthermore, the morphology of the microglia present in the ipsilateral side was readily distinguishable from that of sham mice. For example, in sham-operated mice, the microglial cells seemed to be at the "quiescent" stage with ramified structure, whereas in the CCI-induced mice, the Iba1-positive cells were transformed into the "activated" morphology amoeboid-like shape (66). In the pCK-HGF-X7-treated group, however, the number of Iba1-positive microglia was highly reduced, and the morphology was similar to that of microglia in its "resting" state. This result correlated well with the above data showing the inhibition of injury-mediated induction of CSF1 and pain reduction by pCK-HGF-X7.

Since microglial activation is known to release ligands that also promote astrocyte activation (66-69), the effect of pCK-HGF-X7 on astrocytes was also assessed. As shown in **figure IV-9**, CCI caused a marked increase in the number of GFAP-positive astrocytes on the ipsilateral side of spinal cord dorsal horn. These changes were significantly attenuated in the pCK-HGF-X7 treated group.

To test such morphological changes in the glial cells are accompanied with actual glial cell activation, the expression of various genes involved in the glial cell activation after peripheral nerve injury such as Iba1, CSF1R, cathepsin S, IRF8, IRF5, as well as CX3CR1 was measured. Total RNAs were isolated from the dorsal horn of the spinal cord (L4-5) 4 days after

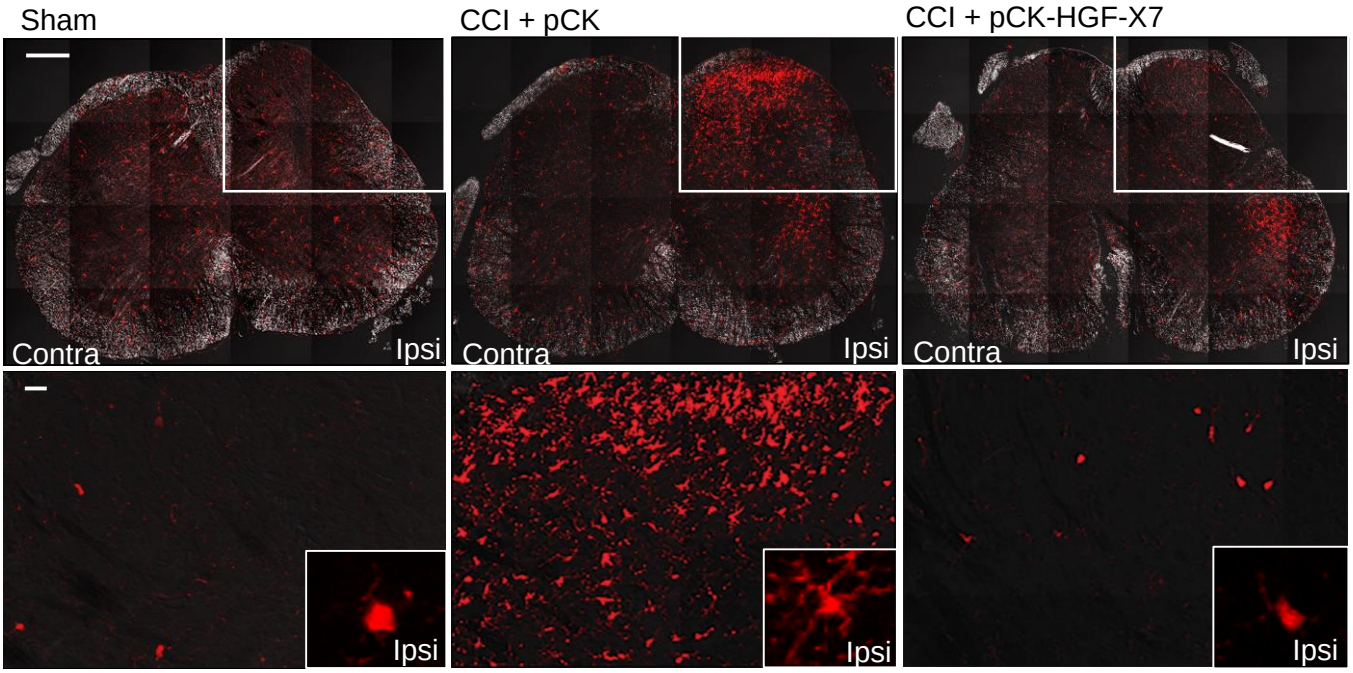

Figure IV-8. Effect of pCK-HGF-X7 on nerve injury-induced spinal cord microgliosis. CCI and *i.m.* injection of 200 μg of pCK or pCK-HGF-X7 were performed. The affected spinal cords were removed 4 days later, and cryocut sections were prepared. Spinal cord sections were subjected to IHC assay, using antibodies specific to Iba1 (in red; marker for microglia) (n=4; scale bar, 200 μm, 20 μm; Contra = contralateral, Ipsi = ipsilateral).

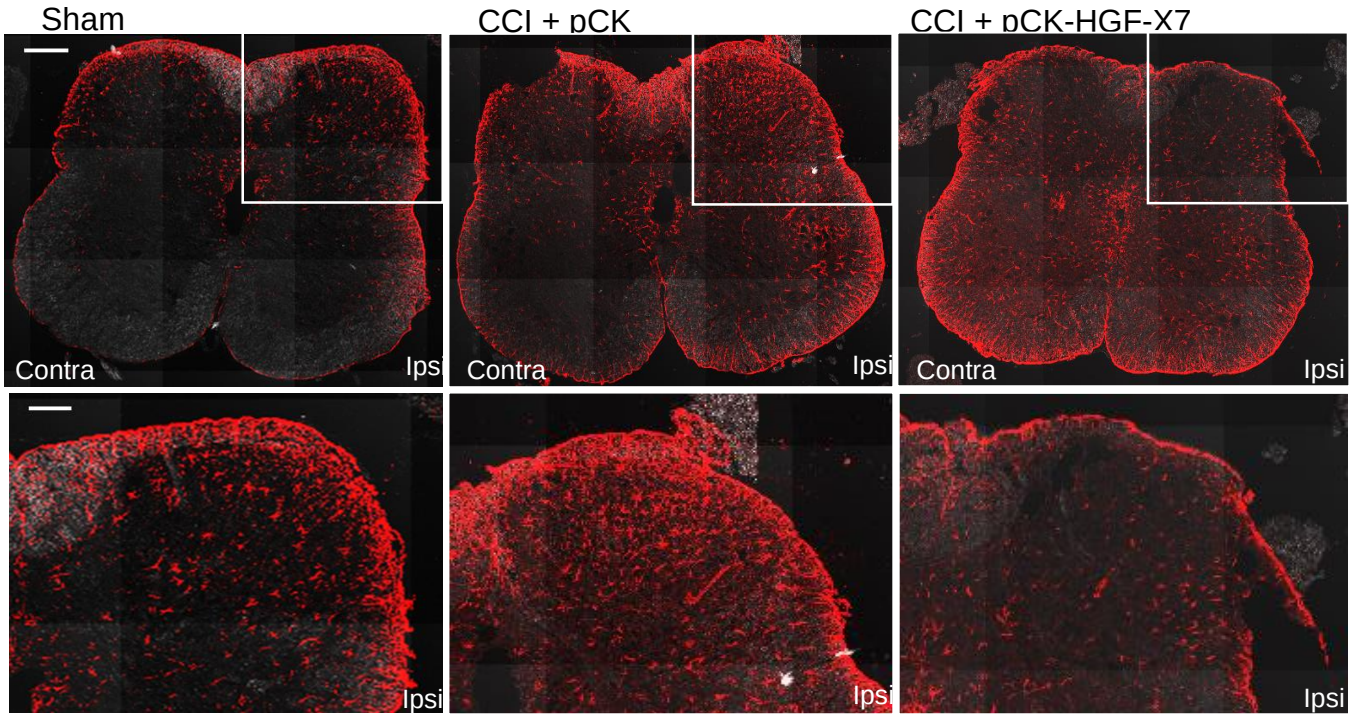

Figure IV-9. Effect of pCK-HGF-X7 on nerve injury-induced spinal cord astrogliosis. CCI and *i.m.* injection of 200 μg of pCK or pCK-HGF-X7 were performed. The affected spinal cords were removed 4 days later, and cryocut sections were prepared. Spinal cord sections were subjected to IHC assay, using antibodies specific to Iba1 (in red; marker for microglia) (n=4; scale bar, 200 μm, 20 μm; Contra = contralateral, Ipsi = ipsilateral).

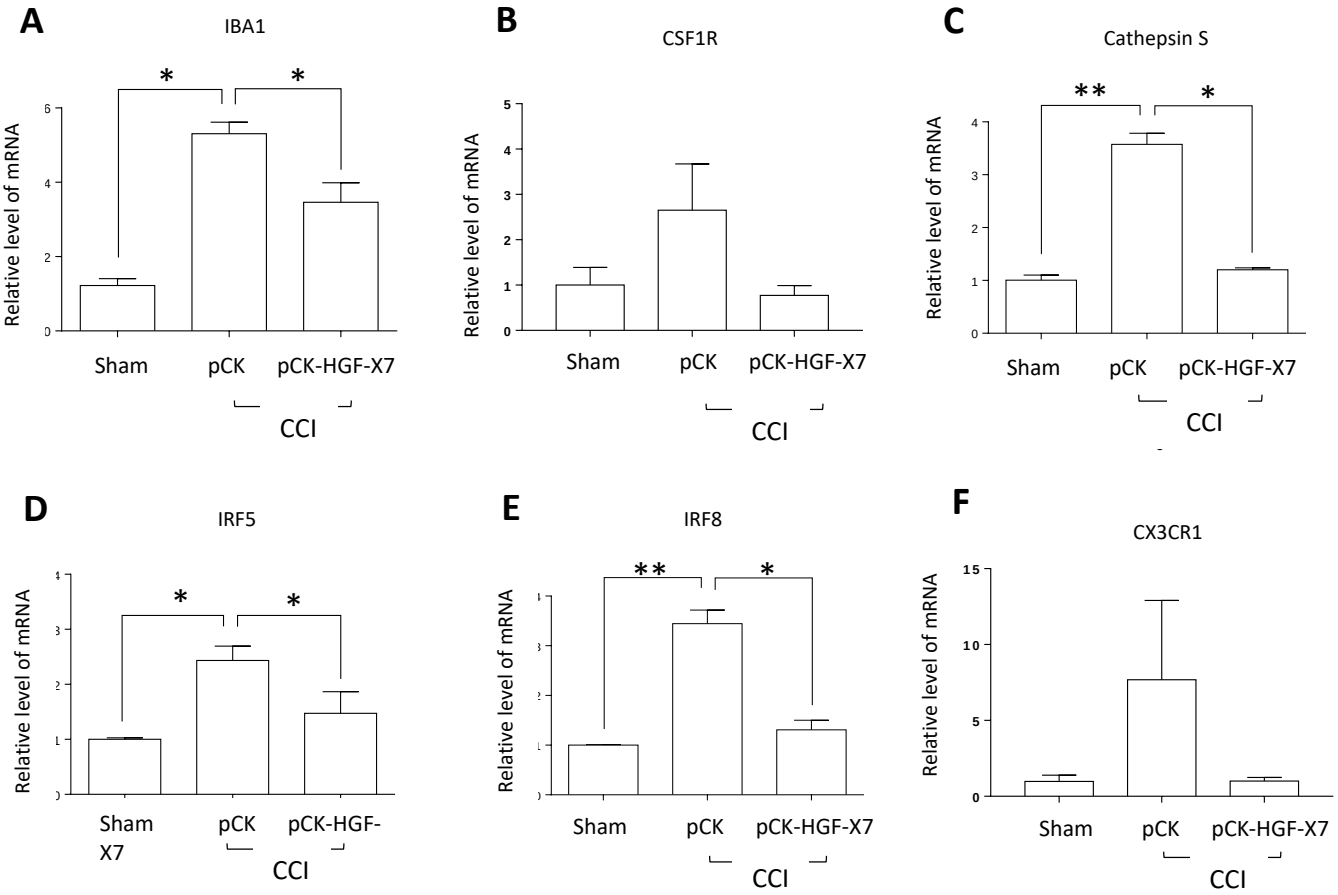

Figure IV-10. Effect of pCK-HGF-X7 on nerve injury-induced glial cell activations in ipsilateral dorsal horn. Four days after CCI and plasmid injection, the ipsilateral spinal cord dorsal horn was isolated, and total RNAs were prepared, followed by RT-qPCR analysis for respective genes. The level of mRNA was normalized against GAPDH (n = 3 for each group; mean + SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with sham; one-way ANOVA).

CCI followed by qRT-PCR (**Fig. IV-10A~F**). The RNA level of these genes associated with microglial cell activation was increased in the ipsilateral dorsal horn after CCI, but was reduced in the pCK-HGF-X7-treated group. These results suggested that the control of microglia and astrocyte activation in the spinal cord dorsal horn might be a part of the pCK-HGF-X7's pain control mechanism in the CCI model.

3. Discussion

In the previous chapter, it was shown that pCK-HGF-X7 could effectively control neuropathic pain when measured by von Frey filaments and Hargreaves tests. In this chapter, I investigated the effects of pCK-HGF-X7 on the expression of various cellular factors involved in nerve injury and neuroinflamation within the peripheral and central nervous system, specifically in the injured DRG neurons and spinal cord dorsal horn. Firstly, microarray analysis was performed to get an overview of the transcriptional changes that occur in parallel with the pathological insults and pCK-HGF-X7 injection. Based on the results from microarray analysis, I have chosen several proteins which are well-known to play important roles in the genesis of neuropathic pain. In depth analyses showed that the expression levels of various injury- or pain-related genes such as ATF3, α2δ1, and 5-HTT were changed in a manner consistent with the analgesic effect of pCK-HGF-X7. Furthermore, HGF have exerted anti-inflammatory effect in peripheral nervous system via suppressing the production of inflammatory cytokines, such as IL-6 and CSF1. *I.m.* injection of pCK-HGF-X7 also resulted in the decreased number of infiltrated macrophages in DRG neurons and activated glial cells in the dorsal horn spinal cord. Taken together, the results presented in this chapter strongly suggested that HGF produced from pCK-

HGF-X7 might effectively reduce pain in mouse CCI model by controlling the expression of pain-related genes.

ATF3, and calcium channel subunit, α2δ1, have been well established to be upregulated when peripheral nerves are damaged in various ways such as sciatic nerve ligation or transection and CCI (15, 19, 61, 65, 70). However, little has been known about how their expressions are controlled in injured sensory neurons, but results described in this chapter indicate that HGF may play a role(s) in the regulation of these pain-related markers. Promoter sequence analysis predicted that the most common nucleotide sequences shared by ATF3, and α2δ1 is the binding site for AP-1 family. AP-1 is a heterodimeric protein that has been well known to be involved in the development of neuropathic pain, dedifferentiation of Schwann cells, and regeneration of the injured neurons (71, 72). Also, it is possible that HGF may somehow affect the expression of c-fos or c-jun and/or the activity of the DNA-binding protein. Further investigations are warranted for understanding of the exact mechanism underlying the reduced level of these pain-related genes after pCK-HGF-X7 was introduced.

HGF have shown significant anti-inflammatory effect in both the peripheral and central nervous system. In particular, the CCI-induced expression of CSF1 and IL-6 was downregulated in the pCK-HGF-X7 treated group, which also implies the suppression of CCI-mediated inflammation. it was recent reported that CSF1 is highly induced in the ipsilateral sensory neurons after peripheral nerve injury, and it is transported and secreted into the dorsal horn spinal cord, leading to activation and proliferation of microglial cells through DAP12-dependent signaling pathways. IL-6 was also reported to be a typical injury-induced mediator, inducing and maintaining neuropathic pain (73). IL-6 and IL-6 receptors are known to be highly upregulated

in sensory neurons after chronic constriction injury of sciatic nerve (73-76). In this study, I have shown that the pCK-HGF-X7 treatment can downregulate the injury-induced upregulation of CSF1 and IL-6 in ipsilateral DRG neurons. The exact mechanism(s) has yet to be revealed, however, it is clear that HGF produced from pCK-HGF-X7 can effectively reduce neuropathic pain through regulation of these factors.

Macrophage infiltrations into DRG neurons plays a crucial role in the development of neuropathic pain after CCI. It is generally known that induction of chemokines after peripheral nerve injury, such as CCL2 and CCL3, is a major factor leading to the promotion of macrophage infiltrations (77). These chemokines have been shown to be regulated via IL-6, activating JAK2 (janus kinase 2)-signal transducer and STAT3 (activator of transcription) cascade. It is possible that suppressed level of IL-6 may be a result of altered downstream JAK2-STAT3 pathway, and that as a consequence, the chemokine productions were suppressed in injured DRG neurons, and the level of macrophage infiltration was lowered. Further detailed studies are warranted to understand the roles of HGF in the control of inflammatory cell infiltration.

Another factor contributing to the development of neuropathic pain is activation of glial cells, such as microglia and astrocytes. When peripheral nerves are injured, microglial cells are known to undergo proliferation and activation, releasing various cytokines and chemokines. These molecules act on respective cellular receptors present on astrocytes to stimulate various glial subtype cells (67-69). Once astrocytes acquire reactive phenotypes, they are known to contribute to the maintenance of neuropathic pain (78, 79). Results present in this chapter show a significant reduction in the number of activated microglia and astrocytes. This may be the reason why there is such a long lasting pain-relieving effect when pCK-HGF-X7 is administered.

In summary, these results showed that an *i.m.* injection of plasmid DNA expressing HGF could produce sustained neuropathic pain relief in mice after CCI. Data presented in this chapter supports the notion that HGF may exert neuroprotective effects via direct interaction with neurons and glia (80). Data from phase I and II studies for DPN demonstrated that pCK-HGF-X7 was safe and produces a remarkably high pain reducing effect (52). Results from my study indicated that the control of expression of cellular genes associated with nerve injury and neuroinflammation is one of the key mechanism(s) through which pCK-HGF-X7 exerts such potent and long lasting analgesic effects.

Chapter V

Regulation of the expression of anti-inflammatory genes by hepatocyte growth factor in primary sensory neurons and Schwann cells

1. Background

HGF is known to exert potent anti-inflammatory effects in the neuronal system, suggesting a possible role in the inhibition of neuroinflammation associated with nerve injury (81-83). In the previous chapters, I showed that HGF produced from pCK-HGF-X7 could exert anti-inflammatory effects after CCI by downregulating inflammatory cytokines in injured DRG neurons. In particular, pCK-HGF-X7 suppressed injury-induced IL-6 expression to an extent where macrophage infiltrations in ipsilateral DRG neurons were also highly reduced. pCK-HGF-X7 injection also inhibited the expression of nerve injury-induced colony stimulating factor-1 (CSF1) in the injured DRGs, which might lead to a change in the functions of microglia and astrocytes in the spinal cord. To understand the mechanism(s) underlying the anti-neuroinflammatory activity of HGF at the molecular level, I used primary sensory neurons and Schwann cells (SCs) in this chapter. The results reveal that in both primary sensory neurons and SCs, the LPS-mediated increase in the RNA levels of pro-inflammatory cytokines were effectively suppressed by treatment with recombinant human HGF protein. In particular, HGF has shown strong anti-inflammatory effects in SCs by controlling the ERK pathway.

2. Results

2.1. Expression and activation of c-Met in primary sensory neurons

Before analyzing the anti-inflammatory effects of HGF, the expression and activation of c-Met in primary sensory neurons were first analyzed using immunofluorescence and Western blot analyses. Primary DRG neurons were isolated from adult mice and grown in a condition where proliferating cells like satellite glial cells were eliminated. In immunofluorescence assay, the expression of c-Met (red) in primary sensory neurons was detected, and c-Met was shown to

co-localize with TUJ1 (green) (**Fig. V-1A)**. In Western blot analysis, it was found that the basal level of total c-Met was not changed by the treatment with 25ng/mL of recombinant HGF protein (**Fig. V-1B and C).** Phosphorylated c-Met (p-cMet) was barely detected in the absence of HGF treatment, but its level was highly enhanced when treated with 25ng/mL of recombinant HGF. Time course analysis revealed that the level of p-cMet peaked after 5 minutes of HGF stimulation and then gradually decreased to its basal level at 180 min. Furthermore, a dose-dependent increase in the intensity of p-cMet was observed after 10 min of HGF stimulation without any changes in the expression level of total c-Met (**Fig. V-1C**). Taken together, these results suggest that c-Met was expressed in primary sensory neurons, and that treatment with recombinant HGF protein highly promoted an increase in the level of phosphorylated c-Met.

2.2. Effect of HGF on LPS-induced production of pro-inflammatory cytokines in primary sensory neurons

Peripheral nerve injuries often encourage nociceptive neurons to produce pro-inflammatory cytokines, but through a mechanism that remains unknown. In chapter IV, I demonstrate that pCK-HGF-X7 is able to suppress the production of pain-causing cytokines such as CSF1 and IL-6 in injured DRG neurons. In order to investigate the anti-inflammatory effect of HGF, I have chosen to use the TLR4 agonist LPS to mimic neuroinflammatory responses of neurons producing pro-inflammatory cytokines and chemokines. DRG neurons were isolated from adult ICR mice and cultured for 3 days with cytosine arabinoside (remov-C) to remove proliferating cells (84). Primary sensory neurons were subjected to a 6-hour stimulation with an increasing dose of LPS. This resulted in significant increases in the levels of IL-1β, IL-6, TNFα, and CSF1 in a dose-dependent manner (**Fig. V-2A~D**). These results confirmed the effect of LPS on the induction of pro-inflammatory cytokines in primary sensory neurons.

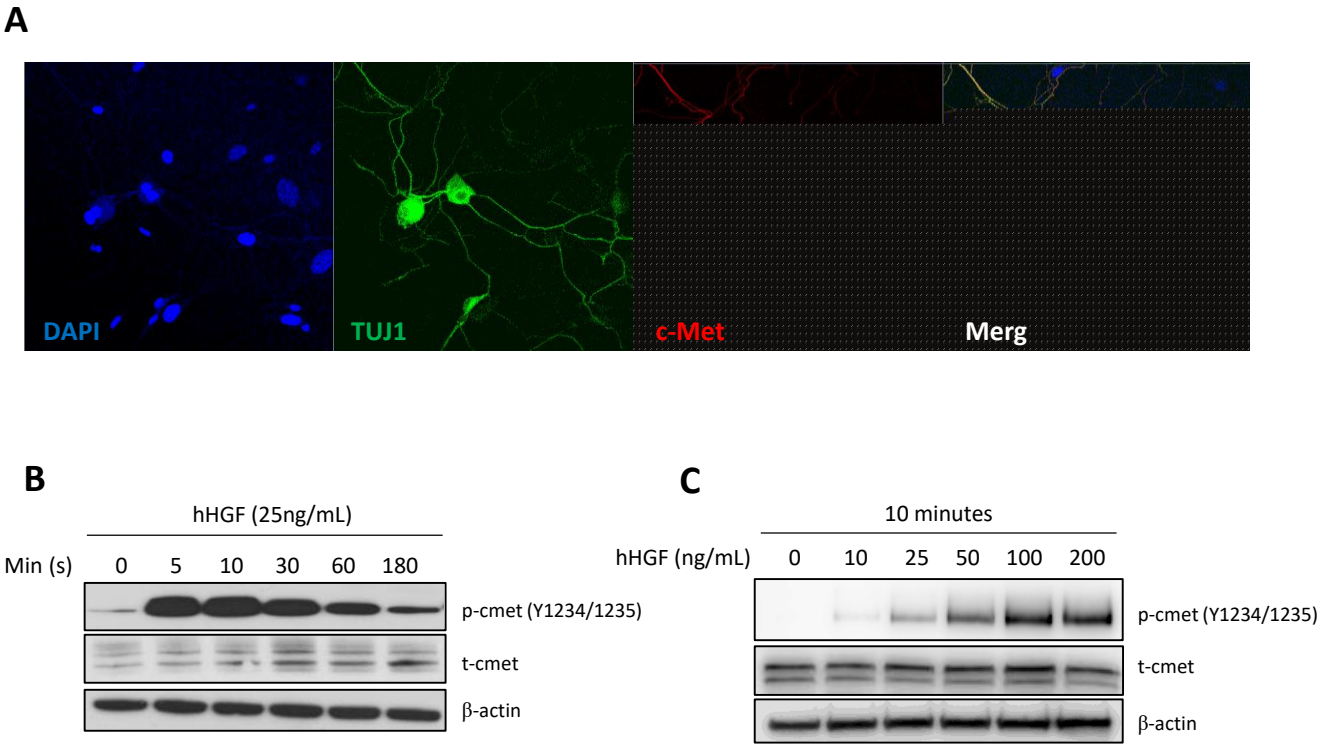

Figure V-1. Expression and activation of c-Met in primary sensory neurons. Primary DRG neurons were isolated from adult mice, and they were cultured in the presence of Ara-C for 3 days to remove proliferating cells such as satellite glial cells. **(A)** Primary sensory neurons were subjected to immunofluorescence assay, using antibodies specific to TUJ1 (green) and c-Met (red). **(B, C)** Total proteins were prepared for western blot analysis using antibodies against p-cMet (Y1234/1235) and t-cMet. In Western blot analysis, β-actin was used as a loading control.