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Molecular Characterization of Hepatocyte Growth Factor Functions in Muscle Atrophy and Regeneration

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ABSTRACT

Hepatocyte growth factor (HGF) has been well characterized in terms of its roles in the migration of embryonic muscle progenitors and the activation of adult muscle stem cells, yet it remains unclear whether HGF affects the adult muscle regeneration process or neurogenic muscle atrophic conditions. In response to this gap in scientific knowledge, my thesis focused on investigating the roles of HGF/c-met signaling in the skeletal muscle wasting and injury-induced regeneration processes.

The first step was to test the effects of HGF on neurogenic muscle atrophy. It was found that HGF expression was upregulated in skeletal muscle tissue following surgical denervation, as well as in hSOD1-G93A transgenic mice showing severe muscle loss. Pharmacological inhibition of c-met receptors decreased the expression level of pri-miR-206 and enhanced that of HDAC4 and atrogenes, resulting in increased muscle atrophy. In C2C12 cells, HGF inhibited phosphorylation of Smad3 and relieved TGF-β-mediated suppression of miR-206 expression via JNK. When extra HGF was exogenously provided through the intramuscular injection of plasmid DNA expressing human HGF protein, the extent of muscle atrophy was reduced and the levels of all affected biochemical markers were changed accordingly, including those of primary and mature miR-206, HDAC4, and various atrogenes.

This study also investigated the roles of HGF in cardiotoxin-induced muscle regeneration. It was found that HGF/c-met signaling was activated during muscle regeneration, and that among various infiltrated cells, the macrophage was the major cell type affected by HGF. Pharmacological inhibition of the c-met receptor by PHA-665752 increased the expression levels of pro-inflammatory (M1) macrophage markers like IL-1β and iNOS while lowering those of pro-regenerative (M2) macrophage markers like IL-10 and TGF-β, resulting in compromised muscle repair. In Raw 264.7 cells, HGF decreased the RNA level of

LPS-induced TNF-α, IL-1β, and iNOS, while enhancing that of IL-10. HGF was also shown to increase the phosphorylation of AMPKα through CaMKKβ, thereby overcoming the effects of the LPS-induced deactivation of AMPKα. Transfection with specific siRNA to AMPKα diminished the effects of HGF on the LPS-induced gene expressions of M1 and M2 markers. Exogenous delivery of HGF through intramuscular injection of the HGF-expressing plasmid vector promoted the transition to M2 macrophage and facilitated muscle regeneration.

Taken together, my findings suggested that HGF may play important roles in the progression of neurogenic muscle atrophy and macrophage transition during adult muscle regeneration. The results described in this thesis provide very useful information in developing innovative therapeutics based in HGF for a variety of neuromuscular disorders and muscle degenerative diseases.

Keywords: Hepatocyte growth factor, skeletal muscle, neurogenic muscle atrophy, muscle regeneration

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ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
AMPK	5' Adenosine monophosphate-activated protein kinase
AMV	Avian myeloblastosis virus
ATP	Adenosine triphosphate
BMDM	Bone marrow-derived macrophage
C/EBPβ	CCAAT/enhancer-binding protein β
CaMKKβ	Calcium/calmodulin-dependent protein kinase kinase
CCL	Chemokine (C-C motif) ligand
CMA	Chaperone-mediated autophagy
COPD	Chronic obstructive pulmonary disorder
CREB	cAMP response element binding
CTX	Cardiotoxin
DMEM	Dulbecco's modified Eagle medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
Erk	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FoxO	Forkhead box O
HDAC4	Histone deacetylase 4
HGF	Hepatocyte growth factor
HL	Hairpin loop
IGF-1	Insulin-like growth factor 1

IL	Interleukin
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LKB1	Liver kinase B1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MH	Mad homology
MMP	Matrixmetalloproteinase
mTOR	mammalian target of rapamycin
MuRF1	Muscle RING-finger protein-1
NF-κB	Nuclear factor-kappa B
Pax7	Paired and homeodomain box 7
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
RING	Really interesting new gene
Smad	Suppressor of mothers against decapentaplegic
SOD	Superoxide dismutase
SPH	Serine protease homology
TA	Tibialis anterior
TGF-β	Transforming growth factor β
TNFα	Tumor necrosis factor α
TWEAK	Tumor necrosis factor-like weak inducer of apoptosis
Ub	Ubiquitin
UBC	Ubiquitin-conjugating enzyme
uPA	urokinase plasminogen activator

UPS	Ubiquitin-proteasome system

CHAPTER I

Introduction

1. Skeletal muscle remodeling

Skeletal muscle undergoes adaptive modifications in muscle size, architecture, protein compositions, and functions in response to different environmental demands and catabolic cues. Skeletal muscle remodeling occurs in several myopathic abnormalities such as muscle atrophy, muscle injury and regeneration, metabolic disorders, muscle dystrophy, and myositis. This study focuses on the understanding of upstream events during muscle remodeling in muscle atrophy and injury-induced regeneration.

Skeletal muscle atrophy or muscle wasting is an exceedingly debilitating condition that occurs in response to several stress stimuli and chronic diseases. Muscle atrophy is characterized by a reduction of the cytoplasmatic volume and by the significant loss of organelles and myofibrillar proteins, eventually leading to a reduced muscle tone and compromised contractile ability (Jackman and Kandarian, 2004).

Muscle atrophy can occur as a common secondary consequence of a wide array of catabolic conditions, including cancer, diabetes, sepsis, starvation, aging, chronic heart failure, chronic obstructive pulmonary disorder (COPD), renal failure, and cystic fibrosis (Jackman and Kandarian, 2004). An atrophic response affects millions of people annually, leading to reduced independence and survival rate, poor functional status and health-related quality of life (Kandarian and Stevenson, 2002; Lecker et al., 2004). Metabolic alterations such as starvation or nutrient deprivation can also induce atrophy. Regardless of the various stimulating events, several key facets of the pathogenesis of atrophy are commonly determined in all atrophic programs.

Loss of muscle protein is a common characteristic in almost all atrophic programs. Skeletal muscles are known to maintain the balance between protein synthesis and degradation in normal physiological conditions. However, in atrophic conditions, there is a shift toward catabolism caused by an imbalance between rate of protein synthesis and protein

degradation, leading to a significant reduction in muscle protein content. Though it is now well established that intracellular pathways of proteolytic degradation are the major contributors of muscle protein loss in atrophic conditions, the upstream mechanisms leading to activation of proteolytic systems are less understood at molecular levels due to the diversity of their extracellular stimuli. Therefore, it is urgent to investigate these catabolic signaling mechanisms, which would greatly contribute to the development of effective medical therapies.

Skeletal muscle damages could result from a variety of pathophysiological conditions including dystrophy, degenerative diseases and severe injuries such as contusions and strains. Under these situations, skeletal muscle undergoes a highly orchestrated regeneration process that involves the degeneration of damaged muscle fibers, inflammation, and satellite cell proliferation and differentiation into new myofibers(myogenesis). Skeletal muscle injuries can be classified into two types, acute and chronic. In acute injury such as sports or exercise injuries and toxin-induced injuries, necrotic tissue is removed by a transient infiltration of inflammatory cells followed by repair mediated by resident stem cells. However, in chronic injuries such as in several dystrophies, the causal molecular defects retain the degenerative microenvironment, and thus make inflammatory infiltration persistent, further causing degeneration of newly regenerated fibers (Porter et al., 2002). In spite of the diverse types and severity of muscle injuries, all regeneration programs follows a stereotype, such as the phagocytosis of necrotic fibers by inflammatory cells, activation of inflammatory responses, and the generation of new muscle fibers mediated by muscle-resident stem cells (Zammit et al., 2002).

2. Common signaling pathways in muscle atrophy and regeneration

All the major tissues contain several proteolytic pathways that are recruited in a context-dependent manner either to maintain homeostasis or to restore normal physiological conditions in a catabolic state. Similarly, skeletal muscle also contains at least five different proteolytic pathways that include ubiquitin-proteasome system, autophagy-lysosome system, Ca^{2+}-dependent calpains, caspase systems and matrixmetalloproteinases (MMPs) (Figure I-1). Among these, Ca^{2+}-dependent calpains may have a minor role in basal skeletal muscle myofibrillar turnover (Bartoli and Richard, 2005). Calpains are neither systematically activated in different models of atrophy nor they degrade major contractile proteins (Bartoli and Richard, 2005). Role of caspases and MMPs in the context of skeletal muscle atrophy are not well understood and poorly documented. They are not involved in gross protein turnover, but instead are involved in ordered proteolytic pathways such as apoptosis. On the other hand, there are several compelling evidences that two well-studied systems including ubiquitin-proteasome (UPS) and autophagy-lysosome systems are responsible for modulating mass of skeletal muscles in atrophic conditions (Cohen et al., 2015).

Ubiquitin-proteasomal degradation is based on proteolysis of proteins, which are labeled with poly-ubiquitin chains. Protein degradation mediated by UPS requires two important steps. First, protein substrates are tagged with multiple ubiquitin moelcules by covalent attachment and then tagged proteins are degraded by the 26S proteasome complex. Ubiquitin is a highly evolutionarily conserved 76-residue polypeptide, which is conjugated to the target protein substrate via a three-step cascade mechanism. Initially, ubiquitin is converted to a high energy thioester mediate, by the ubiquitin-activating enzyme, El. When activated by E1, ubiquitin-conjugating enzymes (UBCs) or E2 enzymes transfer the activated ubiquitin moiety to a member of the E3, to which the substrate protein is specifically bound. A class of E3-ligases that contain RING finger, catalyzes the conjugation of ubiquitin to the protein substrate. A polyubiqutin chain is synthesized in a successive reactions and the poly-

Ub tagged substrates are recognized by downstream 26S proteasome complex and degraded. However, within UPS, the only specificity is introduced by the E3 ubiquitin ligases and therefore E3s play a key role in the ubiquitin mediated proteolytic cascade since they serve as the specific recognition factors of the system (Chen, 2005; Hershko and Ciechanover, 1998; Jackson et al., 2000).

The other major pathway for degradation of cellular proteins is autophagy lysosomal system. This system also contributes significantly in the maintenance of the balance between protein synthesis and protein turnover. It involves the initial sequestration of protein substrates into the vacuolar system, called lysosomes, and their subsequent hydrolysis by lysosomal hydrolases (Bechet et al., 2005). Diverse subtypes of autophagy such as macroautophagy, chaperone mediated autophagy, and microautophagy may be used to deliver intracellular protein substrates to lysosomes (Dehoux et al., 2004). Microautophagy is not yet well characterized in eukaryotic systems, and is thought to be a more selective process involving multivescular body invagination. Macroautophagy is involved in bulk non-selective degadation of cellular compartments, the chaperone-mediated autophagy (CMA) is known to be primarily involved in the degradation of cytosolic proteins (Dehoux et al., 2004). In the context of skeletal muscle remodeling, both macroautophagy and CMA play a major role in the regulation of degradation of defunct cellular compartments and poly-Ub tagged protein aggregates. Many of the catabolic conditions that cause muscle atrophy, are also known to induce autophagy (Bhatnagar et al., 2012; Mammucari et al., 2007; Sandri, 2010). Upon autophagic induction, target recognition and delivery is carried out by different mechanisms depending on the type of target cells and their cellular conditions (Doyle et al., 2011; Ebert et al., 2010). During macroautophagic process, cytosolic cargo is sequestered by pre-autophagosomal structure, eventually generate double membrane vesicle or autophagosome. The autophagosome formation is preceded by recruitment of autophagic machinery to the

poly-Ub tagged protein and protein aggregates (Foletta et al., 2011). The autophagosome then fuses with a lysosome to generate an autolysosome, where sequestered protein aggregates are degraded. (Bechet et al., 2005). In CMA, substrates for lysosomal proteolysis are cytoplasmic proteins bearing a certain pentapeptide motif, Lys-Phe-Glu-Arg-Gln (KFERQ) or a closely related sequence. KFERQ motif containing proteins are recognized and bound to cytosol by prp73 (73-kda peptide recognition protein), and subsequently targeted to lysosome. (Doherty and Mayer, 1992). Although autophagy is extensively investigated in other systems, it is only recently reported that autophagy play major roles in skeletal muscle remodeling (Romanello et al., 2010; Sandri, 2010). Recent evidence has suggested that the autophagy-lysosomal system has a role in diverse pathological situations including fasting, denervation, disuse, sepsis, and cancer-induced muscle proteolysis and atrophy. Therefore, autophagy constitutes an essential component of cellular quality control system in skeletal muscle along with UPS. In addition, there are other points of convergence between these two proteolytic systems, indicating that these two pathways may share a common regulator that dictates their coordinated actions in catabolic conditions including those leading to skeletal muscle remodeling (Gomes et al., 2001).

Several other signaling pathways are shown to be involved in skeletal muscle remodeling. Nuclear factor-kappa B (NF-κB) is one of the central regulator of skeletal muscle remodeling. NF-κB not only is implicated downstream of several catabolic pathways in skeletal muscles (Adhikari et al., 2007; Lamothe et al., 2007), but also has been found to be upregulated in a vast majority of skeletal muscle disorders (Cai et al., 2004; Li et al., 2008). NF-κB upregulates expression of muscle specific E3 ubiquitin-ligases, interacts with components of autophagy lysosomal system, promotes myogenesis and inhibits myoblast differentiation (Paul et al., 2010).

Among the factors that influence skeletal muscle, the most important are growth

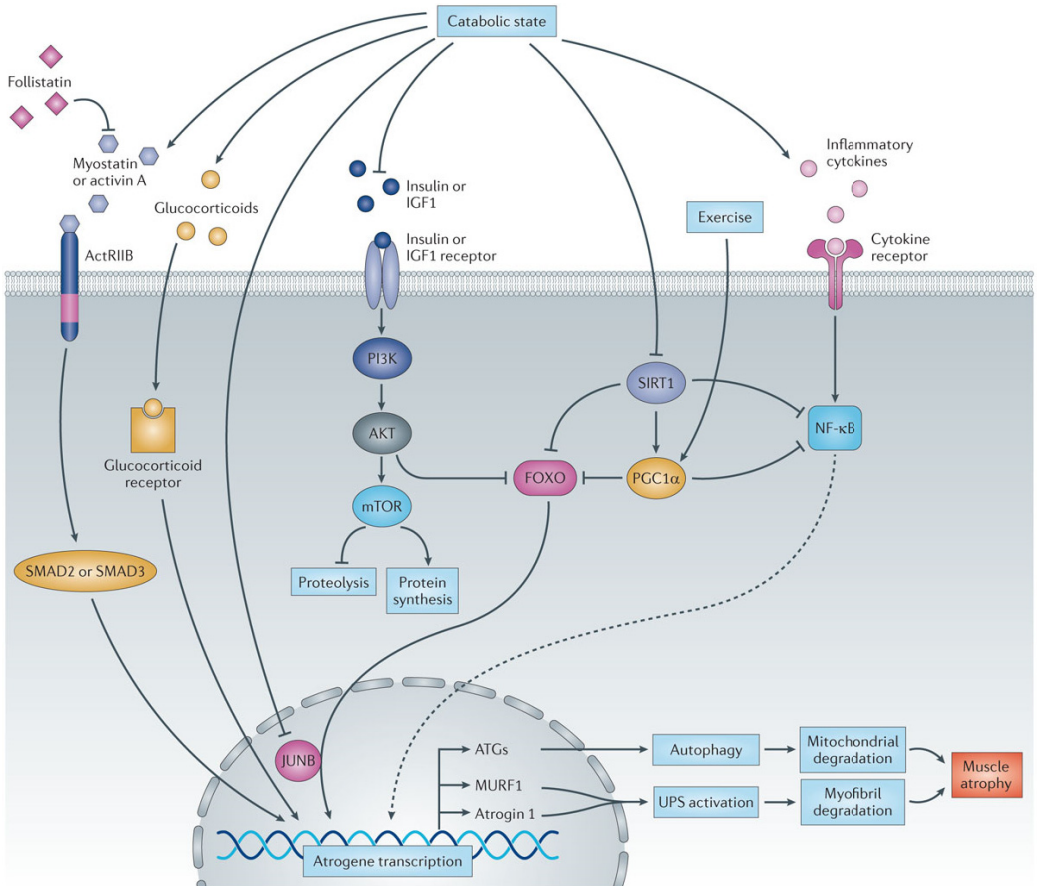

Figure I-1. Signaling pathways leading to muscle atrophy. (Taken from Cohen et.al, 2015.)

factors and cytokines. One large group of growth factors (such as IGF-1, EGF, FGF, and HGF) act by binding to and activating surface receptors with intrinsic protein tyrosine kinase activity (Schlessinger and Ullrich, 1992). They utilize a variety of signaling pathways (such as p38 MAPK, Erk1/2, JNK, PI3K/Akt and AMPK-FoxO) to induce several cellular responses (Cantley, 2002; Johnson and Lapadat, 2002; Mihaylova and Shaw, 2011). An important kinase p38 MAPK is reported to induce skeletal muscle atrophy downstream of TNFα and is also known to function as a molecular switch to activate the quiescent satellite cells. (Harding et al., 1999; Jones et al., 2005). Extracellular regulated kinase (Erk1/2) is reported to be involved in the development of denervation-induced muscle atrophy downstream of HDAC4 (Hishiya et al., 2006; Choi et al., 2012). c-Jun N-terminal kinase (JNK) has also been reported to negatively regulate skeletal muscle mass through insulin signaling and caspases, or positively regulate it via myostatin/Smad inhibition (Aguirre et al., 2000; Lessard et al., 2018). PI3K/Akt kinase is a canonical downstream regulator of insulin-like growth factor-1 (IGF-1), and is reported to promote muscle hypertrophy and prevent the expression of muscle specific E3 ubiquitin-ligases by inhibiting forkhead transcription factors (FoxO) (Stitt et al., 2004). AMP-activated kinase (AMPK) is an upstream regulator of FoxO, and is shown to augment muscle atrophy by promoting autophagy, and inhibit myoblast differentiation (Krawiec et al., 2007; Meley et al., 2006). Downstream of AMPK, FoxO transcription factors have recently been reported to be involved in development of muscle atrophy by activating some key components of UPS and autophagy lysosomal system. (Mammucari et al., 2007; Sandri et al., 2004).

Growth factor signaling has been reported to play an important role(s) in the regulation of many inflammatory or myogenic cytokines. Majority of cytokines are known to be a negative regulator of skeletal muscle growth, proliferation and differentiation. While TWEAK, TNFα and IL-1β are well known to negatively regulate skeletal muscle mass (Choi

et al., 2012; De Larichaudy et al., 2012; Yahiaoui et al., 2008), IL-4, IL-6 and IL-10 have been shown to have beneficial effects on skeletal muscle remodeling (Novak et al., 2009; Serrano et al., 2008). Pro-inflammatory and pro-myogenic cytokines (IFN-γ and TNFα), chemokines (CCL2 and CCL5), and NF-κB play important roles in skeletal muscle injury and regeneration since they both act on myogenic and various types of myeloid cells. Cooperation of these factors allows for a significant interplay between myeloid and myogenic cells that regulates the impairment or improvement in injury-induced regeneration (Tidball, 2017) (Figure I-2). Taken together, the diverse interactions of growth factors make it a point of convergence for all the major signaling pathways that regulate skeletal muscle remodeling.

3. The HGF/c-met signaling and muscle remodeling

Hepatocyte growth factor (HGF) was initially discovered in 1984, as a mitogen for hepatocytes after liver damage (Michalopoulos et al., 1984); thereby this factor was named the hepatocyte growth factor. HGF is a multidomain glycoprotein synthesized and secreted mainly by mesenchymal cells, including fibroblasts, endothelial cells, vascular smooth muscle cells, and muscle satellite cells (Birchmeier and Gherardi, 1998). Upon synthesis, HGF is stored in the extracellular matrix (ECM) as a single-chain, biologically inactive glycoprotein precursor (approximately 90kDa) (Grierson et al., 2000). However, in response to stimuli such as skeletal muscle injury, pro-HGF is proteolytically cleaved into an active heterodimer by serine proteases at Arg494-Val495 (Tatsumi and Allen, 2004). In the activated form, HGF is composed of contains an α (60kDa) and β-chain (30kDa). The α-chain, which is also the heavy chain, contains an amino-terminal hairpin loop domain (HL) homologous to the activation peptide of plasminogen and four kringle domains (K1-K4), while the β-chain (light chain) includes a serine protease homology (SPH) domain (Grierson et al., 2000)

(Figure I-3). The HL region of HGF consists of clusters of amino acid sequences necessary for efficient binding of HGF to its receptor, c-met. The kringle domains (K1, K2, K3 and K4) play a pivotal role in the activation of the c-met, and are important for the mitogenic activity of HGF. The SPH domain contributes additional binding sites which are essential for efficient activation of HGF receptor (Holmes et al., 2007) (Figure I-3).

It has been reported that the interaction of HGF with its receptor, c-met, stimulates a wide variety of cellular responses including angiogenesis, cellular motility, growth, invasion, morphogenesis, embryological development, tissue regeneration, and wound healing (Ding et al., 2003; Mizuno et al., 2001; Ohmichi et al., 1996; Pelicci et al., 1995; Xiao et al., 2001). This signaling cascades also play important roles during the skeletal muscle remodeling, directly and indirectly.

3.1. Roles of HGF in skeletal muscle development during embryogenesis

In the skeletal muscle, HGF and c-met genes are expressed throughout embryogenesis, particularly during early organogenesis and gastrulation (Andermarcher et al., 1996). During early organogenesis, skeletal muscles develop from migratory and non-migratory muscle precursor cells that are produced by the lateral lip of the dermomyotome. Mice that lack c-met and HGF die during the second part of gestation and lack muscle groups which derive from migratory muscle precursor cells (Dietrich et al., 1999). During gastrulation, the expression of HGF and c-met overlap; they are initially expressed in the endoderm and mesoderm and later in the node and notochord. During this phase, HGF regulates detachment of specific myogenic muscle precursor cells and subsequent migration from the somites into limb bud and diaphragm (Andermarcher et al., 1996; van der Voort et al., 2000). The migration of these myoblasts is a crucial step in the development of skeletal

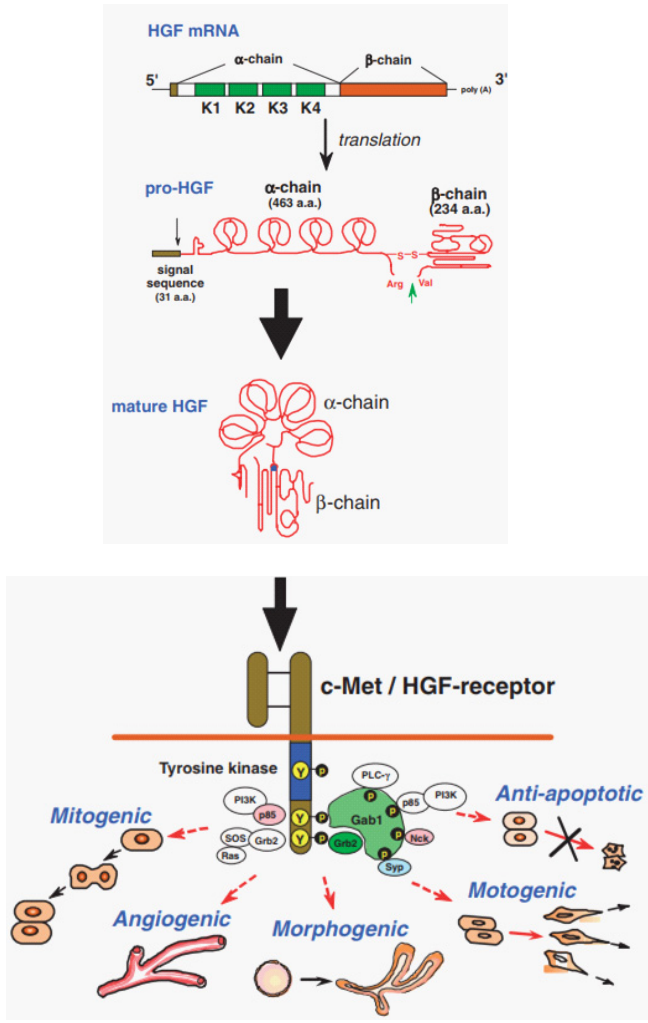

Figure I-2. Structure and biological function of HGF. (Taken from Nakamura et.al, 2010.)

muscle during embryogenesis, and is therefore largely regulated by HGF. The absence of HGF and c-met during embryogenesis results in the deficient development of limb muscle, shoulder muscle, and the diaphragm (Andermarcher et al., 1996; Birchmeier and Gherardi, 1998).

3.2. Roles of HGF during adult skeletal muscle repair

Satellite cells are considered to play critical roles in the maintenance, repair and remodeling of the muscle fiber. Activation of satellite cells is pivotal for skeletal muscle repair and is triggered primarily by HGF following skeletal muscle injury (Le Grand and Rudnicki, 2007). Extrinsic mechanical stretch of the myofibers can trigger a variety of signaling cascades, including nitric oxide synthesis that results in HGF activation. The interaction between HGF and c-met has been reported to induce pro-myogenic mitogen activated protein kinase (MAPK) signaling cascades, such as p38α/β MAPK, which functions as a molecular switch to activate quiescent satellite cells (Jones et al., 2005; Karalaki et al., 2009). For proper activation, satellite cells require spatio-temporal regulation of muscle transcription factors and muscle-specific genes (Karalaki et al., 2009). Among them, Paired and homeodomain box 7 (Pax7) works as a master regulator, as it is expressed in both quiescent and activated satellite cells, and is also important for specification, survival and self-renewal of satellite cells and activation of myogenic genes (Le Grand and Rudnicki, 2007). It was previously reported that the expression of Pax7 during satellite cell activation and proliferation is largely regulated by HGF (Gill et al., 2010). Recent study has also shown that HGF/c-met/mTOR cascade is responsible for the transition of quiescent muscle stem cells into GAlert, a cellular state in which they have an increased ability to participate in tissue repair (Rodgers et al., 2014).

Satellite cell proliferation is the other key processes involved in myogenesis. Throughout embryogenesis, and in the adult skeletal muscle, the ability of satellite cells to proliferate is regulated by growth factors such as HGF and FGF (O'reilly et al., 2008). A number of factors are expressed by adult skeletal muscle to guide the initiation and maintenance of the proliferation status of satellite cells, including transmembrane receptors (e.g. c-met), intracellular signaling molecules and transcription factors (e.g. Pax7). However, in adult skeletal muscle, the self-renewal ability of satellite cells is eventually limited as the proliferation capacity becomes restricted as a result of progressive shortening of their telomere during each cell cycle (Zammit et al., 2006). To date, studies on the effects of HGF on satellite cell proliferation are still controversial depending on the experimental conditions. Studies including C2C12 murine myoblasts and rat skeletal myoblasts have indicated that the cell growth was promoted upon HGF treatment (Anastasi et al., 1997; O'Blenes et al., 2010). Studies by Sisson et al. have also shown that HGF activated by urokinase plasminogen activator (uPA) promotes myoblast proliferation, which leads to improved muscle regeneration (Sisson et al., 2009). In contrast, Yamada et al. demonstrated that high levels of HGF even suppress the proliferative activity and induce quiescence of rat satellite cells (Yamada et al., 2010). These findings correlated with similar studies involving chicken skeletal muscle cells and C2 cells, where the activation of satellite cells were reduced, and the regeneration of muscle fiber was delayed upon the addition of exogenous recombinant HGF protein (Gal-Levi et al., 1998).

3.3. Roles of HGF in skeletal muscle atrophy

The role of HGF in skeletal muscle remodeling remains largely unexplored. Its involvement in skeletal muscle injury and regeneration led to a hypothesis that HGF might

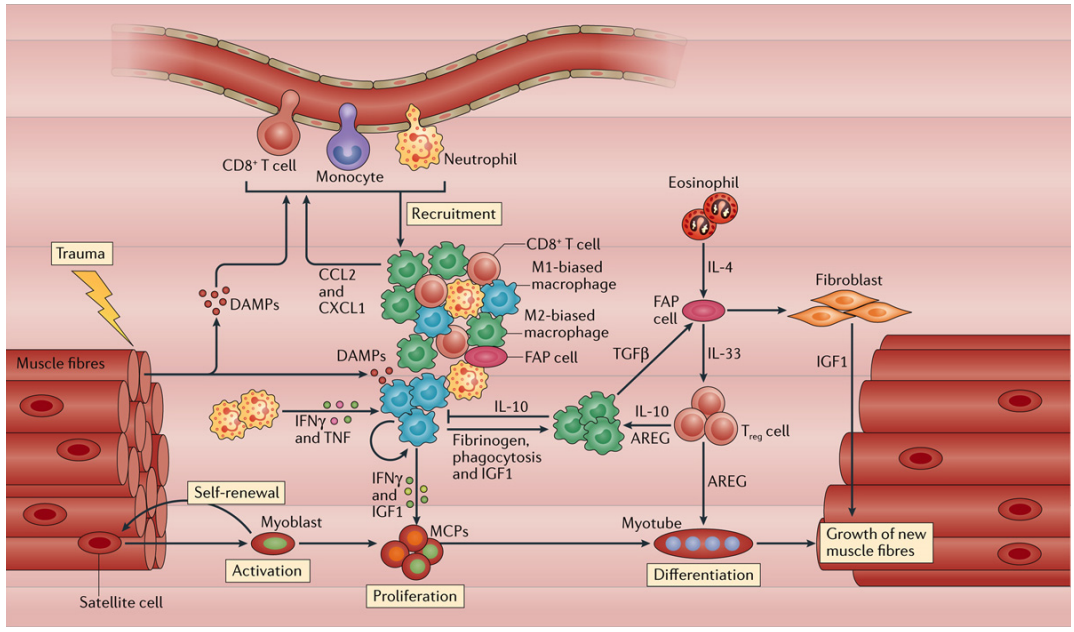

Figure I-3. Molecular mechanisms of muscle regeneration. (Taken and modified from Tidball et.al, 2017.)

act positive regulator of skeletal muscle mass, and thus influence skeletal muscle remodeling. Indeed, recent study has shown that HGF partially prevented the progression of hypoxia-induced muscle atrophy by activating protein synthesis through PI3K/Akt/mTOR pathway and inducing satellite cell proliferation (Hauerslev et al., 2014). However, the involvement of HGF in the neurogenic muscle atrophy process has not been understood at molecular levels, except for one report describing that the expression of HGF is increased in the muscle following denervation (Yamaguchi et al., 2004).

4. HGF gene therapy based on plasmid DNA and implications of data from clinical studies

A wide array of existing literature suggests that HGF could potentially have therapeutic effects on various diseases. However, the instability of the HGF protein *in vivo* (less than 5 minutes in serum) has made it difficult to use its recombinant protein itself as a therapeutic agent (Kawaida et al., 1994). Inducing high-level, long-term expression of HGF is not desirable in the context of gene therapy, as its potent angiogenic properties may induce carcinogenesis (Bussolino et al., 1992). In order to overcome these obstacles, there is a need for developing a method for expressing a low but still therapeutically meaningful level of HGF protein *in vivo,* for a relatively short period of time. Among various methods of delivering HGF, the use of a plasmid DNA expression vector as a means of expressing the protein has received a particular attention from my predecessors. Plasmid DNA is known to be relatively safe: it does not integrate into the host chromosome, and it has been reported that almost all transfected plasmid DNA disappear within 3 weeks (Son et al., 2016).

The plasmid developed by my predecessors, called pCK-HGF-X7 (or VM202), is an expression vector designed to produce two isoforms of human HGF: HGF_{723} (or dHGF) and HGF_{728} (or cHGF), respectively, at high levels *in vivo* when delivered by i.m. injection. This

plasmid consists of not only the replication origins (ColE1) and selectable marker (kanamycin resistant gene), but also the full-length immediate-early (IE) promoter of human cytomegalovirus and the genomic-complementary DNA (cDNA) hybrid of human HGF gene (Lee et al., 2000; Pyun et al., 2010). This hybrid sequence contains truncated intron 4 in the junction of exons 4 and 5 of HGF cDNA, which was engineered to produce the two isoforms of HGF, HGF_{723} and HGF_{728},at high levels (Pyun et al., 2010).

pCK-HGF-X7 has been used in a variety of clinical studies and animal models for ischemic diseases, such as critical limb ischemia and coronary artery disease. Data from these studies clearly show that the injection of pCK-HGF-X7 (1) could efficiently increase the number of angiographically recognizable collateral vessels, as well as improve intra-arterial blood flow in the rabbit model of hindlimb ischemia (Pyun et al., 2010); (2) yielded clinically meaningful ulcer area reductions in patients with critical limb ischemia (Gu et al., 2011; Kibbe et al., 2016); (3) improved cardiac function by increasing left ventricular ejection fraction and fractional shortening compared to the control subjects in porcine models (Hahn et al., 2011; Perin et al., 2011); (4) promoted angiogenesis (as evidenced by increased capillary density compared to the control) while decreasing detrimental remodeling of the tissue at the site of injury in ischemia–induced porcine (Cho et al., 2008); (5) when injected into the right coronary artery, ameliorated regional myocardial perfusion and wall thickness in the injected region in patients with ischemic heart disease (Kim et al., 2013);

pCK-HGF-X7 has also been tested in clinical studies for diabetic peripheral neuropathy and in various animal models of nerve injury. In these studies, it was found that the administration of pCK-HGF-X7 (1) significantly reduced the mean pain score and improved quality of life in patients with diabetic peripheral neuropathy (Kessler et al., 2015); (2) promoted peripheral nerve regeneration by inducing the activation and migration of schwann cells in mice after nerve injury (Ko et al., 2018); (3) ameliorated nerve injury-

induced neuropathic pain by inhibiting pain-related factors in DRG neurons and subsequent spinal cord glial activation in mouse models (Nho et al., 2018).

These data indicate that pCK-HGF-X7 is able to exert beneficial effects in various cardiovascular and neurological diseases, and might also have the therapeutic potential to be applied to other degenerative disorders.

5. Rationale and overall procedures of thesis research

When I initiated my thesis research, the understanding of the *in vivo* role(s) of HGF in skeletal muscle was very limited, despite its interesting therapeutic potential. HGF has been shown to activate muscle stem cells residing in muscle fiber, causing them to make a myogenic commitment (Allen et al., 1995; Jennische et al., 1993; Sheehan et al., 2000; Tatsumi et al., 1998). Exogenously added recombinant HGF protein was reported to increase myoblast proliferation while inhibiting differentiation, resulting in the delayed regeneration of damaged muscle (Miller et al., 2000). However, it has since been shown that HGF activated by urokinase plasminogen activator (uPA) can promote muscle regeneration (Sisson et al., 2009) and that its receptor, c-met, is responsible for the transition of quiescent muscle stem cells into GAlert, a cellular state in which the stem cells' ability to participate in tissue repair is enhanced (Rodgers et al., 2014). Thus, the role of HGF in muscle regeneration remains unclear. While it was found that the immune system plays important role(s) in the muscle regeneration process, the role of HGF in immune cells infiltrating the injured muscle was not yet clearly understood at that time.

As such, I investigated the role(s) of HGF during muscle regeneration in the context of infiltrated immune cells. My initial data indicated that HGF/c-met signaling was activated following muscle injury, and that among various infiltrated cells, the macrophage was the

major cell type affected by HGF. Based on these observations, I investigated whether activated HGF/c-met signaling in macrophages was indeed involved in the muscle regeneration process. This was done by blocking c-met activity with a specific chemical inhibitor or by overexpressing the HGF protein with plasmid DNA in muscle. I found that HGF indeed played important roles in the regulation of macrophage transition, which could lead to improved muscle regeneration. Further molecular details were then studied using Raw 264.7 macrophage cell lines.

Before my work, the role(s) of HGF in neurogenic muscle atrophy was poorly understood. The results described in this thesis focus on understanding the role(s) of HGF and the underlying mechanisms of the development of atrophic responses in skeletal muscle. I first found that HGF/c-met signaling was activated in the process of neurogenic muscle atrophy. Next, I investigated whether changes in HGF/c-met signaling affects the progression of muscle atrophy by controlling c-met activity or overexpressing HGF protein. Data from this study indicated that HGF/c-met signaling regulated the miR-206-HDAC4 cascade and atrogenes, thereby ameliorating the progression of muscle atrophy. C2C12 myoblast cell lines were used to confirm these results and study detailed molecular mechanisms.

It is my belief that my work will contribute to a deeper understanding of the role(s) of HGF in muscle wasting and injury-induced regeneration, leading to the identification of potential therapeutic candidates for the treatment of human diseases associated with skeletal muscle anomalies.

CHAPTER II

Materials &Methods

1. Cell culture and Reagents

1.1 Chemical and biological reagents

For C2C12 cell line experiments, recombinant human HGF (R&D systems, MN) and recombinant TGF-β (eBioscience, MA) were used at appropriate concentrations. U0126 (MEK1/2 inhibitor, Sigma Aldrich, MO), SB203580 (p38 inhibitor, Calbiochem, MA), SP600125 (JNK inhibitor, Sigma Aldrich, MO), Akti1/2 (Akt inhibitor, Sigma Aldrich, MO) were used at 10 μM, and rapamycin (mTOR inhibitor, Sigma Aldrich, MO) was used at 100 nM for experiments.

For Raw 264.7 cell line and primary mouse bone marrow macrophage experiments, recombinant human HGF protein (R&D systems, MN) and LPS (Sigma Aldrich, MO) were used at appropriate concentrations. STO-609 (CaMKKβ inhibitor, Tocris Bioscience, MO) was used at 5 or 20 μM for experiments.

1.2 C2C12 cell line

C2C12 cells were purchased from the American Type Culture Collection (Manassas, VA). C2C12 myoblasts were grown in DMEM (Welgene, Gyeongsan, Korea) supplemented with 10% FBS (Corning, NY) and antibiotics (100 U/ml penicillin and 100μg/ml streptomycin (Sigma Aldrich, MO)) at 37℃ under 5% CO_2.

1.3 Raw 264.7 cell line

Raw 264.7 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM (Welgene, Gyeongsan, Korea) supplemented with 10% FBS (Corning, NY) and antibiotics (100 U/ml penicillin and 100μg/ml

streptomycin (Sigma Aldrich, MO)) at 37℃ under 5% CO_2.

1.4 Primary mouse bone marrow derived macrophage isolation

Bone marrow cells were prepared and differentiated into macrophages using M-CSF as described previously (Zhang et al., 2008). Briefly, femurs and tibias from male C57BL/6 mice were prepared and the bone marrow cavity of each femur was flushed out using a 25 g syringe with 5 ml of PBS without calcium and magnesium. Cells were collected by centrifugation at 453× *g* for 3 min and red blood cells were removed using red blood cell lysis buffer (Sigma Aldrich, MO). Cells were then washed twice with PBS and cultured in sterile plastic Petri dishes in DMEM/F12 (Invitrogen, CA) containing 10% FBS (Hyclone, IL), penicillin-streptomycin-glutamin (Gibco, CA) and 20 ng/ml of M-CSF. After 3 days, 5 ml of fresh culture media were added. On day 7, fully differentiated macrophages were washed twice with PBS and used for experiments. The purity of BMDM was over 95% as determined by FACS analysis using an antibody against CD11b (eBioscience, CA).

1.5 Myotube differentiation *in vitro*

For myotube differentiation, cells were differentiated in DMEM supplemented with 2% horse serum (Sigma Aldrich, MO) for four days, and the differentiation of C2C12 into myotube was evaluated by staining with differentiation marker, Myosin heavy chain.

1.6 Macrophage activation *in vitro*

For macrophage activation, Raw 264.7 cells or primary mouse bone marrow macrophages were treated with 100 ng/ml of LPS for 24 hours, and the activation of

macrophage was evaluated by measuring the expression of activation marker, iNOS and IL-1β.

2. Molecular and cellular biological techniques

2.1 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

TAs were prepared and mechanistically homogenized using Bullet Blender Storm (Next Advance, NY), and total RNA was extracted from homogenized TA or cultured cells with RNAiso (Takara, Kusatsu, Japan) following the manufacturer's instructions. One microgram of RNA was converted to cDNA using oligo dT primers (Qiagen, Hilden, Germany) and Reverse Transcriptase XL (AMV) (Takara, Kusatsu, Japan). Gene expression was assessed using quantitative real-time PCR with Thermal Cycler Dice Real Time System TP800 (Takara, Kusatsu, Japan) and SYBR Premix Ex Taq (Takara, Kusatsu, Japan). For miRNA analysis, RNA was converted to cDNA using miRCURY LNA Universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark). Gene expression was measured using quantitative real-time PCR with ExiLENT SYBR Green master mix kit (Exiqon, Vedbaek, Denmark). Mature miR-206 and miR-103-3p specific primers were purchased from Exiqon.

2.2 Western blot analysis

For immunoblotting, TAs or cultured cells were prepared and homogenized in RIPA lysis buffer (Sigma Aldrich, MO) containing a protease inhibitor (Roche, Basel, Switzerland), and phosphatase inhibitor (Roche, Basel, Switzerland) using Bullet Blender Storm (Next Advance, NY). Equal amounts of protein were then separated by 10% SDS-polyacrylamide gel and electrophoretically transferred to polyvinylidene fluoride membranes (Millipore,

Gene **(Mouse)**	**Primer Sequence** **Forward (5'→3')**	**Primer Sequence** **Reverse (5'→3')**
Hgf	ATCCACGATGTTCATGAGAG	GCTGACTGCATTTCTCATTC
Murf1	TGCCTGGAGATGTTTACCAAGC	AAACGACCTCCAGACATGGACA
Atrogin1	AAGGCTGTTGGAGCTGATAGCA	CACCCACATGTTAATGTTGCCC
Pri-miR-206	ACCCAGTGCCCTGTGTTCCCA	AGCGCCTCTTCTCGGTTTCCCT
Hdac4	CACACCTCTTGGAGGGTACAA	AGCCCATCAGCTGTTTTGTC
Myod1	CCACTCCGGGACATAGACTTG	AAAAGCGCAGGTCTGGTGAG
Myog	GAGACATCCCCCTATTTCTACCA	GCTCAGTCCGCTCATAGCC
IL1b	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
Nos2	CACGGACGAGACGGATAG	GGGAGGAGCTGATGGAGT
IL10	TACCTGGTAGAAGTGATGCC	CATCATGTATGCTTCTATGC
Tgfb1	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
Tnfa	GCCACCACGCTCTTCTGTCT	GTCTGGGCCATGGAACTGAT
Myh3	CACCTGGAGAGGATGAAGAAGAA	AAGACTTGACTTTCACTTGGAGTTTATC
Cebpb	GGAGACGCAGCACAAGGT	AGCTGCTTGAACAAGTTCCG
Gapdh	CTGGAAAGCTGTGGCGTGAT	CCAGGCGGCACGTCAGATCC

Table II-1. Nucleotide sequences of primers used for RT-qPCR analysis

Antibody	Host	Company	Catolog #	Notes
pMet (Y1234/1235)	rabbit	Cell Signaling	#3077	WB (1:500), IF (1:100)
pMet (Y1349)	rabbit	Cell Signaling	#3133	WB, 1:500
Met	rabbit	Sigma Aldrich	SAB4300599	WB, 1:2000
HDAC4	mouse	Cell Signaling	#5392	WB, 1:1000
pSmad3 (S423/425)	rabbit	Cell Signaling	#9520	WB, 1:1000
Smad3	rabbit	Cell Signaling	#9523	WB, 1:1000
Laminin	rabbit	Sigma Aldrich	L9393	IF, 1:1000
GFP	mouse	Millipore	MAB3580	IF, 1:500
CD11b	rat	Biolegend	101202	IF (1:200)
CD31	rat	BD Biosciences	550274	IF (1:200)
Ly6G	rat	Abcam	ab25377	IF (1:200)
MyoD	rabbit	Santacruz	sc-304	WB (1:500)
pLKB1 (S428)	rabbit	Cell Signaling	#3482	WB (1:1000)
LKB1	rabbit	Cell Signaling	#3047	WB (1:1000)
pCREB (S133)	rabbit	Cell Signaling	#9198	WB (1:1000)
CREB	rabbit	Cell Signaling	#4820	WB (1:1000)
pAMPKα (T172)	rabbit	Cell Signaling	#2535	WB (1:1000)
AMPKα	mouse	Cell Signaling	#2793	WB (1:1000)
pCaMKKβ (S511)	rabbit	Cell Signaling	#12818	WB (1:1000)
CaMKKβ	mouse	Abcam	ab124096	WB (1:500)
Myh3	mouse	DSHB	BF-45	IF (1:50)
β-tubulin	rabbit	Abcam	ab6046	WB (1:1000)
GAPDH	mouse	Abcam	ab8245	WB (1:5000)

Table II-2. Antibodies used for Western blot hybridization and Immunofluorescence

MA). The membranes were blocked with 5% BSA (Gibco, MA) in TBST (1M Tris-HCl, pH 7.4, 0.9% NaCl and 0.1% Tween-20) for 1 hour and probed with antibodies diluted in 3% BSA blocking solution overnight at 4˚C. Membranes were then incubated with HRP-conjugated anti-mouse or anti-rabbit IgG (1: 100,000; Sigma Aldrich, MO) for 1 hour, and the protein bands were visualized with the enhanced chemiluminescence system (Millipore, MA). Quantification of the band intensity was done by Image J software (National Institute of Health, MD).

2.3 Enzyme-linked immunosorbent assay (ELISA)

TAs were prepared and mechanistically homogenized using polypropylene pestles (Bel-Art Scienceware, NJ) and total proteins were extracted in RIPA lysis buffer (Sigma Aldrich, MO) containing a protease inhibitor (Roche, Basel, Switzerland), phosphatase inhibitor (Roche, Basel, Switzerland), and PMSF (Sigma Aldrich, MO). Samples were centrifuged at 12,000 rpm for 15 mins at 4˚C and the supernatants containing total protein were subjected to mHGF or hHGF ELISA (R&D systems, MN) following the manufacturer's protocol.

2.4. siRNA transfection

Raw 264.7 cells were transfected with siRNA specific to AMPKα or scramble siRNA (Santa Cruz Biotechnology, TX) using RNAiMAX (ThermoFisher Scientific, MA) according to the manufacturer's protocol. After 24 hours, cells were subjected to the analysis. Knock-down efficiency was evaluated by Western Blot using antibodies against AMPKα (Cell Signaling, MA).

2.5 Immunohistochemistry and H&E staining

TAs were fixed in 10% normalized buffered formalin (Sigma Aldrich, MA) and dehydrated with a gradient series of ethanol from 70% to 100%. Samples were embedded in the paraffin block and sectioned to 6 μm thickness. A paraffin section of the TA was stained by hematoxylin and eosin, and the morphology of each cross-section was analyzed by Image J software (National Institutes of Health, MD). More than 300 myofibers were assessed from 4 individual mice in each group.

2.6 Immunofluorescence

Immunofluorescence analyses were performed as previously described (Ahn et al., 2014). Briefly, TAs were isolated and fixed in 4% paraformaldehyde in PBS and cryo-sectioned to 6 μm thickness. Sections were washed in 0.1M PBS (pH 7.4) twice, then blocked for 1 hour with PBS containing 5% fetal bovine serum (Corning, NY), 5% donkey serum (Jackson ImmunoResearch Laboratories, PA), 2% BSA (Sigma Aldrich, MA) and 0.1% Triton X-100 (Sigma Aldrich, MA). Samples were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Sections were washed 4 times in PBS and incubated for 1 hour at room temperature with secondary antibodies (Invitrogen, CA) diluted in PBS. Immunostained samples were further washed 6 times and counterstained with DAPI (Sigma Aldrich, MA) for nuclear staining. The fluorescence images were obtained using a Zeiss LSM 700 confocal microscope (Zeiss, Oberkochen, Germany).

3. *In vivo* mouse models

3.1 Animal cares

Ten-week-old male C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Korea) for animal studies. Mice were housed at 24˚C with a 12h light-dark cycle. All experiments were performed in compliance with the guideline set by the International Animal Care and Use Committee at Seoul National University.

3.2 Sciatic nerve transection model

All surgical protocols were approved by the International Animal Care and Use Committee at Seoul National University. For sciatic nerve transection, ten-week-old male C57BL/6 mice were anesthetized with isoflurane. The sciatic nerve of the right leg was cut and a 3 mm piece was excised. To prevent nerve reattachment, severed nerve endings were tied with 6-0 black silk suture (AILEE, Pusan, Korea). Then the incision was sutured using 5-0 silk suture (AILEE, Pusan, Korea). Sham surgery was performed by following the same procedure except severing the sciatic nerve. PHA-665752 (Tocris Bioscience, MO), a c-met inhibitor, was dissolved in DMSO (Sigma Aldrich, MO) and i.p. administered in each mouse on a daily basis with a dose of 20 mg/kg. For i.m. injection, 0.3 mm needle size, 0.5 ml insulin syringe (BD, NJ) was used. pCK or pCK-HGF-X7 plasmid expression vector was dissolved in 50 μl PBS (2 μg/μl). The injection procedure was performed by injecting the needle parallel to the tibia and then delivering plasmid into the middle of the TA.

3.3. CTX-induced skeletal muscle regeneration model

All surgical protocols were approved by the International Animal Care and Use Committee at Seoul National University. For muscle injury, ten-week-old male C57BL/6 mice were anesthetized with isoflurane and the TAs were injected with 50μl CTX (Latoxan, Valence, France), diluted to 10μM in phosphate buffered saline (PBS). PHA-665752 (Tocris

Bioscience, MO), a c-met inhibitor, was dissolved in DMSO (Sigma Aldrich, MO) and i.p. administered in each mouse on a daily basis with a dose of 20 mg/kg. For i.m. injection, 0.3 mm needle size, 0.5 ml insulin syringe (BD, NJ) was used. pCK or pCK-HGF-X7 plasmid expression vector was dissolved in 50 μl PBS (2 μg/μl). The injection procedure was performed by injecting the needle parallel to the tibia and then delivering plasmid into the middle of the TA.

CHAPTER III

Roles of HGF in

neurogenic muscle atrophy

1. Background

The skeletal muscle is a highly dynamic tissue which can vary in size, structure, and contractile force under different conditions. Innervation of the motor neuron provides various trophic factors to the target muscle, which are essential to maintain the skeletal muscle function. One of the pathological hallmarks of motor neuron diseases, such as amyotrophic lateral sclerosis (ALS) or poliomyelitis, is deterioration of the muscle innervation. In these diseases, the skeletal muscle undergoes severe physiological changes such as debilitating muscle loss due to the deficiency in neural input. Loss of nerve supply to muscle fiber could activate the muscle atrophy program, including activation of ubiquitin-dependent proteasomal or autophagosomal lysis of the muscle components (Beehler et al., 2006). The muscle-specific E3-ubiquitin ligases, MuRF1 and Atrogin-1/MAFbx, are known to be responsible for proteasomal degradation of the muscle. Histone deacetylase 4 (HDAC4) was reported to positively regulate the expression of these E3-ubiquitin ligases, via two independent mechanisms, especially in neurogenic muscle atrophy (Choi et al., 2012; Moresi et al., 2010).

miRNAs are single-stranded 21-22 nucleotide noncoding RNAs that can control gene expression via a post-transcriptional mechanism. Specific miRNAs have recently been discovered as critical regulatory factors controlling skeletal muscle metabolism, including muscle differentiation and homeostasis. For example, miR-206, a member of muscle-enriched miRNAs (myo-miR), is known to facilitate muscle differentiation by regulating the expression of myogenic regulatory factors *in vitro* (Dey et al., 2011; Kim et al., 2006) and *in vivo* (Taulli et al., 2009). It was recently shown that miR-206 could delay the progression of ALS by suppressing the expression of HDAC4 and thereby promoting regeneration of the neuromuscular synapse, suggesting that miR-206 might affect the course of the neurogenic muscle atrophic condition (Williams et al., 2009).

Hepatocyte growth factor (HGF) was first discovered as a potent mitogen for

hepatocytes, and later found to also contain mitogenic, morphogenic, angiogenic, anti-apoptotic, and anti-fibrotic activities (Ding et al., 2003; Mizuno et al., 2001; Ohmichi et al., 1996; Pelicci et al., 1995; Xiao et al., 2001). It is well known that the interaction of HGF with its cellular receptor, c–met, turns on a variety of signaling pathways, such as Stat3, Erk, and Akt, depending on the cell types. In the skeletal muscle, HGF is known to be secreted by activated muscle stem cells (also known as satellite cells) *in vivo* (Jennische et al., 1993; Tatsumi et al., 1998) as well as *in vitro* (Allen et al., 1995; Sheehan et al., 2000). Upon muscle injury, HGF activates muscle stem cells that reside in muscle fiber, leading to regeneration of damaged muscle (Miller et al., 2000; Tatsumi et al., 1998). Exogenously added recombinant HGF protein has been shown to ameliorate pathological conditions in mouse models for hypoxia-induced muscle atrophy (Hauerslev et al., 2014) and polymyositis/dermatomyositis (Sugiura et al., 2010). It was reported that HGF could promote the survival of motor neurons *in vitro* (Wong et al., 1997), and that HGF overexpression might attenuate the death of motor neurons and axon degeneration in ALS mice (Sun et al., 2002). Despite its interesting biological characteristics, the role(s) of HGF regarding muscles under denervation conditions remains poorly understood.

Here, we report that the role of HGF is partially compensational in neurogenic muscle atrophy. HGF expression was upregulated following surgical denervation. When mice were treated with PHA-665752, an inhibitor of c-met receptor, muscle atrophy was exacerbated. Consistently, the expression level of HDAC4 was further increased, whereas it was the opposite for miR-206. HGF overexpression by intramuscular (i.m.) injection of plasmid expression vector slowed down the progression of muscle atrophy. Data from the C2C12 cell culture experiments indicated that HGF regulated the expression of miR-206 by suppressing TGF-β-mediated phosphorylation of Smad3. Taken together, our data suggested that HGF might be used as a platform for developing therapeutic agents to treat neurogenic

muscle atrophy.

2. Results

2.1 HGF/c-met signaling is upregulated in denervated muscle

To investigate the possible involvement of HGF in neurogenic muscle atrophy, a sciatic nerve transection model, in which irreversible damage is made to the nerve by cutting the sciatic nerve, was used. Denervation was induced by severing the sciatic nerve of a 10-week-old C57BL/6 mouse, and total proteins were prepared from the tibialis anterior (TA) muscle of the injury site at appropriate time points followed by ELISA. The basal level of the HGF protein in the control side was maintained at 50-80 pg/mg of total cellular protein in the TA. After denervation, the level of HGF protein in the ipsilateral side was rapidly increased, reaching a plateau at approximately 250 pg/mg of total cellular protein at day 10 (Figure III-1A). A similar magnitude of RNA induction was observed after denervation as measured by RT-qPCR (Figure III-1B). These data suggested that HGF expression was induced by 3-5 folds after denervation at both RNA and protein levels compared to the normal, uninjured situation.

C-met is the only known receptor for HGF. When HGF is expressed, its receptor, c-met, becomes activated by phosphorylation. Therefore, the level and content of the c-met protein was analyzed after nerve injury in the same sciatic nerve transection model. Total proteins were prepared from the TA followed by Western blot using antibodies to total c-met or phosphorylated form (Figure III-2). After denervation, the level of total c-met protein rapidly increased, and the phosphorylated form of c-met protein was also upregulated in the denervated muscle.

The effect of denervation on HGF expression was also measured in hSOD1-G93A

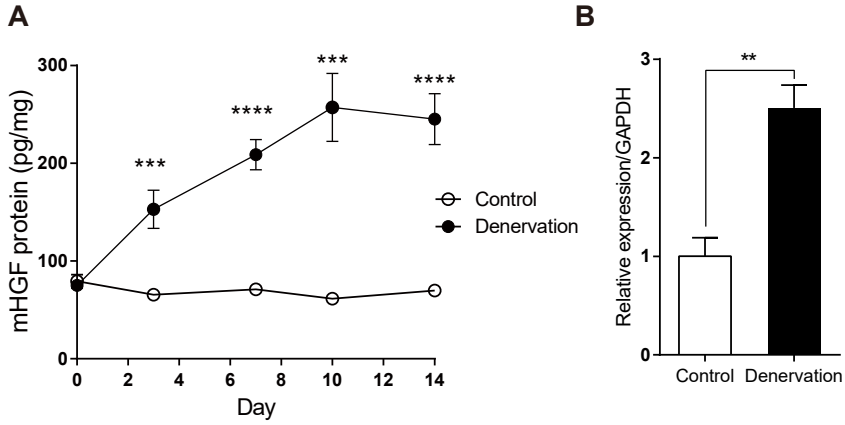

Figure III-1. Expression kinetics of HGF in denervated muscle.

(A) Expression kinetics of HGF protein after denervation. The muscle was isolated at 3, 7, 10, and 14 days after denervation, and total proteins were analyzed by ELISA to measure the protein level of HGF. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus control muscle (unpaired student's t test), n=4 per group. (B) Change in the RNA level of HGF after denervation. RNAs were prepared from TAs 3 days after denervation followed by RT-qPCR, *p<0.05 (unpaired student's t test), n=4 per group. The values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

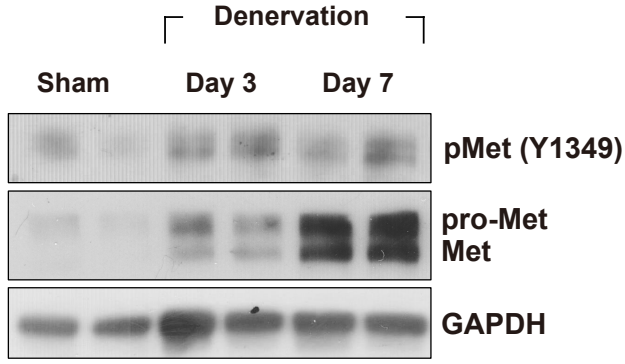

Figure III-2. Expression and phosphorylation kinetics of c-met in denervated muscle.

Expression kinetics of c-met and phosphorylated c-met proteins in denervated TA. Muscle was isolated at days 3 and 7, and total proteins were prepared followed by Western blot using specific antibodies to total or phosphorylated c-met. Each lane represents represented a sample from an individual mouse. Two representative results are are shown here. Two independent experiments were performed (n=4), and similar results were obtained.

transgenic mice, a widely used model for ALS. These mice overexpress the mutated superoxide dismutase (SOD1) protein, resulting in motor neuron death and severe muscle wasting throughout the entire body (Gurney et al., 1994). Total proteins were prepared from the TA of hSOD1-G93A transgenic mice at day 150 after birth when the muscle atrophy progressed severely, and the HGF protein level was measured using ELISA. Wild-type mice produced 70-80 pg/mg of HGF in the TA. In hSOD1-G93A transgenic mice, the amount of the HGF protein was higher by approximately 2-fold (Figure III-3).

2.2 Inhibition of c-met signaling aggravates neurogenic muscle atrophy

It was tested whether denervation-induced expression of HGF played a pathological or compensational role, using an inhibitor specific to the c-met receptor, PHA-665752. After sciatic nerve transection, mice were intraperitoneally (i.p.) injected with PHA-665752 on a daily basis. Treatment of PHA-665752 effectively suppressed c-met phosphorylation in denervated muscle (Figure III-9). Ten days later, TA mass from vehicle (DMSO)-treated animals was found to be reduced by 24±2% from 50.1±1.1 mg to 38.1±1.0 mg, compared to that of the sham-operated group, while PHA-665752 treated mice showed a larger reduction, by 34±3% (Figure III-4A and 4B). The skeletal muscle cross-section was analyzed by hematoxylin and eosin (H&E) staining of the TA. In vehicle-treated mice, muscle fiber size was decreased by 41±1% from 1671±128 μm^2 to 972±14 μm^2 compared with that of the sham-operated animals. In PHA-665752 treated mice, it was further reduced, by 51±1%, compared to the sham-operated group (Figure III-5). These data indicated that the inhibition of c-met signaling could worsen muscle mass and cross-sectional area during neurogenic muscle atrophy, suggesting that HGF worked as part of the compensatory system.

MuRF1 and Atrogin-1 are involved in proteasomal degradation of muscle components, and their expression is highly increased at the RNA level after denervation. The

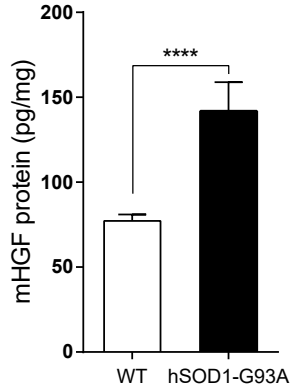

Figure III-3. Expression kinetics of HGF in hSOD1-G93A mice muscle.

Comparison of the HGF protein level in TAs between wild type (WT) and 150 day-old hSOD1-G93A transgenic mice. The TA was isolated and total proteins were analyzed by ELISA to measure the protein level of HGF. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus WT mice (unpaired student's t test), n=6 per group. All data were represented as mean ± SEM.

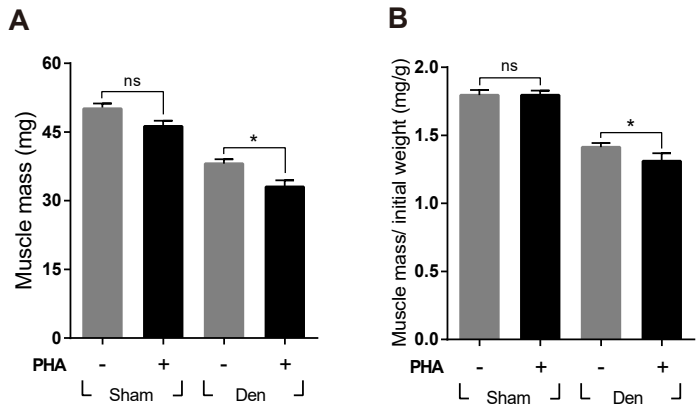

Figure III-4. Effect of c-met inhibitor, PHA-665752, on muscle weight in sciatic nerve transection model.
After sciatic nerve transection, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. (A) Effect on actual muscle weight. (B) Effect on normalized muscle weight. muscle mass was normalized with the initial weight of mice. Den=denervation, PHA=PHA-665752. ns=not significant, *p<0.05 (one-way ANOVA), n=5 per group.

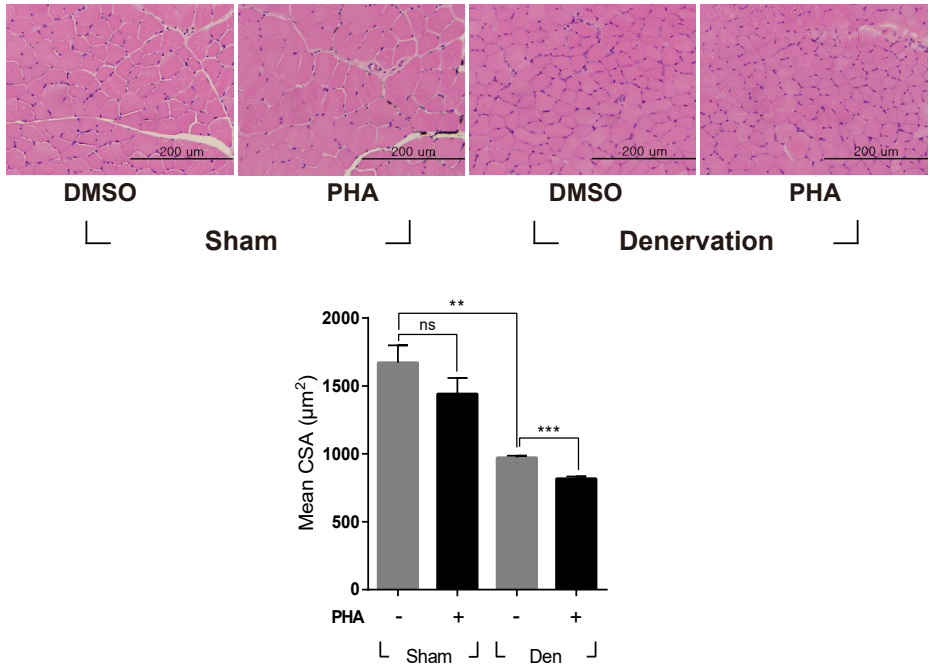

Figure III-5. Effect of PHA-665752 on muscle cross-sectional area in sciatic nerve transection model.

After sciatic nerve transection, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. Effect on cross-sectional area (CSA) of TA was analyzed 10 days after denervation. At least 300 muscle fibers areas were counted per sample. Mean CSA was indicated in the graph. ns=not significant, **p<0.01, ***p<0.001 (one-way ANOVA), n=4 per group. Scale bars, 200 μm.

sciatic nerve was severed to induce denervation of the TA. Three days later, RNAs were isolated from TAs of mice when the RNA level of MuRF1 and Atrogin-1 was greatly induced. In animals treated with PHA-665752, the expression of MuRF1 and Atrogin-1 was even further increased (Figure III-6). In sham-operated animals, PHA-665752 did not have significant effects on either gene. These data suggested that the HGF/c-met signaling pathway might counteract the process of neurogenic muscle atrophy by controlling the expression of genes involved in muscle breakdown.

2.3 C-met signaling controls miR-206 - HDAC4 cascade

Since HDAC4 is a key player in the regulation of MuRF1 and Atrogin-1 during neurogenic muscle atrophy (Moresi et al., 2010), the effect of PHA-665752 on the denervation-mediated increase of HDAC4 expression was studied by RT-qPCR and Western blot. As shown in Figure III-7A, the RNA level of HDAC4 was highly increased after denervation, while treatment with PHA-665752 did not have any effect. The protein level of HDAC4 showed a similar pattern, that is, a sharp increase after denervation, while treatment with PHA-665752 always gave a small, but highly reproducible, increase in the level of the HDAC4 compared to the untreated but denervated animals (Figure III-8; compare lanes 5 and 6 with 7 and 8). These data indicated that HDAC4 expression might be controlled at the post-transcriptional level.

HDAC4 expression has previously been shown to be regulated by miR-206 under muscle atrophic conditions (Williams et al., 2009). To test whether miR-206 expression was affected by c-met signaling, the level of primary miR-206 transcript was analyzed in TAs by RT-qPCR 3 days after nerve transection in the presence or absence of PHA-665752 administration. Denervation markedly increased the level of pri-miR-206. When animals were treated with PHA-665752, however, the level of pri-miR-206 transcript was reduced in

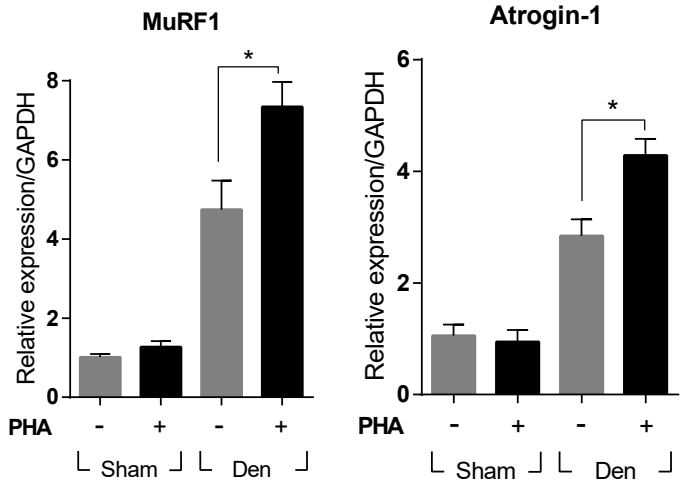

Figure III-6. Effect of PHA-665752 on atrogenes in sciatic nerve transection model.

After sciatic nerve transection, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. Effect on the expression of MuRF1 and Atrogin-1. The RNA level of two genes was determined by real time RT-qPCR using TAs isolated 3 days after denervation. *$p<0.05$ (one-way ANOVA), n=4 per group.

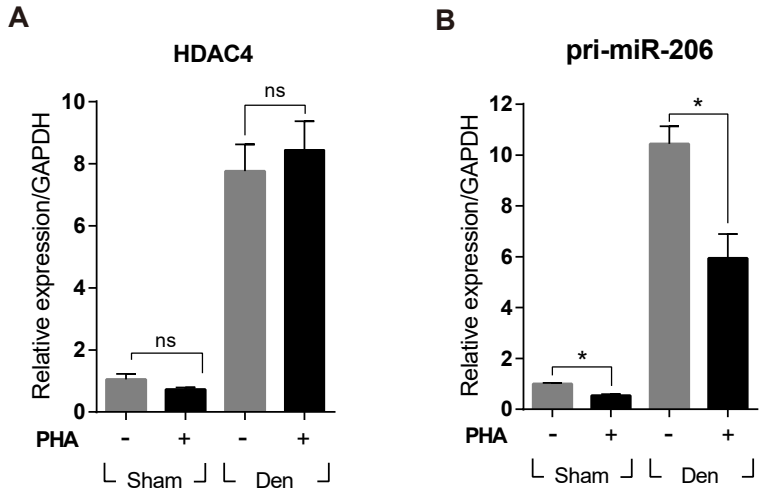

Figure III-7. Effect of PHA-665752 on miR-206-HDAC4 cascade.

After denervation by sciatic nerve transection, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. Three days later, TAs were prepared and total RNAs were isolated followed by RT-qPCR. (A) Effect on HDAC4 RNA. ns=not significant, n=4 per group. (B) Effect on miR-206 primary transcript. *$p<0.05$ (one-way ANOVA), n=4 per group.

both sham and denervated mice (Figure III-7B). The magnitude of reduction was approximately 2-fold in both cases. Taken together, these data suggested that c-met signaling could downregulate HDAC4 expression by upregulating miR-206, not only under denervation but also in the uninjured situation.

MiR-206 expression is known to be controlled by two different pathways; one is E-box transcription factors including myoD and myogenin (Rao et al., 2006; Sweetman et al., 2008), and the other is TGF-β signaling (Ozawa et al., 2013; Winbanks et al., 2011). We found that treatment with PHA-665752 had little or no effect on the former (Figures III-10B and III-10C). TGF-β is highly induced in denervated muscle and participates in developing pathological conditions (Ozawa et al., 2013). The antagonistic relationship between HGF and TGF-β signaling has already been reported in fibrotic conditions (Dong et al., 2013; Mizuno and Nakamura, 2004). Therefore, it was tested whether HGF regulates the expression of miRNA by interacting with TGF-β signaling. Since TGF-β signaling is already known to downregulate the expression of miR-206 through its canonical pathway, Smad2/3 signaling (Winbanks et al., 2011), the effect of PHA-665752 on Smad3 phosphorylation was tested. Total proteins were prepared from TAs followed by Western blot using antibodies detecting Smad3 or its phosphorylated form. As expected, denervation significantly increased the level of total and phosphorylated Smad3 (Figure III-8, compare lanes 1 and 2 with 5 and 6). However, when animals were treated with PHA-665752, Smad3 phosphorylation was even more increased in both sham and denervated mice (Figure III-8, compare lanes 5 and 6 with 7 and 8). These data indicated that HGF/c-met signaling might regulate the expression of miR-206 through the Smad3-dependent pathway.

2.4 HGF regulates miRNA-206 expression via suppressing TGF-β signaling

To understand the mechanism(s) underlying the effect of HGF at the molecular and

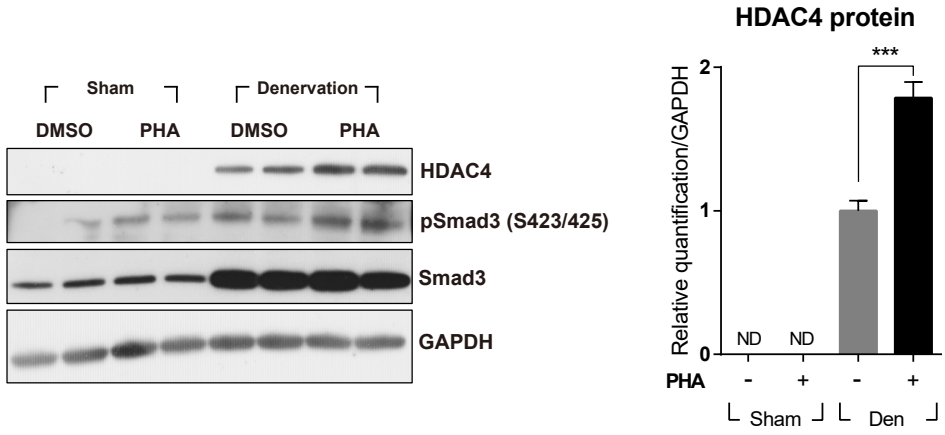

Figure III-8. Effect of PHA-665752 on HDAC4 and Smad3.

After denervation by sciatic nerve transection, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. Three days later, TAs were prepared and total proteins were isolated followed by Western blot. Effect on HDAC4, total and phosphorylated Smad3 protein. This presents two representative results from two independent experiments, with a total number of mice being 4. The graph shows the result of quantification of HDAC4 protein. Values were normalized to GAPDH. ND=not detected, ***$p<0.001$ (unpaired student's t test).

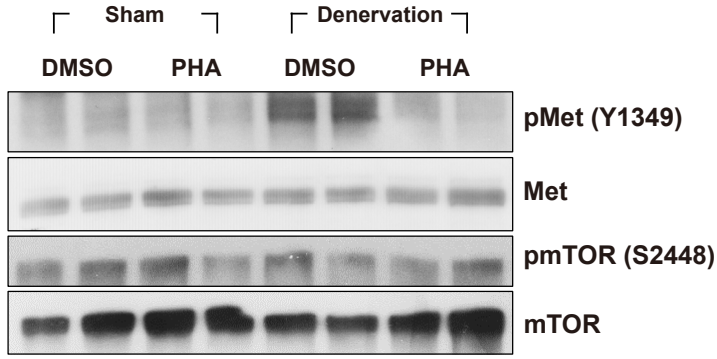

Figure III-9. Effect of PHA-665752 on c-met, mTOR phosphorylation.
After denervation by sciatic nerve transection, mice were intraperitoneally injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. Three days later, TA muscles were prepared and total proteins were isolated followed by Western blot. (A) Effect on total and phosphorylated c-met, mTOR protein. n=4 per group.

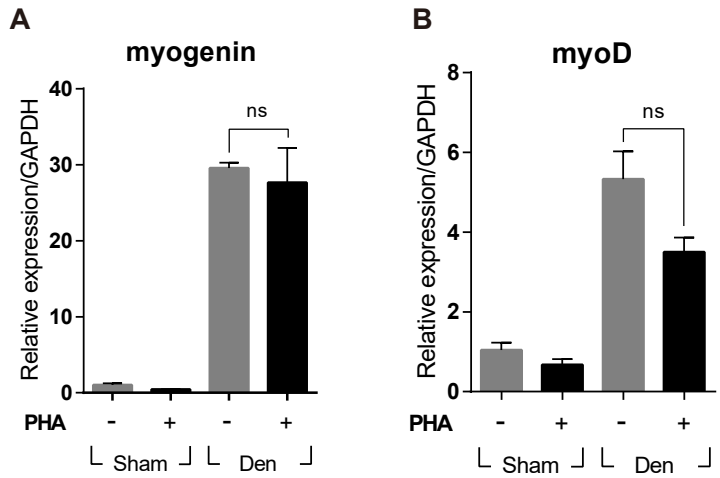

Figure III-10. Effect of PHA-665752 on the expression of E-box transcription factors.

After denervation by sciatic nerve transection, mice were intraperitoneally injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. Three days later, TA muscles were prepared and total RNAs were isolated followed by RT-qPCR . (A) Effect on myogenin RNA. (B) Effect on myoD RNA. Values were normalized to GAPDH. ns=not significant. n=4 per group.

cellular levels *in vitro*, C2C12, a murine myoblast cell line, was used. Cells were differentiated to myotube by changing media to DMEM supplemented with 2% horse serum. Four days later, cells were treated with various concentrations of the recombinant human HGF (hHGF) protein in the presence of 1 ng/ml of recombinant TGF-β for 24 hours. When differentiated C2C12 myotubes were treated with TGF-β only, the expression level of pri-miR-206 was reduced to about 40%, compared to the untreated control. Cotreatment with HGF 10 ng/ml inhibited a TGF-β-mediated decrease in the level of pri-miR-206 transcript, resulting in a 1.5-fold increase compared to the TGF-β only group (Figure III-11A). Similar patterns were observed when the expression level of mature miR-206 was measured (Figure III-11B). The level of miR-206 was not affected by HGF in the absence of TGF-β, suggesting that HGF might upregulate the expression of miR-206 by suppressing the TGF-β signaling.

Next, the effect of HGF on Smad3 phosphorylation was tested. C2C12 cells were pretreated with various concentrations of the hHGF protein for 30 minutes followed by incubation with 2 ng/ml TGF-β for an additional 30 minutes. Treatment with TGF-β increased the level of phosphorylated Smad3 up to 4-fold (Figure III-12, compare lanes 1 with 5). The presence of HGF lowered it in a dose-dependent manner while the level of total Smad3 remained unchanged (Figure III-12). These results indicated that HGF might control the expression of miR-206 by inhibiting Smad3 phosphorylation induced by TGF-β.

It is well known that HGF/c-met signaling utilizes downstream effectors such as Erk1/2, p38, JNK, Akt, and mTOR, to induce various cellular responses. It was tested which downstream effectors of HGF/c-met signaling would be involved in the suppression of Smad3 phosphorylation. C2C12 cells were pretreated with pharmacological inhibitors of Erk1/2, p38, JNK, Akt, and mTOR for 30 minutes, followed by treatment with 10 ng/ml of hHGF for 30 minutes and then by incubation with 2 ng/ml of TGF-β for an additional 30 minutes. Again, HGF inhibited TGF-β-induced Smad3 phosphorylation (Figure III-13,

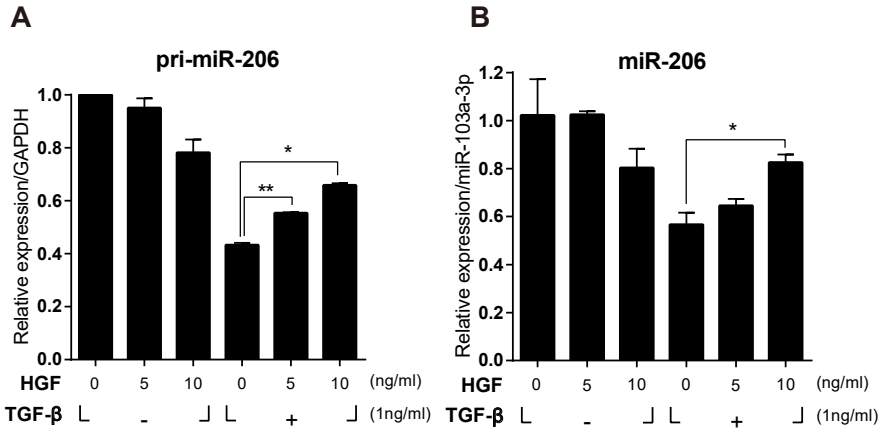

Figure III-11. Effect of recombinant HGF protein on miR-206 expression in C2C12 cells.

C2C12 cells were plated and then cultured in differentiation medium in the presence or absence of recombinant TGF-β and HGF proteins. Total RNAs were prepared and analyzed by RT-qPCR. (A) Effect on pri-miR-206 transcript. Values were normalized to GAPDH. *p<0.05, **p<0.01 (unpaired student's t test), n=3 per group. (B) Effect on mature miR-206. Values were normalized to miR-103a-3p. *p<0.05, (unpaired student's t test), n=3 per group.

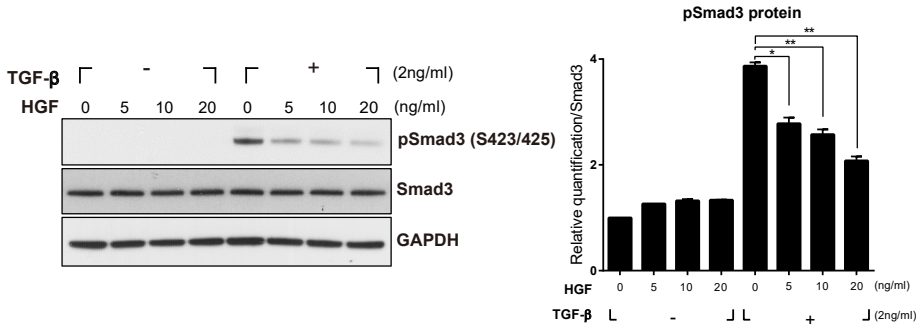

Figure III-12. Effect of recombinant HGF protein on Smad3 phosphorylation in C2C12 cells.

C2C12 cells were plated and then cultured in differentiation medium in the presence or absence of recombinant TGF-β and HGF proteins. Total proteins were prepared and analyzed for Smad3 by Western blot. For Western blot, two independent experiments were performed, one representative result was shown. The graph displays the result of the protein band quantification. Effect of HGF on Smad3 phosphorylation. The graph shows the result of protein band quantification. Values were normalized to total Smad3. *p<0.05, **p<0.01 (unpaired student's t test).

compare lanes 2 with 3). Among different inhibitors, and SP600125, an inhibitor of JNK, seems to be the only one that could rescue the HGF-mediated suppression of Smad3 phosphorylation (Figure III-13, compare lanes 3 with 6). Consistent with these data, treatment with SP600125 significantly reduced the effect of HGF on the pri-miR-206 expression suppressed by TGF-β (Figure III-14A). Taken together, these data suggested that JNK might act as a downstream signal of the HGF/c-met pathway to inhibit Smad3 phosphorylation.

Taken together with data from previous publications, JNK appears to control Smad3 in two ways, by the transcriptional regulation of TGF-β (Ventura et al., 2004) and through phosphorylation of the linker region between Mad homology-1 (MH1) and MH2 (Li et al., 2015). The former is the not case for HGF as the RNA level of TGF-β was not changed by HGF treatment. Therefore, it was tested whether HGF could inhibit the activity of Smad3 by phosphorylating the linker region and subsequently, suppresses phosphorylation of serine 423/425 residues at the C-terminus. C2C12 cells were pretreated with SP600125, followed by followed by treatment with hHGF for 30 minutes, and TGF-β for an additional 30 minutes. As previously reported, treatment with TGF-β induced phosphorylation at both serine 204 and serine 213 residues, two phosphorylation sites in the linker region of Smad3. Cotreatment with HGF increased the phosphorylation of serine 204 residue through JNK, while the phosphorylation of serine 213 residue was not affected by either HGF or SP600125 treatment (Figure III-14B). These results indicated that HGF/JNK signaling might inhibit the activity of Smad3 by phosphorylating the serine 204 residue of Smad3.

2.5 Exogenous introduction of HGF alleviates neurogenic muscle atrophy

Based on the above data indicating a positive role(s) of HGF in muscle atrophy, we tested the effects of the exogenous addition of HGF in the same model. Since HGF has a very short

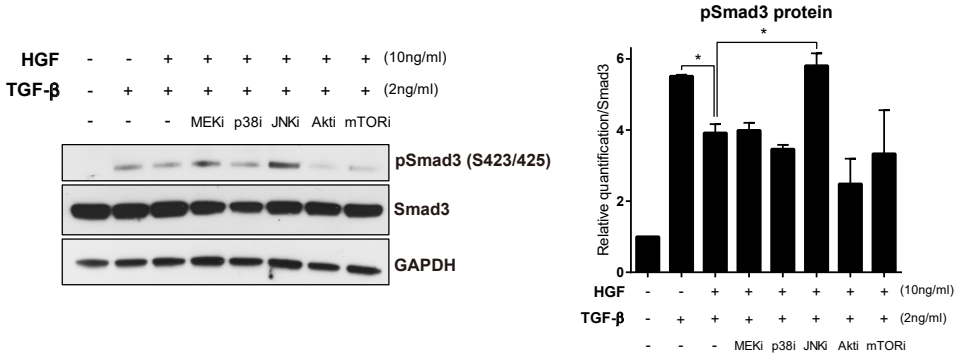

Figure III-13. Effect of recombinant HGF protein on miR-206 and Smad3 in C2C12 cells.

C2C12 cells were plated and then cultured in differentiation medium in the presence or absence of recombinant TGF-β and HGF proteins. Total proteins were prepared and analyzed for Smad3 by Western blot. For Western blot, two independent experiments were performed, one representative result was shown. The graph displays the result of the protein band quantification. Inhibitors of five known downstream effectors, Erk1/2, p38, JNK, Akt, and mTOR were pretreated and HGF-mediated suppression of phosphorylated Smad3 was measured. Values were normalized to total Smad3. *$p<0.05$ (unpaired student's t test).

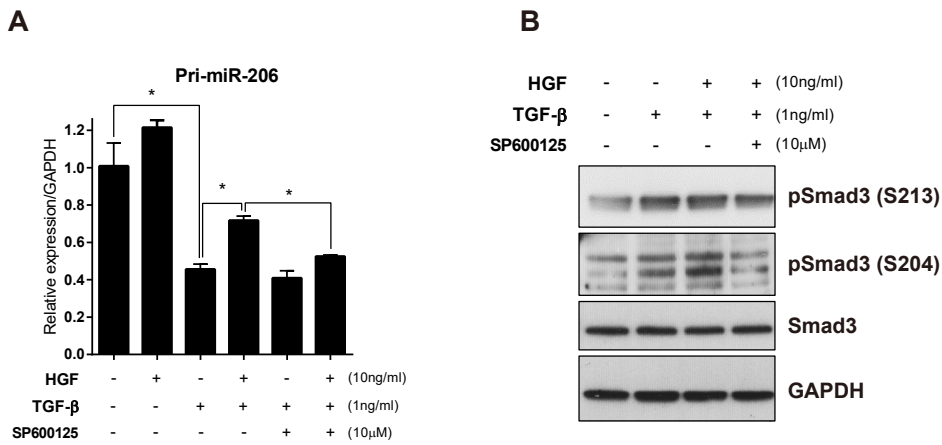

Figure III-14. Effect of JNK inhibitor, SP600125, on the HGF-mediation regulation of pri-miR-206 and Smad3 linker phosphorylation in C2C12 cells.

C2C12 cells were plated and then cultured in differentiation medium in the presence or absence of recombinant TGF-β and HGF proteins. Total RNAs and proteins were prepared and analyzed for miR-206 and Smad3 by RT-qPCR and Western blot, respectively. For Western blot, two independent experiments were performed, one representative result was shown. (A) Effect of JNK inhibitor on the HGF-mediated regulation of pri-miR-206 transcript expression. Values were normalized to GAPDH. *$p<0.05$, (unpaired student's t test), n=3 per group. (B) Effect of JNK inhibitor on the HGF-mediated regulation of the phosphorylation of two linker residues, serine 204 and serine 213, in Smad3.

half-life, less than 5 minutes in serum, the use of recombinant HGF protein for this purpose was not thought to be a viable approach (Kawaida et al., 1994). In the following experiments, we delivered HGF by using a plasmid DNA expression vector. pCK-HGF-X7 (or VM202) is a plasmid designed to express two isoforms of human HGF, HGF_{723} (or dHGF) and HGF_{728} (or cHGF), at high levels *in vivo* (Cho et al., 2008; Hahn et al., 2011; Pyun et al., 2010), and it has been used in a variety of clinical studies and animal models (Cho et al., 2008; Hahn et al., 2011; Kessler et al., 2015; Kibbe et al., 2016; Pyun et al., 2010).

Denervation was induced by severing the sciatic nerve of a 10-week-old C57BL/6 mouse, and 100 μg of pCK-HGF-X7 or pCK control vector lacking the HGF sequence was i.m. administered into the ipsilateral TA, followed by a second injection seven days later. The in vivo protein expression kinetics of this plasmid have been well established previously (Cho et al., 2008; Hahn et al., 2011; Pyun et al., 2010); whereby which the protein level of hHGF produced from pCK-HGF-X7 gradually increases upon injection, reaching a peak (about 30 ng/mg) 7 days after the first injection, then steadily decreases before returning to the control level after approximately 2 weeks (Hahn et al., 2011; Pyun et al., 2010). The hHGF protein is detectable within 5-10 mm from an injection needle point (KR Ko, unpublished data). The *in vivo* time kinetics of hHGF production by pCK-HGF-X7 in denervated muscle were also investigated. The expression level of the hHGF protein produced by pCK-HGF-X7 (about 15-35 ng/mg) was about 100 times higher than mHGF protein induced by denervation (about 150-250 pg/mg) (Figure III-15A). Next, it was tested whether hHGF expressed from pCK-HGF-X7 injected into TA was detected in other tissues. The hHGF protein was detectable in the injected area of TA, but not in sciatic nerve (proximal, distal) and serum (Figure III-15B). Lastly, to test the transfection efficiency of pCK plasmid vector, pCK-eGFP plasmid was used. It was i.m. injected to the denervated TA using the same method as for pCK-HGF-X7. When GFP expression was measured 3 and 7

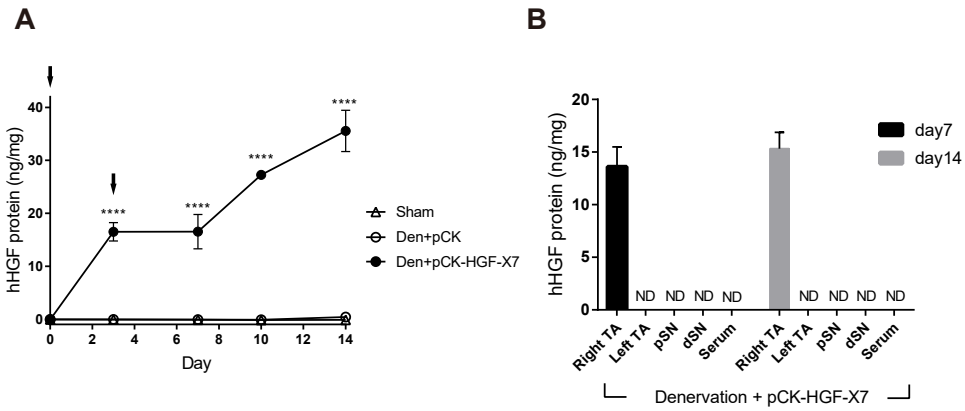

Figure III-15. hHGF expression by pCK-HGF-X7 in denervated mice.

pCK-HGF-X7 was i.m. injected at the time(day 0) of sciatic nerve transection followed by one more injection on day 7. (A) Expression kinetics of the hHGF protein after denervation. Two arrows indicate i.m. injection of pCK-HGF-X7 on days 0 and 7. The muscle was isolated at 3, 7, 10, and 14 days after denervation, and total proteins were analyzed by ELISA to measure the protein level of human HGF. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus pCK-treated muscle (unpaired student's t test), n=4 per group. (B) Expression kinetics of hHGF protein in TA, proximal and distal sciatic nerve, and serum. ND=not detected, n=2 per group.

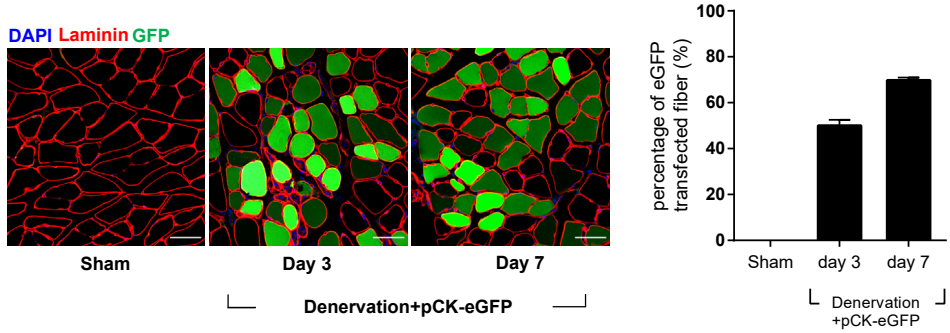

Figure III-16. Transfection efficiency of pCK-eGFP in denervated muscle.

pCK-eGFP was i.m. injected at the time(day 0) of sciatic nerve transection. pCK-eGFP was i.m. injected at the time of sciatic nerve transection. TAs were costained with GFP and the membrane marker laminin (red), 3 and 7 days after injection. n=3 per group. Scale bars, 50μm.

days after denervation and plasmid injection, about 50% of fibers were GFP positive at day3, and 70% at day7. (Figure III-16).

The TA was isolated and quantitated at different time points after denervation. As shown in Figure III-17, in denervated mice injected with the pCK control vector, muscle mass was decreased by 32% and 42% at days 10 and 14, respectively. When mice were injected with pCK-HGF-X7, the reduction of muscle weight was slowed down, to 21% and 34%, compared to the control, at days 10 and 14, respectively.

The muscle cross-section was analyzed by H&E staining to measure muscle fiber size 10 days after denervation. In pCK-treated animals, muscle fiber size was decreased by 61±1% compared to that of the sham-operated group, from 1750±173 μm^2 to 688±11 μm^2. When mice were i.m. injected with pCK-HGF-X7, the magnitude of denervation-induced muscle loss was reduced from 61% to 41% (Figure III-18). Overall, our data showed that the exogenous addition of HGF, delivered in the form of plasmid expression vector, could slow down the progress of neurogenic muscle atrophy.

The effects of i.m. injection of pCK-HGF-X7 on atrogenes were also measured. Denervation was induced, and pCK or pCK-HGF-X7 was i.m. injected into the TA. Three days after denervation, TAs were isolated and the expression level was measured using RT-qPCR. The level of MuRF1 and Atrogin-1 were highly increased after denervation, but pCK-HGF-X7 treatment reduced the denervation-mediated induction of these genes (Figure III-21). Consistent with these observations, the amount of ubiquitination in muscle was also decreased in pCK-HGF-X7 treated group in comparison to pCK-treated animal (Figure III-22). The effect on HDAC4 was also analyzed by measuring the RNA and protein levels, 3 days after denervation and plasmid injection. Denervation greatly increased the RNA level of HDAC4, but i.m. injections of pCK-HGF-X7 had no significant effect (Figure III-20A). When the protein level was measured, however, a completely different picture emerged;

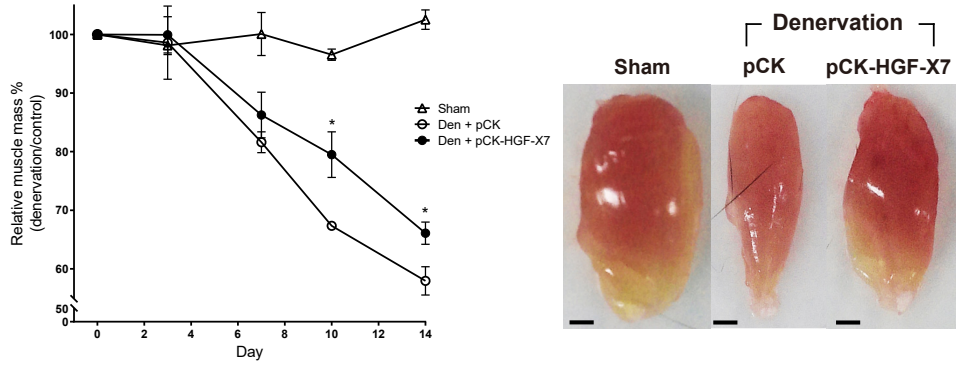

Figure III-17. Effect of HGF overexpression by intramuscular injection of HGF expressing plasmid on muscle weight following surgical denervation.

pCK-HGF-X7 was i.m. injected at the time of sciatic nerve transection followed by one repeat injection 7 days later. TAs were prepared at appropriate time points. Effect on TA weight. Representative TAs from 14 days after denervation are shown in the photos. *$p<0.05$ versus Den+pCK group (one-way ANOVA), n=4 per group. Scale bars, 1mm.

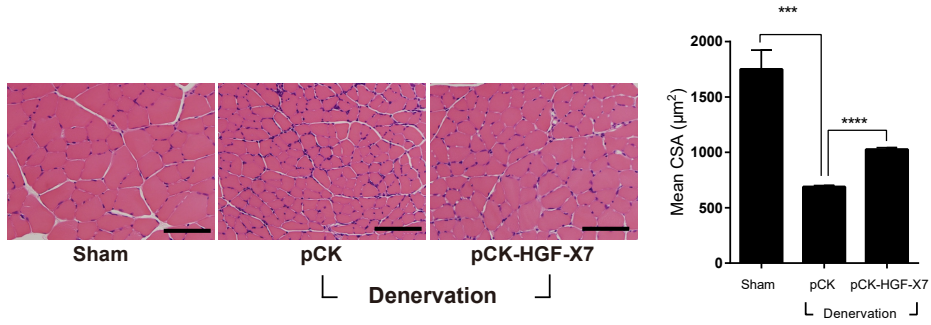

Figure III-18. Effect of HGF overexpression on muscle cross-sectional area following surgical denervation.
pCK-HGF-X7 was i.m. injected at the time of sciatic nerve transection followed by one repeat injection 7 days later. TAs were prepared at appropriate time points. Effect on cross-sectional area of TAs. TAs were analyzed 10 days after denervation. At least 300 muscle fiber areas were counted per sample. Mean CSA was indicated in the graph. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (one-way ANOVA), n=4 per group. Scale bar, 100μm.

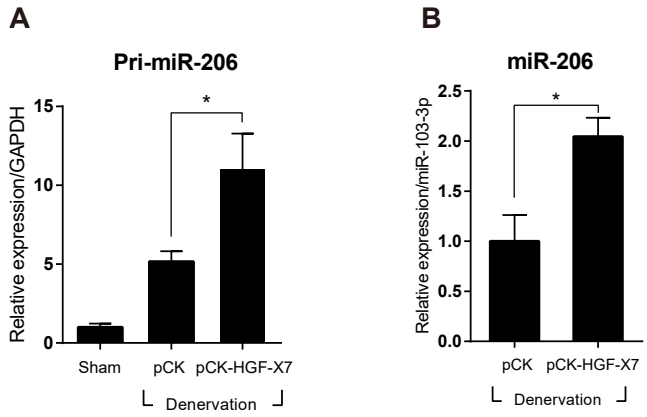

Figure III-19. Effect of HGF overexpression on the expression of miR-206.

pCK-HGF-X7 was i.m. administered at the time of sciatic nerve transection. Three days after denervation, the TA was isolated and total RNAs were analyzed by RT-qPCR. (A) Effect on pri-miR-206 transcript. Values were normalized to GAPDH. *$p<0.05$, n=4 per group. (B) Effect on mature miR-206. Values were normalized to miR-103a-3p. *$p<0.05$ (unpaired student's t test). n=3 per group. All data were represented as mean ± SEM.

pCK-HGF-X7 administration significantly reduced the denervation-mediated increase in the HDAC4 protein level (Figure III-20B).

The effects of pCK-HGF-X7 on primary and mature miR-206 RNAs were determined by RT-qPCR. The level of miR-206 primary transcript was increased by denervation, and became even higher by i.m. injection of pCK-HGF-X7 (Figure III-19A). A similar observation was made with the level of mature miR-206 (Figure III-19B). These data strongly indicated that HGF overexpression by gene transfer technology could reduce the RNA level of atrogenes by controlling miR-206 and HDAC4.

3. Discussion

In this study, I demonstrated that the HGF/c-met signaling plays a compensatory role(s) in mitigating muscle atrophy due to denervation. The HGF level was increased by 3 - 5 folds following denervation. Treating denervated mice with a specific inhibitor for c-met, PHA-665752, aggravated muscle atrophy as measured by muscle mass and its cross-sectional area. Consistent with this observation, treatment with PHA-665752 further increased the expression level of atrogenes like MuRF1 and Atrogin-1, while reducing that of miR-206. Exogenous supply of the HGF protein to the affected region, by i.m. injection of a highly efficient plasmid expression vector, improved muscle atrophy by all measurements, including muscle weight, cross-sectional area, and expression levels of miR-206, HDAC4, and atrogenes. Taken together, HGF/c-met signaling appears to modulate miR-206-HDAC4 cascade in denervated muscle.

TGF-β has been reported to downregulate the expression of miR-206 through Smad3 (Winbanks et al., 2011). We found that treatment of C2C12 cells with recombinant hHGF protein increased the RNA level of miR-206, while decreasing the amount of phosphorylated

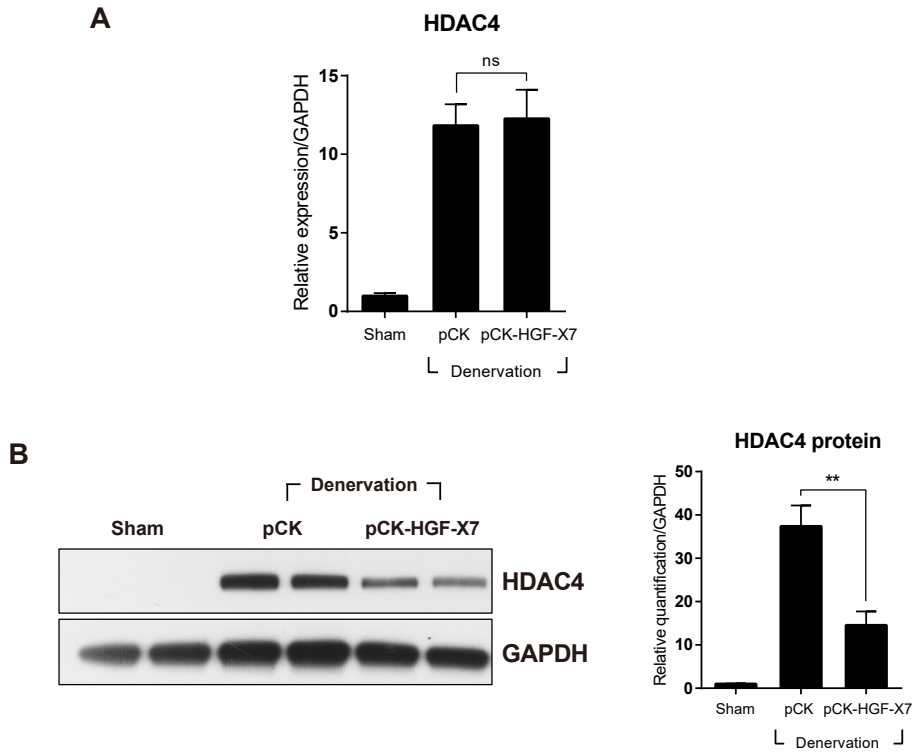

Figure III-20. Effect of HGF overexpression on the expression of HDAC4.

pCK-HGF-X7 was i.m. administered at the time of sciatic nerve transection. Three days after denervation, the TA was isolated and total RNAs and proteins were analyzed by RT-qPCR and Western blot. (A) Effect on HDAC4 RNA. ns=not significant. (B) Effect on HDAC4 protein. For Western blot, two representative results are shown here. Two independent experiments were performed (n=4). Values were normalized to GAPDH for both RNA and protein analysis. **p<0.01 (one-way ANOVA).

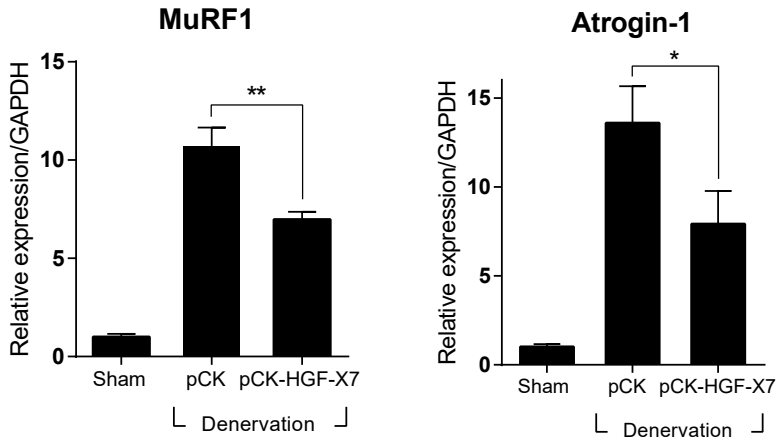

Figure III-21. Effect of HGF overexpression on the expression of atrogenes.

pCK-HGF-X7 was i.m. administered at the time of sciatic nerve transection. Three days after denervation, the TA was isolated and total RNAs were analyzed by RT-qPCR. Effect on the expression of MuRF1 and Atrogin-1. *$p<0.05$, **$p<0.01$ (one-way ANOVA), n=4 per group.

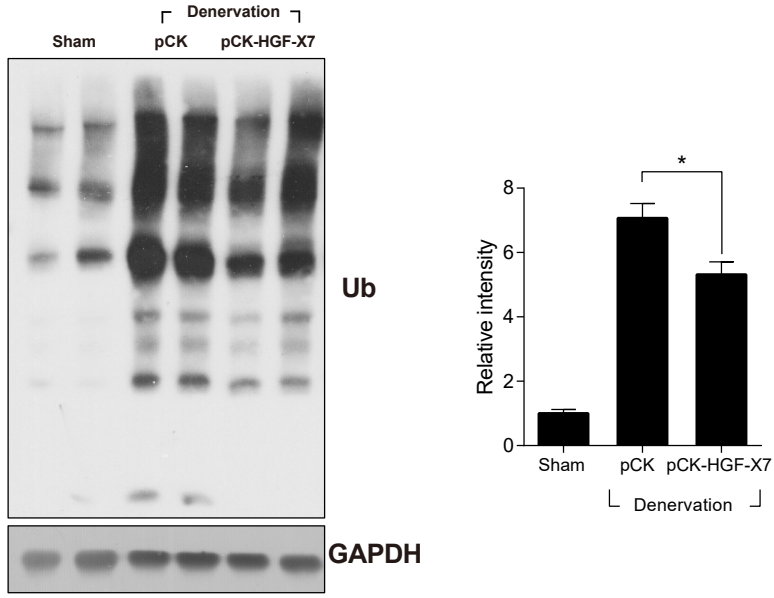

Figure III-22. Effect of HGF overexpression on the ubiquitination of muscle.

pCK-HGF-X7 was i.m. administered at the time of sciatic nerve transection. Seven days after denervation, the TA was isolated and total proteins were analyzed by Western blot. Effect on the ubiquitination of muscle. Total protein extracts from TAs were immunoblotted with anti-ubiquitin (Ub) antibody. The graph shows the result of protein band quantification. Values were normalized to GAPDH.*$p<0.05$ (one-way ANOVA), n=4 per group.

Smad3 protein induced by TGF-β, indicating that HGF might counteract biological consequences generated by TGF-β. Consistently, HGF has been reported to increase the activity of TGIF and galectin-7, both of which act as repressors of TGF-β-stimulated signal transduction by inhibiting transcriptional activity or translocation of Smad3 from the cytoplasm to the nucleus, respectively (Dai and Liu, 2004; Inagaki et al., 2008; Yang et al., 2003). These data suggest that HGF may be used as a basis for developing therapeutics for diseases where TGF-β is a major pathologic factor.

JNK appears to play a key role(s) in the control by HGF of TGF-β-mediated smad3 phosphorylation. Among several pharmacological inhibitors, SP600125, a JNK inhibitor, was the only one that could relieve the HGF-mediated suppression of Smad3 phosphorylation. Together with data from previous publications, JNK appears to control Smad3 in two ways, by the transcriptional regulation of TGF-β (Ventura et al., 2004) and through phosphorylation of the linker region of Smad3 (Li et al., 2015). The former is not the case for HGF as the RNA level of TGF-β was not changed by HGF treatment in our experiments. Therefore, HGF may follow the case of EGF which inhibits the activity of Smad3 by phosphorylating the linker region between Mad homology-1 (MH1) and MH2, and subsequently suppresses phosphorylation of serine 423/425 residues at the C-terminus (Wang et al., 2009). The final outcome is the reduction in the amount of transcriptionally active form of Smad3. It remains to be elucidated whether HGF also regulates TGF-β signaling by controlling the phosphorylation of the linker region of Smad3.

Muscle atrophy results from the imbalance between synthesis and breakdown of muscle proteins. Data from our study suggested that HGF/c-met signaling might improve atrophic conditions by slowing down the breakdown process through the suppression of atrogene expressions. It is interesting to note a difference between our data and those by Hauerslev et al. who used the mouse hypoxia-induced muscle atrophic model (Hauerslev et

al., 2014). In the latter study, mouse recombinant HGF protein was i.p. administered once, and it was observed that the mTOR-S6K pathway was activated, while muscle protein synthesis was facilitated, within a few hours. These results suggest that mTOR pathway might be involved in the effect of HGF on neurogenic muscle atrophy. However, mTOR seemed to play little role in our case. For example, inhibition of HGF/c-met signaling, by daily i.p. injection of c-met inhibitor PHA-665752, did not affect the phosphorylation status of mTOR (Figure III-9), and also, mTOR inhibition did not affect the HGF-mediated upregulation of pri-miR-206 expression in C2C12 cells (Figure III-14C). Taken together, HGF may work differently in these two different muscle atrophy models, each induced by hypoxia or denervation.

HGF is a growth factor binding to the c-met receptor. The interaction between the ligand and the receptor turns on a series of signaling pathways, triggering biological reactions that vary depending on the types of cells. For example, in muscle atrophy described in this report, HGF reduced the expression of HDAC4 that facilitates disease progression, and increased the level of miR-206 which has been reported to delay ALS progression (Williams et al., 2009). Therefore, HGF may be able to produce multiple effects in various diseases associated with muscle atrophy following denervation.

Since the HGF protein has a short half-life, gene transfer technology may provide a powerful way to deliver the HGF protein. Using naked DNA is a method particularly attractive because high-level HGF gene expression for a long-term is undesirable due to its angiogenic, thus potentially oncogenic property (Bussolino et al., 1992). All that is needed is an amount of the HGF protein that can trigger reactions and then disappear, rather than lingering for a long time. In our study, pCK-HGF-X7 (VM202) seems to be generating an amount of the HGF protein sufficient to provide visible therapeutic effects. Our results are consistent with positive data observed in several clinical studies done for peripheral and

coronary artery diseases and neurological diseases as well as in respective animal models involving pCK-HGF-X7 (Ajroud-Driss et al., 2013; Carlsson et al., 2008; Gu et al., 2011; Hahn et al., 2011; Kessler et al., 2015; Kibbe et al., 2016; Kim et al., 2013; Perin et al., 2011; Pyun et al., 2010).

In summary, we demonstrated that HGF/c-met signaling could improve muscle atrophic conditions by upregulating the expression of miR-206. MiR-206 is now well known to play important roles in a majority of neurogenic muscle atrophy cases including ALS. Current treatment methods for these diseases are extremely limited; their efficacy, if any, is marginal and safety is questioned as in the case of riluzole or valproic acid, respectively (Groeneveld et al., 2003; Tong et al., 2005). Given the safety and efficacy records of pCK-HGF-X7 (VM202) shown in several clinical studies for other indications, further studies are warranted to investigate the potential of using HGF, and in particular, plasmid DNA vector expressing HGF, for various neuromuscular diseases.

CHAPTER IV

Effects of HGF on

skeletal muscle regeneration

1. Background

Resolution of tissue damage requires tight interaction between the immune system and the target tissue undergoing repair. Immune cells detect the injury, remove damaged tissues, and then promote repair mechanisms to restore tissue integrity. In addition, they strongly influence the growth and differentiation of stem cells and progenitors to repair the inflicted damage (Wynn and Vannella, 2016). Upon muscle injury, macrophages have been reported to play critical roles in this process as they occupy major cell population infiltrated in injured muscle. They are responsible for the removal of damaged myofibers and also contribute to subsequent regrowth and differentiation of muscle progenitors (Tidball, 2017). These procedures are tightly regulated by the coordinated transition of macrophage between pro-inflammatory (M1) and pro-regenerative (M2) phenotypes in the immune environment (Wang et al., 2014).

Hepatocyte growth factor (HGF) is a multifunctional protein which contains mitogenic, morphogenic, motogenic, and angiogenic activities by interacting with its cellular receptor, c–met (Nakamura and Mizuno, 2010). This interaction subsequently turns on a variety of signaling pathways depending on cell types. HGF has been shown to play important roles in the regeneration process of various tissues by stimulating the proliferation and migration of respective progenitor cells (Huh et al., 2004; Kawaida et al., 1994; Watanabe et al., 2005; Wu et al., 2004). For example, HGF has been implicated in both skeletal muscle development and its regeneration after injury (Sisson et al., 2009).

The interaction between HGF and c-met is required for migration of myogenic progenitors into the limb buds during embryogenesis (Dietrich et al., 1999). Upon muscle injury, HGF activates muscle stem cells that reside in muscle fiber, rendering them to make a myogenic commitment (Allen et al., 1995; Jennische et al., 1993; Sheehan et al., 2000; Tatsumi et al., 1998). Exogenously added recombinant HGF protein was reported to increase

myoblast proliferation while inhibiting differentiation, resulting in delayed regeneration of damaged muscle (Miller et al., 2000). However, recent studies have shown that HGF activated by urokinase plasminogen activator (uPA) promotes muscle regeneration (Sisson et al., 2009) and its receptor, c-met, is responsible for the transition of quiescent muscle stem cells into GAlert, a cellular state in which they have an increased ability to participate in tissue repair (Rodgers et al., 2014). Thus, the role of HGF in muscle regeneration remains to be clarified. In particular, although the immune system plays an instrumental role in the muscle regeneration process, the effect of HGF on immune cells infiltrated in injured muscle tissue is not yet clearly understood.

Here, we report the role of HGF in the transition of infiltrated macrophages during muscle regeneration. HGF expression was upregulated following muscle injury. When mice were treated with PHA-665752, an inhibitor of the c-met receptor, muscle regeneration was delayed. Consistently, the population of M1 and M2 macrophages during muscle regeneration was deregulated. HGF overexpression by intramuscular (i.m.) injection of plasmid expression vector facilitated muscle regeneration. Data from experiments involving Raw 264.7 cells indicated that HGF might regulate the transition of macrophage to the M2 phenotype through CaMKKβ –AMPK signaling. Taken together, our data suggested that HGF might be used as a platform for developing therapeutic agents to treat diseases associated with defects in muscle regeneration.

2. Results

2.1 HGF/c-met signaling is upregulated during muscle regeneration

To investigate the possible involvement of HGF during muscle regeneration, cardiotoxin (CTX)-induced muscle injury model was used. Injection of CTX provides

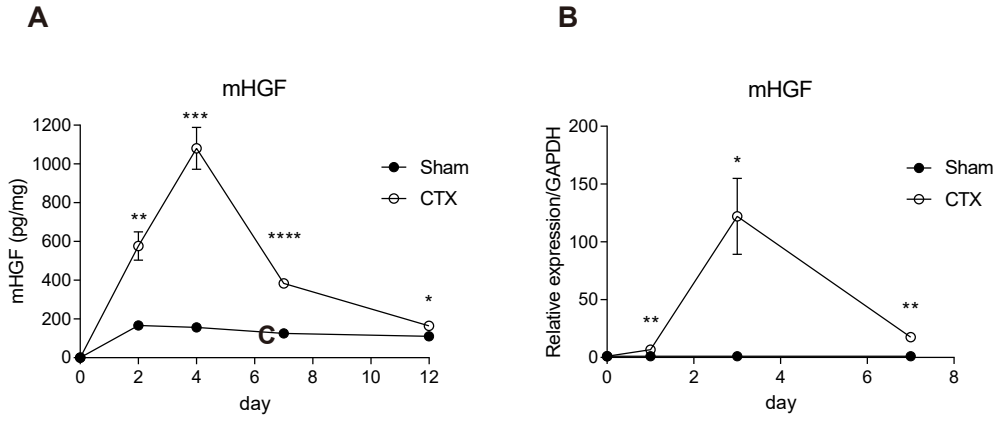

Figure IV-1. Expression kinetics of HGF during muscle regeneration.

(A) Expression kinetics of HGF protein during muscle injury by CTX and regeneration. The muscle was isolated at 2, 4, 7, and 12 days after CTX injection, and total proteins were analyzed by ELISA to measure the protein levels of HGF. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus sham-treated muscles (unpaired student's t test), n=4 per group.

(B) Expression kinetics of the RNA levels of HGF during muscle injury and regeneration. RNA was prepared from TAs 1, 3, and 7 days after CTX injection followed by RT-qPCR, *p<0.05, **p<0.01 versus sham-treated muscles (unpaired student's t test), n=4 per group. The values were normalized to GAPDH.

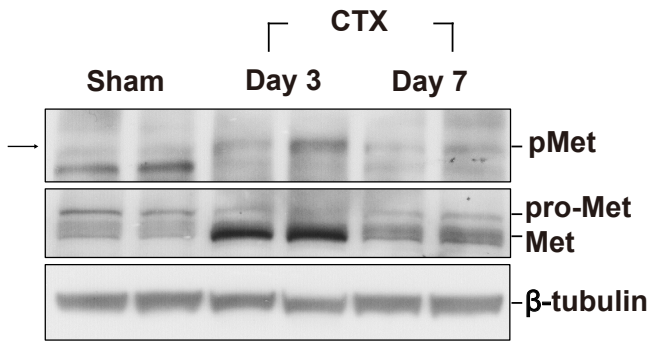

Figure IV-2. Expression and phosphorylation kinetics of c-met during muscle regeneration.

Expression kinetics of total and phosphorylated c-met proteins in CTX-injured TAs. Muscles were isolated at 3 and 7 days postinjury, and total proteins were prepared followed by Western blot using specific antibodies to total or phosphorylated c-met. β-tubulin was used as a loading control. Each lane represents a sample from an individual mouse. Two representative mice are shown here. Two independent experiments were performed (with a total of 4 mice), and similar results were obtained. Arrow indicates the protein of interest in blots.

homogenous damage to the whole muscle and induces the infiltration of various immune cells including monocytes and macrophages into the regenerating muscle until the repair is completed (Arnold et al., 2007). CTX was injected into the tibialis anterior (TA) muscle of a 10-week-old C57BL/6 mouse and total proteins were prepared from the injury site at appropriate time points followed by ELISA. The basal level of the HGF protein in the sham (PBS)-treated side was maintained at 120 to 160 pg/mg of total cellular protein in the TA (Figure IV-1A). After muscle injury, the level of the HGF protein in the injured side was gradually increased, reaching a peak at approximately 1.1 ng/mg of total cellular protein at day 4, then steadily decreased before returning to the sham level at day 12. A similar magnitude of HGF RNA induction was observed during muscle regeneration as measured by RT-qPCR (Figure IV-1B). These data suggested that HGF expression was highly induced after muscle injury at both RNA and protein levels.

c-met is the only known receptor for HGF. When HGF is expressed, the c-met protein becomes phosphorylated to be activated. Therefore, the level and content of the c-met protein was analyzed after muscle injury. Total proteins were prepared from the TA followed by Western blot using antibodies to total c-met or phosphorylated form (Figure IV-2). During muscle regeneration, the level of total c-met protein rapidly increased and the phosphorylated form of the c-met protein was also upregulated in the damaged muscle.

To identify the cell types that express c-met, cells known to be infiltrated in the injured muscle were analyzed by IHC using antibodies to phosphorylated c-met, CD11b for macrophages, CD31 for endothelial cells, and Ly6G for neutrophils, 3 days after muscle injury. As shown in Figure IV-3, the major cell type containing activated c-met was macrophages. In sham-treated muscle, there were no cells expressing phosphorylated c-met (Figure IV-3). Since macrophages are known to be a primary source of HGF after injury (Sisson et al., 2009), these data indicated that in an injured muscle, HGF might act on

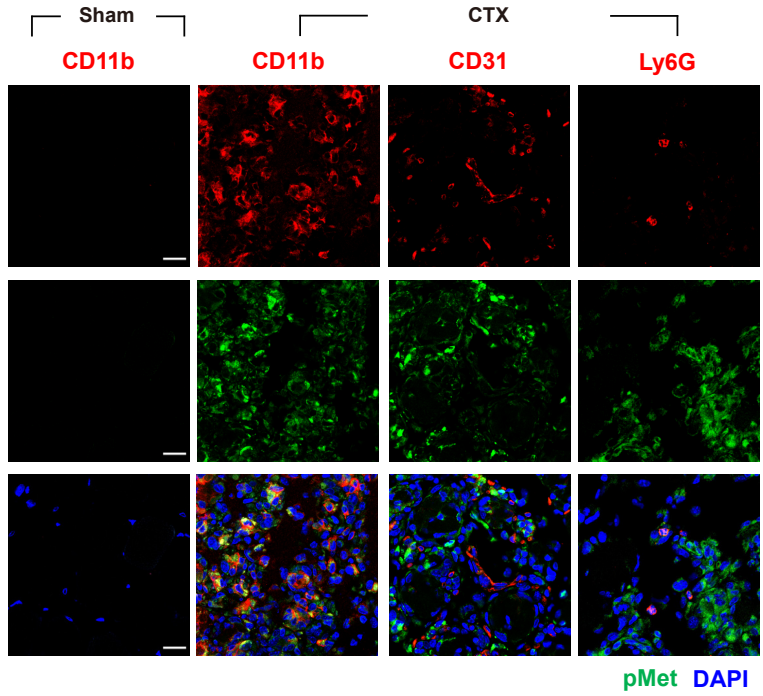

Figure IV-3. Identification of cell types expressing c-met during muscle regeneration.

CTX-injured TA was isolated 3 days postinjury and subjected to immunofluorescence assay using antibodies to CD11b for macrophages, CD31 for endothelial cells, Ly6G for neutrophils (all red), and phosphorylated c-met (green). Nuclei were counterstained with DAPI (blue). n=4 per group. Scale bars, 20 μm.

macrophage in an autocrine manner.

2.2 Inhibition of c-met signaling delayed muscle regeneration

It was tested whether increased expression of HGF would contribute to or inhibit muscle regeneration using an inhibitor specific to the c-met receptor, PHA-665752. After CTX injection, mice were intraperitoneally (i.p.) injected with PHA-665752 on a daily basis. We have previously reported that i.p. injection of PHA-665752 could effectively inhibit the phosphorylation of c-met *in vivo* including in the muscle tissue (Choi et al., 2018; Ko et al., 2018). Treatment with PHA-665752 also suppressed c-met phosphorylation in macrophages infiltrating the injured muscle (Figure IV-6). Seven days later, TA mass from vehicle (DMSO)-treated animals was found to be reduced by 12% from 38.7 ± 0.7 mg to 33.9 ± 0.9 mg, compared to that of the sham-operated group, while PHA-665752 treated mice showed a larger reduction, by 19% (Figure IV-4). The skeletal muscle cross-section was analyzed by hematoxylin and eosin (H&E) staining of the TA (Figure IV-5A). In vehicle-treated mice, muscle fiber size was decreased by 40 ± 3% from 1690 ± 134 μm^2 to 1014 ± 55 μm^2 compared with that of the sham-operated animals. In PHA-665752 treated mice, it was further reduced, by 60 ± 3% compared to the sham-operated group (Figure IV-5B). In addition, the number of necrotic or damaged phagocytic fibers in PHA-treated animal was significantly higher in comparison to the vehicle-treated group (Figure IV-5C). These data indicated that the inhibition of c-met signaling could delay the restoration of muscle mass and muscle fiber regeneration, suggesting that HGF might play a positive role(s) in muscle repair.

It was reported that defects in the process of removing damaged muscle fibers are associated with the improper transition of macrophage from M1 toward M2 phenotype (Mounier et al., 2013). Therefore, the effect of PHA-665752 on the expression of M1 and M2 macrophage markers were tested. Three days after muscle injury, RNAs were isolated from

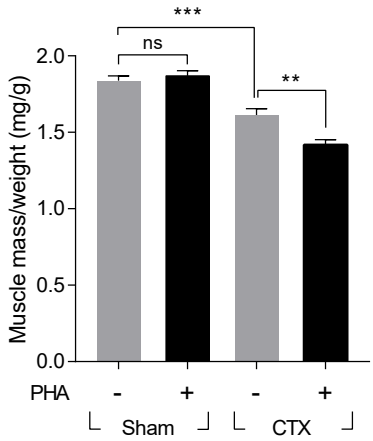

Figure IV-4. Effects of c-met inhibitor, PHA-665752, on muscle weight during regeneration.

After CTX injury, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. CTX injured TAs were analyzed 7 days postinjury. Effect on muscle weight. Muscle mass was normalized with the weight of mice. PHA, PHA-665752. ns, not significant, **p<0.01, ***p<0.001 (one-way ANOVA), n=4 per group.

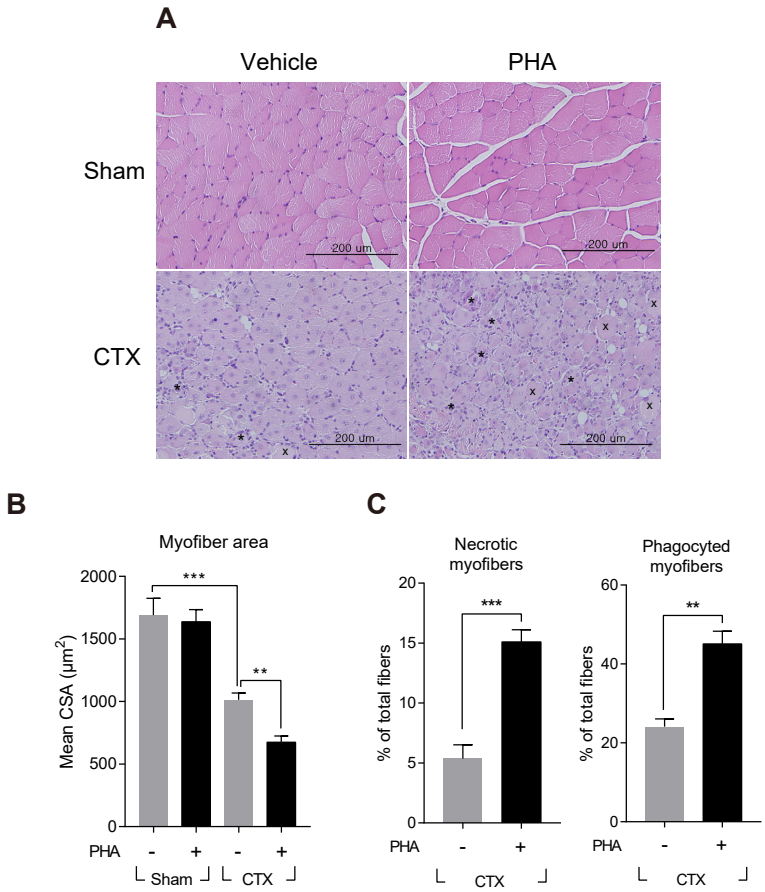

Figure IV-5. Effects of PHA-665752 on muscle morphology during regeneration.

After CTX injury, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. CTX injured TAs were analyzed 7 days postinjury. (A) H&E staining of regenerating muscle. Crosses (x) and asterisks (*) indicate necrotic and phagocyted myofibers, respectively. Scale bars, 200 μm. (B) Effect on cross-sectional areas of muscle fibers. Mean value of area sizes is indicated in the graph. **p<0.01, ***p<0.001 (one-way ANOVA), n=4 per group. (C) Quantification of necrotic or phagocyted fibers expressed as a percentage of total myofibers. At least 300 muscle fiber areas were counted per sample. **p<0.01, ***p<0.001 (unpaired student's t test), n=4 per group.

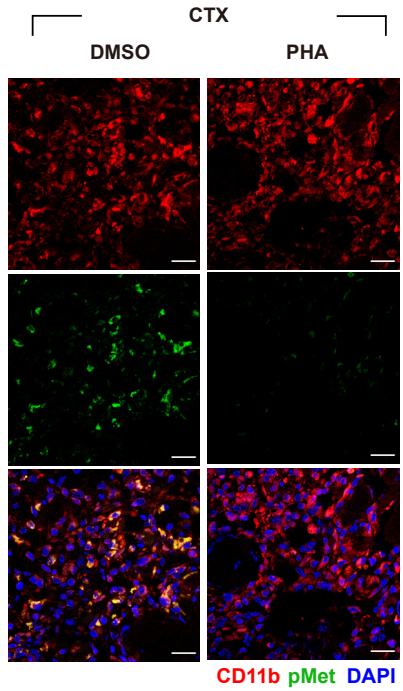

Figure IV-6. Effect of PHA-665752 on c-met phosphorylation in macrophages in CTX-injured muscle.
After CTX injury, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. CTX injured TAs were analyzed 3 days postinjury. Effect on c-met phosphorylation in macrophages. TAs were analyzed by immunofluorescence assay using antibodies to CD11b (red), and phosphorylated c-met (green). Nuclei were counterstained with DAPI (blue). n=4 per group. Scale bars, 20 μm.

TAs of mice when the RNA level of M1 and M2 macrophage markers were greatly induced. In animals treated with PHA-665752, the level of IL-1β, iNOS, and CCL2 (M1 markers) further increased, while that of IL-10, TGF-β, Arginase 1 (Arg1), CD163, and Resistin-like alpha (Retnla) (M2 markers) was reduced, in comparison to the vehicle-treated group (Figure IV-7).

To identify whether the regulation of M1 and M2 marker genes was caused by the changes in the population of macrophage, injured muscle was analyzed by immunofluorescence assay using antibodies to iNOS for M1 macrophages, and CD206 for M2 macrophages. Three days after muscle injury, the number of iNOS-positive macrophages was highly increased, and was further enhanced in animals treated with PHA-665752 (Figure IV-8A). The number of CD206-positive macrophages was also increased upon injury, but PHA-665752 treatment significantly reduced it (Figure IV-8B). These results indicated that the HGF/c-met signaling pathway might be involved in the transition of macrophages to an appropriate type during muscle regeneration.

Muscle regeneration is controlled by spatio-temporal regulation of various myogenic regulatory factors (MRFs) during which infiltrated immune cells play key roles (Di Marco et al., 2005; Tidball and Villalta, 2010). Therefore, the expression of myoD, the key player in the regulation of myogenic commitment, was tested. Three days after muscle injury, the RNA level of myoD was markedly increased in CTX-injured TA, but treatment with PHA-665752 inhibited injury-induced myoD expression (Figure IV-9A). The protein level of myoD showed a similar pattern (Figure IV-9B). Taken together, our results implied that HGF/c-met signaling would act to regulate macrophage M1-M2 transition, thereby contributing to regeneration of injured muscle.

2.3 HGF promotes M2 macrophage transition via CaMKKβ –AMPK signaling

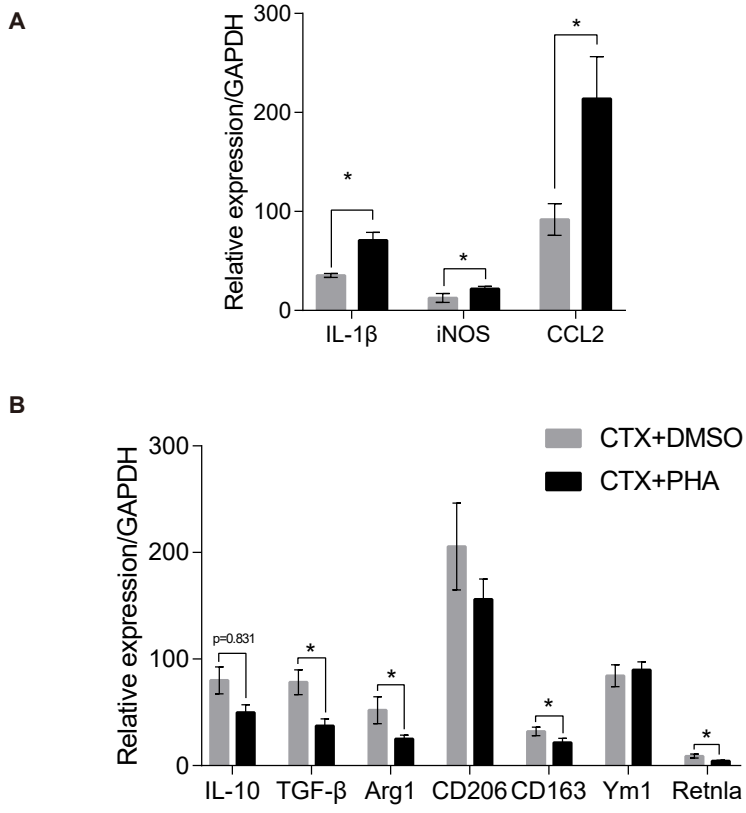

Figure IV-7. Effects of PHA-665752 on the expression of M1 and M2 macrophage markers.

After CTX injury, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. (A) Effects on the RNA levels of M1 markers (iNOS, IL-1β, and CCL2). (B) Effects on the RNA levels of M2 markers (IL-10, TGF-β, Arg1, CD206, CD163, Ym1, and Retnla). The relative expression level of sham-operated, vehicle-treated mice is presented as 1. *$p<0.05$ (unpaired student's t test), n=4 per group.

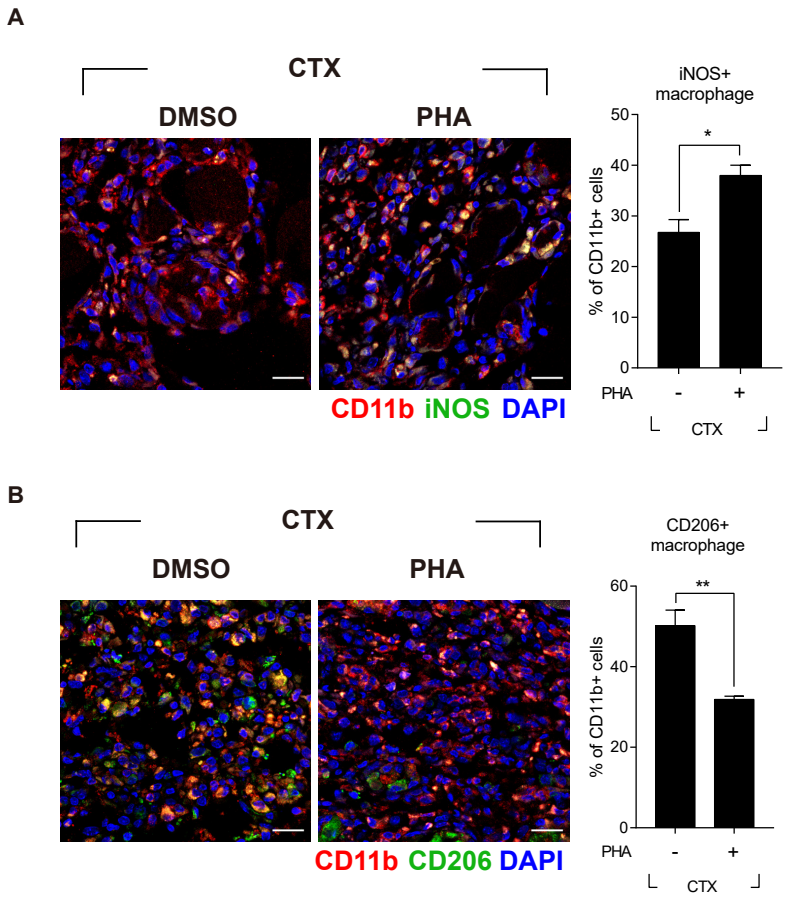

Figure IV-8. Effects of PHA-665752 on the population of M1 and M2 macrophage in injured muscle.
After CTX injury, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. (A) Effect on iNOS-positive macrophages and (B) CD206-positive macrophages. CTX-injured TA was isolated 3 days postinjury and subjected to immunofluorescence assay using antibodies to CD11b (red) and iNOS or CD206 (green). Nuclei were counterstained with DAPI (blue). Percentage of iNOS+ or CD206+ macrophages was indicated in the graph. *p<0.05, **p<0.01 (unpaired student's t test), n=4 per group. Scale bars, 20 μm.

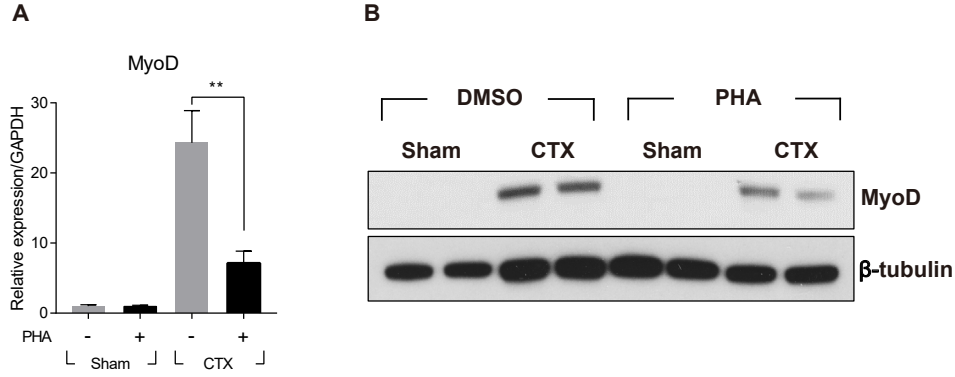

Figure IV-9. Effects of PHA-665752 on the expression of MyoD.

After CTX injury, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. CTX injured TAs were analyzed 7 days postinjury. (A) Effects on MyoD RNA. **p<0.01 (unpaired student's t test), n=4 per group. (B) Effects on MyoD protein. Two representative results are shown here, n=4 per group.

To understand the mechanism(s) underlying the effect of HGF on macrophage transition at the molecular and cellular levels, Raw 264.7 cells, a murine macrophage line, was used. Lipopolysaccharide (LPS) was used to activate these cells to the pro-inflammatory phenotype (M1) in order to mimic *in vivo* muscle injury. Cells were treated with various concentrations of the recombinant human HGF (hHGF) protein in the presence of 100 ng/ml of LPS for 24 hours. When Raw 264.7 cells were treated with LPS only, the expression level of M1 markers (IL-1β, iNOS, and TNFα) was greatly induced compared to the untreated control. Co-treatment with HGF inhibited an LPS-mediated increase in the level of M1 maker genes in a dose-dependent manner resulting in an approximate 40% decrease compared to the LPS only group (Figure IV-10A). On the other hand, LPS stimulation increased the expression level of IL-10 and Arg1, representative M2 marker genes, and HGF treatment further increased it in a dose-dependent manner (Figure IV-10B), suggesting that HGF might indeed control macrophage M2 transition.

The transition of macrophages from M1 to M2 during muscle regeneration is known to be controlled by two different pathways; CREB-C/EBPβ pathway (Ruffell et al., 2009) or AMPK signaling (Mounier et al., 2013). HGF had little or no effect on the former, since treatment with HGF did not affect the phosphorylation of CREB and the expression of C/EBPβ (Figure IV-11, A and B). To test whether HGF regulates the macrophage transition through AMPK, Raw 264.7 cells were treated with 50 ng/ml of the hHGF protein and 100 ng/ml LPS for 30 minutes, followed by Western blot, using antibodies to total or phosphorylated AMPKα. LPS stimulation decreased the level of phosphorylated AMPKα as previously reported (Sag et al., 2008), but the presence of HGF restored the phosphorylation of AMPKα to the normal level. The level of total AMPKα remained unchanged in all conditions (Figure IV-11A).

To test if AMPK is involved in the effects of HGF on inflammatory cytokines

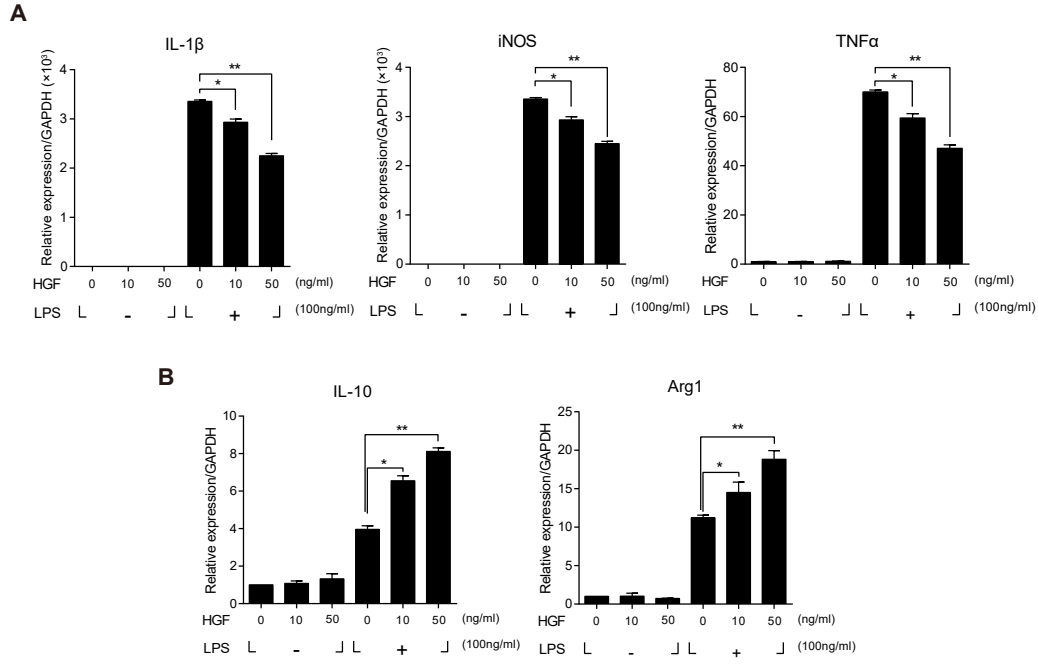

Figure IV-10. Effects of recombinant HGF protein on the expression of M1 and M2 markers in Raw 264.7 cells.
Raw 264.7 cells were cultured in the presence or absence of LPS and recombinant HGF proteins. Total RNAs were prepared and analyzed by RT-qPCR. (A) Effects of HGF on the RNA levels of M1 markers (IL-1β, iNOS, and TNFα). *p<0.05, **p<0.01 (unpaired student's t test), n=3 per group. (B) Effects of HGF on RNA levels of M2 markers (IL-10 and Arg1). Values were normalized to GAPDH. *p<0.05, **p<0.01 (unpaired student's t test), n=3 per group.

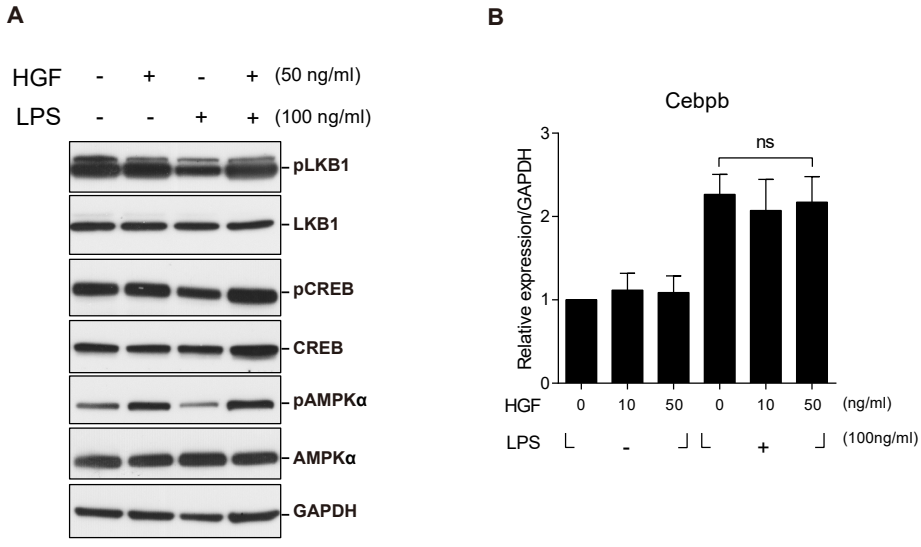

Figure IV-11. Effects of recombinant HGF protein on signaling pathways related to macrophage polarization in Raw 264.7 cells.

Raw 264.7 cells were cultured in the presence or absence of LPS and recombinant HGF proteins. Total RNA and proteins were prepared and analyzed by RT-qPCR and Western blot, respectively. (A) Effects of HGF on the expression and phosphorylation of LKB1, CREB, and AMPKα. (B) Effects of HGF on the RNA level of C/EBPβ. ns, not significant, n=3 per group. Values were normalized to GAPDH.

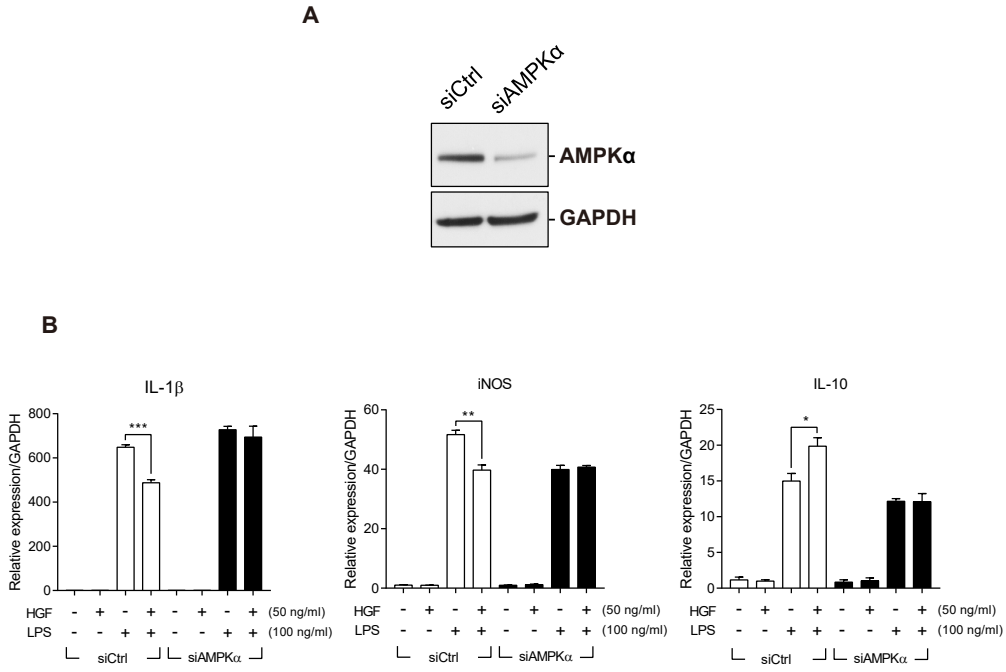

Figure IV-12. Roles of AMPKα on HGF-mediated control of the expression of M1 and M2 markers in Raw 264.7 cells.

(A) The protein level of AMPKα knocked-down by siRNA was determined by Western blot. (B) Effects of AMPKα knockdown on HGF-mediated regulation of the RNA level of IL-1β, iNOS, and IL-10, marker genes of M1 and M2. Raw 264.7 cells were transfected with AMPKα or control siRNAs, and then treated with LPS and HGF. Total RNAs were prepared and analyzed by RT-qPCR.Values were normalized to GAPDH. *p<0.05, **p<0.01, ***p<0.001 (unpaired student's t test), n=3 per group.

production, Raw 264.7 cells were transfected with siRNA against AMPKα followed by treatment with LPS and HGF. The protein level of AMPKα was highly reduced by siRNA transfection (Figure IV-12A). Raw 264.7 cells transfected with siAMPKα had no effect on the HGF-mediated control of M1 and M2 marker gene expressions (Figure IV-12B). These results indicated that HGF controlled the expression of M1 markers (IL-1β and iNOS) and IL-10 by upregulating AMPK phosphorylation.

AMPK phosphorylation is known to be regulated by upstream effectors such as LKB1 and CaMKKβ (Carling et al., 2008). It was tested as to which upstream effectors would be involved in the phosphorylation of AMPK mediated by HGF. LKB1 did not seem to play a role as HGF did not induce the phosphorylation of LKB1 in Raw 264.7 cells (Figure IV-11A). However, treatment with HGF induced the phosphorylation of CaMKKβ and treatment with STO-609, CaMKKβ inhibitor, lowered the level of HGF-mediated phosphorylation of AMPKα (Figure IV-13). Therefore, in Raw 264.7 cells, CaMKKβ, but not LKB1, appeared to act as an upstream effector of HGF-mediated control of AMPK.

Finally, it was investigated whether the phosphorylation of AMPK was regulated by HGF during skeletal muscle regeneration. At day 3 postinjury, TAs were isolated and subjected to Western blot and immunofluorescence. The level of phosphorylated AMPKα was highly increased in CTX-injured TA, and treatment with PHA-665752 had no significant effect (Figure IV-14A). When the phosphorylation of AMPK was measured in situ specifically in macrophages, however, a completely different picture emerged; PHA-665752 treatment significantly reduced the injury-mediated phosphorylation of AMPK in macrophages (Figure IV-14B). Taken together, these data suggested that HGF/c-met pathway regulate M2 macrophage transition by regulating CAMKKβ and AMPK.

2.4 Exogenous delivery of HGF facilitated muscle regeneration

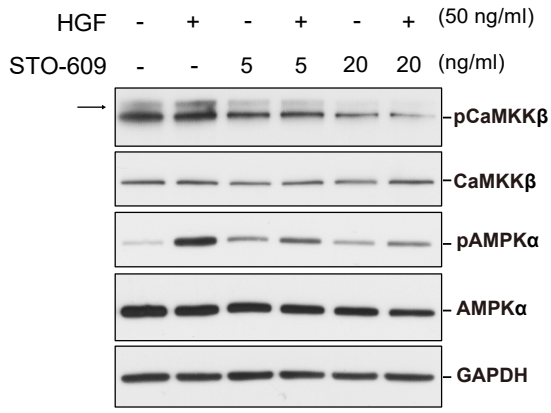

Figure IV-13. Roles of CaMKKβ on HGF-mediated phosphorylation of AMPKα in Raw 264.7 cells.

Raw 264.7 cells were cultured in the presence or absence of STO-609, a CaMKKβ inhibitor, and recombinant HGF proteins followed by Western blot. Arrow indicates the protein of interest in blots. In Western blot hybridization, GAPDH was used as a loading control.

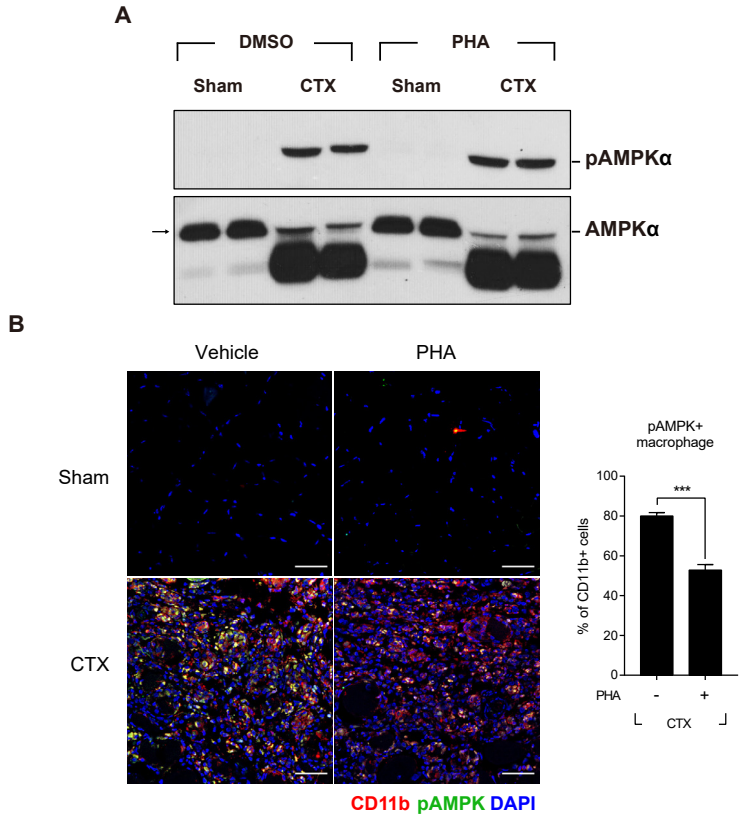

Figure IV-14. Effect of PHA-665752 on AMPK phosphorylation in CTX-injured muscle.

After CTX injury, mice were i.p. injected with 20 mg/kg of PHA-665752 on a daily basis until sacrificed. CTX injured TAs were analyzed 3 days postinjury. (A) Effect on total and phosphorylated AMPK in TA muscle. TA muscles were prepared and total proteins were isolated followed by Western blot using antibodies to total and phosphorylated AMPK. Two representative results are shown here, n=4 per group. (B) Effect on AMPK phosphorylation in macrophages. TAs were analyzed by immunofluorescence assay using antibodies to CD11b (red), and phosphorylated AMPK (green). Nuclei were counterstained with DAPI (blue). n=4 per group. Scale bars, 50 μm. Percentage of iNOS+ or CD206+ macrophages was indicated in the graph. ***p<0.001 (unpaired student's t test)

Based on the above results indicating a positive role(s) of HGF in macrophage transition during muscle regeneration, we tested the effects of the exogenous addition of HGF in the same CTX muscle injury model. Since HGF has a very short half-life, less than 5 minutes in serum (Kawaida et al., 1994), we delivered HGF in the form of a plasmid DNA expression vector. pCK-HGF-X7 (or VM202) is a plasmid designed to produce two isoforms of human HGF, HGF_{723} (or dHGF) and HGF_{728} (or cHGF), at high levels *in vivo* (Cho et al., 2008; Hahn et al., 2011; Pyun et al., 2010), and it has been known to work in a variety of clinical studies and animal models (Cho et al., 2008; Hahn et al., 2011; Kessler et al., 2015; Kibbe et al., 2016; Pyun et al., 2010). The *in vivo* protein expression kinetics of this plasmid in the muscle have been well established previously (Cho et al., 2008; Hahn et al., 2011; Nho et al., 2018; Pyun et al., 2010). The *in vivo* time kinetics of hHGF production by pCK-HGF-X7 in CTX-injured muscle was also investigated. The protein level of hHGF produced from pCK-HGF-X7 gradually increases upon injection, reaching a peak (about 20 ng/mg) 7 days after the plasmid injection (4 days after CTX injection), then steadily decreases to about 8 ng/mg 10 days after injection. The expression level of the hHGF protein produced by pCK-HGF-X7 (about 8-20 ng/mg) was about 20 times higher than mHGF protein induced by denervation (about 400-1100 pg/mg) (Figure IV-15).

Three days prior to the muscle injury, 100 μg of pCK-HGF-X7 or pCK control vector lacking the HGF sequence was i.m. administered into the TA and muscle injury was introduced by injecting CTX to the TA of a 10-week-old C57BL/6 mouse. The TA was isolated and muscle mass was quantitated at different time points after muscle injury. As shown in Figure IV-16, in CTX-injured mice injected with pCK control vector, muscle mass was decreased by 18% and 22% at days 3 and 7, respectively. When mice were injected with pCK-HGF-X7, the restoration of muscle weight was facilitated, to 17% and 13% decrease, compared to the control at days 3 and 7, respectively.

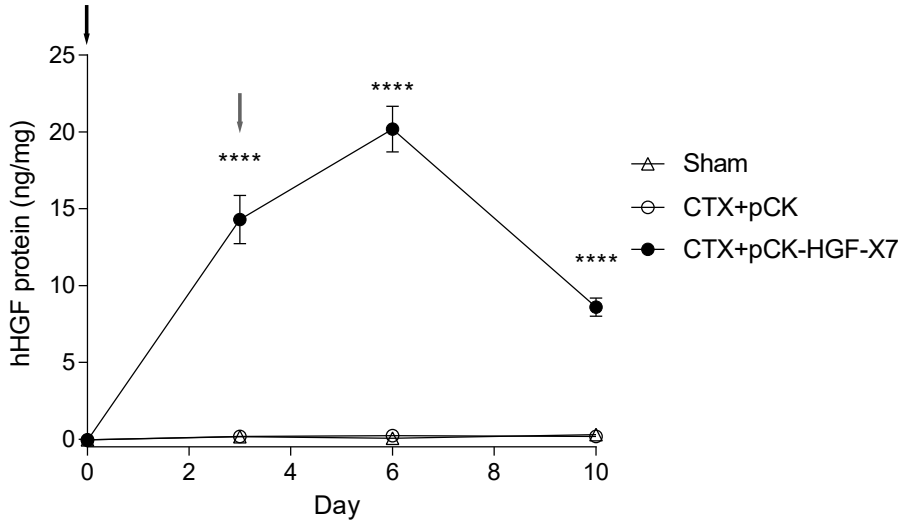

Figure IV-15. hHGF expression by pCK-HGF-X7 in CTX-injected mice.

pCK-HGF-X7 was i.m. injected at three days prior (day 0) to the CTX injection (day 3). Expression kinetics of the hHGF protein after denervation. Black arrow indicate i.m. injection of pCK-HGF-X7 on day 0 and gray arrow indicate i.m. injection of CTX on day 3. The muscle was isolated at 3, 7, and 10 days after plasmid injection, and total proteins were analyzed by ELISA to measure the protein level of human HGF. ****$p<0.0001$ versus pCK-treated muscle (one-way ANOVA), n=4 per group.

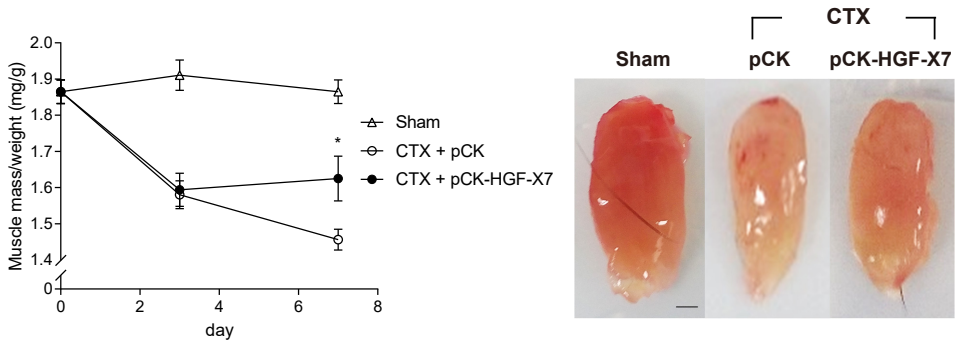

Figure IV-16. Effects of HGF overexpression by intramuscular injection of HGF expressing plasmid on muscle weight during regeneration.

pCK or pCK-HGF-X7 was i.m. injected 3 days prior to the CTX injection. TAs were prepared at appropriate times after injury. Effect on TA weight. Representative TAs from 7 days postinjury are shown in the photos. *p<0.05 versus CTX+p-CK group (one-way ANOVA), n=4 per group. Scale bar, 1 mm.

The regenerating muscle fibers were analyzed by immunostaining embryonic myosin heavy chain (eMHC), 3 days after muscle injury. In pCK-treated animals, the percentage of eMHC+ myocytes were about 20%, while it increased to 37% when treated with pCK-HGF-X7 (Figure IV-17A). Similar patterns were observed when the RNA level of eMHC (Myh3) was measured by qRT-PCR (Figure IV-17B). Overall, our data showed that the exogenous addition of HGF, delivered in the form of plasmid expression vector, could facilitate muscle regeneration.

The effects of i.m. injection of pCK-HGF-X7 on M1 and M2 markers were also measured. Muscle injury was induced and pCK or pCK-HGF-X7 was i.m. injected into the TA. Three days after muscle injury, TA was isolated and the RNA level of M1 and M2 markers were measured using RT-qPCR. The level of M1 markers, IL1β, iNOS, and CCL2 was highly increased after muscle injury, but pCK-HGF-X7 injection reduced the expression of these genes (Figure IV-18A). The level of M2 markers, IL-10, Arg1, and CD163, also increased upon muscle injury and further increased in animals treated with PCK-HGF-X7 (Figure IV-18B). These data strongly indicated that HGF overexpression by gene transfer technology could trigger transition of macrophages to the M2 phenotype to promote muscle regeneration.

3. Discussion

In this thesis works, I demonstrated that HGF/c-met signaling plays a key role in the regulation of macrophage transition during muscle regeneration after necrotic injury. The HGF/c-met signaling was highly activated after muscle damage, and the macrophage was the major cell type affected by HGF among cells that infiltrated the muscle. Treating CTX-injured mice with PHA-665752, a specific inhibitor for c-met, deregulated the population of

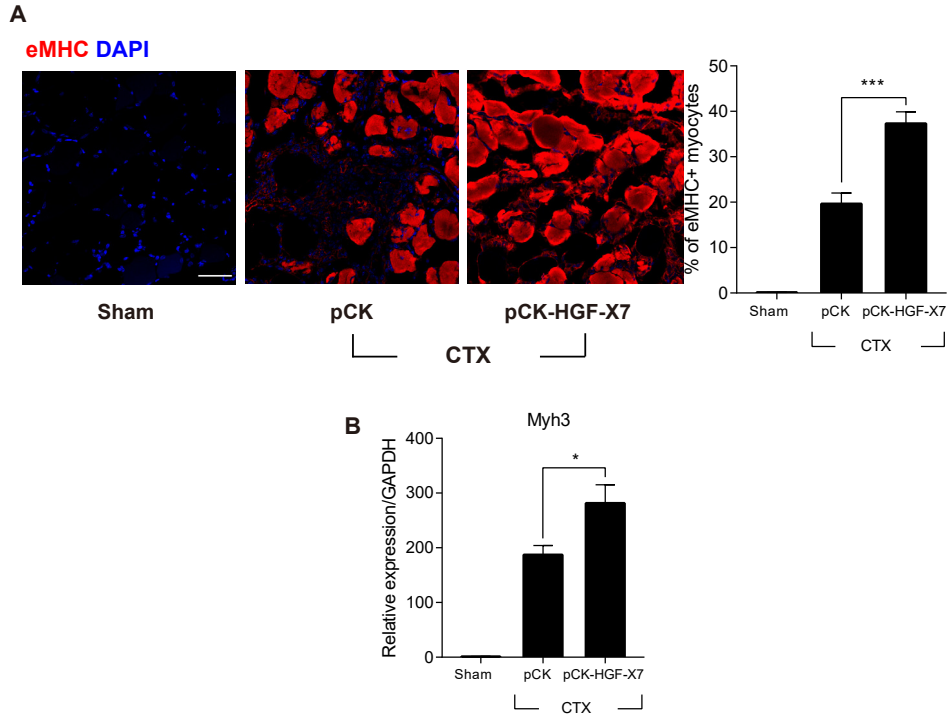

Figure IV-17. Effects of HGF overexpression on the percentage of regenerating fibers.

pCK or pCK-HGF-X7 was i.m. injected 3 days prior to the CTX injection. TAs were prepared at appropriate times after injury. (A) Effects on cross-sectional areas of regenerating fibers. CTX-injured TA was isolated 3 days postinjury and subjected to immunofluorescence assay using antibodies to eMHC (red) for regenerating myofibers. Percentage of eMHC+ myocytes was indicated in the graph. ***$p<0.001$ (one-way ANOVA), n=4 per group. Scale bar, 50 μm. (B) Three days after injury, the TA was isolated, and total RNAs were analyzed by RT-qPCR. Effects on the expression of Myh3. *$p<0.05$ (one-way ANOVA), n=4 per group. Values were normalized to GAPDH.

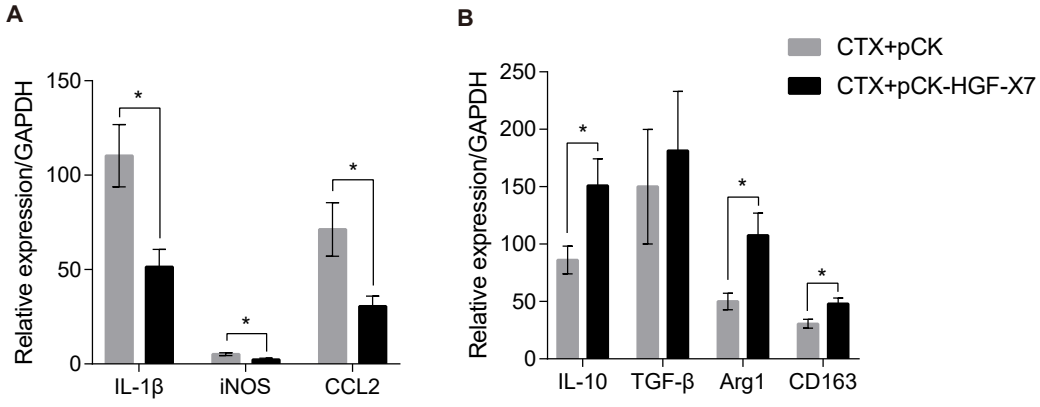

Figure IV-18. Effects of HGF overexpression on the expression of M1 and M2 macrophage markers during regeneration.

pCK or pCK-HGF-X7 was i.m. injected 3 days prior to the CTX injection. TAs were prepared at appropriate times after injury. Three days after injury, the TA was isolated, and total RNAs were analyzed by RT-qPCR. (A) Effects on the RNA levels of M1 markers (IL-1β, iNOS, and CCL2). *$p<0.05$ (one-way ANOVA), n=4 per group. (B) Effects on the RNA levels of M2 markers (IL-10, TGF-β, Arg1, and CD163). *$p<0.05$ (one-way ANOVA), n=4 per group. Values were normalized to GAPDH.

macrophages and delayed muscle regeneration. Exogenous supply of the HGF protein to the affected region through i.m. injection of a highly efficient plasmid expression vector promoted the transition of macrophage to the M2 phenotype and facilitated muscle regeneration. Data from Raw 264.7 cells showed that HGF controls macrophage transition via CaMKKβ-AMPK signaling. Taken together, HGF/c-met signaling plays a key role in the transition of the macrophage infiltrated during muscle regeneration.

HGF is secreted by a variety of cell types in cases of muscle injury. Relevant to our work is that fact that, macrophages are known to be the primary source of HGF during muscle regeneration (Sisson et al., 2009). Consistent with previous reports, we found that the kinetics of the RNA and protein levels of HGF paralleled the curve of macrophagic appearance in regenerating muscle (Mounier et al., 2013). However, the underlying mechanism by which macrophages produce HGF has not yet been understood. One possibility is that HGF may be secreted when these cells are exposed to apoptotic neutrophils. It was previously reported that when macrophages encounter apoptotic cells, HGF is produced via a RhoA-dependent signal *in vitro* (Park et al., 2011). Interestingly, our study showed that the expression level of HGF peaked at 3-4 days postinjury, and this is the time when neutrophils induce an early immune response and became apoptotic and cleared by macrophages (Arnold et al., 2007; Nguyen et al., 2011). It remains to be elucidated whether HGF expression in macrophages would indeed be regulated by their exposure to apoptotic neutrophils *in vivo*.

We found that HGF regulated phosphorylation of AMPK through the CaMKKβ upstream regulator. Since HGF/c-met signaling promotes the influx of calcium ions into a variety of cells, it is possible that calcium-dependent kinases are activated (Baffy et al., 1992; Gomes et al., 2008; Tyndall et al., 2007). In our study involving Raw 264.7 cells, another well-known upstream regulator, LKB1, was not phosphorylated by HGF. This is different

from the previous study showing that HGF regulates the phosphorylation of AMPK through LKB1 in primary hepatocytes (Vázquez-Chantada et al., 2009). In cases of muscle injury, however, it was previously reported that mice lacking LKB1 in their myeloid cells did not show any significant defects during muscle regeneration (Mounier et al., 2013). Therefore, LKB1 in macrophages may play a negligible role in the HGF/AMPK-mediated regulation of macrophage transition during muscle repair. These data imply that HGF might regulate the phosphorylation of AMPK through a different pathway depending on cell type.

In this study, we focused on the role of HGF/c-met signaling on macrophages during muscle regeneration, as they are the major cell type infiltrated after muscle injury. We found that HGF regulated the transition of the macrophage phenotype to promote muscle repair, while others reported that when c-met is knocked out specifically in muscle stem cells, mice showed delayed stem cell activation, decreased myoblast motility, abnormalities in myocyte fusion, and impaired muscle regeneration (Webster and Fan, 2013). These results suggest that HGF/c-met signaling could act on various cell types, including muscle progenitors and invading immune cells, to coordinate muscle repair during muscle regeneration. Also, HGF may be able to produce multiple positive effects in various cells during muscle regeneration.

In summary, HGF/c-met signaling appears to play a role(s) in muscle repair by promoting macrophage transition to the M2 phenotype. Various muscle diseases, including muscular dystrophy or myositis, are accompanied by a high inflammatory burden, leading to tissue lysis and compromise of the regeneration process. Currently, no drug can discriminate between the different phenotypes of the macrophage. Without such specificity, there is a chance of indiscriminate macrophage depletion, which can lead to undesirable systemic side effects (Balaban et al., 2005). Given the safety and efficacy records of pCK-HGF-X7 (VM202) observed in several clinical studies for other indications, further studies are warranted to investigate the possibility of using HGF, and in particular the plasmid DNA

vector expressing HGF, for various muscle degenerative diseases.

CHAPTER V

Concluding Remarks

Skeletal muscle remodeling is an extremely complex response to a number of catabolic cues. These signaling cues activate various intracellular pathways that eventually lead to transient or persistent remodeling of skeletal muscle. Although many studies have been conducted to understand the intracellular mechanisms of skeletal muscle atrophy or regeneration, it still remains largely unknown how proximal signaling events stimulate the activation of crucial downstream cascades. In this thesis, I investigated the roles of HGF, an important upstream signaling molecule, in the process of atrophy and injury-induced regeneration in skeletal muscle. I believe that this research has made valuable contributions to the study of skeletal muscle metabolism by systematically characterizing, for the first time, the role(s) of HGF during neurogenic atrophy and injury-induced regeneration of the skeletal muscle.

In Chapter 3, I investigated the role(s) of HGF in neurogenic muscle atrophy. I found that HGF expression was upregulated in skeletal muscle tissue following surgical denervation and in hSOD1-G93A transgenic mice. Inhibition of c-met signaling aggravated muscle atrophy as measured by muscle mass and its cross-sectional area. The most interesting finding of this study was the identification of the compensatory role(s) of HGF in neurogenic muscle atrophy by increasing the expression of miR-206, thereby regulating the expression of HDAC4 and atrogenes such as MuRF1 and Atrogin-1. Data from C2C12 cell lines showed that the regulation of miR-206 by HGF was mediated through JNK, presumably by suppressing the phosphorylation of Smad3. Finally, exogenous supply of the HGF protein to the affected region by i.m. injection of a plasmid expression vector expressing HGF mitigated muscle atrophy by all measurements, including muscle weight, cross-sectional area, and expression levels of miR-206, HDAC4, and atrogenes. These results are significant in that a novel signaling axis was found that controls the homeostasis of skeletal muscle in catabolic conditions, namely in neurogenic muscle atrophy. They shed light on the unknown upstream

regulator of miR-206 expression and the novel crosstalk between HGF and TGF-β signaling pathways in denervated muscle.

In chapter 4, I studied the role(s) of HGF during injury-induced muscle regeneration, especially in the context of immune cells infiltrating the muscle. In an injured muscle microenvironment, cells of the immune system are known to play roles in the removal of necrotic tissue and the restoration of muscle architecture. Data from my study showed that HGF expression was induced in response to the injury, and that the macrophage was the major cell type affected by HGF. It was found that HGF significantly inhibited the expression of pro-inflammatory (M1) macrophage markers such as TNF-α, IL-1β, and iNOS, while enhancing those of pro-regenerative (M2) macrophage markers like IL-10 and TGF-β in both regenerating muscle and Raw 264.7 cell lines. In addition, inhibition of HGF/c-met signaling by using a specific inhibitor compromised the transition capability of the macrophage phenotype, resulting in delayed muscle regeneration as measured by muscle mass and cross-sectional area. Results from Raw 264.7 cell lines showed that HGF regulated the transition of macrophage to the M2 phenotype via CaMKKβ –AMPK signaling. Finally, exogenous delivery of HGF through i.m. injection of the HGF-expressing plasmid vector was found to promote the transition to M2 macrophage and contribute to an improved regeneration of skeletal muscle (Figure V-1). These findings are important in that they revealed the previously uncharacterized upstream regulators of CaMKKβ, which is an important molecule regulating the macrophage phenotype during muscle regeneration.

There are several questions to be further explored. Firstly, it remains to be seen exactly how the expression of HGF is regulated in response to neurogenic atrophy and injury of the muscle. Secondly, we should also investigate whether other mediators are involved in the execution of the downstream effects of HGF. Thirdly, since a systemic chemical inhibitor was used to suppress HGF/c-met signaling in my study, it was difficult to identify exactly on

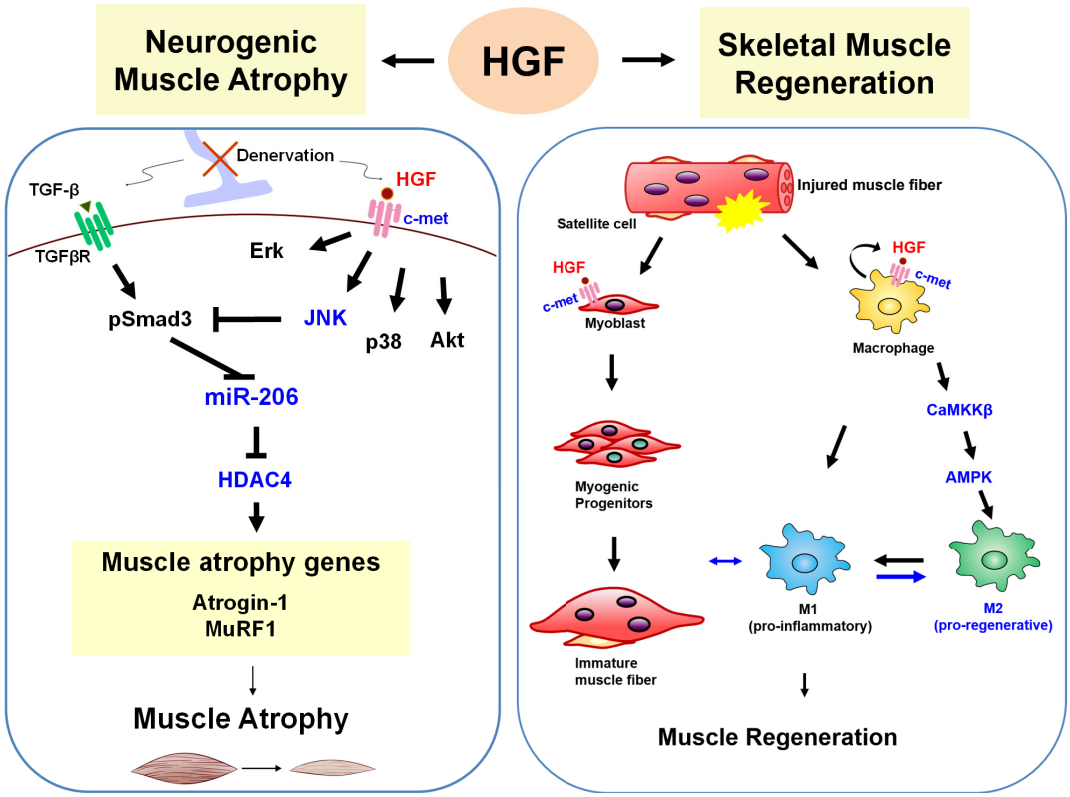

Figure V-1. Schematic diagram of mechanism(s) of HGF in neurogenic muscle atrophy and muscle regeneration process in this thesis work

which cell types HGF acts in muscle remodeling situations. Using the muscle-specific c-met knockout system could be one possible approach to addressing this issue. Fourthly, a more precise mechanism must be developed to closely probe how HGF/c-met signaling inhibits the phosphorylation of Smad3 in the case of neurogenic muscle atrophy. My thesis provides preliminary evidence that HGF may inhibit the activity of Smad3 by phosphorylating serine 204 residue, the linker region between Mad homology-1 (MH1) and MH2, via the JNK downstream mediator. Further research is also needed to delineate whether the phosphorylation of serine 204 residue by HGF has indeed a direct effect on the activity of Smad3, *in vitro* and *in vivo*.

In Chapter 4, I showed that HGF may cause a transition of the macrophage toward M2 phenotypes during muscle regeneration via CaMKKβ and AMPK. If I were to continue this research, I would address the following issues in the context of HGF and macrophage transition: firstly, I would test whether this is a direct result of the action of these downstream factors, or whether other unknown mediators are involved. I would then further investigate whether the regulation of macrophage transition of macrophage phenotypes by HGF indeed plays pivotal role(s) in the promotion of muscle regeneration. To address this issue, genetically modified mouse models showing the deregulated transition of the macrophage would have to be employed. Based on current data, there is also a possibility that the effect of HGF on macrophage transition may be a result of the interaction between myeloid and myogenic cells in injured or regenerating muscle, rather than the roles of various intrinsic factors produced from macrophages. These two scenarios can be differentiated by experimenting with *in vivo* cell type (especially macrophage and myogenic cells)-specific ablation of the c-met signaling system.

In conclusion, this thesis found that HGF/c-met signaling controls the progression of neurogenic muscle atrophy by regulating the miR-206-HDAC4 cascade, and is involved in

the adult muscle regeneration process by coordinating the transition of the macrophage. Data from this research have provided new and interesting insights into the field of skeletal muscle metabolism, as well as useful information for developing innovative therapeutic approaches in the area of a wide range of muscle abnormalities.

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국문 초록

간세포 성장 인자(hepatocyte growth factor: HGF)는 배 (embryo) 발생 과정에서의 근육 전구세포의 이동과 성체 근육 줄기세포의 활성화에 관여한다고 알려져 있다. 그러나 성체의 근육에서 HGF가 어떤 역할을 하는지는 명확히 밝혀지지 않았다. 본 연구에서는 HGF가 신경성 근위축 (neurogenic muscle atrophy) 과정에서 어떠한 기능을 수행하며, 근육 재생에 어떤 영향을 미치는지 조사하였다.

먼저 HGF가 신경성 근위축에 미치는 영향을 조사하였다. 마우스의 좌골 신경을 절단하여 근 위축을 유도하는 sciatic nerve transection 모델과 근위축성 축삭경화증을 모방하는 hSOD1-G93A 마우스 모델의 전경골근 (TA)에서 HGF의 발현과 c-met 수용체의 인산화를 조사하였다. 그 결과 두 모델 모두에서 대조군 대비 HGF의 발현이 증가하고, c-met이 인산화 되는 것을 관찰하였다. 이때 발현이 증가한 HGF가 신경성 근 위축에 관여하는지 알아보기 위하여 c-met 수용체를 억제하는 PHA-665752를 마우스에 경구투여 하였다. 그 결과, PHA-665752를 투여한 마우스에서는 miRNA-206의 1차 전사체 발현이 감소하고, HDAC4 및 근 위축 유전자의 발현은 증가하며 근육 위축이 심화되는 것을 관찰하였다. 이에 대한 분자세포생물학적 메커니즘을 이해하기 위해 C2C12 근육세포주를 사용하였다. C2C12 세포에서 HGF는 하위 신호전달 매개자인 JNK를 통하여 TGF-β에 의해 유도되는 Smad3의 인산화를 저해하였고, 그 결과 miR-206 발현도 증가시킴을 관찰하였다. 또한 간세포 성장인자를 발현하는 플라스미드 DNA를 마우스 근육에 주사하였을 때, 이로부터 발현된 HGF 단백질이 miR-206-HDAC4 cascade를 조절하여 근육 위축을 완화함을 관찰하였다.

다음으로 간세포 성장인자가 근육 재생 과정에 미치는 영향을 연구하였다. 이를 위해 코브라 독에서 분리한 심장 독소 (CTX)를 전경골근에 투여하여 근 손상을 유도하고, 근육의 재생을 관찰하는 시스템을 사용하였다. 근육 손상 시 HGF/c-met 신호전달경로가 활성화되며, 이때 근육으로 침투한 다양한 면역세포 중 대식세포가 HGF에 의해 활성화됨을 확인하였다. PHA-665752를 마우스에 경구투여 하였을 때, 근육에서 친(親)염증성 (pro-inflammatory) 대식세포인 M1 세포의 표지자인 IL-1β와 iNOS의 발현이 증가하였고, 친(親)재생성 (pro-regenerative) 대식세포인 M2 세포의 표지자인 IL-10과 TGF-β의 발현은 감소하여 근육 재생이 지연됨을 관찰하였다. 이 과정의 분자세포생물학적 메커니즘을 이해하기 위해 Raw 264.7 대식세포주를 사용하였다. Raw 264.7 세포를 LPS로 처리할 경우, 대식세포가 활성화되어 염증성, 재생성 표지자들의 발현이 증가한다. 이때 HGF를 가하면 LPS에 의해 유도된 친-염증성 대식세포 표지자 (TNFα, IL-1β, 및 iNOS)의 유전자발현이 농도 의존적으로 감소하였고, 반면 IL-10의 유전자 발현은 증가하였다. 이때 HGF는 CaMKKβ를 하위 신호 전달 매개자로 이용하여 AMPKα의 인산화를 증가시켜 대식세포 표지자들의 유전자 발현을 조절하는 것을 밝혔다. 이러한 현상은 동물 모델에서도 관찰되었다. 즉 간세포 성장인자를 발현하는 플라스미드 DNA를 마우스 근육에 주사하였을 때 이로부터 발현된 HGF에 의하여 친재생성 대식세포로의 전이가 촉진되었으며, 근육 재생을 촉진됨을 관찰하였다.

본 연구를 통하여 HGF가 마이크로RNA 및 근 위축 유전자의 발현을 조절하여 신경성 근위축의 진행을 억제하고, 대식세포 표현형 전이에 관여하여 근육 재생을 촉진한다는 사실을 밝혔다. 이 논문에 실린 연구결과들은 신경성 근위

축 및 근육 재생 과정에서 간세포 성장인자의 역할을 이해하는 데 도움을 줄 뿐 아니라, HGF를 활용하여 신경근육 질환 및 근육 퇴행성 질환에 대한 새로운 치료제를 개발하는 데 유용한 자료로 사용될 수 있을 것으로 생각된다.

중심어: 간세포 성장인자, 골격근, 신경성 근위축, 근육 재생

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지난 4년간 진심 어린 조언과 많은 가르침을 주셨던 김선영 교수님 감사드립니다.

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제가 어떤 길을 가던 항상 힘이 되어주시고 응원해 주셨던 우리 가족들,

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