

© 2021 Dongwook Kim

MYOGENIC FUNCTIONS OF MUSCLE-DERIVED CYTOKINES

BY

DONGWOOK KIM

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Cell and Developmental Biology
in the Graduate College of the
University of Illinois Urbana-Champaign, 2021

Urbana, Illinois

Doctoral Committee:

Professor Jie Chen, Chair and Director of Research
Professor Lisa J. Stubbs
Assistant Professor Xin Li
Professor Marni D. Boppart

ABSTRACT

Adult skeletal muscle has a robust capacity for regeneration, partially recapitulating the embryonic development process. Upon injury, quiescent muscle stem cells, known as satellite cells, are activated, and they proliferate to produce myoblasts at the injury site. These myoblasts withdraw from the cell cycle and enter the myogenic differentiation process. Differentiated myoblasts further fuse together to form new multi-nucleated myofibers or repair existing ones. Many of the regulators of this highly orchestrated myogenic process are still unknown or incompletely understood.

It was long assumed that the immune cells that infiltrate muscle injury sites secrete cytokines that can regulate not only inflammatory responses but also muscle regeneration. However, there have been multiple reports of cytokine secretion by muscle cells, and our lab's functional screening using RNAi identified numerous cytokines that can potentially regulate myogenic differentiation of C2C12 mouse myoblasts.

TNF-related apoptosis-inducing ligand (TRAIL) emerged from our RNAi screen as a potential negative regulator of myogenic differentiation. In Chapter 2, we demonstrate that TRAIL inhibits cell cycle withdrawal, thereby preventing subsequent myogenic differentiation through its cognate receptor, TRAIL-R2. This action is mediated by the ERK signaling pathway. Remarkably, depletion of TRAIL using shRNA delivery enhances *in vivo* muscle regeneration upon injury.

To date, there have been very few studies that directly examine the source of cytokines (i.e., muscle versus immune cells) that can regulate skeletal muscle regeneration *in vivo*. To address this major gap in the field, we have generated myogenic progenitor cell (MPC)-specific Cas9 knock-in mice as a system to examine the function of muscle-derived cytokines in skeletal muscle regeneration as described in Chapter 3. From two types of Cas9 knock-in transgenic mice, expression of Cas9 is driven by Pax7 and

Myf5, respectively. I have validated that Cas9 is predominantly expressed in skeletal muscle in those mice, and intramuscular delivery of an AAV9-packaged sgRNA has led to successful editing of a target gene.

Lastly, in Chapter 4, we investigate the function of chemokine (C-C motif) ligand-8 (Ccl8) in skeletal myogenesis. Although it was previously reported that a cocktail of CCR2 ligands (recombinant Ccl2, Ccl7 and Ccl8) inhibited skeletal myogenesis, the endogenous Ccl8's function had not been investigated. In C2C12 cells, I find knockdown of Ccl8 to enhance myogenic differentiation. Moreover, utilizing the CRISPR mouse system established in Chapter 3, I have depleted Ccl8 specifically in MPCs and observed accelerated injury-induced muscle regeneration, which provides definitive evidence for the role of muscle-derived Ccl8 in vivo.

With the MPC-specific Cas9 mouse model successfully established, we are in the position to investigate many putative muscle-derived cytokines in vivo. A comprehensive understanding of these muscle-specific regulators can contribute to therapeutic development such as stem cell therapy to treat muscle diseases.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deep sense of thanks and gratitude to my advisor, Dr. Jie Chen for her continuous support and encouragement during my Ph.D study. It was my privilege to learn from her immense knowledge and enthusiasm. Her guidance helped me get through all the challenges and broaden my horizon as a scientist. I am also grateful for her kindness and patience when I was in need of help outside of research. She is not only my research mentor, but also my life mentor.

My sincere thanks also go to my thesis committee members, Drs. Jonathan Henry, Lisa Stubbs, Marni Boppart, and Xin Li for their insightful comments and critical questions that helped me make progress on my projects and improve this dissertation.

I would like to thank my fellow Chen lab members who made this journey joyful and memorable. I have been very lucky to be surrounded by such talented colleagues. I especially want to thank to Drs. Rachel Waldemer-Streyer and Nilmani Singh for teaching me what to do and how to do from day one in Chen lab. I also want to thank Allison Boss-Kennedy for helping me with Ccl8 project in many ways.

Last, but certainly not least, I am very grateful for my family and friends for their support. My parents have been so supportive with selfless love and endless prayers. Without their sacrifice, I would not be here. I also thank my aunt and uncle in Chicago whose support and love were immeasurable. And my brother always supports our family with prayers. My wife has showed constant love and wisdom even in the middle of many difficulties, and now we are comrade in arms. My in-laws' overflowing love has been the greatest support to continue this journey. I especially thank Dr. Younguk Sun for helping us continuously and loving two boys. My brothers and sisters in Christ who encouraged and motivated me with words and prayers. My heavenly Father who led me till today and will guide me till the end. It was all His glory that I have survived here. Praise the Lord!

To my parents who have always supported me and taught me to trust in God.

To my wife, Jihye, my support, soul mate, and inspiration.

To my two little (bigger) boys, my greatest accomplishment and gift during this journey.

To the Lord, my God, my source of strength and my source of hope.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION.....	1
1.1. Overview of Skeletal Myogenesis and Cytokines.....	1
1.2. Cytokines in Skeletal Muscle.....	3
1.3. Regulation of Myogenic Processes by Muscle-Derived Cytokines	7
1.4. In vivo Myogenic Function of Muscle-Derived Cytokines	13
1.5. Cytokines in Muscle Disease	15
1.6. Table and Figures	19
1.7. References	25
 CHAPTER 2. MUSCLE-DERIVED TRAIL NEGATIVELY REGULATES MYOGENIC DIFFERENTIATION	 34
2.1. Introduction	34
2.2. Materials and Methods	36
2.3. Results	41
2.4. Discussion	47
2.5. Figures	51
2.6. References	65
 CHAPTER 3. ESTABLISHING A MOUSE MODEL FOR SKELETAL MYOGENIC PROGENITOR CELL-SPECIFIC TARGETED GENE DISRUPTION	 68
3.1. Introduction	68
3.2. Materials and Methods	70
3.3. Results	76
3.4. Discussion	78
3.5. Figures	80
3.6. References	83
 CHAPTER 4. MYOGENIC PROGENITOR CELL-DERIVED CCL8 NEGATIVELY REGULATES MUSCLE REGENERATION IN VIVO.....	 85
4.1. Introduction	85
4.2. Materials and Methods	86
4.3. Results	94
4.4. Discussion	98
4.5. Figures and Tables.....	100
4.6. References	111
 CHAPTER 5. CONCLUSIONS AND PERSPECTIVES.....	 113
5.1. References	116

CHAPTER 1. INTRODUCTION¹

1.1. Overview of Skeletal Myogenesis and Cytokines

Skeletal muscle is one of the few adult mammalian tissues that can undergo robust regeneration after injury, owing at least partly to a resident population of stem cells that imparts a great regenerative capacity. These myogenic progenitor cells, called satellite cells, exist in a quiescent state under the basal lamina of myofibers until stimulated to divide by muscle injury as a result of acute trauma, toxin, exercise, or diseases. A subset of the activated satellite cells returns to quiescence, allowing for self-renewal of the muscle stem cell pool, while the remaining activated cells proliferate at the site of injury to allow for new myofiber formation or repairing of injured myofibers [1, 2]. Effective regeneration depends on the new myoblasts successfully completing a number of different processes, including expression of many of the same myogenic genes seen in embryonic development, such as the MEF2 and MyoD families of transcription factors [3, 4]. The differentiating myoblasts must also undergo massive cytoskeletal rearrangement, migration, cell-cell adhesion and alignment, and finally membrane fusion in order to form a mature multinucleated myofiber [5] (Figure 1.1). Myocyte fusion occurs in two stages that are regulated by distinct molecular pathways. The first stage entails fusion between differentiated myoblasts, leading to small, nascent myotubes. The second stage of fusion occurs between myoblasts and these nascent myotubes, resulting in mature myofibers [5]. This well-orchestrated myogenic process is regulated by numerous intracellular signaling pathways, including Jak/STAT, p38, mTOR, and PI3K/Akt, to name just a few.

¹ **This chapter is adapted from:** a manuscript co-written with Rachel J. Waldemer-Streyer and Jie Chen.

The exact definition of what constitutes a cytokine has been evolving since their discovery in purulent exudates in the 1940s [6]. The earliest identified cytokines were of hematopoietic origin and thought to be dedicated to modulating inflammatory responses. Today, it is well established that nearly every cell type can produce secreted factors to some degree, and that even the original cytokines have other sources and pleiotropic functions beyond the immune system. Biochemical, structural, and functional understanding of these secreted factors have led to various, sometimes overlapping, forms of nomenclature. For instance, most of the interleukins and all interferons signal through cytokine receptors associated with Jak-STAT signaling and they are still considered by some to be the only bona fide “cytokines”. Most growth factors (e.g., EGF, IGF, FGF, VEGF, etc.) act through receptor tyrosine kinases. Chemokines, so named for their chemoattractant properties, are ligands for G protein coupled receptors. TGF β 's and TNFs bind distinct families of receptors known as TGF β receptors and TNF receptors, respectively. For all practical purposes, the term “cytokine” has been extended to include all small (<30 kDa) secreted proteins that function via cell surface receptors, although there are certainly exceptions to this already broad definition. For instance, almost all TNF family members exist in a transmembrane form in addition to the extracellular soluble form resulting from proteolytic cleavage, both biologically active; some cytokines are larger than the 30 kDa molecular weight cutoff (e.g. IL-12, VEGF).

A large number of infiltrating immune cells at the site of skeletal muscle injury play a critical role in not only clearing the damaged tissue but also promoting muscle regeneration [7, 8]. It has long been accepted that these immune cells produce a milieu of secreted factors that modulate every stage of injury-induced muscle regeneration. However, in the past two decades the skeletal muscle has also gained increasing attention as a secretory organ. Originally coined by Pedersen et al. [9], the term “myokine”

refers to a cytokine that is released by skeletal muscle cells (often as a result of exercise) into the circulation to exert endocrine effects and metabolic regulation [10, 11]. IL-6, for which the term myokine was first proposed [9], is secreted by exercising muscles and may exert a multitude of beneficial effects on metabolism [10]. At an ever-growing number, myokines have attracted intense interests and been the subject of many excellent reviews (e.g., [10, 11]).

1.2. Cytokines in Skeletal Muscle

It has long been known that conditioned medium of differentiating skeletal myoblasts or myotubes in culture contain factors that influence myogenic differentiation, implicating muscle-derived cytokines in the autocrine/paracrine regulation of myogenesis. Whereas muscle tissue homogenates can contain proteins from non-muscle sources, analyses of in vitro muscle cell cultures have provided definitive evidence for muscle cells as prolific secretors of cytokines. About 10 years ago, significant efforts by several research groups led to the first proteome-wide glimpse of the skeletal muscle secretome. Two groups independently performed stable isotope labeling by amino acids in cell culture (SILAC) followed by proteomic analysis of the conditioned media of mouse C2C12 myoblasts at various stages of differentiation [12-14]. While Henningsen et al. identified 635 secreted proteins among which 75 were cytokines/growth factors [14], Chan et al. reported 34 secreted proteins [12]. Both studies concluded that a large portion of the proteins were secreted differentially during the course of differentiation, providing potential support for the idea that those secreted factors may have regulatory roles in the myogenic process. Another commonly used myogenic cell line, the rat L6 myoblast, was studied by Yoon et al., and they identified 254 proteins in the conditioned medium of fully differentiated myotubes, a small subset of which were up- or down-regulated by short-

term (5 hours) insulin treatment [15]. Myotubes differentiated from human myoblasts were also analyzed by Norheim et al. and 18 classically annotated secreted proteins were reported [16]. Around the same time as the cluster of proteomic studies, Griffin et al. examined the mRNA expression of 84 chemokines and genes encoding proteins involved in chemokine signaling during differentiation of mouse primary myoblasts. They found 80 of those genes to be expressed, with the majority peaking at the time of myocyte fusion, consistent with a potential role of these chemokines in cell migration necessary for fusion [17]. It is worth noting that Henningsen et al. did not observe a marked correlation between the levels of cytokine proteins in their secretome and the levels of the corresponding mRNAs [14], suggesting that a significant part of regulation of cytokine secretion by muscle cells may be at a post-transcriptional level.

Many of the identified muscle-secreted cytokines remain to be characterized for their potential functions in myogenesis. Nevertheless, the collective evidence raised the intriguing possibility that muscle cell-secreted proteins might have a previously under-appreciated role in modulating myogenic differentiation and muscle regeneration. Our lab conducted a functional screen of 134 mouse cytokines for their impact on myogenic differentiation in C2C12 myoblasts using RNAi, which led us to identify 29 potential regulators of myogenesis in distinct functional groups [18]. Several of those candidates have since been characterized for their myogenic functions and mechanisms (discussed later).

The concept of autocrine regulation of myogenesis by muscle-secreted factors is not new. Below we briefly discuss a few of such cytokines first reported.

IGFs

The insulin-like growth factors IGF1 and IGF2 were among the earliest characterized and most extensively studied cytokines that promote myogenesis in an autocrine fashion [19-21]. IGFs are critically involved in skeletal muscle development, hypertrophy, and regeneration [19, 22, 23]. The autocrine function of IGF was first reported 30 years ago in cultured myoblasts which were found to secrete IGF2 that was indispensable for the initiation of differentiation [24]. Indeed, C2C12 myoblasts secrete high levels of both IGF1 and IGF2 [25, 26], and both stimulate the expression of myogenin, an early marker of myogenic differentiation [19]. Autocrine IGF2 is also a survival factor for muscle cells [27].

FGF, PDGF, HGF

Around the time when IGFs emerged as positive regulators of myogenesis, it became clear that fibroblast growth factors (FGFs) were autocrine inhibitors of myogenic differentiation in vitro [20]. FGF2 (basic FGF) is more potent than FGF1 (acidic FGF) in this regard, both possibly acting as mitogens to prevent cell cycle withdrawal and consequently suppress differentiation, although direct inhibition of myogenic gene expression is also proposed [20]. Several FGFs (at least FGF1, 2, 4 and 6) are expressed in satellite cells and, not surprisingly, they can also stimulate cultured satellite cell proliferation in an autocrine manner [28]. It would therefore appear that FGFs could have dual roles in muscle regeneration – promoting satellite cell expansion and suppressing myogenic differentiation. However, in vivo evidence for FGF function in myogenesis is limited (to be discussed later).

Another growth factor, platelet derived growth factor (PDGF), is expressed by myoblasts and exerts inhibitory effects on myogenic differentiation by promoting cell

proliferation and inhibiting cell cycle exit [29-31]. Likewise, hepatic growth factor (HGF) is found to be expressed in satellite cells during rat muscle regeneration [32], stimulate satellite cell proliferation, and inhibit differentiation [33, 34]. Although IGFs are also growth factors that stimulate myoblast proliferation, unlike FGF, PDGF and HGF, the mitogenic activities of IGFs do not interfere with their myogenic activities at the time of differentiation [19].

TGF β

In 1986 transforming growth factor β (TGF β) was reported independently by three groups to be an inhibitor of myogenic differentiation in vitro [35-37]. Those initial reports concluded that the effect of TGF β was independent of cell proliferation [35-37]. However, it is now confirmed in at least C2C12 cultures that TGF β stimulates myoblast proliferation through Smad2 activation and consequently suppresses cell cycle exit [38, 39]. On the other hand, TGF β activation of Smad3 leads to direct suppression of the transcriptional activity of MyoD and possibly other myogenic regulatory factors [40]. Consistent with an autocrine function, TGF β is found to be secreted by myoblasts in vitro and its mRNA is expressed in mouse skeletal muscles [41].

Myostatin

A member of the TGF β family, myostatin (also known as growth/differentiation factor-8 or GDF8) was reported to be an autocrine inhibitor of muscle mass in mice in 1997 [42]. Deletion of the myostatin gene leads to drastic muscle hypertrophy, manifesting the double-muscled phenotype in mice, cattle, and humans [43]. The mechanism by which myostatin inhibits muscle growth and myogenesis is multifold and will be discussed later. Two other TGF β family members closely related to myostatin, activin A and GDF11, are also secreted by muscle cells and inhibit myogenic differentiation [44, 45]. All three

ligands signal through the same activin receptors, and the receptor binding is antagonized by these ligands' physical interaction with muscle-secreted follistatin [44, 46]. Consistent with its anti-myostatin function, follistatin is a potent inducer of muscle hypertrophy [47, 48], although a myostatin-independent mechanism has also been suggested for follistatin's hypertrophic effects [49].

1.3. Regulation of Myogenic Processes by Muscle-Derived Cytokines

To date, dozens of cytokines that are expressed in muscle cells with myogenic function have been identified as summarized in Table 1.1. These cytokines encompass several major classes of extracellular ligands based on the types of their cognate receptors, including members of TGF β family, members of TNF family, chemokines, ligands for receptor tyrosine kinases, and interleukins/interferons that signal through cytokine receptors. Although not all these cytokines have been defined for their mechanisms of myogenic action, there is a sizable number of reports on the cellular mechanisms of autocrine regulation in myogenesis by many of the cytokines. Remarkably, every stage of the myogenic process involves regulation by muscle-derived cytokines, and many cytokines contribute to myogenic regulation at more than one stage (Figure 1.2).

It is noteworthy that in some cases a cytokine is reported to play opposing roles in the regulation of myogenic differentiation. While it is possible that a cytokine may exert different functions at different stages of differentiation, it is equally likely that the source of the cytokine may dictate the function. TNF α serves as a perfect example. Implicated as a major inflammatory mediator of muscle wasting in aging or disease [50-52], recombinant TNF α inhibits myoblast differentiation in human and mouse cultures [53-55], and an elevated level of TNF α in the mdx dystrophic muscles is reported to suppress

regeneration by inhibiting satellite cell activation [56, 57]. However, interfering with endogenous $\text{TNF}\alpha$ expression or signaling leads to inhibition of myoblast differentiation in vitro and reduction of muscle regeneration in vivo [58-61]. A model to potentially reconcile these paradoxical observations is that normal muscle-derived $\text{TNF}\alpha$ has a positive role in myogenic differentiation whereas immune cell- or dystrophic muscle-derived $\text{TNF}\alpha$ has a negative role. Concentrations of the cytokine and/or activation of distinct downstream signaling pathways may determine the outcome. Indeed, the MAP kinase p38 is a relevant target of $\text{TNF}\alpha$ signaling that supports myogenic differentiation [60, 61], whereas $\text{NF-}\kappa\text{B}$ mostly mediates the inhibitory function of $\text{TNF}\alpha$ [55, 56, 62] (Figure 1.3).

Satellite cell activation and proliferation

Cytokines that have been reported to positively regulate satellite cell activation and myoblast proliferation in a muscle-autonomous manner include Ccl2 [63], Cxcl16 [64], FGFs (1, 2, 4, 6 and possibly others) [20, 28], G-CSF [65], HGF [33, 34], $\text{IFN}\gamma$ [66], IL-6 [67], LIF [68], NGF [69], PDGF [29-31], $\text{TGF}\beta$ [38, 39], and Sonic hedgehog (SHH) [70, 71]. SHH plays a critical role in embryonic myogenesis [72] and its expression is absent in adult muscles. Muscle injury reactivates SHH signaling, and pharmacological inhibition of the signaling is reported to impair regeneration [73]. While its receptor Ptch1 and downstream target Gli1 are expressed in mouse primary myoblasts and C2C12 cells, SHH itself is not produced by those cells [71]. Instead, injured myofibers appear to be the source of SHH production in vivo [73], making SHH a bona fide muscle-derived cytokine.

Myostatin, among its many reported inhibitory functions in muscle, negatively regulates satellite cell self-renewal and proliferation by impinging on the levels of the G1 cell cycle regulators p21^{CIP} and Cdk2 [74, 75]. Similarly, activin A is also reported to negatively regulate satellite cell activation and proliferation [76]. $\text{TNF}\alpha$ inhibits

regeneration of mdx muscles by suppressing satellite cell activation via NF- κ B signaling and epigenetic silencing of Notch1 [56, 62].

Cell cycle withdrawal and initiation of differentiation

Two events during the earliest stage of myogenic differentiation – cell cycle withdrawal and initiation of differentiation – are regulated by a large number of cytokines (Figure 1.2). This is perhaps not surprising, as the commitment to differentiation is a point of no return for the cell fate. Muscle-derived Flt3L promotes C2C12 myoblast exit from the cell cycle, and knockdown of Flt3L impairs myoblast differentiation in vitro and muscle regeneration in vivo [77]. Through its receptor Flt3 (an RTK), Flt3L stimulates hematopoietic cell proliferation via activation of the MAP kinase Erk signaling [78], but in myoblasts Flt3L-Flt3 signaling activates cell cycle withdrawal by suppressing Erk through a non-canonical p120RasGAP pathway [77] (Figure 1.4). Brain derived neurotrophic factor (BDNF) is reported to be expressed in L6 [79], C2C12 [69], and both mouse [80] and human [81] primary myoblasts. Depletion or deletion of BDNF in primary myoblasts results in impaired cell cycle withdrawal and differentiation in vitro as well as muscle regeneration in vivo [80, 81].

Interestingly, a large number of muscle-derived cytokines have been found to block cell cycle withdrawal and negatively regulate myogenic differentiation including, not surprisingly, many of the growth factors that promote myoblast proliferation – FGFs [28, 82, 83], PDGF [29-31], HGF [33, 34] and TGF β [38, 39], as well as TNF α [55], BMP4/7 [84-87], CXCL14 [88], and TNFSF10/TRAIL [89]. These cytokines belong to different classes and signal through different types of receptors, and yet, Erk signaling has emerged as a common downstream pathway responsible for modulating the cell cycle. It has long been established that the ubiquitous mitogenic signaling by Erk suppresses myogenic differentiation by preventing cell cycle withdrawal [90, 91], which also requires

the cyclin dependent kinase inhibitor p21^{CIP} [92, 93]. In principle, it is also possible that distinct downstream pathways can transduce the signals from different cytokines and converge on the cell cycle machinery.

Not all published studies reporting effects of cytokines on the early stage of differentiation directly examined their involvement in the cell cycle, but some of those cytokines may well be regulating cell cycle withdrawal. For instance, LIF is expressed in mouse primary myoblasts [94]; recombinant LIF inhibits myoblast differentiation at an early stage accompanied by reduced p21^{CIP} expression and increased Erk activation, and inhibition of Erk signaling rescues differentiation in LIF-treated cells [95]. An earlier study in fact had reported LIF stimulating mouse primary myoblast proliferation [68]. These observations taken together strongly suggest that LIF negatively regulates myogenic differentiation through suppressing cell cycle withdrawal. Our RNAi screen [18] has led to the identification of additional 10 or so cytokines (belonging to several distinct families) that may negatively regulate myogenic differentiation by blocking cell cycle withdrawal (D. Kim & J. Chen, unpublished observations). It is not clear why myocytes secrete such a large number of distinct cytokines to modulate a single step of myogenesis. Do these cytokines support myoblast/satellite cell proliferation and simply need to be removed at the time of differentiation? The results of our unpublished preliminary studies do not seem to support this notion (R. Waldemer-Streyer, D. Kim, & J. Chen). Or is this arsenal of inhibitors of differentiation necessary for the maintenance of satellite cell pool? The knockdown of any one of those cytokines in myoblasts results in a differentiation phenotype, suggesting that these regulators are not redundant. How do they functionally interact? Do their signaling pathways interact and if so how?

Whereas the initiation of differentiation is affected as a direct consequence of cell cycle withdrawal regulated by the cytokines discussed above, some cytokines may

regulate this process independently of cell cycle withdrawal. IGF1 and IGF2, through their receptor IGF1R, signal through the PI3K-Akt pathway to robustly activate the myogenic differentiation program [96, 97]. SHH was also reported to promote C2C12 myoblast differentiation through the PI3K pathway [70], although another study found recombinant SHH to *inhibit* differentiation in the same cell line [71]. This discrepancy could be explained by potential difference in culturing conditions. Since SHH also stimulates myoblast/satellite cell proliferation [70, 71], the timing of SHH addition to cell culture could determine the outcome of proliferation versus differentiation.

Myostatin, acting through the activin type II receptor, has been shown to reduce MyoD expression and activity via Smad3, hence inhibiting the initiation of differentiation [98]. Signaling from the mammalian target of rapamycin complex 1 and 2 (mTORC1 and mTORC2) may also be involved in mediating myostatin inhibition of differentiation of human myoblasts [99]. Several other TGF β family members may have a similar role as myostatin, including TGF- β 1, GDF11, and activin A [44, 45, 99].

Myoblast migration and fusion

Upon cell cycle withdrawal and activation of the myogenic gene expression program, mononucleated myocytes fuse to form multi-nucleated myotubes/myofibers. Proper positioning or alignment of the cells is necessary for this fusion, which likely requires myocyte migration. Known to induce cell migration in a number of cell types, VEGF is reported to stimulate myoblast migration and differentiation while also inhibiting myoblast proliferation [100, 101]. Both IL-6 [67] and IL-7 [102] also play a positive role in myoblast migration. The chemokine CXCL12 and its receptor CXCR4 are demonstrated to be critical regulators of myocyte migration and fusion [17]. CXCL14 is also reported to stimulate migration of C2C12 cells [103]. In addition, Griffin et al. found a larger number of

chemokines to be expressed at the mRNA levels during myoblast fusion, raising the possibility that a network of chemokines may regulate the fusion process [17].

There are two distinct phases of skeletal myocyte fusion during both embryonic and adult myogenesis in mammals – myoblast-myoblast fusion to form nascent myotubes and myoblast-myotube fusion to form mature myotubes/myofibers [5, 104]. Remarkably, the two fusion processes are separable at the molecular level, with a distinct set of molecules and signaling pathways regulating each [5, 104]. IL-4 was the first example of a muscle-derived cytokine that specifically regulates the second stage of myocytes fusion, reported by Horsley et al. [105]. However, the origin of this cytokine in vivo has been disputed [106]. Heredia et al., detected no IL-4 expression in regenerating muscles in vivo; instead, they demonstrated that eosinophils recruited to the muscle injury site produce IL-4 to regulate muscle resident fibro/adipocyte progenitor cells essential for myofiber regeneration [106].

Cell survival

Although not a direct step in the myogenic process, maintenance of myocyte survival is obviously necessary for the process. Among the muscle-derived cytokines, CNTF [107], IGF2 [27], SHH [71], TNFSF14 [108], and VEGF [100, 101] have been reported to support the survival of myoblasts. Signaling by the Ser/Thr kinase AKT is a major survival pathway in various cell types [109, 110] and, indeed, at least CNTF and Tnfsf14 have been shown to signal through AKT to regulate myoblast survival [107, 108]. Other muscle-derived cytokines may exert pro-apoptotic effects. One example is IL-1, which has been reported to be expressed by human primary myoblasts; exogenous IL-1 β induces myoblast apoptosis without affecting cell proliferation or fusion [111]. It is possible that optimal cell number/density is controlled by counter-acting cytokine signaling to regulate muscle size during myogenesis.

1.4. In vivo Myogenic Function of Muscle-Derived Cytokines

It should be noted that much of what we know to date about muscle-derived cytokines in regulating myogenesis came from in vitro studies. In the literature there is surprisingly a paucity of in vivo studies of myogenesis that entail modulating cytokine expression in a muscle-specific manner. Such studies would be essential for parsing out the relative contributions of cytokines produced by muscle cells versus other cell types (especially immune cells).

BDNF

The expression of BDNF is upregulated in mouse regenerating muscles upon injury. Satellite cell-specific deletion of the BDNF gene in mice using Myf5-Cre leads to impaired regeneration, confirming a physiological role for muscle-derived BDNF [80].

FGF6

The expression of FGF6 is restricted to developing skeletal muscle during embryogenesis [112, 113] and also found in regenerating muscles post-injury [114, 115]. It is reported that FGF6-null mice have impaired muscle regeneration upon injury, most likely due to diminished satellite cell expansion [114], although another study found no defect in injury-induced muscle regeneration with FGF6^{-/-} mice [116]. The phenotype of impaired regeneration in FGF6-null mice is corroborated by accelerated regeneration of injured mouse soleus muscle upon intramuscular delivery of recombinant FGF6 [115]. While these observations combined provide support for a function of muscle-derived FGF6, definitive evidence is yet to come from muscle-specific ablation of the cytokine.

FGF21

Mice with muscle-specific knockout of FGF21 do not display any phenotype in muscle homeostasis under basal conditions, but they are protected from muscle loss during fasting [117]. Conversely, overexpression of FGF21 in muscles induces muscle loss [117]. Hence, FGF21 appears to be an important autocrine regulator of fasting-induced muscle atrophy. It will be interesting to examine regeneration/myogenesis in these mice, which has not been reported.

IGF

The expression of both IGF1 and IGF2 in regenerating muscles has long been associated with regenerative capacity [118, 119]. Muscle-specific transgene expression of IGF1 in mice has been well studied, the effects of which include hypertrophy, accelerated regeneration upon injury, prevention of muscle decline in mdx mice, and enhanced muscle maintenance and regenerative capacity during aging [120-123]. Muscle-specific deletion of IGFR1 (hence impairment of both IGF1 and IGF2 signaling) impairs muscle growth [124, 125]. However, muscle-specific knockout of IGF1 or IGF2 has not been reported and hence definitive genetic evidence for the autocrine function of IGF in vivo is still lacking.

Myostatin

Systemic knockout of myostatin in mice leads to drastically enhanced muscle growth [42], and a mutation in the bovine myostatin gene is responsible for the double-muscling phenotype in cattle [126]. Since myostatin is almost exclusively expressed in skeletal muscles in mice [42], its systemic knockout provides sufficient evidence for a muscle-autonomous function. Muscle-specific expression of follistatin, which antagonizes

myostatin, leads to enhanced muscle growth in mice to a similar degree as myostatin knockout [127].

1.5. Cytokines in Muscle Disease

Dysregulation of cytokine signaling has long been associated with human diseases, especially those that involve acute or chronic inflammation. Cytokine involvement in muscle diseases has been widely reported, but the focus has been on inflammation and immune cell-derived cytokines [128, 129]. Few studies have probed muscle-derived cytokines in muscle disease. Here we briefly survey the involvement of cytokines in three muscle disorders – muscular dystrophy, cachexia, and sarcopenia, and we focus exclusively on cytokines that are expressed in muscle cells with myogenic function. It must be emphasized that whether skeletal muscle is the source of these cytokines relevant to their involvement in the pathologies is an under-studied question that requires future investigations.

Muscular dystrophy

Muscular dystrophy is a broad term for the suite of diseases that are characterized by progressive loss of skeletal muscle function and strength. The variants of muscular dystrophy include Duchenne, limb-girdle, Becker, Walker-Warburg, and Emery-Dreifuss, to name a few. These dystrophies are caused by different genetic mutations, and they feature distinct patterns of muscle weakness [130]. The most common and perhaps most studied variant, Duchenne muscular dystrophy (DMD), is caused by mutations in the *dystrophin* gene leading to loss of the functional dystrophin protein [131]. Dystrophin is found in the membranous sarcolemma of muscle fibers and is an essential component of the dystrophin-associated protein complex, which provides structural integrity to

contracting myofibers [132]. As such, patients with DMD generally present in late childhood increasing weakness in the limbs and shoulders, which progressively worsens with age [130].

While not directly responsible for the development of muscular dystrophies, cytokines may indeed be involved in the pathogenesis of dystrophic muscle symptoms. The muscles of patients with Duchenne or Becker muscular dystrophy contain higher levels of **TGF β** [133], which promotes both the fibrosis and impaired regeneration phenotypes seen in muscular dystrophies [133, 134]. **TNF α** is reported to be expressed in regenerating myofibers of mdx, a commonly used (albeit imperfect) mouse model of DMD, although it is believed that the primary source of this cytokine in DMD is immune cells [129]. Indeed, a common therapy for DMD is low-dose treatment with the corticosteroid prednisolone, which slows progressive loss of muscle strength and function [135], likely through immunosuppression and tamping down of pro-inflammatory cytokines.

Cachexia

Cachexia is characterized by extreme wasting of lean body mass that occurs with an underlying chronic illness, such as cancer, congestive heart failure, or AIDS [136]. The development of cachexia in a chronically ill patient is associated with a poorer overall prognosis and survival rate. Indeed, cachexia is the cause of ~20% of all cancer-related deaths [137]. Muscle atrophy during cachexic states ultimately stems from ubiquitin-mediated breakdown of myofibrils [50].

Significantly, a well-documented association exists between cachexia and the dysregulation of cytokines, most notably the pro-inflammatory cytokines **TNF α** , **IL-1**, and **IL-6** [50, 51]. However, clinical trials aimed at pharmacologically inhibiting each of these cytokines individually have had limited success in reversing the symptoms of cachexia [50]. It is possible that TNF α , IL-1, and IL-6 play an important, interconnected role in

muscle wasting, but other factors—perhaps other cytokines—are also at work. This idea is bolstered by a study demonstrating that inhibition of Fn14, the receptor for the TNF family member **TWEAK** (Tnfsf12), can ameliorate cachexia and prolong survival in tumor-bearing mice [138]. However, this effect was only achieved by inhibiting Fn14 in the tumor, not TWEAK-Fn14 signaling in the host [138], indicating that Fn14+ tumors may be secreting a yet-to-be-identified factor that signals muscle atrophy. The exact mechanism and physiological source of pro-inflammatory cytokines in cachexic skeletal muscle atrophy is still unclear.

Sarcopenia

The progressive loss of muscle mass that comes with advancing age is termed sarcopenia. This form of muscular atrophy is quite common, affecting approximately 50% of people over the age of 80 [50]. Sarcopenia occurs in the absence of any underlying illness, and is correlated with physical frailty, which is closely associated with increased incidence of falls and fractures, hospitalization, and mortality in the aging population [50, 52].

Similar to cachexia, the levels of pro-inflammatory cytokines **TNF α** and **IL-6** closely correlate with loss of muscle mass, osteoporosis, and overall increased markers of frailty [50, 52]. Indeed, TNF α alone is capable of inducing muscle breakdown, possibly via both NF- κ B activation and upregulation of ubiquitin ligases [139]. However, it is likely that other cytokines also play a role in pathological muscle breakdown. **TWEAK**, for example, can induce muscle wasting in mice, also through NF- κ B [140]. GDF11 expression is increased in rat muscles as well as in rat and human sera with aging, and exogenous GDF11 delivered to young adult mice impairs muscle regeneration [45]. A better understanding of the network of cytokines involved in regeneration and the maintenance

of muscle mass—as well as how those cytokines are regulated—could shed some light on how muscle catabolism increases with age and how to prevent it.

1.6. Table and Figures

Table 1.1

<u>Cytokine</u>	<u>Myogenic function</u>	<u>References</u>
<i><u>Ligands for RTKs:</u></i>		
BDNF	+ cell cycle withdrawal; + differentiation	[80, 81]
FGF1,2,4,6	+ proliferation; - cell cycle withdrawal; - differentiation	[20, 28]
Flt3L	+ cell cycle withdrawal; + differentiation	[77]
HGF (Scatter factor)	+ proliferation; - cell cycle withdrawal; - differentiation	[33, 34]
IGF1, IGF2	+ initiation of differentiation; + survival	[19-21, 27]
NGF	+ satellite cell activation/proliferation	[69]
PDGF	+ proliferation; - cell cycle withdrawal; - differentiation	[29-31]
VEGF	- proliferation; + migration; + differentiation; + survival	[100, 101]
<i><u>Ligands for cytokine receptors:</u></i>		
CNTF	+ survival; - differentiation; + dedifferentiation	[107, 141]
CT-1	- initiation of differentiation	[142, 143]
G-CSF	+ proliferation	[144-147]
IFN γ	+ proliferation; + differentiation	[66]
IL-4	+ second-stage fusion	[105]
IL-6	+ proliferation; + migration	[67]
IL-7	+ migration; - differentiation	[102]
IL-12	+ differentiation	[148, 149]
IL-13	+ second-stage fusion	[105, 150, 151]
IL-15	+ differentiation	[152-154]
LIF	+ proliferation; - cell cycle withdrawal; - differentiation	[68, 94, 95]
OSM	+ satellite cell quiescence; - differentiation	[155, 156]
<i><u>Chemokines (ligands for GPCR):</u></i>		
CCL2 (MCP1)	+ proliferation	[13, 63, 157]
CXCL12 (SDF1)	+ differentiation; + migration; + fusion	[17, 158, 159]
CXCL14 (BRAK)	- cell cycle withdrawal; + migration	[88, 103]
CXCL16	+ proliferation; + modulating immune cells	[64]
IL-8	+ differentiation	[160]
<i><u>TGFβ family:</u></i>		
Activin A	- proliferation; - differentiation	[44, 47, 76]
BMP4, 7	- cell cycle withdrawal; - differentiation	[84-87]
GDF11	- differentiation	[45, 99]
Myostatin (GDF8)	- proliferation; - initiation of differentiation	[74, 75, 98, 99]
TGF β	+ proliferation; - cell cycle withdrawal; - differentiation	[35-39]

Table 1.1 (cont.)

TNF family:

TNF α (Cachectin)	- satellite cell activation; - cell cycle withdrawal; -/+ differentiation	[53-55, 58-61]
TNFSF10 (TRAIL)	- cell cycle withdrawal; - differentiation	[89]
TNFSF14 (LIGHT)	+ survival; + differentiation	[108]

Others:

CCN2 (CTGF)	+ proliferation; - differentiation; + dedifferentiation	[161, 162]
CCN3 (NOV) [163]	- myogenic cell lineage; - cell cycle withdrawal; - differentiation	
IL-1	+ proliferation; - differentiation; - survival	[55, 111, 164]
SHH survival	+ satellite cell activation and proliferation; +/- differentiation; +	[70, 71, 73]

Table 1.1 Muscle-derived cytokines and their reported functions in myogenic regulation.

Cytokines reported to be expressed in muscle cells and have a myogenic function are listed. “+” indicates positive regulation of the process whereas “-” indicates negative regulation.

Figure 1.1

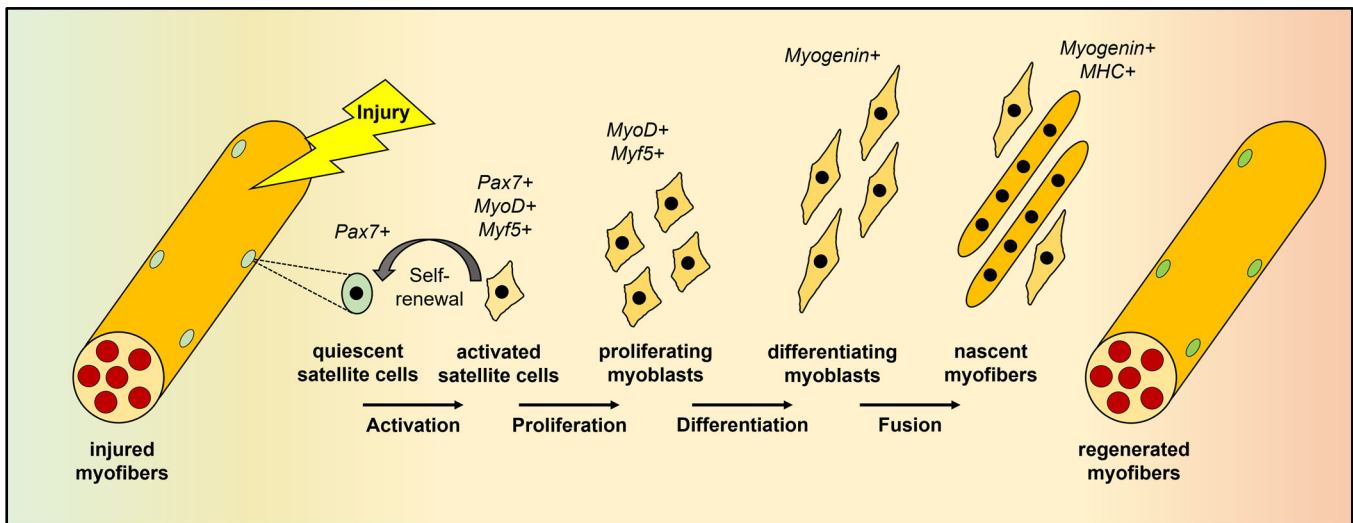


Figure 1.1 Skeletal muscle regeneration.

In mature muscle, quiescent satellite cells are activated in response to injury. A subset of the activated satellite cells returns to quiescence to maintain the muscle stem cell pool, while the remaining activated cells divide to form myoblasts, followed by myogenic differentiation. Differentiated myoblast further form myofibers via fusion processes. Expression of key myogenic regulators are shown at each stage of muscle regeneration.

Figure 1.2

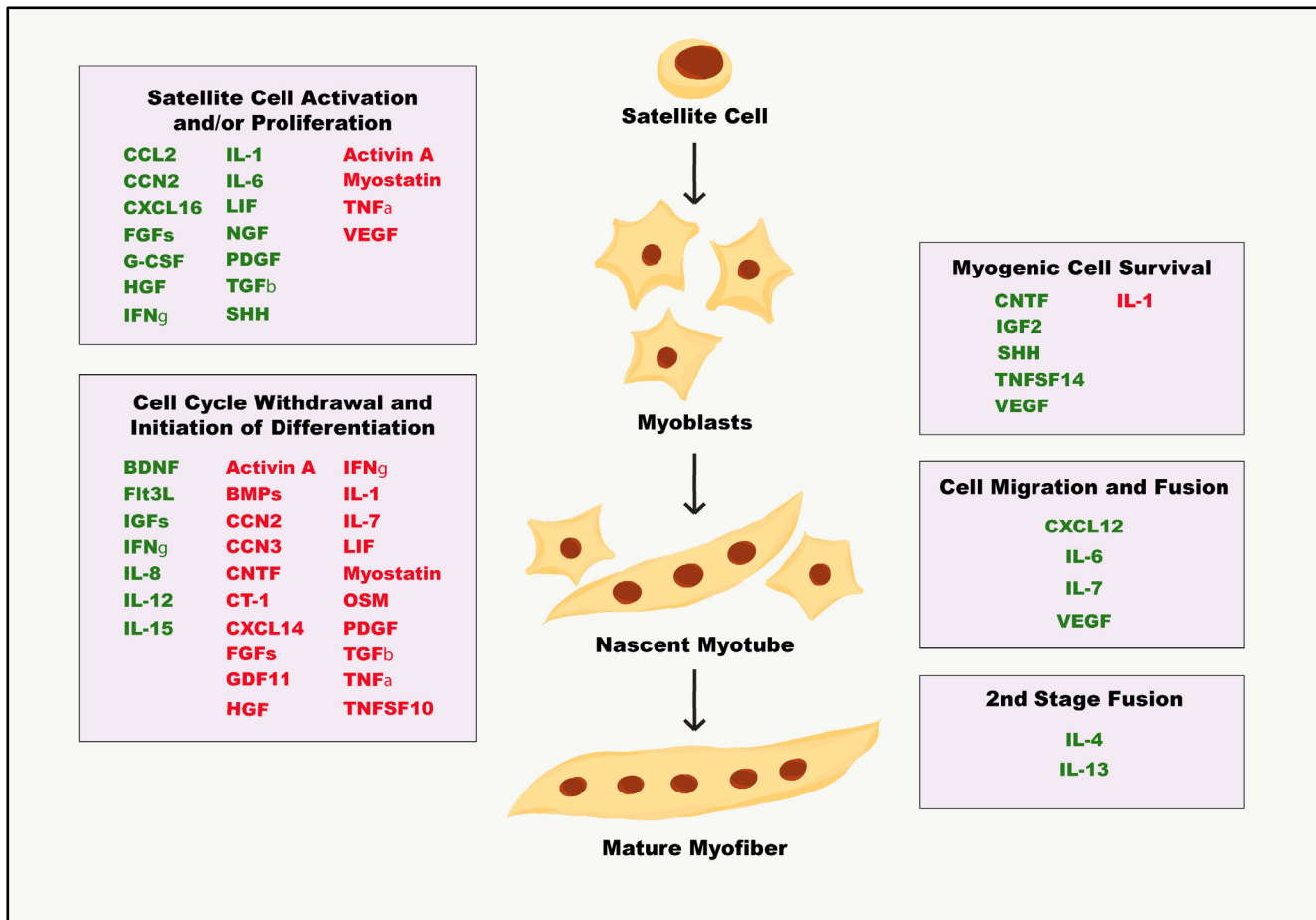


Figure 1.2 Muscle-derived cytokines regulate myogenic processes.

Cytokines reported to regulate each stage of myogenic processes are listed. Cytokines with green color indicate positive regulators of each stage whereas the cytokines with red color indicate negative regulators.

Figure 1.3

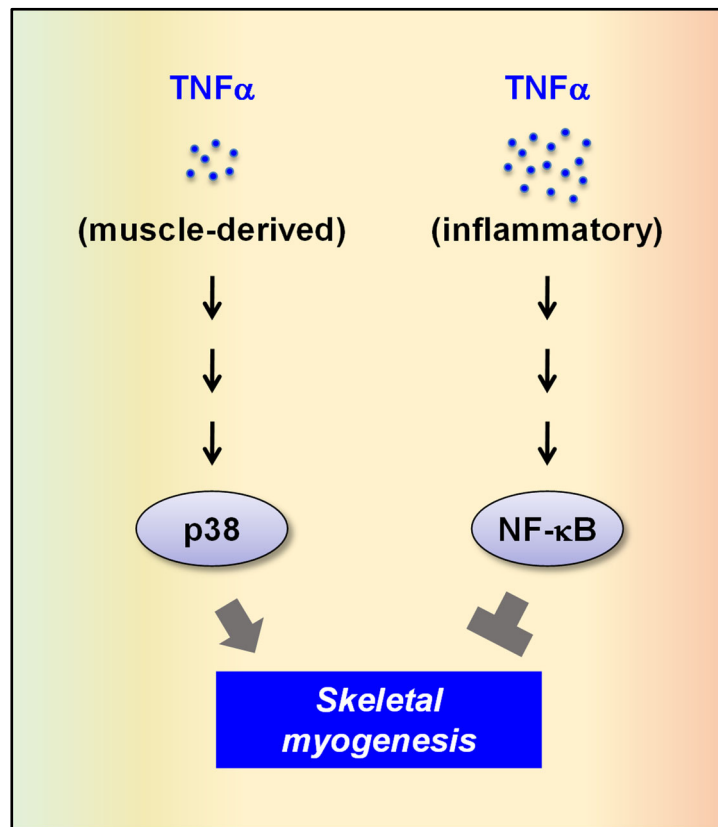


Figure 1.3 A model for TNF α 's opposing roles in the regulation of skeletal myogenesis.

The source of TNF α (muscle-derived or inflammatory) or the concentration of TNF α can culminate in opposite myogenic responses, mediated by different signaling pathways such as p38 MAPK and NF κ B.

Figure 1.4

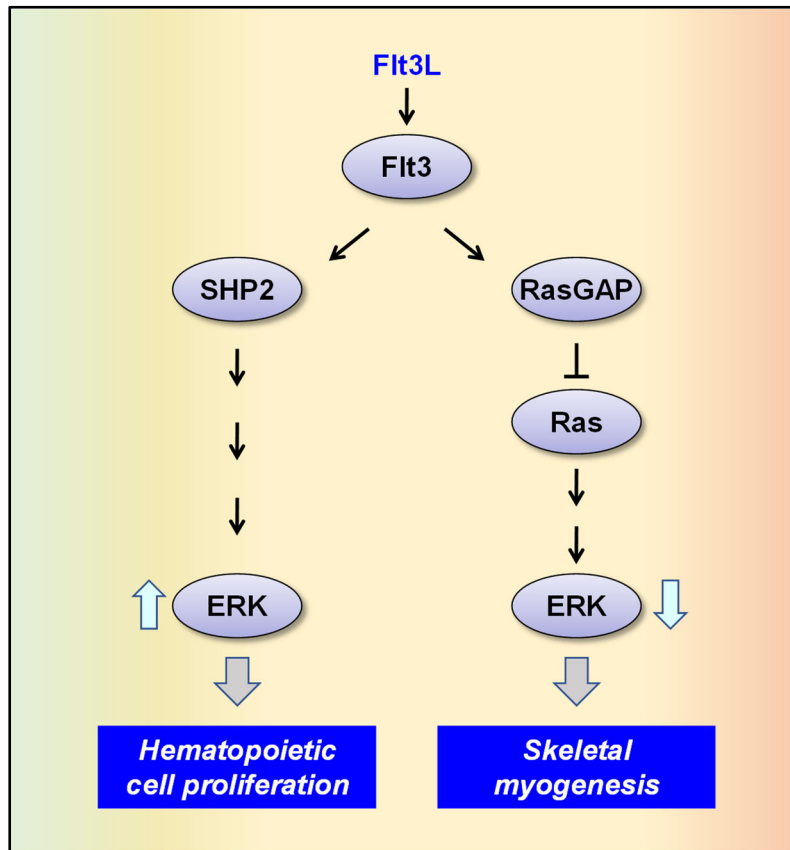


Figure 1.4 Distinct FIt3L signaling through ERK in hematopoietic cells and skeletal muscle cells.

In hematopoietic cells, FIt3L-FIt3 signaling activates ERK through SHP2 recruitment resulting in cell proliferation. In contrast, FIt3L-FIt3 signaling inhibits ERK, mediated by p120RasGAP, promoting cell cycle withdrawal and subsequently differentiation in muscle cells.

1.7 References

1. Montarras, D., A. L'honoré, and M. Buckingham, *Lying low but ready for action: the quiescent muscle satellite cell*. FEBS Journal, 2013. **280**: p. 4036-4050.
2. Tidball, J.G., *Mechanisms of muscle injury, repair, and regeneration*. Compr Physiol, 2011. **1**(4): p. 2029-62.
3. Buckingham, M. and P.W. Rigby, *Gene regulatory networks and transcriptional mechanisms that control myogenesis*. Developmental Cell, 2014. **28**(3): p. 225-238.
4. Parker, M.H., P. Seale, and M.A. Rudnicki, *Looking back to the embryo: defining transcriptional networks in adult myogenesis*. Nature Reviews, Genetics, 2003. **4**: p. 495-505.
5. Simionescu, A. and G.K. Pavlath, *Molecular mechanisms of myoblast fusion across species*. Advances in Experimental Medicine and Biology, 2011. **713**: p. 113-135.
6. Dinarello, C.A., *Historical insights into cytokines*. European journal of immunology, 2007. **37**(S1): p. S34-S45.
7. Tidball, J.G., *Regulation of muscle growth and regeneration by the immune system*. Nat Rev Immunol, 2017. **17**(3): p. 165-178.
8. Tidball, J.G. and S.A. Villalta, *Regulatory interactions between muscle and the immune system during muscle regeneration*. Am J Physiol Regul Integr Comp Physiol., 2010. **298**(5): p. R1173-87.
9. Pedersen, B.K., et al., *Searching for the exercise factor: is IL-6 a candidate?* J Muscle Res Cell Motil, 2003. **24**(2-3): p. 113-9.
10. Pedersen, B.K. and M.A. Febbraio, *Muscles, exercise and obesity: skeletal muscle as a secretory organ*. Nature Reviews Endocrinology, 2012. **8**(8): p. 457-465.
11. Whitham, M. and M.A. Febbraio, *The ever-expanding myokinome: discovery challenges and therapeutic implications*. Nat Rev Drug Discov, 2016. **15**(10): p. 719-29.
12. Chan, C.Y., et al., *Identification of differentially regulated secretome components during skeletal myogenesis*. Mol Cell Proteomics, 2011. **10**(5): p. M110.004804.
13. Henningsen, J., B.K. Pedersen, and I. Kratchmarova, *Quantitative analysis of the secretion of the MCP family of chemokines by muscle cells*. Mol Biosyst., 2011. **7**(2): p. 311-21.
14. Henningsen, J., et al., *Dynamics of the skeletal muscle secretome during myoblast differentiation*. Mol Cell Proteomics., 2010. **9**(11): p. 2482-96.
15. Yoon, J.H., et al., *Comparative proteomic analysis of the insulin-induced L6 myotube secretome*. Proteomics, 2009. **9**(1): p. 51-60.
16. Norheim, F., et al., *Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training*. Am J Physiol Endocrinol Metab, 2011. **301**(5): p. E1013-21.
17. Griffin, C.A., et al., *Chemokine expression and control of muscle cell migration during myogenesis*. Journal of Cell Science, 2010. **123**: p. 3052-3060.
18. Ge, Y., et al., *RNAi Screen Reveals Potentially Novel Roles of Cytokines in Myoblast Differentiation*. PLoS ONE, 2013. **8**(7): p. e68068 (PMC3699544).
19. Florini, J.R., D.Z. Ewton, and S.A. Coolican, *Growth hormone and the insulin-like growth factor system in myogenesis*. Endocr Rev., 1996. **17**(5): p. 481-517.
20. Florini, J.R., D.Z. Ewton, and K.A. Magri, *Hormones, growth factors, and myogenic differentiation*. Annu Rev Physiol, 1991. **53**: p. 201-16.
21. Stewart, C.E. and P. Rotwein, *Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors*. Physiol Rev, 1996. **76**(4): p. 1005-26.

22. Saini, A., N. Al-Shanti, and C.E. Stewart, *Waste management - cytokines, growth factors and cachexia*. Cytokine Growth Factor Rev, 2006. **17**(6): p. 475-86.
23. Glass, D.J., *Molecular mechanisms modulating muscle mass*. Trends Mol Med., 2003. **9**(8): p. 344-50.
24. Florini, J.R., et al., "*Spontaneous*" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. J Biol Chem, 1991. **266**(24): p. 15917-23.
25. Tollefsen, S.E., et al., *Insulin-like growth factors (IGF) in muscle development. Expression of IGF-I, the IGF-I receptor, and an IGF binding protein during myoblast differentiation*. J Biol Chem, 1989. **264**(23): p. 13810-7.
26. Tollefsen, S.E., J.L. Sadow, and P. Rotwein, *Coordinate expression of insulin-like growth factor II and its receptor during muscle differentiation*. Proc Natl Acad Sci U S A, 1989. **86**(5): p. 1543-7.
27. Stewart, C.E. and P. Rotwein, *Insulin-like growth factor-II is an autocrine survival factor for differentiating myoblasts*. J Biol Chem., 1996. **271**(19): p. 11330-8.
28. Pawlikowski, B., et al., *Regulation of skeletal muscle stem cells by fibroblast growth factors*. Dev Dyn, 2017. **246**(5): p. 359-367.
29. Yablonka-Reuveni, Z., T.M. Balestreri, and D.F. Bowen-Pope, *Regulation of proliferation and differentiation of myoblasts derived from adult mouse skeletal muscle by specific isoforms of PDGF*. J Cell Biol, 1990. **111**(4): p. 1623-9.
30. Jin, P., T. Sejersen, and N.R. Ringertz, *Recombinant platelet-derived growth factor-BB stimulates growth and inhibits differentiation of rat L6 myoblasts*. Journal of Biological Chemistry, 1991. **266**(2): p. 1245-1249.
31. Sejersen, T., et al., *Rat skeletal myoblasts and arterial smooth muscle cells express the gene for the A chain but not the gene for the B chain (c-sis) of platelet-derived growth factor (PDGF) and produce a PDGF-like protein*. Proceedings of the National Academy of Sciences, 1986. **83**(18): p. 6844-6848.
32. Jennische, E., S. Ekberg, and G.L. Matejka, *Expression of hepatocyte growth factor in growing and regenerating rat skeletal muscle*. American Journal of Physiology-Cell Physiology, 1993. **265**(1): p. C122-C128.
33. Gal-Levi, R., et al., *Hepatocyte growth factor plays a dual role in regulating skeletal muscle satellite cell proliferation and differentiation*. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1998. **1402**(1): p. 39-51.
34. Sheehan, S.M., et al., *HGF is an autocrine growth factor for skeletal muscle satellite cells in vitro*. Muscle & Nerve, 2000. **23**(2): p. 239-245.
35. Florini, J.R., et al., *Transforming growth factor-beta. A very potent inhibitor of myoblast differentiation, identical to the differentiation inhibitor secreted by Buffalo rat liver cells*. J Biol Chem, 1986. **261**(35): p. 16509-13.
36. Massagué, J., et al., *Type beta transforming growth factor is an inhibitor of myogenic differentiation*. Proceedings of the National Academy of Sciences, 1986. **83**(21): p. 8206-8210.
37. Olson, E.N., et al., *Regulation of myogenic differentiation by type beta transforming growth factor*. J Cell Biol, 1986. **103**(5): p. 1799-805.
38. Rodgers, B.D., et al., *Myostatin stimulates, not inhibits, C2C12 myoblast proliferation*. Endocrinology, 2014. **155**(3): p. 670-5.
39. Schabort, E.J., et al., *TGF-beta's delay skeletal muscle progenitor cell differentiation in an isoform-independent manner*. Exp Cell Res, 2009. **315**(3): p. 373-84.
40. Liu, D., B.L. Black, and R. Derynck, *TGF- β inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3*. Genes & Development, 2001. **15**(22): p. 2950-2966.

41. Lafyatis, R., et al., *Secretion and transcriptional regulation of transforming growth factor-beta 3 during myogenesis*. Molecular and cellular biology, 1991. **11**(7): p. 3795-3803.
42. McPherron, A.C., A.M. Lawler, and S.J. Lee, *Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member*. Nature, 1997. **387**(6628): p. 83-90.
43. Rodgers, B.D. and D.K. Garikipati, *Clinical, agricultural, and evolutionary biology of myostatin: a comparative review*. Endocr Rev, 2008. **29**(5): p. 513-34.
44. Link, B.A. and R. Nishi, *Opposing effects of activin A and follistatin on developing skeletal muscle cells*. Experimental cell research, 1997. **233**(2): p. 350-362.
45. Egerman, M.A., et al., *GDF11 Increases with Age and Inhibits Skeletal Muscle Regeneration*. Cell Metab, 2015. **22**(1): p. 164-74.
46. Rodino-Klapac, L.R., et al., *Inhibition of myostatin with emphasis on follistatin as a therapy for muscle disease*. Muscle Nerve, 2009. **39**(3): p. 283-96.
47. Gilson, H., et al., *Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin*. American Journal of Physiology-Endocrinology and Metabolism, 2009. **297**(1): p. E157-E164.
48. Kota, J., et al., *Follistatin gene delivery enhances muscle growth and strength in nonhuman primates*. Science translational medicine, 2009. **1**(6): p. 6ra15-6ra15.
49. Winbanks, C.E., et al., *Follistatin-mediated skeletal muscle hypertrophy is regulated by Smad3 and mTOR independently of myostatin*. J Cell Biol., 2012. **197**(7): p. 997-1008. Epub 2012 Jun 18.
50. Cohen, S., J.A. Nathan, and A.L. Goldberg, *Muscle wasting in disease: molecular mechanisms and promising therapies*. Nature Reviews Drug Discovery, 2015. **14**(1): p. 58-74.
51. Fearon, K.C., D.J. Glass, and D.C. Guttridge, *Cancer cachexia: mediators, signaling, and metabolic pathways*. Cell Metabolism, 2012. **16**(2): p. 153-166.
52. Michaud, M., et al., *Proinflammatory cytokines, aging, and age-related diseases*. Journal of the American Medical Directors Association, 2013. **14**(12): p. 877-882.
53. Miller, S., et al., *Tumor necrosis factor inhibits human myogenesis in vitro*. Molecular and cellular biology, 1988. **8**(6): p. 2295-2301.
54. Langen, R.C., et al., *Tumor necrosis factor- α inhibits myogenesis through redox-dependent and-independent pathways*. American Journal of Physiology-Cell Physiology, 2002. **283**(3): p. C714-C721.
55. Langen, R.C., et al., *Inflammatory cytokines inhibit myogenic differentiation through activation of nuclear factor-kappaB*. Faseb J., 2001. **15**(7): p. 1169-80.
56. Acharyya, S., et al., *TNF inhibits Notch-1 in skeletal muscle cells by Ezh2 and DNA methylation mediated repression: implications in duchenne muscular dystrophy*. PLoS one, 2010. **5**(8): p. e12479.
57. Porter, J.D., et al., *A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice*. Hum Mol Genet, 2002. **11**(3): p. 263-72.
58. Li, Y.P. and R.J. Schwartz, *TNF-alpha regulates early differentiation of C2C12 myoblasts in an autocrine fashion*. Faseb j, 2001. **15**(8): p. 1413-5.
59. Warren, G.L., et al., *Physiological role of tumor necrosis factor alpha in traumatic muscle injury*. Faseb j, 2002. **16**(12): p. 1630-2.
60. Chen, S.-E., et al., *Role of TNF- α signaling in regeneration of cardiotoxin-injured muscle*. American Journal of Physiology-Cell Physiology, 2005. **289**(5): p. C1179-C1187.
61. Chen, S.-E., B. Jin, and Y.-P. Li, *TNF- α regulates myogenesis and muscle regeneration by activating p38 MAPK*. American Journal of Physiology-Cell Physiology, 2007. **292**(5): p. C1660-C1671.

62. Acharyya, S., et al., *Interplay of IKK/NF- κ B signaling in macrophages and myofibers promotes muscle degeneration in Duchenne muscular dystrophy*. The Journal of clinical investigation, 2007. **117**(4): p. 889.
63. Yahiaoui, L., et al., *CC family chemokines directly regulate myoblast responses to skeletal muscle injury*. The Journal of physiology, 2008. **586**(16): p. 3991-4004.
64. Zhang, L., et al., *Chemokine CXCL16 regulates neutrophil and macrophage infiltration into injured muscle, promoting muscle regeneration*. The American journal of pathology, 2009. **175**(6): p. 2518-2527.
65. Hara, M., et al., *G-CSF influences mouse skeletal muscle development and regeneration by stimulating myoblast proliferation*. The Journal of experimental medicine, 2011. **208**(4): p. 715-727.
66. Cheng, M., et al., *Endogenous interferon- γ is required for efficient skeletal muscle regeneration*. American Journal of Physiology-Cell Physiology, 2008. **294**(5): p. C1183-C1191.
67. Serrano, A.L., et al., *Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy*. Cell Metab., 2008. **7**(1): p. 33-44.
68. Austin, L. and A.W. Burgess, *Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines*. J Neurol Sci, 1991. **101**(2): p. 193-7.
69. Seidl, K., C. Erck, and A. Buchberger, *Evidence for the participation of nerve growth factor and its low-affinity receptor (p75NTR) in the regulation of the myogenic program*. J Cell Physiol, 1998. **176**(1): p. 10-21.
70. Elia, D., et al., *Sonic hedgehog promotes proliferation and differentiation of adult muscle cells: Involvement of MAPK/ERK and PI3K/Akt pathways*. Biochim Biophys Acta, 2007. **1773**(9): p. 1438-46.
71. Koleva, M., et al., *Pleiotropic effects of sonic hedgehog on muscle satellite cells*. Cell Mol Life Sci, 2005. **62**(16): p. 1863-70.
72. Borycki, A.G., et al., *Sonic hedgehog controls epaxial muscle determination through Myf5 activation*. Development, 1999. **126**(18): p. 4053-63.
73. Straface, G., et al., *Sonic hedgehog regulates angiogenesis and myogenesis during post-natal skeletal muscle regeneration*. J Cell Mol Med, 2009. **13**(8b): p. 2424-2435.
74. Thomas, M., et al., *Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation*. Journal of Biological Chemistry, 2000. **275**(51): p. 40235-40243.
75. McCroskery, S., et al., *Myostatin negatively regulates satellite cell activation and self-renewal*. The Journal of cell biology, 2003. **162**(6): p. 1135-1147.
76. Yaden, B.C., et al., *Inhibition of Activin A Ameliorates Skeletal Muscle Injury and Rescues Contractile Properties by Inducing Efficient Remodeling in Female Mice*. American Journal of Pathology, 2014. **184**(4): p. 1152-1166.
77. Ge, Y., et al., *Flt3L is a novel regulator of skeletal myogenesis*. J Cell Sci., 2013. **126**(Pt 15): p. 3370-9 (PMC3730246).
78. Masson, K. and L. Ronnstrand, *Oncogenic signaling from the hematopoietic growth factor receptors c-Kit and Flt3*. Cell Signal., 2009. **21**(12): p. 1717-26. Epub 2009 Jun 18.
79. Mousavi, K. and B.J. Jasmin, *BDNF is expressed in skeletal muscle satellite cells and inhibits myogenic differentiation*. J Neurosci, 2006. **26**(21): p. 5739-49.
80. Clow, C. and B.J. Jasmin, *Brain-derived neurotrophic factor regulates satellite cell differentiation and skeletal muscle regeneration*. Mol Biol Cell, 2010. **21**(13): p. 2182-90.

81. Colombo, E., et al., *Autocrine and immune cell-derived BDNF in human skeletal muscle: implications for myogenesis and tissue regeneration*. The Journal of Pathology, 2013. **231**(2): p. 190-198.
82. Sheehan, S.M. and R.E. Allen, *Skeletal muscle satellite cell proliferation in response to members of the fibroblast growth factor family and hepatocyte growth factor*. J Cell Physiol, 1999. **181**(3): p. 499-506.
83. Hannon, K., et al., *Differentially expressed fibroblast growth factors regulate skeletal muscle development through autocrine and paracrine mechanisms*. J Cell Biol, 1996. **132**(6): p. 1151-9.
84. Ono, Y., et al., *BMP signalling permits population expansion by preventing premature myogenic differentiation in muscle satellite cells*. Cell Death Differ, 2011. **18**(2): p. 222-34.
85. Sterrenburg, E., et al., *Gene expression profiling highlights defective myogenesis in DMD patients and a possible role for bone morphogenetic protein 4*. Neurobiology of disease, 2006. **23**(1): p. 228-236.
86. Friedrichs, M., et al., *BMP signaling balances proliferation and differentiation of muscle satellite cell descendants*. BMC Cell Biol, 2011. **12**: p. 26.
87. Gozo, M., et al., *Foxc2 induces Wnt4 and Bmp4 expression during muscle regeneration and osteogenesis*. Cell Death & Differentiation, 2013. **20**(8): p. 1031-1042.
88. Waldemer-Streyer, R.J., et al., *Cxcl14 depletion accelerates skeletal myogenesis by promoting cell cycle withdrawal*. NPJ Regen Med, 2017. **2**: p. pii: 16017 (PMC553773).
89. Kim, D., et al., *Muscle-derived TRAIL negatively regulates myogenic differentiation*. Exp Cell Res, 2020. **394**(1): p. 112165 (PMC7434709).
90. Bennett, A.M. and N.K. Tonks, *Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases*. Science, 1997. **278**(5341): p. 1288-1291.
91. Coolican, S.A., et al., *The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways*. J Biol Chem, 1997. **272**(10): p. 6653-62.
92. Guo, K., et al., *MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation*. Mol Cell Biol., 1995. **15**(7): p. 3823-9.
93. Halevy, O., et al., *Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD*. Science., 1995. **267**(5200): p. 1018-21.
94. Kurek, J.B., et al., *Leukemia inhibitory factor and interleukin-6 are produced by diseased and regenerating skeletal muscle*. Muscle & nerve, 1996. **19**(10): p. 1291-1301.
95. Jo, C., et al., *Leukemia inhibitory factor blocks early differentiation of skeletal muscle cells by activating ERK*. Biochim Biophys Acta, 2005. **1743**(3): p. 187-97.
96. Jiang, B.H., et al., *Myogenic signaling of phosphatidylinositol 3-kinase requires the serine-threonine kinase Akt/protein kinase B*. Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2077-81.
97. Kaliman, P., et al., *Insulin-like growth factors require phosphatidylinositol 3-kinase to signal myogenesis: dominant negative p85 expression blocks differentiation of L6E9 muscle cells*. Mol Endocrinol, 1998. **12**(1): p. 66-77.
98. Langley, B., et al., *Myostatin inhibits myoblast differentiation by down-regulating MyoD expression*. Journal of Biological Chemistry, 2002. **277**(51): p. 49831-49840.
99. Trendelenburg, A.U., et al., *Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size*. Am J Physiol Cell Physiol., 2009. **296**(6): p. C1258-70. Epub 2009 Apr 8.

100. Bryan, B.A., et al., *Coordinated vascular endothelial growth factor expression and signaling during skeletal myogenic differentiation*. Mol Biol Cell., 2008. **19**(3): p. 994-1006. Epub 2007 Dec 19.
101. Arsic, N., et al., *Vascular endothelial growth factor stimulates skeletal muscle regeneration in vivo*. Molecular Therapy, 2004. **10**(5): p. 844-854.
102. Haugen, F., et al., *IL-7 is expressed and secreted by human skeletal muscle cells*. American Journal of Physiology-Cell Physiology, 2010. **298**(4): p. C807-C816.
103. Hayashi, Y., et al., *CXCL14 and MCP1 are potent trophic factors associated with cell migration and angiogenesis leading to higher regenerative potential of dental pulp side population cells*. Stem Cell Research & Therapy, 2015. **6**(1): p. 1-19.
104. Jansen, K.M. and G.K. Pavlath, *Molecular Control of Mammalian Myoblast Fusion*. Methods in Molecular Biology, 2008. **475**: p. 115-133.
105. Horsley, V., et al., *IL-4 Acts as a Myoblast Recruitment Factor during Mammalian Muscle Growth*. Cell, 2003. **113**(4): p. 483-94.
106. Heredia, J.E., et al., *Type 2 Innate Signals Stimulate Fibro/Adipogenic Progenitors to Facilitate Muscle Regeneration*. Cell, 2013. **153**(2): p. 376-388.
107. Hiatt, K., et al., *Ciliary neurotrophic factor (CNTF) promotes skeletal muscle progenitor cell (MPC) viability via the phosphatidylinositol 3-kinase–Akt pathway*. Journal of tissue engineering and regenerative medicine, 2014. **8**(12): p. 963-968.
108. Waldemer-Streyer, R. and J. Chen, *Myocyte-derived Tnfsf14 is a survival factor necessary for myoblast differentiation and skeletal muscle regeneration*. Cell Death & Disease, 2015. **6**(12): p. e2026 (PMC4720906).
109. Manning, B.D. and A. Toker, *AKT/PKB Signaling: Navigating the Network*. Cell, 2017. **169**(3): p. 381-405.
110. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: navigating downstream*. Cell., 2007. **129**(7): p. 1261-74.
111. Plonquet, A., et al., *Differential expression of the IL-1 system components during in vitro myogenesis: Implication of IL-1b in induction of myogenic cell apoptosis*. Cell death and differentiation, 1999. **6**: p. 1012-1021.
112. deLapeyrière, O., et al., *Expression of the Fgf6 gene is restricted to developing skeletal muscle in the mouse embryo*. Development, 1993. **118**(2): p. 601-11.
113. Han, J.K. and G.R. Martin, *Embryonic expression of Fgf-6 is restricted to the skeletal muscle lineage*. Dev Biol, 1993. **158**(2): p. 549-54.
114. Floss, T., H.-H. Arnold, and T. Braun, *A role for FGF-6 in skeletal muscle regeneration*. Genes & development, 1997. **11**(16): p. 2040-2051.
115. Armand, A.-S., et al., *Injection of FGF6 accelerates regeneration of the soleus muscle in adult mice*. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2003. **1642**(1): p. 97-105.
116. Fiore, F., A. Sébille, and D. Birnbaum, *Skeletal muscle regeneration is not impaired in Fgf6 -/- mutant mice*. Biochem Biophys Res Commun, 2000. **272**(1): p. 138-43.
117. Oost, L.J., et al., *Fibroblast growth factor 21 controls mitophagy and muscle mass*. J Cachexia Sarcopenia Muscle, 2019. **10**(3): p. 630-642.
118. Jennische, E. and G.L. Matejka, *IGF-I binding and IGF-I expression in regenerating muscle of normal and hypophysectomized rats*. Acta Physiol Scand, 1992. **146**(1): p. 79-86.
119. Levinovitz, A., et al., *Activation of insulin-like growth factor II expression during skeletal muscle regeneration in the rat: correlation with myotube formation*. Mol Endocrinol, 1992. **6**(8): p. 1227-34.
120. Barton, E.R., et al., *Muscle-specific expression of insulin-like growth factor I counters muscle decline in mdx mice*. J Cell Biol, 2002. **157**(1): p. 137-48.

121. Barton-Davis, E.R., et al., *Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function*. Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15603-7.
122. Musaro, A., et al., *Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle*. Nat Genet, 2001. **27**(2): p. 195-200.
123. Coleman, M.E., et al., *Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice*. J Biol Chem, 1995. **270**(20): p. 12109-16.
124. Mavalli, M.D., et al., *Distinct growth hormone receptor signaling modes regulate skeletal muscle development and insulin sensitivity in mice*. J Clin Invest, 2010. **120**(11): p. 4007-20.
125. O'Neill, B.T., et al., *Differential Role of Insulin/IGF-1 Receptor Signaling in Muscle Growth and Glucose Homeostasis*. Cell Rep, 2015. **11**(8): p. 1220-35.
126. Grobet, L., et al., *A deletion in the bovine myostatin gene causes the double-muscling phenotype in cattle*. Nature genetics, 1997. **17**(1): p. 71-74.
127. Lee, S.J. and A.C. McPherron, *Regulation of myostatin activity and muscle growth*. Proc Natl Acad Sci U S A, 2001. **98**(16): p. 9306-11.
128. Zhou, J., et al., *Cytokine Signaling in Skeletal Muscle Wasting*. Trends Endocrinol Metab, 2016. **27**(5): p. 335-347.
129. De Paepe, B. and J.L. De Bleecker, *Cytokines and chemokines as regulators of skeletal muscle inflammation: presenting the case of Duchenne muscular dystrophy*. Mediators Inflamm, 2013. **2013**: p. 540370.
130. Mercuri, E. and F. Muntoni, *Muscular dystrophies*. The Lancet, 2013. **381**(9869): p. 845-860.
131. Hoffman, E.P., R.H. Brown, and L.M. Kunkel, *Dystrophin: the protein product of the Duchenne muscular dystrophy locus*. Cell, 1987. **51**(6): p. 919-928.
132. Rahimov, F. and L.M. Kunkel, *Cellular and molecular mechanisms underlying muscular dystrophy*. The Journal of cell biology, 2013. **201**(4): p. 499-510.
133. Bernasconi, P., et al., *Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine*. Journal of Clinical Investigation, 1995. **96**(2): p. 1137.
134. Cohn, R.D., et al., *Angiotensin II type 1 receptor blockade attenuates TGF- β -induced failure of muscle regeneration in multiple myopathic states*. Nature medicine, 2007. **13**(2): p. 204-210.
135. Matthews, E., et al., *Corticosteroids for the treatment of Duchenne muscular dystrophy*. Cochrane Database Syst Rev, 2016(5): p. CD003725.
136. Evans, W.J., et al., *Cachexia: a new definition*. Clin Nutr, 2008. **27**(6): p. 793-9.
137. Tisdale, M.J., *Cachexia in cancer patients*. Nature Reviews Cancer, 2002. **2**(11): p. 862-871.
138. Johnston, A.J., et al., *Targeting of Fn14 Prevents Cancer-Induced Cachexia and Prolongs Survival*. Cell, 2015. **162**(6): p. 1365-78.
139. Cai, D., et al., *IKK β /NF- κ B activation causes severe muscle wasting in mice*. Cell, 2004. **119**(2): p. 285-298.
140. Dogra, C., et al., *TNF-related weak inducer of apoptosis (TWEAK) is a potent skeletal muscle-wasting cytokine*. The FASEB Journal, 2007. **21**(8): p. 1857-1869.
141. Johnson, R.W., et al., *Myokines (muscle-derived cytokines and chemokines) including ciliary neurotrophic factor (CNTF) inhibit osteoblast differentiation*. Bone, 2014. **64**: p. 47-56.
142. Miyake, T., et al., *Cardiotrophin-1 maintains the undifferentiated state in skeletal myoblasts*. J Biol Chem., 2009. **284**(29): p. 19679-93.

143. Pennica, D., et al., *Cardiotrophin-1, a cytokine present in embryonic muscle, supports long-term survival of spinal motoneurons*. Neuron, 1996. **17**(1): p. 63-74.
144. Wright, C.R., et al., *G-CSF does not influence C2C12 myogenesis despite receptor expression in healthy and dystrophic skeletal muscle*. Front Physiol, 2014. **5**: p. 170.
145. Hara, M., et al., *G-CSF influences mouse skeletal muscle development and regeneration by stimulating myoblast proliferation*. J Exp Med., 2011. **208**(4): p. 715-27. Epub 2011 Mar 21.
146. Naito, T., et al., *Administration of granulocyte colony-stimulating factor facilitates the regenerative process of injured mice skeletal muscle via the activation of Akt/GSK3 α β signals*. European journal of applied physiology, 2009. **105**(4): p. 643-651.
147. Hayashiji, N., et al., *G-CSF supports long-term muscle regeneration in mouse models of muscular dystrophy*. Nature communications, 2015. **6**.
148. Gomez-Merino, D., et al., *Effects of chronic exercise on cytokine production in white adipose tissue and skeletal muscle of rats*. Cytokine, 2007. **40**(1): p. 23-29.
149. Romanazzo, S., et al., *IL-12 involvement in myogenic differentiation of C2C12 in vitro*. Biomaterials science, 2015. **3**(3): p. 469-479.
150. Jiang, L.Q., et al., *Autocrine role of interleukin-13 on skeletal muscle glucose metabolism in type 2 diabetic patients involves microRNA let-7*. Am J Physiol Endocrinol Metab, 2013. **305**(11): p. E1359-66.
151. Jacquemin, V., et al., *IL-13 mediates the recruitment of reserve cells for fusion during IGF-1-induced hypertrophy of human myotubes*. J Cell Sci., 2007. **120**(Pt 4): p. 670-81. Epub 2007 Jan 30.
152. Quinn, L.S., K.L. Haugk, and S.E. Damon, *Interleukin-15 stimulates C2 skeletal myoblast differentiation*. Biochemical and biophysical research communications, 1997. **239**(1): p. 6-10.
153. Quinn, L.S., K.L. Haugk, and K.H. Grabstein, *Interleukin-15: a novel anabolic cytokine for skeletal muscle*. Endocrinology, 1995. **136**(8): p. 3669-72.
154. Grabstein, K.H., et al., *Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor*. Science, 1994. **264**(5161): p. 965-8.
155. Xiao, F., et al., *Oncostatin M inhibits myoblast differentiation and regulates muscle regeneration*. Cell Res., 2011. **21**(2): p. 350-64.
156. Sampath, S.C., et al., *Induction of muscle stem cell quiescence by the secreted niche factor Oncostatin M*. Nat Commun, 2018. **9**(1): p. 1531.
157. Lu, H., et al., *Acute skeletal muscle injury: CCL2 expression by both monocytes and injured muscle is required for repair*. Faseb J., 2011. **25**(10): p. 3344-55.
158. Vasyutina, E., et al., *CXCR4 and Gab1 cooperate to control the development of migrating muscle progenitor cells*. Genes & Development, 2005. **19**(18): p. 2187-2198.
159. Bae, G.U., et al., *Regulation of myoblast motility and fusion by the CXCR4-associated sialomucin, CD164*. J Biol Chem., 2008. **283**(13): p. 8301-9. Epub 2008 Jan 27.
160. Polesskaya, A., et al., *Post-transcriptional modulation of interleukin 8 by CNOT6L regulates skeletal muscle differentiation*. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2016. **1863**(2): p. 263-270.
161. Vial, C., et al., *Skeletal muscle cells express the profibrotic cytokine connective tissue growth factor (CTGF/CCN2), which induces their dedifferentiation*. Journal of cellular physiology, 2008. **215**(2): p. 410-421.
162. Nishida, T., et al., *CCN family protein 2 (CCN2) promotes the early differentiation, but inhibits the terminal differentiation of skeletal myoblasts*. Journal of biochemistry, 2014: p. mvu056.

163. Calhabeu, F., et al., *NOV/CCN3 impairs muscle cell commitment and differentiation*. Experimental cell research, 2006. **312**(10): p. 1876-1889.
164. Otis, J.S., et al., *Pro-inflammatory mediation of myoblast proliferation*. PloS one, 2014. **9**(3): p. e92363.

CHAPTER 2. MUSCLE-DERIVED TRAIL NEGATIVELY REGULATES MYOGENIC DIFFERENTIATION²

2.1 Introduction

TRAIL, also known as TNFSF10 (tumor necrosis factor superfamily member 10) and Apo2L, was initially identified as a member of the TNF family with a capacity to induce apoptosis in various types of transformed cells [1, 2]. Since then, TRAIL has been widely studied in oncology because of its antitumor properties. For example, suppression of tumor growth by treatment with TRAIL has been reported from mouse xenograft models [3, 4]. Conversely, TRAIL knockout mice were more susceptible to transplanted lymphoma, as indicated by the knockout animals' lower survival rates than the wild-type counterparts due to tumor burden [5].

TRAIL is expressed as a type II transmembrane protein, which can be subsequently cleaved to release a soluble form that retains biological activities of full-length TRAIL. Both membrane-bound TRAIL and soluble TRAIL are present as homotrimers. In humans, five TRAIL receptors have been identified [6, 7]. TRAIL receptors 1-4 (TRAIL-R1-4) are type I transmembrane proteins. TRAIL-R1 (also known as DR4 and TNFRSF10A) and TRAIL-R2 (also known as DR5 and TNFRSF10B) can induce apoptosis through their cytoplasmic death domain [8]. Thus, binding of TRAIL by TRAIL-R1 or TRAIL-R2 leads to formation of the death-inducing signaling complex (DISC) and triggers a caspase cascade, resulting in apoptosis [6]. TRAIL can also bind TRAIL-R3

² **This work was published as:** Dongwook Kim, Nilmani Singh, Rachel J. Waldemer-Streyer, Mee-Sup Yoon, and Jie Chen. *Muscle-derived TRAIL negatively regulates myogenic differentiation*. Experimental Cell Research, 2020. 394(1): p.112165 (PMC7434709).

(also known as DCR1 and TNFRSF10C) and TRAIL-R4 (also known as DCR2 and TNFRSF10D). However, due to the lack of a cytoplasmic death domain, these two receptors cannot further transduce apoptotic signals and they act as decoy receptors by competing for ligand binding [9]. Lastly, TRAIL can bind to a soluble decoy receptor, osteoprotegerin (OPG, also known as TNFRSF11B), which also functions as a decoy receptor for receptor activator of NF-kappaB ligand (RANKL) [10, 11]. In mice, TRAIL-R2 is the only apoptosis-related receptor displaying marked sequence homology with human TRAIL-R1 (43%) and TRAIL-R2 (49%) [12]. Two decoy receptors without signaling capacity, mDcTRAIL-R1 (or TNFRSF23) and mDcTRAIL-R2 (or TNFRSF22), have been identified in mice [13, 14]. In addition, mouse OPG is believed to also function as a soluble decoy receptor [6].

Aside from the pro-apoptotic signaling through DISC, TRAIL and its receptors can induce apoptosis-independent signaling [15]. It has been reported that TRAIL can activate NF- κ B, leading to proliferation of Jurkat cells [16] or migration and invasion of human cholangiocarcinoma (CCA) cells [17]. TRAIL also activates the Jun N-terminal kinase (JNK) and p38 Mitogen-Activated Protein Kinase (MAPK) pathways in HT1080 cells [15]. Moreover, it has been shown that another member of the MAPK family, ERK1/2 is activated by TRAIL, leading to human endothelial cell proliferation [18] or proliferation and migration of vascular smooth muscle cells [19].

TRAIL and TRAIL-R2 were reported to express in cultured mouse myoblasts, in which a recombinant TRAIL induced apoptosis through TRAIL-R2 [20]. TRAIL is also expressed in human myositis skeletal muscles, and it is implicated in apoptotic or autophagic cell death of the diseased muscles [21, 22]. Whether muscle-derived TRAIL plays a non-cell death role in myogenic differentiation had not been explored. In the current study, we find that TRAIL functions as a negative regulator of myogenic

differentiation, and that TRAIL signals through TRAIL-R2 and ERK to suppress cell cycle withdrawal of myoblasts.

2.2 Materials and Methods

2.2.1 Antibodies and other reagents

Anti-MHC (MF20, used at 1:500), anti-myogenin (F5D, 1:500) and Ad5-luciferase adenovirus were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health and maintained by the University of Iowa, Department of Biological Sciences. Anti-TRAIL (sc-7877, 1:500) for detection of human TRAIL was from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-TRAIL (PA5-47073, 1:500) for detection of mouse TRAIL, ELISA kit for detection of mouse TRAIL (EMTNFSF10), peroxidase-conjugated anti-goat IgG antibody (A27014, 1:1000), Alexa Fluor 594-conjugated goat anti rabbit secondary antibody (A-11072, 1:500) and U0126 were from Thermo Fisher Scientific (Waltham, MA, USA). Anti-tubulin (ab11304, 1:10000) and anti-p21 (ab109199, 1:500) were from Abcam (Cambridge, MA, USA). Anti-TRAIL-R2 (AF721, 1:1000) and human TRAIL recombinant protein (375-TEC) were from R&D Systems (Minneapolis, MN, USA). Anti-pERK (4370, 1:500), anti-ERK (4696, 1:1000) and anti-Ki-67 (12202, 1:400) were from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated anti-rabbit (115-036-003, 1:1000) and anti-mouse (111-036-003, 1:1000) IgG antibodies and FITC-conjugated anti-mouse IgG (115-096-003, 1:100) were from Jackson Immuno Research Laboratories (West Grove, PA, USA). 5-ethynyl-2'-deoxyuridine (EdU) was from Carbosynth Limited (Compton, UK). Fluorescein azide 5-isomer was from Lumiprobe (Hunt Valley, MD, USA). Cytosine β -D-arabinofuranoside hydrochloride (Ara-C), gelatin and collagen type I from calf skin were

from Sigma-Aldrich (St. Louis, MO, USA). Adenovirus expressing human full-length TRAIL (140841A) was from Applied Biological Materials (Richmond, BC, Canada). pCMV-untagged empty vector (CV011) and pCMV-untagged mouse full-length TRAIL (MG50166-UT) were from Sino Biological (Wayne, PA, USA).

2.2.2 Cell culture

C2C12 myoblasts were a gift from S. Kaufman at the University of Illinois and originally obtained from ATCC. Cells were maintained in DMEM containing 4.5 g/L glucose, 10% fetal bovine serum, and 1% penicillin-streptomycin at 37°C with 7.5% CO₂. To induce differentiation, cells were plated on tissue culture plates coated with 0.2% gelatin, grown to 100% confluence, and then cultured in differentiation medium (DMEM containing 2% horse serum). The cells were replenished with fresh differentiation medium daily for 3 days. Myoblasts were transfected by using TransIT-LT1 (Mirus, Madison, WI) according to the manufacturer's recommendations. Cells were selected in 0.5 mg/mL Hygromycin.

2.2.3 Mouse primary myoblast isolation and differentiation

Primary myoblasts were isolated from 2 to 5-day-old FVB mice as described previously [23], and cultured on 0.1 mg/mL collagen-coated tissue culture plates. Differentiation was induced at 50-70% cell density in differentiation medium for 2 days.

2.2.4 Immunofluorescence microscopy and quantitative analysis of myocytes in vitro

C2C12 cells and primary myoblasts differentiated in 12-well plates were fixed with 3.7% formaldehyde and incubated with anti-MHC, -Ki-67 or -myogenin antibody, followed by incubation with DAPI and FITC- or Alexa-conjugated secondary antibody. Cells were observed with a Leica DMI 4000B fluorescence microscope (Leica, Wetzlar, Germany).

The fluorescence images were captured using a RETIGA EXi camera (QImaging, Surry, BC, Canada) and Image Pro Express software (Media Cybernetics, Rockville, MD, USA). Images were analyzed using ImageJ software (NIH, Bethesda, MD, USA). The differentiation index or fusion index was calculated as the percentage of nuclei in MHC-positive cells or in MHC-positive cells with more than one nucleus, respectively. The percentage of Ki-67- or myogenin-positive cells was calculated by taking DAPI signals as the total number of cells. Each data point was generated from quantifying five randomly chosen microscopic fields.

2.2.5 Lentivirus-delivered RNA interference

shRNAs in the pLKO.1-puro vector were purchased from Sigma-Aldrich (MISSION TRC). Clone IDs are: shTRAIL#1, TRCN0000066235; shTRAIL#2, TRCN0000066236; shTRAIL-R2#1, TRCN0000012323; shTRAIL-R2#2, TRCN0000012327. A hairpin of scrambled sequence (shScramble) used for a negative control and lentivirus packaging were previously described [24]. C2C12 cells were transduced with lentiviruses in growth medium with 8 µg/mL polybrene and selected in 3 µg/mL puromycin for 2 days, followed by plating into 12-well plates to induce differentiation. For transduction of primary myoblasts, polybrene and puromycin selection were omitted to preserve cell viability.

2.2.6 Quantitative RT-PCR

C2C12 cells or isolated muscle tissues were lysed in Trizol (Invitrogen, Carlsbad, CA, USA), and RNA was isolated following the manufacturer's protocol. cDNA was synthesized from 1 µg RNA using the qScript™ cDNA Synthesis kit (Quantabio, Beverly, MA, USA) following the manufacturer's protocol. Quantitative PCR was performed with a StepOne Plus (Applied Biosystems, Foster City, CA, USA) using gene-specific primers. β -actin or Hprt was used as a reference to obtain the relative fold change for target samples

using the comparative C_T method. Mouse β -actin primers: forward 5'-ttgctgacaggatgcagaag-3'; reverse 5'-atccacatctgctggaagg-3'. Mouse Hprt primers: forward 5'-atggactgattatggacaggactg-3'; reverse 5'-tccagcaggtcagcaaagaac-3'. Mouse TRAIL primers: forward 5'-tccaatctccaaggatggaaaga-3'; reverse 5'-gatgtaatacaggccctcctgctc-3'.

2.2.7 Measurement of secreted TRAIL

Media were collected from differentiating C2C12 cultures, and the TRAIL amount was measured using TRAIL Mouse ELISA Kit (Thermo Fisher Scientific) following the manufacturer's protocol.

2.2.8 Western blotting

Cells were lysed in SDS sample buffer with 5% β -mercaptoethanol. Proteins were resolved on SDS-PAGE, transferred onto polyvinylidene fluoride membrane (EMD Millipore, Darmstadt, Germany) and incubated with various antibodies in accordance with manufacturers' recommendations. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA), and images were captured on an iBright CL1000 imaging system (Thermo Fisher Scientific, Waltham, MA, USA). Quantification of western blot band intensities was performed with densitometry of images using ImageJ software (NIH, Bethesda, MD, USA).

2.2.9 Cell proliferation and apoptosis assays

To measure proliferation of C2C12 cells, EdU labelling was performed as previously described [24]. To examine apoptosis, TUNEL assays were performed according to manufacturer's protocol (Promega, Madison, WI, USA).

2.2.10 Injury-induced muscle regeneration and intramuscular TRAIL knockdown in mice

Male (D7AI and D14AI) or female (D3AI) C57BL/6 wild-type mice aged 10-12 weeks were used in all the regeneration experiments. Animals were randomly allocated to the different experimental groups. Muscle injury was induced by injection of 50 μ L of 1.2% (w/v) BaCl₂ dissolved in saline into mouse TA muscles as previously described [25]. To knockdown TRAIL, lentivirus expressing shTRAIL (and shScramble as a negative control) was concentrated to $1 \times 10^7 - 1 \times 10^8$ IU/mL via ultracentrifugation and co-injected with BaCl₂ into mouse TA muscles. The injured muscles were collected at 3, 7 or 14 days after injury and subjected to RNA isolation or cryosection and staining.

2.2.11 Muscle tissue cryosection, hematoxylin and eosin staining, and immunohistochemistry

TA muscles were isolated and frozen in liquid nitrogen-cooled 2-methylbutane and embedded in TBS tissue freezing medium (Thermo Fisher Scientific, Waltham, MA, USA). Sections of 10 μ m thickness were obtained with a Microm HM550 (Thermo Fisher Scientific, Waltham, MA, USA) at -20°C , placed on uncoated slides, and stained with hematoxylin and eosin (H&E). Separately, the sections from D3AI were fixed by 1.5% paraformaldehyde, incubated with anti-Ki-67 antibody, followed by incubation with DAPI and Alexa-conjugated secondary antibody. Images were obtained by DMI 4000B fluorescence microscope (Leica, Wetzlar, Germany) with a 20X dry objective (Fluotar, numerical aperture 0.4; Leica). Five bright field or fluorescence images were randomly captured using RETIGA EXi camera (QImaging, Surrey, BC, Canada) and Image Pro Express software (Media Cybernetics, Rockville, MD, USA). The images were analyzed for cross-sectional area of all centrally nucleated regenerating myofibers (H&E staining) or

counting Ki-67-positive nuclei using ImageJ. All procedures were performed by an investigator that were blinded to sample identification.

2.2.12 Statistical analysis

All the data shown are representative results of at least three independent experiments with cells, or 5-8 mice for each data point in animal experiments. The exact sample size for each experiment is described in figure legends. All the quantitative values are presented as mean $\pm$ SEM. Whenever necessary, statistical significance of the data was analyzed by performing two-tailed paired Student's *t*-tests. The specific types of test and the *P*-values, when applicable, are indicated in the figure legends. Statistical comparison was considered significant when $P < 0.05$. All statistical analysis was performed using Excel.

2.2.13 Study approval

All the animal experiments in this study followed protocols approved by the Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

2.3 Results

2.3.1 TRAIL is a negative regulator of myoblast differentiation

Since the expression of TRAIL had been reported in myoblasts [20], we wondered whether its expression level changed during myogenesis. C2C12 mouse myoblasts were induced to differentiate by serum deprivation for up to 72 hrs. We observed that the level of TRAIL mRNA increased by ~3-fold at 24 hrs of differentiation and returned to pre-differentiation level by 72 hrs (Figure 2.1 A). The secreted TRAIL protein level increased

by ~2-fold at 24 hrs and this level was largely maintained throughout differentiation (Figure 2.1 B).

To examine a potential role of endogenous TRAIL in myogenesis, we delivered shRNAs by lentiviral transduction into C2C12 myoblasts. Two independent shRNAs (shTRAIL) knocked down TRAIL mRNA by ~50% and ~70% compared to a negative control shRNA with a scrambled hairpin sequence (shScramble) (Figure 2.1 C). Increased differentiation was observed in correlation with the knockdown, as quantified by differentiation index (% of myosin heavy chain (MHC)-positive cells) and fusion index (% of MHC-positive cells with two or more nuclei) (Figure 2.1 D). Knockdown of TRAIL in mouse primary myoblasts also resulted in statistically significant increase in fusion index (Figure 2.1 E). However, unlike C2C12 myoblasts, which mimic satellite cells in vivo and maintain their stemness by limiting the degree of differentiation [26], primary myoblasts achieved a differentiation index of near 100%, precluding any further increase by TRAIL knockdown (Figure 2.1 E).

Expression of the early myogenic marker myogenin was found to be up-regulated upon TRAIL knockdown in C2C12 cells from 0 to 48 hrs of differentiation, and the late myogenic marker MHC was elevated by the knockdown at 48 and 72 hrs (Figure 2.1 F). These data suggest that TRAIL may have a negative role in myogenic differentiation.

Consistent with a negative function of TRAIL, overexpression of TRAIL from a cDNA construct inhibited differentiation of C2C12 cells treated with the shScramble virus (Figure 2.2 A). In addition, the effect of TRAIL knockdown was reversed by TRAIL overexpression (Figure 2.2 A), validating the shRNA to be on-target. Endogenous TRAIL was not detected by western blotting of the cell lysates (Figure 2.2 A) although it was detected in the medium by ELISA (Figure 2.1 B). Similar to overexpression of TRAIL, a soluble recombinant TRAIL (sTRAIL) added to the cell medium also inhibited myoblast

differentiation and countered the effect of TRAIL knockdown (Figure 2.2 B). Taken together, our observations suggest that TRAIL is likely a muscle cell-derived negative regulator of myogenic differentiation.

2.3.2 TRAIL-R2, the receptor of TRAIL, negatively regulates myoblast differentiation

Next, we wished to test whether the myogenic function of TRAIL was mediated by TRAIL-R2, the only known functional receptor for TRAIL in mice [12]. With lentivirus-delivered shRNAs we knocked down the TRAIL-R2 protein in C2C12 cells by 50-70% (Figure 2.3 A) and observed that, similar to TRAIL knockdown, depletion of TRAIL-R2 increased both differentiation and fusion indices (Figure 2.3 B). Myogenin expression was upregulated by TRAIL-R2 knockdown during the early phase of differentiation (0-24 hrs), whereas elevated MHC expression was observed later through 72 hrs (Figure 2.3 C). Interestingly, the protein level of TRAIL-R2 decreased during differentiation (see shScramble in Figure 2.3 C), in contrast to the increase of TRAIL (Figure 2.1 A&2.1 B). Importantly, upon TRAIL-R2 knockdown, TRAIL overexpression no longer had an effect on myoblast differentiation (Figure 2.3 D). These results are consistent with the notion that TRAIL functions through TRAIL-R2 to negatively regulate myogenic differentiation.

2.3.3 TRAIL inhibits myoblast differentiation through impeding cell cycle withdrawal

We set out to investigate the mechanism by which TRAIL/TRAIL-R2 regulates myogenic differentiation. In the early stage of myogenesis, proliferating myoblasts exit the cell cycle, which is a requisite step prior to myoblast fusion into myotubes. Since TRAIL had been reported to promote proliferation of other types of cells [18, 19], we wondered whether it might impact cell cycle withdrawal of myoblasts. Knockdown of TRAIL decreased the number of EdU-labeled C2C12 cells at the onset of differentiation (Figure 2.4 A), indicating a reduced rate of proliferation. TRAIL knockdown in primary myoblasts

at the time of differentiation also led to a modest but statistically significant decrease in EdU-labeled cells (Figure 2.4 B). Conversely, overexpression of TRAIL (Figure 2.4 C), as well as addition of sTRAIL in cell medium (Figure 2.4 D), increased the number of EdU-labeled C2C12 cells. Interestingly, neither the effect of TRAIL knockdown (Figure 2.5.4E) nor that of sTRAIL addition (Figure 2.4 F) on EdU-labeling was observed when C2C12 cells were actively proliferating, suggesting that TRAIL may impact cell cycle specifically at the time of differentiation.

As a marker of cell proliferation, Ki-67 was also examined in C2C12 cells by immunostaining. We found a reduced number of Ki-67-positive cells upon TRAIL knockdown at the onset of differentiation (Figure 2.4 G), which was accompanied by an increased number of C2C12 cells expressing the early myogenic marker myogenin (Figure 2.4 H).

Knockdown of the receptor TRAIL-R2 in C2C12 had an effect mirroring that of TRAIL knockdown: decreased numbers of EdU-positive (Figure 2.4 I) and Ki-67-positive cells (Figure 2.4 J), and an increased number of myogenin-positive cells (Figure 2.4 K). Taken together, the observations thus far suggest that muscle-derived TRAIL may signal to suppress cell cycle withdrawal that is necessary for myogenic differentiation.

It has been well established that p21^{CIP}, a cell cycle inhibitor, plays a critical role in myogenic differentiation by promoting cell cycle withdrawal [27, 28]. We observed that p21 expression was upregulated when TRAIL (Figure 2.5 A) or TRAIL-R2 (Figure 2.5 B) was knocked down in C2C12 cells. TRAIL knockdown in primary myoblasts also resulted in a modest but statistically significant increase of p21 expression (Figure 2.5 C). Conversely, TRAIL overexpression in C2C12 cells decreased p21 expression, and reversed the effect of TRAIL knockdown on p21 upregulation (Figure 2.5 D). Adding sTRAIL to C2C12 cells revealed an acute (and thus potentially direct) effect – p21 expression was reduced as

early as 30 min after sTRAIL addition (Figure 2.5 E). These observations are consistent with p21, and subsequently cell cycle withdrawal, being a mediator of the myogenic effects of TRAIL.

To directly probe a causal role of cell cycle withdrawal in the regulation of myogenesis by TRAIL, we treated C2C12 cells with the cell cycle inhibitor Ara-C. As shown in Figure 2.5 F, Ara-C treatment rescued differentiation from the inhibition by overexpression of TRAIL, as reflected in both differentiation and fusion indices. Collectively, our data suggest that TRAIL inhibits myogenic differentiation by preventing myoblasts from exiting the cell cycle.

2.3.4 TRAIL depletion enhances muscle regeneration in vivo

To probe a physiological relevance of the myogenic function of TRAIL discovered above in cell culture, we turned to a well-established mouse model of injury-induced muscle regeneration. Barium chloride (BaCl_2) injection into the tibialis anterior (TA) muscle of the hindlimb leads to confined muscle necrosis, which is followed by de novo regeneration [25, 29]. Here, we co-injected lentivirus expressing TRAIL shRNA with BaCl_2 into TA muscles and isolated the injected muscles for analysis on various days after injury (AI). Knockdown of TRAIL was confirmed by analyzing the mRNA isolated from injected muscles on Day 3 AI. Because lentivirus delivery was only expected in the injured part, which was typically 1/3 to 1/2 of the TA muscle, the observed knockdown efficiency of 20% in the entire TA was fully expected (Figure 2.6 A). Importantly, we observed statistically significant, ~25% increase in the average regenerating myofiber size (cross sectional area) upon TRAIL knockdown on both Day 7 and Day 14 AI (Figure 2.6 B), suggesting that endogenous TRAIL has a negative role in muscle regeneration.

To further test the possibility that the upregulated muscle regeneration upon TRAIL knockdown *in vivo* was due to enhanced cell cycle withdrawal as we observed *in vitro*, we probed Day 3 AI TA muscle sections for the proliferation marker Ki-67. As shown in Figure 2.6 C, TRAIL knockdown muscles had fewer Ki-67-positive cells, indicating a lower level of proliferation at the early stage of muscle regeneration. This observation is consistent with TRAIL preventing proliferating cells from exiting the cell cycle.

2.3.5 ERK mediates myogenic function of TRAIL

We next searched for a potential signaling mechanism that mediates the myogenic function of TRAIL. The MAP kinase ERK1/2, a well-known regulator of cell proliferation, suppresses differentiation, likely by preventing cell cycle withdrawal [30]. In addition, TRAIL has been reported to activate ERK in non-muscle cells [18, 31]. Therefore, we decided to test a potential role of ERK1/2 signaling in the regulation of myogenic differentiation by TRAIL. First, we treated myoblasts with recombinant TRAIL and examined phosphorylation of ERK1/2 at various time points. As shown in Figure 2.7 A, after 2, 4 and 6 hrs of TRAIL treatment, phosphorylation of ERK1/2 was found to increase modestly. Consistently, TRAIL knockdown in primary myoblasts led to a decrease in pERK (Figure 2.7 B). The specificity of the anti-pERK antibody was confirmed with lysates of serum-starved and serum-stimulated C2C12 cells (Figure 2.8).

To directly probe a functional relevance of ERK1/2 signaling in mediating TRAIL's effect, we utilized the highly specific MEK inhibitor U0126. Treatment by U0126 reversed the inhibitory effect of overexpressed TRAIL (Figure 2.7 C) and sTRAIL (Figure 2.7 D) on myoblast differentiation such that differentiation and fusion indices were restored to the control levels (Figure 2.7 E). In cells overexpressing TRAIL, the reduced p21 expression was restored by U0126 treatment to a level no longer significantly different from the control (without TRAIL overexpression), consistent with p21 being a mediator of the effect

of ERK signaling on cell cycle withdrawal. Finally, expression of the differentiation marker myogenin was also restored in TRAIL-overexpressing cells by U0126 (Figure 2.7 F). Taken together, our observations support the notion that TRAIL negatively regulates myogenic differentiation in an ERK1/2-dependent manner.

2.4 Discussion

In this Chapter, we have provided evidence for a role of TRAIL in negatively regulating skeletal myogenesis both in vitro and in vivo, a non-canonical function distinct from the widely studied pro-apoptotic signaling of TRAIL. Our data suggest that TRAIL exerts this myogenic function by suppressing cell cycle withdrawal necessary for myoblast differentiation. We further show that TRAIL-R2, the only known functional TRAIL receptor in mice, ERK signaling, and the cell cycle inhibitor p21 likely mediate TRAIL's myogenic function (see Figure 2.7 G for a proposed model). Previously TRAIL-R2 (DR5) was reported to have a *positive* role in myoblast differentiation through the FADD/Caspase pathway that regulates MyoD expression [32]. This discrepancy with our findings could be due to the different myoblast cell lines utilized – 23A2 in that study and C2C12 in ours. It is important to note that our in vitro findings have been recapitulated in vivo by our observations in a muscle regeneration model.

It was reported that a soluble TRAIL induced apoptosis in murine myoblasts [20]. However, we did not observe any change in apoptosis of C2C12 cells upon either TRAIL knockdown (Figure 2.9 A) or recombinant sTRAIL addition at the concentration (100 ng/mL) found to inhibit differentiation (Figure 2.9 B). In the aforementioned study induction of apoptosis was observed in 23A2 myoblasts treated with a soluble TRAIL at 0.5-2 µg/mL, and indeed we found that apoptosis was induced in C2C12 cells by TRAIL at

those high concentrations (Figure 2.9 B). Therefore, it is likely that TRAIL has dose-dependent dual effects on skeletal muscle cells – at low doses it suppresses myogenic differentiation, and it can induce apoptosis at high concentrations. Dose-dependent pleiotropic functions have been reported for other TNF superfamily members. For instance, in primary culture of Schwann cells, treatment with low dosages (1-2 ng/mL) of TNF- α stimulated cell proliferation, whereas higher dosages (10-40 ng/mL) induced apoptosis [33]. Similarly, low dosages (0.156-1.56 ng/mL) of Fas ligand (FasL, also known as CD95 and Apo-1L) stimulated proliferation of brain endothelial cells, and higher dosages (100-1000 ng/mL) induced apoptosis [34]. Hence, it appears to be a common theme that TNF factors promote cell proliferation and induce cell death at low and high dosages, respectively. However, our data show that TRAIL does not affect the proliferation of myoblasts per se; only when myoblasts are poised for differentiation does TRAIL prevent their withdrawal from the cell cycle.

We observed enhanced muscle regeneration in mice that received intramuscular delivery of TRAIL shRNA. However, neither TRAIL^{-/-} mice [35] nor TRAIL-R2^{-/-} mice [36] displayed any developmental phenotype, although they have not been studied in any context of adult skeletal muscle functions. Compensatory effects could take place in the knockout mice, given the known redundancy of functions of TNF superfamily members. Alternatively, it is possible that TRAIL has a specific function in adult muscle regeneration that is distinct from embryonic muscle development. It also cannot be ruled out that the knockdown phenotype is dependent on the residual TRAIL, which is absent upon knockout.

It is important to acknowledge that our intramuscular RNAi delivery method would result in the knockdown of TRAIL in both muscle cells and infiltrating immune cells (macrophages, in particular). Hence, we are not able to conclude that it is the muscle-

derived TRAIL that acts as a brake on muscle regeneration. In fact, there is a general paucity of experimental evidence in the literature that definitively proves the function of muscle-secreted cytokines (or myokines) on myogenesis *in vivo*. Studies with muscle-specific manipulation of endogenous gene expression would be necessary to establish the physiological relevance of those muscle-derived cytokines, including TRAIL.

TRAIL has long been studied as a candidate anti-cancer agent [6]. Our current findings implicate TRAIL in a potentially new therapeutic strategy in muscle disease, regardless of the relevant source of TRAIL (muscle versus infiltrating immune cells). In a recent study, injection of an anti-TRAIL antibody resulted in improved strength of dystrophic EDL muscles in *mdx* mice, a model for Duchenne muscular dystrophy (DMD) [37]. Although this effect was postulated to be due to suppressed apoptosis, in light of our results a role of the anti-TRAIL antibody on myogenic differentiation in the regeneration of dystrophic muscles is also possible. Indeed, DMD muscles contain elevated numbers of satellite cells, which fail to enter the myogenic program, possibly due to prolonged cell division [38]. TRAIL may contribute to preventing the satellite cells from exiting the cell cycle.

Our previous RNAi screen identified a number of cytokines as candidates of autocrine regulators of C2C12 myoblast differentiation [39]. Two of them, the chemokine C-X-C motif ligand 14 (Cxcl14) and Fms-like tyrosine kinase 3 ligand (Flt3L) have been found to regulate myogenic differentiation via suppressing and promoting cell cycle withdrawal, respectively, both mediated by ERK signaling and expression of the cell cycle inhibitor p21 [40, 41]. Additional muscle-derived cytokines with a similar function and mechanism are likely to continue to emerge. It is reasonable to speculate that nature has devised a cytokine network to ensure fail-proof and/or finely-tuned control over a point of

no return in the myogenic process such as exiting the cell cycle. Future investigation of these cytokines at the systems level is warranted.

2.5 Figures

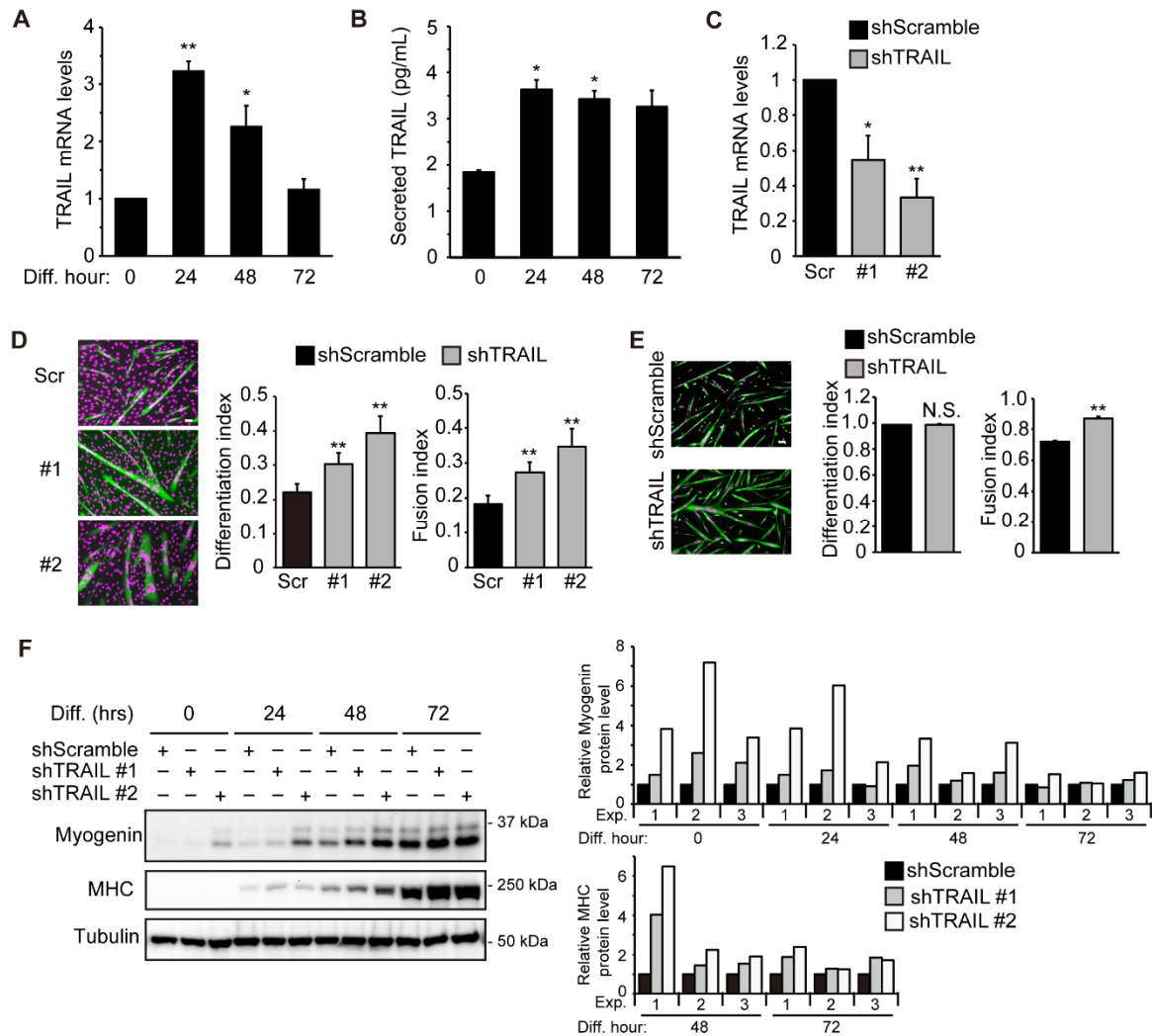


Figure 2.1 Depletion of TRAIL promotes myoblast differentiation.

(A) RNA was isolated from differentiating C2C12 cells at different time points followed by qRT-PCR analysis (n=4).

(B) Culture medium during C2C12 differentiation was collected at different time points and ELISA assay was performed to determine secreted TRAIL levels (n=3).

(C) C2C12 cells were transduced with lentiviruses expressing shTRAIL or shScramble (Scr) followed by 2 days of puromycin selection. Cell lysates were then collected and subjected to qRT-PCR analysis (n=5).

(D) Cells treated as in (C) were induced to differentiate for 72 hrs, followed by staining for MHC (green) and DAPI (magenta), and differentiation and fusion indices were quantified (n=7).

Figure 2.1 (cont.)

(E) Mouse primary myoblasts were transduced with lentiviruses expressing shRNAs overnight, cultured in growth medium for 1 day, and then induced to differentiate for 48 hrs, followed by staining for MHC (green) and DAPI (magenta). Differentiation and fusion indices were quantified (n=3).

(F) C2C12 cells treated as in (C) were induced to differentiate, and at different time points, cells were lysed and subjected to western analysis. Representative blots are shown. Results of quantification by densitometry are shown for 3 independent experiments. The MHC and myogenin protein levels were determined relative to the level of tubulin. Data were normalized to shScramble at each differentiation time point. When applicable, data are presented as mean with error bars representing SEM. Two-tailed *t*-test was performed to compare each data to control. **P* < 0.05; ***P* < 0.01. NS, not significant. Scale bars: 50 μ m.

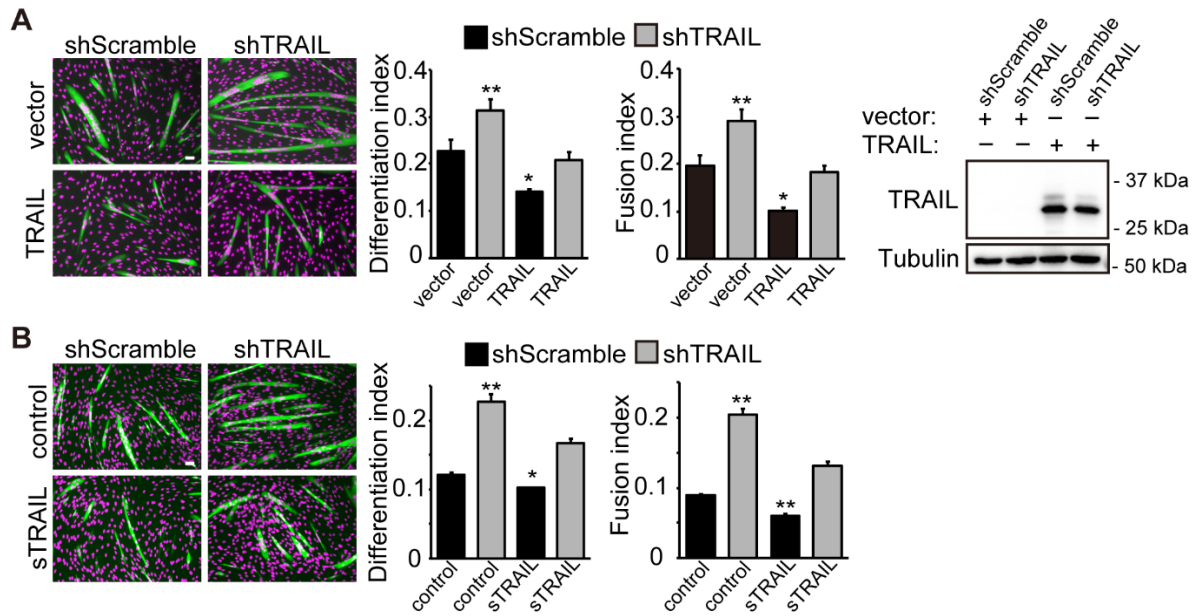


Figure 2.2 Exogenous TRAIL inhibits myoblast differentiation.

(A) C2C12 cells were transduced with lentiviruses expressing shTRAIL or shScramble followed by 2 days of puromycin selection. Cells were then transfected with a mouse full-length TRAIL expression construct (TRAIL) or the corresponding empty vector (vector), selected with hygromycin, and differentiated for 72 hrs, followed by staining for MHC (green) and DAPI (magenta). Differentiation and fusion indices were quantified ($n=4$). Overexpression of TRAIL was confirmed by western blotting.

(B) Cells treated as in (A) were cultured with 100 ng/mL sTRAIL or vehicle (control) for 6 to 9 hrs and then induced to differentiate for 72 hrs with sTRAIL present for the first 24 hrs, followed by staining for MHC (green) and DAPI (magenta). Differentiation and fusion indices were quantified ($n=5$). All error bars represent SEM. Two-tailed t -test was performed to compare each data to control (first bar in each graph). * $P < 0.05$; ** $P < 0.01$.

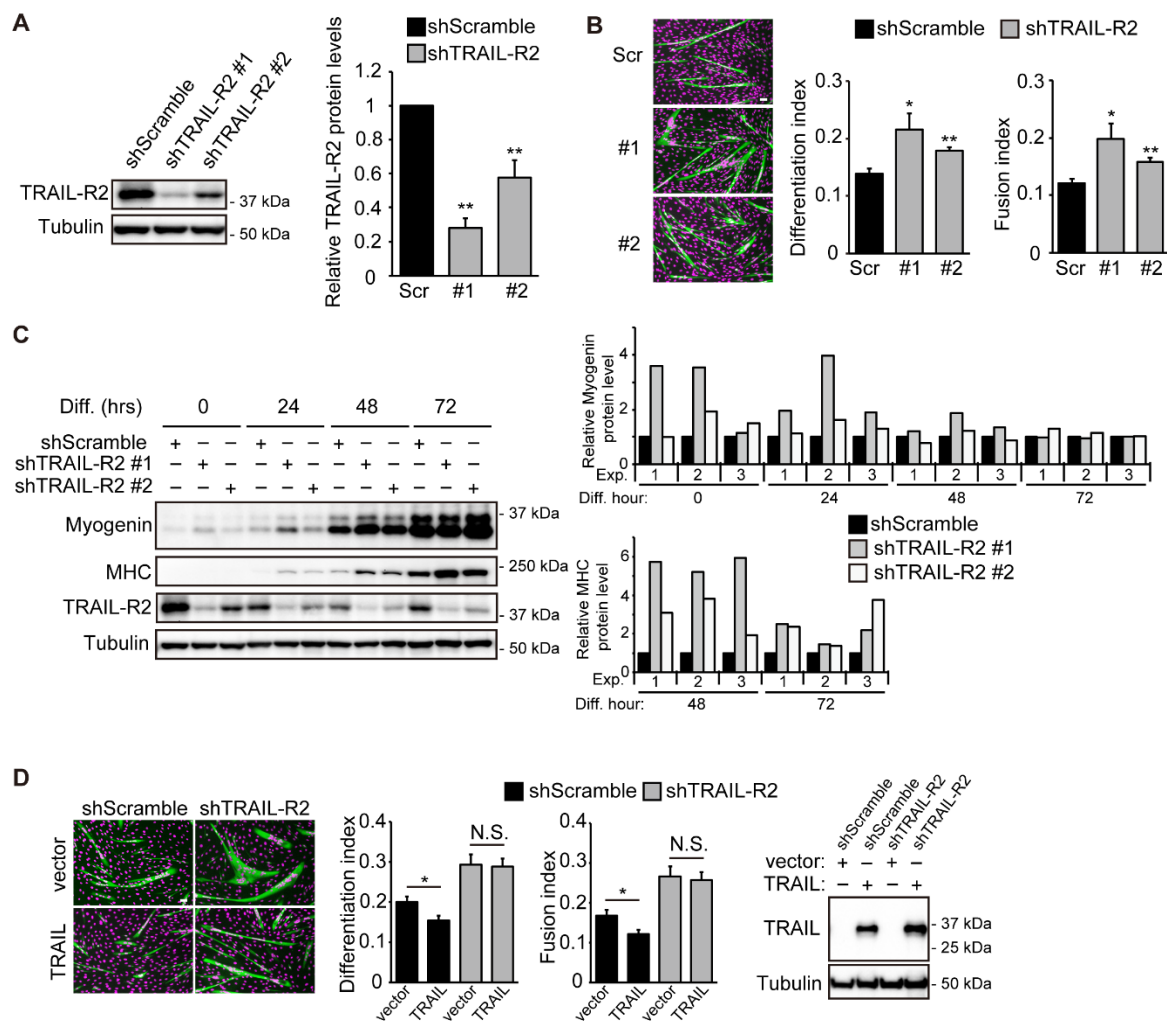


Figure 2.3 Depletion of TRAIL-R2 promotes myoblast differentiation.

(A) C2C12 cells were transduced with lentiviruses expressing shTRAIL-R2 or shScramble followed by 2 days of puromycin selection. Cell lysates were then collected and subjected to western analysis. Representative blots and results of quantification are shown for 5 independent experiments.

(B) Cells treated as in (A) were induced to differentiate for 72 hrs, followed by staining for MHC (green) and DAPI (magenta), and differentiation and fusion indices were quantified (n=5).

(C) Cells treated as in (A) were induced to differentiate, and at different time points, cells were lysed and subjected to western analysis. Representative blots and results of quantification are shown for 3 independent experiments.

(D) Cells treated as in (A) were transfected with TRAIL or empty vector, selected with hygromycin, and differentiated for 72 hrs, followed by staining for MHC (green) and DAPI (magenta). Differentiation and fusion indices were quantified (n=4). Expression of TRAIL

Figure 2.3 (cont.)

was confirmed by western blotting. For (A) and (C), quantification of western blots was performed by densitometry. The level of each specific protein was determined relative to the level of tubulin, and the data were normalized to shScramble at each time point. When applicable, data are presented as mean with error bars representing SEM. Two-tailed *t*-test was performed to compare each data to control. **P* < 0.05; ***P* < 0.01. NS, not significant. Scale bars: 50 μm.

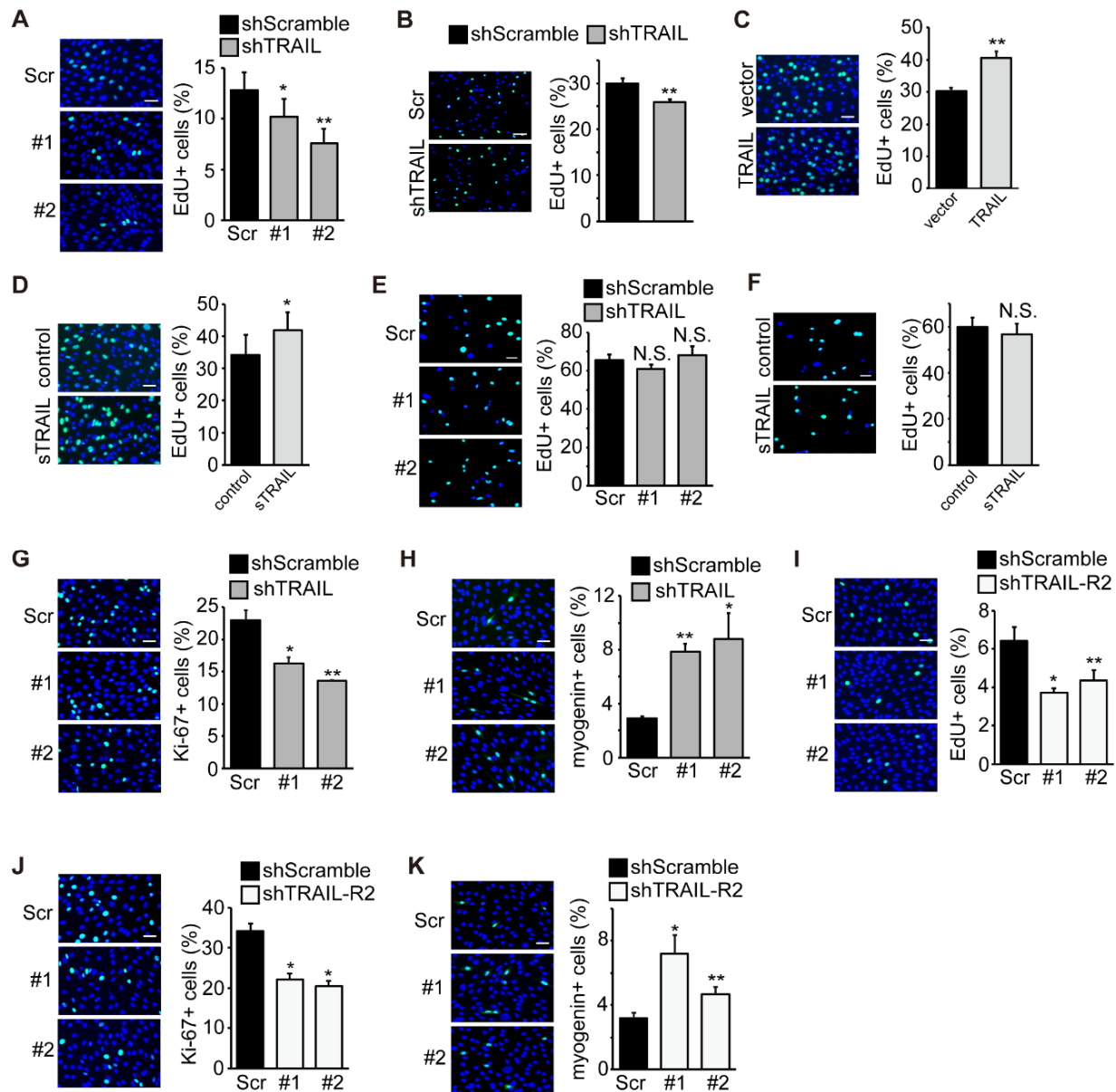


Figure 2.4 TRAIL and TRAIL-R2 promote myoblast proliferation at the onset of differentiation.

(A) C2C12 cells were transduced with lentiviruses expressing shTRAIL or shScramble followed by 2 days of puromycin selection. At 24 hr of differentiation, cells were labeled with EdU for 2 hrs in differentiation medium, stained for EdU (green) and DAPI (blue), and the percentage of cells positive for EdU staining was quantified (n=6).

(B) Mouse primary myoblasts were transduced with lentiviruses expressing shRNAs overnight, cultured in growth medium for 1 day, and then labeled with EdU for 2 hrs in differentiation medium at 0 hr of differentiation and processed as in (A) (n=5).

Figure 2.4 (cont.)

(C) C2C12 cells were transfected with TRAIL or empty vector and selected with hygromycin for 2 days. At 0 hr of differentiation, cells were labeled with EdU for 2 hrs in differentiation medium, and processed as in (A) (n=5).

(D) C2C12 cells were grown in the presence of vehicle (Control) or 100 ng/mL sTRAIL for 24 hrs. At 0 hr of differentiation, cells were labeled with EdU for 2 hrs in differentiation medium, and processed as in (A) (n=6).

(E) C2C12 cells were transduced with lentiviruses expressing shTRAIL or shScramble followed by 2 days of puromycin selection. Cells were then grown to ~20% confluence, labeled with EdU for 2 hrs in growth medium, and processed as in (A) (n=5).

(F) C2C12 cells were grown to ~20% in the presence of vehicle (Control) or 100 ng/mL sTRAIL for 24 hrs, labeled with EdU for 2 hrs in growth medium, and processed as in (A) (n=4).

(G) C2C12 cells treated as in (A) were stained for Ki-67 (green) and DAPI (blue), and the percentage of cells positive for Ki-67 staining was quantified (n=4).

(H) C2C12 cells treated as in (A) were stained for myogenin (green) and DAPI (blue), and the percentage of cells positive for myogenin staining was quantified (n=4).

(I) C2C12 cells were transduced with lentiviruses expressing shTRAIL-R2 or shScramble followed by 2 days of puromycin selection. At 24 hr of differentiation, cells were labeled with EdU for 2 hrs in differentiation medium and processed as in (A) (n=5).

(J) C2C12 cells treated as in (I) were stained for Ki-67 (green) and DAPI (blue), and the percentage of cells positive for Ki-67 staining was quantified (n=4).

(K) C2C12 cells treated as in (I) were stained for myogenin (green) and DAPI (blue), and the percentage of cells positive for myogenin staining was quantified (n=4). All error bars represent SEM. Two-tailed *t*-test was performed to compare each data to control. **P* < 0.05; ***P* < 0.01. NS, not significant. Scale bars: 50 μ m.

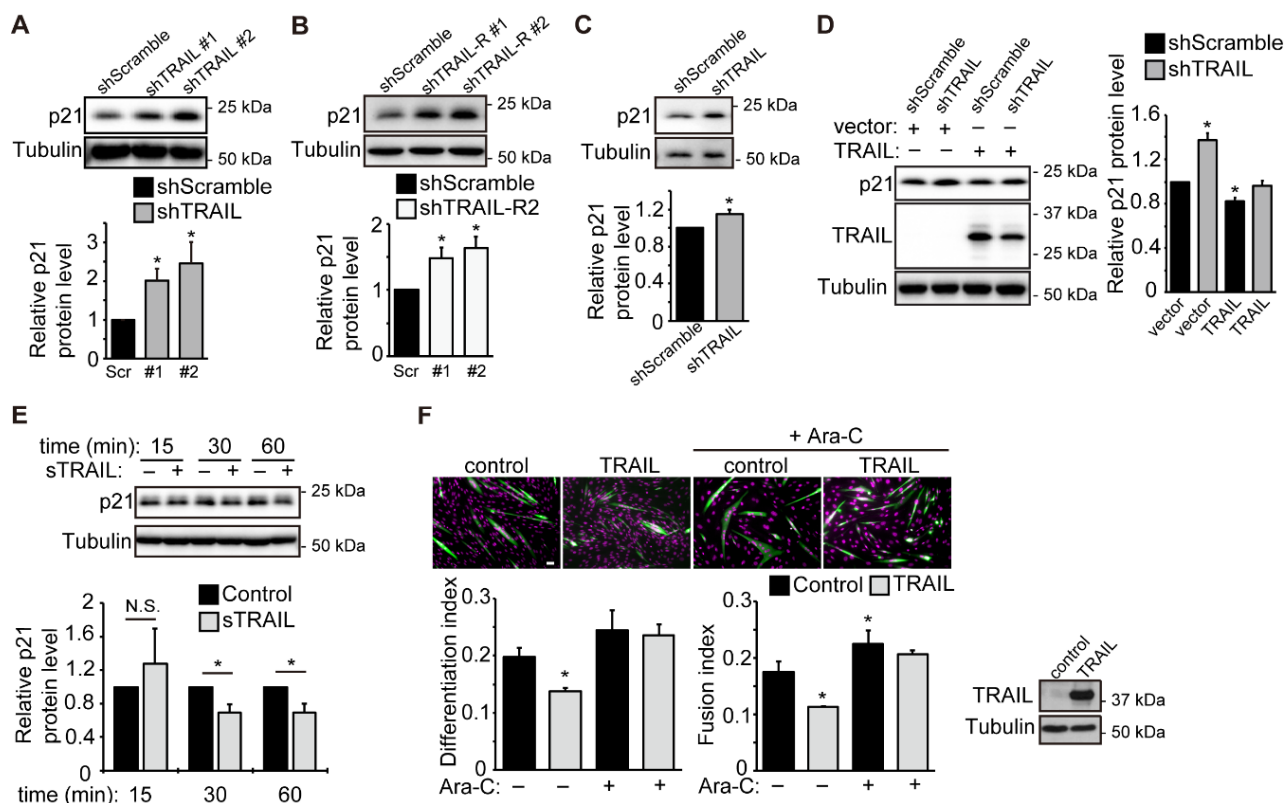


Figure 2.5 TRAIL inhibits p21 expression.

(A) C2C12 cells were transduced with lentiviruses expressing shTRAIL or shScramble followed by 2 days of puromycin selection. Cells were then lysed at 0 hr of differentiation followed by western analysis (n=6).

(B) C2C12 cells were transduced with lentiviruses expressing shTRAIL-R2 or shScramble followed by 2 days of puromycin selection. Cells were then lysed at 0 hr of differentiation and subjected to western analysis (n=7).

(C) Mouse primary myoblasts were transduced with lentiviruses expressing shRNAs overnight, cultured in growth medium for 1 day, and then lysed at 0 hr of differentiation followed by western analysis (n=4).

(D) C2C12 cells treated as in (A) were transfected with TRAIL or empty vector, selected with hygromycin and lysed at 0 hr of differentiation, followed by western analysis (n=4).

(E) C2C12 cells were induced to differentiate for 6 to 9 hrs, and then treated with vehicle or 100 ng/mL sTRAIL for 15, 30 or 60 min, followed by cell lysis and western analysis (n=5).

(F) C2C12 cells were transduced with adenoviruses expressing human full-length TRAIL or luciferase (Control), followed by differentiation for 72 hrs with or without 2 μ M Ara-C during the first 24 hr of differentiation. Cells were stained for MHC (green) and DAPI (magenta), and differentiation and fusion indices were quantified (n=3). Expression of TRAIL was confirmed by western blotting. Quantification of p21 protein was performed by densitometry analysis of western blots. The level of p21 protein was determined relative to the level of tubulin, and the data were always normalized to control at each time point. All

Figure 2.5 (cont.)

error bars represent SEM. For (F), one-tailed t -test was performed to compare each data point to control (without Ara-C). For all other panels, two-tailed t -test was performed to compare each data to control (first bar in graph unless otherwise noted). * $P < 0.05$; ** $P < 0.01$. NS, not significant. Scale bar: 50 μm .

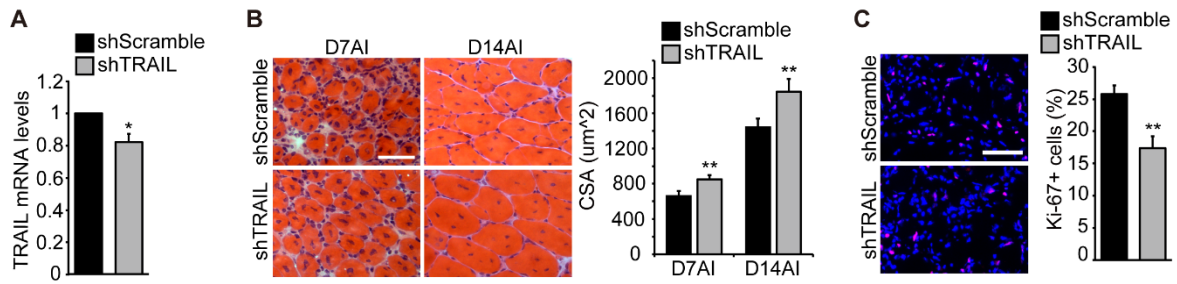


Figure 2.6 TRAIL knockdown enhances muscle regeneration post-injury.

(A) Mouse TA muscles were co-injected with BaCl₂ and lentiviruses expressing shTRAIL or shScramble, isolated on Day 3 AI and subjected to RNA isolation and qRT-PCR (n=5).

(B) TA muscles injected as in (A) were isolated on Day 7 or Day 14 AI and subjected to cryosection. Upon H&E staining, cross-sectional area (CSA) was quantified for regenerating myofibers (n=6 for D7AI, n=8 for D14AI).

(C) TA muscles injected as in (A) were isolated on Day 3 AI, cryosectioned and immunostained for Ki-67 (red) along with DAPI (blue). The percentage of Ki-67 positive cells was quantified (n=5). All error bars represent SEM. Two-tailed *t*-test was performed to compare shTRAIL to shScramble. **P* < 0.05; ***P* < 0.01. Scale bars: 50 μm.

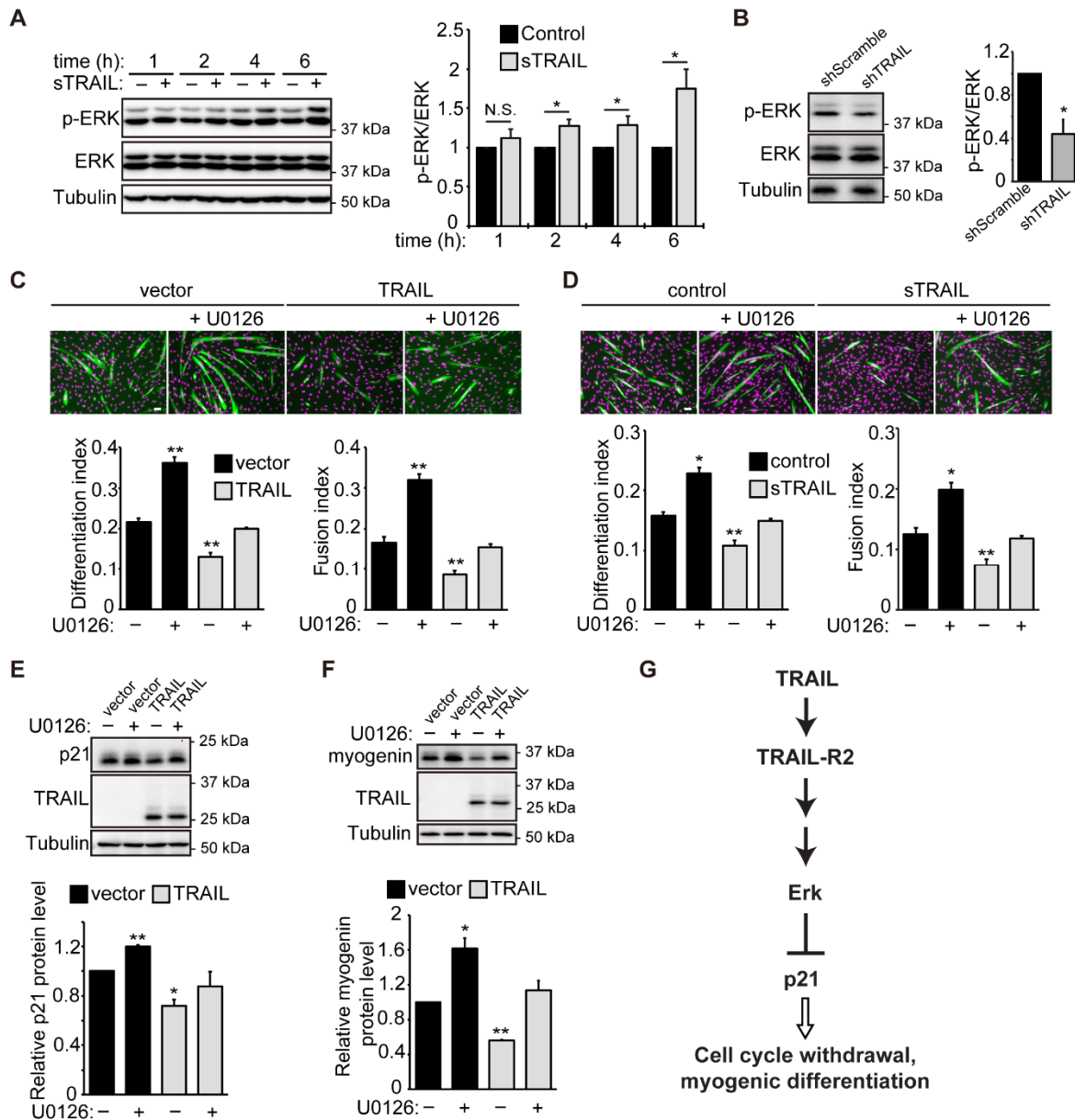


Figure 2.7 TRAIL inhibits differentiation through ERK signaling.

(A) C2C12 cells were induced to differentiation for 6 to 9 hrs, and then treated with 100 ng/mL sTRAIL or vehicle (control). Cells were lysed at 1, 2, 4 or 6 hrs, followed by western analysis (n=5).

(B) Mouse primary myoblasts were transduced with lentiviruses expressing shRNAs overnight, cultured in growth medium for 1 day, and then induced to differentiate for 24 hrs, followed by lysis and western analysis (n=4).

(C) C2C12 cells were transfected with TRAIL or empty vector, selected with hygromycin, and induced to differentiate for 72 hrs in the presence or absence of U0126 (3 μ M) for the first 24 hrs of differentiation. Cells were then stained for MHC (green) and DAPI (magenta). Differentiation and fusion indices were quantified (n=4).

Figure 2.7 (cont.)

(D) C2C12 cells were grown with 100 ng/mL sTRAIL or vehicle (Control) for 6 to 9 hrs and induced to differentiate for 72 hrs in the presence or absence of sTRAIL for the first 24 hrs of differentiation. U0126 (3 μ M) was also added for the first 24 hrs of differentiation. Cells were then stained for MHC (green) and DAPI (magenta). Differentiation and fusion indices were quantified (n=3).

(E) Cells treated as in (C) were induced to differentiate for 6 hrs in the presence or absence of U0126 (3 μ M). Cells were then lysed and subjected to western analysis (n=4).

(F) Cells treated as in (C) were induced to differentiate for 48 hrs in the presence or absence of U0126 (3 μ M) for the first 24 hrs of differentiation. Cells were then lysed and subjected to western analysis (n=4). Quantification of western blots was performed by densitometry. The level of each specific protein was determined relative to the level of tubulin, and the data were normalized to control. All error bars represent SEM. Two-tailed *t*-test was performed to compare each data to control. **P* < 0.05; ***P* < 0.01. Scale bars: 50 μ m.

(G) A proposed model: TRAIL/TRAIL-R2 negatively regulates myogenesis by inhibiting cell cycle withdrawal through activation of ERK signaling and inhibition of p21 expression.

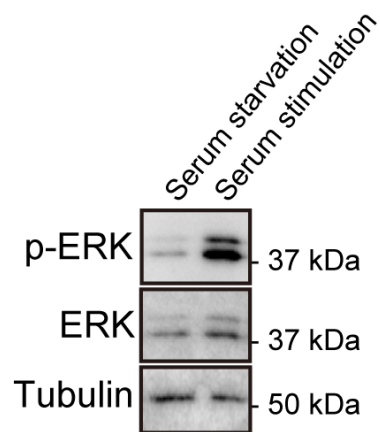


Figure 2.8 pERK antibody validation

C2C12 cells at 50% confluence were serum starved overnight (serum starvation), and then stimulated with 10% fetal bovine serum for 30 min (serum stimulation). Cells were lysed and subjected to western analysis.

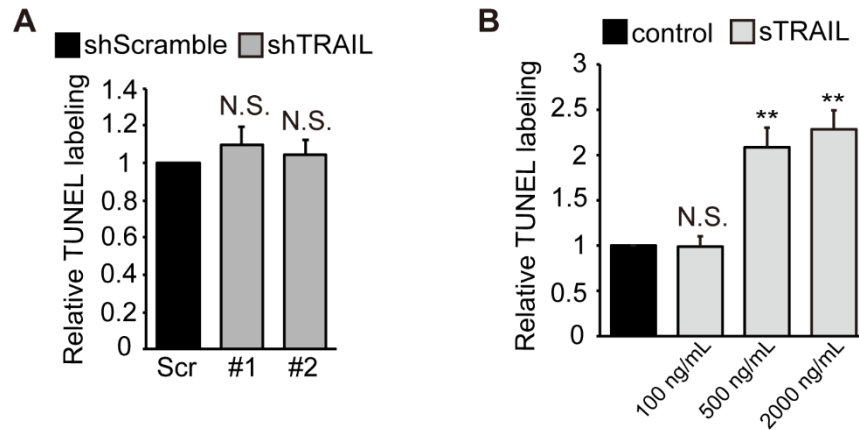


Figure 2.9 TRAIL at high concentrations induces apoptosis in myocytes.

(A) C2C12 cells were transduced with lentiviruses expressing shTRAIL or shScramble (Scr) followed by 2 days of puromycin selection. Cells were then induced to differentiate for 24 hrs, followed by TUNEL assay. Data were normalized to shScramble (n=4).

(B) C2C12 cells were grown with vehicle (Control) or various concentrations of sTRAIL for 6 to 9 hours and induced to differentiate for 24 hrs in the presence or absence of sTRAIL, followed by TUNEL assay. Data were normalized to control (n=5). All error bars represent SEM. Two tailed *t*-test was performed to compare each data to control (first bar). NS: not significant ($P > 0.05$). ** $P < 0.01$.

2.6 References

1. Pitti, R.M., et al., *Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family*. J Biol Chem, 1996. **271**(22): p. 12687-90.
2. Wiley, S.R., et al., *Identification and characterization of a new member of the TNF family that induces apoptosis*. Immunity, 1995. **3**(6): p. 673-82.
3. Walczak, H., et al., *Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo*. Nat Med, 1999. **5**(2): p. 157-63.
4. Naka, T., et al., *Effects of tumor necrosis factor-related apoptosis-inducing ligand alone and in combination with chemotherapeutic agents on patients' colon tumors grown in SCID mice*. Cancer Res, 2002. **62**(20): p. 5800-6.
5. Sedger, L.M., et al., *Characterization of the in vivo function of TNF-alpha-related apoptosis-inducing ligand, TRAIL/Apo2L, using TRAIL/Apo2L gene-deficient mice*. Eur J Immunol, 2002. **32**(8): p. 2246-54.
6. von Karstedt, S., A. Montinaro, and H. Walczak, *Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy*. Nat Rev Cancer, 2017. **17**(6): p. 352-366.
7. Kimberley, F.C. and G.R. Screaton, *Following a TRAIL: update on a ligand and its five receptors*. Cell Res, 2004. **14**(5): p. 359-72.
8. Pan, G., et al., *An antagonist decoy receptor and a death domain-containing receptor for TRAIL*. Science, 1997. **277**(5327): p. 815-8.
9. LeBlanc, H.N. and A. Ashkenazi, *Apo2L/TRAIL and its death and decoy receptors*. Cell Death Differ, 2003. **10**(1): p. 66-75.
10. Vitovski, S., et al., *Investigating the interaction between osteoprotegerin and receptor activator of NF-kappa B or tumor necrosis factor-related apoptosis-inducing ligand*. Journal of Biological Chemistry, 2007. **282**(43): p. 31601-31609.
11. Emery, J.G., et al., *Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL*. J Biol Chem, 1998. **273**(23): p. 14363-7.
12. Wu, G.S., et al., *Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor*. Cancer Res, 1999. **59**(12): p. 2770-5.
13. Schneider, P., et al., *Identification of a new murine tumor necrosis factor receptor locus that contains two novel murine receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)*. J Biol Chem, 2003. **278**(7): p. 5444-54.
14. Esperon, E.D., G. Cordier, and N. Engel, *A genomic reservoir for Tnfrsf genes is developmentally regulated and imprinted in the mouse*. Epigenetics, 2012. **7**(6): p. 626-634.
15. Varfolomeev, E., et al., *Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand*. J Biol Chem, 2005. **280**(49): p. 40599-608.
16. Ehrhardt, H., et al., *TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-kappaB*. Oncogene, 2003. **22**(25): p. 3842-52.
17. Ishimura, N., et al., *Trail induces cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells*. Am J Physiol Gastrointest Liver Physiol, 2006. **290**(1): p. G129-36.
18. Secchiero, P., et al., *TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways*. Circulation, 2003. **107**(17): p. 2250-6.

19. Secchiero, P., et al., *TRAIL promotes the survival, migration and proliferation of vascular smooth muscle cells*. Cell Mol Life Sci, 2004. **61**(15): p. 1965-74.
20. O'Flaherty, J., et al., *Signaling through the TRAIL receptor DR5/FADD pathway plays a role in the apoptosis associated with skeletal myoblast differentiation*. Apoptosis, 2006. **11**(12): p. 2103-13.
21. Zhao, Y., et al., *Apoptosis in the skeletal muscle of untreated children with juvenile dermatomyositis: impact of duration of untreated disease*. Clin Immunol, 2007. **125**(2): p. 165-72.
22. Alger, H.M., et al., *The role of TRAIL in mediating autophagy in myositis skeletal muscle: a potential nonimmune mechanism of muscle damage*. Arthritis Rheum, 2011. **63**(11): p. 3448-57.
23. Ge, Y., Y. Sun, and J. Chen, *IGF-II is regulated by microRNA-125b in skeletal myogenesis*. J Cell Biol, 2011. **192**(1): p. 69-81.
24. Son, K., et al., *Nontranslational function of leucyl-tRNA synthetase regulates myogenic differentiation and skeletal muscle regeneration*. Journal of Clinical Investigation, 2019. **129**(5): p. 2088-2093.
25. Ge, Y., et al., *mTOR regulates skeletal muscle regeneration in vivo through kinase-dependent and kinase-independent mechanisms*. Am J Physiol Cell Physiol, 2009. **297**(6): p. C1434-44.
26. Yoshida, N., et al., *Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'*. J Cell Sci, 1998. **111**(Pt 6): p. 769-79.
27. Guo, K., et al., *MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation*. Mol Cell Biol, 1995. **15**(7): p. 3823-9.
28. Halevy, O., et al., *Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD*. Science, 1995. **267**(5200): p. 1018-21.
29. Caldwell, C.J., D.L. Matthey, and R.O. Weller, *Role of the basement membrane in the regeneration of skeletal muscle*. Neuropathol Appl Neurobiol, 1990. **16**(3): p. 225-38.
30. Bennett, A.M. and N.K. Tonks, *Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases*. Science, 1997. **278**(5341): p. 1288-91.
31. Zoller, V., et al., *Trail (TNF-related apoptosis-inducing ligand) induces an inflammatory response in human adipocytes*. Sci Rep, 2017. **7**(1): p. 5691.
32. Freer-Prokop, M., et al., *Non-canonical role for the TRAIL receptor DR5/FADD/caspase pathway in the regulation of MyoD expression and skeletal myoblast differentiation*. Differentiation., 2009. **78**(4): p. 205-12.
33. Tang, X., et al., *Signaling pathways regulating dose-dependent dual effects of TNF-alpha on primary cultured Schwann cells*. Mol Cell Biochem, 2013. **378**(1-2): p. 237-46.
34. Zhang, C., et al., *Fas/FasL Complex Promotes Proliferation and Migration of Brain Endothelial Cells Via FADD-FLIP-TRAF-NF-kappaB Pathway*. Cell Biochem Biophys, 2015. **71**(3): p. 1319-23.
35. Cretney, E., et al., *Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice*. J Immunol, 2002. **168**(3): p. 1356-61.
36. Finnberg, N., et al., *DR5 knockout mice are compromised in radiation-induced apoptosis*. Mol Cell Biol, 2005. **25**(5): p. 2000-13.
37. Dufresne, S.S., et al., *Genetic deletion of muscle RANK or selective inhibition of RANKL is not as effective as full-length OPG-fc in mitigating muscular dystrophy*. Acta Neuropathol Commun, 2018. **6**(1): p. 31.

38. Chang, N.C., F.P. Chevalier, and M.A. Rudnicki, *Satellite Cells in Muscular Dystrophy - Lot in Polarity*. Trends in Molecular Medicine, 2016. **22**(6): p. 479-496.
39. Ge, Y., et al., *RNAi screen reveals potentially novel roles of cytokines in myoblast differentiation*. PLoS One, 2013. **8**(7): p. e68068.
40. Waldemer-Streyer, R.J., et al., *Cxcl14 depletion accelerates skeletal myogenesis by promoting cell cycle withdrawal*. NPJ Regen Med, 2017. **2**.
41. Ge, Y., et al., *Flt3L is a novel regulator of skeletal myogenesis*. J Cell Sci, 2013. **126**(Pt 15): p. 3370-9.

CHAPTER 3. ESTABLISHING A MOUSE MODEL FOR SKELETAL MYOGENIC PROGENITOR CELL-SPECIFIC TARGETED GENE DISRUPTION

3.1 Introduction

Skeletal muscle is composed of multinucleated myofibers, satellite cells, a resident pool of muscle stem cells, and other cell populations including fibro-adipogenic progenitors (FAPs), endothelial cells, tenocytes, and resident immune cells [1]. Moreover, upon acute muscle injury, immune cells are activated or infiltrated in well-controlled and time-dependent manner. For example, muscle-resident mast cells are activated upon injury, resulting in their degranulation and release of inflammatory mediators [2]. In addition, neutrophils invade the damaged tissue within 2 h of the injury followed by M1 (phagocytic) macrophage invasion. After the number of M1 macrophages begins to decline about 2 days post injury, the concentration of M2 (nonphagocytic) macrophages peaks about 4 days post injury [3]. During these processes, immune cells secrete various cytokines. The muscle-resident mast cells, for example, release $\text{TNF}\alpha$ and IL-1 for immune cell recruitment. Infiltrated neutrophil produces IL-1 and IL-8, which stimulate the differentiation of monocytes into macrophages [2]. Furthermore, M1 macrophages secrete pro-inflammatory cytokines such as $\text{TNF}\alpha$, IL-1 β and IFN- γ to facilitate the removal of cell debris [4]. Although there have been various studies, including our own [5-8], on the functions of cytokines in skeletal muscle regeneration, evidence that distinguishes between muscle cells or immune cells as the relevant source of those cytokines is still entirely lacking. A convenient method to deplete a cytokine in vivo in a myogenic progenitor cell (MPC)-specific manner would enable us to probe the functions of muscle cell-derived cytokines in muscle regeneration.

CRISPR/Cas9 technology has facilitated gene editing in various cell types and organisms. The RNA-guided endonuclease Cas9 can be utilized to generate targeted DNA double-strand breaks (DSBs), which can lead to specific genome editing via nonhomologous end-joining (NHEJ) or homology-directed repairs (HDR), resulting in insertion-deletion mutations (indels) or precise sequence substitutions respectively [9]. Recently, *in vivo* delivery of Cas9/single guide RNA (sgRNA) has enabled the creation of various mouse models or correction of genetic diseases [10]. For instance, many studies have been carried out to correct Duchenne muscular dystrophy (DMD) in the *mdx* mouse, a model for DMD [11]. In those studies, the postnatal editing in *mdx* muscle was achieved by delivering adeno-associated virus (AAV)-mediated Cas9 and sgRNAs, and the tissue specificity was determined by the AAV serotype or muscle-specific promoter-driven Cas9 expression. However, the packaging capacity of AAV (~4.7 kb) renders the MPC- or muscle-specific delivery of CRISPR/Cas9 components challenging because the size of *Streptococcus pyogenes* Cas9 (*SpCas9*) alone is approximately 4.2 kb [12]. Furthermore, despite the use of dual-AAV system (one for Cas9 and the second for sgRNAs), the gene editing efficiency is still limited since each cell needs to be infected by all the components simultaneously. The generation of transgenic mice, in which the Cas9 expression is limited to muscle, can be one of the ways to circumvent those hurdles. In this chapter I will describe my efforts of creating transgenic mice with MPC-specific Cas9 expression by utilizing the Cre-*LoxP* system.

3.2 Materials and Methods

3.2.1 Antibodies

Anti-FLAG antibody (F9219) was from Sigma-Aldrich (St. Louis, MO, USA) and anti-GAPDH antibody (2118) was from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated anti-rabbit (115-036-003) and anti-mouse (111-036-003) IgGs were from Jackson Immuno Research Laboratories (West Grove, PA, USA).

3.2.2 Generation of Cas9 transgenic mice

The Cre recombinase-dependent Rosa26-loxP-Stop (3x polyA signal)-loxP-Cas9 (or LSL-Cas9) knock-in mice (B6J.129 (B6N)-Gt(ROSA)26Sor^{tm1(CAG-Cas9*, -EGFP)Fezh/J}; stock# 026175), Pax7-Cre mice (Pax7^{tm1(cre)Mrc}/J; stock# 010530) and Myf5-Cre mice (B6.129S4-Myf5^{tm3(cre)Sor}/J; stock# 007893) were obtained from Jackson Laboratory. To establish MPC-specific Cas9 knock-in mice, Pax7-Cre or Myf5-Cre mice were crossed with LSL-Cas9 knock-in mice. Genotyping was performed by PCR using genomic DNA extracted from mouse tails.

3.2.3 Cell culture

C2C12 myoblasts were a gift from S. Kaufman at the University of Illinois and originally obtained from ATCC. Cells were maintained in DMEM containing 4.5 g/L glucose, 10% fetal bovine serum, and 1% penicillin-streptomycin at 37°C with 7.5% CO₂. To induce differentiation, cells were plated on tissue culture plates coated with 0.2% gelatin, grown to 100% confluence, and then cultured in differentiation medium (DMEM containing 2% horse serum). The cells were replenished with fresh differentiation medium daily for 3 days.

3.2.4 Western blotting

Isolated mouse tissues were immediately frozen in liquid nitrogen and homogenized with a Polytron in ice-cold MIPT buffer [13] with 1 x protease inhibitor cocktail (P8340, Sigma-Aldrich). The homogenates were incubated on ice for 30 min and centrifugated at 16,200 *g* for 10 min at 4°C to collect the supernatant. The protein concentration in each supernatant sample was determined with the DC protein assay kit (Bio-Rad).

For western blotting, an equal amount of protein from each sample was boiled in SDS sample buffer with 5% β -mercaptoethanol. Proteins were resolved on SDS-PAGE, transferred onto polyvinylidene fluoride membrane (EMD Millipore, Darmstadt, Germany) and incubated with various antibodies in accordance with manufacturers' recommendations. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA), and images were captured on an iBright CL1000 imaging system (Thermo Fisher Scientific, Waltham, MA, USA).

3.2.5 SgRNA design and DNA construct

Sequences of sgRNAs targeting *Arhgef3* exon 4 (sgArhgef3) were identified using CRISPick (<https://portals.broadinstitute.org/gppx/crispick/public>), and the control sgRNA (sgControl) was adopted from Wang et al. [14].

sgArhgef3 #1: 5'- AGCGGAGAGACAGCAAAGTG -3'

sgArhgef3 #2: 5'- TGTCGGGGCGGCTCTCGCTG -3'

sgArhgef3 #3: 5'- ATTTCTGGACCAAGCTCGGG -3'

sgArhgef3 #4: 5'- CTGATTGACACACACATCGA -3'

sgControl: 5'- GTAGCGAACGTGTCCGGCGT -3'

To validate the sgRNAs *in vitro*, the top and bottom strands of the sgRNAs were synthesized (Sigma-Aldrich) and cloned into lentiCRISPR v2 (a gift from Feng Zhang, Addgene plasmid #52961) [15]. For AAV-mediated sgRNA delivery, sgRNA strands of the same sequence were cloned into pX552 (a gift from Feng Zhang, Addgene plasmid #60958) [16].

3.2.6 Lentivirus production and transduction

For lentiviral packaging, lentiCRISPR-sgRNAs, psPAX2 (a gift from Didier Trono, Addgene plasmid #12260) and pMD2.G (a gift from Didier Trono, Addgene plasmid #12259) were cotransfected into 293T cells using PEI at 1 µg, 0.9 µg, and 0.1 µg respectively in a six-well plate. The medium was changed 12 – 18 h post-transfection, and the medium containing viruses were collected 36 – 42 h posttransfection.

C2C12 cells were infected with viruses in the presence of 8 µg/mL polybrene (Sigma-Aldrich) overnight, and then subjected to selection by 3 µg/mL puromycin (InvivoGen) for 72 hours prior to downstream analysis.

3.2.7 T7 Endonuclease assay

From C2C12 cells, genomic DNA was isolated based on a previously described protocol [17]. Briefly, cells were lysed with 50 mM NaOH and heated to 95°C for 30 min. After heating, 1 M Tris (pH 7.5) was added to neutralize the NaOH. Supernatant was recovered after centrifugation. Genomic DNA was isolated from mouse TA muscles using a DNeasy Blood Tissue Kit (Qiagen) according to the manufacturer's instructions.

To detect on-target mutations, the genomic region was amplified using Q5® High-Fidelity DNA Polymerase (New England Biolabs) with the following primers:

Arhgef3 on-target-Forward: 5'- CATCAAAGGGTGTGTGTTGG -3'

Arhgef3 on-target-Reverse: 5'- ATTATCCCTCCCCACGAGAC -3'

PCR products were re-annealed and subsequently digested with T7 Endonuclease I (New England Biolabs) followed by agarose gel electrophoresis (2%) according to the manufacturer's instructions.

3.2.8 AAV9 virus production

AAV9 viral particles were produced according to the previously described methods [18, 19]. HEK293T cells in 15-cm plate were transfected with 10 µg of pX552-sgRNA vector, 10 µg of pAAV2/9n vector (a gift from James M. Wilson, Addgene plasmid #112865), and 10 µg of pAdDeltaF6 (a gift from James M. Wilson, Addgene plasmid #112867) at a ratio of 1:1:1 using PEI. Eighteen to 24 h after transfection, the cells were changed to growth medium. Cells were harvested 72 h post-transfection by dissociating from plates using a cell scraper in the presence of 2 mL of lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl), followed by three cycles of freezing-thawing using liquid nitrogen and 37°C water bath. The lysate was incubated with benzonase (Sigma-Aldrich) for 30 min at 37°C followed by centrifugation at 10,000 g for 10 min at room temperature. The supernatant was filtered with a 0.45 µm sterile filter and added with 0.5 mL of PEG solution (4 M NaCl and 40% PEG8000 (w/v), Sigma-Aldrich) to each 2 mL of supernatant. The mixtures were gently inverted to mix and then kept at 4°C overnight to precipitate the virus particles, followed by centrifugation at 12,000 g for 30 min at 4°C. The pellet was re-suspended with sterile PBS. Equal volume of chloroform was added and vortexed 2 min, followed by centrifugation at 12,000 g for 15 min at 4°C. Top aqueous layer containing AAV particles was collected and placed under a chemical hood for 30 min to evaporate the remaining chloroform. The aqueous layer, which contains AAV particles, was filtered

through a 0.22 μm sterile filter and then using a 100 kDa MWCO centrifugal filter unit (Millipore). After removing the filtrate, 5 mL of PBS was added inside the cup of the centrifugal filter unit and centrifuged. The PBS addition and filtration were repeated two more times to wash the AAV solution, and 200 – 250 μL of virus in PBS was collected after the third wash. AAV9 titer was determined by qRT-PCR using qPCR AAV Titer Kit (Applied Biological Materials Inc.) according to the manufacturer's instructions.

3.2.9 Injury-induced muscle regeneration, cryosection, and hematoxylin and eosin staining

Female mice aged 12 weeks were used in all the regeneration experiments to compare cross section area (CSA) between different mouse lines. Muscle injury was induced by injection of 40 μL of 1% (w/v) BaCl_2 dissolved in saline into mouse TA muscles. Injured muscles were isolated at 7 days after injury (AI) and frozen in liquid nitrogen-cooled 2-methylbutane and embedded in TBS tissue freezing medium (Thermo Fisher Scientific, Waltham, MA, USA). Sections of 10 μm thickness were obtained with a Microm HM550 (Thermo Fisher Scientific, Waltham, MA, USA) at -20°C , placed on uncoated slides, and stained with hematoxylin and eosin (H&E). Images were obtained by DMI 4000B fluorescence microscope (Leica, Wetzlar, Germany) with a 20X dry objective (Fluotar, numerical aperture 0.4; Leica). Five bright-field images were randomly captured using RETIGA EXi camera (QImaging, Surrey, BC, Canada) and Image Pro Express software (Media Cybernetics, Rockville, MD, USA). The images were analyzed for CSA of all centrally nucleated regenerating myofibers (H&E staining) using ImageJ. Image acquisition and CSA quantification were performed by investigators that were blinded to sample identification.

To deliver sgRNAs, $4 \times 10^8 - 1 \times 10^9$ vg of AAV9 expressing sgArhgef3 (or sgControl as a negative control) was co-injected with 20 μ l of 2% (w/v) BaCl₂ into mouse TA muscles. The injured muscles were collected 7 days after injury (AI) and subjected to RNA isolation or genomic DNA isolation.

3.2.10 Quantitative RT-PCR

C2C12 cells or isolated muscle tissues were lysed in Trizol (Invitrogen, Carlsbad, CA, USA), and RNA was isolated following the manufacturer's protocol. cDNA was synthesized from 1 μ g RNA using the qScript™ cDNA Synthesis kit (Quantabio, Beverly, MA, USA) following the manufacturer's protocol. Quantitative PCR was performed with a StepOne Plus (Applied Biosystems, Foster City, CA, USA) using gene-specific primers. Hypoxanthine guanine phosphoribosyltransferase (Hprt) was used as a reference to obtain the relative fold change for target samples using the comparative C_T method. The following primers were used:

Mouse Hprt-Forward: 5'- ATGGACTGATTATGGACAGGACTG -3'

Mouse Hprt-Reverse: 5'- TCCAGCAGGTCAGCAAAGAAC -3'

Mouse Arhgef3-Forward: 5'- CTTTCAGCAACCACGAGAGAGT -3'

Mouse Arhgef3-Reverse: 5'- GAAGGTGTCGTTGGCTTGTA -3'

3.2.11 Statistical analysis

The sample size for each experiment is described in figure legends. All the quantitative values are presented as mean $\pm$ SEM. Whenever necessary, statistical significance of the data was analyzed by performing two-tailed paired Student's *t*-tests. The specific types of test and the *P*-values, when applicable, are indicated in the figure

legends. Statistical comparison was considered significant when $P < 0.05$. All statistical analysis was performed using Excel.

3.2.12 Study approval

All the animal experiments in this study followed protocols approved by the Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

3.3 Results

3.3.1 Generation of myogenic progenitor cell-specific Cas9 transgenic mice

Conditional knockout mice have been widely used to study gene of interest in a cell- or tissue- specific manner, and a principal method to achieve conditional knockout is to use the Cre-*LoxP* system. However, it will be both laborious and expensive if a mouse model needs to be generated for each gene of interest. Hence, we decided to take advantage of the CRISPR/Cas9 technology and establish a mouse model with MPC-specific gene editing. To that end, we obtained a Cas9 knock-in mouse line, in which Cas9 gene expression is interrupted by a “stop (3x polyA)” signal flanked by LoxP sites (LSL-Cas9) [9]. In these mice Cas9 would only be expressed in the presence of a Cre recombinase. For the expression of Cre recombinase, we decided to use two types of mice: Pax7-Cre [20] and Myf5-Cre [21]. Both Pax7 and Myf5 are transcription factors expressed in satellite cells [22].

We crossed LSL-Cas9 mice with Pax7-Cre or Myf5-Cre mice, resulting in Pax7-Cas9 or Myf5-Cas9 mice, respectively (Figure 3.1 A). To confirm that Cas9 is correctly expressed in skeletal muscle, I carried out western blotting to detect Flag-tagged Cas9. As expected, Cas9 was highly expressed in muscle tissues from both Pax7-Cas9 and

Myf5-Cas9 mice; a weak expression was also observed in brain and lung from Myf5-Cas9 mice (Figure 3.1 B). The activity of Myf5-Cre in the brain was previously reported [23]. In addition, previous lineage tracing of Pax7-descendant cells using Pax7-Cre demonstrated that not only skeletal muscle but also brain, lung and liver express Pax7 [24]. However, this 'non-muscle' expression of Cas9 does not affect the MPC-specific study of gene of interest because the sgRNAs will be delivered by intramuscular injection in our systems.

3.3.2 Cas9 expression does not affect skeletal muscle regeneration in vivo

Constitutive Cas9 expression as a result of crossing LSL-Cas9 and β -actin-Cre mice has been reported to have no observable effect on mice including fertility, litter size, and morphology, nor upregulation in apoptosis and DNA damage [9]. However, muscle regeneration has not been examined in those mice. To rule out any potential effect of Cas9 expression on muscle regeneration, I injected BaCl₂ into TA muscles of Pax7-Cas9 and Myf5-Cas9 mice. No significant difference in regenerating myofiber CSA was observed among the mouse strains (Figure 3.2), suggesting that ectopic expression of Cas9 in skeletal muscle does not affect muscle regeneration.

3.3.3 Editing of *Arhgef3* in skeletal MPC-specific Cas9 mice

As proof of concept, I chose to use the mouse system described above to edit the *Arhgef3* gene. ARHGEF3, a GEF for RhoA/B, plays a critical role in skeletal muscle regeneration [25]. Four sgRNAs against exon 4 of *Arhgef3* were designed using CRISPick (<https://portals.broadinstitute.org/gppx/crispick/public>). Individual sgRNA was cloned into Cas9-expressing vector followed by lentivirus packaging. C2C12 cells were transduced with the lentiviruses, and indels were detected by T7 Endonuclease assay. As shown in Figure 3.3 A, sgArhgef3 #3 showed the highest on-target editing efficiency and was chosen for further in vivo validation.

To deliver sgArhgef3 in vivo, AAV9 was used since its skeletal muscle tropism has been widely reported [26, 27]. I co-injected AAV9 expressing sgArhgef3 with BaCl₂ into TA muscles of Pax7-Cas9 and Myf5-Cas9 mice and isolated the injected muscles for analysis on day 7 AI. Similar to the in vitro results, on-target gene editing was detected in the injected muscle (Figure 3.3 B). Moreover, I observed that Arhgef3 mRNA expression was reduced in the TA muscles from AAV9-sgArhgef3-injected mice (Figure 3.3 C). Taken together, my data show that the Cas9 transgenic mice we have generated can be utilized to edit target genes specifically in skeletal myogenic progenitor cells.

3.4 Discussion

We have established a mouse model with MPC-specific Cas9 expression using two satellite cell-specific Cre lines (Pax7-Cre and Myf5-Cre). I demonstrate that Flag-Cas9 is expressed predominantly in skeletal muscle in these mice and that ectopic Cas9 expression does not affect muscle regeneration. I have further validated the utility of these Cas9 mice by targeting Arhgef3 in regenerating muscles upon injury.

Most recently, two other groups reported generation of skeletal muscle-specific Cas9 transgenic mice. Li et al. used muscle creatine kinase (MCK)-Cre to drive Cas9 expression in order to study the function of prolyl hydroxylases in skeletal muscle atrophy [28]. Bond et al. created Cas9 transgenic mice using muscle actin (ACTA1)-Cre and found the transgenic mice to display no abnormality in body weight, tissue weight and the expression of myogenic transcription factors [29]. Although the type of Cre driver is different, these studies corroborate our own Cas9 transgenic mice system.

Although some tissues other than skeletal muscle showed expression of Pax7 or Myf5 when each line of Cre mice was used for lineage tracing [23, 24], there is no

evidence thus far that these myogenic transcription factors would be expressed in immune cells. Nevertheless, because our main motivation of generating these Cas9 mice is to differentiate the function of MPC-derived cytokines from immune cell-derived cytokines, it will be essential to rule out Cas9 expression or gene editing upon sgRNA delivery in immune cells while verifying the expression and editing in muscle. To this end, flow cytometry with satellite cell- and immune cell-specific markers can be performed to aid the analysis. For example, we can analyze cells with GFP+ (proxy for Cas9) and Vascular cell adhesion molecule 1 (VCAM1)+ (marker for satellite cells [30]) and cells with GFP+ and F4/80+ (pan-macrophage marker [31]) or CD45+ (hematopoietic cell marker [32]) for Cas9 expression and gene editing.

The skeletal muscle regeneration process can be divided into multiple steps including activation of muscle stem cells, proliferation of the activated muscle stem cells, differentiation of the muscle stem cells, and formation of multinucleated muscle fibers [33]. Each stage of muscle regeneration is controlled by distinct regulators. For example, Cxcl16 [34] and Ccl2 [35] have been reported to regulate satellite cell activation and proliferation. On the other hand, Cxcl12 is believed to regulate myocyte migration and fusion [36]. Various Cre driver mice including inducible ones such as HSA-rtTA/TRE-Cre [37] and Pax7^{creER} [38], when crossed with the LSL-Cas9 mice, can provide a complete toolkit for spatiotemporal investigation of myogenic regulators.

3.5 Figures

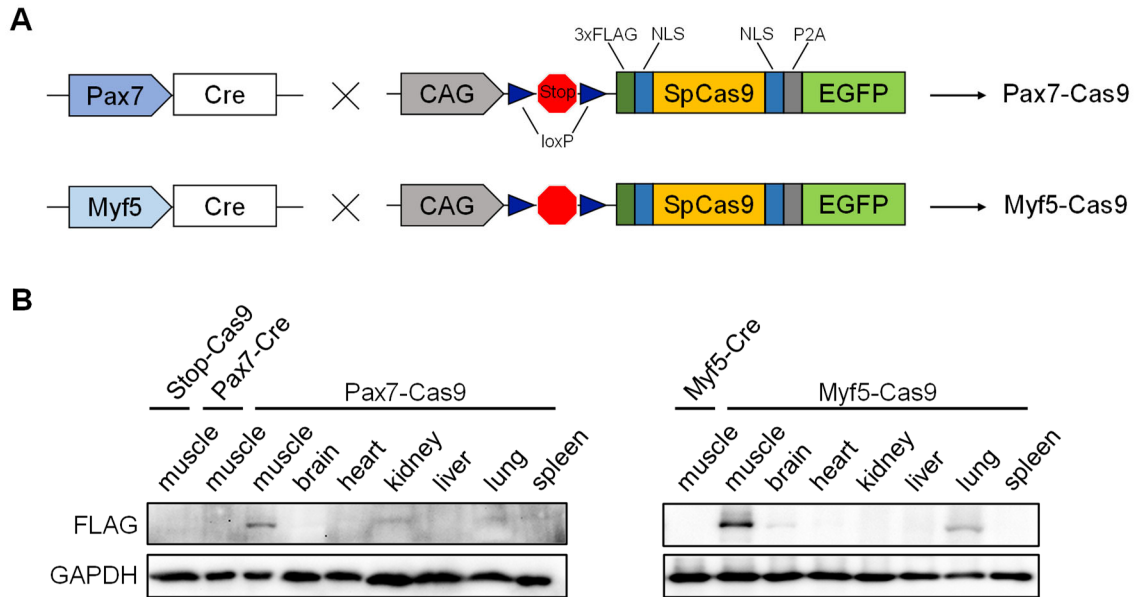


Figure 3.1 Generation of MPC-specific Cas9 transgenic mice

(A) Schematic depiction of the strategy for Cas9 transgenic mice generation. CAG: CAG promoter; Stop: 3x polyA signal; NLS: Nuclear localization sequence; P2A: Porcine teschovirus-1 2A.

(B) The expression of Flag-tagged Cas9 in tissues from Pax7-Cas9 or Myf5-Cas9 mice was evaluated by western blotting. TA muscles from Rosa26-LSL-Cas9 knock-in mice (Stop-Cas9) and Cre mice were used as a control.

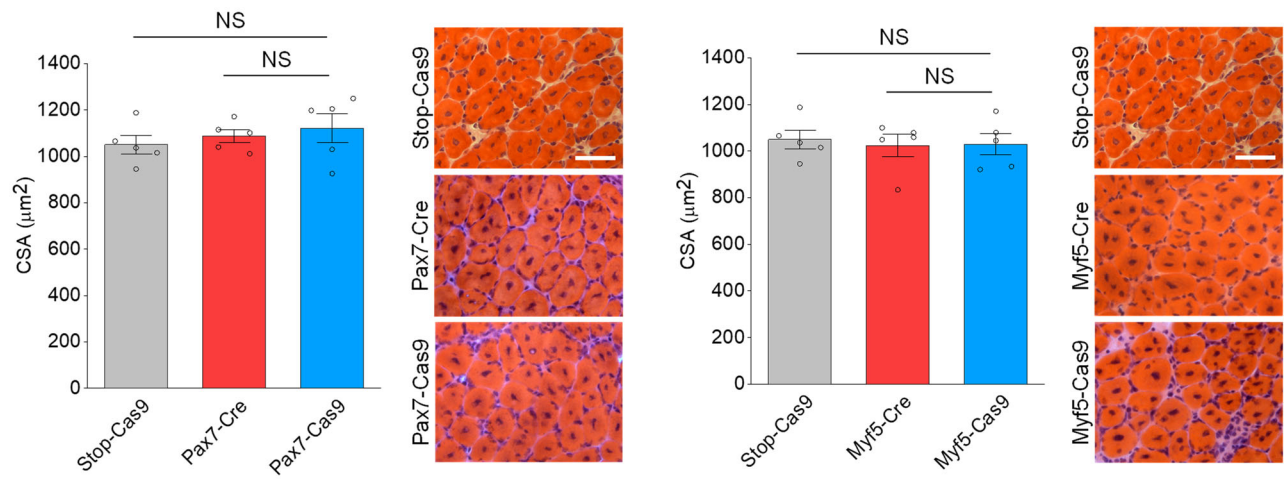


Figure 3.2 Cas9 expression does not affect skeletal muscle regeneration.

TA muscles of indicated mouse lines were injected with BaCl₂. On Day 7 AI, TA muscles were isolated and subjected to cryosection. Upon H&E staining, cross-section area (CSA) was quantified for regenerating myofibers (n=5 for each mouse line). All error bars represent SEM. Two-tailed *t*-test was performed to compare the Cas9 transgenic mice to control (Stop-Cas9 or Cre mice). NS, not significant. Scale bars: 50 μm.

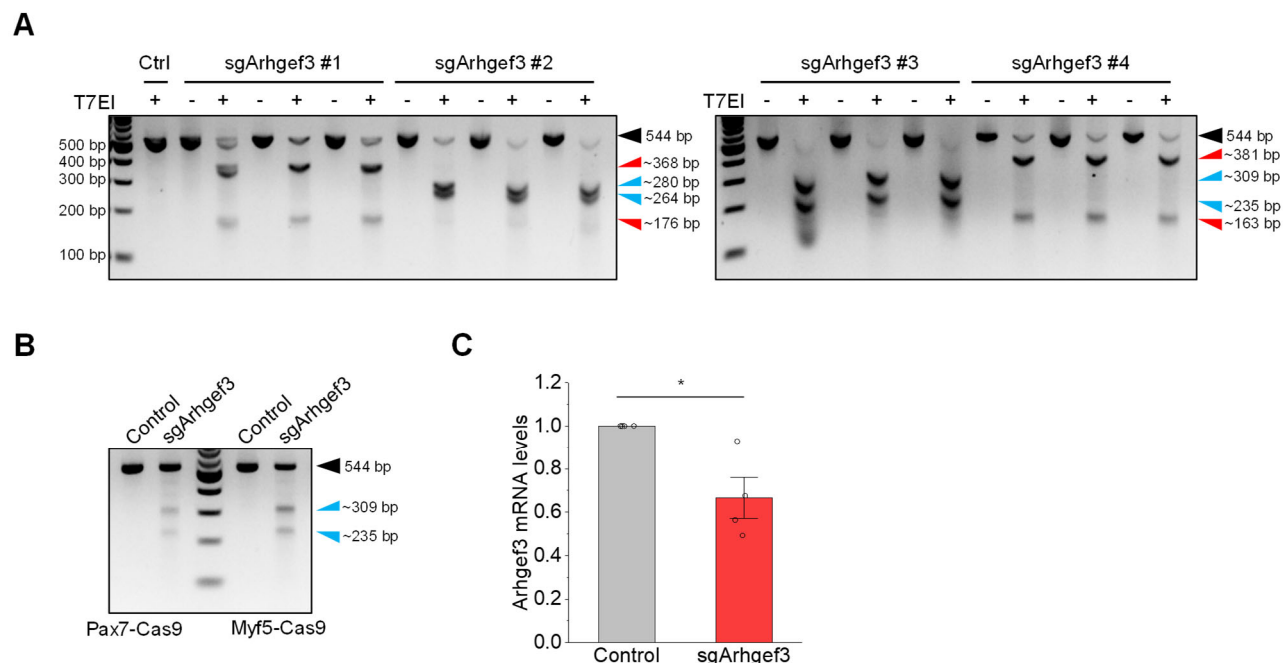


Figure 3.3 Downregulation of Arhgef3 expression upon delivery of AAV9-sgArhgef3 in Cas9 transgenic mice.

(A) C2C12 cells were transduced with lentiviruses expressing Cas9 and sgRNA (sgArhgef3 #1 to #4 or Control, Ctrl) followed by 3 days of puromycin selection. Genomic DNAs were isolated and subjected to T7 Endonuclease I assay upon PCR amplification of on-target region (n=3). Undigested (544 bp) and digested fragments are marked.

(B) TA muscles of Cas9 transgenic mice (Pax7-Cas9 or Myf5-Cas9 mice) were co-injected with BaCl₂ and AAV9 expressing sgArhgef3 or Control, isolated on Day 7 AI and subjected to genomic DNA isolation. On-target region was amplified by PCR followed by T7 Endonuclease I assay. Undigested (544 bp) and digested fragments are marked.

(C) RNAs were isolated from TA muscles of Myf5-Cas9 mice injected as in (B) and subjected to qRT-PCR (n=4). All error bars represent SEM. Two-tailed *t*-test was performed to compare the data to control. **P* < 0.05.

3.6 References

1. Paylor, B., et al., *Nonmyogenic cells in skeletal muscle regeneration*. Curr Top Dev Biol, 2011. **96**: p. 139-65.
2. Howard, E.E., et al., *Divergent Roles of Inflammation in Skeletal Muscle Recovery From Injury*. Front Physiol, 2020. **11**: p. 87.
3. Tidball, J.G. and S.A. Villalta, *Regulatory interactions between muscle and the immune system during muscle regeneration*. Am J Physiol Regul Integr Comp Physiol, 2010. **298**(5): p. R1173-87.
4. Dort, J., et al., *Macrophages Are Key Regulators of Stem Cells during Skeletal Muscle Regeneration and Diseases*. Stem Cells Int, 2019. **2019**: p. 4761427.
5. Ge, Y., et al., *Flt3L is a novel regulator of skeletal myogenesis*. J Cell Sci, 2013. **126**(Pt 15): p. 3370-9.
6. Waldemer-Streyer, R.J. and J. Chen, *Myocyte-derived Tnfsf14 is a survival factor necessary for myoblast differentiation and skeletal muscle regeneration*. Cell Death & Disease, 2015. **6**.
7. Waldemer-Streyer, R.J., et al., *Cxcl14 depletion accelerates skeletal myogenesis by promoting cell cycle withdrawal*. NPJ Regen Med, 2017. **2**.
8. Kim, D., et al., *Muscle-derived TRAIL negatively regulates myogenic differentiation*. Experimental Cell Research, 2020. **394**(1).
9. Platt, R.J., et al., *CRISPR-Cas9 knockin mice for genome editing and cancer modeling*. Cell, 2014. **159**(2): p. 440-55.
10. Komor, A.C., A.H. Badran, and D.R. Liu, *CRISPR-Based Technologies for the Manipulation of Eukaryotic Genomes*. Cell, 2017. **168**(1-2): p. 20-36.
11. Min, Y.L., R. Bassel-Duby, and E.N. Olson, *CRISPR Correction of Duchenne Muscular Dystrophy*. Annu Rev Med, 2019. **70**: p. 239-255.
12. Chemello, F., R. Bassel-Duby, and E.N. Olson, *Correction of muscular dystrophies by CRISPR gene editing*. J Clin Invest, 2020. **130**(6): p. 2766-2776.
13. Yoon, M.S. and J. Chen, *Distinct amino acid-sensing mTOR pathways regulate skeletal myogenesis*. Molecular Biology of the Cell, 2013. **24**(23): p. 3754-3763.
14. Wang, T., et al., *Genetic Screens in Human Cells Using the CRISPR-Cas9 System*. Science, 2014. **343**(6166): p. 80-84.
15. Ran, F.A., et al., *Genome engineering using the CRISPR-Cas9 system*. Nature Protocols, 2013. **8**(11): p. 2281-2308.
16. Swiech, L., et al., *In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9*. Nat Biotechnol, 2015. **33**(1): p. 102-6.
17. Behringer, R., et al., *Preparation of Polymerase Chain Reaction Template DNA from Mouse Tail Tissue*. Cold Spring Harb Protoc, 2019. **2019**(4).
18. Kimura, T., et al., *Production of adeno-associated virus vectors for in vitro and in vivo applications*. Sci Rep, 2019. **9**(1): p. 13601.
19. Gaj, T. and D.V. Schaffer, *Adeno-Associated Virus-Mediated Delivery of CRISPR-Cas Systems for Genome Engineering in Mammalian Cells*. Cold Spring Harb Protoc, 2016. **2016**(11).
20. Keller, C., et al., *Pax3:Fkhr interferes with embryonic Pax3 and Pax7 function: implications for alveolar rhabdomyosarcoma cell of origin*. Genes Dev, 2004. **18**(21): p. 2608-13.
21. Tallquist, M.D., et al., *Early myotome specification regulates PDGFA expression and axial skeleton development*. Development, 2000. **127**(23): p. 5059-70.
22. Motohashi, N. and A. Asakura, *Muscle satellite cell heterogeneity and self-renewal*. Front Cell Dev Biol, 2014. **2**: p. 1.

23. Berry, D.C., Y. Jiang, and J.M. Graff, *Mouse strains to study cold-inducible beige progenitors and beige adipocyte formation and function*. Nat Commun, 2016. **7**: p. 10184.
24. Murdoch, B., C. DelConte, and M.I. Garcia-Castro, *Pax7 lineage contributions to the mammalian neural crest*. PLoS One, 2012. **7**(7): p. e41089.
25. You, J.S., et al., *ARHGEF3 Regulates Skeletal Muscle Regeneration and Strength through Autophagy*. Cell Rep, 2021. **34**(1): p. 108594.
26. Nance, M.E., et al., *AAV9 Edits Muscle Stem Cells in Normal and Dystrophic Adult Mice*. Mol Ther, 2019. **27**(9): p. 1568-1585.
27. Muraine, L., et al., *Transduction Efficiency of Adeno-Associated Virus Serotypes After Local Injection in Mouse and Human Skeletal Muscle*. Hum Gene Ther, 2020. **31**(3-4): p. 233-240.
28. Li, F., et al., *PHD3 mediates denervation skeletal muscle atrophy through Nf-kappaB signal pathway*. FASEB J, 2021. **35**(4): p. e21444.
29. Bond, S.T., et al., *Tissue-specific expression of Cas9 has no impact on whole-body metabolism in four transgenic mouse lines*. Mol Metab, 2021. **53**: p. 101292.
30. Choo, H.J., et al., *A tale of two niches: differential functions for VCAM-1 in satellite cells under basal and injured conditions*. Am J Physiol Cell Physiol, 2017. **313**(4): p. C392-C404.
31. Cassado, A.D., *F4/80 as a Major Macrophage Marker: The Case of the Peritoneum and Spleen*. Macrophages: Origin, Functions and Biointervention, 2017. **62**: p. 161-179.
32. Trowbridge, I.S. and M.L. Thomas, *Cd45 - an Emerging Role as a Protein-Tyrosine-Phosphatase Required for Lymphocyte-Activation and Development*. Annual Review of Immunology, 1994. **12**: p. 85-116.
33. Yang, W.J. and P. Hu, *Skeletal muscle regeneration is modulated by inflammation*. Journal of Orthopaedic Translation, 2018. **13**: p. 25-32.
34. Zhang, L., et al., *Chemokine CXCL16 regulates neutrophil and macrophage infiltration into injured muscle, promoting muscle regeneration*. Am J Pathol, 2009. **175**(6): p. 2518-27.
35. Yahiaoui, L., et al., *CC family chemokines directly regulate myoblast responses to skeletal muscle injury*. J Physiol, 2008. **586**(16): p. 3991-4004.
36. Griffin, C.A., et al., *Chemokine expression and control of muscle cell migration during myogenesis*. Journal of Cell Science, 2010. **123**(18): p. 3052-3060.
37. Rao, P. and D.A. Monks, *A tetracycline-inducible and skeletal muscle-specific Cre recombinase transgenic mouse*. Dev Neurobiol, 2009. **69**(6): p. 401-6.
38. Murphy, M.M., et al., *Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration*. Development, 2011. **138**(17): p. 3625-3637.

CHAPTER 4. MYOGENIC PROGENITOR CELL-DERIVED CCL8 NEGATIVELY REGULATES MUSCLE REGENERATION IN VIVO

4.1 Introduction

Chemokine (C-C motif) ligand 8 (Ccl8), also known as monocyte chemoattractant protein2 (MCP-2), was first identified in human osteosarcoma cells [1]. Since then, it has been identified as a chemoattractant for various immune cells including monocytes, lymphocytes, natural killer cells, dendritic cells, basophils, and eosinophils, and it is therefore involved in inflammatory responses [2]. Moreover, the role of Ccl8 in tumor cells has been studied. For example, Ccl8 contributes to breast cancer dissemination [3] and inhibits cervical carcinoma tumor growth [4].

The redundant and promiscuous relationships between ligands and receptors render the signaling of CC chemokine sub-family complex [5]. In particular, human Ccl8 has been identified as an agonist for several C-C motif chemokine receptors (CCRs), including CCR1, CCR2, CCR3, and CCR5 [5-7]. In mouse, however, Ccl8 was also identified as a CCR8 agonist to promote atopic dermatitis [8]. Furthermore, Ccl8 can form either homodimer or heterodimer with Ccl2 [9], which makes its signaling study challenging.

Expression of Ccl8 in skeletal muscle has been reported in C2C12 cells [10] and mouse primary myoblasts [11]. Moreover, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α) induced Ccl8 expression in human skeletal muscle cells [12], and irisin, an exercise-induced myokine, induced Ccl8 expression in C2C12 cells [13]. Interestingly, in *mdx* mice, a model of Duchenne muscular dystrophy, the

expression of both Ccl8 and its receptor, CCR2, is upregulated compared to WT [14]. Ccl8 expression was also increased in aged muscle during regeneration [15].

Myogenic function of Ccl8 had been poorly understood until Blanc et al. reported that a cocktail of CCR2-ligands (recombinant Ccl2, Ccl7 and Ccl8) inhibits myogenic differentiation of isolated mouse satellite cells and mouse regeneration *in vivo* [15]. CCR2 signaling plays a key role in inflammation [16], which is a necessary step for muscle regeneration upon injury, and all the data shown in this study are consistent with immune cell-derived CCR2 ligands regulating skeletal myogenesis. The authors' conclusions are drawn based on observations made with recombinant Ccl's rather than manipulating the endogenous ligands. Moreover, Ccl8 has not been examined directly, aside from the 3-ligand cocktail. Therefore, the specific role of Ccl8 in skeletal myogenesis and its relevant source (immune versus muscle cells) remain unclear. Here, I set out to further investigate the myogenic function of myogenic progenitor cell-expressed Ccl8, one of the CCR2 ligands, using the CRISPR/Cas9 mouse model described in Chapter 3.

4.2 Materials and Methods

4.2.1 Antibodies and other reagents

Anti-MHC (MF20) and anti-myogenin (F5D) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health and maintained by the University of Iowa, Department of Biological Sciences. Allophycocyanin (APC)-conjugated anti-mouse F4/80 (123116) and Phycoerythrin (PE)-conjugated anti-mouse Vascular cell adhesion protein 1 (VCAM1) antibodies were from BioLegend (San Diego, CA, USA). Peroxidase-conjugated anti-rabbit (115-036-003) and anti-mouse (111-036-003) IgG antibodies and FITC-conjugated anti-mouse IgG (115-096-

003) were from Jackson Immuno Research Laboratories (West Grove, PA, USA). Anti-tubulin (ab11304) was from Abcam (Cambridge, MA, USA). ELISA kit for detection of Ccl8 (DY790) was from R&D Systems (Minneapolis, MN, USA). 5-ethynyl-2'-deoxyuridine (EdU) was from Carbosynth Limited (Compton, UK). Fluorescein azide 5-isomer was from Lumiprobe (Hunt Valley, MD, USA). BMS CCR2 22 was from TOCRIS (Minneapolis, MN, USA).

4.2.2 Mice

The Cre recombinase-dependent Rosa26-loxP-Stop (3x polyA signal)-loxP-Cas9 (or LSL-Cas9) knock-in mice (B6J.129 (B6N)-Gt(ROSA)26Sor^{tm1(CAG-Cas9*, -EGFP)Fezh/J}; stock# 026175), Pax7-Cre mice (Pax7^{tm1(crc)Mrc}/J; stock# 010530) and Myf5-Cre mice (B6.129S4-Myf5^{tm3(crc)Sor}/J; stock# 007893) were obtained from Jackson Laboratory. To establish MPC-specific Cas9 knock-in mice, Pax7-Cre or Myf5-Cre mice were crossed with LSL-Cas9 knock-in mice. Genotyping was performed by PCR using genomic DNA extracted from mouse tails.

4.2.3 Cell culture

C2C12 myoblasts were a gift from S. Kaufman at the University of Illinois and originally obtained from ATCC. Cells were maintained in DMEM containing 4.5 g/L glucose, 10% fetal bovine serum, and 1% penicillin-streptomycin at 37°C with 7.5% CO₂. To induce differentiation, cells were plated on tissue culture plates coated with 0.2% gelatin, grown to 100% confluence, and then cultured in differentiation medium (DMEM containing 2% horse serum). The cells were replenished with fresh differentiation medium daily for 3 days.

4.2.4 Immunofluorescence microscopy and quantitative analysis of myocytes in vitro

C2C12 cells differentiated in 12-well plates were fixed with 3.7% formaldehyde and incubated with anti-MHC antibody, followed by incubation with DAPI and FITC-conjugated secondary antibody. Cells were observed with a Leica DMI 4000B fluorescence microscope (Leica, Wetzlar, Germany). The fluorescence images were captured using a RETIGA EXi camera (Qimaging, Surry, BC, Canada) and Image Pro Express software (Media Cybernetics, Rockville, MD, USA). Images were analyzed using ImageJ software (NIH, Bethesda, MD, USA). The fusion index was calculated as the percentage of nuclei in MHC-positive cells with more than one nucleus. Each data point was generated from quantifying five randomly chosen microscopic fields.

4.2.5 Western blotting

Cells were lysed in SDS sample buffer with 5% β -mercaptoethanol. Proteins were resolved on SDS-PAGE, transferred onto polyvinylidene fluoride membrane (EMD Millipore, Darmstadt, Germany) and incubated with various antibodies in accordance with manufacturers' recommendations. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA), and images were captured on an iBright CL1000 imaging system (Thermo Fisher Scientific, Waltham, MA, USA). Quantification of western blot band intensities was performed with densitometry of images using ImageJ software (NIH, Bethesda, MD, USA).

4.2.6 Measurement of secreted Ccl8

Media were collected from differentiating C2C12 cultures, and the Ccl8 amount was measured using the Mouse CCL8 DuoSet ELISA kit (R&D Systems) following the manufacturer's instructions.

4.2.7 SgRNA design and DNA construct

Short guide RNA (sgRNA) sequences targeting *Ccl8* exon 2 (sgCcl8) were identified using CRISPick (<https://portals.broadinstitute.org/gppx/crispick/public>), and the control sgRNA (sgControl) was adopted from Wang et al. [17]

sgCcl8 #1: 5'- CTTACACCACAGCTTCCATG -3'

sgCcl8 #2: 5'- TAAAGCTGAAGATCCCCCTT -3'

sgControl: 5'- GTAGCGAACGTGTCCGGCGT -3'

To validate the sgRNAs *in vitro*, the top and bottom strands of the sgRNAs were synthesized (Sigma-Aldrich) and cloned into lentiCRISPR v2 (a gift from Feng Zhang, Addgene plasmid #52961) [18]. For AAV-mediated sgRNA delivery, the same sequence of sgRNA strands were cloned into pX552 (a gift from Feng Zhang, Addgene plasmid #60958) [19].

4.2.8 Lentivirus production and transduction

For lentiviral packaging, lentiCRISPR-sgRNAs, psPAX2 (a gift from Didier Trono, Addgene plasmid #12260) and pMD2.G (a gift from Didier Trono, Addgene plasmid #12259) were co-transfected into 293T cells using PEI at 1 µg, 0.9 µg, and 0.1 µg respectively in a six-well plate. The medium was changed 12 – 18 h post-transfection, and the medium containing viruses were collected 36 – 42 h post-transfection. shRNAs in the

pLKO.1-puro vector were purchased from Sigma-Aldrich (MISSION TRC) and packaged into lentiviruses as described above. shRNA constructs are listed in Table 4.1.

C2C12 cells were infected with viruses in the presence of 8 $\mu\text{g/mL}$ polybrene (Sigma-Aldrich) overnight, and then subjected to selection by 3 $\mu\text{g/mL}$ puromycin (InvivoGen) for 48 – 72 h prior to inducing differentiation or downstream analysis.

4.2.9 Cell proliferation assay

C2C12 cells were incubated with EdU (final concentration of 1 μM) for 2 hours and then fixed with 3.7% formaldehyde. Cells were treated with 10 μM FAM azide 5-isomer, 1 mM CuSO_4 , and 100 mM Ascorbic acid in PBS for 30 minutes. After the treatment, nuclei were stained by DAPI for 30 minutes. Fluorescence microscopy and quantification were performed as described in “Immunofluorescence microscopy and quantitative analysis of myocytes” above.

4.2.10 T7 Endonuclease assay

From C2C12 cells, genomic DNA was isolated based on the protocol [20]. Briefly, cells were lysed with 50 mM NaOH and heated to 95°C for 30 min. After heating, 1 M Tris (pH 7.5) was added to neutralize the NaOH. Supernatant was used after centrifugation. Genomic DNA was isolated from mouse TA muscles using a DNeasy Blood Tissue Kit (Qiagen) according to the manufacturer’s instructions.

To detect the on-target or off-target (OT) mutations, each genomic region was amplified using Q5® High-Fidelity DNA Polymerase (New England Biolabs) with the primers listed in Table 4.2. For off-target mutation analysis, top five-ranked potential off-target sites were identified using crispr.mit.edu [21]. PCR products were re-annealed and subsequently digested with T7 Endonuclease I (New England Biolabs) followed by agarose gel electrophoresis (2%) according to the manufacturer’s instructions.

4.2.11 AAV9 virus production

AAV9 viral particles were produced according to the previously described methods [22, 23]. HEK293T cells in 15-cm plate were transfected with 10 µg of pX552-sgRNA vector, 10 µg of pAAV2/9n vector (a gift from James M. Wilson, Addgene plasmid #112865), and 10 µg of pAdDeltaF6 (a gift from James M. Wilson, Addgene plasmid #112867) at a ratio of 1:1:1 using PEI. Eighteen to 24 h after transfection, the cells were changed to growth medium. Cells were harvested 72 h post-transfection by dissociating from plates using a cell scraper in the presence of 2 mL of lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl), followed by three cycles of freezing-thawing using liquid nitrogen and 37°C water bath. The lysate was incubated with benzonase (Sigma-Aldrich) for 30 min at 37°C followed by centrifugation at 10,000 *g* for 10 min at room temperature. The supernatant was filtered with a 0.45 µm sterile filter and added with 0.5 mL of PEG solution (4 M NaCl and 40% PEG8000 (w/v), Sigma-Aldrich) to each 2 mL of supernatant. The mixtures were gently inverted to mix and then kept at 4°C overnight to precipitate the virus particles, followed by centrifugation at 12,000 *g* for 30 min at 4°C. The pellet was re-suspended with sterile PBS. Equal volume of chloroform was added and vortexed 2 min, followed by centrifugation at 12,000 *g* for 15 min at 4°C. Top aqueous layer containing AAV particles was collected and placed under a chemical hood for 30 min to evaporate the remaining chloroform. The aqueous layer, which contains AAV particles, was filtered through a 0.22 µm sterile filter and then using a 100 kDa MWCO centrifugal filter unit (Millipore). After removing the filtrate, 5 mL of PBS was added inside the cup of the centrifugal filter unit and centrifuged. The PBS addition and filtration were repeated two more times to wash the AAV solution, and 200 – 250 µl of virus in PBS was collected after the third wash. AAV9 titer was determined by qRT-PCR using qPCR AAV Titer Kit (Applied Biological Materials Inc.) according to the manufacturer's instructions.

4.2.12 Injury-induced muscle regeneration, cryosection, and hematoxylin and eosin staining

Male or female Pax7-Cas9 or Myf-Cas9 mice aged 10 – 12 weeks were used in all the regeneration experiments. Muscle injury was induced by injection of 20 μ l of 2% (w/v) BaCl₂ dissolved in saline into mouse TA muscles. To deliver sgRNAs, 4 x 10⁸ – 1 x 10⁹ vg of AAV9 expressing sgCcl8 #1 (and sgControl as a negative control) was co-injected with BaCl₂ into mouse TA muscles. The injured muscles were collected 7 or 14 days after injury (AI) and subjected to RNA isolation, genomic DNA isolation or cryosection and staining. Isolated injured muscles were frozen in liquid nitrogen-cooled 2-methylbutane and embedded in TBS tissue freezing medium (Thermo Fisher Scientific, Waltham, MA, USA). Sections of 10 μ m thickness were obtained with a Microm HM550 (Thermo Fisher Scientific, Waltham, MA, USA) at -20°C, placed on uncoated slides, and stained with hematoxylin and eosin (H&E). Images were obtained by DMI 4000B fluorescence microscope (Leica, Wetzlar, Germany) with a 20X dry objective (Fluotar, numerical aperture 0.4; Leica). Five bright-field images were randomly captured using RETIGA Exi camera (Qimaging, Surry, BC, Canada) and Image Pro Express software (Media Cybernetics, Rockville, MD, USA). The images were analyzed for CSA of all centrally nucleated regenerating myofibers (H&E staining) using ImageJ. Image acquisition and CSA quantification were performed by investigators that were blinded to sample identification.

4.2.13 Quantitative RT-PCR

C2C12 cells or isolated muscle tissues were lysed in Trizol (Invitrogen, Carlsbad, CA, USA), and RNA was isolated following the manufacturer's protocol. cDNA was synthesized from 1 μ g RNA using the qScript™ cDNA Synthesis kit (Quantabio, Beverly,

MA, USA) following the manufacturer's protocol. Quantitative PCR was performed with a StepOne Plus (Applied Biosystems, Foster City, CA, USA) using gene-specific primers. Hypoxanthine guanine phosphoribosyltransferase (Hprt) or β -actin or were used as a reference to obtain the relative fold change for target samples using the comparative C_T method. Primers used for quantitative RT-PCR are listed in Table 4.3.

4.2.14 Isolation of Cas9+, VCAM1+ cells and macrophages by FACS

Injured TA muscles of Myf5-Cas9 mice were collected 5 days AI and digested with Collagenase II (Worthington Biochemical Corporation, Lakewood, NJ, USA) and Dispase II (Roche Diagnostics, Mannheim, Germany) as reported previously [24], followed by incubation with APC-conjugated mouse F4/80 antibody. Injured TA muscles of Pax7-Cas9 mice were collected 5 days AI and digested as described above, followed by incubation with APC-conjugated mouse F4/80 antibody and PE-conjugated mouse VCAM1 antibody. These stained cells were subjected to fluorescence activated cell sorting (FACS). Single-stained positive controls were used for setting compensation. BD FACS Aria II cell sorting system (BD Bioscience, San Jose, CA) was used for collecting the cells with GFP+ (proxy of Cas9) or APC+ from Myf5-Cas9 mice and APC+ or PE+ from Pax7-Cas9 mice. The collected cells were subjected to RNA extraction and qRT-PCR analysis.

4.2.15 Statistical analysis

The sample size for each experiment is described in figure legends. All the quantitative values are presented as mean $\pm$ SEM. Whenever necessary, statistical significance of the data was analyzed by performing two-tailed paired Student's *t*-tests. The specific types of test and the *P*-values, when applicable, are indicated in the figure legends. Statistical comparison was considered significant when *P* < 0.05. All statistical analysis was performed using Excel.

4.2.16 Study approval

All the animal experiments in this study followed protocols approved by the Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

4.3 Results

4.3.1 Depletion of endogenous Ccl8 enhances differentiation in C2C12 cells

The expression of Ccl8 had been reported in myoblasts [10, 11]. We wondered whether Ccl8 expression level changed during myogenic differentiation. Indeed, we observed that the level of secreted Ccl8 increased by ~3-fold at 24 h of differentiation and returned to pre-differentiation level by 72 h (Figure 4.1 A).

To examine a potential role of endogenous Ccl8 in myogenic differentiation, we delivered shRNAs by lentiviral transduction into C2C12 myoblasts. As shown in Figure 4.1 B, two independent shRNAs (shCcl8) significantly knocked down Ccl8 mRNA compared to a negative control shRNA with a scrambled hairpin sequence (shScr). In addition, increased differentiation was observed, which was quantified by fusion index (Figure 4.1 C). Expression of the myogenin (early myogenic marker) or MHC (late myogenic marker) was found to be upregulated upon Ccl8 knockdown in C2C12 cell (Figure 4.1 D).

We then asked whether Ccl8 affected cell cycle withdrawal, which is one of the earliest events of myogenesis. As shown in Figure 4.1 E, the two shRNAs did not elicit a consistent effect on EdU incorporation at 0-hour or 24-hour differentiation, or during myoblast proliferation (Figure 4.1 F). Therefore, it does not seem likely that the myogenic effect of Ccl8 is through modulation of cell cycle withdrawal. Taken together, our

observations suggest that Ccl8 is likely a myogenic progenitor cell-derived negative regulator of myogenic differentiation.

4.3.2 Ccl8 may not use CCR2 as a cognate receptor to regulate myogenic differentiation

Although a cocktail of three cytokines (Ccl2/7/8) negatively regulates skeletal myogenesis [15], to the best of our knowledge none of the individual cytokine has been examined for its endogenous function on myogenic differentiation. Therefore, we reasoned that it could not be assumed Ccl2 or Ccl7 would play a similar role as Ccl8, and we set out to investigate Ccl2 and Ccl7. We were able to detect the mRNA expression of both cytokines in C2C12 cells; expression of Ccl2 was upregulated by ~2.5-fold at 24 h of differentiation, whereas Ccl7 expression throughout differentiation did not change with any statistical significance (Figure 4.2 A).

To knock down Ccl2 and Ccl7, I tested 5 previously unvalidated shRNAs for Ccl2 and 2 pre-validated shRNAs for Ccl7 via lentiviral transduction into C2C12 myoblasts. Each shRNA significantly knocked down the target cytokine (Figure 4.2 B). Interestingly, knockdown of Ccl2 did not consistently impact myogenic differentiation, whereas knockdown of Ccl7 inhibited differentiation (Figure 4.2 C). This observation suggests that Ccl2 and Ccl7 may not have the same function as Ccl8 in myogenesis.

To probe a potential role of CCR2, a receptor for Ccl2/7/8, I first measured mRNA expression of CCR2 in C2C12 myoblasts and found it to be upregulated upon differentiation (Figure 4.3 A). Three independent shRNAs significantly knocked down CCR2 (Figure 4.3 B), but they did not result in a consistent differentiation phenotype (Figure 4.3 C). As an alternative means to investigate CCR2 signaling, I treated C2C12 cells with BMS CCR2 22 (Ccr2i), a small molecule inhibitor of CCR2 [25]. Blanc et al.

previously reported that 1 nM of Ccr2i rescued primary myoblast differentiation from the inhibitory effect of a cocktail of Ccl2/7/8 [15]. However, I did not observe a significant effect with Ccr2i at up to 100 nM (Figure 4.3 D).

There is a redundant and promiscuous relationship among the ligands and receptors in the CC chemokine sub-family [5]. It is possible that Ccl8 can bind to not only CCR2, but also other receptor(s) to regulate myogenic differentiation. For example, Ccl8 was previously reported as a CCR8 agonist in mouse dermatitis [8]. Similar to CCR2, I also observed upregulated CCR8 expression during C2C12 differentiation (Figure 4.3 E). To date I have not been able to examine the effect of CCR8 knockdown on differentiation because the two available shRNAs were not effective in knockdown (Figure 4.3 F). Nevertheless, our collective data hint at the possibility that muscle-derived Ccl8 has a function distinct from Ccl2 and Ccl7, and that Ccl8 may signal through a receptor other than CCR2 to regulate myogenic differentiation.

4.3.3 MPC-specific depletion of Ccl8 accelerates muscle regeneration

Next, to investigate the potential role of MPC-derived Ccl8 in muscle regeneration in vivo, we wanted to make use of the CRISPR/Cas9 system established in Chapter 3. To first validate sgRNA in vitro, we designed two sgRNAs targeting the *Ccl8* exon 2 region (sgCcl8 #1 and sgCcl8 #2). To test the on-target editing efficiency of each sgRNA, we expressed Cas9 and sgRNA by lentiviral transduction into C2C12 myoblasts. Using T7 Endonuclease I assay, we found that sgCcl8 #1 displayed higher on-target editing efficiency (Figure 4.4 A) with no evidence of off-target mutagenesis (Figure 4.4 B). Therefore, sgCcl8 #1 was chosen for further study.

To further validate sgCcl8 #1, we transduced C2C12 cells with lentiviruses co-expressing Cas9 and sgCcl8 #1 followed by differentiation. As shown in Figure 4.4 C, the

secreted Ccl8 level was significantly dampened upon the viral transduction and enhanced myogenic differentiation was also observed as measured by fusion index (Figure 4.4 D).

We then set out to probe MPC-derived Ccl8 function in vivo using an injury-induced muscle regeneration model in mice. We co-injected AAV9 expressing sgCcl8 with BaCl₂ into TA muscles Pax7-Cas9 and Myf5-Cas9 mice and isolated the injected muscles for analysis 7 days or 14 days AI. Similar to the in vitro results, delivery of AAV9-sgCcl8 into mouse TA muscle led to on-target gene editing (Figure 4.5 A) without detectable off-target editing effect (Figure 4.5 B) from both types of Cas9 transgenic mice. Moreover, we observed that Ccl8 mRNA expression was downregulated upon the delivery of sgRNA targeting Ccl8 (Figure 4.5 C). We wondered whether this downregulation of Ccl8 expression is limited to muscle cell. As preliminary data (n=1), I used FACS to sort out either GFP+ (proxy for Cas9) or F4/80+ (pan-macrophage marker) 5 days after co-injection of BaCl₂ and AAV9-sgCcl8 (or sgControl), followed by RNA isolation and qRT-PCR. As shown in Figure 4.5 D, Ccl8 knockdown was observed in GFP+ cells, but not in F4/80 (APC) + cells. However, the knockdown level of Ccl8 in GFP+ cells was similar to cells before sorting. This could be partly due to inconsistent injury induction throughout 4 mice whose TA muscles were pooled for FACS. Considering the possibility of GFP expression in non-muscle cells, better sorting strategy might be necessary. One example is sorting out VCAM1 (marker for satellite cells [26]). Indeed, VCAM1 (PE) + cells showed higher knockdown level of Ccl8 compared to cells before sorting from another preliminary data (Figure 4.5 E). Future investigation of CD45+ (hematopoietic cell marker [27]) besides F4/80+ cells can also corroborate myogenic cell-specific Ccl8 depletion.

We next examined the degree of regeneration by measuring the average cross section area (CSA) of regenerating myofibers. Interestingly, delivery of sgCcl8 into Pax7-Cas9 transgenic mice significantly increased CSA of regenerating myofiber size on Day 7

(but not Day 14) AI (Figure 4.5 F). Similarly, injection of AAV-sgCcl8 into Myf5-Cas9 TA muscles also increased the CSA of regenerating myofibers on Day 7 AI and not on Day 14 AI (Figure 4.5 G). Taken together, our data show that depletion of MPC-derived Ccl8 accelerates muscle regeneration.

4.4 Discussion

Ccl8 has been identified as a chemoattractant for various types of immune cells, which are involved in inflammatory responses [2]. Since muscle injury elicits an inflammatory response, which coincides with muscle regeneration [28], reported upregulation of Ccl8 expression on Day 2 AI [15] could come from both muscle cells and immune cells. We have provided evidence for a role of Ccl8 in negatively regulating skeletal myogenesis in both C2C12 myoblasts and an in vivo muscle regeneration model. Furthermore, taking advantage of a myogenic progenitor cell-specific CRISPR/Cas9 mouse model I have developed, my study is the first to probe a relevant source of the cytokine in vivo and to offer direct evidence for a negative role of MPC-derived Ccl8 in muscle regeneration. Our finding reveals an autocrine function of Ccl8 in vivo, in contrast to the widely presumed (but not proven) role of immune cells as a source of this cytokine in vivo.

Although CCR2 is shown to mediate the effect of a cocktail of Ccl2, Ccl7 and Ccl8 on muscle regeneration [15], our results suggest that muscle-derived Ccl8 may use another receptor to exert a unique function not shared with Ccl2 and Ccl7. CCR8 as the potential receptor for Ccl8 in this context should be studied in future investigations. Additional candidates to be investigated include CCR1 and CCR5, which were identified

as the receptors for Ccl8 in human glioblastoma cells [29], and interaction between human Ccl8 and human CCR3 using in vitro binding assay was also reported [30].

Ccl8 expression has been reported to increase in aged muscle [15] and dystrophic muscles of *mdx* mice [14], and it is widely assumed that elevated inflammation contributes to the increased Ccl8 expression. However, muscle as a source of this cytokine in vivo has not been ruled out. The mouse model I have established can facilitate future studies to distinguish the impacts of MPC- or muscle-derived and immune cell-derived Ccl8 to regeneration during aging and/or muscular dystrophy. Many other muscle-derived cytokines can be investigated with the same approach.

4.5 Figures and Tables

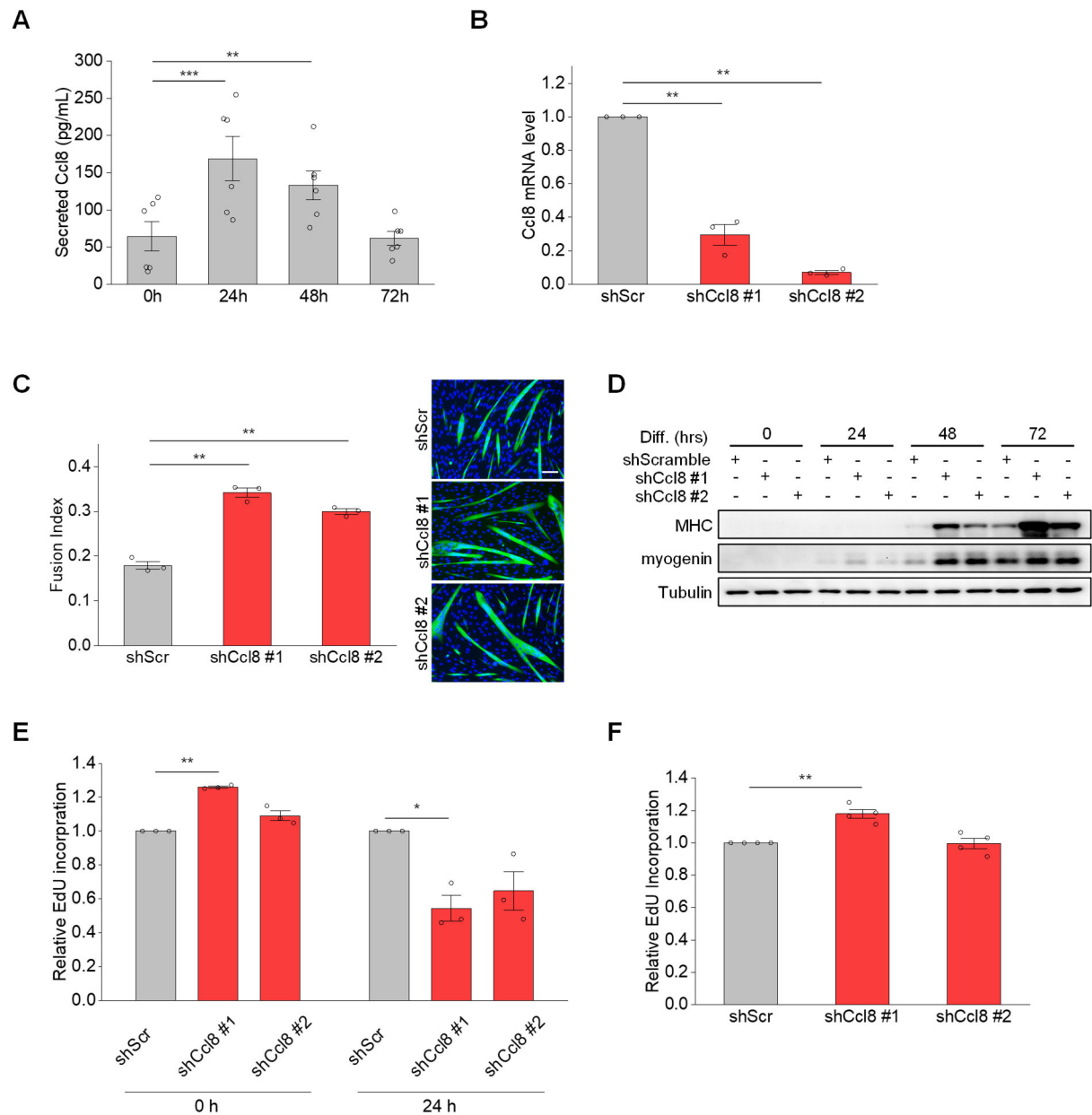


Figure 4.1 shRNA mediated Ccl8 knockdown promotes myoblast differentiation.

(A) Culture medium during C2C12 differentiation was collected at different time points, and ELISA assay was performed to determine secreted Ccl8 levels (n=6).

(B) C2C12 cells were transduced with lentiviruses expressing shCcl8 or shScramble (shScr) followed by 2 days of puromycin selection. Cell lysates were then collected and subjected to qRT-PCR analysis (n=3).

(C) Cells treated as in (B) were induced to differentiate for 72 hrs, followed by staining for MHC (green) and DAPI (blue), and fusion index was quantified (n=3).

Figure 4.1 (cont.)

(D) Cells treated as in (B) were induced to differentiate, and at different time points, cells were lysed and subjected to western analysis. Representative blots are shown (n=3).

(E) At 0 or 24 hr of differentiation, cells treated as in (B) were labeled with EdU for 2 hrs in differentiation medium, stained for EdU and DAPI, and the percentage of cells positive for EdU staining was quantified. Data were normalized to shScr (n=3).

(F) Cells treated as in (B) were grown ~20% confluence and labeled with EdU for 2 hrs in growth medium and processed as (E) (n=4). Data are presented as mean with error bars representing SEM. Two-tailed *t*-test was performed to compare each data to control. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Scale bars: 100 μ m.

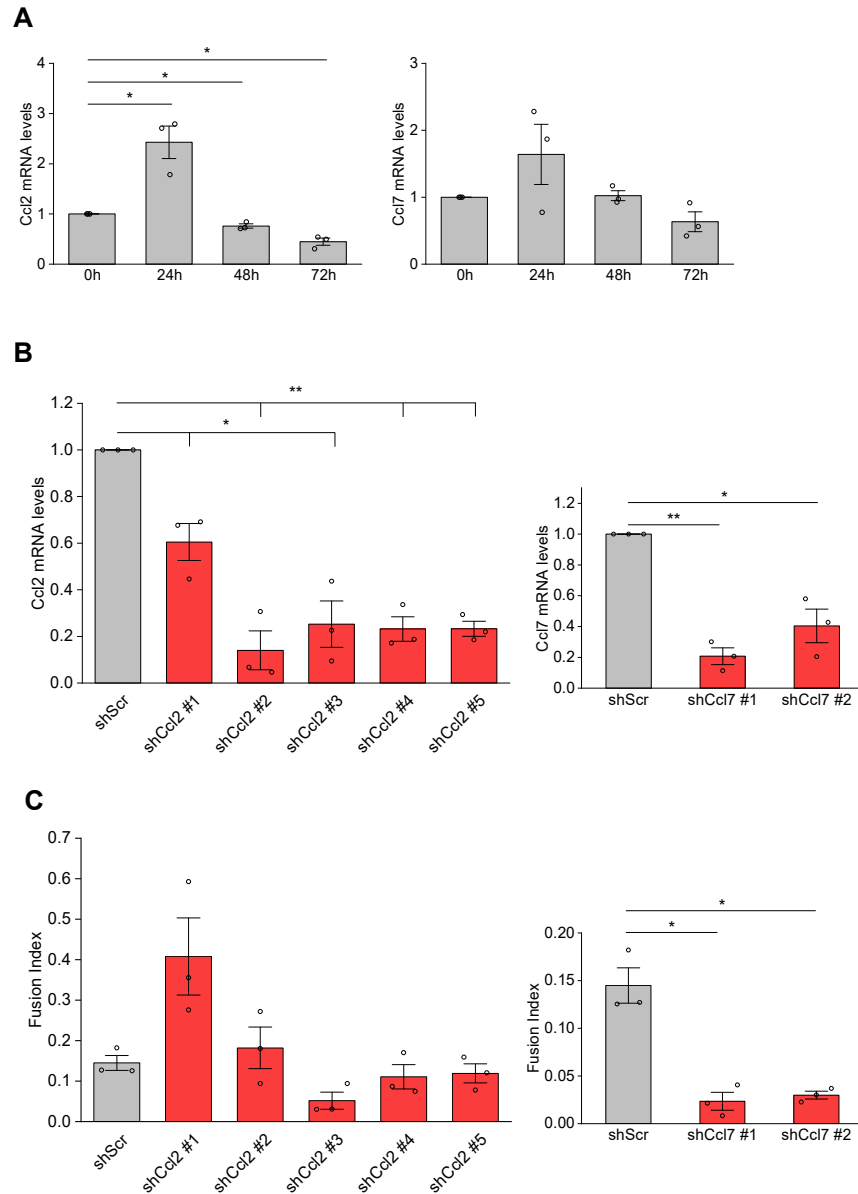


Figure 4.2 Potential effect of endogenous Ccl2 or Ccl7 on myogenic differentiation.

(A) RNA was isolated from differentiating C2C12 cells at different time points followed by qRT-PCR analysis (n=3).

(B) C2C12 cells were transduced with lentiviruses expressing shCcl2, shCcl7 or shScramble (shScr) followed by 2 days of puromycin selection. Cell lysates were then collected and subjected to qRT-PCR analysis (n=3).

(C) Cells treated as in (B) were induced to differentiate for 72 hrs, followed by staining for MHC and DAPI, and fusion index were quantified (n=3). Data are presented as mean with error bars representing SEM. Two-tailed *t*-test was performed to compare each data to control. **P* < 0.05; ***P* < 0.01.

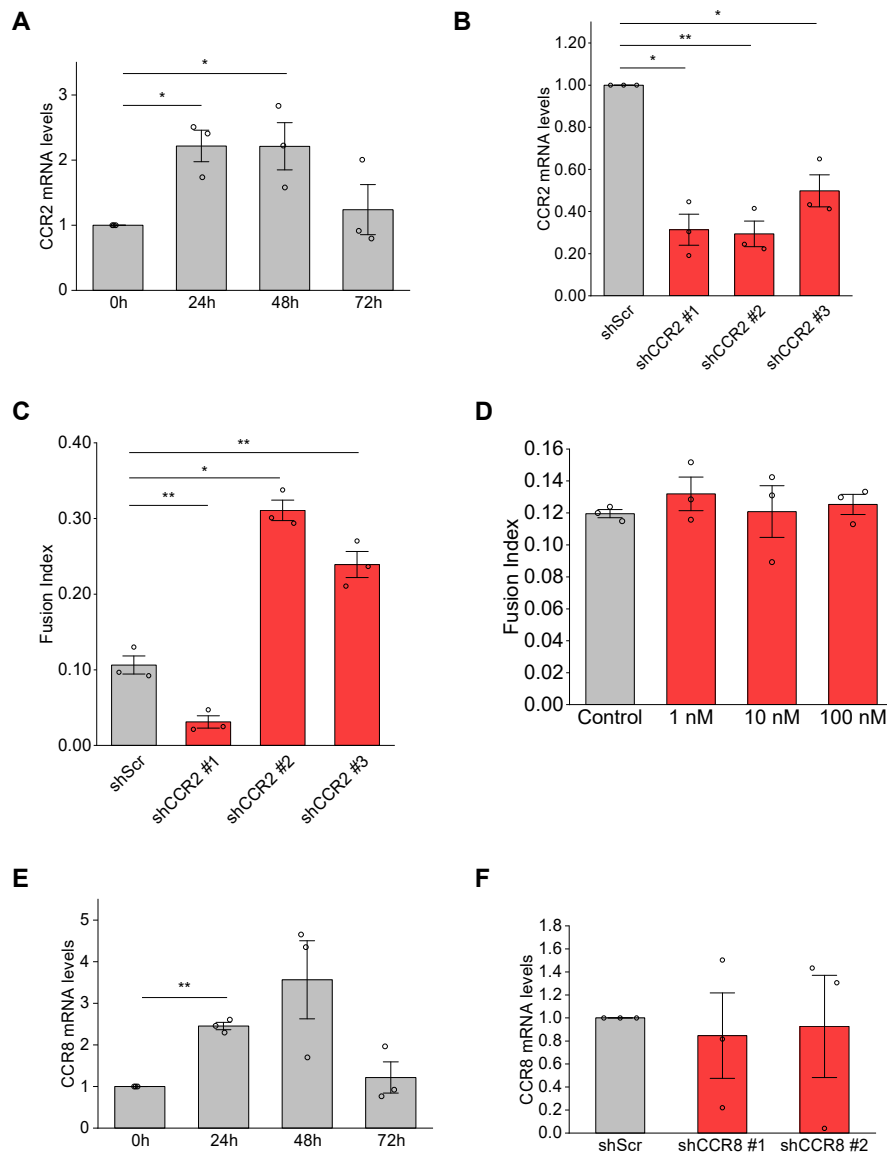


Figure 4.3 Potential effect of CCR2 on myogenic differentiation.

(A) RNA was isolated from differentiating C2C12 cells at different time points followed by qRT-PCR analysis (n=3).

(B) C2C12 cells were transduced with lentiviruses expressing shCCR2 or shScramble (shScr) followed by 2 days of puromycin selection. Cell lysates were then collected and subjected to qRT-PCR analysis (n=3).

(C) Cells treated as in (B) were induced to differentiate for 72 hrs, followed by staining for MHC and DAPI and fusion index were quantified (n=3).

(D) C2C12 cells were induced to differentiation for 72 hrs with indicated concentration of Ccr2i, followed by staining for MHC and DAPI and fusion index were quantified (n=3).

Figure 4.3 (cont.)

(E) RNA was isolated from differentiating C2C12 cells at different time points followed by qRT-PCR analysis (n=3).

(F) C2C12 cells were transduced with lentiviruses expressing shCCR8 or shScramble (shScr) followed by 2 days of puromycin selection. Cell lysates were then collected and subjected to qRT-PCR analysis (n=3). Data are presented as mean with error bars representing SEM. Two-tailed *t*-test was performed to compare each data to control. **P* < 0.05; ***P* < 0.01.

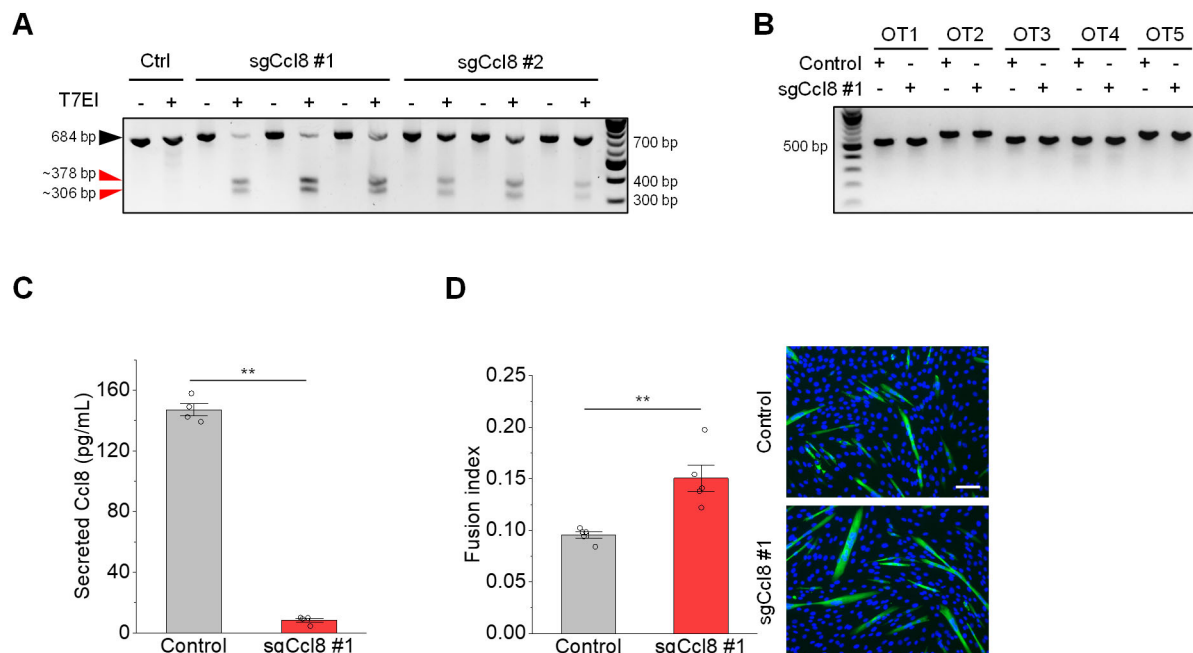


Figure 4.4 sgRNA mediated Ccl8 depletion promotes myoblast differentiation.

(A) C2C12 cells were transduced with lentiviruses expressing Cas9 and sgRNA (sgCcl8 #1, sgCcl8 #2 or Control, Ctrl) followed by 3 days of puromycin selection. Genomic DNAs were isolated and subjected to T7 Endonuclease I assay upon PCR amplification of on-target region (n=3).

(B) C2C12 cells were transduced with lentiviruses expressing Cas9 and sgRNA (sgCcl8 #1 or Control) followed by 3 days of puromycin selection. Isolated genomic DNAs were subjected to PCR amplification targeting 5 predicted off-target (OT) sites, followed by T7 Endonuclease I assay.

(C) Cells treated as in (B) were induced to differentiate for 24 hrs. Then the culture medium was collected, and ELISA assay was performed to determine secreted Ccl8 level (n=4).

(D) Cells treated as in (B) were induced to differentiate for 72 hrs, followed by staining for MHC (green) and DAPI (blue), and fusion index was quantified (n=5). Data are presented as mean with error bars representing SEM. Two-tailed *t*-test was performed to compare each data to control. *******P* < 0.01. Scale bars: 100 μ m.

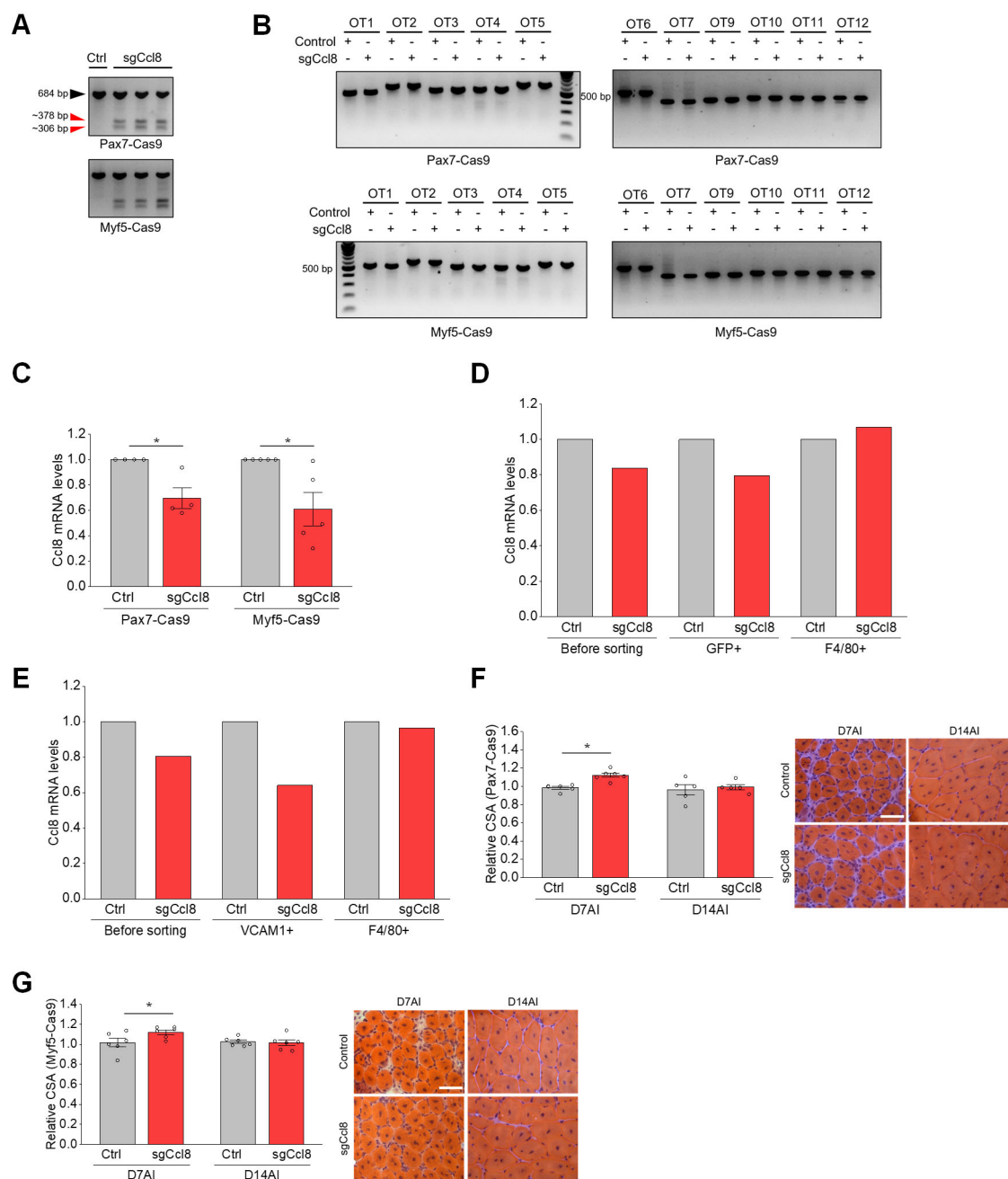


Figure 4.5 Myogenic progenitor cell-specific depletion of Ccl8 accelerates muscle regeneration.

(A) Mouse TA muscles (Pax7-Cas9 or Myf5-Cas9 mice) were co-injected with BaCl₂ and AAV9 expressing sgCcl8 or Control (Ctrl), isolated on Day 7 AI and subjected to genomic DNA isolation. On-target region was amplified by PCR followed by T7 Endonuclease I assay (n=3).

(B) Genomic DNAs isolated as in (A) were subjected to PCR amplification targeting 11 predicted off-target (OT) sites, followed by T7 Endonuclease I assay. Note: OT8 is not included because its genomic region could not be amplified.

Figure 4.5 (cont.)

(C) RNAs were isolated from TA muscles injected as in (A) and subjected to qRT-PCR (n=4 for Pax7-Cas9 mice, n=5 for Myf5-Cas9 mice).

(D) Mouse TA muscles of four Myf5-Cas9 mice were co-injected with BaCl₂ and AAV9 expressing sgCcl8 or Control (Ctrl), isolated on Day 5 AI and pooled for digestion. Digested cells were stained with APC-conjugated mouse F4/80 antibody, followed by FACS to sort out GFP+ or APC+ cells. Cells were then subject to qRT-PCR. Cells before and after FACS were analyzed (n=1).

(E) Mouse TA muscles of four Pax7-Cas9 mice were co-injected with BaCl₂ and AAV9 expressing sgCcl8 or Control (Ctrl), isolated on Day 5 AI and pooled for digestion. Digested cells were stained with APC-conjugated mouse F4/80 and PE-conjugated mouse VCAM1 antibodies followed by FACS to isolate APC+ or PE+ cells. Cells were then subject to qRT-PCR. Cells before and after FACS were analyzed (n=1).

(F) TA muscles (Pax7-Cas9 mice) injected as in (A) were isolated on Day 7 or Day 14 AI and subjected to cryosection. Upon H&E staining, cross-sectional area (CSA) was quantified for regenerating myofibers (n=6 for D7AI, n=5 for D14AI).

(G) TA muscles (Myf5-Cas9 mice) injected as in (A) were isolated on Day 7 or Day 14 AI and subjected to cryosection. Upon H&E staining, cross-sectional area (CSA) was quantified for regenerating myofibers (n=6 for each time point). Data were normalized to Control at each time point. Data are presented as mean with error bars representing SEM. Two-tailed *t*-test was performed to compare each data to control. **P* < 0.05. Scale bars: 50 μm.

Name	Clone ID	Clone Name
shCcl2 #1	TRCN0000034469	NM_011333.1-362s1c1
shCcl2 #2	TRCN0000034470	NM_011333.1-207s1c1
shCcl2 #3	TRCN0000034471	NM_011333.1-433s1c1
shCcl2 #4	TRCN0000034472	NM_011333.1-190s1c1
shCcl2 #5	TRCN0000034473	NM_011333.1-329s1c1
shCcl7 #1	TRCN0000068135	NM_013654.2-313s1c1
shCcl7 #2	TRCN0000068136	NM_013654.2-110s1c1
shCcl8 #1	TRCN0000068173	NM_021443.1-322s1c1
shCcl8 #2	TRCN0000068177	NM_021443.1-312s1c1
shCCR2 #1	TRCN0000001680	NM_009915.x-684s1c1
shCCR2 #2	TRCN0000001681	NM_009915.x-1065s1c1
shCCR2 #3	TRCN0000001682	NM_009915.x-714s1c1
shCCR8 #1	TRCN0000027957	NM_007720.1-408s1c1
shCCR8 #2	TRCN0000028001	NM_007720.1-125s1c1

Table 4.1 List of shRNA constructs

Name	Forward (5' to 3')	Reverse (5' to 3')
Ccl8 on-target	AGGTGATGGACAGCGAGTTC	CTTGCTCCAGAGGTGAAGGA
Ccl8 OT1	TACCTTCCAATCCCCTACCC	CTGGACTTTGCCATTGGATT
Ccl8 OT2	TGTTGGAGTTCTGCAACCTG	CTTGAGCTGATGCCTTCCTC
Ccl8 OT3	CTTGCAGTTGCTGGTGTGAT	AGCTCTGCGACTTCATCTCC
Ccl8 OT4	CACTCACACACAACGCACAC	TGCCTCACTCTCAGGAGGAT
Ccl8 OT5	AAGGCTGTTGGTGCTCATCT	GCCTCCTGGTTGTATTCTCG
Ccl8 OT6	GATGAAAGCAGAGGGAGGTG	AAGAGGCTACCCAGGGTGTT
Ccl8 OT7	TGGGATTTGAACTCCAGACC	CAGAGAAAGCAGGGGTATGC
Ccl8 OT9	CAGACCCACACCTTCCAGTT	CATGGCACTTTGTGAGATGG
Ccl8 OT10	TTGAAGAAGAGTGGGGGTTAGA	CCAGTGGGGACCTCAAAGTA
Ccl8 OT11	GTCTGGAAGGAACGCTTGTC	TGACCATCCAACAGGAGTGA
Ccl8 OT12	CCGCAGCTGTCACAAGTAAA	TCAAAGGCCAGAAGAAGGAA

Table 4.2 List of PCR primers used for on-target and off-target (OT) mutation detection

Name	Forward (5' to 3')	Reverse (5' to 3')
Ccl2	GTTGGCTCAGCCAGATGCA	AGCCTACTCATTGGGATCATCTTG
Ccl7	TCAAGAGCTACAGAAGGATCACC	ATAGCCTCCTCGACCCACTT
Ccl8	CACCTGCTGCTTTCATGTAC	TTGGTCTGGAAAACCACAGC
CCR2	ACAGCTCAGGATTAACAGGGACTTG	ACCACTTGCATGCACACATGAC
CCR8	TTCCTCTACTTAGGGAGACAAATGC	CATCCAGGGTGGAGAATGG
Hprt	ATGGACTGATTATGGACAGGACTG	TCCAGCAGGTCAGCAAAGAAC
β -Actin	TTGCTGACAGGATGCAGAAG	ATCCACATCTGCTGGAAGGT

Table 4.3 List of PCR primers used for quantitative RT-PCR

4.6 References

1. Decock, B., et al., *Identification of the monocyte chemotactic protein from human osteosarcoma cells and monocytes: detection of a novel N-terminally processed form*. Biochem Biophys Res Commun, 1990. **167**(3): p. 904-9.
2. Van Coillie, E., et al., *The human MCP-2 gene (SCYA8): cloning, sequence analysis, tissue expression, and assignment to the CC chemokine gene contig on chromosome 17q11.2*. Genomics, 1997. **40**(2): p. 323-31.
3. Farmaki, E., et al., *A CCL8 gradient drives breast cancer cell dissemination*. Oncogene, 2016. **35**(49): p. 6309-6318.
4. Struyf, S., et al., *Synergistic up-regulation of MCP-2/CCL8 activity is counteracted by chemokine cleavage, limiting its inflammatory and anti-tumoral effects*. Eur J Immunol, 2009. **39**(3): p. 843-57.
5. Zlotnik, A. and O. Yoshie, *The chemokine superfamily revisited*. Immunity, 2012. **36**(5): p. 705-16.
6. Gong, W., et al., *Monocyte chemotactic protein-2 activates CCR5 and blocks CD4/CCR5-mediated HIV-1 entry/replication*. J Biol Chem, 1998. **273**(8): p. 4289-92.
7. Gong, X., et al., *Monocyte chemotactic protein-2 (MCP-2) uses CCR1 and CCR2B as its functional receptors*. J Biol Chem, 1997. **272**(18): p. 11682-5.
8. Islam, S.A., et al., *Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5+ T(H)2 cells*. Nat Immunol, 2011. **12**(2): p. 167-77.
9. Crown, S.E., et al., *Heterodimerization of CCR2 chemokines and regulation by glycosaminoglycan binding*. J Biol Chem, 2006. **281**(35): p. 25438-46.
10. Henningsen, J., B.K. Pedersen, and I. Kratchmarova, *Quantitative analysis of the secretion of the MCP family of chemokines by muscle cells*. Molecular Biosystems, 2011. **7**(2): p. 311-321.
11. Griffin, C.A., et al., *Chemokine expression and control of muscle cell migration during myogenesis*. Journal of Cell Science, 2010. **123**(18): p. 3052-3060.
12. Mormeneo, E., et al., *PGC-1 alpha Induces Mitochondrial and Myokine Transcriptional Programs and Lipid Droplet and Glycogen Accumulation in Cultured Human Skeletal Muscle Cells*. Plos One, 2012. **7**(1).
13. Lee, J., et al., *Irisin promotes C2C12 myoblast proliferation via ERK-dependent CCL7 upregulation*. PLoS One, 2019. **14**(9): p. e0222559.
14. Mojumdar, K., et al., *Inflammatory monocytes promote progression of Duchenne muscular dystrophy and can be therapeutically targeted via CCR2*. EMBO Mol Med, 2014. **6**(11): p. 1476-92.
15. Blanc, R.S., et al., *Inhibition of inflammatory CCR2 signaling promotes aged muscle regeneration and strength recovery after injury*. Nat Commun, 2020. **11**(1): p. 4167.
16. Boniakowski, A.E., et al., *Murine macrophage chemokine receptor CCR2 plays a crucial role in macrophage recruitment and regulated inflammation in wound healing*. Eur J Immunol, 2018. **48**(9): p. 1445-1455.
17. Wang, T., et al., *Genetic Screens in Human Cells Using the CRISPR-Cas9 System*. Science, 2014. **343**(6166): p. 80-84.
18. Ran, F.A., et al., *Genome engineering using the CRISPR-Cas9 system*. Nature Protocols, 2013. **8**(11): p. 2281-2308.
19. Swiech, L., et al., *In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9*. Nat Biotechnol, 2015. **33**(1): p. 102-6.
20. Behringer, R., et al., *Preparation of Polymerase Chain Reaction Template DNA from Mouse Tail Tissue*. Cold Spring Harb Protoc, 2019. **2019**(4).

21. Hsu, P.D., et al., *DNA targeting specificity of RNA-guided Cas9 nucleases*. Nat Biotechnol, 2013. **31**(9): p. 827-32.
22. Kimura, T., et al., *Production of adeno-associated virus vectors for in vitro and in vivo applications*. Sci Rep, 2019. **9**(1): p. 13601.
23. Gaj, T. and D.V. Schaffer, *Adeno-Associated Virus-Mediated Delivery of CRISPR-Cas Systems for Genome Engineering in Mammalian Cells*. Cold Spring Harb Protoc, 2016. **2016**(11).
24. Liu, L., et al., *Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting*. Nature Protocols, 2015. **10**(10): p. 1612-1624.
25. Kredel, S., et al., *High-content analysis of CCR2 antagonists on human primary monocytes*. J Biomol Screen, 2011. **16**(7): p. 683-93.
26. Choo, H.J., et al., *A tale of two niches: differential functions for VCAM-1 in satellite cells under basal and injured conditions*. Am J Physiol Cell Physiol, 2017. **313**(4): p. C392-C404.
27. Trowbridge, I.S. and M.L. Thomas, *Cd45 - an Emerging Role as a Protein-Tyrosine-Phosphatase Required for Lymphocyte-Activation and Development*. Annual Review of Immunology, 1994. **12**: p. 85-116.
28. Tidball, J.G., *Inflammatory processes in muscle injury and repair*. Am J Physiol Regul Integr Comp Physiol, 2005. **288**(2): p. R345-53.
29. Zhang, X., et al., *CCL8 secreted by tumor-associated macrophages promotes invasion and stemness of glioblastoma cells via ERK1/2 signaling*. Lab Invest, 2020. **100**(4): p. 619-629.
30. Ge, B., et al., *Functional expression of CCL8 and its interaction with chemokine receptor CCR3*. BMC Immunol, 2017. **18**(1): p. 54.

CHAPTER 5. CONCLUSIONS AND PERSPECTIVES

Immune cells have always been considered a major source of cytokines that can regulate skeletal myogenesis. However, over the last two decades, skeletal muscles have been recognized as another source of cytokines with myogenic functions. During this time there have been various proteomic studies cataloging cytokines secreted by muscle cells [1-5]. The lack of functional studies of those muscle-derived cytokines prompted our lab to perform an RNAi-based screen of mouse cytokines in C2C12 myoblasts [6]. TRAIL emerged from this RNAi screen as a potential negative regulator of myogenic differentiation. As described in Chapter 2 of this thesis, we identified the negative role of TRAIL in skeletal myogenesis through inhibition of cell cycle withdrawal, and we also found that depletion of TRAIL using shRNA enhanced muscle regeneration upon injury [7]. Expression of TRAIL in immune cells such as neutrophils [8] or macrophages [9] and its role in inflammatory responses have been reported [10, 11]. Therefore, shRNA injected into muscle may affect TRAIL that is potentially expressed from immune cells. For the same reason, TRAIL knockout mice [12] may not be a proper model to study muscle-derived TRAIL's function on muscle regeneration.

While seeking a better system to study muscle cell-derived cytokines' function, we turned to CRISPR/Cas9 technology. In Chapter 3, we established a CRISPR/Cas9 mouse system to disrupt genes in a myogenic progenitor cell-specific manner. Cas9 was predominantly expressed in skeletal muscle from Cas9 transgenic mice (Pax7-Cas9 or Myf5-Cas9), and on-target editing and gene disruption were observed when I delivered AAV9-mediated sgRNA into TA muscles. In Chapter 4, I examined the potential function of MPC- or muscle-expressed Ccl8 on skeletal myogenesis. Since it has been reported that Ccl8 plays a role in inflammatory responses [13], it is necessary to distinguish the

source of Ccl8 to assess its MPC- or muscle-specific function. The Cas9 transgenic mice we generated are appropriate models for this purpose. Interestingly, AAV9-sgCcl8 mediated depletion of Ccl8 led to accelerated muscle regeneration in both Cas9 transgenic mice. Although there was a previous report that a cocktail of CCR2 ligands (Ccl2/7/8) inhibits muscle regeneration, our study is the first to reveal a function of the MPC-derived endogenous Ccl8 in skeletal myogenesis.

MPC- or muscle-specific gene activation using the CRISPR activation (CRISPRa) technology would be another useful tool for not only functional studies of the cytokines but also therapeutic applications. CRISPRa utilizes an endonuclease deficient Cas9 (dCas9) fused with transcription activators. By delivering sgRNAs targeting upstream region, gene of interest can be induced to overexpress [14]. For example, using the Cre-dependent dCas9-SunTag mouse system, in which transcription activators are bound to dCas9-fused protein scaffold termed SunTag [15, 16], cytokines can be overexpressed when combined with MPC- or muscle-specific promoters to drive Cre recombinase.

There have been various studies that show potential involvement of cytokines in muscle diseases such as muscular dystrophy [17], cachexia [18, 19], and sarcopenia [18, 20]. However, the focus is primarily on immune cell-derived cytokines since most of the muscle diseases are closely associated with inflammation. For example, elevated expression of IL-6, a pro-inflammatory cytokine, was reported in Duchenne muscular dystrophy (DMD) muscles [21], and involvement of IL-6 in cachexia was also previously reported [22]. Interestingly, elevated levels of Ccl8 together with Ccl2 and Ccl7 were observed in aged skeletal muscles, and inhibition of CCR2, which is a shared receptor known for its role in inflammatory signaling [23], led to enhanced muscle regeneration in older mice [24]. By crossing our Cas9 transgenic mice with relevant muscle disease models (e.g., mdx mice), potential roles of MPC-derived cytokines in the muscle diseases

can be further examined. In addition, aged Cas9 transgenic mice would be a convenient tool to investigate the potential impact of depleting MPC-derived cytokines on age-related muscle disorders.

5.1 References

1. Norheim, F., et al., *Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training*. Am J Physiol Endocrinol Metab, 2011. **301**(5): p. E1013-21.
2. Henningsen, J., B.K. Pedersen, and I. Kratchmarova, *Quantitative analysis of the secretion of the MCP family of chemokines by muscle cells*. Molecular Biosystems, 2011. **7**(2): p. 311-321.
3. Henningsen, J., et al., *Dynamics of the skeletal muscle secretome during myoblast differentiation*. Mol Cell Proteomics, 2010. **9**(11): p. 2482-96.
4. Yoon, J.H., et al., *Comparative proteomic analysis of the insulin-induced L6 myotube secretome*. Proteomics, 2009. **9**(1): p. 51-60.
5. Chan, C.Y., et al., *Identification of differentially regulated secretome components during skeletal myogenesis*. Mol Cell Proteomics, 2011. **10**(5): p. M110 004804.
6. Ge, Y., et al., *RNAi screen reveals potentially novel roles of cytokines in myoblast differentiation*. PLoS One, 2013. **8**(7): p. e68068.
7. Kim, D., et al., *Muscle-derived TRAIL negatively regulates myogenic differentiation*. Experimental Cell Research, 2020. **394**(1).
8. Koga, Y., et al., *Neutrophil-derived TNF-related apoptosis-inducing ligand (TRAIL): a novel mechanism of antitumor effect by neutrophils*. Cancer Res, 2004. **64**(3): p. 1037-43.
9. Kaplan, M.J., et al., *TRAIL (Apo2 ligand) and TWEAK (Apo3 ligand) mediate CD4+ T cell killing of antigen-presenting macrophages*. J Immunol, 2000. **164**(6): p. 2897-904.
10. Zoller, V., et al., *Trail (TNF-related apoptosis-inducing ligand) induces an inflammatory response in human adipocytes*. Sci Rep, 2017. **7**(1): p. 5691.
11. Cartland, S.P., et al., *TRAIL-Expressing Monocyte/Macrophages Are Critical for Reducing Inflammation and Atherosclerosis*. iScience, 2019. **12**: p. 41-52.
12. Sedger, L.M., et al., *Characterization of the in vivo function of TNF-alpha-related apoptosis-inducing ligand, TRAIL/Apo2L, using TRAIL/Apo2L gene-deficient mice*. Eur J Immunol, 2002. **32**(8): p. 2246-54.
13. Van Coillie, E., et al., *The human MCP-2 gene (SCYA8): cloning, sequence analysis, tissue expression, and assignment to the CC chemokine gene contig on chromosome 17q11.2*. Genomics, 1997. **40**(2): p. 323-31.
14. Shakirova, K.M., V.Y. Ovchinnikova, and E.B. Dashinimaev, *Cell Reprogramming With CRISPR/Cas9 Based Transcriptional Regulation Systems*. Front Bioeng Biotechnol, 2020. **8**: p. 882.
15. Tanenbaum, M.E., et al., *A Protein-Tagging System for Signal Amplification in Gene Expression and Fluorescence Imaging*. Cell, 2014. **159**(3): p. 635-646.
16. Zhou, H., et al., *In vivo simultaneous transcriptional activation of multiple genes in the brain using CRISPR-dCas9-activator transgenic mice*. Nat Neurosci, 2018. **21**(3): p. 440-446.
17. Bernasconi, P., et al., *Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine*. Journal of Clinical Investigation, 1995. **96**(2): p. 1137.
18. Cohen, S., J.A. Nathan, and A.L. Goldberg, *Muscle wasting in disease: molecular mechanisms and promising therapies*. Nature Reviews Drug Discovery, 2015. **14**(1): p. 58-74.
19. Fearon, K.C., D.J. Glass, and D.C. Guttridge, *Cancer cachexia: mediators, signaling, and metabolic pathways*. Cell Metabolism, 2012. **16**(2): p. 153-166.

20. Michaud, M., et al., *Proinflammatory cytokines, aging, and age-related diseases*. Journal of the American Medical Directors Association, 2013. **14**(12): p. 877-882.
21. Kostek, M.C., et al., *IL-6 signaling blockade increases inflammation but does not affect muscle function in the mdx mouse*. (1471-2474 (Electronic)).
22. Fearon, K.C., D.J. Glass, and D.C. Guttridge, *Cancer cachexia: mediators, signaling, and metabolic pathways*. Cell Metab, 2012. **16**(2): p. 153-66.
23. Gschwandtner, M., R. Derler, and K.S. Midwood, *More Than Just Attractive: How CCL2 Influences Myeloid Cell Behavior Beyond Chemotaxis*. Front Immunol, 2019. **10**: p. 2759.
24. Blanc, R.S., et al., *Inhibition of inflammatory CCR2 signaling promotes aged muscle regeneration and strength recovery after injury*. Nat Commun, 2020. **11**(1): p. 4167.