REGULATION OF SKELETAL MUSCLE INNERVATION AND ALS PATHOGENESIS BY MICRORNA 206

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To my wife Michelle,

and my Parents.

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REGULATION OF SKELETAL MUSCLE INNERVATION AND ALS PATHOGENESIS BY MICRORNA 206

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REGULATION OF SKELETAL MUSCLE INNERVATION AND ALS PATHOGENESIS BY MICRORNA 206

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Mentor: Eric N. Olson, Ph.D.

Motor neurons and the skeletal muscle fibers they innervate maintain an intimate relationship that requires bidirectional signaling for the establishment and maintenance of neuromuscular synapses and muscle function. Abnormalities in the regulation of neuromuscular gene expression often result in neuropathies and myopathies, reflecting the intimate communication between muscle and motor nerve. In this thesis, I present my studies on the function of microRNAs in neuromuscular synapse regeneration and neurodegenerative disease.

First, I show that the expression of a muscle-specific microRNA (miRNA), miR-206, is dramatically upregulated following surgical denervation of skeletal muscle and in a mouse model of amyotrophic lateral sclerosis (ALS). The responsiveness of the miR-206 gene to the state of motor innervation is dependent on binding sites for MyoD in an

upstream enhancer. Based on the upregulation of miR-206 following denervation and its synapse-enriched expression pattern, I hypothesized that miR-206 is an important regulator of neuromuscular junction (NMJ) physiology and I generated miR-206 mutant mice. Using these mice, I demonstrated that miR-206 is an essential regulator of neuromuscular synapse reinnervation following nerve injury. The requirement of miR-206 for efficient reinnervation reflects, at least in part, its repressive influence on histone deacetylase 4 (HDAC4). I also explored another function of miR-206, as an essential modulator of retrograde growth factor signaling during the progression of neurodegenerative disease. By crossing miR-206 mutant mice to G93A-SOD1 transgenic mice, which express a mutant form of superoxide dismutase (SOD), I determined that the loss of miR-206 accelerates the pathogenesis of ALS due to the loss of functional NMJs. Thus, the results of my thesis research demonstrate that miR-206 functions as a sensor of motor innervation and regulates a retrograde signaling pathway required for nerve-muscle interactions during stress and disease.

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Van Rooij, E., Sutherland, L.B., Liu, N., **Williams, A.H**., McAnally, J., Gerard, R.D., Richardson, J.A., and Olson, E.N. (2006). A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc. Natl. Acad. Sci. USA* **103(48)**, 18255-18260.

*equal contribution

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List of Abbreviations

ACh	acetylcholine
AChR	acetylcholine receptor
ALS	amyotrophic lateral sclerosis
BDNF	brain derived neurotrophic factor
BTX	bungarotoxin
bHLH	basic helix-loop-helix
BP	base pair
cDNA	complementary DNA
CMV	cytomegalovirus
CNS	central nervous system
Cx	connexin
DNA	deoxyribonucleic acid
E	embryonic day
EBD	Evan's blue dye
EDL	extensor digitorum longus
ERα	estrogen receptor α
ES	embryonic stem
FGF	fibroblast growth factor
FGFBP1	fibroblast growth factor binding protein 1
FSTL1	follistatin-like 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G/P	gastrocnemius/plantaris
H&E	hematoxylin and eosin
HDAC	histone deacetylase
hGH	human growth hormone
IGF	insulin-like growth factor
KB	kilobase
Hif1α	hypoxia inducible factor 1, alpha subunit
MADS	MCM1, agamous, deficiens, SRF

MDX	dystrophin-deficient mice
MEF2	myocyte enhancer factor 2
miRNA	microRNA
MRF	myogenic regulatory factor
NF	neurofilament
NMJ	neuromuscular junction
n.s.	non-significant
ORF	open reading frame
P	postnatal day
PBS	phosphate buffered saline
PCR	polymerase chain reaction
POLA1	Dna polymerase alpha 1
Q-PCR	quantitative PCR
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SIRPα	signal regulatory protein alpha
SOD1	superoxide dismutase 1
SRF	serum response factor
TA	tibialis anterior
UTR	untranslated region
UTRN	utrophin
ZNP	synaptotagmin

Chapter I

Introduction

Cardiac and skeletal muscle development are controlled by evolutionarily conserved networks of transcription factors that coordinate the expression of genes involved in muscle growth, morphogenesis, differentiation, and contractility. In addition to regulating the expression of protein-coding genes, recent studies have revealed that myogenic transcription factors control the expression of a collection of microRNAs (miRNAs), which act through multiple mechanisms to modulate muscle development and function. In most cases, miRNAs fine-tune the expression of target mRNAs, whereas in other cases they function as 'on-off' switches. MicroRNA control of gene expression appears to be especially important during muscle diseases, in which miRNAs participate in stress-dependent remodeling of striated tissues. The integration of miRNAs into the core muscle transcriptional program expands the precision and complexity of gene regulation in muscle cells because individual miRNAs are capable of regulating hundreds of mRNAs, and individual mRNAs can be targeted by many miRNAs.

Here I will review how miRNAs modulate the function of muscle cells. First, I will review the biogenesis and function of miRNAs in skeletal muscle development and disease. Then, I will describe the molecular mechanisms regulating neuromuscular synaptogenesis and maintenance during development and neurodegenerative disease.

Transcriptional regulation of skeletal muscle development

As has been shown for the development of other organ systems, skeletal muscle development is orchestrated by evolutionarily conserved networks of transcription factors (Olson, 2006). Skeletal muscle development is primarily controlled by interactions between myogenic regulatory factors (MRFs) and myocyte enhancer factor 2 (MEF2) family members on the promoters of muscle-specfic genes (Berkes and Tapscott, 2005).

MRFs are evolutionarily conserved members of a large family of DNA-binding transcription factors that contain a basic helix-loop-helix (bHLH) domain (Molkentin and Olson, 1996). MRFs dimerize with ubiquitous E proteins and bind to a consensus binding site (CANNTG) termed an E-box, which is present in the promoters of most muscle-specific genes (Olson, 1990). MyoD is the founding member of the MRF family of transcription factors and was discovered by its ability to convert a variety of cell types to myoblasts (Lassar et al., 1986). Subsequently, three closely related proteins; Myf5, myogenin, and MRF4, were identified based on their homology to MyoD and the ability to convert non-muscle cells to myoblasts (Braun et al., 1989; Edmondson and Olson, 1989; Miner and Wold, 1990). Mice lacking expression of both MyoD and Myf5 lack myoblasts, whereas deletion of either gene by itself results in normal skeletal muscle, reflecting the functional redundancy between these factors (Rudnicki et al., 1993). Mice lacking expression of myogenin die perinatally due to a lack of terminal differentiation of myoblasts (Hasty et al., 1993; Nabeshima et al., 1993). Thus, the MRFs constitute a family of transcription factors necessary and sufficient for myogenic differentiation.

Members of the MEF2 family of transcription factors interact with MRFs directly and indirectly to activate myogenic gene expression (Molkentin and Olson, 1996;

Potthoff and Olson, 2007). MEF2 proteins belong to the MADS (MCM1, agamous, deficiens, SRF) family of transcription factors that bind the consensus sequence YTA(A/T)$_4$TAR (Shore and Sharrocks, 1995). MEF2 was originally identified as a DNA-binding activity at the muscle creatine kinase (MCK) enhancer (Gossett et al., 1989). MEF2 factors alone are not sufficient to induce myogenesis, but cooperate with MRFs and other factors to amplify the myogenic differentiation program (Molkentin et al., 1995). While MEF2 factors are not sufficient to induce myogenesis, they do appear to be necessary. Muscle lineages are properly patterned and specified in *Drosophila Mef2* mutant embryos, but there is a complete block in differentiation of all muscle lineages, demonstrating the obligate role of MEF2 in myogenesis (Lilly et al., 1995).

Vertebrate genomes contain four *Mef2* genes- *Mef2a*, *b*, *c*, and *d* (Potthoff and Olson, 2007). Mice lacking expression of *Mef2c* specifically in skeletal muscle die perinatally due to the deterioration of myofibers and disorganized sarcomeres (Potthoff et al., 2007a). Knockdown of *mef2c* and *mef2d* expression in zebrafish also results in disorganization of skeletal muscle myofibers (Hinits and Hughes, 2007), reflecting the evolutionarily conserved function of MEF2 in activating the expression of structural and contractile proteins. In addition to activating the expression of protein-coding genes, MRFs and MEF2 factors have been shown to directly activate the expression of noncoding genes, such of miRNAs.

miRNA biogenesis and function

MiRNAs are small evolutionarily conserved non-coding RNAs that are transcribed by RNA polymerase II as long pri-miRNAs encoding one or more miRNAs (Bartel, 2004). Most miRNAs are transcribed as independent transcripts, but approximately a third are embedded within introns of protein-coding genes and processed following splicing of pre-messenger RNAs (van Rooij and Olson, 2007). Pri-miRNAs are processed in the nucleus by the proteins Drosha and DGCR8, which produce an ~70 nucleotide hairpin RNA, termed the pre-miRNA, which is subsequently exported to the cytoplasm where it is processed by Dicer to yield a duplex of RNA ~22 nucleotides long. This duplex is released from Dicer and the single-stranded mature miRNA is incorporated into the RNA-induced silencing complex (RISC) where it associates with complementary target mRNAs to induce gene silencing (Bartel, 2009) (**Figure 1.1**).

The processing and subcellular localization of miRNAs is beginning to be recognized as an additional layer of potential regulation by controlling the availability of mature miRNAs (Balzer and Moss, 2007; Leung and Sharp, 2006; Piskounova et al., 2008; Viswanathan et al., 2008). The importance of accessory proteins regulating miRNA biogenesis is underscored by discoveries of mutations in these proteins associated with human diseases (Melo et al., 2009; Perron and Provost, 2009).

MiRNAs regulate gene expression through the sequence-specific interactions with the 3' untranslated regions (UTRs) of target mRNAs resulting in translational repression or mRNA destabilization through an incompletely characterized mechanism (Bartel, 2009). The primary determinant of binding specificity to complementary target mRNAs is Watson-Crick base-pairing of nucleotides 2-8 at the 5' end of the

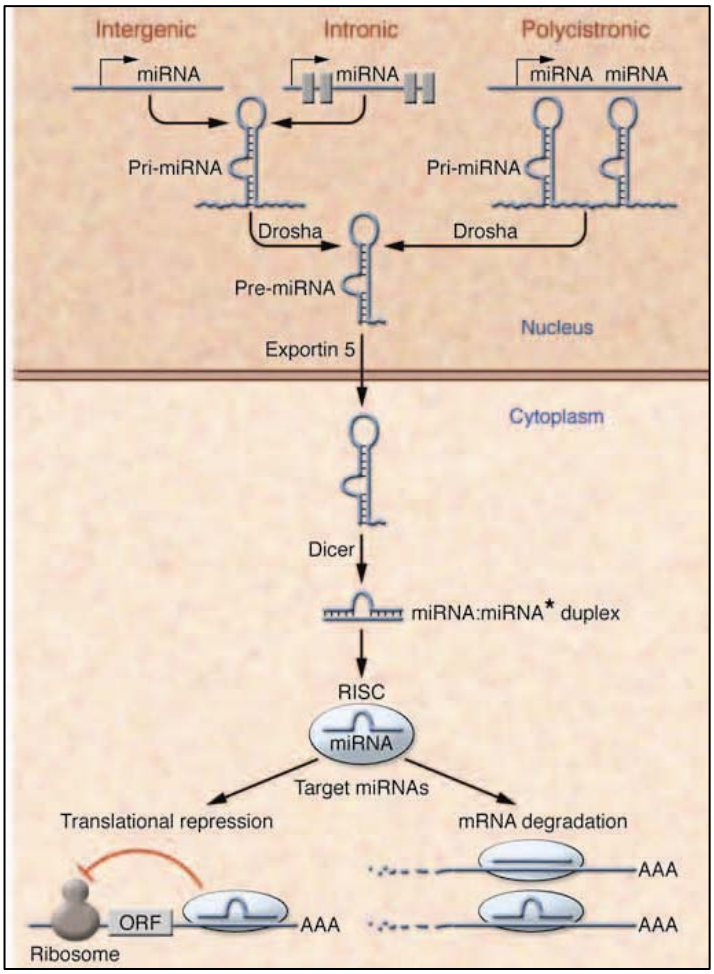

Figure 1.1. miRNA biogenesis and function. The primary transcripts of miRNA genes, termed pri-miRNAs and are transcribed from intergenic regions of the genome, the introns of protein-coding genes, or from polycistronic transcripts. The RNase enzyme Drosha processes pri-miRNAs into hairpin-shaped pre-miRNAs which are exported from the nucleus by Exportin 5. The enzyme Dicer cleaves the pre-miRNA into a double-stranded duplex, which is incorporated into the RISC complex and associates with target mRNAs to negatively regulate the target gene expression through translational repression or mRNA degradation. (Adapted from Van Rooij and Olson, 2007).

miRNA, referred to as the 'seed-sequence'. However, nucleotides outside of this region also influence mRNA repression, as does the secondary structure of the surrounding regions of the mRNA 3' UTR sequence (Lewis et al., 2005).

The identification and validation of *in vivo* targets of miRNAs is a significant challenge in the field. The degenerate base-pairing of miRNAs to target mRNAs means that individual miRNAs can potentially have hundreds of targets. In most cases, the effect of individual miRNAs on mRNA targets is generally quite modest (~2-fold). Thus, the summation of small changes in multiple mRNAs is likely responsible for the phenotypic effects of miRNAs, which is supported by proteomic profiling studies involving the over-expression and loss-of-function of individual miRNAs (Baek et al., 2008; Selbach et al., 2008).

Recent studies have also identified potentially new regulatory functions of miRNAs. One study demonstrated the ability of a miRNA to enhance the translation of target mRNAs in cells that have exited the cell cycle (Vasudevan et al., 2007). There is also evidence that small RNAs can directly control transcription through sequence-specific interactions with promoter elements of target genes (Schwartz et al., 2008). Perhaps, miRNAs can also function in a similar setting by directly conferring transcriptional, as well as post-transcriptional regulation on target genes.

Muscles without miRNAs

An essential role for miRNAs in mouse development was shown by a loss-of-function mutation in the miRNA-generating enzyme, Dicer, which results in embryonic lethality by day 7.5 (Bernstein et al., 2003). In order to circumvent the lethality associated with

the deletion of Dicer and to study the roles of Dicer in specific tissues, several groups have generated conditional null alleles of *Dicer*. Deletion of a conditional *Dicer* allele in embryonic skeletal muscle using a *MyoD* Cre recombinase transgene results in perinatal lethality due to skeletal muscle hypoplasia, demonstrating an essential role of miRNAs in muscle development (O'Rourke et al., 2007). While these studies illustrate the importance of miRNA processing for muscle development and function, they do not indicate whether this essential function of Dicer reflects requisite roles of specific miRNAs or multiple miRNAs in these processes.

Muscle-specific miRNAs

Several individual miRNAs are specifically expressed in cardiac and skeletal muscle (McCarthy, 2008) (**Table 1.1**). Of these, the most widely studied are members of the miR-1/206 and miR-133a/133b families, which originate from bicistronic transcripts on three separate chromosomes (Chen et al., 2006) (**Figure 1.2**). MiR-1-1 and miR-1-2 are identical and differ from miR-206 by four nucleotides, and miR-133a-1 and miR-133a-2 are identical and differ from miR-133b by two nucleotides (**Figure 1.2**). Cardiac and skeletal muscle-specific transcription of miR-1-1/133a-2 and miR-1-2/133a-1 in vertebrates appears to be controlled by two separate enhancers, one upstream and the other intronic (Liu et al., 2007; Rao et al., 2006; Zhao et al., 2005) (**Figure 1.2**). The myogenic transcription factors serum response factor (SRF), MEF2, and MyoD control the expression of miR-1 and miR-133a in cardiac and skeletal muscle. In the case of the *miR-1-2/133a-1* locus, SRF directs cardiac-specific expression through the upstream

miR	Target Gene(s)	Function
miR-1	*Hdac4*	Myoblast Differentiation
	Mef2	Neuromuscular Synapse Function
miR-133	*SRF*	Myoblast Proliferation, Smooth Muscle Gene Expression
miR-206	*Pola1, Connexin 43, Fst1, Utrn*	Myoblast Differentiation

Table 1.1. Table of muscle-specific miRNAs. Table of muscle-specific miRNAs with a list of experimentally determined target genes and proposed cellular functions.

enhancer and MEF2 directs ventricular expression through the intronic enhancer (Liu et al., 2007; Zhao et al., 2005). In addition, a miRNA microarray showed miR-1 and miR-133a to be among the most significantly downregulated miRNAs in *Srf* deficient hearts (Niu et al., 2008).

The third cluster of muscle-specific miRNAs encoding miR-206 and miR-133b is expressed specifically in skeletal muscle (Chen et al., 2006). Skeletal muscle-specific transcription of the *miR-206/133b* transcript is thought to be controlled by an upstream regulatory region that is enriched for MyoD binding, as assessed by ChIP-on-chip assays using chromatin from C2C12 muscle cells. Also, MyoD was shown to directly activate the transcription of *miR-206/133b* in an in vitro *MyoD* deficient fibroblast cell line (Rosenberg et al., 2006).

In vitro and *in vivo* studies have demonstrated that miR-1 and miR-133a regulate fundamental aspects of muscle biology such as differentiation and proliferation (**Figure I.3**). In C2C12 skeletal muscle cells, miR-1 represses the expression of HDAC4, a negative regulator of differentiation and a repressor of the MEF2 transcription factor (Chen et al., 2006). Thus, the repression of HDAC4 by miR-1 establishes a positive feed-forward loop in which the upregulation of miR-1 by MEF2 causes repression of HDAC4 and increased activity of MEF2, which drives myocyte differentiation.

In C2C12 myoblasts, miR-133a promotes proliferation, at least partly, by repressing SRF (Chen et al., 2006). The genetic interaction between miR-133a and SRF constitutes a negative feedback loop in which the upregulation of miR-133a by SRF results in increased repression of SRF. Genetic deletion of both *miR-133a-1* and *miR-133a-2* showed that SRF is a direct target of miR-133a *in vivo* and suggested that miR-

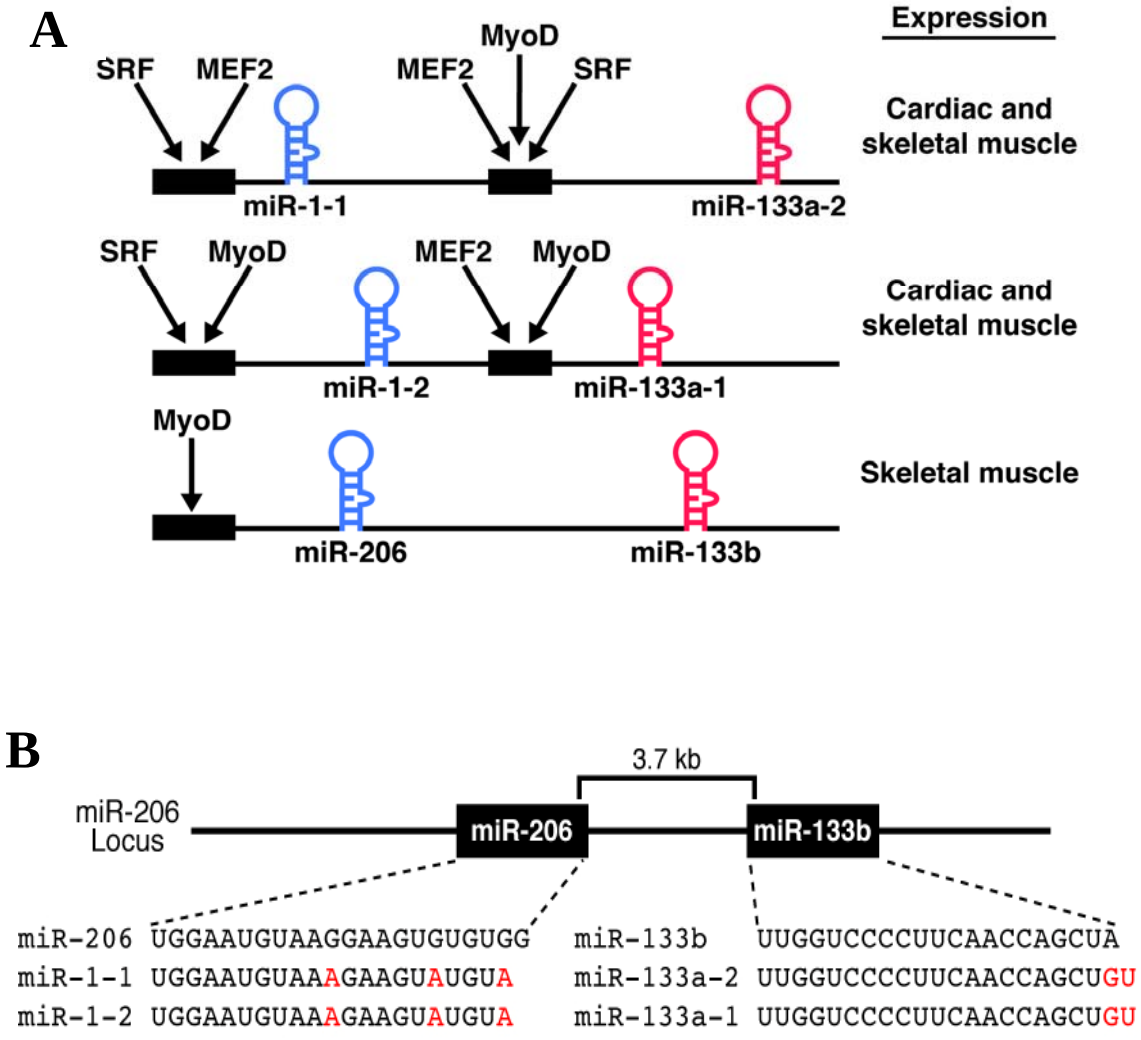

Figure 1.2. Muscle-specific miRNAs. (A) Three bicistronic clusters of muscle-specific miRNAs. Three bicistronic gene clusters each encoding two miRNAs are shown. miR-1-1, miR-1-2, andmiR-206 are nearly identical in sequence, as are miR-133a-2, miR-133-a-1 and miR-133b. Cis-regulatory elements that direct muscle-specific expression of each locus are indicated by black boxes, and the transcription factors that act through these elements are shown. **(B)** Schematic diagram of the miR-206/133b locus and sequence homologies among muscle-specific miRNAs. (Adapted from Williams et al., 2009)

133a suppresses proliferation (Liu et al., 2008). Indeed, the *in vitro* and *in vivo* results seem conflicting regarding a role for miR-133a in regulating proliferation; however, SRF has previously been shown to be capable of functioning as an activator of proliferation and differentiation depending on its association with co-factors such as myocardin, HOP, and Elk-1 (Pipes et al., 2006).

Like miR-1, miR-206 has been shown to promote differentiation of C2C12 myoblasts *in vitro* (Anderson et al., 2006; Kim et al., 2006). MiR-206 induces muscle differentiation by repressing the expression of a subunit of DNA polymerase alpha (Pola1), connexin 43 (Cx43), as well as follistatin-like 1 (Fstl1) and utrophin (Utrn) (Anderson et al., 2006; Kim et al., 2006; Rosenberg et al., 2006). Also, miR-206 was reported to repress estrogen receptor alpha (ERα) protein expression in ER negative breast cancer cells (Adams et al., 2007). Although the function of miR-206 is currently unknown, many functions have been proposed, such as a regulator of slow muscle fiber identity, a mediator of skeletal muscle hypertrophy, and a regulator of skeletal muscle regeneration (Clop et al., 2006; McCarthy and Esser, 2007; McCarthy et al., 2007).

miRNAs in muscle disease

Several important studies have indicated that miRNA expression is dysregulated in cardiac and skeletal muscle disease and in some cases individual miRNAs have been shown to cause or alleviate disease. The first series of such studies focused on the profiling of miRNAs in hypertrophic murine and human hearts and revealed a common set of miRNAs that are elevated in hypertrophic hearts

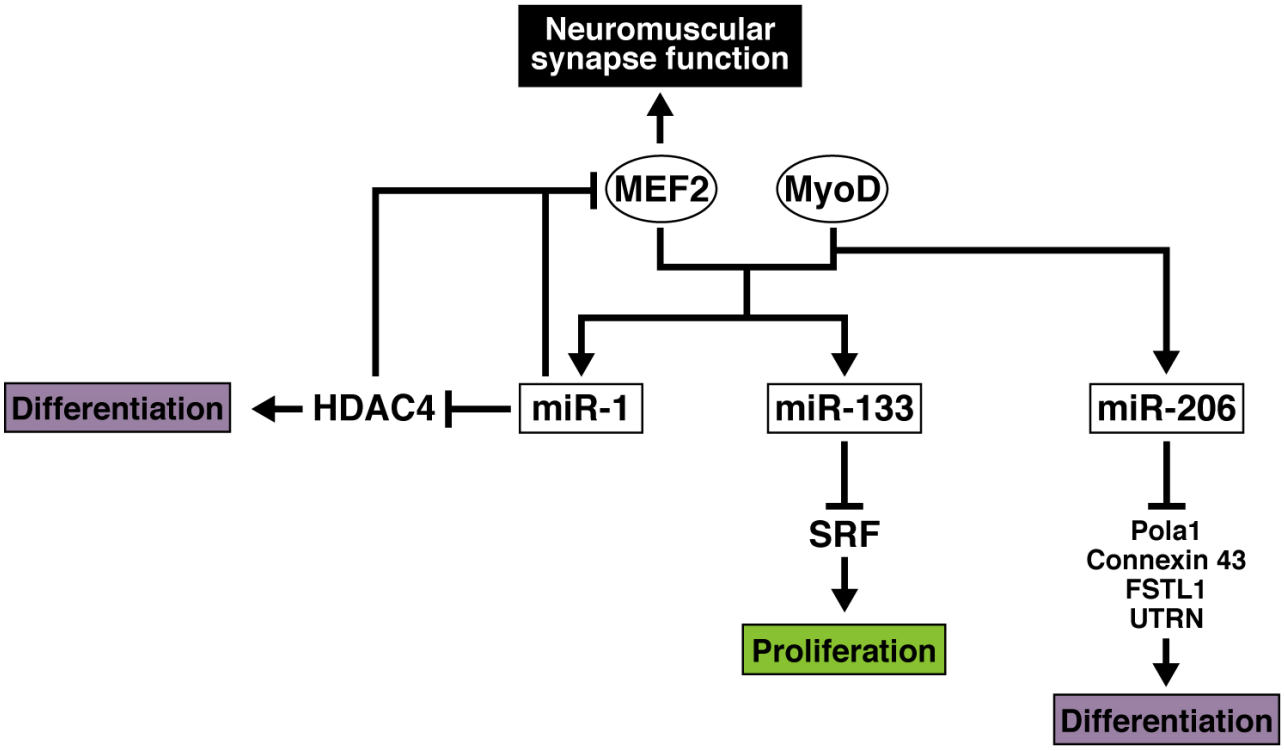

Figure 1.3. miRNA-transcription factor circuits involved in skeletal muscle development. MEF2 and MyoD control expression of miR-1, miR-133, and miR-206 in skeletal muscle. Targets for repression by these miRNAs, and the processes they regulate during skeletal muscle development, are shown. (Adapted from Williams et al., 2009)

(Cheng et al., 2007; Lohof et al., 1993; Sayed et al., 2007; Tatsuguchi et al., 2007; van Rooij et al., 2006). Several other studies have focused on the identification of miRNAs dysregulated in skeletal muscle regeneration and muscular dystrophy. Profiling of miRNAs in muscle samples from a variety of human patients with primary muscle disorders revealed a collection of miRNAs commonly dysregulated among patients with different types of muscle disorders (Eisenberg et al., 2007). The muscle-specific miRNA, miR-206, was found to be upregulated in the diaphragm of dystrophin deficient (mdx) mice, a model of muscular dystrophy (McCarthy et al., 2007). Another recent study demonstrated that miR-206 is upregulated in the skeletal muscle of mdx mice and also upon injection of cardiotoxin, a potent inducer of muscle regeneration; however, the expression of miR-1 and miR-133a was not changed (Yuasa et al., 2008). Collectively, these studies demonstrate that the expression patterns of miRNAs are dramatically and distinctly altered during various types of muscle disease and that the manipulation of disease-associated miRNAs represents a potentially powerful diagnostic and therapeutic approach to treat muscle disease.

Neuromuscular Synaptogenesis

The precise alignment and differentiation of presynaptic and postsynaptic structures of synapses is an essential step in the proper wiring of neuronal circuits (Hippenmeyer et al., 2007). Due to its large size and accessibility, the synapse forming at the neuromuscular junction (NMJ) is by far the most comprehensively studied (Sanes and Lichtman, 2001). Several of the features of synapses comprising the NMJ are shared with synapses in the

central nervous system (CNS), thus the principles governing synaptogenesis at the NMJ are likely to be similar to synapses in the CNS.

The NMJ is primarily composed of two cell-types, motor neurons (presynapse) and muscle fibers (postsynapse). Motor axons reach target muscles as myoblasts are fusing to form myotubes, which induces the expression of synaptic proteins (Ontell et al., 1995) (**Figure 1.4**). Nerve axons contact muscles in a central end-plate band along the myofiber, leading to the formation of specialized structures on the nerve for synaptic transmission via acetylcholine (ACh) release and enrichment of proteins in the postsynaptic region of muscle fibers for propagation of the neuronal signal (Sanes and Lichtman, 2001). At the synapse, acetylcholine receptors (AChRs) accumulate, reaching extremely high levels (>10,000-fold) compared to extra-synaptic regions of muscle, demonstrating that nuclei associated with the synapse become transcriptionally specialized following the initiation of synaptogenesis (Salpeter and Loring, 1985).

Postsynaptic differentiation

The identification and characterization of three molecules has primarily contributed to our understanding of the mechanisms governing the formation of the postsynapse. The first molecule is the heparan sulfate proteoglycan, agrin (Tsen et al., 1995). Agrin is a molecule synthesized and released by motor neurons that is necessary and sufficient for the formation of AChR clusters. Injection of plasmids expressing agrin into muscles resulted in accumulation of AChR clusters in direct apposition to the exogenously applied agrin (Jones et al., 1997). Conversely, differentiation and accumulation of AChR clusters was dramatically impaired in agrin mutant mice (Gautam et al., 1996). The second

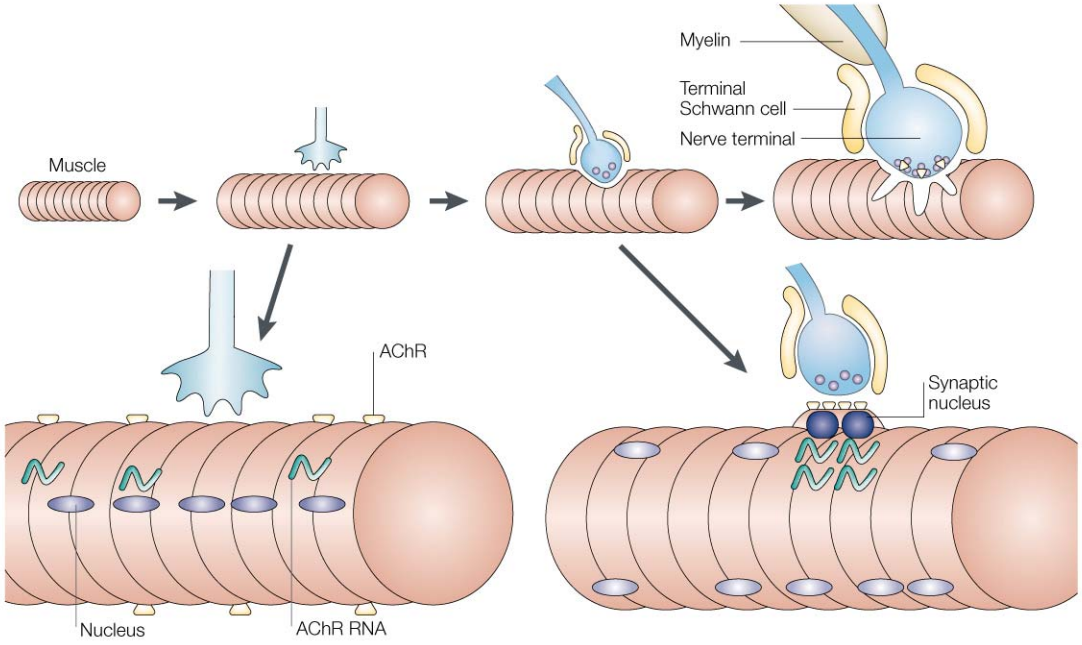

Figure 1.4. Outline of neuromuscular synaptogenesis. As the motor axon reaches the maturing myotube, differentiation and specialization of pre- and postsynaptic structures occurs. In immature muscle, AChRs are present diffusely throughout the myofiber; however, in adult muscle AChRs are selectively transcribed by synaptic nuclei and become concentrated at the synapse. (Adapted from Sanes and Lichtman, 2001).

molecule is the receptor tyrosine kinase, MuSK. MuSK is expressed specifically in skeletal muscle and co-localizes with AChR clusters (Valenzuela et al., 1995). MuSK mutant mice also have a severe AChR clustering defect (DeChiara et al., 1996). The third molecule is the cytoplasmic protein, rapsyn. No AChR clusters form in the skeletal muscle of rapsyn mutant mice (Gautam et al., 1995). Thus, agrin, MuSK, and rapsyn form the foundation of a signaling network required for clustering of postsynaptic proteins (**Figure 1.4**).

Presynaptic differentiation

Observations that motor neurons differentiate only at sites of contact with myofibers suggested that factors secreted from the muscle regulate and actively participate in presynaptic differentiation (Lupa et al., 1990). Several of these molecules have recently been identified. Members of the fibroblast growth factor (FGF) family were shown to regulate presynaptic differentiation of motor neurons (Fox et al., 2007). FGF family members 7, 10, and 22 are expressed and secreted specifically by skeletal muscle and the FGF receptor, FGFR2b is expressed specifically by motor neurons. Deletion of FGFR2b specifically in motor neurons results in a significant delay in presynaptic differentiation, demonstrating the importance of retrograde FGF signaling (Fox et al., 2007). In addition, deletion of beta-catenin specifically in skeletal muscle results in a similar phenotype in which there is a lack of presynaptic differentiation, demonstrating the importance of retrograde Wnt signaling (Li et al., 2008). These studies illustrate the principle that muscle-derived factors are required for the proper differentiation of motor nerve

terminals. However, whether or not these same developmental pathways regulate presynapse differentiation in the adult is currently unknown.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is the most common adult neuromuscular disease (Bruijn et al., 2004). The hallmark of the disease is the dysfunction and eventual death of motor neurons, leading to muscle weakness, muscle atrophy, paralysis, and eventually death. Although the causes for most cases of ALS are unknown, and the disease course is highly variable, the common initial symptom for all patients is the denervation and atrophy of target muscle (Bruijn et al., 2004). Most cases (90%) of ALS are sporadic in nature and approximately 10% are inherited dominant mutations with 20% of inherited ALS cases resulting from mutations in the superoxide dismutase protein (SOD1).

Although, the initiation and progression of the disease arises from the death of motor neurons, it is clear that the damage from mutant proteins occurs in a non-cell-autonomous manner. Expression of mutant SOD1 protein specifically in motor neuron or astrocytes did not produce motor neuron degeneration (Gong et al., 2000; Lino et al., 2002). Mice with a "floxed" mutant *Sod1* gene have permitted the identification of specific cell types that are responsible for disease pathogenesis (Boillee et al., 2006b). For example deletion of mutant SOD1 in motor neurons extends survival by delaying the early disease progression; whereas, deletion of mutant SOD1 in microglia delays later disease progression and significantly extends survival (Boillee et al., 2006b). In addition, deletion of mutant SOD1 specifically in skeletal muscle had little effect on overall

survival (Miller et al., 2006). However, treatment of ALS mice with molecules that can induce hypertrophy such as insulin-like growth factor-1 (IGF-1) or growth hormone (GH) can delay the pathogenesis of ALS (Dobrowolny et al., 2005; Kaspar et al., 2005; Kaspar et al., 2003). Thus, investigations into the molecular mechanisms regulating neurodegeneration in multiple cell types could prove useful for designing novel therapeutics.

Recently, mutations in two DNA/RNA binding proteins have been shown to be involved in inherited forms of ALS (Kwiatkowski et al., 2009; Neumann et al., 2006). Interestingly, both of these proteins FUS and TDP-43 have been shown to biochemically interact with the RNA-processing protein Drosha (Gregory et al., 2004). These discoveries have catalyzed a new interest in RNA metabolism and possibly miRNAs as new mechanisms involved in the pathogenesis of ALS.

This dissertation describes my experiments to determine the molecular mechanisms of neuromuscular synaptogenesis during stress and disease. My specific aims were:

1. To determine the function of miR-206 in skeletal muscle development and disease using miR-206 mutant mice.

2. To determine the function of miRNAs and miR-206 during the progression of ALS.

Chapter II

miR-206 Promotes Neuromuscular Synapse Reinnervation

ABSTRACT

Following transection of motor nerves, axons degenerate distal to the point of injury, leaving muscles denervated. Subsequently, the motor axons regenerate through the nerve stump to the muscle and, under favorable circumstances, form new neuromuscular NMJs that look and perform like the original ones. The efficacy of reinnervation is regulated by factors in the nerve stump, by muscle-derived factors that attract axons to muscle fibers, and by components of the basal lamina that occupies the synaptic cleft at the NMJ. We discovered that miR-206, regulates a retrograde signal essential for efficient skeletal muscle reinnervation. The expression of miR-206 is robustly increased following denervation of skeletal muscle. Genetic deletion of *miR-206* results in impaired reinnervation following nerve injury. The delay in reinnervation in miR-206 mutant mice is due to a lack of retrograde growth factor signaling. These results identify the molecular basis for reinnervation, in which muscle can respond to denervation through the upregulation of protein-coding factors and miRNAs to induce nerve regeneration.

RESULTS

miR-206 expression is upregulated following skeletal muscle denervation

In light of recent studies implicating miRNAs in stress responses in muscle cells (Van Rooij et al., 2008), we compared the miRNA expression profiles of skeletal muscles from the lower limbs of normal adult mice and mice subjected to surgical resection of the sciatic nerve for 10 days (**Figure 2.1**). Of 320 miRNAs tested, the levels of 16 miRNAs were significantly affected (up or downregulated > 2-fold) in response to denervation. MiR-206 was one of the most dramatically upregulated miRNAs in denervated muscle (**Table 2.1 and Table 2.2**). Northern blot and real time PCR confirmed the miR-206 is muscle-specific and that it is upregulated following denervation (**Figure 2.2**). Upregulation of miR-206 was dramatic in three muscles that contain predominantly fast-twitch fibers, extensor digitorum longus (EDL), tibialis anterior (TA), and gastrocnemius/plantaris (G/P) (**Figure 2.2**). At baseline, miR-206 levels were higher in normally innervated soleus, which contains predominantly slow myofibers, and upregulation following denervation was correspondingly less striking. Consistent with its transcription from the same promoter, miR-133b was also upregulated following denervation, whereas miR-1 and miR-133a were downregulated approximately 2-fold in response to denervation (**Figure 2.2**).

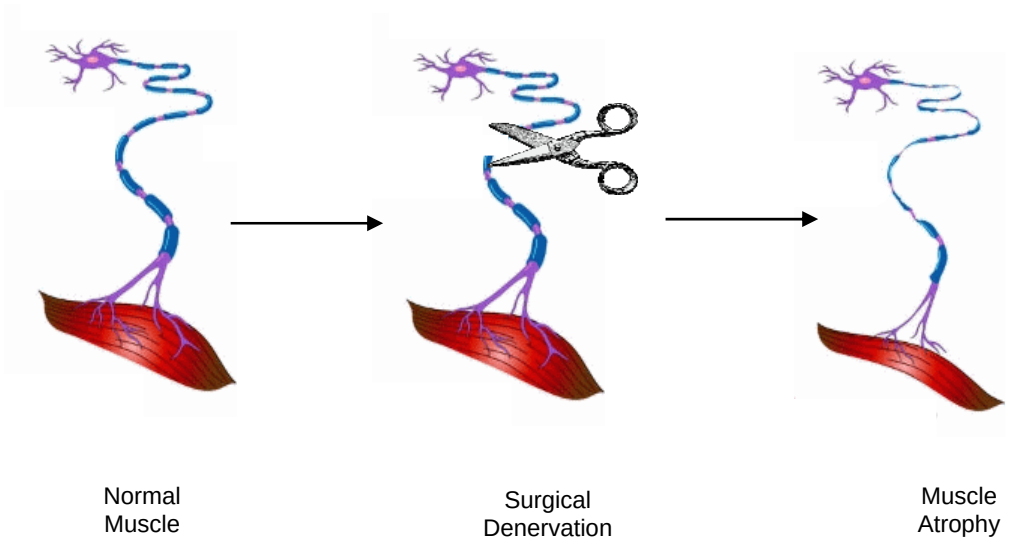

Figure 2.1. Schematic of surgical denervation resulting in muscle atrophy.

Table 2.1. Upregulated miRNAs in response to denervation.

miRNA	Fold Change
miR-34c-3p	5.86
miR-206	5.24
miR-21	2.93
miR-709	2.11
miR-155	2.06
miR-92b	2.03

Table 2.2. Downregulated miRNAs in response to denervation.

miRNA	Fold Change
miR-451	-15.7
miR-133a*	-2.71
miR-486-3p	-2.68
miR-422a	-2.51
miR-181b	-2.48
miR-30a-3p	-2.48
miR-101	-2.36
miR-101b	-2.27
miR-1b	-2.06
miR-30e	-2.04

Tables 2.1 and 2.2. Dysregulation of miRNAs in response to denervation. (2.1) Upregulated miRNAs (>2-fold) in response to denervation compared to wild-type (WT) animals. Data represent fold-change compared to WT animals. **(2.2)** Downregulated miRNAs (>2-fold) in response to denervation compared to wild-type animals. Data represent fold-change compared to WT animals.

Denervation-responsiveness of the miR-206 gene is mediated by E-boxes

Prior studies have implicated the myogenic bHLH protein MyoD and myogenin in the regulation of denervation-dependent gene expression (Bodine et al., 2001; Eftimie et al., 1991). Therefore, we analyzed the 5' flanking region of the miR-206 gene for evolutionarily conserved E-boxes (CANNTG), which are binding sites for MyoD and myogenin. We identified three conserved E-boxes between -910 and -765 bp from the start of pre-miR-206, within a genomic region enriched for MyoD binding in ChIP-on-Chip assays using chromatin from muscle cells (Rao et al., 2006) (**Figure 2.3**). In gel mobility shift assays, these sites were bound by heterodimers of MyoD and the ubiquitous bHLH protein E12 (**Figure 2.3**). Increasing levels of MyoD potently activated the expression of a luciferase reporter controlled by an 837 bp genomic fragment encompassing the conserved E-boxes, and mutations introduced into the E-boxes abolished the responsiveness of the promoter to MyoD (**Figure 2.3**).

To address whether these E-boxes mediate denervation-dependent upregulation of miR-206, we generated transgenic mice in which the miR-206 5' regulatory region containing these sites controlled expression of an *E. coli* β-galactosidase (lacZ) reporter. LacZ expression was low in muscles from adult transgenic mice, but 10 days following resection of the sciatic nerve, the miR-206-lacZ transgene was dramatically upregulated in denervated skeletal muscle fibers (**Figure 2.4**). Mutations in the three E-boxes abolished MyoD/E12 binding and abrogated responsiveness of the miR-206 enhancer to denervation (**Figure 2.4**). Transgenic mice harboring a lacZ reporter under control of the hsp68 basal promoter had minimal upregulation of lacZ activity in response to denervation, similar to that of the E-box mutant transgenic (**Figure 2.4**), demonstrating

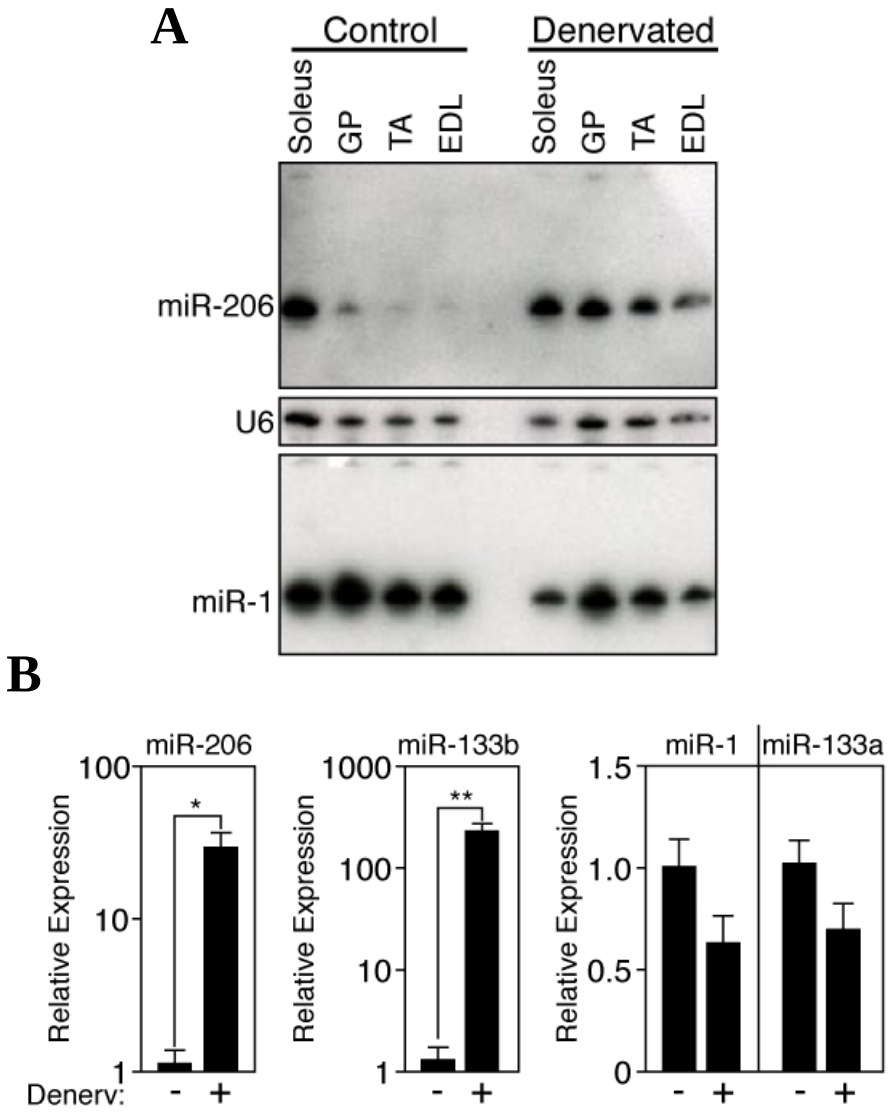

Figure 2.2. Regulation of miR-206 by denervation. (A) Northern blot analysis of miR-1 and miR-206 expression in adult mouse muscle tissues 10-days after sciatic nerve transection. The contralateral leg was used as a control. U6 was used as a loading control. **(B)** Transcripts of miR-206, miR-133b, miR-1, and miR-133a were detected by real time PCR in TA muscles following 10-days of denervation (+). The contralateral muscle was used as a control (-). *$p < 0.02$, **$p < 0.005$. n=3-4 per group.

that the upregulation of lacZ in response to denervation is specifically due to sequences in the miR-206 enhancer. These experiments identify a miR-206 enhancer that is a target for transcriptional activation in response to denervation, as a consequence of its direct regulation by MyoD or related bHLH factors.

Generation of miR-206 mutant mice

To determine the *in vivo* function of miR-206, we generated miR-206 null mice. The targeting strategy replaced miR-206 and flanking sequence with a neomycin cassette flanked by loxP sites (**Figure 2.5**). Southern blot with a 5' external probe demonstrated targeting and germline transmission of the mutant allele (**Figure 2.5**). Mice homozygous for the targeted deletion of miR-206 were viable and showed no gross abnormalities in weight, behavior, or the overall architecture of skeletal muscles. The absence of mature miR-206 in mutant mice was confirmed by Northern blot analysis and RT-PCR (**Figure 2.5**). In addition, deletion of miR-206 had no effect on the expression of linked pre-miR-133b or the closely related miR-1-1 or miR-1-2 (**Figure 2.5**).

Deletion of miR-206 does not affect muscle fiber-type or muscle atrophy

Previous studies have demonstrated that the deletion of genes enriched in slow myofibers or genes upregulated upon denervation typically results in a fiber-type switch or change in muscle atrophy following denervation, respectively (Bodine et al., 2001; Handschin et al., 2007; Potthoff et al., 2007b). Metachromatic ATPase staining on sections of soleus muscles from wild-type and miR-206 mutant mice demonstrated that the deletion of miR-

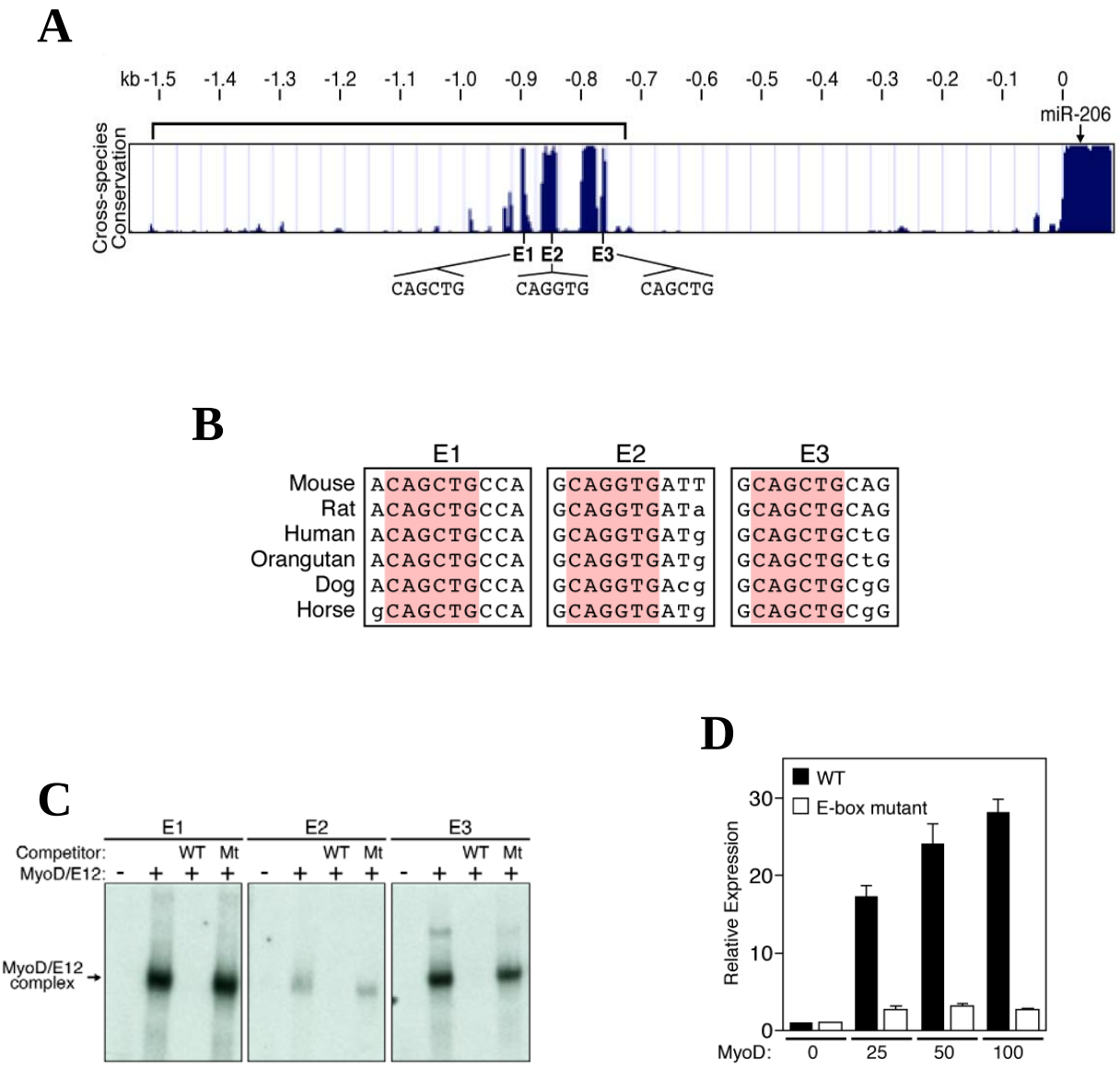

Figure 2.3. Regulation of miR-206 by MyoD. (A) Sequence alignment of the mouse *miR-206* 5' flanking sequence from different species shows the conserved upstream region containing E-boxes (CANNTG). Position (0) denotes the start of pre-miR-206. Bracketed region represents the identified denervation-response element. **(B)** Sequence alignment of the three conserved E-boxes in the miR-206 upstream region. **(C)** Electrophoretic mobility shift assays demonstrate direct binding of MyoD/E12 to the three conserved E-boxes. Unlabeled wild-type (WT) oligonucleotides compete for binding, but mutant (Mt) E-box oligonucleotides do not compete. The region of the gel with the shifted probe is shown. (-) refers to extract containing protein lysate from untransfected cells. **(D)** COS1 cells were transfected with increasing amounts (0-100 ng) of MyoD expression plasmid and a luciferase reporter containing the 837 bp enhancer upstream of the *miR-206* gene. Mutations in the E-boxes abolished responsiveness of the luciferase reporter to MyoD.

206 does not effect fiber-type distribution (**Figure 2.6**). Additionally, we denervated wild-type and miR-206 mutant mice for 10 days and measured the weight of G/P muscles as a measure of the relative amount of muscle atrophy. Denervation of miR-206 mutant mice resulted in a significant reduction (~60%) in muscle weight, similar to what was seen in wild-type mice (**Figure 2.6**). These studies demonstrate that miR-206 is not a regulator of muscle fiber-type or muscle atrophy in response to denervation.

Synaptic enrichment of miR-206 expression

A transcript derived from the miR-206/133b locus was originally identified as a synapse-associated non-coding RNA (referred to as 7H4) (Velleca et al., 1994). Presumably 7H4 is selectively transcribed by myonuclei associated with the NMJ, as has been shown for genes encoding neurotransmitter receptor genes and other components of the postsynaptic apparatus (Sanes et al., 1991; Schaeffer et al., 2001). Utilizing transgenic mice that express yellow fluorescent protein (YFP) in motor neurons, synapse-rich and synapse-free regions of muscle were isolated by micro-dissection (**Figure 2.7**) (Feng et al., 2000). RT-PCR demonstrated that miR-206 sequences are included in the 7H4 transcript and confirmed that the expression of miR-206 is enriched in synaptic regions of muscle fibers (**Figure 2.7**). Therefore, the original 7H4 transcript appears to represent a partially processed pri-miRNA from the miR-206/133b locus (Velleca et al., 1994). These results, along with the lack of any obvious phenotype in muscle structure or function, focused our attention on the neuromuscular junction.

To determine if miR-206 regulates the formation and/or maturation of NMJs, we examined the architecture of NMJs in the TA, EDL, and soleus muscles of embryonic,

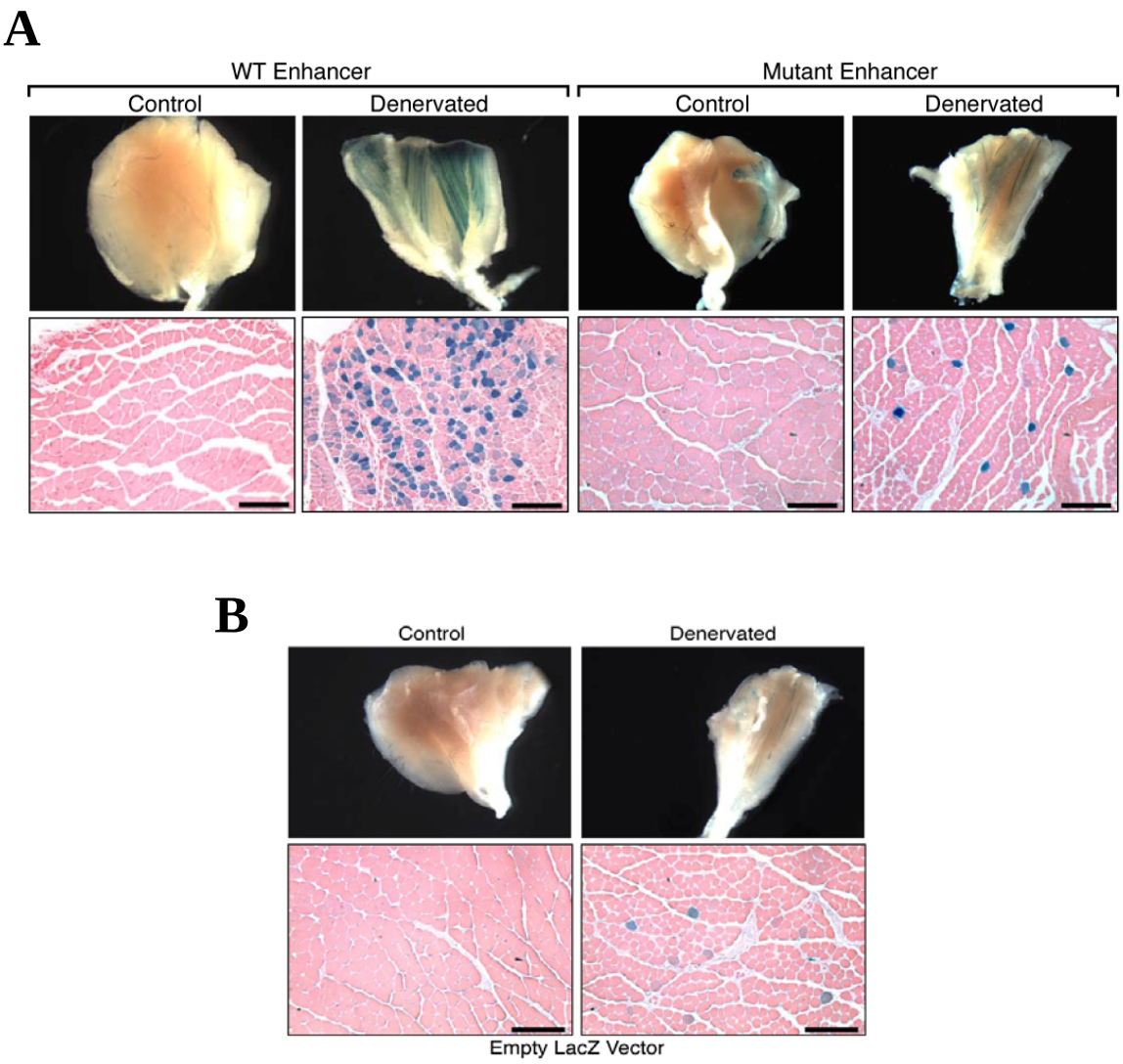

Figure 2.4. Regulation of miR-206 by denervation. (A) β-galactosidase staining of gastrocnemius/plantaris muscle isolated from denervated transgenic mice containing a lacZ transgene controlled by the 837 bp genomic region upstream of the *miR-206* gene (WT Enhancer) or the 837 bp genomic region upstream of the *miR-206* gene with mutations in the conserved E-boxes (Mutant Enhancer). Contra-lateral muscle was used as a control. Lower panels show transverse section of muscle. Scale bar =200 μm. **(B)** Denervation of transgenic mice containing a transgene with the hsp68 basal promoter controlling lacZ expression shows minimal β-galactosidase activity. Lower panels show transverse sections of muscle. Scale bar =200 μm.

neonatal, and adult wild-type and miR-206 mutant mice. We visualized the post-synaptic membrane using fluorescently-tagged Bungarotoxin (BTX), which binds to acetylcholine receptors (AChRs), the motor axon with antibodies to neurofilaments, and the nerve terminal with antibodies to the synaptic vesicle protein, synaptotagmin 2 (ZNP) (Fox et al., 2007). The NMJs of embryonic, neonatal, and adult mutant mice showed no obvious differences when compared to age-matched wild-type NMJs (**Figure 2.8**). Thus, miR-206 is dispensable for formation and maturation of the NMJ.

Requirement of miR-206 for efficient reinnervation following nerve injury

Given the robust upregulation of miR-206 in denervated muscle, we next asked whether miR-206 might regulate reinnervation following nerve injury. We cut the sciatic nerve of miR-206 mutant and control littermates in the mid-thigh and assessed reinnervation of the TA muscle 1-8 weeks later. The TA muscle was chosen due to the reproducible identification and reinnervation of NMJs compared to the other muscles (Magill et al., 2007). Regenerating axons preferentially reinnervate original synaptic sites following denervation (Bennett and Pettigrew, 1976; Sanes and Lichtman, 1999), so we quantified the number of postsynaptic sites apposed by nerve. Because the postsynaptic AChRs remain largely intact following denervation (Frank et al., 1976), reinnervation can be accurately assessed by the superimposition of BTX staining (red) with ZNP staining (green). In wild-type mice, reinnervation began between 2 and 3 weeks after denervation, and was nearly complete by 5 weeks (**Figure 2.9**). In contrast, reinnervation of miR-206 mutant TA muscles did not begin until 3 weeks post-injury, and remained retarded at 5 weeks post-injury (**Figure 2.9**).

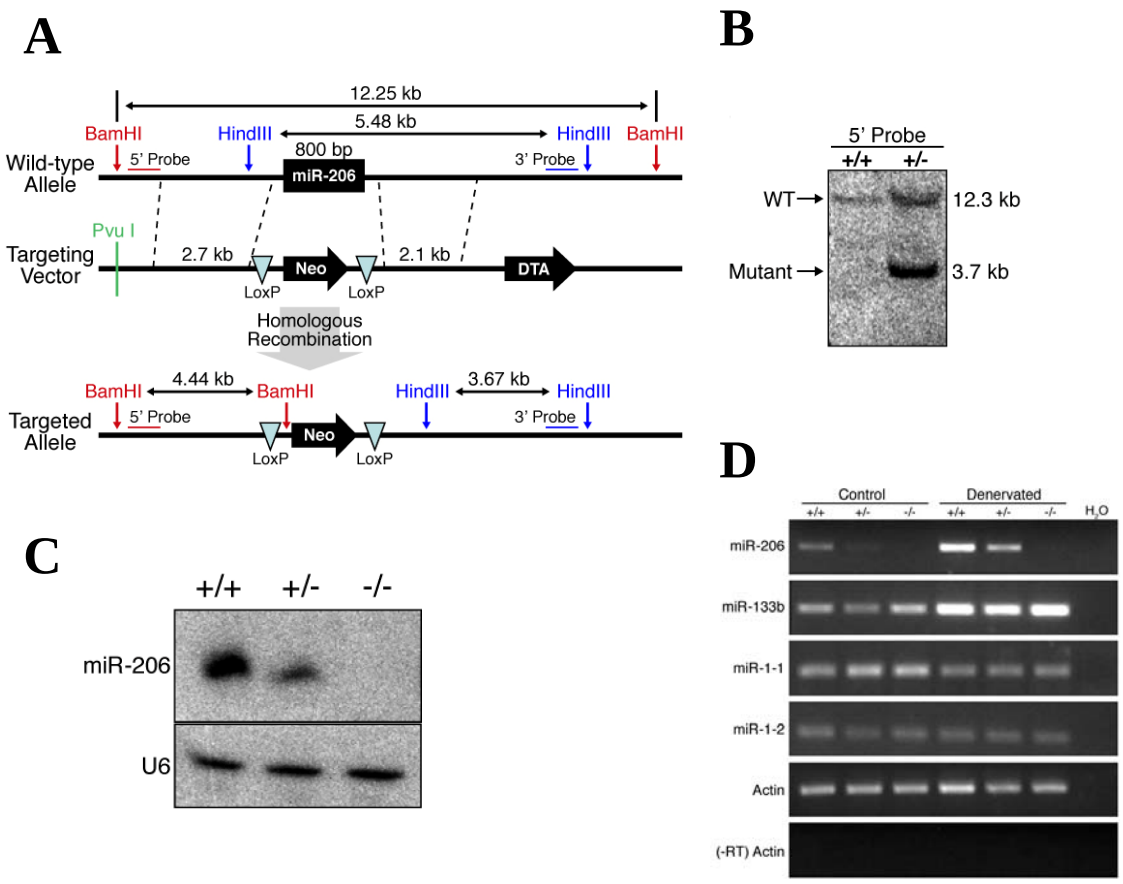

Figure 2.5. Generation of miR-206 mutant mice. (A) Targeting strategy to delete miR-206 from the *miR-206/133b* locus by replacing the pre-miR-206 sequence with a neomycin cassette flanked by loxP sites. Positions of probes used for Southern blots are shown. **(B)** Southern blot analysis of genomic DNA from wild-type and heterozygous mice using an external 5' probe. Genomic DNA was digested with BamHI. **(C)** Northern blot analysis of mature miR-206 transcript expression in gastrocnemius/plantaris muscle of the indicated miR-206 genotypes. U6 was used as loading control. **(D)** Detection of miRNA transcripts in soleus muscles of wild type (+/+) or miR-206 mutant mice (+/- and -/-) after 5-weeks of denervation using RT-PCR. Contralateral leg muscle was used as control. Actin was used as a loading control. Reactions with no reverse transcriptase (-RT) were a negative control for the assay. (H_2O) refers to PCR performed without the addition of cDNA.

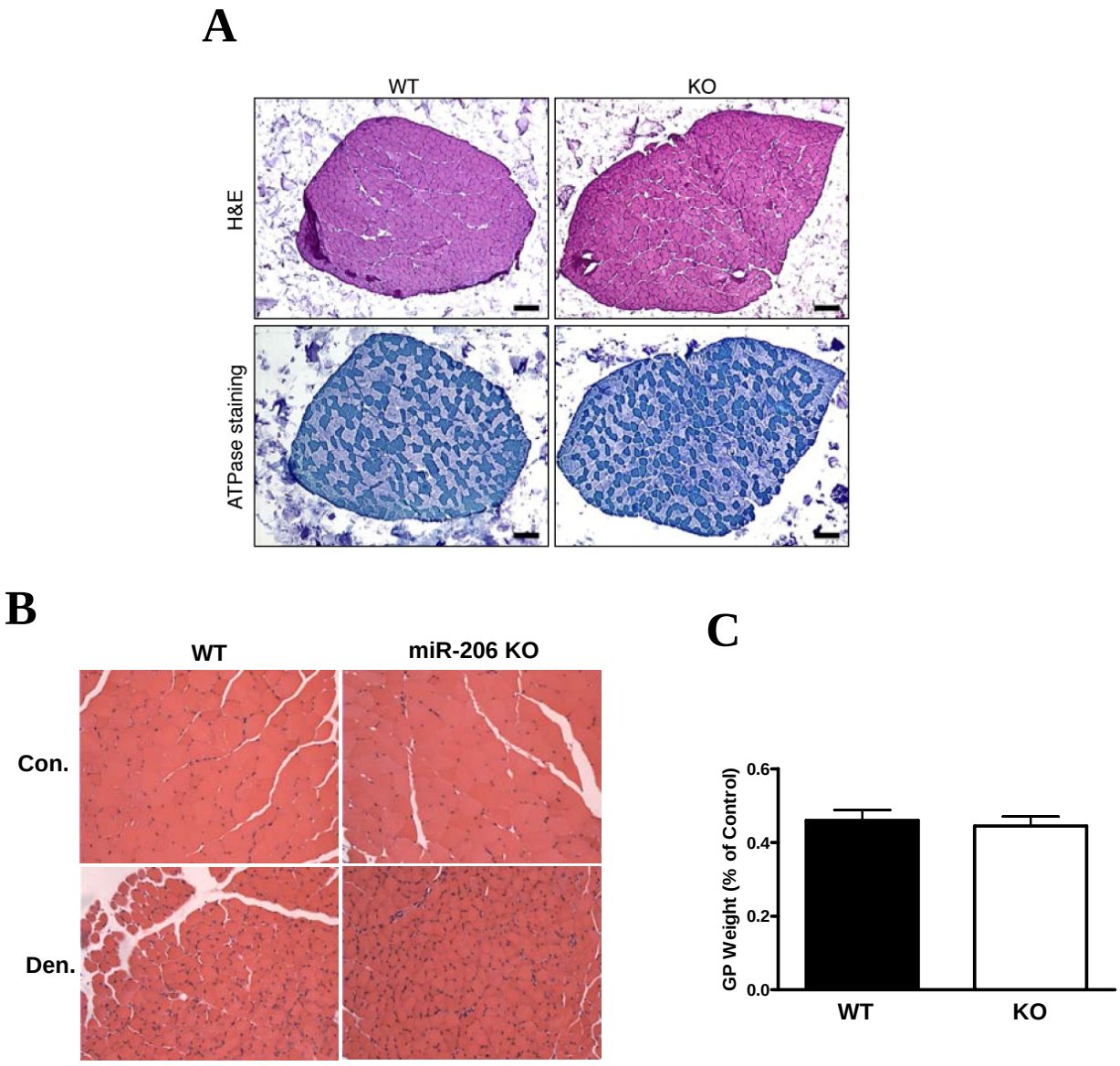

Figure 2.6. miR-206 does not regulate muscle atrophy or fiber-type switching. (A) Hematoxylin and eosin (H&E) and metachromatic ATPase staining show no difference in the skeletal muscle architecture and distribution of Type I (dark blue) and Type II (light blue) skeletal myofibers in the soleus muscles of wild-type (WT) and miR-206$^{-/-}$ (KO) mice. Scale bar=200 μm. **(B)** H&E staining shows no difference in muscle atrophy following surgical denervation for three weeks. **(C)** Quantitation of the amount of muscle atrophy in wild-type (WT) and miR-206$^{-/-}$ (KO) mice following denervation.

To confirm the delay in reinnervation in a second model of nerve injury, the sciatic nerve was crushed rather than cut; in this procedure, no gap is generated and regeneration to targets occurs more rapidly and reliably than after nerve cut. Again, a significant delay in the reinnervation of NMJs was seen in miR-206 mutant mice compared to wild-type littermates (**Figure 2.9**). Similar results were obtained in the gastrocnemius and EDL muscles. Thus, in several muscles and following two types of nerve injury, reinnervation was significantly delayed in the absence of miR-206.

miR-206 regulates motoneuron branching and differentiation

Successful reinnervation of NMJs following nerve transection involves a series of steps. First, axotomized neurons turn on a growth program and their axons regenerate through the distal stump to reach the muscle. We expected these steps would be unaffected in miR-206 mutant animals given that miR-206 is expressed specifically in muscle. To experimentally address this, we visualized the nerves near the muscle entry point at 3 weeks after nerve transaction and found similar numbers of nerve fibers in wild-type and miR-206 mutant nerves, even though few NMJs were reinnervated in the mutant muscle (**Figure 2.10**). These results indicate that axonal regeneration per se was unimpaired.

Another set of steps occurs intramuscularly when axons branch, contact and re-occupy muscle fibers, and finally differentiate into new nerve terminals specialized for neurotransmitter release. The prolonged delay in reinnervation in the absence of miR-206 suggests that miR-206 regulates the expression of a signal emanating from muscle that influences interaction of the presynaptic motor nerve with the muscle fibers

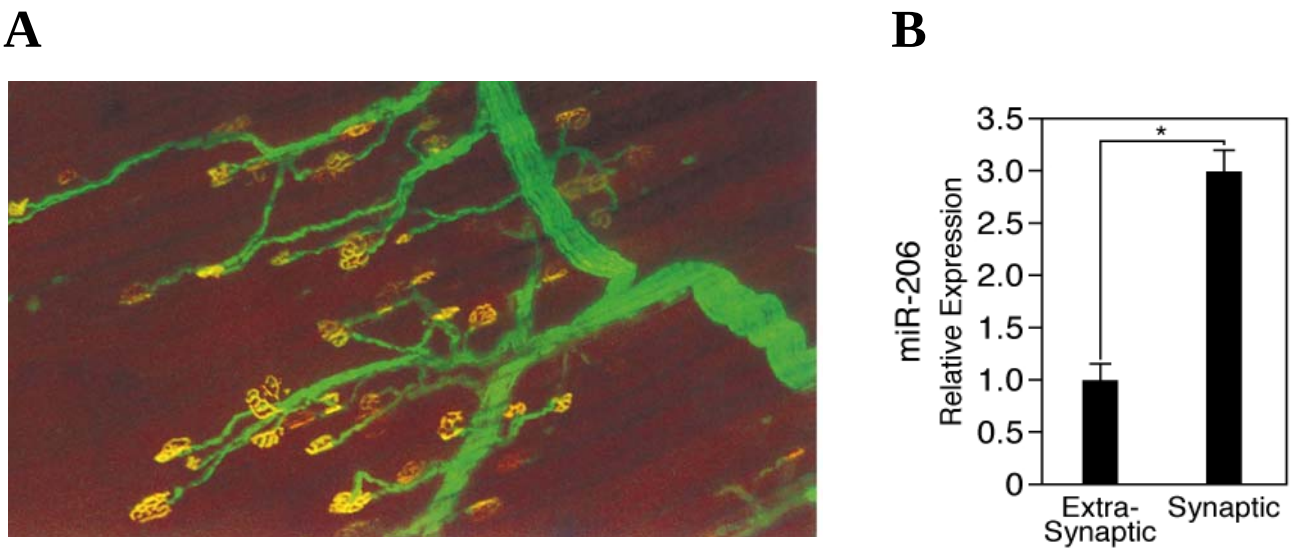

Figure 2.7. Synaptic enrichment of miR-206. (A) Microscopic view of NMJs in thy1-YFP transgenic mice. Motor axons (green) branch and innervate AChRs (red) on muscle fibers. **(B)** Quantitative real time PCR reveals miR-206 expression is enriched in synaptic regions of muscle fibers in thy1-YFP transgenic mice. *$p < 0.0002$. n=3 per group. (Figure A adapted from Feng et al., 2000).

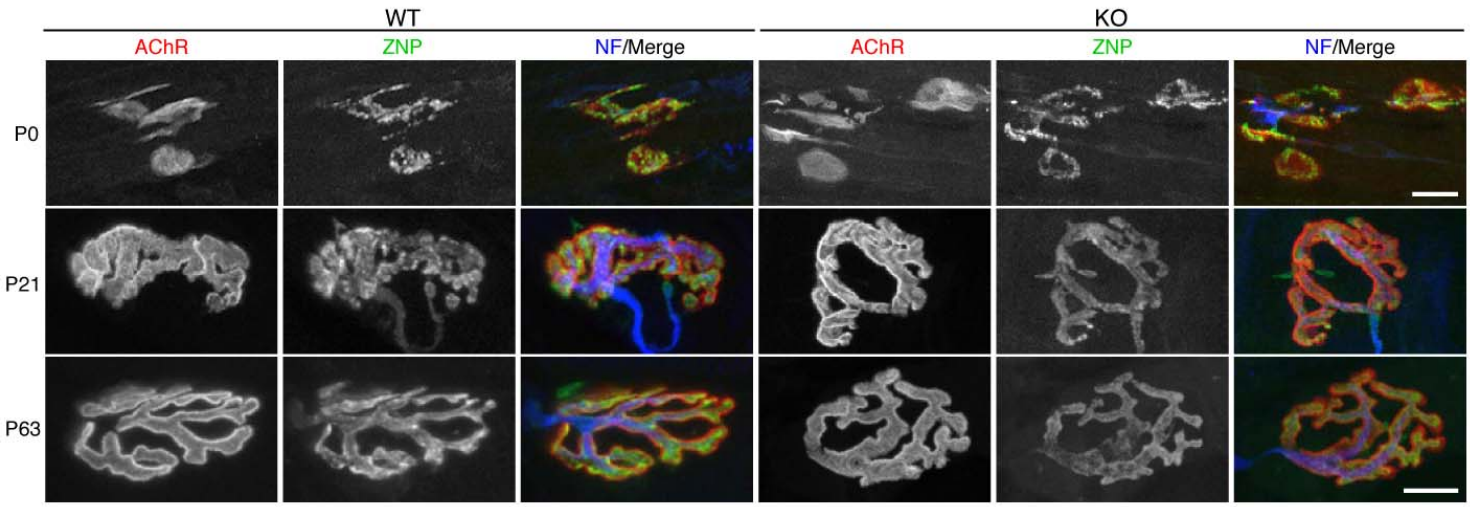

Figure 2.8. Normal NMJ Development in miR-206 mutant mice. NMJ development of miR-206$^{-/-}$ mice (KO) is similar to wild type (WT) mice at postnatal days (P) 0, 21, and 63. Thick longitudinal sections of the NMJ from WT and miR-206$^{-/-}$ TA muscle were co-stained with α-bungarotoxin (BTX) to visualize the post-synaptic acetylcholine receptor (AChR) (red), anti-synaptotagmin (ZNP) to detect pre-synaptic vesicles (green), and anti-neurofilament (NF) to detect nerve axons (blue). Scale bar=10μm. n=3 for each genotype and time point.

following injury. Consistent with this conclusion, reinnervation of original synaptic sites on miR-206 mutant muscle fibers was aberrant in multiple ways. First, many synaptic sites were only partially re-occupied by the regenerated nerve in the mutant mice (**Figure 2.10**). Second, levels of synaptotagmin 2 (ZNP) were lower in the terminal regions of miR-206 mutant NMJs than in control mice. Thus, the vesicles fail to aggregate properly in regenerated nerve terminals of mutants. Similar defects have been documented in mutants lacking muscle-derived organizers of presynaptic differentiation and maturation (Fox et al., 2007). Thus, although we cannot exclude the possibility that these aberrant features are secondary consequences of the delayed reinnervation in miR-206 mutant animals, they are consistent with a lack of muscle-derived factors that promote reinnervation once axons approach muscle fibers.

miR-206 represses histone deacetylase 4 (HDAC4) translation

Bioinformatic programs predict that miR-206 has many potential mRNA targets (~450). Among the predicted targets, *Hdac4* mRNA is one of the strongest (Chen et al., 2006; Lewis et al., 2005). The 3' untranslated region (UTR) of the mouse *Hdac4* mRNA contains two evolutionarily conserved sequences with perfect complementarity to the seed sequence of miR-206 (**Figure 2.11**). Also, the closely related miRNA, miR-1, has been shown to inhibit translation of *Hdac4* mRNA in vitro (Chen et al., 2006). To test if miR-206 was capable of repressing *Hdac4* translation, we cloned the 3' UTR of *Hdac4* mRNA downstream of a luciferase reporter under control of the CMV promoter. Transfection of increasing amounts of miR-206 resulted in a decrease in luciferase

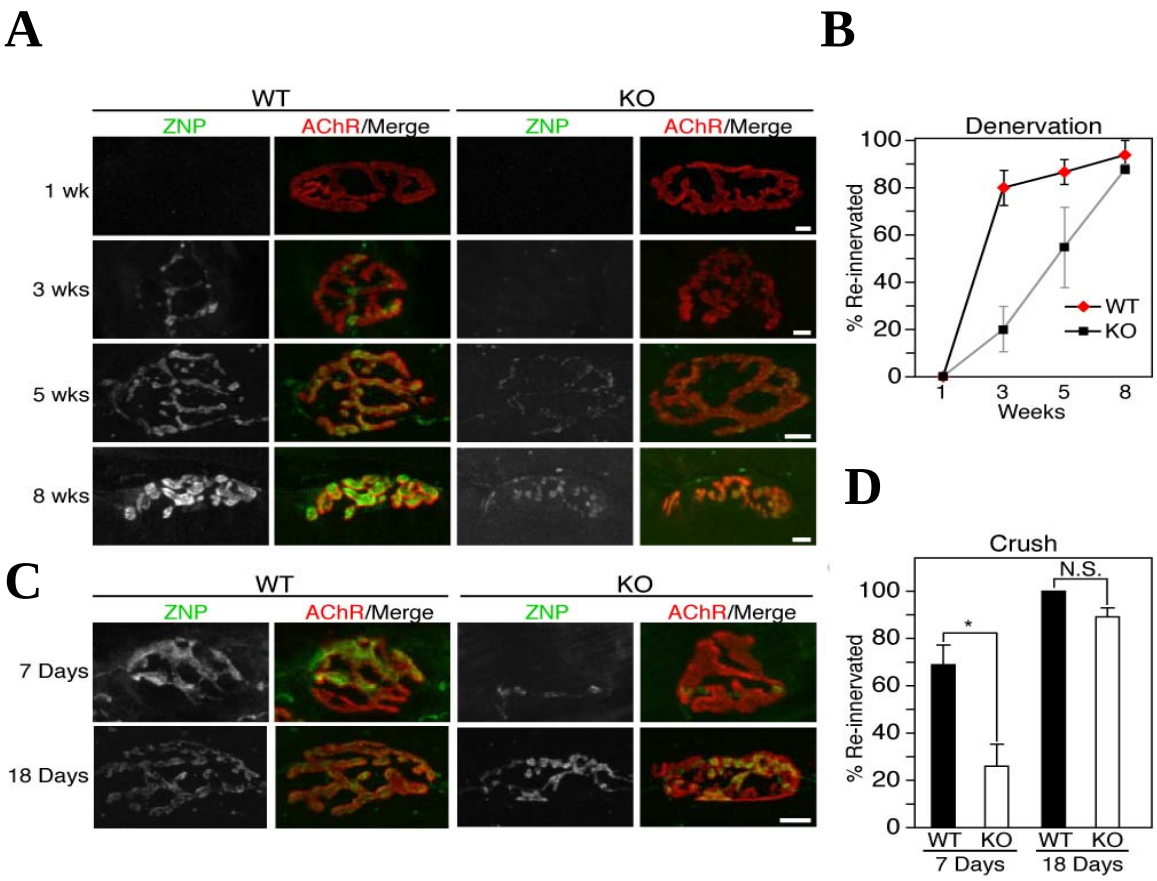

Figure 2.9. Delayed NMJ reinnervation in miR-206 mutant mice. (A) Following sciatic nerve transection (as indicated in weeks) a delay in reinnervation is seen in miR-206$^{-/-}$ mice (KO) compared to WT mice as detected by the superimposition of anti-ZNP staining (green) with BTX (red). Note the lack of anti-ZNP (green) staining in miR-206$^{-/-}$ mice 3-weeks after nerve transection. Scale bar=10μm. **(B)** Time course and quantification of the number of reinnervated NMJs in WT and miR-206$^{-/-}$ (KO) mice following sciatic nerve transection. n=2-6 for each genotype and time point. **(C)** Immunohistochemistry using BTX (red) and anti-ZNP (green) shows a delay in reinnervation of NMJs in miR-206$^{-/-}$ (KO) mice compared to WT mice 7- and 18-days after nerve crush. Note the incomplete nerve terminal coverage, marked by anti-ZNP (green) staining in miR-206$^{-/-}$ (KO) NMJs. Scale bar=10μm. **(D)** Number of reinnervated NMJs in WT and miR-206$^{-/-}$ (KO) mice following sciatic nerve crush for the indicated number of days. *$p < 0.02$. n=3-5 for each genotype and time point.

activity, and mutation of the miR-206 target sequences in the *Hdac4* 3' UTR prevented repression by miR-206 (**Figure 2.11**). Conversely, HDAC4 protein expression was increased in the skeletal muscle of miR-206 mutant animals compared to wild-type controls after denervation (**Figure 2.11**). HDAC4 protein expression was similar in miR-206 mutant and control uninjured animals. In addition, *Hdac4* mRNA levels were not changed in miR-206 mutant mice, indicating that miR-206 acts in this instance by translational inhibition rather than by mRNA destabilization (**Figure 2.11**) (Valencia-Sanchez et al., 2006). Previous work has demonstrated that HDAC4 induces *myogenin* expression through the repression of *Dach2* expression, a repressor of *myogenin* (Cohen et al., 2007; Tang et al., 2009). As expected, Dach2 transcripts were decreased and myogenin transcripts were increased in miR-206 mutant mice, consistent with increased HDAC4 protein expression and enhanced repression of signaling downstream of HDAC4 in denervated miR-206 mutant mice (**Figure 2.11**).

HDAC4 regulates reinnervation following nerve injury

Previous work has implicated HDAC4 in the control of neuromuscular gene expression (Cohen et al., 2007; Tang et al., 2009). To ask whether HDAC4 mediates the effects of miR-206 in muscle, we generated mice with a conditional Hdac4 null allele in which loxP sites flanked exon 6 of the Hdac4 gene, and deleted the allele in skeletal muscle using transgenic mice that express Cre recombinase specifically in this tissue (referred to as HDAC4 mKO) (Li et al., 2005; Potthoff et al., 2007b). NMJs formed and matured normally in the absence of HDAC4 (**Figure 2.12**). In contrast to miR-206 mutant

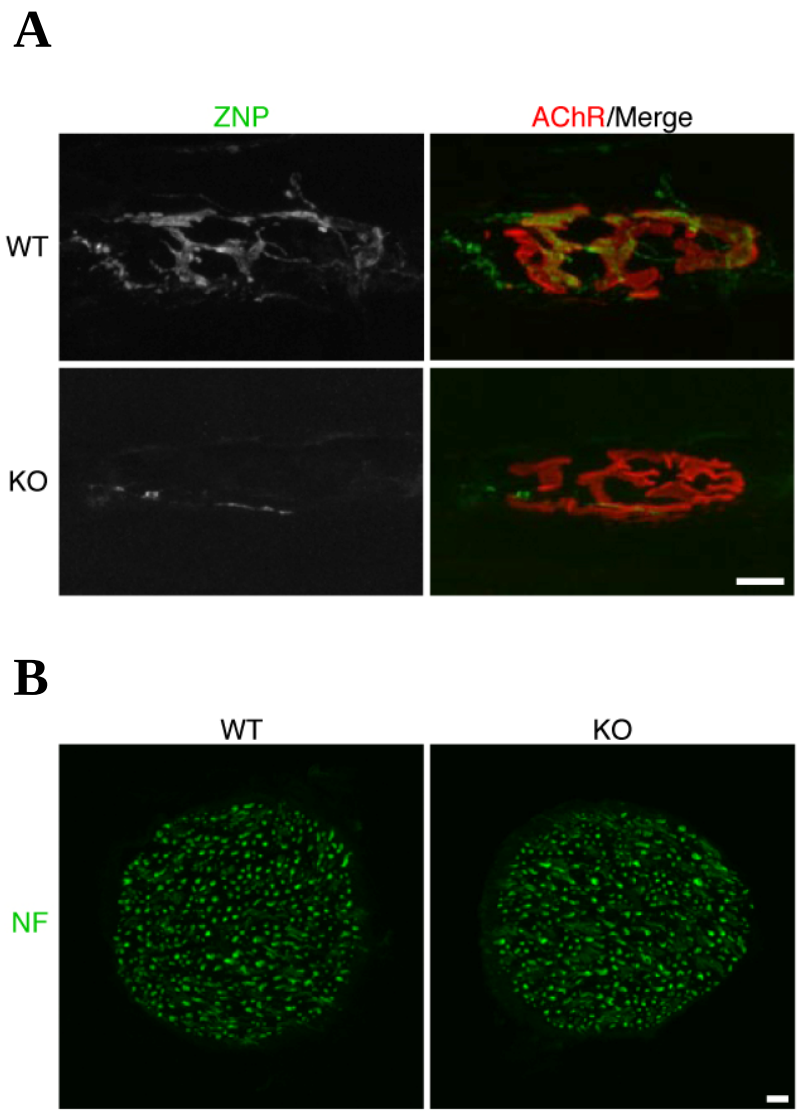

Figure 2.10. Delayed presynaptic differentiation but normal axon regeneration in miR-206$^{-/-}$ mice. (A) Immunohistochemistry using BTX (red) and anti-ZNP (green) shows a delay in presynaptic differentiation and parital re-occupancy of postsynaptic sites in miR-206-/- NMJs compared to WT NMJs 3-weeks after sciatic nerve transection. Scale bar =10 μm. **(B)** Transverse sections of sciatic nerves show similar numbers of axons proximal to the muscle entry point in miR-206$^{-/-}$ (KO) and WT mice 3-weeks after sciatic nerve transection. Nerve fibers were labeled with an antibody to neurofilament (NF). Scale bar =10 μm.

animals, where reinnervation is delayed, HDAC4 mutant animals displayed an acceleration of reinnevation following injury compared to control animals. Following nerve cut or nerve crush, NMJs were more rapidly reinnervated in HDAC4 mKO mice compared to wild- type mice (**Figure 2.12**). Likewise, synaptic sites were better covered by regenerating nerve terminals in HDAC4 mutant than in controls, whereas deletion of miR-206 hampered complete occupancy of synaptic sites (**Figure 2.10**). These findings are consistent with the conclusion that miR-206 functions to counter-act the negative influence of HDAC4 on reinnervation following nerve injury.

Retrograde regulation of neuromuscular synaptogenesis

Both motoneurons and muscle fibers secrete molecules essential for NMJ formation and maintenance (Sanes and Lichtman, 1999). Much is known about the molecules secreted from motor nerves that regulate neuromuscular synaptogenesis, such as agrin (Gautam et al., 1996); however, less is known about the identity of retrograde molecules that are secreted from the muscle to regulate motoneuron differentiation. The reinnervation phenotypes of miR-206 and HDAC4 mutant mice suggested that these molecules regulate the expression of a secreted factor from muscle. Using a candidate approach, we searched for retrograde factors downstream of miR-206 and HDAC4 that could potentially regulate neuromuscular synaptogenesis. The mRNA expression of several regulators of synapse formation including *Fgf7*, *Fgf10*, *Fgf22*, *Bdnf*, *Igf*, and *Sirpα* was not significantly changed in miR-206 mutant mice compared to wild-type mice after denervation (Lohof et al., 1993) (**Figure 2.13**). The similarities in neuromuscular

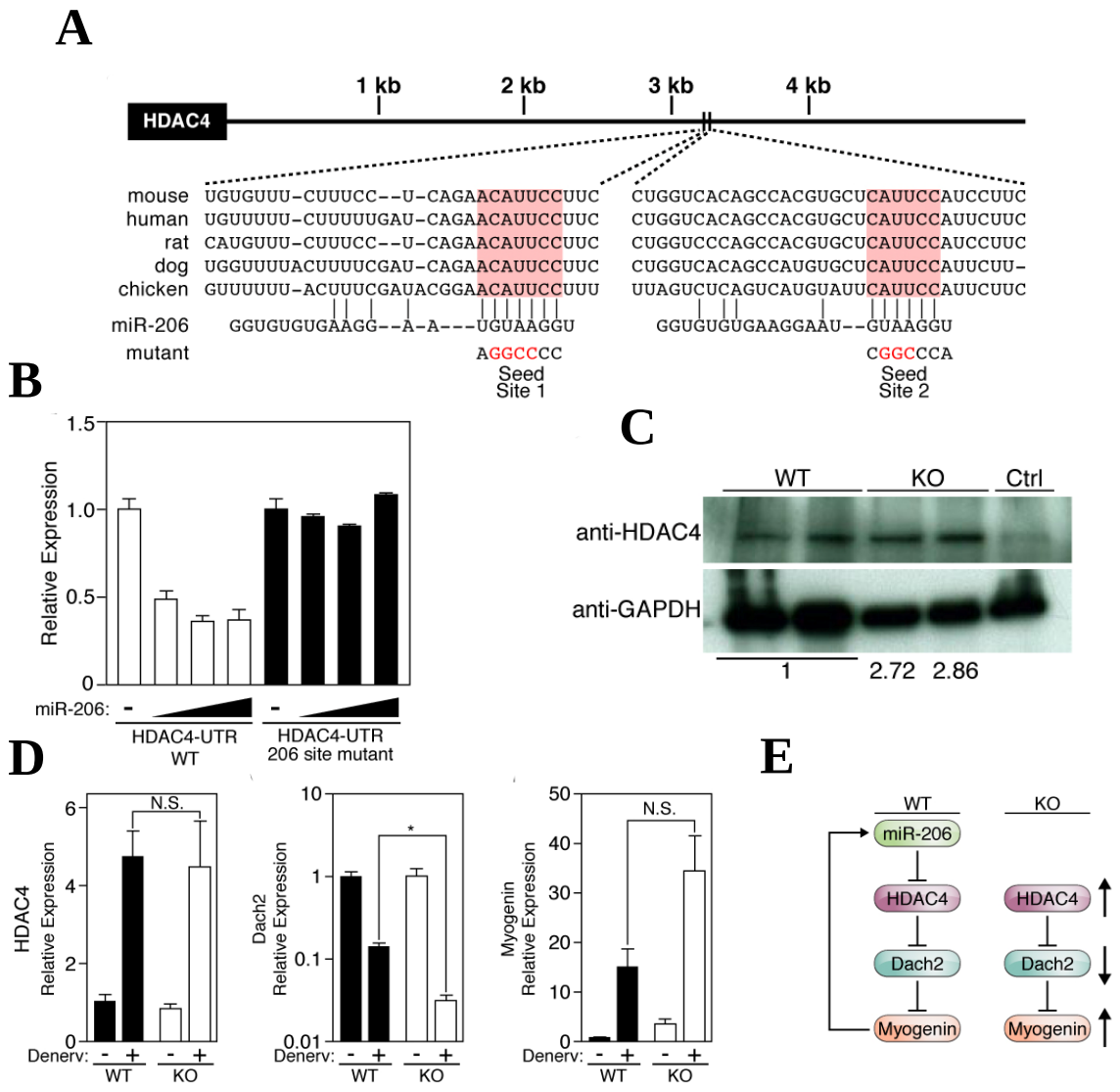

Figure 2.11. miR-206 targets HDAC4. (A) Schematic diagram of the *Hdac4* 3' UTR with sequence homologies of predicted miR-206 binding sites. **(B)** Luciferase activity of COS1 cells co-transfected with WT or mutant HDAC4 3'UTR-luciferase constructs with increasing amounts of miR-206 expression plasmid. Mutation of the predicted miR-206 binding sites in the 3'UTR alleviates the inhibitory activity of miR-206. Values are normalized to β-galactosidase activity. **(C)** Western blot analysis showing increased HDAC4 expression in muscle lysates isolated from wild-type (WT) and miR-206$^{-/-}$ (KO) mice following 3-weeks of denervation. (Ctrl.) refers to HDAC4 mKO protein lysate. GAPDH protein was detected as a control. Relative expression of HDAC4 protein compared to WT lysate is indicated below. **(D)** Transcripts of *Hdac4, Dach2, and myogenin* were detected in wild-type (WT) and miR-206$^{-/-}$ (KO) muscles after 3-weeks of denervation. n=3-5 per group. *$p < 0.001$. **(E)** Model describing the interactions of miR-206 with the HDAC4/Dach2/myogenin pathway. The observed changes in gene expression in the pathway are consistent with miR-206 regulating HDAC4.

synapse phenotypes between miR-206 mutant mice and mice lacking retrograde FGF signaling (Fox et al., 2007), prompted us to look at the expression of molecules capable of regulating the activity of FGF ligands. We discovered that the expression of an FGF binding protein, *Fgfbp1* (Abuharbeid et al., 2006), was significantly downregulated in the muscles of miR-206 mutant mice and upregulated in the muscles of HDAC4 mKO mice following denervation (**Figure 2.13**). FGFBP1 is a secreted factor that directly interacts with FGF7/10/22 family members and potentiates the bioactivity of FGF7 in rat L6 myoblasts (Abuharbeid et al., 2006; Beer et al., 2005). In that FGF7/10/22, which are close relatives, are muscle derived organizers of presynaptic differentiation at the embryonic NMJ (Fox et al., 2007), we hypothesized that FGFBP1 could also potentiate FGF action during motoneuron synaptogenesis. Consistent with this idea, recombinant FGFBP1 potentiated the ability of FGF10 to promote differentiation of vesicle-rich varicosities in cultured motor neurons (**Figure 2.13**). These findings implicate FGFBP1 as a novel regulator of synapse formation and as a downstream effector of miR-206 and HDAC4.

DISCUSSION

The results of this study provide several key molecular insights into the biological functions of miRNAs and the reinnervation of skeletal muscle following nerve injury. For over 30 years it has been known that denervated muscle is readily reinnervated, whereas innervated muscle cannot be hyperinnervated (Frank et al., 1975). This finding suggested that muscle fibers can sense whether or not they are innervated and respond to denervation by enhancing their susceptibility to reinnervation. The molecular basis for

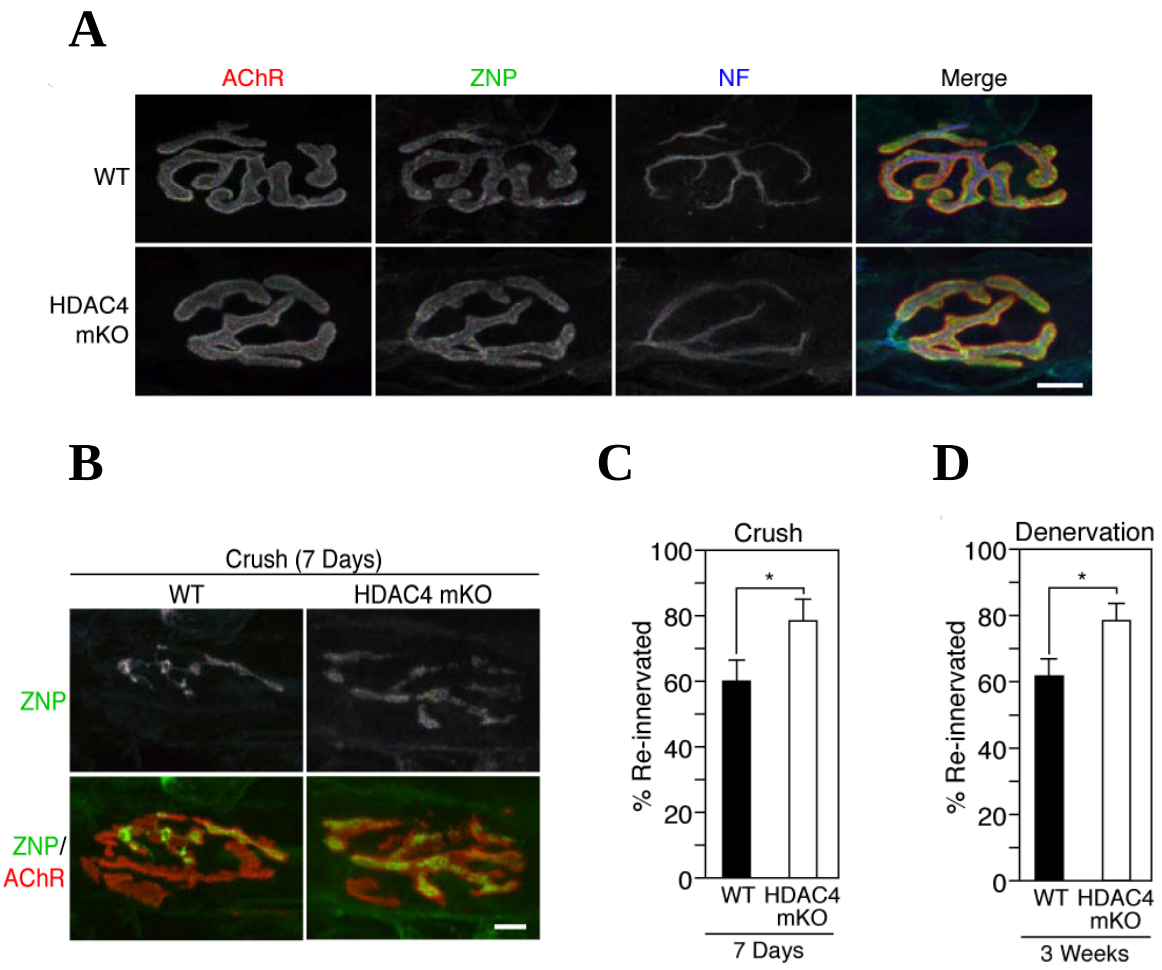

Figure 2.12. Regulation of reinnervation by muscle-derived HDAC4. (A) NMJ development in HDAC4 mutant (mKO) mice is similar to wild type (WT) mice at postnatal day (P) 71. Thick longitudinal sections from WT and HDAC4 mKO TA muscle were co-stained with α-bungarotoxin (BTX) to visualize the post-synaptic acetylcholine receptor (AChR) (red), anti-synaptotagmin (ZNP) to detect pre-synaptic vesicles (green), and anti-neurofilament (NF) to detect nerve axons (blue). Scale bar=10μm. **(B)** Immunohistochemistry using BTX (red) and anti-ZNP (green) shows an increase in reinnervation in HDAC4 mKO mutant mice compared to WT mice 7-days following nerve crush. Note the increase in anti-ZNP (green) staining in HDAC4 mKO mice. Scale bar=10μm. **(C)** Number of reinnervated NMJs in WT and HDAC4 mKO mice following sciatic nerve crush for 7-days. *$p < 0.05$. n=3-8 for each genotype and time point. **(D)** Number of reinnervated NMJs in WT and HDAC4 mKO mice following sciatic nerve cut for 3-weeks. *$p < 0.05$. n=3-8 for each genotype and time point.

this finding has not been identified. MiR-206 and its' associated up- and downstream pathways are ideal molecules that fit this paradigm. We identified the molecular basis for the upregulation of miR-206 following denervation through the direct binding of myogenic bHLH factors to an upstream enhancer. Next, we identified and characterized the physiological function of miR-206 in denervated muscle as an essential regulator of reinnervation. Finally, we identified a downstream pathway that accounts for the delay in reinnervation in miR-206 mutants involving the regulation of a retrograde growth factor signaling pathway. Thus, miR-206 serves as a sensor of motor innervation and regulates a retrograde signaling pathway required for nerve-muscle interactions.

Regulation of miR-206 expression

Following denervation, increases in intracellular calcium levels depolarize muscle fibers and activate downstream pathways that induce MyoD and myogenin protein expression, which activate the expression of many protein-coding genes involved in neuromuscular synaptogenesis. Our results identify miRNAs, and more specifically miR-206, as new transcriptional targets of MyoD and myogenin following denervation of skeletal muscle. We identified an 837 bp upstream enhancer that is responsible for the upregulation of miR-206 expression following denervation. The presence of three conserved and three non-conserved E-boxes in this upstream enhancer confers exquisite sensitivity to the state of motor innervation to the miR-206 locus. Presumably, one of the reasons for the induction of MyoD and myogenin expression is as a protective mechanism to transcriptionally induce the expression of a cadre of genes that signal the motor nerve to

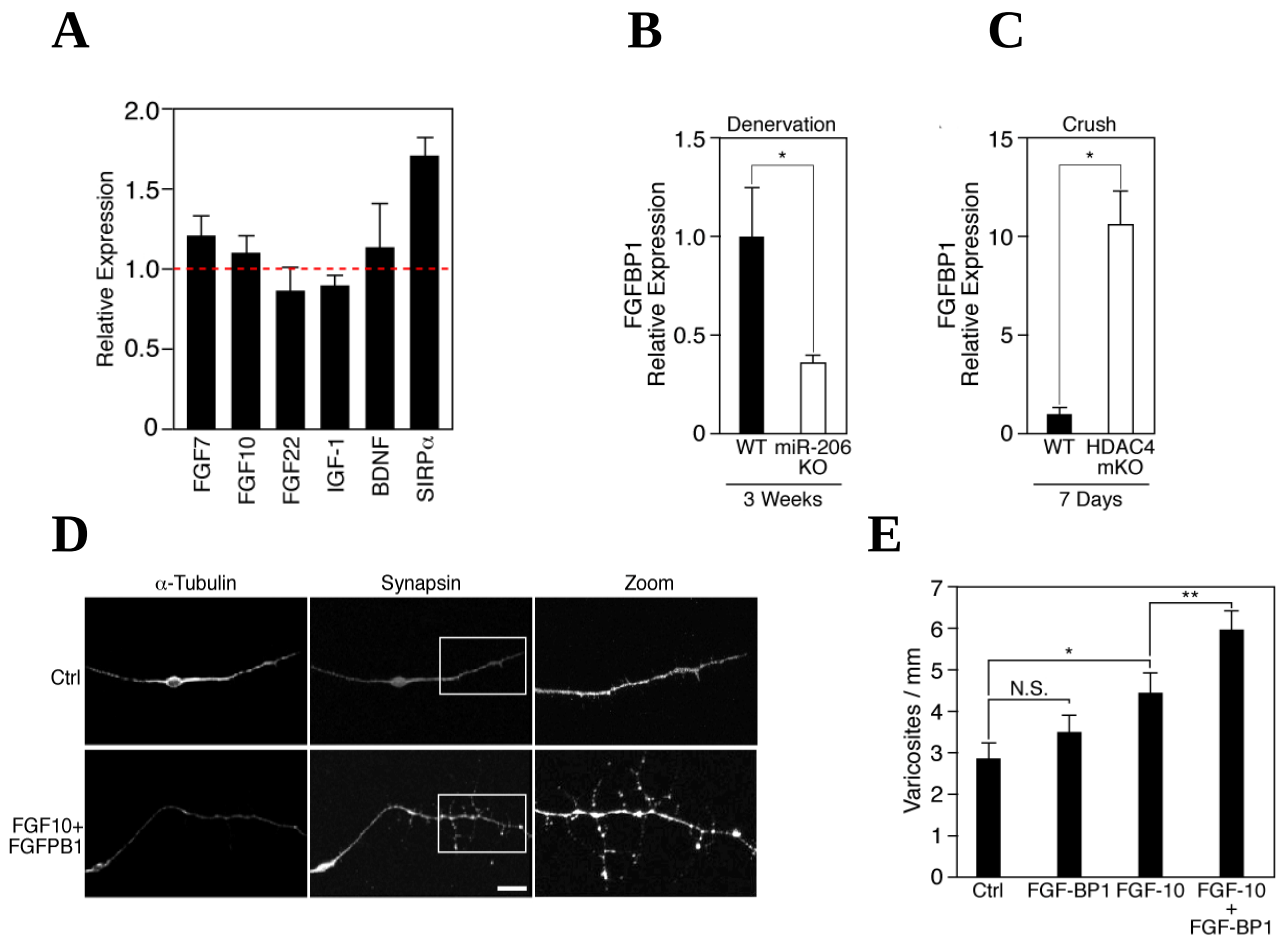

Figure 2.13. Retrograde regulation of synaptogenesis by FGFBP1. (A) Transcripts of candidate secreted retrograde factors were detected in miR-206$^{-/-}$ muscles 3-weeks after sciatic nerve transection. Expression was normalized to wild-type muscle following sciatic nerve transection. n=3-5 per group. **(B)** Decrease in expression of *Fgfbp1* transcripts in wild-type (WT) and miR-206$^{-/-}$ (KO) muscles 3-weeks after nerve transection. *$p < 0.02$. n=3-5 per group. **(C)** Increase in expression of *Fgfbp1* transcripts in wild-type (WT) and HDAC4 mKO muscles 7-days after nerve crush. *$p < 0.0005$. n=3-5 per group. **(D)** Motoneuron cultures were treated with FGFBP1 and FGF10 for 48 hours and stained for synapsin and acetylated tubulin. FGFBP1 potentiates the ability of FGF10 to induce synapsin-positive varicosities. Scale bar=20μm. **(E)** Quantification of the results from the motoneuron culture in (i). Data were obtained from 3 independent experiments. *$p < 0.01$, **$p < 0.02$.

transcriptionally induce the ereinnervate. Our results conclusively demonstrate that miR-206 is one of the transcriptional targets of these bHLH proteins and is involved in the regulation of retrograde signaling to the motor nerve.

Regulation of reinnervation by miR-206

Some of the molecules that promote innervation in embryos or reinnervation in adults have been identified, but little is known about how their expression is regulated (Bennett and Pettigrew, 1976; Chevrel et al., 2006; Fox et al., 2007; Lohof et al., 1993; Sanes and Lichtman, 1999; Umemori and Sanes, 2008). These studies identify miR-206 as another molecule that regulates the reinnervation of NMJs in adult animals. Our results also suggest that one of the functions of miR-206 is to 'fine-tune' the expression level of HDAC4. Indeed, 'fine-tuning' of mRNA targets by miRNAs appears to be a general principle in gene-regulatory networks (Baek et al., 2008; Selbach et al., 2008).

Previous work has shown that over-expression and partial loss-of-function of HDAC4 results in dramatic changes in synaptic gene expression (Cohen et al., 2007); however, effects on retrograde regulation of reinnervation were never examined. In addition, recent work has shown in *C. elegans* the closely related miRNA, miR-1, regulates a muscle-derived signal required for presynaptic function at neuromuscular junctions (Simon et al., 2008). It is noteworthy that both miR-206 and HDAC4 are upregulated upon similar physiological and pathological signals. We suspect that miR-206 is required to titrate the increasing levels of HDAC4 that result from the loss of neural signaling. This idea is supported by the HDAC4 mKO mice, in which the loss of

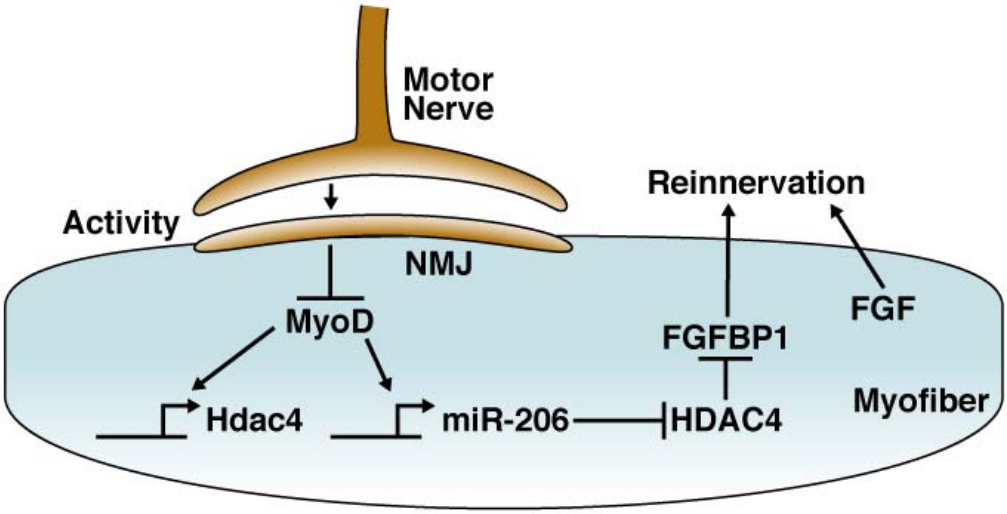

Figure 2.14. Model of miR-206 function. Model of the signaling system of miR-206 and HDAC4 that regulates reinnervation of NMJs.

HDAC4 in skeletal muscle promotes reinnervation following injury. Indeed, local protein accumulation and depletion is regarded as a central regulatory mechanism in the function of synapses (Hippenmeyer et al., 2007). Thus, as a synapse-enriched factor, miR-206 is poised to regulate the local accumulation of proteins at the neuromuscular synapse.

Several lines of evidence indicate that FGFBP1 is a retrograde signal downstream of miR-206 and HDAC4. 1) The expression of FGFBP1 is downregulated in miR-206 mutant mice after denervation. 2) The expression of FGFBP1 is upregulated in HDAC4 mKO mice after denervation. 3) Loss of muscle-derived FGF signaling results in a phenotype very similar to that of miR-206 mutant mice (Fox et al., 2007). 4) The ability of FGFBP1 to potentiate the activity of FGF ligands to induce motoneuron differentiation in culture implicates FGFBP1 as a mediator of neuromuscular synaptogenesis, schematized in (**Figure 2.14**).

FGFBP1 has been extensively characterized as an essential regulator of angiogenesis (Czubayko et al., 1997; Tassi and Wellstein, 2006). Also, it is widely accepted that the molecular mechanisms regulating angiogenesis and nerve growth and regeneration are evolutionarily conserved (Carmeliet and Tessier-Lavigne, 2005). These observations are consistent with and support our discovery of FGFBP1 as a regulator of nerve growth and differentiation.

Stress-dependent functions of miRNAs

The phenotype of miR-206 mutant mice is mechanistically similar to that of an endothelial cell-specific miRNA knock-out, miR-126. There was lack of an overt cellular

phenotype in the endothelium of miR-126 mutant animals; however, miR-126 mutants were unable to respond and induce angiogenesis after several stresses that normally induce angiogenesis, such as myocardial infarction (Wang et al., 2008). In the absence of a neuromuscular stress, miR-206 mutant mice have normal skeletal muscle and neuromuscular synapse structure and function, whereas after a neuromuscular stress, the mice show a severely compromised ability to re-innervate their neuromuscular junctions. These data are highly suggestive of a stress-dependent function for miR-206; however, we cannot rule out the possibility that a phenotype is seen in miR-206 mutant mice only after stress because of redundancy with miR-1. The generation of miR-1-2 knock-out mice has previously been published, but the authors reported no abnormalities in the skeletal muscle of these mice (Zhao et al., 2007). Perhaps, genetic deletion of both miR-1-2 and miR-206 would result in more severe neuromuscular deficits after nerve injury.

This concept of stress-dependent functions for miRNAs is further supported by a large-scale genetic analysis of miRNA function in *C. elegans* that revealed deletion of most individual miRNAs results in no phenotype or subtle phenotypes in the absence of stress (Miska et al., 2007). The fact that they commonly function as regulators of mRNA translation supports a role for miRNAs as important regulators of stress responses because of their potential to facilitate rapid changes in gene expression. Exposure to different types of cellular stress has been shown to result in the localization of miRNAs, target mRNAs, and Argonaute proteins to stress granules, which would result in increased miRNA activity in times of stress (Leung and Sharp, 2007). Whether or not miR-206 or its target mRNAs are subject to this type regulation during neuromuscular stress remains to be determined. Thus, the upregulation of miRNAs following cellular

stress is likely a general signaling principle to allow cells to properly sense and respond to pathological and physiological stimuli.

METHODS

Generation of miR-206 mutant mice

The 2.7-kb 5' arm was amplified from 129SvEv genomic DNA and digested with Sac II and Not I and ligated into pGKNeo-F2L2DTA targeting vector. The 2.1-kb 3'arm was digested with Hind III and Eco RV and ligated between the neomycin and DTA cassettes of the targeting vector. Targeted ES-cells were identified by Southern blotting with 5' and 3' external probes. One clone with a properly targeted miR-206 allele was used for injection into 3.5 day C57Bl/6 blastocysts and the resulting chimeras were bred to C57Bl/6 females for germline transmission. All breedings were performed in the 129SvEv and C57Bl/6 backgrounds.

Generation of HDAC4 mutant mice

Mice with loxP sites flanking exon 6 of the *Hdac4* gene have been previously described (Potthoff et al., 2007). These mice were bred to mice expressing Cre recombinase under control of the muscle-specific *myogenin* promoter and *Mef2c* enhancer to delete HDAC4 specifically in skeletal muscle (Li et al., 2005). All breedings were performed in the 129SvEv and C57Bl/6 mixed backgrounds.

RNA analyses

Total RNA was isolated from tissues using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Northern blots to detect miRNAs generally used 8-10 μg of total RNA and were run on 20% denaturing acrylamide gels. Oligonucleotide probes antisense to the mature miRNA were generated using the Starfire Labeling Kit (IDT). A Starfire oligonucleotide probe for U6 was used as a loading control.

RNA was treated with Turbo DNase (Ambion) prior to reverse transcription. RT-PCR with random hexamer primers was performed on RNA samples using SuperScript III (Invitrogen). Quantitative real time PCR was performed using Taqman probes or SYBR green primers. mRNA levels of genes of interest were normalized to *Gapdh* levels.

For synapse enrichment studies, the diaphragms from thy1-YFP transgenic mice were isolated (Feng et al., 2000). The endplate band, delineated by the YFP rich nerve terminals, was separated. RNA was extracted from synapse-rich and synapse-free regions using TRIzol. Quantitative real time PCR was used to assess miR-206 levels using primers specific for miR-206 and primers for miR-206 and 7H4. *Gapdh* mRNA levels were used as a loading control. Three diaphragms were examined and each quantitative real time PCR experiment was done in triplicate.

For miRNA microarray, total RNA was extracted from gastrocnemius/plantaris (G/P) muscles of wild-type mice following resection of the sciatic nerve for 10 days, and used for miRNA microarray analysis at LC Sciences (Houston, TX).

Generation and analysis of lacZ reporter mice

An 837 bp genomic fragment upstream of the *miR-206* gene (from -1576 to -739) containing conserved E-boxes was fused to the hsp68 basal promoter upstream of a lacZ reporter gene. Mutations were introduced into the three conserved E-boxes using QuikChange II Site-Directed Mutagenesis Kit (Stratagene). LacZ reporter transgenes were injected into the pronuclei of fertilized oocytes by standard techniques. Muscles of P10 transgenic mice were denervated for 10 days by sciatic nerve transection followed by staining for β-galactosidase activity (Cheng et al., 1993). Two independent transgenic wild-type (WT) and mutant (Mt) lines were examined.

Surgical procedures

Surgical procedures were performed on anaesthetized 6- to 10-week old mice. Denervation was performed by cutting the left sciatic nerve in the mid-thigh region. Nerve crush was performed by crushing the sciatic nerve for 15 or 30 seconds and analyses were performed 7 and 18 days later. All animal protocols and procedures in this study were reviewed and approved by the UT Southwestern Institutional Animal Care and Use Committee.

Plasmids and transfection assays

The 1100 bp genomic fragment containing the miR-206 coding sequence was ligated into the pCMV6 expression vector. A 1200 bp genomic fragment of the mouse *Hdac4* 3' UTR containing the miR-206 predicted binding sites was ligated into luciferase reporter pMIR-REPORT (Ambion). Mutations in the miR-206 predicted binding sites were introduced using QuikChange II Site-Directed Mutagenesis Kit (Stratagene). Increasing

amounts of the miR-206 expression vector (0ng, 50ng, 100ng, and 200ng) were co-transfected with the HDAC4-UTR luciferase vector and CMV-LacZ vector. Assays were performed in triplicate and luciferase activity was determined 40 hours later and normalized to lacZ activity. The 837 bp miR-206 denervation response element was ligated into the luciferase reporter pGL3 containing a minimal hsp70 TATA element. Increasing amounts of pcDNA-MyoD (0ng, 25ng, 50ng, 100ng) were co-transfected with miR-206-enhancer luciferase vector and CMV-LacZ vector. Luciferase activity was determined 40 hours later and normalized to lacZ activity.

Gel shift assay

Oligonucleotides corresponding to the conserved E-boxes and mutated binding sites were synthesized (IDT). Annealed oligonucleotides were labeled with ^{32}P-dCTP using Klenow. Whole-cell lysates from COS1 cells transfected with pcDNA-myc-MyoD and pcDNA-E12 or empty vector pcDNA were isolated and used in DNA binding assays. Unlabeled oligonucleotides were used as competitors.

Western blot analysis

Protein lysates were resolved on 12% SDS-PAGE gels using standard procedures. Antibodies against HDAC4 (H-92 Santa Cruz, 1:500) and GAPDH (MAB374 Chemicon, 1:5000) were used. HDAC4 protein expression was quantified by densitometry and normalized to GAPDH protein expression using ImageQuant 5.0 software.

Histology and immunostaining

Mice were anesthetized with avertin and trans-cardially perfused first with PBS followed with 4% paraformaldehyde. Muscles were removed, immersed in 30% sucrose for cryoprotection, frozen in Tissue-Tex OCT reagent and cryosectioned into 30-40 um-thick longitudinal sections. Before immunostaining, sections were washed 3 x 10 minutes with PBS and blocked (5% normal goat serum, 2% BSA and 0.1% TritonX-100 in PBS) for 1hr. The sections were then incubated with Alexa488-conjugated Bungarotoxin (BTX) (A488-BTX; Invitrogen), anti-synaptotagmin-2 (ZNP-1; Developmental Hybridoma Bank (University of Iowa; Iowa City, IA)), and anti-neurofilament (SMI312; Covance) diluted in blocking solution and incubated overnight at 4 degrees. The sections were then washed 3 x 10 minutes and incubated with fluorescently tagged secondary antibodies for 1hr at room temperature followed by three PBS washes and coverslip in Vectashield mounting medium. All images were acquired using an FV-100 confocal microscope. Hematoxylin and eosin (H&E) and metachromatic ATPase staining were performed using standard procedures (Oh et al., 2005).

Motor neuron culture

Chicken motoneurons were purified at embryonic day 5.5 as previously described (Fox et al. 2007). Factors (2nM FGF10 and 25 ng/mL FGFBP-1) were added 3 hours after culture and incubated for 48 hours. Motoneurons were fixed and stained with anti-synapsin and acetylated-tubulin. The synapsin puncta, representing nascent synaptic sites, were counted on an epifluorescence microscope (Zeiss). High magnifaction images were obtained using an Olympus FV1000 microscope.

Statistical analysis. Statistical significance was determined using an Unpaired *t* test (Graphpad). P values < 0.05 were considered to be statistically significant. All error bars represent ± SEM.

ACKNOWLEDGEMENTS

This research was conducted in collaboration with Greg Valdez and Joshua Sanes at Harvard University.

Chapter III

Regulation of ALS Pathogenesis by miR-206

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is the most common adult motor neuron disease, affecting approximately 30,000 persons in the United States (Bruijn et al., 2004). About 90% of ALS cases are sporadic and the other 10% occur as the result of an inherited mutation is several genes (Boillee et al., 2006a). Regardless of the etiology, the disease involves common symptoms, which include the denervation of target muscle through the selective loss and degeneration of motor neurons, resulting in muscle atrophy and paralysis of lower limb and respiratory muscles. The discovery of the upregulation of miR-206 expression in denervated muscle prompted us to investigate if miR-206 is regulated in a genetic model of muscle denervation. To define the potential functions of miRNAs in ALS pathogenesis, we compared the miRNA expression profiles of skeletal muscles of wild-type and G93A-SOD1 transgenic mice. We discovered that miR-206 is the most upregulated miRNA in G93A-SOD1 muscle and that the upregulation coincides with the progression of the disease. To determine if miR-206 expression influences the pathogenesis of ALS, we crossed G93A-SOD1 transgenic and miR-206 mutant mice. We show that the loss of miR-206 expression significantly accelerates the onset of symptoms of ALS. These results demonstrate that the upregulation of miR-206 expression is required to delay the pathogenesis of ALS and point to miR-206 as a potential therapeutic target to treat the clinical symptoms associated with ALS.

RESULTS

Upregulation of miR-206 in ALS mice

To determine if miR-206 is also regulated in a genetic model of skeletal muscle denervation, we performed miRNA array profiling of muscles of G93A-SOD1 transgenic mice, a common mouse model of ALS (Son et al., 2007) (**Figure 3.1**). These mice harbor a gain-of-function mutation in superoxide dismutase (SOD) in which glycine-93 is replaced with alanine, as seen in a subset of human ALS patients. The array was performed on the muscles of symptomatic G93A-SOD1 transgenic and non-symptomatic wild-type mice. Among the many miRNAs that were up and down-regulated in the muscles of G93A-SOD1 mice, miR-206 was found to be the most upregulated (**Figure 3.2**). Northern blot analysis confirmed the array results and revealed an approximate 9-fold increase in miR-206 expression (**Figure 3.2**). The upregulation of miR-206 coincided with the onset of neurological symptoms in G93A-SOD1 mice as younger, healthy transgenic mice displayed no upregulation of miR-206 when compared to wild-type littermates (**Figure 3.2**).

miR-206 regulates the pathogenesis of ALS

The upregulation of miR-206 in ALS mice could simply be a consequence of the initiation and/or the progression of the disease. To determine if miR-206 has a functional role in the pathogenesis of ALS, we crossed G93A-SOD1 transgenic and miR-206 mutant mice. We discovered that the loss of miR-206 significantly accelerated the onset

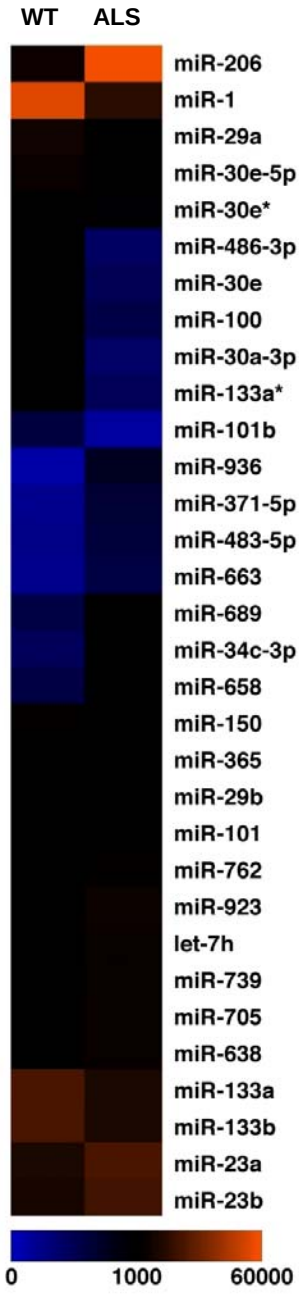

Figure 3.1. Profiling miRNAs in ALS mice. Heat map of miRNAs up- or downregulated (> 2-fold) in the GP muscle of 7-month old wild-type (WT) and G93A-SOD1 mice.

of symptoms of ALS. G93A-SOD1 mice in a wild-type background developed neurological symptoms at approximately 240 days, with a mean survival of 274 days (**Figure 3.3**). In contrast, contemporaneously generated littermates of G93A-SOD1 mice in a miR-206 mutant background developed neurological symptoms at approximately 210 days, with a mean survival of 244 days, an 11% decrease in typical disease survival (**Figure 3.3**). The acceleration of disease symptoms was seen by excessive denervation and dysfunction of NMJs (**Figure 3.4**). This was accompanied by accelerated atrophy of skeletal muscle, leading to accelerated kyphosis and paralysis (**Figure 3.3**). MiR-206 mutant mice showed no overt phenotype or decrease in survival up to 500 days (**Figure 3.3**). These results conclusively demonstrate a role for skeletal-muscle derived factors and miR-206 in the pathogenesis of ALS and further substantiate the function of miR-206 in regulating nerve-muscle signaling during stress and disease.

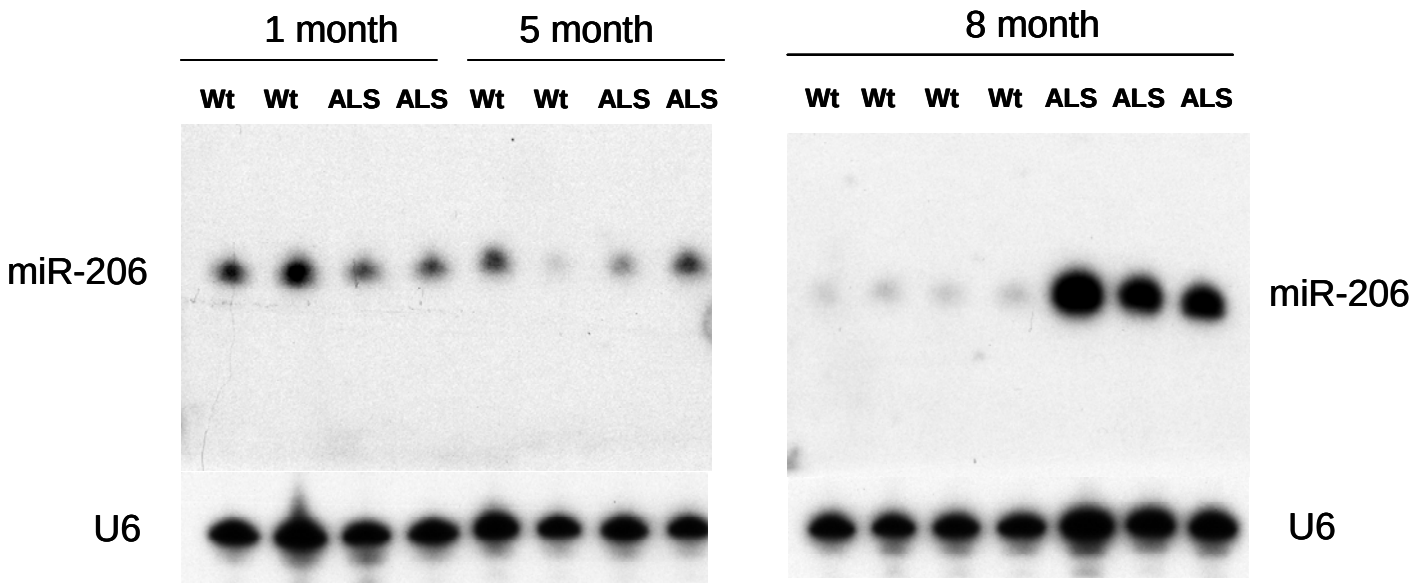

Figure 3.2. Upregulation of miR-206 in ALS mice. Northern blot of miR-206 expression in TA muscle of wild-type (WT) and G93A-SOD1 (ALS) mice at the indicated ages. U6 was used as a loading control.

DISCUSSION

The results of this study demonstrate a unique function of miRNAs and miR-206 as essential regulators of the pathogenesis of ALS. The absence of miR-206 results in an acceleration of the initiation of symptoms and a decrease in survival in ALS mice. These results also identify a previously unidentified and unappreciated role of muscle-derived factors in the pathogenesis of ALS.

miRNAs and RNA processing in ALS pathogenesis

The dysregulation of protein-coding genes in mouse models and patients with ALS has been well described (Boillee et al., 2006a; Gonzalez de Aguilar et al., 2008). However, to date the expression profile of miRNAs in ALS has not been reported . We found that the expression of several miRNAs, most notably miR-206, is significantly changed in the muscles of G93A-SOD1 mice. Although there appears to be a requirement for damage to motor neurons to initiate the ALS phenotype, other cell types are clearly involved in the pathological progression of the disease (Boillee et al., 2006b). These observations support a role for non-neuronal mRNAs; as well as miRNAs in impacting the pathological gene networks involved in ALS.

Previous data has shown that G93A-SOD1 toxicity in skeletal muscle is probably not a cell-autonomous determinant of the pathogenesis of ALS (Dobrowolny et al., 2008; Miller et al., 2006). However, retrograde transport and delivery of various growth factors,

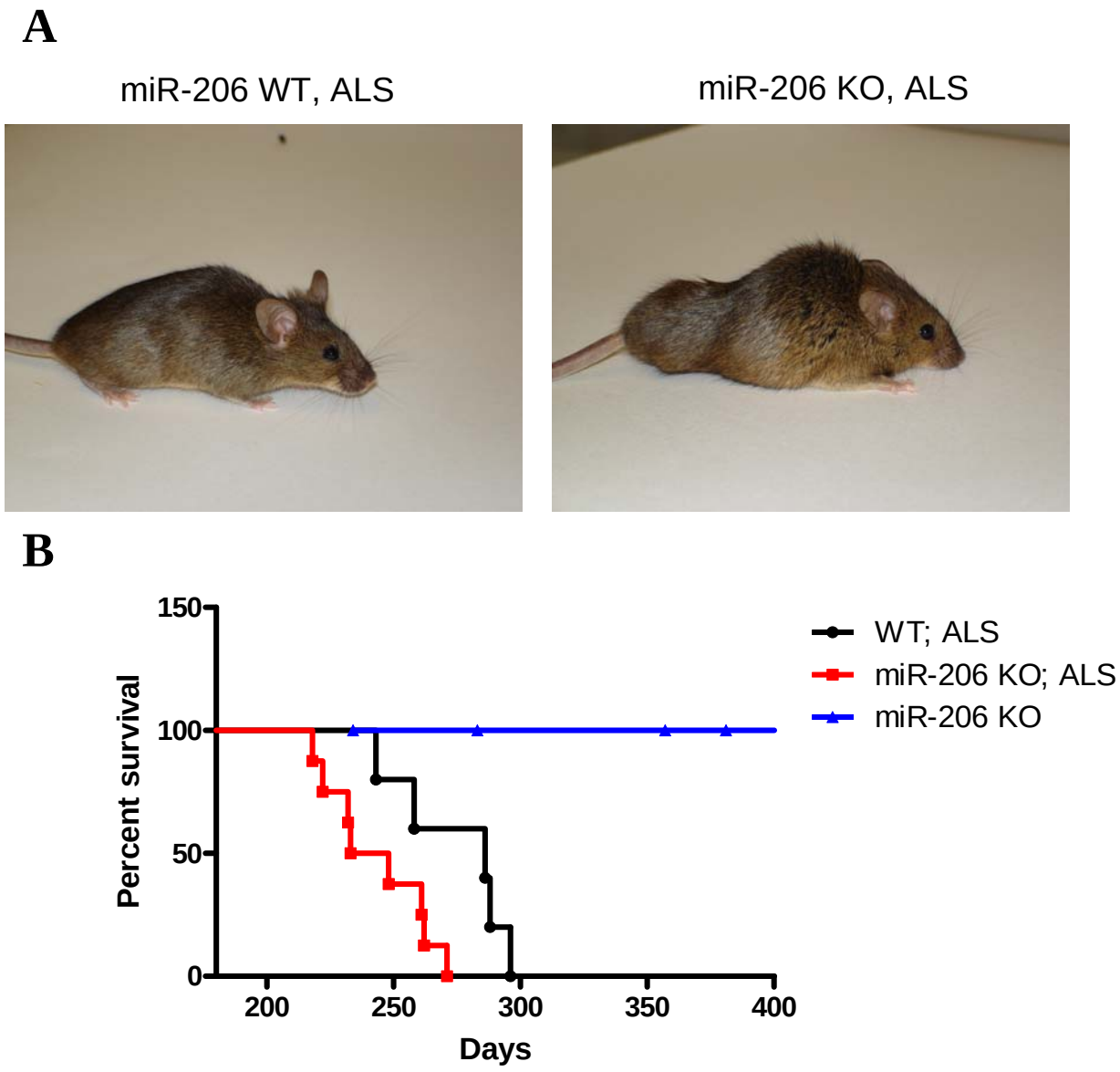

Figure 3.3. Decreased survival of ALS mice lacking expression of miR-206. (A) Acceleration of symptoms and disease onset in G93A-SOD1 mice lacking expression of miR-206 as shown by paralysis and kyphosis. **(B)** Survival curve of G93A-SOD1 mice in wild-type background and G93A-SOD1 mice in a miR-206 KO mutant background demonstrates the loss of miR-206 accelerates disease progression.

including IGF1 has been shown to delay the pathogenesis of ALS (Dobrowolny et al., 2005; Kaspar et al., 2003). Our data and the phenotype of the miR-206 null mice genetically demonstrate a function for muscle-derived factors, including miR-206, in the pathogenesis of ALS. Several mechanisms have been proposed to contribute to the progression of ALS, including oxidative damage, glutamate excitotoxicity, and axonal retrograde transport defects (Dunckley et al., 2007). Our findings demonstrate that a change in the expression of miRNAs is also a likely mechanism contributing to the progression of ALS.

Recent studies have identified human mutations in the genes TDP-43 and Fus, which are proteins involved in various aspects RNA metabolism (Kwiatkowski et al., 2009; Neumann et al., 2006). Interestingly, both of these proteins were identified to biochemically interact with the miRNA processing enzyme Drosha (Gregory et al., 2004). These studies further corroborate our results and conclusions that the dysregulation of miRNA expression is an additional mechanism involved in the pathogenesis of ALS.

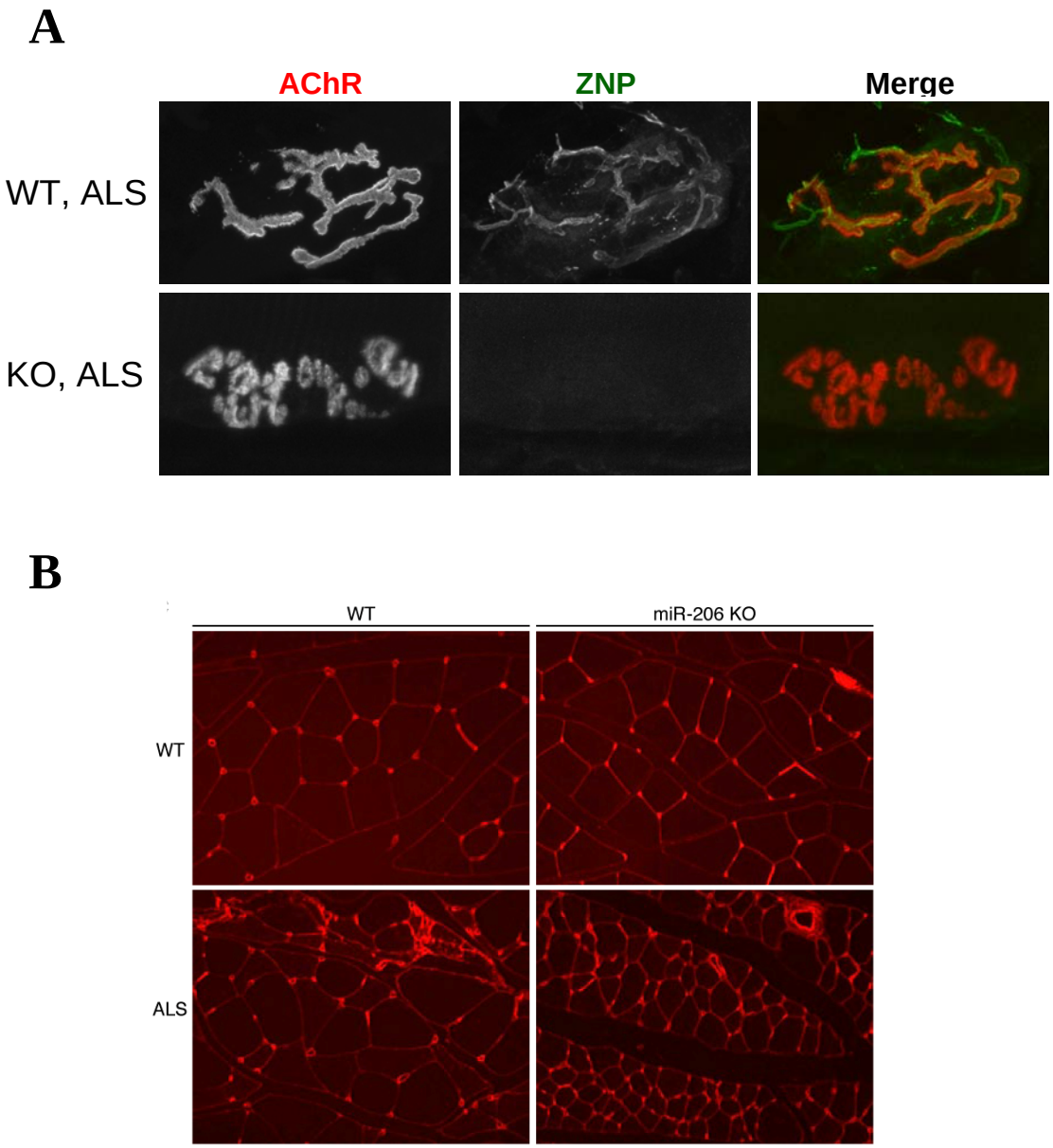

Figure 3.4. Acceleration of disease pathogenesis in mice lacking miR-206. (A) Immunohistochemistry using BTX (red) and anti-ZNP (green) shows dysfunction and denervation of NMJs in G93A-SOD1 (ALS) mice lacking expression of miR-206. **(B)** Wheat-germ agglutinin staining (red) shows acceleration of muscle atrophy in G93A-SOD1 (ALS) mice lacking expression of miR-206.

METHODS

G93A-SOD1 Mice

Mice expressing a low-copy number of mutant SOD1 B6SJL-TgNSOD1-G93A were obtained from Dr. Jeffrey Elliott (Son et al., 2007). These mice were crossed to miR-206 mutant mice in a mixed SvEv129 and C57Bl/6 background. For survival analysis, contemporaneously produced littermates were compared to avoid differences due to genetic background. Mice were sacrificed once they were unable to right themselves within 15 seconds of being turned over.

RNA analyses

Total RNA was isolated from tissues using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Northern blots to detect miRNAs generally used 8-10 µg of total RNA and were run on 20% denaturing acrylamide gels. Oligonucleotide probes antisense to the mature miRNA were generated using the Starfire Labeling Kit (IDT). A Starfire oligonucleotide probe for U6 was used as a loading control.

For miRNA microarray, total RNA was extracted from tibialis anterior (TA) muscles of wild-type and symptomatic G93A-SOD1 mice, and used for miRNA microarray analysis at LC Science (Houston, TX).

Histology and immunostaining

Mice were anesthetized with avertin and trans-cardially perfused first with PBS followed with 4% paraformaldehyde. Muscles were removed, immersed in 30% sucrose for

cryoprotection, frozen in Tissue-Tex OCT reagent and cryosectioned into 30-40 um-thick longitudinal sections. Before immunostaining, sections were washed 3 x 10 minutes with PBS and blocked (5% normal goat serum, 2% BSA and 0.1% TritonX-100 in PBS) for 1hr. The sections were then incubated with Alexa488-conjugated Bungarotoxin (BTX) (A488-BTX; Invitrogen), anti-synaptotagmin-2 (ZNP-1; Developmental Hybridoma Bank (University of Iowa; Iowa City, IA)), and anti-neurofilament (SMI312; Covance) diluted in blocking solution and incubated overnight at 4 degrees. The sections were then washed 3 x 10 minutes and incubated with fluorescently tagged secondary antibodies for 1hr at room temperature followed by three PBS washes and coverslip in Vectashield mounting medium. All images were acquired using an FV-100 confocal microscope. Alexa-fluor 594-conjugated wheat-germ agglutinin (WGA) staining was performed on paraffin-embedded sections of muscles at a concentration of 50 μg/mL.

Chapter IV

Summary and Future Directions

SUMMARY

Utilizing several mouse models, I have identified a new signaling pathway required for efficient reinnervation of neuromuscular synapses following injury. I found that denervation of skeletal muscle induces the expression of a muscle-specific miRNA, miR-206, which is required for efficient reinnervation. After an injury, skeletal muscle induces the expression of genes involved in signaling to the nerve to reinnervate. We identified miR-206 as one of these genes. Additionally, miR-206 appears to function by positively regulating FGF activity to regulate the rate of reinnervation of neuromuscular synapses.

These discoveries motivated us to determine if these same molecular mechanisms are deployed during the progression of neurodegenerative disease. To this end, using a mouse model of neurodegenerative disease, I have discovered a new molecular mechanism regulating the pathogenesis of ALS that involves the upregulation of miR-206 and possibly other protein-coding genes in skeletal muscle. Currently, there is no cure or effective treatment for ALS patients that can prolong survival or the onset of symptoms. Thus, there is an intense effort to identify the signaling pathways involved in the progression of the disease. The results of this thesis present evidence to pursue miR-206 and its associated pathways as new therapeutic targets to treat neurodegenerative disease.

FUTURE DIRECTIONS

Currently, I have demonstrated that the expression of miR-206 is essential for efficient reinnervation of neuromuscular synapses and to delay the pathogenesis of ALS. Current studies are aimed at determining if the over-expression of miR-206 is sufficient to accelerate reinnervation and/or delay the pathogenesis of ALS. We have taken two approaches to address these hypotheses. 1) We have genenerated transgenic mice that over-express miR-206 specifically in skeletal muscle ~3-fold and crossed them to the G93A-SOD1 transgenic mice (**Figure 4.1**). 2) We have started to inject modified oligonucleotides (miR-mimics) that contain the mature miR-206 sequence into G93A-SOD1 mice to attempt to therapeutically delay the pathogenesis of ALS (**Figure 4.1**). Indeed, delivery of antisense oligonucleotides against mutant SOD1 has previously been shown to delay disease pathogenesis (Smith et al., 2006). Since, G93A-SOD1 mice survive to at least 250 days, the results of these experiments are still pending. In support of this approach; however, preliminary data indicates that genetic over-expression of miR-206 specifically in skeletal muscle is sufficient to accelerate reinnervation following denervation (**Figure 4.2**). The molecular basis for these observations is under investigation.

In addition, we have started exploring another function of miR-206 as a regulator of skeletal muscle satellite cell physiology. Satellite cells are stem-cells in skeletal muscle that can give rise and re-populate muscle cells in response to injury or disease like muscular dystrophy (Buckingham and Montarras, 2008). We discovered that the expression of miR-206 is upregulated upon treatment of muscle with cardiotoxin, a

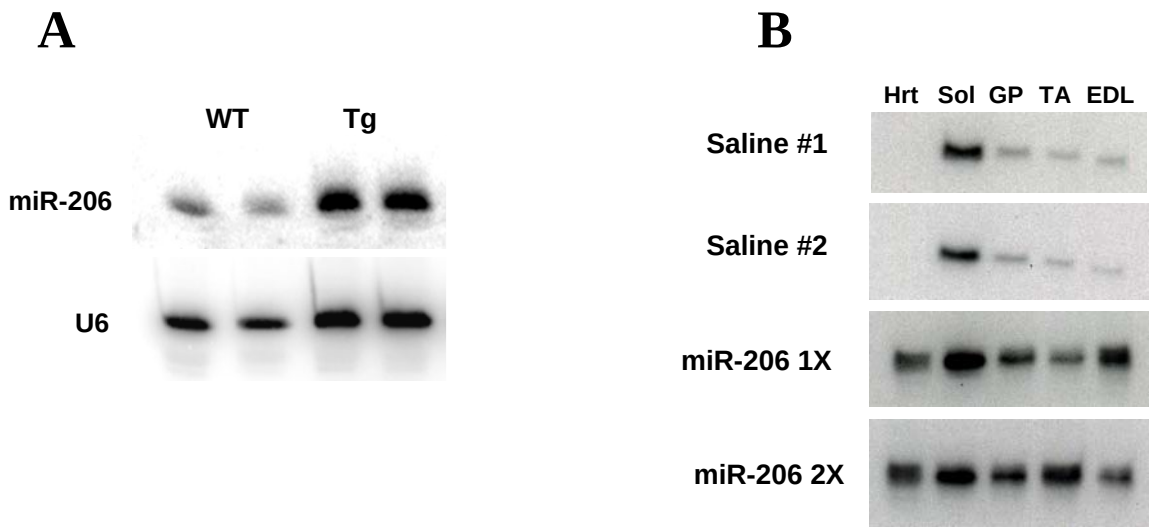

Figure 4.1. Over-expressing miR-206 in skeletal muscle. (A) Northern blot analysis of skeletal muscle of wild-type (WT) and miR-206 transgenic (Tg) mice. **(B)** Northern blot analysis of different muscles of saline injected and miR-206-mimic injected mice. Mice were injected with 100 mg/kg of mimic for 4-days.

potent inducer of muscle regeneration, suggesting that miR-206 may have an important function during muscle regeneration (**Figure 4.3**). A genetic model of muscle regeneration is the dystrophin deficient (mdx) mouse, which harbors a point mutation introducing a stop codon in the dystrophin gene resulting in muscular dystrophy (Bulfield et al., 1984). Muscular dystrophy is characterized by progressive weakness and decline of muscle function due to the depletion of muscle satellite cells. To determine if miR-206 regulates muscle regeneration, we crossed miR-206 mutant and mdx mice. We found that the loss of miR-206 expression results in increased pathogenesis of the mdx phenotype. This was shown by increased uptake of Evan's blue dye (EBD) into the myofibers of miR-206; mdx double mutant mice with a lack EBD uptake in mdx mutant mice (**Figure 4.3**). Also, trichrome staining revealed a significant worsening of muscle pathology shown by myofiber disorganization, degeneration, and necrosis (**Figure 4.3**). The molecular basis for these observations is currently under investigation; however, these results solidify the upregulation of miR-206 as an essential component in the response of muscle to a variety of stresses and diseases.

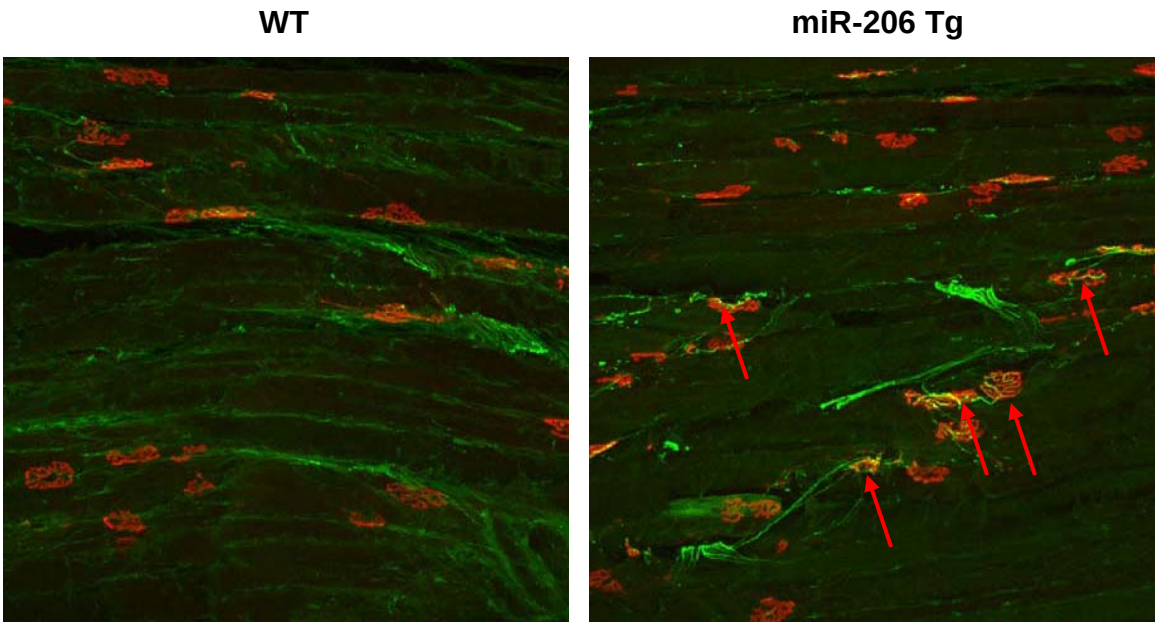

Figure 4.2. Acceleration of reinnervation by over-expressing miR-206. Immunohistochemistry using BTX (red) and anti-ZNP (green) shows increased numbers of reinnervated neuromuscular synapses following 3-weeks of denervation in mice over-expressing miR-206 specifically in skeletal muscle (miR-206 Tg) compared to wild-type (WT) mice. Red arrows indicate reinnervation of neuromuscular synapses.

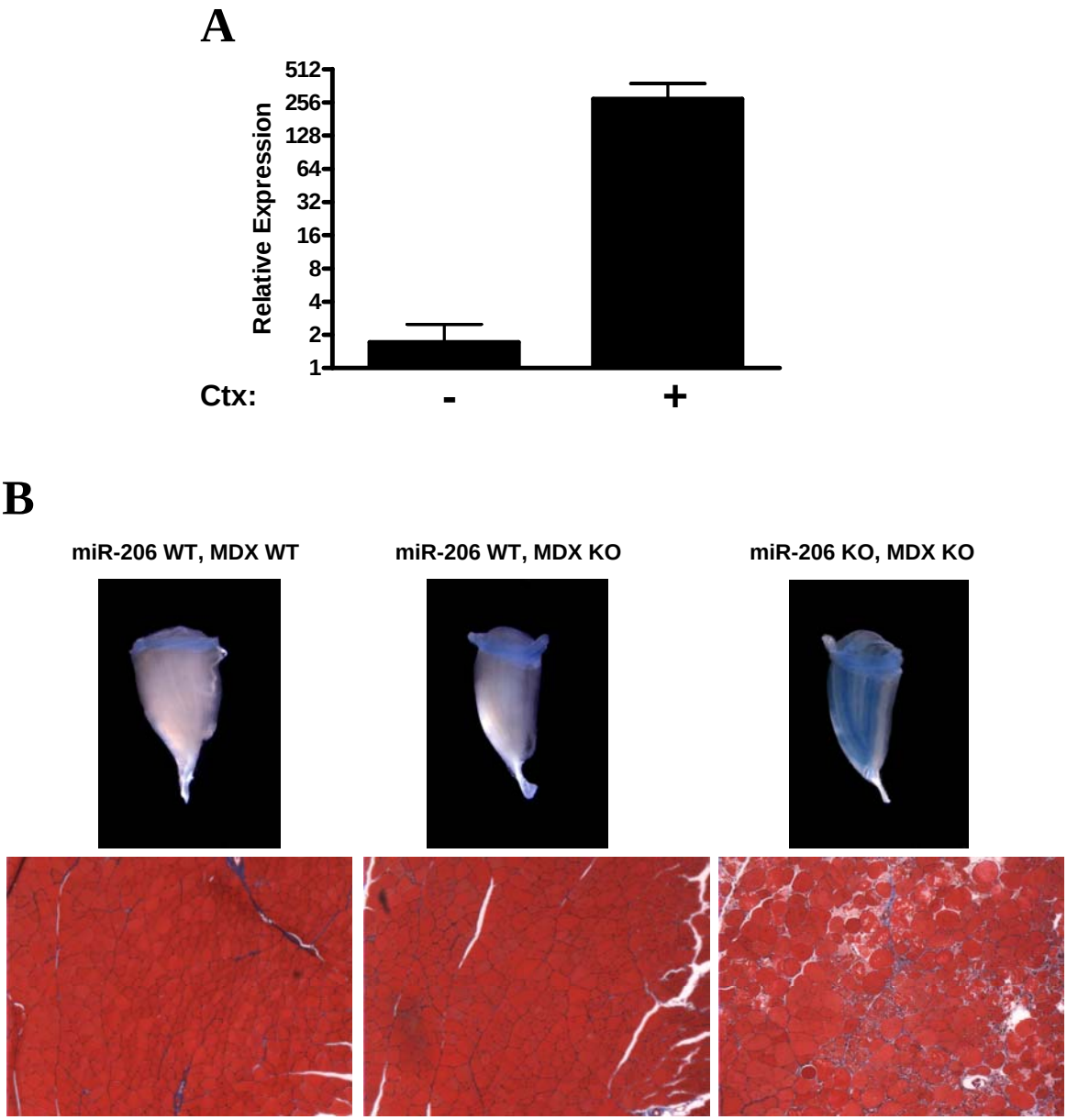

Figure 4.3. miR-206 regulates muscle regeneration. (A) Real-time PCR from skeletal muscle of untreated (-) and cardiotoxin-injected (+) tibialis anterior (TA) muscles demonstrates an increase in miR-206 expression during skeletal muscle regeneration. **(B)** Upper panel shows increase in EBD uptake in TA muscle of miR-206, mdx double mutant mice compared to mdx mutant alone. Lower panels show increased muscle degeneration and myofiber disorganization in miR-206, mdx double mutant mice by trichrome staining.

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