The Regulation of Myogenic Transcription Factors by MicroRNAs

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Abstract

Molecular biology has been a field of study with a constantly expanding frontier. Short RNAs in particular were once thought to be cellular debris. They are in fact critical regulators of other nucleic acids. Early studies showed that short RNAs are enriched for a specific length and sequence. A class of such short RNAs was called microRNAs, and their function is to bind and repress messenger RNA. MicroRNAs regulate cellular processes by repressing multiple genes. Studying the transcriptional regulation of microRNAs as well as the messenger RNAs that they target can give insight into how cells govern cell fate decisions.

The genetics of skeletal muscle lineage commitment are at first glance relatively simple. MyoD overexpression is sufficient to convert fibroblasts into skeletal muscle myotubes. *In vivo*, there is a spectrum of differentiation that requires a large regulatory network to maintain the proper balance of progenitor and differentiating cells. The understanding of the mechanisms regulating transitions between various steps of differentiation remains incomplete. In this work, I will describe how microRNA plays a critical role in skeletal muscle differentiation by targeting repressors of myogenic transcription factors.

Pax7 is a marker of myogenic stem cells, called satellite cells. The downregulation of Pax7 is a critical step to permit differentiation of satellite cells. In the first part, I will show that miR-206 and miR-486 give negative feedback from MyoD to Pax7. To prevent premature differentiation, MyoD is inhibited by other proteins besides Pax7. In the second part, I will describe how MyoD also promotes the transcription of miR-378. This microRNA represses the competitive inhibitor of MyoD, known as MyoR.

The expression of a number of structural proteins associated with terminal differentiation is dependent on the transcription factor Mef2c. In the third part I will demonstrate that the intracellular domain of Notch3 is a negative regulator of Mef2c. Furthermore, I will show that Notch3 is regulated by a combination of a unique enhancer and miR-206. In summation, I will show that microRNAs regulate multiple steps in myogenesis, setting up and reinforcing bistable switches between different stages of differentiation in myogenic stem cells.

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"Try to learn something about everything and everything about something." - T.H. Huxley

My greatest strength and weakness is that once I become infatuated with something, I want to know everything about it. Luckily I have had a remarkable run of people in my life who have allowed me to spend every day in pursuit of new knowledge.

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Chapter 1: Introduction

There has been a lot of excitement about using our rapidly growing knowledge of stem cells to treat an array of degenerative diseases. Directing stem cells to the proper place in a condition that allows for therapy has been a challenge in every system. Skeletal muscle should be an attractive candidate for cell therapy as it has an innate capacity for repair, albeit one that declines with age. Chronic degeneration and regeneration are a central part of the pathophysiology (1) of diseases, such as Duchene Muscular Dystrophy,. While the cause of the damage is still debated, there is increasing evidence that the disease becomes symptomatic once the resident progenitor cells become exhausted (2). The progenitor cells of mature skeletal muscle, known as satellite cells, were first identified more than 50 years ago (3). An obvious question is whether satellite cells can be expanded *ex vivo* and used as therapy for myopathies or for age-related degeneration. Initial tests performed 20 years ago showed very limited clinical utility (4).

Since then, it has been demonstrated that both biochemical (5) and mechanical factors (6) can impact a satellite cell's regenerative capacity. There are many obstacles that remain to be overcome before we can begin to redouble our efforts on clinical therapies. First, we must identify the cell state that will permit therapy, and then be able to direct a large cohort of cells to that state. The latter goal will require the identification of factors important for myogenesis that have many downstream effects with functional roles, not one or two "key" targets. Therefore, we must begin to integrate our currently dispersed knowledge. Many signaling pathways have been shown to be important for myogenesis, but we still need to discover how they cross-talk and converge. By

combining computational systems biology with rigorous biochemical data, we will eventually properly harness the potential for cell based therapy.

A. Dissertation Overview

In this dissertation, I will show that microRNAs and myogenic transcription factors can form feed forward loops to regulate myogenic differentiation. I have helped to elucidate three loops where a microRNA gene is induced and in turn downregulates an inhibitor of its own transcription factor.

In the first loop, we show that Pax7 is repressed by microRNA (miR) -206 and miR-486 (7). We noticed that the inverse was also true; Pax7 expression represses microRNA-206 levels. We inferred that Pax7 inhibits the myogenic transcription factor MyoD. These findings suggest that microRNA is important for setting up and regulating the jump from one metastable state to another.

In discovering the second loop, we utilize publically available data to find new microRNAs that are regulated by MyoD. We found miR-378 as a novel regulator of myogenesis (8). miR-378 targets the competitive antagonist of MyoD, MyoR. This complemented our earlier work and established MyoD as a critical regulator of its own inhibitors.

The third loop shows that Notch3 is regulated by miR-206. Unlike earlier targets, Notch3 does not affect MyoD activity. Instead it inhibits differentiation by inhibiting Mef2c. This work showed that multiple steps in the myogenic progression are regulated by microRNAs. We think that most cell fate transitions will have microRNAs reinforcing the transition from one metastable state to another.

B. Skeletal Muscle Genetics

Skeletal muscle precursor cells originate in the dermomyotome during early development. These proliferating and undifferentiated progenitor cells express the paired-box transcription factors Pax3 and Pax7 (9). Pax3 is critical for initiating the myogenic fate of cells in the dermomyotome (10). It is also critical for the migration of myogenic progenitor cells to the developing limbs (11). The Pax genes induce commitment to the myogenic cell fate by inducing expression of a group of basic helix-loop-helix (bHLH) transcription factors referred to as the muscle regulatory factors (MRFs) in the dermomyotome (12). Forced expression of Pax3 is sufficient to induce MRFs in embryonic mesoderm (13).

The four MRFs are MyoD, Myf5, Myogenin and Mrf4. The MRFs were first identified for their ability to transdifferentiate fibroblasts into myotubes (14). All of the MRFs heterodimerize with other bHLH proteins known as E proteins. The dimer then binds to the consensus DNA sequence CANNTG. Knocking out either Myf5 (15) or MyoD (16) resulted in mice with relatively minimal phenotypes. Knocking out both resulted in a complete absence of skeletal muscle tissue (17). This was the first demonstration of compensatory regulation giving robustness to the overall process of myogenesis. Myogenin is upregulated later, and is required for terminal myogenesis (18). It is not a functional homolog for Myf-5 or MyoD (19). Mice carrying different targeted MRF4 mutations display a range of phenotypes consistent with a role for MRF4 late in the myogenic pathway (20). Interestingly, mice lacking both MyoD and MRF4 display a phenotype similar to the myogenin-null phenotype (21). Therefore, MRF4 function may be substituted by the presence of myogenin but only in the presence of MyoD. Notably,

MRF4 appears to have a role as a lineage determination factor in the early somite development and as a differentiation factor in later myogenesis (22).

The MADS-box transcription factor Mef2c also has an important role in myogenic differentiation (23). It is expressed in somites and limb buds during embryonic development, generally after the expression of MRFs begin (24). It works in concert with the MRFs to promote myogenesis on two levels. First, many myogenic gene loci have an MRF binding element (E-box) and a Mef2 binding element (MADS-box) in close proximity. The simultaneous binding of both transcription factors is required for maximal expression of genes including myogenin (25), MRF4 (26) and Mef2c itself (27). Mef2c also seems to increase the transactivation ability of MRFs independent of its own DNA-binding (28). Skeletal muscle specific knockout of the Mef2c gene in mice resulted in disorganized myofibers and perinatal lethality (29).

After lineage specification in the dermomyotome, the muscle progenitors then migrate to the appropriate anatomical compartment and begin to form the skeletal muscle architecture. Skeletal muscle retains the ability to regenerate in adulthood due to the presence of a reservoir of progenitor cells. These cells are referred to as satellite cells due to their anatomical location outside of the mature myofiber, but below the basement membrane (3). These cells express Pax7, but usually not Pax3. The expression of Pax7 is crucial to proper satellite cell formation in development (30). Satellite cells are normally quiescent but can divide either symmetrically to create two new quiescent satellite cells, or asymmetrically to create one differentiating cell and one progenitor cell (31). When these cells divide asymmetrically, the differentiating cell begins to express Myf5. Myf5 directs the transcription of several genes that both initiate differentiation and signal back

to the non-differentiating cell via the Notch ligand Dll1. The ligand signals to Notch1 receptor on the progenitor cell to maintain it in the stem cell mode. Among the myogenic genes turned on by Myf5 are the MRFs MyoD and Myogenin. Myogenin and Mef2c reciprocally induce each other (32) (Fig. 1). Together, these two transcription factors drive the maturation of myocytes into mature myotubes by regulating expression of myosin heavy chain proteins and proteins of other appartuses. The regulation of this transcriptional network involves a number of posttranscriptional pathways, including microRNAs. A basic summary of this hierarchy is shown in Figure 1.

C. MicroRNAs Biogenesis and Basic Biology

MicroRNAs (miRNAs) are small non-coding regulatory RNAs with sizes of 17-25 nucleotides. Their biogenesis is summarized in Figure 2 and many reviews (33,34). MicroRNAs are coded either as unique genes, or found within an intron of a protein coding gene. They are abundant in animals accounting for 1-2% of known genes in C. elegans, Drosophila and Humans (35). Regulation by miRNAs is likely to be found in many biological processes: more than 60% of protein-coding genes are computationally predicted targets of miRNAs (36). Primary miRNAs are processed into hairpins by the Drosha/Dgcr8 complex (37). The hairpin is then exported from the nucleus and processed by Dicer in the cytoplasm (38). The mature miRNA then associates with the RISC complex and binds to the 3’UTR of target genes (35). The specificity of miRNA–mRNA interaction is mainly conferred by the first eight nucleotides of a miRNA, known as the seed sequence (39). The likelihood that a predicted target is a bona fide target is influenced not only by the seed pairing but also by other factors such as the number of target sites, the context of surrounding sequence in the mRNA (40), and the occlusion of

target sites by RNA-binding proteins (41). Recently, it was demonstrated that extensive central pairing can compensate for the lack of a perfect seed sequence (42). The mechanism of repression is predominately via a decrease in target mRNA stability and inhibition of translation (43). The repression of a single gene by a single miRNA is not likely to be very strong, although this can be increased by having multiple target sites within the same 3'UTR. MiRNAs can also potently influence broader signal transduction pathways by targeting multiple players (44).

D. MicroRNAs and Skeletal Muscle

I. Genetics

There are several microRNAs whose expression is restricted to skeletal muscle tissue. For example miR-1, miR-133, and miR-206 are specifically expressed in striated muscle tissues (45). These muscle-specific miRNA are encoded at three genomic loci, each featuring a pair of miRNAs. Two encode miR-1/133a, and the third miR-206/133b. All three are thought to be evolutionary homologs. Knockouts of these genes individually had varying, but generally mild, phenotypes. MiR-206 is important for neuromuscular junction formation(46) and response to chronic injury(47). MiR-1 is important for cardiomyocyte formation (48), but its role in skeletal muscle remains unknown. Lack of miR-133a resulted in a mild myopathy (49). One mitigating factor in each of these situations could be compensation from the homologous loci. MiR-1 and miR-206 share a similar seed sequence, and therefore repress many of the same targets. Similarly, the miR-133a knockout phenotype could have been ameliorated by functional miR-133b. If all microRNAs are removed, via skeletal-muscle specific knock-out of the Dicer gene in mice, there are gross abnormalities of fiber morphology and perinatal lethality (50).

However, there was no change in the levels of MRFs in Dicer knockout muscles when compared to control muscles. Therefore, it is probable that although MRFs themselves are not targeted by miRNA, proteins that control MRF activity are.

II. Regulators of MRF activity and miRNA

Our hypothesis that microRNA regulates MRF activity by targeting inhibitors raises the possibility of feed forward regulation. Transcriptional upregulation of miRNAs by MRFs would reinforce a more robust cell fate decision. The potential for up regulation of miRNA loci by MRFs was suggested by genome-wide Chromatin Immunoprecipitation (ChIP). There was increased binding for both MyoD and Myogenin at all three miR-1/133/206 loci (51). Genetic evidence reinforced this observation; in mouse embryos that lack Myf5, but not MyoD, there was a complete absence of miR-1 and miR-206 expression (52). Mef2c also plays a critical role in inducing the expression of miR-1 and miR-133 (53). Figure 3 illustrates all of the interactions that have been found between MRFs/Mef2 and the miR-1/133/206 loci. The full scope of miRNA function in skeletal muscle is not encompassed in these three loci, and further interactions are likely to be found.

The first level of regulation of MRFs in skeletal muscle development is the induction of their expression by Pax3. However, Pax3 must also be downregulated during terminal differentiation (54). The Pax3 gene utilizes multiple polyadenylation sequences, altering the length of the 3'UTR of its mRNA (55). The shorter version is not regulated by miRNA, but the longer version has two binding sites for miR-206. The shorter version is expressed early in embryonic development, when Pax3 is directing

myogenesis, but later in embryogenesis the long version predominates. In theory this allows for terminal differentiation to myotubes to proceed.

The 3'UTR of Pax3 also contains a binding site for miR-27b, and transgenic overexpression of the microRNA decreases the expression of Pax3 in the myotome (56). Inhibiting miR-27b in regenerating muscle led to an increase in Pax3 expression and the appearance of fibers with smaller diameters. Although correlative, this result suggests that regulation of Pax3 by miR-27b is important in adult muscle regeneration. This conclusion could be strengthened by discovering the mechanism of miR-27b up-regulation during myogenesis. Work in cardiomyocytes has shown that miR-27b is repressed by the TGF-beta pathway (57). However, this has not been demonstrated for skeletal muscle, nor is there any link between TGF-beta and Pax3 expression. In adult myoblasts, Pax3 is also down-regulated by the proteasome (54). Expression of myogenin and Pax3/7 was mutually exclusive by immunofluorescence (58), but the complete mechanism of feedback control is unknown. The relative contribution of proteasomes and miRNA, and their co-ordination, to Pax3 downregulation is an outstanding question.

Differentiation of skeletal muscle, as in many other tissues, involves changes in the cell cycle machinery. Prior to differentiation into myocytes, cells must leave the cell cycle and arrest in the G0 phase. Either overexpression of cyclin-dependent kinases (Cdk) (59) or knockout of their inhibitors p27 and p57 (60) is sufficient to prevent myogenesis. This is thought to occur because MyoD is normally phosphorylated and destabilized by the Cdk, thereby blocking differentiation and promoting cell proliferation (61). During differentiation, the expression of p27 and p57 increases, preventing the degradation of MyoD (62). Upon stabilization, MyoD reinforces the cell-cycle exit by

increasing expression of p21 (63), another Cdk inhibitor. However, unlike some of the loops discussed here, the induction of p27 and p57 is not dependent on MyoD and there are no known MyoD binding sites in their respective promoters. Both p27 and p57 are known targets of miR-221/222 (64,65), which decrease dramatically during skeletal muscle differentiation, thereby providing a microRNA-based mechanism for the increase of p27 and p57 (66). While the inhibition of MyoD is specific to skeletal muscle, the decrease in miR-221/222 is seen in other differentiation pathways. Therefore the downregulation of these microRNAs may not be a direct consequence of MyoD action, but serve as another example of how microRNAs may regulate skeletal muscle differentiation.

There are additional ways by which microRNAs induced during muscle differentiation inhibit the cell-cycle. The activity of Cdk is repressed by phosphorylation on threonine 14 and tyrosine 15, and the phosphatase Cdc25a increases Cdk kinase activity by removing these inhibitory phosphates. MiR-322 and miR-503, induced during muscle differentiation, both target Cdc25a, thereby inhibiting Cdk (67). MiR-322 and -503 both increase non-specifically in cells that are in the G0 phase of the cell cycle (68), indicating that their induction may be a general feature of post-mitotic differentiation. In addition, the MyoD induced microRNA miR-206 inhibits the DNA polymerase involved in DNA replication, the p180 subunit of DNA polymerase alpha, further inhibiting cell cycle progression and promoting differentiation (45).

III. Regulation of Mef2c and Myosin Heavy Chain by miRNA

In differentiating myoblasts, Mef2c's activity is regulated by association with Hdac4 and Hdac5. These histone deacetylases attach to the DNA-binding domain and

inhibit Mef2c's transcriptional activity (69). MiR-1 forms a similar loop with Mef2c and Hdac4 as miR-206 does with MyoD and Pax7. The expression of miR-1 from both genomic loci requires Mef2c (53). Expression of miR-1 then decreases levels of its target, Hdac4, thereby further promoting the transcription activity of Mef2c (70). There is one interesting example where a microRNA is repressed to protect the activity of Mef2c. Mef2c and its cofactor Maml1 can be targeted by miR-135 and miR-133, respectively (71). While the fold change of miR-135 is minimal, miR-133 is significantly increased during myoblast differentiation. Therefore a decrease of Maml1 during differentiation could be expected. However, the repression of Maml1 proteins is limited because of the simultaneous induction of a long non-coding RNA that serves as a decoy target for miR-133, preventing miR-133 from targeting MamL1 mRNA. Although this phenomenon has been shown in other contexts, it is still not clear exactly why the cell would express both a miRNA and a non-coding RNA to block its activity.

Skeletal muscle is not a completely homogenous organ. Fibers are commonly referred to as "fast" or "slow" and this is defined by the forms of myosin heavy chain (Myh) that are predominately expressed. Type I slow fibers express the Myh7 and Myh7b genes. Each of these genes encodes a miRNA found within an intron, miR-208b and miR-499, respectively. These miRNAs are homologous and mice with a knockout of one microRNA or the other display little phenotype (72). The soleus (a slow-twitch oxidative muscle) of the double knockout mouse, however, shows a profound shift towards fast-twitch type IIa and IIx fibers. Transgenic mice with muscle-specific overexpression of miR-499 have uniformly type I soleus muscles. In addition, the extensor digitorum longus muscle lost the very fast-twitch glycolytic type IIb fibers. This phenomenon can

be explained by the fact that both miR-208b and miR-499 repress the transcription factor Sox6, which promotes the expression of fast-twitch fiber Myh genes. Thus the expression of Myh7 and Myh7b in the slow fibers is accompanied by expression of miR-208b and -499. This prevents spurious expression of Sox6 and thus represses the fast fiber Myh genes. So in the same way that miRNAs are critical for earlier cell fate decisions of progenitors, they can also reinforce decisions of fiber type.

E. Muscle-differentiation-induced microRNAs for disease therapy

A clinical problem that could benefit from microRNA therapy is rhabdomyosarcoma (RMS), a cancer that shares many characteristics with skeletal muscle. This cancer commonly features a fusion between Pax3 or Pax7 and the gene Foxo1 (73,74). These function as constitutively active Pax genes, preventing terminal differentiation (75). Cell lines derived from this cancer can be differentiated into normal skeletal muscle with a MyoD-E12 tethered dimer, forcing the tumor cells to differentiate (76). Forced expression of miR-206 can also attenuate tumor growth of RMS in mouse xenograft models (77). In addition, miR-29 (78) and miR-26a (79) also induce differentiation of RMS by targeting components of the polycomb group complex. Delivery of microRNAs has been proposed as a way to control the proliferation of cancers, including RMS. We suspect, however, that if microRNA is directly used for therapy, the tumor could escape by selecting for mutations in miRNA binding sites. Knowing the network of genes inhibited by these miRNAs will allow us to find novel therapeutic targets against which we could develop chemical inhibitors that are likely to be more effective for therapy. In addition, critical microRNA targets often escape from

microRNA control during the pathogenesis of RMS, e.g. chromosomal translocations that separate the open reading frame of Pax3 or Pax7 from the microRNA-responsive 3'UTRs. MicroRNA delivery to such tumors is then expected to be futile for inhibiting Pax3 or Pax7. However, miRNAs may have some use for analysis of RMS aside from direct therapy. Expression of miR-206 has been found to be inversely correlated with the severity of the disease (80). Patients with RMS also have higher levels of myogenic miRNAs in their plasma (81), which could be a minimally invasive way of monitoring response to therapy.

The scientific community has already begun the work of taking advantage of our knowledge of microRNAs and applying it to modifying tissue engineering. Some studies have shown that miR-133 actually prevents terminal differentiation (70), and inhibition of miR-133 in myoblasts improved the function of artificially grown skeletal muscle tissue (82). Others have shown that overexpression of miR-1 or -206 similarly improved the differentiation of human satellite cells in bioartificial scaffolds (83). Embryonic stem cells are a tantalizing source for any form of cell therapy, but standard embryoid body culture forms skeletal muscle very poorly (84). Treatment with miRNA is being used to increase the myogenic differentiation of embryonic stem cells by inhibiting the chromatin remodeler Ezh2 (85).

F. Description of Current Research

Reciprocal Inhibition of Pax7 and miRNA

This project began by identifying Pax7 as a predicted target of miR-206 and miR-486. We confirmed that inhibition of the miRNAs alters the dynamics of Pax7 downregulation in myoblast differentiation. Pax7 itself also seemed to prevent this

regulation. Overexpression of exogenous Pax7 lowered the levels of both miRNAs. We demonstrate that both miRNAs are upregulated by MyoD, and that inhibition of MyoD by Pax7 is sufficient to prevent their transcription. These observations suggest that microRNA plays a role in two metastable states, one with the transcriptional targets of Pax7 inhibiting MyoD, and the second with the opposite. This work is described in Chapter II.

Feed Forward Activation of MyoD via miR-378

The goal of this project was to utilize the new, high-throughput sequencing data that described the genomic location of all MyoD binding sites. We intersected those sites with known miRNA loci, and found multiple sites binding close to miR-378. We found that MyoD acts as a transcription factor for miR-378. We also identified MyoR, a competitive antagonist of MyoD, as a novel target of miR-378. This led to a feed-forward loop whereby MyoD upregulates miR-378 and thereby increases its own activity by repressing MyoR. This work is described in Chapter III.

miR-1/206 Promote Mef2c Activity by Targeting Notch3

The goal of this project was to elucidate how miRNA regulates satellite cell fate choice. We found a predicted multiple miR-206 binding site in Notch3, which was previously shown to be expressed in quiescent satellite cells. To our surprise, we found that Notch3 had increased expression early in myoblast differentiation, and it failed to inhibit the MRFs. In fact, MyoD was involved in activating the expression of Notch3. We showed Notch3 decreases Mef2c activity by preventing its phosphorylation. It appears that the expression of Notch3 during myoblast differentiation, orchestrates a pause to

prevent premature differentiation and that microRNA is critical for regulating that balance. This work is described in Chapter IV.

G. References

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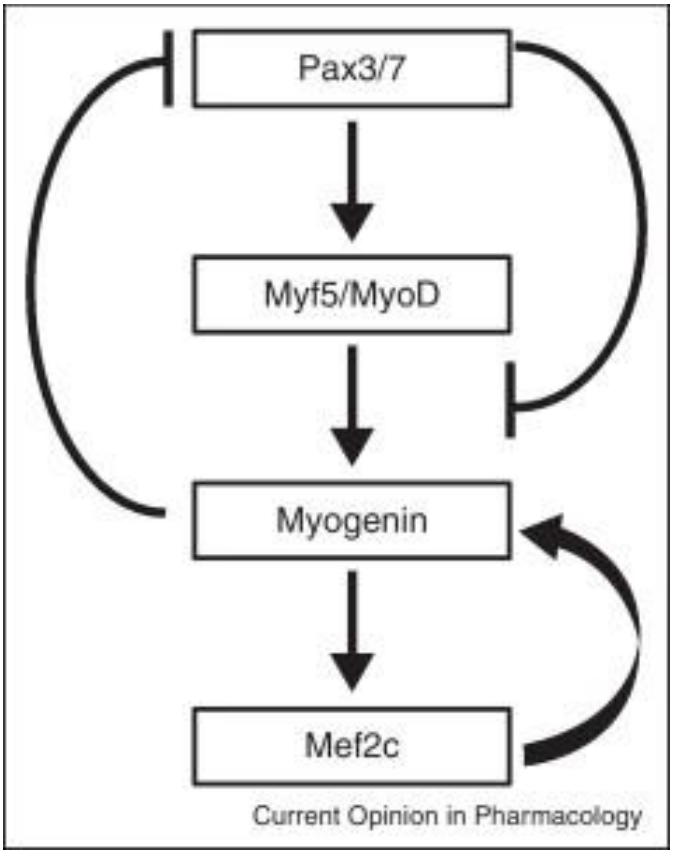
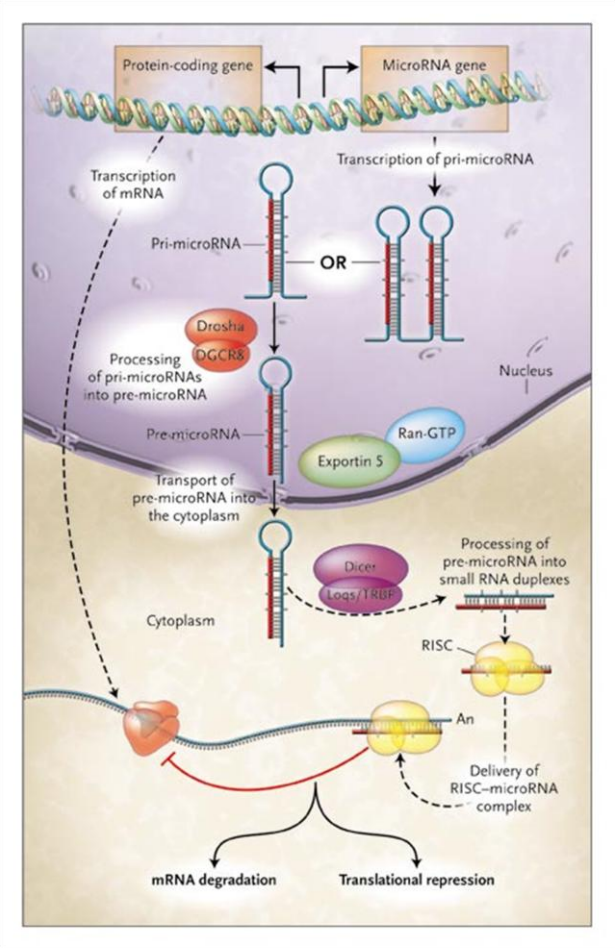
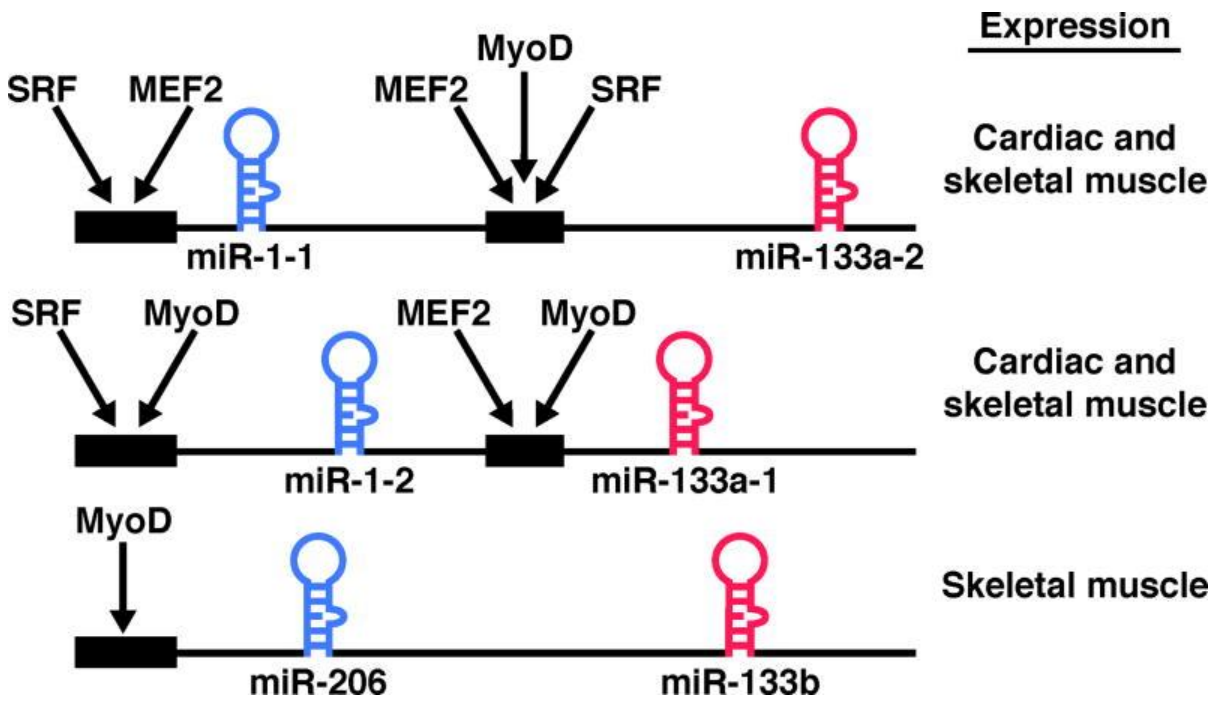


Figure 1. A simplified flow chart of the order of expression and activation of transcription factors in secondary myogenesis. These interactions were all established before the discovery of the regulatory roles of miRNA. (Gagan, Curr Opin Pharmacol. 2012)



Chen, N Engl J Med. 2005

Figure 2. MicroRNA is transcribed from either the introns of protein-coding genes or dedicated microRNA genes. They fold into hairpins that are excised from the rest of the primary transcript. The hairpins are then exported to the cytoplasm, where they are further processed into unlinked double-stranded RNA. One strand is loaded into the RISC complex and then binds to and represses messenger RNA.



Williams et al., Curr Opin Cell Biol. 2009

Figure 3. The best characterized microRNA loci in skeletal muscle are the 1/206-133 loci. Each has been shown to bind MyoD, as well as Mef2 and SRF binding in the miR-1 loci. Many more microRNAs are likely to be regulated by tissue specific transcription factors.

Chapter 2: Regulation of MyoD via Pax7

Adapted From:
Dey BK, **Gagan J**, Dutta A, "*miR-206 and -486 induce myoblast differentiation by downregulating Pax7*", **Molecular and Cellular Biology,** Jan 2011

ABSTRACT:

The Pax7 transcription factor is required for muscle satellite cell biogenesis and specification of the myogenic precursor lineage. Pax7 is expressed in proliferating myoblasts but is rapidly downregulated during differentiation. Here we report that miR-206 and -486 are induced during myoblast differentiation and downregulate Pax7 by directly targeting its 3′ untranslated region (UTR). Expression of either of these microRNAs in myoblasts accelerates differentiation, whereas inhibition of these microRNAs causes persistence of Pax7 protein and delays differentiation. A microRNA-resistant form of Pax7 is sufficient to inhibit differentiation. Since both these microRNAs are induced by MyoD and since Pax7 promotes the expression of Id2, an inhibitor of MyoD, our results revealed a bistable switch that exists either in a Pax7-driven myoblast state or a MyoD-driven myotube state.

CONTRIBUTION:

The work presented in this chapter was the result of a collaboration between Dr. Bijan Dey, a postdoc, and I. The work was published in January 2011 in the journal Molecular and Cellular Biology. Dr. Dey performed the experiments confirming the targeting of Pax7 by miR-206 and miR-486. I came up with the hypothesis that Pax7 inhibited microRNA levels by inhibiting MyoD activity via Id2/3. I also mapped the MyoD binding site near miR-486, cloned the site and confirmed that it could function as a MyoD activated enhancer. Anindya Dutta supervised all aspects of the project.

miR-206 and -486 Induce Myoblast Differentiation by Downregulating Pax7

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INTRODUCTION:

Skeletal muscle is largely composed of multinucleated muscle fibers. Postnatal growth or the regeneration capacity of adult skeletal muscle is dependent on satellite cells (1,2). Satellite cells give rise to myoblast cells that undergo multiple rounds of division before terminal differentiation. Skeletal muscle development during embryogenesis and during regeneration in adults requires a delicate balance between myogenic differentiation and self renewal (3).

The paired-box family of transcription factors, specifically Pax3 and Pax7, are important for regulation of the development and differentiation of diverse cell lineages, including skeletal muscle during embryogenesis (4). Pax3 is extensively expressed in the somite, whereas Pax7 expression is restricted to the central part of the dermomyotome (5). The Pax7 transcription factor is required for satellite cell biogenesis, survival, and self renewal and has a crucial role in specifying the satellite cell myogenic lineage, functioning upstream of the MyoD family of basic helix-loop-helix (bHLH) transcription factors (6,7). Most of the activated satellite cells proliferate, downregulate Pax7, and maintain MyoD to go into differentiation. However, another group of proliferating cells maintain Pax7 expression but downregulate MyoD to remain in the undifferentiated self-

renewing state (8,9). Pax7 upregulation inhibits myogenesis by suppressing MyoD expression and myogenin induction (10). The mechanism by which Pax7 is downregulated during muscle differentiation has not been studied in detail.

Most of our current understanding of muscle differentiation is based on transcriptional regulation by, for example, the MyoD and MEF2 families of transcription factors (2,11,12). More recently, we have discovered that specific microRNAs play fundamental roles during muscle proliferation and differentiation by modulating a number of transcription factors and signaling molecules (13-17). MicroRNAs are a novel class of small, noncoding RNAs of 18 to 25 nucleotides that modulate gene expression by translational repression and mRNA cleavage caused by microRNA-guided rapid deadenylation (18-20).

The role of microRNAs in muscle differentiation has been recently reviewed (16,21). In mammals, miR-1 and -133 are expressed in both skeletal and cardiac muscles and miR-206 is specifically expressed in skeletal muscles (14,15). Overexpression and knockdown experiments investigated the function of these microRNAs in muscle differentiation in a C2C12 model system (14,15). The effects of miR-1 and -206 were partly mediated by repression of histone deacetylase 4 (HDAC4) and DNA polymerase α (Pola1), respectively. Additional direct targets for miR-206 such as connexin 43 (cx43), follistatin-like 1(Fstl1), utrophin (Utrn), estrogen receptor alpha (ERα), butyrate-induced transcript 1 (Bind1), monocyte-to-macrophage differentiation-associated protein (Mmd), and cMET were identified (13,15,22-26). Targeted deletion of Dicer, an enzyme critical for microRNA biogenesis, in the myogenic compartment caused perinatal lethality with reduced skeletal muscle mass and abnormalities in muscle fiber morphology (27).

Intriguingly, deficiency of miR-206 in the amyotrophic lateral sclerosis mouse model accelerated disease progression (25).

For this study, we screened for additional microRNAs and alternative targets involved in skeletal muscle differentiation. Here we report that miR-486 is also upregulated during myoblast differentiation and that miR-206 and -486 accelerate myogenic differentiation by inhibiting Pax7 expression. Pax7 is expressed in proliferating myoblast cells and is rapidly downregulated as these cells differentiate (7,10). Both the microRNAs are induced by MyoD. The link between MyoD and Pax7 through these microRNAs reveals a bistable switch that distinguishes between two fates: myoblasts and myotubes.

MATERIALS AND METHODS

Cell culture.

Mouse skeletal myoblast cell line C2C12 was obtained from the American Type Culture Collection (28) and maintained at subconfluent densities in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (growth medium [GM]) and 1% penicillin-streptomycin. For myogenic differentiation (from myoblasts into myotubes), DMEM containing 2% heat-inactivated horse serum (differentiation medium [DM]) and 1% penicillin-streptomycin was used (29). Mouse primary myoblast cells (a kind gift from Denis Guttridge, Ohio State University) were cultured in Ham's F-10 medium supplemented with 20% fetal bovine serum (FBS), HEPES (20 nM), basic fibroblast growth factor (bFGF; 2.5 ng/ml), and 1× penicillin-streptomycin in a collagen type 1-coated plate and differentiated using DM. C3H10T1/2

fibroblast cells were grown in DMEM supplemented with 10% FBS and 1× penicillin-streptomycin.

RNA isolation and RT-PCR.

Cells were collected at different days of differentiation, and total RNA was extracted using Trizol reagent (Invitrogen) by following the manufacturer's instructions. Total RNA from human atrium, breast, brain, colon, heart, kidney, liver, lung, ovary, small intestine, skeletal muscle, and uterus was purchased from Clontech Laboratories, Inc. Ncode microRNA first-strand cDNA synthesis and a quantitative reverse transcriptase PCR (qRT-PCR) kit (Invitrogen) were used to perform RT-PCR for microRNA detection. For mRNA detection, cDNA synthesis was carried out using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen). Then, quantitative PCR (qPCR) was carried out using Sybr green PCR master mix in an ABI cycler. ABI 7300 software was used for quantification (Applied Biosystems).

Plasmid construction.

The *Pax7* open reading frame (ORF) (30) and *Pax7* ORF with wild-type or mutated untranslated region 2 (UTR2; bp 2521 to 4196) were subcloned to the pMSCV retroviral vector using an EcoRI/NotI site. Retrovirus was made in HEK-293T cells cotransfected with virus packaging plasmids using a standard protocol. The mouse*Pax7* 3′ UTR was PCR amplified from C2C12 myoblast genomic DNA and cloned into modified pRL-CMV as described previously (15) using EcoRI and XhoI restriction sites. Mutations in the *Pax7* 3′ UTRs cloned into the pRL-CMV vector were created using a site-directed

mutagenesis kit (Stratagene). pCMV-MyoD and pBABE-E12 constructs were obtained from Addgene.

Plasmid, siRNA, microRNA mimic, and antisense microRNA transfection.

Plasmids were transfected using Lipofectamine 2000 transfection reagent (Invitrogen), and small interfering RNA (siRNA) and microRNA mimics were transfected into U2OS or C2C12 cells using RNAiMAX (Invitrogen) by following the manufacturer's instructions. The antisense (2′-*O*-methyl) oligonucleotides were transfected into C2C12 cells at 0, 24, and 48 h in serum-containing medium. At 72 h cells were differentiated by serum depletion.

Luciferase reporter assays.

U2OS cells were transfected with microRNAs using RNAiMAX transfection reagent twice in a 24-h interval. Six hours after the last transfection, luciferase plasmids were transfected using Lipofectamine 2000. Control vector pGL3 (Promega) was transfected as an internal control. At 48 h after plasmid transfection, luciferase assays were performed with the Dual-Luciferase reporter assay system (Promega) by following the manufacturer's instructions. The luminescent signal was quantified with a luminometer (Monolight 3020; BD Biosciences). Each value from the *Renilla*luciferase construct (rr) was first normalized to the firefly (*Photinus pyralis*) luciferase value (pp) from the cotransfected pGL3 control vector. Each rr/pp value in the microRNA transfections was again normalized to the rr/pp value obtained in control GL2-transfected cells.

Western blotting and antibodies.

For Western blotting cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 10% glycerol) supplemented with protease inhibitor mix (Sigma). Proteins were resolved by SDS-PAGE, transferred, and immunoblotted with various antibodies. The antibodies used were mouse monoclonal antibody (MAb), anti-myosin heavy chain (anti-MHC), anti-GAPDH (anti-glyceraldehyde-3-phosphate dehydrogenase; Sigma), and antimyogenin (Santa Cruz). The mouse Pax7 hybridoma clone was obtained from the Developmental Studies Hybridoma Bank (DSHB), University of Iowa, and the Pax7 MAb was produced by following the DSHB protocol.

Fluorescence-activated cell sorter (FACS) analysis.

Cells were harvested by trypsinization and fixed with 70% ethanol for 24 h at 4°C. Fixed cells were stained in 1 ml of propidium iodide solution (0.05% NP-40, 50 μg/ml propidium iodide, and 10 μg/ml RNase A) for at least 2 h at 4°C. Stained cells were analyzed with a Becton Dickinson flow cytometer using Cellquest software.

Microarray profiling of microRNA.

Total RNA was extracted from C2C12 cells growing in GM and DM on day 5 separately using Trizol reagent as described above. The samples were further purified using a Qiagen RNA column, and 5 μg of each sample was send to Exiqon. Microarray profiling of microRNA was carried out in a locked nucleic acid-based platform by Exiqon.

Immunocytochemistry.

Immunostaining was performed as described previously (14,15). Cells on a sterile glass coverslip were fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 15 min and were permeabilized with 0.2% Triton X-100 and 1% normal goat serum (NGS) in ice-cold PBS for 5 min. After cells were blocked with 1% NGS in PBS two times for 15 min, incubation with primary antibody (dilutions in 1% NGS: Pax7, 1:10; myogenin, 1:50; MHC,1:400) for 1 h was followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (dilution, 1:500; Dako Cytomation) for 1 h except for Pax7. The Pax7 primary antibody was incubated for 16 h, and secondary antibody was incubated for 2 h. After washes, nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (H-1200; Vector Laboratories) for 1 min before mounting. Images were visualized using a microscope (Olympus; Hi-Mag).

RESULTS

miR-206 and -486 are upregulated during myoblast differentiation and are expressed in skeletal muscles.

Mouse C2C12 myoblast cells are derived from adult skeletal muscles and mimic skeletal muscle differentiation in cell culture. C2C12 myoblast cells proliferate in the presence of serum and differentiate upon serum withdrawal with upregulation of specific markers such as cell cycle inhibitor p21, myogenin, and myosin heavy chain (MHC) (15,29). Therefore, C2C12 serves as an excellent model system to study the molecular mechanism underlying skeletal muscle differentiation. We carried out microarray profiling of microRNAs and found that a number of microRNAs were induced more than 2-fold (see Table S1 in the supplemental material). These include miR-206, previously

identified as involved in myogenic differentiation, and miR-486. The role of miR-486 in skeletal muscle differentiation was not reported earlier.

qRT-PCR analysis showed that miR-206 and -486 were significantly upregulated during myoblast differentiation (Fig. 1a and b). Consistent with earlier studies (15), a tissue survey showed that miR-206 was expressed only in the skeletal muscle. miR-486 was also expressed abundantly in skeletal muscle as well as in heart and liver (Fig.1c).

miR-206 and -486 promote myogenesis and cell cycle quiescence.

To assess the function of these microRNAs in myogenesis, we transfected synthetic RNA duplexes mimicking the microRNAs into C2C12 cells cultured in growth medium (GM). GL2, a siRNA to luciferase, was used as a negative control. The GM was replaced by differentiation medium (DM), and the cells were collected for immunocytochemistry, RT-PCR, and Western blot analysis at different time intervals.

Transfection of miR-206 increased the number of myogenin- and MHC-positive cells more than 2-fold compared to the GL2 control (Fig. 2a and b; Table Table1).1). Similarly, miR-486 also increased the number of myogenin- and MHC-positive cells compared to GL2 control cells (Fig. 2a and b; Table Table1).1). In both cases the microRNA-transfected cells were elongated and often multinucleated and yielded a brighter immunofluorescence signal than the GL2-transfected cells. Similarly qRT-PCR and Western blot analysis reveal that both myogenin and MHC mRNA and protein levels were upregulated in the miR-206- and -486-transfected cells compared to the GL2 control (Fig. 2c and d). Cell cycle profiling shows that transfection of miR-206 or -486 independently increased the G1 population of cells by 20 to 30% and decreased the S-phase population by 20% (Fig. 2e and f).

In a reciprocal experiment, we transfected C2C12 myoblasts with 2′-O-methyl antisense inhibitors of miR-206 and -486 (antimix) or 2′-O-methyl antisense GL2 (anti-GL2) as a negative control. By a procedure similar to that for microRNA mimics, we transfected antimix or anti-GL2 into C2C12 cells cultured in GM. The GM was replaced by DM, and the cells were collected for immunocytochemistry and Western blot analysis at different time intervals. Cells transfected with antimix showed inhibition of myogenesis, as indicated by a decrease in the appearance of myogenic differentiation markers myogenin and MHC (Fig. 3a to d). Transfection of antimix decreased the myogenin- and MHC-positive cells about 2-fold compared to transfection with the anti-GL2 control (Fig. 3a and b; Table Table1).1). Similarly, qRT-PCR and Western blot analyses revealed that both myogenin and MHC mRNA and protein levels were downregulated in the antimix-transfected cells compared to the anti-GL2-transfected control (Fig. 3c and d). Taken together, these data suggest that miR-206 and -486 are induced during differentiation and promote and are required for optimal differentiation of skeletal muscles.

Pax7 is downregulated by miR-206 and -486 during myoblast differentiation.

Pax7 is expressed in nearly 100% of C2C12 myoblast cells (Fig.4a), and by qRT-PCR we have shown that Pax7 mRNA in C2C12 cells is comparable to that in mouse primary myoblasts (Fig.4b). Similar to what is found for C2C12 cells, when mouse primary myoblasts are induced to differentiate by serum withdrawal, miR-206 and -486 increased and Pax7 mRNA decreased gradually (Fig. 4c and d). Given the similarities between these cell lines, we carried out our experiment using the C2C12 line.

When C2C12 cells are induced to differentiate by serum withdrawal, Pax7 protein decreases start from day 1, with the level going below detection by day 4 (Fig. 5a and c). The decrease in Pax7 protein is accompanied by an increased level of MHC expression, indicating that the cells are differentiating (Fig.5b). Interestingly, qRT-PCR of mRNA shows that Pax7 mRNA declines in a more gradual fashion, with only a 40% reduction after day 3 of serum withdrawal (Fig.5d). The faster kinetics of repression of Pax7 protein relative to that of the mRNA suggests that posttranscriptional mechanisms may take part in repressing Pax7 during muscle differentiation.

The microRNA target prediction algorithm miRanda suggests that Pax7 is a potential target of miR-206 and -486 (Fig.6a). We have found two predicted target sites for each of these microRNAs on the basis of seed match (first 8 nucleotides) and one predicted target site for each of these microRNAs on the basis of nonseed match (high negative energy binding, −19.35 kCal/mol and −27.88 kCal/mol, respectively) (Fig.6a). The microRNA binding through nonseed match has recently been described (31). miR-206 target sites in the Pax7 3′ UTR span nucleotides 2817 to 2842 (Fig.6a, line 2a), 3541 to 3566 (line 2b), and 3757 to 3779 (line 2c), and miR-486 target sites in the Pax7 3′ UTR span nucleotides 1851 to 1875 (line 1a), 2375 to 2403 (line 1b), and 2671 to 2696 (line 1c).

To ascertain whether the 3′ UTR of Pax7 mediates the downregulation of the protein, the two parts of the 3′ UTR (UTR1, bp 1640 to 2800; UTR2, bp 2521 to 4196) were separately fused to a luciferase reporter gene driven by the cytomegalovirus (CMV) promoter and transfected into C2C12 cells in GM. The relative luciferase activity was gradually downregulated when cells were moved to DM, indicating posttranscriptional

regulation through the 3′ UTR (Fig. 6b). The importance of the 3′ UTR for this microRNA action was corroborated by the relative persistence of the luciferase signal in cells in DM for 1 day and 3 days (DM1 and DM3 cells, respectively) when miR-206-responsive sites were mutated in UTR2 (Fig. 6b). The remaining decrease of luciferase activity with the mutated UTR2 is likely due to the presence of the miR-486 target site (Fig.6a, line 1c). A luciferase reporter containing the site complementary to miR-206 was similarly downregulated, showing that the assay was capable of detecting an upregulation of miR-206 during differentiation (Fig. 6b).

Only UTR2 has the target sites for miR-206, and both UTR1 and UTR2 have the target site(s) for miR-486. Cotransfection of miR-206 repressed the luciferase activity of the construct containing UTR2 (Fig.6c). Mutation of each of three miR-206 target sites in UTR2 partially relieved the repression, and three mutations together relieved the repression almost entirely, suggesting that the miR-206 target sites at 2a, 2b, and 2c are each responsible for the direct repression of Pax7 (Fig. 6c). In a similar experiment, UTR1 or UTR2 conferred responsiveness to miR-486 (Fig. 6d). Mutation at the UTR1b site alone (MutUTR1b) relieved the repression by miR-486 (Fig. 6d), and there was no additive effect from adding a mutation at UTR1a (data not shown). Like MutUTR1b, MutUTR2-1c (1c site mutated in the UTR2 fragment) also relieved the repression by miR-486 (Fig. 6d). Thus, the miR-486 target sites at 1a and 1c mediate repression by this microRNA. Collectively these results show that Pax7 is a bona fide direct target of miR-206 and -486.

Consistent with this, when C2C12 cells in GM were transfected by miR-206 and -486, endogenous Pax7 protein and mRNA were downregulated by these microRNAs

independently (Fig. 6e and f). MicroRNAs are known to decrease the levels of target mRNAs (32,33). The downregulation of protein level was much greater than the mRNA repression, suggesting that these microRNAs also repress translation of Pax7.

Finally, inhibition of these microRNAs using 2′-O-methyl antisense inhibitors of miR-206 and -486 caused longer persistence of endogenous Pax7 protein during differentiation (Fig. 6g and h). Therefore, the two microRNAs are indeed responsible for the optimal repression of Pax7 protein during differentiation.

MicroRNA-resistant form of Pax7 slows myoblast differentiation.

Having demonstrated that Pax7 is a cognate target of miR-206 and -486, we next tested whether transfection of the Pax7 ORF, which is resistant to miR-206 and -486 due to the absence of its 3′ UTR, suppresses microRNA-mediated myogenesis. The Pax7 ORF increased the level of Pax7 protein in C2C12 cells (Fig. 7a). We first ensured that the exogenous Pax7 persisted in DM and was resistant to miR-206 and -486 in GM. In DM, Pax7 protein in the parental vector control cells started decreasing on day1 and no Pax7 protein was detectable from day 3, whereas no significant changes of Pax7 protein were seen in the Pax7 ORF-expressing cells up to day 5 (Fig. 7b). In addition, transfection of miR-206 and -486 in C2C12 (parental vector- and Pax7 ORF-expressing) cells held in GM showed that endogenous Pax7 containing the 3′ UTR disappeared at 72 h after transfection, whereas the Pax7 protein expressed from a gene devoid of its 3′ UTR was not repressed (Fig. 7c).

Upon transfer of the two types of cells to DM, MHC- or myogenin-positive cells appeared with normal kinetics among the parental vector control cells, whereas no MHC- or myogenin-positive cells were seen among Pax7 ORF-expressing cells as late as day 3

or day 2, respectively (Fig. 7d and e). Similarly, Western blot analysis revealed no detectable myogenin and MHC protein levels in Pax7 ORF-expressing cells up to differentiation day 5 (Fig. 7f). These results are consistent with the previous findings that overexpression of Pax7 devoid of its 3′ UTR delays C2C12 differentiation (30,34).

To further demonstrate that microRNA target sites were important, we overexpressed Pax7 containing UTR2, either wild type or mutant for miR-206-reponsive sites in C2C12 cells (Fig. 8a). When these cells were transfected with miR-206 and kept in DM for 48 h, the wild-type UTR2-expressing cells downregulated Pax7 mRNA (Fig. 8b) and differentiated with normal kinetics (Fig. 8c and d). In contrast, cells expressing Pax7 with mutated UTR2 had less downregulation of Pax7 mRNA (Fig. 8b) and less upregulation of myogenin and MHC (Fig. 8c and d).

These data suggest that the 3′ UTR of Pax7 is critical for the proper differentiation of myoblasts. Since miR-206 and -486 are induced during differentiation and target the Pax7 3′ UTR, we suggest that the microRNAs contribute to myoblast differentiation by specifically repressing Pax7 protein and mRNA.

miR-206 and -486 downregulate Pax7 target genes.

If miR-206 and -486 repress Pax7, one expects them to repress known targets of Pax7 (30,35,36). The mRNA levels of four such targets, PLagL1, Id1, Id2, and Id3, were measured after transfection of miR-206 or miR-486 into myoblasts in GM (Fig. 9a to d). Consistent with the downregulation of Pax7 during differentiation, moving myoblasts from GM to DM decreased the levels of all four mRNAs. Interestingly, all four genes were downregulated by the transfection of miR-206 or -486 into myoblasts growing in

GM, consistent with the hypothesis that the microRNAs downregulate the Pax7 transcription factor.

To test whether the downregulation of these four genes was mediated by the repression of Pax7 by these microRNAs, we transfected Pax7 ORF-expressing cells with miR-206 and -486 or with GL2 as a negative control and held the cells in GM for 72 h (Fig. 9e). PlagL1, Id1, Id2, and Id3 were no longer repressed (Fig. 9e). These findings demonstrate that downregulation of PlagL1 and Id1 to -3 in the control cells by miR-206 and -486 is through Pax7 downregulation. Earlier we had shown that miR-206 downregulates Id1 to -3 in C2C12 cells but that the Id genes were not direct targets of this microRNA (15). Here, we demonstrate that the downregulation of Id1 to -3 by miR-206 (and miR-486) is mediated indirectly through Pax7 downregulation.

A bistable switch exists between Pax7 and MyoD via miR-206 and -486.

The next question we addressed was how these microRNAs were regulated during myogenesis. miR-206 has already been shown to be responsive to MyoD (23,25,37,38), and we wondered if that was true for miR-486 as well. Ank1.5 is a variant transcript that is found exclusively in muscle (39), and miR-486 is found in an intron of Ank1.5. The mRNA level of Ank1.5 increases during C2C12 differentiation as we found for miR-486 (Fig. 10a). We therefore thought that miR-486 may be controlled at the transcriptional level along with of Ank1.5.

By analyzing previously published MyoD chromatin immunoprecipitation-DNA sequencing (ChIP-seq) data (40), we found a MyoD binding peak in the promoter of Ank1.5 (Fig. 10b). Within that binding peak there were two well-conserved E boxes (Fig. 10c). The enhancer activity of this region was tested by cloning the fragment upstream

from a simian virus 40 (SV40) promoter-driven luciferase. Transcription was greatly activated by transfecting MyoD and E12 into C3H10T1/2 fibroblasts (Fig. 10d). This activation was greatly attenuated by mutating either of the E boxes. This result is consistent with previous findings that MyoD requires multiple E boxes to function as a transcriptional activator (41). We conclude that miR-486 levels are regulated directly by MyoD activity. In cells overexpressing Pax7, the Ank1.5 level accumulated much more slowly during differentiation (Fig. 10e).

Our next question was whether Pax7 prevented its own repression via inhibition of miR-206 and -486. Pax7 directly upregulates a number of repressors of myogenesis, including Id2 and Id3 (35) (Fig.9). Id2 binds to E12/47 and sequesters it away from MyoD binding sites, thereby repressing MyoD activity (42). Therefore, we expected and observed that overexpression of Pax7 prevented the increase of MyoD targets miR-206 and -486 (Fig. 11a and b). Knockdown of Id2 in Flag-Pax7 ORF-expressing C2C12 cells relieved the repression of miR-206 and -486 (Fig. 11c and d) and traditional markers of terminal myogenesis like myogenin and MHC (Fig. 11e and f). These results are consistent with the hypothesis that Pax7 inhibits miR-206 and -486 through the inhibition of MyoD by the activation of Id2.

DISCUSSION

Most of the current understanding of skeletal muscle differentiation is based on transcriptional regulation by the MyoD family of myogenic transcription factors and the MEF2 family of MADS box transcription factors (2,11,12). More recently, studies have shown that specific microRNAs play fundamental roles during muscle proliferation and differentiation by modulating a number of transcription factors and signaling molecules

(13-15,17). Here we report that miR-206 and -486 are induced during myoblast differentiation and promote muscle differentiation by directly targeting and downregulating Pax7 protein and mRNA. Transfecting miR-206 or -486 independently increased the G1 phase population and decreased the S phase population of myoblast cells, indicating that these microRNAs also promote cell cycle quiescence (Fig. 2). Expression of either of these microRNAs in myoblasts accelerates differentiation, whereas inhibition of these microRNAs causes persistence of Pax7 protein and inhibits differentiation (Fig.2 to 6). Furthermore, we have shown that a microRNA-resistant form of Pax7 that lacks its 3′ UTR or is mutated in all miR-206 sites delays the differentiation (Fig. (Fig.7 and 8). Thus, microRNAs contribute to myoblast differentiation by specifically repressing Pax7 protein and mRNA.

Pax7 is an important regulator of skeletal muscle development required for maintenance of the satellite cells that are responsible for postnatal muscle growth and regeneration (6,43). Pax7 is upregulated in progenitor cells that have migrated to the limbs to activate the myogenic program. Pax7-positive myoblast cells proliferate rapidly, but they downregulate Pax7 at the onset of differentiation. However, the mechanism of Pax7 downregulation during myoblast differentiation was not clearly understood. Our study strongly suggests that Pax7 downregulation is microRNA mediated. While our paper was under review another paper reported that miR-1 and -206 repressed Pax7 during muscle differentiation (44).

The potential role of microRNAs in regulating Pax7 adds a new dimension to how microRNAs sculpt the myogenic gene expression program. Very recently miR-27 was reported to repress Pax3, suggesting that the direct repression of antidifferentiation

transcription factors could be quite widespread (45). Due to the repression of Pax7, we find that several inhibitors of differentiation, Id1, -2, and -3, are also repressed by the muscle differentiation-induced microRNAs (Fig. 9). These data suggest that extensive cooperation between several microRNAs and several transcription factors is necessary to execute the complete differentiation program.

We have seen in our previous study that miR-206 promotes myogenesis by inhibiting DNA polymerase α (15). B-ind1, c-MET, Cx43, HDAC4, Fstl1, and Mmd were the other identified targets for miR-206, although repression of some of these did not stimulate muscle differentiation. For example, a block in DNA synthesis through the direct downregulation of DNA polymerase α affected DNA replication but was not sufficient to promote differentiation (our unpublished data). This result suggested that there were undiscovered targets for miR-206. The results in this paper suggest that Pax7 is one such target.

We have also found an intricate regulatory network between Pax7 and miR-206 and -486 that is at least partially mediated via the Pax7 target gene Id2 and muscle regulatory factor MyoD (Fig. 11). This finding suggests a tight control in the muscle differentiation pathways for normal development and function. Clearly, the cells can be in a Pax7-positive myoblast state when Id2 is activated and MyoD and the muscle differentiation-induced microRNAs are repressed. Conversely, once MyoD has gained the upper hand, the induction of miR-206 and -486 and repression of Pax7 shift the equilibrium toward the MyoD-active myotube state.

Chromosomal translocation of Pax7 and aberrant expression of the fusion of Pax7 with FKHR cause rhabdomyosarcoma, again indicating that fine tuning of Pax7

expression and its target genes may be important for normal skeletal muscle differentiation (46-48). The translocation not only deletes the C-terminal transactivation domain of Pax7 to replace it with a more active FKHR transactivation domain but also deletes the 3′ UTR of Pax7. Interestingly, the miRanda target prediction algorithm can detect putative miR-206 binding sites in the human Pax7 3′ UTR (see Fig. S1 in the supplemental material). Our studies suggest that the deletion of the Pax7 3′ UTR and escape of the fusion transcript from repression by muscle differentiation-induced microRNAs could be important players in the deregulation of Pax7. This is very similar to the way that oncogene HMGA2 escapes from repression by let-7 (49,50) and may be a common mode by which potential oncogenes escape repression from microRNAs.

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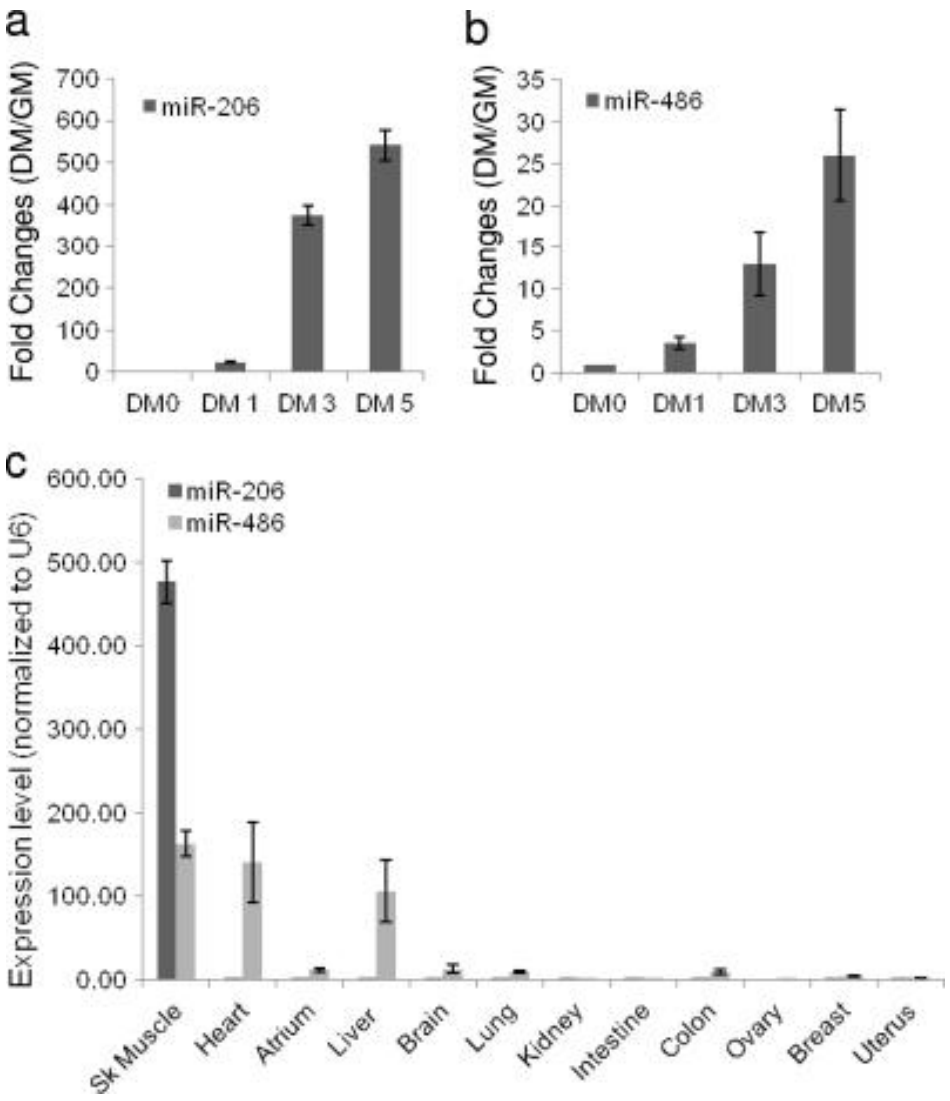

Figure 1. Expression of miR-206 and -486 during myoblast differentiation and in skeletal muscles. (a and b) miR-206 and -486 expression is gradually upregulated during myoblast differentiation. qRT-PCR of miR-206 and -486 during differentiation of C2C12 myoblasts is shown. Days of serum deprivation are indicated. Fold changes of these microRNAs, normalized to U6 snRNA, are expressed relative to day 0 of serum depletion. All data shown represent means ± standard deviations of three replicates. (c) miR-206 and -486 are abundantly expressed in the skeletal (Sk) muscles. qRT-PCR was performed with RNA from various tissues to detect miR-206 and -486. Relative expression levels of these microRNAs are normalized to U6 snRNA. Data shown are means ± standard deviations of three replicates.

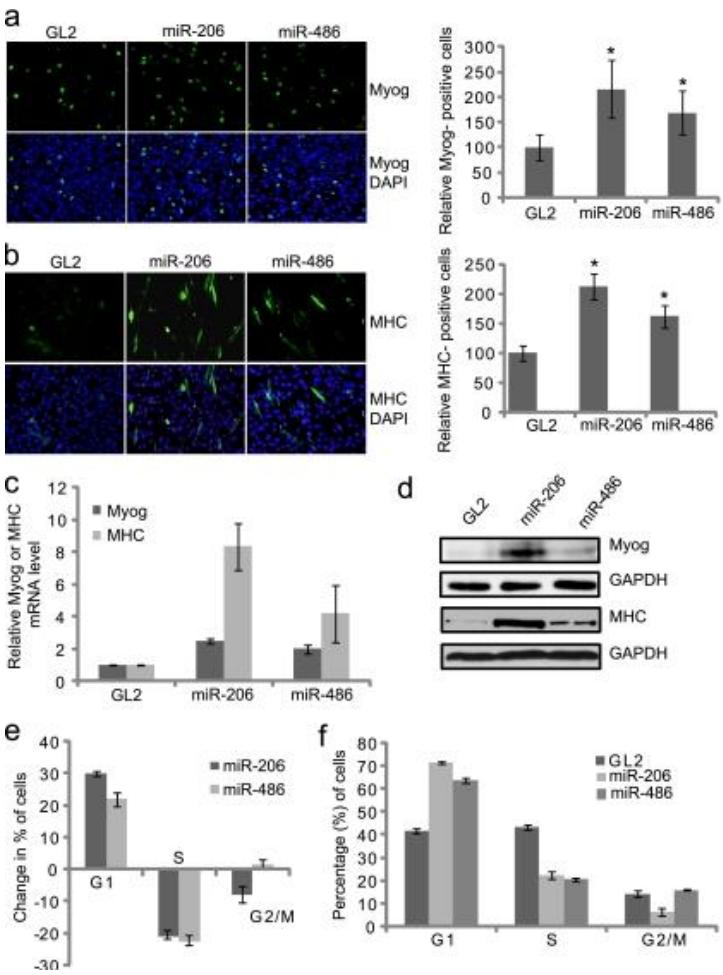

Figure 2. miR-206 and -486 promote myogenesis and cell cycle quiescence. (a and b) C2C12 cells were transfected three times at 24-h intervals separately with synthetic RNA duplexes mimicking miR-206, miR-486, or GL2 as a negative control in proliferating medium. After an additional 24 h in GM, the cells were transferred to DM and were stained for myogenin (myog) at 24 h (a) or MHC at 36 h (b). Blue indicates nuclei stained by DAPI. (Graphs) Fractions of myogenin- and MHC-positive cells were determined, and data are presented relative to the GL2 control (100%). Values are means ± standard deviations of 10 measurements. *, $P \leq 0.001$. (c and d) qRT-PCR (c) and Western blotting (d) for myogenin and MHC after transfection of miR-206, miR-486, or negative control GL2. C2C12 cells were held in DM for 1 day for myogenin and for 3 days for MHC. The RT-PCR results were normalized to GAPDH in the same sample and then again to the level in GL2-transfected cells. For the Western blot, GAPDH served as loading control. (e) Changes in cell cycle stage after miR-206 and -486 were transfected in C2C12 cells. Propidium iodide staining for DNA content and FACS analysis data are shown. The results are expressed as % changes of cells in a given phase of the cell cycle in the microRNA-transfected cells relative to that in the GL2 control. Values are means ± standard deviations of three measurements. (f) Primary FACS results for panel e.

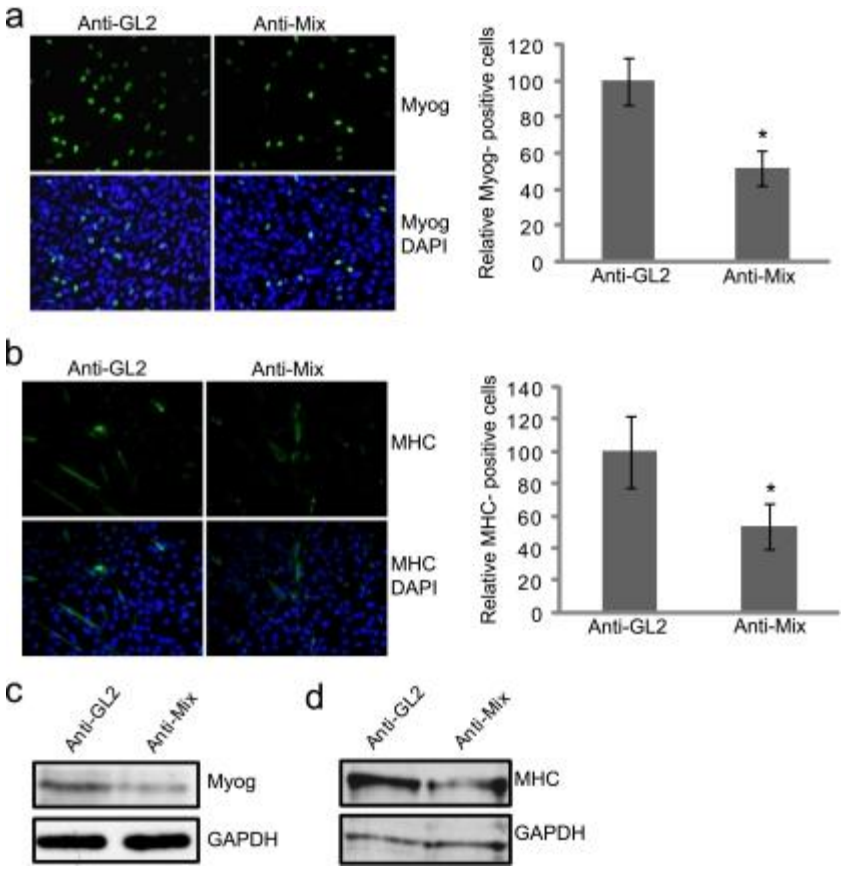

Figure 3. Inhibitors of miR-206 and -486 inhibit the myogenesis. (a and b) C2C12 myoblast cells were transfected three times at 24-h intervals with 2′-*O*-methyl antisense oligonucleotide against GL2 (Anti-GL2) or antisense oligonucleotides against miR-206 and -486 (Anti-Mix). Cells were continuously grown in GM for 24 h and then transferred to DM before staining for MHC (a) or myogenin (b) as in Fig. 2 except that immunofluorescence was done after 48 h. Histograms show fractions of cells staining for myogenin or MHC relative to the fraction in cells transfected with the anti-GL2 control (100%). Values are means ± standard deviations of 10 measurements. *, $P \leq 0.001$. (c, d) Western blotting for myogenin (c) and MHC (d) after transfection of a 2′-*O*-methyl antisense oligonucleotide against GL2 or antisense oligonucleotides against miR-206 and -486. C2C12 cells were held in DM for 2 days for myogenin and 3 days for MHC. GAPDH served as the loading control.

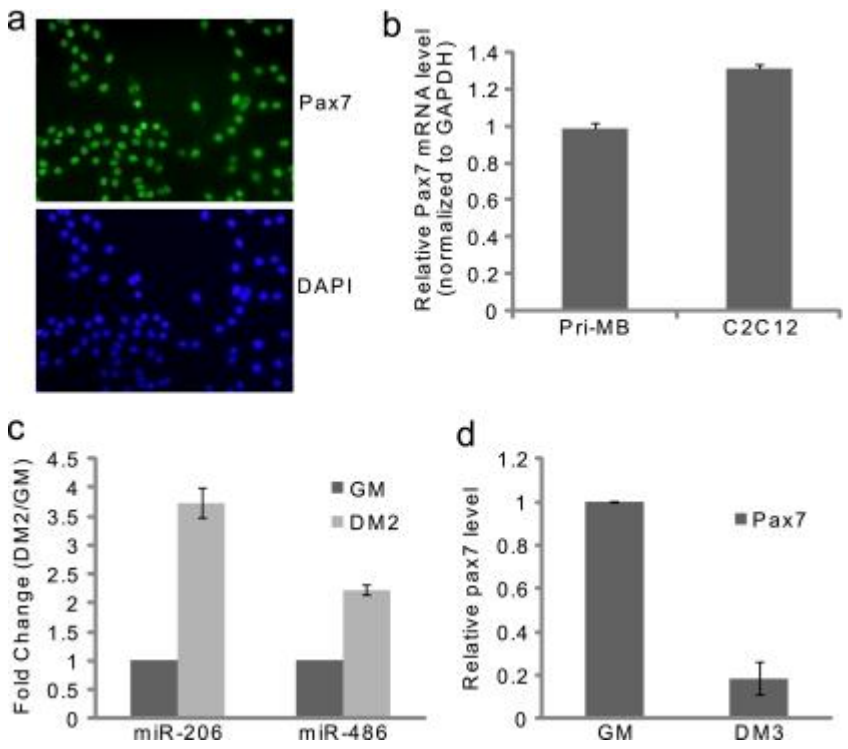

Figure 4. Expression of Pax7 in C2C12 and mouse primary myoblasts and upregulation of miR-206 and -486 and downregulation of *Pax7* mRNA in differentiated mouse primary myoblasts. (a) C2C12 cells were cultured in GM on top of a coverslip, fixed, and immunostained with monoclonal anti-Pax7. DAPI staining marks the nuclei. (b) qRT-PCR was carried out to compare the *Pax7* mRNA levels in C2C12 and mouse primary myoblasts. *Pax7* mRNA levels were comparable in both the cell lines. (c, d) qRT-PCR was carried out to compare the miR-206 and -486 and *Pax7* mRNA levels in DM2 and DM3 mouse primary myoblast cells, respectively. miR-206 and -486 were upregulated (c), and conversely *Pax7* mRNA was downregulated.

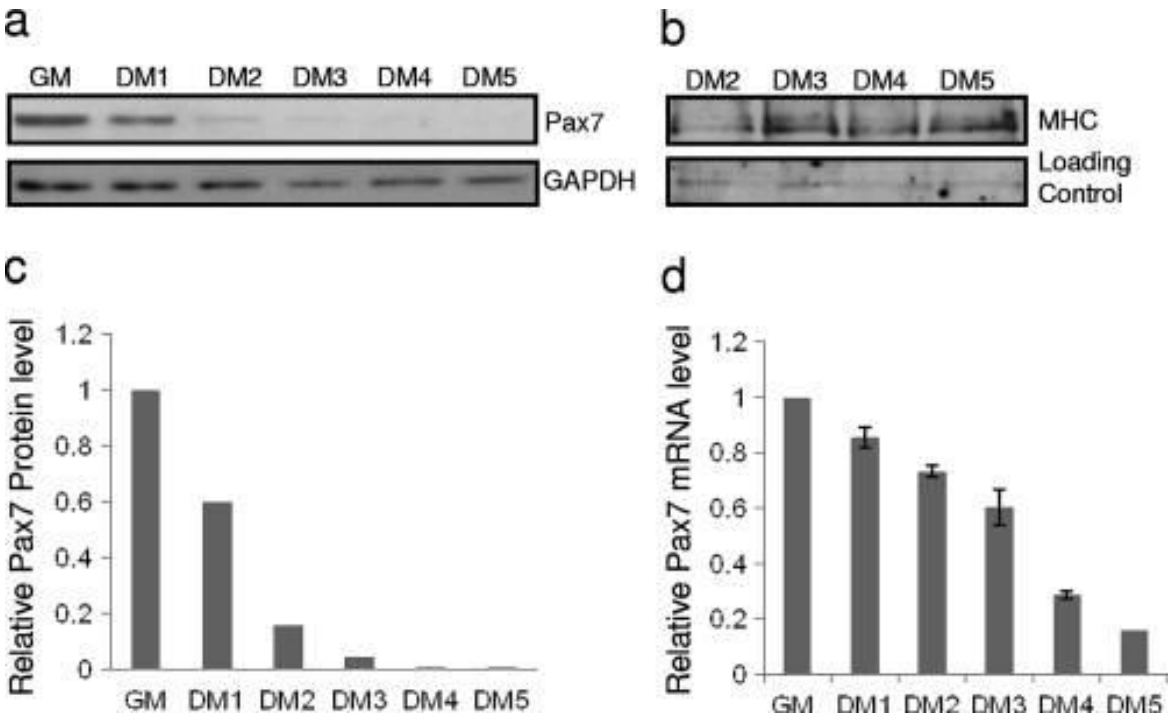

Figure 5. miR-206 and -486 downregulate Pax7 during myoblast differentiation. (a) Western blot analysis of Pax7 protein in C2C12 cells at the indicated times following transfer to DM. GAPDH served as loading control. (b) Western blot of MHC expressed in the samples shown in panel a starting from DM day 2. Cross-reactive bands served as the loading control. (c) Relative expression of Pax7 protein normalized to GAPDH. (d) Relative *Pax7* mRNA level normalized to GAPDH mRNA. Values are means ± standard deviations of three biological replicates.

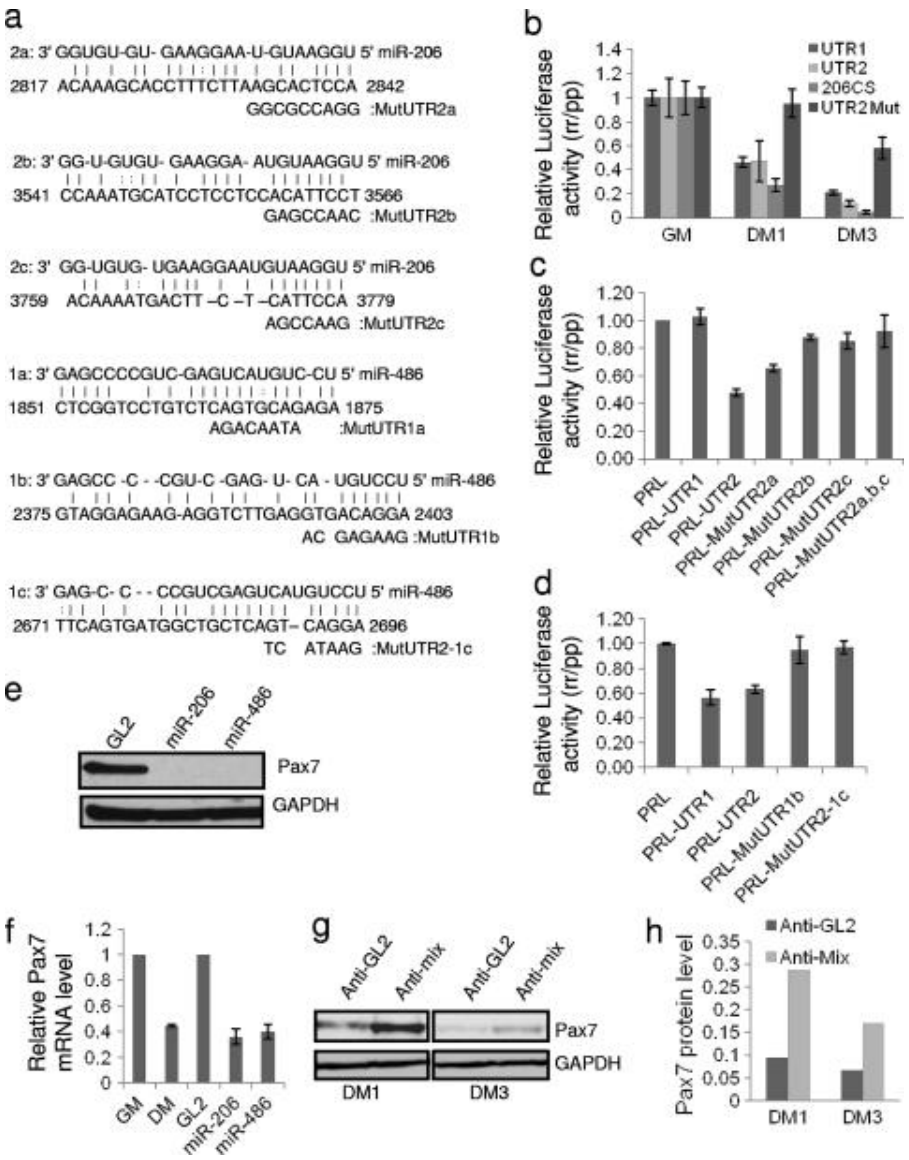

Figure 6. miR-206 and -486 downregulate *Pax7* by directly targeting its 3′ UTR. (a) miR-206 and -486 target sequence alignment in the *Pax7* 3′ UTR and mutations created. Three predicted target sites for miR-206 (2a, 2b, and 2c) and -486 (1a, 1b, and 1c) in the 3′ UTR of mouse *Pax7* are shown. Mutations in the target sites are indicated. (b) Activity of a*Renilla* luciferase reporter fused to *Pax7* 3′ UTR fragments transfected into C2C12 cells that were kept in GM or moved to DM. UTR1, bp 1640 to 2800; UTR2, bp 2521 to 4196; 206cs, complementary sequence to miR-206 in the 3′ UTR of the luciferase gene; UTR2 Mut, bp 2521 to 4196, where miR-206-responsive sites were mutated. A firefly luciferase plasmid was cotransfected with a *Renilla* luciferase construct as a transfection control, and the results are expressed as *Renilla* luciferase activity (rr) relative to firefly luciferase activity (pp). The ratio of rr/pp for a given plasmid is expressed after normalizing to the ratio in GM. Values are means ± standard deviations of three measurements. (c and d) Luciferase assays were performed to measure the effect of miR-206 (c) and -486 (d) on a *Renilla* luciferase reporter fused to the indicated segments of the *Pax7* 3′ UTR as described in Materials and Methods. The results are expressed

relative to the ratio of rr/pp for the control *Renilla* luciferase plasmid without a *Pax7* 3′ UTR segment. Values are means ± standard deviations of three measurements. The UTR2 fragment was mutated as follows: MutUTR2a, -2b, and -2c were mutated at miR-206 target sites 2a, 2b, and 2c, respectively, and MutUTR2-1c was mutated at miR-486 target site 1c. UTR1 fragment mutants were MutUTR1a and MutUTR1b. (e) Transfection of miR-206 and -486 downregulates Pax7 protein as detected by immunoblotting. GAPDH served as a loading control. (f) Transfection of miR-206 and -486 downregulated *Pax7* mRNA, as detected by qRT-PCR. Results are expressed relative to the GAPDH mRNA level and normalized to the relative *Pax7* level seen in C2C12 cells in GM or GL2 transfection. GL2-, miR-206-, and miR-486-transfected cells were held in GM. Values are means ± standard deviations of three measurements. (g) Western blot for Pax7 and GAPDH. 2′*O*-Methyl antisense oligonucleotides against miR-206, and -486 (Anti-mix) cause longer persistence of Pax7 protein level in C2C12 cells held in DM for 1 or 3 days compared to cells transfected with the anti-GL2 control oligonucleotide. (h) Quantification of Pax7 protein normalized to GAPDH from panel g.

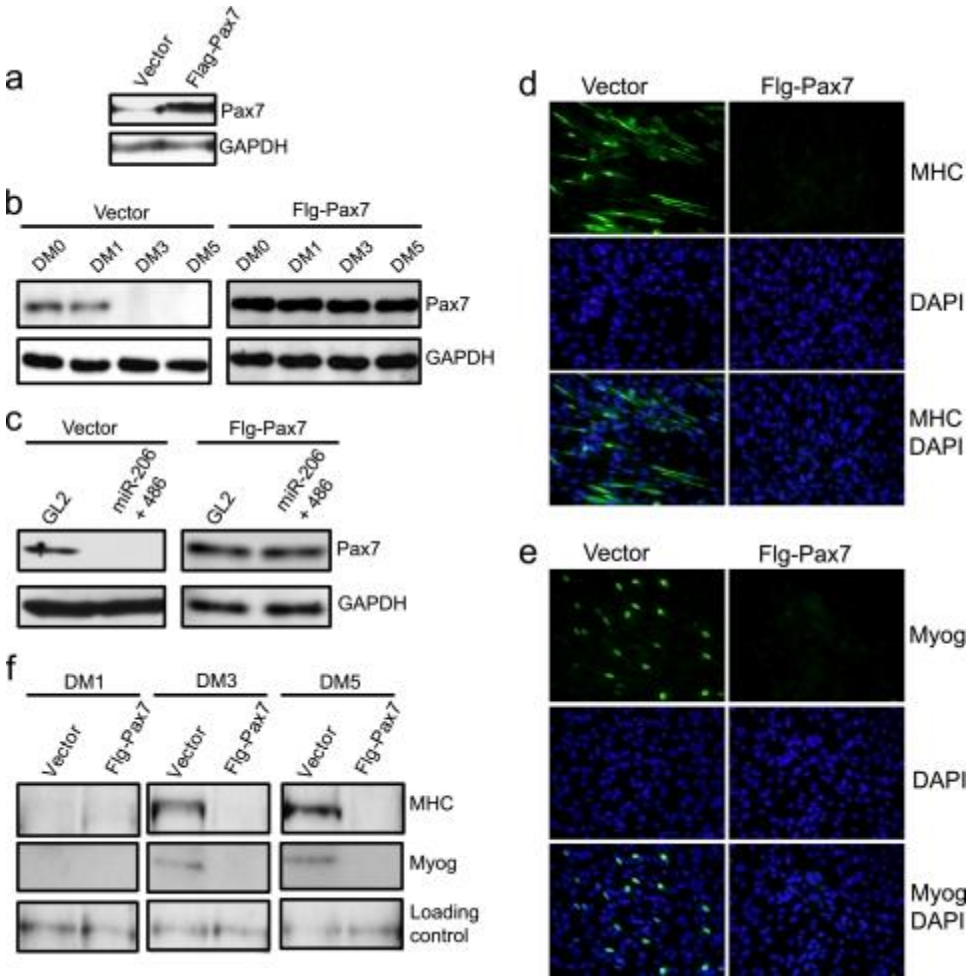

Figure 7. MicroRNA-resistant form of Pax7 delays myoblast differentiation. (a) Western blot of C2C12 cells infected with empty retroviral vector or retrovirus expressing the Flag-tagged*Pax7* ORF. GAPDH represents the loading control. (b) Levels of Pax7 protein after transfer to DM of C2C12 cells infected with empty vector virus or with virus expressing the*Pax7* ORF. GAPDH serves as the loading control. Flg, Flag. (c) Transfection of miR-206 and -486 into the two types of C2C12 cells maintained in GM. Shown is a Western blot of Pax7 and GAPDH (loading control) on day 3. (d and e) C2C12 cells stably infected with retroviruses were induced to differentiate by transferring them to DM. Overexpression of the *Pax7* ORF inhibited differentiation in DM, as shown by MHC immunostaining on day 3 (d) or myogenin immunostaining on day 2 (e). Blue, DAPI staining of nuclei. (f) Western blot analysis revealed no detectable myogenin or MHC protein up to differentiation day 5 in the *Pax7* ORF-expressing cells, whereas in the vector-infected cells both myogenin and MHC appear with normal kinetics. Nonspecific bands in the MHC Western blot served as the loading control.

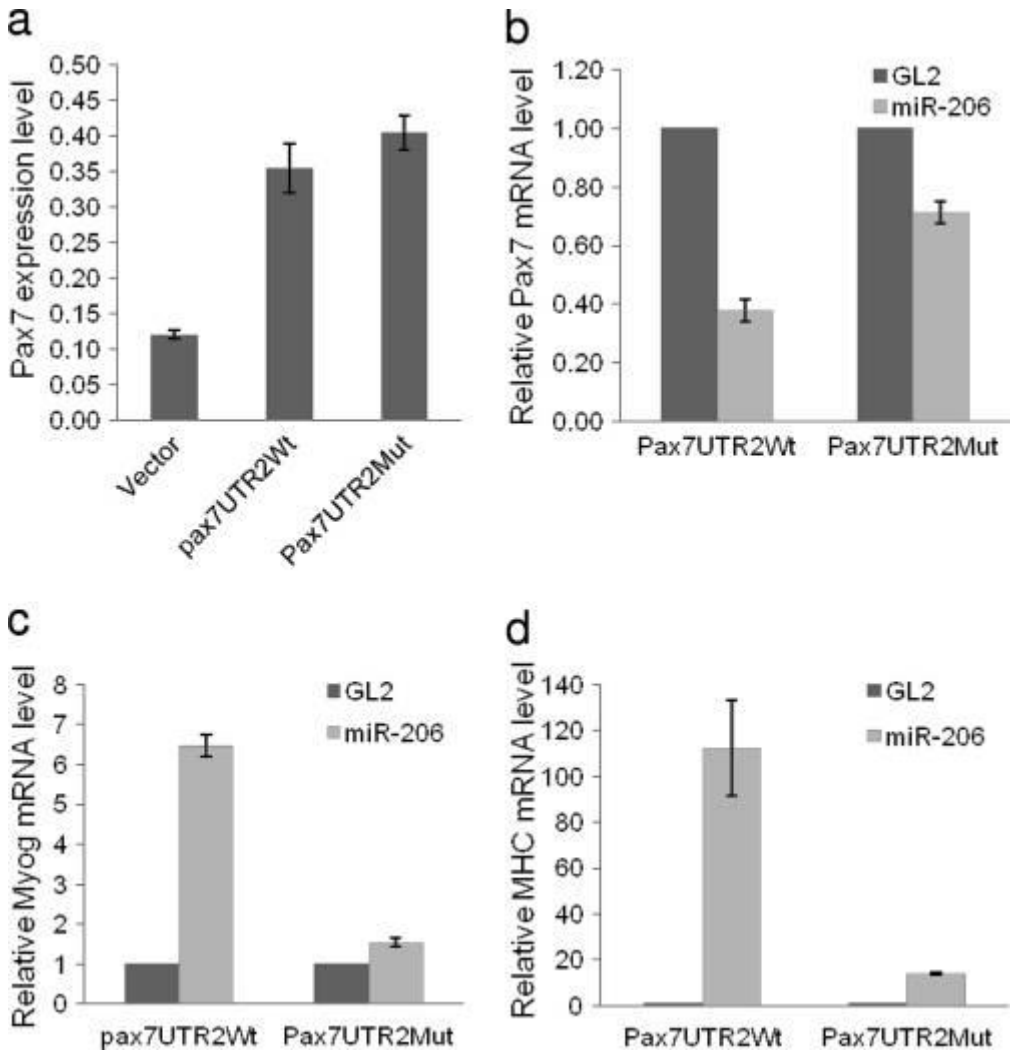

Figure 8. *Pax7* containing the 3′ UTR2 mutant for miR-206-responsive sites but not *Pax7* containing the wild-type UTR2 delays differentiation. (a) *Pax7* mRNA levels in stable C2C12 cells expressing the vector or expressing *Pax7* containing either wild-type UTR2 (pax7UTR2Wt) or UTR2 with mutations in miR-206-responsive sites (Pax7UTR2Mut). (b) Relative change of *Pax7* mRNA in response to miR-206 transfection in DM. C2C12 cells expressed *Pax7* containing wild-type UTR2 or UTR2 with mutations in miR-206-responsive sites. (c and d) Relative changes of mRNA levels of differentiation markers myogenin (c) and MHC (d) in C2C12 cells expressing *Pax7* containing wild-type UTR2 or UTR2 with mutations in miR-206-responsive sites. In panel d the GL2 bar is 0.01 times less than the highest bar in the graph and therefore lies very close to the baseline.

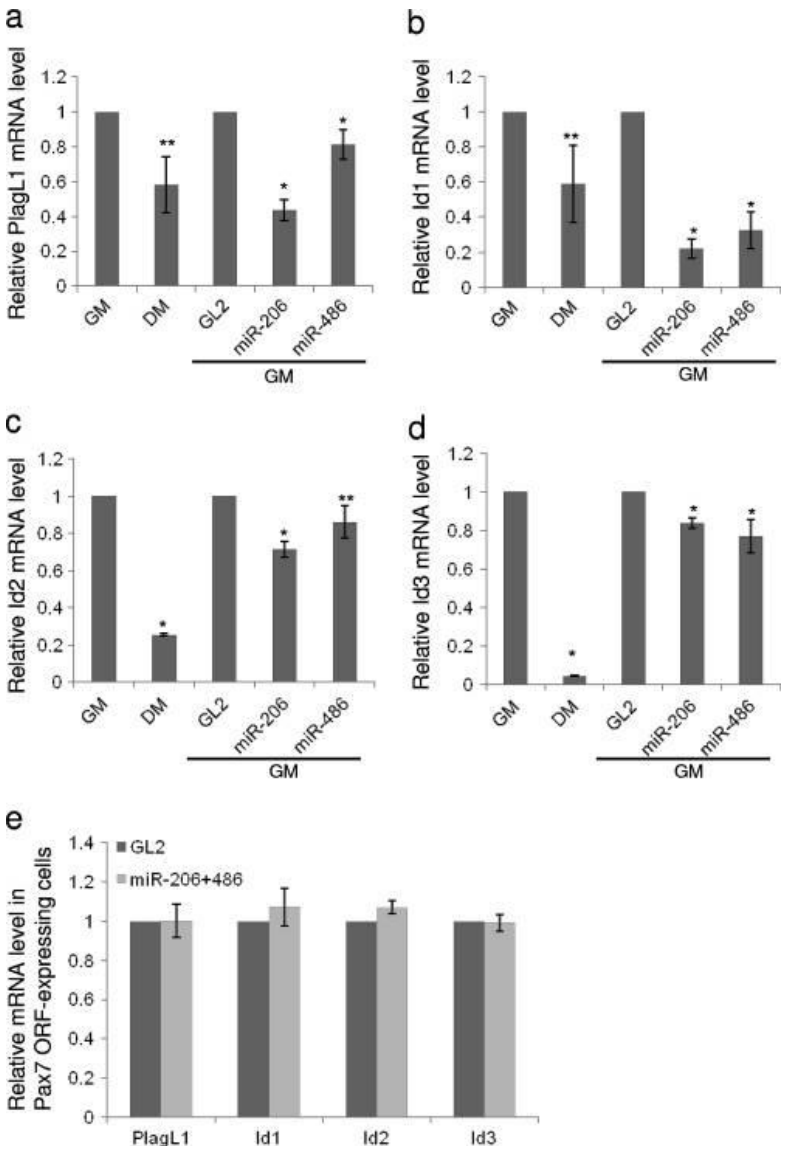

Figure 9. miR-206 or -486 downregulates genes that are activated by Pax7. (a to d) qRT-PCR measurement of the mRNA levels of Pax7 target genes in C2C12 cells in DM compared to GM and in C2C12 cells held in GM after transfection with the GL2 negative control, miR-206, or miR-486. The measured mRNA was normalized to GAPDH mRNA and expressed relative to the level in cells held in GM or transfected with GL2 in GM. Values are means ± standard deviations of three measurements. *, $P \leq 0.01$; **, $P \leq 0.05$. (e) qRT-PCR was performed to measure the mRNA levels of Pax7 target genes in *Pax7* ORF-expressing C2C12 cells transfected with either GL2 or miR-206 and -486. The measured mRNA was normalized to GAPDH mRNA and expressed relative to the level in cells transfected with GL2. Values are means ± standard deviations of three measurements.

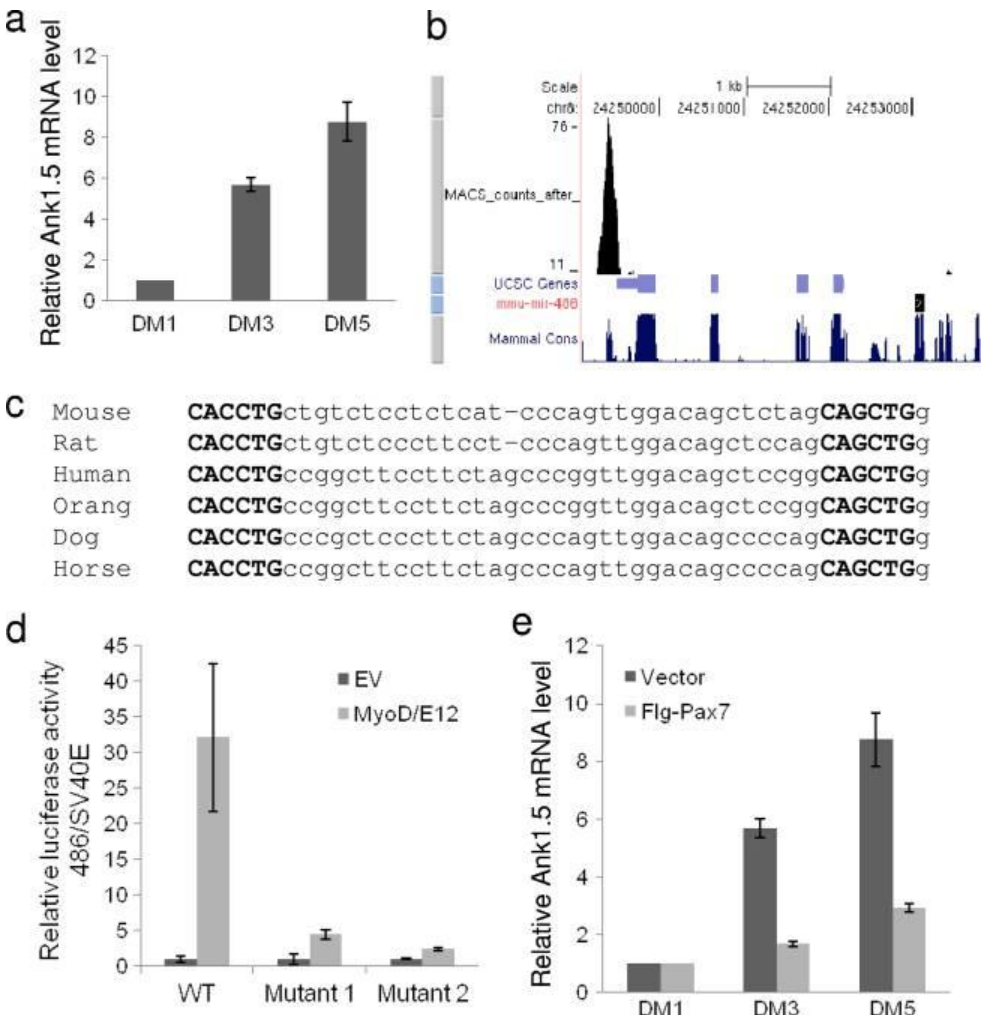

Figure 10. The miR-486 host gene *Ank1.5* promoter contains two conserved E boxes, responds to MyoD, and is upregulated during differentiation. (a) qRT-PCR showing that the mRNA level of *Ank1.5* increases during C2C12 differentiation. (b) University of California, Santa Cruz (UCSC), genome browser shot showing a MyoD binding peak in the promoter of*Ank1.5* that we obtained by analyzing previously published MyoD ChIP-seq data (10). MACS, model-based analysis for cell sorting; Cons, conservation. (c) The MyoD binding peak contains two conserved E boxes. (d) Relative luciferase activity showing the enhancer activity of the MyoD binding peak containing two E boxes. Transcription was activated by transfecting MyoD and E12 into C3H10T1/2 fibroblasts, and this activation was attenuated by mutating either of the E boxes. EV, empty vector; WT, wild type. (e)*Ank1.5* upregulation in Pax7 ORF-overexpressing cells is lower than in vector-expressing cells, as shown by qRT-PCR.

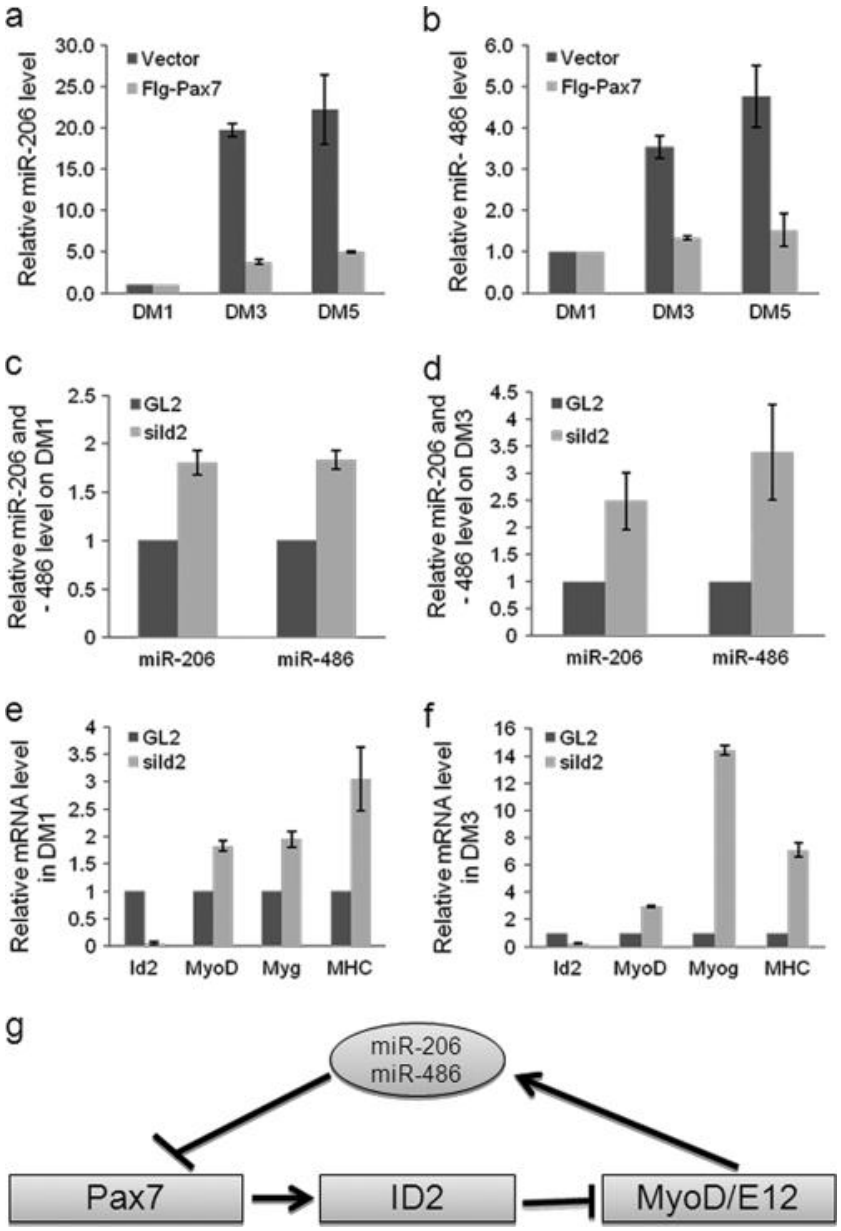

Figure 11. Regulatory network between Pax7 and miR-206 and -486 is mediated by MyoD. (a and b) qRT-PCR shows that miR-206 (a) and -486 (b) upregulation in Pax7 ORF-overexpressing cells is lower than in vector-expressing cells. (c and d) siRNA against Id2 relieved the repression of miR-206 and -486 in Pax7 ORF-overexpressing cells in DM1 (c) and DM3 (d). (e and f) siRNA against Id2 in Pax7-overexpressing cells downregulates Id2 mRNA level and upregulates MyoD and markers of terminal myogenesis, myogenin and MHC in DM1 (e) and DM3 (f). (g) A model for a bistable switch of the Pax7-dominated state and MyoD-dominated state. miR-206 and -486 are expressed by active MyoD and repress Pax7, which lowers levels of inhibitors such as Id2. This allows more E12 to heterodimerize with MyoD and allows transcription of MyoD-responsive genes.

Chapter 3: Regulation of MyoD via MyoR

Adapted From:
Gagan J, Dey BK, Layer R, Yan Z, Dutta A, "*MicroRNA-378 targets the myogenic repressor MyoR during myoblast differentiation*", **Journal of Biological Chemistry,** Jun 2011

ABSTRACT

MicroRNAs play important roles in many cell processes including the differentiation process in several different lineages. For example, microRNAs can promote differentiation by repressing negative regulators of transcriptional activity. These regulated transcription factors can further upregulate levels of the miRNA in a feed forward mechanism. Here we show that MyoD upregulates miR-378 during myogenic differentiation in C2C12 cells. ChIP-seq analysis shows that MyoD binds in close proximity to the miR-378 gene and causes both transactivation and chromatin remodeling. Overexpression of miR-378 increases the transcriptional activity of MyoD, in part by repressing an antagonist, MyoR. The 3'UTR of MyoR contains a direct binding site for miR-378. The presence of this binding site significantly reduces the ability of MyoR to prevent the MyoD driven transdifferentiation of fibroblasts. MyoR and miR-378 were anticorrelated during cardiotoxin induced adult muscle regeneration in mice. Taken together, this shows a feed-forward loop where MyoD indirectly downregulates MyoR via miR-378.

CONTRIBUTION:

The work presented in this chapter was the result of a collaboration between Dr. Bijan Dey and I. The work was published in June 2011 in the Journal of Biological Chemistry. I performed almost all of the cloning, luciferase and qPCR reactions. Dr. Dey

originally identified the miR-378 binding site by bioinformatic prediction and performed one of the miR-378 inhibitor experiments. In addition, we jointly conducted the cardiotoxin experiments. Ryan Layer performed all of the bioinformatic analysis of the ChIP-seq data sets. Zhen Yan performed the injection of cardiotoxin. Anindya Dutta supervised all aspects of the project.

Microrna-378 Targets The Myogenic Repressor Myor During Myoblast Differentiation

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INTRODUCTION

Skeletal muscle differentiation from specification of the myotome through the formation of mature myofibers is driven by basic Helix-Loop-Helix (bHLH) transcription factors referred to as the muscle regulatory factors (MRFs). MRFs heterodimerize with ubiquitous E-proteins E12/E47 and bind to DNA motifs known as E-boxes (1). Although all MRFs have similar DNA binding motifs, their spatiotemporal regulation and role in development is unique. A well-characterized MRF is MyoD, which was noted for its ability to differentiate fibroblasts into myotubes (2). MyoD has roles in both lineage specification and terminal differentiation. MyoD has a broad range of targets and has been shown to bind to thousands of locations in the genome (3). In the C2C12 myoblast cell line, MyoD is expressed, but is not active as a transactivator at loci of differentiation associated genes until myotube formation is induced by low serum conditions. There are a number of inhibitors of MyoD that prevent its premature activation. Among these are

the Inhibitor of DNA binding proteins (4), Twist (5), and a competing bHLH called MyoR or Musculin (6). MyoR inhibits MyoD by binding to E-proteins, and binding directly to MyoD target DNA sequences. MyoR is present during early embryonic stages, but downregulated during secondary embryogenesis. MyoR is expressed in C2C12 myoblasts, and downregulated upon differentiation to myotubes.

A second level of regulation of the myogenic program involves microRNAs. They are initially transcribed as a long transcript by Pol II or Pol III, which is referred to as the pri-miRNA. The RNA then folds into a hairpin and is cut by Drosha/DGCR8 into a hairpin shaped pre-miRNA and exported to the cytoplasm. Most miRNAs are then processed by Dicer into a 19-24 bp single stranded mature miRNA. One strand of the hairpin is then preferentially loaded into the RNA Induced Silencing Complex (RISC). Silencing is achieved through destabilizing target mRNAs and blocking translation. Several miRNAs that are normally induced during myogenic differentiation can initiate the myogenic program even in the presence of high serum in C2C12 (7,8). The targets of these myogenic miRNAs include cell cycle machinery (9), Pax family transcription factors (10-12), and chromatin remodelers (13). Since the activity of MyoD as a transactivator increases during differentiation, we explored whether differentiation-induced microRNAs have an impact on targets that regulate MyoD activity. In this paper we show that miR-378, a micoRNA that is upregulated during differentiation by MyoD, plays a role in the activation of MyoD by targeting its inhibitor MyoR. This interaction illustrates a new mechanism that allows MyoD to change its transcriptional program in the transition from proliferating myoblasts to differentiating myotubes.

Materials and Methods

Cell Culture

C2C12 myoblasts were cultured in DMEM with 20% fetal bovine serum (FBS) and 1% penicillin/ streptomycin (GM). 10T1/2 fibroblasts were cultured similarly but with 10% FBS. Cells were induced to differentiate by replacing 20% FBS with DMEM containing 2% horse serum (Differention Media or DM). Transfection with miRNA mimics or inhibitors was performed as described before (12). Retroviruses for the creation of stable cells were generated in 293T cells. All transfections were performed with Lipofectamine 2000 (Invitrogen). Cells were transfected with the viral vector, as well as a VSVG and a gag/pol encoding plasmid. After 48 hours, the supernatant was removed, centrifuged to pellet any 293T cells, and passed through a .45 μm filter. This viral supernatant was then added to the target cells in the presence of polybrene. Stable cell lines were selected by puromycin (1.5 mg/ml) for 48 hours.

Plasmids

Mir-378 encoding oligonucleotides were cloned into a plasmid based on the miR-30 conformation as described previously (14). This was done to eliminate any effect from the -378* strand. The miR-378 inhibitor design was based on previous work (15), and ligated into tough decoy plasmids that were a kind gift from Dr. Hideo Iba. The short hairpin for MyoD knockdown was cloned into pLKO.1 plasmid. The target sequence on MyoD was described before (16). MyoR expression plasmids were cloned by PCR from C2C12 cDNA, and ligated into pBabe-puro vectors. Mutagenesis was performed by PCR amplification and Dpn I digestion to remove parental DNA. MyoR 3'UTR sequence was PCR amplified from C2C12 genomic DNA and cloned into pRL-CMV vector. Enhancer

activity was tested by cloning a ~500 base pair region around the microRNA binding site and ligating it into a pGL3 promoter plasmid. MCK luciferase was a kind gift from Dr. Stephen Tapscott. The 4RE plasmid was ordered from Addgene, plamsid 16057.

RT-PCR

Cells were lysed and total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. Ncode miRNA first-strand cDNA synthesis and qRT-PCR kit (Invitrogen) were used to perform quantitative RT-PCR for microRNA detection. For mRNA detection, cDNA synthesis was carried out using Superscript III first-strand synthesis system for RT-PCR (Invitrogen). Then, quantitative PCR (qPCR) was carried out using Sybr-green PCR master mix in an ABI cycler. ABI 7300 software was used for quantification (Applied Biosystem).

Luciferase Assays

For the assay of repression of the MyoR 3'UTR (Fig. 4D), a miR-378 expressing plasmid, a 3'UTR containing renilla luciferase reporter (2 ng) and a firefly luciferase control (5 ng) were co-transfected into NIH3T3 cells. Transcriptonal experiments were performed in 10T1/2 fibroblasts. Experiments were analyzed with Dual-luciferase reporter assay system (Promega) following the manufacturer's instructions. Luminescent signal was quantified by luminometer (Monolight 3020; BD Biosciences).

ChIP-seq mapping

We used Novoalign (version 2.05.04) to align reads from the Sequence Read Archive (SRP001761) to the mouse reference genome (NCBI v37, mm9). MACS (version 1.3.6.1) was used to identify areas enriched over background without a control sample. From MACS, we created wig files with peaks of at least ten-fold enrichment and a

window size of ten base pairs. For the H4ac experiment, the MyoD and PCLBABE samples were aligned with Novoalign using default parameters. For each sample, we collected unique reads with an alignment score of at least 50, and generated wig files of the enrichment using MACS without a control sample. The log2 of MyoD to PCLBABE enrichment for H4Ac bound DNA was calculated for the chr18 1400000-61600000 region. To ensure the ratio was always defined, we set the enrichment at each position to be at least one.

Animal Experiments

The use of animals was approved by the Animal Care and Use Committee of University of Virginia. Eight-week-old male C57BL/6 mice were anesthetized with isoflurane and sacrificed by cervical dislocation to harvest muscle. For the regeneration assay, the injury was performed on Tibialis Anterior muscles of mice by injecting 100 μl of 10 μM cardiotoxin (CTX). In groups of N=5, mice were sacrificed at 1, 3, 5, 7 and 14 days post-injection to collect the TA muscles.

Results

Identifying miR-378 as a myomiR, and that its level is regulated by MyoD

In order to fully catalog the miRNA species that are induced in myogenic differentiation, we hybridized short RNAs from proliferating and differentiating C2C12 cells to a locked nucleic acid array of probes for miRNA (12). miR-378 was one of the species that was upregulated more than 4-fold during differentiation. We confirmed this change of mature miRNA levels by qPCR. As shown in Fig. 1A, there was a slight increase on Day 1, a larger increase on Day 2 and no further increase up to Day 4. The

miR-378 coding sequence lies in the first intron of the Ppargc1b gene. There is a fair amount of conserved DNA sequence in the first intron adjacent to the coding sequence of the miRNA (Fig. 1B). We hypothesized that some of the conserved sequence is important for regulating miR-378 expression.

Of the myogenic transcription factors to consider as possible inducers of miR-378, the first candidate was MyoD because the increase of miR-378 was observed early in differentiation concurrent with MyoD transactivation activity. This would not be consistent with myogenin or other factors that are induced later during differentiation. We analyzed the sequencing data published from a MyoD ChIP-Seq experiment in C2C12 (3). A conclusion of the authors was that areas with a higher read density in myotubes than myoblasts were correlated with increased expression of associated genes. Less than 1 kb downstream of miR-378, we mapped a ChIP-seq peak that was below our threshold for calling a peak in myoblasts (10 reads per 10 bp window), but had a maximum read density of 38 per 10 bp window in myotubes (Fig. 1b). MyoD has been known to bind to a DNA sequence, called E-box, with the canonical sequence CANNTG. The ChIP-Seq study showed that CAGCTG and CAGGTG are the sequences that are specifically enriched within MyoD bound regions. The binding peak downstream of miR-378 contained three such E-boxes, all of which had at least partial conservation among mammals (Fig. 1C).

Since not all of the MyoD-bound regions are correlated with increased gene expression during differentiation, we first tested whether MyoD binding at this peak regulates miR-378 transcription. We knocked down MyoD by an shRNA expressed from a lentiviral vector in C2C12 myoblasts (Fig. 2A). The primary miR-378 transcript was

then measured because mature sequences are very stable and the pri-miRNA level would more accurately reflect recent changes in transcriptional activity. There was significantly less pri-miR-378 in myoblasts expressing shMyoD versus a control hairpin vector (Fig. 2B). From this we conclude that MyoD is involved in regulating miR-378 levels in myoblasts.

MyoD Regulates miR-378 by both Chromatin Remodeling and Transcriptional Enhancer Activity

One of the ways MyoD potentiates myogenic transdifferentiation is by recruiting chromatin remodeling proteins. Specifically, MyoD is known to bind to histone acetyltransferases p300 and PCAF (17). This leads to the local enrichment of acetylated histone H4, which is a marker of transcriptional activity. The same group that performed the MyoD ChIP-seq in C2C12 also performed a ChIP-seq with a pan acetyl-H4 antibody in fibroblasts transfected with MyoD or a control vector (3). Identifying changes in histone modifications in MyoD transfected samples will reveal which regions are epigenetically modified during myogenic transdifferentiation. As shown in Fig. 3A, mapping the reads from this experiment showed a significant enrichment of acetylated H4 in the cells transfected with MyoD over control in the region of miR-378. To determine whether MRFs other than MyoD are activating differentiation induced genes, C3H10T1/2 fibroblasts were used because they have no inherent myogenic properties, but can readily differentiate into myotubes when MyoD is expressed exogenously (2). Full differentiation does not occur until the cells are placed in low serum, differentiating media (DM). Therefore, a gene directly responsive to MyoD should increase once the

cells are transfected with a MyoD-expressing plasmid, with a larger increase upon serum withdrawal. A gene that is activated by another MRF or a secondary response will only be turned on after being placed in DM. miR-378 showed a pattern of directly induced genes, with a 2-fold induction after co-transfection with MyoD and E12, and another 2 fold induction after switching to DM (Fig. 3B). The MyoD binding site near miR-378 was PCR amplified out of genomic DNA and cloned into an enhancer-less luciferase plasmid that contained a core promoter. Co-transfection of MyoD and E12 with this luciferase reporter stimulated luciferase activity by 4-fold (Fig. 3C), exactly corresponding with the increase of miR-378 detected by qPCR in Fig. 3B. In order to prove that this is a direct function of MyoD, increasing amounts of MyoR were added. MyoR (100ng) reduced activation to baseline levels. In addition, mutation of the E-boxes in the miR-378 enhancer prevented activation of the luciferase reporter by MyoD. Taken together, these results suggest that the E-boxes near miR-378 constitute both a site for chromatin remodeling and a MyoD-responsive enhancer that can be inhibited by MyoR.

miR-378 modulates MyoD activity by repressing MyoR

In order to elucidate the biological function of miR-378 we first tested whether it had any effect on MyoD transcriptional activity. One of the simple ways to measure MyoD transcriptional activity is to use a firefly luciferase-expressing plasmid that contains a multimerized E-box upstream from a minimal promoter (4RE). When C2C12 cells were co-transfected with this reporter and a plasmid that constitutively expresses miR-378, there was an increase in luminescence (Fig. 4A). A more physiologically

relevant target sequence, the promoter of muscle creatine kinase (MCK) fused to firefly luciferase, was also activated by miR-378 expression.

We then wanted to test if modulation of miR-378 levels can have an effect on the overall kinetics of C2C12 differentiation. After 24 hours in DM, overexpression increased mRNA levels of myogenin (Fig. 4B and 4C). Likewise, expression of Myosin heavy chain was increased after 72 hours in DM. Inhibition of miR-378 with 2'-O-methyl antisense was associated with a decrease in levels of myogenin mRNA, but a minor decrease in MHC that was not statistically significant. These data suggest that while miR-378 can promote differentiation, it is not the only factor that can do so. Thus induction of the microRNA promotes differentiation, but inhibition of the microRNA has a minor effect on differentiation.

By using bioinformatic prediction software, we discovered a putative target site of miR-378 in the 3' UTR of MyoR (Fig. 4C). There is perfect complementarity to base pairs 2-8 of the miRNA. Perfect Watson-Crick pairing in this "seed sequence" is the most important property for miRNA targeting (18). We cloned the 3'UTR of MyoR into a reporter as the UTR of luciferase. Adding a plasmid encoding miR-378 reduced the luminescence of the MyoR 3'UTR containing luciferase to 60% of the control, consistent with the notion that it contains a functional miR-378 target site. When the miR-378 binding site is mutated, luminescence returned to control levels (Fig. 4D), indicating that this site was indeed the unique sequence targeted by miR-378 in the MyoR 3'UTR.

To test if this binding site could be used to functionally regulate MyoR, we tested the ability of MyoR to prevent MyoD-mediated transdifferentiation of 10T1/2 fibroblasts, where MyoR is normally transcribed at a very low level. 10T1/2 cells were infected with

a retrovirus encoding different constructs of MyoR and stable cell lines were created with puromycin selection. These cells were then transfected with MyoD, and differentiation was assessed quantitatively by co-transfection of MCK-luciferase. Cells were placed in differentiation media 24 hours after transfection and harvested 48 hours later. The MyoR open reading frame (ORF) without the 3'UTR dramatically reduced differentiation, consistent with previously published work (6). A vector with both the ORF and the 3'UTR was less effective in blocking MCK luciferase activity. Mutation of the seed sequence of the binding site, thereby preventing repression by miR-378, mostly restored this block of MyoD activity (Fig. 5A). The finding was confirmed by a more physiological readout, the endogenous MHC mRNA levels were measured by suing qRT-PCR (Fig. 5B). As judged by MHC mRNA, differentiation was decreased by MyoR. The 3'UTR with an intact miR-378 target site mediated miR-378 inhibition of MyoR activity and so allowed better differentiation.

The inhibitory effect of microRNAs on protein level is achieved primarily through destabilization of target mRNA (19). To show that a change in MyoR expression was behind the differences observed above, we assayed MyoR mRNA in 10T1/2 cells after 2 days in growth and differentiation conditions. The ORF showed a slight reduction, and including the 3'UTR significantly decreased MyoR mRNA levels. Mutating the miR-378 binding site alleviated some, but not all of this repression (Fig. 5C). This suggests the possibility of a separate repressor that binds the 3'UTR, possibly another miRNA. We expect the MyoR protein levels to follow the change in mRNA, but the available MyoR antibodies did not give specific signals on Western blots. These

results suggest that the repression of MyoR by miR-378 is sufficient to affect the ability of MyoR to inhibit differentiation.

MyoR and miR-378 levels are anticorrelated during muscle regeneration

In order to show the regulation of MyoR by miR-378 in skeletal muscle regeneration, we measured RNA level in mouse skeletal muscle recovering from cardiotoxin treatment. We injected a cohort of mice in the tibialis anterior simultaneously and harvested 5 mice per time point up to two weeks after injection. By qRT-PCR we analyzed miR-378 levels along with MyoD and MyoR. miR-378 levels decreased dramatically after injury, down to 0.5% of pre-injection levels, as was expected for a miRNA that is induced during myogenic differentiation. By day 14 it had returned to 50% of saline injected mice (Fig 6a). The regulation follows a similar pattern as canonical myomiRs, miR-1 and -133, but unlike -206 whose level saturates in the first week (20). mir-378 levels were roughly anticorrelated with MyoR, which peaked on day 5 of regeneration and then continually declined at each subsequent time point (Fig. 6b). This decrease in MyoR occurred at the same time that miR-378 levels increased, suggesting that our findings in C2C12 cells likely reflect the regulation of MyoR by miR-378 in vivo.

It is interesting to note that miR-378 levels were not correlated at all with MyoD transcript levels or even the MyoD:MyoR ratio. This makes it unlikely that MyoD is the only regulator of miR-378 levels in regenerating skeletal muscle. However, the pattern of expression of miR-378 is similar to miR-1 and -133, two microRNAs known to be regulated by MyoD, Myogenin, MEF2 (21) and Serum Response Factor (22).

Discussion

In this study, we identify miR-378 as another miRNA that plays a role in skeletal muscle differentiation. MiR-378 forms the basis of a simple positive feedback loop whereby it is upregulated by MyoD and then targets MyoR, a repressor of MyoD transcriptional activity (Fig. 7).

MyoD has often been called the "master switch" of skeletal muscle differentiation due to its ability to singularly transdifferentiate fibroblasts. This simplicity of a single reprogramming factor has not been reproduced in other systems such as cardiomyocyte differentiation (23). It has been known that miRNAs are regulated by the MRFs, specifically MyoD and Myogenin, (24-27) though most of the work has focused on miRs -1, -133 and -206. Additionally, miRs -22, -100, -138 and -191 have been identified as having MyoD binding peaks in their promoters (28), but their biology remains largely unknown, and most were shown not to have altered expression levels in MyoD knockout myoblasts (29). This miR-mediated regulation was missed in the earlier studies because they were based upon CHIP-Chip experiments where the probes on the array were restricted to promoter sequences. Here we have identified a MyoD binding region that is very proximate to miR-378 with properties consistent with a role in transcriptional control. There is a large increase in the amount of histone H4 acetylation at the miR-378 locus when naïve fibroblasts are transfected with MyoD. The MyoD binding site functions as an enhancer in a luciferase assay, and that the function is ablated when MyoR is added or the E-boxes are mutated. Knockdown of MyoD reduces levels of miR-378 primary transcript in myoblasts. miR-378 levels during cardiotoxin-induced injury

and regeneration mimics established MyoD responsive miRNA genes miR-1 and -133. We conclude that the MyoD-binding site near miR-378 is functional, and results in increased transcription early in myoblast differentiation. The fact that miR-378 levels lag behind MyoD in cardiotoxin-induced muscle regeneration should not be interpreted that this regulation is not functional in vivo; rather that understanding the regulation of miR-378 is incomplete. Myogenic cells have many cell signaling pathways that cross-talk with MyoD including Notch, FGF and TGF-beta(30), and these cross-talks could possibly delay the upregulation of MyoD targets. As miR-378 levels so closely mimic other myomiRs during cardiotoxin-mediated muscle regeneration, it will be interesting to see if transcription factors such as MEF2 and SRF, also affect miR-378 levels.

The downregulation of MyoR during C2C12 differentiation was established soon after it was identified as an inhibitor of myogenesis (6), but the mechanism was not established. Further studies have suggested the importance of Hes6 (31), but the evidence was correlative without demonstrating direct binding of Hes6 to MyoR gene. Notch has been postulated as a positive regulator of MyoR (32), but no binding site for the canonical Notch DNA-binding protein Rbpj has been located in the promoter. Here, we have demonstrated the first direct interaction of a differentiation induced repressor of MyoR within the MyoR gene or transcript. Interestingly, Hes6 is also thought to be upregulated by MyoD (33), and therefore could be acting redundantly with miR-378 in a feed-forward loop from MyoD to repress MyoR. This could also explain why the repression of MyoR and induction of miR-378 after cardiotoxin injury weren't perfectly correlated. Notch signaling inhibits MyoD (34); therefore it is possible that the upregulation of MyoR that was reported after stimulation of the Notch pathway is just

reflective of the loss of miR-378 and Hes6, two repressors of MyoR that are normally induced by MyoD.

Myogenic transcription factors and the repressors have long been accepted as forming regulatory loops that allow the graduated induction of myogenesis interspersed with metastable progenitors. Two recent discoveries now begin to interpolate microRNAs in these regulatory loops. miR-206 was recently shown to be induced by MyoD to inhibit Pax7 and thus decrease Id2, a repressor of MyoD (12). In this report we demonstrate that miR-378 plays a similar role in that it is induced by MyoD to directly repress MyoR. It will be interesting to learn of other such examples in myogenic differentiation. Also, similar regulatory loops involving the interaction of transcription factors, microRNA, and inhibitors of differentiation may occur in other types of tissue.

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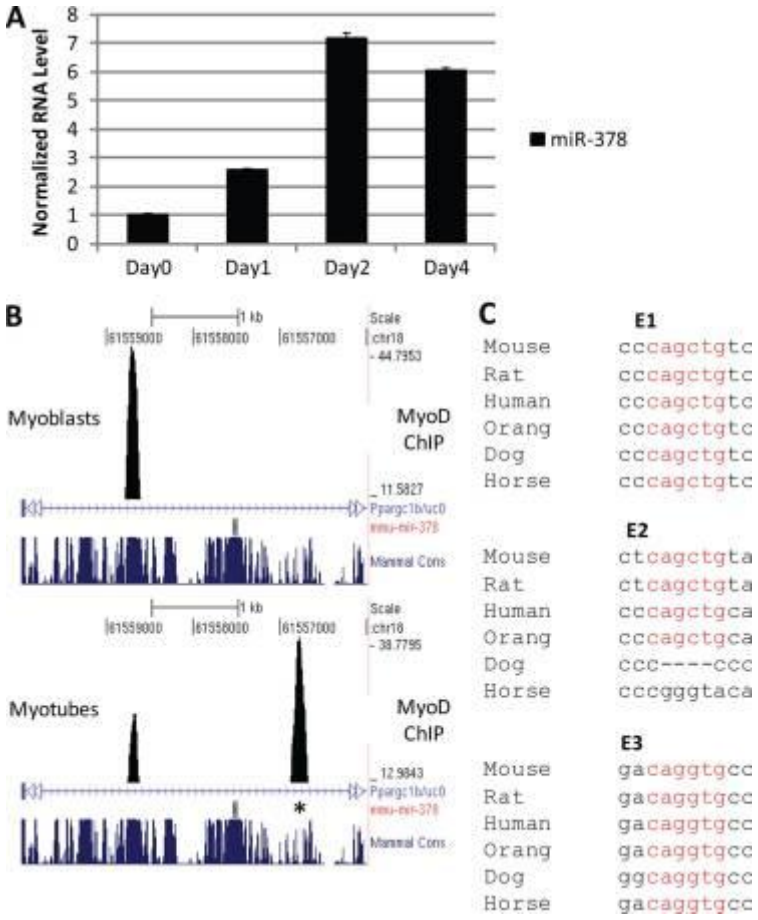

Figure. 1. miR-378 is upregulated during C2C12 differentiation. **a**) miR-378 levels increase during C2C12 differentiation, predominately in the first two days after serum withdrawal. miR-378 level was normalized to that of snU6 and the ratio on day 0 set to 1. Mean ± SD of three measurements. **b**) MyoD CHIP-seq shows MyoD binding relative to miR-378 locus in C2C12 myoblasts (top) and myotubes (bottom). The relevant portion of the Ppargc1b gene containing the miR-378 locus is shown. The Y axis represents the number of independent sequence reads that overalp within a given 10b window in the MyoD ChIP. The primary data is from (3). **c**) The 3' binding peak of MyoD that was detected only in myotubes (* in b) contained three E-box sequences consistent with the MyoD consensus sequence. All three showed significant mammalian conservation.

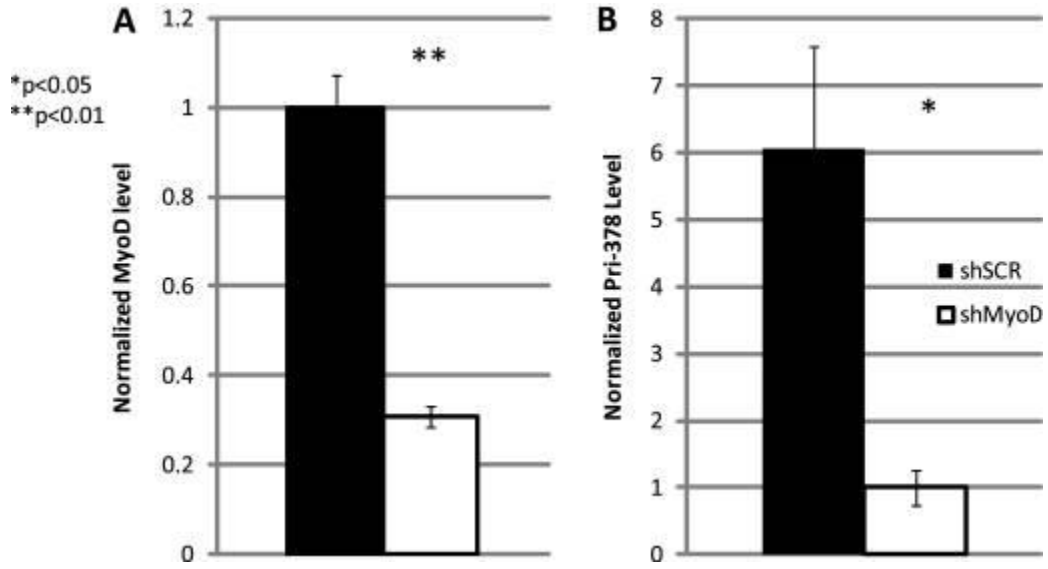

Figure 2. Knockdown of MyoD in C2C12 decreases miR-378 levels. **a**) shMyoD infected cells decreased MyoD transcript level to 30% of short hairpin scramble control infected cells. MyoD level normalized to level of GAPDH. Mean ± SD of three measurements. Statistical significance as determined by 2-sided student's t-test. **b**) MyoD knockdown caused a decrease in primary transcript levels of miR-378 in myoblasts. Details as in 2a.

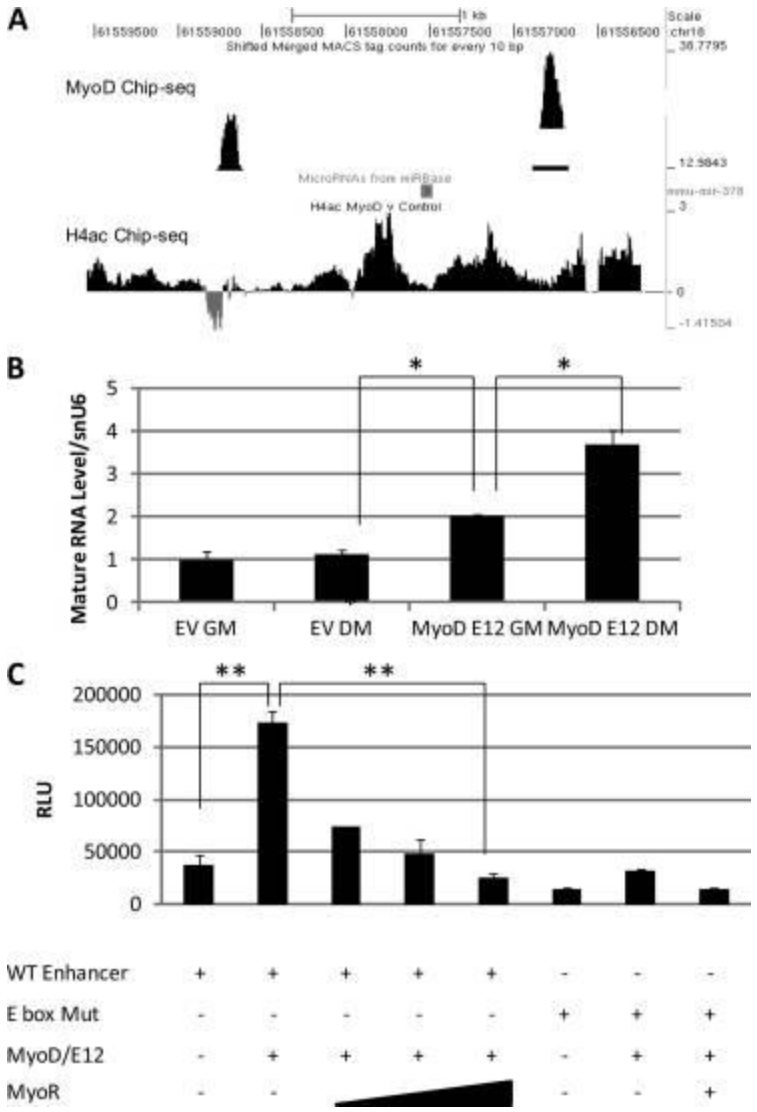

Figure 3. The MyoD binding peak near miR-378 function for both chromatin remodeling and as a tranactivator in naïve fibroblasts. **a**) Log base 2 ratio of histone h4 acetylation between 10T1/2 fibroblasts infected with a MyoD expressing virus relative to control fibroblasts. For reference the MyoD binding sites in C2C12 myotubes (Fig 1b) are shown above. **b**) Mature miR-378 levels in 10T1/2 fibroblasts in growth media (GM) and low serum, differentiating media (DM) when transfected with empty vector (EV) or MyoD and E12 expressing plasmids. miR-378 levels were increased by MyoD/E12 transfection and further increased by DM. Details as in Fig. 1a. **c**) The MyoD binding site from miR-378 (Fig 1b*) was cloned into an enhancer-less luciferase vector. Luminescence increased 4 fold with MyoD/E12, but this was abrogated by adding exogenous MyoR or mutating the E-boxes. RLU: Relative Light Unit. Mean ± SD of three experiments.

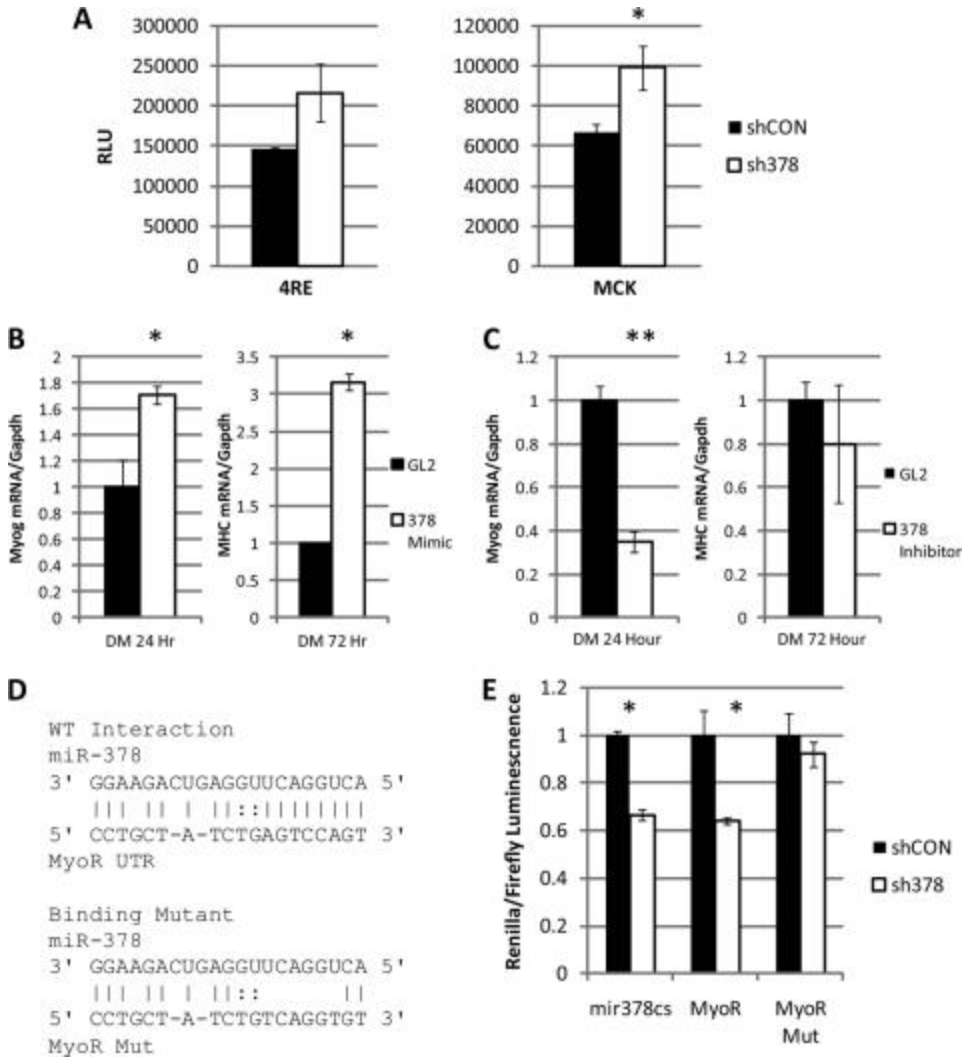

Figure 4. miR-378 activates MyoD and targets MyoR. **a**) Addition of exogenous miR-378 increases MyoD transcriptional activity as measured by 4RE and MCK luciferase reporters. Details as in Fig. 3c. **b)** Addition of a synthetic miR-378 mimic increases markers of myogenic differentiation in C2C12. The ratio of myogein or MHC to Gadph in cells infected with control (GL2) is set to 1. Mean ± SD of three experiments. **c**) Blocking miR-378 by addition of an inhibitor decreases the mRNA levels of myogenin in C2C12 differentiation. **d**) Schematic of the WT binding site in the MyoR 3'UTR for miR-378. The mutated seed sequence was used as a negative control. **e**) A luciferase construct containing the MyoR 3'UTR is repressed by addition of miR-378, and the repression is relieved by mutating the seed sequence binding site (MyoR Mut). miR-378cs is a positive control that contains a perfect match of miR-378 in the 3'UTR of luciferase. Mean ± SD of three experiments.

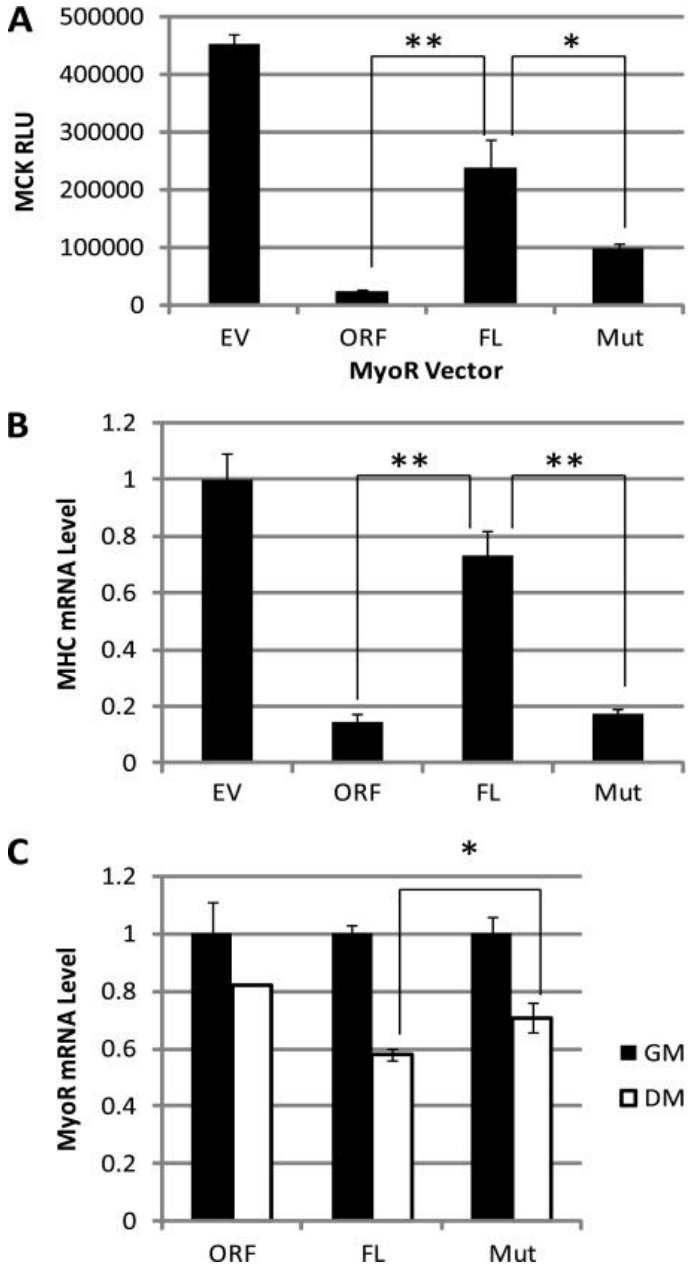

Figure 5. The miR-378 binding site can serve as an important regulator of MyoR function. **a**) The ORF of MyoR blocks the transdifferentiation of 10T1/2 fibroblasts by addition of MyoD, as measured by MCK luciferase. The inclusion of the 3'UTR greatly diminishes the inhibitory effect of MyoR, which is restored by mutating the miR-378 binding site. MCK RLU: Mean ± SD of three experiments. **b**) This result is confirmed by qRT-PCR of myosin heavy chain mRNA normalized to GAPDH, rest as in 5a. **c**) The 3'UTR promotes the ability of MyoD to decrease expression of exogenous MyoR. At least part of this ability is via the miR-378 binding site. MyoR mRNA normalized to GAPDH and the ratio in GM is set to 1. Mean ± SD of three experiments. ORF: only the open reading frame of MyoR. FL: ORF of MyoR with the wild type 3'UTR. Mut: Same as FL with the miR-378 target site mutated as in Fig. 4c.

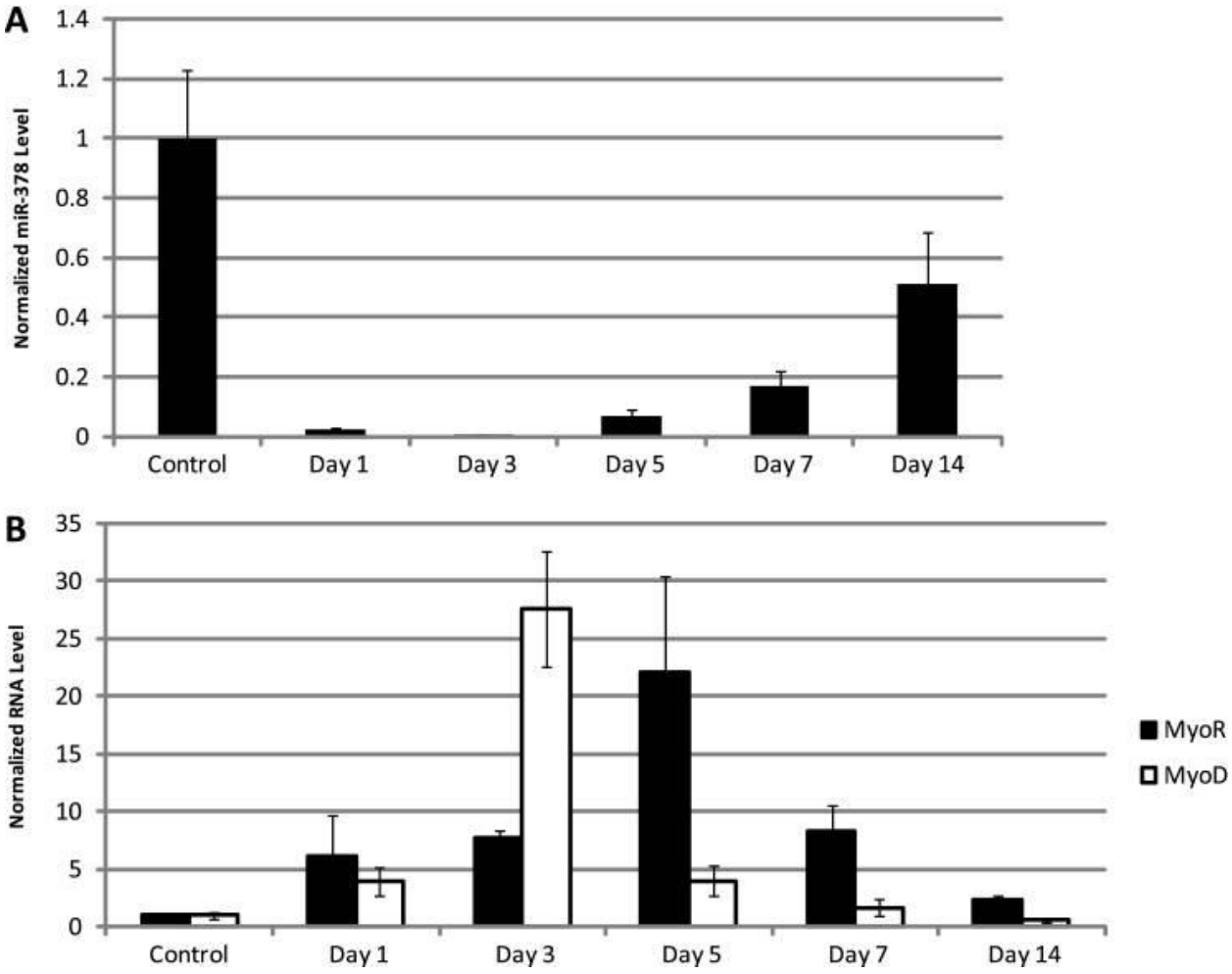

Figure 6. miR-378 is repressed in skeletal muscle during regeneration after snake venom cardiotoxin treatment. **a**) Mature miR-378 decreases dramatically to 0.5% of control three days after venom injection and then gradually recovers. Normlized to snU6 transcript level, ratio in control injected animals set to 1. Average of technical triplicate, n=5, ± SEM. **b**) MyoR transcript levels peak 5 days after injection and then decreases, with a general anticorrelation to miR-378. MyoD transcript peaks on day 3. Both transcripts normalized to GAPDH. Other details as in 6a.

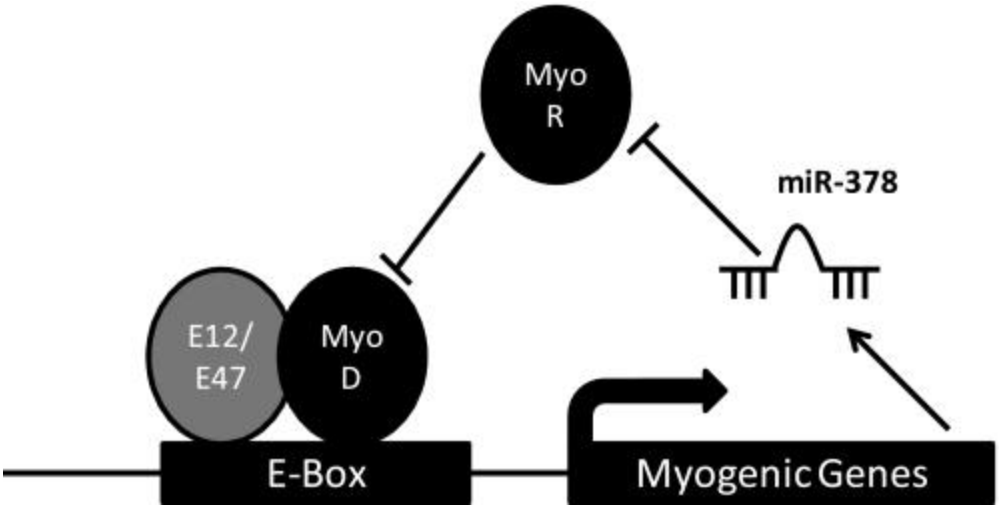

Figure 7. The working model for the feedback loop. MyoD induces miR-378, which represses MyoR and thus feeds back to further activate MyoD.

Chapter 4: Regulation of Mef2c via Notch3

Adapted From:
Gagan J, Dey BK, Layer R, Yan Z, Dutta A, "*Notch3 and Mef2c Are Mutually Antagonistic via Mkp1 and miR-1/206 in Differentiating Myoblasts*", **Journal of Biological Chemistry,** In Revision

ABSTRACT

The Notch signaling pathway is a well-known regulator of skeletal muscle stem cells known as satellite cells. Loss of Notch1 signaling leads to spontaneous myogenic differentiation. Notch1, normally expressed in satellite cells, is targeted for proteasomal degradation by Numb during differentiation. A homolog of Notch1, Notch3, is also expressed in these cells but is not inhibited by Numb. We find that Notch3 is paradoxically upregulated during the early stages of differentiation by an enhancer that requires both MyoD and activated Notch1. Notch3 itself strongly inhibits the myogenic transcription factor Mef2c, most likely by stabilizing the p38 phosphatase Mkp1, which inhibits the Mef2c activator p38 Map kinase. Active Notch3 decreases differentiation. Mef2c, however, induces microRNAs miR-1 and miR-206, which directly downregulate Notch3 and allow differentiation to proceed. Thus the myogenic differentiation-induced microRNAs miR-1 and -206 are critical for differentiation at least partly by turning off Notch3. We suggest that the transient expression of Notch3 early in differentiation generates a temporal lag between myoblast activation by MyoD and terminal differentiation into myotubes directed by Mef2c.

CONTRIBUTION:

The work is in revision for publication in the Journal of Biological Chemistry. I performed all of the cloning, luciferase, western blot and qPCR reactions. Dr. Dey jointly conducted the cardiotoxin experiments. Ryan Layer performed all of the bioinformatic analysis of the ChIP-seq data sets. Zhen Yan performed the injection of cardiotoxin. Anindya Dutta supervised all aspects of the project.

Introduction:

Notch signaling has been shown to be critical for both skeletal muscle development and response to injury in adults. Classically, Notch signaling involves the interaction of a ligand on one cell and a receptor on its neighbor (reviewed in (1)). The receptor is proteolytically cleaved, releasing the intracellular domain (ICD[1]), which then shuttles into the nucleus. The DNA-binding protein for Notch is Rbpj, which is bound to DNA even in the absence of Notch ICD. Without the ICD, Rbpj is associated with transcriptional repressors (2,3). When ICD interacts with Rbpj along with the coactivator MamL (4), it displaces the repressors, and initiates transcription. Genes promoted by the ICD are generally inhibitory to terminal differentiation of skeletal muscle (5). If Rbpj is deleted in skeletal muscle by tissue-specific knockout, the result is uncontrolled differentiation of myogenic progenitors resulting in severe hypotrophy (6).

After embryonic development, adult post-mitotic skeletal muscle retains a reservoir of progenitor cells that are referred to as satellite cells due to their anatomical location outside of the fiber, but underneath the basement membrane (7,8). Deletion of Rbpj in satellite cells by inducible recombination results in spontaneous differentiation of quiescent satellite cells (9,10) indicating that Notch signaling is continually required to maintain quiescence. Notch has been shown to antagonize the activity of the bHLH transcription factors collectively called Muscle Regulatory Factors (MRFs) (11,12). These are four genes (Myf5, MyoD, Myog, Myf6) that promote myogenic progression and transdifferentiation. They can work in concert with transcription factor Mef2c (13), which is also known to be antagonized by Notch (14).

The activity of MRFs and Mef2c can be regulated via microRNAs (Reviewed in (15)). MicroRNAs are genes that are initially transcribed as a long transcript by Pol II or Pol III, which is referred to as the pri-miRNA. The RNA then folds into a hairpin and is cut by Drosha/DGCR8 into a hairpin shaped pre-miRNA and exported to the cytoplasm. Most miRNAs are then processed by Dicer into a 19-24 bp single stranded mature miRNA. One strand of the hairpin is then preferentially loaded into the RNA Induced Silencing Complex (RISC). Silencing is achieved through destabilizing target mRNAs and blocking translation. Among the first microRNAs to be identified in skeletal muscle are miR-1 and miR-206 (16,17). They are highly upregulated in differentiation, and their expression is much higher in striated muscle than all other tissue.

Satellite cells, like all stem cells, undergo asymmetric cell divisions; one cell daughter cell begins to differentiate; the other returns to quiescence as a progenitor. Notch1 is normally expressed in progenitor cells, while the differentiating cell turns off Notch1 by targeting it for degradation by Numb (18) via the E3 ubiquitin ligase Itch (19). A subset of satellite cells has never expressed the myogenic transcription factor Myf5 and these stem-like cells express Notch3 at a higher level than those that have expressed Myf5 (20). After a cell division, the differentiating daughter cell begins to express Myf5 as well as the Notch ligand Dll1. Aside from high expression in Myf5-negative satellite cells, the role of Notch3 in skeletal muscle is not well known. The expression of Notch3 was shown to be higher in quiescent than actively dividing satellite cells (21). Notch3 (22) has been shown to be targeted by miR-206, but in the context of HeLa cells. We set out to investigate whether miRNA regulation of Notch3 has important effects on myogenic differentiation in skeletal muscle. To our surprise, we discovered that Notch3

is initially induced during differentiation even though activated Notch3 normally inhibits differentiation. The subsequent inhibition of Notch3 by miR-1 and miR-206 is therefore critical for differentiation. Intriguingly Notch3 and the myogenic transcription factor Mef2c set up a mutually antagonistic network that is dependent on the actions of the microRNAs and that acts as a bistable switch. In one position the switch is antagonistic to differentiation, while in the opposite position it promotes differentiation.

MATERIALS AND METHODS

Cell Culture

C2C12 myoblasts were cultured in DMEM with 20% fetal bovine serum (FBS) and 1% penicillin/ streptomycin (GM). 10T1/2 fibroblasts were cultured similarly but with 10% FBS. Cells were induced to differentiate by replacing 20% FBS with DMEM containing 2% horse serum (Differention Media or DM). Transfection with miRNA mimics or inhibitors was performed as described before (23). Retroviruses for the creation of stable cells were generated in 293T cells. All transfections were performed with Lipofectamine 2000 (Invitrogen). Cells were transfected with the viral vector, as well as a VSVG and a gag/pol encoding plasmid. After 48 hours, the supernatant was removed, centrifuged to pellet any 293T cells, and passed through a .45 μm filter. This viral supernatant was then added to the target cells in the presence of polybrene. Stable cell lines were selected by puromycin (1.5 mg/ml) for 48 hours.

Plasmids

Mir-1/206 encoding oligonucleotides were cloned into a plasmid based on the miR-30 conformation as described previously (24). The short hairpin for Notch3 knockdown was

designed by The RNAi Consortium (clone TRCN0000075569) cloned into pLKO.1 plasmid. shSCR was a predesigned control (Addgene plasmids 10878 & 10879). Notch3-ICD expression plasmids were cloned by PCR from C2C12 cDNA, and ligated into pBabe-puro vectors. Mutagenesis was performed by PCR amplification and Dpn I digestion to remove parental DNA. MamL1 cDNA was a kind gift from Dr. Lizi Wu. Notch3 3'UTR sequence was PCR amplified from C2C12 genomic DNA and cloned into pRL-CMV vector. Enhancer activity was tested by cloning a ~500 base pair region around the Chip-seq binding site and ligating it into a pGL3 promoter plasmid. MCK luciferase was a kind gift from Dr. Stephen Tapscott. The 4RE plasmid was ordered from Addgene plamsid 16057.

RT-PCR

Cells were lysed and total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. Ncode miRNA first-strand cDNA synthesis and qRT-PCR kit (Invitrogen) were used to perform quantitative RT-PCR for microRNA detection. For mRNA detection, cDNA synthesis was carried out using Superscript III first-strand synthesis system for RT-PCR (Invitrogen). Then, quantitative PCR (qPCR) was carried out using Sybr-green PCR master mix in an ABI cycler. ABI 7300 software was used for quantification (Applied Biosystem).

Luciferase Assays

For the assay of repression of the Notch3 3'UTR (Fig. 4D), a miRNA expression plasmid, a 3'UTR containing renilla luciferase reporter (2 ng) and a firefly luciferase control (5 ng) were co-transfected into NIH3T3 cells. Transcriptonal experiments were performed in NIH3T3 or 10T1/2 fibroblasts. Experiments were analyzed with Dual-

luciferase reporter assay system (Promega) following the manufacturer's instructions. Luminescent signal was quantified by luminometer (Monolight 3020; BD Biosciences).

ChIP-seq mapping

We used Novoalign (version 2.05.04) to align reads from the Sequence Read Archive to the mouse reference genome (NCBI v37, mm9). PeakSeq (version 1.1) was used to identify enriched areas of Notch1 (SRR243559 and SRR243551) over control (SRR243561). From the PeakSeq results, we created BedGraph files containing peaks with a p-value of at most 0.0001. MyoD ChIP-seq mapping was described previously (25).

Western blotting and antibodies.

For Western blotting cells were lysed in RIPA buffer supplemented with protease inhibitor mix (Sigma). Proteins were resolved by SDS-PAGE, transferred, and immunoblotted with various antibodies. The antibodies used anti-myosin heavy chain (Mouse, Sigma), anti-GAPDH (Mouse, Sigma), anti-Mef2c (5030, Cell Signaling), anti-Mkp1 (M-18, Santa Cruz) and anti-Notch3 (M-134, Santa Cruz).

Animal Experiments

Animal experiments were performed as described previously (25).

RESULTS

Notch3 Levels Increase Early During Differentiation

Based on previous reports we had expected Notch3 to have higher expression in quiescent cells. We used mouse model of muscle injury where the tibialis anterior is injected with snake venom cardiotoxin. Following extensive degeneration of myofibers

during the first two days, there is rapid proliferation of myogenic precursors followed by differentiation so that the muscle heals and resembles uninjured tissue again by day 14 after injection. In this context we surprisingly found that the expression of Notch3 increases very early in the differentiation process, on days 1-3 (Fig. 1A). The pattern of expression mimicked, albeit with lower fold change, the pattern observed for MyoD (Fig. 1B). It is worth noting, that in regenerating skeletal muscle, the miR-206 microRNA decreased on day 1, but rapidly increases on day 3 to a level above baseline after day 4 as differentiation supersedes stem cell proliferation (26).

In another model of differentiation, C2C12 myoblasts are induced to differentiate by transfer from serum-rich growth medium (GM) to serum-poor differentiation medium (DM). In this model, there was also a transient increase of Notch3 mRNA (Fig. 1C) and protein (Fig. 6A) early in the differentiation program. miR-206 begins to rise on day 1 after transfer to DM (23).

Notch3 is induced by an enhancer that responds to the combined presence of MyoD and activated Notch

The mRNA expression pattern of Notch3 was not consistent with previously described miR-206 targets DNA polymerase α (17) and Pax7 (Supp Fig. 1 and (23)). These targets decrease precipitously from day 1 in DM. Notch3 has previously been shown to have increased expression in C2C12 co-culture with NIH3T3 fibroblasts that stably express Notch ligand (27). Consistent with the established regulation of Notch3 by canonical Notch signaling, we found that overexpression of the Notch3 intracellular domain (N3-ICD) induces endogenous Notch3 expression at all time points in C2C12 differentiation. However, other canonical Notch targets, such as Hes1, have minimal

changes in mRNA expression during C2C12 differentiation (28). Therefore, we hypothesized that Notch3 is induced via myogenic transcription factors. Analysis of previously published ChIP-seq data (29), showed the presence of a MyoD binding site near the 3' end of intron 1 of the Notch3 gene that is close to several E boxes (Supp. Fig. S2B and Fig. 2A).

We thus tested whether the 3' end of intron 1 of Notch3 has a MyoD responsive enhancer by cloning this site into an enhancerless luciferase reporter. When we found that the putative enhancer was not activated by co-transfection of MyoD in fibroblasts (Fig. 2B, MyoD alone), we therefore considered the possibility that Notch and MyoD were both necessary for induction. We examined a Notch1 and Rbpj ChIP-seq data (30) for evidence of canonical Notch binding within the Notch3 locus. Surprisingly, there was a Notch1 bound peak at the same locus as MyoD (Supp. Fig. S2A) in published ChIP-seq data (30). We also found this site to be marked by H3K27ac in ENCODE project data set in human skeletal muscle myoblasts (Supp. Fig. S2C). Furthermore, there seems to be some specificity as only the myoblasts and lymphoblasts have H3K27ac enrichment at this locus. The Notch1 binding peak is composed of two local maxima corresponding to two canonical Rbpj binding sequences (TGGGAA) with the tails facing the E-box pair (Fig. 2A).

All this ChIP-seq data is consistent with the possibility that the enhancer at the 3' end of intron 1 of Notch3 responds to the combined action of Notch and MyoD. Indeed, in subconfluent fibroblasts, co-transfection of the enhancer-driven luciferase reporter with either Notch1-ICD (not shown) or Notch3-ICD (Fig. 2B) failed to activate the enhancer. However, when either Notch1-ICD or Notch3-ICD was co-transfected with

MyoD, there was a significant activation of the Notch3 enhancer (Fig. 2B). We confirmed that the E boxes (for attracting MyoD) and the Rbpj boxes (for attracting Rbpj and Notch were independently required for this stimulation by site directed mutatgenesis. Either set of mutations was sufficient to decrease the activation of the enhancer by MyoD or N1-ICD (Fig. 2C). In C2C12, the WT reporter was not active at all in undifferentiated myoblasts, but each mutant had weak activity (Fig. 2D). After 48 hours in differentiation media, the WT enhancer was the most active, while each mutant had significantly less activity. However, in C2C12, the Ebox mutant displayed lower activity than the Rbpj mutant. Together, this suggests that the Notch3 enhancer is a specialized response element that responds to the combined presence of MyoD and activated Notch, accounting for the specific induction of Notch3 early during the differentiation process.

Notch3 Is Targeted by miR-1/206

The induction of Notch3 in both the in vivo and in vitro differentiation models was transient. Since Notch3 can activate itself, we reasoned that the repression of Notch3 by muscle differentiation induced microRNAs could switch the equilibrium in the direction of Notch3 repression. The earlier report in HeLa cells showed one miR-206 binding site in the Notch3 3'UTR (22) (Fig. 3A, site 2). In addition, we found that there is a second binding site for miR-1/206 in the Notch3 3'UTR (Fig. 3A, site 1). This new site uses central base pairing with the microRNA (31) in contrast to the traditional seed pairing found in site 2 (Fig. 3A). As recent reports have shown a difference in activity for miR-1 and miR-206 in certain cases (32), we tested whether both miR-1 and miR-206 repress the 3'UTR of Notch3 by co-transfecting the luciferase-Notch3 3'UTR constructs with plasmids expressing short hairpins that were control, miR-1 or miR-206 (Fig. 3B).

Both microRNAs effectively repressed the 3'UTR, and the repression was mediated by both sites 1 and 2 in the 3'UTR.

To show that the repression occurs at endogenous miRNA levels, the 3'UTR constructs were transfected into differentiating C2C12. The wild type 3'UTR was repressed on Day 3 of differentiation, but the 3'UTR with the miR-1/206 sites mutated was not (Fig. 3C). We created a C2C12 cell line that stably expresses an inhibitor (33) of miR-206. In both myoblasts and differentiated cultures, the levels of Notch3 mRNA were higher in the presence of the miR-206 inhibitor (Fig. 3D).

Notch3 represses miR-1 and miR-206

In multiple instances, we have found a muscle differentiation-induced microRNA represses an inhibitor of differentiation, and we also discovered that the inhibitor of differentiation directly or indirectly represses the microRNA (15). We therefore tested whether in addition to promoting its own transcription by transactivation, Notch3 could reduce the expression of miR-1/206. We first tested this by making C2C12 cell lines that stably expressed the ICD of Notch3 (N3-ICD). On the fourth day of differentiation (DM4) both miR-1 and miR-206 were significantly repressed in the C2C12 cells expressing the N3-ICD (Fig. 4A). Conversely, knockdown of Notch3 by a shRNA targeting Notch3 increased the levels of miR-1 and miR-206 several fold higher than control (Fig. 4B). Therefore Notch3 represses miR-1 and -206.

Notch3 Prevents Premature Differentiation via Inhibition of Mef2c

We next tested whether Notch3 interacts with and inhibits the myogenic transcription factors that induce the myogenic-differentiation-induced microRNAs. Activated Notch3 delayed but did not block myoblast differentiation, as evidenced by the

consistently reduced expression of the myosin heavy chain Myh3 on all days during differentiation (Supp. Fig. S3A). In contrast, the bHLH myogenic transcription factors, MyoD and myogenin were lower initially on DM1 in the C2C12 cells stably expressing N3-ICD compared to empty vector controls, but then recovered and were higher at later time points (Supp. Fig. S3B and S3C). Mef2c mRNA, on the other hand, was consistently repressed at all time points of differentiation (Fig. 5A). Mef2c protein was also significantly repressed in differentiating C2C12 by N3-ICD (Fig. 5B). These results suggested that Notch3 may delay differentiation through the inhibition of Mef2c mRNA and protein expression.

The mutual exclusivity of Notch3 and Mef2c expression is also seen from examining published microarray data of gene expression (Supp. Table S1). In C2C12 culture, it is possible to separate the differentiated myotubes from the undifferentiated reserve cells by brief treatment with diluted trypsin (34). On DM4, Notch3 mRNA was only about one-tenth the level in myotubes compared to the reserve cells, while Mef2c mRNA was nearly ten times more abundant in the myotubes compared to reserve cells (Supp. Table S1, last column). Notch1, in contrast was nearly equally abundant in the two populations of cells.

In order to ascertain whether the effect of Notch3 could be rescued by increased expression of Mef2c, we co-transfected exogenous Mef2c with N3-ICD. The myogenic activity was read by MCK luciferase activity. on DM2. By itself, the exogenous N3-ICD had a small but not statistically significant reduction on MCK activity (Fig. 5C). Addition of exogenous Mef2c greatly increased MCK activity, but was completely blocked by the addition of N3-ICD. This indicates that the activity of Mef2c is the more likely target of

inhibition rather than transcription. The decrease in Mef2c mRNA is most likely explained by Mef2c auto-activating its own promoter (35).

We thought that Mef2c could repress Notch3 via its previously characterized regulation of miR-1 (36). We transfected equal amounts of constructs expressing just the N3-ICD, the ICD and the 3'UTR, or the ICD and UTR with the miR-1/206 sites mutated. All three repressed Mef2 transcriptional activity, but the construct with the wild type 3'UTR was significantly poorer than the other two (Fig. 5D).

Notch3 Delays Differentiation

If Notch3 inhibits Mef2c, it is expected to inhibit differentiation. To test this hypothesis, we created stable C2C12 cell lines infected with lentiviral vectors that express shRNA to Notch3 or a scrambled hairpin (shSCR). These cells suppressed the transient induction of Notch3 early in differentiation (DM1 and 2, Fig. 6A). The knockdown of Notch3 induced earlier differentiation as seen from the earlier and higher expression of Myosin Heavy Chain compared to controls(Fig. 6A). Knockdown of Notch3 promoted the formation of larger, wider myotubes (Fig. 6B). Consistent with the idea that endogenous Notch3 represses Mef2c mRNA, Mef2c was induced in the cells where Notch3 was knocked down (Fig. 6C). Taken together, it appears that endogenous Notch3 causes a delay in differentiation, and that it could be important for maintaining the undifferentiated reserve cells.

The delay of differentiation was through the inhibition of Mapk p38

Although it is possible that Notch3 delays differentiation through weak activation of canonical Notch target genes (37), few of these showed any change in expression in the muscle of Notch3-/- mice (38). We considered direct binding of Notch-ICD to the Mef2c

promoter unlikely as neither Rbpj ChIP-seq nor Notch1 ChIP-seq demonstrated any binding at the Mef2c locus. Since we knew that Mef2c is inhibited by Notch3, we focused on signal transduction pathways that activate Mef2c. Mef2c is known to be activated by Map kinase p38 (39). In skeletal muscle, p38 is activated by the kinase Mkk6 (40), and antagonized by the phosphatase Mkp1 (41). Mkp1 is induced by Notch1 (41), leading us to test whether the inhibition of differentiation by Notch3 could be mediated by an inhibition of p38 Mapk. Overexpression of Notch3-ICD led to an increase in Mkp1 at both the protein (Fig. 7A), and mRNA level (Fig. 7B). If the inhibition of p38 Mapk is the primary mechanism by which N3-ICD inhibits muscle differentiation, we anticipated that an activator of p38 Mapk, Mkk6 will overcome the inhibition of muscle differentiation by N3-ICD. To follow the trans-differentiation of 10T1/2 cells into muscle by transfection of MyoD, we measured the activation of a luciferase reporter driven by the muscle creatine kinase promoter (MCK luciferase). N3-ICD repressed the MCK-luciferase (Fig. 7C). Co-transfection of increasing doses of Mkk6, the activator of p38 Mapk, overcame the inhibition of differentiation by N3-ICD (Fig. 7C). These results show that Notch3 inhibits differentiation through inhibition of p38 Mapk, which presumably leads to inhibition of Mef2c.

DISCUSSION

In this study we found that Notch 3 and Mef2c are antagonistic with each other in a closed circle that requires the action of the microRNAs miR-1 and -206 to eventually switch the equilibrium in favor of Mef2c and differentiation (Fig. 8). This is uncannily

similar to the bistable switches that we have described involving other inhibitors of differentiation such as Pax7 or MyoR and the myogenic activators such as MyoD (23,25).

Notch3 is transiently stimulated during differentiation through an enhancer at the end of intron 1 that requires the concurrent activity of MyoD and activated Notch. Notch3 inhibits differentiation of myoblasts through the inhibition of p38 Mapk. The latter enzyme is known to be important for myogenesis via phosphorylation and activation of Mef2c (26,27). Notch3, therefore, represses Mef2c activity and via its auto-activation Mef2c mRNA accumulation.

Although Notch3 may have effects through weak activation of canonical Notch target genes (28), few of these genes showed any change in the muscle of Notch3 knockout mice (29). We chose to look at p38 due to recent findings that in Rhabdomyosarcoma, Notch3 overexpression leads to a decrease in the phosphorylation of p38 (30). We chose to investigate Mkp1 because it is the only other gene to our knowledge whose expression in myoblasts is induced by both Notch1 (31) and MyoD (32). We were unable to find a significant change of Mkp1 mRNA in our shNotch3 C2C12 cells, but Mkp1 protein was clearly decreased. Notch3 has been shown to decrease proteolytic turnover of MKP1 without significantly altering mRNA in lymphoblastic leukemia (33), so it is conceivable that Notch3 stabilizes the Mkp1 protein and thus inhibits p38 Mapk. Mkp1 has already been shown to be important for proper skeletal muscle regeneration (32). Although p38 Mapk has several effects in myogenesis, we believe that in the context of Notch3, its major role is as an activator of Mef2c. Thus Notch3 inhibits Mef2c (and differentiation) by stabilizing Mkp1, inhibiting p38 Mapk and thus inhibiting Mef2c.

Since Notch3 is not inhibited by Numb, and since Notch3 clearly inhibits Mef2c and differentiation, there must be another mechanism by which Notch3 is turned off as cells proceed to differentiate. We suggest that this is one of many important roles of miR-1/206: repression of Notch3 in differentiating myoblasts. Despite the active enhancer on DM1, Notch3 levels do not continue to rise on DM2, presumably due to the appearance of the muscle-differentiation induced miRNAs miR-1/206. Beginning on Day3, Notch3 level begins to decrease due to the continued increase of miR-1 and 206 and the consequent inactivation of the enhancer because of the disappearance of active Notch. Thus the microRNAs are very important for switching the equilibrium towards loss of Notch3 and differentiation.

Mef2c can feedback and inhibit Notch3 through the induction of miR-1 (34). The regulation of miR-206 is thought to be almost entirely by the MRFs. However, Mef2c can increase the activity of the MRFs independent of its own DNA binding (35) and this could stimulate miR-206. Another pathway by which miR-1/206 could be induced during differentiation is through the activity of p38 Mapk, the very same kinase that activates Mef2c. p38 Mapk is known to activate Akt in myogenesis (36), and Akt has a significant role in controlling processing of miR-1/206 (37). The two microRNAs inhibit Notch3 by acting on its 3' UTR and thus switch the balance in favor of Mef2c and differentiation. Besides stimulating Mef2c by inhibiting Notch3, miR-1/206 can also stimulate Mef2c by inhibiting the histone deacetylase, Hdac4 (16,38). Thus there are multiple pathways by which miR-1/206, once induced, can stimulate the Mef2c activity and thus promote differentiation.

Unlike MyoD or Myogenin, which are widely expressed in differentiating cultures, Mef2c is exclusively detected in myotubes. In these cells, levels of Mef2c enhanced miR-1 (34) could force Notch3 levels below the threshold necessary to act on the Notch3 enhancer. In reserve cells, Mef2c is not expressed, and Notch3 mRNA is maintained at a high level. The combination of the miRNAs and the enhancer explains the expression pattern seen with Notch3 and Mef2c being mutually exclusive during muscle differentiation. The slower inhibition of Notch3 by miRNA repression is better suited than fast degradation by Numb to allow Notch3 to persist long enough to contribute to a lag in the induction and activation of Mef2c.

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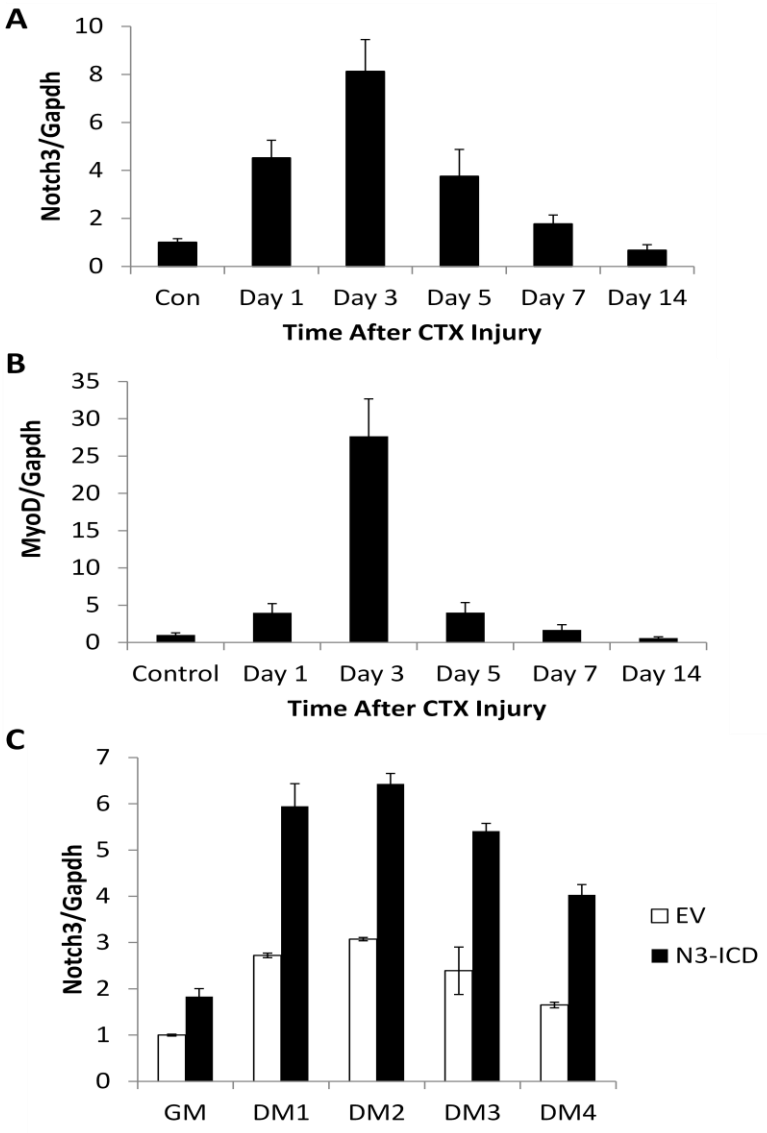


FIGURE 1: Levels of Notch3 increase and then decrease during myogenesis. (A) Levels of Notch3 mRNA from the Tibialis Anterior of mice recovering from injection of snake venom cardiotoxin. Levels measured by qRT-PCR and normalized to Gapdh transcript level, ratio in control injected animals set to 1. Average of technical triplicate, n=5, ± SEM. (B) Levels of Myod1 show a similar pattern of regulation as Notch3, details as in A. (C) The expression of Notch3 mRNA as measured by qRT-PCR also showed an initial increase before decreasing later in differentiation of C2C12 in vitro. Expression of endogenous Notch3 was increased at all time points by stable expression of exogenous N3-ICD. Values on Y-axis are mean ± SD of three measurements. GM: Growth medium. DM1-4: Days 1-4 after transfer to differentiation medium.

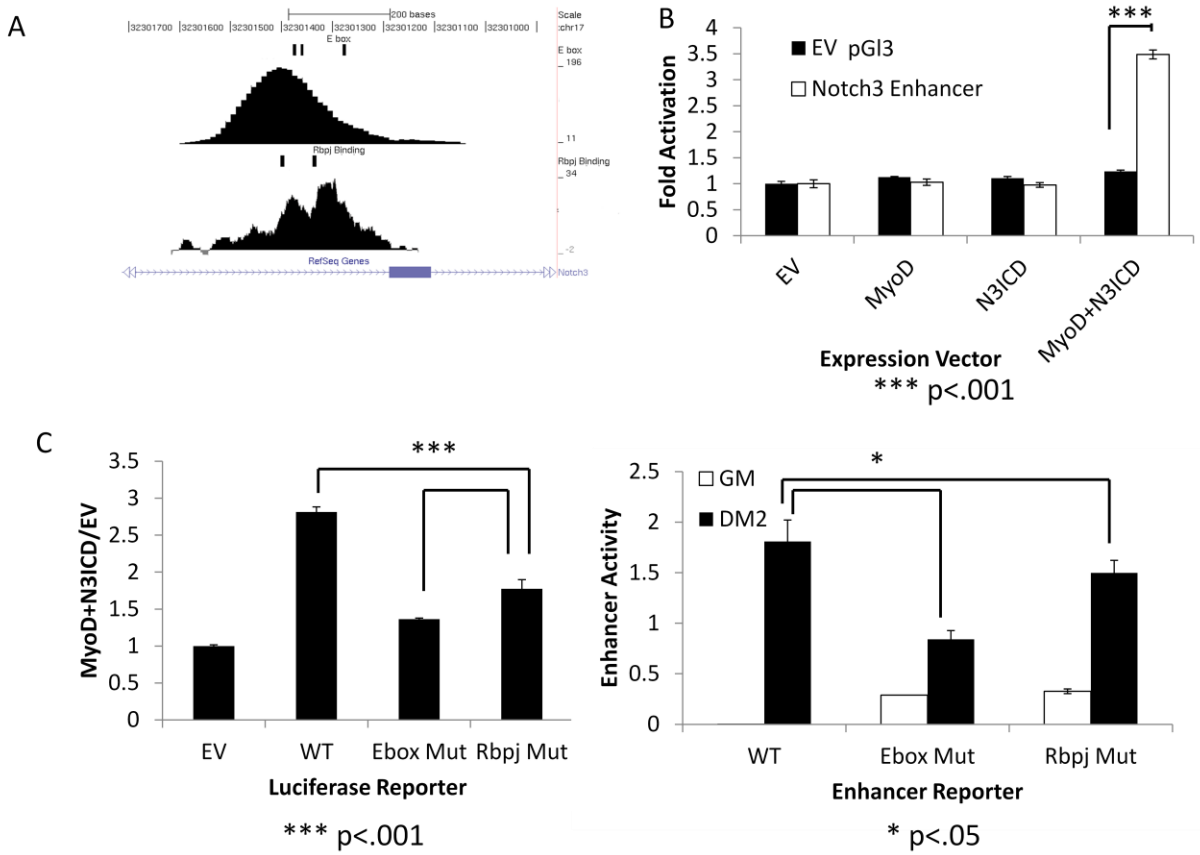


FIGURE 2: Transcription of Notch3 is controlled by an enhancer with both MyoD and Notch1 binding. (A) Top: ChIP-seq shows MyoD binding relative to end of intron 1 of the Notch3 locus in C2C12 myoblasts. The Y axis represents the number of independent sequence reads that overlap within a given 10b window in the MyoD ChIP. The primary data is from (39). The locations of canonical E-boxes are represented by black bars at the top of the graph. Bottom: ChIP-seq shows Notch1 binding to the same locus. The window size has been reduced to 1bp to show two distinct binding, seeming to correspond with the locations of two canonical Rbpj binding sequences (TGGGAA). (B) This region was cloned into an enhancerless luciferase reporter with a minimal promoter. It was co-transfected into NIH3T3 along with N3-ICD, MyoD or both. Only when both were transfected was there activation. Values were normalized to a Renilla Luciferase reporter driven by thymidine kinase (TK). Values on y-axis are mean ± SD of three measurements of the ratio of Enhancer/TK. Key: EV pGl3, Enhancerless luciferase plasmid; Notch3 Enhancer, Binding region from intron 1 of Notch3 cloned into pGl3 as an enhancer. X-axis: EV, empty pcDNA3.1; Other genes also expressed from pcDNA3.1 (C) Mutations were made in the Notch3 Enhancer plasmid of either the 3 E-boxes (Ebox mut) or the 2 Rbpj sites (Rbpj mut). The transfections were repeated as in B. The ratio of luciferase activity upon co-transfection with MyoD+N3-ICD/pcDNA Empty vector is plotted on the Y-axis. (D) The activation of the enhancer reporters in C2C12 Differentiation. Enhancer reporters were transfected into C2C12 and assayed after 24 hours in GM, or after 48 additional hours in DM. Values on y-axis are mean ± SD of three measurements of the ratio of Enhancer/TK.

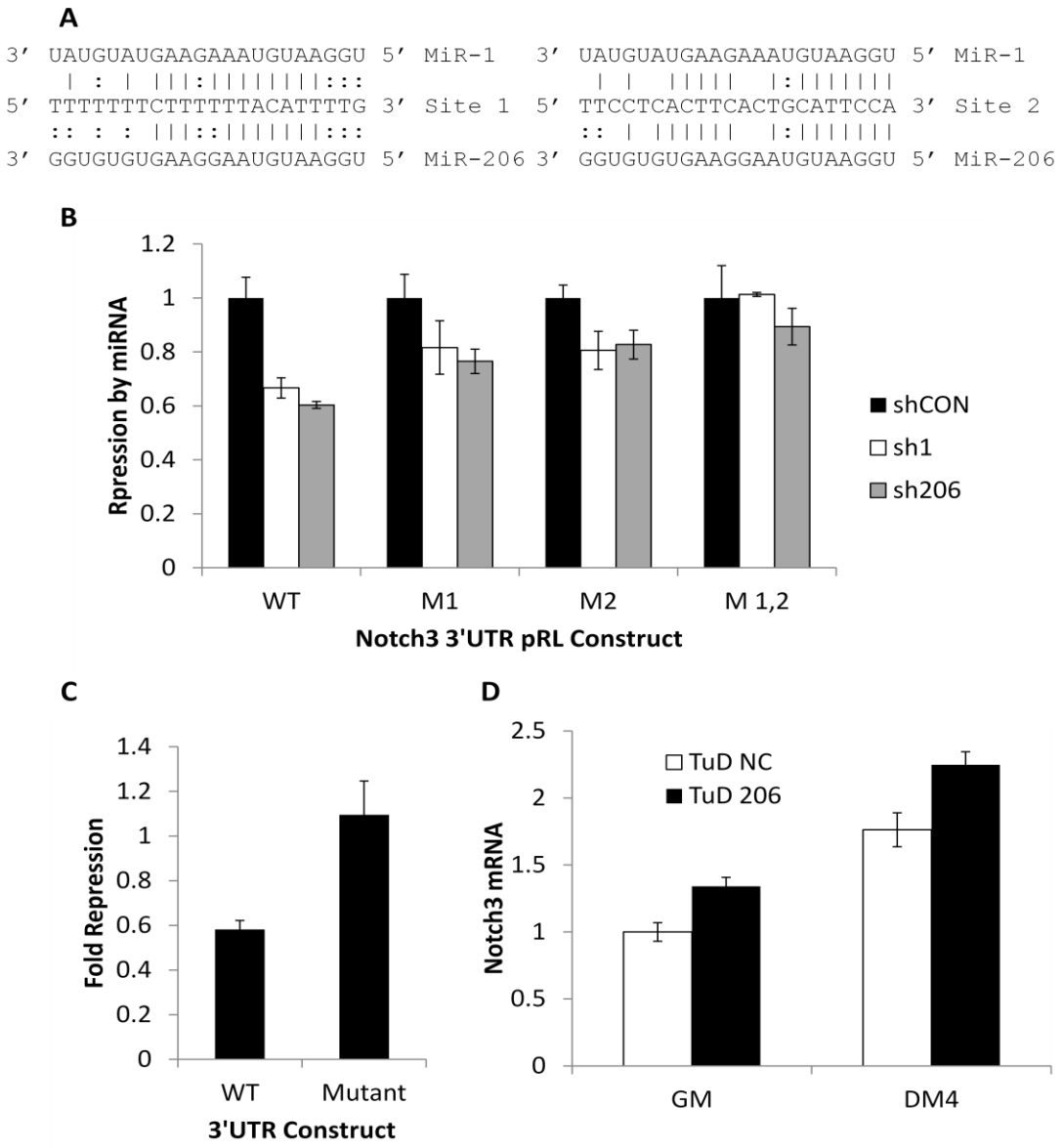


FIGURE 3: Targeting of the Notch3 3’UTR by miR-1/206. (A) miR-1 and miR-206 binding sites within the Notch3 3’UTR with complementation are represented with lines indicating complementary base pairing. (B) A renilla luciferase construct containing the Notch3 3’UTR or mutants of binding sites 1, 2, or both was transfected into into NIH3T3 cells along with constructs expressing hairpin precursors for miR-1 and miR-206 and a firefly luciferase transfection control. The results confirm that both miR-1 and miR-206 are capable of repressing the Notch3 3’UTR and that both of the target sites in the Notch3 3’UTR are utilized. Values on Y-axis represent Mean ± SD of three measurements. (C) Repression of 3’UTR constructs in differentiating C2C12. The mutant construct has both miRNA sites mutated. Cells were transfected in GM, and then allowed to differentiate for 72 hours. Values represent the average of three biological replicates of the ratio of reporter activity in DM3/GM ± SD. (D) Inhibition of miR-206 leads to higher expression of Notch3. Stable expression of an inhibitor (40) of miR-206 increases Notch3 mRNA both in GM and DM4.

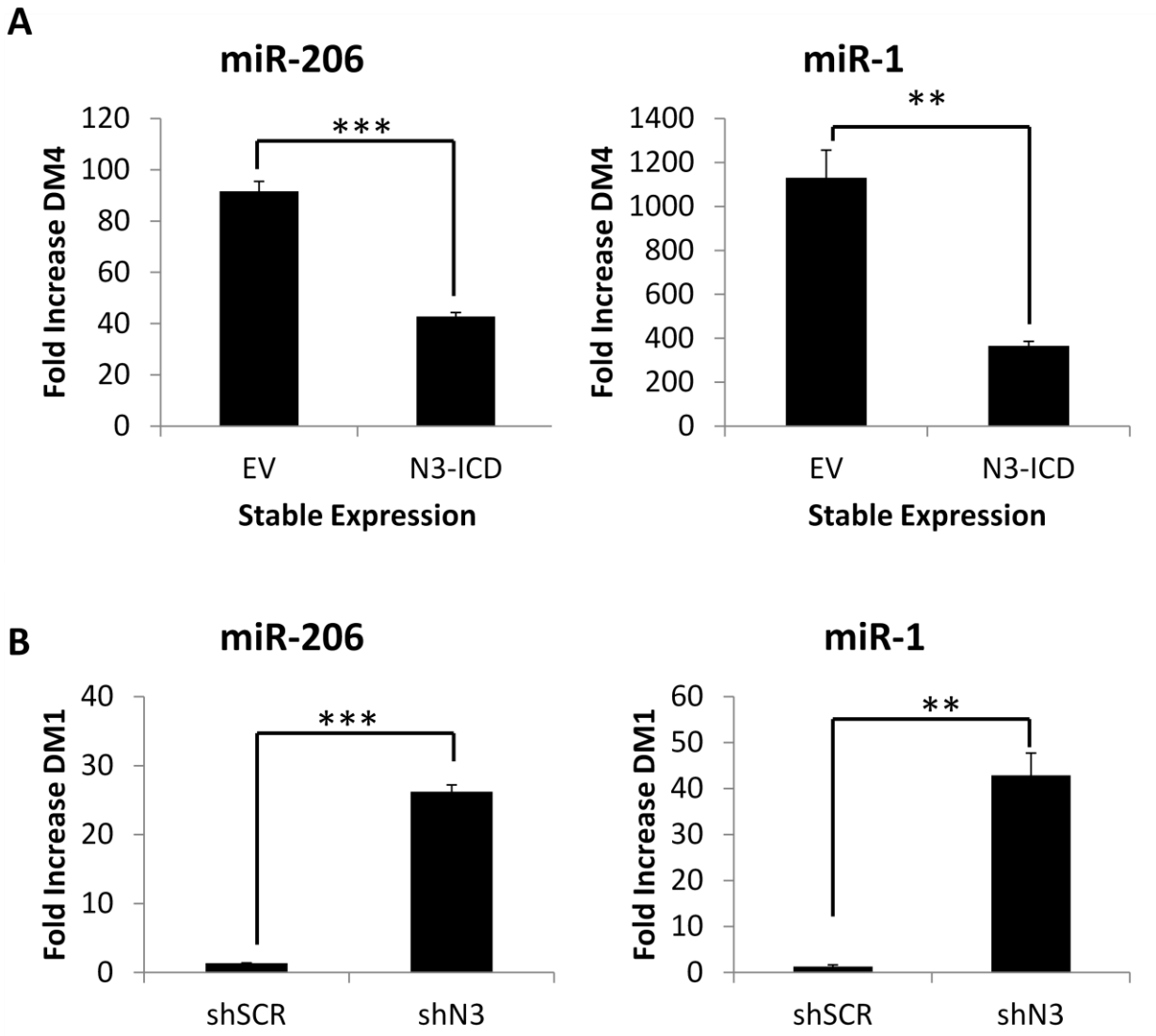


FIGURE 4: Notch3 ICD has a negative feedback on miR-1/206 levels. (A) Expression of miR-1/206 as measured by qPCR is blocked by overexpression of Notch3 ICD in C2C12 cells. Y axis represents the increase in miRNA relative to snu6 as measured by qRT-PCR from samples collected on DM4 versus GM. Values are a mean ± SD of three measurements. (B) Knockdown of Notch3 leads to precocious expression of miR-1/206 on DM1 in C2C12 cells. Cells were infected with a virus encoding either a short hairpin with scrambled sequence (shSCR) or a hairpin targeting Notch3 (shN3). Y axis represents the increase in miRNA relative to snu6 as measured by qRT-PCR from samples collected on DM1 versus GM. Values are a mean ± SD of three measurements.

Figure 5

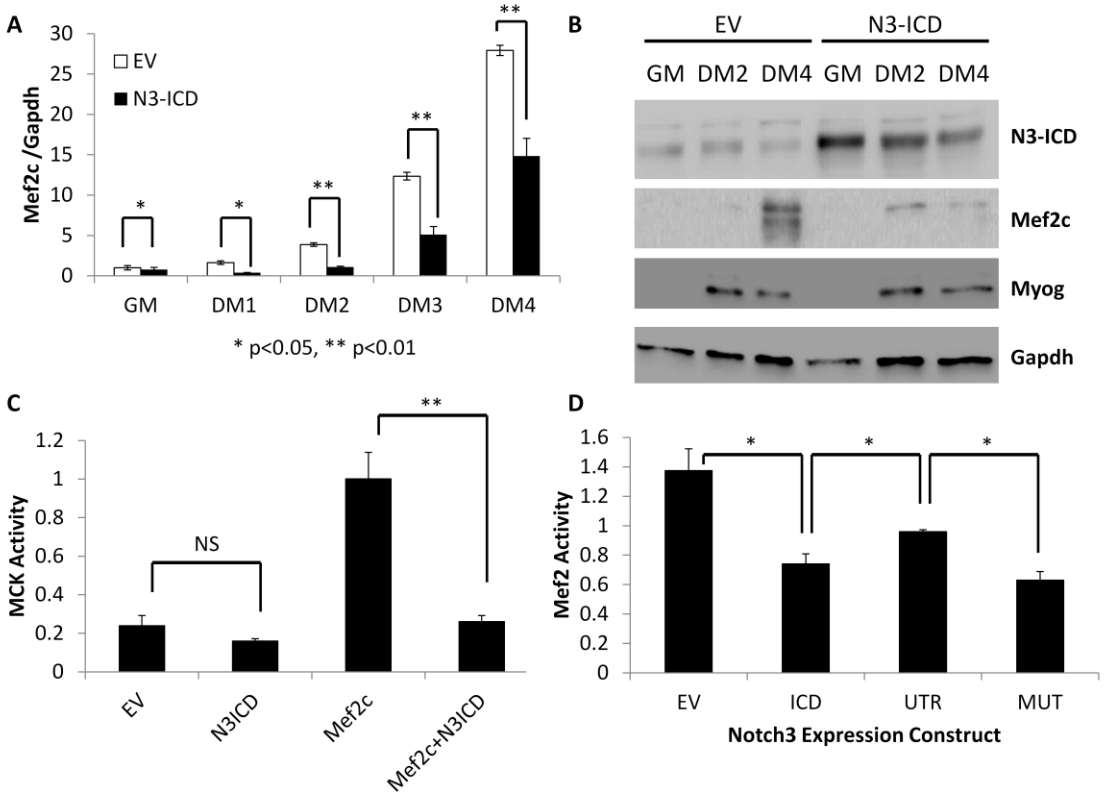


FIGURE 5: Notch3 specifically antagonizes Mef2c activity. (A) qPCR of Mef2c levels were consistently lower in N3-ICD expressing C2C12 as compared to empty vector pBabe. Conducted as in 1C. (B) Stable expression of Notch3-ICD also blocks the induction of Mef2c at the protein level in differentiating C2C12. The level of myogenin, upstream of Mef2c in the myogenic cascade, was not changed. (C) Notch3-ICD blocks increased activity by exogenous Mef2c. Activity of Mef2 was assayed by the luciferase reporter pGl3 the promoter and enhancer from muscle creatine kinase (MCK). C2C12 cells were transiently transfected with the reporter and then pcDNA3.1 plasmids encoding Mef2c and/or Notch3-ICD. Cells were switched to DM and measurements were made on DM2. The reading was normalized to TK promoter as a transfection control. Values are a mean ± SD of three measurements. (D) Mef2 can antagonize Notch3 via its 3'UTR. Activity of Mef2 was assayed by the luciferase reporter pGl3 with a multimerized Mef2 response element (41). C2C12 cells were transiently transfected with the reporter and then pcDNA3.1 plasmids encoding Notch3-ICD alone or with WT and miRNA site mutated 3'UTR. Cells were switched to DM and measurements were made on DM2. The reading was normalized to TK promoter as a transfection control. Values are a mean ± SD of three measurements.

Figure 6

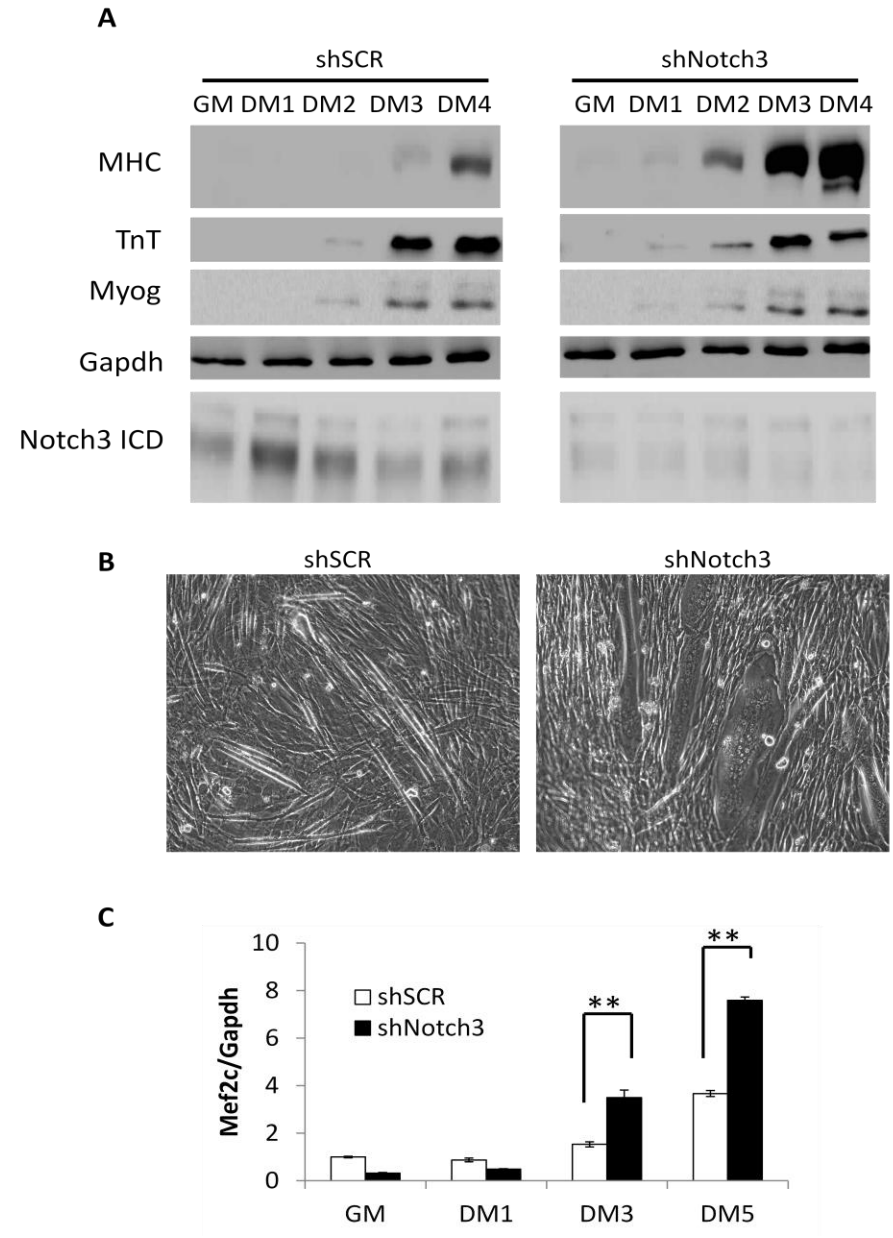


FIGURE 6: Knockdown of Notch3 accelerates differentiation. (A) Western blot showing that C2C12 with stable knockdown of Notch3 (shNotch3) leads to a consistently higher amount of Myosin Heavy Chain (MHC) across differentiation time points than cells with a hairpin scrambled control (shSCR). Earlier expression is also seen in Troponin T (TnT). However, Myogenin is not changed between the two. Images between shNotch3 and shSCR are from the same gel with central ladder spliced out. (B) Representative image showing the morphology of the shNotch3 fibers as larger and wider than control. (C) Mef2c shows a precocious increase in mRNA in the shNotch3 C2C12 as measured by qRT-PCR compared to scrambled control. Values on Y-axis are normalized to Gapdh, and are the mean ± SD of three measurements.

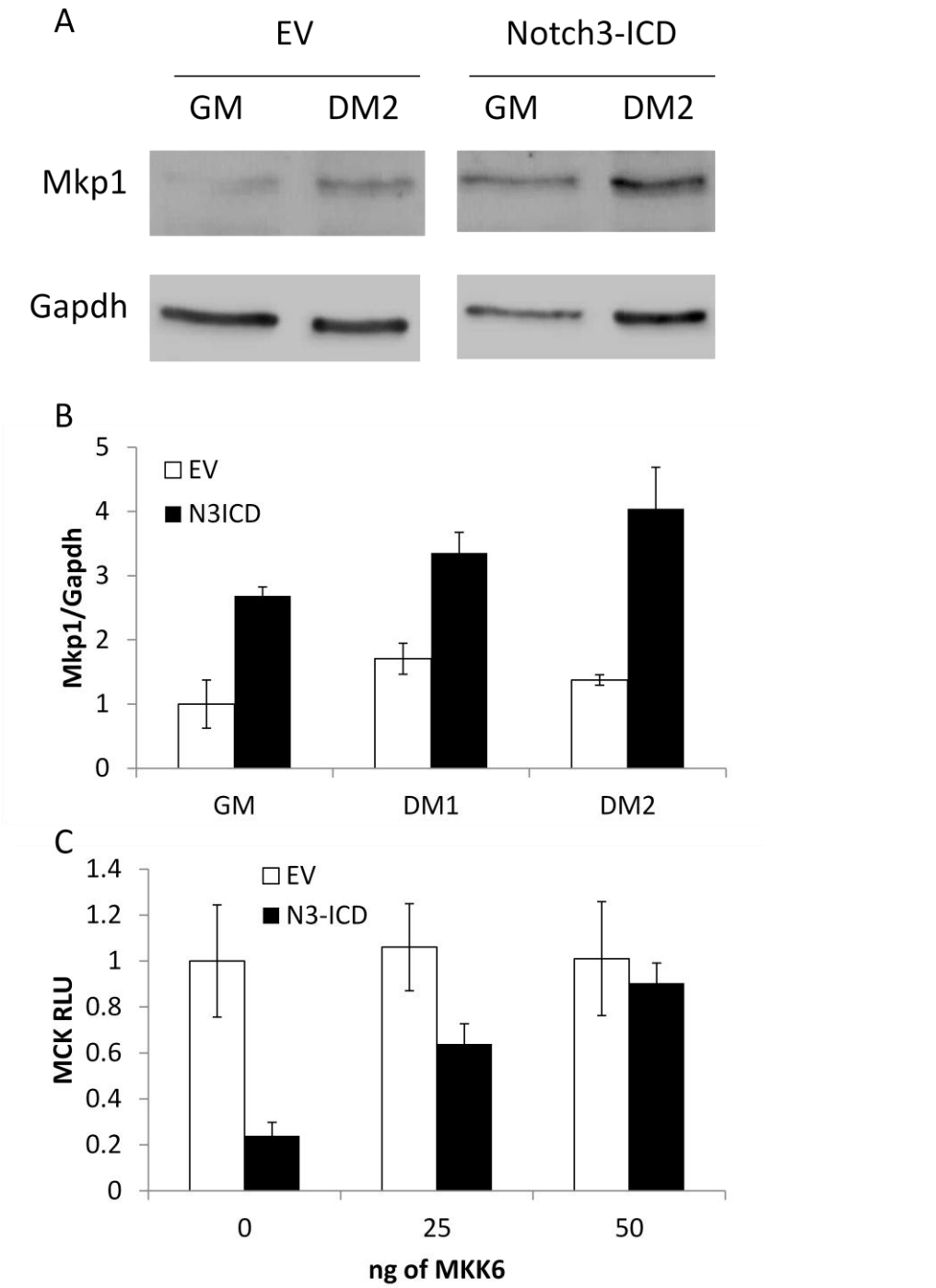


FIGURE 7: Notch3 influences Mkp1 expression. (A) Western blot showing overexpression of Notch3-ICD increased levels of Mkp1 in differentiating C2C12. Images are from the same gel with central lanes spliced out. (B) mRNA of Mkp1 was higher in Notch3-ICD expressing C2C12 cells as measured by qRT-PCR. Values on Y-axis are normalized to Gapdh, and are the mean ± SD of three measurements. (C) The ability of Notch3-ICD to block transdifferentiation of 10T1/2 fibroblasts by MyoD was overcome by expression of increasing amounts of Mkk6. Measured by MCK luciferase.

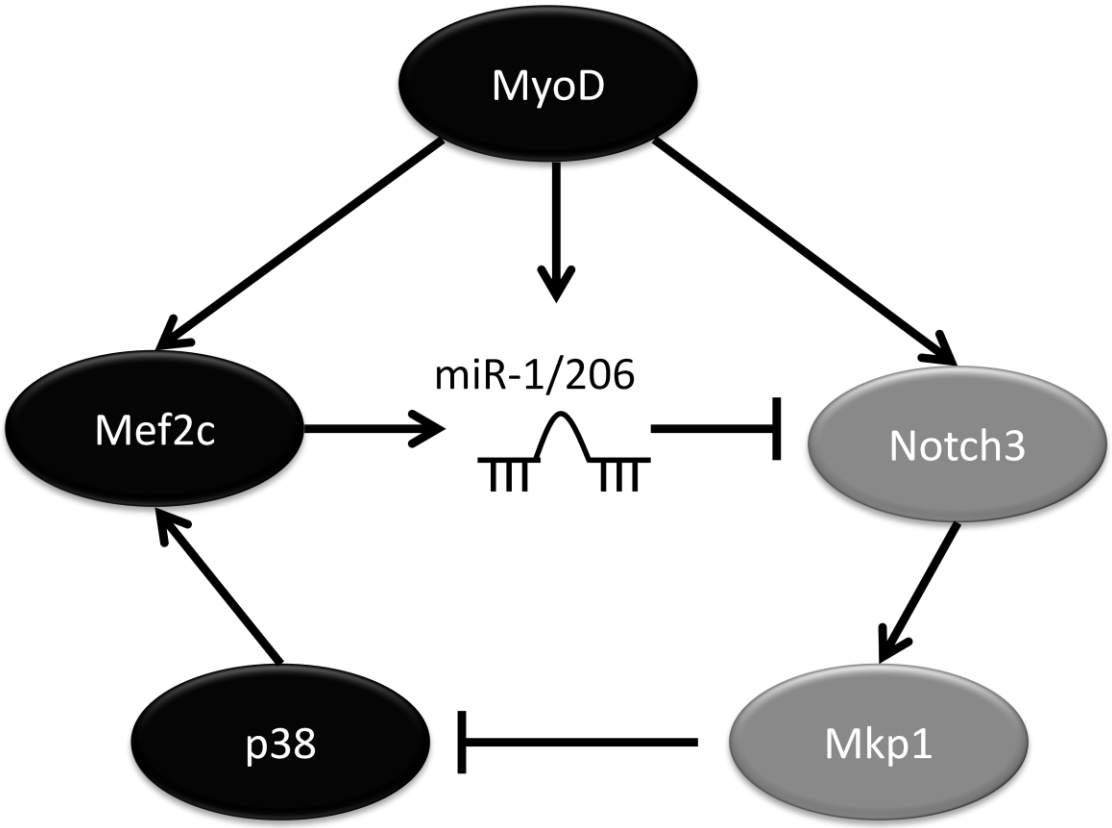
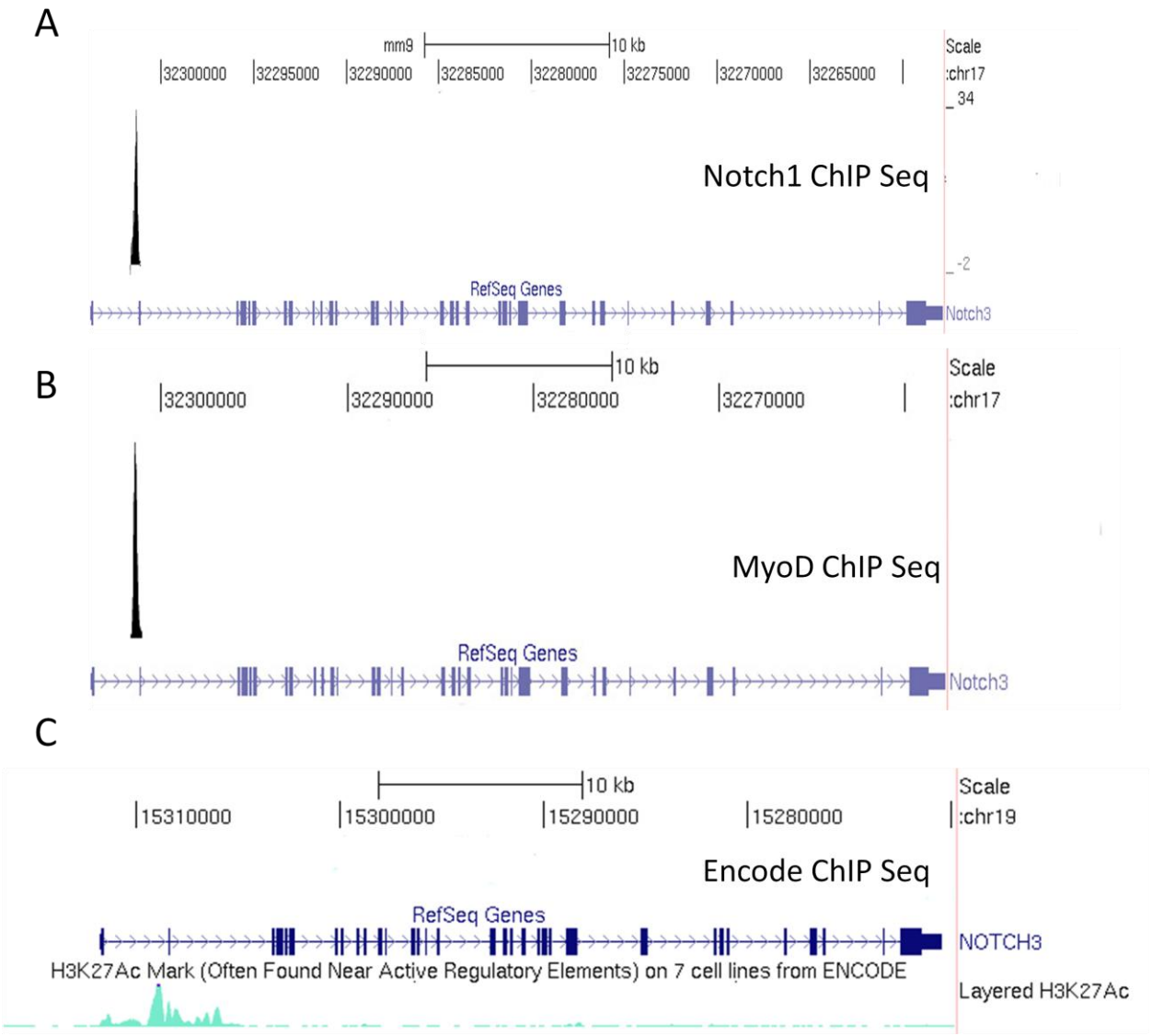
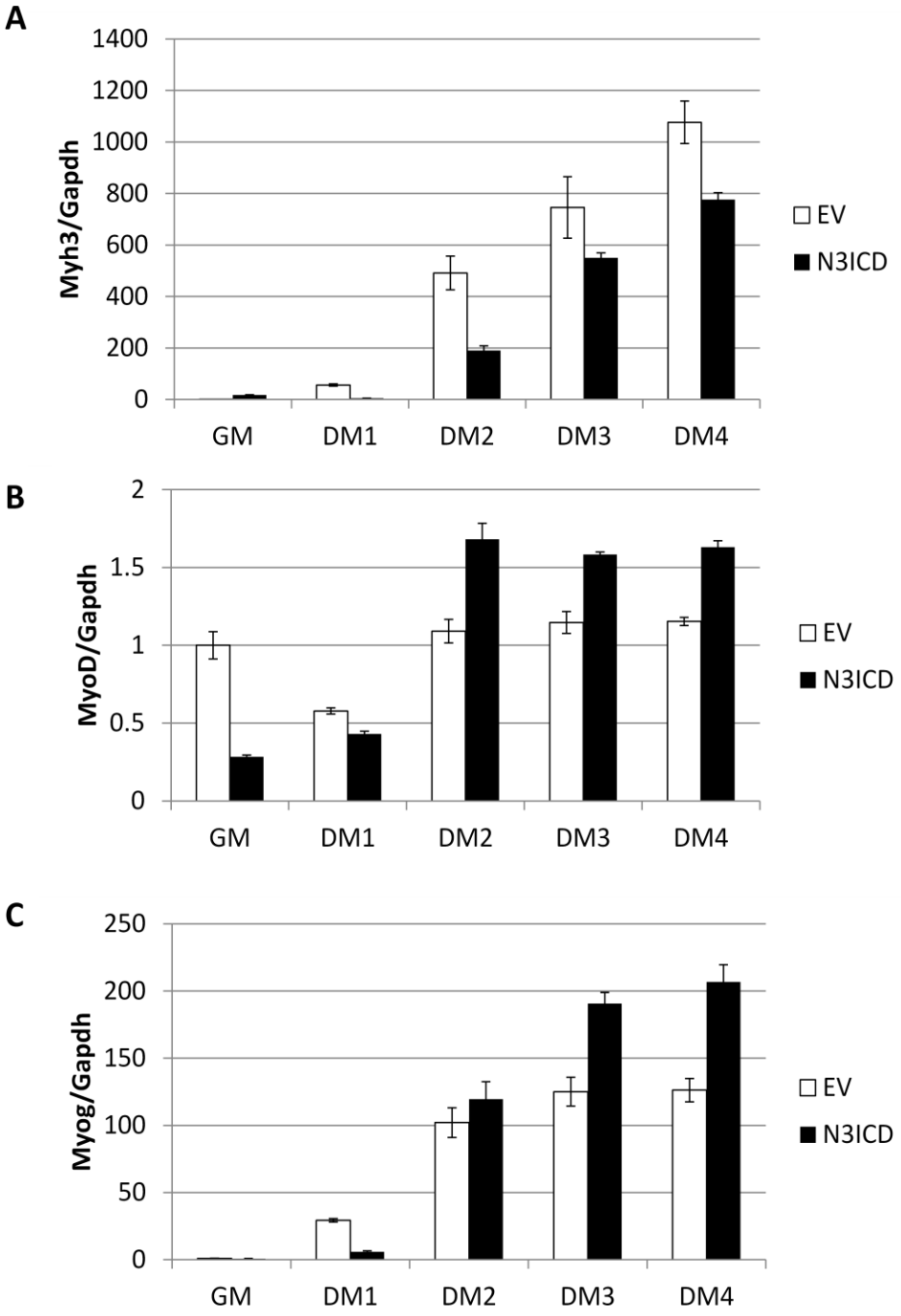


Figure 8. The working model for the feedback loop. MyoD promotes the transcription of Notch3 in conjunction with Notch-ICD. This prevents the increase in transcription of Mef2c by blocking its activity via p38. miR-1/206 eventually inhibit Notch3 and allow activation of Mef2c.



SUPPLEMENTARY FIGURE 1: The locations of Chip-seq binding peaks within the whole Notch3 locus. (A) Notch1 ChIP-seq peak described in Figure 2 zoomed out to show the entire Notch3 gene locus. (B) MyoD binding peak described from Figure 2 also showing the entire Notch3 locus. (C) Both peaks overlap with the ENCODE H3K27ac ChIP-seq from Human skeletal muscle myoblasts. The H3K27ac is thought to mark areas of active regulation. Shot captured from UCSC Genome Browser



SUPPLEMENTARY FIGURE 2: The overexpression of Notch3-ICD in C2C12 results in a delay, but not a block in the accumulation of Myosin Heavy Chain Myh3 mRNA levels. Measured by qRT-PCR and normalized to Gapdh. Mean ± SD of three measurements. fHowever, overexpression of Notch3 did not lower the mRNA levels of either Myod1 (B) or Myogenin (C). All experiments were performed as in Fig 1C.

Probe Label	Accession	GM	DM 24 HR	MT	Reserve Cell	MT/ Reserve
Notch gene homolog 3	NM_008716	391.117	1188.15	147.341	1159.81	**0.13**
Notch gene homolog 3	NM_008716	800.627	2245.24	342.916	2194.22	**0.16**
MAPK phosphatase 1	NM_013642	1055.5	1360.32	750.649	2013.29	**0.37**
Myocyte enhancer factor 2C	NM_025282	132.746	178.134	2753.1	281.752	**9.77**
Myocyte enhancer factor 2C	NM_025282	87.4229	154.299	3473.1	309.379	**11.23**
Notch gene homolog 1	NM_008714	331.796	395.991	394.185	355.131	**1.11**

SUPPLEMENTARY TABLE: Expression of Notch3 and Mef2c. Values represent hybridization intensity from Affymetrix array of C2C12 from 4 samples. Growth Media (GM), 24 hours of Differentiation Media (DM 24 HR), and samples from DM4 that have separated myotubes (MT) from Reserve Cells. The ratio of signal from Myotubes over reserve cells is shown in the last column. Mef2c is much higher in the myotubes, as expected, and Notch3 is much higher in the reserve cells. Mkp1 showed a pattern that resembled Notch3, though the fold change was less extreme. Notch1 showed almost no change between the myotubes and reserve cells. Adapted from (42).

Chapter Five: Unpublished Results

Regulation of Dll1 in Myogenesis

Abstract:

The Notch ligand Dll1 plays an important part in maintaining satellite cells. It is transiently induced during the recovery from injury. The mechanisms by which it increases during injury recovery and decreases after full healing are not known. Here we show that Dll1 is a direct transcriptional target of MyoD. We furthermore show preliminary data that the Dll1 3'UTR is repressed late in myoblast differentiation, but not by microRNA. Rather, we find that Mef2 appears to bind to and repress Dll1.

Introduction:

While the Notch pathway is been shown to be important for regulation of muscle satellite cells, the molecular mechanism is not well understood. There are 5 known Notch ligands and 4 Notch receptors. Rather than separate the effects of each many researchers block the processing of all Notch ligands by adding a gamma secretase inhibitor, which causes the premature differentiation of satellite cells (1). The regulation of Delta-like Ligand 1 (Dll1) is of interest because its expression is associated with effective recovery from injury in aged muscle (2). Satellite cells are normally quiescent in the absence of outside stimuli. When they become activated, they must asymmetrically divide. One daughter cell remains a stem cell and the other is committed to differentiating (3). The daughter cell that differentiates induces the expression of Dll1 (1). When Dll1 is knocked out in skeletal muscle by Cre-lox recombination, satellite cells are exhausted by the end of embryogenesis (4). In addition to causing activation of the Notch receptor on an adjacent cell, there are some direct effects of the Dll1 intracellular domain (5,6).

Furthermore, Delta ligand was shown to be a target for miR-1 in Drosophila (7), and the interaction was suggested in humans (8). When MyoD is overexpressed in Xenopus embryos, it induces ectopic expression of Dll1 (9). As an extension of the project on Notch3, we looked at whether we could verify and expand upon these previous findings.

Materials and Methods:

The mapping of the MyoD binding site in the Dll1 locus was as described in other chapters. Transdifferentiation of 10T1/2 fibroblasts was done by transiently transfecting 1μg of pCMV-MyoD. The DM samples were harvested 48 hours after switching the media. A 500bp putative enhancer was cloned exactly as the other enhancers were cloned in Chapter 2/3/4. The E-boxes were all mutated from CANNTG to CGNNTA. The enhancer was tested in 10T1/2 fibroblasts.

The 3' UTR was cloned and tested for responsiveness to miR-1/206 as described in Chapters 2/3/4. Putative Mef2 regulation was found manually assuming a Mef2 binding motif of TAWWWWTA (10). The identified putative binding site was TAAAAATA and mutated to TACGCATA. Repression by Mef2c was performed in C2C12 myoblasts. The 3'UTR constructs were cotransfected with a Mef2c expression plasmid. 24 hours after transfection, media was changed to DM. Samples were assayed on DM2.

The interaction with Notch3 was done with samples described in Chapter 4.

Results:

We found a MyoD enriched site in Intron 4 of the Dll1 gene (Fig. 1A). The enriched site contained 3 E-boxes all of which were conserved perfectly in all vertebrates

that we analyzed. We believe the binding site was missed in earlier studies (11) that relied on ChIP-Chip technology with arrays only probed for proximal promoters. Analysis of ENCODE data showed that there is an enrichment of H3K27ac in human fibroblasts adjacent to the binding site (Fig. 1B). This mark is thought to be enriched around active regulatory elements. When 10T1/2 fibroblasts are transfected with MyoD and put into low serum media (DM), they greatly induce the expression of Dll1 mRNA. We cloned a 500bp region around the binding site was into an enhancerless luciferase reporter. When 10T1/2 Fibroblasts are transfected with MyoD, the reporter was strongly activated (Figure 2B). When the E-boxes in the reporter were mutated, preventing MyoD binding, the reporter was not activated.

In addition, we cloned the 3'UTR of Dll1 to see if we could reproduce its repression by miR-1 (8). Like earlier studies, we were unable to find repression by dual luciferase assay. Thinking that perhaps something was wrong with my miR-1 expression construct, I instead transfected Mef2c expression vector, thinking that would activate endogenous miR-1 in C2C12. To our surprise, miR-1 still did not repress, but Mef2c did. To locate exactly where Mef2c or its downstream effectors were binding, I split the Dll1 3'UTR. The majority of the repression appeared to come from the 3' half (Figure 3A). This was surprising because most of the predicted miRNA sites were in the 5' half. The only motif I could discern was a putative direct Mef2 binding site. When it is transfected into C2C12, there is a repression of the Dll1 3'UTR by some endogenous mechanism (Figure 3B). Mutating the single putative binding site prevents most of the in C2C12 differentiation.

As Dll1 was shown to be activated by MyoD and repressed by Mef2c, we hypothesized that Notch3 would alter Dll1 mRNA levels. Notch3 has relatively little effect on MyoD, but inhibits Mef2c (Chapter IV). Therefore, overexpression of Notch3 should increase Dll1 late in differentiation. In fact, Dll1 was significantly higher in N3-ICD expressing C2C12 than control on DM2-4 (Fig. 4A). When Notch3 was knocked down by shRNA, the levels were higher at DM1, but failed to have increase further on DM3 and DM5 (Fig. 4B). We speculate that this is due to premature activity of Mef2c.

Discussion:

The induction of Dll1 is important for asymmetric division of satellite cells (1), presumably due to the requirement that the differentiating daughter signal back to the other daughter to remain a stem cell. Therefore, it follows logically why Dll1 would be activated by MRFs. In aged muscle, Dll1 is not activated in response to injury (2). This inability to properly activate Notch signaling leads to poorer wound healing in aged muscle, but can be restored by an antibody that causes Notch receptor cleavage in the absence of ligand (2).

However, constitutive activation of Notch is not a desirable result in quiescent muscle, so there would have to be a mechanism to repress Dll1 transcription. While we originally hoped it would be a microRNA, the second half of the Dll1 3'UTR is A/T rich and lacks and predicted miRNA seed sequences that could plausibly explain its expression pattern. Although Mef2c is thought of as a transcription factor is also known to form inhibitory complexes with Histone Deacetylase proteins (12,13). Further studies could try to confirm the presence of Mef2 or Hdac proteins at this locus. As these results

were not compatible with microRNA regulation, they were not pursued actively, but the regulation of Dll1 during myogenesis is important to understand.

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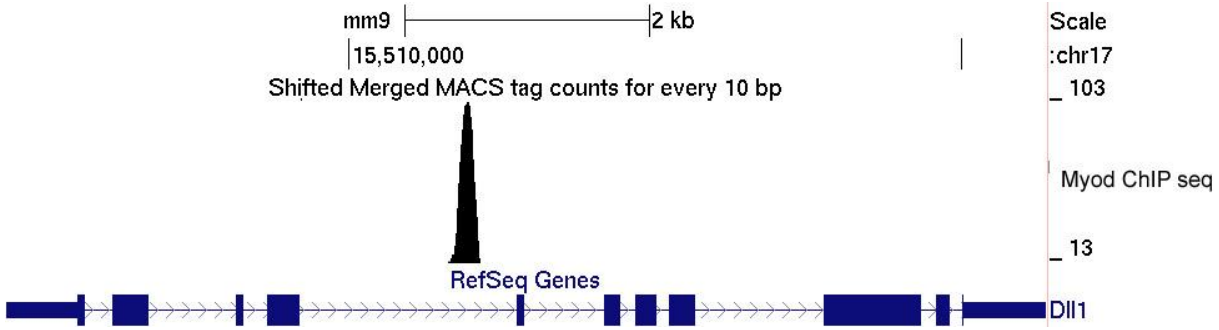
Figures

A



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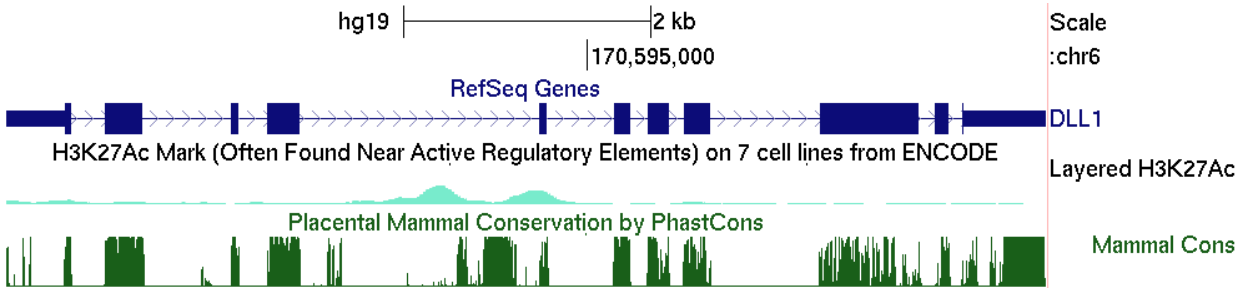
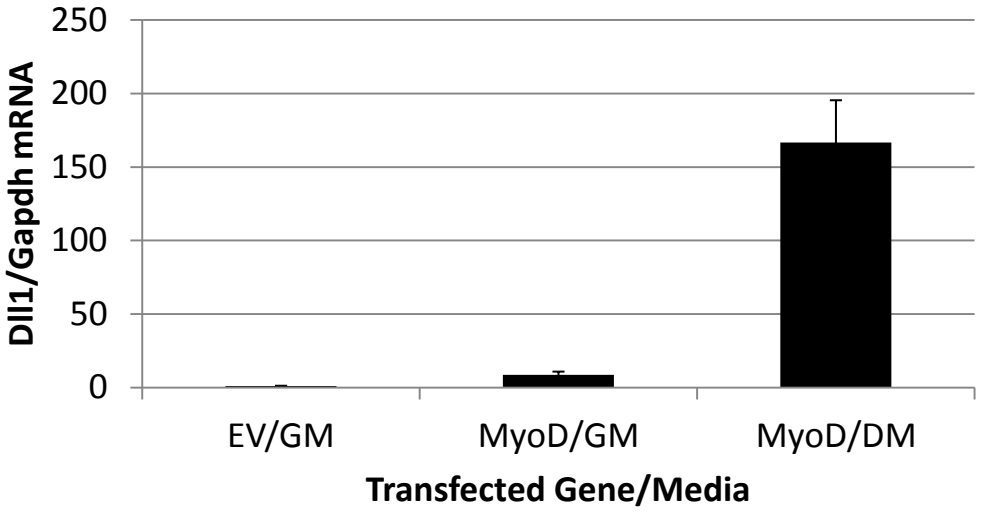


Figure 1. The MyoD binding site in Intron 4 of Dll1. (A) ChIP-seq of MyoD shows a large enrichemtn of reads in intron 4. This was present both in Myoblasts and Myotubes. (B) ENCODE consortium data shows that there is an enrichment of K3K27ac, a marker associated with enhancers, in human skeletal muscle myoblasts at the same locus.

A



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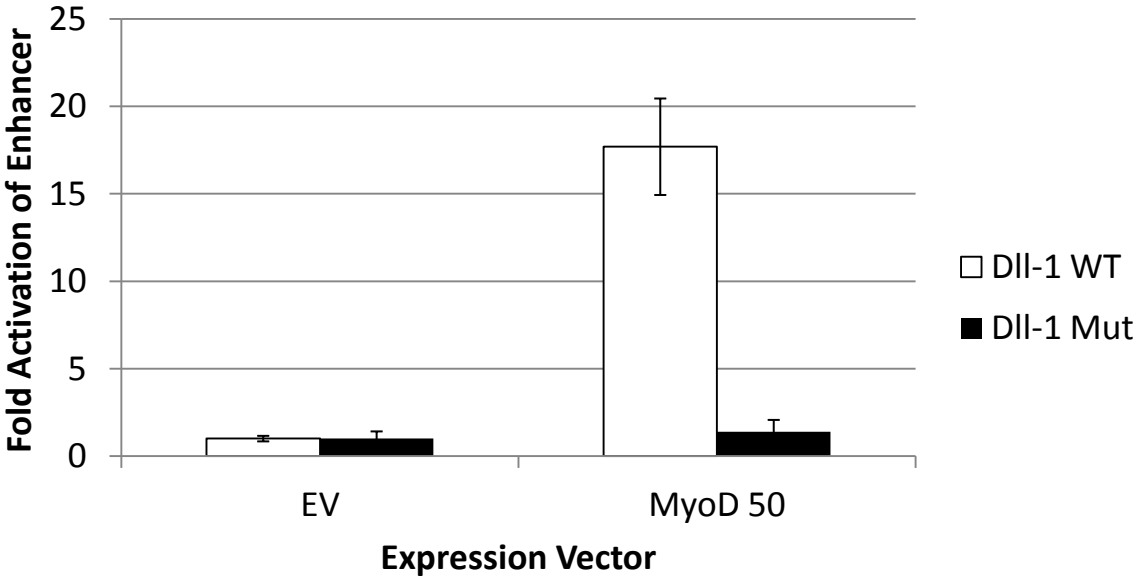
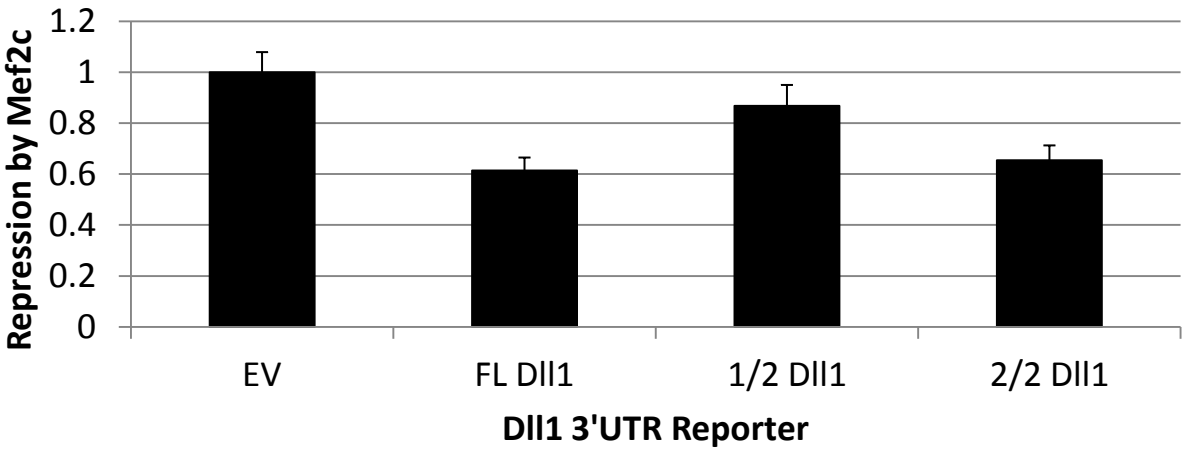


Figure 2: Response of Dll1 Enhancer to MyoD. (A) 10T1/2 Fibroblasts were transiently transfected with MyoD and RNA was harvested in normal growth media (GM) and low serum differentiation media (DM). RNA was assayed by qRT-PCR, and normalized to Gapdh. The levels on the y-axis are with the empty vector (EV) control set to 1. (B) A 500bp region encompassing the MyoD binding site was cloned into an enhancerless luciferase reporter. The E-boxes were mutated to prevent MyoD binding in

the Mutant construct. The reporters were transfected into 10T1/2 fibroblasts along with a MyoD expression vector (50 ng).

A



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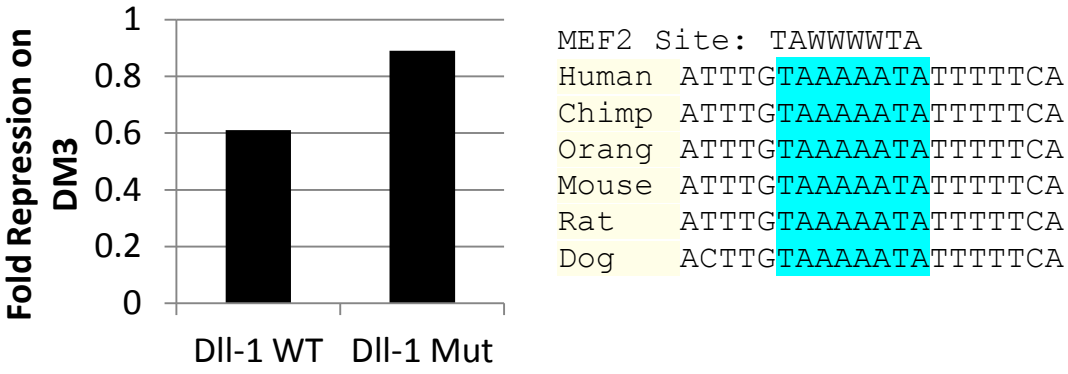

Figure 3. Mef2c Represses the Dll1 3'UTR. (A) Addition of exogenous Mef2c repressed the full length 3'UTR of Dll1 in 10T1/2 fibroblasts. In order to identify the likely mechanism, the UTR was split in half. and the majority of the repression was found to reside in the second half. (B) A putative direct binding site was found in the second half of the Dll1 3'UTR. When the wild type UTR is transfected, it is repressed by day 3 of C2C12 differentiation. When the putative Mef2 site was mutated, the repression was ameliorated.

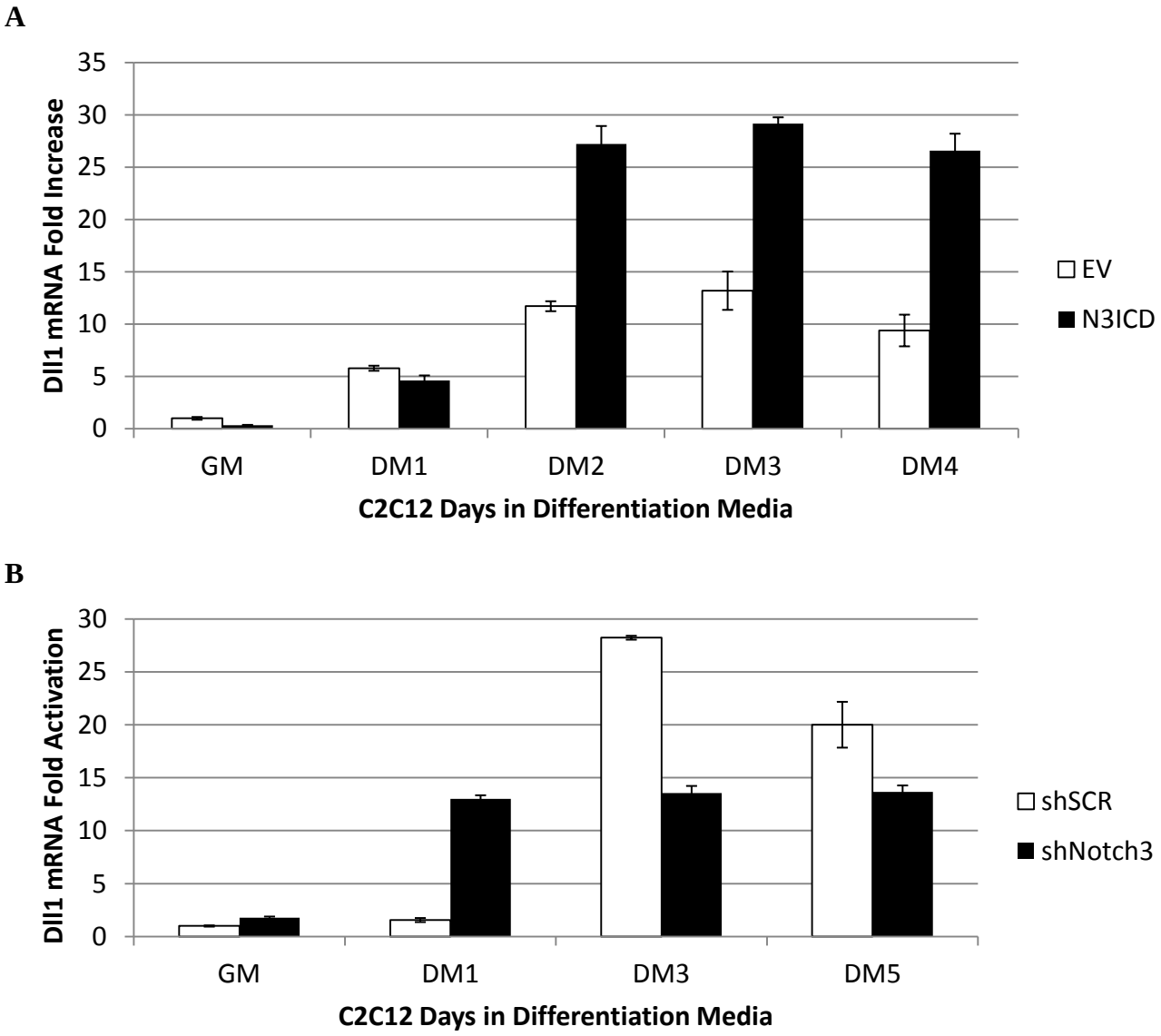

Figure 4. The effect of Notch3 on Dll1. (A) Overexpression of the Notch3 ICD in C2C12 myoblasts resulted in higher expression of Dll1 at later time points. RNA was assayed by qRT-PCR, and normalized to Gapdh. The levels on the y-axis are with the empty vector (EV) control in GM set to 1. (B) Knockdown of Notch3 by shRNA also altered Dll1 mRNA levels. Although initially lower, they failed to reach the proper peak of expression on DM3.

Chapter 6: Discussion and Future Plans

The work here describes how microRNA regulates the transition from myoblast to myotube. The choice of this differentiation system for our project was made for several reasons. The genetics of myogenesis were well established prior to this work, and we wanted to avoid having to decipher both the biology of miRNA and starting from scratch on the target genes. Skeletal muscle already had a robust cell culture model of differentiation as well as accessible in vivo models. Lastly, the pathogenesis of chronic injury diseases such as Duchene Muscular Dystrophy makes understanding skeletal muscle regeneration clinically important.

A. Use in therapy

The regulation of Pax7 by microRNA could turn out to be very important for the overall behavior of cells used in therapy. A recent paper showed that a subpopulation of satellite cells with higher expression of Pax7 has a low metabolic state, is transcriptionally less primed for myogenic commitment, and takes longer to execute the first mitosis after being stimulated to enter the cell cycle (1). These cells also undergo asymmetric distribution of parental DNA, meaning that Pax7 could be a master regulator of stem cell behavior. However, the paper fails to address whether the higher expression of Pax7 is a driver or an effect or another regulator of satellite cells. If miR-206 and miR-486 are critical for altering the state to one that is inhibitory to Pax7 and in favor of differentiation, modification by microRNA inhibitors could be useful for improving clinical efficacy by sustaining a pool of Pax7+ stem cells.

The regulation of MyoR by miR-378 has fewer obvious implications, as both genes are not well studied. MyoR has been shown to be critical for the embryonic

formation of the muscles of mastication, but not those further posterior (2). Furthermore, it has been implicated as one of the mechanisms by which rhabdomyosarcomas resist differentiation (3). The host gene for miR-378, Pgc-1β, has an important role in specifying skeletal muscle fiber type (4). It has furthermore been shown to be controlled by the alternative NF-κB pathway (5). Therefore stimulation of NF-κB pathways to stimulate miR-378 production in RMS could induce differentiation and promote therapy. Of course, since miR-378 expression also requires MyoD activity, the integratation of multiple pathways, such as NF-κB and MyoD, will be an interesting challenge in future works that exploit this knowledge for therapy.

Although the role of the Notch signaling pathway as a whole has been well established for satellite cell behavior (6), the regulation of individual Notch components remains poorly understood. There are four Notch receptors and five ligands, yet we don't have a very good idea of their contributions relative to one another. Our demonstration that Notch3 is upregulated in differentiating myoblasts adds on to previous work that only focused on how it contributes to fate decisions in satellite cells (7). Furthermore, most work previously focused on proteolytic degradation of Notch receptors (8,9), not the slower regulation of miRNA. A transient stimulation of Notch3 levels by inhibiting miR-206, will only pause differentiation by repressing Mef2c, without completely block myogenic commitment. Such a transient manipulation of Notch3 levels may be very useful for directing donor cells to the proper state for cell therapy.

There are a number of directions that logically follow this work both within and apart from skeletal muscle genetics.

B. Alterations in Developmental versus Adult versus Aged Networks

There is mounting evidence that the process of myogenesis is not the same during development and in adult regeneration. Pax7 is critical for maintaining satellite cells through the neonatal period, but apparently dispensable in mature adults (10). Myogenin was shown to be critical for muscle fusion in the developing embryo (11,12), but inducible recombination based knockout of Myogenesis in adults showed mice with histologically normal muscle (13). This disparity in the regulatory networks and rate-limiting nodes needs to be properly understood because they may explain why diseases like DMD are typically not diagnosed until approximately 5 years of age.

The changes that occur during aging that inhibit proper skeletal muscle repair are not well understood and may underlie age-related sarcopenia. If a shunt is constructed between a young mouse and an old mouse, the young serum seems to be sufficient to reinvigorate the old satellite cells (14). This suggests that old satellite cells possess the capacity to function, but either young serum contains a necessary growth signal or elderly serum has an inhibitory signal. If these results are correct, this would add another layer of difficulty for the use of cell therapy in the elderly utilizing myogenic progenitors. It may prove more useful to treat with cells that would properly condition the serum by removing the inhibitor present in elderly serum. This does not obviate the necessity of understanding the transcriptional networks governing myogenesis but reinforces the need to be carefully examine biological outputs that integrate various inputs that are cell-autonomous, or are from the serum and the surrounding tissue microenvironment.

C. Further Utilization of High Throughput Data to Produce More Complete Networks of Regulation

In our work, we made liberal use of a ChIP-seq data set for MyoD (15), as well as Notch1 (16). This data allowed us to make hypotheses on transcriptional targets more easily than we could by traditional methods of sequence analysis for binding sites of transcriptional factors and ChIP-PCR specifically directed to potential promoters. Previous efforts to analyze MyoD binding made use of ChIP-Chip (17), but this technique is restricted to the probes that are put on the array and was therefore limited to discovering MyoD sites proximal in promoters of known genes. The genome wide survey done by ChIP-seq has allowed us to discover newly identified transcriptional targets of MyoD including miR-378, miR-486 and Notch3. All of these featured intronic binding sites that were not covered in the promoter-specific arrays of the previous generation of genome-wide ChIP studies.

Already, I have helped initiate a project that used ChIP-seq to identify potential long non-coding RNAs. By integrating histone marks associated with transcription (18) and RNA-seq (19) during C2C12 differentiation, we have found several novel putative non-coding transcripts. This project is now being headed by Adam Mueller and Magdalena Wegrzynska. However, ChIP-seq studies of transcription factors give a number of genomic binding sites in vast excess of their direct target genes (20). Like many high throughput methodologies it does not obviate traditional targeted biochemical approaches. In addition, we must find a way to integrate sets of different factors. While in our data, MyoD and Notch bind to the exact same locus in Notch3, this is likely to be an unusual case. Furthermore, we must begin to take additional steps in vivo. In

collaboration with Dr. Bijan Dey, we have been experimenting with delivering miRNA inhibitors to skeletal muscle via adeno-associated viruses. Delivery of miRNA by these episomal viruses may be a major tool for manipulating microRNAs in satellite cells.

Recent studies have published ChIP-seq data sets for Pax3 and Pax7 (21), opening up a new opportunity to examine how Pax3 and Pax7 restrain MRFs in the stem-cell like satellite cells and how microRNA regulate this activity. Although our data show that Id proteins are critical for the inhibition of MyoD by Pax7, this is unlikely to account for all of the inhibition. Intersecting Pax regulation with MyoD will also give more insight into how the transition from quiescent to activated satellite cells is regulated. A recent publication implicated miR-489 as being important for keeping quiescence satellite cells in the quiescent state (22). It will be interesting to examine the genome-wide datasets discussed above with microRNA target prediction data to determine whether miR-489 promotes satellite cell quiescence by supporting Pax7 activity. It will also be useful to integrate knowledge of the chromatin modifications at different stages of myogenesis with the transcription factor binding. One of the primary ways MyoD is distinct from Myogenin is that only MyoD can interact with histone acetyltransferases (23).

Bioinformatic predictions of miRNA-mRNA binding have been very useful, and were used for every part of this work. However, they are weakened by a high number of false positives (24). In addition, investigators looking at those lists are restricted to looking at known genes, and are more likely to follow up on targets whose biological properties have already been studied. This bias has been identified in a number of fields (25), and in the future we must attempt to purge our *a priori* assumptions of which targets are "interesting." There are efforts underway to sample the entire spectrum of miRNA

bound targets by cross-linking RNA to the miRNA effector protein Ago2 and performing high throughput sequencing (26). This type of data allows us to delineate true microRNA targets before we ever do an experiment. If a target site for a relevant miRNA is conserved across species, it is likely to be a method for identifying novel myogenic regulators. The paradigm of studying one microRNA and identifying one or two targets in a given study is frankly too slow. According to miRBase, there are currently 741 miRNA precursors and 1157 mature miRNAs identified in mouse, and more in humans. If the goal is to construct a model of myogenesis that includes a robust yet perturbable network, the nodes and edges connecting microRNAs and transcription factors need to be identified in a shorter time frame.

D. Other Steps in Myogenesis Subject to MicroRNA Mediated Regulation

In this work we have chosen to focus on how microRNAs target direct regulators of the myogenic transcription factors. We also performed most of our work on myoblasts in culture. This creates a homogenous environment for our cells, but we know that in vivo regulation of satellite cells is much more complex. There are a large number of signaling pathways that affect stem cell function. Notch signaling is important for proper muscle repair (27), and it is antagonized by TGF-beta family members like BMP (28). BMP ligands (29), including myostatin (30), are negative regulators of satellite cell differentiation. Wnt signaling has been implicated for being a driver in age-related inhibition of myogenesis (31). All of these pathways utilize many different genes, and represent a large number of possible miRNA target genes. There is increasing evidence that microRNAs target multiple steps in a given pathway (32).

There are other steps involved in myogenesis besides the myoblast to myotube transition. While some microRNAs may fluctuate in multiple steps of myogenesis, it is more likely that there are distinct sets of microRNA and target genes. Other studies have begun, such as the transition from muscle stem cell to myoblast (33). The microRNAs with the largest change in expression are completely different than those that change in the transitions studied here (34). Satellite cells are not homogeneous, and their molecular signature depends on whether they are quiescent or actively dividing (35). When the processing enzyme Dicer is deleted by conditional knockout in satellite cells, they enter the cell cycle and are at an increased risk for apoptosis (22). The authors chose to focus on only one particular gene, miR-489. It was shown to be critical for maintaining quiescence, its expression is lost in active satellite cells and all further differentiation. Clearly there is significant work to be done to understand how microRNAs regulate the step of satellite cell activation.

Similarly, further analysis could be performed on embryonic lineage specification. Embryonic stem cells (ESC) have the potential to form every somatic tissue, making them wonderful candidates for cell therapy. In a step that circumvents possible immune incompatibility, somatic cells have also been reprogrammed towards an ES cell like state, known as induced pluripotent stem cells (iPSC) (36). However, ESCs do not efficiently differentiate to the skeletal muscle lineage (37). Differentiation of myocytes within a stem cell culture known as an embryoid body can be increased by adding exogenous MyoD (38). Those cells would be too differentiated to be clinically useful. More recently Pax3 (39) and Pax7 (40) have been used to drive mouse ESCs and iPSCs towards the myogenic lineage. Human stem cell lines have can also be induced

towards myogenic differentiation with exogenous expression of Pax7 (41). Importantly, in a mouse model of Duchenne Muscular Dystrophy, the human cells were shown to have integrated into the satellite cell niche. However, having Pax7 integrated into the genome by retrovirus makes the cells incompatible with direct clinical application. Rather we should look to transient medications that are less likely to cause unwanted effects such as tumorigenesis. MicroRNAs serve an important role in maintaining pluripotency of embryonic stem (ES) cells (42), as well as in the differentiation of mesoderm (43), ectoderm (44), or endoderm (45). Already miR-1 has been shown to promote the differentiation of cardiomyocytes from ES cells (43), therefore it should be possible to also find microRNAs that promote skeletal myogenesis.

In each of these examples of cell fate transitions, while a large number of microRNAs were identified by sequencing or microarray, only a few were followed up in detail. While this is useful as a resource for future work, what is really required is a fully developed network. The expression of microRNAs must be tied to their controlling transcription factors and the set of target genes for each microRNA must be more complete. With the proper synthesis of systems biology, genetics and biochemistry, future work with predictions of myogenic behavior made in silico could allow for much more rapid advances in the use of cell therapies in vivo.

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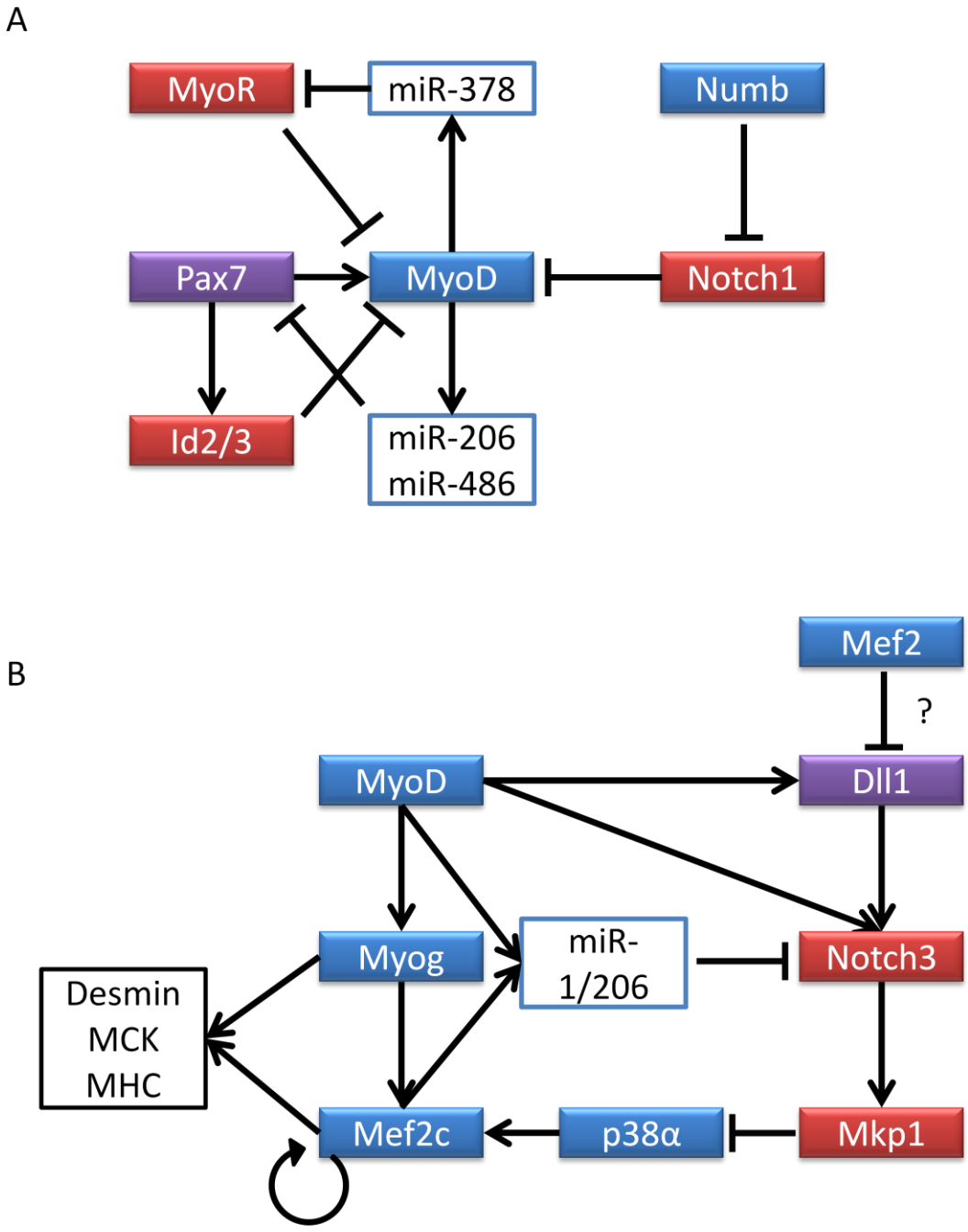

Figure 1. Summary of Interactions. (A) The interactions governing the activation of early myoblast differentiation. Other signaling pathways not described in this dissertation also will affect the balance between Pax7 and MyoD. The eventual output is induction of Myogenin and its downstream effects, which are detailed in (B). The network governing late myoblast differentiation eventually leads to the structural proteins such as Desmin, creatine kinase (MCK) and muscle myosin heavy chain (MHC).