Concise Review: Epigenetic Regulation of Myogenesis in Health and Disease

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ABSTRACT

Skeletal muscle regeneration is initiated by satellite cells, a population of adult stem cells that reside in the muscle tissue. The ability of satellite cells to self-renew and to differentiate into the muscle lineage is under transcriptional and epigenetic control. Satellite cells are characterized by an open and permissive chromatin state. The transcription factor Pax7 is necessary for satellite cell function. Pax7 is a nodal factor regulating the expression of genes associated with satellite cell growth and proliferation, while preventing differentiation. Pax7 recruits chromatin modifiers to DNA to induce expression of specific target genes involved in myogenic commitment following asymmetric division of muscle stem cells. Emerging evidence suggests that replacement of canonical histones with histone variants is an important regulatory mechanism controlling the ability of satellite cells and myoblasts to differentiate. Differentiation into the muscle lineage is associated with a global gene repression characterized by a decrease in histone acetylation with an increase in repressive histone marks. However, genes important for differentiation are upregulated by the specific action of histone acetyltransferases and other chromatin modifiers, in combination with several transcription factors, including MyoD and Mef2. Treatment with histone deacetylase (HDAC) inhibitors enhances muscle regeneration and is considered as a therapeutic approach in the treatment of muscular dystrophy. This review describes the recent findings on epigenetic regulation in satellite stem cells and committed myoblasts. The potential of epigenetic drugs, such as HDAC inhibitors, as well as their molecular mechanism of action in muscle cells, will be addressed. STEM CELLS TRANSLATIONAL MEDICINE *2016;5:282–290*

SIGNIFICANCE

This review summarizes recent findings concerning the epigenetic regulation of satellite cells in skeletal muscle.

INTRODUCTION

Skeletal muscle tissue is mainly composed of post-mitotic, multinucleated muscle fibers. Upon disease or injury, skeletal muscle has the remarkable ability to regenerate. The regenerative capacity of skeletal muscle relies on a subpopulation of satellite cells, termed satellite stem cells, that function as muscle stem cells [1]. Satellite stem cells reside between the basal lamina and the sarcolemma of the muscle fiber. Study of satellite stem cell self-renewal and differentiation has revealed the importance of the environment and the cellular interactions during homeostasis and regeneration [1]. In addition, a combination of specific transcription factors and epigenetic regulators are critical in the determination of satellite cell identity and functions, which is discussed in this review.

Epigenetics involve the regulation of gene expression without altering the primary DNA nucleotide sequence. Epigenetic modifications include nucleosome positioning, histone post-translational modifications, and DNA methylation. Although those modifications can be heritable, changes in the epigenetic status are usually a dynamic and reversible process. Studying satellite cell epigenetic regulation is a technical challenge for two main reasons. First, satellite stem cells represent a relatively rare population, and their prospective isolation yields small cell numbers. Therefore, classical molecular biology techniques are inefficient because they necessitate large numbers of cells. Second, stem cells reside in a specific environment or niche required to maintain their quiescence. Thus, isolation of quiescent satellite cells from their niche, amplifying them in culture, or using myogenic cell lines introduces a proliferation artifact that may not represent the intrinsic biology of muscle stem cells. Nevertheless, multiple in vitro studies have provided useful information about the epigenetic regulation of myogenesis. Recent findings indicate that epigenetic drugs can

favor myogenic differentiation and ameliorate the muscle phenotype of dystrophic mice. Of particular interest, the use of histone deacetylase (HDAC) inhibitors has been suggested to be a potential therapeutic candidate in the treatment of muscular dystrophy.

This review discusses recent discoveries in the epigenetic regulation of satellite stem cells and myogenesis. The potential use of epigenetic drugs in the treatment of muscular dystrophies is also addressed.

EPIGENETIC STATUS OF SATELLITE CELLS AND MYOBLASTS

Satellite cells are the adult muscle stem cells responsible for muscle regeneration upon injury or disease. After activation, satellite stem cells enter the cell cycle and can generate both self-renewing stem cells and progenitor daughter cells committed to muscle differentiation, a process achieved through asymmetric cell division [2–4]. Committed muscle cells, or myoblasts, are highly proliferative and can differentiate and fuse into multinucleated cells, or myotubes, that finally mature into muscle fibers. In vitro, primary myoblasts can proliferate over multiple passages, and they can also be induced to differentiate into myotubes [5]. Therefore, many epigenetics studies have been performed in primary myoblasts. Here, we summarize the recent findings on the epigenetic status of satellite cells and committed myoblasts.

Chromatin Status in Satellite Cells

One key feature of stem cells is the poised chromatin state of genes involved in lineage progression [6]. Indeed, genes controlling differentiation programs are repressed in stem cells, but they also harbor active histone marks keeping them primed to be expressed upon differentiation cues [6]. These genes possess a distinct epigenetic signature combining both active (methylation of histone H3 lysine 4 [H3K4me3]) and repressive marks (methylation of histone H3 lysine 27, H3K27me3), called bivalent histone modifications [7]. Nearly half of the bivalent genes in embryonic stem cells are also marked by bivalent domains in quiescent satellite cells [8]. When satellite cells are activated, these genes remain bivalent and are not or are barely transcribed, consistent with the concept of a poised transcriptional state [8]. In myoblasts, a majority of the genes resolve to a monovalent state, but a small subset of genes still possesses bivalent histone marks at their promoters [9]. This group of genes encodes mostly developmental regulators and genes associated with neuronal differentiation, as well as transcription factors. Surprisingly, most of these genes keep their bivalent histone marks after differentiation.

Another characteristic of stem cells is their chromatin state, which is less compact and more permissive for transcription [6]. Accordingly, it has been established that quiescent satellite cells possess only a small subset of genes marked with the repressive H3K27me3 mark, whereas the permissive H3K4me3 mark is present on half of the annotated genes [8]. Upon activation, many genes gain the repressive H3K27me3 mark (without losing the H3K4me3 mark), leading to their repression. In agreement with these observations, the expression of Ezh2, the methyltransferase responsible for the deposition of the repressive H3K27me3 mark, is increased when satellite cells become activated [8, 10]. Consistently, $Ezh2^{-/-}$ satellite cells have impaired proliferation and differentiation [10, 11]. Taken together, these results suggest that upon activation, satellite cells do not lose their bivalent chromatin state. Rather, they are subjected to an increase in the

number of bivalent genes by the addition of the repressive H3K27me3 mark on genes that are rapidly downregulated at the transcriptional level [8]. When cells commit into the myoblast stage, the bivalence of the chromatin state is mostly resolved.

Transcriptional Regulation of Satellite Cells

Pax7 is a master transcriptional regulator of satellite cells. In $Pax7^{-/-}$ mice, satellite cells are completely absent, resulting in muscle atrophy and ultimately to death [12, 13]. Pax7 is critical for cell cycle progression of satellite cells and myoblasts [12]. In agreement with these observations, Pax7 chromatin immunoprecipitation sequencing in primary myoblasts revealed that Pax7 regulates target genes involved in cell growth and proliferation [14]. In addition, Pax7 represses genes important for muscle differentiation [14]. One well-studied Pax7 target gene is the myogenic regulatory factor (MRF) Myf5 [15]. In quiescent satellite cells, Myf5 is the only MRF expressed at the protein level. Pax7 activates Myf5 expression via different binding sites located at the −57.5 kilobase (kb), −111 kb, and −129 kb enhancers, relative to the Myf5 transcriptional start site. Whereas the −111 kb enhancer drives the expression of Myf5 in quiescent satellite cells, the −57.5 kb enhancer is more related to Myf5 expression in activated satellite cells and proliferating myoblasts [14, 16–18].

Pax7 recruits the Trithorax complex, composed of Ash2l, Wdr5, Rbbp5, and MLL1/2 on Myf5 regulatory sequences, through direct interaction with MLL1/2 [15, 19]. The Trithorax complex possesses methyltransferase activity and specifically methylates histone H3 lysine 4 (H3K4). In agreement with these findings, the Myf5 gene harbors the active H3K4me3 mark in quiescent satellite cells as well as in primary myoblasts [8, 15]. Pax7 itself is methylated in the amino terminus by the action of the arginine methyltransferase Carm1 [19] (Fig. 1). Arginine methylation, as lysine methylation, regulates many cellular processes by modulating protein-protein interactions as well as protein function (reviewed in [20]). The methylation of Pax7 by Carm1 is required for the recruitment of MLL1/2 and the Trithorax complex to the Myf5 promoter. Disruption of Carm1 levels using small interfering RNA in muscle fibers dramatically impairs the capacity of satellite stem cells to perform asymmetric cell division [19]. In a normal asymmetric cell division, one daughter cell retains the stem cell potential and never expresses Myf5, whereas the other daughter cell is more committed and expresses Myf5 [4]. Carm1 interacts with Pax7 specifically in the committed daughter cell, leading to Myf5 expression. Carm1 depletion results in a decrease of Myf5 expression, a defect in asymmetric cell division, and an impaired muscle regeneration capacity [19].

Epigenetic Regulation of Myogenic Transcription Factors

Muscle differentiation is orchestrated by the sequential activation of MRFs: Myf5, MyoD, myogenin (MYOG) , and Myf6. In quiescent satellite cells, Myf5 and MyoD transcripts are detected [8]. Pax7, Myf5, and Myod1 genes possess the active H3K4me3 mark in quiescent satellite cells. In contrast, Myog and Myf6 promoters are devoid of the active H3K4me3 or the repressive H3K27me3 marks [8]. In activated satellite cells, the Myog promoter gains the active H3K4me3 mark, as well as other genes whose function is associated with muscle contraction [8]. However, these genes are not expressed at the protein level in activated satellite cells. This suggests that upon satellite cell activation, the transcriptional
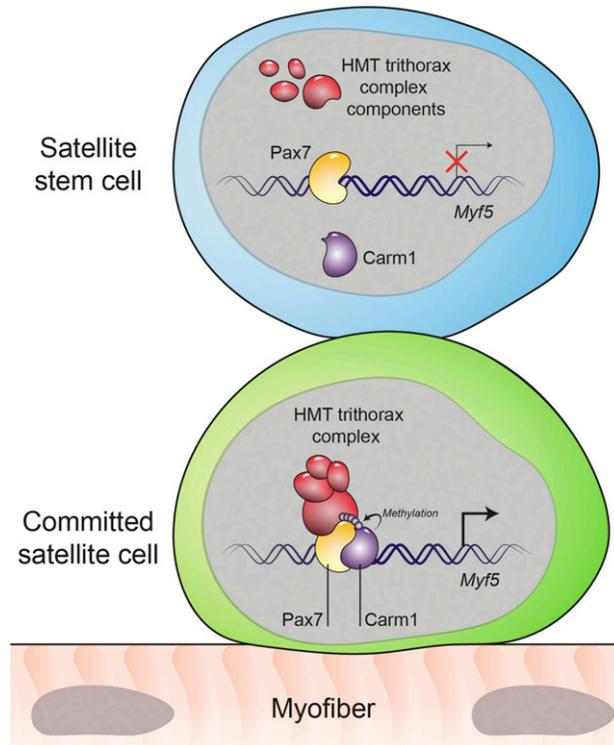
Figure 1. Carm1 regulates Pax7 transcriptional activity. Satellite stem cells (top) express high levels of Pax7 whereas transcription of Myf5 is repressed. Carm1 binding to Pax7 is inhibited in satellite stem cells to maintain the stem cell state. In committed satellite cells (bottom), expression of Myf5 requires the post-translational activation of Pax7. The protein arginine methyltransferase Carm1 binds and methylates Pax7. Arginine methylation of Pax7 allows the recruitment of the Trithorax complex, encompassing MLL1/2, ASH2L, WDR5, and RBBP5 proteins, at the *Myf5* locus, followed by transcription of Myf5 and myogenic commitment.

machinery primes the satellite cell for myogenic differentiation. In quiescent satellite cells, the protein expression of Myf5 is prevented by sequestration of Myf5 mRNA in messenger ribonucleoprotein (mRNP) granules, as well as by the action of microRNA-31, which blocks Myf5 translation [21]. After satellite cell activation, mRNP granules are disassembled, leading to Myf5 protein expression [21]. The protein expression of MyoD is also prevented in quiescent satellite cells by the action of tristetraprolin, a protein mediating the decay of MyoD mRNA [22]. During satellite cell activation, signaling by p38 α leads to inactivation of tristetraprolin and stabilization of MyoD mRNA [22]. Therefore, although in quiescent satellite cells Myf5 and MyoD translation is not detected, their protein expression becomes abundant after satellite cell activation.

Histone Acetylation in Satellite Cells

The genome-wide status of histone acetylation (associated with an active chromatin state) has not been studied so far in quiescent or activated satellite cells. Studies in C2C12 myoblasts suggest that histone acetylation is globally higher in the undifferentiated state, compared with cells induced to differentiate into myotubes [9]. However, an increase in histone H4 acetylation is observed during differentiation specifically on MyoD target genes, suggesting that histone acetylation is increased in a subset of genes important for myogenic differentiation [23].

CHROMATIN REMODELING UPON DIFFERENTIATION CUES

Role of Polycomb Repressive Complex 2 in Myogenic Differentiation

When myoblasts are induced to differentiate into myotubes, the expression of genes that prevent the differentiation program must be decreased. Gene repression is mediated in part by the polycomb repressive complex 2, comprising the histone H3K27 methyltransferase Ezh2 [24]. One example is the *Pax7* promoter, which presents increased levels of H3K27 trimethylation and reduced levels of expression when cells are induced to differentiate [25]. Of interest, Ezh2 is phosphorylated by the promyogenic kinase p38 α. This phosphorylation is important to repress Pax7 expression upon differentiation [25].

Although Ezh2 is required to repress the expression of certain genes during myogenesis, Ezh2 expression decreases progressively upon differentiation cues, and overexpression of Ezh2 prevents terminal muscle differentiation [26]. This suggests that Ezh2 expression must be turned off to enable proper myogenesis. Ezh2 expression is also decreased by the action of specific microRNAs (miRNAs) that are expressed when cells are induced to differentiate [27, 28].

In undifferentiated myoblasts, Ezh2 is present on the regulatory regions of genes required for muscle differentiation (*Myh10*, *Ckm*, *Myog*), thus preventing their expression [26, 29]. When myoblasts initiate differentiation, Ezh2 leaves the promoters of muscle-specific genes. The histone demethylase ubiquitously transcribed tetratricopeptide repeat X chromosome (UTX, Kdm6a) is recruited to these promoters to remove the methylation mark on histone H3 lysine 27 [26, 29]. In parallel, the combined action of JMJD2A (Kdm4a) and JMJD2C (Kdm4c) allows the removal of the repressive H3K9 methylation mark deposited by Suv39h1 at the myoblast stage [30].

Activation of Gene Transcription During Myogenesis and Role of MyoD

Other chromatin modifiers that deposit histone marks associated with active gene transcription are also recruited on muscle-specific genes. For example, the Ash2l/Wdr5/Mll2 (Trithorax) methyltransferase complex occupies the *Myog* and *Ckm* regulatory regions during myogenesis, and this complex deposits the H3K4me3 mark associated with active gene transcription [31]. The Trithorax complex is recruited via its interaction with the transcription factor MEF2D, phosphorylated by the promyogenic kinase p38 α [31, 32].

One master regulator of myogenic differentiation is the basic helix-loop-helix transcription factor MyoD [33]. MyoD regulates the expression of muscle-specific genes following the induction of differentiation. Thus, MyoD expression is low in undifferentiated myoblasts, and its expression is increased early during the differentiation process. One mechanism by which MyoD expression is repressed in myoblasts is the presence of the homeodomain repressor Msx1 on the *Myod1* locus [34, 35]. Msx1 interacts with the repressive histone methyltransferase G9a (Ehmt2) to repress MyoD expression via deposition of the repressive H3K9me2 mark [35]. G9a is required for the inhibition of myogenic differentiation by Msx1 [35]. During differentiation, the expression of the histone demethylase Lsd1 (Kdm1a) is increased, leading to the removal of the H3K9me2 mark on muscle-specific promoters [36]. Histone acetyltransferases (HAT), namely p300, are recruited at the *Myod1*

regulatory elements upon differentiation, and the increase of histone acetylation correlates with MyoD expression [37].

In undifferentiated myoblasts, MyoD is present on the chromatin of only a small subset of its target genes. MyoD is excluded from the promoters/enhancers of muscle-specific genes by the presence of the repressor Snai1, which prevents MyoD binding and recruits HDAC1/2 [38]. Overexpression of Snai1/2 prevents myogenic differentiation, whereas Snai1/2 knockdown using small interfering RNAs leads to precocious differentiation of myoblasts. During differentiation, the expression of Snai1/2 is down-regulated by the action of specific miRNAs, allowing MyoD to reach its target genes [38]. Other groups detected the presence of HDACs on the regulatory elements of muscle-specific genes in undifferentiated myoblasts, some of which were also occupied by MyoD. For example, HDAC1 is present on *Myog*, *Myh10*, and *Ckm* promoters in myoblasts [26, 39, 40], whereas HDAC2 is detected on the *Ckm* and *Des* (desmin) loci [41]. HDACs also associate with Suv39h1 and Hp1 (Cbx5) to repress MyoD and Mef2 target genes in undifferentiated myoblasts [42–44].

Upon differentiation, HDACs leave the promoters of muscle-specific genes, allowing the recruitment of transcription factors (MyoD, Mef2, Myogenin), which in turn recruit chromatin-remodeling complexes (i.e., (SWItch/sucrose nonfermentable [SWI/SNF] complex, HATs), leading to active transcription [39–41, 45]. There are multiple mechanisms by which HDACs leave muscle-specific promoters upon induction of differentiation: their decreased expression, their nuclear export, and a redistribution to other binding proteins and promoters. For example, HDAC1 is bound to MyoD in myoblasts, whereas its expression is reduced during differentiation [46]. Furthermore, the MyoD-HDAC1 complex is disassembled during differentiation, coinciding with the formation of a pRb-HDAC1 complex in myotubes [46]. In addition, the interaction between class II HDACs and Hp1 observed in myoblasts is disrupted following promyogenic signaling mediated by calcium/calmodulin-dependent protein kinase (CaMK), which allows the recruitment of histone acetyltransferases on muscle-specific gene promoters [44, 47]. Moreover, HDAC4 and HDAC5 are direct phosphorylation targets of CaMK, leading to their export from the nucleus upon differentiation signals [47].

Nucleosome Positioning

In addition to changes in histone post-translational modifications, changes in the DNA methylation status and in nucleosome positioning on different genomic loci regulate gene expression during muscle differentiation. When myoblasts differentiate into myotubes, a subset of genes display changes in their DNA methylation status, most of them becoming hypomethylated [48]. The loss of methylation is normally associated with active gene expression. This observation correlates with pioneer experiments showing that treatment of fibroblasts with 5-azacytidine, an inhibitor of DNA methylation, leads to spontaneous transdifferentiation into myotubes [49]. Chromatin remodeling is also important for myogenesis. For example, initiation of the myogenic program by MyoD is abrogated in the absence of a functional SWI/SNF chromatin-remodeling complex [50]. The promyogenic kinase p38 binds chromatin at the *Myog* locus during muscle differentiation and is important for the recruitment of the SWI/SNF complex to this gene, leading to myogenin expression [51]. Interestingly, p38 directly phosphorylates BAF60c (Smarcd3), a structural component of the SWI/SNF complex that interacts with MyoD [51, 52]. MyoD and BAF60c are present on the *Myog* promoter at the

myoblast stage, whereas Brg1 (Smarca4), the ATPase subunit of the SWI/SNF chromatin-remodeling complex, is recruited only when cells are induced to differentiate [52]. Both MyoD and myogenin have the capacity to recruit chromatin-remodeling complexes to their target genes [40, 41, 53]. In addition, the histone arginine methyltransferases Prmt5 and Carm1 are also involved in the recruitment of the SWI/SNF complex at different gene loci (*Myog, Ckm, Des*) [54, 55]. During differentiation, higher-order chromatin structure is also detected, in which muscle-specific gene promoters, located on different chromosomes, lie in close proximity [56]. These long-range chromatin interactions require MyoD as well as Brg1 [56].

Histones Variants in Myogenesis

Emerging evidence also suggests that the replacement of canonical histones with histone variants could influence gene expression and regulate muscle differentiation (Fig. 2). For example, the homeoprotein Msx1 interacts specifically with the histone variant H1b, and both are found on the *Myod1* promoter in undifferentiated C2C12 myoblasts [34]. This interaction represses *Myod1* gene expression, leading to the inhibition of myogenesis (Fig. 2A) [34]. In contrast, histone H3.3 is recruited at the *Myod1* promoter upon differentiation induction [57–59] and H3.3 is associated with active transcription. Both histone H3.3, Asf1, and histone cell cycle regulator (HIRA), the histone chaperones responsible for H3.3 deposition at *Myod1* regulatory elements, are required for muscle differentiation, as treatment with an small hairpin RNA against HIRA or against H3.3 prevents myotube formation (Fig. 2B) [57, 58].

Interestingly, the presence or absence of histone H3.3 does not affect other covalent histone modifications (H3K4me3, H3K9Ac) at the *Myod1* regulatory regions [57]. In *Xenopus*, the presence of histone H3.3 at the *Myod1* promoter is associated with the maintenance of transcriptionally active MyoD expression throughout cell divisions [59]. The presence of histone H3.3 is also detected on muscle-specific genes (*Myog, Myl3, Dmd*) in myoblasts and myotubes (Fig. 2C) [58]. Interestingly, the mechanism of histone H3.3 deposition at *Myod1* regulatory elements differs from that at other muscle-specific genes. Indeed, whereas H3.3 deposition at muscle-specific genes depends on the histone chaperone Chd2 and on MyoD itself, the presence of H3.3 at the *Myod1* gene is independent of Chd2 and is mediated by HIRA [57, 58].

Increasing the ratio of the major H3 isoform histone H3.1 over H3.3 inhibits myogenic differentiation, whereas decreasing this ratio has the opposite effect [60]. In differentiating C2C12, a cleavage of histone H3 is observed, suggesting that the major H3 isoform, H3.1, and possibly other isoforms, are regulated by proteolysis [9]. The H3 cleavage was also observed in differentiating embryonic stem cells, suggesting that it could be a common feature of differentiation [61]. However, the function of this cleavage and whether this cleavage occurs in other cell types or in other histones remain largely unexplored.

The histone variant H2A.Z is also associated with active transcription and has been shown to accumulate at the *Myog* promoter when cells are differentiating (Fig. 2C) [62]. This event is regulated by p38 signaling, as p18[Hamlet], a subunit of the Snf2-related CREB-binding protein activator protein complex (SRCAP complex) responsible for H2AZ deposition, is phosphorylated by p38. Moreover, treatment with inhibitors of p38 signaling impedes H2A.Z deposition at the *Myog* promoter [62].
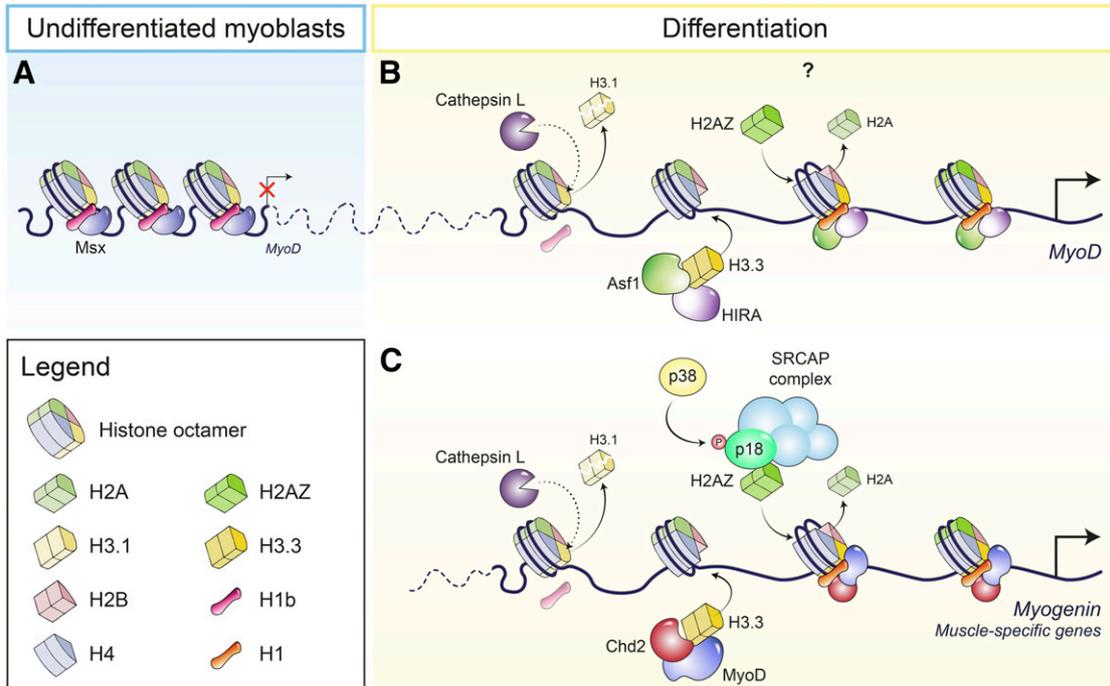
Figure 2. Schematic representation of transcriptional activation involving histone variant deposition at the myogenic loci upon differentiation. **(A):** In undifferentiated myoblasts, both Msx protein and histone variant H1b bind to the *Myod1* promoter, leading to repressed chromatin and the consequent repression of MyoD expression. **(B):** Upon differentiation, the canonical histone H3 is cleaved and this cleavage could rely on the Cathepsin L activity. The histone H3 is replaced by the variant histone H3.3 at the *Myod1* promoter thanks to the histone chaperones Asf1 and HIRA, then allowing MyoD transcriptional activation. The mechanism leading to H2A replacement by H2A.Z, a variant histone associated with active transcription, at the *Myod1* locus, remains unknown. **(C):** In contrast, deposition of histone H3.3 at *Myog* and other muscle-specific promoters depends on MyoD and the histone chaperone Chd2. Moreover, p38 kinase phosphorylates p18[Hamlet], a subunit of the Snf2-related CREB-binding protein activator protein complex that is responsible for H2A.Z deposition at the *Myog* promoter. Abbreviation: SRCAP, Snf2-related CREB-binding protein activator protein.

These results suggest that myogenic differentiation is regulated at the epigenetic level by the interplay between transcription factors, histone modification enzymes, histone variants, and nucleosome remodeling factors. Several independent studies point to a role of p38 signaling in promoting muscle differentiation, by targeting different proteins for phosphorylation. This was confirmed in satellite cells prospectively isolated from human samples, in which p38 was upregulated in activated satellite cells compared with quiescent satellite cells [63]. Furthermore, ex vivo treatment of human satellite cells with a p38 inhibitor prevented satellite cell activation and increased their proliferation, while retaining their ability to differentiate. Another particularly well-studied molecular switch in myogenesis is the antagonistic role of HATs and HDACs during myogenesis, which led to the identification of HDACs as potential therapeutic targets in Duchenne muscular dystrophy as discussed in the next section.

TREATMENT OF MUSCULAR DYSTROPHIES USING EPIGENETIC DRUGS

The increasing data on epigenetics in various tissues have led to the discovery that aberrant epigenetic regulation can be associated with various diseases, notably cancer [64, 65]. Therefore, treatment with epigenetic drugs to cure these diseases is appealing. Several ongoing phase I and phase II clinical trials are using HDAC inhibitors for the treatment of hematologic and solid malignancies, as well as other diseases such as HIV infection and arthritis [66–68]. Two molecules have been approved in the United States for treatment of T-cell lymphomas [69]. HDAC inhibitors also synergize with other anticancer drugs [66]. Moreover, treatment with HDAC inhibitors surprisingly ameliorates the phenotype of dystrophin-deficient (*mdx*) mice (Fig. 3) [70].

Rationale for the Use of HDAC Inhibitors in Targeting Duchenne Muscular Dystrophy

Duchenne muscular dystrophy, the most common and severe form of muscular dystrophy, is an X-linked disease characterized by progressive muscle weakness and degeneration that ultimately leads to death by the second decade of life. Duchenne muscular dystrophy is characterized by the lack of dystrophin (Dmd), a structural protein that links myofibers to the extracellular matrix [71]. Treatment of wild-type myoblasts (murine or human origin) with various HDAC inhibitors, such as trichostatin A (TSA), valproic acid, or sodium butyrate, all pan-inhibitors of class I and II HDACs, increases the efficiency of myoblast fusion and favors myogenic differentiation [72, 73]. Indeed, treatment with TSA does not lead to hypertrophy of pre-formed myotubes and does not increase cell proliferation, but favors myoblasts fusion into pre-existing myofibers [73].

Because HDAC inhibitor treatment improves muscle differentiation, one could expect that histone acetylation is increased during this process. However, it is quite the opposite. Acetylation of histone H3 (lysine 9, lysine 18) and H4 (lysine 12) is globally decreased during myogenesis [9]. These results suggest that treatment with HDAC inhibitors targets specific genes that will favor
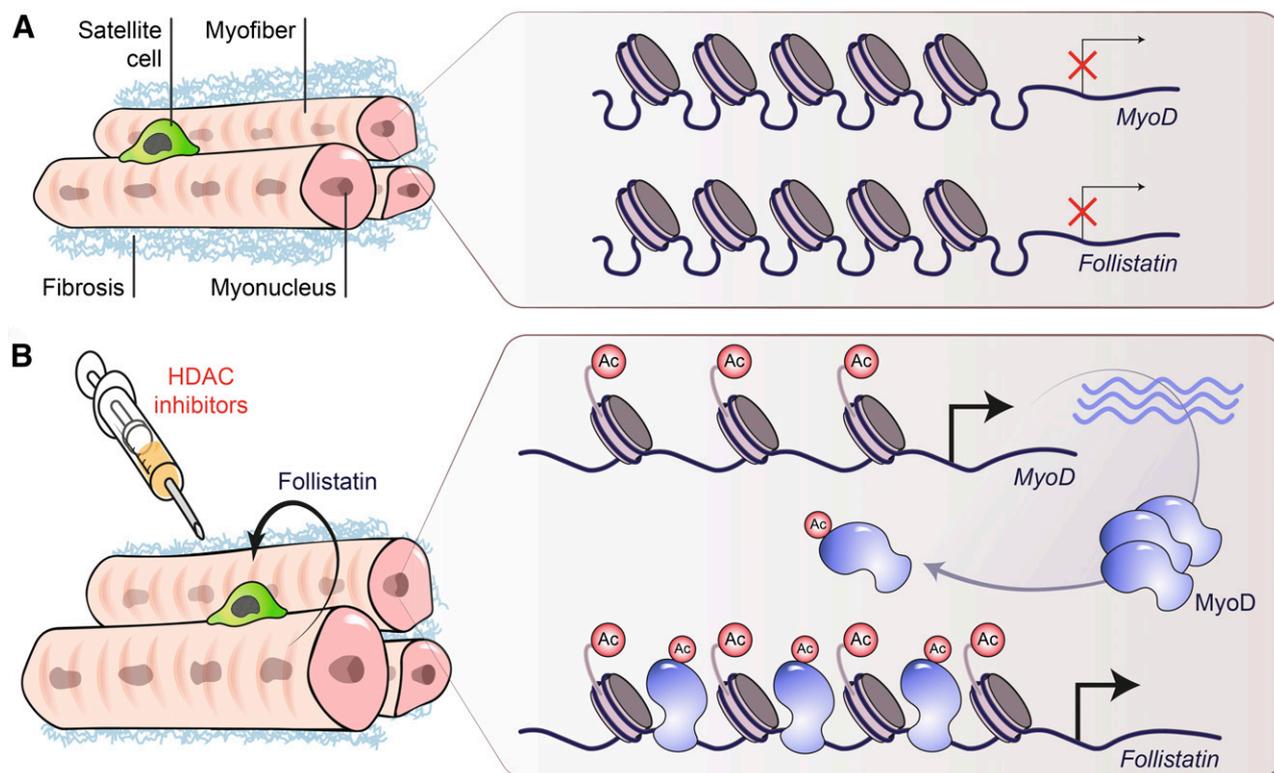
Figure 3. Therapeutic benefits of HDAC inhibitor treatment of muscular dystrophy. **(A):** Duchenne muscular dystrophy is characterized by the absence of dystrophin, leading to extreme myofiber weakness and, subsequently, cycles of degeneration/regeneration, inflammation, fibrosis, and progressive loss of muscle mass and function. HDAC activity leads to repressed chromatin and repression of MyoD and follistatin expression. **(B):** HDAC inhibitor treatment favors muscle regeneration in dystrophic muscles by increasing acetylation of both *Myod1* and *Fst* promoters, which triggers their transcriptional activation. Moreover, HDAC inhibitors increase acetylated MyoD levels and its subsequent recruitment to the *Fst* promoter. Therefore, follistatin upregulation leads to increased myofiber size and restored muscle function. It also decreases fibrosis and muscle necrosis. Abbreviation: HDAC, histone deacetylase.

myogenic differentiation. In addition, nonhistone proteins are also targeted by HDAC inhibitors. For example, the transcription factors Mef2 and MyoD are acetylated during myogenic differentiation, and their acetylation promotes myogenesis [74–80]. Treatment with HDAC inhibitors increases the acetylation of MyoD in myoblasts [72]. In addition, treatment with HDAC inhibitors increases the acetylation of Mef2 in HEK293 cells, suggesting that this increase may also take place in myoblasts [81].

Molecular Targets of HDAC Inhibitors

At the transcriptional level, treatment with HDAC inhibitors dramatically increases the expression of follistatin (Fst) [73], a glycoprotein that binds to members of the transforming growth factor β superfamily to inhibit their activity. In myogenic cells, follistatin antagonizes the function of many transforming growth factor β members, including myostatin, an inhibitor of muscle growth and differentiation. Exogenous expression of follistatin in myoblasts is sufficient to recapitulate the increased fusion index observed upon HDAC inhibitor treatment. Importantly, treatment of injured muscles with TSA induces the expression of follistatin and other myogenic markers, supporting the idea that HDAC inhibitor treatment could improve muscle regeneration [73].

Exposure to HDAC inhibitors increases the levels of acetylated MyoD in wild-type myoblasts [72]. Follistatin upregulation is due to an increase in the recruitment of MyoD to the *Fst* (follistatin) promoter, together with an increase in the level of histone

acetylation at the *Fst* promoter [73]. In addition, treatment with HDAC inhibitors increased histone acetylation on the regulatory elements of MyoD target genes, as exemplified by the *Ckm* enhancer [72]. Treatment with HDAC inhibitors before the onset of differentiation also increases the expression of myogenin and Myf6 in myoblasts [73]. Taken together, these results suggest that HDAC inhibitor treatment uses different mechanisms to enhance muscle regeneration, including MyoD acetylation, modification of histone acetylation at specific gene promoters, and increased expression of the promyogenic follistatin protein.

These results obtained in wild-type cells suggest that treatment with HDAC inhibitors may favor myoblast fusion and muscle regeneration in the context of a disease such as muscular dystrophy. Because of the absence of dystrophin, muscles deteriorate, leading to recurring cycles of muscle degeneration and regeneration. Therefore, treatment with HDAC inhibitors favors muscle regeneration in *mdx* mice, using a similar mechanism as observed in wild-type mice (Fig. 3). Notably, treatment with HDAC inhibitors increased myofiber size and restored muscle force of *mdx* mice to a similar level than the one observed in wild-type mice [70]. It also reduces both fibrosis and muscle necrosis in *mdx* mice. In addition, the same improvement is observed in α-sarcoglycan (*Sgca*)-deficient mice, a model of limb girdle muscular dystrophy, confirming the positive effect of HDAC inhibitor treatment on muscular dystrophy.

Follistatin is considered as a main target of HDAC inhibitors in *mdx* and wild-type mice because *mdx* myofibers knocked-down

for follistatin are unable to respond to TSA treatment [70]. Accordingly, blocking the action of myostatin ameliorates the dystrophic phenotype in *mdx* mice [82–86], confirming that follistatin is the main effector of muscle regeneration upon TSA treatment. In addition, HDAC2 is upregulated in *mdx* muscles and *mdx* primary myoblasts, and global deacetylase activity is higher in *mdx* samples. This suggests that TSA treatment could also mediate its effect by restoring HDAC activity to normal levels [87]. Importantly, HDAC2 is recruited to the *Fst* promoter in undifferentiated myoblasts [70]. Decreasing the levels of HDAC2 in vitro and in vivo enhances the expression of follistatin and leads to a phenotype similar to that observed after treatment with HDAC inhibitors [87].

Preclinical Study Using HDAC Inhibitors

Taken together, these studies highlight the potential of HDAC inhibitors as a potential therapeutic approach in the treatment of Duchenne muscular dystrophy. Preclinical studies using *mdx* mice have been successful [88] and paved the way for ongoing phase I and phase II clinical trials [89]. In the preclinical study [88], *mdx* mice were treated with the HDAC inhibitor givinostat, which was administered safely to pediatric patients in a previous study [90]. After 3.5 months of givinostat treatment, *mdx* mice showed a global increase in muscle mass and size, with reduced fibrosis, inflammation, and fat deposition [88]. Givinostat treatment also led to an amelioration of muscle endurance in *mdx* mice. It must be noted that treatment with epigenetic drugs, such as HDAC inhibitors, targets the downstream effects of the genetic defect in dystrophin expression, slowing down the disease progression. However, the pharmacologic treatment to cure the disease still needs to be found. The use of HDAC inhibitors could also be coupled with other drugs targeting muscular dystrophy, as the combination of different treatments can synergize together to rescue muscle force in mdx mice [91].

HDAC inhibitors are promising molecules for the treatment of other neuromuscular diseases. Specifically, different HDAC inhibitors have proven to be effective in slowing down the progression of the disease in mouse models of amyotrophic lateral sclerosis [92–94]. In addition, HDAC inhibitors synergize with current anticancer drugs to induce apoptosis in rhabdomyosarcoma cells [95].

Epigenetic Drugs in the Treatment or Other Neuromuscular Diseases

Other epigenetic drugs are considered in the treatment of rhabdomyosarcoma and facioscapulohumeral muscular dystrophy (FSHD). Indeed, pharmacological inhibition of EZH2 results in decreased cell proliferation of rhabdomyosarcoma cells, accompanied by an increase in myogenic differentiation [96, 97]. Whether the use of EZH2 inhibitors also favors myogenic differentiation in dystrophic muscles is unknown. However, in *mdx* mice, inflammation-activated signaling induced by tumor necrosis α results in the recruitment of EZH2 on genes regulating satellite cell activity, namely *Pax7* and *Notch1* [25, 98]. Downregulation or pharmacological inhibition of EZH2 promotes satellite cell proliferation in *mdx* mice [25], pointing out EZH2 as a potential therapeutic target for the promotion of muscle regeneration in Duchenne muscular dystrophy. FSHD is a disease characterized by aberrant epigenetic regulation of the chromosome 4q35 D4Z4 macrosatellite repeat array, leading to de-repression of the *DUX4* gene encoded within this region (reviewed in [99]). There is no effective treatment for this disease, partly because of the poor understanding of the pathophysiology and the molecular events occurring at the cellular level. One interesting avenue in the treatment of FSHD is the use of epigenetic drugs to restore chromatin compaction at the 4q35 locus because the epigenetic status at this locus is correlated with the disease [100].

CONCLUSION

In satellite cells as well as in other types of stem cells, the chromatin is in a poised conformation and generally permissive for transcription. The commitment of satellite cells and their differentiation into muscle cells is controlled by the combinatorial activity of many transcription factors, including Pax7, Myf5, MyoD, and MYOG. Chromatin-modifying enzymes also control gene expression programs in satellite cells, myoblasts, and myotubes. Treatment with HDAC inhibitors favors muscle differentiation and ameliorates the disease phenotype of muscular dystrophy in different mouse models. It will be of great interest to deepen our knowledge about the efficiency and the possible adverse effects of HDAC inhibitors in clinical trials. In addition, development of more epigenetic drugs targeting other chromatin modifiers could be used in the treatment of muscular dystrophy and other neuromuscular diseases.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

M.A.R. has compensated intellectual property rights, is a compensated consultant, and has compensated ownership interest in Fate Therapeutics. The other authors indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

M.-C.S., C.E.B., and M.A.R.: manuscript writing, final approval of manuscript.

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