Muscle-specific microRNAs as biomarkers of Duchenne Muscular Dystrophy progression and response to therapies

L Giordani 1,1, M Sandona 2,1, A Rotini 2, PL Puri 1,2, S Consalvi 2,* and V Saccone 2,*
1Sanford-Burnham Medical Research Institute; Sanford Children’s Health; Research Center, La Jolla, CA USA; 2IRCCS Fondazione Santa Lucia, Rome, Italy

These authors have equally contributed to this work.

Recent studies have revealed the contribution of fibro-adipogenic progenitors (FAPs) to the pathogenesis and progression of Duchenne Muscular Dystrophy (DMD). While FAPs direct compensatory regeneration at early stages of disease, as the disease progresses they contribute to the progressive replacement of contractile myofibers with fibrotic scars and fatty infiltration. Using the mouse model of DMD – the mdx mice – we have recently reported that FAPs mediate the ability of HDAC inhibitors (HDACi) to promote muscle regeneration and prevent fibro-adipogenic degeneration at early stages of disease. This effect is mediated by the induction of myomiRs that, in turn, target the SWI/SNF components BAF60A and B, thereby favoring the formation of BAF60C-based SWI/SNF complex, which directs the switch from the fibro-adipogenic to the myogenic lineage. Here we show direct evidence of induction of miR-206 and BAF60C, and reduction of BAF60A, in FAPs isolated from mdx muscles exposed to the HDACi Trichostatin A (TSA). We also discuss how increased expression of myomiRs in dystrophic muscles can be integrated with circulating myomiRs to provide accurate biomarkers of disease progression and response to treatment.

DMD is caused by mutations in the dystrophin gene, located on the X-chromosome, that lead to the absence of dystrophin protein.1 Dystrophin-deficient muscles are vulnerable to mechanical damage, leading to myofiber degeneration and necrosis following contractile activity.2 At early stages of disease, the cycles of muscle contraction/degeneration are counterbalanced by “compensatory” repair, which is characterized by proliferation and differentiation of muscle (satellite) stem cells (MuSCs) to form new myofibers. These stages coincide with a “clinical latency” of DMD, with attenuated symptoms of disease – the so-called “Honeymoon” stage. The progression of the disease coincides with qualitative changes in muscle tissue composition, with ensuing deposition of fibrotic tissue and fat, and exhaustion of the regeneration potential that bias muscle repair toward a progressive replacement of contractile myofibers with fibrotic scars and fat infiltration.3,5

While the progressive decline of compensatory regeneration has been historically attributed to the functional exhaustion of muscle satellite cells and is currently regarded as a key event in the pathogenesis of DMD, its relationship with fibrosis and fat deposition has only recently begun to be appreciated, owing to the seminal discovery of a population of muscle interstitial cells, originally termed fibro/adipogenic progenitors (FAPs).6-9 These cells can contribute to either muscle regeneration and fibro-adipogenic degeneration. In healthy muscles, FAPs provide the support to satellite cell-mediated regeneration during acute injury, by integrating the signals released from inflammatory infiltrate with the...
activation of muscle satellite cells. This response is typically active in muscles of the murine model of DMD (the mdx mice) at early stages of disease. However, as the disease progresses, these cells become biased toward their intrinsic fibro-adipogenic activity and repress satellite cell-mediated regeneration, while promoting fibrosis and fat infiltration. As such, FAPs are currently considered a heterogeneous population of functionally versatile cells that contribute to the pathogenesis of DMD and possibly other chronic degenerative muscle disorders, and might provide valuable target for therapeutic interventions toward promoting compensatory regeneration, while inhibiting fibro-adipogenic degeneration of diseased muscles.

In recent studies, we have investigated FAPs as potential cellular mediators of the beneficial effects of HDAC inhibitors (HDACi) that have been observed in animal models of DMD, such as mdx mice, and Zebralfish. Interestingly, HDACi exert beneficial effects in mdx mice at early, but not late, stages of disease and this coincides with striking changes of FAP function and phenotype observed along with disease progression. Within the “permissive” regenerative environment of young mdx mice, FAPs display a latent pro-myogenic phenotype that is fully expressed upon the exposure to HDACi, in concomitance with the inhibition of the fibro-adipogenic potential. By contrast, at late stages of DMD progression, FAPs adopt a constitutive fibro-adipogenic phenotype that appears to be both “dominant” over the pro-myogenic potential and resistant to HDACi.

The mutually exclusive phenotypes adopted by FAPs at different stages of disease progression and the related differential response to HDACi can be explained by the activation of alternative transcription networks. In our recent work, we have performed an integrated genome-wide approach to address this issue. Combinatorial analysis of gene expression microarray, genome-wide chromatin remodeling by nuclease accessibility site sequencing (NA-seq), and small RNA sequencing (RNA-seq) revealed that HDACi derepress the “latent” myogenic program in FAPs from muscles of mdx mice at early stages, while FAPs from muscles of mdx mice at late stages of disease are refractory to HDACi. In particular, we observed reduced chromatin accessibility in response to HDACi at muscle-specific loci in FAPs from old mdx mice, as compared to FAPs isolated from muscles of young mdx mice. This finding suggests that in FAPs from old dystrophic muscles the chromatin adopts a configuration that precludes the reprogramming by epigenetic drugs, such as HDACi. It also indicates that changes in chromatin remodeling activity, observed at early stages of disease, are regulated by HDAC-mediated control of the chromatin remodeling machinery.

Chromatin remodeling is typically catalyzed by specialized complexes that are endowed with specific enzymatic activities. The SWI/SNF chromatin remodeling complex has been previously shown to regulate lineage determination in many cell types and to be required for the activation of skeletal myogenesis in muscle progenitors. SWI/SNF complexes show heterogeneous composition, with mutually exclusive incorporation of 2 enzymatic subunits (the ATPases BRG1 and BRM) and a number of structural subunits, collectively referred to as BRG1/BRM-associated factors (BAFs). Among them, there are 3 alternative variants of the 60 kDa subunit - BAF60a, BAF60b, and BAF60c - which confer the affinity for tissue-specific transcription factors. Despite the large sequence homology shared by BAF60 variants, they appear to exert specialized functions in lineage determination. While BAF60c plays a key role in both cardiac and skeletal muscle, BAF60a and BAF60b appear to be involved in the activation of alternative pathways, including lipid metabolism. Thus, the finding that pharmacological blockade of HDAC in FAPs of young mdx mice induces BAF60c and the muscle-specific transcriptional activator MyoD, while down-regulating the expression of BAF60a and b, supports the conclusion that HDAC-mediated control of SWI/SNF composition regulates chromatin remodeling and lineage determination in FAPs.

Interestingly, the vast majority of HDACi-induced nuclease accessibility sites (NAS) annotated in FAPs from young mdx mice associates with non-coding regions of the genome, including long non-coding RNA (LncRNA), long and short interspersed non-coding elements (LINE and SINE), and microRNA (miRs). Previous work from the Bozzoni lab has shown an HDAC-dependent control of miR expression in mdx mice. Based on this report, and with the support of microRNA high-throughput screening (HTS) against SWI/SNF BAF60 variants, we identified HDACi-induced miRs that establish a reciprocal network with the SWI/SNF complex to promote “localized” chromatin remodelling at muscle loci and impart the pro-myogenic phenotype in FAPs of young mdx mice exposed to HDACi. Specifically, we have shown that HDACi upregulate muscle-specific miRs - the myomiRs miR-1, miR-133, and miR-206 - that promote muscle progenitor proliferation and differentiation, and could be detected in the serum in patients affected by DMD and other muscular dystrophies. Indeed, studies from our group and others have established a direct relationship between myomiRs and selection of BAF60 subunits, by showing that myomiRs selectively target BAF60a and b.

We sought to correlate the relative expression of BAF60 variants with the expression of myomiRs in FAPs from mdx mice exposed to HDACi. Figure 1 shows that after exposure to the HDACi Trichostatin A (TSA), cultured FAPs isolated from young dystrophic muscles show an increased nuclear expression of BAF60c (Fig. 1A), reduced levels of BAF60a (Fig. 1B), and up-regulation of miR-206 (Fig. 1C, D). These events underline the activation of the myogenic program, at the expense of the fibro-adipogenic one, as FAPs from young mdx mice exposed to TSA and cultured in adipogenic medium form miR-206 and MyHC-positive myotubes (Fig. 1D, bottom panel), rather than differentiating into Oil red O-positive adipocytes (Fig. 1D, upper panel). The activation of the MyoD/BAF60c/myomiR network is also required for the increased ability of FAPs to support MuSC differentiation after TSA treatment. The relationship between HDAC-mediated control of muscle lineage and the ability of FAPs to promote satellite cell-mediated muscle regeneration is currently unclear and...
deserves further investigation. Likewise, how HDAC-regulated chromatin remodelling and gene expression control soluble cues that mediate functional interactions between FAPs and satellite cells is still unclear. The increasing evidence on miR-mediated cell-to-cell communication process in skeletal muscle and, in particular, their clustering into exosomes,39,40 suggest that miR can also mediate functional interactions between FAPs, MuSCs and possibly other cell types that contribute to the regeneration process (Fig. 2).

Collectively these data indicate that HDAC-regulated myomiR-BAF60 variant network "shapes" the composition of the SWI/SNF complex and directs its activity to determine whether FAPs supports compensatory regeneration or fibro-adipogenic degeneration of dystrophic muscles. This model is in striking analogy with previously reported miR-mediated control of BAF53 and 45 variants that regulate sequential stages of neurogenic differentiation.41,42

While this data provide new insight into the molecular pathogenesis of DMD, they also shed new light on the detection and use miRs as clinical biomarkers of disease progression. Beyond their active role in regulating myogenesis and skeletal muscle homeostasis, myomiRs are passively released in the blood as a consequence of degeneration of dystrophic myofibers.34,43 Circulating myomiRs can be detected in the peripheral blood of dystrophic patients and their increase correlates with the severity of the disease. Recently, myomiR quantification in blood has been proposed as a diagnostic and prognostic marker more accurate than the conventional detection of creatine kinase (CK) in DMD patients34, because miRs are more stable and less susceptible to variables, such as exercise.43 The stability of circulating miRs is likely to be preserved by liposomes,44 protein complexes,45 and exosome vesicles.46 In particular, exosomes are emerging as soluble mediators of cell-to-cell communications that might broadcast...
regulatory signals in a protected environment insulated from the oxidative stress generated during tissue regeneration.\cite{47,48}

While the accurate detection of circulating myomiRs and their diagnostic and prognostic interpretation in DMD is under evaluation,\cite{49,50} we propose that FAP-specific expression of miRs could also be considered as an independent biomarker that correlates with compensatory regeneration of DMD muscles at early stages of disease or following therapeutic interventions, such as treatment with HDACi. We speculate that changes in the expression profile of miRs in FAPs could reflect histological changes during disease progression and be used to monitor the transition from the compensatory early stages to the fibrotic late stages of DMD. As FAPs are key targets of therapeutic interventions in DMD,\cite{4,18} and possibly other degenerative muscular disorders, measuring myomiR levels in FAPs could also predict the response to treatments aimed at promoting regeneration and reducing fibrosis. Thus, we propose that integrating the levels of circulating myomiRs with the expression levels of myomiRs in FAPs might offer a reliable marker of disease progression, by correlating myofiber degeneration (circulating miRs) with muscle potential to regenerate or undergo fibrosis and fat infiltration (local, intramuscular miRs). Importantly, the levels of circulating miRs have been proposed as a non-invasive biomarker of myofiber degeneration in DMD patients and their reduction appears to correlate with dystrophin re-expression by exon skipping.\cite{33,34,45,51} By contrast, the measurement of myomiR expression in FAPs is currently relying on the availability of muscle biopsies, and future studies should explore the possibility to detect FAP myomiRs using semi-invasive methods – e.g., taking advantage of their interstitial position to isolate FAPs from muscle interstitial bio-fluids by fine needle aspiration.

The availability of FAPs from muscles of DMD patients and the relative measurement of myomiRs levels would offer an unprecedented molecular readout of muscle tendency to regenerate or undergo fibro-adipogenic degeneration that can be used not only to monitor the disease progression, but also as an inclusion criteria to select DMD patients for clinical trials and as biomarkers of treatment efficacy. Overall, the integration of passively released (circulating) and locally produced (intramuscular) myomiRs should be regarded as a novel outcome measure of DMD progression and response to therapeutic intervention of potential interest for the upcoming clinical trials.

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No potential conflicts of interest were disclosed.

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