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DOTTORATO DI RICERCA
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Multiple approaches for Duchenne Muscular Dystrophy therapy

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ABSTRACT

Exon Skipping has been demonstrated to be a successful strategy for the gene therapy of Duchenne Muscular Dystrophy (DMD): the rational is to convert severe Duchenne forms into milder Becker ones. Here we show the selection of U1 snRNA-antisense constructs able to confer effective rescue of dystrophin synthesis in a Δ44 Duchenne genetic background, through skipping of exon 45; moreover, we demonstrate that the resulting dystrophin is able to recover the correct timing of myogenic marker expression, to re-localize nNOS and to rescue expression of miRNAs previously shown to be sensitive to the Dystrophin-nNOS-HDAC2 pathway.

Becker mutations display different phenotypes, likely depending on whether the shorter protein is able to reconstitute the wide range of wild type functions. Among them, efficient assembly of the dystrophin associated protein complex (DAPC) and Nitric Oxide Synthase (nNOS) localization are important. Comparing different Becker deletions we demonstrate the correlation between the ability of the mutant dystrophin to re-localize nNOS and the expression levels of two miRNAs, miR-1 and miR29c, known to be involved in muscle homeostasis and to be controlled by the Dys-nNOS-HDAC2 pathway.

Since the gene responsible for the disease has been identified, several aberrant pathways have been characterized and many therapeutic approaches have been proposed to face all the symptoms associated to the pathology. What is now quite clear is that the best way to cure the disease is to apply different strategies in parallel, to enhance the beneficial effect that could be obtained from a single treatment.

With this concept in mind we identified a microRNA, miR-31, that is deregulated in DMD conditions if compared to a healthy control. This miRNA represses dystrophin expression by targeting its 3′UTR region. In human DMD myoblasts treated with exon skipping, we demonstrate that miR-31 inhibition increases dystrophin rescue.

These results indicate that interfering with miR-31 activity can provide an ameliorating strategy for those DMD therapies that are aimed at efficiently recovering dystrophin synthesis.

INTRODUCTION

1. DUCHENNE MUSCULAR DYSTROPHY

Duchenne Muscular Dystrophy (DMD) is a severe, X-linked neuromuscular disorder affecting 1 in 3500 live males in their early childhood (Emery, 1993). It is due to mutations in the dystrophin gene that, spanning over 2.5Mb, is one of the largest in the human genome.
DMD has been deeply studied in the last twenty years, since 1986, when two independent research groups identified this gene as the one responsible for the pathology (Koenig et al., 1987; Monaco et al., 1986). Studying dystrophin gene and protein, many aspects of its structure and functional role have been elucidated, as well as the molecular aberrant processes that are involved in the severe phenotype of patients. They are usually diagnosed as DMD during the first five years of life, when they show difficulties in running, maintaining balance, climbing stairs. Then progressive weakness and decrease of strength in the limb muscles, force them to the wheelchair by the age of twelve. Finally cardiac and respiratory failures lead to death within the third decade of life.
From a histologically point of view, DMD biopsies are characterized by the presence of necrotic and degenerating fibers, subject to phagocytosis (FIGURE 1). This leads to the infiltration of inflammatory cells (macrophages and lymphocytes) and to the attempt to replace the damaged tissue with newly formed fibers, characterized by a smaller diameter and centrally localized nuclei. This regeneration process is possible thanks to a pool of quiescent cells, named satellite cells, that undergo activation after muscle damage and are able to differentiate in myoblasts and fuse to pre-existing myotubes. Unfortunately, when the regenerative capacity is lost, for the exhaustion of the satellite cell pool, necrotic fibers are replaced by adipose and fibrous connective tissue, causing the atrophy characteristic of the DMD phenotype.

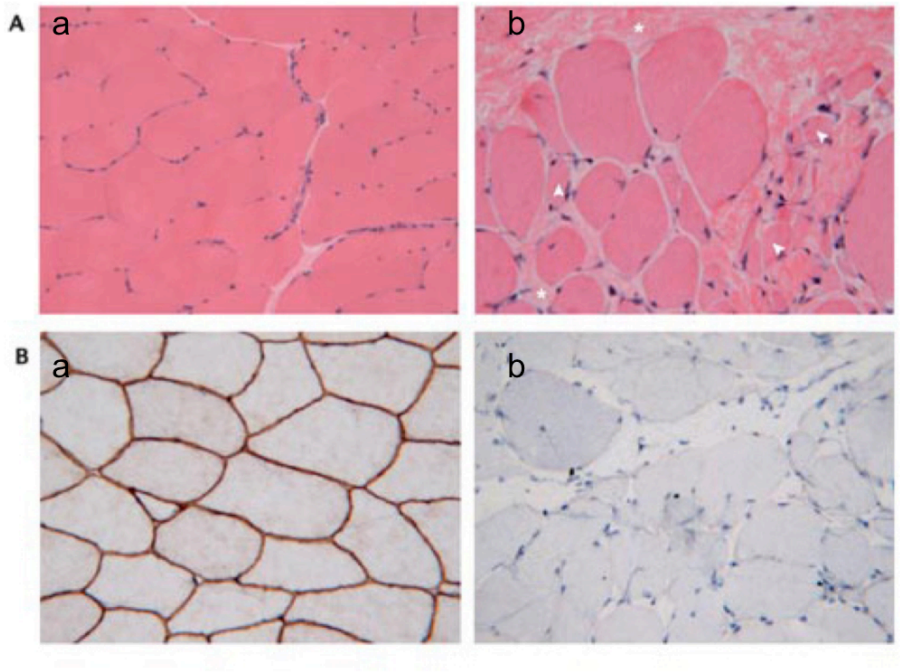


FIGURE 1 - Histological analysis of DMD muscle. A. Haematoxylin and eosin staining of normal muscle (a) and dystrophic muscle (b). In normal muscle, myofibres are approximately equal in diameter with nuclei (stained blue) located around the periphery. The characteristic features of dystrophic muscle are reflective of successive rounds of degeneration and regeneration. These include central nuclei (indicated by arrows), a variation in fibre size with smaller myofibres present (indicated by arrowheads) and a build-up of connective tissue between muscle fibres (indicated by asterisks). B. Immunostaining of normal muscle (a) and dystrophic muscle (b) using an antibody against the dystrophin protein. Dystrophin is present in the normal muscle section (brown staining) and is localized at the sarcolemma (a). Nuclei staining in blue. (Adapted from Davies KE et al., 2006).

Another form of muscular dystrophy exists, the Becker Muscular Dystrophy (BMD, Becker et al., 1955), which is again due to mutations in the dystrophin gene but, unlike the DMD mutations, they don't affect the open reading frame of the mature mRNA. As a consequence it is usually characterized by less severe symptoms and a higher life expectancy. Despite this, a great variance in disease severity has been documented over the years (Norman et al., 1990; Muntoni et al., 1994): in fact there are patients with a Becker genetic background but a Duchenne phenotype, that cannot be justify if we don't go deep inside the cause of the disease.
So, after the discovery of this milder allelic form of dystrophy, many efforts have been done to understand why there is such heterogeneity of phenotypes among patients affected by the same pathology. To answer these questions it is necessary to understand the molecular role of the protein and its involvement in cellular pathways deregulated in the disease.

2. THE DMD GENE AND THE MUTATIONS

Dystrophin protein is encoded by a gene localized to Xp21: it spans over 2.5Mb, is transcribed in a 14 Kb messenger RNA (mRNA) made of 79 exons. It was identified in 1986 by two groups who were studying rare DMD females with translocations X:autosome with the breakpoint in Xp21: this identification represented the first example of positional cloning (Koenig et al., 1987; Monaco et al., 1986).
The DMD gene presents three independently regulated and tissue specific promoters: they give rise to three full length proteins indicated as Dp427M, expressed in skeletal and cardiac muscles; Dp427B, active in cortical neurons and hippocampus of the brain; Dp427P, present in cerebellar Purkinje cells (Blake et al., 2002).
There are also four more internal promoters, encoding for smaller isoforms that are thought to be involved in the stabilization and function of non-muscle dystrophin-like protein complexes (FIGURE 2). Each of these promoters utilizes a unique first exon that splices into exon 30, 45, 56 and 63 respectively, to generate protein products of 260 kDa (Dp260), 140 kDa (Dp 140), 116 kDa (Dp116) and 71 kDa (Dp71). Dp71 is detected in most non-muscle tissues including brain, kidney, liver and lung, while the remaining short isoforms are primarily expressed in the central and peripheral nervous system (Ervasti, 2007; Waite et al., 2012)

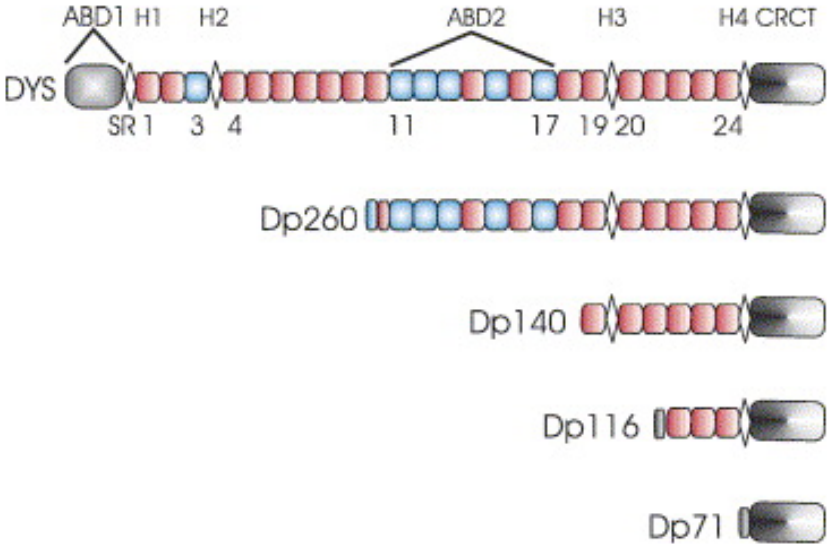


FIGURE 2 - Protein products of the DMD gene. Schematic diagram illustrating the domain structure of the protein isoforms encoded by the DMD gene. Dystrophin (DYS) contains an amino-terminal actin binding domain (ABD1) consisting of tandem CH domains, a spectrin-like triple-helical repeat (SR) domain with 4 putative hinge modules (H1–H4) interspersed throughout its length, a cysteine-rich (CR) domain critical for binding β-dystroglycan, and a carboxy-terminal domain (CT) important for binding syntrophins and α-dystrobrevin-2. Acidic spectrin repeats are colored red, basic repeats colored blue, and a cluster of basic repeats form a second independent actin binding domain (ABD2). Alternate promoters drive the expression of four truncated non-muscle isoforms, Dp260, Dp140, Dp116, and Dp71 each with unique amino-terminal sequences and the indicated domains in common with full-length dystrophin. (Adapted from Ervasti et al., 2007).

Dystrophin gene is subject to a high mutation rate due to its huge size. Most of them are deletions of one or more exons (around 65% of cases) or duplications (6%). These mutations usually occur in two regions: a major hotspot between exon 45-52, which removes part of

the rod domain, and a minor hotspot between exons 2-19, that deletes part of the actin-binding and rod domain (Gillard et al., 1989; Oudet et al., 1992).
In 30% of cases, when neither exonic deletion nor duplication can be detected, the most common mutations are changes of one or a few nucleotides leading to a truncated transcript, either via a premature stop codon or the disruption of the reading frame.
Surprisingly all the mutations described can be responsible of both a Duchenne and a Becker phenotype, without a direct correlation between the extent of the deletion and the severity of the pathology. For example, in several cases the deletion of a single exon is enough to cause DMD, whereas deletion of nearly half of the gene (46%) has been found in BMD patients (England et al., 1990; Love et al., 1991). In the beginning, this observation led to the hypothesis that the rod domain could act as a spacer between the actin and the C-terminal domain and that it was dispensable. According to this theory mutations affecting the size of the rod-domain should be responsible only for mild Becker phenotypes. But again it was not enough to justify what emerged from clinical studies on BMD and DMD patients. This lack of correlation between mutations and phenotypes, suggested the so called reading frame hypothesis (Monaco et al., 1988): if the mutation responsible for the pathology causes a frame shift in the open reading frame, the formation of random and premature stop codons leads to the degradation of the mRNA and to the absence of the protein; on the contrary if the mutation doesn't change the correct reading frame, a shorter but still functional protein is produced, a protein that is still able to fulfil its structural role.
However, exceptions to this theory have been described, both for DMD patients with in-frame deletions and BMD with out-of-frame deletions (Winnard et al., 1993). In-frame DMD-causing mutations are either large or remove a domain essential for dystrophin function, whereas out-of-frame mutations causing BMD are presumably compensated for, either by the use of an alternative start codon or exon-skipping leading to an in-frame product (Winnard et al., 1995;

Prior et al., 1997). These observations altogether suggest that there are some domains of the protein that are not dispensable, whose absence causes the inability of a shorten protein to carry out all its functions, both structural and not functions.
The structural functions of the protein can be easily inferred from its cellular localization and its peculiar rod shape. On the contrary, its role in the intracellular signalling has been elucidated after the characterization of the so called Dystrophin Associated Protein Complex (DAPC; FIGURE 3). Each member of this complex interacts with a specific domain of dystrophin and is involved in the multiple functions exerted by the protein.

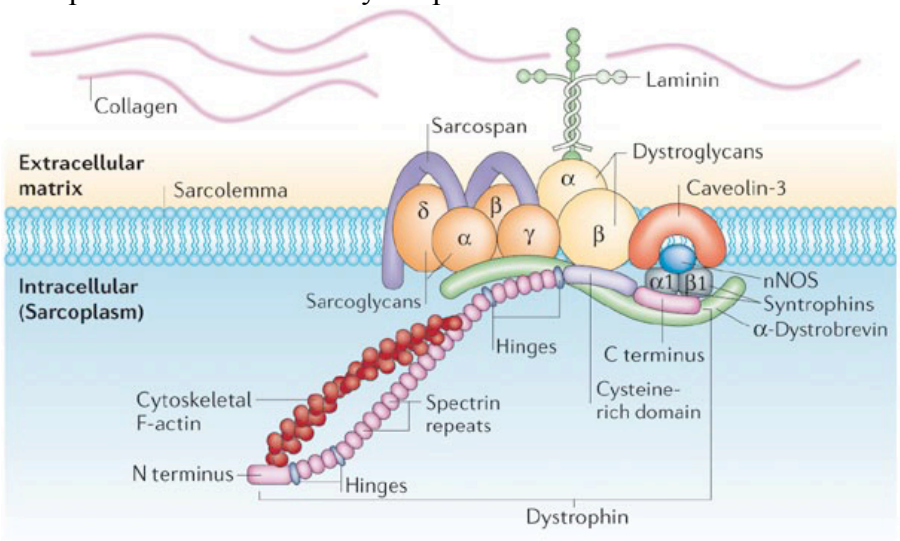


FIGURE 3 - Dystrophin Associated Protein Complex (DAPC). Dystrophin binds to the dystrophin-associated protein complex (DAPC) through its C terminus. The DAPC is comprised of sarcoplasmic proteins (-dystrobrevin, syntrophins and neuronal nitric oxide synthase (nNOS)), transmembrane proteins (-dystroglycan, the sarcoglycans, caveolin-3 and sarcospan) and extracellular proteins (-dystroglycan and laminin). (Davies KE et al., 2006)

3. DYSTROPHIN ASSOCIATED PROTEIN COMPLEX (DAPC) AND ITS ROLE IN MUSCLE PHYSIOLOGY AND PATHOLOGY.

The DAPC consists of cytoplasmic, transmembrane and extracellular proteins; it provides a strong mechanical link and mediates interactions between the intracellular cytoskeleton and the Extracellular Matrix (ECM - Rando, 2001). It is known that mutant protein members of the DAPC cause a loss of sarcolemmal integrity and therefore render muscle fibers more vulnerable to damage. In the absence of dystrophin, for example, the DAPC becomes destabilized; the sarcolemma anchorage to the internal actin cytoskeleton and to the ECM is lost, with the consequent reduction of the protective role of the complex. The increasingly fragile sarcolemma is subjected to mechanical injury, which results in progressive muscle-fibre damage and sarcolemmal leakage.
Most of the protein members of the complex are involved in this protective and mechanical role: dystroglycans, α-dystrobrevin, sarcoglycans, dysferlin and laminin (Cohn et al., 2002; Barton et al., 2006; Michele et al., 2003). As a consequence, mutations in the genes encoding for these proteins, or aberrant post translational modifications, are responsible for different muscular dystrophies: Limb-Girdle Muscular Dystrophy Type 2D (LGMD 2D, Roberds et al., 1994), Left Ventricular Non-Compaction (LVNC, Ichida et al., 2001), Miyoshi Myopathy (MM, Liu et al., 1998), Merosin-Deficient Congenital muscular dystrophy (MDC1A Helbling-Leclerc et al., 1995).
Some members of the DAPC, anyway, seem to be involved also in intracellular signalling, even if only some pathways have been characterized. Among them, the one that has been recently studied involves the neuronal Nitric Oxide Synthase (nNOS) and the Histone Deacetylases 2 (HDAC2).
Nitric Oxide (NO) has emerged as a key second messenger molecule in several cellular pathways. Its production in muscle is granted by

the activity of a neuronal/muscular specific isoform, nNOS, which directly interacts with dystrophin in the DAPC (FIGURE 3, Davies KE et al., 2006). In DMD muscles dystrophin absence at sarcolemma causes the mislocalization of the entire DAPC, including nNOS, and the consequent impairing in NO production (Brenman et al., 1995). Nitric oxide (NO) is an important vasodilator and regulator in the cardiovascular system; it also plays many other regulatory roles, including immune function, neural activity, and gastrointestinal function. Within skeletal muscle, NO is thought to regulate glucose uptake and possibly blood flow during and following exercise (McConell et al., 2012). Moreover it is responsible for nitrosylation of different proteins, among which HDAC2 has been described (Stamler et al., 2001; Colussi et al., 2008).

Histone deacetylases (HDACs) are a family of enzymes divided in four sub-classes based on structural similarities. They are principally involved in the withdrawal of acetyl-groups from a large number of proteins including nuclear core histones. Their pivotal role in regulating the acetylation state of chromatin resulted in a direct control of chromatin accessibility and dinamics.

In particular HDAC2 activity in repressing gene target transcription, is negatively regulated by S-nitrosylation on two Cys residues (Cys262 and Cys274). These modifications determine conformational changes in the enzyme inducing its release from chromatin and a consequent increase in chromatin accessibility (Nott. et al., 2008; Cacchiarelli et al., 2010).

In normal skeletal muscles the correct regulation of this enzyme is guaranteed by nNOS activity at the sarcolemma. In Duchenne pathology, instead, the reduction of NO production due to DAPC delocalization, results in a reduced HDAC2 S-nitrosylation, with a consequent aberrant deacetylase activity (Colussi et al., 2008). As a consequence, different target genes, included follistatin and some myomiRs, as mir-1 and miR-29, are down-regulated, promoting fibrosis and oxidative stress, usually observed in dystrophic muscles (FIGURE 4, Cacchiarelli et al., 2010; Minetti et al., 2006).

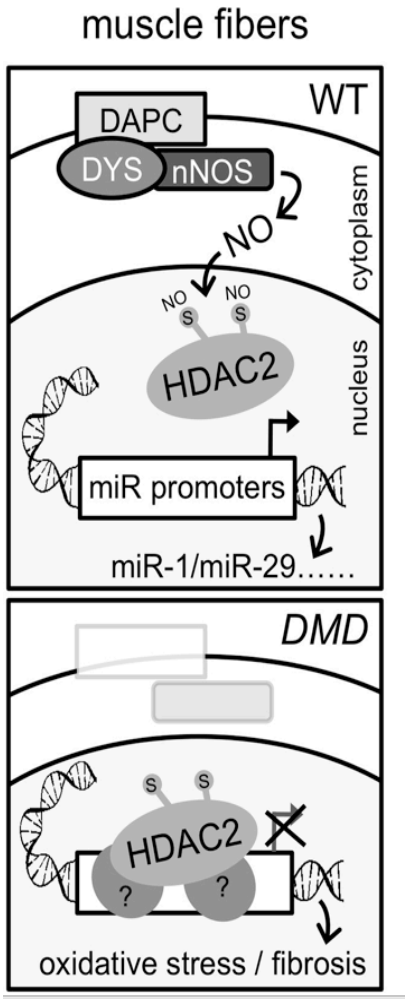


FIGURE 4 - Model of the pathway connecting dystrophin to specific miRNA expression
In WT muscle fibers the dystrophin/DAPC complex activates nNOS and S-nitrosylation of HDAC2. The modified HDAC2 is released from the chromatin and activation of a specific subset of miRNA occurs. In DMD this circuitry is deregulated due to impaired nNOS activity and decrease in HDAC2 nitrosylation, causing its retention to miRNA promoters. (Cacchiarelli et al., 2010)

Dystrophin domain responsible for nNOS correct localization at the sarcolemma has been localized in the rod domain (spectrin-like repeats 16 and 17) and is encoded by exons 41-46 (Lai et al, 2009). As a consequence, BMD patients lacking this part of the protein present much more severe DMD-like symptoms than BMD lacking other spectrin-like portions of the rod domain.
So we can conclude that the severity of the pathology can be justified by the effect of the mutation on the open reading frame and, among in frame mutations, by the role of the lacking domain of the protein in ensuring the correct interaction between dystrophin and DAPC members.

4. CURRENT STATUS OF PHARMACEUTICAL AND GENETIC THERAPEUTIC APPROACHES TO TREAT DMD

As described above, DMD and BMD symptoms can be very severe. Unfortunately a therapy able to restore muscle strength and therefore to return a normal life to patients doesn't exist. Thus, even if there are currently no curative treatments for this disease, at least the medical monitoring and the care coverage of these patients contribute to prevention of some complications and to the improvement in their quality of life.
In parallel many efforts have been done to develop a resolutive therapeutic strategy for the majority of patients. Among them we can distinguish pharmaceutical approaches from gene and cell therapy approaches (Pichavant et al., 2011).

Pharmaceutical approaches - The great advantage of a pharmacological approach is that nearly all drugs can be delivered systemically (orally, intravenously, sub-cutaneously) and thus will reach and potentially treat all muscles. Most of the strategies included in this category are focused on endogenous dystrophin synthesis restoration.

Stop codon read-through. About 10–15% of DMD patients have a mutation that converts an amino acid into a premature nonsense codon, while the rest of the mRNA is unaffected. Some drugs have been shown to enable stop codon read-through by introducing an amino acid at the premature stop codon to continue the mRNA translation (FIGURE 5). This phenomenon is called “stop codon read-through”. Gentamicin and Ataluren are two molecules able to interact with the ribosome and to induce the inclusion of an aminoacid when a premature stop codon is present. Even if many studies on the *mdx* mouse (murine model of DMD) have been successful for both drugs,

phase I and II clinical trials on humans showed less efficacy and some toxicity (Hirawat et al., 2007).

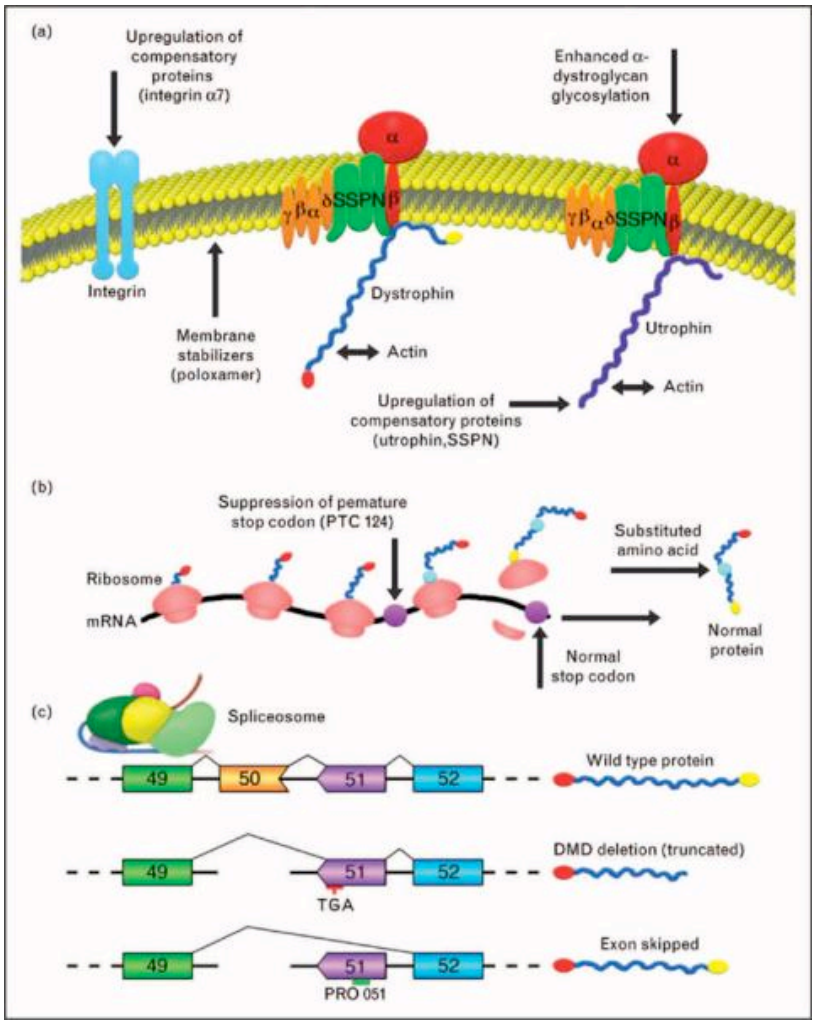


FIGURE 5 – Schematic representation of the current therapeutic strategies for the cure of DMD. (Adapted from Nelson et al., 2009).

Exon skipping. In DMD the deletion of one or more exons can cause the formation of a premature stop codon, due to the shift of the correct reading frame. But we know that BMD patients lacking even wider portions of the gene, can live a normal life, because the resulting protein is shorter than the normal one but still functional. The exon skipping strategy (FIGURE 6) is based on the use of antisense molecules able to interfere with splicing signals, inducing the skipping of a specific exon; its exclusion from the dystrophin mRNA can restore the open reading frame and allow the expression of an internally deleted but functional dystrophin in DMD patients. So exon skipping can convert a severe DMD phenotype into a milder Becker one. The validity of this approach has been widely demonstrated both in the mdx mouse (Mann et al., 2001) and in in-vitro experiments performed on human DMD myoblasts (Aartsma-Rus et al., 2005). A great advantage of this approach is the possibility of curing different deletions with the skipping of the same exon. This way one antisense molecule can be therapeutic for a percentage of different patients (see TABLE 1, Aartsma-Rus et al., 2009).
The molecules used for this purpose are small synthetic modified RNAs or DNAs called antisense oligonucleotides (AOs) able to bind specific intronic or exonic sites of pre-mRNA. Annealing to selected splice motifs, the AO essentially masks the target exon from the splicing machinery, thereby promoting specific exon exclusion from the mature mRNA. Two types of AO are mainly used: 2′-O-methyl-phosphorothioate (2OMP, Goemans et al., 2011) and phosphorodiamidate morpholino oligomer (PMO, Cirak et al., 2011). Both entered clinical trials of phase I and II, with promising results in inducing dystrophin synthesis with low toxic effects.
The use of AOs presents some limits: they are not stable, so reiterated administrations are necessary. Moreover it is desirable that antisense molecules localize in the correct cellular compartment to obtain efficient exon skipping. To fulfill all these requests antisense sequences were cloned in the small nuclear RNA U1 or U7 (snRNA) and expressed as chimeric molecules. AAV1 coding for the U7 or U1

snRNA modified genes targeting the mouse dystrophin exon 23 were injected in *mdx* mice. The expression of the internally deleted dystrophin was observed up to 3 months following the injection of an AAV1 coding for the U7 snRNA (Goyenvalle et al., 2004) and for at least 1 year and half with an AAV1 coding for the U1 snRNA (Denti et al., 2008). The efficiency of the approach was demonstrated also in human DMD derived myoblasts (Incitti et al., 2010). Clinical data about this approach are not yet available.

Myostatin. A potential therapeutic method to improve muscle strength is to block myostatin. Myostatin is a member of the transforming growth factor-β family implicated in muscle size regulation. Indeed, in the myostatin gene knockout mouse, robust muscular hypertrophy and hyperplasia are observed. The idea is to use antibodies or knock-down approaches to inhibit myostatin pathway and increase muscle size, perhaps in parallel with one of the treatments described above (Malerba et al., 2012).

Pharmacological upregulation of the dystrophin-related protein, utrophin. Utrophin is a protein encoded by a gene on chromosome 6 in humans. The primary structure of utrophin is very similar to that of dystrophin, with the N-terminal, cysteine-rich and C-terminal domains displaying significant structural similarity, being 80% identical to each other (Tinsley et al. 1992). Utrophin shares many of the same binding partners as dystrophin. The C-terminus of utrophin has been shown to bind to members of the DAPC, such as α-dystrobrevin-1 (Peters et al. 1998) and β-dystroglycan (Ishikawa-Sakurai et al. 2004). Unlike dystrophin, which is expressed in muscle, with lower levels in brain, utrophin is ubiquitously expressed.
A range of strategies has been used to upregulate utrophin by pharmacological means, and clinical success has been documented (Tinsley et al., 2011); the main problem is that this protein can't fulfil all dystrophin functions.

HDAC inhibitors. Since the discovery of HDAC2 aberrant activation in DMD pathogenesis, different drugs able to inhibit these enzymes entered in clinical trials (Consalvi et al., 2011) and have been successfully used in order to promote regeneration in dystrophic muscle.

Classifica	Esone	Mutazioni tot.	Delezioni	Mutaz. Punt.	duplicazioni
1	51	13.0%	19.1%	0.3%	3.0%
2	45	8.1%	11.8%	0.2%	2.2%
3	53	7.7%	11.4%	0.1%	1.5%
4	44	6.2%	8.8%	0.4%	2.7%
5	46	4.3%	6.2%	0.2%	1.6%
6	52	4.1%	5.7%	0.5%	2.3%
7	50	4.0%	5.6%	0.2%	1.9%
8	43	3.8%	5.3%	0.2%	2.6%
9	6 e 7	3.0%	3.6%	0.1%	6.3%
10	8	2.3%	2.3%	-	8.0%
11	55	2.0%	2.7%	0.8%	0.2%
12	2	1.9%	1.3%	-	12.7%
13	69 e 70	1.4%	-	5.6%	-
14	19 e 20	1.1%	1.7%	4.6%	-
15	45 e 51	1.1%	-	-	-
16	58 e 59	1.1%	1.1%	4.5%	-
17	17	1.0%	1.4%	0.1%	3.1%
18	7	1.0%	-	-	0.8%
19	65 e 66	1.0%	-	3.9%	-
20	43 e 44	0.9%	-	3.5%	-

TABLE 1.
Overview of the Applicability of Exon Skipping for DMD Mutations

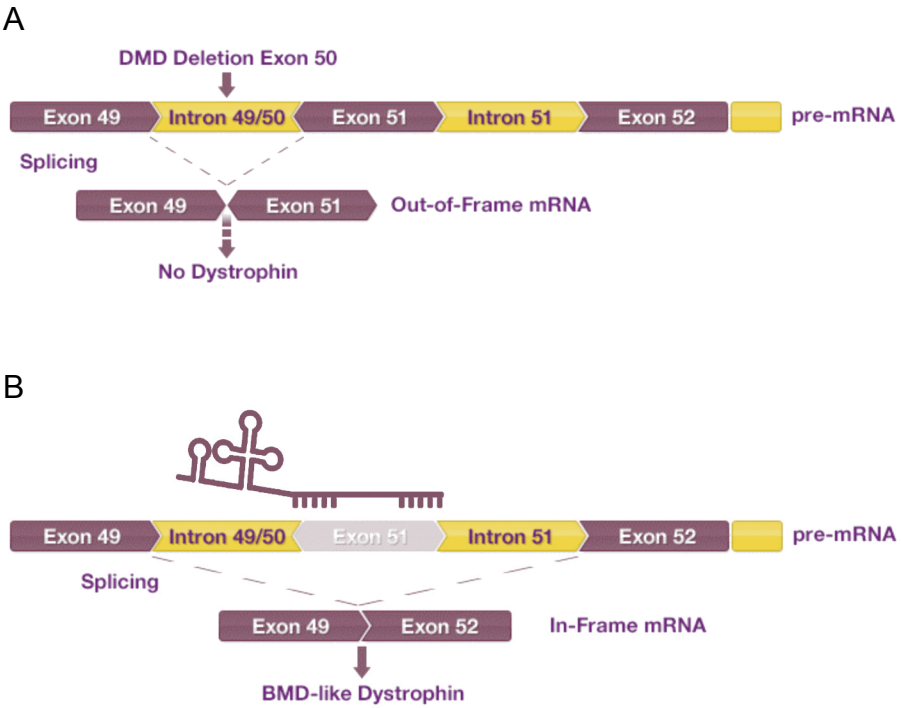


FIGURE 6 - Example of exon-skipping strategy.
A. Deletion of exon 50 in DMD gene results in an out-of-frame mRNA transcript and a prematurely aborted dystrophin synthesis. B. Employing of antisense molecules against exon 51 splice sites produce an in frame mRNA transcript resulting in a shortened BMD-like dystrophin protein.

Gene therapy approaches - Gene therapy in DMD consists of the introduction of a functional copy of the DMD gene in muscle fibers, with the aim of restoring muscle function, including force generation and resistance to muscle contraction induced damage. The concept of dystrophin internally deleted genes that would fit the packaging capacity of small viral vectors came from clinical observations that some BMD patients with internally deleted dystrophins could maintain ambulation for many decades. This gave rise to the concept of mini-dystrophin (mDYS) or micro-dystrophin (μDys). Gene therapy is divided in two distinct categories: those using viral vectors to transfer the gene are referred to as "viral gene therapy"; those employing naked DNA as "non viral gene therapy". In both the cases the main issue that has to be solved regards the immune response of the host organism against the new protein and/or the viral capsid used for the delivery (Wang et al., 2000; Acsadi et al., 1991)

Cell therapy approaches - Cell therapy involves the delivery of cells able to produce new muscle to diseased areas. These can be either muscle precursor cells or stem cells that have the ability to differentiate into muscle cells. The cell therapy strategy that has experienced the most success so far has been the transplantation of myoblasts into diseased tissue; anyway the percentage of colonized fiber in different clinical trials was very low, due to the immune response of the host organism. Current hopes are based on the use of donor genetically modified myoblasts to reduce the patient immune response. Moreover the attention of researchers is moving from myoblasts to muscle stem cells: satellite cells and mesoangioblasts (Dellavalle et al., 2007), the only ones able to repopulate the muscle successfully. A big limit of these cell-based strategies is the delivery of the cells in every muscle district, in particular heart and diaphragm. Stem cell therapy in clinical trial for DMD includes CD133+ (Torrente et al., 2007) and mesoangioblasts (Giulio Cossu, in progress).

AIMS

Duchenne Muscular Dystrophy is a rare and severe genetic disease whose gene has been identified since 1986; however, a cure has not yet been developed. The main issue to be solved is related to the huge size of the entire dystrophin gene (14Kb) that renders quite difficult the use of a classical gene therapy approach consisting in gene transfer. Moreover, it is becoming clear from recent studies that, even if DMD is a monogenic disease, the lack of dystrophin has consequences on different pathways involved in muscle homeostasis and differentiation. These findings confirm that the protein has not only a mere structural role in preserving muscle from degeneration, but is involved in intracellular signalling, for example orchestrating gene expression through the nNOS-HDAC2 pathway.

Taking into account all these data, when thinking to a therapeutic approach for a so complex disease, researchers should develop a main strategy aimed to rescue dystrophin synthesis, applying in parallel other treatment able to help the muscle in recovering its physiological homeostasis.

In my PhD I focused my attention on the selection of antisense molecules able to induce the skipping of exon 45 of the dystrophin gene; this exon is one of the most common, after exon 51, in terms of number of patients that can be cured (8.1% of known DMD mutations). Δ44 DMD myoblasts, treated with the skipping of exon 45, were then analyzed to verify the beneficial effects of recovering dystrophin synthesis on muscle differentiation. Indeed we demonstrated that rescue of dystrophin was able to recover the expression of late muscle differentiation marker; moreover even the expression of some myomiRs depending on the nNOS-HDAC2 pathway was rescued, underlying that exon 44 and 45 are dispensable for the recruitment of nNOS to the sarcolemma. BMD biopsies were then analyzed to correlate the mis-localization of nNOS with the expression of miR-1/miR29 and with the clinical phenotype of 14 patients.

Finally a miRNA, miR-31 was identified that is over expressed in pathological condition (both in the mdx mouse and in DMD myoblasts) and targets dystrophin 3'UTR. We demonstrated that decoy of miR-31 could be a valid parallel approach to enhance the effect of all those therapies that aim to rescue endogenous dystrophin synthesis.

RESULTS

Design and expression analysis of antisense molecules against exon 45 of the dystrophin gene

Eight constructs, in the backbone of the U1 snRNA, were designed and produced for the skipping of exon 45 of the dystrophin pre-mRNA (FIGURE 7a, TABLE 1). Nucleotides from position 3 to 10 at the 5′end of U1 snRNA, required for the recognition of 5′splice sites (Zhuang et al., 1986), were substituted with antisense sequences complementary to different portions of exon 45 and splice sites. The initial construct (#1) contained antisense sequences against both splice junctions, since we previously showed that both splice sites have to be targeted in order to induce efficient exon skipping when using U1 snRNA (Incitti et al., 2010). Additional constructs were produced that contained also antisense sequences against putative ESEs, known to represent effective target substrates for efficient exon skipping (Aartsma-Rus et al., 2006; Cartegni et al., 2003; Aartsma-Rus et al., 2005).
The chimeric U1-antisense sequences were cloned under the control of the strong polII U1 snRNA gene promoter and termination sequences, and subsequently inserted in the dU3 portion of the 3′ long-terminal repeat region of a lentiviral vector plasmid (Bonci et al., 2003).
The expression and stability of the different constructs were tested by transfection into the murine myogenic $C_2$7 cell line. The relative expression was measured by co-transfection with the U16RBE (Buonomo et al., 1999) plasmid and normalized for the endogenous U2 snRNA. Northern blot analysis indicated that all chimeric molecules accumulated at fairly similar levels (FIGURE 7b).

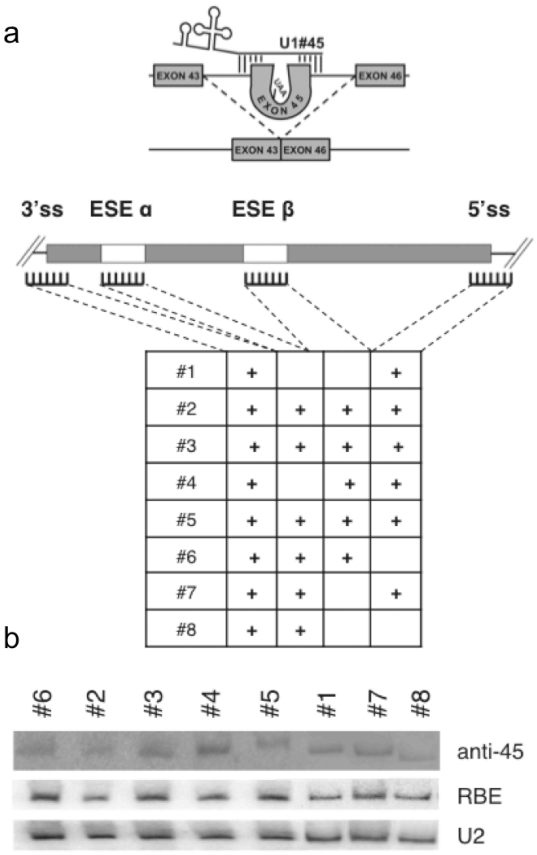


FIGURE 7 - Exon 45 skipping
(a) Schematic representation of the exon-skipping strategy for the human Δ44 Duchenne Muscular Dystrophy (DMD) mutation. The table summarizes the eight different constructs produced together with the corresponding target regions on exon 45 and flanking intron (ss, splice site; ESE, exonic splicing enhancer); (b) $C_2$7 myoblasts were transfected with the different antisense constructs together with the U16-RBE plasmid [expressing a 143-nucleotide (nt) long modified U16 snoRNA]. Northern blot analysis was performed with probes against the 3′ splice site (anti-45), U16-RBE (RBE), and U2 snRNA (U2). The two latter hybridizations are used to normalize for transfection efficiency and as loading control, respectively.

	3' ss	ESE α	ESE β	5'ss
U1-45#1	cttacagGAACT	CCAGGATGGCATTGGGCAGC	CAGCAATCCTCAAAAACAGATGC	
U1-45#2	cagGAACT	CCAGGATGGCATTGGGC	TCCTCAAAAACAGATGCCAG	AAGAGgtagggcg
U1-45#3	cagGAACT	CCAGGATGGCATTGGGCAGC	CAGCAATCCTCAAAAACAGATGC	GAGgtaggg
U1-45#4	cttacagGAACTCCAGG		CAGCAATCCTCAAAAACAGA	GAAAAAAGAGgtagggcg
U1-45#5	acagGAACT	CCAGGATGGCATTGG	GCAATCCTCAAAAACAGA	GAAAAAAGAGgtaggg
U1-45#6	gccttttggtatcttacagGAACTCCAGG			CAGAAAAAAGAGgtagggcgacag
U1-45#7	cttacagGAACT	CCAGGATGGCATTGGGCAGC		CAGAAAAAAGAGgtagggcgacag
U1-45#8	gccttttggtatcttacagGAACT	CCAGGATGGCATTGGGCAGC		

TABLE 1 – Description of antisense construct.
Description of antisense constructs. Table summarizes the 8 different constructs produced together with the corresponding target regions on exon 45 and flanking intron sequences (uppercase – exonic regions; lowercase – intron sequences). ss= splice site; ESE= Exonic Splicing Enhancer.

Study of exon skipping activity in human DMD myoblasts

Lentiviral particles for each construct were produced in 293T cells and used to transduce DMD myoblasts carrying the deletion of exon 44 (Δ44, provided by the Telethon Neuromuscular Biobank). In this case, skipping of exon 45 allows restoration of the correct reading frame and the production of a dystrophin protein 106 aminoacid shorter than the wild type. Δ44 cells were infected with comparable amounts of the different recombinant lentiviruses. After infection, they were induced to differentiate and samples were collected after 10 days for RNA and protein analysis. Exon 45 skipping (FIGURE 8a, upper panel) and antisense expression (FIGURE 8a, middle panel) were assessed by RT-PCR analysis, utilizing GAPDH as normalizer (FIGURE 8a, lower panel). Dystrophin rescue was assessed by Western blot (FIGURE 8b, upper panel) and the relative values, normalized on actinin levels, are reported in the histogram. Due to the low efficiency of exon skipping and in order to have more comparable signals, 50 μg of proteins from Δ44-treated cells (PGK and #1 - #8) were loaded in parallel with 10 μg of proteins from control cells (CTRL). Myosin heavy chain (MHC) detection was used as a marker of muscle terminal differentiation. This analysis revealed that constructs #6 and #8, targeting only the 3′splice junction and ESE sequences, are poorly active in terms of dystrophin recovery. Moreover, #1, targeting the two splice junctions, provided very low skipping activity and dystrophin rescue. The importance of the simultaneous targeting of splice sites and ESE is shown by constructs #3 and #4 that provided the highest rescue of dystrophin synthesis (around 5%, see the histogram of FIGURE 8b). Interestingly higher levels of dystrophin correlated with increase in the MHC terminal differentiation marker (FIGURE 8b, panel MHC).

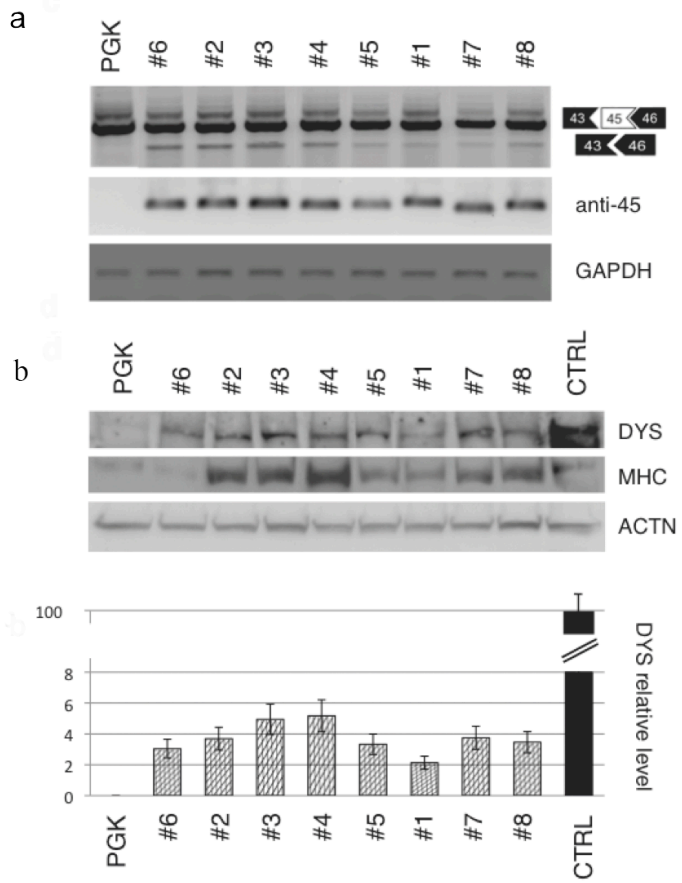


FIGURE 8 - Exon skipping activity.
a) The amount of exon skipping was calibrated by nested reverse transcription-PCR (RT-PCR) on RNA extracted from Δ44 cells infected with the antisense-expressing lentiviruses. Unskipped and skipped products are indicated on the right. Anti-45 refers to the expression of the antisense molecules; GAPDH is used as an internal control. b) Rescue of dystrophin synthesis. Upper panel: western blot on proteins from CTRL (10µg + 40µg PGK), mock infected Δ44 (PGK-50µg) and exon-skipping Δ44 treated cells (#1 to #8–50 µg) probed with anti-dystrophin (DYS), anti-myosin heavy chain (MHC) and anti-actinin (ACTN) antibodies. Lower panel: the histogram indicates dystrophin levels normalized on actinin signals and expressed as percentage of CTRL.

Dystrophin levels affect myogenic differentiation

In vitro cultured DMD myoblasts display a delay in the appearance of typical myogenic markers (Delaporte et al., 1984); this can be easily appreciated when comparing in vitro differentiation of Δ44 myoblasts with control ones derived from a healthy individual: FIGURE 9a shows that the terminal differentiation marker MHC, already present at day 6 of differentiation in control cells, is poorly detectable in Δ44 cells even at day 10. Notably, when exon skipping was applied, the treated DMD myoblasts showed an effective recovery of the timing of myogenic marker appearance: FIGURE 9b shows a time course (3, 6, and 10 days of differentiation) on mock infected (Δ44-PGK) and exon-skipping treated (Δ44#4) Δ44 cells. Dystrophin rescue in treated cells was paralleled by a relevant temporal restoration of late/terminal differentiation markers; in particular, MHC appeared at day 6 similarly to control cells, even if at lower levels, and muscle creatine kinase (MCK) at day 3. These results clearly demonstrate that, with respect to healthy myoblasts, the delay of appearance of late myogenic markers (MHC and MCK) in untreated (Δ44) and mock infected (Δ44-PGK) DMD cells is recovered in exon-skipping treated (Δ44#4) cells. Moreover, immunostaining analysis of FIGURE 9c indicates that the terminal differentiation marker MHC, already present at day 8 of differentiation in control cells (CTRL), is almost undetectable in mock infected Δ44 cells (Δ44-PGK) at day 12. At this time point, efficient recovery of MHC staining was detected when exon skipping was applied (Δ44#4).

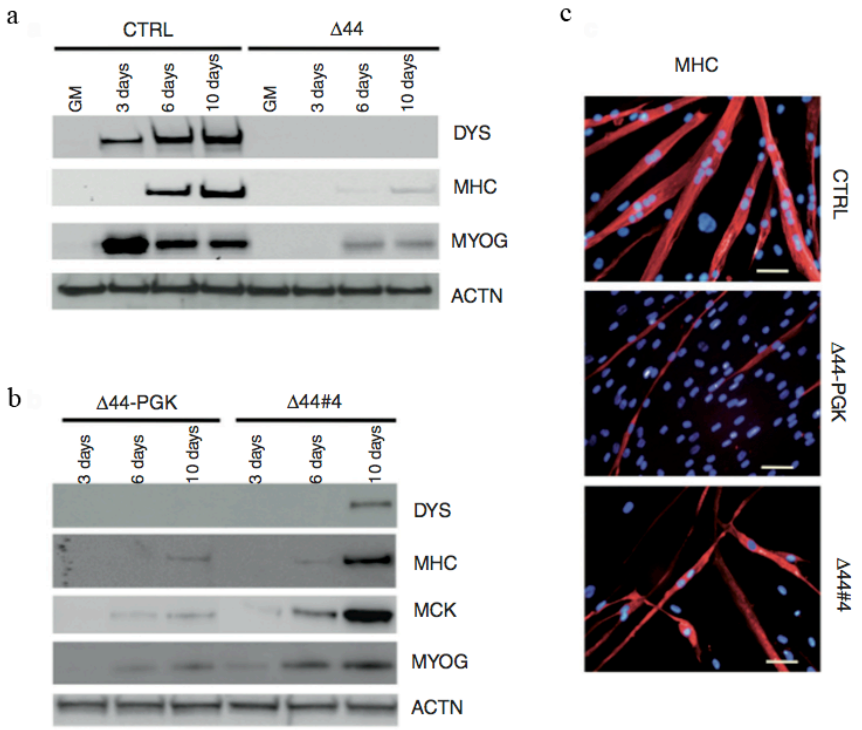
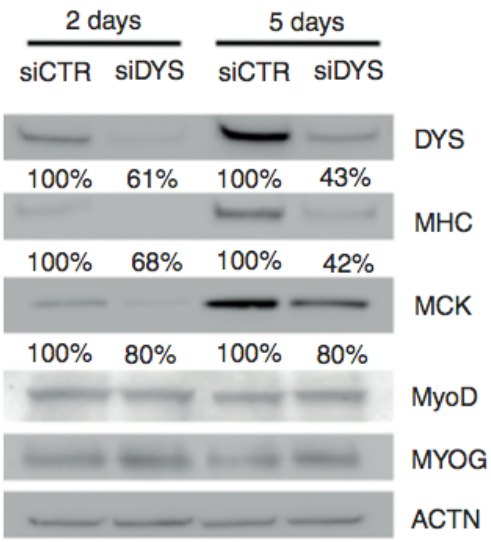


FIGURE 9 - Analysis of muscle differentiation markers.
(a) Western blot on proteins (10µg) extracted from CTRL and Δ44 cells probed with anti-dystrophin (DYS), anti-myosin heavy chain (MHC), and anti-myogenin (MYOG) antibodies in growth medium (GM) and at different days upon shift to differentiation conditions. Actinin (ACTN) was used as a loading control. (b) Western blot on proteins (50 µg) extracted from Δ44 cells, infected with lentivirus expressing GFP (Δ44-PGK) or with the #4 construct (Δ44#4), at different days upon shift to differentiation conditions. Western blot was probed with anti-dystrophin (DYS), anti-MHC, anti-muscle creatine kinase (MCK), and anti-myogenin (MYOG) antibodies. Actinin (ACTN) was used as a loading control. (c) Immunofluorescence for MHC localization (red) on control myoblasts (CTRL) at 8 days of differentiation, in parallel with mock infected (Δ44-PGK) and exon-skipping treated (Δ44#4) Δ44 DMD cells at 12 days of differentiation. DAPI staining for nuclei detection is also shown.

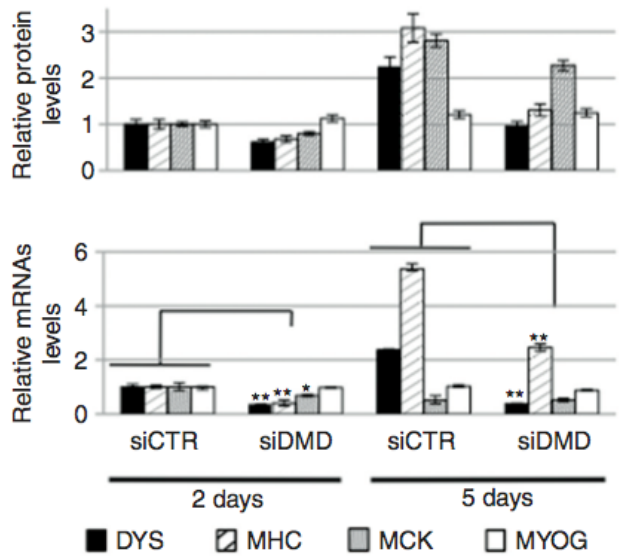
To further analyze the link between dystrophin expression and muscle differentiation, RNA interference against dystrophin was performed on human myoblasts from a healthy control. siRNAs were transfected in myoblasts in grow medium and one day after shift to differentiation medium. RNA and protein samples were collected at 2 and 5 days of differentiation. After 2 days of treatment, dys-siRNA treated cells (siDYS) showed a significant reduction of dystrophin mRNA (residual 61%) that, after 5 days, reached a residual value of 43% (FIGURE 10a). Dystrophin decrease paralleled that of mRNA levels, even if at a lower extent (FIGURE 10b), probably due to the well known long half-life of the dystrophin protein. Notably, in RNAi-treated cells, both MHC and MCK accumulated at lower levels, with MHC being reduced to 42% of control levels at day 5 of differentiation; in contrast, early myogenic factors such as myogenin and myoD, were not affected by dystrophin reduction both in terms of protein and mRNA (FIGURE 10a, b).
Altogether, these data support the hypothesis that dystrophin is a crucial myogenic factor regulating the progression from early to late phases of differentiation.

FIGURE 10 - RNA interference against dystrophin.
Control human myoblasts were transfected with scramble (siCTR) or with anti-dystrophin (siDYS) siRNAs and differentiated for 2 and 5 days. (a) Western blot was performed with anti-DYS, anti-MHC, anti-MCK, anti-MyoD, and anti-MYOG antibodies. Actinin (ACTN) was used as loading control. (b) Upper panel: The histogram indicates DYS, MHC, MCK, and MyoG protein levels normalized on actinin signals. Error bars: means ± SD. Lower panel: DYS, MHC, MCK, and MyoG mRNAs relative expression in mock (siCTR) MCK, and DMD-siRNA treated (siDYS) human myoblasts after 2 and 5 days of differentiation assessed by quantitative reverse transcriptase-PCR (qRT-PCR). GAPDH was used as internal control. Relative expressions are shown with respect to mock (siCTR) cells, set to a value of 1. *$P < 0.05$, **$P < 0.01$.

a


2 days
5 days
siCTR
siDYS
siCTR
siDYS
DYS
100%
61%
100%
43%
MHC
100%
68%
100%
42%
MCK
100%
80%
100%
80%
MyoD
MYOG
ACTN

b


Relative protein levels
3
2
1
0
Relative mRNAs levels
6
4
2
0
siCTR
siDMD
siCTR
siDMD
2 days
5 days
DYS
MHC
MCK
MYOG

Dystrophin rescue and miRNAs expression levels

In order to test whether the dystrophin protein produced in Δ44 myoblasts through skipping of exon 45, relocalizes and stabilizes nNOS, an immunofluorescence assay was performed with nNOS antibodies on myoblasts from: healthy control (CTRL), mock-infected (Δ44-PGK), and exon-skipping treated (Δ44#4) Δ44 cells. FIGURE 11 shows the correct localization of nNOS in control myotubes, revealed by well-defined increase in sarcolemmal labelling. Indeed, this striated staining is almost absent in Δ44-untreated myoblasts (Δ44-PGK), where only faint diffused cytoplasmic staining is visible. These results are in line with the well-known decrease and delocalization of nNOS in DMD patients (Brenman et al., 1995; Kobayashi et al., 2008). Notably, when Δ44 cells were treated with exon skipping (Δ44#4), they displayed a well visible nNOS signal distributed along the fibers. These results indicate that the recovery of dystrophin synthesis allows the correct relocalization and stabilization of nNOS at the sarcolemma and that exons 44 and 45 are dispensable for this function.

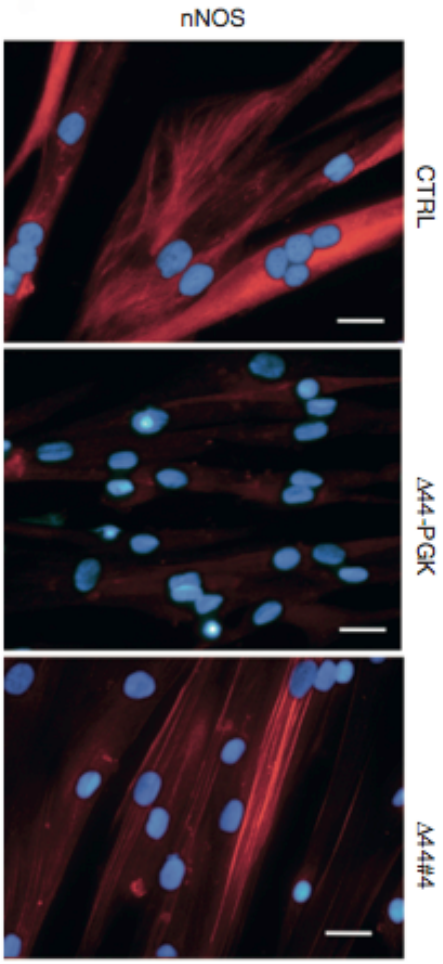


FIGURE 11 - Analysis of neuronal nitric oxide synthase (nNOS) localization exon-skipping treatment.
nNOS localization analysed by immunofluorescence with nNOS antibodies on CTRL, mock-infected Δ44 (Δ44-PGK) and exon skipping Δ44-treated cells (Δ44#4) after 10 days of differentiation. Original magnification,×40. Bar = 25 μm.

It has been previously shown (Cacchiarelli et al., 2010) that the DYS-nNOS pathway regulates, through the control of HDAC2 activity, the expression of a specific subset of miRNAs with specific function in muscle terminal differentiation (miR-1 and miR-133) or in muscle-degenerative processes such as fibrosis (miR-29 and miR-30). Conversely, miRNAs expressed in regenerating fibres, such as miR-31 and miR-206, were shown not to be controlled through this pathway. In order to test the dependence of these miRNAs from nNOS rescue in exon skipping treated Δ44 myoblasts, quantitative reverse transcriptase-PCR was performed on total RNA extracted from WT and Δ44 cells either mock infected (PGK) or treated with the different antisense constructs (#1 through #8). FIGURE 12 indicates that in Δ44 myoblasts miR-1 and miR-133 levels are strongly reduced with respect to healthy myoblasts (CTRL), in agreement with previous observation in Δ48–50 DMD myoblasts; however, when exon skipping recovered dystrophin synthesis, increase in the levels of both miRNAs was obtained.

At difference with these miRNAs, miR-31 was upregulated in Δ44 cells and, upon dystrophin rescue, its expression remained high (FIGURE 12). Recently, it has been shown that Duchenne myoblasts contain reduced levels of linc-MD1 (Cesana et al., 2011), a long noncoding RNA required for muscle differentiation. FIGURE 12 (lower panel) indicates that also Δ44 myoblasts have low levels of linc-MD1; notably, recovery of dystrophin synthesis induced increase in linc-MD1 levels. ChIP experiments on linc-MD1 promoter in mouse myoblasts indicated that this transcript is not under the control of HDAC2 (C. Pinnarò, personal communication); therefore, the increase of linc-MD1 upon dystrophin restoration does not directly depend on the DYS-nNOS pathway but it is likely due to an indirect effect on muscle differentiation, as previously discussed.

Altogether, these data confirm that the recovery of dystrophin in Δ44 cells restores nNOS sarcolemmal localization and in turn the biosynthesis of those miRNAs that depend on the DYS-nNOS pathway.

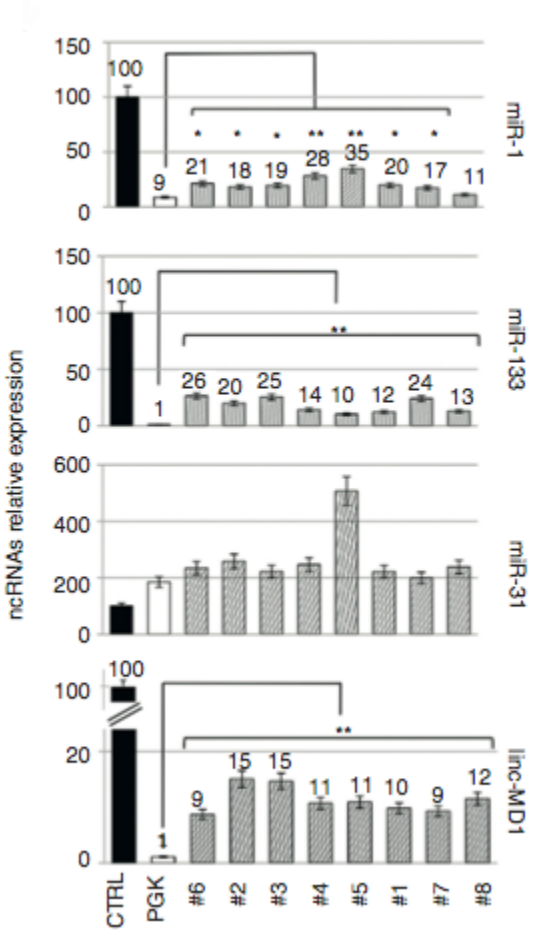
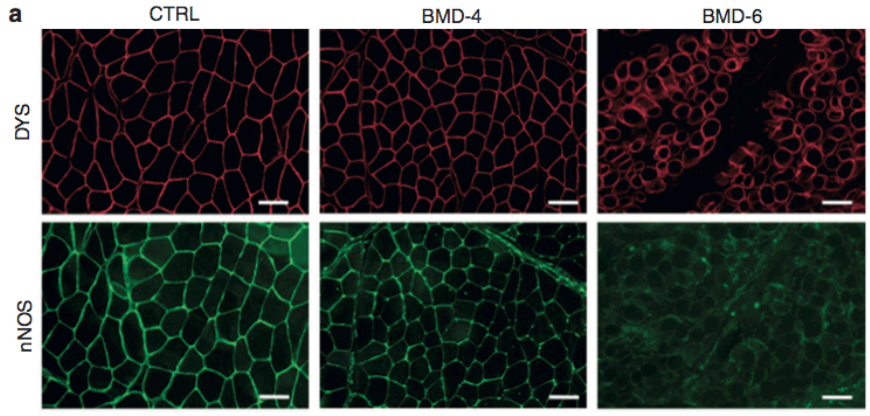


FIGURE 12 - ncRNAs expression upon exon-skipping treatment.
miRNAs and linc-MD1 expression analysis performed by quantitative reverse transcriptase-PCR (qRT-PCR) on RNA extracted from CTRL, mock-infected Δ44 (PGK), and Δ44 cells treated with the different antisense constructs (#1 – #8) after 10 days of differentiation. U6 small nuclear RNA (snRNA) is used as endogenous control. Relative expressions are shown with respect to CTRL cells, set to a value of 100 and ncRNA relative quantifications are shown on the top of each lane. *P < 0.05, **P < 0

Becker Muscular Dystrophy and nNOS

To further correlate the beneficial effects of dystrophin with the integrity of the DYS-nNOS pathway, we analysed 14 biopsies from Becker patients carrying mutations differently affecting nNOS localization (representative examples are shown in FIGURE 13a). The expression of miR-1 and miR-29, previously shown to respond to the DYS-nNOS pathway, was analysed from total RNA obtained from eight of such biopsies. Figure 13c shows that BMD samples lacking nNOS (BMD-5, -6, -7, and -8) display low levels of miR-1 and miR-29 if compared to BMD samples with reduced or normal levels of nNOS (BMD-1, -2, -3, and -4), confirming the direct correlation between nNOS and specific miRNA expression.
Interestingly, in agreement with previous work, biopsies lacking or having trace levels of nNOS corresponded to patients displaying an overall more severe clinical phenotype (FIGURE 13b and Table 2), measured as Hammersmith functional motor scale. This scale, that is routinely performed in the clinical follow-up of patients, consist in 20 consecutive motor activities each scored on a 3-point scale (2 to 0) and the total test score can range from 0 if the child cannot perform any of the items to 40 if all the items are fully achieved. The table indicates that in BMD patients the absence of nNOS correlates with a worse outcome (patients BMD-5, -6, -9, and -14) with the only exception of BMD-7 that show traces of nNOS, low miRNA levels but unaffected HFMS score.
Becker mutations completely lacking nNOS are those missing exons 45-47 and 45-49 as they more severely disrupt spectrin like repeats 16 and 17. However, the finding that deletion 45-51 has visible levels of nNOS indicates that a direct relationship between primary sequence and protein activity cannot be simply made.



b

ID	Deletion	nNOS
CTRL	/	Present
BMD-1	39	Present
BMD-2	74	Reduced
BMD-3	48–49	Present
BMD-4	45–51	Present
BMD-5	45–47	Absent
BMD-6	45–49	Absent
BMD-7	45–49	Absent
BMD-8	42–53	Traces

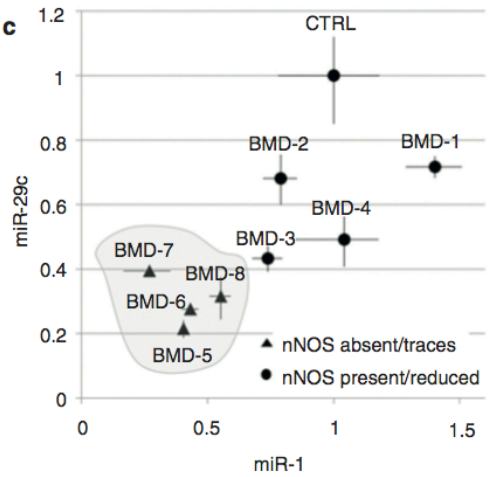


FIGURE 13 – neuronal Nitric Oxide Synthase (nNOS) localization and miRNAs expression in Becker biopsies.
(a) Dystrophin (DYS) and nNOS localization analyzed by immunofluorescence on control (CTRL), BMD-4, and BMD-6 human biopsies. Original magnification, ×10. Bar = 100μm. (b) Table summarizes exon deletions of the different BMD patients and corresponding nNOS levels. (c) Scatterplot showing the expression levels of miR-29c (Y axis) and miR-1 (X axis) analyzed by quantitative reverse transcriptase-PCR (qRT-PCR) on RNAs extracted from the different BMD biopsies. U6 small nuclear RNA (snRNA) is used as endogenous control. Relative expressions are shown with respect to healthy individual (CTRL), set to a value of 1. Error bars: means ± SD.

ID	Centre	Deletion	nNOS	Age and HFMS score at biopsy		Age and score at last HFMS	
Control	R	/	present	12	/	/	
BMD-1	L	39	present	6	40/40	9	40/40
BMD-3	R	48 - 49	present	3	33/40	5	33/40
BMD-4	R	45 - 51	present	13	40/40	14	40/40
BMD-10	R	45 - 55	present	8	40/40	11	40/40
BMD-11	R	point mutation	present	9	40/40	9	40/40
BMD-12	R	48	present	8	40/40	9	40/40
BMD-13	R	48 - 53	present	8	40/40	10	40/40
BMD-2	L	74	reduced	7	40/40	11	40/40
BMD-7	L	42 - 53	traces	7	40/40	15	40/40
BMD-8	L	9 - 12	traces	15	34-36/40		
BMD-5	L	45 - 49	absent	7	*		*
BMD-6	R	45 - 49	absent	2	37/40	7	21/40
BMD-9	R	point mutation	absent	1	**	2	35/40
BMD-14	L	45 - 47	absent	4	38/40	11	34/40

R= Rome, L=London, HFMS= Hammersmith functional motor scale.
*This boy had no physiotherapy assessments, however he presented with a severe phenotype, almost resembling an intermediate DMD. At 17 years he walks 100 meters before stopping, he requires a wheelchair outdoors and in college
** too young to have motor functional assessment but he presented proximal weakness with positive gowers manouvre and waddling gait
In bold biopsies for which RNA was available and analyzed (see Fig. 4c)

TABLE 2- Clinical information of BMD patients

miR-31 is upregulated in DMD myoblasts

Microarray analysis on total RNA from WT and *mdx* muscle revealed the deregulation, in pathologic condition, of different miRNAs. Among them miR-31 is upregulated more than 60-fold in *mdx* mice if compared with control ones. In situ hybridization (FIGURE 14) showed that miR-31 has a preferential localization in regenerating myoblasts identified by the characteristic phenotype of mononucleated fibres, which are abundant in mdx conditions and almost absent in wild type.

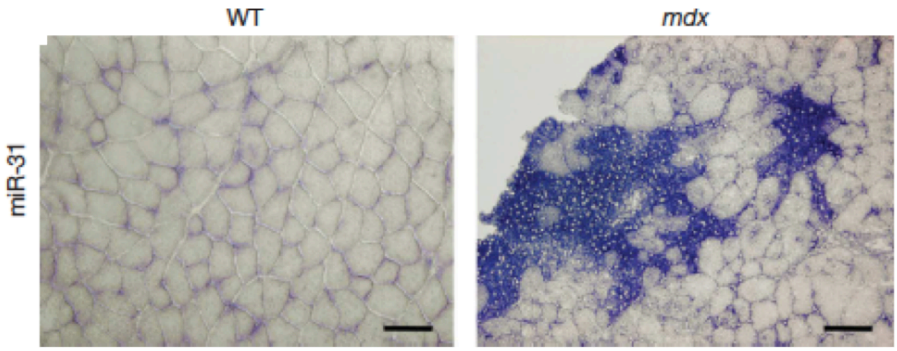


FIGURE 14 – miR-31 in-situ hybridization on WT and *mdx* gastrocnemius sections.

We analyzed its expression in our human DMD biopsies and data confirmed that miR-31 is more abundant in a dystrophic condition if compared to healthy and Becker muscles (FIGURE 15a). miR-31 remained high in DMD myoblasts induced to differentiate *in vitro,* whereas in human healthy controls its levels decreased with progression of differentiation (FIGURE 15b). DMD myoblasts appeared to have a higher proliferating ability and a lower differentiation potential than control cells. This was shown by the presence of PAX7 in growth conditions and by the delayed appearance of differentiation markers upon serum withdrawal (FIGURE 15c). In control myoblasts, myogenin peaked already at day 2 of differentiation, preceding myosin heavy chain and dystrophin synthesis; whereas in DMD cells, myogenin appeared only at day 4 preceding MHC synthesis, clearly visible at day 6. Immunostaining of the same samples confirmed that DMD cells express MHC protein at lower levels and later than control cells (FIGURE 15c and d).
These data indicate that the high levels of miR-31 in Duchenne muscles is due to the intensive regeneration involving activated satellite cells and to the reduced ability of Duchenne myoblasts to complete the differentiation programme.

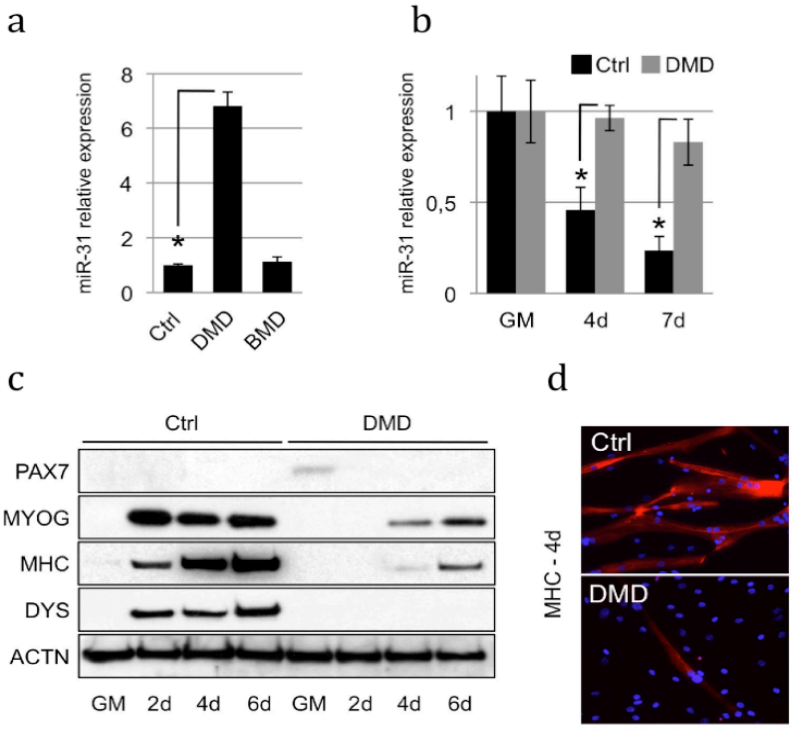


FIGURE 15 - miR-31 is expressed in delayed DMD myoblasts. (a) qRT-PCR of miR-31 relative expression in human muscle biopsies from healthy (Ctrl), Duchenne (DMD) and Becker (BMD) donors. (b) qRT-PCR of miR-31 relative expression in human primary myoblasts from healthy donors (Ctrl, black bars) or DMD patients (DMD, grey bars) in growth medium (GM) and at the indicated time points after shift to differentiation medium. (c) The same cells were analyzed by Western blot for PAX7, myogenin (MyoG), Myosin Heavy Chain (MHC) and Dystrophin (DYS). Actinin (ACTN) was used as a loading control. The values are derived from at least three independent experiments. Asterisks: $p<0.05$. (d) Immunofluorescence staining with MHC antibodies (red) and DAPI (blue) on Ctrl and DMD myoblasts at 4 days after induction of differentiation.

miR-31 targets dystrophin mRNA

In silico analysis, performed by comparing mRNA expression profiles in wild type versus *mdx* animals (data not shown), identified a large number of predicted targets of miR-31. Notably, the most down-regulated mRNAs in *mdx* muscles encode for proteins involved in terminal differentiation, including dystrophin. One putative binding site, embedded in a 40 nucleotide region perfectly conserved among mammalian species, was identified in the 3'UTR of the dystrophin mRNA (FIGURE 16a). Wild type dystrophin 3'UTR (DMD-WT) and a derivative mutated in the miR-31 target site (DMD-*mut*) were fused to the luciferase ORF (FIGURE 16b). Enzyme activity was measured in C2 myoblasts in endogenous conditions and upon miR-31 overexpression (FIGURE 16c). The results indicate that miR-31 repressed luciferase activity only on DMD-WT and that de-repression was obtained with LNA oligos against miR-31 and not with control scramble LNA. Scramble LNA transfection *per se* did not affect luciferase activities (not shown). Moreover, when cells were treated with a sponge-construct containing four binding sites for miR-31 (Sponge-31, FIGURE 16a and b), luciferase activity resulted higher with respect to cells treated with a control construct (FIGURE 17d). Release from miR-31 repressing activity was also obtained when cells were treated with a LNA oligo complementary to 23 nucleotides across the miR-31 target site (protector-31) both in the presence of the endogenous miR-31 and in conditions of miR-31 overexpression (FIGURE 16e).
These data indicate that miR-31 targets the 3'UTR of the dystrophin mRNA and that repression is prevented either by the use of miR-31 decoys or by protecting the miR-31 binding site on dystrophin mRNA.

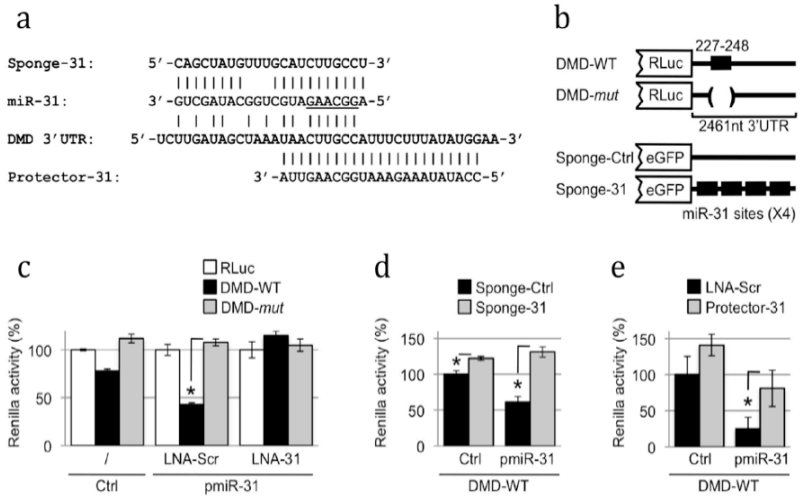


FIGURE 16 - miR-31 targets dystrophin mRNA.
(a) Sequences of: the mature miR-31, the conserved 40 nucleotide region of the DMD 3'UTR, the decoy sequence of the sponge-31 construct and the 23 nucleotide LNA protector (Protector-31) covering miR-31 binding site on the DMD 3'UTR. miR-31 seed sequence is underlined. (b) Schematic representation of: the luciferase constructs containing the wild type 3'UTR of the dystrophin mRNA (DMD-WT) and its mutant derivative with a deletion of the miR-31 target site (DMD-*mut*), the sponge constructs containing the GFP gene with a control 3'UTR (Sponge-Ctrl) or with the same 3'UTR containing four miR-31 decoy sites (Sponge-31). (c) Renilla Luciferase activity in proliferating C2 myoblasts transfected with the control Rluc vector or DMD-WT or DMD-*mut.* in combination with the plasmid overexpressing miR-31 (pmiR-31) or a control plasmid (Ctrl). Cells were also transfected with control (LNA-Scr) or anti-miR-31 (LNA-31) LNA oligos. Renilla activity of the Rluc vector was set to a value of 100% in all treatments. (d) Renilla Luciferase activity in C2 myoblasts transfected with DMD-WT, in the presence of Sponge-Ctrl or Sponge-31 constructs, with pmiR-31, or a control plasmid. (e) Renilla Luciferase activity in C2 myoblasts transfected with DMD-WT alone (Ctrl) or upon miR-31 overexpression (pmiR-31) in the presence of control (LNA-Scr) or protector (Protector-31) LNAs. The values of all experiments are derived from at least three independent experiments. Asterisks: $p<0.05$.

miR-31 inhibition enhances exon skipping treatment

In vitro differentiation of C2 mouse myoblasts indicated that miR-31 was expressed at good levels in proliferating conditions (GM) and its levels decreased upon differentiation (FIGURE 17a). Synthesis of dystrophin was prominent only at day 5 even though transcription was already consolidated at day 3. These data reflected the expected inverse correlation between the miRNA and its putative target. We next tested in C2 myoblasts whether the endogenous synthesis of dystrophin correlated to altered levels of miR-31 (FIGURE 17b): overexpression was obtained through infection with a lentivirus containing the pri-miR-31 expression cassette (miR-31), while depletion was obtained via administration of LNA-31 or sponge-31. When cells were induced to differentiate, a consistent decrease of dystrophin (almost 3-fold) was observed in conditions of persistent overexpression of miR-31; on the contrary, increase in dystrophin levels was detected when cells were treated with LNA-31 or sponge-31. In the last two cases, the limited increase (50 and 40% respectively) of dystrophin synthesis is likely due to the fact that miR-31 levels already start to decrease at 3 days of differentiation (FIGURE 17a). In the lower panels of Figure 17b the levels of the dystrophin protein are compared with those of its mRNA; in all cases dystrophin mRNA levels were not affected by miR-31 modulation, indicating that the miRNA acts by repressing translation rather than controlling dystrophin mRNA stability.
DMD myoblasts, from patient with deletion of exons 48-50 were infected with the U1#51 construct able to induce skipping of exon 51 and to rescue dystrophin synthesis (FIGURE 18a and b; Incitti et al., 2010). In order to check whether dystrophin synthesis could be further improved by reducing miR-31 levels, we tested the ability of the sponge-31 construct to increase dystrophin levels when infected in Δ48-50 DMD myoblasts. Figure 18b shows that sponge construct was indeed able to increase by 3-fold dystrophin synthesis when exon skipping was applied to these cells. Moreover, also the anti-miR-31

LNA oligos were able to increase dystrophin synthesis (2-fold) when transfected into exon skipping-treated cells. qRT-PCR showed that dystrophin mRNA levels were the same in the different conditions, similarly to the MCK mRNA.

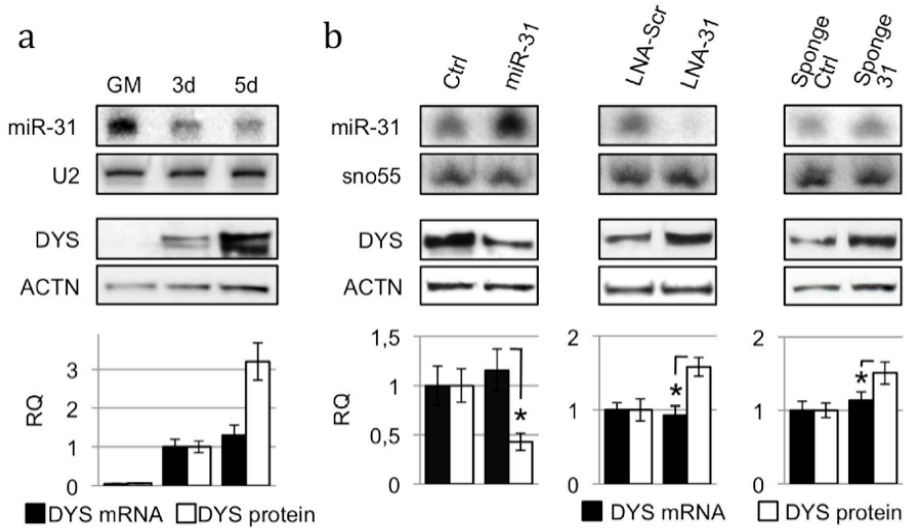


FIGURE 17 - Effects of miR-31 modulation on dystrophin expression. (a) Northern (miR-31 and U2) and Western (DYS and ACTN) analyses of C2 mouse myoblasts in growth medium (GM) and at 3 and 5 days after shift to differentiation medium. The histogram at the bottom shows the relative quantification (RQ) of dystrophin protein and mRNA levels referred to the 3 day point set to a value of 1. (b) Northern (miR-31 and sno55) and Western (DYS and ACTN) analyses on C2 myoblasts treated with: lentiviruses with control (Ctrl) or miR-31 (miR-31) expression cassettes, control (LNA-Scr) or anti-miR-31 (LNA-31) LNA oligos and lentiviruses carrying Sponge-Ctrl or Sponge-31. Differentiation was allowed to proceed for 3 days. Dystrophin mRNA (DYS mRNA, black bars) and protein (DYS protein, white bars) levels were measured from three independent experiments. Histograms at the bottom show the relative quantifications (RQ) of dystrophin protein and mRNA levels referred to the values of control experiments (Ctrl, LNA-Scr and Sponge-Ctrl) set to 1.

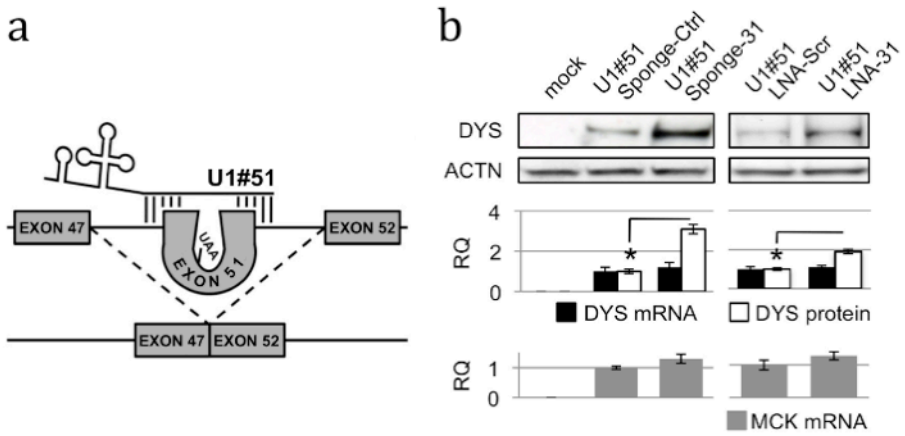


FIGURE 18 - miR-31 decoy enhances the beneficial effects of exon skipping

(a) Schematic representation of the exon skipping strategy for the human Δ48-50 DMD mutation. (b) human Δ48-50 DMD myoblasts were infected with control (mock) or U1#51 lentiviruses alone or in combination with the miR-31 sponge (U1#51 Sponge-31). Alternatively, cells treated with the exon skipping construct (U1#51) were transfected with control (LNA-Scr) or anti-miR-31 (LNA-31) LNA oligos. Cells were induced to differentiate for 5 days and dystrophin quantified by Western blot. Histograms show the RQ of dystrophin protein and mRNA levels as well as MCK mRNA levels respectively referred to the values of control experiments set to 1. Asterisks: $p<0.05$.

DISCUSSION

Inducing exon skipping as a therapeutic approach of DMD relies on the concept of converting severe Duchenne phenotypes into the milder Becker ones. However, the latter display a large range of phenotypes likely reflecting the ability of shorter forms of dystrophin to accomplish only a subset of the different functions of the wild-type full-length protein. Multiple variables play a role in determining the efficiency of internally shortened dystrophins produced in Becker patients: these relate to structural properties of the residual dystrophin molecules as well as to their capacity to correctly assemble DAPC complexes and to relocalize nNOS (Anthony et al., 2011).
Regarding the former, the spectrin-like repeat region is composed of 24 modular repeats found in the rod domain. While removing an integral repeat is mostly dispensable, deletions that affect the phasing of the repeats are associated with less stable and functional dystrophin molecules (Harper et al., 2009). Concerning the role that different internal dystrophin deletions have on assembly of DAPC proteins, we have recently demonstrated that BMD dystrophins of patients with deletions located around exon 51 and 53 are more efficient in re-localising at the sarcolemma β–destroglycan and α–sarcoglycan compared to other deletions that remove spectrin-like repeats 16 and 17 (Anthony et al., 2011). There is also a strong correlation between deletions of spectrin-like repeats 16 and 17 and ability to localize nNOS at the sarcolemma (Wells et al., 2003; Lai et al., 2009).
These are crucial points when thinking of possible therapeutic benefits of exon skipping in different DMD mutations and suggest that dystrophin rescue should be tested together with correct recovery of the entire complex of proteins at the sarcolemma. We have recently shown that in DMD mutants lacking exons 48–50, further skipping of exon 51 is able to relocalize α-sarcoglycan and nNOS (Cirak et al., 2012).
The relevance of nNOS was previously shown to rely on its ability to control gene expression through nitrosylation of HDAC2.

The rescue of this pathway in dystrophic animals has been proved to ameliorate the dystrophic phenotype and to influence the expression of a specific subset of genes playing a crucial role in the control of muscle terminal differentiation and tissue homeostasis (Cacchiarelli et al., 2010): among them, those coding for relevant myogenic miRNAs (miR-1 and miR-133) and for species controlling fibro-adipocitic degeneration (miR-29 and miR-30). These data suggested that nNOS activity plays an important role in muscle homeostasis via its ability to directly regulate the expression of specific HDAC2 target genes and to have a positive effect on differentiation.

In this work, we produced antisense molecules, raised in the U1 snRNA backbone, able to induce effective skipping of exon 45 that is one of the most common, after exon 51, in terms of number of patients that can be cured (8.1% of known DMD mutations). We showed that rescue of dystrophin provided a considerable enhancement of differentiation in DMD myoblasts, well characterized for their delay in reaching a mature molecular and morphological phenotype. This evidence was further supported by experiments of RNAi against dystrophin in myoblasts from healthy individuals. In this case an evident delay in timing of myogenic markers synthesis was observed. Notably, while having such a clear effect on late differentiation markers, dystrophin did not show any specific effect on timing and levels of early myogenic markers such as Myogenin and MyoD.

The dystrophin protein, obtained through skipping of exon 45 in a Δ44 genetic background, produced efficient rescue of nNOS localization at the membrane, together with recovery of miR-1 and miR-133 expression, indicating the reactivation of the DYS-nNOS pathway. In line with the relevance of this pathway, nNOS analysis of different Becker individuals indicated that those lacking correct localization of the enzyme had a more severe clinical phenotype as well as reduction of those marker miRNAs depending on the DYS-nNOS pathway.

Altogether, these data demonstrate a crucial role of dystrophin in regulating the switch between early and late phases of muscle differentiation and point to the relevance of the DYS-nNOS pathway in DMD and BMD.
It is well known that DMD is a monogenic disease due to mutations in the dystrophin gene; but the absence of the protein, as discussed before, has multiple effects on different pathways involved in muscle homeostasis and differentiation. For this reason, when thinking of a possible therapeutic approach, we should prefer a strategy able to restore dystrophin synthesis, exon skipping for example, but we can combine it with other corollary treatments (HDAC inhibitors, corticosteroids, physiotherapy) that can improve the effect of the main approach.
We described miR-31 as part of circuitries controlling late muscle differentiation by repressing dystrophin synthesis and, likely, many other terminal differentiation markers: for example miR-31 was described to repress Myf5 both in muscle and brain (Daubas et al., 2009; Crist et al., 2012).
miR-31 repressing activity was detected in early phases of myoblasts differentiation supporting the idea that this control is necessary in normal muscle cells to avoid early expression of late differentiation markers and, specifically, dystrophin.
The intense and localized expression of miR-31 in regenerating myoblasts of dystrophic muscles indicated that the high levels of miR-31 found in dystrophic conditions (both in mouse and human biopsies) are due to the intensive regeneration program that is mediated by the activation of satellite cells. Interestingly, in dystrophic myoblasts and satellite cells the lack of dystrophin correlated with a delay of the maturation process of the cells.
In this study we have also shown that in dystrophic conditions, when dystrophin synthesis is rescued through the exon skipping strategy, the inhibition of miR-31 activity increased dystrophin production. Since in a compromised muscle the contribution to dystrophin production by regenerating fibers is quite relevant, miR-31 repression

in this compartment can represent an improvement to current therapeutic treatments aimed to increase the levels of dystrophin synthesis. Rescue of consistent levels of dystrophin will also have additional benefits such as the completion of muscle fiber maturation process.

MATERIALS AND METHODS

Antisense clones construction. Clones U1-45 were obtained by inverse PCR on the construct pCCL-5′3′Esx (Incitti et al., 2010). The oligos utilized are:

U1-45#1:
F:(5'CAGAAAAAAGAGGTAGGGCGACAGGGCAGGGGAGATACCATGATC 3')
R:(5'CCTGGAGTTCCTGTAAGATACCAAAAAGGCATGAGATCTTGGGCCTCTGC3')

U1-45#2:
F:(5'GCCCAATGCCATCCTGGAGTTCCTGGGCAGGGGAGATACCATGATC 3')
R:(5'TCCTCAAAAACAGATGCCAGAAGAGGTAGGGCGATGAG ATCTTGGGCCTCTGC 3')

U1-45#3:
F:(5'GCTGCCCAATGCCATCCTGGAGTTCCTGGGCAGGGGAGATACCATGATC 3')
R:(5'CAGCAATCCTCAAAAACAGATGCGAGGTAGGGATGAGATCTTGGGCCTCTGC 3')

U1-45#4:
F:(5'GAGGTTGCTGCCTGGAGTTCCTGTAAGGGCAGGGGAGATACCATGATC 3')
R:(5'AAAAACAGAGAAAAAAGAGGTAGGGCGATGAGATCTTGGGCCTCTGC 3')

U1-45#5:
F:(5'ATTGCCCAATGCCATCCTGGAGTTCCTGTGGCAGGGGAGATACCATGATC 3')
R:(5'CCTCAAAAACAGAGAAAAAAGAGGTAGGGATGAGATCTTGGGCCTCTGC 3')

U1-45#6:
F:(5'GCTGCCCAATGCCATCCTGGAGTTCCTGTAAGGGCAGG
GGAGATACCATGATC 3')
R:(5'CAGCAATCCTCAAAAACAGATGCATGAGATCTTGGGC
CTCTGC 3')

U1-45#7:
F:(5'GCTGCCCTGCCTCCTGGAGTTCCTGTAAGGGCAGGGGA
GATACCATGATC 3')
R:(5'CAGAAAAAAGAGGTAGGGCGACAGATGAGATCTTGGG
CCTCTGC 3')

U1-45#8:
F:(5'CCTGGAGTTCCTGTAAGATACCAAAAAGGCGGCAGGG
GAGATACCATGATC 3')
R: (5'ATGGCATTGGGCAGCATGAGATCTTGGGCCTCTGC 3')

C_27 transfections
C27 cells were plated on 3,5 cm diameter plates and co-transfected with 3 μg of the lentiviral vector carrying the antisense expression cassette and 1 μg of U16RBE plasmid used as a transfection efficiency control. Transfection was performed according to the Lipofectamine 2000 protocol (Invitrogen, Carlsbad, CA). Cells were grown in Dulbecco's modified Eagle's medium 10% fetal bovine serum for 36 hours and then harvested with 1 ml of QIAzol Lysis Reagent (Qiagen, West Sussex, UK).

RNA preparation and analysis
Cells were harvested with 1 ml of QIAzol Lysis Reagent (Qiagen) and biopsies were homogenized with a rotor-stator homogenizer in the presence of QIAzol Lysis Reagent (Qiagen). RNAs were extracted by miRNeasy (Qiagen), following manufacturer's specifications; concentration was assessed with Nanodrop ND-1000 Spectrophotometer (CELBIO, Pero, Milan, Italy).

qRT-PCR were performed using miScript System (Qiagen).

Northern Blot
Antisense expression was analyzed by Northern blot as previously described (Incitti et al., 2010) using the following probe: anti-45 (5'-CAGGAACTCCAGG-3').

Primary myoblasts cultures
Cultures of primary myoblasts (WT-9808 and Δ44-9981 from the Telethon Neuromuscular Biobank) were first pre-plated in order to separate fibroblasts from the primary line, then seeded in Human Skeletal Muscle Growth Medium (PromoCell, Haidelberg, Germany) and grown in a humidified incubator, at 5% CO2 and 37°C.

Virus preparation and cell transduction
Viruses were prepared as described (Incitti et al., 2010). The day before transduction, myoblasts were seeded in Growth Medium, on 6 cm plates (at least two for each different virus), at a density of 5 x 105 cells per plate. The next day cells were infected once with lentiviruses and polybrene (4 mg/ml). Two days after infection, cells were induced to differentiate with Human Skeletal Muscle Differentiation Medium (PromoCell). After 10 days of differentiation, cells were washed twice with complete PBS buffer (PromoCell) and collected with 300 microliters of protein buffer (100 mM Tris-HCl pH 7.4, 1 mM EDTA, 2% SDS, 1x Complete EDTA- free Protease Inhibitor Cocktail (Roche, Applied Science, Mannheim, Germany) for protein extraction, or with 1 ml of QIAzol Lysis Reagent (Qiagen) for RNA extraction.

RT-PCR
Dystrophin mRNA was analyzed by RT-PCR (Access RT-PCR system – Promega, Madison, WI) on 200 ng of total RNA with oligos E42F (5'-GAAGACATGCCTTTGGAAATTTCT-3') and E48R (5'-CTGAACGTCAAATGGTCCTTC-3'). 4µl of the RT-PCR products

were then used as template for a nested reaction performed with oligo E43F (5'-CTACAACAAAGCTCAGGTCG-3') and E46R (5'-CTCTTTTCCAGGTTCAAGTGG-3').
Antisense expression was analyzed by RT-PCR (SuperScript® VILOTM cDNA Synthesis Kit -Invitrogen) on 50 ng of total RNA with oligos U1primer (5'- CAGGGGAAAGCGCGAACG-3') and RT45 (5'- ATCCTGGAGTTCCTGTAA-3'). GAPDH was used as loading control (oligos: F 5'- GGAAGGTGAAGGTCGGAGTC-3';R 5'-TTACCAGAGTTAAAAGCAGCCC-3').
10 µl of the reactions were run on a 2% agarose-ethidium bromide gel and signals were revealed on a UV transilluminator.

Western blot analysis
Western blot analyses were carried out as previously described.28 Primary antibodies: anti dystrophyn (NCL-DYS1 Novocastra, New Castle upon Tyne, UK, 1:40 in 3% milk); anti myosin (anti-MF-20, 1:20 in TBST), anti muscle creatine kinase (MCK sc-15161, Santa Cruz biotecnology, Santa Cruz, CA) 1:500 in TBST); anti myogenin (MyoG sc-12732, 1:1000 in TBST); anti MyoD (DAKO, Glostrup, Denmark, 1:500 in 3% milk); anti actinin (ACTN sc-15335, 1:1000 in TBST). Secondary antibodies: ImmunoPure®Goat Anti-Rabbit IgG Peroxidase Conjugated (PIERCE, Rockford, IL, 1:5000 in 5% milk); ImmunoPure®Goat Anti-Mouse IgG Peroxidase Conjugated (PIERCE, 1:10000 in 5% milk); donkey anti-goat IgG-HRP (sc-2020, diluted 1:5000 in 3% milk).

RNA interference against dystrophin
The WT 9808 cell line, obtained from Telethon Genetic Biobank Network, was grown to 70% confluence on 6 cm plates in Dulbecco's modified Eagle's medium with 18% fetal bovine serum. Transfection was performed twice with 200 pmol of siRNA oligonucleotides targeting the dystrophin gene (Qiagen, target sequence: 5'-AATAACTTGCCATTTCTTTAT-3') or 200 pmol of siRNA negative control using lipofectamine (Invitrogen) according to

manufacturer's specification. After 18 hours, the medium was replaced. RNA and protein samples were collected after 2 and 5 days of differentiation.

Patients

Fourteen BMD patients, aged from 2 to 15 years, were assessed by physiotherapists to quantify muscular strength through Hammersmith motor ability score (Scott et al., 1982). Skeletal muscle biopsies were obtained, with informed consent, from all BMD patients along with non- myopathic control. BMD-3, BMD-4, BMD-6, BMD-9, BMD-10, BMD-11, BMD-12, BMD-13, BMD-14 and control were obtained from Laboratory of Molecular Medicine, Department of Neuroscience, Bambino Gesù Children's Hospital, Rome whereas BMD-1, BMD-2, BMD-5, BMD-7, BMD-8, BMD-15 biopsies were obtained from Dubowitz Neuromuscular Centre, Institute of Child Health and Great Ormond Street Hospital, London.

Immunohistochemistry

Muscle biopsies were mounted in OCT medium. Serial 7 μm transverse cryosections were fixed for 15 minutes at 4 °C in 4% paraformaldehyde (Electron microscopy Sciences, Hatfield, PA), permeabilized with Triton 0,2%/1%BSA/PBS for 15 minutes and subsequently blocked in 10% Goat Serum/1%BSA/PBS for 1 h at room temperature. Sections were incubated at 4°C with primary antibodies diluted in 5% Goat Serum/PBS (incubation buffer) for 16h. After serial washes in 0,2% Triton /PBS, secondary antibodies (Goat anti-Mouse IgG AlexaFluor 488, Invitrogen or Cy3 conjugated, Jackson ImmunoResearch, Inc., Pennsylvania) diluted 1:500 with incubation buffer were added for 1h at room temperature. The specificity of immunolabeling was verified in control samples prepared with the incubation buffer alone, followed by the second conjugated antibody. The sections were counterstained with 1.5 mg/ml 4′,6-Diamidino-2 phenylindole in Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA). Cultured cells

were fixed in Methanol/Acetone for 20 minutes at -20°C, subsequently air-dried and after brief rehydration with PBS processed for immunostaining with primary antibodies (diluted in 1% Goat Serum/PBS) as described above.
Primary monoclonal antibodies used in this study were diluted as follows: anti-Dystrophin NCL-DYS2, Novocastra Laboratories (diluted 1:12.5); anti-Nos-1 sc-55521 (diluted 1:50), anti-MHC from hybridoma supernatants.

Image acquisition and analysis
Immunostained cells and muscle sections were examined using a Zeiss AxioObserver A1 inverted fluorescence microscope equipped with Axiocam MRM R camera and Plan-Neofluar EC 10X/0,3 M27 and LD 40X/0,6 M27 objectives. The images were acquired with AxioVision Rel.4.8 imaging software.

Luciferase constructs and assays.
Full-length murine DMD- 3' UTR sequence (DMD-WT, 2,461 bp) was amplified by polymerase chain reaction and then cloned in NotI restriction site of the psicheck2 plasmid (Promega), downstream from the renilla luciferase (RLuc) gene. The same plasmid also contains the firefly luciferase (FLuc) to normalize transfection efficiency. Mutant derivative DMD-mut was obtained by deletion of miR-31 binding sites. RLuc and FLuc activities were measured by Dual Glo luciferase assay (Promega).

miRNA overexpression and sponge constructs.
Plasmid pmiR-31 was produced by cloning a pri-miR-31 with 100 nucleotides upstream and downstream from the pre-miRNA into the U1snRNA expression cassette (Denti et al, 2004). Sponge-31 construct was generated by cloning-annealed oligonucleotides containing four artificial miR-31 binding sites into the WPRE SacII restriction site downstream from the green fluorescent protein open reading frame, according to Gentner et al., (2009).

miR-31 sponge was combined with exon skipping by cloning-annealed oligos into the WPRE sequence of the green fluorescent protein reporter of lentiviral PCCL-U1#51 reported to induce skipping of DMD exon 51 (Incitti et al, 2010).

Statistical analyses

Each data shown in qRT-PCR is the result of at least three independent experiments. Data are shown as mean ± standard deviation (SD). Unless specifically stated, statistical significance of differences between means was assessed by two-tailed t-test and a $p < 0.05$ was considered significant.

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GLOSSARY

BMD: Becker Muscular Dystrophy
DAPC: Dystrophin Associated Protein Complex
DYS: Dystrophin
DMD: Duchenne Muscular Dystrophy
GFP: Green Fluorescent Protein
HDAC: Histon Deacetylase
LNA: Locked Nuclei Acid
mdx: murine model of Duchenne Muscular Dystrophy
miR: microRNA
mRNA: messenger RNA
nNOS: neuronal Nitric Oxide Synthase
snRNA: small nuclear RNA

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