miR-31 modulates dystrophin expression: new implications for Duchenne muscular dystrophy therapy

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Duchenne muscular dystrophy (DMD)—which is caused by mutations in the dystrophin gene—is one of the most severe myopathies. Among therapeutic strategies, exon skipping allows the rescue of dystrophin synthesis through the production of a shorter but functional messenger RNA. Here, we report the identification of a microRNA—miR-31—that represses dystrophin expression by targeting its 3' untranslated 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.

Keywords: DMD; dystrophin; miRNA; myoblasts; gene therapy
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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a genetic disorder caused by mutations in the dystrophin gene. With a few exceptions, all DMD mutations (substitutions, deletions and duplications) lead to frameshifts, resulting in the formation of premature termination codons that impair dystrophin translation. When mutations do not perturb the reading frame, as in the case of Becker muscular dystrophy (BMD), the translation of a partly functional protein still occurs. As internal in-frame deletions in the protein produce only mild myopathic symptoms, a therapeutic approach based on the skipping of specific mutated exons has been conceived. This can be obtained by antisense molecules that, by pairing with splice junctions or exonic splicing enhancers, prevent exon recognition by the splicing machinery (Dunckley *et al*, 1998). It has been demonstrated that both chemically modified antisense oligonucleotides and antisense RNAs—derived from expression cassettes delivered through viral vectors—can rescue dystrophin synthesis

in vitro (De Angelis *et al*, 2002; Aartsma-Rus *et al*, 2003) and *in vivo* (Bremmer-Bout *et al*, 2004; Goyenvall *et al*, 2004; Alter *et al*, 2006; Denti *et al*, 2006, 2008). Some of these studies have now entered clinical trials (Fletcher *et al*, 2006; van Deutekom *et al*, 2007).

Recent works have shown that microRNAs (miRNAs) have a crucial role in muscle development and function (Chen *et al*, 2006; Naguibneva *et al*, 2006). Moreover, altered levels of miRNAs were found in several muscular disorders, such as myocardial infarction (Van Rooij *et al*, 2008), DMD and other myopathies (Eisenberg *et al*, 2007; Greco *et al*, 2009). In line with this, it has been demonstrated recently that deregulated expression of miRNAs due to a lack of dystrophin accounts for several characteristics of DMD pathogenesis (Cacchiarelli *et al*, 2010).

In this work we show that a specific miRNA that is highly expressed in DMD conditions represses dystrophin translation, and is therefore a potential therapeutic target for improving current strategies aimed at rescuing dystrophin synthesis and promoting fibre maturation.

RESULTS AND DISCUSSION

Profile analysis of Duchenne compared with wild-type muscles indicated that several classes of miRNAs are differently expressed in *mdx* mice (Greco *et al*, 2009; Cacchiarelli *et al*, 2010). Among these, miR-31 showed a 50-fold enrichment compared with control (Fig 1A; supplementary Fig S1A online). *mdx* muscles are characterized by reduced fibre maturation, indicated by the decrease in miR-1 levels, and intensive regeneration, shown by the upregulation of miR-206 (supplementary Fig S1A online; Cacchiarelli *et al*, 2010). miR-31 and miR-206 abundance correlated with degeneration and regeneration processes, as, in muscles of 10-day-old *mdx* mice no upregulation was observed before the onset of dystrophic symptoms (supplementary Fig S1B online).

In situ hybridization (Fig 1B) 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. In line with this, miR-31 localized with miR-206 (a marker of activated muscle progenitors; Cacchiarelli *et al*, 2010) in MyoD⁺/MyoG⁺/MHC (myosin heavy chain)⁺ cells (supplementary

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Fig 1 | miR-31 expression. (A) miR-31 relative expression in WT and *mdx* mice, measured by qRT-PCR. (B) An miR-31 digoxigenin (DIG)-labelled probe was hybridized on WT and *mdx* gastrocnemius sections. Original magnification $\times 20$; scale bar, 100 μm . (C) miR-31 relative expression in WT and *mdx* mouse satellite cells, measured by qRT-PCR in GM and at the indicated time points after shift to differentiation medium. (D) qRT-PCR of miR-31 relative expression in human muscle biopsies from healthy (Ctrl), DMD and BMD donors. (E) qRT-PCR of miR-31 relative expression in human primary myoblasts from healthy donors (Ctrl) or DMD patients (DMD) in GM and at the indicated time points after shift to differentiation medium. (F) The same cells were analysed by western blot for PAX7, MYOG, MHC and DYS. ACTN was used as a loading control. The values are derived from at least three independent experiments. $*P < 0.05$. (G) Immunofluorescent staining with MHC antibodies (red) and 4'-6-diamidino-2-phenylindole (blue) on Ctrl and DMD myoblasts at 4 days after induction of differentiation. ACTN, actinin; BMD, Becker muscular dystrophy; Ctrl, control; DMD, Duchenne muscular dystrophy; DYS, dystrophin; GM, growth medium; MHC, myosin heavy chain; MYOG, myogenin; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; WT, wild type.

Fig S1C online). miR-31 was highly expressed at early stages of *in vitro* differentiation of wild-type mouse satellite cells, and its expression decreased progressively at later differentiation stages; by contrast, in *mdx*-derived satellite cells, the levels of miR-31 remained high even at prolonged differentiation times (Fig 1C). Persistent upregulation of miR-31 in *mdx* conditions was linked to delay of the muscle differentiation programme, as shown by longer persistence of Pax7, lower levels of MyoD and a delay in the appearance of myogenin expression in comparison with wild-type cells (supplementary Fig S2A online).

miR-31 was also more abundant in human DMD biopsies than in healthy and Becker muscles (Fig 1D). miR-31 levels remained high in DMD myoblasts induced to differentiate *in vitro*, whereas in healthy human controls its levels decreased with the progression of differentiation (Fig 1E). DMD myoblasts seemed to have a higher proliferating capacity and a lower differentiation potential than control cells. This was shown by the presence of PAX7 in growth conditions and the delayed appearance of differentiation markers after serum deprivation (Fig 1F). In control myoblasts, myogenin peaked at day 2 of differentiation, preceding MHC and dystrophin synthesis; in DMD cells, myogenin only appeared at day 4 preceding MHC synthesis, and was clearly visible at day 6. Immunostaining of the same samples confirmed that DMD cells express the MHC protein at lower levels and later times than wild-type cells (Fig 1G; supplementary Fig S2B online).

Altogether, these data indicate that the high levels of miR-31 in Duchenne muscles are partly due to intensive regeneration involving activated satellite cells and partly to the reduced ability of Duchenne myoblasts to complete the differentiation programme.

In silico analysis—performed by comparing messenger RNA (mRNA) expression profiles in wild-type and *mdx* animals (see supplementary information online)—identified many predicted targets of miR-31. Notably, the most downregulated mRNAs in *mdx* muscles code for proteins involved in terminal differentiation, including dystrophin (supplementary Fig S3 online). One putative binding site, embedded in a 40-nucleotide region conserved in mammalian species, was identified in the 3' untranslated region (UTR) of the dystrophin mRNA (Fig 2A). Wild-type dystrophin 3'UTR (DMD-WT) and a derivative mutated in the miR-31 target site (DMD-*mut*) were fused to the luciferase open reading frame (Fig 2B). Enzyme activity was measured in C2 myoblasts in endogenous conditions and, on miR-31 overexpression, obtained through transfection of a plasmid carrying the pri-miR-31 sequence under the strong and constitutive U1snRNA promoter (pmiR-31, Fig 2C). The results indicate that miR-31 repressed luciferase activity only on DMD-WT and that depression was obtained with locked nucleic acid (LNA) oligos against miR-31 (LNA-31) and not with control scramble LNA. Scramble LNA transfection did not affect luciferase activities

DMD myoblasts, derived from a patient with deletion of exons 48–50, were infected with the U1#51 construct able to induce skipping of exon 51 and rescue of dystrophin synthesis (Fig 3C; Incitti *et al*, 2010). To determine 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 Δ 48–50 DMD myoblasts. The sponge construct was indeed able to increase dystrophin synthesis threefold when exon skipping was applied to these cells (Fig 3D, U1#51-Sponge-31). Moreover, the anti-miR-31 LNA oligos were able to increase dystrophin synthesis twofold, when transfected into exon-skipping-treated cells (Fig 3D). Quantitative reverse transcription-polymerase chain reaction showed that dystrophin mRNA levels were the same in the different conditions, similarly to the case in muscle creatine kinase (MCK) mRNA (Fig 3D). These results indicate that dystrophin increase is mainly due to translational de-repression as a consequence of miR-31 depletion.

Fig 3 | Effects of miR-31 modulation on dystrophin expression. (A) Northern (miR-31 and U2) and western (DYS and ACTN) blot 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 RQs of dystrophin protein and mRNA levels referred to the 3-day points 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 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. DYS mRNA and DYS protein levels were measured from three independent experiments. Histograms at the bottom show the RQs of dystrophin protein and mRNA levels referred to the values of control experiments (Ctrl, LNA-Scr and Sponge-Ctrl) set to 1. (C) Schematic representation of the exon-skipping strategy for the human $\Delta 48-50$ DMD mutation. (D) Human $\Delta 48-50$ 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 DYS quantified by western blot. Histograms show the RQs of dystrophin protein and mRNA levels as well as MCK mRNA levels, respectively, referred to the values of control experiments set to 1. * $P < 0.05$. ACTN, actinin; DMD, Duchenne muscular dystrophy; DYS, dystrophin; MCK, muscle creatine kinase; mRNA, messenger RNA; RQ, relative quantification.

In conclusion, we suggest that miR-31, previously linked to neoplastic development and tumour metastasis (Valastyan & Weinberg, 2010), is part of the circuits controlling late muscle differentiation by repressing dystrophin synthesis and probably many other terminal differentiation markers. In line with this, miR-31 was previously shown to repress Myf5, a key element of muscle differentiation, in the central nervous system (Daubas *et al*, 2009).

miR-31-repressing activity was detected in the early phases of myoblast 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 indicates that the high levels of miR-31 found in dystrophic conditions—in both mouse and human biopsies—are due to the intensive regeneration programme that is mediated by the activation of satellite cells. Interestingly, in dystrophic myoblasts and satellite cells the lack of dystrophin correlates 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 exon skipping, the inhibition of miR-31 activity increased dystrophin production.

This might reflect both enhanced differentiation as well as enhanced translation of the dystrophin mRNA.

The contribution to dystrophin production by regenerating fibres in a compromised muscle is important. miR-31 repression in this compartment could therefore improve therapeutic treatments aimed at increasing levels of dystrophin synthesis. Rescue of consistent levels of dystrophin will have additional benefits, such as completion of the muscle fibre maturation process.

METHODS

RNA and protein analyses. Total RNA was prepared from liquid nitrogen-powdered tissues homogenized in Qiazol reagent (QIAGEN). miRNA and mRNA analyses were performed using miScript System (QIAGEN). Relative quantification was performed using, as endogenous controls, U6 snRNA for miRNAs and HPRT1 for mRNAs. Northern blots for miRNAs were performed using LNA detection probes (Exiqon). miRNA *in situ* hybridization was performed in formaldehyde and carbodiimide (EDC)-fixed gastrocnemius cryosections, according to Pena *et al* (2009). Western blot on total extracts and immunohistochemistries were performed as described in Denti *et al* (2006).

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 (sequence in Fig 2A) into the WPRE *SacI* 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). Oligonucleotide sequences and plasmids are available on request.

Cell culture. Muscle satellite cells were cultured and differentiated as described in Rando & Blau (1994). The Duchenne primary myoblasts carrying deletion of exons 48–50 were obtained from Telethon Biobank and infected with a lentiviral construct containing antisense sequences against the splice junctions of exon 51. LNA miRNA knockdown (Exiqon) treatments were performed according to Taulli *et al* (2009).

Statistical analyses. The data shown in the histograms are the result of at least three independent experiments performed on at least three samples or animals. Data are shown as mean $\pm$ standard deviation. Unless stated otherwise, the statistical significance of differences between means was assessed by two-tailed *t*-test and $P < 0.05$ was considered significant.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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