Altered Levels of MicroRNA-9, -206, and -132 in Spinal Muscular Atrophy and Their Response to Antisense Oligonucleotide Therapy

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The identification of noninvasive biomarkers to monitor the disease progression in spinal muscular atrophy (SMA) is becoming increasingly important. MicroRNAs (miRNAs) regulate gene expression and are implicated in the pathogenesis of neuromuscular diseases, including motor neuron degeneration. In this study, we selectively characterized the expression of miR-9, miR-206, and miR-132 in spinal cord, skeletal muscle, and serum from SMA transgenic mice, and in serum from SMA patients. A systematic analysis of miRNA expression was conducted in SMA mice with different disease severities (severe type I-like and mild type III-like) at different disease stages (pre-, mid-, and late-symptomatic stages), and in morpholino antisense oligonucleotide-treated mice. There was differential expression of all three miRNAs in spinal cord, skeletal muscle and serum samples in SMA mice. Serum miRNAs were altered prior to the changes in spinal cord and skeletal muscle at the presymptomatic stage. The altered miR-132 levels in spinal cord, muscle, and serum transiently reversed to normal level after a single-dose morpholino antisense oligomer PMO25 treatment in SMA mice. We also confirmed a significant alteration of miR-9 and miR-132 level in serum samples from SMA patients. Our study indicates the potential of developing miRNAs as noninvasive biomarkers in SMA.

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Introduction

Spinal muscular atrophy (SMA) is an autosomal recessively inherited neuromuscular disorder caused, in the majority of patients (~95%), by homozygous deletion in the Survival Motor Neuron gene 1 (SMN1). It is characterized by the selective degeneration of lower motor neurons and limb and trunk muscle weakness due to the deficiency of SMN protein. SMN protein is ubiquitously expressed. While SMA is generally described as a lower motor neuron disease with spinal motor neurons being the primary pathological target, increasing numbers of clinical and experimental reports indicate the involvement of additional peripheral organs which may contribute to the pathogenesis of the disease, especially in severe cases. SMA is currently incurable, however, experimental therapies, i.e., antisense oligonucleotide (AON) therapy,2-4 viral-vector-mediated gene therapy,7-10 and small molecule therapy,11,12 have shown promising preclinical efficacy. AON therapy on IONIS-SMNRx (Nusinersen) is now in phase 3 clinical trial, with encouraging data on safety and clinical outcomes from the earlier phase 1 clinical trial.13 Small molecule and SMN1 gene therapy (AVXS-101) are also in early phase clinical trials.

MicroRNAs (miRNAs) are a class of small (~22nt) endogenous non-protein-coding RNA molecules that are involved in fine regulation of gene and protein expression across multiple tissues. Dysregulation of specific miRNAs is associated with pathological processes and various disease states. In neuromuscular diseases, a number of miRNAs have been studied as biomarkers in Duchenne muscular dystrophy (DMD) and to measure the response of the mdx mouse model of DMD to AON therapy.14-16 Interestingly, specific serum miRNA profiles have been found to be associated with distinct muscular dystrophies in mouse models.17

The hallmark in SMA mouse models is the loss of lower motor neurons following the defects in neuromuscular junctions due to the primary SMN deficiency. In vitro, the loss of SMN function disrupts axonal extension and pathfinding.18 Numerous studies have shown that miRNAs are key factors in regulating the development of the nervous system. The most direct evidence comes from a study in which disrupting miRNA biogenesis pathway by deleting Dicer1 from spinal motor neurons in mice caused an SMA-like phenotype and downregulation of miR-9 (ref. 19). miR-9 also regulates axon extension and branching in mouse cortical neurons.20 In addition to its role in the central nervous system, the dicer-miRNA pathway is also required for peripheral nerve regeneration and functional recovery in vivo and axonal outgrowth in vitro.21 Indeed, deletion of Dicer in the mouse sciatic nerve suggested that functional miRNAs are crucial for nerve regeneration and axonal regrowth.21 Another miRNA that plays an essential role in neuron dendritic outgrowth22 and synaptic function is miR-132. Both miR-9 and miR-132 can delay neurite outgrowth in vitro and impair the radial neuronal
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The muscular dystrophic phenotype in SMA is thought to be caused by skeletal muscle denervation secondary to spinal motor neuron defects. Direct correlation has also been identified between SMN deficiency and disruption of the myogenic program. miR-206 is a skeletal muscle specific miRNA involved in the formation of myotubes and differentiation of satellite cells. It plays a neuroprotective role in the regeneration of neuromuscular junction in amyotrophic lateral sclerosis. Knocking-out miR-206 in the amyotrophic lateral sclerosis mouse model accelerates disease progression. miR-206 is required for regeneration of neuromuscular junctions after acute nerve injury. The serum levels of miR-206 are increased in mouse models of muscular dystrophy and amyotrophic lateral sclerosis. In a SMA mouse model, miR-206 levels in muscle was found to be activated and provided a survival mechanism to delay SMA neurodegeneration, although this was not sufficient to rescue the integrity of motor neurons.

Here, we examined the abundance of three miRNAs, miR-9, miR-206, and miR-132, in spinal cord, skeletal muscle, and serum samples from two SMA transgenic mouse models with severe and mild phenotypes, respectively, at different disease stages. These miRNAs were selected based on their potential involvement in motor neuron survival and differentiation, skeletal muscle development and in the pathogenesis of SMA. miR-9 was selected as a representative neuron-specific miRNA; miR-206 was selected as a representative muscle-specific miRNA; miR-132 was selected as a representative miRNA which is expressed in both central nervous and peripheral tissues. In addition, we studied the response of these miRNAs to effective AON therapy. Finally, we measured the level of the same miRNAs in serum samples from patients affected by type II and type III SMA. Our study indicates that miRNAs in serum have the potential to serve as informative biomarkers for SMA, to monitor disease progression and response to effective experimental therapy in future clinical studies.

Results

Alteration of miR-9, miR-206, and miR-132 in spinal cord and skeletal muscle tissues from SMA mice and their response to AON treatment

To measure the abundance of the three candidate miRNAs, reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) analyses were performed on RNA prepared from spinal cord and tibialis anterior (TA) muscles harvested from 10-day-old SMA mice, including the severe type I SMA-like mice (SMA-I), the mild type III SMA-like mice (SMA-III), and heterozygous littermate controls (control) which presented no phenotype. Spinal cords and muscles were also collected from 10 days old SMA-I mice that received a single dose of 40 µg/g PMO25 via subcutaneous injection at postnatal day 0 (PND0), the AON strategy we have reported previously. The treated SMA-I mice showed dramatic improvement in their general condition: the average survival was increased from ~10 days to over 200 days; the body weight was increased to approximately 80% of the unaffected littermate controls; and motor functions such as righting reflex in young mice was completely corrected to normal level. The neuromuscular pathologies, including muscle fibre size and neuromuscular junction maturation, were both significantly improved although still slightly inferior to those in unaffected control mice.

Small RNA TaqMan assays designed to detect only the mature miRNA species were used for all the miRNAs. Significant differences among the groups of SMA-I, SMA-III and controls in all the three miRNAs, and in samples collected from both spinal cords and TA muscles were detected (Figure 1).

There was significant downregulation of miR-9 in the spinal cord of SMA-I mice compared with the controls (P < 0.05, Figure 1a). In contrast, in skeletal muscle, miR-9 was increased over threefold in SMA-I mice compared with controls (P < 0.05, Figure 1b). After systemic PMO25 treatment, miR-9 was reduced to near-normal level in skeletal muscle but not in spinal cord.

In both spinal cord and skeletal muscle, miR-206 was increased in SMA-I mice compared with controls (P < 0.05 and P < 0.01, respectively). There was no significant change in miR-206 level in SMA-I mice in response to PMO25 treatment, either in spinal cord or in skeletal muscle.

Reduction of miR-132 occurred in the spinal cord tissue of SMA-I mice (P < 0.05, Figure 1a), while the level in skeletal muscle was significantly increased (approximately 2.5-fold) compared to controls (P < 0.05, Figure 1b). After PMO25 treatment, the altered miR-132 level in both spinal cord and skeletal muscle were completely reversed to normal levels (P < 0.01 and P < 0.05 compared to SMA-I mice; no significant difference to control mice).

In mild SMA-III mice, the level of the three miRNAs in spinal cord and skeletal muscle was altered at intermediate levels between what found in the severe SMA-I and the control mice (Figure 1). One-way analysis of variance showed that there was a significant difference in the level of the three miRNAs in skeletal muscle and spinal cord among groups of SMA-I, SMA-III and control mice (P < 0.05).

In addition, all three miRNAs showed a tissue-dependent abundance pattern. In keeping with its predominant central nervous system expression pattern, miR-9 was far more abundant in spinal cord than in skeletal muscle. Using real-time PCR assay, in control mice we detected approximately 500 times higher miR-9 levels in spinal cord (Ct value ~23) than in skeletal muscle (Ct value ~32); miR-206 had the highest level in skeletal muscle compared to the other two miRNAs. The level of miR-206 in skeletal muscle (Ct value ~20) was over 1,000 times higher than that in spinal cord (Ct value ~30). In contrast to miR-9 and miR-206, miR-132 in skeletal muscle (Ct value ~27) and spinal cord (Ct value ~26) showed similar levels.

Serum miRNAs change dynamically with disease progression and following AON treatment

Serum miRNAs are promising biomarkers in Duchenne and Becker muscular dystrophy and in other types of muscular dystrophies and myopathies. They provide a noninvasive
In SMA-I mice, one-way analysis of variance showed no significant difference in serum miRNAs at the three time points measured in this study. Significant difference was only detected between PND2 and PND7 in all three miRNAs in serum, analyzed by t-test. There was 2.1-fold reduction in serum miR-9 (P < 0.05), 3.2-fold reduction in miR-206, and 2.7-fold reduction in miR-132 (P < 0.05). No significant difference was detected between PND7 and PND10 in all three miRNAs. miR-9 and miR-132 had a tendency towards increased levels at PND10 compared to PND7, but failed to reach statistical significance due to the high variation in the level of these miRNAs at the late symptomatic stage (Figure 2a).

At PND7, the mid-symptomatic stage in SMA-I, all three miRNAs were dramatically increased in SMA-I mice relative to controls, at approximately 4.4-fold in miR-9, 10-fold in miR-206, and 5-fold in miR-132 (Figure 2b). All three serum miRNAs responded remarkably well to systemic PMO25 treatment and showed significant reduction to near-normal levels compared to the untreated SMA-I mice (P < 0.05, Figure 2b).

At PND10, the late-symptomatic stage in SMA-I, there were higher levels of all three miRNAs in SMA-I mice compared to controls (Figure 2), although only miR-132 reached significance (P < 0.05, threefold change). The higher variation in serum miR-9 and miR-206 at the late stage of the disease could explain the lack of significant difference. Significant abundance of all three miRNAs was observed in PMO25-treated SMA-I mice.
The different levels of serum miR-9, miR-206, and miR-132 on PND2, PND7, and PND10 suggests that the abundance of miRNAs changes with disease progression and that the alteration of miRNAs in serum is more significant at mid-symptomatic than at the late-symptomatic stage. While serum miRNAs responded well at PND7 to PMO25 treatment, all three miRNAs increased dramatically at PND10 in PMO25 treated SMA-I mice compared to controls (P < 0.01 and P < 0.001, Figure 2c). This result suggests that after a single-dose PMO25 treatment at PND0, the altered level of serum miRNAs can be corrected but only for a limited period, between 7–10 days in severe SMA mice, before they rebound to the active disease state levels. This is consistent with the clinical phenotype of the PMO25 single-dose treated mice that had partially rescued body weight, but that developed ear and tail necrosis, indicating active disease, at a later stage.5

Altered level of miRNAs in serum occurs prior to their change in spinal cord and skeletal muscle

Our study showed that alteration in serum miRNAs occurred much earlier than spinal cord and skeletal muscle miRNAs. At PND2, the presymptomatic stage in severe SMA-I mice, there was no significant difference in the abundance of all three miRNAs in either spinal cord or skeletal muscle between the SMA-I mice and controls. However, there was significant higher level of miR-9 and miR-132 in serum samples from SMA-I (3.5- and 2.5-fold, respectively) than that of control mice at PND7. miR-9, miR-206, and miR-132 responded dramatically to PMO25 treatment (P < 0.01), and were significantly reduced relative to untreated SMA-I mice. No significance in the difference between PMO25-treated SMA-I and the control group. The level is relative to the average miRNA level in heterozygous controls. Data are presented as mean ± standard error of the mean. *P < 0.05; **P < 0.01; ***P < 0.001.

The mild SMA-III mice show no loss of motor neurons in the anterior horn in comparison with normal littermates. The SMN protein expression in spinal cord from SMA-III mice is lower than that in control mice.35 We have previously shown that the SMA-III mice have the same body weight and righting reflex motor function as the controls on PND10, albeit
with slightly decreased size of skeletal muscle fibers and neuromuscular junction end plates. 36 Ear and tail necrosis commence in these mice from 2–3 weeks old. In this study, SMA-III mice showed no significant alteration of miRNAs in spinal cord and skeletal muscle samples at PND10, compared to control mice. However, the miRNAs in serum of SMA-III were all significantly increased at PND10 (miR-9 to threefold, miR-206 to eightfold, and miR-132 to sixfold control levels) (Figure 3b). These results suggest that the change of miRNA level in serum occurs before the change in spinal cord and muscle, in both SMA-I and SMA-III mice.

To detect the window in which the serum miRNA starts to change in SMA-III mice, we measured the level of serum miRNAs in SMA-III mice at PND7 and no change was detected (data not shown). This suggests the alteration of miRNA abundance in serum may commence between PND7-10 in SMA-III mice.

### Altered levels of miRNAs in serum samples from SMA patients

To further evaluate the potential of miR-9, miR-206, and miR-132 as noninvasive biomarkers, we examined the expression of these miRNAs in serum samples from SMA patients with type II (n = 6) and type III SMA (n = 4), aged between 4–14 years old (Figure 4). Serum miRNAs were measured by real-time quantitative PCR, relative to age-matched healthy controls (n = 7). We found upregulation of miR-9 (2.36-fold) and miR-132 (1.54-fold) expression in type II SMA serum relative to healthy controls (P < 0.05, Figure 4a). In serum from type III SMA patients, these two miRNAs were at intermediate levels between type II SMA and controls without significance in difference. There was no significant difference in the level of serum miR-206 from SMA patients compared to healthy controls (Figure 4a). There was no significant correlation between the level of the three miRNAs in serum and the motor functional ability of the patients, measured using the Hammersmith Functional Motor Scale (Figure 4b).

To determine if there is an association between the expression level of miRNAs in serum and the age of SMA patients, linear regression and correlation analyses were performed in serum samples from SMA patients and healthy controls (Figure 4c). Although there was a decrease in serum miR-9 expression in SMA patients with increasing age, in contrast to the stable expression in healthy controls, there was no significant correlation between the expression of miR-9 and the age of SMA patients. Similarly, there was no correlation between the expression level of miR-206 and miR-132 with age in SMA patients and healthy controls. Our data therefore suggest that there is no significant correlation between the expression levels of the three miRNAs in serum samples with age in SMA patients.

### Discussion

We have characterized the differential expression of miR-9, miR-206, and miR-132 in spinal cord, skeletal muscle and serum from SMA and control mice, and in serum samples from SMA and control patients. A systematic analysis of miRNA expression was conducted in SMA mice with different severities (severe SMA-I and mild SMA-III) at different disease stages (presymptomatic, mid-symptomatic and late stage) and their responses to therapeutic AON treatment. This is the first study to assess the potential of selected miRNAs as biomarkers in SMA.

As a highly conserved neuronal-specific miRNA, miR-9 is involved in the neuronal development, including the regulation of neuronal progenitor proliferation and the maturation
and differentiation of postmitotic neurons.\textsuperscript{37} miR-9 has also been found to be altered in other neurodegenerative disorders. It is decreased in the brain of Alzheimer’s disease patients\textsuperscript{38} and increased in the cortex of Parkinson’s disease patients.\textsuperscript{39} The reduction of miR-9 level in spinal cord tissue in severe SMA mice in our study is consistent with the phenotype of motor neuron degeneration which characterizes the late stage of the disease in the mice, and also in line with the finding in \textit{SMN1} mutated motor neurons, where miR-9 is decreased in cultured motor neurons harboring \textit{SMN1} mutation.\textsuperscript{19} The conversely increased level of miR-9 in TA muscles is consistent with a report that miR-9 is upregulated in fibroblasts with SMN protein deficiency.\textsuperscript{30} miR-132 also has contrary abundance in spinal cord and skeletal muscle. miR-132 plays an important role in neurite outgrowth.\textsuperscript{22} The reason for the contrary tendency of miR-9 and miR-132 level in spinal cord and skeletal muscle in SMA mice is not known.

miR-206 is increased in skeletal muscle in \textit{SMN}Δ7 mice, at the early phase (PND5).\textsuperscript{34} Similarly, we found that miR-206 was significantly increased in both spinal cord and TA muscle in the severe SMA mice at the late stage of the disease. It has been speculated that miR-206 upregulation is a protective response to the severe defect in neuromuscular junction maturation in SMA mice.\textsuperscript{34}

In SMA-I mice, there were clearly detectable altered levels of miR-9 and miR-132 in serum as early as at PND2, but there was no change of these two miRNAs in either spinal cord or skeletal muscle at this presymptomatic time point (Figure 3a). Similarly, at PND10, the alterations of miRNAs in serum of SMA-III mice were more dramatic than in spinal cord and skeletal muscle (Figure 3b). These results indicate that the change in abundance of miR-9 and miR-132 in serum are more prominent than that in spinal cord or muscle, and show the potential of these two miRNAs as noninvasive candidate biomarkers for this disease.

miRNAs that are highly enriched in serum than in skeletal muscle have also been reported in \textit{mdx} mice and DMD patients relative to controls.\textsuperscript{16,41,42} It has been proposed that,
in DMD, the serum dystromiRs are released by regenerating and/or degenerating mature fibres and the miRNAs in circulation are protected from RNase-mediated degradation by either associated with Argonaute-2 protein or complexing with lipoproteins. This is possible for miR-206, as it is enriched in skeletal muscle. miR-9 and miR-132 are not muscle-specific. The more dramatic change of miR-9 and miR-132 in serum than in skeletal muscle and spinal cord may suggest that there might be organs other than, or in addition to, spinal cord and skeletal muscle, from which these serum miRNAs originate. This may imply that additional organs, beyond spinal cord and skeletal muscle, could contribute to the SMA disease pathogenesis. Indeed, increasing numbers of clinical and experimental studies indicate the involvement of additional peripheral organs in the pathogenesis of the disease in severe cases. We have previously shown that regular systemic administration of morpholino AON in an intermediate SMA mouse model from birth until adulthood provided therapeutic benefit and rescued the SMA phenotypes, presumably via peripheral SMN restoration. Possible candidates for the peripheral involvement include perisynaptic Schwann cells at neuromuscular junctions and dorsal root ganglia, as they are likely to benefit more from the peripheral rather than the exclusive CNS delivery of morpholino AON. Further investigation of miR-9 and miR-132 in the peripheral neuronal system will help to understand the molecular mechanism of their involvement in SMA.

Vascular defects have been reported in some severe cases of SMA infants and mouse models. These include digital necrosis and distal vascular thrombosis in severe SMA infants, decreased vascular density in skeletal muscle, spinal cord and intestine in SMA mice, and defective microvascular development in skeletal muscle in severe SMA patients. miRNAs play a crucial role in the biogenesis and function of endothelial cells and are implicated as dynamic regulators of cardiac and vascular signalling and pathophysiology. miR-132 is involved in the proliferation of endothelial cells and has been implicated in neovascularisation. Further studies on miR-132 in vascular function in SMA are required to understand its involvement in the pathogenesis of SMA vasculopathy.

We have previously reported the successful rescue of severe SMA mice by PMO25 treatment when delivered in newborn litters. While all parameters measured were dramatically improved, some features, for example, the body weight, skeletal muscle fibre size, and neuromuscular junction maturation, were still not completely rescued when compared to the unaffected controls. The single-dose treated mice also displayed ear and tail necrosis at a later stage. It is therefore not surprising to see more elevated miRNAs levels at PND10 in the treated SMA mice than in unaffected controls (Figure 2c). However, the early significant response of serum miR-206 and miR-132 to PMO25 treatment at PND7 (Figure 2b) indicates the potential of miRNAs as biomarker of response to therapeutic intervention, although the response is transient and time limited. This result may also suggest a requirement for repeated AON treatment in SMA in order to maintain the therapeutic effect. Indeed, the clinical benefit of repeated administrations of AON has already been confirmed in a preclinical study and such regime is now being applied in clinical trial (www.clinicaltrials.org, ID: NCT02386553).

In SMA patients, there was significantly higher level of miR-9 and miR-132 in serum compared to healthy controls, and SMA-II patients had higher level of miRNAs than SMA-III patients (Figure 4). However, the lack of correlation between the level of serum miRNAs and Hammersmith Functional Motor Scale indicates the possibility that high interpatient variability may limit the use of these miRNAs as biomarkers for SMA, especially given the modest fold changes observed. In addition, the fact that the serum miR-206 level change is significant in SMA mice but not in SMA patients suggests the differential abundance of some miRNAs between patients and mouse models. While this study examined only three miRNAs selected based on literature reports, it provides useful information for future more in-depth studies on developing miRNAs as biomarkers in SMA. In the future, deep microRNA profiling in SMA patients, using the advanced next generation sequencing technology, may provide more potential biomarker candidates.

In summary, we have shown that miR-9, miR-206, and miR-132 are differentially expressed in spinal cord, skeletal muscle, and serum in SMA mouse models. Serum miRNAs in SMA may be altered prior to the change in spinal cord and skeletal muscle tissues and before the appearance of any clinical phenotypes. miR-132 in spinal cord, skeletal muscle, and serum responds dramatically, although transiently, to systemic AON treatment in the severe mouse model. Serum miR-132 shows the greatest potential, among the three miRNAs tested in this study, as a candidate biomarker of disease progression and therapeutic response in SMA. Given that encouraging clinical trials are currently undertaking in SMA patients, our findings indicate the potential clinical application of miRNA as promising noninvasive biomarker candidate for this disease.

Materials and methods

Oligonucleotides. The 25-mer therapeutic antisense Phosphorodiamidate Morpholino Oligomer PMO25, to augment exon 7 inclusion in SMN2, was synthesized by Gene Tools LLC with agreement for research usage only. The sequence of PMO25 has been previously described. Products were dissolved to standard concentrations and stored according to the manufacturer’s instructions.

Animals. All mouse experiments were performed according to protocols approved by University College London Biological Services and UK Home Office under the Animals (Scientific Procedures) Act 1986. The initial breeding pairs of SMA transgenic mice FVB.Cg-Tg(SMN2)2Hung Smn1+/+; Smn2+/-; the mild type III SMA-like mice, referred as “SMA-I”, carry two copies of human SMN2 transgene with genotype (SMN2)2; Smn2+/-; Smn2+/–; and the heterozygous progeny with genotype (SMN2)2; Smn2+/-; Smn2+/- is phenotypically unaffected and was
used as unaffected littermate control, referred as "control". Subcutaneous injections in severe type I SMA-like mice were performed as described previously. 40 µg/g PMO25 was injected subcutaneously in SMA-I mice at PND0. The treated mice were referred as “SMA-I + PMO25”.

**SMA patients and healthy controls serum cohort.** The study was approved by the Berkshire Research Ethics Committee (REC reference 05/MRE12/32). Serum samples were supplied by the MRC Center for Neuromuscular Diseases Biobank London (REC reference number 06/Q0406/33, http://www.cmnd.ac.uk) and were collected from individuals under written informed consent of parents or legal guardians. The Declaration of Helsinki protocols were followed. Serum samples were collected from six SMA type II patients and four SMA type III patients (age range between 4 to 14 years) from the SMA clinic at Great Ormond Street Hospital, London. All patients have confirmed genetic diagnosis of SMA with homozygous genomic deletion in SMN1 gene and two or three copies of SMN2 gene.

**RNA preparation in spinal cord and skeletal muscle samples from SMA mice.** Spinal cord and TA skeletal muscle samples were collected from 2 day old (n = 5 for SMA-I, n = 5 for control), 7 day old (n = 4 for SMA-I, n = 4 for SMA-III, n = 5 for control), and 10 day old (n = 6 in each group of mice). Samples were immediately snap frozen in liquid nitrogen after dissection and stored at −80 °C for RNA processing. Mouse tissues were homogenized using Precellys Homogenizer (Bertin Technologies) in lysis buffer. Total RNA was extracted using mirNeasy kit (QIAGEN) following the manufacturer's protocol for tissue samples.

**RNA extraction in serum samples collected from SMA mice and patients.** In mice, blood samples were collected from the right atrium immediately after the mice were culled. In patients, serum samples were prepared from 1.5 to 4 ml of blood taken in BD vacutainer tubes. The blood sample was allowed to clot at room temperature for 30 minutes, followed by centrifugation at 2,850 g for 10 minutes. Serum supernatant was carefully collected and stored at −80 °C until use.

Total RNA was extracted from serum samples using mirNeasy Serum/Plasma kit (QIAGEN) following the manufacturer’s protocol for liquid samples. For data normalization, we used an artificial *Caenorhabditis elegans* cel-miR-39 added at 25 fmol in a 5 µl volume as previously described.4,53

**Reverse transcription and qRT-PCR analysis.** Quantitative RT-PCR was performed using hsa-miR-9 (Cat. 4427975_002231), hsa-miR-206-3p (Cat. 4427975_000510), hsa-miR-132-3p (Cat. 4427975_000457), cel-miR-39 (Cat. 4427975_002000) and U6 snRNA (Cat. 4427975_001973) Taqman small RNA Assay (Life Technology) following the manufacturer's protocol and the methods previously described.14 Briefly, a fixed volume of 5 µl of total RNA (10 ng) of a given sample was reverse transcribed using TaqMan MicroRNA Reverse Transcription kit. The PCR amplification of a volume of 1.33 µl cDNA was carried out using TaqMan Universal PCR master mix and miRNA-specific stem-loop primers (Life Technology). The relative expression of miRs was analyzed by ΔΔCt method. U6 snRNA was used as endogenous control for data normalization in spinal cord and muscle tissues, and the artificial spiked in cel-miR-39 was used as endogenous control in serum samples.

**Statistical analysis.** One-way analysis of variance and post t-test were used to determine statistical significance when comparing three and four groups of mice. Student t-test was used for statistical analysis of two groups of data. Data are presented as mean ± standard error of the mean. GraphPad Prism 5.0 software (GraphPad Software) was used for statistical analysis and graph design.

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