MicroRNA-206 Delays ALS Progression and Promotes Regeneration of Neuromuscular Synapses in Mice

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by loss of motor neurons, denervation of target muscles, muscle atrophy, and paralysis. Understanding ALS pathogenesis may require a fuller understanding of the bidirectional signaling between motor neurons and skeletal muscle fibers at neuromuscular junctions. Here, we show that a key regulator of this signaling is miR-206, a skeletal muscle–specific microRNA that is dramatically induced in a mouse model of ALS. Mice that are genetically deficient in miR-206 form normal neuromuscular synapses during development, but deficiency of miR-206 in the ALS mouse model accelerates disease progression. miR-206 is required for efficient regeneration of neuromuscular synapses after acute nerve injury, which probably accounts for its salutary effects in ALS. miR-206 mediates these effects at least in part through histone deacetylase 4 and fibroblast growth factor signaling pathways. Thus, miR-206 slows ALS progression by sensing motor neuron injury and promoting the compensatory regeneration of neuromuscular synapses.

Amyotrophic lateral sclerosis (ALS) is the most common adult motor neuron disease (1). Symptoms of the disease include atrophy and paralysis of lower limb and respiratory muscles because of the degeneration of motor neurons. There is currently no effective treatment. Thus, identification of the signaling pathways and cellular mediators of ALS remains a major challenge in the search for novel therapeutics (2).

In light of recent studies implicating microRNAs (miRNAs) in stress responses in muscle (3), we investigated whether disease progression in a mouse model of ALS was accompanied by changes in expression of miRNAs. We compared miRNA expression in skeletal muscles from the lower limbs of normal adult mice and G93A-SOD1 transgenic mice (4, 5) that express a low copy number of a mutant form of superoxide dismutase (SOD1) in which glycine-93 is replaced with alanine (G93A-SOD1), as seen in a subset of human ALS patients. These mice recapitulate the progression of human ALS symptoms (4, 5). Of 320 miRNAs tested, the muscle-specific miRNA miR-206 (6, 7) was the most dramatically up-regulated in G93A-SOD1 muscles (Fig. 1A and fig. S1, A and B) (8). Up-regulation of miR-206 coincided with the onset of neurological symptoms, as indicated by levels of miR-206 in healthy G93A-SOD1 transgenic mice being similar to those in wild-type littermates (Fig. 1A and fig. S1B).

Because ALS leads to denervation of skeletal muscle (1), we determined whether miR-206 up-regulation was a consequence of denervation. Indeed, 10 days after severing the sciatic nerve of wild-type mice to denervate lower leg muscles, levels of mature and primary miR-206 (pri-miR-206) transcripts were robustly increased in three muscles that contain predominantly fast-twitch fibers, extensor digitorum longus (EDL), tibialis anterior (TA), and gastrocnemius/plantaris (G/P) (Fig. 1B and fig. S2) (9). miR-206 levels were higher in normally innervated soleus, which contains predominantly slow myofibers, and up-regulation after denervation was correspondingly less substantial (fig. S2).

miR-206 is a skeletal muscle–specific miRNA in humans and mice that is generated from a bicistronic transcript that also encodes miR-133b (fig. S3, A and B) (7, 10). Two other homologous miRNA pairs, miR-1-1/133a-2 and miR-1-2/133a-1, are encoded on separate chromosomes and are expressed in skeletal and cardiac muscle (6, 10). Consistent with its transcription from the same promoter, miR-133b was also up-regulated after denervation, whereas miR-1 and miR-133a were down-regulated (Fig. 1B and fig. S2).

Previous studies have implicated the myogenic basic helix-loop-helix (bHLH) proteins MyoD and myogenin in denervation-dependent gene expression (fig. S4A) (11). Three evolutionarily conserved E-boxes (CANNTG), which are binding sites for MyoD and myogenin, are located between –910 and –765 base pairs (bp) upstream of the start of the precursor (pre-miR-206) stem loop, within a genomic region that was previously shown to be enriched for MyoD binding by using chromatin from muscle cells (Fig. 1C and fig. S4B) (7). Heterodimers of MyoD and its bHLH partner E12 bind these sites (fig. S4C). In cultured cells, MyoD activated the
Fig. 1. Regulation of miR-206 in response to ALS and denervation. (A) Up-regulation of miR-206 during the progression of ALS in G93A-SOD1 mice as determined by means of Northern blot and quantified by densitometry. *P < 0.0001, Student’s t test; n = 2 to 4 mice. (B) Transcripts of miR-206, miR-133b, miR-1, and miR-133a were detected by means of real-time PCR in TA muscles after 10 days of denervation (+). The contralateral muscle was used as a control (–). *P < 0.02, **P < 0.005, Student’s t test; n = 3 to 4 mice. (C) Sequence alignment of the mouse miR-206 5’ flanking sequence from different species shows the conserved upstream region containing E-boxes. Position (0) denotes the start of pre-miR-206. Bracketed region represents the identified enhancer region. (D) β-galactosidase staining of G/P muscle isolated from denervated transgenic mice containing a lacZ transgene controlled by the wild-type (WT) enhancer or the mutant enhancer (containing mutated E-boxes) [C], bracket. Contralateral muscle was used as a control. Lower panels show transverse section of muscle. Scale bar, 200 μm. Values represent mean ± SEM.

Fig. 2. Regulation of ALS pathogenesis by miR-206. (A) Age of disease onset for G93A-SOD1 (black) (n = 9 mice) (mean, 188 days), miR-206−/−; G93A-SOD1 (red) (n = 10 mice) (mean, 187 days), and miR-206 KO (blue) littermates. (B) Days of disease progression for G93A-SOD1 (mean, 78 days) and miR-206−/−; G93A-SOD1 littermates (mean, 56 days). P < 0.005, log-rank test. (C) Survival curve for G93A-SOD1 (mean, 266 days), miR-206−/−; G93A-SOD1 (mean, 244 days). P < 0.05, log-rank test. (D) Survival of G93A-SOD1 and miR-206−/−; G93A-SOD1 mice. *P < 0.02, Student’s t test. (E) G93A-SOD1 and miR-206−/−; G93A-SOD1 mice at approximately 7.5 months of age. There is severe atrophy of hindlimb muscles in miR-206−/−; G93A-SOD1 mouse. (F) X-ray reveals kyphosis in miR-206−/−; G93A-SOD1 mice. (G) Wheat-germ agglutinin (WGA) staining of transverse sections of muscle show accelerated muscle atrophy in miR-206−/−; G93A-SOD1 mice as compared with that of G93A-SOD1 littermates. Scale bar, 100 μm. Values represent mean ± SEM.
expression of a reporter controlled by this region, and mutations of the E-boxes abolished the responsiveness to MyoD (fig. S4D).

In transgenic mice harboring an Escherichia coli β-galactosidase (lacZ) reporter controlled by the miR-206 S′ regulatory region, lacZ expression was dramatically up-regulated in muscle after surgical denervation (Fig. 1D). Mutations in the three E-boxes reduced responsiveness of the miR-206 enhancer to the minimal level of the hsp68 basal promoter (Fig. 1D and fig. S4E). Thus, direct regulation by myogenic bHLH factors leads to transcriptional activation of miR-206 in response to skeletal muscle denervation.

The increased level of miR-206 in ALS might be an innocuous correlate, a contributor to pathology, or part of an ultimately inadequate compensatory effort. To distinguish these possibilities, we generated targeted mutants in which miR-206 expression was abolished without affecting miR-133b expression (fig. S5, A to D). Mice that were homozygous for the targeted deletion of miR-206 showed no obvious abnormalities in weight, behavior, the architecture and fiber-type distribution of skeletal muscles, or expression of pri-miR-133b and pri-miR-1 (fig. S5, E and F).

Next, we generated miR-206−/− mice that express a low copy number of G93A-SOD1. Loss of miR-206 did not affect disease onset but did accelerate disease progression and diminish survival (Fig. 2, A to D). The exacerbation of disease symptoms in miR-206−/− mice was accompanied by an accelerated atrophy of skeletal muscle, leading to kyphosis, paralysis, and death (Fig. 2, E to G, and movies S1 and S2). MiR-206−/− mice lacking the G93A-SOD1 transgene showed no overt phenotype or decrease in survival up to 300 days (Fig. 2C). Thus, increased miR-206 expression in response to denervation counteracts, albeit ultimately unsuccessfully, the pathogenesis of ALS.

How does miR-206 act to extend survival in ALS? In that motor neuron pathology plays a key role in ALS, whereas miR-206 is expressed exclusively in muscles, we suspected that the miRNA affects nerve-muscle interactions. Indeed, a transcript derived from the miR-206/133b locus was originally identified as a synapse-associated noncoding RNA called 7H4 (12), as has been seen for other genes encoding components of the postsynaptic apparatus (13). Although the reported 7H4 sequence did not include miR-206, reverse transcription polymerase chain reaction (RT-PCR) demonstrated that miR-206 sequences are in fact included in this synapse-enriched transcript (Fig. 3A and fig. S6, A and B) (12). This expression pattern focused our attention on the neuromuscular junction (NMJ).

We examined the architecture of NMJs in neonatal and adult wild-type and miR-206−/− mice using labels for the postsynaptic membrane [α-bungarotoxin (BTX)], motor axons (antibodies to neurofilaments), and nerve terminals (antibodies to the synaptic vesicle protein synaptotagmin 2) (14). NMJs of embryonic, neonatal, and adult mutant mice showed no obvious differences when compared with age-matched wild-type NMJs (fig. S7, A and B). Thus, miR-206 is dispensable for formation and maturation of the NMJ.

In contrast, miR-206 profoundly influenced formation of new NMJs after nerve injury, which denervates muscle. Three weeks after surgical denervation, both wild-type and miR-206−/− mice exhibited similar degrees of muscle atrophy (fig. S8, A and B), but reinnervation of denervated muscles by motor axons was delayed in the absence of miR-206. Regenerating axons preferentially reinnervate original synaptic sites after denervation (15, 16), so we quantified the

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**Fig. 3.** Delayed NMJ reinnervation in miR-206 mutant mice. (A) Quantitative real-time PCR reveals miR-206 expression is enriched in synaptic regions of muscle fibers. *P* < 0.0002, Student’s t test; *n* = 3 mice. (B) After sciatic nerve transection (as indicated in weeks), a delay in reinnervation is seen in miR-206−/− mice (KO) as compared with WT mice as detected by the superimposition of synaptotagmin 2 (Syt 2) staining (green) with BTX (red). Scale bar, 10 μm. (C) Time course and quantification of the number of reinervated NMJs in WT and miR-206−/− (KO) mice after sciatic nerve transection. *n* = 2 to 6 mice. (D) Immunohistochemistry shows a delay in presynaptic differentiation and partial reoccupancy of postsynaptic sites in miR-206−/− NMJs 5 weeks after sciatic nerve transection. Scale bar, 10 μm. (E) Postsynaptic area occupied by the reinnervating nerve (in percent) 5 weeks after cutting the sciatic nerve in WT and miR-206−/− (KO) mice. *P* = 0.02, Student’s t test. (F) Immunohistochemistry shows increased NMJ dysfunction and denervation in miR-206−/−;G93A-SOD1 as compared with G93A-SOD1 littermates. Scale bar, 10 μm. (G) Number of innervated NMJs in G93A-SOD1 and miR-206−/−;G93A-SOD1 mice at 7 months of age. *P* < 0.0005, Student’s t test. (H) Number of fragmented NMJs in G93A-SOD1 and miR-206−/−;G93A-SOD1 mice at 7 months of age. *P* < 0.005, Student’s t test. Values represent mean ± SEM.
number of postsynaptic sites apposed by nerve. Because postsynaptic acetylcholine receptor (AChR) aggregates remain largely intact after denervation (17), reinnervation can be accurately assessed by the superimposition of BTX (red) and synaptotagmin (green) staining. In wild-type mice, reinnervation began between 2 and 3 weeks after nerve cut and was nearly complete by 5 weeks after injury (Fig. 3, B and C, and fig. S9A). In contrast, reinnervation of miR-206−/− TA muscles did not begin until 3 weeks after injury and remained retarded for at least 2 more weeks (Fig. 3, B and C, and fig. S9A). Reinnervation was also delayed when the nerve was crushed rather than cut; in this procedure, no gap is generated, and re regeneration to targets occurs more rapidly and reliably than after nerve cut (fig. S9, B to E).

Fig. 4. MiR-206 regulates reinnervation through HDAC4 and FGFBP1. (A) Luciferase activity of COS1 cells cotransfected with WT or mutant HDAC4 3′ untranslated region (3′UTR)–luciferase constructs with a miR-206 expression plasmid. (B) Quantitation of HDAC4 protein expression in muscle lysates isolated from WT and miR-206 KO (miR-206 KO) mice after 3 weeks of denervation. *P < 0.02, Student’s t test; n = 3 mice. (C) Immunohistochemistry shows an increase in reinnervation in HDAC4 mKO mutant mice as compared with that of WT mice 7 days after nerve crush. Scale bar, 10 μm. (D) Number of reinnervated NMJs in WT and HDAC4 mKO mice after sciatic nerve crush for 7 days. *P < 0.05, Student’s t test; n = 3 to 8 mice. (E) Postsynaptic area occupied by the reinnervating nerve (in percent) 7 days after crushing the sciatic nerve in WT and HDAC4 mKO mice. *P < 0.02. (F) Decrease in expression of Fgfbp1 transcripts in miR-206−/− (KO) muscles 3 weeks after nerve transaction. *P < 0.02, Student’s t test; n = 3 to 5 mice. (G) Increase in expression of Fgfbp1 transcripts in HDAC4 mKO muscles 7 days after nerve crush. *P < 0.0005, Student’s t test; n = 3 to 5 mice. (H) Immunohistochemistry shows an inhibition of synaptic-vesicle clustering in neonatal NMJs upon knockdown of FGFBP1. Scale bar, 10 μm. (I) NMJ size in muscle fibers expressing LacZ or FGFBP1 shRNAs. Mice were electroporated at 0 days after birth (P0) and analyzed at P8. *P < 0.02, Student’s t test; n = 4 mice. Values represent mean ± SEM. (J) Schematic of miR-206 up-regulation and function after denervation. (K) Proposed mechanism of miR-206–dependent reinnervation.

To rule out defects in axonal regeneration, we visualized the nerves near the muscle entry point at 3 weeks after transection and found similar numbers of nerve fibers in wild-type and miR-206−/− nerves, indicating that axonal growth was unimpaired in the mutants (fig. S9F). Thus, the prolonged delay in reinnervation in the absence of miR-206 may result from the lack of a local signal emanating from muscle that influences...
interaction of regenerated motor axons with muscle fibers. Consistent with this conclusion, many mutant synaptic sites were only partially reoccupied by the regenerated nerve (Fig. 3, D and E, and fig. S9E). Moreover, synaptic vesicles failed to aggregate properly in regenerated mutant nerve terminals, and motor axons often sprouted beyond miR-206−/− NMJs, suggesting a possible lack of “stop and differentiate” signals emanating from the muscle (Fig. 3, D and E, and fig. S9G). Similar defects have been documented in mutant mice that lack muscle-derived organizers of presynaptic differentiation and maturation (14), suggesting decreased levels of muscle-derived factors that promote reinnervation once axons approach muscle fibers.

The role of miR-206 in reinnervation after nerve damage may explain its salutary function in ALS. As motor neurons die in ALS, denervated muscle fibers are reinnervated by the axon branches of the surviving motor neurons. Compensatory reinnervation may account for the clinical observations that ALS is nearly asymptomatic in humans until a large fraction of motor neurons have died, at which point the few remaining ones cannot sufficiently compensate (18, 19). Consistent with this idea, NMJs were remarkably similar with this idea, NMJs were remarkably similar in mutant mice that lack muscle-derived factors that promote nerve-muscle interactions in response to motor neuron injury. We therefore searched for muscle-derived synaptic organizing factors that were affected in opposite ways by miR-206 and HDAC4. The mRNA levels of several known regulators of synapse formation (14, 24, 25), including fibroblast growth factor 7 (FGF-7), FGF-10, and FGF-22, were not changed between miR-206−/− and wild-type mice (fig. S13). However, an FGF binding protein, FGFBP1, was down-regulated in muscles of miR-206−/− mice and up-regulated in muscles of HDAC4 mKO mice after denervation (Fig. 4, F and G). FGFBP1 is a secreted factor that interacts with FGF-7, FGF-10, and FGF-22 family members and potentiates the bioactivity of FGF-7 in rat L6 myoblasts by releasing sequestered FGF from the extracellular matrix (26). Because FGF-7, FGF-10, and FGF-22 are muscle-derived regulators that promote presynaptic differentiation at the NMJ (14), we hypothesized that FGFBP1 could potentiate the effects of FGFs during reinnervation. Consistent with this idea, recombinant FGFBP1 enhanced the ability of FGF10 to promote differentiation of vesicle-rich varicosities in cultured motor neurons (fig. S14, A and B).

To probe the role of FGFBP1 in vivo, we used an interfering RNA (fig. S15). Knockdown of FGFBP1 in vivo inhibited the maturation of neonatal NMJs; AChR clusters were smaller than those in control myofibers, and presynaptic vesicle clustering was perturbed (Fig. 4, H and I). These defects resembled those documented above in miR-206−/− mice after denervation, suggesting that miR-206 and HDAC4 promote and impede NMJ innervation, respectively, via opposing effects on FGFBP1.

It has long been known that denervated muscle is readily reinnervated, whereas innervated muscle cannot be hyperinnervated (27, 28), suggesting that muscle fibers can sense whether or not they are innervated and respond to denervation by enhancing their susceptibility to reinnervation. Our results indicate that miR-206 regulates one important pathway involved in this bidirectional signaling (Fig. 4, J and K).

Our results reveal miR-206 as a modifier of ALS pathogenesis and suggest that the salutary actions of miR-206 are mediated by muscle-derived factors that promote nerve-muscle interactions in response to motor neuron injury. miR-206 expression is highly enriched in slow muscles, which are resistant to denervation in mouse models of ALS (29). Perhaps increased miR-206−/−dependent retrograde signaling helps protect slow myofibers. Recent studies have identified mutations in humans with ALS in the genes encoding TDP-43 and FUS, which regulate various aspects of RNA metabolism (30–32) and biochemically interact with the miRNA processing enzyme Drosha (33, 34). Moreover, the related Caenorhabditis elegans miRNA, miR-1, also regulates nerve terminal function and retrograde signaling (35). Together with these results, the identification of miR-206 as a modifier of ALS pathogenesis provides a new perspective on the mechanisms underlying this disease and suggests opportunities for intervention through the modulation of miR-206 or the downstream pathways it regulates.

References and Notes
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Norbin Is an Endogenous Regulator of Metabotropic Glutamate Receptor 5 Signaling

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Metabotropic glutamate receptor 5 (mGluR5) is highly expressed in the mammalian central nervous system (CNS). It is involved in multiple physiological functions and is a target for treatment of various CNS disorders, including schizophrenia. We report that Norbin, a neuron-specific protein, physically interacts with mGluR5 in vivo, increases the cell surface localization of the receptor, and positively regulates mGluR5 signaling. Genetic deletion of Norbin attenuates mGluR5-dependent stable synaptic transmission in the hippocampus. As with mGluR5 knockout mice or mice treated with mGluR5-selective antagonists, Norbin knockout mice showed a behavioral phenotype associated with a rodent model of schizophrenia, as indexed by alterations both in sensorimotor gating and psychotomimetic-induced locomotor activity.

In view of the numerous important roles of mGluR5 in the CNS (1–5), we searched for endogenous regulators of this receptor. We used the carboxy-terminal domain of mGluR5a (Ala12 to Leu1171) as bait in a yeast two-hybrid screen. Sixteen interacting clones (6) were isolated, including several known mGluR5-interacting proteins, such as Homer1 (7) and calmodulin (8). Three clones encoded the C terminus of Norbin (Fig. 1A, top, and fig. S1A) (9). Norbin, also known as Neurochondrin (10), is a 75-KD neuronal protein without any known functional domain (11). When tested with all known mGluR receptors (mGluR1 to mGluR8) (12), Norbin (Glu499 to Pro729) specifically interacted with a subset of group I mGluRs, namely mGluR1a, mGluR5a, and mGluR5b (Fig. 1A, bottom).

Direct interaction of Norbin and mGluR5 was confirmed by GST (glutathione S-transferase) pull-down (fig. S1B) and coimmunoprecipitation experiments (fig. S1C). Endogenous Norbin and mGluR5 proteins coimmunoprecipitated from rat brain lysates (Fig. 1B). Experiments with truncated mutants of mGluR5 indicated that the membrane proximal region of mGluR5a (Ala12 to Gly90) interacted with Norbin (fig. S2A). Further studies narrowed the binding sites to two small regions, region A (Arg857 to Arg867) and region B (Gly90 to His90) (fig. S2B). Synthetic peptides covering either region A or B interfered with the interaction between mGluR5 and Norbin in cell lysates (fig. S2C). Replacement of the key amino acids in either region A (mGluR5-mut1) or B (mGluR5-mut2) or both (mGluR5-mut1/2) with alanine abolished binding of mGluR5 to Norbin (Fig. S2D). Norbin-binding regions partially overlapped with identified calmodulin-binding sites (fig. S3, A and B) (8). However, mGluR5-mut1 was defective in Norbin binding (fig. S2D), but not in calmodulin binding (fig. S3C) and, therefore, was used to study the specific role of Norbin in the regulation of mGluR5 function. Homer and Norbin did not affect each other’s binding to mGluR5 (fig. S3D).

Using an affinity-purified antibody to Norbin (fig. S4A), prominent expression of Norbin in adult mouse brain was observed in the hippocampus, amygdala, septum, and nucleus accumbens, with moderate expression in the dorsal striatum (Fig. 1C). This distribution of Norbin resembles that of mGluR5 (13). A synaptosomal fraction purified from mouse brain contained both Norbin and mGluR5 (Fig. 1D). In primary hippocampal neurons, Norbin and mGluR5 were localized together in dendrites and had a punctate appearance (Fig. 1E). Double staining with antibodies to microtubule-associated protein 2 (MAP2) or spinophilin (Fig. 1F) indicated that Norbin was localized with the dendritic spine marker spinophilin.

We assessed the physiological consequence of Norbin expression on signaling through the mGluR5 receptor. mGluR5 is coupled to the heterotrimeric guanine nucleotide-binding protein (G protein) α subunit Gz, that activates phospholipase C, causes generation of inositol 1,4,5-triphosphate (InsP3), and leads to calcium release and calcium oscillations (14). After a 30-min exposure to a group I mGluR agonist (10 μM 1,2,5-triazosilacacid), more inositol phosphates were formed in human embryonic kidney (HEK) 293T cells doubly transfected with mGluR5 and Norbin (84.6 ± 5.7% increase above basal level) than in cells transfected with mGluR5 alone (57.0 ± 3.1% increase above basal level) (fig. S4B). Activation of mGluR5 leads to extracellular signal-regulated kinase ERK1/2 phosphorylation, and this was also enhanced when Norbin was coexpressed in HEK293T cells (fig. S4C).

We tested whether Norbin transfection affected mGluR5-elicited calcium oscillations in HEK293T cells stably expressing mGluR5. The average length of the calcium oscillations was significantly longer in Norbin-expressing cells than in control cells (12.5 ± 0.8 min versus 8.2 ± 1.1 min, P < 0.05, Wilcoxon rank sum test) (Fig. 2, A and B). The mean number of calcium peaks was significantly higher in Norbin-expressing cells (11.2 ± 0.9 peaks; n = 196) than in control cells (6.7 ± 0.8 peaks; n = 105; P < 0.05, Wilcoxon rank sum test) (dashed lines in Fig. 2B). Additionally, 16% of the control cells failed to respond to (S)-3,5-dihydroxyphenylglycine (DHPG), whereas only 7% of the Norbin-transfected cells failed to do so [fig. 2B (bottom), yellow and blue bars]. However, the effect of Norbin was not seen in cells expressing the Norbin-binding defective mutants, mGluR5-mut1 and mGluR5-mut1/2 (Fig. 2C).

The fact that Norbin binds to the membrane proximal region of mGluR5 prompted us to test whether Norbin might influence cell surface expression of mGluR5. The amount of cell surface mGluR5 was significantly increased in the presence of cotransfected Norbin in N2a cells (a cell line derived from mouse neural crest), whereas