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Expression of MicroRNAs in Human Post-mortem Amyotrophic Lateral Sclerosis Spinal Cords Provides Insight into Disease Mechanisms

Claudia Figueroa-Romero[#a], **Junguk Hur**[#a,1], **J. Simon Lunn**[a,2], **Ximena Paez-Colasante**[a], **Diane E. Bender**[a,3], **Raymond Yung**[b,c], **Stacey A. Sakowski**[d], and **Eva L. Feldman**[a,d,*]

[a] Department of Neurology, University of Michigan, Ann Arbor, MI 48109 USA

[b] Division of Geriatrics and Palliative Medicine, Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA

[c] Geriatric Research, Education and Clinical Care Center, VA Ann Arbor Health System, Ann Arbor, MI 48105, USA

[d] A. Alfred Taubman Medical Research Institute, University of Michigan, Ann Arbor, MI 48109, USA

[#] These authors contributed equally to this work.

Abstract

Amyotrophic lateral sclerosis is a late-onset and terminal neurodegenerative disease. The majority of cases are sporadic with unknown causes and only a small number of cases are genetically linked. Recent evidence suggests that post-transcriptional regulation and epigenetic mechanisms, such as microRNAs, underlie the onset and progression of neurodegenerative disorders; therefore, altered microRNA expression may result in the dysregulation of key genes and biological pathways that contribute to the development of sporadic amyotrophic lateral sclerosis. Using systems biology analyses on postmortem human spinal cord tissue, we identified dysregulated mature microRNAs and their potential targets previously implicated in functional process and pathways associated with the pathogenesis of ALS. Furthermore, we report a global reduction of mature microRNAs, alterations in microRNA processing, and support for a role of the nucleotide binding protein, TAR DNA binding protein 43, in regulating sporadic amyotrophic lateral sclerosis-associated microRNAs, thereby offering a potential underlying mechanism for sporadic amyotrophic lateral sclerosis.

[*]Corresponding author: Eva L. Feldman, MD, PhD, 109 Zina Pitcher Place, 5017 AAT-BSRB, Ann Arbor, MI 48109 USA; Phone: 001 734-763-7274; Fax 001 734-763-7275; efeldman@umich.edu.

[1]Department of Basic Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND 58202, USA;

[2]Renovo Neural, Inc., Cleveland, OH 44106, USA;

[3]Center for Human Immunology and Immunotherapy Programs, Washington University School of Medicine, St. Louis, MO, USA.

Keywords

Amyotrophic lateral sclerosis; Epigenetics; MicroRNA

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disease characterized by motor neuron degeneration (de Carvalho and Swash, 2011; Robberecht and Philips, 2013). While some (~15%) cases are familial (fALS), the majority (~85%) are sporadic (sALS) and several cell types and mechanisms contribute to the pathogenesis of the disease (Siddique and Ajroud-Driss, 2011; van Blitterswijk et al., 2012). This complexity has hindered therapeutic development, and Riluzole, the only approved therapy for ALS, exerts a modest benefit at best (Bensimon et al., 1994; Gordon, 2013). Thus, identification of the molecular mechanisms underlying ALS is critical to support effective therapy development.

Evidence suggests that environmental exposures, physical stress, and altered immunity may promote epigenetic changes and contribute to ALS (Ahmed and Wicklund, 2011; Callaghan et al., 2011; Qureshi and Mehler, 2013). Epigenetic mechanisms, including DNA methylation, histone remodeling, and microRNAs (miRNAs), reversibly regulate gene expression without altering the basic genetic code (Qureshi and Mehler, 2011). These mechanisms are induced by the same environmental/occupational risk factors associated with ALS (Callaghan et al., 2011; Cox et al., 2009; Furby et al., 2010), can accumulate throughout life, and their reversibility supports their potential utility as therapeutic targets. Our group and others previously examined how DNA methylation/hydroxymethylation and chromatin modifications contribute to the altered regulation of gene expression in spinal cord tissue, brain tissue, and whole blood from sALS subjects (Chestnut et al., 2011; Figueroa-Romero et al., 2012; Morahan et al., 2009; Tremolizzo et al., 2014). We found that only a subset of altered genes were regulated by methylation and hydroxymethylation (Figueroa-Romero et al., 2012), and while these modifications have been recently shown to play an important role in the development of neurodegeneration and mental disorders (Kato and Iwamoto, 2014), our data further suggest that other epigenetic mechanisms may contribute to altered gene expression in ALS.

miRNAs are small non-coding RNAs that negatively regulate up to 60% of the human genome by destabilizing mRNA or inhibiting translational efficiency. Canonical miRNA biogenesis begins in the nucleus where long primary miRNAs (pri-miRNAs) are encoded by the genome and cleaved by the microprocessor complex comprised of Drosha and DiGorge syndrome critical region gene 8 (DGCR8) to generate precursor miRNAs (pre-miRNAs) (Gregory et al., 2006). The pre-miRNAs are then transported to the cytoplasm by exportin 5 (Lund et al., 2004)and further processed by the endoribonuclease DICER-1 into mature 17-22-nucleotide duplex miRNAs. A single strand of the mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which includes DICER, eukaryotic translation initiation factor 2C, 2/argonaute RISC catalytic component 2 (EIF2C2/AGO2), TAR (HIV-1) RNA binding protein 2 (TRBP), and protein kinase, interferon-inducible double-stranded RNA dependent activator (PACT) (Chendrimada et al., 2005; Emde et al., 2015;

Lee et al., 2006). This complex facilitates binding of the mature miRNA to complementary nucleotide sequences in the 3' untranslated region (UTR) of the mRNA targets.

Importantly, a single miRNA can regulate several hundred mRNA targets via RNA-dependent post-transcriptional silencing mechanisms, and mRNA transcripts can be regulated by multiple miRNAs (Paez-Colasante et al., 2015). Hence, altered expression of only a few miRNAs can impact many genes and promote aberrant effects on multiple biological pathways (Friedman et al., 2009; Liang, 2009). There are currently over 2,000 annotated human miRNAs that regulate many biological processes, including development, cell proliferation, and organ system homeostasis (Friedlander et al., 2014; Haramati et al., 2010). In the central nervous system, miRNAs are highly expressed, and their dysregulation is linked to several neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), and multiple sclerosis (Goodall et al., 2013; Hartl and Grunwald Kadow, 2013; Ksiazek-Winiarek et al., 2013; Maciotta et al., 2013). Notably, toxicants linked to sALS onset, such as heavy metals and pollution, are also associated with altered miRNA expression (Ahmed and Wicklund, 2011; Callaghan et al., 2011; Wang and Cui, 2012), and two fALS genes, fused in sarcoma (*FUS*) and transactive response DNA binding protein 43 (*TARDBP*) encoding the TDP-43 protein, are involved in RNA processing and miRNA biogenesis (Buratti et al., 2010; Kawahara and Mieda-Sato, 2012; Mackenzie et al., 2010).

Here, we investigated the contribution of miRNA dysregulation to ALS pathogenesis using a systems biology approach on postmortem spinal cord tissue from sALS and control subjects. We hypothesized that differential miRNA expression results in the dysregulation of key genes and biological pathways, contributing to sALS development. We identified differentially expressed mature miRNAs (DEmiRNAs) and their predicted mRNA targets in human postmortem spinal cord, and further examined biologically relevant pathways and functions affected by the miRNA-target pairs to gain insight into disease pathogenesis. Finally, we examined miRNA biogenesis and the role of TDP-43 as potential novel mechanisms underlying miRNA dysregulation in sALS.

Materials and Methods

Subjects and tissue

Frozen human spinal cord samples from 12 Caucasian ALS subjects without a family history of ALS and 12 age- and gender-matched neurologically-normal controls were obtained from the National Institute for Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD (contract HHSN275200900011C, Ref. No NO1-HD-9-0011) and from the Michigan Brain Bank and Michigan ALS Consortium at the University of Michigan (Table 1). All autopsy participants at the University of Michigan signed a written informed consent under a protocol reviewed and approved by the University of Michigan Medical School Institutional Review Board (Protocol # HUM00028826). sALS samples were screened for abnormal GGGGCC hexanuclotide repeat expansions within the chromosome 9 open reading frame 72 (*C9ORF72)* gene, the most common genetic cause for fALS and sALS to date, using the

repeat-primed PCR method as previously described (Meisler et al., 2013; Renton et al., 2011).

Nucleic acid extraction

Total RNA was extracted from postmortem human spinal cord with a miRNeasy kit and treated with RNAse-free DNAse1 according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). As previously reported, genomic DNA from ALS postmortem human spinal cord was isolated using the Promega Maxwell 16 Tissue DNA Purification kit and a Maxwell instrument (Promega Co, Madison, WI, USA) (Figueroa-Romero et al., 2012). Nucleotide concentration was assessed using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA).

miRNA array profiling and data analysis

High-throughput analysis of mature miRNAs in human spinal cord was performed using TaqMan OpenArray according to the manufacturer's instructions (Applied Biosytems/Life Technologies, Carlsbad, CA, USA). Stem-loop reverse transcription (RT) was obtained with the TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT primers and a pre-amplification utilized TaqMan PreAmp Master Mix and Megaplex PreAmp Primers. The RT and pre-amplification reactions were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosytems/Life Technologies). Diluted pre-amplification product was mixed with the TaqMan Open Array Real-Time PCR Master Mix and loaded in a 384-well OpenArray Plate. TaqMan OpenArray miRNA assays were performed at the University of Michigan's DNA Sequencing Core by the Affymetrix and Microarray Core Group to comprehensively measure miRNA abundance levels.

Raw array data were analyzed using DataAssist™ v3.01 (Applied Biosystems/Life Technologies). Briefly, the PCR plate was comprised of 817 miRNAs, including multiple copies of endogenous controls. Samples with non-available values in more than 50% of data were excluded from further analyses. To identify additional poor-quality samples and to determine the most appropriate reference miRNA for normalization (RNU48, RNU44, and U6), a box-plot of un-normalized data with all endogenous control data marked was examined. Samples with high levels of variability in each endogenous control (cycle threshold (CT) value range > 3) were not included for further analyses. RNU48 was selected as the internal reference for calculating delta-CT, as it showed the least variation across all samples. The maximum CT value was set to 40 across all samples.

DEmiRNAs were determined using a two-sample t-test on the delta-CT data between the ALS and control samples. The False Discovery Rate (FDR) for each miRNA was obtained after adjusting the corresponding *P*-value with the Benjamini-Hochberg multiple testing correction method. A FDR < 0.05 was used as the cutoff for significant DEmiRNAs between ALS and control samples.

Identifying potential miRNA targets

Potential functional gene targets of the DEmiRNAs were identified by collecting a comprehensive list of both experimentally validated and predicted targets. Validated targets

were obtained from TarBase, a comprehensive database of experimentally supported animal miRNA targets (Sethupathy et al., 2006). Predicted targets were obtained using both conserved and non-conserved sites from TargetScan (Score > −0.2) (Kawahara and Mieda-Sato, 2012; Lewis et al., 2005) and microRNA.org (mirSVR score < −0.1) (Betel et al., 2008; Meisler et al., 2013). To filter out potentially non-functional miRNA targets in sALS spinal cord samples, our previous microarray gene expression data from the same samples (Figueroa-Romero et al., 2012) were used to limit the targets to those among the differentially expressed genes (DEGs) that exhibited inversely correlated regulation. For each DEmiRNA-target gene pair, a Pearson correlation coefficient was calculated using the delta-CT values in the OpenArray and $\log_2$-transformed normalized intensity values in the microarray. Selection criteria for miRNA-target pairs included a Pearson correlation coefficient greater than 0.5 (P-value < 0.05) and a gene expression fold-change of at least 2.

Functional enrichment analysis and miRNA-target network analysis

miRNA-target pairs that passed the above filtering steps were subjected to functional enrichment analyses. For each miRNA, the collected high-quality targets were evaluated for functional enrichment, and a locally implemented version of the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang da et al., 2009; Renton et al., 2011) was used to identify over-represented biological functions among the targets related to Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes pathways (Kanehisa and Goto, 2000). A FDR < 0.05 was used as the cutoff for significantly enriched terms. A heat map was generated to visualize the most significantly enriched terms, and the 10 most significantly enriched terms per gene set were collectively selected and their P-values were transformed by $-\log_{10}(P\text{-value})$ and color-indexed. Finally, a regulatory network that included the miRNA-target gene interactions identified above and the protein-protein interactions among the gene targets identified based on the Biological General Repository for Interaction Datasets (BioGRID) (Chatr-Aryamontri et al., 2013) was generated in Cytoscape (Huang da et al., 2009; Smoot et al., 2011).

Expression of miRNA and mRNA by quantitative PCR (qPCR)

Differential expression of selected miRNAs was confirmed by qPCR analysis using TaqMan universal PCR master mix (without Uracil-N glycosylase), TaqMan miRNA assays, and the pre-amplified product used for the array analysis (1:200 dilution in water) according to the manufacturer's instructions and following the standard protocol for TaqMan using a StepOnePlus™ Real-Time PCR System (Applied Biosystems/Life Technologies). The fluorescence threshold CT value representing miRNA expression in sALS samples was calculated using StepOnePlus system software. miRNA levels were first normalized to RNU48 as the reference gene (delta-CT) and then relative to the control group (delta-delta-CT). Levels of miRNA PCR products are expressed as mean ± SEM and a two-sample equal variance t-test was performed using GraphPad Prism 5 to indicate significant miRNA differences between sALS and control.

To validate mRNA target expression levels, cDNA from the same total RNA extracted for the array was generated using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). qPCR was performed in triplicate using sequence-specific primers with Power SYBR®

Green PCR Master Mix (Applied Biosystems/Life Technologies). Primer sequences for chemokine C-C Motif ligand 2 (*CCL2*) and cluster of differentiation 4 (*CD4*), TNF receptor superfamily, member 6 or Fas ligand (*FAS*), eukaryotic translation initiation factor 2C, 4/argonaute RISC catalytic component 4 (*EIF2C4/AGO4*), and aquaporin 1 (*AQP1*) are described in Table S1. The PCR amplification profile was as follows: 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 30 s, and a final phase of 72°C for 5 min. Differential expression of downstream targets was determined and analyzed as indicated for validation of miRNA expression using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the reference gene for normalization. Expression levels of pri-let-7e and pri-miR-577 were analyzed using TaqMan primary miRNA (pri-miRNA) assay probes using *GAPDH* for normalization (Applied Biosystems/Life Technologies, Carlsbad, CA) (Table S2).

Plasmid construction

To establish an inducible N-terminus (N)-Flag-tagged TDP-43 mouse embryonic stem (mES) cell line, we modified the pLox vector (Kyba et al., 2002) by inserting a Flag tag. Briefly, 6.25 nM each of the forward and reverse adaptor primers (Table S1) were annealed in 1X annealing buffer (100 mM Tris-HCl pH 8.0, 1 M NaCL) and incubated in boiling water that was gradually cooled to room temperature. The annealed adaptors were diluted 1:8000 in 0.1 X TE, pH 8.0, and 3 μl were cloned into the Sal1 site of the 5'-multicloning site (MCS) and the Xba1 site of the 3'MCS of the vector to introduce a Flag tag (MDYKDDDDK) followed by the restriction sites for Xho1, EcoR1, Kpn1, Xba1, Sal1/Acc1, Sma1, and Not1. This resulted in the elimination of the neo cassette and the eGFP gene sequence (the Xba1 site is not unique, as there is an Xba1 site upstream of the 5' MCS). Human TDP-43 was PCR amplified using SH-SY5Y-derived cDNA as template and Phusion High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, MA, USA) following the manufacturer's protocol. TDP-43 was cloned into the Xho1-Xba1 sites of the CS2+ vector (generously provided by Dr. Dave Turner at the University of Michigan) and then subcloned into the Xho1-Not1 sites of the Flag-pLox vector.

To create the constructs required for the luciferase assays (pmirGlo-AGO4), the 3' UTR of EIF2C4/AGO4 (nucleotides 75-929 3' to the stop codon) was amplified in 25 μl reactions using 100 ng of spinal cord genomic DNA as template (Figueroa-Romero et al., 2012), specific primers (Table S1), and Q5® High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) following the manufacturer's protocol. The reaction was performed in a Genemap PCR system 9700 thermocycler (Applied Biosystems/Life Technologies) with the following settings: 98°C for 30s, 35 cycles of denaturation at 98°C for 5 s, annealing at 70°C for 20 s, and extension at 72°C for 30 s, and a final phase of 72°C for 2 min. Amplicons were subcloned into the Xho1-Sbf1 sites of the pmirGlo Dual-Luciferase vector (Promega, Madison, WI, USA). All constructs were verified by sequencing at the University of Michigan Sequencing Core.

Establishment, culture, and transfection of Flag-TDP-43 inducible mES cells

The AinV15 cell line has been previously described (Kyba et al., 2002; Lunn et al., 2012; Reyes et al., 2008). Briefly, this mES cell line contains a reverse tet transactivator upstream

of a puromycin selectable cassette and a truncated neomycin resistance cassette downstream of the interaction site allowing for puromycin and geneticin (G418) selection (Kyba et al., 2002; Lunn et al., 2012; Reyes et al., 2008). N-Flag-TDP-43 was integrated into the genome of the parental AinV15 cell line upstream of the tet operon to generate the tetracycline-inducible N-Flag-TDP-43 mES line as previously described (Lunn et al., 2012). Briefly, AinV15 cells were plated in 0.1% gelatin (from porcine skin Type A, Sigma-Aldrich, St. Louis, MO, USA)-coated 12-well plates (Corning/Cellgro, Manassas, VA, USA) at 8×10^4 cells/well in complete media [4.5 mM glucose DMEM (Gibco, Life Technologies), 10% heat inactivated mES-tested fetal bovine serum (FBS; University of Michigan Transgenic Core), 1000 units/mL human recombinant Leukemia inhibitory factor (Chemicon/Millipore, Billerica, MA, USA), and 1.5μg/mL puromycin (Sigma-Aldrich)] and maintained at 37 °C with 5% CO_2. The cells were transfected with 1 μg of DNA and TransIT®-2020 transfection reagent (Mirus Bio LLC, Madison, WI) following the manufacturer's protocol 24 h after plating. Three days later, the cells were expanded into a gelatin-coated 60 mm plate in complete media and incubated for 2 days. Selection of stably transfected cells was achieved by culturing the cells in complete media supplemented with 300 μg/mL G418 (Gibco/Life Technologies). A total of 6 colonies were picked from each selection plate and expanded for 10 passages. The N-Flag-TDP-43 mES cell lines were validated for N-Flag-TDP-43 insertion by incubating with increasing concentrations of doxycycline (Dox; 1-2 mg/ml) for 3 days prior to DNA and protein extraction for sequencing by the University of Michigan Sequencing Core and western blotting, respectively. One validated line was used for all subsequent experiments.

Luciferase reporter assays

N-Flag-TDP-43-expressing AinV15 cells were plated at ~2×10^5 cells/well in 0.1% gelatin-coated 6-well plates (Corning/Cellgro) in complete media on day 1. On day 2, media was changed to complete media ± 1μg/mL Dox to induce expression of TDP-43 (Kelloff and Sigman, 2005; Lunn et al., 2012). The cells were split into a 24-well plate on day 6 at ~7×10^4 cells/well and transfected using Lipofectamine 2000 reagent (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA, USA) with 150 ng of pmirGlo (empty vector) or pmirGlo-AGO4 on day 7. Luciferase reporter activity was assayed 24 h after transfection in duplicate by using the Dual-Light system (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to Renilla luciferase activity to account for transfection efficiency. Analysis of data was performed as previously reported (Van Etten et al., 2013) and expressed as mean ± SEM of percent repression from 3 independent experiments using a two-sample equal variance t-test with GraphPad Prism 5.

Western blot analysis

Laemmli sample buffer was added to luciferase assay protein extracts and the samples were boiled, centrifuged, and run in a SDS-polyacrylamide gel and the proteins were transferred to a PVDF membrane (EMB Millipore, Darmstadt, Germany). Immunoblot analysis was performed using rabbit polyclonal antibody for Flag (G400) (Cat# F1804; Sigma-Aldrich) and goat polyclonal antibody for Actin (1-19) (Cat# sc-1616; Santa Cruz Technology, Dallas, Texas, USA) as well as Santa Cruz Technology secondary antibodies: horseradish peroxidase-conjugated goat anti-mouse (sc-2005) and goat anti-rabbit (Cat# sc-2020).

Results

Mature miRNA dysregulation in postmortem human spinal cord

To determine whether altered miRNA regulation plays a role in the dysregulation of DEGs previously identified in postmortem human spinal cord tissue (Figueroa-Romero et al., 2012), we performed TaqMan OpenArray miRNA profiling on spinal cord tissue from 12 sALS and 12 control subjects (Table 1). Array profiling identified 90 mature DEmiRNAs in sALS, among which 98% were decreased and only miR-155 and miR-142-5p were increased (Table S3). Functional enrichment analyses provided insight into over-represented functions among the DEmiRNAs, identifying functional categories associated with functions known to be altered in ALS, such as cell death, the immune response, and brain development (Fig. 1A).

The sALS postmortem spinal cord samples used in this study corresponded to subjects without a family history of ALS. Abnormal GGGGCC hexanuclotide repeat expansions within the *C9ORF72* gene underlie the most common form of fALS, affecting about 40% of cases, and expansions are also found in 6-8% of sALS cases. Mutations in other genes, such as Cu^{2+}/Zn^{2+} superoxide dismutase (*SOD1*), *TARDBP*, and *FUS*, are only found in 1-2% of sALS cases (Boylan, 2015). Based on the size of our cohort, we screened only for abnormal GGGGCC hexanuclotide repeat expansions within the *C9ORF72* gene and identified one subject with an abnormal *C9ORF72* expansion (Table 1; Fig. S1). Follow-up analyses on the array profiling results, however, revealed that the levels of selected dysregulated mature miRNAs and their predicted targets were similar across all samples, suggesting that the inclusion of the subject with an abnormal *C9ORF72* expansion did not impact the outcomes of the present analyses.

DEmiRNA gene targets and affected biological functions in postmortem human spinal cord

Potential relevant DEG targets of the 90 DEmiRNAs were obtained by compiling multiple miRNA prediction target databases that included both experimentally validated and predicted targets. Since miRNAs negatively regulate gene expression, pairs were limited by identifying inverse correlations between DEmiRNAs and mRNAs (1,182 DEGs from our previous work (Figueroa-Romero et al., 2012)). Examination of enriched biological functions among the potential DEmiRNA gene targets using heat map analysis indicated enrichment of defense and immune responses (Fig. 1B). Connections among the target genes and their corresponding miRNAs were next identified using target network analysis. This indicated that the increased (n=2) and decreased (n=88) DEmiRNAs and their targets (n=237) formed two large sub-networks (Fig. 2A) that are connected via protein-protein interactions between 8 targets (Fig. 2B).

Our analysis indicates that the two increased miRNAs in sALS spinal cord, miR-155 and miR-142, are predicted regulators of dysregulated sALS and neurodegeneration-related gene transcripts, which include ubiquilin2 (*UBQLN2*), RNA binding protein, fox-1 (*RBFOX1*), and reelin (*RELN*) (Fig. 2B). miR-155 and miR142 are also potential regulators of several genes, such as *RBFOX1*, crystallin-mu (*CRYM*), tyrosin kinase-related protein 1 (*TYRP1*),

and indirectly through protein-protein interactions, with Rap guanine nucleotide exchange factor 2 (RAPGEF2) and kinesin family member 5A (KIF5A). These data suggest that several disease targets are likely regulated by these two miRNAs (Fig. 2B).

For the larger subnetwork of decreased miRNAs, we selected miRNAs to be further analyzed based on 1) high delta-CT (fold expression) in the array or high correlation expressions of miRNA and predicted mRNA pairs, or 2) an ALS candidate gene approach using the known biological function for the miRNA, the predicted mRNA candidate functions (focusing on ALS-related functions such as immune response, cell death, and neurodegeneration), or interconnection with ALS-related pathways in miRNA-mRNA predicted target networks. Based on these criteria, the miRNAs let-7e, miR-148b-5p, miR-577, miR-133b, and miR-140-3p (Fig. 3, blue octagons) were selected. These five DEmiRNAs collectively may regulate 86 different genes in human spinal cord (Fig. 3, white/yellow circles), among which we selected *FAS*, *CD4*, *EIF2C4/AGO4*, *CCL2*, and *AQP1* for experimental validation, as they have been implicated in neuronal homeostasis and/or the pathogenesis of ALS (Fig. 3, yellow circles). Inverse correlation between the five selected miRNAs and the five predicted targets in sALS was confirmed by qPCR (Fig. 4). Together, these results demonstrate the identification and validation of differentially expressed miRNA-target pairs and provide insight into the potential biological implications of their dysregulation.

Expression of immature miRNA transcripts are not altered in sALS

Given that the global decrease of most mature DEmiRNAs in sALS spinal cord may be explained by alterations either at the level of gene expression or at the level of miRNA processing, we examined intermediates in miRNA biogenesis for two of our selected DEmiRNAs. To determine if miRNA processing defects could explain the differences in mature miRNA levels, we examined the primary transcript expression levels of pri-let-7e, which is an evolutionarily conserved miRNA, and pri-miR-577, a predicted regulator of several genes. While the levels of the mature miRNAs for these two transcripts are differentially expressed in our array data (Fig. 4; Table S3), the levels of the two pri-miRNAs are the same between sALS and control samples (Fig. 5). These data suggest that differential expression of mature miRNAs in sALS is likely not secondary to alterations in gene expression, but instead, that miRNA processing is halted and/or premature miRNAs are “trapped” while undergoing processing.

Over-expression of TDP-43 alters miRNA activity

TDP-43 plays a role in miRNA biogenesis, has been linked to the inhibitory effect of miRNAs on their targets by interacting with mature and pri-miRNAs, and is known to prevent the incorporation of selected miRNAs into the RISC (Buratti et al., 2010; Emde et al., 2015; Kawahara and Mieda-Sato, 2012; King et al., 2014; Li et al., 2013b; Zhang et al., 2013); therefore, we were interested in determining whether TDP-43 plays a role in the miRNA-induced regulation of one of our selected target genes, EIF2C4/AGO4. AinV15 mES cells conditionally expressing N-Flag-TDP-43 under control of a Dox-inducible promoter were transiently transfected with a luciferase reporter expressing the 3’ UTR of EIF2C4/AGO4 in the presence or absence of Dox. While endogenous miRNAs negatively

regulated the expression of the luciferase reporter, over-expression of TDP-43 attenuated the endogenous negative regulation of this reporter (Fig. 6). These data suggest that TDP-43 may alter the expression or function of endogenous miRNAs, offering a potential mechanism by which miRNA dysregulation occurs in ALS.

Discussion

The lack of an effective treatment for ALS and the emerging association of epigenetic alterations stemming from environmental exposures has fueled increased interest in understanding miRNA regulation in the nervous system in ALS, especially since miRNAs may be potential therapeutic targets (Buratti et al., 2010; Kawahara, 2010; Mackenzie et al., 2010; Williams et al., 2009). Here, we identified 90 DEmiRNAs in sALS postmortem spinal cord that associate with the differential expression of 237 predicted gene targets. These DEmiRNA:DEG pairs affect functional processes and pathways including cell death, defense responses, immune responses, and inflammation, thereby supporting their biological relevance and providing insights into potential pathogenic mechanisms. Furthermore, we demonstrate global reduction of mature miRNAs but no changes in two pri-miRNA transcripts, indicating that miRNA biogenesis and/or miRNA turnover are likely compromised in sALS. Finally, our data support for a role of TDP-43 in miRNA regulation and offers insight into a potential pathogenic mechanism for sALS.

The potential contribution of epigenetic mechanisms, including DNA methylation, chromatin remodeling, and miRNAs, to neurodegeneration is an emerging area of intense investigation. Of particular interest, miRNA dysregulation is linked to aging and neurodegenerative disorders, including PD, AD, and multiple sclerosis (Goodall et al., 2013; Li et al., 2011; Ma et al., 2014). miRNA alterations are also present in microglial and neuronal cells in rodent ALS models and in peripheral blood mononuclear cells (PBMCs), fibroblasts, cerebrospinal fluid, and postmortem tissue from ALS subjects (Butovsky et al., 2012; Campos-Melo et al., 2013; De Felice et al., 2012; Freischmidt et al., 2014; Koval et al., 2013a; Kye and Goncalves Ido, 2014; Shinde et al., 2013), results which all support our observed DEmiRNAs in postmortem ALS spinal cord tissue. DEmiRNAs are likewise present in serum from pre-manifest fALS mutation carriers, suggesting both a role for miRNAs in ALS pathogenesis and as potential diagnostic biomarkers (Freischmidt et al., 2014). Therapeutic strategies targeting miRNAs (DeVos and Miller, 2013; Koval et al., 2013a; Nolan et al., 2014) further endorse their role in ALS pathogenesis; both intraventricular delivery of a miR-155 inhibitor as well as intracerebroventricular injection of an antagomir to miR-29a increases lifespan in ALS transgenic mice. We contend that understanding the contribution of miRNAs to sALS has the potential to enhance our understanding of disease mechanisms and offer novel therapeutic targets.

The majority of the identified DEmiRNAs in postmortem ALS spinal cord are decreased and may collectively promote the up-regulation of over 200 gene targets. This is consistent with recent reports demonstrating overall decreases of miRNAs in ALS spinal cord tissue (Campos-Melo et al., 2013; Emde et al., 2015). Interestingly, functional analysis of our DEmiRNA:DEGs reveals enriched biological functions, including cell death, defense and immune responses, and brain development, suggesting alterations in the neuronal

microenvironment. Notably, human ALS research is restricted by the unavailability of nervous tissue to study during disease development, and while postmortem tissue provides a direct way to analyze spinal cord and brain, it is difficult to differentiate whether molecular alterations are due to pre-mortem events, post-mortem handling of the tissue, or tissue region (Ferrer et al., 2008) rather than the pathogenic mechanisms responsible for disease onset and progression. Our study most likely represents alterations in miRNA levels within the neuronal microenvironment rather than in motor neurons themselves. ALS is a non-cell autonomous multifactorial disease, and end-stage ALS spinal cord presents considerable reactivity and proliferation of astrocytes and microglia, respectively, in concert with a significant reduction of motor neurons and oligodendrocytes (Philips and Rothstein, 2014). Interestingly, Emde *et al* recently reported a similar global reduction of mature miRNAs in laser-captured microdissected ALS postmortem spinal cord motor neurons. Thus, although our study may reflect a lack of motor neurons at end-stage, the high representation of dysregulated miRNAs involved in the regulation of immune and inflammation response-related genes strongly argues that our profiling embraces mostly non-neuronal cells (Jiang et al., 2005) or that the miRNA profile of non-neuronal cells is similar to that of motor neurons that have survived degeneration. Previous studies assessing miRNA alterations in ALS mouse microglia similarly identified immune-related miRNAs (Parisi et al., 2013), and a number of groups are investigating the immunological aspects underlying ALS pathogenesis (Evans et al., 2013; Zhao et al., 2013). Whether our observed findings reflect alterations in endogenous or infiltrating immune cells requires additional investigation; however, these data do support continued research on understanding a potential immunological component to neurodegeneration in ALS.

In our dataset, only miR-155 and miR-142-5p were increased. These miRNAs show the same trend in multiple sclerosis PBMCs, ALS mouse spinal cord, and human ALS monocytes (Butovsky et al., 2012; Koval et al., 2013b; Ma et al., 2014); however, a decrease in miR-142-5p levels was reported in ALS subject fibroblasts (Raman et al., 2014), indicating potential tissue-specific regulation. Interestingly, miR-155 and miR-142-5p are predicted to regulate *UBQLN2* and *RELN*, respectively, genes down-regulated among our DEGs (Figueroa-Romero et al., 2012). *UBQLN2* mutations are associated with fALS and its malfunction is associated with altered clearance of protein aggregates (Zhang et al., 2014). RELN is an extracellular matrix glycoprotein important for neural circuit formation and maintenance and cortical neuron migration. RELN dysfunction or loss is associated with neurodegeneration in AD, epilepsy, and autism (Folsom and Fatemi, 2013), and reports demonstrate *RELN* regulation by heavy metals and methylation (Zhubi et al., 2014). Given the previous association of both targets with neurodegeneration in conjunction with the loss of connectivity along the corticospinal tract in ALS, additional investigations into potential pathogenic pathways affected by these two miRNAs are warranted.

For further analyses, we next selected five decreased DEmiRNAs and five gene targets based on both fold change/high correlation between the DEmiRNA:DEG pairs and an ALS candidate gene approach. The miRNAs let-7e, miR-148b-5p, miR-577, miR-133b, and miR-140-3p, as well as the targets *FAS*, *CD4*, *EIF2C4/AGO4*, *CCL2*, and *AQP1*, were selected. Interestingly, we observed decreased levels of several members of the let-7

miRNA family in sALS spinal cord. Alterations in let-7 are present in animal models of PD and serum from subjects with multiple sclerosis and AD (Asikainen et al., 2010; Gandhi et al., 2013; Gehrke et al., 2010; Tan et al., 2014). Let-7b processing is also regulated by TDP-43 and its expression is altered in lymphoblast cell lines as well as cerebrospinal fluid, serum, and PBMCs from sALS patients (Buratti et al., 2010; Butovsky et al., 2012; Freischmidt et al., 2013; Kawahara and Mieda-Sato, 2012). These observations provide further support for a potential pathological role for let-7 family members in ALS.

Similarly, the predicted gene targets *FAS*, *CCL2*, and *CD4* are implicated in ALS and neurodegeneration. Microglia express increased CCL2 leading up to disease onset in ALS mice, and CD4+ T cells mediate dopaminergic toxicity in a mouse model of PD and are present in the spinal cord of ALS subjects (Brochard et al., 2009; Butovsky et al., 2012; Engelhardt et al., 1993; Kawamata et al., 1992). Importantly, the association of EIF2C4/AGO4 is also of particular interest given its role as a member of the argonaute family of proteins, a component of the RISC in miRNA function (Modzelewski et al., 2012; Sasaki et al., 2003; Winter and Diederichs, 2011). Indeed, recent studies demonstrate that argonaute proteins contribute to miRNA processing and function and are dysregulated in ALS and Huntington's disease (Raman et al., 2014; Savas et al., 2008; Tan et al., 2014), suggesting that miRNA biogenesis may be altered in neurodegeneration.

Along these lines, our results identified a global reduction in levels of mature DEmiRNAs and altered regulation of their corresponding targets in ALS that may underlie a potential defect in miRNA biogenesis or RNA stability. We found that the levels of two immature pri-miRNAs, pri-miR-577 and pri-miR-let-7e, were not altered in postmortem sALS spinal cord tissue compared to their mature forms. Recent evidence suggests that alterations of miRNA biogenesis lead to motor neuron degeneration, in part, due to the aberrant catalytic activity of the cytoplasmic RNAse Dicer and protein-protein interactions between molecular components responsible for stress granule formation and miRNA biogenesis (Emde et al., 2015; Haramati et al., 2010). We argue that dysregylation of non-canonical miRNA biogenesis may also play a role in sALS pathogenesis, as we observe increased levels of miR-155 and miR-142-5p in sALS while the rest of the dysregulated miRNAs are decreased (Abdelfattah et al., 2014).

Mutations in genes encoding the nucleotide binding proteins FUS and TDP-43 and abnormal hexanucleotide repeat expansions in *C9ORF72*, as well as the pathological aggregation of FUS and TDP-43 in stress granules and processing bodies (p-bodies) in the cytoplasm (Li et al., 2013a) or disruption of nuclear paraspeckle formation (Shelkovnikova et al., 2014) in neurons and glia, further highlights the role of altered RNA processing in ALS pathogenesis (Belzil et al., 2013; Duan et al., 2014). Of interest, TDP-43 binds to miRNAs and components of the miRNA-processing pathway at different steps, exhibiting direct interactions with mature miRNAs, nuclear pri-miRNAs, the nuclear RNAse Drosha, and the cytoplasmic RNAse Dicer (Buratti et al., 2010; Emde et al., 2015; Kawahara and Mieda-Sato, 2012; King et al., 2014; Li et al., 2013b; Zhang et al., 2013). In addition, TDP-43 also affects how miR-206, a skeletal muscle-specific miRNA involved in neuromuscular junction re-innervation in ALS animal models and following nerve injury, incorporates into the RISC (King et al., 2014). Our data suggests that TDP-43, in part, may regulate the function of

miRNAs decreased in sALS spinal cord as well as their downstream targets, perhaps by the sequestration of miRNA biogenesis components to stress granules upon stress (Emde et al., 2015). Furthermore, it is important to note that post-transcriptional RNA modifications could also play a role in the global reduction of dysregulated RNAs by altering the stability of the premature miRNAs. Although miRNAs are thought to be more stable than mRNAs, global and rapid turnover of miRNAs is more striking in neuronal cells compared to other cell types (Krol et al., 2010). Post-transcriptional O-methylation is a well-known mechanism that increases the stability of small RNAs (Ji and Chen, 2012), while on the other hand, O-methylation of the 5' mono-phosphate end of pre-miR-145 by the BCDIN3 domain-containing methyltransferase (BCDIN3D) interferes with further maturation by Dicer (Xhemalce et al., 2012). Collectively, our data and these studies suggest that global reduction of miRNA levels in ALS may result from their sequestration within RNA-binding protein aggregates, accelerated RNA turn over, or disruption of miRNA biogenesis (Paez-Colasante et al., 2015).

We acknowledge that the size of the cohort analyzed in this study was relatively small due to the limited availability of human postmortem samples. Nevertheless, others have reported a similar global decrease in mature miRNAs using smaller cohorts (Campos-Melo et al., 2013; Emde et al., 2015), suggesting that this observation is relevant to the disease, as it has been reported in three different cohorts. With the advent of multicenter biobanks, these results should be validated in larger cohorts in the future. Moreover, the rapid development of stem cell technology to generate human motor neurons from patient-derived induced pluripotent stem cells will allow us to determine whether the drastic decline in miRNAs is responsible for the onset of the disease, facilitates the progression of the disease, or whether it results from end-stage complications.

Conclusions

This study combines gene and miRNA expression profiling/bioinformatics analyses to address the hypothesis that miRNA dysregulation promotes aberrant effects on biologically relevant pathways that underlie ALS. Our data suggest a potential mechanism for altered miRNA expression in ALS and justify further studies examining the mechanisms and consequences of altered miRNA and target gene expression, as well as the utility of miRNA profiling as a potential diagnostic, pathogenic, and prognostic biomarker for ALS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AQP1	aquaporin 1
BCDIN3D	BCDIN3 domain-containing methyltransferase
BioGRID	Biological Genomic Repository for Interaction Datasets
C9ORF72	chromosome 9 open reading frame 72
CCL2	chemokine C-C motif ligand 2
CD4	cluster of differentiation 4
CRYM	crystallin-mu
CT	cycle threshold
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DEG	differentially expressed gene
DEmiRNA	differentially expressed microRNA
DGCR8	DiGorge syndrome critical region gene 8
EIF2C4/AGO2	eukaryotic translation initiation factor 2C,2/argonaute 2
EIF2C4/AGO4	eukaryotic translation initiation factor 2C,4/argonaute 4
fALS	familial amyotrophic lateral sclerosis
FAS	TNF receptor superfamily member 6/Fas ligand
FDR	false discovery rate
FUS	fused in sarcoma
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GO	gene ontology
KIF5A	kinesin family member 5A
miRNA	microRNA
PACT	protein kinase, interferon-inducible double-stranded RNA dependent activator
PBMC	peripheral blood mononuclear cell
PD	Parkinson's disease
qPCR	quantitative polymerase chain reaction
RAPGEF2	Rap guanine nucleotide exchange factor 2

RBFOX1	RNA binding protein, fox 1
RELN	reelin
RISC	RNA induced silencing complex
RT	reverse transcription
sALS	sporadic amyotrophic lateral sclerosis
TAM	tool for annotations of human miRNAs
TDP-43	TAR DNA binding protein 43
TYRP1	tyrosinase-related protein 1
UBQLN2	ubiquilin 2
UTR	untranslated region

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Highlights

- Mature miRNA are globally dysregulated in postmortem sporadic ALS spinal cord
- Altered genes affect neuronal homeostasis, ALS pathogenesis, and miRNA biogenesis
- Altered processes include cell death, defense/immune responses, and inflammation
- Primary transcripts of a subset of dysregulated mature mRNAs are unchanged in sALS

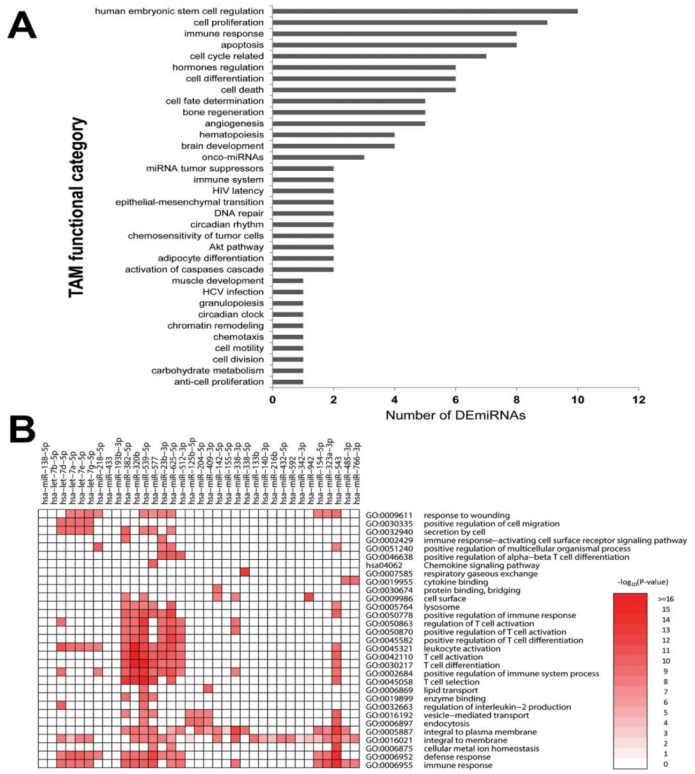

Fig. 1. Enriched biological functions of DEmiRNAs and targets in sALS spinal cord

(A) To examine the distribution of functional categories for the DEmiRNAs, a bar chart was created based on the "Functions" categories of the tool for annotations of human miRNAs (TAM) database. The grey bars indicate the number of DEmiRNAs in each functional category. Only those functions with at least 5 DEmiRNAs are included. **(B)** For each DEmiRNA with a minimum of five target genes in the current study, we performed gene set enrichment analysis to identify the enriched biological functions among the collected high-quality targets (both validated and predicted). A heat map was generated to visualize the most significantly enriched terms. *P*-values were transformed by $-\log_{10}(P\text{-value})$ and color-indexed to indicate significance levels. Significance ranged from no significance (white) to highest significance (dark red).

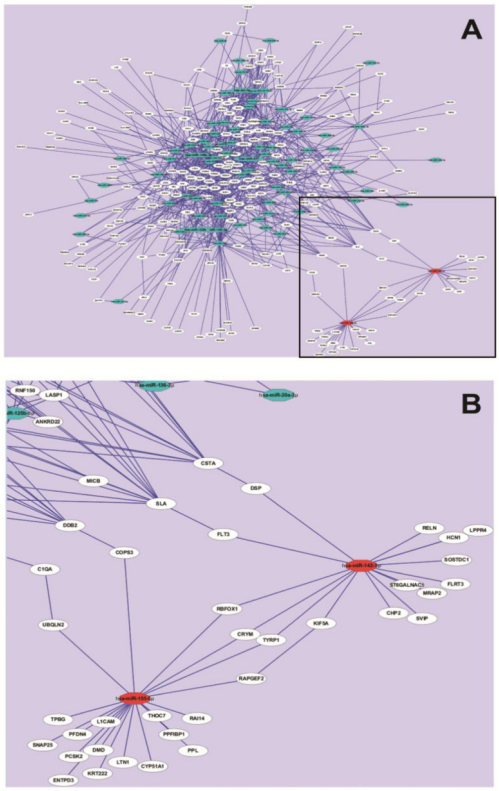

Fig. 2. Association network of miRNA-targets in sALS human spinal cord

(A) The complete miRNA-target network representing all 90 DEmiRNAs, and their targets. **(B)** Zoomed image of the boxed region in (A) depicting the two increased miRNAs and their predicted mRNA targets and interactions. Blue octagons represent decreased DEmiRNAs and red octagons represent increased DEmiRNAs. White circles represent the predicted miRNA target genes. Lines connecting two nodes represent miRNA-target or target-target interactions.

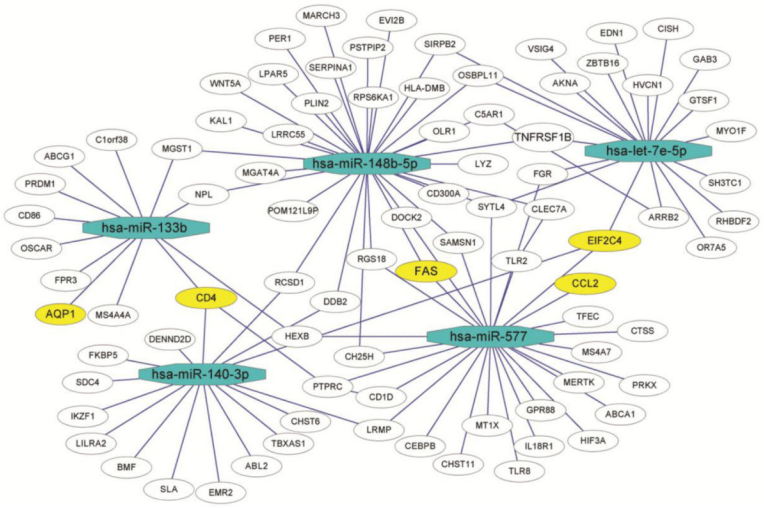

Fig. 3. Association network of select decreased miRNAs and their candidate mRNA targets in sALS human spinal cord

Five decreased miRNAs were selected from the DEmiRNAs based on 1) array delta-CT (fold expression) or high correlation expression of miRNA and predicted mRNA pairs, or 2) by the known biological function for the miRNA or the predicted mRNA candidate (immune response, cell death, and neurodegeneration) or interconnection with several ALS related pathways by miRNA-mRNA predicted target network. Selected miRNA-mRNA interactions were then visualized using Cytoscape. Blue octagons represent decreased DEmiRNAs. White and yellow circles represent predicted candidate target genes, with the yellow circles representing genes selected for further investigation. Lines connecting two nodes represent miRNA-target or target-target interactions.

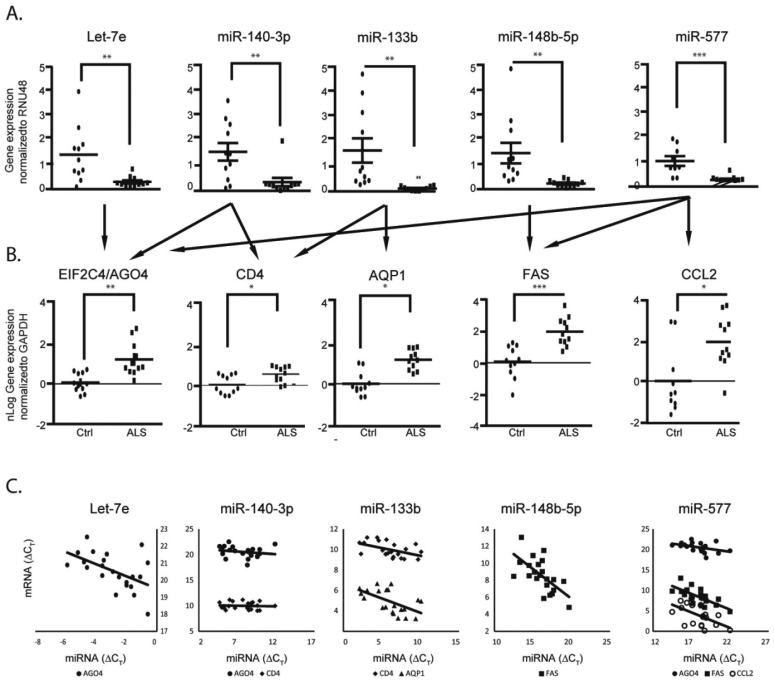

Fig. 4. Confirmation of selected DEmiRNAs and potential mRNAs targets in spinal cord

(A) Preamplified miRNA cDNA from postmortem human spinal cord used for the array from sALS (n=11) and control (n=11) subjects was analyzed by qPCR using TaqMan technology. Results were normalized to RNU48 as the reference gene (delta-CT) and then relative to the control group (delta-delta-CT). Levels of miRNA PCR products are expressed as mean ± SEM. **(B)** For mRNA, cDNA was generated from total RNA extracted from the same postmortem human spinal cord samples as in (A) and analyzed by qPCR. mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and presented as fold change. Individual data points are plotted as nLog of mean ± SEM. Arrows indicate the predicted targets for the selected miRNAs. **P*<0.05, ** *P*<0.01, ****P*<0.001 compared to the control group (Ctrl). **(C)** X-Y scatter plot of miRNA and predicted target mRNA values from individual samples using their delta-Ct values referenced respectively from RNU48 miRNA (A) and GAPDH (B) mRNA levels indicate inverse correlation.

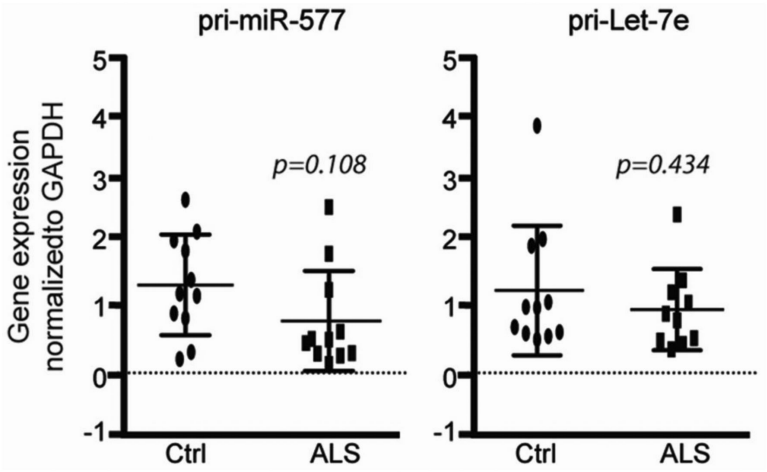

Fig. 5. Let-7e and miR-577 primary transcripts are not altered in sALS

Gene expression levels of pri-miR-577 and pri-Let-7e were analyzed in sALS and control samples by qPCR and normalized to *GAPDH*. The data are presented as fold change and plotted as mean ± SEM.

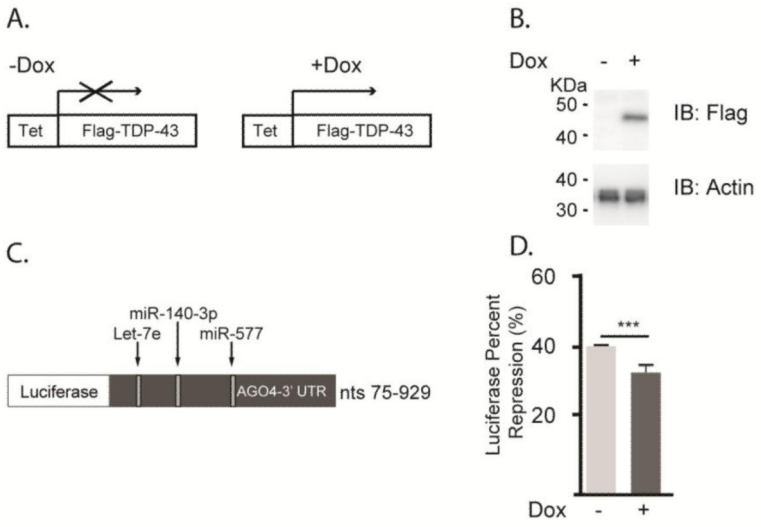

Figure 6. Over-expression of TDP-43 reduces miRNA regulation of EIF2C4/AGO4 stability

(A) Schematic representation of protein expression when N-Flag-TDP-43 AinV15 cells containing an inducible tetracycline response element (Tet) are grown in the presence or absence of Dox for 5 days. **(B)** Total cell lysates were immunoblotted for the Flag tag to confirm the expression of Flag-TDP-43 in the presence or absence of Dox. Actin levels were assessed to ensure equal protein loading. **(C)** At day 3 of Dox induction, the cells were transiently transfected with a luciferase reporter fused to the 3' UTR of EIF2C4/AGO4 (75-929 nt). The reporter harbors seed sequences recognized by three of the 5 selected DEmiRNAs, including miR-140-3p, miR-577, and Let-7e. **(D)** Relative luciferase activity was quantified in duplicate to determine the effect of endogenous miRNAs on luciferase reporter expression in the presence or absence of TDP-43 over-expression. Data are presented as the mean ± SEM of three independent experiments. ***P<0.001.

Table 1

Clinical characteristics

		ALS	Control	*p-value*
n		12	12	-
Age (years)[(a)]		56 (35-71)	55 (36-73)	NS
Gender	Male	10	10	NS
	Female	2	2	
SC location	Cervical	9	8	NS
	Thoracic	3	4	NS
Onset	Bulbar	2	-	-
	Limb	8	-	-
	NA	2	-	-
Postmortem interval[(b)]		14.5±7.0 h	15.2±6.2 h	NS
C9ORF72		1	-	-
Cause of death	ALS	12	-	<0.001
	Accident	-	2	-
	PC	-	2	-
	ASCVD	-	5	-
	Cancer	-	1	-
	Cardiac arrest	-	1	-
	NA	-	1	-

[(a)] median (range);

[(b)] mean ± standard deviation; ALS, amyotrophic lateral sclerosis; ASCVD, arteriosclerotic cardiovascular disease; NA, no data available; NS, not significant; PC, pulmonary complications; SC, spinal cord