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Altered Purkinje cell miRNA expression and SCA1 pathogenesis

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ABSTRACT

Spinocerebellar ataxia type 1 (SCA1) is a dominantly inherited neurodegenerative disorder caused by polyglutamine repeat expansions in Ataxin-1. Recent evidence supports a role for microRNAs (miRNAs) deregulation in SCA1 pathogenesis. However, the extent to which miRNAs may modulate the onset, progression or severity of SCA1 remains largely unknown. In this study, we used a mouse model of SCA1 to determine if miRNAs are misregulated in pre- and post-symptomatic SCA1 cerebellum. We found a significant alteration in the steady-state levels of numerous miRNAs prior to and following phenotypic onset. In addition, we provide evidence that increased miR-150 levels in SCA1 Purkinje neurons may modulate disease pathogenesis by targeting the expression of *Rgs8* and *Vegfa*.

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Introduction

Spinocerebellar ataxia type 1 (SCA1) is a member of the polyglutamine (polyQ) family of diseases, a group of dominantly inherited neurodegenerative disorders caused by the expansion of translated CAG trinucleotide repeats (Orr, 2012; Zoghbi and Orr, 2009). In SCA1, the expanded polyQ tract resides near the *N*-terminus of the Ataxin-1 (Atxn1) protein (Banfi et al., 1994). Although mutant Atxn1 is expressed throughout the brain, SCA1 is primarily characterized by the loss of cerebellar Purkinje cells and degeneration of the spinocerebellar tracts (Durr, 2010; Seidel et al., 2012). While the exact molecular mechanisms underlying this selective neurodegeneration remain largely unknown, it has been suggested that the deregulation of gene expression programs may, in part, explain this cell and region-specific vulnerability (Crespo-Barreto et al., 2010; Matilla-Duenas et al., 2010; Serra et al., 2004).

Altered neuronal transcriptional activity is an early and persistent pathomolecular feature of most polyglutamine diseases (Orr and Zoghbi, 2007; Takahashi et al., 2010; Verbeek and van de Warrenburg, 2011). Changes in the steady-state levels of numerous mRNA transcripts are seen in the cerebella of SCA1 patients and mouse models of the disease (Crespo-Barreto et al., 2010; Cvetanovic et al., 2011; Fernandez-Funez et al., 2000; Lin et al., 2000; Serra et al., 2004). Since wild-type Atxn1 functions within a transcriptional repressor complex that includes the DNA binding protein Capicua (Lam et al., 2006), transcriptional deregulation in SCA1 is thought to result from both a partial loss of Atxn1 function and a gain of toxic function in mutant Atxn1 (Lim et al., 2008; Orr, 2012). In fact, SCA1 mouse models have reduced the levels of the Atxn1-Capicua transcriptional repressor complex and a corresponding increase in the levels of some Atxn1-Capicua-regulated mRNAs (Crespo-Barreto et al., 2010). However, the steady-state levels of numerous other mRNAs not directly regulated by the Atxn1-Capicua complex are also altered early in the pathogenesis of SCA1. This suggests that other cellular pathways may play key roles in the pathogenesis of SCA1.

MicroRNAs (miRNAs) are small non-coding RNAs employed to post-transcriptionally regulate the steady-state levels of mRNA transcripts in the cell (Kim et al., 2009). Mounting evidence supports a role for microRNAs in the pathogenesis of SCA1. First, changes in steady-state levels of several miRNAs are observed in human SCA1 brains (Persengiev et al., 2011). Second, disrupting miRNA biogenesis in Purkinje neurons leads to ataxia and cerebellar degeneration reminiscent of SCA1 and other dominantly inherited ataxias (Schaefer et

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al., 2007). Finally, the Atxn1 mRNA itself is posttranscriptionally regulated by several miRNAs, some of which display increased activity in the human SCA1 brain (Lee et al., 2008; Persengiev et al., 2011). Nevertheless, the extent to which miRNA deregulation in SCA1 may drive early events in the pathogenesis of the disease remains largely unknown.

Here, we profiled global miRNA expression in the cerebellum of pre- and post-symptomatic SCA1 transgenic mice. As was reported for human SCA1 brains, we find significant changes in the expression of several miRNAs. We also provide evidence that a number of miRNAs display altered steady-state levels prior to the onset of measurable phenotypes. In addition, we establish a connection between increased miR-150 levels and the loss of *Vegfa* mRNA in SCA1 mouse brain. These results shed new light into the role that miRNAs play in the pathogenesis of SCA1 and provide new opportunities for the development of disease biomarkers and therapies.

Materials and methods

Animals

The SCA1 BO5 transgenic line used in this study was maintained in an FVB background. The mouse colony was bred and maintained at the University of Iowa animal vivarium. Mice were exposed to a 12-hour light-dark cycle and had access to food and water ad libitum. Transgenic and littermate control mice were genetically identified using established PCR-based protocols. All experiments involving animals were approved by Animal Care and Use Committee at the University of Iowa.

RNA collection, microRNA array and quantitative PCR

Cerebella were collected from wild-type and age matched SCA1 mice at 4 and 12 weeks of age (n = 4, per group) using TRIzol (Invitrogen) according to the manufacturer instructions. MiRNA expression profiles were obtained using miRCURY LNA™ all species microRNA arrays, miRBase version 9.2 (Exiqon, Vedbaek, Denmark). Individual miRNAs were analyzed by quantitative PCR (Q-PCR) using a high-capacity cDNA archive kit and TaqMan® MiRNA Assays (both from ABI). Gene specific Q-PCR was performed as above using random hexamer primers and TaqMan® Gene-Specific Expression Assays on an ABI-7900 instrument (ABI).

In situ hybridization

MicroRNA in situ hybridization was performed using short digoxigenin-labeled DNA–LNA probes (Exiqon). Briefly, fresh frozen 12 μm sections were post-fixed in 4% paraformaldehyde and washed in PBS. Prior to hybridization, sections were acetylated by washing in 1.32% triethanolamine solution followed by acetic anhydride treatment. Sections were prehybridized for 2 h at 55 °C, then hybridized at 55 °C overnight (prehybridization and hybridization buffer were purchased from Ambion, mRNA locator kit). After hybridization, sections were washed in 2× SSC at 55 °C three times for 90 min, and then briefly rinsed in PBST (0.1% Tween 20 in PBS). Sections were blocked with 2% sheep serum in PBST at room temperature for 1 h, and incubated in alkaline phosphatase (AP) conjugated anti-digoxigenin antibody (Roche) at 1:1000 at 4 °C overnight. Color reaction was carried in AP buffer (100 mM Tris–HCl, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween-20, pH 9.5) containing NBT/BCIP (50× stock solution, Roche).

Anti-Vegfa immunohistochemistry

Mice were transcardially perfused with saline solution followed by 4% paraformaldehyde (PFA, pH 7.4). Dissected brains were fixed overnight in 4% PFA and immersed in cryoprotectant (30% sucrose/0.1 M PBS) for 48-h at 4 °C. Sagittal cerebellar sections (30 μm) were obtained on a sliding microtome and stored frozen in a 30% sucrose–30% ethylene glycol/0.1 M PBS solution. All sections were washed in 0.1 M PBS, overnight, prior to histological processing. The anti-Vegfa antibody stain was performed following manufacturer's recommendations (Abcam, ab39250). Briefly, sections were incubated for 20 min in a 10 mM sodium citrate buffer (pH 6.0), 0.05% Tween 20 solution preheated to and maintained at 100 °C. Following the antigen retrieval step, sections were blocked using 0.1 M PBS with 0.05% Tween 20 and 5% normal goat serum. Sections were next incubated for 3 days at 4 °C with anti-Vegfa antibody (1:100) diluted in blocking solution. Finally, an Alexa Fluor®488-conjugated goat anti-rabbit secondary antibody was used at a 1:500 dilution. Images were captured using a Leica DM RBE fluorescent stereoscope.

N2A cell culture and transfections

Mouse Neuro2a cells were maintained in DMEM/F12 mix supplemented with 10% fetal bovine serum, L-glutamine (5 mM) and non-essential amino acids (0.1 mM). A *MirVana*™ miR-150 miRNA mimic and a *MirVana*™ miRNA control mimic were purchased from Applied Biosystems (Life Technologies, Carlsbad, CA). Transfection of the double-stranded miRNA mimics into Neuro2a cells was performed using RNAimax reagent (Life Technologies) following the manufacturer's recommendations. Total RNA or protein lysates were obtained as previously described.

The first 1.7 kb of the 3′ untranslated region of mouse Vegfa was amplified using conventional RT-PCR and the following primers: 5′-CCATAGATGTGACAAGCCAAGGC-3′ and 5′-CTGCTCTAGAGACAAAGACGTG-3′. To generate the mutant Vegfa-3′UTR the conserved miR-150 binding site (5′-TGCTGTGGACTTGTGTTGGGAGG-3′) was disrupted by deleting the underlined 12-nucleotide sequence using a previously described site-directed mutagenesis approach (Tsou et al., 2011). The resulting Vegfa-3′UTR sequences were cloned into the XhoI–NotI sites of the psiCHECK-2™ vector system (Promega, Fitchburg, WI). *Renilla* luciferase activity was measured following the manufacturer's recommendations (Promega) and normalized using the intraplasmid *firefly* luciferase normalization reporter.

Western blot analyses

Vegfa protein expression was detected in western blots using a rabbit polyclonal antibody (ab39250, 1:500 dilution; Abcam, Cambridge, UK). Tubulin levels (anti-α-tubulin, 1:10,000; Sigma, St Louis, MO) were analyzed and used to normalize the levels of Vegfa in western blots. Following 48-h of primary antibody incubation at 4 °C, membranes were washed and incubated with peroxidase-conjugated with either anti-rabbit or anti-mouse secondary antibodies (1:20,000 dilution; Jackson Immuno Research Laboratories, West Grove, PA). The signal was obtained using the ECL-plus reagent (*Western Lighting*, PerkinElmer, Waltham, MA) as previously described (Tsou et al., 2011). Quantification of band intensities was performed using the Quantity One Software analysis tool. We quantified Vegfa expression in three independent experiments by normalizing the Vegfa signal to the Tubulin signal and calculating the mean expression level in experimental groups (miR-150 mimic treatment) relative to the control groups (control miRNA mimic treatment).

AAV RNAi vectors

Artificial miRNAs targeting human ATXN1 or a control sequence were designed and cloned into shuttle recombinant adeno-associated viral vectors (rAAV) as previously described (Boudreau et al., 2009, 2011). High-titer rAAV virus used in this study was produced at the University of Iowa Gene Transfer Vector Core (Iowa City, IA) following previously described methods.

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