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Analysis of piRNA-like small noncoding RNAs present in axons of adult sensory neurons

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Abstract

Small noncoding RNAs (sncRNAs) have been shown to play pivotal roles in spatiotemporal-specific gene regulation that is linked to many different biological functions. PIWI-interacting RNAs (piRNAs), typically 25–34 nucleotide long, are originally identified and thought to be restricted in germline cells. However, recent studies suggest that piRNAs associate with neuronal PIWI proteins, contributing to neuronal development and function. Here, we identify a cohort of piRNA-like sncRNAs (piLRNAs) in rat sciatic nerve axoplasm, and directly contrast temporal changes of piLRNA levels in the nerve following injury, as compared to those in an uninjured nerve using deep sequencing. We find that 32 of a total 53 annotated piLRNAs show significant changes in their levels in the regenerating nerve, suggesting that individual axonal piLRNAs may play important regulatory roles in local mRNA translation during regeneration. Bioinformatics and biochemical analyses show that these piLRNAs carry characteristic features of mammalian piRNAs, including sizes, a sequence bias for uracil at the 5' end, and a 2'-O-methylation at the 3' end. Their axonal expression is directly visualized by fluorescence *in situ* hybridization in cultured dorsal root ganglion neurons as well as immunoprecipitation with MIWI. Further, depletion of MIWI protein using RNAi from cultured sensory neurons increases axon growth rates, decreases axon retraction after injury, and increases axon regrowth after injury. All these data suggest more general roles for MIWI/piLRNA pathway that could confer a unique advantage for coordinately altering the population of proteins generated in growth cones and axons of neurons by targeting mRNA cohorts.

Keywords

small noncoding RNA; neuronal piRNA; intra-axonal translation; regenerating nerve; small RNA sequencing; axon growth

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Introduction

Over the past decade, small noncoding RNAs (sncRNAs) have emerged as key regulators in the control of gene expression in the nervous system (1–4). sncRNAs are typically classified into functional groups, including microRNAs (miRNAs), short interfering RNAs (siRNAs), and PIWI (P-element induced wimpy testis)-interacting RNAs (piRNAs), based on the rather arbitrary criteria of size and limited information of their biological functions in the cell. Among these, piRNAs were originally identified in germ cells of *Drosophila* as small RNAs that bind to PIWI protein and the related proteins Aubergine (Aub) and Ago3 (5–8). Rodent homologs are MIWI, MILI, and MIWI2, respectively. Mature piRNAs are longer than miRNAs, roughly 25 to 34 nucleotides (nts) long rather than 21–24 nts, and are much less characterized regarding their biogenesis and biological functions. However, piRNAs are, by far, the most numerous species of all known RNAs in all Metazoa, and can pair with all types of genomic sequences including intergenic, intronic and exonic regions, implying diverse roles in gene regulation (9–11).

piRNAs are primarily known for their roles in germline cells, where they repress transposable elements *via* heterochromatin formation and post-transcriptional silencing. Some piRNAs are predominantly found in spermatids during pachytene stage of meiosis I, where their function is not yet understood (12,13). Relatively recently, PIWI/piRNA complexes were detected in mouse and *Aplysia* CNS neurons, suggesting more general roles for piRNAs in regulation of gene expression (14,15). For example, Lee et al. showed that detected piRNAs by deep sequencing of small RNA libraries (14). Knockdown of piRNAs detected in the cytoplasm of mouse hippocampal neurons led to a significant decrease of dendrite spine area, suggesting a modulator role for dendritic spine development. Similarly, knockdown of *Aplysia* PIWI proteins in the CNS showed that a neuronal PIWI/piRNA pathway causes methylation of the promoter of CREB2 for promoting long-term synaptic facilitation (15). While these early studies require more in-depth verification, they clearly show that piRNAs can play important roles in regulation of gene expression in both germline and somatic cells, including neurons.

We recently reported profiles for miRNAs in distal axons of sciatic nerve after crush injury (16). From deep sequencing of small RNAs isolated from the sciatic nerve axoplasm, we also detected a set of sncRNAs in the size of mammalian piRNAs (piRNA-like sncRNAs). Although there is strong evidence that these neuronally expressed piRNAs show gene-regulatory capability in the proximity of cell bodies, no report has yet been made to suggest the presence of piRNA-like sncRNAs (piLRNAs) in distal axons of neurons and link to their endogenous capacity to alter axon growth in sensory neurons. Here, we demonstrated that at least a subset of the piLRNAs we identified from sciatic nerve axoplasm present characteristic features of a sequence bias for uracil at the 5' end and a 2'-O-methylation at the 3' end. These piLRNAs associate with MIWI protein, and depletion of MIWI protein using siRNA increased axon growth rates, decreased axon retraction after injury, and increased axon regrowth after injury. This work indicates more general roles of neuronal MIWI/piRNA-like sncRNA pathway for axon growth and/or regeneration.

Materials and Methods

Animal Surgery and Tissue Preparation

Animal procedures were approved by the Institutional Animal Care and Use Committees (IACUC), and the experiments were conducted under the IACUC at Alfred I. DuPont Hospital for Children. The sciatic nerve of 150–225 g male (approximately 5–6 weeks old) Sprague Dawley rats (Harlan) was crush-injured at mid-thigh level. Briefly, the sciatic nerve was surgically exposed by gentle separation of the quadriceps muscle and manually crushed using fine jewelers' forceps twice for 15 sec. The muscle and skin overlying the nerve were closed in layers and animals were placed in prewarmed cages. Animals were euthanized at 1, 4, 7, and 14 days post-injury (DPI), and injured and uninjured sciatic nerves were collected and processed as previously described (16).

RNA Extraction, fractionation and Illumina Sequencing

A mechanical squeezing method was used to isolate total RNA from nerves as previously described (16). Briefly, 10 mm segments of nerve were cut into small pieces, transferred into Eppendorf tubes, and manually squeezed with a plastic pestle on ice in QIAzol lysis reagent (Qiagen) according to the manufacturer's instructions. Total RNA was extracted and ethanol precipitated. Using *iScript RT kit* (BioRad), cDNA was synthesized from 100 ng RNA. To assess purity of RNA isolation, standard extended PCR (35 cycles) was subsequently carried out for β -actin, cell body-restricted [microtubule-associated protein 2 (MAP2) and H1 histone family member 0 (H1F0)], and non-neuronal cell [glial fibrillary acidic protein (GFAP) and receptor tyrosine-protein kinase ErbB family-3 (ErbB-3)] mRNAs. After testing the purity of the axoplasm through extended cycle RT-PCR, purified RNA was further fractionated to <200 nucleotides in length by using an *RNeasy Mini spin column* followed by the *RNeasy MinElute Cleanup Kit* (Qiagen).

A total of 15 samples of sciatic nerves collected at different time points (1, 4, 7, and 14 DPI) including uninjured nerves were processed to Illumina HiSeq 2500 sequencing for generating small RNA libraries by the University of Delaware DNA Sequencing and Genotyping Center (UDSGC) located in the Delaware Biotechnology Institute (DBI) in Newark, DE, as previously described (16). Sequence data were analyzed by the CLC Genomics Workbench (Qiagen) software package with support from the University of Delaware Center for Bioinformatics and Computational Biology (CBCB) Bioinformatics Core. After trimming off adapters and counting reads, sequences ranging from 26 – 36 nt were annotated to rat piRNADatabase and sequences ranging from 26 – 36 nt were annotated to piRNABank rat database (pirnabank. ibab.ac.in). Only sequences containing 50 reads or greater were used for differential expression analysis. To facilitate a comparison of expression profiles, we chose to normalize our data using the Trimmed mean of M (TMM) method because the TMM method produced less variation between the biological replicates in our data set. All analyses were done using *edgeR* bioconductor package. Only small RNAs with expression in all 3 samples of each time point were included in the analysis.

Clustering methods

Hierarchical clustering method using *Cluster 3.0* open source clustering software was utilized to determine the relationship between significantly altered piRNA-like sncRNAs in response to injury. The similarity metrics across as a function of time after injury were calculated using average linkage and Euclidean distances metric.

Validation of piRNA-like sncRNA levels in sciatic nerve axoplasm by quantitative real time PCR (qPCR)

Illumina deep sequencing data were validated by quantitative PCR (qPCR) analysis using piRNA-specific LNA primers (Exiqon) as previously described (16). In brief, using *NCode™ VILO™ miRNA cDNA Synthesis Kit* (Invitrogen) enabling poly(A) tailing of the small RNAs according to the manufacturer's instructions with a universal RT primer, the purified small RNA was converted to cDNA. Subsequently, the resulting cDNA was used as a template for qPCR analyses using *NCode™ EXPRESS® SYBR GreenER™ miRNA qRT-PCR Kit* (Invitrogen) on the ABI 7900 Real-time PCR system by the Nemours Biomolecular Core using standard workflows and operating procedures. The final results of qPCR in relative level of piRNA-like sncRNAs normalized to that of U6 were expressed as the ratio of the injured levels to the uninjured level. To verify piRNA-specific primer specificity, we confirmed a single peak in the melting-curve analysis, as well as run the reaction products on a 5% agarose gel (NuSieve 3: 1; Lonza) for all genes analyzed.

Primers, probes, siRNAs and oligonucleotides

Sequences of the primers, probes and oligonucleotides used in this study are listed in Supplementary Table S1.

NaIO₄ treatment and β -elimination of RNA

Total RNA was extracted from sciatic nerve axoplasm followed by RNA fractionation to enrich small RNAs (<200 nt). Synthetic RNA in a 32-nucleotide length without modification was used as a control. Briefly, a portion of the small RNA enriched sample (2 μ g) was incubated with 25 mM NaIO₄ in borate buffer in the dark at 24°C for 30 min. The reaction was quenched by adding to final concentration of 10% (v/v) glycerol and concentrated in a speedvac to 5 μ L. β -elimination reaction was carried out with borate buffer at pH 9.5 for 90 min at 45°C. Treated neuronal RNA was then precipitated in three volumes of ethanol, washed once in 70% ethanol, and run on a 15% urea-acrylamide gel along with untreated RNA. For positive control with synthetic RNA, the gel was stained with GelStar™.

Northern blot

NaIO₄/ β -elimination treated and untreated small RNAs fractionated on 15% urea-acrylamide gels were transferred onto a nylon membrane in a 0.5× TBE for 30 min at 40 mA followed by UV-crosslinking. Northern blot was performed using biotin-labeled LNA antisense probes specific for piLRNA-1199 (Integrated DNF Technologies) and detected by the Chemiluminescent Nucleic Acid Detection Module (Pierce). Briefly, the blot was pre-washed in Wash buffer and then hybridized with the labelled probe in Hybridization buffer overnight at 55°C. The blot was washed in Wash buffer at room temperature for three times

followed by incubation for 15 min in blocking buffer. After washing the membrane four times for 5 min, the blot was incubated in Substrate Equilibration Buffer for 5 min followed by incubating in the Substrate working solution before imaging to phosphorImager screen in cassette.

Reverse transcription at low [dNTP] followed by PCR (RTL-P)

To amplify sncRNAs, the extracted small RNA was ligated to a 3' RNA adapter using T4 RNA ligase (Takara). The ligation product was then reverse-transcribed into cDNA with anchored or unanchored RT primers in either a low (0.4 μ M) or high (40 μ M) dNTP concentration. SuperScript III First-Strand Synthesis System (Invitrogen) was used according to the manufacturer's instructions with piRNA-1199-specific primer. Primer specific for miRNA-433-3p was used as a control. For PCR, a universal reverse primer and primers specific for piRNA-1199 and miRNA-433-3p were used. 25 cycles were performed consisting of denaturation at 95°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 1 min (2min for the last cycle).

Densitometric analysis of bands on agarose gels and western blots

The gels and blots were imaged with AlphaImager HP System (Cell Biosciences) and analyzed using NIH ImageJ. Briefly, after checking the boxes for Label with percentages and Invert peaks under the menu of *Gel Analyzer Options*, each of the bands across the lanes was selected as a region of interest (ROI) by drawing a tight boundary around the band. Using the same ROI across a row, intensities of the selected bands were measured by enclosing the area under each peak and used for further statistical analysis.

Cell culture and knockdown of MIWI using siRNA

L2-6 dorsal root ganglia (DRGs) were extracted and dissociated with 50 U collagenase type XI (Sigma) for 25 min at 37°C followed by trituration. The dissociated DRG neurons were transfected using an AMAXA 4D-Nucleofector system (Lonza) with initially three siRNAs (siRNAs #1, #2, and #3) (Integrated DNA Technologies) targeting different sequences in *MIWI* (a rodent homolog of the *Drosophila PIWI*) mRNA to deplete the mRNA or nontargeting scrambled siRNA. The siRNAs were individually tested for knock-down efficiency and a single most efficient siRNA was subsequently used for the functional experiments. DRG neurons were plated on glass-bottom culture dishes coated with poly-L-lysine (Sigma) and laminin (Millipore) at 37°C, 5% CO₂. mRNA and protein depletion were assessed at 24 and 48 hr, respectively, after transfection. For assessing axon regrowth from the cut end, neurons were plated in tissue culture inserts with polyethylene tetraphthalate membrane etched with 3 μ m diameter pores (Falcon). After pre-injury images of all neurons were acquired, distal axons were physically scraped away from the lower membrane surface. The inserts with the injured cell bodies were placed back into the culture media for further incubation. Images of regenerating axonal processes of the same neurons were then taken at 2 hrs, 1 d, and 2 d after axotomy. The length of axonal regrowth was measured from the cut ends by comparing to the pre-injury images by blinded assessor to the experiments.

Fluorescent *in situ* hybridization (FISH) and immunofluorescence (IF)

FISH for DRG cultures was similar to previously described methods (17). Briefly, Digoxigenin (DIG)-labeled antisense LNA probes (Exiqon) to rodent piRNAs were used to detect endogenous piRNA-like sncRNAs. DIG-labeled, 'scrambled' LNA probe was also included as a negative control. All steps were carried out at room temperature unless otherwise indicated. Coverslips with DRG neurons were rinsed in phosphate buffered saline (PBS), fixed in buffered 2% paraformaldehyde for 20 min, and then permeabilized in 0.3% Triton X-100 for 5 min. After being rinsed in PBS, hybridization was performed at 55°C for 4 hrs. Coverslips were then washed and processed for subsequent IF with chicken anti-neurofilament (NF) H (1:1,000; Chemicon) and sheep anti-digoxigenin (1:1,000; Roche). Secondary antibodies were as follow: Alexa488-conjugated anti-chicken IgG antibody (1:2,000; Life Technologies) and Cy3-conjugated anti-sheep IgG antibody (1:2,000; Life Technologies). Coverslips were mounted using PVA-DABCO (Sigma) anti-fading mounting medium and visualized with Leica DMRXA2 epifluorescent microscope. All images were matched for acquisition parameters.

Immunoprecipitation and RNA co-immunoprecipitation (RNA co-IP)

For analysis of associations of endogenous piRNA-like sncRNAs and MIWI protein, RNA co-IP was performed as described previously with a few modifications (17). Whole tissue lysates of testis and sciatic nerve were prepared in 'polysome buffer' (100 mM KCl, 5 mM MgCl₂, 0.5% NP40, 1 mM DTT, 100 U/ml RNase Out, 400 μ M vanadyl ribonucleoside complexes, protease inhibitor cocktail, 10 mM HEPES, pH 7.0). Primary MIWI antibody (Abcam) was absorbed to protein G *Dynabeads* (Life Technologies). *Dynabeads* coated with anti-mouse IgG were used as a negative control. Lysates were then immunoprecipitated using anti-MIWI coated protein G *Dynabeads* in NT2 buffer (150 mM NaCl, 1 mM MgCl₂, 0.05% NP40, 50 mM Tris-HCl, pH 7.4) for 1 h at 4°C, followed by vigorously washing with the polysome buffer. The precipitates resolved by SDS/PAGE were analyzed by immunoblotting with MIWI antibody (Thermo) using standard methods (18). To isolate RNA from the precipitates, beads were incubated in TRIzol (Life Technologies) and RNA extracted by phenol-chloroform. Extended RT-PCR ($\times$ 35 cycles) was used to detect bound piRNA-like sncRNAs over at least 3 separate experiments. To determine specificity of the precipitation, antisense oligonucleotides to piRNA-1 or piRNA-5567 were preincubated with the lysates prior to IP.

Statistical analysis

The *edgeR* program was used for statistical analyses of small RNA deep sequencing data. Differences in altered piRNA-like sncRNA levels across different time points after injury was assessed using the Chi-squared test, and the false discovery rate (FDR) was calculated to correct the *p* values; the smaller FDR indicates the smaller error in judging the *p* value. All experiments were performed in at least 3 separate experiments and reported as mean $\pm$ SD. Graphpad Prism 5 software package (GraphPad) was used for statistical analyses of qPCR and axon growth data. Student's *t*-test was used to determine significance differences between groups.

Results

Endogenous piRNA-like sncRNAs in adult rat sciatic nerve

We previously reported profiles for temporal changes in miRNA levels in axons of adult rat sciatic nerve in response to crush injury (16,19). From deep sequencing data, we found that $52.8 \pm 6.6\%$ of the total reads were either <20 nt or >25 nt in length and could not be mapped as miRNAs based on size criteria. We also unexpectedly found a distinct subpopulation of small RNAs ranging from 26–36 nt in length (predominantly 30–33 nt) (Fig 1A). Since known piRNAs in mammals correspond to this size range, we asked whether these sequences would map to the piRNA bank (pirnabank. ibab.ac.in), discovering that 33.1% of the total reads from the uninjured small RNA have been reported as piRNA sequences (Supplementary Fig 1). Several lines of evidence have also shown that mammalian piRNAs have a sequence bias for uracil at the 5' end (5'U preference) and are 2'-O-methylated at their 3' end (20–22). We examined further these annotated piRNA-like sncRNA sequences. We found that $33.5 \pm 5.9\%$ of these annotated piRNAs had a 5'U preference, a characteristic feature of mammalian piRNAs (Fig 1B).

To explore whether these piRNA-like sncRNAs contain a 2'-O-methylation at the 3' terminal, a characteristic feature of canonical piRNAs, we used two different methods. First, we treated RNA isolated from axoplasm and 3' non-modified synthetic RNA, as a control, with sodium periodate (NaIO_4) followed by β -elimination. When the synthetic RNA was subjected to the treatment and separated on the gel, GelStar^{TM} stain for detecting RNA revealed a reduction in size (Fig 1C, top), indicating the sensitivity of the synthetic RNA to the treatment. Next, total RNA from axoplasmic RNA underwent to the identical treatment followed by northern blot using a biotin-labeled probe specific for either miRNA-433 or piRNA-1199. Whereas non-modified miRNA-433 was sensitive to the treatment showing a reduction in size (Fig 1C, middle), we observed no detectable change for piRNA-1199 (Fig 1C, bottom). Second, we utilized reverse transcription (RT) at low dNTP concentration followed by PCR (RTL-P) approach (23). This recently developed methodology exploits the biochemical property of reverse transcriptase that pauses and terminates cDNA synthesis at a 2'-O-methylated site in a RNA template at a low dNTP concentration (e.g., 0.4 μM , instead of 40 μM). Consistent with previous reports (23,24), the band intensity of the RTL-P products of piRNA-1199 produced with an unanchored RT primer in the RT reaction at the low concentration of dNTPs was only 64.8% of the products with anchored RT primer, suggesting that the 3' end of the piRNA-like small RNA-1199 carries a 2'-O-methylation. In contrast, the signal intensity of the RTL-P product for 3' non-modified miRNA-433 showed no significant differences regardless of the dNTP concentration or primers used in the RT reaction (Fig 1D).

Taken together, these data indicate that at least a subset of the piRNA-like sncRNAs detected in sciatic nerve axoplasm represent characteristic features of canonical piRNAs, as have been described for mammalian piRNAs found in germline cells.

Validation and visualization of piRNA-like sncRNAs in the distal axons of neurons

To validate the levels of the piRNA-like sncRNAs in sciatic nerve identified from the deep sequencing data, we performed quantitative real-time PCR (qPCR) using piRNA-specific primers. We also included primer sets specific to germline piRNAs (piRs-2 and -3) that previously showed no expression in the nervous system to ascertain the specificity of the qPCR reactions (25). Since sciatic nerves include not only afferent and efferent axons, but also non-neuronal Schwann cells, non-neuronal contamination in the axonal RNA preparations from sciatic nerve was first assessed by extended cycle PCR using gene-specific primer sets for mRNAs that are restricted in cell body and glia (17,26–32). Figure 2A inset showed highly enriched axonal RNAs isolated from sciatic nerve axoplasm with undetectable levels of glial cell mRNAs.

Selecting specific piRNA-like sncRNAs for qPCR, we chose three most (piRs-5567, -1199, and -5901) and two less abundant piRNAs (piRs-4288 and -5597) that were identified from the deep sequencing data (Supplementary Table S2). Consistent with the deep sequencing data, the results of qPCR analysis for the piRNAs tested followed a similar pattern over the relative abundance of the piRNA-like sncRNAs isolated from the sciatic nerve axoplasm (Fig 2A). In contrast, we could not detect the germline specific piRNAs, piRs-2 and -3.

To confirm and directly visualize piRNA-like sncRNAs present in distal axons of adult neurons identified by both deep sequencing and qPCR analyses, we used fluorescence *in situ* hybridization (FISH) with locked nucleic acid (LNA) probes specific for piRNAs-5567 and -4288, and a scrambled probe as a negative control to determine if we could visualize these piRNAs in both cell bodies and axons. Consistent with the deep sequencing and qPCR analyses, we were able to detect piRNA-5567 and -4288 in the cell body as punctate signals (Fig 2B, right panel, and Supplementary Fig S2). In contrast, no fluorescent signals were visualized in neurons with the scrambled probe (Fig 2B, inset panel).

Temporal patterns of differential piRNA-like sncRNA expression in rat sciatic nerve following injury

We used deep sequencing to measure levels of small RNAs in regenerating nerves following crush injury. Based on the size criterion for mammalian piRNAs and mapping to the piRNA bank, we were able to annotate a total of 31 rat piRNA-like sncRNAs with read counts greater than 50 (Supplementary Table S2). The differential changes in piRNA-like sncRNA levels responding to injury in a temporal manner were then classified into 6 groups, profile 1 with no changes (e.g., piR-5595), profile 2 being first up-regulated then back to preinjury level (e.g., piR-1414), profile 3 being first down-regulated then up-regulated (e.g., piR-1200), profile 4 being first down-regulated then back to preinjury level (e.g., piRs-219), profile 5 being down-regulated along the time series (e.g., piR-5781), and profile 6 being slowly up-regulated along the time series (e.g., piR-1199) with different false detection rate (FDR) when compared with that for the uninjured nerve. A total of 18 piRNA-like sncRNAs showed significant changes in their levels in the regenerating nerve (profiles 2–6) (*edgeR* program, $p < 0.05$ and $FDR < 0.05$) (Supplementary Table S3), and 13 piRNAs did not change significantly along the time series tested. To further determine if levels of 31 axoplasmic piRNA-like sncRNAs could be distinguished, we performed cluster analyses using *Cluster*

3.0 open source clustering software. Figure 3 showed the results of hierarchical cluster analysis in the heatmap. In our previous miRNA profiling study (16), the uninjured control and injured nerves at 7 DPI were clustered together close to the injured nerve at 1 DPI. However, piRNA-like sncRNAs in the injured nerves at 7 and 14 DPI were closely clustered and those in the uninjured control and injured nerve at 1 DPI were clustered together (Fig 3B), suggesting that the regulatory roles of piRNA-like sncRNAs are likely distinct from those of miRNAs in axons of neurons.

Association of MIWI protein and piRNA-like sncRNAs in axoplasm of sciatic nerve

Given that piRNAs play a functional role in posttranscriptional gene silencing by forming functional complexes with PIWI proteins, we speculated that PIWI proteins are present and bind to piRNA-like sncRNAs in adult neurons. To examine expression of murine PIWI clade members, MIWI, MILI, and MIWI2, we first examined the expression of the mRNAs encoding murine PIWI clade proteins in total RNA from normal adult rat heart, brain, DRG, kidney, and liver tissues compared with adult testes by qPCR (Supplementary Fig 3A). *MIWI* mRNA was detected in all tissues tested, but at much lower levels than in testes. Consistent with MIWI protein expression previously documented in the mouse somatic tissue lysates (14), we also detected MIWI protein in rat nervous tissue lysates (Supplementary Fig 3B). In contrast, we could not detect MILI and MIWI2 proteins in the nervous tissues (*data not shown*), suggesting that only MIWI forms a functional complex with neuronal piRNA-like sncRNAs in neurons. To examine whether endogenous piRNA-like sncRNAs associates with MIWI protein to form a functional complex, MIWI protein from testes and sciatic nerve whole tissue lysates were coimmunoprecipitated using a MIWI antibody; immunoprecipitation with mouse IgG was also included as a control for non-specific binding (Fig 4A). Associated RNAs were extracted as described previously (17), and extended PCR using piRNA specific primers was used to determine association with the specific piRNA-like sncRNAs compared to IgG control (Fig 4B). The neuronal piRNA-like sncRNAs identified in our deep sequencing study were detected only in nerve tissue, but not in testes tissue lysate except piRNA-1200. Consistent with prior studies showing germline specific piRNA-1 (25), we found a high expression level of piRNA-1 in testis, but not in neuronal tissues.

To determine the specificity of immunoprecipitation, the nerve extract was preincubated with either antisense piR-1 or piR-5567 oligos prior to IP. We speculated that the preincubation of the nerve lysate with antisense oligos would inhibit the association of the corresponding piRNA-like sncRNAs with MIWI protein. As expected, precipitation of piR-5567 was significantly and specifically reduced by preincubation with its corresponding antisense oligo (Fig 4C). In contrast, preincubation with piRNA-1 did not affect on the formation of MIWI/piRNA-like sncRNAs complexes. No RNAs were detected when IP was performed with mouse IgG.

Biological impact of neuronal MIWI/piRNA-like sncRNA complexes in axon growth

The presence of MIWI/piRNA-like sncRNA complexes in axons of rat sciatic nerve indicates that axonally localized mRNAs could be posttranscriptionally regulated. Although the functional significance of PIWI and the PIWI-bound piRNAs in somatic cells is not yet

clear, increasing evidence indicates that piRNAs interact directly with PIWI proteins for translational repression and degradation of certain target mRNAs *via* a miRNA-like mechanism (33–36). To determine whether functional piRNA-like sncRNAs in adult neurons require expression of the MIWI protein that partners with piRNA-like sncRNAs, we targeted *MIWI* mRNA using RNAi and evaluated the effects of MIWI knockdown on axon growth of rat DRG neurons in culture. Three separate siRNAs (siRNAs #1, #2, and #3) were used targeting separate regions of the target *MIWI* mRNA (Supplementary Fig S4). In the analysis of the siRNA target gene knockdown efficiency, MIWI protein and mRNA levels were determined using western blot and qPCR methods (Fig 5A and 5B). In the case of qPCR analysis of *MIWI* mRNA level following siRNA knockdown, all 3 siRNAs showed a significant knockdown efficiency (Fig 5B). However, only siRNAs #1 and #2 were actually able to reduce the protein level in transfected DRG neurons (Fig 5A). To test whether knockdown effect of MIWI gene on growth of DRG neurites, siRNA #1 from the siRNA pool was subsequently tested in cultures of transfected DRG neurons. Knockdown of MIWI with siRNA #1 significantly enhanced axon length by >60% as measured 24 hours after initial plating, when compared with scrambled siRNA control (Fig 5C). To determine whether depletion of neuronal MIWI protein from DRG neurons affects on axonal regeneration, we performed single-axon axotomy on DRG neurons to observe morphological changes during the first hour after axotomy using a glass micropipette mounted into a needle holder of a electric micromanipulator placed on the inverted confocal microscope as described previously (37–40). Although we were not able to detect axon regrowth from the cut end within the time window of this assay, we observed a significant reduction in axonal retraction from either the proximal or distal cut ends of PIWI knockdown axons ($12.6 \pm 2.4 \mu\text{m}$), when compared with retraction from the cut end of control axons ($79.6 \pm 8.8 \mu\text{m}$, $p < 0.001$), suggesting a protective effect of MIWI knockdown on axonal degeneration.

To further determine whether knockdown of MIWI affected regrowth of axons from proximal cut ends to the injury, we used a culture system that allowed us to physically axotomize distal axons (41). DRG neurons transfected with siRNA targeting *MIWI* mRNA were cultured onto tissue culture inserts with pores (3 μm in diameter) for 24 hrs. After the individual neurons were imaged prior to axotomy, distal axons were scraped away from the lower membrane surface and the inserts were placed back into the culture media for further incubation. Images of regenerating axons of the same neurons were taken at 2 hrs, 1 d, and 2 d after axotomy. Measurement of axon length from the cut ends revealed a significant promotion in regeneration of the knockdown cells when compared with control by 2 days after axotomy (Fig 5D). Taken together, these data indicate that functional MIWI/piRNA-like sncRNA complexes present in neurons appear to attenuate axonal regeneration.

Discussion

The work presented here provides the first evidence for axonal piRNA-like sncRNAs in adult sensory neurons. We previously reported that 63 rat miRNAs identified in sciatic nerve show differential changes in their levels after nerve injury, indicating a direct role in regenerative response presumably *via* axonal mRNA translation regulation. Similarly to axonal miRNAs, we found that axonal levels of 18 piRNA-like sncRNAs were significantly

altered by nerve injury, implying their distinct regulatory roles in autonomous axonal response to local extracellular signals including injury.

Differential changes in piRNA-like sncRNA levels in axoplasm responding to injury could be achieved by either differential gene expression in the cell body followed by symmetrical redistribution of the piRNA-like sncRNAs or differential transport of specific piRNA-like sncRNAs into axons. Recent studies identified a subset of miRNAs that are differentially distributed in neurons with some miRNAs being particularly enriched in axons of sensory neurons (16,42) and that play roles in axon regeneration (43–46). Among those axonally abundant miRNAs, miR-16 is known to regulate axonal mRNA translation by downregulating synthesis of the eukaryotic translation initiation factors, eIF2B2 and eIF4G2 (47). In addition, miR-338 controls translation of nuclear-encoded mRNAs for mitochondrial proteins Cytochrome C oxidase IV (COXIV) and ATP synthase 5 gamma 1 (AP5G1). Through this mechanism, miR-338 modulates mitochondrial oxygen consumption and ATP generation in axons (48,49). Taken together, these reports indicate that axonally enriched miRNAs regulate the local protein synthesis of axonally targeted mRNAs to play critical roles in axonal function and development. Furthermore, alterations in axonal miRNA levels significantly contribute to axon outgrowth of sympathetic neurons (43,44,46–49). Selective miRNA enrichment in axon is notable and its important roles in regulating intra-axonal protein synthesis are beginning to emerge. Similarly, piRNA-like sncRNAs that are important for axonal function and growth might be located/enriched in distal axons to locally regulate the expression of target mRNAs in the axons. Using a culture system (modified Boyden chamber) that allows for separation of axonal processes from cell bodies of neurons followed by qPCR approach, we were able to detect the axonal enrichment of piR-1199 while others including piRs-5567, 4288, and 1200 showed the cell body enrichment (*unpublished preliminary results*), supporting our hypothesis that a subset of piRNA-like sncRNAs present in axons of sciatic nerve is altered locally in nerve following injury to actively and autonomously regulate *de novo* protein synthesis to promote regeneration after injury. Future studies on the role of individual piRNA-like sncRNAs enriched in distal axons for axon growth and function will greatly increase our understanding of the mechanisms that determine the specificity of axonal mRNA translation.

Unlike miRNAs and siRNAs, piRNAs have a strong bias for uridine at position 1 (5'-U bias) (20–22). While the exact molecular mechanism of generation of 5'-U bias is not well understood, it appears to be important for binding to PIWI (50). Kawaoka et al showed that SIWI, silk worm homolog of PIWI, preferentially binds to synthetic piRNA precursors with a 5'-uridine *in vitro*, suggesting important roles of this characteristic in the function of piRNAs (51). From the bioinformatics analysis, we found that only about 33% of the annotated piLRNAs had a 5'-U bias, but found that >60% of the piLRNAs started with guanosine. Although our data suggest that a subset of these piLRNAs carry characteristic features of piRNAs, the actual number of piRNAs/piLRNAs expressed in sciatic nerve axoplasm would be lower. Further studies will be needed to determine whether these piLRNAs are biologically important in the neurons, particularly those with a 5'-guanosine.

Local translation of axonally present mRNAs has been linked to regenerative responses in PNS neurons (27,52–57), and injury triggers an increase in anterograde transport of mRNAs

from the cell body to distal axons and a rapid local translation (17,27,29,31,40,53,56–61). All these data indicate that these mRNAs are delivered to axons from the cell body and that intra-axonal translation of these mRNAs are precisely regulated as needed for autonomous response to local stimuli including nerve injury. However, it is not currently known how this intra-axonal translation is initiated, nor how this activated translation is down-regulated once the stimuli disappear. Recently, miRNAs have been shown to regulate translation in the nervous system and suggested as a local translation regulator in axons and dendrites (3,44,62–68). In addition to miRNAs, expression of piRNAs and orthologs of PIWI proteins in somatic tissues including regenerative tissues across phyla suggests more conserved regulatory functions of PIWI in regenerating nerve beyond germline cells and mouse hematopoietic cells and human cancer cells than what has previously been anticipated (8,11,69,70). However, biological functions of axonal MIWI/piRNA-like sncRNA complexes in regenerating axons have not been assessed. We demonstrated here for the first time that knockdown of neuronal MIWI protein increases rates of axonal regrowth of DRG neurons and decreases axon retraction after mechanical injury (Fig 5). Combining previous reports on presence and potential functions of piRNAs in neuronal cells (14,15) with our data suggest that functional MIWI/piRNA pathways are present in nerves to precisely regulate *de novo* protein synthesis in axons and thereby control neural repair and regenerative processes following injury. Although the MIWI knockdown experiments shown in the present study are global in nature and we cannot identify which specific piRNA-like sncRNAs or axonally-localized piRNA-like sncRNAs are critical to regulating nerve regeneration, our results show clearly that functional MIWI/piRNA-like sncRNA complexes present in neurons significantly contribute to axonal growth and regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. piRNA-like small noncoding RNAs are found in adult rat sciatic nerve

A. Size and frequency distribution of piRNA-like sncRNAs in regenerating nerve from Illumina deep sequencing data. **B.** Nucleotide preference for a 5' start site of neuronal piRNA-like sncRNAs. Detection of a 2'-O-methylation at the 3'-ends of piRNA-1199 using NaIO₄/β-elimination reaction (**C**) and RTL-P (**D**). Note that synthetic and total RNAs were separated and detected by GelStar™ (top) and northern blot (middle and bottom), respectively, using a biotin-labeled miRNA-433-specific (middle) or piRNA-1199-specific (bottom) probes. Arrowhead and arrow indicate untreated and treated sncRNA band, respectively. For piRNA-1199, the band intensity of the RTL-P product in a low concentration of dNTPs (0.4 μM) with an unanchored RT primer was significantly lower than that with anchored RT primer in the RT reaction. Non-modified miRNA-433 as a control showed no differences in the band intensities of the RTL-P product. Data are represented as mean ± SD. n=3. *** indicates p<0.001 by Student's *t*-test.

Figure 2. Neuronal piRNA-like sncRNAs are identified and visualized in axons of DRG neurons
A. Relative level of several piRNA-like sncRNAs found in sciatic nerve using qPCR. Inset panel shows that extended PCR products using gene-specific primer sets for cell body restricted (MAP2 and H1FO) and non-neuronal glial cells (GFAP and ErbB-3), indicating axonally enriched RNA extraction. **B.** Representative FISH/IF images of soma (left panel) and distal axon (right panel) of cultured DRG neuron using LNA RNA probes specific for the piRNAs indicated. Exposure matched image for scrambled probe is shown in the inset panel.

Figure 3. Neuronal piRNA-like sncRNAs show differential expression patterns in rat sciatic nerve following injury

A. The heatmap showed changes in levels of 31 piRNA-like sncRNAs in rat sciatic nerve that received a crush injury and were sacrificed at 1, 4, 7, and 14 days post-injury (DPI), as compared to the sham-operated uninjured control (n=3/group). **B.** The color scale shown on the top left denotes the relative expression level of the indicated piRNA-like sncRNAs across all time points (log₂ scale): red represents an increased change in level and green denotes a decreased level. Clustering analysis was performed using *Cluster 3.0* with an average linkage and Euclidean distances metric and visualized using Java *Tree View*.

Figure 4. Neuronal piRNA-like sncRNAs bind to MIWI protein in axons of sciatic nerve
A. Western blot of immunoprecipitates with anti-MIWI antibody. Arrow and arrowhead indicate MIWI-IP and IgG Heavy chain, respectively. **B.** Extended PCR products of small RNAs from MIWI-IP and IgG control using piRNA specific primers. **C.** Quantification of piRNA-like sncRNAs (mean $\pm$ SD, n=3) in MIWI-IPs that were preincubated with either antisense piR-1 or piR-5567 oligos. No RNAs were detected when co-immunoprecipitation was employed with mouse IgG (IgG control). Note that the enrichment of piR-5567 in MIWI-IP preincubated with antisense piR-1 oligo was significantly decreased by preincubating with antisense piR-5567 oligo. ** represents p 0.01 by Student's t -test.

Figure 5.

Neuronal function of MIWI/piRNA-like snRNA complex in DRG axon growth. **A.** Immunoblot of MIWI in lysates of DRGs transfected with either control scrambled siRNA or three separate MIWI-specific siRNAs #1, #2, and #3, showing a significant knockdown of PIWI (arrow). Numbers below the immunoblot are the intensity normalized to GAPDH. Knockdown efficiency of MIWI expression was quantified and plotted after normalization to GAPDH from three separate experiments (Bottom graph). **B.** Relative quantity of *MIWI* mRNA measured by qPCR from DRG transfected with control or three separate MIWI

siRNAs #1, #2, and #3. **C.** Representative images and quantification of axon length of cultured DRG neuron transfected with either control or MIWI siRNA #1. AcGFP was cotransfected with siRNA to confirm transfection and aid in visualizing axons. **D.** Quantification of axon regrowth from cut ends. Quantifications are shown as average $\pm$ SD (B, n=3) or SEM (A, C and D, n = 9). *** p < 0.001 for MIWI siRNA #1 vs. control siRNA by Student's t -test.