The *Bombyx* ovary-derived cell line endogenously expresses PIWI/PIWI-interacting RNA complexes

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ABSTRACT

Genetic studies and large-scale sequencing experiments have revealed that the PIWI subfamily proteins and PIWI-interacting RNAs (piRNAs) play an important role in germ line development and transposon control. Biochemical studies in vitro have greatly contributed to the understanding of small interfering RNA (siRNA) and microRNA (miRNA) pathways. However, in vitro analyses of the piRNA pathway have been thus far quite challenging, because their expression is largely restricted to the germ line. Here we report that *Bombyx mori* ovary-derived cultured cell line, BmN4, endogenously expresses two PIWI subfamily proteins, silkworm Piwi (Siwi) and Ago3 (BmAgo3), and piRNAs associated with them. Siwi-bound piRNAs have a strong bias for uridine at their 5’ end and BmAgo3-bound piRNAs are enriched for adenine at position 10. In addition, Siwi preferentially binds antisense piRNAs, whereas BmAgo3 binds sense piRNAs. Moreover, we identified many pairs in which Siwi-bound antisense and BmAgo3-bound sense piRNAs are overlapped by precisely 10 nt at their 5’ ends. These signatures are known to be important for secondary piRNA biogenesis in other organisms. Taken together, BmN4 is a unique cell line in which both primary and secondary steps of piRNA biogenesis pathways are active. This cell line would provide useful tools for analysis of piRNA biogenesis and function.

Keywords: PIWI; piRNA; cultured cell; *Bombyx mori*; BmN4

INTRODUCTION

PIWI subfamily proteins bind to a class of small RNAs, called PIWI-interacting RNAs (piRNAs). Insights into PIWI and piRNAs have been obtained mainly from genetic studies and large-scale sequencing. Genetic studies have implicated PIWI proteins in germ line development in both invertebrates and vertebrates. In *Drosophila*, piwi mutations cause sterility and loss of germ line stem cells. The requirement of *piwi* for transposon control has been also shown genetically in *Drosophila* and mouse (Hartig et al. 2007; Klattenhoff and Theurkauf 2008). Large-scale sequencing efforts in several organisms including mammals have identified the presence of piRNAs matching transposons (O’Donell and Boeke 2007). Thus, PIWI proteins and piRNAs are thought to exert an essential role in germ line development, stem cell self-renewal, and transposon control (Hartig et al. 2007; O’Donell and Boeke 2007; Klattenhoff and Theurkauf 2008). Unlike small interfering RNAs (siRNAs) and microRNAs (miRNAs), piRNAs are produced by a Dicer-independent mechanism (Vagin et al. 2006; Houwing et al. 2007). Brennecke et al. (2007) and Gunawardane et al. (2007) proposed a ping-pong model of piRNA production. *D. melanogaster* encode three PIWI subfamily proteins; Piwi, Aubergine (Aub), and Arga-nautae3 (Ago3) (Brennecke et al. 2007; Gunawardane et al. 2007). In the ping-pong model, Piwi/Aub-bound antisense piRNAs direct sense piRNA cleavage, resulting in the production of Ago3-bound sense piRNAs, and vice versa (Brennecke et al. 2007; Gunawardane et al. 2007).
Biochemical analysis using lysates from *Drosophila* embryos and S2 cells has provided many valuable insights into the biogenesis and functions of siRNAs and miRNAs (Tuschl et al. 1999; Zamore et al. 2000; Bernstein et al. 2001; Elbashir et al. 2001; Hutvághner et al. 2001; Zamore 2001). However, piRNA expression is largely restricted to the germ line, which makes it difficult to obtain enough lysate for in vitro experiments. Here we report that the silkworm cell line BmN4 endogenously expresses two PIWI proteins and piRNAs associated with them. This cell line can be used as a robust model system for monitoring the piRNA-associated pathway.

## RESULTS AND DISCUSSION

### Identification and characterization of piRNAs in BmN4 cells

Based on our previous observations of a huge amount of piRNAs in the silkworm *Bombyx mori* ovary (Kawaoka et al. 2008b), we sought for piRNA-like small RNA-expressing cell lines derived from ovaries of several lepidopteran insect species to establish an in vitro experimental system for piRNA research.

As shown in Figure 1A, we found that a distinct population of small RNAs around 27–28 nucleotides (nt) long are expressed in *Bombyx*-derived BmN4, *Trichoplusia ni*-derived High Five, and *Spodoptera frugiperda*-derived Sf9 cells. In this study, we focused on BmN4-derived small RNAs, as the *Bombyx* genome has been almost completely sequenced (The International Silkworm Genome Consortium 2008). Solexa sequencing technology was used to generate 416,177 species (1,058,466 clones) of unique small RNAs with 0–2 mismatches in the *Bombyx* genome. Seventy-five percent of cloned small RNAs were read only once, suggesting that they are highly diverse. Cloned small RNAs showed a Gaussian distribution in size with a peak at 27 nt (Fig. 1B). Of these, 76% contained uridine (U) at the 5’ end (Fig. 1C), a bias similar to that observed in piRNAs from other organisms. Classification of cloned small RNAs in Figure 1D shows that 31% of them were associated with repetitive sequences. Relationships between transposons and BmN4-derived small RNAs were indicated in Supplemental Table 1. We observed differences in small RNA profiles derived from BmN4 cells and *Bombyx* ovary. In the case of SART1, ovary-derived small RNAs were mapped almost exclusively to ORF2 (Kawaoka et al. 2008b), while this feature was not observed in BmN4-derived small RNAs (Supplemental Fig. 1). In addition, the number of BmN4-derived small RNAs matching HOPEBm2 was much less than that of ovary-derived small RNAs (Supplemental Fig. 1). These differences might be due to different activities or copy numbers of transposons in each source.

Previous studies have shown that the 3’ ends of piRNAs are 2’-O-methylated (Horwich et al. 2007; Kirino and Mourelatos 2007; Ohara et al. 2007; Saito et al. 2007). To investigate properties of the 3’ ends of BmN4-derived small
RNAs, we performed NaIO4-mediated oxidation followed by β-elimination reaction to ovary- and BmN4-derived total RNAs. As shown in Figure 1E, Northern blot analysis of piRNA-1, one of the abundant small RNAs in BmN4 cells, and Bombyx let-7 revealed that these reactions did not affect the mobility of piRNA-1, while the reaction product of let-7 migrated faster than that without reaction (Fig. 1E), indicating that the 3’-end of small RNAs cloned from BmN4 cells is blocked. Together with these results, we refer to cloned small RNA as piRNA in the following sections.

Expression of two Bombyx PIWI proteins, Siwi and BmAgo3, in BmN4 cells

Next, we investigated whether PIWI proteins exist in BmN4 cells. In Bombyx, the PIWI subfamily consists of two members, Siwi and BmAgo3 (Kawaoka et al. 2008a). To examine the endogenous expression of Siwi and BmAgo3, we generated rabbit polyclonal antibody against each protein. Western blot analysis using anti-Siwi and anti-BmAgo3 antibodies revealed that endogenous Siwi and BmAgo3 were expressed as ~100 kDa and 110 kDa proteins in BmN4 cells, respectively (Fig. 2). In addition, cell lines stably expressing FLAG-tagged Siwi or FLAG-tagged BmAgo3 were generated. The specificities of anti-Siwi and anti-BmAgo3 antibodies were verified using these cell lines (Supplemental Fig. S2). Anti-Siwi or anti-BmAgo3 antibody did not detect any bands from the anti-FLAG immunoprecipitates from extracts of BmN4 cells stably expressing FLAG-BmAgo3 or FLAG-Siwi, respectively. Therefore, we concluded that anti-Siwi and anti-BmAgo3 antibodies could specifically recognize Siwi and BmAgo3, respectively, and both proteins were not coimmunoprecipitated. Collectively, piRNAs and PIWI subfamily proteins exist in BmN4 cells.

Evidence for piRNA biogenesis in BmN4 cells

To characterize the piRNA population in each PIWI complex in BmN4 cells, we immunopurified FLAG-Siwi- or FLAG-BmAgo3-containing complexes from cells stably expressing FLAG-Siwi or FLAG-BmAgo3, respectively, using anti-FLAG antibody, as antibodies against endogenous Siwi and BmAgo3 were not suitable for coimmunoprecipitation of PIWI-bound piRNAs (data not shown). Subsequently, small RNAs in each complex were cloned and deeply sequenced. We obtained 6,034,838 and 4,398,344 clones of Siwi- and BmAgo3-bound piRNAs, respectively. Siwi-bound piRNAs showed a strong enrichment for U at position 1 (77%), while BmAgo3-bound piRNAs lacked this bias (Fig. 3A). In contrast, position 10 of BmAgo3-bound piRNAs was enriched for A (75%) (Fig. 3B). These biases are quite similar to those observed in Drosophila PIWI subfamily-bound piRNAs (Brennecke et al. 2007).

Next, to investigate relationships between Siwi- or BmAgo3-bound piRNAs and transposons, we focused on piRNAs that matched 120 transposons (Fig. 4A; Supplemental Tables 2, 3). We observed a clear trend toward antisense and sense preferences in Siwi- and BmAgo3-bound piRNAs, respectively (Fig. 4A,B). Density maps of piRNAs on Aquila and SART1 transposons clearly showed that antisense piRNAs are preferentially incorporated into Siwi-containing complexes (Fig. 4C, red line), while sense piRNAs are preferentially incorporated into BmAgo3-containing complexes (Fig. 4C, blue line). In addition, as reported in zebrafish (Houwing et al. 2008), we observed an opposite piRNA polarity in some transposons (Fig. 4A,C).

Sense–antisense piRNA pairs overlapping by precisely 10 nt at their 5’ ends are characteristic of secondary piRNA biogenesis in the ping-pong model (Brennecke et al. 2007; Gunawardane et al. 2007). To verify whether secondary piRNA biogenesis pathway exists in BmN4 cells, we focused on the complementary relationship between Siwi-bound piRNAs and BmAgo3-bound piRNAs. As shown in Figure 5A, many Siwi-bound antisense and BmAgo3-bound sense piRNA pairs are overlapped by precisely 10 nt from their 5’ ends. Also, we found the strongest complementarity between piRNAs in Siwi and BmAgo3 (Fig. 5B). 5’-10 nt overlapping was also observed both within Siwi-bound and BmAgo3-bound piRNAs. Such self-complementarity has been observed in Drosophila ovary-derived piRNAs (Brennecke et al. 2007). A recent report suggests that piRNAs

![Figure 2](image-url)

**FIGURE 2.** Expressions of Siwi and BmAgo3 in BmN4 cells. (A) Expression of Siwi in BmN4 cells. Western blotting was performed using total protein extract from BmN4 cells. The immunoprecipitates prepared with anti-FLAG antibody from extracts of BmN4 cells stably expressing FLAG-Siwi were used as a positive control. Siwi was expressed as an ~100-kDa protein in BmN4 cells. (B) Expression of BmAgo3 in BmN4 cells. BmAgo3 was not detected by anti-BmAgo3 antibody using total protein lysate from BmN4 cells (data not shown). Thus, Western blotting was performed using the immunoprecipitates prepared with anti-BmAgo3 antibody from extracts of BmN4 cells. The immunoprecipitates prepared with anti-FLAG antibody from extracts of BmN4 cells stably expressing FLAG-BmAgo3 were used as a positive control. BmAgo3 was expressed as an ~110 kDa protein in BmN4 cells.
which have U at position 1 but do not have A at position 10 (1U but not 10A) are putative primary piRNAs, and those showing 10A but not 1U are secondary piRNAs (Aravin et al. 2008). Strikingly, we found that 80% of Siwi-bound antisense piRNAs that contain 1U (but not 10A) form 10 nt overlapping pairs with BmAgo3-bound sense piRNAs having 10A (but not 1U). These results strongly indicated that both primary and secondary steps of piRNA biogenesis pathways are active in BmN4 cells.

CONCLUSION

Here we identified PIWI proteins and piRNAs endogenously expressed in the Bombyx-derived cell line, BmN4. Our data show that both of the primary and secondary piRNA biogenesis pathways are active in BmN4 cells. This is the first discovery of the cultured cell line expressing the PIWI/piRNA complexes. Just as cultured cell lines including Drosophila S2 and human HeLa cells have largely contributed to progress in the study on siRNAs and miRNAs, BmN4 cells would provide extremely valuable tools for biochemical understanding of the piRNA pathway.

MATERIALS AND METHODS

Cell lines

BmN4 cells were cultured at 27°C in IPL-41 medium (Gibco) supplemented with 10% fetal bovine serum. The Sf9 cells were cultured at 27°C in TC-100 medium (Invitrogen) supplemented with 10% fetal bovine serum and High Five cells were cultured at 27°C in Express Five medium (Invitrogen), respectively.

RNA isolation

Total RNAs were prepared using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. For small RNA preparation, miRVana miRNA isolation kit (Ambion) was used according to the manufacturer’s protocol.

Detection of small RNAs and preparation of small RNA libraries

Total RNA (10 μg) was loaded onto a 15% denaturing polyacrylamide gel containing 10 M urea, electrophoresed, and then stained with SYBRGold (Invitrogen). Signals were visualized using LAS-1000 film (Fujifilm). Small RNA libraries were constructed using a Small RNA cloning kit (Takara), DNA sequencing was performed using the Solexa genetic analysis system (Illumina) (Bently 2006). A total of 1 ng of the prepared cDNA was used for the sequencing reactions with the Illumina GA; 10,000–15,000 clusters were generated per “tile” and 36 cycles of the sequencing reactions were performed. The protocols of the cluster generation and sequence reactions were according to the manufacturer’s instructions.

Sequence analysis of cloned small RNAs

Solexa sequencing generated reads of up to 36 nt in length. The 3’ adaptor sequences were identified and removed, allowing for up to two mismatches. Reads without adaptor sequences were discarded. Reads shorter than 23 nt or longer than 30 nt were excluded, resulting in reads of 23–30 nt. The trimming described above and alignment to the Bombyx genome (The International Silkworm Genome Consortium 2008) were performed using SOAPaligner/soap2 (Li et al. 2008). Reads that could be aligned to the genome up to two mismatches were used for further analysis. Alignments of the extracted reads onto the annotated transposable elements in the Bombyx genome were performed using BLAST, allowing no mismatch.

b Elimination

NaIO4 reaction was performed by incubating total RNA in 25 mM NaIO4 at room temperature for 30 min. Ten microliters of glycerol were added to quench unreacted NaIO4. b Elimination was then performed by adding 10 μL of 500 mM NaOH followed by incubation at 45°C for 90 min. The resultant RNA was collected by ethanol precipitation.

Northern blot

Total RNA was separated on urea-containing 15% polyacrylamide gel and transferred to Hybond-N+ (GE Healthcare Biosciences) using the Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad). For the detection of piRNA-1 and Bombyx let-7, DNA probes (5’-ATTCGAAAAACATTCCGTTAGTGGTTTTGA-3’ for piRNA-1 and 5’-TACTATACAACTACTACTACCTCA-3’ for let-7) were radiolabeled with T4 polynucleotide kinase (Takara Bio, Inc.) and [γ-32P] ATP. The probes were hybridized using PerfectHyb Plus (Sigma) at 37°C.
Antibody generation
Amplification products corresponding to amino acids 1–200 of Siwi and amino acids 80–230 of BmAgo3 were cloned into the pET24b vector (Novagen) and expressed in *Escherichia coli* BL21 DE3 at 37°C in the presence of 1 mM IPTG. Recombinant proteins were purified using a HisGraviTrap column (GE Healthcare Bioscience) according to the manufacturer’s protocol. The purified recombinant proteins were used to generate anti-Siwi and anti-BmAgo3 rabbit polyclonal antibodies (Sigma).

Generation of BmN4 cells stably expressing FLAG/His-tagged PIWI proteins
BmN4 cells stably expressing FLAG/His-tagged Siwi or FLAG/His-tagged BmAgo3 were generated as described previously (Katsuma et al. 2006). The full-length cDNA of Siwi was cloned into pIZ/V5-His (Invitrogen) and PCR-mediated mutagenesis was performed to attach an N-terminal FLAG/His-tagged sequence (primers are indicated in Supplemental Table 4). The coding region of BmAgo3 with an N-terminal FLAG/His-tag sequence was amplified by PCR with the gene-specific primers (Supplemental Table 4) using the cDNA clone as a template. The PCR products were cloned into the pIZ/V5-His vector, which possesses the *ie2* promoter of *Orgyia pseudotsugata* nucleopolyhedrovirus for the constitutive expression of the gene of interest and the zeocin-resistant gene for selection of stable cell lines. BmN4 cells were transfected with pIZ/V5-His, pIZ/FLAG-Siwi, or FLAG-BmAgo3 by using Cellfectin reagent (Invitrogen). Two days after transfection, zeocin (final concentration, 500 μg/mL) was added to the medium. Two weeks after drug selection, the expression of FLAG-tagged Siwi and BmAgo3 was verified using the previously described Western blot analysis (Katsuma et al. 2005) with anti-FLAG monoclonal (1:1000, Sigma), anti-Siwi (1:10,000), or anti-BmAgo3 (1:5000) antibodies.

Immunoprecipitation of Siwi-bound and BmAgo3-bound piRNAs
We used anti-FLAG antibody to immunoprecipitate Siwi and BmAgo3 from cells stably expressing FLAG-Siwi or FLAG-BmAgo3. The cells were cultured in a 150-mm dish and collected with RIPA buffer (50 mM Tris at pH 8.0, 100 mM NaCl, 3 mM MgCl₂, 1% NP-40, protease inhibitor cocktail [Roche], 100 U/mL RNasin ribonuclease inhibitor [Promega]. The lysate was cleared by centrifugation at 15,000 rpm for 15 min at 4°C. The total protein concentration was determined using Coomassie Plus Protein assay reagent (Pierce) according to the manufacturer’s protocol. The resulting supernatant was collected and incubated with anti-FLAG antibody (1:100) or normal mouse IgG (Santa Cruz; 1:40) for 2 h with gentle agitation. Then, 40 μL of Protein A Sepharose 4 Fast Flow beads (GE Healthcare Biosciences) was added to the lysate and the incubation was continued for 2 h at 4°C, followed by three washes with ice-cold RIPA buffer.

Nucleic acids that were coimmunoprecipitated were then isolated using a miRVana miRNA isolation kit (Ambion) according to the manufacturer’s protocol. Briefly, the beads were suspended in 500 μL of lysis/binding buffer. Total RNA was extracted by equal volumes of phenol:chloroform treatment. The fraction of small RNA was extracted from 400 μL of total RNA using a filter cartridge with 50 μL of preheated (95°C) elution solution. Small RNAs were was loaded onto a 15% denaturing PAGE gel after electrophoresis.
polyacrylamide gel containing 10 M urea, electrophoresed, and then stained with SYBRGold. Small RNA libraries were constructed as described above.

**Data deposition**

The nucleotide sequences reported in this study have been submitted to the DDBJ/EMBL/GenBank data bank under accession numbers of AHAAB0000001-AHAAB0547473 (BmN4-derived piRNAs), AHAAC0000001-AHAAC1704525 (BmN4-derived Siwi-bound piRNAs), and AHAAD0000001-AHAAD2196220 (BmN-derived BmAGO3-bound piRNAs).

**SUPPLEMENTAL MATERIAL**

Supplemental material can be found at http://www.rnajournal.org.

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**REFERENCES**


