gyri and also saw less ventral stream temporal activation during whistle speech than during speech processing. Now, we also show left- and right hemispheric contributions that result in an absence of cerebral asymmetries during whistle language encoding. Thus, a natural but acoustically different language can create a radical change in the organizational dynamics of language asymmetries.

SUPPLEMENTAL INFORMATION

Supplemental Information containing a one figure, two tables and a movie can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.06.067.

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Is the expression of sense and antisense transgenes really sufficient for artificial piRNA production?

Keisuke Shoji and Susumu Katsuma*

Animals have evolved an elegant defense system against a diverse range of selfish elements such as transposons. In animal germ line cells, PIWI proteins and small RNAs associated with PIWI proteins (piRNAs) are at the heart of this defense system. piRNAs are 23–30-nt-long small RNAs that act as sequence-specific guides for PIWI proteins. PIWI proteins possess a slicer activity that is guided by piRNAs; the PIWI–piRNA complex thus silences transposon activity by cleaving transposon RNAs [1]. At present, how *de novo* piRNA production occurs against a new non-self element is largely unknown. A recent study by Itou *et al*. [2] using reporter transgenic mice concluded that the concomitant expression of sense and antisense RNA transcripts is sufficient for piRNA production. Our bioinformatic analysis using the same piRNA datasets, however, demonstrates that the introduction of the antisense reporter construct alone produces transgene-derived piRNAs, which is inconsistent with a part of the conclusions of Itou *et al*. [2].

The biogenesis of piRNAs initiates with the fragmentation of putative long, single-stranded piRNA precursors by Zucchini endoribonuclease [3,4]. The fragmented RNAs are incorporated into a subset of PIWI proteins with a specific nucleotide preference for uracil (1U) at the 5′ end of the associated RNA [5]. The 3′ end of the associated RNA is then trimmed by an unidentified nuclease called Trimmer. This process is called the primary processing pathway. The PIWI–primary piRNA complexes then cleave their complementary targets across from positions 10 and 11 from the guide piRNAs [1]. The 3′ RNA fragments are in turn incorporated into another subset of PIWI proteins, and again processed into mature secondary piRNAs with adenine at position 10 (10A) that precisely overlaps with 1U piRNAs by 10 nt [1]. Next, these secondary 10A piRNAs generate secondary 1U piRNAs by cleaving their complementary target RNAs. This cleavage-dependent piRNA biogenesis is called a ping-pong amplification cycle [1]. The system has been shown to be broadly conserved among animals including flies, mice, zebrafish, and silkworm.

How does *de novo* piRNA production initiate against a new invading non-self element? To solve this issue, transgenic approaches using *EGFP*-expressing transgene cassettes have been utilized in flies, mice, and silkworm [6–8]. The results of these studies clearly show that the integration of a transgene cassette into the specific, active piRNA cluster is critical for *de novo* piRNA production. The recent study by Itou *et al.* [2] published in *Current Biology*, however, appears to upend this concept. They developed an artificial piRNA production system in mice by expressing sense and antisense EGFP mRNAs in embryonic male germ cells during the piRNA biogenesis stage. On the basis of these experiments, they argue that concomitant expression of sense and antisense RNA transcripts is necessary and sufficient for piRNA production and subsequent piRNA-dependent gene silencing [2].

To investigate why such a discrepancy is observed between the study by Itou *et al*. [2] and previous studies [6–8], we performed bioinformatic analysis using piRNA data that were deposited in the public database by Itou *et al*. We note that we only used a part of the deposited data because the data included variable length sequences both with and without adaptor trimming (the data marked in Figure 1A were used in our analysis). We therefore focused on mapping patterns of piRNAs but not on amounts of mapped piRNA reads (normalized mapped reads are shown in Table S1 in Supplemental

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Information, published with this article online). This does not affect the conclusion that *de novo* piRNA production occurs or not. We simply map the piRNAs from each library onto the transgene cassette (*Miwi2* promoter-antisense *EGFP*-polyA). As shown in Figure 1B and 1C, the piRNAs from wild-type (non-transgenic) and the *Oct4* promoter-sense *EGFP*-introduced mice do not map to the transgene sequence, indicating that artificial piRNA production does not occur in these mice. However, surprisingly, piRNAs clearly map to the transgene sequence of both strands of the *Miwi2* promoter and *EGFP* regions when the piRNA library derived from the *Miwi2* promoter-antisense *EGFP* strain is used (Figure 1D). This demonstrates that this cassette transcription is driven at both directions by unknown promoters that are located outside this cassette, and that expression of sense *EGFP* mRNA is not required for artificial piRNA production in this transgenic strain. This conclusion is also supported by the similar mapping patterns that are observed in the *Miwi2* promoter-antisense *EGFP* strain (Figure 1D) and sense/antisense *EGFP*-expressing strain (Figure 1E).

Our bioinformatic results strongly suggest the possibility that some of the transgene fragments are integrated into the active piRNA clusters in the *Miwi2* promoter-antisense *EGFP* strain. If this is true, the results by Itou *et al*. simply support the previous findings in flies, mice, and silkworm that show that insertion of the transgene cassette into the active piRNA clusters is critical for *de novo* piRNA production against new, non-self elements [6–8]. In order to exclude this possibility, Itou *et al*. [2] attempted to identify the genomic loci where the transgene is integrated by capture sequence analysis. They used the 'typical' 821 piRNA clusters in the mouse genome in their *in silico* analysis. As pointed out by Hirano and Siomi [9], this strategy does not find 'unknown' piRNA clusters in the mouse genome. Thus, the results obtained by Itou *et al.* [2] do not fully exclude the possibility that the transgene fragments are integrated into active piRNA clusters in the *Miwi2* promoter-antisense *EGFP* strain.

A

Filename	Strain	Min length	Max length	Reads	Adaptor trimmed?
ERR746959.fastq	*Miwi2asEGFP*	1	101	22626689	yes
ERR746960.fastq	*Double-mutant*	101	101	241137	no
ERR746961.fastq	*Double-mutant*	101	101	3794729	no
ERR746962.fastq	*wild*	16	36	41780889	yes
ERR746963.fastq	*Double-mutant*	101	101	3887288	no
ERR746964.fastq	*Miwi2-asDnmt3L*	16	36	42812580	yes
ERR746965.fastq	*Oct4-EGFP*	1	101	23221125	yes
ERR746966.fastq	*Double-mutant*	101	101	3787295	no
ERR746967.fastq	*Double-mutant*	101	101	3888312	no

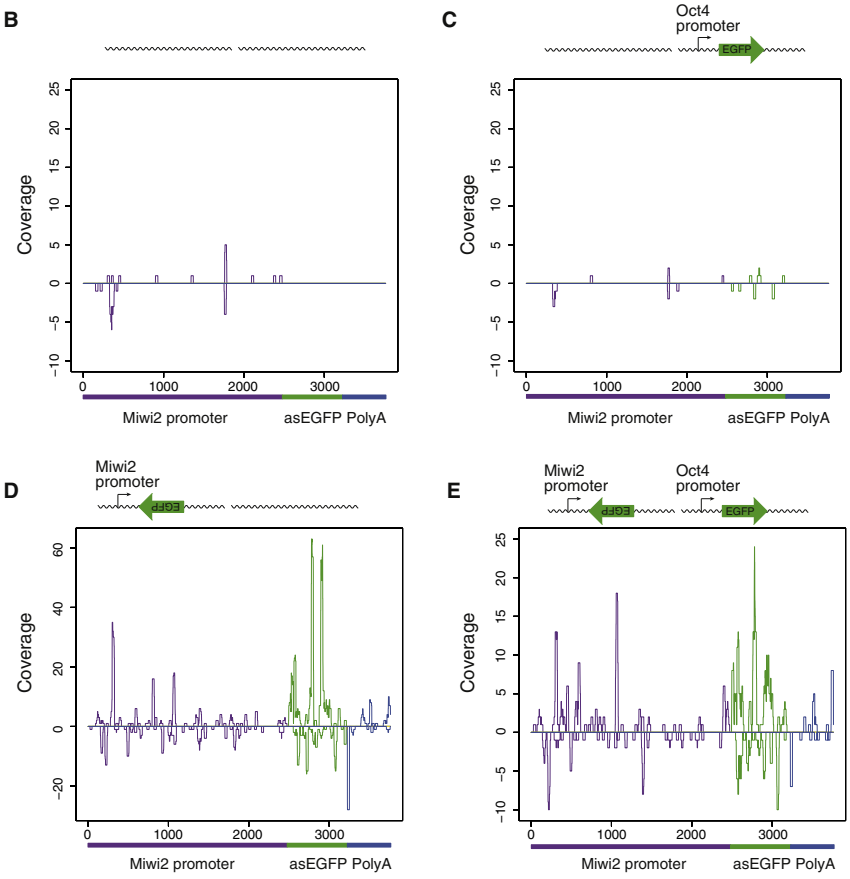

Figure 1. Properties of the transgene-derived piRNAs reported by Itou *et al*.
(A) The datasets used for our bioinformatic analysis. (B–E) Mapping pattern of piRNAs from each library onto the transgene cassette.

On the basis of our re-analysis of the data of Itou *et al*. [2], we believe that their initial conclusion that the concomitant expression of sense and antisense RNA transcripts is sufficient for piRNA production is unwarranted. Further studies are required to understand the requirements for detection of non-self sequences in the animal genome and *de novo* generation of piRNAs against such sequences. Introduction of reporter constructs into the active piRNA clusters results in the production of piRNAs with ping-pong signatures [6–8]. However, proof-of-concept evidence for the ping-pong amplification cycle of piRNAs has not been obtained yet. At present, the ping-pong pathway is just a proposed model on the bases of the bioinformatic analyses of the

resultant piRNAs. If we can monitor the behavior of each piRNA in cells, we will be able to show whether this pathway actually exists or not. Towards this goal, it will be necessary to develop a system in which piRNAs can be traced individually in their amplification cycle.

SUPPLEMENTAL INFORMATION

Supplemental Information contains one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.07.001.

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Reply to Shoji and Katsuma

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Our paper reports a novel strategy for the artificial introduction of DNA methylation in mouse gonocytes [1]. The manuscript presents data showing that the concomitant expression of sense and anti-sense of EGFP transgenes in embryonic male germ cells induces gene silencing via the piRNA pathway and that the expression of an antisense Dnmt3L transgene induces silencing of the endogenous Dnmt3L gene.

Based on our study of EGFP transgenic mice, we concluded that the expression of sense and anti-sense EGFP genes is necessary and sufficient for piRNA production. Analyzing the data on piRNA expression from the Miwi2 promoter region of the antisense EGFP transgenic mice, Shoji and Katsuma raised a serious concern regarding this conclusion [2]. Given the data on the piRNAs of the Miwi2 promoter region, we would like to withdraw one of our conclusions, "concomitant expression of sense and antisense RNA transcripts is necessary and sufficient for piRNA production."

We concluded based on gene capture analysis that the transgene was not integrated into a 'typical' piRNA cluster, because the number of piRNAs corresponding to the integrated region was not significant. That is, we adopted a kind of 'prospective' definition of piRNA clusters. Meanwhile, Shoji and Katsuma adopted criteria to define piRNA clusters based on bioinformatic analysis of transgenic mice. For transgenes that produce piRNAs corresponding to their own sequences, the loci into which the transgenes are integrated are interpreted as belonging to the piRNA clusters. We agree with their claim based on this definition; however, at the same time, we consider that a more precise definition of piRNA clusters would be necessary.

There are still some ambiguities to be addressed. Although we did not perform deep sequencing analysis of small RNAs, similar gene silencing by the antisense EGFP transgene was found in two other transgenic lines. These transgenic lines silenced the expression of Oct4–EGFP in the double transgenic mice, in which both sense and antisense EGFP were expressed concomitantly, as well as transgenic line #1. In order to draw a robust conclusion, we will further analyze piRNA production in these two antisense transgenic animals and report the results in the near future.

There are two possible results. In the first case, these transgenic mice may also produce piRNAs corresponding to the MIWI2 promoter and EGFP transgene. If this is the case, we are willing to conclude that they are integrated into 'minor' or 'atypical' piRNA clusters. In this case, comparison of the expression of the amount of piRNA corresponding to the transgene will allow us to determine whether the amount of piRNAs or another factor is necessary for gene silencing via the piRNA pathway. This is because the simple existence of transgene-related piRNAs did not induce gene silencing in antisense transgenic mice (Figure 1C in [1]). In the second case, at least one of the two transgenic mouse lines may not produce a significant amount of piRNAs corresponding to the transgene. This would confirm our initial interpretation.

The main source of our misunderstanding was the discrepancy between artificial piRNA production and gene silencing. During the course of our experiments and publication process, we mistakenly believed that they should be concomitant. This discrepancy raises an interesting and important question about the relationship between piRNA production and gene silencing. Abundant piRNAs and/or piRNAs with some unknown characteristics may be necessary for DNA methylation and subsequent gene silencing. Finally, we would like to emphasize that all of the data presented in our paper and the main conclusion are robust.

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