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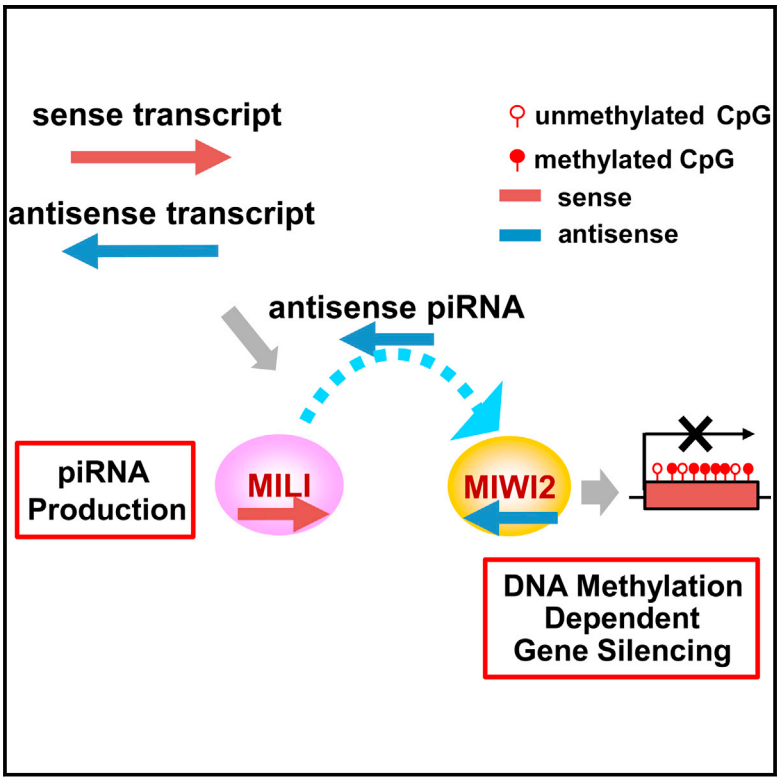
Induction of DNA Methylation by Artificial piRNA Production in Male Germ Cells

Graphical Abstract



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In Brief

Itou et al. established an artificial piRNA production system by the concomitant expression of sense and antisense mRNAs in murine embryonic testes. This system made it possible to induce gene-specific DNA methylation by piRNAs in male germ cells and would be useful for the study of transgenerational epigenetic inheritance.

Highlights

- Concomitant expression of sense and antisense EGFP transgenes induced EGFP piRNAs
- EGFP transgenes were silenced by piRNA pathway-dependent DNA methylation
- piRNA-dependent gene silencing was introduced into the endogenous Dnmt3L gene
- Our piRNA induction system should be useful for the study of epigenetic inheritance

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Induction of DNA Methylation by Artificial piRNA Production in Male Germ Cells

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SUMMARY

Global DNA demethylation and subsequent de novo DNA methylation take place in mammalian male embryonic germ cells [1–3]. P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs), which are germline-specific small RNAs, have been postulated to be critically important for de novo DNA methylation of retrotransposon genes, and many proteins, including PIWI family proteins, play pivotal roles in this process [4–6]. In the embryonic mouse testis, two mouse PIWI proteins, mouse PIWI-like (MILI) and mouse PIWI2 (MIWI2), are involved in the biogenesis of piRNAs through the so-called ping-pong amplification cycle [7–10], and long single-stranded RNAs transcribed from the gene regions of piRNA clusters have been proposed to be the initial material [11–16]. However, it remains unclear whether transcription from the piRNA clusters is required for the biogenesis of piRNAs. To answer this question, we developed a novel artificial piRNA production system by simple expression of sense and antisense *EGFP* mRNAs in embryonic male germ cells in the piRNA biogenesis phase. EGFP expression was silenced by piRNA-dependent DNA methylation, indicating that concomitant expression of sense and antisense RNA transcripts is necessary and sufficient for piRNA production and subsequent piRNA-dependent gene silencing. In addition, we demonstrated that this artificial piRNA induction paradigm could be applied to an endogenous gene essential for spermatogenesis, DNM T3L [3, 17, 18]. This study not only provides novel insights into the molecular mechanisms of piRNA production, but also presents an innovative strategy for inducing epigenetic modification in germ cells.

RESULTS AND DISCUSSION

DNA Methylation of *EGFP* Transgene by Concomitant Expression of Antisense Transcripts

Comprehensive sequencing of piRNAs and genomic mapping of piRNAs suggest that long transcripts from piRNA clusters are required as the precursors for piRNAs [11–16]. There are two classes of murine piRNA clusters. One is embryonic piRNA clusters, which are important for MILI- and MIWI2-dependent retrotransposon-related ping-pong amplification of piRNAs. The other is pachytene piRNA clusters, which are MILI and MIWI dependent and related to intergenic sequences in general. Previous papers suggested that RNA transcripts containing retrotransposon sequences derived from the embryonic piRNA clusters were important for piRNA biogenesis [4]. However, how they are utilized as the substrate of piRNA and even whether they are a prerequisite for piRNA production have not yet been elucidated. It is also unknown why piRNAs corresponding to retrotransposons are preferentially produced. Our hypothesis, that neither long transcripts nor sequence preference is important for piRNA production, challenges the aforementioned two unproven general beliefs. In this study, we adopted a simple experimental system wherein sense and antisense *EGFP* transgenes were expressed in embryonic male germ cells during de novo DNA methylation. We used this paradigm to assess the induction of piRNA-dependent DNA methylation.

In the *Oct4-EGFP* mouse testis, EGFP expression was detectable from embryonic day 7 [19] to at least 2 weeks after birth (Figures 1A and S1B). Three lines of *Miwi2-asEGFP* transgenic mice (#1, #6, and #8), in which antisense *EGFP* mRNA expression was controlled by the 2.5-kb *Miwi2* promoter, expressed antisense *EGFP* RNA transcripts in embryonic day 16.5 testes (Figures S1A and S1B). The antisense *EGFP* transcript was only expressed in the embryonic testis of the *Miwi2-asEGFP* mouse, consistent with the expression of MIWI2 [5, 6] (Figures S1B and S1C). We primarily used transgenic line #1 in subsequent experiments because of its high expression of antisense *EGFP* RNAs.

In double transgenic mice bearing both *Oct4-EGFP* and *Miwi2-asEGFP* transgenes, expression of EGFP was silenced

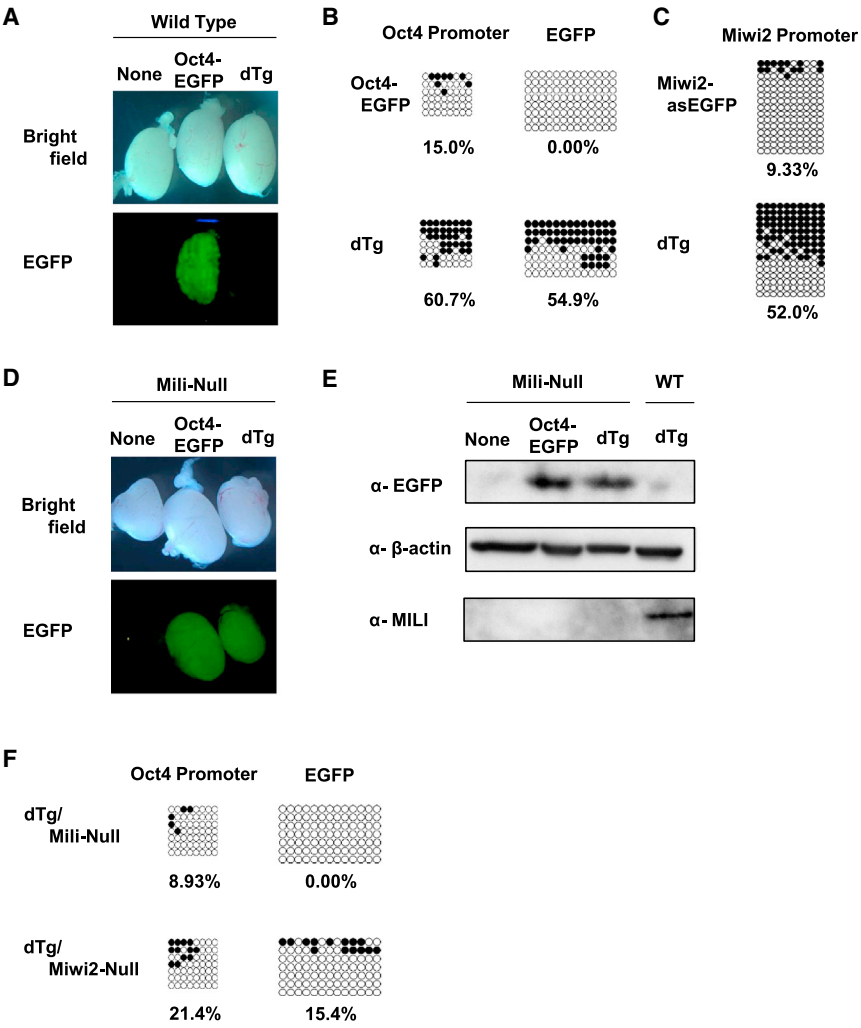

Figure 1. Gene Silencing of EGFP by DNA Methylation in a piRNA-Dependent Manner

(A) Bright-field and fluorescent photographs of day 14 testes of wild-type, *Oct4-EGFP*, and double transgenic (dTg) mice, which were produced by crossing *Oct4-EGFP* mice with *Miwi2-asEGFP* mice.

(B) Bisulfite sequencing analysis of the *Oct4* promoter and *EGFP* gene of the *Oct4-EGFP* transgenic mice. Genomic DNA from day 14 EpCAM-positive germ cells from *Oct4-EGFP* transgenic mice and double transgenic mice was used to analyze *Oct4-EGFP*. White and black circles represent unmethylated and methylated cytosine, respectively.

(C) Bisulfite sequencing analysis of the *Miwi2-asEGFP* transgenes. Genomic DNA from E-CADHERIN-positive germ cells was used to analyze the *Miwi2*-promoter.

(D) Bright-field and fluorescent photographs of *Oct4-EGFP* and double transgenic mice testes under the MILI null condition. Data of the mice bearing no transgene (None), *Oct4-EGFP* transgene, and both *Oct4-EGFP* and *Miwi2-asEGFP* transgenes (dTg) are shown.

(E) Western blot analysis of day 14 *Oct4-EGFP* and double transgenic testes under the MILI null condition, using anti-EGFP and anti-MILI antibodies. β-ACTIN was used as an internal control.

(F) Bisulfite sequencing analysis of day 14 EpCAM-positive germ cells of double transgenic mice under MILI and MIWI2 null conditions.

at embryonic day 16.5 (Figure S1D) and at day 14 after birth (Figure 1A). Not only the representative *EGFP* antisense transgenic line (#1) but also the other lines (#6 and #8) showed essentially same silencing (Figure S1E). This suppression is unlikely to be a result of a direct effect of the antisense *EGFP* transcript because antisense *EGFP* was minimally or not at all expressed in male germ cells at day 14 (Figure S1C).

Next, we examined the DNA methylation status of the *Oct4-EGFP* transgene by bisulfite sequencing (Figure 1B). In male germ cells of double transgenic mice, methylation of the *Oct4-EGFP* promoter and the *EGFP*-coding region was significantly higher than that of the *Oct4-EGFP* mice. Although the expression of EGFP was utilized as a marker to visualize *EGFP* gene silencing, sense and antisense *EGFP* transgenes can be considered equivalent from the point of view of gene silencing through piRNA-dependent DNA methylation. Next, we examined the expression and DNA methylation of the *Miwi2-asEGFP* transgene (Figures S1F and 1C). Similar to the results for the *Oct4-EGFP* transgene, silenced expression of antisense *EGFP* and high DNA methylation of its promoter were detected in the double transgenic male germ cells. These data clearly demonstrate that expression of both sense and antisense transgenes was silenced by DNA methylation of their promoters. To exclude the possibility that the activity of the endogenous *Oct4* and *Miwi2* promoters in the double transgenic mice were affected by some unknown reasons, we carried out western blotting of the OCT4 and MIWI2 proteins (Figure S1G). The amounts of OCT4 and MIWI2 proteins in dTg embryonic testes were essentially the same as those in the control testes.

Involvement of Artificially Induced *EGFP*-Related piRNAs in the Gene Silencing of *EGFP* Transgenes

A critical question to answer was whether or not DNA methylation and subsequent gene silencing were dependent upon the piRNA pathway. To resolve this, we examined the expression of EGFP in double transgenic mice under *Mili*- and *Miwi2*-deficient conditions (described as dTg/*Mili* Null and dTg/*Miwi2* Null mice, respectively). Gross examination and western blotting analysis clearly demonstrated that the expression of EGFP, which was abrogated in the double transgenic mice, was recovered under *Mili* and *Miwi2* null conditions (Figures 1D and 1E and data not shown). Levels of methylation of the *Oct4-EGFP* promoter in the dTg/*Mili* Null and dTg/*Miwi2* Null mice were quite low, compared to the simple double transgenic mice (Figures 1B and 1F). These data demonstrate that gene silencing of *Oct4-EGFP* was dependent on MILI and MIWI2, i.e., the piRNA pathway.

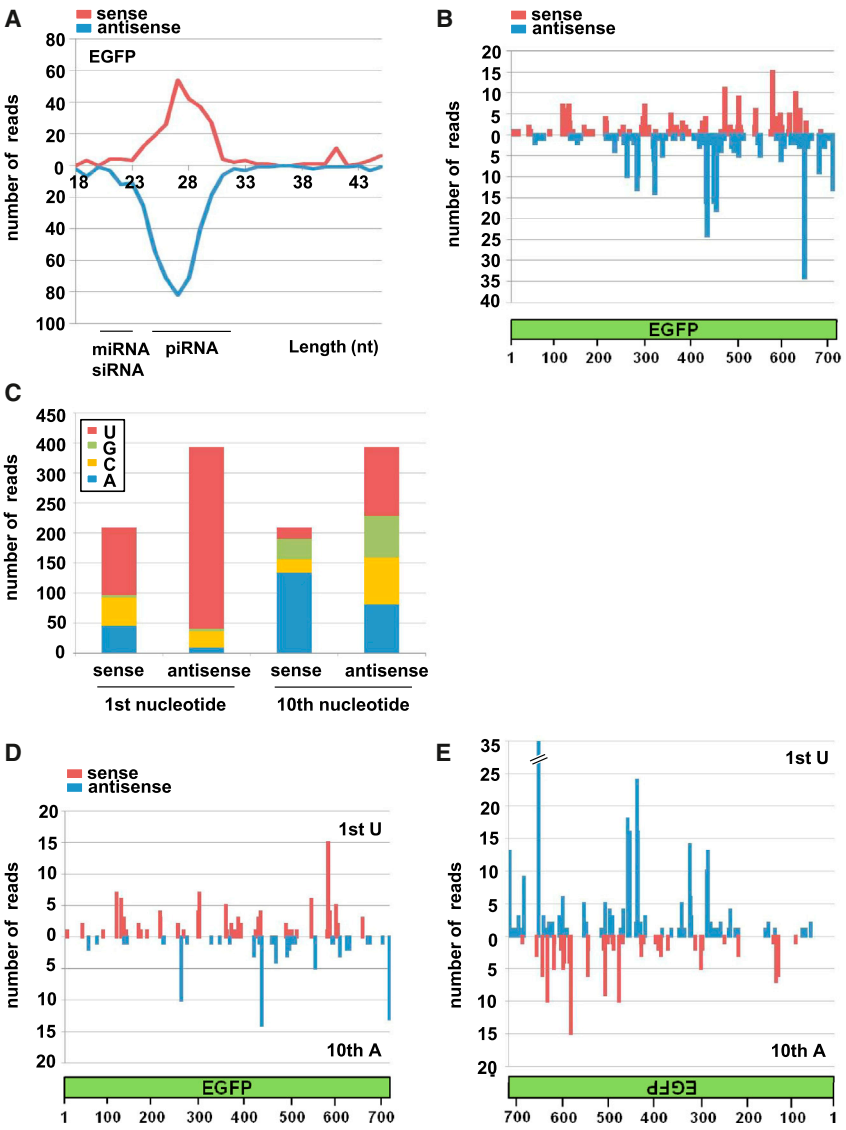

Figure 2. Deep Sequencing Analysis of Small RNAs in the Whole Embryonic Gonads of *EGFP* Double Transgenic

(A) Length distribution of the small RNAs corresponding to *EGFP*.

(B) Mapping of *EGFP* piRNAs. Red and blue bars indicate sense and antisense *EGFP* piRNAs, respectively.

(C) Numbers of the first and tenth nucleotides in *EGFP* piRNAs.

(D and E) Mapping of 1st U and 10th A *EGFP* piRNAs. Sense 1st U and antisense 10th A piRNAs corresponding to the sense *EGFP* are shown in (D). Antisense 1st U and sense 10th A piRNAs corresponding to the antisense *EGFP* are shown in (E).

To examine the production of *EGFP*-related piRNAs, we carried out deep sequencing analysis of small RNAs in the whole embryonic gonads of double transgenic mice. A total of 552 reads of *EGFP*-related RNAs were obtained from the RNA sequence data (11,747,822) of 18–45 nt in length. The length of *EGFP*-related small RNAs showed a single peak of 25–31 nt (Figure 2A), which was consistent with the length of piRNAs. Both sense and antisense piRNAs related to *EGFP* were mapped to the entire *EGFP* sequence (Figure 2B). A strong sequence bias, namely, uracil in the first position (1st U) and adenine in the tenth position (10th A), has been reported as a signature of piRNA production [8–10]. As shown in Figure 2C, the majority of both sense and antisense *EGFP* piRNAs demonstrated a high 1st U bias (54% [113/209] and 88% [302/343], respectively). A strong 10th A bias was only evident in sense *EGFP*-piRNAs (64% [134/209]).

The distributions of 1st U and 10th A piRNAs against sense and antisense *EGFP* transgenes are shown in Figures 2D and 2E. These piRNAs were screened for the ping-pong signature (ten bases matching between 1st U and 10th A piRNAs with reverse orientations, and the results are shown in Figure S2. Approximately 50%–60% of the piRNAs matched the ping-pong signature (Figures S2A and S2C). Representative ping-pong signature data for sense and antisense *EGFP* sequences are shown in Figures S2B and S2D, respectively. These data clearly show that piRNAs for *EGFP* were produced via the ping-pong amplification cycle.

Silencing of *Dnmt3L* Gene through DNA Methylation Introduced by the Expression of Antisense *Dnmt3L*

Next, we aimed to establish whether this piRNA-dependent germ cell-specific gene silencing was applicable to endogenous genes. We selected *Dnmt3L* (DNA methyltransferase 3-like) as a model gene because it is expressed in embryonic male germ cells, and null mutant mice show defective DNA methylation of retrotransposon genes and impairment of spermatogenesis [3, 17, 18], similar to the *Mili* or *Miwi2* null mice [5, 6, 20]. We produced *Miwi2-asDnmt3L* transgenic mice expressing the antisense mRNA of *Dnmt3L* under the control of the *Miwi2* promoter. These transgenic mouse lines (#3 and #6, described as *asDnmt3L#3* and *asDnmt3L#6*, respectively) had significantly smaller testes than control mice (Figures 3A and S3A).

We used the *asDnmt3L#3* line in further experiments because it showed the more severe impairment of spermatogenesis (Figure S3B). Although *asDnmt3L#6* did not produce offsprings by natural mating, microscopic analysis showed some spermatogenesis in the mice. These germ cells possessed the fertilization ability when tested by intracytoplasmic sperm injection (ICSI) and elongating spermatid injection (ELSI) (Figure S3C). We assumed that this insufficient spermatogenesis was due to the lower DNA methylation level of the mice, presumably caused by the lower expression of the antisense transgene (Figure S3D).

DNMT3L proteins were drastically reduced in *asDnmt3L* embryonic testes, and spermatogenesis was severely impaired

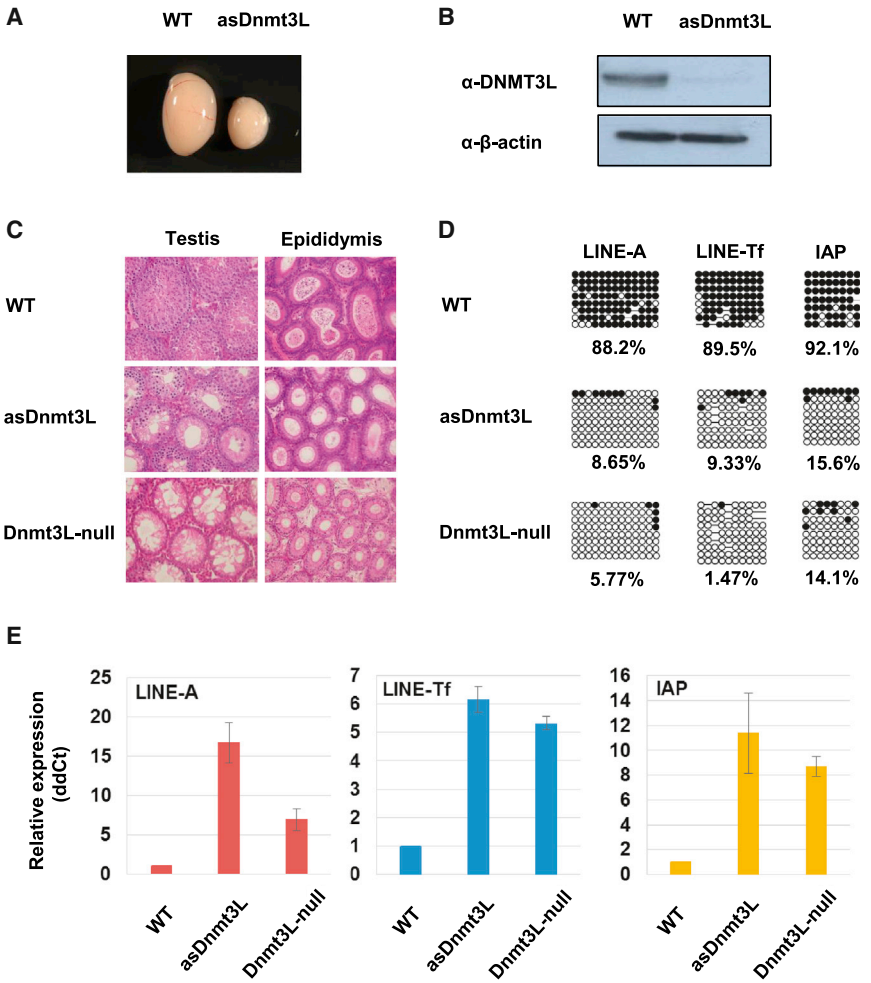

Figure 3. Impaired Spermatogenesis, Silencing of *Dnmt3L* Gene, and Increased Retrotransposon Expression in the Antisense *Dnmt3L* Transgenic Mouse

(A) Testes of 5-week-old wild-type and *asDnmt3L* mice.

(B) Western blotting analysis of embryonic day 16.5 wild-type and *asDnmt3L* testes, using anti-DNMT3L and anti-β-ACTIN antibodies.

(C) Hematoxylin-eosin staining of 5-week-old wild-type, *asDnmt3L*, and *Dnmt3L*-null mice.

(D) Bisulfite sequencing analysis of *LINE-A*, *LINE-Tf*, and *IAP* retrotransposon promoter regions. Genomic DNA was extracted from the EpCAM-positive germ cells of 2-week-old wild-type, *asDnmt3L*, and *Dnmt3L*-null mice.

(E) Quantitative RT-PCR analysis of *LINE-A*, *LINE-Tf*, and *IAP* retrotransposon expression. RNAs were extracted from whole testes of 2-week-old wild-type, *asDnmt3L*, and *Dnmt3L*-null mice. Error bars indicate SD of n = 3 biological replicates. *β-actin* was used as an internal control.

(Figures 3B and 3C). DNA hypomethylation of the promoter regions of *LINE-1* and *IAP* retrotransposons and subsequent abrogation of gene silencing were observed in *asDnmt3L* male germ cells (Figures 3D and 3E). This phenotype was essentially identical to that of the *Dnmt3L*-null mice, strongly suggesting that piRNA-mediated gene silencing of *Dnmt3L* takes place in *asDnmt3L* embryonic testes. The DNA methylation status of control regions of the *Dnmt3L* gene, spanning from the promoter to the second exon [21–23], was significantly increased in the *asDnmt3L* male germ cells (Figure 4A). It is quite likely that the observed phenotype is manifested by DNA methylation of *Dnmt3L* in a piRNA-dependent manner. Otherwise, the phenotype of these transgenic mice cannot be explained.

Comprehensive Analysis of *Dnmt3L*-Related piRNAs

Next, we carried out deep sequencing analysis of small RNAs in the *asDnmt3L* whole embryonic gonads. Although there were very few *Dnmt3L*-related piRNAs in the control male embryonic germ cells, a significant number of piRNAs were observed in the transgenic mice (Figures 4B, 4C, and S4A–S4C). Mapping of *Dnmt3L*-associated piRNAs demonstrated that these piRNAs were produced from various regions of the *Dnmt3L* mRNAs (Figure 4D). In addition, both sense and antisense *Dnmt3L* piRNAs harbor high 1st U (43.6% [5,323/12,205] sense; 63.2% [14,069/22,266] antisense) and 10th A bias (70.7% [8,625/12,205] sense; 43.4% [9,670/22,266] antisense) (Figure 4E), and approximately 90% of *Dnmt3L* piRNAs harbored the ping-pong signature (Figure S4D). These data clearly demonstrate that expression of antisense *Dnmt3L* mRNA induces the production of corresponding *Dnmt3L* piRNAs and subsequent DNA methylation. We cannot exclude the possibility of post-transcriptional degradation of *Dnmt3L* because there exist antisense RNAs, which can potentially introduce the post-transcriptional modification. Transcriptional repression and post-transcriptional degradation are mutually unexclusive, and our results clearly showed that significant DNA methylation and subsequent transcriptional repression took place.

The absolute reads number of *Dnmt3L* piRNAs was much larger than that of *EGFP* piRNAs (34,471 and 552, respectively). Even taking the reads of miRNAs as an internal control (the reads of the dTg and *asDnmt3L* cells were 1,204,399 and 8,092,774, respectively), the relative amount of *Dnmt3L* piRNAs was still nine times higher compared to that of *EGFP* piRNAs (Figure S4E). Meanwhile, the characteristics of *Dnmt3L* piRNAs and *EGFP* piRNAs were a little different. Although sense and antisense *Dnmt3L* piRNAs showed the tendency of 10th A bias (Figure 4E), only antisense, but not sense, *EGFP* piRNAs possessed this preponderance (Figure 2C). These differences would be due to the amount of RNA transcripts, the balance between sense and antisense transcripts, and/or the sequence differences of the two kinds of genes.

Usefulness and Utility of Artificial piRNA Induction System

Our data support two important concepts. One is that retrotransposon sequences and transcription from piRNA clusters

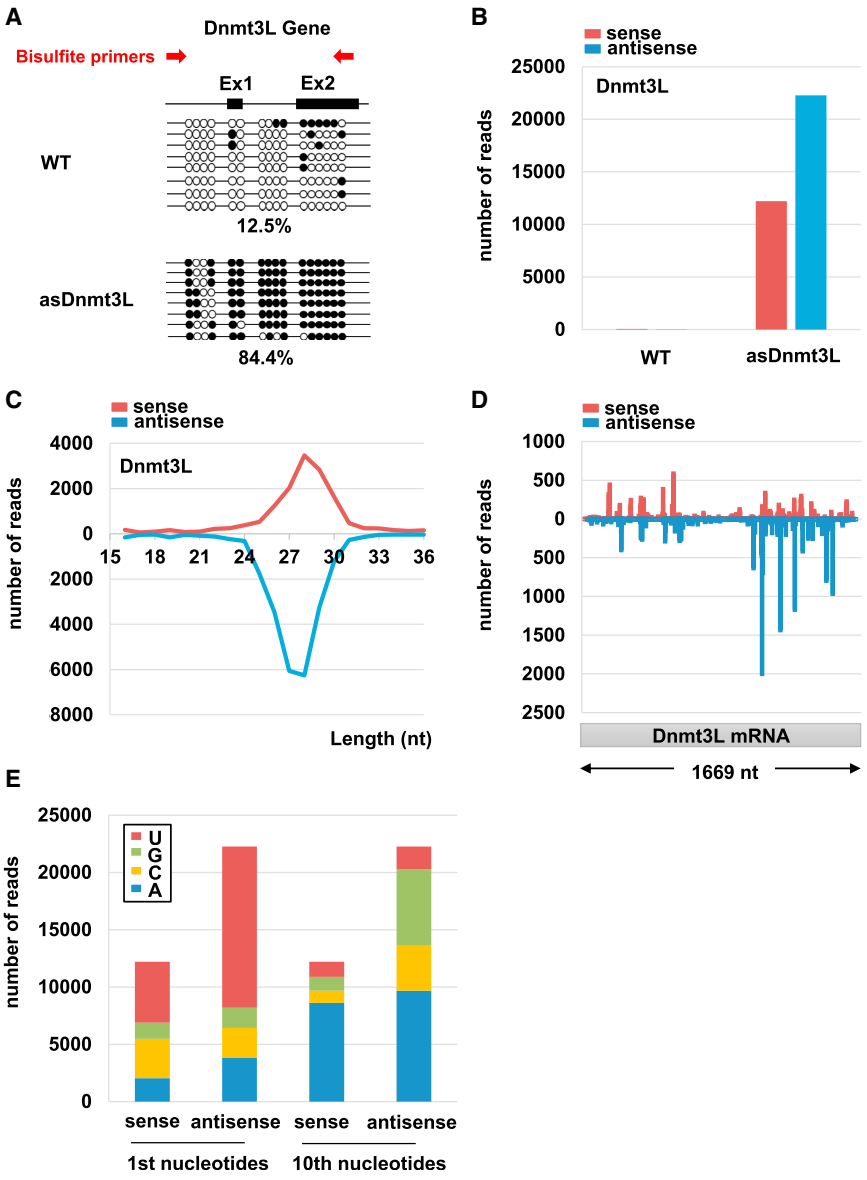

Figure 4. Induction of *Dnmt3L* Gene piRNA Production and DNA Methylation by Expression of Antisense *Dnmt3L* mRNA

(A) Bisulfite sequencing of the control region of the *Dnmt3L* gene. Genomic DNA was extracted from EpCAM-positive germ cells of 2-day-old wild-type and *asDnmt3L* mice. Black bars indicate exons in the *Dnmt3L* promoter. Red arrows represent the primers used for the bisulfite sequence.

(B–E) Deep sequencing analysis of small RNAs in *asDnmt3L* whole embryonic gonads. Numbers of small RNAs with 25–31 nt length corresponding to the *Dnmt3L* sequence are shown in (B). Length distribution of small RNAs corresponding to the *Dnmt3L* sequence is shown in (C). Mapping of *Dnmt3L* piRNAs is shown in (D). Red and blue bars indicate the sense and antisense strands, respectively. Numbers of the first and tenth nucleotides in *Dnmt3L* piRNAs are shown in (E).

are irrelevant to the piRNA biosynthesis of embryonic mouse male germ cells. Recently, it was demonstrated that *EGFP*-related piRNAs were produced in flies, mice, and cultured silkworm ovary cells, as shown by inserting an *EGFP* sequence into their piRNA clusters, suggesting that the locus of the gene is important for piRNA production [24–26]. These papers highlighted the importance of the piRNA cluster region in piRNA production. To identify the insertion sites of *Miwi2-asEGFP* transgenes, capture sequence analysis was performed as described in Figure S2E and Table S1. In silico piRNA cluster analysis by proTRAC [27] showed that there were 821 typical piRNA clusters in the dTg mice and that the genomic insertion sites of the transgenes were not in the piRNA clusters (Table S2). RT-PCR analysis using the primers between the exogenous *Miwi2* promoter and the antisense *EGFP* revealed that there were no RNA transcripts from the upstream regions of antisense *EGFP* (data not shown). These data supported the notion that the transgene integration site was not in the active piRNA clusters. Thus, our data suggest that the transcription from the piRNA cluster is not necessarily a critical factor for piRNA biogenesis if sense and antisense RNAs are co-expressed.

There were *EGFP*-related piRNAs in the *Oct4-EGFP* and *Miwi2-asEGFP* transgenic mice embryonic testes (8 in 22,098,877 and 273 in 18,792,650 of the RNAs with 18 to 45 nt length, respectively). Similarly, although the numbers were quite low, there were endogenous sense piRNAs including the piRNAs corresponding to *Dnmt3L* in the control embryonic male germ cells (Figures S4A–S4C). Such "seed" *EGFP* and *Dnmt3L* piRNAs may have some roles in the initial step of ping-pong cycle in the dTg and *Dnmt3L* antisense transgenic mice. However, considering that there were many more abundant sense piRNAs in these transgenic mice embryonic testes, both sense and antisense transcripts should have been necessary for the efficient piRNA production even in this case.

The second important finding is the utility of piRNA-dependent silencing of endogenous gene(s). Our simple experimental system for inducing artificial piRNAs and subsequent DNA methylation-dependent gene silencing provides a novel procedure for induction of DNA methylation to inhibit gene expression during spermatogenesis. Now we are producing transgenic mouse lines that express antisense RNAs of various genes in the embryonic germ cells under the control of *Miwi2* promoter. Moreover, we believe that sperm containing abnormal DNA methylation patterns introduced by piRNA represent a useful tool for the study of transgenerational epigenetic inheritance.

EXPERIMENTAL PROCEDURES

Details are shown in the Supplemental Experimental Procedures. All animal experiments were performed in accordance with the general guidelines of The Institute of Experimental Animal Sciences, Osaka University Medical School.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.01.060.

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REFERENCES

1. Aravin, A.A., and Bourc'his, D. (2008). Small RNA guides for de novo DNA methylation in mammalian germ cells. Genes Dev. *22*, 970–975.
2. Saitou, M., Kagiwada, S., and Kurimoto, K. (2012). Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development *139*, 15–31.
3. Bourc'his, D., and Bestor, T.H. (2004). Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. Nature *431*, 96–99.
4. Aravin, A.A., Sachidanandam, R., Bourc'his, D., Schaefer, C., Pezic, D., Toth, K.F., Bestor, T., and Hannon, G.J. (2008). A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. Mol. Cell *31*, 785–799.
5. Carmell, M.A., Girard, A., van de Kant, H.J., Bourc'his, D., Bestor, T.H., de Rooij, D.G., and Hannon, G.J. (2007). MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev. Cell *12*, 503–514.
6. Kuramochi-Miyagawa, S., Watanabe, T., Gotoh, K., Totoki, Y., Toyoda, A., Ikawa, M., Asada, N., Kojima, K., Yamaguchi, Y., Ijiri, T.W., et al. (2008). DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. Genes Dev. *22*, 908–917.
7. Castel, S.E., and Martienssen, R.A. (2013). RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. Nat. Rev. Genet. *14*, 100–112.
8. Siomi, M.C., Sato, K., Pezic, D., and Aravin, A.A. (2011). PIWI-interacting small RNAs: the vanguard of genome defence. Nat. Rev. Mol. Cell Biol. *12*, 246–258.
9. Ishizu, H., Siomi, H., and Siomi, M.C. (2012). Biology of PIWI-interacting RNAs: new insights into biogenesis and function inside and outside of germlines. Genes Dev. *26*, 2361–2373.
10. Aravin, A.A., Hannon, G.J., and Brennecke, J. (2007). The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. Science *318*, 761–764.
11. Aravin, A.A., Sachidanandam, R., Girard, A., Fejes-Toth, K., and Hannon, G.J. (2007). Developmentally regulated piRNA clusters implicate MILI in transposon control. Science *316*, 744–747.
12. Rangan, P., Malone, C.D., Navarro, C., Newbold, S.P., Hayes, P.S., Sachidanandam, R., Hannon, G.J., and Lehmann, R. (2011). piRNA production requires heterochromatin formation in Drosophila. Curr. Biol. *21*, 1373–1379.
13. Zhang, F., Wang, J., Xu, J., Zhang, Z., Koppetsch, B.S., Schultz, N., Vreven, T., Meignin, C., Davis, I., Zamore, P.D., et al. (2012). UAP56 couples piRNA clusters to the perinuclear transposon silencing machinery. Cell *151*, 871–884.
14. Pane, A., Jiang, P., Zhao, D.Y., Singh, M., and Schüpbach, T. (2011). The Cutoff protein regulates piRNA cluster expression and piRNA production in the Drosophila germline. EMBO J. *30*, 4601–4615.
15. Watanabe, T., Tomizawa, S., Mitsuya, K., Totoki, Y., Yamamoto, Y., Kuramochi-Miyagawa, S., Iida, N., Hoki, Y., Murphy, P.J., Toyoda, A., et al. (2011). Role for piRNAs and noncoding RNA in de novo DNA methylation of the imprinted mouse Rasgrf1 locus. Science *332*, 848–852.
16. Gu, W., Lee, H.C., Chaves, D., Youngman, E.M., Pazour, G.J., Conte, D., Jr., and Mello, C.C. (2012). CapSeq and CIP-TAP identify Pol II start sites and reveal capped small RNAs as C. elegans piRNA precursors. Cell *151*, 1488–1500.
17. Hata, K., Kusumi, M., Yokomine, T., Li, E., and Sasaki, H. (2006). Meiotic and epigenetic aberrations in Dnmt3L-deficient male germ cells. Mol. Reprod. Dev. *73*, 116–122.
18. Webster, K.E., O'Bryan, M.K., Fletcher, S., Crewther, P.E., Aapola, U., Craig, J., Harrison, D.K., Aung, H., Phutikanit, N., Lyle, R., et al. (2005). Meiotic and epigenetic defects in Dnmt3L-knockout mouse spermatogenesis. Proc. Natl. Acad. Sci. USA *102*, 4068–4073.
19. Yoshimizu, T., Sugiyama, N., De Felice, M., Yeom, Y.I., Ohbo, K., Masuko, K., Obinata, M., Abe, K., Schöler, H.R., and Matsui, Y. (1999). Germline-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in mice. Dev. Growth Differ. *41*, 675–684.
20. Kuramochi-Miyagawa, S., Kimura, T., Ijiri, T.W., Isobe, T., Asada, N., Fujita, Y., Ikawa, M., Iwai, N., Okabe, M., Deng, W., et al. (2004). Mili, a mammalian member of piwi family gene, is essential for spermatogenesis. Development *131*, 839–849.
21. Hu, Y.G., Hirasawa, R., Hu, J.L., Hata, K., Li, C.L., Jin, Y., Chen, T., Li, E., Rigolet, M., Viegas-Péquignot, E., et al. (2008). Regulation of DNA methylation activity through Dnmt3L promoter methylation by Dnmt3 enzymes in embryonic development. Hum. Mol. Genet. *17*, 2654–2664.
22. Shovlin, T.C., Bourc'his, D., La Salle, S., O'Doherty, A., Trasler, J.M., Bestor, T.H., and Walsh, C.P. (2007). Sex-specific promoters regulate Dnmt3L expression in mouse germ cells. Hum. Reprod. *22*, 457–467.
23. O'Doherty, A.M., Rutledge, C.E., Sato, S., Thakur, A., Lees-Murdock, D.J., Hata, K., and Walsh, C.P. (2011). DNA methylation plays an important role in promoter choice and protein production at the mouse Dnmt3L locus. Dev. Biol. *356*, 411–420.
24. Kawaoka, S., Mitsutake, H., Kiuchi, T., Kobayashi, M., Yoshikawa, M., Suzuki, Y., Sugano, S., Shimada, T., Kobayashi, J., Tomari, Y., and Katsuma, S. (2012). A role for transcription from a piRNA cluster in de novo piRNA production. RNA *18*, 265–273.
25. Muerdter, F., Olovnikov, I., Molaro, A., Rozhkov, N.V., Czech, B., Gordon, A., Hannon, G.J., and Aravin, A.A. (2012). Production of artificial piRNAs in flies and mice. RNA *18*, 42–52.
26. Yamamoto, Y., Watanabe, T., Hoki, Y., Shirane, K., Li, Y., Ichiiyanagi, K., Kuramochi-Miyagawa, S., Toyoda, A., Fujiyama, A., Oginuma, M., et al. (2013). Targeted gene silencing in mouse germ cells by insertion of a homologous DNA into a piRNA generating locus. Genome Res. *23*, 292–299.
27. Rosenkranz, D., and Zischler, H. (2012). proTRAC—a software for probabilistic piRNA cluster detection, visualization and analysis. BMC Bioinformatics *13*, 5.

