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LETTER TO THE EDITOR

Effective gene silencing in *Drosophila* ovarian germline by artificial microRNAs

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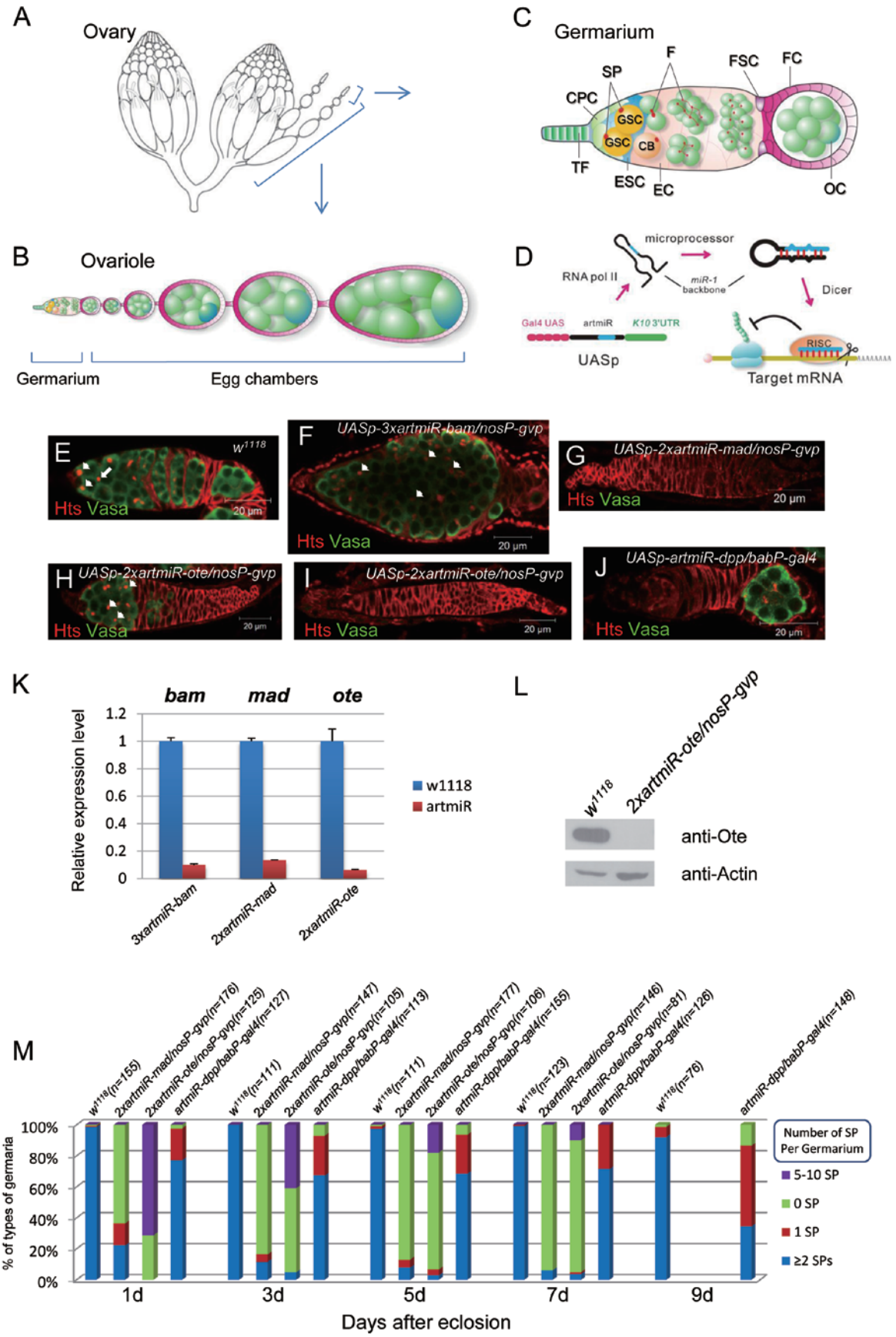
Dear Editor,

Drosophila oogenesis is of great interest because it represents an excellent model system to study a number of fascinating biological processes, such as stem cell regulation, germ cell meiosis and oocyte determination, as well as signal interactions between germline and soma. A typical *Drosophila* ovary is composed of 16-20 ovarioles, each consisting of an anterior functional unit called a germarium and a linear string of differentiated egg chambers posterior to the germarium [1] (Figure 1A and 1B). *Drosophila* oogenesis initiates at the tip of the germarium, when a germline stem cell (GSC) divides asymmetrically to generate a daughter GSC and a cystoblast that eventually develops into a mature egg [2] (Figure 1C and 1E). During the last decades, much progress has been made on identifying the key regulators controlling oogenesis, using traditional forward genetic screening via ethyl methanesulfonate and P-element mutagenesis. Such approaches are undoubtedly fruitful. To date, many sterile mutations that disrupt genes necessary for oogenesis have been generated. However, systematic genetic screening and subsequent identification of the role of genes in oogenesis largely depend on phenotypic analysis; therefore, the effects of many unidentified genes on oogenesis are often masked by lethality. Thus, given the limitation of inherent bias in traditional forward genetics, it would be advantageous to develop alternative methods to remove a gene's function in a tissue-specific manner for screening and characterization of novel gene functions in oogenesis.

Small RNA-mediated gene silencing controls a wide range of processes during development. So far, three classes of small RNAs have been characterized in *Drosophila*, including small interfering RNAs (siRNAs), microRNAs (miRNAs) and Piwi-interacting RNAs [3]. Double-stranded siRNA-mediated gene silencing, as a potent tool, has been widely used for knocking down gene expression in S2 cell cultures and in multiple tissues in flies. In *Drosophila* oogenesis, the siRNA pathway is apparently activated during egg activation, because the introduction of exogenous dsRNAs results in degradation of target mRNAs. However, in the early stage of germ cell development these cells fail to support dsRNA-mediated RNA interference because of unknown inhibitory mechanisms. To our knowledge no practical protocol for siRNA-mediated gene knockdown has yet been established for the *Drosophila* ovarian germline.

The miRNAs are structurally similar to siRNAs, but have a distinct biogenesis mechanism and execute gene silencing either through degradation of target mRNAs or by translation repression of target genes. There is increasing evidence that the miRNA pathway plays critical roles in the regulation of GSCs and germ cell development, suggesting that miRNA-mediated gene silencing functions as an important mechanism in regulating *Drosophila* germline development [4, 5]. Haley *et al*. [6] have now successfully established a simplified gene silencing method by using artificial miRNAs in *Drosophila* S2 cell cultures and in wing imaginal discs. Therefore, we sought to test whether artificial miRNAs could also be applied in germ cells for knocking down genes of interest and for systematic reverse genetic screening to identify hitherto undiscovered genes involved in germline development.

To explore whether ectopic expression of the artificial miRNAs could affect germline development, we chose *bam*, *mad*, *ote* and *dpp* as target genes (Supplementary information, Data S1). These genes have been demonstrated to play critical roles in determining the fate of GSCs and germ cell development. Using an earlier described method [6], we used the precursor of miR-1, which is enriched in *Drosophila* ovaries, as the backbone to design the pre-miRNA stem–loop base of artificial miRNAs (Figure 1D and Supplementary information, Figure S1). The sequence of pre-artmiRNA, 71 nucleotides (nt) long, contains 21-nt oligos that are complementary to the mRNA sequence of target genes, and the detailed information is shown in Supplementary information, Figure S1B. This design allows pre-artmiRNA to mimic the natural structure of pre-miR-1 that ensures the accurate processing and efficient production of the ma-


A
Ovary
B
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Germarium
Egg chambers
C
Germarium
SP
F
FSC
FC
CPC
GSC
GSC
CB
TF
ESC
EC
OC
D
microprocessor
RNA pol II
miR-1 backbone
Dicer
Gal4 UAS
artmiR
K10 3'UTR
UASp
RISC
Target mRNA
E
w1118
Hts Vasa
20 μm
F
UASp-3xartmiR-bam/nosP-gvp
Hts Vasa
20 μm
G
UASp-2xartmiR-mad/nosP-gvp
Hts Vasa
20 μm
H
UASp-2xartmiR-ote/nosP-gvp
Hts Vasa
20 μm
I
UASp-2xartmiR-ote/nosP-gvp
Hts Vasa
20 μm
J
UASp-artmiR-dpp/babP-gal4
Hts Vasa
20 μm
K
bam
mad
ote
Relative expression level
1.2
1
0.8
0.6
0.4
0.2
0
w1118
artmiR
3xartmiR-bam
2xartmiR-mad
2xartmiR-ote
L
w1118
2xartmiR-ote/nosP-gvp
anti-Ote
anti-Actin
M
w1118(n=155)
2xartmiR-mad/nosP-gvp(n=176)
2xartmiR-ote/nosP-gvp(n=125)
artmiR-dpp/babP-gal4(n=127)
w1118(n=111)
2xartmiR-mad/nosP-gvp(n=147)
2xartmiR-ote/nosP-gvp(n=105)
artmiR-dpp/babP-gal4(n=113)
w1118(n=111)
2xartmiR-mad/nosP-gvp(n=177)
2xartmiR-ote/nosP-gvp(n=106)
artmiR-dpp/babP-gal4(n=155)
w1118(n=123)
2xartmiR-mad/nosP-gvp(n=146)
2xartmiR-ote/nosP-gvp(n=81)
artmiR-dpp/babP-gal4(n=126)
w1118(n=76)
artmiR-dpp/babP-gal4(n=148)
% of types of germaria
100%
80%
60%
40%
20%
0%
1d
3d
5d
7d
9d
Days after eclosion
Number of SP Per Germarium
5-10 SP
0 SP
1 SP
≥2 SPs

ture artmiRNAs by Dicer-1/Ago1 complexes.

To construct the artificial miRNA expression vectors, we used a modified *UASp* vector as the backbone to generate P{*UASp-artmiRNA*}, in which one or two or three hairpin sequences were placed under the control of the *UASp* promoter. The *UASp* promoter is the only active *UAS* promoter in germ cells when it is combined with P{*nosP-gal4:vp16*}, a germ cell-specific driver [7]. To explore whether the artificial miRNAs would efficiently knock down the expression of target genes in germ cells, we first chose *bam* as a target gene and generated *bam* knockdown transgenic lines, P{*UASp-3XartmiR-bam*}, in which three different hairpins targeting *bam* mRNA were under the control of the *UASp* promoter. This gene has been shown to play key roles in germline development, given that loss of *bam* blocks germ cell differentiation resulting in GSC hyperplasia, while ectopic expression of *bam* in GSCs results in their elimination [8]. To assess the effectiveness of expression of *artmiR-bam* in germ cells, we employed the Gal4-UAS system to express it by combining P{*UASp-3XartmiR-bam*} with P{*nosP-gal4:vp16*}. Notably, as shown in Figure 1F and 1K, expression of *3XartmiR-bam* significantly knocked down the endogenous *bam* and blocked germ stem cell differentiation leading to germ cell hyperplasia, which simply phenocopied *Drosophila* with a loss of *bam* background, suggesting that artificial miRNAs could potentially silence the target genes of interest in germ cells. Additionally, we found that expression of one copy of *bam* hairpin (*1XartmiR-bam*) resulted in a weaker *bam*-like phenotype (data not shown), compared to *3XartmiR-bam*, suggesting that artificial miRNAs act in a dose-dependent manner to down-regulate target gene expression. To test whether artificial miRNAs also knock down expression of other genes in germ cells, we then generated two more artificial miRNA transgenes, P{*UASp-2XartmiR-mad*} and P{*UASp-2XartmiR-ote*}, for knocking down the endogenous *mad* and *ote* genes, respectively. Both *mad* and *ote* genes have been shown to function as key regulators in the bone morphogenetic protein/decapentaplegic (BMP/Dpp) pathway and are essential for the maintenance of germline cells [7, 9]. We first examined whether the artificial miRNAs of *mad* would affect adult GSC function by combining P{*UASp-2XartmiR-mad*} with P{*nosP-gal4:vp16*} to express artmiR-*mad* in adult germ cells. We took advantage of the temperature-dependent activity of Gal4:Vp16 to control the expression level of *2XartmiR-mad*. According to the method described previously [10], the crosses were performed and the resulting progenies were initially raised at 18 °C until adult eclosion, then they were shifted to the standard culture temperature (25 °C). The numbers of GSCs per germarium from the controls and from female flies expressing *2XartmiR-mad* were quantified at days 1, 3, 5 and 7 after eclosion. As shown in Figure 1G, 1K and 1M, compared with controls, the numbers of GSCs per germarium reduced progressively in the ovaries from the females expressing *2XartmiR-mad* during the testing period. Together with quantitative RT-PCR analysis, our findings further demonstrated that expression of *2XartmiR-mad* efficiently knocked down expression of the endogenous *mad*, resulting in loss of GSCs. Similar results were obtained when we knocked down the *ote* gene by expression of the artificial miRNAs of 2X*artmiR-ote* in germ cells (Figure 1H, 1I, 1K-1M).

Previous studies have revealed that maintenance of GSCs requires both extrinsic and intrinsic mechanisms. Dpp functions as an extrinsic niche factor to maintain

Figure 1 Effective gene silencing in *Drosophila* ovarian germline by artificial microRNAs. **(A)** A schematic diagram of a *Drosophila* ovary. A single female fly has a pair of ovaries, each of which consists of 16-20 ovarioles. **(B)** Diagram of an ovariole containing an anterior functional unit called a germarium and a linear string of differentiated egg chambers posterior to the germarium. **(C)** Diagram of the germarium consisting of different cell types and organelles indicated as follows: terminal filaments (TFs, dark green) and niche cap cells (CPCs, light green) are in close contact with germline stem cells (GSCs, yellow) that divide in coordination with somatic escort stem cells (ESCs, blue). ESCs produce squamous daughter escort cells (ECs, light pink), which encyst the differentiating GSC daughter cystoblasts (CB, orange). As a CB divides, it develops into cyst cells (green), one of which is determined to form the oocyte (OC). A 16-cell cyst is surrounded by follicle cells (FCs), generated by follicle stem cells (FSCs, violet). GSCs and CBs carry a spectrosome (SP, red), whereas cysts have extending or branched fusomes (F, red). **(D)** Construction and biogenesis of artificial microRNAs. **(E-J)** Characterization of the function of the microRNAs in *Drosophila* ovary. Ovaries from wild-type w^{1118} **(E)**, P{*UASp-3XartmiR-bam*}/P{*nosP-gal4:vp16*} **(F)**, P{*UASp-2XartmiR-mad*}/P{*nosP-gal6:vp16*} **(G)**, P{*UASp-2XartmiR-ote*}/P{*nosP-gal4:vp16*} **(H**, **I)** and P{*UASp-artmiR-dpp*}/P{*bab1-gal4*} **(J)** flies were stained with anti-Vasa (green) and anti-Hts (red) antibodies. Anti-Hts was used to outline the germarium and the morphology of the spectrosomes (as indicated by arrows or arrowheads) and fusomes, whereas the staining of anti-Vasa was used to visualize all germ cells in the germarium and egg chambers. **(K)** Quantitative RT-PCR experiments were used to analyze the effectiveness of target gene knockdown in the examined fly ovaries as indicated. **(L)** Western blot result showing the effectiveness of knockdown by *2XartmiR-ote* in the ovary. **(M)** Quantitative analysis of the numbers of germ cells carrying spectrosomes in w^{1118} and *artmiR* transgenic flies.

GSCs by silencing *bam* transcription. To test whether artificial miRNA-mediated gene silencing could also be applied to somatic niche cells in the ovary we constructed *dpp* knockdown vectors, P{*UASp-artmiR-dpp*} and P{*bab1-gal4*}. As shown in Figure 1J, 1M and Supplementary information, Figure S2, specific knockdown of *dpp* expression in the cap cells of germaria resulted in loss of GSCs, which mimics the phenotype in *dpp* mutants [9], suggesting that artificial miRNAs could also interfere with target gene functions in somatic cells in ovaries.

In summary, our results support the idea that artificial miRNAs can effectively down-regulate endogenous target genes of interest in *Drosophila* germline, as well as in somatic cells in *Drosophila* ovary. We believe that the technique established in this study is not only useful for characterization of the functions of genes of interest, but also provides a powerful tool for systematic reverse genetic screening for identifying hitherto undiscovered genes involved in GSC regulation and germline development.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)