Argonaute 1 regulates the fate of germline stem cells in Drosophila

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INTRODUCTION

The Argonaute-family proteins play crucial roles in small-RNA-mediated gene regulation. In Drosophila, previous studies have demonstrated that Piwi, one member of the PIWI subfamily of Argonaute proteins, plays an essential role in regulating the fate of germline stem cells (GSCs). However, whether other Argonaute proteins also play similar roles remains elusive. Here, we show that overexpression of Argonaute 1 (AGO1) protein, another subfamily (AGO) of the Argonaute proteins, leads to GSC overproliferation, whereas loss of Ago1 results in the loss of GSCs. Combined with germline clonal analyses of Ago1, these findings strongly support the argument that Ago1 plays an essential and intrinsic role in the maintenance of GSCs. In contrast to previous observations of Piwi function in the maintenance of GSCs, we show that AGO1 is not required for bag of marbles (bam) silencing and probably acts downstream or parallel of bam in the regulation of GSC fate. Given that Ago1 serves as a key component of the miRNA pathway, we propose that an AGO1-dependent miRNA pathway probably plays an instructive role in repressing GSC/cystoblast differentiation.

KEY WORDS: Argonaute protein, Ago1, miRNA, GSC self-renewal, Drosophila
MATERIALS AND METHODS

Drosophila genetics

All flies were maintained under standard culture conditions. *bam*^Bo^ is a null allele for *bam* as described previously (McKearin and Ohstein, 1995; Ohstein et al., 2000). *bam*^Bo^ is a strong allele for *bam* as described previously (Chen and McKearin, 2005). *piwi*^EP^ and *P{hs-gal4}* have been described previously (Chen and McKearin, 2005; Cox et al., 2000) and *Ago1*^loqsf00791^ has been described previously (Jin et al., 2004; Kataoka et al., 2001; Williams and Rubin, 2002), and *loq*^G07546^ was a strong allele for *loq* from the Exelixis Collection and described previously (Forstemann et al., 2005; Jiang et al., 2005). *P{bam-gfp}* was described by Chen and McKearin (Chen and McKearin, 2003b), and *P{hs-Ago1}* (a gift from Dr T. Uemura, Kyoto University, Japan) was used for rescuing *Ago1* mutants (Kataoka et al., 2001). *Ago1*^EMS^ is generated through an EMS mutagenesis; sequencing results showed that the *Ago1*^EMS^ allele contains a C-T switch, which changes amino acid 212 Q to a stop codon in the AGO1 B form and changes amino acid 246 Q to a stop codon in the putative AGO1 A and C forms. The resulting truncated proteins lack both the PAZ and PIWI domains, which are essential for Argonaute protein function (Miyoshi et al., 2005; Okamura et al., 2004; Shi et al., 2004), indicating that *Ago1*^EMS^ is a null allele for the *Ago1* gene; to obtain more mutants for the further analysis of *Ago1* function in GSCs, we performed a mutagenesis through imprecise mobilization of the P-element in *Ago1*^loqsf00791^, Fifteen alleles, which we referred to as *Ago1*-^loqsf00791^-^*Ago1*^15^, were recovered, and one of them, *Ago1*^14^, was characterized as having a 16 kb deletion that covers the *Ago1* coding sequence, indicating that *Ago1*^14^ is another null allele for the *Ago1* gene.

Immunohistochemistry and microscopy

Ovaries were prepared for reaction with antibodies as described previously (McKearin and Ohstein, 1995). Monoclonal anti-Bam antibody (McKearin and Ohstein, 1995) was used at a 1:500 dilution, polyclonal anti-Vasa antibody (Santa) was used at 1:200 dilution, polyclonal anti-GFP antibody (Invitrogen) was used at a 1:5000 dilution, monoclonal anti-Hts antibody was used at a 1:500 dilution, and mouse anti-AGO1 (a gift from Dr M. Siomi and Dr H. Siomi, Institute for Genome Research, University of Tokushima, Japan) (Miyoshi et al., 2005) was used at a 1:200 dilution. Secondary antibodies used were goat anti-mouse Alexa 568, goat anti-rabbit Alexa 488, and goat anti-rat Cy3 (Molecular Probes), all at 1:200. All samples were examined by Zeiss microscope and images were captured using the Zeiss Two Photon Confocal LSM510 META system supported by the State Key Laboratory of Biomembrane and Membrane Biotechnology and Institute of Zoology, CAS. Images were further processed with Adobe Photoshop 6.0.

Phenotypic assay for quantification of GSC maintenance in mutant adult ovaries

Ovaries isolated from wild-type and different mutant flies of different ages were incubated with anti-Hts antibody, anti-Vasa antibody and DNA dyes to identify terminal filament cells, fusomes and germ cells. We scored as GSCs any Vasa-positive germ cells at the anterior position that appeared close to cap cells or to the basal cells of terminal filaments and also carried spherical fusomes (at the anterior position or extending fusomes when a GSC was dividing.

Germline clonal analysis

FLP/FRT-mediated recombination was used to generate *Ago1* mutant GSC and PGC clones. To generate GSC clones, *w; FRTG13, Ago1/Cyo* (*w; FRTG13/Cyo as the control*) males were crossed to virgin females of *w hsFlp; FRTG13, ubi-gfp*, and 3-day-old female progenies lacking the *Cyo* chromosome underwent heat-shock treatment at 37°C for 60 min twice daily at 12 hourly intervals. Ovaries dissected from *hs-flp; frtG13, /frtG13, ubi-gfp* or *hs-flp; frtG13, Ago1/ frtG13, ubi-gfp* were stained with anti-GFP and anti-Hts antibodies for quantification of GSC clones. GSC clones were identified by the lack of GFP expression and carrying anterior-positioned spectrosome. To analyze GSC establishment, for adult GSC assay, the heat-shock treatment was started at the early third larval stage or early to induce PGC clones. GSC clones with negative GFP in *hs-flp; frtG13, Ago1/ frtG13, ubi-gfp* newly eclosed females were quantified to calculate the rate of GSC clones. In this experiment, *hs-flp; frtG13, / frtG13, ubi-gfp* was used as the FRT control. For pupa GSC clonal assay, the progenies from the cross of *w; FRTG13, Ago1/Cyo and w hsFlp; FRTG13, ubi-gfp* began to be treated by constitutive heat-shock from the first instar larval stage; meanwhile, the progenies from the cross of *w; FRTG13/Cyo and w hsFlp; FRTG13, ubi-gfp* were used as FRT control. After staining with anti-GFP and anti-Hts antibodies, female gonads containing GFP-negative germ cells were examined and putative GSC clones were identified by their anterior position close to the terminal filament and their lack of GFP expression.

RESULTS

Ectopic AGO1 expression increases the number of GSC-like cells

To explore the potential role of *Ago1* in the regulation of GSC fate, we overexpressed *Ago1* in germaria by expressing an *Ago1* cDNA under the control of the heat-shock promoter *P{hs-Ago1}* and then applying daily heat-shock treatment (Kataoka et al., 2001). In this study, we distinguished GSCs from differentiated germ cells by using anti-Vasa and anti-Hts antibodies to visualize germ cells and fusomes, respectively. The fusome (also called a ‘spectrosome’ in GSCs/CBs) is a germ-cell-specific organelle that is morphologically spherical in GSCs/CBs or extends from anterior position to posterior position when a GSC is dividing, but is branched in differentiated cysts (Fig. 1A). To assess the potential role of ectopic AGO1, we scored the number of germ cells carrying spectrosomes per germarium that came from wild type, and *P{hs-Ago1}* flies at 0, 6 and 10 days heat-shock treatment. As a positive control we used *piwi*^EP^, *P{hs-gal4}* to induce ectopic GSC-like cells by overexpression of *piwi* as described previously (Cox et al., 2000). As shown in Fig. 1B, before heat-shock treatment, we observed averages of 3.3 (*n*=128), 3.2 (*n*=102) and 3.2 (*n*=95) spectrosome-containing germ cells per germarium in wild-type, *P{hs-Ago1}*, and *piwi*^EP^, *P{hs-gal4}*, respectively, suggesting that there was no difference in the number of spectrosome-containing germ cells among wild-type, *P{hs-Ago1}*, and *piwi*^EP^, *P{hs-gal4}*. However, for wild-type control under the same conditions, the number of spectrosome-containing germ cells in *P{hs-Ago1}*) flies was increased to 5.9 (Fig. 1A), and 6.7 (*n*=95) spectrosome-containing germ cells per germarium in *P{hs-Ago1})* flies without heat-shock treatment. However, after 6 days of heat-shock treatment (three times per day), we observed averages of 4.9 (*n*=105) and 5.6 (*n*=123) spectrosome-containing germ cells per germarium in *P{hs-Ago1})* and *piwi*^EP^, *P{hs-gal4})* ovaries, respectively. We noted that 25.7% (*n*=105) of *P{hs-Ago1})* and 43.7% (*n*=145) of *piwi*^EP^, *P{hs-gal4})* germaria contained more than six spectrosome-containing germ cells (Fig. 1B). As ovaries from wild-type control females undergoing the same treatment carried only 3.3 spectrosome-containing germ cells per germarium (*n*=131), we excluded the possibility that the increase in the number of spectrosome-containing germ cells could be due to heat-shock treatment. We also found that, by extending heat-shock induction to 10 days, the average number of spectrosome-containing germ cells in *P{hs-Ago1})* and *piwi*^EP^, *P{hs-gal4})* ovaries was increased to 5.9 (*n*=176) and 6.5 (*n*=103) per morphological normal germarium (tumorous germaria found in *P{hs-gal4})* were excluded in this quantification). In this case, we found that 56.5% (*n*=176) of *P{hs-Ago1})* and 64.2% (*n*=112) *piwi*^EP^, *P{hs-gal4})* germaria contained more than six spectrosome-containing germ cells. However, for wild-type control under the same conditions, the number of spectrosome-containing germ cells was still maintained at 3.2 (*n*=115) per germarium. Thus, similar to *piwi*, the ectopic expression of AGO1 could also increase the number of GSC-like cells. Interestingly, in contrast to overexpression of *piwi*, a certain percentage (variable at 5-10%, *n*=100) of *P{hs-Ago1})* germaria were morphologically tumorous-like, when they were treated with 10 day heat-shock. As shown in Fig. 1C, GSC-like cells (Fig. 1C left and right panels and see Fig. S3 in the supplementary material) and differentiated cysts (Fig. 1C...
right panel) were filled in these tumorous germaria. Thus, the increased AGO1 activity could also potentially induce the overproliferation of GSC-like cells, probably by delaying GSC/CB differentiation, and disrupt normal oogenesis.

**The loss of Ago1 leads to defects in GSC maintenance**

The increase in GSC-like cells induced by Ago1 overexpression suggests that Ago1 could play a role in preventing GSC differentiation for their self-renewal. To explore this possibility, we analyzed the phenotype in Ago1 loss-of-function mutants. One well-characterized Ago1 mutant, Ago1k08121 (Jin et al., 2004; Kataoka et al., 2001; Williams and Rubin, 2002), leads to a loss of function of Ago1 that causes homozygous mutant lethality at the embryonic stage. To analyze the specificity of the Ago1k08121 allele in germ cell development we generated revertants of the Ago1k08121 allele. We found that the homozygous animals of precise excision lines containing Ago1k08121 exhibited strong defects in GSC maintenance. As illustrated in Fig. 2 and Table 1, for 15-day-old mutant ovaries (from hs-Ago1-k08121/Ago1k08121 flies carrying spectrosomes were undifferentiated germ cells (GSCs/CB and GSC-like cells). (C) A germarium from 10-day heat shock was morphologically tumorous; many GSC-like cells carrying spectrosomes (both panels) and differentiated germ cells carrying branched fusomes (right panel) were observed. Scale bar: 10 μm.

![Image](image-url)

**Table 1. Phenotypic assay for Ago1-deficient flies**

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>% germaria containing 2 or 3 GSCs</th>
<th>% germaria containing 1 GSC</th>
<th>% germaria containing only cysts</th>
<th>% empty germaria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control wild-type</td>
<td>99.2 (n=245)</td>
<td>0.8 (n=245)</td>
<td>0 (n=245)</td>
<td>0 (n=245)</td>
</tr>
<tr>
<td>Hs-Ago1;Ago1k08121/Ago1k08121</td>
<td>6.3 (n=79)</td>
<td>19.0 (n=79)</td>
<td>45.6 (n=79)</td>
<td>29.1 (n=79)</td>
</tr>
<tr>
<td>Hs-Ago1;Ago1k08121/Ago114</td>
<td>10.8 (n=113)</td>
<td>23.9 (n=113)</td>
<td>60.1 (n=113)</td>
<td>5.3 (n=113)</td>
</tr>
<tr>
<td>Hs-Ago1;Ago1k08121/Ago1EMS</td>
<td>10.3 (n=68)</td>
<td>26.5 (n=68)</td>
<td>60.3 (n=68)</td>
<td>2.9 (n=68)</td>
</tr>
</tbody>
</table>

*n = Number of germaria examined.

*Progeny from wild-type and different crosses (hs-Ago1;Ago1/Cyo × hs-Ago1;Ago1/Cyo) were treated with daily heat shock until adult eclosion. 15-day-old mutant and wild-type control flies were analyzed for quantification of GSC number.
cells, and about 19.0% (n=79) of germaria contained a single stem cell; most of the germaria (74.6%, n=79) contained either differentiated germ cells with branched fusomes only (45.6%) or no germ cells at all (29.1%). In the latter case, germaria composed of somatic cells were still visible by staining with Hoechst and anti-Hts antibodies. We observed similar results with the other two combinations of different Ago1 alleles (hs-Ago1; Ago1EMS and hs-Ago1; Ago1EMS and Ago1k08121) undergoing the same treatment (Fig. 2 and Table 1). It would therefore appear that, in contrast to controls, progressively reducing Ago1 activity causes the loss of GSCs. To further exclude the possibility that the phenotype seen in Ago1 deficiency could be rescued by exogenous AGO1 protein (Fig. 2E). Taken together, these results suggest that AGO1 plays an important role in germline stem cell maintenance and possibly controls other aspects of germ cell development as well.

AGO1 is required intrinsically for the establishment and maintenance of GSCs

The loss of germline stem cells in Ago1 mutant ovaries suggests that Ago1 is required in either GSCs or somatic cells (or possibly both). To test whether Ago1 is required intrinsically for GSC maintenance, we first examined the expression pattern of AGO1 in germlarium. We found via immunostaining that the AGO1 protein is ubiquitously expressed in both germ cells and somatic cells, indicating that AGO1 could function in either cell type (see Fig. S1A in the supplementary material). To explore whether AGO1 functions as a cell-autonomous factor for maintaining GSC fate, we used an FLP-FRT-mediated mitotic recombination technique to generate marked mutant GSCs, then calculated the life span of the marked mutant GSCs by quantifying their loss rate (Xie and Spradling, 1998). The marked mutant GSCs were identified by the lack of GFP expression and by carrying an anterior-positioned spectrosome. The three alleles (Ago1k08121, Ago1EMS and Ago1K1) of Ago1 were used to generate marked mutant GSC clones for analysis of Ago1 function in GSCs. The percentages of Ubi-GFP-marked GSCs were measured at 2, 10 and 20 days after heat-shock treatment (AHST). As shown in Fig. 3A-C and Table 2, for wild-type control GSC clones, the percentage of marked clones was about 30.0% at day 2, which we considered the initial percentage, and this was reduced to 19.2% by day 20 AHST, suggesting that the marked clone loss rate was roughly 36% over the 20 day period; however, for Ago1k08121 mutant GSC clones under the same conditions, the initial percentage of marked clones was measured at 30.3%, but this fell to 3.9% at day 20 AHST, suggesting a loss rate for Ago1k08121 of about 90% during the testing period (Fig. 3D-F and Table 2). We obtained similar results with the two other alleles, Ago1K1 and Ago1EMS. We observed that all the marked GSC clones were lost (100% loss rate) in both these alleles over the 20 day testing period, as shown in Table 2. Taken together, these findings support the argument that AGO1 is intrinsically required for GSC maintenance.

The loss of GSCs in the Ago1 mutants could be due to either differentiation or a reduction in cell viability. We noticed that the marked Ago1 mutant stem cells had normal DNA staining and could also develop into normal cysts; thereby we excluded the possibility

Table 2. Clonal analyses of Ago1 deficiency in GSCs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Days after stopping heat shock</th>
<th>% marked GSCs</th>
<th>Relative % marked GSCs</th>
<th>Total number of germaria examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control hs-flp;FRTG13,ubi-gfp/FRTG13</td>
<td>2</td>
<td>30.0</td>
<td>100</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>25.0</td>
<td>83.3</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>19.2</td>
<td>64.0</td>
<td>73</td>
</tr>
<tr>
<td>Ago1k08121 hs-flp;FRTG13,ubi-gfp/FRTG13, Ago1k08121</td>
<td>2</td>
<td>30.3</td>
<td>100</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12.5</td>
<td>41.3</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.9</td>
<td>12.9</td>
<td>128</td>
</tr>
<tr>
<td>Ago1K1 hs-flp;FRTG13,ubi-gfp/FRTG13, Ago1K1</td>
<td>2</td>
<td>30.0</td>
<td>100</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12.6</td>
<td>41.6</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>137</td>
</tr>
<tr>
<td>Ago1EMS hs-flp;FRTG13,ubi-gfp/FRTG G13, Ago1EMS</td>
<td>2</td>
<td>15</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>102</td>
</tr>
</tbody>
</table>
of stem cell loss due to cell death. To further confirm this conclusion, we examined the apoptosis of Ago1 mutant GSC clones. In more than 200 GSC clones examined, there were no dying GSCs observed by TUNEL assay (see Fig. S2 in the supplementary material). We therefore concluded that Ago1 is essential for regulating GSC self-renewal in an intrinsic manner and not essential for GSC survival.

We then investigated whether Ago1 could be involved in the establishment of GSCs. We marked wild-type, mutant Ago1EMS and Ago1primordial germ cells (PGCs) before the early third instar stage by the FLP-FRT-mediated recombination technique and examined the efficiency of these marked PGCs to differentiate into GSCs after adult eclosion. We observed that, for wild type, 17.9% (40/223) of GSCs were marked; however, only about 4.9% of Ago1EMS (10/206) and 1.8% of Ago1EMS/GSC clones were marked under the same experimental conditions. Interestingly, we also found that, when we started to induce Ago1PSC clones as early as the first instar larva stage and gave constitutive heat-shock induction until pupa formation, 23.2% (n=82) of adult germaria were empty, without any germ cells in the case of Ago1PSC clone induction. Under the same conditions, the rate of the marked GSC clones was observed at only 1.2% (n=82), in contrast to 13.7% (n=51) of that for wild-type FRT controls under exactly the same experimental conditions. We also examined the gonads in the early pupa stage. As shown in Fig. 3G,G’,H,H’, we observed that only 2.6% (n=79) of Ago1PSC anterior germ cells (putative GSCs) (Asaoaka and Lin, 2004; Zhu and Xie, 2003) were marked (GFP-), in contrast to 24.2% (n=99) of anterior germ cells (putative GSCs) in FRT controls.

Given that the half-life of Ago1PSC mutant GSCs is no less than 5 days (based on our Ago1 clonal assay), and that very low rate of Ago1PSC GSC clones from PGC clones were marked compared with FRT controls, we excluded the possibility that Ago1 contributed only to GSC maintenance and proposed that Ago1 is important for GSC establishment.

To explore whether Ago1 is involved in controlling the rate of GSC division, we investigated the ability of Ago1 mutant GSC-producing cysts at day 10 post-heat-shock inductions. For FRT controls, we found 52 germline cyst clones in the presence of 19 GSC clones, whereas for Ago1PSC mutants we observed 30 cyst clones in the presence of 13 GSC clones. As the relative percentages of marked GSCs were 83.3 and 41.6% for FRT controls and Ago1PSC at day 10 after heat-shock induction, respectively (shown in Table 2), this suggests that 83.3% of FRT control cysts and 41.6% of cysts came from the examined GSC clones. So we deduced that each FRT control GSC clone could produce an average of 2.3 cyst clones, whereas each Ago1PSC GSC clone could produce an average of 0.96 cyst clones. Consistent with the previous findings of the clonal assay for Dcr1 mutant GSCs (Hatfield et al., 2005), it appears that the loss of Ago1 reduces the rate of GSC division.

**The microRNA pathway is not required for bam silencing and probably acts downstream of or parallel to bam action**

It has been shown previously that BMP/Dpp-dependent bam silencing represents the primary pathway for GSC self-renewal (Chen and McKearin, 2003a; Song et al., 2004). To test whether Ago1 is involved in bam silencing in GSCs, we examined BamC expression in both wild-type and marked Ago1 mutant GSC clones. As shown in Fig. 4A,B, both wild-type (n=100) and Ago1GSC clones (n=100) were BamC-negative, suggesting that Ago1 is not required for bam silencing. As Ago1 is a key component of the microRNA pathway, and given the potential role of miRNAs in stem cell biology, we decided to explore whether other components of the microRNA pathway might modulate GSC fate in a similar manner as well. One good candidate for such a modulator is Loquacious (Log), which functions together with Dcr1 and AGO1 to guide miRNA biogenesis (Forstemann et al., 2005; Jiang et al., 2005; Saito...
Fig. 4. Two components of the miRNA pathway, AGO1 and Loqs, are not required for bam silencing in Drosophila. Ovaries dissected from flies hs-flp; frtG13, Ago1^{108121}/ frtG13, ubi-gfpl with heat-shock treatment were stained with anti-BamC (red). Two wild-type GSCs that are GFP-positive are negative for BamC (indicated by an arrow) (A), and a negatively marked GFP GSC, indicated by an arrow, was also BamC-negative (B). Ovaries dissected from P[bamP-GFP] (C), loqs; P[bamP-GFP] (D), bam, P[bamP-GFP] (E), loqs; bam, P[bamP-GFP] (F) were stained with anti-GFP (green) and anti-Hts (red) antibodies. Branched fusomes in loqs and bam mutant germaria are indicated by arrows. Scale bar: 10 µm.

Fig. 5. In Drosophila, germ cells could differentiate in both Ago1, bam and loqs, bam double mutants. Ovaries dissected from bam<sup>86</sup>/bam<sup>BG</sup> mutants (15 days old) (A), hs-dAgo1; Ago1<sup>k08121</sup> bam<sup>86</sup>/bam<sup>BG</sup> mutants (15 days old) (B), bam<sup>86</sup> mutant (15 days old) (C) and loqs<sup>00971</sup>; bam<sup>86</sup> double mutants (15 days old) (D) were stained with anti-Vasa (green) and anti-Hts (red) antibodies. Branched fusomes in loqs and bam mutant germaria are indicated by arrows. Scale bar: 10 µm.

et al., 2005). Consistent with two previous studies, we also found that the loqs<sup>00971</sup> mutant displays defects in GSC maintenance (see Table S1 in the supplementary material) (Forstemann et al., 2005; Jiang et al., 2005). To determine whether loqs is required for bam silencing, we then examined the expression of bam reporters in loqs mutant GSCs, as described previously (Chen and McKearin, 2005). As shown in Fig. 4D, 85.5% (n=101) of newly eclosed loqs-deficient flies carrying P[bamP-GFP] reporters showed a completely negative GFP pattern in putative GSCs (Fig. 4D). As loqs mutants cause complete GSC loss in some cases (about 36.3% at day 2 after eclosion), we further examined P[bamP-GFP] reporters (Chen and McKearin, 2003b) in loqs and bam double mutant flies that preserve GSCs in a majority of cases. In contrast to what was observed in pin<sup>i</sup> and bsgc double mutants (Chen and McKearin, 2005), our results revealed that, as with GFP patterns in the wild-type and bam single mutants (Fig. 4C,E), 87% of GSCs (n=150 germaria) exhibited a completely GFP-negative pattern in loqs and bam double mutants (Fig. 4F), indicating that the regulation of GSCs mediated by loqs does not require bam<sup>m</sup> activity.

To investigate the genetic relationship between loqs and bam, we began by examining the fusome behavior of germ cells in bam single mutants and loqs, bam double mutants. As described previously, in bam single mutants, we stained ovaries with anti-Hts and anti-Vasa antibodies from 7-day-old flies and found that all the germ cells were non-differentiated, GSC-like cells carrying either spherical fusomes or associated spherical fusomes between two germ cells (Fig. 5C). By contrast, the same-age ovaries lacking both loqs and bam produced a much more complex phenotype. We found that, even though most of the germaria were also tumorous, about 50% (n=100) of the germaria contained some germ cell clusters with highly branched fusomes (Fig. 5D). The appearance of germ cell clusters with branched fusomes suggested that these cells were undergoing differentiation. It appears that germ cells can still differentiate, provided that they lack loqs, even in a bam mutant background. This finding was different from the recent study showing that none of germ cells can differentiate in loqs and bam double mutant ovaries (Park et al., 2007). In order to further verify this, we next generated Ago1 and bam double mutant flies by using the transgene P[hs-Ago1] with daily heat-shock treatment. Consistent with the analysis of loqs and bam double mutants in this study, we found that about 80% of tumorous germaria (n=102) (Fig. 5B) from 15-day-old flies with {hs-dAgo1; Ago1<sup>k08121</sup>; bam<sup>86</sup>/bam<sup>BG</sup>} (immediately withdrawing the heat-shock treatment after adult eclosion) contained differentiated germ cells with highly branched fusomes (Fig. 5B); by contrast, in ovaries (n>100) from bam single mutants at the same age {bam<sup>86</sup>/bam<sup>BG</sup>}, no differentiated germ cell was observed (Fig. 5A).

Taking these results together, we conclude that Ago1 and loqs are not involved in a bam-silencing pathway to regulate GSC fate. Given that bam<sup>m</sup> is dispensable for the loss of GSCs in loqs or Ago1 mutants, we propose that the microRNA pathway probably represses GSC differentiation downstream of or parallel to bam.

**DISCUSSION**

In Drosophila, five members of Argonaute proteins have been characterized as constituting two distinct subfamilies (Gunawardane et al., 2007). As members of the PIWI subfamily, Aubergine (Aub) and Piwi play important roles for pole cell formation (Harris and Macdonald, 2001; Megosh et al., 2006). Piwi has been shown to be crucial for the maintenance of GSCs. A recent study showed that AGO3, another member of PIWI subfamily, is required for pole cell formation (Harris and Macdonald, 2001; Megosh et al., 2006). These findings suggest that PIWI subfamily Argonaut proteins play important roles in development. In this study, we analyzed the function of AGO1, a member of the AGO subfamily of Argonaut proteins in GSCs. We showed that overexpression of AGO1 leads to GSC overproliferation, whereas loss of AGO1 results in the loss of GSCs. Combined with germline clonal analyses of AGO1, these findings strongly suggest that AGO1, as a member of the AGO subfamily also plays an essential role in the maintenance of GSCs. Given that an AGO1 serves as an important component in the
miRNA pathway, we propose that the AGO1-dependent miRNA pathway plays at least a partial instructive role in repressing GSC/CB differentiation. Furthermore, in contrast to previous observations of Piwi function in GSCs, we found that Ago1 is not required for bam silencing and probably acts downstream of or parallel to bam action in the regulation of GSC maintenance.

Previous work has shown that Dcr1, another key component in the miRNA pathway, is important for controlling the GSC division rate but is dispensable for maintaining GSC self-renewal (Hatfield et al., 2005). Based on the data that Loqs functions selectively in the biogenesis of specific miRNAs (Park et al., 2007), and the recent results showing that Ago1 and Ago2 act in a partially redundant manner to control key steps in the midblastula transition and segmental patterning (Meyer et al., 2006), we speculate that Dcr1 may have more functions than either loqs or Ago1 alone (or together). It is possible that Dcr1, loqs and Ago1 are all required for GSC maintenance; however, in some cases, even in the absence of Loqs and AGO1, Dcr1 can collaborate with AGO2 to execute some specific miRNA functions. Recent data have shown that the Notch/Delta signal plays an important role in controlling both niche and GSC fates (Ward et al., 2006). Previous data also demonstrated that Notch signaling was negatively regulated by the miRNA pathway (Kwon et al., 2005). Therefore, it is possible that Dcr1 is not only required for GSC maintenance, but also required for some specific miRNA function to promote GSC differentiation. In Dcr1-null GSCs, the loss of certain classes of miRNAs causes GSCs to differentiate; however, the loss of different miRNAs might lead to the upregulation of Delta activity in GSCs, which in turn upregulates Notch activity in somatic cells. Conversely, as a feedback signal, overexpression of Notch in somatic cells represses or delays GSC differentiation; therefore the determination of Dcr1-null GSC fate is balanced back to normal. Hence it is likely that the miRNAs play key roles in GSC maintenance.

Importantly in this study, we showed, for the first time, that overexpression of Ago1 can potentially repress GSC/CB differentiation and result in the over-proliferation of GSC-like cells, suggesting that AGO1-dependent miRNAs play at least a partial instructive role in regulating GSC fate. Given the multiple functions of AGO1 in the miRNA pathway, the increase in GSC-like cells could be interpreted to mean that the overexpression of Ago1 probably enhances either the efficiency of specific miRNA(s) production and/or the stability of mature miRNAs to repress the transcriptional or translational activity of the target miRNAs required for the differentiation of pre-cystoblasts (pre-CBs)/CBs, thereby resulting in delayed differentiation of GSCs/CBs.

In the previous model, both BMP/Dpp-dependent bam transcriptional silencing and the bam-independent pathway are required for GSC maintenance (Chen and McKearin, 2005; Maines et al., 2007; Szakmary et al., 2005; Xi et al., 2005). Our genetic evidence suggests that the regulation of GSC self-renewal mediated by the miRNA pathway acts in a bam-silencing-independent manner. Given the role of miRNAs in translational regulation, we favor a model in which the translational control of GSC fate determination may be partially via the miRNA pathway, although the possibility remains that some selective miRNAs could directly modulate the stability of specific miRNAs required for GSC/CB differentiation. Similarly, two other groups reported that Dcr-1 and Loqs, both important components of the miRNA pathway, are also required for GSC maintenance (Jin and Xie, 2007; Park et al., 2007). The question becomes how the microRNA pathway regulates the fate of GSC. Previous and current studies showed that Dcr1, loqs and Ago1 are all not involved in bam transcriptional silencing (Hatfield et al., 2005; Park et al., 2007), suggesting that regulation of GSC fate by microRNAs does not go through a dpp-dependent bam silencing pathway. A recent study (Park et al., 2007) showed that no germ cells can differentiate in loqs and bam; however, in our study, we observed at least 10% of germ cells started to differentiate in loqs; bam double mutants (this study), as well as in loqs; bgcn double mutant ovaries (data not shown). Consistently, a similar phenotype was observed in the analysis of Ago1; bam double mutants, suggesting that Loqs and AGO1 probably act independently of Bam action.

**Fig. 6. Model of Ago1-dependent miRNAs in GSC fate determination.** In the tip of the Drosophila germarium, BMP/Dpp, as short-range signals from niche cells perceived directly by GSCs, represses bam transcription and results in Bam/Bgcn complex activity loss, thereby depressing both the Pumilio/Nanos complex (Nos, Pum) and GSC-specific miRNA activities. The Pumilio/Nanos complex and GSC-specific miRNAs could function together to repress the translation of mRNAs for GSC/CB differentiation (A), or they might function separately to repress the translation of different groups of mRNA for GSC/CB differentiation (B).
Given that the Ago1-dependent microRNA pathway plays a major role in translational control, we propose that, aside from the bam silencing pathway, the Ago1 contributes to GSC fate determination either in conjunction (Fig. 6A) or in parallel (Fig. 6B) with the pathway of translational control of Nos/Pum. Overall, our data suggest that miRNA, as an important global regulatory mechanism, plays vital roles in stem cell biology.

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