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LETTER TO THE EDITOR

Argonaute-2 regulates the proliferation of adult stem cells in planarian

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Dear Editor,

Planarian *Schmidtea mediterranea* has extraordinary regeneration capabilities due to the abundance of adult stem cells (ASCs) known as neoblasts, which make planarian a powerful *in vivo* system to study ASC biology [1]. The Argonaute (AGO) family proteins are defined by the presence of Piwi-Argonaute-Zwille (PAZ)) and PIWI domains [2], and mediate silencing via cleavage of mRNAs [3] or inhibition of translation [4]. The AGO family proteins fall into two subfamilies, one named after *Arabidopsis* Argonaute and the other after *Drosophila* PIWI [5]. In most organisms investigated so far, PIWI proteins bind Piwi-interacting RNAs (piRNAs) and are functionally involved in the regulation of germ cells [6, 7]. By contrast, AGO proteins have distinct roles in the small-RNA-mediated gene silencing pathway. In *Drosophila*, AGO1 has been illustrated to be engaged in the miRNA pathway, while AGO2 plays an important role in the siRNA-mediated gene regulation [4].

Perturbations in AGO-protein function affect stem cell properties in a variety of tissues or organisms, indicating that this protein family could be one of the most basic regulators of stem cell states [5]. The foregoing studies in *Drosophila* and *Caenorhabditis elegans* reveal that *piwi* subfamily members have important roles in germline stem cell (GSC) maintenance [8]. *Drosophila Ago1* has been demonstrated to determine the GSC fate [9] and homozygous mutants of some *Ago1* alleles in *Arabidopsis*-induced premature differentiation of the shoot apical meristem [10]. Moreover, the miRNA pathway has been integrated into the regulatory network governing the self-renewal and fate of embryonic stem cell [11, 12]. In planarian, the role of PIWI subfamily genes has been demonstrated to be crucial for the differentiation of ASCs and regeneration [13, 14]. However, the roles of AGO and miRNA pathway in the regulation of regeneration and ASC functions remain to be explored.

To investigate the function of miRNA pathway in the regulation of regeneration, we focused on the *Ago* genes. The protein sequences of human AGO2 and *Drosophila* AGO1 were used as queries to retrieve their orthologs in planarian by a tblastn search in the Expressed Sequence Tag (EST) database of *S. mediterranea* [15]. We first identified one EST sequence with the most significant similarity to queries. Then we acquired the full-length sequence corresponding to the EST using 5′ and 3′ rapid amplification of cDNA ends (RACE) (Supplementary information, Data S1). It encodes a predicted protein of 927 amino acids, which clustered to the AGO subfamily and was closely related to *Drosophila* AGO1 and human AGO2 as revealed by phylogenic analysis (Supplementary information, Figure S1A). Therefore, it was named as *smed-Ago-2* according to the nomenclature guidelines. Domain homology analysis of the predicted protein sequence of *smed-Ago-2* showed that it contains highly conserved PAZ and PIWI domains (Supplementary information, Figure S1B and S1C). The PIWI domain of SMED-AGO-2, similar to human *Ago2*, also contains the catalytic residues DDH [16] (Supplementary information, Figure S1B), which are essential for cleavage activity. In addition, we also acquired another EST sequence of which the predicted protein sequence is more similar to human AGO1 (Supplementary information, Figure S1D), and we named it *smed-Ago-1*. Taken together, we identified two *argonaute-like* genes, *smed-Ago-1* and *smed-Ago-2*, in planarian EST database (hereafter referred to in the text in short as *Ago1* and *Ago2*, respectively).

To elucidate the function of *Ago1* and *Ago2* during planarian regeneration, we performed RNA interference (RNAi) experiments in planarian (Supplementary information, Data S1). Whole-mount *in situ* hybridization (WISH) and quantitative real-time RT-PCR (qRT-PCR) showed that both *Ago1* and *Ago2* were knocked down efficiently and specifically (Supplementary information, Figure S2A and S2B). Severe regeneration defects were observed after knockdown of *Ago2* (Figure 1A). The amputated fragments of *Ago2(RNAi)* worms did not regenerate their missing body parts, in contrast to amputated *control(RNAi)* and *Ago1(RNAi)* animals in which the resulted fragments completely regenerated the missing body parts in 7 days. Most fragments of animals lysed at

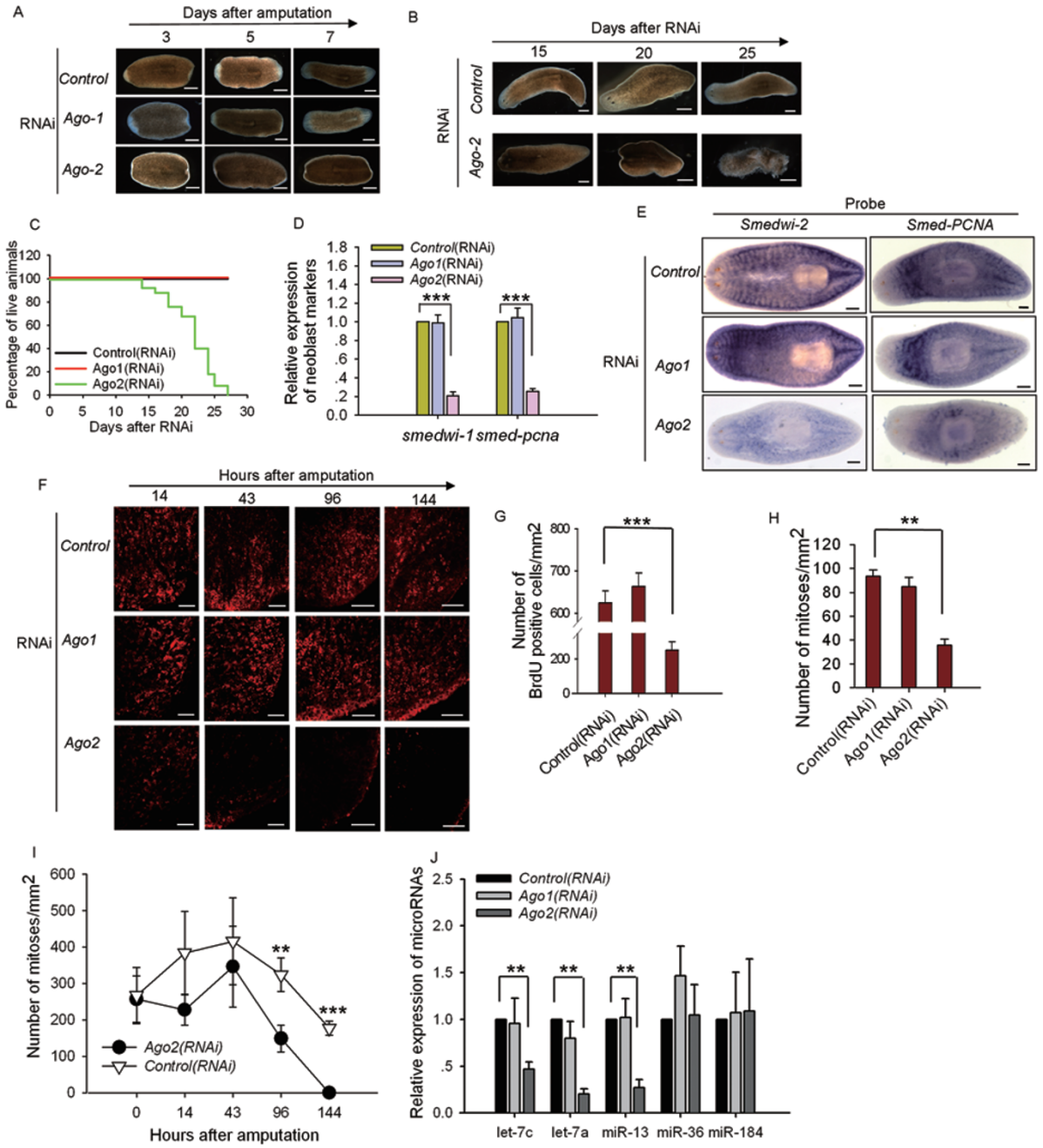

Figure 1 Argonaute-2 regulates the proliferation of adult stem cells in planarian. **(A)** Regeneration defects after knockdown of *Ago2*. Worms were amputated at 9 daf. Each panel shows a dorsal view of trunk fragments regenerating a new head and tail, with anterior at the left. Scale bar: 250 μm. **(B)** Tissue regression of intact *Ago2(RNAi)* animals. Each panel shows a dorsal view. Scale bar: 250 μm. **(C)** Survival curve. Percentage of live animals after control, *Ago1* and *Ago2* RNAi, $n = 50$. **(D)** qRT-PCR showing the relative expression level of stem cell markers in planarians at 15 daf. **(E)** Analysis of the expression of stem cell markers at 10 daf by WISH. *Smedwi-1* and *Smed-PCNA* were used as stem cell markers. Scale bar: 50 μm. **(F)** Immunofluorescence for SMEDWI-1 in amputated worms at 14, 43, 96 and 144 h after amputation. Each panel shows a dorsal view of tail fragments regenerating a new head, with anterior at the bottom. All images are single confocal sections. Scale bar: 50 μm. **(G)** Numbers of cells labeled with an anti-Brdu antibody were divided by animal surface area. All worms were examined at 15 daf. **(H)** Numbers of mitoses labeled with an anti-phospho histone 3 (H3P) antibody were divided by animal surface area. All worms were examined at 15 daf. **(I)** Numbers of mitoses labeled with anti-H3-S10P were divided by animal surface area. Animals were fed three times and amputated at 1 day after the last feeding, then animals were fixed at various time points after amputation. **(J)** qRT-PCR showing the relative expression of several miRNAs in planarians at 15 daf. **(D**, **G**-**J)** Data are means ± sd ($n = 3$ experiments, > 4 worms per time point). $^{**}P < 0.001$, $^{***}P < 0.0001$, Student's *t*-test.

14 days after initial *Ago2* RNAi feeding (daf) (data not shown). Intact *Ago2(RNAi)* worms started to display a head-regression phenotype by 15 daf, showing a characteristic sign of ASC dysfunction. Then, *Ago2(RNAi)* animals eventually lysed and all the animals died by 4 weeks after RNAi treatment (Figure 1B and 1C), while *control(RNAi)* and *Ago1(RNAi)* animals survived well. Together, these observations demonstrated that *Ago2* was required for regeneration and normal tissue homeostasis.

Since *Ago2* is required for planarian regeneration and tissue homeostasis, we explored whether it is expressed in ASCs. WISH revealed that stem cell gene, such as *smedwi-2*, was expressed in a discrete, cell-specific pattern, posterior to photoreceptors and were excluded from the pharynx (Supplementary information, Figure S3A). However, *Ago1* and *Ago2* were expressed ubiquitously, which indicated that they were expressed both in ASCs and in differentiated cells (Supplementary information, Figure S3B and S3C). Moreover, ASCs are quickly and specifically eliminated after irradiation in planarian and the genes expressed in ASCs decreased dramatically. Indeed, the expression of ASC marker *smedwi-2* was decreased after irradiation (Supplementary information, Data S1 and Figure S3D). The expression levels of *Ago1* and *Ago2* were still detectable after irradiation, especially in the differentiated tissues, but the expression levels were decreased in mesenchymal tissues (Supplementary information, Figure S3E and S3F). These results demonstrate that both *Ago1* and *Ago2* are expressed ubiquitously in planarian.

Given that the regeneration capacities and the maintenance of normal tissue turnover depend on the abundance of ASCs in planarian, we considered that the defects of regeneration and tissue turnover in *Ago2(RNAi)* worms might have resulted from the malfunction of ASCs. Then we used qRT-PCR (Supplementary information, Table S1) and WISH to detect the expression of the ASC markers (*smedwi-1*, *smedwi-2* and *smed-PCNA*), and found that the expression of these genes was decreased dramatically in *Ago2(RNAi)* animals but not in *control(RNAi)* and *Ago1(RNAi)* animals (Figure 1D and 1E). In addition, immunofluorescence of the ASC marker SMEDWI-1 was performed to assess the number of ASCs in regenerating and intact RNAi worms. After 10 days of *Ago2* RNAi, animals showed a significant decrease in the number of SMEDWI-1-positive cells and then the SMEDWI-1-positive cells were barely detectable at 15 daf (Supplementary information, Figure S4). This phenotype was observed in the regenerating worms as well. The number of SMEDWI-1-positive cells in *Ago2(RNAi)* worms was slightly less than that in *control(RNAi)* and *Ago1(RNAi)* worms at 14 h after amputation. However, the number of SMEDWI-1-positive cells gradually decreased to an undetectable level at 144 h after amputation in *Ago2(RNAi)* worms (Figure 1F), which may be the reason that the regeneration was arrested and the blastema began to regress by 5 days after amputation in *Ago2(RNAi)* worms (Figure 1A). These results suggest that *Ago2* is required for the maintenance of ASC population in both intact and regenerating animals.

The reduction of ASCs in the *Ago2(RNAi)* worms could be due to either differentiation or proliferation defects. Previous studies identified three categories of lineage-related genes, and Category 1 gene-expressing cells (ASCs) differentiate into cells expressing Category 2 and 3 genes [17]. We examined the expression of the Category 2 genes (*NB.32.1*, *NB.21.11e* and *p53*) and the Category 3 genes (*ras-related, AGAT, MCP1 and CYP1A1*) by qRT-PCR (Supplementary information, Table S1). During normal tissue turnover, the expression of stem cell progeny markers in *Ago2(RNAi)* worms was normal compared with the expression in *control(RNAi)* and *Ago1(RNAi)* worms at 10 daf in intact worms (Supplementary information, Figure S5A). By 15 daf, the expression of category 2 genes in *Ago2(RNAi)* worms was less than that in *control(RNAi)* and *Ago1(RNAi)* worms (Supplementary information, Figure S5C). As the number of ASCs in *Ago2(RNAi)* worms was barely detectable by 15 daf, the decrease in the expression of category 2 genes was possibly due to the reason that the progeny cells differentiated with no further replacement. We also examined the expression of *NB.32.1 and AGAT* by WISH (Supplementary information, Figure S5B and S5D), which further substantiated the results of qRT-PCR. These results suggest that the reduction of ASCs after knockdown of *Ago2* is not a result of ASC overdifferentiation.

In both intact and regenerating planarians, ASC proliferation constantly occurs to replenish cells lost during normal physiological turnover and injury [1]. The decrease in the number of ASCs induced by knockdown of *Ago2* suggests that *Ago2* might play a role in regulating the proliferation of ASCs. To investigate that, we performed BrdU incorporation experiments at 15 daf. The number of BrdU-positive cells in *Ago2(RNAi)* worms was much less than that in *Ago1*(RNAi) worms and *control(RNAi)* worms (Figure 1G and Supplementary information, Figure S6A), which revealed that knockdown of *Ago2* caused a dramatic reduction of ASCs in S phase.

The subset of the ASC population that undergoes mitosis can be labeled with anti-phospho-Histone H3-S10 (H3-S10P), which can be used to monitor the mitotic level in planarian. Immunostaining with anti-H3-S10P demonstrated that the number of mitotic cells was dra-

matically reduced in intact *Ago2(RNAi)* worms (35/mm^2) at 15 daf, in comparison to *Ago1(RNAi)* worms (84/mm^2) and *control(RNAi)* worms (93/mm^2) (Figure 1H and Supplementary information, Figure S6B). These results showed that knockdown of *Ago2* led to a decrease in the number of mitotic cells.

We further used flow cytometry to study the distribution of cells in G0/G1, S, G2/M phases at 11 daf in planarian (Supplementary information, Data S1). Consistent with the results of BrdU labeling experiments and immunostaining experiments with anti-H3-S10P, the percentage of cells found in S and G2/M phases in *Ago2(RNAi)* worms was significantly less than that in the *control(RNAi)* worms (Supplementary information, Figure S6C). Taken together, *Ago2* is indispensible for the proliferation of ASCs during normal tissue turnover in planarian.

In addition to the sustained proliferation in normal planarians, ASCs produce a mitotic burst after wounding [18]. We used anti-H3-S10P to detect the number of ASCs in *Ago2(RNAi)* worms at different time points after amputation. Although ASCs in *Ago2(RNAi)* worms were able to respond to wounding by slightly elevating mitotic numbers at 14 h and 43 h following amputation, the response was impaired compared with *control(RNAi)* animals. The mitotic level decreased to undetectable level in *Ago2(RNAi)* worms, while the proliferation returns to the previous steady-state levels in *control(RNAi)* and *Ago1(RNAi)* worms at 6 daf (Figure 1I and Supplementary information, Figure S7). These results indicate that *Ago2* is also essential for the proliferation of ASCs in response to wounding.

Although *Ago2* is a core component of the miRNA pathway in humans, the link between the *Ago2* phenotype and miRNAs in planarian was not illustrated. We analyzed the expression level of several miRNAs by qRT-PCR (Supplementary information, Table S1) after knockdown of *Ago2*, and the results showed that some miRNAs (let-7c, let-7a and miR-13) were influenced upon *Ago2* depletion (Figure 1J). In addition, a previous study using deep sequencing showed that some miRNA expression level is altered upon deprivation of regeneration [19]. Collectively, these results indicate that *Ago2* might mediate the miRNA pathway and be functionally involved in the regeneration process and ASC proliferation in planarian.

In conclusion, our results demonstrate that *Ago2*, but not *Ago1*, is required for regeneration and tissue turnover through regulation of ASC proliferation in planarian. Considering that *Ago2* is expressed both in ASCs and in differentiated cells, it will be interesting in future work to determine whether *Ago2* regulates ASC proliferation in an intrinsic or extrinsic way. Since *Ago2* affects the expression of several miRNAs, investigating whether planarian *Ago2* regulates miRNA-mediated gene regulation and which miRNAs are involved in regeneration will provide important insights into the function of miRNAs in stem cell biology and tissue regeneration.

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