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Reduction of germ cells in the *Odysseus* null mutant causes male fertility defect in *Drosophila melanogaster*

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Odysseus (*OdsH*) has been identified as a hybrid male sterility gene between *Drosophila mauritiana* and *D. simulans* with accelerated evolutionary rate in both expression and DNA sequence. Loss of a testis-specific expression of *OdsH* causes male fertility defect in *D. melanogaster*. Yet, the underlying mechanisms at the cellular level are unknown. In an attempt to identify the possible mechanisms and functional roles of *OdsH* in spermatogenesis, the cell numbers at different developmental stages during spermatogenesis between the *OdsH* null mutant and wild-type flies were compared. The results showed that the early developing germ cells, including spermatogonia and spermatocytes, were reduced in the *OdsH* mutant males. In addition, the number of germline stem cells in aged males was also reduced, presumably due to the disruption of germline stem cell maintenance, which resulted in more severe fertility defect. These results suggest that the function of the enhancement of sperm production by *OdsH* acted across males of all ages.

Key words: hybrid sterility gene, male-specific transcript, speciation, spermatogenesis

Odysseus-site homeobox (*OdsH*) was identified as one of the hybrid male sterility genes between *Drosophila mauritiana* and *D. simulans* by introgression and subsequently recombinant mapping (Perez et al., 1993; Perez and Wu, 1995; Ting et al., 1998). When the *D. mauritiana OdsH* allele was introgressed into *D. simulans*, misexpression of $OdsH^{mau}$ in testis caused hybrid male sterility (Perez and Wu, 1995; Sun et al., 2004). *OdsH* encodes a homeodomain protein, which contains a DNA binding domain, and has evolved rapidly at both sequence and expression levels between closely related species (Ting et al., 1998, 2004). In *D. melanogaster*, *OdsH* is a testis-specific transcript which can be detected in early spermatogenesis (Ting et al., 2004). Loss of *OdsH* expression in males caused fertility defect during the first three days after eclosion (Sun et al., 2004). Although OdsH can be found in pre-meiotic spermatocytes of *D. simulans* (Bayes and Malik, 2009), the cell-specific localization of OdsH in *D. melanogaster* is not known. The heterochromatic localization of OdsH in *D. melanogaster* and its sibling species, viz *D. mauritiana, D. sechellia*, and *D. simulans*, suggests that it functions as a heterochromatin binding protein and that the gene *OdsH* has diverged from its paralogous copy, *unc-4*, which encodes

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a transcription factor (Bayes and Malik, 2009). Meanwhile, a large number of mis-regulated genes in testes from the sterile introgression line between *D. mauritiana* and *D. simulans* suggest that the mis-expression of *OdsH* affects the spermatogenesis program (Lu et al., 2010). As most of these studies focused on the hybrid incompatibility and divergence among sibling species, which stages of spermatogenesis were affected for lack of *OdsH* expression in *D. melanogaster* is not clear. In this study, we examined the effect of the *OdsH* null mutation, $OdsH^0$, at the cellular level during early spermatogenesis to identify the functional role of *OdsH* in *D. melanogaster*.

As *OdsH* is mainly expressed in late spermatogonia and early spermatocytes (Bayes and Malik, 2009), we focus on the comparison between $OdsH^0$ and $OdsH^+$ lines at these two stages. These two lines were generated by gene targeting method and therefore shared the same genetic background except for the *OdsH* locus (Sun et al., 2004). All germ cells of 3-day old males were stained by anti-VASA antibody (Decotto and Spradling, 2005). The number of germ cells estimated by the size of the areas in both spermatogonia and spermatocytes were noticeably reduced (Fig. 1, A and B). The boundary between spermatogonia and spermatocytes (white dotted lines in Fig. 1, A and B) was determined by the changes in cell sizes

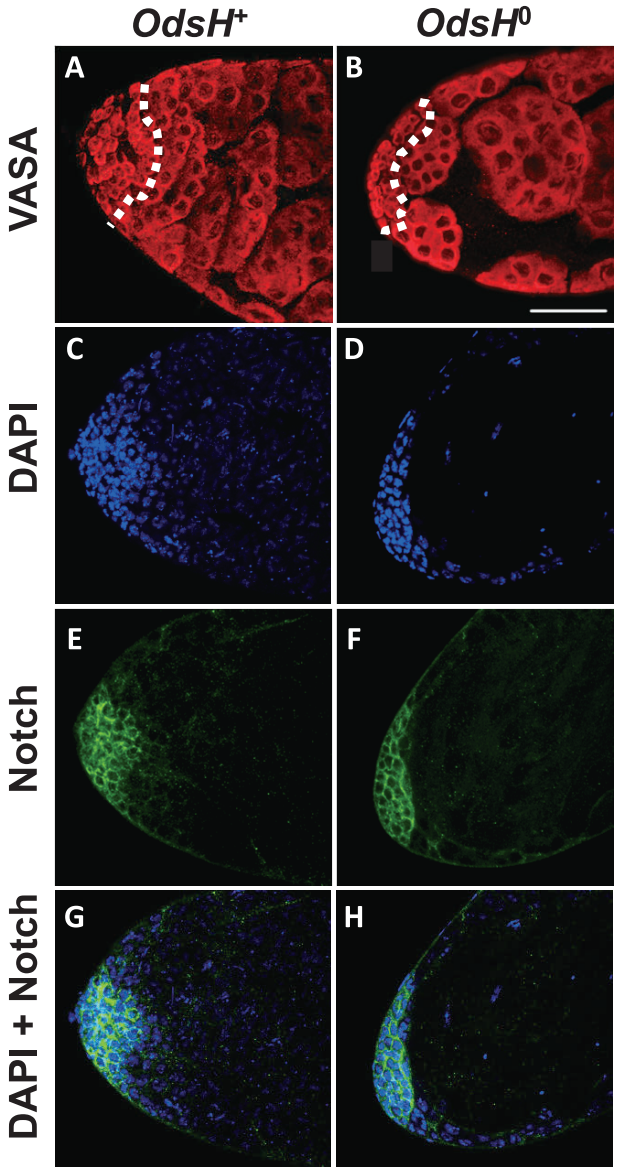

Fig. 1. Comparison of the germ cells between $OdsH^+$ and $OdsH^0$ testes. (A-B) All germ cells of $OdsH^+$ (A) and $OdsH^0$ (B) testes were labeled using anti-VASA antibody. Spermatogonia and spermatocytes were separated by dotted white lines based on the changes in cell size. (C-D) All nuclei of $OdsH^+$ (C) and $OdsH^0$ (D) testes were stained with DAPI. (E-F) The stem cells, goniablasts and early spermatogonia of $OdsH^+$ (E) and $OdsH^0$ (F) testes were labeled by anti-Notch antibody. (G-H) Merged images of anti-Notch (green) and DAPI (blue) from $OdsH^+$ (C and E) and $OdsH^0$ (D and F).

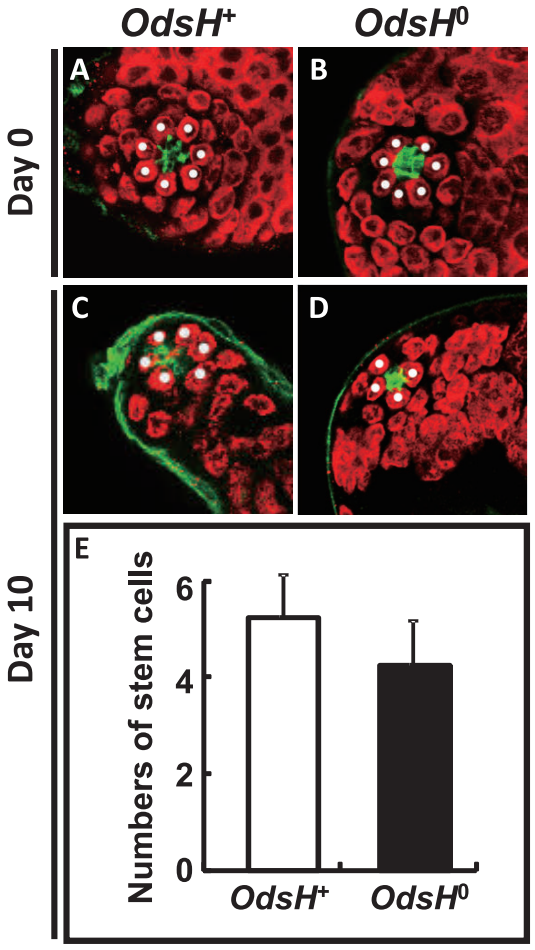

Fig. 2. Comparison of the germline stem cells between $OdsH^+$ and $OdsH^0$ testis. (A-D) The stem cells of the $OdsH^+$ (A, C) and $OdsH^0$ (B, D) testes of 0- and 10-day old males were marked with white dots. The hub cells and the germ cells were labeled with anti-Fasciclin III antibody (green) and anti-VASA antibody (red), respectively. (E) The average numbers (mean ± SE) of stem cells of the 10-day-old males between $OdsH^+$ (n = 12) and $OdsH^0$ (n = 23) are significantly different (Mann-Whitney *U* test, $P < 0.01$).

because germ cells were enlarged in the spermatocyte stage. As shown in Fig. 1B, the regions of germ cells were greatly reduced in both spermatogonia and spermatocyte stages. We then quantified the reduction in early germ cells by DAPI and anti-Notch antibody staining. With DAPI staining nuclei, the cell density was high in the apical region which comprises germline stem cells, goniablasts, and spermatogonia whereas the nuclei density became lower in the region of spermatocytes where cells increased in size (Fuller, 1993). The size of the apical region containing early germ cells was quantified by Adobe Photoshop image analysis. The apical region of the $OdsH^0$ testis was reduced more than 50% of that in the wild-type testis (Fig. 1, C and D) and the reduction was statistically significant (Mann-Whitney U test, $P < 0.01$). Using anti-Notch antibody labeling stem cells, gonialblasts and early spermatogonia (Kiger et al., 2000; Singh and Hou, 2008), we observed that the Notch positive region in the $OdsH^0$ testis was about 30% less than that in the $OdsH^+$ testis (Fig. 1, E and F). Since the late spermatogonia and stages beyond could not be labeled with anti-Notch antibody, the difference between mutant and wild-type in the Notch positive region was smaller than that in the high DAPI density area as shown in the DAPI-Notch merged photographs (Fig. 1, G and H). Taken together, the area of the early germ cells (stem cells, gonialblasts, and spermatogonia) was dramatically decreased in the mutant testis.

To further address whether the germ cell reduction is due to the difference in the number of germline stem cells, we compared the number of stem cells between *OdsH* mutant and wild-type testes. Using anti-Fasciclin III and anti-VASA antibodies to label hub cells and germ cells, respectively, the germline stem cells were recognized as the germ cells surrounding the hub cells (Hardy et al., 1979). The results showed that both $OdsH^0$ and $OdsH^+$ testes consisted of 7 stem cells when flies were just emerged, *i.e.*, 0-day old (Fig. 2, A and B), suggesting that the fertility defect in young males was unrelated to different numbers of germline stem cells. Since the number of germline stem cell declined with age (Cheng et al., 2008), the numbers of germline stem cell were examined in aged males (10-day old). Only 3–5 cells were observed in $OdsH^0$ testes while 4–6 stem cells could be seen in the $OdsH^+$ testes in aged males (Fig. 2, C and D). The differences in the stem cell numbers between the two genotypes were statistically significant (Mann-Whitney U test, $P < 0.01$; Fig. 2E). These results suggest that causes of fertility reduction in young and aged $OdsH^0$ males are different. The fertility reduction in young males is mainly owing to the reduction in the number of developing germ cells but not of stem cell during spermatogenesis. On the other hand, the reduction in both germline stem cells and developing germ cells led to the low fertility in aged males.

Since reduction in germline stem cells were observed in aged flies, we then asked if older males also suffered from fertility defect. To address this question, the daily fertility differences between $OdsH^+$ and $OdsH^0$ males were measured by the total offspring numbers of three w^{1118} females mated with a single male each day for 10 consecutive days after eclosion. Nineteen males of each genotype were used in this assay. The results showed that the daily fertility of $OdsH^+$ males was significantly higher than that of $OdsH^0$ males for all 10 days (Student's t-test, $df = 36$, $P < 0.001$ for all 10 comparisons; Fig. 3). The total number of offspring produced by $OdsH^0$ males was 40% to 50% of that produced by $OdsH^+$ males from day 1 to day 3. The fertility reduction in $OdsH^0$ males aged more than 3-day-old was more severe, and the total number of offspring produced by $OdsH^0$ was only 20% to 30% of that produced by wild-type males. The result on the male fertility is in concert with our finding at the cellular level.

OdsH was classified as a dispensable gene as the only phenotype detected in the *OdsH* knockout strain was reduction in male fertility (Sun et al., 2004). The sperm-exhaustion fertility assay from day 2 to day 4 showed that the male fertility of $OdsH^0$ was reduced for all three days and it is statistically significant for days 2 and 3. It was proposed that the function of *OdsH* is probably the enhancement of the fertility of young males. In this study, we showed that the declines in germ cells were not limited to the young males but extended to the aged males. The reduction in germline stem cells in aged (10-day-old) mutant flies (Fig. 2) motivated us to compare the 10-day fertility between mutant and wild-type flies. It shows that males suffer fertility defect from day 1 to day 10 (Fig. 3), which is concordant with the result of Sun et al. (2004). Our new findings suggest that the function of

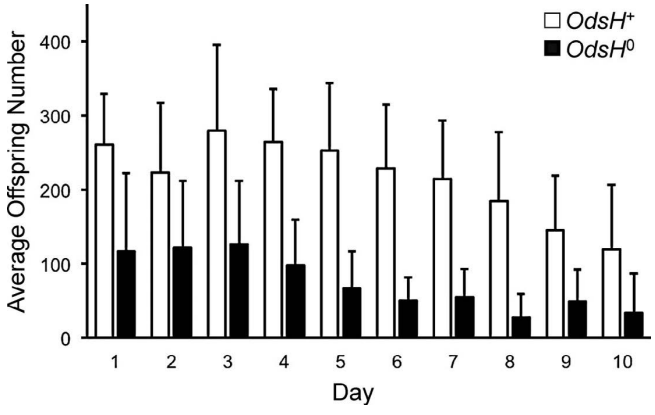

Fig. 3. Fertility comparison between $OdsH^+$ and $OdsH^0$ males. The daily fertility was calculated by averaging offspring numbers (mean ± SE, Y-axis) of 19 tested males for 10 consecutive days after eclosion (X-axis). All 10 comparisons between the two genotypes were significantly different (Student's t-test, $df = 36$, $P < 0.001$).

OdsH is to enhance sperm production not only in young males but also for males in all ages.

By characterizing mutant phenotype at the cellular level, we show that the fertility reduction in *OdsH* mutant males is because of the reduction in developing germ cells and additional loss of germline stem cells in aged males. The rapidly evolving OdsH has gained a new function to bind the heterochromatin region and result in the chromosome decondensation (Bayes and Malik, 2009). *OdsH* transcripts could be detected in the late spermatogonia and early spermatocyte stages in *D. melanogaster* (Ting et al., 2004). Although OdsH is expressed in the pre-meiotic spermatocytes in *D. simulans* (Bayes and Malik, 2009), the cell-specific localization of OdsH in *D. melanogaster* is still unknown. Based on the RNA expression pattern, it is possible that OdsH is expressed in both the late spermatogonia and spermatocyte stages. If this is the case, lack of OdsH in the null mutant testis would affect the decondensation in the heterochromatic regions, leading to the prolonged mitotic stages of the early germ cells. As a consequence, the cell division rate decreases, and fewer functional sperms are produced in the mutant males. Further studies will be required to elucidate its underlying molecular mechanism.

The reduction in male fertility of the *OdsH* null mutant becomes more severe when the flies are aged over 3-day old due to the loss of germline stem cells. The number of germline stem cells is known to be maintained either by self-renewal or by the reversion of differentiating goniablast or spermatogonia cells (Wong et al., 2005; Davies and Fuller, 2008; de Cuevas and Matunis, 2011). The fact that the germline stem cells of the aged testes are fewer in the *OdsH* mutant males than in the wildtype males suggests that the maintenance of germline stem cells is disrupted in some way while lacking *OdsH* expression. How *OdsH* interacts with genes involved in the stem cell maintenance remains to be further investigated. In conclusion, loss of *OdsH* expression in *D. melanogaster* males causes fertility defect, and the reduction in fertility is mainly attributed to the reduction of germ cell number in early spermatogenesis. These results provide a foundation in understanding the molecular function of *OdsH* in *D. melanogaster*.

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