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Stem Cell Aging Is Controlled Both Intrinsically and Extrinsically in the *Drosophila* Ovary

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SUMMARY

It is widely postulated that tissue aging could be, at least partially, caused by reduction of stem cell number, activity, or both. However, the mechanisms of controlling stem cell aging remain largely a mystery. Here, we use *Drosophila* ovarian germline stem cells (GSCs) as a model to demonstrate that age-dependent decline in the functions of stem cells and their niche contributes to overall stem cell aging. BMP signaling activity from the niche significantly decreases with age, and increasing BMP signaling can prolong GSC life span and promote their proliferation. In addition, the age-dependent E-cadherin decline in the stem cell-niche junction also contributes to stem cell aging. Finally, overexpression of SOD, an enzyme that helps eliminate free oxygen species, in either GSCs or their niche alone can prolong GSC life span and increase GSC proliferation. Therefore, this study demonstrates that stem cell aging is controlled extrinsically and intrinsically in the *Drosophila* ovary.

INTRODUCTION

Efficient tissue repair is critical for maintaining tissue function and deterring the aging process. Adult stem cells/progenitor cells are responsible for producing new cells to replace worn-out ones. The age-dependent decline in the activities and/or number of adult stem cells has been observed in several systems. Using GSC transplantation, it has been demonstrated that the GSC number or function in the mouse testis declines as the age of male mice increases (Ryu et al., 2006). In the hematopoietic system, although the hematopoietic stem cells (HSCs) are more frequently found in older mice, their ability to home to and engraft the bone marrow is significantly diminished, suggesting that there is a functional reduction in the aged HSCs (Morrison et al., 1996). In the central nervous system, the hematopoietic system, and the pancreas, aging leads to upregulation of $p16^{INK4a}$, a gene responsible for cell senescence, while the deletion of this gene increases the proliferation capacity and prolongs the life span of stem cells (Krishnamurthy et al., 2006; Molofsky et al., 2006). However, the molecular and cellular mechanisms underlying stem cell aging remain largely unexplored.

Germline stem cells (GSCs) in the *Drosophila* ovary represent an attractive system for studying the intimate relationships between stem cells and their niche (Lin, 2002; Xie et al., 2005). Well-defined stem cells and niche structure and the availability of abundant mutants also constitute an advantage for genetically dissecting molecular pathways that control stem cell aging in the *Drosophila* ovary. In the *Drosophila* ovary, cap cells, possibly along with escort stem cells (ESCs), form a functional GSC niche (Decotto and Spradling, 2005; Xie and Spradling, 2000), and the niche-expressing Dpp/BMP2-4, Gbb/BMP5-8, and PIWI are essential for controlling GSC self-renewal (Cox et al., 2000; Song et al., 2004; Xie and Spradling, 1998). The niche signals directly act on the GSCs to repress the expression of *bag-of-marbles* (*bam*), a GSC differentiation-promoting gene (Chen and McKearin, 2003; Song et al., 2004). The GSCs and their niche are intimately connected by E-cadherin-rich adherens junctions. It is known that E-cadherin expressed by both GSCs and the niche is essential for GSC anchorage in the niche and long-term self-renewal (Song et al., 2002).

Reactive oxygen species (ROS), the by-products of oxidative energy metabolism, have long been suggested to be a main cause of aging in different organisms, including *Drosophila* (Tower, 2000). One of the well-known antioxidant enzymes is superoxide dismutase (SOD), a copper/zinc-dependent superoxide dismutase, which helps remove ROS. *SOD* overexpression can extend life span in *Drosophila* (Klichko et al., 1999; Orr and Sohal, 1994; Parkes et al., 1998; Sun et al., 2002; Tower, 2000). In this study, we show that *SOD* overexpression can also deter the aging of stem cells and their niche in the *Drosophila* ovary.

One previous study shows that the number of cap cells undergoes very limited decline at 5 weeks of age, but the contribution of cap cell reduction to stem cell aging has not been directly tested (Xie and Spradling, 2000). Similarly, it has been shown recently that hub cells (niche cells) and GSCs decline with age in the *Drosophila* testis (Wallenfang et al., 2006). However, it remains largely unclear how changes in the niche structure and function and inside stem cells themselves with age contribute to stem cell aging. In this study, we have investigated how niche and intrinsic stem cell aging contribute to age-related decline of stem cell number and proliferation.

RESULTS

The Number and Functions of GSCs and Niche Cells Undergo Age-Related Decline

Although cap cells and escort cells (ESCs) are shown to form the niche for the GSCs in the *Drosophila* ovary, here we only used cap cell number to study the niche structure change with age because cap cells are a reliably identifiable niche component but ESCs are not (Decotto and Spradling, 2005; Xie and Spradling, 2000). Cap cells can be reliably identified according to their location (between terminal filament and GSCs), size (the smallest cells in the tip of the germarium), and molecular markers such as lamin C (Xie and Spradling, 2000). The two or three GSCs in a niche are identified by their anteriorly anchored spectrosome and direct contact with cap cells (Figures 1A and 1B). The spectrosomes in GSCs and cystoblasts can be labeled with a monoclonal anti-Huli tai-shao (Hts) antibody. In contrast with its special location inside the GSC, the spectrosome is randomly distributed in the cytoplasm of the cystoblast. In developing cysts, the fusome becomes branched and is still labeled by Hts expression (Lin et al., 1994).

The numbers of cap cells and GSCs in the germaria were determined at 7 days, 21 days, 35 days, 49 days, and 63 days after eclosion. The 7-day-old control germaria carried 6.35 ± 0.92 cap cells (n = 60) and 2.62 ± 0.56 GSCs (n = 60) (Figures 1B and 1C). In the 63-day-old germaria, there were 5.33 ± 1.07 cap cells ($p < 0.005$; n = 60) and 1.90 ± 0.48 GSCs ($p < 0.005$; n = 60), which both are significantly less than those of the 7-day-old germaria. At the age of 63 days, the majority of the germaria had one or two GSCs (Figures 1D and 1E). To facilitate comparisons between controls and experimental genotypes, we normalized cap cells and GSCs to 100% in the 7-day-old germaria for the controls and experimental genotypes. Compared to 7-day-old germaria, 83.9% of cap cells and 72.5% of GSCs were maintained in the 63-day-old germaria (Figures 1F and 1G). These results demonstrate that the numbers of niche cells and GSCs indeed significantly decline with age. This is consistent with the recent observation that *Drosophila* testicular GSCs and their niche cells undergo an age-related decline in numbers (Wallenfang et al., 2006).

In addition to the age-dependent decline in stem cell number, it remains unclear whether or not GSC proliferation capacity also declines with age. Because every GSC division generates a differentiated cyst, the number of cysts in a given germarium can be used as an indicator of the GSC proliferation rate, given that there is no obvious increase in cyst apoptosis with age (based on condensed chromatin revealed by DAPI staining). To facilitate the analysis, we quantified cyst numbers only in the germaria containing two GSCs for subsequent comparisons of cyst numbers because the germaria carrying two or three GSCs could potentially have different numbers of cysts. In the control, the 63-day-old germaria contained 8.5 ± 1.6 cysts, which is significantly less than the 10.7 ± 1.5 of the 7-day-old ones (Figure 1H), indicating that GSC proliferation capacity also decreases with age. The results from our later BrdU labeling experiments also confirm the idea that GSC proliferation capacity declines with age (Table 1). Therefore, GSC aging is composed of age-related decreases in GSC persistence in the niche and proliferation capacity.

The Compromised BMP Signal Production from the Aged Niche Contributes to GSC Aging

gbb and *dpp* are expressed in the niche and encode BMP-like proteins essential for GSC self-renewal (Song et al., 2004; Xie and Spradling, 1998). To investigate the possibility that weakening BMP signaling in the aged niche contributes to GSC aging, we examined heterozygous mutant *dpp* and *gbb* females to investigate the effect of partial removal of *dpp* and *gbb* gene function on GSC aging by using two moderate *dpp* alleles, dpp^{hr56} and dpp^{hr4}, and one strong or null *gbb* allele, gbb^{D4}. The 7-day-old heterozygous *dpp* and *gbb* mutant germaria had normal or close to the normal numbers of GSCs, suggesting that GSCs form normally in the *dpp* or *gbb* heterozygous females. For example, the 7-day-old dpp^{hr4} and gbb^{D4} heterozygous germaria respectively had 2.63 ± 0.58 GSCs ($p = 0.25$; n = 60) and 2.72 ± 0.56 GSCs ($p = 0.25$; n = 60), which are not significantly different from the 7-day-old control germaria (2.62 ± 0.56 GSCs) (Figures 1I and 1J). By contrast, the number of GSCs in the 63-day-old dpp^{hr56}, dpp^{hr4}, and gbb^{D4} heterozygous germaria only accounts for 39.5% ($p < 0.0001$; n = 60), 33.1% ($p < 0.0001$; n = 60), and 40.4% ($p < 0.0001$; n = 60) of that of the 7-day-old germaria, respectively, which is significantly different from the 72.5% for the control (Figure 1F). This indicates that partial reduction of the BMP signal from the niche significantly enhances age-related GSC loss. Consequently, most of these 63-day-old *dpp* and *gbb* heterozygous mutant germaria had one or no GSC (Figures 1K and 1L). As for cyst production, the 35-day-old and 63-day-old dpp^{hr4} and gbb^{D4} heterozygous germaria carrying two GSCs contained significantly fewer cysts than the control germaria of the same ages (Figure 1H). These results indicate that partial reduction of BMP signaling also enhances age-related decline in GSC proliferation. Interestingly, the maintenance rates for the cap cells in the 63-day-old *dpp* (dpp^{hr56}, 88.0%; dpp^{hr4}, 85.7%) and *gbb* (gbb^{D4}, 92.5%) heterozygous germaria were similar to that of the control germaria (83.9%) (those p values > 0.12), indicating that

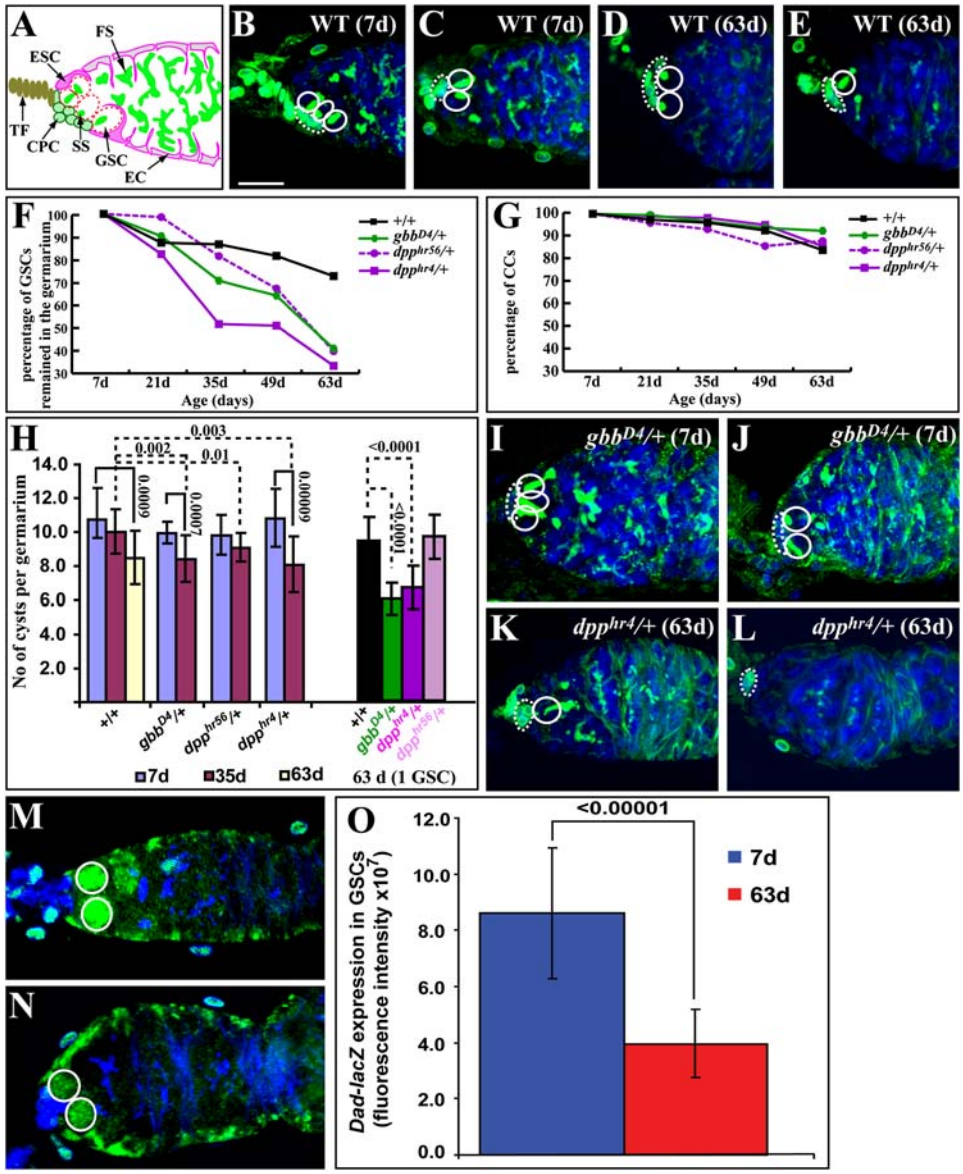

Figure 1. Partial Interference of BMP Signaling Does Not Affect GSC Formation but Has Severe Consequences on GSC Aging

(A) A schematic diagram of a germarial tip showing different cell types in the proximity of GSCs. Abbreviations: TF, terminal filament (dark yellow); CPC, cap cells (light green); ESC, escort stem cells (purple); GSC, germline stem cells (highlighted by broken red lines); EC, escort cells (light purple); SS, spectrosome (green, round or elongated); and FS, fusomes (green, branched). All the germaria in (B)–(E) and (I)–(L) are labeled for lamin C (TFs and cap cells, green), Hts (spectrosome and fusome, green), and DNA (blue). All the genotypes described in this figure, including the wild-type control, are in the *hh-lacZ* heterozygous background.

(B and C) Seven-day-old control germarial tips showing three (B) and two (C) GSCs (solid circles) attached to the cap cells (highlighted by broken lines).

(D and E) Sixty-three-day-old wild-type germarial tips showing two (D) and one (E) GSCs (solid circles) attached to the cap cells (highlighted by broken lines).

(F and G) Graphs showing that the GSC (F) or cap cell (G) maintenance rates for the control and heterozygous mutants for the BMP pathway (percentages, the y axis) change with age (days, shown in the x axis). The GSC (F) or cap cell (G) numbers for the 7-day-old control and heterozygous mutant for *dpp* and *gbb* are normalized to 100% for comparison. The GSC (F) or cap cell (G) maintenance rate for a given genotype at a given time point is determined by the average GSC or cap cell number for the genotype at the time point divided by that for the 7-day-old germaria of the same genotype. All the subsequent graphs are generated using the same method.

(H) The bar graph showing the changes in the numbers of cysts per germarium (y axis) in the two-GSC-containing germaria of different genotypes (+/+ control, $gbb^{D4/+}$, $dpp^{hr56/+}$, and $dpp^{hr4/+}$) with age (7 days, blue; 35 days, purple; and 63 days, yellow). Because there were not enough *dpp* and *gbb* mutant heterozygous germaria containing two GSCs at the age of 63 days, we compared the cyst numbers in the 63-day-old germaria containing one GSC between the control and mutants. p values that are less than 0.05 between two comparisons (solid lines, among the same genotypes; broken lines, between different genotypes) are indicated, while 15 germaria are examined for each genotype at a given time point.

Table 1. Increasing BMP Signal Transduction and SOD Overexpression Can Promote Proliferation of the Aged GSCs

	Age		
Genotypes	7 days	35 days	63 days
hh-gal4 UAS-gal80^{ts}/+	86.7%[a] (100%[b])	56.7% (65.4%)	36.7% (42.3%)
UASp-gbb/hh-gal4 UAS-gal80^{ts}	70.0% (100%)	60.0% (85.7%)	50.0% (71.4%)
nos-gal4/+	66.7% (100%)	70.0% (105.0%)	16.7% (25.0%)
UASp-Sax/nos-gal4*	46.7% (100%)	43.3% (92.9%)	26.7% (57.1%)
UASp-SOD-2/nos-gal4	63.3% (100%)	50.0% (78.9%)	43.3% (68.4%)
UASp-SOD-1/nos-gal4	60.0% (100%)	46.7% (77.8%)	43.3% (72.2%)
hh-gal4/+	90.0% (100%)	90.0% (100%)	43.3% (48.1%)
UASp-SOD-1/hh-gal4	63.3% (100%)	60.0% (94.7%)	46.7% (73.7%)
UASp-SOD-2/hh-gal4	70.0% (100%)	60.0% (85.7%)	46.7% (66.7%)
UASp-SOD-1/+	81.8% (100%)	50.0% (61.1%)	33.3% (40.7%)
UASp-SOD-2/+	81.8% (100%)	73.3% (89.6%)	36.7% (44.8%)

[a] Percentage of BrdU-positive GSCs for a given genotype at a particular age is determined by the number of BrdU-positive GSCs divided by the number of total GSCs examined. Because the GSCs divide differently in the germaria carrying different numbers of GSCs, we have only analyzed the germaria carrying two GSCs for reliable comparisons. For each time point, we analyzed 15 germaria (30 GSCs).
[b] To facilitate comparisons among different age groups, we have normalized the percentage at the first time point (7 days) to 100%, and the percentages at two later time points (35 and 63 days) are normalized to the first time point.

compromising niche signal production does not significantly enhance age-related cap cell decline (Figure 1G). Taken together, these results suggest that a moderate decrease in BMP dosage enhances stem cell aging but has little effect on GSC formation and age-related niche structural changes.

To determine whether BMP signaling activity in GSCs undergoes the age-related decreases as the above experiments suggested, we examined the expression of *Dad-lacZ*, an indicator of BMP signaling activity, in the *Dad-lacZ*/+ young (7-day-old) and old (63-day-old) ovaries. *Dad* is a direct BMP target gene and can negatively regulate BMP signaling in *Drosophila* (Tsuneizumi et al., 1997), while expression of a *Dad-lacZ* reporter can recapitulate BMP signaling activity in the ovarian GSCs (Casanueva and Ferguson, 2004; Kai and Spradling, 2003; Song et al., 2004) (Figure 1M). As expected, in comparison with the 7-day-old GSCs (Figure 1M), *Dad-lacZ* expression significantly decreased in the 63-day-old GSCs (Figures 1N and 1O). Thus, these results further support the idea that BMP signaling activity in the GSC undergoes an age-dependent decline.

We then used *hh-gal4*-driven *UAS-dpp* or *UAS-gbb* expression in cap cells to test whether moderate overexpression of *dpp* and *gbb* in the niche cells could slow down GSC aging. Because *hh-gal4*-driven *UAS-dpp* expression leads to embryonic lethality (data not shown), we used *hh-gal4* together with *UAS-gal80^{ts}* (a temperature-sensitive version of *gal80*) to temporally control *dpp* expression since GAL80^{ts} is functional in repressing the UAS-containing promoter at 18°C but not fully functional at room temperature (Lee and Luo, 1999). The control germaria carrying either *hh-gal4 UAS-gal80^{ts}* or *UAS-dpp* at different ages behaved similarly to the control used in earlier experiments, and most of the 63-day-old germaria still had one or two GSCs (Figures 2A and 2C). Because *dpp* overexpression causes GSC-like tumor formation (Song et al., 2004; Xie and Spradling, 1998), the germaria of the *hh-gal4 UAS-gal80^{ts}/UAS-dpp* females raised at room temperature did not accumulate GSC-like cells, and thus likely had moderate levels of *dpp* expression. Surprisingly, the germaria of the 63-day-old *hh-gal4 UAS-gal80^{ts}/UAS-dpp* females cultured at room temperature carried only 45.5% of the GSCs and 49.7% of the cap cells in the 7-day-old germaria, which is significantly lower than those for the control (both p values < 0.0001; Figures 2A, 2B, and 2D), suggesting that continuous exposure to higher levels of *dpp* has adverse effects on the maintenance of niche cells and GSCs. In mammalian systems, accelerated stem cell proliferation leads to early depletion of the stem cell pool,

(I and J) Seven-day-old *gbb$^{D4/+}$* germarial tips showing three (I) and two (J) GSCs (solid circles) attached to the cap cells (highlighted by broken lines).
(K and L) Sixy-three-day-old *dpp$^{hr4/+}$* germarial tips showing one (K) (solid circle) or no (L) GSC attached to the cap cells (highlighted by broken lines).
(M and N) Seven-day-old (M) and 63-day-old (N) *Dad-lacZ*/+ germarial tips showing that *Dad-lacZ* expression is lower in the older GSCs (circles) than in the younger GSCs (circles).
(O) A bar graph showing that the 63-day-old GSCs (red) have significantly lower *Dad-lacZ* expression than the 7-day-old GSCs. The error bars represent standard derivation calculated using EXCEL. All the micrographs represent overlayed multiple confocal sections and are shown in the same scale, and the scale bar at (B) represents 10 μm.

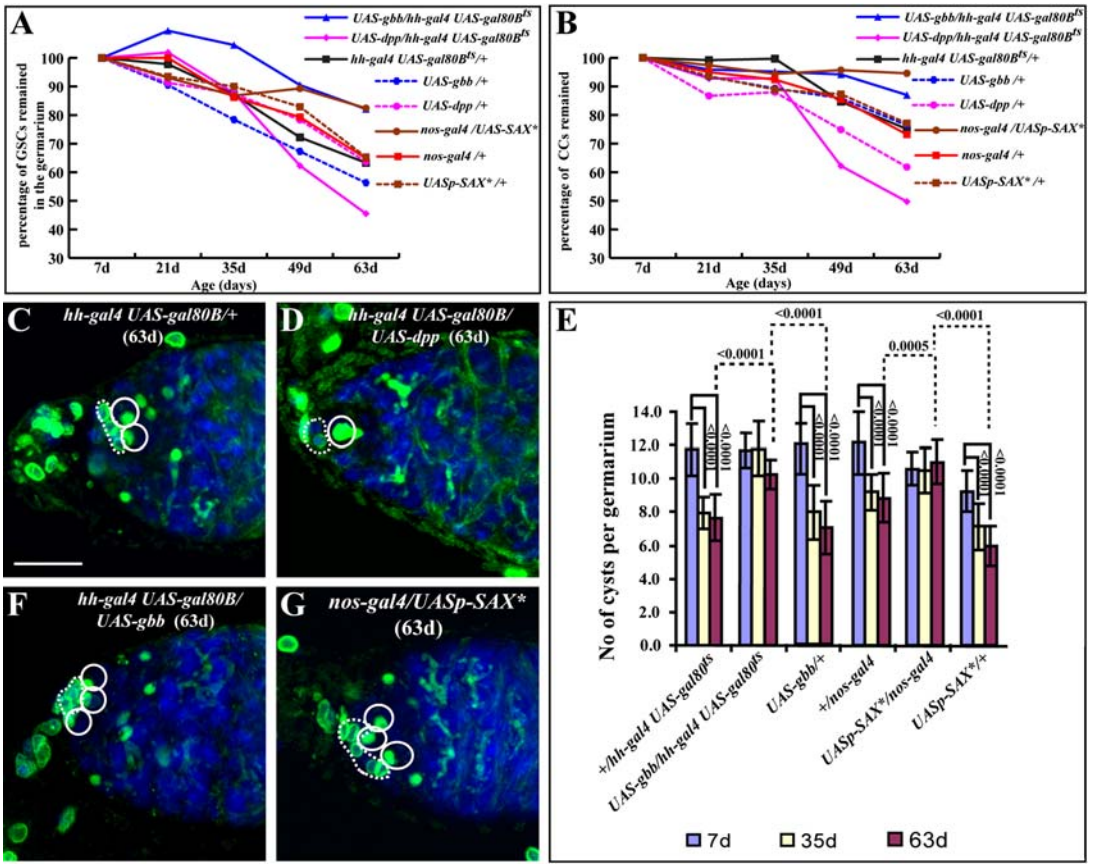

Figure 2. Strengthening BMP Signaling in Aged Ovaries Can Slow Down GSC Aging

(A and B) Graphs showing that the GSC (A) or cap cell (B) maintenance rates for the controls (*hh-gal4 UAS-gal80^{ts}*/+ and *nos-gal4*/+) and *gbb*-, *dpp*-, and *SAX**-overexpressing ovaries (percentages, the y axis) change with age (days, shown in the x axis). All the germaria in (C), (D), (F), and (G) are labeled for lamin C (TFs and cap cells, green), Hts (spectrosome and fusome, green), and DNA (blue).

(C) A 63-day-old *hh-gal4 UAS-gal80^{ts}*/+ control germarial tip showing two GSCs (solid circles) in the niche (broken lines).

(D) A 63-day-old *hh-gal4 UAS-gal80^{ts}*/+; *UAS-dpp*/+ germarial tip showing one GSC (solid circle) in the niche (broken lines).

(E) The bar graph showing the changes in the numbers of cysts per germarium in the two-GSC-containing germaria of different genotypes and ages (7 days, blue; 35 days, yellow; and 63 days, purple). p values that are less than 0.05 between two comparisons (solid lines, among the same genotypes; broken lines, between different genotypes) are indicated, while 15 germaria are examined for each genotype at a given time point.

(F) A 63-day-old *hh-gal4 UAS-gal80^{ts}*/+; *UAS-gbb*/+ germarial tip showing three GSCs (solid circles) in the niche (broken lines).

(G) A 63-day-old *nos-gal4*/+; *UASp-SAX**/+ germarial tip showing three GSCs (solid circles) in the niche (broken lines). All the micrographs represent overlayed multiple confocal sections and are shown in the same scale, and the scale bar at (C) represents 10 μm.

which is thought to be a built-in protection mechanism for preventing tumorigenesis (Li and Xie, 2005). Although the underlying mechanism remains unclear in this case, we suspect that this is a similar protection mechanism for GSCs since Dpp is a potent mitogenic factor for GSCs (Song et al., 2004; Xie and Spradling, 1998).

Because Gbb is also a niche signal for GSC self-renewal (Song et al., 2004), we used the same strategy to overexpress *gbb* in the niche cells to test its potential in preventing stem cell aging. Similarly, the 7-day-old *hh-gal4 UAS-gal80^{ts};UAS-gbb* germaria had normal numbers of cap cells (6.92 ± 1.04) and GSCs (2.98 ± 0.60), indicating that moderate *gbb* overexpression does not have any obvious effect on GSC and niche formation. In contrast with the 63-day-old *UAS-gbb*/+ control germaria that had the maintenance rate of 76.5% for cap cells and 56.3% for GSCs, the maintenance rates significantly increased to 87.0% for cap cells and 82.2% for GSCs in the 63-day-old *hh-gal4 UAS-gal80^{ts};UAS-gbb* germaria (both p values < 0.0001; n = 60; Figures 2A and 2B). Those 63-day-old *gbb*-overexpressing germaria still had two or three GSCs (Figure 2F). For cyst production, the 63-day-old *gbb*-overexpressing germaria carried significantly more cysts than the 63-day-old control germaria (Figure 2E), indicating that Gbb overexpression can promote GSC proliferation. To further test the idea, we used BrdU labeling to detect the GSCs in the S phase of the cell cycle. In the controls, the number of the BrdU-labeled GSCs declined with age, supporting our conclusion that GSC proliferation declines with age (Table 1). Interestingly, *gbb* overexpression did not affect the BrdU labeling rate at young ages; however, there were significantly more

BrdU-positive GSCs in the 63-day-old *gbb*-overexpressing germaria than the controls of the same age (Table 1). These results demonstrate that *gbb* overexpression in the aged niche can decrease age-related GSC loss (or prolong GSC persistence in the niche or life span) and promote proliferation of the aged GSCs.

Increased BMP Signaling Activity in Aged GSCs Can Enhance Their Life Span and Proliferation

To further test whether niche-expressed Gbb/BMP directly acts on GSCs to slow down stem cell aging, we overexpressed activated *Drosophila* type I BMP receptors to boost BMP signaling intrinsically in GSCs. TKV and SAX are BMP type I receptors that work synergistically to transduce the BMP signal in *Drosophila*, but TKV plays a more important role than SAX (Haerry et al., 1998; Nellen et al., 1994; Neul and Ferguson, 1998; Penton et al., 1994). Germline-specific expression of an active form of TKV (TKV*) leads to pronounced repression of cystoblast differentiation (Casanueva and Ferguson, 2004), which prevented us from using it in this study. In order to moderately stimulate BMP signaling specifically in the germline without interfering with cystoblast differentiation, we generated a transgenic line, *UASp-SAX** (an active form of *sax*, *SAX**, under the control of the *UASp* promoter), whose expression in the germline can be achieved in combination with *nos-gal4* (Rorth, 1998; Van Doren et al., 1998). The *nos-gal4*-driven *UASp-SAX** expression did not prevent cystoblast differentiation but increased the size of GSC-like tumors caused by *TKV** expression, indicating that SAX* expression can enhance BMP signaling (G.C., S.C., and T.X., unpublished data). The controls (*nos-gal4*/+ or *UASp-SAX**/+) behaved like the other controls regarding the GSC and cap cell maintenance rates during the period of 63 days (Figures 2A and 2B). The 7-day-old germaria overexpressing *SAX** specifically in the germline had a normal number of cap cells (6.53 ± 0.98) and GSCs (2.65 ± 0.58), indicating that *SAX** overexpression in the germline does not affect formation of the niche and GSCs. Consistent with the *gbb* overexpression results, 82.3% of the GSCs (2.18 ± 0.68; n = 60) and 94.6% of cap cells (6.18 ± 0.95; n = 60) detected at 7 days of age remained in the 63-day-old *SAX**-overexpressing germaria, and these maintenance rates were significantly higher than those of the controls (both p values < 0.0001) (Figures 2A and 2B). Consequently, the SAX*-overexpressing germaria carried two or three GSCs (Figure 2G). Note that preventing GSC loss in the aged niche also benefits niche maintenance, which is consistent with recent findings that the GSCs can signal back to the niche cells for controlling their maintenance (Song et al., 2007; Ward et al., 2006).

For cyst production, the *nos-gal4*/+ or *UAS-SAX**/+ control germaria also showed the age-related decline like the other controls (Figure 2E). In contrast, the number of cysts in the 63-day-old *SAX**-overexpressing germaria was significantly higher than that of the 63-day-old control germaria (Figure 2E). These results are also further supported by our BrdU labeling results. There were more BrdU-positive GSCs in the 63-day-old *SAX**-overexpressing germaria than in the controls of the same age (Table 1). Corroborating the *gbb* overexpression results, these data establish that a limited increase in BMP signaling in the aged GSCs can significantly slow down aging of stem cells and niche cells and promote stem cell proliferation.

Strengthening Physical Interactions between GSCs and Their Niche Can Prolong GSC Life Span

Because E-cadherin-mediated cell adhesion is essential for GSC anchorage in the niche (Song et al., 2002), one would expect that any change in expression or function of E-cadherin could have a consequence on stem cell persistence in the niche or life span. We first examined whether or not partial reduction of E-cadherin has any effect on GSC aging by studying GSC and cyst numbers in the germaria from heterozygous *shg* mutant females at different ages. *shg* encodes a *Drosophila* E-cadherin; shg^{10469} and shg^{R69} represent hypomorphic and null *shg* mutant alleles, respectively (Tepass et al., 1996; Uemura et al., 1996). As expected, the 7-day-old heterozygous shg^{10469} and shg^{R69} mutant germaria had normal numbers of cap cells (5.38 ± 1.03 for shg^{10469}; 5.48 ± 0.75 for shg^{R69}) and GSCs (2.55 ± 0.54 for shg^{10469}; 2.57 ± 0.53 for shg^{R69}), suggesting that niche cells and GSCs form normally in the *shg* heterozygous females. In contrast, only 49.0% and 54.5% of the GSCs detected in the 7-day-old germaria were maintained in the 63-day-old shg^{10469} and shg^{R69} heterozygous germaria, respectively, which was significantly lower than that for the control at the same age (72.5%) (Figures 3A and 3B). The 63-day-old *shg* heterozygous mutant germaria had one or two GSCs (Figures 3C and 3D). As for GSC proliferation, the cyst numbers in both 63-day-old heterozygous shg^{10469} and shg^{R69} mutant germaria were significantly lower than that of the 63-day-old control germaria (Figure 3E), indicating that partial reduction of E-cadherin expression enhances the age-related decline in GSC proliferation. This suggests that E-cadherin may help enhance, or be directly involved in, niche signaling for promoting GSC proliferation, which requires further investigation. Meanwhile, the cap cell maintenance rates for shg^{10469} and shg^{R69} heterozygotes were not significantly different from the control, suggesting that the decline in cap cell number in *shg* mutant heterozygotes could not be responsible for a significant decline in GSC number (Figure 3B). These results suggest that the E-cadherin-mediated cell adhesion may be weakened in the aged ovary.

To determine whether E-cadherin accumulation in the stem cell-niche junction indeed declines with age, we compared E-cadherin expression in the junction between the 7-day-old and 63-day-old wild-type ovaries. In comparison with the 7-day-old germaria (Figure 3F), the E-cadherin accumulation in the junction had more than a 3-fold decrease in the 63-day-old germaria (Figures 3G and 3H). Thus, these results show that E-cadherin accumulation between GSCs and their niche dramatically declines in an age-dependent manner, which supports the idea that the adhesion between GSCs and the niche declines with age.

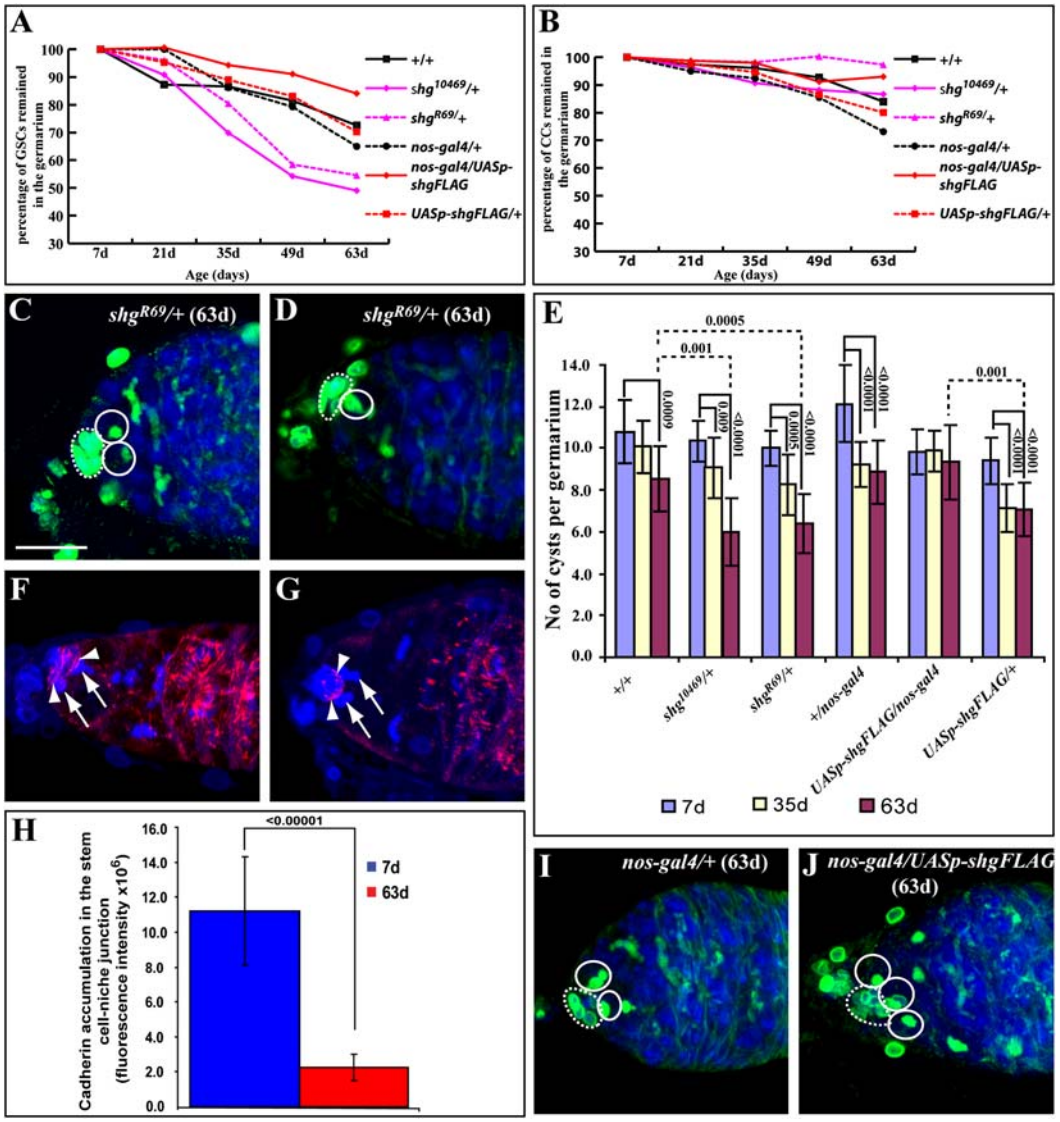

Figure 3. E-Cadherin Participates in GSC Aging

(A and B) Graphs showing that the GSC (A) or cap cell (B) maintenance rates for the controls (*hh-lacZ*/+ and *nos-gal4*/+), *shg* heterozygous mutant, and E-cadherin-overexpressing ovaries (percentages, the y axis) change with age (days, shown in the x axis). All the germaria are labeled for lamin C (TFs and cap cells, green), Hts (spectrosome and fusome, green), and DNA (blue).

(C and D) Sixty-three-day-old shg^{R69}/+ germarial tips showing two GSCs (solid circles) (C) and one GSC (solid circle) (D) in the niche (broken lines).

(E) The bar graph showing the changes in the numbers of cysts per germarium in the two-GSC-containing germaria of different genotypes and ages (7 days, blue; 35 days, yellow; and 63 days, purple). p values that are less than 0.05 between two comparisons (solid lines, among the same genotypes; broken lines, between different genotypes) are indicated, while 15 germaria are examined for each genotype at a given time point.

(F and G) Seven-day-old (F) and 63-day-old (G) germarial tips showing that E-cadherin accumulation in the junctions (arrowheads) between GSCs (their fusomes indicated by arrows) and their niche is less in the older germarium than in the younger one.

(H) A bar graph showing that E-cadherin accumulation in the stem cell-niche junction of the 63-day-old wild-type germaria (red) is significantly lower than that in the 7-day-old germaria (blue). The error bars represent standard derivation calculated using EXCEL.

(I) A 63-day-old *nos-gal4*/+ control germarial tip showing two GSCs (solid circles) in the niche (broken lines).

(J) A 63-day-old *nos-gal4*/+; *UAS-E-cad*/+ germarial tip showing three GSCs (solid circles) in the niche (broken lines). All the micrographs represent overlayed multiple confocal sections and are shown in the same scale, and the scale bar at (C) represents 10 μm.

To investigate whether or not increasing E-cadherin expression can slow down stem cell aging, we used *nos-gal4* and *UASp-shgFLAG* (a FLAG-tagged E-cadherin) to express E-cadherin specifically in the germline. The 7-day-old germaria overexpressing E-cadherin in the germline had normal numbers of cap cells (6.65 ± 1.00; n = 60) and GSCs (2.62 ± 0.74; n = 60), indicating that overexpression of E-cadherin does not affect formation of the niche

and GSCs. As expected, the *UASp-shgFLAG*/+ and *nos-gal4*/+ controls behaved just like other controls, and most of them had two GSCs (Figure 3I). However, 84.1% of the GSCs detected in the 7-day-old germaria overexpressing E-cadherin still remained in the 63-day-old germaria, which was significantly higher than the 70.3% for the controls ($p < 0.001$). Consequently, many 63-day-old E-cadherin-overexpressing germaria still carried three GSCs (Figure 3J), indicating that increasing E-cadherin expression can strengthen interactions between the niche and the GSCs and thus prolong GSC life span. As for cyst production, the *UASp-shgFLAG*/+ and *nos-gal4*/+ controls also manifested the age-related decline similar to other controls (Figure 3E). Interestingly, the germaria overexpressing E-cadherin in the germline did not show significant age-related decline in cyst production from 7 days to 63 days ($p = 0.20$; Figure 3E), indicating that E-cadherin overexpression in GSCs can promote cyst production in the aged ovary. Note that the cap cell maintenance rate for E-cadherin-overexpressing germaria was significantly higher than those for the control germaria (Figure 3D), further supporting the idea that persistent GSCs in the aged niche can stabilize the niche. Taken together, our findings demonstrate that the age-dependent decline in E-cadherin expression in the stem cell-niche junction contributes to GSC aging. Unfortunately, *hh-gal4*-driven expression of E-cadherin leads to death of young adults, precluding us from directly testing the idea of whether an increase in expression of E-cadherin in the aged niche could also stabilize GSCs (data not shown).

Overexpression of SOD in Either the Niche or GSCs Can Prolong GSC Life Span

SOD overexpression can lead to life span extension possibly by reducing ROS in *Drosophila* (Tower, 1996). To test whether SOD overexpression can rejuvenate niche function and prolong GSC life span, we used *hh-gal4* and two independent transgenic strains, *UASp-SOD-1* or *UASp-SOD-2*, to overexpress the SOD gene, encoding the Cu^{2+}/Zn^{2+}-dependent SOD enzyme, in the cap cells. As expected, the 7-day-old *hh-gal4*/+, *UASp-SOD-1*/+, and *UASp-SOD-2*/+ control germaria had normal numbers of cap cells and GSCs and exhibited similar maintenance rates for cap cells and GSCs (Figures 4A and 4B). The 7-day-old germaria overexpressing SOD had normal numbers of cap cells (SOD-1, 6.38 ± 1.34; SOD-2, 6.30 ± 0.87; n = 60) and GSCs (SOD-1, 2.6 ± 0.49; SOD-2, 2.55 ± 0.53; n = 60), indicating that SOD overexpression in the niche does not affect niche and GSC formation (Figures 4A and 4B). Because enough *hh-gal4 UAS-SOD* flies lived to the age of 77 days, we analyzed the numbers of cap cells and GSCs of the 77-day-old flies instead of the 63-day-old ones. Interestingly, the 77-day-old germaria overexpressing SOD in the niche had significantly higher GSC and cap cell maintenance rates than those for the 77-day-old control germaria carrying *hh-gal4* or *UASp-SOD-1* (all p values < 0.0001) (Figures 4A and 4B). In contrast with the 77-day-old control germaria carrying one or two GSCs (Figure 4C), those 77-day-old SOD-overexpressing germaria had two or three GSCs (Figures 4D and 4E), indicating that SOD overexpression in cap cells can slow down deterioration of their function, and thereby prolong GSC life span. Furthermore, the 77-day-old germaria overexpressing SOD in the niche contained significantly more cysts than the *UASp-SOD-1*/+, *UASp-SOD-2*/+, or *hh-gal4*/+ control germaria of the same age, indicating that SOD overexpression in the niche can deter age-related decline in stem cell proliferation (those p values < 0.0001; Figure 4F). In addition, there were more BrdU-positive GSCs in the 63-day-old germaria overexpressing SOD in the niche cells than those in the control germaria of the same age (Table 1). Taken together, these results suggest that SOD overexpression in the niche can ameliorate niche and stem cell aging. Because SOD expression helps remove cellular ROS, these results also suggest that age-related accumulation of ROS-induced cellular damage contributes to niche aging.

To investigate whether or not SOD overexpression in GSCs themselves can also slow down their aging, we used *nos-gal4* to drive *UAS-SOD* expression in GSCs. The 7-day-old germaria carrying both *nos-gal4* and *UASp-SOD* had a normal number of cap cells (SOD-1, 6.52 ± 0.62; SOD-2, 6.63 ± 0.98; n = 60) and GSCs (SOD-1, 2.78 ± 0.52; SOD-2, 2.82 ± 0.54; n = 60), indicating that germline-specific SOD overexpression does not affect formation of the niche and GSCs. Interestingly, the 63-day-old germaria overexpressing SOD in the germline (SOD-1, 2.35 ± 0.73; SOD-2, 2.33 ± 0.71; both p < 0.0001; n = 60) had significantly more GSCs than the 63-day-old germaria carrying only *nos-gal4* (1.88 ± 0.52; n = 60) or *UASp-SOD* (SOD-1, 2.08 ± 0.72; SOD-2, 1.83 ± 0.72; n = 60), indicating that SOD expression in GSCs can extend their life span (Figures 4A and 4B). Those 63-day-old germaria overexpressing SOD in the germline had two or three GSCs (Figure 4G). As for cyst production, the 63-day-old GSC-specific SOD-overexpressing germaria contained significantly more cysts than the control germaria of the same age (Figure 4F), indicating that SOD expression in GSCs can obviate the age-related decline in their proliferation. Therefore, these results show that intrinsic SOD overexpression can extend GSC life span, and also suggest that the ROS-induced cellular damage contributes to intrinsic stem cell aging.

As in the germaria overexpressing SAX* and E-cadherin, the 63-day-old germaria overexpressing SOD in the germline had significantly more cap cells than the 63-day-old germaria carrying only *nos-gal4* or *UASp-SOD* ($p < 0.0001$), further supporting the idea that stabilization of aged GSCs has a beneficial effect on the maintenance of the aged niche and its function (Figures 4A and 4B). It is worth noting that the effect of SOD overexpression in the germline is less pronounced than that of SOD overexpression in the niche (Figures 4A and 4B), suggesting that the intervention of aging in the niche may be more effective than in the GSCs themselves. In sum, our results demonstrate that SOD overexpression in either the niche or the stem cell represents an effective approach to preventing stem cell aging.

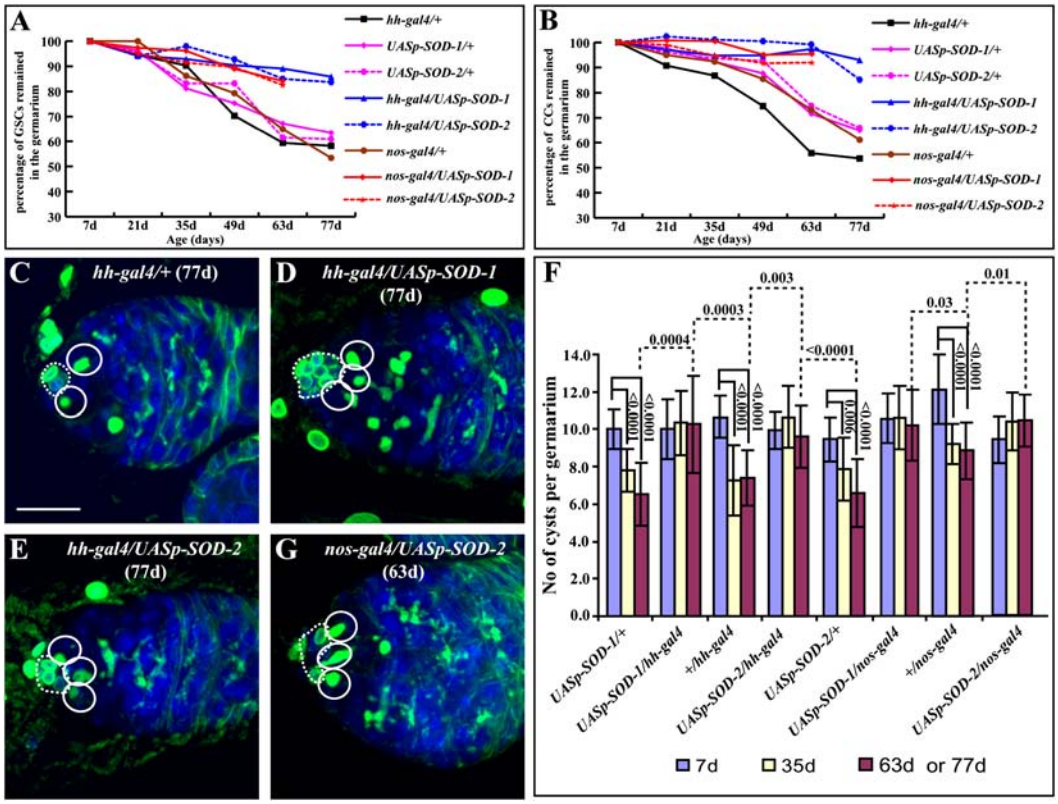

Figure 4. SOD Overexpression in GSCs or Their Niche Can Extend GSC Life Span and Stimulate Proliferation of Aged GSCs

(A and B) Graphs showing that the GSC (A) or cap cell (B) maintenance rates for the controls (*hh-gal4*/+ and *nos-gal4*/+) and for niche-specific and germline-specific SOD-overexpressing ovaries (percentages, the y axis) change with age (days, shown in the x axis). All the germaria in (C)–(F) are labeled for lamin C (TFs and cap cells, green), Hts (spectrosome and fusome, green), and DNA (blue).

(C) A 77-day-old *hh-gal4*/+ germarial tip showing two GSCs (solid circles) in the niche (broken lines).

(D) A 77-day-old *hh-gal4*/+; *UASp-SOD-1*/+ germarial tip showing three GSCs (solid circles) in the niche (broken lines).

(E) A 77-day-old *hh-gal4*/+; *UASp-SOD-2*/+ germarial tip showing three GSCs (solid circles) in the niche (broken lines).

(F) The bar graph showing the changes in the numbers of cysts per germarium in the two-GSC-containing germaria of different genotypes and ages (7 days, blue; 35 days, yellow; and 63 or 77 days, purple). p values that are less than 0.05 between two comparisons (solid lines, among the same genotypes; broken lines, between different genotypes) are indicated, while 15 germaria are examined for each genotype at a given time point.

(G) A 63-day-old *nos-gal4*/+; *UASp-SOD-2*/+ germarial tip showing three GSCs (solid circles) in the niche (broken lines). All the micrographs represent overlayed multiple confocal sections and are shown in the same scale, and the scale bar at (C) represents 10 μm.

DISCUSSION

As an organism ages, stem cells and their niche cells undergo similar age-dependent loss, and thus the stem cell loss is likely attributed to both age-dependent decline in signal communication and physical adhesive interaction between stem cells and their niche. In this study, we show that in the *Drosophila* ovary aging leads to gradual loss of GSCs and their niche cells and reduction of GSC proliferation. BMP signaling activity and E-cadherin-mediated cell adhesion decrease with age. Furthermore, strengthening BMP signaling in GSCs and E-cadherin-mediated cell adhesion between GSCs and their niche can significantly prolong GSC life span as well as increase proliferation capacity of the aged GSCs. Finally, SOD overexpression, which likely leads to reduction of the ROS-induced cellular damage, in either GSCs or the niche can also retard GSC aging. Therefore, this study has, for the first time to our knowledge, shown that the experimental manipulation of niche signaling, stem cell-niche interaction, or their functions can drastically influence the stem cell aging process. This study has also demonstrated that stem cell aging is controlled intrinsically and extrinsically (Figure 5).

The Declined Niche Function Contributes to Stem Cell Aging

Drosophila ovarian GSCs have been shown to be controlled by the signals from the surrounding cells, including cap cells and ESCs (Kirilly and Xie, 2007; Lin, 2002). Although it is conceivable that any age-related reduction in the production of niche signals could lead to age-dependent stem cell loss and age-related decline in stem cell proliferation, it remains unclear whether manipulation of niche functions in aged organisms could influence the stem cell aging process. In this study, we have provided

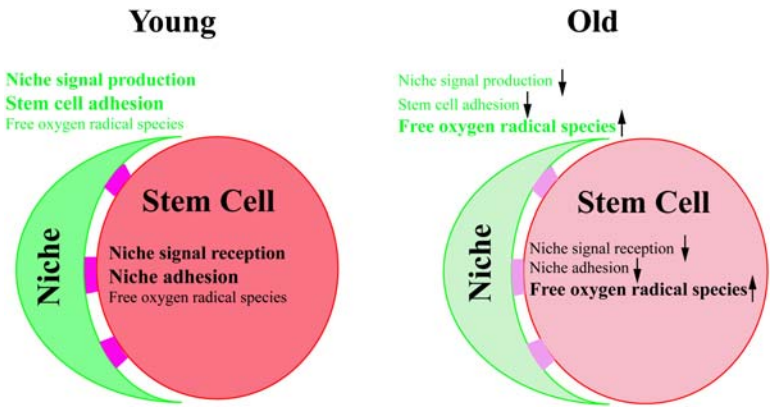

Figure 5. A Model Depicting the Control Mechanism of GSC Aging

The young niche (green) has high levels of niche signals and E-cadherin (purple) and possibly low ROS damage, while the young GSC (red) also has high levels of niche signal reception and E-cadherin expression and possibly low ROS damage. In contrast, the old niche cell (green) has decreased levels of niche signals and E-cadherin (purple) and has accumulated more ROS damage, while the old GSC (red) also has decreased niche signal reception and E-cadherin expression and also has accumulated more ROS damage. Self-renewal and proliferation capacities of the GSC are greatly reduced in aged individuals.

two pieces of experimental evidence indicating that niche function declines with age, and intervention in age-dependent decline of niche functions can increase stem cell life span.

First, we show that partial reduction of the BMP signal by removing one copy of *dpp* or *gbb* does not have any obvious impact on stem cell formation and self-renewal at young ages but significantly accelerates age-related loss and functional deterioration of GSCs (Figure 1). In addition, we show that BMP signaling activity in GSCs, which is detected by *Dad-lacZ* expression, declines with age. The specificity of the BMP signal in regulating the GSC aging process is further demonstrated by *gbb* overexpression in the niche cells. The *gbb* overexpression in the niche and Sax* overexpression in GSCs leads to a prolonged GSC life span and enhances the proliferation capacity of the aged GSCs (Figure 2). Together, our results suggest that the BMP signal from the niche weakens as the GSC ages. Unfortunately, we could not directly investigate whether aging regulates Dpp and Gbb production, secretion, or maturation due to the lack of good antibodies against Dpp and Gbb. In this study, our observation that partial reduction of the niche signal dosage by mutations does not affect the formation and maintenance of stem cells and their niche at young ages but significantly enhances stem cell aging could have important implications in understanding stem cell aging in human tissues. For example, individuals who are heterozygous for a mutation in certain genes important for adult stem cell maintenance may be predisposed to accelerated age-dependent stem cell loss and tissue degeneration although they do not show such obvious defects at a young age.

Second, we show that overexpression of SOD in the niche can significantly slow down age-dependent GSC loss and promote proliferation of the aged GSC. It has been proposed that SOD overexpression can prolong organismal life span in *Drosophila*, possibly through reducing ROS-induced cellular damage (Tower, 1996). Our study raises an interesting possibility that SOD overexpression can extend the life span of both the niche and GSCs, perhaps through reducing ROS and its induced cellular damage. This can be directly tested by developing in vivo ROS-responsive reporters in the future. It appears that SOD overexpression can extend GSC life span as efficiently as upregulation of BMP signaling and E-cadherin, raising an interesting possibility that SOD overexpression in the niche might promote BMP and/or E-cadherin production. In the future, it will also be important to investigate whether SOD overexpression in the niche indeed enhances its functions by increasing BMP production and/or E-cadherin expression.

The experimental results from this study have demonstrated that GSC aging is controlled extrinsically due to the age-related deterioration in niche structure and function. One mammalian study shows that Notch signaling activity declines in the resident muscle precursor cells and forced Notch activation can lead to rejuvenation of the aged precursor cells (Conboy et al., 2003). Therefore, the decline in niche function most likely represents a general mechanism for stem cell aging. However, the cause of the functional decline of the niche in aged tissues remains unknown. Because this study shows that SOD overexpression can prolong GSC life span and promote proliferation of the aged GSCs, it is possible that the ROS-induced cellular damage to the niche actually modulates its signaling capacity and adhesive property.

Stem Cells Also Undergo Intrinsic Aging

Although it is not surprising to discover that stem cells also undergo intrinsic aging, it is still important to know what major factors contribute to intrinsic aging. In this study, we show that the age-dependent reduction of adhesion between GSCs and niche as well as the possible age-dependent accumulation of the ROS-induced cellular damage contributes to GSC aging. First, we show that E-cadherin accumulation in the stem cell-niche junction, which likely reflects strength of adhesion between GSCs and their niche, decreases in an age-dependent manner, and the reduction of E-cadherin dosage by half does not affect GSC formation and maintenance at young ages but can significantly enhance GSC aging. E-cadherin-mediated physical interaction between GSCs and the niche is known to be essential for keeping stem cells in the niche for long-term self-renewal (Song and Xie, 2002). As expected, overexpression of E-cadherin in GSCs themselves can prolong their life span. Surprisingly, the aged GSCs

overexpressing E-cadherin also proliferate faster than those that do not overexpress it. This suggests that the adhesion between GSCs and their niche may strengthen the niche-mediated signal transduction or E-cadherin may directly initiate a signal transduction for regulating GSC proliferation. Second, intrinsic overexpression of SOD leads to a significant increase in GSC life span and proliferation, suggesting that the ROS-induced cellular damage likely contributes to intrinsic stem cell aging. In summary, declining intrinsic functions contribute to overall stem cell aging.

Recent studies have shown that stem cells can signal back to the niche and regulate its maintenance and function (Song et al., 2007; Ward et al., 2006). In this study, we have also observed that the cap cell maintenance rates in the germaria overexpressing SAX*, E-cadherin, and SOD in GSCs are significantly higher than those for the control germaria, supporting the idea that persistent stem cells in the aged niche can provide feedback and slow down niche aging. These findings further highlight intimate relationships between the stem cells and their niche. In the future, it would be of great importance to investigate whether aged stem cells can be rejuvenated by manipulating niche signal transduction and physical interactions between stem cells and their niche in other systems, including humans.

EXPERIMENTAL PROCEDURES

Drosophila Stocks

The following *Drosophila* stocks used in this study are described in Flybase: dpp^{hr56}, dpp^{hr4}, gbb^{D4}, shg^{R69}, shg^{10469}, *Dad-lacZ*, *hh-lacZ*, *UAS-dpp*, and *UAS-gbb*. All stocks were cultured at room temperature on standard cornmeal/molasses/agar media unless specified.

Generation of Transgenic *UASp* Lines

To generate the *UASp-SOD* construct, a 5′ primer with a KpnI site and a 3′ primer with an XbaI site were used to amplify the 697 bp region of *SOD* including the coding region by PCR, which was then cloned into the KpnI-XbaI site of the UASp vector. To generate a *UASp-SAX** construct, the 2.4 kb of *sax* cDNA carrying a constitutively active mutation at the 263th amino acid residue (Q to D) was cloned into a shuttle vector with XhoI and EcoRI sites, and the cDNA fragment was excised with NotI and was then cloned into the NotI site of the pUASp vector. The Gateway cloning strategy (Invitrogen) was used to generate a *UASp-shg-FLAG* construct. The coding region for *shg* was amplified from an EST by PCR and was then cloned into the ENTR vector using a TOPO Gateway cloning kit (Invitrogen). The *shg* coding region was recombined into a destination vector pPWF using the LR reaction kit (Invitrogen). All the clones were completely sequenced and confirmed. These DNA constructs were injected into w^{1118} embryos for generating transgenic lines.

Experimental Genotypes

To determine whether a moderate reduction of BMP signaling and adhesion has any effect on GSC aging, the *hh-lacZ/TM3 Sb* virgins were crossed with dpp^{hr56}/CyO, dpp^{hr4}/CyO, gbb^{D4}/CyO, shg^{R69}/CyO, shg^{10469}/CyO, and *y w* males. To investigate how *dpp* and *gbb* overexpression affects GSC aging, *hh-gal4 UAS-gal80^{ts}/TM3 Sb* virgin females were mated with *UAS-dpp* (at 18°C), *UAS-gbb*, and *y w* males. To test whether SOD, SAX*, or E-cadherin overexpression in the germline has any effect on GSC aging, *nos-GAL4* virgin females were crossed with *UASp-SOD-1*, *UASp-SOD-2*, *UASp-SAX**, *UASp-shg-FLAG*, and *y w* males. To test whether niche-specific expression of SOD can slow down GSC aging, *hh-gal4/TM 6B* virgin females were crossed with *UASp-SOD-1*, *UASp-SOD-2*, and *y w* males. The adult females of appropriate genotypes were cultured with males at room temperature for 7, 21, 35, 49, and 63 days (up to 77 days for SOD overexpression) and were transferred daily to fresh food.

BrdU Labeling of GSCs

The *Drosophila* females of appropriate genotypes at different ages were fed on wet yeast paste made from 10 mg/ml BrdU solution for 24 hr. The ovaries were dissected from those females and were then processed for immunostaining with anti-BrdU and anti-Hts/anti-α-spectrin antibodies according to our published procedures (Song et al., 2007).

Quantification of Cap Cells, GSCs, and Cysts

For each genotype and each time point (7, 21, 35, 49, 63, and/or 77 days), cap cells and GSCs were carefully determined in 60 randomly chosen germaria under a fluorescent microscope, and average numbers of cap cells and GSCs and their standard deviations were calculated using Microsoft Excel. Multivariate profile analysis techniques were used to compare the temporal profiles of treatment and control groups for five or six time points. Both groups were measured over five or six consecutive and evenly spaced time points with 60 (randomly sampled) replicates for each group at each time point. p value of each comparison was calculated based on the null hypothesis that the treatment has the same effect as the control over time. The data analysis was done using PROC GLM of SAS 9.1. For a comparison between two time points of the same genotype, the standard student's t test was used.

Quantification of *Dad-lacZ* and E-Cadherin Expression in GSCs

The ovaries of 7-day-old and 63-day-old *Dad-lacZ/TM3 Sb* females were immunolabeled for β-galactosidase (LacZ, green), E-cadherin (red), and Hts (blue). For quantification, the germaria carrying two GSCs were sectioned through with 0.5 μm thickness with a Leica confocal microscope. E-cadherin in the stem cell-niche junction was quantified for every section, and the total value for each germarium was achieved by adding up the value from each section. Similarly, nuclear β-galactosidase expression was also quantified on each section, and the total value for each germarium was achieved by adding up the value from each section. For a comparison between the two time points, the standard student's t test was used.

Immunohistochemistry

The following antisera were used: monoclonal anti-α-spectrin antibody (1:10, Developmental Studies Hybridoma Bank or DSHB); anti-lamin C antibody (1:3, DSHB); rabbit polyclonal anti-β-galactosidase antibody (1:100, Molecular Probes), and rabbit polyclonal anti-BrdU antibodies (1:150, Megabase Research). Secondary antibodies, including goat anti-rabbit or anti-mouse IgG conjugated with Alexa 488 or 568 (Molecular Probes), were used as 1:200. The immunostaining protocol used in this study has been described previously (Song and Xie, 2002). All micrographs were taken with the Leica TCS SP2 confocal microscope.

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