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Dynamics of the male germline stem cell population during aging of *Drosophila melanogaster*

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Summary

Drosophila melanogaster **has emerged as an important model system for the study of both stem cell biology and aging. Much is known about how molecular signals from the somatic niche regulate adult stem cells in the germline, and a variety of environmental factors as well as single point mutations have been shown to affect lifespan. Relatively little is known, however, about how aging affects specific populations of cells, particularly adult stem cells that may be susceptible to aging-related damage. Here we show that male germline stem cells (GSCs) are lost from the stem cell niche during aging, but are efficiently replaced to maintain overall stem cell number. We also find that the division rate of GSCs slows significantly during aging, and that this slowing correlates with a reduction in the number of somatic hub cells that contribute to the stem cell niche. Interestingly, slowing of stem cell division rate was not observed in long-lived** ***methuselah*** **mutant flies. We finally investigated whether two mechanisms that are thought to be used in other adult stem cell types to minimize the effects of aging were operative in this system. First, in many adult tissues stem cells exhibit markedly fewer cell cycles relative to transit-amplifying cells, presumably protecting the stem cell pool from replication-associated damage. Second, at any given time not all stem cells actively cycle, leading to 'clonal succession' from the reserve pool of initially quiescent stem cells. We find that neither of these mechanisms is used in** ***Drosophila*** **male GSCs.**

Key words: aging; cell cycle; *Drosophila*; germline stem cell; *methuselah*; spermatogenesis.

Introduction

Aging is a process fundamental to all organisms, and phenotypic effects of aging can be observed in most organ systems (reviewed in Chien & Karsenty, 2005). One hypothesis to explain at least some aspects of the aging of organ systems is that defects within the adult stem cell population that maintain specific lineages might contribute to aging of those systems (reviewed in Van Zant & Liang, 2003). In contrast to many mature cell types that undergo rapid turnover, stem cells persist throughout the lifetime of the organism and thus may be more susceptible to accumulated damage from the cellular environment. Additionally, because most stem cells divide many more times over their lifetime than non-stem-cell types, genetic damage may have a greater chance of accumulating to detrimental levels. Although adult stem cells likely have evolved mechanisms that aid in protecting them from such damage (Morrison *et al*., 1996a; Park *et al*., 2002; Zhou *et al*., 2002), evidence exists that stem cells do indeed show aging-related defects. It has been known for some time that serially transplanted hematopoietic stem cells show greatly decreased ability to repopulate the niche of a lethally irritated host after just a few generations, suggesting that their function may become impaired as a result of aging (Siminovitch *et al*., 1964). This, however, is contradicted by the fact that the age of the initial donor does not seem to affect the efficiency of transplantation (Harrison & Astle, 1982). In skeletal muscle stem cells (satellite cells), increased age is associated with a decrease in Notch signaling in these cells, which leads to a decreased rate of proliferation and differentiation (Conboy *et al*., 2003). More recently, it has been shown that hair graying, a prominent phenotype of aging in mammals, is due to loss of melanocyte stem cells from the niche due to either differentiation or senescence (Nishimura *et al*., 2005). It thus appears that, at least in the case of the soma, reduced function and/or a lack of maintenance of adult stem cell populations may indeed underlie some aspects of organismal aging. The effect of aging on germline stem cells, which due to their immortality likely have mechanisms to protect them from aging-related damage, has not been extensively studied.

The male germline of *Drosophila melanogaster* offers a unique opportunity to study stem cell aging *in vivo*. In contrast to most other stem cell systems, *Drosophila* germline stem cells (GSCs) are easily identifiable within the stem cell niche (Gonczy & DiNardo, 1996). These stem cells function throughout adulthood to produce many thousands of mature spermatids, and much has been described about the molecular mechanisms that are used to maintain a steady-state balance between self-renewal and differentiation (reviewed in Yamashita *et al*., 2005). Flies have a relatively short lifespan, which has facilitated the isolation of a number of genetic mutations that are known to increase lifespan. The ways in which these genes affect aging at the cellular level, however, are largely unknown (reviewed in Helfand & Rogina, 2003). It has, however, been observed that expression

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of nuclear anillin, which is correlated with interphase of actively cycling cells, but not cells that have left the cell cycle, is significantly reduced in older GSCs compared to GSCs of young adult flies, and GSCs from older flies show altered expression of marker genes (Tran *et al.*, 2000). This demonstrates that GSCs do indeed change during the course of aging. We thus set out to further examine dynamics of the germline stem cell population during aging and asked whether these properties might be altered in long-lived flies.

The ability of stem cell populations to produce large numbers of differentiating progeny is ultimately limited by two factors: the number of stem cells present within the niche and the frequency with which they divide to give differentiating progeny. We were interested to find whether either of these aspects changes in the male GSC population during the course of normal aging in *Drosophila*. By marking individual stem cells and their progeny, and by using S-phase labeling of cells, we describe in detail the dynamics of the stem cell population during normal aging, and in a long-lived mutant strain. We then examine whether mechanisms that have previously been proposed to maintain adult stem cell populations during aging are acting in this system. Taken together, our data support the hypothesis that aging of organ systems may indeed be due in part to changes in adult stem cell populations.

Results

Individual GSCs are lost from the stem cell niche over time

The *Drosophila* testis contains ~5–11 germline stem cells that are located adjacent to a nondividing cluster of somatic cells known as the hub (Fig. 1A). A GSC divides asymmetrically to give rise to one daughter that remains adjacent to the hub, retaining stem cell identity, and one daughter that is displaced from the hub and becomes a spermatogonium (Yamashita *et al.*, 2003). This spermatogonium then undergoes transit amplification by mitotically dividing four times to give rise to a cyst of 16 interconnected spermatogonia. These cells typically go on to enter meiosis and differentiate into spermatids, although a recent study demonstrates that spermatogonia also have the potential to dedifferentiate into functional stem cells (Brawley & Matunis, 2004). The stem cell identity of those germ cells adjacent to the hub was first confirmed by lineage tracing experiments, in which flip recombinase (Flp)-mediated recombination was used to combine a ubiquitously active tubulin promoter followed by an flip recombinase target (FRT) site with a promotorless lacZ open reading frame preceded by an FRT site (Gonczy & DiNardo, 1996). Upon induction of Flp recombinase by heat-shock, recombination can occur in dividing cells to activate marker gene expression. When activated in transit-amplifying gonia, lacZ expression is restricted to a single cyst of germ cells, which eventually moves away from the hub as it differentiates. However when recombination occurs in a stem cell, a functional lacZ gene is inherited by every gonial cyst produced by that stem cell, resulting in a marked clone of cells all derived from a single cell (the GSC) at the apical tip of the testis adjacent to the hub (Fig. 1B,C).

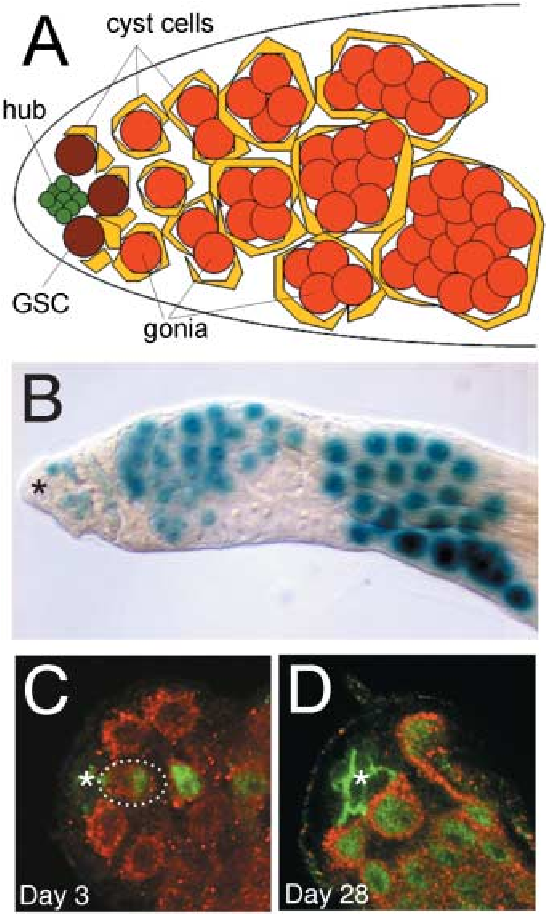

Fig. 1 Lineage tracing analysis of male germline stem cells during aging. (A) Schematic showing the organization of germline stem cells (GSCs), spermatogonia, and somatic cells within the apical tip of the testis. Typically 5–11 germline stem cells (dark red, 3 shown here for clarity) are found in contact with the hub (green). A GSC divides asymmetrically to produce one daughter GSC and one primary spermatogonium (red). The primary spermatogonium divides with incomplete cytokinesis four times to produce a cyst of 16 interconnected spermatogonia, which will undergo meiosis and differentiate as 64 spermatids; the meiotic and differentiation stages are not shown. Each GSC and gonial cyst is flanked by two somatic cyst cells (yellow) which play a role in regulating stem cell and gonial development. (B) Flp-FRT mediated recombination creates a functional lacZ gene, which can be used to mark individual stem cells and their progeny, resulting in a marked clone of cells as shown here, revealed by X-gal activity stain (blue). (C) The majority of testes containing marked germ cells had only one marked GSC (outlined) 3 days following a 30-min heat-shock. Anti-FascIII marks hub (green, asterisk), anti-vasa marks germ cells (red), and anti-β-galactosidase stains marked GSCs (green). One z-slice containing three visible GSCs (one of which is marked) is shown from a confocal micrograph z-series – no other marked GSCs were found in other sections. (D) After 28 days of aging, testes that still contained marked GSCs typically had a greater number of marked GSCs than un-aged testes (cf. three marked stem cells in the testis in Fig. 1D vs. one marked stem cell shown in C).

We took advantage of this ability to individually mark GSCs to investigate the rate at which these cells are lost from the stem cell niche during adulthood. Newly eclosed adult flies carrying one copy each of the tub-FRT promoter and FRT-lacZ open reading frame on homologous chromosomes were subjected to a 30-min heat-shock to induce the Flp recombinase. Testes were then dissected and fixed at various times after heat-shock to analyze lacZ marker gene expression. After 3 days of aging, transit-amplifying gonia that had undergone recombination at the time of Flp induction had moved away from the gonial proliferation center and begun spermatocyte development, such that only gonial clones derived from marked stem cells remained in this region (Gonczy & DiNardo, 1996). Control flies that received no heat-shock showed a low basal level of stem cell labeling that did not increase as flies were aged (Fig. 2A). In heat-shock treated flies, we found that after 3 days 41% ($n = 39$) of testes contained a marked germ cell that included marked progeny gonial cells. In flies that had been aged for longer times following heat-shock, however, progressively fewer testes containing marked GSCs were identified (Fig. 2B). In many of these testes, marked cysts of late gonia or early spermatocytes could be seen, but with no identifiable marked cell near the hub, suggesting that the marked GSC that had given rise to those cells had recently either died or differentiated and exited the niche. Consistent with previous studies (Tulina & Matunis, 2001; Kawase *et al.*, 2004), we found that after 14 days the number of testes containing marked GSCs was reduced by almost half (from 41% to 25%, $n = 57$), suggesting that there is a significant loss of stem cells from the niche during the normal course of aging.

Total GSC number decreases at a rate less than that predicted by the rate of individual stem cell loss

Given the approximately 14-day half-life for individual GSCs, as testes age we expected only ~50% of the GSCs to be present after one half-life, and a further decrease in GSC number by 28 days (Fig. 2C, dashed line). Instead we observed only a modest decrease in the number of GSCs. Testes from flies used in our germ cell marking experiments contained on average 6.9 GSCs shortly after eclosion. After one half-life, 81% of the initial GSC number remained (5.6 GSCs), and 70% still remained after 28 days (4.3 GSCs, Fig. 2C solid line). A similar slight decrease in stem cell number was observed in control flies of the genotype w^{1118} (Table 1). More dramatically, testes from a third wild-type strain of flies, *mthEx28* (see below), showed no decrease in stem cell number after 35 days of aging (Table 1). These results are significantly different from what would be expected if stem cell number was solely dependent on the rate at which stem cells are lost from the niche as determined by the lineage-tracing analysis described above ($P < 0.01$), and suggests that a mechanism exists to replace lost GSCs in order to maintain the stem cell niche.

Individual GSCs can give rise to multiple GSCs within the niche during the normal course of aging

Further evidence that stem cells within the niche might be replaced over time came from analysis of the tub-lacZ marked GSCs generated for lineage tracing. As previously described, after a 30-min heat-shock to induce Flp recombinase and 3 days of aging, 41% of flies contained gonial clones that included a marked GSC. By examining such clones using immunofluorescence to localize marked stem cells adjacent to the hub (Fig. 1C), we determined that these testes contained on average 1.6 ± 0.18

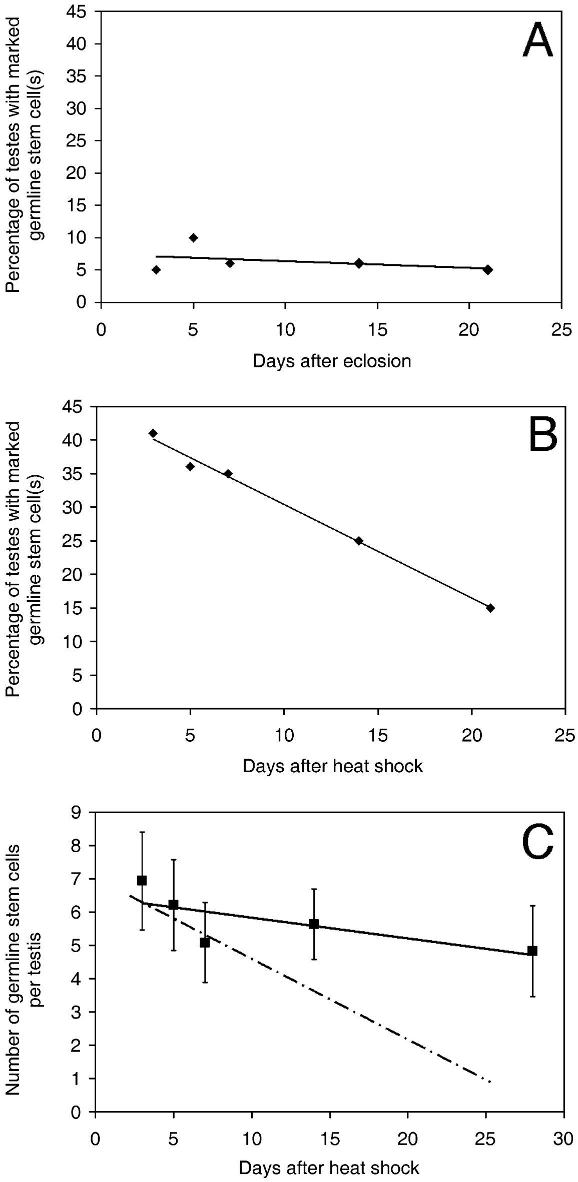

Fig. 2 Stem cell loss during aging is balanced by replacement. (A) Testes from the clonal marking strain were analyzed for clone induction in the absence of heat-shock. The percentage of testes containing at least one marked clone of stem cells is indicated. Although a low level of heat-shock-independent clone induction is observed, this number does not increase over time. (B) The percentage of testes containing at least one marked clone of stem cells after a 30-min heat-shock to induce Flp recombinase is indicated. This percentage decreases in aged flies, indicating a loss of stem cells. (C) The average number of germ cells adjacent to the hub at each time point is indicated. Although this number decreases over time (solid line), the rate of decrease is significantly different from what would be expected if stem cell loss were occurring without replacement (dashed line). Error bars represent standard deviation.

Table 1 Proliferation rates of stem cells in young and old flies determined by 30-min *in vitro* BrdU labeling

	0 day				35 days			
	GSCs	BrdU(+) GSCs	BrdU Index	Hub cells	GSCs	BrdU(+) GSCs	BrdU Index	Hub cells
w^{1118}	5.6 ± 1.1	1.6 ± 1.0	0.28	9.6 ± 2.6	4.2 ± 1.3*	0.77 ± 0.84*	0.18*	6.4 ± 2.3*
mthEx28	11.1 ± 2.3	2.9 ± 1.4	0.27	11.1 ± 2.4	11.4 ± 3.0	2.2 ± 1.7	0.19*	7.5 ± 2.3*
mth	8.7 ± 1.3	2.2 ± 1.6	0.25	N.D.†	8.1 ± 1.5	2.1 ± 1.5	0.26	N.D.†

> 20 testes were examined for each sample. Error is standard deviation.*Significantly different from 0 day ($P < 0.01$).
†ND, not determined due to differences in staining of *mth* hub cells (see text and Fig. 3).

β-galactosidase-expressing cells adjacent to the hub. In contrast, after 28 days, although only 22% of testes now contained stem cell clones, those which did contain clones had significantly more marked GSCs per testis, with an average of 3.6 ± 0.66 β-galactosidase-expressing cells adjacent to the hub. Control experiments using aging flies in the absence of heat-shock show that although there is a low level of heat-shock-independent recombination observed in this strain, the number of marked testes does not increase over time (Fig. 2A). It is thus unlikely that the observed increase in the number of marked GSCs in particular testes during aging is due to additional heat-shock-independent recombination events. Instead, the extra marked GSCs are likely to be derived from a previously marked GSC or its progeny, resulting in a GSC population in older adults that is becoming increasingly clonally derived. Such an increase in monoclonality over time is reminiscent of population genetics models of loss and replacement within a population over time (Crow & Kimura, 1970), and these data are consistent with a model in which all cells within the niche divide at similar rates to replace lost stem cells (see Discussion).

Cell cycle activity of GSCs decreases during normal aging

In addition to the number of stem cells present within the niche, the cell cycle activity of stem cells affects the ultimate potential of a stem cell population to produce large numbers of progeny. A decrease in cell cycle activity during aging was previously suggested by observing a decrease in the expression of nuclear anillin (Tran *et al.*, 2000), a marker that has been correlated with actively cycling cells (Field & Alberts, 1995). To directly test whether GSC cycling decreases during normal aging, we pulse labeled testes using the thymidine analog bromo-deoxy-uridine (BrdU) for 30 min and then measured the percentage of GSCs undergoing S-phase during that time period as indicated by BrdU incorporation. Testes from newly eclosed wild-type flies had an average S-phase index of 28% of GSCs that had incorporated BrdU after a 30-min pulse (Table 1). In contrast after 5 weeks of aging, the S-phase index had decreased significantly to 18% ($P < 0.01$), demonstrating that GSC cycling rates do indeed decrease during the course of normal aging.

Cell cycle activity of GSCs remains constant during aging of long-lived *mth* mutant flies

To assess whether the decreased cell cycle activity of stem cells might contribute to phenotypes associated with adult aging, we next examined the S-phase index in *methuselah* (*mth*) mutant flies, which have a significantly longer lifespan than wild-type (Lin *et al.*, 1998). GSCs from newly eclosed *mth* mutant adult flies showed an S-phase index similar to wild-type (25%, Table 1). Strikingly, no decrease in S-phase index was observed in GSCs from *mth* mutant flies that had been aged for 35 days (26%). GSCs from a revertant strain, containing a precise excision of the transposable P-element used to create the *mth* mutation (*mthEx28*), behaved similarly to wild-type, exhibiting a decreased S-phase index in aged flies (27% at 0 day vs. 19% at 35 days, $P < 0.01$). This control demonstrates that the observed change was indeed due to the *mth* mutation. We suspect that the S-phase index would eventually drop as *mth* mutant flies age for longer than 35 days. While preliminary unpublished data support this, the structure of testes from these very old flies has frustrated our attempts at a detailed enough analysis for statistical significance.

The somatic hub population decreases with age

Because signaling from the somatic hub cells has been shown to be an important regulator of GSC behavior, we were interested in determining whether the changes we observed in GSC activity correlated with changes in the hub population. We determined the total number of cells contributing to the hub by Fasciclin III staining, which is enriched at the border between hub cells. Testes from newly eclosed w^{1118} flies had on average 9.6 ± 2.6 hub cells (Table 1, Fig. 3A). By 35 days, the number of hub cells had decreased significantly to 6.4 ± 2.3 (Fig. 3B, $P < 0.01$). A similar decrease in hub cell number over time was observed in the wild-type strain *mthEx28*. The age-dependent decrease in hub cell number thus correlates with slowed cell cycling (see Discussion). Interestingly, attempts to determine hub cell number in *mth* mutant testes were confounded by the fact that FascIII staining was typically limited to just a few cells in the region of the presumed hub, based on the arrangement of surrounding GSCs (Fig. 3C). It is unclear whether this difference in hub architecture in *mth* mutants contributes to observed differences in *mth* GSC aging.

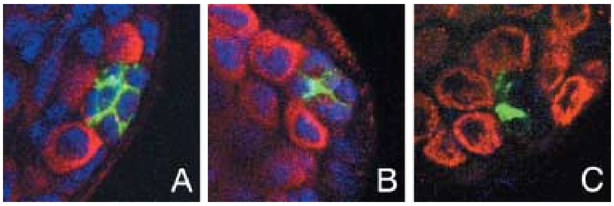

Fig. 3 The somatic hub population decreases during aging. (A) Confocal slice through the center of the hub region of a typical w^{1118} testis shortly after eclosion. This testis has a total of 13 hub cells; six are visible in this slice. Anti-FascIII (green) outlines hub cells, antivasa (red) marks germ cells, Hoechst (blue) stains DNA. (B) Confocal slice through the center of a typical 35-day-old w^{1118} testis. This testis has a total of six hub cells; four are visible in this slice. (C) Confocal slice through the center of a typical newly eclosed *mth* testis. FascIII staining marks only a few of the presumed hub cells, based on the arrangement of germ cells surrounding this region.

Relative quiescence and clonal succession do not maintain GSC activity

One proposed mechanism that may permit stem cells to function throughout the lifetime of the organism is clonal succession, in which only a few stem cells within the stem cell niche actively divide at a given time, while the others remain quiescent (Kay, 1965). In this way a population of stem cells is maintained that might be less susceptible to stresses such as DNA coding errors incorporated over multiple rounds of replication. We wished to investigate whether such a mechanism might be responsible for maintaining GSC activity in the *Drosophila* testis. First, we estimated the rate at which active GSCs divide by determining the average number of marked gonia produced by a marked stem cell 3 days after cell marking, using the tub-lacZ marking system described above. We found that 3 days after clone induction, testes that had a single marked stem cell contained on average 2.2 marked gonial progeny cysts, suggesting a GSC division rate of ~32 h (Fig. 4A). Notably, the oldest gonial cysts produced by these marked GSCs were on average at the 4-cell stage (6 of 10 marked clones examined), suggesting that the transit-amplifying gonial progeny divide at a similar rate to GSCs (two gonial divisions are required to produce a 4-cell cyst). We next induced marked stem cell clones in 35-day-old flies and similarly examined the number of stem cell progeny produced after 3 days. Although the number of aged flies that survived the heat-shock regimen was low, we found that in the few testes in which we observed a marked stem cell significantly fewer marked gonial cysts were produced (average of 1.25 marked progeny cysts, $P < 0.05$, Fig. 4C), confirming the decrease in cell cycle activity we had observed in our BrdU pulse experiments described above.

In separate experiments, we found no evidence of BrdU-positive ‘label retaining cells’, which have been observed within adult tissues such as the mouse epidermis, indicative of the relative quiescence of stem cells within this tissue (Bickenbach, 1981). Flies pulse labeled with BrdU via intra-abdominal injection contained on average 21.6% labeled GSCs. After 3 days, the number of GSCs containing detectable label had dropped to 3.5% ($P < 0.01$). These results confirm that GSCs within the *Drosophila* testis are not relatively quiescent.

We next labeled all dividing stem cells *in vivo* using continuous application of BrdU via feeding. If all stem cells divide at a similar rate, we would ideally expect to find the majority of stem cells labeled with BrdU after ~32 h. In practice, flies did not always begin to feed and incorporate BrdU immediately upon being transferred to BrdU-containing food, based on the observation that after 12 h 60% of testes did not contain any BrdU-positive germ cells. Nevertheless, we found that after 36 h, 63% of all GSCs were labeled. By 48 h, this had increased to 89% of all GSCs, including 7 of the 10 testes with 100% of their GSCs labeled (Fig. 4B,C). These results are similar to what has been observed for mouse hematopoetic stem cells (HSCs), where BrdU is seen in virtually all cells after just a few months of

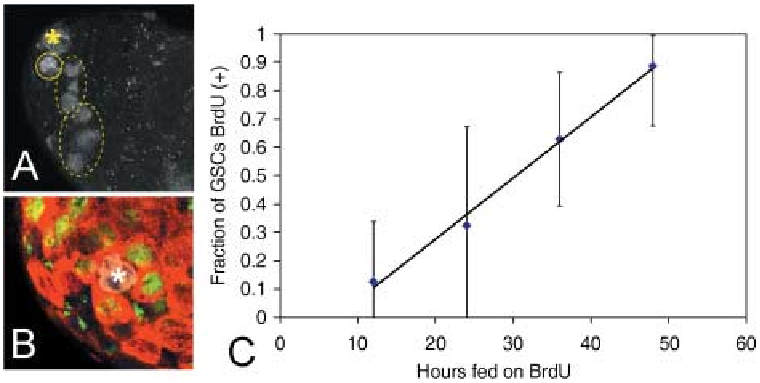

Fig. 4 All germline stem cells (GSC) divide at a similar rate. (A) Confocal projection of a testis with a single marked GSC (solid outline) adjacent to the hub (asterisk). Three days following clone induction, this GSC has divided twice to produce two gonial cysts (dashed lines) – one 2-cell cyst and one 4-cell cyst. The outlined 2-cell cyst includes a marked somatic cell between the two marked gonia, which can be identified based on its distinctive triangular shaped nucleus. In the outlined 4-cell cyst, two nuclei are in different focal planes, and overlap in this projection. (B) Example of a testis after 48 h of continuous BrdU labeling in which all six GSCs have been labeled. GSCs are those cells containing vasa-positive (red) cytoplasm adjacent to the hub (marked with anti-FascIII, tan, asterisk). Other BrdU-positive (green) cells include spermatogonia and cyst cells away from the hub. (c) Flies were continually labeled with BrdU by feeding for the indicated lengths of time. After dissection and fixation, the fraction of GSCs labeling with BrdU was determined. Error bars represent standard deviation.

labeling (Cheshier *et al*., 1999). These data demonstrate that in these systems all stem cells within the niche divide at a similar rate, and thus clonal succession does not play a significant role.

Discussion

Using male GSCs in *Drosophila* as a model, we have been able to directly examine *in vivo* the effects of aging on a stem cell population. Although stem cells are lost from their niche at a significant rate during aging, the total number of stem cells present remains high as a result of stem cell replacement by neighboring stem cells and/or their daughters. In contrast, the cell cycle activity of GSCs decreases significantly during aging, and this correlates with a reduction in the number of somatic hub cells that serve to anchor the GSC population. This decrease in GSC cell cycle activity is not observed in long-lived *methusalah* mutant flies, suggesting that changes in the activity of stem cell populations might lead to at least some of the phenotypes associated with aging. It will be interesting to examine whether a similar stem cell phenotype is observed in other long-lived *Drosophila* strains.

In our stem cell marking experiments, we found that in those cases where a single, randomly marked GSC was retained as a testis ages, its siblings tended to make up a substantial population of GSCs in the niche. We considered whether these cells represented a distinct subpopulation of GSCs that are more capable of replacing lost GSCs than neighboring unmarked cells. If this were the case, we would expect that, over time, the proportion of marked GSCs within the total population would increase significantly. This is not what we observe. At 3 days following marking, testes containing marked cells had an average of 1.6 marked GSCs per testis, but such marked testes were only 41% of the total testis population sampled. Therefore, over the total population, there were 0.65 marked GSCs per testis. At 28 days, the representation of marked GSCs in the total testis population sampled was only 0.79, even though in those few testes that retained a marked GSC there were now an average of 3.6 marked GSCs, as these rare testes moved toward monoclonality. Thus, in the overall population, the frequency of a marked GSC did not increase significantly (0.65–0.79), demonstrating that the likelihood of a particular marked or unmarked GSC to replace a lost stem cell is simply proportional to the initial frequency of marked vs. unmarked cells within the niche. Thus, in the majority of older testes where the initially marked GSC had been lost, we assume that progeny of one of the other (unmarked) GSCs now produce the clonal descendants that repopulate the niche, while in a small number of testes a marked cell is observed replacing lost unmarked cells. This tendency of a given unit to win out in a subpopulation while its frequency remains roughly constant over the whole population is reminiscent of ‘random drift’ observed in population genetics (Crow & Kimura, 1970).

Stem cell loss during aging accompanied by replacement to maintain overall stem cell number has previously been described for GSCs in the *Drosophila* ovary (Xie & Spradling, 2000), and likely also occurs in female somatic stem cells in the ovary, based on an increased monoclonality of follicle cells during aging (Margolis & Spradling, 1995). Outside of the germline, adult stem cells that maintain the *Drosophila* gut have also recently been shown to undergo loss during aging, although replacement of lost stem cells was not addressed in this study (Ohlstein & Spradling, 2006). Given the likely differences in signaling pathways and cellular architecture used in each of these niches, examining how stem cell loss and replacement occurs in each case could reveal quite different regulatory mechanisms used to maintain stem cells during aging.

Drosophila germline and somatic stem cell loss and replacement during aging are in contrast to what has been observed for melanocyte stem cells in mice, where stem cells are lost and are apparently not replaced, resulting in hair graying (Nishimura *et al*., 2005). There is, however, a suggestion that stem cell loss and replacement may occur in the HSCs of mammals. Our observation that a single marked stem cell has the ability to populate the entire niche when neighboring unmarked stem cells are lost is remarkably similar to what has been observed for HSCs (Gale *et al*., 1997; Hatakeyama *et al*., 2004). In these studies, it was found that X-chromosome inactivation patterns in blood cells, which are randomly distributed at birth and in younger individuals, become markedly skewed in elderly individuals, suggesting that activity of just a few HSCs persists into old age. Although data on HSC numbers during aging are not clear-cut, existing data from mice suggest that HSC number decreases only modestly (de Haan & Van Zant, 1999) or even increases (Morrison *et al*., 1996b) during aging. Thus the situation in HSCs may be analogous to that in *Drosophila* male and female GSCs where stem cell loss over time is balanced by replacement by neighboring stem cells and/or their progeny, and that this leads to cell populations in older individuals that are derived from a small subset of the initial stem cell population.

We have not addressed the mechanism of stem cell replacement. GSCs have been shown to consistently orient their division such that one daughter remains adjacent to the somatic hub cells and remains a stem cell, while one daughter is born away from the hub, and presumably begins to differentiate (Hardy *et al*., 1979; Yamashita *et al*., 2003). One possibility is that the occasional loss of a stem cell from the niche leads to a re-orientation of the division of a neighboring stem cell, such that it produces two stem cell daughters in direct contact with the hub. Such reorientation of cell division has been suggested as the mechanism for GSC replacement in the *Drosophila* ovary (Xie & Spradling, 2000). From our observations of the rate of stem cell loss from the niche (loss of about half the stem cells over 2 weeks) and the rate of stem cell division (one per ~32 h), we might expect to see such symmetric divisions occurring in ~5% of dividing GSCs. Yamashita *et al*. (2003), however, reported seeing no such examples in over 500 male GSC divisions examined in testes from young flies. It is possible that such reorientation only occurs in older flies that have begun to lose a significant number of stem cells from their niche. An alternative possibility is suggested by the fact that differentiating spermatogonia have the ability to reenter the stem cell niche (i.e. move to lie adjacent to the hub) and dedifferentiate into functional GSCs (Brawley & Matunis, 2004). Although this behavior was observed in testes in which the niche had been depleted of stem cells due to a conditional mutation in one of the stem cell-promoting niche signals, it is possible that such dedifferentiation occurs as a normal consequence of aging as stem cells become lost from the niche.

Given the requirement for stem cells throughout the lifetime of the organism – and in the case of GSCs, into future generations – and given that stem cells appear to have mechanisms to prevent aging-related damage (Morrison *et al*., 1996a; Park *et al*., 2002; Zhou *et al*., 2002), it might seem surprising that the effects of aging can so readily be observed in multiple stem cell systems. One possibility is that even in stem cells, mechanisms to counter aging are not robust enough to completely prevent damage, and that in cells that have persisted into late adulthood such damage begins to have phenotypic consequences. The immortality of the germ lineage, however, demonstrates that such damage can in fact be effectively countered. A more likely explanation is that deterioration in supporting cell populations, in which such protective mechanisms are not as prevalent, negatively affects the activity of stem cells. In skeletal muscle, it appears that age of the surrounding environment affects stem cell behavior. If aged stem cells in an older mouse are exposed to circulating factors from a young mouse through parabiotic pairing, notch signaling, a key regulator of stem cell activity that declines with age, is restored, and skeletal muscle stem cell proliferation increases (Conboy *et al*., 2005). In a second example, in rodent testes, spermatogonial stem cells from aged mice can function normally when transplated into testes of younger mice (Ryu *et al*., 2006). Given the demonstrated importance of the somatic niche in regulating *Drosophila* GSC activity (reviewed in Yamashita *et al*., 2005), it is quite likely that aging of the stem cell environment in this tissue would play an important role in determining the activity of GSCs during aging. Here we have shown that the population of somatic hub cells decreases during aging, and we have previously found that compromising a EGFR/raf-dependent signal in the somatic cyst cells led to increased activity of the GSC population, as judged by prolonged marker gene activity and cycling (Tran *et al*., 2000). Exploring the activity of this and other previously described regulatory signals emanating from the soma (Kiger *et al*., 2000, 2001; Tran *et al*., 2000; Tulina & Matunis, 2001; Schulz *et al*., 2002) during the course of aging should provide much ground for future study.

Experimental procedures

Fly stocks and antibodies

Flies were maintained using standard techniques at 25 °C unless otherwise indicated. The following stocks were used: w^{1118}, yw *hsp70-Flp*; *P[w +; tub-FRT]*, *yw; P[y +; FRT-lacZ]* (Harrison & Perrimon, 1993), *w*; *mth*^{1}, *w*; *mthEx28* (Lin et al., 1998). Antibodies and dilutions used were rabbit anti-vasa (1 : 5000, gift of R. Lehmann), mouse anti-β-galactosidase (1 : 500, Sigma, St Louis, MO, USA), mouse anti-Fasciclin III (1 : 100, Developmental Studies Hybridoma Bank), rat anti-Brdu (1 : 1000, Accurate Chemical, Westbury, NY, USA), mouse anti-BrdU (1 : 4, Becton Dickinson, Franklin Lakes, NY, USA). Alexa-488-, Cy3-, and Cy5-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA and Jackson Immunoresearch, West Grove, PA, USA) were used at 1 : 400.

Clonal marking of GSCs

Male *yw*; *P[y +*; *FRT-lacZ]* flies were crossed to virgin female *yw hsp70-Flp; P[w +; tub-FRT]*. Young male progeny (0–2 days after eclosion) were collected and heat-shocked at 37 °C in a circulating water bath for 30 min. Males were aged in the presence of females at 25 °C. Either X-gal activity stains or immunofluorescence were used to detect lacZ expression. X-gal activity stains were performed as described (Gonczy *et al*., 1992). For immunofluorescence, dissected testes were fixed with 4% formaldehyde in PBS for 20 min and blocked for 1 h. Testes were incubated with primary antibodies overnight at 4 °C and secondary antibodies for 2 h at room temperature.

BrdU labeling

For short *in vitro* labeling, testes were dissected in Ringer's buffer and transferred to a tube containing 10 μм BrdU in Ringer's on ice. The pulse commenced with a shift to room temperature for 30 min. For *in vivo* pulse and chase, young adult males were anesthetized by CO_2, and injected intra-abdominally using a mouth pipet attached to an injection needle. The injection solution contained 50 mм BrdU in 5 mм KCl, 0.1 mм NaH_2PO_4, pH 6.8. Green food coloring was used to monitor injection, and, while the amount per fly was difficult to control precisely, 2–3 μL of labeling solution was loaded into the needle, and used up for 40 flies, thus averaging 0.05–0.075 μL per fly. Injected flies were either fixed after 3 h (pulse) or aged for 3 days and then processed (chase). To visualize BrdU incorporation, testes were fixed with 4% formaldehyde in PBS for 1 h and then blocked for 1 h. Primary antibody incubation was for 90 min at room temperature, diluted in 66 mм Tris pH 8, 2.66 mм $MgCl_2$, 1 mм β-mercaptoethanol and 50 units mL^{-1} DNAse I. Secondary antibody incubation was for 2 h at room temperature or overnight at 4 °C. Immunostained testes were mounted with ProLong Gold (Invitrogen), and visualized on a Zeiss LSM 510 confocal microscope (Zeiss Microimaging Inc., Thornwood, NY, USA). For continuous BrdU application, flies were fed yeast paste containing 5 mм BrdU. Flies were transferred onto freshly prepared yeast paste every 12 h. For BrdU pulse labeling, flies were injected intra-abdominally with 50 mм BrdU in PBS. Primary and secondary antibody staining and visualization was done as for *in vitro* labeling. Statistical analyses were performed using a two-tailed *t*-test.

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