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Germline stem cell aging in the *Drosophila* ovary

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The age-related decline of adult stem cells leads to loss of tissue homeostasis and contributes to organismal aging. Though the phenotypic hallmarks of aging are well-characterized at the organ or tissue level, the molecular processes that govern stem cell aging remain unclear. This review seeks to highlight recent research in stem cell aging in the *Drosophila* ovary and connect the discoveries in the fly to ongoing questions in stem cell aging.

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Introduction

As complex multicellular organisms age, organ function declines, leading to an overall reduction in health over time. The limited capacity for the adult soma to maintain itself contributes to the pathogenesis of diverse diseases and therefore limits health span. While resident populations of adult stem cells reliably support tissue homeostasis in young animals, stem cell dysfunction in old animals corresponds to the gradual loss of tissue homeostasis. Though experimental evidence has largely refuted the existence of a conserved, genetically encoded aging program, mechanistic theories of aging revolve around the pleiotropic antagonism hypothesis and the disposable soma theory. Pleotropic antagonism posits that if a given gene is beneficial during youth and deleterious in old age then it will persist in the gene pool. The disposable soma theory argues that overinvestment in somatic maintenance comes at the expense of germline resource allocation and would therefore be disadvantageous [1]. However, studies in nutritional geometry have elucidated that, though flies will opt for dietary nutrition that maximizes fertility, this does not come at the expense of total lifespan [2]. This would suggest a more nuanced, context-specific role of germline activity in *Drosophila* somatic aging.

In diverse eukaryotes, caloric restriction or pharmacological inhibition of nutrient-sensing pathways through drugs like rapamycin can extend lifespan, predominantly mediated by the Target-of-Rapamycin (Tor) and insulin signaling pathways [3–5]. The centrality of insulin and ecdysone signaling pathways in GSC proliferation and maintenance has been reviewed recently [6]. The *Drosophila* male and female gonads are among the best-characterized stem cell niches and have elucidated aspects of stem cell signal transduction, niche–stem cell interactions, and stem cell quality control mechanisms [7–9]. The *Drosophila* ovary is an especially valuable tool for aging research due to the presence of mitotically active maternal germline stem cells and somatic follicle stem cells [10]. Though it was previously believed that human oocytes were post-mitotic and incapable of proliferating after birth, researchers recently successfully isolated viable oocytes from mitotic germ cells taken from adult ovaries [11]. The relative longevity of the maternal germline allows for the comparison of aging in a theoretically immortal cell lineage (germline) right next to a somatic cell lineage (follicle). This conveniently facilitates interrogation of the conservation of stem cell maintenance and aging between somatic and germline stem cells. The effects of intrinsic, extrinsic, and systemic signaling in stem cell aging have been reviewed in the past [12–14,15•]. However, few have made use of an injury model of aging in order to investigate the earliest causes of stem cell dysfunction in aging animals.

Drosophila oogenesis efficiently illustrates several key principles of stem cell organization, maintenance, and aging [16]. Each ovary comprised ~18 ovarioles, each of which is a functional egg-producing unit. At the anterior apex of each ovariole, two to three germline stem cells (GSCs) retain their identity through contact with the somatic cap cells. The cap cells physically anchor the GSCs while providing them extrinsic signals such as decapentaplegic (Dpp) and glass bottom boat (Gbb) ligands to inhibit differentiation by activating BMP signaling and downstream transcription factor, Mothers against decapentaplegic (Mad), which transcriptionally represses bag-of-marbles (*bam*), a gene essential for differentiation. Similar to other stem cell types, the GSC divides asymmetrically to produce one stem cell and one transit-amplifying daughter called a cystoblast. Within region 1 of the germarium, somatic escort cells push the precursor to the presumptive oocyte posteriorly

as it undergoes four rounds of cell division with incomplete cytokinesis, forming a 16-cell cyst. When the cyst reaches the region 2a/2b border, the escort cells pass the cyst onto the multipotent follicle stem cells (FSCs) whose progeny encase the cyst. As the egg chamber moves into region 3, the follicle cells intercalate between neighboring cysts, culminating in stalk formation and separation of the stage 1 egg chamber from the germarium. The stage 1 egg chamber is remodeled into an orb, with the oocyte positioned at the posterior end of the egg chamber. This review will focus on the regulation of GSC aging at the cellular level, primarily in an injury model of aging.

Earliest signs of aging in the fruit fly ovary

Definitions of aging depend on the biological context. At the organismal level, aging can be inferred from performance in athletic and mental challenges. For instance, zebrafish aging can be monitored by swimming performance, which decreases with age at least partly due to telomere attrition-induced apoptosis of muscle cells [17,18]. In contrast to organismal aging, the age-related decline of organs is often measured by morphological and functional assays. Specifically, aged mouse intestines have a lower number of intestinal stem cells and reduced capacity to regenerate following irradiation, phenotypes that can be ameliorated in aged animals through Wnt agonism [19]. Though accumulated DNA damage is appreciated as a characteristic of aged cells, the cellular events that predispose certain stem cells to aging or longevity remain unclear, urging deeper understanding of the earliest observable consequences of aging.

A variety of fly models exist for studying the mechanisms of aging. In the ovary, the age-associated decline in fertility [1,20] and the effects of dietary nutrition on fecundity have been reviewed recently [2,21]. However, the first cellular events that cascade into the reduced fecundity observed in old flies remain elusive. DNA damage, epigenetic remodeling, and systemic metabolism can all contribute to stem cell senescence in aged animals [13,22]. Utilizing an injury model of aging, the *Drosophila* ovary provides a tractable system for investigation into the effects of aging on stem cell division and differentiation in an *in vivo* stem cell niche with similar characteristics to mammalian stem cell niches. Moreover, recent in-depth molecular characterization of FSC niche signaling and maintenance [23••] opens up the possibility for parallel analysis of both germline and epithelial stem cell regulation in the same tissue.

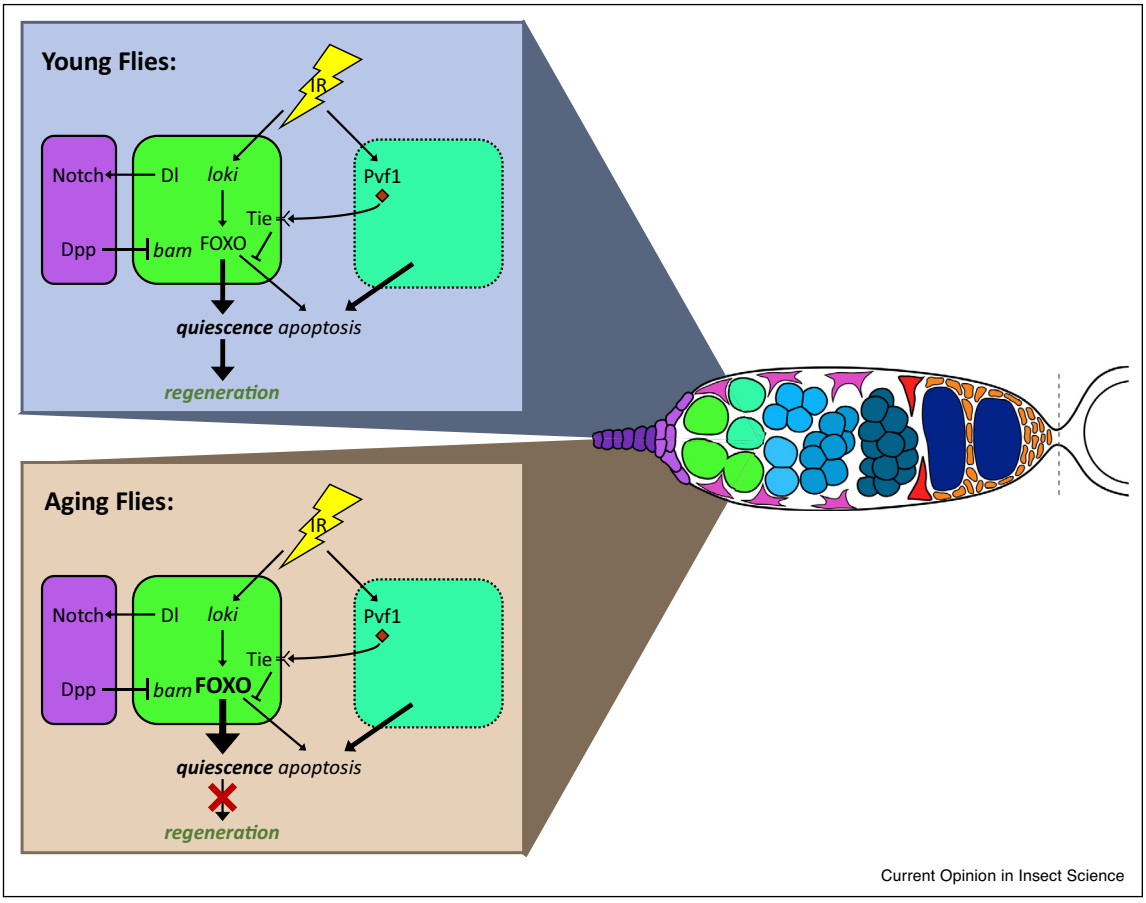
Stem cell quiescence is an important component of stem cell plasticity in adult animals and can allow for recovery from an acute stress that may have otherwise induced apoptosis [24]. Following gamma irradiation, GSCs will briefly pause cell cycle and become 'quiescent' before resuming cell cycle and regenerating lost progeny [25,26•]. Irradiation induces DNA damage, triggering a Loki/Chk2-dependent checkpoint in GSCs [27] (Figure 1). As shown by gamma-H2AV staining, DNA damage is high 30 min after irradiation and drops to moderate within 12 hours; DNA damage is almost completely eradicated [26•]. In young flies, cell division has fully resumed by 48 hours after injury — at least 24 hours after Loki activation, indicating the action of additional mechanisms of pausing regeneration. This injury model of aging was then used to determine the earliest signs of GSC aging. The big surprise in these analyses was that the earliest aging phenotype in GSCs is permanent quiescence. Four-week-old female GSCs retain the normal capacity to avoid apoptosis, enter quiescence and exit quiescence a day later, regenerating the ovary after injury. Conversely, six-week-old female GSCs resist apoptosis and enter quiescence after irradiation as usual, but they fail to re-enter cell cycle and regenerate the tissue [26•]. Therefore, the earliest sign of aging in *Drosophila* germline stem cells is not stem cell apoptosis or terminal differentiation, but failure to re-enter cell cycle and regenerate. These results demonstrate than an injury model of aging can reveal subtle differences in stem cell biology, thereby providing an opportunity to identify the earliest age-dependent changes that occur in regenerating stem cells.

A dynamic niche in the ovary

A necessary part of stem cell biology is the environment, or niche in which the stem cell resides. In stem cell biology, loss of niche adhesion is associated with stem cell aging and terminal differentiation [3,22,28]. In the *Drosophila* ovary, the anterior end of the GSC remains in contact with a somatic niche cells, also called cap cells that anchor the GSC in place and provide cytokines such as BMP ligands, Dpp and Gbb (Figure 1). The gradual decrease of BMP ligand secretion by the cap cell with increasing age contributes to stem cell loss [29] and, likely, declines in fertility in aging flies. Anteriorly, the GSC may be in contact with either escort cells or cystoblasts. Once a GSC or its progeny exits the niche, distance from the cap cell limits BMP ligand availability and promotes the *bam*-mediated differentiation program.

In contrast to the traditional model of stem cell biology in which the niche recruits and maintains the identity of its resident stem cells through cytokines, previous work has showcased that Dl ligand secreted by GSCs activates Notch receptors on cap cells in order to induce cap cell proliferation [30]. This role in niche establishment and maintenance places the GSCs in a more active role than passive niche occupancy. A group recently reported that enhanced YAP/Yorkie activity in the *Drosophila* testis promotes cap cell proliferation, suggesting a similar bilateral relationship [31•]. If YAP/Yorkie functions similarly in the female GSCs, then YAP/Yorkie could be acting in the GSCs to promote Dl ligand secretion. Today, it is not known whether Notch/Delta signaling is regulated in GSC aging, nor is it well understood how, if at all, Notch activation in the cap cells interacts with *dpp* ligand secretion for the maintenance of GSCs.

Figure 1



Female germline stem cells (green) maintain contact with the somatic cap cells (light purple) in order to anchor themselves apically. Germline stem cells promote Notch activation in the cap cell through secreted Dl ligand, and the cap cell promotes GSC identity through secreted Dpp ligand. Distance from the cap cells reduces Dpp ligand availability and promotes differentiation in the daughter cystoblast (aqua). Acute genotoxic insult such as gamma irradiation promotes *loki*/Chk2 activation and subsequently FOXO transcriptional activation. In young flies, FOXO is necessary for germline stem cell quiescence. Pvf1 ligand released by apoptotic daughter cystoblasts (aqua) activates Tie receptors on the germline stem cell and inhibits apoptosis. In young flies, FOXO-mediated quiescence is temporary and is followed by successful regeneration of the germline. Conversely, in aging flies, elevated FOXO leads to irreversible cell cycle exit, or senescence.

Following irradiation, GSCs evade apoptosis while GSC daughters, cystoblasts and more differentiated cysts, often succumb to apoptosis. Remarkably, it turns out that GSCs are protected by an 'SOS' signal sent by neighboring apoptotic daughter cells. The apoptotic GSC daughters release Pvf1, orthologous to angiopoietin, which binds to Tie receptor on the GSCs; Tie signaling activates *bantam* miRNA which binds and inhibits HID mRNA, limiting apoptosis in the mother GSC [25] (Figure 1). In mammals, Tie2 signaling is central to angiogenesis [32], making it an important pharmaceutical target for cancer treatment and organoid vascularization alike. This protective community effect mirrors the phenomenon of a small number of cancer stem cells surviving chemoradiation therapy only to metastasize later. Drosophila GSCs therefore serve as an excellent model to understand the dynamic cellular mechanisms that coordinate the successful evasion of apoptosis before returning to proliferation. Curiously, aged GSCs successfully evade apoptosis and repair DNA damage, but fail to re-enter cell cycle [26•]. These data refute the belief that DNA damage alone causes cellular senescence, and instead suggest that cell signaling and intrinsic factors such as epigenetic alterations may be serving as the brake on cell division.

Cell cycle arrest and senescence

DNA damage is a hallmark of aging, as well as a likely accelerant of the aging cascade that has been reviewed in the context of stem cell aging before [33]. If DNA damage is repaired within 24 hours after irradiation, then why do GSCs only exit quiescence 48 hours after irradiation? DNA damage upregulates FOXO [27], a transcription factor necessary for quiescence [26•], but it's unclear

which genes under regulation by FOXO are necessary for quiescence. FOXO target genes include Tor signaling repressors, cell cycle repressors, and metabolic genes [34]. However, *dacapo*, *Drosophila* ortholog of p21, is not necessary for irradiation-induced quiescence, indicating that G1 arrest is likely not the mechanism by which FOXO is enabling quiescence [26•]. Enticingly, Tor knockdown is sufficient to impair the exit of quiescence and the return to proliferation. Taken together, these results suggest that FOXO effects GSC quiescence by repressing Tor signaling, and that Tor signaling is derepressed in order to exit quiescence.

FOXO transcription factors are promising candidates for genes that may play conserved roles in aging across diverse higher order eukaryotes, as FOXO levels tend to increase in aged tissues and may have evolved in order to protect against oncogenic transformation. Though FOXO appears to be largely protective in a young fly, higher levels of FOXO in aged flies results in an inability to return to cell division after quiescence [26•] (Figure 1). Such a genetic interaction, in which young animals benefit from the cyto-protection associated with a gene that in aged animals leads to senescence, fits the theory of pleiotropic antagonism, in which genetically encoded aging programs are not selected against in a population because they're advantageous in youth, when fecundity is highest and sexual selection is the most relevant. The molecular mechanisms by which FOXO leads to reversible cell cycle arrest in young animals and senescence in old animals are unknown, but may rely on metabolic remodeling. There is precedent in mammalian stem cells, as FOXO3A helps maintain quiescent hematopoietic stem cells through activating autophagy [34]. It's plausible that FOXO-mediated epigenetic modifications and/or FOXO-mediated metabolic remodeling may be involved in the cell autonomous choice to re-enter cell cycle or not.

Cellular metabolism

One of the most functionally relevant causes and effects of aging is accumulated protein damage [3,13]. Studies in dental pulp stem cells *in vitro* have shown that metabolic signature can predict age-related senescence [35], supporting the notion that metabolism regulates stem cell aging. As such, protein turnover through proteasome degradation and macroautophagy, hereby referred to as autophagy, may be critical to slowing the tick of each cell's biological clock. On the other end of protein metabolism, protein synthesis is governed by Tor signaling, which can sense intracellular amino acids and reactive oxygen species (ROS) [4]. Tor signaling inhibits autophagy, and Tor repression often activates autophagy [36]. Due to the centrality of FOXO in GSC quiescence and Tor in the exit from quiescence, it follows that autophagy would be high during quiescence in response to FOXO activity and repressed Tor signaling. The functional relevance of autophagy in GSC survival and quiescence following irradiation has yet to be studied.

In addition to catabolic processes, the mitochondrial network serves as a key regulatory hub in stem cell aging. In addition to housing the machinery for oxidative metabolism, the mitochondria also regulates transcription via epigenetic cofactors such as *S*-adenosylmethionine and α-ketoglutarate [22,37]. Additionally, GSCs are believed to asymmetrically segregate newer mitochondria towards the daughter cell, allowing for the accumulation of old, damaged mitochondria in the GSC over time and contributing to intrinsic aging. This decline in GSC mitochondrial quality may not be detrimental to off-spring, given recent work demonstrating that eight-cell germ cell cysts undergo a mitochondrial remodeling event in which in fission of the mitochondrial network precedes a preferential expansion of healthy mitochondria [38••], presenting one possible mechanism for rejuvenation of the presumptive oocyte in order to reset the aging clock. However, mitochondrial dysregulation in the GSC may still influence GSC proliferation and differentiation cell-autonomously, such that they contribute to the eventual loss of GSCs seen in the aged *Drosophila* ovary. It is generally accepted that four-cell and eight-cell cysts retain the capacity to dedifferentiate and reoccupy the stem cell niche given the proper cues [39,40]. Indeed, innate stem cell heterogeneity is hypothesized to be a facet of stem cell plasticity and regeneration [41]. In the context of genotoxic insult from gamma irradiation, a dedifferentiated GSC that has already remodeled its mitochondria may be functionally different from a GSC that has not left the niche in its lifetime. Further research is needed to connect mitochondrial dynamics to regulation of GSC metabolism and epigenetic state.

Future perspectives

The *Drosophila* ovary provides a tractable model for the study of regeneration in aged adult animals. By synthesizing the cutting-edge genetic tools available to the *Drosophila* community with the canon of knowledge on *Drosophila* oogenesis, stem cell biologists and aging researchers alike have the opportunity to ask very precise questions of the mechanisms of stem cell quiescence and regeneration. For example, the recent generation of GeneSwitch GAL4 lines in the stem cell niche provides a less harsh alternative to heatshock for temporal transgene expression, allowing for previously unfeasible investigation into the regulation of GSC aging by cap cells [42••]. Alternatively, the production of UASz, a purported replacement to the popular UASt for somatic expression and UASp for germline expression, enables efficient transgene expression in both germline and somatic tissues [43]. Should these tools be integrated with other common fly tools, then more precise and stronger target gene expression can be achieved for discerning the molecular regulation of stem cell aging.

Furthermore, advance *in vivo* reporter lines, such as those for mitochondrial ROS and autophagic flux open the possibility of real-time analysis of proteostasis and redox homeostasis at the single cell resolution [44••]. Live cell imaging and optogenetics have been successfully employed to study mitochondrial dynamics in germline stem cells and their progeny [38••]. Perhaps taking inspiration from groundbreaking parabiosis experiments in mammals [45], it may be possible to study stem cell aging by transplanting ovaries into female flies with no germline, such as *ovoD* females [46] of different ages in order to probe the effects of systemic factors in stem cell behavior and aging. A comparison between transplanted GSCs and FSCs will help disambiguate how circulated factors affect different stem cell types.

In this review, we characterize a highly sensitive injury model of aging using *Drosophila* female germline stem cells and discuss some of the major insights into the molecular mechanisms of aging learned from this system. Moreover, we propose a framework for addressing future inquiries into stem cell aging and regeneration in the *Drosophila* ovary.

Conflict of interest statement

Nothing declared.

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