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Regulation of Stem Cells in Their Niche

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Abstract Stem cells are characterized by their ability to asymmetrically divide, generating another self-renewing stem cell and a differentiating daughter cell. They reside within the local niche microenvironment, which, together with systemic signals, regulates intrinsic stem cell function. Stem cells are finely regulated, with even slight deregulation leading to gain of stem cell function and tumorigenesis or loss of stem cell function and tissue degeneration in aging. In this review, we highlight how stem cells are maintained within their niche and what is known about their deregulation in aging. To highlight these points, we look at the *Drosophila* male germline stem cell niche as a model system, specifically focusing on cell cycle progression, signaling pathways, epigenetics, and the control of spindle orientation by centrosomes. Finally, we sum up pertinent questions to be addressed in understanding stem cell function and their malfunction in aging.

Keywords Stem cells · *Drosophila* male germline · Cell cycle · Centrosomes · Aging · Epigenetics · Signaling

Introduction

Stem cells are characterized by two properties, namely their ability to self-renew and their potency in forming other cell types, which is achieved through the process of asymmetric division where a stem cell divides to produce another self-renewing stem cell and a daughter cell that is one step closer to differentiation. The number of steps required prior to terminal differentiation varies for different tissues [1]. Stem cells are first encountered in the embryo where totipotent stem cells that can divide to form all cell types of an organism give rise to a fully functioning adult.

In adults, certain tissues and organs retain unipotent stem cells that only differentiate to form mature cells of one lineage. They thus partake in homeostatic cell replacement and tissue regeneration, maintaining organ function throughout an organism's lifetime. As an organism ages, tissues and organs start to deteriorate. One of the postulated hallmarks of aging is stem cell exhaustion, which can include both the loss of stem cells from their niche, as well as a decline in their regenerative potential, and is usually the combined consequence of different factors [2]. However, the underlying mechanisms leading to this loss of stem cells remain poorly understood.

How stem cells are regulated and the changes in aging remain unclear. Stem cells reside within a microenvironment known as the niche. The stem cell niche provides extrinsic extracellular cues, which then initiate intrinsic changes to control cellular dynamics through which stem cells are maintained in their undifferentiated and self-renewable state [3]. As a stem cell leaves its niche, either due to the axis of division, loss of adhesion or space constraints, it is then exposed to differentiation signals, altering internal genetic programs, which guide it to form particular cell lineages. Thus, dissecting the intrinsic, extrinsic, and systemic factors that regulate stem cells and their alterations during aging requires a genetically tractable model system [4].

The fruit fly *Drosophila melanogaster* is a well-established model organism with biological mechanisms and pathways that are conserved with mammals. Research in recent years

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has identified various stem cell systems in developing as well as adult flies. One of these is the male germline stem cell (GSC) niche, which is quite well described, thus making it suitable for the study of stem cells, the factors that maintain them and their deregulation in aging.

Establishment and Structure of the Stem Cell Niche

Fertilization between the ovum and sperm results in a totipotent zygote, which develops into a complete organism. During development, various stem cell niches are established, providing anatomical and functional spaces for adult stem cells. Stem cell establishment usually precedes or coincides with niche formation and involves a series of actions through which non-self-renewing precursor cells acquire stem cell characteristics.

The establishment of the male GSC niche in *Drosophila* starts at stage 13 to 14 of embryogenesis, when primordial germ cells (PGCs) and somatic gonadal precursors (SGPs) come together to form the testis. Two populations of SGPs exist, *Abdominal-B*-specified male specific SGPs (msSGPs) at the posterior end of the testis and *escargot* expressing SGPs at the anterior end [5]. It is the escargot expressing SGPs that then give rise to the embryonic hub. At stage 13 of embryogenesis, all germ cells throughout the testis display JAK-STAT (janus kinase-signal transducer and activator of transcription) activation. However, at stage 17, JAK-STAT activation is restricted to the germ cells at the anterior end of the testis where hub cells secreting the JAK-STAT ligand, Upd, reside [5]. Studies have shown that JAK-STAT signaling is essential for conferring stemness to the PGCs [5]. By the middle of stage 17 embryos, these initial GSCs are already observed to divide with a plane perpendicular to the hub, typically characteristic of adult male GSCs.

As mentioned above, at the tip of the testis lies a cluster of 10 to 18 somatic post-mitotic cells forming the hub. In young adults, these hub cells are surrounded by 8 to 15 GSCs, each of which is flanked by a pair of somatic cyst stem cells (CySCs). Asymmetric division of the GSC produces a differentiating daughter gonialblast (GB), which is also surrounded by a pair of cyst cells (CyCs) formed from CySCs. The GB then initiates differentiation, undergoing four rounds of mitotic division with incomplete cytokinesis, resulting in the stepwise formation of 2-, 4-, 8- and 16-cell interconnected spermatogonial cysts (Fig. 1a) [6]. The 16 interconnected spermatogonia then differentiate into spermatocytes, the whole process lasting around 2 days. Spermatocytes enter into an extended G2-meiotic prophase I transition lasting 3.5 days, during which they undergo DNA synthesis, increasing 25-fold in volume and initiating an extensive transcription program to prepare for spermatid formation [7]. From metaphase I onwards, spermatocytes rapidly undergo 2 meiotic divisions generating a cyst of 64 haploid spermatids, which mature to form motile sperm [8].

The Cell Cycle in *Drosophila* Male GSCs

Although general cell cycle components and progression are well characterized and conserved across many different species and cell types, the exact regulation of cell cycle progression in GSCs still remains to be elucidated. The cell cycle is composed of four successive phases: mitosis (M), gap 1 (G1), synthesis (S), and gap 2 (G2), each characterized by specific events, and culminating in the successful division of a cell (Fig. 1b). At the transition between phases, various switch-like checkpoints exist to ensure that preceding cell cycle events are correctly completed before allowing later events to occur [9].

Cell cycle progression involves the complex interplay of various cyclin-dependent kinases (CDKs) and cyclins. CDKs contain a protein kinase subunit, the activation of which requires binding of a partner cyclin and an activating phosphorylation on a threonine residue in the vicinity of its active site. In the classical model of cell cycle control, specific cyclin-CDK combinations facilitate the transition between the different phases (Fig. 1b), with the main events controlled by CDK1, whose activation promotes entry into mitosis, and APC, whose activation drives exit from mitosis [10]. Although the cell cycle network is well characterized, cell type specific differences remain to be discovered.

In addition to cyclins and CDKs, other major regulator proteins include Wee1, Cdc25, and Plk1. Wee1 inactivates cyclin-CDK1 by phosphorylation of a tyrosine residue (Y15) and a threonine residue (T14) to restrain entry into mitosis. On the other hand, the protein phosphatase Cdc25 dephosphorylates CDK1 on these residues to initiate G2/M transition and thus entry into mitosis. Both Wee1 and Cdc25 are regulated by signaling pathways controlling G2/M-transition, thus providing a feedback regulatory mechanism to ensure accurate cell cycle progression [9].

There has been an emergence of research linking the cell cycle to stem cell states and fates. Certain stem cells possess a cell cycle uniquely different to that of differentiating cells, leading to the question of how and what role the cell cycle plays in stem cells [11, 12]. In the *Drosophila* testis, GSCs divide frequently in an asynchronous manner. Previous studies show them to divide every 12–16 h with mitosis lasting ~30 min [13, 14] (Fig. 1b). At any given time, ~75 % of GSCs were observed to be in the G2 phase, while ~3–4 % of GSCs were in mitosis [15]. After mitosis, GSCs enter an extremely short G1 phase. Both GSCs and GBs then appear to synchronously enter S phase without undergoing cytokinesis, with cytokinesis being completed at early G2 [16].

The components of the cell cycle appear to be conserved in the male GSC niche. In mutants of cyclin B, which binds to

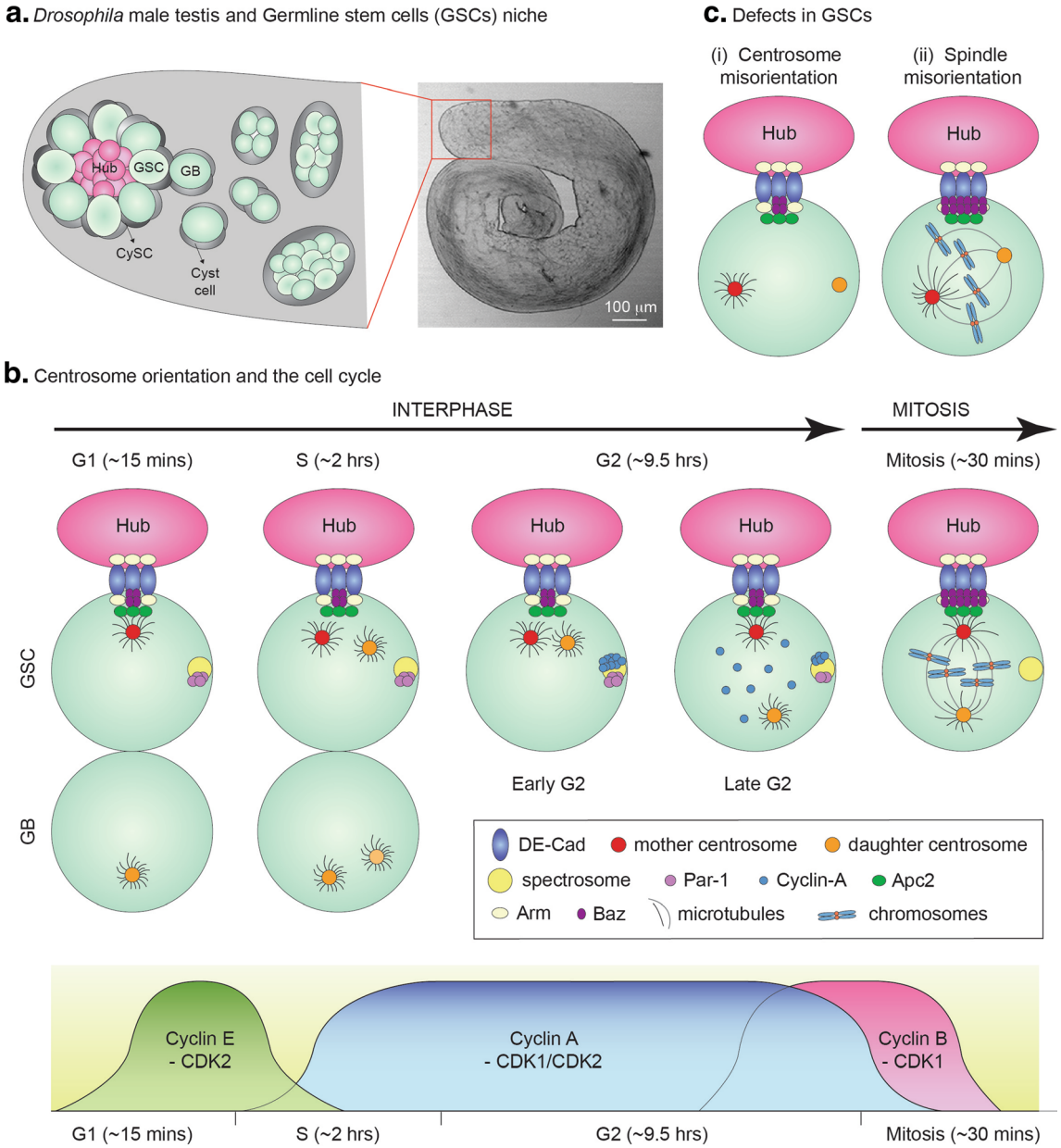

Fig. 1 **a** A young *Drosophila* testis with the male germline stem cell niche highlighted by a *red box* and shown on the *left*. Germline stem cells (*GSCs*) surround post-mitotic hub cells to form a rosette structure. These GSCs then asymmetrically divide to form 2-, 4-, 8- and 16-cell interconnected spermatogonial cysts, which undergo meiosis and maturation to form sperm. **b** Coordination of centrosome orientation in GSCs with cell cycle progression. The mother centrosome remains in the vicinity of the hub-GSC interface, while the daughter centrosome moves away to the hub-distal pole to be segregated into the daughter GB upon cellular division. Various components of the centrosome orientation checkpoint (*COC*) and the changes they undergo during the cell cycle are shown. The corresponding changes in cyclin-dependent kinases (*CDKs*) and cyclins are also shown in the graph at the *bottom*. Cyclin E-CDK2 initiates S phase. Cyclin A and either CDK1 or 2 then plays a role in the continuation of S phase and B-type cyclins, together with CDK1 is responsible for entry into mitosis. **c** (*i*) Centrosome misorientation where neither centrosome is located in the proximity of the hub-GSC junction. (*ii*) Centrosome misorientation and inactivation of the COC results in spindle misorientation during mitosis

CDK1 to initiate entry into mitosis, a reduction and loss of GSCs is observed. However, testicular somatic cells do not seem to require cyclin B as cyst and hub cells appear normal in cyclin B mutants. The observed defects in the GSCs could not be rescued by cyclin A or cyclin B3, thus showing the non-redundant functions of different cyclins [17]. String (Stg), the *Drosophila* homolog of Cdc25, is highly expressed in GSCs and CySCs and rapidly downregulated in their differentiating daughters (GBs, spermatogonia and cyst cells, CC). It is required in both GSCs and CySCs where increase or decrease in

Stg activity in each stem cells affects its neighboring stem cell depicting the complex interplay between stem cells in their niches [18].

Together, these studies illustrate the complexity of the cell cycle in different stem cells within their niche, and how the cell cycle of a stem cell influences and controls neighboring stem cells. The male GSC niche thus provides a simplified system to study this in detail.

Centrosomes and the Plane of Cell Division

Centrosomes regulate mitotic spindle orientation to determine the plane of cell division thus ensuring accurate stem cell division. The *Drosophila* male GSCs in their niche provide one of the best-characterized systems to study this. In male GSCs, the mitotic spindle is oriented perpendicular to the GSC-hub interface, thus ensuring that one daughter cell stays in contact with the hub to receive sustaining signals and maintain its stemness, while the daughter cell is displaced away from the niche and initiates differentiation. Various checkpoints are in place to halt cell division when the requirements are not met for accurate cell division. One of these checkpoints includes the centrosome orientation checkpoint (COC), which monitors centrosome orientation and functions during the G2-M transition of the cell cycle.

Centrosomes, highly conserved organelles that are the main microtubule organizing centers (MTOCs) of cells, play a role in establishing the stereotypical spindle orientation in GSCs. During G1, a single centrosome is situated at the hub-GSC interface [14]. This centrosome duplicates, and by G2, separates. The mother centrosome, which is faithfully retained by GSCs across many cellular divisions, remains in the vicinity of the hub-GSC interface, while the daughter centrosome moves away to the hub-distal pole to be segregated into the daughter GB upon cellular division [19••]. However, mother and daughter centrosomes possess different characteristics. Hub-distal daughter centrosomes have very little associated microtubules during interphase, allowing their free movement around the GSC and developing robust microtubule asters during mitosis. Hub proximal mother centrosomes have robust microtubule arrays even during interphase, with astral microtubules extending towards the adherens junctions, composed of DE-cadherin and Armadillo, which provide a scaffold for the localization of Apc2 (Adenomatous Polyposis Coli 2), thus enabling astral microtubule docking to orient centrosome positioning (Fig. 1b).

The COC in GSCs ensures correct centrosome orientation before mitosis, where in misoriented GSCs, where neither centrosome is located adjacent to the hub, cell cycle progression is delayed until centrosome positioning is re-oriented, thus preventing the formation of misoriented spindles. Various components of the COC have been described. Among them is the polarity protein Bazooka (Baz), also known as Par-3 [20, 21]. Baz-centrosome association is potentially the key physical event monitored by the COC in addition to Baz being required for centrosome orientation itself. Baz forms a confined ~1.5 μm subcellular structure (Baz-patch) at the hub-GSC interface during interphase. Docking of the apical centrosome at the Baz-patch occurs during late G2 of the cell cycle, coinciding with Par-1-dependent phosphorylation of Baz at serine 151 (S151). Upon entry into mitosis, Baz localization diffuses, phosphorylation signals become undetectable, and Baz-centrosome docking could no longer be defined. Centrosome-Baz association is thus interpreted by GSCs to indicate "correct centrosome orientation" resulting in the inactivation of the COC and allowing mitotic progression (Fig. 1b) [16].

Another COC component is the cell polarity gene par-1, which links the COC to cell cycle progression. Par-1 localizes to the spectrosome during interphase, with diminished localization during mitosis. Cyclin A is barely detectable during the G1/S stage of the cell cycle, while in G2, cyclin A levels increase, initially localizing to the spectrosome, then spreading to the cytoplasm. At prophase to prometaphase, cyclin A is observed both at the nucleus and in the cytoplasm, and by metaphase cyclin A is degraded, signifying the onset of anaphase. In wild type flies, cytoplasmic cyclin A is not observed when centrosomes are misoriented. However, in *par-1* mutants, cytoplasmic cyclin A is observed even when centrosomes were misoriented, with a corresponding increase in spindle misorientation. Par-1 thus acts as a gatekeeper of the COC, keeping cyclin A at the spectrosome when centrosomes are misoriented. When centrosomes reach the right orientation, Par-1 releases cyclin A to the cytoplasm and nucleus, allowing entry into mitosis (Fig. 1b) [22].

Apart from the COC, the SAC (spindle assembly checkpoint) also exists in GSCs and monitors the tension at kinetochores generated by microtubule attachments during the metaphase-anaphase transition [23]. Together, these checkpoints ensure that correct centrosome and spindle orientation is achieved to accurately segregate chromosomes and cell fate determinants for asymmetric cell division.

Epigenetic Regulation of Stem Cells

Epigenetics refer to a wide range of phenomena, which result in a usually heritable change of gene expression without affecting primary DNA sequence. *Drosophila* male GSCs show a unique segregation of epigenetic cues, where existing H3 histones are distinctly retained by GSCs, while newly synthesized H3s are partitioned into differentiating GBs [24]. This is achieved by phosphorylation of existing H3 at threonine 3, preceding mitotic entry. During mitosis, phosphorylated H3 is then separated into GSCs, while newly synthesized, unphosphorylated H3 is separated into GBs [25••]. The

Haspin protein, which also regulates mitotic spindle polarity [26, 27], has been shown to be potentially responsible for this phosphorylation [28].

It should be noted that although histones are non-randomly segregated based on age, the genetic material they package does not necessarily share the same fate. While X and Y chromosomes are non-randomly segregated between GSCs and GBs, the autosomes (chromosomes 2 and 3) appear to be randomly segregated [29•]. The proteins cnn, KOI, KLAR and Dnmt2 play a role in this segregation pattern, where cnn is an essential centrosomal protein also partaking in the COC, and KOI and KLAR are components of the LINC (linker of nucleoskeleton and cytoskeleton) complex connecting the nucleus to the cytoskeleton [30–32]. Dnmt2 is postulated to facilitate non-random X and Y chromosome segregation through DNA sequence independent modifications during gametogenesis in parent flies [33]. It would thus be interesting to elucidate the mechanisms correlating this difference in segregation of histones and sister chromatids.

Another histone modifier, Enhancer of Zeste (E(z)), is also involved in maintaining male germline identity through its involvement in the Polycomb repressive complex 2 (PRC2) [34]. E(z), a histone methyltransferase, trimethylates lysine 27 of histone H3 (H3K27me3) [35]. H3K27me3 has then been shown to be enriched at the gene locus of Zinc-finger homeodomain protein 1 (Zfh-1), which marks CySCs and early cyst cells. Interestingly, E(z) functions in CySCs in a non-cell autonomous manner to convert early stage-germ cells (GSCs, GBs, and/or 2-cell spermatogonia) to cyst cells. It has thus been hypothesized that lineage conversion occurs through E(z)-mediated changes to EGFR and Wg signaling between both germ- and cyst-cells, as H3K27me3 is enriched at the gene loci of components of both pathways, with removal of one copy of egfr (EGF receptor) sufficient to suppress E(z) null phenotypes [36].

Little imaginal discs (lid) is a transcriptional repressor that demethylates H3k4me3, which is generally associated with transcriptionally active genes [37, 38]. In male GSCs, lid cell - autonomously regulates JAK-STAT activity through the control of Stat92E transcript levels to maintain GSC number and mitotic index and prevent premature GSC differentiation [39]. While lid is required for GSC maintenance, ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome (dUTX) regulates the proper behavior of CySCs and early cyst cells, preventing them from overpopulating the niche, thus maintaining the proper architecture of the niche. dUTX regulation of cyst cells is exerted through its demethylase activity that abolishes trimethylation on lysine 27 of histone H3 (H3K27me3) at the gene locus of Socs36E, which suppresses JAK-STAT signaling [40].

Undoubtedly, epigenetics play important roles in the *Drosophila* male GSC niche. However, much work is still needed to clarify how they regulate and maintain the different stem cells in the GSC niche.

Signaling Pathways in the *Drosophila* Male GSC Niche

Signaling pathways are activated through binding of specific ligands to transmembrane receptors that initiate intracellular signaling cascades, resulting in the up- or down-regulation of target genes, thus controlling protein levels and activation to alter cellular dynamics and determine cell fate and activity.

The JAK-STAT pathway is well characterized in the *Drosophila* male GSC niche with pathway components shown to be involved in regulating cell-cycle progression [41]. JAK-STAT pathway activation requires binding of its secreted ligand Unpaired (either Upd1, Upd2 or Upd3) to its transmembrane receptor Domeless (Dome), which undergoes a conformational change, allowing the phosphorylation of the intracellular JAK kinase homolog Hopscotch (Hop). This allows the binding of the cytoplasmic STAT homolog Stat92E to the activated Dome-Hop complex, leading to phosphorylation of Stat92E, which dimerizes and translocates to the nucleus to control target gene expression [42].

Hub cells produce and secrete glycosylated Upd, thus restricting its diffusion from the hub [43]. This is crucial in the accurate maintenance and self-renewal of both GSCs and CySCs. Loss of JAK-STAT activity in GSCs and CySCs lead to their loss from the hub, while ectopic expression of Upd was sufficient to increase the number of GSC- and CySC-like cells. JAK-STAT activity is differentially regulated in the three cell types of the niche. CySCs express higher levels of the JAK-STAT signaling inhibitor, suppressor of cytokine singling 36E (SOCS36E) in comparison to GSCs [44]. High SOCS36E expression levels are also observed in the hub cells. Thus, although Upd secreted from hub cells binds to the JAK-STAT receptor Dome in all cell types of the niche, higher levels of JAK-STAT pathway activation are observed in GSCs. This is required for ensuring a proper ratio of the GSC and CySC lineages in the niche [45]. In GSCs, JAK-STAT signaling mediates hub-GSC adhesion via DE-cadherin, and reduced pathway activation has been shown to result in mislocalization of DE-cadherin and GSC loss from the hub.

In addition to JAK-STAT signaling, BMP signaling also plays an essential role in preventing GSCs from differentiating [46, 47]. This is achieved through its ligands Gbb (Glass bottom boat) and Dpp (Decapentaplegic), which are secreted from hub cells and CySCs to initiate BMP signaling in GSCs. BMP signaling in GSCs represses expression of the differentiation factor *bam* thus maintaining GSCs and their ability to self-renew [48]. Interestingly, recently identified nanotubes, which are microtubule-based structures, were shown to specifically play a role in BMP signaling in GSCs. Nanotubes extend into the hub, possibly allowing GSCs to selectively access the high threshold level of BMP ligand necessary for self-renewal [49•].

Other signaling pathways are also involved in regulation of the *Drosophila* male GSC niche, including the Insulin-, TOR (target of Rapamycin)-, EGFR (epidermal growth factor receptor)-, MAPK (mitogen-activated protein kinase)-, and Hh (Hedgehog)-signaling pathways [50–53]. However, how these pathways interact with each other to regulate male GSCs is beyond the scope of this review.

Deregulation of the *Drosophila* Male GSC Niche in Aging

How the young *Drosophila* male GSC niche is maintained is well characterized. Recent studies have started elucidating the changes observed in this system upon aging, thus providing a firm foundation on which to further explore how stem cells age in vivo.

The male GSC niche undergoes progressive aging leading to a gradual decrease in overall function. In the initial stages of aging, germ cell numbers decrease, resulting in the shrinkage of overall testis size. There is however no drastic change in GSC numbers. Instead, this decline is caused by a decrease in the rate of GSC asymmetric divisions, due to a reduction in cell cycle activators and increased centrosome misorientation [13].

Stg is the *Drosophila* homolog of Cdc25, a phosphatase that activates CDKs. During initial aging (day 20), the levels of Stg decrease in GSCs. Surprisingly, this decrease in Stg is not observed in neighboring CySCs, which highlights the differences in stem cell maintenance, even within the same niche. GSCs exhibit decreased Stg and cell division while maintaining its numbers, whereas CySCs decrease in numbers yet show no decrease in Stg levels or division rate. It should also be noted that at this stage of aging, the decrease in Stg levels did not lead to centrosome misorientation [18].

Decreased GSC division rates are also caused by centrosome misorientation, which increases to its maximum by day 30. This level of centrosome misorientation is observed even in 50-day-old flies. Centrosome misorientation is defined as the condition where neither centrosome is situated next to the hub. Misoriented centrosomes then lead to arrest of GSCs or a transient cell cycle delay. GSCs with misoriented centrosomes rarely overcome the G1/S transition to progress on to mitosis, and thus misoriented spindles are rarely observed. Concurrently, misoriented GSCs divide less frequently. However, upon correction of centrosome orientation, GSCs re-enter cell cycle. Research has shown misoriented GSCs to arise from dedifferentiated spermatogonia (Fig. 1c) [13, 54].

Aging also leads to a decrease in Upd expression by hub cells, resulting in a loss of GSCs from its niche. This is due to the decrease in levels of the IGF-II messenger RNA binding protein (Imp), which functions by preventing endogenous siRNAs present in hub cells from degrading *upd* RNAs. The decrease in Imp is caused by an increase in *let-7* miRNA during aging which destabilizes *Imp* through seed sequences on *Imp's* 3'UTR. Constitutive expression of *upd* only in hub cells could restore the GSC numbers in aging. However, these GSCs showed reduced proliferation, lower than that of aged controls, and the flies displayed decreased lifespan [55].

Despite the wealth of studies on epigenetics in aging as a whole, epigenetic changes in the male GSC niche during aging are not well studied. However, the epigenetic regulators of the male GSC niche do play a role in lifespan at the whole organism level. Mutations in E(z) lead to an increased lifespan in flies [56]. Male Lid mutants possess shortened lifespans [57]. In fact, overexpression of RBR-2, the orthologue of *Drosophila* Lid, in the germline of *C. elegans* led to an increased lifespan, giving rise to the notion that H3k4me3 demethylation in the germline can maintain somatic cells [58]. Overexpression of histones H3 and H4 extends the replicative lifespan of yeast, leading to the question of whether asymmetric segregation of old and new H3 in the male GSC niche are affected in aging [59].

The above-mentioned aspects highlight changes observed in the aging male GSC niche. However, many questions still remain unanswered. Although increased centrosome misorientation in aging leads to a delayed cell cycle, it is not well elucidated how and if that is the only cause leading to delays in cell cycle progression. In addition, despite JAK-STAT signaling having been shown to play a role in the cell cycle, it is also not well elucidated if and how this is connected to centrosome misorientation and delayed cell cycle progression. Finally, what are the epigenetic changes observed in the male GSC niche? Further studies are thus warranted to identify if these changes observed in aging are causative or rather just coincidental, and how they interact with and influence each other, leading to aging.

Conclusions

Recent years have seen a huge interest in stem cell research and its potential therapeutic possibilities [60]. Despite what is already known, much remains to be studied about how systemic, extrinsic, and intrinsic factors coordinate to regulate stem cell function. The fruit fly *Drosophila melanogaster* provides a simplified model organism to study these in vivo, providing fundamental concepts which can be further extrapolated to accelerate research in mammalian systems. This will be greatly helpful in facilitating medical applications of stem cells by improving safety, efficacy, and cost, with the end aim of revolutionizing medicine to benefit mankind.

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Compliance with Ethical Standards

Conflict of Interest Li Ming Gooi and Jay Gopalakrishnan declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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