Regulation of adult stem cell behavior by nutrient signaling

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Adult stem cells play an essential role throughout life, maintaining tissue and organ function by providing a reservoir of cells for homeostasis and repair. Maintenance and activity of adult stem cells have been the focus of numerous studies that have revealed stem cell-intrinsic factors and signals from the local microenvironment that regulate stem cell behavior. A growing body of work has provided evidence that circulating, systemic factors also contribute to the regulation of stem cell behavior in numerous tissues. We have demonstrated that *Drosophila* male germline stem cells (GSCs) and intestinal stem cells (ISCs) respond to changes in nutrient availability, specifically amino acids. Furthermore, we have shown that insulin signaling plays an important role in mediating the effects of changes in nutritional conditions. Notably, insulin signaling is cell-autonomously required within male GSCs for maintenance. Here we discuss our data regarding the effects and mechanisms by which changes in systemic nutritional conditions may influence the maintenance and activity of adult stem cells via insulin signaling.

Introduction

Many adult tissues are actively maintained by stem cells,¹⁻³ which are defined by their ability to divide asymmetrically to generate new stem cells (self-renewal) as well as daughter cells that undergo differentiation. The decision between self-renewal and differentiation is regulated by intrinsic factors, extrinsic signals from the local microenvironment (niche) and systemic signals originating from other tissues. Organisms utilize a range of strategies to

cope with adverse environmental conditions, such as extreme fluctuations in temperature or scarcity of food, until favorable conditions resume;⁴⁻⁷ thus, it is likely that stem cell behavior is regulated by systemic signals to coordinate an appropriate, tissue-specific response to chronic changes in metabolism. To begin to explore this question, we have investigated how stem cells respond to chronic nutritional stress, specifically amino acid deficiency.

Nutritional Stress Induces Reversible Losses of Active Stem Cells

In *Drosophila*, early studies indicated that egg production dramatically declines in response to poor nutritional conditions, due to reduced proliferation of both germline and follicle stem cells and an increase in apoptosis in the ovary.^{8,9} However, it was unclear whether such responses were specific to oogenesis or represented conserved mechanisms shared by other adult stem cells as a response to nutritional stress. Recently, we demonstrated that stem cells in the *Drosophila* testis and intestine both respond dynamically to changes in nutrient availability.¹⁰

In the *Drosophila* testis, germline stem cells (GSCs) and cyst stem cells (CySCs) reside at the tip, in close contact with a group of non-dividing somatic cells known as the apical hub. Hub cells express and secrete the self-renewal factor Unpaired (Upd), which activates the JAK-STAT pathway in adjacent stem cells to specify their maintenance.¹¹⁻¹³ Testes from flies that were fed a diet lacking protein (protein starvation) became progressively thinner and displayed a dramatic reduction in spermatogenesis. This was

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Figure 1. Starvation-induced changes are distinct from aging-related changes to the niche. (A) Quantification of the average number of GSCs per testis in flies fed (dark green) or starved (light green) for 15 d. Genotypes analyzed were *w*, *upd-GAL4* (hub driver) outcrossed to *w¹¹¹⁸* (*upd-GAL4/+*) and *w*, *upd-GAL4, UAS-GFP; UAS-upd, TM2*. Starvation-induced GSC loss was not significantly different between the two genotypes using two-way ANOVA. Error bars represent 95% confidence interval of the mean. Number of testes analyzed for each genotype is as follows: *upd-GAL4/+* fed *n* = 36, starved *n* = 37; *upd-GAL4, UAS-GFP; UAS-upd, TM2* fed *n* = 27, starved *n* = 35. (B and C) Immunofluorescence images of the testes from flies fed (B) or starved (C) for 15 d and stained with antibodies against E-cadherin (E-cad). Note expression of E-cad in hub cells is not affected by starvation. (D) Immunofluorescence image of the testis containing *dlnR³³⁹* mutant clones stained with antibodies against FasIII (green), GFP (green), STAT92E (red) and DAPI (blue). Arrow indicates a GFP⁺ *dlnR³³⁹/dlnR³³⁹* mutant GSC (D') that is also positive for STAT92E (D''). Arrowhead indicates a wild-type (GFP) GSC that is also STAT92E⁺. Scale bars, 20 μ m.

accompanied by a significant decrease in the average number of GSCs per testis, as well as a decrease in GSC proliferation. The number of early cyst cells in the testis, including CySCs, exhibited similar behavior in response to starvation.¹⁰ In contrast to what was observed

in the ovary,^{8,9} no significant increase in apoptosis was detected in testes of starved males, and inhibition of apoptosis did not prevent GSC loss upon starvation,¹⁰ suggesting that stem cell loss occurs through alternative mechanisms, such as direct differentiation.¹⁴

To determine whether spermatogenesis could return to baseline levels in starved flies when optimal nutritional conditions were restored, starved flies were placed onto normal food (refed). Soon after refeeding, testes increased in size, and GSCs and spermatogonia repopulated the testis. The number of GSCs and early cyst cells increased to levels seen in flies that were continuously fed,¹⁰ demonstrating their ability to respond quickly to changes in nutrient availability.

Testes from starved flies morphologically resembled testes from significantly older flies raised on a normal diet, as both showed a decrease in spermatogenesis, fewer GSCs and a slower rate of GSC proliferation.^{15,16} Therefore, we speculated that changes similar to those associated with aging, such as reduced expression of the self-renewal factor *upd* or the cell-cell adhesion molecule E-cadherin (E-cad) in hub cells,¹⁵ could be occurring upon starvation to contribute to loss of GSCs and reduced spermatogenesis. However, constitutive overexpression of *upd* in hub cells, which was sufficient to suppress GSC loss during aging,¹⁵ did not prevent GSC loss upon starvation (Fig. 1A), suggesting that diminished secretion of self-renewal signals is unlikely to be the major cause of loss of GSCs upon starvation. In addition, we observed no decrease in E-cad levels within hub cells in testes from starved males (Fig. 1B and C).

Because GSCs are specialized unipotent stem cells that are required for proper transmission of genetic information to the next generation, we wanted to determine whether the behavior of GSCs represents a unique strategy to protect stem cells under severe stress or a general mechanism utilized in other adult tissues. Therefore, we also examined the response of *Drosophila* intestinal stem cells (ISCs) to starvation. In the *Drosophila* midgut, active ISCs divide to self-renew and give rise to daughter cells called enteroblasts (EBs), which do not divide again but undergo terminal differentiation.^{17,18} Both ISCs and EBs express a gene called *escargot* (*esg*), which can be used for their identification. We observed significantly fewer *esg*⁺ ISCs/EBs in the midguts from flies that were starved when compared with guts from age-matched flies that were continuously

fed. In addition, the frequency of actively dividing ISC, identified by the mitotic marker phosphorylated histone H3, was significantly lower in starved flies, similar to what was observed for GSCs in the testis. Strikingly, the number of actively dividing ISCs increased dramatically within 24 h of refeeding, to a level considerably higher than in flies that were never starved.¹⁰ These data indicate that ISCs in the posterior midgut are sensitive to protein availability and can quickly respond to changes in systemic nutrient availability.

Given that *esg* is not restricted to ISCs, it was unclear whether the decrease in *esg*⁺ cells represented a selective decrease in ISCs or EBs or whether both stem cell and daughter cell numbers were reduced similarly. In order to discriminate between ISCs and EBs, we used a reporter expressed in EBs in combination with *esg*. The Notch (N) signaling pathway is required for proper differentiation of EBs: the N ligand Delta (Dl) is expressed in ISCs, which results in activation of N signaling in EBs, as revealed by the activation of a Su(H) reporter construct [Su(H)-*lacZ*]. Therefore, we quantified the number of ISCs by measuring the number of *esg*⁺/β-galactosidase (β-gal) cells in midguts from flies exposed to our starvation paradigm (Fig. 2A). Consistent with our previous findings, we found that the total number of both ISCs and *esg*⁺ cells in the posterior midgut (PMG) was significantly reduced upon starvation; the decrease in total ISCs was likely due to a dramatic shrinkage of the entire PMG, as the density of *esg*⁺ cells appeared to remain unchanged (Fig. 2B and E and data not shown). Upon a short period of refeeding, the midgut returned to normal size, and the total number of ISCs and *esg*⁺ cells quickly increased to a level comparable to the number observed in flies that were never starved (Fig. 2C–E).

Taken together, our findings indicate that reducing the pool of active stem cells available for homeostasis and repair may represent a conserved mechanism utilized by different tissues in *Drosophila* to cope with adverse nutritional conditions. Once favorable nutritional conditions return, stem cell number and activity increases, leading to robust tissue recovery. A similar

Figure 2. The total number of intestinal stem cells (ISCs) and *esg*⁺ progenitor cells in the posterior midguts (PMG) respond dynamically to nutrient availability. (A–D) Immunofluorescence images of PMGs from *GbeSu(H)lacZ; esg-GFP* flies stained with antibodies against GFP to mark ISC/EBs (green), antibodies against βGAL to mark activated Notch signaling (red) in EBs and DAPI to mark DNA (blue). ISCs are identified as GFP⁺/βGAL⁻ cells as indicated by arrowheads in (A) and its inset showing the βGAL channel only. Guts shown were from newly eclosed flies fed for 3 d as control (A), control flies that were starved for additional 15 d (B), control flies that were continuously fed for additional 15 d (C) or control flies that were starved 15 d then re-fed for 4 d (D). Scale bars, 50 μm. (E) Quantification of the total number of ISCs and *esg*-GFP⁺ cells in the PMGs from flies described in (A–D). To estimate the total number of cells per PMG, the number of ISCs and *esg*-GFP⁺ cells in each immunofluorescence image were measured, normalized to a defined gut area, then adjusted to the total area of each PMG. Number of guts examined, from left to right: 32, 32, 32, 31. Error bars represent 95% confidence interval of the mean. **p < 0.01 by Student t-test.

phenomenon was reported in the nematode *C. elegans*,⁴ where GSCs were able to recover after a period of prolonged starvation, suggesting that the plasticity of tissue stem cells might represent an evolutionarily conserved mechanism employed by animals to adapt to fluctuations in nutritional conditions.

Insulin-Mediated Direct Regulation of Adult Stem Cells

Numerous studies have indicated that stem cells within a variety of tissues and species are influenced by insulin/IGF signaling.¹⁹⁻²³ The insulin signaling pathway is conserved in *Drosophila*, which encodes a single insulin/IGF-like receptor (*dInR*) and seven insulin-like peptides (*dILPs*) in its genome.²⁴⁻²⁶ Experiments have clearly demonstrated that insulin signaling directly regulates the rate of GSC proliferation in the *Drosophila* ovary^{9,25,27} and regulates the maintenance of female GSCs indirectly by preservation of the niche.²¹ ISC in the *Drosophila* midgut are also sensitive to insulin signaling, as *dInR* mutant flies show a decrease in pHH3 staining, and homozygous *dInR* mutant ISCs fail to proliferate following injury.^{19,28} However, no effect on maintenance of ISCs has been reported.

In our recent study, we demonstrated that *dInR* is required cell-autonomously for GSC maintenance in the *Drosophila* testis, suggesting that male GSCs are competent to receive and respond to insulin signaling directly. Our data are consistent with a previous study showing that male flies that are deficient in insulin signaling have fewer GSCs and spermatocytes that are arrested in the G₂/M phase of the cell cycle.²² In addition, our studies demonstrated a role for dInR in regulating GSC maintenance independently of the JAK-STAT pathway. GSC clones homozygous mutant for the null allele of *dInR* strongly expressed STAT92E (Fig. 1D), the downstream transcriptional effector of the JAK-STAT pathway that is critical for stem cell maintenance.¹¹⁻¹³ As STAT92E is a target of itself in the testis, these data indicate that *dInR* mutant GSCs are still capable of responding to Upd secretion from the hub. Therefore, diminished JAK-STAT activation within GSCs is unlikely to be

the cause of the loss of maintenance of *dInR* mutant GSCs.

To determine whether the effects of starvation on stem cells is mediated by diminished insulin signaling, we measured the levels of dILPs in starved flies. Three dILPs, *dILP2*, *dILP3* and *dILP5*, are expressed in insulin-producing cells (IPCs) in the brain, secreted into the circulating hemolymph, acting to regulate InR activity systemically.^{24-26,29} We showed that expression of *dILPs 2* and *5*, the most abundant dILPs produced by IPCs, decreased in the heads of starved flies.¹⁰ The level of *dILP3* expression increased, which is consistent with a previous report describing compensatory increases in *dILP3* in response to loss of *dILPs 2* and *5*.³⁰

While a significant source of dILPs is in the brain, expression of *dILP5* and *dILP7* has been reported in the ovary and female reproductive tract, where they regulate the onset of vitellogenesis and coordinate the strong and swift response of the female reproductive program to nutrition.^{31,32} In addition, *dILP6* was found to be predominantly expressed in the fat body, a functional equivalent of the vertebrate liver and adipocytes.³³ To examine whether any dILPs are expressed in the male gonad and could act as a local source to influence GSC behavior, we examined testes from flies carrying *dILP* reporter lines. No expression of *dILP5* or *dILP7* was detected in the testis (data not shown); however, both *dILP2* and *dILP3* are expressed in somatic cyst cells that are encapsulating spermatocytes and differentiating cysts at the basal end of the testis (Fig. 3A, B and B'). Decreases in both *dILP2* and *dILP3* expression were observed in testes from starved flies (Fig. 3C). Furthermore, we also assayed expression of the translational inhibitor 4E-binding protein (4E-BP), as it is a well-characterized downstream target of the transcription factor dFoxo, which is inactivated by insulin signaling.³⁴ Consistent with a drop in insulin signaling, *4E-BP* transcript levels increased in the testes of starved flies, indicative of Foxo activation (Fig. 3D). Upon refeeding, we observed an increase in *dILP2* and *dILP3* expression levels (Fig. 3C) and a decrease in the expression level of *4E-BP* (Fig. 3D),

reversing the gene expression changes that occurred during starvation.

Therefore, our studies suggest that decreased production of both brain-derived and locally produced dILPs, as a consequence of starvation, likely contributes to a reduction of dInR signaling in GSCs in the testis, which leads to a loss of GSCs and a decrease in their proliferation rate. It would be interesting to dissect the relative contribution of local dILPs vs. brain-derived dILPs in regulating tissue stem cells upon changes in nutritional conditions.

Non-Autonomous Regulation of Stem Cells by Insulin Signaling

To directly test whether decreased insulin signaling leads to GSC loss upon starvation, we activated insulin signaling specifically in the testis using a constitutively activated form of dInR (*dInR^{CA}*). Expression of endogenous dInR was detected at the tip of the testis in early germ cells and in hub cells with an enrichment at the interface between the hub and GSCs. Consistent with this, we observed only a mild rescue of GSC loss in starved flies when *dInR^{CA}* was expressed in early germ cells. In contrast, expression of *dInR^{CA}* in both hub cells and early germ cells resulted in a robust suppression of GSC loss upon starvation.¹⁰ Therefore, although GSCs can receive and respond to InR signaling directly, hub cells likely play an important role in sensing fluctuations in dILP levels and coordinating changes in GSC behavior through secondary signals.

In accordance with these observations, we observed strong expression of dFOXO, a critical downstream target of the insulin signaling pathway,^{26,29} in hub cells (Fig. 4B). To test whether dFOXO plays a role in regulating GSC behavior upon starvation, we analyzed *dFOXO* mutant flies for GSC number and their response to starvation. Newly eclosed flies mutant for *dFOXO* had fewer male GSCs under normal feeding conditions, and the number of GSCs did not decline in response to starvation (Fig. 4A). To rule out non-specific effects caused by loss of dFOXO in other tissues, we inactivated dFOXO locally in the hub cells via RNAi-mediated

Figure 3. *dILP* expression in the *Drosophila* testis. (A) Immunofluorescence image of a testis (genotype *w; dILP2-GAL4;UAS-GFP^{NLS}*) showing GFP⁺, *dILP2* expressing cells (green) and DAPI marking DNA (blue). Apical tip (*), mitotic amplification zone (bar), sperm (arrowhead), late cyst cells expressing *dILP2* (arrows). Scale bar, 100 μ m. (B and B') Immunofluorescence image of the basal end of a testis (genotype: *w; dILP3-LacZ*) stained with antibodies against β -galactosidase (β -gal, green) to highlight *dILP3*-expressing cells, Eyes absent (*Eya*; red) to mark late cyst cells and DAPI (blue). (B') β -gal expression. *Eya*⁺/ β -gal⁺ late cyst cell (arrowhead). *Eya*⁺/ β -gal⁺ late cyst cell (arrow). Scale bars, 50 μ m. (C and D) Quantitative RT-PCR (qPCR) showing relative mRNA abundance for *dILP2* and 3 (C) and *4E-BP* (D) in testes from fed, starved and refed flies. mRNA levels were normalized to *GAPDH2* and expression is shown relative to the level in fed flies at 20 d (C) or to the level in fed flies at 15 d (D). Error bars represent the statistical range across triplicate measurements.

knockdown, which also led to fewer GSCs in the testis that appear to be insensitive to starvation (Fig. 4A). It is not clear whether *dFOXO* is required for the maintenance of GSCs, as fewer GSCs in *dFOXO* mutant flies could be a result of developmental defects. More specific tests of *dFOXO* function during adulthood are needed to address these questions.

Taken together, our data suggest that insulin signaling directly regulates male GSC proliferation and maintenance and also indirectly regulates GSC maintenance by acting on local support cells. This is similar to the manner in which *dInR* directly regulates the integrity of the female GSC

niche to non-autonomously impact female GSC maintenance. Specifically, *dInR* signaling affects the number of somatic niche support cells in the ovary, known as cap cells, and expression of cell-cell adhesion molecules between cap cells and GSCs.^{21,35} The exact mechanisms underlying the non-autonomous regulation of GSCs by insulin signaling in the testis remain to be identified.

Future Directions

Interestingly, we found that an extended period of protein deprivation did not lead to any further decline in the number of

GSCs per testis. A similar phenomenon was reported for *C. elegans* adult reproductive diapause, during which a fixed number of GSCs appear to remain, even after a long period of starvation.⁴ How are the remaining stem cells selected from a pool of stem cells before starvation? Is it a stochastic process, and are the remaining cells more resilient to stress? Regardless of whether an active selection mechanism is involved, the small number of remaining stem cells must be protected from further loss. How do they survive extended period of protein deprivation? Answering these questions will not only shed light on the basic mechanism of how stem cells adapt

Figure 4. Inactivating *dFOXO* results in fewer GSCs in the Drosophila testis that are insensitive to starvation. (A) Quantification of the average number of GSCs per testis in flies fed (dark green) or starved (light green) for 20 d. Flies analyzed were control (*dFOXO*^{21/+}), transheterozygous *dFOXO* mutants (*dFOXO*^{21/dFOXO}²⁵) and those expressing an RNAi transgene against *dFOXO* in the hub using the *upd*-GAL4 driver (*upd* > *dFOXO*^{RNAi}). The total numbers of testes analyzed (n) were: control fed n = 37, starved n = 33; *dFOXO*^{21/dFOXO}²⁵ fed n = 26, starved n = 42; *upd* > *dFOXO*^{RNAi} fed n = 32, starved n = 32. Error bars represent 95% confidence interval of the mean. Asterisks indicate statistically significant difference (*p* < 0.001) using Student *t*-test. (B) Immunofluorescence image of testes, in which early germ cells are expressing GFP (green; B and B'') stained with antibodies that recognize *dFOXO* (red; B and B''). *dFOXO* are expressed in germ cells, including GSCs (GFP⁺, arrows), in somatic cells (GFP, arrowheads) and in the hub (asterisk). Note the strong enrichment of *dFOXO* in the hub (B''). (Genotype: *w*;UAS-GFPmCD8;*nanos*-GAL4).

to nutritional stress, but will also provide insights for the therapeutic use of stem cells in the course of regenerative medicine, particularly in cases where patients may be suffering from metabolic disease.

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