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MINI-REVIEW

Hedgehog in the *Drosophila* testis niche: what does it do there?

Zhao Zhang✉, **Chenyu Pan, Yun Zhao**✉

State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
✉ Correspondence: yunzhao@sibcb.ac.cn (Y. Zhao), zhangzhao@sibcb.ac.cn (Z. Zhang)
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ABSTRACT

Stem cell niche is a specialized microenvironment crucial to self-renewal. The testis in *Drosophila* contains two different types of stem cells, the germline stem cells and the somatic cyst stem cells that are sustained by their respective niche signals, thus is a good system for studying the interaction between the stem cells and their hosting niche. The JAK-STAT and BMP pathways are known to play critical roles in the self-renewal of different kinds of stem cells, but the roles of several other pathways have emerged recently in a complex signaling network in the testis niche. Reports of independent observations from three research groups have uncovered an important role of Hedgehog (Hh) in the *Drosophila* testis niche. In this review, we summarize these recent findings and discuss the interplay between the Hh signaling mechanisms and those of the JAK-STAT and BMP pathways. We also discuss directions for further investigation.

KEYWORDS *Drosophila*, testis, stem cell, Hedgehog, JAK-STAT

INTRODUCTION

Stem cells are a type of undifferentiated cells that have unique abilities to renew themselves and differentiate into specialized cell types while maintaining a stable population. Stem cells are critical during embryonic development, adult organ homeostasis, and tissue repair. Germline stem cells (GSCs) are adult stem cells responsible for production of gametes. The proper maintenance of GSCs depends on surrounding microenvironment, or the niche, in which multiple signals instruct stem cells to either renew themselves or differentiate (Li and Xie, 2005; Jones and Wagers, 2008). Advances in the knowledge of niche-stem cell interaction have important implication for regenerative medicine. In higher organisms, the regulatory network underpinning niche-stem cell interaction is still not well understood due to its intrinsic complexity. In this regard, the *Drosophila* testis offers a good system to address this issue because it harbors one of the best characterized stem cell niches, in which GSCs and somatic cyst stem cells (CySCs) co-exist (Fuller and Spradling, 2007). This system ensures that the number and the relative ratio of GSCs and CySCs are kept steady from one individual fly to another, thus providing an excellent model for studying multiple signaling pathways, including those of the bone morphogenetic protein (BMP) and Janus kinase-signal transducer and activator of transcription (JAK-STAT) that controls this homeostasis (Issigonis and Matunis, 2011). Here, we review several recent findings that generate insights into the role of Hh signaling in the *Drosophila* testis niche. We also discuss several remaining questions for future investigation.

THE MECHANISM OF HH SIGNALING AND ITS FUNCTION IN THE *DROSOPHILA* OVARY

The Hh pathway plays an important role in cell growth, patterning and stem cell maintenance during metazoan development (Jiang and Hui, 2008). Hh ligands are morphogens whose concentration gradient provides positional cues that dictate target gene expression (Heemskerk and DiNardo, 1994; Tabata and Kornberg, 1994). In *Drosophila*, Hh binds its receptor Patched (Ptc) (Ingham et al., 1991; Chen and Struhl, 1996) and co-receptor iHog/Boi (Yao et al., 2006, 2010; Zheng et al., 2010) to relieve an inhibitory effect of Ptc on Smoothened (Smo), a signal transducer with seven transmembrane domains. Hh induces phosphorylation and stabilization of Smo at the plasma membrane (Denef et al., 2000; Taipale et al., 2002; Jia et al., 2004; Zhang et al., 2004; Apionishev et al., 2005), leading to

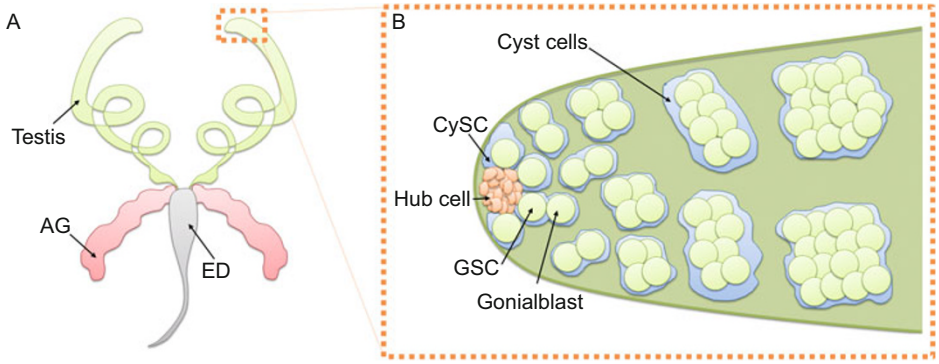

Figure 1. The structure of the reproductive system and stem cells in male *Drosophila*. (A) A cartoon of the reproductive system of a male *Drosophila*, which harbors a pair of testes which produce sperms, and accessory glands (AG) which produce seminal fluid components that mix with the sperm to protect and preserve them. Semen passes through ejaculatory duct (ED) during mating. (B) An enlarged view of testis tip where GSCs and CySCs are arranged around the hub cells.

a conformational switch at the C-terminus of Smo (Zhao et al., 2007). This in turn promotes the dimerization of a downstream kinase, Fused (Fu), causing it to be phosphorylated and thereby activated, and leading to phosphorylation of kinesin Costal2 (Cos2) (Therond et al., 1996; Nybakken et al., 2002; Ruel et al., 2007; Shi et al., 2011; Zhang et al., 2011; Zhou and Kalderon, 2011; Ranieri et al., 2012). The signal is further propagated to the pathway transcription factor, Cubitus interruptus (Ci), which is phosphorylated and converted into a truncated, 75-kDa Ci repressor (Ci75) in the absence of Hh. (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998; Methot and Basler, 1999). Hh signal stimulates the formation and activation of the full-length, 155-kDa Ci activator (Ci155), which enters the nuclear and turns on target gene expression.

Previous studies in *Drosophila* ovary showed that Hh signaling mainly functions in the somatic stem cells (Forbes et al., 1996a, 1996b; King et al., 2001; Zhang and Kalderon, 2001). Hh protein is secreted by the terminal filament cells and cap cells, which are present at the anterior tip of the germarium, to regulate the self-renewal of follicle stem cells, a kind of somatic stem cell functions to differentiate into follicle cells to encapsulate passing cysts (Margolis and Spradling, 1995; Forbes et al., 1996a; Zhang and Kalderon, 2001). Balanced Hh pathway activity is critical for normal egg chamber formation. Inactivation of Hh signaling reduces the number of follicle stem cells and stops egg chamber budding (Forbes et al., 1996a), while overactivation of Hh signaling leads to over-proliferation of follicle stem cells, which accumulate between egg chambers (Forbes et al., 1996a, 1996b; Zhang and Kalderon, 2001). In both kinds of situation, GSCs could not develop properly to form mature eggs, suggesting a critical role of Hh signaling in female egg formation. The stem cell niche of ovary is defined by several signaling pathways, including BMP, JAK-STAT, Wnt and Hh signaling. Using follicle stem cells as a model, it has been shown that Hh, JAK-STAT and Wnt signaling cooperate to define an intersection of gradients of long-range niche signals which regulates the behavior of stem cells (Vied et al., 2012). A recent paper reported that Hh regulates the expression of BMP family ligands to maintain GSCs in *Drosophila* ovary, extending the roles of Hh signaling in the GSC niche (Rojas-Rios et al., 2012). As some similarities are shared by stem cells in different systems (Decotto and Spradling, 2005; Losick et al., 2011), these observations in the ovary suggested a possible role for Hh signaling in the testis system.

DROSOPHILA TESTIS, A TALE OF TWO STEM CELLS

Adult male *Drosophila* harbors a pair of testes that are a tube-like coiled structure attaching to seminal vesicle (Fig. 1). At the apex of the testis tube, 10–15 non-mitotic somatic cells form a tightly folded structure called the hub. About 10 GSCs, which are round shaped, make direct contact with the hub cells to form a rose-like structure. GSCs undergo asymmetric divisions, one of the daughter cells remains GSC, and the other one (gonialblast) detaches itself from the hub cells and begins to differentiate. 16-cells spermatogonial clusters are formed after four mitotic cycles with incomplete cytokinesis. Spermatogonia differentiate into spermatocytes, which undergo meiosis to finally form sperm. Two irregularly shaped CySCs enwrap one GSC and make a narrow contact with the hub cells. CySCs differentiate into cyst cells, which enwrap and accompany GSC daughter cells during spermatogenesis. Both of GSCs and CySCs require niche for self-renewal. CySCs rely on the hub cells for niche signal, while the niches of GSCs are composed of signals from both the hub cells and CySCs (Voog et al., 2008; Issigonis and Matunis, 2011).

JAK-STAT signaling is critical to stem cell maintenance in the *Drosophila* testis (Kiger et al., 2001; Tulina and Matunis, 2001). The ligand Unpaired (Upd) is produced by the hub cells and received by GSCs and CySCs to activate the transcriptional factor STAT (Stat92E in *Drosophila*), which enters the nucleus to turn on different targets in GSCs and CySCs. STAT activation in CySCs leads to the expression of *zfh-1* (Leather-

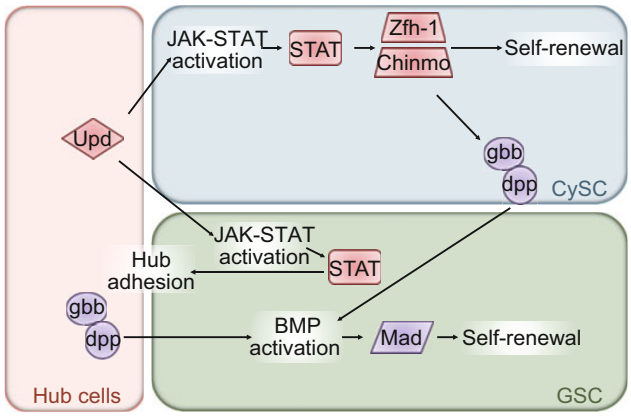

Figure 2. JAK-STAT signaling and BMP signaling in the regulation of stem cells in *Drosophila* testis. JAK-STAT ligand Upd is secreted by hub cells and received by both CySCs and GSCs. STAT activation in CySCs leads to expression of *zfh-1* and *chinmo* to regulate the self-renewal of CySCs, while activated STAT in GSCs only regulates the hub adhesion of GSCs. Hub cells and CySCs derived BMP ligands, gbb and dpp, are received by GSCs which is critical for the GSC self-renewal.

man and Dinardo, 2008) and *chinmo* (Flaherty et al., 2010), two factors sufficient for CySC self-renewal. JAK-STAT signaling does not primarily regulate self-renewal of GSCs, but governs niche adhesion (Leatherman and Dinardo, 2010; Singh et al., 2010). GSC self-renewal requires BMP signaling activation during which Mothers against dpp (Mad) is phosphorylated and translocates to the nucleus to repress transcription of the differentiation factor *bag of marbles* (*bam*) (Affolter and Basler, 2007). Two BMP ligands, Decapentaplegic (Dpp) and Glass bottom boat (Gbb) are secreted from the hub cells, as well as CySCs with activated JAK-STAT signaling (Shivdasani and Ingham, 2003; Kawase et al., 2004; Schulz et al., 2004) (Fig. 2).

HH SIGNALING PATHWAY, A NEW PLAYER IN THE *DROSOPHILA* TESTIS

Although a Hh enhancer trap fly named hh^{p30} (*hh-lacZ*) was observed a specific and strong lacZ expression in the hub cells 17 years ago (Forbes et al., 1996a), the knowledge of Hh signaling in the testis is still poor. Until very recently, three groups reported their independent findings to draw a picture of a functional and critical role of Hh signaling in the testis (Michel et al., 2012; Amoyel et al., 2013; Zhang et al., 2013). In a fashion similar with the role of Hh signaling in the ovary, Hh ligands are secreted by somatic cells in both ovary and testis system (cap cells in the ovary and hub cells in the testis). Although GSCs tightly attach to the Hh secreting cells (hub cells) in the testis, Hh ligands are not received by GSCs. Hh signaling regulates the self-renewal of somatic stem cells (follicle stem cells in the ovary and CySCs in the testis). Hh signaling contributes to the GSC niche through regulating BMP signaling (Rojas-Rios et al., 2012; Zhang et al., 2013). The revealed roles of Hh signaling in the testis extend the conserved regulation of Hh signaling in stem cell biology.

HH IS ONLY RECEIVED BY CYSCS TO REGULATE ITS SELF-RENEWAL, NOT GSCS

Both GSCs and CySCs make direct contact with hub cells, Hh proteins derived by hub cells are specifically received by CySCs. The components of Hh signaling, Ptc, Smo and Ci are detected in CySCs (Michel et al., 2012; Amoyel et al., 2013; Zhang et al., 2013). By using lacZ enhancer trap flies or GFP reporter flies, the transcriptions of *ptc* and *ci* are shown to exist in CySCs (Michel et al., 2012; Amoyel et al., 2013; Zhang et al., 2013). *Ptc* expression responds to different Hh signaling activity in a similar way with Hh cascade in the wing disc, suggesting that Hh signaling in CySCs is functional (Zhang et al., 2013). Clonal assay showed that CySCs loss of *smo* are unable to self-renew and begin to differentiate into Eya positive daughter cells, while CySCs loss of *ptc* over-proliferate (Michel et al., 2012; Amoyel et al., 2013; Zhang et al., 2013). Those results indicate that a proper activity of Hh signaling is critical for CySC maintenance. Manipulation of Hh signaling in GSCs by RNAi or clonal assay has no effect on GSC number, which is consistent with the absent of Hh signaling components in GSCs (Michel et al., 2012; Amoyel et al., 2013; Zhang et al., 2013). In summary, the independent findings of three groups lead to a consistent conclusion that Hh signaling regulates CySC number and maintenance in a cell-autonomous manner.

THE RELATIONSHIP BETWEEN HH SIGNALING AND JAK-STAT SIGNALING

In CySCs, JAK-STAT signaling is the primary pathway to regulate self-renewal through targeting on *zfh-1* (Leatherman and Dinardo, 2008) and *chinmo* (Flaherty et al., 2010). What is the relationship between the emerging Hh signaling and JAK-STAT signaling? Two groups (Amoyel et al., 2013; Zhang et al., 2013) investigated it with different approaches and got a similar conclusion that Hh signaling and JAK-STAT signaling work in parallel to regulate CySC self-renewal, with minor differences. Amoyel et al. (2013) found that the readouts of JAK-STAT and Hh signaling are not affected by each other. MARCM assay showed that ectopic activation of the JAK-STAT signaling cannot rescue CySCs lacking *smo*. Hh activation is not sufficient for the maintenance of CySCs loss of *Stat92E* or *chinmo*, though the CySCs differentiation is delayed. They finally argued that Hh signaling and JAK-STAT signaling may target on different factors to regulate the self-renewal of CySCs (Fig. 3). Zhang et al. (2013) showed that Stat92E activity is not altered by different Hh signaling activity. Genetic interaction experiments were performed with *Stat92E temperature sensitive* (*ts*) and UAS-Smo mutants. Activated Smo rescues the CySCs number by loss of *Stat92E*, while overexpression of Smo dominant negative form in $Stat92E^{ts}$ background causes a complete loss of CySCs number. On the other hand, Stat92E overex-

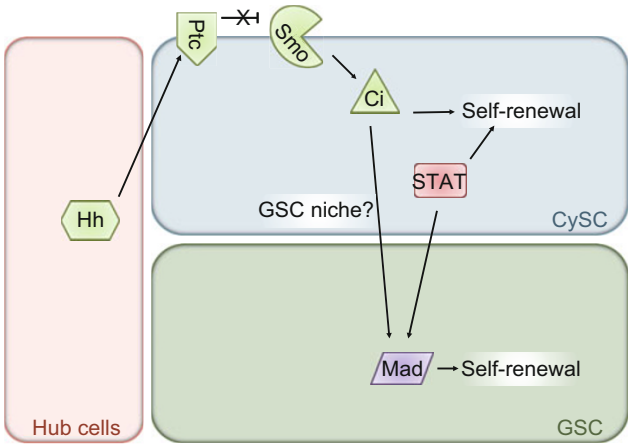

Figure 3. The emerging role of Hh signaling in the testis. Hh ligands derived from hub cells are received by CySCs. Activated Hh signaling regulates the self-renewal of CySCs, which is independent of STAT activity. BMP signaling in the GSCs are regulated by Hh activity in the CySCs, thus dual roles of Hh signaling in the testis niche are revealed.

pression partially rescues CySC loss induced by *smo* RNAi. In summary, they indicated that Hh signaling might share similar self-renewal factors with JAK-STAT signaling (Fig. 3). The diversity of conclusions comes from different approaches used, indicating the complexity of interactions between signaling pathways than expected.

THE CONTRIBUTION OF HH SIGNALING TO GSC NICHE

CySCs play another role to function as a part of GSC niche, probably through regulating BMPs expression (Kawase et al., 2004; Leatherman and Dinardo, 2008, 2010). Hh signaling regulates the self-renewal of CySCs, raising a possibility that Hh signaling in CySCs contributes to niche function. All of the three groups observed that loss of Hh signaling in CySCs or whole testis results in the reduced GSC number (Michel et al., 2012; Amoyel et al., 2013; Zhang et al., 2013). Based on the observations that Hh signaling is not received by GSCs, the GSC loss is likely a secondary effect of Hh signaling in CySCs. Different views were pointed out on the niche function of Hh signaling. Amoyel et al. (2013) found that overexpression of Ci active form (Ci^{act}) cannot rescue GSC loss in $Stat92E^{ts}$ testes. They conducted that Hh signaling does not contribute to the GSC niche. Zhang et al. (2013) showed that BMP signaling in GSCs is affected by Hh signaling activity from CySCs. They also found that activated Hh signaling in CySCs reduce GSC number, probability through cell competition. Hh signaling thus plays dual roles in testis niche. Facing those two different conclusions, people may want to compare their experiment details. They used different approaches to activate Hh signaling, one is Ci^{act}, another one is Smo^{SD123}. Though both of them are shown to activate Hh signaling in wing discs (Price and Kalderon, 2002; Jia et al., 2004), the extent of pathway activity is not even, especially in testis. Further investigation is needed to test the relationship between the degree of Hh pathway activity and niche function.

SUMMARY AND PERSPECTIVES

The emerging role of Hh signaling in testis draws a beautiful image of how different signaling pathways work together to maintain the homeostasis of stem cell niche. Hub cells derived Hh ligands are received by cyst cells to primarily regulate the self-renewal of CySCs, and a secondary effect of Hh signaling on BMP signaling in GSCs is mediated by CySCs. One source of Hh signaling contributes to the regulation of two different populations of stem cells, implying a novel niche-stem cell interaction. Desert hedgehog (Dhh), which is one of the three Hh proteins in mammal, is specifically expressed in the testis but not the ovary (Bitgood et al., 1996). Dhh knockout mice are sterile (Bitgood et al., 1996) and somatic Leydig cells are severely defective (Yao et al., 2002). Despite the investigation that Dhh signaling is critical for a normal function of testis, the inside mechanism of spermatogenesis is still poorly understood due to the complex of niche-stem cell interactions in mammal. The knowledge of niche-stem cell regulations in fly testis may provide us some clues for better understanding of mammalian spermatogenesis. However, the new findings also raise many important questions that need to be addressed in the future.

Why the major members of Hh signaling are expressed in cyst cells? Although *ptc*, *smo* and *ci* are found to express in cyst cells including CySCs, the inside mechanism of this specific distribution is not clear. According to the experience in wing discs, the expression pattern of *ptc* is defined by Ci activity, it seems reasonable that *ptc* is not expressed in GSCs due to lack of Ci activity. Engrailed (En) directly represses *ci* expression in posterior compartment cells of wing discs (Schwartz et al., 1995), providing a possibility of the absence of Ci in GSCs. There are other two possibilities. First, there is a cyst specific protein turns on the expression of *ci* or *smo* in CySCs. Second, there is a germline specific protein represses the transcription of *ci* or *smo* in GSCs. Further whole genome RNAi screening may give some answers to those questions.

Are there other targets of Ci to regulate the self-renewal of CySCs? The finding of Amoyel et al. (2013) raises some hints that, besides *zfh-1* and *chinmo*, there may exist other targets of Ci in CySCs. This unknown Ci targeting factor likely regulates CySCs self-renewal. Ci/Gli family transcriptional factors bind to a specific DNA motif (GACCACCCA) (Kinzler and Vogelstein, 1990) in the regulation area of target genes, which may help us to better identify those unknown factors in the future. Hh is a morphogen to turn on different target genes in a concentration dependent manner, such as *dpp*, *ptc* and *en* in wing discs (Jiang and Hui, 2008). A predictable Hh dosage from hub cells to surrounding cells may also result in different Ci targets expression. Further investigation is needed to give us a better

understanding of morphogen in stem cell niche.

Till now, we know that hub cells secrete at least three kinds of ligands: Upd for JAK-STAT signaling, Gbb/Dpp for BMP signaling and Hh for Hh signaling. How can the hub cells express exact amount of different ligands? The proper amount of different ligands is critical for individual signaling activities. There are several possible approaches for maintaining the testis niche homeostasis. One is that hub cells secrete a programmed amount of diverse ligands during different developmental stage, and the interaction between different signaling pathways buffers the abnormal of niche environment. Conclusion from Zhang et al. (2013) that Hh signaling plays dual roles in testis niche is an example to show how a single signaling buffers the whole niche. Another possible approach is that hub cells could sense the demands of different ligands from CySCs and GSCs to make a corresponding responds. Further study on Hh signaling in testis should therefore not only provide insight into how Hh signaling functions in testis niche but also shed light on how different signaling pathways are connected in niche-stem cell biology.

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ABBREVIATIONS

BMP, bone morphogenetic protein; Ci, Cubitus interruptus; Ci155, 155-kDa Ci activator; Ci75, 75 kDa Ci repressor; Ci^{act}, Ci active form; Cos2, Costal2; CySC, cyst stem cell; Dhh, Desert hedgehog; En, Engrailed; Fu, Fused; Gbb, Glass bottom boat; GSC, germline stem cell; Hh, Hedgehog; JAK-STAT, Janus kinase-signal transducer and activator of transcription; Mad, Mothers against dpp; Ptc, Patched; Smo, Smoothened

COMPLIANCE WITH ETHICS GUIDELINES

Zhao Zhang, Chenyu Pan, and Yun Zhao declare that they have no conflict of interest.

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