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FGF signaling supports *Drosophila* fertility by regulating development of ovarian muscle tissues

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ABSTRACT

The *thisbe* (*ths*) gene encodes a *Drosophila* fibroblast growth factor (FGF), and mutant females are viable but sterile suggesting a link between FGF signaling and fertility. Ovaries exhibit abnormal morphology including lack of epithelial sheaths and muscle tissues that surround ovarioles. Here we investigated how FGF influences *Drosophila* ovary morphogenesis and identified several roles. Heartless (Htl) FGF receptor was found to be expressed within somatic cells at the larval and pupal stages, and phenotypes were uncovered using RNAi. Differentiation of terminal filament cells was affected, but this effect did not alter the ovariole number. In addition, proliferation of epithelial sheath progenitors, the apical cells, was decreased in both *htl* and *ths* mutants, while ectopic expression of the Ths ligand led to these cells' over-proliferation suggesting that FGF signaling supports ovarian muscle sheath formation by controlling apical cell number in the developing gonad. Additionally, live imaging of adult ovaries was used to show that *htl* RNAi mutants, hypomorphic mutants in which epithelial sheaths are present, exhibit abnormal muscle contractions. Collectively, our results demonstrate that proper formation of ovarian muscle tissues is regulated by FGF signaling in the larval and pupal stages through control of apical cell proliferation and is required to support fertility.

Introduction

The *Drosophila melanogaster* ovary is a highly studied developmental system that has already provided many important insights into the biology of organ development. In particular, the *Drosophila* adult ovary has served as an excellent model for the interaction of germline stem cells (GSCs) with their somatic support cells known as the niche. In the ovary, the GSCs and niche facilitate egg production throughout the lifetime of the *Drosophila* female (Song et al., 2007; Xie and Spradling, 2000). Less is known regarding how the ovary is formed, but tight regulation of cell proliferation, differentiation, and survival by signaling pathways appears critical. Regulated signaling ensures that all cell types within this organ develop in a balanced manner during this period of major growth of the ovary at the larval and pupal stages.

Each ovary in the *Drosophila* adult consists of 15–20 ovarioles that contain GSCs, their associated niche, and a chain of oocytes at various stages of development. At the apical region of an ovariole, a unique structure called the germarium resides. It is within this structure that two to three GCSs reside at the apical tip next to their niche composed of terminal filament (TF) cells and cap cells (Eliazer and Buszczak, 2011). Much insight into the mechanisms controlling GSC maintenance and differentiation has been uncovered in *Drosophila* owing to the ease of accessibility of these cells within adult ovaries and because the system is amenable to genetic manipulation (Kirilly and Xie, 2007). In contrast, less is known regarding how GSCs, their somatic niche, and muscle tissues that encapsulate the ovarioles (the epithelial and peritoneal sheaths) are formed as these events occur earlier, at the larval and pupal stages, as the ovaries develop.

Previous studies of ovary morphogenesis at the larval and pupal stages have focused on the role of signaling pathways in regulating cell number, proliferation, differentiation, and survival (review in Gilboa (2015); Sarikaya and Extavour (2015)). EGFR, JAK/STAT, and Hippo signaling is important in mediating cellular homeostasis during the period of extreme cell growth of the gonad at the larval stage. Specifically, EGFR regulates the number of primordial germ cells (PGCs) as well as their somatic support cells, the interstitial cells (ICs) (Gilboa and Lehmann, 2006; Matsuoka et al., 2013). Ecdysone hormone also has been shown to trigger cell proliferation and to control growth of the ovary through effects on the insulin receptor (InR) and Target of rapamycin (Tor) pathway, as well (Gancz and Gilboa, 2013). Additionally, Bone morphogenetic protein (BMP) and Hedgehog (Hh) signaling pathways positively regulate PGC cell division at the larval stage (Sato et al.,

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2010). However, whether FGF signaling impacts ovary homeostasis and morphogenesis had not been previously investigated.

FGF signaling is involved in a multitude of important biological processes. FGF receptors (FGFRs) are a family of receptor tyrosine kinases. Upon receptor activation by ligand binding, various intracellular signaling pathways are induced (Feldman et al., 1995; Powers et al., 2000; Rottinger et al., 2008). To define a role for FGF signaling or to identify the specific molecular mechanisms involved can be challenging due to the complexity of the pathway. In humans and mice, for instance, 24 FGF and four FGFR genes have been discovered (Ornitz and Itoh, 2001), which support over one hundred possible FGF–FGFR complexes (Ornitz et al., 1996). Conversely, invertebrate systems have much simpler FGF signaling systems (Tulin and Stathopoulos, 2010a). In the case of *Drosophila*, three FGF and two FGFR genes have been discovered, supporting only three functional FGF–FGFR combinations (Kadam et al., 2009). The role of FGFR signaling in *Drosophila* as well as ligand choice varies and is context-specific (review in Bae et al. (2012)).

A role for fibroblast growth factor (FGF) signaling pathway in supporting ovarian development has been suggested in vertebrates, but no previous study has directly examined the role of FGF signaling in the *Drosophila* ovary. In vertebrates, it has been shown that both FGF ligands and receptors are expressed within follicular cells of vertebrate ovaries, including human (Berisha et al., 2006; Buratini et al., 2007). Furthermore, FGF addition to cultured ovarian tissues leads to cells' proliferation, and high levels of FGF signaling are correlated with many cancers including that of the ovary (Basu et al., 2014; Ropiquet et al., 2000). In particular, vertebrate FGF-8 exhibits gonad-specific expression, within the ovary and testes, suggesting that this signaling pathway plays an important, yet currently uncharacterized role in supporting gonad development (Valve et al., 1997). Keeping FGF signaling properly regulated is important for normal ovary development, but its exact role in supporting gonad development is unclear. Furthermore, FGF signaling is conserved as its biological roles and structural properties appear similar in *Drosophila* and higher vertebrates (Huang and Stern, 2005; Tulin and Stathopoulos, 2010b). Studies of how FGF signaling impacts *Drosophila* ovary morphogenesis have the potential to provide novel insights into conserved functions and/or regulatory mechanisms acting in other organisms, including vertebrates.

In the current study, we investigated the role of FGF signaling in supporting *Drosophila* ovary morphogenesis and found that this signaling pathway has several roles spanning multiple stages of development. At the larval stage, our results demonstrate a role for the Htl FGFR in controlling specification of the adult stem cell niche through regulation of TF cell differentiation; in the larval and pupal stages, this pathway also supports migration of a somatic cell population in the ovary, the apical cells, through regulation of these cells' proliferation. These earlier functions are necessary for the proper specification of the epithelial sheaths that surround individual ovarioles to support proper oocyte development and, thus, fertility.

Materials and methods

Fly stocks

Drosophila stocks were kept at 25 °C, unless otherwise noted. *yw* Stock was used as wildtype. To generate *ths* mutant viable flies, *ths*^{e02026}*/Cyo ftz–lacZ* (CFLZ) (Stathopoulos et al., 2004) and *Df(2R) ths238/CFLZ* (Kadam et al., 2009) were crossed to generate trans-heterozygotes. GAL4 lines used for genetic analysis were: *c587*.GAL4 (Kai and Spradling, 2003) and *nos*.GAL4vp16 (Bloomington Drosophila Stock Center, BDSC). UAS lines utilized for genetic analysis were: UAS.*htl*.RNAi40627 [Vienna Drosophila Research Center (VDRC); reported to have one off target]; (Dietzl et al., 2007; Kadam et al., 2012)], UAS.*htl*.RNAi6692 (VDRC; reported to have no off targets), UAS.*ths*.RNAi24538/*CyO* (VDRC; reported to have one off target), UAS.*pyr*.RNAi36523 (VDRC; reported to have over two hundred off targets), UAS.*ths* [AMS289–22; (Stathopoulos et al., 2004)], UAS.*htl.lambda* (#5367, BDSC). For temporal control, UAS.*htl.RNAi40627* was crossed with w^{+}; *Sco/Cyo;tub*-GAL80^{ts} (#7018, BDSC). As necessary, *If/CyO,actin-gfp*; *MKRS/Tm3,Ser,actin-gfp* (from Dr. Kai Zinn, California Institute of Technology, US) was used as a marked balancer at the larval stage.

To examine expression patterns of *htl* or *ths*, *htl*.GAL4 or *ths*.GAL4 lines (Pfeiffer et al., 2008) were crossed with UAS.GFP. The following GAL4 lines from BDSC were assayed but only a subset (bold) drove expression in the ovary: *htl*.GAL4 lines 47240, 40668, ***40669***, 48004, 40706, 47277, 40707, 40708, 48431, 47278, 47279; and *ths*.GAL4 lines ***40051***, ***47051***, 40049, 40050, 40052, 48624, 48355.

We also generated five *pyr*-GAL4 lines by cloning 1–3 kB fragments of non-coding DNA sequence flanking the *pyr* gene into the Gateway donor vector and pBGUw vector (Pfeiffer et al., 2008 #1883) to create GAL4 drivers HV01-05; of these, only HV03 and HV04 supported expression in the developing ovary. Primer sequences are provided (Table S1).

To examine Htl localization, an inframe insertion of the Cherry reporter was inserted into a construct "*htl-mcherry*" able to rescue the *htl* mutant. The 52 kb *htl* P[acman] construct was generated using recombineering-mediated gap repair performed as described (Venken et al., 2006). Insertion of the *cherry* gene just before the stop codon of *htl* was performed by standard recombineering techniques, using the Cherry-SV40-frt-kan-frt plasmid modified from the GFP-SV40-frt-kan-frt plasmid kindly provided by Dr. Eric Davidson (Caltech). The kan cassette was flipped out by arabinose induction of Flp in the SW105 cells (Warming et al., 2005).

The Ths rescue construct contains 33 kB of sequence spanning the *ths* gene and is able to rescue the *ths* mutant. Primer sequences used to construct the *htl* and *ths* rescue constructs are provided (Table S1).

Collection and aging

Eggs were collected in fresh vials for two hours to prevent overcrowding. Once flies were removed, vials were incubated at 25 °C for various lengths of time: 72 h for early-larval third instar, 96 h for mid-larval third instar, 120 h for late-larval third instar, 144 h for early pupae, 168 h for middle pupae, and 192 h for late pupae. For UAS.*htl*.RNAi;GAL80^{ts}, after a two-hour-egg collection, vials were incubated at 18 °C until development to adult. The adult flies were transferred to 29 °C, and incubated for an additional, appropriate length of time. Before dissection, adult flies were well fed with yeast paste for one day.

For fertility assays, five female flies of each genotype were crossed with two *yw* male flies. The eggs were collected on apple juice plates, and number of eggs deposited counted after 24 h.

Fixation, immunocytochemistry, and in-situ hybridization

Dissected ovaries were fixed in 33% paraformaldehyde in PBT solution for 20 min at room temperature (RT). The fixed ovaries were washed with PBS three times and incubated in blocking solution (10% BSA in PBT) for 1 h. After the blocking, samples were incubated with primary antibodies for ~18 h at 4 °C and, subsequently, were washed with 1:10 diluted blocking solution 4×, with 30 min incubation for each wash. Secondary antibodies diluted 1:100 in blocking solution were added to the sample, and incubated further for ~18 h at 4 °C. The samples were washed