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# Ecdysone signaling opposes epidermal growth factor signaling in regulating cyst differentiation in the male gonad of *Drosophila melanogaster*

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ABSTRACT

The development of stem cell daughters into the differentiated state normally requires a cascade of proliferation and differentiation steps that are typically regulated by external signals. The germline cells of most animals, in specific, are associated with somatic support cells and depend on them for normal development. In the male gonad of *Drosophila melanogaster*, germline cells are completely enclosed by cytoplasmic extensions of somatic cyst cells, and these cysts form a functional unit. Signaling from the germline to the cyst cells via the Epidermal Growth Factor Receptor (EGFR) is required for germline enclosure and has been proposed to provide a temporal signature promoting early steps of differentiation. A temperature-sensitive allele of the EGFR ligand Spitz (Spi) provides a powerful tool for probing the function of the EGRF pathway in this context and for identifying other pathways regulating cyst differentiation via genetic interaction studies. Using this tool, we show that signaling via the Ecdysone Receptor (EcR), a known regulator of developmental timing during larval and pupal development, opposes EGF signaling in testes. In *spi* mutant animals, reducing either Ecdysone synthesis or the expression of Ecdysone signal transducers or targets in the cyst cells resulted in a rescue of cyst formation and cyst differentiation. Despite of this striking effect in the *spi* mutant background and the expression of EcR signaling components within the cyst cells, activity of the EcR pathway appears to be dispensable in a wildtype background. We propose that EcR signaling modulates the effects of EGFR signaling by promoting an undifferentiated state in early stage cyst cells.

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## Introduction

Throughout metazoan life, adult tissues are in constant flux between the loss and the replacement of highly specialized cells. These cells are derived from the activity of adult stem cells. Over the past decade, considerable progress has been made in our understanding of how stem cells are organized. It has become clear that the specification and maintenance of most stem cell populations depend on signals from their natural cellular microenvironment (Conover and Notti, 2008; de Rooij, 2009; Ju et al., 2007; Takakura, 2012). For example, blood stem cells in the bone marrow depend on signals from the mesenchymal osteoblasts, including angiopoietin, thrombopoietin, and the chemokine Cxcl12 (Luis et al., 2012; Park et al., 2012). Similarly, gut and skin stem cells depend on signaling via the highly conserved Wnt pathway (Choi et al., 2013; Krausova and Korinek, 2014; Lim and Nusse, 2013). Less is known about the mechanisms that regulate differentiation of stem cell daughters. When cultured, embryonic stem cells can be induced to differentiate into a plethora of cell types (Keller, 2005). Comparably few signals that act *in situ* have been identified. In mouse skin, conserved molecules such as p63, Mitogen Activated Protein Kinase (MAPK), Notch, and $\beta$-Catenin are essential for skin cell development (Blanpain and Fuchs, 2006; Sotiropoulou and Blanpain, 2012).

The male gonad of *Drosophila melanogaster* provides an excellent model for studying the signaling events regulating differentiation processes. The testis houses two distinct stem cell lineages, a germline stem cell (GSC) lineage and a somatic stem cell lineage, called the cyst stem cell (CySC) lineage. Both stem cell populations are attached to a group of somatic cells at the apical tip, the hub cells, which serve as an organizing center. Each GSC is enclosed by cytoplasmic extensions from two CySCs that extend around the GSC and into the hub. The divisions of GSCs and CySCs are formative, producing one daughter that remains a stem cell and one daughter that becomes a gonialblast or a cyst cell, respectively. Gonialblasts

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subsequently become fully enclosed by the cytoplasmic extensions of two cyst cells. Analogous to mammalian stem cell populations, *Drosophila* gonialblasts first undergo mitotic transit amplifying divisions before they differentiate into sperm. Throughout this process, the cyst cells continue to enclose the germline cells and co-differentiate with them (Fuller, 1993; Hardy et al., 1979; Zoller and Schulz, 2012). This developmental sequence is tightly regulated by signaling between the two lineages. For example, the exit of the germline cells from amplifying mitotic divisions depends on Transforming Growth Factor-$\beta$ signaling in the surrounding cyst cells (Bunt and Hime, 2004; Matunis et al., 1997).

The EGF signaling pathway is involved in embryonic development, cancer, stem cell proliferation, and gametogenesis in numerous species (Moghal and Sternberg, 2003; Normanno et al., 2006; Parrott et al., 2012; Shilo, 2003; Strand and Micchelli, 2013; Wiley et al., 1995). The enclosure of the germline cells by the cyst cells is regulated via EGF signaling (Sarkar et al., 2007; Schulz et al., 2002). Germline cells signal to the cyst cells via Spi, a transmembrane protein that is activated by the germline-specific protease Stet. Once cleaved, Spi stimulates the EGFR on the cyst cells. Mutations in either *spi*, *stet*, or *Egfr* disrupt germline enclosure and result in a failure of the germline cells to differentiate. Testes from *spi* or *stet* mutant animals are tiny compared to wildtype testes and contain tumor-like accumulations of proliferating early-stage germline cells (Kiger et al., 2000; Schulz et al., 2002; Urban et al., 2002). Recent findings further show that the progression of the cysts (germline and surrounding cyst cells) through the early stages of spermatogenesis is also promoted by EGF signaling. By studying genetic backgrounds in which EGF signaling was reduced but not completely abolished, we were able to show that enclosed germline cells depend on continued EGF signaling for progressing through all four rounds of transit amplifying divisions. Conversely, an increase in EGF signaling caused the cysts to initiate terminal differentiation prior to completing all four rounds of mitosis. These results implied that EGF signaling provides an instructive signal, or a temporal signature that guides the progression of the cysts through the early stages of spermatogenesis (Hudson et al., 2013).

Factors modulating EGF signaling in the gonad have been identified by virtue of their genetic interactions with the temperature-sensitive allele, $spi^{77\text{-}20}$. For example, reducing the expression of the small monomeric GTPase, *rac1*, in cyst cells exacerbated the germline enclosure defects observed in testes of $spi^{77\text{-}20}$ mutant males (*spi*/*spi*-testes) suggesting that Rac1 acts downstream of the EGFR. Conversely, reducing the expression of the small monomeric GTPase, *rho1*, in the cyst cells had the opposite effect on *spi*/*spi*-testes, rescuing the germline enclosure defects. This indicated that Rho1 acts in a pathway opposing EGF for germline enclosure. In conjunction with ultrastructural data and protein binding studies, these findings suggested that EGF signaling from the germline cells organizes a differential of Rac- and Rho-activities in the cyst cells, leading to polarization of the actin cytoskeleton and directional growth around the germline cells (Sarkar et al., 2007).

EcR signaling regulates the timing of key developmental transition, such as molting and metamorphosis. The ligands of the pathway, the Ecdysteroids, are polyhydroxylated compounds that are synthesized from dietary cholesterol in a multi-step biosynthetic process that produces 20-hydroxyecdysone (20E). During larval development, pulses of ecdysone and other ecdysteroids are released from the prothoracic gland portion of the ring gland and further converted to 20E, the most active form of the ligand, in peripheral tissues (Warren et al., 2006). The prothoracic gland degenerates early in pupal development and the source of ecdysteroids in adult flies is not clear (Dai and Gilbert, 1991). However, 20E is still detectable and active in both the adult male and female (Brownes et al., 1984; Schwedes et al., 2011). In the adult male, 20E signaling has been implicated in long-term memory and male-to-male courtship (Ganter et al., 2011; Simon et al., 2006). In the adult female, 20E signaling is essential for oogenesis, long-term courtship memory, and for regulating the wake-sleep cycle (Carney and Bender, 2000; Ishimoto and Kitamoto, 2010; Ishimoto et al., 2009; Schwedes and Carney, 2012).

The EcR is a member of the nuclear steroid receptor superfamily that contains DNA- and hormone-binding domains, indicating that it is a ligand-regulated transcription factor. In order to bind to DNA, EcR forms a heterodimer with Ultraspiracle (Usp), a homolog of the human retinoid X receptor (RXR). This complex binds to 20E and also recruits co-regulators, such as Hsp70/90, Taiman (Tai), and Trithorax-related (Bai et al., 2000; Koelle et al., 1991; Sedkov et al., 2003; Yao et al., 1993). EcR can bind to DNA independent of ligand but displays the highest transcriptional activation when bound to 20E (Braun et al., 2009; Buszczak and Segraves, 1998; Dela Cruz et al., 2000; Hall and Thummel, 1998). Within DNA, EcR binds to specific target sites, known as ecdysone response elements (Perera et al., 2005; Riddihough and Pelham, 1987). During larval development, the EcR complex induces expression of a small group of early regulatory genes. During embryonic and larval development, the complex induces the expression of Eip74 and Eip75, and at pupariation, also the expression of Broad-Complex (BrC). The protein products of these "early genes" repress their own transcription and also activate or induce the transcription of a larger set of downstream "early-late" and "late-late" genes, producing a genetic hierarchy of transcription (Ashburner and Richards, 1976).

Here, we identify a novel function for EcR signaling in testes, where it acts antagonistically to EGF signaling. We show that reducing the production of 20E restored cyst formation and cyst development in *spi*/*spi*-testes. Furthermore, EcR signaling components are expressed in cyst cells and reducing EcR signaling specifically in cyst cells of *spi*/*spi*-testes also resulted in a rescue of the defects caused by the $spi^{77\text{-}20}$ mutation. While EcR signaling is dispensable for normal development of the cysts in a wildtype background, overexpression of EcR in cyst cells induced cyst death. On the basis of our observations, we propose that EcR modulates cyst development in the male gonad of *Drosophila* by promoting an undifferentiated state of the cyst cells.

## Material and methods

### *Fly Stocks & UAS-Gal4 expression Studies*

All fly stocks in this study were raised and maintained on standard cornmeal molasses medium at room temperature. Fly stocks used in this study include $spi^{77\text{-}20}$ (Sarkar et al., 2007), the cyst cell drivers EyaA3-*gal4* (Leatherman and Dinardo, 2008) and C587-*gal4* (Hrdlicka et al., 2002), and UAS-*dnEcR* (Cherbas et al., 2003). The following flies carrying RNAi constructs, alleles of 20E synthesis genes, and overexpression constructs were obtained from the Bloomington Stock Center (The Flybase Consortium, 2003): UAS-$EcRi^{97}$ [BL#9326]; UAS-$EcRi^{104}$ [BL#9327]; UAS-$Uspi^{TRiP.HMS01620}$ [BL#36729]; UAS-$Eip74EFi^{TRiP.JF02515}$ [BL#29353]; UAS-$BrCi^{TRiP.JF02585}$ [BL#27272]; UAS-$Taii^{TRiP.HMS00673}$ [BL32885]; $spo^{1}$ [BL#3276]; $dib^{2}$ [BL#2776]; $sad^{1}$ [BL#2087]; UAS-*dcr* [BL#24651]; UAS-*EcR-B1* [BL#6469]; UAS-*EcR-B2* [BL#6468]; FRT-$tai^{61G1}$ [BL#6379]; $EcR^{M554fs}$ [BL#4894]; $EcR^{V559fs}$ [BL#4901]; $EcR^{A483T}$ [BL#5799]; $EcR^{Q50ST}$ [BL#4895]; $EcR^{W53ST}$ [BL#5604]. All *spi* mutant flies were raised and maintained at 26.5 °C. Flies for overexpression of *EcR* and expression of RNAi in otherwise wildtype animals were raised at 18 °C and shifted to 29 °C as adult for seven to ten days. *P*-values were calculated using Fisher's and chi-squared exact test.

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