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Developmental Biology 294 (2006) 92–103
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Development of the male germline stem cell niche in *Drosophila*

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Received for publication 22 November 2005; revised 15 February 2006; accepted 17 February 2006
Available online 29 March 2006

Abstract

Stem cells are found in specialized microenvironments, or "niches", which regulate stem cell identity and behavior. The adult testis and ovary in *Drosophila* contain germline stem cells (GSCs) with well-defined niches, and are excellent models for studying niche development. Here, we investigate the formation of the testis GSC niche, or "hub", during the late stages of embryogenesis. By morphological and molecular criteria, we identify and follow the development of an embryonic hub that forms from a subset of anterior somatic gonadal precursors (SGPs) in the male gonad. Embryonic hub cells form a discrete cluster apart from other SGPs, express several molecular markers in common with the adult hub and organize anterior-most germ cells in a rosette pattern characteristic of GSCs in the adult. The sex determination genes *transformer* and *doublesex* ensure that hub formation occurs only in males. Interestingly, hub formation occurs in both XX and XY gonads mutant for *doublesex*, indicating that *doublesex* is required to repress hub formation in females. This work establishes the *Drosophila* male GSC niche as a model for understanding the mechanisms controlling niche formation and initial stem cell recruitment, as well as the development of sexual dimorphism in the gonad.

Keywords: Stem cell niche; Sex determination; *doublesex*; *transformer*; Gonad; Testis; *Drosophila*; *escargot*; *center divider*; Fasciclin 3; DE-cadherin; DN-cadherin; *unpaired*

Introduction

Stem cells provide a continuous source of undifferentiated progenitor cells due to their remarkable ability to produce daughter cells that retain stem cell identity while other daughter cells go on to differentiate. Stem cells in vivo reside in cellular microenvironments, known as "niches", that maintain stem cell identity and influence stem cell behavior [reviewed in (Ohlstein et al., 2004)]. Recent work indicates that stem cell niches can also act to determine stem cell identity, and can recruit cells to become stem cells and populate the niche (Brawley and Matunis, 2004; Kai and Spradling, 2004). Thus, the development of the stem cell niche is a critical aspect of any stem cell system.

One important class of stem cells are germline stem cells (GSCs) that reside in testes, and often in ovaries, and produce the large number of germ cell precursors necessary for the continuous production of sperm or eggs. Therefore, the proper function of GSCs is essential for the reproductive health of an organism. *Drosophila melanogaster* has emerged as a powerful model in which to study GSC niches in both the testis and ovary. Work by many labs has defined the respective niches and GSCs, and characterized aspects of how the niches influence GSCs through cell–cell contact and signaling [reviewed in (Gilboa and Lehmann, 2004; Lin, 2002; Spradling et al., 2001; Xie et al., 2005; Yamashita et al., 2005)]. Although the adult male and female GSC niches share many commonalities, they are composed of different cell types and show differences in how GSC maintenance and differentiation are regulated. They therefore represent an important divergence in the development of males vs. females (sexual dimorphism).

The adult *Drosophila* testis is a coiled tube closed at the apical end and connected to the rest of genital tract at the basal end [for a review of testis structure and function, see (Fuller, 1993)]. At the apical tip resides a group of somatic cells, called "the hub", which forms the male GSC niche (Kiger et al., 2001; Tulina and Matunis, 2001) and contacts an average of nine GSCs distributed in a characteristic rosette arrangement (Hardy et al., 1979) (though the true "niche" includes the environment surrounding the hub that the GSCs contact, we will use the terms GSC niche and hub interchangeably since the hub is

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doi:10.1016/j.ydbio.2006.02.030

thought to create this environment). The GSCs divide perpendicular to the hub (Hardy et al., 1979; Yamashita et al., 2003) to give rise to one daughter cell that remains adjacent to the hub and retains GSC identity, while the other daughter is displaced from the hub and initiates spermatogenesis. Hub cells express the ligand Unpaired (Upd), which activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway in adjacent germ cells to maintain them as GSCs (Kiger et al., 2001; Tulina and Matunis, 2001). Signaling through the TGF-β pathway is also important for maintaining GSCs (Kawase et al., 2004; Shivdasani and Ingham, 2003; Schulz et al., 2004). In addition, a somatic stem cell population, the cyst progenitor cells, also resides adjacent to the hub and produces cyst cells that nurture the germ cells during spermatogenesis (Aboïm, 1945; Hardy et al., 1979). The adult testis is formed from the embryonic gonad, but little is known about how this occurs or even what embryonic cells give rise to the different cells of the testis, such those that form the hub.

The embryonic gonad is created from two specialized cell types, germ cells and somatic gonadal precursors (SGPs). Germ cells form at the posterior pole of the blastoderm embryo and migrate through the embryo to reach the SGPs by stage 12 of embryogenesis [stages as in (Campos-Ortega and Hartenstein, 1985), for a review of germ cell migration see (Santos and Lehmann, 2004)]. SGPs are mesodermal cells that are specified in bilateral clusters within abdominal parasegments (PS) 10 to 13 (Boyle and DiNardo, 1995; Boyle et al., 1997; Brookman et al., 1992; DeFalco et al., 2003). The homeotic genes *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) promote formation of SGPs in the proper parasegments (DeFalco et al., 2004; Moore et al., 1998a; Riechmann et al., 1998). In addition, *abd-A* and *Abd-B* act to provide distinct identities to the SGP clusters: *abd-A* specifies anterior SGP identity (PS10 and likely PS11), a combination of *abd-A* and *Abd-B* specifies posterior SGP identity (PS12) and *Abd-B* alone specifies male-specific SGP (msSGP) identity (PS13) (Boyle and DiNardo, 1995; DeFalco et al., 2004). By stage 13, the germ cells and SGPs have associated to form a contiguous tissue, which coalesces into the embryonic gonad during stage 14. Gonad coalescence involves the two distinct processes of germ cell ensheathment, whereby SGPs extend cellular processes to surround the germ cells (Jenkins et al., 2003), and gonad compaction, in which the SGPs and germ cells form an organized, spherical gonad in PS10 (Boyle and DiNardo, 1995; Brookman et al., 1992).

The male and female gonads are already developing differently at the time of gonad coalescence, since msSGPs join the posterior of the male gonad but die by apoptosis in females (DeFalco et al., 2003). In addition, the germ cells are receiving sex-specific signals from the SGPs (Wawersik et al., 2005) and exhibit a sexually dimorphic pattern of gene expression (Staab et al., 1996; Wawersik et al., 2005) at the time of gonad formation. The first signs of spermatogenesis are observed as early as the first instar larval stage and a structure reminiscent of the adult hub has formed by this time (Aboïm, 1945). Furthermore, molecular evidence indicates that presumptive hub cells may already be present in the embryonic gonad (Gönczy et al., 1992). Thus, it appears that the male GSC niche is likely to form during embryogenesis, and may contain functioning GSCs soon after.

Here, we examine the development of the male GSC niche or hub. Our work indicates that the hub is formed during the final stage of embryogenesis (stage 17), and already makes specific contacts with a subset of germ cells. We further study the origins of embryonic hub cells, and how sex determination influences the development of these cells to ensure that GSC niche formation is sexually dimorphic.

Materials and methods

Fly stocks

The following fly stocks were used: w^{1118} (as wild type), *68–77* [D. Godt, (Simon et al., 1990)], esg^{K00606}, esg^{G66B} (Whiteley et al., 1992), *esg-GFP*^{P01986} (Flytrap), cdi^{Q29} (C. Samakovlis), cdi^{07013}, Pc^{3}, Pc^{XT109} (R. Paro), *Abd-B*^{M5} (M. Akam), $foi^{20.71}$, $foi^{16.33}$ (Moore et al., 1998b), shg^{R69} (P. Rorth), tra^{1}, *Df(3L) st-j7* (*tra* deficiency), dsx^{1}, dsx^{23}, dsx^{D}, UAS-*traF*-20J7, UAS-*mCD8::GFP*-LL6 (L. Luo), UAS-*GAL4*-12B, UAS-*GFP.nls*-14, *unpaired*-GAL4 (T. Xie), *paired*-GAL4-RG1, *tubulin*-GAL4-LL7, *twist 24B*-GAL4 (Brand and Perrimon, 1993), *nanos*-GAL4-VP16 [germ cells, (Van Doren et al., 1998)]. osk^{301}/osk^{CE4} females (Lehmann and Nüsslein-Volhard, 1986) were mated at 18°C to esg^{G66B}, *68–77* or cdi^{Q29} males to produce agametic embryos. Unspecified fly stocks are from the Bloomington Stock Center.

Immunohistochemistry and whole-mount in situ hybridization

The following antibodies (dilution, source) were used: mouse anti-β-GAL (1:10,000, Promega), rabbit anti-β-GAL (1:10,000, Cappel), rabbit anti-cleaved Caspase 3 (1:50, Cell Signaling Technology), rat anti-DE-cadherin DCAD2 (1: 20, Developmental Studies Hybridoma Bank/DSHB; T. Uemura), mouse anti-EYA 10H6 (1: 25, DSHB; N. Bonini), mouse anti-Fasciclin 3 7G10 (1: 30, DSHB; C. Goodman), mouse anti-GFP B-2 (1: 50, Santa Cruz), rabbit anti-GFP (1: 2,000, Torrey Pines Biolabs), rat anti-DN-cadherin Ex#8 (1: 20, DSHB; T. Uemura), rabbit anti-SOX100B (1: 1,000, S. Russell), mouse anti-SXL M18 (1: 50, DSHB; P. Schedl), chick anti-VAS (1:10,000, K. Howard), rabbit anti-VAS (1:10,000, R. Lehmann). Fluorescently conjugated 488-, 546-, 633- and Cy5-secondary antibodies were used at 1: 500 (Molecular Probes, Rockland and Amersham Pharmacia Biotech).

Adult testes were dissected, fixed and immunostained as previously described (Gönczy et al., 1997). Embryos were fixed, devitellinized and immunostained as previously described (Patel, 1994), with modifications as in (DeFalco et al., 2003). For stage 17 embryos, sonication was used to render embryos accessible to immunostaining (Patel, 1994). Embryos were rehydrated and washed twice for 3 min in 1 ml PBS containing 0.1% Tween-20 (PBTw), sonicated in 500 μl of PBTw with a 3 second constant pulse using a Branson Sonifier 250 (set at 100% duty cycle and output setting 1), washed twice for 3 min with PBTw, and immunostained as above. For anti-DCAD2 staining, embryos were fixed as described (Jenkins et al., 2003; Rothwell and Sullivan, 2000) but were sonicated as above rather than hand-devitellinized. Following staining, embryos were mounted in 2.5% DABCO (Sigma) on slides and viewed with a Zeiss 510 Meta confocal microscope.

Embryos were fixed as above for whole-mount in situ hybridization which was performed as described (Lehmann and Tautz, 1994). The *esg* antisense riboprobe was synthesized by digesting pBS-SK-*esg* (a gift from N. Fuse and S. Hayashi) with *Xba*I and transcribing with T7 RNA polymerase (Promega) using digoxigenin-labeled UTP (Boehringer-Mannheim).

Genotyping and sex identification of embryos

We used balancer chromosomes containing a P{*Kr-GFP*} transgene to identify homozygous mutant embryos. Sex of embryos was identified as previously described (DeFalco et al., 2003) using a female-specific anti-SXL antibody (Figs. 3G, 3G, 6C–D, 6F–G, 6I–M), an X chromosome carrying a P