Development of the male germline stem cell niche in Drosophila

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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...
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 (Aboim, 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 as 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 GSC clusters: abd-A specifies anterior SGP identity (PS10 and likely PS11), and 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 enshainment, whereby GSCs 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 (Aboim, 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)], esgX0066, esgG068 (Whiteley et al., 1992), esg-GFPoH10 (Flytrap), cd0229 (C. Samakovlis), cd0701, PcA, PcB (R. Paro), Abd-BMS (M. Akam), foi20.71, foi4.63 (Moore et al., 1998b), shg80G (P. Borst), tra1, Df(3L) st1 (tra deficiency), dsxGAL4, dsxE, UAS-tra-F07, UAS-mCD8–GFP–LL6 (L. Luo), UAS-GAL4-12B, UAS-GFP-14, unpaired-GAL4 (T. Xie), paired-GAL4-RG1, tubulin-GAL4-LL7, twist-GAL4-B (Brand and Perrimon, 1993), nanos-GAL4-V16 [germ cells, (Van Doren et al., 1998)], oskH102G004 females (Lehmann and Nüsslein-Volhard, 1986) were mated at 18°C to esgG068, 68–77 or cd0229 males to produce agamic 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-DCAD2 (1: 2,000, Torrey Pines Bioslabs), rat anti-DN-cadherin Ex8/1 (1: 20, DSHB; T. Uemura), rabbit anti-SOX100B (1: 1,000, S. Russell), mouse anti-MLX1 (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 egs antisense riboprobe was synthesized by digesting PBS-SK-egs (a gift from N. Fuse and S. Hayashi) with XhoI 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, 3H, 6C–D, 6F–G, 6L–M), an X chromosome carrying a P
A hub is present in the male gonad by the end of embryogenesis

Results

To determine if the hub forms during embryogenesis, we first analyzed the embryonic expression of molecular markers that are specific to adult hub cells. We observed expression of an enhancer trap for the transcription factor *escargot* [esg<sup>G66B</sup>, (Whiteley et al., 1992)] in a tight cluster of SGPs at the anterior of late stage (stage 17) male embryonic gonads (Fig. 1A), and also in the adult hub (Fig. 1B), as previously observed (Gönczy et al., 1992). Similarly, examination of other previously identified markers for the adult hub, Fasciclin 3, *Drosophila* E-cadherin (DE-cadherin), and an enhancer trap for *upd* (Figs. 1D, F) (Brower et al., 1981; Kawase et al., 2004; Tazuke et al., 2002), revealed that they are also expressed in the anterior cluster of SGPs in the late embryonic gonad (Figs. 1C, E and 4H) (Jenkins et al., 2003). In addition, we were able to identify new genes expressed in the anterior cluster of SGPs in the embryo, such as the serine/threonine kinase *center divider* [cdi, (Matthews and Crews, 1999)] and the cell adhesion molecule DN-cadherin (Iwai et al., 1997) (Figs. 1G, I), and found that these markers are expressed in the adult hub (Figs. 1H, J). Thus, all molecular markers tested that are expressed in the adult hub are also detected in a specific cluster of anterior SGPs in the stage 17 male embryonic gonad. To determine the number of anterior SGPs that have this identity in the embryo, we used two independent nuclear enhancer-traps (cdi<sup>Q29</sup> and cdi<sup>07013</sup>), and found an average of 8.3 cdi-expressing cells (*n* = 23, range = 6–12) per male gonad in stage 17 embryos.

Another distinguishing feature of the adult hub is the specific, radial arrangement (rosette) of GSCs around this structure (Fig. 1L) (Hardy et al., 1979). We looked at the distribution of germ cells in late embryos and found that the anterior-most germ cells in males adopt a rosette organization around the putative hub and orient their nuclei toward the hub (Fig. 1K), while the distribution of the female germ cells remains unaltered (data not shown). Since a subset of SGPs in the stage 17 embryonic male gonad exhibits a compact, hub-like morphology, expresses multiple markers in common with adult hub cells, and specifically organizes anterior germ cells, we...

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Fig. 1. A hub is present in the male gonad at the end of embryogenesis. Stage 17 embryonic male gonads (A, C, E, G, I, K) and adult testes (B, D, F, H, J, L, M, N). Sex of embryos was determined as indicated in Materials and methods. Anterior is left in embryonic panels. Scale bar in panel A represents 10 μm in panels A–M. (A–J) Germ cells are labeled with anti-VAS (red), along with the following markers (green): (A–B) esg<sup>G66B</sup> enhancer trap (anti-β-GAL), (C–D) anti-Fasciclin 3, (E–F) upd-GAL4 driven UAS-mCD8:GFP expression (anti-GFP), (G–H) cdi<sup>G29</sup> (anti-β-GAL), (I–J) anti-DN-cadherin. Note that a discrete cluster of SGPs in the stage 17 embryo expresses the same molecular markers as the adult hub. (K–L) Anti-VAS (white). Anterior-most germ cells adopt a specific rosette distribution around hub cells (green arrow), (M–N) upd-GAL4 permanently driven UAS-GFP expression (upd-GAL4; UAS-GAL4, UAS-GFP). Germ cells are labeled with anti-VAS (red). upd-GAL4 drives GFP expression (anti-GFP, green) specifically in hub cells (M and green arrow in panel N), while non-specific green fluorescence can be detected in other parts of testis (N), as observed in control testes (UAS-GAL4, UAS-GFP, data not shown).
conclude that an “embryonic hub” is present by the end of embryogenesis and is likely to be the precursor of the adult hub.

To further understand the relationship between the embryonic hub and the adult hub, we used the GAL4/UAS system (Brand and Perrimon, 1993) to irreversibly label embryonic hub cells and analyzed their contribution to the adult testis [using UAS-GAL4 in combination with upd-GAL4 and UAS-GFP so that, once a cell expresses upd-GAL4, that cell and its progeny will permanently express GFP (Hassan et al., 2000)]. When adult testes of this genotype were analyzed, only the cells of the adult hub were observed to express GFP (Figs. 1M–N). Although we cannot exclude the possibility that the cells of the embryonic hub die or migrate out of the testis to be replaced by an adult hub of separate origin, the simplest interpretation of this result is that the cells of the embryonic hub give rise to the adult hub and no other cell types in the testis.

**Hub cell identity is specified from sexually dimorphic, anterior SGPs**

We next wanted to determine which cells give rise to the embryonic hub. esg is a marker of anterior SGP identity in the gonad at earlier stages (Boyle and DiNardo, 1995), and so we investigated whether anterior SGPs give rise to the hub. Since hub formation only occurs in males, we first examined if this early expression of esg in the newly formed gonad is sex-specific. By in situ hybridization, two patterns of esg expression were observed in the coalesced gonad: half of stage 15 and 16 embryos (82/158) exhibited esg expression in the anterior of the gonad (Fig. 2A), while the other half (76/158) did not (Fig. 2B).

Using an esg enhancer trap, and determining embryonic sex by examining the msSGPs [anti-EYA, (DeFalco et al., 2003)], we observed that esg is expressed only in male gonads. Prior to gonad coalescence, esg expression was not observed in SGPs of either sex (Fig. 2C). After coalescence (stage 15), esg expression was observed in male gonads (Fig. 2D) but was never seen in female gonads (Fig. 2E). esg expression in the male gonad was present in anterior SGPs, as characterized by a lower level of Eyes Absent (EYA) expression (Fig. 2D inset). Male-specific expression of esg in anterior SGPs indicates that these cells are already sexually dimorphic soon after gonad formation. Later in development (stage 17), esg expression becomes restricted to a subset of anterior SGPs (Figs. 2F–G) that form the embryonic hub.

To define which SGPs are specified to form the hub, we used the GAL4/UAS system to express GFP in subsets of SGPs and followed esg enhancer trap expression in these cells. The paired-GAL4 driver expresses UAS-GFP in odd parasegments, including in SGPs from PS11 and msSGPs from PS13. In stage 15 coalesced male gonads, most or all SGPs originating from PS11 exhibited co-expression of GFP and esg (Figs. 3A–A’). esg was also observed in more anterior SGPs (PS10), but was weak in posterior SGPs (PS12) and undetectable in msSGPs. When esg expression becomes restricted to a subset of SGPs during stage 17, some of these SGPs were observed to co-express GFP while others did not, indicating that they likely came from both PS10 and 11 (Fig. 3B). Additionally, Fasciclin 3 expression was observed in a similar subset of SGPs (Fig. 3C) suggesting that hub cell identity is specified only in those anterior SGPs that maintain esg expression.

If hub cells are derived from anterior SGPs, we would expect that specification of anterior SGP identity would be a prerequisite for hub formation. Previously, it has been shown that anterior SGP identity, and esg expression, are promoted by abd-A and repressed by Abd-B (Boyle and DiNardo, 1995; DeFalco et al., 2004). We examined hub cell specification in
Polycomb (Pc) mutants in which Abd-B is ectopically expressed, abolishing anterior SGP identity (DeFalco et al., 2004). Hub cell-specific expression of esg, DN-cadherin, Fasciclin 3 and DE-cadherin was no longer observed in the anterior of male stage 17 gonads in Pc mutants (Figs. 3D–E and data not shown), indicating that hub cell specification is indeed blocked when anterior SGP identity is repressed. We also examined hub cell specification in Abd-B mutants, which lack posterior SGP identity and show expanded anterior identity and esg expression at earlier stages (Boyle and DiNardo, 1995; DeFalco et al., 2004). In late stages (stage 17), we still observed expression of esg, Fasciclin 3, DN-cadherin and DE-cadherin in a similar cluster of SGPs (Figs. 3F–G and data not shown) indicating that hub cell identity is still specified in Abd-B mutants. In addition, since hub cell identity was not expanded to all SGPs, we conclude that restriction of hub cell identity to a subset of anterior SGPs does not require Abd-B. Abd-B does appear to be required for proper positioning of the hub, since the hub was often disorganized and its location was more variable in Abd-B mutants.

The formation of the embryonic hub

Anterior SGPs in males must undergo dramatic changes in morphology and cell–cell interactions to form the hub. These changes occur during the last stage of embryogenesis (stage 17) which encompasses one third of embryonic development (8h) (Campos-Ortega and Hartenstein, 1985). Using both general markers for cell morphology [cell-surface GFP, UAS-mCD8::GFP, (Lee and Luo, 1999)], and specific molecular markers for the embryonic hub, we can divide hub formation into three steps that appear to occur sequentially. During Step 1, anterior SGPs are morphologically similar in males and females (Figs. 4A–B) and, while esg and upd are expressed only in males [Fig. 2, (Wawersik et al., 2005) and data not shown], no additional hub-specific gene expression is observed (Figs. 4F, J, N). During Step 2, the male gonad initiates morphological changes in which an anterior “cap” of somatic cells becomes visible (Fig. 4C, arrow) and additional markers of hub identity begin to be expressed in these cells (see below). Finally, during Step 3, a highly compacted cluster of SGPs is visible in the anterior of the gonad (Fig. 4D, arrow) that maintains esg expression (Fig. 2G), and anterior-most germ cells adopt a rosette organization around this cluster in a manner reminiscent of the GSCs around the adult hub (Hardy et al., 1979).

The morphological changes observed during hub formation are likely to require changes in cell–cell contact and adhesion. Indeed, three markers of hub cell identity, DE-cadherin, DN-cadherin and Fasciclin 3 are homophilic adhesion molecules whose expression changes dramatically during hub formation. DE-cadherin is expressed in both female and male gonads during gonad coalescence [stage 14, (Jenkins et al., 2003)], and was still observed at stage 17 in both female and Step 1 male gonads (Figs. 4E–F), particularly in the msSGPs (Fig. 4F, open arrow). However, DE-cadherin expression increases significantly in embryonic hub cells during Step 2 (Fig. 4G, closed arrow), and by Step 3, DE-cadherin is strongly enriched in hub cells.
Fig. 4. The embryonic hub forms during stage 17. Anterior is left. All panels show wild type, stage 17 male gonads except for (A, E, I, M, Q) which are female. Sex of embryos was determined as indicated in Materials and methods. Perimeter of gonads are outlined in panels E–T. Scale bar in panel A represents 10 μm in panels A–T and 33 μm in insets. (A–D) Embryos expressing cell surface GFP throughout the mesoderm (UAS-mCD8::GFP, twist 24B-GAL4) labeled with anti-GFP (green) and anti-VAS (germ cells, red). White arrows indicate the formation of an anterior SGP cluster. (E–H) Anti-DE-cadherin shows increased labeling in presumptive hub cells (G, closed arrow) compared to msSGPs (F, open arrow) during Step 2. Insets show the same images with two channels to reveal anti-DE-cadherin (green) and anti-VAS (red). (I–L) Anti-DN-cadherin exhibits increased immunoreactivity in presumptive hub cells from Step 2 to Step 3. Insets show the same images with two channels to reveal anti-DN-cadherin (green) and anti-VAS (red). Note that DE- and DN-cadherin are present at the hub cell–germ cell interface (H, L, green arrowheads). (M–P) Anti-Fasciclin 3 labels presumptive hub cells from Step 2 to Step 3, and is only present where hub cells contact other hub cells and not where hub cells contact germ cells (P, red arrowhead). Insets show the same images with all three channels to reveal anti-Fasciclin 3 (red), anti-VAS (blue) and anti-SOX100B (green). (Q–T) Embryos labeled to reveal upd-GAL4 driven UAS-mCD8::GFP (anti-GFP, green) expression only in presumptive hub cells from Step 2 to Step 3. Insets show the same images with all three channels to reveal anti-GFP (red), anti-VAS (blue) and anti-SOX100B (green).
compared to other SGPs (Fig. 4H). Similarly, DN-cadherin was seen at only low levels in both female gonads (Fig. 4I) and Step 1 male gonads (Fig. 4J). Its expression was found to increase in embryonic hub cells in males during Step 2 and was most clearly seen in Step 3 (Figs. 4K–L). Finally, Fasciclin 3 was not observed in stage 17 female or Step 1 male gonads (Figs. 4M–N), but was first seen in embryonic hub cells during Step 2 (Fig. 4O) and is maintained in the tight cluster of SGPs in Step 3 (Fig. 4P). During this time, esg expression is being restricted to a subset of anterior SGPs (Fig. 3) and co-localizes with these other hub markers (data not shown). Both DE- and DN-cadherin were observed at sites of hub cell–germ cell contact (e.g., green arrowheads in Figs. 4H, L) as well as sites of hub cell–hub cell contact. In contrast, Fasciclin 3 was only seen at sites of hub cell–hub cell contact, and was notably missing from sites of hub cell–germ cell contact (e.g., Fig. 4P, red arrowhead). When a cell-surface marker (mCD8::GFP) was specifically expressed in the embryonic hub (Figs. 4S–T), no ensheathment of germ cells by embryonic hub cells was observed, which is in contrast to the dramatic ensheathment of germ cells by SGPs observed at earlier stages (Jenkins et al., 2003).

The initiation of hub-specific gene expression during Step 2, along with the restriction of esg expression to these cells, suggests that a subset of anterior SGPs are adopting the hub cell identity at this time. In further support of this, expression of a particular upd enhancer trap is only first detected during Step 2 in the embryonic hub (Figs. 4Q–T), even though upd is expressed in anterior SGPs in males beginning at earlier stages (Wawersik et al., 2005). Similarly, two esg enhancer traps (esg\textsuperscript{X06606} and esg-\textsuperscript{GFPP01986}) are not expressed when esg RNA is first observed in the stage 15 gonad, and were only observed at stage 17 in the embryonic hub (data not shown). Expression of the upd and esg enhancer traps likely reveals a change in gene regulation that occurs within those anterior SGPs that form the embryonic hub. In summary, our data indicate that the male GSC niche forms during the last stage of embryogenesis as a subset of anterior SGPs acquire hub cell identity, specifically express several cell adhesion molecules, change their morphology to form a tight cluster and organize anterior-most germ cells.

Hub cell identity does not depend on gonad coalescence or the presence of germ cells

We next wanted to determine whether hub formation requires earlier aspects of gonad formation. We first examined whether proper gonad coalescence is required for hub formation. In mutants for shotgun (shg), which encodes DE-cadherin (Tepass et al., 1996; Uemura et al., 1996), both gonad compaction and germ cell ensheathment are disrupted (Jenkins et al., 2003). However, expression of DN-cadherin and Fasciclin 3 was still observed in a cluster of SGPs in shg mutant gonads, even in embryos where gonad coalescence was severely disrupted (Fig. 5A and data not shown), indicating that gonad coalescence is

Fig. 5. Hub cell identity does not require gonad coalescence or germ cells. Anterior is left. All embryos are male, as determined as indicated in Materials and methods. Scale bar in panel A represents 10 μm in panels A, E–H, 7 μm in panels B–D and 33 μm in insets. (A) shg\textsuperscript{69} mutant male gonad labeled with anti-V AS (red) and anti-DN-cadherin (green). Note that hub cells are present despite a defect in gonad coalescence. (B–D) Stage 15 (B) and 17 (C, D) for\textsuperscript{671}/foi\textsuperscript{63} mutant male gonads also heterozygous for esg\textsuperscript{68-77}, labeled to reveal the germ cells (anti-V AS, blue). (B, C) Embryos also labeled to reveal the esg\textsuperscript{68-77} enhancer trap (anti-\textit{β}-GAL, green) and SGPs (anti-EYA, green). Note that proper male-specific expression of esg in the anterior SGPs and SOX100B in the msSGPs occurs despite a lack of gonad coalescence (B), but esg is not restricted to a subset of anterior SGPs at stage 17 (C). (D) Embryo also labeled with anti-DN-cadherin (green in inset), and anti-SOX100B (red in inset). Note that DE-cadherin expression is missing where the hub would normally form but is still present in the msSGPs (green arrow). The gonad is outlined as judged by the location of the germ cells. (E, F) Stage 15 (E) and 17 (F) agamic male gonads of progeny from osk\textsuperscript{βi-63}/osk\textsuperscript{βi-63} females crossed to esg\textsuperscript{68-77}+/+ males labeled to reveal the esg\textsuperscript{68-77} enhancer trap (anti-\textit{β}-GAL, red) and SGPs (anti-EYA, green). Note that esg is expressed normally in anterior SGPs and becomes restricted to a subset of these cells at stage 17. (G, H) Stage 17, agamic, male gonads of progeny from osk\textsuperscript{βi-63}/osk\textsuperscript{βi-63} females crossed to 68-77/68-77 (G) or cdi\textsuperscript{βi-63}/+ (H). (G) Embryo labeled for anti-DN-cadherin (green) and anti-\textit{β}-GAL (68-77 SGP enhancer trap, red). (H) Embryo labeled for anti-DE-cadherin (green) and anti-\textit{β}-GAL (cdi enhancer trap, red). Note that the hub markers DN-cadherin, DE-cadherin and cdi are properly expressed in a subset of anterior SGPs in agamic gonads.
not required for hub formation. Gonad coalescence is similarly disrupted in fear of intimacy (foi) mutants (Van Doren et al., 2003), however, we did observe hub formation defects in these embryos. Proper male-specific expression of esg in anterior SGPs and SOX100B in posterior msSGPs was still observed in stage 15 foi-mutant gonads (Fig. 5B) (DeFalco et al., 2003), but esg expression was not restricted to a subset of SGPs (the hub) at stage 17 (Fig. 5C). In addition, hub expression of Fasciclin 3 and DN-cadherin were never observed in foi mutant gonads (data not shown), and DE-cadherin was still observed in the msSGPs, but remained absent from the anterior of stage 17 foi mutant gonads where hub cells should have been specified (Fig. 5D). Since the analysis of shg mutants suggests that gonad coalescence itself is not required for hub formation, this may indicate that foi plays a more direct role in this process.

We next investigated whether germ cells play a role in hub formation. Many aspects of somatic gonad development occur normally in embryos lacking germ cells, including gonad coalescence (Brookman et al., 1992; Jenkins et al., 2003), anterior–posterior patterning (Boyle and DiNardo, 1995) and posterior sexual dimorphism (DeFalco et al., 2003). In addition, the hub appears to be present in agametic adult testes (Geigy, 1931; Gönczy and DiNardo, 1996), indicating that germ cells are not required for hub formation. To examine hub development in embryos that lack germ cells, we used a hypomorphic mutation in oskar, which blocks germ cell formation but does not affect somatic patterning (Lehmann and Nüsslein-Volhard, 1986). We observed that esg expression in agametic gonads is similar to wild type: esg was found in anterior SGPs after gonad coalescence (Fig. 5E) and became restricted to a subset of anterior SGPs in stage 17 (Fig. 5F). In addition, hub markers such as cdi, DN-cadherin and DE-cadherin were still seen in a similar subset of anterior SGPs at stage 17 (Figs. 5G–H), while the general SGP marker 68–77 (Fig. 5G) was expressed in all SGPs, but not msSGPs, as in wild type. We conclude that embryonic hub cell specification does not require germ cells, although the embryonic hub might not be as small and constricted in agametic gonads as it is in wild type male gonads.

The control of sexual dimorphism in the developing hub

Since a hub is formed only in male gonads, we investigated how these cells receive information about their sexual identity. In Drosophila somatic sex determination, the ratio of X chromosomes to autosomes regulates an alternative RNA splicing cascade that results in the production of functional Sex lethal and Transformer (TRA) proteins only in females [reviewed in (Cline and Meyer, 1996), see Fig. 6A for a simplified view of the sex determination cascade]. Thus, we expect tra mutant females to be masculinized since they no longer produce TRA. Indeed, we observed an increased percentage of stage 17 tra mutant embryos that exhibited Fasciclin 3 expression in the gonad. In wild type, 45% (n = 33) of stage 17 embryos exhibited Fasciclin 3 expression in the hub (i.e., males that are Step 2 or older). In contrast, this was the case for 84% (n = 26) of tra mutant embryos, indicating that both XX and XY embryos initiate Fasciclin 3 expression at Step 2 (Fig. 6B). In addition, both XX and XY embryos exhibited embryonic hubs that express DN- and DE-cadherin and organize the anterior germ cells in a rosette pattern (Figs. 6C–D and data not shown). Furthermore, ectopic expression of TRA (using UAS-traF) was sufficient to block hub formation in males. When TRA was expressed either ubiquitously (tub-GAL4) or in the mesoderm (twist 24B-GAL4), hub markers such as Fasciclin 3, DN-cadherin and DE-cadherin were absent from the gonad in all embryos (Figs. 6E–F and data not shown).

Outside the nervous system, doublesex (dsx) is the principle somatic sex determination factor downstream of tra. The male form of DSX (DSXM) promotes male development and represses female development, while the female form (DSXF) does the opposite (Fig. 6A). Consequently, dsx null mutant adults appear neither fully male nor female (Hildreth, 1965). However, we observed that hub formation can occur in both XX and XY dsx mutant embryos. Fasciclin 3 was expressed in a typical male hub-specific pattern in an increased percentage of dsx mutant embryos (Fig. 6H, 76%, n = 46 for dsx vs. 45%, n = 33 for wild type). In addition both XX and XY dsx mutants expressed DN- and DE-cadherin in the hub and exhibited a rosette organization of anterior germ cells (Figs. 6l–J and data not shown). A similar result is obtained when examining the earlier aspects of sexual dimorphism in the anterior somatic gonad: esg is expressed in anterior SGPs in both XX and XY dsx mutant embryos (Figs. 6K–L). Thus, with respect to hub formation, XX and XY dsx-mutant embryos appear identical and both resemble wild type males. This indicates that DSXM is not required for hub formation in males, but DSXF is required to repress hub formation in females. However, DSXM is able to partially block repression of male development by DSXF when the two are co-expressed, since we observed esg expression in anterior SGPs in dsx/F+/XX embryos (Fig. 6M).

Discussion

Development of the male germline stem cell niche

Our evidence indicates that an embryonic hub, which appears to give rise to the adult hub and create the male GSC niche, forms during the late stages of embryogenesis. A subset of anterior SGPs initiates expression of several molecular markers that are also expressed in the adult hub. These SGPs segregate into a tight cluster in a distinct region of the gonad, and a subset of germ cells organizes around these SGPs in a manner similar to the organization of GSCs around the adult hub. Since spermatogenesis begins by early larval stages (Aboim, 1945), it is possible that the embryonic hub already forms a functional GSC niche. The formation of the hub, or indeed any stem cell niche, can be divided into the distinct issues of niche cell identity, niche morphogenesis, and stem cell recruitment:

Hub cell identity

Our data indicate that the specification of hub cell identity occurs in two stages. During the first stage, some SGPs acquire an anterior identity that is sexually dimorphic, as indicated by
the male-specific expression of *esg* (Fig. 2) and *upd* (Wawersik et al., 2005). Anterior SGP identity is positively regulated by *abd-A*, and is repressed by *Abd-B* (Boyle and DiNardo, 1995; DeFalco et al., 2004), while sexual identity is regulated by *tra* and *dsx*. During the second stage of hub cell specification, a subset of these anterior SGPs acquires hub cell identity during stage 17 of embryogenesis. Only some anterior SGPs maintain *esg* expression, and the control of late gene expression in the hub appears to be distinct from early expression in anterior SGPs, since some *esg* and *upd*
enhancer traps only exhibit gonad expression in the hub at this later stage. Furthermore, cells that maintain esg expression during stage 17 also express every other marker of adult hub identity tested, including Fasciclin 3, cdi, DN-cadherin and DE-cadherin. We conclude that these cells are specified as hub cells at this time. The fate of the anterior SGPs that lose esg expression and do not form part of the hub is unknown. An intriguing possibility is that these cells could form another important somatic cell type: the cyst progenitor cells (somatic stem cells) that associate with the hub along with the GSCs.

Based on its expression pattern, the transcription factor esg would seem to be an excellent candidate for specifying hub cell identity. However, we observed no changes in the expression of our other hub markers in esg null mutants (S. Le Bras and M. Van Doren, unpublished); this includes expression of DE-cadherin, which is known to be regulated by esg in other tissues (Tanaka-Matukatsu et al., 1996). It has been reported, however, that esg is required for hub maintenance, and that the hub is severely defective at later stages in esg mutants that survive embryogenesis (L. Jones and M. Fuller, personal communication). Thus, esg is critical for the male GSC niche, but is either not important for the initial formation of this structure, or acts redundantly with another factor.

**Hub morphogenesis**

We have been able to follow the morphogenesis of the hub from the time of gonad formation until the embryonic hub is fully formed. At the time of gonad coalescence, anterior SGPs interact with other SGPs, and with the germ cells, in a manner that is indistinguishable from posterior SGPs (Jenkins et al., 2003). However, during stage 17, the hub cells undergo dramatic changes in their relationship to other SGPs and germ cells. Hub cells segregate away from other SGPs to one pole of the gonad, and coalesce tightly with one another (Fig. 4). In addition, hub cells do not ensheath the germ cells at this stage (Figs. 4S–T). Instead, a defined interface between hub cells and germ cells forms which is labeled by DE- and DN-cadherin, but not Fasciclin 3 (Fig. 4). Thus, hub cells appear to maximize their interactions with one another, and minimize their interactions with other cells in the gonad, although they clearly still contact a subset of germ cells.

It is apparent that the changes in cell–cell contact and morphology that occur during hub formation require changes in cell adhesion. Indeed, we have found that characteristic changes in expression of the homophilic adhesion molecules Fasciclin 3, DN-cadherin and DE-cadherin occur during hub formation; all three are significantly upregulated in the embryonic and adult hub. Increased homophilic adhesion among hub cells could account for their ability to maximize their contacts with one another, and sort away from other SGPs. However, we have not yet observed changes in embryonic hub formation in mutants for these cell adhesion molecules (S. Le Bras and M. Van Doren, unpublished). Thus, these proteins, and possibly others, may act redundantly in this process.

**Stem cell recruitment**

While we have not focused on the GSCs in this study, it is clear that a subset of germ cells organizes specifically with the developing hub as it forms. During the last stage of hub formation (Step 3), germ cells become oriented in a rosette distribution around the developing hub in a manner characteristic of GSCs in the adult (Hardy et al., 1979). These may represent the subset of germ cells that will become GSCs. The presence of DE- and DN-cadherin at sites of hub–germ cell contact suggests that cadherin-mediated adhesion may be important for niche–GSC interaction in the testis, as has been observed in the ovary (Song et al., 2002). Interestingly, germ cells are not required for hub formation (Fig. 5) (Aböim, 1945; Geigy, 1941; Gönçzy and DiNardo, 1996). Analysis of a number of hub identity markers indicates that these cell form normally from a subset of anterior SGPs in embryos that lack germ cells (Fig. 5). The hub does not appear as well compacted in these embryos, consistent with observations of the adult hub (Gönçzy and DiNardo, 1996), indicating that hub–germ cell contact (or hub–germ cell signaling) affects the final shape of the hub. Nevertheless, the GSC niche can form in the absence of one of its stem cell populations (somatic stem cells may still be present). It will be of great interest in the future to determine if the subset of germ cells organized around the male embryonic hub are, indeed, developing GSCs, and to study how their transition to stem cell identity might be regulated by the niche.

**Sexual dimorphism in the gonad**

We have shown that the formation of the male GSC niche is a sex-specific characteristic of anterior SGPs. Male-specific expression of esg and hub formation both require the sex determination genes tra and dsx (Fig. 6). In some tissues, DSXM is required to promote male development and repress female development, while the opposite is true for DSX̄ (Fig. 6A). Interestingly, we find that embryonic hub development is entirely masculinized in dsx null mutants; XX and XY individuals appear identical when mutant for dsx and both resemble wild type males. Thus, we see no role for DSXM in promoting embryonic hub formation, while DSX̄ is required in females to repress hub formation. Since esg is expressed male-specifically, it is one candidate for being directly regulated by DSX.

We can compare the development of the anterior SGPs and hub with the development of another sexually dimorphic cell type, the msSGPs that join the posterior of the male gonad (DeFalco et al., 2003). First of all, these two cell types are distinct and do not depend on one another for their proper development. The hub still forms in Abd-B mutants that lack msSGPs, while msSGPs are still found in the gonad in Pc mutants, in which no anterior SGPs or hub cells form (Fig. 3) (DeFalco et al., 2004). Second, the mechanism for how sexual dimorphism is created differs between the two cell types. msSGPs are present only in males because they have undergone sex-specific apoptosis in females (DeFalco et al., 2003). In contrast, we observe no apoptosis in anterior SGPs (S. Le Bras and M. Van Doren, unpublished). These cells appear to remain
present in both sexes, but only form a hub in males. Thus, although the sex determination genes \textit{tra} and \textit{dsx} regulate sex-specific development of both cell types, the cellular mechanisms employed are different. Finally, as we observed for the hub, development of the msSGPs is completely masculinized in \textit{dsx} mutant embryos (DeFalco et al., 2003). Thus, for both of these cell types, the male pattern of development in the embryonic gonad is the default state in the absence of \textit{dsx} function, and it is the role of DSX\textsuperscript{M} to repress male development in females. However, DSX\textsuperscript{M} may well play a role in development of one or both of these gonad cell types at later stages, since proper testis development in males clearly requires \textit{dsx} (Hildreth, 1965).

The sex determination pathway must also ensure that GSC niches form in females and are different from those in males. Recently, it has been shown that germ cells populating the anterior of the gonad in female embryos are predisposed to become GSCs in the adult ovary, while germ cells populating the posterior rarely become GSCs (Asaoka and Lin, 2004). This suggests that anterior SGPs in the female embryonic gonad may promote GSC identity, similar to what we propose happens in the male during hub formation. One possibility is that anterior SGPs give rise to GSC niches in both sexes, while genes such as \textit{tra} and \textit{dsx} control whether these niches will be male or female.

In conclusion, we have been successful in following the development of the embryonic hub, which may represent the nascent GSC niche for the testis. This work provides a basis for further understanding the mechanisms controlling niche formation and GSC recruitment in \textit{Drosophila}, and determining if these mechanisms are conserved in other stem cell systems, including the GSC niche of the mammalian testis.

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