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Clonal expansion of ovarian germline stem cells during niche formation in *Drosophila*

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SUMMARY

Stem cell niches are specific regulatory microenvironments formed by neighboring stromal cells. Owing to difficulties in identifying stem cells and their niches in many systems, mechanisms that control niche formation and stem cell recruitment remain elusive. In the *Drosophila* ovary, two or three germline stem cells (GSCs) have recently been shown to reside in a niche, in which terminal filaments (TFs) and cap cells are two major components. We report that signals from newly formed niches promote clonal expansion of GSCs during niche formation in the *Drosophila* ovary. After the formation of TFs and cap cells, anterior primordial germ cells (PGCs) adjacent to TFs/cap cells can develop into GSCs at the early pupal stage while the rest directly differentiate. The anterior PGCs are very mitotically active and exhibit two division patterns with respect to cap cells. One of these patterns generates two daughters that both contact cap cells and potentially become GSCs. Our lineage tracing study confirms that one PGC can generate two or three GSCs to occupy a whole niche ('clonal expansion'). *decapentaplegic* (*dpp*), the *Drosophila* homolog of human bone morphogenetic protein 2/4, is expressed in anterior somatic cells of the gonad, including TFs/cap cells. *dpp* overexpression promotes PGC proliferation and causes the accumulation of more PGCs in the gonad. A single PGC mutant for *thick veins*, encoding an essential *dpp* receptor, loses the ability to clonally populate a niche. Therefore, *dpp* is probably one of the mitotic signals that promote the clonal expansion of GSCs in a niche. This study also suggests that signals from newly formed niche cells are important for expanding stem cells and populating niches.

Key words: Germline stem cell, Niche, *Drosophila*, Ovary

INTRODUCTION

Adult stem cells are defined by their ability to self-renew and to yield differentiated cells that replace lost cells. Upon stem cell division, one daughter cell goes through self-renewal and retains stem cell identity. The other daughter cell differentiates into one or more terminal cell types that maintain adult tissues. Stem cell self-renewal and differentiation have recently been shown to be controlled by microenvironments or niches (Watt and Hogan, 2000; Spradling et al., 2001). However, it appears that the proper functioning of niches also depends on the continued presence of stem cells and that empty niches are likely to degenerate (Xie and Spradling, 2000). Therefore, it may be necessary to rejuvenate or replace niches as well as stem cells in stem cell-based therapies. Understanding how niches form and what signals control this process will provide novel insights for future medical applications. Unfortunately, the difficulty in identifying and manipulating stem cells and their niches *in vivo* is a major obstacle for answering these important stem cell questions in many systems.

The *Drosophila* ovary is an excellent system to study stem cells and their relationships to niches (Lin, 1998; Xie and Spradling, 2001). Each ovary is composed of 12-16 individual ovarioles, in which both germline and somatic stem cells are located at the tip of the ovariole also known as the germarium. Two or three somatic stem cells are located at the middle of each germarium and are responsible for producing somatic follicle cells that surround germline cells in developing egg chambers (Margolis and Spradling, 1995; Zhang and Kalderon, 2001). Two or three GSCs that situate at the tip of each germarium generate differentiated germline cysts (Wieschaus and Szabad, 1979; Lin and Spradling, 1993). These GSCs have recently been shown to be located in a niche, which is composed of three differentiated somatic cell types: terminal filament (TF) cells, cap cells and inner germarium sheath (IGS) cells (Cox et al., 1998; Cox et al., 2000; King and Lin, 1999; King et al., 2001; Xie and Spradling, 1998; Xie and Spradling, 2000). A GSC divides asymmetrically to generate one stem cell and one cystoblast daughter. The cystoblast divides precisely four times to produce 16 interconnected cystocytes that are then encapsulated by a layer of follicle cells to form an egg chamber (Spradling, 1993).

Germline stem cells in each niche can be easily recognized by their size, location and the presence of a special intracellular

organelle known as a spectrosome. Spectrosomes, like their counterparts in differentiated germ cell cysts known as fusomes, are rich in cytoskeletal proteins such as Huli-tai shao (Hts) (Lin et al., 1994). The spectrosome is found in GSCs and cystoblasts usually as a spherical structure; the fusome in cysts is branched thereby connecting individual cystocytes. In the germarium, GSCs are in direct contact with cap cells, and their spectrosome is invariably anchored to the cap cell contact site. Adherens junctions exist in the interface between cap cells and GSCs, and their disruption causes GSC loss (Song et al., 2002). Furthermore, GSCs divide along the anteroposterior germarial axis so that the anterior GSC daughter remains anchored to cap cells and maintains stem cell identity, while the posterior daughter that fails to contact cap cells differentiates into a cystoblast. However, when one of the GSCs in a niche is lost, its neighboring stem cell divides perpendicular to the germarial axis, causing both daughter cells to contact cap cells and to retain stem cell identity and thus repopulate the niche (Xie and Spradling, 2000). Consistently, it has been shown that in the adult ovary, TFs/cap cells express many genes that are known to be important for maintaining GSCs, such as *hedgehog* (*hh*), *piwi*, *fs(1) Yb* and *dpp* (Cox et al., 1998; Cox et al., 2000; King and Lin, 1999; King et al., 2001; Xie and Spradling, 1998; Xie and Spradling, 2000). Therefore, direct interactions with niche cells, especially cap cells, are essential for maintaining GSC identity.

Relatively little is known about germ cell development in the female gonads of larvae and pupae. GSCs in the adult *Drosophila* ovary originate from embryonic pole cells. The pole cells proliferate and migrate from the posterior end of the embryo to the gonadal mesoderm to form the primitive gonad (Mueller, 2002). In females, the primordial germ cells (PGCs) and somatic cells in the gonad increase dramatically in number during the larval period. Individual TFs that consist of eight or nine disc-shaped cells are formed 2 hours before pupation (King, 1970). With the aid of molecular markers, it has been shown that TFs form in a progressive manner from medial to lateral across the ovary, with the number of terminal filament cells increasing gradually during the second half of the third instar larval stage (Sahut-Barnola et al., 1995). We have recently shown that adherens junctions establish between cap cells and newly formed GSCs during early pupation (Song et al., 2002). A genetic study suggests that the establishment of GSCs takes place during the early pupal stage (Bhat and Schedl, 1997). As there are more PGCs than needed for the formation of 12-16 ovarioles, each of which contains two or three GSCs, it has been proposed that the extra PGCs directly enter the germ cell differentiation pathway without passing through the stem cell stage (King, 1970; Bhat and Schedl, 1997). However, nothing is known about how PGCs are selected to become GSCs or to differentiate directly, or how the selected PGCs populate the niche. In this study, we show that PGCs are selected to become GSCs based on their juxtaposition to TFs/cap cells. We further demonstrate that GSCs in a niche can come from one PGC and that *dpp* signaling probably controls the proliferation of GSCs in the niche.

MATERIALS AND METHODS

Fly culture and stocks

The following fly stocks used in this study were described either in FlyBase or as otherwise specified: *Bam-GFP* (a generous gift from D. McKearin before publication) (Chen and McKearin, 2003); *hh-lacZ* (Forbes et al., 1996); X-15-29 and X-15-33 (*tub-lacZ* lines) (Harrison and Perrimon, 1993); FRT_{40A} *armadillo-lacZ* (Lecuit and Cohen, 1997); *C587-gal4* (a generous gift from D. Drummond-Barbosa and A. Spradling), *UAS-GFP*; *UAS-dpp*; FRT_{40A} tkv^{8} (Das et al., 1998); and *Dad-lacZ* (Tsuneizumi et al., 1997). All stocks were maintained at 25°C on standard medium, except the C587-gal4;*UAS-dpp* strain, which was kept at 18°C.

Developmental staging of larvae and pupae

Morphological definitions of the developmental stages of *Drosophila* followed those of King (King, 1970). In this study, late third instar larvae were referred to as the third instar larvae that remained in food. At this stage, terminal filament stacks started to form. The larvae at the larval-to-pupal transition were referred to as the larvae that moved out of food but pupation had not started. At this stage, most of TFs were still forming and cap cells were starting to form. The early pupal stage was the stage at which pupation had already started but pupae were still pale and clear. At this stage, all TFs were finished and a few cap cells had already formed.

Immunohistochemistry and microscopy

Ovary dissection, fixation and immunohistochemistry were performed as described previously (Song et al., 2002). The following antibodies were used: monoclonal anti-Hts antibody 1B1(1:3) (Developmental Studies Hybridoma Bank at the University of Iowa); rat anti-Bam antibody (1:100) (McKearin and Ohlstein, 1995); polyclonal anti-β-galactosidase antibody (1:100) (Molecular Probes); polyclonal anti-GFP antibody (1:100) (Molecular Probes); and monoclonal anti-BrdU antibody (1:20) (Oncogene). All micrographs were taken using a Leica confocal NT II microscope.

Clonal analysis and calculations

To generate positively marked germ cells by *tubulin-lacZ* in the developing female gonads, larvae from the cross between X-15-29 females and *hsFLP*; X-15-33/CyO males were heatshocked at or before the late third instar larval stage in a 37°C water bath for 4 hours, and were then allowed to develop into adults. The ovaries from one- to two-day-old females of *hsFLP*; X-15-33/X-15-29 were dissected and immunostained with anti-Hts and anti-β-gal antibodies. The percentage of germaria containing only marked GSCs was determined by dividing the number of germaria containing only marked GSCs by the number of germaria containing any marked GSCs.

To generate marked germ cells by loss of *armadillo-lacZ* expression in the developing female gonads, larvae from the cross between FRT_{40A} males and *hsFLP*; FRT_{40A} *armadillo-lacZ* females were heatshocked at or before the late third instar stage in a 37°C water bath, and were then allowed to develop into adults. The ovaries from one- to two-day-old females of *hsFLP*; FRT_{40A}/FRT_{40A} *armadillo-lacZ* were dissected and immunostained with anti-Hts and anti- β-gal antibodies. For generating the marked mutant *tkv* germ cells at the late third instar larval stage, the FRT_{40A} tkv^{8} strain was used instead of the FRT_{40A} strain.

Whole-mount mRNA in situ hybridization

The *dpp* probe was labeled with digoxigenin following the DIG RNA Labeling Kit instruction (Roche). The mRNA in situ hybridization was performed according to the protocol described by Tautz and Pfeifle (Tautz and Pfeifle, 1989), except fluorescent tyramide detection was used. The combination of immunostaining with an anti-Hts antibody and fluorescent *dpp* mRNA in situ hybridization was performed according to a published protocol (Wilkie and Davis, 2001).

BrdU and TUNEL labeling

BrdU labeling was performed for 1 hour in Grace's medium as described previously (Lilly and Spradling, 1996). The TUNEL cell

death assay was performed following the ApopTag apoptosis detection kit manual (Intergen Company).

RESULTS

GSCs develop from the anterior PGCs that are adjacent to TFs/cap cells

Even though previous studies suggest that some of the PGCs develop into GSCs while the rest differentiate during the early pupal stage (King, 1970; Bhat and Schedl, 1997), it remains unclear how PGCs are selected to develop into GSCs. To answer this question, we examined the expression of *bag of marbles* (*bam*) in the female gonads at the stages from late third instar larvae to early pupae using a *bam-green fluorescent protein* (*GFP*) transgene (GFP expression controlled by the *bam* promoter). The *bam-GFP* transgene is expressed at high levels in differentiating germ cells but at background or very low levels in GSCs (Chen and McKearin, 2003), consistent with the role of *bam* in germ cell differentiation (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995; Ohlstein and McKearin, 1997). The female gonads of the *bam-GFP* strain were labeled with anti-GFP and anti-Hts antibodies. *bam-GFP* was expressed at background levels in all the PGCs at the late third-instar larval stage like in adult ovarian GSCs, suggesting that all PGCs at this stage have not begun to differentiate and retain the potential to become GSCs (Fig. 1A). At the larval-pupal transition stage, we observed that *bam-GFP* was sporadically expressed in a posterior region of PGCs (Fig. 1B). At the early pupal stage, only the anterior PGCs adjacent to somatic cells maintained background levels of GFP expression like GSCs, although almost all of the remaining PGCs expressed GFP at high levels like differentiating germ cells in the adult ovary (Fig. 1C).

To further confirm this observation, we also examined the expression of the cytoplasmic form of Bam protein using an anti-BamC antibody and a *vasa*-GFP strain that labels all germ cells with GFP (Ohlstein and McKearin, 1997; Nakamura et al., 2001). The *vasa* gene, encoding an RNA helicase, is expressed only in germ cells throughout *Drosophila* development (Lasko and Ashburner, 1988; Hay et al., 1988). The expression of the cytoplasmic form of Bam protein (BamC) is correlated with germ cell differentiation (McKearin and Ohlstein, 1995). Consistently, BamC protein is not expressed in PGCs at the late third-instar larval stage (data not shown). BamC is present in some of the posterior PGCs, but remains absent in anterior PGCs at the early pupal stage (Fig. 1D). These results indicate that only the anterior PGCs juxtaposing the anterior TFs/cap cells can become GSCs, while the rest of them differentiate. In the adult ovary, signals from TFs/cap cells are essential for maintaining the undifferentiated state of GSCs (Cox et al., 1998; Cox et al., 2000; King and Lin, 1999; King et al., 2001; Xie and Spradling, 1998; Xie and Spradling, 2000). Possibly, signals from newly formed TFs/cap cells prevent the anterior PGCs from differentiation and thus from expressing *bam*.

To determine whether TF/cap cell formation correlates with initial PGC differentiation during *Drosophila* ovarian development, we used a *hh-lacZ* line to mark TFs/cap cells in the developing female gonads in addition to using the *bam-GFP* as a marker for germ cell differentiation. In the *hh-lacZ* line (the bacterial *lacZ* gene expression controlled by the *hh* promoter), *lacZ* is specifically expressed in TFs and cap cells of the adult ovary (Forbes et al., 1996). As expected, *hh-lacZ* was expressed in newly formed TF cells and cap cells (Fig. 1E,F). As *hh-lacZ* was expressed in both TF cells and cap cells, we distinguished them based on their morphology. Eight or

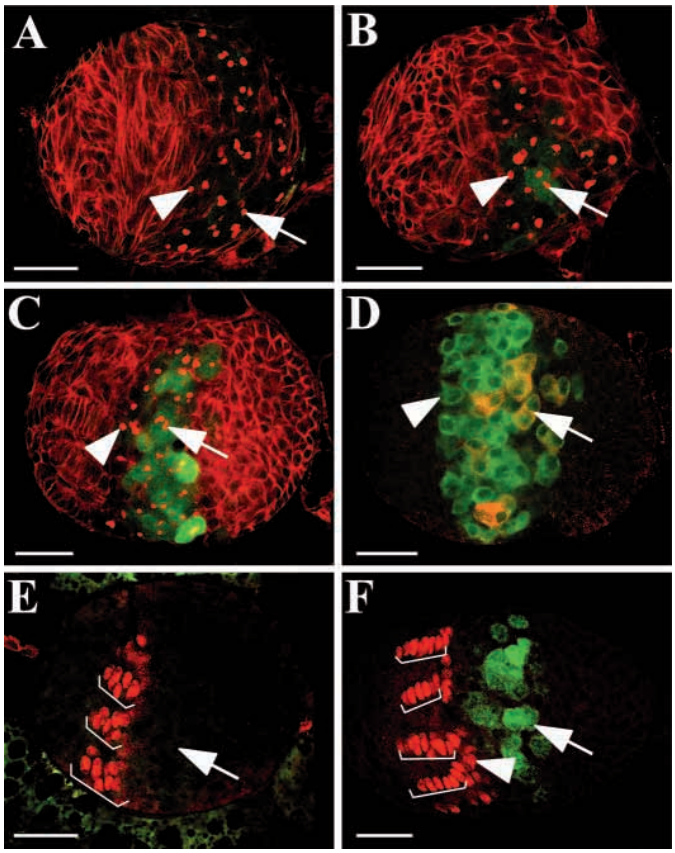

Fig. 1. Related developmental timing between GSC establishment and TF/cap cell formation during ovarian development. The female gonads in A-C were labeled for Hts (red) and GFP (green). D was labeled for Bam (red) and GFP (green). E and F were labeled for *lacZ* (red) and GFP (green). All the images represent one confocal section and the anterior end is towards the left. (A) A *bam-GFP* gonad at the late third-instar larval stage showing no significant GFP expression in PGCs. The arrowhead indicates an anterior PGC and the arrow indicates a posterior PGC. (B) A *bam-GFP* gonad at the larval-pupal transition stage showing sporadic GFP-positive PGCs. The arrowhead indicates one anterior PGC negative for GFP, while the arrow indicates a posterior PGC positive for GFP. (C) A *bam-GFP* gonad at the early pupal stage showing that all posterior PGCs (arrow) are positive for GFP and anterior PGCs (arrowhead) are negative for GFP. (D) A *vasa-GFP* gonad at the early pupal stage showing that some of the posterior PGCs (arrow) are BamC-positive and anterior PGCs (arrowhead) are BamC-negative. *vasa-GFP* labels all germ cells. (E) A *hh-lacZ*;*bam-GFP* gonad at the late third instar larval stage showing TF stacks (brackets) forming in a progressive manner across the gonad and no *bam-GFP* expression in PGCs (arrow). The TF stack indicated by the bottom bracket has more cells than the ones indicated by the upper brackets. (F) A *hh-lacZ*;*bam-GFP* gonad at the early pupal stage showing that posterior PGCs (arrow) are *bam-GFP*-positive and anterior PGCs close to TFs (brackets) and cap cells (arrowhead) are *bam-GFP*-negative. Scale bars: 20 μm.

nine oval shaped TF cells were packed very tightly against each other in each stack, while cap cells were rounder and did not line up with TF cells. Consistent with previous studies (King, 1970; Sahut-Barnola, 1995), TF cells started to form at the late third-instar larval stage, and the number of TF cells in a developing stack increased gradually in a progressive manner across the ovary until early pupal stage (Fig. 1E). *bam-GFP* was not expressed in the PGCs at the late third instar larval stage regardless of their location (Fig. 1E; 12 female gonads examined). During the larval-pupal transition, *bam-GFP* was expressed in 1.5% of the PGCs adjacent to TFs/cap cells, but its expression in the rest of the PGCs was heterogenous, ranging from 3.5% to 99.0% with an average of 71.5% (12 female gonads examined). After TF formation, cap cell differentiation occurred from the larval-pupal transitional stage to the early pupal stage (about 0-4 hours after pupation) (Fig. 1F). At the early pupal stage, all eight or nine oval TF cells were packed tightly against each other along their anteroposterior axis, and rounder *lacZ*-positive cap cells accumulated at the posterior end of TFs. By then, *bam-GFP* had been expressed at high levels in 93.0% of the germ cells that were not in contact with TFs/cap cells, but only 2.7% of the PGCs that were close to TFs/cap cells expressed *bam-GFP* (Fig. 1F; 12 female gonads examined). The remaining 7.0% of the posterior germ cells probably represented newly produced germ cells from anterior PGCs. Consistent with this interpretation, newly produced cystoblasts also fail to express bam-GFP at high levels in the adult ovary (Chen and McKearin, 2003). The formation of TF/cap cells prior to *bam* expression during early ovarian development suggests that signals from TFs/cap cells are important for preventing anterior PGCs from differentiating (Bam expression) and for then allowing them to become GSCs.

GSCs in one niche can originate from one PGC

To gain further evidence supporting GSC establishment at the early pupal stage, we carefully examined spectrosome positioning and division patterns of PGCs that were in contact with cap cells. Owing to the lack of a definitive GSC marker, two criteria are often used to determine GSC identity in the adult ovary (Lin, 1998; Xie and Spradling, 2001). One is that the spectrosome of GSCs is anchored to the cap cell contact site; the other is that a GSC divides asymmetrically and generates two daughters with only one of them remaining in contact with cap cells. At the larval-pupal transitional stage, most of the female gonads did not have obvious cap cells but had only eight or nine precisely packed oval TF cells, and the spectrosome of the germ cells in the anterior row was not positioned to the anterior side (Fig. 2A,B). This observation indicates that before the early pupal stage, GSCs have not yet been established. At the early pupal stage, cap cells were evident by a few rounder *lacZ*-positive cells that were positioned posterior to the eight or nine nicely packed TF cells (Fig. 2C,D). After cap cell formation, spectrosomes in some of the PGCs that were juxtaposed with cap cells started to be anchored to the anterior side that was in contact with cap cells (Fig. 2C), suggesting that the establishment of GSCs takes place during this period. Furthermore, the putative GSCs juxtaposing cap cells continued to divide (Fig. 2D), exhibiting two distinct division patterns: one division pattern generated two daughters in which only one was in contact with cap cells, while the other pattern generated two daughters that were both in contact with cap cells. To exclude the possibility that two germ cells connected by an elongated fusome in either division pattern are a two-cell cyst, we examined the expression of *bam-*

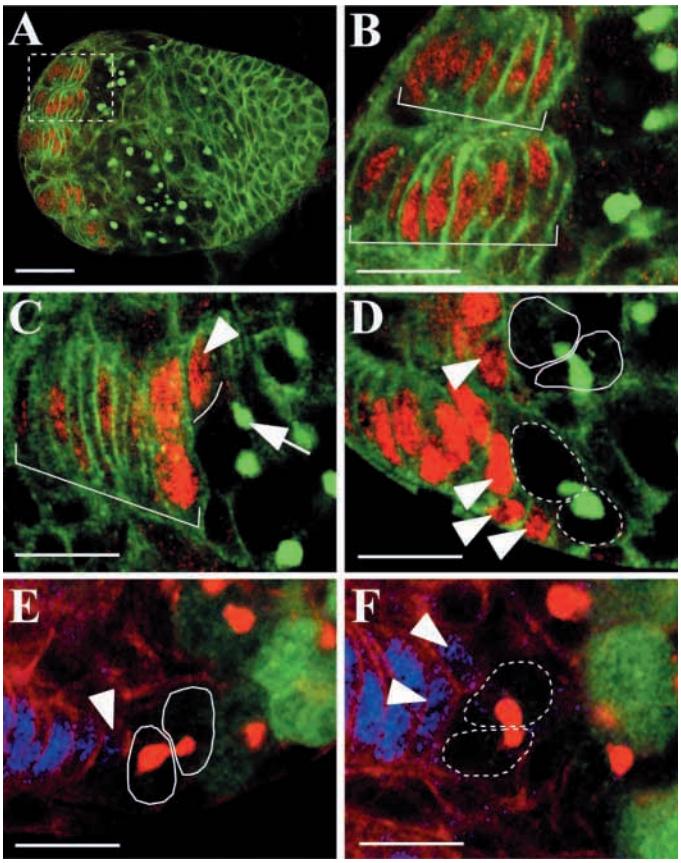

Fig. 2. The establishment of GSCs during the early pupal stage. All images represent one confocal section. *Hh-lacZ* is expressed in the nuclei of TFs/cap cells, whereas Hts staining highlights fusomes in germ cells and membranes of TFs/cap cells and other somatic cells. The *lacZ*-positive disc-like cells that are packed against each other in a line with their longitudinal axis are TFs, whereas the posterior *lacZ*-positive cells that do not align with TFs are cap cells. The gonads in A-D are labeled for *lacZ* (red) and Hts (green); the gonads in E and F are labeled for *lacZ* (blue), GFP (green) and Hts (red). (A,B) A *hh-lacZ* gonad at the larval-pupal transition stage showing only TFs. B shows two tightly packed *hh-lacZ*-positive TFs (brackets) without the appearance of cap cells at a higher magnification in the boxed area in A. (C) Part of a *hh-lacZ* female gonad at the transitional stage from larva to pupa showing finished TF cells (bracket) and newly formed cap cells. The fusome (arrow) in one of the PGCs moves close to the interface (white line) with the adjacent cap cell (arrowhead). (D) Part of a *hh-lacZ* female gonad also at the transitional stage showing two different PGC division patterns. In the top part, only one of the two PGC daughter cells (unbroken outline) contacts cap cells (arrowhead); in the lower part, both of the PGC daughters (broken outline) contact cap cells (arrowheads). (E,F) *hh-lacZ*;*bam-GFP* gonads showing that the two-cell clusters close to cap cells (arrowheads) are not two-cell cysts but two PGC/GSC daughters. Only one of the two GSC daughters (unbroken lines) is in juxtaposition to TFs/cap cells (arrowhead) in E, whereas both the GSC daughters (broken lines) are in close contact with TFs/cap cells (arrowheads) in F. Scale bars: 20 μm in A; 10 μm in B-F.

GFP in the gonad in which TFs/cap cells were identified by *hh-lacZ* expression. The differentiated cysts always express *bam-GFP* in the adult ovary (Chen and McKearin, 2003). Interestingly, the two cells generated by either division pattern failed to express *bam-GFP* (Fig. 2E,F), indicating they were two daughters of a newly established GSC rather than a two-cell cyst. The division pattern that generates two daughters contacting cap cells that then become two GSCs can be seen in the adult ovary during stem cell replacement (Xie and Spradling, 2000). Therefore, we predicted that GSCs in some niches might come from one PGC.

To investigate whether GSCs in a niche can originate from one PGC, we examined the integration of marked PGCs into niches before niches are occupied. Two nonfunctional but complementary *tubulin* (*tub*)-*lacZ* (nuclear) alleles were induced by FLP-mediated FRT recombination to generate a functional *tub-lacZ* gene (Harrison and Perrimon, 1993; Margolis and Spradling, 1995) and thus positively mark PGCs at or before the late third instar larval stage (see Materials and Methods). The marked PGCs were incorporated into individual ovarioles and their progeny in one- to two-day-old ovaries were identified by *lacZ* expression (Fig. 3A). We predicted that all progeny would be marked if GSCs in the same niche came from one marked PGC; otherwise only a fraction of progeny would be marked if GSCs in the same niche came from multiple PGCs. PGCs were positively marked at a very low frequency (to prevent two individually marked PGCs from entering the same niche) before PGCs gained their stem cell identity. Our results showed that 50.6% of the one- to two-day-old ovarioles that had marked GSCs were wholly populated by marked germ cells, while the rest contained a fraction of marked germ cells (Fig. 3B,C; total 79 *lacZ*-positive GSC clones examined), indicating that GSCs in the same niche can originate from one or multiple PGCs. The clonal expansion of GSCs cannot be accounted for by the accidental recruitment of two or three marked PGCs into the same niche because, in our experiment, only 2.0% of the total ovarioles carried one or more marked GSCs (total 3955 ovarioles examined). GSCs in one niche can develop from one PGC, indicating the existence of clonal GSC expansion during niche formation.

To rule out the possibility that the clonal expansion of GSCs in a niche was due to the specific genetic background we used, we marked PGCs by the loss of *armadillo-lacZ* (nuclear and cytoplasmic) expression under a different genetic background (Xie and Spradling, 1998; Song et al., 2002). Consistently, after randomly marking PGCs at or before the late third-instar larval stage, 36.5% of the ovarioles carrying marked GSCs contained only marked germ cells (total 126 marked GSC clones examined) (Fig. 3D,E). In this experiment, only 8.7% of total ovarioles carried one or more marked GSCs (total 1456 ovarioles examined). Note that the percentage of clonal niches in this experiment is lower than that of the previous experiment. We reason that this may be due to the *arm-lacZ* genetic background having a slightly bigger GSC niche. Because more GSCs in one niche require more divisions to populate, there is less chance for clonal GSC expansion. Alternatively, this would make it less likely for a given nascent niche to receive only one GSC precursor during organization of the germarium. These results demonstrate that GSCs in one niche originate from either one PGC or multiple PGCs.

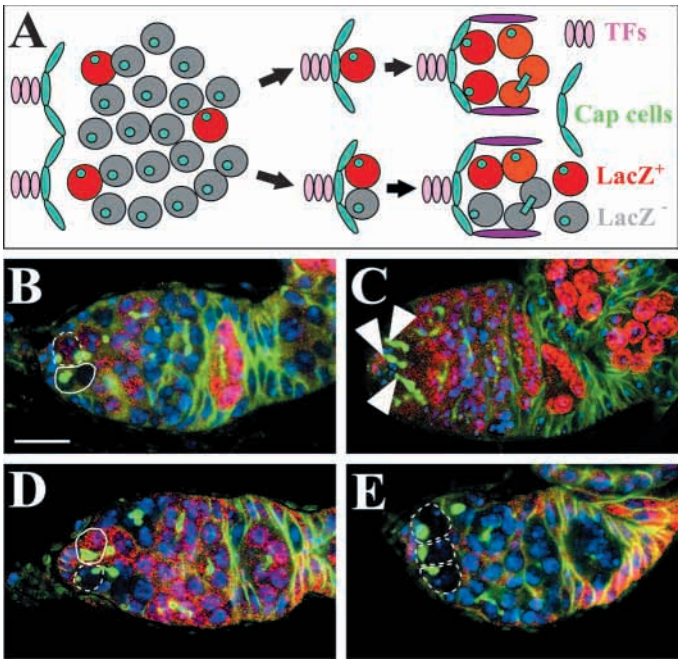

Fig. 3. Two mechanisms used by GSCs for populating their niches. (A) An explanatory diagram for two potential mechanisms populating GSC niches: GSCs in one niche come from one PGC (top); GSCs in one niche come from multiple PGCs (bottom). PGCs are marked by gain of *lacZ* (nuclear) expression (B,C) or loss of *lacZ* (nuclear and cytoplasmic) expression (D,E) before the late third instar larval stage. (B-E) Germaria are labeled for *lacZ* (red), Hts (green) and DNA (blue). (B) A germarium with one *lacZ*-positive marked GSC (broken outline) and a *lacZ*-negative unmarked GSC (unbroken outline) (i.e. only some of the GSC progeny are marked by *lacZ* expression). (C) A germarium with three GSCs (their spectrosomes indicated by arrowheads) and their progeny are marked by *lacZ* expression (an overlaid image). (D) A germarium with one *lacZ*-negative marked GSC (broken outline) and one *lacZ*-positive unmarked GSC (unbroken outline) (i.e. only a fraction of differentiated germ cells are marked by loss of *lacZ* expression). (E) A germarium with three GSCs (broken outline) marked by the loss of *lacZ* expression, in which all differentiated germ cells are *lacZ* negative. Scale bar: 10 μm.

Dpp signaling is capable of stimulating PGC proliferation

Among many possible explanations for clonal expansion of GSCs in a niche, the most attractive is that mitogenic signals from TFs/cap cells stimulate PGCs to divide and produce two daughters that will directly contact cap cells and become GSCs before other PGCs enter the niche. If this prediction is correct, we would expect that the PGCs adjacent to TFs/cap cells are more mitotically active than the rest of the PGCs. To determine the mitotic activity of PGCs during niche formation, we examined the distribution of PGCs in the S phase of the cell cycle after the incorporation of a nucleotide analog, bromodeoxyuridine (BrdU), into the gonads ranging from the late third instar larval stage to the early pupal stage. The gonads were further immunostained with anti-BrdU and anti-Vasa antibodies to visualize BrdU-positive cells and germ cells, respectively. The presence of BrdU in the nucleus indicates a cell in the S phase of the cell cycle. At the late third-instar

larval stage, a few PGCs were positive for BrdU and were randomly distributed in the PGC zone (data not shown). At the early pupal stage, we observed that more PGCs were positive for BrdU and that those cells were located preferentially close to TFs/cap cells (Fig. 4A,B). Of the PGCs that were adjacent to TFs/cap cells, 31.1±5.5% were BrdU-positive, in contrast to only 11.6±1.6% for the remaining PGCs (total of nine gonads examined), indicating that the anterior PGCs are more mitotically active than the rest of the PGCs. Interestingly, many of the somatic cells throughout the gonads, including the somatic cells mingled with the PGCs, were positive for BrdU, indicating that the somatic cells are also very active in proliferation at this developmental stage (Fig. 4A,B). These results suggest that a mitogenic signal(s) from TF/cap cells stimulates PGC division during niche formation.

To identify the signals involved in stimulating the division of anterior PGCs during the process of niche formation, we examined the expression of *dpp*. *dpp* has been shown to prevent GSCs from differentiation and stimulate GSC division in the adult ovary (Xie and Spradling, 1998). *dpp* is also known to stimulate cell proliferation in the developing wing (Burke and Basler, 1996). Owing to the lack of suitable anti-Dpp antibodies to determine the distribution of Dpp protein, we applied fluorescent mRNA in situ hybridization to determine where *dpp* mRNA is expressed at the early pupal stage. The female gonads were also immunostained with an anti-Hts antibody to visualize TFs/cap cells and spectrosomes. *dpp* mRNA was present in TFs/cap cells and other somatic cells in the anterior half of the gonad (Fig. 4C,D), suggesting that *dpp* is a candidate signal to stimulate PGC division. To determine whether *dpp* directly acts on PGCs, we examined the expression pattern of *Daughters against dpp* (*Dad*), one of the *dpp* responsive genes. The *Dad-lacZ* line is commonly used to determine *Dad* expression and to indicate *dpp* activity in many different developmental processes in which *dpp* is known to play a role (Tsuneizumi et al., 1997). *Dad-lacZ* was expressed at higher levels in the anterior PGCs than the other PGCs (Fig. 4E,F). Interestingly, *Dad-lacZ* heterozygous gonads appeared to have more PGCs than wild type (compare Fig. 4E with 4C). The germ cells also had a tendency to spread throughout the posterior half of the *Dad-lacZ* gonad instead of staying in the PGC zone in the middle of the wild-type gonad (Fig. 4E). The *Dad-lacZ* line contains a P-element insertion in the *Dad* locus, and thus is a *Dad* mutant (Tsuneizumi et al., 1997). *Dad* is directly induced by *dpp* but negatively regulates *dpp* signaling. The increase in the PGC number in the *Dad-lacZ* heterozygous gonads is probably caused by the increase in *dpp* signaling in PGCs. This result suggests that *dpp* directly signals to PGCs and potentially regulates their proliferation during early ovarian development.

To investigate further whether *dpp* can stimulate PGC proliferation, we used the GAL4-UAS expression system to overexpress *dpp* in the somatic cells of female gonads and examined PGC proliferation. The GAL4 line, *C587-gal4*, was used to overexpress *dpp* in the somatic cells throughout the developing female gonad. The *C587-gal4* line drives expression of UAS-GFP in most of the somatic cells but not in germ cells in the developing female gonads (Fig. 5A,B). To test whether we could achieve *dpp* overexpression throughout the developing female gonads, we used the *Dad-lacZ* line to detect *dpp* action in gonadal cells. *dpp* overexpression caused *Dad-lacZ* to be expressed in all the somatic cells and PGCs, indicating *dpp* activity everywhere in the gonads (Fig. 5C,D). It appeared that the somatic cells expressed *Dad-lacZ* at higher levels than the germ cells after *dpp* was overexpressed (Fig. 5C,D). However, most of the somatic cells normally express *Dad-lacZ* at much lower levels than anterior PGCs (Fig. 4E), suggesting that the somatic cells are more sensitive to elevated *dpp* expression. This also raises a possibility that *dpp* could indirectly affect germ cell proliferation by regulating the production of other signals that are important for germ cell

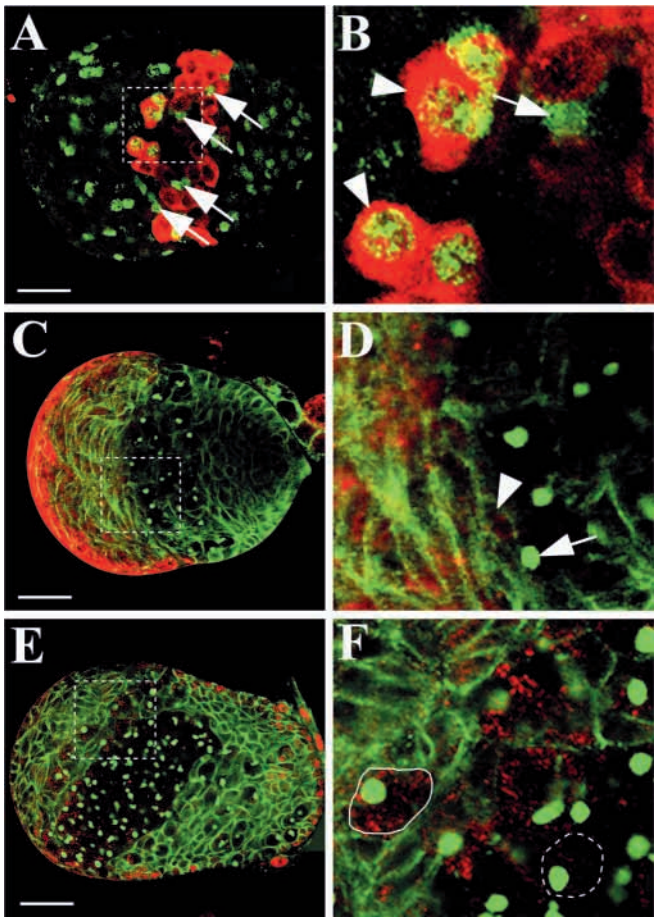

Fig. 4. *dpp* directly signals to anterior PGCs at the early pupal stage of ovarian development. All female gonads were isolated from early pupa. (A,B) A wild-type gonad labeled for BrdU (green, nuclei) and Vasa (red, cytoplasm), showing that the PGCs close to TFs/cap cells have a higher tendency to be BrdU-positive than the other PGCs. Several BrdU-positive somatic cells in the germ cell zone are indicated by arrows. (B) Area in A outlined by broken lines, showing anterior BrdU-positive PGCs (arrowheads) and a BrdU-positive somatic cell (arrow). (C,D) A gonad labeled for *dpp* mRNA (red) and Hts (green), showing the expression of *dpp* mRNA in anterior somatic cells. (D) Boxed area in C at higher magnification, showing *dpp* mRNA in TFs and cap cells (arrowhead) but not in PGCs indicated by spectrosomes (arrow). (E,F) A *Dad-lacZ*/+ gonad labeled for *lacZ* (red) and Hts (green), showing that *Dad* is preferentially expressed in the PGCs close to TFs/cap cells. (F) Boxed area in E at higher magnification, showing a *Dad*-positive PGC (outlined by an unbroken line) adjacent to TFs/cap cells and a *Dad*-negative PGC lying more posterior (outlined by a broken line). Scale bars: 20 μm for A,C,E.

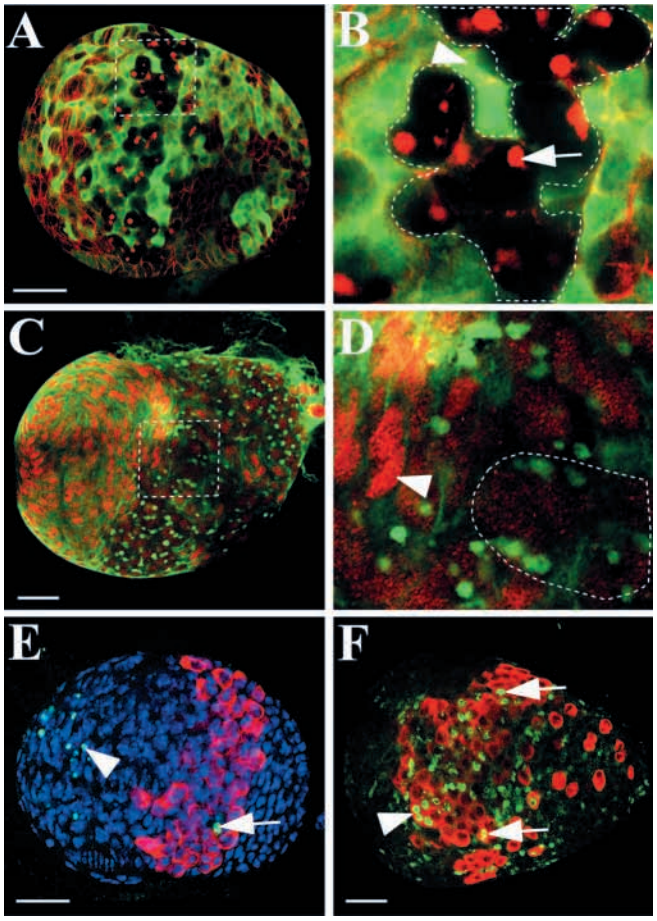

Fig. 5. *dpp* is capable of stimulating PGC proliferation during early ovarian development. (A,B) A *C587-gal4/UAS-GFP* gonad labeled for GFP (green) and Hts (red), showing the expression pattern of a target gene controlled by the GAL4 line in the majority of the somatic cells. (B) The boxed area in A, showing that GFP is only expressed in most of the somatic cells (arrowhead) but not in the PGCs (outlined) that are identified by the presence of spectrosomes (arrow). (C,D) A *C587-gal4/UAS-dpp*; *Dad-lacZ/+* gonad labeled for *lacZ* (red) and Hts (green), showing that overexpressed *dpp* induces expression of *Dad-lacZ* in all the PGCs as well as somatic cells. (D) The boxed area in C, showing that all the PGCs (some PGCs not adjacent to TFs/cap cells are outlined) and somatic cells (arrowhead) express *Dad*. (E) An early pupal wild-type gonad labeled for dead cells by ApoTag (green, nucleus) and Vasa (red, cytoplasm), showing a dying germ cell (arrow) and dying somatic cells in the anterior (arrowhead). (F) A *C587-gal4/UAS-dpp* gonad at the early pupal stage labeled for BrdU (green, nucleus) and Vasa (red, cytoplasm), showing that in the *dpp*-overexpressing gonad BrdU no longer preferentially labels anterior PGCs (arrowhead) and also labels more posterior PGCs (arrows) than in the control. Scale bars: 20 μm for A,C,E,F.

proliferation. To determine quantitatively the effect of *dpp* overexpression on the accumulation of PGCs in the gonad, we counted PGCs based on the number of spectrosomes in the gonad. The female gonads of the early pupae overexpressing *dpp* or GFP (control) by *C587-gal4* were labeled with an anti-Hts antibody to visualize the spectrosomes. In female gonads overexpressing *dpp*, the number of PGCs per gonad was increased, averaging 245±92 per gonad (total of 10 gonads examined), in contrast to 136±26 (total of 14 gonads examined) observed in control female gonads. These results indicate that the increase in *dpp* signaling causes the accumulation of more PGCs in the developing female gonads.

To rule out the possibility that the increase in PGC numbers in the *dpp*-overexpressing gonad is due to the ability of *dpp* to promote PGC survival rather than to stimulate proliferation, we also examined PGC death in wild-type gonads using the TUNEL assay. No significant levels of PGC death were observed in the gonads at the stages from the late third-instar larvae to the early pupae. For example, we were able to detect on average only one dying PGC per gonad at the early pupal stage (total of 10 gonads examined; Fig. 5E). The dying somatic cells in the anterior tip of the gonad at this stage were readily detected, suggesting that cell death might help get rid of extra somatic cells in the anterior gonad. This result indicates that *dpp* overexpression increases PGC numbers in the developing gonads by promoting PGC proliferation rather than by inhibiting cell death. To confirm further that *dpp* overexpression increases the mitotic activities of PGCs, we quantified PGCs in S phase by BrdU incorporation and also examined the distribution of BrdU-positive PGCs in the *dpp*-overexpressing gonads. The gonads from the early pupae overexpressing *dpp* were incubated with BrdU and then immunostained with anti-BrdU and anti-Vasa antibodies to visualize BrdU-positive cells and germ cells, respectively. In the early pupal gonad that overexpressed *dpp*, 20.5±3.2% of PGCs were BrdU-positive (total of 13 gonads examined), in comparison with 15.8%±2.0% of BrdU-positive PGCs in the control gonad (total of 9 gonads examined), indicating that there is a 30% increase of BrdU-positive PGCs (P<0.005). Even though the gonad overexpressing *dpp* has 80% more PGCs than does wild type, the 30% increase of PGC mitotic activity could account for the increase in PGC numbers during a long period of time because *dpp* is continuously expressed. Interestingly, even though the mitotic activity of anterior PGCs decreased (25.8±6.0% of BrdU-positive anterior PGCs in the gonad overexpressing *dpp* in comparison with 31.1±5.5% in wild type, P<0.05), the posterior PGCs did become more mitotically active when *dpp* was expressed throughout the entire gonad [18.6±2.0% were BrdU positive (Fig. 5F) in comparison with 11.6±1.6% in wild type; P<0.005). Overexpressed *dpp* slightly lowered the mitotic activity of anterior PGCs possibly by inducing unknown local inhibitory mechanisms. As in the *Dad-lacZ* heterozygous gonad, the germ cells were also found throughout the posterior half of the *dpp*-overexpressing gonad (Fig. 5F). All of these results indicate that *dpp* is capable of stimulating PGC proliferation.

tkv is essential for GSC clonal expansion in a niche

Owing to the stringent requirement of *dpp* during embryogenesis, it is difficult to test directly the effect of *dpp* mutations on the PGC proliferation and clonal expansion of GSCs. To determine whether *dpp* is required for GSC clonal expansion, we tested the requirement of *dpp* downstream components, *thick veins* (*tkv*) and *mothers against dpp* (*mad*), for populating adult GSC niches. *tkv*, which encodes a serine/threonine kinase receptor, is essential for transducing the *dpp* signal in all tissues that require *dpp* (Nellen et al., 1994; Penton et al., 1994; Brummel et al., 1994). Mad is a transcription factor that is phosphorylated upon *dpp* signaling

and is responsible for activating *dpp* target genes (Sekelsky et al., 1995; Newfeld et al., 1997). To test the requirement of *tkv* and *mad* for clonal GSC expansion, we removed their function from PGCs just before they were recruited into their niches by using the FLP-mediated FRT recombination and strong *tkv* and *mad* alleles, tkv^{8} and mad^{12}. In the control, 11.2% of the ovarioles carried marked wild-type GSC clones (total 331 ovarioles examined). Under the exactly same conditions, only 2.5% of the ovarioles carried mutant *tkv* GSC clones, and instead many marked mutant *tkv* GSCs were lost before adulthood, which was evident by the presence of mutant *tkv* cysts but the absence of marked mutant *tkv* GSCs in the germarium (total 937 ovarioles examined; Fig. 6A,B). No ovarioles carrying mutant mad^{12} GSC clones were recovered but the ovarioles with mutant mad^{12} germ cells in egg chambers were observed (total 500 ovarioles examined), indicating that marked mad^{12} GSCs could not be maintained before adulthood. These results suggest that *dpp* signaling may be involved in maintaining GSCs before adulthood. In the ovarioles that carried marked wild-type GSC clones (total of 37 marked GSC clones examined), 36.5% of them contained only marked GSCs, indicating the clonal expansion of GSCs. By contrast, none of the ovarioles that carried marked *tkv* mutant GSC clones were clonally populated (total of 23 marked *tkv* GSC clones examined; Fig. 6B). *tkv* mutant GSCs divide slower than the wild type in the adult ovary (Xie and Spradling, 1998). The mitotic potential of *tkv* mutant PGCs in the developing gonad is probably also compromised. A slow division rate of mutant *tkv* PGCs allows more time for adjacent wild-type PGCs to contact cap cells directly and become GSCs. Therefore, a mutant *tkv* PGC cannot effectively populate a niche by itself probably because of its proliferation defects. This result demonstrates that *tkv* is essential for GSC expansion, thus allowing one PGC to populate a niche. It also further suggests that *dpp* signaling is required for this process.

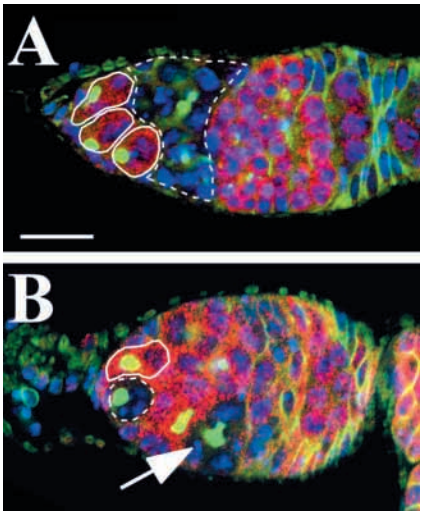

Fig. 6. The requirement of *tkv* for clonal expansion of GSCs during niche formation. Both germaria are labeled for *lacZ* (red), Hts (green) and DNA (blue). *lacZ*-negative mutant *tkv* PGCs were generated before the late third instar larval stage, and mutant *tkv* GSCs in the adult ovary were subsequently identified by loss of *lacZ* expression. (A) A germarium carrying three *lacZ*-positive wild type GSCs (outlined by unbroken lines) and *lacZ*-negative *tkv* mutant germline cysts (outlined by a broken line). The mutant tkv cysts were most likely derived from a lost mutant *tkv* GSC. (B) A germarium carrying a *lacZ*-negative *tkv* mutant GSC (outlined by a broken line), a *lacZ*-positive wild type GSC (outlined by an unbroken line), and a mutant *tkv* cyst (arrow). Scale bar: 10 μm.

DISCUSSION

Our study has, for the first time, demonstrated how an adult GSC niche is populated with stem cells in the *Drosophila* ovary. Before niche formation, all PGCs proliferate as pre-stem cells and are undifferentiated. As niche formation starts, PGCs divide into two distinct subpopulations: anterior PGCs adjacent to cap cells start to acquire stem cell identity, and the remaining PGCs directly proceed to differentiation. GSCs in one niche can come from one PGC. We further show that Dpp is likely involved in stimulating clonal expansion of GSCs during niche formation. This study suggests that signals from newly formed niches are important for expanding GSCs and most likely for populating nascent adult GSC niches.

Positional information helps select stem cells from GSC precursors

How stem cell identity is established initially remains elusive even in the well-studied stem cell systems: *Drosophila* ovary and testis. In the primitive female gonads before the pupal stage, PGCs appear to undergo symmetric division to generate germ cells with the identical pre-stem cell fate. Several studies suggest that GSCs were established at the early pupal stage (Bhat and Schedl, 1997; Song et al., 2002). At the early pupal stage, there are 136 germ cells on average in each gonad. The adult ovary, which is composed of 12-16 ovarioles with two or three GSCs per ovariole (average of 2.5), contains about 30 to 40 GSCs. Therefore, at the most, 20-30% of PGCs in the early pupal gonad are recruited to niches and turn into GSCs.

How is a particular germ cell selected and recruited to niches, and how does it become a GSC? Positional information is known to be very important for cell-fate determination in various developmental processes. In this study, we have taken a developmental approach to investigate when key niche components form, and how PGCs are subdivided into GSCs and differentiated germ cells. The expression of *bam* is associated with germ cell differentiation in the adult ovary (McKearin and Ohlstein, 1995; Ohlstein and McKearin, 1997). Using *bam* expression as an indicator for germ cell differentiation, we have shown that no PGCs in late third instar larval gonads have differentiated. In early pupal gonads (about 0-4 hours after pupation), all the PGCs that are not in contact with TFs/cap cells are differentiated; therefore, the PGCs that contact newly formed cap cells remain undifferentiated and become GSCs (Fig. 1F). Possibly, newly formed TFs/cap cells directly prevent the most anterior PGCs from differentiation when an unknown developmental signal triggers PGC differentiation around the larval-pupal transition stage. Our study demonstrates that the stem cell fate of PGCs is determined by their position, i.e. juxtaposition to TFs/cap cells.

Stem cells can originate from one GSC precursor by clonal expansion

The next important question is how these anterior PGCs

populate niches. In this study, we show that the PGCs in contact with newly formed cap cells at the early pupal stage divide more frequently than the rest of the PGCs. The division patterns are very interesting: one division pattern generates two daughters that are both in contact with cap cells; the other pattern generates only one daughter that is in contact with cap cells. As in the adult ovary, two daughters that are in contact with cap cells can both become GSCs. This is verified by the observation that one marked PGC in the gonad at the late third-instar larval stage can generate two or three GSCs in a niche. Our results also indicate that the stem cells in a niche can come from multiple PGCs. Whether GSCs in a niche come from one or multiple PGCs probably depends on whether one or multiple PGCs directly contact cap cells within the developing niche. If only one PGC contacts cap cells, it probably has an opportunity to generate two or three germ cells that contact cap cells and become GSCs. This study shows that newly formed niches do not simply recruit existing PGCs and turn them into GSCs, but also stimulate PGCs to proliferate and produce more GSCs.

The orientation of stem cell divisions seems to be very important for self-renewal and expansion of a stem cell pool. This strategy does not seem unique to the *Drosophila* ovarian GSCs. In the ventricular zone of the developing mammalian brain, neural stem cells divide either parallel or perpendicular to the ventricular surface (Chenn and McConnell, 1995). Normally, neural stem cells are in close contact with the surface of the ventricular zone, and differentiated daughters move away from the ventricular zone. It has been suggested that the stem cell divides along the ventricular surface to give rise to two stem cells, while the perpendicular division generates one stem cell and one differentiated neuronal cell. Controlling the orientation of the stem cell division plane could be a general mechanism for maintaining stem cell homeostasis and generating needed differentiated cells.

BMP-like signaling stimulates clonal expansion of GSCs during early ovarian development

The clonal expansion of GSCs in a niche clearly requires the newly established stem cell to divide rapidly and generate a daughter that occupies the same niche, which further prevents other neighboring precursor cells from entering it. Consistent with this prediction, we observed that the anterior row of germ cells at the early pupal stage was more mitotically active than the rest of the germ cells based on the BrdU incorporation assay. *dpp* is known to be important for maintaining GSCs and stimulating their division in the adult ovary (Xie and Spradling, 1998). We show that *dpp* is expressed in TFs/cap cells and other anterior somatic cells, and that PGCs close to cap cells are capable of responding to *dpp*. Furthermore, overexpressing *dpp* promotes PGC proliferation. To demonstrate the necessity of *dpp* signaling in stimulating GSC clonal expansion, we have shown that a PGC mutant for *tkv*, an essential *dpp* receptor, fails to clonally populate a niche. All these results demonstrate that *dpp* is probably a signal for stimulating GSC clonal expansion. However, we cannot rule out that other BMP-like molecules, such as *gbb-60A*, could also play a similar role because *tkv* could also be involved in other BMP-like signaling pathways.

As in the adult ovary, *hh* is also expressed in terminal filaments and cap cells in developing female gonads. Hh has recently been shown to play a minor role in modulating GSC division (King et al., 2001). Wingless (Wg) protein is expressed in terminal filaments and cap cells (X. Song and T.X., unpublished). Its expression in developing female gonads has not been examined. Because *wg*, *dpp* and *hh* often work together to regulate many developmental processes in *Drosophila*, it is possible that *hh* and *wg* could also cooperate with *dpp* to regulate PGC proliferation and modulate GSC clonal expansion in niches.

What signal(s) keeps anterior GSC precursors from differentiation?

PGCs in the gonad do not show any signs of differentiation until the larval-to-pupal transition. At the early pupal stage, only the PGCs in the anterior row remain undifferentiated, but the rest have already differentiated. It seems that a developmental signal(s) starts to appear and then induces the differentiation of PGCs during the transition from larva to pupa. Such a developmental signal could be mediated by a steroid-like hormone ecdysone. Interestingly, during most of the third instar larval stage, the ecdysteroid levels are very low but begin to rise and peak just before pupation (Riddiford, 1993). The ecdysteroid peak could be potentially responsible for the initial differentiation of germ cells in the gonad of the larva ready for pupation. It is also possible that the hormone is not a direct signal but controls the production of the signal(s). Somehow, the signals from the anterior somatic cells antagonize the differentiating signals and thus prevent the anterior row of the PGCs from differentiation. One of the signals that prevent PGCs from differentiation could be encoded by *dpp*. Dpp is known to prevent GSCs from differentiation in the adult ovary (Xie and Spradling, 1998). In this study, 2.5% of the marked *tkv* mutant PGCs and none of the marked *mad* mutant PGCs before the third instar larval stage were recruited to niches or were maintained as GSCs before adulthood. The failure of *tkv* and *mad* mutant GSCs to be maintained in niches could be explained by the role of *dpp* in preventing PGCs from differentiation. It could also be explained by other possibilities, such as defects in the formation of adherens junctions between cap cells and GSCs. Whether *dpp* is a signal for maintaining the undifferentiated state of PGCs during early ovarian development remains undetermined. Therefore, the signals that maintain the undifferentiated state of PGCs from TFs/cap cells remain to be identified.

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