Long-term live imaging provides new insight into stem cell regulation and germline-soma coordination in the *Drosophila* ovary

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SUMMARY

The *Drosophila* ovariole tip produces new ovarian follicles on a 12-hour cycle by controlling niche-based germline and follicle stem cell divisions and nurturing their developing daughters. Static images provide a thumbnail view of folliculogenesis but imperfectly capture the dynamic cellular interactions that underlie follicle production. We describe a live-imaging culture system that supports normal ovarian stem cell activity, cyst movement and intercellular interaction over 14 hours, which is long enough to visualize all the steps of follicle generation. Our results show that live imaging has unique potential to address diverse aspects of stem cell biology and gametogenesis. Stem cells in cultured tissue respond to insulin and orient their mitotic spindles. Somatic escort cells, the glial-like partners of early germ cells, do not adhere to and migrate along with germline stem cell daughters as previously proposed. Instead, dynamic, microtubule-rich cell membranes pass cysts from one escort cell to the next. Additionally, escort cells are not replenished by the regular division of escort stem cells as previously suggested. Rather, escort cells remain quiescent and divide only to maintain a constant germ cell:escort cell ratio.

KEY WORDS: Live imaging, Stem cells, Germarium, *Drosophila*

INTRODUCTION

Stem cells respond continuously to ongoing signals from within their local microenvironment (Morrison and Spradling, 2008). These sophisticated interactions must ultimately be analyzed at the level of individual cells, but the size and complexity of many tissues makes this process extremely difficult. The *Drosophila* germarium, a germinal structure found at the anterior of each ovariole, is favorable for such studies because it comprises only ~300 cells that are further divided into four subregions (Fig. 1A). Operating at steady state throughout adulthood, the germarium houses both germline and follicle stem cells within distinct niches and controls the development of their daughters into a new ovarian follicle every 12 hours. This cycle encompasses cellular processes ranging from stem cell division to cyst formation, cell migration, oocyte specification, meiotic initiation and follicle construction. Moreover, all these events respond homeostatically to changes in nutrition, sperm availability, oviposition sites and other variables that impact the need for gametes (Allemand and Bouletreau-Merle, 1989; Chapman et al., 1995; Drummond-Barbosa and Spradling, 2001). Consequently, the germarium provides exceptional opportunities for studying how tissue homeostasis is controlled.

A diverse array of cellular interactions mediates follicle formation in the germarium. At the anterior, non-dividing terminal filament and cap cells specify a niche that houses two germline stem cells (GSCs), which are retained by cadherin-mediated adhesion and regulated by Jak-Stat and BMP signals (Kirilly and Xie, 2007; Morrison and Spradling, 2008). Downstream,

throughout the anterior half of the germarium (regions 1-2a), squamous escort cells wrap GSC daughters (cystoblasts) as they divide synchronously to form 16-cell cysts and move towards the posterior. Germ cells and escort cells communicate via Notch signals, EGFR ligands, Jak-Stat and BMP signals (Decotto and Spradling, 2005; Schulz et al., 2002; Wang et al., 2008). Soon after entering region 2b, cysts exchange their escort cell covering for a monolayer of follicle cells and begin to specify a few of these as polar cells using germline Notch signaling (Assa-Kunik et al., 2007; Bender et al., 1993; Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001; Margolis and Spradling, 1995; Nystul and Spradling, 2010; Ruohola et al., 1991; Torres et al., 2003). Polar cells signal via the Jak-Stat ligand Unpaired (Outstretched – FlyBase) to stimulate follicle budding and to specify stalk formation (Assa-Kunik et al., 2007). As a result of these interactions, cells develop in a sequential manner from stem cell to follicle while moving steadily away from the tip over a period of ~4 days, with each successive 16-cell cyst corresponding to ~12 hours of developmental time.

How germ cells exchange their covering of escort cell membranes for a monolayer of follicle cells midway through the germarium remains poorly understood. Escort cells have been proposed to arise by the division of 5-6 anterior cells termed 'escort stem cells' that move along with the cysts as they traverse the anterior germarium and then undergo apoptosis as they are replaced by follicle cells (Decotto and Spradling, 2005). The incoming follicle cells are generated by two follicle stem cells (FSCs) that reside within lateral niches near the boundary of regions 2a and 2b (Nystul and Spradling, 2007) (Fig. 1A). The complex cellular behavior that must occur in the mid-germarium as a result of these events has been difficult to decipher at single-cell resolution using fixed material. Consequently, the anatomy and molecular regulation of the FSC niche are still poorly understood. FSCs require both cadherin-mediated adhesion and multiple intercellular

signals in order to function (Kirilly and Xie, 2007). However, stable niche cells have not been identified and it remains unclear on which cells these pathways act (Nystul and Spradling, 2007; Nystul and Spradling, 2010).

The direct observation of cellular activity in living tissue preparations expressing fluorescent reporter molecules can provide insights that are impossible to deduce from fixed material. However, it is often difficult to maintain normal cellular activity in tissues cultured in artificial media and subjected to frequent illumination. Developing *Drosophila* follicles have been successfully imaged to study border cell migration, dorsal appendage formation, mRNA and mitochondrial transport and cell cycle progression (Bianco et al., 2007; Cox and Spradling, 2003; Dorman et al., 2004; Forrest and Gavis, 2003; Gilliland et al., 2007; Haigo and Bilder, 2011; Prasad et al., 2007; Theurkauf and Hazelrigg, 1998; Wang et al., 2010). However, early oogenesis entails slower and far more complex cellular behavior. Germaria have been imaged for short periods (30-40 minutes) under oil (Fichelson et al., 2009); however, this method cannot be used for longer culture periods. Here, we describe extended live imaging of GSC activity and early cyst development within cultured germaria. Both germline and somatic cells exhibit near normal levels of activity for a prolonged period. Our experiments provide some of the first direct observations of dividing stem cells in their natural environment and clarify the escort cell-follicle cell exchange in the mid-germarium. We find that escort cells are not replenished by constitutively active anterior stem cells, but divide only as needed to maintain a constant escort cell:germ cell ratio. Escort cells remain fixed in the anterior germarium and pass cysts from one cell to the next by dynamic membrane activity. The development of an efficient in vitro imaging system to simultaneously examine two types of active stem cells and their niches provides a valuable new tool with which to address many remaining questions in stem cell biology and gametogenesis.

MATERIALS AND METHODS
Drosophila strains
Drosophila strains were maintained at 22-25°C on standard medium, except for *upd* overexpression when strains were kept at 29°C for 7 days. Canton-S strain was used as wild type. For experiments on adult flies, flies were fed yeast paste every other day. 11A12 GAL4 driver is expressed in the terminal filament, cap and escort cells (Pfeiffer et al., 2008), UAS-Upd is a lab stock. All other stocks came from the Bloomington Stock Center. Stocks used for live imaging were: Df31-GFP (Buszczak et al., 2007); Jupiter-GFP (Karpova et al., 2006); UAS-Tubulin-GFP; FLP-out system, *hsFlp; Tub FRT-CD2-FRT-GAL4; UAS-GFP* (G. Struhl, Columbia University); and His2Av-mRFP (Bloomington Stock Center). For 'FLP-out' labeling of germarial cells, flies of genotype *hsFlp; Tub FRT-CD2-FRT-GAL4, UAS-GFP; UAS-Tubulin-GFP* were heat shocked at 37°C for 45 minutes as pupae.

Immunostaining and fluorescence microscopy
Dissected ovaries were fixed for 10 minutes in 4% paraformaldehyde (Sigma) diluted in Graces medium (Lonza Walkersville). Staining and confocal analysis were then performed as described (Cox and Spradling, 2003). Primary antibodies were diluted as follows: mouse anti-phosphohistone H3 (Ser10) (1:2000, Cell Signaling Technology); guinea-pig anti-Traffic jam (Tj) (1:1000, this work). Secondary antibodies used were generated in goat against mouse and guinea pig and conjugated with Alexa Fluor 488 and 568 (Invitrogen) and used at 1:2000.

Antibody production
Anti-Tj was produced by raising antibodies in guinea pigs against amino acids 1-412 as described (Buszczak and Spradling, 2006). Antibody production was carried out by Covance.

EdU detection
EdU staining was carried out using the Click-iT EdU Alexa Fluor 594 Imaging Kit (C10339, Invitrogen). Dissected ovaries were soaked for 1 hour in 20 µM EdU diluted in Graces medium (Lonza Walkersville), rinsed in Graces medium and fixed and immunostained as described above. Samples were then washed six times in antibody wash (1× PBS, 0.1% Triton X-100, 1 mg/ml BSA), rinsed in 1× PBS and incubated with EdU reaction cocktail for 30 minutes with agitation. Ovaries were washed overnight at 4°C in antibody wash, stained with 1 mg/ml DAPI (Sigma) for 30 minutes and mounted in Vectashield (Vector Laboratories).

Live imaging
The culture and imaging protocol was based on methods developed previously for analyzing border cell migration (Bianco et al., 2007; Prasad et al., 2007; Wang et al., 2010). Several aspects of the protocol appeared to be critical for success. First, only ovarioles from newly eclosed flies of the appropriate genotype were used because ovarioles with large, more mature follicles underwent greater movement. Ovarioles were dissected in Schneider's medium (Invitrogen) supplemented with 15% fetal calf serum, 0.6× penicillin:streptomycin (Invitrogen) and 200 µg/ml bovine insulin (Sigma); the muscle sheath was removed manually. Great care was required at this step to avoid damaging the germarium, but when the sheath was present movement was excessive. Several individual ovarioles lacking sheaths were separated and placed in a small drop of the same medium on a glass-bottom dish (P35G-1.0-14-C, MatTeck) containing a wet Kimwipe for humidification and covered (Fig. 1B). Germaria were not physically restrained by a gas-permeable membrane or an artificial substrate as these techniques either inhibited development or failed to significantly limit germarial movement. We found that it was necessary to accept some movement during the incubation; indeed, only ~10% of germaria remained within the field of view and in focus for at least 11 hours. Imaging was carried out using a 63× (NA 1.32) PlanApo lens (Leica) on a Yokogawa CSU-10 spinning disk confocal or Leica DMIRE2 inverted microscope controlled with MetaMorph software (Molecular Devices). Finally, confocal images (110 0.5-µm sections per time point) were collected no more frequently than every 10 minutes. Imaging at higher frame rates failed to sustain the normal cell division program over an extended period. Subsequently, each cell was tracked manually across each frame using Imaris 7.0 software (Bitplane) for image visualization.

Calculation of cell migration distances
Cell nuclei were tracked as described above and the position of each nucleus after 0 and 14 hours in culture was marked. Cell movement was calculated from the anteroposterior position of the nuclei relative to the cap cells. Cell process movement was measured by tracking one end of a Tubulin-GFP process and recording its position after 0 and 7 hours in culture. A shorter time interval was necessary for tracking the processes than the nuclei as the microtubule bundles appeared to breakdown and reform over time. Values are shown with ±s.d.

Expansion of germarial germ cell number
Germ cell number was expanded by *upd* overexpression. Flies (gal80ts;11A12 × UAS-upd) were grown at 18°C and, after eclosion, adult flies were transferred to 29°C (overexpression) or kept at 18°C (controls) for 7 days. Ovaries were then stained with: 1B1 (DSHB, University of Iowa), which marks an endoplasmic reticulum-like structure present in germ cells that allows GSC and cyst number and type to be determined; anti-phosphohistone H3; and anti-Tj, which labels all somatic cells that contact the germline – escort cells are distinguishable from other somatic cells by their position and nuclear morphology.

RESULTS
Imaging cellular activity in cultured *Drosophila* germaria
We investigated whether the entire *Drosophila* germarium can be cultured in vitro like a single follicle and still maintain normal cellular activity. Germaria were dissected from adult females, removing the muscle sheath, and incubated in vitro in media

previously shown to support border cell migration (Bianco et al., 2007; Prasad et al., 2007; Wang et al., 2010). Cellular activity was visualized using multiple fluorescently labeled reporters and a spinning disk confocal microscope (Fig. 1B; see Materials and methods). Under ideal conditions, we observed that cellular activity persists in germaria cultured in vitro for up to 14 hours. We could record images every 10 minutes at a resolution of 110 0.5-μm sections per time point throughout this period. Overall, we analyzed more than 120 cultured germaria and recorded more than 1300 hours of movies using different fluorescent reporters (see Table S1 in the supplementary material). Forty-seven of these contributed to the data that are reported here.

A subset of movies was analyzed by identifying and tracking specific cells and subcellular structures. Initial studies utilized a fluorescent marker that labels all cell nuclei with a GFP protein trap in Decondensation factor 31 (Df31-GFP) to characterize the division and migration behavior of germarial cells during in vitro culture (Table 1; Fig. 1C; see Movie 1 in the supplementary material). Under these labeling conditions, cell types were readily distinguished by their nuclear morphology and position within the ovariole. For example, GSCs (Fig. 1C′, magenta) contain extremely large, round nuclei and are located adjacent to cap cells (Fig. 1C′, yellow). Escort cell nuclei are smaller, flattened and curved in shape and reside along the edges of the germarium near

the basement membrane (Fig. 1C′, blue). Follicle cells surround more mature germline cysts and display distinctive oblong nuclei (Fig. 1C′, green). The positions of specific cells were then tracked manually, frame by frame, throughout entire movies using Imaris software (Fig. 1D,D′; see Materials and methods). In each of the eight germaria listed in Table 1, we recorded every germ cell in region 1 (which contains stem cells and forming cysts), all 16-cell germline cysts, 6-12 of ~25 escort cells, as well as 6-20 of ~130 germarial follicle cells. Virtually any cell of interest could be followed using these methods. Additionally, the division behavior of all germarial cells was recorded. Using Df31-GFP, mitotic cells were easily identified because, upon division, the GFP is released into the cytoplasm during nuclear membrane breakdown (Fig. 2A).

Germ cell division proceeds normally in vitro

To investigate whether germ cells develop normally during in vitro incubation and imaging we first identified all cell divisions and estimated their duration (Table 1; see Fig. S1D in the supplementary material). Eighty-nine percent (50 of 56) of the mitotically competent germ cells that were present at the onset of incubation divided within the next 12-14 hours, including 88% (14 of 16) of GSCs. The duration of M phase was estimated by recording the time from initial GFP diffusion into the cytoplasm to the re-establishment of nuclear GFP (Fig. 2A). M-phase duration was found to be similar for GSCs (41±10 minutes) and forming cysts (42±14 minutes) (see Fig. S1D in the supplementary material). Germ cell divisions took place throughout the entire period of imaging, although the frequency varied and declined moderately during the final several hours (Fig. 2B).

The behavior of cyst cells provided further evidence that development proceeds normally during in vitro culture. Cysts form by undergoing four rapid, synchronous mitoses in which cytokinesis remains incomplete. In culture, cysts divided rapidly as we sometimes observed two successive divisions (Fig. 2C). Cyst divisions were also highly synchronous, as they are in vivo, as all cyst nuclei broke down and reformed almost simultaneously, based on GFP localization (Fig. 2D). Movies of germaria labeled with Jupiter-GFP (a microtubule-binding protein) also showed that spindle behavior was highly synchronized within all the cells of a mitotic cyst (Fig. 2E).

Germline stem cells undergo normal, oriented divisions and respond to insulin

Our movies made it possible to characterize whether female GSC mitotic spindles orient non-randomly in cultured germaria. Mitotic spindle angle has been found to be important in regulating GSCs
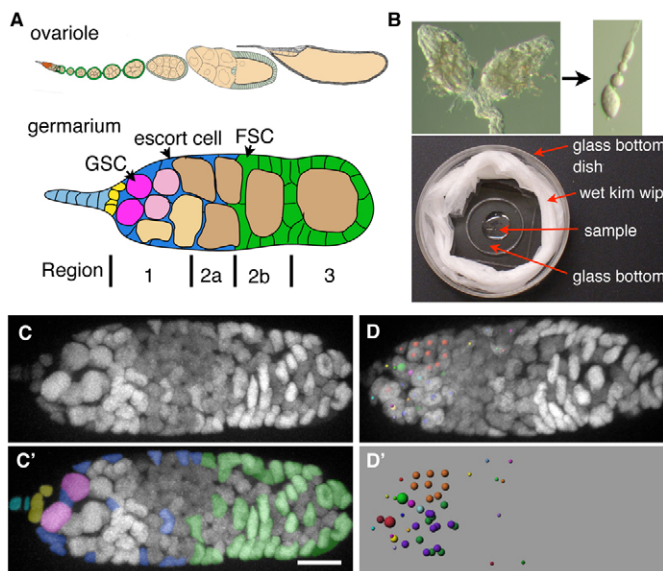

Fig. 1. The *Drosophila* germarium can undergo development in vitro. (**A**) The *Drosophila* ovariole and germarium, showing regions 1, 2a, 2b and 3 referred to in the text, terminal filament cells (light blue), cap cells (yellow), germline stem cells (GSCs, magenta), escort cells (dark blue) and follicle cells including the follicle stem cells (FSCs, green). (**B**) Experimental set up for live imaging. Ovarioles are dissected out of ovaries from newly eclosed *Drosophila* and placed in a drop of medium in a glass-bottomed dish. (**C,C′**) Df31-GFP marks the nuclei of all cells in the germarium. Individual nuclei were identified as to cell type based on their position and morphology and are pseudocolored in C′: terminal filament cells, turquoise; cap cells, yellow; GSCs, magenta; downstream germ cells, gray; escort cells, blue; follicle cells, green. (**D,D′**) Workflow for analysis of movie data using Imaris software. Germ and somatic cell positions were tracked by marking each cell at each time point. The largest red and green dots are GSCs, multiple medium-sized dots of the same color are germline cysts, and the smallest dots are somatic cells. Scale bar: 10 μm.

Table 1. Germline and follicle cell division in culture

Ovariole	GSCs		Cysts		Follicle cells	
	Total	Divided	Total	Divided	Total analyzed	Divided
1	2	2	7	7	38	19
2	2	2	4	5	–	–
3	2	2	3	2	42	10
4	3	2	3	3	40	2
5	2	2	6	5	58	23
6	2	2	6	5	80	13
7	1	0	7	5	22	9
8	2	2	4	4	–	–

Numbers and division behavior of germline stem cells (GSCs), mitotically competent germline cysts (1 to 8 cell cysts) and follicle cells in eight germaria monitored over 14 hours (ovarioles 1, 2, 5-7) or 11 hours (ovarioles 3, 4). Follicle cells could not be analyzed in ovarioles 2 and 8 because they moved outside the field of view during part of the movie.
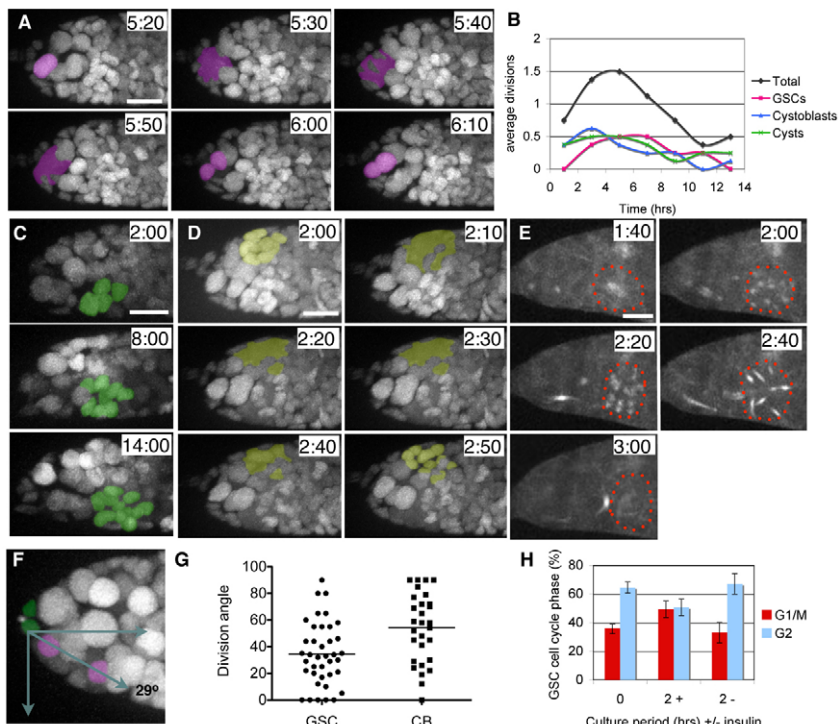
Fig. 2. GSC and germline cyst divisions occur in culture. (**A**) GSC division in culture, pseudocolored in magenta (for non-pseudocolored images, see Fig. S1A in the supplementary material). (**B**) Graph of germ cell divisions during each hour of in vitro culture. Divisions are more frequent during the initial 8 hours of imaging. (**C-E**) Germline cyst divisions occur in vitro and are synchronized. (C) A germline cyst that divides twice during imaging from a 4-cell to a 16-cell cyst, pseudocolored in green (for non-pseudocolored images, see Fig. S1B in the supplementary material). (D,E) Germline cyst divisions from 8- to 16-cell cysts showing synchrony. (D) Synchrony demonstrated by simultaneous loss and re-establishment of nuclear GFP using line Df31-GFP. The cyst is pseudocolored yellow (for non-pseudocolored images, see Fig. S1C in the supplementary material). (E) Jupiter-GFP demonstrates synchronous establishment and loss of mitotic spindles during an 8- to 16-cell cyst division (outlined). (**F,G**) Division axis of GSCs and cystoblasts (CBs) relative to cap cells (GSCs, n=39; CBs, n=29). An example of axis measurement is shown in F. Cap cells, green; GSC daughters, magenta. The bar in G indicates the mean. (**H**) In vitro culture of ovaries in insulin-rich culture media forces GSCs out of G2 phase of the cell cycle and into mitosis and G1. Five-day-old ovaries were cultured in medium with (+) or without (–) 200 μg/ml insulin. Error bars indicate s.d. Times are shown as hours:minutes. Scale bars: 10 μm.

of the *Drosophila* testis (Yamashita et al., 2003) and in vivo data from the *Drosophila* ovary suggest that GSCs divide parallel to the anteroposterior (a/p) axis (Deng and Lin, 1997). We plotted a line between the two daughter nuclei of each GSC division at the time that they reform and determined its angle relative to the a/p axis of the germarium and perpendicular to the cap cells (Fig. 2F). Very rarely was division perpendicular or parallel to the a/p axis (90° or 0°, respectively), but instead showed an average of 35±24° (Fig. 2G). By contrast, the divisional angle of the cystoblast, when calculated in an identical manner, was random (Fig. 2G). Thus, stem cells do orient divisions during culture but at a slightly different angle than that reported previously.

Insulin signaling plays a major role in controlling the rate of GSC division and in coordinating germ cell production with available nutritional resources (Drummond-Barbosa and Spradling, 2001; Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005; LaFever et al., 2010). We supplemented the medium used for in vitro culture with insulin at a level (200 μg/ml bovine insulin) that maximally stimulated the division of GSCs and of cells generally (see Fig. S1E in the supplementary material). Unexpectedly, incubation of germaria with added insulin affected the coordination of stem cell division. In the absence of added insulin, only one GSC within each germarium divided during the period of culture (see Fig. S1F in the supplementary material). However, in the presence of insulin, two GSCs divided 88% of the time (see Fig. S1F in the supplementary material). This represents a higher incidence of stem cell division than would be expected based on an average GSC cell cycle of 24 hours and clearly demonstrates that GSCs respond to insulin during in vitro culture.

The fact that at high insulin levels GSC division is more frequent than in vivo suggests that a significant number of GSCs are normally in a state susceptible to insulin stimulation. Five-day-old ovaries were used to determine the cell cycle stage of normal GSCs and GSCs in germaria that have been subjected to supplemental insulin for 2 hours (Fig. 2H). GSC cell cycle was established by fusome morphology (de Cuevas and Spradling, 1998) (see Fig. S1G in the supplementary material). Around 65% of GSCs are in G2 of the cell cycle under standard conditions in vivo (Fig. 2H, 0 hours in culture) and this did not change after 2 hours of incubation in vitro in the absence of high insulin. By contrast, the fraction of GSCs in G2 fell to 50% within 2 hours of incubating germaria with supplemental insulin. These observations suggest that many GSCs are normally arrested in G2 and can be stimulated to enter M phase by high insulin levels.

Follicle cells divide in vitro but cycling slows down after 9 hours

In vivo, follicle cells divide robustly downstream from the FSCs in order to produce the 60-80 cells that are present on each newly budded follicle. Polar cells, which are the first differentiated cells of the FSC lineage, form in region 2b and additional polar cells and stalk cells must be specified in region 3 prior to follicle separation (Margolis and Spradling, 1995; Nystul and Spradling, 2010; Torres et al., 2003). This follicle cell divisional and developmental program continued in germaria incubated in vitro. An average of 51 follicle cells in six separate germaria were analyzed for the transient loss of nuclear GFP that indicates division (Fig. 3A,B; Table 1). Our results showed that follicle cells, like germ cells, continue to divide during germarial culture and imaging. Divisions took place in the vicinity of the FSCs (Fig. 3A), throughout region 2b (Fig. 3B), as well as in the vicinity of the next-forming follicle (Fig. 3B-D). Follicle cell divisions were slightly shorter than those of the germ cells (on average 30±5 minutes) based on the interval between GFP loss in the mother nucleus and re-uptake in the two daughters. Quantitation of the rate of follicle cell division throughout the culture period showed that, in contrast to germ cells, follicle cells significantly slowed their cycling after 9 hours in culture (Fig. 3D).
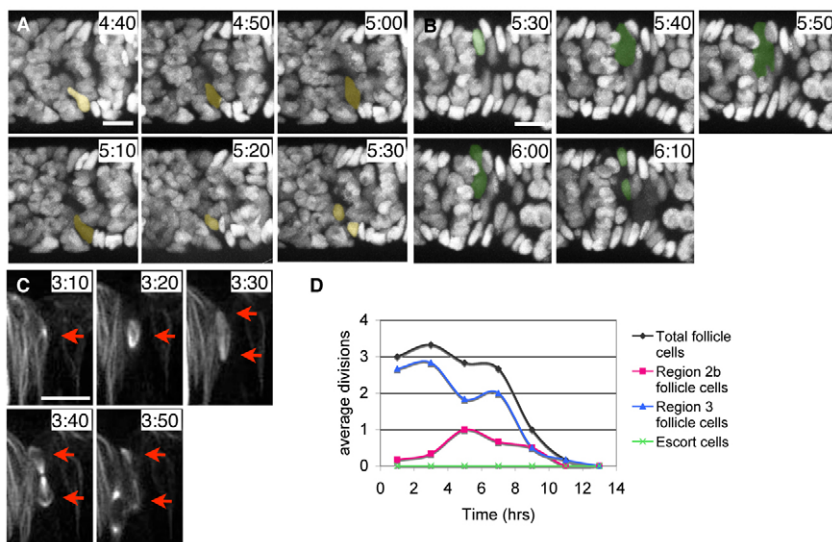
Fig. 3. Follicle cell divisions take place in culture. (**A-C**) Kinetics of follicle cell divisions in culture. (A,B) The Df31-GFP line, showing a dividing follicle cell (yellow) in region 2b (A) and a dividing follicle cell (green) in region 3 (B) (for non-pseudocolored images, see Fig. S2A,B in the supplementary material). (C) A dividing follicle cell (arrow) in region 3 labeled with Tubulin-GFP. (**D**) Graph of follicle cell divisions during in vitro culture. The rate of division falls after the first 9 hours. Note the complete absence of escort cell division, as based on the loss of nuclear GFP. Times are shown as hours:minutes. Scale bars: 10 µm.

Several candidate FSC divisions were recorded. For example, we recorded the division of a cell located at the 2a/2b junction that exhibited several properties expected of an FSC (Fig. 3A), including the movement of the daughter cell across the germarium as expected for a 'cross-migrating' cell (Nystul and Spradling, 2007). Such divisions were rare, however. The continued proliferation of daughter cells means that dividing FSCs will constitute only 6% of total follicle cell divisions (Margolis and Spradling, 1995). Thus, we would expect to see at most two FSC divisions per movie. It was not possible to determine definitively that these divisions actually represent FSCs as no distinctive anatomical features or genetic markers characteristic of FSCs or their niche are known at present.

According to the escort stem cell model, two escort cell divisions are expected for every one GSC division as the ratio of escort cells to cysts is ~2:1 (Decotto and Spradling, 2005), and so two to four escort cell divisions should be observed per movie. Contrary to expectation, we observed no escort cell divisions based on the criterion of transient nuclear GFP loss (Fig. 3D). We considered whether escort cell divisions might have been missed owing to a more rapid M phase or to the relatively weak expression of the Df31-GFP marker. Consequently, we manually tracked many escort cells using Imaris software. Divisions would have been apparent by the appearance of new cells. However, no such division events were observed and we concluded that escort cells do not divide within germaria cultured in vitro.

Cysts move towards the posterior and new follicles form in vitro

Organized cell movement plays many crucial roles in the steps leading up to follicle formation. Germline cysts move in a single file towards the posterior about one cyst position every 12 hours (corresponding to the budding of one new follicle). According to the escort stem cell model, escort cells are expected to accompany cysts throughout the anterior half of the germarium. Follicle cells undergo the most complicated movements. In region 2b, some follicle cell daughters migrate from one side of the germarium to the other and others migrate between cysts and contribute to the forming cellular monolayer. In region 3, at the posterior end of the germarium, a new subgroup of follicle cells, the pre-stalk cells, forms in response to signaling and helps

separate the new follicle from the germarium by budding, leaving only a small interconnecting stalk. We examined whether each of these characteristic movements takes place in cultured germaria.

To determine whether cyst movement takes place normally in vitro we followed the position of individual germ cells over the course of development (Fig. 4A). Cysts moved in vitro much like they have been deduced to behave based on observations of fixed material. For example, the most posterior 2a cyst (Fig. 4A, blue), which was not initially surrounded by follicle cells, gradually moved into region 2b and took on the characteristic lens shape during the 12 hours recorded in the movie. Despite the complexity of these events, they took place in an apparently normal manner and required ~10-12 hours, which is close to the estimated time of 12 hours in vivo (King, 1970). Similar normal behavior was observed for germ cell cysts throughout the germarium. Based on analyses of 30 germline cysts in our movies, we determined that they move 6.3±3.3 µm on average towards the posterior during the 14 hours of each experiment (Fig. 4B).

Follicle cells also underwent regular movement during culture that corresponded closely with known movements in vivo (Fig. 4B,C), including migration both across the germaria (Fig. 4C, green) and towards the posterior (Fig. 4C, blue and magenta). The average posterior component of such movement was 10.9±10.2 µm (Fig. 4B). Follicle cells between regions 2b and 3 form an organized structure that gradually constricts the width of the germarium at this point, an early step in follicle budding. The movement of follicle cells in this region took place normally in vitro during the first 9 hours of incubation (Fig. 4D). However, after 9 hours, the budding process arrested and the follicle eventually ruptured (Fig. 4D, asterisk). We believe that these problems are caused by the cessation of follicle cell proliferation that is observed after 9 hours.

Escort cells do not accompany germline cysts during development in vitro

When we carried out cell tracking experiments on individual escort cells the results were surprising. Although individual escort cell nuclei could move relatively large distances in both the anterior and posterior directions, they underwent very little net movement:
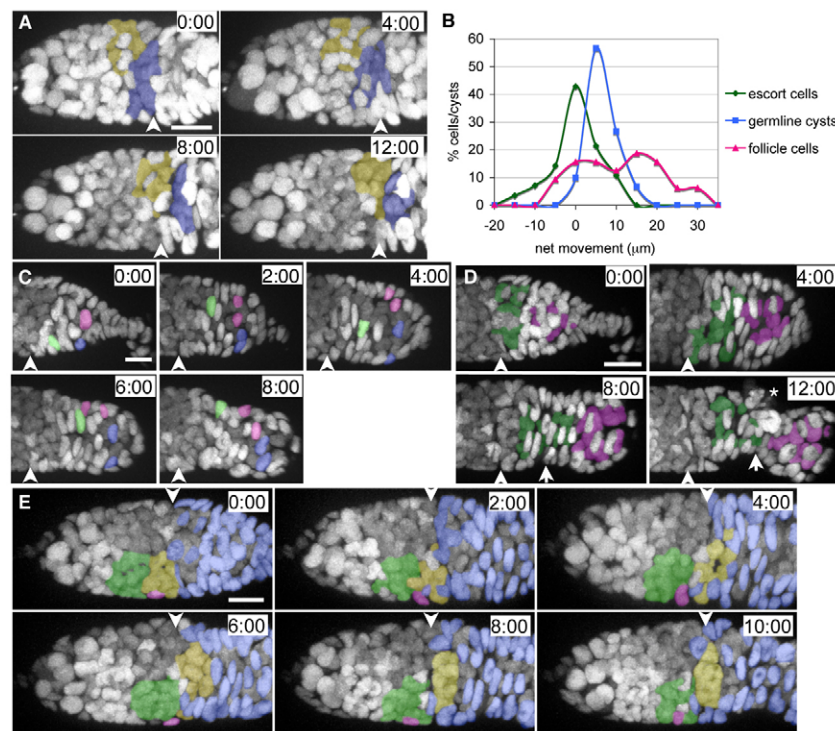
Fig. 4. Germline cysts migrate normally during culture but escort cells do not accompany them. (**A**) Germline cysts migrate through the 2a/b border in vitro. The last 2a cyst (blue) at time 0 migrates to region 2b during the course of imaging, while the penultimate 2a cyst (yellow) moves to the posterior 2a cyst position. Arrowhead indicates 2a/2b border. (**B**) Summary distribution showing that germline cysts migrate on average 6.3±3.3 μm (*n*=30) during 12 hours in culture and follicle cells 10.9±10.2 μm (*n*=32), whereas escort cells migrate only 0.2±5.1 μm (*n*=28). The anteroposterior position of cell nuclei was calculated relative to the position of the cap cells at the anterior of the germarium. (**C**) The position of three follicle cells (green, magenta, blue) surrounding the budding stage 1 follicle over 8 hours of in vitro culture. Two of the cells (magenta, blue) undergo division during this time and one (green) migrates across the germarium. Arrowhead indicates 2a/2b border. (**D**) Stage 3 follicles bud off germaria after stalk formation. Two stage 3 cysts are pseudocolored in magenta and green. After stalk formation, follicle cells delaminate from the follicle cell layer (asterisk). The arrow indicates the position of the forming stalk. Arrowhead indicates 2a/2b border. (**E**) At the start of the imaging period, a region 2b germline cyst (yellow) is associated with an escort cell (magenta). During 10 hours in vitro, the cyst migrates into region 2b, acquires a characteristic lens shape and becomes surrounded by follicle cells (blue). The escort cell now associates with a younger, adjacent germline cyst (green). Arrowhead indicates 2a/2b border. Times are shown in hours:minutes. Scale bars: 10 μm.

0.2±5.1 μm (Fig. 4B). For example, the escort cell in Fig. 4E (magenta) is initially associated with the last region 2a cyst (Fig. 4E, yellow) that will soon leave region 2a and acquire a follicle cell layer (Fig. 4E, blue). However, rather than undergoing apoptosis, as predicted by the escort stem cell model, this cell remains in the same location within the germarium after the cyst moves away and becomes associated with the next-most posterior cyst (Fig. 4E, green). The lack of escort cell movement, the low level of detectable escort cell apoptosis and the absence of escort cell division all indicate that, during development in vitro, escort cells are stationary. Despite this, cysts moved at a normal rate towards the posterior by associating with multiple escort cells in succession. This raised the question of whether this was the normal mechanism of cyst movement in vivo.

Escort cells do not normally divide or accompany germline cysts in vivo

In light of our movies in which no escort cell mitoses were evident we re-investigated the behavior of escort cells in vivo. First, we simply counted the number of escort cells per germarium as a function of age in the strain of flies used for these experiments (Fig. 5A). Escort cell number declined only slightly over the course of 30 days, but this corresponded to a slight reduction in the total number of GSCs plus cysts, such that the ratio of escort cells to GSCs plus cysts remained constant (Fig. 5B). To determine whether additional escort cell divisions were taking place in vivo that were balanced by apoptosis, we isolated germaria from flies at 4-30 days of age and stained their germaria for mitotic escort cells. As a positive control, we carried out similar staining on germaria from flies bearing the 11A12 GAL4 line and UAS-*upd*, which drives expression of the Jak-Stat ligand *unpaired* (*upd*) in escort, cap and terminal filament cells, causing germ cell and escort cell proliferation (Decotto and Spradling, 2005). Additionally, we carried out similar staining on testis, the cyst progenitor cells (CPCs) of which morphologically resemble escort cells and undergo constitutive division to produce cyst cells. We observed no mitotic escort cells in more than 1000 normal germaria examined, whereas mitotic escort cells were easily detected in germaria expressing *upd* (Fig. 5C,D; see Table S2 in the supplementary material) and in testis CPCs (Fig. 5C; see Table S2 in the supplementary material). Attempts to detect escort cell cycling by EdU incorporation identified only a very low level (0.5%; Fig. 5E, arrow; see Table S2 in the supplementary material) of escort cells in S phase. We concluded that escort cells are capable of division, but are normally quiescent unless the ratio of escort cells to germ cells/cysts increases.
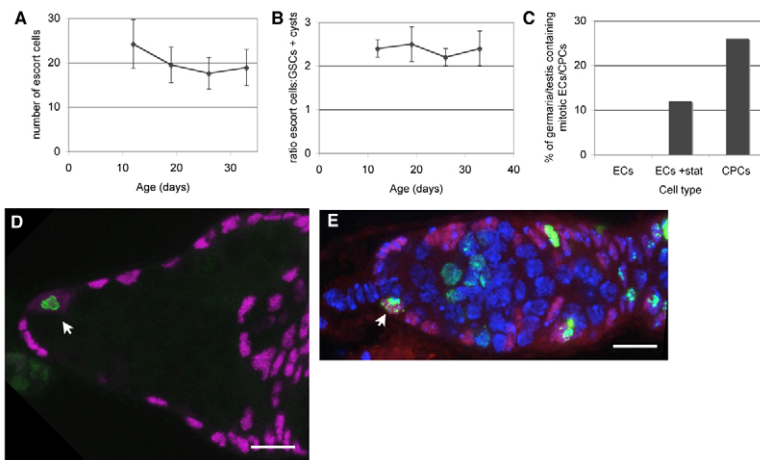
Fig. 5. Unlike their testis counterparts, escort cells are normally quiescent in vivo but do divide when germ cell number increases. (**A**) The average number of escort cells per germarium declines slowly with age (*n*=10). (**B**) The ratio of escort cells to GSCs plus germline cysts remains constant as flies age (*n*=10). (**C,D**) Although mitotic (phosphohistone H3 staining) cyst progenitor cells (CPCs) of the *Drosophila* testis are readily detected (9/35 testis), no mitotic escort cells (ECs) were identified (*n*≥1000 germaria). However, increasing the number of germ cells in the germaria by overexpression of the Jak-Stat ligand *upd* in terminal filament, cap and escort cells, caused escort cells to re-enter the cell cycle and 16/116 germaria contained mitotic escort cells (D, arrow). Magenta, somatic cells (anti-Tj); green, anti-phosphohistone H3. (**E**) Although mitotic escort cells were not found in wild-type flies, escort cells in S phase of the cell cycle can be detected in vivo at a very low level (2/400 germaria) by EdU incorporation (arrow). Magenta, anti-Tj; green, EdU; blue, DAPI. Error bars indicate s.d. Scale bars: 10 µm.

Escort cells transfer germline cysts using dynamic cytoplasmic processes

Escort cells extend thin cytoplasmic processes that entirely surround GSCs and cysts, precluding any direct germ cell-germ cell contact except between sister cyst cells (King, 1970). We carried out live imaging of the microtubules in these cell processes to gain insight into how germline cysts are passed from one escort cell to another. Escort cells contain elaborate microtubule networks that were seen to be highly dynamic (Fig. 6A). To build a clearer picture of how the shape of each escort cell changes over time, Tubulin-GFP was expressed in a subset of cells within the germarium (Fig. 6B,B′). As predicted, escort cell shape changed continuously, including the production of long, thin membrane protrusions (Fig. 6B,B′, yellow cell and blue cell). However, there was no net migration of the microtubule bundles (0.2±6.7 µm net posterior migration after 7 hours; *n*=18 processes from six germaria). The presence of these dynamic bundles suggests that they play a role in maintaining the squamous covering of the germline cysts.

DISCUSSION

Drosophila germaria support a remarkably complex and diverse array of multicellular processes within a confined space: stem cells divide asymmetrically, daughters migrate and differentiate, cysts form, enter meiosis, choose an oocyte and form new follicles. Our results show that even these complex, interdependent, hormonally regulated events can proceed with remarkable fidelity during in vitro culture and in the presence of regular illumination. The ability to image cells under these conditions has revised and clarified several important aspects of our understanding of stem cell and escort cell behavior.

Live imaging has provided, for the first time, the ability to visualize active pairs of GSCs within their niche. Our system allows the direction of division to be unambiguously determined based on the relative location of the daughter nuclei immediately after nuclear membranes reform. The results confirm that divisions are oriented with respect to the a/p axis. We typically observed GSC divisions at ~35° off axis, which is very similar to those in published images of GSC mitosis (Deng and Lin, 1997; Lin and Spradling, 1995), although we did observe a higher level of variation in spindle direction (±24°) than previously reported (Deng and Lin, 1997). Given that no differences in the shape of the GSC

niche region or in any other aspect of GSC division were observed in vitro, it is unlikely that GSC divisions in vitro differ in orientation from those in vivo.

Drosophila female GSCs reside in a well-characterized niche, the operational mechanisms of which allow stem cell activity to respond to levels of environmental resources (Kirilly and Xie, 2007). Our results indicate that at least one important aspect of environmental regulation, namely insulin-mediated control of GSC division, takes place in cultured germaria. Previous studies have demonstrated that the movement of GSCs through the G2 phase of the cell cycle is controlled by both insulin (Hsu et al., 2008) and the TOR pathway (LaFever et al., 2010). In vivo, under normal conditions, each GSC divides approximately once per 24 hours. Without added insulin we observed a rate of GSC division in vitro that was consistent with this expectation. However, in the presence of supplemental insulin, nearly every GSC underwent division within 12-14 hours, confirming that exogenous insulin removes constraints that normally limit G2 progression. In line with this observation, we observed a substantial increase in the rate of GSC division, which peaked just 2-4 hours after the onset of incubation, indicating that many GSCs were arrested within 2-4 hours of M phase at the onset of the experiment. Additionally, after incubation in culture for 2 hours the proportion of cells in G2 decreased, as would be expected if G2-arrested cells were activated by insulin treatment. Consequently, GSCs respond to exogenous insulin during in vitro culture in the same manner as in living animals. The ability to study the response of stem cells to insulin (and very likely to other serum factors) within germaria developing in vitro provides many experimental advantages that can be exploited toward a better understanding of this crucial and widespread aspect of stem cell control.

Previously, it was proposed that escort cells are maintained by six to eight anterior escort stem cells that divide to provide an average of two new escort cells for each new cystoblast (Decotto and Spradling, 2005). Similar stem cells, the CPCs, do exist in close association with the germline stem cells of the *Drosophila* testis and their daughters accompany male gametes throughout their subsequent development (Fuller, 1993). However, the experiments reported here rule out this model in the case of the ovariole. Since escort cells do not move along with cysts or turn over regularly at the 2a/2b junction, the need for new escort cells is much lower than the need for new germ and follicle cells. New
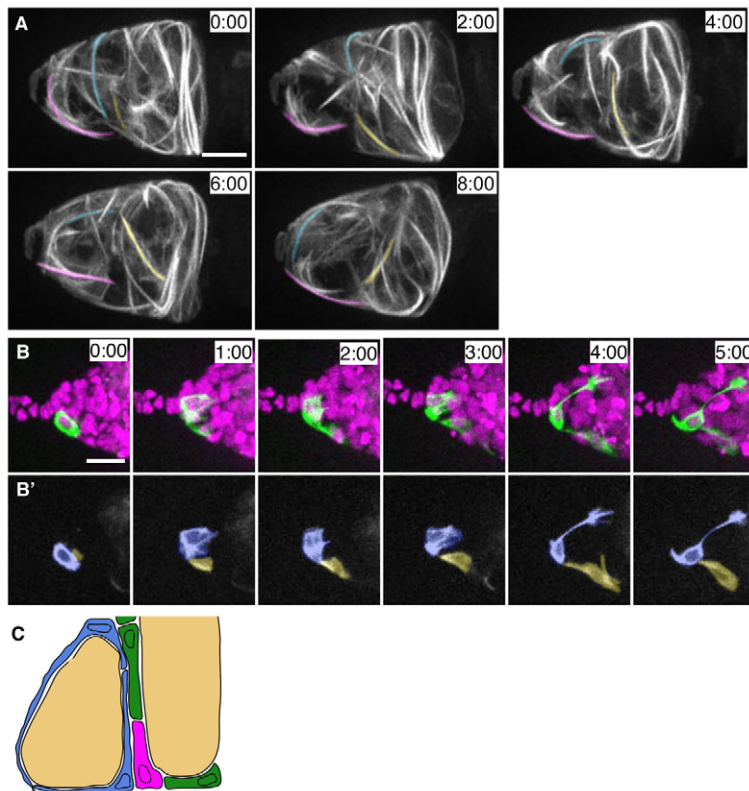
Fig. 6. Escort cells undergo dramatic shape changes to allow germline cysts to be passed from one escort cell to another. (**A-B′**) Tubulin-GFP expressed in escort cells illustrates their cell and kinetic behavior. (**A**) Expression of Tubulin-GFP in the majority of escort cells by the FLP-out system demonstrates that these cells contain dynamic microtubule processes. Three processes within different escort cells are pseudocolored (magenta, yellow, turquoise), highlighting the movements that these processes undergo. (**B,B′**) FLP-out-driven expression of Tubulin-GFP (B′, green in B) in a small number of cells allows the shape of individual escort cells to be analyzed. His2Av-mRFP (B, magenta) is expressed in all cells. (**B′**) Two labeled escort cells are pseudocolored blue or yellow. (**C**) The cellular environment surrounding the FSC (magenta). An escort cell (blue) contacts the FSC, suggesting that a stable escort cell(s) is an essential component of the FSC niche. Beige, germline cysts; green, downstream follicle cells. Scale bars: 10 μm.

escort cells are sometimes required, as we confirmed that a low level of escort cell apoptosis takes place even under optimal nutritional conditions (Drummond-Barbosa and Spradling, 2001). Furthermore, as reported previously (Xie and Spradling, 2000), escort cell numbers maintain a fixed relationship to the number of stem cells, cystoblasts and cysts. However, parity is not maintained by upregulating anterior escort stem cells but rather by inducing differentiated escort cells at multiple positions within the germarium to divide. In this regard, escort cells resemble the differentiated cells of many mammalian tissues, such as liver and pancreas, which retain the ability to divide following injury (Fausto and Campbell, 2003; Xu et al., 2008).

Our experiments provide new insight into the longstanding issue of what mechanism moves cysts towards the posterior of the germarium. The division of stem cells and cysts at the anterior is not required for movement, as all the existing cysts continue to mature and exit the germarium over several days following the forced differentiation of stem cells by Bam expression (Ohlstein and McKearin, 1997). Each ovariole is surrounded by a muscular epithelial sheath and this tissue might play a role in posterior germline cyst movement; indeed, waves of contraction are observed in sheath-covered ovarioles cultured in vitro (Middleton et al., 2006). However, germaria appeared to be fully functional when transplanted into hosts in the absence of the muscle sheath (Lin and Spradling, 1993) and we observed germline cyst migration in vitro after the muscle sheath had been removed. Consequently, we propose that the dynamic activity of the escort cells provides the force that moves cysts through the germarium.

Finally, our results also have important implications for the nature of the FSC niche. Each germarium contains two FSCs that occupy separate niches on opposite lateral walls near the region 2a/2b border (Nystul and Spradling, 2007). Although much has been learned about these cells, like most adult mammalian stem cells they have remained difficult to study at the single-cell level (Nystul and Spradling, 2007; Nystul and Spradling, 2010). Our finding that escort cells function in a fixed location suggests that one or more specific escort cells at the 2a/2b junction act to define and maintain a specialized microenvironment at the niche sites (Fig. 6C). We followed such a candidate cell (Fig. 4E) and showed that it remains at the 2a/2b junction despite cyst passage. The ability to study follicle cell production in living germaria cultured in vitro should make it possible to gain a more detailed understanding of the role of specific escort cells in this model epithelial stem cell niche.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.065508/-/DC1

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