Self-maintained escort cells form a germline stem cell differentiation niche  
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SUMMARY  
Stem cell self-renewal is controlled by concerted actions of niche signals and intrinsic factors in a variety of systems. In the Drosophila ovary, germline stem cells (GSCs) in the niche continuously self-renew and generate differentiated germ cells that interact physically with escort cells (ECs). It has been proposed that escort stem cells (ESCs), which directly contact GSCs, generate differentiated ECs to maintain the EC population. However, it remains unclear whether the differentiation status of germ cells affects EC behavior and how the interaction between ECs and germ cells is regulated. In this study, we have found that ECs can undergo slow cell turnover regardless of their positions, and the lost cells are replenished by their neighboring ECs via self-duplication rather than via stem cells. ECs extend elaborate cellular processes that exhibit extensive interactions with differentiated germ cells. Interestingly, long cellular processes of ECs are absent when GSC progeny fail to differentiate, suggesting that differentiated germ cells are required for the formation or maintenance of EC cellular processes. Disruption of Rho functions leads to the disruption of long EC cellular processes and the accumulation of ill-differentiated single germ cells by increasing BMP signaling activity outside the GSC niche, and also causes gradual EC loss. Therefore, our findings indicate that ECs interact extensively with differentiated germ cells through their elaborate cellular processes and control proper germ cell differentiation. Here, we propose that ECs form a niche that controls GSC lineage differentiation and is maintained by a non-stem cell mechanism.

KEY WORDS: Drosophila, Germline stem cell, Niche, Differentiation, Escort cell

INTRODUCTION  
Adult tissues are often maintained by a population of tissue-specific stem cells, which have the capacity to self-renew and generate differentiated cells throughout the organism’s lifetime. Many fast turnover tissues, such as blood, skin, intestine and testis, are known to be maintained by stem cells (Li and Xie, 2005; Morrison and Spradling, 2008). Some slow turnover tissues are either maintained by stem cells (Li and Xie, 2005; Morrison and Spradling, 2008). Adult tissues are often maintained by a population of tissue-specific stem cells, which have the capacity to self-renew and generate differentiated cells throughout the organism’s lifetime. Many fast turnover tissues, such as blood, skin, intestine and testis, are known to be maintained by stem cells (Li and Xie, 2005; Morrison and Spradling, 2008). Some slow turnover tissues are either maintained by stem cells (Li and Xie, 2005; Morrison and Spradling, 2008). These adult stem cells are maintained in the niche for long-term self-renewal (Li and Xie, 2005; Morrison and Spradling, 2008). However, it remains unclear whether stem cell lineage differentiation is also controlled by a niche mechanism.

In the Drosophila ovary, two or three germline stem cells (GSCs) are anchored physically to their niche, which is composed of five to seven cap cells, through E-cadherin-mediated cell adhesion at the tip of the germarium (Song et al., 2002). Recently, a population of escort stem cells (ESCs), which directly contacts GSCs and cap cells, has been proposed to generate the differentiated escort cells (ECs) that accompany differentiated germ cells to the middle region of the germarium where ECs undergo apoptosis (Decotto and Spradling, 2005). The germ cells released from ECs are subsequently surrounded by follicle cells, which are produced by two follicular stem cells (FSCs), to form individual egg chambers (Margolis and Spradling, 1995; Song and Xie, 2002). Therefore, ESCs have been proposed to behave similarly to cyst progenitor cells in the Drosophila testis, which produce differentiated somatic cells that wrap around differentiated germ cells to support their differentiation (Gonczy et al., 1992; Kiger et al., 2000; Tran et al., 2000; Schultz et al., 2002; Decotto and Spradling, 2005). A previous study has shown that stem cell tumor (ster) encodes a germ cell-specific protein related to Rhomboid, which is required for the formation or maintenance of the long EC cellular processes and germ cell differentiation (Schultz et al., 2002). The Rhomboid family of transmembrane proteases is involved in processing EGF (epidermal growth factor) ligands (Urban et al., 2001). Indeed, another recent study showed that three EGF ligands function in germ cells to activate EGF receptor (EGFR) signaling in ECs, which in turn represses Dally expression (Liu et al., 2010). Dally is a type I transmembrane glycoprotein that binds bone morphogenetic protein (BMP) and facilitates its signaling (Jackson et al., 1997; Belenkaya et al., 2004; Akiyama et al., 2008). In addition, Dally is also important for restricting BMPs inside the GSC niche in the Drosophila ovary (Guo and Wang, 2009; Hayashi et al., 2009). These findings suggest a model in which differentiated germ cells activate EGFR signaling in ECs, which prevents BMP diffusion from the GSC niche to ECs and thus promotes germ cell differentiation. However, it remains unclear how EC cellular process-mediated interactions between ECs and germ cells are regulated. In this study, we show that ECs are maintained by self-duplication instead of by ESCs. Furthermore, EC cellular processes are dependent on differentiated germ cells, and the physical interactions between ECs and germ cells are essential for germ cell differentiation. Therefore, we propose that self-maintained ECs form a niche that controls germ cell differentiation.
MATERIALS AND METHODS

Drosophila strains and culture

Information about the Drosophila stocks used in this study is either available from Flybase (http://www.flybase.org) or specified here: X-15-29 (Harrison and Perrimon, 1993), X-15-33 (Harrison and Perrimon, 1993), UAS-mEGFP (a membrane-tethered GFP generated by a fusion of the Src membrane signal peptide with GFP) (Kirilly et al., 2005), hsFLP, UAS-RhoDN (a dominant-negative Rho) (Strutt et al., 1997), P(TEP1)GAL4 (Morgulis and Spradling, 1995), UAS-dsRed, UAS-dcr2, UAS-dallyRNAi (on chromosome 2 and 3; kindly provided by Dr Xinhua Lin, Cincinnati Children’s Hospital Medical Center, Cincinnati, USA), UAS-dppRNAi (TR00047A: 47A; TR00047R: 47R; kindly provided by Dr Norbert Perrimon, Harvard Medical School, Boston, USA), UAS-capnRNAi (VDC#110404), dppRNAi and dppRNAi56. Flies were maintained and crossed at room temperature on standard commen/molasses/agar media unless specified. For maximizing the effect of RNAi-mediated knockdown or gene overexpression, newly eclosed flies were shifted to 29°C for a week before the analysis of ovarian phenotypes.

Generating positively labeled EC clones

Three different positive marking systems were used for labeling ECs in the Drosophila ovary: the lacZ-based positive labeling system (Harrison and Perrimon, 1993), the UAS-GAL4-based positively marked mosaic lineage (PMML) system (Kirilly et al., 2005) and the GAL80-based mosaic analysis with a repressible cell marker (MARCM) system (Lee et al., 2000). We generated mitotic clones according to our previously published procedures for labeling the ESC lineage (Kirilly et al., 2005). One- to three-day-old females with genotypes hs-FLP: X-15-13/X-15-29 and hs-FLP UAS-mEGFP; FRT52B/FRT52B UAS-EGFP and hs-FLP FRT194 gaI80/ FRT194; tub-gal4 UAS-GFP were subjected to incubation in a water bath at 37°C for 30 or 60 minutes to induce FLFP expression and FRT-mediated mitotic recombination. Flies were transferred daily to the fresh yeast-containing food, and the marked EC clones were detected one week, two weeks and three weeks after the heatshock treatment.

BrdU labeling and retention assays

Two different types of BrdU incorporation assays were utilized to investigate the proliferation patterns of ECs: two-hour BrdU labeling and BrdU retention. For the two-hour BrdU labeling, the ovaries were incubated in Grace’s medium containing 75 μM BrdU for 2 hours at 25°C. For the BrdU retention assay, female flies were fed on food with yeast paste containing BrdU (10 mg/ml) for three consecutive days, and then on food with BrdU-free yeast paste for 14 days. The ovaries from these two types of assays were then fixed and processed for BrdU label detection along with other protein markers according to our previously published procedures (Xie and Spradling, 1998).

Immunohistochemistry

Antibody staining was performed according to our previously published procedures (Xie and Spradling, 1998; Song and Xie, 2002). The following antibodies were used in this study: rabbit polyclonal anti-β−galactosidase antibody (1:100, Cappel), rabbit polyclonal anti-GFP antibody (1:200, Molecular Probes), mouse monoclonal anti-FasciclinIII antibody (1:3, Developmental Studies Hybridoma Bank (DSHB)), mouse monoclonal anti-Hts antibody (1:3, DSHB), rabbit monoclonal anti-Vasa antibody (1:3, DSHB) and monoclonal anti-BrdU antibody (1:20, Oncogene). All images were taken with either a Leica TCS SP2 or a Leica TCS SP5 confocal microscope.

RESULTS

ECs in different regions of the germarium undergo slow cell turnover and proliferation

The ESC model predicts that only ESCs at the tip of the germarium are mitotically active and that ESCs undergo apoptosis at the 2a/2b junction (Decotto and Spradling, 2005). To investigate whether ECs die only at the 2a/2b junction, we used Apoptag labeling to detect apoptotic ECs. c587 is a GAL4 line that drives UAS-GFP expression in ESCs, ECs and early follicle cell progenitors (Song et al., 2004) (Fig. 1A). PZ1444 is a lacZ enhancer trap line that is expressed in cap cells and ECs (Margolis and Spradling, 1995; Xie and Spradling, 2000) (Fig. 1B). lacZ-positive ECs can be easily identified in the germarium in which germ cells are labeled with vasa-GFP (Sano et al., 2002) (Fig. 1B). Here, we used PZ1444, c587-driven UAS-dsRed and vasa-GFP to assist in the identification of ECs. One or more dying EC is present in the 2a/2b junction area of 14.7% of the 8-day-old germaria (n=116), whereas only 4.3% of those harbor one or more dying ECs in region 1 or 2a (Fig. 1C-F). These results indicate that ECs at different positions can undergo slow turnover but with the highest turnover rate for the most posterior ECs.

To investigate whether those lost ECs in regions 1 and 2a are repopulated by their neighboring ECs, we performed two-hour BrdU labeling of PZ1444 ovaries in vitro to detect proliferative ECs. Following two hours of BrdU incorporation, 9.6% of the germaria contain at least one BrdU-positive EC at the 2a/2b boundary, and only 0.8% of them contain a BrdU-positive EC in the region 1 or 2a (n=125) (Fig. 1G). This result indicates that ECs are slow cycling cells and that the ones at the 2a/2b boundary proliferate faster than those in more anterior regions. To investigate further the slow cycling property, we also performed the BrdU retention assay by feeding PZ1444 or vasa-GFP flies for 3 days with BrdU-containing food and then 15 days with BrdU-free food. In the vasa-GFP germarium, ECs are reliably identified in the periphery of the anterior half of the germarium by absence of GFP expression, whereas ECs in the PZ1444 germarium are readily identified by lacZ-expression (Fig. 1B). After 3 days of BrdU feeding, 40% of the GSCs in the PZ1444 germaria (n=155 germaria) and 48.9% of the GSCs in the vasa-GFP germaria become BrdU-positive because of their fast proliferative nature (Fig. 1H-I). For ECs, only a small number of them in either a PZ1444 germarium or a vasa-GFP germarium are BrdU-positive, and most of those BrdU-positive ECs are localized in the 2a/2b junction (Fig. 1H-I). After 15 days of chase, no GSCs in either PZ1444 (n=212 germaria) or vasa-GFP (n=154 germaria) germaria retain the BrdU label, indicating that fast cycling GSCs and FSCs do not retain BrdU label (Fig. 1J-K). This result is consistent with the notion that not all adult stem cells retain BrdU label as is proposed for some adult stem cell types in mammalian systems (Morrison and Spradling, 2008). For the PZ1444 germarium, 34.3% of them harbor one or more BrdU-labeled ECs in the 2a/2b junction area, and only 13.8% of them have one or more BrdU-positive ECs in region 1 or 2a (n=137; Fig. 1J). The vasa-GFP germaria exhibit a similar BrdU labeling pattern among the ECs (Fig. 1K-L). The previously defined ESCs are rarely BrdU-positive (Fig. 1L), suggesting that ESCs are unlikely to be responsible for generating ECs. These results show that ECs proliferate infrequently owing to slow cell turnover and most of the proliferative cells are restricted to the 2a/2b junction area, which is consistent with the EC turnover pattern.

ECs are not maintained by ESCs

In the previous study, the lacZ-based positive labeling system was used to show that the most anterior ECs, which directly contact cap cells and GSCs, were proposed to function as ESCs to generate slow cycling ECs. Following two hours of BrdU incorporation, 9.6% of the germaria contain at least one BrdU-positive EC at the 2a/2b boundary, and only 0.8% of them contain a BrdU-positive EC in the region 1 or 2a (n=125) (Fig. 1G). This result indicates that ECs are slow cycling cells and that the ones at the 2a/2b boundary proliferate faster than those in more anterior regions. To investigate further the slow cycling property, we also performed the BrdU retention assay by feeding PZ1444 or vasa-GFP flies for 3 days with BrdU-containing food and then 15 days with BrdU-free food. In the vasa-GFP germarium, ECs are reliably identified in the periphery of the anterior half of the germarium by absence of GFP expression, whereas ECs in the PZ1444 germarium are readily identified by lacZ-expression (Fig. 1B). After 3 days of BrdU feeding, 40% of the GSCs in the PZ1444 germaria (n=155 germaria) and 48.9% of the GSCs in the vasa-GFP germaria become BrdU-positive because of their fast proliferative nature (Fig. 1H-I). For ECs, only a small number of them in either a PZ1444 germarium or a vasa-GFP germarium are BrdU-positive, and most of those BrdU-positive ECs are localized in the 2a/2b junction (Fig. 1H-I). After 15 days of chase, no GSCs in either PZ1444 (n=212 germaria) or vasa-GFP (n=154 germaria) germaria retain the BrdU label, indicating that fast cycling GSCs and FSCs do not retain BrdU label (Fig. 1J-K). This result is consistent with the notion that not all adult stem cells retain BrdU label as is proposed for some adult stem cell types in mammalian systems (Morrison and Spradling, 2008). For the PZ1444 germarium, 34.3% of them harbor one or more BrdU-labeled ECs in the 2a/2b junction area, and only 13.8% of them have one or more BrdU-positive ECs in region 1 or 2a (n=137; Fig. 1J). The vasa-GFP germaria exhibit a similar BrdU labeling pattern among the ECs (Fig. 1K-L). The previously defined ESCs are rarely BrdU-positive (Fig. 1L), suggesting that ESCs are unlikely to be responsible for generating ECs. These results show that ECs proliferate infrequently owing to slow cell turnover and most of the proliferative cells are restricted to the 2a/2b junction area, which is consistent with the EC turnover pattern.
marks mitotic cells and their progeny (Harrison and Perrimon, 1993). This system has been used to label GSCs, FSCs, ESCs and their progeny (Margolis and Spradling, 1995; Decotto and Spradling, 2005). In the current study, to ensure that *lacZ*-labeled somatic cells that surround differentiated germ cells are indeed ECs, we focused on the germaria only carrying *lacZ*-labeled ESCs or ECs and disregarded other germaria carrying *lacZ*-positive FSCs or GSC clones. To verify whether ESCs are indeed responsible for producing ECs, we used the same system to repeat the *lacZ*-based EC lineage tracing experiments using half-an-hour and one-hour heatshock treatments. These germaria are also labeled for Fasciclin 3 (Fas3), which is expressed in follicle cell progenitors but not in ECs (Zhang and Kalderon, 2001). As the previous study reported (Decotto and Spradling, 2005), both heatshock regimes can efficiently induce *lacZ*-labeled ESCs and ECs although the one-hour treatment generates higher *lacZ*-labeling efficiencies than the half-an-hour treatment (Fig. 2A, A'). For example, one week after clone induction (ACI), 34.5% and 56.2% of the germaria carry at least one *lacZ*-marked ESC after the half-an-hour and one-hour heatshock treatments, respectively (Fig. 2A). These *lacZ*-labeled ESCs are relatively stable three weeks ACI, which is consistent with the prediction of the ESC model. In addition, 82.4% of the germaria carrying one or more *lacZ*-marked ESCs also have one or more *lacZ*-positive ECs for the half-an-hour heatshock treatment, and 99.1% of the germaria carrying one or more *lacZ*-marked ESCs have one or more *lacZ*-positive ECs for the one-hour heatshock treatment (Fig. 2B). These observations could explain why the previous study concludes that ECs are produced by ESCs (Decotto and Spradling, 2005) (Fig. 2A, B). However, careful analyses of the *lacZ*-marked ECs have revealed the serious inherent problem of the *lacZ* labeling system. First, the percentages of germaria carrying only marked ECs but not ESCs, which are induced by either of the heatshock treatments, remain constant one week, two weeks and three weeks ACI (Fig. 2A', C). This result indicates that *lacZ*-labeled ECs can be stably maintained in the absence of *lacZ*-labeled ESCs. Second, some germaria containing one *lacZ*-labeled ESC do not contain any *lacZ*-positive ECs (supplementary material Fig. S1A), suggesting that they might not be responsible for EC production. Third, high *lacZ* labeling rates for ESCs and ECs are not supported by our BrdU labeling results. This system has recently been shown to generate *lacZ*-positive cells even in mitotically inactive *Drosophila* polyplloid intestinal cells (Fox and Spradling, 2009). Our results also suggest that this system can reconstitute the *tub-lacZ* gene in an FLP-dependent but division-independent manner in ECs, and, therefore, is not a suitable system for determining the presence of ESCs.

To investigate further whether ESCs are responsible for producing ECs, we used two other FLP-FRT-mediated positive lineage labeling systems, positively marked mosaic lineage (PMML) and mosaic analysis with a repressible cell marker (MARCM), to generate positive GFP-marked EC clones and study their behavior. In the PMML system, FLP-induced FRT recombination can randomly mark mitotic cells and their progeny (Harrison and Perrimon, 1993). This system has been used to label GSCs, FSCs, ESCs and their progeny (Margolis and Spradling, 1995; Decotto and Spradling, 2005). In the current study, to ensure that *lacZ*-labeled somatic cells that surround differentiated germ cells are indeed ECs, we focused on the germaria only carrying *lacZ*-labeled ESCs or ECs and disregarded other germaria carrying *lacZ*-positive FSCs or GSC clones. To verify whether ESCs are indeed responsible for producing ECs, we used the same system to repeat the *lacZ*-based EC lineage tracing experiments using half-an-hour and one-hour heatshock treatments. These germaria are also labeled for Fasciclin 3 (Fas3), which is expressed in follicle cell progenitors but not in ECs (Zhang and Kalderon, 2001). As the previous study reported (Decotto and Spradling, 2005), both heatshock regimes can efficiently induce *lacZ*-labeled ESCs and ECs although the one-hour treatment generates higher *lacZ*-labeling efficiencies than the half-an-hour treatment (Fig. 2A, A'). For example, one week after clone induction (ACI), 34.5% and 56.2% of the germaria carry at least one *lacZ*-marked ESC after the half-an-hour and one-hour heatshock treatments, respectively (Fig. 2A). These *lacZ*-labeled ESCs are relatively stable three weeks ACI, which is consistent with the prediction of the ESC model. In addition, 82.4% of the germaria carrying one or more *lacZ*-marked ESCs also have one or more *lacZ*-positive ECs for the half-an-hour heatshock treatment, and 99.1% of the germaria carrying one or more *lacZ*-marked ESCs have one or more *lacZ*-positive ECs for the one-hour heatshock treatment (Fig. 2B). These observations could explain why the previous study concludes that ECs are produced by ESCs (Decotto and Spradling, 2005) (Fig. 2A, B). However, careful analyses of the *lacZ*-marked ECs have revealed the serious inherent problem of the *lacZ* labeling system. First, the percentages of germaria carrying only marked ECs but not ESCs, which are induced by either of the heatshock treatments, remain constant one week, two weeks and three weeks ACI (Fig. 2A', C). This result indicates that *lacZ*-labeled ECs can be stably maintained in the absence of *lacZ*-labeled ESCs. Second, some germaria containing one *lacZ*-labeled ESC do not contain any *lacZ*-positive ECs (supplementary material Fig. S1A), suggesting that they might not be responsible for EC production. Third, high *lacZ* labeling rates for ESCs and ECs are not supported by our BrdU labeling results. This system has recently been shown to generate *lacZ*-positive cells even in mitotically inactive *Drosophila* polyplloid intestinal cells (Fox and Spradling, 2009). Our results also suggest that this system can reconstitute the *tub-lacZ* gene in an FLP-dependent but division-independent manner in ECs, and, therefore, is not a suitable system for determining the presence of ESCs.

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In the MARCM system, the FLP-induced FRT recombination can generate cells that lose the expression of tub-gal80, a transcriptional repressor for GAL4, and consequently allow actin-gal4 to drive UAS-GFP expression in mitotic cells (Lee et al., 2000). In contrast with the lacZ-based labeling system, the two GFP-based labeling systems do not produce GFP-labeled GSCs and mitotic germ cell cysts owing to the inability of the GAL4-UAS system to be expressed in GSCs and early mitotic cysts. To ensure that GFP-labeled somatic cells that surround differentiated germ cells are indeed ECs, we focused on the germaria carrying only ESC and ECs by ignoring the germaria carrying a FSC clone. Interestingly, the MARCM and PMML systems produce much lower percentages of GFP-positive ESCs than the lacZ-based system (Fig. 2D; supplementary material Fig. S1B). For the PMML system, the half-an-hour and one-hour heatshock treatments can label one ESC with GFP in 0.9% (n=1482) and 2.5% (n=1456) of the germaria, respectively (the data from three time points are pooled together; Fig. 2D). These GFP-labeled ESCs are stably maintained in the germaria with time. There are similar observations using the MARCM system (supplementary material Fig. S1B). These results support the findings from BrdU-labeling experiments that ESCs proliferate rarely. In addition, among the total 76 GFP-positive ESCs examined (produced by either MARCM or PMML), 66 do not have any GFP-positive ECs in the same germaria, indicating that these ESCs are not responsible for producing ECs (Fig. 2E; supplementary material Fig. S1D). All of these observations do not support the previously established ESC model (Decotto and Spradling, 2005).

The MARCM and PMML systems also produce much lower percentages of GFP-positive ECs than the lacZ-based system (Fig. 2D'; supplementary material Fig. S1C). Following the half-an-hour and one-hour heatshock treatments, the PMML system can label at least one EC with GFP in the absence of marked ESC labeling in 10.6% and 19.7% of the germaria one week ACI, respectively (Fig. 2D',F,G). Although those germaria do not have any GFP-labeled ESCs, the GFP-labeled ECs are stably maintained three weeks ACI. Similar findings have been made for the MARCM-labeled ECs (supplementary material Fig. S1C). These results indicate that GFP-labeled ECs are not produced by GFP-labeled ESCs. Along with the earlier results from the BrdU and TUNEL labeling experiments, our findings strongly argue against the idea that the previously defined ESCs are responsible for generating ECs, and instead support the new model in which ECs undergo slow turnover and lost ECs can be replenished by their proliferative neighboring ECs. Recently, a live imaging study has also shown that escort cells do not move along with differentiated cells and, thus, are not maintained by stem cells (Morris and Spradling, 2011).

**Long cellular processes of escort cells wrap up differentiated germ cells**

ECs have been shown to wrap around germ cells in the anterior half of the germarium (Schultz et al., 2002; Decotto and Spradling, 2005), and these long cellular processes appear to be involved in passing differentiated germline cysts posteriorly (Morris and...
Spradling, 2011). To investigate further to what extent germ cells interact with ECs, we used c587-driven UAS-GFP expression to label ECs with GFP, which allows the visualization of EC cellular processes. In the whole-mount germaria immunostained for GFP and Hts, the long cellular processes of ECs wrap around differentiated germ cells, which are identified by the presence of branched fusomes labeled by Hts protein (Fig. 3A). Hts protein is rich in spherical spectrosomes in GSCs and cystoblasts (CBs) and in branched fusomes in differentiated germ cells (Lin et al., 1994). In cryosections of c587-gal4/UAS-GFP germaria labeled for GFP and Vasa, EC long cellular processes are shown to separate individual germ cells from one another (Fig. 3B). Vasa is a commonly used germ cell-specific marker (Hay et al., 1988; Lasko and Ashburner, 1988). Our results further support the previous finding that individual early differentiated germline cysts interact extensively with long EC cellular processes.

Previous studies were unable to determine the morphology of individual ECs using nuclear lacZ-labeled ECs and c587-driven or patch-gal4-driven UAS-GFP expression (Margolis and Spradling, 1995; Schultz et al., 2002; Song et al., 2004; Decotto and Spradling, 2005). To investigate the EC morphology of individual ECs, we used the PMML system described above to label one or a few ECs with tub-gal4-driven expression of UAS-mGFP (a membrane-targeted GFP; the fusion between the Src membrane localization signal and GFP). In the ovariole that lacks a GFP-labeled FSC clone, the GFP-positive ECs in regions 1, 2a and 2a/2b can be readily identified (Fig. 3C-I). The most anterior GFP-labeled ECs, which correspond to the previously identified ESCs, directly contact GSCs and wrap around GSCs with their cellular processes as previously described (Decotto and Spradling, 2005) (Fig. 3C). The ECs in region 1 that interact with CBs and mitotic germ cysts have short cellular processes (Fig. 3D,E; supplementary material Movie 1). In region 2a, where 16-cell cysts occupy half the diameter of the germaria, individual ECs have slightly longer cellular processes to encase individual 16-cell cysts (Fig. 3F,G; supplementary material Movie 2). In the 2a/2b junction areas, where germline cysts start to adopt a lens shape crossing the germarium, the ECs extend their cellular processes across the whole width of the germarium to wrap around individual 16-cell cysts (Fig. 3H,I; supplementary material Movie 3). These results show that ECs at different positions adopt distinct morphologies based on the size and morphology of underlying differentiated germ cell cysts, further suggesting that EC cellular processes are likely to be regulated by the underlying differentiated germ cell cysts.

**EC cellular processes might be regulated by underlying differentiated germ cells**

Although EC long cellular processes are only associated with differentiated germ cells, it remains unclear whether differentiated germ cells are required for the formation of long cellular processes in ECs. **bag of marbles (bam)** is required for germ cell
development, and its mutant ovaries only contain undifferentiated cystoblast-like single germ cells (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995). \textit{bam}86 (a \textit{bam} deletion allele) mutant germaria are filled with undifferentiated germ cells, which are identified by spherical spectrosomes (Fig. 4A). Interestingly, in \textit{bam} mutant germaria in which ECs are labeled by \textit{c587}-driven \textit{UAS-GFP} expression, the long cellular processes of most ECs fail to form or to penetrate inside the germ cells (Fig. 4A). To rule out the possibility of poor antibody penetration into tightly packed germ cells, the \textit{c857; UAS-GFP; bam}86/\textit{bam}56 germarial cryosections are labeled for GFP and Vasa to detect EC cellular processes and germ cells, respectively. Consistently, long EC cellular processes are not observed, although some ECs have small EC protrusions (Fig. 4B, arrows). These results suggest that differentiated germ cells might be required for the formation or maintenance of long EC cellular processes.

To determine further whether the formation of long EC cellular processes is dependent on differentiated germ cells or Bam function in germ cells, we examined the formation of long EC cellular processes in germaria carrying \textit{Dpp} overexpression-induced GSC-like cells. \textit{c587}-driven \textit{UAS-dpp} results in the formation of ovaries filled with GSC-like cells (Fig. 4C), as previously reported (Song et al., 2004). In the \textit{dpp}-induced tumorous germaria, EC cellular processes, which are visualized by \textit{c587}-driven \textit{UAS-GFP} expression, fail to penetrate into individual GSC-like aggregates and to wrap around individual single germ cells (Fig. 4C). As \textit{c587}-driven \textit{dpp} overexpression cannot completely inhibit GSC differentiation owing to mosaic expression, some differentiated cysts form but some GFP-positive cellular processes can still be detected to wrap up the differentiated germ cells (Fig. 4D). Along with the \textit{bam} mutant results, these results further support the idea that differentiated germ cells might support the formation of EC cellular processes.

\textbf{Rho functions in ECs to maintain cellular processes and promote germ cell differentiation}

The small GTPase Rho has been extensively studied in different organisms for its role in the regulation of cell protrusion formation and cell-cell adhesion (Jaffe and Hall, 2005). To investigate further whether EC cellular process-mediated interactions with germ cells are important for germ cell differentiation, we overexpressed a dominant negative form of Rho (\textit{Rho}DN) in ECs using \textit{c587}-driven \textit{UAS-Rho}DN expression. \textit{UAS-Rho}DN has previously been used to disrupt specifically Rho function in different \textit{Drosophila} cell types (Strutt et al., 1997; Hacker and Perrimon, 1998; Prokopenko et al., 1999; Billuart et al., 2001; Bloor and Kiehart, 2002; Magie et al., 2002). Interestingly, in the germaria in which \textit{Rho}DN is expressed in ECs, differentiated germ cells are not wrapped up by EC cellular processes, suggesting that Rho might regulate EC cellular process formation or maintenance (Fig. 5A-C). In those germaria, spherical spectrosome-containing single germ cells often accumulate (Fig. 5A,C). In contrast with the wild-type germaria, which contain ~5.5 spectrosome-containing single germ cells including both GSCs and cystoblasts (n=91), the \textit{Rho}DN-expressing germaria contain an average of 8.9 spectrosome-containing germ cells (n=69). Also, the disruption severity of EC cellular processes is related to the increase in the number of spectrosome-containing single germ cells, suggesting that long EC cellular processes might regulate germ cell differentiation.

One of the potential mechanisms for regulation by Rho of EC cellular processes is by modulation of physical interactions between ECs and germ cells through regulating actin cytoskeleton because Rho is known to regulate actin dynamics and cell-cell interaction in different \textit{Drosophila} tissues (Hacker and Perrimon, 1998; Prokopenko et al., 1999; Bloor and Kiehart, 2002; Magie et al., 2002). To determine whether actin regulation is important for EC function and germ cell differentiation, we sought to inactivate the function of the Formin-like actin regulator \textit{capuccino} (\textit{capu}) in ECs using \textit{c587}-driven \textit{UAS-capuRNAi} expression. \textit{Capu} is known to regulate actin nucleation and the formation of actin meshwork (Dahlgaard et al., 2007; Quinlan et al., 2007). Following \textit{capu} knockdown in ECs, the ECs located in the anterior half of the germaria generally lack cellular processes, and the anterior part of the germaria accumulate more spectrosome-containing single germ cells, suggesting that actin regulation is crucial for maintaining EC cellular process and, thus, CB differentiation (supplementary material Fig. S2A,B). However, all of the germaria appear to be normal in size, indicating that disruption of actin dynamics does not affect EC survival. These results further support the idea that EC cellular processes regulated by actin dynamics are important for germ cell differentiation.

Because some germaria expressing \textit{Rho}DN in ECs are small in comparison with wild-type germaria (Fig. 5A,B), we used the \textit{PZ1444} EC marker to examine whether the number of ECs following \textit{Rho}DN expression is indeed reduced. The control week-old \textit{PZ1444} germaria contain an average of 42 ECs (n=31). Only very few of the week-old \textit{Rho}DN-expressing germaria have the normal or close to normal number of ECs (Fig. 5D); most of them
have fewer ECs (Fig. 5E,F). The week-old RhoDN-expressing germaria have an average of 21 ECs (n=50), significantly fewer than those in control germaria (Fig. 5G). Those germaria with fewer ECs tend to have more spectrosome-containing single germ cells than the germaria with more ECs, suggesting that ECs are important for germ cell differentiation. The RhoDN-expressing germarium in Fig. 5D has a close to normal number of ECs, but contains excess spectrosome-containing single germ cells (arrowheads) located anteriorly to differentiated cysts (arrows). (E, F) Germaria show a moderate (E) or severe (F) reduction of ECs and excess spectrosome-containing single germ cells (arrowheads). (G) RhoDN-expressing germaria have significantly fewer ECs than do control germaria. P-value is indicated. (H) A control germarium contains no apoptotic ECs. (I) A RhoDN-expressing germarium contains an apoptotic EC (arrowhead). Scale bar: 10 μm.

Fig. 5. Defective Rho signaling in ECs disrupts EC-germ cell interactions and germ cell differentiation. The c587;UAS-RhoDN/UAS-GFP germaria (single confocal section) in A-C are labeled for GFP, Hts and DNA, whereas c587;UAS-RhoDN/PZ1444 germaria (overlaid confocal sections) in D-F and H-I are labeled for β-galactosidase (PZ1444), Hts and DNA, and ApopTag, β-galactosidase (PZ1444) and DNA, respectively. (A) The GFP-positive cellular processes of RhoDN-expressing ECs fail to wrap around a differentiated cyst (dashed lines). (B) RhoDN expression in ECs leads to a reduction of regions 1 and 2a of the germarium and a defect in wrapping a differentiated cyst (dashed lines) by EC cellular processes. (C) Spectrosome-containing single germ cells and a 16-cell cyst (dashed lines) are clustered together and are not individually wrapped by RhoDN, expressing EC cellular processes. Arrowheads indicate spectrosomes. (D) Germarium contains a normal number of ECs but excess single germ cells (arrowheads) located anteriorly to differentiated cysts (arrows). (E, F) Germaria show a moderate (E) or severe (F) reduction of ECs and excess spectrosome-containing single germ cells (arrowheads). (G) RhoDN-expressing germaria have significantly fewer ECs than do control germaria. P-value is indicated. (H) A control germarium contains no apoptotic ECs. (I) A RhoDN-expressing germarium contains an apoptotic EC (arrowhead). Scale bar: 10 μm.

Defective Rho in ECs causes the germ cell differentiation defect by upregulating BMP signaling activity
To characterize further the identity of accumulated spectrosome-containing single germ cells in the RhoDN-overexpressing germaria, we examined expression of bam-GFP and Dad-lacZ in these extra single germ cells. In the wild-type germarium, bam-GFP is a reporter construct for monitoring bam transcription and is mainly expressed in differentiated germ cells but is repressed in GSCs by BMP signaling (Chen and McKearin, 2003), whereas Dad-lacZ is a BMP signaling activity reporter and is activated in GSCs and repressed in differentiated germ cells (Kai and Spradling, 2003; Casanueva and Ferguson, 2004; Song et al., 2004). As expected, GSCs close to cap cells have low bam-GFP and high Dad-lacZ expression in control germaria (Fig. 6A,D). Most of the single
germ cells express high levels of bam-GFP and low levels Dad-lacZ, suggesting that those single germ cells resemble CBs instead of GSCs (Fig. 6B,C,E,F). However, some spectrosome-containing single germ cells that are located several cells away from cap cells express Dad-lacZ and repress bam-GFP expression (Fig. 6C,F). This observation indicates that BMP signaling activity has expanded outside the GSC niche and thereby interferes with germ cell differentiation. Therefore, these results demonstrate that Rho function is required in ECs to control CB differentiation possibly by restricting BMP signaling to the GSC niche.

Rho in ECs helps restrict BMP signaling activity to the GSC niche

Activation of EGFR signaling leads to phosphorylation of ERK (pERK) in ECs, and defective EGFR signaling leads to the absence of long EC cellular processes and defective germ cell differentiation (Schultz et al., 2002). EGFR signaling in ECs promotes germ cell differentiation by repressing the expression of daily, encoding a protein important for Dpp diffusion and function, in ECs (Liu et al., 2010). To investigate whether the disruption of Rho function affects EGFR signaling in ECs, we examined the expression of pERK in the ECs of RhoDN-expressing germaria. Our quantitative result indicates that RhoDN-expressing ECs (n=160) have significantly, though not dramatically, less pERK expression than control ECs (n=160) (Fig. 6G-I). This finding demonstrates that defective Rho function moderately decreases EGFR signaling in ECs.

To determine whether the germ cell differentiation defect in RhoDN-expressing germaria is caused by upregulation of BMP signaling outside the GSC niche, we tested whether the removal of a copy of the dpp gene could suppress the differentiation defect. In contrast with the results that 65% of the RhoDN-expressing germaria accumulate excessive spectrosome-containing single germ cells outside the niche (Fig. 7A,B), only 15% and 20% of the Rho DN-expressing dpp^{hr56} and dpp^{hr4} heterozygous germaria, respectively, have extra single germ cells (Fig. 7C,D). These results indicate that upregulation of Dpp/BMP signaling in the RhoDN-expressing germarium contributes to the germ cell differentiation defect. To knock down specifically daily function in ECs, two independent daily RNAi lines were used to be expressed in ECs using c587. Interestingly, EC-specific knockdown of daily in the RhoDN-expressing germaria can also slightly suppress the differentiation defect based on the accumulation of single germ cells, indicating that downregulation of EGFR signaling makes a slight contribution to the germ cell differentiation defect (Fig. 7C,E). Although EC-specific knockdown of dpp mRNAs in ECs using two independent RNAi lines produces no discernible phenotype in wild-type germaria, it leads to strong suppression of the germ cell differentiation defect in the RhoDN-expressing germaria, suggesting that RhoDN-expressing germaria fail to control CB differentiation.
ECs might also upregulate dpp mRNAs via transcription or stabilization (Fig. 7C,F). Taken together, Rho is required in ECs to control CB differentiation by restricting BMP signaling activity to the GSC niche.

**DISCUSSION**

In the *Drosophila* ovary, ECs are thought to be produced by a population of ESCs and to move along with differentiated germ cells and commit apoptosis after differentiated germ cell cysts are surrounded by follicle cells (Decotto and Spradling, 2005). In this study, we have used three positive labeling systems and BrdU labeling experiments to demonstrate that ECs are maintained by self-duplication but not by the previously identified ESCs (Fig. 7G). Furthermore, ECs exhibit extensive interactions with differentiated germ cells through their long cellular processes, and differentiated germ cells are likely to be responsible for maintaining long cellular processes. Interestingly, EC cellular process-mediated interactions are required for germ cell differentiation by restricting BMP signaling to the GSC niche. Therefore, we propose that ECs form a germ cell differentiation niche by preventing BMP signaling from interfering with CB differentiation (Fig. 7G).

In this study, we have provided three pieces of experimental evidence demonstrating that ECs are maintained by self-duplication but not by stem cells. First, contrary to one of the predictions by the ESC model that ECs can only undergo apoptosis at the junction between ECs and follicle cell progenitors, we show that ECs in any position can undergo apoptosis although those at the junction die relatively more frequently. Second, contrary to another prediction of the ESC model that only ESCs can proliferate, we show that ECs in any position can proliferate slowly although those at the posterior divide relatively more frequently. Third, we have used three independent lineage-labeling methods to demonstrate that the previously defined ESCs do not produce new ECs (Fig. 7G). Therefore, the ECs in the *Drosophila* ovary behave similarly to the differentiated β cells in the pancreas that are maintained by self-duplication (Dor et al., 2004). Possibly, in other slow turnover mammalian tissues, stem cells are not required for maintaining tissue homeostasis.

Long EC cellular processes might help pass on a differentiated germ cell cluster from one EC to other (Morris and Spradling, 2011). In this study, we propose that long cellular process-mediated intimate interactions between ECs and differentiation are required for proper germ cell differentiation probably by restricting BMP signalization.
signaling activity to the GSC niche (Fig. 7G). EC cellular processes extensively wrap around differentiated germ cells, and are modeled according to the size, shape and differentiated status of the underlying differentiated germ cells. Forced expression of a dominant-negative Rho or knocking down expression of the actin regulator capu causes defects in EC cellular extension and germ cell differentiation. This is consistent with the finding in the previous study that defective EGFR signaling causes loss of EC cellular processes and germ cell differentiation defects (Schultz et al., 2002). Our genetic results have revealed two possible ways for Rho in ECs to control germ cell differentiation by restricting BMP signaling within the GSC niche. One of them is to repress daily expression in ECs via EGFR signaling and thereby prevent BMP diffusion to outside the GSC niche because EGFR signaling-mediated repression of daily expression has been shown to be essential for preventing BMP diffusion and germ cell differentiation (Guo and Wang, 2009; Hayashi et al., 2009; Liu et al., 2010). The other is to prevent BMP expression in ECs by repressing dpp transcription or degrading dpp mRNAs through unknown mechanisms. In addition to the regulation of EC-germ cell interaction (Ridley, 2006), this study also shows that Rho is also required for promoting EC survival. Therefore, we propose that ECs form a niche for promoting germ cell differentiation, and that Rho functions in ECs to promote germ cell differentiation at least partly by preventing BMP signaling in the differentiation niche (Fig. 7G).

The differentiation niche is likely to be a conserved feature for different adult stem cell systems. In the Drosophila testis, somatic cyst cells, which are produced by cyst stem cells, encase differentiated germ cells and move together to accompany the differentiated germ cells (Gonczy and DiNardo, 1996). EGFR signaling is required in somatic cyst cells, as it is in ECs, to control germ cell differentiation because defective EGFR signaling in the Drosophila testis causes germ cell differentiation defects (Kiger et al., 2000; Tran et al., 2000). Although, unlike ECs, somatic cyst cells are not stationary, they function like ECs to control germ cell differentiation. Although equivalent cells for ECs in mammalian adult stem cell systems have not been defined, they are likely to exist and to play important roles in the regulation of lineage-specific differentiation because cultured adult stem cells often require a cocktail of growth factors for their proper differentiation. As easily defined stem cell niches in Drosophila contribute to a better understanding of mammalian stem cell regulation (Li and Xie, 2005; Morrison and Spradling, 2008), we anticipate that what we will learn from studies on the Drosophila GSC differentiation niche will be equally important for defining differentiation niches and studying their functions in lineage differentiation in mammalian systems.

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Competing interests statement

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Supplementary material

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