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# *wingless* signaling regulates the maintenance of ovarian somatic stem cells in *Drosophila*

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## SUMMARY

**Identifying the signals involved in maintaining stem cells is critical to understanding stem cell biology and to using stem cells in future regenerative medicine. In the *Drosophila* ovary, Hedgehog is the only known signal for maintaining somatic stem cells (SSCs). Here we report that Wingless (Wg) signaling is also essential for SSC maintenance in the *Drosophila* ovary. Wg is expressed in terminal filament and cap cells, a few cells away from SSCs. Downregulation of Wg signaling in SSCs through removal of positive regulators of Wg signaling, *dishevelled* and *armadillo*, results in rapid SSC loss. Constitutive Wg signaling in SSCs through the removal of its negative regulators, *Axin* and *shaggy*, also causes SSC loss. Also, constitutive *wg* signaling causes over-proliferation and abnormal differentiation of somatic follicle cells. This work demonstrates that *wg* signaling regulates SSC maintenance and that its constitutive signaling influences follicle cell proliferation and differentiation. In mammals, constitutive β-catenin causes over-proliferation and abnormal differentiation of skin cells, resulting in skin cancer formation. Possibly, mechanisms regulating proliferation and differentiation of epithelial cells, including epithelial stem cells, is conserved from *Drosophila* to man.**

Key words: Somatic stem cells, Ovary, *Drosophila*, *wingless*

## INTRODUCTION

Stem cells are distinguished from other cells by a unique ability to self-renew and continuously produce one or several differentiated cell types throughout the lifetime of an organism (Donovan and Gearhart, 2001; Spradling et al., 2001). Increasing evidence shows that stem cells are controlled by a specialized regulatory microenvironment or 'niche' (Watt and Hogan, 2000; Spradling et al., 2001; Nishimura et al., 2002). It has been demonstrated that germline stem cells (GSCs) in the *Drosophila* ovary are located in niches (Cox et al., 1998; King and Lin, 1999; Xie and Spradling, 1998; Cox et al., 2000; Xie and Spradling, 2000; King et al., 2001). Identification of the signals involved in the communication between stem cells and their niches is important for understanding stem cell biology and its role in future regenerative medicine.

The *Drosophila* ovary is an excellent system in which to study two different stem cell types that are responsible for egg production during adult life (Lin, 1998; Xie and Spradling, 2001). The adult ovary contains 12-16 ovarioles, each with a germarium at the tip, in which GSCs and somatic stem cells (SSCs) are located (Fig. 1A). Two or three GSCs, surrounded by three groups of somatic cells at the germarial tip [terminal filament cells (TFs), cap cells (CPCs) and inner germarial sheath (IGS) cells] produce all germline cells in the ovariole. These stem cells directly contact cap cells and are posterior to terminal filament cells. After a GSC divides, the daughter still in contact with cap cells remains a stem cell, whereas the daughter that is more distant from cap cells differentiates into a cystoblast. However, if both daughters remain in contact with cap cells, they both become stem cells (Xie and Spradling, 2000). Consistent with the existence of niches, terminal filament, cap cells and IGS cells express several genes that are important for GSC function (Cox et al., 1998; King and Lin, 1999; Xie and Spradling, 1998; Xie and Spradling, 2000). In addition, the failure of GSCs to stay in their niches, because of defects in DE-cadherin-mediated cell adhesion, results in stem cell loss (Song et al., 2002).

Two or three SSCs, located in the middle of the germarium (Fig. 1A), generate several different types of somatic follicle cells in egg chambers (Margolis and Spradling, 1995; Zhang and Kalderon, 2001). These stem cells directly interact with a posterior group of IGS cells that may function as the niche for SSCs. Their immediate progeny, also known as follicle cell progenitors, can divide and generate differentiated follicle cells that encapsulate germ cysts to form individual egg chambers. *hedgehog* (*hh*) is expressed in terminal filament and cap cells, and directly regulates SSC maintenance and proliferation (Forbes et al., 1996a; King et al., 2001; Zhang and Kalderon, 2001). Reduction of *hh* signaling causes stem cell loss, whereas constitutive *hh* signaling causes SSC proliferation (Zhang and Kalderon, 2001). Recently, *fs(1)Yb* has been shown to regulate

follicle cell proliferation through the regulation of *hh* expression in the *Drosophila* ovary (King et al., 2001). Similarly, in mice and humans, constitutive sonic hedgehog (*Shh*) signaling can cause basal cell carcinoma (Johnson et al., 1996; Oro et al., 1997). These studies suggest that *hh* is probably a general growth factor for regulating epithelial cell proliferation and differentiation from *Drosophila* to humans. *Delta* is expressed in germline cells and directly activates *Notch* in somatic follicle cells to control their proliferation and differentiation through regulating *decapentaplegic* (*dpp*) expression (Jordan et al., 2000). It remains unclear whether *Notch* or *dpp* also regulates SSCs in the *Drosophila* ovary.

In mammals, increasing evidence shows that Wnt/β-catenin signaling is important for regulating epithelial cells (Oshima et al., 2001; Taylor et al., 2000; Watt, 2001). Two populations of stem cells are localized in the basal cell layer and the deep rete ridges. Recent evidence indicates that long-lived stem cells reside apically within the bulge region and are responsible for re-populating matrix stem cells and replacing epidermal stem cells (Oshima et al., 2001; Taylor et al., 2000). The dermal papilla seems to be a key niche component, producing signals that stimulate matrix stem cell activity. Signals such as fibroblast growth factor (FGF)-7, β1-integrin, bone morphogenetic protein (BMP)-4 and *shh* have been shown to regulate different aspects of hair follicle cell proliferation and differentiation (Spradling et al., 2001; Watt and Hogan, 2000). Furthermore, increasing evidence shows that Wnt/β-catenin signaling is important for regulating epithelial cells in mammals. For example, Wnt/β-catenin signaling is required for hair follicle cells to form and for matrix-derived cells to differentiate into follicular rather than epidermal keratinocytes (DasGupta and Fuchs, 1999; Huelsken et al., 2001). Constitutive activation of Wnt signaling increases hair follicle and skin tumor formation in mice and humans (Chan et al., 1999; Gat et al., 1998). Defective maintenance of crypts in the small intestines of Tcf-4 knockout mice suggests that Wnt signaling also regulates intestinal stem cell activity (Korinek et al., 1998).

In *Drosophila*, Wg signal is transduced by Frizzled receptors, Frizzled (Fz) and Frizzled 2 (Fz2), resulting in the phosphorylation of the cytoplasmic protein Dishevelled (Dsh), inhibition of Shaggy (Sgg) and Axin (Axn), stabilization of Armadillo (Arm) and activation of target gene expression (reviewed by Peifer and Polakis, 2000). *wg* is involved in regulating many different cellular processes during *Drosophila* development, and it is transcribed in cells close to GSCs and SSCs, but its role in stem cell regulation in the *Drosophila* has not been previously demonstrated (Forbes et al., 1996b). Here, we report that *wg* signaling directly regulates SSC maintenance and that constitutive *wg* signaling causes over-proliferation and improper differentiation of their progeny in the *Drosophila* ovary.

## MATERIALS AND METHODS

### *Drosophila* stocks and genetics

The following fly stocks were used in this study and are described in Flybase: X-15-29, X-15-33, MKRS hs-FLP, $FRT_{18A}$ $sgg^{M1\text{-}1}$, $FRT_{18A}$ $dsh^{VA135}$, $FRT_{18A}$ $arm^{2}$, $FRT_{18A}$ $arm^{8}$, $FRT_{18A}$ $arm^{3}$, $FRT_{18A}$ $arm^{4}$, $FRT_{18A}$ +, $FRT_{82B}$ $Axn^{S044230}$, $FRT_{82B}$ +, *hs-GAL4*, *C587-GAL4*, *UAS-dsh*, *UAS-Fz2*, *UAS-arm*$^{S10}$, $wg^{ts}$ and $w^{1118}$. All *Drosophila* stocks were maintained at room temperature on standard cornmeal/molasses/agar media. The $wg^{ts}$/CyO flies were grown at 18°C to obtain homozygous *wg* mutant females. For more severe ovarian phenotypes, *wg* homozygous mutant flies and wild-type control flies were cultured at 30°C for a week before their ovaries were isolated.

### Generating mutant somatic stem cell clones

Clones of mutant SSCs were generated by FLP-mediated mitotic recombination. To generate the stocks for *arm*, *dsh* or *sgg* clonal analysis, $FRT_{18A}$ $sgg^{M1\text{-}1}$/FM7, $FRT_{18A}$ $dsh^{VA135}$/FM7, $FRT_{18A}$ $arm^{2}$/FM7, $FRT_{18A}$ $arm^{8}$/FM7, $FRT_{18A}$ $arm^{3}$/FM7, $FRT_{18A}$ $arm^{4}$/FM7 and $FRT_{18A}$ + virgin females were mated with males $FRT_{18A}$ *armadillo-lacZ*; *hs-FLP*, respectively. To generate the stocks for *Axn* clonal analysis, $FRT_{82B}$ $Axn^{S044230}$/TM3 Sb and $FRT_{82B}$ + males were mated with virgin females *yw hs-FLP*; $FRT_{82B}$ *armadillo-lacZ*, respectively. One- or two-day-old adult non-FM7 or non-Sb females carrying an *armadillo-lacZ* transgene in trans to the mutant-bearing chromosome were heat-shocked six times at 37°C for 1 hour (8-12 hour intervals). The females were transferred to fresh food daily at room temperature, and ovaries were removed from them one, two or three weeks after the last heat-shock treatment and then further processed for antibody staining. To determine stem cell maintenance, the percentages of the ovarioles carrying a marked SSC clone at different time points were calculated by dividing the number of germaria carrying marked follicles by the total number of germaria examined.

### Overexpression of *wg* downstream components in the ovary

To construct the stocks for over-expressing *wg* downstream components in the ovary, the *hs-Gal4* virgin females were crossed with males carrying *UAS-Fz2*, *UAS-dsh* and *UAS-arm*$^{S10}$. The females that did not carry balancer chromosomes were heat-shocked eight times at 37°C for 1 hour (8-12 hour intervals for 4 days). The females were further cultured at room temperature for 1 day before their ovaries were isolated for analysis.

### Immunohistochemistry

The following antisera were used: monoclonal anti-Hts antibody 1B1 (1:4), monoclonal anti-Fas3 antibody 7G10 (1:4), monoclonal anti-Wg antibody 4D4 (1:2), rabbit polyclonal anti-β-galactosidase (1:150; Molecular Probes) and rabbit poly anti-Vasa (1:1000) antibodies.

To detect secreted Wg protein, we followed the published procedures (Strigini and Cohen, 2000). To detect other proteins, ovaries were processed according to the published procedures (Song et al., 2002). All micrographs were taken using a Leica SPII confocal microscope.

## RESULTS

### *wg* signaling regulates the production of somatic follicle cells during early *Drosophila* oogenesis

*wg* is expressed in the cap cells of the *Drosophila* ovary based on an enhancer trap line in which the bacterial *lacZ* gene with a minimal basal promoter is directly inserted into the *wg* locus (Forbes et al., 1996b). To directly determine Wg protein distribution in the germarium, wild-type ovaries were immunostained using an anti-Wg monoclonal antibody. We observed that Wg proteins were present in high levels at the tip of the germarium (including terminal filament cells and cap cells) (Fig. 1B). We also observed that low levels of Wg proteins were present in the germ cells in the egg chambers (data not shown). GSCs and SSCs are located in the regions of

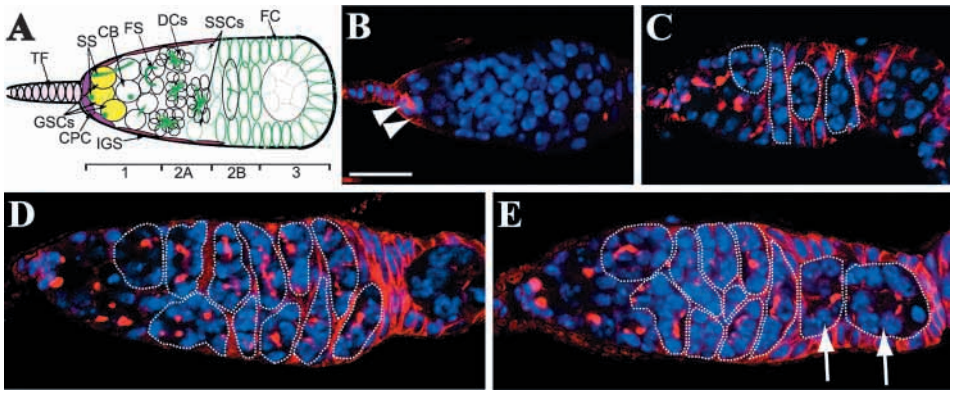


**Fig. 1.** *wg* signaling regulates follicle cell production or differentiation in the *Drosophila* ovary. (A) Cross-sectional diagram of a *Drosophila* germarium. Two or three SSCs, follicle cell progenitors and differentiated follicle cells are located in regions 2A, 2B and 3, respectively. The micrographs in B-E represent one confocal cross section of the germarium or ovariole. (B) A wild-type germarium labeled for Wg (red) and nuclei (blue). Terminal filament and cap cells in the germarium express Wg proteins (cap cells indicated by arrowheads). (C) A wild-type germarium labeled for Hts (red) and nuclei (blue) showing 16-cell cysts (outlined). (D) A mutant $wg^{ts}$ germarium labeled for Hts (red) and nuclei (blue) showing accumulation of extra 16-cell cysts (outlined). (E) A mutant $wg^{ts}$ germarium labeled for Hts (red) and nuclei (blue) showing ongoing encapsulation of two cysts (outlined and indicated by arrows) into an egg chamber. All the germaria are shown at the same scale. Scale bar in B: 10 μm. CB, cystoblast; CPC, cap cell; DCs, developing cysts; FC, follicle cell; FS, fusome; GSCs, germline stem cells; IGS, inner germarial sheath cell; SS, spectrosomes; SSCs, somatic stem cells; TF, terminal filament cell.

the germarium that express Wg, suggesting a possible role of Wg in stem cell regulation.

To directly determine whether *wg* regulates somatic follicle cell production, we studied the mutant *wg* ovary using a temperature-sensitive allele $wg^{ts}$. The $wg^{ts}$ flies can survive to adulthood at 18°C (Bejsovec and Martinez Arias, 1991), which allows us to examine the role of *wg* in the adult ovary. One week after the shift to a restrictive temperature (30°C), the ovaries were immunostained with an anti-Huli tai shao (Hts) antibody. The Hts protein is present not only on spectrosomes in GSCs, cystoblasts and fusomes in early germline cysts, but also on the membranes of somatic follicle cells (Lin et al., 1994). Both $wg^{ts}$ and wild-type females were raised under the same conditions (incubation at the restrictive temperature, 30°C, for one week). The $wg^{ts}$ mutant germarium carried many more germline cysts (20.0±3.8; $n$=40) in comparison with the wild-type one (7.9±1.2; $n$=49) (Fig. 1C,D). Sometimes, in the $wg^{ts}$ mutant germarium, two 16-cell cysts were packed into an egg chamber because of over-crowded germline cysts or defects in follicle cells (Fig. 1E). These swollen germaria could result from over-production of germline cysts, reduction of somatic follicle cell production or defects in follicle cell differentiation. *wg* signaling does not seem to directly regulate the maintenance or division of GSCs (Song et al., 2002) (data not shown). This raises the possibility that *wg* could regulate proliferation and differentiation of follicle cells.

To further determine whether *wg* signaling is capable of regulating follicle cell production, we over-expressed *Fz2*, *dsh* and activated *arm* in the adult ovary using transgenes *UAS-Fz2*, *UAS-dsh* and *UAS-*$arm^{S10}$. These transgenes have been used to increase *wg* signaling by increasing the amount of downstream components using the GAL4-driven UAS target gene expression system (Brand and Perrimon, 1993; Axelrod et al., 1998; Cadigan et al., 1998; Pai et al., 1997). The *hs-gal4* transgene allows us to induce transcription of target genes in the adult *Drosophila* ovary by heat-shock treatments. Normally, a row of 5-7 stalk cells link two adjacent egg chambers (Fig. 2A). After four days of pulsed heat-shock treatment, over-expression of *Fz2*, *dsh* and the activated *arm* consistently produced more follicle cells that accumulated between egg chambers (Fig. 2B-D). These extra follicle cells also formed long stalks with multiple rows of cells instead of one row in wild type. These follicle cell over-proliferation phenotypes are similar to, but weaker than, those caused by increasing *hh* and *Notch* signaling activities (Forbes et al., 1996b; Larkin et al., 1996; Zhang and Kalderon, 2000). These results raise the possibility that *wg* signaling modulates somatic follicle cell production in the adult *Drosophila* ovary.

### *dsh*, *arm*, *sgg* and *Axn* are required for maintaining somatic stem cells in the adult ovary

The above experiments suggest that *wg* signaling could regulate SSC function. However, Hh has a similar expression pattern to that of *wg*, and is known to directly regulate SSCs (Forbes et al., 1996a; King et al., 2001; Zhang and Kalderon, 2001). To determine whether *wg* signaling directly regulates SSCs, we first tested whether removal of *dsh*, *sgg*, *Axn* and *arm* from SSCs affects their behavior using the FLP-mediated FRT mitotic recombination technique (Xu and Rubin, 1993). Marked

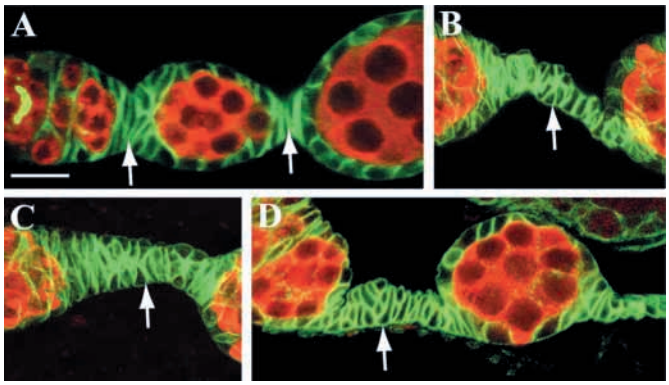


**Fig. 2.** Increasing *wg* signaling by over-expressing *wg* downstream components can promote follicle cell proliferation. The ovariole from the females over-expressing (A) no genes (control), (B) *Fz2*, (C) *dsh* and (D) an activated *arm* for four days are labeled for Vasa (red) and Hts (green). A shows normal stalk cells (arrows), whereas B-D show extra stalk cells (arrows). A and D represent one confocal section, whereas B and C show images of multiple sections. All the germaria are shown at the same scale. Scale bar in A: 10 μm.

clones homozygous for loss-of-function mutations in SSCs were generated according to experimental procedures and identified by loss of expression of the *arm-lacZ* transgene that is strongly expressed in all the cells within the germarium (Xie and Spradling, 1998; Song and Xie, 2002). This method not only induces recombination events in SSCs but also in follicle cells, and thus marks both SSCs and follicle cells. Marked follicle cell clones are only transiently present in the germarium following their induction, and completely leave the germarium one week after clone induction. Therefore, marked follicle cells present in the germarium one week after clone induction must surely derive from marked SSCs induced by heat-shock treatments. Two or three SSCs have been mapped to the 2A/2B junction of the germarium (Margolis and Spradling, 1995; Zhang and Kalderon, 2001) (Fig. 1A). We consistently observed marked SSCs in the 2A/2B junction that also produced marked follicle cells at the posterior part of the germarium (Fig. 3A). Inner sheath cells in the same region as SSCs can also be labeled by this technique but at lower rates than SSCs (Song and Xie, 2002) (X.S. and T.X., unpublished). Because IGS cells do not give rise to follicle cells, we differentiated between SSC and IGS clones by the presence of marked follicle cells in the germarium. In addition, germaria carrying both IGS and SSC clones were rare, and thus IGS clones did not impact the analysis of our data. Many aspects of SSCs and their progeny can be determined through this mutant clonal analysis. Loss rates of marked mutant SSC clones over a period of three weeks can be compared to those of marked wild-type SSC clones to determine whether a mutation has any effect on SSC maintenance. The effect a particular mutation has on follicle cell proliferation can be determined by analyzing the sizes of mutant follicle cell clones in egg chambers. Finally, the mutant follicle cells can be studied side-by-side with wild-type ones in the same egg chamber to determine whether a mutation affects follicle cell differentiation.

SSC clones of *dsh*$^{-}$, *arm*$^{-}$, *sgg*$^{-}$ and *Axn*$^{-}$ were generated by subjecting females of the appropriate genotype to heat-shock and examining their ovaries one week later. *dsh*$^{VA135}$ is a strong loss-of-function allele that severely disrupts the *wg* signaling pathway (Heslip et al., 1997). In the control, marked wild-type SSCs were well maintained within the three-week testing period. It has been previously shown that wild-type SSCs have a limited lifespan (Margolis and Spradling, 1995; Zhang and Kalderon, 2001). Our previous study demonstrated that SSCs have a half-life of approximately two weeks (Song and Xie, 2002). Consistently, this study indicates that marked wild-type SSCs exhibited a similar half-life, approximately two weeks, with only 56% of the marked SSCs observed within the first week after clone induction being maintained for two more weeks (Fig. 3A,B, Table 1). In contrast to wild-type clones, mutant *dsh* SSC clones were lost rapidly (Table 1); 92% and 100% of the marked mutant *dsh*$^{VA135}$ SSC clones observed one week after clone induction were lost two or three weeks after clone induction, respectively (Fig. 3C,D, Table 1). Consistent with the important role of Dsh in maintaining SSCs, we observed that some ovarioles did not carry marked follicle cells in the germarium but carried marked follicle cells in mid- or late-stage egg chambers two or three weeks after clone induction (Fig. 3D, inset). Because it only takes approximately 7 days for newly produced follicle cells from SSCs to appear on mature stage 14 eggs, those ovarioles must have carried marked mutant SSC clones during the first week after clone induction but lost them afterward. These results indicate that *dsh* is required to maintain SSCs in the adult *Drosophila* ovary.

We have recently shown that DE-cadherin-mediated cell adhesion is essential for anchoring SSCs in their niches (Song and Xie, 2002). To determine whether *arm* is involved in regulating SSCs through transducing the *wg* signal, we used four different *arm* alleles: *arm*$^{2}$, *arm*$^{3}$, *arm*$^{4}$ and *arm*$^{8}$. *arm* is required for DE-cadherin-mediated cell adhesion and *wg* signaling in *Drosophila*. Here, two classes of *arm* alleles were used to differentiate its role in *wg* signaling and cell adhesion. *arm*$^{2}$ and

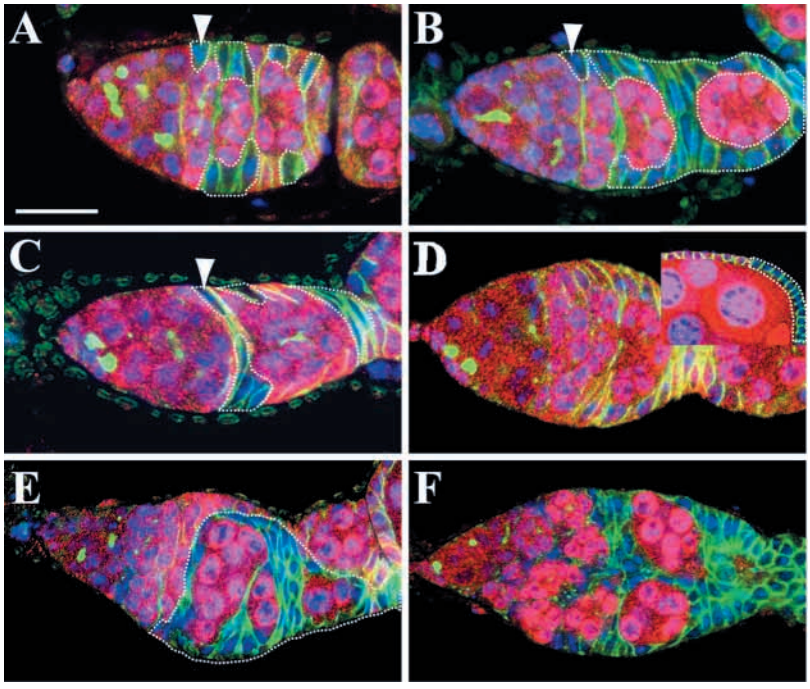


**Fig. 3.** *wg* downstream components are required for SSC maintenance. Germaria are labeled for *lacZ* (red), Hts (green), nuclei (blue). All the micrographs represent one confocal cross section of the germarium. Marked wild-type (A,B) or mutant (C-E) clones are outlined and are identified by loss of *lacZ* expression. (A) A germarium showing a one-week-old wild-type SSC clone and its follicle cell progeny (outlined). The putative marked SSC is indicated by an arrowhead. (B) A germarium showing a three-week-old wild-type SSC clone in which all follicle cells (outlined) are marked, indicating that all the SSCs are marked. One of the putative marked SSCs is indicated by an arrowhead. (C) A germarium showing a one-week-old mutant *dsh*$^{VA135}$ SSC clone (outlined). The putative marked *dsh* mutant SSC is indicated by an arrowhead. (D) A germarium showing loss of a marked mutant *dsh*$^{VA135}$ SSC clone three weeks after clone induction indicated by absence of marked follicle cells in the germarium but their presence in a late egg chamber (outlined in the inset). (E) A germarium with a marked mutant *sgg* clone three weeks after clone induction. (F) A germarium with all SSCs mutant for *sgg* three weeks after clone induction, indicated by loss of *lacZ* expression in all the follicle cells. All the germaria are shown at the same scale. Scale bar in A: 10 μm. SSCs, somatic stem cells.

**Table 1. Downstream components of the *wg* signaling pathway are essential in maintaining SSCs in the *Drosophila* ovary**

| Genotypes | Percentage of germaria carrying a marked SSC after 1 week | Percentage of germaria carrying a marked SSC after 2 weeks | Percentage of germaria carrying a marked SSC after 3 weeks |
|---|---|---|---|
| Wild type | 26.9% (145) | 21.6% (268) | 15.1% (218) |
| $dsh^{VA135}$ | 18.4% (208) | 1.5% (328) | 0.0% (340) |
| $Arm^{2}$ | 14.8% (162) | 0.5% (357) | 0.0% (272) |
| $Arm^{8}$ | 24.3% (218) | 12.7% (283) | 6.0% (283) |
| $Arm^{3}$ | 6.7% (150) | 0.2% (416) | 0.0% (461) |
| $arm^{4}$ | 13.0% (192) | 0.7% (282) | 0.0% (282) |
| $Axn^{S044230}$ | 23.6% (127) | 3.7% (107) | 0.5% (203) |
| $sgg^{M1-1}$ | 21.2% (396) | 5.1% (297) | 0.4% (242) |

The percentage of germaria carrying a marked SSC at a given time point=the number of germaria carrying a marked SSC/total germaria examined ×100. Total germaria examined for each genotype at that time point are give in parentheses.

$arm^{8}$ are defective in *wg* signaling but have no obvious effect on DE-cadherin-mediated cell adhesion (Llimargas, 2000); whereas, $arm^{3}$ and $arm^{4}$ are defective in both *wg* signaling and DE-cadherin-mediated adhesion (Peifer et al., 1993). Consistent with *dsh* clonal analysis results, mutant $arm^{2}$ SSC clones were also lost faster than wild type (Table 1). Compared to $arm^{2}$ mutant SSCs, $arm^{8}$ mutant SSCs were lost at a slower rate. Supporting the idea that DE-cadherin-mediated cell adhesion is also required for maintaining SSCs, mutant $arm^{3}$ SSCs were lost more quickly than $arm^{2}$. Along with the mutant *dsh* clonal analysis, these results demonstrate that *wg* signaling is required for maintaining SSCs in the *Drosophila* ovary.

Unlike *dsh* and *arm*, *Axn* and *sgg* are downstream negative regulators of *wg* signaling, and removal of their function from SSCs can cause constitutive *wg* signaling. $Axn^{S044230}$ is a transposon-induced loss-of-function allele in which a P-element is inserted in the first exon, 135bp downstream of the transcription start site (Hamada et al., 1999); $sgg^{M1-1}$ is a strong loss-of-function allele (Heslip et al., 1997). Surprisingly, mutant *sgg* and *Axn* SSC clones were also lost much faster than the marked wild-type SSC clones. Three weeks after clone induction, 98% of mutant $Axn^{S044230}$ and $sgg^{M1-1}$ SSC clones were lost, in contrast to the 44% loss of marked wild-type control clones (Table 1). These results indicate that constitutive *wg* signaling also shortens the SSC lifespan.

In the germaria carrying both wild-type and mutant SSCs, marked (*lacZ*-negative) mutant *Axn* or *sgg*, follicle cells appeared more in number than wild-type (*lacZ*-positive) ones (Fig. 3E), indicating that mutant *Axn* or *sgg* SSCs and/or follicle progenitor cells are over-proliferative. In other germaria carrying only *lacZ*-negative mutant *sgg* or *Axn* follicle cells (Fig. 3F), which is because of the natural loss of *lacZ*-positive wild-type SSCs, more mutant follicle cells accumulated. Interestingly, mutant *sgg* or *Axn* follicle cells appeared to express higher levels of Hts than wild-type follicle cells. In the germaria, where most or all follicle cells were mutant for *sgg* or *Axn*, germline cysts could not undergo normal morphological changes from ball-like structures in region 2a to lens-shape cysts in region 2b, and thus still remained in ball-like structures in region 2b (Fig. 3E,F). These results indicate that constitutive *wg* signaling can modulate proliferation and differentiation of SSCs and/or follicle cell progenitors.

### *sgg* and *Axn* mutant follicle cells over-proliferate and accumulate between egg chambers

To further investigate whether *wg* signaling regulates follicle cell proliferation, we examined mutant *dsh*, *arm*, *sgg* and *Axn* follicle cell clone size. During follicle epithelial development, a marked early progenitor proliferates and generates a population of follicle cells that stay together throughout development. Thus, the sizes of follicle cell clones mutant for a particular gene can be used to determine whether its function is required for the proliferation of follicle cells. Follicle cell clones mutant for either *arm* or *dsh* formed big patches similar to those formed by marked wild-type clones (Fig. 4A-C). The mutant $arm^{2}$ and $arm^{8}$ follicle cell clones had normal morphology in comparison with neighboring wild-type follicle cells (Fig. 4A). The mutant $arm^{4}$ or $arm^{3}$ follicle cells lost wild-type regular columnar shape, and there was abnormal accumulation of Hts proteins on apical membranes instead of on lateral membranes in wild type (Fig. 4B). These results indicate that DE-cadherin-mediated cell adhesion, but not *wg* signaling, is very important for maintaining the integrity of follicle cell epithelia. Consistent with the idea that *wg* signaling does not affect morphology of follicle cells, mutant $dsh^{VA135}$ follicle cells had normal morphology and expression of Hts in their apical membranes and normal sizes of mutant *dsh* or *arm* clones in the germarium (Fig. 4C). To further determine whether *dsh* is required for proliferation of follicle cells in egg chambers, we generated twin clones derived from the same follicle cell during the follicle cell development. Because the twin clones were produced at the same time, sizes of homozygous mutant clones and corresponding wild-type twin clones could be used to demonstrate the requirement of a particular gene in cell proliferation. *dsh* mutant clones and their corresponding wild-type twin clones had similar sizes (Fig. 4D), supporting that *wg* signaling does not regulate the proliferation of follicle cells in egg chambers. These results demonstrate that *wg* signaling is not essential for the proliferation of follicle cells in egg chambers.

In contrast to *dsh* or *arm* mutant follicle cells, the mutant *Axn* or *sgg* follicle cells could not properly integrate into egg chambers, accumulating instead as a disorganized cell mass between egg chambers. The mutant follicle cells also expressed higher levels of Hts proteins on their membranes (Fig. 5A-D). The mutant *Axn* or *sgg* mutant follicle cells appeared to be over-produced. As mentioned earlier, the wild-type cysts surrounded by mutant *Axn* or *sgg* follicle cells could not undergo normal shape changes, and thus, we often observed that wild-type germline cysts were broken into clusters by mutant *Axn* or *sgg* follicle cells, with less than 16 germ cells per cluster (Fig. 5B). This phenotype may be attributed to

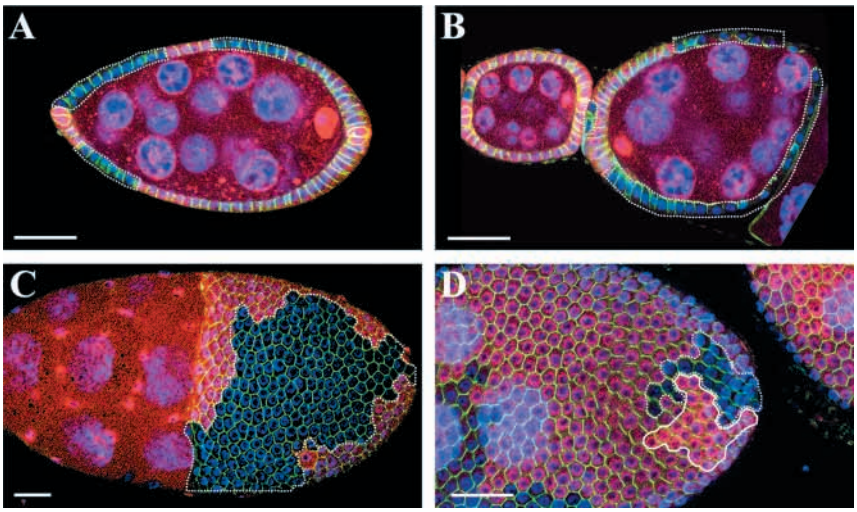


**Fig. 4.** *wg* signaling is not required for the proliferation of follicle cells on egg chambers. All egg chambers are labeled for *lacZ* (red), Hts (green) and nuclei (blue). The micrographs in A and B represent one confocal cross section of an egg chamber, whereas the micrograph in C is one confocal section along the surface of an egg chamber. Marked mutant follicle cell clones are identified by loss of *lacZ* expression (outlined in panels A-D). (A) A stage-8 egg chamber carrying marked mutant $arm^{2}$ follicle cell clones. The $arm^{2}$ mutant follicle cells have normal accumulation of Hts in lateral membranes. (B) A stage-6 egg chamber carrying mutant $arm^{4}$ follicle cell clones in which Hts accumulates abnormally on apical membranes. In this egg chamber the oocyte is mislocalized to the anterior end because of a defect in DE-cadherin-mediated cell adhesion. However, the mutant follicle cell clones appear to have a normal size. (C) A stage-10 egg chamber showing a mutant $dsh^{VA135}$ follicle cell clone. The mutant clone appears to have a normal size and normal expression of Hts on lateral membranes. (D) A stage-9 egg chamber carrying twin follicle cell clones. The mutant *dsh* follicle cell clone, which is identified by loss of *lacZ* expression and highlighted by broken lines, appears to proliferate normally in comparison with the corresponding wild-type twin clone. The wild-type clone (highlighted by a solid line) carries two copies of the *lacZ* gene and thus shows stronger *lacZ* expression than the rest of *lacZ*-positive follicle cells (carrying one copy of the *lacZ* gene). The mutant clone appears to have a normal size and normal expression of Hts on lateral membranes. Scale bars: 10 μm.

differentiation defects of mutant *sgg* or *Axn* follicle cells because the germline cysts were wild type. The mutant *sgg* or *Axn* follicle cells in egg chambers still maintained higher levels of Hts on their membranes with abnormal morphologies (e.g. smaller and irregular) (Fig. 5C,D). This phenotype is restricted only to mutant follicle cells, but neighboring wild-type cells had normal expression of Hts and normal morphology (Fig. 5C,D), suggesting that *sgg* and *Axn* function in a cell-autonomous manner.

The extra mutant *sgg* or *Axn* follicle cells that accumulated between egg chambers could be derived from over-proliferation of follicle cells in the germarium or from continuous proliferation of mutant follicle cells after exiting the germarium. Usually, stalk cells (one row of somatic cells that link two adjacent egg chambers) are not mitotically active. To determine whether mutant *sgg* or *Axn* follicle cells that exit the germarium are still capable of dividing, we examined phosphorylated histone H3 (pH3) expression in mutant *sgg* or *Axn* follicle cell clones. The presence of pH3 indicates a cell in the mitotic phase of the cell cycle (Hendzel et al., 1997). Mutant *Axn* follicle cells in the germarium and in egg chambers were pH3-positive, indicating that they are capable of dividing (Fig. 6A). However, we did not observe pH3-positive cells when they accumulated between egg chambers (Fig. 6B), suggesting that they are not actively dividing. Observations of mutant *sgg* follicle cells were similar (data not shown). These

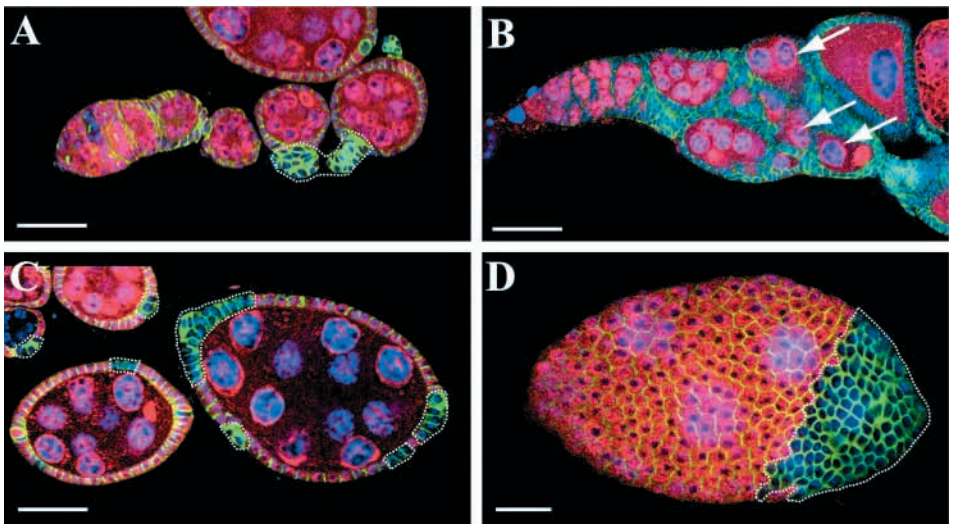


**Fig. 5.** Constitutive *wg* signaling causes follicle cell over-proliferation and abnormal accumulation of Hts protein on the membranes of follicle cells. All egg chambers and ovarioles are labeled for *lacZ* (red), Hts (green) and nuclei (blue). The micrographs in A-C represent one confocal cross section of an ovariole or an egg chamber, whereas the micrograph in D is one confocal section along the surface of an egg chamber. Marked mutant follicle cell clones are identified by loss of *lacZ* expression (outlined in A,C,D). (A) An ovariole showing abnormal accumulation of extra mutant *Axn* somatic cells (outlined) between egg chambers that also express higher levels of Hts than wild-type ones. (B) An ovariole carrying only marked mutant $sgg^{M1-1}$ follicle cells. Extra mutant *sgg* follicle cells accumulate abnormally between egg chambers and break germline cysts into small clusters (arrows). (C) Egg chambers with mutant *Axn* follicle cell clones that express higher levels of Hts (outlined). (D) A stage-8 egg chamber with a mutant *sgg* clone in which mutant follicle cells have abnormal morphology and higher levels of Hts expression. Scale bars: 10 μm.

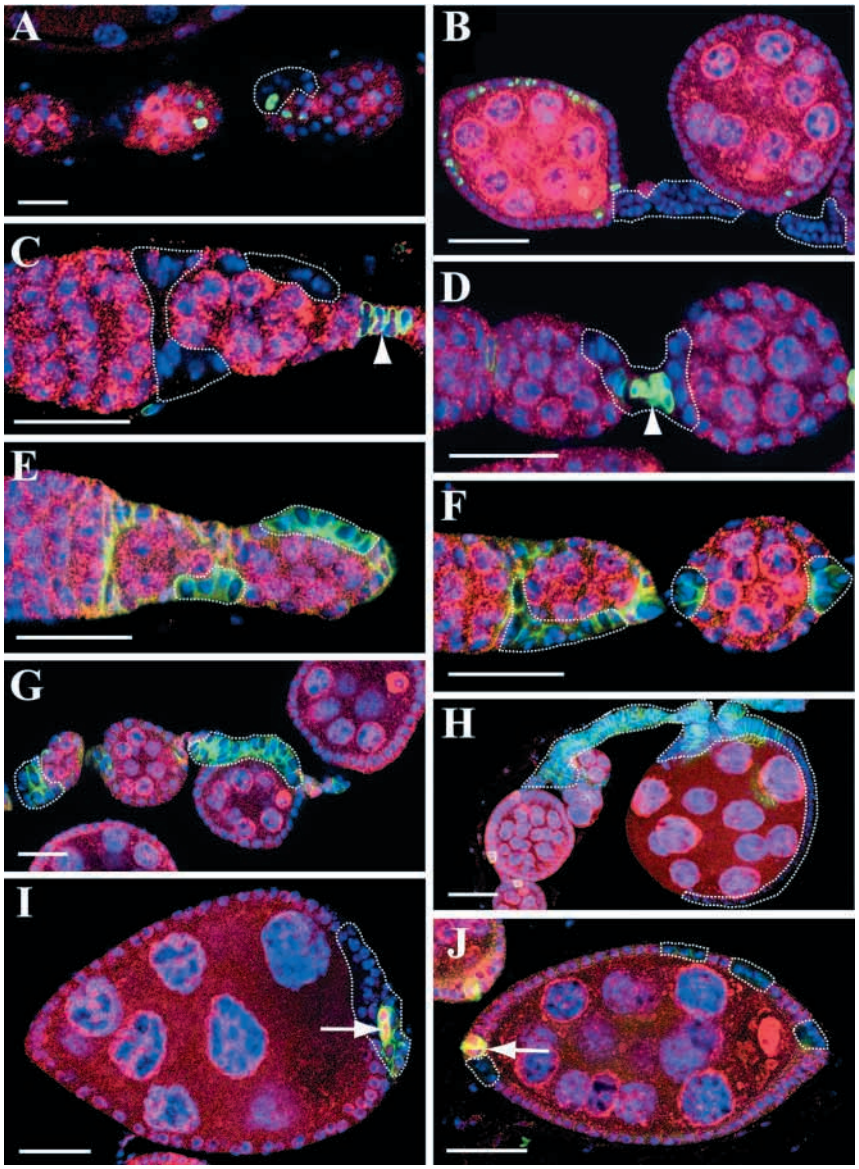


**Fig. 6.** Constitutive *wg* signaling blocks normal differentiation pathways of follicle cells. The micrograph in A represents a confocal section along the surface of an ovariole, whereas the rest are one confocal cross section of an egg chamber or an ovariole. (A,B) Parts of two ovarioles labeled for *lacZ* (red) and pH3 (green) showing marked mutant *Axn* follicle cell clones (outlined). (C,D) Parts of two ovarioles labeled for *lacZ* (red) and Lamin C (green), showing marked mutant *Axn* follicle cell clones (outlined) and normal (C, arrowhead) or mutant (D, arrowhead) *Axn* stalk cells. (E-J) Parts of ovarioles labeled for *lacZ* (red) and Fas3 (green) showing (E,G,I) marked mutant *Axn* (outlined) or (F,H,J) *sgg* follicle cell clones (outlined). The mutant *Axn* follicle cells between egg chambers express high levels of Fas3, but those that are integrated into egg follicle epithelium fail to express Fas3 at high levels. Arrows in I and J indicate wild-type polar cells. Scale bars: 10 μm.

results suggest that the extra follicle cells lying between egg chambers are generated directly from over-proliferation of SSCs and/or follicle progenitor cells induced by hyperactive *wg* signaling.

### Mutant *sgg* and *Axn* follicle cell clones have defects in differentiation

Because we observed defects in egg chamber budding and abnormal morphology associated with mutant *Axn* or *sgg* follicle cells, we used different molecular markers to investigate whether mutant follicle cells had defects in differentiation. Because mutant *sgg* or *Axn* follicle cells often accumulated between egg chambers and were not mitotically active, we investigated whether mutant follicle cells adopted the same cell fates as stalk cells using lamin C antibodies. Lamin C is highly expressed in nuclear membranes of cap cells and stalk cells (Fig. 6C) (Xie and Spradling, 2000) (this study). Mutant *Axn* follicle cells that were integrated into egg chambers did not express Lamin C similar to their wild-type counterparts (Figs 6C,D). All the follicle cells that accumulated outside egg chambers expressed Lamin C just like normal stalk cells (Fig. 6D). Mutant *sgg* follicle cells behaved exactly like mutant *Axn* follicle cells in terms of Lamin C expression (data not shown), suggesting that the extra mutant *Axn* or *sgg* follicle cells in-between egg chambers adopt some stalk cell characteristics. However, mutant *Axn* or *sgg* stalk cells were organized differently to wild-type stalk cells. Normally, a row of 5-7 stalk cells is organized into a chain-like structure. The excessive mutant *Axn* or *sgg* follicle cells were often organized into a chain-like structure but with multiple rows in contrast to one row in wild type (Fig. 5A), and they were sometimes completely disorganized (Fig. 5B).

Follicle cell clones mutant for *patched* (*ptc*) or *protein kinase A* (*pka*), two negative regulators of the *hh* pathway, have similar mutant phenotypes to those of mutant *Axn* or *sgg* follicle cells in the *Drosophila* ovary (Forbes et al., 1996a; Zhang and Kalderon, 2001). Some mutant *ptc* or *pka* follicle cells can adopt polar cell fates. Normally, one pair of polar cells situates at each end of an egg chamber and expresses high levels of Fas3. Polar cells play an important role in regulating egg chamber budding and generating different follicle cell types by expressing important signaling molecules such as *unpaired* (Xi et al., 2003). To investigate whether mutant *Axn* or *sgg* follicle cells in egg chambers can adopt polar cell fates, we examined Fas3 expression in mutant *Axn* or *sgg* follicle clones. Mutant *Axn* or *sgg* follicle cells expressed higher levels of Fas3 than wild-type ones in the germaria and stage 2 chambers (Fig. 6E,F). Extra mutant *Axn* or *sgg* follicle cells that accumulated between egg chambers also continuously expressed high levels of Fas3 (Fig. 6G,H). In this respect, the mutant follicle cells do not behave like stalk cells because stalk cells do not normally express high levels of Fas3. However, mutant *Axn* or *sgg* follicle cells that were integrated into late egg chambers did not express high levels of Fas3, similar to their neighboring wild-type follicle cells

(Fig. 6H-J). Only two pairs of polar cells located at both ends of an egg chamber continuously expressed high levels of Fas3 (Fig. 6I,J). Interestingly, in some cases a few mutant follicle cells adjacent to wild-type polar cells expressed slightly higher levels of Fas3 than wild-type follicle cells neighboring polar cells (Fig. 6I), suggesting that mutant *Axn* follicle cells on egg chambers also do not differentiate normally. All these results indicate that mutant *Axn* or *sgg* follicle cells have differentiation defects.

## DISCUSSION

Stem cells have been thought to be maintained by signals from niches in which they reside. Identifying these signals is critical for understanding how stem cell maintenance, differentiation and division are controlled. Here we report that *wg* signaling directly regulates SSC maintenance and follicle cell differentiation in the *Drosophila* ovary. The Wg protein is expressed in terminal filament and cap cells, a few cells away from SSCs. Elimination of the *wg* signaling cascade from SSCs results in rapid SSC loss; whereas, its constitutive signaling also destabilizes them, also causing their loss. Constitutive *wg* signaling in follicle progenitors causes over-proliferation and abnormal differentiation of follicle cells. This study demonstrates that *wg* signaling regulates SSC maintenance and constitutive *wg* signaling disrupts follicle cell differentiation in the *Drosophila* ovary.

Similarly, constitutively active β-catenin in mice and humans disrupts hair follicle differentiation, resulting in the formation of skin cancers (DasGupta and Fuchs, 1999; Huelsken et al., 2001). No Wnt-like molecules have been directly implicated in hair follicle stem cell regulation. Because several aspects of epithelial cell regulation have been conserved from *Drosophila* to humans (Jordan et al., 2000; Zhang and Kalderon, 2001), our study suggests that the ability of Wnt-like signaling to regulate maintenance of epithelial stem cells and the differentiation of their progeny may be conserved.

### *wg* signaling regulates the maintenance of somatic stem cells

Wg produced from terminal filament and cap cells may reach SSCs at a distance of a few cells by either diffusion or active transport, and then directly controls SSC maintenance. Furthermore, correct intermediate levels of *wg* signaling seem to be important for maintaining SSCs in the *Drosophila* ovary. We show that reduction of *wg* signaling in SSCs by removal of positive regulators such as *arm* and *dsh* causes rapid SSC loss, as does constitutive *wg* signaling in SSCs by removal of negative regulators such as *Axn* and *sgg*. *wg* signaling maintains SSCs through several possible mechanisms. First, *wg* signaling could be required for SSC self-renewal and/or survival. Second, it could maintain the association of SSCs with IGS cells. Finally, both mechanisms could work simultaneously. DE-cadherin-mediated cell adhesion has been shown to be important for keeping SSCs in their niche (Song and Xie, 2002), and also shares *arm* as a common component with *wg* signaling (Peifer and Polakis, 2000). *wg* signaling is known to regulate levels of *arm*, which are also important for DE-cadherin-mediated cell adhesion. Thus, it is possible that *wg* signaling regulates cell adhesion between SSCs and their niches. In addition, our *arm* mutant clonal analysis strongly argues that *wg* signaling must also directly regulate SSC self-renewal and/or survival. $arm^2$ mutant SSC clones are lost very quickly over time in comparison with wild-type SSC clones, and the $arm^2$ mutation primarily affects *wg* signaling but does not disrupt DE-cadherin-mediated cell adhesion. Therefore, *wg* signaling controls SSC maintenance through regulating SSC self-renewal/survival and/or cell adhesion between SSCs and their niche cells. The temperature-sensitive allele of *wg* gives very mild phenotypes in follicle cell production, however, removal of *wg* downstream components has a dramatic impact on SSC maintenance. In *Drosophila*, there are six other *wg*-related genes. This raises an interesting possibility that other *wg*-like molecules could also be involved in regulating SSC maintenance.

In addition to *wg* signaling, *hh* signaling is also essential for SSC maintenance and proliferation. Hyperactive *hh* signaling causes follicle cell over-proliferation and abnormal differentiation of follicle cells (Forbes et al., 1996a; Zhang and Kalderon, 2000). Disrupting *hh* signaling in SSCs by removing the function of *hh* downstream components such as *smoothen* and *Cubitus interruptus* results in rapid SSC loss (Zhang and Kalderon, 2001). Similarly, reduction or elimination of *wg* signaling also causes rapid SSC loss. Removal of *patched*, a negative regulator of the *hh* pathway, stabilizes SSCs (Zhang and Kalderon, 2001). However, SSCs mutant for negative regulators for the *wg* pathway, *sgg* and *Axn*, are destabilized. All the evidence indicates that *wg* and *hh* may use different mechanisms to regulate SSCs in the *Drosophila* ovary.

### Constitutive *wg* signaling causes over-proliferation and abnormal differentiation of follicle cells

Constitutive *wg* signaling increases the division rates of early follicle cell progenitors in the germarium. When *Fz2*, *dsh* and activated *arm* are over-expressed, extra follicle cells accumulate in the ovarioles, suggesting that hyper-activation of *wg* signaling causes over-proliferation of follicle cells. Furthermore, *sgg* or *Axn* mutations cause over-proliferation of follicle cells, resulting in the formation of extra follicle cells that accumulate outside egg chambers. These cells are not mitotically active and usually assume some stalk cell characteristics. These results suggest that production of extra follicle cells by excessive *wg* signaling is because of higher mitotic activities of progenitors and/or SSCs in the germarium. It is important to note that *sgg* mutations are more potent than *Axn* in stimulating the proliferation of follicle cell progenitors. The different potencies may be because of differences in how these mutations affect *wg* signaling. Alternatively, because *sgg* negatively regulates *hh* signaling (Jia et al., 2002), *sgg* could be involved in negatively regulating both *hh* and *wg* signaling in the ovary. It has been demonstrated that excessive *hh* signaling causes extra follicle cells to accumulate outside egg chambers (Forbes et al., 1996a; King et al., 2001; Zhang and Kalderon, 2001). Therefore, it might be probable that *sgg* is involved in regulating both *hh* and *wg* signaling pathways in follicle cells of the *Drosophila* ovary.

This study also demonstrates that constitutive *wg* signaling disrupts the normal differentiation of somatic follicle cells. Mutant *Axn* or *sgg* follicle cells in and outside the germarium express higher levels of Hts in their membranes and tend to accumulate between egg chambers. In ovarioles that contain a

majority of mutant follicle cells, germline cysts fail to undergo normal morphological changes necessary for proper encapsulation by follicle cells, although they are wild type, suggesting that the mutant follicle cells are defective in their interactions with germ cells. Although some of them are recruited to egg chambers, these mutant follicle cells have abnormal morphologies (e.g. smaller and irregular sizes). This phenotype may be because of abnormal levels of Hts, which may prevent follicle cells from shape changes and growth. The extra mutant follicle cells accumulating outside egg chambers express Lamin C and do not divide similar to stalk cells. However, unlike stalk cells, they express high levels of Fas3. Similar to the mutant follicle cells in the germarium, the mutant follicle cells that are recruited to egg chambers also express high levels of Hts. Unlike the follicle cells in the germarium, the cells fail to express high levels of Fas3. These results indicate that constitutive *wg* signaling in follicle cells disrupts proper follicle cell differentiation.

### Follicle cells in the *Drosophila* ovary could potentially be a great model to study epithelial stem cells and skin cancers

In mice and humans, excessive *hh* signaling disrupts the normal differentiation processes of hair follicles, resulting in the formation of skin cancers (Johnson et al., 1996; Oro et al., 1997). In the *Drosophila* ovary, excessive *hh* signaling disrupts the normal differentiation of somatic follicle cells and produces extra follicle cells (Forbes et al., 1996a; King et al., 2001; Zhang and Kalderon, 2000). *hh* also directly regulates the maintenance and number of SSCs in the *Drosophila* ovary (Zhang and Kalderon, 2001). In humans and mice, constitutively active β-catenin also disrupts hair follicle cell differentiation, causing skin cancer formation (Chan et al., 1999; Gat et al., 1998; Zhu and Watt, 1999). Here we show that *wg* signaling directly controls SSC maintenance, whereas constitutive *wg* signaling disrupts somatic follicle cell differentiation, resulting in extra follicle cell production and abnormal morphological changes. Notch signaling has been implicated in regulating somatic follicle cell differentiation in the *Drosophila* ovary and epithelial cells in the mouse skin (Jordan et al., 2000; Lowell et al., 2000). All of the evidence suggests that mechanisms regulating the maintenance of epithelial stem cells and differentiation of their progeny could be conserved from *Drosophila* to human. Therefore, the *Drosophila* ovarian follicle epithelial cells represent a model system in which to study epithelial stem cell regulation, and possibly skin cancer formation in humans by identifying major signaling pathways and their targets.

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