BMP Signaling Is Required for Controlling Somatic Stem Cell Self-Renewal in the *Drosophila* Ovary

Daniel Kirilly,1,2 Eric P. Spana,3,5 Norbert Perrimon,3 Richard W. Padgett,4 and Ting Xie1,2,*
1Stowers Institute for Medical Research
1000 East 50th Street
Kansas City, Missouri 64110
2Department of Anatomy and Cell Biology
University of Kansas Medical Center
3901 Rainbow Boulevard
Kansas City, Kansas 66160
3Howard Hughes Medical Institute
Department of Genetics
Harvard Medical School
77 Avenue Louis Pasteur
Boston, Massachusetts 02115
4Waksman Institute
Rutgers University
190 Frelinghuysen Road
Piscataway, New Jersey 08854-8020

Summary

BMP signaling is essential for promoting self-renewal of mouse embryonic stem cells and *Drosophila* germ-line stem cells and for repressing stem cell proliferation in the mouse intestine and skin. However, it remains unknown whether BMP signaling can promote self-renewal of adult somatic stem cells. In this study, we show that BMP signaling is necessary and sufficient for promoting self-renewal and proliferation of somatic stem cells (SSCs) in the *Drosophila* ovary. BMP signaling is required in SSCs to directly control their maintenance and division, but is dispensable for proliferation of their differentiated progeny. Furthermore, BMP signaling is required to control SSC self-renewal, but not survival. Moreover, constitutive BMP signaling prolongs the SSC lifespan. Therefore, our study clearly demonstrates that BMP signaling directly promotes SSC self-renewal and proliferation in the *Drosophila* ovary. Our work further suggests that BMP signaling could promote self-renewal of adult stem cells in other systems.

Introduction

Stem cells maintain adult tissue homeostasis by their ability to self-renew and continuously generate differentiated cells throughout life. This unique property makes stem cells an ideal medical reagent for treating many different degenerative diseases. They are thought to be regulated by extrinsic signals from their surrounding microenvironments or niches and intrinsic factors that respond to the signals (Lin, 2002; Spradling et al., 2001; Watt and Hogan, 2000). However, extrinsic signals and intrinsic factors that directly control stem cell function still remain poorly defined. The *Drosophila* ovary represents a powerful system for studying stem cells at the molecular and cellular level (Lin, 2002; Xie et al., 2005). Since the self-renewal property of stem cells is conserved from *Drosophila* to humans, some aspects of the molecular mechanisms controlling stem cell function may be conserved from *Drosophila* to humans.

The continuous production of egg chambers in the *Drosophila* female depends on two types of stem cells, germline stem cells (GSCs) and somatic stem cells (SSCs), which are responsible for producing differentiated germ cells and somatic follicle cells, respectively (Lin, 2002; Xie et al., 2005). These stem cells are located at the tip of the ovariole, also known as the germarium, which is a tubular structure in which stem cells and surrounding supporting niche cells can be easily identified (Figure 1A). At the very end of the germarium, 2–3 GSCs directly contact cap cells and are also close to terminal filament (TF) cells and inner gerarial sheath (IGS) cells. They divide and give rise to cystoblasts, which divide four times synchronously with incomplete cytokinesis to form 16-cell cysts. As germline cysts move to the middle of the germarium, they become surrounded by epithelial cell-like follicle cells and bud off from the germarium to form individual egg chambers separated by 5–7 stalk cells.

Follicle cells surrounding the egg chamber and stalk cells linking two adjacent egg chambers are produced by SSCs that reside in the halfway point of the germarium (Margolis and Spradling, 1995). Margolis and Spradling (1995) used FLP-mediated FRT mitotic recombination to positively label SSC lineages and identified two SSCs as the most anterior marked cells in the halfway point of the germarium that generate marked follicle cells in the posterior germarium and its subsequent egg chambers. Both SSCs divide once every 10 hr, on average, followed by three rounds of division of their progeny to generate 16 cells that initially cover each cyst. Just like GSCs, SSCs are also anchored to their neighboring supporting cells (posterior IGS cells) through DE-cadherin-mediated cell adhesion (Song and Xie, 2002). Such anchorage is important for maintaining SSC identity. As a SSC divides, one daughter that retains stem cell identity remains in its position, and the other daughter moves posteriorly to proliferate and then generate differentiated follicle cells and stalk cells.

Hedgehog (Hh) and Wingless (Wg) have been identified as two critical signals for SSC maintenance and proliferation. Hh is primarily expressed in TF cells and cap cells in the germarium, and it appears to function as a long-range signal for directly controlling SSC maintenance and proliferation (Forbes et al., 1996; King et al., 2001; Zhang and Kalderon, 2001). Overexpression of *hh* causes follicle cell overproliferation (Forbes et al., 2001;...
Figure 1. A New Positive Labeling System to Mark SSCs and Their Progeny

(A) A schematic diagram of an ovariole (top left) and a germarium (ovariolar tip; bottom right). Abbreviations: TF, terminal filament cells; CC, cap cell; GSC, germline stem cell; CB, cystoblast; IGS, inner germarial sheath cell; FS, fusome; SSC, somatic stem cell; FC, follicle cell. The germarium is divided into four regions: 1, 2a, 2b, and 3.

(B) A schematic diagram showing how to generate a functional actin5C-gal4 gene by using the FLP-mediated FRT recombination technique. A functional actin5C-gal4 gene is reconstituted by heat shock-induced FLP-mediated recombination between inactive but complimentary alleles, actin5C FRT and FRT gal4. The daughter cell that inherits the actin5C-gal4 gene expresses UAS-GFP or any other transgene constructs.

(C–E) An ovariole and germaria are labeled for Fas3 (red), GFP (green), and DNA (blue). (C) An ovariole containing a GFP-marked SSC clone in which only its marked descendants are shown in the germarium and egg chambers. (D) A GFP-marked SSC (arrow) and progeny in the germarium 1 week ACI. (E) A germarium showing a GFP-marked IGS cell (arrow). The images in (D) and (E) are shown at the same scale, and the bars in (C) and (D) represent 10 μm. All of the images in this and subsequent figures are shown as a single confocal section.

1996), whereas disruption of the hh signaling cascade in SSCs results in their loss (King et al., 2001; Zhang and Kalderon, 2001). Similarly, Wg protein is expressed in TFs and cap cells, and disruption of Wg signaling in SSCs abolishes SSC self-renewal (Song and Xie, 2003). Hyperactive wg signaling resulting from removal of negative regulators, such as Axin and shaggy (sgg), causes excessive follicle cell proliferation and abnormal differentiation of follicle cells, and intriguingly also destabilizes SSCs. Interestingly, in mammals, Wnt and Shh signaling has been implicated in the regulation of epithelial stem cell/precursor cell maintenance and proliferation in the intestine and airway (He et al., 2004; Korinek et al., 1998; Watkins et al., 2003). The findings from Drosophila and mammals suggest that some of the molecular mechanisms regulating epithelial stem cells are likely conserved.

BMP signaling pathways have diverse functions in the development of multicellular organisms (Hogan, 1996). Recently, BMP signaling has been shown to promote self-renewal of mouse embryonic stem cells and repress proliferation of skin and intestinal stem cells (Haramis et al., 2004; He et al., 2004; Kobielak et al., 2003; Qi et al., 2004; Ying et al., 2003). In the Drosophila ovary and testis, BMP signaling directly controls GSC self-renewal by repressing expression of a differentiation-promoting gene, bam (Chen and McKeerin, 2003; Kawase et al., 2004; Shivdasani and Ingham, 2003; Song et al., 2004). In the germarium, dpp and gbb are expressed in the somatic cells, including cap cells and inner sheath cells, but not in germ cells (Song et al., 2004). However, it remains unclear whether BMP signaling can promote self-renewal of adult stem cells other than GSCs. In this study, our genetic and cell biological
studies have shown that BMP signaling is required for promoting self-renewal of adult SSCs by preventing differentiation in the Drosophila ovary.

Results

Developing a Positively Marked Mosaic Lineage Labeling Technique for Lineage Tracing and Lineage-Specific Gene Overexpression

FLP-mediated FRT recombination has revolutionized studies on diverse developmental processes in Drosophila (Chou and Perrimon, 1996; Golic and Lindquist, 1989; Xu and Rubin, 1993). The mosaic clones marked by loss of armadillo (arm)-lacZ or ubiquitin (ubi)-GFP are routinely used to study Drosophila oogenesis (Xie and Spradling, 1999; Xu and Rubin, 1993). Two positive labeling methods, the tubulin-lacZ positive labeling system (Harrison and Perrimon, 1993) and the gal80-based mosaic analysis with a repressible cell marker (MARC) (Lee and Luo, 1999), have been developed to facilitate visualization of marked cells. The lacZ-positive labeling system is effective for identification of marked cells, but it is not ideal for manipulating gene function, while stable GAL80 protein may not allow rapid visualization of marked cells after one or two divisions due to its persistence. Here, we report a new, to our knowledge, positively marked mosaic lineage (PMML) method to positively mark cells and allow for rapid expression of the UAS-GFP marker and any other UAS construct in the marked cells by using a combination of the GAL4-UAS and FLP-FRT systems. This PMML system uses the heat shock-inducible FLP to reconstitute a functional actin5C-gal4 gene from two complementary inactive alleles, actin5C FRT52B and FRT52B gal4 (see the Suplemental Data available with this article online for details on generating these lines). The actin5C-gal4 gene drives GFP expression to mark cells and can also activate or knock down gene function by using UAS constructs in the marked cells (Figure 1B).

To test whether PMML is also suitable for marking SSCs and assisting in SSC identification in the Drosophila ovary, we immunostained ovaries with anti-GFP and anti-Fasciclin III (Fas3) antibodies 1 week after clone induction (ACI). Fas3 is expressed in SSCs at low levels and in differentiated follicle cell progenitor cells at higher levels (Zhang and Kalderon, 2001). Since the PMML system works similarly to the one described in Margolis and Spradling (1995) in terms of positively marking mitotic cells, we applied similar criteria to those used by Margolis and Spradling (1995) to identify positively marked SSCs in this study. It takes about 4–5 days for transiently labeled GFP-positive follicle cells to completely exit the germarium (Margolis and Spradling, 1995). One week ACI, a typical GFP-positive SSC clone was easily observed with the GFP-marked follicle cells present in regions 2b and 3 of the germarium and in egg chambers (Figure 1C). The marked SSC could be identified by its location (the GFP-positive somatic cell at the 2a/2b junction), low Fas3 expression, and the presence of GFP-marked follicle cells in the germarium and/or in the egg chambers (Figure 1D). The GFP-marked IGS cells could also be readily identified by their location (the germarial regions 1 and 2a), the absence of marked differentiated follicle cells in the same ovarioles, and also the absence of Fas3 expression, since the IGS descendants do not pass beyond the 2a/2b junction (Figure 1E). Therefore, this system can be applied effectively for labeling SSCs and their progeny and for further studying the function of any gene in the marked SSCs and the germarium.

SSCs in the Germarium Are Capable of Responding to BMP Signaling

In Drosophila, Dpp and Gbb likely bind to receptor complexes composed of type II receptor, Put, and one or two of the type I receptors, Tkv and Sax, resulting in phosphorylation of Mad, which is then associated with Med and translocated into the nucleus (Raftery and Sutherland, 1999). The Mad and Med protein complexes in the nucleus control their target gene expression, including Daughters against dpp (Dad). To determine whether SSCs are capable of responding to BMPs, we examined the expression of Dad in GFP-marked SSCs by using PMML and a Dad-lacZ enhancer trap line. The Dad-lacZ line can recapitulate the endogenous expression of the Dad gene in several different tissues, including the ovary (Kai and Spradling, 2003; Song et al., 2004; Tsuneizumi et al., 1997). Surprisingly and interestingly, Dad was found to be expressed in only 5% of the marked SSCs (Figures 2A and 2B, arrow; n = 86), while the rest of the marked SSCs did not express detectable Dad (Figures 2C and 2D). Dad was strongly expressed in anterior IGS cells close to cap cells, but it was weakly expressed or not expressed at all in other IGS cells (Figures 2A and 2B). This observation that Dad-lacZ is only expressed in a small fraction of SSCs could reflect periodic BMP signaling activity in SSCs or the nature of the enhancer trap line.

To further test whether all SSCs are indeed capable of responding to BMP signaling, we examined Dad expression in the GFP-marked SSCs that overexpressed an activated tkv receptor (tkv*) under the control of the actin5C promoter by using PMML. Expression of tkv* can cause ligand-independent BMP pathway activation (Neil and Ferguson, 1998; Nguyen et al., 1998). Four days ACI, all marked SSCs and follicle cells expressing the tkv* also expressed very high levels of lacZ (Figures 2E and 2F; n = 38). Normally, follicle cells do not express Dad-lacZ (Figures 2A and 2B). Taken together, these results indicate that SSCs express all essential BMP downstream components for responding to BMPs.

gbb Regulates SSC Maintenance and SSC/Follicle Cell Progenitor Proliferation in the Agametic Ovary

Once the germ cells and IGS cells completely disappear from the germarium following complete GSC loss, SSCs/follicle progenitor cells occupy the vacated GSC niche and still respond to Dpp from the GSC niche, as determined by the expression of Dad-lacZ (Kai and Spradling, 2003). To determine whether mutations in dpp and gbb affect Dad-lacZ expression in ectopic SSCs in the GSC niche, we generated empty GSC
Developmental Cell
654

Figure 2. SSCs in the Ovary Respond to BMP Signaling
(A–F) Germaria in (A)–(F) are labeled for GFP (green), LacZ (red), and DNA (blue). (A and B) A GFP-marked wild-type SSC (arrow) and marked IGS cells (arrowhead) that express Dad-lacZ (red). (C and D) A GFP-marked wild-type (arrow) that does not express Dad-lacZ. (E and F) A GFP-marked SSC (arrow) and its progeny that expresses tkv* show Dad-lacZ expression at high levels. All of the images are shown at the same scale, and the bar in (A) represents 10 μm.

Figure 3. Gbb Signaling Is Required for Proliferation of SSCs/Follicle Cell Progenitors in the GSC Niche
(A–G') (A–D) Agametic germaria are labeled with Hts (green), LacZ (red), and DNA (blue). (A) A wild-type agametic germarium showing that Dad-lacZ is highly expressed in a SSC (arrowhead) that is relocated to the GSC niche 10 days after GSCs are induced to differentiate. (B) A dppl56/dppr4 mutant germarium showing no or extremely low Dad-lacZ expression in the SSCs located in the GSC niche. (C and D) (C) gbb1/gbbD4 and (D) gbb1/gbbD20 germaria do not express Dad-lacZ in the SSCs located in the GSC niche. (E and F') 10-day-old wild-type control agametic germaria. (F and F') 10-day-old dppl56/dppr40 agametic germaria showing slightly reduced sizes. (G and G') 10-day-old gbb1/gbbD4 agametic germaria showing severely reduced sizes. For the germaria in (E)–(G'), their GSCs were ablated by bam overexpression during the early pupal stage. The images in (A)–(D), (E)–(G), and (E')–(G') are shown at the same scale, while the bars in (A), (E), and (E') represent 10 μm, 20 μm, and 10 μm, respectively.

niches in the germarium by forced bam expression by using a hs (a heat shock protein 70 promoter)-bam transgene. Forced bam expression in GSCs causes them to differentiate and exit the germarium (Ohlstein and McKearin, 1997). As expected, no GSCs and their differentiated progeny remained in the germaria 10 days after two 2 hr heat shock treatments. In 53% of the wild-type agametic germaria, most of the anterior cells (presumably SSCs) in direct contact with cap cells highly upregulated Dad-lacZ expression (Figure 3A; n = 51), suggesting that the SSCs in the GSC niche can still respond to BMP. As mentioned earlier, only about 5% of SSCs in their normal niche also appear to respond to BMP signaling at a given time. This observation also suggests that the SSC niche provides low, possibly oscillating, expression of the BMP signal, which results in the activation of BMP signaling in some, but not all, SSCs.

For determining which BMP is important for Dad-lacZ response in SSCs, dppl56/dppr4, gbb1/gbbD4, and gbb1/gbbD20 temperature-sensitive dpp and gbb mutant females were generated at the permissive temperature (18°C) and were then shifted to the restrictive temperature (29°C) for 10–12 days. Our previous studies have shown that mutations in dpp and gbb cause premature GSC loss (Song et al., 2004; Xie and Spradling, 1998).

After the dpp or gbb mutant germaria lost all of their germ cells, including GSCs, the putative SSCs in contact with cap cells were examined for Dad-lacZ expression. In the dpp mutant agametic germaria, Dad-lacZ expression was dramatically reduced in all of the putative SSCs (Figure 3B; n = 30). Similarly, no obvious Dad-lacZ expression in the putative SSCs was detected in the gbb mutant agametic germaria (Figures 3C and 3D; n = 32). One of the caveats in these experiments is that no Dad-lacZ expression could be due to complete loss of SSCs in the dpp or gbb mutant germaria. In any case, our results suggest that gbb and dpp could be involved in either mediating BMP signaling in SSCs or maintaining SSCs in the agametic ovary.
After SSCs are relocated to the GSC niche, they continue to proliferate and form a bag of follicle cells (Kai and Spradling, 2003). The size of an agamic germarium reflects the proliferation of its SSCs and their progeny. To effectively compare the sizes of agamic germaria between wild-type and dpp mutant genotypes, we synchronized GSC loss by inducing GSC differentiation by using the hs-bam transgene. The wild-type and the dpphr56/dpphr4, dpphr56/dpphr4, gbb4/gbbD4, and gbb4/gbbD4 mutant late third-instar larvae carrying the hs-bam transgene received four 2 hr heat shock treatments, and the emerged adult wild-type, mutant dpp, or mutant gbb females were cultured at a restrictive temperature (29°C) for an additional 10 days, since dpp and gbb mutants are temperature sensitive. In the control ovaries, the majority of germaria contained many follicle cells (Figures 3E and 3F; n = 130). Since a stronger allelic combination, dpphr56/dpphr4, failed to reach adulthood after heat shock treatments, we only examined a weaker heteroallelic combination, dpphr56/dpphr4, and Spradling, 2003), which is similar to our results with the gbb mutants. These dpp mutant agamic germaria contained slightly less follicle cells than the control agamic germaria (Figures 3F and 3F; n = 156). It has been reported that dpphr56/dpphr4 mutant ovaries do not show dramatic SSC proliferation defects after they are shifted to a restrictive temperature (Kai and Spradling, 2003), which is similar to our results with the dpp allelic combination. In both gbb mutant combinations, follicle cell proliferation and/or survival were greatly reduced (Figures 3G and 3G). From 12 gbb4/gbbD4 mutant ovaries, we only obtained 64 recognizable germaria, indicating that most of the germaria have degenerated, since we expected a total of 144–192 germaria (12–16 ovarioles/ovary). Among them, only 26 germaria contained only a few follicle cells, while the rest had no follicle cells but did have terminal filament cells (Figure 3G). In gbb4/gbbD4 mutant agamic germaria, all of the germaria contained no follicle cells but contained terminal filament cells (data not shown). Since gbb mutant germaria contain a few or no follicle cells, SSC self-renewal and/or proliferation must be compromised in gbb mutants. These results indicate that gbb is required for maintaining SSCs in the ectopic GSC niche. However, we could not rule out the possibility that dpp is also required for maintaining SSCs in the ectopic niche since we are not able to test strong dpp alleles.

The SSCs that Are Defective in BMP Signal Transduction Have a Shorter Lifespan in the Adult Ovary

The experiments described above demonstrate that BMP signaling mediated by gbb and perhaps dpp is required for promoting proliferation of SSCs and follicle cells in the ectopic niche. We then sought to investigate whether BMP signaling functions to control SSC maintenance and proliferation in the native niche. To disrupt BMP signaling in SSCs, we generated marked SSCs mutant for BMP receptors (punt, tkv, and sax) and intracellular signaling transducers (mad and Med) by using the FLP-mediated FRT mitotic recombination technique (Golic and Lindquist, 1989; Xu and Rubin, 1993). Marked wild-type and mutant SSC clones were generated by subjecting females of the appropriate genotype to heat shock treatments and identified by loss of arm-lacZ expression, and the percentages of germaria carrying one or more marked SSC clones mutant for a given gene were determined 1, 2, and 3 weeks ACI. The marked SSC clones were identified according to the morphological criteria that they reside in the middle of the germarium and generate marked differentiated follicle cells in regions 2b and 3 of the germarium (Margolis and Spradling, 1995; Song and Xie, 2002; Zhang and Kalderon, 2001). The changes in the percentages of the germaria carrying one or more marked SSCs with time can then be used to deduce whether a given gene is important for maintaining SSCs.

The majority of wild-type clones (72%) were maintained in the germaria 3 weeks ACI, indicating that there is a slow, spontaneous SSC turnover (Figures 4A and 4B; Group A of Table 1). This has been previously observed in several independent studies (Margolis and Spradling, 1995; Song and Xie, 2002; Zhang and Kalderon, 2001). Interestingly, SSCs mutant for punt, tkv, mad, and Med were lost much faster than the wild-type SSC clones (Figures 4C and 4D; Group A of Table 1). For example, only 19.9% of the marked SSCs mutant for punt135, the BMP type II receptor, remained in the germaria 3 weeks ACI, while 23.8% of the marked SSCs mutant for mad12, a Drosophila homolog of SMAD1, 5, 8, still persisted. Surprisingly, about 60% of the SSCs mutant for sax+, a null allele for the BMP type I receptor sax, were maintained 3 weeks ACI, while only 24% of the SSCs mutant for tkv8, a strong allele for another BMP type I receptor, tkv, remained in the germaria 3 weeks ACI. Though previous studies have suggested that the Gbb signal is primarily transduced through Sax, our results strongly support a different model: that the Gbb signal in SSCs is primarily transduced through Tkv. Together, these results demonstrate that BMP signaling is required for maintaining SSCs.

Unexpectedly, both Med26 and MedAF33 mutant SSC clones were lost much faster than the control wild-type SSCs and the SSCs mutant for the other BMP downstream components. Only 3.9% of the Med26 mutant SSC clones and none of the MedAF33 mutant SSC clones were maintained 3 weeks ACI (Group A of Table 1). Since Med26, MedAF33, tkv8, and mad12 are strong or null alleles, one of the likely explanations is that Med participates not only in BMP signaling, but also in another signaling pathway for maintaining SSCs. Med encodes a co-SMAD, SMAD4, which is known to be involved in all TGFr-like signaling pathways in mammals. This observation suggests that a TGFr-like signal other than BMP is also involved in regulating SSC maintenance.

BMP Signaling-Defective SSCs Are Likely Lost Due to Differentiation, but Not Apoptosis

The observation that SSCs that are defective in BMP signaling are lost much faster than wild-type ones prompted us to investigate whether the premature SSC loss is due to differentiation or apoptosis, p35, a baculovirus antiapoptotic gene, has been shown to suppress spontaneous or environmental insults-induced apoptosis in Drosophila when it is overexpressed (Hay...
Figure 4. BMP Signaling Is Required for Controlling SSC Self-Renewal

(A–N) Germlia in (A)–(D) are labeled for Hts (green), LacZ (red), and DNA (blue), whereas germlia in (E)–(N) are labeled for GFP (green), Fas3 (red), and DNA (blue). Putative (A–C) LacZ-negative or (E–H, I, and K) GFP-positive SSCs are indicated by arrowheads. (A and B) Germarium showing a (A) 1-week-old or (B) 3-week-old wild-type SSC clone in which the SSC and its early progeny are highlighted by dashed lines. (C) A germarium showing a 1-week-old tkv* mutant SSC clone in which the SSC and its early progeny are highlighted by dashed lines. (D) A germarium showing a lost tkv* mutant SSC clone 3 weeks ACI. The lost SSC is still evident by the presence of a patch of marked follicle cells (highlighted by dashed lines) in an egg chamber (insert) from the same ovariole. (E and F) Germlia carrying (E) 1-week-old and (F) 3-week-old GFP-marked wild-type SSC clones. (G and H) Germlia carrying (G) 1-week-old and (H) 3-week-old GFP-marked wild-type SSC clones that also overexpress p35. (I) A germarium carrying a GFP-marked 1-week-old punt135 mutant SSC clone. (J) A germarium showing loss of a marked punct135 SSC clone evident by the presence of a patch of GFP-positive follicle cells in a late egg chamber (insert) from the same ovariole 3 weeks ACI. (K) A germarium carrying a GFP-marked 1-week-old punt135 mutant SSC clone that also overexpresses p35. (L) A germarium showing loss of a marked punt135 SSC clone that also overexpresses p35, which is evident by the presence of a patch of GFP-positive follicle cells in a late-stage egg chamber (insert) 3 weeks ACI. (M) A germarium carrying a 1-week-old GFP-positive SSC clone that also overexpresses tkv*. (N) An

Hyperactive BMP Signaling Prolongs SSC Lifespan

So far, we have shown that BMP signaling is required for controlling SSC self-renewal. Our previous report that Dpp signaling is not only necessary, but also sufficient, to control GSC self-renewal in the Drosophila ovary prompted us to investigate whether BMP signal-

ovariole tip carrying a 1-week-old full SSC clone that also overexpresses tkv*. All of the SSCs in the gerarium are marked by GFP, which is probably due to the replacement of lost unmarked SSC(s) by GFP-marked follicle progenitor cells. All of the images are shown at the same scale, and the bar in (A) represents 10 μm.
ing is sufficient for promoting SSC self-renewal (Xie and Spradling, 1998). Given the evidence supporting the idea that tkv is likely a major type I receptor for BMP signaling in SSCs, we focused on investigating the effect of tkv* expression on SSC self-renewal. To further investigate whether expression of tkv* can promote SSC self-renewal and thus prolong the stem cell lifespan, we measured the maintenance of SSC clones expressing tkv* in comparison with that of the marked wild-type clones. In this experiment, a mild heat shock regimen was used to generate SSC clones so that all of the marked germaria should carry only one marked SSC clone (like ones in Figure 4M) decreased with time, while the number of germaria carrying only marked SSCs (full SSC clones, like ones in Figure 4N; the marked SSCs replaced the lost unmarked SSCs) increased with time. If the marked tkv*-expressing full clones, respectively, while 3.5% (-expressing SSC progeny are likely to be recruited to the empty niches and become SSCs. All of the results from the tkv* overexpression experiments support the model that BMP signaling promotes SSC self-renewal and

<table>
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<th>Genotypes</th>
<th>1 Week ACI</th>
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<td>16.3% (306)</td>
<td>26.9%</td>
</tr>
<tr>
<td>Med26</td>
<td>52.2% (431)</td>
<td>100%</td>
<td>18.8% (240)</td>
<td>36.0%</td>
<td>3.3% (269)</td>
<td>6.3%</td>
</tr>
<tr>
<td>MedAF3;UAS-p35</td>
<td>47.6% (275)</td>
<td>100%</td>
<td>23.8% (378)</td>
<td>50.0%</td>
<td>10.7% (412)</td>
<td>22.5%</td>
</tr>
<tr>
<td><strong>Group C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UAS-GFP (control)</td>
<td>37.4% (329)</td>
<td>100%</td>
<td>20.4% (460)</td>
<td>54.5%</td>
<td>15.2% (488)</td>
<td>40.6%</td>
</tr>
<tr>
<td>UAS-tkv*</td>
<td>26.2% (430)</td>
<td>100%</td>
<td>22.6% (354)</td>
<td>86.3%</td>
<td>17.2% (326)</td>
<td>65.6%</td>
</tr>
</tbody>
</table>

*The marked SSC clones in Groups A, B, and C are produced by using different genetic techniques and different heat shock induction protocols: A, standard FLP/FRT and strong heat shock induction; B, MARCM and strong heat shock induction; and C, PMML and moderate heat shock induction.

*bThe percentage of germaria bearing a marked SSC(s) at a given time point equals the number of germaria bearing a marked SSC(s)/total germaria examined.

*cThe number of total germaria examined for a given genotype at a given time point is shown in parentheses.

*dThe normalized percentage of germaria bearing a marked SSC at a given time, since different FRT chromosomes produced different percentages of germaria bearing a marked SSC(s) 1 week ACI, which are normalized to 100%. The percentages for the following time points are calculated by the actual percentages divided by the percentages at the first week ACI for each genotype.
proliferation, which is consistent with the results from our mutant clonal analyses.

**BMP Signaling Is Required for SSC Division, but Not for Follicle Cell Proliferation, in the Drosophila Ovary**

As one SSC daughter moves posteriorly to continue its proliferation and differentiation, all of its progeny will stay together as a patch on the surface of the egg chambers. The number of marked mutant follicle patches on egg chambers in comparison with that of marked wild-type control patches can be used to estimate the effect of a particular mutation on SSC division, while the size of marked mutant follicle cell patches in comparison with that of the marked wild-type controls can be used to delineate the effect of a particular mutation on follicle cell proliferation. To facilitate our data collection and analysis, we only counted the marked follicle cell patches on the first five egg chambers of the ovarioles. A marked wild-type SSC produced 3.7 ± 0.95 patches (n = 30), while punt<sup>135</sup>, tkv<sup>3</sup>, mad<sup>12</sup>, and Med<sup>26</sup> mutant SSCs generated 3.2 ± 1.20 (n = 32; p < 0.045), 3.1 ± 0.83 (n = 30; p < 0.034), 2.9 ± 1.35 (n = 36; p < 0.0025), and 2.1 ± 1.22 (n = 37; p < 0.0001) patches, respectively, indicating that SSCs defective in BMP signaling divide significantly slower than wild-type ones. Notably, a SSC mutant for Med produced significantly fewer patches than the punt, tkv, and mad mutant SSCs. Along with the fact that mad<sup>12</sup>, tkv<sup>3</sup>, and Med<sup>26</sup> carry strong or null mutations, this result suggests that Med might be involved in another BMP-independent pathway to regulate SSC division.

To further determine whether BMP signaling controls follicle cell proliferation, we used FLP-mediated FRT recombination to generate twin-spot clones in which the wild-type one is marked by two copies of the arm-lacZ construct and the mutant one is marked by loss of arm-lacZ expression. The cells carrying two copies of construct can be easily distinguished from the cells carrying one copy (Figures 5A–5C). Since twin-spot clones are derived from one follicle cell progenitor (a differentiated SSC progeny), the numbers of follicle cells in the wild-type clone and its twin mutant clone can be reliably quantified; thus, their relative division rate (rdr) can be calculated by the number of lacZ<sup>+</sup> follicle cells divided by the number of 2xLacZ<sup>+</sup> follicle cells. As expected, the marked wild-type follicle cells had an rdr of 0.94 (n = 17). The marked tkv<sup>3</sup> mutant follicle cells had an rdr of 0.96 (n = 11), and the marked tkv twin clones had similar sizes, supporting the idea that BMP signaling is not required for controlling follicle cell proliferation (Figure 5A). In contrast, the division rates of Med<sup>26</sup> and Med<sup>AF33</sup> mutant follicle cells were 0.52 (n = 22) and 0.68 (n = 10), respectively. The Med mutant clones were much smaller than their corresponding twin-spot wild-type clones, and the cell size in the Med mutant clones was smaller than that of wild-type ones (Figures 5B and 5C), indicating that Med is required for controlling follicle cell proliferation and size. To further determine whether Med is involved in the regulation of the mitotic cycle of follicle cells, we used 5-bromo-2-deoxyuridine (BrdU) incorporation to label S phase cells to investigate mitotic activities of wild-type and punt and Med mutant follicle cell clones. A total of 10.5% of the marked wild-type follicle cells (n = 1096) and 10.0% of the marked punt<sup>135</sup> mutant follicle cells (n = 1147) were BrdU positive, further supporting the idea that BMP signaling is not required for follicle cell proliferation. In contrast, 7.8% of the marked Med<sup>26</sup> mutant follicle cells were BrdU positive (n = 1273), indicating that Med is required for follicle cell proliferation. This result demonstrates that BMP signaling is not required for controlling follicle cell proliferation, and that another undefined TGF-β-like signaling pathway(s) mediated by Med is involved in the regulation of follicle cell proliferation and size.

**Hyperactive BMP Signaling Can Partially Rescue SSC Loss Caused by Defective Wg Signaling, but Not by Defective Hh Signaling**

Since Hh and Wg signaling pathways have been shown to control SSC self-renewal, we then investigated whether hyperactive BMP signaling can bypass requirements of Hh or Wg signaling in SSC regulation. We used the MARCM system to generate GFP-positive marked smoothened (smo) or disheveled (dsh) mutant SSCs as well as smo or dsh mutant SSC clones that also express tkv*. smo encodes an essential receptor for Hh signaling (van den Heuvel and Ingham, 1996), while dsh encodes an essential downstream component for Wg signaling in Drosophila (Klingensmith et al., 1994). In this experiment, two strong smo alleles, smo<sup>3</sup> and smo<sup>156</sup>, and one strong dsh allele, dsh<sup>2</sup>, were used to block Hh and Wg signaling in SSCs, respectively. As reported previously, SSCs mutant for smo (King et al., 2001; Zhang and Kalderon, 2001) and dsh (Song and...
Xie, 2003) are lost rapidly. Consistently, GFP-positive marked SSC clones mutant for smo, smoD16, and dsh2 were lost quickly in comparison with marked wild-type SSCs (Table 2). The marked smo and smoD16 mutant SSC clones that expressed tkv∗ showed no dramatic improvement in SSC maintenance in comparison with the marked smo mutant SSC clones that did not express tkv∗, indicating that hyperactive BMP signaling cannot bypass the requirement of Hh signaling in maintaining SSCs (Table 2). Interestingly, the marked dsh2 mutant SSC clones that expressed tkv∗ showed dramatic improvement in SSC maintenance in comparison with the marked dsh mutant SSC clones that did not express tkv∗, indicating that hyperactive BMP signaling can, at least partially, substitute for Wg signaling in SSC self-renewal. Together, these results suggest that the BMP pathway works as one of downstream branches of or in parallel with the Wg pathway in the control of SSC self-renewal.

### Discussion

In this study, we show that SSCs in the adult Drosophila ovary are capable of responding to BMP signaling. Our genetic mosaic analyses demonstrate that known BMP downstream components are also required for SSC self-renewal, but not survival. Hyperactive BMP signaling enhances SSC self-renewal capacity. Gbb is essential for controlling SSC maintenance, at least in the GSC niche. Furthermore, BMP signaling appears to be specific to stem cells, since follicle cells mutant for BMP-specific downstream components proliferate and differentiate normally. In addition to participation in BMP signaling, Med is likely involved in other TGF-β-like pathway(s) to control proliferation and size of differentiated follicle cells. The results from this study lead us to propose a working model that Gbb perhaps as well as Dpp from neighboring somatic cells function as stem cell growth factors in vivo for promoting self-renewal of ovarian SSCs.

### BMP Signaling Directly Controls SSC Self-Renewal and Division

gbb and dpp are expressed in cap cells, IGS cells, and follicle cells (Song et al., 2004; Xie and Spradling, 2000). SSCs are located in the middle of the germarium and are likely exposed to both BMPs, since both Dpp and Gbb are diffusible molecules. gbb mutants exhibit severe SSC/follicle cell proliferation defects and SSC loss. Furthermore, SSCs mutant for BMP downstream components such as tkv, punt, and mad are lost faster and divide slower than wild-type ones. Although dpp mutants show much weaker mutant defects, it is still possible that it plays an important role as does gbb, since only weak dpp mutations could be used for studying the regulation of adult SSCs due to its stringent requirements during early development. Therefore, these findings support the idea that Gbb, perhaps together with Dpp, controls SSC self-renewal and division. Studies on GSCs in the Drosophila ovary have shown that BMPs control GSC self-renewal by directly repressing transcription of differentiation-promoting genes such as bam (Chen and McKearin, 2003; Song et al., 2004). Possibly, BMP signaling also represses differentiation-promoting genes and thereby maintains SSC self-renewal. Meanwhile, BMP signaling could also positively regulate other genes that are important for maintaining the undifferentiated state of SSCs. This study also shows that BMP signaling promotes SSC self-renewal and division, it is critical to identify the BMP target genes in SSCs, which are either repressed or activated by BMP signaling.

This study also shows that tkv is a major type I BMP receptor for controlling SSC self-renewal in the Drosophila ovary. The SSCs mutant for sax, a null allele of sax (Twombly et al., 1996), behave close to normal wild-type ones, while the SSCs mutant for a strong tkv allele, tkv8, are lost rapidly, indicating that tkv is a major functional receptor to control SSC self-renewal. Given the evidence that gbb signaling is essential for maintaining SSCs, our study strongly supports the idea that Gbb signals mainly through Tkv to control SSC self-renewal in the Drosophila ovary. Our recent study on Drosophila spermatogenesis also suggests that Gbb signaling primarily functions through Tkv, but not Sax (Kawase et al., 2004). In the Drosophila testis, gbb and tkv are both

### Table 2. Hyperactive BMP Signaling Can Ameliorate SSC Loss Caused by Defective Wg Signaling, but Not Defective Hh Signaling

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>1 Week ACI</th>
<th>Relative Percentage to 1 Week ACI</th>
<th>2 Weeks ACI</th>
<th>Relative Percentage to 1 Week ACI</th>
<th>3 Weeks ACI</th>
<th>Relative Percentage to 1 Week ACI</th>
</tr>
</thead>
<tbody>
<tr>
<td>smoD16</td>
<td>16.9%a (243)b</td>
<td>100%c</td>
<td>2.8% (288)</td>
<td>16.5%</td>
<td>0.0% (312)</td>
<td>0.0%</td>
</tr>
<tr>
<td>smoD16; UAS-tkv*</td>
<td>30.2% (222)</td>
<td>100%</td>
<td>2.2% (187)</td>
<td>7.3%</td>
<td>2.0% (245)</td>
<td>6.6%</td>
</tr>
<tr>
<td>smo2</td>
<td>26.9% (249)</td>
<td>100%</td>
<td>3.2% (310)</td>
<td>11.9%</td>
<td>0.9% (221)</td>
<td>3.3%</td>
</tr>
<tr>
<td>smo2; UAS-tkv*</td>
<td>23.8% (223)</td>
<td>100%</td>
<td>9.7% (195)</td>
<td>40.8%</td>
<td>3.9% (255)</td>
<td>16.4%</td>
</tr>
<tr>
<td>dsh2</td>
<td>32.9% (219)</td>
<td>100%</td>
<td>5.6% (360)</td>
<td>17.6%</td>
<td>3.8% (314)</td>
<td>11.5%</td>
</tr>
<tr>
<td>dsh2; UAS-tkv*</td>
<td>28.5% (249)</td>
<td>100%</td>
<td>21.9% (215)</td>
<td>76.8%</td>
<td>11.5% (191)</td>
<td>40.3%</td>
</tr>
</tbody>
</table>

a The percentage of germaria carrying a marked SSC(s) at a given time point equals the number of germaria carrying a marked SSC(s)/total germaria examined.

b The number of total germaria examined for a given genotype at a given time point is shown in parentheses.

c The normalized percentage of germaria carrying a marked SSC at a given time point is calculated by the actual percentages divided by the percentages at the first week for each genotype.
essential for maintaining SSCs, but sax is not. Although one study on dominant-negative tkv and sax receptors suggests that dpp and gbb signal preferentially through tkv and sax, respectively (Haery et al., 1998; Khalsa et al., 1998), a recent study has shown that both dpp and gbb use tkv, but not sax, to control the process of vein promotion during pupal development and disc proliferation and vein specification during larval development (Ray and Wharton, 2001). Taken together, the results from this study and the previous studies indicate that Gbb can use Tkv as a major receptor for its signal transduction in Drosophila.

Med Regulates Proliferation and Growth of Follicle Cells, Possibly through Participating in BMP-Independent Pathway(s) in the Drosophila Ovary

Although Gbb/BMP signaling plays a critical role in controlling SSC self-renewal and division, it appears that it is dispensable for SSC survival, follicle cell proliferation, and cell size control. For example, p35 expression could not rescue the mutant punt SSC loss; the follicle cell clones mutant for strong tkv and mad alleles, tkv and mad, proliferate normally, and the sizes of the mutant follicle cell clones are quite normal. In contrast, p35 expression can rescue the Med transgenic SSC loss to the levels of the mutant punt, tkv, and mad mutant SSC losses. The partial rescue indicates that Med is required for SSC survival in a BMP-independent pathway. The Med mutant follicle cell clones proliferate slower than wild-type, and the size of follicle cell is also smaller than that of wild-type, suggesting that Med is required for follicle cell proliferation and growth. Since BMP signaling is not involved in the control of SSC survival, follicle cell proliferation, and growth, our findings further suggest that Med must participate in other TGF-β-like pathways controlling these processes. In mammalian systems, SMAD4 has been shown to be a common SMAD for all TGF-β-like signaling pathways including TGF-β, Activin, and BMP (Shi and Massague, 2003). A likely candidate TGF-β-like signaling pathway includes Activin and TGF-β. Activin and TGF-β molecules exist in Drosophila (Raftery and Sutherland, 1999). Activin-like signaling has been shown to be involved in regulating growth control and neuronal remodeling (Brummel et al., 1999; Raftery and Sutherland, 1999). However, the role of TGF-β signaling in Drosophila remains a mystery. We could not completely rule out, however, that Med is involved in other signaling pathways unrelated to TGF-β-like pathways to control SSC survival, follicle cell proliferation, and growth. In the future, it is very important to figure out which pathway Med takes part in for controlling SSC survival, follicle cell proliferation, and growth control.

BMP, Hedgehog, and Wnt Signaling Pathways Work Together to Control Stem Cell Behavior from Drosophila to Mammals

In a variety of systems, stem cells have been proposed to be regulated by signals from niches. SSCs are anchored to the posterior group of IGS cells through DE-cadherin-mediated cell adhesion (Song and Xie, 2002). Elimination of the anchorage leads to rapid SSC loss, suggesting that the posterior IGS cells function as a SSC niche (Song and Xie, 2002). In this study, we show that gbb is expressed in the somatic cells, including IGS cells and follicle cells, and plays an important role in maintaining SSCs. Hh and Wg are expressed in the cap cells and play essential roles in controlling SSC self-renewal, suggesting that the SSC niche is composed of IGS cells and cap cells. In Drosophila imaginal development, these three pathways often regulate one another to control patterning, cell proliferation, and differentiation (Chen and Baker, 1997; Jiang and Struhl, 1996). In the Drosophila ovary, disruption of Hh, Wg, and BMP signaling cascades causes rapid SSC loss, while hyperactive signaling results in abnormal proliferation and differentiation of SSC progeny (Forbes et al., 1996; King et al., 2001; Song and Xie, 2003; Zhang and Kalderon, 2001; this study). Interestingly, their downstream transcriptional factors are also required for controlling SSC maintenance, suggesting that integration of these pathways likely takes place at or after transcription of their target genes. In this study, we also show that hyperactive BMP signaling can substitute for Wg signaling, but not Hh signaling, in controlling SSC self-renewal. However, it still remains unclear how hyperactive BMP signaling bypasses Wg signaling in SSCs. An important task in the future is to define their target genes in SSCs and to further figure out how these three signal transduction pathways interact with each other to control expression of these target genes.

In mammals, Shh, Wnt, and BMP pathways have been shown to regulate stem cell behavior directly or indirectly. BMP signaling directly represses activities of stem cells in the intestine and the hair follicle and promotes self-renewal of ES cells and spermatogonial stem cells (Haramis et al., 2004; He et al., 2004; Kobiljak et al., 2003; Qi et al., 2004; Ying et al., 2003; Zhao et al., 1998). BMP signaling can also indirectly regulate hematopoietic stem cells (HSCs) by controlling niche size (Calvi et al., 2003; Zhang et al., 2003). Wnt signaling has been shown to control self-renewal of HSCs, ES cells, intestinal stem cells, and possibly hair follicle stem cells (Alonso and Fuchs, 2003; He et al., 2004; Korinek et al., 1998; Rey et al., 2003; Sato et al., 2004). Shh signaling is required for proliferation of stem cells/progenitor cells in the lung airway (Watkins et al., 2003). Studies from Drosophila and mice have shown that different stem cell types may utilize a combination of different growth factors to control their self-renewal, proliferation, and differentiation. Interestingly, Wnt and BMP signaling pathways promote ES self-renewal in mice and ovarian SSC self-renewal in Drosophila. Future studies of how different signaling pathways are integrated in Drosophila ovarian SSCs may also shed light on how these same pathways control stem cell self-renewal in mammals.

Experimental Procedures

Drosophila Stocks and Experimental Genotypes

The following fly stocks were used in this study and are described either in Flybase or as specified: tkv, mad, sax, Med, Med, p35 (Das et al., 1998); punt, FRT, hs-FLP, armadillo(lam)-lacZ, UAS-GFP, UAS-tkv*, UAS-sax*, Dad-lacZ (Tsunezumi et al., 1997); dpp, dpp, dpp, gbb, gbb, gbb, smo, smo, dash, c58, gal4 (Song et al., 2004), hs-bam, UAS-arcEGFP.
FRT52B(y) (yellow-FRT-GAL4), and FRT52B(w) (white-Actin5C-FRT) (see the Supplemental Data for generation of last stocks). The genotypes and detailed heat shock protocols used in this study are provided as Supplemental Data. All Drosophila stocks were maintained at room temperature on standard cornmeal/molasses/sugar media.

BrdU Labeling
BrdU labeling was performed for 1 hr in Grace's medium as described previously (Lilly and Spradling, 1996).

Immunohistochemistry
The following antisera were used: monoclonal anti-Fasciclin III antibody 7G10 (1:3, DSHB), monoclonal anti-Hts antibody 1B1 (1:3, DSHB), polyclonal anti-β-galactosidase antibody (1:500, Cappel), monoclonal anti-β-galactosidase antibody (1:200, Promega), polyclonal anti-GFP antibody (1:200; Molecular Probes), and Alexa 488- and Alexa 568-conjugated to goat anti-mouse and anti-rabbit IgG (1:300, Molecular Probes). The immunostaining protocol used in this study has been described previously (Song and Xie, 2002). All micrographs were taken with a Leica TCS SP2 confocal microscope.

Supplemental Data
Supplemental Data including Supplemental Experimental Procedures and information on PMML stocks are available at http://www.devst.org/cgi/content/full/9/S/651/DC1/.

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