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

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Summary

BMP signaling is essential for promoting self-renewal of mouse embryonic stem cells and *Drosophila* germline 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 germarial 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.,

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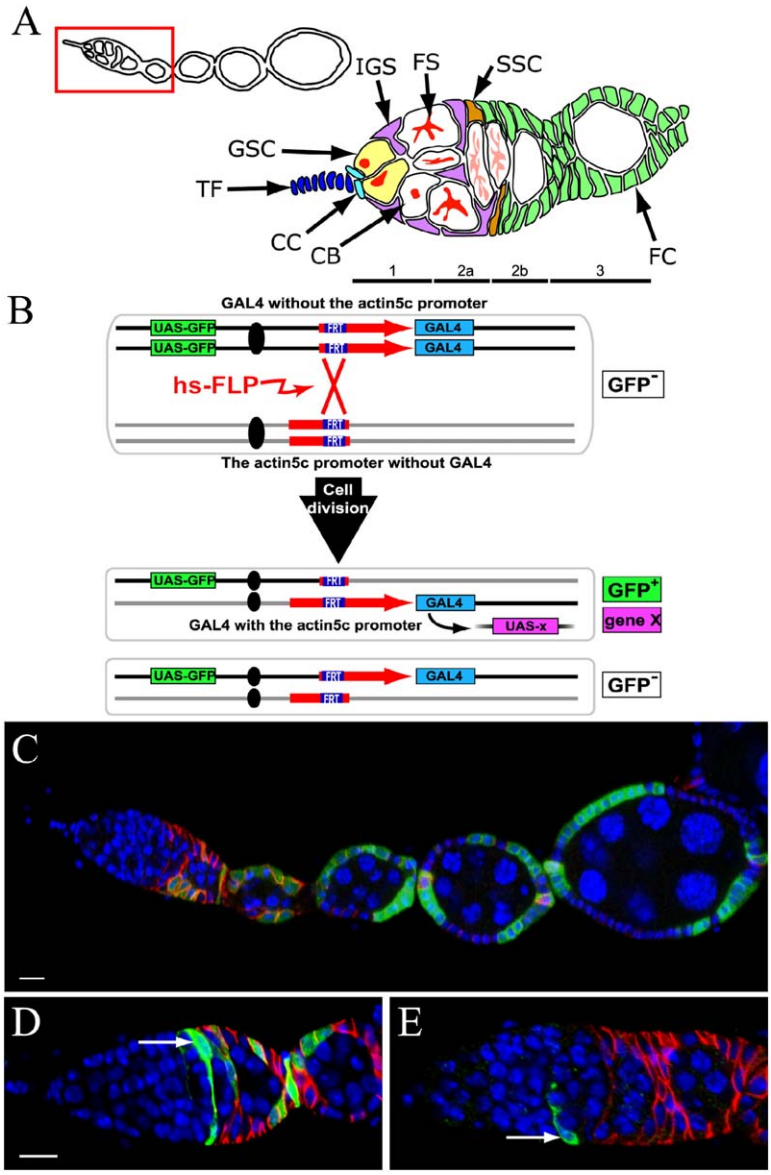

Figure 1. A New Positive Labeling System to Mark SSCs and Their Progeny

(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 (C) ovariole and (D and E) 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 McKearin, 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, 1998; 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 (MARCM) (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* FRT_{52B} and FRT_{52B} *gal4* (see the Supplemental 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 their progeny by overexpression.

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 (Neul 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

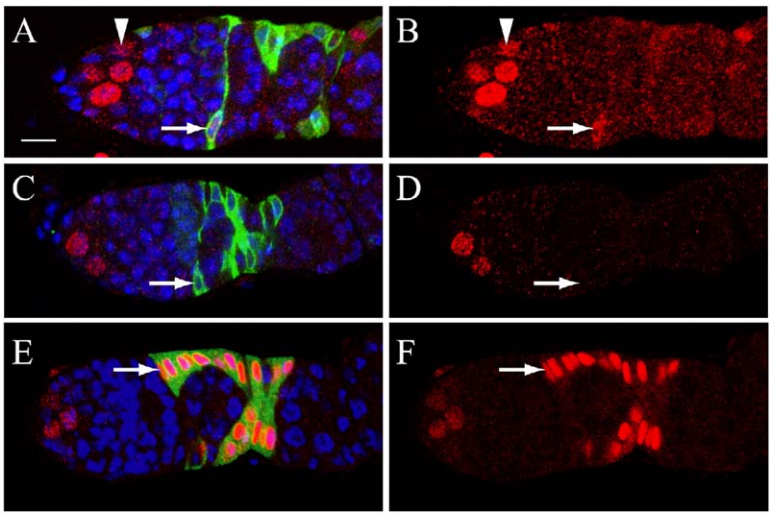

Figure 2. SSCs in the Ovary Respond to BMP Signaling

(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 SSC (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.

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, dpp^{hr56}/dpp^{hr4}, gbb^{4}/gbb^{D4}, and gbb^{4}/gbb^{D20} 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.

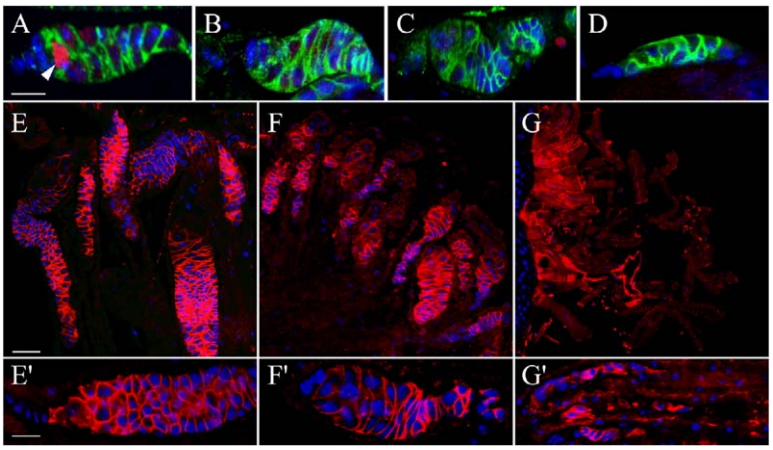

Figure 3. Gbb Signaling Is Required for Proliferation of SSCs/Follicle Cell Progenitors in the GSC Niche

(A–G′) (A–D) Agametic germaria are labeled with Hts (green), LacZ (red), and DNA (blue), while (E–G) agametic germaria are labeled with Hts (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 dpp^{hr4}/dpp^{hr56} mutant germarium showing no or extremely low *Dad-lacZ* expression in the SSCs located in the GSC niche. (C and D) (C) gbb^{4}/gbb^{D4} and (D) gbb^{4}/gbb^{D20} germaria do not express *Dad-lacZ* in the SSCs located in the GSC niche. (E and E′) 10-day-old wild-type control agametic germaria. (F and F′) 10-day-old dpp^{hr56}/dpp^{e90} agametic germaria showing slightly reduced sizes. (G and G′) 10-day-old gbb^{4}/gbb^{D4} 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.

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 agametic germarium reflects the proliferation of its SSCs and their progeny. To effectively compare the sizes of agametic germaria between wild-type and *dpp* or *gbb* mutants, we synchronized GSC loss by inducing GSC differentiation by using the *hs-bam* transgene. The wild-type and the dpp^{hr56}/dpp^{e90}, dpp^{hr56}/dpp^{hr4}, gbb^{4}/gbb^{D4}, and gbb^{4}/gbb^{D20} 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 3E′; n = 130). Since a stronger allelic combination, dpp^{hr56}/dpp^{hr4}, failed to reach adulthood after heat shock treatments, we only examined a weaker heteroallelic combination, dpp^{hr56}/dpp^{e90}. These *dpp* mutant agametic germaria contained slightly less follicle cells than the control agametic germaria (Figures 3F and 3F′; n = 156). It has been reported that dpp^{hr56}/dpp^{hr4} agametic 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 gbb^{4}/gbb^{D4} 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 gbb^{4}/gbb^{D20} mutant agametic ovaries, 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/or follicle cells in the ectopic niche. We then sought to investigate whether BMP signaling functions to control SSC maintenance and proliferation in their 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 SSCs were identified according to the published 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 $punt^{135}$, the BMP type II receptor, remained in the germaria 3 weeks ACI, while 23.8% of the marked SSCs mutant for mad^{12}, a *Drosophila* homolog of SMAD1, 5, 8, still persisted. Surprisingly, about 60% of the SSCs mutant for sax^{4}, a null allele for the BMP type I receptor *sax*, were maintained 3 weeks ACI, while only 24% of the SSCs mutant for tkv^{8}, 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 Med^{26} and Med^{AF33} 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 Med^{26} mutant SSC clones and none of the Med^{AF33} mutant SSC clones were maintained 3 weeks ACI (Group A of Table 1). Since Med^{26}, Med^{AF33}, tkv^{8}, and mad^{12} 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 TGF-β-like signaling pathways in mammals. This observation suggests that a TGF-β-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

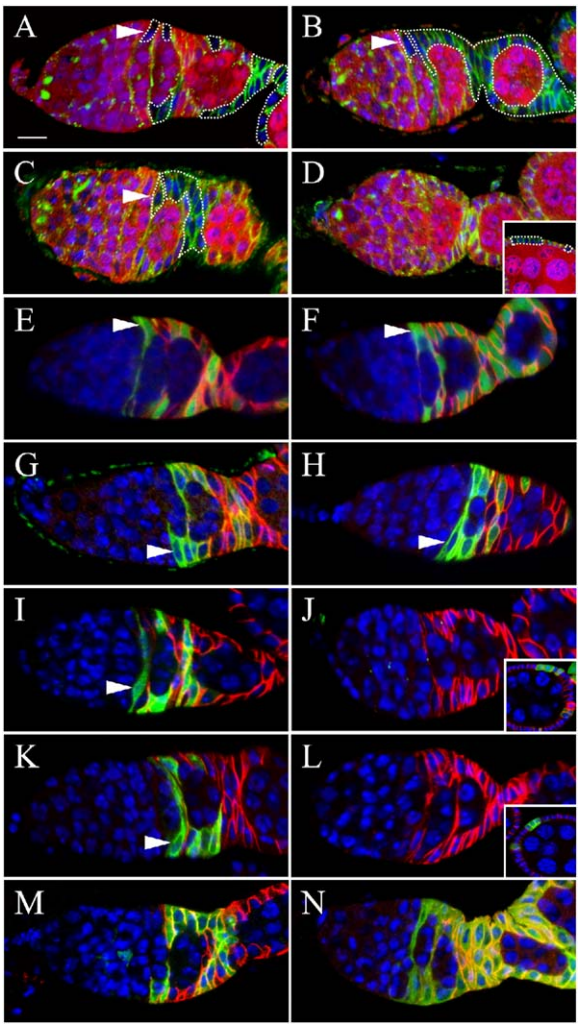

Figure 4. BMP Signaling Is Required for Controlling SSC Self-Renewal

(A–N) Germaria in (A)–(D) are labeled for Hts (green), LacZ (red), and DNA (blue), whereas germaria 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*^{8} mutant SSC clone in which the SSC and its early progeny are highlighted by dashed lines. (D) A germarium showing a lost *tkv*^{8} 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) Germaria carrying (E) 1-week-old and (F) 3-week-old GFP-marked wild-type SSC clones. (G and H) Germaria 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 *put*^{135} mutant SSC clone. (J) A germarium showing loss of a marked *put*^{135} 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 *put*^{135} mutant SSC clone that also overexpresses *p35*. (L) A germarium showing loss of a marked *put*^{135} 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 ovariole tip carrying a 1-week-old full SSC clone that also overexpresses *tkv**. All of the SSCs in the germarium 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.

et al., 1994; Sah et al., 1999). Recently, its overexpression has been demonstrated to inhibit apoptosis caused by defective Dpp signaling in the wing imaginal disc (Moreno et al., 2002). To investigate whether defective BMP signaling causes apoptosis of SSCs, we used the MARCM system to generate positively marked mutant SSC clones that also overexpress *p35* (Hay et al., 1994; Lee and Luo, 1999). In the MARCM system, mitotic recombination events between homologous chromosomes generate homozygous mutant clones, which are exclusively labeled by *tubulin-gal4*-driven UAS-GFP expression due to the loss of a *gal4* repressor, *tubulin-gal80*. We generated GFP-labeled SSC clones mutant for *punt*^{135} and *Med*^{26} and GFP-labeled *punt*^{135} and *Med*^{26} mutant SSC clones that also expressed *p35* to determine whether *p35* expression could prevent SSC loss caused by defective BMP signaling. Positive GFP-marked wild-type SSCs were maintained just like SSCs that are marked by loss of LacZ expression (Figures 4E and 4F; Group B of Table 1). Interestingly, the GFP-marked wild-type SSCs that expressed *p35* were maintained as the GFP-marked wild-type SSCs, indicating that the normal spontaneous SSC loss is likely due to differentiation, but not apoptosis (Figures 4G and 4H; Group B of Table 1). As expected, 26.5% of the GFP-marked *punt*^{135} SSC clones detected in the first week ACI were present in the germaria 3 weeks ACI, and they behave similarly to those that were labeled by loss of *lacZ* expression (Figures 4I and 4J; Group B of Table 1). A total of 26.9% of the *punt*^{135} SSCs that expressed *p35* were maintained 3 weeks ACI, which is comparable with that of the marked *punt* SSCs alone, suggesting that *p35* expression appears to have no dramatic effect on loss of *punt* mutant SSCs (Figures 4K and 4L). Together, these results suggest that SSC loss caused by defective BMP signaling is not likely due to apoptosis, but rather due to differentiation. On the other hand, *p35* overexpression appeared to partially mitigate the *Med* mutant SSC loss. Almost 94% of the GFP-marked *Med*^{26} SSCs were lost 3 weeks ACI (Group B of Table 1). *p35* expression reduced the SSC loss from 94% to 77%, which is very close to the loss rates for mutant *mad*^{12} and *tkv*^{8} SSCs. Along with the result that *p35* cannot alleviate SSC loss caused by the *punt* mutation, this result suggests that *Med* is involved in regulating SSC survival, likely not through modulating BMP signaling. This result further suggests that a TGF-β-like signal other than BMP is involved in controlling SSC survival.

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-

Table 1. Downstream Components of the BMP Signaling Pathway Are Required for SSC Maintenance

Genotypes	1 Week ACI		2 Weeks ACI		3 Weeks ACI	
	Percentage of Germaria Carrying a Marked SSC(s)	Relative Percentage to that of 1 Week ACI	Percentage of Germaria Carrying a Marked SSC(s)	Relative Percentage to that of 1 Week ACI	Percentage of Germaria Carrying a Marked SSC(s)	Relative Percentage to that of 1 Week ACI
Group A[a]						
Wild-type (control)	52.3%[b] (731)[c]	100%[d]	40.7% (626)	77.8%	38.4% (406)	73.4%
punt^{135}	35.2% (671)	100%	19.9% (652)	56.5%	7.0% (770)	19.9%
tkv^{8}	49.5% (390)	100%	21.7% (322)	43.8%	12.3% (318)	24.8%
sax^{4}	24.5% (151)	100%	20.0% (180)	81.6%	14.8% (142)	60.4%
mad^{12}	52.9% (293)	100%	27.1% (251)	51.2%	12.6% (388)	23.8%
Med^{26}	47.9% (409)	100%	17.2% (535)	35.9%	1.9% (534)	3.9%
Med$^{\Delta F3}$	21.9% (219)	100%	2.4% (211)	10.8%	0.0% (211)	0.0%
Group B						
Wild-type (control)	65.3% (357)	100%	57.4% (397)	87.9%	48.2% (570)	73.8%
UAS-p35	52.0% (198)	100%	44.2% (453)	85.0%	32.4% (389)	62.3%
punt^{135}	58.6% (382)	100%	26.3% (228)	44.8%	15.5% (225)	26.5%
punt^{135}*;UAS-p35*	60.5% (357)	100%	27.5% (291)	45.4%	16.3% (306)	26.9%
Med^{26}	52.2% (431)	100%	18.8% (240)	36.0%	3.3% (269)	6.3%
Med^{26}*;UAS-p35*	47.6% (275)	100%	23.8% (378)	50.0%	10.7% (412)	22.5%
Group C						
UAS-GFP (control)	37.4% (329)	100%	20.4% (460)	54.5%	15.2% (488)	40.6%
*UAS-tkv**	26.2% (420)	100%	22.6% (354)	86.3%	17.2% (326)	65.6%

[a] 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.

[b] 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.

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

[d] The normalized percentage of germaria carrying a marked SSC at a given time, since different FRT chromosomes produced different percentages of germaria carrying 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.

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 almost all of the marked germaria should carry only one marked SSC, resulting in partial labeling of follicle cells in the egg chamber (Figure 4M). In earlier experiments (Groups A and B), we noticed that quite a high percentage of germaria 2 weeks ACI had already carried "full" clones, in which all follicle cells are marked. In the germaria carrying marked full clones, loss of marked SSCs could not be detected any more since the germaria losing a marked SSC are not able to be distinguished from the germaria that do not lose a marked SSC. As a result, our earlier experiments likely overestimate maintenance rates of marked wild-type as well as mutant SSCs. However, since we can compare maintenance rates between marked wild-type SSCs and marked mutant ones under the same conditions, the information gained from earlier experiments is still valid. As expected, only 40.6% of the marked wild-type SSCs were maintained 3 weeks ACI when the marked SSC clone frequency was reduced, which is in contrast with the over 70% maintenance rates for marked wild-type SSCs in earlier experiments. Interestingly, 65.6% of the marked SSCs that expressed *tkv** were maintained 3 weeks ACI, suggesting that strengthening BMP signaling can promote SSC self-renewal and thus prolong the SSC lifespan (Group C of Table 1).

As reported previously (Margolis and Spradling, 1995), the number of the germaria carrying a 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 SSC progeny can maintain their stem cell property longer, interact better with niches, or are abundant in number, they might be preferentially recruited to empty niche spaces left by lost SSCs. One week ACI, 0.9% (n = 329) and 0.5% (n = 420) of the germaria carried wild-type or *tkv**-expressing full clones, respectively, while 3.5% (n = 488) and 8.9% (n = 326) of the germaria carried wild-type and *tkv**-expressing full clones, respectively, 3 weeks ACI. These findings suggest that the *tkv**-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

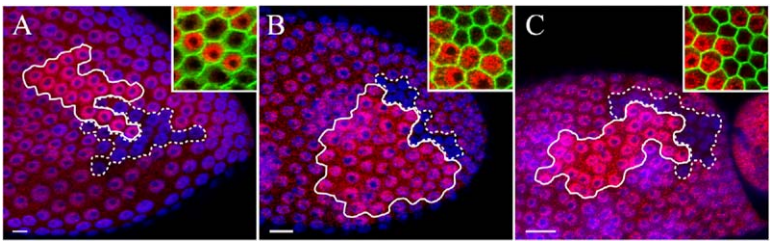

Figure 5. BMP Downstream Components, Except *Med*, Are Dispensable in Controlling Follicle Cell Proliferation

(A–C) The egg chambers are labeled for LacZ (red) and DNA (blue), whereas inserts in (A)–(C) are labeled for membrane skeletal protein Hts (green) and LacZ (red). (A) A twin-spot clone showing similar sizes of *tkv*^{8} mutant follicle cell patch (broken lines, *lacZ*$^{-}$) and its twin wild-type counterpart (solid lines, two copies of *lacZ*$^{+}$) in a stage-10B egg chamber. The insert shows the normal size of *tkv* mutant follicle cells. (B and C) Twin-spot clones showing that the (B) *Med*^{26} and (C) *Med*^{AF33} mutant follicle cell patches (broken lines, *lacZ*$^{-}$) are smaller than those of the corresponding wild-type counterparts (solid lines, two copies of *lacZ*$^{+}$) in egg chambers (stage 8 for [B] and stage 6 for [C]). The inserts in (B) and (C) show that *Med* mutant follicle cells are smaller than wild-type ones. The bars represent 10 μm.

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*^{135}, *tkv*^{8}, *mad*^{12}, and *Med*^{26} 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*^{12}, *tkv*^{8}, and *Med*^{26} 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*$^{-}$ follicle cells divided by the number of 2xLacZ^{+} follicle cells. As expected, the marked wild-type follicle cells had an rdr of 0.94 (n = 17). The marked *tkv*^{8} 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*^{26} and *Med*^{AF33} 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*^{135} 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*^{26} 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*^{3} and *smo*^{D16}, and one strong *dsh* allele, *dsh*^{3}, 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

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

	1 Week ACI		2 Weeks ACI		3 Weeks ACI	
Genotypes	Percentage of Germaria Carrying a Marked SSC(s)	Relative Percentage to that of 1 Week ACI	Percentage of Germaria Carrying a Marked SSC(s)	Relative Percentage to that of 1 Week ACI	Percentage of Germaria Carrying a Marked SSC(s)	Relative Percentage to that of 1 Week ACI
smo^{D16}	16.9%[a] (243)[b]	100%[c]	2.8% (288)	16.5%	0.0% (312)	0.0%
*smo^{D16}; UAS-tkv**	30.2% (222)	100%	2.2% (187)	7.3%	2.0% (245)	6.6%
smo^{3}	26.9% (249)	100%	3.2% (310)	11.9%	0.9% (221)	3.3%
*smo^{3}; UAS-tkv**	23.8% (223)	100%	9.7% (195)	40.8%	3.9% (255)	16.4%
dsh^{3}	32.9% (219)	100%	5.8% (360)	17.6%	3.8% (314)	11.5%
*dsh^{3}; UAS-tkv**	28.5% (249)	100%	21.9% (215)	76.8%	11.5% (191)	40.3%

[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. Since different FRT chromosomes produced different percentages of germaria carrying a marked SSC(s) 1 week ACI, the percentages at the first week are normalized to 100%, and the percentages for the following time points are calculated by the actual percentages divided by the percentages at the first week for each genotype.

Xie, 2003) are lost rapidly. Consistently, GFP-positive marked SSC clones mutant for *smo^{3}*, *smo^{D16}*, and *dsh^{3}* were lost quickly in comparison with marked wild-type SSCs (Table 2). The marked *smo^{3}* and *smo^{D16}* 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 *dsh^{3}* 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 regulation. Taken together, our results suggest that the BMP pathway works as one of downstream braches 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 as important a 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 also promotes SSC division. Our previous studies have shown that BMP signaling promotes GSC division (Xie and Spradling, 1998). In order to better understand how BMP signaling controls 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^{4}*, 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, *tkv^{8}*, 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

essential for maintaining GSCs, 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 (Haerry 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^{8} and mad^{12}, proliferate normally, and the sizes of the mutant follicle cells are quite normal. In contrast, *p35* expression can rescue the Med^{26} SSC loss to the levels of the mutant *punt*, *tkv*, and *mad* mutant SSC loss. 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 cells 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; Kobielak et al., 2003; Qi et al., 2004; Ying et al., 2003; Zhao et al., 1998). BMP signaling can also indirectly regulate haematopoeitic 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; Reya 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^{8}, mad^{12}, sax^{4}, Med^{26}, Med^{AF33} (Das et al., 1998); $punt^{135}$, FRT_{40A}, FRT_{82B}, *hs-FLP*, *armadillo*(*arm*)-*lacZ*, *UAS-GFP*, *UAS-tkv**, *UAS-sax**, *Dad-lacZ* (Tsuneizumi et al., 1997); dpp^{hr56}, dpp^{hr4}, dpp^{e90}, gbb^{4}, gbb^{D4}, gbb^{D20}, smo^{D16}, smo^{3}, dsh^{3}, *c587-gal4* (Song et al., 2004), *hs-bam*, *UAS-srcEGFP*,

FRT_{52B}(y) (*yellow-FRT-GAL4*), and *FRT_{52B}(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 the generation of PMML stocks are available at http://www.developmentalcell.com/cgi/content/full/9/5/651/DC1/.

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