Establishment of stable cell lines of *Drosophila* germ-line stem cells

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Each *Drosophila* ovariole has three independent sets of stem cells: germ-line stem cells (GSCs) and escort stem cells, located at the anterior tip of the germarium, and somatic stem cells (SSCs), located adjacent to the newly formed 16-cell cysts. Decapentaplegic (Dpp) is required to maintain the anterior stem cells, whereas Hedgehog is required for maintenance and cell division of the SCCs. In an effort to establish a new *in vitro* system to analyze intrinsic and extrinsic factors regulating the division and differentiation of GSCs of *Drosophila*, we tested various culture conditions for growing GSCs, derived from *bag of marbles* (*bam*) mutant ovaries. We have shown that *bam*$^-$ GSCs can be maintained and promoted to divide *in vitro* in media containing Dpp. These cells retain the morphological features of GSCs, i.e., expression of Vasa and Nanos and spectrosomes, even after several months of culture. Somatic cells are induced to grow in culture by the presence of sonic Hedgehog. The somatic cells produce Dpp. GSCs associate with the somatic cells via DE-cadherin, features that are also prominent at the niche of a normal germarium. Finally, we have established stable cell cultures consisting of GSCs and sheets of somatic cells, which are dependent on the addition of fly extract. A somatic cell line, lacking GSCs, has also been established. These cells are thought to be descendants of SCCs. Our *in vitro* system may provide the opportunity to manipulate GSCs genetically and to analyze the interaction of germ-line stem cells and soma.

tissue culture | niche | somatic stem cell | decapentaplegic | DE-cadherin

Stem cells are characterized by the ability to divide asymmetrically to produce daughter cells of two types, one fated for differentiation and one to regenerate a stem cell. This cell division takes place within a cellular niche or environment where the stem cell maintains contact with the cells composing the niche, whereas the differentiating cell moves away from the niche (reviewed in ref. 1). The *Drosophila* ovary provides an excellent system for studying factors required to establish and maintain stem cells (1, 2). Three sets of stem cells are located in the germarium of each ovariole (Fig. 1*a*) (3–5). One set, composed of two or three germ-line stem cells (GSCs), is located adjacent to the cap cells at the anterior tip of each ovariole. After a stem-cell division, one daughter cell retains its attachment to the cap cells whereas the other daughter (called a cystoblast) moves away from the cap cells and begins a series of differentiation steps (Fig. 1*a*). Maintenance of GSCs requires contact with the cap cells by means of adherens (6) and gap (7, 8) junctions, and the continuous secretion of the bone morphogenetic protein (BMP)2/4-like growth factor decapentaplegic (Dpp) (9) by the cells of the niche. The Dpp signal represses the transcription of *bag of marbles* (*bam*) in the GSC, but this repression is released in the cystoblast (10). A second set of stem cells, adjacent to the GSCs, has recently been shown to produce sets of transitory escort cells that envelope cystocytes during the four nuclear divisions (5). Immediately posterior to the inner gonial sheath cells (Fig. 1*a*) is located a third set of stem cells responsible for the somatic lineages of the ovary [somatic stem cell (SSC)] (4, 11). Proliferation of SSCs depends upon Hedgehog (Hh), secreted by the terminal filament and cap cells (12, 13), and maintenance of SSCs requires adherens junctions (14). The mechanism by which the Hh downstream pathway controls SSC maintenance and proliferation is not known. Recently both Dpp and Gbb have also been shown to play a role in maintaining SSCs (15).

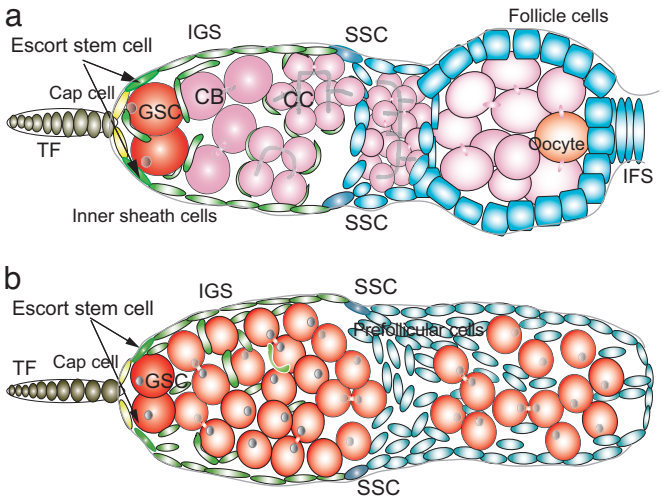

Fig. 1. Diagrams of a germarium, the anteriormost structure of each ovariole. (*a*) Wild-type germarium. GSCs are located adjacent to the terminal filament (TF) and cap cells, and they divide to produce another GSC and a cystoblast (CB). The CB will subsequently divide synchronously four times to produce a cyst of 16 cystocytes (CC), one of which will differentiate into the oocyte. A number of inner gonial sheaths (IGS) line the outside of the germarium in the region where the initial four divisions of the CB occur. The transitory escort cells derive from IGSs adjacent to the cap cells. The SSCs are located posterior to the IGSs and are responsible for producing the follicular epithelium. Each chamber is separated by interfollicular stalks (IFS), which are derived from SSCs. (*b*) *bam*$^-$ germarium. Germ-line stem cells, escort stem cells, and SSCs continue to divide. Escort cells and prefollicular cells populate regions at the anterior and posterior of the germarium, respectively.

Expression of Bam is sufficient to initiate the differentiation of either GSCs or cystoblasts, even in the presence of excess Dpp (10). In ovaries lacking Bam GSC-like cells accumulate. These cells display a novel cytoplasmic structure, the spectrosome, which characterizes both GSCs and daughter CBs, and is enriched for membrane skeletal proteins such as α- and β-spectrin, the adducin-like Hts protein and ankyrin (16, 17, 18). When the cystoblast undergoes four synchronous nuclear divisions with incomplete cytokinesis, the spectrosome enlarges into the fu-

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Abbreviations: Dpp, decapentaplegic; GSC, germ-line stem cell; SSC, somatic stem cell.

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some that stretches through each of the cytoplasmic bridges connecting the 16 daughter cells (19). In *bam*$^-$ ovarioles, the GSC-like cells retain the spherical spectrosome, without developing the elongated fusome structure (20). The restriction of the Dpp signal to the GSCs depends on a series of genetically redundant mechanisms to inhibit Dpp signaling (21).

Because of the complexity of these interactions between germ-line and surrounding somatic cells, it would be very useful to have a culture system that provides the opportunity for direct analysis of the interactions occurring during these processes. In various model animals, a few *in vitro* systems have been reported. For example, PGCs have been cultured in the mouse (cf. recent reviews in refs. 22 and 23) and the chicken (24). Recently, adult murine spermatogonial stem cells have been cultured and shown to be pluripotent (25). So far as we know, however, in *Drosophila* there are no reports of culturing germ-line cells. A major reason for this absence is the difficulty in obtaining sufficient numbers of germ-line cells to culture and the lack of adequate culturing methods. Here, we report our experiments to culture germ-line cells of *Drosophila*. To facilitate the collection of sufficient number of cells, we used expanded GSCs derived from the *bam*$^-$ mutant. These cells resemble GSCs by the presence of a spectrosome and the ability to divide continually. They are different, however, inasmuch as the *bam*-promoter is active in *bam*$^-$ cells whereas it is not active in wild-type GSCs (26). Two lines of evidence suggest that *bam*$^-$ GSCs are comparable to stem cells. First, they can be incorporated into the embryonic gonad and differentiate as stem cells and produce fertile oocytes if the wild-type *bam* gene is provided (27). Second, differentiating cystocytes (i.e., descendants of cystoblast) can revert to GSCs (28), which suggests that cystoblasts probably have the same capability. Over-produced *bam*$^-$ GSCs are thought to have the status of precystoblasts because of their fusome morphology (29) and lower pMad activity than GSCs (30, 31).

Our experiments demonstrate that *bam*$^-$ GSCs can be cultured for long periods in media supplemented with Dpp. In addition, we show that somatic cells, probably derived from SCCs, are induced to grow in culture in the presence of sHh to form sheets of cells. Growth of GSCs is enhanced by the presence of these somatic cells. The somatic cells produce Dpp. GSCs and the somatic cells are associated with each other via DE-cadherin, resembling that seen at the niche in the normal germarium. We have successfully established a stable cell culture, named fGS/OSS (female germ-line stem cells/ovarian somatic sheet), which contains both germ-line and somatic cells. We have also produced a somatic cell line (OSS), obtained by removing the GSCs.

Results

Effect of BMP4 and Dpp on the Proliferation of *bam*$^-$ GSCs. We collected *bam*$^-$ GSCs from females 30–40 days old by first breaking open the enlarged germaria by homogenization, which released the loosely organized germ-line cells, followed by filtration through nylon meshes to remove tissue debris (muscle sheaths, etc.). After concentrating the cells by centrifugation, they were dispersed into M3 (BF) media with the addition of glutathione, FBS and insulin at initial cell concentrations of $\approx 2 \times 10^5$ cells per ml. GSCs and somatic cells are present in almost equal numbers in these preparations. The somatic cells are flat and smaller than *bam*$^-$ GSCs and can be easily distinguished (Fig. 7*a*, which is published as supporting information on the PNAS web site). Many of the *bam*$^-$ GSCs showed cell pairs as reported (32). Cells survived for only two or three weeks in this medium (Fig. 7*b*). Because Dpp has been shown to be a critical growth factor for germ-line stem-cell maintenance and cell division, we added commercially available human BMP4 to M3 (BF) medium. Initially, BMP4 stimulated growth of cells but subsequently the number of cells did not increase further (Fig. 7*b*). At all concentrations used, cells disappeared after 20 days in culture. These results indicate that BMP4 alone does not support the continuous cell division of either the *bam*$^-$ GSCs or somatic cells. The addition of Dpp (provided by supernatants from S2 cells, stimulated to secrete Dpp into the medium), effectively maintained GSCs but was insufficient for continued growth (Fig. 8, which is published as supporting information on the PNAS web site). It is possible that the procedure of dispersing cell clumps to collect and count cells seriously affects the ability of cells to continue to divide. To test this possibility, we cultured cell clusters without splitting them. Both somatic and germ-line cells maintained their association in these clusters. The presence of Dpp provided much greater growth (cf. Fig. 9, which is published as supporting information on the PNAS web site), but nevertheless the cell clusters ceased growing after one month in culture despite periodic media changes.

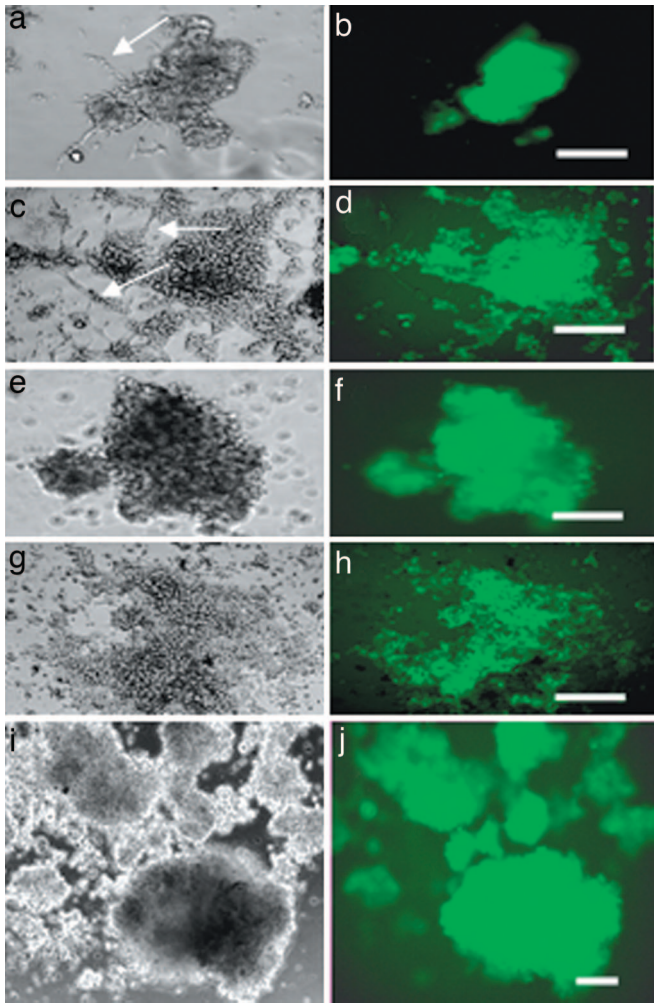

Fig. 2. Comparison of cell clusters after culturing 5–7 days in media supplemented with supernatants from embryonic cells of Oregon-R (*a* and *b*), of *actin-gal4/UAS* cells overexpressing sHh (*c* and *d*), Dpp (*e* and *f*), or sHh plus Dpp (*g* and *h*). *a*, *c*, *e*, and *g* are phase-contrast, and *b*, *d*, *f*, and *h* are fluorescent figures of *a*, *c*, *e* and *g*, respectively. *bam*$^-$ GSCs are from females of genotype w^{1118}; *P[w*$^+$ *hsp-70 bam*$^+$*]*$^{11\text{-}d}$ $bam^{\Delta 86}$ / $bam^{\Delta 86}$ *P[ovo-lacZ] P[vas-EGFP]*. Somatic cells (arrows) were more prominent when cultured in the media containing Shh. Dpp and combination of Shh plus Dpp supported considerable growth of *bam*$^-$ GSCs. Shown are phase contrast (*i*) and fluorescent (*j*) photographs of 50-day cultures of *bam*$^-$ GSCs in media supplemented with sHh plus Dpp. Clusters of >500 μm in diameter were observed, each containing at least several thousands cells based on the size of a *bam*$^-$ GSC. Most cells are *vas*-EGFP-positive, thus indicating germ-line origin. (Scale Bar: 100 μm.)

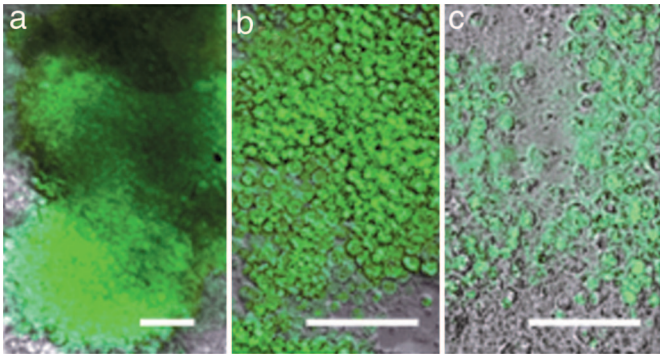

Fig. 3. Clusters of *bam*$^-$ cells cultured in the media with fly extract and various growth factors. (*a*) Ten percent fly extract (FE) plus Shh plus Dpp. (*b*) FE plus Shh. (*c*) Ten percent FE only. Growth of somatic cells varied considerably in these different conditions. Cell clusters were larger in media supplemented with FE, Shh, and Dpp but not in media containing only FE. In the latter medium, somatic cells spread to form a continuous sheet. GSCs located on the somatic sheet frequently formed pairs of cells (data not shown). (Scale bar: 100 μm.)

Effects of Growth Factors from Embryonic Primary Cultures on the Proliferation of *bam*$^-$ GSCs. To achieve continued growth of GSCs in culture, we introduced two modifications: first, we avoided homogenization of the ovaries and instead fragmented the ovarioles with tungsten needles to release GSCs; second, we obtained growth factors by deriving primary embryonic cells from embryos expressing either Dpp, Wg or Shh (see *Materials and Methods* for protocols). In this series of experiments, we prepared *bam*$^-$ GSCs from two to three ovaries without dispersing the cells into single cell suspensions. In each medium, both *vas*-EGFP-positive cells (GSCs) and *vas*-EGFP negative cells (somatic cells) were present at the start of the culture. The growth pattern of cell clumps, however, showed major differences in the various culture media within several days. Wg had little effect on the growth of either somatic cells or GSCs (data not shown). In media with Shh, somatic cells became prominent and spread around the GSCs (Fig. 2 *c* and *d*). Dpp was most effective in increasing the size of GSC clusters (Fig. 2 *e* and *f*). The growth was similar to that seen with conditioned media from S2-Dpp-HA expressing cells. Furthermore, *bam*$^-$ GSCs in these cell clumps tended to become more tightly compacted than occurred with other media. Most cell clumps continued to grow at least one month in culture. The mixture of Dpp and Shh was more effective than only Dpp (Fig. 2 *g* and *h*). Growing cell clumps sometimes fused with each other to form larger clumps. In some instances, cell clumps reached >500 μm in diameter. Moreover, *bam*$^-$ GSCs in these cultures frequently spread with the somatic cells. Fig. 2*i* shows an example of a large cell clump cultured in media with Dpp plus Shh for 50 days. We estimate, based on the 10-μm diameter of individual cells, that there are at least 5,000 cells present in the bottom layer of each clump, and many more in the whole clump.

During the preparation of these cultures, germaria were fragmented into pieces and an effort was made to remove non-germ-line tissues. Nevertheless, somatic cells always appeared together with GSCs. To examine whether niche cells, cap cells or terminal filaments at the anterior tip of the germarium were included among these somatic cells, we cultured separately pieces of the anterior tip of germaria. Cap cells and terminal filament did not incorporate BrdU, indicating they did not enter the cell cycle (Fig. 10, which is published as supporting information on the PNAS web site). Thus, the cells of the niche are probably not the source of the somatic cells. We cannot exclude the contribution of escort cells.

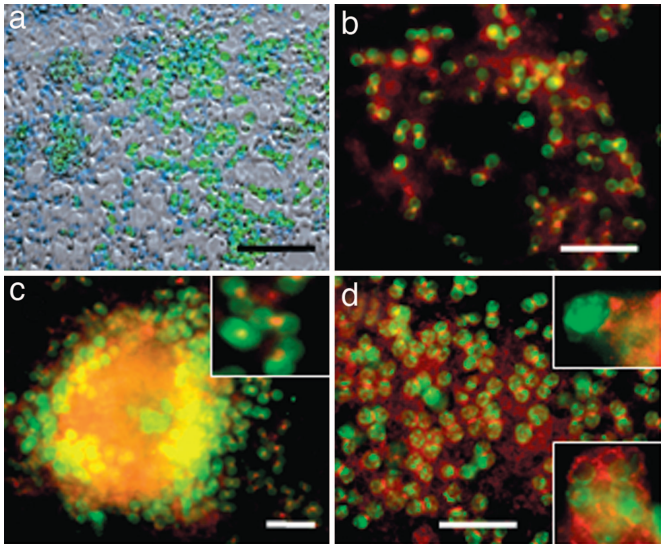

Fig. 4. Characteristics of *bam*$^-$ GSCs in fGS/OSS cultures. (*a*) Live fGS/OSS cells, cultured for 3 months after freezing and thawing. *vas*-EGFP-positive cells are easily discriminated from the nonstained somatic cells. (*b*) fGS/OSS cells stained with anti-Spectrin (red) and anti-Vasa (green). (*c*) fGS/OSS cells stained with anti-spectrin (red) and anti-Nanos (green). GSCs continued to express Vasa and Nanos and showed dot spectrosome (*Inset*). (*d*) Cell junctions between the somatic cells and GSCs in fGS/OSS cultures, stained with anti-DE-Cadherin (red) and anti-Vasa (green). Strong DE-cadherin staining was found between somatic cells and GSCs (*Upper Inset*) and Armadillo (*Lower Inset*). (Scale bar: 100 μm.)

Establishment of Stable Cell Lines, Using Fly Extract as a Supplement. To stimulate the growth of the somatic cells that appear together with *bam*$^-$ GSCs, fly extract (FE), which is known to be effective in many kinds of *Drosophila* cells in primary cultures, was added to the media (33). Clusters of cells obtained from minced ovaries formed large clumps in media supplemented with FE and conditioned medium containing Dpp and Shh (Fig. 3*a*). The clumps continued to get larger for several months. In addition, the growth of somatic cells is more prominent than when culture medium is supplemented with Dpp and Shh only (cf. Fig. 2 *i* and *j*). The cell clumps continued to grow stably for several months. When Dpp conditioned medium is left out, GSCs continue to grow (Fig. 3*b*) without forming the large clumps seen when Dpp is present (Fig. 3*a*). This result suggests that Shh stimulates the growth of the somatic cells and that these somatic cells support the maintenance and division of GSCs.

When cells from minced ovaries were cultured in media supplemented with fly extract only, initially there was little growth without the added growth factors. Usually >30 days were needed before the cells began to grow in these cultures. As the somatic cells expanded, they formed a monolayer sheet (Fig. 3*c*). Clusters of GSCs were found associated with these somatic cells, frequently in pairs reminiscent of that seen in initial growth of *bam*$^-$ GSCs. This growth pattern strongly indicates that the somatic sheets can maintain and stimulate the proliferation of *bam*$^-$ GSCs without the addition of Dpp or sHh. Both somatic cells and *bam*$^-$ GSCs divided frequently. Thirty to fifty percent of cells were mitotically active, as evidenced by BrdU incorporation (data not shown).

Both somatic cells and GSCs continued to grow stably for >1 year. We split the cultures every 5 to 7 days to avoid confluence. If the cells were allowed to reach confluence, somatic cell clumps appeared and *bam*$^-$ GSCs were found clustered within the clumps (Fig. 11*a*, which is published as supporting information on the PNAS web site). These somatic/GSC clumps were easily detached from the sheet by pipetting and they would continue to

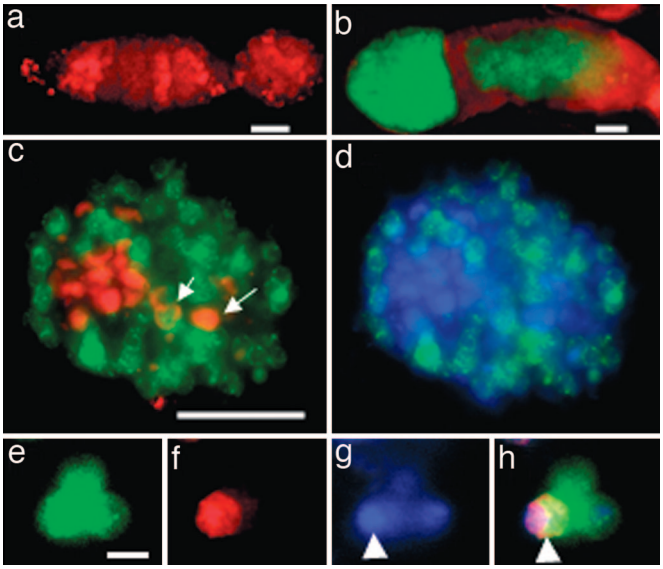

Fig. 5. Dpp and pMad staining of wild-type and *bam*$^{-}$ tumorous germaria and fGS/OSS cultures. Anti-Dpp antibody (red) staining of wild-type (*a*) and *bam*$^{-}$ tumorous germaria (*b*). In wild-type germarium, cap cells and prefollicular cells produce Dpp. Strong signals of Dpp were seen in the posterior somatic cells of *bam*$^{-}$ tumorous germaria. They are assumed to be prefollicular cells. (*c* and *d*) fGS/OSS cultures, expressing *vas*-EGFP, stained with anti-Dpp (red) and anti-pMad antibody (blue). Somatic cells are strongly positive for both Dpp and pMad. A lower level of pMad is also seen in some GSCs. Some GSCs are also Dpp-positive (arrows). (Scale bar: 100 μm.) (*e–h*) Dissociated fGS/OSS cells, cultured for 15 months, stained as follows: *vas*-EGFP (*e*); anti-Dpp (*f*); anti-pMad (*g*); merge of *e*, *f*, and *g* (*h*). One GSC accumulated pMad in the nucleus (arrowhead). The fGS/OSS cells were cultured for 15 months. (Scale bar: 10 μm.)

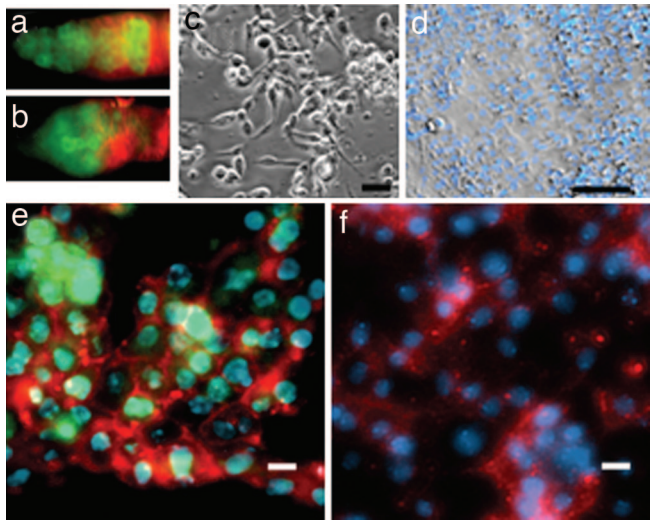

Fig. 6. Characteristics of OSS cells and FasIII-positive cells. Shown is anti-FasIII antibody staining of *vas*-EGFP germarium (*a*) and tumorous *bam*$^{-}$ germarium (*b*) (red). Overproduced prefollicular cells populate the posterior region of the germarium and display strong FasIII staining. (*c*) Living OSS cells at low cell density. The somatic cells are small and have cytoplasmic processes. (Scale bar: 10 μm.) (*d*) Somatic sheet stained with Hoechst 33342. Cells became tightly associated and cannot be discriminated from each other. Round and *vas*-EGFP-positive GSCs are absent (compare with Fig. 4*a*). (Scale bar: 100 μm.) (*e*). Freshly prepared *bam* cells were stained with anti-Vasa (green) and anti-FasIII (red) antibodies and Hoechst 33342 (blue). Most of somatic cells are FasIII-positive, indicating that they are prefollicular cells. (Scale bar: 10 μm.) (*f*) OSS cells stained with anti-FasIII antibody (red) and Hoechst 33342 (blue). The OSS cells have been cultured for 15 months. Many somatic cells are FasIII-positive. (Scale bar: 10 μm.)

grow and make large spherical structures up to 1 mm in diameter (Fig. 11*b*).

bam^{-} GSCs sometimes showed higher rates of cell death in the media with growth factors whereas cell death is rare in the cultures supplemented only with FE. Thus, we used the FE medium without growth factors to establish stable cell lines.

Establishment of a Stable Cell Line. Both *bam*$^{-}$ GSCs and the accompanying somatic cells can be cultured stably >1 year and require splitting every 5–7 days. We have successfully stored 6-month-old cultured cells at −80°C for 6 months, and reestablished cultures after thawing. Thus, this coexistent culture of *bam*$^{-}$ GSCs and soma can be regard as a stable cell line and named fGS/OSS.

The *bam*$^{-}$ GSCs in fGS/OSS cultures are *vas*-EGFP-positive. They continue to express Vasa and Nanos and show the characteristic spectrosome (Fig. 4 *b* and *c*). To examine whether GSCs form cell junctions with neighboring somatic cells, we stained the cultures with anti-DE-cadherin antibody (Fig. 4*d*). DE-cadherin is present between somatic cells and GSCs, indicative of the presence of adherens junctions. Actin and Armadillo (β-catenin) were also abundant in the cytoplasm of somatic cells and at the site of association with GSCs (Fig. 4*d Insets*).

Dpp Signaling of *bam*$^{-}$ GSCs and Somatic Cells. *dpp* mRNA is detected by *in situ* hybridization in cap cells, inner sheath cells and somatic cells in region 2B and 3 of the wild-type germarium (9). We used commercially available *Drosophila* anti-Dpp antibody to detect the localization of Dpp in the cultured cells. The staining patterns of wild type germaria by this antibody agreed with the *in situ* hybridization pattern (Fig. 5*a*). In *bam*$^{-}$ germaria, the cluster of prefollicular cells at the posterior end of the tumorous germarium stained heavily with the anti-Dpp antibody (Fig. 5*b*). Dpp and pMad were detected in some *bam*$^{-}$ GSCs (data not shown). To examine whether there were somatic cells producing Dpp in the fGS/OSS cell line, we stained cultures with anti-Dpp and anti-pMad antibodies (Fig. 5 *c* and *d*). Some somatic cells strongly stained with the anti-Dpp antibody. The somatic cells producing Dpp also stained strongly with anti-pMad antibody, which indicates that there might be a positive feedback loop affecting the somatic cells. Some *bam*$^{-}$ GSCs were Dpp-positive (Fig. 5*f*) and pMad were found to accumulate in the nuclei (Fig. 5*g*). This result indicates that the Dpp signal pathway was activated in *bam*$^{-}$ GSCs by receiving Dpp from the surrounding somatic cells.

Characteristics of Somatic Cells and Establishment of a Somatic Cell Line. We wanted to know whether the somatic cells in fGS/OSS cultures were heterogeneous. If they are heterogeneous, we predict that the morphology of subpopulations should vary from each other. Between 1 and 50 cells from the fGS/OSS line were inoculated into individual wells of a 96-well culture plate. We selected subpopulations consisting of only somatic cells by checking for *vas*-Egfp expression. We repeated the dilution several times and established >20 independent subpopulations of somatic cells. There are no conspicuous variations of cellular morphology among these subpopulations. The somatic cells are flat and <10 μm in diameter. They show cytoplasmic processes like epithelial cells (Fig. 6*c*). After the formation of a continuous sheet of cells, there is no clear boundary visible between cells (Fig. 6*d*). The uniformity of the subcultures indicates that the somatic cells are probably of one type. We named the somatic cell line OSS.

Prefollicular cells were found to populate the posterior region of *bam*$^{-}$ germaria (Fig. 6*b*). We assume that the somatic

component of the cultures is derived from the somatic stem cells (cf. Fig. 1*b*). Although there is no diagnostic stain for these cells, it is known that prefollicular cells stain intensely for FasIII, whereas SSCs stain faintly for FasIII (14, 34). Fig. 6*e* shows an example of cell masses stained with anti-FasIII antibody. About 63% of somatic cells (195/294 cells) in the cell masses were FasIII-positive. Most (if not all) of the OSS cells were also FasIII-positive but many stained faintly (Fig. 6*f*). From the lower signal of FasIII in these cells and their continuous division, at least some OSS cells are presumably stem cells, dedifferentiated from prefollicular cells.

Discussion

bam$^-$ GSCs of *Drosophila* can be cultured successfully in media supplemented with Dpp. These results are consistent with *in vivo* experiments showing that Dpp has an essential role for the maintenance and division of GSCs (9). Wg and Shh did not stimulate growth of *bam*$^-$ GSCs, which is consistent with previous *in vivo* results. The human homologue of Dpp, BMP4, was not effective in promoting the continuous growth of cells, although it stimulated GSC division during the first few days in culture. A mixture of Dpp and Shh promoted the growth of *bam*$^-$ GSCs better than media with only Dpp. Because somatic cells are also prominent under this culture condition, it is reasonable to postulate that these somatic cells support the maintenance of *bam*$^-$ GSCs by producing some factors and/or by the association with the germ-line cells. If we consider that *bam*$^-$ GSCs in cell clumps survive and continue to divide, whereas single cells die, it seems that *bam*$^-$ GSCs need to be associated with each other and these somatic cells to survive. Conditioned media from primary embryonic cells were more effective in maintaining *bam*$^-$ GSCs for long-term culture than media obtained from S2 cells secreting Dpp. There may be quantitative differences in the amount of Dpp present in these two types of cultures, and it is possible that additional growth factors are produced by the primary cell cultures. Finally, fly extract was sufficient by itself to support long term culture either of a mixture of GSCs and somatic cells, presumably derived from SSCs, or the somatic cell line by itself.

In the mouse, primordial germ cells (PGCs) have been successfully cultured. All available culture methods for mouse PGCs rely upon the presence of cell feeder layers (reviewed in 35). The life span of these PGCs is short, and the cells soon die. However, if an additional mitotic regulator, basic fibroblast growth factor (bFGF), is added, the cells continue to proliferate and then produce pluripotent embryonic stem cells with characteristics resembling the cells derived from the inner cell mass (36, 37). Besides soluble growth factors and compounds, adhesion to cell monolayer *in vitro* and neighboring cells *in vivo* is believed to be critical for optimal PGC growth (reviewed in ref. 24). A mixture of soluble growth factors [Kit ligand, leukemia inhibitory factor (LIF), BMP-4, stroma derived factor-1, bFGF] and compounds (*N*-acetyl-cysteine, forskolin, retinoic acid) were needed to sustain the survival and self-renewal of mouse PGCs in the absence of somatic cell support (38). Recently, long-term proliferation was reported for male germ-line stem cells of the mouse in the presence of glial cell line-derived neurotrophic factor, epidermal growth factor, bFGF, and LIF without any feeder cells (39).

In the present culture conditions, *bam*$^-$ GSCs required presence of somatic cells for long-term culture. Possibly some components required to prevent cell death are supplied by these associated somatic cells. Many extrinsic factors participate in the maintenance and the division of GSCs and SSCs in *Drosophila* (40), but it is difficult to elucidate quantitative aspects of each factor *in vivo*. To elucidate how many factors are needed and what are the appropriate concentrations, further study of these cultures of *Drosophila* GSCs will provide the opportunity to identify which additional factors are needed and other conditions for improving the culture medium.

Somatic cells were prominent in the media with Shh and fly extract. Indeed, they formed extended sheets in the latter media. There are several types of mesodermal cells present in ovaries. Because the homogeneous phenotype displayed by all subpopulations of somatic cells, we assume that they are derived from the same progenitor. The muscular ovariole sheaths and ovarian cell layer of the germaria were removed after the GSCs clusters of cells were freed during the mincing step, so they are probably not the source of the somatic cells. Moreover, the morphology of the somatic sheet was much different from those of muscle tissues. When we cultured the anterior tips of germaria, terminal filament and cap cells did not divide. Thus, they are not the source of the somatic cells. Another possible source could be the escort stem cells adjacent to the niche and/or prefollicular cells at the posterior of *bam*$^-$ germaria. There was a mixture of FasIII-positive and negative cells in the cell masses from *bam*$^-$ germaria before culture. After subcloning the somatic cells from fGS/OSS, only FasIII-positive cells are detected. Thus, the most plausible candidates for the somatic cells are descendents of the SSCs (40, 34). Although we cannot exclude the possibility that escort cells contribute to the somatic cell populations, we assume that the primary source is from the large number of cells derived from the SSCs in the *bam*$^-$ germaria. This OSS line would be very useful as a cell feeder layer for culturing various types of genetically marked germ-line cells including primordial germ cells.

The cell line composed of both germ-line and ovarian somatic cells (fGS/OSS) has been established in media supplemented by fly extract, but without the addition of any specific growth factors. So far as we know, this study is the first report of the long-term culture and the establishment of a stable cell line of female germ-line cells in animals. Growth of the germ-line cells depends upon the simultaneous presence of somatic cells that are also positive for producing Dpp. Considering that Dpp is essential for the survival and division of *bam*$^-$ GSCs in culture, Dpp produced by the somatic cells may support the maintenance and proliferation of *bam*$^-$ GSCs. *bam*$^-$ GSCs are apparently attached to the underlying somatic cells by adherens junctions. This relationship resembles the niche for germ-line stem cells at the anterior tip of the germarium *in vivo*. It seems that the association of GSCs and ovarian somatic cells *in vitro* reflects the cellular environment seen in the tumorous germarium in which there is considerable overgrowth of somatic cells at the posterior of the tumorous germarium. The present *in vitro* results suggest that Dpp from prefollicular cells can be another source for Dpp for the expanded GSCs in *bam*$^-$ tumorous ovarioles.

Cultured *bam*$^-$ GSCs retain the normal cellular characteristics of germ-line stem cells. *vasa* and *nanos* are expressed and spectrosome is present in every cell. Furthermore, they are pMad-positive. However, it still is not clear whether these cultured *bam*$^-$ GSCs retain the ability to differentiate into gametes. To elucidate this property, cultured *bam*$^-$ GSCs need to be transplanted into host embryos to determine whether they are functional (27).

Materials and Methods

Detailed description of fly strains used, and staining methods can be found in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site. Either Oregon-RP or flies expressing *vas-egfp* were used as wild-type. *bam*$^-$ GSCs were obtained from flies expressing either *ovo-lacZ* or *vas-egfp* to mark germ-line cells. To overexpress growth factors, *P[actin-Gal4]* flies (obtained from Y. Hiromi, National Institute of Genetics, Mishima, Japan) were mated with *P[UAS-dpp]* (supplied by N. Perrimon, Harvard University), *P[UAS-wg]* (Bloomington stock center), and

P[UAS-shh] (M. V. D. Heuvel, Oxford University, Oxford, U.K.). *shh* of mammalian origin was used instead of *hh* of *Drosophila*, because embryos with *actin-Gal4 UAS-hh* were not viable.

Culture Media. Culture medium used in this study was Cross and Sang's M3 (BF) medium (41), which was prepared from Shields and Sang M3 Insect Medium according to the manufacturer's protocol (Sigma, St Louis, MO). We added 0.6 mg/ml glutathione, 10% FBS (Invitrogen, Carlsbad, CA), 10 milliunits/ml insulin (Sigma), 100 units/ml penicillin (Sigma), and 757 unit/mg streptomycin (Sigma). Embryonic primary cultures were prepared according to the method of Ui *et al*. (42). Fly extract was prepared according to the method of Currie *et al*. (43).

Culturing *bam*$^-$ Germ-Line Cells. Thirty- to forty-day-old female flies were sterilized with 70% ethanol for 10 min, and then their ovaries were removed in bivalent free medium or PBS and washed several times with M3 (BF). *bam*$^-$ GSCs were obtained by either of two methods. First, dissected ovarioles were transferred into Eppendorf tubes and homogenized with a microhomogenizer, and then strained through a 150 nylon mesh, followed by centrifugation. Cells were washed several times by repeating resuspension and centrifugation. Finally, the cells were resuspended in M3 (BF) media and inoculated into a 96-well tissue culture dish. Second, ovarioles were dissociated into ovarioles and each ovariole was fragmented into pieces with fine tungsten needles. After removal of any membranous debris, the cell masses from fragmented pieces were washed several times with M3 (BF) and inoculated into a 96-well tissue culture plate.

Microscopy and Immunohistochemistry. For immunostaining, ovaries or cells were fixed with 4% formaldehyde in PBS for 15 min or 5–10 min, respectively, and washed with PBS or 0.1% PBT for 50 min. After blocking with 5% BSA in PBS or 0.1% PBT, they were incubated overnight at 4°C with the primary antibody. Cells from *bam*$^-$ ovarioles were prepared after ovarioles were fragmented into pieces with fine tungsten needles in PBS and then fixed with the above fixative for 5–10 min. The immunologically stained samples were examined by using phase contrast and epifluorescence microscopy (Olympus IX71, Tokyo, Japan). Images were obtained with Cool SNAP fx (Roper Scientific Inc., Trenton, NJ) and Metamorph (Molecular Devices, Union City, CA).

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