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# The somatic sex determines the requirement for *ovarian tumor* gene activity in the proliferation of the *Drosophila* germline

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## SUMMARY

**Gametogenesis in *Drosophila* requires sex-specific interactions between the soma and germline to control germ cell viability, proliferation, and differentiation. To determine what genetic components are involved in this interaction, we examined whether changes in the sexual identity of the soma affected the function of the *ovarian tumor* (*otu*) and *ovo* genes. These genes are required cell autonomously in the female germline for germ cell proliferation and differentiation. Mutations in *otu* and *ovo* cause a range of ovarian defects, including agametic ovaries and tumorous egg cysts, but do not affect spermatogenesis. We demonstrate that *XY* germ cells do not require *otu* when developing in testes, but become dependent on *otu* function for proliferation when placed in an ovary. This soma-induced requirement can be satisfied by the induced expression of the $98\times10^3$ $M_r$ OTU product, one of two isoforms produced by differential RNA splicing. These results indicate that the female somatic gonad can induce *XY* germ cells to become 'female-like' because they require an oogenesis-specific gene. In contrast, the requirement for *ovo* is dependent on a cell autonomous signal derived from the X:A ratio. We propose that differential regulation of the *otu* and *ovo* genes provides a mechanism for the female germline to incorporate both somatic and cell autonomous inputs required for oogenesis.**

Key words: ovarian tumor, oogenesis, sex determination, *otu*, *ovo*, *Drosophila*

## INTRODUCTION

To examine mechanisms of genetic regulatory pathways during development we are studying the interactions that occur between the somatic- and germline-dependent genes that control oogenesis in *Drosophila melanogaster*. The development of the egg chamber requires the coordinate development of both germline and somatic components of the ovary. During the pupal and adult stages, the germline stem cells divide assymmetrically to produce a cystoblast and a daughter stem cell. Each cystoblast undergoes four mitotic divisions to produce 16 cystocytes (King, 1970). One cystocyte differentiates into the oocyte while the other 15 form large, polyploid nurse cells. The 16 germ cells are surrounded by somatically derived follicle cells that migrate from the wall of the germarium to form the egg cyst. Later in oogenesis, the follicle cells migrate to surround the oocyte and take on specialized fates necessary for egg chamber maturation.

The differentiation of the ovarian soma and germline is dependent on the X:A ratio (the comparison of the number of X-chromosomes to sets of autosomes) of these tissues. In the soma, the X:A ratio regulates the activity of the *Sex-lethal* (*Sxl*) gene, which in turn controls the expression of the somatic sex regulatory pathway that includes the genes *transformer (tra*), *transformer-2* (*tra-2*), and *doublesex* (*dsx*) (reviewed by Baker, 1989; Parkhurst and Meneely, 1994). The *tra* and *tra-2* genes control the regulation of the sexually dimorphic *dsx* gene (Nagoshi et al., 1988; Inoue et al., 1990; Hoshijama et al., 1991). Sexual differentiation of much of the soma depends on the activity of *dsx* (Baker and Ridge, 1980). In contrast, neither *tra*, *tra-2*, or *dsx* gene expression are required in the *XX* germline for oogenesis to occur (Marsh and Wieschaus, 1978; Schüpbach, 1982).

Less is known about the genes required in the germline for sex determination. Recent studies have implicated the *Sxl*, *sans fille (snf)*, *ovarian tumor (otu)*, and *ovo* genes in the regulation of female-specific differentiation of *XX* germ cells (reviewed by Steinmann-Zwicky, 1992). Certain alleles of these genes produce ovarian tumors, a phenotype in which the egg cysts are filled with thousands of small germ cells (King and Riley, 1982). It has been proposed that ovarian tumors result from the disruption of sex determination such that the *XX* germ cells undergo abnormal male development (Oliver et al., 1988, 1990; Steinmann-Zwicky, 1988, 1992). This hypothesis is based on observations that the tumorous *XX* germ cells ectopically express some male-specific gene products and develop morphologies similar to early spermatocytes, including a spherical nucleus, prominent nucleolus, and the assemblage of mitochondria surrounding the nucleus (Oliver et al., 1988, 1990, 1993; Steinmann-Zwicky, 1992; Bopp et al., 1993; Pauli et al., 1993; Wei et al., 1994). However, these tumorous cells also retain some female-specific gene expression (Bae et al.,

1994). Null mutations in *otu* or *ovo* produce agametic ovaries, suggestive of a role in the early proliferation or viability of *XX* germ cells (King and Riley, 1982; Busson et al., 1983; Oliver et al., 1987; Mèvel-Ninio et al., 1989). This makes both genes potential candidates for controlling the initial determination of sexual identity in the *XX* germline.

Complicating our understanding of the genetic regulation of oogenesis is the finding that the soma influences the proliferation and differentiation of the germline in a sex-specific manner. When *XX* germ cells are transplanted into a male soma, they appear to undergo early stages of spermatogenesis (Schüpbach, 1985; Steinmann-Zwicky et al., 1989). Similarly, when *XX* germ cells develop in an *XX* soma genetically transformed to a male differentiated state, they can also undergo male-like differentiation (Seidel, 1963; Nöthiger et al., 1989; Steinmann-Zwicky, 1994). From these results, it is evident that the sexual identity of the soma influences oogenesis, perhaps by affecting the sexual differentiation of the *XX* germ cell.

Soma-germline interactions are also important for spermatogenesis. When *XY* germ cells are transplanted into ovaries they produce ovarian tumors. The tumorous germ cells have morphologies similar to primary spermatocytes, indicating that they may still retain some male identity (Steinmann-Zwicky et al., 1989). However, because these germ cells do not advance to later spermatogenic stages, the female soma must either antagonize further spermatogenic development or lack a necessary factor required to support male germline differentiation. These soma-germline interactions occur relatively early in development as alterations in the somatic sex by *tra* and *dsx* mutations affect the morphology of the germ cells during larval stages (Steimann-Zwicky, 1994).

To better understand the molecular and genetic mechanisms that underlie soma-germline interactions, we are interested in identifying germline genes whose regulation and function depend on the sexual identity of the soma. In this manuscript, we examine whether the regulation and function of *otu* and the phenotypically similar *ovo* genes are controlled by the sexual identity of the somatic tissue. We demonstrate that the presence of a female soma causes *XY* germ cells to require what is normally an oogenesis-specific *otu* product for cell proliferation. This indicates that the *XY* germline has become physiologically more female-like. In contrast, we find that *ovo* activity is required only in *XX* germ cells for viability and proliferation irrespective of the somatic sexual environment.

## MATERIALS AND METHODS

### Fly strains and crosses

Flies were grown on standard corn meal/molasses medium at 25°C. Three classes of *otu* alleles were examined: $otu^{P\Delta 1}$, $otu^{10}$ eliminate *otu* activity and result in primarily agametic *XX* ovaries, $otu^{P\Delta 3}$, $otu^{11}$, $otu^{13}$ produce ovarian tumors, and $otu^{14}$ and $otu^{P\Delta 5}$ are hypomorphic alleles that arrest oogenesis at late stages (King and Riley, 1982; King et al., 1986; Geyer et al., 1993). The molecular characterization of most of these alleles has been previously described (Steinhauer and Kalfayan, 1992; Geyer et al., 1993; Sass et al., 1993). Genetic loci and chromosomes not described are found in Lindsley and Zimm (1992).

*hs-otu* flies carry a second chromosome insertion of a P-element containing a genomic *otu* fragment fused to the *hsp 70* promoter. Under heat shock conditions, this construct suppresses the ovarian defects resulting from *otu* mutations (data not shown). *hs-OTU98* carries a second chromosome P-element insertion which contains the *otu* cDNA encoding the $98\times10^3$ $M_r$ OTU isoform fused to the *hsp70* promoter. *hs-tra* flies carry a P-element containing a fusion of the *tra* female-specific cDNA to the *hsp83* promoter (gift from P. Schedl). Two strains were used, *hs-tra47.2* and *hs-tra53.7*, in which the *hs-tra* construct was inserted on the 2nd and 3rd chromosomes, respectively. At temperatures ≥25°C, *XY* flies carrying this construct differentiate as somatic females.

Pseudofemales of the different genotypes were produced by crossing females carrying the *hs-tra* construct and heterozygous for an *otu*, or *ovo* allele to males with a Y chromosome carrying the dominant eye mutation $B^s$. *XY hs-tra* females were identified as bar-eyed flies that had a female somatic morphology.

Pseudomales were produced by mutant combinations of different *tra*, *tra-2*, of *dsx* mutations. *tra* mutant pseudomales were obtained by crossing $tra^1$ heterozygous mothers with $tra^{v1}$ *kar ry red* heterozygous fathers. $tra^1/tra^{v1}$ flies develop as somatic males. *tra-2* pseudomales were obtained by making the *tra-2* allele homozygous or heterozygous with a second allele, *tra-2B*. *dsx* pseudomales were obtained by mating a dominant *dsx* allele, ie. $dsx^T$, with the loss of function $dsx^1$ mutation (Baker and Ridge, 1980). $dsx^T$ is constitutive for the male-specific *dsx* product required for male somatic differentiation (Nagoshi and Baker, 1990).

### Culture conditions and nuclei staining

Flies of the appropriate genotypes were aged 2-5 days after eclosion at 25°C. The gonads were dissected in phosphate-buffered saline (PBS; 130 mM NaCl, 7 mM $Na_2HPO_4{\cdot}2H_2O$, 3 mM $NaH_2PO_4{\cdot}2H_2O$) then stained by either DAPI or Feulgen reaction. Feulgen staining was carried out using a modification of a published procedure (Galigher and Kozloff, 1971). Ovaries were fixed in Carnoy's solution (1:4 acetic acid: ethanol) for 2-3 minutes, followed by incubation in 1 N HCl for 3-4 minutes. The specimen was washed in PBS and stained in Feulgen reagent until the nuclei were appropriately stained. Staining was stopped by a 5 minute incubation in dilute sulfuric acid. The ovaries were dehydrated by a series of washes in 20%, 50%, 70%, 90%, 100% ethanol. The stained ovaries were cleared in xylene and mounted in Permount.

The fluorescent dye DAPI provided a convenient method for staining nuclei. Isolated gonads were permeablized by incubation in a 1:2 mixture of PBS:heptane for 5 minutes with agitation on a circular shaker. The gonads were rinsed with PBS, then stained with freshly dilute DAPI at 0.5 μg/ml in PBS for 15-20 minutes. Gonads were washed several times in PBS and mounted in glycerol.

## RESULTS

### The requirement for *otu* activity in the germline is dependent on the sex of the soma

Oogenesis in *Drosophila* is influenced by the state of somatic sexual differentiation as well as the X:A ratio of the germline. Sexual incompatibility between the soma and germline results in aberrant germ cell proliferation and differentiation, two processes controlled in females by the germline-dependent *ovo* and *otu* genes. We were interested in determining whether the requirements for *otu* and *ovo* activities in oogenesis depend on the X:A ratio of the germ cells or are influenced by sex-specific interactions with the somatic ovary.

To address this issue, we examined the regulation and function of *otu* and *ovo* in *XY* germ cells developing in a female soma. An *XY* soma can undergo female differentiation due to the ectopic expression of the somatic sex regulatory gene, *tra* (McKeown et al., 1988). We obtained flies carrying

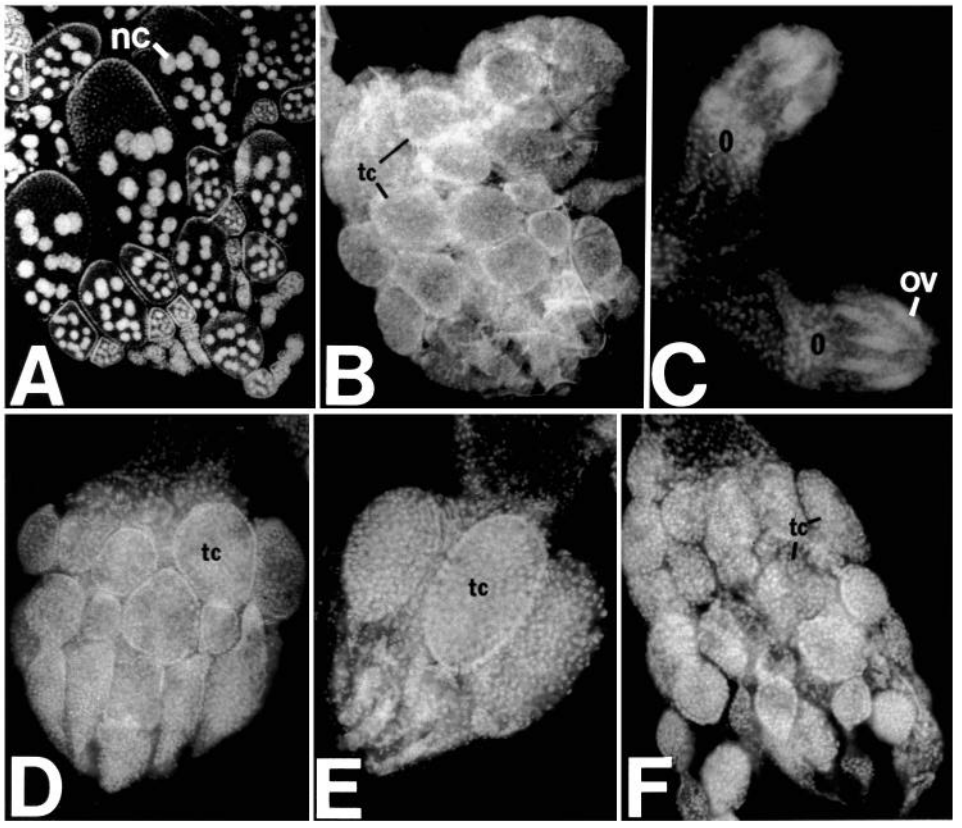


**Fig. 1.** Photomicrographs of Feulgen-stained *XY* pseudo-ovaries. *XY* flies were transformed to somatic females by the ectopic expression of *tra* using the *hs-tra* minigene. The isolated gonads were visualized under fluorescence using a green absorption filter. Feulgen-stained nuclei fluoresce under these conditions. Magnification is the same for all plates. (A) Portion of a wild-type *XX* ovary containing egg chambers with 15 prominent, polyploid nurse cells (nc). (B) $otu^{+}/Y$ pseudo-ovary containing many tumorous cysts (tc). The egg chambers are filled with thousands of small cells. (C) Two $otu^{10}/Y$ pseudo-ovary lobes (o). Ovarioles are present (ov) but are devoid of egg chambers. (D) $otu^{11}/Y$ pseudo-ovary contains several tumorous chambers. $otu^{11}$ is an allele that specifically disrupts the $104\times10^3$ $M_r$ OTU isoform. (E) $otu^{10}/Y$ pseudo-ovary carrying the *hs-OTU98* minigene that expresses the OTU $98\times10^3$ $M_r$ isoform. Three large tumorous egg chambers are visible. Flies were grown under heat shock conditions throughout development. (F) $ovo^{D1rS1}/Y$ pseudo-ovary containing many tumorous egg chambers (tc).

a construct in which *tra* activity is controlled by the *hsp83* heat shock promoter (*hs-tra*). At temperatures ≥25°C, all *XY* flies carrying one copy of this construct differentiate as somatic females. We designate these sexually transformed *XY* flies as 'pseudofemales' and the gonads they produce 'pseudo-ovaries'. To facilitate the phenotypic comparisons, the gonads of pseudofemales were categorized into three groups based on the number of egg cysts present. These groups include agametic pseudo-ovaries (no cysts), gonads containing 1-5 cysts, and gonads with ≥5 cysts. In a wild type female ovary, the developing egg chamber contains 15 large, polyploid nurse cells (nc) and an oocyte surrounded by somatically derived follicle cells (Fig. 1A). A very different phenotype is seen in *XY* pseudofemales produced by the *hs-tra* construct. These sexually transformed flies produce ovaries that are predominantly tumorous (>90%; Table 1), containing egg chambers filled with thousands of small germ cells surrounded by apparently normal follicle cells (Fig. 1B; McKeown et al., 1988; Steinmann-Zwicky et al., 1989).

We examined the effects of two severe *otu* alleles on the pseudo-ovary phenotype. $otu^{10}$ is a point mutation that lacks almost all *otu* function (King et al., 1986), and $otu^{P\Delta1}$ is a deletion of the entire *otu* coding region (Geyer et al., 1993; Sass et al., 1993). Homozygotes of either allele generally result in *XX* ovaries without egg chambers (King et al., 1986; Geyer et al., 1993). We found that *XY* pseudofemales mutant for $otu^{10}$ or $otu^{P\Delta1}$ contain gonads that are similarly agametic (Fig. 1C). Approximately 90% of $otu^{10}/Y$ and $otu^{P\Delta1}/Y$ pseudo-ovaries lack egg cysts with the rest containing fewer than five chambers (Table 1). If *otu* function is restored by introducing a P element carrying a duplication of the *otu* gene, over 90% of $otu^{10}/Y$ pseudofemale ovaries now contain tumorous egg cysts (Table 1). These results demonstrate that *otu* activity is essential for *XY* germ cells developing in an ovary but not in a testis. The presence of a female soma, or the consequent absence of a male soma, causes *XY* germ cells to become more female-like in their physiology as defined by their requirement for the normally oogenic-specific *otu* function.

## The ectopic requirement for *otu* activity in *XY* germ cells is dosage-sensitive and specific to one *otu* isoform

The *XX* ovary morphology is sensitive to changes in the level of *otu* activity. Hypomorphic *otu* alleles can produce tumorous egg cysts ($otu^{P\Delta3}$) or arrest at late stages of egg maturation ($otu^{P\Delta5}$, $otu^{14}$). The difference in phenotype reflects reduced activity of one or both OTU isoforms (King and Storto, 1988; Steinhauer and Kalfayan, 1992; Geyer et al., 1993; Sass et al., 1993). We examined whether the *XY* pseudo-ovary germ cells exhibited a similar dosage-sensitivity.

When *XY* flies mutant for $otu^{P\Delta5}$or $otu^{14}$ were transformed into pseudofemales by introduction of *hs-tra*, they produced sufficient *otu* activity in the *XY* germ cells to allow proliferation, consistent with their relatively mild phenotype in *XX* ovaries (Table 1). The frequency of tumorous egg cysts in pseudofemales mutant for $otu^{14}$ is approximately equal to $otu^{+}/Y$ pseudofemales, while $otu^{P\Delta5}/Y$ pseudofemales have a higher frequency of agametic ovaries. In contrast, pseudofemales mutant for the more severe $otu^{P\Delta3}$ allele ($otu^{P\Delta3}/Y$) produced primarily agametic pseudo-ovaries at frequencies similar to that obtained with $otu^{10}$ and $otu^{P\Delta1}$. These data indicate that the requirement for *otu* activity in *XY* germ cells

**Table 1. The effect of *otu* and *ovo* mutations on X/Y pseudofemales**

| Genotype | Description of *otu* or *ovo* mutation | Phenotype of pseudo-ovaries | | | |
|---|---|---|---|---|---|
| | | agametic | 1-5 cysts* | >5 cysts* | n† |
| $\frac{+}{Y}$ ; $\frac{hs\text{-}tra}{+}$ | wild-type | 0.08 | 0.21 | 0.71 | 164 |
| $\frac{otu^{P\Delta1}}{Y}$ ; $\frac{hs\text{-}tra}{+}$ | deletion of *otu* coding region | 0.92 | 0.06 | 0.02 | 48 |
| $\frac{otu^{10}}{Y}$ ; $\frac{hs\text{-}tra}{+}$ | eliminate all *otu* activity | 0.90 | 0.10 | 0.00 | 102 |
| $\frac{otu^{10}}{Y}$ ; $\frac{hs\text{-}tra}{Dp(otu^{+})}$ | $otu^{10}$ has no activity, $Dp(otu^{+})$ has wild type *otu* activity | 0.01 | 0.27 | 0.72 | 81 |
| $\frac{otu^{10}}{Y}$ ; $\frac{hs\text{-}tra}{hs\text{-}OTU98}$ | $otu^{10}$ has no activity, *hs-OTU98* makes only the $98\times10^3$ $M_r$ isoform | 0.23 | 0.58 | 0.20 | 40 |
| $\frac{otu^{P\Delta3}}{Y}$ ; $\frac{hs\text{-}tra}{+}$ | greatly reduced $98\times10^3$ $M_r$ and $104\times10^3$ $M_r$ activities | 0.93 | 0.07 | 0.00 | 71 |
| $\frac{otu^{11}}{Y}$ ; $\frac{hs\text{-}tra}{+}$ | active $98\times10^3$ $M_r$ but defective $104\times10^3$ $M_r$ isoform. | 0.07 | 0.32 | 0.61 | 110 |
| $\frac{otu^{13}}{Y}$ ; $\frac{hs\text{-}tra}{+}$ | active $98\times10^3$ $M_r$ but no $104\times10^3$ $M_r$ isoform. | 0.05 | 0.22 | 0.73 | 41 |
| $\frac{otu^{14}}{Y}$ ; $\frac{hs\text{-}tra}{+}$ | intermediate $98\times10^3$ $M_r$ and $104\times10^3$ $M_r$ activities | 0.05 | 0.22 | 0.73 | 83 |
| $\frac{otu^{P\Delta5}}{Y}$ ; $\frac{hs\text{-}tra}{+}$ | intermediate $98\times10^3$ $M_r$ and $104\times10^3$ $M_r$ activities | 0.50 | 0.40 | 0.10 | 160 |
| $\frac{ovo^{D1rs1}}{Y}$ ; $\frac{hs\text{-}tra}{+}$ | severe *ovo* allele | 0.09 | 0.05 | 0.87 | 105 |
| $\frac{lzl^{G}}{Y}$ ; $\frac{hs\text{-}tra}{+}$ | severe *ovo* allele | 0.01 | 0.03 | 0.96 | 109 |

*cysts are tumorous
†represents number of gonads examined

developing in a female soma is dosage-dependent in a manner similar to that seen for *XX* germ cells.

The *otu* gene produces two polypeptides of $98\times10^3$ and $104\times10^3$ $M_r$ that result from alternative RNA splicing. *XX* germ cells display differential expression of these isoforms at different stages of oogenesis (Steinhauer and Kalfayan, 1992). We investigated whether the *XY* germ cells developing in pseudo-ovaries required one or both *otu* products. In *XX* flies, the $otu^{11}$, $otu^{13}$, and $otu^{P\Delta3}$ alleles give rise to similar ovarian tumors indicating that all three mutations block oogenesis at about the same stage. Surprisingly the $otu^{11}$and $otu^{13}$ mutations have a different effect on *XY* germ cell development than $otu^{P\Delta3}$. While $otu^{P\Delta3}/Y$ pseudofemales contained predominantly agametic gonads, $otu^{11}/Y$ and $otu^{13}/Y$ pseudo-ovaries produced tumorous egg cysts at frequencies similar to that found in $otu^{+}$ pseudofemales (Fig. 1D, Table 1). The explanation for these differences may be explained by the molecular defects associated with these alleles. $otu^{11}$ and $otu^{13}$ are lesions specific to the $104\times10^3$ $M_r$ OTU product (Steinhauer and Kalfayan, 1992), while $otu^{P\Delta3}$ reduces the expression of both isoforms (Geyer et al., 1993; Sass et al., 1993). These results indicate that wild-type levels of the $104\times10^3$ $M_r$ OTU product is not required for *XY* germ cell proliferation (i.e. tumorous chambers can occur in the absence of this activity), implicating the $98\times10^3$ $M_r$ OTU isoform in this process.

To confirm that the $98\times10^3$ $M_r$ product could function in *XY* germ cells, we tested a construct in which a cDNA specific for the $98\times10^3$ $M_r$ isoform was fused to the *hsp70* promoter (*hs-OTU98*). The presence of *hs-OTU98* caused a significant shift in the pseudo-ovary phenotype. Over 75% of $otu^{10}/Y$ pseudofemales carrying the construct had gonads with tumorous egg chambers, compared to 90% agametic gonads in $otu^{10}/Y$ pseudofemales without *hs-OTU98* (compare Fig. 1C with 1E, Table 1). These results demonstrate that the expression of the OTU $98\times10^3$ $M_r$ product alone can restore *XY* germ cell proliferation in agametic *otu* mutant pseudofemales.

## *XY* germ cells do not require *ovo* activity for viability in pseudo-ovaries

We next examined the effect of *ovo* mutations on germ cell proliferation and development in pseudofemales. The *ovo* gene has a similar range of mutant phenotypes as *otu* (Mohler, 1977; Busson et al., 1983; Komitopoulou et al., 1983; Oliver et al., 1987), suggesting that the two genes may be regulating the same processes in oogenesis. Two severe *ovo* alleles, $ovo^{D1rS1}$ and $lzl^{G}$, produce agametic ovaries in *XX* females that are identical to the mutant gonads in $otu^{10}$ females, and have no effect on spermatogenesis. This indicates that *XX*, but not *XY*, germ cells absolutely require *ovo* activity. We found that sexual transformation of the soma does not alter the sex-specific requirement for *ovo* function. $ovo^{D1rS1}/Y$ and $lzl^{G}/Y$ pseudofemales produce ovaries containing large numbers of tumorous egg cysts (Fig. 1F, Table 1), indicating that $ovo^{-}$ *XY* germ cells can proliferate and form egg cysts when developing in a female soma. Therefore, the requirement for *ovo* activity is primarily dependent on the X:A ratio in the germline. These results represent a significant difference between the regulation of *ovo* and *otu* activities.

### The *otu* and *ovo* genes are required in *XX* germ cells that develop in a male somatic environment

To complement the pseudofemale experiments, we examined how *XX* germ cells mutant for *otu* or *ovo* developed in a male somatic background. Null mutations in *tra* or *tra-2* transform *XX* somatic tissue to a male-like phenotype (Baker and Ridge, 1980; Belote and Baker, 1982). A similar transformation is obtained when a *dsx* null mutation is made heterozygous with a *dsx* allele that is constitutive for the male-specific *dsx* function (Baker and Ridge, 1980). These sexually transformed *XX* flies are denoted as 'pseudomales' (Sturtevant, 1945; Watanabe, 1975; Nöthiger et al., 1989), and we term the male-like gonads produced 'pseudotestes'. The direct control of sexual differentiation by *tra*, *tra-2*, and *dsx* is limited to somatic tissue, as their activities are not required in the germline for normal gametogenesis (Marsh and Wieschaus, 1978; Schüpbach, 1982).

Previous studies suggested that when *XX* germ cells develop in a male somatic environment, they can be induced to undergo early stages of spermatogenesis (Nöthiger et al., 1989; Steinmann-Zwicky et al., 1989). We tested whether these pseudomale germ cells still required *otu* and *ovo* for their proliferation. To compare the effects of *otu* and *ovo* mutations on pseudotestes development, we divided the gonads into three groups based on morphological criteria that allowed relatively quick and unambiguous categorization. Group 1 pseudotestes resemble narrow tubes that have an extended apical lumen. These gonads are either deficient in germ cells or have a disorganized group of undifferentiated cells localized at the apical tip of the testes (Fig. 2A,B). Group 1 most likely represents defects in the proliferation, viability, or the early differentiation of the *XX* germ cells. Group 2 pseudotestes are approximately 2-4 times the volume of group 1 gonads. They are characterized by a bulging lumen that can contain a number of different cell types, although all have small nuclei. These germ cells are usually organized in clusters of approximately 10-30 cells located primarily in the apical half of the testes (Fig. 2C). Group 3 testes are defined by one or more clusters of cells containing larger nuclei that label intensely with nucleic acid-specific stains (i.e. DAPI or Feulgen; Fig. 2D). These nuclei are of varying size and some of the larger ones resemble the polyploid nurse cells found in developing egg cysts. In groups 2 and 3, the germ cells have undergone substantial proliferation but appear to be differentiating abnormally.

When $otu^+$, $ovo^+$ *XX* flies are transformed to a male somatic identity by mutations in *tra*, *tra-2*, or *dsx*, substantial proliferation of the *XX* germ cells usually occurs (Table 2). We find that less than half of the *tra* mutant pseudomales and no more than 26% of the the *tra-2* and *dsx* mutants produce gonads of the severe group 1 category. These observations are in agreement with previous studies indicating that *XX* germ cells are viable and can differentiate (albeit abnormally) in a male soma (Nöthiger et al., 1989). We tested the effect of two severe *otu* alleles, $otu^{10}$ and $otu^{P\Delta 1}$ on the pseudotestes phenotype. In $otu^{10}$ or $otu^{P\Delta 1}$ pseudomales, over 70% of the resulting gonads were of group

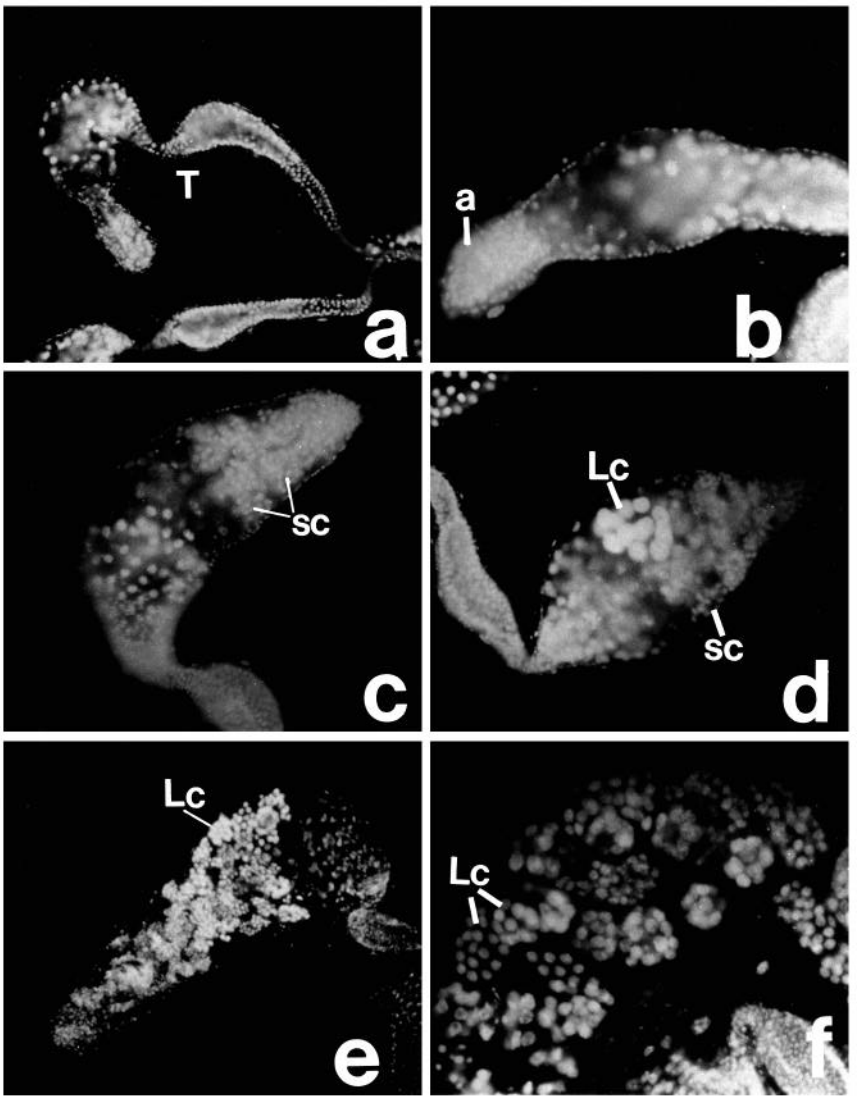


**Fig. 2.** Photomicrographs of *XX* pseudotestes. *XX* flies were transformed to somatic females by mutations in *tra*, *tra-2*, or *dsx*. The isolated gonads were stained with DAPI and visualized under ultraviolet light. (A) *tra* mutant *XX* pseudotestis (T) representing the group 1 phenotype (20× magnification). The gonad is largely devoid of germ cells. (B) Higher magnification view of a typical group 1 pseudotestis showing collection of small cells at the apical (a) tip (40× magnification). (C) *tra* mutant *XX* pseudotestis from group 2 with clusters of small cells (sc) generally localized to the apical half of the gonad (20× magnification). (D) Group 3 *tra* mutant *XX* pseudotestis with one cluster of large nuclei cells (Lc) amid small cell (sc) clusters (20× magnification). (E) *tra* mutant *XX* pseudotestis carrying the *hs-otu* construct (10× magnification). Overexpression of *otu* results in many large cell clusters (Lc) and a much larger gonad. (F) Higher magnification view of large cell clusters (20× magnification).

**Table 2. The effect of *ovo* and *otu* mutations on X/X pseudomales**

| Genotype | *ovo* activity | *otu* activity | Group 1 | Group 2 | Group 3 | *n* |
|---|---|---|---|---|---|---|
| $\frac{otu^{P\Delta 1}}{otu^{+}};\frac{tra}{tra^{v1}}$ | + | + | 0.31 | 0.53 | 0.16 | 55 |
| $\frac{otu^{P\Delta 1}}{otu^{P\Delta 1}};\frac{tra}{tra^{v1}}$ | + | – | 0.85 | 0.09 | 0.05 | 66 |
| $\frac{otu^{10}}{otu^{+}};\frac{tra\text{-}2}{tra\text{-}2}$ | + | + | 0.26 | 0.60 | 0.14 | 42 |
| $\frac{otu^{10}}{otu^{10}};\frac{tra\text{-}2}{tra\text{-}2}$ | + | – | 0.86 | 0.06 | 0.08 | 51 |
| $\frac{otu^{10}}{otu^{+}};\frac{tra\text{-}2}{tra\text{-}2B}$ | + | + | 0.05 | 0.86 | 0.09 | 65 |
| $\frac{otu^{10}}{otu^{10}};\frac{tra\text{-}2}{tra\text{-}2B}$ | + | – | 0.74 | 0.24 | 0.02 | 46 |
| $\frac{otu^{10}}{otu^{+}};\frac{dsx^{T}}{dsx^{1}}$ | + | + | 0.13 | 0.66 | 0.21 | 65 |
| $\frac{otu^{10}}{otu^{10}};\frac{dsx^{T}}{dsx^{1}}$ | + | – | 0.87 | 0.13 | 0.00 | 46 |
| $\frac{ovo^{D1rs1}}{ovo^{+}};\frac{tra\text{-}2}{tra\text{-}2}$ | + | + | 0.12 | 0.64 | 0.24 | 33 |
| $\frac{ovo^{D1rs1}}{ovo^{D1rs1}};\frac{tra\text{-}2}{tra\text{-}2}$ | – | + | 0.89 | 0.10 | 0.01 | 90 |
| $\frac{ovo^{D1rs1}}{ovo^{+}};\frac{dsx^{T}}{dsx^{1}}$ | + | + | 0.00 | 0.35 | 0.65 | 51 |
| $\frac{ovo^{D1rs1}}{ovo^{D1rs1}};\frac{dsx^{T}}{dsx^{1}}$ | – | + | 0.86 | 0.14 | 0.00 | 44 |
| $\frac{ovo^{D1rs1}}{ovo^{+}};\frac{tra}{tra^{v1}}$ | + | + | 0.45 | 0.46 | 0.09 | 118 |
| $\frac{ovo^{D1rs1}}{ovo^{D1rs1}};\frac{tra}{tra^{v1}}$ | – | + | 0.84 | 0.15 | 0.01 | 143 |
| $\frac{hs\text{-}otu}{+};\frac{tra}{tra^{v1}}$ | + | + | 0.00 | 0.04 | 0.96 | 67 |

1 (Table 2). These were significantly smaller than the typical *otu*$^{+}$ pseudotestes, containing mostly abortive or undifferentiated germ cells. This change in pseudotestis phenotype indicates a continued requirement in the *XX* germline for *otu* activity irrespective of the presence of the male somatic gonad.

That the pseudomale *XX* germ cells are still responsive to changes in *otu* activity is further demonstrated by experiments in which *otu* is overexpressed in pseudotestes. By fusing the *hsp70* promoter to the genomic *otu* sequence, we placed *otu* activity under heat shock control (*hs-otu*). Following a daily heat shock regime this construct suppresses the mutant phenotype of the most severe *otu* allele combinations (data not shown). *tra*$^{-}$ pseudomales were constructed that carried one copy of the *hs-otu* fusion construct (*XX ; hs-otu/+ ; tra/tra*). The increase in *otu* activity led to elongated pseudotestes resulting from the presence of many cysts containing 20-30 cells (Fig. 2E,F). These cells had large nuclei that stained intensely with nucleic acid-specific dyes (Feulgen or DAPI), a characteristic of polyploid cells. Although we categorized these as group 3 gonads, they differed from the typical group 3 pseudotestes found in *otu*$^{+}$ pseudomales. The *otu*$^{+}$ group 3 gonads have only 1-2 clusters of polyploid cells (Fig. 2D), and many have only 2-3 such cells in the testes lumen.

A parallel series of experiments were performed to examine the effect of *ovo* mutations on pseudotestis development. As previously noted, *XX* females homozygous for *ovo*$^{D1rS1}$ produce agametic ovaries while *ovo*$^{D1rS1}$*/Y* males undergo normal spermatogenesis. When *XX* flies homozygous for *ovo*$^{D1rS1}$ were transformed into pseudomales, the pseudotestes produced were characteristic of group 1, being either mostly empty or containing only undifferentiated germ cells (Table 2). These results demonstrate that the *XX* germ cells developing in a somatically male environment are still physiologically female in that they require the normally female-specific *ovo* activity for their proliferation and differentiation. However, the *ovo*$^{-}$ pseudomale gonads are not completely agametic. Under phase contrast microscopy, we detected germ cells in *ovo*$^{-}$ pseudomales that were morphologically similar to those found in *ovo*$^{+}$ pseudomales and in the ovarian tumors present in *XY* pseudofemales (Fig. 3). Cells of this type have been identified in previous studies on pseudomales and were classified as spermatogenic (Nöthiger et al., 1989). This indicates that some *ovo*$^{-}$ *XX* germ cells can survive, proliferate, and undergo apparent male differentiation when developing in a male-like soma.

## DISCUSSION

### The female soma alters the physiology of *XY* germ cells

The development of the male and female germlines depends on interactions with the somatic gonad that influence germ cell viability, proliferation, sexual differentiation, and gamete maturation. Complete gametogenesis only occurs when the germline and soma have compatible sexual identities. The nature of the somatic interaction and the identity of the responsive germline genes are not known. In these experiments, we examined how changes in the somatic sex affect the functions and germline requirements for two germline-dependent genes,

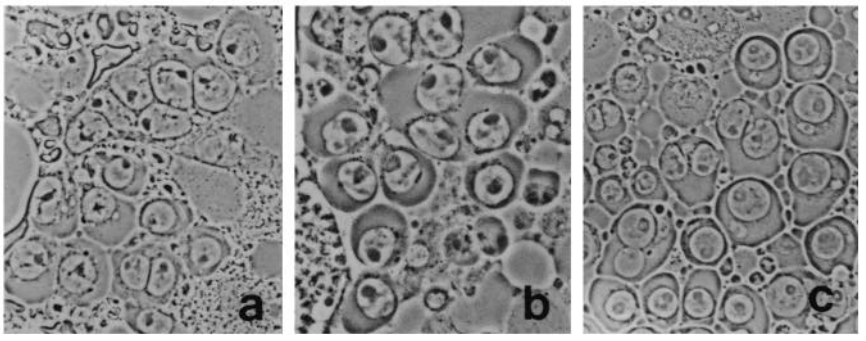


**Fig. 3.** Phase-contrast photomicrographs of germ cells. (A) *XX* pseudomale germ cells homozygous for *ovo*$^{D1rS1}$. The pseudomales were produced using loss of function *tra* mutations. (B) *XX ovo*$^{+}$ germ cells isolated from a *tra*$^{-}$pseudotestis. (C) Germ cells from *XY* pseudo-ovary. The pseudo-ovary was produced by the ectopic expression of *tra* from the *hs-tra* construct.

*otu*, and *ovo*. We found that the function of one gene, *otu*, was sensitive to the identity of the somatic sex.

*otu* is required cell autonomously for several stages of oogenesis (King and Riley, 1982; King and Storto, 1988). Severe *otu* alleles can completely block the development of *XX* germ cells, producing agametic ovaries, but have no significant effect on *XY* spermatogenesis. We demonstrate that the transformation of an *XY* soma to a female differentiated state alters the physiology of *XY* germ cells such that they require *otu* activity in order to proliferate. These data indicate that the sex-specific requirement for *otu* function is not dependent solely on cell autonomous regulation initiated by the X:A ratio, but is influenced by the sexual identity of the soma. Furthermore, the *XY* germline dependency for *otu* function occurs by ectopic expression of only *tra* in the soma. This finding is consistent with recent studies indicating that an *XY* soma transformed to a female state can direct *XX* germ cells to undergo oogenesis (Steinmann-Zwicky, 1994).

A male soma does not eliminate the requirement for *otu* function. *XX* germ cells developing in a pseudotestis require *otu* activity for optimal development, signifying that they still retain some oogenic identity. This may be due to the incomplete male transformation of *XX* somatic tissue caused by mutations in *tra, tra-2*, or *dsx*. When *XY* germ cells are placed in *XX* pseudomales, spermatogenesis is arrested, indicating that the *XX* pseudomale soma is not equivalent to *XY* male tissue (Marsh and Wieschaus, 1978; Schüpbach, 1985). Therefore, the *XX* pseudomale germ cells may be receiving inaccurate or incomplete input from the soma, thereby preventing complete male germline transformation. Nevertheless, it appears that *otu* expression in *XX* germ cells is affected in pseudomales. Experimentally increasing *otu* function by the *hs-otu* construct, causes a significant alteration in the mophology of pseudomale germ cells (Fig. 2E,F), suggesting that germline *otu* activity may be limiting in the genetically transformed pseudomales.

### The proliferation of *XY* germ cells in pseudo-ovaries requires the 98×10$^3$ $M_r$ OTU isoform

The *otu* gene produces at least two protein products of 98×10$^3$ and 104×10$^3$ $M_r$ that differ by an alternatively spliced exon (Steinhauer and Kalfayan, 1992). It has been shown that the 104×10$^3$ $M_r$ isoform controls the differentiation of cystocytes to nurse cells and oocytes by interactions with *Sxl* (Bae et al., 1994), while a role for the 98×10$^3$ $M_r$ isoform has not been directly demonstrated. We show that in *XY* pseudofemales, the 98×10$^3$ $M_r$ isoform is sufficient to support proliferation of the *XY* germline. This suggests that this product may be required for the early proliferation of oogenic germ cells and may be functionally distinct from the 104×10$^3$ $M_r$ isoform.

### The regulation of oogenesis involves both somatic and germline factors

Germ cells are unable to complete gametogenesis if made to develop in gonads of the opposite sex (Schüpbach, 1985; Steinmann-Zwicky et al., 1989). This indicates that there are two components that interact to control the development of the germline. First, there must exist sex-specific somatic factors that can influence gametogenesis. Second, cell autonomous germline factors controlled by the X:A ratio cause *XY* and *XX* germ cells to respond differently to the male or female soma. These two components ensure that complete gametogenesis can only occur if there is compatibility between the sexual identities of the soma and germline. Our results indicate that in *XX* germ cells, the interaction with the soma is mediated, at least in part, by the action of the *otu* gene. In contrast, the requirement for *ovo* function is primarily dependent on the germline X:A ratio. We believe that these two genes act in parallel to control germ cell proliferation and differentiation at different stages of oogenesis. Therefore, their differential regulation provides a simple genetic mechanism for how somatic and germline factors interact to regulate female germ cell development.

In wild-type *XX* flies the somatic ovary forms through the action of *tra* and the other somatic sex regulatory genes. A model detailing these interactions is described in Fig. 4A. We propose that female-specific somatic factors induce *XX* germ cells to produce and require the OTU 98×10$^3$ $M_r$ isoform. In addition, the *ovo* gene is also needed for XX germ cell proliferation in a manner dependent on the germline X:A ratio. The combined functions of the OTU 98×10$^3$ $M_r$ product and *ovo* allow germ cell proliferation and early stages of oogenic differentiation. Subsequent phases of oogenesis require other activities, including *ovo*, *Sxl*, and the 104×10$^3$ $M_r$ OTU isoform.

We speculate that an analogous mechanism occurs in *XY* males (Fig. 4B). The male soma induces the *XY* germ cells to require an as yet unidentified male-specific factor(s) that serves as the male equivalent to the female OTU 98×10$^3$ $M_r$ function. We propose that this factor acts with an *XY* germline autonomous function to direct the proliferation and early differentiation of the *XY* germline. Therefore, as in females, the requirement for soma-germline sexual compatibility occurs because gametogenesis requires the coordinate action of germline genes that respond to somatic influences with germline autonomous genes.

In *XY* pseudofemales, sexual incompatibility between the soma and germline creates an unusual situation. In the soma, the expression of the *hs-tra* construct directs the formation of somatic ovaries despite a male X:A ratio. We propose that the presence of the female somatic gonad induces the *XY* germ cells to express and require the normally female-specific OTU 98×10$^3$ $M_r$ product (Fig. 4C). At the same time, the male X:A ratio in the germ cells causes the production of male-specific germline factors. We suggest that this results in the pseudofemale germ cells receiving a mix of male- and female-specific regulatory inputs that allow proliferation, but cannot support normal differentiation in either a spermatogenic or oogenic pathway. The consequence of this misregulation is the production of ovarian tumors, ie. hyperplastic germ cells capable expressing both male- and female-specific genes. This model provides an explanation for why *ovo* mutations have no effect on the *XY* pseudo-ovary phenotype, while mutations in the OTU 98×10$^3$ $M_r$ product result in agametic gonads.

The studies with *XX* pseudomales are more difficult to interpret because mutations in *tra, tra-2,* or *dsx* lead to incomplete sexual transformation (Marsh and Wieschaus, 1978; Schüpbach, 1985). It is therefore possible that the presence of an interfering female-specific factor or the absence of a necessary male function causes the germline to receive both male and female somatic signals (Fig. 4D). This would explain the variable phenotype associated with *XX* pseudotestes in different genetic backgrounds (Table 2). *otu*$^+$, *ovo*$^+$ *XX* pseudotestes are rudimentary and contain a degenerating germline. Occasionally

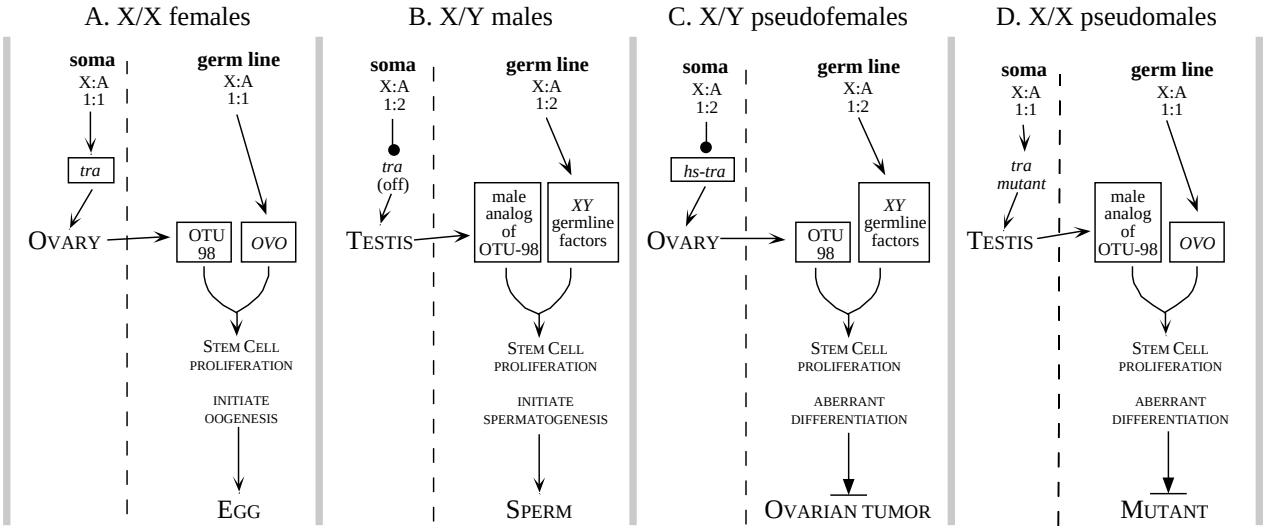


**Fig. 4.** A model for the function of *otu* and *ovo* in oogenesis. Hypothetical mechanisms for the action of *otu* and *ovo* in different genetic backgrounds. (A) The soma-germ line interactions that occur in *XX* ovaries. A somatic X:A ratio equal to 1:1 results in somatically active *tra* function that leads to the formation of the ovary. The female soma induces the germ line to require the OTU $98\times10^3$ $M_r$ isoform. The female X:A ratio in the germ line causes the expression of the female-specific *ovo* gene. Together the OTU $98\times10^3$ $M_r$ product and the *ovo* activity allow *XX* germ line proliferation. (B) In an *XY* soma the *tra* gene is inactive allowing the formation of the male somatic gonad. The male soma induces the germ line to required a male equivalent of the OTU $98\times10^3$ $M_r$ product. The male X:A ratio activates one or more germline-specific factors. The two sets of male-specific activities initiate germ cell proliferation and spermatogenesis. (C) In *XY* pseudofemales the ectopic expression of *tra* by the *hs-otu* construct causes the development of an *XY* somatic ovary. This induces the germ line to express and require the OTU $98\times10^3$ $M_r$ product. The male X:A ratio activates the male germline-specific factors. The OTU $98\times10^3$ $M_r$ isoform and the male germline-specific factors allow germ cell proliferation, but cannot support female differentiation, resulting in ovarian tumors. (D) The absence of *tra* activity (or mutations in *tra-2* or *dsx*) partially transforms the *XX* soma to a male phenotype. The incomplete transformation leads to a mixed signal from the soma that may activate both the OTU $98\times10^3$ $M_r$ product as well as its male equivalent. This mixed regulatory signal leads to the variable germ line phenotype associated with pseudotestes.

cells are arrested at what appears to be early oogenic or spermatogenic stages (Seidel, 1963; Nöthiger et al., 1989). We propose that the partial somatic transformation in pseudomales reduces *otu* activity in the *XX* germline such that only a subset of cells can initiate oogenesis. Mutations in *otu* or *ovo* will further prevent pseudomale germ cells from attempting female development, shifting the pseudotestes phenotype toward a more degenerative appearance. As predicted by this hypothesis, the additional *otu* activity derived from the *hs-otu* construct alters the pseudotestis morphology (Fig. 2E-F, Table 2). The polyploid cells produced in pseudomales by *hs-otu* are reminiscent of the female-specific nurse cells, indicating a more female-like differentiation. However, from our data we cannot determine the degree of sexual differentiation of these cells with any certainty.

## Reconsideration of the criteria used to determine the sexual identity of germ cells.

Pole cell transplantation studies indicate that the development of *XY* germ cells in ovaries is characterized by the large nuclei, prominent nucleoli, and localized mitochondria diagnostic of primary spermatocytes (Steinmann-Zwicky et al., 1989). This was interpreted as indicating that the *XY* germline develop along a male developmental pathway regardless of the somatic sex. A similar morphology is produced by the *XY* germ cells in pseudofemales (Fig. 2), suggesting that these cells may also be spermatogenic (Steinmann-Zwicky et al., 1989). However, this conclusion is inconsistent with our finding that these pseudofemale *XY* germ cells require the female-specific *otu* product in order to proliferate (Fig. 1, Table 1). The requirement for *otu* can be considered a physiological marker of sexual identity, indicating that despite their morphology, the *XY* germ cells in pseudofemales have undergone some female differentiation. We believe this observation suggests that morphological characteristics used to define a ‘spermatogenic’ or ‘oogenic’ state of differentiation may not be completely accurate in appraising the sexual identity of the germline.

This conclusion is consistent with previous observations that the process of sex determination in the germline is mechanistically distinct from that which occurs in the soma (Bae et al., 1994). Somatic sex is determined by the activity state of the *Sxl* gene that is regulated by cell autonomous signals derived from the reading of the X:A ratio. In contrast, we find that germline sex determination occurs by the parallel action of multiple pathways that are initiated by both somatic and germline signals and control nonoverlapping subsets of sexually dimorphic processes. Therefore, a ‘master’ sex determination gene whose expression determines all aspects of sexual dimorphic differentiation, does not exist in the germline. Based on these considerations, we believe that mutations in a single germline gene can cause only partial sexual transformation. This results in an ambiguous sexual phenotype in which a subset of male and female-specific genes are expressed simultaneously. Thus, the ovarian tumor phenotype most likely reflects a sexually ambiguous or dimorphic state in which the proliferation of the tumorous cells occurs by a female-specific

mechanism requiring *otu* activity, while subsequent stages of oogenic differentiation are blocked. This model is consistent with previous results demonstrating that tumorous ovaries mutant for *otu* or *Sxl* still express genes required for oogenesis (Bae et al., 1994).

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