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13. ABSTRACT (Maximum 200 Words) Three Specific Aims (SA) were proposed to test in mice if accelerated oocyte loss caused by Bclw deficiency or Bax gain-of-function drives ovarian surface mesothelial cell (OSMC) transformation: 1) characterize preneoplastic changes in OSMC of bclw ^{-/-} mice with increasing age; 2) determine if disruption of the gene encoding Bax rescues the compromised oocyte survival and the OSMC transformation phenotype in aging bclw ^{-/-} mice; and, 3) test if targeting overexpression of bax to only growing oocytes accelerates oocyte depletion and causes OSMC transformation. To date, we have confirmed the occurrence of OSMC transformation in aging bclw mutants, but there is no progression to invasive carcinoma by 20+ months of age (SA 1). Inactivation of bax restores the compromised oocyte endowment in neonatal bclw mutants to normal, but this protective effect on the oocyte pool is lost in young adult females (SA2). We have constructed a zp3-bax minigene and have generated transgenic mice expressing Bax only in growing oocytes (SA 3). Finally, we have shown in another mouse model that accelerated oocyte loss is directly involved in ovarian tumorigenesis. In addition, we have demonstrated a direct growth inhibitory effect of oocytes on human ovarian cancer cells in vitro.				
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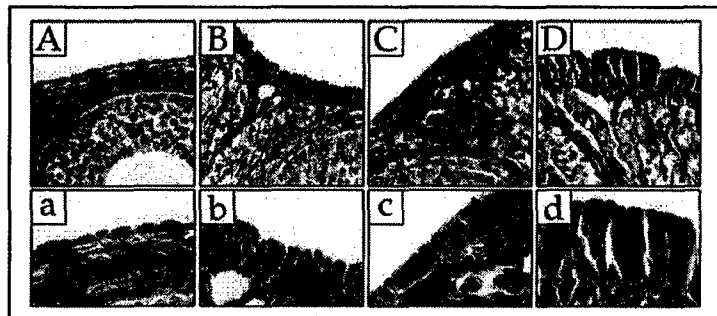
INTRODUCTION: Ovarian cancer accounts for approximately 4% of the total cancers diagnosed in humans per year, and is the seventh most common cause of tumors in women. The majority of patients diagnosed with ovarian cancer are between 50-60 years of age, and 60-70% of these women present with advanced stages of the disease (stage III or IV) at the time of diagnosis. It is widely accepted that the vast majority (85-90% or more) of ovarian cancers in humans originate from the ovarian surface mesothelium (OSM). However the mechanisms underlying the onset of the disease, as well as the early pre-neoplastic changes that occur prior to invasive (stage I) ovarian carcinoma, remain poorly described. In the original application for this *Idea Award*, the investigators provided evidence supporting a novel etiology for OSM cell transformation, that being accelerated depletion of the female germ cell (oocyte) population. Preliminary studies in mice revealed that targeted disruption of the gene encoding Bclw, an anti-apoptotic member of the Bcl2 family of programmed cell death regulators, leads to premature loss of the oocyte pool followed by pre-neoplastic transformation of OSM cells around mid-life (similar to humans). The process appears to phenotypically copy many of the pre-cancerous cellular changes observed in other gynecologic epithelial lineages (e.g., cervical and uterine tissues) prior to the development of stage I carcinoma. Therefore, the following *Specific Aims* were proposed to test the hypothesis that accelerated oocyte loss drives transformation of OSM cells: 1) analyze the pre-neoplastic changes in OSM cells of *bclw*^{-/-} female mice with increasing postnatal age using histopathology and immunohistochemical screening of known antigens expressed by normal versus transformed human OSM cells; 2) determine if disruption of the gene encoding Bax, a Bclw interacting partner required for oocyte apoptosis to proceed, rescues the compromised oocyte survival and the OSM cell transformation phenotype observed in aging *bclw*^{-/-} female mice; and, 3) test if targeting over-expression of the *bax* cell death-promoting gene to *only* growing oocytes of postnatal female mice leads to accelerated oocyte depletion followed by OSM cell transformation.

BODY: As indicated in our annual report for the period between 1 October 2002-30 September 2003, Dr. Grant MacGregor, the Co-Principal Investigator who is overseeing much of the hands-on work with animals, accepted a new faculty position at the University of California-Irvine (UCI) at the end of Year 2 of the proposed studies. At UCI, Dr. MacGregor was provided with temporary laboratory space until ongoing renovations to his dedicated research space were completed – in effect necessitating that Dr. MacGregor re-assemble his laboratory twice in one year. As a consequence, Dr. MacGregor was unable to perform experiments related to this project for the majority of Year 3. This unanticipated delay required us to request a one-year no-cost extension in October 2003, which was processed and approved by Patricia A. Evans (Grants Officer, U.S. Army) on 5 November 2003, to allow us sufficient time to generate the transgenic mice needed for completion of Specific Aim 3 (this has now been successfully accomplished as described below).

Despite this delay, we have: 1) finalized collection of ovarian tissues from all relevant genotypic combinations of aged female mice as outlined for Specific Aim 1; 2) completed all studies outlined for Specific Aim 2; and, 3) conducted additional experiments that further substantiate the central hypothesis of our *Idea Award*, which is to test if a causative relationship exists between oocyte loss and development of ovarian cancer. The results generated thus far from these experiments are detailed below.

For *Specific Aim 1* (analyze the pre-neoplastic changes in OSM cells of *bclw*^{-/-} female mice with increasing post-natal age), we have generated all of the mice needed to investigate changes in the ovaries of *bclw* mutant females during post-natal life into advanced chronological age (20+ months). We have confirmed the occurrence of the pre-cancerous OSM cell transformation in the *bclw*-null females at 9+ months of age (Figure 1); however, the phenotype does not progress into invasive carcinoma by 20+ months of age (not shown). Therefore, we have concluded that the accelerated oocyte depletion caused by *bclw* gene disruption in female mice facilitates hyperplastic growth and transformation of the OSM cells without progression to invasive carcinoma. However, given the multi-step nature of tumor development, in retrospect such a finding may not be surprising. Accordingly, the generation of mice simultaneously lacking the tumor suppressor protein, p53, in the context of accelerated oocyte depletion (see *Specific Aim 3* below) may provide the 'genetic' environment needed to allow the hyperplastic OSM cells, produced as a consequence of oocyte loss, to commit to carcinoma development.

FIGURE 1. Histological analysis of the ovaries of wild-type (A, a) and *bclw*-null (B-D, b-d) female mice at 9-9.5 months of age. Note the dramatic change in the morphology of the OSM cell population from a squamous (wild-type) to a columnar (mutants) phenotype. Further, note the increase in the nuclear-to-cytoplasmic ratio in, and the progressively dysplastic aggregation of, the OSM cells of *bclw* mutant mice. Panels designated by lower case letters are higher magnifications of the images shown in the corresponding panels designated by capital letters.



For *Specific Aim 2* (determine if disruption of the gene encoding Bax, a Bclw interacting partner required for oocyte apoptosis to proceed, rescues the compromised oocyte survival and the OSM cell transformation phenotype observed in aging *bclw*^{-/-} female mice), we have generated all of the mice needed to investigate changes in the ovaries of *bclw/bax* double-null females, along with wild-type and single gene mutant controls, during post-natal life into advanced chronological age (16+ months). We have completed the histomorphometric quantitation of oocyte numbers in wild-type, *bclw* mutant/*bax* wild-type, *bax* mutant/*bclw* wild-type, and *bclw/bax* double mutant females at day 4 postpartum (Figure 2), and have now also finished our series of oocyte counts at day 42 postpartum (Figure 3). These experiments show that *bax* gene knockout prevents the loss of oocytes caused by Bclw-deficiency in neonatal female mice (Figure 2). However, primordial oocyte counts in young adult Bclw/Bax double-deficient females are comparable to those observed in female mice lacking Bclw alone (Figure 3), suggesting that the protective effect of Bax deficiency is lost as the females mature. If our hypothesis is true, *bax* gene knockout should not prevent the occurrence of OSM cell transformation in *bclw* mutant females with age. Histopathological analysis of ovaries collected from the double-null females at 9+ months of age, which were finalized this year, has indeed shown this to be the case (data not shown). In light of these data, generation of the transgenic

mice described in Specific Aim 3 will be critical for us to test if a direct link exists between accelerated oocyte depletion and OSM cell transformation *in vivo*.

FIGURE 2. Quantitative analysis of non-atretic primordial oocyte-containing follicle numbers in wild-type ($bclw^{+/+}/bax^{+/+}$; WT/WT), $bclw$ -null ($bclw^{-/-}/bax^{+/+}$; KO/WT) and $bclw/bax$ double-null ($bclw^{-/-}/bax^{-/-}$; KO/KO) female mice at day 4 postpartum. These data represent the mean $\pm$ SEM of combined results from analyzing oocyte numbers in a minimum of three mice per genotype.

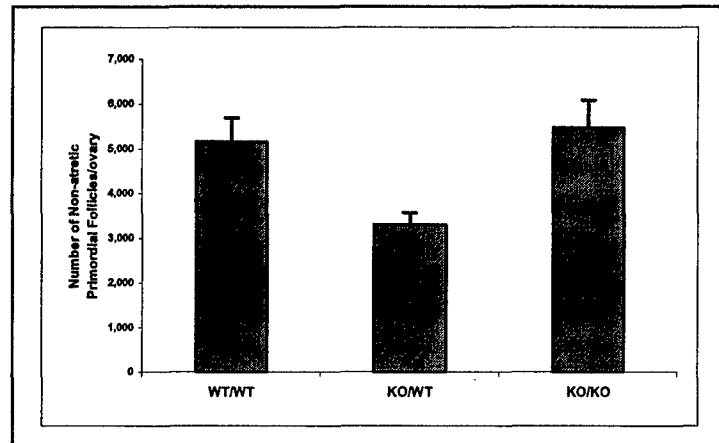
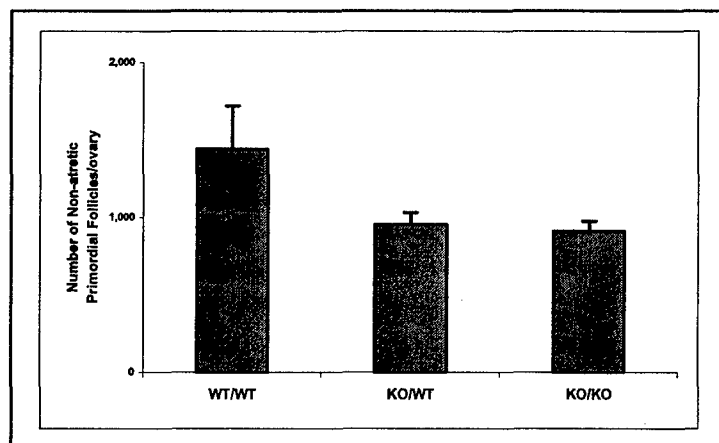


FIGURE 3. Quantitative analysis of non-atretic primordial oocyte-containing follicle numbers in wild-type ($bclw^{+/+}/bax^{+/+}$; WT/WT), $bclw$ -null ($bclw^{-/-}/bax^{+/+}$; KO/WT) and $bclw/bax$ double-null ($bclw^{-/-}/bax^{-/-}$; KO/KO) female mice at day 42 postpartum. These data represent the mean $\pm$ SEM of combined results from analyzing oocyte numbers in three mice per genotype.



For *Specific Aim 3* (test if targeting over-expression of the *bax* cell death-promoting gene to only growing oocytes of post-natal female mice leads to accelerated oocyte depletion followed by OSM cell transformation), we successfully completed construction of the transgene vector containing a fragment of the murine *zona pellucida protein-3 (zp3)* gene promoter (for oocyte-specific expression) upstream of the cDNA coding sequence of human *bax*. The transgene was grown to large scale, sequenced and restriction enzyme-mapped to confirm its fidelity, and purified for pronuclear injection to generate transgenic mice expressing recombinant human Bax protein only in growing oocytes. The first round of pronuclear injections of the transgene into one-cell embryos was conducted in Year 2 of the project, and 8 transgene-positive or founder (F0) animals were generated. These animals were allowed to reach adulthood and then tested for germline transmission of the transgene by mating trials. Unfortunately, none of the 8 founders were capable of germline transmission, necessitating that we repeat the pronuclear injections and produce additional founder animals.

At that time, however, Dr. MacGregor, moved from Emory University to UC-Irvine (see above), which essentially put the project on hold for almost a year until his new lab became fully functional. With an approved no-cost extension for 2003-2004, we have repeated the pronuclear injections and obtained 6 new founder animals (three females and three males), one of which (a female) unfortunately runted and died before adulthood. These animals were matured to adulthood and placed in mating trials to test for germline transmission of the transgene. To date, we have observed that all three transgenic founder males mated with wild-type females have produced transgenic offspring (17 total thus far; 8 males, 9 females), indicating that germline transmission of the transgene has been successfully achieved. Southern blot analyses are now underway to determine if there are multiple sites of integration of the transgene in the founder males (preliminary PCR-based studies have suggested this to be the case). Importantly from a functional standpoint, the transgene appears to be active in that repeated mating of transgenic founder females with wild-type males have produced no transgenic offspring to date. One of the F0 females is appears sterile (no offspring at all) and the other is most likely mosaic for expression of the transgene in that this female has produced wild-type but no transgenic offspring (14 total from two mating trials).

The F1 female offspring have already been placed in mating trials with wild-type males, and we anticipate no offspring from these crosses if the transgene is expressed as expected, leading to premature oocyte death. Should this be the case over at least three mating attempts for each female, the F1 females will be euthanized to assess: 1) transgene expression by immunohistochemistry using a human Bax-specific antibody; 2) oocyte numbers and atresia rates. These results will be compared against those obtained from parallel assessment of offspring derived from non-transgenic founder females mated with wild-type males ('negative' controls). Given the data obtained thus far, we are confident that we have produced the transgenic mouse line needed for completion of Specific Aim 3. Although the project is now formally completed (end date of 1 November 2004), we fully plan to see these experiments to completion over the next year so that all of the data generated under this *Idea Award* can be prepared for publication sometime in 2005.

Also related to *Specific Aim 3*, we plan to test if simultaneous inactivation of the *p53* gene in these transgenic mice either accelerates the time frame for OSM cell transformation or enables the OSM transformation process to progress to invasive carcinoma. To do this, the resultant transgenic mice will be outcrossed with *p53*^{-/-} mice to produce *zp3-bax* transgenic/*p53*-null females for parallel analysis with the *zp3-bax* transgenic/*p53* wild-type mice. We have acquired several breeding pairs of heterozygous *p53* animals, and are breeding these to maintain a small colony of these mice for the crosses. Once again, although the project is now formally completed, we fully plan to pursue these experiments in the coming year. As with the studies described above, all publications resulting from these studies will of course acknowledge DAMD17-00-1-0567 as the source of support. In addition, the Department of The Army (US Army Medical Research and Materiel Command) will be kept up to date on all future outcomes and publications supported by this *Idea Award*.

In addition to completion of the work described above, we have performed and are following up on a number of additional experiments, which fully support the central hypothesis of this *Idea Award*. These experiments are briefly outlined below:

1. Pathological models of oocyte loss and ovarian cancer. As discussed in the original application (*i.e.*, as part of the rationale for this work), other but more noxious methods of induced oocyte loss in female mice, such as irradiation, lead to early ovarian failure that is often followed by the development of pre-cancerous (tubular mesothelial adenomas) and cancerous (complex mixed cell lineage tumors, often containing granulosa cells) lesions in the ovaries later in life (reviewed in *J Exp Pathol* 1987 3:115-145). While our approach is much 'cleaner' with respect to causing premature oocyte elimination in the absence of collateral damage to other cell types (and thus establishing cause-effect relationships are much easier), the radiation-induced ovarian cancer model is nonetheless intriguing because of its association with accelerated oocyte loss. To determine if the development of ovarian cancer in irradiated female mice is related to the oocyte depletion, we utilized an approach recently validated by our lab which protects the female germ line from destruction following exposure to radiation (*Nature Medicine* 2000 6:1109-1114). This entails pretreatment of young adult female mice with a single intrabursal injection of the anti-apoptotic lipid molecule, sphingosine-1-phosphate (S1P), prior to irradiation. The massive oocyte loss observed in vehicle-pretreated irradiated animals does not occur in S1P-pretreated irradiated females, thus providing us with a unique model to explore the contribution of oocyte loss versus global (collateral) radiation-induced ovarian cell damage to the development of ovarian cancer. For these experiments, the mice were treated and irradiated at 2 months of age, and ovaries were then collected at 12 months of age for histopathological analysis.

As shown in Table 1 (see next page), all seven vehicle-pretreated irradiated female mice exhibited nearly complete (n=1) or complete (n=6) ovarian failure – defined by the absence of multi-layer follicles containing viable oocytes. This was correlated with pre-cancerous lesion formation in all seven animals and cancerous lesion formation in three of the seven animals (incidence of tumor formation = 43%). In striking contrast, only three of the seven S1P-pretreated irradiated females exhibited ovarian failure. And while these three animals showed evidence of pre-cancerous lesion formation, none exhibited tumor development. Furthermore, of the four remaining S1P-pretreated irradiated female mice still retaining multi-layer follicles (oocytes), only one exhibited evidence of tubular mesothelial adenoma formation, but again none exhibited evidence of tumor development (Table 1; see next page)). Although additional mice are needed to increase the sample size before we can draw final conclusions, these data strongly support the validity of our central hypothesis that accelerated oocyte loss is a critical factor involved in the pathogenesis of ovarian cancer.

2. Oocytes directly repress human ovarian cancer cell growth. In a second series of experiments, we sought to determine if oocytes possess the capacity to directly regulate the growth of human ovarian cancer cells, using an *in vitro* model. To do this, OVCAR-3 cells (a human ovarian cancer cell line), which are highly resistant to the chemotherapeutic drug doxorubicin *in vitro* (unpublished findings), were grown in the presence of 10% serum *in vitro* without or with 15-100 oocytes collected from prepubertal female mice. The oocytes utilized were either immature (germinal vesicle-intact, GV) or mature (metaphase-II, MII), and in both cases the oocytes were not denuded of the most proximal layer of granulosa cells that form tight junctions with the germ cell. After 48 hours, the numbers of OVCAR-3 cells present per well were counted, and remaining cells were also scored for the extent of apoptosis. These experiments demonstrated that GV-stage, but not MII-stage, oocytes reduced the number of OVCAR-3 cells per well by

approximately 50% versus control cultures. This effect was observed in the absence of OVCAR-3 cell apoptosis, suggesting that the ability of oocytes to reduce ovarian cancer cell growth *in vitro* is due to a suppression of proliferation rather than an induction of apoptosis. Of further interest, the specificity of the growth inhibitory effect for immature (GV-stage) oocytes is intriguing as these are representative of the germ cells present in the immature follicle pool that becomes depleted with age or following pathological insults (such as radiation; see above). These data not only add further support to our central hypothesis, but also begin to extend this hypothesis to human ovarian cancer cells, at least *in vitro*.

TABLE 1. Incidence of ovarian failure, pre-cancerous ovarian lesion (tubular mesothelial adenoma) formation, and ovarian cancer (mixed cell/granulosa cell tumor) development in 12-month old female mice pretreated with vehicle or S1P prior to irradiation at 2 months of age.

<u>Treatment Group</u>	<u>Mouse</u>	<u>Ovarian Failure</u>	<u>Adenomas</u>	<u>Tumors</u>
No Radiation	n=7	No (many oocytes)	No	No
Vehicle+Radiation	SII-1	Yes	Yes	Yes
	SII-2	Yes	Yes	No
	SII-3	Yes	Yes	Yes
	SII-4	No (few oocytes)	Yes	No
	SII-5	Yes	Yes	No
	SII-6	Yes	Yes	No
	SII-7	Yes	Yes	Yes
S1P+Radiation	SII-10	No	No	No
	SII-11	Yes	Yes	No
	SII-12	No	Yes	No
	SII-13	Yes	Yes	No
	SII-14	No	No	No
	SII-15	Yes	Yes	No
	SII-16	No	No	No

KEY RESEARCH ACCOMPLISHMENTS (Bulleted List):

- Generated all single and double gene mutant female mice needed, at all of the appropriate ages, to satisfy the objectives of Specific Aims 1 and 2
- Collected sera and ovaries from all single and double gene mutant female mice needed, at all of the appropriate ages, to satisfy the objectives of Specific Aims 1 and 2
- Confirmed the occurrence of OSM cell transformation in 9+ month *bclw* mutant females, and found that there is no evidence of progression to invasive carcinoma by 20+ months of age (Specific Aim 1)
- Confirmed that simultaneous inactivation of *bax* restores the compromised oocyte endowment in *bclw* mutant females at day 4 postpartum to wild-type levels (Specific Aim 2),

- Demonstrated that the protective effect of Bax deficiency on the oocyte pool of neonatal Bclw deficient females is lost as the animals mature into adulthood (Specific Aim 2)
- Showed that simultaneous inactivation of the *bax* gene does not prevent the occurrence of OSM cell transformation in aging (9+ month) *bclw*-null females (Specific Aim 2)
- Completed construction of the *zp3-bax* transgene vector needed for generation of mice with targeted over-expression of the pro-apoptotic Bax protein to only growing oocytes (Specific Aim 3)
- Confirmed fidelity of the *zp3-bax* transgene vector by sequence analysis and restriction enzyme mapping, and purified transgene for pronuclear injections (Specific Aim 3)
- Performed first round of pronuclear injections and generated 8 founder offspring exhibiting incorporation of the transgene in the genome (Specific Aim 3)
- Tested if any of the 8 founder *zp3-bax* transgenic animals were capable of germline transmission of the transgene to offspring (Specific Aim 3)
- Generated a colony of p53 mutant mice for future crossing with the *Zp3-bax* transgenic mice (Specific Aim 3)
- Determined that ovarian cancer development in aging female mice resulting from gonadal irradiation in young adult life was related to accelerated oocyte depletion (additional experiments not originally proposed)
- Demonstrated that small numbers of immature, but not mature, oocytes contained within granulosa cell complexes significantly repress the proliferative potential of human ovarian cancer cells *in vitro* (additional experiments not originally proposed)
- Performed second round of pronuclear injections and generated 6 founder (F0) offspring exhibiting incorporation of the transgene in the genome (Specific Aim 3)
- Conducted mating trials with the 5 remaining founder *zp3-bax* transgenic founder animals from the second round of pronuclear injections (Specific Aim 3)
- Established the occurrence of germline transmission of the *zp3-bax* transgene to F1 offspring from all three F0 transgenic males (Specific Aim 3)
- Demonstrated that the transgene is likely expressed in, and functioning to kill, growing oocytes as expected based on the inability of either transgenic F0 female to produce transgenic offspring when mated with wild-type males (Specific Aim 3)

REPORTABLE OUTCOMES:

Abstracts

- Maravei DV, Ross A, Waymire K, Morita Y, Robles R, Korsmeyer SJ, MacGregor GR, Tilly JL. *Bax* gene inactivation rescues gametogenic failure caused by Bcl-w-deficiency but not *ataxia telangiectasia mutated (Atm)* gene knockout. Proceedings of the 82nd Annual Meeting of the Endocrine Society, Toronto, Ontario, Canada 2000; pp 317-318.
- Tilly JL. Genes and mechanisms of ovarian failure. Proceedings of the 84th Annual Meeting of the Endocrine Society, San Francisco, CA 2002; p 41.

Book Chapters

- Pru JK, Tilly JL. Apoptosis gene knockouts. In: Henry AL, Norman AW, editors. Encyclopedia of Hormones. San Diego: Academic Press; 2003. p. 157-65
- Tilly JL, Pru JK, Rueda BR. Apoptosis in ovarian development, function and failure. In: Leung PCK, Adashi EY, editors. The Ovary (2nd Edition). San Diego: Academic Press; 2003. p. 321-52.

Presentations/Invited Lectures

- NIH Workshop on The Ovary: Genesis, Function and Failure, Bethesda, MD; "Genetics of Programmed Cell Death (PCD) in Normal and Premature Ovarian Failure"; March 2000.
- Department of Cell Biology Distinguished Lecturer Series, University of Medicine and Dentistry of New Jersey, Stratford, NJ; "Use of Gene Knockouts to Navigate the Muddy Waters of Oocyte Apoptosis"; April 2000.
- NICHD-Sponsored 3rd Symposium on Frontiers in Reproduction, The Oocyte and Human Reproduction, Boston, MA; "Prenatal Oocyte Apoptosis: A Genetic Balancing Act"; June 2000.
- Symposium on Oocyte Development, 82nd Annual Meeting of the Endocrine Society, Toronto, Ontario, Canada; "Gene Knockout Analysis of Oocyte Apoptosis During Gametogenesis"; June 2000.
- Gordon Research Conference on Mammalian Gametogenesis and Embryogenesis, New London, CT; "Programmed Cell Death Signaling Pathways in Developing Oocytes"; July 2000.
- 18th Annual Meeting of the Japanese Society for Fertilization and Implantation, Okazaki, Japan; "Genetics of Programmed Cell Death in Mammalian Oocytes"; July 2000.
- Institut de Recherches Servier, Suresnes, Paris, France; "Ovarian Failure and Ovarian Cancer: Two Sides of the Same Coin?"; November 2000.
- Annual Meeting of the Triangle Consortium for Reproductive Biology (TCRB), NIEHS, Research Triangle Park, NC; "Mouse and Human Models to Study the Molecular Genetics of Ovarian Cell Death"; January 2001.
- Keystone Symposium on the Molecular Mechanisms of Apoptosis, Keystone, CO; "Apoptosis in Mouse and Human Models of Ovarian Failure"; January 2001.
- Symposium on Ovarian Failure, 84th Annual Meeting of the Endocrine Society, San Francisco, CA; "Genes and Mechanisms of Ovarian Failure"; June 2002.
- Oregon Regional Primate Research Center, Portland, OR; "Communing the Death Sentence: How Oocytes Strive to Survive"; November 2002.
- Derald H. Rittenberg Cancer Center, Mount Sinai School of Medicine, New York, NY; "Strategies to Preserve Fertility in Cancer Patients Uncover a Novel 'Tumor Suppressor' Function of the Female Germ Line"; February 2003.

Animal Models

- Mutant female mice lacking *bclw*
- Double-mutant female mice lacking both *bclw* and *bax*
- Irradiated female mice with SIP-based preservation of the oocyte pool
- Transgenic mice with targeted expression of the pro-apoptotic *bax* gene in growing oocytes

REFERENCES: no data have been published in *manuscript* form as of yet.

APPENDICES: none.