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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 begun to generate transgenic mice expresson 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.

14. SUBJECT TERMS
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Table of Contents

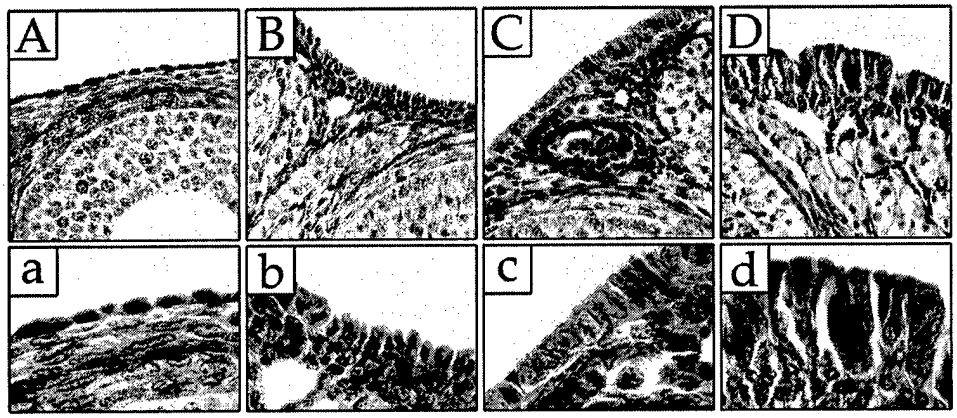
INTRODUCTION: Ovarian cancer accounts for approximately 4% of the total cancers diagnosed in humans per year, and is the seventh most common cause of tumors in women. The majority of patients diagnosed with ovarian cancer are between 50-60 years of age, and 60-70% of these women present with advanced 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 a mystery. 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 post-natal 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 post-natal 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 2001-30 September 2002, 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 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 that we request a one-year no-cost extension in October 2003, which has been processed and approved by Patricia A. Evans (Grants Officer, U.S. Army) on 5 November 2003, to allow us sufficient time to complete the studies proposed.

Despite this delay in being able to complete Specific Aim 3, we have: 1) finalized collection of ovarian tissues from all relevant genotypic combinations of aged female mice for immunohistochemical screening of known tumor antigens 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 related to the completion of this *Idea Award* 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 (data not shown). Therefore, we must conclude 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 multifactorial/multistep 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.

Fig 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. Furthermore, note the increase in the nuclear:cytoplasmic ratio in, and the 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. As a consequence, it is highly unlikely that *bax* gene knockout would subsequently prevent the occurrence of OSM cell transformation in *bclw* mutant females with age, a conclusion now supported by histopathological analysis of ovaries collected from the double-null females at 9+months of age (data not shown), completed this year. In light of these data, generation of the transgenic mice described in Specific Aim 3 is critical to establishing a direct link between accelerated oocyte depletion and OSM cell transformation *in vivo*.

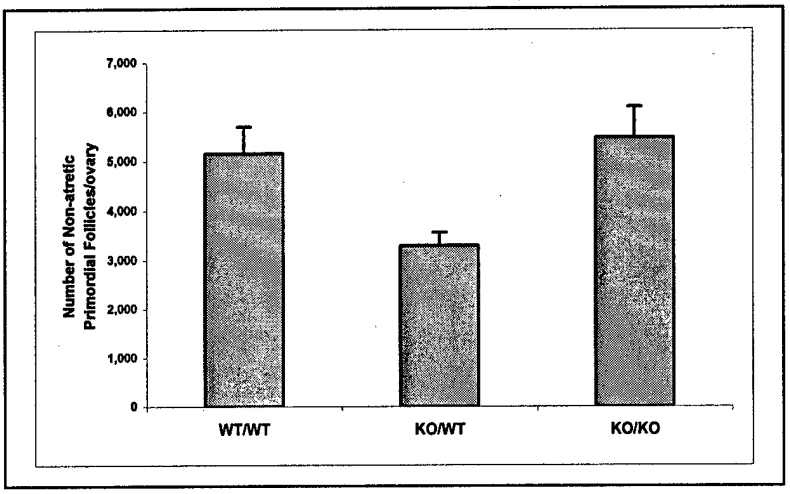


Fig. 2. Quantitative analysis of primordial oocyte-containing follicle endowment in wild-type ($bclw^{+/+}/bax^{+/+}$ or WT/WT), *bclw*-null ($bclw^{-/-}/bax^{+/+}$ or KO/WT) and *bclw*/*bax* double-null ($bclw^{-/-}/bax^{-/-}$ or KO/KO) female mice at day 4 postpartum. These data represent the mean±SEM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

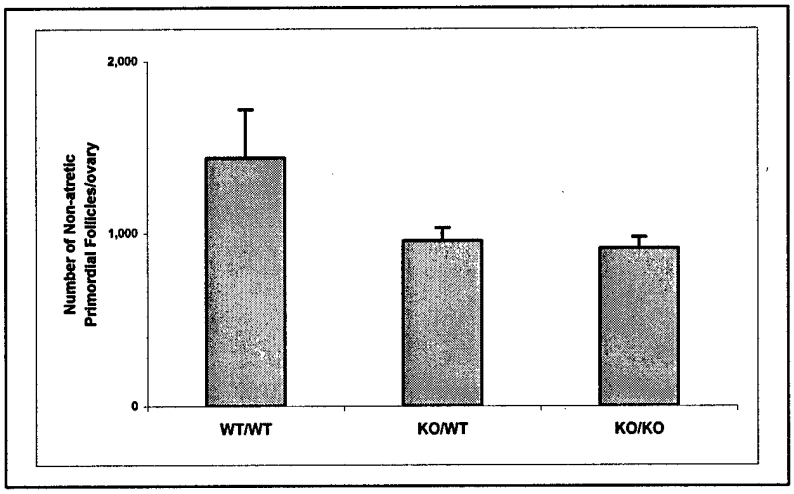


Fig 3. Quantitative analysis of primordial follicle numbers in wild-type ($bclw^{+/+}/bax^{+/+}$ or WT/WT), *bclw*-null ($bclw^{-/-}/bax^{+/+}$ or KO/WT) and *bclw*/*bax* double-null ($bclw^{-/-}/bax^{-/-}$ or KO/KO) female mice at day 42 postpartum. These data represent the mean±SEM of combined results 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 have 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 animals were generated. These animals were allowed to reach adulthood and then tested for germline transmission of the transgene. Unfortunately, none of the 8 founders were capable of germline transmission, necessitating that we repeat the pronuclear injections and produce additional founder animals. At this time, however, Dr. MacGregor, moved from Emory University to UC-Irvine (see above), and thus has been as-yet unable to complete the generation of transgenic mice with germline transmission. With Dr. MacGregor's lab now fully functional, we are confident that these mice will be generated and that the histopathology can be conducted on ovaries collected from *zp3-bax* transgenic mice at 9+ months of age over this fourth and final year of the project under the requested no-cost extension. Also related to *Specific Aim 3*, and as indicated in our previous annual report, 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, once generated, will be immediately outcrossed with $p53^{-/-}$ mice (Jackson Laboratories) to produce *zp3-bax* transgenic/*p53*-null females for parallel analysis with the *zp3-bax* transgenic/p53 wild-type mice. We acquired several breeding pairs of heterozygous *p53* animals, and are breeding these to maintain a small colony of these mice in anticipation of generating of the transgenic mice.

Additional experiments conducted in support of the central hypothesis (note that these studies, along with the *p53* mutant mouse studies described above, will be submitted in a revised Statement of Work to the Contracting Officer Representative):

1. Pathological models of oocyte loss and ovarian cancer. As discussed in the original application (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 laboratory 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, 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 (*viz.* 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). Although additional mice are needed to increase the sample size before we can draw final conclusions (these experiments are ongoing), 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.

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.

Treatment Group	Mouse	Ovarian Failure	**Adenomas**	**Tumors**
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

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*.

KEY RESEARCH ACCOMPLISHMENTS (Bulleted List; Changes and Additions from 2001-2002 Annual Report are Highlighted by Underlining):

•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 the *bax* gene restores the compromised oocyte endowment in *bclw* mutant females at day 4 postpartum to normal (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 Aim2)

•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, as needed for 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 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

•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*

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

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; "Commuting the Death Sentence: How Oocytes Strive to Survive"; November 2002.
- Derald H. Ruttenberg 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 S1P-based preservation of the oocyte pool

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

APPENDICES: none.