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14. ABSTRACT
Women with germline mutations in BRCA1 are strongly predisposed to cancers of the ovary and fallopian tubes. Given the strong link between menstrual activity and risk of ovarian cancer in the general population, we hypothesized that BRCA1 might predispose to ovarian cancer indirectly, by influencing ovarian granulosa cells, which play an important role in controlling menstrual cycle progression. We used the Cre-lox system to inactivate the mouse Brca1 gene in granulosa cells. A truncated form of the FSH receptor promoter was used as Cre driver. Our most recent results show that a majority (40 of 59) of mutant mice develop grossly visible cystic tumors either attached to the ovary or the uterine horns. These tumors resembled human serous cystadenomas, which are benign tumors made up of the same cell type as ovarian serous carcinomas. We confirmed that these tumors carried only the wild type allele of the floxed Brca1 allele while the mutant form was present in granulosa cells. These findings strongly support our initial hypothesis that Brca1 influences tumor development cell non-autonomously, through an effector secreted by granulosa cells. We developed tools such as long-term cultures of human granulosa cells, which will be used to compare the gene expression patterns of wild type and mutant granulosa cells in the second year. We also obtained preliminary data suggesting that the dynamics of the hormonal changes associated with the estrous cycle are slightly different in mutant mice, suggesting that the influence of granulosa cells on tumor predisposition in this animal model may be mediated through their role in the ovulatory cycle. Finally, we show evidence that the mutant mice show increased proliferative activity in epithelial cells lining the uterus and endometrium and endometrial glands, strongly supporting our view that ovarian epithelial tumors are derived from components of the mullerian tract.

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Table of Contents

Introduction

Most individuals with familial predisposition to ovarian carcinoma carry a germline mutation in the *BRCA1* gene. In spite of extensive efforts to understand the normal function of the BRCA1 gene product during the last decade, the reason for the association between *BRCA1* mutations and disease predisposition are still unclear. In particular, there is no good explanation for the site specificity of the cancers that develop in these individuals. Indeed, although the *BRCA1* gene is expressed in most cell types, mutation carriers develop primarily breast and ovarian/fallopian tube tumors. We hypothesized that BRCA1 controls ovarian cancer predisposition in a cell non-autonomous manner, through a factor secreted by ovarian granulosa cells. The idea is that reduction in BRCA1 activity in granulosa cells results in changes in the secretion, by those cells, of one or several circulating or paracrine factors that influence the cell of origin of ovarian tumors. We tested this hypothesis by inactivating the *Brca1* gene in mouse ovarian granulosa cells specifically. We had reported, in our initial grant application, that over 50% of the mice carrying this targeted gene knockout developed ovarian/tubal tumors morphologically very similar to human ovarian serous cystadenomas in strong support of our hypothesis. We proposed to elucidate the mechanism of tumor predisposition in this mouse model by identifying the signaling molecules downstream of Brca1 that control tumorigenesis (aim #1) and to test the hypothesis that tumor development in this animal model results from an effect of Brca1 on the epithelial cells lining the entire mullerian tract. This latter hypothesis has important implications on the understanding of the exact site of origin of human ovarian epithelial tumors.

Body

The progress related to each task mentioned in the original statement of work is summarized below:

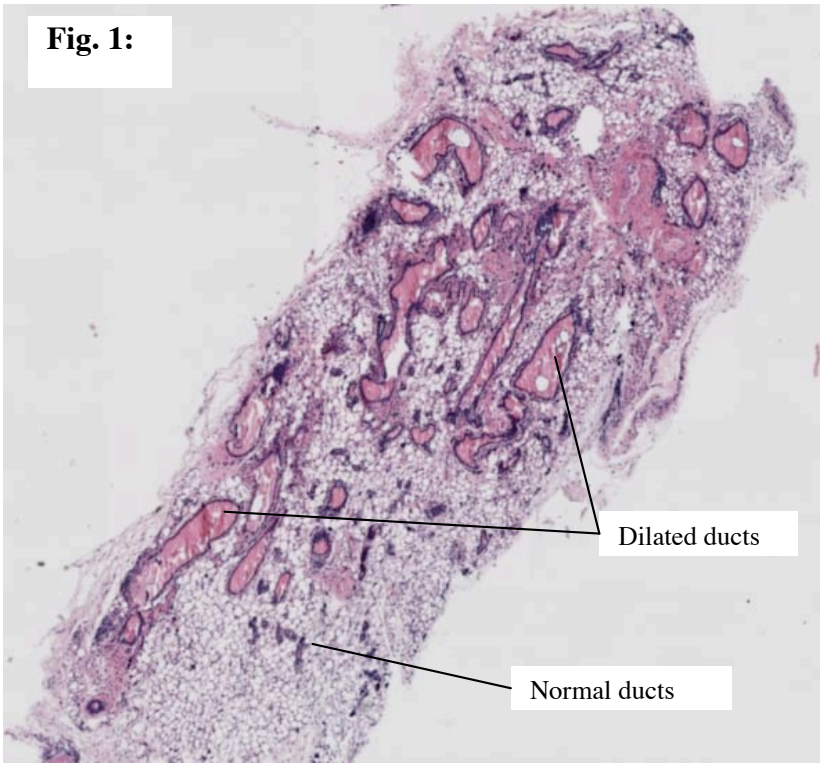
Task #1: Breed and genotype mice to support aims #1 and #2. According to our initial statement, this task was essentially meant to maintain our mouse colonies in order to secure enough mice to support the other tasks. We also continued to examine the phenotype of mutant *versus* wild type mice and, in that regard, expanded the number of wild type (control) mice examined in order to increase the strength of our argument. The results confirmed our earlier findings that the *Brca1* knockout in our mutant mouse population was restricted to granulosa cells, at least in the area of the pelvis. When genomic DNA from a total of 5 ovarian cysts and 3 uterine cysts was amplified enzymatically using primers specific for the mutant *Brca1* alleles, the only specific product seen was with one of the ovarian cysts. The presence of a product in this sample was probably due to the fact that this lesion had not been microdissected and was probably contaminated with ovarian stromal elements. None of the microdissected ovarian or uterine tumors examined showed evidence of *Brca1* rearrangement although all tumors showed a product when primers specific for the wild type allele were used. In contrast, products for the rearranged allele were readily obtainable with genomic DNA

from ovarian stroma. The authenticity of these products were verified by DNA sequencing.

As of now, about 68% of the mutant mice developed either ovarian or uterine tumors or tumors in both of these organs. The tumors expressed nonsquamous keratins and did not express mullerian inhibiting substance (a marker of granulosa cells), attesting to their epithelial nature. In addition, the tumors expressed estrogen and progesterone receptors. This work has now been published in Current Biology (see attached manuscript by Chodankar et al). The potential impact of this work is underscored by the fact that it was featured in the News & View section of Nature, April 14, 2005 issue.

Given that humans with germline BRCA1 mutations are predisposed to breast carcinoma in addition to cancers of the mullerian tract, we also started examining the mammary glands of wild type and mutant animals. The number of mammary glands examined is still small, as we first needed to train ourselves at finding and examining this organ, especially since we wanted to examine older mice, where mammary glands are often atrophic and not readily seen. We examined the fourth mammary gland pair in eight mutant and eight wild type mice with ages ranging between 12 and 18 months. All mice used in this study were virgins. Mammary glands from three of the mutant mice showed dilated breast ducts filled with proteinaceous fluid alternating with areas showing small, inactive ducts. The remaining mutant and all wild type mice showed only small inactive ducts. A representative section of dilated ducts from one mutant is shown in Fig. 1. The presence of such cysts is clearly abnormal and is in fact reminiscent of fibrocystic disease in humans. The fact that mutant mice develop abnormalities in the breast in addition to the reproductive tract argues strongly in favor of the relevance of our animal model to humans.

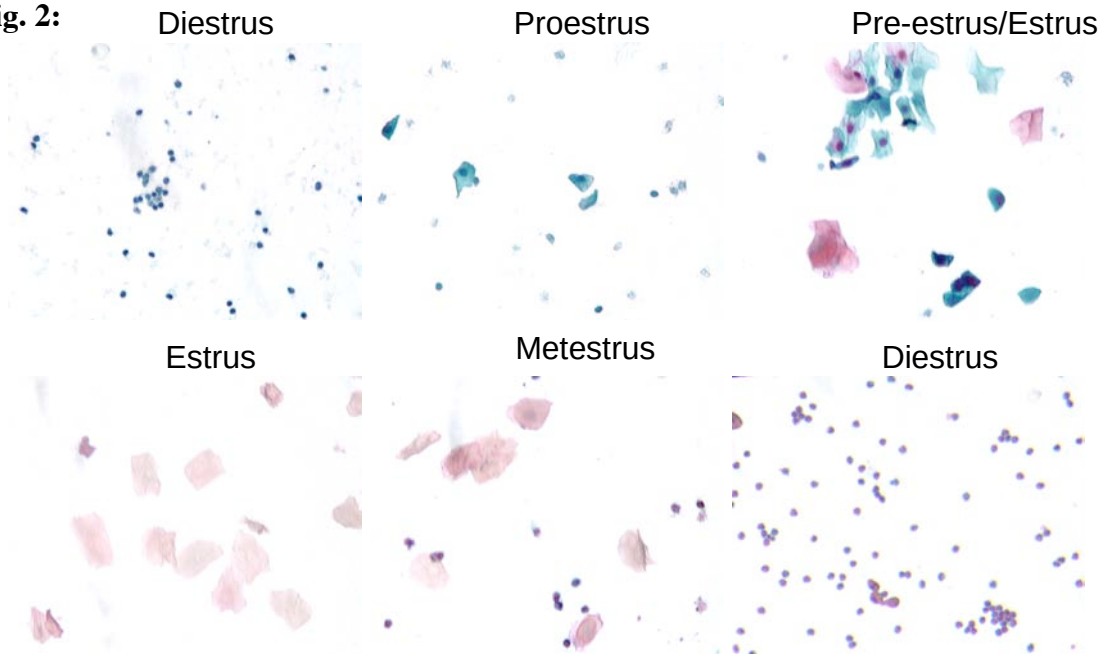
Fig. 1:



We consider this task as completed.

Task #2: Test specific candidate hormones for their potential regulation by BRCA1 in vivo. The original plan was to measure circulating levels of various hormones at specific stages of the estrous cycle, which we proposed to evaluate based on the color of the vaginal mucosa. It turned out that this method of determining the stage of the estrous cycle is very inaccurate and subjective. We therefore learned to perform Papanicoulou stains (PAP stains) on vaginal lavages of mice and trained ourselves on interpreting these stains in order to more accurately evaluate the stage of the cycle. This method turned out to be very reliable and reproducible. We plan to report it in the "Method" section of a future manuscript comparing the differences in circulating hormonal levels between normal and mutant mice and anticipate that this technique will attract the interest of several scientists. An illustration of the cytopathological changes associated with each stage of the estrous cycle is illustrated in Fig. 2:

Fig. 2:



The diestrus and proestrus phases correspond to the follicular phase of the menstrual cycle in humans. Estrus corresponds to ovulation while metestrus corresponds to the luteal phase. Vaginal smears obtained at the diestrus phase show primarily inflammatory cells. Immature (green) epithelial cells start appearing at proestrus. By pre-estrus, the inflammatory cells have completely disappeared and an admixture of immature (green) and mature (orange) epithelial cells are seen. Estrus is characterized exclusively by mature epithelial cells. Metestrus, which is often subdivided into metestrus I and metestrus II, shows mature epithelial cells admixed with an increasing number of inflammatory cells. Although these changes have been described in the past using other staining techniques such as Giemsa, we are not aware of any previous report based on PAP stain, which greatly facilitates evaluation of parameters such as cellular maturity.

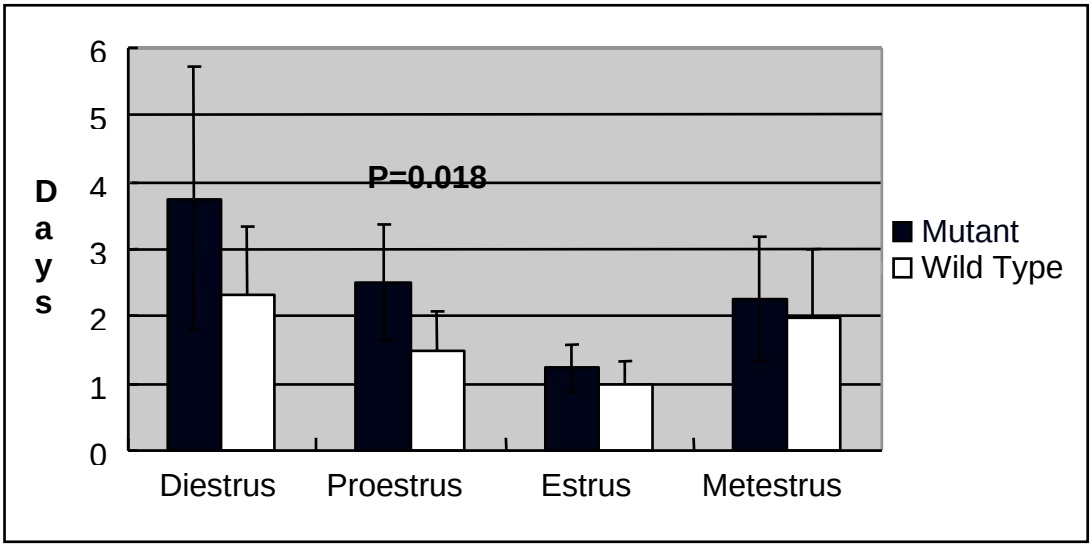
We used a group of mice in which the length of the different phases of the estrus cycle had been measured at 3-4, 7-8, and 14 months of age because we wanted to examine potential correlations between changes in circulating hormone levels, if present, and changes in the dynamics of the estrus cycle. Thus, all mice were kept alive for 14 months and measurement of hormone levels were done at this time point. We could not perform these measurements at the earlier time points because it turns out that the amount of blood needed to measure those two hormones alone is too large to be compatible with survival. Thus, all blood samples were drawn from cardiac puncture just before the mice were sacrificed. In retrospect, we now realize that this may not have been the best strategy because hormone levels vary significantly between individual mice at this older age. In addition, many of the mice had stopped cycling at 14 months. Finally, interpretation of the significance of the results is complicated by the fact that the levels of this hormone rise substantially after one year of age. Thus, the levels that we measured in 14 months old mice may not reflect the levels that were present when the mice were younger. In addition, any influence of circulating hormone levels on tumor predisposition presumably happened when the mice were younger.

The average level of FSH in mutant mice was found to be 34.7 +/- 28.5 ng/ml compared to 21.1 +/- 16.8 ng/ml in wild type mice. These differences were not statistically significant. The average number of circulating estradiol levels were 11.3 +/- 5.6 pg/ml in mutant mice compared to 21.3 +/- 14.4 pg/ml in wild type mice. These results were of borderline statistical significance ($P = 0.07$, t test). There was no significant difference in mutant mice that had developed cysts compared to those in which no cysts were found in this age group.

Although we have completed the studies initially included in this task, we decided not to take these results at face value given the limitations mentioned above. We are repeating these measurements using younger mice. We hope that the differences in estradiol levels will be reproducible in this younger group of mice and that the statistical significance will be greater. We also hope that some differences can be demonstrated between circulating FSH levels in the two groups of mice.

As explained in yearly progress reports, we also expanded this task to include measurements of the average length of each phase of the estrus cycle in mutant versus wild type mice. Figure 3 shows the results of measurements of the length of each phase of the estrus cycle in 3-4 month old mice. These measurements were calculated by determining the stage of the cycle each day over a period of 3 weeks.

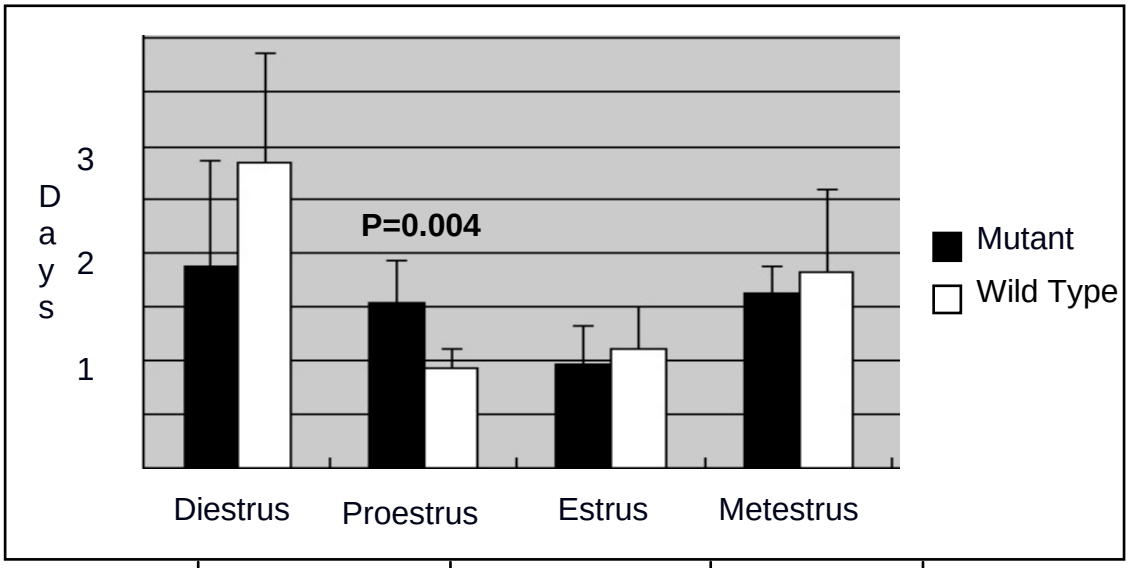
Fig. 3:



Avg ± SD (days)	Diestrus	**Proestrus**	Estrus	Metestrus
Wild Type **(N=7)**	2.3 ± 1.0	**1.5 ± 0.6**	1.0 ± 0.3	2.0 ± 1.0
Mutant (N=9)	3.8 ± 1.9	**2.5 ± 0.9**	1.2 ± 0.4	2.2 ± 0.9

Figure 4 shows results of a similar experiment performed in 7-8 month old mice:

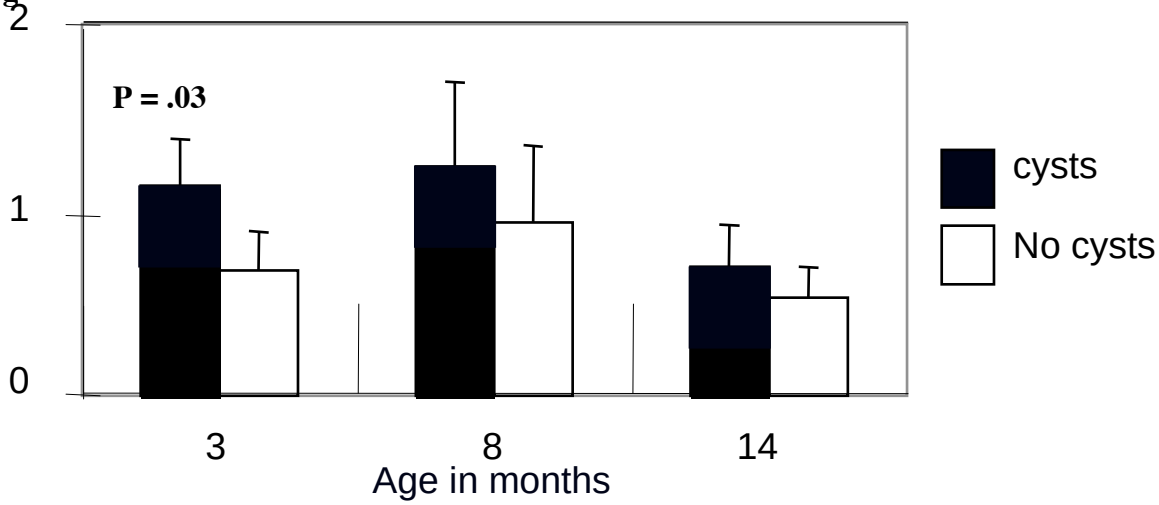
Fig. 4:



Avg ± SD (days)	Diestrus	**Proestrus**	Estrus	**Metestrus**
Wild Type (N=6)	2.833 + 1.03	**0.9166 + 0.17**	1.1 ± 0.4	1.83 ± 0.75
Mutant (N=8)	1.875 + 0.99	**1.53 ± 0.39**	0.98 ± 0.35	1.62 ± 0.25

Both of these studies show a statistically significant elongation of the proestrus period in mutant mice. Similar studies were also repeated in 12-15 month old mice but the results although showing a similar trend, were not statistically significant in this age group probably because mice in general do not cycle very regularly after one year. We sacrificed the mice after this life long study, which allowed us to test the hypothesis that the changes in the average length of specific phases of the cycle that we have now demonstrated to be present in mutant mice can be correlated with tumor predisposition. Figure 5 shows the average ratio of the length of proestrus over that of metestrus in mutant mice that developed tumors versus those that did not develop such tumors at three different time points. The results show that there was a trend toward a higher ratio in all three age groups. Although the results did not reach statistical significance in the older age groups probably due to lack of statistical power given the small number of mice available, statistical significance (P = 0.03) was demonstrated in the younger age group. These results will be published in the near future.

Fig. 5:



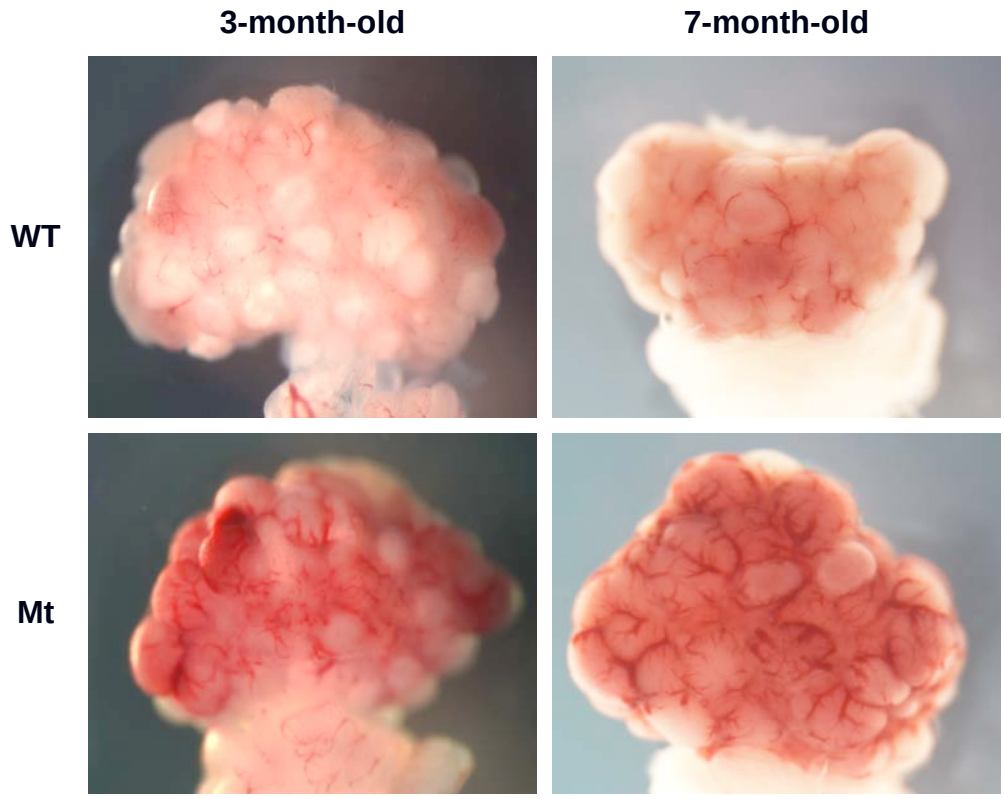
In summary, we consider our original task, which was expanded during the course of this grant, to be essentially completed. We plan to repeat studies with 3-4 month old mice and to repeat circulating hormone measurements in younger mice before submitting the data for publication. We anticipate that these results will generate significant interest in the ovarian cancer community.

Task #3: Test specific candidate hormones for their potential regulation by BRCA1 in vitro. The initial proposal was to remove both ovaries from one mutant mouse and one control mouse, harvest granulosa cells, and initiate several granulosa cell cultures per mouse in vitro. After ensuring purity of our cell cultures and verifying their authenticity, we would measure and compare the levels of hormones secreted in the conditioned medium in normal *versus* mutant mice. We have succeeded in obtaining cultures of granulosa cells as initially proposed. We were able to obtain primary cultures of such cells by using sterile needles to puncture ovarian follicles and aspirating the cell rich fluid with a syringe, followed by dispersing the cells and plating them in tissue culture dishes. We showed, in our previous progress report, that the cultured cells expressed mullerian inhibiting substance and developed a strategy to look at the consequences of various hormones of interest on Brca1 expression. Unfortunately, in spite of much effort, we were unable to obtain conclusive results because of a problem that we were not familiar with initially and that we had not anticipated. It turns out that once in culture, granulosa cells, whether from human or mice, differentiate and stop expressing receptors for steroid and gonadotropin hormones. We have contacted reproductive biologists, who were only able to confirm that this is a problem. Although we first decided on an alternative strategy, this task was extensively modified during year 3 because we were asked to submit a revised statement of work in order to eliminate potential overlaps with a new NIH grant that started during that year. The revised task was focused on testing specific candidate hormones for their potential regulation by BRCA1 in vivo. Mutant and wild type mice were inoculated with PMSG +/- hCG to induce follicular growth +/- ovulation. The ovaries were harvested. We are still in the

process of developing real-time PCR primers and probes in order to measure and compare Brca1 levels in ovarian follicles from mutant versus wild type mice. Thus, this task is still in progress, but is in its final stages of completion.

We examined the ovaries removed in the context of this task as well as task #4 in an effort not to miss any previously unrecognized phenotypic characteristic associated with the mutant phenotype. This led to the observation that graffian follicles in mutant ovaries tend to have a much more prominent vasculature than similar follicles from wild type mice. Figure 6 shows photographs taken under a dissecting microscope from ovaries from wild type (WT) and mutant (Mt) mice harvested following PMSG administration followed, 24 hours later, by hCG administration:

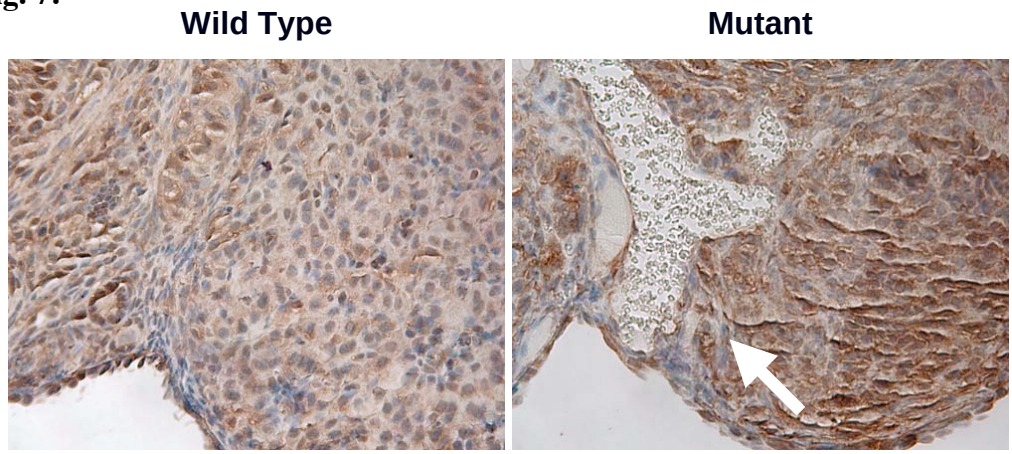
Fig. 6:



The nodular appearance of the ovarian surface reflects underlying follicles that developed in response to the hormonal manipulations. The difference in vascularization of the follicles between wild type and mutant ovaries is striking in both age groups. These studies were repeated and were found to be reproducible. This finding stimulated us to examine graffian follicles from wild type and mutant mice not treated with hormones and the observation that vascularization of the mutant follicles is more prominent seems to be reproducible in these animals as well (not shown). We performed immunohistochemistry on sections from wild type and mutant ovaries from mice treated with PMSG + hCG using a polyclonal antibody against VEGF in order to test the hypothesis that these differences are mediated, at least in part, by differences in secretion of this vascular growth factor by follicular cells. Indeed, follicular cells from mutant ovaries stained

much more strongly than follicular cells from wild type ovaries as shown in the FIGURE 7, which shows representative results from such immunostains:

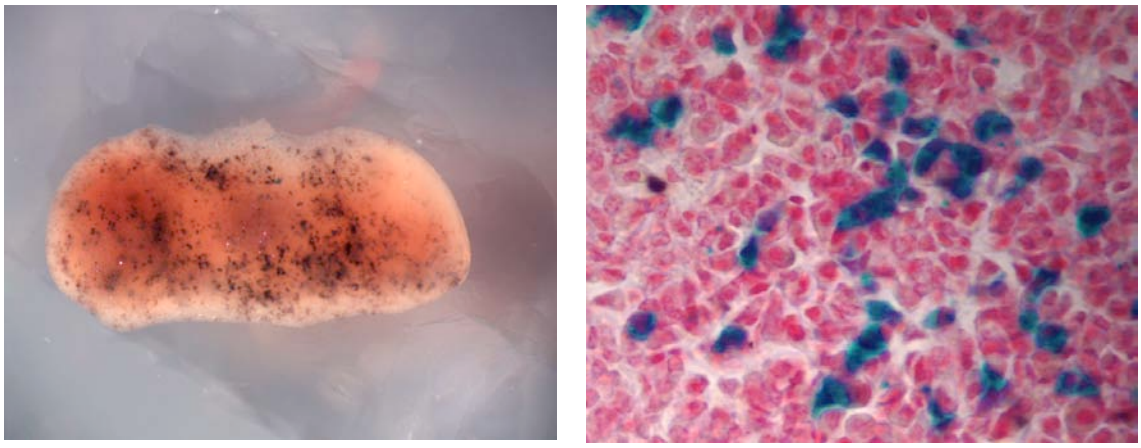
Fig. 7:



Please also note the dilated capillary, indicated by the arrow, in the mutant ovary. The exact significance of these findings is still unclear. However, these differences are clearly associated with the mutant phenotype in our mouse model. It is very likely that the increased vascularization of mutant follicles results in increased uptake of follicular hormone by the blood stream as well as in increased delivery of hormones such as pituitary hormones to the follicles. This could, in part, be responsible for the differences in estrus cycle dynamics that we already demonstrated in the mutant animals.

Tasks #4 and 5: Expression microarray analyses to compare gene expression in normal and mutant pituitary cells (task 4) and verification of the data (task 5). These tasks were also revised during the course of the grant period in order to avoid potential overlap with our new NIH grant that started during year 3 of the DOD grant period. The revised tasks were based on an observation that we made during the DOD grant period that was not known at the time of our initial application. We obtained data suggesting that Brca1 recombination in our Fshr-Cre mice carrying floxed Brtca1 alleles did not only take place in granulosa cells, but also in the pituitary. This is shown in figure 8, which is the results of *lacZ* staining of the pituitary gland of a Fshr-Cre transgenic mouse crossed with the R26R reporter strain.

Fig. 8:



The photograph on the left in the above illustration is that of a whole pituitary gland while the one on the right is that of a histological section of the same gland. The results show that the Fshr promoter that was used to drive Cre in our mouse model is active in at least some cells within the anterior pituitary gland. However, the results also suggest that only a small fraction of the total cell population in the anterior pituitary express this promoter. We then reasoned that in order for our proposed expression profiling studies to be meaningful, we should characterize the cell population that expresses *lacZ* in the above studies and perform our proposed studies on those cells only. We became concerned that if we were to perform our expression profiling studies using whole pituitaries, any change in cells carrying a mutant Brca1 would be masked by the much larger population of cells that does not carry a mutant Brca1. We are in the process of using fluorescence-activated cell sorting (FACS) techniques to purify the *lacZ* positive cells and plan to use immunostains and RT-PCR to identify which pituitary cell type is represented. We will then purify these cells from the anterior pituitary of mutant versus wild type mice and use these purified populations for our gene profiling studies. We were not able to complete all of these studies during the grant period because it is only within the last year that we became aware of the fact that our Fshr-Cre transgene is expressed in the pituitary and it took several months to cross our transgenic mice with the R26R reporter mouse and generate sexually mature progeny. However, we plan to pursue these studies and are working on a new grant that will be submitted during 2007 to support these experiments.

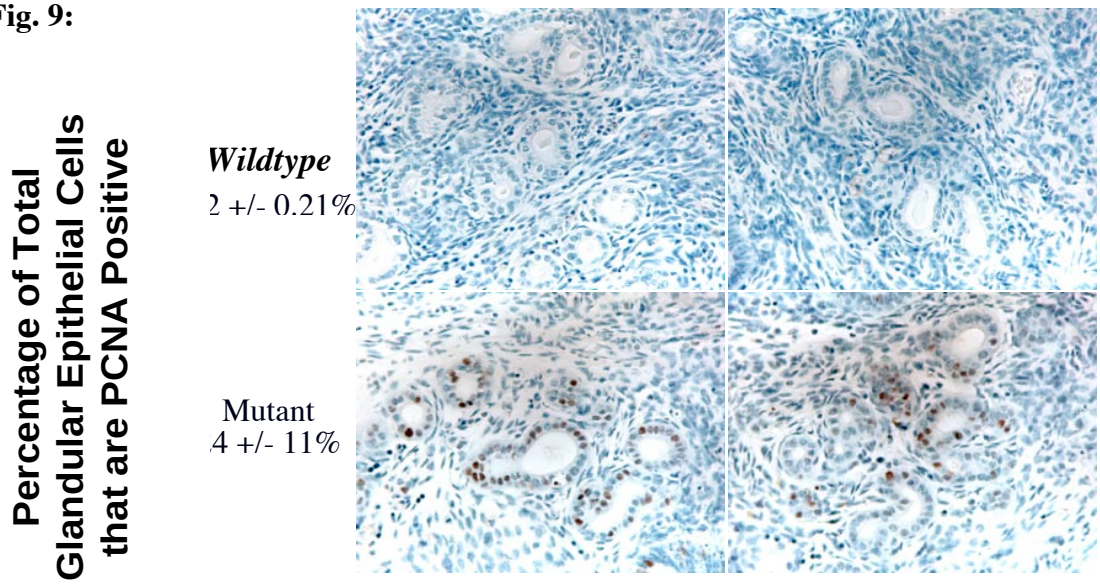
In summary, these tasks were modified on several occasions during the course of this grant due to new developments. Although the studies that were initially proposed have not been completed, we made progress with alternative studies that will form the basis of a new grant application.

Task #6: Comparing proliferation of specific mullerian-derived tissues and ovarian tissues in mutant versus normal mice. Our original plan was to compare the proliferation rate of various tissues of interest in the reproductive tract in normal and mutant mice. We compared cellular proliferation activity in the uterus of 5 wild type and 5 mutant mice at the diestrus ad estrus phases of the estrus cycle. Histological cross-

sections were taken from each horn at the junction between the proximal (closest to the ovary) and middle thirds. All mice were 3-4 months old. The sections were stained with antibodies against PCNA, a marker of cell cycle activity. A digital photomicrograph of representative areas of each uterus was taken under 40X objective and the total number of endometrial stromal cells showing either positive or negative nuclear staining for PCNA was determined. In accordance with the results shown in last year's progress report, there was a 50% increase in the average percentage of PCNA positive glandular epithelial cells in mutant mice, although there was great variation among the different samples, including one mutant sample that showed almost total lack of PCNA staining, and the results were not statistically significant. There were significant differences in the endometrial stroma. An average of 716 +/- 60 stromal cells were examined in the 5 mutant mice and of 846 +/- 154 cells in the 5 wild type mice. The percentage of PCNA positive stromal cells was 67.8 +/- 6.7% in mutant compared to 36.1 +/- 25% in wild type mice ($P = .025$, t test). This provides further support for the idea that measurable phenotypic differences exist between cells derived from the mullerian tract in wild type *versus* mutant animals in addition to raising interesting issues about the importance of epithelial-stromal interactions in the uterus. Given that a wild type Brca1 was expressed in the uterus of mutant animals, the results also provide additional support a cell non-autonomous mechanism. We have already set up experiments to examine other stages of the estrus cycle and plan to use other markers listed in the application.

Another set of experiments investigated differences in cellular proliferation in the uterus of wild type *versus* mutant mice in animals synchronized with regard to their estrus cycles. In the experiment shown in the next illustration, two mutant mice and two wild type mice were inoculated with PMSG to stimulate follicular growth, followed 48 hours later by inoculation of hCG to induce ovulation. The mice were sacrificed 24 hours after receiving the latter hormone and the uteri were examined for PCNA immunoreactivity. The total number of glandular cells in the entire uterine sections was determined and the percentage of cells that were positive for PCNA was calculated. As shown in figure 9, there were practically no glandular cells that were positive for this marker in the wild type mice. In contrast, an average of 24% of the glandular cells in mutant mice were positive. These results need to be regarded as preliminary until a larger number of mice is examined. However, the sharp differences between mutant and wild type animals are striking.

Fig. 9:



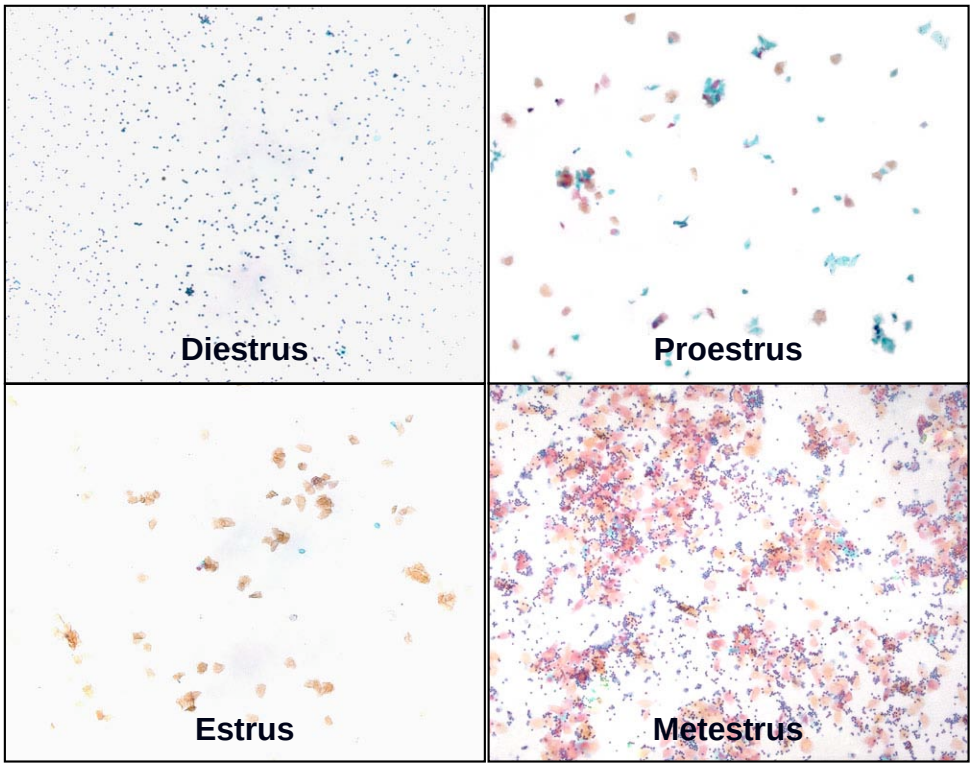
These results are the subject of a manuscript that is currently in preparation. We consider this task to be completed.

Task #7: Comparing proliferation of uterine cysts in mutant mice ovariectomized at specific time points. We ovariectomized mice at 3-4 months and 7-8 months of age. Several of these mice have now reached the age of 14 months. These mice have been sacrificed and their ovaries have been collected. We need another 3 months before all of our mice reach that age, at which point immunostains will be performed to compare the proliferative rate of epithelial cells lining uterine cysts in these mice versus control mice with intact ovaries. Thus, this task has been initiated but is still in progress.

Additional Studies that follow up on recent findings but that were not part of the submitted Statement of Work: Our finding that the anterior pituitary carries a mutated Brca1 in our mouse model implies that interpretation of data using this model is complicated by the fact that any phenotypic change associated with the mutant phenotype is not necessarily a reflection of the role of Brca1 in ovarian granulosa cells only, but possibly also in the pituitary. This has important implications regarding the use of our mouse model in future studies. We therefore perfected the technique of transplanting ovaries from mutant mice into wild type mice and vice versa in order to generate animals in which mutated Brca1 is present either only in granulosa cells or only in the pituitary. This will allow us not only to determine the understand the differential roles of the pituitary and granulosa cells in driving the phenotype in our mouse model, but will also provide us with a final proof that tumor development in our mouse model is entirely driven by cell non-autonomous mechanisms. Figure 10 shows vaginal cytology from specimens obtained on separate days in a mouse that had received an ovarian transplant. The transplanted ovary was placed under the renal capsule. This mouse had previously

undergone a bilateral oophorectomy so that any sign of ovarian function in this mouse could only be attributed to the transplanted ovary.

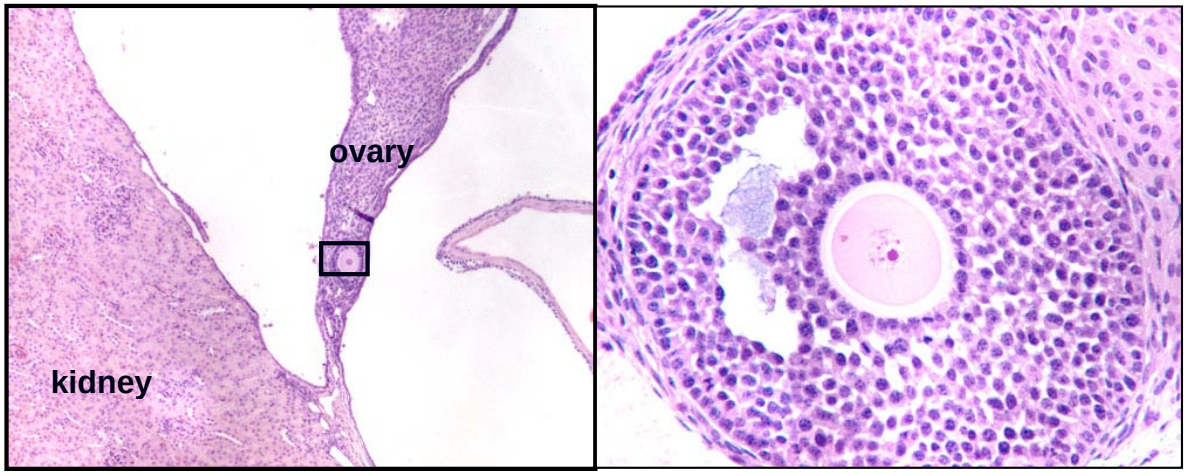
Fig. 10:



The presence of changes representing all phases of the normal estrus cycle in the above illustration indicates that this mouse was cycling normally in spite of the fact that its ovaries had been removed and the only remaining ovary was the one transplanted into the renal capsule. The ovary was transplanted when the mouse was 3 weeks old (before sexual maturity) and the photographs were obtained from specimens collected when it was 3 months old.

A photograph of an ovary transplanted into the renal capsule is shown in figure 11. The area in the box in the left panel is magnified in the right panel. This area shows a secondary follicle. The presence of such follicles confirms that the transplanted ovary was functioning normally.

Fig. 11:



Key Research Accomplishments

- We overcame the embryonic lethality of Brca1 knock out mice by targeting such knock out specifically to granulosa cells.
- We showed that mice carrying a mutant *Brca1* in their granulosa cells developed tumors not in granulosa cells, but in epithelial cells lining the entire mullerian tract. We further showed that the tumors did not carry mutant *Brca1* alleles.
- We showed that mice carrying the mutant Brca1 allele also develop cystic dilatations of ducts in their mammary glands, in a manner reminiscent of fibrocystic disease in humans.
- We showed that mutant mice carry of Brca1 rearrangement not only in their granulosa cells, but also in a subset of pituitary cells.
- We improved the techniques allowing accurate identification of the stage of the estrous cycle in mice by adapting staining techniques used for PAP tests in humans to these animals.
- We showed that mice carrying a mutant *Brca1* in their granulosa cells have a longer estrus cycle due primarily to increases in he length of the diestrus and proestrus phases. This is interesting in light of extensive epidemiological data showing that continuous (uninterrupted) menstrual cycles in humans are associated with increased ovarian cancer risk.
- We showed that mutant mice with an increase in the length of proestrus to metestrus are more likely to develop tumors than those who do not show such increase.
- We showed that mice carrying a mutant *Brca1* in their granulosa cells show increased cell proliferation in the endometrial stroma as well as in endometrial glandular epithelium, at least at specific phases of the estrus cycle. A similar increase in endometrial glandular proliferation was seen in mutant mice treated with Pregnant Mare Serum Gonadotropins (PMSG) compared to normal mice treated with the same dose of PMSG.

- We showed that ovarian follicles in mutant mice show increased vascularization immediately after ovulation.

Reportable Outcomes

1. A manuscript describing our mouse model and arguing that Brca1 controls cancer predisposition in a cell non-autonomous manner was published in Current Biology (Curr Biol 15:561-565, 2005). The potential impact of this work is underscored by the fact that it was featured in the News & View section of Nature, April 14, 2005 issue.

2. Dubeau, L. BRCA1 induced ovarian oncogenesis. Published in Proceedings of the 1st International Conference on Ovarian Cancer: State of the Art and Future Directions. Springler Science, New York, 2007 (in press).

3. Dr. Dubeau was a guest speaker at the Mouse Model of Human Cancers Consortium – Gynecological Models Meeting in Puerto Rico in February 2004 This abstract was submitted following a formal invitation by the organizers of this meeting, who had heard about our work. A copy of the abstract is shown in the appendix.

4. Dr. Dubeau was a guest speaker at a symposium entitled "Ovarian Cancer: Prevention and detection of the Disease and its recurrence" held in Pittsburgh, PA in October 2005. The official handout is appended.

5. Seminar presentation based on this work given at University of Southern Florida entitled "Prospects of Identifying a Precursor Lesion for Ovarian Carcinoma", January 8, 2004.

6. Dr. Dubeau was a guest speaker at The Lynne Cohen Foundation Symposium on the Emerging Role of Screening and Prevention in Women's Cancers, New York, NY, April 23, 2004. Title of the presentation was: "Mechanism of Ovarian Cancer Predisposition in BRCA1 Mutation Carriers – Implications for Ovarian Cancer Prevention".

7. Seminar presentation based on this work given at Fox Chase Cancer Center entitled "Mechanism of Ovarian Cancer Predisposition in Individuals with Germline BRCA1 Mutations", December 7, 2004

8. Seminar presentation based on this work entitled "Mechanism of Cancer Predisposition in Individuals Carrying Germline BRCA1 Mutations", University of Virginia School of Medicine, Charlottesville, VA, October 27, 2005.

9. Dr. Dubeau was a guest speaker at the "1st International Conference on Ovarian Cancer: State of the Art and Future Directions" held in Crete (Greece) in June 2006. The official handout is appended. This presentation will also be published in the book of proceedings of this meeting (see #2 above).

10. Dr. Dubeau will be a guest speaker at the XIII Charles Heeidelberger International Symposium to be held at New York University on September 5-8, 2007. He will speak on a session entitled "Models of Cancer Causation".

Note: additional manuscripts based on the work done in the context of this grant are currently in preparation. Support for the DOD will be acknowledged when these are published.

Conclusions

Our results provide strong support for the idea that the reason why individuals with germline mutations in the *BRCA1* gene are predisposed to ovarian cancer is that the ensuing decrease in *BRCA1* gene dosage results in a disruption of normal cellular interactions between ovarian granulosa cells and the cells from which ovarian epithelial tumors originate. In other words, BRCA1 controls the secretion of one or several hormonal or paracrine factor(s) by granulosa cells that can influence ovarian tumor predisposition. In addition to granulosa cells, the anterior pituitary gland may also contribute to ovarian tumorigenesis in a BRCA1 dependent manner. Given that the pituitary gland as well as granulosa cells are both important for the control of the menstrual/estrus cycle, these conclusions are interesting in the context of the well documented association between menstrual cycle activity and ovarian cancer risk in humans. In that regard, our findings that mice with higher proestrus/metestrus length ratios are more likely to develop ovarian tumors is likely to generate significant interest in the scientific community and to stimulate the development of human population based studies aimed at testing the hypothesis that similar alterations in the menstrual cycle are associated with ovarian cancer predisposition as well as with the presence of germline BRCA1 mutations in humans. Our finding that ovarian follicles in mutant ovaries have more prominent vascularization points to a potential mechanism contributing to the establishment of the mutant phenotype including tumor predisposition that has so far been overlooked by the scientific community.

References

None.

Appendices

Manuscripts:

Chodankar R, Kwang S, Sangiorgi F, Hong H, Yen H-Y, Deng C, Pike MC, Shuler CF, Maxson R, Dubeau L: Inactivation of Brca1 in mouse ovarian granulosa cells causes serous epithelial cystadenomas carrying functional Brca1alleles in the ovary and uterus. Curr. Biol. 15:561-565, 2005.

Dubeau, L. BRCA1 induced ovarian oncogenesis. Published in Proceedings of the 1st International Conference on Ovarian Cancer: State of the Art and Future Directions. Springler Science, New York, 2007 (in press).

Text of a News & Views article that appeared in the April 14, 2005 issue of Nature.

Abstracts:

Chodankar R, Kwang S, Yen H-Y, Hong H, Deng C, Sangiorgi F,Yu MC, Maxson R, Dubeau L: Homozygous Knock Out of *Brca1* in mouse ovarian granulosa cells results in benign and malignant ovarian epithelial tumors. Presented at the Mouse Model of Human Cancers Consortium – Gynecological Models Meeting in Puerto Rico in February 2004.

Handout of lecture given at Symposium entitled: "Ovarian Cancer: Prevention and Detection of the Disease and its Recurrence" held in Pittsburgh, PA on October 24-25, 2005

Abstract of a lecture given at the "1st International Conference on Ovarian Cancer: State of the Art and Future Directions" held in Crete (Greece) from June 26 to July 1, 2006

Note: The text of the abstract of the lecture given at the Lynne Cohen Symposium on the Emerging Role of Screening and Prevention in Women's Cancers, New York, NY, April 23, 2004 was lost due to a computer crash.

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Cell-Nonautonomous Induction of Ovarian and Uterine Serous Cystadenomas in Mice Lacking a Functional Brca1 in Ovarian Granulosa Cells

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Summary

Women with germline mutations in *BRCA1* have a 40% risk of developing ovarian cancer by age 70 [1] and are also predisposed to cancers of the fallopian tubes [2–4]. Given that ovulatory activity is a strong risk factor for sporadic ovarian cancer [5], we hypothesized that reduced BRCA1 expression might predispose to gynecological cancers indirectly, by influencing ovarian granulosa cells. These cells secrete sex steroids that control the ovulatory cycle and influence the growth of ovarian epithelial tumors [6, 7]. Granulosa cells also secrete mullerian inhibiting substance (MIS), a hormone that inhibits both the formation of female reproductive organs in male embryos [8] and the proliferation of ovarian epithelial tumor cells [9, 10]. We tested this hypothesis by using the *Cre-lox* system to inactivate the *Brca1* gene in mouse ovarian granulosa cells. A truncated form of the Fsh receptor promoter [11] served as the Cre driver. Here, we show that indeed, inactivation of the *Brca1* gene in granulosa cells led to the development of cystic tumors in the ovaries and uterine horns. These tumors carried normal *Brca1* alleles, supporting the view that Brca1 may influence tumor development indirectly, possibly through an effector secreted by granulosa cells.

Results

Granulosa Cell Specificity of Truncated Fsh Receptor Promoter

We verified the cell-type specificity of a truncated Fsh receptor promoter form shown previously to direct expression exclusively in granulosa cells [11]. We crossed a transgenic mouse expressing the Cre recombinase under the control of this promoter fragment with the ROSA26R Cre reporter mouse strain [12]. Examination of the pelvic organs of mice carrying the Cre driver and reporter showed β-galactosidase activity exclusively in granulosa cells (Figure 1).

Consequences of Loss of Brca1 in Granulosa Cells on Tumor Formation

Fshr-Cre transgenic mice were crossed with mice carrying a floxed *Brca1* allele [13] to create a *Brca1* homozygous knockout restricted to granulosa cells. One ovary was removed from each of 30 *Brca1 flox/flox; Fshr-Cre* mice at 2 months of age. Histological examination revealed that these ovaries were morphologically normal (not shown). The mice were fertile and, at least during the first 6 months of life, produced litters of normal size.

Fifty-nine *Brca1 flox/flox;Fshr-Cre* mice, including the 30 mice that had a unilateral oophorectomy at two months, were sacrificed between the ages of 12 and 20 months. Of these 59, 40 (68%) homozygous mutant mice had grossly visible cysts attached to the ovary, within the wall of the uterine horns, or on the external surface of the uterine horns (Figure 2). The ovarian cysts were occasionally bilateral (Figure 2). The uterine cysts were usually multiple and most concentrated near the ovaries. All cysts were lined by cuboidal to columnar cells and were occasionally papillary (see Figure 3E, below). The cysts resembled human serous cystadenomas, which are benign tumors composed of the same cell type as ovarian serous carcinomas.

A solid tumor contiguous to a morphologically benign cyst was observed in a single case. Although the complexity and cellular atypia levels seen in the solid component were compatible with a malignant process, the malignant potential of this tumor remains unclear because it showed no evidence of either invasion of surrounding structures or metastasis (Figure 2D). Renal cysts were also observed in two mutant mice. No abnormality was seen in any of 36 age-matched littermate controls lacking *Cre* recombinase.

Evidence for a Cell-Nonautonomous Mechanism of Tumor Induction

Our studies with the R26R reporter mouse (Figure 1) suggested that rearrangement of the *Brca1* gene in response to Fshr-Cre occurred primarily in ovarian granulosa cells, our intended target. That all tumors exhibited an epithelial morphology suggested that they were not derived from granulosa cells. Further support for this possibility came from findings that the tumor cells (1) expressed keratins (Figure 3), which are markers of epithelial cells, and (2) did not express mullerian inhibiting substance, a marker of granulosa cells (Figure 3). The tumor cells also expressed estrogen (Figure 3) and progesterone (not shown) receptor proteins, further supporting the view that they were functionally similar to human ovarian epithelial tumors.

The conclusion that the tumors did not originate in granulosa cells was also supported by the fact that they were often localized in the uterine horns, which do not contain granulosa cells. The possibility remained

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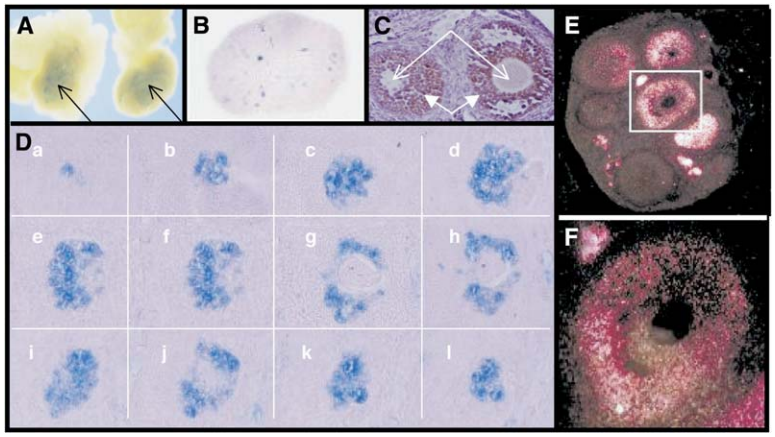

Figure 1. Specificity of Fsh Receptor Promoter for Granulosa Cells

Two transgenic mouse lines expressing Cre recombinase under the control of a truncated form [11] of the Fsh receptor promoter (285 bp) were crossed with a ROSA26R Cre reporter mouse. The pelvic organs were removed at 8 weeks postnatal and examined for the presence of *lacZ* under bright-field (A, B, and D) or dark-field (E and F) microscopy. Shown here are representative results from one transgenic line.

(A) Ovaries with portion of adjacent uterine horns; *lac*Z staining is restricted to the ovaries (arrows).

(B) Whole-mount section of one ovary showing scattered foci of *lac*Z.

(C) Histological section of a mouse ovary stained with an antibody against mullerian inhibiting substance, a marker of granulosa cells; this panel is meant to illustrate the normal histology of ovarian follicles for use as reference when examining panels (D)–(F); it shows two ovarian follicles (short arrows), each with a central oocyte (long arrows) surrounded by immunopositive granulosa cells. Cells outside the two ovarian follicles are ovarian stromal cells.

(D) Serial sections through an entire ovarian follicle morphologically similar to those shown in (C) and showing *lac*Z staining confined to the granulosa cells.

(E) Whole-mount section through an entire ovary seen under dark-field microscopy showing the presence of *lac*Z in ovarian follicles; the area within the rectangle, which shows a cross-section through the center of one follicle as well as small portions of adjacent follicles, is enlarged in (F).

that the Fshr-Cre transgene produced a Cre level that was sufficient to cause recombination of the floxed *Brca1* allele but was too low to cause recombination of the R26R allele. If this were the case, then the tumor cells should carry the recombined form of the floxed *Brca1* allele. However, although the expected 530 bp product from the unrearranged *Brca1* allele could be amplified readily from all tissues examined with PCR primers specific for this allele, the only tissues from which the expected 640 bp product from the rearranged allele could be amplified were whole ovaries, the site of granulosa cells, as well as one of four ovarian cysts that had been separated from the adjacent ovaries with scissors under a dissecting microscope (Figure 4, bottom panel). The weak amplification product obtained with primers specific for the rearranged allele (pair e-d) in this ovarian cyst most likely reflects the presence of admixed ovarian stroma, either in the cyst wall or in contaminating fragments of normal ovary. It is highly unlikely that this allele played a role in tumor development owing to its absence in most cystic tumors examined. A fifth ovarian cyst, subjected to laser capture microdissection to ensure absence of admixed granulosa cells, did not contain the rearranged allele either in the lining epithelium or in the cyst wall (Figure 4, middle panel). We detected only the unrearraged allele in the epithelial lining of two additional uterine cysts examined after laser capture microdissection (not shown).

Discussion

Our results strongly support our hypothesis that inactivation of the *Brca1* gene in granulosa cells acts cell-nonautonomously by altering the activity of an effector that influences tumorigenesis in cells from which ovarian epithelial tumors originate. This conclusion is based on the fact that inactivation of *Brca1* in ovarian granulosa cells led to the formation of epithelial tumors carrying normal *Brca1* alleles. An earlier report showed similarly that breast tumors resulting from inactivation of *Brca1* in a subset of mammary cells (with MMTV-Cre or Wap-Cre) did not carry the mutant form of *Brca1* [13]. Although we did not examine breast tissue in *Brca1 flox/flox;Fshr-cre* mice, we note that ovulatory activity, which is controlled largely by the activity of granulosa cells, is a well-established risk factor for breast cancer in humans [14].

That mice lacking a functional Brca1 protein in their granulosa cells developed lesions involving the uterine horns in addition to the ovaries is consistent with the observation that precancerous changes are frequently seen in the fallopian tubes of women who are asymptomatic carriers of *BRCA1* mutations [2–4]. This is also compatible with L.D.'s earlier suggestion that ovarian epithelial tumors do not originate from the mesothelial layer lining the ovarian surface, the site favored by most authors, but from mullerian duct derivatives surrounding the ovary or abutting this organ [15].

Most tumors that develop in individuals with germline *BRCA1* mutations and show loss of heterozygosity at the *BRCA1* locus have retained the mutant allele [16–18], suggesting that BRCA1 may act as a classical tumor suppressor. However, not all tumors that develop in this group of patients carry losses of heterozygosity at this locus [18], and there is little evidence for the notion that the wild-type allele in these tumors is silenced by epigenetic mechanisms [19]. In addition, a number of observations suggest that total loss of BRCA1 activity does not promote, but interferes with cellular growth. The small number of breast or ovarian cancer cell lines so far isolated that lack a functional BRCA1 protein typically have long doubling times. Primary cultures derived from *Brca1*$^{-/-}$ mouse embryos

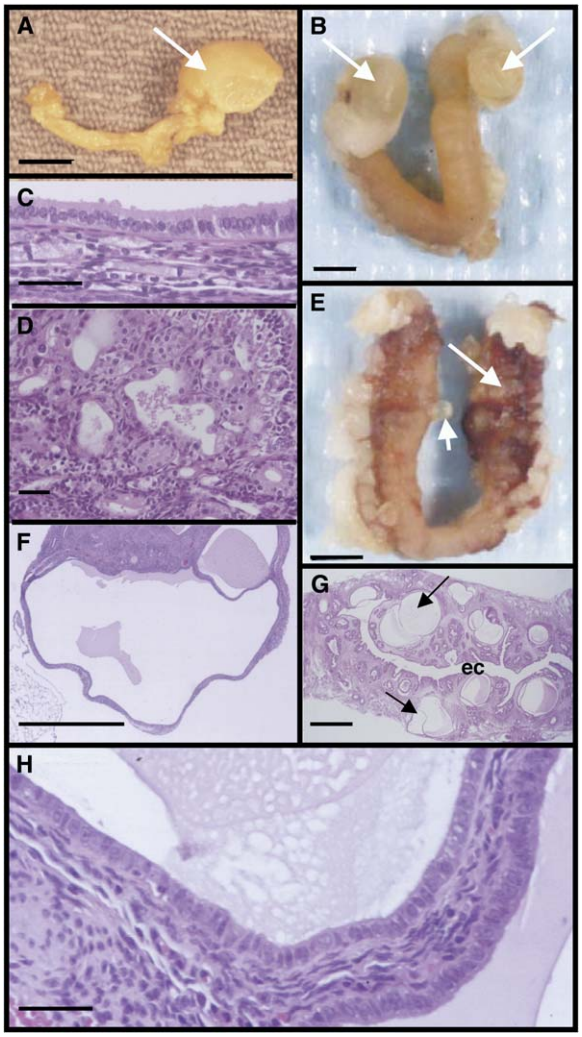

Figure 2. Examples of Ovarian and Uterine Lesions Observed in Mutant Mice

Shown are gross photographs of ovarian (arrows in [A] and [B]) and uterine (arrows in [E]) cysts and histological sections from ovarian (C and D) and uterine (F–H) lesions. The ovarian tumor shown in (A) was 80% cystic and 20% solid. Histological sections of both of these components are shown in (C) and (D), respectively. A bilocular cyst on the external surface of a uterine horn is shown under low and high magnification in panels (F) and (H), respectively. A uterine horn containing multiple epithelial cysts (arrows) is shown at low magnification in (G); ec denotes endometrial cavity. The scale bars in (A), (B), and (E) represent 5 mm. The scale bars in (C), (D), and (H) represent 40 μm. The scale bars in (F) and (G) represent 1000 μm. Stain: hematoxylin and eosin.

do not proliferate unless the embryos also carry a p53 knockout. Given that cells from such embryos grow only clonally, additional events must occur to ensure their viability [20, 21]. Recent evidence suggests that downregulation of BRCA1 results in growth arrest at the G2 to M transition [22], a finding clearly inconsistent with the view that Brca1 functions as a classical tumor suppressor. That mutations in this ubiquitously expressed gene lead mainly to predisposition to breast and ovarian cancer is also difficult to reconcile with this view.

It is possible that the Fshr promoter used in our studies is expressed in cells other than granulosa cells at levels undetectable with the R26R reporter mouse. Thus, non-granulosa cells may control ovarian and uterine tumorigenesis. However, we favor the hypothesis that it is the granulosa cells that act at a distance to control mullerian epithelial tumorigenesis via a mechanism regulated by Brca1. At least in reproductive organs, these cells appear to be the principal site of Brca1 inactivation. The idea that a specific effector released by granulosa cells and regulated by Brca1 influences tumor predisposition in the mullerian tract is both the simplest and biologically most attractive hypothesis that follows from our data. Another possibility is that an abnormal Brca1 expression might result in alterations in the dynamics of the estrus cycle. An example would be changes in the length of a specific phase of this cycle. Such changes, in turn, might influence tumor predisposition.

The finding that loss of Brca1 in mouse ovarian granulosa cells causes epithelial tumors in wild-type cells of the ovary and uterus raises the prospect that reduced levels of functional BRCA1 protein in humans carrying a germline *BRCA1* mutation could lead to the development of cancer by modulating the ability of granulosa cells to act on distant target tissues. This hypothesis has important implications for the clinical management of individuals with a familial predisposition to ovarian tumors owing to germline *BRCA1* mutations. Knowing the identity of the endocrine or paracrine factor(s) that mediates such actions at a distance could provide a new way to identify individuals predisposed to ovarian cancer and could also form the basis for novel strategies based on manipulations of the levels of the factor(s) in question and aimed at preventing ovarian cancer in individuals with familial predisposition to this disease.

Experimental Procedures

Immunohistochemical Analyses

The mouse monoclonal antibody against nonsquamous keratins was purchased from Chemicon International (Temecula, CA, MAB 1611). The polyclonal goat antibody against mouse mullerian inhibiting substance was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, catalog number sc-6886). Goat polyclonal antibodies against mouse estrogen receptor α and progesterone receptor proteins were purchased from Santa Cruz Biotechnology (catalog numbers sc-542 and sc-2018, respectively). All primary antibodies were diluted 1:200. For secondary antibodies, we used either anti-mouse IgG purchased from Chemicon International (catalog number AP124B) diluted 1:500 or anti-goat IgG purchased from Santa Cruz Biotechnology and diluted 1:200. Antibody binding was detected with the ABC kit (Vector Laboratories, Burlingame, CA).

Examination of *Brca1* Rearrangement Status by PCR

Tissues of interest were either microdissected with a Pixcell II laser-capture microdissection instrument (Arcturus Bioscience, Mountain View, CA) or were sampled under a dissecting microscope. All laser-capture microdissections were performed on tissues fixed in ethanol only, embedded in paraffin, and either unstained or lightly stained with hematoxylin. Genomic DNA was amplified by PCR with primers specific for either the unrearranged (primers a-b) or rearranged (primers e-d) alleles. The sequences of primers a and b were as published [13]. The sequence of primer e (forward) was: 5′-GCAGTGAAGAGAACTTGTTCCT-3′. The se-

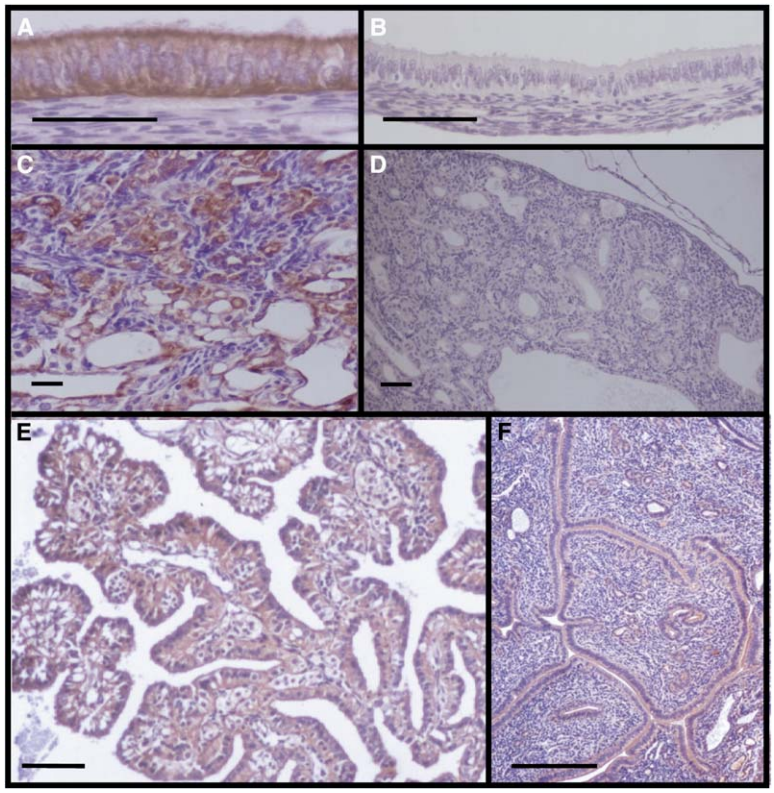

Figure 3. Immunohistochemical Characterization of Ovarian Tumors

(A)–(E) are various portions of the ovarian tumor shown in Figure 2A. (E) shows a papillary area of the cystic component. The sections were stained with a polyclonal antibody against nonsquamous keratins (A and C) and with monoclonal antibodies against either mullerian inhibiting substance (B and D) or the estrogen receptor protein (E). The cytoplasmic staining pattern seen in (E) is similar to that seen in sections of normal endometrium from wild-type mice (F). Secondary mouse ovarian follicles stained with an antibody against mullerian inhibiting substance were shown in Figure 1C. The scale bars represent 40 μm in all panels except (E), in which it is 100 μm.

quence of primer d (reverse) was: 5′-CTGCGAGCAGTCTTCAG AAAG-3′. PCR cycling profiles were 30 s at 94°C, 60 s at 58°C, and 60 s at 72°C over 35 cycles.

Generation of Mice with *Brca1* Knockout Targeted to Granulosa Cells

A transgene composed of the Cre recombinase protein-coding sequence (1.1 kb) and a 900 bp SV40 fragment containing an untranslated exon sequence and polyadenylation signal fused with a truncated form [11] of the FSH receptor promoter (285 bp) was used to produce transgenic mice. The initial parental mice were from a cross between C57 and Black 6 strains. Six lines were initially created, two of which were crossed with R26R reporter mice and found to be equally effective at driving *Cre*. One line was selected randomly for breeding with a mouse carrying a floxed *Brca1* allele described earlier [21]. The mouse genotypes were determined by amplifying tail DNA with primers specific for either the floxed *Brca1* allele or for *Cre*.

Acknowledgments

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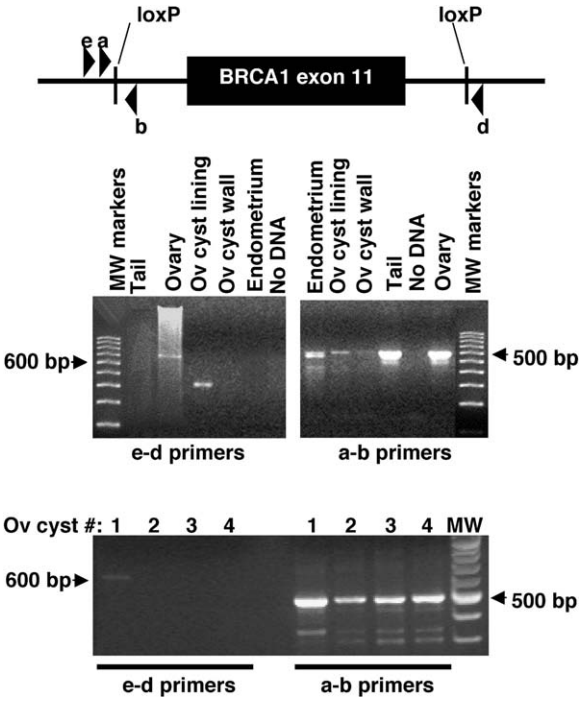

Figure 4. Recombination Status of Floxed Brca1 Alleles in Normal and Neoplastic Tissues

The top diagram shows *lox*P sites flanking exon 11 of the *Brca1* gene. The arrowheads represent the position and orientation of oligonucleotide primers in relation to the *lox*P sites. Genomic DNA from various normal or neoplastic tissues was amplified by PCR with either primer pair e-d, specific for the rearranged *Brca1* allele, or pair a-b, specific for the unrearranged allele. The PCR products were resolved on agarose gels and visualized under UV after staining with ethidium bromide (middle and bottom panels). Lanes labeled "Ov cyst lining" and "endometrium" in the middle panel represent, respectively, the epithelial lining of an ovarian cyst and of endometrium separated from adjacent tissues by laser-capture microdissection. The stromal cells underlying the ovarian cyst epithelium were likewise microdissected and examined ("Ov cyst wall"). The bottom panel shows four ovarian cysts not subjected to laser-capture microdissection but separated from the adjacent ovaries with scissors under a dissecting microscope. Although the expected 530 bp unrearranged *Brca1* allele (primer pair a-b) was detected in all tissues examined, the expected 647 bp amplification product from the rearranged allele (primer pair e-d) was only seen in normal ovarian tissues (which contain granulosa cells) as well as in one of the four ovarian cysts not subjected to microdissection (bottom panel). The product of approximately 300 bp in "Ov cyst lining" (primer pair e-d, middle panel) was sequenced, confirming that it represents a nonspecific product. The 647 bp product from normal ovaries with the same primers was also sequenced and shown to be authentic.

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BRCA1 INDUCED OVARIAN ONCOGENESIS

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Text based on presentation made at the 1st International Conference on Ovarian Cancer: State of the Art and Future Directions, Crete, June 2006

November 30th, 2006

Abstract

Women with germline mutations in *BRCA1* have a 40% risk of developing ovarian cancer by age 70 and are also predisposed to cancers of the fallopian tubes and breast. Although some observations are supportive of the currently favored notion that BRCA1 functions as a classical tumor suppressor gene, others are inconsistent with this notion. Given that ovulatory activity is a strong risk factor for sporadic ovarian cancer, we hypothesized that reduced BRCA1 expression might predispose to gynecological cancers indirectly by influencing ovarian granulosa cells. These cells secrete sex steroids that control the ovulatory cycle and influence the growth of ovarian epithelial tumors. We tested this hypothesis by using the *Cre-lox* system to inactivate the *Brca1* gene in mouse ovarian granulosa cells. A truncated form of the FSH receptor promoter served as the Cre driver in order to achieve such tissue specificity. Indeed, inactivation of the *Brca1* gene in granulosa cells led to the development of benign cystic tumors in the ovaries and uterine horns in approximately one third of the mutant mice. The mutant mice also developed epithelial cysts in their mammary glands. These tumors were of epithelial origin and carried normal *Brca1* alleles, supporting the view that Brca1 influenced their development indirectly, through an effector secreted by granulosa cells. The fact that the cystic tumors were not confined to the ovaries, but involved the entire mullerian tract, is also consistent with the view that ovarian tumors are not of mesothelial origin, but are derived from the mullerian tract. We measured the length of each phase of the estrus cycle in a group of mutant and wild type mice at 3-4 months and 7-8 months of age. In both age groups, there was a significant increase in the length of proestrus, which corresponds to the follicular phase of the human menstrual cycle.

We suggest that the increased cancer predisposition seen in individuals carrying a germline *BRCA1* mutation is due, at least in part, to reduced levels of BRCA1 expression in granulosa cells. This, in turn, may lead to alterations in the length of the follicular phase of the menstrual cycle and ensuing increased unopposed estrogen stimulation as well as, perhaps, to disruption of additional interactions between granulosa cells and mullerian epithelial cells resulting in promotion of neoplastic transformation.

Women carrying a germline mutation in *BRCA1* have a 40% risk of developing ovarian cancer by age 70 and are also predisposed to cancers of the fallopian tubes and breast [1]. The molecular mechanisms responsible for cancer predisposition in these individuals remain unclear in spite the huge effort focused on understanding the normal function of the BRCA1 protein since the encoding gene was first isolated. Particularly intriguing is the site specificity of the cancers that develop in such individuals. Indeed, although BRCA1 is expressed ubiquitously in most cell types, individuals carrying germline *BRCA1* mutations are predisposed primarily to cancers of the breast and female reproductive tract. This chapter focuses on observations with an experimental model that not only provide a potential explanation as to why germline BRCA1 mutations are associated almost exclusively with predisposition to breast and ovarian cancers, but also sheds light into an underlying mechanism contributing to such predisposition.

Evidence for and against the idea that BRCA1 functions as a classical tumor suppressor

The concept that certain genes act as suppressors of cancer development originated largely from observations made in the context of familial cancer predisposition. Knudsen proposed over three decades ago that two genetic hits are needed for retinoblastoma development and further suggested that one of these two hits is inherited through the germline in individuals with familial predisposition to this disease [2]. It has since been established that the two hits referred to in this hypothesis correspond to inactivation of the two alleles of RB, the first tumor suppressor gene ever identified. A similar scenario where two alleles of a tumor suppressor are inactivated independently, one from a germline mutation and the

other from a somatic event, has been applied to other familial cancer predisposition syndromes and has become a central dogma in cancer genetics. Implied in this scenario is the idea that loss of both alleles of a given tumor suppressor provides an inherent growth survival advantage to cells harboring such loss.

BRCA1 is involved in a variety of important cellular processes such as cell cycle regulation, control of apoptosis, DNA repair, chromatin remodeling, transcriptional regulation, X chromosome inactivation, and post-translational protein modification [3-7]. Functions associated with cell growth or DNA repair are especially supportive of the idea that this protein functions as a classical tumor suppressor based on Knudsen's hypothesis. Earlier reports [8-10] that tumors developing in individuals with germline *BRCA1* mutations, if showing loss of heterozygosity, almost always show loss of the wild type allele provide further support for this notion, as these observations suggest that cancer cells that lack a normal BRCA1 allele have a survival advantage over those in which such an allele is present. A number of observations are not readily reconciled with the idea that BRCA1 functions a classical tumor suppressor in spite of these arguments. Tumor cell lines lacking a functional *BRCA1* gene have been extremely difficult to establish from cancers arising in individuals with germline mutations in this gene. Only a handful of such cell lines is available, which surprisingly have very long doubling times and are difficult to work with [11, 12]. The idea that loss of BRCA1 function provides a survival advantage is hard to defend in light of these observations. Furthermore, primary cultures derived from mouse embryos with homozygous knockouts of the *Brca1* gene do not proliferate. Such cultures are only successful from embryos carrying double *Brca1* and *p53* knockouts and, given that cells from such embryos grow only clonally,

additional events have to occur to ensure viability of the cells [13, 14]. In addition, cells from Brca1 knock out embryos show evidence of cell cycle arrest at the G2/M phase [15], which is consistent with findings that the BRCA1 gene product is important for regulation of progression through this cell cycle checkpoint [16]. All these findings are at odd with the notion that this gene is a tumor suppressor gene. Finally, the idea that BRCA1 functions as a classical tumor suppressor gene is difficult to reconcile with the observation that mutations in this ubiquitously expressed gene lead mainly to predisposition to breast and gynecological cancers.

Support for the existence of a link between BRCA1 expression and menstrual cycle regulation

Several epidemiological studies have demonstrated that the normal menstrual cycle is an important risk factor for ovarian cancer [17]. In fact, this cycle is probably the most important determinant of ovarian cancer risk in individuals who do not carry a genetic predisposition to this disease. Interruption of ovulatory activity protects against the development of this disease independently of whether such interruption is achieved through pregnancy or oral contraceptives. For example, use of oral contraceptives for 5 years results in an approximately 60% decrease in ovarian cancer risk, which is similar to the protective effect of 5 pregnancies after the first [18]. More recent studies suggest that late pregnancies are more protective than those occurring at early ages [19].

We hypothesized that the molecular mechanisms underlying familial ovarian cancer predisposition in individuals carrying germline BRCA1 mutations could be directly linked to those mediating cancer predisposition associated with the ovulatory

cycle. The fact that pregnancy or oral contraceptive use, which both confer strong protection against ovarian cancer in the general population, also provide a similar protection in BRCA1 mutation carriers [20] is supportive of this hypothesis. We therefore reasoned that BRCA1 might, at least in part, influence ovarian tumorigenesis indirectly, by controlling an effector secreted by cells important for the control of menstrual cycle progression. In other words, loss of BRCA1 function could influence ovarian tumorigenesis cell non-autonomously, by disrupting interactions between cells that control the menstrual cycle an cells from which ovarian epithelial tumors originate. Thus, it is the role of BRCA1 as a regulator of transcription and in cell to cell signaling rather than its role in DNA repair that is the basis for our hypothesis, which is illustrated schematically in figure 1.

Given the central role of granulosa cells in regulating progression through the normal menstrual cycle and the role of this cycle in predisposition to ovarian cancer, we further hypothesized that these cells might interact with the cell of origin of ovarian tumors and influence their neoplastic transformation as suggested in the model shown in figure 1. Indeed, granulosa cells secrete a variety of hormones thought to influence growth and signal transduction in ovarian tumors. Such hormones include estrogens, progesterone, and the peptide hormone mullerian inhibiting substance (MIS). MIS belongs to the TGF-beta family [21]. It is secreted by Sertoli cells of the testes in male embryos, functioning to prevent the development of mullerian ducts, from which female reproductive organs other than the ovaries are derived [21]. It is also secreted by granulosa cells in adult ovaries, resulting in detectable levels of MIS in the serum of pre-menopausal women [22-24]. The function of this hormone in women of reproductive age is unknown, although a role in

controlling follicular growth has recently been suggested [25]. A possible role for MIS in controlling ovarian cancer development is suggested by the facts that (i) MIS prevents the development of mullerian ducts in the embryo and (ii) that ovarian epithelial tumors bear a close resemblance to tissues derived from mullerian ducts, which include the fallopian tubes, uterus, and cervix [26]. In support of this idea, MIS can inhibit ovarian epithelial tumor cell growth *in vitro* and *in vivo* [27, 28]. The MIS receptor has a very limited normal tissue distribution, as it is present exclusively in the uterus, fallopian tubes (or uterine horns in mice), granulosa cells of the ovaries, and Sertoli cells [29]. Conolly *et al.* [30] recently took advantage of this tissue specificity to develop a transgenic mouse model for ovarian carcinoma.

Granulosa cell-specific inactivation of Brca1 in a mouse model

We used the *cre-lox* system to inactivate the *Brca1* gene in mouse granulosa cells specifically [31]. This tissue-specific gene knock out was attempted by crossing mice carrying a floxed *Brca1* allele with mice carrying a cre recombinase transgene driven by a truncated form of the FSH receptor promoter, which was reported to drive expression exclusively in granulosa cells [32]. Although the mutant mice indeed showed inactivation of Brca1 in secondary and tertiary ovarian follicles [31], further characterization also showed low frequency of Brca1 rearrangement in 10-20% of cells within the anterior pituitary gland, implying that the entire pituitary-gonadal axis might have been affected (unpublished observations from the author's laboratory). The exact significance of Brca1 inactivation in the pituitary gland remains unclear because of the small proportion of cells that are affected in that organ.

The mutant mice were fertile and their litters were of normal size, at least in the first 4 months of life. Two thirds of the mice developed epithelial cysts in their reproductive organs by the time they reached the age of 12-18 months [31]. Some of those cysts involved the ovary and were very similar to human ovarian cystadenomas. These tumors were not confined to the ovary, but were seen along the entire mullerian tract in a manner reminiscent of para-ovarian and para-tubal epithelial cysts in humans [31]. The finding of abnormalities in the uterine horns in addition to the ovaries is compatible with reports that women undergoing prophylactic oophorectomy for familial predisposition to ovarian cancer have a high incidence of preneoplastic lesions in the fallopian tube epithelium [33-36]. Although the tumors were benign, preliminary results suggest that crossing the mutant mice with mice carrying a homozygous knock out of p53 increases the rate of malignant transformation. The fact that the cystic tumors showed no evidence of rearrangement of Brca1, implying that they expressed a functional Brca1 protein, strongly supports the hypothesis that cells that control the ovulatory cycle, including ovarian granulosa cells and a possibly a subset of cells from the anterior pituitary gland, use signaling pathways dependent on the presence of a normal Brca1 gene product to influence the development of ovarian epithelial tumors.

The mutant mice would be expected to develop lesions in their mammary glands in addition to their reproductive tract if this experimental model was relevant to familial cancer predisposition in human BRCA1 mutation carriers. Although the mammary glands of these animals has not yet been systematically examined, preliminary findings show that mutant animals show prominent large ectatic ducts, suggesting that the phenotypic consequences of Brca1 inactivation in the pituitary-

gonadal axis include abnormalities in the breasts in addition to the ovaries and uterine horns (unpublished observations from the author's lab). Thus, the distribution of the lesions seen in this mouse model closely mimic the distribution of cancers developing in human BRCA1 mutation carriers as indicated in Table 1.

Consequences of Brca1 inactivation on the estrus cycle

Much of the rationale for creating this mouse model was based on the idea that cancer predisposition in BRCA1 mutation carriers is mediated through mechanisms similar to those responsible for such predisposition in incessantly ovulating women. We therefore tested the hypothesis that the mutant mice showed differences in their estrus cycle and that such differences could be in part responsible for increased predisposition to epithelial cysts in their reproductive tract. Daily vaginal cytology specimens were obtained from mutant and littermate control mice over 3-5 weeks when the animals were 3-4 and 7-8 months old. Given the fact that characteristic cytological changes are associated with each phase of the estrus cycle, microscopic examination of each sample allowed determination of the phase of the cycle present at each time point in each mouse. We used these data to calculate and compare the average length of each phase of the cycle in mutant *versus* normal mice. There was a statistically significant elongation of the proestrus phase in mutant mice compared to wild type mice in both age groups that was most marked in the 7-8 month old group ($P = .003$). Given that this proestrus is characterized by unopposed estrogens, these results support the idea that tumor predisposition in mutant mice is mediated, at least in part, by increased estrogen stimulation due to an increase in the average length of the proestrus phase. A recent report that down-regulation of BRCA1 expression in human granulosa cells leads to increased

expression of aromatase, the rate limiting enzyme in estradiol biosynthesis, is well in line with this idea [37]. These results also raise the possibility that women harboring germline BRCA1 mutations could similarly have differences in their menstrual cycle such as elongation of the follicular phase, which is the equivalent of the proestrus phase in the estrus cycle. Whether mice showing an increase in the length of their proestrus phase are more likely to develop epithelial cysts is still unclear because although the current data suggests that such an association indeed exists, the results do not reach statistical significance and have low statistical power due to the small number of animals so far examined.

Cancer predisposition in BRCA1 mutation carriers

Given that the epithelial cysts that develop in mutant mice in our animal model do not harbor mutant *Brca1* alleles, a strong argument can be made that at least in this experimental model, a Brca1 mutation acts cell non-autonomously to cause proliferative lesions in the epithelium of the entire mullerian tract. Although the relevance of our animal model to cancer predisposition in humans is still unclear, the fact that mutant animals develop abnormalities in the same organs that are at risk in women harboring BRCA1 mutations (Table 1) argues in favor of such relevance. We therefore propose, based on this evidence, that predisposition to breast and gynecological cancers in women with germline BRCA1 mutations is mediated, at least in part, by an overall decrease in BRCA1 gene dosage that is the direct result of this germline mutation. Such decrease in BRCA1 expression affects granulosa cells as well as perhaps in other components of the pituitary-gonadal axis by interfering with endocrine or paracrine interactions take that normally occur between those cells and the epithelial cells lining the mullerian tract, resulting in predisposition to neoplastic

transformation. This hypothesis does not rule out a cell autonomous mechanism based on the idea that BRCA1 also functions as a classical tumor suppressor, as those two scenarios are not mutually exclusive and both could cooperate with each other to promote cancer development. However, the idea of a cell non-autonomous mechanism not only provides a straightforward explanation for the site specificity of the cancers that develop in individuals carrying germline BRCA1 mutations, but also accounts for the protective effect of surgical ablation of the ovaries, the site of granulosa cells, on breast cancer predisposition in these patients [38].

Implications for the Identification of the Cell of Origin of Ovarian epithelial Tumors

Ovarian epithelial tumors are thought to arise from the mesothelial layer that covers the ovarian surface according to the favored hypothesis. An argument has been made that these tumors could instead originate from derivatives of the mullerian tract based on their morphological and functional characteristics [26]. Indeed, serous ovarian carcinoma, which is the ovarian tumor subtype that typically develops in BRCA1 mutation carriers, is morphologically indistinguishable from neoplasms of the fallopian tubes, which are part of the mullerian tract. This resemblance is so striking that pathologists, by convention, have for decades been diagnosed all serous tumors from the tubo-ovarian area as serous ovarian neoplasms unless they were dealing with lesions small enough to be confined to the tubes or distributed in such a way that an origin from the tubes could clearly be demonstrated. It seems unlikely that cells that are as different in their function and embryological origin as the ovarian surface mesothelium and the fallopian tube epithelium could give rise to identical tumors. In addition, if serous ovarian tumors indeed developed in the cell layer lining the ovarian

surface, these tumors would be the only example of tumor of somatic cells that shows a greater degree of differentiation than the cell type from which it originates. I have argued that all tumors currently classified as ovarian epithelial tumors originate in components of the mullerian tract, either the fallopian tube or the numerous mullerian derivatives found within and around the ovary such as endosalpingiosis, endometriosis, and endocervicosis, which have also been referred to as secondary mullerian system [26]. The fact that the epithelial cysts that develop in mice lacking a functional Brca1 in their pituitary-gonadal axis are not confined to the ovary, but distributed along the entire mullerian tract is not only supportive of this hypothesis, but provides an attractive experimental model to test it further.

Concluding remarks

Our results strongly suggest that a circulating factor secreted by granulosa cells and under the control of Brca1 can influence predisposition to tumor development in the ovary as well as in components of the mullerian tract in rodents. Our hypotyhesis, in line with our views regarding the site of origin of ovarian epithelial tumors in humans, is that the lesions involving the ovary in this animal model originate in cells derived from the mullerian tract. At this point, it is not clear whether the mechanism of tumor predisposition in this model is similar to that in humans with germline *BRCA1* mutations. Even if the mechanisms are not identical, it is likely that there are significant overlaps because the tumors in both species involve similar organs and tissues and are driven by inactivation of a similar gene. This has potentially important translational potential because knowledge of a circulating factor secreted by granulosa cells or other components of the pituitary-gonadal axis and associated with ovarian cancer predisposition could lead to the development of a

novel approach, possibly based on a simple blood test, for screening for ovarian cancer predisposition. This knowledge may also form the basis for novel strategies, based on manipulations of the levels of the factor in question, for ovarian cancer prevention in individuals with familial predisposition to this disease.

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Table 1

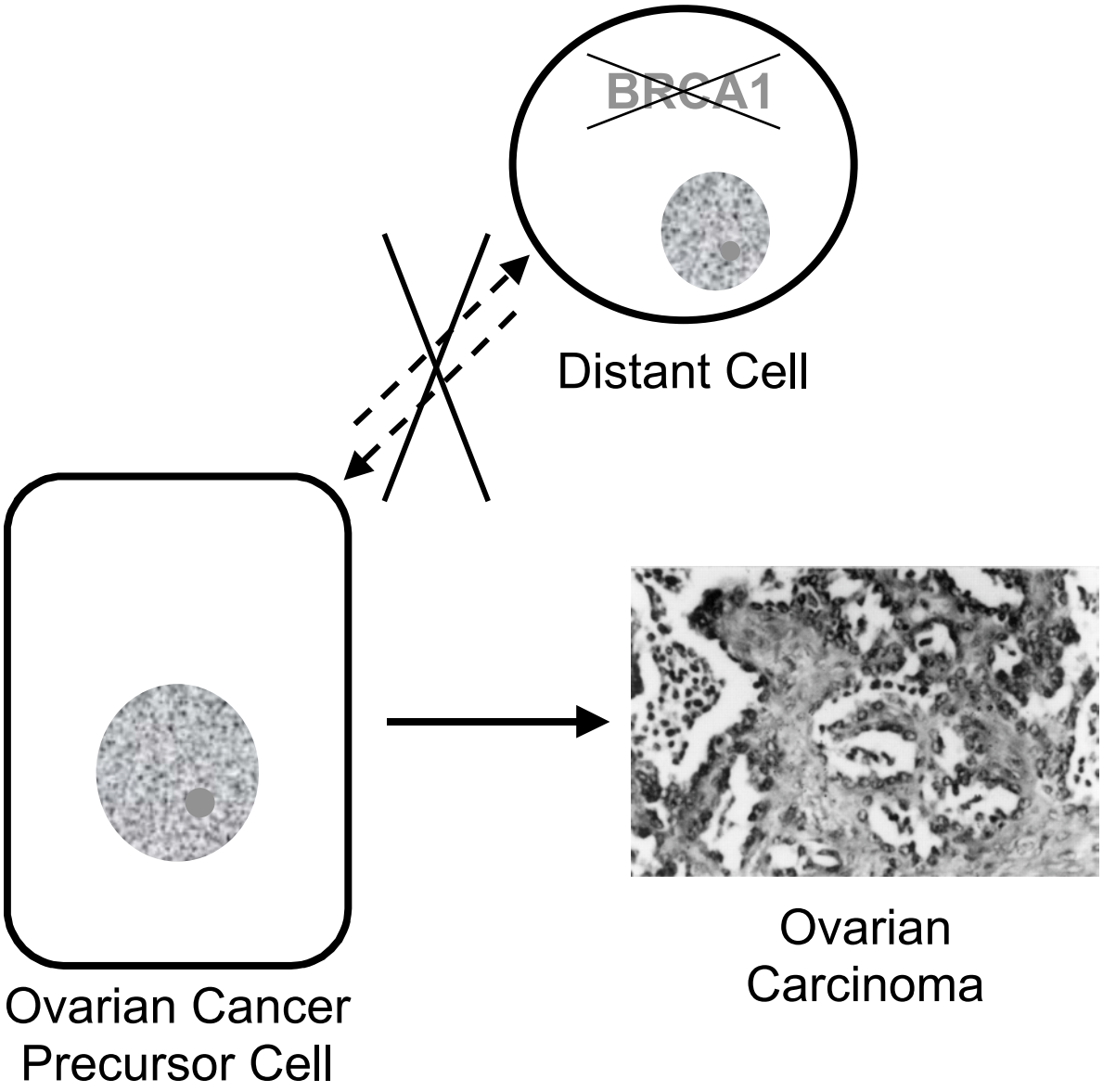
Comparison of lesions in mice with granulosa cell specific inactivation of Brca1 to cancers in human with germline BRCA1 mutations

ORGAN	HUMAN	*Brca1* $^{flox/flox}$; *Fshr-Cre* MICE
Ovary	Serous carcinoma	Serous cystadenoma
Fallopian tube/Uterine horn	Serous carcinoma	Multiple serous cysts
Breast	Ductal carcinoma	Ductal ectasia

Figure Legends

Figure 1: Cell non-autonomous hypothesis for cancer predisposition in BRCA1 mutation carriers. This model stipulates that in the presence of normal BRCA1 function, the cell type from which ovarian epithelial tumors originate (ovarian cancer precursor cell) interacts with another cell type (distant cell) from a distance, either via endocrine or paracrine mechanisms. Loss of normal BRCA1 function in the distant cells leads to disruption of these normal intercellular interactions, resulting in predisposition to neoplastic transformation.

Figure 1:


BRCA1
Distant Cell
Ovarian Cancer
Precursor Cell
Ovarian
Carcinoma

Cancer

Remote control

Curr. Biol. **15,** 561–565 (2005)

BRCA1 is notorious as the first gene to be linked with inherited susceptibility to breast and ovarian cancer. It has been thought of as a classic 'tumour suppressor', but Rajas Chodankar *et al.* suggest that it may have another, more subtle, effect.

Granulosa cells in the ovary produce the sex hormones that regulate the ovulatory cycle — and the growth of ovarian tumours. Given that repeated ovulations (that is, fewer pregnancies or reduced oral contraceptive use) are known to increase the risk of non-hereditary ovarian cancer, the researchers wondered whether decreased levels of BRCA1 protein in granulosa cells are involved. Using mice, they inactivated the gene specifically in these cells. The animals developed tumours in the ovaries and uterine horns. But the tumour cells looked like epithelial cells and had normal copies of the gene, implying that they had not developed from granulosa cells.

Inactivating *BRCA1* seems, therefore, to be controlling some intermediary produced by the granulosa cells. It is this unidentified factor that appears to promote tumours in the ovary epithelium, so providing a lead for further investigation. **Helen Dell**

ABSTRACT OF A LECTURE GIVEN AT THE MOUSE MODEL OF HUMAN CANCERS CONSORTIUM – GYNECOLOGICAL MODELS MEETING IN PUERTO RICO IN FEBRUARY 2004

Homozygous Knock Out of *Brca1* in mouse ovarian granulosa cells results in benign and malignant ovarian epithelial tumors

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University of Southern California/Norris Comprehensive Cancer Center and Center for Ceaniofacial Molecular Biology, University of Southern California; National Institute of Diabetes and Digestive and Kidney Disease

Ovarian granulosa cells secrete several hormones thought to influence the growth of ovarian epithelial tumors including sex steroids and and mullerian inhibiting substance. We hypothesized that such intercellular interactions between granulosa cells and cells from which these tumors originate are controlled by the BRCA1 protein. We further hypothesized that disruption of these intercellular interactions in the absence of a fully functional BRCA1 protein are, at least in part, responsible for the ensuing predisposition to ovarian cancer. We tested this hypothesis by creating a *Brca1* knock out targeted to granulosa cells specifically. This cellular specificity was achieved by introducing a transgene for the cre recombinase under the control of a truncated form of the FSH receptor promoter into a mouse homozygous for a floxed *Brca1* allele. The specificity of this promoter for granulosa cells was verified by crossing the transgenic line with the ROSA26R Cre reporter mouse strain.

Mice homozygous for mutant *Brca1* in their granulosa cells were fertile and had normal litter sizes, at least during the first 6 months of life. Histological examination of the ovaries and other organs of the female reproductive tract in two-month old animals revealed no apparent abnormality. One third of the mice developed large ovarian cysts by the time they reached the age of one year. Most of these cysts were histologically benign and similar to human ovarian serous cystadenomas. Of the 10 such tumors so far examined, one was malignant. Crossing the mice with a strain carrying a p53 knock out increased the rate of malignant transformation. Approximately 50% of the mutant mice also developed cysts in the outer wall of their uterine horns. The epithelial lining of these cysts was similar to that of the ovarian tumors.

These results strongly suggest that disruption of BRCA1 function in granulosa cells leads to change in the levels of one or more circulating factor(s), which in turn leads to proliferative lesions within the entire mullerian tract, which we regard as the site of origin of ovarian epithelial tumors. The nature of this (these) circulating factor(s) and the consequences of the mutant alleles on the length and characteristics of the estrus cycle are currently under investigation. This animal model not only provides a novel tool to elucidate the mechanisms of ovarian epithelial tumor predisposition and development, but should also prove valuable in elucidating the nature of their precursor lesions and of their exact cell of origin.

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HANDOUT OF LECTURE GIVEN AT SYMPOSIUM ENTITLED: "OVARIAN CANCER: PREVENTION AND DETECTION OF THE DISEASE AND ITS RECURRENCE" HELD IN PITTSBURGH, PA ON OCTOBER 24-25, 2005

MECHANISM OF OVARIAN CANCER PREDISPOSITION IN INDIVIDUALS WITH GERMLINE *BRCA1* MUTATIONS

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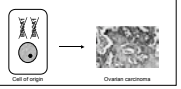
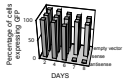
MECHANISM OF OVARIAN CANCER PREDISPOSITION IN BRCA1 MUTATION CARRIERS

Tumor suppressor hypothesis:

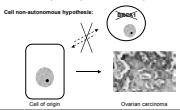


SELECTIVE DISADVANTAGE OF REDUCED *BRCA1* EXPRESSION



MECHANISM OF OVARIAN CANCER PREDISPOSITION IN BRCA1 MUTATION CARRIERS

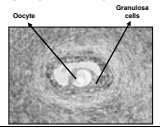
Cell non-autonomous hypothesis:



INFLUENCE OF PARITY AND ORAL CONTRACEPTIVE USE ON OVARIAN CANCER RISK

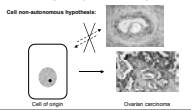
Risk Factors		Relative Risk
Parity	0	1.00
	1	0.81
	2	0.42
	3	0.33
	4+	0.31
Oral Cont. Contraceptive Use (yr)	0	1.0
	<1	0.9
	1-3	0.8
	3-5	0.5
	5+	0.4

SECONDARY FOLLICLE

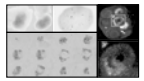


MECHANISM OF OVARIAN CANCER PREDISPOSITION IN BRCA1 MUTATION CARRIERS

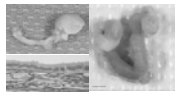
Cell non-autonomous hypothesis:



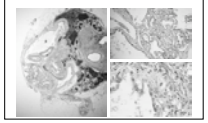
DISTRIBUTION OF Fshr PROMOTER ACTIVITY IN R26R MICE



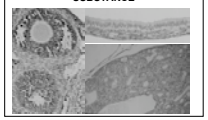
MUTANT MICE DEVELOP OVARIAN CYSTADENOMAS



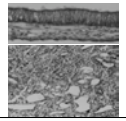
INVASIVE CARCINOMA IN A P53/BRCA1 DOUBLE MUTANT



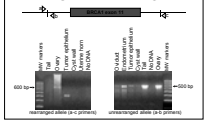
IMMUNOHISTOCHEMICAL STAINING FOR MULLERIAN INHIBITING SUBSTANCE



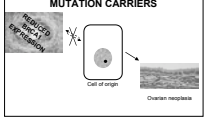
IMMUNOHISTOCHEMICAL STAINING FOR NON-SQUAMOUS KERATINS



TISSUE DISTRIBUTION OF MUTANT ALLELES

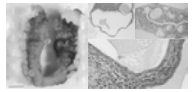


MECHANISM OF OVARIAN CANCER PREDISPOSITION IN BRCA1 MUTATION CARRIERS

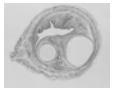


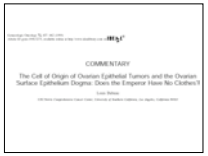
PERI-TUBAL CYST IN BRCA1 KNOCK-OUT MICE

MUTANT MICE DEVELOP EPITHELIAL CYSTS ALONG THE ENTIRE MULLERIAN TRACT



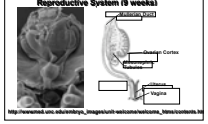
UTERINE CYST




COMMENTARY

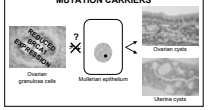
The Cell of Origin of Ovarian Epithelial Tumors and the Ovarian Surface Epithelium Dogma: Does the Emperor Have No Clothes?

Embryological Development of the Female Reproductive System (9 weeks)

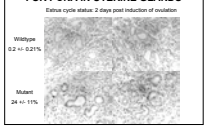


http://www.med.unc.edu/embryo_images/unh-welcome/welcome_htms/contents.htm

CELL NON-AUTONOMOUS MECHANISM OF PREDISPOSITION TO CANCERS OF THE REPRODUCTIVE TRACT IN BRCA1 MUTATION CARRIERS



IMMUNOHISTOCHEMICAL STAINING FOR PCNA IN UTERINE GLANDS



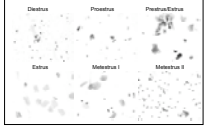
7

POSSIBLE MECHANISMS FOR OVARIAN TUMOR PREDISPOSITION BASED ON A CELL NON-AUTONOMOUS SCENARIO

- Direct mechanism
  - An effector secreted by granulosa cells is regulated by Brca1
- Indirect mechanism
  - Brca1 inactivation results in alterations in the ovulatory cycle, which in turn influences ovarian epithelial tumor development

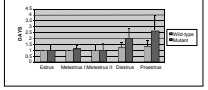
Oncogene, in press

Classification of estrus cycle stages in vaginal smears using the Papanicolaou stain



8

COMPARISON OF LENGTH OF ESTRUS CYCLE STAGES IN MUTANT *VERSUS* WILD-TYPE LITTERMATE MICE



SUMMARY

- The embryonic lethality of BRCA1 general knock out was avoided by knocking out this gene in granulosa cells specifically
- Mouse carrying mutant alleles of BRCA1 in their granulosa cells had morphologically normal ovarian follicles and were fertile, but developed benign and malignant epithelial ovarian tumors as well as extra-ovarian cysts in their mullerian tract
- Alterations in interactions between granulosa cells and the cell of origin of ovarian epithelial tumors may be the key to familial ovarian cancer predisposition in individuals carrying germline BRCA1 mutations.

SIGNIFICANCE

- A better understanding of the normal interactions between ovarian granulosa cells and the cell of origin of ovarian epithelial tumors may lead to novel strategies for the identification of individuals at risk and for ovarian cancer prevention.
- The results support the hypothesis that ovarian epithelial tumors are of mullerian origin, which is important for the understanding of their precursor lesions and for the development of effective screening strategies for their early detection.

9

MAMMARY GLAND OF 1.5 YEAR OLD VIRGIN MUTANT MOUSE

ACKNOWLEDGEMENTS

- Rajas Chodankar, Hai-Yun (Helen) Yen, Hao Hong , Sepideh Karimi (Dubeau Lab)
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- Axel Schönthal (USC)
- French Anderson (USC)
- Nori Kasahara (USC/UCLA)
- Chu-Xia Deng (NIH)

10

Aegean Conferences Abstract Submission Form

IMPORTANT

1) Please ensure that you have filled out the Title, Authors, Affiliations and Abstract sections.
2) The maximum number of words allowed in your entire abstracts 400 words. This includes the Title, Author(s), Affiliation(s) AND Abstract body.
3) Save the completed for upload using Microsoft Word 2000 or lower version.

I. Contact Information:

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Meeting:	1st International Conference on Ovarian Cancer: State of the Art and Future Directions

II. Abstracts

IMPORTANT: Please select the grey text below *(by clicking once on the grey text)* and start typing in the designated section. The text is pre-formatted in Times New Roman 12pt. font single line spacing. You may alter these values only to add symbols or superscripts etc. Use Symbol font for Greek and other special characters.
Do **NOT** include tables, figures or references. These will be removed before your abstract is published.

Title:
BRCA1 INDUCED OVARIAN ONCOGENESIS

Louis Dubeau

University of Southern California Keck school of Medicine
USC/Norris Comprehensive Cancer Center

Women with germline mutations in *BRCA1* have a 40% risk of developing ovarian cancer by age 70 and are also predisposed to cancers of the fallopian tubes and breast. Although some observations are supportive of the currently favored notion that BRCA1 functions as a classical tumor suppressor gene, others are inconsistent with this notion. Given that ovulatory activity is a strong risk factor for sporadic ovarian cancer, we hypothesized that reduced BRCA1 expression might predispose to gynecological cancers indirectly by influencing ovarian granulosa cells. These cells secrete sex steroids that control the ovulatory cycle and influence the growth of ovarian epithelial tumors. We tested this hypothesis by using the *Cre-lox* system to inactivate the *Brca1* gene in mouse ovarian granulosa cells. A

truncated form of the FSH receptor promoter served as the Cre driver in order to achieve such tissue specificity. Indeed, inactivation of the *Brca1* gene in granulosa cells led to the development of benign cystic tumors in the ovaries and uterine horns in approximately one third of the mutant mice. The mutant mice also developed epithelial cysts in their mammary glands. These tumors were of epithelial origin and carried normal *Brca1* alleles, supporting the view that Brca1 influenced their development indirectly, through an effector secreted by granulosa cells. The fact that the cystic tumors were not confined to the ovaries, but involved the entire mullerian tract, is also consistent with the view that ovarian tumors are not of mesothelial origin, but are derived from the mullerian tract. We measured the length of each phase of the estrus cycle in a group of mutant and wild type mice at 3-4 months and 7-8 months of age. In both age groups, there was a significant increase in the length of proestrus, which corresponds to the follicular phase of the human menstrual cycle. We suggest that the increased cancer predisposition seen in individuals carrying a germline *BRCA1* mutation is due, at least in part, to reduced levels of BRCA1 expression in granulosa cells. This, in turn, may lead to alterations in the length of the follicular phase of the menstrual cycle and ensuing increased unopposed estrogen stimulation as well as, perhaps, to disruption of additional interactions between granulosa cells and mullerian epithelial cells resulting in promotion of neoplastic transformation.