Award Number: W81XWH-04-1-0485

TITLE: Epithelial-Stromal Interactions in Induction of Ovarian Cancer in a Mouse Model

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REPORT DATE: April 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20050927 077
### REPORT DOCUMENTATION PAGE

**1. AGENCY USE ONLY**  
(Leave blank)

**2. REPORT DATE**  
April 2005

**3. REPORT TYPE AND DATES COVERED**  
Annual (1 Apr 2004 - 31 Mar 2005)

**4. TITLE AND SUBTITLE**  
Epithelial-Stromal Interactions in Induction of Ovarian Cancer in a Mouse Model

**5. FUNDING NUMBERS**  
W81XWH-04-1-0485

**6. AUTHOR(S)**  
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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**  
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT (Maximum 200 Words)**

Ovarian cancer can be effectively treated if detected early, but it is generally diagnosed after it has metastasized. This seriously impacts the survival rate of patients, and has also limited our knowledge of the early genetic changes that induce ovarian cancer. Our goal is to develop experimental systems that recapitulate genetic changes that occur during ovarian carcinoma initiation and simulate the complex interactions between ovarian surface epithelial and stromal cells. By introducing various combinations of genetic alterations into the ovarian surface epithelial cells *ex vivo* and *in situ*, we demonstrated that certain biochemical pathways can collaborate in ovarian cancer initiation and progression. We also explored the role of stromal cells in ovarian cancer progression and determined that both transformed and untransformed stromal cells are inhibitory to tumor growth. Since ovulation is thought to be directly associated with ovarian tumor initiation, we studied cell proliferation during ovulatory wound repair. We determined that ovulation increases the overall rate of ovarian surface epithelial cell proliferation. However, the ovulatory wound is mostly repaired by cell migration and not by local cell proliferation. Induced ovulation in mice also contributed to the formation of ovarian inclusion cysts, which are thought to be the precursor lesions in ovarian cancer.

**14. SUBJECT TERMS**  
No subject terms provided.

**15. NUMBER OF PAGES**  
10

**16. PRICE CODE**

**17. SECURITY CLASSIFICATION OF REPORT**  
Unclassified

**18. SECURITY CLASSIFICATION OF THIS PAGE**  
Unclassified

**19. SECURITY CLASSIFICATION OF ABSTRACT**  
Unclassified

**20. LIMITATION OF ABSTRACT**  
Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102
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Introduction

The tumor environment is thought to influence many of the steps of tumor progression (Hanahan and Weinberg, 2000). It has been demonstrated that xenografted human tumor cell lines give rise to tumors more efficiently when mixed with normal fibroblasts (Elenbaas et al., 2001; Elenbaas and Weinberg, 2001). It has also been demonstrated that fibroblasts associated with cancer cells may undergo functional changes that, in turn, enhance their ability to promote tumor formation (Olumi et al., 1999). These results suggest that the presence of normal fibroblasts provides a milieu that facilitates growth and proliferation of tumorigenic epithelial cells. However, it is also possible that fibroblasts, when transformed, play a more direct role in promoting tumor initiation in epithelial cells.

Ovarian carcinoma is most frequently diagnosed at a late stage. Hence, the precursor lesions for ovarian carcinomas have not been detected, and the series of morphologic changes that occur as the benign epithelium becomes malignant is not well defined in ovarian cancer. Architectural aberrations of the surface epithelium, such as pseudostratification, papillomatosis, cortical invaginations and epithelial inclusion cysts, are commonly seen in human ovarian samples. It is thought that these atypical histologic features represent precursor lesions to ovarian carcinoma. In a normal ovary, the monolayered ovarian surface epithelium lies adjacent to the basement membrane (Nicosia et al., 1989) that separates the epithelium from the ovarian stroma. It is thought that upon malignant transformation, the rapidly proliferating surface epithelial cells lose their contact with the basement membrane and disseminate into the peritoneal cavity and/or invade the stroma and grow inside the ovarian cortex. The development of a model for ovarian cancer biogenesis would aid in the identification of molecular markers or recognizable premalignant histologic alterations.

We are developing mouse models in which defined multiple genetic alterations can be introduced into mouse ovarian stromal and/or surface epithelial cells in culture or in mouse ovaries. This system is based on avian RCAS virus delivery to the cells that are programmed to express the avian TVA receptor under the control of a tissue-specific promoter. The expression of the TVA receptor in mouse ovarian cells renders the cells susceptible to infection with RCAS viruses. RCAS vectors can be designed to carry oncogenes, marker genes, Cre recombinase, or activators of inducible systems into the TVA receptor-expressing cells. Various candidate genes that are thought to play a role in ovarian cancer can be introduced simultaneously or sequentially into mouse ovarian cells. Since multiple genes can be delivered to the same cell, it is possible to study the collaboration of biochemical pathways in ovarian cancer induction and progression.

Body

SPECIFIC AIM 1: Characterize epithelial-stromal interactions in ovarian tumor initiation and progression.

In order to determine whether stromal cells play a role in ovarian cancer formation, we assayed transformed and untransformed MEFs for the ability to alter the growth of ovarian cancer cell lines with defined genetic alterations. We introduced various combinations of genes that are known to play a role in human ovarian cancer into the mouse ovarian surface epithelial cells from K5-TVA; p53−/− mice. After generating a large number of cell lines (Appendix), our results confirm that multiple genetic alterations are necessary for the transformation of mouse ovarian surface epithelial cells. Conversely, p53+/− MEFs could be transformed with a single oncogene, such as Akt, myc, or K-ras (Appendix).

We combined MEFs with transformed ovarian surface epithelial cells in order to determine whether tumor formation would be enhanced, unchanged, or inhibited. Varying numbers (0, 10^5, 10^6 or 10^7) of
ovarian epithelial cells and MEFs were mixed in vitro and injected subcutaneously into nude mice in order to screen for tumor formation (Table 2). As expected, injection of p53\(^{-}\) MEFs alone did not give rise to tumors. However, injection of transformed ovarian surface epithelial cells resulted in 0.5 cm\(^3\) tumors in 2 to 4 weeks (depending on the combination of genetic alterations). Immunohistochemical staining with antibodies against the HA-tag and Keratin 8 were used to determine the predominant cell type in the tumor. Based on tumor volume and the contribution of epithelial cells (Keratin 8 positive and HA-negative), we determined that both transformed and untransformed stroma were inhibitory to ovarian tumor proliferation. Since the bi-directional signaling between adjacent stromal and epithelial cells has a greater relevance to ovarian cancer formation, we will repeat these experiments with transformed and untransformed ovarian stromal cells.

**SPECIFIC AIM 2: Identify molecular events associated with neoplastic transformation of ovarian surface epithelium.**

Epidemiologic studies suggest a direct correlation between the number of ovulatory cycles and the risk of ovarian cancer (Bernal et al., 1995, Perez et al., 1991, Purdie et al., 2003). The first theory about the role that ovulation could play in ovarian carcinogenesis was put forward by Fathalla (Fathalla, 1971). He speculated that the rupture of a follicle increases the risk of ovarian cancer by causing trauma and exposing the ovarian surface epithelium to high levels of steroid hormones and gonadotropins. Also, repair of the ovulatory wound in the ovarian surface likely results in the rapid proliferation of epithelial cells, which may increase the frequency and accumulation of spontaneous mutations. Additionally, ovulation may lead to the entrapment of epithelial cells in the underlying stroma with the subsequent formation of inclusion cysts. These inclusion cysts could be the precursor ovarian cancer lesions in which the surrounding stromal environment facilitates neoplastic transformation (Ghahremani, 1999).

In order to test the role of ovulation on cell proliferation, we superovulated 4-week old FVB/N female mice and isolated their ovaries at different time points during ovulatory wound repair (12 h, 16 h and 24 h after hCG injection). The mice were injected with BrdU 2 h before ovary isolation and the levels of incorporated BrdU in the ovarian surface epithelial cells were measured by staining paraffin-embedded ovary sections with antibodies against BrdU. We demonstrated that ovarian surface epithelial cells do not proliferate locally to repair the ovulatory wound. Instead, the wound is repaired by overall cell proliferation and migration to the wound site (Figure 1).

![Figure 1. BrdU incorporation in ovarian cells 16 h after hCG injection. A) BrdU positive surface epithelial cells are not specifically localized to the areas of ovulatory wound repair. B) Enlarged view of an ovulatory wound site stained with antibodies against keratin 8 and BrdU.](image-url)
In addition to rapid cell proliferation after ovulation, there is evidence that inclusion cysts may contribute to ovarian carcinoma initiation. Inclusion cysts are common in the human ovaries, and have also been observed in aged mouse ovaries. Upon superovulation, we observed inclusion cyst formation in young mouse ovaries. Such inclusion cysts were lined with epithelial cells that resembled the Müllerian morphology (Figure 2A). Based on low BrdU incorporation, the epithelial cells lining the cyst did not appear to proliferate rapidly (Figure 2B).

A B

Figure 2. Inclusion cyst formation in the superovulated ovary of a 4-week old FVB/N mouse. A) The cyst is lined with Keratin 8 positive epithelial cells that resemble the cells of the Müllerian tract. B) The epithelial cells in the cyst incorporate low levels of BrdU.

We have now demonstrated that the RCAS-TVA approach can be used to induce neoplastic changes in the mouse ovarian surface epithelium in situ by direct injection of concentrated viruses under the ovarian capsule (Figure 3). Also, we have combined the RCAS-TVA system with the Cre-loxP system in order to generate mice in which the p53 and/or Brca1 tumor suppressor genes can be conditionally inactivated in the cells of the ovarian surface epithelium. These additional features improve the flexibility and experimental control of gene expression in the mouse model and also more closely mirror the process that is thought to occur in human ovarian carcinogenesis.

A B

Figure 3. Direct injection of RCAS viruses carrying c-myc and Akt oncogenes into the mouse ovarian capsule of K5-TVA/p53-/- mice. A) Control ovary. B) Neoplastic proliferation of the ovarian surface epithelium was detected by staining paraffin-embedded sections of the infected ovaries with the Keratin 8 antibody.

We will use expression profiling in order to identify differences in gene expression between normal and morphologically aberrant ovarian surface epithelial cells. Candidate markers from gene expression and histochemical analyses will be subsequently validated in human ovarian tumor samples. These tissue microarrays will be simultaneously screened for the expression of differentially expressed genes for which commercial antibodies are available.
Key Research Accomplishments

- Distinct biochemical pathways collaborate in the transformation of mouse ovarian epithelial cells.

- Transformed and untransformed stromal cells are inhibitory to ovarian tumor proliferation.

- Ovarian surface epithelial cells do not proliferate locally to repair the ovulatory wound. Instead, the wound is repaired by overall cell proliferation and migration to the wound site.

- Ovulation induces inclusion cyst formation. Such inclusion cysts are typically lined with Müllerian-like epithelial cells that display normal rates of proliferation.

- Neoplastic premalignant changes can be induced in the mouse ovarian surface epithelium in situ by direct injection of concentrated viruses under the ovarian capsule.

Reportable Outcomes

The following reagents were generated:

1. Mouse ovarian cancer cell lines with defined genetic alterations (see Appendix).

2. K5-TVA; p53<sub>lox/lox</sub>; Brca1<sub>lox/lox</sub> triple transgenic mice in which Brca1 and p53 can be conditionally inactivated by the introduction of RCAS-Cre recombinase.

3. Custom tissue microarray that contain 78 ovarian tumors of different histological types (serous, mucinous, endometrioid, clear cell, transitional), grade (1-3), and diagnosis (primary, metastatic, borderline).

Conclusions

Unlike many other epithelial tumors that progress gradually and display identifiable preneoplastic lesions, the majority of ovarian epithelial cancers display characteristics of invasive carcinoma without any evidence of intermediate phases of benign and/or borderline neoplastic lesions. Consequently, very little is known about precursor lesions for ovarian carcinoma and the underlying genetic events that induce transformation of the normal ovarian surface epithelium. The lack of understanding of the molecular and morphological steps involved in ovarian carcinogenesis limits our ability to screen for early stage ovarian cancer. Therefore, it is expected that the development of animal models in which ovarian cancer can be induced and studied during its early stages will help better understanding of early ovarian cancer lesions and elucidate molecular events that support ovarian cancer progression. We have developed a mouse model that allows us to study the molecular mechanisms by which combinations of genetic alterations contribute to the initiation and progression of ovarian cancer. Using this model, we have determined that distinct genetic alterations collaborate in ovarian cancer initiation. Our results support the hypothesis that ovulation and ovulatory wound repair may be directly involved in ovarian cancer initiation.
References


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<th>Cell line</th>
<th>Genetic alterations</th>
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<td>C1, C11, C111</td>
<td>p53&lt;sup&gt;+&lt;/sup&gt;, myc, K-ras</td>
<td>Ovaries from K5-TVA; p53&lt;sup&gt;+&lt;/sup&gt; mice were infected with RCAS-myc and RCAS-K-ras in vitro</td>
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<td>T1, T11, T111</td>
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<td>C1, C11, and C111 cell lines were injected IP into nude mice; T1, T11, and T111 cell lines were derived from IP tumor cells</td>
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<td>mC1 + GFP (4 cell lines)</td>
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<td>C1 cell line was injected IP into four nude mice; RCAS-GFP was injected IP; one mC1+GFP cell line was derived from IP tumor cells from each mouse</td>
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<td>pBmyc</td>
<td>p53&lt;sup&gt;+&lt;/sup&gt;F&lt;sup&gt;f&lt;/sup&gt;, Brca1&lt;sup&gt;f&lt;sup&gt;f&lt;/sup&gt;&lt;/sup&gt;, myc</td>
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## Appendices, continued

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