Mutagenic, surviving and tumorigenic effects of follicular fluid in the context of p53 loss: initiation of fimbria carcinogenesis

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Abstract

Ovulation is the strongest risk factor for ovarian high-grade serous carcinoma (HGSC) that largely originates from the fallopian tube fimbriae and always carries loss-of-function mutations of TP53 in both early and late lesions. Mature ovarian follicle contains high level of reactive oxygen species (ROS). When released from ovulation, follicular fluid (FF) bathes the fimbriae and may lead to DNA double-strand break (DSB) and neoplastic transformation. In this study, we examined the mutagenic and tumorigenic activities of human pre-ovulatory FFs. A subset (6/11) of FFs was found with high levels of ROS whereas the antioxidant capacities were indifferent. These ROS high FFs induced intracellular ROS and DSBs in the secretory cell population of fimbriae epithelium. When p53 and Rb were turned down, the FF-exposed secretory cells overcame apoptosis and expanded the population carrying ROS and DSB. The cancer initiation and promotion effects of FF were further recapitulated in Trp53−/− mice. When introduced into the mammary fat pad, ROS high but not ROS low FFs induced early-onset B-cell lymphoma. Cotreatment with physiological concentration of melatonin, a potent antioxidant, ameliorated the mutagenic and tumorigenic effect of ROS high FF in vitro and in vivo. The study revealed ROS and mitogens in mature ovarian follicles could initiate the transformation of fimbria epithelium in the context of p53 loss and melatonin is a potent preventive agent.

Introduction

High-grade serous carcinoma (HGSC) of the ovary represents over 70% of epithelial ovarian cancers and is the leading cause of death from gynecologic cancers in Western world (1,2). The high prevalence and mortality of HGSC is mainly attributed to lack of prevention and early detection target, since both the origin and etiology are elusive (3). Studies in recent years have clarified that a large proportion of HGSC originates in the fallopian tube fimbriae (4,5). Examinations of the fallopian tubes and ovaries prophylactically removed from women who carry BRCA1 or BRCA2 mutations and are at high risk for HGSC have revealed early lesions, namely serous tubal intraepithelial carcinoma (STIC), in the fallopian tube but not in the ovarian surface (6,7).

This early lesion is associated with 60% of women with HGSC of the ovary or peritoneum (8). Two folds of evidence further support this non-ovarian origin of HGSC: the resemblance of xenograft tumors of transformed human fimbria epithelial cells and HGSCs in aspects of histology, immunophenotype and genomic profile (9,10), and the de novo development of both HGSC and STIC in transgenic mice with targeted disruption of the Tp53, Brc and Pten genes in oviduct secretory cells (11). However, the etiological agents that drive early transformation of fimbria epithelial cells remained unclear.

Somatic mutation of TP53 is believed to be the earliest event of fimbria transformation. Mutation of TP53 has been observed...
ubiquitously in ovarian HGSC and in STIC lesions (12,13), with identical forms of mutation in reported cases with concomitant HGSC and STIC lesions (14). Furthermore, the earliest lesion of p53 mutation, presenting as foci of strong p53 immunostaining, has been found in histologically normal tubal epithelium (12,15). This so-called ‘p53 signature’ was found in about one-third of normal appearing tubes from women with or without BRCA mutations, and in one half of tubes carrying STIC lesions (12,16). The prevalence of p53 signature increased with age, and is associated with low parity and less duration of oral contraceptive (OC) use, suggesting an association with ovulation (17). Meanwhile, about two-third of the p53 signature lesions were also positive for γH2AX indicating DNA double-strand break (DSB) (12).

Numerous epidemiological studies have suggested that incessant ovulation is a strong risk factor for ovarian cancer and that using OCs strongly protects from ovarian cancer in a dose- and time-dependent manner (18). Each year of OC use confers a 5% reduction (95% CI = 2–8%) of risk, up to odds ratio of 0.18 (95% CI = 0.08–0.39) (19). The risk-reducing effect of OC is long-lasting and does not attenuate until three decades after the discontinuation of use (20). All these evidences point to ovulation as the very early cause of ovarian carcinogenesis.

Exposure of fimbria epithelial cells to mature follicular fluid (FF) could induce upregulation of inflammatory and DNA repair genes, and accumulation of p53 (21). Accumulation of DNA DSBs was also observed in cultured human fimbria tissues or fimbria epithelial cells after exposure to ionizing radiation (22) or human FF (21). However, the mutagens in FF have not been identified yet, and whether the mutagen in FF is tumorigenic in vivo is unknown. Besides, the role TP53 in this initiation stage of transformation is unclear.

We propose that the main cause of transformational initiation in the fimbriae is the reactive oxygen species (ROS) in preovulatory follicles. High level of ROS in FF is essential for the physiological process of ovulation (23). FF released from ovulation bathes the surrounding fimbriae and may lead to mutagenesis and neoplastic transformation of the epithelium (24). The present study investigated ROS and antioxidants in human FF and explored the mutagenic, mitogenic and tumorigenic effects in the context of wild type and null TP53.

### Materials and methods

#### Primary culture and immortalization of fimbria or ovarian epithelial cells

For primary culture of human fallopian tube epithelial cells, the method reported by Paik et al. (25) was adopted. After soaking in 1% trypsin and 5mM EDTA at 37°C for 30min, the epithelium of fimbriae was peeled off, digested with 1.5 mg/ml of collagenase (c2674, Sigma) for 1h, and cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, with 5 μg/ml insulin, 100 IU/ml penicillin, and 100 μg/ml streptomycin on a 0.1% gelatin-coated plate. For cell immortalization, primary fimbria epithelial cells at passage 2 were transduced with an HPV16 E6/E7 lentiviral construct to generate the FTE-E6E7 cells. FTE-E6E7 cells were subsequently transduced with a lentiviral hTERT (Applied Biological Materials) to generate the FE25 cell. Cells were analyzed expressing pancytokeratin (AE1/AE3) (53-9001-80, ebioscience) and secretory cell specific PAX8 (ab53490, Abcam) marker, and were negative for the TUBB8 (GTX102095, GeneTex) ciliated cell marker, indicating a secretory cell line. FE25 cells had a doubling time of 35.8h, did not grow a colony in soft agar, and did not produce a tumor in NOD-SCID mice following subcutaneous and intraperitoneal injections (n = 4 each with 10³ cells) (data not shown). The cell line was maintained in MCDB105 and M199 medium (Sigma) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. This cell line has been authenticated by ATCC short tandem repeat profiling (www.atcc.org/STR%20Database.aspx) by Center for Genomic Medicine, National Cheng Kung University, Taiwan and exhibited no evidence of cross-contamination with known ATCC cell lines.

#### FFs and fallopian tube specimens

Procurement of clinical specimens for this study was approved by the Institutional Review Board (TGH-IRB #93-025) of Tzu Chi General Hospital, Hualien, Taiwan. Informed consent was signed by each donor. FF aspirates were collected from 11 women (aged 30–47 years) underwent oocyte retrieval and in vitro fertilization. After centrifugation to removal cell debris (1200g, 10min), they were subjected to spectrophotometry to identify aspirates with minimal contamination with blood and flush medium (Supplementary Figure 1A, available at Carcinogenesis Online). Fallopian tube were procured from patients who underwent prophylactic salpingectomy during operations for benign neoplasm of the uterus or ovary. To collect epithelial scrapings, the lumen of the fimbriae and isthmus parts of the fallopian tube were each gently brushed in a single circle with a cervical Cytobrush and transferred to the phosphate-buffered saline or medium before use (Supplementary Figure 2A, available at Carcinogenesis Online).

#### Cell viability and cell cycle assays

Cell proliferation was evaluated with XTT assay (20-300-1000A, Biolegend) as described before (26). Commercial kits of flow cytometry (FACSCalibur™), including Click-iT® EdU Alexa Fluor® 488 (C10425, Invitrogen) and Annexin V (556420, BD Biosciences), were used for cell cycle and cell apoptosis analyses, respectively.

#### ROS assay

We used an ROS detection kit from Enzo Life (ENZ-51011, Enzo Life Sciences) to measure ROS level. The Oxidative Stress Detection Reagent in the kit reacts with a wide range of ROS, including hydrogen peroxide (H₂O₂), peroxynitrite and hydroxyl radicals, and yields a green fluorescent product. In the analysis, FF samples were diluted in 1:16 with the provided buffer and a concentration gradient (62.5–500 μM) of H₂O₂ was included as the standard. After a reaction at room temperature for 5h, the level of ROS in the samples was determined using an ELISA reader. The ROS level was indicated by the equivalent concentration of H₂O₂ based on the standard curve (Supplementary Figure 1B, available at Carcinogenesis Online). To analyze intracellular ROS after FF treatment, cells were treated with FF for 30min, detached, incubated with the Oxidative Stress Reagent at 37 °C for 30min, and analyzed using flow cytometry (FACSCalibur™) and CELLQuest software (Becton Dickinson).

#### Total antioxidant capacity analysis

Total antioxidant capacity (TAC) was analyzed with the TEAC (Trollox equivalent antioxidant capacity) assay by using the Antioxidant Assay Kit (709001, Cayman). The extent of oxidation inhibition of ABTS® (2,2’-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS® (709001, Cayman) was included as the standard. After a reaction at room temperature for 5h, the level of TAC in the samples was determined using an ELISA reader. The ROS level was indicated by the equivalent concentration of H₂O₂ based on the standard curve (Supplementary Figure 1B, available at Carcinogenesis Online). To analyze intracellular ROS after FF treatment, cells were treated with FF for 30min, detached, incubated with the Oxidative Stress Reagent at 37 °C for 30min, and analyzed using flow cytometry (FACSCalibur™) and CELLQuest software (Becton Dickinson).

#### DNA DSB assay

DNA DSB was detected using immunofluorescence staining. Cells at subconfluence were added with FF and incubated for 3h. After detachment,
the cells were fixed in 4% paraformaldehyde for 15 min, permeabilized by 0.1% Triton X 100 for 30 min, blocked with 2% bovine serum albumin and incubated with mouse monoclonal anti-\( \gamma \)-H\(_2\)Ax antibody (1:200) (05-636, Millipore) at room temperature for 2 h. This was followed by adding FITC-conjugated anti-mouse IgG (1:400) (11-6010-82, Biolegend), incubating for 1 h and examination by using flow cytometry or confocal laser-scanning microscopy (LSM5 PASCAL, Carl Zeiss) with the nucleus counterstained with DAPI.

**Ex vivo fimbria tissue analysis**

For ex vivo fimbria tissue analyses, the fimbria segments of the fallopian tube were opened longitudinally along the lumen, dissected in 1 cm × 1 cm pieces, and cultured in RPMI-1640 with 10% fetal bovine serum. They were then treated with 3% FF or 400 μM \( \mathrm{H}_2\mathrm{O}_2 \) for 3 h before being prepared as paraffin sections. To detect superoxide ROS, sections were stained with 5 μM dihydroethidium (D7008, Sigma). To detect DSB, the same protocol of \( \gamma \)H\(_2\)Ax staining for cells was followed. After staining, sections were observed under light and confocal microscopes.

**Melatonin assay**

Melatonin assay of FF aspirates was evaluated with Melatonin ELISA Kit (RS4021, IBL International). Briefly, 0.5 ml of each sample was extracted using extraction column by the Kit standard version. Evaporate the eluting solution (methanol) by evaporator centrifuge to recovery the dryness melatonin. Extracted melatonin was reconstituted with 150 μl of ddH\(_2\)O and subjected to test procedure immediately according to manufacturer’s instructions.

**Tumorigenesis in TP53-null (Trp53\(^{-/-}\)) mice**

The Trp53\(^{-/-}\) mice (STOCK tm1Brd/J) were purchased from Jackson Laboratory (Stock No. 012620). This type of mouse has a conventional knockout of Trp53 and a conditionally floxed Brca1 with no phenotype in the absence of Cre. To analyze FF-induced tumorigenesis, 3% FFKos mice and into the mammary fat pads of 7-week-old female mice, with or without a coinjection with 800 pM of melatonin. The injection was done in a volume of 200 μl weekly for a total of 8 weeks or until a tumor had obviously developed. Tumors were dissected, weighted, and subjected to paraffin section and stained with hematoxylin and eosin (HE), and with CD20 antibody (sc-7735, Santa Cruz). As controls, phosphate-buffered saline was used to replace FF, and the same FF was injected subcutaneously into the Trp53\(^{-/-}\) mice and into the mammary fat pads of female C57BL/6 mice. All mouse experimental procedures were conducted under the approved guidelines of the Animal Care and Use Committee of Tzu-Chi University (Approval ID: 101–21).

**Data analysis**

The results are presented as mean ± SD of at least three independent experiments. Statistical analysis was carried out using Prism Software (GraphPad), Microsoft Office Excel 2007 and SigmaPlot 10. For statistical comparison, the data were analyzed using Student’s t test between two groups or paired t test of matched samples. Significant differences were defined as \( P < 0.05 \).

**Results**

**Various ROS levels and invariable antioxidant capacity in human FFs**

FF in different aspirates of the same individual and from different individuals exhibited varying levels of ROS. Out of 11 aspirates of 11 consecutive patients, six (55%) happened to have high level of ROS (400 ± 25 μM \( \mathrm{H}_2\mathrm{O}_2 \) equivalent in average), and the remaineders happened to have low level of ROS (56 ± 4 μM \( \mathrm{H}_2\mathrm{O}_2 \) equivalent in average) (Figure 1A). ROS levels in these aspirates were not related to age, cause of infertility, protocol of induction of superovulation, estradiol (E2) level or IVF outcome (Table 1). Remarkably, both TAC and melatonin, a potent antioxidant in ovarian follicles that protects the maturing oocyte, of various FFs (27) were invariable, regardless of the various ROS level (Figure 1B).

**ROS\(^{\text{high}}\) FF is mutagenic and cytotoxic to secretory cells of human fimbria epithelium**

Intracellular ROS in epithelial scrapings from the fimbriae and isthmus of the same tubes were compared pairwise (Figure 2A; Supplementary Figure 2A and B, available at Carcinogenesis Online). In 10 of 11 fallopian tubes, scrapings from the fimbriae exhibited a higher level of intracellular ROS than that from the isthmus (Figure 2B). By using fluorescence-activated cell sorting analysis, the secretory cell population in the tubal scrapings was identified by PAX8 staining (22) (Supplementary Figure 3, available at Carcinogenesis Online) and determined for DSB. In accordance with the finding of ROS level, in five of six individuals, secretory cells in the fimbriae carried more \( \gamma \)H\(_2\)Ax-positive cells than those in the isthmus counterpart.
## Table 1. ROS, total antioxidant capacity (TAC) and estradiol (E2) levels in ovarian follicles of patients receiving in vitro fertilization (IVF) and embryo transfer (ET) protocol

<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Etiology of infertility</th>
<th>Controlled ovarian hyper-stimulation protocol</th>
<th>ROS level (mM)</th>
<th>TAC (ng/ml)</th>
<th>Fertilization rate (%)</th>
<th>No of ET</th>
<th>Clinical outcome</th>
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<tr>
<td>ROS\textsuperscript{high} FF</td>
<td>130083-02</td>
<td>47</td>
<td>Advanced age</td>
<td>CC + rFSH + Menopur + GnRH antagonist</td>
<td>70.9</td>
<td>3.8</td>
<td>53.2</td>
<td>100% (2/2)</td>
</tr>
<tr>
<td>130084-02</td>
<td>33</td>
<td>Male factor</td>
<td>Long protocol GnRH analog with rFSH</td>
<td>67.1</td>
<td>2.4</td>
<td>266.6</td>
<td>43% (3/7)</td>
<td>2</td>
</tr>
<tr>
<td>130112-03</td>
<td>35</td>
<td>PCOS, tubal factor</td>
<td>CC + Menopur + GnRH antagonist</td>
<td>29.5</td>
<td>3.3</td>
<td>224.8</td>
<td>70% (7/10)</td>
<td>2</td>
</tr>
<tr>
<td>130119-11</td>
<td>42</td>
<td>Tubal factor</td>
<td>Long protocol GnRH analog with CC + rFSH</td>
<td>56.8</td>
<td>3.8</td>
<td>621</td>
<td>86% (12/14)</td>
<td>3</td>
</tr>
<tr>
<td>ROS\textsuperscript{low} FF</td>
<td>130120-05</td>
<td>31</td>
<td>PCOS, hyperprolactinemia</td>
<td>Long protocol GnRH analog with Menopur</td>
<td>57.1</td>
<td>3.1</td>
<td>489</td>
<td>66% (10/15)</td>
</tr>
<tr>
<td>130044-01</td>
<td>39</td>
<td>Advanced age</td>
<td>Long protocol GnRH analog with CC + rFSH</td>
<td>364.5</td>
<td>4</td>
<td>456.4</td>
<td>50% (3/6)</td>
<td>3</td>
</tr>
<tr>
<td>130049-01</td>
<td>34</td>
<td>PCOS, tubal factor</td>
<td>Long protocol GnRH analog with CC + rFSH</td>
<td>327.5</td>
<td>3.2</td>
<td>220.2</td>
<td>75% (30/40)</td>
<td>All frozen</td>
</tr>
<tr>
<td>130058-03</td>
<td>38</td>
<td>Tubal factor</td>
<td>Long protocol GnRH analog with CC + rFSH</td>
<td>288.5</td>
<td>3.1</td>
<td>41.5</td>
<td>100% (13/13)</td>
<td>4</td>
</tr>
<tr>
<td>130110-02</td>
<td>34</td>
<td>Tubal factor</td>
<td>Long protocol GnRH analog with CC + rFSH</td>
<td>236.1</td>
<td>3.3</td>
<td>24</td>
<td>75% (6/8)</td>
<td>2</td>
</tr>
<tr>
<td>130110-08</td>
<td>34</td>
<td>Tubal factor</td>
<td>Long protocol GnRH analog with CC + rFSH</td>
<td>510.9</td>
<td>2.8</td>
<td>380.4</td>
<td>75% (6/8)</td>
<td>2</td>
</tr>
<tr>
<td>130157-08</td>
<td>30</td>
<td>PCOS, tubal factor</td>
<td>Long protocol GnRH analog with rFSH</td>
<td>708</td>
<td>3.5</td>
<td>272.6</td>
<td>68% (23/34)</td>
<td>2</td>
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</table>

\*No endometriosis case included, no oral contraceptives were given prior to IVF cycle.
\*All PCOS (polycystic ovary syndrome) patients were given with Metformin 500mg three times per day at least 1 month before IVF cycle.
\*CC: clomiphene citrate; Menopur: FSH 75 IU + LH 75 IU; GnRH: gonadotropin releasing hormone; rFSH: Recombinant FSH. When the number of 18 mm-sized leading follicles reached three, hCG was given as following: hCG 10000 for GnRH analogue and hCG 5000 + Ovidrel (rLH) for GnRH antagonist.
\*ROS level equivalent to \( \text{H}_2\text{O}_2 \) \((\mu\text{M})\).
\*Total antioxidant capacity (TAC) was analyzed with the TEAC (Trolox equivalent antioxidant capacity assay).
\*Fertilization rate = no. of zygote/no. of oocyte retrieved.
\*Number of embryo transferred (ET).

(Figure 2C). We assume the higher intracellular ROS level and more frequent \( \gamma \text{H}_2\text{O}_2 \)-staining in the fimbriae is due to a frequent exposure to FF. Indeed, treatment of isolated fimbria tissues with a pool of six ROS-high FFs (ROS\textsuperscript{high} FF), but not a pool of five ROS-low FF (ROS\textsuperscript{low} FF), resulted in a systemic increase of ROS stress in the epithelium (Figure 2D; Supplementary Figure 2C, available at Carcinogenesis Online) and nuclear \( \gamma \text{H}_2\text{O}_2 \) expression in a small part of epithelial cells where annexin V was also induced (Figure 2D). The results suggested that ROS\textsuperscript{high} FF could induce ROS stress, DSB and cell apoptosis to fimbria secretory cells.

Fimbria epithelial cells with p53 loss are more resistant to the cytotoxicity of FF

Because both loss of p53 and deregulation of cyclin E1 (28), the major Rb suppressor, are the earliest event in fimbria secretory cell transformation, we immortalized human fimbria epithelial cells with HPV16 E6/E7, known to shut down p53 and Rb, and hTERT to overcome replicative senescence (Figure 3A, upper left). The immortalized cells, named FE25, exhibited a cobblestone-like epithelial morphology, expressed pancytokeratin (AE1/AE3) and secretory cell-specific PAX8, and were negative for the TUBB4 ciliated cell marker, indicating a secretory cell line. The p53 protein were much lower than primary cell under \( \text{H}_2\text{O}_2 \) stress (Figure 3A). Compared with primary fimbria epithelial cells, FE25 were more resistant to the cytotoxicity of FF, and ROS\textsuperscript{high} FF were more cytotoxic than ROS\textsuperscript{low} FF (Figure 3B). A fluorescence-activated cell sorting analysis of annexin V revealed considerably less apoptosis in FE25 than in primary cells when treated with ROS\textsuperscript{high} FF (Figure 3C). Cell apoptotic level was associated to FF concentration (Figure 3D, Supplementary Figure 4, available at Carcinogenesis Online).

Expansion of fimbria epithelial cells with accumulation of DSB after repeated exposure of sublethal dose of ROS\textsuperscript{high} FF

Since the ovulated FF is soon diluted in the peritoneum cavity and its cytotoxicity to FE25 cells was concentration-dependent, we tested the possibility that fimbria cells may survive the cytotoxic effect of FF at lower concentrations and accumulate DSB. After repeated treatment with low or sublethal concentrations (6.25 and 3%) of ROS\textsuperscript{high} FF, a surviving effect was observed in FE25 cells (Figure 4A). As shown, a high proportion of cells were in the S phase of the cell cycle (Figure 4B; Supplementary Figure 5, available at Carcinogenesis Online). Higher levels of intracellular ROS and \( \gamma \text{H}_2\text{O}_2 \) were also noted as compared with those treated with the vehicle or ROS\textsuperscript{low} FF (Figure 4C–E, Supplementary Figure 6), and all these changes were completely ameliorated by adding the ROS inhibitor N-acetyl-L-cysteine (NAC) (Figure 4C and D). To clarify
whether the effect of FF on this increase of intracellular ROS is to be exogenous, cells were pre-treated with diphenyleneiodonium (DPI), a NAD(P)H oxidase and mitochondrion inhibitor, to block the endogenous source of ROS. This difference remained, suggesting that it was an effect of the exogenous ROS stress from FF (Figure 4C).

**ROS**<sup>high</sup> FF induces early tumorigenesis in **Trp53**<sup>−/−</sup> mice

Knowing that there is no spontaneous tubo-ovarian tumorigenesis model other than the laying hen (30) in which genetic manipulation is difficult, we chose to test the tumor initiation effect of FF in **Trp53**<sup>−/−</sup> mice. Since long term, repeated intra-bursa injection is impractical and since trials of induction of superovulation in **Trp53**<sup>−/−</sup> mice did not induce ovarian cancer (data not shown), we used the mammary fat pad injection model for further in vivo validation. Upon weekly injection of ROS<sup>high</sup> FF or ROS<sup>low</sup> FF to the mammary fat pad, 7 of 12 (58%) and 1 of 6 (16.7%) mice grew CD20-positive B cell lymphoma by the end of the 8th week, respectively (Figure 5A and B). Moreover, no tumorigenesis was noted if the same injection was conducted in WT mice or dorsal subcutaneously in the **Trp53**<sup>−/−</sup> mice (Figure 5B). The results indicated a carcinogenic effect of ROS<sup>high</sup> FF in the context of p53 loss and a dependence on the mammary gland microenvironment for tumorigenesis.
Melatonin ameliorates the ROS\textsuperscript{high} FF-inducing carcinogenic effect

Melatonin, with a concentration 4-fold higher than in serum\textsuperscript{(31)}, is a potent antioxidant in ovarian follicles that protects oocytes from excessive ROS damage and ameliorates excessive ROS-inducing complications during oocyte maturation\textsuperscript{(27)}. Melatonin activates cell antioxidative responses through its receptors and directly functions as a multifaceted free radical scavenger to reduce ROS stress; it also activates antioxidative enzymes in the FFs\textsuperscript{(32)}. When a physiological concentration (400 pM) of melatonin was added before the treatment of FE25 cells with ROS\textsuperscript{high} FF, both intracellular ROS and DSBs diminished (Figure 3C). Additionally, in the breast lymphoma model of Tprp53\textsuperscript{−/−} mice, adding 800 pM of melatonin to the FFs largely prevented tumorigenesis. Only one of the six injected mice developed a small nodule (Figure 5A and B). The results suggested that melatonin effectively eliminates the ROS, DSB and tumorigenic effects of FF.

Discussion

Since incessant ovulation was proposed as the cause of ovarian cancer\textsuperscript{(33)}, few studies have determined the underlying mechanisms of carcinogenesis. This study discovered a dichotomous pattern of ROS level in different FF aspirates and tumor initiating and promotion activities of the ROS\textsuperscript{high} FF. The average ROS level in ROS\textsuperscript{high} group was 7.2 folds higher than that of ROS\textsuperscript{low} FF. There was no correlation of FF ROS level with known clinical characteristics such as age, number of follicles, estradiol level or IVF outcome. Further epidemiological investigation is warranted to identify risk factors as well as surrogate markers for this high follicular ROS during ovulation.

The ovulatory process is initiated by an acute inflammatory response in the follicle immediately after LH surge\textsuperscript{(34)}. A high level of ROS is a major characteristic of the preovulatory follicle and is indispensable for normal ovulation\textsuperscript{(23)}. Administering broad-range ROS scavengers into the ovarian bursa of mice significantly reduced the rate of ovulation\textsuperscript{(23)}. The study found ROS...
in FF could induce apoptosis of fimbria epithelial cells. We speculate that the fallopian tube fimbria epithelium, which is rich in stem cells (35), readily regenerates after the ovulatory injury.

ROS stress is typically counteracted by antioxidants in FF (32,36). However, we observed in this study an invariable TAC among different FFs. A genome-wide profiling of induced genes in cells treated with H$_2$O$_2$ has revealed unexpectedly few increase of antioxidant gene expression (37). This is in consistence with a constitutive antioxidant defense mechanism in mammals (38,39). Thus, the key factor in unbalanced oxidative stress in FFs is the overproduction of ROS, but not the loss of antioxidant capacity.

Considering the mutagenic activity of FF, the fimbriae of fallopian tubes should accumulate more ROS and DSBs than those in the proximal part. We demonstrated this anatomical difference in human fallopian tubes and identified secretory cells in the proximal fimbria epithelium to be targets of irradiation-induced DSB (22). We discovered in this study that ROS in mature FF could exert DSB of fimbria secretory cells, and these cells are prone to apoptosis.

In the context of p53 loss, the immortalized fimbria cells survived the cytotoxicity of FF-ROS and rapidly accumulated intracellular ROS and DSBs. These cellular effects were no longer found when NAC antioxidant was added in the treatment, suggesting it is the ROS in FF that is responsible for the mutagenesis in fimbria epithelial cells. Meanwhile, it is known that both DPI and NAC have multiple effects on cells (29,40). The study also

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Figure 4. Expansion of fimbria epithelial cells with accumulation of DSB after repeated exposures to sublethal dose of ROS$^{\text{high}}$ FF. (A) FE25 cells were subjected to single (adding FF at 0 h and assayed 24 h later) or double treatment (adding FF at 0 h and 24 h) of ROS$^{\text{high}}$ FF of different concentrations, and cell viability was checked. H$_2$O$_2$ at a concentration (400 μM) equivalent to the average ROS level of ROS$^{\text{high}}$ FF and medium (vehicle) served as controls. (B) Cell cycle was characterized in FE25 cells by EdU incorporation analysis after double treatment with ROS$^{\text{high}}$ FF. (C) Intracellular ROS were analyzed in FE25 cells after single treatment with ROS$^{\text{low}}$ or ROS$^{\text{high}}$ FF with or without cotreatment with 200 μM H$_2$O$_2$ (H) or 500 μM N-acetyl-L-cysteine (N), or pretreatment with 10 μM diphenyleneiodonium (DPI) for 30 min. (D) Accumulation of γH$_2$Ax was quantified by FACS in primary fimbria epithelial cells and FE25 cells with the same cotreatments. (E) Accumulation of γH$_2$Ax (green) was observed in the nucleus (blue) of FE25 cells after double treatment with ROS$^{\text{high}}$ FF. Scale bar: 10 μm. All results were from three independent experiments performed in duplicate.

* P < 0.05, ** P < 0.01; # P < 0.05, compared to vehicle.
showed melatonin, also a potent antioxidant, could also abolished the mutagenic effect of FF. The mutagenic effect of ROS$_{high}$ FF was specifically evaluated at low concentrations (6.5% or 3%) in this study. After ovulation, FF is rapidly diluted by peritoneal fluid, which has an estimated volume of 23 ml (41), and an absorption rate of 0.04 ml/min (42). The extent of dilution of FF in peritoneal fluid can be calculated by dividing the mean concentration of estradiol in the peritoneal fluid in the luteal phase [2500 pmol/l] (43) and that in FF [277 ng/ml or 10$^6$ pmol/l (this study)], which has a final dilution of 0.25% in a stable state. Thus, the effective concentrations noted in our experiments are reasonably within the range of dilution.

Upon repeatedly exposure to 3% ROS$_{high}$ FF, FE25 cells survived and expanded with progression of cell cycle. This is consistent with the compensatory increase in cell proliferation following acute oxidative injury (44), and the abundant surviving and growth factors in mature FF (45) may be responsible for this clonal expansion. These expanded FE25 cells harbored a higher level of intracellular ROS. Level of DSB also increased by 50%. The effect of FF on this increase of intracellular ROS was clarified to be exogenous. Cells treated with DPI (29) still showed
the same increase of intracellular ROS after ROS\textsuperscript{high} FF treatment (Figure 4C). Thus, repeated exposure of p53-defective fimbria secretory cells with sublethal concentration of ROS\textsuperscript{high} FF could induce a serial of events of carcinogenesis including ROS stress, DSB and cell expansion. The tumorigenic effect of ROS\textsuperscript{high} FF in the context of p53 loss was demonstrated in the Trp53\textsuperscript{-/-} transgenic mice model. Although in the C57BL/6 background, over 70% of the Trp53\textsuperscript{-/-} mice will develop lymphoma in old age (19±4.5 weeks) (46–48), the onset of FF-induced lymphoma was at least 5 weeks earlier. The tumorigenesis seemed to be dependent on the ROS level in FF. Compared to a tumorigenic rate of 58% and average tumor size of 1778mg in ROS\textsuperscript{high} FF group, only one of six mice injected with ROS\textsuperscript{low} FF grew a small tumor of 49mg. The same injection in the wild-type mice did not produce any tumor, suggesting that the loss of p53 is essential for FF-induced carcinogenesis. Previous studies on a skin carcinogenesis model in the same transgenic strain have concluded that Trp53 loss is responsible for tumor progression but not for tumor initiation or promotion (49). Given with mutagenic and mitogenic activities of FF-ROS, lymphocytes recruited to the injection site may be subjected to these early steps of transformation. In the absence of p53, initiated cells may evade the apoptosis, accumulate more genetic changes and lead to tumor progression. Meanwhile, subcutaneous injection of the same FF did not produce a tumor, suggesting that a microenvironment such as hormone responsiveness may also be essential.

Women carrying mutant TP53 (Li–Fraumeni syndrome) are prone to breast cancer but are not susceptible to ovarian cancer. Distal fallopian tubes in two Li–Fraumeni women were determined to contain abundant p53 signatures with evidence of DNA damage. These fimbriae were also prone to focal epithelial p53 gene inactivation, mutation, and allele loss (50). We speculated that DSB repair systems in the fimbria cells are sufficient to overcome the carcinogenic effects of FF, even without p53. Provided that the genetic and epigenetic loss of genes involved in homologous recombination is observed in over half of HGSCs (51), impairment of DSB repair can be a key driver toward STIC and HGSC following initiation and in the context of p53 loss. Melatonin is a potent and abundant antioxidant in human FF. In clinical practice, the correct level of melatonin is crucial for protecting the oocyte from oxidative stress and, ultimately, fertility (27). Upon cotreatment with a sub-nM level of melatonin, both the in vitro DSBs and in vivo tumorigenesis effects of ROS\textsuperscript{high} FF were diminished. The result suggests melatonin can be a testable medicine to prevent the occurrence of HGSC.

Based on present and previous findings, a pathway of ovulation-induced carcinogenesis can be proposed. The pathway first involves the exposure of secretory cells of fallopian tube fimbriae with ROS\textsuperscript{high} FF during ovulation, causing DSB and cell apoptosis. Upon repeated exposure, a progenitor cell may acquire TP53 mutations, evade cell death, and expand to a signature of p53. When machinery involving DSB repair, such as BRCA1/2, are further mutated, more severe lesions, such as STIC, are evolved (50) (Figure 5D).

Taken together, the study revealed the mutagenic and tumorigenic activity of a subset of FF with high ROS. Both the initiation and promotion of carcinogenesis in the fallopian tube fimbriae can be induced by this FF in the context of p53 loss, and melatonin could be an effective preventive treatment.

**Supplementary material**

Supplementary Figures 1–6 can be found at http://carcin.oxfordjournals.org/