



Original Article

Efficient analyses of DNA double-strand breaks and the cell cycle in the secretory epithelial cells of fallopian tube fimbriae

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ABSTRACT

Objective: Reactive oxygen species (ROS)-induced DNA double-strand breaks (DSBs) are a feature of cancer initiation. Recently, the cells of origin of ovarian high-grade serous carcinoma (HGSC) have been identified as secretory cells in fallopian tube fimbriae, which might be heavily exposed to ROS after ovulation. Establishing a sensitive detection method for measuring DSBs and the cell cycle in fallopian tube secretory cells after ovulation is essential for facilitating research on ovarian cancer formation.

Materials and methods: Fimbrial epithelial cells from a prophylactically removed human fallopian tube were primarily cultured and immortalized by TP53/Rb-null and hTERT overexpression (FE25 cell line). Hydrogen peroxide (H₂O₂) was used to treat the FE25 cells to induce DNA DSBs. The DSBs and cell cycle were analyzed using flow cytometry. In addition, human specimens of fimbrial scrapings were subjected to the flow cytometry-based analysis.

Results: We report an efficient flow cytometry-based method for analyzing the DSBs and cell cycle in immortalized fallopian tube secretory cells as well as in clinical specimens.

Conclusion: This analysis method would facilitate the investigation of cancer initiation in fallopian tube fimbriae and other tissues with DSBs.

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1. Introduction

Cellular DNA is continuously damaged because of normal cellular physiology and environmental exposures. Reactive oxygen species (ROS) are generated as byproducts of cellular metabolism. Furthermore, exposure to radiation, chemicals, and UV light can damage DNA bases and backbones and cause DNA double-strand breaks (DSBs) [1].

Cellular DSBs are traditionally analyzed by immunostaining γ H2AX, a phosphorylated form of the histone protein H2AX. On encountering DSBs, damage-sensing kinases, ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related), accumulate

rapidly at the sites of DSBs and phosphorylate H2AX [2], enabling the identification of DSBs through immunostaining.

The secretory epithelial cells of fallopian tube fimbriae have recently been identified as the cells of origin of ovarian high-grade serous carcinoma (HGSC) [3,4]. Precursor lesions of HGSC, such as “p53 signature” and serous tubal intraepithelial carcinoma, have been observed in the secretory epithelial cells of fallopian tubes but not on the ovarian surface [5,6].

We hypothesized that ROS released from ovulated follicular fluid (FF) are the main cause of cancer initiation in the secretory cells of fallopian tube fimbriae. Exposure of fimbrial epithelial cells to mature FF could induce the upregulation of DNA repair genes and the accumulation of p53 [7]. Furthermore, the accumulation of DSBs was observed in cultured fimbrial epithelial cells after exposure to ionic radiation [8] or human FF [7]. To study the ROS-induced transformation of fallopian tube fimbriae, we established a flow cytometry-based method for quantitatively analyzing γ H2AX and the cell cycle in immortalized fallopian tube secretory cells, as well as in the secretory cell population obtained from

Conflict of interest: none.

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fallopian tube epithelial cell scrapings. By using the flow cytometry-based method, we anticipate a highly efficient analysis of the vulnerability of fallopian tube fimbriae to ROS and cancer initiation.

2. Materials and methods

2.1. Primary culture and immortalization of fimbrial epithelial cells

The procurement of clinical specimens for this study was approved by the Institutional Review Board (TCGH-IRB #93-025) of Tzu Chi General Hospital, Hualien, Taiwan. A human fallopian tube was procured from a 35-year-old woman who underwent prophylactic salpingectomy during an operation for uterine myoma. The primary culture of human fallopian tube epithelial cells was modified from Paik et al [9]. In brief, the human fallopian tube was soaked in 1% trypsin and 5 mM EDTA at 37°C for 30 minutes. The fimbrial epithelium was peeled off and digested with 1.5 mg/mL of collagenase (c2674, Sigma, St. Louis, MO, USA) for 1 hour, and isolated cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal bovine serum), 5 µg/mL insulin, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin in a 0.1% gelatin-coated plate. To immortalize the cultured fimbrial epithelial cells, an HPV16 E6/E7 lentiviral construct was introduced into the primary cells during the second passage. Subsequently, the viable cells were transduced with a lentiviral hTERT (Applied Biological Materials Inc., Richmond, BC, CA) to generate FE25 cells (Fig. 1). The FE25 cells were maintained in MCD105 and M199 media

(Sigma) supplemented with 10% FBS, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. The FE25 cells were authenticated by ATCC short tandem repeat profiling (www.atcc.org/STR%20Database.aspx) at the Center for Genomic Medicine, National Cheng Kung University, Tainan, Taiwan, and exhibited no evidence of cross-contamination with the known ATCC cell lines.

2.2. Fallopian tube fimbrial scraping

To collect epithelial cell scrapings, the fimbrial lumen of the tube was gently brushed in a single circle by using a cervical cytobrush, and transferred to 3 mL of PBS (Phosphate buffered saline) before immunostaining and flow cytometry.

2.3. DNA DSB analysis by flow cytometry

DNA DSBs were induced by hydrogen peroxide (H₂O₂) treatment and detected by immunostaining γH2AX. In brief, subconfluent culture cells were treated with H₂O₂ and incubated for 3 hours. After the cells were detached, they were fixed in 4% paraformaldehyde for 15 minutes, permeabilized by 0.1% Triton X-100 for 30 minutes, blocked with 2% BSA (Bovine serum albumin), and incubated with a mouse monoclonal antiphospho-γH2AX antibody (05-636, Millipore, Billerica, Massachusetts, USA) (1:200 dilution) at room temperature for 2 hours. Subsequently, FITC-conjugated (Fluorescein isothiocyanate) antimouse immunoglobulin G (11-4010-82, Biologend, Taipei, Taiwan; 1:400) was added and incubated for 1 hour. The analysis was performed using a FACSCalibur equipped with BD CELLQuist software or by examination with

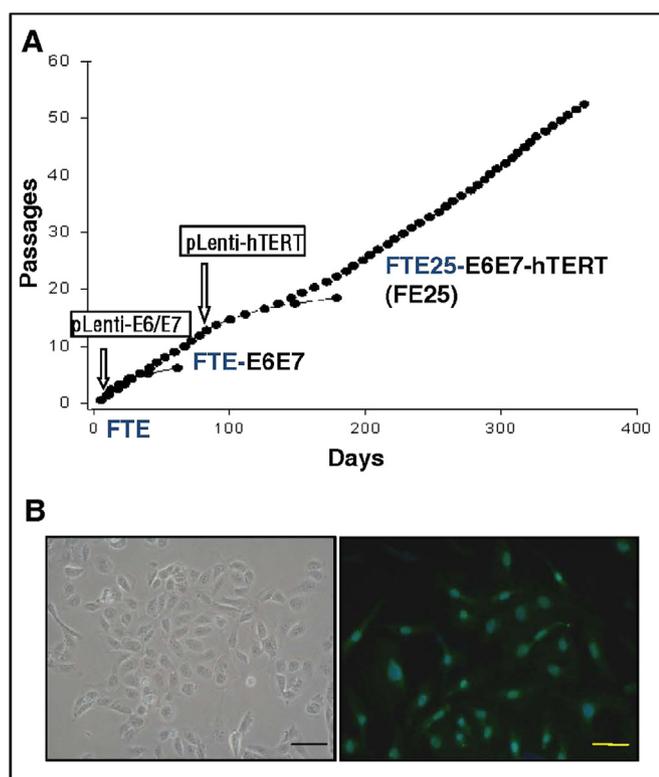


Fig. 1. Immortalization of human fimbrial secretory epithelial cells. (A) The sequence of immortalization of primarily cultured human fimbrial epithelial cells is shown. (B) Immortalized FE25 cells have a cobblestone-like morphology (left) and are positive for the secretory cell marker PAX-8 (green), as revealed through immunofluorescent staining (right). Cell nuclei were counterstained with DAPI (blue), scar bar: 100 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

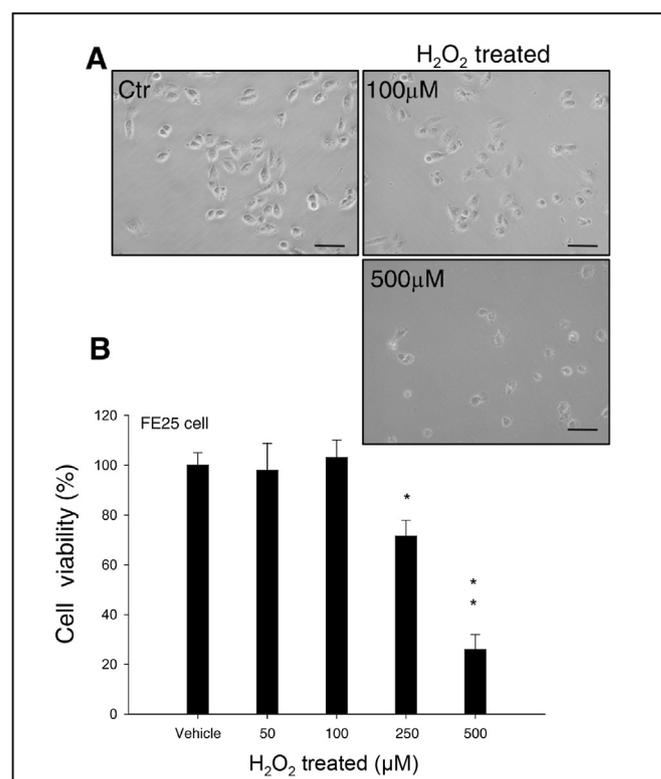


Fig. 2. Analysis of the FE25 cells in response to reactive oxygen species (ROS) stress. (A) Morphology of the FE25 cells under the stress of sublethal (100 µM H₂O₂) or lethal (500 µM H₂O₂) dose of ROS compared to vehicle-treated (Ctr; PBS), scar bar: 100 µm. (B) Cell viability analysis by using an XTT assay under H₂O₂ treatment condition. Results were obtained from three independent experiments performed in duplicate. **p* < 0.05 and ***p* < 0.01, compared to vehicle-treated (PBS).

confocal laser-scanning microscopy (LSM5 PASCAL; Carl Zeiss, Oberkochen, Germany), with the nucleus counterstained with DAPI (4',6-diamidino-2-phenylindole). To compare the DSB levels in the cells through BD CELLQuest analysis, the background γ H2AX-FITC fluorescence was set at < 10% of the fluorescence intensity (<10% of the M1 region).

2.4. Cell viability and cell cycle assays

Cell proliferation was evaluated using an XTT assay (20-300-1000A, Biolegend) 24 hours after H_2O_2 treatments, as described previously [10]. For cell cycle analysis, the cells were detached from the culture dish, fixed in 70% ethanol at $-20^\circ C$ overnight, and washed with PBS. Approximately 1×10^5 cells were resuspended in 500 μL of propidium iodide (PI) solution (25 $\mu g/mL$ of PI reagent, 0.1 mg/mL of RNase, 0.05% Triton X-100) for 30 minutes in a dark room. The cells were filtered in a Falcon 5 mL round-bottom polystyrene test tube by using a cell strainer snap cap and analyzed using flow cytometry.

2.5. Data analysis

The results are presented as mean \pm standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using Microsoft Office Excel 2007 and SigmaPlot 10. For statistical comparison, the data were analyzed using the Student *t* test between the two groups. Significant differences were defined as $p < 0.05$.

3. Results

3.1. Detection of DSBs in immortalized fimbrial epithelial cells treated with a sublethal dose of H_2O_2

We investigated the viability of FE25 (Fig. 1A and B) after treatment with different doses of H_2O_2 . In contrast to drastic cell death observed after treatment with 500 μM H_2O_2 , the FE25 cells survived with a sublethal dose of 100 μM and maintained a normal cell morphology (Fig. 2A and B). Confocal microscopy revealed an

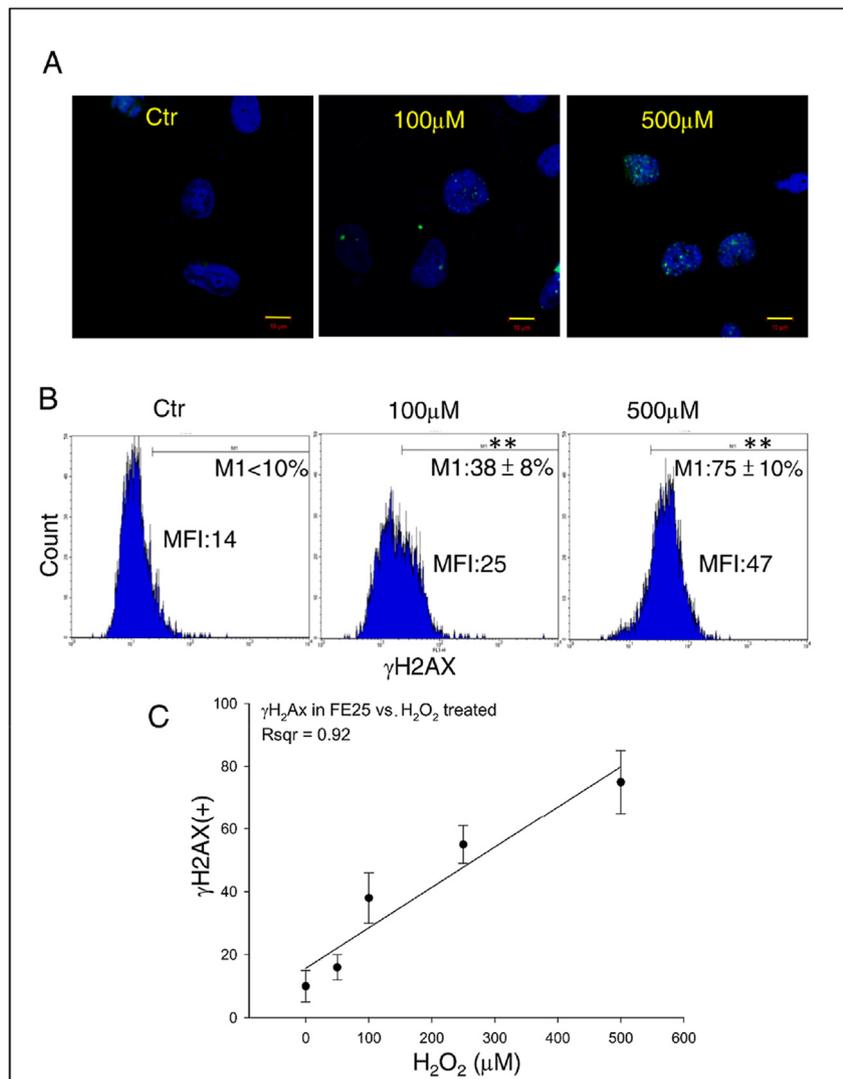


Fig. 3. H_2O_2 -induced double-strand breaks (DSBs) in fimbrial secretory cells measured using confocal microscopy and flow cytometry. (A) Confocal microscopy analysis of DSBs (green dot) in the FE25 cells treated with H_2O_2 . Cell nuclei were counterstained with DAPI (blue), scale bar: 10 μm . (B) Flow cytometry analysis of DSBs in the FE25 cells treated with H_2O_2 . (C) The R-square value obtained from Pearson correlation coefficient for analyzing the relation between DSB levels and H_2O_2 concentrations. Results were obtained from three independent experiments. Figures are representative of one of the experimental results. ** $p < 0.01$, compared to vehicle-treated control (Ctr). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

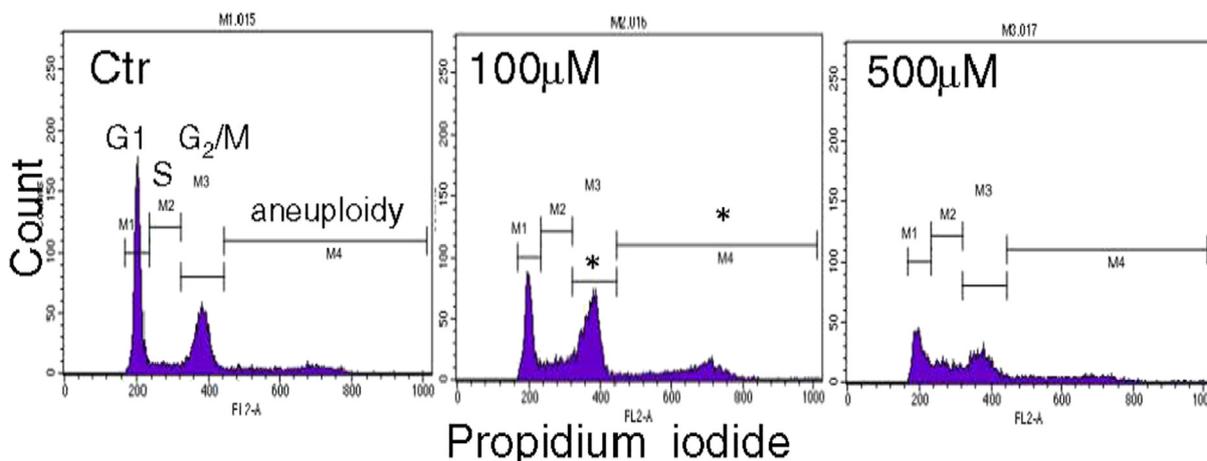


Fig. 4. Flow cytometry analysis of cell cycle changes in fallopian tube fimbrial secretory cells under reactive oxygen species (ROS) stress. Flow cytometry analysis of the cell cycle of FE25 cells treated with H_2O_2 . Results were obtained from three independent experiments. Figures are representative of one of the experimental results. * $p < 0.05$, compared to control (Ctr).

intracellular immunostaining of γ H2AX, a DSB marker (Fig. 3A). The number of cells harboring γ H2AX after H_2O_2 treatment was counted using flow cytometry analysis. At an H_2O_2 dose of 500 μ M, $75 \pm 10\%$ of the cells were found with DSBs; at a lower dose of 100 μ M, $38 \pm 8\%$ of the cells were positive (Fig. 3B). Data acquired using flow cytometry revealed that the FE25 cells treated with five H_2O_2 concentrations showed a sensitive dose response to DSB accumulation. The R-square value obtained from Pearson correlation coefficient analysis was 0.92, indicating a strong relation between DSB levels and H_2O_2 concentrations (Fig. 3C).

3.2. Flow cytometry analysis shows cell cycle changes under ROS stress

In addition to the DSB measurement, the same H_2O_2 -treated cells were also stained with PI and analyzed for cell cycle changes on the same flow cytometer. As shown in Fig. 4A and Table 1, the FE25 cells had a background aneuploidy rate of 9.6%. After H_2O_2 treatment under sublethal conditions (100 μ M), the aneuploidy rate of the viable cells increased to 18.4%, and 43% of the cells were arrested at the G2/M phase of the cell cycle (Table 1).

3.3. Analysis of secretory cell DSBs in human fallopian tube epithelial cell scrapings

To study the susceptibility of physiological ROS-induced DNA damage in the secretory cell population of human fallopian tube fimbriae (Fig. 5A), we established a method for analyzing γ H2AX in the PAX-8-positive cells, a secretory cell-specific marker [8], of human fallopian tube epithelia. With the FE25 cells used as a reference, the cell scrapings of fallopian tube fimbriae were first gated for epithelial cell population (Fig. 5B, a and b). The secretory cells carrying DSBs were then analyzed on the basis of PAX-8 and

γ H2AX, simultaneously (Fig. 5B, c and d). In an example of a postovulatory fimbrial scraping of a 35-year-old woman, 71% of the gated epithelial cell population was PAX-8⁺/ γ H2AX⁺, and 22.6% was PAX-8⁺/ γ H2AX⁻ (Fig. 5B, d). By contrast, in the PAX-8 low population (Fig. 5B, c), most cells were γ H2AX negative. These results are consistent with those of a previous study reporting that secretory cells are the major population harboring DSBs in fimbriae after ionic irradiation [8].

4. Discussion

DNA damage in individual cells is typically detected by performing a comet assay in which a single-cell DNA gel electrophoresis technique shows the extent and length of the tail of the gel, indicating the severity of DNA damage. Recent studies have shown that DSBs are more frequently analyzed by detecting the phosphorylation of histone H2AX at Ser-139, defined as γ H2AX, providing a sensitive and reliable immunoassay for detecting DNA damage [7,11–13]. In addition, γ H2AX and p53 nuclear expression are essential markers observed in ovarian and fallopian tube epithelia derived from risk-reducing salpingo-oophorectomy in HGSC high-risk patients with BRCA 1/2 mutation [14,15]. In this study, we used flow cytometry to measure γ H2AX immunofluorescence in the cell, leading to a quantitative and efficient measurement of DSBs.

The analysis of the secretory cell specific marker PAX-8 enabled quantifying DSBs in the secretory cell population of the fallopian tube scrapings. This method was sensitive in detecting DSBs in the immortalized fallopian tube secretory epithelial cell line and fallopian tube epithelial cell scrapings after ovulation. A simultaneous analysis of the DNA content by PI-staining enabled characterizing the DNA ploidy status of the cells subjected to DSBs. Thus, after the treatment of the immortalized fimbrial secretory cells, FE25, with a sublethal dose of 100 μ M H_2O_2 , the DNA aneuploidy rate was measured at 18.4%, in contrast to the background aneuploidy rate of 9.6%. These ROS-exposed cells did not change in cell viability, but they accumulated DSBs and were arrested in the G2/M phase of the cell cycle in 37% and 43% of the cell population, respectively.

Hence, we established an efficient qualitative method for DSB measurement and cell cycle analysis in the secretory cell population of human fallopian tube fimbriae. Our analysis confirmed that the fallopian tube fimbrial secretory cells possess high levels of DSBs after ovulation, indicating that ovulation can be a source of DSBs in the adjacent fallopian tube fimbria.

Table 1

Cell cycle analysis by flow cytometry.

Treated phase	Vehicle	100 μ M ^a	500 μ M ^a
G1	47	27	32.7
S	6.78	12	21.9
G2/M	36.9	42.9	31.7
Aneuploidy	9.62	18.4	14.6

Data are presented as %.

^a FE25 cells treated with hydrogen peroxide.

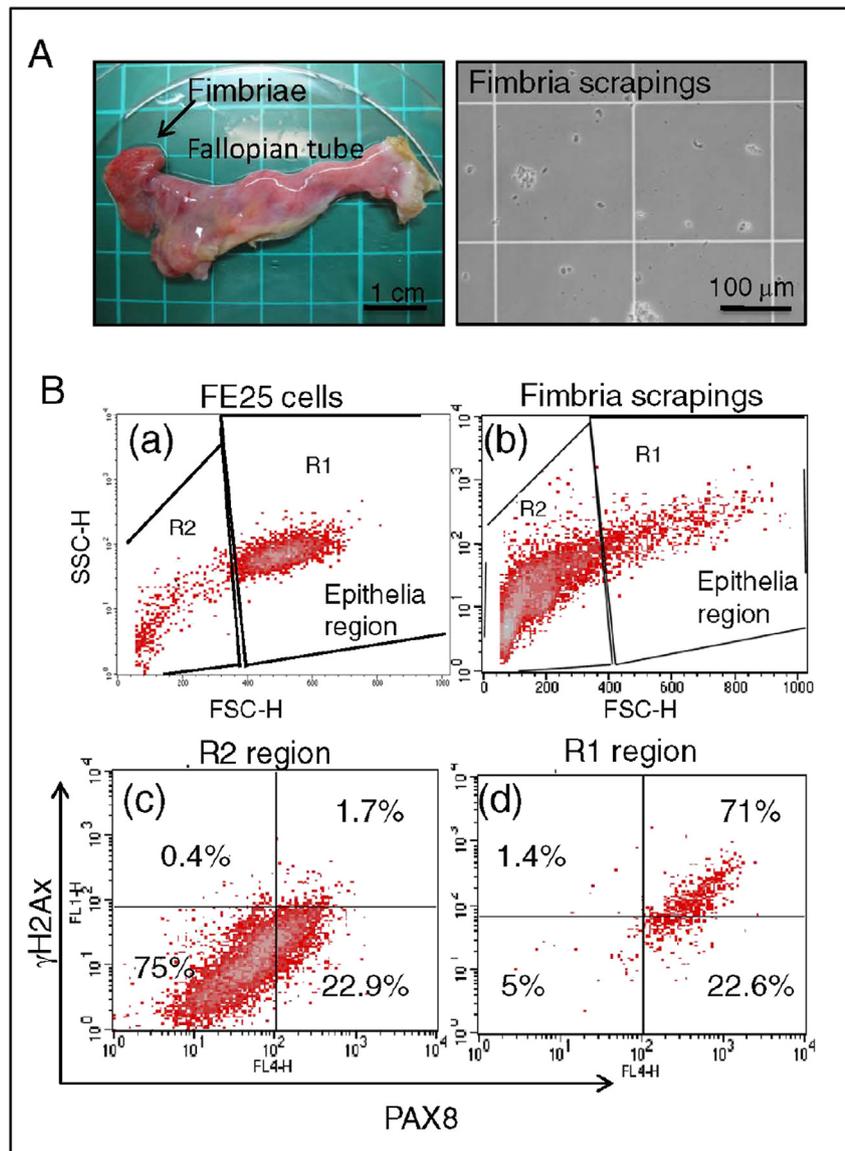


Fig. 5. Application of flow cytometry for double-strand breaks (DSB) analysis in fallopian tube fimbrial scraping. (A) Image shows a human fallopian tube specimen (left) and the scraping of epithelial cells of the fimbrial portion of the tube viewed under a microscope (right). (B) Flow cytometry analysis of DSBs in the secretory cell population of fimbrial scraping by dual staining with γ H2AX and PAX-8 markers. Results were obtained from three independent experiments. Figures are representative of one of the experimental results.

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