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ORIGINAL ARTICLE

Primary *ex vivo* cultures of human fallopian tube epithelium as a model for serous ovarian carcinogenesis

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Recent studies suggest that some serous ovarian carcinomas (SOCs) arise from the fallopian tube (FT) epithelium rather than the ovarian surface epithelium. This hypothesis places emphasis on the FT secretory epithelial cell as a cell-of-origin. Herein, we report the development of a novel *ex vivo* primary human FT epithelium culture system that faithfully recapitulates the *in vivo* epithelium, as shown by morphological, ultrastructural and immunophenotypic analyses. Mass spectrometry-based proteomics reveal that these cultures secrete proteins previously identified as biomarkers for ovarian cancer. We also use this culture system to study the response of the FT epithelium to genotoxic stress and find that the secretory cells exhibit a distinct response to DNA damage when compared with neighboring ciliated cells. The secretory cells show a limited ability to resolve the damage over time, potentially leaving them more susceptible to accumulation of additional mutagenic injury. This divergent response is confirmed with *in situ* studies using tissue samples, further supporting the use of this *ex vivo* culture system to investigate FT epithelial pathobiology. We anticipate that this novel culture system will facilitate the study of SOC pathogenesis, and propose that similar culture systems could be developed for other organ site-specific epithelia.
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Introduction

Epithelial ovarian cancer (EOC) is a leading cause of mortality in developed countries, with an incidence of about 190 000 new cases diagnosed annually worldwide and 114 000 fatalities (Jemal *et al*., 2008). Epithelial ovarian cancers are generally subclassified into two types based on biological behavior and histology: low-grade tumors (also known as type-I tumors), which typically present at an earlier stage and have an indolent natural history, and high-grade or type-II tumors, which typically have a more disseminated and aggressive behavior (Landen *et al*., 2008). Serous ovarian carcinoma (SOC) is a type-II tumor, and is the most aggressive and most prevalent histological subtype of this disease (Cannistra, 2004). The poor prognosis of SOC is a direct consequence of advanced-stage disease in a majority of newly diagnosed patients, and the eventual development of resistance to currently available chemotherapy.

Until recently, the ovarian surface epithelium (OSE) was considered the principal cell-of-origin for both type-I and type-II ovarian tumors. However, models depicting the transformation of OSE have not been consistently corroborated by pathological observations of transitions from OSE to malignancy. Recent studies raise a compelling hypothesis that the fallopian tube (FT) may harbor a cell-of-origin, the FT secretory epithelial cell (FTSEC), for serous tumors of the ovary and peritoneum (Jarboe *et al*., 2008). Evidence that supports this hypothesis includes (1) 5–10% of BRCA + women undergoing prophylactic salpingo-oophorectomy will have an early lesion, termed serous tubal intraepithelial carcinoma, and ~80% of these early cancers are found in the distal (fimbriated) end of the FT; (2) > 50% of women with stage III/IV pelvic serous cancer also have a neoplastic lesion in their FT mucosa; (3) identical *TP53* mutations have been identified in early FT neoplasia and the corresponding SOC (Kindelberger *et al*., 2007); (4) non-neoplastic FTSEC and SOC share similar morphological and immunophenotypic features; and (5) a candidate precursor lesion (termed the 'p53 signature'), composed of benign appearing FTSECs that harbor DNA damage and p53 mutations, has been described in the FT (Kindelberger *et al*., 2007; Levanon *et al*., 2008; Crum, 2009; Folkins *et al*., 2009). These observations suggest that the different pelvic serous carcinomas previously assigned to different origins (ovary, FT and peritoneum), share a common carcinogenic pathway not previously appreciated and originate in the secretory cell of the fallopian tube epithelium (FTE) (Levanon *et al*., 2008). Whether this model is correct remains to be determined.

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With the identification of a candidate cell-of-origin for serous carcinomas comes the opportunity for basic and translational research aimed at deciphering the molecular mechanism linking the previously known risk factors and the actual serous carcinogenic process; developing new strategies for detection of the early lesions; and proposing biologically targeted therapy for pelvic serous carcinomas. At the same time, the need for tools to study the benign FTE becomes more obvious and urgent, as all of these studies require reproducible *in vitro* and *in vivo* model systems that involve human FTSECs. To accomplish this goal, we developed a reproducible *ex vivo* culture system of primary benign cells that is capable of recapitulating the histology and function of the human FT fimbria epithelium. This model system has a number of advantages over previously reported cultures of FTE (Kervancioglu *et al*., 1994; Rajagopal *et al*., 2006). First, it represents a true co-culture of primary ciliated and secretory cells. Second, the cultures maintain polarity and morphology with each cell type expressing unique markers. Finally, it is a dynamic system as illustrated by the ability of the cultures to secrete factors unique to this epithelium and to respond to injury by proliferating and healing a wound. Herein, we report the use of this system to study the unique properties and differences between ciliated and secretory cells of the FT and how they respond to genotoxic stress. The unique features of this *ex vivo* primary cell culture system lay the foundation for studies aimed at defining the molecular mechanism of serous tumorigenesis and support the potential use of similar *ex vivo* culture system in other organ systems.

Results

The ex vivo *culture system is a phenocopy of the FTE*

The FTE is composed of two cell populations: the secretory cells and the ciliated cells (Crow *et al*., 1994). Previous efforts to recapitulate primary human FTE in culture have produced suboptimal results because of the rapid dedifferentiation and loss of polarized morphology and biological behavior of the two sub-populations of cells within the epithelium (Henriksen *et al*., 1990; Kervancioglu *et al*., 1994; Comer *et al*., 1998; Ando *et al*., 2000).

As described in Materials and methods, epithelial cells are dissociated from fresh surgical samples of FT fimbria and grown at the air–liquid interface in the upper compartment of a porous polyester membrane of a Transwell insert (Corning, Corning, NY, USA; Figure 1a). Our experience with over 100 samples from different subjects shows that we can obtain highly reproducible and viable cultures that can be maintained for over 28 days. As the cells are derived from primary tissue, they will senesce on subsequent passage or when the cells are plated at a very low concentration (data not shown). Importantly, our specific dissociation and culturing conditions result in cultures that are highly enriched for epithelial cells (data not shown).

Our first goal was to characterize the *ex vivo* cultures by analyzing their composition, immunoprofile and ultrastructural components. We examined the cultures using scanning electron microscopy and transmission electron microscopy. These techniques revealed that the *ex vivo* cultures are composed of true ciliated cells (Figure 1b,c) with the classical arrangement of $9+2$ microtubules typically seen in motile cilia (Figure 1d) (Satir and Christensen, 2008), as well as secretory cells which are characterized by abundant microvilli (Figure 1c).

We found that FTSECs, both *in vivo* and in the *ex vivo* cultures, uniquely expressed several lineage-specific markers: Pax-8, which is a paired-box transcription factor that is involved in lineage determination of the thyroid and reproductive systems (Bowen *et al*., 2007), Bcl-2, an anti-apoptotic mitochondrial protein (Piek *et al*., 2001) and TNFαIP2, a poorly characterized protein, which is induced by tumor necrosis factor-α (TNF-α) and retinoic acid (Rusiniak *et al*., 2000)(Figure 1e). We identified TNFαIP2 by mining the Human Protein Atlas database for factors that are expressed by only one of the two cell types in the FTE (Ponten *et al*., 2008). To our knowledge, this is the first study showing that TNFαIP2 is a FTSEC marker, and that this specificity is also preserved in our *ex vivo* cultures. Conversely, the motile cilia stain positive for acetylated α-tubulin (Seeley *et al*., 2009). In addition, ciliated cells expressed Foxj1, a member of the Forkhead transcription factors family implicated in programming ciliogenesis (Okada *et al*., 2004; You *et al*., 2004), Sall2, a zinc finger transcription factor involved in the cell senescence program (Li *et al*., 2004; Liu *et al*., 2007) and SELENBP1, the selenium-binding protein (Huang *et al*., 2006), which was also identified by searching the archives of the Human Protein Atlas (Figure 1e) (Ponten *et al*., 2008). The two cell lineages share several common pan-epithelial markers, such as Ep-CAM and E-Cadherin (Figure 1e); E-Cadherin being critical for the proper formation and maintenance of adherent junctions and polarity in areas of cell–cell contact. On the basis of these observations, we conclude that the *ex vivo* system mirrors the morphological and immunophenotypic properties of FTE *in vivo*.

The ex vivo *culture is a viable and responsive model system*

We next set out to characterize the dynamic behavior of the culture system. Ideally, we want our cultures to be responsive to biologically relevant stimuli and to be suitable for manipulation over an extended period of time as an essential prerequisite of a model system used to study factors that lead to carcinogenesis. Toward this end, we examined the proliferative and restorative capacity and the *trans*-differentiation potential of the cells in the *ex vivo* cultures by performing tissue wounding assays. Specifically, we wanted to know whether disruption of the tissue would lead to restoration of integrity through migration, proliferation or a combination, and most importantly, which cell type,

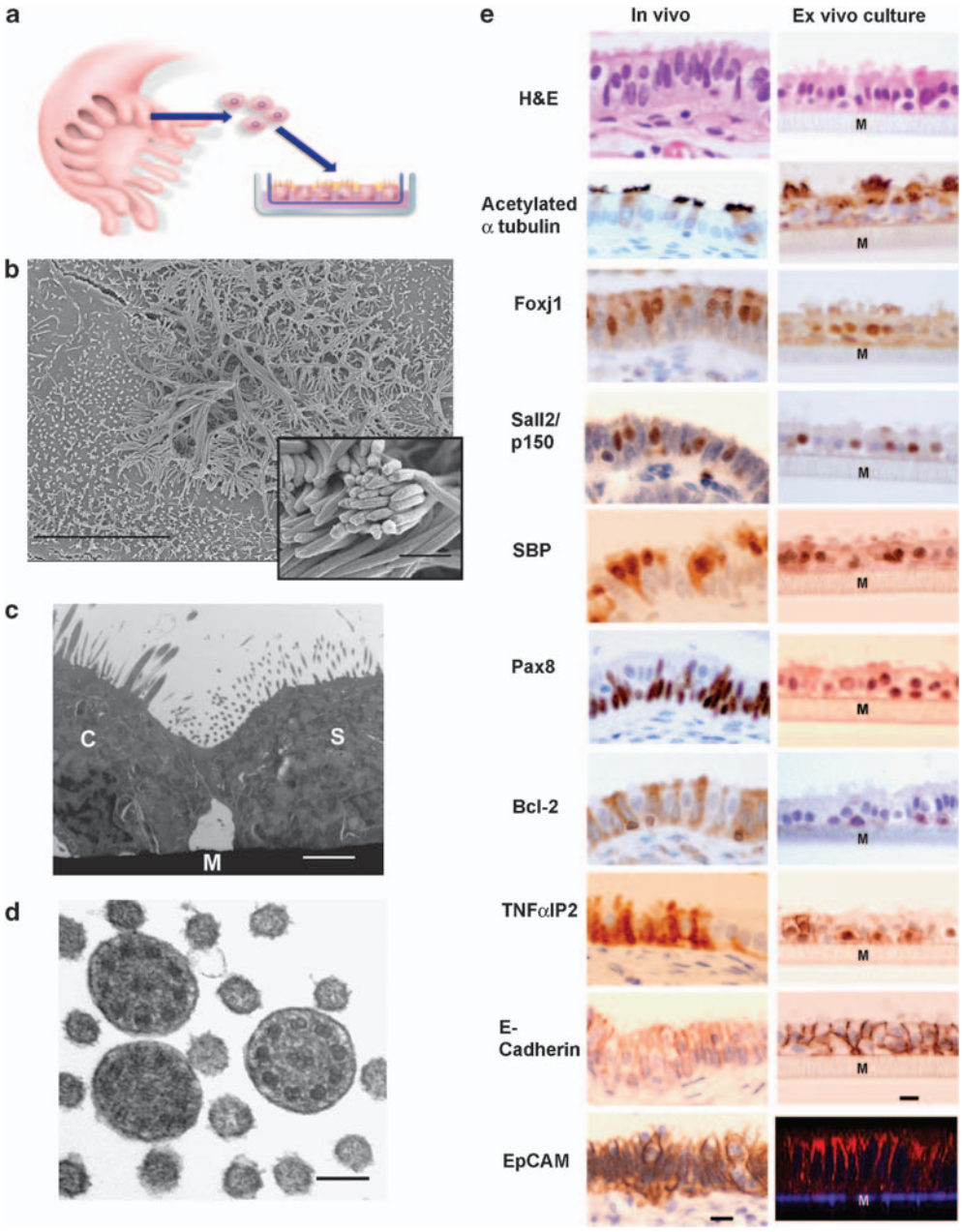


Figure 1 The FTE *ex vivo* culture system displays morphology and immunomarkers of both fallopian tube secretory epithelial cells (FTSECs) and ciliated cells. (**a**) A schematic illustration of the *ex vivo* co-culture system. The co-culture is composed of primary human FTE cells grown in the air–liquid interface on Transwell inserts. The cells form a polarized monolayer similar to that seen in normal human fallopian tube. (**b**) Scanning electron microscope image of a confluent culture showing both ciliated cells and secretory cells with microvilli (bar, 10 μm). Inset shows cilia in higher power (bar, 1 μm). (**c**) Transmission electron microscope image showing a ciliated cell (C) and a secretory cell (S) and the Transwell membrane (M) (bar, 1 μm). (**d**) Transmission electron microscopy cross-section through cilia showing the 9 + 2 arrangement of axial microtubules (bar, 100 nm). (**e**) Hematoxylin and eosin staining and immunohistochemistry of normal human fallopian tube fimbria and cross sections of formalin-fixed paraffin-embedded *ex vivo* cultures with the ciliated lineage markers, namely, acetylated α-tubulin, FoxJ1, Sall2/p150, SELENBP1 (SBP); secretory lineage markers, namely, Pax-8, Bcl-2 and TNFαIP2; and pan-epithelial surface markers, namely, E-Cadherin and Ep-CAM (immunofluorescence, Z-stack reconstitution of confocal microscopy images). The gray matrix beneath the cell layer represents the Transwell membrane (marked by M). Bar, 10 μm.

FTSECs or ciliated cells, participates in the healing of the 'wound'. Confluent *ex vivo* cultures were disrupted using a sterile pipette tip and washed with phosphate-buffered saline (PBS) (Figure 2a). The cultures were allowed to recover for 48 h (Figures 2a–c). At the end of the recovery period, the wound had healed (Figures 2b, c). This wound repair was at least in part due to proliferation of cells at the edges of the disrupted tissue, as shown by Ki67 staining of the healing cultures on day 2 post wounding (Figures 2d). This was confirmed by

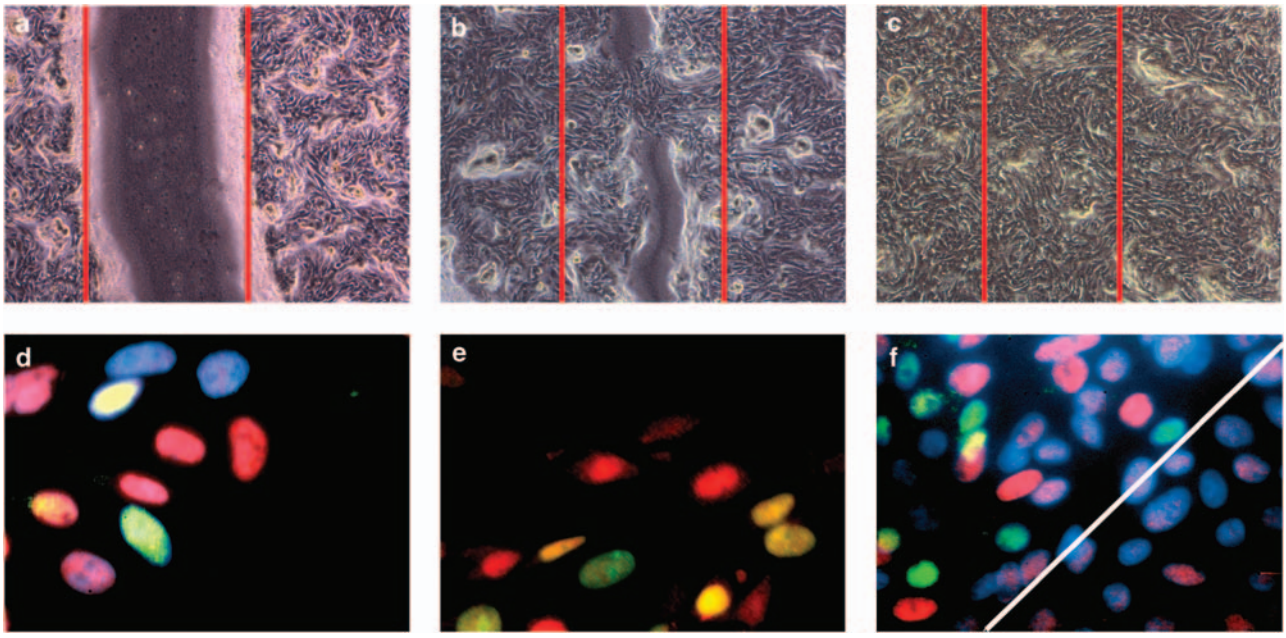


Figure 2 Wound healing in the fallopian tube (FT) *ex vivo* culture system. (**a**–**c**) Bright field images of the healing of a scratch in a confluent *ex vivo* culture, at day 0 (**a**), day 1 (**b**) and day 2 (**c**) post scratching (red lines mark the borders of the scratch). Proliferating cells on the perimeter of the healing wound, as shown by immunofluorescence staining for either the proliferation factor Ki67 (**d**, green) or BrdU (**e**, green), which co-localize with nuclei of secretory cells (Pax-8-positive nuclei, red), giving a yellow signal. (**f**) Although both cell types exist in the culture, the wound is 'healed' predominantly by FT secretory cells (right of the white line), as shown by co-staining for the lineage markers for secretory cells (Pax-8, red) and for ciliated cells (Foxj1, green).

BrdU labeling (Figure 2e). The cells that occupied the gap were secretory cells, as determined by co-staining for the lineage markers, Pax-8 and Foxj1 (Figure 2f). As a control, we fully scraped a matched *ex vivo* culture and returned the cells back to an identical Transwell insert, but in this case, wherein the architecture of the tissue was completely disrupted, none of the cells adhered to the collagen-coated insert after 7 days (data not shown). It is noteworthy that when pure FTSECs were derived from the mixed FTE cells and then plated onto the Transwell inserts, we did not observe *trans*-differentiation into ciliated cells (data not shown). Consistent with our observation *in vivo*, the FTSECs in our *ex vivo* cultures retain their proliferative capacity (see Supplementary information (SI) and Supplementary Figure 1), and this property is preserved over 15 days in culture (Supplementary Figure S2). The proportion of secretory versus ciliated cells is variable between specimens, but remains constant during the 15 days in culture (Supplementary Figure S3). These findings suggest that the *ex vivo* culture system, which is composed of primary cells, is a dynamic model that does not senesce after becoming confluent, but rather retains a proliferative potential.

The ex vivo *culture system recapitulates the secretome of the human FT epithelium*

Finally, we wanted to confirm the functional similarity of our system to FTE *in vivo*. For this purpose, we probed the conditioned medium from the cultured cells

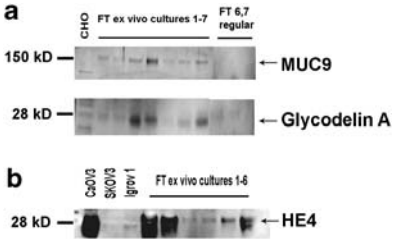


Figure 3 Fallopian tube (FT) *ex vivo* cultures secrete known FT and ovarian carcinoma proteins. **(a)** Detection of oviductin (MUC9, ~150 kDa) and Glycodelin A (PP14, ~28 kDa) by western blot in conditioned medium of FTE from seven different donors grown in *ex vivo* cultures, whereas there is no detectable protein secretion when grown in regular culture conditions. **(b)** Detection of HE4 (~28 kDa), an ovarian cancer serum biomarker, in some established ovarian cancer cell lines and some FTE *ex vivo* cultures.

for the presence of proteins that are known to be secreted by human FT, including Oviductin (MUC9) and Glycodelin A (PP14) (Saridogan *et al*., 1997; Briton-Jones *et al*., 2002). We detected both Oviductin and Glycodelin A in serum-free conditioned medium collected after 24–48 h of incubation with confluent *ex vivo* cultures (Figure 3a). These proteins were not detected when the medium was collected after 2 h of incubation, suggesting that they did not originate from the medium. We also did not detect these proteins when the cells were grown in standard submerged cultures on plastic (Figure 3a). Interestingly, the cultures also secreted

HE4 (Figure 3b), a recently approved serum biomarker of ovarian carcinoma (Hellstrom *et al*., 2003; Drapkin *et al*., 2005; Moore *et al*., 2009). As the *ex vivo* culture system is a polarized cell layer, we wanted to test our ability to detect secreted proteins that are present in the apical (lumen facing) side of the cells. We noted that once totally confluent, the cultures formed a leak-proof fluid barrier, and that none of the serum-containing feeding media in the lower compartment of the culture vessel could permeate the upper compartment. We exploited this property to isolate fluid from the apical aspect of the culture. To obtain the conditioned media, the upper chamber of the filters was washed with PBS, which was collected 24 h later. We profiled 11 different culture samples using liquid chromatography tandem mass spectrometry and found several established ovarian cancer serum biomarkers in the secretions of normal FTE (Table 1). Several of these biomarkers were further validated by immunohistochemical staining of normal FT fimbria sections (Supplementary Figure 4). Moreover, secreted proteins that were collected and analyzed in an identical manner from two different OSE cell lines grown under the same conditions did not include the major ovarian cancer biomarkers (Table 1 and Supplementary Table 2). Although the number of FTE and OSE cultures tested in this manner is small, these data further support the notion that the FTE may be the tissue-of-origin for a significant subset of patients with serous carcinoma. This data also suggests that the *ex vivo* culture system can be uniquely interrogated to yield potentially valuable information regarding biomarkers for this disease.

Secretory cells and ciliated cells exhibit different DNA repair kinetics

Having established the structural and functional validity of our FTE model system, we wanted to ask whether the system could be used to start deciphering the mechanisms underlying the apparent predisposition of the secretory cell to neoplastic transformation. We chose to address the effect of genotoxic stress on our cultures. *Ex vivo* cultures were exposed to ionizing radiation (IR) at a dose of 500 rad (5 Gy), and the activation of the dsDNA repair pathway was monitored by co-staining for repair foci containing either phosphorylated ATM (pS1981) (Branzei and Foiani, 2008), a protein kinase that is activated in response to dsDNA breaks, or γH2A.X (pS139), a histone isoform that is phosphorylated by activated ATM and recruits DNA repair

Table 1 Selected serous carcinoma biomarkers in the FT *ex-vivo* culture secretome

Accession number	Protein description	*Incidence*[a]	*Expressed by OSE*
gi|115298678|ref|NP_000055.2|	Complement component-3 precursor (*Homo sapiens*)	8	
gi|4557871|ref|NP_001054.1|	Transferrin (*Homo sapiens*)	8	
gi|5032057|ref|NP_005611.1|	S100 calcium-binding protein-A11 (*Homo sapiens*)	7	
gi|22208982|ref|NP_002767.2|	Kallikrein-related peptidase-10 preproprotein (*Homo sapiens*)	6	
gi|4507509|ref|NP_003245.1|	Tissue inhibitor of metalloproteinase-1 precursor (*Homo sapiens*)	6	+
gi|83367077|ref|NP_078966.2|	Mucin-16 (*Homo sapiens*)	6	
gi|4504919|ref|NP_002264.1|	Keratin-8 (*Homo sapiens*)	5	
gi|62414289|ref|NP_003371.2|	Vimentin (*Homo sapiens*)	5	+
gi|4505185|ref|NP_002406.1|	Macrophage migration inhibitory factor (glycosylation-inhibiting factor) (*Homo sapiens*)	5	+
gi|4505773|ref|NP_002625.1|	Prohibitin (*Homo sapiens*)	5	
gi|53988378|ref|NP_005814.2|	Mesothelin isoform 1 preproprotein (*Homo sapiens*)	5	
gi|4502693|ref|NP_001760.1|	CD9 antigen (*Homo sapiens*)	5	
gi|4557485|ref|NP_000087.1|	Ceruloplasmin precursor (*Homo sapiens*)	4	
gi|56699495|ref|NP_006094.3|	WAP four-disulfide core domain 2 precursor (*Homo sapiens*)	4	
gi|48255935|ref|NP_000601.3|	CD44 antigen isoform 1 precursor (*Homo sapiens*)	4	
gi|5031701|ref|NP_005851.1|	Follistatin-like-3 glycoprotein precursor (*Homo sapiens*)	4	
gi|91984773|ref|NP_658985.2|	Apolipoprotein A-I-binding protein precursor (*Homo sapiens*)	3	
gi|67782365|ref|NP_005547.3|	Keratin-7 (*Homo sapiens*)	3	
gi|5453678|ref|NP_006423.1|	Epididymal secretory protein E1 precursor (*Homo sapiens*)	3	
gi|21237748|ref|NP_004348.2|	CD151 antigen (*Homo sapiens*)	3	
gi|4507065|ref|NP_003055.1|	Secretory leukocyte peptidase inhibitor precursor (*Homo sapiens*)	3	
gi|4505787|ref|NP_002629.1|	Elafin preproprotein (*Homo sapiens*)	3	
gi|5453541|ref|NP_006399.1|	Anterior gradient 2 homolog (*Homo sapiens*)	2	
gi|65301117|ref|NP_002447.4|	Mucin 1 isoform 1 precursor (*Homo sapiens*)	2	
gi|65507501|ref|NP_001018059.1|	Glycodelin precursor (*Homo sapiens*)	2	
gi|4505583	Progestagen-associated endometrial protein (placental protein 14)	2	
gi|4505059|ref|NP_002345.1|	Pregnancy-associated endometrial tumor-associated calcium signal transducer 1 precursor (*Homo sapiens*)	2	
gi|58386720|ref|NP_002548.3|	Oviductal glycoprotein 1 precursor (*Homo sapiens*)	1	

A representative subset of proteins that are secreted by FTE cells in the *ex vivo* cultures and were previously reported to be detectable in ovarian cancer or fallopian tube secretions. The incidence is calculated out of 11 different patients' samples. Proteins secreted by at least one of two ovarian surface epithelium (OSE) samples analyzed in an identical manner are marked.

[a]A total of 11 fallopian tube culture secretions were analyzed using liquid chromatography tandem mass spectrometry (MS). This column represents the number of samples (out of 11) in which this particular protein was identified by MS.

factors, and for the lineage-specific markers, either Pax8 (for secretory cells) or FoxJ1 (for ciliated cells) (Figure 4a and Supplementary Figures S5–8). Analysis of a time course revealed an early activation of the dsDNA breakage repair pathway in the secretory cells 1–2 h after induction with IR, in contrast to late response by the ciliated cells, which peaked after 8–10 h. Although the dsDNA breaks in the ciliated cells were almost completely repaired by 24 h, as measured by resolution of the γH2A.X foci, these foci persisted in FTSECs even after 48 h (Figure 4b). No increase in apoptosis or necrotic cell death, or change in the proportions of secretory versus ciliated cells was encountered during the course of these experiments (data not shown). Importantly, when the DNA damage was induced by other agents, including Mitomycin C (3 μM for 16 h), cisplatinum (5 μM for 4 h) or H_2O_2 (50 μM for 10 min), similar results were observed (Supplementary Figure 8). Therefore, the DNA damage response (DDR) kinetics of FTSECs is characterized by an early and rapid activation that persists beyond the time in which ciliated cells resolve the damage. These results suggest that FTSECs may be more prone to accumulating DNA lesions that could potentially lead to mutations and the genesis of p53 signatures.

To validate these *ex vivo* findings, we asked whether fresh FT tissue exhibits a similar differential kinetics in the DDR between the FTSECs and the ciliated cells. Fresh FT tissue fragments were exposed to low doses of IR (125 rad). We recorded the response shortly after the exposure (1 h) and again 24 h later, during which time the tissue was maintained in culture medium. This time point represents a complete DNA repair cycle in many cell types (Keogh *et al*., 2006). Although higher doses of IR (500 rad, as used for the *ex vivo* cultures) resulted in dsDNA breaks in all epithelial cells, as determined by staining for γ-H2A.X, exposure to low doses (125–250 rad) resulted in a markedly different response: early activation of DDR in the secretory cells, with sustained signaling even after 24 h, while the ciliated cells had recovered by the later time point (Figures 5a and b). The activation of the DDR is displayed as preferential phosphorylation of ATM, histone H2A.X and Chk2, and accumulation of p53 in FTSECs (Figure 5). The general morphology of the tissue was preserved over the 24 h incubation period (hematoxylin and eosin staining in Figure 5). Although an average of 53% of the secretory cells in these experiments displayed γ-H2A.X foci 24 h after exposure to IR, only 22% of the ciliated cells still retained such foci (Figure 5b). The difference between the two cell lineages became more evident when p53 was analysed: 71% of the secretory cells and only 4% of the ciliated cells had accumulation of p53 at the 24-h time point (Figure 5b). These results further corroborate the validity of the *ex vivo* culture system as a tool for the characterization and manipulation of the FTE, and suggest that persistent DNA damage in FTSECs may underlie its susceptibility to genomic instability.

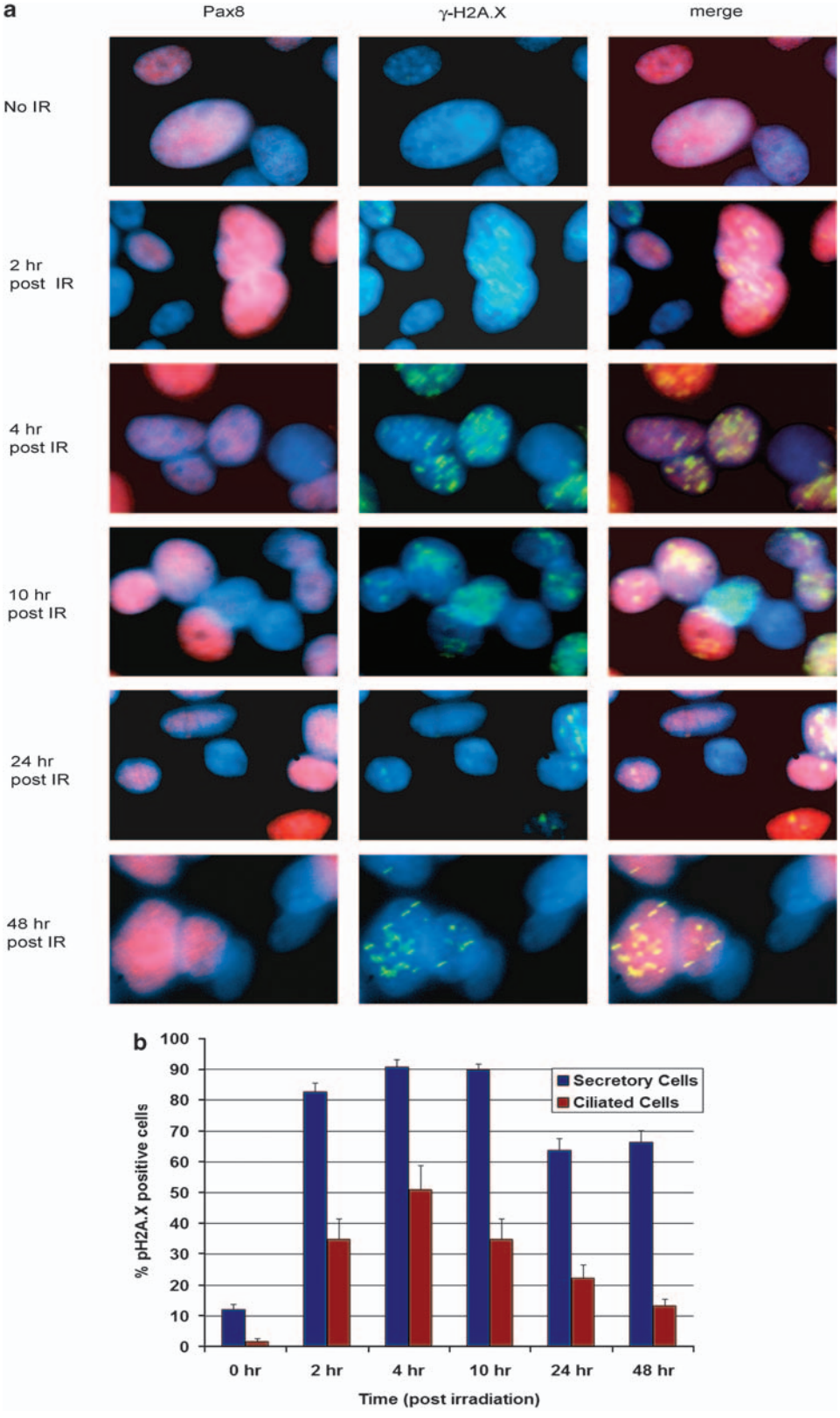
Discussion

Developing a reliable model system for studying the early events in SOC pathogenesis has been particularly challenging, largely because of the uncertainty regarding the cell-of-origin of this disease. Given the recent data implicating the FTSEC in this process, it became important to develop an *in vitro* model system that would recapitulate the morphology and biology of these cells. Our first goal was to culture normal human primary FTE in a manner that would preserve the composition of both secretory and ciliated cells, as well as the degree of differentiation and tissue polarity. We also expected the system to be able to overcome inherent problems of working with benign primary cells, such as early senescence, narrow time window for manipulation and experimentation, lack of reproducibility and loss of plasticity over time. The model that we have developed permits the isolation and growth of both secretory and ciliated epithelial cells, which retain their phenotypic properties over an extended period of *in vitro* culture. The cultures are viable over a period of several weeks, and retain a proliferative capacity. The cells also function in a way that recapitulates the complex biology of the FT, both by secreting specific proteins and by responding to stimuli, such as cell injury and DNA damage, in a manner that is observed *in vivo*.

In addition to validating our culture system as a faithful replicate of FTE, our data also lend support to the notion that the FTE is a field-of-origin for serous carcinomas. The identification of 'cancer' biomarkers (Hellstrom *et al*., 2003; Bast *et al*., 2005; Drapkin *et al*., 2005; Kozak *et al*., 2005) in the secretome of benign FTE supports the hypothesis that the cell-of-origin of ovarian tumors is likely Müllerian (Drapkin *et al*., 2005). Furthermore, the fact that these biomarkers are below detectable levels in normal subjects does not necessarily suggest that they are expressed *de novo*, as

Figure 4 Differential double-stranded DNA (dsDNA) break response in the fallopian tube epithelium *ex vivo* cultures. (**a**) Immunofluorescence (en-face images) of representative cells in the *ex vivo* culture system stained for the secretory lineage marker Pax8 (red) and γH2A.X (green); the nuclei are outlined with DAPI (4',6-diamidino-2-phenylindole) (blue). The formation of γH2A.X foci was recorded 2, 4, 10, 24 and 48 h after exposure of the co-cultures to ionizing radiation (500 rad). γH2A.X foci are preferentially detectable in fallopian tube secretory epithelial cells (FTSECs) 2 h after irradiation and persist for at least 48 h. Original magnification, × 60. (**b**) The kinetics of dsDNA break pathway activation in FTSECs versus ciliated cells *ex vivo*. γH2A.X foci form in FTSECs early after exposure to ionizing radiation, and persist in 70% of the cells for at least 48 h. Under the same conditions, γH2A.X foci formation in ciliated cells peaks after 10 h and returns to baseline within 24 h. The graph represents integration of four experiments.

part of the carcinogenic process, but rather reflect the increase in mass of the tissue that normally secretes them. Importantly, as a control for specificity, we show that the profile of proteins secreted by the FT *ex vivo* cultures is divergent from that of OSE cultured under identical conditions. Although the significance of these findings is compromised by the small number of samples, it is tempting to postulate that manipulating



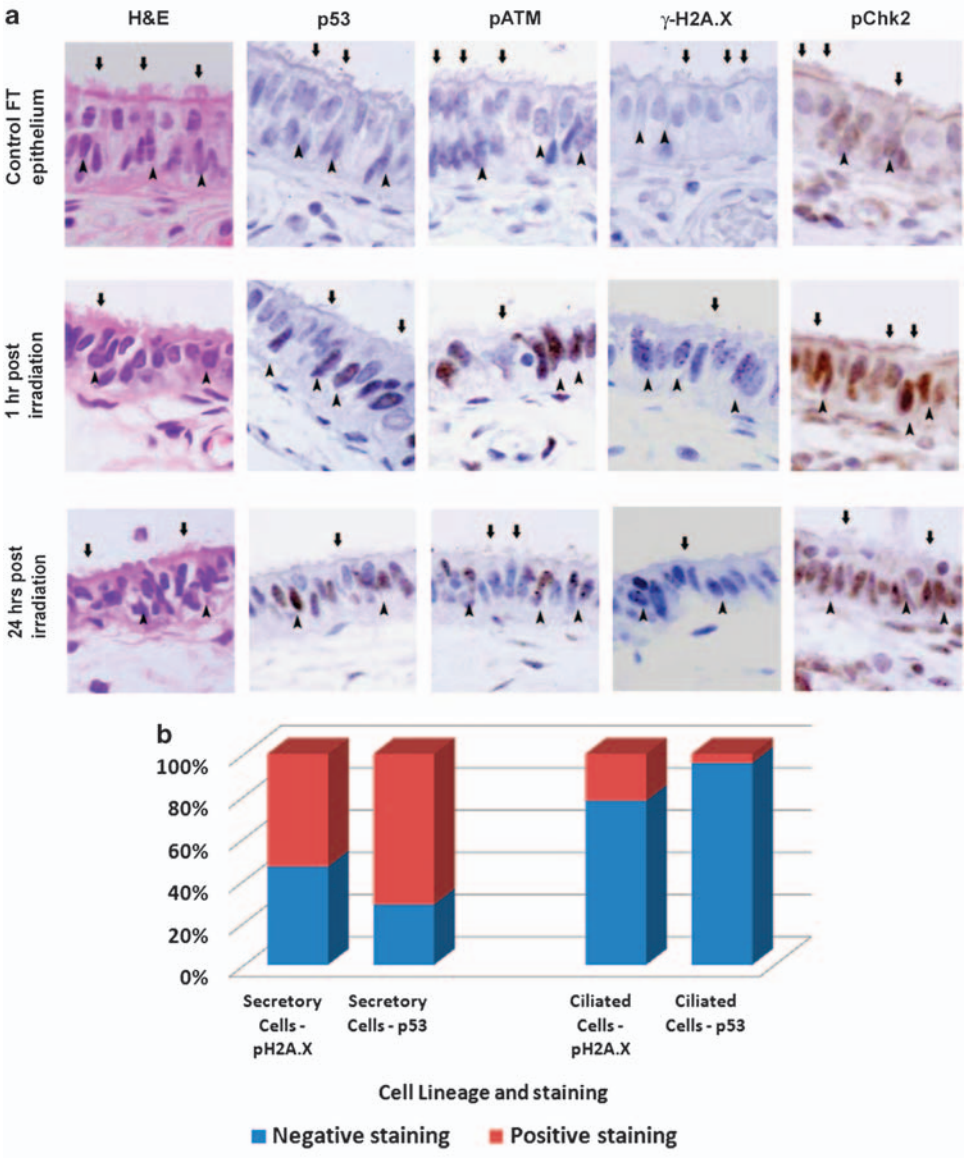


Figure 5 Preferential activation of the double-stranded DNA (dsDNA) break checkpoint pathway in fallopian tube secretory epithelial cell (FTSEC) *in situ*. (**a**) Hematoxylin and eosin staining and immunohistochemistry of normal human fallopian tube fimbria after exposure to ionizing radiation, showing preferential activation of the dsDNA break signaling pathway in secretory cells (marked by arrowheads), compared with ciliated cells (marked by arrows). Original magnification, ×60. (**b**) Quantitative analysis of the number of adjacent secretory cells and ciliated cells that stained positive for γH2A.X foci and p53, 24 h after exposure to ionizing radiation *in situ* (125–250 rad). No staining was seen in non-irradiated cells.

this system to undergo changes that are typical of serous carcinoma, such as loss of *BRCA1* or of *TP53*, may result in genomic instability and the development of serous precursor lesions that could alter the signature of secreted proteins. We intend to use this model to study the early events in serous oncogenesis, focusing on identification of candidate targets for drug development and secreted biomarkers for early detection.

Secretory and ciliated epithelial cells are intimately mixed within the FT inner lining. Although they may run two different transcriptional and translational programs, they are essentially exposed to the same microenvironment. We used the *ex vivo* culture system to study how these two cell lineages respond to DNA damage. DNA damage has a major role in malignant transformation, and the DDR is not only committed to repairing the DNA but also serves as a barrier to transformation by inducing cell cycle arrest, apoptosis or senescence. This general pattern is observed in multiple tumor types (Bartkova *et al*., 2005; Halazonetis *et al*., 2008). We focused our study on the dsDNA repair pathway because it is a common pathway to several other repair mechanisms, and as such, provides an insight into the genotoxic stress in a cell or a tissue regardless of the cause. We found that the FTSECs display a rapid, yet prolong, activation of the DDR after minimal damage. In contrast, the ciliated cells appear to activate the DDR more slowly, but resolve the injury

within the time frame of our studies. This phenomenon may reflect the mere fact that the ciliated cells are terminally differentiated and that most of these cells are quiescent and in G0/G1. We currently do not know whether the mechanism by which ciliated cells and FTSECs repair dsDNA breaks is different because of their proliferative capacities, but one could imagine that non-homologous end-joining and homologous recombination may be preferentially used in each cell type (Branzei and Foiani, 2008). In this regard, the recent report indicating that the interaction between CtIP and BRCA1 acts as a molecular switch to shift the balance of DNA break repair from error-prone non-homologous end-joining to error-free homologous recombination in cell transitioning out of G0/G1 (Yun and Hiom, 2009) is compelling in light of the role that BRCA1 has in genome integrity control and in hereditary forms of serous ovarian cancer. More importantly, our *in situ* studies confirmed the findings in the *ex vivo* cultures and lend support to biological integrity of the culture system.

We believe that the *ex vivo* culture system will enable us to begin testing the hypothesis that the FTSEC is a cell-of-origin for serous carcinomas. Exposure of FTE in these cultures to hormonal changes and inflammatory mediators released during ovulation may provide insights into the genetic aberrations that culminate in pelvic serous carcinomas. These discoveries can in turn be used to develop early detection biomarkers, preventive approaches and novel drugs. It is also likely that a similar system can be broadly applied to study the normal biology of other epithelia in which precursor lesions and malignant counterparts are not entirely understood. For example, similar systems have been described to study airway biology and disease (Karp *et al*., 2002; Vermeer *et al*., 2003), and a system in monkeys has been described to study reproductive biology (Rajagopal *et al*., 2006). Using such model systems to study the tumorigenic process may greatly impact our understanding of epithelial biology and oncogenesis.

Materials and methods

Tissue samples

Fresh FT fimbria specimens were obtained from the Brigham and Women's Hospital Department of Pathology with the approval of the institutional review board. The fimbrial tissues used in this study are collected from surgical procedures for benign gynecological indications. Specifically, cases of inflammatory disease, infection and extensive adhesions were excluded.

Primary human FT epithelium ex vivo *culture system*

The FT tissue was kept in cold PBS at 4 °C until processing. Once minced, the tissue is incubated in a dissociation medium composed of Eagle's Minimum Essential Medium (EMEM, Cellgro, Manassas, VA, USA) supplemented with 3.5 mg/ml Pronase (Roche Diagnostics, Indianapolis, IN, USA) and 0.25 mg/ml DNase (Sigma, St Louis, MO, USA) for 48–72 h in 4 °C with constant mild agitation. Human collagen IV (Sigma) is dissolved in H_2O to a concentration of 0.06 mg/ml. All culture wares used for FTE cell culture are coated with collagen before use and washed thoroughly with PBS before cell plating. The dissociated epithelial cells are harvested by centrifugation and re-suspended in growth medium (Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 1:1 (Cellgro), supplemented with 2% serum substitute Ultroser G (Pall France, St-Germain-en-Laye Cedex, France) and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY, USA)). The number of cells from a single fimbria specimen is estimated to range between 10^5 and 5×10^6 epithelial cells. Cells are plated onto collagen IV-coated Costar polyester Transwell-clear inserts placed in 24-well tissue culture plates (Corning, Corning, NY, USA) at a density of 1×10^4 cells per insert. Growth medium is then added to the lower compartment and the cells are incubated at 37 °C in a humidified 5% CO_2 incubator. In our experience using over 100 different FT samples, greater than 85% of the samples were viable and yielded pure epithelial cell cultures, with no evidence of other contaminant cell types. The medium is removed from the upper compartment 48 h after plating of the cells.

Electron Microscopy

The cell cultures were prepared for electron microscopy using standard procedures. Briefly, the cell cultures were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer followed by post fixation in 1% osmium tetroxide. The samples were then dehydrated in a graded series of ethanol. For scanning electron microscopy, the *ex vivo* cultures were transitioned to hexamethyldisilazane and air-dried overnight. The filters were cut from their support and mounted on aluminum stubs. After sputter coating with gold/palladium, the samples were imaged in a Hitachi S-4800 scanning electron microscope (Hitachi, Pleasanton, CA, USA).

For transmission electron microscopy, the tissue is cleared in propylene oxide and infiltrated with Poly-Bed 812 (Polysciences, Warrington, PA, USA). Ultrathin sections with silver to light golden interference color are stained with saturated uranyl acetate solution followed by lead citrate. The sections are examined in a JEM 1010 electron microscope (JEOL, Peabody, MA, USA) at 80 kV acceleration voltage. Images are recorded with an AMT digital camera (Advanced Microscopy Techniques, Corp., Danvers, MA, USA).

Wound healing assay

Confluent cultures on Transwell inserts were gently disrupted using a sterile plastic pipette tip and washed with PBS. The cultures were monitored until restoration of tissue confluence by serial imaging, using an OLYMPUS inverted microscope (OLYMPUS, Center Valley, PA, USA) and the QCapture imaging software (Qimaging, Surrey, BC, Canada). The cultures were then fixed, and immunofluorescence staining for lineage markers of secretory versus ciliated cells, Ki67 and 5-bromo-2-deoxyuridine (BrdU) labeling were performed. For BrdU labeling experiments, cells at different time points after plating were incubated with 32.5 μM BrdU (Sigma) in growth medium for 2 h, followed by fixation with 2% paraformaldehyde and denaturation with 2 M hydrochloric acid for 30 min. BrdU incorporation was detected by immunofluorescence staining. These experiments were repeated thrice with cells from different patients.

Mass spectometry

The upper compartment of confluent *ex vivo* cultures was washed with PBS. Fresh PBS was then added to the upper compartment for 16–24 h. The proteins present in the sample were reduced with 5 mM dithiothreitol at 56 °C for 1 h, and digested with 10 μg of trypsin (Promega, Madison, WI, USA) at 37 °C overnight. Peptides were extracted using C18 cartridge (Waters, Milford, MA, USA) and reconstituted with 200 μl of

incubation buffer (50 mM HEPES, 50 mM NaCl, pH 7.4). Cysteine-containing peptides were immobilized on activated thiol sepharose 4B resin (GE Healthcare, Piscataway, NJ, USA), eluted with 10 mM dithiothreitol and alkylated with 55 mm iodoacetamide. Peptides were then analyzed by liquid chromatography tandem mass spectroscopy on an Orbitrap-XL mass spectrometer (Thermo Scientific, Waltham, MA, USA). MS/MS spectra were searched against Human database using Mascot (Matrix Science, Boston, MA, USA). Peptides with score over 25 (FDR (false discovery rate) $<1\%$) were accepted for protein identification.

DNA damage experiments

Confluent FTE *ex vivo* cultures and whole FT tissues were treated with IR in a Gammacell–1000 irradiator (Best Theratronics, Ottawa, Canada). Radiation dose was 500 rad for the *ex vivo* cultures and 125 rad for the whole tissue pieces.

Alternatively, cells or tissue were treated with 3 μM mitomycin C (Sigma) in growth medium overnight, 5 μM cisplatinum in growth medium for 4 h (Sigma, stock solution dissolved in dimethylformamide) or with 50 μM H_2O_2 (Sigma) for 10 min, followed by washing. DNA damage was detected by means of immunofluorescent or immunohistochemical staining for factors in the dsDNA repair pathway at different time points. The *ex vivo* culture experiments have been repeated five times, with cells from five different donors. For quantitative analysis of each experiment, at least 500 nuclei were evaluated for each time point. The experiments with whole tissue pieces have been repeated thrice, with tissues from different donors. The quantitative analysis was performed by counting the secretory and ciliated cells on three different fields per experiment on γH2A.X-stained and p53-stained slides.

Conflict of interest

Dr Drapkin's work is funded by the NIH. He receives compensation as a consultant to Novartis Pharmaceuticals. No other authors declare any potential conflicts of interest.

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