Gynecologic Oncology 136 (2015) 348–354

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Three-dimensional modeling of the human fallopian tube fimbriae

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HIGHLIGHTS

- 3D fimbriae cultures retain tissue architecture and remain metabolically active.
- H_2O_2 and insulin promote epithelial proliferation and E_2 induces IL8 secretion.
- p53 expression in secretory cells can be induced artificially in culture.

ARTICLE INFO

Article history:
Received 6 September 2014
Accepted 10 December 2014
Available online 16 December 2014

Keywords:
fallopian tube
fimbriae
microphysiological modeling

ABSTRACT

Objective. Ovarian cancer is the most lethal gynecological malignancy that affects women. Recent data suggests that the disease may originate in the fallopian fimbriae; however, the anatomical origin of ovarian carcinogenesis remains unclear. This is largely driven by our lack of knowledge regarding the structure and function of normal fimbriae and the relative paucity of models that accurately recapitulate the *in vivo* fallopian tube. Therefore, a human three-dimensional (3D) culture system was developed to examine the role of the fallopian fimbriae in serous tumorigenesis.

Methods. Alginate matrix was utilized to support human fallopian fimbriae *ex vivo*. Fimbriae were cultured with factors hypothesized to contribute to carcinogenesis, namely; H_2O_2 (1 mM) a mimetic of oxidative stress, insulin (5 μg/ml) to stimulate glycolysis, and estradiol (E_2, 10 nM) which peaks before ovulation. Cultures were evaluated for changes in proliferation and p53 expression, criteria utilized to identify potential precursor lesions. Further, secretory factors were assessed after treatment with E_2 to identify if steroid signaling induces a pro-tumorigenic microenvironment.

Results. 3D fimbriae cultures maintained normal tissue architecture up to 7 days, retaining both epithelial subtypes. Treatment of cultures with H_2O_2 or insulin significantly induced proliferation. However, p53 stabilization was unaffected by any particular treatment, although it was induced by *ex vivo* culturing. Moreover, E_2-alone treatment significantly induced its canonical target PR and expression of IL8, a factor linked to poor outcome.

Conclusions. 3D alginate cultures of human fallopian fimbriae provide an important microphysiological model, which can be further utilized to investigate serous tumorigenesis originating from the fallopian tube.

Background

High-grade serous cancer (HGSC), the most lethal histotype of ovarian cancer, has been postulated to originate from the epithelium lining the fallopian tube fimbriae [1]. Traditionally, HGSC was thought to arise from the ovarian surface epithelium (OSE). However, a clear ovarian precursor has yet to be identified, and screening of high-risk patients has not improved recurrence and survival in several decades [2]. A potential precursor has recently been described in the secretory epithelium of the fallopian tube fimbriae; the 'p53 signature' [3]. These lesions are classified by stabilized p53, a protein dysregulated in ~96% of HGSCs [4]. p53 signatures are thought to transform into serous tubal intraepithelial carcinomas (STICs) [3], which have been found concomitant with HGSC tumors and often harbor identical mutations in p53, suggesting a common origin [5].

Despite a potential role in the origin of HGSC, there are few models for the investigation of the human fallopian tube epithelium (FTE) that accurately recapitulate the *in vivo* environment. Although cell

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http://dx.doi.org/10.1016/j.ygyno.2014.12.015
0090-8258/

culture is a valuable model and allows for continual passage of human FTE and for targeted genetic manipulation, traditional two-dimensional culture is unable to simulate interaction with the fallopian stroma and generally does not allow for maintenance of ciliated FTE [6–8]. Although an advanced FTE model that retains ciliated cells has been reported, this model manipulates the architecture and eliminates stromal cells [9]. Further, human FTE cells require artificial immortalization via SV40-T antigen [6–8], which sequesters p53 to the nucleus, functionally silencing p53 or equivalent siRNA molecules [10]. This is counter to the majority of p53 alterations seen in HGSC, where mutation allows for p53 gain-of-function rather than silencing [11]. Thus, by immortalizing human FTE for *in vitro* research, the cells become a less accurate model of important preneoplastic changes suggested to occur in HGSC carcinogenesis. Moreover, although transgenic murine models have been developed using fallopian-specific promoters driving Cre recombinase [12], the murine anatomy is different from that of the human, with continuation of the oviduct into a bursal sac and the absence of a fimbriated end. Thus, the development of a human 3D fallopian model is a critical goal for our field.

Irrespective of the site of origin, reducing ovulation through the use of oral contraception is protective against ovarian cancer [13], suggesting a role for an ovulatory factor(s) in the initiation of the disease. However, the impact of ovulatory factors on FTE and how they might promote HGSC is unclear. Unopposed estrogen (E_2) signaling is an aspect of ovulation linked to increased risk of ovarian cancer, as compared to combined E_2 and progesterone signaling experienced during pregnancy, breastfeeding, and while taking oral contraceptives [13]. Oxidative stress is also enhanced during ovulation and is known to induce DNA damage in oviductal epithelium [14]. Yet, the impact of oxidative stress and E_2 signaling on p53 stabilization and proliferation in the fimbriae, hypothesized to be the earliest transformative changes in the FTE [3], is unknown.

The purpose of this study was to develop an *ex vivo* three-dimensional (3D) model of the human fallopian tube that maintains tissue architecture and more accurately recapitulates the *in vivo* environment. This model allows for the investigation of specific ovulatory components and their effects on FTE proliferation. Insulin, a common culture supplement and a known mitogen, was utilized as a positive control to validate the system. The impact of E_2 on fallopian samples was further investigated to define the impact of ovarian hormones and how E_2 might promote a transformative microenvironment. Finally, p53 stabilization, the hallmark of the purported precursor to HGSC, was evaluated after extended culture and treatment with ovulatory factors.

Materials and methods

Tissue collection

Fallopian fimbriae were collected with consent prior to surgery at the University of Illinois at Chicago (UIC IRB #2012-0539). Patients utilized in this study were undergoing salpingectomy for a variety of gynecological purposes (outlined in Supplemental Table 1). Resulting tissues were deemed morphologically normal and considered benign as determined by gross examination by the University of Illinois at Chicago Pathology Department. A total of 12 samples from patients ranging from 28 to 62 years of age (average age of 43).

3D culture optimization and treatment

Tissues were micro-dissected in alpha-MEM (Gibco, Carlsbad, CA) with 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Individual fimbriae were separated into ~50 mm^3 pieces. A portion of the tissue was fixed in 2% paraformaldehyde for use as uncultured controls. For optimization studies, fimbriae were cultured without matrix, encapsulated in 0.5% alginate, or encapsulated in 0.5% alginate with 1 mg/ml collagen and 0.1% fibronectin, as previously described for murine cultures [15]. For subsequent treatments, 0.5% alginate encapsulated fimbriae were randomly assigned to treatment groups, with at least five fimbriae per condition, per patient, in a 24-well plate containing alpha-MEM and 1% penicillin-streptomycin. Tissues were treated with 1 μl/ml ethanol (vehicle), 10 nM E_2 (Sigma-Aldrich, St. Louis, USA), 1 mM H_2O_2 (Fisher Scientific, Pittsburgh, PA), or 5 μg/ml insulin [via ITS (insulin; transferrin, 5 μg/ml; selenite, 5 ng/ml); Roche, Indianapolis, IN], and cultured for 2 or 7 days. Prior to fixation, fimbriae were labeled with 10 μM bromodeoxyurine (BrdU; Sigma-Aldrich) for 24 hours to denote proliferating cells. Fimbriae cultures were fixed (2% paraformaldehyde) followed by dehydration in ethanol and xylene, and embedded in paraffin.

Tissue preparation and immunohistochemistry

Sections (5 μm) were cut and stained via hematoxylin and eosin for morphological analysis, and immunohistochemistry was performed to localize proteins of interest as previously described [14]. Briefly, slides were rehydrated through an ethanol gradient, prior to 0.1 M sodium citrate retrieval and peroxidase block. Tissues probed for BrdU were exposed to 4 M HCl and 0.1 M NaB_4O_7 (Fisher Scientific) to denature DNA. All immunohistochemical reagents were obtained from Vector Laboratories, Inc. (Burlingame, CA) unless otherwise stated. Tissues were blocked in 3% bovine serum albumin (Gemini, West Sacramento, CA)-TBS/10% serum and incubated with a primary antibody 1:50 acetylated tubulin (Cell Signaling, Cambridge, MA); 1:200 BrdU (AbCam, Cambridge, MA); 1:100 cytokeratin 8 (CK8, Developmental Studies Hybridoma Bank, Iowa City, IA); 1:50 p53 (Santa Cruz, Santa Cruz, CA); 1:100 PAX8 (Proteintech, Chicago, IL); 1:100 pH2AX (Cell Signaling); or 1:75 PR (Santa Cruz)) overnight at 4 °C. Tissues were washed in TBS-0.1% Tween and incubated with a secondary antibody (1:200), before being probed with ABC peroxidase standard, followed by detection with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin.

Image capture and analysis

Immunohistochemistry images were taken via a Nikon E600 microscope, DXM1200 digital camera and NIS Elements software (Nikon Instruments, Melville, NY). For proliferation analysis, concurrent sections were stained for CK8 and BrdU. BrdU sections were imaged and epithelial cells (CK8 positive) were quantified for proliferation via ImageJ software (NIH, Bethesda, MD). At least three fimbriae with 200 or more FTE were quantified for each treatment. Analysis of p53 staining was similar, with at least three fimbriae per treatment, per patient analyzed. Samples with p53 expression were quantified utilizing adjacent sections stained for the secretory cell marker PAX8 in a qualitative manner as described.

ELISA

IL8, VEGF-A, and FGF2 were detected in fallopian culture medium by enzyme-linked immunosorbent assay for human IL8 (EMD Millipore, Billerica, MA, USA), VEGF-A (RayBiotech, GA, USA), or FGF2 (Abcam) respectively, using the manufacturer's protocols. The sensitivity for IL8, VEGF-A, and FGF2 are 4.4 pg/ml, 10 pg/ml, and 2 pg/ml, respectively. Results were normalized to total protein content as determined by Western blotting and Ponceau staining to account for differences in tissue size between treatment groups. Briefly, conditioned medium (20 μl) was run on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane (Fisher Scientific). Ponceau (Sigma-Aldrich) staining and subsequent densitometry via ImageJ software was performed.