Increased expression of uteroglobin associated with tubal inflammation and ectopic pregnancy

Amado Alfredo Quintar, M.D.,[a] *Jorge Humberto Mukdsi, M.D., Ph.D.,*[a] *Mónica del Valle Bonaterra, Ph.D.,*[a] *Agustín Aoki, M.D., Ph.D.,*[a] *Cristina Alicia Maldonado, Ph.D.,*[a] *and José Pérez Alzaa, M.D.*[b]

[a] Center of Electron Microscopy, School of Medical Sciences, and [b] Department of Gynecology, University Hospital, School of Medicine, National University of Cordoba, Cordoba, Argentina

Objective: Evaluation of uteroglobin (UG) expression in the fallopian tube in different tubal diseases.
Design: The UG was screened and quantified in samples of fallopian tubes from patients with salpingitis, hydrosalpinx, and ectopic pregnancy by exposing the UG with immunohistochemical techniques.
Setting: University hospital and electron microscopy center.
Patient(s): Women with pelvic inflammatory disease (PID) and complicated tubal ectopic pregnancy consulting for medical care.
Intervention(s): Salpingectomy.
Main Outcome Measure(s): Tubal tissues were collected and examined using regular pathologic techniques. The UG immunoreactivity in the tubal epithelium was also assessed.
Result(s): Fallopian tube epithelium displayed an increased UG expression in patients with PID and complicated tubal pregnancy compared with control patients.
Conclusion(s): Uteroglobin is present in the human fallopian tube as a secretory protein and appears to be involved in immunosuppressive responses in the fallopian tube. (Fertil Steril® 2008;89:1613–7. ©2008 by American Society for Reproductive Medicine.)

Key Words: Uteroglobin expression, fallopian tube, ectopic pregnancy, inflammation, secretoglobin

The fallopian tube is lined by an epithelium comprising ciliated and secretory cells. This epithelial compartment plays critical roles in mammalian reproduction, supporting the viability of gametes and preimplantation embryos during their journey into the uterus (1). Therefore, pathologies leading to tubal damage are important causes of infertility, with tubal factors accounting for 14%–38% of female infertility cases (2) and forming a substantial part of the cost of health policies worldwide. Pelvic inflammatory disease (PID), including salpingitis and its sequel hydrosalpinx, is the most important cause of tubal pathology leading to infertility.

Ectopic pregnancy is also a noteworthy factor of morbidity and mortality in women, especially in the first trimester of pregnancy. Although the precise cause and the mechanism by which an embryo implants in the fallopian tube remains poorly understood, ductal obliteration and a defectively developed corpus luteum have been proposed as central factors contributing to tubal ectopic pregnancy (3). Occlusion of the fallopian tube tends to be a frequent consequence of inflammation due to bacterial infection. According to Hunter (3), even if tubal patency was not critically compromised by infection, the salpingitis may modify the oviductal epithelium, leading to a pseudoendometrial microenvironment that could facilitate the attachment and abnormal implantation of the egg.

Uteroglobin (UG) is a homodimeric peptide of low molecular weight, which was initially detected in uterine secretion and forms a major component of the blastocyst fluid in the rabbit (4). Also, it was found to be implicated in the endometrial preparation for implantation (4–6). It belongs to the secretoglobin superfamily of proteins that comprises many molecules secreted by the epithelium, the majority of which bear antiinflammatory and immunomodulatory properties (7). Several biologic effects have been accredited to UG (8), in particular, the inhibition of the soluble phospholipase A2 (9) and the suppression of sperm and embryo antigenicity (10) involved in the modulation of inflammatory processes and in the endometrial receptivity of the embryo. Taking into consideration that the fallopian tube is the most frequent site for ectopic egg implantation, the aim of the present study was to examine the occurrence of tubal UG and its

Received November 20, 2006; revised and accepted March 16, 2007.
Supported by research grants from Consejo Nacional de Investigaciones Cientìficas y Técnicas (CONICET), FONCYT, and Agencia Córdoba Ciencia (ACC).
The last two authors contributed equally to this work.
Reprint requests: José Pérez Alzaa, M.D., Fundación Fecundart, Chacabuco 1016, 5000 Córdoba, Argentina (FAX: +54 351 44694219; E-mail: jperezalzaa@yahoo.com).

differential expression in inflammatory processes and tubal pregnancy.

MATERIALS AND METHODS

Patients and Tissue Sampling

Fifteen women at reproductive age (range 20–43 years old) undergoing salpingectomy were selected for this study and divided into four groups: group 1 with chronic salpingitis (n = 4), group 2 with hydrosalpinx (n = 4), group 3 with tubal ectopic pregnancy (n= 3), and a control group with normal fallopian tubes (n = 4). Patients from groups 1 and 2 presented PID and infertility, diagnosed clinically and ultrasonographically, and were affected mainly at the tubal level. Indications for salpingectomy in patients from group 3 were rupture of the fallopian tube (n = 2) and severe abdominal pain (n = 1). In group 4, normal fallopian tubes were obtained from patients subjected to adnexal hysterectomy for treatment of uterine fibroids. Pathologic conditions were confirmed by examination of biopsies, and no other diseases were detected during physical examination or in routine biochemical tests. Except for women from group 3, all patients were also subjected to biopsy of endometrium for histologic dating, which was performed by a pathologist following standard criteria. None of the patients received any hormonal treatment during the 3-month period before the study. The patients were informed in detail about the study to be performed, and written consent was obtained. The protocols applied in this investigation were approved by the University Hospital and Medical School Ethics Committees.

The samples of fallopian tubes were obtained at the midluteal phase of the cycle (22–24 days, according to the histopathologic dating of endometrial biopsies) within 10 minutes after performing surgery, and were then washed in 0.1 mol/L phosphate-buffered saline (PBS) before being immersed in 4% formaldehyde for fixation. The pieces processed for immunohistochemistry were fixed in the same fixative for a maximum of 12 hours and then stored in 70% ethanol until processing. Isthmus (proximal and medial) and ampullar (distal) tubal segments were analyzed. In ectopic pregnancies, specimens from the implantation site were also studied.

Histopathology

Formalin-fixed oviductal samples were processed for conventional light microscopy. Tissues were embedded in paraffin, cut into 4-μm-thick sections, and stained with hematoxylin-eosin.

Immunohistochemical Analysis

Paraffin-embedded tubal tissue sections (3 μm) were deparaffinized in xylene and rehydrated in ethanol series (100%, 96%, and 80%). Antigen retrieval was performed in a microwave oven in 10 mmol/L citric acid buffer, pH 6.0, for 14 minutes. Slides were allowed to cool to room temperature and were washed three times in PBS before being incubated with methanol-H_2O_2 (96:4, v/v) for 15 minutes to inhibit endogenous peroxidase. Nonspecific binding was blocked with PBS-milk containing 5% (v/v) normal goat serum (Sigma, St. Louis, MO) for 30 minutes. Then the sections were incubated overnight at 4°C with two rabbit polyclonal antibodies able to recognize human UG (anti–urine protein 1 diluted 1:200; Dako, Carpinteria, CA; or anti-CC10 diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies were detected by an indirect technique using a biotin-labeled goat-antirabbit antibody (Vector Laboratories, Burlingame, CA) diluted 1:150 in PBS containing 1% normal goat serum. After incubation for 30 minutes at room temperature, the sections were treated with Vectastain Elite ABC complex (Vector), following the manufacturer's protocol. Staining was developed with 3,3′-diaminobenzidin (Sigma) in a 0.1 mol/L Tris buffer, pH 7.2, containing 0.03% H_2O_2. Slides were dehydrated, counterstained with Harris hematoxylin, and mounted with Entellan (Merck, Darmstadt, Germany).

To validate the specificity of the immunostaining, the following controls were carried out: 1) negative controls were incubated with PBS alone or containing 1% normal goat or rabbit serum; 2) normal human lung and human endometrial sections (retrieved from pathology files) were used as positive controls of UG antigenicity.

Immunohistochemical Scoring and Statistics

Evaluation of staining was performed with a computer-assisted imaging system (Image Tool, version 1.27; University of Texas Health Science Center at San Antonio; available at: ftp://maxrad6.usthsca.edu) of digital images taken from slides with no counterstaining and converted to the grayscale mode. Scoring of UG immunoreactivity was performed based on the intensity and the stained area measured in one field of vision at ×25 magnification. In the same field, the UG immunostained area was measured per total area of the epithelium and multiplied by 100. At least five fields per level and at three different levels were examined per tube in each group.

Data from more than two groups were examined by applying analysis of variance with Tukey as a post test. Statistical testing and calculation of the immunohistochemical scoring data were performed using the InStat V2.05 program from GraphPad (San Diego, CA).

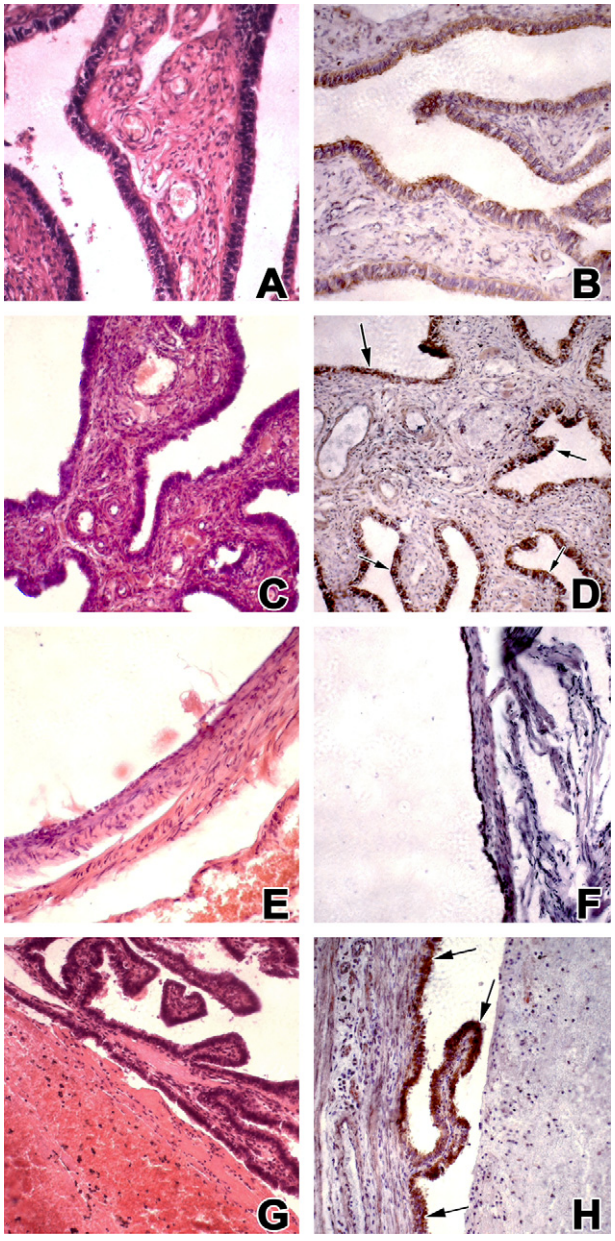
RESULTS

Morphologic Analysis

In control fallopian tubes, the endosalpinx displayed longitudinal folds lined by a columnar epithelium comprising ciliated and secretory cells with no cellular atypia (Fig. 1A). The myosalpinx exhibited an inner circular, as well as an outer longitudinal, muscular layer.

FIGURE 1

Photomicrographs of oviductal sections from control women (**A, B**) and patients with chronic salpingitis (**C, D**), hydrosalpinx (**E, F**), and tubal ectopic pregnancy (**G, H**). Panels A, C, E, and G show sections stained with hematoxylin-eosin. To detect uteroglobin (UG) immunoreactivity, slides were incubated with a specific anti-UG antibody and then counterstained (B, D, F, and H). *Arrows* in D and H denote the strong immunostaining in the epithelium. All photographs were taken at the same magnification (×25).



Quintar. Uteroglobin in the human fallopian tube. Fertil Steril 2008.

Chronic salpingitis was diagnosed histopathologically, taking the presence of mononuclear cell infiltration in the lamina propria (Fig. 1C) as diagnostic parameter. In addition, edema and subsequent shortening, as well as atrophy and fibrosis of the plica, were observed in the fallopian tube. Hydrosalpinx was characterized by a marked dilatation of the fallopian tube, the lining epithelium of which became flat and accompanied by a considerable cellular atrophy. Intense vascular congestion was observed in the tubal stroma (Fig. 1E).

The fallopian tube from women with tubal ectopic pregnancy showed the typical signs of implantation, such as chorionic villi, intraluminal extravillous trophoblast, and decidual changes with infiltration of the lamina propria with neutrophilic leukocytes. In these patients, the chorionic villi were formed by trophoblastic cells without cellular atypia. Fibrin-hematic clots in tubal lumen, vascular congestion, and edema were also frequently observed. The epithelium from remaining segments exhibited a slight hyperplasia with cellular features similar to those of the controls (Fig. 1G).

Expression of UG

The localization of UG in the human fallopian tube was carried out with immunocytochemistry at light microscope level. All tubal samples showed a positive immunoreactivity for UG, mainly in the oviductal epithelium. Both antihuman UG antibodies tested, i.e., from Dako and from Santa Cruz, exhibited similar immunostaining patterns.

In fallopian tubes from the control group, UG was present in secretory cells whereas interspersed ciliated cells were not immunoreactive (Fig. 1B). The UG was localized in the apical compartment and frequently in the lumen. Some isolated stromal cells were also stained. The distribution of UG-positive cells was variable: some zones of the epithelium were devoid of these cells, particularly in the proximal isthmic region, where the immunolabeling for UG was weaker than in the remaining tube. Compared with lung and endometrium controls, the tubal epithelium exhibited a lower UG expression. When specific anti-UG antibody was replaced by PBS or normal serum, no labeling was revealed (data not shown).

Increased expression of UG was detected in tubal epithelial cells from patients with chronic salpingitis (Figs. 1D and 2). Isthmic, as well as medial regions of the fallopian tube, showed a similar intense immunolabeling. Interestingly, fallopian tubes from patients with hydrosalpinx (group 2) also exhibited a strong UG immunostaining in the atrophic epithelial cells (Fig. 1F).

In patients with ectopic pregnancy, the UG expression in fallopian tubes was found in two well differentiated areas, namely, the implantation site and the rest of the epithelial fallopian tube. The epithelial UG immunostaining at the site of implantation was comparable with that in normal tubes.

In contrast, a stronger staining occurred in the epithelium of remaining segments (Fig. 1H). As shown in Figure 2, the highest expression of tubal UG was found in this group in the area surrounding the implantation site.

DISCUSSION

Most of the studies dealing with human uteroglobin have concentrated mainly on the lung (11–13) and endometrium (14, 15). In the field of reproductive biology, UG has been shown to be involved in blastocyst formation, expansion, and/or attachment, by its detection in blastocyst fluid as well as in the uterine milieu (5). Moreover, in the rabbit, UG has been suggested to be a valuable factor implicated in the endometrial preparation for implantation owing to the high levels found at the midsecretory phase controlled by progesterone (4–6). Although UG was found previously in rabbit fallopian tube (16), data presented here are the first to show UG expression in the human fallopian tube from normal patients, as well as from those with different tubal pathologic conditions.

The fallopian tube is the main predicted site for fecundation. Therefore, proteins generated by oviductal epithelial cells are proposed to be responsible for creating an appropriate microenvironment that facilitates gamete functions, fertilization, and early embryo development (1). The UG immunostaining of the apical tubal epithelium reported in the present work suggests that UG could be released via apical extrusion into the luminal compartment, as occurs in the endometrium (17), and as a result be incorporated into tubal secretions. Although the specific physiologic role of UG remains largely unknown, several researchers have hypothesized that UG might act as a cytokine with potent immunosuppressive actions (8).

It has been reported that spermatozoa and tubal epithelial cells have a paracrine interaction that affects the function of both cell types. Moreover, sperm-to-epithelial cell contact seems to be a crucial step in the capacitation of human spermatozoa in the fallopian tube (18). Because UG may exert its immunosuppressive effects by masking the cell surface antigens of the male gametes (10), the secretion of this protein by oviductal cells could be a key factor supporting a permissive milieu, necessary for the physical contact between the tubal epithelium and spermatozoa required for successful capacitation. Once the embryo is developed, tubal UG could also be responsible for immunologic protection during its journey throughout the fallopian tube.

Steroid hormones have been described as major regulators of the UG gene. In the female reproductive tract, UG is modulated by ovarian estrogen and progesterone (19, 20). However, cytokines involved in the modulation of the inflammatory response, such as interferon (IFN) γ (21), tumor necrosis factor α (22), and interleukin (IL) 4 (23), also increased the UG expression in human bronchiolar cells. Because *Chlamydia trachomatis*, the main cause of salpingitis and hydrosalpinx, induced the release of high levels of IFN-γ (24), it seems likely that cytokines involved in tubal inflammation could also increase the UG secretion by oviductal cells, probably as a modulating mechanism of the inflammatory responses in the human fallopian tube.

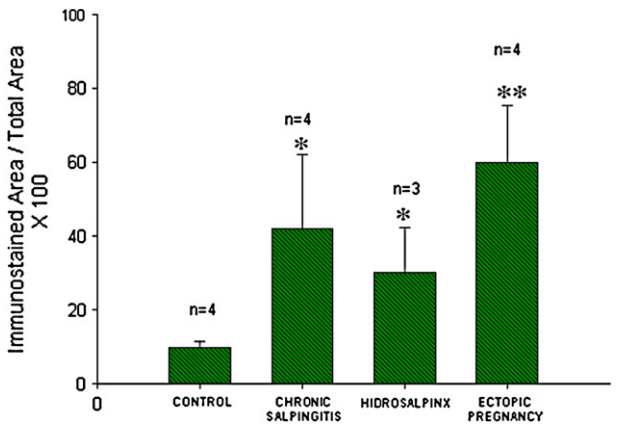
Luconi et al. (25) reported that UG exerts a "decapacitating factor" of seminal plasma, i.e., reducing sperm motility and responsiveness to progesterone. Accordingly, abnormally elevated levels of UG from an inflamed fallopian tube might also compromise negatively the sperm functions, thus inhibiting the fertilizing ability of spermatozoa. This hypothesis is in agreement with the high incidence of infertility observed in patients with salpingitis and PID (26, 27), and with the increased endometrial UG associated with *Chlamydia trachomatis* infections in infertile patients (unpublished observations).

Tubal ectopic pregnancy is usually a result of an abnormal tubal patency, and frequently a sequel to one or more infections (3). Consequently, PID has long been accredited to be the chief risk factor for ectopic pregnancy (28). In both the salpingitis and the hydrosalpinx studied here, there was an increased immunoexpression of tubal UG, reinforcing the hypothesis that inflammatory responses in the fallopian tube are essentially dampened by immunosuppressive factors (29) but unfortunately lead to persistent infections (30). In addition to classic concepts, such as the conditions that prevent or retard migration of the fertilized ovum to the uterus, another possible factor that may contribute to tubal pregnancy could be the immunosuppressive status produced by the increased levels of tubal UG.

In the oviductal epithelium from patients with tubal ectopic pregnancy, we found an interesting differential

FIGURE 2

Expression of uteroglobin in tubal epithelium analyzed by immunocytochemistry and semiquantified using densitometry. Values represent averages ± SEM. Significant values as determined by analysis of variance relative to control: *$P<.05$; **$P<.01$.



Quintar. Uteroglobin in the human fallopian tube. Fertil Steril 2008.

expression of UG. At the site of implantation, the UG increased slightly, in contrast to the sharp rise occurring in the surrounding implantation site and in the rest of the tube. The process of embryo implantation and placentation requires a complex signaling mechanism which shares characteristics with the immune response, i.e., cytokines are involved in both processes. Consequently, increased UG expression could be attributed, among others, to IFN-γ and IL-4, normally present at the embryo-maternal interface (6). On the other hand, the relatively low immunoreactivity of UG at the implantation site suggests that the local expression of this protein is regulated by several signals from the trophoblast.

The present results demonstrate the presence of UG as a secretory protein from epithelial cells of the human fallopian tube. The high expression of UG in patients with PID and ectopic pregnancy suggests a possible increased immunosuppressive response that would contribute to an abnormal improved receptivity for ectopic embryo implantation in fallopian tubes.

Acknowledgments: The authors gratefully acknowledge the excellent technical assistance of Cristian Giacomelli and Lucia Artino. Thanks are also due to Team-18 for their constant advice. The authors thank Dr. Luque from the LETH (Santa Fé, Argentina) for assistance with immunostaining and Dr. Paul Hobson for revision of the manuscript.

REFERENCES

1. Killian GJ. Evidence for the role of oviduct secretions in sperm function, fertilization and embryo development. Anim Reprod Sci 2004;82–83: 141–53.
2. Dabekausen YA, Evers JL, Land JA, Stals FS. *Chlamydia trachomatis* antibody testing is more accurate than hysterosalpingography in predicting tubal factor infertility. Fertil Steril 1994;61:833–7.
3. Hunter RH. Tubal ectopic pregnancy: a patho-physiological explanation involving endometriosis. Hum Reprod 2002;17:1688–91.
4. Beier HM. Uteroglobin: a hormone-sensitive endometrial protein involved in blastocyst development. Biochim Biophys Acta 1968;160: 289–91.
5. Beier HM. The discovery of uteroglobin and its significance for reproductive biology and endocrinology. Ann N Y Acad Sci 2000;923:9–24.
6. Herrler A, von Rango U, Beier HM. Embryo-maternal signalling: how the embryo starts talking to its mother to accomplish implantation. Reprod Biomed Online 2003;6:244–56.
7. Klug J, Beier HM, Bernard A, Chilton BS, Fleming TP, Lehrer RI, et al. Uteroglobin/Clara cell 10-kDa family of proteins: nomenclature committee report. Ann N Y Acad Sci 2000;923:348–54.
8. Mukherjee AB, Kundu GC, Mantile-Selvaggi G, Yuan CJ, Mandal AK, Chattopadhyay S, et al. Uteroglobin: a novel cytokine? Cell Mol Life Sci 1999;55:771–87.
9. Levin SW, Butler JD, Schumacher UK, Wightman PD, Mukherjee AB. Uteroglobin inhibits phospholipase A2 activity. Life Sci 1986;38: 1813–9.
10. Mukherjee DC, Agrawal AK, Manjunath R, Mukherjee AB. Suppression of epididymal sperm antigenicity in the rabbit by uteroglobin and transglutaminase in vitro. Science 1983;219:989–91.
11. Singh G, Katyal SL. Clara cell proteins. Ann N Y Acad Sci 2000;923: 43–58.
12. Ryerse JS, Hoffmann JW, Mahmoud S, Nagel BA, deMello DE. Immunolocalization of CC10 in Clara cells in mouse and human lung. Histochem Cell Biol 2001;115:325–32.
13. Dhanireddy R, Kikukawa T, Mukherjee AB. Detection of a rabbit uteroglobin-like protein in human neonatal tracheobronchial washings. Biochem Biophys Res Commun 1988;152:1447–54.
14. Muller-Schottle F, Classen-Linke I, Alfer J, Krusche C, Beier-Hellwig K, Sterzik K, et al. Expression of uteroglobin in the human endometrium. Mol Hum Reprod 1999;5:1155–61.
15. Cowan BD, North DH, Whitworth NS, Fujita R, Shumacher EK, Mukherjee AB. Identification of a uteroglobin-like antigen in human uterine washings. Fertil Steril 1986;45:820–3.
16. Gonzalez M, Garcia C, Nieto A. Regional differences in uteroglobin biosynthesis along the rabbit oviduct: immunohistochemical and biochemical studies. Histochem J 1996;28:209–15.
17. de la Torre J, Lopez de Haro MS, Nieto A. Ultrastructural and kinetic studies on uteroglobin secretion in the uterus and oviduct of the pseudopregnant rabbit. Histochem J 1987;19:572–8.
18. Kervancioglu ME, Saridogan E, Aitken RJ, Djahanbakhch O. Importance of sperm-to-epithelial cell contact for the capacitation of human spermatozoa in fallopian tube epithelial cell cocultures. Fertil Steril 2000;74:780–4.
19. Shen X, Tsai M, Bullock D, Woo S. Hormonal regulation of rabbit uteroglobin gene transcription. Endocrinology 1983;112:871–6.
20. Miele L, Cordella-Miele E, Mukherjee AB. Uteroglobin: structure, molecular biology, and new perspectives on its function as a phospholipase A2 inhibitor. Endocr Rev 1987;8:474–90.
21. Yao XL, Ikezono T, Cowan M, Logun C, Angus CW, Shelhamer JH. Interferon-gamma stimulates human Clara cell secretory protein production by human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 1998;274:L864–9.
22. Yao XL, Levine SJ, Cowan MJ, Logun C, Shelhamer JH. Tumor necrosis factor-alpha stimulates human clara cell secretory protein production by human airway epithelial cells. Am J Respir Cell Mol Biol 1998;19: 629–35.
23. Jain-Vora S, Wert SE, Temann U-A, Rankin JA, Whitsett JA. Interleukin-4 alters epithelial cell differentiation and surfactant homeostasis in the postnatal mouse lung. Am J Respir Cell Mol Biol 1997;17:541–51.
24. Reddy BS, Rastogi S, Das B, Salhan S, Verma S, Mittal A. Cytokine expression pattern in the genital tract of *Chlamydia trachomatis* positive infertile women—implication for T-cell responses. Clin Exp Immunol 2004;137:552–8.
25. Luconi M, Muratori M, Maggi M, Pecchioli P, Peri A, Mancini M, et al. Uteroglobin and transglutaminase modulate human sperm functions. J Androl 2000;21:676–88.
26. Patton DL, Askienazy-Elbhar M, Henry-Suchet J, Campbell LA, Cappuccio A, Tannous W, et al. Detection of *Chlamydia trachomatis* in fallopian tube tissue in women with postinfectious tubal infertility. Am J Obstet Gynecol 1994;171:95–101.
27. Henry-Suchet J, Utzmann C, De Brux J, Ardoin P, Catalan F. Microbiologic study of chronic inflammation associated with tubal factor infertility: role of *Chlamydia trachomatis*. Fertil Steril 1987;47:274–7.
28. Bakken IJ, Skjeldestad FE, Nordbo SA. *Chlamydia trachomatis* infections increase the risk for ectopic pregnancy: a population-based, nested case-control study. Sex Transm Dis 2006.
29. Cardenas H, Corvalan L, Imarai M. Is there a mucosal immune system associated with the mammalian oviduct? Biol Res 1998;31:329–38.
30. Ness RB. The consequences for human reproduction of a robust inflammatory response. Q Rev Biol 2004;79:383–93.