

RESEARCH ARTICLE

OVGP1 Expression in BOEC and Oviduct: An Immunohistochemical and Immunocytochemical Study ^{[1][2]}

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Abstract

Oviduct is an important tubular organ fostering critical physiological processes such as transport of gametes and embryos, capacitation, fertilization, early embryo development, and maturation of gametes. The aim of the present study was to evaluate oviduct specific glycoprotein-1 (OVGP1) expression in the oviduct regions at different phases of the sexual cycle and bovine oviduct epithelial cells (BOEC). In the study, oviduct samples collected from 6 cows in estral and luteal phases were used. The oviduct samples were collected from the ampulla, isthmus and fimbria and evaluated through routine histology and immunohistochemical studies for OVGP1. The primary BOEC were obtained from the ampulla region and characterized by cytokeratin expression. The immunohistochemistry assay indicated that OVGP1 is expressed in secretory cells of the bovine oviduct. OVGP1 expression varies by the oviduct regions and phases of the sexual cycle. Changes in OVGP1 expression during the sexual cycle suggestively indicates a hormonal influence. Regional difference in OVGP1 expression is most likely related to the physiological events that occur in different regions of the oviduct. BOEC isolated from the oviduct of estral and luteal phases also expresses OVGP1. Further studies should focus on possible role of OVGP1 in adaption of BOEC to very tedious condition like cell culture.

Keywords: BOEC, OVGP1, Oviduct, Cow, Sexual cycle

BOEC ve Ovidukt'ta OVGP1 Ekspresyonu: İmmunohistokimyasal ve İmmunositokimyasal Çalışma

Öz

Ovidukt, gametlerin olgunlaşması, erken dönemde embriyo gelişimi fertilizasyon, kapasitasyon embriyo ve gametlerin taşınması gibi kritik fizyolojik süreçleri destekleyen önemli bir tubuler organdır. Bu çalışmanın amacı, seksüel siklusun farklı evrelerinde oviduktun bölgelerinde ve siğir ovidukt epitel hücrelerinde (BOEC) oviduktta özgü glikoprotein-1 (OVGP1) ekspresyonunu değerlendirmektir. Bu çalışmada östral ve luteal dönemdeki 6'şar inekten alınan ovidukt örnekleri kullanıldı. Oviduktun ampulla, istmus ve fimbriya bölgelerinden alınan örneklerde, OVGP1 immünohistokimyasal çalışmalarla değerlendirildi. BOEC ampulla bölgesinden elde edildi ve sitokeratin ekspresyonu ile karakterize edildi. İmmünohistokimya ile, OVGP1'in siğir oviduktunda özellikle sekretorik hücrelerde bulunduğu gösterildi. OVGP1 ekspresyonu, oviduktun bölgelerine ve seksüel siklusun dönemlerine göre değişmektedir. Seksüel siklus sırasında OVGP1 ekspresyonundaki değişiklikler muhtemel bir hormonal bir etkiyi düşündürür. OVGP1 ekspresyonundaki bölgesel farklılık, büyük olasılıkla yumurta kanalının farklı bölgelerinde meydana gelen fizyolojik olaylarla ilgilidir. Estral ve luteal dönemlerde oviduktan izole edilen BOEC'te OVGP1'i ekspresse etmektedir. Bundan sonraki çalışmalar, OVGP1'in BOEC'in zorlu hücre kültürü şartlarına, adaptasyonundaki rolüne odaklanmalıdır.

Anahtar sözcükler: BOEC, OVGP1, Oviduk, Siğir, Seksüel siklus

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INTRODUCTION

Oviduct is a tubular organ and known as the fertilization site, which provides optimal conditions for sperm capacitation, fertilization and early embryonic development as a consequence connection between oocyte and spermatozoon coming in opposite directions ^[1]. Oviduct is divided into three parts: fimbria (infundibulum), ampulla and isthmus ^[2,3]. The oviduct epithelial lining consists of ciliated and secretory cells, the proportions and morphologies of which change during the sexual cycle ^[4,5]. Following cell culturing, the morphological properties of these cells are defined electron microscopically and immunohistochemically ^[6]. Secretory activities of the secretory cells are important for gametes and embryos. It is also reported that the secretory modalities of these cells differ by the phases of the cycle ^[7]. The oviduct is under the influence of ovarian hormones (estrogen and progesterone). Estrogen is undoubtedly active in the differentiation of the epithelium during the follicular (estral) phase and the addition of various macromolecules to the development and secretion of secretory cells ^[8]. Impairment of oviduct function or deficiency may result in infertility or develop conditions such as ectopic pregnancy with vital consequences ^[9].

Oviduct-specific glycoproteins have been reported to have positive effects on sperm capacitation, sperm-ovum binding, ovum penetration and embryo development *in vitro* ^[10]. It has been suggested that they contribute to sperm viability and motility as well as sperm capacitation ^[11]. Oviduct-specific glycoproteins have been reported to increase in the estral phase ^[8-12]. One of them is oviduct glycoprotein 1 (OVGP1). OVGP1, also known as oviductin in some species, is a high molecular weight protein similar to chitinase. Synthesized in secretory cells of the oviduct during estrous cycle ^[13], OVGP1 has been reported to have positive effects on sperm capacitation, ovum-sperm binding, sperm penetration to the ovum ^[14] and prevention of polyspermia ^[15] and early embryonic development ^[14]. In a study conducted in buffaloes, both recombinant and natural buffalo OVGP1 had a significant effect on sperm characteristics and *in vitro* embryo development ^[16]. It has been proposed that as an embryotrophic protein OVGP1 causes molecular changes in the zona pellucida of oocyte, especially during fertilization ^[15]. Oviduct-specific glycoprotein 1 is reported to increase monospermia in pigs in addition to its embryotrophic activities ^[17,18].

In vitro fertilization and embryo production have been an important tool in determining the effects of oviduct secretions in the presence of gametes, fertilization and embryo development ^[10]. Early embryonic deaths is one of the well-known fertility problems especially in cattle that causes significant economic losses ^[19]. Assisted reproductive techniques have provided a very useful tool for studying early embryonic development. Examining the relationship between the mother and the embryo

or gametes is very valuable in terms of fertilization and early embryonic deaths. However, *in vivo* studies are particularly expensive in cattle ^[20]. In this regard, *in vitro* systems represent an alternative tool for explaining *in vivo* pathways and mechanisms, especially in farm animals ^[21]. The low success rate in the production of bovine embryos using *in vitro* techniques has led researchers to examine *in vitro* conditions in more detail. Various somatic cells were used as co-cultures in bovine embryos. Bovine oviduct epithelial cells (BOEC) are the most popular choice among them ^[22]. Thus, there is a continuous effort to characterize BOEC.

In this study, we aimed to investigate the OVGP1 expression in different parts of the oviduct tissues during estral and luteal phases as well as in BOEC.

MATERIAL AND METHODS

BOEC Isolation

For BOEC cultures, the oviduct samples were collected from 6 estral and 6 luteal cows slaughtered in a regional slaughterhouse. To determine the phase of the sexual cycle in a slaughtered animal, both ovaries were macroscopically examined ^[23]. The ampulla region of the oviduct was separated and brought to the laboratory in sterile conditions in a transport solution, DMEM (Dulbecco's Modified Eagle Medium) medium containing penicillin-streptomycin and Fetal Bovine Serum (FBS). In a laminar flow cabin, the ampulla region was cleaned from the surrounding tissues, the oviduct lumen was cut open, and then the epithelial tissue was scraped mechanically into a flask. The scrapped epithelial tissue was gently homogenized in DMEM containing 10% FBS, 1% Penicillin-Streptomycin and transferred into sterile chambered coverslip with 6-wells and then incubated at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium was changed every 2-3 days. Cell cultures exhibiting a 90-95% confluence were used for immunocytochemistry. Furthermore, BOEC were characterized by cytokeratin expression based on their epithelial origin ^[24].

Immunocytochemistry for Cytokeratin-5 and OVGP-1 in BOEC

Cytokeratin-5 and OVGP1 immunocytochemistry procedures were conducted using the routine immunocytochemistry. The cells on the cell culture coverslip were fixed in 4% paraformaldehyde for 30 min. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide solution prepared in methanol. Upon treating cells with normal goat serum to prevent nonspecific binding, either a monoclonal cytokeratin-5 antiserum (ab194135 at a dilution of 1/200) or a monoclonal anti-OVGP1 antibody (ab118590, 1 µg/mL) were applied to cells and then treated with a horse radish peroxidase (HRP) labeled secondary antibody (Thermo Scientific TP-125-HL) followed by AEC

chromogen (Thermo Scientific TA-125-HA) incubation, and then covered with water-based adhesive (Abcam Mounting Medium ab64230).

OVGP1 Immunohistochemistry in Oviduct Tissues

The bovine oviduct samples were collected during slaughter. The oviducts were then separated into three parts: ampulla, isthmus and fimbria and processed for routine immunohistochemistry. For antigen retrieval, sections were incubated in citrate buffer for 4 times with an interval of 5 min at 700-800 W in a microwave oven. Sections were placed in 0.3% triton-X in PBS for permeabilization for 15 min. Endogenous peroxidase activity was quenched in 3% H₂O₂ for 20 min. Following blocking solution steps, sections were incubated with the primary antibody (anti-OVGP1 antibody ab118590, 5 µg/mL) for an hour, biotinylated secondary antibody (Thermo Scientific TP-125-HL) and streptavidin peroxidase solution for 30 min each at room temperature. Sections were then treated with chromogen (Thermo Scientific TA-125-HA), counterstained with Gill's hematoxylin for 20 sec, washed in tap water and then coverslipped using a water based mounting solution (Abcam Mounting Medium ab64230). Sections were examined using a light microscope (Nicon Eclipsi 50i, Tokyo, JAPAN). The intensity and localization of OVGP1 expressions

were assessed with a semiquantitative scoring system: (-) negative, (+) weak, (++) moderate, (+++) strong [25].

RESULTS

Immunocytochemistry for Cytokeratin-5 and OVGP-1 in BOEC OVGP-1 Immunohistochemistry in Oviduct Tissues

The cultured BOEC cells were cytokeratin immunopositive to a significant degree in both estral and luteal phases (Fig. 1-A,B). The OVGP1 expression was observed in some BOEC cells in both phases of the sexual cycle (Fig. 2-A,B).

Immunohistochemistry for OVGP1

In oviduct samples, OVGP1 immunoreactivity was observed in the epithelial lining during both phases of sexual cycle. It has been determined that OVGP1 immunoreactivity was limited to the secretory cells as ciliated cells expressed no immunoreactivity. The immunoreactivity was observed especially in the fimbria and ampulla of the oviduct. In samples of the luteal phase, the immunoreactivity was common in the cytoplasm of secretory cells in all oviduct regions (Fig. 3-A,B,C,D,E), except in the isthmus (Fig. 3-F). The cytoplasmic immunoreactivity was especially seen at the apical site of the cells and there even were immuno-

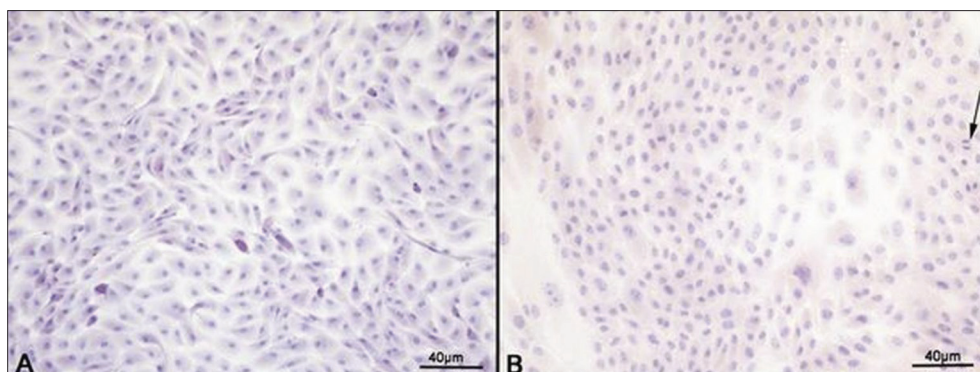


Fig 1. Cytokeratin immunohistochemistry in BOEC cell culture. Cytokeratin immunoreactivity was observed in both estral (A) and luteal (B) phases. Immunoperoxidase staining, hematoxylin counterstaining

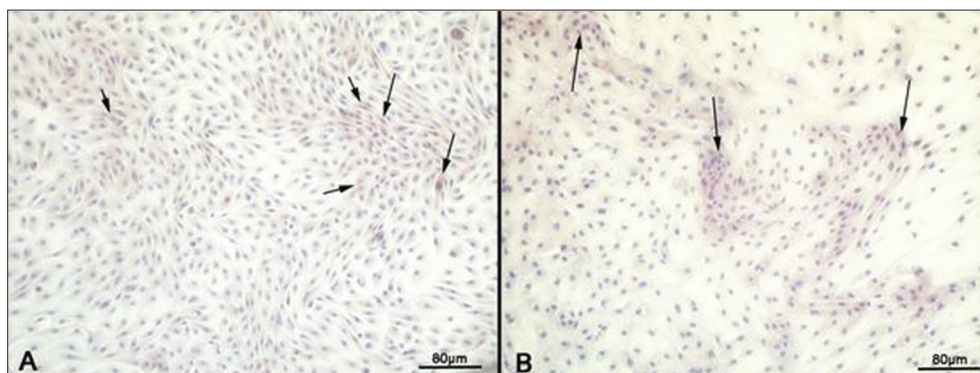


Fig 2. OVGP-1 immunocytochemistry in BOEC. The OVGP-1 immunoreactivity (arrows) was seen in some cells of both the luteal (A) and estral (B) phases of the sexual cycle. Immunoperoxidase staining, hematoxylin counter staining

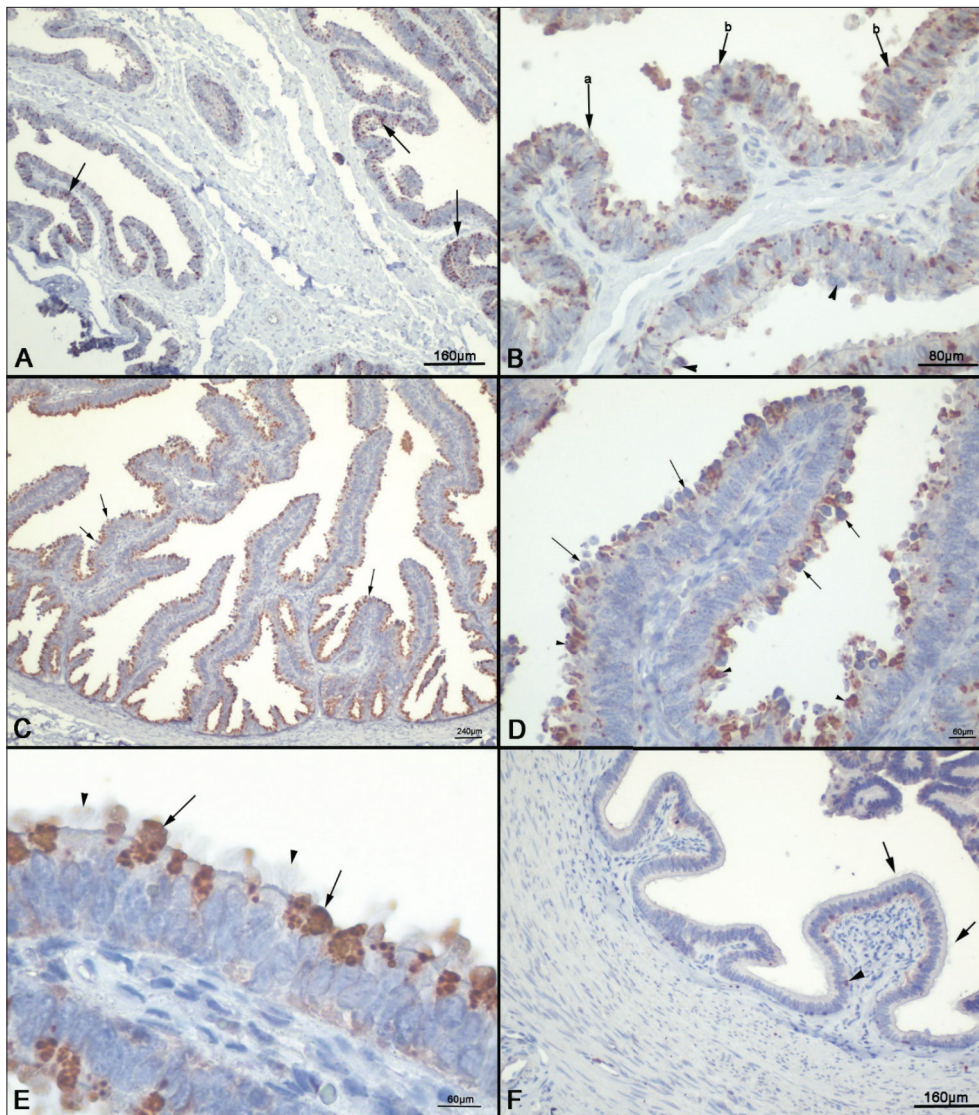


Fig 3. OVGP1 immunoreactivity in the fimbria, ampulla and isthmus regions of the bovine oviduct at the luteal phase. A) In the fimbria, immunoreactivity (arrow) was present in the epithelial lining, B) Ciliated cells are immunonegative (a) while secretory cells are immune positive (b). Nuclear extrusion (arrowheads) can also be seen. C) Immunoreactivity (arrows) is also present in the ampulla in a similar manner, D) In the ampulla at the late luteal phase, the immunoreactivity (arrowheads) is localized in the apical sites of the secretory cells, characterized by numerous nucleus extrusions (arrows), E) Immunoreactive secretory cells (arrows) can be seen, F) Immunoreactivity was usually absent in the epithelial lining (arrows); however, it can rarely be seen in a very limited number of cells. Immunoperoxidase staining and hematoxylin counterstain

reactive secretory protrusions towards the lumen along with the nucleus (Fig. 3-E). In the samples of the estral phase, OVGP-1 immunoreactivity distribution was similar to those the luteal phase (Fig. 4-A,B,C,D,E); however, there was a limited immunoreactivity in a very short region of the isthmus, neighboring to the ampulla (Fig. 4-F). The intensity of immunoreactivity by regions and periods of the sexual cycle is given in Table 1.

DISCUSSION

Oviduct is a dynamic organ and influenced by the sexual cycle. It undergoes through some physiological and biochemical changes while creating an optimal micro-

environment required for the early phase of the embryo cleavage, fertilization and ovulation. These changes are hormonally controlled by ovarian steroids, especially estrogen^[26] as the oviduct expresses hormone receptors that were also up/down regulated during the sexual cycle^[27] Oviduct epithelial cells and oviduct fluid originating from blood plasma contains various proteins and enzymes. These are of great importance for the maturation of gametes, fertilization and embryo development in the oviduct^[24]. Cattle embryos are in close contact with the oviduct fluid in the first 4-5 days of pregnancy^[28]. It has been reported that the effects of oviduct fluid on gamete functions and embryo development vary according to the cycle phase and region of the oviduct^[10]. Boice et

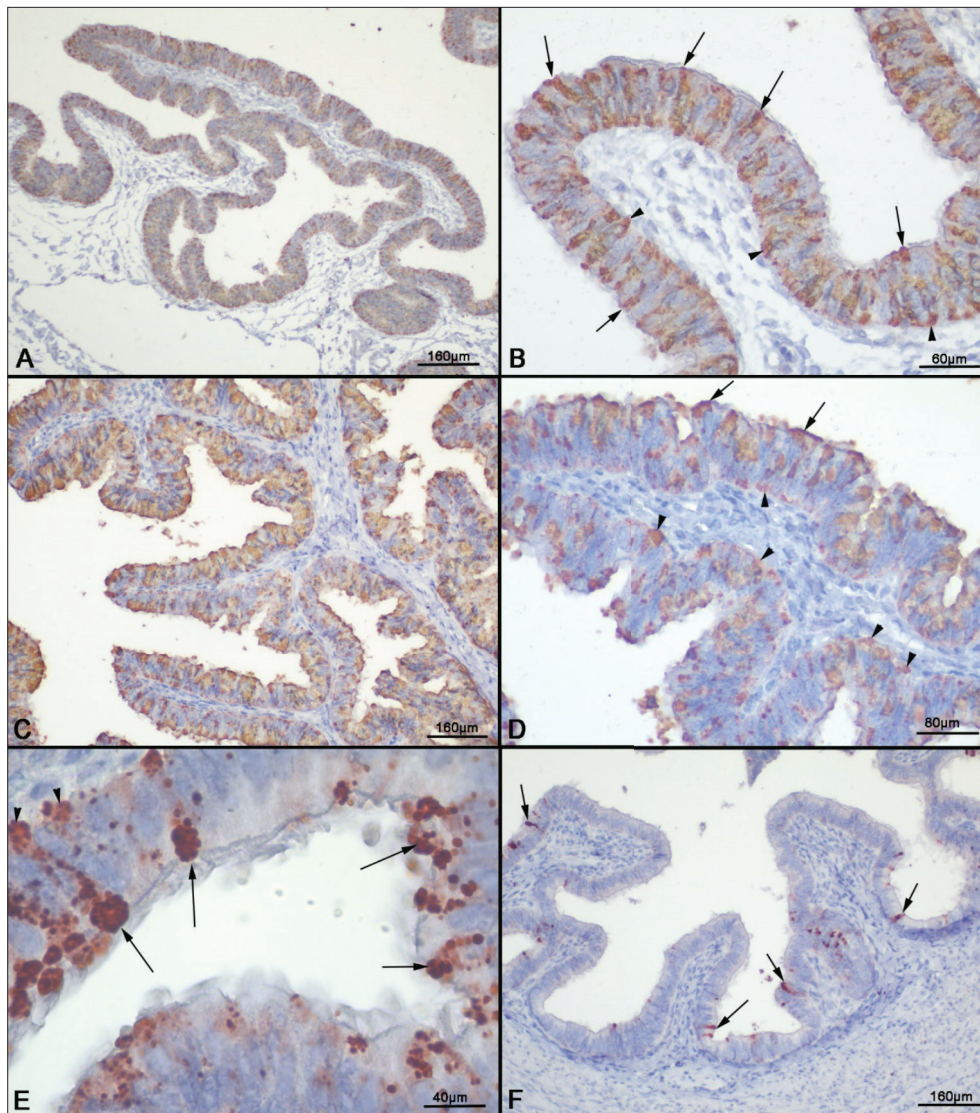


Fig 4. OVGP1 immunoreactivity in fimbria, ampulla and isthmus regions at the estral phase. **A)** Immunoreactivity is present in epithelial lining of the fimbria, **B)** Immunoreactivity can be seen not only in the apical region (*arrows*) but also in the basal region (*arrowheads*), **C-D)** Similarly, immunoreactivity is seen in secretory cells (*arrow*) of the epithelial layer of the ampulla, filling the whole cytoplasm (*arrowheads*), **E)** In addition, the apical (*arrows*) and basal sites (*arrowheads*) of the secretory cells of the ampulla had intense immunoreactivity, **F)** In the isthmus, immunoreactivity (*arrows*) is very limited to the regions adjacent to the ampulla. Immunoperoxidase staining, hematoxylin counterstaining

al.^[29] reported that a group of oviduct-specific glycoproteins including OVGP1 synthesized from bovine oviduct epithelium are present in the oviduct fluid, especially during fertilization. They also reported that these glycoproteins are oviduct specific, not present in other reproductive organs^[29]. OVGP1 is reported to be intensely secreted from secretory cells in cattle, especially during the phase of estrogen predominance^[30] and supports fertilization and embryonic development^[9]. It has also been suggested that OVGP1 prevents polyspermy by preserving the zona pellucida stability^[31]. In support to previous studies, the present study also found the OVGP1 immunoreactivity in bovine oviduct epithelial cells, specifically in the secretory cells. Therefore, the present study strongly supports the

OVGP1 involvement in various physiological processes in the oviduct during the sexual cycle. The sexual cycle is a continuous and repeating dynamic process, and the oviduct is one of the organ sites influenced by the cycle^[10]. Likewise, expression of secreted molecules by the oviduct cells can be influenced as a consequence of hormonal changes^[32]. Abe et al.^[33] reported that oviduct-specific glycoproteins expressions were concentrated in the follicular phase, but the expressions were weaker in the luteal phase. The present study also found that OVGP1 immunoreactivity exhibited some regional and periodical variation. The OVGP1 immunoreactivity was intense especially in samples of the estral phase, especially in the fimbria and ampulla, and it was weaker in samples of the

Table 1. Intensity distribution of OVGP-1 immunoreactivity in the oviduct by regions and phases of the sexual cycle

Oviduct Region	The Phase of the Sexual Cycle			
	Luteal		Oestral	
	Ciliated Cell	Secretory Cell	Ciliated Cell	Secretory Cell
Fimbria	-	+	-	++
Ampulla	-	+	-	+++
Isthmus	-	-	-	-

Semiquantitative scoring of immunostaining intensity; - negative, + weak, ++ moderate, +++ strong

luteal phase. There was also some difference in the mode of expression. The immunoreactivity was seen throughout the cytoplasm of the secretory cells in samples of the estral phase while it was generally limited to the apical site of the same cells in the samples of the luteal phase. Furthermore, there were immunoreactive protrusions at the luteal phase, suggesting secretory cells are emptying OVGP1 along with nucleus and other substances. The Abe et al.^[33] study also found a regional variation for OVGP1 immunoreactivity such that the immunoreactivity was intense in the fimbria and ampulla of the estral phases, but weaker in the isthmus of both phases. The present study found also found some regional difference by means OVGP1 immunoreactivity, similar to Abe et al.^[33] study with some differences such that the immunoreactivity was absent in the isthmus of the luteal phase, but present at the estral phase, especially in the isthmus sites near the ampulla. Thus, not only the Abe et al.^[33] study but also the present study present suggest that synthesis of OVGP1 by secretory cells changes regionally and periodically. In particular, the OVGP1 immunoreactivity is intense in samples of the estral phase, during which estrogen is the dominant sexual hormone. Thus, further studies should focus on how sexual hormones affect gene expression, proteome and secretion in the oviduct epithelium by managing a series of changes through genomic or non-genomic means. As a general concept, not specifically for OVGP1, such a notion has been previously argued by Coy et al.^[34] for the oviduct epithelial cells.

In vitro methods of fertilization and embryo production provide a very favorable experimental environment for investigation of biological effects of oviduct secretions on gametes, fertilization and embryo development^[10]. In particular, the oviduct and uterine epithelial cell cultures have gained importance in terms of evaluating the paracrine effects^[35]. Short-term BOEC culture systems are instruments for studies examining embryo-mother interaction by mimicking the *in vivo* oviduct environment^[36]. Co-culture of embryo with BOEC is a method to support the development of bovine embryos derived from IVM/IVF procedures. In this concept, BOEC have been reported to stimulate embryos to produce IFNT, which is in turn influence immune cells to promote an anti-inflammatory response in the oviduct^[37]. In this respect, BOEC creates

a favorable environment for embryonic development^[38]. In the present study, the presence of OVGP1 expression in some BOEC cells suggests that OVGP1 may contribute to the adaptation of BOEC to *in vitro* environments or cell survival.

The OVGP1 expression in the oviduct is influenced by hormonal changes. Progesterone addition suppresses the OVGP1 transcription in BOEC^[5]. Expression of oviductin increases in human mucosal cells in response to estrogen *in vitro*^[39]. In the present study, cytoplasmic expression of OVGP1 in secretory cells is more prominent and present throughout cytoplasm in samples of the estral phase. OVGP1 immunoreactivity in the luteal phase was limited to the apical site of the secretory cells. Furthermore, secretory cells seemed to empty OVGP1 probably along with other others. Thus, our study also suggests that OVGP1 expression is the highest in estrogen predominance.

In BOEC culture, cytokeratin expression is generally evaluated for fibroblast contamination and BOEC purity or ratio^[24]. Similarly, we evaluated cytokeratin immun-expression in the present study which resulted in intensive positive immune reaction in BOEC cultures of both phases. The appearance of cytokeratin expression without any addition of hormones is consisted with the findings of Comer et al.^[40].

It has been proposed that exosomes and microvesicles found in the oviduct fluid may module the relationship between embryo and mother^[41]. The researchers claimed that exosomes were the key components of the oviduct secretion *in vitro* and *in vivo*. The exosomes contained OVGP1 *in vivo*. In the present study, secretory cells expressed strong immunoreactivity at the apical site especially at the estral period. Thus, a further study may focus on presence of OVGP1 exosomes and their extrusions from the cells.

As a result, OVGP1 is expressed in secretory cells of the bovine oviduct. It varies by regions and phases of the sexual cycle. Changes in OVGP1 expression during the sexual cycle suggestively indicates a hormonal influence. Regional difference in OVGP1 expression is most likely related to the physiological events that occur in different regions of the oviduct. BOEC isolated from the oviduct

of estral and luteal phases also expresses OVGP1. Further studies should focus on a possible involvement of OVGP1 in BOEC adaption to very tedious condition like cell culture.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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AUTHOR CONTRIBUTIONS

The design of the study and evaluation of the results were executed by the contribution of A. KÜRÜM, S. KARAHAN, H. KOCAMIŞ and M. TÜRK. The oviduct samples were collected by A. KÜRÜM and Y. ÖZKABADAYI. Cell culture studies were implemented by M. TÜRK, A. KÜRÜM and Y. ÖZKABADAYI. The Immunohistochemistry and immunocytochemistry studies were executed by A. KÜRÜM, S. KARAHAN, H. KOCAMIŞ and Y. ÖZKABADAYI. All authors also contributed to the preparation of the manuscript.

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