ENDOMETRIAL CELLS IN A PRIMARY CULTURE MODEL – PHYSIOLOGICAL AND MOLECULAR ASPECTS

KOMÓRKI BŁONY ŚLUZOWEJ MACICY W HODOWLI PIERWOTNEJ – ASPEKTY FIZJOLOGICZNE I MOLEKULARNE

Wiesława KRANC[1], Adrian CHACHUŁA[2], Sylwia CIESIÓŁKA[2], Sylwia BORYS[1], Dominika KAŹMIERCZAK[2], Maciej JANKOWSKI[3], Małgorzata BRUSKA[1], Bartosz KEMPISTY[1,2]

[1]Department of Anatomy,
[2]Department of Histology and Embryology,
Medicine Faculty II, Poznan University of Medical Sciences
[3]Department of Oncology and Chemiotherapy, Piła

Summary: Mammalian endometrial tissue is permanently modified and undergoes several morphological, biochemical, and molecular modifications. The growth and differentiation of the endometrium is significantly regulated by the secretion of steroids, mainly sex hormones including progesterone (P4) and estradiol (E2). Additionally, these modifications lead to preparation of the tissue, which is highly specialized both morphologically and functionally. The main function of the endometrium, located within the uterus, is embryo implantation, a highly coordinated process that is orchestrated by several complex mechanisms. It has been suggested that endometrial receptivity and/or sensitivity is dependent on aquaporin (AQP) and connexin (Cx) expression. Moreover, both of these protein families are involved in endometrial pathogenesis, which is related to their function as ions and water micro-channels.

It is possible that the development of the primary culture model of endometrial tissue can be a basis on which to focus new physiological aspects of cellular growth and/or differentiation. Additionally, these culture models may prove to be important in diagnosing uterine related diseases in mammals.

Key words: endometrium, fertilization, sperm-ovum interactions, embryo implantation

Streszczenie: Błona śluzowa macicy u ssaków podlega ciągłym modyfikacjom zarówno morfologicznym, biochemicznym, jak i molekularnym. Doświadczenia ostatnich lat dowodzą, że wz-

rost i różnicowanie się komórek endometrium są ściśle regulowane przez wydzielanie steroidów, a w szczególności hormonów płciowych jak progesteron (P4) oraz estradiol (E2). Wszystkie te modyfikacje prowadzą do uformowania w pełni wyspecjalizowanej morfologicznie i funkcjonalnie tkanki. Najważniejsza rola endometrium polega na umożliwieniu implantacji zarodka, która należy do wysoce złożonych i koordynowanych procesów. Ostatnie wyniki badań wykazały, że receptywność endometrium jest także uzależniona od ekspresji genów i białek z grupy akwapuryn (AQPs) i koneksyn (Cxs). Ponadto obie wspomniane rodziny białek są związane z niektórymi stanami patologicznymi endometrium, w których pełnią swoją funkcję, jako mikro-kanały dla jonów i wody.
Podsumowując, sugeruje się, że rozwój techniki pierwotnej hodowli komórek endometrium może stać się podstawą poznawania nowych aspektów wzrostu i różnicowania się tej tkanki oraz zyskać znaczenie w diagnostyce chorób macicy u ssaków.

Słowa kluczowe: błona śluzowa macicy, zapłodnienie, reakcja plemnik-komórka jajowa, implantacja zarodka

INTRODUCTION

The mammalian uterus is formed by the endometrium which is composed of three layers: luminal, glandular, and stromal epithelium. During the reproductive lifespan of females, the endometrial epithelium undergoes several morphological and molecular modifications [4, 9, 2]. The most important modifications include changes of endometrial epithelium during different stages of the oestrus cycle, preparation of the endometrium for proper embryo implantation, formation of the trophoblast, and fetal development [10]. The oestrus cycle and implantation are regulated by secretion of the hormones LH and FSH during ovulation and progesterone (P4), a hormone which change the structure and function of the endometrium. The moment when the blastocyst reaches the endometrium is considered the beginning of embryo implantation [6]. While the mechanisms concerning fetal growth have been previously described, the processes associated with embryo-luminal epithelium interactions are not entirely known. Moreover, the specific morphological changes and/or molecular-biochemical factors that trigger specific-site embryo implantation in the uterus are still unknown.

In this article, we present data that assess the embryo-maternal (blastocyst-endometrium) interaction. This data is based on the primary culture of luminal epithelial cells in relation to the proliferation index as measured during long-term, *in vitro* real-time proliferation. The expression of luminal epithelial cell markers such as cytokeratin 8 and 18 were also analyzed under the same conditions.

The expression, activity, and regulation of aquaporin (AQP) in the mammalian endometrium were recently investigated in relation to morphological changes and preparation of the endometrium prior to embryo implantation [30]. Since AQP build water channels, they may be involved in several processes associated

with the water-ion balance in endometrial tissue during physiological and pathological stages. Moreover, data presented here examines tissue specific expression and regulation of connexins (Cx) as alternate channels whose activity may be involved in the function and/or dysfunction of endometrial tissues.

MORPHOLOGICAL CHANGES WITHIN ENDOMETRIAL TISSUE DURING THE OESTRUS CYCLE

During the oestrus cycle, progesterone (P_4), oestrogen, and oxytocin induce functional and morphological changes of the endometrium in bovines [28]. The entire bovine oestrus cycle can be divided into four stages: preoestrus, oestrus, metoestrus, and dioestrus. At each stage, hormones are expressed at different levels, leading to various histological and functional endometrial modifications. At the oestrus stage, progesterone levels are low while oestrogen is elevated. This hormonal combination increases blood circulation, resulting in oedematisation of the mucosa and high contractility of the smooth muscles. The endometrial epithelium is made up of cuboidal cells consisting of ciliated and secretory cells, which are responsible for the production of oeastral mucus. Moreover, as an effect of endometrial mucosa oedema, the uterine glands are elongated as compared to their shape in the preoestrus stage. Oestradiol levels start to decrease before the surge of LH, leading to ovulation. After ovulation, throughout the three days of the metoestrus stage, the progesterone level increases due to the formation of a new corpus luteum; however, P4 and oestradiol levels in peripheral blood remain low. During the metoestrus stage, contractility of smooth muscles and mucosal oedema decreases. During the dioestrus stage, progesterone levels are elevated and oestradiol levels are low, thus epithelial cells take on a flat shape. If fertilization does not occur during the oestrus cycle, luteolytic pulses of prostaglandin F2α cause regression of the corpus luteum, resulting in a rapid decrease of progesterone during the preoestrus stage [10].

INDUCTION OF PATHWAYS IMPORTANT FOR ENDOMETRIAL TISSUE ACTIVITY

In recent years, several studies have examined the induction of endometrial molecular pathways during the oestrus cycle by analyzing gene expression of several species, e.g.: mouse, bovine, and rhesus monkey. [31, 5, 27]. Bauersachs et al. reported in 2005 that upregulation of mRNAs from different functional groups was predominant at the oestrus or dioestrus stage . During the oestrus stage, expression of mRNAs of ECM proteins and those involved in ECM remodeling was

highly elevated. This upregulation indicated changes in endometrial tissue composition and cytoskeletal alterations throughout the oestrus cycle. While ECM synthesis and remodeling were increased, genes responsible for protein folding and secretion were upregulated, e.g.: SERPINH1 (serpin peptidase inhibitor, clade H, member 1), a collagen-binding protein of the endoplasmic reticulum, which is involved in collagen secretion and processing within cells.

Furthermore, mRNAs expression of several IGF-binding proteins was also elevated. The IGF-binding proteins are involved in cell growth regulation, and they may be regulated by oestrogen, which itself mediates the expression of TGFB1 (transforming growth factor β1) and retinoic acid (RA) [26]. During complex remodeling and growth of the endometrial tissue, the process of angiogenesis becomes important in order to ensure sufficient uterine blood flow. This process is regulated by ANGPTL2 (angiopoietin-like 2; cluster 5). Moreover, in late-oestrus, expression of mRNAs which code proteins that negatively regulate proliferation and cell growth are elevated. It has been suggested that inhibitory protein expression is the sign of the end of proliferation of endometrial tissue. During dioestrus, the uterine fluid transport is regulated by different ion channel genes. Proteins and enzymes responsible for transport between endometrial cells are also upregulated. The elevated expression of many enzymes may be a sign of increased prostaglandin metabolism [10]. Furthermore, the mRNAs coding for ECM degradation proteases are upregulated while collagen mRNA levels are decreased. During the oestrus cycle, the TGF-β signaling pathway is the most prominent pathway, with 17 genes upregulated in the oestrus and dioestrus stage. Throughout the oestrus stage, proteins that inhibit TGF-β signaling are upregulated, and during the dioestrus stage, only three genes are involved in TGF-β signaling. At the late oestrus phase, when the oestrogen level is low, target genes of the TGF-β signaling pathway are upregulated while negative pathway regulators are also present. Another pathway important for endometrial tissue differentiation is the retinoic acid (RA) signaling pathway. Throughout the dioestrus stage, expression of proteins responsible for RA metabolism is elevated, while in the oestrus stage, expression of genes involved in tissue differentiation is higher.

EXPRESSION OF AQP AND ITS REGULATION IN THE ENDOMETRIUM

Aquaporins are a family of proteins which conduct water movement across cell membranes [1]. They are small, hydrophobic proteins with six transmembrane spanning domains that are integral to plasma membranes. Aquaporins are divided into groups based on their permeability: classical AQPs, which are permeable only to water (AQPs 0, 1, 4, 5, 6); aquaglyceroporins, which transports water, urea, and glycerol

(AQP 3, 7, 8, 10); and AQP 9, which allows the passage of water, urea, glycerol, purines, pyrimidines, and monocarboxylates [32]. In 1994, Li et al. discovered that the AQP1 gene is present in cDNA from the human uterus. Various studies have thus been conducted describing the role and expression of AQP in reproductive tissues [21].

In the rat myometrium, AQP1 expression is increased during pregnancy and is suggested to play an important role in uterine closure, stromal oedema, and blastocyst orientation [23]. Furthermore, Lindsay and Murphy reported in 2007 that AQP 5 and 9 are expressed in glandular epithelial cells during the time of implantation in the rat uterus and are responsible for transcellular fluid transport across the glandular epithelium [24]. Aquaporin 2 expression is elevated in human endometrium, therefore implying a role of AQP2 in uterine receptivity [12]. Expression of various AQPs is regulated by sex hormones, i.e. progesterone, oestrogen, and/or a combination. It has been reported that mouse AQP5 contains a functional element responsible for the oestrogen response and is directly regulated by this steroid [20]. The combination of progesterone and oestrogen increases AQP5 expression in uterine epithelial cells of ovariectomized rats [25]. Conversely, oestrogen alone induces expression of AQP1 in the immature rat uterus [22]. The expression and role of AQP 1, 2, and 5 during the oestrus cycle of a bitch has shown that AQP1 was expressed only in blood vessels and its expression level did not change during the oestrus cycle [3]. On the other hand, expression of AQP 2 and 5 changed throughout the oestrus cycle, suggesting these two AQP isoforms are influenced by sex steroid levels. Thus, AQP2 expression is elevated during the oestrogen-dominant stage, and AQP5 expression is increased during the progesterone-dominant phase of the oestrus cycle. It has been reported that oestrogen enhances the expression of AQP 1, 5 and 9 in the porcine uterus [29]. In 2013, Klein et al. described the expression of AQPs in the equine endometrium throughout the oestrus cycle [19]. Aquaporins 3, 5, and 7 levels were highest after 8 days after ovulation, while AQP 0 and 2 were elevated at the 14th day of the oestrus cycle. Moreover, treatment with progesterone did not change expression levels of AQPs in anoestrus mares; therefore, it has been suggested that the factors other than progesterone are required for higher expression of AQPs during the oestrus cycle. Changes in AQP expression in reproductive tissues could be induced not only by sex hormones but also by alterations in uterine pathologies, such as unovulatory uterine bleeding in women [8].

EXPRESSION AND REGULATION OF CX IN ENDOMETRIAL TISSUES

The connexins are a family of transmembrane proteins which form gap junction connections (GJCs) between cells in vertebrates. The GJCs are intercellular channels that allow communication within cells [17, 16]. Communication on the molecular level occurs because of the cytoplasmic exchange of small molecules,

i.e.: metabolites, second messengers, and ions. Therefore, electric impulses among neighboring cells are transmitted through gap junctions [7]. Each gap junction is formed from two hemi-channels, known as connexons, and each connexon is built from six protein subunits (connexins) ordered around the pore. Currently, the connexin family has included 20 members in the murine genome and 21 members in the human genome. Connexin sequences are highly conserved among different species, and their size varies from 23 to 62 kDa. Connexin expression requires tight regulation as any aberration in expression could potentially lead to a number of diseases, and even to cancer growth induction [13]. Cx43 and Cx26 are mainly expressed in human, baboon, and rodent endometrium. Cx43 is present between the stromal cells while Cx26 is expressed in the luminal and glandular epithelium. During the menstrual/oestrus cycle, expression levels of Cxs vary due to different levels of ovarian hormones. In the human endometrium during the early follicular phase, Cx43 has very low expression while Cx26 is not expressed in the epithelium. Subsequently, with elevation of serum estrogen levels during the follicular phase, expression of both connexins is increased. Afterwards, throughout the luteal phase, progesterone levels are increased and connexin expression is down-regulated. Connexin 26 is more sensitive to hormonal regulation as it isn't detected during the luteal phase, while Cx43 is still weakly expressed [14]. Hence, during the receptive-phase, gap junction-mediated intercellular communication is down-regulated in the human endometrium.

A similar hormone-sensitivity was also demonstrated for rodents [11]. During the progesterone-dominant stage of the oestrus cycle, expression of Cx26 and Cx43 is down-regulated, but as opposed to humans, the endometrium of rodents is transformed into the receptive state only during pregnancy. During early pregnancy, Cx26 and Cx43 expression is decreased then ultimately ceases shortly before implantation during the receptive phase [11].

FURTHER PERSPECTIVES – APPLICATION OF ENDOMETRIAL TISSUE PRIMARY CULTURE

The growth and differentiation of endometrial tissues during the oestrus cycle are regulated by a coordinated secretion of sex steroids hormones such as progesterone and estradiol. Therefore, the cultivation of endometrial cell lines and/or processing of endometrial cell primary culture requires highly specialized conditions as well as various agent supplementations. In prior research, the proliferation activity of porcine granulosa cells (GCs) and cumulus oophorus cells (CCs) during real-time primary culture was analyzed. Results have shown that GCs and CCs proliferation begins shortly after 24 and 48 hours of culture, respectively [17,

16, 15, 18]. Contrary to these experiments, our research indicated that porcine luminal epithelial cells (LECs) proliferation began after 96-120 hours of primary culture. Moreover, the morphology of GCs-CCs and LECs differs significantly in the primary culture model, from spindle-shaped granulosa cells to cuboidal colonies for endometrial cells. The physiology of endometrial cells in primary culture may be a utilized as a representative model for research of several uterine diseases such as endometritis-pyometra in domestic bitches. Moreover, the molecular mechanisms as related genes expression profiles in canine endometrium has been well recognized, however, there is still lack of information regarding physiological and/or pathophysiological aspects of this disease. Therefore, it has been suggested that the endometrial tissue primary culture model may be successfully used in the recognition of new features of several uterine diseases, creating new applications in routine veterinary practice and diagnostics.

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Bartosz Kempisty
Department of Histology and Embryology, Department of Anatomy
Poznan University of Medical Sciences
6 Święcickiego St., 60-781 Poznań, Poland
tel./fax: (61) 854 64 19/ (61) 854 64 55
e-mail: bkempisty@ump.edu.pl