

Effect of Matrigel on Function and Morphology of Human Endometrial Epithelial Cell *in vitro*

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ABSTRACT

Introduction: The importance of extra cellular matrix (ECM) in development and function of different cells has been reported but little is known about its role in human endometrial epithelial cells. The aim of the present study was to examine effects of artificial ECM (Matrigel) and progesterone on the function and morphology of human endometrial epithelial cells *in vitro*. **Methods:** Endometrial samples were removed, with informed patients consent and Ethics Committee approval, from 17 previously fertile women undergoing total abdominal hysterectomy. The tissue was dissociated and centrifuged to provide an epithelial rich suspension which was cultured either on plastic or seeded into Matrigel to produce polarized cells and then supplemented with or without progesterone (10^{-6} M). The amount of nucleic acid content of the cells in both *in vitro* model systems was examined by DNA, RNA extraction methods. The DNA and RNA content were later measured by spectrophotometry. **Results:** The amount of total RNA in cells grown on Matrigel (23 ± 1.5 pg/cell) was more than double that in cells grown on plastic (9.1 ± 1.4 pg/cell). Cells cultured on both *in vitro* model systems had RNA induced by steroid hormones, but the extent of induction was greater in cells grown on Matrigel (30 ± 2 pg/cell) than those on plastic (12 ± 1.9 pg/cell). Cells cultured on Matrigel were differentiated and became polarized but cells grown on plastic proliferated to full confluency. Cells grown on Matrigel with progesterone supplementation were highly polarized, euchromatic and had greater mitochondria and accumulation of glycogen, when compared to unsupplemented cultures. **Conclusion:** These results suggest that ECM plays an important role in gene expression, polarization and differentiation of human endometrial epithelial cells *in vitro*. Endometrial cells grown on ECM responded to steroid hormone in a manner to that reported in endometrial cells *in vivo*. *Iran. Biomed. J. 11 (2): 87-94, 2007*

Keywords: Matrigel, Epithelial endometrium, Function and morphology, *in vitro*

INTRODUCTION

There are basically two types of extra cellular matrix (ECM): the interstitial matrix, which is the collagen-rich ground substance in which stromal fibroblast are located, and the basement membrane (BM) to which epithelial cells attach. BM *in vivo* is about 20-100 nm thick and is located under the basal surface of epithelial cells, around individual muscles cells, fat cells, nerve and capillaries [1]. It contains type IV collagen, the glycoproteins laminin, entactin, nidogen and heparin sulphate [2].

The importance of BM in development and adult tissue function has been found from a number of

observations. For example: cells often migrate along BM during development; BM are required for the polarization of cells in both embryo and adult; and BM serves as substrates for cell adhesion and migration during wound healing and nerve regeneration [3, 4]. A large body of work *in vitro* with isolated BM components and extracts of BM has shown that the behavior of cells is greatly influenced by these components. BM components promote cell adhesions via integrin, proteoglycans and lectins, and modulate the *in vitro* phenotype of the cell [2, 5]. BM also serves as depositories of growth factors, and may thereby modulate access to, and activity of, such growth factors [5]. Most recently, several groups have shown that disruption

of cell adhesion to ECM *in vitro* may induce programmed cell death, apoptosis, in the cells [6, 7]. BM functions, as a substrate, for combining and movement of the cells during the renovation of wounds and reconstruction of neurons [8].

Morphology of human endometrial cells which have been grown on artificial ECM (Matrigel), has been reported else where [9]. However, It appears to be no published information on the combined function and *in vitro* ultrastructure of human endometrium obtained from fertile women in different phases of the menstrual cycle. In addition, although it is well documented that progesterone induces extensive morphological changes in human endometrium *in vivo*, it is little published data available on the effect of progesterone on the ultrastructural changes of endometrial cells *in vitro*. In this study, Matrigel, very similar to BM, was used; it was taken from the mice with Engelbreth-Holm-Swarm sarcoma. In the present study, epithelial endometrial cells obtained from fertile women in different phases of the menstrual cycle were cultured on Matrigel both with progesterone supplementation (10^{-6} M) and without progesterone supplementation to examine effects of Matrigel and progesterone on the function and morphology of human endometrial epithelial cells *in vitro*.

MATERIALS AND METHODS

Endometrial tissues were obtained at the Jessop Hospital for Women (Sheffield, UK) from 17 previously fertile women, aged 32-40 years with regular menstrual cycles of 25-35 days undergoing total abdominal hysterectomy in Sheffield, UK. All tissues were collected with informed patient consent and Ethics Committee approval. Patients had not used oral contraceptives or IUD for at least 3 months prior to the operation and no tissue was used from patients with carcinoma of ovary, uterus, tube or cervix. All patients had one or more prior successful pregnancies. Using a Sharman's biopsy curette (Downs Surgical LTD, Sheffield, UK), a single specimen was taken from the fundus and upper part of the body of the uterus. Then tissue was divided into two pieces: one piece of tissue (called original biopsy [OB]) was processed for light and electron microscopy to determine normality and dating of tissue and the other piece was used for cell culture. The day of cycle of OB was determined by the first day of the last period (LMP) and electronic and light microscope.

Preparation for cell culture. After one pieces of tissue () was taken for the study of OB *in vivo*, the remaining tissue was sliced finely with a scalpel blade (No. 24 size, Swann-Morten Ltd., Sheffield, England) and placed into the centrifuge tube containing 5 ml of 0.25% collagenase (grade IV, Sigma: Aldrich, Poole, England), in 10% HBSS and incubated in a water bath at 37°C for 1-2 hours, vortexing intermittently. Glands were allowed to settle and the supernatant (containing collagenase and the smaller stromal cell rich compartment) was pipetted off and discarded. 5 ml of 10% HBSS was added into the tube to stop further collagenase digestion and then the tube contents were vortexed again. After standing for a few minutes, the supernatant (now rich in epithelial cells) was removed from the heavier gland fragments which had settled down in the bottom of the tube. The supernatant was centrifuged at 1,000 ×g for 5 min (using a Beckman TJ-6 centrifuge). The resulting supernatant was decanted leaving the pellet of epithelial cells. The pellet was resuspended in 0.5 ml of Dulbecco's/Hams F 12 (EMEM-F12, Life Technologies Ltd., Paisley, Scotland) culturing media. The epithelial-rich suspension was cultured either on plastic for further study or seeded into the artificial ECM Matrigel (Uniscience Ltd., Collaborative Biomedical Products, Bedford, USA) to produce polarized cells.

Polarised endometrial cell cultures. Type HA millicell inserts pore size 0.5 µm (Millipore, UK) were placed into wells of a Sterilin 24 (1 cm) well culture plate. Approximately 0.15 ml of Matrigel (Uniscience, Collaborative Biomedical products, Bedford, USA) in gel form was layered onto the insert, ensuring the bottom was completely covered. This procedure was carried out on a 'cold block' over ice. The culture plate was then removed from the cold block and 0.5 ml of epithelial cell suspension was carefully layered on top. Half ml of the culture medium, alone or supplemented with progesterone, was added to the well around the insert. The multi-well culture plate was then placed in an incubator gassed with 5% CO₂ and 95% air at 36.5°C. Culturing time for epithelial cells was approximately 5-7 days. The culture medium was removed every 48 hours and replaced with fresh culture medium and any appropriate supplement. Cells have been cultured for 7 days and assessed under microscope.

Culture of endometrial cells on plastic and Matrigel with progesterone. In order to assess the effects of progesterone on all amounts of RNA produced by endometrial cells in laboratory environment, the endometrial cells were obtained from the early stage of luteal phase and cultured on plastic and Matrigel. These were either unsupplemented (serving as a control) or supplemented with 10^{-6} M progesterone (Sigma, Aldrich, Poole, England) for 7 days and then cells were collected and studied under molecular assessment.

Cell collection of polarised endometrial cell cultures. When polarised cells were formed, after 5 days, the culture medium was removed and stored. The filter part of the insert was cut using a sterile blade and the gel containing the cells was gently removed and with the aid of a sterile tip placed in a 2 ml cryovial (Nalge company, England); 0.5 ml of culture medium was added to the cryovial which was stored in liquid nitrogen (-196°C) for further study.

Cell collection of unpolarized endometrial cell cultures. When a confluent monolayer of cells was formed, after 5 days the cells were detached from the well using trypsin. For trypsinization, the culture medium was removed and stored, and 200 μl of 5% trypsin and 2% EDTA solution (Gibco: BRL, Paisley, Scotland) was added to each well and pipetted gently back and forth several times. The tissue culture plates were incubated at 37°C and examined under a phase contrast microscope (Cambridge Instruments, England) every 5 minutes for signs of cell detachment. After 20 minutes, 0.5 ml of culture medium was added to each well and pipetted back and forth vigorously to a form cell suspension. This cell suspension was quickly removed and placed in a 2 ml cryovial which was centrifuged at $1,000 \times g$ for 5 minutes. The supernatant was decanted and the cell pellet was resuspended in 0.5 ml of culture medium and stored in liquid nitrogen for further study.

RNA extraction. The endometrial epithelial cell cultures, which were put in liquid nitrogen for long-term storage, were removed and thawed on ice before transferring to a sterile 1.5 ml microcentrifuge tube (Sarstedt, Numbrecht, Germany). The cells were spun down in a microfuge (Eppendorf, USA, Model 5415C.) for a few seconds and the media was then removed. TRIzol Reagent (1

ml, Life Technologies TM, USA) was added to the cells. The cells were then incubated at room temperature for 5 minutes. Chloroform (200 μl , Fisons, UK) was added to samples prior to shaking vigorously for 15 seconds and then incubated at room temperature for 3 minutes. The samples were centrifuged at $12,000 \times g$ at 4°C for 15 minutes (Beckman, USA, Model: 32-21 M/E) and top phase carefully removed to fresh, sterile 1.5-ml microcentrifuge tubes. Isopropanol (500 μl , Fisons, UK) was added and incubated at room temperature for 10 minutes, prior to further centrifugation at $12,000 \times g$ at 4°C for 10 minutes. The supernatants were then discarded and the pellets washed in 1,000 μl of 75% ethanol (BDH, UK) and spun down at $7,500 \times g$ at 4°C for 5 minutes. Pellets were then dried at room temperature for 5 minutes, resuspended in 25 μl of ddH_2O and incubated at 60°C for 10 minutes. Total RNA was then stored at -70°C and the sample of total RNA yield and purity was assessed by spectro-photometry (Beckman, USA, Model: DU-62).

DNA extraction. The remaining aqueous phase overlying the interphase was removed to fresh, sterile 1.5-ml microcentrifuge tubes. A volume of 300 μl of 100% ethanol was added and mixed by inversion. The samples were incubated at room temperature for 3 minutes, prior to centrifugation at $2,000 \times g$ at 4°C for 5 minutes. The supernatants were removed and the pellets were washed twice in a solution containing 0.1 M sodium citrate in 10% ethanol. At each wash, pellets were stored in washing solution for 30 minutes at room temperature and then centrifuged at $2,000 \times g$ at 4°C for 5 minutes. Following these two washes, the supernatants were discarded and pellets were suspended in 1.5 ml of 75% ethanol and incubated at room temperature for 20 minutes, prior to centrifugation at $2,000 \times g$ at 4°C for 5 minutes. Pellets were dried at room temperature for 5 minutes and then dissolved in 500 μl of 8 mM NaOH, prior to centrifugation at $12,000 \times g$ at room temperature for 10 minutes. The supernatant containing DNA was transferred to a fresh tube and stored at 4°C . Total DNA yield was assessed by spectrophotometry (Beckman, USA, Model: DU-62) at 260 nm. The formula $\text{OD}_{260} \times 0.05 = \text{total DNA yield } (\mu\text{g}/\mu\text{l})$ was used: where 0.05 - 1.2 = the accurate, linear range of absorbance at 260 nm [2]. Since the amount of DNA per each diploid cell of human equals 7.1 pg, the amount of DNA (pg) was divided by 7.1 for calculation of cell number in the samples (Life

Technologies TM, USA). The amount of total RNA for each cell was obtained by dividing the amount of total RNA by the number of cells.

Tissue preparation for histological dating of the endometrium. Endometrial biopsies were fixed in 3% glutaraldehyde (Taab Laboratories, Aldermaston, England) in 0.1 M phosphate buffer (pH 7.4) and placed in a refrigerator at 4°C for a maximum of 6 hours. The tissue was washed in 0.1 M phosphate buffer thoroughly, and then transferred into 2% aqueous osmium tetroxide (Agar Aids Ltd, Stanstead, Essex, England) for 2 hours. Following this secondary fixation, the tissue was again washed in 0.1 M phosphate buffer and dehydrated through a series of 75%, 90%, 95% and 100% ethanol solutions and then put in propylene oxide (1.2-peoxy propane) (Fisons Scientific Equipment, Loughborough, England) for 40 minutes followed by a 50:50 propylene oxide: Epon resin mixture overnight, then in fresh Epon resin. Tissue pieces were embedded in fresh Epon resin in plastic moulds (Agar Aids Ltd, Stanstead, England) and left to polymerase in an embedding oven (Taab Laboratories, Aldermaston, England) at 60°C for 2-3 days. Histological characterization of the endometrium was performed according to Noyes [10] and Dockery and Rogers [11] criteria.

Ultra-thin sectioning and staining for electron microscopy. After the “semi-thin”, sections were viewed under a Leitz Dialux 22 light microscope, areas of polarised epithelium were chosen for “ultra-thin” microtomy. Blocks were re-trimmed and the “ultra-thin” sections were cut with an interference colour of grey to silver (approximately 70 nm). The sections were picked up onto 3.05 mm 100 mesh copper grids (Agar Aids, Essex, UK) coated with 2% pyroxlene (Collodion) in amyl acetate. Grids were double stained in saturated 50% alcoholic uranyl acetate (15 minutes in the dark) and Reynolds lead citrate. Then, the sections were examined using Phillips 301 transmission electron microscope (Phillips, Holland) at an operating voltage of 60 Kv. Using the technically best parts of the section, 10 electron micrographs per section were taken in a systemic random manner. A grating replica of 2,160 lines per mm (Agar Ltd, Essex, UK) was used as a magnification standard on each film. Negatives were developed in a dark room and viewed using a projecting microscope (Carl-Zeiss, Germany).

RESULTS

Effects of ECM on the function of endometrial epithelial cells. In order to assess the effects of ECM on the function of human endometrial epithelial cells *in vitro*, the endometrial epithelial cells were prepared from all stage of menstrual cycle and cultured on ECM (polarized Method) and plastic (standard method) for 7 days. The findings of this study indicated that despite the equality of number of used cells in two methods (standard and polarized), the number of obtained cells using the trypsin in standard cultured method was several times more than polarized cultured method after 7 days. Indeed, epithelial cells cultured on plastic continue to multiply and proliferate to the extent that they occupy the whole surface of plastic and get confluent form. While, epithelial cells cultured on ECM undergoes several mitosis, and after a couple of days they establish glands similar to those are seen *in vivo* (Fig. 1).

Moreover, in terms of structure, epithelial cells in standard culture were squamous forms, vice a versa; cells in polarized culture were polar and all similar to morphology of cells *in vivo* (Fig. 2). With measurement of total RNA in every cell, it was observed that the amount of total RNA in cultured cells on ECM in each cell was 23 ± 1.5 pg; this amount was about 2.5 times more than the amount of RNA isolated from cells grown on plastics (in each cell, 9.1 ± 1.4 pg).

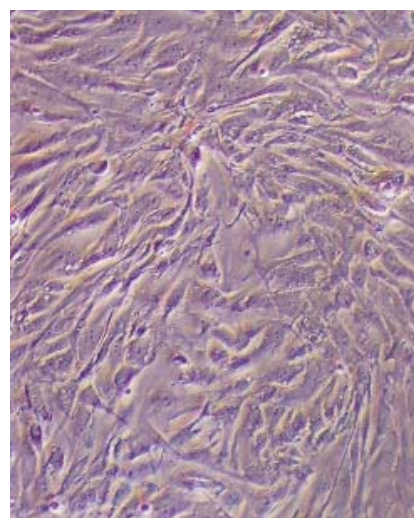


Fig. 1. Microscopic image of endometrial cells cultured on plastic.

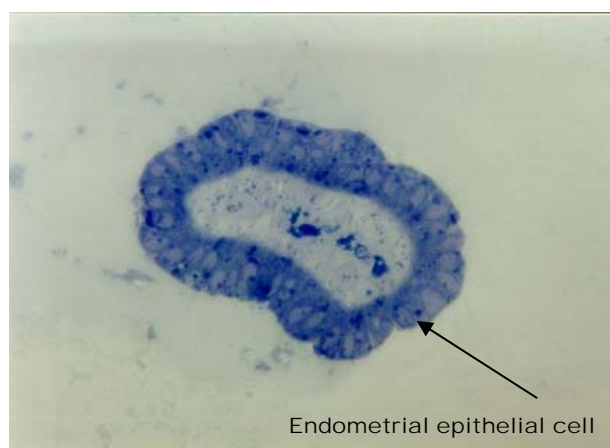


Fig. 2. Microscopic image of semi-thin section of human endometrial epithelial cells cultured on Matrigel and stained with Toluidine Blue. Cells are polar and establish glands similar to those of seen *in vivo*.

Ultra structure of epithelial cells *in vitro*. Cells were grown in culture (polarised) without P supplementation were low columnar in profile. All nuclei were seen basally and contained a thin rim of heterochromatin; a few nucleoli were also seen. The nucleus was variable in shape, but tended to be plump and moderate in size, with a regular membrane profile. Long, thin mitochondria,

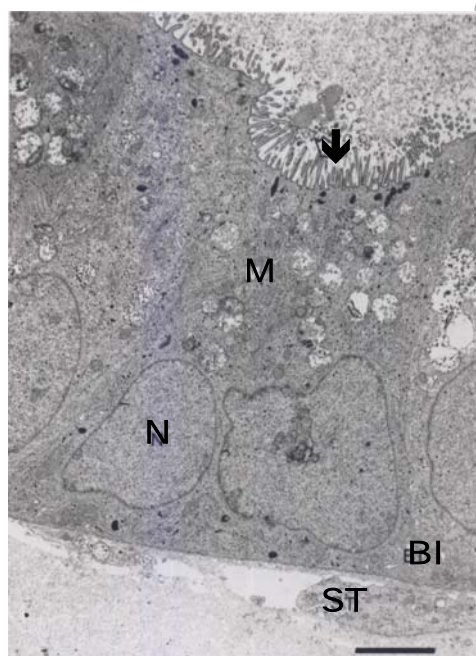


Fig. 3. Electron micrograph of cells taken from the proliferative phase and grown on ECM for 7 days without steroid hormone supplementation showing columnar polarized epithelial cells. Nuclei (N) are variable in shape and many mitochondria (M) can be seen. Basolateral interdigitations (BI) and long, regular microvilli (↓) are present. A few stromal cells (ST) can also be seen. Bar represents 2 μ m.

especially at the apical part of the cells were seen. Patches of glycogen were common, mainly above the nucleus. Many Golgi bodies were found apically, mostly lying horizontally and supranuclearly. Rough endoplasmic reticulum was seen in cells, often lying parallel to the long-axis of the cell. There were many lateral membrane connections between cells (near the cell apex) and many baso-lateral interdigitations. Apically long, regular microvilli were present which had a relatively thick glycocalyx (Fig. 3).

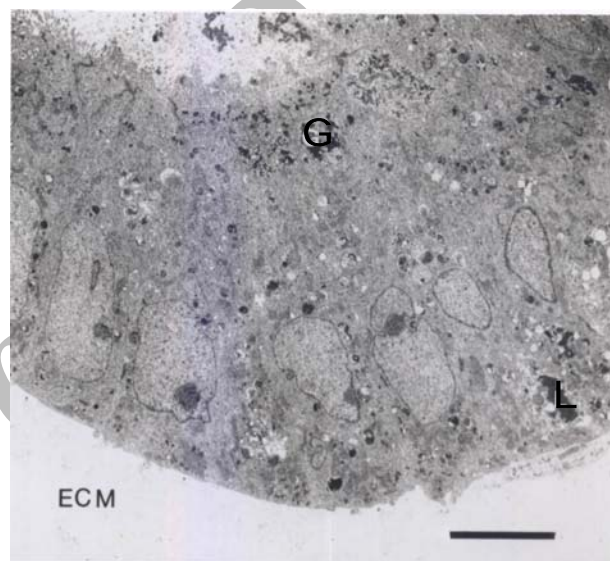


Fig. 4. Electron micrograph of cells taken from the late proliferative phase, grown on ECM for 7 days and supplemented with progesterone, showing highly polarized cells. Glycogen (G) is distributed throughout the cells and lipid droplets (L) are also seen in the base of the cells. Bar represents 4 μ m.

Cells were grown in culture and supplemented with P produced consistently highly polarized cells (Fig. 4). Nuclei were oval in shape with occasional infoldings of nuclear membrane. These were euchromatic with a rim of heterochromatin and prominent nucleoli. Supra and infra nuclear accumulations of glycogen were seen in most cells. Numerous thin and long mitochondria were observed throughout the cytoplasm. Sparse rough endoplasmic reticulum was seen close to mitochondria. There were some lateral membrane interdigitations and a few lipid droplets. There was extensive attachment of the lateral wall of cells through desmosomes and many long, thin microvilli were seen on the apical surface. A notable feature of these cells was their uniformity.

Effect of progesterone on total RNA expression of endometrial cell cultures grown on plastic and ECM.

In order to determine the effect of progesterone on total RNA expression in endometrial enriched-epithelial cells prepared from early stage of luteal phase cultured on plastic ($n = 6$) and ECM ($n = 6$), total RNA and DNA were isolated using the TRIzol method. The amount of total RNA in cells grown on ECM (23 ± 1.5 pg per cell) was about three times more than those grown on plastic (9.1 ± 1.4 pg per cell). Progesterone supplementation increased total RNA synthesis in cells grown on both *in vitro* model systems compared to cells unsupplemented with progesterone. This induction was greater in cells supplemented with P which were cultured on ECM (each cell has 30 ± 2 pg) than those grown on plastic (each cell has 12 ± 1.9 pg).

DISCUSSION

This study indicated that ECM effects essentially on the function and development of human endometrial epithelial cells. There are numerous publications indicating that ECM induces tissue-specific gene expression in mammary epithelial cell [12, 13]. Li *et al.* [14] found that primary mouse mammary epithelial cells cultured on Matrigel produced high levels of mRNA for beta-casein. Chen and Bissell [15] showed that mouse mammary epithelial cells cultured on ECM expressed high levels of whey acidic protein mRNA. Parry *et al.* [16] found that the mouse mammary gland cultured on ECM (Matrigel) had 5- to 10 fold more Muc-1 mucin mRNA than those cultured on plastic tissue culture dishes. Streuli *et al.* [3] have also reported that activation of transcription of the milk-protein genes requires the interaction of the epithelial cells with BM laminin through cell surface integrin receptors. They, using anti-integrin antibody as a blocker of function, showed that the ability of production of casein beta is reduced by epithelial cells of mice breast. The results of the present study, which showed that ECM induces overall gene expression, are in general agreement with the above reports.

This study indicated that ECM prevents the mitosis and proliferation of the cells and causes the development of cells and organelles of the cell. In addition, by combining ECM to the adhesion molecules on the surface of cell, some signals transmit the cell which results in reconstruction of

the cytoskeleton of inside the cell and cause the polarization of the cell [17-19]. The polarization of endometrial cells plays an essential role in impanation of embryo. In polarized cell, the produced proteins and molecules of cell, under an especial programme, are transmitted to the various surface of the cell or secreted from those surfaces. For example, at the implantation time, endometrial epithelial cells produce special adhesion molecules called integrins which are transmitted to the apical surface of cell [20]. The expression of these adhesion molecules and their lignads on the embryo resulted in the attachment of embryo to the endometrium.

At the implantation time, the endometrial epithelial cells produce internal factors (cytokine, and growth factors) which are secreted from the apical surface of cell and cause to the embryo development [20]. In addition, the polarization of cell causes the endometrial epithelial cell at the implantation time to produce especial internal factors which are secreted from the basal (underneath) surface of cell and cause to stroma decidualization of endometrial [21].

The findings of this study indicated that the endometrial epithelial cells in the laboratory environment, without regarding culture type, response to the progesterone hormone with the enhancement of gene expression in cell. We, using electronic microscope and morphometry technique, showed that progesterone *in vitro* causes to the enhancement of the euchromatin to the heterochromatin ratio in the endometrial epithelial cell nucleus which is indicative of cell activity to enhance the gene expression and approval the enhancement of RNA amount of whole cell. In sum, these findings depict the existence of progesterone receptors (PR) inside the cells *in vitro*.

Under effect of estrogen hormone, the amount of PR in the human endometrial epithelial cells *in vivo* enhances in the proliferative stage of menstrual cycle, and reaches to its maximum in the early stage of luteal phase. By increasing progesterone after the ovulation, the amount of PR in these cells decrease under the effect of progesterone hormone and reaches to its minimum at the late stage of luteal phase [22]. For this reason, endometrial samples were taken form the early luteal phase which cells have the maximum amount of PR.

After ovulation, corpus luteum produces progesterone which causes dramatic changes in both structure and function of endometrial cells in preparation for embryo implantation [23, 24]. The

cells become tall and much polarized with ovoid, rounded and euchromatic nuclei. Glycogen begins to be synthesized around ovulation time and accumulates basally, then moves apically, finally to be secreted into the lumen. Lipid-like vacuoles are often seen scattered within and around the glycogen. Giant mitochondria, which are believed to provide for the massive energy demands of implantation, appear around day 17 of menstrual cycle [23, 24]. The increase of metabolic (mitochondria) and secretory (Glycogen) activity of epithelial cells supplemented with progesterone when compared to unsupplemented cells, suggested that epithelial cells *in vitro* respond to progesterone in a similar way to that of seen *in vivo* and so this model could be used to study the effects of progesterone and growth factor supplementation.

The findings of this study indicated that the cells cultured on the plastic (producing non-polarized cells) responded to the progesterone hormone lesser than the cells cultured on the ECM (producing polarized cells). This is indicator of low number of PR in the non-polarized cells. The difference between the PR numbers can be due to the disorder in the production of these receptors in the non-polarized cells.

Lee *et al.* [25] showed that the epithelial cell of mouse mammary epithelial cell cultured on the plastic in the post translation are deficient due to the disorder in the cell skeleton system. Therefore, the low number of PR in the non-polarized cells can be resulted from the disorder in the post translation and low production of PR. The studies showed that the PR in cells, after production, transmit inside the nucleus actively and with the waste of energy (nucleocytoplasmic shuttling) in order to enhance the duplication of especial genes after combining progesterone inside nucleus [26]. The present study showed the number of mitochondria in non-polarized cells in less than polarized cell. This low number of mitochondria produces less energy to transmit progesterone receptor into nucleus which causes weaker response of non-polarized cell to the progesterone compared with polarized cells.

In conclusion, ECM plays an essential role in the development and functions of human endometrial epithelial cells and the study of endometrial epithelial cell function in laboratory environment without using ECM can not the indicator of its real function in the body.

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