



UPPSALA  
UNIVERSITET

**Evaluation of an *in vitro*-model  
for standardized testing of  
endocrine disrupting chemicals  
and their effects on female reproduction**

Michelle Zetterström  
Bachelor Thesis  
Bachelor of Medical Science, Biomedicine  
30 hp

Department of Women's and Children's Health  
Uppsala University  
Supervisor: Matts Olovsson, Professor and Senior Consultant

2011

## Table of contents

<b>Abstract</b> .....	3
<b>1 Introduction</b> .....	4
1.1 The female reproductive tract.....	4
1.2 The endometrium.....	5
1.3 Human endometrial endothelial cells.....	5
1.4 Endometrial stromal cells.....	6
1.5 The menstrual cycle.....	6
1.6 Steroid hormones: estradiol and progesterone.....	7
1.7 Antiprogestins.....	8
1.8 Endocrine disrupting chemicals.....	8
1.9 Aim of the study.....	9
<b>2 Materials and methods</b> .....	9
2.1 Subjects.....	9
2.2 Establishment of cell cultures.....	9
2.3 Exposure of cells to test substance.....	10
2.4 Migration assay.....	10
2.5 Tube formation assay.....	11
<b>3 Results</b> .....	11
<b>4 Discussion</b> .....	12
<b>5 References</b> .....	13

## Abstract

**Background:** Some of the polluting chemicals present in our environment are classified as endocrine disrupting chemicals (EDCs), which have the ability to interfere with the physiology of normal endocrine-regulated events. Since the structure and function of the endometrium is hormonally regulated, it is highly sensitive to hormonally active substances such as EDCs, where exposure might result in female reproductive disturbances. Identifying a suitable *in vitro*-model would facilitate the identification of EDCs, as well as the investigation of their potentially harmful effects.

**Aim:** To evaluate whether this *in vitro*-model can be used as a method for identifying EDCs that might have the capacity to cause reproductive disturbances, and thereby facilitating the search for potentially harmful EDCs.

**Methods:** Human endometrial endothelial cells (HEECs) collected from endometrial biopsies were co-cultured with endometrial stromal cells. At subconfluency in passage two the HEECs were exposed to the progesterone receptor modulator mifepristone, which was used as a model substance to evaluate whether this type of hormonally active substances might affect endometrial angiogenesis. After exposure, two angiogenesis-related processes were tested: HEEC migration and tube formation.

**Results and hypothetical conclusion:** The circumstances during which this particular *in vitro*-model was evaluated made it hard to predict whether it is a suitable model for standardized testing of EDCs or not. Using primary cells, difficulties attaining a pure monoculture of HEECs were experienced, hence a precise setup of the *in vitro*-model failed to succeed. Replacing the primary cells with cell lines would most probably help to overcome this obstacle, making the *in vitro*-model functional, however there are no commercially available HEEC lines. In addition, both the cell migration assay and the tube formation assay involved several practical problems and therefore ought to be replaced or simplified. Thus, the possibility to standardize the use *in vitro*-model cannot be eliminated; however further development and revision would be required.

# 1 Introduction

## *1.1 The female reproductive tract*

The female reproductive tract includes the ovaries, the fallopian tubes, the uterus, the vagina and the vulva.

The ovaries are two oval structures lateral to and behind the uterus [1]. This is the site of germ cell production and steroid hormone biosynthesis. Germ cell support occurs in the ovarian follicles. The resting follicles enclose a primordial oocyte surrounded by an inner layer of granulosa cells and an outer layer of theca cells. Androgen production takes place in the theca cells, while the granulosa cells convert these into estrogens. As the follicles grow, they develop into a structure known as the Graffian follicle. About 20 follicles mature during each cycle, however generally only one precedes to ovulation, the release of the oocyte from the ovary. As the oocyte is released from the follicle, the granulosa cells proliferate and transform into an endocrinologically active structure recognized as the corpus luteum [2].

The fallopian tubes are two hollow structures attached to the uterus. They can be divided into four main sections: 1) the uterine or intramural part that opens into the uterine cavity, 2) the isthmus which is the thick-walled part that enters the uterine horn, 3) the ampulla which constitutes the major part where fertilization occurs, and 4) the infundibulum which is the distal end that opens into the abdominal cavity in close proximity to the ovaries. The infundibulum has finger-like processes, the fimbriae [3], that move in a sweeping manner to capture ovulated eggs [1]. The epithelium of the fallopian tube is ciliated, and the cilia beat toward the uterus [2] transferring the fertilized zygote to the uterine cavity for implantation [1].

The uterus, a pear-shaped, hollow, muscular structure, is the site of normal pregnancy. The uterine wall is built up of three layers: the perimetrium, the myometrium and the endometrium. The perimetrium is the peritoneal outer serous coat supported by a thin layer of connective tissue [3]. It encloses the myometrium, the major part of the uterine wall composed of smooth muscle cells [2]. During pregnancy, the myometrium becomes greatly distended [3]. The inner layer of uterus is referred to as the endometrium [4] which is the site of implantation.

The vagina is a tubular structure that stretches from its opening at the introitus of the perineum to the cervix. The exterior of the female reproductive tract is referred to as the vulva [1].

## *1.2 The endometrium*

The endometrium is the inner layer of the uterus. It is composed of two main parts: a germinal layer adjacent to the myometrium, which remains from cycle to cycle, and a transient functional layer adjacent to the uterine cavity. The functional layer provides a site for implantation of the blastocyst as well as the maternal component of the placenta. The endometrium is constructed of various tissue compartments: a lining surface of epithelium and associated glands with a connective tissue stroma with a complex vascular tree. The basal arteries supply the germinal layer whereas the spiral arteries, branching into a plexus, supply the functional layer [4]. The endometrium is influenced by the cyclic production of sex steroid hormones (estradiol and progesterone) in the ovaries [2], as well as paracrine factors, such as various growth factors and cytokines [5]. In the case of no conception, the functional layer of the endometrium is shed through menstruation [3].

## *1.3 Human endometrial endothelial cells*

The human endometrial endothelial cells (HEECs) are vital for the normal function of the endometrium, and disturbances of the normal functions of these cells are probably related to several pathological conditions. The structure and function of the endometrium is delicately regulated by the ovarian sex hormones estrogen and progesterone. Quantitative RT-PCR and microarray analyses have demonstrated that HEECs express the sex hormone receptors ER- $\beta$  (estrogen receptor  $\beta$ ) and PR (progesterone receptor), a feature making them potential targets for endocrine disrupting chemicals (EDCs). As HEECs are important for normal endometrial function, studies of their possible involvement in several reproductive disturbances that might be caused by EDCs are of interest [6, 7].

The vascular tree is an important feature of the endometrium. For this reason, a normal vascular morphology and function are essential for fertility, implantation and placentation. Endothelial cells, lining the lumen of all blood vessels, control vascular events such as angiogenesis, vascular remodeling and functional changes [6], a process involving an intense interaction with stromal cells. Endometrial angiogenesis is controlled by the ovarian sex steroid hormones and might thus also be influenced by EDCs [8]. In addition, environmental contaminants can affect angiogenic factors such as vascular endothelial growth factor (VEGF) [6].

In contrast to normal vascular beds, the endometrial vascular network is dynamic. Endometrial angiogenesis most likely occurs by elongation and expansion of preexisting

blood vessels. Beginning during menstruation with vascular repair, it continues during the estrogen-driven proliferative phase as well as during the secretory phase, when the spiral arteries are fully developed and the sub-epithelial capillary network is formed [4, 9].

#### *1.4 Endometrial stromal cells*

The stroma is the connective tissue of the endometrium. It contains fibroblast-like cells and a complex extracellular matrix with fibrillar components and ground substance. The fibroblast-like cells produce the majority of the matrix components, and therefore are involved in the cyclic remodeling of the extracellular matrix. Other cell types present in the endometrial stroma are numerous natural killer cells and lymphocytes.

Coincident with the menstrual cycle, the stromal cells undergo morphological changes that can be observed as proliferation, differentiation and maturation [4].

#### *1.5 The menstrual cycle*

The menstrual cycle is the dynamic reorganization of the endometrium [10], controlled by the cyclic changes in the hypothalamic-pituitary-ovarian axis. The cycle lasts for approximately 28 days and consists of three phases: the proliferative phase (days 5-14), the secretory phase (days 14-28) and the menstrual phase (days 1-4). The proliferative phase is characterized by post-menstrual re-epithelization and growth of stromal and glandular elements, resulting in a thickening of the endometrium. Ovarian estradiol is the dominant hormone during this phase and its production is stimulated by the follicle-stimulating hormone (FSH) [11]. Once ovulation occurs, the corpus luteum in the ovary starts producing progesterone [2] the dominant hormone during the secretory phase. At this point the endometrium experiences structural and functional changes. The most evident change can be observed in the glands, which obtain a secretory character. In the absence of conception the levels of progesterone and estrogen fall, causing the secretory phase to transcend into the menstrual phase. This largely endometrial event, a stage of shedding, destruction and degeneration, is triggered by a decrease in the levels of progesterone and estradiol as well as local factors. Spasm and relaxation of spiral arteries caused by an increase in the availability of prostaglandin PGL2 $\alpha$  induces impairment of the vascular elements and consequently hemorrhage [4, 11].

### *1.6 Steroid hormones: Estradiol and progesterone*

The menstrual cycle is controlled by the cyclic changes in the hypothalamic-pituitary-ovarian axis: gonadotropin-releasing hormone (GnRH) produced in hypothalamus, the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) produced in the pituitary gland, and finally estradiol and progesterone synthesized in the ovaries [12].

Gonadotropins regulate steroidogenesis [13], the intracellular enzymatic conversion of cholesterol into all classes of steroid hormones (progestins, androgens, estrogens, glucocorticoids and mineralocorticoids). The tissue specific expression of enzymes required for the conversion of cholesterol into specific hormones makes the steroid hormone synthesis limited to certain tissues [14].

Steroid hormones exert their effects by inducing protein synthesis in target cells, where the proteins serve to produce the hormone activity. The hormones diffuse into cells, producing responses by binding to specific intracellular receptors. This binding activates the receptor by altering its conformation, allowing it to interact with regulatory elements of DNA, inducing protein synthesis [15].

Estradiol and progesterone, both responsible for the classical histological changes of the endometrium [10], operate by means of their associated receptors [16]. Two estrogen receptors have been identified, ER- $\alpha$  and ER- $\beta$ , which respond to estrogens in a comparable manner. Estrogens induce the expression of progesterone receptors [17], thus the action of progesterone depends of the preceding exposure of estrogen [10]. The progesterone receptor exists in two isoforms, the PR-A and the PR-B [18]. The A and B receptors have different molecular functions, exerting their individual effects on different genes. Hence, the target tissue response to progesterone is controlled by the expression of each isoform and their relative concentration [17].

Even though the overall control of endometrium is through estrogen and progesterone, these two hormones operate indirectly via a range of different local factors, such as vascular endothelial growth factor A (VEGF-A), fibroblast growth factor (FGF), transforming growth factor  $\alpha$  and  $\beta$  (TGF- $\alpha$ , TGF- $\beta$ ) and relaxin. Dependent on the actions of these factors, steroid hormones are capable to either promote or inhibit angiogenesis. While the estrogenic effects on the endometrium promote growth of endometrial vasculature, the effects of progesterone have been focused on to a lesser extend. However, studies indicate that progesterone has multiple and essential influences on the endometrial vasculature [4, 19].

### *1.7 Antiprogestins*

Analogous to progesterone, antiprogestins induce conformational changes within the progesterone receptor. The antiprogestin mifepristone (RU 486) [17], known for its abortifacient potential, is a so-called progesterone receptor modulator [10], which binds to the receptor with an affinity five times greater than that of progesterone [17]. The compound possesses both antagonist and agonist activity [10], however it acts primarily as a potent antagonist, resulting in a down regulation of progesterone-dependent genes [20]. The agonistic effect is produced in the absence of progesterone [17]. The interaction of mifepristone with the progesterone receptor prevents the transcription of progesterone-dependent genes, causing softening of the uterine cervix and increasing the contractions of the myometrium, e.g. by increasing the myometrial sensitivity to prostaglandins [18]. By blocking the biological effects of progesterone, the endometrium transforms the endometrium into a non-receptive character, with changes in expression of receptors (e.g. PRs) as well as factors such as VEGF and TGF- $\beta$  [21].

### *1.8 Endocrine disrupting chemicals*

Some of the polluting chemicals present in our environment are classified as endocrine disrupting chemicals, EDCs [6]. These have the ability to interfere with the physiology of normal endocrine-regulated events such as reproduction.

The endometrium, the inner lining of the uterus, is vital for implantation and other reproductive processes. The structure and function of the endometrium is regulated by the female sex hormones progesterone and estradiol. As a result, it is highly sensitive to hormonally active substances such as EDCs. EDCs have been associated with female reproductive disturbances such as infertility and spontaneous abortions [22, 23].

Several studies imply that many indices of female reproduction have declined over the past half century. Conception rates have been proven to decrease in Danish and US women and hormone-related diseases such as endometriosis are unfortunately not exceptional. Environmental exposures may be a contributing factor [22], emphasizing the need for further studies of endocrine disrupting chemicals and their effects on female reproductive health. Identifying suitable *in vitro* models would facilitate the investigation of the potential harmful effects of EDCs, in addition to reducing or replacing the unethical use of animal models and thereby overcoming inter-species variations.

The endothelial cells of the endometrium are vital for the normal function of the female reproductive tract [6], however HEECs are the least studied cells of the human uterus [9].

### *1.9 Aim of the study*

In this study, human endometrial endothelial cells, HEECs, were co cultured with stromal cells in a specialized *in vitro*-system. The two cell types were separated, however still allowed to communicate. Primary cultures of cells collected from human endometrial biopsies were used in order to come as close as possible to their normal function, and overcoming the course of normal differentiation as when using cell lines [5]. A monoculture of HEECs was used as a negative control to investigate whether stromal cells alter the HEEC response or not.

The aim of the study was to evaluate whether this *in vitro*-model could be used as a predictive method to identify EDCs, endocrine disrupting chemicals, and their ability to cause reproductive disturbances. The cells were exposed to the progesterone receptor modulator mifepristone, in this experiment used as a model substance to evaluate the effects of hormonally active substances. If mifepristone was to influence the HEECs *in vitro*, one could also suspect that the endometrial angiogenesis would be affected *in vivo*, hence affect the reproductive ability. HEEC migration and tube formation, two events involved in angiogenesis [4], were studied after exposure of mifepristone.

## **2 Materials and methods**

### *2.1 Subjects*

Endometrial biopsy samples were obtained by curettage from women undergoing hysterectomies (removal of the uterus) for benign medical conditions at Uppsala University hospital. They were all in the secretory phase of the menstrual cycle and declared to be non-smokers.

### *2.2 Establishment of cell cultures*

The biopsies were immediately put in ice-cold endothelial cell medium (EGM-2 MV, Lonza, Basel, Switzerland) and transported to the laboratory. They were cut into 1mm pieces and washed in PBS three times. A 5ml enzyme solution containing Collagenase II (500µl), DNAase (500µl), Hyaluronidase (1ml), Gentamycin (200µl) and PBS was prepared and divided into two 2,5ml test tubes. The minced endometrium was placed in one of the test

tubes with enzyme solution and incubated for one hour (5% CO<sub>2</sub>, 37°C). The content was passed through a 40µm cell strainer into a 50ml test tube with 5ml PBS. The non-digested tissue was placed in the remaining tube with enzyme solution and incubated for an additional hour, and then passed through the cell strainer into the same tube as previous. The cells were centrifuged for 10 minutes at 110xg, and the supernatant was removed. Subsequently the cells were suspended in 2ml sterile, cold 0,1% BSA-PBS and transferred to two 3,6ml cryotubes (1ml/tube). 20µl endothelial cell specific CD31 Dynabeads® (Invitrogen™) was added to each cryotube. The tubes were placed on a rocking table at 2-8°C for 30 minutes, followed by addition of 2-3ml refrigerated 0,1% BSA-PBS to each tube. The tubes were placed in a special magnetic holder for approximately 2 minutes, causing the CD31-bound endothelial cells to assemble at the back edge of the tube. The supernatant containing the stromal cells was moved to another 3,6ml tube. Freshly prepared 0,1% BSA-PBS was added to the endothelial cells and the washing procedure was repeated. This procedure was also done with the stromal cells in the other 3,6ml tube. The endothelial cells were suspended in 10ml cell medium (EGM-2 MV, Lonza) and put into two T25 flasks. The stromal cells were centrifuged for 10 minutes at 110xg and suspended in 15ml cell medium. The medium was changed 2-3 times weekly. Subcultivation of cells was carried out at subconfluency in passage 0 and 1 following standard procedures.

### *2.3 Exposure of cells to test substance*

Both the monocultured and co-cultured cells were treated identically. Two different concentrations of Mifepristone (0.1 and 10µM) were used. Negative controls were treated with ethanol (vehicle) and endothelial cell medium (EGM-2 MV).

### *2.4 Migration assay*

A modified Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD, USA) was used for assessing the ability of HEECs to migrate. Cells with a confluence of 80-90% were re-suspended in trypsin and transferred to two 6-well plates as monocultures and co-cultures. Regarding the co-cultures, the HEECs were allowed to attach to the wells for a couple of hours before adding the stromal cells in inserts.

The following day the medium was removed and exchanged to different media either containing mifepristone (0.1 and 10µM) or ethanol. The cells were incubated for 48 hours.

The incubation was followed by trypsin treatment and re-suspension of the endothelial cells in their respective medium. The desired cell concentration was  $0,5 \times 10^6$  cells/ml. A volume of 26  $\mu$ l of the negative control (basal cell culture medium), the positive control (basal medium with foetal bovine serum) and the positive control together with attractant (basal medium with foetal bovine serum and VEGF) were added to the wells in the lower chamber of the Boyden chamber. An 8  $\mu$ m pore size polycarbonate filter coated with collagen (100  $\mu$ g/ml PBS) was carefully placed on top of the wells in the lower chamber, and the upper chamber was installed. 50  $\mu$ l cell suspension was added to each well in the upper chamber.

The cells in the Boyden chamber were incubated for 5 hours. After incubation the filter was removed and fixed in pure methanol for 15 minutes. This was followed by Giemsa staining (2ml/25ml PBS) for 10 minutes. After rinsing in water the non-migrated cells were wiped off with a cotton pad. The examination of the migration assay was performed at a magnification of 20x and the migrated cells were quantified. All assays were done in triplicate.

### *2.5 Tube formation assay*

Cells with a confluence of 80-90% were harvested and added to a 6-well plate as monocultures and co-cultures. In the co-cultured plate, the HEECs were allowed to attach to the wells for a couple of hours before adding the stromal cells in inserts. The preferred confluence of the mono-cultured cells the day after subcultivation was 50-60%.

The following day the medium was exchanged to medium containing the test substance and vehicles. The cells were incubated for 48 hours.

Geltrex™ was thawed in a refrigerator over night. Endothelial cells with a preferred confluence of approximately 50-60% were harvested with 0,5ml 0,05% trypsin/EDTA and centrifuged for 10 minutes at 110xg. The cells were re-suspended in 100  $\mu$ l of the previous used media with test substance and negative controls. The desired cell concentration of 9000 cells/well was achieved by counting the cells in a Bürker chamber and diluted accordingly. 100  $\mu$ l Geltrex™ was added to each well in a 48-well plate, and the plate was incubated for 30 minutes at 37° C, allowing the gel to solidify. 100  $\mu$ l cell suspension was gently added to the gel-coated wells. Each of the cells exposed to the different substances was assayed in triplicate; as a result at least 350  $\mu$ l of each sample was needed. The plate was incubated for 8 hours and the result was observed in a microscope. The wells were photographed at a magnification of 10 and the number of branch points was quantified.

### 3 Results

The study indicates that this *in vitro*-model needs to be further developed before it can be used as a standardized model for *in vitro*-testing of the effects of EDCs on reproductive function. In addition, the methodology used appeared to be very complex and sensitive, and the usage of these assays should therefore be revised.

Regarding the *in vitro*-model, difficulties were experienced. The isolation process failed to separate the endothelial cells from the stromal cells; consequently no pure monoculture of HEECs could be established. A pure monoculture was requisite for a successful establishment of the *in vitro*-model itself as well as for the two assays. Despite this inadequacy the cell migration assay and the tube formation assay were carried out. Both were shown to involve complex procedures, where the slightest mistake eliminated the chance of attaining useful data.

As a result, no calculable data could be generated from the assays and the *in vitro*-effects of mifepristone on HEECs could therefore not be examined, emphasizing the need of further developing the *in vitro*-model as well as the two assays.

### 4 Discussion

The aim of the study was to evaluate if this *in vitro*-model could be used as a method to identify EDCs that might have the capacity to cause reproductive disturbances such as infertility, endometriosis, and spontaneous abortions. Not only does this facilitate the search for potentially harmful EDCs, it also aspires to reduce or even replace the unethical use of animal models, and thereby also overcoming inter-species variations.

Human endometrial endothelial cells (HEECs) attained from endometrial biopsy samples were co cultured with endometrial stromal cells in order to optimally mimic the physiological milieu and to investigate whether their presence influences the HEEC response. This two-chamber arrangement separated the two cell types, however it still allowed communication and signaling between them. Hence the results attained from the two assays were expected to represent the *in vivo*-effects on angiogenesis to quite some extent.

The progesterone receptor modulator mifepristone was used as a model substance to evaluate whether this type of hormonally active substances might affect endometrial angiogenesis and if the possible effects could be identified using the methodology of this study. Two angiogenesis related processes were tested; cell migration and tube formation. The effects of mifepristone on HEEC migration or tube formation *in vitro* would indicate that it might also

affect endometrial angiogenesis *in vivo*, and thus have effects on endometrial fertility functions.

This *in vitro*-model and the assays used did not generate any calculable data. Since primary cells are more complex to deal with than cell lines, difficulties were experienced attaining pure monocultures of HEECs, a necessity for the setup of the *in vitro* model as well as the two assays. However, the advantages of using primary cell cultures motivate the usage of these cells, since they better mimic the *in vivo*-state and would therefore generate more relevant data than cell lines, in addition to the lack of commercially available HEEC lines [8]. Practical problems with the assays were also experienced, this probably due to lack of experience and the sensitivity of the execution. Simplifying the assays or even replacing them would be necessary for standardized usage.

An identical *in vitro*-model has been used in a previous study, in which the effects of cadmium chloride were proven to alter the mRNA levels of angiogenesis related genes. The effects of cadmium exposure on HEEC were shown to depend on the presence or absence of stromal cells, emphasizing the significance of an *in vitro* environment that resembles that of the *in vivo* state. [8]. Adjusting the technical shortcomings, this *in vitro*-model might be an appropriate method for predicting the potential harmful effects of EDCs on the human endometrium.

## 5 References

1. Linda J. Heffner, D.J.S., *The Reproductive System at a Glance*, 2010, Blackwell Publishing. p. 28-29.
2. Linda J. Heffner, D.J.S., *The Reproductive System at a Glance*, 2010, Blackwell Publishing. p. 30-31.
3. Keith L. Moore, A.M.R.A., *Essential Clinical Anatomy*, 2006, Lippincott Williams & Wilkins.
4. *The Endometrium: Molecular, Cellular, and Clinical Perspectives*, A.T.F. John D Aplin, Stanley R Glasser, Linda C Giudice, Editor 2008, Informa Healthcare p. 46-62.
5. Bremer, S., et al., *In vitro tests for detecting chemicals affecting the embryo implantation process. The report and recommendations of ECVAM workshop 62 -- a strategic workshop of the EU ReProTect project*. Altern Lab Anim, 2007. **35**(4): p. 421-39.
6. Bredhult, C., B.M. Backlin, and M. Olovsson, *Effects of some endocrine disruptors on the proliferation and viability of human endometrial endothelial cells in vitro*. Reprod Toxicol, 2007. **23**(4): p. 550-9.
7. Krikun, G., et al., *Endometrial endothelial cell steroid receptor expression and steroid effects on gene expression*. J Clin Endocrinol Metab, 2005. **90**(3): p. 1812-8.

8. Helmestam, M., A. Stavreus-Evers, and M. Olovsson, *Cadmium chloride alters mRNA levels of angiogenesis related genes in primary human endometrial endothelial cells grown in vitro*. Reprod Toxicol, 2010. **30**(3): p. 370-6.
9. Kayisli, U.A., et al., *Regulation of angiogenic activity of human endometrial endothelial cells in culture by ovarian steroids*. J Clin Endocrinol Metab, 2004. **89**(11): p. 5794-802.
10. *The Endometrium: Molecular, Cellular, and Clinical Perspectives*, A.T.F. John D Aplin, Stanley R Glasser, Linda C Giudice, Editor 2008, Informa Healthcare. p. 601-610.
11. Linda J. Heffner, D.J.S., *The Reproductive System at a Glance*, 2010, Blackwell Publishing. p. 38-39.
12. Boron, W.F., *Medical Physiology*. 2nd ed 2008, Philadelphia, Pa.
13. Linda J. Heffner, D.J.S., *The Reproductive System at a Glance*, 2010, Blackwell Publishing. p. 12-13.
14. Linda J. Heffner, D.J.S., *The Reproductive System at a Glance*, 2010, Blackwell Publishing. p. 14-15.
15. Linda J. Heffner, D.J.S., *The Reproductive System at a Glance*, 2010, Blackwell Publishing. p. 16-17.
16. *The Endometrium: Molecular, Cellular, and Clinical Perspectives*, A.T.F. John D Aplin, Stanley R Glasser, Linda C Giudice, Editor 2008, Informa Healthcare p. 99-116.
17. Leon Speroff, M.A.F., *Clinical gynecologic endocrinology and infertility*, 2004, Lippincott Williams & Wilkins. p. Chapter 2.
18. Benagiano, G., C. Bastianelli, and M. Farris, *Selective progesterone receptor modulators 1: use during pregnancy*. Expert Opin Pharmacother, 2008. **9**(14): p. 2459-72.
19. *The Endometrium: Molecular, Cellular, and Clinical Perspectives*, A.T.F. John D Aplin, Stanley R Glasser, Linda C Giudice, Editor 2008, Informa Healthcare.
20. Im, A. and L.J. Appleman, *Mifepristone: pharmacology and clinical impact in reproductive medicine, endocrinology and oncology*. Expert Opin Pharmacother, 2010. **11**(3): p. 481-8.
21. Meng, C.X., et al., *Effect of levonorgestrel and mifepristone on endometrial receptivity markers in a three-dimensional human endometrial cell culture model*. Fertil Steril, 2009. **91**(1): p. 256-64.
22. Crain, D.A., et al., *Female reproductive disorders: the roles of endocrine-disrupting compounds and developmental timing*. Fertil Steril, 2008. **90**(4): p. 911-40.
23. Toft, G., et al., *Epidemiological evidence on reproductive effects of persistent organochlorines in humans*. Reprod Toxicol, 2004. **19**(1): p. 5-26.
24. Vazquez, F., et al., *Progesterone regulates proliferation of endothelial cells*. J Biol Chem, 1999. **274**(4): p. 2185-92.
25. Walter, L.M., P.A. Rogers, and J.E. Girling, *The role of progesterone in endometrial angiogenesis in pregnant and ovariectomised mice*. Reproduction, 2005. **129**(6): p. 765-77.