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The Tissue Specific Role of Estrogen and Progesterone in Human Endometrium and Mammary Gland

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1. Introduction

The purpose of this chapter is to review the tissue-specific role of estrogen (E2) and progesterone (P4) in human endometrium and mammary gland. It is well known that both E2 and P4 are essential for the development and differentiation of human endometrium and mammary gland, but the exact basis for differential tissue-specific signalling of E2 and P4 are still not fully understood. This chapter explores observed functions of two major female steroid hormones and their cognate receptors in normal physiology of human reproductive system but also in assisted reproductive technology and breast cancer treatment.

The normal reproductive physiology requires tightly coordinated action of hypothalamus, pituitary gland, ovaries and endometrium. Also functioning of other endocrine units such as the thyroid and adrenal glands are essential for regular ovulation and cyclic changes. The production of ovarian steroid hormones is coordinated by the hypothalamic-pituitary-gonadal axis which is activated in puberty (Figure 1). The hypothalamus produces and secretes luteinizing hormone-releasing hormone (LHRH), which binds to its receptors in pituitary gland. This causes cascade of biochemical events culminating in the production of two hormones in pituitary gland, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH are secreted into the general blood circulation and attach to receptors on the ovary, where they trigger ovulation and stimulate the production of E2 and P4. Ovarian steroid hormones themselves have direct role in the development of the inner lining of the uterus but they also act as a positive feedback system to hypothalamus and pituitary gland for continuous cyclic changes until the beginning of menopause (Kanis and Stevenson, 1994).

Cholesterol is the building block for all steroid hormones, which is carried into the bloodstream and through a sequence of enzymatic changes is synthesized into final products. In the bloodstream steroid hormones are distributed rapidly throughout the tissues and act on distant targets. This secretory process is called endocrine action and the function of many target tissues as mammary gland, brain, bones, liver and heart are affected by circulating hormones. Steroid hormones can also act very close to their site of secretion

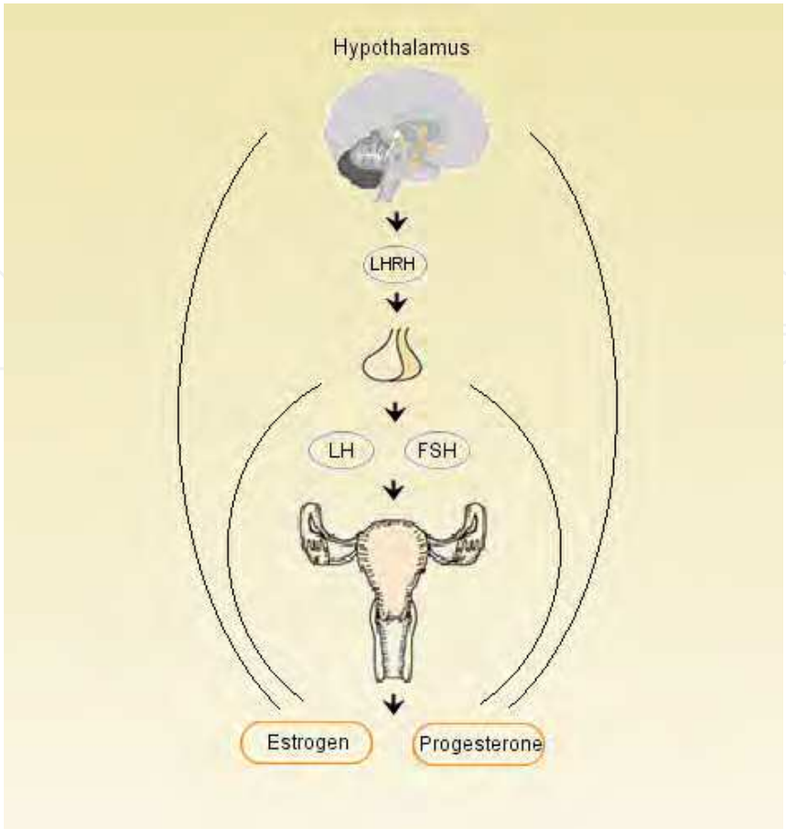

Fig. 1. The female hypothalamic–pituitary–gonadal axis. The hypothalamus produces and secretes luteinizing hormone–releasing hormone (LHRH) into a system of blood vessels that link the hypothalamus and the pituitary gland. LHRH stimulates the pituitary gland by attaching to specific molecules (i.e., receptors). After the coupling of LHRH with these receptors, a cascade of biochemical events causes the pituitary gland to produce and secrete two hormones, luteinizing hormone (LH) and follicle–stimulating hormone (FSH). LH and FSH are two of a class of hormones commonly known as gonadotropins. They are secreted into the general circulation and attach to receptors on the ovary, where they trigger ovulation and stimulate ovarian production of the hormones estrogen and progesterone. These female hormones cause monthly menstrual cycling and have multiple effects throughout the body. In particular, estrogen has profound effects on the skeletal system and is crucial to maintaining normal bone health (Figure adapted from Kanis and Stevenson, 1994).

on adjacent cells and tissues as it happens in gonads, testis and ovaries- paracrine action. Gonads produce only three classes of steroids: progestins, androgens and estrogens where progestins are obligatory precursors of both androgens and estrogens. Likewise, androgens are obligatory precursors of estrogens. Steroidogenesis in the ovary is compartmentalized in a cell-specific manner: the theca cells primarily producing androstenedione and the granulosa cells completing the synthesis of E2. After the ovulation the corpus luteum of the ovary starts to produce P4. Albeit the vast amount of sex steroids are synthesized locally in peripheral tissue, providing individual target tissues with the means to adjust synthesis and metabolism to their local requirements (Venken et al., 2008).

Beside the reproductive system, one of the most widely recognized effects of E2 is the prevention of the osteoporosis. Adequate E2 levels through E2 replacement therapy has shown to prevent or diminish calcium loss from bones in menopausal women (Venken et al., 2008). In the nervous system both estrogens and androgens have been reported to influence verbal fluency, performance of spatial tasks, verbal memory capacity and fine motor skills (Kelly and Ronnekleiv, 2008). The major role for P4 in humans is related to initiation and maintenance of the pregnancy. P4 is essential for milk preparation and secretion in mammary gland and for mediating signals required for sexually responsive behaviour. Recent evidence also supports a role for P4 in the modulation of bone mass (Seifert-Kaluss and Prior, 2010).

NRs function as transcription factors. The biological activities of E2 and P4 are mediated mainly by nuclear receptors (NRs). Binding of a steroid hormone to its cognate receptor results in a conformational change in the nuclear receptor that allows the ligand-receptor complex to bind with high affinity to response elements in DNA and regulate transcription of target genes. In the absence of ligand, NRs are held in a multi-subunit complex containing heatshock proteins such as Hsp90, SP70, HSP40, Hop, and p23 (Wolf et al., 2008). After binding to ligand, these receptors, undergo conformational changes, dissociate themselves from chaperone proteins, dimerize and in some cases translocate into the nucleus (if not already locked into the nucleus) (Bain et al., 2007). The differences in specificity of molecular mechanisms result from receptor subcellular location and binding to genomic DNA as homo- or heterodimers in either head-to-tail or head-to-head orientation to different consensus sequences known as hormone response elements (HREs) (Bain et al., 2007). Upon NR activation a hydrophobic pocket is created in their tertiary structure for interaction with co-activators such as members of the steroid receptor co-activator (SRC) protein family or co-repressors such as NR co-repressor (NCoR) and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) (Hall et al., 2005). The recruitment of co-regulators leads to alterations in the rate of gene expression via modification of initiation complex formation process. Two types of estrogen receptors, ERα and ERβ, encoded by separate genes, are found in humans (Enmark etal., 1997; Kuiper and Gustafsson, 1997). P4 signalling is also mediated by two receptors, PRA and PRB, which are encoded by the same gene but transcribed from different promoters, resulting in a PRB that has an additional 164 amino acids at the N-terminus (Wen et al., 1994; Kastner et al., 1990). PRB is a stronger transcriptional activator in most cell types, while PRA acts often as a dominant negative repressor for PRB activity (Tung et al., 1993; Vegeto et al., 1993).

In addition to operating as TFs in the nucleus, NRs have been shown to possess non-genomic action which is usually characterized by a shorter lag time required to elicit a biological response following steroid hormone stimulation. For instance ERs can regulate gene expression independent of estrogen responsive element (ERE) through tethering different TFs and by membrane- initiated ER interference with other intracellular pathways. Examples of motifs recognized by ER other than ERE is the activator protein-1 site (AP-1 site) commonly occupied by the TFs c-Fos/c- Jun B (Björnström and Sjöberg., 2004).

2. The role of E2 and P4 in human endometrium

Human endometrium is the inner tissue lining of uterine cavity that undergoes monthly cyclic changes dictated by ovarian steroid hormones E2 and P4 (Figure 2). As endometrium is a regenerative tissue it is subjected to proliferation, secretion and degeneration on

monthly basis. Nearly all morphologic and biochemical processes that the uterus undergoes during its acquisition of receptivity are directly or indirectly regulated by ovarian steroid hormones (Lim et al., 2002). The development of human endometrium is divided into follicular and luteal phase. During the follicular phase ovarian E2 is produced with increasing quantities until ovulation, stimulating the proliferation and growth of the epithelial and stromal components of the endometrium. During the luteal phase the increasing amounts of the P4 and secondary maintaining levels of E2 are both involved in the differentiation of the endometrium but P4 reverses the proliferative effects of E2 (Lim et al., 2002). Together, coordinated action of steroid hormones produced by the follicle and corpus luteum prepare the endometrium every month for potential embryo implantation. In the event of embryo implantation P4 predominantly facilitates and permits decidualization of the endometrium and supports maintenance of pregnancy. On the contrary, in the absence of implantation declining levels of E2 and P4 lead to degeneration of the endometrial tissue, which is followed by regeneration during the next cycle. In addition to cell differentiation, P4 plays the key role in the decision of cell survival or death prior to the menstruation. Three proteins related to apoptotic activation in endometrial cells are proto-oncogene p53, FOXO1 (forkhead box-O) and BIM which act as a switches between apoptosis and survival (Brosens and Gellersen, 2006).

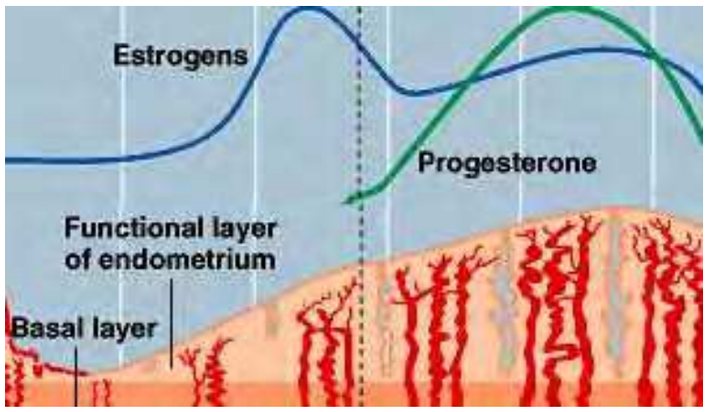

Fig. 2. **E2 and P4 in human endometrium.** E2 causes the growth or proliferation of the endometrium during the first two weeks of the menstrual cycle. After ovulation, the corpus luteum produces P4. This hormone causes the endometrial glands to secrete nutritive substances required by the embryo and to allow it to implant into the endometrial lining (figure adapted from internet http://www.tubal-reversal.net/uterus-menstrual-cycle.htm).

Ovarian steroids mediate their signals through genomic or non-genomic pathways. The genomic signal is passed on by cognate receptors, ERs and PRs, in endometrial cells. As E2 is dominant hormone during the follicular phase of the cycle genes regulated by E2 are also more often related to tissue proliferation. Under the actions of E2 the epithelial cells respond by rapid induction of gene expression that promotes DNA synthesis and cell replication (Lessey et al., 2010). During the luteal phase P4 induces the genes related to differentiation. Clinically used steroid hormone analogues (Tamoxifen, Fluvestrant, Progestin, Mifepristone) could have a suppressive or repressive impact on normal steroid hormone signalling in endometrial cells (Figure 2). The expression of ERs and PRs in spatiotemporal manner is crucial for the successful implantation process (Lessey et al., 2003). Although ERα

and ERβ are present in all endometrial cell types throughout the entire menstrual cycle, they are expressed at higher levels during the proliferative phase and show lower activity during the secretory phase because of the suppressive effect of P4. After the proliferative phase P4 takes the E2-primed endometrium towards a state of receptivity. P4, acting through its cognate receptors, is absolutely mandatory for successful implantation and post-implantation embryo survival. PRA and PRB levels are similar during the follicular phase of the menstrual cycle while the PRA is down-regulated at the time of implantation but higher stromal PRB levels during the mid-luteal phase have been reported (Arnett-Mansfield et al., 2004). The expression of the PR gene in endometrial glands is controlled by E2 and P4, where E2 induces PR synthesis and P4 down-regulates the expression of its own receptor (Graham et al., 1990). The actions of P4 counter the effects of P4 in the endometrium through paracrine regulators from the stromal part. Recent studies about small non-protein coding RNAs (microRNA, miRNA) have revealed their important role in gene regulation in endometrium (Kuokkanen et al., 2010; Li et al., 2011). The regulation of specific microRNAs is a mechanism that appears to fine tune gene expression by blocking cell proliferation at the time of implantation P4 dependently (Lessey et al., 2010).

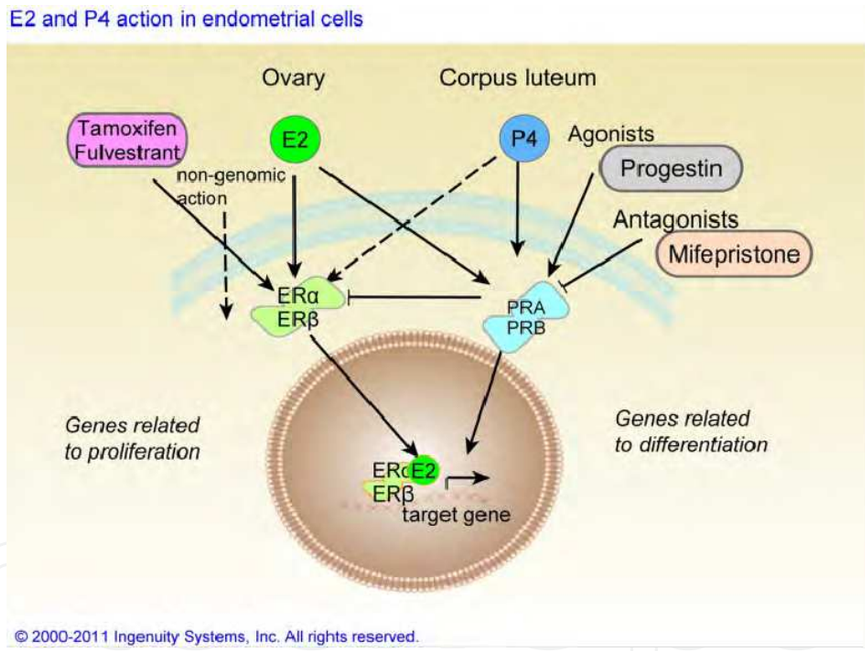

Fig. 3. **E2 and P4 genomic signalling in human endometrial cell.** E2 produced by ovary enters into the cell and binds to its specific receptors ERα or ERβ. Formed complex moves into the cell and has an impact on target gene expression. After the ovulation corpus luteum starts to produce P4 which also diffuses into the endometrial cells and through its receptors regulate gene expression. Steroid hormone analogues (Tamoxifen, Fluvestrant, Progestin, Mifepristone) have a suppressive or repressive impact on ER , PR signalling.

2.1 Endometrial gene expression during the time of embryo implantation

In a restricted period, called implantation window (IW), endometrium is most receptive for the embryo attachment. In humans IW is temporally confined to days 20-24 of menstrual

cycle (8-10 days after ovulation). During this time period corpus luteum induces high level of P4 and stable level of E2 expression. For successful pregnancy the apposition, adhesion and invasion of developing embryo is needed which can only happen if the endometrium is at the right developmental stage posessing a receptive atmosphere.

There is a certain group of women who repeatedly fail to achieve pregnancy in spite of good quality embryos transferred during IVF (*in vitro* fertilisation) treatments. This has led to the search for better solutions to improve implantation rates. In a molecular level embryo implantation is a dialog between blastocyst and receptive endometrium which is mediated by various growth factors, cytokines, lipid mediators, transcription factors and other putative molecules often regulated by steroid hormones. In recent years, numerous studies applying global gene expression analysis have found a wide range of genes up- or down regulated in human endometrium during the IW (Carson et al., 2002; Kao et al., 2002; Riesewijk et al., 2003; Horcajadas et al., 2004; Krikun et al., 2005; Mirkin et al., 2005; Simon et al., 2005; Punyadeera et al., 2005; Talbi et al., 2006; Horcajadas et al., 2008; Haouzi et al., 2009a,b; Altmäe et al., 2010). Each study has brought out candidate genes believed to be crucial in embryo implantation process but the overlap of potential marker genes between different publications has still remained relatively low. However, today there are already some biomarkers confirmed in separate studies which are pivotial during implantation process. For example, the most potential endometrial marker identified is leukemia inhibitory factor (LIF) and its importance has been proven in animal and human studies (*Stewart et al., 1994;* Arici et al., 1995; Steck et al., 2004). Unfortunately, the development of recombinant human LIF (r-fLIF) has not met the expectations of increasing implantation rates in infertile women (Brinsden et al., 2009). The localization of immune system related molecules like cytokines, IL-6 and IL-11, has been identified in endometrial cells and they have shown coincidental expression changes at the time of high levels of E2 and P4 (Tabibzadeh et al., 1995; Robertson et al., 2000; Vandermolen and Gu, 1996; Cork et al., 2001; Dimitriadis et al., 2000; von Rango et al., 2004). The two integrins, α4βl and αvβ3, appear to be good markers of the receptive endometrium in normal fertile women (Lessey et al., 1994). Recognized growth factors related to endometrial receptivity and implantation are transforming growth factor β (TGF-β), epidermal growth factor (EGF), heparin binding-epidermal growth factor (HB-EGF) and inlsulin like growth factor (IGF) (Jones et al., 2006b; Hofmann et al., 1991; Dadi et al., 2007; Lessey et al., 2002; Stavreus-Evers et al., 2002). Growth factors and their respective receptors have shown to enhance embryo development and improve implantation rates in IVF cycles (Kabir-Salmani et al., 2004).

It is more likely that there is no single molecule, which could solve the implantation issue and help patients with recurrent implantation failures. As a complex process implantation seems to depend on many factors, which influence the development of the embryo and endometrial dating in synchronized manner. Moreover, the individual differences and monthly cyclic changes of the regenerative tissue make the search for universal markers relevant to implantation complex.

2.2 The influence of the IVF treatment on endometrial receptivity

Since the first announcement of successful IVF treatment in 1978 (Steptoe and Edwards, 1978) assessed fertilization procedures have been increasingly used world-wide. Based on the report by European Society of Human Reproduction and Embryology (ESHRE) in 2008, more than three million babies have been born with the help of IVF (ESHRE 2008).

Nowadays the number of couples seeking for aid to achieve pregnancy is constantly increasing as at least every tenth couple requires infertility treatments.
Ovarian stimulation and ovulation induction with gonadotrophin administration has been a success from the 1960s (Fowler and Edwards, 1957). Ovulation induction leads to multi-follicular growth instead of a single follicle in natural cycles escalating possible successful fertilisation. Still, the general success rates for clinical pregnancies have stayed around 30-40% for more than three decades (Department of Health and Human Services Centres for Disease Control and Prevention Report 2001). The focus to develop more effective ovarian stimulation protocols to increase the number of oocytes and embryos obtained from one cycle has by some means overlooked the relevance of supraphysiological levels of ovarian steroid hormones and their collateral effect on the endometrium (Simon et al., 2008). In modern IVF, drugs used to stimulate ovaries during the follicular phase include clomiphene citrate, urinary and recombinant gonadotrophins and gonadotrophin releasing hormone (GnRH) agonists and antagonists (Edwards et al., 2005). The usage of ovarian stimulating drugs often results in shorter luteal phase of the endometrium, which is therefore no longer synchronized with embryo development. The use of GnRH agonists may have a negative effect on implantation. Several studies observing endometrial biopsies from patients undergoing IVF treatment show 1-3 day advancement in endometrial development (Lass et al., 1998; Nikas et al 1999). The formation of pinopodes, considered as morphological markers for receptive endometrium, has also been shifted to day 17 or 18 compared to day 20 in normal cycle (Stavreus-Evers et al., 2001). Elevated concentrations of E2 and subtle P4 increases in the late follicular phase lead to modulated steroid hormone receptor profile (Papanikolau et al., 2005). Histological study has shown down-regulation of the ERs and PRs and pinopode expression in stimulated cycles compared to natural cycles (Develioglu et al., 1999). There is some evidence of a negative impact of supraphysiological steroid levels on endometrium because increased pregnancy rates have been observed in the presence of reduced production of serum E2. This explains the fact that there are higher pregnancy and implantation rates recorded for oocyte recipients versus donors who have only P4 support prior to embryo transfer (Check et al., 1995). A premature reduction in PRs in the early luteal phase has been found after ovarian stimulation. Horcajadas and colleagues have demonstrated that gene expression profiling of the endometrium is different between natural and controlled ovarian stimulation cycles in the receptive phase (Horcajadas et al., 2008).
There are ways to restore the length of luteal phase by stimulating corpus luteum with hCG or by supplementing the luteal phase with steroids, such as E2 and P4 (Smitz et al., 1992). Also, to overcome the side effects caused by high doses of drugs milder stimulation protocols have been developed (Olivennes et al., 2002; Nargund and Frydman, 2007; Pennings and Ombelet, 2007; Ubaldi et al., 2007). The evidence regarding a potentially negative effect of supraphysiological steroid levels on endometrial receptivity (Simon et al., 1995; Devroey et al., 2004), corpus luteum function (Fauser and Devroey, 2003; Beckers et al., 2006), oocyte and embryo quality (Valbuena et al., 2001; Baart et al., 2007) indicate that limited ovarian stimulation and response might have a beneficial effect on implantation potential.

2.3 E2 and P4 endometriosis

The ovarian steroid hormones play also a central role in pathogenesis of several uterine disorders, including endometriosis, which is characterized by the presence of endometrial tissue outside the uterine cavity like the peritoneum and ovary. It has been shown that both

eutopic and ectopic endometrial tissues expresses ERs and PRs and they respond to ovarian steroid hormones but the predominance of ERα and PRA receptors have been described in cases of ectopic lesions (Matsuzaki et al., 2001; Attia et al., 2000). Despite the obvious importance of E2/P4 in the development of the endometriosis, the exact aetiology and pathogenesis of it are still unclear. It is predicted that in general endometriosis could affect about 10% of women of reproductive age and up to 25-50% of women seeking infertility treatment. There is still uncertainty whether the decreased fertility is related to reduction of the oocyte/embryo quality or dysregulation of the endometrium (Kim et al., 2007). Aberrant gene expression in endometrium which is suboptimal for implanting blastocyst has been shown by several studies in cases of endometriosis (Giudice et al., 2002; Kao et al., 2003).
Even though endometriosis has been characterized as E2-dependent gynaecological disease, where E2 favours the growth of the tissue, the dysregulation of the P4 response on the molecular level is suggested in endometriosis. It has been noticed that endometriotic tissue does not respond to P4 as normal endometrium does. Altered PR expression or diminished activity predictably results in differential gene expression compared to eutopic tissue (Cakmak et al., 2010). For example, altered P4 signalling can cause unpaired regulation of *HOXA 11, HOXA12* genes in ectopic tissue which are expressed in high levels during the IW in normal tissue (Cakmak et al., 2010). The up-regulation of *HOXA10* and *HOXA11* expression fails to occur in women with endometriosis (Taylor et al., 1999). Recent studies looking for functional miRNA-s have shown up-regulation of miR-21 in eutopic endometrium of women with versus without endometriosis (Luo et al., 2010; Aghajanova et al., 2011).
Hopefully further studies in the future help us understand the molecular mechanisms, which are responsible for the development of endometriosis.

3. The role of E2 and P4 in mammary gland

The development and physiology of human mammary gland is also under the strict control of steroid hormones, including E2 and P4. The mammary gland is not completely formed at birth, but begins to develop in early puberty when the primitive ductal structures enlarge and branch (Russo et al., 1987). From that point ovarian E2 and P4 are fundamental for the growth and differentiation of the duct system. There are slight cyclical changes during each menstrual cycle caused by ovarian steroid hormones where E2 is increasing the volume of the tissue and P4 is responsible of the acinar growth of breast tissue. During pregnancy, the mammary gland epithelium experiences its greatest and most rapid proliferation initially as a response to the hormones produced by corpus luteum, following by placental hormones.
Due to difficulties in studying developing mammary gland there is relatively small amount of information about normal ER and PR expression in breast tissue. It has been confirmed that PRs and ERs are found in a minority population (7–10%) of luminal, non-dividing epithelial cells. As E2 is required to induce progesterone receptor (PR) expression it is difficult to separate the effects of P4 alone from E2. However, the obligate role of the ERs and PRs in mammary gland development has been confirmed with knocked out mice studies (Bocchinfuso and Korach, 1997; Humphreys et al., 1997).

3.1 E2 and P4 in breast cancer development

Broad spectrum of physiological activity of steroid hormones displays its dark side in cases when cells in steroid hormone guided organs lose their normal responsiveness to hormone.

Third of female malignancies are hormone dependent in their growth. Most prominent leading death causing factors for women under age of 50 are breast cancer and also various cancers of reproductive system. Many factors are involved in the development of breast cancer, including genetics, lifestyle, diet, endogenous hormone status and environment. Demographic risk factors for breast cancer are early age of menarche, nulliparity, late full-term pregnancy, higher social class and increasing age. Known factors with protective effects on breast cancer development are early full-term pregnancy, increasing number of births, longer periods of anovulation and more physical activity (Bernstein et al., 1994). The incidence of this lethal cancer has steadily increased during the last centuries in part due to the better and more widespread screening procedures. Increased ERα expression is one of the earliest changes occurring in the tumorigenic process and is associated with uncontrolled proliferation of the breast tissue (Khan et al., 1994). Some data is showing that ERβ could negatively modulate the effects of ERα but the prognosis for endocrine therapy are still under the question because of the somehow contradictory outcomes (Roger et al., 2001; Speirs et al., 2002). Similarly PR isoform ratio also seems to have a role in breast tumorigenesis as the ration of PRA and PRB has been altered with PRA prevalence (Mote et al., 2002).

Currently, only the expression level of ERα is measured for clinical decision-making and treatment of breast cancer patients as a favourable prognosis in primary tumours. Still, only 50% of ERα-positive tumours respond well to hormonal therapy. Large research programs are dedicated to search for better and more specific clinical breast cancer markers. The significance of ERβ status is still controversial and further analysis of the role it plays in the pathogenesis of breast cancer is required. As more experimental information on E2-mediated signalling accumulates, new possibilities emerge for breast cancer therapy.

3.2 Selective ER and PR modulators

Selective ER modulators (SERMs) function through ERs, acting as agonists or antagonists of E2 depending on the target tissue and modulate the signal transduction pathway to E2-responsive genes. The implementation of SERMs in clinical aspects is wide. They are used to treat or prevent breast cancer and osteoporosis, to cure ovulatory dysfunction in women but also for contraceptive purposes. SERMs have an ability to differently regulate many ER-regulated genes (Berrodin et al., 2009; Chang et al., 2010). In general, most SERMs have E2 agonist activity in bone and antagonist activity in the breast, while the activity in the uterus varies among the molecules. The tissue specificity depends on various co-activators (CoA) and co-repressors (CoR) expressed and recruited in different tissues (Riggs et al., 2003). E2 binds to either ERα or ERβ and subsequently binds CoA molecules required to form a transcription complex at EREs located in the promoter region of estrogen-responsive genes. The antiestrogenic action of a SERM results from the inappropriate folding of an ERα or ERβ complex that either cannot recruit CoA molecules or instead recruits CoR molecules. This programmed change in conformation produces antiestrogen action at specific sites like the breast, but estrogen-like effects in the uterus if an excess of CoA molecules is present. SERM–ER complexes may initiate gene transcription to produce an estrogen-like effect, by forming a protein–protein interaction at fos/jun that activates AP-1 sites (Jordan et al., 2001) (Figure 4). Although widely used and with many beneficial effects in treating breast cancer SERMs still battle with several side effects where most common is the stimulation of the endometrium.

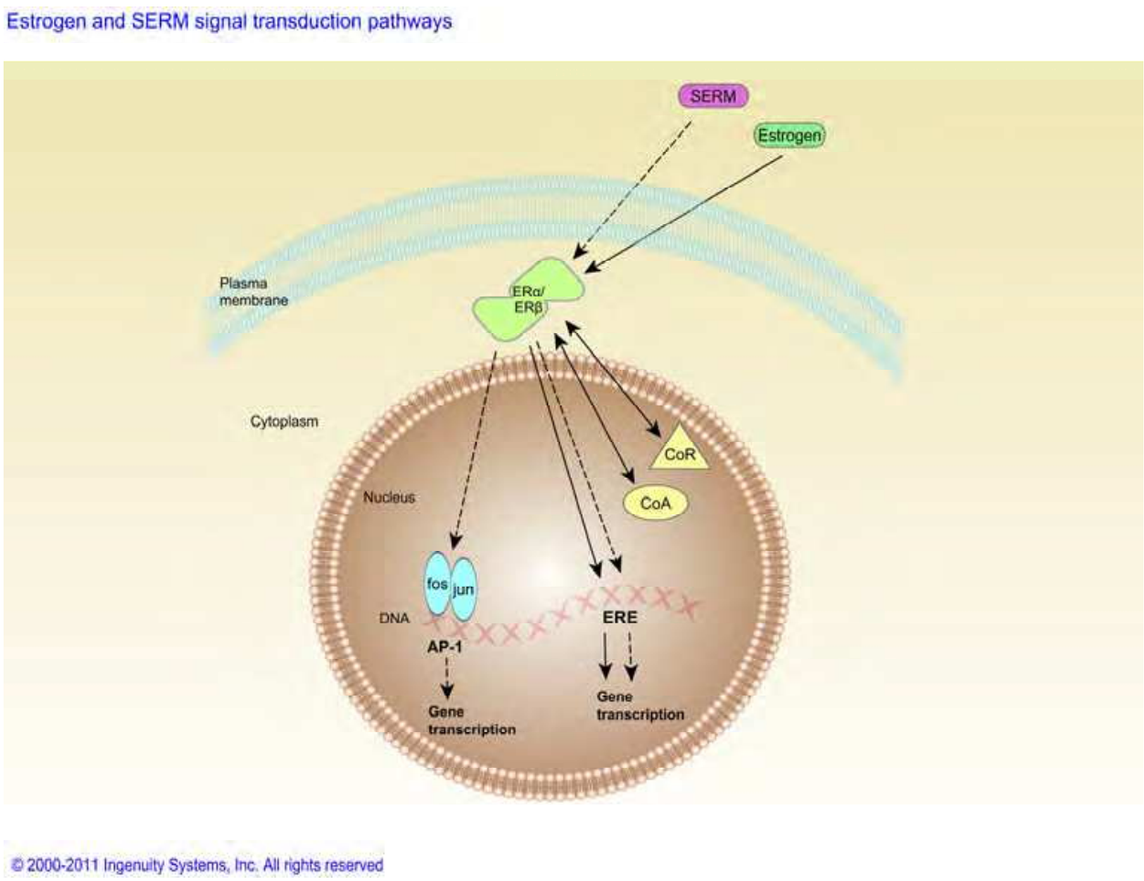

Fig. 4. The signal transduction pathways available to E2 or a SERM to initiate gene transcription. E2 receptors – ERα, ERβ, selective ER modulator-SERM, coactivator –CoA, corepressor CoR, E2 response element-ERE, activating protein -1-AP-1 (Figure adapted from Jordan et al., 2001).

Tamoxifen (TAM), the first SERM available for clinical use, is regarded as a highly effective agent for the prevention and treatment of breast cancer in premenopausal and postmenopausal women. TAM has been used in women to treat breast cancer for over 40 years (Fisher et al., 1998; Fisher et al., 2005). This compound binds with high affinity to ER, thereby blocking the action of native E2. Subsequently it inhibits or modifies the interaction of ER with DNA, which impedes the transcriptional activation of target genes (Berry et al., 2005). TAM strongly counteracts E2 effects, including secretion of several growth factors and growth controlling enzymes, so that a woman's own E2 cannot stimulate growth of the tumor cells. TAM has been a successful drug especially in treating hormone-responsive breast cancer, being one of the main reasons why ER-positive breast cancer patients have a better prognosis compared to those with an ERα-negative breast tumours. Another positive effect was noticed when postmenopausal women's bone density increased after breast cancer treatment (Love et al., 1992). One of the most significant side effects of the treatment with the TAM appears to be its proliferative effect on the endometrium (estrogen-agonistic effect; Buzdar et al., 1998; Bergman et al., 2000). The use of TAM results significant 3.3 fold increase of endometrial cancer (Fisher 2005). The repression of the cell proliferation during

breast cancer treatment could lead to endometrial cell proliferation later on (Figure 5). Endometrial pathologies associated with TAM use include hyperplasia, polyps, carcinomas and sarcomas (Cohen et al., 2004). The full mechanism of this paradox remains still undiscovered.

Another well-studied antiestrogen, Raloxifen, has an E2-antagonistic effect similarly to TAM but it is reported to have small or no proliferative effect on uterus (Fugere et al., 2000). Although Raloxifen was developed initially for breast cancer treatment, its use was abandoned in the late 1980s because clinical trials showed no activity in TAM-resistant patients (Buzdar et al, 1998). Today, Raloxifen is used specifically to reduce the risk of osteoporosis in postmenopausal women at high risk for osteoporosis (Jordan et al., 2001, Cohen et al., 2000).

As SERMs have the ability to provide mixed functional ER agonist or antagonist activity, depending on the target tissue, compaunds devoid of agonist activity have been developed. The most known „pure" antiestrogen is Fulverstant (aslo known as ICI 182780) (Bowler et al., 1989; Wakeling et al., 1991). In addition to blocking the ER activity Fulvestrant induces ER degradation by changing its conformation (Dauvois et al., 1993; Gibson et al., 1991; Reese and Katzenellenbogen 1992). This forces the receptor into conformation that it is recognized as being misfolded, which induces its rapid degradation (Wu et al 2005). Fulvestrant is currently licensed for the use in postmenopausal women with ER-positive recurrent disease (Johnston et al., 2010). However, the lack of agonist activity limits its beneficial effects in bone.

SERM might inhibit the ER found in breast cells but activate the ER present in uterine endometrial cells. That would inhibit cell proliferation in breast cells, but stimulate the proliferation of uterine endometrial cells (Figure 5). There are number of decision points that determine the biological response to a SERM, which is linked to its E2-like ability to recruit CoA-s and CoR-s. ER contains a ligand-binding domain, called Activating Function-2 (AF-2), which is essential for the activation of genes that mediate the E2 effect in tissues like breast and uterus. Therefore, the different ligands can induce distinct gene transcription processes. For example, the union of the ligand binding domain with TAM results in partial agonistic effect in the uterus, whereas the same interaction is fully antagonistic in the breast (Perez et al., 2006).

Similarly to anti-estrogens, anti-progestins or Selective Progesterone Receptor Modulators (SPRMs) are developed in order to antagonize the processes activated by P4. Mifepristone (RU-486) acts as a P4 antagonist by competing with endogenous P4 for receptor binding and has three primary pharmacological effects: endometrial, gonadotropic, and adrenocortical (Goldberg et al., 1998). It has 2 to 10 time higher affinity compared to P4 to bind PRs (Brogden et al., 1993). Because PRs are found primarily in reproductive organs, Mifepristone exerts its principal effect on the uterus. More precisely, Mifepristone blocks the effects of natural P4 on the endometrium and decidua. While P4 is supposed to support the pregnancy, anti-P4 leads to degeneration and shedding of the endometrial lining, thereby preventing or disrupting implantation of the conceptus. Mifepristone also increases both uterine production of prostaglandins and uterine sensitivity to the contractile effects of prostaglandins, stimulating uterine contractions. It is postulated that Mifepristone acts directly on the uterine muscle through an entirely separate mechanism, perhaps by increasing gap junctions in the myometrium (Weiss et al., 1993). Tissue culture studies have shown that Mifepristone continues to display procontractile effects on the uterus even when

the effects of prostaglandins are neutralized (Brogden et al., 1993). Most research and clinical experience with Mifepristone involves its use as an aborted material. Several studies reported its effectiveness in softening and dilation of the cervix prior to surgical abortion, decrease of pain in women with diagnosed endometriosis and in labour inducement (Goldberg et al., 1998). In the absence of P4, however, Mifepristone can act as a partial agonist (Spitz et al., 1993) and upregulate P4-responsive genes, such as p53, and through this possesses a slight anticarcinogenic effect.

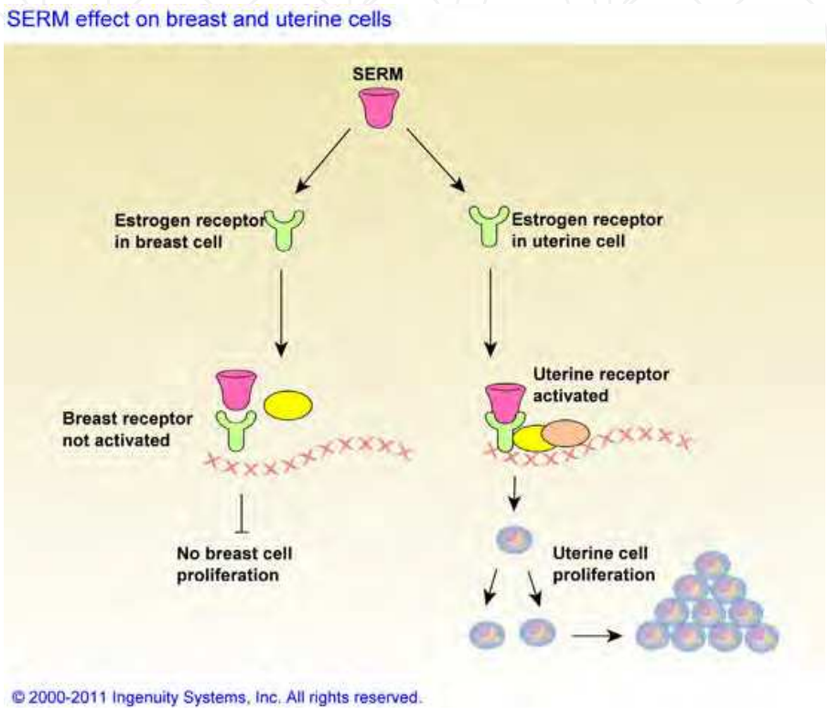

Fig. 5. **The opposite effect of SERM on breast and uterine cell proliferation.** SERM might inhibit the ER found in breast cancer cells but activate the ER present in uterine endometrial cells. A SERM of this type would inhibit cell proliferation in breast cells, but stimulate the proliferation of uterine endometrial cells. (Figure adapted from internet: http://www.cancer.gov/cancertopics/understandingcancer/estrogenreceptors/page14)

3.3 The risk for cancer development after IVF treatment

The impact of infertility and fertility treatments on cancer risk has become more and more prevalent since the increasing need to use IVF treatment in current society. Relatively low number of studies has been published to investigate the relation between IVF treatment and developing cancer risk. The administration of high doses of gonadotrophin analogues during the induction of the ovaries and synthetic E2 and P4 preparations in order to support the endometrium has raised the question of a possible contribution of supraphysiological levels of hormones to the development of breast or other cancer types. Previous studies have demonstrated a possible association between infertility treatments and breast cancer for women treated with at least six cycles with clomiphene citrate, or within the first year after starting IVF (Venn et al., 1995, 2001). Also women who start IVF after the age of 30 appear to be at increased risk of developing breast cancer (Katz et al., 2008; Pappo et al., 2008). Other

studies have found elevated risk for ovarian cancer too but probably the risk was already higher prior to the first IVF (Källen et al., 2005; Kristiansson et al., 2007). However, there are publications, which have not found a relation between infertility treatments and any cancer development (Potashnik et al., 1999, Doyle et al., 2002; Dor et al., 2002; Lerner-Geva et al., 2010; Brinton et al., 2004). For example, a case-control study (1380 pairs) showed no risk for IVF treatment even among women who carry mutations in breast cancer susceptibility gene 1 (BRCA1) or BRCA2 gene (Kotsopoulos et al., 2008). The common opinion today is that the use of fertility medications does not increase the risk of breast cancer among those with family history of BRCA mutations.

A more recent study, published by Källen and colleagues using Swedish cancer register, showed that there was no or significantly low cancer risk among women udergoing IVF treatment compared to general population. The study included 24 058 women who had been treated with IVF where 1279 women later appeared in the cancer register. For comparison, total of 1 394 061 women in the general population were studied as a control group where 95 775 women had registered cancer (Källen et al., 2011).

The phrase "healthy patient effect" has emerged saying that women who choose IVF treatment might be more aware of risks or more health conscious at the time of conception compared to non-IVF women (Venn et al., 2001). In addition, there are numerous confounding factors which could influence the outcome of the study like the age at the time of the first IVF cycle or the first delivery, the number of the unsuccessful cycles and the follow up time after last IVF treatment. It is obvious that the question needs to be studied in more detail involving large number of women and with attention to precise subgroups.

4. Genome-wide E2 and P4 signalling

There are hundreds of studies presenting how expression of a single gene could change upon E2 or P4 treatment in different cell culture. Knock out studies with transgenic animals have confirmed the importance of ERs and PRs in reproductive system and cancer development. To understand the broad role of steroid hormones in humans it is mandatory to study their action in genome-wide level. Recently, the development of large-scale genomic methods to analyse gene expression and factor binding to DNA enable us to study steroid hormone dependent gene expression changes and transcription regulation in the entire genome. As ERs and PRs are acting as TFs they have an ability to regulate the expression of proximal and distal genes by binding hormone responsive elements. Chromatin immunoprecipitation (ChIP) analysis has been broadly used for identification TF binding regions on DNA. ChIP assay can be followed by polymerase chain reaction (PCR), hybridization the probes on a microarray (ChIP-on-chip) or high throughput (HTP)-sequencing (ChIP-Seq) to establish the genomic regions occupied by a specific TF. To understand whether TF binding has a positive or negative impact on gene expression microarrays, sequencing (RNA-Seq) and RT-qPCR are commonly used followed to mRNA extraction.

4.1 Genome-wide identification of TF binding regions, ChIP-Seq

ChIP is a technique for assaying protein-DNA interactions *in vivo* (Weinmann et al., 2002). This analysis allows identifying regions of the genome bound directly to ERs or PRs as well as regions bound indirectly via other TFs or co-regulators. During the procedure proteins are cross-linked to DNA and the chromatin is thereafter sonicated to small fragments

around 150-1000bp depending on which application is used below. After immunoprecipitation of protein-DNA complexes, the cross-links are reversed and the DNA fragments purified. Extracted DNA could be analyzed with either PCR, ChIP-on-chip or direct sequencing. Regions significantly overrepresented in the immunoprecipitated DNA relative to control DNA are regarded as epigenetically modified or protein-bound, depending on the antibody used (Bock et al., 2008). Computational algorithms are used to infer the information from the array data or sequencing output. ChIP has two main drawbacks. First and the main problem is the specificity of antibodies used. The second problem is aggregation of chromatin that contaminates the purified specific chromatin fraction and raises unspecific background of isolated DNA. In case of ChIP-on-chip ChIP-enriched DNA is spotted on glass slide microarrays (chip) to study how regulatory proteins interact with the genome of living cells (Lin Z et al., 2007, Liu et al., 2008). ChIP-on-Chip has many modifications such as ChIP-linked target site cloning (Lin Z et al., 2007) and ChIP coupled with a DNA selection and ligation (ChIP-DSL) strategy for direct target genes, permitting analysis of fewer cells than required by the conventional ChIP-on-chip method (Kwon et al., 2007). The ChIP-DSL technology is distinct from the latter assay. Besides it being more specific and sensitive, the immunoprecipitated DNA is used to template oligonucleotide ligation, instead of being directly amplified for hybridization, which makes it possible to bypass incomplete decrosslinking. There is also the paired-end ditag (PET) approach, which directly links the 5' terminal tags of genomic sequences with their corresponding 3' terminal tags to form PET ditags and concatenates them for efficient sequencing (Bock et al., 2008).

ChIP-Seq is emerging as the method of choice for genome-wide identification of TF binding sites. The ChIP-Seq involves immunoselecting an enriched population of transcription factor-bound chromatin fragments, which are purified and resolved via next-generation sequencing. Today, several DNA sequencing technologies are available - the ABI SOLiD platform utilizes oligonucleotide ligation and detection methodology (Dietz and Carroll, 2008), the sequencing-by-synthesis methods of 454 Life Sciences and Solexa/Illumina technology utilize, an emulsion based PCR followed by HTP sequencing and reversible terminator sequencing respectively. Also it is possible to sequence on single-molecule sequencing platforms such as the HeliScope by Helicos where, fluorescent nucleotides incorporated into templates can be imaged at the level of single molecules (Figure 6). A typical dataset generated from the Illumina Genome Analyzer yields several million short sequence reads with typical length 36-75 bp. These are aligned to a reference genome, and the resulting trace read placements are used to infer the locations of transcription factor binding in a global fashion. ChIP-seq provides clearly interpretable binding information. Even more, compared to ChIP-on-chip data normalization is not an issue because the sequencing results in absolute read counts (Barski et al., 2007). Also, the repetitive portion of DNA is not a hindrance. One limitation is that the process of mapping tags to the reference genome can bias the analysis toward genomic regions with unique and complex sequence patterns. This is because short sequencing reads that overlap with low-complexity regions or with interspersed repeats stand a higher chance of being discarded for lack of unique genomic alignment (Bock et al., 2008). Even though ChIP-seq shares ChIP-on-chip's dependence on high-quality antibodies, the unparalleled throughput makes ChIP-seq superior for whole genome mapping of DNAprotein interactions. The latest results show that ChIP-Seq method could detect more than 10 000 binding regions for ERα in MCF7 cells (Carroll et al., 2006; Hurtado et al., 2011). Nevertheless, linking the binding regions to the

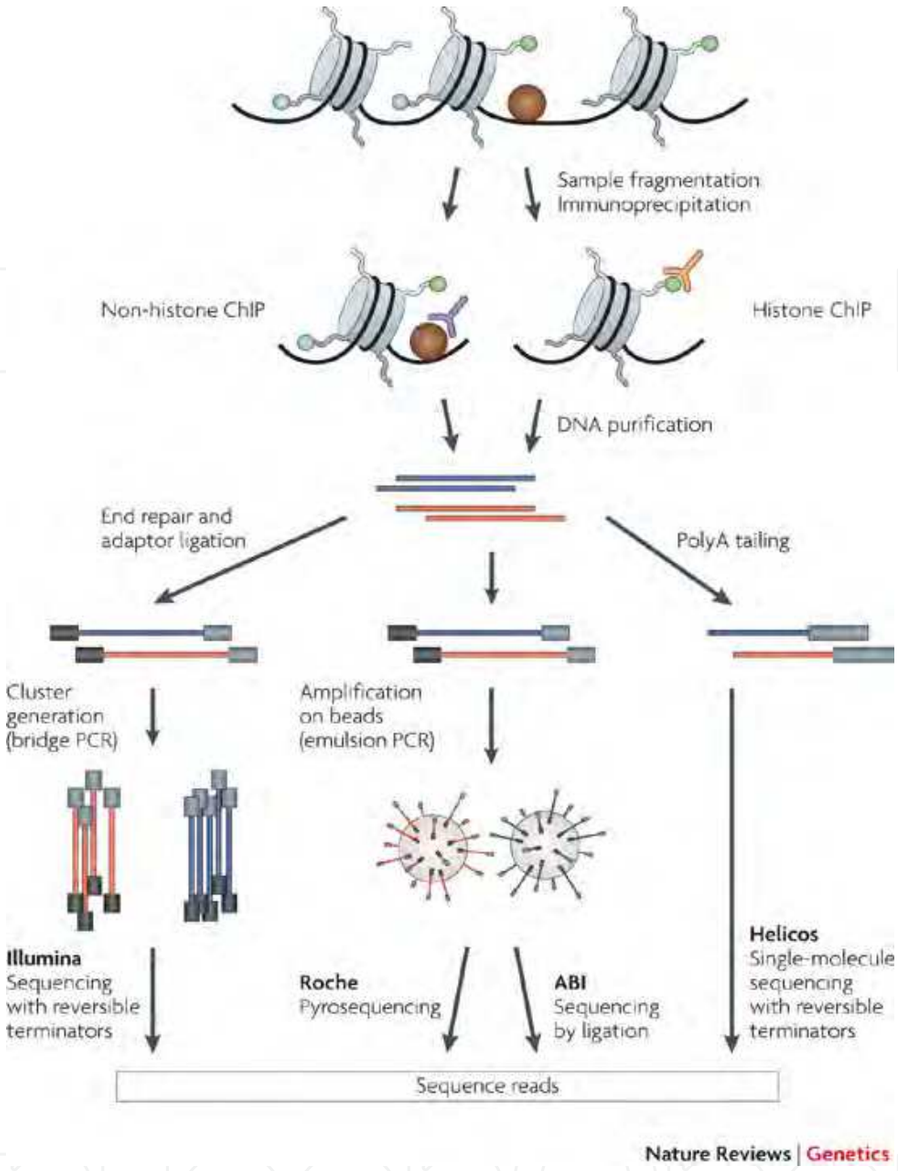

Fig. 6. **ChIP followed by highthroughput sequencing.** The ChIP process enriches the crosslinked proteins or modified nucleosomes using an antibody specific to the protein or the histone modification of interest. Purified DNA can be sequenced using different next-generation sequencing platforms. On the Illumina Solexa Genome Analyzer (bottom left) clusters of clonal sequences are generated by bridge PCR, and sequencing is performed by sequencing-by-synthesis. On the Roche 454 and Applied Biosystems (ABI) SOLiD platforms (bottom middle), clonal sequencing features are generated by emulsion PCR and amplicons are captured on the surface of micrometre-scale beads. Beads with amplicons are then recovered and immobilized to a planar substrate to be sequenced by pyrosequencing (for the 454 platform) or by DNA ligase-driven synthesis (for the SOLiD platform). On single-molecule sequencing platforms such as the HeliScope by Helicos (bottom right), fluorescent nucleotides incorporated into templates can be imaged at the level of single molecules, which makes clonal amplification unnecessary (adapted from Nature Reviews, Park 2009).

target genes has been an on-going struggle as majority of binding regions can be separated by hundreds of kilobases and in some cases megabases. In many cases the biological functionality of the TF binding is still unrevealed.

Fullwood and colleagues have developed a technique called ChIA-PET (chromatin interaction analysis using paired-end tag sequencing) (Fullwood et al., 2009), which couples chromosome conformation capture (Dekker et al., 2002), a method for identifying interacting chromatin regions, with high-throughput sequencing. The authors found 689 ER-associated chromatin interaction complexes made up of duplexes and more complex interactions. These tend to involve stronger ER-binding events, which are biased toward specific histone marks and other transcriptional regulators more imperative for ER function.

Endometrial cell lines seem to be less hormone responsive compared to MCF7. In our previous study we used ChIP-qPCR to identifying ER and PR targets in two endometrial cell lines. We found 137 target genes for ERs in HEC1A and 83 target genes for PRs in RL95-2 from 382 pre-selected genes. The results confirmed the *in vitro* model of non-receptive (HEC1A) and receptive (RL95-2) endometrium in steroid hormone manner (Tamm et al., 2009).

4.2 Expression analysis, RNA-Seq

The transcriptome is the complete set of transcripts in a cell or tissue at a specific developmental stage or physiological condition. Expression microarrays are currently the most widely used methodology for transcriptome analysis. Breast cancer cell line MCF7 is most extensively used cell line in terms of studying E2 responsiveness and ERα localization. The number of genes which could be regulated by E2 has expanded extensively during the last decade from ~100 to ~1500 genes (Frasor et al., 2003, Carroll and Brown, 2006, Kininis et al., 2007, Levenson et al., 2002, Lin et al., 2004, Lin et al., 2007). It is likely that in the near future RNA-Seq, more sensitive technique, will introduce even more genes which show significant change in their activity after E2 or P4 treatment. Gene expression studies investigating endometrial receptivity using human biopsy samples have searched for genes differentially expressed in follicular and luteal phase (Kao et al., 2002; Carson et al., 2002; Riesewijk et al., 2003; Mirkin et al., 2005). The highest number of regulatory genes was brought out in Carson´s study with 323 up-regulated and 370 down-regulated genes comparing follicular phase to the luteal phase. As mentioned before, the overlap of genes identified in different publications is relatively low. The difference could be due to variations is study design and limiting factors of microarray analysis. Microarray is hybridization-based approach, which involves incubating fluorescently labelled cDNA with custom made microarrays. Prominent limitations with this method include hybridization, cross-hybridization artefacts, different data analysis and low coverage of all possible genes in large genomes (Casneuf et al., 2007). Comparing expression levels across different experiments is often difficult and requite complicated normalization methods. The newer and potentially more comprehensive way to measure the whole active transcriptome is by direct ultra-high-throughput sequencing named RNA-Seq. The resulting sequence reads are individually mapped to the source genome and counted to obtain the number and density of reads corresponding to RNA from each known exon, splice event or new candidate gene (Mortazavi et al., 2008). RNA-Seq uses recently developed deep-sequencing technologies where RNA is converted to a library of cDNA fragments with adaptors attached to one or both ends. Each molecule is sequenced from single end or paired end. The reads are typically 30-400bp, depending on the DNA-sequencing technology used. Similarly to ChIP extracted

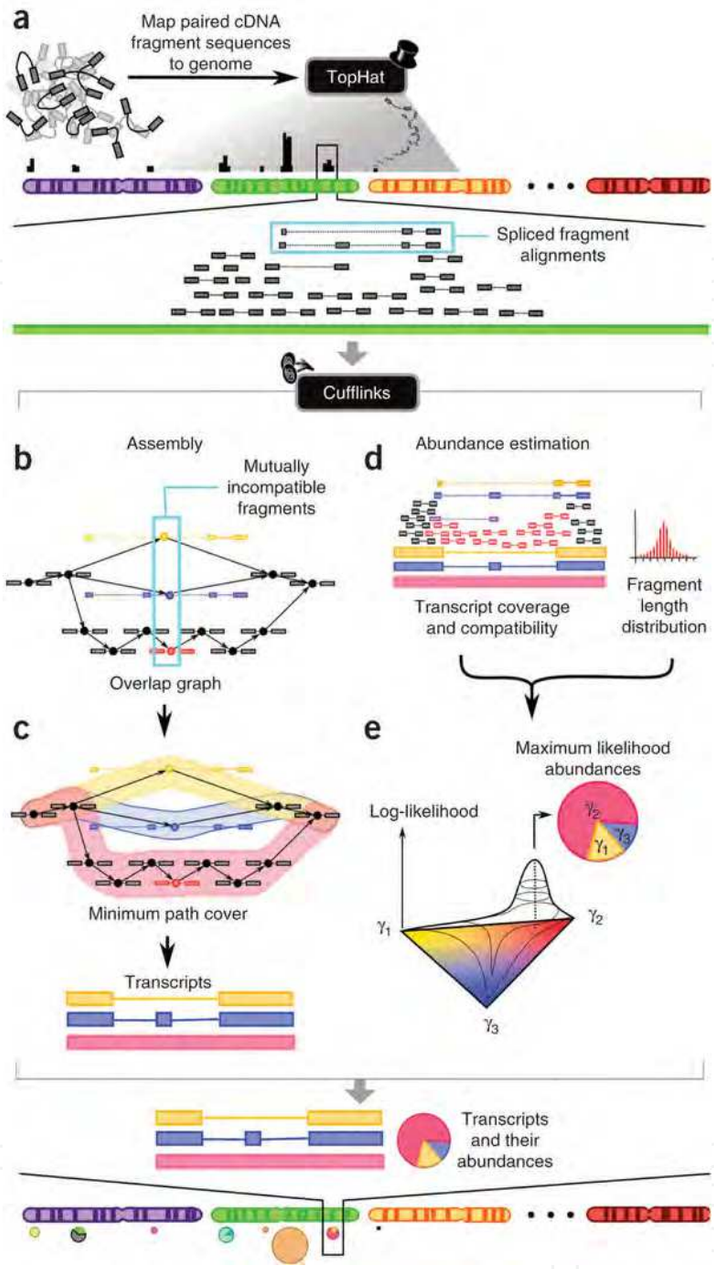

Fig. 7. RNA-Seq method. a) Paired cDNA fragments are mapped to genome using TopHat software b) Each pair of fragment is treated as a single alignment and the abundances of the aasembled transcripts are estimated (b-e). First the fragments from distinct spliced mRNA isoforms are identified (b). Isoforms are then assembled from the overlap graph (c) and transcript abundance is estimated (d). Cufflinks estimates transcript abundances using a statistical model in which the probability of observing each fragment is a linear function of the abundances of the transcripts from which it could be originated. The program numerically maximizes a function that assigns a likelyhood to all possible sets of relative abundances of different isoforms (e), producing the abundances that best explain the observed fragments (adapted from Trapnell et al., 2010).

RNA can be used with Illumina, Applied Biosystems SOLIiD and Roche 454 Life Science platforms (Wang et al., 2009, Rev). RNA-Seq has very low, if any, background signal because cDNA sequences can be mapped to unique regions of the genome. It does not have any upper limit of quantification like DNA microarrays which lack sensitivity for genes expressed either at low or very high levels. Like other HTP Sequencing technologies, RNA-seq faces several bioinformatics challenges in data processing.

The analysis of RNA-Seq data starts from raw cDNA sequences, usually having lengths of 40-70bp, depending on the platform. The general goal is to find which sites in human genome the RNA was transcribed from and determine the expression levels of these transcripts. Additionally, RNA-Seq data can be used to study expression levels of alternative splicing isoforms.

The usual analysis consists of three main steps:

1. Map each RNA sequence to the human genome. Mapping program has to enable spliced alignments because the sequences can come from separated exons. For this a fast and open source tool TopHat can be used (Trapnell et al., 2009)
2. Count mappings to every site and measure the expression level of the sites. The expression level is usually measured in **F**ragments **P**er **K**ilobase of exon per **M**illion fragments mapped (FPKM) which permits comparison of results across experiments. FPKM of a site shows how frequently the mapped fragments fall on that site. This analysis is possible using Cufflinks software, which can measure FPKM for whole genes and also for specific spliced isoforms (Trapnell et al., 2010)
3. Compare experiments and find genes (or spliced isoforms) that have statistically significant different FPKM across experiments. This is also possible using commercial or open source software as Cufflinks.

More detailed explanation of the analysis is depicted in the Figure 7.

5. Conclusion

This chapter summarised the current knowledge of E2 and P4 action in human endometrium and mammary gland. To understand both sides of steroid hormone action - in normal physiology and especially in pathology it is important to understand the molecular intracellular events of E2 and P4 in tissue-type manner. The same hormones could have different or even opposite effects in different tissues. Thus, attention should be applied to steroid hormones' or their analogues' possible side effects before using them in clinical treatments. Questions still remain about the aberrations of the endometrium leading to implantation failure, endometriosis and other dys-regulations. Even though studies of breast cancer development continuously unravel new information about the mechanisms leading from normal to malignant tissue proliferation, breast cancer is still the most fatal cancer type among women. The number of couples seeking for aid to achieve fertility is constantly increasing and thus a better understanding of factors needed for successful treatment and possible side effects is crucial.

We would like to punctuate the importance of the next generation sequencing technologies which we believe are the key in understanding hormone dependent action in whole organism. With today´s knowledge of nearly entire human genome sequence and the development of new technologies based on HTP sequencing it has become possible to define all targets for the TF in vivo and establish entire transcriptome in a single experiment. Data

analysis is still complicated in a way and needs excellent computational skills but the data collected today will become the knowledge of tomorrow.

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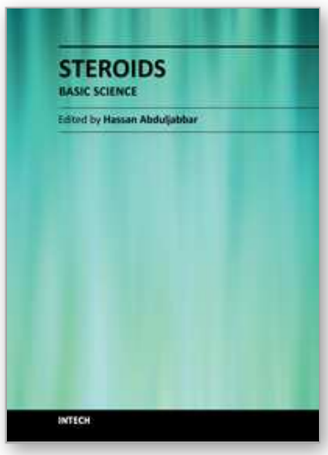

Steroids - Basic Science
Edited by Prof. Hassan Abduljabbar

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This book explains the basic science of steroids and is targeted towards professionals engaged in health services. It should be noted that medical science evolves rapidly and some information like the understanding of steroids and their therapeutic use may change with new concepts quickly. Steroids are either naturally occurring or synthetic fat-soluble organic compounds. They are found in plants, animals, and fungi. They mediate a very diverse set of biological responses. The most widespread steroid in the body is cholesterol, an essential component of cell membranes, and the starting point for the synthesis of other steroids. Since the science of steroids has an enormous scope, we decided to put the clinical aspects of steroids in a different book titled "Steroids-Clinical Aspects". The two books complete each other. We hope that the reader will gain valuable information from both books and enrich their knowledge about this fascinating topic.

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