Estrogen and the endometrium: lessons learned from gene expression profiling in rodents and human

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To date, research into the biological processes and molecular mechanisms associated with endometrial receptivity and embryo implantation has been a focus of attention, whereas the complex events that occur in the human endometrium during the menstrual and proliferative phase under the influence of estrogen have received little attention. The objective of this review is to provide an update of our current understanding of the actions of estrogen on both human and rodent endometrium, with special emphasis on the regulation of uterine growth and cell proliferation, and the value of global gene expression analysis, in increasing understanding of these processes.

Keywords: estradiol; endometrium; uterus; gene profiling; proliferation

Introduction

The human endometrium is an amazingly plastic tissue. Throughout the adult reproductive life, monthly steroid hormone controlled cycles of proliferation, differentiation and degeneration occur continuously. Even after menopause, the tissue retains its responsiveness to steroid hormones and endometrial cycles can also be induced. In each menstrual cycle, if no embryo implantation occurs, the functional layer of the endometrium is shed and within 2 weeks the complete functional layer is restored. The events underlying this phenomenon are highly complex and include repair of the endometrium surface, proliferation, angiogenesis, vasculogenesis, cell differentiation and extracellular matrix remodelling. Once the functional layer has successfully been rebuilt, the actions of progesterone change the estrogen primed endometrium into a receptive state (Martin *et al.*, 2002; Navot *et al.*, 1989; Riesewijk *et al.*, 2003).

During the menstrual cycle, two phases of elevated estrogen concentrations can be distinguished. During the proliferative phase, the growing follicles produce increasing amounts of estradiol (E_2) that peak at ovulation. After ovulation the corpus luteum continues to produce significant amounts of estrogens, in addition to progesterone. However, it has been shown that this mid-luteal rise in estrogen is not essential for successful implantation in the human (Ghosh *et al.*, 1994; Smitz *et al.*, 1993).

Prior to ovulation, the role of estrogen is considered to be important in the regeneration and growth of the endometrium and to prepare the tissue to respond to progesterone post-ovulation. Until the advent of microarray technology, the study of complex biological mechanisms was hindered by the fact that only the expression of individual genes could be investigated. Genome wide gene expression analysis has proven to be a powerful approach to revealing individual genes and signalling cascades that are directly or indirectly affected by the steroid hormones.

Estrogen regulation of uterine and endometrial growth

Estrogen regulation of uterine growth in rodents

The uterus in rodents and the human undergoes cyclical changes of growth and degeneration. In both species, estrogens produced from the developing follicles stimulate endometrial growth, and progesterone is responsible for converting the estrogen-primed endometrium into a receptive state. In rodents, if pregnancy does not occur, diestrous (secretory phase in humans, cycle days 15–28) terminates with regression of the corpus luteum, and the endometrium is resorbed (menstruation in humans, cycle days 1–5). During proestrous (proliferative phase in humans, cycle days 6–14) follicles develop and start to produce estrogens that stimulate endometrial growth. During estrous (peri-ovulatory period in humans, cycle days 13–15) ovarian follicles mature.

Rodents are versatile animal models that allow precise hormonal manipulation of the endometrium, usually after ovariectomy. In the classical sense, the uterine growth responses in rodents are grouped as early and late responses in relationship to a single dose of E_2 (reviewed by Barton *et al.*, 1998). The early responses that usually occur during the first 6 h after administration of estrogen

include increases in RNA and protein synthesis as well as water imbibition. Late estrogen responses include cycles of DNA synthesis and epithelial cell mitosis, which begin 10–16 h after E_2 administration. Two waves of mitotic activity are generally seen. One wave after ∼16 h and one after ∼24 h.

The magnitude of uterine growth stimulation is largely dependent upon the duration of bioavailable E_2 and receptor interaction (Agarwal *et al.*, 1982). Oestriol, which is a short-acting estrogen agonist, stimulates the early events following a single aqueous dose, but does not stimulate cell proliferation. The inability of oestriol to stimulate cell proliferation could be due to a rapid clearance of this steroid and low affinity to estrogen receptors. Similarly, administration of single doses of both E_2 and oestriol immediately activate early genes such as MYC and FOS, but additional estrogen is required for a cell to complete G1 and enter the S-phase (Hyder *et al.*, 1994; Loose-Mitchell *et al.*, 1988).

Administration of a low dose of E_2 (0.25 or 2.5 µg/animal) to immature rats caused nuclear translocation after 1–3 h and maintenance of uterine growth occurred after 24 h. At a higher dose (10.0 µg/rat), circulatory E_2 levels were maintained longer and a biphasic nuclear translocation occurred. The uterus continued to grow until 72 h, reaching five times its original wet weight. Administration of one dose of oestriol, a short-acting estrogen, induces the same early responses as E_2, however, no uterine proliferative response is induced (Cheng *et al.*, 1985). These studies show that a single injection of sufficient amounts of E_2 induces endometrial growth and maturation in rats, provided estrogen levels remain elevated in the circulation for a long period of time. This is supported by reports which show that the administration of increasing doses of E_2 is required to sustain a full uterine response. Treating immature rats with a single bolus of a long-acting estrogen (17α-ethinyl oestriol-3-cyclopentyl ether, EE3CPE) did not result in further increases in uterine weight beyond 24–48 h. In contrast, multiple injections of EE3CPE for 72 h produced a progressive increase in tissue and uterine weight markedly above the 24 h level, and responsiveness to E_2 is maintained (Katzenellenbogen *et al.*, 1977). Medlock *et al.* (1991) observed that rats receiving silastic implants with a pharmacological dose of E_2 (5.0 mg/ml) did maintain the maximal uterine weight gain through 24 h, whereas subcutaneous injections of a single dose (1.0 and 10.0 µg) caused only a significant and equivalent increase in uterine weight at 6 h, but the weight gain could not be maintained for long. Treating adult mice and immature female rats for several consecutive days with the same dose, also renders the uterine epithelial cells 'refractory' (Newbold *et al.*, 2001; Stormshak *et al.*, 1976), and only when challenged with a higher dose this 'refractory' state could be overcome (Stormshak *et al.*, 1976).

The minimal dose of E_2 that is required to induce uterine sensitivity for implantation was determined to be in the range of 1.5–3 ng (Ma *et al.*, 2003; Milligan *et al.*, 1995). Ma *et al.* (2003) showed that the concentration of E_2 also controls the length of the window of uterine receptivity. At different physiological concentrations implantation can be initiated, however, high doses shorten this period. This is associated with aberrant expression of implantation-related genes including *LIF*, *PTGS1* (cyclooxygenase 1) and *AREG* (amphiregulin). Finn *et al.* (1995) showed that administration of high doses of E_2 stimulated cell division, but no decidualization occurred.

Collectively, these findings suggest that uterine levels of E_2 must exceed a certain level to initiate the early events associated with the induction of uterine growth. However, sustained bioavailability of estrogens as well as the receptors is required to induce a full uterine proliferative response, and excessive levels of estrogen may have adverse effects on implantation.

Estrogen regulation of endometrial growth in the human

The role of estrogen in the regulation of human endometrium is still elusive. In contrast to the rodent uterus, endometrial growth in humans is not a result of water imbibition, but mostly a result of cellular amplification. Repair of the endometrial surface is already initiated during the menstruation process in the remaining basal layer, prior to any increase in estrogen concentrations (Ferenczy, 1976). This process was also shown to involve recruitment of bone marrow derived cells (Taylor, 2004). Proliferative activity in the basal layer remains constantly low, and once estrogen concentrations increase, proliferative activity in the developing functional layer of the human endometrium is induced. Proliferative activity peaks between cycle days 8 and 10 (Ferenczy *et al.*, 1979).

In humans, a minimum of 5 days of estrogen exposure is required to build a sufficiently thick endometrium to allow implantation of the embryo (Michalas *et al.*, 1996; Navot and Bergh, 1991). In this regard, it worth mentioning the recent study from Kurita *et al.* (2005) who made tissue recombinants from uterine stroma of newborn mice and epithelial cells either from newborn murine uteri or from adult human endometrium. These were placed under kidney capsules of female nude mice, which were ovariectomized 4 weeks later. After two additional weeks the animals were treated with E_2. Similar to what is observed *in vivo* in the mouse and human, the proliferative response in the mouse epithelium was visible after 1 day, whereas the human epithelium required 5 days of E_2 exposure to show a maximum response. In addition, the human uterine epithelial cells responded to E_2 by up-regulation of progesterone receptor (PR), whereas in the mouse epithelium PR expression was down-regulated.

Previous studies from the same group have shown that the proliferative response in endometrial epithelium is regulated by the stromal compartment. The fact that uterine stroma from a mouse shows the same response as adult endometrial stroma from the human, indicates that these stroma-mediated effects are not species specific. The distinct responses in the mouse and human uterine epithelial cells suggest that the epithelial cells respond differently to the cues from the stromal compartment. This means that it is likely that the early responses of the stromal compartments of mouse and human endometrium show similarities, maybe allowing careful extrapolation of findings in mouse studies to the human, whereas the late responses in the epithelial compartments show more disparities.

Studying the effect of estrogens on human endometrium is complicated. Most information about the role of estrogen in the regulation of endometrial development have been obtained in IVF patients in either natural or artificially induced cycles. During IVF, high estrogen concentrations as a result of the hyperstimulation of the ovaries are thought to result in a disparity in maturation between the epithelium and the stroma which is more advanced in

its development (Noci *et al.*, 1997), possibly as a result of premature steroid receptor down-regulation (Develioglu *et al.*, 1999; Noci *et al.*, 1997). When E_2 concentrations were >2500 pg/ml on the day of human chorionic gonadotrophin injection, significant decreases in pregnancy and implantation rates were observed compared with patients having low E_2 concentrations, whereas embryo quality was unaffected (Pellicer *et al.*, 1996; Simon *et al.*, 1995). Reducing the E_2 levels using a step-down protocol significantly improved implantation and pregnancy rates compared to the patients that received the standard protocol, without affecting the fertilization rate and the number of good-quality embryos (Simon *et al.*, 1998). In addition, optimal pregnancy rates were achieved when estrogen was administered for 6–11 days (Michalas *et al.*, 1996; Navot and Bergh, 1991) or 12–19 days (Younis *et al.*, 1992) before progesterone administration.

Others, however, have not find abnormal endometrial morphology or reduced implantation and pregnancy rates at high hormone levels (de Ziegler *et al.*, 1991; de Ziegler and Bouchard, 1993; Sauer *et al.*, 1990; Serhal and Craft, 1987; Sharara and McClamrock, 2000). Increased implantation and pregnancy rates per embryo transfer were found in cycles with high E_2 levels (>5000 pg/ml) compared with controls (Gelety and Buyalos, 1995). The length of estrogen exposure was also shown to be flexible, ranging from as short as 6 days to as long as 60 days without affecting receptivity or pregnancy rates (Borini *et al.*, 2001; Navot *et al.*, 1984; Serhal and Craft, 1987; Yaron *et al.*, 1995). In line with these findings, Remohi *et al.* (1997) observed that implantation and pregnancy rates were also normal at very low concentrations (<50 pg/ml).

Obviously our knowledge about the regulation of endometrial growth and differentiation by estrogen shows dramatic lacunas. Studying estrogen regulation from a genomics perspective may provide new insights into the cellular regulatory mechanisms involved.

Global gene expression profiling

Gene expression studies in rodent uterus

The outcome and interpretation of global gene expression profiling studies is influenced by the use of different array platforms, the use of different protocols for sample and probe preparation, differences between mouse strains, the manner of application of the steroids and differences in data processing and analysis. (Table 1 presents a summary of gene expression studies aimed at evaluating the effects of estrogen). For human endometrium, this was first demonstrated by Horcajadas *et al.* (2004). The authors compared the results of four studies on gene expression in human endometrium collected during the implantation window and reported only three genes that were up-regulated in all four studies (osteopontin, apolipoprotein D, Dickkopf) and one down-regulated gene (olfactomedin-1) (Horcajadas *et al.*, 2004).

Similar disagreements among array studies are also present among rodent studies. For instance, both Hong *et al.* (2004) and Watanabe *et al.* (2003, 2004) treated ovariectomized mice for 6 h with E_2 and of the reported genes that were most affected, only seven were commonly regulated (Table 2). When including the findings of the study from Hewitt *et al.* (2003), only *MAD2*

and Small proline-rich protein 2A were up-regulated by E_2 in wild-type mice in all three studies.

The choice of animal model system will also have an impact on the outcome. For studying the effects of steroid hormones on steroid-responsive tissues, ovariectomized animals receiving hormone replacement is an established animal model. Alternatively, researchers have employed immature animals. Immature animals were shown to be more sensitive with regard to the detection of estrogenic effects than the ovariectomized adult animals (Kang *et al.*, 2000). In the study of Naciff *et al.* (2003) prepubertal rats received increasing doses of ethynyl E_2 (EE, 0.001–10 μg/kg) and the changes in gene expression were monitored using the Affymetrix Rat Genome U34A high-density oligonucleotide array. In this study, 24-h exposure to a high dose (10 μg/kg) of EE induced a 5-fold increase in uterine wet weight and a 2-fold increase in uterine height. In contrast, treating adult ovariectomized rats with 500 μg/kg of EE did not even increase uterine wet weight (Wu *et al.*, 2003). Moreover, the magnitude of gene expression induced by E_2 was higher in the immature rats than in the adult ovariectomized rats, i.e. complement component 3 and *CD24* were induced 300- and 7.5-fold in the study of Naciff *et al.* versus only 3.6- and 2.8-fold in the study of Wu *et al.* (2003). Whether the immature rat model or the ovariectomized/E_2 supplemented adult rat model is the most reliable model for studying the role of E_2 in the endometrium and for extrapolating findings to the human situation has yet to be decided.

The major advantage of animal models is their flexibility. Using recombinant DNA technologies, the animals can be genetically altered, and it is possible to perform longitudinal studies. For example, the mouse models were proven to be very illustrative in demonstrating the roles of the two ER isoforms, ERα and ERβ, in endometrial regulation. The availability of mouse strains in which the ERα (αERKO mice) or ERβ (βERKO mice) has been ablated, paved the way to study the selective actions of ERα and ERβ (Hewitt *et al.*, 2003). The early and late responses of the βERKO mice were indistinguishable from those of wild-type samples, whereas the αERKO mice showed little response to E_2 (Hewitt *et al.*, 2003). These observations indicate that ERα is essential for mediating the actions of E_2.

Using the same mouse models, Hewitt *et al.* (2005) also showed that IGF-1 and ER signalling pathways act in parallel with regard to the regulation of gene expression, and that treating αERKO mice with IGF-1 elicited certain responses that closely resembled the response induced by E_2 in wild-type mice. Certain genes were regulated similarly (up: *IGFBP5*, *CYR61*, *p21*, *c-fos*; down: *Txnip*, *IGFBP3*, *SOX4*) by E_2 and growth factors in wild-type mice, and retained growth factor responsiveness in the αERKO mice. However, another group of genes was only regulated by E_2 and only in the wild-type mice (*MAD2*, *RAMP3*, *LF*, *IGF-1*, *KRT1-19*), and they therefore depend on the presence of ERα. A third group of genes was regulated only by the growth factors (Baiap2, Kruppel-like factor 9). This confirms earlier findings that growth factors and ER signalling pathways converge in the regulation of some uterine functions that still depend on a correct ER pathway. EGF and IGF-1 treatment of ovariectomized mice resulted in increased uterine weight and proliferation of uterine epithelial cells (Klotz *et al.*, 2002; Nelson *et al.*, 1991), but these responses were not observed in the αERKO mice (Klotz *et al.*, 2000, 2002).

Table 1: Summary of gene profiling studies in rodents and humans to study estrogen regulation of gene expression in uterus and endometrium

A. Changes in menstrual and oestrous cycles

Human endometrium

    Menstrual (*CD3-4*) versus LP (*CD12-13*) phase endometrium (Punyadeera *et al.*, 2005)

    Proliferative phase endometrium, LCM of epithelium and stroma (Yanaihara *et al.*, 2005)

    Menstrual and proliferative phase endometrium chemokine array (Jones *et al.*, 2004)

    Various stages of the proliferative and secretory phase (Ponnampalam *et al.*, 2004)

Mouse endometrium

    Oestrous versus diestrous (Tan *et al.*, 2003)

    Wild-type (Wt) ovariectomized (ovex) mice, estradiol (E_2) 100 µg/animal for 6 and 24 h, followed by LCM epithelial glands and stroma (Hong *et al.*, 2004)

B. Ovariectomized rodent models

    Wt mice sacrificed 2, 8, 12, 24 h after one dose of EE (100 µg/kg), or 72 h (dosed 3×, every 24 h) (Fertuck *et al.*, 2003)

    Wt mice sacrificed 0, 1, 2, 6, 12, 24, 48 h after one injection of E_2 (5 µg/kg). Extra control, αERKO mice treated with E_2 for 6 h (Watanabe *et al.*, 2003)

    Wt and αERKO mice sacrificed 6 h after treatment with E_2, dose range 0.5–50 µg/kg (Watanabe *et al.*, 2002)

    Wt, αERKO, βERKO mice sacrificed 0.5 and 2 h after 1 µg/kg E_2 i.p. in saline, or 6, 12, 24 h after 1 µg/kg E_2 sc in oil; additional groups received 45 µg/kg ICI in DMSO i.p., 30 min prior to E_2 (Hewitt *et al.*, 2003)

    Wt and αERKO mice sacrificed 2 and 24 h after administration of 1 µg/kg E_2, or sacrificed 2 h after i.p. injection with 200 µg EGF or IGF-1 analogue 2 h after i.p. injection, or 24 h after administration by osmotic pumps (Hewitt *et al.*, 2005)

    Rats sacrificed after 3 days after receiving 1 µg/animal/day E_2 (Rochett *et al.*, 2002)

    Rats sacrificed after 1, 4, and 7 days after receiving 2.5 mg/animal/day E_2 (Wu *et al.*, 2003)

C. Immature rodent models

    Wt mice, 20–21 days old, sacrificed 1, 2, 4, 8, 24, 72 h after administration of a high dose E_2 400 µg/kg (Moggs *et al.*, 2004)

    Wt mice treated with 50 µg/kg E_2 for 3 consecutive days (Waters *et al.*, 2001)

    Wt rats treated sc with 0.001–10 µg/kg EE for 4 consecutive days (Naciff *et al.*, 2003)

D. *In vitro* models

    Primary cultures epithelium and stroma treated with E_2, tamoxifen and raloxifene (Pole *et al.*, 2005)

Table 2: Genes regulated by E_2 in endometrium of ovariectomized mice after 6 h (Hong *et al.*, 2004; Watanabe *et al.*, 2003, 2004)

Thioether-*S*-methyltransferase
Serum-inducible kinase
Mitotic checkpoint component *MAD2*
Small proline-rich protein 2A
Chemokine orphan receptor 1
GTPase (Ran)
Kruppel-like factor 4 (gut)

Phenotypic anchoring

To understand complex mechanisms, relationships must be defined between the changes in gene expression and the alterations that occur in the cells or tissues (Moggs *et al.*, 2004). In toxicogenomics this is termed 'phenotypic anchoring' (Paules, 2003). Moggs *et al.* (2004) applied this approach to define the transcriptional program associated with the response of the rodent uterus to E_2 and to identify groups of genes that result in specific histological changes. A single high dose of E_2 (400 µg/ml) was administered to immature mice, which induced a sustained increase in uterine weight. In addition, uterine expression profiles were assessed after 1, 2, 4, 8, 24, 48 and 72 h. The 3538 E_2-responsive genes were subjected to hierarchical clustering to identify the temporarily co-regulated genes and the clustered genes were further interrogated using the GOStat gene ontology mining tool to gain an overview of the predominant molecular functions and biological pathways that were regulated at the transcriptional level. The temporal associations in gene expression were anchored to distinct alterations in uterine phenotype. The authors found that E_2 regulates different classes of genes during narrow time windows, and suggested that E_2 induces uterine growth and maturation by successively regulating the activities of different biological pathways. In the first 4 h after injection of E_2, a major influx of fluid into the uterus is seen, most likely due to an increase in vascular permeability due to increased expression of VEGF. Many genes that have roles in the regulation of vascular permeability were up-regulated (e.g. angiogenic/vascular cell growth factors *VEGF*, *PlGF*, *ADM*, *ANG2*, *TGFβ2*, the vasoactive serine protease *KLK*2, *-6*, *-9* and *-22*, and vascular endothelial receptors *IL17R*, *BDKRB1*, *ENG* and *GNA13*). Next to these vasoactive substances, a rapid induction of transcriptional regulators and signalling components involved in regulating growth and differentiation is observed.

Moggs *et al.* (2004) also reported that between 4 and 8 h after E_2 injection, no obvious changes in uterine histology occurred. However, many genes involved in mRNA (and protein synthesis) are induced, whereas a number of known transcriptional repressors (i.e. *TGIF*, *MAD4*, *EZH1*) are suppressed. These changes are required to increase the mass of uterine cells to provide sufficient cellular components required for survival of the daughter cells (Norbury and Nurse, 1992). These events occur immediately preceding the up-regulation of genes involved in controlling chromosome replication and the cell cycle. Luminal epithelial height doubled between 8 and 24 h, and mitotic activity was dramatically increased 24 h after E_2 injection and decreased again at 48 h. This agrees with the contention that most cells in the rodent uterus are stimulated to leave their quiescent state and divide synchronously after exposure to E_2 (Quarmby and Korach, 1984). After induction, the expression of most genes

decreased to levels well below that of the control animals, suggesting active repression to prevent further rounds of proliferation.

In parallel E_2 appeared to suppress the apoptotic process by inducing the expression of anti-apoptotic genes and simultaneously down-regulating the expression of pro-apoptotic genes. The investigations were able to provide more insight into the possible mechanisms that may be involved in the various events that lead to a receptive endometrium.

In this particular study (Moggs *et al.*, 2004), the experiment was performed three times, and the observed uterine weight responses as well as the expression profiles of the estrogen-responsive reference genes including *FOS* and *LTF* were highly reproducible. The combination of independent experiments and the use of stringent selection criteria have increased the reliability of the findings. One would expect than that in independent studies performed in other laboratories some agreement exists. In a similar study also performed in ovariectomized immature mice by Fertuck *et al.* (2003), temporal patterns of gene expression were identified after oral administration of EE instead of E_2. Using functional gene annotation information from public databases, Fertuck *et al.* (2003) established associations between changes in gene expression and the pathways involved in the uterotrophic response. After K-means clustering, seven temporal gene expression patterns could be distinguished: genes induced at 2, 8, 12, 24 and 72 h, genes induced at 8 and 3×24 h, and genes induced at 24 and 3×24 h. Even these rigorous experimental and bioinformatics approaches resulted in only 31 genes that were also reported by Moggs *et al.* (Table 3). Of these 31 estrogen-regulated genes, only 14 showed similar temporal changes in gene expression. Two of the genes that were down-regulated were cell cycle-related, *CCNG2* and *GADD45*. These genes were also reported to be down-regulated prior to the induction of proliferation in other mouse studies (Watanabe *et al.*, 2003). The difference in gene expression profiles could be explained by the fact that Fertuck *et al.*, used EE, a synthetic steroid which is significantly more stable than E_2. Others, however, showed that the transcriptional profile in the rat uterus induced by EE is very similar to that of the endogenous E_2 (Hyder *et al.*, 1999). This illustrates clearly that despite rigorous statistical procedures and validated experimental designs, the value of the findings for the scientific community is limited due to the large inter-laboratory variation.

Estrogen regulation of gene expression in human endometrium

The exposure of the human endometrium to estrogen increases after ~5–6 days after the onset of menstruation. At this point, the endometrial surface repair is already completed (Ferenczy, 1976), and a major role has been indicated for the bone marrow-derived cells, which constitute about half the endometrial cell population (Taylor, 2004). The major role of estrogen is supposedly to modulate the growth of the human endometrium by inducing proliferation. The proliferation index peaks between 8 and 10 days after the onset of menstruation in the upper one-third of the functionalis layer (Ferenczy, 1976). At the same time, blood vessels have to develop to supply the growing tissue with nutrients and oxygen. This process appears to occur mostly as the result of

vessel elongation rather than increased endothelial cell proliferation. The fact that endothelial cells only express ERβ and not ERα (Critchley *et al.*, 2001) supports the contention that these cells are not primary targets of the proliferative effects of E_2, which is mostly mediated by ERα and not ERβ. Following the increase in mitotic activity in the endometrium, stromal oedema increases (Dubowy *et al.*, 2003), which is indicative of increased biological activity of *VEGF-A* leading to increased vascular permeability. However, we were not able to confirm that the expression of *VEGF-A* and its receptors show significant increases during this time (Punyadeera *et al.*, 2006).

When searching the literature for microarray studies aimed at understanding the role of estrogen in the regulation of endometrial function, only a few studies are useful. Ethical restrictions limit the design of clinical studies, whereas *in vitro* models based on human endometrial cells have shown loss of steroid responsiveness. Only one study attempted to use a genomics approach to study the effects of estrogens on gene expression in cultured endometrial cells. Pole *et al.* (2005) sought to compare and characterize the transcript profile of tamoxifen, raloxifene and the agonist E_2 in human endometrial cells. Tissues ($n = 3$) were collected in the proliferative phase of the menstrual cycle. The authors found 230 significant changes in gene expression for epithelial cells and 83 for stromal cultures, either specific to E_2, tamoxifen or raloxifene, or changing across more than one treatment. Remarkable findings of this study are that (i) there were limited fold-changes observed, not exceeding 2.5-fold, (ii) there were a limited number of target genes shared by E_2 and tamoxifen (7/118) and E_2 and raloxifene (6/94), (iii) there were more genes comparably regulated between the SERMs tamoxifen and raloxifene than between E_2 and either tamoxifen or raloxifene and (iv) only three genes were also differentially expressed in our study in endometrium tissues (Punyadeera *et al.*, 2005). A drawback of this study is that no validation experiments were performed to confirm the findings of the microarray analysis, which limits the reliability of the data.

In vitro tests offer several advantages including a low intra-assay variability; however, they do not reflect the sophisticated processes that occur in an intact tissue or animal, and therefore often show impaired steroid-responsiveness. *In vitro* studies based on whole tissue as we showed in an earlier study (Punyadeera *et al.*, 2004), and *in vivo* studies have the added advantage that they may offer the opportunity to project the findings to the human situation.

Yanaihara *et al.* (2005) used laser capture microdissection (LCM) to study gene expression in epithelial and stromal cells of proliferative endometrium (CDs 6–9) of normal human endometrium from fertile women. This approach also allows the study of gene expression in individual cell populations at a given time point. Unfortunately, the investigators used BD Atlas Nylon cDNA Expression Arrays with a limited number of probes that resulted in the identification of only 14 and 12 genes that were strongly expressed in epithelial and stromal cells, respectively. Three of these genes are known cell cycle regulators, *CDC28* protein kinase 2 (*CKS2*), *CCNA1* and *CCNB1*. The objective of the study was to evaluate the gene expression profiles in epithelial and stromal cells, therefore no inferences could be made with regard to the actions of E_2. However, one gene, decorin, was subsequently shown to be regulated by estrogen in stromal cells.

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Table 3: E$_2$-regulated genes in uteri of ovariectomized mice after phenotypic anchoring (Moggs *et al.*, 2004; Fertuck *et al.*, 2003)

Gene symbol	Gene name	Fertuck *et al.*				Moggs *et al.*			
		2	8	24	72	2	8	24	72
ATF4	Activating transcription factor 4	1.38	2.23	0.52	1.35	3	1	1	0
CCNG2	Cyclin G2[a]	0.98	0.09	0.18	0.68	0	−2.5	−2.5	0
CFI	Complement component factor i[a]	0.43	0.43	0.33	23.12	0.5	0	3	3
CLCA3	Chloride channel calcium activated 3[a]	1.05	0.33	5.26	8.34	−1.5	−3	3	3
CRTR1-pending	*TCFCP2*-related transcriptional repressor 1[a]	1.42	0.53	0.44	0.43	2	1	−1	0
CTSH	Cathepsin H[a]	0.74	0.59	0.93	1.59	0	0	1	1.5
DDX21	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 21 (RNA helicase II-Gu)	1.00	3.40	0.93	4.68	2	1.5	1	0
EIF1A	Eukaryotic translation initiation factor 1A	1.60	2.66	1.16	1.89	2	2	2.5	0.5
EZH1	Enhancer of zeste homolog 1 (Drosophila)	0.76	0.59	2.56	0.84	−2	−1.5	−1	0.5
GADD45A	Growth arrest and DNA-damage-inducible 45 alpha[a]	5.42	2.97	1.12	2.54	3	3	2	3
H2-D1	Histocompatibility 2, D region locus 1	0.37	1.48	0.70	1.41	0	0	0	2.5
HRMT1/2	Heterogeneous nuclear ribonucleoproteins methyltransferase-like 2 (*S. cerevisiae*)[a]	0.49	7.28	4.57	0.70	0.5	1.5	1.5	0
LCN2	Lipocalin 2	1.32	0.72	0.92	0.86	1	3	3	3
MUC1	Mucin 1, transmembrane[a]	0.54	2.18	3.73	7.20	0	0.5	3	3
MX1	Myxovirus (influenza virus) resistance 1	1.01	1.42	1.41	10.94	2	3	3	3
NCL	Nucleolin[a]	2.04	2.79	3.61	1.85	0	1	1	0
PSMB2	Proteasome (prosome, macropain) subunit, beta type 2	1.04	2.92	1.95	1.43	0.5	0.5	1	0
PSMB3	Proteasome (prosome, macropain) subunit, beta type 3[a]	0.74	2.58	2.15	1.27	0	1	1.5	0
PSMB4	Proteasome (prosome, macropain) subunit, beta type 4	0.95	1.27	0.70	1.26	1	2.5	1	0
PSMB6	Proteasome (prosome, macropain) subunit, beta type 6	0.99	2.10	1.14	1.25	0.5	2	2	0
RAMP1	Receptor (calcitonin) activity modifying protein 1	0.73	0.25	0.28	0.42	−1	−2	−3	0
RRM1	Ribonucleotide reductase M1	1.16	3.58	2.90	3.22	−0.5	0.5	0.5	−2
RRM2	Ribonucleotide reductase M2	2.58	1.31	4.14	2.65	0.5	0.5	1	−3
SFRS10	Splicing factor, arginine/serine-rich 10	1.91	1.56	1.34	2.24	3	3	3	0
SMN	Survival motor neuron[a]	1.68	2.39	2.28	0.85	0.5	0.5	1	0
SNRK	SNF related kinase[a]	2.01	0.61	0.73	0.44	3	−1	0	1
SOCS1	Suppressor of cytokine signaling 1[a]	4.81	5.64	2.29	0.56	3	3	3	0
SOCS3	Suppressor of cytokine signaling 3	8.85	4.26	1.09	1.39	3	3	3	3
SPRR2A	Small proline-rich protein 2A[a]	0.37	1.22	32.23	31.19	0	3	3	3
TAF10	*TAF10* RNA polymerase II, TATA box binding protein (TBP)-associated factor, 30 kDa	0.96	1.04	0.66	1.00	0	0.5	1	0
THBS1	Thrombospondin 1	1.26	1.57	1.64	1.27	0.5	2	0	2

[a]Genes that show similar response profiles in both studies.

There is only one study which has focussed on elucidating the actions of estrogen in the human endometrium (Punyadeera *et al.*, 2005). Gene expression profiles were compared between late proliferative (LP) and menstrual (M) phase endometrium. Genes expressed or suppressed in LP endometrium would reflect genes that are expressed at a late stage of endometrium development. We identified 282 gene transcripts that were up-regulated and 512 gene transcripts that were down-regulated in the LP phase compared with the M phase endometrium. As expected, some gene transcripts were elevated during menstruation (M phase endometrium) for example, inflammatory cytokines, enzymes involved in eicosanoid biosynthesis and immunomodulators and their receptors. Also angiogenic modulators, hypoxia-induced proteins (i.e. heamoxygenase-1, adrenomedullin, carbonic anhydrase II, *VEGF*, *CYR61* and hypoxia-induced protein-1) and MMP's were highly expressed in M phase endometrium (Punyadeera *et al.*, 2005). In turn, the expression of different cell cycle regulators was overexpressed in LP phase endometrium (Table 4).

We compared these profiles also with the profiles of explant cultures prepared from the same biopsies (M and LP phase endometrium) treated with E$_2$ for 24 h. This approach would theoretically distinguish genes that are directly regulated by estrogen from those that require extended exposure to estrogen. We found 148 and 45 gene transcripts to be up- and down-regulated, respectively, by E$_2$ in M phase endometrium. In LP phase endometrium only 12 transcripts were up-regulated and four transcripts were down-regulated by E$_2$. This clearly demonstrates that the responsiveness of the human endometrium is reduced after prolonged exposure to E$_2$ *in vivo*, probably because all relevant genes have already been activated at this time.

In contrast, when these tissues are treated with progesterone, LP phase endometrium responds much better (219 versus 117 genes in M phase endometrium; Dassen *et al.*, submitted), indicating that the responsiveness of the endometrium to progesterone increases after extended periods of exposure to estrogen.

Indirectly, potential estrogen regulated genes should also be extractable from studies investigating changes in gene expression throughout the menstrual cycle. Particularly from studies which have being performed on global gene profiling using endometrium collected in early and late the proliferative phase. Only one study meets this criterion (Ponnampalam *et al.*, 2004), even though the

Table 4: Cell cycle regulators differentially expressed in late proliferative (LP) versus menstrual (M) phase endometrium (Punyadeera *et al.*, 2005)

Gene	Fold-change
A. In vivo—LP phase versus M phase endometrium	
CCNA1	3.4[a]
CCNB1	4.3[a]
CCNB2	4.3[a]
CCNL1	−3.3
CDC2	3.2[a]
CDC20	6.8[a]
CDC6	3.0[a]
CDCA3	4.8
CKS2 (CDC28 kinase 2)	2.3
CDKN1A(p21, CIP1)	−3.8
CDKN2C	3.4[a]
CDKN3	2.4[a]
GADD45B	−6.2[a]
B. In vitro—M phase endometrium treated with 17β-E$_2$	
CCNA1	3.5[a]
CCNL2	2.2
CDK10	4.4
CDKN2B	3.3

[a]Genes also found oppositely regulated in secretory when compared to proliferative endometrium.

Table 5: Genes common to the studies of Ponnampalam *et al.* and Punydeera *et al.*

Ponnampalam *et al.* (2004)	Punyadeera *et al.* (2005)
High menstrual, low proliferative (Clusters 2, 5, 6, 7)	LP versus menstrual
CENPF	+9.7
NCR3	−6.6[a]
SOX4	−4.58[a]
TYMS	+5.2
TAC1	−5.9[a]
DNAJB1	−4.2[a]
STC1	−20.4[a]
DTR	−12.6[a]
CD59	−2.5[a]
EDN2	−2.4[a]
S100P	−6.8[a]
TGFA	−3.0[a]
IL7R	−4.9[a]
RAI3	−16.6[a]
ITGA2	−5.0[a]
Low menstrual, up proliferative (Cluster 3)	
DCI	+3.5[a]
TRIP13	+2.0[a]
Low early, high LP (Cluster 4)	
HMGB2	+3.5[a]
CSTF2	−3.2
PDEF	+5.4[a]

[a]Genes displaying similar changes in both studies.

attention of these authors was mostly focussed on the secretory phase. With regard to the proliferative phase endometrium, the authors stated that there is little evidence of major changes in gene expression that correlates with the rise in estrogen during the proliferative phase of the cycle. However close examination of the clusters defined by the investigators clearly show differences between the menstrual, early/mid-proliferative, mid-proliferative and LP/early secretory stages. Comparing the genes to those identified in our study (Punyadeera *et al.*, 2005), we found 20 genes to be common to both studies (Table 5). In contrast to our expectations, none of these genes were cell cycle regulators.

Estrogen regulation of cell cycle regulators

Rodent uterus

Estrogen is the most important regulator of proliferation in endometrium. Yet, we know surprisingly little about the subcellular processes involved in estrogen regulation of proliferation. Even in the microarray studies mentioned above, little attention has been given to this aspect of endometrial development. Four studies have attempted to extract information from the array data to understand how estrogens affect the cell cycle: (Fertuck *et al.*, 2003; Hewitt *et al.*, 2003, 2005; Moggs *et al.*, 2004). Only a small subset of genes was frequently affected by E$_2$ in the murine or rat uterus. As indicated earlier, the expression of the cell cycle inhibitors *GADD45* and *CCNG2* is suppressed by E$_2$ treatment prior to the up-regulation of various cell cycle inducers. A direct involvement of E$_2$-occupied ERα in the down-regulation of *CCNG2* was recently shown by Stossi *et al.* (2006). They observed that the suppression of *CCNG2* is associated with the recruitment of the co-repressor N-CoR and histone deacetylases, leading to a hypoacetylated state of the chromatin (Stossi *et al.*, 2006). Although *GADD45* suppression by estrogen

in the rodent uterus has not yet been shown, these observations point to the fact that the proliferative response induced by estrogen is initiated by the down-regulation of cell cycle inhibitors, rather than the induction or activation of cell cycle stimulators. This is further substantiated by the studies of Hewitt *et al.* (2003, 2005), Watanabe *et al.* (2003), Moggs *et al.* (2004) and Hong *et al.* (2004). The common denominators in these studies next to *CCNG2*, are p27^{KIP1} (*CDKN1B*) and *GAS1*. These cell cycle inhibitors are down-regulated during the first 1–8 h after estrogen administration. Other negative regulators of the cell cycle reported in more than one study are p21^{CIP1}(*CDKN1A*) and *MAD*2. Upon administration of E$_2$, both p21^{CIP1}(*CDKN1A*) and *MAD*2 expression peak during the first 6 h, after which the expression decreases again. *MAD*2 interacts with the anaphase-promoting complex (APC) which is required for anaphase initiation and exit of mitosis (Fang *et al.*, 1999). Upon binding of *MAD*2, activation of the APC is inhibited and the cells are arrested at the prometaphase. Upon the decrease in *MAD*2 these cells will enter mitosis that may result in the first wave of cell divisions observed after about 16 h. Parallel to the decrease in *MAD*2, levels of *CCNE1* (which is involved in the G1 to S transition in the cell cycle) increase dramatically (Hewitt *et al.*, 2003, 2005). At this point levels of p21^{CIP1}, which inhibits S-phase entry, are still elevated. The levels start decreasing 15 h post-E$_2$, at the same time that *CCNE1* and *CCNG1* levels increase. This may allow cells to progress from the G1 to the S-phase and initiate the second wave of mitotic divisions. The increase in *CCNE1* was also observed in response to IGF, indicating that this cyclin also mediates the growth factor induced proliferative response (Hewitt *et al.*, 2005).

Groothuis *et al.*

Human endometrium

When comparing the gene expression profiles of LP phase and M phase endometrium, we observed that the expression of a subset of cell cycle regulators was differentially expressed (Table 4) (Punyadeera *et al.*, 2005). Particularly interesting is the down-regulation of the cell cycle inhibitors p21$^{\text{CIP}}$ and *GADD45*, which were also implicated in the regulation of murine uterine growth by E$_2$. In addition, the expression of the cell cycle inducers *CKS2*, *CCNA1* and *CCNB1*, also reported by Yanaihara *et al.* (2005), was elevated in LP phase endometrium as compared with M phase endometrium.

An alternative way to deduce candidate genes involved in estrogen-regulation of proliferation, is to evaluate expression profiles after exposure to the natural antagonist of E$_2$, progesterone.

We generated gene expression profiles for two biopsies collected on cycle day 23 of the menstrual cycle, the end of the implantation window, and compared them with the profiles of two biopsies collected on cycle day 9 of the menstrual cycle, the mid-proliferative phase. We extracted the most common cell cycle regulators (more than 2-fold difference), and found a total of 43 genes differentially expressed: 11 genes were up-regulated and 32 genes were down-regulated in secretory phase endometrium (Table 6; unpublished data). These genes were compared with the genes which were found to be elevated in LP phase endometrium when compared with M phase endometrium (Punyadeera *et al.*, 2005) and presumably induced by estrogen. Eight genes were down-regulated and one gene was up-regulated (*GADD45*) in the secretory phase endometrium (Table 6). We also compared these findings with the

Table 6: Cell cycle regulators differentially expressed in secretory versus proliferative phase endometrium

Gene symbol	Gene name	>2-fold Prol/Secr
CDC2	Cell division cycle 2, G1 to S and G2 to M	13.1[a]
CDC45L	*CDC45* cell division cycle 45-like (*S. cerevisiae*)	11.77
CCNA1	Cyclin A1	10.79[a]
CCNA2	Cyclin A2	10.64[a]
CCNB2	Cyclin B2	8.6[a]
CCNB1	Cyclin B1	7.94[a]
CCNE2	Cyclin E2	6.84
CDC25C	Cell division cycle 25C	6.63[a]
CDCA8	Cell division cycle associated 8	6.56
CKS2	*CDC28* protein kinase regulatory subunit 2	5.99[a]
CDK5R2	Cyclin-dependent kinase 5, regulatory subunit 2 (p39)	5.81
CDC6	*CDC6* cell division cycle 6 homolog (*S. cerevisiae*)	5.44[a]
CDKN3	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificit)	4.9[a]
PCNA	Proliferating cell nuclear antigen	4.13[a]
CDKL2	Cyclin-dependent kinase like 2 (*CDC2*-related kinase)	4.09
CDC20	*CDC20* cell division cycle 20 homolog (*S. cerevisiae*)	3.88[a]
GDF1	Growth differentiation factor 1	3.77
GDF3	Growth differentiation factor 3	3.7
GAS2	Growth arrest-specific 2	3.65
CKS1B	*CDC28* protein kinase regulatory subunit 1B	3.62
CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18, inhibits *CDK4*)	3.27[a]
CDCA3	Cell division cycle associated 3	3.18
GDF5	Growth differentiation factor 5 (cartilage-derived morphogenetic prote)	3.08
GAS41	Growth arrest-specific 41	2.82
CDC25A	Cell division cycle 25A	2.66
CDK2	Cyclin-dependent kinase 2	2.55
CDK5R1	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	2.54
CDC7	*CDC7* cell division cycle 7 (*S. cerevisiae*)	2.53
CCNF	Cyclin F	2.39
CDKL3	Cyclin-dependent kinase-like 3	2.26
GDF11	Growth differentiation factor 11	2.09
CDKN2D	Cyclin-Dependent kinase inhibitor 2D (p19, inhibits *CDK4*)	2.03
CDC42EP3	*CDC42* effecotr protein (Rho GTPase binding) 3	0.49
CDC34	Cell division cycle 34	0.48
CDC42EP4	*CDC42* binding protein (Rho GTPase binding) 4	0.45
CCNI	Cyclin I	0.45
CGR11	Cell growth regulatory with EF-hand domain	0.43
CDC42BPA	*CDC42* binding protein kinae alpha (DMPK-like)	0.42
GADD45B	Growth arrest and DNA-damage-inducible, beta	0.31[a]
GDF8	Growth differentiation factor 8	0.23
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, *Kip2*)	0.15
GADD45A	Growth arrest and DNA-damage-inducible, alpha	0.12
GAS1	Growth arrest-specific 1	0.11[a]

[a]Genes indicated are also reported in the study of Talbi *et al.* (2006).

results of the extensive study by Talbi *et al.* (2006), who studied gene expression in histologically well-defined biopsies of human endometrium throughout the menstrual cycle. In the gene list resulting from the comparison between the secretory and proliferative endometrium, 13 cell cycle related genes corresponded to our findings: 11 genes were down-regulated and two genes were up-regulated in the secretory endometrium (Table 6). Interestingly, the expression of various cell cycle inhibitors, i.e. *GADD45*, *GAS1*, *CDKN1C* (*KIP2*), is dramatically induced in secretory endometrium, supporting the findings from the mouse studies indicating that the role of cell cycle inhibitors in the regulation of proliferation may have been underestimated thus far.

Estrogen regulation of the cell cycle has been extensively studied in breast cancer cell lines (reviewed by Doisneau-Sixou *et al.*, 2003), and central roles have been identified for *CMYC*, *CCND1* and its binding partners *CDK4* and *CDK6*, *CCNE* and its binding partner *CDK2*, and the *CDK* inhibitor p21^{CIP}. In brief, *CMYC* and *CCND1* can independently mediate the effects of estrogen on cell cycle progression. These pathways converge at the *CCNE–CDK2* complex that is activated by stimulating the dissociation of p21^{CIP} from this complex. This dissociation is mostly a result of the down-regulation of p21^{CIP} gene transcription. After up-regulation by estrogen, *CCND1* complexes with *CDK4* and *CDK6*, which are potent kinases, which, in turn,
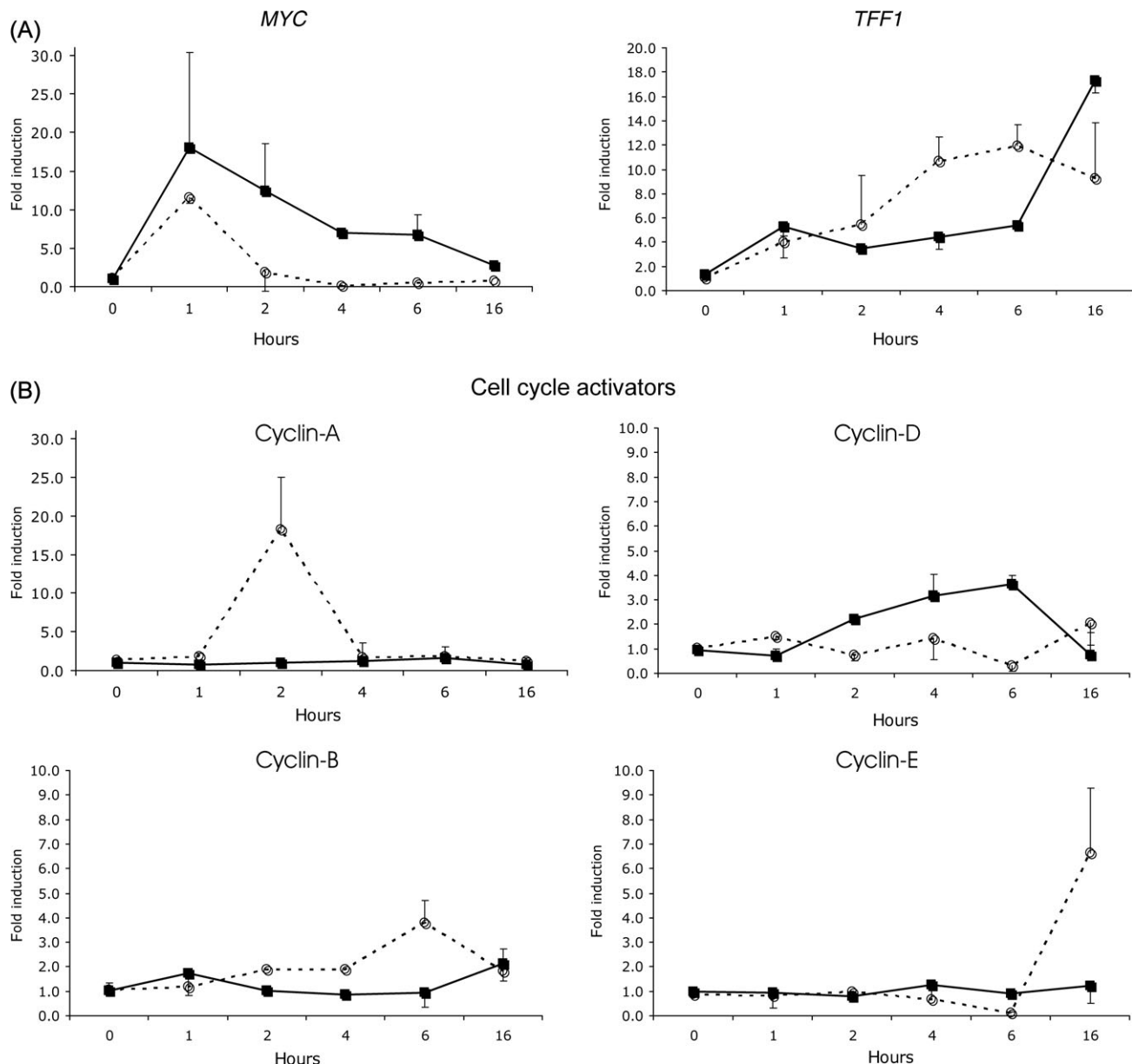

Figure 1: Estrogen regulation of the expression of cell cycle regulators in the endometrial carcinoma cells ECC1 (solid line) and breast carcinoma cells T47D (dotted line). Gene transcript levels were assessed by quantitative real-time PCR after 0, 1, 2, 4, 6 and 16 h incubation with E$_2$. Presented are the results for (**A**) the estrogen-responsive genes *TFF1* and *MYC*, (**B**) the cell-cycle activators *CCNA*, *CCNB* and *CCNE*
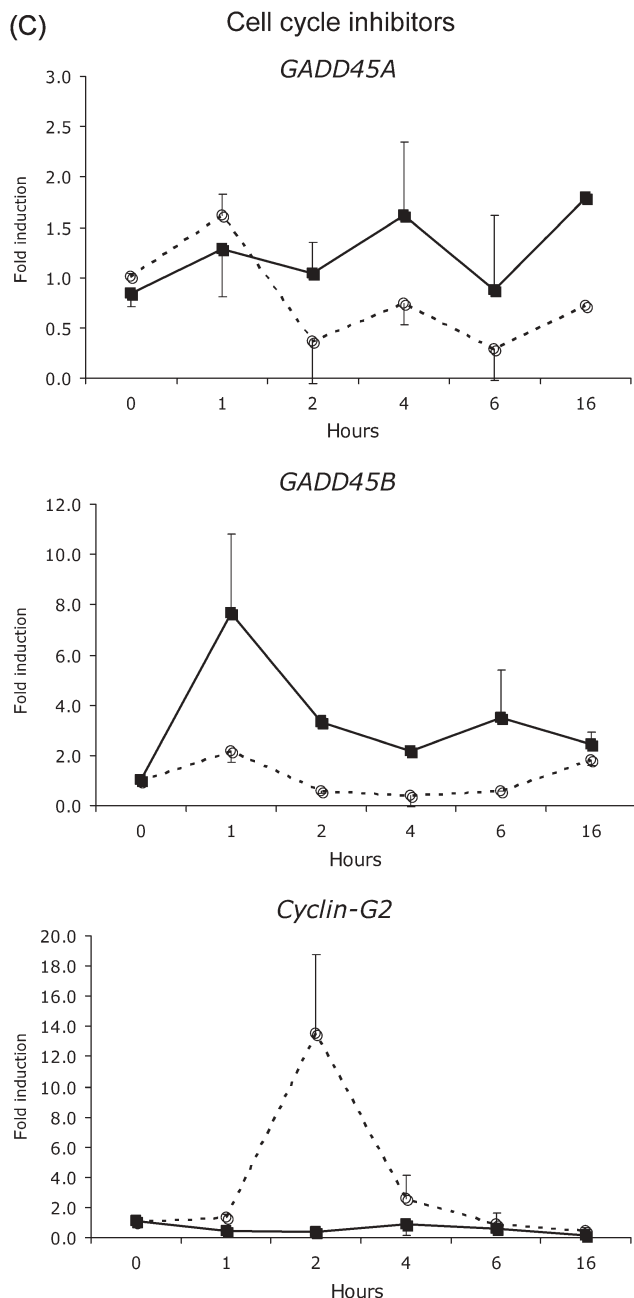
(C)

Figure

Figure 1 *continued*: (C) the cell cycle inhibitors *GADD45A*, *GADD45B* and *CCNG2*

phosphorylate pRb and, within 9 h, the cell enters in the S-phase. Even though *MYC* does not enhance the expression of *CCND1*, suppression of *MYC* expression reduced *CCND1* expression and prevents estrogen-stimulated cell cycle progression.

As a model to elucidate the mechanism of estrogen-stimulated cell proliferation, we have investigated the expression of a subset of these cell cycle regulators in endometrial and breast cancer cell lines treated with E_2 (1.0 nM). The endometrial cancer cell line ECC1 and the breast cancer cell line T47D respond nicely to E_2 treatment as illustrated by the induction of the transcription of the estrogen-responsive gene *Tff1* in both cell lines (Fig. 1A). Yet, T47D cells respond to estrogen stimulation with strong cell proliferation, whereas ECC1 cells do not. To explain this, we

evaluated the expression of various cell cycle regulators in both cell lines. In both cell lines, the key gene in the regulation of proliferation, *MYC* is strongly induced in both cell lines with maximal expression 1 h after E_2 stimulation (Fig. 1A). Subsequently, in T47D cells the expression of the cell cycle activators *CCNA* ($\sim$2 h), *CCNB* (6 h) and *CCND* and *CCNE* (16 h) is also induced (Fig. 1B). When evaluating the expression of cell cycle suppressors, it was apparent that in the T47D cells the expression of *GADD45A* and *GADD45B* was down-regulated after 2 h, whereas in the ECC-1 cells the expression of *GADD45A* was not inhibited, whereas the expression of *GADD45B* was highly induced (Fig. 1C). These evidences support the aforementioned hypothesis that the down-regulation of cell-cycle inhibitors is one of the first actions in E_2-induced cell proliferation.

Surprisingly, however, we observed that in T47D cells the expression of *CCNG2* is induced after 6 h of E_2 treatment (1.0 nM). As this was not seen in the ECC-1 cells, we tend to believe that the *CCNG2* is a cell cycle activator. This contradicts however, the finding of Stossi *et al.* (2006) in MCF-7 cells, which clearly indicated *CCNG2* as a cell cycle suppressor. These observations show that mechanisms of estrogen control of proliferation as revealed in one breast cancer cell line cannot habitually be extrapolated to other breast cancer cell lines or any other model system, without extensive validation.

Variations in the expression of cell cycle regulators in tissues may very well be masked during genomic profiling by their cell-specific expression patterns. There are multiple cell types present in the human endometrium that contribute to the growth of the endometrium tissue and studying gene expression on a tissue provides no information about the individual cell types. Moreover, expression has also to be confirmed at the protein level in order to draw any conclusions. For instance, expression of *CCNE* implicated as a key regulator of estrogen induced cell cycle progression, is most prominent in the glandular epithelium, and migrates from the cytoplasm in the mid-proliferative phase to the nucleus in the secretory phase. This dramatic change in intra-cellular distribution may occur without alterations in mRNA levels. In addition, it is also strongly expressed in blood vessels, yet it is almost absent in the stromal cells. This would implicate a role for *CCNE* in endothelial and epithelial cells rather than the stromal cells.

It is apparent that the mechanism by which estrogen regulates endometrial cell proliferation and differentiation is far from elucidated. Close examination of the gene expression data and validation of cell cycle regulators may facilitate this.

Is extrapolation of rodent genomic profiling data to humans feasible?

The analogy between the role of estrogen in the rodent and human uterus is restricted to the major growth promoting effect. Besides this, there are major differences between the actions of estrogen in the rodent and human uterus. For example, in rodents estrogen is essential for both epithelial proliferation and embryo implantation (Curtis Hewitt *et al.*, 2002), whereas in humans prolonged estrogen exposure is required for endometrial growth, but its presence is not required during embryo implantation. In addition, the first effects of estrogen in the rodent uterus are effects on the vasculature resulting in increased vascular permeability and leakage of

Table 7: Genes reported in both human endometrium (Punyadeera *et al.*, 2005) and murine uterus (Moggs *et al.*, 2004)

Gene symbol	Gene title	>2-fold Prol/Secr	Moggs *et al.*			
			1 h	4 h	8 h	24 h
AFT3	Activating transcription factor 3[a]	−17.4	3	2	0	−0.5
NR4A1	Nuclear receptor subfamily 4, group A, member 1[a]	−14.8	3	3	0.5	−0.5
ADM	Adrenomedullin[a]	−13.8	2	2.5	2	0
LCN2	Lipocalin 2 (oncogene 24p3)	−6.5	0	3	3	3
GADD45B	Growth arrest and DNA-damage-inducible, beta	−6.2	3	3	3	2
SUI1	Putative translation initiation factor	−5.8	0	2.5	2	2
VEGF	Vascular endothelial growth factor[a]	−4.9	3	3	0.5	−0.5
SOCS3	Suppressor of cytokine signaling 3	−4.8	3	3	3	3
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide[a]	−3.9	0	3	0	0
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)[a]	−3.8	3	3	3	0.5
AMGPT2	Angiopoietin 2	−3.6	0	2.5	2	2
CD68	*CD68* antigen	−3.1	0	0.5	0.5	0.5
SNK	Serum-inducible kinase	−2.6	2.5	3	3	3
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta[a]	−2.6	3	0.5	−1	−0.5
SPP1	Secreted phosphoprotein 1 (osteopontin)	−2.6	−1	1	2	3
CD14	*CD14* antigen	−2.4	−0.5	0	−1	0.5
C3	Complement component 3	−2.3	0	1.5	3	3
MAP2K3	Motogen-activated protein kinase kinase 3	−2.1	0	2	0.5	0.5
CTSS	Cathepsin S	−2.0	0	0	0	0.5
FEN1	Flap structure-specific endonuclease 1	2.2	1	0	0.5	3
PC4	Activated RNA polymerase II transcription cofactor 4[a]	2.2	0	3	2.5	0.5
PCNA	Proliferating cell nuclear antigen[a]	2.3	0	1	0.5	1
MCM2	*MCM2* minichromosome maintenance deficient 2[a]	2.5	0	0	2	3
CDC6	*CDC6* cell division cycle 6 homolog (*S. cerevisiae*)[a]	3.0	0	0	2	3
MCM4	MCM4 minichromosome maintenance deficient 4 (*S. cerevisiae*)[a]	3.2	0	0	2	3
CCNB1	Cyclin B1[a]	4.3	0	0	−1	3
RRM2	Ribonucleotide reductase M2 polypeptide[a]	4.9	0.5	0.5	1	3

[a]Some genes show similar response patterns: low ratio in proliferative/secretory phase endometrium and down-regulated by 17ß-E2 in ovariectomized mice, or vice versa.

fluids into the interstitial space, whereas in the human endometrium angiogenic activity is initially triggered by the postmenstrual hypoxic milieu which results in the up-regulation of angiogenic factors, including *VEGF-A* (Punyadeera *et al.*, 2006; Sharkey *et al.*, 2000).

The differences with regard to the effects of estrogen on endometrial function in rodents and human also become apparent when comparing rodent and human gene expression profiling studies. Gene expression profiling is generally performed in preparations of the whole uterus rather than only the endometrium. Therefore, certain differences may be masked and also result in false positives due to the inclusion of the myometrial tissue.

In a first attempt to make such a comparison, we compared expression profiles reported in the rodent uterus and human endometrium in similar physiological conditions. We have compared the gene expression profiles of LP phase endometrium, during which estrogen exposure of the endometrium has reached its maximum, with M phase endometrium (cycle day 3 and 4), during which estrogen levels are at their lowest point during the menstrual cycle (Punyadeera *et al.*, 2005). In analogy, these genes would have to be compared with genes which are modulated during the peak levels of pre-ovulatory estrogen, such as in ovariectomized mice treated with E_2 (Moggs *et al.*, 2004). When comparing the results of Moggs *et al.* with our own data, we observed only 27 common genes, of which 14 were regulated similarly in both species. Some genes involved in DNA replication and cell division were also up-regulated in LP phase endometrium

(i.e. *PCNA*, *CDC6*, *CCNB1*; Table 7). Interestingly, the expression of the cell cycle inhibitors, *CDKN1A* (p21^{CIP}) and *GADD45*, was also down-regulated in the human endometrium during periods of high proliferative activity. None of the genes involved in the RNA and protein synthesis reported by Moggs *et al.* was common, whereas we did find various transcriptional regulators and signalling genes in our arrays (Table 7). Despite the fact that rodent models allow close investigation of the actions of ovarian steroid hormones with regard to the regulation of uterine function, the limited analogy with the actions of estrogen in the human endometrium hampers extrapolation of the findings to the human situation. The limited agreement between rodent and human findings may also be caused by differences in nomenclature of human and murine genes. Initiatives are ongoing to synchronize the naming of homologous genes (reviewed by Wright and Bruford, 2006).

Concluding remarks

We are far from understanding the mechanisms by which E_2 regulates endometrial growth and differentiation. Even though gene expression profiling can be beneficial in revealing the biological processes and cellular functions involved, they have not yet significantly contributed to better insights in the role of E_2. Thus far, we have learned more from 'hypothesis-driven' research approaches, whereas the validity of the findings of the 'hypothesis-generating' genomics approaches have not yet improved our

understanding of the regulation of endometrial function in humans.

The infrequent similarities between the responses of rodent and human uterus to estrogen, limits extrapolation of findings in rodent models. In addition, the large differences between studies performed in different laboratories and the biological variation that exists between samples is a major issue of concern and calls for large multicenter collaborations to increase numbers of samples and reduce variations with regard to the choice of platform, sample and probe preparation and hybridization. Also the use of 'clean' samples consisting of individual cell types, such as those collected with Laser capture microdissection, would provide more sensible information. However, technical restrictions have thus far prohibited such studies.

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