This is to certify that the

dissertation entitled

In vitro and in vivo evaluation of the potential estrogenic effects of polycyclic aromatic hydrocarbons

presented by

Kirsten Cecilia Fertuck

has been accepted towards fulfillment
of the requirements for

PhD degree in Biochemistry and Molecular Biology

Major professor

Date 5/8/03

IN VITRO AND *IN VIVO* EVALUATION OF THE POTENTIAL ESTROGENIC EFFECTS OF POLYCYCLIC AROMATIC HYDROCARBONS

By

Kirsten Cecilia Fertuck

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Molecular Biology

2003

ABSTRACT

IN VITRO AND *IN VIVO* EVALUATION OF THE POTENTIAL ESTROGENIC EFFECTS OF POLYCYCLIC AROMATIC HYDROCARBONS

By

Kirsten Cecilia Fertuck

A large number of exogenous compounds have been found to possess the ability to bind to the α and β isoforms of the estrogen receptor (ER). The identification and characterization of these compounds is typically achieved through in vitro assays and, when concern is warranted, by assessing the ability of the compound to stimulate uterine proliferation in the rodent uterus. Benzo[a]pyrene (B[a]P) is a ubiquitous pollutant and shares some structural similarity with endogenous estrogens, particularly when hydroxyl groups are introduced during metabolism. Specific B[a]P metabolites, as well as other compounds of interest, were identified that were able to bind to ERα and ERβ in vitro, and to induce ERα- and ERβ-mediated reporter gene expression in MCF-7 breast cancer cells. B[a]P was also shown to interact in an additive manner in vitro when cotreated with estrogen. However, administration of B[a]P or of its most active metabolites to immature, ovariectomized mice was not sufficient to stimulate uterine proliferation or induction of uterine lactoferrin mRNA expression. Because the transcriptional targets of estrogen in the uterus are numerous and many are still undiscovered, a GeneChip microarray approach was used in order to characterize the temporal transcriptional responses to estrogen in the uterus. This approach was able to confirm previously characterized effects as well as to identify novel responses. For example, the arginine and ornithine utilization

pathway was found to be particularly highly regulated in the uterus in response to estrogen, and while induction of some of these enzymes had been shown previously, this provided the first evidence that numerous aspects of this pathway could be monitored at the transcription level in the estrogen-stimulated uterus. Expression of arginase 1 was found to be particularly high in response to prolonged estrogen exposure. The ability of B[a]P to alter the expression of arginase 1 and other chosen genes was then assessed in the rodent uterus. While B[a]P was recently reported to antagonize the ability of estrogen to stimulate the proliferation of MCF-7 breast cancer cells *in vitro*, we showed that B[a]P was not able to interfere with estrogen-induced uterine proliferation or with expression of estrogen-regulated genes including arginase 1. Finally, the extensive literature searching required in the analysis of the microarray data made it clear that the lack of a comprehensive review of previously characterized estrogen-responsive genes is a great impediment to the analysis of new large-scale transcriptional profiling studies. Accordingly, a comprehensive review of estrogen-regulated transcription in the uterus is provided.

ACKNOWLEDGEMENTS

My advisor, Tim Zacharewski, deserves my heartfelt thanks for both professional and personal guidance, and for providing me with countless opportunities that I never expected to have. It is largely due to his mentorship that this experience has been so much more rewarding than I could ever have predicted.

The members of the Zacharewski lab have been very generous in providing patient advice, sympathetic assistance, and plenty of comic relief. I have learned a great deal from everyone that I have come in contact with, including numerous postdocs and undergraduate students, and have received particular support and empathy from the graduate students, Jason Matthews, Mark Fielden, Lyle Burgoon, Darrell Boverhof, and Cora Fong. The Zacharewski family naturally counts among the lab members, since afternoons are always best when Jana, Lara, and Nick make a surprise appearance.

My experience here has been shaped very much by the energy and kindness of numerous other members of the department over these years. I would like to thank my first friends here, Craig Seaver, Janet Filek, and Matt Larson, who helped me to get started. I was sad to see them move on, but through that, met Anu Kumar and Beth Strunk, who were my favorite neighbors. Then as they were leaving, I became friends with Dawn Greensides, who was an endless source of energy and treats, not to mention new friends. I would particularly like to thank Francisco Herrera for making me laugh so much, Soren Ottosen for giving me the best photo that I have ever seen, Joe Graber for being funny, Brandon

Hespenheide for being on my team, Rich Gray for all of the etiquette lessons, Pete Davidson and Yu-wen Chung for the Shakespeare, Heather Hirsch and Jim Mann for the baking support, Sheldon Leung for having his own special sense of humor, Nikki LeBrasseur for standing up for me, Bryan Schmidt and Josh Edwards for inviting us all over so generously and often, Setsuko Wakao for being my great new neighbor, and Dean Shooltz for being willing to juggle just about anything.

My committee members also receive my deep thanks for the guidance that they have provided, in addition to countless reference letters over the years. I am very grateful also for the help of the departmental staff, and in particular that of Julie Oesterle, whom I have relied on repeatedly.

My parents have been extremely helpful over the years in many ways, and their phone calls while I have been here have been especially appreciated. My brother and sister are extremely skilled at making me laugh, and their energy is always welcome. And finally, Chris has been an immense source of support from far away, and talking to him is my favorite part of every day. His steady help during difficult times has been critical, as has his sharing of the many good time, and all of it has been an enormous part of my time here.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

ABBREVIATIONS

AhR:	Aryl hydrocarbon receptor
ANOVA:	Analysis of variance
AP-1:	Activation protein 1
AR:	Androgen receptor
ATP:	Adenosine triphosphate
B[a]P:	Benzo[a]pyrene
CTGF:	Connective tissue growth factor
DBD:	DNA binding domain
E2:	17β-Estradiol
EE:	17α-Ethynyl estradiol
EGF:	Epidermal growth factor
EST:	Expressed sequence tag
ER:	Estrogen receptor
ERE:	Estrogen responsive element
FGF:	Fibroblast growth factor
FSH:	Follicle stimulating hormone
GAG:	Glycosaminoglycan
GnRH:	Gonadotropin releasing hormone
GR:	Glucocorticoid receptor
HSP:	Heat shock protein
IGF:	Insulin-like growth factor
IL:	Interleukin
LBD:	Ligand binding domain
LH:	Luteinizing hormone
MAS5:	Affymetrix MicroArray Suite version 5.0
MMP:	Matrix metalloproteinase
PAH:	Polycyclic aromatic hydrocarbon
PDGF:	Platelet-derived growth factor
PR:	Progesterone receptor
RT-PCR:	Reverse transcriptase-polymerase chain reaction
SD:	Standard deviation
TCDD:	Tetrachlorodibenzo-*p*-dioxin
TNF:	Tumor necrosis factor
TGF:	Transforming growth factor
TR:	Thyroid hormone receptor
VEGF:	Vascular endothelial growth factor

CHAPTER 1

REVIEW OF THE LITERATURE: TRANSCRIPTIONAL RESPONSES TO ESTROGEN IN THE UTERUS

INTRODUCTION

The profound actions of estrogens on the uterus were first described in detail in the 1920s, and great advances in understanding the nature of these effects were made in the following decades. In the 1960s, the first studies characterizing the transcriptional responses in the estrogen-stimulated uterus were initiated, and in the years since then, thousands of additional studies have used humans, various model species, and cultured cells to further refine our understanding of these processes. Many uterine responses to estrogen have a transcriptional component, and therefore microarrays have become an appealing tool for further characterizing these immensely complex effects, an endeavour that is of great interest to pharmacologic and toxicologic researchers. A difficulty in this approach however is in the data interpretation, since this body of published information has not been comprehensively reviewed, and it is therefore difficult to discern novel responses from those that have been previously reported, and laborious to associate these responses with known associated physiological effects. Furthermore, many of the affected transcripts are associated with numerous gene names, further hampering data analysis.

The following review addresses the deficiency in the organization of this information by discussing the known estrogen-induced changes in levels of specific transcripts in the uterus of humans and of common model organisms, as well as their

relevance to uterine biology. Citations of pertinent reviews are provided. As well, recent research that has greatly expanded the understanding of mechanisms of estrogen signaling is discussed. Transcripts here are identified by their italicized official mouse gene abbreviation, as identified in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/LocusLink/).

OVERVIEW: ESTROGENS, THEIR RECEPTORS, AND THEIR RESPONSE ELEMENTS

Estrogens

Estrogens are steroid hormones that are formed by the aromatization hydroxylation of the A ring of androgens. 17β-Estradiol and its weaker estrogen metabolites are the predominant form of circulating estrogen, though endogenous non-aromatized steroids, including testosterone and several of its precursors and derivatives, have also been shown to possess direct estrogen receptor-mediated transcriptional activity and cell proliferation (1, 2). Biosynthesis of steroid estrogens occurs in all vertebrates and in protochordates (3), and estrogen availability is controlled by associations with sex hormone binding globulins (4, 5).

In premenopausal women, the ovaries are the primary source of estrogen, which is secreted and acts in an endocrine manner on target tissues. In postmenopausal women and in men, estrogen is produced in extragonadal sites, including specific cell types within fat, bone, blood vessels, and brain, where it can act locally as a paracrine or intracrine factor (6). Aromatase is encoded by a single gene with a complex promoter,

and gonadal aromatase expression is regulated by cAMP and gonadotropins (7), while at other sites it can be regulated by prostaglandins and cytokines (8).

Estrogens are oxidatively metabolized by specific cytochrome P450s, and the metabolites retain varying levels of estrogenic activity depending on the oxygenation position within the steroid ring (9). Certain unstable metabolites also possess mutagenic activity and are implicated in carcinogenesis in estrogen target tissues (10). Cytochrome P450 polymorphisms that affect the formation of reactive metabolites have also been associated with cancer risk (11).

Estrogen receptors

The ER was first cloned in a human breast cancer cell line (MCF-7) more than 15 years ago (12). ERs were cloned soon after from numerous species, and phylogenetic analysis of these sequences suggested that the ER is the ancestral steroid receptor, found in all vertebrates, from which the other members (progesterone, androgen, glucocorticoid, and minerallocorticoid) subsequently evolved (13). Moreover, the ERs from different species were found to be highly similar in the ligand binding and DNA binding regions, consistent with the critical importance of both ligand and DNA binding to the mechanism of ER action.

The ER was renamed ERα following the discovery a decade later of a second isoform, denoted ERβ (14). The sequence similarity is quite high in the ligand and DNA binding domains, and at the dimerization interface, and differs mainly in the N and C terminal regions. A third receptor, ERγ, has also been identified in fish (15), although no homologous sequence has been identified in the human or mouse genome (16). The length of ERα is well established (595 amino acids), whereas the extent of the ERβ

protein sequence has been revised several times, and is now believed to most commonly contain 530 residues (17).

ER crystal structures and predictions indicate that the ERs, as well as the ancestral steroid receptor, all possess the critical binding pocket residues that accept estrogen and discriminate against the 3-keto group of all other steroid hormones as well as the 17-methylketo group of progesterone and corticoids (18-20). In the classic mechanism, cytoplasmic ligand binding promotes a conformational change that exposes the dimerization surface, and the liganded dimer can bind to specific response elements to modulate transcription of target genes in the nucleus.

Estrogen response elements

The ancestral steroid receptor activated genes by binding to the same motif as does the modern estrogen receptor, the estrogen responsive element (EREs; an inverted repeat of the sequence AGGTCA separated by three unspecified residues), while the other steroid receptors later evolved to interact with a distinct motif (a direct or inverted repeat of AGAACA). The repeated sequence in nuclear receptor response elements is important for the binding of the liganded receptor dimer (21).

Coactivators

A rapidly expanding area of research in the action of ERs and other nuclear receptors has been in determining the identity and nature of key coactivators and corepressors, which modulate the transactivational activity of the ligand-receptor dimer complexes. The existence of coactivators was first suggested after a 'squelching,' or damped response was observed when estrogen and progesterone receptors were

concurrently activated, suggesting that shared factors, present in limiting amounts, were required for their action (22). Corepressors were first identified during efforts to explain the repression of thyroid hormone transcription in the absence of ligand (23).

The specific nature of the assembled modulatory proteins that are recruited by the ER is tissue-, ligand-, and receptor isoform-specific, and this area has been reviewed extensively (e.g. (24, 25)). Since nuclear receptors act primarily as transcription factors, corepressors typically act in the absence of ligand to block receptor-mediated transcription. Certain coactivators, by contrast, can potentiate nuclear receptor-mediated transcription by promoting chromatin accessibility and interactions with the basal transcriptional machinery. *In vitro* methods such as yeast two-hybrid studies have been instrumental in identifying numerous novel nuclear receptor-interacting cofactors (24, 26). Interestingly, ERα has recently been shown to be much more effective than ERβ at activating transcription on ERE-containing chromatin templates, which has been attributed to the N-terminal activation function (AF-1) of ERα (17).

Nonclassical ER signaling

A rapidly expanding area of study is broadly termed nonclassical nuclear receptor signaling. ERs, like the other family members, have been reported to stimulate signaling by numerous pathways that diverge from the classical ER-ERE-mediated signaling pathway. For example, a subclass of non-nuclear, membrane-localized receptors has been identified, which are believed to be responsible for many of the effects of estrogen that are too rapid to be consistent with *de novo* transcript and protein synthesis. Ligand-independent activation of nuclear ERs has also been described, as well as the ability of ERs to influence transcription at non-ERE sites in association with other transcription

factors such as AP-1 and Sp1. This rapidly expanding area of research has been the subject of numerous reviews (e.g. (27, 28)).

To contribute to the understanding of nontraditional modes of estrogen signaling, a nonclassical ER knock-in (NERKI) mouse strain was developed. The NERKI mice contain an ER that bears a mutation in the DNA binding domain that specifically abolishes ER binding to DNA, therefore ablating the classical ER-ERE signaling but retaining all other ER functions (29). Homozygotes do not survive, and heterozygote females are viable but infertile. The uteri are enlarged and hyperplastic, with greatly enlarged endometrial glands filled with secretory material, and uteri are in constant diestrus and progesterone levels were reduced, though LH signaling is normal. Estrogen is still able to induce epithelial proliferation and hyperemia. The severity of the phenotype in the heterozygote, in particular compared to the ERαKO heterozygote (30), is surprising. It indicates that nonclassical estrogen signaling is crucial to female reproductive physiology, though uterine estrogen responsiveness is still preserved, and that the classical and nonclassical pathways may interact in a complex manner.

OVERVIEW OF ESTROGEN EFFECTS IN THE UTERUS

Estrogen acts as an important regulator in numerous tissues, of which the uterus is a prime example. Influences of circulating, ovarian-derived estrogen in the uterus of the intact animal are difficult to discern, however, because of the confounding presence of numerous other hormones and growth factors. As a result, a multitude of studies have examined uterine responses to exogenous estrogens in rodent models possessing low circulating estrogen levels either due to prepubertal age or to ovariectomy. These widely used models have a dual purpose, being aimed at further elucidating the basic

understanding estrogen action or at evaluating the estrogen-like activity of other ligands of interest (31).

In placental mammals, the embryo (trophoblast) implants in the maternal uterus and is protected there until it is capable of surviving independently. Successful implantation is therefore critical to reproductive success, and the carefully coordinated uterine changes, stimulated by cycling and carefully controlled levels of ovarian estrogen and progesterone, allow the trophoblast to penetrate the uterine epithelium into the stroma (32).

Cyclic changes in the rodent uterus were first described in detail more than 80 years ago (33, 34), and in the decades since then, the understanding of these changes in response to oscillating levels of circulating hormones has been greatly refined, though many uncertainties still remain.

Uterine cell types

The uterus is a complex and highly specialized organ, composed of numerous diverse cell types. Although there are some differences in structural organization between primates and rodents, such as the dual uterine horns and multiple implantation sites in rodents, and the menstrual shedding phase in primates, the cell types and compartments overall are comparable between the species, and the utility of using rodents to model human uterine responses is well established (35). Similarities and differences between species have been reviewed extensively (36).

The intrauterine space, or lumen, is physically defined by the luminal epithelial monolayer. Below this, underneath a basement membrane, lies the supporting stromal layer, which is composed of fibroblast-like stromal cells as well as various lymphoid

cells. Numerous tubular glands lined with a specialized glandular epithelial layer penetrate the stromal layer. Adjacent to the stroma, the outer region is the myometrial region, composed of inner circular and outer longitudinal smooth muscle layers, which together support uterine contractility. The outermost uterine lining is the serosal layer, formed from a thin layer of fibroblast-derived mesothelial cells. As well the uterus is highly vascularized, and therefore also contains numerous endothelial cells (36).

The endometrium, comprised of the epithelial and stromal layers, is functionally distinct from the surrounding muscular myometrium. Within the endometrium, the luminal epithelial cells regulate the capacity of the luminal walls to act as either a barrier or as a receptive surface for embryonic implantation, the glands formed by invaginations of the lumen into the stroma develop progressively during the uterine cycle and secrete factors that are critical in promoting uterine receptivity and embryonic implantation (37), and the stromal layer provides other crucial functions including structural support, growth factor production, and immune protection (36). During estrogen-stimulated uterine growth, the cell type composition remains relatively constant by volume, approximately 70% stroma, 10% luminal epithelium, 15% glandular epithelium, 5% endothelium, and a small proportion occupied by uterine, glandular, and vascular lumens, as well as interstitial space (38).

The reproductive cycle

The uterine cycle here refers to the menstrual cycle in primates as well as to the estrous cycle in rodents. An overview of the nature of cyclic uterine changes is useful to the understanding of the role that estrogen plays in uterine biology.

The uterus undergoes extensive cyclic changes in preparation for containing and nurturing the developing fetus in the event of conception and implantation. Changes involved in promoting uterine receptivity include proliferation and differentiation of specific cell types, extracellular matrix (ECM) alterations, cell surface changes, and immunological changes, and must occur in synchrony with the development of the preimplantation embryo. Essential stimuli in these events are cyclic changes in estrogen and progesterone levels, and their absence causes uterine regression and infertility (39). An important role of estrogen is to stimulate proliferation of the epithelium of the lumen and glands, while stromal proliferation requires the carefully timed and sequential presence of both estrogen and progesterone.

The menstrual cycle

During the proliferative phase, rising estrogen levels stimulate intensive endometrial cell proliferation and postmenstrual structural repair. At ovulation, ovarian steroid production switches from estrogen to progesterone predominance. Progesterone action is mediated through PRs in epithelial, stromal, and vascular cells, and induces numerous changes in preparation for pregnancy, including stromal thickening and differentiation to decidual cells, flattening of the luminal surface, and glandular secretion of specific products. In the absence of conception and implantation, the outer endometrial layers are shed and the endometrium then regenerates in preparation for a new cycle (40-42).

The estrous cycle

The rodent estrous cycle has many features in common with the primate menstrual cycle, though key differences in the rodent are the short duration (4-5 days) and the lack of endometrial shedding. Analogous to the proliferative phase in humans, proestrus and estrus are characterized by rising estrogen levels and epithelial proliferation, while progesterone dominates during the diestrus phase, inducing stromal differentiation (35). As a result the physiological and biochemical features of the rodent estrous cycle have been carefully studied, providing key insights into the responses to estrogen exposure (43).

In the rodent, proestrus is marked by maximal LH levels, followed shortly after by maximum estrogen levels in mid-proestrus, while FSH levels rise slightly and progesterone levels are at their minimum. During this time, the luminal epithelial surface is smooth and densely covered with microvilli. Glands are plentiful and protrude into the uterine lumen, but the glandular openings are poorly developed and often blocked, and so glandular secretions are scarce.

At estrus, the LH level is at its minimum and FSH briefly reaches its maximum. Estrogen reaches a minimum in mid-estrus and then begins to increase, while progesterone is low. The epithelium is proliferating maximally, and the epithelial cells have reached maximal size and an elongated shape. The epithelial surface is no longer smooth, and instead is characterized by deep folds and ridges. Microvilli and secretions cover the epithelial surface, and pseudoglands, which are depressions caused by necrosis or apoptosis of one or two cells beneath, and are not connected by a glandular duct to the underlying stroma, are much more numerous than are fully formed glands.

In early diestrus, LH and FSH levels are low, estrogen again declines, and progesterone reaches a maximum and then rapidly begins to decline. The epithelial cells are diminished in size and the epithelial surface is nearly flat, and glands and pseudoglands are becoming fewer and smaller. Microvilli are less dense. In late diestrus, LH and FSH are at their minimum, estrogen is at a minimum and then begins to increase, and progesterone decreases to a minimum. The epithelial surface is very flat, secretory products are scarce, pseudoglands are regenerating into normal surface epithelium, and glands are decreased in size. Epithelial cell height is minimal, microvilli are scarce, and the overall appearance resembles a resting stage.

Circulating hormone levels

Ovarian steroids are produced primarily in the ovary, and ovariectomized mice have undetectable levels of circulating estrogens and androgens. Although a basic understanding of gonadotropin and estrogen synthesis is well established, recent studies employing ERα, ERβ, and double knockouts have allowed further refinement (Couse 2003). Hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the anterior pituitary to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH), which act on the ovarian theca and granulosa cells respectively to promote estrogen (17β-estradiol) synthesis from cholesterol and through several intermediates including testosterone. Estrogen from the ovaries, in addition to acting on its numerous other targets, negatively regulates further GnRH release. ERα was shown to be critical in negatively regulating circulating levels of estrogen, testosterone, and LH, while FSH levels are instead influenced by activin and inhibin levels. The role of ERβ in regulating

these hormone levels is minor, however ERβ is implicated in the control of GnRH secretion and of granulosa cell function.

Although uterine cells have been reported to express aromatase and to possess the capability to synthesize estrogens (44), estrogen predominantly acts on the uterus in an endocrine manner following secretion from the ovaries (16). Estrogen is very potent, and serum levels are measured in pg/ml, compared with ng/ml levels of progesterone. Serum estrogen levels vary from approximately 10 pg/ml at metestrus to 60 pg/ml at proestrus. The peak at proestrus stimulates the LH surge, which is accompanied by FSH secretion, and is rapidly followed by a surge in progesterone levels.

Although a major surge in estrogen levels during the uterine cycle is well documented in numerous species, other aspects of estrogen secretion show distinct variability between species. For example in humans, a sharp peak in estrogen release immediately prior to ovulation is followed by a second peak in the middle of the luteal phase, while in the rodent an estrogen surge is associated with late proestrus but other spikes of estrogen secretion throughout the cycle are also observed (45).

Overview of responses to exogenous estrogen administration

Because of the confounding effects of the fluctuations of numerous hormones in the intact animal, the responses modulated by estrogen in the uterus have typically been studied using estrogen-treated noncycling (immature and/or ovariectomized) animal models. 17β-estradiol and 17α-ethynyl estradiol are commonly used, and their responses have been shown to be highly similar (46). The effects modulated by a single dose of estrogen have been shown to encompass a large number of physiological processes, but are roughly divided into two phases (47, 48). Phase I responses include increased uterine

vascular permeability and edema, as well as increased blood flow, cAMP production, changes in electrolyte levels which drive water imbibition, and histamine release that are evident by 6 hr after estrogen exposure. Phase II responses occur at approximately 24 to 30 hr after estrogen exposure and are particularly associated with dramatic increases in epithelial and stromal cell size (hypertrophy) and cell number (hyperplasia) (48-50). The phase II response is also characterized by large uterine glands containing secretory products, by accumulation of uterine luminal fluid, by large interstitial spaces between the stromal cells, by increased metabolic activity and increased RNA and protein synthesis, by increased cytoplasmic volume and RER, and by immune cell recruitment (38, 51-53). The volume fraction occupied by the epithelium, stroma, endothelium, and extracellular space is unchanged, indicating that all endometrial compartments increase in size uniformly during phase II (38). Interestingly, the stromal proliferative response is greatly reduced as animals reach maturity, indicating that choice of model is important to the results that will be generated (54). The mitotic response is maximal at approximately 24 hr after estrogen administration, and has returned to baseline levels by 36-48 hr (50, 55), while apoptosis in response to estrogen withdrawal has become prominent (56). Continuous and unopposed estrogen exposure, by contrast, is associated with neoplasia, with prolonged estrus in the cycling rodent, and in the developing rodent is associated with abnormal uterine gland development, possibly due to an inability of glandular epithelial differentiation and gland invagination into the stroma to keep pace with luminal epithelial proliferation (36).

Many of the effects mentioned above have been studied for several decades, and many are known to be mediated by the estrogen-ER signaling pathway. However, some

extremely rapid and likely nongenomic effects have also been observed, such as an increase in the number of luminal surface microvilli within 30 s after estrogen injection, and great increase in their number and density within approximately 5 min (57).

Although differences in uterine responses to estrogen can occur even between different mouse strains (58), overall, the responses described above, including proliferation (59), stromal edema (60), and immune cell recruitment, have also been observed in estrogen-exposed humans, confirming the merit of the estrogen-treated rodent model in discerning estrogen effects.

Nuclear receptor signaling

Estrogen receptor

ERα and ERβ exhibit distinct tissue distributions, which have been well characterized. Overall, ERα is predominantly expressed in the uterus, mammary gland, liver, kidney, and heart, while ERβ expression which is highest in the ovary and prostate, but is also present in other tissues such as uterus (16).

Within the uterus, ERα (*Esr1*) and ERβ (*Esr2*) are both expressed in the epithelial, stromal, and vascular cells in a menstrual cycle-dependent manner that is distinct for each isoform and has been recently described in detail (61). Estrogen has recently been shown to affect *Esr1* expression in a compartment-specific manner. In the epithelium, estrogen administration causes a rapid (3-6 hr) decrease in *Esr1* expression that is not blocked by cycloheximide, and then an increase at 24 hr (62). By contrast, *Esr1* was rapidly upregulated in the stroma, while another report indicated that stromal and endometrial *Esr1* levels were unchanged (63)

The specifics of the interregulation between ERα and ERβ are not currently well understood, though differences in the isoform ratio have been reported in uterine disease (64). Furthermore, despite high sequence similarity between the two isoforms, differences in their ability to activate transcription at specific response elements and in complex with particular ligands have been reported (65). As well, *in vitro* ERβ has been shown to repress ERα-induced transcriptional activity (66, 67).

There are few documented cases of humans possessing mutations in the ERα or aromatase genes, recently reviewed (8), and therefore targeted disruptions of the ER and aromatase genes in mice have been particularly valuable in understanding the specific role of these genes in estrogen action (16). The uterus of the ERαKO mouse is highly hypoplastic, although it appears normal at the histological level (68). A marked aspect of the ERαKO uterine phenotype is the absence of the uterine edema and increased uterine weight that is normally observed in the early and late phase responses to three consecutive daily estrogen doses (68), though after ten daily treatments a detectable but submaximal induction of uterine weight has been observed (69). Heterozygotes responded normally (68). Estrogen-induced mitogenic activity in the ERαKO uterus is also much lower than in the wild-type, though still detectable (70).

Tissue recombination experiments, in which the uterine epithelium and stroma from WT and ERαKO mice were recombined, showed that that luminal and glandular epithelium proliferation depended on the presence of ERα in the stroma, suggesting that the binding of estrogen to stromal ERα stimulates the production and release of factors that stimulate mitogenesis of the adjacent epithelium (71, 72), which had been suggested by much earlier *in vitro* studies (73). A similar study showed that estrogen stimulation of

stromal ERα results in downregulation of epithelial progesterone receptor expression (74).

The uterus of the ERβKO mouse displays an exaggerated response to estrogen, including hyperstimulated secretion and higher progesterone receptor and Ki67 levels, suggesting that ERβ normally acts to oppose some of the effects of ERα (75).

The similarity in uterine phenotype between the ERαβKO and ERαKO mouse was expected, given the much higher levels of the α isoform normally present in that tissue (76). The uterine phenotype of the aromatase knockout mouse, ArKO, was also similar to that of the ERαKO mouse (77), consistent with the belief that estrogen is required for uterine proliferation and differentiation.

Estrogen receptor-related orphan receptor α1 (ERRα; *Esrra1*) is a transcription factor that can bind DNA at SF-1 response elements (SFREs). Though it shares sequence similarity with the ER, it does not bind estrogen (78). The transcript has been found to be upregulated after 3x24 hr (three consecutive daily doses) estrogen exposure in the uterus (79). Several downstream targets have been identified, including lactoferrin, osteopontin, medium-chain acyl coenzyme A dehydrogenase (MCAD) and thyroid hormone receptor (80), however specific ligands and roles are not currently known. Importantly, though, ERRα has been found to also bind to EREs and to compete with ERs for coactivators, which could profoundly alter ER-mediated signaling (81).

Progesterone receptor

Estrogen and progesterone signaling pathways interact in crucial and complex ways to regulate uterine function. A key function of progesterone, however, is to modify

the action of estrogen on the uterus by redirecting the proliferative response from the epithelia to the stroma. Progesterone also antagonizes the inflammatory action of estrogen by preventing the recruitment of leukocytes that is induced by estrogen (82).

Progesterone has been called the hormone of pregnancy, since maternal production is crucial in all mammals for conceptus survival and development (83). Progesterone acts primarily through the progesterone receptors PR-B and its shorter form PR-A, which are transcribed from the same gene. PR-B is believed to be the dominant PR isoform in the uterus, similar to how ERα is believed to be the dominant uterine ER isoform, and furthermore the ratio of PR-B:PR-A can be associated with uterine pathologies in the same way that alterations to ERα:ERβ levels can (64, 84). Progesterone is widely considered to oppose the effects of estrogen, and in support of this, the uteri of *Pgr* null mice show an exaggerated response to estrogen believed to be due to the lack of progesterone signaling to depress the effects of estrogen. It is believed that progesterone is able to repress the estrogen-stimulated epithelial response through a mode that depends on stromal PR, and involves the repression of estrogen-inducible stromal growth factor production (74).

Estrogen induction of PR (*Pgr*) expression is believed to be mediated by the binding of both AP-1 and E2-ER to a promoter region containing an adjacent AP-1 site and in imperfect ERE half-site (85). Stromal cells require progesterone in order to divide (86, 87) and progesterone accelerates stromal transit through G_1 to S. Progesterone in conjunction with FGF induces stromal cyclin D1, comparable to the actions of estrogen on the epithelial cells (88).

Interestingly, estrogen is required to prime the uterus for progesterone action in the ovariectomized animal, since PR content is low until induced by estrogen. Estrogen exerts both direct and indirect effects on *Pgr* expression, since estrogen has been shown to directly induce *Pgr* in the stroma while indirectly downregulating epithelial *Pgr* by means of stromal ERα (74). Furthermore, during pregnancy, the epithelium manufactures numerous secretory products in response to progesterone despite a lack of epithelial PRs during much of the gestation period, and paracrine signaling from the PR-positive stroma in the form of progesterone-induced HGF, FGF7, and FGF10, for which the epithelium has receptors, has been suggested (83).

Pgr is expressed in the uterine epithelium in the absence of estrogen, while estrogen has been shown to abrogate *Pgr* expression in the LE and upregulate it in the stroma (89), which primes the uterus to respond to progesterone. PR-dependent actions of progesterone in the uterus are described as opposing estrogen, since progesterone can induce stromal cell proliferation, inhibit edema, and affect infiltration of immune cells in various ways. Studies in PR null (PRKO) mice confirmed that PR is required for most aspects of female reproduction, and the PRKO uterus is highly inflamed in response to estrogen exposure.

Androgen receptor

Induction of the rodent uterotrophic response by androgens, which could be inhibited by antiandrogens, was first reported several decades ago (90, 91). Estrogen-induced uterotrophy could be blocked by antiestrogens but not antiandrogens, indicating that the AR may be one of several downstream effectors of the ER that mediate uterine growth.

A specific mechanism defining the importance of uterine androgens and androgen receptor (AR) has recently been proposed (63). It was recently demonstrated that estrogen exposure stimulates *Ar* expression in the uterine stroma and myometrium, and this is believed to be achieved by the interaction of ER and AP-1 at AP-1 response elements (63, 92). AR in turn induces IGF-I secretion, which stimulates epithelial cell proliferation in a paracrine manner (63, 93). Furthermore, it was shown that estrogen-induced luminal epithelial cell proliferation could be prevented not only by antiestrogen administration but also by antiandrogen administration (63). Also androgens are able to induce phase II uterotrophy in a manner similar to estrogen, although at high doses they inhibit estrogen-induced phase I uterine edema and eosinophilia (94).

AR induces gene expression by binding to a specific response element (95), however many genes have multiple response elements and in particular, some target genes are common to estrogen and androgen. For example *Igf1* (93) and thioredoxin (*Txn1*) (96) are each inducible through both ER and AR, with different kinetics.

Glucocorticoid receptor

Glucocorticoids are associated with growth repression, and their ability to inhibit estrogen-induced uterine proliferation and function is believed to promote energy conservation in stressful environments. Glucocorticoids appear to have little effect when administered alone (97), but can prevent estrogen-induced uterine proliferation (97-101), which may be due to its ability to decrease vascular permeability (102) and to the attenuation of estrogen-stimulated induction of growth factors such as IGF-I (103). The glucocorticoid receptor (GR) is expressed exclusively in the uterine stroma, particularly

in fibroblasts and uNK cells, as well as in endothelial cells, suggesting its role in decidualization (104, 105).

Retinoic acid receptor

Retinoic acid (RA) synthesis is stimulated at estrus in the uterine epithelium, and is also observed following exogenous estrogen exposure (106). As a result, it is believed that, since proliferation of the uterine epithelium is required only in the event of pregnancy, the estrogen-induced stimulation of RA at estrus is to be prepared for epithelial proliferation in the event of conception and pregnancy (107). Furthermore, in other tissues retinoids have been found to promote epithelial cells to differentiate to secretory phenotypes (108).

Retinoid signaling is controlled at several levels. Serum transport is effected by retinol-binding protein (*Rbp4*), while cellular carriers include cellular retinol-binding protein (CRBP; *Rbp1*) and two cellular retinoic acid-binding proteins, CRABP-I and CRABP-II. RA induces transcription primarily by heterodimers of the receptors RAR and the multifunctional RXR, which transactivate genes after binding to RA-responsive element sequences (109).

Levels of RAR and RXR have been shown to fluctuate only slightly in epithelial and stromal cells during the uterine cycle (110), and therefore it is believed that regulation takes place primarily at the levels of RA availability and metabolism. *Crabp1* in the uterine stroma is induced transiently by estrogen (4-24 hr), while *Crabp2* expression in the luminal epithelium is sustained to 48 hr (111). Stromal *Rbp1* and *Rbp4* expression, as well as retinol levels, are reduced following estrogen exposure (111). Differences in regulation and function between *Crabp1* and *Crabp2* have been studied

(106, 112), and only *Crabp2* induction has been associated with acquisition of the ability to synthesize RA from retinol (113). Transcripts for proteins involved in RA action, including the cellular RA binding protein *Crabp2*, the retinol binding protein *Rbp1*, the RA biosynthetic enzyme *Rdhl*, and possibly the retinaldehyde dehydrogenase *Aldh1a2*, have also been reported to be induced at estrus (107).

Thyroid hormone receptor

It was first reported 25 years ago that estrogen-induced phase II responses could be diminished in hypothyroid rats, and that the late responses could be restored by thyroid hormone or iodide replacement (114, 115). It was further observed that thyroid hormones were able to repress estrogen-induced edema and eosinophilia phase I responses (116). Although there have not been further studies concerning thyroid actions in the uterus, crosstalk between ER and TR signaling has been recently reviewed (117).

Vitamin D receptor

There is little information available about vitamin D signaling in the uterus, though estrogen has been reported to increase vitamin D receptor levels concurrently with estrogen-induced uterine growth (118, 119). Though the significance is not well understood, the estrogen-mediated control of calcium homeostasis has been suggested. Vitamin D has also been reported to induce endometrial cell proliferation (120) and to potentiate the estrogen-stimulated increase in alkaline phosphatase activity *in vitro* (121).

TRANSCRIPTIONAL EFFECTS OF ESTROGEN IN THE UTERUS

The numerous uterine changes in response to estrogen that are described above are achieved in a variety of ways, but many are associated with changes in gene expression either directly in response to estrogen-ER acting as a transcription factor or indirectly due to an estrogen-induced changes in the levels or activity of other transcription and growth factors. While a multitude of studies have identified and examined transcriptional responses to estrogen in the uterus, the data have not been well reviewed and summarized. Given the recent interest in using large-scale microarray studies to examine estrogen-induced changes in uterine gene expression (122-125), subsequent attempts at the biological interpretation of these large data sets is extremely challenging, and a review of knowledge to date in this area is timely. This review is of benefit both to those seeking to appreciate the effects of estrogen action in the uterus, and to serve as a baseline for those aiming to evaluate the estrogen-like properties of exogenous compounds.

DNA, RNA, and protein synthesis

The remarkable extent of the estrogen-induced uterine proliferative response must be supported by the rapid induction of mitosis and of proteins that physically direct and support growth. Consequently, a swift and coordinated increase in available precursors for DNA, RNA, and protein synthesis must be mounted.

DNA synthesis

Estrogen stimulates DNA polymerase activity in the uterus (126), and DNA polymerase α (*Pola*) expression levels have been shown to be estrogen inducible by 6 hr

in MCF-7 cells, by the interaction of Sp1 and liganded ER at an upstream Sp1 site (127). Recent microarray studies have identified numerous other estrogen-regulated genes that are involved in the DNA replication machinery (128). DNA synthesis has been shown to be stimulated only by long-acting estrogens that are retained for at least 15 hr, such as E2. A short-acting estrogen such as the metabolite estriol is not able to potently induce uterine DNA synthesis unless it is administered again during a critical period 9-15 hr after the initial exposure (49, 129). This long period of stimulation required to induce uterine DNA synthesis, an important effect of estrogen, may be crucial in explaining the apparent disparity with some rapidly inactivated ligands in their ability to rapidly induce ER-mediated gene expression *in vitro* but their inability to induce uterine growth *in vivo*.

Maintenance of a nucleotide pool for DNA and RNA synthesis is critical to cell growth, and while not all associated transcripts have been studied in the estrogen-stimulated uterus, they are believed to be characteristic features of that and other proliferative events. For example thymidine availability is essential for cell proliferation, and its removal in cultured breast cancer cells causes cell cycle arrest that can be relieved by the addition of estrogen (130). Thymidine kinase is critical in the pyrimidine salvage pathway, catalyzing formation of dTMP from deoxythymidine. High levels of activity are associated with rapidly proliferating tissues, and increased thymidine kinase activity and increased thymidine incorporation in response to estrogen are concurrent and well established responses to estrogen in the uterus (131). Activity of the cytosolic isozyme (Tk1) is highly induced 24-36 hr after estrogen exposure, while induction of the mitochondrial form (Tk2) is slight during the same period (132). At the transcriptional level, the induction of *Tk1* (133) and adenosine deaminase (*Ada*) (134) by estrogen is

well established in MCF-7 cells, and the involvement of EREs and of ER-Sp1 interactions, respectively, have been established. Thymidine phosphorylase (platelet-derived endothelial cell growth factor; *Ecgf1*) expression, which is associated with cell cycle arrest and angiogenesis (135, 136), was reported to be inversely correlated with estrogen levels (135, 137). Several other responses may also be associated with transcriptional changes in the uterus that have not yet been studied. The activity of aspartate carbamoyltransferase (*Cad*), a multifunctional enzyme of *de novo* pyrimidine synthesis, is also induced after estrogen exposure, while uridine kinase activity is unaffected and is believed to be present in excess in the unstimulated uterus (138).

Estrogen has long been known to stimulate the covalent modification of histones (139), which can facilitate transcription (140). Estrogen also rapidly stimulates protein synthesis of histones and high mobility group chromatin proteins, and maximal levels (9 hr) are reached several hours before DNA synthesis begins (141). Histone transcript levels, by contrast, lag behind protein levels, beginning to increase at 9 hr and reaching maximal levels at 18 hr (142). While the mechanism by which estrogen stimulates uterine histone transcription is not known, the increased transcript levels just prior to the beginning of S phase is comparable to that observed in HeLa cells *in vitro* (143). Unfortunately, the specific histone transcripts remain to be identified.

RNA synthesis

An early observation in the estrogen-stimulated uterus was the selective synthesis of numerous distinct transcripts (144). In the uterus, estrogen administration is associated with a burst in RNA PolII activity within all uterine cell types, and a subsequent increase in the ratio of RNA to DNA in all major uterine cell types (145). In the classical mode of

estrogen action, the estrogen-ER complex acts as a transcription factor to induce the expression of numerous key ERE-containing genes. While this mechanism of action has been highly studied and reviewed, some important recent advances in this area have focused on alternate modes, such as through associations with AP-1 and Sp1 and with alternative response elements (28). In addition, intensive efforts have been focused on identifying the nature and consequences of coactivator and corepressor interactions with the transcriptional complex recruited by estrogen-ER. Alterations in the expression of relevant coactivators and corepressors in the estrogen-stimulated uterus are not yet well characterized, although their levels and localization during the uterine cycle have recently been shown to be complex. For example, mRNA levels of the coactivator SRC1 and the corepressors SMRT and NCoR have been shown to vary during the human and rodent uterine cycle in a cell type-specific manner, while CBP/p300 levels were found to be constant (146, 147).

The estrogen-induced uterine expression of transcription factors, which can in turn act as effectors of estrogen action by modulating the expression of numerous target genes, has been highly studied (148-153). Results suggest that these gene products play key roles in the early phase uterine growth promotion induced by estrogen (154), though short-acting estrogens are able to induce immediate early gene expression (Fos, Jun, Myc) without being able to stimulate later DNA synthesis (129, 155).

AP-1

Members of the Fos, Jun, Jun dimerization partner (JDP), activating transcription factor (ATF), and Maf families can homo- or heterodimerize in specific ways to form the AP-1 transcription factor, which activates gene expression at response elements that

depend on the components of the dimer. AP-1 components are transcriptionally induced by numerous diverse stimuli, including several growth factors and cytokines, and the complex nature of their regulation and roles in cell cycle progression have been recently reviewed (156).

Fos can be upregulated by a downstream ERE sequence (157), and shows rapid and well-characterized transcriptional inducibility and short half-life in the uterus (150, 158). Estrogen rapidly induces *Fos* expression specifically in the luminal and glandular epithelia (155), while other fos-related genes (fra-1, fra-2, and fos B) have not been detected (158). Transcriptional regulation of Jun family members by estrogen is also complex and cell type-specific. For example, estrogen induces a rapid increase in jun-B (*Junb*) and jun-D (*Jund*) expression mainly in the epithelial cells, while inducing c-jun (*Jun*) expression in the myometrium and repressing it in the epithelium (159). Temporal expression is also subtype specific, with *Jun* being induced most rapidly (within 30 min), followed shortly after by *Jund* and then *Junb* (160). In addition to inducing the expression of Fos and Jun family members, the ER can affect AP-1 mediated signaling by interacting directly the Jun or Junb subunit of AP-1 (161).

Myc

Myc plays a central and highly complex role in pathways regulating G_1 progression, differentiation, and apoptosis, with many features that are currently not understood (162, 163). The heterodimeric transcription factor Myc/Max typically binds to motifs termed E-boxes (5'-CACGTG-3'), and regulates transcription of numerous target genes involved in cell proliferation, while alternate Max binding partners (e.g. Mad, Mxi) block these responses, though the mechanism is now believed to be more complex (164).

Direct targets have recently been shown to include cyclins and cyclin-dependent kinases (165), and numerous other well characterized responses of importance to estrogen proliferation include Myc-mediated induction of *Cdc25a* (166), ornithine decarboxylase (*Odc1*) (167), and the E2F transcription factors, and repression of growth arrest gene Gas1 and of cell cycle inhibitors such as p15, p21, p27 (168). Microarray studies are now identifying numerous other targets (169 , 170 , 171).

Myc transcription is upregulated by numerous mitogens, and in the uterus rapid upregulation in response to estrogen is specifically associated with the cell types that have been stimulated to divide (172). Uterine N-myc (*Mycn*) expression can be observed within minutes after estrogen administration, while *Myc* expression increases after 2-4 hr exposure and then again after 28 hr (148, 173).

E2F

Transcriptional regulation of E2F genes has not been reported to date in the estrogen-stimulated uterus. However these factors have been shown to be critical to G_1/S progression (174, 175), and *E2f1* has been shown to be estrogen inducible *in vitro* by a mechanism dependent on ER and Sp1 (176).

Protein synthesis

Estrogen not only promotes protein synthesis indirectly by inducing the transcription of genes involved in estrogen action, but has several other actions as well. Activation of numerous tRNA synthetases in the estrogen-exposed uterus is almost immediate (177), and is followed by a very rapidly (1-2 hr) induced rate of mRNA translation (178, 179). In addition, later accelerated formation of ribosomes and increased

amounts of tRNAs (178) have been observed. All of these factors contribute to an enhanced capability of the stimulated uterus to synthesize proteins, and it is believed that the mRNA, rather than translational components, is the rate limiting component in uterine growth responses following estrogen stimulation (178).

Heat shock proteins (HSPs) can act as chaperones to promote correct protein folding. They also associate with unliganded ER and are believed to repress transcription while promoting ligand binding (180). A recent model describes the association of unliganded ER with a 'foldosome' complex composed of a specific arrangement of HSPs 40, 60, 70, and 90 (181). The expression of several HSPs in response to estrogen stimulation in the uterus have been described. HSP70 and HSP90 have been found to be upregulated in the myometrium during the proliferative phase (182), while expression of an HSP70 (183), of the HSP90 isoforms *Hsp86-1* and *Hspcb*, and of the glucose regulated chaperones GRP94 (*Tra1*) (184) and GRP78 (BiP; *Hspa5*) (185) have been shown to be upregulated in the rodent uterus within 4-18 hr after estrogen exposure. Induction of HSP expression is typically achieved by stimulation of the heat shock transcription factors (HSFs), and *Hsf1* and *Hsf2* have themselves been shown to be upregulated in the uterus by estrogen within 6 hr after administration (186). Regulation by estrogen of Hsp27 has not been well studied in the uterus to date, however protein levels have been shown to be induced by estrogen in glandular epithelial cells (187), and to be induced by estrogen through cooperative binding of ER and Sp1 (188).

Proliferation

It is been established for several decades that estrogen is able to rapidly recruit quiescent uterine cells to enter the cell cycle (189), with 50% of cells in the luminal

epithelium in mitosis by 24 hr after estrogen exposure (38), similar to the proportion of MCF-7 cells in S phase after 24 hr estrogen treatment (190). As a result, the estrogen-stimulated uterus has been used by some investigators as a model system to examine cell cycle entry and progression in an intact system. Current understanding of the mechanisms by which estrogen stimulates cell cycle entry and progression is based on a variety of systems, particularly estrogen-treated cultured cells. Research on the molecular mechanisms governing cell cycle control continues to be extensive, and to be supported and extended by large-scale microarray studies (e.g. (191-196)), and while many pathways have been clarified, differences clearly exist in different cell types and environments.

Uterine cell proliferation (hyperplasia) is a classic response to estrogen exposure, and excessive stimulation by estrogen has also been implicated in uterine abnormalities, infertility, and cancer (99). Abnormal uterine gland development in response to chronic estrogen stimulation is believed to be due to the rapid rate of epithelial proliferation outpacing and physically preventing the normal formation of glandular invaginations, as well as a reorientation of mitosis to be perpendicular to the basement membrane, which promotes the formation of glandular growths that are associated with precancerous changes (99). Furthermore, hyperplasticity itself can be associated with an increased risk of dysplasia (abnormal growth and cancer). Interestingly, expression of proliferating cell nuclear antigen (*Pcna*), which is a DNA polymerase processivity factor essential for DNA replication and which is induced approximately 24 hr after estrogen exposure, has been found to be an ineffective indicator of uterine proliferation, since it is also highly expressed during estrogen withdrawal-induced apoptosis (197).

Estrogen stimulation is known to be required throughout G_1 (198), while *in vitro* it has also been demonstrated that late G_1, G_1/S, and G_2 are refractory to estrogen exposure (130). A key effect of estrogen exposure is the dramatic shortening of G_1, which decreases from approximately 100 hr to 15 hr in the immature mouse (54). While earlier models suggested that estrogen-induced uterine mitogenesis is mediated solely or primarily by autocrine growth factor secretion, it has now been shown that estrogens can also promote G_1 progression directly by activating cell cycle genes or indirectly by activation of the ras-MAPK pathway. Consequently, a growing number of pathways by which estrogens can promote G_1 are being identified, which may serve to potentiate the estrogen induction of cell cycle progression.

Cell cycle progression

It has been shown *in vitro* that estrogen treatment causes a rapid activation of responses associated with preparation for the G_1/S transition. These include activation of Cdk4 and Cyclin E-Cdk2, hyperphosphorylation of pRB, and increased cyclin D1 (*Ccnd1*) and *Myc* transcript and protein levels within the first 3-6 hr (190, 199). Cyclin D1 and Myc by different mechanisms activate cyclin E-Cdk2, which then associates with hyperphosphroylated p130 (199). Numerous other recent studies have identified direct actions of the ER, in the presence or absence of ligand, on the activity of integral mediators of cell cycle progression (e.g. (200-203)).

Many of the complex responses stimulated by estrogen that promote uterine proliferation are reflective of transcriptional changes. In particular, the cyclins act as the regulatory subunits of the cyclin-Cdk complexes, and are transcriptionally regulated by numerous stimuli. Cyclin levels are closely linked to cell cycle progression, and they

therefore have short half-lives and are rapidly degraded by a ubiquitin-dependent pathway. A highly studied example is *Ccnd1*, which is expressed in most cell types and is induced rapidly during G_1 by stimuli such as estrogen (204). Estrogen induces Cyclin D1 expression and activity by numerous diverse pathways (205). Interestingly, in addition to interactions with Cdks, Cyclin D1 has also been shown to potentiate ER-induced transcription *in vitro* by directly binding to at least one ER coactivator (206).

In the estrogen-stimulated rodent uterus, *Ccnd2* was found to be induced most highly and rapidly, at 1-8 hr after exposure, while *Ccnd3* was also induced at approximately 2-8 hr, and *Ccnd1* was only slightly induced, at approximately 8-24hr. The functional significance of the differential regulation of the D-type cyclins is not currently known, though the existence of single and double knockouts is assisting in this (207). Cyclin E (which could be *Ccne1* or *Ccne2*) was induced slightly at 8-24hr, while cyclin A (which could be *Ccna1* or *Ccna2*) was highly induced at 24hr, and more modestly at other times in the 16-28hr range (208).

Estrogen-responsive finger protein (Efp; Zinc finger protein 147; Trim25) is a ubiquitin ligase of the tripartite motif (TRIM) family, and is able to degrade 14-3-3σ (Sfn; also known as Stratifin or Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein σ), a factor that promotes cell cycle arrest in G_2 (209). *Trim25* is expressed in the uterine luminal epithelium (210), is rapidly induced by estrogen exposure, and is critical to estrogen-induced endometrial proliferation and water imbibition responses (211). ERα and Trim25 have been shown to colocalize, and the *Trim25* genomic sequence contains an ERE motif (210). Targeted *Trim25* disruption results in underdeveloped uteri, decreased response to estrogen, and decreased proportion

of uterine cells reaching S phase and beyond (211), although the phenotype is not as severe as in the ERα null mouse.

Importantly, estrogen is concurrently able to suppress transcription of cell cycle inhibitors. For example, the growth arrest specific gene *Gas1*, which blocks S-phase entry and is actively transcribed in the unstimulated uterus, is suppressed in a rapid and prolonged manner following estrogen exposure (212). However, the cell cyclin inhibitor p27 is localized to the uterine stroma in the adult rodent, which may help to explain why stromal cells to not proliferate extensively in the adult in response to estrogen (213).

Telomerase (*Tert*) is a ribonucleoprotein polymerase containing a short RNA template (*Terc*) that directs synthesis of telomeric repeats at chromosomal ends to prevent telomere shortening. While most normal somatic cells are not associated with telomerase activity, those with high regenerative potential, including the late proliferative phase endometrium, can express high levels. In the cycling uterus, *Terc* expression was found to be highest in proliferating glandular epithelial cells (214). Although *Terc* was not induced by estrogen in uterine epithelial cells *in vitro*, expression of the catalytic subunit *Tert* has been shown to be stimulated in MCF-7 cells by estrogen both directly at ERE and ERE/Sp1 elements, and indirectly by estrogen-induced Myc (215).

Peptide growth factors

The importance of growth factors in uterine growth and the crosstalk in signaling between growth factors and the ER is well established, but many aspects remain poorly understood. It has long been established that growth factor overexpression can stimulate uterine growth and reciprocally that defects in growth factors signaling can result in hypoplasia, as will be described below.

It was long believed that all or most of the actions of estrogen on the uterine epithelium were mediated by the direct action of estrogen in this cell type. However, recent evidence indicates that many vital actions of estrogen on the epithelium are mediated by stromally derived paracrine factors. First, it was observed that estrogen was still able to induce uterine epithelial proliferation in neonatal mice, which lack epithelial ERs (216), and a paracrine growth response emanating from within the ER-positive stroma was suggested. Another important advance in this area came with the development of uterine tissue recombinants differentially possessing epithelium and stromal from wild-type or ERα knockout mice (71). Using these recombinant tissues it was possible to differentiate between responses mediated through epithelial vs. stromal ERs, and it was demonstrated that induction of epithelial proliferation was a paracrine event requiring ERα-positive stroma, whereas epithelial ERα was not necessary or sufficient to permit estrogen-induced epithelial proliferation. Epithelial-stromal interactions have also been shown to be critical to growth in uterine cells *in vitro* (217) and in other tissues *in vivo* (218-220).

It has now been demonstrated that numerous secreted factors can induce epithelial cell proliferation, and their involvement is discussed in the following sections. While the roles of growth factors in the uterine response to estrogen are not fully understood, factors such as EGF, FGFs, and PDGFs have been termed 'competence factors' due to their ability to stimulate quiescent cells to enter G_1 (221, 222), while IGF-1 has been characterized as a G_2/M progression factor (223).

EGF and TGFα

Epidermal growth factor is a member of the EGF family of ligands, which also includes TGFα, HB-EGF (heparin-binding EGF; DTR), amphiregulin, epiregulin, and heregulins. EGF functions as a progression factor, and can act as a potent mitogen in certain cell types. Like other growth factors, EGF binding to its membrane receptor (EGFR; ErbB1) induces dimerization and intrinsic receptor tyrosine kinase activity, which in turn induces phosphorylation cascades involving PLCγ, MAPK, and the Ras GTPase-activating protein (GAP). These signaling pathways can then induce expression of immediate early genes such as *Fos*, *Jun*, and *Myc* (206), *Ccnd1* (224), and other genes involved in further transcription and in adhesion, such as actin (225) and tyrosine hydroxylase (*Th*) (226). Heterodimerization to other EGFR family members (ErbB2-4) may produce other distinct actions (227).

EGF rapidly induces DNA synthesis in all major uterine compartments, and estrogen-induced uterine proliferation can be repressed by coadministration of an EGF antibody (228). Furthermore, proliferation induced by either EGF or estrogen can be blocked by coadministration with antiestrogen (229), and is absent in the ERαKO mouse (230). Experiments in *Egfr* null mice were able to demonstrate the paracrine signaling of EGF (231). This study revealed that the epithelium was not a direct target for EGFR ligands, in contrast to previous findings *in vitro* (232). *Egfr* is induced rapidly in the uterus (233, 234), in particular in the stroma and myometrium (235), and deficiency is associated with severe hypoplasia due to an inability of estrogen to elicit growth of the stromal cells. The predominant localization of *Egf* itself to the epithelium (236), suggests that in response to estrogen, newly synthesized *Egf* in the epithelium either translocates to

the stroma to induce EGFR-mediated stromal growth, or else may interact with epithelial ErbB2. *Erbb2* expression is induced in the epithelium in response to estrogen and may be involved in epithelial proliferation (237).

Recent research has shown crosstalk between estrogen and EGF signaling to be highly complex, which has been recently reviewed (238). In addition to estrogen stimulating expression of *Egf* and its receptor, estrogen and EGF interact in other ways. In particular, the EGF pathway can induce phosphorylation of ER or its coregulators to alter its activity, and in the nucleus has even recently been shown to be directly associated with the *Ccnd1* promoter (239). As well, the estrogen signaling pathway can induce specific matrix metalloproteinases by a nongenomic mechanism, and these can activate HB-EGF, which then binds to and activates EGFR (240).

Transforming growth factor α is a member of the EGF ligand family, and is able to bind to EGFR (241). *Tgfa* transcript levels in the epithelial and mast cells are stimulated in the uterine epithelium by 6 hr following estrogen exposure, and the protein is secreted in the uterine luminal fluid at much higher levels than is EGF (242). Overexpression of *Tgfa in vitro* is associated with enhanced proliferation (243), while transgenic mice overexpressing *Tgfa* exhibit increases in estrogen-induced uterine hyperplasia (244) and delayed implantation (245). Furthermore, administration of antibodies to TGFα significantly reduces estrogen-induced uterine growth (242). The significance of the estrogen-mediated induction of two EGFR ligands (i.e. EGF and TGFα) is not clear, although TGFα has been reported to be more potent, and both are able to induce *Tgfa* expression *in vitro* (246). This overlapping function is consistent with

the lack of severe reproductive phenotype in the *Tgfa* null mouse (247). TGFα is also associated with stromal decidualization and embryo implantation during pregnancy (248).

IGFs

The role of insulin-like growth factors and their binding proteins in female reproductive function have received much attention (249). IGF-I is believed to be the dominant IGF in the reproductive tract, and to exert most of its effects through the membrane type I receptor (IGF1R). Strong evidence also suggests that IGF-I acts a paracrine factor produced in the stroma and acting on IGF1R in the epithelium to induce mitogenesis, though recent data suggests that systemic IGF-I levels are sufficient and that local synthesis is not required (250). The importance of IGF-I in uterine function was strongly suggested by numerous lines of evidence, including the colocalization of estrogen-induced *Igf1* expression and cellular proliferation (251) and the hypoplastic uteri of the *Igf1* null mouse (252). The known roles of IGF-I in uterine function have been recently reviewed (253).

The single IGF-I protein can be encoded by different transcripts that are subjected to complex differential regulation (249). *Igf1* has been shown to be regulated by numerous pathways, including directly by estrogen-ER in the uterus (253, 254). Uterine expression of *Igf1* is induced by estrogen primarily in the epithelium, while it is induced by progesterone and androgen primarily in the stroma (255).

Targeted disruption of the mouse *Igf1* gene has demonstrated that IGF-I plays a critical role in G_2 progression, consistent with the observed reduction in cell size in the null mouse. In addition, the lack of apoptotic response to uterine estrogen withdrawal in the *Igf1* null mouse has been proposed to be due in some way to a lack of completion of

the mitotic cycle (223). Furthermore, it was then shown that IGF-I, like estrogen, can elicit a uterine proliferative response that depends on the presence of ERα expression, and that these two pathways crosstalk *in vivo*, since IGF-I can induce ER-mediated transcription in the absence of estrogen. It has been proposed that activation of ERα by either E2 or IGF-I is essential for uterine proliferation (256). However, IGF-I is not required for the induction of epithelial cell height or expression of lactoferrin (*Ltf*) or *Pgr* (250).

IGF-I is considered to be one of several estromedins (i.e. mediators of estrogen signal) during the proliferative phase, acting as a mitogenic stimulus to effect rapid endometrial growth, while IGF-II is highly expressed in the mid-secretory phase, and is suggested to be a progestomedin acting on the stroma. IGF1R is expressed primarily in the glandular epithelium with lower amounts in stroma; since IGFs are expressed by stroma, they are believed to participate in both stromal proliferation (by autocrine mechanisms) and in epithelial proliferation (by paracrine mechanisms) (42). IGF-II is believed to be involved in stromal differentiation, and to be induced by progesterone (253).

The receptors *Igf1r* and *Igf2r* are both expressed in the endometrium, predominantly in the LE and GE, although IGF1R is believed to be the isoform that mediates IGF-I signaling (253). Receptor levels do not appear to fluctuate in the uterine cycle, indicating that control is at the level of the IGFs and IGF binding proteins (IGFBPs).

The actions of IGFs can be modulated by interactions with a diverse class of high affinity insulin-like growth factor binding proteins (IGFBPs). By competing with the IGF

receptors for IGF binding, they are able to influence IGF availability and thus its signaling potential, while their proteolysis reduces their ability to sequester IGF (249). The subcellular localization of the IGFBPs during the uterine cycle has been recently reviewed (249), and their patterns of expression and regulation appear to be very divergent. *Igfbp1* has been shown to be repressed by estrogen and induced by progesterone, and estrogen exposure is associated with decreased *Igfbp3* and increased *Igfbp4* expression (257). Progesterone, by contrast, has been shown to induce stromal *Igfbp1* production and decrease IGF receptor levels during the secretory phase, which then decreases IGF-I bioactivity to inhibit stromal growth and promote differentiation and decidualization (249). Furthermore, mice overexpressing *Igfbp1*, which localizes to the epithelium and to luminal secretions, exhibit an impaired uterotrophic response to estrogen, IGF-I, and EGF, attributed to a reduction in the availability of free IGF-I (258).

TGFβ

The three transforming growth factor β isoforms are involved in a highly complex array of distinct and overlapping biological effects, including effects on regulation of cell growth and differentiation, apoptosis, angiogenesis, immunomodulation, and ECM formation, and the numerous null mutants that have been generated are greatly furthering this knowledge (259). TGFβs have been recently shown to have numerous direct effects on cell cycle regulatory components (206).

Transduction of TGFβ signaling, following receptor binding and phosphorylation, is mediated by Smad cascades, which act to regulate expression of numerous target genes (260). Several modulators of TGFβ signaling have also been shown to have altered expression in the uterus in response to estrogen. For example, repression of *Ski*

expression by estrogen in the uterine epithelium has been observed as early as 2-6 hr after exposure (261). Ski has been identified as a corepressor of Smad signaling, stabilizing the association of other corepressors and histone deacetylases in the absence of the TGFβ signal (262, 263). Jun has also been shown to cooperate in this mechanism, following activation of the JNK signaling pathway. Furthermore, Myc has been shown to bind to Sp1-Smad complexes thereby preventing transcription of Sp1 and TGFβ targets, such as the cell cycle inhibitor p15 (264).

Tgfb2 is rapidly induced in the estrogen-stimulated rodent uterus, either transiently (265, 266) or in a sustained manner, and the protein is also detected in the uterine luminal fluid (267). *Tgfb1* shows a similar pattern of expression to *Tgfb2* (265). *Tgfb3* was found to be unaffected in the uterus, although it has been shown to be induced by ERα in a ras-dependent manner (266, 268). Protein levels of all three are induced in a sustained manner following estrogen exposure (265).

Estrogen withdrawal in the mouse is associated with epithelial apoptosis and markedly increased expression of the TGFβ receptor *Tgfbr2*. Epithelial apoptosis can be alternatively induced by stromal administration of exogenous TGFβ1 (269).

FGFs

Fibroblast growth factors are a large family of polypeptides with roles in cell proliferation, migration, implantation, and other processes. They bind heparan sulfate proteoglycans with high affinity, which assists in their activation of surface FGF receptor tyrosine kinases, a receptor family with complex patterns of alternative splicing that yield specificity for specific FGF family members (270).

Fgf9 expression has been studied in detail, and is localized mainly in the stroma, along with its high-affinity receptors (271). FGF9 was also shown be necessary in the induction of stromal proliferation and be induced by estrogen, implicating FGF9 as an important effector in linking the rising levels of estrogen signal in the late proliferative phase to the subsequent rapid endometrial proliferation (271). Similarly, the stromally associated keratinocyte growth factor (KGF; *Fgf7*) in the uterus can be induced by estrogen (272), and has been shown to highly induce uterine epithelial proliferation and surface changes (273), although the role is not critical since *Fgf7* null mice are reproductively normal (274). Basic FGF (*Fgf2*) expression has also been shown to be elevated rapidly following estrogen exposure, although induction by progesterone was more pronounced (275). In the sheep uterus, *Fgf2* expression has been localized to the endometrial glands in response to estrogen (8-24 hr), and is thought to be involved in the uterine angiogenic response (276, 277).

VEGF

Vascular endothelial growth factor A was first discovered as a vascular permeability agent, but is also recognized as an important inducer of endothelial cell proliferation, migration, and differentiation. *Vegfa* has multiple splice variants that give rise to different isoforms, but in most species $VEGF_{165}$ is the more potent endothelial cell mitogen and the dominant variant in the uterus (278). Most VEGF isoforms can bind to the ECM through heparin-binding domains (278). The VEGF glycoprotein is active when associated with dimerized surface receptor tyrosine kinases, Flt-1 and Flk-1, which are localized on endothelial cell surfaces. These activated receptors activate signal transduction pathways to stimulate cellular migration and endothelial cell mitosis

respectively. Recently $VEGF_{165}$ has also been shown to bind to the neuropilins NRP1 and NRP2, surface receptors that are differentially expressed in the uterine endothelial and glandular epithelial cells (278). The functional significance of VEGF associations with neuropilins is not yet well understood, but it has been suggested that neuropilins act as coreceptors along with Flt-1 or Flk-1 to enhance their action (279).

Vegfa is expressed in cells lining the endometrial and myometrial vessels as well as in neutrophils and macrophages, as reviewed in (276, 280). Other angiogenic factors, including *Vegfc*, are expressed in uNK cells. *In vitro*, *Vegfa* induction by estrogen has been shown to be mediated by both ERα and ERβ, while *Vegfc* is repressed. Many aspects of the control of VEGF family expression in the uterus are not currently well understood, although there appear to be great cell type-specific differences (281). In whole uterine studies, the rapid induction of *Vegfa* expression in response to estrogen is very well studied (282). Recent comprehensive studies with knockouts including ERα and PR null mice, however have given rise to the suggestion that *Vegfa* is primarily progesterone-induced, since stromal expression of *Vegfa* and its receptor VEGF receptor 2 (*Flk1*) is inducible by progesterone alone, and is preserved in the PRKO and abrogated in the ERαKO (282). Additional studies with an *Flk1* null mouse indicated that estrogen inhibits, while progesterone stimulates, uterine angiogenesis, and that estrogen is able to inhibit the effect of progesterone, furthermore that these responses could be eliminated by specific ER and PR inhibitors respectively (282). Similarly, estrogen inhibits while progesterone stimulates uterine *Nrp1* and *Nrp2* expression (278).

PDGF

Platelet-derived growth factor consists of homo- or heterodimers of PDGF isoforms that display distinct functional properties and affinities for the PDGF receptors. Estrogen rapidly induces expression of *Pdgfa* and *Pdgfb*, as well as of the receptors *Pdgfra* and *Pdgfrb*, in the uterine epithelium and stroma (283). PDGF has also been shown to stimulate stromal and myometrial cell proliferation *in vitro* (284 , 285). The important role of PDGF in uteine leiomyoma cell cycle progression has also been well characterized (286).

TNFα

Tumor necrosis factor α is a pleiotropic cytokine produced by numerous cell types, and with many important roles in reproduction that are modulated by interaction with two TNFRSF1 receptor isoforms (287). *Tnfa* is expressed in many cell types, including all uterine leukocyte types, although estrogen regulation has particularly been studied in uterine mast as well as myometrial cells (288, 289). TNFα is also a component of secreted uterine fluid (290). In MCF-7 cells, TNFα administration inhibits estrogen-induced G_1/S phase progression (291).

Transcriptional regulation of *Tnfa* appears to be complex. *Tnfa* expression in the mouse uterus is rapidly induced (1-6 hr) by estrogen in the epithelium, and then later is localized to both the epithelium and stroma (72 hr) (292). Estrogen withdrawal is also associated with induced epithelial *Tnfa* expression *in vitro*, and TNFα can also induce its own synthesis (293). Epithelial and stromal transcript levels of the receptor *Tnfrsf1a* have also been shown to be elevated 24 hr after estrogen exposure or after prolonged progesterone exposure (294).

In addition, uterine transcript levels of the TNFα-induced protein 6 (*Tnfaip6*) have been shown to be highly elevated at 8 hr after estrogen stimulation (183). Tnfaip6 is a member of a secretory hyaluronan binding protein family that can be rapidly transcribed in response to TNFα or IL1β, which can potentiate the binding of AP-1 to its response element, and by the active isoform of NF-IL6 (CCAAT/enhancer binding protein β) (295). It has been shown to be involved in cell migration, proteoglycan synthesis, and ECM stability, and it can bind to and promote the activity of a serine protease inhibitor involved in inflammation.

CCN family

The ctgf/cyr1/nov (CCN) family is currently composed of six structurally related members, all of which contain an IGF binding domain as well as a cell attachment domain. Numerous functions have been ascribed to the CCN family, although CTGF and Cyr61 have been most often associated with promoting cell growth and the others with growth inhibition (296), and have also been associated with estrogen-stimulated uterine growth (below). Wisp2 is also a member of the CTGF family, although it lacks the heparin binding domain that the others possess, and is believed to be a secreted protein (297). Estrogen regulation of Wisp2 expression has not yet been reported in the uterus, although it has been recently identified as a robust estrogen-inducible gene in breast cancer cells (298).

Connective tissue growth factor CTGF is the best studied member of the CCN family, and was first isolated as a peptide growth factor capable of stimulating endothelial cell DNA synthesis and chemotaxis, and as an immediate early gene that could be transcriptionally induced by serum or TGFβ in fibroblasts. While CTGF was

named for these actions, its roles are now known to be very complex, and its involvement in numerous other cell types including epithelial, endothelial, and vascular smooth muscle cells, have been recently reviewed (296). CTGF has numerous aliases, including IGFBP8, although its interaction with the IGF signaling system is not currently well understood. CTGF can bind to cell surface integrins to promote adhesion of numerous cell types, and can also bind heparin, and consequently interactions with the ECM are believed to regulate CTGF bioavailability. In addition, heparin has been implicated in the regulation of CTGF-mediated mitogenic activity.

Ctgf expression has been shown to be strongly stimulated by TGFβ or serum, but to also be induced in a weaker manner by other growth factors (PDGF, EGF, and FGF). The *Ctgf* gene contains an unusual TGFβ response element that has not been found in other CCN family members or TGFβ-inducible genes. It has also been shown to be induced directly by factor VIIa, factor Xa, or thrombin in fibroblasts in a TGFβ-independent manner. CTGF can then induce transcription of numerous ECM components, but other targets have been found to include cyclin A and Bcl-2. *Ctgf* expression in uterine epithelial cells is decreased at implantation, consistent with the degradation of subepithelial ECM that is coincident with the establishment of neovascularization of maternal stromal tissues during placentation. Furthermore, *Ctgf* is expressed at relatively high levels in the epithelial cells of the uterus, and expression levels change during the estrous cycle, suggesting that it plays a dynamic role as a function of reproductive status, possibly in regulating subepithelial ECM stability. CTGF is also present in uterine secretory fluid.

CTGF can stimulate the synthesis of numerous ECM components, including fibronectin, collagen, integrins, lysyl oxidase, and CTGF itself in fibroblasts, and Tgfb stimulation of these CTGF-mediated processes is implicated in both normal tissue remodeling as well as in fibrotic lesions. CTGF has also been shown to be critical to the G_1/S phase transition in fibroblasts (299). Little is known, by contrast, about the role of CTGF in epithelial cells, but it has been implicated in epithelial cell adhesion.

Ctgf is weakly expressed in the unstimulated uterine epithelium, and is transiently further induced by estrogen treatment and to a lesser extent by progesterone, in a manner that has been proposed to be both TGFβ1 dependent and independent (300).

The cysteine-rich protein Cyr61, also identified as IGFBP10, was first identified as a serum-activated immediate-early gene in fibroblasts (301). *Cyr61* was later reported to be highly induced at 4-8 hr in the estrogen-stimulated rodent uterus (183). Recently, it has also been shown to be an estrogen-inducible factor required for cell cycle progression in MCF-7 cells, and also to be induced by EGF treatment (302). Secreted Cyr61 associates with cell surfaces and ECM and acts as an adhesion substrate for integrin receptors, and promotes angiogenesis by regulating expression of specific genes (303, 304). The importance of Cyr61 was further demonstrated when the null mouse was found to be embryonic lethal due to impaired vascular development (305).

Polyamine utilization

The endogenous polyamines, which consist of spermine, spermidine, and putrescine, are ubiquitous small aliphatic amines that are critical for mitotic spindle formation and chromosome condensation, and that serve numerous other roles in cell growth, differentiation, and apoptosis that have been extensively reviewed (306, 307). A

highly regulated group of enzymes act to constantly modify the intracellular polyamine pool to meet current cellular needs, which is believed to be a universal requirement of proliferating cells.

Ornithine decarboxylase (ODC), a rate limiting enzyme, converts the TCA cycle intermediate ornithine to putrescine, which can be further converted to spermine and spermidine. ODC activity is controlled at the transcriptional, translational, and posttranslational levels and is under strict negative feedback control by its polyamine products. Rapid and dramatic regulation of *Odc1* expression is possible because of its extremely short half-life of several minutes to 1 hr (the shortest half-life of any known enzyme). Its degradation is regulated by a unique regulatory protein named antizyme, which binds reversibly to ODC monomers and promotes its proteolytic degradation by the 26S proteasome. Antizyme also downregulates polyamine uptake by cells. Activity of antizyme is regulated by another unique protein, the antizyme inhibitor, which is highly homologous to ODC but lacks its activity completely and is believed to prevent ODC degradation by trapping the antizyme (308).

Odc1 expression is very low in quiescent cells, and expression can be induced as an early response to numerous stimuli, being maximal in mid-G_1, and decreasing as the cells enter S phase (309). Sustained *Odc1* expression involves Myc/Max-induced transcription (310), whereas expression of the Myc/Max inhibitor Mxi is inversely correlated with proliferative activity (311).

Energy utilization and storage

ATP

The predominant component of a rapidly inducible protein originally designated estrogen-induced uterine protein (IP) was later identified as brain-type creatine kinase (CKB), while the second component was identified as brain-type gamma enolase (Eno2) (312). Estrogen induction of uterine activity of CKB and the glycolytic enzymes Eno2, phosphoglycerate kinase (PGK), and pyruvate kinase (PK), but not phosphoglycerate mutase (PGM), was then confirmed (313).

Creatine kinase can reversibly catalyze ATP production, using creatine phosphate as a phosphate donor. It has been shown that creatine phosphate, and to a lesser extent ATP levels in the rodent uterus begin to be depleted within the first two hours after estrogen stimulation, and that initial levels are reestablished at approximately 6-12 hr, then increasing to maximal levels at 24 hr (314, 315).

Ckb transcript levels are rapidly and highly induced in the uterus and other tissues following estrogen exposure (316), particularly in the uterine smooth muscle (317). The *Ckb* promoter has been extensively studied, and while numerous transcription factor binding sites have been identified, of particular interest to uterine responses is that the transcript has been found to be upregulated by binding of ER to an imperfect ERE and an Sp1 site in certain cell types (318). Upregulation of the ubiquitous mitochondrial creatine kinase, *Ckmt1*, has also been demonstrated in the presence of estrogen *in vitro* (319), and it is believed to be regulated coordinately with *Ckb* in the uterus (320).

Glucose

An early effect of estrogen exposure observed in the rodent uterus is the rapid uptake of glucose, which is an important component of numerous vital pathways. It has been shown that estrogen stimulates uterine glucose utilization for the pathways of protein, lipid, GABA, lactate, and carbon dioxide formation (321). In conjunction with this, the levels of intermediates of glycolysis, TCA cycle, and pentose phosphate pathway were measured, and many were found to be elevated in the uterus at 4-16 hr after estrogen administration (321).

It was recently shown that uterine increases in glucose uptake, which are already detectable at 15 minutes after estrogen injection and begin to plateau at 4-8 hr, are partially attributable to increased transcription of the glucose transporter GLUT1 (*Slc2a1*), while other glucose transporters were found to be unaffected or undetectable (322). Since increases in *Glut1* transcript and protein levels are not induced before the observed stimulation of glucose uptake, however, it is believed that other mechanisms, as yet undefined, also contribute to this response (322).

Rapid induction of glucose utilization is accompanied by stimulation of lactate production and glycogen and glutamate synthesis (323). Numerous studies have shown that fructose-2,6-bisphosphate and other glycolytic intermediates are upregulated in the uterus after estrogen exposure, paralleled by gradually increased activity in glycolytic and pentose phosphate pathway enzymes (321). The rate of glycolysis has been shown to increase within 3 hr (324).

Lipoproteins and fatty acids

A widely studied effect of estrogen administration in humans is the beneficial lowering of total and LDL cholesterol, while increasing HDL and VLDL levels. However several important differences in response are observed in rodents, such as the lowering of both LDL and HDL, indicating that some results of this type of study in rodents may be difficult to extrapolate to humans (325). In addition to transiently depressing food intake, estrogen causes a sustained repression of lipoprotein lipase (LPL) activity in adipose tissue, thus inhibiting LPL-mediated free fatty acid uptake and triglyceride deposition, and also stimulates fatty acid synthase and acetyl CoA carboxylase activities (326). However in the uterus, estrogen promotes an increase in its activity (327), which may cause a local increase in free fatty acids and monoacylglycerols and in triglyceride deposition, although the effects of this local stimulation are not fully understood.

Further evidence of the tissue-specific regulation of lipoprotein levels by estrogen is that the mechanism of induction of apoVLDLII induction by estrogen has been well studied, but is believed to be specific to the liver (328). The high-density lipoprotein scavenging receptor (Scarb1) has also been found to induced in a complex ERE-dependent manner (329), and although its regulation in the uterus is not well defined, it is believed to be both the selective transfer of cholesteryl ester into cells and cholesterol efflux from cells, and has been detected in the endometrium (330). As well, estrogen has been found to induce uterine expression of a high density lipoprotein binding protein (*Hdlbp*; vigilin), which becomes localized predominantly to the epithelial cells (331).

The periimplantation uterus contains high levels of the cannabinoid anandamide (ANA), and its availability is modulated by its metabolism by fatty acide amide hydrolase

(FAAH), which produces arachidonic acid and ethanolamine. Cannabinoids have been associated with trophoblast differentiation and implantation, while arachadonic acid can then be converted to vasodilatory eicosanoids to cause vasorelaxation. While downregulation of FAAH activity by both estrogen and progesterone has been reported, *Faah* transcription has been shown to be induced by 24 hr estrogen exposure in rat uterine epithelial cells, and to be maximally expressed at estrus in the epithelium and circular myometrium (332).

Structural

Structural components are critical to organ shape and volume. Epithelial and stromal cell layers are separated by a basement membrane (BM), and the stroma also contains an extensive extracellular matrix (ECM). The BM and ECM are composed of numerous proteins and modified proteins, including fibrillar proteins such as collagens and elastin, proteoglycans, and large multidomain glycoproteins such as laminin and fibronectin (333).

The structural composition of the uterus is remodeled cyclically as a component of the menstrual cycle, with proteolytic degradation being a major feature of menses, followed by regrowth during the proliferative phase. These changes involve rapid and extensive remodeling of the ECM and connective tissue in response to changes in ovarian hormone levels. Importantly, due to the nature of the differences between uterine structural changes in menstruating and nonmenstruating species, close concordance between changes in remodeling enzymes in humans and in rodent models is not expected or observed (334, 335). Furthermore, the extent of the remodeling necessary in primates

has been postulated to be linked to the occurrence of endometriosis only in menstruating species (334).

In addition to the structural changes associated with menstrual shedding, prior structural changes to the luminal surface are critical for implantation. These include both acquisition of adhesion ligands as well as the loss of inhibitory barrier structures, and these are more highly conserved between primates and rodents.

The ECM acts both as a scaffold and as a dynamic regulator of alterations in cell shape, proliferation, differentiation, and apoptosis (336). For example, ECM alterations or degradation can release growth factors that can impact cell proliferation (337). As well, glycosaminoglycan (GAG) oligosaccharides can function by regulating access of growth factors and other signals to cell surface receptors. During glandular growth (adenogenesis), nonsulfated GAGs, such as hyaluronic acid, are localized to proliferating regions of the glands, while sulfated GAGs such as heparans and chondroitins are localized at inactive sites (36).

Structural components

Glycosaminoglycans

Glycosaminoglycans (GAGs) are the carboxyhydrate side chains of glycoproteins. Estrogen stimulates an increase in chondroitin 4- and 6-sulfates while decreasing hyaluronic acid and dermatan sulfate levels (338), so that in the estrogen-stimulated uterus GAGs consist predominantly of chondroitin sulfates (A and C; 40%), dermatan sulfate (25%), heparan sulfate (19%), hyaluronic acid (13%), and sialoglycoprotein (4%) (339, 340). Sulfation of sulfated glycoconjugates is also stimulated by estrogen (341).

Collagens

Estrogen has been shown to rapidly stimulate both the formation and breakdown of collagen in the rodent uterus, and the types of collagen synthesized in the estrogen-stimulated uterus have been identified as types I, III, and V (342-344). The effect of estrogen on collagen content and organization is biphasic and associated with uterine hypertrophy (345), with early effects including a loosening and fragmenting of collagen bundles resulting in large spaces opening in the ECM and increased spacing between stromal cells, while late effects include reappearance of collagen bundles in close association with stromal cell membranes (346). Estrogen is also able to reverse the collagen degradation associated with uterine atrophy (347).

Increased transcription of specific type I, III, and V procollagens, *Col1a1*, *Col1a2*, *Col3a1*, and *Col5a2* has been reported both early (2-4 hr) and late (18-36 hr) in the estrogen-stimulated uterus (348, 349).

Dermatan sulfate proteoglycans

Dermatan sulfate proteoglycans are small proteoglycans that bind to collagen fibrils and other ECM proteins in connective tissue to influence matrix assembly. This family includes decorin, biglycan, and heparan sulfate proteoglycans 3. Of these, decorin (*Dcn*) mRNA has been shown to be upregulated by estrogen in the sheep uterus (350).

N-linked glycoproteins

Estrogen has been reported to rapidly increase the rate of synthesis of N-linked glycoproteins such as laminins, fibronectin, and E-cadherin. An analysis of transcript and protein levels in response to estrogen exposure, however, showed that levels of these

transcripts were unaffected. However, an increase in transcription of mannosylphosphoryldolichol synthase (*Dpm1*), which is involved in N-linked glycoprotein assembly, was increased by estrogen stimulation, indicating that N-linked glycoprotein synthesis in response to estrogen is induced by increased levels of a key glycosylating enzyme (351).

Fibronectin and laminins are N-linked glycoproteins associated with stromal differentiation (351). No production is associated with stromal differentiation (352), and no evidence has been reported that estrogen increases uterine fibronectin production, although it has been reported to be posttranscriptionally repressed by estrogen *in vitro* (353).

Keratins

The presence of sufficient retinoid levels in the uterus prevents squamous differentiation of the epithelium, and induced expression of gene products involved in keratinization and cornification is not normally observed in response to estrogen (354).

Syndecans

Syndecans, also known as heparin sulfate proteoglycans (HSPGs), are small membrane-anchored proteins with extensive glycosaminoglycans modifications that facilitate binding of growth factors such as VEGF and FGF2 to their receptors, as well as providing binding sites for ECM adhesion proteins such as laminin and fibronectin to facilitate their action. Syndecans are implicated in cell adhesion and morphogenesis, and it is believed that their downregulation is important for uterine receptivity (355).

Estrogen treatment induces endocytosis of uterine membrane-associated syndecans, particularly from the basolateral epithelial surface, and this is followed by their cleavage and degradation (356). The syndecan Sdc1 was not found to be altered by estrogen exposure (357), while *Sdc3* was observed to be induced in the uterus at 4-12 hr after E2 exposure (355). Interestingly, surface Sdc3 protein was dramatically redistributed away from the apical surface of the luminal epithelial cells following E2 treatment, which may be involved in altering epithelial cell morphology (355). Estrogen-induced syndecan expression is believed to involve paracrine signaling from the underlying stroma (358).

Mucins, cadherins and catenins in the regulation of adhesion

Maintenance and regulation of cell-matrix and cell-cell adhesion connections are critical to tissue architecture and remodeling. For example, in the uterine epithelium, cadherins are localized to the lateral surfaces and mediate cell-cell adhesion, integrins are basally located and mediate cell-substrate binding, and different integrins are exposed at the luminal surface during the receptive period to facilitate embryo attachment (359). The cytoplasmic domains of cadherins and integrins can interact with specific linker proteins that attach to cytoskeletal actin or intermediate filaments.

Mucin glycoproteins are bulky, highly hydrated structures that serve as a barrier layer at many apical epithelial cell surfaces, extending much farther into the luminal spaces than most other membrane components. Mucins contain numerous tandem repeat sequences containing many serine and threonine residues which are very highly modified by O-linked oligosaccharides (50-90% by weight), and many proline residues contributing to their nonlinearity, making them very resistant to proteolysis and inhibiting

adhesion of foreign cells to the tissue surface. These glycocalyx linings are believed to provide a steric block to microbial attack in the female reproductive tract, as an initial mechanism of defense against infections while a host defense is being mounted in order to protect the tissue from infections that can threaten reproductive success. Furthermore, it also appears that bacterial proteins termed adhesins act as receptors for the mucin carbohydrate chains, to promote specific binding and trapping of the invading bacteria. In this way, bacteria can be trapped in the mucosal layer, after which the immune system can promote neutralization and expulsion from the body (360, 361).

Luminal surface mucins are associated with blocking of uterine receptivity, by obstructing integrin- and cadherin-mediated interactions (362). In particular, expression of the predominantly membrane-associated mucin episialin (Muc1) is associated with the non-receptive stages of the uterine cycle in rodents (363), while the marked downregulation during the brief receptive period is associated with permitting embryo attachment to the luminal surface (364). In humans, the regulation of Muc1 expression appears to be more complex, with focal changes in expression at the implantation site, as well as decreased levels of associated keratan sulfates that are believed to promote embryo implantation (40). *Muc1* transcript expression in response to steroid administration has been extensively studied. *Muc1* induction by estrogen can be blocked by antiestrogen or by progesterone (365). Muc1 however, accounts for less than 10% of the total mucin composition in mouse uterine epithelia (364). As well, expression of the sialomucin complex, encoded by the *Cd164* gene, has been shown to be similarly upregulated in the uterine luminal epithelium in response to estrogen (366), although the direct reason for the stimulation is believed to be due to downregulation by stromally

derived TGFβ1 (362). Since estrogen induction of *Muc1* has been shown to be ER-dependent but no evidence was found for direct binding of ER to the *Muc1* promoter, it has been suggested that *Muc1* expression is similarly mediated by stromal factors (367).

Integrins are a class of heterodimeric cell adhesion molecules expressed as glycoproteins at cell surfaces. They promote cell-cell and cell-matrix interactions and thus play a critical role both in normal cell architecture and polarity and in the receptivity of the uterine luminal surface to trophoblast implantation, and have additionally been implicated in the promotion of signal transduction pathways that induce transcription of other genes necessary to implantation, which has been recently reviewed (359). In the uterus, integrins anchor the epithelial cells to the basement membrane and promote trophoblast attachment, and also promote cell-cell interactions in the stroma. Complex, isoform-specific regulation of integrin expression in the uterus has been shown to be mediated by numerous paracrine factors including IL1, EGF, TGFα, TGFβ1, and TNFα, recently reviewed (359). In addition, estrogen and progesterone have both been shown to be associated with repressed expression of β_3 integrin (*Itgb3*) in endometrial cells *in vitro*, whereas EGF and FGF2 are highly stimulatory (359, 368).

Cadherins are transmembrane glycoproteins that mediate cell-cell adhesion in a calcium-dependent manner, while catenins act as a bridge between cadherins and the actin cytoskeleton. The estrogen-stimulated prevention of uterine surface adhesion was prevented by inactivation of E-cadherin (Cdh1), and estrogen promotes E-cadherin cleavage and inactivation, while inhibition of this process permits adhesion (369). Estrogen is also associated with decreased expression of α- (which could be *Catna1* or *Catna2*) and β-catenin (*Catnb*) as well as *Cdh1* (370), however overall, Muc1 is believed

to be able to disrupt cell-cell and cell-matrix interactions by providing steric hindrance to integrin access (371) and by inhibiting the formation of Cdh1-Catnb complexes (372).

Another adhesion molecule important to uterine function is cellCAM-105 (Ceacam1; also known as biliary glycoprotein), an integral membrane glycoprotein and member of the immunoglobulin superfamily. *Ceacam1* is localized to the apical surface of the uterine epithelium, and is induced by estrogen in the luminal epithelium and by progesterone in the glandular epithelium (373), and its involvement in embryo attachment has been proposed (374). Recently, *Ceacam1* has also been localized to endometrial blood vessels, and identified as a chemotactic and angiogenic factor that is inducible by VEGF and that may act as an effector of VEGF to promote blood vessel maturation (375).

Modifications to luminal surface carbohydrate chains, including fucosylation and sialylation, have been observed to be regulated during the reproductive cycle also, reviewed in (32). Transcriptionally, α1-2fucosyltransferase (*Fut1*), which catalyzes the final step in the formation of the H-type-1 oligosaccharide, is implicated as an epithelial attachment site for the blastocyst and has been shown to be induced by estrogen and repressed by progesterone in uterine epithelial cells (376, 377). By contrast, no sialyltransferases examined to date have been shown to be modulated by estrogen, reviewed in (32).

Proteases and inhibitors

Structural remodeling of the uterus is an intrinsic feature of the uterine cycle, and as with normal growth and differentiation processes, involves extensive and controlled proteolysis events. In addition, embryo implantation requires the highly controlled

invasion of uterine stroma by the trophoblast, and this complex event is also supported by the secretion and activation of numerous proteases, which degrade the ECM. Important proteinase families involved in this include the serine and cysteine proteinases, and the matrix metalloproteinases. Normal tissue functioning requires fine control over these processes, and these degradative enzymes are controlled at several levels of regulation, including transcriptional regulation, proenzyme activation, and regulation of enzyme inhibitor levels, in response to estrogen signaling.

An early observation in the estrogen-stimulated uterus was a marked induction of peroxidase activity (378). The properties of this peroxidase activity have been avidly studied, and this activity, referred to as estrogen-induced peroxidase (EIP) and found at high levels in the estrogen-stimulated epithelium and luminal fluid, was found to have several components (379). EIP is localized to the epithelial microvilli and uterine fluid (380) and is distinct from eosinophil peroxidase, which is concurrently induced but is localized to the stroma and myometrium (381). A recent report then indicated that cathepsin B and complement C3 were major components of EIP (382).

The cathepsins B, H, K, L, and S are lysosomal proteases involved in degradation of ECM and other proteolytic processes. They are secreted as proenzymes that are activated either autocatalytically or by MMPs or other cathepsins. They are active in acidic environments, and are able to degrade matrix molecules such as collagens, laminin, fibronectin, and proteoglycans. Cathepsins and other proteases have been found to be interregulated in a complex manner, since certain cathepsins can activate matrix metalloproteases (MMPs) and urokinase plasminogen activator (uPA), while they in turn can be activated by MMPs. As a result, they can digest matrix proteins and activate other

proteases involved in matrix degradation. The cathepsins have been found to be localized in both epithelial and stromal cells, particularly at the apical the luminal epithelium and in lymphoid cells in the stroma. The details of their isoform-specific localization and variation with menstrual phase have been reported in detail, and their localization suggests that they act within the uterine tissue and are also secreted into the lumen, where they may act to modify surface adhesion molecules on the luminal surface (383).

Cathepsin D (Ctsd), an aspartyl protease, is present in higher levels in the secretory than in the proliferative endometrium. Initially its production in the uterus was thought to be controlled only by progesterone, but weak induction by estrogen has more recently been reported (384). ERE-dependent estrogen responsiveness of *Ctsd* has been studied extensively in MCF-7 cells, and analysis of its responsiveness in different cell types has led to the proposal that in the uterus a local factor interferes with access to the ERE (385). *Ctsb* has also been shown to be weakly inducible by estrogen in the rodent uterus (124). Cathepsins can be inhibited by cystatins, however there are no reports to date of their regulation by estrogen.

Secretory leukocyte protease inhibitor (Antileukoproteinase; SLPI) is a serine proteinase inhibitor of the chelonianin class. SLPI can inhibit trypsin, chymorypsin, elastases, cathepsin G, and mast cell chymase, and in addition to antiprotease activity it has anti-inflammatory, antibacterial, and antifungal activities, and has been implicated in suppressing MMP activation and in fertility. More recently it has been described as a uterine epithelial growth factor, associated with uterine epithelial cell proliferation, with induction of DNA synthesis and direct induction of cyclin D1 expression, and concurrent suppression of genes involved in growth suppression, which together favor epithelial cell

growth (386). *Slpi* is predominantly expressed in glandular epithelial cells in various tissues. It is typically localized in the ECM, and is considered to be a contributing but nonessential growth factor, since *Slpi* null mice appear normal (387). Uterine *Slpi* expression to date has been investigated only in pigs, and has been found to be induced by estrogen as well as by progesterone (388).

Matrix metalloproteases (MMPs) are a large family of locally secreted zinc-dependent enzymes that play a major role in tissue turnover, primarily by degrading ECM and BM components. They are divided into several subgroups with distinct specificities based on similarities in sequence and in specificity for various substrates, a complex system which has been recently reviewed (334). Generally, however, the MMPs can be subdivided into collagenases, gelatinases, stromelysins, and membrane-type enzymes, and the substrates include collagens as well as fibronectin, elastin, laminin, and aggrecan, though stromelysins are also active towards other MMPs as well as growth factors and cytokines such as IGFBPs and TNFα (334).

Because of their actions in the degradation of the extracellular architecture, MMPs have been implicated in numerous processes such as angiogenesis, wound healing, and tumor infiltration and metastasis (334). In addition, because of their tightly regulated expression and their potent effects on the ECM, they have been intensively studied in the cycling and pregnant uterus. In the reproductive organs, MMPs are typically synthesized by stromal connective tissue cells, including resident fibroblasts, endothelial cells, and infiltrating macrophages and neutrophils, and their importance in female and male reproductive function have been extensively reviewed (333, 334). While estrogen does not appear to influence MMP expression directly, certain estrogen-

stimulated growth factors such as EGF and cytokines do influence MMP expression (389). Progesterone also exerts strong influences, being associated with potent repression of MMP expression both directly and by repressing cytokine action (39).

Tissue MMP activity is controlled at many levels, including transcription, activation, and levels of specific inhibitors. To date, there is no evidence for regulation of these inhibitors by estrogen.

Blood flow and angiogenesis

Estrogen has been found to possess both rapid and delayed effects on the vasculature, which have been recently reviewed (390). It is currently believed that the delayed effects are due to ER-mediated induction of specific genes such as prostacyclin cycloogenases and synthases, nitric oxide synthases, endothelin, VEGF, BCAM, and elastin. By contrast, the rapid vasodilatory effects are believed to be mediated by endothelial nitric oxide synthase (NOS3) activation by signal transduction pathways downstream of membrane ERs. Specifically, both MAPK and PI3K/Akt signal transduction cascades are believed to stimulate Nos3 in the vascular caveolae to produce nitric oxide, a signaling molecule which induces cGMP-mediated VSMC relaxation by (390).

Expression of the PTH-like peptide *Pthlh* is induced rapidly in the uterus after estrogen administration, declining by 24 hr (391), a response which can be inhibited by either glucocorticoids or vitamin D3 (392). Interestingly, expression of its receptor, which also serves as the parathyroid hormone (PTH) receptor (*Pthr1*), was found to be concurrently downregulated (392). PTHLH is believed to stimulate uterine blood flow (393) and possibly prevent uterine smooth muscle contractions (394).

Adrenomedullin (Adm) is a peptide with many known functions, including an ability to potently induce vasodilation, as well as to promote angiogenesis and reduce blood pressure, in part through the generation of nitric oxide. The proadrenomedullin gene also codes for an alternate peptide, PAMP, which is also able to reduce arterial pressure. Although Adm is known to act through the receptor Admr, it has been shown to also interact with calcitonin gene-related peptide (CGRP) receptors (395). In addition to stimulation of endometrial vasodilation and angiogenesis, a role for Adm in attenuating myometrial contractility induced by galanin has also been suggested (45). *Adm* transcription can be induced by various hormones, growth factors, and cytokines, as well as oxidative stress. In the uterus, *Adm* is highly expressed in the female reproductive tract, with cyclic variation during the uterine cycle, and is induced following estrogen administration. TGFβ1 has been proposed as a mediator of the estrogen-induced Adm transcription, although this regulatory pathway is not yet well defined (45).

Angiogenesis is the process in which new blood vessels are derived from preexisting ones, and can occur by several distinct mechanisms that have been recently reviewed (396). In the adult, angiogenesis primarily occurs in the uterus and ovary as a necessary component of the reproductive cycle and during pregnancy, as well as in pathologic processes such as wound healing, chronic inflammatory disorders, and malignant tumor growth (397). The role of angiogenesis in the female reproductive tract is critical, however, since the endometrium is one of the most dynamic adult tissues, and the rapid increase in endometrial thickness depends critically on the efficient establishment of vascularization. Angiogenesis is also critical to placentation during pregnancy. The cyclical regrowth of the endometrium is dependent on its ability to

rapidly and efficiently regenerate a vascular capillary network to accompany and support the proliferation and differentiation of the endometrial lining. This neovascularization, involving the formation of new capillaries from preexisting blood vessels, is stimulated by both endocrine and paracrine pathways, and requires proteolytic dissolution of endometrial extracellular matrix, proliferation and migration of endothelial cells, permitting the formation of new capillary tubules to supply the growing endometrial tissue (398). Blood vessel growth and regression in the human endometrium differs both temporally and spatially during the menstrual cycle in response to changes in the needs of the other cell types within the tissue. Specifically, it occurs in the functionalis to support the rapid four-fold increase in endometrial thickness during the proliferative phase (399), during the growth and coiling of spiral arterioles in the functionalis in the secretory phase, and during repair of the vascular bed in the superficial layer of the remaining basalis in the menstrual phase (396). During the menstrual cycle a capillary plexus also develops just below the luminal epithelial surface, and blood flow through this plexus is maximal during the early and mid-secretory phases in preparation for implantation (400)

Estrogen has long been implicated in the promotion of increased uterine blood flow caused by angiogenesis and associated increases in vasodilation and in vascular permeability to water, small molecules, and proteins, but its role is not fully understood (69). Evidence has recently been provided, however, that estrogen, through the induction of factors such as VEGF, is involved in the promotion of vascular permeability and inhibition of angiogenesis, while progesterone conversely stimulates angiogenesis though has little effect on vascular permeability (282). Furthermore, both ERα and ERβ are expressed in vascular smooth muscle and endothelial cells in a uterine cycle-dependent

manner. In particular, ERα expression is increased in the late proliferative and early secretory phases (401).

Erythropoiesis and coagulation

The soluble factor erythropoietin (Epo) is the primary stimulator of proliferation and differentiation of erythroid precursors, after binding to its receptor (EPOR), although additional roles have been proposed. For example, the epithelial and stromal localization of *Epo* and *Epor* in the uterus, and the glandular increases in both proteins in the late proliferative phase, suggest additional roles for these proteins that have not yet been discovered (402). *Epo* expression is typically induced by the hypoxia inducible factor HIF-1α in response to hypoxia, and the stimulation of red blood cell formation results in improved oxygen supply, which then acts negatively on *Epo* expression (403).

Reports of estrogen regulation of *Epo* synthesis under hypoxic conditions have been controversial over the last several decades. Recently it was shown the estrogen inhibits *Epo* expression during hypoxia (404), although uterine *Epo* is also reported to be inducible by estrogen in the uterus (403, 405).

Tissue factor (F3) is a cell surface glycoprotein receptor for coagulation factor VII (F7), which as a complex initiate the extrinsic coagulation protease cascade. In this cascade, prothrombin (F2) is activated, stimulating fibrin formation. In addition, thrombin can act as a mitogen in numerous cell types including fibroblasts, VSMCs, and immune cells, as well as in cultured uterine stromal cells (406), and its function as an estrogen-regulated uterine growth factor has been proposed (407).

At the protein level, estrogen rapidly induces both F3 and F2 levels. F3 is found in both epithelial and stromal cells within 3 hr in the uterus, with F2 being localized to

the basement membrane that separates the epithelium and stroma, and to the apical surface of the epithelium (407). *F3* is rapidly synthesized in the uterus following estrogen exposure (408), while thrombin levels are increased due to infiltration into the uterus through capillaries that are made hyperpermeable following estrogen exposure, similar to albumin, plasminogen, and alpha-1-protease inhibitor (409). Thrombin has also been shown *in vitro* to stimulate uterine stromal cell proliferation (406).

Oxidative stress

During normal metabolic activity the cells of all aerobic organisms produce reactive oxygen species, which include superoxide, hydrogen peroxide, and hydroxyl radical, and the toxicity of these compounds is regulated by various antioxidant mechanisms. Moreover, estrogen can further induce oxidative stress through its metabolism to reactive quinines, semiquinones, and free radicals (410, 411). In addition, reactive oxygen species or their lipid peroxide products can stimulate prostaglandin F2α synthesis, which is involved in uterine contraction and endometrial breakdown (412). Estrogen has been shown to promote oxidative stress in uterine tissues (413).

Superoxide levels have been shown to vary during the rodent uterine cycle, with NADPH oxidase-dependent superoxide levels highest at proestrus and lowest at estrus (414), while in humans they are increased in the endometrium in the late secretory phase just before menstruation (415). Superoxide levels and NADPH oxidase activity was also shown to be highly induced by estrogen treatment, whereas SOD activity was slightly repressed (416). Furthermore, hydride and dienyl radical levels were elevated following estrogen treatment, and cell membrane fluidity was enhanced, possibly due to perturbations associated with the partitioning of the estrogens into the cells membranes

(416). In addition, estrogen treatment is associated with increased membrane lipid peroxidation, decreased progesterone concentration, and enhanced catalase activity (413). It has been proposed that the enhanced catalase activity partially mutes the extent of lipid peroxidation, which would otherwise be greater following estrogen exposure (411).

At the transcript level, mitochondrial manganese SOD (*Sod2*), has been shown to be upregulated at estrus in the luminal epithelium and at proestrus in the leukocytes (417), and both *Sod2* and the cytosolic Cu,Zn-SOD (*Sod3*) in human endometrial cells at the time of decidualization or by combined estrogen and progesterone administration (418). Regulation is isoform-specific, and although the functional significance of these changing levels is not fully clear, it has been suggested that Sod3 controls PGF2α production by preventing cytosolic accumulation of oxygen radicals (419), while Sod2 promotes successful decidualization by protecting the epithelium from free radical-induced damage (415). Specific transcriptional regulation by estrogen has not yet been established, however estrogen and progesterone have been shown to induce both enzymes by cAMP-mediated mechanisms (415), and promoter analyses also indicate potential direct regulation by numerous factors, including AP-1, Sp1, and progesterone (420, 421).

Solute and fluid regulation

Estrogen induces a rapid increase in uterine microvascular permeability, and this, in conjunction with estrogen-induced increases in uterine blood flow, leads to stromal edema. This effect is believed to promote an environment supportive of the endometrial growth and remodeling that is necessary for implantation and pregnancy, and that is similar to the edema observed in the rodent proestrus stage and human midproliferative

and midsecretory stages. In addition, it has been proposed that uterine edema promotes blastocyst attachment by decreasing the size of the luminal space (422).

This edema response is associated with increased VEGF production. In the rodent, the inactivation of *Vegfa* with a specific antibody or with an Flt-1 inhibitor has confirmed that VEGF is the dominant mediator of the estrogen-induced changes in vascular permeability, and that the VEGF-induced edema is essential for successful implantation (38, 422).

An important function of the endometrial epithelium is the production and secretion of uterine fluid into the lumen. Cyclic variations in solute levels, including K+ and Ca2+ levels have been observed (423). In addition, Na+ levels are critical modulators of fluid balance, and the depression in fluid Na+ levels and consequently of uterine fluid volume directly prior to implantation are believed to promote luminal closure, a flattening of the epithelial surface that is important to implantation (424). IGF-I has been shown to induce many of these parameters in a manner similar to estrogen (425).

Sodium and Chloride

The regulation of water intake and export is dependent on the regulation of specific solute levels. In the uterus, recent studies indicate that fluid accumulation is due to estrogen-induced increases in the Cl^- channel CFTR coupled with downregulation of the ENaC (Scnn1) Na^+ channel isoforms (426). It is believed that when Cl^- channel expression is favored, active Cl^- secretion drives Na^+ and fluid from the plasma into the uterine lumen, while the reverse is true under conditions that favor Na^+ channel expression. This model is consistent with the patterns of CFTR and ENaC expression as well as fluid dynamics that are observed during the estrous cycle (426). The upregulation

of *Cftr* 12 hr after estrogen administration has been reported (427), and indirect evidence strongly supports the upregulation of *Scnn1a*, *Scnn1b*, and *Scnn1g* also (426).

Potassium

Large-conductance Ca^{2+}/voltage-activated K^+ channels, also known as Kcnm or maxi-K channels, are important in regulating myometrial contractility (428). They are known to be regulated at various levels, including transcriptionally. For example, estrogen has been shown to induce expression of a specific splice variant of a pore-forming α Kcnm subunit, *Kcnma1*, with reduced sensitivity to Ca^{2+} and voltage, though total transcript levels and channel density remain relatively constant (429). This is believed to attenuate K^+ current and possibly allow increased uterine excitability and contractility, contributing to changes in conductance observed during the estrous cycle. The regulatory β subunits Kcnmb3 and Kcnmb4 have been shown to be activated by E2 (430), but their transcriptional regulation is not currently known, while E2-induced increases in myometrial *Kcnmb1* expression have been recently reported (431). Furthermore, Kcnd3, and specifically the long transcript isoform, has recently been shown to be the dominant isoform of the Kcnd, or Kv4, voltage-gated K^+ channel family in the uterine myometrium, and E2 was shown to be associated with transcriptional downregulation of *Kcnd3*, in addition to altering its delivery to the cell membrane (432).

Aquaporins and NHE proton pumps

Proton transporters play important roles in regulating intracellular pH, cell volume, and osmolarity. In addition, exit of HCO3- requires that H+ also be pumped out of the epithelial cells to maintain intracellular pH. It is believed that basolateral Na^+/H^+

exchangers (NHEs) in the uterine epithelium may be involved in allowing HCO3- secretion, while apical NHEs may mediate luminal Na+ absorption in a manner similar to the Scnn1 channels. In the mouse endometrium, three NHEs, Slc9a1, Slc9a2, and Slc9a4, have been identified, though the specific contributions of each are not presently known. During HCO3- secretion, basolateral NHE activity is high while apical NHE activity is low (433)

Aquaporins are vasopressin-insensitive water channels. The aquaporin AQP1, or CHIP28, has been detected in the human uterus (434), and *Aqp1* transcript levels are induced in the rat uterus approximately 9 hr after estrogen exposure (435). Details of its regulation and action in the uterus are currently unknown, however AQP1 is known to be expressed in secretory tissues, and has been shown to be an important component of fluid regulation in the male reproductive tract, secondary to export by the sodium/hydrogen exchanger NHE3 (Slc9a3) of protons formed by the carbonic anhydrase Car2 (436). A similar mechanism may act in the female reproductive tract. *Slc9a3* expression in the efferent ductules of the male reproductive tract was shown to be dependent on ERα in that system, and the promoter sequence was reported to contain EREs (436). Furthermore, the expression of Slc9a3r1 (NHE-RF1; EBP50), which is believed to function as a regulatory component or scaffold for many transporters including Slc9a3, CFTR, and a sodium bicarbonate cotransporter, has been shown to be strongly expressed in nearly all epithelial cells of the human proliferative phase uterus, and to be highly abundant in cells rich in microvilli (437). The human *Slc9a3r1* promoter has been shown to contain a high number of functional half ERE sites, through which ER upregulates

Slc9a3r1 transcription through a complex mechanism, and participation of Slc9a3r1 in modulating uterine cell ultrastructure has been proposed (438).

Immunologic responses

Estrogen has been described as a proinflammatory mediator, since it promotes leukocyte influx, uterine edema, and epithelial proliferation. A characteristic feature of the uterus is the cyclic changes in levels of immune cells, which play roles in phagocytosis of apoptotic cells and in combating infections introduced during mating (439, 440), as well as numerous critical functions during pregnancy (441).

Uterine leukocytes consist predominantly of lymphocytes, macrophages, and mast cells, as well as a uterine-specific type of natural killer cell (uNK). These cells have been shown to have distinct localizations, with mast cells associated predominantly with the inner myometrium, and macrophages and uNK cells throughout the endometrium and myometrium (289, 442). Immune cells are also detected cyclically in the uterine luminal fluid (443).

Macrophages and neutrophils are an important component of the stromal mass and function, and macrophages alone, which are normal components of all connective tissue, constitute as much as 10% of uterine mass (444). Macrophages and neutrophils are able to phagocytize invading organisms, and macrophages can also present processed antigens to lymphocytes to initiate the immune response (289). Macrophages bear a wide range of receptors, including ERs, which make them highly susceptible to numerous stimuli including estrogen (35). Myometrial mast cells are believed to be involved in proliferation, vasodilation, and edema through the release of mediators such as nitric

oxide, TNFα, and histamine (289). uNK cells, which have been shown to express ERβ, have been implicated in both implantation and in initiating endometrial breakdown (104).

Eosinophils contain potent peroxidase granules, and the estrogen-induced recruitment of eosinophilic leukocytes has been shown to contribute substantially to the peroxidase activity that is characteristic of the estrogen-stimulated uterus (445). Eosinophils can express Fc and C3b receptors, allowing them to bind to Ig- and C3b-presenting invading cells, and can initiate complement and coagulation cascades. Eotaxin (SCYA11; Ccl11) has recently been shown to be the signal stimulating eosinophil recruitment to the uterine stroma, and *Ccl11* expression is highly upregulated following several consecutive days of estrogen treatment (446). It has been suggested that eotaxin may be the uterine eosinophil chemotactic factor referred to as ECF-U (447). Eosinophil recruitment is not required for estrogen-stimulated uterine growth (445), and *Ccl11* null mice display delayed onset of estrous cycling but possess normal fertility (446).

Secreted immunoglobulins provide an important early defense against pathogen attack at the uterine luminal surface. An association between estrogen stimulation of the uterus and increased immunoglobulin (IgA and IgG) content in the uterine luminal fluid is well established (448). IgA, unlike IgG was found to be secreted against its concentration gradient, and the involvement of an active carrier that transports IgA from the uterine epithelium into the lumen was postulated (449). This carrier was then found to be a protein called secretory component (SC), which was later identified as a domain of the polymeric IgA receptor (Pigr), a transmembrane glycoprotein which is essential for normal mucosal barrier function (450). Uterine *Pigr* expression follows a similar pattern to IgA levels, and is highly induced by estrogen after 3x24 hr (451). Direct regulation of

Pigr expression in the uterus is not well established, but *in vitro* studies indicate direct induction of *Pigr* expression by numerous cytokines, including interferon γ, TNFα, IL1β, and IL4, as well as the involvement of numerous factors in its basal transcription (452).

Interleukin 4 receptor (*Il4ra*) has been shown be induced at 4-8 hr after estrogen exposure in the uterus (183), while expression of the interferon-inducible gene HEM45 (*Isg20*), which has recently been shown to be an antiviral RNase (453), was upregulated 3-15 hr after estrogen treatment in the uterus (454).

Cytokines and chemokines

Cytokines stimulate the proliferation and differentiation of numerous cell types. The unstimulated uterus contains little cytokine activity (455), while cytokines known to be induced by estrogen in the uterus, and further increased by cotreatment with progesterone, include TNFα and the interleukins IL1 and IL6 (455).

Chemokines are a subclass of chemotactic cytokines, and are expressed in leukocytes as well as in other endometrial cells and in the trophoblast. In addition, however, chemokines can mediate vital cyclic changes within the uterus, including changes associated with proliferation, differentiation, angiogenesis, apoptosis, and structural changes. Chemokine expression is induced by cytokines or by other local growth factors, and their dysregulation is associated with cancer and other pathologies. The complex nature of chemokine regulation and signaling has been recently reviewed (456).

Chemokines known to be expressed in the uterus include macrophage colony stimulating factor (M-CSF; Csf1), monocyte chemotactic protein 1 (MCP1; JE; Ccl2), and the granulocyte chemotactic protein IL8 (GCP1, for which no rodent homolog has

been found) (457). Induction of *Csf1* appears to be directly by estrogen (458), while *Ccl2* induction by estrogen is believed to be mediated by IL1β (459), and increased estrogen appears to be associated with *Il8* repression (457). Interestingly, in *Csf1* null mice the cyclic estrogen surge is impaired, and is associated with abnormal estrous cycles and reduced fertility (460).

Complement

Complement plays numerous roles in immune and inflammatory responses, including in the recruitment of phagocytes as well as direct involvement in phagocytosis and other roles that have been recently reviewed (461). The alternative pathway of complement activation is induced in the estrogen-treated uterus, although the exact role is not currently known. Cleavage of complement component C3 and factor B (Bf) yields C3b and Bb, which form a complex that acts as a C3 convertase to promote the formation of the membrane attack complex on the surface of invading cells.

C3 and *Bf* are highly and concurrently expressed in the uterine epithelium and secreted into the lumen in proestrus and estrus or after estrogen exposure, where they are believed to defend against microbial invasion (462). The induction of C3 by estrogen has been particularly well studied. It is generally considered to be a late phase response and has been shown to be highly induced at 24 hr (463), although it was also identified in a screen for transcripts that are induced by estrogen at 4 hr (183).

Iron sequestration

Lactoferrin is a glycoprotein of the serum transferrin family, and its many functions described to date have been the subject of numerous reviews (e.g. (464)). In

tissues such as the uterus, these include multiple roles in defense against infection and inflammation. Its multifunctional antimicrobial activity is directed towards bacteria, viruses, and fungi, and is attributed to its ability to sequester iron away from iron-requiring bacteria, to its potently bactericidal lactoferricin domain, to its serine protease activity which is able to degrade bacterial-host adhesion proteins, and to its pH-dependent ability to donate the iron atoms used in catalysis of hydroxyl radical production within neutrophils (464). Furthermore, lactoferrin is able to modulate the proliferation and differentiation of immune cells, suppress inflammation, and downregulate the production of specific cytokines (464). In the uterus, lactoferrin is found in both the luminal and glandular epithelium and in neutrophil granules, and is secreted during the estrus cycle concurrently with immunoglobulins.

Ltf had already been examined in many other tissues before it was identified in 1987 as the major component of uterine fluid (465, 466). The induction of Ltf mRNA and protein expression by estrogen has been studied extensively, and complex species and strain differences have been observed. For example, Ltf has been shown to be highly inducible by estrogen in many mouse strains, induced variably in the rat, limited to the uterine stromal neutrophils and vaginal epithelium in the hamster, and has not been detected at all in the pig (467, 468).

While lactoferrin has been used extensively as a biomarker of estrogenic effect in the uterus, the *Ltf* gene has a highly complex promoter, and lactoferrin transcription occurs in response to many stimuli, as has been recently reviewed (468). Lactoferrin expression can also be induced by EGF, independent of the ovary, adrenal gland, or

hypothalamus, and *in vitro* can also be induced by RA or by changes in cell shape and actin cytoskeleton.

Intercellular communication

Gap junctions

Gap junctions, first identified in the 1970s, act as a type of intercellular channel that permits direct communication between the plasma membranes of adjacent cells, allowing them to share a common pool of small (<1 kDa) regulatory molecules, such as inorganic ions and second messengers. Each channel is composed of one connexon (hemi-channel) from each cell, which in turn is composed of a circular hexameric arrangement of transmembrane connexin subunits to form a pore, and the specific connexin subtypes influences pore size and thus permeability to specific signaling molecules (469). Expression of these connexons has been identified in numerous cell types, and in the uterus, they are found in all of the major cell types, and allow communication between the epithelial, stromal, and myometrial compartments (51, 470), as well as allowing synchronization of contractions within the myometrial smooth muscle cells (470). Many tumors have been found to have lowered connexin expression, and connexins have been implicated as tumor suppressors (471). The roles and importance of various connexins has been recently reviewed (472).

The control of certain connexins by ovarian steroids in the uterus has been studied for more than a decade (470), and although they are a highly homologous family, their regulation has been shown to be very different. The cell type-specific localization of the connexin proteins Cx26, Cx32, and Cx43 in the uterus has been studied in estrogen-

treated immature rats. A marked increase of Cx43 in the stroma and myometrium was observed after a 4x24 hr exposure, which was abrogated by cotreatment with progesterone, whereas increases in connexins 26 and 32 were highly induced by progesterone in the luminal epithelium (51). In the human endometrium, Cx43 protein levels were lowest on days 12-14 of the cycle (i.e. time of implantation), which they think may be due to inhibition by LH, they think that this may reduce cell-to-cell communication, possibly facilitating the invasion of the trophectoderm through the stromal cells (473).

When levels of endometrial transcripts were measured, *Cx26* and *Cx43* were found to be highly induced by estrogen by 14 hr as well as at 3x24 hr, while levels of *Cx32* were low and unchanged (474, 475).

The promoter of the *Cx43* gene has been well studied, and many motifs have been identified. By contrast, the regulation of *Cx26* and *Cx32* are not well understood, although predominant regulation by posttranscriptional mechanisms in the uterus has been suggested (51). Furthermore, there is increasing evidence that induction by estrogen is not mediated by classical ER-ERE binding. *Cx43* induction is believed to be mediated at least in part by AP-1 (476, 477), while regulation of *Cx26* expression has been shown to involve Sp1 and Sp3 (478).

Tight junctions

Reciprocal effects of ovarian steroids on tight junctions have been reported in the uterine luminal epithelium, with estrogen decreasing and progesterone increasing their size and complexity (479, 480). This pattern of regulation appears to mirror the increase in gap junction protein expression that was described above. Transcriptional regulation of

tight junction components (e.g. ZO-1, claudin-1 and occludin) by estrogen in the uterus has not been described to date, but altered protein levels at the apical epithelial membrane during pregnancy have been recently described (481).

Apoptosis

Sex steroid hormones control cell proliferation and differentiation in endometrial epithelia, and removal of estrogen, for example by ovariectomy, is associated with uterine epithelial apoptosis, which can be restored by the readministration of estrogen, or alternatively with progesterone or androgen exposure (269). Further evidence for the involvement of progesterone in the proliferative reaction is that cotreatment of rodents with estrogen and a PR antagonist induces apoptosis (482). Nevertheless, endometrial proliferation in the proliferative phase has been generally related to estrogen action, while progesterone is thought to divert cells into the differentiation pathway.

In humans, apoptotic cells are detected mainly in the glandular epithelium of the endometrium. Very little apoptosis is observed in the stroma at any stage, or in the glands in the late proliferative phase, while the number of glandular apoptotic cells increased during the secretory phase and reached a maximum at menstruation and still high in the early proliferative phase. This is consistent with a possible role of apoptosis in regulating cell turnover and the number of glandular cells that survive to the next menstrual cycle (483). Overall, the pattern of apoptosis and its negative correlation to serum E2 concentrations in the proliferative phase indicated that, in contrast to many other cell types, proliferating endometrial cells do not undergo significant apoptosis. This study found an inverse correlation between apoptosis and Ki-67 (i.e. proliferation), suggesting

that apoptosis in hormone-dependent cells may not be dependent on cell cycle entry (483), in support of previous findings in the human endometrium (484).

Neurotransmitters

The reproductive tract contains an extensive network of nerve fibers, which highly express gastrin-releasing peptide (GRP). GRP is one of several neural signals involved in the control of uterine motor activity, and can stimulate uterine contractions that can be potentiated by estrogen coadministration (485). GRP is also secreted from the glandular epithelium, suggesting a possible endocrine function. In sheep, estrogen was found to dramatically inhibit uterine *Grp* expression and secretion (486).

Galanin (Gal) is a neuropeptide that can directly induce myometrial contractions by modulating calcium levels and by increasing the calcium sensitivity of the contractile apparatus (487). Stromal *Gal* expression is rapidly induced following estrogen administration (488). Galanin acts through the Galr2 receptor isoform, which is localized to the myometrium, suggesting that galanin modulates myometrial contractility in a paracrine manner (487). Galanin protein has been identified in a subset of CGRP-positive myometrial nerve fibers, and CGRP is able to antagonize galanin-stimulated uterine contraction (489).

Prostaglandins

Prostaglandins are able to stimulate myometrial contraction. Myometrial expression of the contractile prostaglandin F receptor (*Ptgfr*) was induced after 24 hr estrogen administration, while the relaxant prostaglandin E receptor isoform *Ptger2* was induced by progesterone (490).

Other

Other transcriptional responses in the estrogen-stimulated uterus have been described for which the biological function is not yet clearly understood, and these responses are summarized below.

Frizzled/Wnt

The uterus of the *Wnt7a* null mouse lacks glands and has a stratified rather than columnar luminal epithelium, as well as a thickened myometrium (491). Estrogen has been reported to inhibit *Wnt7a* expression, as reviewed in (492). The importance of Wnt7a levels are believed to involve its role in maintaining Wnt5a, Hoxa10, and Hoxa11 levels.

Calbindins

Calbindins are calcium binding proteins with highly estrogen inducibility in the uterus, although their precise functions in the uterus are unclear. Calbindins display well characterized estrogen inducibility. The 9 kD calbindin (*Calb3*) has two splice variants that are expressed in an ERE-dependent manner (493), while expression of the 28 kD isoform (*Calb1*) is regulated through multiple imperfect ERE half-sites (494). *Calb3* is induced at 4-24 hr in the estrogen-stimulated uterus (183), while *Calb1* appears to be repressed (495).

Lipocalins

The lipocalin Lcn2 was originally described as 24p3, or neutrophil-gelatinase-associated lipocalin. Lcn2 is involved in the delivery of iron to the cytoplasm, thereby

influencing the transcription of critical iron-responsive genes as well as providing the iron needed for the catalytic mechanism of specific enzymes (496). Lcn2 has been shown to be complementary, but not redundant, to the iron transporter Transferrin. Lcn2, like Ltf, has been detected in endosomes, where the acidic environment is believed to promote iron unloading. *Lcn2* expression is induced in the epithelial cells in response to three consecutive estrogen doses (497) and the glycoprotein is secreted into the luminal fluid (498). Placental protein PP14, a lipocalin also known as glycodelin-A or progestagen-associated endometrial protein (PAEP), by contrast, has recently been shown to not be inducible by estrogen in human endometrial cells in culture (499),

CONCLUSIONS

A vast array of transcriptional responses to estrogen have been described over the past few decades. Although the experimental systems differ with respect to many variables including species, strain, age, treatment regimen, many commonalities have been observed between the different models, and they continue to be employed effectively in the study of uterine responses to endogenous, pharmaceutical, and pollutant estrogens. A rapid increase in the use of microarrays to further characterize uterine responses to estrogen has underscored the need for the vast body of previously published information to be summarized. The preceding review, which discusses the transcriptional regulation of over a hundred transcripts in the estrogen-stimulated uterus, is of benefit to researchers in this area seeking to analyze new data and compare it to previously published work. Furthermore, numerous other researchers who use a single gene response as a marker of estrogen action can address current concerns that this does not incorporate recent information about nonclassical mechanisms of action by readily choosing markers

that represent several different estrogen-induced responses (e.g. cell proliferation, angiogenesis, solute transport, energy production, immune responses).

CHAPTER 2

HYDROXYLATED BENZO[A]PYRENE METABOLITES ARE RESPONSIBLE FOR *IN VITRO* ESTROGEN RECEPTOR-MEDIATED GENE EXPRESSION INDUCED BY BENZO[A]PYRENE, BUT DO NOT ELICIT UTEROTROPHIC EFFECTS

ABSTRACT

The estrogenic activities of benzo[a]pyrene (B[a]P) and 10 metabolites (1, 3-, 7-, and 9-hydroxy-B[a]P; 4,5-, 7,8-, and 9,10-dihydrodihydroxy-B[a]P; and 1,6-, 3,6-, and 6,12-B[a]P-dione) were investigated. *In vitro*, B[a]P did not displace tritiated 17β-estradiol ([^{3}H]E2) from either a bacterially expressed fusion protein consisting of glutathione-S-transferase linked to the D, E, and F domains of human ERα (GST-hERαdef), or from full-length human ERβ (hERβ) at concentrations as high as 60 μM. However, 10 μM B[a]P demonstrated partial agonist activity in human Gal4-ERαdef and mouse Gal4-ERβdef reporter gene assays in transiently transfected MCF-7 cells, relative to 10 nM E2. 1-, 3-, 7-, and 9-hydroxy-B[a]P were found to bind to both receptor isoforms, each showing a higher affinity for the β isoform. At 10 μM the four monohydroxylated metabolites were able to induce Gal4-hERαdef- and Gal4-mERβdef-mediated reporter gene expression to levels 20-100% of that caused by 10 nM E2, suggesting that these metabolites, and not the parent compound, induced reporter gene expression following B[a]P treatment of transiently transfected MCF-7 cells. In addition, the effect of B[a]P on two estrogen-inducible endpoints, uterine weight and lactoferrin mRNA levels, was determined in ovariectomized DBA/2 and C57BL/6 mice. Neither orally administered

B[a]P at doses as high as 10 mg/kg body weight nor subcutaneously injected 3- or 9-hydroxy-B[a]P at doses as high as 20 mg/kg induced uterine wet weight or uterine lactoferrin mRNA levels in either strain. These data suggest that B[a]P metabolites that are estrogenic at high concentrations *in vitro* do not induce estrogenic effects in the mouse uterus.

INTRODUCTION

Interest in identifying natural and synthetic xenobiotic compounds that are able to elicit ER-mediated adverse effects has increased in recent years. It has been suggested that exposure to estrogenic substances at critical times during development may disrupt normal structural and functional development of estrogen-responsive tissues, including the reproductive tract, mammary glands, central nervous system, cardiovascular system, and bone (500, 501). This has resulted in heightened concern that exposure to natural and synthetic estrogenic chemicals may adversely affect human and wildlife health.

Compounds of diverse structure have the ability to bind to the estrogen receptor isoforms ERα and ERβ, though they often share some general structural features with E2 and other endogenous estrogens. Recent crystallization of the LBD of both ERα and ERβ in the presence of agonist or antagonist has revealed the nature of the binding pocket, which is both lined with hydrophobic amino acids and contains specific residues for hydrogen-bonding with the hydroxyl groups of the ligand (18, 502). Interestingly, some 4- or 5-ring carcinogenic polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (B[a]P), are isosteric to sex steroids (see Figure 1) and are able to induce tumor formation in estrogen-responsive tissues such as the mammary gland, ovary, and uterus (503-505). Furthermore, B[a]P has been reported to adversely affect ovarian follicle growth, ovulation, and corpora lutea formation (506, 507) and to produce a weak estrus-like response in the rodent uterus (508).

PAHs are a class of environmental pollutants that are formed during the incomplete combustion of organic matter. As a result, they may be released into the environment during vehicle use, incineration, forest fires, and many other human

activities and natural processes. Although B[a]P and other PAHs can be detected in water, soil, sediments, and adsorbed to air particulates, it has been estimated that 97% of human exposure to B[a]P occurs though ingestion of food, primarily meat, dairy, and produce (509). Human daily intake of B[a]P is estimated to be approximately 1.1 to 2.2 μg/adult/day, with much higher exposure levels occurring in smoking and occupationally exposed individuals (509, 510).

The majority of B[a]P studies to date have examined the mutagenicity and carcinogenicity of B[a]P metabolites. B[a]P is known to be biotransformed to polar metabolites by a subset of the cytochrome P450 metabolizing enzymes. It is predominantly metabolized by the CYP1A1 isozyme, but 1A2, 2A1, 3A4, and 1B1 can also contribute, with at least 4 other isozymes playing minor roles (511-515). The specific metabolites formed in many *in vitro* and *in vivo* systems have been identified, and while there are quantitative differences in the amount of each metabolite formed in cells from different sources, the identities of the metabolites remain essentially constant regardless of the species or tissue type: 1-, 3-, 7-, and 9-OH-B[a]P, 4,5-, 7,8-, and 9,10-diOH-B[a]P, and 1,6-, 3,6-, and 6,12-B[a]P-dione (516-518).

B[a]P has recently been shown to induce *in vitro* gene expression mediated though the D, E, and F domains of human ERα (Gal4-hERαdef) (519), whereas it reportedly acts in an antiestrogenic manner in other *in vitro* systems (520, 521). In this study the possible *in vitro* and *in vivo* estrogenic activity of B[a]P and its major metabolites was further examined.

MATERIALS AND METHODS

Chemicals and biochemicals

B[a]P (97% purity) was obtained from Aldrich (Milwaukee, WI). 4,5-diOH-B[a]P, 7,8-diOH-B[a]P, 9,10-diOH-B[a]P, B[a]P-1,6-dione, B[a]P-3,6-dione, B[a]P 6,12-dione, 3-OH-B[a]P, and 9-OH-B[a]P (all $\geq$ 98% purity) were obtained from NCI Chemical Carcinogen Reference Standard Repository (Kansas City, MO). 1-OH-B[a]P and 7-OH-B[a]P were kindly provided by Dr. Harish Sikka (SUNY Buffalo, NY). 17α-Ethynyl estradiol (EE), 17β-estradiol (E2), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). [2,4,6,7,16,17-^{3}H]-E2 ([^{3}H]E2; 123 Ci/mmol) and [γ^{32}P]dATP (3000 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA). Fetal bovine serum (FBS) was obtained from Intergen (Purchase, NY). Phenol red-free Dulbecco's Modified Eagle Medium (DMEM), antibiotics, Superscript II reverse transcriptase, and Taq DNA polymerase were purchased from Life Technologies (Rockville, MD). T4 polynucleotide kinase (PNK) was obtained from New England Biolabs (Beverly, MA). RNAse inhibitor was obtained from Promega (Madison, WI). Restriction enzymes and dNTPs were obtained from Boerhinger Mannheim (Indianapolis, IN). D-luciferin was purchased from Molecular Probes (Eugene, OR). All other chemicals were of the highest quality available from commercial sources.

Construction of Plasmids.

The plasmid pGEX-hERαdef was constructed by PCR amplification of the human ERα (Dr. P. Chambon, IGBMC, Illkirch, France) DEF domains (amino acids 264-595) as

previously described (522). The pGal4-mERβdef was constructed by PCR amplification of amino acids 159-485 of the mERβ (kindly provided by Dr. V. Giguère, Montreal, Quebec, Canada), using forward primer (5′-aaaagaattcctcgagcctgcgacttcgcaagtgttacgaa-3′) and reverse primer (5′-aaaagcggccgcagatcttcactgtgaatggaggttctggga-3′). The fragment was digested with XhoI and ClaI and ligated into the similarly digested eukaryotic expression vector containing the DNA binding domain of the yeast transcription factor Gal4, pG4MpolyII. PCR amplification was performed essentially as previously described (523) using Vent DNA polymerase in a reaction mixture containing Thermopol buffer, 200 μM dNTPs, 1 mM $MgSO_4$, 500 nM primer and 1.25 IU of polymerase. The mixture was heated to 94°C for 5 min followed by 35 rounds of 94°C for 45 s, 60°C for 45 s and 72°C for 1 min 45 s. The sequence of each construct was confirmed by restriction enzyme digest and ABI/Prism automated sequencing (Perkin Elmer Applied Biosystems; Foster City, CA).

Competitive Ligand Binding Assay

The method used for the competitive binding assays has recently been described in detail (522), but is outlined briefly as follows. Experiments were performed using either a bacterially expressed fusion protein consisting of glutathione-S-transferase and the D, E, and F domains of human ERα (GST-hERαdef, >85% purity (522)) or full length human ERβ (hERβ, >80% purity, Panvera, Madison, WI). The receptor was first diluted in TEGD buffer (10 mM Tris pH 7.6, 1.5 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol) containing 1 mg/ml BSA as a carrier protein. An aliquot (240 μl) was incubated at 4°C for 2 h with 5 μl of 2.5 nM [^{3}H]E2 and 5 μl of unlabeled competitor (1 pM to 1

μM final concentration of E2, or 60 nM to 20 μM final concentration of B[a]P or metabolite). The [^{3}H]E2 and all competitor compounds were dissolved in DMSO, with the DMSO concentration in the final mixture not exceeding 4%. The fusion protein preparation was diluted in order to ensure 10,000 dpm of total binding in each tube.

Each concentration was tested in quadruplicate and at least 3 independent experiments were performed. Results are expressed as percent specific binding of [^{3}H]E2 versus log of competitor concentration. Analysis was performed using nonlinear regression with the single site competitive binding option of GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA). Reported IC_{50} values denote the calculated concentration of test compound required to displace 50% of the [^{3}H]E2 from the fusion protein.

Cell Culture

MCF-7 human breast cancer cells were kindly provided by Dr. L. Murphy (University of Manitoba, Winnipeg, Manitoba, Canada). Cells were maintained in DMEM supplemented with 10% FBS, and with 20 mM HEPES, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml gentamicin. Cells were grown at 37°C in a 4% CO_2 humidified environment.

Transfection and Reporter Gene Assay

Transfections were performed essentially as previously described (519). MCF-7 cells were plated in 6-well culture dishes at approximately 50% confluency, in 2 ml of media supplemented with 6% FBS than had been stripped with dextran-coated charcoal, and allowed to attach for 6 h. Cells were then transiently transfected with three plasmids, using the calcium phosphate co-precipitation method (524): 1) 1.5 μg 17m5-G-Luc

(provided by Dr. P. Chambon), 2) 0.2 μg Gal4-hERαdef (Gal4 linked to D, E, and F domains of hERα; also known as Gal4-HEG0) or Gal4-mERβdef (Gal4 linked to D, E, and F domains of mouse ERβ, and 3) 0.2 μg pCMV-lacZ, a β-galactosidase expression vector used for normalizing transfection efficiency across wells (Amersham Pharmacia). After 18 h cells were rinsed twice with sterile phosphate-buffered saline and fresh media was added. Cells were then treated by adding 2 μl of test compound dissolved in DMSO to 2 ml of media, so that the total concentration of DMSO did not exceed 0.1% unless otherwise noted. The cells were harvested after 24 h of treatment, and luciferase and β-galactosidase activity was measured using standard protocols (524, 525).

Each treatment was performed in duplicate, and two aliquots were assayed from each well, so that means and standard deviations were calculated using four measurements. Independent experiments were performed at least three times, and results are expressed as fold induction, representing luciferase activity of treated cells divided by that of solvent-treated cells, normalized for β-galactosidase activity. GraphPad Prism 3.0 was used for graphical analyses, including EC_{50} values, which denote the concentration of test compound required to cause 50% of the maximal response induced by E2.

Uterotrophic Assay

C57BL/6 and DBA/2 mice ovariectomized by the vendor on postnatal day 20 were obtained from Charles River Laboratories (Raleigh, NC). The mice were kept in cages containing cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) in a 23°C HEPA-filtered environment with 30-40% humidity and a 12 h light/dark cycle. Animals were allowed free access to deionized

water and Harlen Teklad 22/5 Rodent Diet 8640 (Madison, WI). Mice were acclimatized for four days prior to dosing. On the fourth day, animals were weighed, and doses of EE and B[a]P were prepared in sesame oil (Loriva, Ronkonkoma, NY) based on the average weight of the animals. Beginning the following day, each mouse was gavaged with a 0.1 c.c. dose of 0.1 mg/kg/day EE, or 0.1, 1, or 10 mg/kg/day B[a]P for three consecutive days. At 24 h following the third treatment, animals were sacrificed by cervical dislocation, and uteri were excised, separated from attached connective tissue, blotted, weighed, and stored in RNAlater storage solution (Ambion Inc., Austin, TX) at -80°C until further use. Studies with 3- and 9-OH-B[a]P were performed as described above for B[a]P, except that solutions were injected subcutaneously with a 25-gauge needle at a dose level of 1, 5, 10, or 20 mg/kg/day.

RT-PCR of lactoferrin mRNA

Uteri were thawed in the storage solution, transferred to a tube containing 0.5 ml Trizol (Life Technologies), minced with sterile scissors, and homogenized using a Polytron tissue homogenizer (Kinematica, Lucerne, Switzerland) for three pulses of 15 s each at 85% output. Total RNA extraction was performed by the Trizol method according to the manufacturer's instructions.

Samples were reverse transcribed and then PCR-amplified using specific primers for lactoferrin (LF) or for β-actin (A) in separate tubes. The primers used were as follows, with restriction enzyme sites (to allow subcloning and sequencing to verify the identity of the amplified product) underlined and indicated in brackets: LF forward 5'-aaaagaattcggatccgggacacttcgtccatacctgaa-3' (EcoRI, BamHI); LF reverse 5'-aaaactcgaggcggccgcgctatcacatcctgctgctttttat-3' (XhoI, NotI); A forward 5'-

aaaaggatccaagcttctgaagtaccattgaacatggca-3' (BamHI, HindIII); A reverse 5'-aaaactcgaggcggccgctgtcacgcacgatttccctctcag-3' (XhoI, NotI).

The lactoferrin primer pair was designed to amplify a 632 bp product from nt 436 to 1067 of the cDNA, and the actin primer pair was designed to amplify a 436 bp product from nt 121 to 556. A 1 μg aliquot of RNA was incubated with 20 pmol reverse primer for 12 minutes at 70°C, cooled quickly on ice, and then mixed with First Strand Buffer, 200 IU SuperScript II reverse transcriptase, 10 nmol each dNTP, 200 nmol DTT, and 20 IU RNase inhibitor, in a 20 μl final reaction volume. The mixture was then incubated for 50 min at 42°C followed by 15 min at 70°C using a RoboCycler Gradient 96 PCR machine (Stratagene, La Jolla, CA).

Lactoferrin and actin forward primers were end-labeled with [γ^{32}P]dATP to facilitate quantitation of PCR products. A mixture of PNK buffer, 75 IU PNK, 50 nmol of primer, and 5 μl [γ^{32}P]dATP in DEPC-treated water was incubated at 37°C for 30 min, an additional 50 IU of PNK was added, and the mixture was incubated another 30 min. Final reaction volume was 300 μl. Unincorporated nucleotides were removed using a MicroSpin G-25 spin column (Amersham/Pharmacia) according to the manufacturer's directions.

A 2 μl aliquot (10%) of the cDNA product was then combined with PCR Buffer, 2.5 IU Taq DNA polymerase, 75 nmol $MgCl_2$, 10 nmol each dNTP, 20 pmol reverse primer, 5 pmol labeled forward primer, and 15 pmol unlabeled forward primer. The 50 μl reaction mixture was subjected to the following cycling temperature program: 3 min at 94°C; 24 cycles of: 30 sec at 94°C, 30 sec at 67°C, 60 sec at 72°C; 10 min at 72°C. Samples were run on a 5% acrylamide gel, which was dried and exposed to a Molecular

Dynamics (Sunnyvale, CA) storage phosphor screen for 16 hr. The signals were quantitated using a Molecular Dynamics Storm 820 scanner and ImageQuaNT v. 4.2a software. Lactoferrin levels were normalized using β-actin mRNA levels, which were assumed to be unaffected by treatment.

Statistical Analyses

Increases in uterine weight above that of vehicle-treated controls were determined using an analysis of covariance with body weight at time of sacrifice as a covariate, followed by the Fischer's multiple pairwise comparison procedure of Systat v. 5.04 (Systat, Inc., Evanston, IL). A log transformation was performed if group variances (body weight or uterine weight) had $p<0.01$ using Bartlett's test for homogeneity.

RESULTS

Competitive binding to GST-hERαdef and full-length hERβ

Table 1 and Figure 2 summarize the results of the competitive binding assay for E2, B[a]P, and the 10 B[a]P metabolites. E2 exhibited IC_{50} values of approximately 5.5 nM for both ERα and ERβ. Studies of B[a]P and its metabolites showed that only the monohydroxylated B[a]P metabolites possessed any affinity for either receptor isoform over the range of concentrations tested. 10 μM 3-OH-B[a]P caused approximately 60% displacement of [^{3}H]E2 from hERαdef, while 1-, 7-, and 9-OH-B[a]P caused 20-40% displacement. These four compounds all showed higher binding affinity for hERβ, and

IC_{50} values were calculated for 1-, 3-, and 9-OH-B[a]P (2.6 μM, 49 nM, and 1.0 μM respectively), which each caused ~100% displacement of [^{3}H]E2.

Gal4-hERαdef- and Gal4-mERβdef-mediated gene expression

The results of the reporter gene expression assays are summarized in Table 1 and Figure 3. As shown in Figure 3A, 3- and 9-OH-B[a]P caused higher induction of Gal4-hERα-mediated gene expression than did comparable concentrations of parent B[a]P. In particular, at a concentration of 10 μM the two metabolites induced luciferase activity comparable to that obtained with 10 nM E2, while B[a]P itself caused only 30-50% of that level of response. 1- and 7-OH-B[a]P caused a low level of induction, while no other metabolites yielded any detectable response at the concentrations used. When cells were treated simultaneously with E2 and B[a]P, Gal4-hERαdef-mediated induction of reporter activity appeared to be additive, as shown in Figure 4.

Figure 3B shows that, in contrast to results with Gal4-hERαdef, B[a]P and all four monohydroxylated metabolites highly induced Gal4-mERβdef-mediated reporter gene expression. B[a]P and 3-, 7-, and 9-OH-B[a]P all displayed EC_{50} values in the 200-450 nM range, approximately 10^3-fold higher than that of E2 (Table 1). Again, no dihydrodiol or dione metabolites showed any appreciable activity. Interestingly, Gal4-mERβdef consistently did not show as strong an additive response as Gal4-hERαdef when cotreated with B[a]P and 0.1 nM E2 (Figure 4B). Furthermore, no additive response was observed when B[a]P was cotreated with 1 nM E2.

Uterotrophic assay and expression of uterine lactoferrin mRNA

Mice were dosed with B[a]P by oral gavage, in order to simulate dietary exposure. While EE (0.1 mg/kg) caused a statistically significant increase in uterine wet weight (6-fold; $p<0.001$) in both the DBA/2 and C57BL/6 strains, B[a]P was not found to cause an increase in uterine wet weight or uterine lactoferrin mRNA expression at any of the concentrations studied (Table 2 and Figure 5). Similarly, when EE or 3- or 9-OH-B[a]P were administered to mice by subcutaneous injection, in order to bypass first-pass metabolic effects, EE caused a 6-fold and 10-fold increase in uterine weight in DBA/2 and C57BL/6 mice respectively ($p<0.05$), while no response was seen in 3- and 9-OH-B[a]P-treated mice, as summarized in Table 3. This lack of response was also reflected in the absence of changes in uterine lactoferrin mRNA in either mouse strain, as shown in Figure 5. EE, B[a]P, and B[a]P metabolites were not found to adversely affect body weight during the dosing periods (data not shown), and body weight was not found to be a significant covariate in any of the experiments at the $p<0.02$ level of significance.

DISCUSSION

The ability of B[a]P to induce Gal4-hERαdef-mediated gene expression in transiently transfected MCF-7 cells has been previously reported (519). Since patterns of PAH metabolism can vary by gender, species, tissue, individual, dosing route, and duration (526-530), determining whether a compound itself or certain metabolites are responsible for observed effects may help to identify potentially sensitive tissues and organs. Numerous studies have measured the extent of B[a]P metabolism in a variety of tissues and organisms, and although different cell types exhibit characteristic metabolic profiles, the identity of the metabolites formed remains generally constant: 1-, 3-, 7-, and

9-OH-B[a]P; 4,5-, 7,8-, and 9,10-diOH-B[a]P; and B[a]P-1,6-, 3,6-, and 6,12-dione have been repeatedly identified in a number of cell-free and cultured cell systems, and in whole animal metabolism studies. Of these compounds, only 1-, 3-, 7- and 9-OH-B[a]P were found to effectively compete with [^{3}H]E2 for binding to either ER isoform and to cause ER-mediated gene expression in MCF-7 cells. Previous studies have reported that 1-, 2-, 5-, 6-, 11-, and 12-OH-B[a]P partially displace [^{3}H]E2 from the ER in rat uterine cytosol (531), but the affinity of these compounds was very weak compared to the monohydroxylated metabolites examined in the present study.

B[a]P itself was not able to bind to either receptor isoform, but induced hERα- and mERβ-mediated luciferase reporter expression in MCF-7 cells. The ability of monohydroxylated B[a]P metabolites to bind and induce gene expression mediated through both ER isoforms suggests that these metabolites are responsible for the increase in reporter gene expression observed in MCF-7 cells treated with B[a]P. This is consistent with previous studies reporting that B[a]P is not able to compete appreciably with [^{3}H]E2 for binding to recombinant full-length hERα (521), but is able to compete for ER binding in an MCF-7 whole-cell binding assay (520). Similar results would be expected using hERβ rather than mERβ, since these two receptors share a high degree of similarity (89% amino acid identity in the DEF region).

Though it is known that the P450 content of cultured cells may differ greatly from that of the cells of an intact organism (532), MCF-7 cells are known to express several P450 isozymes, including 1A1, 1A2, and 1B1 (533, 534), and to exhibit aryl hydrocarbon hydroxylase and ethoxyresorufin-O-deethylase activity (535). The data presented here suggest that B[a]P-exposed MCF-7 cells are biotransforming the compound to estrogenic

metabolites. It has recently been shown that MCF-7 cells leave much of the B[a]P unmetabolized, but also produce several hydroxylated metabolites including 3- and 9-OH-B[a]P, and that Gal4-hERαdef-mediated induction of reporter gene expression caused by these two metabolites is abrogated by treatment with α-naphthoflavone, a P450 inhibitor (536). Dihydrodiol and dione metabolites were ineffective at either binding or inducing ER-mediated gene expression. The finding that monohydroxylated B[a]P metabolites act as weak ER agonists is consistent with studies showing that high-affinity ER ligands such as E2 and the hydroxylated PAH 3,9-dihydroxy-benz[a]anthracene tend to possess a hydroxyl group at each end of the molecule (537), while the removal of either hydroxyl group from E2 results in detectable but greatly reduced estrogenic activity (31, 538).

The effects of B[a]P were also examined *in vivo*, in order to evaluate its ER-mediated effects in the context of an intact organism, which includes serum binding proteins and a full complement of oxidative and conjugative enzymes of metabolism. Increased uterine weight, which is considered to be a hallmark of E2 activity, and increased lactoferrin mRNA expression are both accepted, sensitive markers of estrogen action (31, 539, 540). Orally administered B[a]P did not cause a detectable increase in uterine weight or lactoferrin mRNA levels in ovariectomized immature C57BL/6 ('PAH-responsive') or DBA/2 ('PAH-nonresponsive') mouse strains at the doses used. The use of differentially responsive mouse strains is reflective of the heterogeneity of metabolic pathways in the human population (541), where responsiveness refers to ability of ligand-aryl hydrocarbon receptor (AhR) complexes to induce the expression of specific P450 isozymes. The resulting increased P450 activity induces metabolism of a variety of PAHs

that are ligands for the AhR, including B[a]P (542, 543). As a result, these compounds are able to induce their own metabolism, particularly in animals possessing highly inducible CYP1A1, such as the C57BL/6 strain (544, 545).

B[a]P is known to be distributed rapidly within the body (546), and its metabolites can readily penetrate cell membranes (518, 547). Although a large proportion of administered B[a]P remains unmetabolized (548), 3- and 9-OH-B[a]P are known to be major metabolites in C57BL/6 and DBA/2 mouse strains, whether B[a]P is given as a single dose or is able to cause induction of P450 enzymes by treatment over a period of several days (528, 549). The lack of uterotrophic or lactoferrin mRNA induction effect observed in B[a]P-treated mice in the present study therefore suggests that either 1) monohydroxylated B[a]P metabolites are not formed by these mice at levels sufficiently high to induce an estrogenic response, 2) the weakly estrogenic B[a]P metabolites are acting as antiestrogens overall *in vivo* by displacing stronger endogenous estrogens from the binding sites, 3) the metabolites are conjugated and excreted before reaching the target tissue, or 4) monohydroxylated B[a]P metabolites exhibit tissue-specific gene expression activities that do not lead to an increase in uterine wet weight or lactoferrin mRNA expression. The lack of response following s.c. injection of up to 20 mg/kg of 3- or 9-OH-B[a]P is evidence against the first possibility, and suggests that the metabolites are excreted quickly or are not acting as estrogens on the specific uterine endpoints examined.

These results show that ER binding and ER-mediated gene expression studies of B[a]P did not accurately predict the lack of observed *in vivo* estrogenicity in the C57BL/6 or DBA/2 mouse uterus. Moreover, though the present study provided little evidence of

non-additive interactions between B[a]P metabolites and E2 in MCF-7 cells, other researchers have reported contrasting findings. For example it has been reported that B[a]P can antagonize the estrogenic activities of E2 in yeast expressing hERα (521) and cause a decrease in E2-induced MCF-7 cell proliferation (520) and nuclear ER levels (550). It has been hypothesized that many of the antiestrogenic effects observed with PAHs are mediated through the AhR. B[a]P and many other PAHs exhibit high binding affinity for the AhR (542, 543), a ligand-dependent transcription factor that modulates the expression of specific genes by binding to the xenobiotic response element (XRE, also known as DRE). Binding of a PAH or other ligand to the AhR could produce an antiestrogenic response by increasing the rate at which E2 is transformed to less active metabolites, or by allowing the AhR-ligand complex to alter the transcription rate of E2-inducible genes by binding to XRE sequences in the promoters of these genes (551-553).

Importantly, the published reports demonstrating an antiestrogenic effect of B[a]P do not account for interactions of B[a]P metabolites with ERβ, which has been shown to be absent or nearly absent in MCF-7 cells (554). hERα and hERβ are fairly well conserved in the regions of the LBD forming the ligand binding pocket, and while some studies have reported that many compounds display a similar affinity for each isoform, others have demonstrated significant differences with certain ligands (502, 554, 555). In addition, ERβ has been found to have a slightly lower affinity for the ERE, as well as for E2, compared with ERα, though their abilities to transactivate reporter genes when bound to E2 are very similar (556). The *in vitro* preference of monohydroxylated B[a]P metabolites for ERβ over ERα in the present study, as well as the distinct behavior of the two isoforms in the cotreatment experiments is therefore of particular interest, since the

extent to which these two isoforms have overlapping or distinct roles is currently an area of intense research. Interestingly, in this study individual metabolites showed distinct behaviors that emphasize the importance of hydroxyl group placement, as illustrated, for example, by the greater EC_{50} value for 3-OH-B[a]P relative to its IC_{50} in the ERβ test systems, while the reverse was true for 7- and 9-OH-B[a]P. The lack of correlation between relative binding affinity and the level of reporter gene induction may be due, in part, to ligand-induced allosteric changes that affect ER complex:DNA interactions (557). The ligand may also induce conformational changes that affect interactions with coactivators (558), which could influence the induction of gene expression. For example, genistein has been found to exhibit a 30-fold greater affinity for hERβ relative to hERα, but induced comparable levels of hERα- and hERβ-mediated reporter gene expression (554, 559). This may partially explain the lack of correlation between relative binding affinity and gene expression observed for some B[a]P metabolites.

Moreover, the heightened combined ability of B[a]P (through its metabolites) and E2 to induce reporter gene expression in the ERα compared to the ERβ test system was surprising. B[a]P affects a number of cellular processes, and therefore the apparent synergistic effects may be due to cross-talk between mechanisms that converge at ERα but not ERβ. Although both receptors have a similar structure, hERβ shares only 58% amino acid sequence identity with hERα in the ligand binding domain (Domain E) (560). This difference may partially account for the enhanced activity with ERα but not ERβ.

No evidence of estrogenic effects of B[a]P was found in the mouse uterus, suggesting that *in vitro* studies of estrogenic activity should be interpreted with caution. Furthermore, these findings suggest that as further information is gained regarding the

biological properties of ERβ, appropriate *in vivo* studies should be designed that examine effects in tissues such as the ovaries and bone, which, unlike the uterus, express high levels of ERβ. Future studies will be designed to clarify these issues, with the aim of developing in vitro assays that accurately predict *in vivo* estrogenic responses.

Table 1. *In vitro* competitive binding IC_{50} and gene expression EC_{50} values for E2, B[a]P, and B[a]P metabolites.

Compound	GST-hERαdef IC_{50}	hERβ IC_{50}	Gal4-hERαdef EC_{50}	Gal4-mERβdef EC_{50}
E2	5.5 ± 1.2 nM	5.6 ± 1.1 nM	350 ± 240 pM	130 ± 24 pM
B[a]P	nb[b]	nb	wi[e]	330 ± 49 nM
1-OH-B[a]P	nb	3.3 ± 0.2 μM	wi	3.2 ± 1.0 μM
3-OH-B[a]P	>10 μM[c]	90 ± 57 nM	3.2 ± 3.3 μM	210 ± 110 nM
7-OH-B[a]P	nb	>10 μM	wi	430 ± 10 nM
9-OH-B[a]P	wb[d]	2.0 ± 1.0 μM	1.2 ± 3.3 μM	430 ± 110 nM
4,5-diOH-B[a]P	nb	nb	ni[f]	ni
7,8-diOH-B[a]P	nb	nb	ni	ni
9,10-diOH-B[a]P	nb	nb	ni	ni
B[a]P-1,6-dione[a]	nb	nb	ni	ni
B[a]P-3,6-dione[a]	nb	nb	ni	ni
B[a]P-6,12-dione[a]	nb	nb	ni	ni

[a]maximum concentration tested was 2.5 μM due to solubility limitations
[b]non-binder, defined as a compound that, at 10 μM, causes <10% displacement of [^{3}H]E2
[c]>10 μM denotes a compound that, at 10 μM, causes >50% but not full displacement of [^{3}H]E2
[d]wb denotes a weak binder, defined as a compound that, at 10 μM, causes 10-50% displacement of [^{3}H]E2
[e]wi denotes a compound that, at 10 μM, induces reporter gene expression at <50% of maximal induction caused by 10 nM E2
[f]ni denotes a compound that, at 10 μM, induces reporter gene expression at <10% of maximal induction caused by 10 nM E2

Table 2. Effect of orally administered ethynyl estradiol (EE) or benzo[a]pyrene (B[a]P) on blotted uterine wet weight (UW) in ovariectomized DBA/2 and C57BL/6 mice.

Strain	Treatment	Dose (mg/kg)	BW (g)	UW (mg)	UW/BW (mg/g)
DBA/2	Sesame oil	-	12.9 ± 1.9	5.9 ± 2.4	0.45 ± 0.15
	EE	0.1	13.3 ± 1.5	33.3 ± 6.6**	2.5 ± 0.44
	B[a]P	0.1	12.9 ± 2.3	6.3 ± 2.5	0.48 ± 0.14
		1	13.1 ± 2.1	4.8 ± 1.9	0.36 ± 0.09
		10	11.8 ± 2.5	5.6 ± 2.3	0.53 ± 0.32
C57BL/6	Sesame oil	-	11.8 ± 1.7	3.7 ± 2.0	0.31 ± 0.14
	EE	0.1	10.0 ± 3.6	20.6 ± 3.5**	1.44 ± 0.82
	B[a]P	1	11.3 ± 1.2	3.5 ± 0.8	0.30 ± 0.06
		10	10.8 ± 2.0	3.6 ± 1.2	0.33 ± 0.09

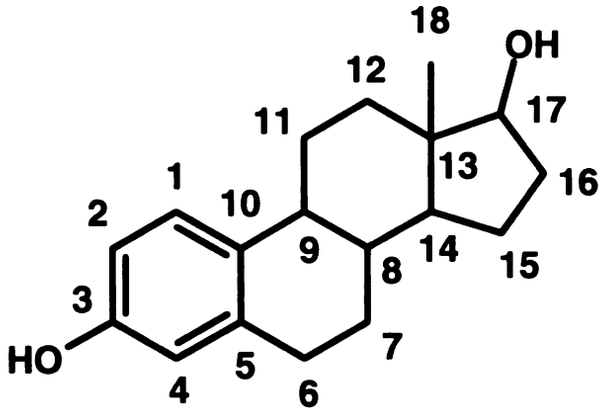
** denotes significant increases in uterine weight above vehicle-treated controls ($p<0.001$, $n=5$).

Table 3. Effect of subcutaneously administered ethynyl estradiol (EE) or 3- or 9-hydroxy-benzo[a]pyrene (OH-B[a]P) on blotted uterine weight (UW) in ovariectomized DBA/2 and C57BL/6 mice.

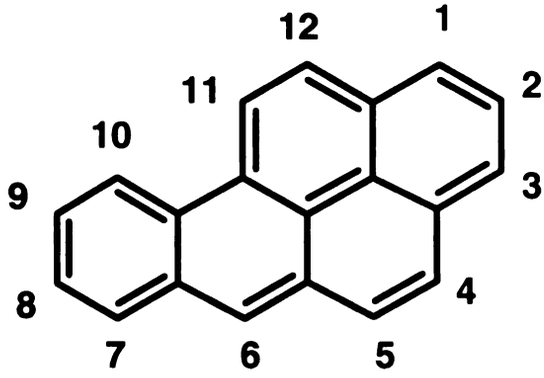
Strain	Treatment	Dose (mg/kg)	BW (g)	UW (mg)	UW/BW (mg/g)
DBA/2	Sesame oil	-	16.1 ± 0.4	4.7 ± 1.5	0.29 ± 0.10
	EE	0.1	17.4 ± 0.7	28.0 ± 8.4**	1.62 ± 0.53
	3-OH-B[a]P	1	15.7 ± 0.7	4.6 ± 2.2	0.25 ± 0.18
		5	15.4 ± 0.9	5.8 ± 1.3	0.38 ± 0.09
		10	15.9 ± 0.9	4.6 ± 1.9	0.29 ± 0.12
		20	16.0 ± 1.1	5.7 ± 2.7	0.35 ± 0.17
	9-OH-B[a]P	1	15.9 ± 1.6	3.8 ± 1.2	0.24 ± 0.07
		5	16.0 ± 0.7	5.8 ±1.4	0.36 ± 0.08
		10	15.6 ± 0.3	6.1 ± 2.1	0.39 ± 0.13
		20	15.8 ± 1.1	6.6 ± 1.5	0.41 ± 0.07
C57BL/6	Sesame oil	-	16.9 ± 0.7	2.2 ± 1.0	0.06 ± 0.06
	EE	0.1	16.7 ± 0.6	22.2 ± 3.9**	1.33 ± 0.26
	3-OH-B[a]P	1	16.1 ± 1.6	4.4 ± 3.4	0.28 ± 0.24
		5	16.6 ± 2.0	5.5 ± 3.9	0.35 ± 0.27
		10	16.7 ± 0.6	4.3 ± 2.4	0.21 ± 0.17
		20	15.5 ± 0.9	3.3 ± 0.9	0.17 ± 0.11
	9-OH-B[a]P	1	16.2 ± 1.8	7.9 ± 5.1*	0.50 ± 0.34
		5	16.3 ± 0.6	9.2 ± 8.7*	0.57 ± 0.54
		10	16.6 ± 0.8	4.0 ± 1.3	0.24 ± 0.07
		20	17.1 ± 1.4	5.7 ± 1.9*	0.34 ± 0.13

** denotes significant increases in uterine weight above vehicle-treated controls ($p<0.001$, $n=5$); * denotes increases at the level $p<0.05$ level.

Figure 1. Chemical structures of 17β-estradiol (E2) and benzo[a]pyrene (B[a]P), with position numbers labeled.



17β-Estradiol



Benzo[a]pyrene

Figure 2. Competitive displacement curves for E2, B[a]P, and B[a]P metabolites displacing 2.5 nM [^{3}H]E2 from (A) GST-hERαdef and (B) hERβ.

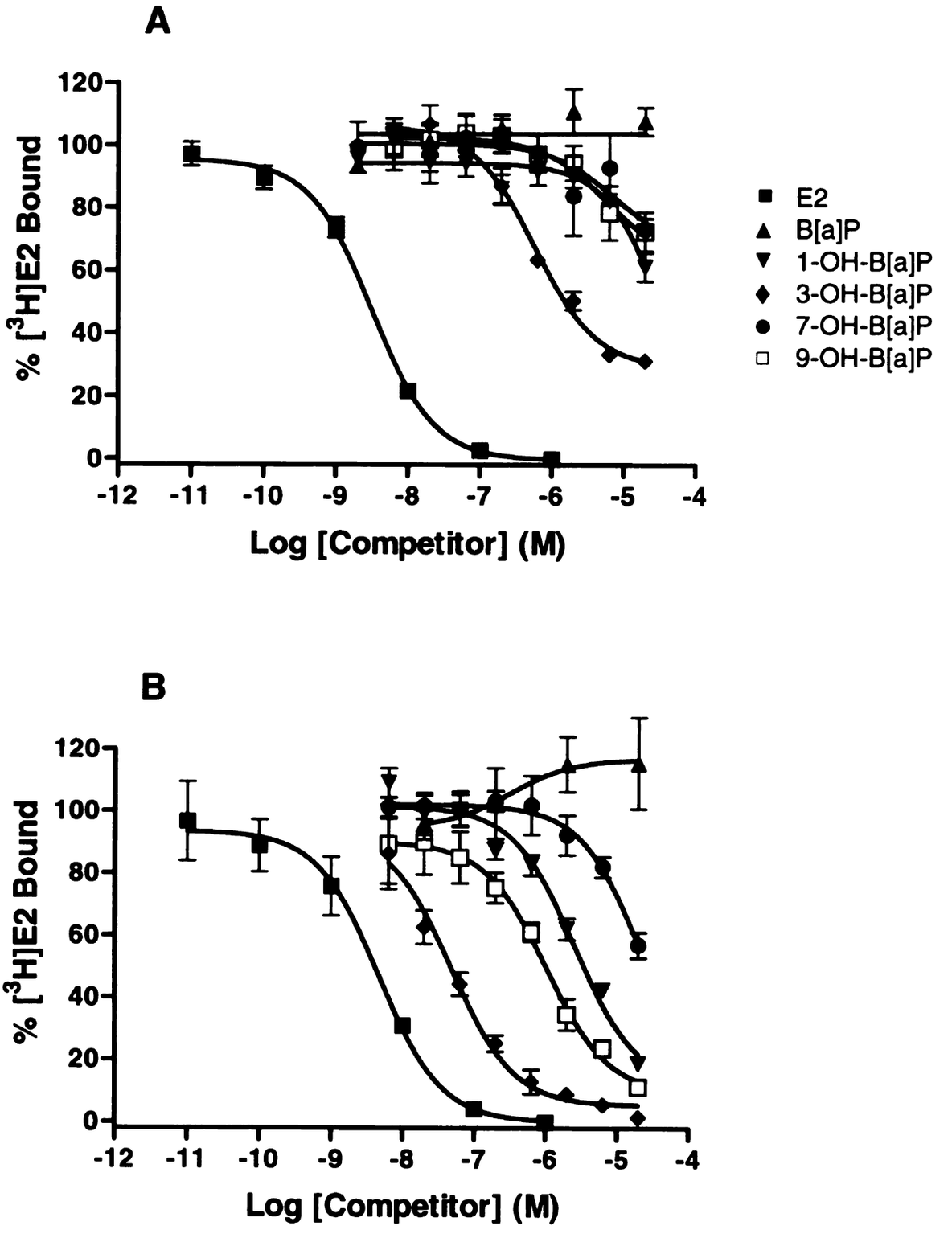


Figure 3. Induction of (A) Gal4-hERαdef- and (B) Gal4-mERβdef-mediated reporter gene expression in transiently transfected MCF-7 cells treated for 24 hrs with E2, B[a]P, or B[a]P metabolites.

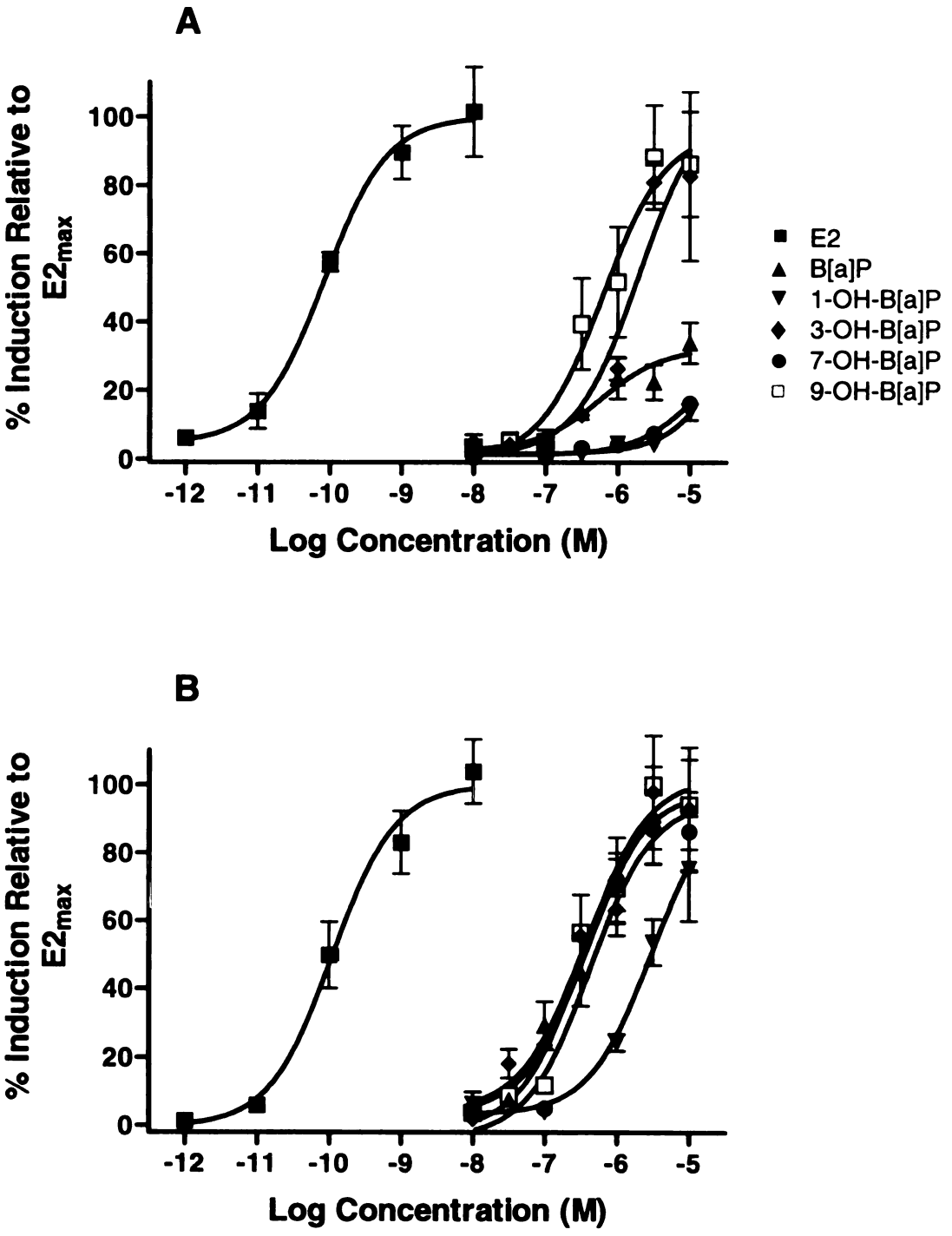


Figure 4. Results of cotreatment of E2 and B[a]P on hERα-mediated reporter gene expression in MCF-7 cells.

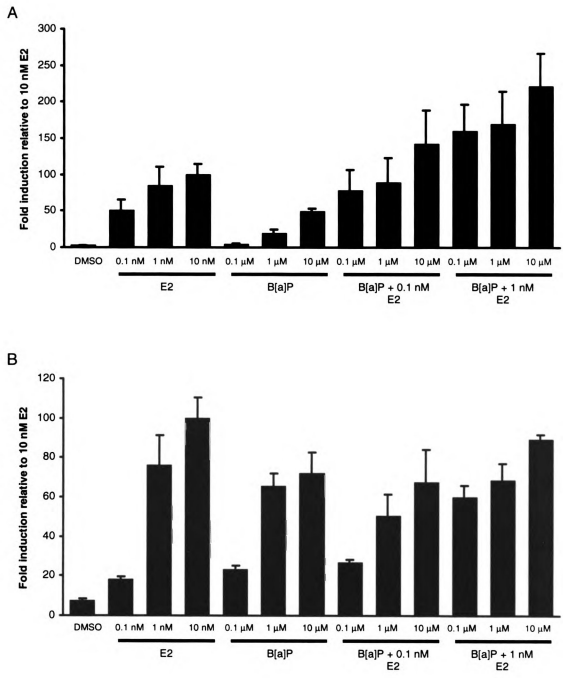
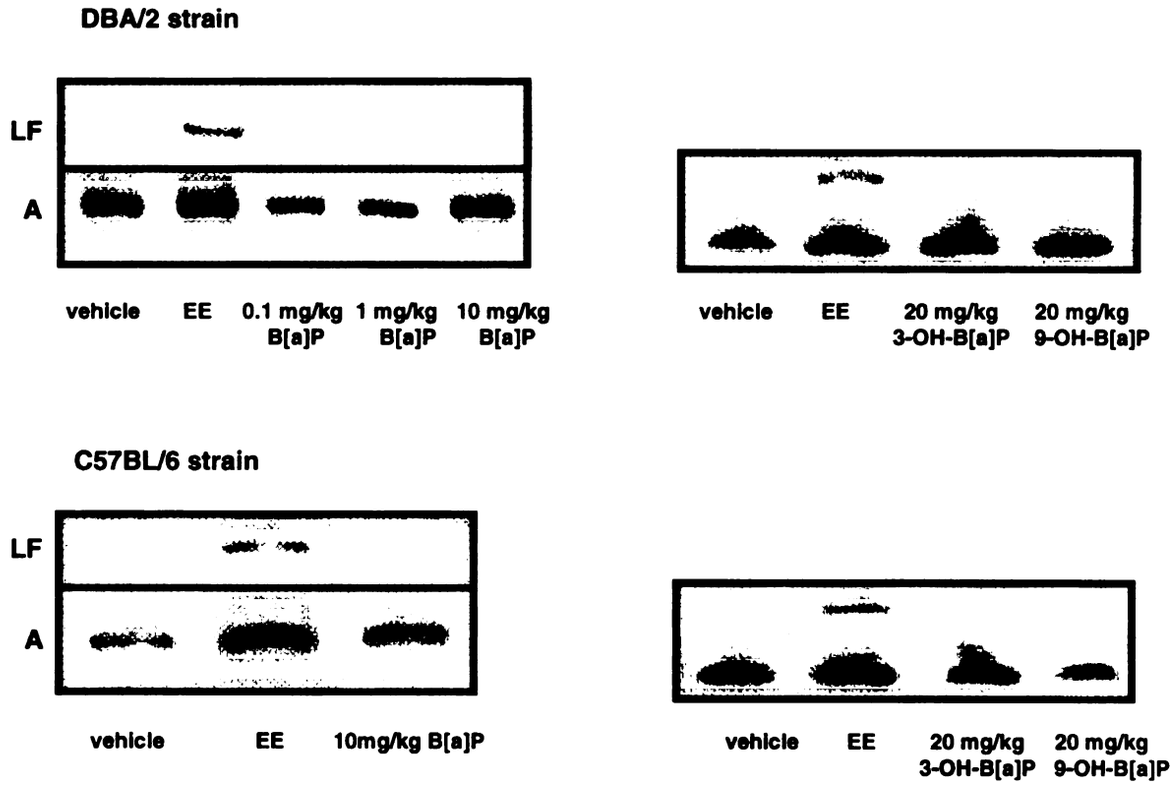


Figure 5. Representative gel showing uterine lactoferrin (LF) and actin (A) expression in ovariectomized D2 and C57 mice treated with vehicle, EE, or 20 mg/kg 3- or 9-OH-B[a]P.



CHAPTER 3

INTERACTION OF PAH-RELATED COMPOUNDS WITH THE α AND β ISOFORMS OF THE ESTROGEN RECEPTOR

ABSTRACT

The ability of several 4- and 5-ring polycyclic aromatic hydrocarbons (PAHs), heterocyclic PAHs, and their monohydroxy derivatives to interact with the estrogen receptor (ER) alpha and beta isoforms was examined. Only compounds possessing a hydroxyl group were able to compete with ^{3}H-labeled 17β-estradiol (E2) for binding to either a glutathione-S-transferase and human ERα D, E, and F domain fusion protein (GST-hERαdef) or to the full-length human ERβ. Competitive binding was comparable for both isoforms, with IC_{50} values ranging from 20-300 nM (E2 IC_{50} approximately 3 nM). However, several compounds were able to induce reporter gene expression preferentially through mERβ, using MCF-7 cells transiently transfected with either a Gal4-human ERαdef or Gal4-mouse ERβdef construct, as well as a Gal4-regulated reporter. These data extend the number and type of PAH-related compounds capable of interacting with ERα and ERβ, and provides additional evidence that even though some compounds may possess a similar affinity for both ER isoforms, the capacity for transcriptional activation can still be isoform-specific.

INTRODUCTION

The impact of polycyclic aromatic hydrocarbons (PAHs) on health is of great interest because these compounds are widely dispersed in the environment. While the mutagenicity and carcinogenicity of this class of compounds has been studied extensively (561, 562), more recent reports have also suggested that certain 4- and 5-ring PAHs can interact with the estrogen receptor (ER) α and β signaling pathways in a variety of test systems (519-521, 550, 563), potentially interfering with estrogen signaling in important developmental and reproductive processes. Though the specific roles of the α and β isoforms of the ER are not yet completely understood, they are currently being explored using binding and gene transactivation studies (554, 555), and by examining the tissue localization of the isoforms (500, 554, 564) and the effects of targeted disruption of the ER genes (76, 501, 565).

While reported interactions between PAHs and the ER are weak, the large size of this class of chemicals and the extent of their environmental distribution suggests that further research into potential health effects is merited. In particular, there are a large number of other polycyclic aromatic compounds (PACs), including heterocyclic PAHs, which contain an N, S, or O in one of the rings, as well as PAHs with alkyl and other substituents. Many of these have been reported to have mutagenic and carcinogenic activity (566-569), but remain poorly characterized with respect to ER interactions. These compounds are formed during the incomplete combustion of organic matter, and are found as a complex mixture in coal furnace and vehicle emissions and cigarette smoke (570, 571), and have been detected at high levels in the air, water, and soil near sites of

emissions or fuel spills (572-575). Because of the large number of PACs isosteric to 4- and 5-ring PAHs, and their ubiquitous distribution in the environment, the possibility of these compounds interacting with ERs is of interest.

In the present study, the ability of several PAHs, heterocyclic PAHs, and their monohydroxy derivatives, to interact with the α and β isoforms of the ER was examined, using *in vitro* competitive binding and reporter gene expression assays.

MATERIALS AND METHODS

Chemicals

Benz[a]anthracene (B[a]A), chrysene (CR), and benzo[b]naphtho[2,3-d]thiophene (B[b]N[2,3-d]T) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Benzo[b]fluorene (B[b]F) was obtained from Accustandard (New Haven, CT). Benzo[c]phenanthrene (B[c]PH) was obtained from the NCI Chemical Carcinogen Reference Standard Repository (Kansas City, MO). Benzo[a]carbazole (B[a]C) and benzo[c]carbazole (B[c]C) were obtained from Chemsyn Science Laboratories (Lenexa, Kansas). Benzo[b]naphtho[2,1-d]thiophene (B[b]N[2,1-d]T) was obtained from Fisher (Pittsburgh, PA). 3-Hydroxybenzo[c]phenanthrene (3-OH-B[c]PH) was synthesized as previously described (Kumar, 1997). 5-Methylchrysene (5-MeCR) and various hydroxylated derivatives of chrysene and 5-methylchrysene were prepared according to published procedures (576, 577). 3-Hydroxybenzo[b]naphtho[2,1-d]thiophene (3-OH-B[b]N[2,1-d]T and 3-hydroxybenzo[b]phenanthro[2,3-d]thiophene (3-OH-B[b]PH[2,3-

d]T) were synthesized as described (578); Kumar, submitted). Structures of test compounds are shown in Figure 1.

17β-estradiol (E2) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO), and [2,4,6,7,16,17-^{3}H]-E2 ([^{3}H]E2; 123 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). Fetal bovine serum (FBS) was obtained from Intergen (Purchase, NY). Phenol red-free Dulbecco's Modified Eagle Medium (DMEM) and antibiotics were purchased from Life Technologies (Rockville, MD), and D-luciferin was purchased from Molecular Probes (Eugene, OR). All other chemicals were of the highest quality available from commercial sources.

Competitive Ligand Binding Assay

The method used for the competitive binding assays has recently been described in detail (522, 563), but is outlined briefly as follows. Experiments were performed using either a bacterially expressed fusion protein consisting of glutathione-S-transferase and the D, E, and F domains of human ERα (GST-hERαdef, >85% purity (522)) or full length human ERβ (hERβ, >80% purity, Panvera, Madison, WI). The receptor was incubated with [^{3}H]E2 (2.5 nM final concentration) and with 10 pM to 1 μM final concentration of E2, or 60 nM to 20 μM final concentration of test compound. Following a 24 hr incubation at 4°C, bound radioactivity was measured using a scintillation counter. The [^{3}H]E2 and all competitor compounds were dissolved in DMSO, with the DMSO concentration in the final mixture not exceeding 4%.

Cell Culture

MCF-7 human breast cancer cells were kindly provided by Dr. L. Murphy (University of Manitoba, Winnipeg, Manitoba, Canada) and maintained as described in (579).

Transfection and Reporter Gene Assay

Transfections were performed as previously described (579), using the following three plasmids: 1) 1.5 μg 17m5-G-Luc (provided by Dr. P.Chambon IGBMC CNRS-LGME, Illkirch Cedex C.U. de Strasbourg, France), 2) 0.2 μg Gal4-hERαdef (Gal4 linked to D, E, and F domains of hERα; also known as Gal4-HEG0) or Gal4-mERβdef (Gal4 linked to D, E, and F domains of mouse ERβ, and 3) 0.2 μg pCMV-lacZ, a β-galactosidase expression vector used for normalizing transfection efficiency across wells (Amersham Pharmacia). After 18 h cells were treated with test compound dissolved in DMSO so that the total solvent concentration did not exceed 0.1%. The cells were harvested after 24 h of treatment, and luciferase and β-galactosidase activity was measured using standard protocols (524, 525).

RESULTS

Preliminary studies indicated that most PAHs with 2-6 rings (naphthalene, acenaphthene, acenaphthylene, anthracene, fluorene, phenanthrene, fluoranthene, cyclopenta[c,d]pyrene, benzo[b]fluoranthene, dibenzo[a,h]anthracene, perylene, benzo[g,h,i]perylene, dibenzo[a,l]pyrene, dibenzo[a,e]fluoranthene, and indeno[1,2,3-cd]pyrene) were not able to bind to GST-hERαdef or to induce hERαdef-mediated gene expression. However, certain 4- and 5-ring compounds, e.g. benzo[a]pyrene (B[a]P)

(579), B[a]A, B[b]F, and B[c]PH (this study) displayed weak agonistic interactions in the ERα test system, and therefore these compounds were also tested in the ERβ assays. All of the PAHs mentioned above were also tested for antiestrogenicity by cotreatment with 0.1 or 1 nM E2, though only additive effects or no effect were observed.

None of the parent PAHs and their heterocyclic analogs listed in Table 1 were able to displace [^{3}H]E2 from either receptor isoform (data not shown). By contrast, the monohydroxylated compounds were able to compete for binding to both receptor isoforms, to varying degrees (Figure 2 and Table 1). For example, chrysene was unable to compete for binding, while the hydroxylated derivatives competed in the order 2-OH-5MeCR > 2-OH-CR > 8-OH-5MeCR, showing similar ability to compete for binding to each receptor isoform. By comparison, the competitive binding ability of 2-OH-B[c]PH, 3-OH-B[b]N[2,1-d]T, and 3-OH-B[b]PH[2,3-d]T was somewhat weaker, but again comparable between the two receptor isoforms.

As shown in Figure 3 and summarized in Table 1, all test compounds were able to induce some degree of ER-mediated reporter gene response with each isoform (10 μM inducing a response 20-60% of that of $E2_{max}$), with the exception of CR and B[c]PH. B[a]A and B[b]F induced a level of reporter gene expression comparable to that of B[a]C and B[c]C, but below that of B[b]N[2,1-d]T and B[b]N[2,3-d]T. Furthermore, B[a]A, B[b]F, B[b]N[2,1-d]T, and B[b]N[2,3-d]T showed induction of reporter gene expression that appeared to be preferentially mediated through the β isoform of the ER.

All of the monohydroxylated compounds studied, with the exception of 3-OH-B[b]PH[2,3-d]T (parent compound not available), exhibited much higher reporter gene induction than did the respective parent compounds. Some preference for the β isoform

of the receptor was seen, particularly in the chrysene-related compounds. The only compounds to achieve a 100% induction relative to that of $E2_{max}$ were 2- and 8-OH-5MeCR, which was seen exclusively in the ERβ test system. All compounds in the gene expression studies were tested at doses of up to 10 μM, but many caused cytotoxicity at the highest concentrations, as evidenced by reduced cell number upon microscopic examination, as well as significantly reduced β-galactosidase activity. Those concentrations that significantly reduced β-galactosidase activity were not included in the graphs (Fig. 3). The most cytotoxic compounds appeared to be 2-OH-5MeCR and 3-OH-B[b]PH[2,3-d]T.

DISCUSSION

In the present study, chrysene was not found to interact with the either ER isoform, though a previous study had reported an agonistic interaction with hERαdef (519). GC-MS analysis of the chrysene samples used in each study revealed that the CR sample used by Clemons *et al.* (1998) was contaminated with B[b]N[2,1-d]T, B[b]N[2,3-d]T, B[a]C, and B[c]C (Dr. B. McCarry, McMaster University, Hamilton, ON, Canada, unpublished data). These findings stimulated interest in determining possible interactions of the ER isoforms with benzonaphthothiophenes and benzocarbazoles, the results of which are discussed below.

The finding that nonhydroxylated compounds were unable to compete for ER binding but were able to induce significant ER-mediated responses agrees with previous studies concerning the estrogenicity of monohydroxylated B[a]P metabolites (536, 579). B[a]P was unable to compete for ER binding but caused a moderate level of gene

expression, comparable to that of B[a]A and B[b]F in the present study, while monohydroxylated derivatives of B[a]P were able to bind and induce gene expression to a much higher degree, with preference for ERβ. Isoform-specific interactions are of particular interest in light of recent information regarding the different roles of ERα and ERβ. For example, ERα has been shown to be critical for maintaining fertility and maternal behavior (501, 580), while ERβ may be more important in the maintenance of bone mineral density (581).

Given the general structural similarity of B[a]P to compounds in the present study, it is likely that the response seen in MCF-7 cells is due to metabolites produced by PAH-inducible cytochrome P450 isozymes (535), which are known to produce monohydroxylated and other metabolites of B[a]P in these cells (515, 536, 582). For example several PACs, including B[a]A, CR, B[b]F, benz[a]acridine, and 10-aza-B[a]P have been found to induce P450 activity following interaction with the Ah receptor, thereby inducing their own metabolism (542, 543, 575). Furthermore, B[a]A has also been shown to cause a decrease in nuclear ER levels suggestive of an antiestrogenic response (550).

In addition to the PACs examined in this study, other PAH and heterocyclic PAH compounds in environmental matrices share some structural similarity with the E2 steroid nucleus, including some furan derivatives and nitro- and amino-PAHs. While the metabolite profiles are likely to be similar to those of the isosteric PAHs, the heterocyclic compounds can also be oxidized at the heteroatom, which may alter the overall activity of the compound (583, 584). However, clear patterns of heteroatom influencing reporter gene expression levels were not observed in the present study. For example, CR is

structurally related to the S-containing B[b]N[2,1-d]T and N-containing B[a]C, but CR did not induce reporter gene activity and B[a]C exhibited little activity, while B[b]N[2,1-d]T caused a modest induction (10 μM caused induction to 40-50% of $E2_{max}$). By contrast, isosteric B[b]F and B[b]N[2,3-d]T exhibited comparable activity (the highest dose in each case causing 30-50% of $E2_{max}$ induction). Unfortunately, the isosteric N-containing PAH, benzo[b]carbazole, was not available for comparison.

For many of the heterocyclic compounds in the present study there is little or no metabolism data available. B[b]N[2,1-d]T is one of the best-studied, with seven monohydroxylated metabolites and two dihydrodiols, as well as oxidation at the heteroatom being reported using the S9 fraction of rat liver homogenate (585). The ratio of metabolites produced, as is true for PAHs, was found to be strongly affected by pretreatment with P450 inducers. Overall, the metabolite pattern produced from B[b]N[2,1-d]T was similar to that of benzo[b]fluorene, with the formation of phenolic metabolites primarily at the benzene ring and dihydrodiols in the naphthalene portion. A separate study on the metabolism of nine thiaarenes, including B[b]N[2,1-d]T and B[b]N[2,3-d]T, concluded that the position of the thiophene ring within the molecule strongly influences whether phenols, dihydrodiols, or S-oxidation products are predominantly formed (573). This is important because to date only monohydroxylated PAHs have been found to interact with the ER (531, 579).

A comparison of the metabolism of chrysene (586) and its isoster B[b]N[2,1-d]T (573, 585) shows that dihydrodiols are the major metabolites of CR, whereas B[b]N[2,1-d]T produces significant amounts of phenolic metabolites. This may explain why B[b]N[2,1-d]T, but not chrysene, is able to induce ER-mediated gene expression.

Similarly, the predominance of dihydrodiol metabolites formed from B[c]PH incubated with rat liver microsomes (574) is consistent with the present finding that B[c]PH shows only a weak reporter gene response in MCF-7 cells.

This is the first report of the agonistic interaction of several PAHs, heterocyclic PAHs, and their monohydroxy derivatives with the ER, though the antiestrogenicity of some B[a]C, benzo[b]thiophene, and benzo[b]furan derivatives has been explored (587, 588). Other investigators have reported antiestrogenic effects of some PAHs, including B[a]P, B[a]A, B[b]F, and 6-OH-CR, in other *in vitro* test systems (520, 521). Since all of these systems, including assays used in the present study, are highly artificial, extrapolation to specific *in vivo* responses is challenging. For example, hydroxylated B[a]P metabolites that were ER agonists *in vitro* were not found to affect mouse uterine weight and uterine lactoferrin mRNA expression, two E2-inducible endpoints (579). Instead, the present data suggest that several commonly occurring PAHs potentially can interact, positively or negatively, with ER signaling *in vivo*, likely in a cell-specific manner, and that other PACs are likely to act similarly. Therefore, due to the widespread and continuous emission of this large group of compounds into natural, domestic, and occupational environments, as well as their potential to bioaccumulate in lipid-rich tissues (574, 589, 590), concern regarding potential health effects of these weak estrogens is warranted.

Table 1. *In vitro* competitive binding IC_{50} and gene expression EC_{50} values for 17β-estradiol (E2) and test compounds.

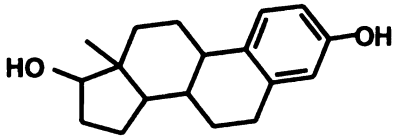
Compound	*GST-hERαdef IC_{50}*	*hERβ IC_{50}*	*Gal4-hERαdef EC_{50}*	*mERβdef EC_{50}*
E2	5.5 ± 1.2 nM	5.6 ± 1.1 nM	350 ± 240 pM	130 ± 24 pM
B[a]A	nb[a]	nb	wi[b]	wi
B[b]F	nb	nb	wi	wi
B[c]PH	nb	nb	ni[c]	ni
CR	nb	nb	ni	ni
B[b]N[2,1-d]T	nb	nb	wi	wi
B[b]N[2,3-d]T	nb	nb	wi	wi
B[a]C	nb	nb	wi	wi
B[c]C	nb	nb	wi	wi
2-OH-CR	95 ± 44 nM	42 ± 14 nM	wi	wi
2-OH-5MeCR	28 ± 12 nM	29 ± 5 nM	wi	63 ± 35 nM
8-OH-5MeCR	180 ± 26 nM	180 ± 32 nM	wi	320 ± 230 nM
2-OH-B[c]PH	250 ± 4 nM	180 ± 100 nM	wi	wi
3-OH-B[b]N[2,1-d]T	300 ± 74 nM	220 ± 82 nM	160 ± 110 nM	40 ± 22 nM
3-OH-B[b]PH[2,3-d]T	230 ± 10 nM	110 ± 38 nM	wi	wi

[a] nb denotes a non-binder, which is defined as a compound that, at 10 μM, causes <10% displacement of [^{3}H]E2

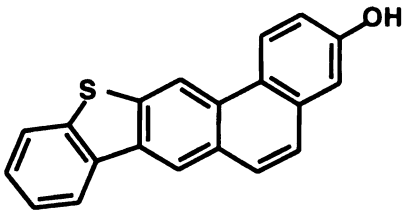
[b] wi denotes a compound that, at 10 μM, induces reporter gene expression at <50% of maximal induction caused by 10 nM E2

[c] ni denotes a compound that, at 10 μM, induces reporter gene expression at <10% of maximal induction caused by 10 nM E2

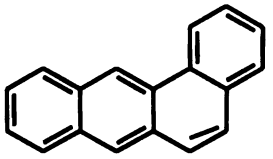
Figure 1. Chemical structures of select polycyclic aromatic compounds under study. Position numbers are labeled for compounds possessing functional groups.



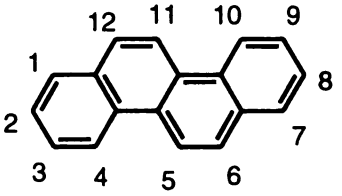
17β-Estradiol (E2)



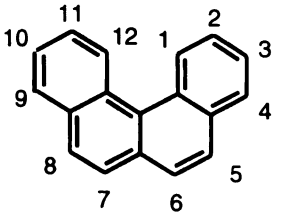
3-OH-Benzo[b]phenanthro[2,3-d]thiophene
(3-OH-B[b]N[2,3-d]T)



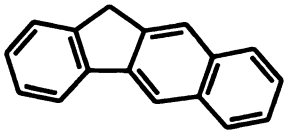
Benz[a]anthracene (B[a]A)



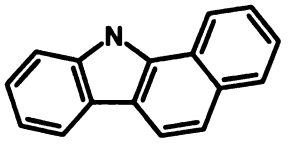
Chrysene (CR)



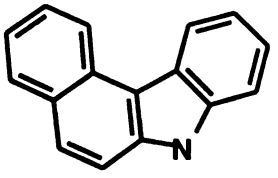
Benzo[c]phenanthrene
(B[c]PH)



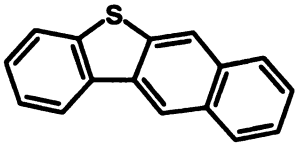
Benzo[b]fluorene (B[b]F)



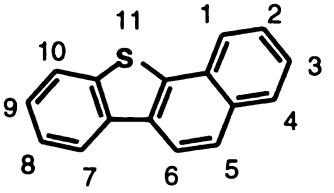
Benzo[a]carbazole (B[a]C)



Benzo[c]carbazole (B[c]C)



Benzo[b]naphtho[2,3-d]thiophene
(B[b]N[2,3-d]T)



Benzo[b]naphtho[2,1-d]thiophene
(B[b]N[2,1-d]T)

Figure 2. Competitive binding curves for (A) chrysene derivatives and (B) benzo[c]phenanthrene and benzothiophene derivatives displacing 2.5 nM [^{3}H]E2 from GST-hERαdef and hERβ.

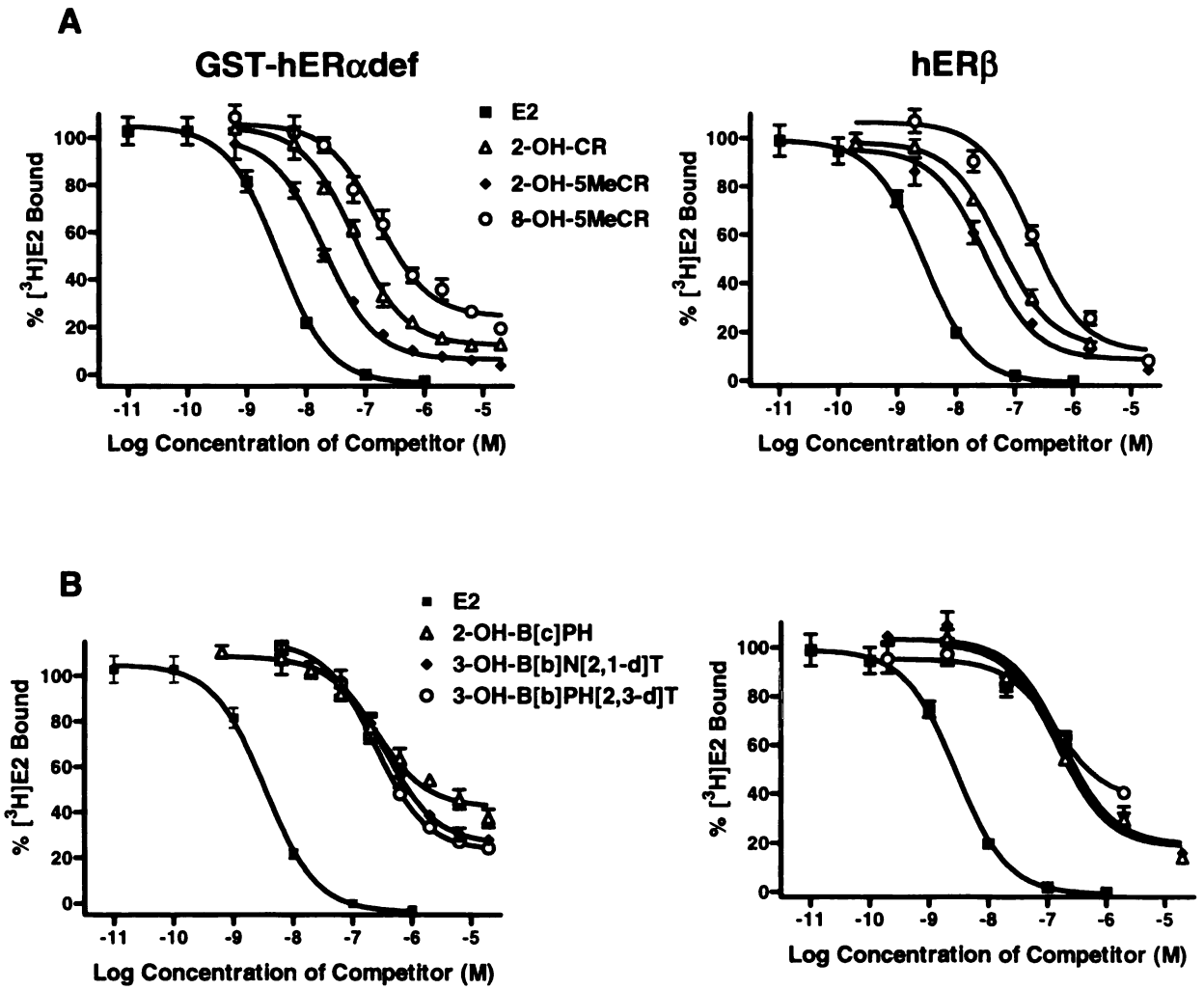
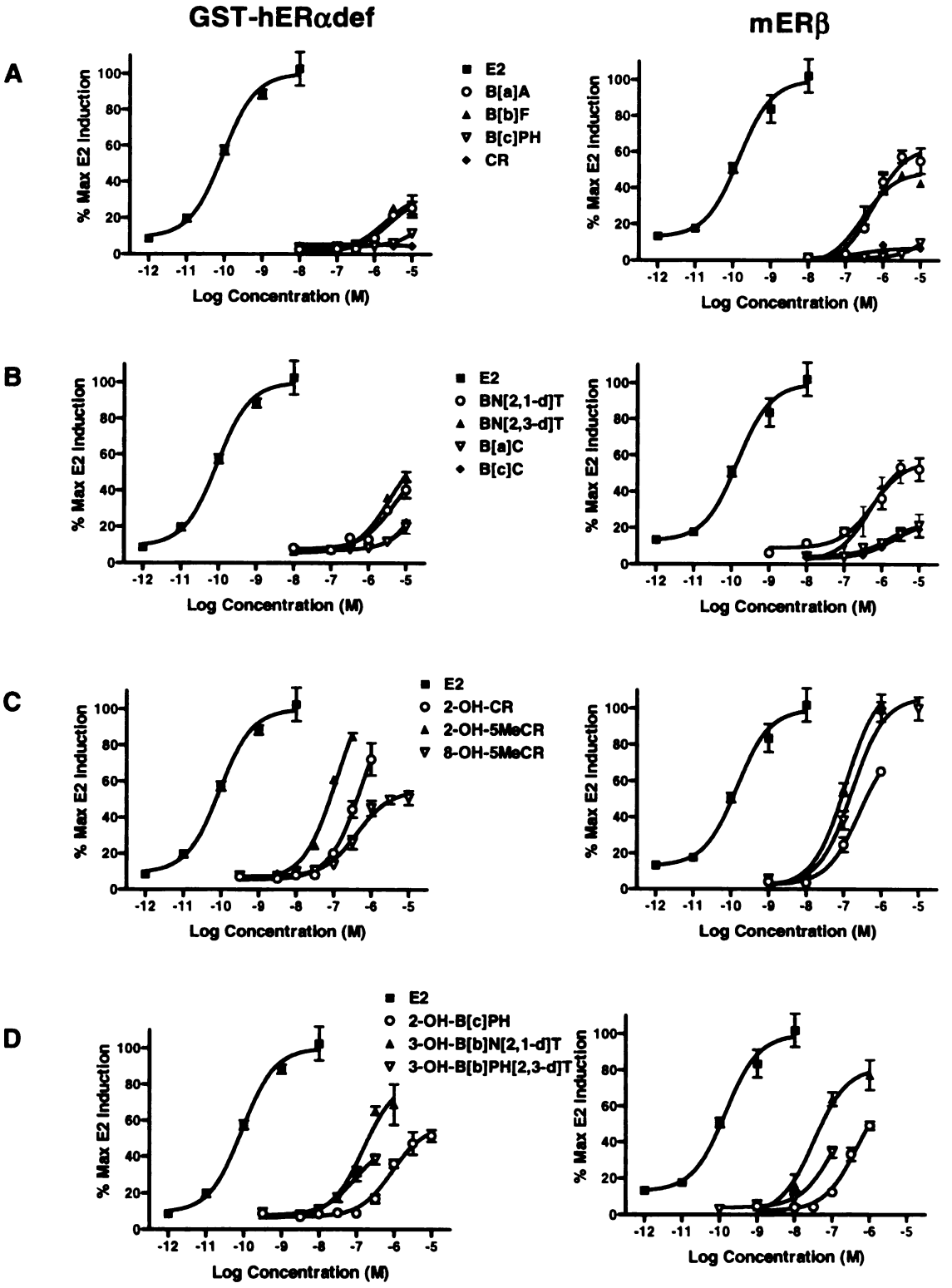


Figure 3. Induction of Gal4-hERαdef- and Gal4-mERβdef-mediated reporter gene expression in transiently transfected MCF-7 cells treated for 24 h with (A) PAHs, (B) heterocyclic PAHs, (C) chrysene derivatives, and (D) benzo[c]phenanthrene and benzothiophene derivatives.



CHAPTER 4

IDENTIFICATION OF TEMPORAL PATTERNS OF GENE EXPRESSION IN THE UTERI OF IMMATURE, OVARIECTOMIZED MICE FOLLOWING EXPOSURE TO ETHYNYL ESTRADIOL

ABSTRACT

The induction of uterine wet weight provides an excellent model to investigate global gene expression effects elicited by estrogens. In this study, time course microarray GeneChip data were analyzed using a novel approach to identify temporal changes in uterine gene expression following treatment of immature ovariectomized C57BL/6 mice with 0.1 mg/kg 17α-ethynyl estradiol. Functional gene annotation information from public databases facilitated the association of changes in gene expression with physiological outcomes, which allowed detailed mechanistic inferences to be drawn regarding cell cycle control and proliferation, transcription and translation, structural remodeling, and immunological responses. An *in silico* search for estrogen response element motifs within the genomic sequence of responsive probe sets provided complementary evidence of primary estrogen-regulated responses. These systematic approaches were able to confirm previously established responses, identify novel estrogen-regulated transcriptional effects, and disclose the coordinated activation of multiple modes of action that support the uterotrophic response elicited by estrogen. For example, it was possible to elucidate the physiological significance of the dramatic induction of arginase, a classical estrogenic response, by elucidating its mechanistic

relevance and delineating the role of arginine and ornithine utilization in the estrogen-stimulated induction of uterine wet weight.

INTRODUCTION

Although estrogens are among the most widely prescribed pharmacological agents (591), many aspects of their action following receptor binding remain unresolved. In the classic model, the estrogen 17β-estradiol (E2) binds to the estrogen receptor (ER), causing displacement of chaperone proteins. Dimers of the estrogen-ER complexes can then act as transcription factors by binding to specific estrogen response element (ERE) sequences in the promoters of target genes, evoking a wide range of transcriptional responses. Recent research has revealed a vast spectrum of roles for estrogen, which, in addition to important roles in female reproduction (592, 593), include regulation of bone growth and mineral density (594), food intake and weight gain (595), vascular function (390), immunological responses (596), various neurological roles (597, 598), and even in male reproductive capacities (599). These divergent actions are mediated through two ER isoforms, each with distinct differences in tissue distribution, coactivator and corepressor interactions, and resulting target responses (600-604). Furthermore, rapid nongenomic responses mediated by short-lived membrane ERs have been recently shown to stimulate a variety of signal transduction pathways (605, 606). In addition to endogenous estrogens and pharmacological agents, an increasing number of natural products, industrial chemicals, and environmental contaminants have also been identified as ligands for ERs, with varying affinities and activities (607-609), but the potential for many of these to cause beneficial or adverse health effects is poorly understood.

Despite these diverse functions, the uterus continues to be a preferred model for investigations aimed at elucidating the effects of estrogenic compounds. The timing of the changes in circulating estrogen levels is critical to the normal progression of the

menstrual cycle, referred to as the estrus cycle in rodents, and stimulates uterine events associated with implantation in the event of ovum fertilization. These changes are numerous, and include the stimulation of cell cycle progression, transcription and translation of specific proteins, cellular water intake (imbibition), vascularization, recruitment of immune cells, and modifications in cell and tissue architecture. The ERα isoform is the predominant isoform in the uterus, while ERβ is detected only at low levels (610). Studies in mice with targeted ER disruptions have confirmed that ERα is required for the early hyperemia and water imbibition responses induced by estrogen, as well as later stimulation of DNA synthesis, induction of specific estrogen-inducible transcripts, and uterine growth and morphogenesis (611).

The profound complexity of estrogen signaling underscores the limitations of current assays aimed at the detection and characterization of exogenous estrogens. Due to the major role of the estrogen-ER complex as a transcriptional activator and proliferative stimulus, current commonly used *in vitro* assays are limited and do not adequately address the consequences of ER binding and subsequent gene expression (612). Typical *in vivo* assays, by contrast, are based on the ability of estrogen to stimulate proliferation of the uterine cell populations, and often consist of the standard rodent uterotrophic assay, in which estrogen markedly induces uterine weight in an immature and/or ovariectomized animal that is typically treated daily for 3-4 days (613). Although this protocol has been used for decades (614), a lack of specificity to estrogens has been observed (615), despite the incorporation of complementary uterine endpoints (i.e. epithelial cell height and number, gland number) (553, 613, 616). Therefore, i*n vitro* tests have some clear advantages, however they underrepresent the sophisticated processes

intrinsic to an intact animal, and conversely *in vivo* studies offer enhanced ability to extrapolate responses to humans as well as to other animals of interest, but frequently focus only on a limited number of uterine-based endpoints (617).

In an attempt to address these weaknesses, several recent studies have examined uterine responses to estrogen using microarrays (122-125). However, the studies vary substantially in design and conditions used, as well as in the specific transcripts under consideration. Furthermore, since the dosing protocol for measuring increased uterine weight (i.e. several consecutive daily doses) may not be optimal for capturing transcriptional responses, a time course experiment examining estrogen response is desired. In the present study, a GeneChip oligonucleotide array experiment was performed using the standardized uterotrophic assay protocol (i.e. three consecutive daily doses) to determine the temporal effects on gene expression responses elicited by 17α-ethynyl estradiol (EE). EE is a pharmaceutical estrogen that has been shown to induce a transcriptional profile similar to that of the endogenous estrogen E2 (46), but has an ethynyl substitution that enhances its oral bioavailability (618). Rigorous statistical approaches were used to identify the most active treatment-induced gene expression responses while accounting for variability between replicates. Significant responses were clustered and then associated with functional annotations extracted from public databases to identify critical pathways involved in the uterotrophic response. In addition, corresponding gene sequences were searched *in silico* to identify possible estrogen response element motifs, providing indirect support for the role of ER in the elicited responses.

MATERIALS AND METHODS

Uterine time course of ethynyl estradiol treatment

C57BL/6 mice, ovariectomized by the vendor on postnatal day 20 and all having body weights within 10% of the average body weight, were obtained from Charles River Laboratories (Raleigh, NC) on postnatal day 26. The mice were kept in cages containing cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) in a 23°C HEPA-filtered environment with 30-40% humidity and a 12 h light/dark cycle. Animals were allowed free access to deionized water and Harlan Teklad 22/5 Rodent Diet 8640 (Madison, WI). Mice were acclimatized for four days prior to dosing. On the fourth day, animals were weighed, and 17α-ethynyl estradiol (Sigma Chemical Co., St. Louis, MO) was dissolved in sesame oil (Loriva, Ronkonkoma, NY) to achieve the desired concentration based on the average weight of the animals (13.6 ± 1.3 g). Animal groups for each dose and time point were housed in separate cages. Beginning the following day, each mouse was dosed by gavage with either a 0.1 ml dose of 0.1 mg/kg EE or with 0.1 ml sesame oil alone. An untreated animal was sacrificed at time zero, the time at which the other animals were dosed. Duplicate animals from both the treated and vehicle-treated groups were then sacrificed at 2, 8, 12, and 24 hr. Additional pairs of animals were treated for three consecutive days with 0.1 mg/kg/day EE or vehicle and then sacrificed 24hr following the final dose, and referred to as the 3x24hr dose time point. In total 21 animals were used. Doses were staggered to ensure that the time of exposure was within 5% of the reported dose length. Animals were sacrificed by cervical dislocation, and uteri were excised, separated from attached connective tissue, blotted, weighed, and stored in RNAlater storage solution (Ambion

Inc., Austin, TX) at -80°C until further use. All procedures were performed with the approval of the Michigan State University All-University Committee on Animal Use and Care.

RNA extraction, sample labeling and GeneChip hybridization

Uteri were homogenized individually as previously described (579), and total RNA was extracted with Trizol (Life Technologies, Rockville, MD) according to the manufacturer's instructions, with yields of approximately 3.0 μg RNA/mg uterine tissue. RNA was further purified using RNeasy spin columns (Qiagen, Valencia, CA) according to the manufacturer's instructions, and eluted in 20 μl water. RNA yield and purity were assessed by spectrophotometer and denaturing gel electrophoresis. Ten μg total RNA from each uterus was then separately reverse transcribed, then transcribed *in vitro* in the presence of biotinylated nucleotides, fragmented, and hybridized to either Test2 or Test3 GeneChip arrays (Affymetrix, Santa Clara, CA) to verify target quality, according to the manufacturer's protocols. The samples were then hybridized to Mu11KSubA oligonucleotide GeneChip arrays, which contain 6,523 probe sets of 25-mer oligonucletides directed at murine targets.

GeneChip data acquisition and clustering

Signal values and detection confidence levels were obtained using MicroArray Suite 5.0 (MAS5; Affymetrix) using the default settings. In accordance with MAS5 absolute call default parameters, a transcript was considered to be 'Present' (P; expressed) within a sample if the detection p-value for the corresponding probe set was below 0.04, and to be 'Marginal' (M) if the p-value fell between 0.04 and 0.06, with all

other observations being considered to be 'Absent' (A). Hierarchical and K-means clustering were performed using GeneSpring 4.2 (Silicon Genetics, Redwood City, CA).

Obtaining updated gene annotation information for probe set accession identifiers

A gene name for each probe set was obtained by using the GenBank accession identifier (GenBank ID) associated with the Affymetrix probe set identifier (probe set ID) to query a local copy of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) mouse UniGene database information (build 118), using a Perl script that returned and saved the corresponding cluster name (UniGene name), cluster identifier (UniGene ID), and gene abbreviation. mRNA RefSeq accession IDs were also saved where available. For GenBank IDs not found within UniGene, the probe set exemplar sequence was obtained from NetAffx (http://www.affymetrix.com), a BLAST search was performed against all other mouse sequences in GenBank, and a probable gene identity was assigned in cases where the five closest sequence matches all had an expect value of $e \leq 10^{-20}$ and were representative of a single UniGene cluster. Probe sets are referred to in the following text by their official gene abbreviation as obtained from NCBI.

RefSeq IDs were then used by another Perl script to query a local copy of the NCBI LocusLink database information, returning information of interest including LocusLink ID, RefSeq category, chromosomal location, Gene Ontology IDs and descriptions, and relevant NCBI PubMed references. The LocusLink ID of the NCBI-MGD human homolog was also noted from the mouse LocusLink record, which in turn provided a link to additional public functional information available in the Online

Mendelian Inheritance in Man (OMIM; NCBI) and GeneCards (Weizmann Institute) databases. For probe sets with a UniGene name but lacking a RefSeq ID, a modified version of the Perl script used the gene name to query the LocusLink database information, and records were inspected manually to verify that the desired record had been returned. As well, additional sequence and annotation information about each probe set was obtained from NetAffx. All Perl scripts are available upon request, in accordance with software availability guidelines proposed by the International Society for Computational Biology (http://www.iscb.org/pr.shtml#software).

Several cases also existed in which two different probe sets interrogated the same GenBank ID (referred to here as a probe set double). Microsoft Excel was used to identify probe set doubles and to determine the concordance of absolute calls on each array between probe set doubles and to examine links between these results and the probe set suffix flags (defined in Supplemental Table A; all supplemental tables are accessible at http://www.bch.msu.edu/~zacharet/data/kfdiss.html) of the probe set double members.

Raw GeneChip data is available in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) as time course experiment GSE280 and sample files GSM3870-90. Probe set IDs, gene names and abbreviations, K-means cluster assignments, fold change calculations, and other supplemental information is available in Supplemental Table B.

Quantitative RT-PCR

RNA was isolated from uteri obtained from two additional replicates of the time course experiment described above. Verification by SYBR Green quantitative real-time PCR (QRT-PCR) of each replicate used samples consisting of equal amounts of uterine

RNA pooled from five identically treated animals. Data for each time point were collected separately from each of the two pools and then averaged.

For each pool, total RNA was reverse transcribed using SuperScript II (Invitrogen, Carlsbad, CA) and an anchored oligo-dT primer as described by the manufacturer. Then 1.6 µl of cDNA was added to a 30 µl PCR reaction containing 0.1 µM each of forward and reverse gene-specific primer, 3 mM Mg^{2+}, 0.33 mM dNTPs, 0.5 IU AmpliTaq Gold, and 1x SYBR Green PCR buffer (Applied Biosystems, Foster City, CA). Primers (Table 1) were selected using Primer3 (619) to obtain an amplicon of approximately 125 bp length with T_m of 60°C. Amplicons were selected from within the Affymetrix exemplar sequence region, and were compared by BLAST to all other sequences in GenBank to ensure specificity. Default cycling parameters were employed, using an ABI7000 Prism Sequence Detection System (Applied Biosystems).

Response element search

NCBI RefSeq IDs were used to query the February 2002 mouse assembly of the University of California Santa Cruz (UCSC) Genome Browser Gateway (http://genome.ucsc.edu). Corresponding gene promoter and transcript sequences were downloaded, and Java applications (available on request) were developed to identify sequence motifs of interest. Each genomic sequence was searched for exact matches to the 13 bp perfect ERE motif (pERE; GGTCAnnnTGACC), to imperfect verified EREs with verified functionality (ivEREs; Supplemental Table C), and to other predicted single bp mismatch imperfect EREs (imEREs). In addition, potential ERE-Sp1 composite sites consisting of one of the two ERE half-sites (GGTCA or TGACC), and an Sp1 site (CCCGCC or GGCGGG) (1/2ERE-Sp1) were identified. A 1-50bp spacer was used in

order to limit the search in accordance with an analysis of spacers present in published ERE-Sp1 composite sites.

EREs can be functional over long distances from the gene promoter region either in the 5' promoter region (620) or the transcribed (621) or 3' untranscribed region (622), however no consensus exists regarding the appropriate distance range in either direction from the transcriptional start site (TSS) for ERE searching. Therefore, all perfect and imperfect EREs located within the 10,000 bp upstream of the TSS, as specified in the UCSC database, and within the transcribed region (i.e. exons, introns, and 3' untranslated region), were identified. In the case of 1/2ERE-Sp1 elements, the search included 1,200 bp in each direction from the location of the TSS, since an initial search of the -2,000 to +2,000 bp range showed a predominance of these sites in relatively close proximity to the TSS, in agreement with previously reported findings (28). Sequence matches were categorized by their location in the upstream region (UR), 5' untranslated region (5'UTR), coding sequence (CDS), or 3' untranslated region (3'UTR).

Statistical analyses

Uterine weights were assessed via a two-way ANOVA model to test for differences in weight both across treatments and across time. If the treatment-by-time interaction was found to be significant, the time effect relationship was tested at fixed levels of the treatment effect.

For the GeneChip data, an empirical Bayes model (623) modified to account for an experimental design with multiple comparative variables, was used as a screening tool to identify genes that have the highest probability of being regulated due to treatment and/or time. This approach reduces the dimensionality of the data to a single summary

statistic per gene while simultaneously accounting for multiple testing issues. The empirical Bayes model estimates the *a posteriori* probability of being active (called p1z) with respect to treatment and/or time for each gene. In other words, the p1z value is a single summary statistic for each probe set, in which a high p1z value is indicative of a high level of response (either induction or repression) of a probe set relative to the variability in the response. The false positive rate for a given rejection region was estimated using Benjamini and Hochberg's false discovery rate (FDR) (624), and was found to be 0.52% at using p1z>0.99 as the selected threshold.

An ANOVA model was then fit to the genes with p1z$\geq$ 0.99 and each response was classified as being active due to either a treatment effect, a time effect, or a treatment-by-time interaction. Responses with a significant ($p<0.01$) treatment-by-time interaction were considered to be of interest, as were gene responses with only a significant treatment effect. Conversely, gene responses with only a significant time effect (indicative of strong vehicle-dependent or circadian responses), or responses in which the treatment and the time effects were separately significant but their interaction was not, were rejected.

For the QRT-PCR data, β-actin-normalized responses were standardized to the average response at the vehicle 2hr time point for each gene to adjust for gene-specific differences in expression level. An ANOVA model was fit to the data using the logarithm transformed standardized response, and gene responses were also classified has having a treatment effect, a time effect, or a treatment-by-time interaction. In addition, the Pearson correlation between the temporal responses observed by QRT-PCR and microarray prior to time-matched normalization for each gene was calculated using Microsoft Excel. A

technical paper describing the details of these statistical methods is available at http://www.bch.msu.edu/~zacharet.

RESULTS

Uterine weights

Blotted uterine weight was induced approximately 4-fold after 3x24 hr exposure to EE, while no significant increase was observed at any other time. Comparable results were obtained when unblotted uterine weights were considered.

GeneChip summary statistics

The proportion of probe sets with an absolute call by MAS5 of either Present (P) or Marginal (M) was relatively uniform among the arrays (45.4 ± 7.9% and 2.8 ± 0.4%, respectively). In addition, 17.3% of probe sets were called P on all 21 arrays, while 28.9% were called Absent (A) on every array. Hierarchical clustering of all of the GeneChip data in GeneSpring using default settings indicated that the untreated sample, the 24 hr EE-treated samples, and the 3x24 hr EE-treated samples formed three distinct groups, while the 8 hr and 12 hr EE-treated samples together formed an additional group, and the 2 hr EE-treated samples could not be distinguished from the vehicle-treated samples (not shown).

A gene name and associated publicly available gene annotation were linked to each probe set where possible by submitting the GenBank ID for the probe set to the NCBI mouse UniGene database. In UniGene Build 118 (December 2002; Figure 1), 57.2% of the probe sets had an associated gene name, while 36.7% were denoted as ESTs in UniGene. The remaining 6.1% were no longer represented in the UniGene database,

but for those which were retained by subsequent screening steps, a tentative gene identity was assigned based on BLAST searching of the GenBank database. In some cases several probe sets were associated with the same UniGene identifier, and as a result, 6261 unique UniGene IDs were found to be represented on the GeneChip (i.e. 4.0% redundancy).

Assessing performance of pairs of probes interrogating the same GenBank accession

Following the GenBank ID, Affymetrix probe set IDs have an appended abbreviation (referred to here as a suffix flag, see Supplemental Table A) which indicates the selection rules used in determining the probe sequences comprising the probe set (625) and therefore can significantly affect data interpretation. Only 31.7% of the probe sets had no suffix flag ('none') and therefore were selected using the optimal set of rules. A majority (55.1%) belonged to the 's' (similar) suffix flag, in which the probe set interrogates a region shared by more than one transcript, while the remaining probe sets were mainly members of the 'f' (family; 6.3%) and 'g' (group; 5.8%) cases.

GeneChips often have GenBank IDs represented by multiple probe sets, with each of the multiple probe sets associated with a different suffix flag. On the Mu11KSubA array, 433 GenBank IDs are represented by two different probe sets (referred to here as probe set doubles), which facilitates a comparison of results obtained by different probe sets within doubles or triplets. All 'g' probes have a corresponding 'none' probe, and this comprises the majority of the probe set doubles (87.8%). The remaining pairs were 'f-i' (5.8%), 'i-s' (2.5%), 'r-s' (2.3%), 'f-r' (1.4%), and 'i-r' (0.2%).

Absolute calls were used to evaluate the performance of probe pair doubles, in which 'Present' (P) and 'Marginal' (M) calls were grouped together and then the absolute

call (i.e. either P/M or 'Absent' (A)) for each probe set within a double was compared across the 21 arrays. At the extremes, 29.1% of probe set doubles had at least 20 identical absolute calls, while 5.1% of doubles had only one or zero identical calls. Upon further examination, no association between the proportion of identical calls within the double and the specific pair of suffix flags within the double was evident (not shown). Interestingly in 22 cases, one probe set within the double was called P/M on all 21 arrays while the other probe set was called A on every array (i.e. all-P/M-all-A probe set doubles). In 16 of the 17 cases (94.1%) of a 'none-g' double, the 'none' array was all-A while the 'g' array was all-P/M. Similarly, for the 34 doubles in which there were between 16 and 19 differences in absolute call across the arrays, there were 20 doubles (58.9%) with a highly-A 'none' probe and a highly-P/M 'g' probe, but only two doubles (5.9%) with the reverse pattern. Overall, this suggests that the 'none' probe set, which was designed using the ideal probe set design rules, is not able to detect the presence of the corresponding transcript, while the permissive 'g' probe set interrogates a transcript which may be similar but not identical to that indicated by the probe set ID.

Cluster analysis – complete dataset

To initially describe temporal patterns of gene expression within the data, K-means clustering was performed on the entire dataset for the five time points using the average of the duplicate responses at each time point divided by the average of the corresponding duplicate vehicle responses (i.e. time-matched normalization). Eight K-means clusters, denoted clusters A-H, appeared to best describe the main temporal patterns in the data based on visual inspection (Table 2). Cluster H, the largest cluster, contained the least responsive probe sets, and had the lowest proportion of all-A probe

sets (17.8%). All clusters had a majority of probe sets corresponding to named genes in UniGene (53.6-59.5%), with the exception of cluster D (46.6%), which had a majority of probe sets being represented by ESTs.

For comparison, the gene expression patterns in each cluster were also viewed in a non-time-normalized form, in which the averages of the duplicate EE-treated responses were graphed separately from the averages of the duplicate vehicle-treated responses. Overall the patterns appeared as expected, with, for example, cluster A being composed mainly of genes that were relatively unchanged over time in the vehicle-treated samples while being highly induced at 2 hr in the EE-treated samples. Importantly, however, other responses were also seen, such as essentially flat responses with slight differences at one time point that were greatly amplified by the ratio calculation during the process of time-matched normalization.

Screening to identify most active gene responses

To eliminate negligible or confounding responses, a reduced dataset was desired in which probe sets with little or highly variable response were removed. The empirical Bayes approach was employed to objectively provide a single summary statistic (denoted the p1z value, ranging from 0 to 1) for each probe set, indicative of the degree of the response relative to its reproducibility. After retaining a subset of probe sets with p1z values greater than a threshold level of interest, and thus having low variability relative to their level of responsiveness, a subsequent ANOVA was performed to identify and remove responses likely due to vehicle or circadian effects.

At a chosen threshold of 0.99, 881 probe sets were retained, of which nearly 500 were associated with a p1z of 0.999, indicative of a robust response with low variability

between replicates. Of the 881 significant probe sets, 537 (62.2%) had a gene name in UniGene, a percentage slightly higher than the proportion of named probe sets represented on the array as a whole (57.2%), suggesting that probe sets found in the present experiment to be significantly regulated were not much more likely to be annotated than randomly selected probe sets on the array.

The second screening step, an ANOVA, was designed to identify probe set responses for which the treatment effect or the treatment-by-time interaction were significant ($p<0.01$), thereby eliminating vehicle or circadian effects for which only the time effect would be significant. Of the 392 that were considered to have a significant treatment effect or treatment-by-time interaction, 257 (63.0%) had an associated UniGene gene name (Figure 1), again indicating that the proportion of uncharacterized ESTs among the highly responsive final probe set list was no less than the proportion in the entire probe set population on the Mu11KSubA arrays. The complete list of 392 highly responsive probe sets is available in Supplemental Table B.

Quantitative RT-PCR

QRT-PCR was used to verify selected response patterns in uteri collected from experiments using the same protocol. Due to the limited sample size and the number of identified robust responses, it was not feasible to verify all affected genes, and a subset of 26 probe sets was randomly selected for verification that spanned the range of p1z values. Overall, while correlation between the GeneChip and QRT-PCR response was high when the response was high, correlation was low in cases where no response was evident, since values then tended to fluctuate without pattern by both methods. Furthermore, in a few cases no transcript was detectable by QRT-PCR, and in these cases no correlation value

was calculated. Therefore, responses by both methods were also compared by careful visual inspection, and a qualitative assessment was provided (Table 3).

Results for Arg1, Egr1, and for a sequence formerly classified in UniGene as isopentenyl-diphosphate delta-isomerase (Ippi; Table 3), all of which passed both GeneChip screening tests, were associated with significantly treatment-affected QRT-PCR results. The magnitude of induction of a classical estrogen induced response, Arg1, at the 3x24 hr treatment was strikingly similar for the GeneChip and QRT-PCR data (228- and 233-fold, respectively). Egr1 was induced 2-3.5-fold at 2-12 hr by both measures, while Iddi was induced up to7-fold at 8-3x24 hr by microarray but up to 12-fold at 8-24 hr as measured by QRT-PCR.

At the other extreme, Saa4, Fcgr1, and Krtap8-2 were all undetectable in every sample when measured by both techniques, and in agreement with this, no change was observed in the GeneChip intensity data that is calculated by MAS5 regardless of the Presence/Absence determination. Nxf1 was also absent or nearly absent in all samples, consistent with the microarray data. By contrast, only QRT-PCR was able to detect Tbx6, C2ta, and Crygs. A treatment effect at or near p=0.05 was observed for all three, corresponding to 8- and 25-fold EE-induced repression at 8-3x24 hr for Tbx6 and C2ta respectively, as well as an approximately 2-fold repression of Crygs, while these responses were muted or not evident in the GeneChip data. Mapk1 was also absent or nearly absent in all samples by microarray, whereas slight induction at 12 hr was measured by QRT-PCR.

Repression of Tgfb3, Lepr, Capn10, and Ltbr was found to be more pronounced in QRT-PCR measurements, and statistical correlation between the two measures was

moderate, although qualitatively there was good agreement between the platforms. Pdi2 responses for the two measures were highly correlated but more pronounced in the GeneChip data, with induction at 12-3x24 hr of 7-17-fold by microarray but 6-9-fold by QRT-PCR. Comparable results were observed with Sftpd and C3 induction at 3x24 hr. Induction of Farsl of approximately 2.5-fold at 8-12 hr was consistent and the response was highly correlated between the two methods, while Idb3 and Vegfb were similarly and slightly repressed at 8-24 hr. Ccnb1 was similarly induced approximately 2-fold at 24 and 3x24 hr, though one 12 hr sample was also highly induced as measured by QRT-PCR, and statistical correlation was low. Csf1, S100a6, Idh2, and Fkbp10 were detectable by both methods but not greatly affected by treatment. Overall, responses were qualitatively similar, while statistical differences appeared to be due to variation between the duplicate measurements in isolated cases, or to the higher sensitivity of QRT-PCR in detecting some transcripts.

Cluster analysis – reduced dataset

K-means clustering requires that the number of clusters be specified *a priori*. Visual inspection of two to fifteen K-means clusters indicated that the time-normalized data for the reduced list of probe sets could be best described using seven K-means clusters (Table 4 and Figure 2). Viewing the corresponding non-time-normalized data revealed, as expected, that overall these probe sets were very responsive to estrogen treatment but showed little response to vehicle alone. Attempts to further stratify the data into subclusters confirmed the clustering of only a single temporal gene expression pattern. While cluster J and cluster L both contained some responses that were induced at both 8 and 12 hr, seven K-means clusters categorized the observed temporal responses

more completely than did six K-means clusters. Clusters corresponding to late responses (i.e. clusters M, N, and O) had the highest proportion of probe sets with UniGene annotation information (72.0-78.6%; Table 4).

Responses not observed

Numerous responses were observed that agree well with published data, as described in the discussion. However, there were also several established estrogen regulated responses that were not observed in this study. These included Cdk5 (p1z=0.130) (208), Ccnd3 (p1z=0.997) (208, 626), estrogen-responsive finger protein (Trim25; p1z=0.999) (210), Rb1 (p1z=0.559) (627), and syndecan 3 (Sdc3; p1z=0.248) (355). In several cases, these responses in the present study were associated with low p1z values or were associated with a lack of significance for the treatment-by-time interaction ANOVA term, and in some cases, considered Absent by the MAS5 software. The lack of concordance between these results and published data may also be due to differences in experimental protocols, sensitivity of the methods and specificity of the probe sets, including the possibility that 's' and 'f' probe sets are interrogating transcript variants that were not detected in previous reports. As a result, it is expected that reporting the full probe set name, including the suffix flags in this study (Supplemental Table B), will facilitate and enhance the validity of comparing the results presented here with future studies. The well characterized estrogen inducible transcripts lactoferrin (540) and progesterone receptor (89) were not represented on the Mu11KSubA array.

Response element search

Genomic actions of estrogens can be mediated by the ER and response elements such as EREs, 1/2ERE-Sp1 composite sites, and AP1 and Sp1 sites. In order to provide supporting evidence for the role of ER/ERE-mediated gene regulation for genes affected by EE in this study, genomic sequence (i.e. 10,000 bp upstream of the TSS as well as the transcribed region) was searched *in silico* for pEREs, ivERE, imEREs and 1/2ERE-Sp1 motifs. Within the UCSC database, 8060 mouse RefSeq IDs were unambiguously associated with single chromosomal locations. Of the 236 unique probe sets which passed the empirical Bayes and ANOVA filtering criteria, 205 genes were associated with a RefSeq ID and a corresponding genomic sequence which was then computationally searched for EREs. EREs were identified in 162 of the 205 genes including 10 pEREs (2%), 110 ivEREs (44%), 111 imEREs (32%) and 64 half ERE/Sp1 composite sites (22%). As many as 12 EREs were found in one gene (the glycerol phosphate dehydrogenase Gdm1) while 57 genes contained one ERE, 39 had two EREs and the remaining 66 had more that two ERE sites. The complete set of results, including the locations of the matching sequences relative to the transcriptional start site, is available in Supplemental Tables D and E. However, when a similar number of 'unresponsive' probe sets (i.e. p1z<0.25 and >0.95 correlation to a flat response) were searched a comparable number of EREs were identified, indicating an inability to identify functional EREs based only on GeneChip and *in silico* approaches.

DISCUSSION

Uterine weight induction

The marked induction of rodent uterine hypertrophy and hyperplasia following a 3x24 hr estrogen dose is well documented with a variety of estrogens and routes of exposure (31). An earlier and more modest induction of uterine weight due to water imbibition has also been reported at 4-6 hr after estrogen exposure, but is not required for the pronounced late phase uterine weight induction (628), and was not assessed in the present study.

Two-step screening method

In comprehensive resource-intensive microarray experiments, it is desirable to maximize the number of dose levels or durations examined, while ensuring sufficient replication to enable meaningful statistical analysis that accounts for biological and technical variability. In the present study, a modified empirical Bayes screening approach (623) was used to identify a subset of active responses, followed by an ANOVA to remove vehicle-induced and circadian effects. This approach reduces the data to single summary statistic (p1z value) for each probe set that is indicative of the overall level of the response relative to its variability, and is comparable to a signal-to-noise ratio calculation. Consequently the p1z value represents the responsiveness and reproducibility of a change in gene expression over time. In the current study, p1z values were used to rank responsive genes in order to reduce the dataset to a smaller subset that would serve as the focus for detailed results interpretation. A threshold value of p1z>0.99 was initially chosen to identify a subset of genes for further functional investigation, resulting in the

selection of approximately 15% of the probe set results. The false discovery rate (FDR), or false positive rate, was calculated and reported in order to address multiple testing issues, and indicated that approximately 0.5% of probe sets passing the threshold, or approximately 4 of the nearly 900 responses, may falsely be classified as a significant response when a threshold of p1z>0.99 was used. The ANOVA was subsequently applied to identify treatment-induced responses while removing vehicle or circadian effects on gene expression. Multiple testing was not taken into consideration in the ANOVA, and therefore a stringent threshold of $p<0.01$ was used. This two-step approach can be readily modified to accommodate a variety of experimental designs, and provides additional flexibility by allowing the user to specify separate thresholds at each screening step.

Specific responses

Responses passing both screening steps were annotated using public databases with functional information for the mouse gene and the human ortholog, where available. Although responses that failed the screening steps were not clustered, certain responses were included in the discussion if they approached the selection criteria and contributed mechanistic information consistent with the EE-induced uterotrophic effect. In addition, estrogen regulation of some transcripts has been reported in other studies, and while the specific design employed (species, strain, age, route of exposure, dose level, type of estrogen and platform used,) may influence the reported results, concordance is seen in many cases, and therefore their inclusion is warranted (122-125).

Cell cycle

It is well established that estrogen treatment of rodents with low circulating estrogen levels (due to sexual immaturity or ovariectomy) causes a synchronized stimulation of uterine cell proliferation (629). Specifically, estrogen exposure causes quiescent (G_0) cells to enter the G_1 growth phase, followed by DNA synthesis (S phase), a second phase of growth (G_2), and finally mitosis (M), after which the daughter cells start a new G_1 phase. Though many cell cycle-related changes are non-transcriptional events, others are wholly or partially regulated at the mRNA level, and are the object of consideration in the present study.

Several of the gene responses that passed the two step screening process serve important roles in cell cycle control (Supplemental Table F). Transcripts upregulated early (2-12 hr) tended to correspond to proteins with essential roles in the G_1/S phase transition, while those directly involved in DNA synthesis or in the G_2/M or M/ G_1 transitions were upregulated at 24 hr and possibly sustained after the 3x24 hr treatment. This is consistent with the finding that in mice injected with E2, only 3% of cells were in S phase at the time of dosing while 75% had progressed to S phase by 15 hr after treatment (629). Moreover the rate of DNA synthesis in immature rats was also found to be highest 24 hr following exposure to E2 (142, 630). Few responses involved in cell cycle control or DNA synthesis were regulated as rapidly as 2 hr (i.e. were members of cluster I) or after maximal uterine proliferation was achieved at 3x24 hr (i.e. were members of cluster O).

Gadd45a (cluster I) induction is an early cell cycle response, providing a crucial link between the p53-dependent cell cycle checkpoint and DNA repair. Its levels are

known to be highest in the G_1 phase of the cell cycle, with a sharp drop during S phase. Gadd45a was also reported to be upregulated following estrogen treatment in other rodent studies (122, 123, 125). Similarly, cyclin D2 (Ccnd2; cluster J), which was upregulated in the G_1/S transition, and is highly induced at both the mRNA and protein level in the early proliferative stage of the human endometrium (631). A time course study has also shown that cyclin D2 is highly induced at 2-8 hr after estrogen injection and returns to baseline levels by 12 hr after exposure (208). As well, the cdc2 activator cyclin B1 (Ccnb1; p1z=0.964), which was previously reported to be upregulated at 16-24 hr as a downstream effect of E2 treatment in rats and is known to be highly upregulated during G_2/M (632), was found here to be upregulated at 24 and 3x24 hr respectively. The Cdc2a transcript itself (cluster N) was found to be upregulated 4.5-fold at the later time points, though it has been reported not to be regulated by estrogen at the protein level (633). Moreover, cyclin G2 (Ccng2; p1z=0.999), which is upregulated in various cell types in late S phase (634), was found to be repressed as much as 10-fold at all treatments between 8-3x24 hr. To our knowledge the other cell cycle-related transcripts listed in Supplemental Table F have not been previously reported to be affected by estrogen.

Chromosome replication is an integral part of the cell cycle, and several of the EE-regulated genes are known to be involved in DNA synthesis. For example, Nme1 (cluster L), a nucleoside diphosphate kinase important in dNTP synthesis, was upregulated 2.5-fold at 8-12 after treatment, consistent with another report (123). The two subunits of ribonucleotide reductase, Rrm1 (cluster K) and Rrm2 (cluster M), were induced at 24 and 3x24 hr, consistent with findings that ribonucleotide reductase is essential for deoxyribonucleotide production prior to DNA synthesis while it is

undetectable in quiescent cells (635). Ribonucleotide reductase is one of the substrates for thioredoxin, which is implicated in many processes including cell growth and uterine function (636), and a thioredoxin-like transcript, Txnl2 (cluster J), was upregulated at 8-12 hr. The activity of thymidine kinase Tk1(cluster M), for which the transcript was found to be upregulated approximately 14- and 4-fold at 24 and 3x24 hr respectively, is well known to be associated with proliferating cells, and serves as a marker for the onset of S phase (132). Tk1 transcript has been reported to be induced at 18-24 hr (626), and at 3x24 hr (125) in other studies, and pronounced induction of enzyme activity from newly synthesized enzyme has been observed at 24-36 hr after estrogen treatment (131, 132). In untreated cycling rats, thymidine kinase enzyme activity is highest in the proestrus stage of the estrus cycle, coincident with the peak in glandular mitosis (138).

Several protooncogenes show rapid and well-characterized estrogen inducibility in the uterus but were not represented on the array, including Fos and several Jun and Myc family members (reviewed in (637)). Myb (cluster N), however, is known to be upregulated at the G_1/S phase transition during cell proliferation (638), and in the present study was upregulated nearly 11-fold at 3x24 hr. In addition, the Myb binding protein Mybbp1a (cluster J) was also upregulated at 8-12 hr, possibly to assist Myb in its actions, which may include a critical role in cell proliferation and development.

Several genes involved in apoptosis were also found to be transcriptionally regulated following EE treatment. These include the early growth response factor Egr1 (cluster J), which was highly upregulated at 2-12 hr, peaking at 8 hr, and repressed below control levels at 3x24 hr. Egr1 has been implicated in growth suppression and induction of apoptosis, and is down regulated in tumor cells. Uterine induction of Egr1 transcript

has been reported at 2 hr in rats (639), and at 6 hr in mice (123). Upregulation of a probe sequence with high similarity (96% identity; expect value e^{-175}) to rat Egr1 in gestationally exposed fetal rats has also been reported (122). As well, the transcription factor Klf4 (cluster L), a suppressor of cell growth that can be activated by p53 to in turn induce p21, was upregulated at all time points. In the present study induction of p53 (Trp53; plz=0.678) was equivocal, while p21 (Cdkn1a; plz=0.980) appeared to be induced up to 9-fold at 2-12 hr. Furthermore p21 was considered to be Absent on 19/21 arrays, in agreement with a report in which it was undetectable in the uteri of E2-injected mice (640). Other transcripts involved in apoptosis included the upregulation of lymphotoxin B receptor (Ltbr; cluster J) at 8-12 hr which can trigger apoptosis following activation, Pea15 (cluster K), which was repressed 2-fold at all time points and is associated with anti-apoptotic function and overexpression in breast cancer cells, and Tia1 (cluster L), which was induced 2-3-fold at 2-24 hr and is thought to be involved in the induction of apoptosis. The upregulation of these proapoptotic genes at 2-24 hr after estrogen exposure may contribute to the attenuation of cell proliferation as maximal growth is achieved, similar to the apoptotic phase that follows endometrial proliferation in the cycling uterus (440, 641). However, the possible significance of apparent, though below threshold, repression of other apoptotic inducers during the same period, such as Bcl2-like 11 (Bcl2l11; plz=0.807; also known as Bim), caspase 2 (Casp2; plz=0.984), caspase 6 (Casp6; plz=0.722), and Bok (plz=0.919) is not known, but possibly indicating complex interactions between cell types within the proliferated uterus.

RNA and protein synthesis and modification

The overall rate of uterine transcription increases 6-8 hr after estrogen exposure (642, 643). In the present study, several transcripts with roles in RNA synthesis were found to be altered significantly following estrogen treatment (Supplemental Table G), few of which have been previously reported to our knowledge. For example, several novel induced responses were identified in cluster J (8-12 hr), including upregulation of arginine methyltransferase (Hrmt1l2), which specifically methylates H4 histones and may be essential to pre-mRNA processing (644). Also observed were upregulation of small nuclear ribonucleoprotein Snrnpa, which functions as a spliceosome component to excise introns from pre-mRNA, EBNA1 binding protein Ebp2, which plays a role in pre-rRNA processing, and poly(A) binding protein Pabpn1, which is required for efficient poly(A) tail formation and control of tail length. Paf53 (p1z=0.973), an activating subunit of RNA polymerase I, was induced at 8-12 hr, consistent with published results (123).

Many genes involved in amino acid synthesis and transfer were also within cluster J. Cysteinyl-tRNA synthetase (Cars), alanyl-tRNA synthetase (Aars), and asparagine synthetase (Asns) all exhibited comparable patterns of induction at 8-12 hr, while asparaginyl-tRNA synthetase (Nars; cluster L) showed an expression pattern that was similar but still elevated at 24 hr. Phenylalanine-tRNA synthetase (Farsl, p1z=0.678), seryl-tRNA synthetase 1 (Sars1; p1z=0.248), and arginyl-tRNA synthetase (Rars; p1z=0.846) transcripts displayed nearly identical patterns of induction at 8-12 hr but with greater variability. No response was observed for a probe set with similarity to aspartyl-tRNA synthetase (Dars; p1z=0.938), or for seryl-tRNA synthetase 2 (Sars2; p1z=0.504 and Absent on all 21 arrays), tyrosyl-tRNA synthetase (Yars; p1z=0.900 and absent on

20/21 arrays), or lysyl-tRNA synthetase (Kars; plz=0.131 and absent on 20 of 21 arrays). EE induction of histidyl-, glutamyl-, lysyl-, phenylalanine-like-, and seryl-tRNA synthetases as well as asparagine synthetase at 6 hr were also reported previously (123), while the activity of tRNA synthetases was reported to be upregulated as early as 1-2 hr (177). In addition, the dehydrogenase Mthfd2 (cluster M), was upregulated at 8-24 hr in the present study, and reported to be induced at 6 hr (123), which may provide the formyltetrahydrofolate for formylmethionyl-tRNA synthesis.

Protein synthesis genes, including the translation initiation factors Eif2a, Eif2s2, Eif4a1, and Itgb4bp (also known as eIF6) were also induced at 8-12 hr (cluster J), while Eif1a and Eif2b1 were classified to the closely related cluster L. Several chaperonin transcripts, which code for proteins that aid in the correct folding and assembly of nascent proteins, were also upregulated at the intermediate time points. These included the heat shock proteins Hspa8 (cluster J), induced in the rat uterus 4 hr after treatment with 30 μg/kg EE (183), as well as Hspa4 (cluster L) and Hspa5 (also known as BiP; cluster K), which is upregulated as a primary transcriptional response in mice at 1-24 hr after exposure (645).

Several genes involved in protein modification or degradation were also induced. Peptidyl arginine deiminase Pdi2 (cluster M), which converts arginine to citrulline residues in proteins, was upregulated 17- and 10-fold during the 24 and 3x24 hr time points respectively, while the transglutaminase Tgm2 (cluster N), which is involved in linking proteins through peptide bond formation and is also implicated in the packaging of apoptotic bodies during apoptosis, was upregulated 6-fold at 3x24 hr. Protease inhibitors such as cystatin B (Cstb; cluster J), an inhibitor of the cathepsin family of thiol

proteases, were also upregulated. Cystatin B is thought to be involved in the prevention of apoptosis, and is upregulated after 3x24 hr exposure to E2 (124). Interestingly one of its target cathepsins (Ctsb; cluster L) was also upregulated 2-fold at 8-12 hr as well as at 3x24 hr. Meanwhile Spi1-3 and Spi1-5 (cluster O), which belong to a family of serine protease inhibitors with similarity to α1-antitrypsin, exhibited significant upregulation at 3x24 hr. Several proteasome subunits were also members of the related clusters J and L based on their induction at 8-12 hr, including Psmb3, Psmd2, Psma3, Psmb6, and Psmb7.

The matrix metalloproteinase Mmp7 (cluster O) was induced 26-fold at 3x24 hr, and contains numerous (how many?) estrogen response motifs within its genomic sequence. Mmp7 degrades proteoglycans, fibronectin, elastin, casein, and gelatins. It is upregulated in processes involved in the breakdown of extracellular matrix, such as during reproduction and tissue remodeling. The importance of numerous MMP family members in the context of structural changes in the cycling human endometrium has been well described (646). Other members of the MMP family including 2, 12, 14, 15, and 24 were unaffected by EE treatment. None of the tissue inhibitor of metalloproteases (TIMP) isoforms were represented on the array.

Immune and complement activation

Early uterine responses to estrogen exhibit traits characteristic of a classical inflammatory response which can be blocked by anti-inflammatory steroids (647). These include edema, enhanced vascular permeability, eosinophil infiltration, and subsequent production of inflammatory mediators (648). Eosinophil infiltration is also greatly stimulated at estrus, with the eosinophils undergoing degranulation at the time of ovulation, and releasing large quantities of eosinophil peroxidase that are characteristic

following estrogen exposure (649, 650). The exact role of the recruited eosinophils is not known. They are not required for induction of uterine wet weight, protein synthesis, or epithelial complement C3 synthesis, although it is believed that they may play a role in endometrial stromal remodeling (445).

The process of menstruation has also been similarly compared to an inflammatory process (651). It is thought that the decline in progesterone levels, rather than direct action of estrogen, may stimulate a cascade of events including the release of uterine cytokines, attraction of eosinophils, release of the degradative MMPs, vasoconstriction, and hypoxia. Hypoxia in turn induces mediators such as vascular endothelial growth factor (Vegf), which is angiogenic and may promote edema by enhancing vascular permeability (648). However, the 15- to 20-fold increase in uterine neutrophil and macrophage content in the uterus of the ovariectomized mouse in response to 4x24 hr E2, independent of progesterone or its receptor, confirms that estrogen also plays a critical role (82). Moreover, the chemokine responsible for estrogen-induced eosinophil infiltration was recently identified as eotaxin (446).

The chemokine orphan receptor Cmkor1 (cluster I) was highly induced at 2 hr, with sustained induction at 8-12 hr (Supplemental Table H), consistent with a previous report of upregulation at 6 hr (123). In addition, two members of the suppressor of cytokine signaling (SOCS) family were highly induced, Socs3 (cluster I) at 2-8 hr, and Socs1 (cluster J) at 2-24 hr. In the interleukin family, Il25 (cluster L) was induced at 8-24 hr, while Il17 (cluster M) appeared to be induced 5-fold at 24 hr, but the signal intensities were low and the probe set was classified as Absent on all 21 arrays. The interleukin-4 receptor Il4ra (p1z=0.999) was upregulated at 24 and 3x24 hr, while previously reported

to be upregulated following 4-8 hr of exposure to EE (183). In addition, the monocyte chemotactic protein Ccl2, also known as MCP1 or JE (cluster L), was highly induced at all time points, similar to a previous report (158). A probe set highly similar to macrophage migration inhibitory factor (Mif; cluster L), a lymphokine that stimulates immune cell activation and cytokine production, was induced in the present study at 8-3x24 hr, consistent with a previous report (125), while the protooncogene tyrosine kinase Lyn (cluster N), which was induced at 24 hr and 3x24 hr, is required in immunoglobulin-mediated signaling. In addition, the well-characterized estrogen-inducible transcript for polymeric immunoglobulin receptor (Pigr; p1z=0.952), formerly referred to as secretory component (451, 652), was found to be induced at 3x24 hr.

Complement component factor H2-Bf (cluster N), a precursor also known as Factor B in the signaling pathway that stimulates the proliferation of activated B-cells, was induced at the later time points. H2-Fb is expressed at high levels in the luteal phase of the uterine cycle in humans and rodents (653). An active cleavage product of H2-Fb associates with complement C3 degradation product C3b to form the C3 convertase of the alternative complement pathway. However, complement component factor Cfi (cluster O), which inactivates complement components including C3b, was also upregulated 23-fold at 3x24 hr. Complement component C3 itself, though associated with a low p1z value (p1z=0.131) due to variability in its response, was upregulated 5 to 9-fold at 3x24 hr. C3 is a well characterized estrogen-inducible transcript and protein (183, 654) regulated by EREs in the human and mouse upstream regulatory region (Supplemental Table C) (462, 655). C3 is involved in phagocytic and immunoregulatory processes, but the significance of the concurrent upregulation here of C3 itself as well as

both an activator (H2-Fb) and an inactivator (Cfi) of C3 activity is confounding. Furthermore, the antigen Cd59a, or protectin (cluster I), which inhibits the terminal step of the complement activation cascade and is expressed on many types of immune cells, as well as the complement component C1r (cluster I), which is involved in activation of the first step of the classical complement system, were significantly repressed at 8-3x24 hr following estrogen exposure. Overall, the complex and concurrent transcriptional regulation of several members of the alternative and classical complement pathways in the uterine response to estrogen is readily detected but poorly understood and requires further examination to determine if changes in gene expression translate into changes in protein levels and in situ studies to identify the cell types involved.

Other responses

Several genes representing a multitude of pathways and functions further illustrate the complex and diverse transcriptional effects of estrogens on the uterus. In general, all of these responses are consistent with the physiology that is experienced by the uterus. For example several genes involved in ion transport, which is critical to uterine fluid balance and therefore to the physiological response to estrogen, were upregulated following estrogen exposure (Supplemental Table I). The amiloride binding protein Abp1, or diamine oxidase (cluster O), is known to both bind the diuretic amiloride which is involved in sodium channel closure and to catalyze the degradation of polyamines, and is thereby implicated in numerous responses including cell proliferation, tissue differentiation, tumor formation, and possibly apoptosis. Abp1 was induced over 100-fold at 3x24 hr, suggesting its importance in the estrogen response, although confirmation would require assessing the effects at the protein level. As well, the cystic fibrosis

transmembrane conductance regulator (Cftr; plz=0.812), a cAMP-activated chloride channel, was upregulated 7-fold at 3x24 hr. An important role of Cftr and the epithelial sodium channel (ENaC, or Scnn1; not represented on the Mu11KSubA array) isoforms in the regulation of uterine chloride and sodium balance, respectively, has been recently clarified (426), and E2-induced upregulation of Cftr mRNA has been reported in the rat uterus (427). Furthermore, the ion channel Fxyd5 (cluster J), was induced 3-fold at 8 hr, and though its ion transport capacity has not been well characterized, it has been recently identified as being the homolog of human dysadherin, which inhibits the function of E-cadherin (Cdh1; not represented on the array), an important component of epithelial cell adhesion (656). Interestingly the mucin Muc1 (cluster N), another inhibitor of E-cadherin, increased steadily beginning at 8 hr to greater than 7-fold at 3x24 hr. Muc1 has been described as an anti-adhesion molecule induced by estrogen through an ERE-independent mechanism (367), and has been shown to be important in the structure and function of the uterine luminal epithelial layer (361).

The hypoxia-inducible factor Hif1a (cluster K), which is implicated in angiogenesis, apoptosis, and energy metabolism, was upregulated at 2-8 hr, in agreement with a previous report of upregulation at approximately 6-12 hr (657), while the poorly characterized hypoxia-induced gene Hig1 (cluster K) was also upregulated at 8 hr. The regulation of Hif1a in the uterus is not well understood, but a direct role of hypoxia in inducing Hif1a in the estrogen-stimulated uterus has been questioned (657). By contrast, the rapid induction of the ERE-containing (Supplemental Table C) vascular endothelial growth factor, Vegf, in the uterus by estrogen is well characterized (621, 657), though in the present study Vegfa was highly induced at 2 hr in only one of the two samples,

resulting in high variability (p1z=0.052). The critical role of Vegf in the induction of vascular permeability has been established, and furthermore Vegf inhibition has been shown to abrogate the estrogen-induced uterine edema response (422). In addition, protein Sα (Pros1; cluster I), which inhibits blood clotting, and the tissue plasminogen activator Plat (Plat; cluster J), a serine protease that activates the fibrinolytic enzyme plasmin, were both highly repressed beyond 8 hr. No change was observed in the plasminogen activator inhibitor known as Pai1 or Serpine1 (p1z=0.846), while probe sets for two other transcripts with important roles in the cycling uterine endometrium, the urokinase plasminogen activator Plau and the potent vasoconstricting endothelin Edn1 (646), were not represented on the array.

The dramatic changes undergone in the rapidly proliferating uterus are associated with an extensive energy requirement. Responses observed included the late upregulation of the carbonic anhydrase Car2 (cluster M), which is involved in many biological processes due to its role in the reversible hydration of carbon dioxide. The creatine kinase Ckb (cluster J), which has well-characterized rapid estrogen inducibility (316) and plays an important role in generating ATP in tissues with high and fluctuating energy demands, was also upregulated at 8 hr in the present study, consistent with other studies in immature ovariectomized rats (46), and in estrogen-exposed fetal rat uteri (122). In addition, two probe sets representing an important ATP transporter known as Slc25a5 or ANT2 were shown to be induced 3-fold at 8-3x24 hr (cluster L), which may also facilitate the supply of ATP needed for uterine growth (125, 658).

Other responses were observed for which their roles in the uterotrophic response are not well understood. For example alkaline phosphatase (Akp2; cluster O), a well-

characterized estrogen-responsive gene (659) for which the exact physiological role is unknown, was upregulated 86-fold at 3x24 hr. Also galanin (Gal; cluster J), which regulates growth hormone and insulin release, was upregulated 11-fold at 8 hr, and the retinoic acid binding protein Crabp2 (cluster N), normally induced by retinoic acid but also reportedly induced by estrogen in rats after 24-48 hr (111) was upregulated at 24 hr and 3x24 hr. The angiotensin II receptor Agtr2 (cluster N), which is the isoform implicated in the induction of apoptosis (660) and is induced by estrogen during the human menstrual cycle (661, 662), was also found to be induced at 3x24 hr in the present study. In addition, the importance in cell cycle progression and the direct inducibility by estrogen of heparin-binding epidermal growth factor-like growth factor (Hegfl; plz=0.846) has been reported at 2-4 hr as well as at 24 hr, (663). In the present study Hegfl was upregulated nearly 6-fold at 8 hr, though variability between replicates at 2 hr likely prevented it from passing the plz screening step. In addition, the ras-related gene Rasd1 (cluster I), which is implicated in altering cell morphology, growth, and interactions between the extracellular matrix and the cell, as well as in nitric oxide signaling, was upregulated 5-fold at 2 hr.

Several diverse responses were also repressed, such as the guanine nucleotide exchange factor Arhgef3 (cluster I), and the receptor activity modifying protein Ramp1 (cluster I) involved in the calcitonin receptor-like receptor signaling pathway which were both repressed after 2 hr. Secreted frizzled-related protein Sfrp2 (cluster I) was also downregulated, and though its function is poorly defined, it appears to function as a Wnt receptor, and may have a role in apoptosis (645). In addition, insulin-like growth factor

receptor Igf2r (cluster N), which functions as a suppressor of cell growth, was repressed at 8-12 hr, and similarly was repressed in mice (123).

A proposed model

When the information presented above is integrated with results from previous studies, compelling evidence suggests the convergence of multiple pathways regulated by estrogens that support the uterotrophic response. Although not fully elucidated, Figure 3 provides a model depicting the estrogen regulation of arginine and ornithine utilization and their roles in the promotion and subsequent mitigation of uterine proliferation.

In this model, the Myc/Max heterodimer induces ornithine decarboxylase expression (Odc; p1z=0.999 and ANOVA p-values near significance threshold) (664, 665), elevating Odc transcript levels at 8-24 hr, a response previously demonstrated in the estrogen-stimulated uterus (627, 666, 667). Odc catalyzes the first and rate limiting step in the tightly regulated synthesis of polyamines, proline, creatine, and GABA (668), which are involved in mitotic spindle formation and chromatin condensation, and plays an essential role in cell proliferation and differentiation (669). Meanwhile the Myc/Max inhibitor Mxi1 (311) (p1z=0.999, and near significance in the ANOVA screening) is concurrently repressed 10-fold to nearly undetectable levels at 8-24 hr. Further, the Odc antizyme (670) Oaz2 (p1z=0.937) is consistently repressed 2-fold from 2-3x24 hr, while transcript levels of the Oaz inhibitor (671) Oazi (p1z=0.242) are nearly undetectable. These conditions (i.e. repression of inhibitors of Odc expression) provide an environment in which Odc transcription is favored, and polyamine synthesis progresses during G_1 (309). Meanwhile, the key multifunctional substrate arginine is being used in protein synthesis by arginyl-tRNA synthetase (Rars). As well, nitric oxide, the potent and

multifunctional signaling molecule formed from arginine by nitric oxide synthase (Nos) is known to be elevated in mice after 24 hr estrogen exposure. This has been shown to be due to induction of Nos3 (672), which was not represented on the microarray, but which was found by QRT-PCR to be induced 2-fold at 2-8 hr (not shown). Though the roles of nitric oxide in uterine function are complex, its critical importance has been established, since a Nos inhibitor prevented estrogen-induced uterine edema and growth, while pretreatment with exogenous arginine restored the response (673).

Activation of these pathways promotes uterine growth and remodeling. When maximal uterine growth is achieved, marked induction of the polyamine acetyltransferase Sat (p1z=0.952) and the arginase Arg1 (cluster O) at 3x24 hr reduce ornithine and arginine availability to slow uterine proliferation. Sat is the rate limiting enzyme in the polyamine degradation pathway, and the involvement of Sat in uterotrophy has been previously demonstrated by the uterine hypoplasia in mice overexpressing Sat (674). Induction of uterine arginase activity is well established and a classical biomarker for estrogen (675). In silico analysis of the Arg1 genomic sequence identified three ivEREs (Supplemental Table D), however it has also been reported to be induced by Tgfb1 (not represented on the array), which itself is known to be induced rapidly in the uterus following estrogen exposure (265). Although the biological significance of Arg1 transcript (230-fold in the present study at 3x24 hr) and enzyme induction has not been previously characterized, results from this study indicate that a stimulus may be depletion of ornithine due to Odc induction as maximum induction of uterine weight is realized. In addition, Arg1 induction, mediated by decreased ornithine levels or by the transcriptional

activator c/EBPβ or the growth factor Tgfb1 (not measured in the present study), is also believed to play a key role in curtailing macrophage nitric oxide synthesis (676, 677).

Estrogen responsive elements

Numerous motifs with either perfect or near-perfect sequence identity to the consensus ERE sequence were identified in the promoters of many of the genes that were the focus of the present study. While the presence of these motifs suggests possible transcriptional regulation by the classical ER-mediated mechanism of estrogen action, the presence of many such elements in the promoters and transcribed regions of genes found to be unresponsive in the present study underscores to the limitation of identifying estrogen responsiveness by *in silico* motif searches and gene expression data alone. Future studies may reveal the estrogen responsiveness of some of these genes in other estrogen-sensitive tissues, while our understanding of non-classical mechanisms of ER-mediated signaling increases (e.g. Ap1, Sp1) (176, 318, 678, 679). Furthermore, the induction by estrogen of many secondary transcription and growth factors in the uterus results in a complex network of transcriptional and posttranscriptional interactions that are difficult to predict based on sequence analysis alone. Complementary approaches that integrate gene expression, transcriptional regulator- response element interaction and *in silico* motif identification data are required in order to fully elucidate the ER uterine regulon.

General conclusions

Estrogen elicits a myriad of gene responses from a multitude of pathways that are exquisitely orchestrated to culminate in the uterotrophic response. This study provides

statistically rigorous gene expression data that support decades of molecular and physiological research into uterine function and the uterine response to estrogens. *In silico* analysis of genomic sequence suggests that many of these responses are estrogen regulated, however more direct functional approaches (e.g. chromatin immunoprecipitation assays) in combination with gene expression data are required in order to distinguish primary and secondary ER-mediated effects as well as to identify actively exploited response elements. Despite the comprehensive nature of microarray technology, many important genes were not represented on the GeneChip, and therefore it was not possible to place all responses into the appropriate mechanistic or biological context. Moreover, a lack of information regarding RNA stability, timing of translation, post-translational modification, and protein-protein interactions, as well as the limited amounted of gene annotation information also confounded the interpretation of the temporal pattern of groups of genes that may share a pathway relationship. Overall, the uterus of the ovariectomized immature mouse provides an ideal model for systematic assessment of the biological effects of estrogens at multiple levels of organization, while also being a key target of estrogen action with numerous physiological implications.

Table 1. Primer sequences used in real-time QRT-PCR verification.

Probe set ID	Abbrev.	Forward	Reverse
U51805_s_at	Arg1	tcacctgagctttgatgtcg	ctgaaaggagccctgtcttg
D16580_s_at	Pdi2	ggaagtgagcacctcccata	agtgagatgggacaggcagt
L40156_s_at	Sftpd	caacaacaatggtggagcag	cctccagtggctcagaactc
u29173_s_at	Ltbr	acacaaggaaggctcgtgac	gaaggtagggatgagcacca
aa267683_s_at	Ippi-like	ggagtagggctgaggaaaca	tgacacacctgtggcaagtt
m22326-2_s_at	Egr1	cttttgtgtgacacgccttg	ccctcttcctcgtttttgct
m21952_s_at	Csf1	atttgccccttggtctcttt	gacagggctgatggagctta
U57331_s_at	Tbx6	ggcagctccatctgtaccat	accgaggctcagtacattgg
AA426917_s_at	Ccnb1	gtctaaggccgtgacaaagg	tcacaacctttattgaagagcaa
m37761_s_at	S100a6	aaggctgatggatgatctgg	catctcaacggtcccatttt
u42467_s_at	Lepr	gtggatcagatgctgagctg	tgtgtccgacagaacctttg
aa616877_s_at	Capn10	gtggaccttggggctaaaat	ctcctggccttcactaagga
U60653_s_at	C2ta	atccctccatcgagacagtg	ctctagcccagctcccactt
M32745_s_at	Tgfb3	ctcctcctgccttccttctt	aacggggtctagggtaggag
aa154688_s_at	Farsl	caaagcattgggaagctagg	gggacactcagagaccaacc
u02554_s_at	Saa4	gggcttctctgaaaagtgga	aacagagaccggttgtgacc
M60523_s_at	Idb3	gcatggatgagcttcgatct	accagcgtgtgctagctctt
aa270881_g_at	Nxf1	ttctgagcatgattcagagca	cagtagtggcatcctccaca
AF032995_g_at	Crygs	cggtcagatgtacgaaacca	ctgccacggtagttgggtag
d10939_s_at	Mapk1	cagtttgtccccttccattg	agggctgccactttatttca
U51167_s_at	Idh2	acatggtagagggtgcctca	atgtccaccgtgaggaaaat
k02782_s_at	C3	gaaaagcccaacaccagcta	ctgtgaatgccccaagttct
L07063_s_at	Fkbp10	cagatccagttcagggagga	tgggaaagtgaaggaccaag
u43836_s_at	Vegfb	ggcttagagctcaacccaga	cctgtgctccactcttctcc
M31314_s_at	Fcgr1	tctgagcagggaaagaaagc	cactgagcttcgaggtccat
D86422_s_at	Krtap8-2	gcacgaggcaaaccataact	ccacagccatagccataacc

Table 2. Summary of K-means clustering of the entire Mu11KSubA dataset. For clarity, probe sets corresponding both to unnamed UniGene clusters and those not represented within UniGene are denoted 'ESTs' in this table.

K-means cluster ID	general pattern	# in cluster	# all-A[1]	% all-A	# with gene name[2]	% with gene name
A	induced at 2hr	597	286	47.9	324	54.3
B	induced at 2hr and 24hr	1023	412	40.3	604	59.0
C	induced at 8hr	912	197	21.6	498	54.6
D	induced at 12hr	266	144	54.1	124	46.6
E	induced at 8hr and 24hr	1025	245	23.9	604	58.9
F	induced at 24hr	262	144	55.0	156	59.5
G	induced at 3x24hr	272	84	30.9	153	56.3
H	no change	2227	397	17.8	1194	53.6

[1] Number of probe sets classified as Absent on every array by MAS5 analysis software.
[2] Number of probe sets with an associated gene name in UniGene (build 118).

Table 3. Comparison of microarray data with corresponding QRT-PCR data.

		ANOVA for GeneChip p1z>0.99					ANOVA for QRT-PCR					
Gene Abbrev.	**# P/M**	**p1z**	**p-value trt**	**p-value time**	**p-value trt*time**	**Overall[1]**	**p-value trt**	**p-value time**	**p-value trt*time**	**Overall[1]**	**Correl.[2]**	**Result[3]**
Arg1	5	1	0	0	0	S	0	0.001	0	S	1.000	+
Pdi2	9	1	0	0	0	S	0.149	0.497	0.404	NS	0.932	+
Sftpd	21	1	0	0	0	S	0.347	0.550	0.742	NS	0.890	+
Ltbr	21	0.999	0.006	0.294	0.105	S	0.855	0.248	0.725	NS	0.300	+
Ippi	19	0.999	0	0.0128	0.026	S	0	0.011	0.063	S	0.599	+/-
Egr1	21	0.997	0.001	0.002	0.003	S	0.928	0.002	0.03	S	0.777	+
Csf1	8	1				NS	0.1	0.377	0.33	NS	0.370	+
Tbx6	0	0.972					0	0.152	0.116	S	0.616	+
Ccnb1	8	0.964					0.289	0.458	0.263	NS	0.180	+/-
S100a6	21	0.951					0.391	0.791	0.603	NS	0.303	+
Lepr	9	0.898					0.001	0.089	0.061	S	0.562	+
Capn10	18	0.872					0.012	0.203	0.459	S	0.599	+
C2ta	0	0.872					0	0.017	0.01	S	0.206	-
Tgfb3	12	0.842					0.02	0.177	0.151	S	0.391	+
Farsl	21	0.678					0.047	0.638	0.971	S	0.817	+
Saa4	0	0.624					N/D	N/D	N/D	N/D	N/A	+
Idb3	20	0.497					0.003	0.119	0.106	S	0.445	+
Nxf1	12	0.497					0.408	0.659	0.451	NS	0.205	+
Crygs	0	0.311					0.053	0.044	0.334	NS	-0.423	+
Mapk1	5	0.190					0.039	0.474	0.169	S	0.302	+
Idh2	21	0.183					0.08	0.23	0.164	NS	0.299	+
C3	21	0.131					0.156	0.1	0.102	NS	0.952	+
Fkbp10	14	0.09					0.052	0.944	0.643	NS	0.319	+
Vegfb	18	0.085					0.039	0.374	0.326	S	0.541	+
Fcgr1	1	0.024					N/D	N/D	N/D	N/D	N/A	+
Krtap8-2	0	0					N/D	N/D	N/D	N/D	N/A	+

[1] A summary of the ANOVA results as significant (S) or non-significant (NS), using a threshold of $p<0.01$ for trt and trt*time.
[2] Pearson correlation coefficient comparing GeneChip and QRT-PCR temporal responses, with 1 indicating perfect correlation.
[3] Qualitative assessment of agreement between GeneChip and QRT-PCR data as strong (+), moderate (+/-), or poor (-).

Table 4. Summary of K-means clustering of the active subset of gene responses.

K-means cluster ID	General Pattern	# in cluster	# all-A[1]	% all-A	# with gene name[2]	% with gene name
I	induced at 2hr	59	6	10.2	35	59.3
J	induced at 8hr	77	3	3.9	47	61.0
K	induced at 8hr and 3x24 hr	47	2	4.3	25	53.2
L	induced at 12hr	101	1	1.0	59	58.4
M	induced at 24hr	69	12	17.4	50	72.5
N	induced at 24hr and 3x24 hr	25	1	4.0	18	72.0
O	induced at 72hr	14	0	0.0	11	78.6

[1] Number of probe sets classified as Absent on every array by MAS5 analysis software.
[2] Number of probe sets with an associated gene name in UniGene (build 118).

Figure 1. Probe sets represented on the Mu11KSubA array were categorized according to the gene name available in UniGene and whether the response passed the two statistical screening steps described in the text. A heavy outline indicates those probe set responses which passed both screening steps based on the defined threshold criteria, and which were subsequently subjected to K-means clustering and response element searching.

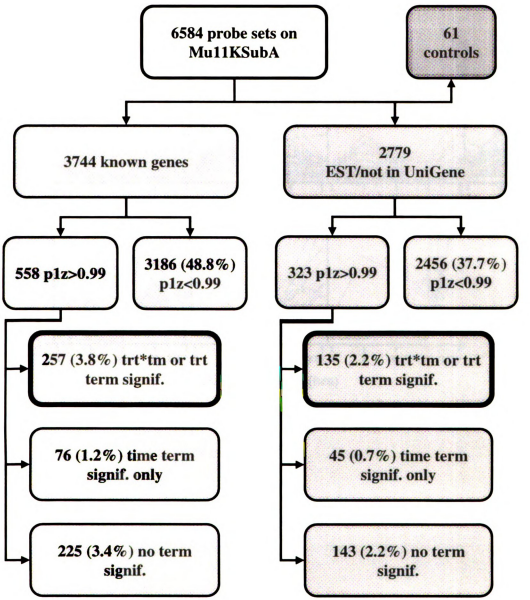


Figure 2. Responses passing the empirical Bayes and ANOVA screening steps, categorized into seven K-means clusters labeled I-O, as described in the text and in Table 4. A pseudogene line is drawn in bold to illustrate a representative response for each cluster.

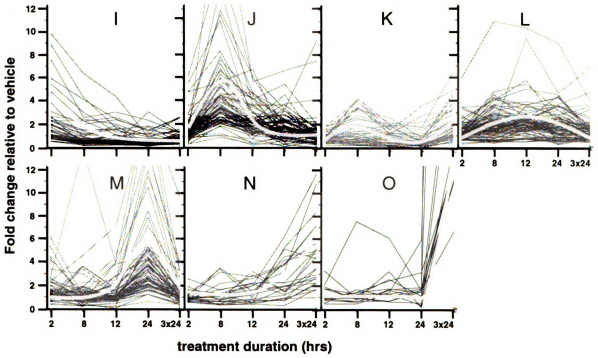
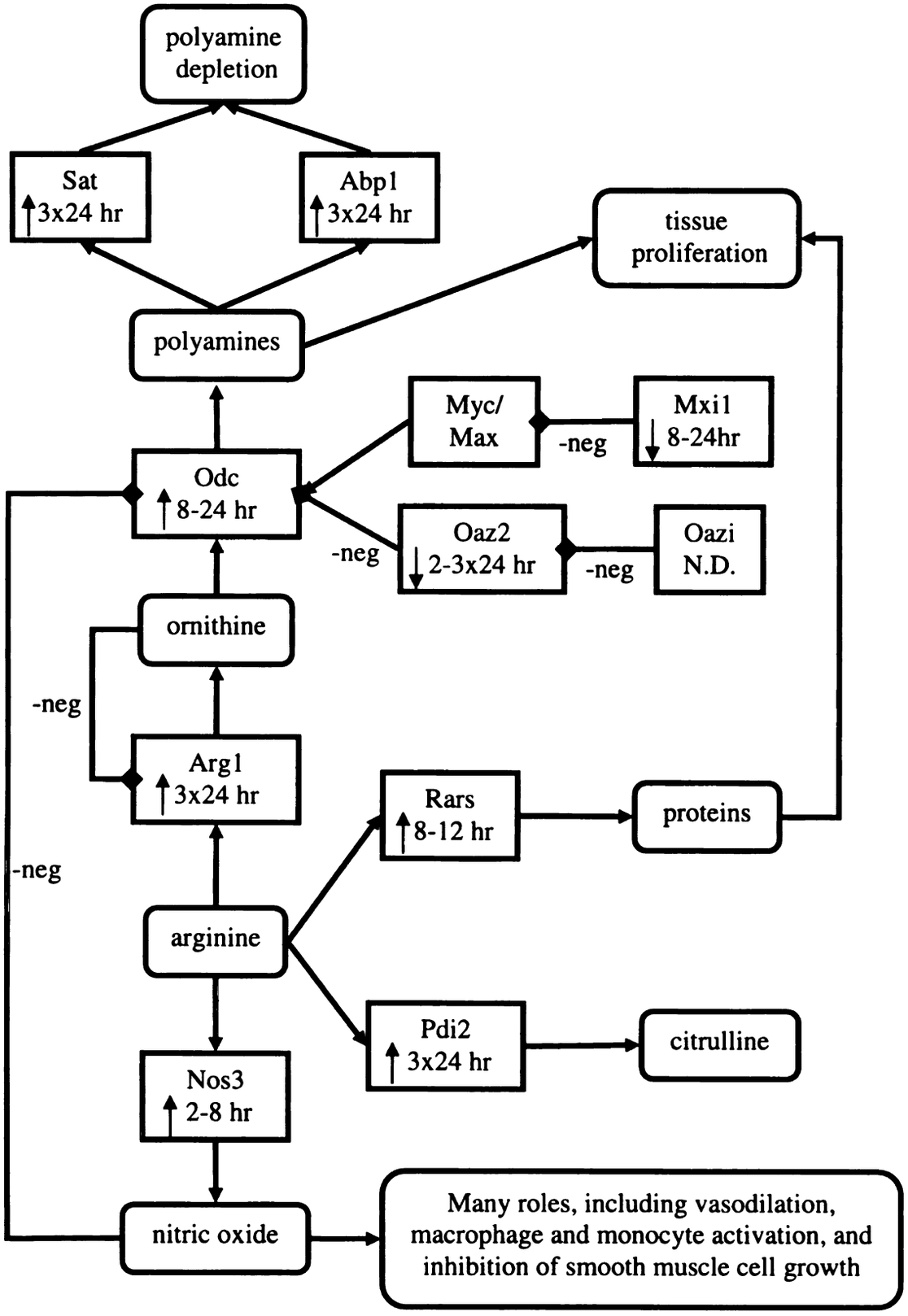


Figure 3. A model for the estrogen-induced promotion of uterine growth through regulation of specific urea cycle product levels.



CHAPTER 5

BENZO[A]PYRENE DOES NOT ELICIT ESTROGENIC OR ANTIESTROGENIC ACTIONS IN THE RODENT UTERUS

ABSTRACT

Numerous methods have been employed in attempts to assess the (anti)estrogenic potential of benzo[a]pyrene (B[a]P), a widely dispersed pollutant with a steroid-like structure. These studies have produced conflicting results which appear to be highly dependent on the nature of the assay being used. A previous study showed that B[a]P was not able to stimulate an increase in uterine weight, a hallmark of estrogen action, however its possible antiestrogenic effects were not evaluated. In the present study, the ability of B[a]P to antagonize an estrogen-induced uterotrophic response was evaluated. In addition, its ability to alter transcription of several known and suspected estrogen-regulated genes representative of diverse estrogen-induced physiological responses was assessed. The results presented here indicate that B[a]P does not possess either estrogenic or antiestrogenic effects in the rodent uterus, and demonstrate the utility of monitoring arginase 1 expression, in addition to other well characterized transcriptional responses.

INTRODUCTION

Benzo[a]pyrene (B[a]P) is a ubiquitous environmental pollutant of the polycyclic aromatic hydrocarbon (PAH) class, and concerns have been raised over the potential of this compound to interact with the estrogen receptor and modulate estrogen signaling either positively or negatively. Hydroxylated metabolites of B[a]P possess a flat hydrophobic core with one or more hydroxyl groups, reminiscent of the chemical structure of endogenous estrogens. Numerous assays can be commonly employed in the assessment of the potential (anti)estrogenic activity of a compound of interest, and although these have been employed with B[a]P, conflicting results have been obtained. These assays assess the ability of a compound to mimic the behavior of estrogen by either binding to the nuclear estrogen receptor (ER) and inducing ER-mediated transcription, or by stimulating proliferation of estrogen-responsive cells.

B[a]P was first isolated in 1932 from coal tar, and its potent carcinogenic activity was described (680). A brief report of its estrogenic activity followed soon after (508), in which an injection of 100 mg B[a]P into ovariectomized rats (corresponding to a dose of over 500 mg/kg in a 175 g animal) was found to greatly prolong estrus in three of ten animals. Several decades later, B[a]P was found to lack estrogenic activity and to somewhat inhibit the activity of 17β-estradiol (E2) in yeast expressing human estrogen receptor α (ERα) and an ERα-mediated reporter gene (521). Next, an *in vitro* study demonstrated the ability of B[a]P to induce ERα-mediated reporter gene expression in the MCF-7 breast cancer cell line (519). This was followed by the identification of specific monohydroxylated metabolites as the compounds that are able to bind to both ERα and ERβ *in vitro*, and to induce ERα- and ERβ-mediated reporter gene expression

(536, 579). In addition, cotreatment of B[a]P with the estrogen 17β-estradiol (E2) was found to produce an additive response (579). However around the same time, B[a]P in a metabolically competent system was shown to bind to endogenous MCF-7 ER but to lack proliferative activity in the MCF-7 cell proliferation assay, whereas it antagonized cell proliferation induced by E2 (520). B[a]P was also found to stimulate E2 metabolism in MCF-7 cells somewhat, which was suggested to be a mechanism for its observed antiestrogenic action (520). *In vivo*, B[a]P was not found to stimulate classical estrogen-inducible endpoints of increased uterine weight (uterotrophy) and uterine lactoferrin expression (579), although its ability to directly affect these parameters in estrogen-treated mice was not evaluated.

The uterotrophic assay has long been considered to be the 'gold standard' for assessing estrogenic effects (553), however more recently its sensitivity and specificity have been questioned (681, 682). Moreover, recent advances in the understanding of estrogen signaling have revealed numerous unexpected deviations from the classical mode of estrogen action. These include ER action in the absence of ligand, ER action at response elements that deviate substantially from the consensus estrogen responsive element (ERE), ER activation by phosphorylation, activation of membrane-bound ER, different effects mediated by ERα versus ERβ, and dependence on the nature and levels of coactivators and corepressors in the cellular system under consideration (28, 602, 606).

In the present study, we examine the possible estrogenic or antiestrogenic activity of B[a]P and TCDD, another AhR ligand, in the rodent uterus. These effects were evaluated by monitoring the uterine weight and the expression of several well characterized estrogen-inducible gene responses representative of diverse pathways that

are modulated by estrogen in the uterus. The animals used in this study were also used for a concurrent study by Darrell Boverhof, in which the objective was to characterize the transcriptional effects of estrogen and of tetrachlorodibenzo-*p*-dioxin (TCDD) in the liver and spleen. As a result, uteri from TCDD-treated mice were available, and were also used in the present study.

MATERIALS AND METHODS

Immature ovariectomized C57BL/6 mice (Charles River Laboratories, Raleigh, NC) were housed in groups of four, with one treatment group per cage. Animals were administered the test compounds by oral gavage in a total 0.1 ml of sesame oil. 17α-ethynyl estradiol (EE) was administered at 0.01 mg/kg, and benzo[a]pyrene (B[a]P) and tetrachlorodibenzo-*p*-dioxin (TCDD) were administered at 10 mg/kg. Animals received one dose for either 12 or 24 hr, or else three consecutive daily doses (3x24 hr) followed by sacrifice on the fourth day. An exception was the TCDD multi-dose group, which received one TCDD treatment, followed by two daily doses of vehicle. The TCDD cotreatment group received initial treatment with EE and TCDD, followed by two daily doses of EE alone, although for simplicity this is referred to as 3x24 hr EE+TCDD.

Uteri were weighed, blotted, reweighed, and stored in RNALater (Ambion) at –80°C until use. Separate RNA isolations, reverse transcription, and quantitative real-time PCR (QRT-PCR) reactions for each tissue sample were performed as previously described (Chapter 4). Primer sequences are listed in Table 1. Each measurement was normalized to its corresponding measured value for β-actin, and then at each time point groups were compared to the vehicle control using a two-tailed Student's t-test ($p<0.05$). This procedure was also used to analyze the uterine weight data. One sample from the 3x24 hr vehicle group and one from the 3x24 hr EE+TCDD group had very low actin values and inflated actin-normalized responses for the other transcripts, compared to the other members of their groups, and were removed from consideration.

RESULTS

Uterine weight

A slight increase in unblotted uterine weight was observed in several of the treatment groups after the 12 hr exposure period, which was not evident in the 24 hr group (Figure 1). After the 3x24 hr exposure regimen, however, unblotted uterine weight relative to the vehicle group was induced approximately 3.5-fold by 3x24 hr EE treatment, and this response was comparable when B[a]P or TCDD were coadministered with EE. After blotting to expel the luminal fluid, the induction of uterine tissue weight was slightly lower, approximately 2-fold (not shown). Treatment with B[a]P or TCDD alone did not stimulate an increase in uterine weight.

Hepatic induction of Cyp1a1

Induction of *Cyp1a1* transcript levels were was determined in the liver (data provided by Darrell Boverhof). None of the EE treatment regimens caused a significant alteration in *Cyp1a1* mRNA levels. B[a]P stimulated *Cyp1a1* transcript levels only after 12 hr treatment, and the induction was high (80-fold), although much more modest than that induced by TCDD (Figure 2A). Cotreatment with EE reduced the 12 hr response to 40-fold. At 24 and 3x24 hr, however, cotreatment of EE and B[a]P caused a slight but nonsignificant increase in *Cyp1a1* transcript levels, and the single treatments did not cause a significant response.

TCDD induced *Cyp1a1* transcript levels approximately 2810-, 4060-, and 13630-fold after 12, 24, and 3x24 hr treatments, respectively, which were somewhat reduced (to 2360-, 2050-, and 4390-fold respectively) by cotreatment with EE (Figure 2B).

Uterine induction of Cyp1a1

No changes in uterine *Cyp1a1* transcript levels reached significance in the present study (not shown), although the lowest *Cyp1a1* levels were consistently observed in the EE and EE+B[a]P treated groups. *Cyp1a1* levels in the 3x24 hr vehicle and TCDD groups were variable but readily detectable, whereas it was expressed at extremely low levels in the other groups.

Uterine induction of other transcripts

Ltf was significantly induced by 3x24 hr EE (132-fold) and EE+B[a]P (91-fold), while no effect was observed at earlier times (Figure 3A). In addition, induction by 3x24 hr EE+TCDD (37-fold) was nearly significant ($p<0.07$).

Similarly, *Arg1* was induced by 3x24 hr EE (10-fold) and EE+B[a]P (7-fold), while the 8-fold increase in the EE+TCDD group was nearly significant ($p<0.08$) (Figure 3B).

C3 induction was significant only in the 3x24 hr EE treatment group (8-fold), while the increase in the EE+B[a]P group (6-fold) was not significant due to variability. *Sftpd* induction was not significant in any group, although increases of 4-fold and 3-fold were observed in the 3x24 hr EE and EE+B[a]P groups respectively (not shown).

No significant changes were observed in the levels of *Rars*, *Muc1*, *Ccnb1*, or *Socs1*.

DISCUSSION

Uterine weight

The 3.5-fold stimulation of uterine weight in response to a 3x24 hr EE treatment is comparable to previous findings (e.g. Chapter 2; (31)). These experiments suggest that B[a]P and TCDD do not exert positive or negative effects on EE-stimulated increases in unblotted or blotted uterine weight, and thus did not affect uterine tissue proliferation or fluid accumulation. By contrast, the slight increase at 12 hr by several treatments was not previously observed in our studies.

Cyp1a1 expression

Induction of hepatic *Cyp1a1* transcript and protein levels by PAHs and dioxins such as B[a]P and TCDD is very well characterized. TCDD is the ligand with the highest known affinity for the aryl hydrocarbon receptor (AhR), and readily stimulates expression of AhR-inducible genes such as *Cyp1a1*. Although CYP1A1 is a metabolizing enzyme that can readily hydroxylate a wide variety of hydrophobic compounds including many of its own inducers, TCDD is extremely refractory to degradation. As a result, in experiments longer than 24 hr, it is rare to administer repeat daily doses, and in the present study the 3x24 hr dose regimen is in fact one dose of TCDD followed by two daily doses of vehicle, as described in the Materials and Methods. Because of the single TCDD dose, it is then particularly interesting that hepatic *Cyp1a1* expression was highest at 72 hr, and may be indicative of continual release during the three days of TCDD that had partitioned into body fat. Cotreatment with EE, interestingly, appeared to cause a depression in TCDD-induced *Cyp1a1* at the later time points.

B[a]P, in contrast, is a somewhat weaker ligand for the AhR, and is also readily metabolized by P450 enzymes including CYP1A1. It is likely that both of these factors contribute to the decline in *Cyp1a1* expression by 24 hr after the first exposure. Interestingly, however, cotreatment of B[a]P and EE at the later time points appeared to potentiate *Cyp1a1* expression.

Expression of other transcripts

An EE dose of 0.01 mg/kg was chosen since previous dose response experiments within the lab (Dr. Yan Sun) had indicated that this dose stimulated a submaximal induction of estrogen-regulated gene expression. This was important so that either an induction or a repression of expression in cotreatment groups relative to the EE group could be detected.

Transcripts were examined that were representative of numerous processes that are known to be regulated by estrogen in the uterus: cell cycle regulation, tRNA synthetase production, cell surface antiadhesion, and immune responses. In the present study, the only transcripts that were highly upregulated by estrogen were lactoferrin and arginase, which are observed as late phase responses in the uterine proliferative process, and complement component C3 and surfactant associated protein D were upregulated in a similar manner. These transcripts were highly upregulated in the 3x24 hr group and little repressive effect was observed in the EE+B[a]P and EE+TCDD groups. Moreover, no significant increase in these transcript levels was observed when B[a]P or TCDD were administered alone.

Selection of specific transcripts as markers of estrogen action

These transcripts, because of their high inducibility, provide a sensitive means of evaluating estrogenic action. By contrast, the other transcripts that were monitored were not as successful in demonstrating robust upregulation in response to an estrogenic stimulus. Although they were chosen because their inducibility has been reported or suspected, they may not be suitable for studies with relatively small numbers of replicates and widely spaced time points, since biological variability in magnitude and timing of response becomes a very important factor.

Because of the rapidly expanding number of mechanisms by which estrogen and/or its receptor can effect its responses, it seems prudent to monitor estrogen-inducible transcripts that are involved in several diverse uterine responses to estrogen. While many additional transcripts involving other aspects of estrogen-induced uterine response (e.g. vasodilation, electrolyte regulation, energy production, proteolysis, and many others) have been identified (Chapter 1), some of these transcripts have been shown to be altered in only one uterine compartment by *in situ* hybridization, and therefore may not be detectable in a whole uterine homogenate. Species and strain differences may also contribute to a lack of concordance between reported and observed responses. Again, the identification of several estrogen-induced responses that are representative of diverse estrogen-regulated physiological events in the uterus will be of great benefit. In the present study, lactoferrin was found, as has been shown previously, to be extremely highly inducible in estrogen-stimulated uterus, however since it is known to be regulated by different mechanisms in mice and in humans (683), the use of additional markers is particularly warranted. Complement component C3 is another well accepted estrogen-

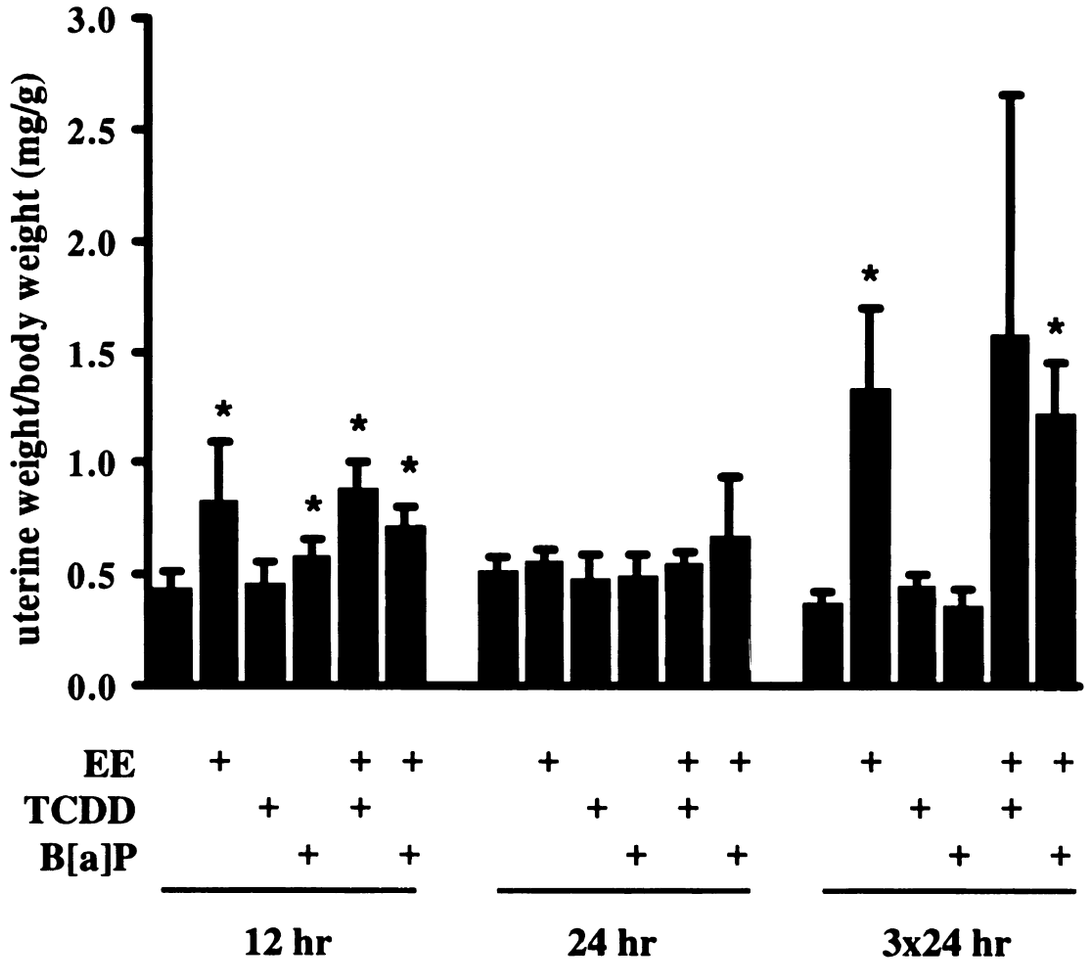
regulated gene in the uterus (463), however like lactoferrin, it is also an immune-related response. Arginase 1, which was long ago shown to be an estrogen-inducible enzyme in the uterus (675), was shown (Chapter 4) to be highly inducible at the transcript level as well, and its utility as an estrogenic marker is confirmed in the present study. Arginase is a key multifunctional enzyme with numerous roles including providing ornithine for the synthesis of polyamines, and polyamine ability is closely linked to cell proliferation (669).

While it is possible to continue measuring additional transcripts indefinitely, the results presented here suggest that B[a]P is not able to initiate a uterotrophic response or to significantly induce the expression of key estrogen-regulated genes (684), in confirmation of results presented earlier (Chapter 2). Moreover, this study addresses previous *in vitro* findings suggesting that B[a]P might possess antiestrogenic activity (520, 521), showing that in the rodent uterus B[a]P is unable to antagonize these estrogen-induced responses, despite it being administered at a 1000-fold excess compared to EE. Overall, these findings highlight the fact that the various *in vitro* assays that are available to assess estrogenic action can not always be reflective of the biological responses in a complex tissue, and that caution is warranted in the extrapolation from *in vitro* results to *in vivo* predictions.

Table 1. Primer sequences used in real-time QRT-PCR verification.

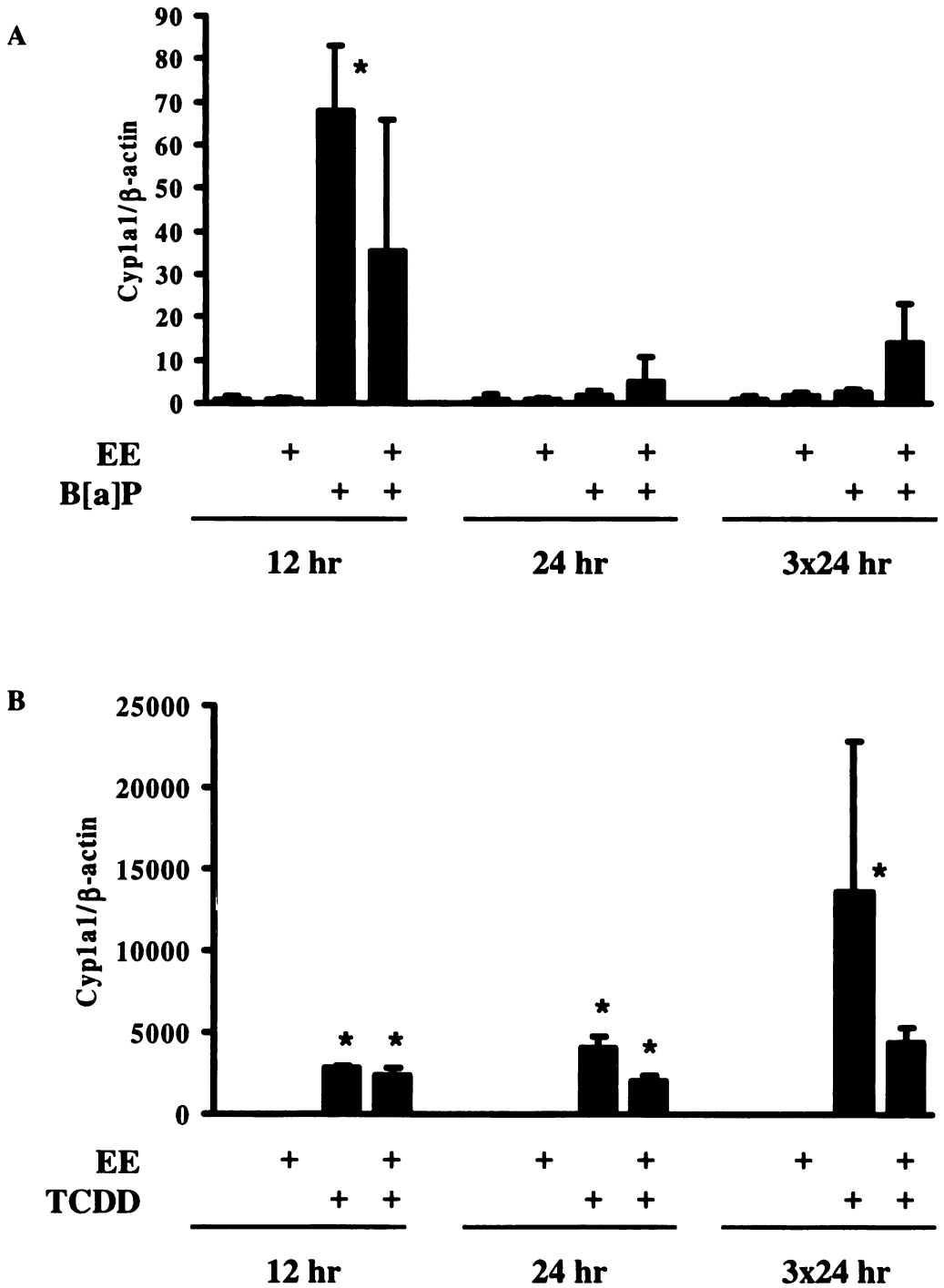
mRNA RefSeq	Abbrev.	Forward	Reverse	Product size
NM_007482	Arg1	tcacctgagctttgatgtcg	ctgaaaggagccctgtcttg	134
NM_009160	Sftpd	caacaacaatggtggagcag	cctccagtggctcagaactc	114
NM_025936	Rars	ttgatgaggcgatgctacag	ggtgtggagaaacaggtcgt	140
NM_013605	Muc1	ccaccagagctcctgaagac	agctgaagaggtgccactgt	185
NM_172301	Ccnb1	gtctaaggccgtgacaaagg	tcacaacctttattgaagagcaa	123
NM_009896	Socs1	acttctggctggagacctca	cccagacacaagctgctaca	100
NM_009992	Cyp1a1	aagtgcagatgcggtcttct	aaagtaggaggcaggcacaa	140
NM_008522	Ltf	gagaagatgctggcttcacc	atttgcaggatctggtctgg	126
NM_009778	C3	gaaaagcccaacaccagcta	ctgtgaatgccccaagttct	121
NM_007393	Actb	gctacagcttcaccaccaca	tctccagggaggaagaggat	123

Figure 1. Uterine weights normalized by body weights at the end of the treatment period.



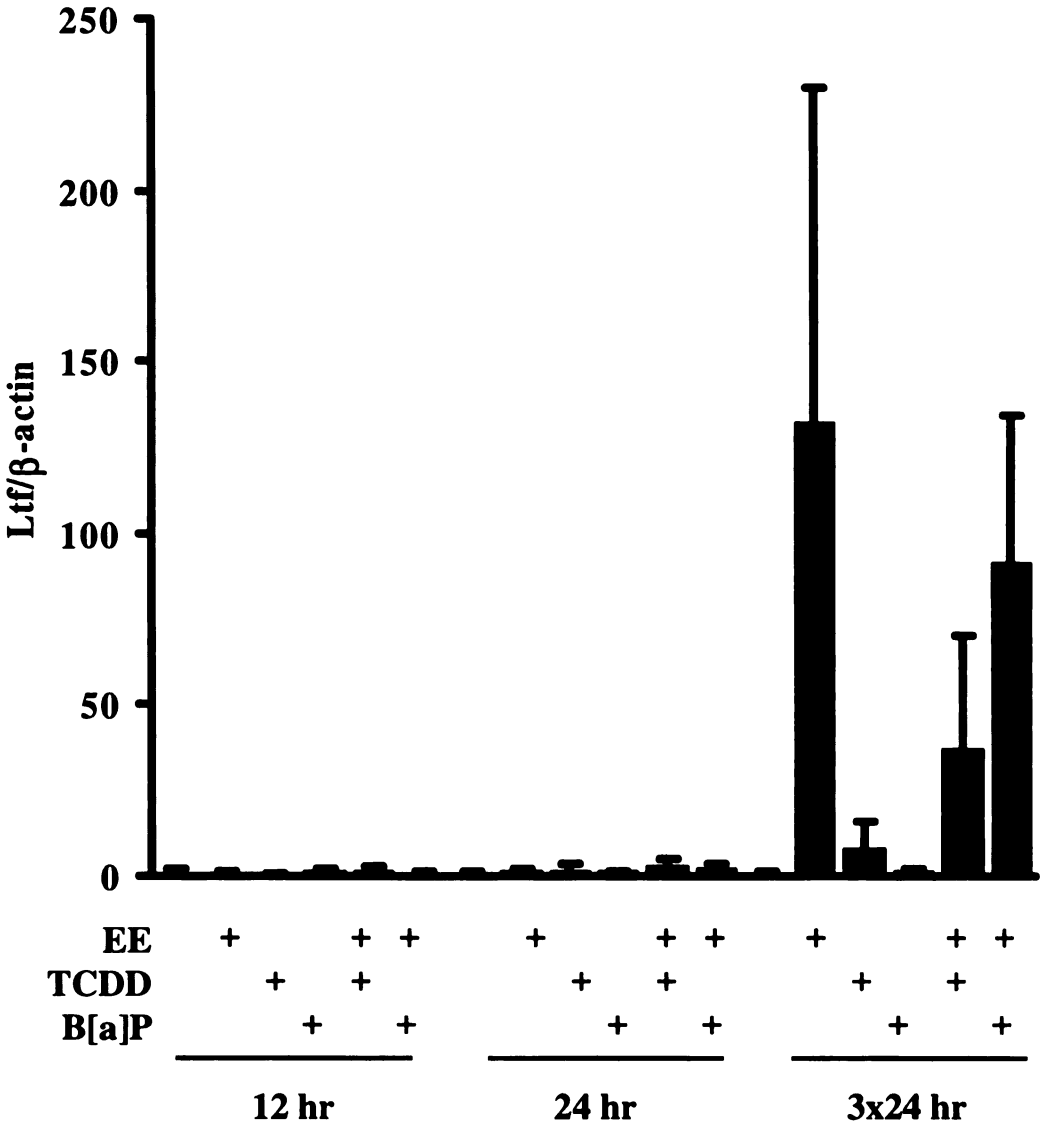
* indicates significant increases ($p<0.05$) relative to the vehicle group for each exposure duration

Figure 2. Hepatic expression of *Cyp1a1* measured by QRT-PCR. Benzo[a]pyrene (A) and TCDD (B) are presented separately due to large differences in inducibility.



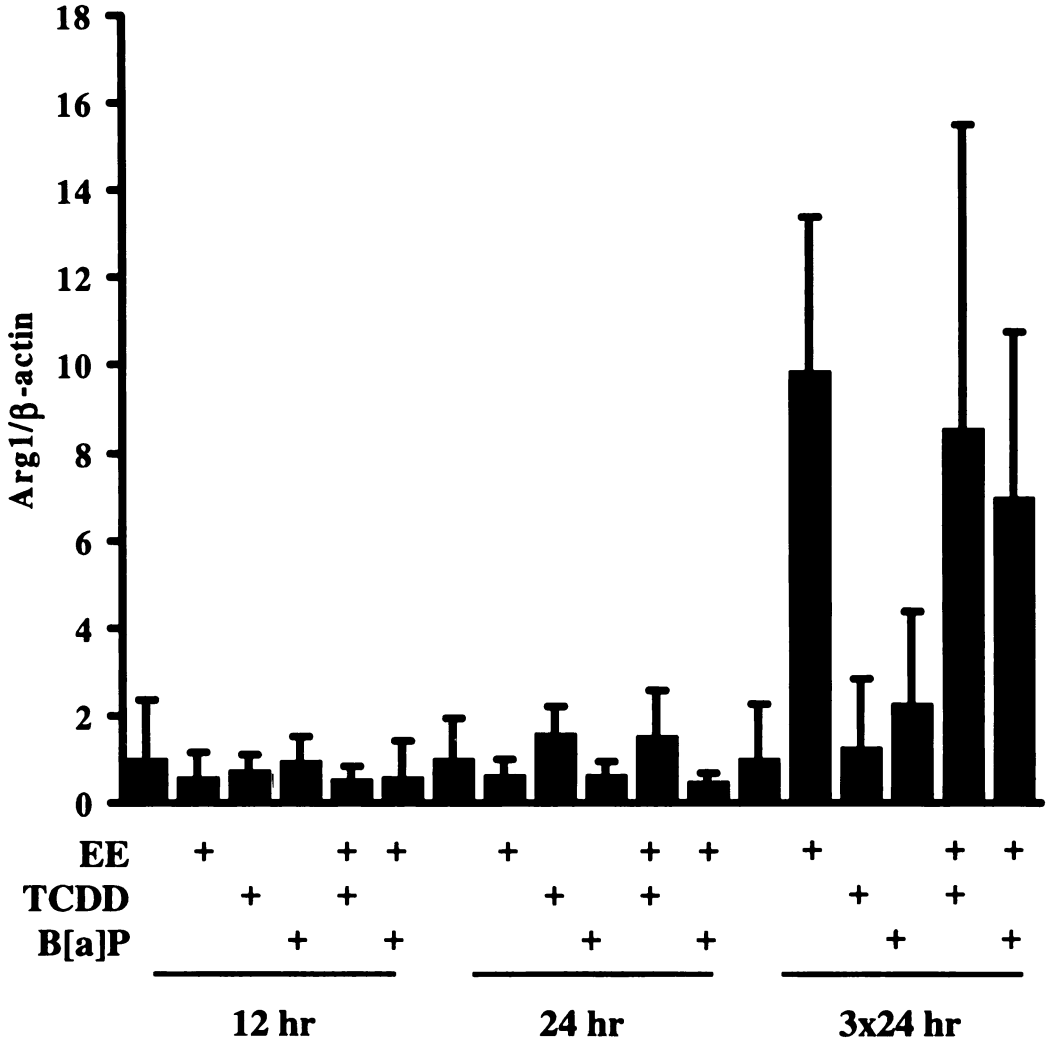
* indicates significant increases ($p<0.05$) relative to the vehicle group for each exposure duration

Figure 3A. Uterine expression of *Ltf* measured by QRT-PCR.



* indicates significant increases ($p<0.05$) relative to the vehicle group for each exposure duration

Figure 3B. Uterine expression of *Arg1* measured by QRT-PCR.



* indicates significant increases ($p<0.05$) relative to the vehicle group for each exposure duration

CHAPTER 6

SUMMARY AND FUTURE PERSPECTIVES

The preceding studies have used B[a]P as a model to examine the activity of a compound with suspected weak estrogenic or antiestrogenic activity. The results of these studies have underscored several critical principles that are emerging in the context of estrogenicity tests: (1) the importance of using systems with defined metabolic potential, since metabolites may be either more or less active than, and moreover species differences and polymorphisms can make an individual or a population more sensitive to a particular metabolite; (2) the lack of agreement that can exist between assays that are designed to measure the same outcome but that are biologically very different; (3) the importance of designing an estrogenicity experiment that will be able to evaluate both additive and antagonistic effects; (4) the necessity, particularly in microarray data analysis, of identifying each gene by one single gene name; and (5) the need to adapt existing assays to accommodate new mechanistic information.

Different *in vitro* estrogenicity tests of B[a]P activity yielded opposite results (i.e. estrogenic or antiestrogenic), however in vivo studies using anestrogenic (i.e. immature, ovariectomized) mice indicated that B[a]P displayed neither estrogenic nor antiestrogenic activity in the uterus at the dose level that was examined. While induction of uterine weight and uterine lactoferrin expression are considered to be hallmarks of the estrogenic response, it is must be remembered that B[a]P metabolites, as well as other PAH metabolites, were found to interact preferentially with the β isoform of the ER, and therefore could still possess (anti)estrogenic activity in a tissue such as ovary, which

expresses ERβ much more highly than does the uterus. An examination of ovarian responses would involve abandoning the ovariectomized model animal, which is important for ensuring a lack of background circulating estrogen, however other lab members are continuing related studies examining the transcriptional effects of estrogen in different tissues. Other lab members are addressing the important issue of dose level, in order to characterize the dose of estrogen needed to provide a robust but submaximal transcriptional response following estrogen administration. The further characterization of transcriptional responses to endogenous estrogens and to both weak and strong exogenous estrogens remains a lab research focus. This, in conjunction with the projects ongoing in other institutions to characterize the transcriptional profiles induced by estrogen exposure, will be important in contributing both to the understanding of the role of estrogen in uterine biology, and to the knowledge of which changes that should be most closely monitored following exposure to suspected (anti)estrogens.

REFERENCES

1. **Maggiolini M, Donze O, Jeannin E, Ando S, Picard D** 1999 Adrenal androgens stimulate the proliferation of breast cancer cells as direct activators of estrogen receptor alpha. Cancer Res 59:4864-9

2. **Poulin R, Labrie F** 1986 Stimulation of cell proliferation and estrogenic response by adrenal C19-delta 5-steroids in the ZR-75-1 human breast cancer cell line. Cancer Res 46:4933-7

3. **Callard GV, Petro Z, Ryan KJ** 1978 Phylogenetic distribution of aromatase and other androgen-converting enzymes in the central nervous system. Endocrinology 103:2283-90

4. **Philip A, Murphy BE** 1984 Does sex hormone-binding globulin play a role in the transport of estradiol in vivo? Am J Obstet Gynecol 148:643-8

5. **Puig-Duran E, Greenstein BD, MacKinnon PC** 1979 The effects of serum oestrogen-binding components on the unbound oestradiol-17 beta fraction in the serum of developing female rats and on inhibition of [3H]oestradiol uptake by uterine tissue in vitro. J Reprod Fertil 56:707-14

6. **Simpson E, Rubin G, Clyne C, et al.** 2000 The role of local estrogen biosynthesis in males and females. Trends Endocrinol Metab 11:184-8

7. **Michael MD, Kilgore MW, Morohashi K, Simpson ER** 1995 Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (CYP19) gene in the ovary. J Biol Chem 270:13561-6

8. **Simpson ER, Davis SR** 2001 Minireview: Aromatase and the Regulation of Estrogen Biosynthesis--Some New Perspectives. Endocrinology 142:4589-4594

9. **Martucci CP, Fishman J** 1993 P450 enzymes of estrogen metabolism. Pharmacol Ther 57:237-57

10. **Jefcoate CR, Liehr JG, Santen RJ, et al.** 2000 Tissue-specific synthesis and oxidative metabolism of estrogens. J Natl Cancer Inst Monogr:95-112

11. **Hanna IH, Dawling S, Roodi N, Guengerich FP, Parl FF** 2000 Cytochrome P450 1B1 (CYP1B1) Pharmacogenetics: Association of Polymorphisms with Functional Differences in Estrogen Hydroxylation Activity. Cancer Res 60:3440-3444

12. **Green S, Walter P, Kumar V, et al.** 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 320:134-9

13. **Thornton JW** 2001 Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. PNAS 98:5671-5676

14. **Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA** 1996 Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93:5925-30

15. **Hawkins MB, Thornton JW, Crews D, Skipper JK, Dotte A, Thomas P** 2000 Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc Natl Acad Sci U S A 97:10751-6.

16. **Rosenfeld CS, Roberts RM, Lubahn DB** 2001 Estrogen receptor- and aromatase-deficient mice provide insight into the roles of estrogen within the ovary and uterus. Mol Reprod Dev 59:336-46

17. **Cheung E, Schwabish MA, Kraus WL** 2003 Chromatin exposes intrinsic differences in the transcriptional activities of estrogen receptors {alpha} and {beta}. EMBO J. 22:600-611

18. **Brzozowski AM, Pike ACW, Dauter Z, et al.** 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753-758

19. **Williams SP, Sigler PB** 1998 Atomic structure of progesterone complexed with its receptor. Nature 393:392-6

20. **Tanenbaum DM, Wang Y, Williams SP, Sigler PB** 1998 Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. Proc Natl Acad Sci U S A 95:5998-6003

21. **Klinge CM** 2001 Estrogen receptor interaction with estrogen response elements. Nucl. Acids. Res. 29:2905-2919

22. **Meyer ME, Gronemeyer H, Turcotte B, Bocquel MT, Tasset D, Chambon P** 1989 Steroid hormone receptors compete for factors that mediate their enhancer function. Cell 57:433-42

23. **Baniahmad A, Leng X, Burris T, Tsai S, Tsai M, O'Malley B** 1995 The tau 4 activation domain of the thyroid hormone receptor is required for release of a putative corepressor(s) necessary for transcriptional silencing. Mol. Cell. Biol. 15:76-86

24. **Ratajczak T** 2001 Protein coregulators that mediate estrogen receptor function. Reprod Fertil Dev 13:221-9

25. **Jordan VC** 2002 The secrets of selective estrogen receptor modulation: cell-specific coregulation. Cancer Cell 1:215-7

26. **Nishikawa J-i, Saito K, Goto J, Dakeyama F, Matsuo M, Nishihara T** 1999 New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicol Appl Pharmacol 154:76-83

27. **Levin ER** 2001 Cell localization, physiology, and nongenomic actions of estrogen receptors. J Appl Physiol 91:1860-7

28. **Sanchez R, Nguyen D, Rocha W, White JH, Mader S** 2002 Diversity in the mechanisms of gene regulation by estrogen receptors. Bioessays 24:244-54

29. **Jakacka M, Ito M, Martinson F, Ishikawa T, Lee EJ, Jameson JL** 2002 An Estrogen Receptor (ER){alpha} Deoxyribonucleic Acid-Binding Domain Knock-In Mutation Provides Evidence for Nonclassical ER Pathway Signaling in Vivo. Mol Endocrinol 16:2188-2201

30. **Couse JF, Korach KS** 1999 Estrogen receptor null mice: what have we learned and where will they lead us? [published erratum appears in Endocr Rev 1999 Aug;20(4):459]. Endocr Rev 20:358-417

31. **Odum J, Lefevre PA, Tittensor S, et al.** 1997 The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. Regul Toxicol Pharmacol 25:176-88

32. **Kimber SJ, Stones RE, Sidhu SS** 2001 Glycosylation changes during differentiation of the murine uterine epithelium. Biochem Soc Trans 29:156-62

33. **Allen E** 1922 The estrous cycle in the mouse. Am J Anat 30:297-371

34. **Long J, Evans H** 1922 The estrous cycle in the rat and its associated phenomena. Mem Univ California 6:1-128

35. **Hunt JS, Miller L, Platt JS** 1998 Hormonal regulation of uterine macrophages. Dev Immunol 6:105-10

36. **Gray CA, Bartol FF, Tarleton BJ, et al.** 2001 Developmental biology of uterine glands. Biol Reprod 65:1311-23

37. **Gray CA, Taylor KM, Ramsey WS, et al.** 2001 Endometrial glands are required for preimplantation conceptus elongation and survival. Biol Reprod 64:1608-1613

38. **Hastings JM, Licence DR, Burton GJ, Charnock-Jones DS, Smith SK** 2003 Soluble Vascular Endothelial Growth Factor Receptor 1 Inhibits Edema and

Epithelial Proliferation Induced by 17{beta}-Estradiol in the Mouse Uterus. Endocrinology 144:326-334

39. **Osteen KG, Bruner-Tran KL, Keller NR, Eisenberg E** 2002 Progesterone-Mediated Endometrial Maturation Limits Matrix Metalloproteinase (MMP) Expression in an Inflammatory-like Environment: A Regulatory System Altered in Endometriosis. Ann NY Acad Sci 955:37-47

40. **Aplin JD, Meseguer M, Simon C, Ortiz ME, Croxatto H, Jones CJ** 2001 MUC1, glycans and the cell-surface barrier to embryo implantation. Biochem Soc Trans 29:153-6

41. **Png FY, Murphy CR** 2000 Closure of the uterine lumen and the plasma membrane transformation do not require blastocyst implantation. Eur J Morphol 38:122-7

42. **Giudice LC, Mark SP, Irwin JC** 1998 Paracrine actions of insulin-like growth factors and IGF binding protein-1 in non-pregnant human endometrium and at the decidual-trophoblast interface. J Reprod Immunol 39:133-48

43. **Spornitz UM, Socin CD, Dravid AA** 1999 Estrous stage determination in rats by means of scanning electron microscopic images of uterine surface epithelium. Anat Rec 254:116-26

44. **Tseng L** 1984 Estrogen synthesis in human endometrial epithelial glands and stromal cells. J Steroid Biochem 20:877-81

45. **Cameron VA, Autelitano DJ, Evans JJ, et al.** 2002 Adrenomedullin expression in rat uterus is correlated with plasma estradiol. Am J Physiol Endocrinol Metab 282:E139-146

46. **Hyder SM, Chiappetta C, Stancel GM** 1999 Synthetic estrogen 17alpha-ethinyl estradiol induces pattern of uterine gene expression similar to endogenous estrogen 17beta-estradiol. J Pharmacol Exp Ther 290:740-7

47. **Astwood EB** 1939 Changes in the weight and water content of the uterus of the normal adult rat. Am J Physiol 126:162-170

48. **Huet YM, Dey SK** 1987 Role of early and late oestrogenic effects on implantation in the mouse. J Reprod Fertil 81:453-8

49. **Harris J, Gorski J** 1978 Evidence for a discontinuous requirement for estrogen in stimulation of deoxyribonucleic acid synthesis in the immature rat uterus. Endocrinology 103:240-5

50. **Kaye AM, Sheratzky D, Lindner HR** 1971 Kinetics of DNA synthesis in immature rat uterus: age dependence and estradiol stimulation. Biochim Biophys Acta 261:475-86

51. **Risek B, Klier F, Phillips A, Hahn D, Gilula N** 1995 Gap junction regulation in the uterus and ovaries of immature rats by estrogen and progesterone. J Cell Sci 108:1017-1032

52. **De M, Wood GW** 1990 Influence of oestrogen and progesterone on macrophage distribution in the mouse uterus. J Endocrinol 126:417-24

53. **Williams T, Rogers AW** 1972 Morphological changes in the luminal epithelium of the rat uterus in response to progesterone and oestradiol. J Anat 111:515

54. **Quarmby VE, Korach KS** 1984 The influence of 17 beta-estradiol on patterns of cell division in the uterus. Endocrinology 114:694-702

55. **Mendoza-Rodriguez CA, Merchant-Larios H, Segura-Valdez ML, et al.** 2003 c-fos and estrogen receptor gene expression pattern in the rat uterine epithelium during the estrous cycle. Mol Reprod Dev 64:379-88

56. **Rotello R, Lieberman R, Lepoff R, Gerschenson L** 1992 Characterization of uterine epithelium apoptotic cell death kinetics and regulation by progesterone and RU 486. Am J Pathol 140:449-456

57. **Rambo CO, Szego CM** 1983 Estrogen action at endometrial membranes: alterations in luminal surface detectable within seconds. J Cell Biol 97:679-85

58. **Greenman DL, Dooley K, Breeden CR** 1977 Strain differences in the response of the mouse to diethylstilbestrol. J Toxicol Environ Health 3:589-97

59. **Friedrich ER, Meyer JS** 1982 Estrogen-progestin pharmacodynamics of the postmenopausal endometrium studied by thymidine labeling. Am J Obstet Gynecol 143:352-9

60. **Klebe U, Moltz L, Pickartz H** 1983 Effects of cyproterone acetate and ethinylestradiol on endometrial histology. Arch Gynecol 234:113-20

61. **Lecce G, Meduri G, Ancelin M, Bergeron C, Perrot-Applanat M** 2001 Presence of Estrogen Receptor {beta} in the Human Endometrium through the Cycle: Expression in Glandular, Stromal, and Vascular Cells. J Clin Endocrinol Metab 86:1379-1386

62. **Nephew KP, Long X, Osborne E, Burke KA, Ahluwalia A, Bigsby RM** 2000 Effect of estradiol on estrogen receptor expression in rat uterine cell types. Biol Reprod 62:168-77

63. **Weihua Z, Ekman J, Almkvist A, et al.** 2002 Involvement of Androgen Receptor in 17{beta}-Estradiol-Induced Cell Proliferation in Rat Uterus. Biol Reprod 67:616-623

64. **Brandenberger AW, Lebovic DI, Tee MK, et al.** 1999 Oestrogen receptor (ER)-alpha and ER-beta isoforms in normal endometrial and endometriosis-derived stromal cells. Mol Hum Reprod 5:651-5

65. **Paech K, Webb P, Kuiper GG, et al.** 1997 Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 277:1508-10

66. **Pettersson K, Delaunay F, Gustafsson JA** 2000 Estrogen receptor beta acts as a dominant regulator of estrogen signaling. Oncogene 19:4970-8

67. **Hall JM, McDonnell DP** 1999 The Estrogen Receptor {beta}-Isoform (ER{beta}) of the Human Estrogen Receptor Modulates ER{alpha} Transcriptional Activity and Is a Key Regulator of the Cellular Response to Estrogens and Antiestrogens. Endocrinology 140:5566-5578

68. **Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O** 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A 90:11162-6

69. **Johns A, Freay AD, Fraser W, Korach KS, Rubanyi GM** 1996 Disruption of estrogen receptor gene prevents 17 beta estradiol-induced angiogenesis in transgenic mice. Endocrinology 137:4511-3

70. **Couse JF, Curtis SW, Washburn TF, et al.** 1995 Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. Molecular Endocrinology 9:1441-1454

71. **Cooke PS, Buchanan DL, Young P, et al.** 1997 Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. Proc Natl Acad Sci U S A 94:6535-40

72. **Buchanan DL, Setiawan T, Lubahn DB, et al.** 1999 Tissue Compartment-Specific Estrogen Receptor-{alpha} Participation in the Mouse Uterine Epithelial Secretory Response. Endocrinology 140:484-491

73. **Cunha GR, Lung B** 1979 The importance of stroma in morphogenesis and functional activity of urogenital epithelium. In Vitro 15:50-71

74. **Kurita T, Lee K-j, Cooke PS, Taylor JA, Lubahn DB, Cunha GR** 2000 Paracrine Regulation of Epithelial Progesterone Receptor by Estradiol in the Mouse Female Reproductive Tract. Biol Reprod 62:821-830

75. **Weihua Z, Saji S, Makinen S, et al.** 2000 Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. Proc Natl Acad Sci U S A 97:5936-41.

76. **Couse JF, Hewitt SC, Bunch DO, et al.** 1999 Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. Science 286:2328-31

77. **Fisher CR, Graves KH, Parlow AF, Simpson ER** 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc Natl Acad Sci U S A 95:6965-70

78. **Vanacker J-M, Bonnelye E, Chopin-Delannoy S, Delmarre C, Cavailles V, Laudet V** 1999 Transcriptional Activities of the Orphan Nuclear Receptor ERR{alpha} (Estrogen Receptor-Related Receptor-{alpha}). Mol Endocrinol 13:764-773

79. **Shigeta H, Zuo W, Yang N, DiAugustine R, Teng CT** 1997 The mouse estrogen receptor-related orphan receptor alpha 1: molecular cloning and estrogen responsiveness. J Mol Endocrinol 19:299-309

80. **Sladek R, Bader J, Giguere V** 1997 The orphan nuclear receptor estrogen-related receptor alpha is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene. Mol. Cell. Biol. 17:5400-5409

81. **Zhang Z, Teng CT** 2001 Estrogen receptor [alpha] and estrogen receptor-related receptor [alpha]1 compete for binding and coactivator. Molecular and Cellular Endocrinology 172:223-233

82. **Tibbetts TA, Conneely OM, O'Malley BW** 1999 Progesterone via Its Receptor Antagonizes the Pro-Inflammatory Activity of Estrogen in the Mouse Uterus. Biol Reprod 60:1158-1165

83. **Spencer TE, Bazer FW** 2002 Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. Front Biosci 7:d1879-98

84. **Attia GR, Zeitoun K, Edwards D, Johns A, Carr BR, Bulun SE** 2000 Progesterone Receptor Isoform A But Not B Is Expressed in Endometriosis. J Clin Endocrinol Metab 85:2897-2902

85. **Petz LN, Ziegler YS, Loven MA, Nardulli AM** 2002 Estrogen Receptor {alpha} and Activating Protein-1 Mediate Estrogen Responsiveness of the Progesterone Receptor Gene in MCF-7 Breast Cancer Cells. Endocrinology 143:4583-4591

86. **Rider V, Wang MY, Finn C, Heap RB, Feinstein A** 1986 Anti-fertility effect of passive immunization against progesterone is influenced by genotype. J Endocrinol 108:117-21

87. **Moulton BC** 1979 Effect of progesterone on DNA, RNA and protein synthesis of deciduoma cell fractions separated by velocity sedimentation. Biol Reprod 21:667-72

88. **Jones SR, Kimler BF, Justice WM, Rider V** 2000 Transit of normal rat uterine stromal cells through G1 phase of the cell cycle requires progesterone-growth factor interactions. Endocrinology 141:637-48

89. **Tibbetts TA, Mendoza-Meneses M, O'Malley BW, Conneely OM** 1998 Mutual and intercompartmental regulation of estrogen receptor and progesterone receptor expression in the mouse uterus. Biol Reprod 59:1143-1152

90. **Armstrong DT, Moon YS, Leung PC** 1976 Uterotrophic effects of testosterone and 5alpha-dihydrotestosterone in intact and ovariectomized immature female rats. Biol Reprod 15:107-14

91. **Schmidt WN, Katzenellenbogen BS** 1979 Androgen-uterine interactions: an assessment of androgen interaction with the testosterone- and estrogen-receptor systems and stimulation of uterine growth and progesterone-receptor synthesis. Mol Cell Endocrinol 15:91-108

92. **Klocker H, Culig Z, Eder IE, et al.** 1999 Mechanism of androgen receptor activation and possible implications for chemoprevention trials. Eur Urol 35:413-9

93. **Sahlin L, Norstedt G, Eriksson H** 1994 Androgen regulation of the insulin-like growth factor-I and the estrogen receptor in rat uterus and liver. J Steroid Biochem Mol Biol 51:57-66

94. **Mena MA, Grunert G, Mansilla MS, et al.** 1985 Inhibition of non-genomic responses to oestrogen in the rat uterus by testosterone propionate. J Reprod Fertil 74:1-7

95. **Roy AK, Chatterjee B** 1995 Androgen action. Crit Rev Eukaryot Gene Expr 5:157-76

96. **Sahlin L, Wang H, Masironi B, Holmgren A, Eriksson H** 1999 Regulation of thioredoxin mRNA in the rat uterus by gonadal steroids. J Steroid Biochem Mol Biol 68:203-9

97. **Gunin AG, Emelianov VU, Tolmachev AS** 2003 Expression of estrogen receptor-alpha, glucocorticoid receptor, beta-catenin and glycogen synthase kinase-3beta in the uterus of mice following long-term treatment with estrogen and glucocorticoid hormones. Eur J Obstet Gynecol Reprod Biol 107:62-7

98. **Rabin DS, Johnson EO, Brandon DD, Liapi C, Chrousos GP** 1990 Glucocorticoids inhibit estradiol-mediated uterine growth: possible role of the uterine estradiol receptor. Biol Reprod 42:74-80

99. **Gunin AG, Mashin IN, Zakharov DA** 2001 Proliferation, mitosis orientation and morphogenetic changes in the uterus of mice following chronic treatment with both estrogen and glucocorticoid hormones. J Endocrinol 169:23-31.

100. **Bigsby RM, Young PC** 1993 Progesterone and dexamethasone inhibition of uterine epithelial cell proliferation: studies with antiprogesterone compounds in the neonatal mouse. J Steroid Biochem Mol Biol 46:253-7

101. **Bigsby RM, Cunha GR** 1985 Effects of progestins and glucocorticoids on deoxyribonucleic acid synthesis in the uterus of the neonatal mouse. Endocrinology 117:2520-6

102. **Campbell PS** 1978 The mechanism of the inhibition of uterotrophic responses by acute dexamethasone pretreatment. Endocrinology 103:716-23

103. **Sahlin L** 1995 Dexamethasone attenuates the estradiol-induced increase of IGF-I mRNA in the rat uterus. J Steroid Biochem Mol Biol 55:9-15

104. **Henderson TA, Saunders PTK, Moffett-King A, Groome NP, Critchley HOD** 2003 Steroid Receptor Expression in Uterine Natural Killer Cells. J Clin Endocrinol Metab 88:440-449

105. **Bamberger A-M, Milde-Langosch K, Loning T, Bamberger CM** 2001 The Glucocorticoid Receptor Is Specifically Expressed in the Stromal Compartment of the Human Endometrium. J Clin Endocrinol Metab 86:5071-

106. **Bucco RA, Zheng WL, Davis JT, et al.** 1997 Cellular retinoic acid-binding protein(II) presence in rat uterine epithelial cells correlates with their synthesis of retinoic acid. Biochemistry 36:4009-14

107. **Rexer BN, Ong DE** 2002 A novel short-chain alcohol dehydrogenase from rats with retinol dehydrogenase activity, cyclically expressed in uterine epithelium. Biol Reprod 67:1555-1564

108. **Marill J, Idres N, Capron CC, Nguyen E, Chabot GG** 2003 Retinoic acid metabolism and mechanism of action: a review. Curr Drug Metab 4:1-10

109. **Chambon P** 1996 A decade of molecular biology of retinoic acid receptors. Faseb J 10:940-54

110. **Kumarendran MK, Loughney AD, Prentice A, Thomas EJ, Redfern CP** 1996 Nuclear retinoid receptor expression in normal human endometrium throughout the menstrual cycle. Mol Hum Reprod 2:123-9

111. **Bucco RA, Zheng WL, Wardlaw SA, et al.** 1996 Regulation and localization of cellular retinol-binding protein, retinol-binding protein, cellular retinoic acid-binding protein (CRABP), and CRABP II in the uterus of the pseudopregnant rat. Endocrinology 137:3111-22

112. **Dong D, Ruuska SE, Levinthal DJ, Noy N** 1999 Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. J. Biol. Chem. 274:23695-23698

113. **Zheng WL, Sierra-Rivera E, Luan J, Osteen KG, Ong DE** 2000 Retinoic Acid Synthesis and Expression of Cellular Retinol-Binding Protein and Cellular Retinoic Acid-Binding Protein Type II Are Concurrent with Decidualization of Rat Uterine Stromal Cells. Endocrinology 141:802-808

114. **Gardner RM, Kirkland JL, Ireland JS, Stancel GM** 1978 Regulation of the uterine response to estrogen by thyroid hormone. Endocrinology 103:1164-72

115. **Keeping HS, Newcombe AM, Jellinck PH** 1982 Modulation of estrogen-induced peroxidase activity in the rat uterus by thyroid hormones. J Steroid Biochem 16:45-9

116. **Steinsapir J, Rojas AM, Mena M, Tchernitchin AN** 1982 Effects of thyroid hormone on some uterine responses to estrogen. Endocrinology 110:1773-9

117. **Vasudevan N, Ogawa S, Pfaff D** 2002 Estrogen and Thyroid Hormone Receptor Interactions: Physiological Flexibility by Molecular Specificity. Physiol. Rev. 82:923-944

118. **Levy J, Zuili I, Yankowitz N, Shany S** 1984 Induction of cytosolic receptors for 1 alpha, 25-dihydroxyvitamin D3 in the immature rat uterus by oestradiol. J Endocrinol 100:265-9

119. **Walters MR, Cuneo DL, Jamison AP** 1983 Possible significance of new target tissues for 1,25-dihydroxyvitamin D3. J Steroid Biochem 19:913-20

120. **Delissalde F, Hernandez MA, Barron A, et al.** 1998 [Vitamin D induces proliferation in rat endometrium cultured cells]. Rev Invest Clin 50:113-8

121. **Lieberherr M, Acker GM, Grosse B, Pesty A, Balsan S** 1984 Rat endometrial cells in primary culture: effects and interaction of sex hormones and vitamin D3 metabolite on alkaline phosphatase. Endocrinology 115:824-9

122. **Naciff JM, Jump ML, Torontali SM, et al.** 2002 Gene expression profile induced by 17alpha-ethynyl estradiol, bisphenol a, and genistein in the developing female reproductive system of the rat. Toxicol Sci 68:184-99

123. **Watanabe H, Suzuki A, Mizutani T, et al.** 2002 Genome-wide analysis of changes in early gene expression induced by oestrogen. Genes Cells 7:497-507

124. **Waters KM, Safe S, Gaido KW** 2001 Differential gene expression in response to methoxychlor and estradiol through ERalpha, ERbeta, and AR in reproductive tissues of female mice. Toxicol Sci 63:47-56.

125. **Rockett JC, Kavlock RJ, Lambright CR, et al.** 2002 DNA Arrays to Monitor Gene Expression in Rat Blood and Uterus following 17beta-Estradiol Exposure: Biomonitoring Environmental Effects Using Surrogate Tissues. Toxicol Sci 69:49-59

126. **Harris JN, Gorski J** 1978 Estrogen stimulation of DNA-dependent DNA polymerase activity in immature rat uterus. Mol Cell Endocrinol 10:293-305

127. **Samudio I, Vyhlidal C, Wang F, et al.** 2001 Transcriptional Activation of Deoxyribonucleic Acid Polymerase {{alpha}} Gene Expression in MCF-7 Cells by 17{{beta}}-Estradiol. Endocrinology 142:1000-1008

128. **Lobenhofer EK, Bennett L, Cable PL, Li L, Bushel PR, Afshari CA** 2002 Regulation of DNA Replication Fork Genes by 17{beta}-Estradiol. Mol Endocrinol 16:1215-1229

129. **Persico E, Scalona M, Cicatiello L, Sica V, Bresciani F, Weisz A** 1990 Activation of 'immediate-early' genes by estrogen is not sufficient to achieve stimulation of DNA synthesis in rat uterus. Biochem Biophys Res Commun 171:287-92

130. **Leung BS, Potter AH** 1987 Mode of estrogen action on cell proliferation in CAMA-1 cells: II. Sensitivity of G1 phase population. J Cell Biochem 34:213-25

131. **Bourtourault M, Mahoudo H, Haras D, Samperez S, Jouan P** 1984 Stimulation by estradiol-17 beta of thymidine kinase activity in the rat uterus. J Steroid Biochem 21:613-20

132. **Sakamoto S, Sawasaki Y, Kudo H, Suzuki S, Sawaki K, Nagasawa H** 1992 Effects of oestrogen on mitochondrial thymidine kinase activity in the immature rat uterus. In Vivo 6:157-60

133. **Kasid A, Davidson NE, Gelmann EP, Lippman ME** 1986 Transcriptional control of thymidine kinase gene expression by estrogen and antiestrogens in MCF-7 human breast cancer cells. J Biol Chem 261:5562-7

134. **Xie W, Duan R, Safe S** 1999 Estrogen induces adenosine deaminase gene expression in MCF-7 human breast cancer cells: role of estrogen receptor-Sp1 interactions. Endocrinology 140:219-27

135. **Sivridis E, Giatromanolaki A, Koukourakis MI, Bicknell R, Harris AL, Gatter KC** 2000 Thymidine phosphorylase expression in normal and hyperplastic endometrium. J Clin Pathol 53:704-708

136. **Rees M, Hague S, Oehler MK, Bicknell R** 1999 Regulation of endometrial angiogenesis. Climacteric 2:52-8

137. **Fujimoto J, Ichigo S, Sakaguchi H, Hirose R, Tamaya T** 1998 Expression of platelet-derived endothelial cell growth factor and its mRNA in uterine endometrium during the menstrual cycle. Mol Hum Reprod 4:509-13

138. **DiBattista WJ, Nishikawara MT, Leung YF** 1978 Activity of enzymes involved in pyrimidine nucleotide synthesis during uterine growth in cyclic rats. J Reprod Fertil 54:249-53

139. **Guo JZ, Gorski J** 1989 Estrogen effects on modifications of chromatin proteins in the rat uterus. J Steroid Biochem 32:13-20

140. **Cavailles V** 2002 Estrogens and receptors: an evolving concept. Climacteric 5 Suppl 2:20-6

141. **Guo JZ, Gorski J** 1988 Differential stimulation of histone and high mobility group chromatin protein synthesis in the rat uterus by estrogen. Mol Endocrinol 2:686-92

142. **Guo JZ, Gorski J** 1988 Estrogen effects on histone messenger ribonucleic acid levels in the rat uterus. Mol Endocrinol 2:693-700

143. **Plumb M, Stein J, Stein G** 1983 Coordinate regulation of multiple histone mRNAs during the cell cycle in HeLa cells. Nucleic Acids Res 11:2391-410

144. **Trachewsky D, Segal SJ** 1967 Selective stimulation of ribonucleic acid synthesis in uterine nuclei by estradiol-17-beta. Biochem Biophys Res Commun 27:588-94

145. **McCormack SA, Glasser SR** 1980 Differential response of individual uterine cell types from immature rats treated with estradiol. Endocrinology 106:1634-49

146. **Shiozawa T, Shih H-C, Miyamoto T, et al.** 2003 Cyclic Changes in the Expression of Steroid Receptor Coactivators and Corepressors in the Normal Human Endometrium. J Clin Endocrinol Metab 88:871-878

147. **Wieser F, Schneeberger C, Hudelist G, et al.** 2002 Endometrial nuclear receptor co-factors SRC-1 and N-CoR are increased in human endometrium during menstruation. Mol Hum Reprod 8:644-50

148. **Travers MT, Knowler JT** 1987 Oestrogen-induced expression of oncogenes in the immature rat uterus. FEBS Lett 211:27-30

149. **Murphy LJ, Murphy LC, Friesen HG** 1987 Estrogen induces insulin-like growth factor-I expression in the rat uterus. Mol Endocrinol 1:445-50

150. **Weisz A, Bresciani F** 1988 Estrogen induces expression of c-fos and c-myc protooncogenes in rat uterus. Mol Endocrinol 2:816-24

151. **Zheng X, Hendry WJ, 3rd** 1997 Neonatal diethylstilbestrol treatment alters the estrogen-regulated expression of both cell proliferation and apoptosis-related proto-oncogenes (c-jun, c-fos, c-myc, bax, bcl-2, and bcl-x) in the hamster uterus. Cell Growth Differ 8:425-34

152. **Weisz A, Cicatiello L, Persico E, Scalona M, Bresciani F** 1990 Estrogen stimulates transcription of c-jun protooncogene. Mol Endocrinol 4:1041-50

153. **Webb DK, Moulton BC, Khan SA** 1990 Estrogen induced expression of the C-jun proto-oncogene in the immature and mature rat uterus. Biochem Biophys Res Commun 168:721-6

154. **Chiappetta C, Kirkland JL, Loose-Mitchell DS, Murthy L, Stancel GM** 1992 Estrogen regulates expression of the jun family of protooncogenes in the uterus. J Steroid Biochem Mol Biol 41:113-23

155. **Nephew KP, Peters GA, Khan SA** 1995 Cellular localization of estradiol-induced c-fos messenger ribonucleic acid in the rat uterus: c-fos expression and uterine cell proliferation do not correlate strictly. Endocrinology 136:3007-15

156. **Shaulian E, Karin M** 2001 AP-1 in cell proliferation and survival. Oncogene 20:2390-400

157. **Hyder SM, Stancel GM, Nawaz Z, McDonnell DP, Loose-Mitchell DS** 1992 Identification of an estrogen response element in the 3'-flanking region of the murine c-fos protooncogene. J Biol Chem 267:18047-54

158. **Cicatiello L, Sica V, Bresciani F, Weisz A** 1993 Identification of a specific pattern of "immediate-early" gene activation induced by estrogen during mitogenic stimulation of rat uterine cells. Receptor 3:17-30

159. **Nephew KP, Tang M, Khan SA** 1994 Estrogen differentially affects c-jun expression in uterine tissue compartments. Endocrinology 134:1827-34

160. **Cicatiello L, Ambrosino C, Coletta B, et al.** 1992 Transcriptional activation of jun and actin genes by estrogen during mitogenic stimulation of rat uterine cells. J Steroid Biochem Mol Biol 41:523-8

161. **Teyssier C, Belguise K, Galtier F, Chalbos D** 2001 Characterization of the Physical Interaction between Estrogen Receptor alpha and JUN Proteins. J. Biol. Chem. 276:36361-36369

162. **Schorl C, Sedivy JM** 2003 Loss of Protooncogene c-Myc Function Impedes G1 Phase Progression Both before and after the Restriction Point. Mol. Biol. Cell 14:823-835

163. **Levens D** 2002 Disentangling the MYC web. PNAS 99:5757-5759

164. **O'Hagan RC, Schreiber-Agus N, Chen K, et al.** 2000 Gene-target recognition among members of the myc superfamily and implications for oncogenesis. Nat Genet 24:113-9

165. **Menssen A, Hermeking H** 2002 From the Cover: Characterization of the c-MYC-regulated transcriptome by SAGE: Identification and analysis of c-MYC target genes. PNAS 99:6274-6279

166. **Galaktionov K, Chen X, Beach D** 1996 Cdc25 cell-cycle phosphatase as a target of c-myc. Nature 382:511-7

167. **Auvinen M, Jarvinen K, Hotti A, et al.** 2003 Transcriptional regulation of the ornithine decarboxylase gene by c-Myc/Max/Mad network and retinoblastoma protein interacting with c-Myc. The International Journal of Biochemistry & Cell Biology 35:496-521

168. **Gartel AL, Shchors K** 2003 Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. Experimental Cell Research 283:17-21

169. **Guo QM, Malek RL, Kim S, et al.** 2000 Identification of c-Myc Responsive Genes Using Rat cDNA Microarray. Cancer Res 60:5922-5928

170. **Coller HA, Grandori C, Tamayo P, et al.** 2000 Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. Proc Natl Acad Sci U S A 97:3260-5.

171. **Schuhmacher M, Kohlhuber F, Holzel M, et al.** 2001 The transcriptional program of a human B cell line in response to Myc. Nucl. Acids. Res. 29:397-406

172. **Huet-Hudson YM, Andrews GK, Dey SK** 1989 Cell type-specific localization of c-myc protein in the mouse uterus: modulation by steroid hormones and analysis of the periimplantation period. Endocrinology 125:1683-90

173. **Murphy LJ, Murphy LC, Friesen HG** 1987 Estrogen induction of N-myc and c-myc proto-oncogene expression in the rat uterus. Endocrinology 120:1882-8

174. **Wu L, Timmers C, Maiti B, et al.** 2001 The E2F1-3 transcription factors are essential for cellular proliferation. Nature 414:457-62

175. **Stanelle J, Stiewe T, Theseling CC, Peter M, Putzer BM** 2002 Gene expression changes in response to E2F1 activation. Nucl. Acids. Res. 30:1859-1867

176. **Safe S** 2001 Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. Vitam Horm 62:231-52

177. **Berg BH** 1977 The early influence of 17-beta-oestradiol on 17 aminoacyl-tRNA synthetases of mouse uterus and liver. Phosphorylation as a regulation mechanism. Biochim Biophys Acta 479:152-71

178. **Lutz WH, Barker KL** 1986 Effect of estradiol on the amino acid-accepting activity of uterine tRNAs and their participation in protein synthesis. J Biol Chem 261:11230-5

179. **Whelly SM, Barker KL** 1985 Inhibition of the aminoacylation of selected tRNA molecules by an estrogen-regulated factor on uterine ribosomes. Regulation of aminoacylation of tRNA by estrogens. Eur J Biochem 146:245-53

180. **Pratt W** 1998 The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. Proc Soc Exp Biol Med 217:420-434

181. **Knoblauch R, Garabedian MJ** 1999 Role for Hsp90-Associated Cochaperone p23 in Estrogen Receptor Signal Transduction. Mol. Cell. Biol. 19:3748-3759

182. **Komatsu T, Konishi I, Fukumoto M, et al.** 1997 Messenger Ribonucleic Acid Expression of Heat Shock Proteins HSP70 and HSP90 in Human Endometrium and Myometrium during the Menstrual Cycle. J Clin Endocrinol Metab 82:1385-1389

183. **Rivera-Gonzalez R, Petersen DN, Tkalcevic G, Thompson DD, Brown TA** 1998 Estrogen-induced genes in the uterus of ovariectomized rats and their regulation by droloxifene and tamoxifen. J Steroid Biochem Mol Biol 64:13-24

184. **Shyamala G, Gauthier Y, Moore SK, Catelli MG, Ullrich SJ** 1989 Estrogenic regulation of murine uterine 90-kilodalton heat shock protein gene expression. Mol Cell Biol 9:3567-70

185. **Simmons DG, Kennedy TG** 2000 Induction of Glucose-Regulated Protein 78 in Rat Uterine Glandular Epithelium During Uterine Sensitization for the Decidual Cell Reaction. Biol Reprod 62:1168-1176

186. **Yang X, Dale EC, Diaz J, Shyamala G** 1995 Estrogen dependent expression of heat shock transcription factor: implications for uterine synthesis of heat shock proteins. J Steroid Biochem Mol Biol 52:415-9

187. **Padwick ML, Whitehead M, King RJ** 1994 Hormonal regulation of HSP27 expression in human endometrial epithelial and stromal cells. Mol Cell Endocrinol 102:9-14

188. **Porter W, Saville B, Hoivik D, Safe S** 1997 Functional Synergy between the Transcription Factor Sp1 and the Estrogen Receptor. Mol Endocrinol 11:1569-1580

189. **Clark BF** 1971 The effects of oestrogen and progesterone on uterine cell division and epithelial morphology in spayed, adrenalectomized rats. J Endocrinol 50:527-8

190. **Prall OW, Sarcevic B, Musgrove EA, Watts CK, Sutherland RL** 1997 Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. J Biol Chem 272:10882-94

191. **Whitfield ML, Sherlock G, Saldanha AJ, et al.** 2002 Identification of Genes Periodically Expressed in the Human Cell Cycle and Their Expression in Tumors. Mol. Biol. Cell 13:1977-2000

192. **van der Meijden CMJ, Lapointe DS, Luong MX, et al.** 2002 Gene Profiling of Cell Cycle Progression through S-Phase Reveals Sequential Expression of Genes Required for DNA Replication and Nucleosome Assembly. Cancer Res 62:3233-3243

193. **Cho RJ, Huang M, Campbell MJ, et al.** 2001 Transcriptional regulation and function during the human cell cycle. Nat Genet 27:48-54

194. **Polager S, Kalma Y, Berkovich E, Ginsberg D** 2002 E2Fs up-regulate expression of genes involved in DNA replication, DNA repair and mitosis. Oncogene 21:437-46

195. **Ishida S, Huang E, Zuzan H, et al.** 2001 Role for E2F in Control of Both DNA Replication and Mitotic Functions as Revealed from DNA Microarray Analysis. Mol. Cell. Biol. 21:4684-4699

196. **Muller H, Bracken AP, Vernell R, et al.** 2001 E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev. 15:267-285

197. **Lai MD, Lee LR, Cheng KS, Wing LY** 2000 Expression of proliferating cell nuclear antigen in luminal epithelium during the growth and regression of rat uterus. J Endocrinol 166:87-93

198. **Stack G, Gorski J** 1985 Estrogen-stimulated deoxyribonucleic acid synthesis: a ratchet model for the prereplicative period. Endocrinology 117:2017-23

199. **Prall OWJ, Rogan EM, Musgrove EA, Watts CKW, Sutherland RL** 1998 c-Myc or Cyclin D1 Mimics Estrogen Effects on Cyclin E-Cdk2 Activation and Cell Cycle Reentry. Mol. Cell. Biol. 18:4499-4508

200. **Trowbridge JM, Rogatsky I, Garabedian MJ** 1997 Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex. PNAS 94:10132-10137

201. **Fero ML, Rivkin M, Tasch M, et al.** 1996 A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell 85:733-44

202. **Zhao J, Dynlacht B, Imai T, Hori T-a, Harlow E** 1998 Expression of NPAT, a novel substrate of cyclin E-CDK2, promotes S-phase entry. Genes Dev. 12:456-461

203. **Krek W, Xu G, Livingston DM** 1995 Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. Cell 83:1149-58

204. **Motokura T, Arnold A** 1993 PRAD1/cyclin D1 proto-oncogene: genomic organization, 5' DNA sequence, and sequence of a tumor-specific rearrangement breakpoint. Genes Chromosomes Cancer 7:89-95

205. **Marino M, Acconcia F, Bresciani F, Weisz A, Trentalance A** 2002 Distinct Nongenomic Signal Transduction Pathways Controlled by 17beta -Estradiol Regulate DNA Synthesis and Cyclin D1 Gene Transcription in HepG2 Cells. Mol. Biol. Cell 13:3720-3729

206. **Pestell RG, Albanese C, Reutens AT, Segall JE, Lee RJ, Arnold A** 1999 The Cyclins and Cyclin-Dependent Kinase Inhibitors in Hormonal Regulation of Proliferation and Differentiation. Endocr Rev 20:501-534

207. **Ciemerych MA, Kenney AM, Sicinska E, et al.** 2002 Development of mice expressing a single D-type cyclin. Genes Dev. 16:3277-3289

208. **Altucci L, Addeo R, Cicatiello L, et al.** 1997 Estrogen induces early and timed activation of cyclin-dependent kinases 4, 5, and 6 and increases cyclin messenger ribonucleic acid expression in rat uterus. Endocrinology 138:978-84

209. **Hermeking H, Lengauer C, Polyak K, et al.** 1997 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell 1:3-11

210. **Orimo A, Inoue S, Ikeda K, Noji S, Muramatsu M** 1995 Molecular cloning, structure, and expression of mouse estrogen-responsive finger protein Efp. Co-localization with estrogen receptor mRNA in target organs. J Biol Chem 270:24406-13

211. **Orimo A, Inoue S, Minowa O, et al.** 1999 Underdeveloped uterus and reduced estrogen responsiveness in mice with disruption of the estrogen-responsive finger protein gene, which is a direct target of estrogen receptor alpha. Proc Natl Acad Sci U S A 96:12027-32

212. **Ferrero M, Cairo G** 1993 Estrogen-regulated expression of a growth arrest specific gene (gas-1) in rat uterus. Cell Biology International 17:857-862

213. **Zhuang Y-H, Sarca D, Weisz A, et al.** 2001 Cell type-specific induction of cyclin D and cyclin-dependent kinase inhibitor p27kip1 expression by estrogen in rat endometrium. The Journal of Steroid Biochemistry and Molecular Biology 78:193-199

214. **Tanaka M, Kyo S, Takakura M, et al.** 1998 Expression of Telomerase Activity in Human Endometrium Is Localized to Epithelial Glandular Cells and Regulated in a Menstrual Phase-Dependent Manner Correlated with Cell Proliferation. Am J Pathol 153:1985-1991

215. **Kyo S, Takakura M, Kanaya T, et al.** 1999 Estrogen Activates Telomerase. Cancer Res 59:5917-5921

216. **Bigsby RM, Cunha GR** 1986 Estrogen stimulation of deoxyribonucleic acid synthesis in uterine epithelial cells which lack estrogen receptors. Endocrinology 119:390-6

217. **Pierro E, Minici F, Alesiani O, et al.** 2001 Stromal-Epithelial Interactions Modulate Estrogen Responsiveness in Normal Human Endometrium. Biol Reprod 64:831-838

218. **Cunha GR, Young P** 1992 Role of stroma in oestrogen-induced epithelial proliferation. Epithelial Cell Biol 1:18-31

219. **Wiesen J, Young P, Werb Z, Cunha G** 1999 Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. Development 126:335-344

220. **Bigsby RM** 2002 Control of Growth and Differentiation of the Endometrium: The Role of Tissue Interactions. Ann NY Acad Sci 955:110-117

221. **Stiles CD, Capone GT, Scher CD, Antoniades HN, Van Wyk JJ, Pledger WJ** 1979 Dual control of cell growth by somatomedins and platelet-derived growth factor. Proc Natl Acad Sci U S A 76:1279-83

222. **Pardee AB** 1989 G1 events and regulation of cell proliferation. Science 246:603-8

223. **Adesanya OO, Zhou J, Samathanam C, Powell-Braxton L, Bondy CA** 1999 Insulin-like growth factor 1 is required for G2 progression in the estradiol-induced mitotic cycle. PNAS 96:3287-3291

224. **Won K, Xiong Y, Beach D, Gilman M** 1992 Growth-Regulated Expression of D-Type Cyclin Genes in Human Diploid Fibroblasts. PNAS 89:9910-9914

225. **Elder PK, Schmidt LJ, Ono T, Getz MJ** 1984 Specific stimulation of actin gene transcription by epidermal growth factor and cycloheximide. Proc Natl Acad Sci U S A 81:7476-80

226. **Lewis EJ, Chikaraishi DM** 1987 Regulated expression of the tyrosine hydroxylase gene by epidermal growth factor. Mol Cell Biol 7:3332-6

227. **Earp HS, Dawson TL, Li X, Yu H** 1995 Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. Breast Cancer Res Treat 35:115-32

228. **Nelson KG, Takahashi T, Bossert NL, Walmer DK** 1991 Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth factor and differentiation. Proceedings of the National Academy of Science 88:21-25

229. **Ignar-Trowbridge D, Nelson K, Bidwell M, et al.** 1992 Coupling of Dual Signaling Pathways: Epidermal Growth Factor Action Involves the Estrogen Receptor. PNAS 89:4658-4662

230. **Curtis SW, Washburn T, Sewall C, et al.** 1996 Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. Proc Natl Acad Sci U S A 93:12626-30

231. **Hom YK, Young P, Wiesen JF, et al.** 1998 Uterine and Vaginal Organ Growth Requires Epidermal Growth Factor Receptor Signaling from Stroma. Endocrinology 139:913-921

232. **Tomooka Y, DiAugustine RP, McLachlan JA** 1986 Proliferation of mouse uterine epithelial cells in vitro. Endocrinology 118:1011-8

233. **Lingham RB, Stancel GM, Loose-Mitchell DS** 1988 Estrogen regulation of epidermal growth factor receptor messenger ribonucleic acid. Mol Endocrinol 2:230-5

234. **Mukku VR, Stancel GM** 1985 Regulation of epidermal growth factor receptor by estrogen. J Biol Chem 260:9820-4

235. **Tong BJ, Das SK, Threadgill D, Magnuson T, Dey SK** 1996 Differential expression of the full-length and truncated forms of the epidermal growth factor receptor in the preimplantation mouse uterus and blastocyst. Endocrinology 137:1492-6

236. **DiAugustine RP, Petrusz P, Bell GI, et al.** 1988 Influence of estrogens on mouse uterine epidermal growth factor precursor protein and messenger ribonucleic acid. Endocrinology 122:2355-63

237. **Lim H, Dey SK, Das SK** 1997 Differential Expression of the erbB2 Gene in the Periimplantation Mouse Uterus: Potential Mediator of Signaling by Epidermal Growth Factor-Like Growth Factors. Endocrinology 138:1328-1337

238. **Levin ER** 2003 Bidirectional Signaling between the Estrogen Receptor and the Epidermal Growth Factor Receptor. Mol Endocrinol 17:309-317

239. **Lin SY, Makino K, Xia W, et al.** 2001 Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat Cell Biol 3:802-8

240. **Prenzel N, Zwick E, Daub H, et al.** 1999 EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402:884-8

241. **Brissenden JE, Derynck R, Francke U** 1985 Mapping of transforming growth factor alpha gene on human chromosome 2 close to the breakpoint of the Burkitt's lymphoma t(2;8) variant translocation. Cancer Res 45:5593-7

242. **Nelson KG, Takahashi T, Lee DC, et al.** 1992 Transforming growth factor-alpha is a potential mediator of estrogen action in the mouse uterus. Endocrinology 131:1657-64

243. **Clarke R, Brunner N, Katz D, et al.** 1989 The effects of a constitutive expression of transforming growth factor-alpha on the growth of MCF-7 human breast cancer cells in vitro and in vivo. Mol Endocrinol 3:372-80

244. **Gray K, Bullock B, Dickson R, et al.** 1996 Potentiation of diethylstilbestrol-induced alterations in the female mouse reproductive tract by transforming growth factor-alpha transgene expression. Mol Carcinog 17:163-73

245. **Das SK, Lim H, Wang J, Paria BC, BazDresch M, Dey SK** 1997 Inappropriate expression of human transforming growth factor (TGF)-alpha in the uterus of transgenic mouse causes downregulation of TGF-beta receptors and delays the blastocyst-attachment reaction. J Mol Endocrinol 18:243-57

246. **Coffey RJ, Jr., Derynck R, Wilcox JN, et al.** 1987 Production and auto-induction of transforming growth factor-alpha in human keratinocytes. Nature 328:817-20

247. **Luetteke NC, Qiu TH, Peiffer RL, Oliver P, Smithies O, Lee DC** 1993 TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. Cell 73:263-78

248. **Tamada H, Das SK, Andrews GK, Dey SK** 1991 Cell-type-specific expression of transforming growth factor-alpha in the mouse uterus during the peri-implantation period. Biol Reprod 45:365-72

249. **Wang HS, Chard T** 1999 IGFs and IGF-binding proteins in the regulation of human ovarian and endometrial function. J Endocrinol 161:1-13

250. **Sato T, Wang G, Hardy MP, Kurita T, Cunha GR, Cooke PS** 2002 Role of Systemic and Local IGF-I in the Effects of Estrogen on Growth and Epithelial Proliferation of Mouse Uterus. Endocrinology 143:2673-2679

251. **Adesanya O, Zhou J, Bondy C** 1996 Sex steroid regulation of insulin-like growth factor system gene expression and proliferation in primate myometrium. J Clin Endocrinol Metab 81:1967-1974

252. **Baker J, Hardy MP, Zhou J, et al.** 1996 Effects of an Igf1 gene null mutation on mouse reproduction. Mol Endocrinol 10:903-18

253. **Rutanen EM** 1998 Insulin-like growth factors in endometrial function. Gynecol Endocrinol 12:399-406

254. **Klotz DM, Hewitt SC, Korach KS, Diaugustine RP** 2000 Activation of a uterine insulin-like growth factor I signaling pathway by clinical and environmental estrogens: requirement of estrogen receptor-alpha. Endocrinology 141:3430-9

255. **Kapur S, Tamada H, Dey S, Andrews G** 1992 Expression of insulin-like growth factor-I (IGF-I) and its receptor in the peri-implantation mouse uterus, and cell-specific regulation of IGF- I gene expression by estradiol and progesterone. Biol Reprod 46:208-219

256. **Klotz DM, Hewitt SC, Ciana P, et al.** 2002 Requirement of estrogen receptor-alpha in insulin-like growth factor-1 (IGF-1)-induced uterine responses and in vivo evidence for IGF-1/estrogen receptor cross-talk. J Biol Chem 277:8531-7

257. **Molnar P, Murphy LJ** 1994 Effects of oestrogen on rat uterine expression of insulin-like growth factor-binding proteins. J Mol Endocrinol 13:59-67

258. **Rajkumar K, Dheen T, Krsek M, Murphy LJ** 1996 Impaired estrogen action in the uterus of insulin-like growth factor binding protein-1 transgenic mice. Endocrinology 137:1258-64

259. **Letterio JJ, Bottinger EP** 1998 TGF-beta knockout and dominant-negative receptor transgenic mice. Miner Electrolyte Metab 24:161-7

260. **Lutz M, Knaus P** 2002 Integration of the TGF-[beta] pathway into the cellular signalling network. Cellular Signalling 14:977-988

261. **Yamanouchi K, Soeta C, Harada R, Naito K, Tojo H** 1999 Endometrial expression of cellular protooncogene c-ski and its regulation by estradiol-17beta. FEBS Lett 449:273-6

262. **Pessah M, Marais J, Prunier C, et al.** 2002 c-Jun Associates with the Oncoprotein Ski and Suppresses Smad2 Transcriptional Activity. J. Biol. Chem. 277:29094-29100

263. **Sun Y, Liu X, Eaton EN, Lane WS, Lodish HF, Weinberg RA** 1999 Interaction of the Ski oncoprotein with Smad3 regulates TGF-beta signaling. Mol Cell 4:499-509

264. **Feng XH, Liang YY, Liang M, Zhai W, Lin X** 2002 Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-beta-mediated induction of the CDK inhibitor p15(Ink4B). Mol Cell 9:133-43

265. **Takahashi T, Eitzman B, Bossert NL, et al.** 1994 Transforming growth factors beta 1, beta 2, and beta 3 messenger RNA and protein expression in mouse uterus and vagina during estrogen-induced growth: a comparison to other estrogen-regulated genes. Cell Growth Differ 5:919-35

266. **Das SK, Flanders KC, Andrews GK, Dey SK** 1992 Expression of transforming growth factor-beta isoforms (beta 2 and beta 3) in the mouse uterus: analysis of

the periimplantation period and effects of ovarian steroids. Endocrinology 130:3459-66

267. **Schneider SL, Gollnick SO, Grande C, Pazik JE, Tomasi TB** 1996 Differential regulation of TGF-[beta]2 by hormones in rat uterus and mammary gland. Journal of Reproductive Immunology 32:125-144

268. **Lu D, Giguere V** 2001 Requirement of Ras-Dependent Pathways for Activation of the Transforming Growth Factor {beta}3 Promoter by Estradiol. Endocrinology 142:751-759

269. **Wada K, Nomura S, Morii E, et al.** 1996 Changes in levels of mRNAs of transforming growth factor (TGF)-beta1, -beta2, -beta3, TGF-beta type II receptor and sulfated glycoprotein-2 during apoptosis of mouse uterine epithelium. J Steroid Biochem Mol Biol 59:367-75

270. **Ornitz DM, Itoh N** 2001 Fibroblast growth factors. Genome Biol 2:REVIEWS3005

271. **Tsai S-J, Wu M-H, Chen H-M, Chuang P-C, Wing L-YC** 2002 Fibroblast Growth Factor-9 Is an Endometrial Stromal Growth Factor. Endocrinology 143:2715-2721

272. **Ka H, Jaeger LA, Johnson GA, Spencer TE, Bazer FW** 2001 Keratinocyte Growth Factor Is Up-Regulated by Estrogen in the Porcine Uterine Endometrium and Functions in Trophectoderm Cell Proliferation and Differentiation. Endocrinology 142:2303-2310

273. **Hom YK, Young P, Thomson AA, Cunha GR** 1998 Keratinocyte Growth Factor Injected into Female Mouse Neonates Stimulates Uterine and Vaginal Epithelial Growth. Endocrinology 139:3772-3779

274. **Guo L, Degenstein L, Fuchs E** 1996 Keratinocyte growth factor is required for hair development but not for wound healing. Genes Dev 10:165-75

275. **Rider V, Carlone DL, Foster RT** 1997 Oestrogen and progesterone control basic fibroblast growth factor mRNA in the rat uterus. J Endocrinol 154:75-84

276. **Reynolds LP, Kirsch JD, Kraft KC, Redmer DA** 1998 Time-course of the uterine response to estradiol-17ß in ovariectomized ewes: expression of angiogenic factors. Biol Reprod 59:613-620

277. **Fujimoto J, Hori M, Ichigo S, Tamaya T** 1997 Ovarian steroids regulate the expression of basic fibroblast growth factor and its mRNA in fibroblasts derived from uterine endometrium. Ann Clin Biochem 34 (Pt 1):91-6

278. **Pavelock K, Braas KM, Ouafik LH, Osol G, May V** 2001 Differential Expression and Regulation of the Vascular Endothelial Growth Factor Receptors Neuropilin-1 and Neuropilin-2 in Rat Uterus. Endocrinology 142:613-622

279. **Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M** 1998 Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92:735-45

280. **Mueller MD, Lebovic DI, Garrett E, Taylor RN** 2000 Neutrophils infiltrating the endometrium express vascular endothelial growth factor: potential role in endometrial angiogenesis. Fertility and Sterility 74:107-112

281. **Hyder SM** 2002 The Role of Steroid Hormones on the Regulation of Vascular Endothelial Growth Factor. Am J Pathol 161:345-346

282. **Ma W, Tan J, Matsumoto H, et al.** 2001 Adult tissue angiogenesis: evidence for negative regulation by estrogen in the uterus. Mol Endocrinol 15:1983-92

283. **Gray K, Eitzman B, Raszmann K, et al.** 1995 Coordinate regulation by diethylstilbestrol of the platelet-derived growth factor-A (PDGF-A) and -B chains and the PDGF receptor alpha- and beta-subunits in the mouse uterus and vagina: potential mediators of estrogen action. Endocrinology 136:2325-40

284. **Chegini N, Rossi MJ, Masterson BJ** 1992 Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and EGF and PDGF beta-receptors in human endometrial tissue: localization and in vitro action. Endocrinology 130:2373-85

285. **Rossi MJ, Chegini N, Masterson BJ** 1992 Presence of epidermal growth factor, platelet-derived growth factor, and their receptors in human myometrial tissue and smooth muscle cells: their action in smooth muscle cells in vitro. Endocrinology 130:1716-27

286. **Barbarisi A, Petillo O, Di Lieto A, et al.** 2001 17-beta estradiol elicits an autocrine leiomyoma cell proliferation: evidence for a stimulation of protein kinase-dependent pathway. J Cell Physiol 186:414-24

287. **Terranova PF, Hunter VJ, Roby KF, Hunt JS** 1995 Tumor necrosis factor-alpha in the female reproductive tract. Proc Soc Exp Biol Med 209:325-42

288. **Roby KF, Hunt JS** 1995 Myometrial tumor necrosis factor alpha: cellular localization and regulation by estradiol and progesterone in the mouse. Biol Reprod 52:509-15

289. **Hunt JS, Miller L, Roby KF, Huang J, Platt JS, DeBrot BL** 1997 Female steroid hormones regulate production of pro-inflammatory molecules in uterine leukocytes. J Reprod Immunol 35:87-99

290. **von Wolff M, Classen-Linke I, Heid D, et al.** 1999 Tumour necrosis factor-alpha (TNF-alpha) in human endometrium and uterine secretion: an evaluation by immunohistochemistry, ELISA and semiquantitative RT-PCR. Mol Hum Reprod 5:146-52

291. **Danforth DN, Jr., Sgagias MK** 1993 Tumour necrosis factor-alpha modulates oestradiol responsiveness of MCF-7 breast cancer cells in vitro. J Endocrinol 138:517-28

292. **Roby KF, Hunt JS** 1994 Mouse endometrial tumor necrosis factor-alpha messenger ribonucleic acid and protein: localization and regulation by estradiol and progesterone. Endocrinology 135:2780-9

293. **Tabibzadeh S, Satyaswaroop PG, von Wolff M, Strowitzki T** 1999 Regulation of TNF-alpha mRNA expression in endometrial cells by TNF-alpha and by oestrogen withdrawal. Mol Hum Reprod 5:1141-9

294. **Roby KF, Laham N, Hunt JS** 1996 Cellular localization and steroid hormone regulation of mRNA encoding tumour necrosis factor receptor I in mouse uterus. J Reprod Fertil 106:285-90

295. **Klampfer L, Lee TH, Hsu W, Vilcek J, Chen-Kiang S** 1994 NF-IL6 and AP-1 cooperatively modulate the activation of the TSG-6 gene by tumor necrosis factor alpha and interleukin-1. Mol Cell Biol 14:6561-9

296. **Moussad EE-DA, Brigstock DR** 2000 Connective Tissue Growth Factor: What's in a Name? Molecular Genetics and Metabolism 71:276-292

297. **Inadera H, Dong H-Y, Matsushima K** 2002 WISP-2 is a secreted protein and can be a marker of estrogen exposure in MCF-7 cells. Biochemical and Biophysical Research Communications 294:602-608

298. **Inadera H, Hashimoto S-i, Dong H-Y, et al.** 2000 WISP-2 as a Novel Estrogen-Responsive Gene in Human Breast Cancer Cells. Biochemical and Biophysical Research Communications 275:108-114

299. **Kothapalli D, Grotendorst GR** 2000 CTGF modulates cell cycle progression in cAMP-arrested NRK fibroblasts. J Cell Physiol 182:119-26

300. **Rageh MA, Moussad EE, Wilson AK, Brigstock DR** 2001 Steroidal regulation of connective tissue growth factor (CCN2; CTGF) synthesis in the mouse uterus. Mol Pathol 54:338-46

301. **Lau L, Nathans D** 1985 Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. EMBO J. 4:3145-3151

302. **Sampath D, Winneker RC, Zhang Z** 2001 Cyr61, a Member of the CCN Family, Is Required for MCF-7 Cell Proliferation: Regulation by 17{beta}-Estradiol and Overexpression in Human Breast Cancer. Endocrinology 142:2540-2548

303. **Chen C-C, Mo F-E, Lau LF** 2001 The Angiogenic Factor Cyr61 Activates a Genetic Program for Wound Healing in Human Skin Fibroblasts. J. Biol. Chem. 276:47329-47337

304. **Babic AM, Kireeva ML, Kolesnikova TV, Lau LF** 1998 CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. PNAS 95:6355-6360

305. **Mo F-E, Muntean AG, Chen C-C, Stolz DB, Watkins SC, Lau LF** 2002 CYR61 (CCN1) Is Essential for Placental Development and Vascular Integrity. Mol. Cell. Biol. 22:8709-8720

306. **Schipper RG, Penning LC, Verhofstad AA** 2000 Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? Semin Cancer Biol 10:55-68

307. **Pegg AE** 1986 Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J 234:249-62

308. **Schipper RG, Verhofstad AAJ** 2002 Distribution Patterns of Ornithine Decarboxylase in Cells and Tissues: Facts, Problems, and Postulates. J. Histochem. Cytochem. 50:1143-1160

309. **Kaczmarek L, Calabretta B, Ferrari S, de Riel JK** 1987 Cell-cycle-dependent expression of human ornithine decarboxylase. J Cell Physiol 132:545-51

310. **Packham G, Cleveland JL** 1997 Induction of ornithine decarboxylase by IL-3 is mediated by sequential c-Myc-independent and c-Myc-dependent pathways. Oncogene 15:1219-32

311. **Wu S, Pena A, Korcz A, Soprano DR, Soprano KJ** 1996 Overexpression of Mxi1 inhibits the induction of the human ornithine decarboxylase gene by the Myc/Max protein complex. Oncogene 12:621-9

312. **Reiss NA, Kaye AM** 1981 Identification of the major component of the estrogen-induced protein of rat uterus as the BB isozyme of creatine kinase. J Biol Chem 256:5741-9

313. **Reiss NA** 1988 Ontogeny and estrogen responsiveness of creatine kinase and glycolytic enzymes in brain and uterus of rat. Neurosci Lett 84:197-202

314. **Degani H, Victor TA, Kaye AM** 1988 Effects of 17 beta-estradiol on high energy phosphate concentrations and the flux catalyzed by creatine kinase in immature rat uteri: 31P nuclear magnetic resonance studies. Endocrinology 122:1631-8

315. **Degani H, Shaer A, Victor TA, Kaye AM** 1984 Estrogen-induced changes in high-energy phosphate metabolism in rat uterus: 31P NMR studies. Biochemistry 23:2572-7

316. **Kaye AM** 1983 Enzyme induction by estrogen. J Steroid Biochem 19:33-40

317. **Bergen HT, Pentecost BT, Dickerman HW, Pfaff DW** 1993 In situ hybridization for creatine kinase-B messenger RNA in rat uterus and brain. Mol Cell Endocrinol 92:111-9

318. **Wang F, Samudio I, Safe S** 2001 Transcriptional activation of rat creatine kinase B by 17beta-estradiol in MCF-7 cells involves an estrogen responsive element and GC-rich sites. J Cell Biochem 84:156-72

319. **Payne RM, Friedman DL, Grant JW, Perryman MB, Strauss AW** 1993 Creatine kinase isoenzymes are highly regulated during pregnancy in rat uterus and placenta. Am J Physiol 265:E624-35

320. **Payne RM, Strauss AW** 1994 Expression of the mitochondrial creatine kinase genes. Mol Cell Biochem 133-134:235-43

321. **Baquer NZ, Sochor M, Kunjara S, McLean P** 1993 Effect of oestradiol on the carbohydrate metabolism of immature rat uterus: the role of fructose-2, 6-bis-phosphate and of phosphoribosyl pyrophosphate. Biochem Mol Biol Int 31:509-19

322. **Welch RD, Gorski J** 1999 Regulation of glucose transporters by estradiol in the immature rat uterus. Endocrinology 140:3602-3608

323. **Shinkarenko L, Kaye AM, Degani H** 1994 13C NMR kinetic studies of the rapid stimulation of glucose metabolism by estrogen in immature rat uterus. NMR Biomed 7:209-17

324. **Kaye AM, Shinkarenko L, Waisman A, Victor T, Degani H** 1989 Estradiol induction of accelerated energy metabolism in prepuberal rat uteri in vitro: mRNA hybridization and [13C]NMR studies. J Steroid Biochem 34:289-92

325. **Lundeen SG, Carver JM, McKean M-L, Winneker RC** 1997 Characterization of the Ovariectomized Rat Model for the Evaluation of Estrogen Effects on Plasma Cholesterol Levels. Endocrinology 138:1552-1558

326. **Gray JM, Greenwood MR** 1982 Time course of effects of ovarian hormones on food intake and metabolism. Am J Physiol 243:E407-12

327. **Gray JM, Greenwood MR** 1983 Uterine and adipose lipoprotein lipase activity in hormone-treated and pregnant rats. Am J Physiol 245:E132-7

328. **Wijnholds J, Muller E, Ab G** 1991 Oestrogen facilitates the binding of ubiquitous and liver-enriched nuclear proteins to the apoVLDL II promoter in vivo. Nucleic Acids Res 19:33-41

329. **Lopez D, Sanchez MD, Shea-Eaton W, McLean MP** 2002 Estrogen Activates the High-Density Lipoprotein Receptor Gene via Binding to Estrogen Response Elements and Interaction with Sterol Regulatory Element Binding Protein-1A. Endocrinology 143:2155-2168

330. **Ramachandran S, Song M, Murphy AA, Parthasarathy S** 2001 Expression of Scavenger Receptor Class B1 in Endometrium and Endometriosis. J Clin Endocrinol Metab 86:3924-3928

331. **Rumpel E, Kruse C, Muller PK, Kuhnel W** 1996 Expression of vigilin in the uterus of ovariectomized steroid-treated rats and during the estrous cycle. Anat Anz 178:337-44

332. **Xiao AZ, Zhao YG, Duan EK** 2002 Expression and regulation of the fatty acid amide hydrolase gene in the rat uterus during the estrous cycle and peri-implantation period. Mol Hum Reprod 8:651-8

333. **Hulboy D, Rudolph L, Matrisian L** 1997 Matrix metalloproteinases as mediators of reproductive function. Mol. Hum. Reprod. 3:27-45

334. **Curry TE, Jr, Osteen KG** 2001 Cyclic Changes in the Matrix Metalloproteinase System in the Ovary and Uterus. Biol Reprod 64:1285-1296

335. **Osteen KG, Bruner-Tran KL, Ong D, Eisenberg E** 2002 Paracrine Mediators of Endometrial Matrix Metalloproteinase Expression: Potential Targets for Progestin-Based Treatment of Endometriosis. Ann NY Acad Sci 955:139-146

336. **Lukashev ME, Werb Z** 1998 ECM signalling: orchestrating cell behaviour and misbehaviour. Trends in Cell Biology 8:437-441

337. **Werb Z** 1997 ECM and cell surface proteolysis: regulating cellular ecology. Cell 91:439-42

338. **Munakata H, Isemura M, Aikawa J, Kodama C, Yosizawa Z** 1985 Changes of glycosaminoglycan composition of uterine myometrium of rabbit induced by female sex steroids. Tohoku J Exp Med 147:77-81

339. **Endo M, Shimizu T, Munakata H, Yosizawa Z** 1978 Composition of acidic glycoconjugates (glycosaminoglycans and glycoprotein) in myometrium of rabbit uterus under estrogenic condition. Tohoku J Exp Med 126:103-10

340. **Takata K, Terayama H** 1977 Hormonal effect on glycosaminoglycans and glycoproteins in uteri of ovariectomized rats. Biochim Biophys Acta 500:333-43

341. **Munakata H, Isemura M, Yosizawa Z** 1981 Hormonal effects on the sulfation of sulfated glycoconjugates in the uterine endometrium of non-ovariectomized rabbit. Tohoku J Exp Med 135:275-9

342. **Henneman DH** 1971 Effect of estradiol-17 on collagen biosynthesis, degradation and re-utilization in vivo. Biochem Biophys Res Commun 44:326-32

343. **Dyer RF, Sodek J, Heersche JN** 1980 The effect of 17 beta-estradiol on collagen and noncollagenous protein synthesis in the uterus and some periodontal tissues. Endocrinology 107:1014-21

344. **Mandell MS, Sodek J** 1982 Metabolism of collagen types I, III, and V in the estradiol-stimulated uterus. J Biol Chem 257:5268-73

345. **Pastore GN, Dicola LP, Dollahon NR, Gardner RM** 1992 Effect of estriol on the structure and organization of collagen in the lamina propria of the immature rat uterus. Biol Reprod 47:83-91

346. **Pastore GN, diCola LP, Dollahon NR, Gardner RM** 1989 The effect of estradiol on collagen structure and organization in the immature rat uterus. Proc Soc Exp Biol Med 191:69-77

347. **Woessner JF, Jr.** 1969 Inhibition by oestrogen of collagen breakdown in the involuting rat uterus. Biochem J 112:637-45

348. **Komm BS, Frankel FR, Myers JC, Lyttle CR** 1987 Estrogen regulation of alpha 1(I)-procollagen messenger ribonucleic acid in the rat uterus. Endocrinology 120:1403-10

349. **Frankel FR, Hsu CY, Myers JC, et al.** 1988 Regulation of alpha 2(I), alpha 1(III), and alpha 2(V) collagen mRNAs by estradiol in the immature rat uterus. DNA 7:347-54

350. **Wu WX, Zhang Q, Unno N, Derks JB, Nathanielsz PW** 2000 Characterization of decorin mRNA in pregnant intrauterine tissues of the ewe and regulation by steroids. Am J Physiol Cell Physiol 278:C199-206

351. **Carson DD, Farrar JD, Laidlaw J, Wright DA** 1990 Selective activation of the N-glycosylation apparatus in uteri by estrogen. J Biol Chem 265:2947-55

352. **Irwin JC, Utian WH, Eckert RL** 1991 Sex steroids and growth factors differentially regulate the growth and differentiation of cultured human endometrial stromal cells. Endocrinology 129:2385-92

353. **Vollmer G, Hopert AC, Ellerbrake N, Wunsche W, Knuppen R** 1995 Fibronectin is an estrogen-repressed protein in RUCA-I rat endometrial adenocarcinoma cells. J Steroid Biochem Mol Biol 54:131-9

354. **Jetten AM, De Luca LM, Nelson K, Schroeder W, Burlingame S, Fujimoto W** 1996 Regulation of cornifin [alpha] expression in the vaginal and uterine epithelium by estrogen and retinoic acid. Molecular and Cellular Endocrinology 123:7-15

355. **Russo LA, Calabro SP, Filler TA, Carey DJ, Gardner RM** 2001 In vivo regulation of syndecan-3 expression in the rat uterus by 17 beta-estradiol. J Biol Chem 276:686-92

356. **Morris J, Potter S, Gaza-Bulseco G** 1988 Estradiol-stimulated turnover of heparan sulfate proteoglycan in mouse uterine epithelium. J. Biol. Chem. 263:4712-4718

357. **Inki P** 1997 Expression of syndecan-1 in female reproductive tract tissues and cultured keratinocytes. Mol Hum Reprod 3:299-305

358. **Boutin EL, Sanderson RD, Bernfield M, Cunha GR** 1991 Epithelial-mesenchymal interactions in uterus and vagina alter the expression of the cell surface proteoglycan, syndecan. Dev Biol 148:63-74

359. **Lessey BA, Arnold JT** 1998 Paracrine signaling in the endometrium: integrins and the establishment of uterine receptivity. J Reprod Immunol 39:105-16

360. **DeSouza MM, Lagow E, Carson DD** 1998 Mucin functions and expression in mammalian reproductive tract tissues. Biochem Biophys Res Commun 247:1-6

361. **Carson DD, DeSouza MM, Kardon R, Zhou X, Lagow E, Julian J** 1998 Mucin expression and function in the female reproductive tract. Hum Reprod Update 4:459-64

362. **Carraway KL, Idris N** 2001 Regulation of sialomucin complex/Muc4 in the female rat reproductive tract. Biochem Soc Trans 29:162-6

363. **Braga V, Gendler S** 1993 Modulation of Muc-1 mucin expression in the mouse uterus during the estrus cycle, early pregnancy and placentation. J Cell Sci 105:397-405

364. **Pimental RA, Julian J, Gendler SJ, Carson DD** 1996 Synthesis and intracellular trafficking of Muc-1 and mucins by polarized mouse uterine epithelial cells. J Biol Chem 271:28128-37

365. **Surveyor GA, Gendler SJ, Pemberton L, et al.** 1995 Expression and steroid hormonal control of Muc-1 in the mouse uterus. Endocrinology 136:3639-47

366. **McNeer RR, Carraway CA, Fregien NL, Carraway KL** 1998 Characterization of the expression and steroid hormone control of sialomucin complex in the rat uterus: implications for uterine receptivity. J Cell Physiol 176:110-9

367. **Zhou X, DeSouza MM, Julian J, Gendler SJ, Carson DD** 1998 Estrogen receptor does not directly regulate the murine Muc-1 promoter. Mol Cell Endocrinol 143:65-78

368. **Somkuti SG, Yuan L, Fritz MA, Lessey BA** 1997 Epidermal Growth Factor and Sex Steroids Dynamically Regulate a Marker of Endometrial Receptivity in Ishikawa Cells. J Clin Endocrinol Metab 82:2192-2197

369. **Potter SW, Gaza G, Morris JE** 1996 Estradiol induces E-cadherin degradation in mouse uterine epithelium during the estrous cycle and early pregnancy. J Cell Physiol 169:1-14

370. **Fujimoto J, Ichigo S, Hori M, Tamaya T** 1996 Alteration of E-cadherin, alpha- and beta-catenin mRNA expression in human uterine endometrium during the menstrual cycle. Gynecol Endocrinol 10:187-91

371. **Wesseling J, van der Valk SW, Hilkens J** 1996 A mechanism for inhibition of E-cadherin-mediated cell-cell adhesion by the membrane-associated mucin episialin/MUC1. Mol Biol Cell 7:565-77

372. **Sivridis E, Giatromanolaki A, Koukourakis MI, Georgiou L, Anastasiadis P** 2002 Patterns of episialin/MUC1 expression in endometrial carcinomas and prognostic relevance. Histopathology 40:92-100

373. **Svalander PC, Odin P, Nilsson BO, Obrink B** 1990 Expression of cellCAM-105 in the apical surface of rat uterine epithelium is controlled by ovarian steroid hormones. J Reprod Fertil 88:213-21

374. **Bamberger A-M, Sudahl S, Loning T, et al.** 2000 The Adhesion Molecule CEACAM1 (CD66a, C-CAM, BGP) Is Specifically Expressed by the Extravillous Intermediate Trophoblast. Am J Pathol 156:1165-1170

375. **Wagener C, Ergun S** 2000 Angiogenic Properties of the Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1. Experimental Cell Research 261:19-24

376. **Sidhu SS, Kimber SJ** 1999 Hormonal Control of H-Type {alpha}(1-2)Fucosyltransferase Messenger Ribonucleic Acid in the Mouse Uterus. Biol Reprod 60:147-157

377. **White S, Kimber SJ** 1994 Changes in alpha (1-2)-fucosyltransferase activity in the murine endometrial epithelium during the estrous cycle, early pregnancy, and after ovariectomy and hormone replacement. Biol Reprod 50:73-81

378. **Brokelmann J, Fawcett DW** 1969 The localization of endogenous peroxidase in the rat uterus and its induction by estradiol. Biol Reprod 1:59-71

379. **Gabriel BW, Eckberg WR, Wyche JH, Meones H, Hanker JS, Anderson WA** 1986 Peroxidases induced in rat uterus by estrogen administration. I. Synthesis, secretion and immunofluorescent demonstration of fluid enzymes. J Submicrosc Cytol 18:673-82

380. **Wang G, Bhattacharyya N, Wilkerson C, Ramsammy RA, Eatman E, Anderson WA** 1994 Estrogen induced peroxidase secretion from the endometrial epithelium: a possible function for the luminal enzyme. J Submicrosc Cytol Pathol 26:405-14

381. **Anderson WA, Gabriel BW, Nerurkar SG, Wyche JH, Yates PE, Hanker JS** 1986 Peroxidases induced in rat uterus by estrogen administration. II. Cytochemical and biochemical heterogeneity. J Submicrosc Cytol 18:683-90

382. **Balan KV, Hollis VW, Eckberg WR, et al.** 2001 Cathepsin B and complement C3 are major comigrants in the estrogen-induced peroxidase fraction of rat uterine fluid. J Submicrosc Cytol Pathol 33:221-30

383. **Jokimaa V, Oksjoki S, Kujari H, Vuorio E, Anttila L** 2001 Expression patterns of cathepsins B, H, K, L and S in the human endometrium. Mol Hum Reprod 7:73-8

384. **Moulton BC, Khan SA** 1992 Progestin and estrogen control of cathepsin D expression and processing in rat uterine luminal epithelium and stroma-myometrium. Proc Soc Exp Biol Med 201:98-105

385. **Miralles F, Gaudelet C, Cavailles V, Rochefort H, Augereau P** 1994 Insensitivity of Cathepsin D Gene to Estradiol in Endometrial Cells Is Determined by the Sequence of Its Estrogen Responsive Element. Biochemical and Biophysical Research Communications 203:711-718

386. **Zhang D, Simmen RCM, Michel FJ, Zhao G, Vale-Cruz D, Simmen FA** 2002 Secretory Leukocyte Protease Inhibitor Mediates Proliferation of Human Endometrial Epithelial Cells by Positive and Negative Regulation of Growth-associated Genes. J. Biol. Chem. 277:29999-30009

387. **Ashcroft GS, Lei K, Jin W, et al.** 2000 Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. Nat Med 6:1147-53

388. **Farmer SJ, Fliss AE, Simmen RC** 1990 Complementary DNA cloning and regulation of expression of the messenger RNA encoding a pregnancy-associated porcine uterine protein related to human antileukoproteinase. Mol Endocrinol 4:1095-104

389. **Osteen K, Rodgers W, Gaire M, Hargrove J, Gorstein F, Matrisian L** 1994 Stromal-Epithelial Interaction Mediates Steroidal Regulation of Metalloproteinase Expression in Human Endometrium. PNAS 91:10129-10133

390. **Mendelsohn ME** 2002 Genomic and nongenomic effects of estrogen in the vasculature. Am J Cardiol 90:3F-6F

391. **Thiede MA, Harm SC, Hasson DM, Gardner RM** 1991 In vivo regulation of parathyroid hormone-related peptide messenger ribonucleic acid in the rat uterus by 17 beta-estradiol. Endocrinology 128:2317-23

392. **Paspaliaris V, Petersen DN, Thiede MA** 1995 Steroid regulation of parathyroid hormone-related protein expression and action in the rat uterus. J Steroid Biochem Mol Biol 53:259-65

393. **Shenberger JS, Dixon PS, Choate J, Helal K, Shew RL, Barth W** 2001 Pregnancy and labor increase the capacity of human myometrial cells to secrete parathyroid hormone-related protein. Life Sciences 68:1557-1566

394. **Morimoto T, Devora GA, Mibe M, Casey ML, MacDonald PC** 1997 Parathyroid hormone-related protein and human myometrial cells: action and regulation. Mol Cell Endocrinol 129:91-9

395. **Hinson JP, Kapas S, Smith DM** 2000 Adrenomedullin, a Multifunctional Regulatory Peptide. Endocr Rev 21:138-167

396. **Gargett CE, Rogers PA** 2001 Human endometrial angiogenesis. Reproduction 121:181-6

397. **Folkman J** 1995 Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1:27-31

398. **Taylor RN, Lebovic DI, Hornung D, Mueller MD** 2001 Endocrine and Paracrine Regulation of Endometrial Angiogenesis. Ann NY Acad Sci 943:109-121

399. **Rogers PA, Gargett CE** 1998 Endometrial angiogenesis. Angiogenesis 2:287-294

400. **Gannon BJ, Carati CJ, Verco CJ** 1997 Endometrial perfusion across the normal human menstrual cycle assessed by laser Doppler fluxmetry. Hum Reprod 12:132-9

401. **Perrot-Applanat M, Deng M, Fernandez H, Lelaidier C, Meduri G, Bouchard P** 1994 Immunohistochemical localization of estradiol and progesterone receptors in human uterus throughout pregnancy: expression in endometrial blood vessels. J Clin Endocrinol Metab 78:216-24

402. **Yokomizo R, Matsuzaki S, Uehara S, Murakami T, Yaegashi N, Okamura K** 2002 Erythropoietin and erythropoietin receptor expression in human endometrium throughout the menstrual cycle. Mol Hum Reprod 8:441-6

403. **Sasaki R, Masuda S, Nagao M** 2001 Pleiotropic Functions and Tissue-Specific Expression of Erythropoietin. News Physiol Sci 16:110-113

404. **Mukundan H, Resta TC, Kanagy NL** 2002 17beta -Estradiol decreases hypoxic induction of erythropoietin gene expression. Am J Physiol Regul Integr Comp Physiol 283:R496-504

405. **Yasuda Y, Masuda S, Chikuma M, Inoue K, Nagao M, Sasaki R** 1998 Estrogen-dependent Production of Erythropoietin in Uterus and Its Implication in Uterine Angiogenesis. J. Biol. Chem. 273:25381-25387

406. **Arena CS, Quirk SM, Zhang YQ, Henrikson KP** 1996 Rat uterine stromal cells: thrombin receptor and growth stimulation by thrombin. Endocrinology 137:3744-9

407. **Henrikson KP, Hall ES, Lin Y** 1994 Cellular localization of tissue factor and prothrombin in the estrogen-treated immature rat uterus. Biol Reprod 50:1145-50

408. **Henrikson KP, Greenwood JA, Pentecost BT, Jazin EE, Dickerman HW** 1992 Estrogen control of uterine tissue factor messenger ribonucleic acid levels. Endocrinology 130:2669-74

409. **Finlay TH, Katz J, Kirsch L, Levitz M, Nathoo SA, Seiler S** 1983 Estrogen-stimulated uptake of plasminogen by the mouse uterus. Endocrinology 112:856-61

410. **Liehr JG, Ulubelen AA, Strobel HW** 1986 Cytochrome P-450-mediated redox cycling of estrogens. J Biol Chem 261:16865-70

411. **Gomez-Zubeldia MA, Hinchado G, Arbues JJ, Nogales AG, Millan JC** 2001 Influence of Estradiol on Oxidative Stress in the Castrated Rat Uterus. Gynecologic Oncology 80:227-232

412. **Baird DT, Cameron ST, Critchley HO, et al.** 1996 Prostaglandins and menstruation. Eur J Obstet Gynecol Reprod Biol 70:15-7

413. **Gomez-Zubeldia MA, Corrales S, Arbues J, Nogales AG, Millan JC** 2002 Influence of estradiol and gestagens on oxidative stress in the rat uterus. Gynecol Oncol 86:250-8

414. **Jain S, Saxena D, Kumar GP, Laloraya M** 2000 NADPH dependent superoxide generation in the ovary and uterus of mice during estrous cycle and early pregnancy. Life Sci 66:1139-46

415. **Sugino N, Karube-Harada A, Sakata A, Takiguchi S, Kato H** 2002 Different mechanisms for the induction of copper-zinc superoxide dismutase and manganese superoxide dismutase by progesterone in human endometrial stromal cells. Hum Reprod 17:1709-14

416. **Jain S, Saxena D, Kumar PG, Koide SS, Laloraya M** 1999 Effect of estradiol and selected antiestrogens on pro- and antioxidant pathways in mammalian uterus. Contraception 60:111-8

417. **Nomura T, Sasaki J, Mori H, et al.** 1996 Expression of manganese superoxide dismutase mRNA in reproductive organs during the ovulatory process and the estrous cycle of the rat. Histochem Cell Biol 105:1-6

418. **Sugino N, Kashida S, Takiguchi S, Nakamura Y, Kato H** 2000 Induction of superoxide dismutase by decidualization in human endometrial stromal cells. Mol Hum Reprod 6:178-84

419. **Sugino N, Karube-Harada A, Kashida S, Takiguchi S, Kato H** 2001 Reactive oxygen species stimulate prostaglandin F2{alpha} production in human endometrial stromal cells in vitro. Hum. Reprod. 16:1797-1801

420. **Kim HT, Kim YH, Nam JW, Lee HJ, Rho HM, Jung GH** 1994 Study of 5'-flanking region of human Cu/Zn superoxide dismutase. Biochemical and Biophysical Research Communications 201:1526-1533

421. **Wan XS, Devalaraja MN, St Clair DK** 1994 Molecular structure and organization of the human manganese superoxide dismutase gene. DNA Cell Biol 13:1127-36

422. **Rockwell LC, Pillai S, Olson CE, Koos RD** 2002 Inhibition of Vascular Endothelial Growth Factor/Vascular Permeability Factor Action Blocks Estrogen-Induced Uterine Edema and Implantation in Rodents. Biol Reprod 67:1804-1810

423. **Casslen B, Nilsson B** 1984 Human uterine fluid, examined in undiluted samples for osmolarity and the concentrations of inorganic ions, albumin, glucose, and urea. Am J Obstet Gynecol 150:877-81

424. **Van Winkle LJ, Campione AL, Webster DP** 1983 Sodium ion concentrations in uterine flushings from "implanting" and "delayed implanting" mice. J Exp Zool 226:321-4

425. **Katagiri S, Ma S, Yuen BH, Moon YS** 1997 Role for insulin-like growth factor I in the regulation of electrolyte composition of uterine luminal fluid. J Reprod Fertil 109:115-20

426. **Chan LN, Tsang LL, Rowlands DK, et al.** 2002 Distribution and regulation of ENaC subunit and CFTR mRNA expression in murine female reproductive tract. J Membr Biol 185:165-76

427. **Rochwerger L, Buchwald M** 1993 Stimulation of the cystic fibrosis transmembrane regulator expression by estrogen in vivo. Endocrinology 133:921-30

428. **Anwer K, Oberti C, Perez GJ, et al.** 1993 Calcium-activated K+ channels as modulators of human myometrial contractile activity. Am J Physiol 265:C976-85

429. **Holdiman AJ, Fergus DJ, England SK** 2002 17[beta]-Estradiol upregulates distinct maxi-K channel transcripts in mouse uterus. Molecular and Cellular Endocrinology 192:1-6

430. **Behrens R, Nolting A, Reimann F, Schwarz M, Waldschutz R, Pongs O** 2000 hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel [beta] subunit family. FEBS Letters 474:99-106

431. **Benkusky NA, Korovkina VP, Brainard AM, England SK** 2002 Myometrial maxi-K channel [beta]1 subunit modulation during pregnancy and after 17[beta]-estradiol stimulation. FEBS Letters 524:97-102

432. **Song M, Helguera G, Eghbali M, et al.** 2001 Remodeling of Kv4.3 potassium channel gene expression under the control of sex hormones. The Journal of Biological Chemistry 276:31883-31890

433. **Wang XF, Yu MK, Lam SY, et al.** 2003 Expression, Immunolocalization, and Functional Activity of Na+/H+ Exchanger Isoforms in Mouse Endometrial Epithelium. Biol Reprod 68:302-308

434. **Li X, Yu H, Koide SS** 1994 The water channel gene in human uterus. Biochem Mol Biol Int 32:371-7

435. **Li XJ, Yu HM, Koide SS** 1997 Regulation of water channel gene (AQP-CHIP) expression by estradiol and anordiol in rat uterus. Yao Xue Xue Bao 32:586-92

436. **Zhou Q, Clarke L, Nie R, et al.** 2001 Estrogen action and male fertility: Roles of the sodium/hydrogen exchanger-3 and fluid reabsorption in reproductive tract function. PNAS 98:14132-14137

437. **Stemmer-Rachamimov AO, Wiederhold T, Nielsen GP, et al.** 2001 NHE-RF, a Merlin-Interacting Protein, Is Primarily Expressed in Luminal Epithelia, Proliferative Endometrium, and Estrogen Receptor-Positive Breast Carcinomas. Am J Pathol 158:57-62

438. **Ediger TR, Park S-E, Katzenellenbogen BS** 2002 Estrogen receptor inducibility of the human Na+/H+ Exchanger Regulatory Factor/Ezrin-Radixin-Moesin Binding Protein 50 (NHE-RF/EBP50) gene involving multiple half-estrogen response elements. Mol Endocrinol 16:1828-1839

439. **McMaster MT, Newton RC, Dey SK, Andrews GK** 1992 Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. J Immunol 148:1699-705

440. **Sato T, Fukazawa Y, Kojima H, Enari M, Iguchi T, Ohta Y** 1997 Apoptotic cell death during the estrous cycle in the rat uterus and vagina. Anat Rec 248:76-83

441. **Hunt JS, Petroff MG, Burnett TG** 2000 Uterine leukocytes: key players in pregnancy. Seminars in Cell and Developmental Biology 11:127-137

442. **Mori A, Zhai YL, Toki T, Nikaido T, Fujii S** 1997 Distribution and heterogeneity of mast cells in the human uterus. Hum Reprod 12:368-72

443. **Casslen B, Kobayashi TK, Stormby N** 1982 Cyclic variation of the cellular components in human uterine fluid. J Reprod Fertil 66:213-8

444. **Wood GW, Hausmann E, Choudhuri R** 1997 Relative role of CSF-1, MCP-1/JE, and RANTES in macrophage recruitment during successful pregnancy. Mol Reprod Dev 46:62-9; discussion 69-70

445. **Perez MC, Furth EE, Matzumura PD, Lyttle CR** 1996 Role of eosinophils in uterine responses to estrogen. Biol Reprod 54:249-54

446. **Gouon-Evans V, Pollard JW** 2001 Eotaxin is required for eosinophil homing into the stroma of the pubertal and cycling uterus. Endocrinology 142:4515-21

447. **Lee YH, Howe RS, Sha SJ, Teuscher C, Sheehan DM, Lyttle CR** 1989 Estrogen regulation of an eosinophil chemotactic factor in the immature rat uterus. Endocrinology 125:3022-8

448. **Wira CR, Sandoe CP** 1977 Sex steroid hormone regulation of IgA and IgG in rat uterine secretions. Nature 268:534-6

449. **Wira CR, Sullivan DA, Sandoe CP** 1983 Epithelial cell involvement in the estradiol-stimulated accumulation of IgA in the rat uterus. J Steroid Biochem 19:469-74

450. **Johansen F-E, Pekna M, Norderhaug IN, et al.** 1999 Absence of Epithelial Immunoglobulin A Transport, with Increased Mucosal Leakiness, in Polymeric Immunoglobulin Receptor/Secretory Component–deficient Mice. J. Exp. Med. 190:915-922

451. **Kaushic C, Richardson JM, Wira CR** 1995 Regulation of polymeric immunoglobulin A receptor messenger ribonucleic acid expression in rodent uteri: effect of sex hormones. Endocrinology 136:2836-44

452. **Hempen PM, Phillips KM, Conway PS, et al.** 2002 Transcriptional Regulation of the Human Polymeric Ig Receptor Gene: Analysis of Basal Promoter Elements. J Immunol 169:1912-1921

453. **Espert L, Degols G, Gongora C, et al.** 2003 ISG20, a new interferon-induced RNase specific for single-stranded RNA, defines an alternative antiviral pathway against RNA genomic viruses. J. Biol. Chem.:M209628200

454. **Pentecost BT** 1998 Expression and estrogen regulation of the HEM45 MRNA in human tumor lines and in the rat uterus. J Steroid Biochem Mol Biol 64:25-33

455. **De M, Sanford TR, Wood GW** 1992 Interleukin-1, interleukin-6, and tumor necrosis factor alpha are produced in the mouse uterus during the estrous cycle and are induced by estrogen and progesterone. Dev Biol 151:297-305

456. **Kayisli UA, Mahutte NG, Arici A** 2002 Uterine chemokines in reproductive physiology and pathology. Am J Reprod Immunol 47:213-21

457. **DeLoia JA, Stewart-Akers AM, Brekosky J, Kubik CJ** 2002 Effects of exogenous estrogen on uterine leukocyte recruitment. Fertility and Sterility 77:548-554

458. **Wood GW, De M, Sanford T, Choudhuri R** 1992 Macrophage colony stimulating factor controls macrophage recruitment to the cycling mouse uterus. Dev Biol 152:336-43

459. **Boucher A, Mourad W, Mailloux J, Lemay A, Akoum A** 2000 Ovarian hormones modulate monocyte chemotactic protein-1 expression in endometrial cells of women with endometriosis. Mol Hum Reprod 6:618-26

460. **Cohen PE, Zhu L, Pollard JW** 1997 Absence of colony stimulating factor-1 in osteopetrotic (csfmop/csfmop) mice disrupts estrous cycles and ovulation. Biol Reprod 56:110-8

461. **Nielsen CH, Leslie RGQ** 2002 Complement's participation in acquired immunity. J Leukoc Biol 72:249-261

462. **Li S-H, Huang H-L, Chen Y-H** 2002 Ovarian steroid-regulated synthesis and secretion of complement C3 and factor B in mouse endometrium during the natural estrous cycle and pregnancy period. Biol Reprod 66:322-332

463. **Sundstrom SA, Komm BS, Ponce-de-Leon H, Yi Z, Teuscher C, Lyttle CR** 1989 Estrogen regulation of tissue-specific expression of complement C3. J Biol Chem 264:16941-7

464. **Ward PP, Uribe-Luna S, Conneely OM** 2002 Lactoferrin and host defense. Biochem Cell Biol 80:95-102

465. **Teng CT, Walker MP, Bhattacharyya SN, Klapper DG, diAugustine RP, McLachlan JA** 1986 Purification and properties of an oestrogen-stimulated mouse uterine glycoprotein (approx. 70 kDa). Biochemical Journal 240:413-322

466. **Pentecost BT, Teng CT** 1987 Lactotransferrin is the major estrogen inducible protein of mouse uterine secretions. Journal of Biological Chemistry 262:10134-10139

467. **Teng CT, Beard C, Gladwell W** 2002 Differential Expression and Estrogen Response of Lactoferrin Gene in the Female Reproductive Tract of Mouse, Rat, and Hamster. Biol Reprod 67:1439-1449

468. **Teng CT** 2002 Lactoferrin gene expression and regulation: an overview. Biochem Cell Biol 80:7-16

469. **Bevans CG, Kordel M, Rhee SK, Harris AL** 1998 Isoform composition of connexin channels determines selectivity among second messengers and uncharged molecules. J Biol Chem 273:2808-16

470. **Garfield RE, Blennerhassett MG, Miller SM** 1988 Control of myometrial contractility: role and regulation of gap junctions. Oxf Rev Reprod Biol 10:436-90

471. **Yamasaki H, Omori Y, Zaidan-Dagli ML, Mironov N, Mesnil M, Krutovskikh V** 1999 Genetic and epigenetic changes of intercellular communication genes during multistage carcinogenesis. Cancer Detect Prev 23:273-9

472. **White TW, Paul DL** 1999 GENETIC DISEASES AND GENE KNOCKOUTS REVEAL DIVERSE CONNEXIN FUNCTIONS. Annu. Rev. Physiol. 61:283-310

473. **Granot I, Dekel N, Bechor E, Segal I, Fieldust S, Barash A** 2000 Temporal analysis of connexin43 protein and gene expression throughout the menstrual cycle in human endometrium. Fertility and Sterility 73:381-386

474. **Grummer R, Traub O, Winterhager E** 1999 Gap Junction Connexin Genes cx26 and cx43 Are Differentially Regulated by Ovarian Steroid Hormones in Rat Endometrium. Endocrinology 140:2509-2516

475. **Petrocelli T, Lye S** 1993 Regulation of transcripts encoding the myometrial gap junction protein, connexin-43, by estrogen and progesterone. Endocrinology 133:284-290

476. **Piersanti M, Lye S** 1995 Increase in messenger ribonucleic acid encoding the myometrial gap junction protein, connexin-43, requires protein synthesis and is associated with increased expression of the activator protein-1, c-fos. Endocrinology 136:3571-3578

477. **Geimonen E, Boylston E, Royek A, Andersen J** 1998 Elevated Connexin-43 Expression in Term Human Myometrium Correlates with Elevated c-Jun Expression and Is Independent of Myometrial Estrogen Receptors. J Clin Endocrinol Metab 83:1177-1185

478. **Tu ZJ, Kollander R, Kiang DT** 1998 Differential Up-Regulation of Gap Junction Connexin 26 Gene in Mammary and Uterine Tissues: The Role of Sp Transcription Factors. Mol Endocrinol 12:1931-1938

479. **Murphy CR, Swift JG, Mukherjee TM, Rogers AW** 1979 Effects of ovarian hormones on cell membranes in the rat uterus. I. Freeze fracture studies of the apical membrane of the liminal epithelium. Cell Biophys 1:181-93

480. **Murphy CR, Swift JG, Mukherjee TM, Rogers AW** 1981 Effects of ovarian hormones on cell membranes in the rat uterus. II. Freeze-fracture studies on tight junctions of the lateral plasma membrane of the luminal epithelium. Cell Biophys 3:57-69

481. **Orchard MD, Murphy CR** 2002 Alterations in tight junction molecules of uterine epithelial cells during early pregnancy in the rat. Acta Histochem 104:149-55

482. **Vinatier D, Dufour P, Subtil D** 1996 Apoptosis: A programmed cell death involved in ovarian and uterine physiology. European Journal of Obstetrics & Gynecology and Reproductive Biology 67:85-102

483. **Vaskivuo TE, Stenback F, Karhumaa P, Risteli J, Dunkel L, Tapanainen JS** 2000 Apoptosis and apoptosis-related proteins in human endometrium. Mol Cell Endocrinol 165:75-83

484. **Coates PJ, Hales SA, Hall PA** 1996 The association between cell proliferation and apoptosis: studies using the cell cycle-associated proteins Ki67 and DNA polymerase alpha. J Pathol 178:71-7

485. **Stjernquist M, Ekblad E, Owman C, Sundler F** 1986 Neuronal localization and motor effects of gastrin-releasing peptide (GRP) in rat uterus. Regul Pept 13:197-205

486. **Whitley JC, Giraud AS, Mahoney AO, Clarke IJ, Shulkes A** 2000 Tissue-specific regulation of gastrin-releasing peptide synthesis, storage and secretion by oestrogen and progesterone. J Endocrinol 166:649-58

487. **Niiro N, Nishimura J, Hirano K, Nakano H, Kanaide H** 1998 Mechanisms of galanin-induced contraction in the rat myometrium. Br. J. Pharmacol. 124:1623-1632

488. **Vrontakis M, Schroedter I, Leite V, Friesen HG** 1993 Estrogen regulation and localization of galanin gene expression in the rat uterus. Biol Reprod 49:1245-50

489. **Shew RL, Papka RE, McNeill DL** 1992 Galanin and calcitonin gene-related peptide immunoreactivity in nerves of the rat uterus: localization, colocalization, and effects on uterine contractility. Peptides 13:273-9

490. **Dong Y-L, Yallampalli C** 2000 Pregnancy and exogenous steroid treatments modulate the expression of relaxant EP2 and contractile FP receptors in the rat uterus. Biol Reprod 62:533-539

491. **Miller C, Sassoon D** 1998 Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract. Development 125:3201-3211

492. **Sassoon D** 1999 Wnt genes and endocrine disruption of the female reproductive tract: a genetic approach. Mol Cell Endocrinol 158:1-5

493. **L'Horset F, Blin C, Colnot S, Lambert M, Thomasset M, Perret C** 1994 Calbindin-D9k gene expression in the uterus: study of the two messenger ribonucleic acid species and analysis of an imperfect estrogen-responsive element. Endocrinology 134:11-8

494. **Gill RK, Christakos S** 1995 Regulation by estrogen through the 5'-flanking region of the mouse calbindin-D28k gene. Mol Endocrinol 9:319-26

495. **Opperman LA, Saunders TJ, Bruns DE, Boyd JC, Mills SE, Bruns ME** 1992 Estrogen inhibits calbindin-D28k expression in mouse uterus. Endocrinology 130:1728-35

496. **Yang J, Goetz D, Li JY, et al.** 2002 An iron delivery pathway mediated by a lipocalin. Mol Cell 10:1045-56

497. **Huang HL, Chu ST, Chen YH** 1999 Ovarian steroids regulate 24p3 expression in mouse uterus during the natural estrous cycle and the preimplantation period. J Endocrinol 162:11-9

498. **Chu ST, Huang HL, Chen JM, Chen YH** 1996 Demonstration of a glycoprotein derived from the 24p3 gene in mouse uterine luminal fluid. Biochem J 316 (Pt 2):545-50

499. **Mueller MD, Vigne JL, Vaisse C, Taylor RN** 2000 Glycodelin: a pane in the implantation window. Semin Reprod Med 18:289-98

500. **Giguère V, Tremblay A, Tremblay GB** 1998 Estrogen receptor beta: re-evaluation of estrogen and antiestrogen signaling. Steroids 63:335-9

501. **Korach KS** 1994 Insights from the study of animals lacking functional estrogen receptor. Science 266:1524-27

502. **Pike AC, Brzozowski AM, Hubbard RE, et al.** 1999 Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. Embo J. 18:4608-4618

503. **Grover PL, MacNicoll AD, Sims P, Easty GC, Neville AM** 1980 Polycyclic hydrocarbon activation and metabolism in epithelial cell aggregates prepared from human mammary tissue. Int J Cancer 26:467-75

504. **Huggins CB, Pataki J, Harvey RG** 1967 Geometry of carcinogenic polycyclic aromatic hydrocarbons. Proc Natl Acad Sci U S A 58:2253-60

505. **Yang NC, Castro AJ, Lewis M, Wong T-W** 1961 Polynuclear aromatic hydrocarbons, steroids and carcinogenesis. Science 134:386

506. **Mattison D, Thorgeirsson SS** 1979 Ovarian aryl hydrocarbon hydroxylase activity and primordial oocyte toxicity of polycyclic aromatic hydrocarbons in mice. Cancer research 39:3471-3475

507. **Swartz WJ, Mattison DR** 1985 Benzo(a)pyrene inhibits ovulation in C57BL/6N mice. Anat Rec 212:268-76

508. **Cook JW, Dodds EC** 1933 Sex hormones and cancer-producing compounds. Nature 131:205-6

509. **Hattemer-Frey HA, Travis CC** 1991 Benzo[a]pyrene: environmental partitioning and human exposure. Toxicol and Ind Health 7:141-157

510. **Jacob J, Grimmer G** 1996 Metabolism and excretion of polycyclic aromatic hydrocarbons in rat and in human. Central European Journal of Public Health 4:33-9

511. **Hall M, Forrester LM, Parker DK, Grover PL, Wolf CR** 1989 Relative contribution of various forms of cytochrome P450 to the metabolism of benzo[a]pyrene by human liver microsomes. Carcinogenesis 10:1815-21

512. **Kim JH, Stansbury KH, Walker NJ, Trush MA, Strickland PT, Sutter TR** 1998 Metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-diol by human cytochrome P450 1B1 [published erratum appears in Carcinogenesis 1999 Mar;20(3):515]. Carcinogenesis 19:1847-53

513. **Larsen MC, Angus WG, Brake PB, Eltom SE, Sukow KA, Jefcoate CR** 1998 Characterization of CYP1B1 and CYP1A1 expression in human mammary epithelial cells: role of the aryl hydrocarbon receptor in polycyclic aromatic hydrocarbon metabolism. Cancer Res 58:2366-74

514. **Parke DV, Ioannides C** 1990 The role of metabolism studies in the safety evaluation of new chemicals. Acta Pharm. Jugosl. 40:363-82

515. **Shou M, Korzekwa KR, Crespi CL, Gonzalez FJ, Gelboin HV** 1994 Metabolism of benzo[a]pyrene by seven cDNA expressed human cytochromes P450. Polycyclic Aromatic Compounds 7:1-9

516. **Estabrook RW, Saeki Y, Chacos N, Capdevila J, Prough RA** 1980 Polycyclic hydrocarbon metabolism: a plethora of phenomena. Adv Enzyme Regul 19:3-17

517. **Gelboin HV** 1980 Benzo[alpha]pyrene metabolism, activation and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. Physiol Rev 60:1107-66

518. **Pelkonen O, Nebert DW** 1982 Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis. Pharmacol Rev 34:189-222

519. **Clemons JH, Allan LM, Marvin CH, et al.** 1998 Evidence of estrogen- and TCDD-like activities in crude and fractionated extracts of PM_{10} air particulate material using in vitro gene expression assays. Environ. Sci. and Technol. 32:1853-1860

520. **Arcaro KF, O'Keefe PW, Yang Y, Clayton W, Gierthy JF** 1999 Antiestrogenicity of environmental polycyclic aromatic hydrocarbons in human breast cancer cells. Toxicology 133:115-27

521. **Tran DQ, Ide CF, McLachlan JA, Arnold SF** 1996 The anti-estrogenic activity of selected polynuclear aromatic hydrocarbons in yeast expressing human estrogen receptor. Biochem. Biophys. Res. Commun. 229:102-108

522. **Matthews J, Zacharewski T** 1999 Differential binding affinities of PCBs, HO-PCBs and aroclors with recombinant human, rainbow trout (*Onchorhynkiss mykiss*) and green anole (*Anolis carolinensis*) estrogen receptors, using a semi-high throughput competitive binding assay. Toxicol. Sci. 53:326-39

523. **Gillesby BE, Zacharewski TR** 1999 pS2 (TFF2) mRNA levels in human breast tumor samples: correlation with clinical and histological prognostic markers. Breast Cancer Res Treat 56:253-265

524. **Sambrook J, Fritsch EF, Maniatis T** 1989 Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, New York

525. **Brasier AR, Tate JE, Habener JF** 1989 Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. Biotechniques 7:1116-1122

526. **Grimmer G, Brune H, Dettbarn G, et al.** 1988 Urinary and faecal excretion of chrysene and chrysene metabolites by rats after oral, intraperitoneal, intratracheal or intrapulmonary application. Archives of Toxicology 62:401-405

527. **Jacob J, Doehmer J, Grimmer G, et al.** 1996 Metabolism of phenanthrene, benz[a]anthracene, benzo[a]pyrene, chrysene and benzo[c]phenanthrene by eight cDNA-expressed human and rat cytochromes P450. Polycyclic Aromatic Compounds 10:1-9

528. **Legraverend C, Guenthner TM, Nebert DW** 1984 Importance of the route of administration for genetic differences in benzo[a]pyrene-induced in utero toxicity and teratogenicity. Teratology 29:35-47

529. **Selkirk JK, MacLeod MC, Mansfield BK, Nikbakht PA, Dearstone KC** 1983 Species heterogeneity in the metabolic processing of benzo[a]pyrene. Basic Life Sci 24:283-94

530. **van Schooten FJ, Moonen EJC, van der Wal L, Levels P, Kleinjans JCS** 1997 Determination of polycyclic aromatic hydrocarbons (PAH) and their metabolites in blood, feces, and urine of rats orally exposed to PAH contaminated soils. Archives of Environmental Contamination and Toxicology 33:317-22

531. **Ebright RH, Wong JR, Chen LB** 1986 Binding of 2-hydroxybenzo[a]pyrene to estrogen receptors in rat cytosol. Cancer Res. 46:2349-51

532. **Dipple A, Bigger CAH** 1983 Metabolic properties of in vitro systems. Ann N Y Acad Sci 407:26-33

533. **Li W, Harper PA, Tang BK, Okey AB** 1998 Regulation of cytochrome P450 enzymes by aryl hydrocarbon receptor in human cells: CYP1A2 expression in the LS180 colon carcinoma cell line after treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin or 3- methylcholanthrene. Biochem Pharmacol 56:599-612

534. **Spink DC, Spink BC, Cao JQ, et al.** 1998 Differential expression of CYP1A1 and CYP1B1 in human breast epithelial cells and breast tumor cells. Carcinogenesis 19:291-8

535. **Harris M, Piskorska-Pliszczynska J, Zacharewski T, Romkes M, Safe S** 1989 Structure-dependent induction of aryl hydrocarbon hydroxylase in human breast cancer cell lines and characterization of the Ah receptor. Cancer Res. 49:4531-5

536. **Charles GD, Bartels MJ, Zacharewski TR, Gollapudi BB, Freshour NL, Carney EW** 2000 Activity of benzo[a]pyrene and its hydroxylated metabolites in an ERα reporter gene assay. Toxicol. Sci. 55:320-6

537. **Schneider SL, Alks V, Morreal CE, Sinha DK, Dao TL** 1976 Estrogenic properties of 3,9-dihydroxybenz[a]anthracene, a potential metabolite of benz[a]anthracene. Journal of the National Cancer Institute 57:1351-4

538. **Chernayaev GA, Barkova TI, Egorova VV, et al.** 1975 A series of optical structural and isomeric analogs of estradiol: a comparative study of the biological activity and affinity to cytosol receptor of rabbit uterus. J Steroid Biochem 6:1483-8

539. **Korach KS, McLachlan JA** 1995 Techniques for detection of estrogenicity. Environmental Health Perspectives 103:5-8

540. **Teng CT, Pentecost BT, Chen YH, Newbold RR, Eddy EM, McLachlan JA** 1989 Lactotransferrin gene expression in the mouse uterus and mammary gland. Endocrinology 124:992-9

541. **Camus A-M, Aitio A, Sabadie N, Wahrendorf J, Bartsch H** 1984 Metabolism and urinary excretion of mutagenic metabolites of benzo[a]pyrene in C57 and DBA mice strains. Carcinogenesis 5:35-9

542. **Bigelow SW, Nebert DW** 1982 The Ah regulatory gene product. Survey of nineteen polycyclic aromatic compounds' and fifteen benzo[a]pyrene metabolites' capacity to bind to the cytosolic receptor. Toxicol. Lett. 10:109-18

543. **Piskorska-Pliszczynska J, Keys B, Safe S, Newman MS** 1986 The cytosolic receptor binding affinities and AHH induction potencies of 29 polynuclear aromatic hydrocarbons. Toxicol. Lett. 34:67-74

544. **Harper PA, Golas CL, Okey AB** 1991 Ah receptor in mice genetically "nonresponsive" for cytochrome P4501A1 induction: cytosolic Ah receptor, transformation to the nuclear binding state, and induction of aryl hydrocarbon hydroxylase by halogenated and nonhalogenated aromatic hydrocarbons in embryonic tissues and cells. Mol Pharmacol 40:818-26

545. **Niwa A, Kumaki K, Nebert DW, Poland AP** 1975 Genetic expression of aryl hydrocarbon hydroxylase activity in the mouse. Distinction between the "responsive" homozygote and heterozygote at the Ah locus. Arch Biochem Biophys 166:559-64

546. **Moir D, Viau A, Chu I, Withey J, McMullen E** 1998 Pharmacokinetics of benzo[a]pyrene in the rat. J Toxicol Environ Health 53:507-30

547. **Brown S, Chee CTY** 1992 Benzopyrene metabolism and transport by cells in culture. Biochem Soc Trans 20:279S

548. **Moore CJ, Gould MN** 1984 Differences in mediated mutagenesis and polycyclic aromatic hydrocarbon metabolism in mammary cells from pregnant and virgin rats. Carcinogenesis 5:103-108

549. **Wang IY, Rasmussen RE, Petrakis NL, Wang A-C** 1976 Enzyme induction and the difference in the metabolite patterns of benzo[a]pyrene produced by various strains of mice. Raven Press, New York

550. **Chaloupka K, Krishnan V, Safe S** 1992 Polynuclear aromatic hydrocarbon carcinogens as antiestrogens in MCF-7 human breast cancer cells: role of the Ah receptor. Carcinogenesis 13:2233-9

551. **Duan R, Porter W, Samudio I, Vyhlidal C, Kladde M, Safe S** 1999 Transcriptional activation of c-fos protooncogene by 17β-estradiol: mechanism of aryl hydrocarbon receptor-mediated inhibition. Molecular Endocrinology 13:1511-21

552. **Gillesby BE, Zacharewski TR** 1998 Exoestrogens: mechanisms of action and strategies for identification and assessment. Environ Tox and Chem 17:3-14

553. **Zacharewski T** 1998 Identification and assessment of endocrine disruptors: limitations of in vivo and in vitro assays. Environ Health Perspect 106 Suppl 2:577-82

554. **Kuiper GG, Carlsson B, Grandien K, et al.** 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138:863-70

555. **Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J-A, Nilsson S** 1998 Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Mol. Pharmacol. 54:105-12

556. **MacGregor JI, Jordan VC** 1998 Basic guide to the mechanisms of antiestrogen action. Pharmacol Rev 50:151-96

557. **Christman JK, Nehls S, Polin L, Brooks SC** 1995 Relationship between estrogen structure and conformational changes in estrogen receptor/DNA complexes. J Steroid Biochem Mol Biol 54:201-10

558. **Paige LA, Christensen DJ, Gron H, et al.** 1999 Estrogen receptor (ER) modulators each induce distinct conformational changes in ER alpha and ER beta. Proc Natl Acad Sci U S A 96:3999-4004

559. **Kuiper GG, Lemmen JG, Carlsson B, et al.** 1998 Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252-63

560. **Pace P, Taylor J, Suntharalingam S, Coombes RC, Ali S** 1997 Human estrogen receptor beta binds DNA in a manner similar to and dimerizes with estrogen receptor alpha. J Biol Chem 272:25832-8

561. **Phillips DH** 1983 Fifty years of benzo(a)pyrene. Nature 303:468-72

562. **Warshawsky D** 1999 Polycyclic aromatic hydrocarbons in carcinogenesis. Environ. Health. Perspect. 107:317-9

563. **Diamond ML, Gingrich SE, Fertuck K, et al.** 2000 Evidence for organic film on an impervious urban surface: characterization and potential teratogenic effects. Environ. Sci. Technol. 34:2900-2908

564. **Tremblay GB, Tremblay A, Copeland NG, et al.** 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. Mol. Endocrinol. 11:353-65

565. **Krege JH, Hodgin JB, Couse JF, et al.** 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc. Natl. Acad. Sci. 95:15677-82

566. **Grimmer G, Jacob J, Dettbarn G, Naujack K-W** 1985 Determination of polycyclic aromatic hydrocarbons, azaarenes, and thiaarenes emitted from coal-fired residential furnaces by gas chromatography/mass spectrometry. Fresenius Zeitschrift Fur Analytische Chemie 322:595-602

567. **Pelroy RA, Stewart DL, Tominaga Y, Iwao M, Castle RN, Lee ML** 1983 Microbial mutagenicity of 3-ring and 4-ring polycyclic aromatic sulfur heterocycles. Mutat. Res. 117:31-40

568. **Croisy A, Mispelter J, Lhoste J-M, Zajdela F, Jacquignon P** 1984 Thiophene analogues of carcinogenic polycyclic hydrocarbons: Elbs pyrolysis of various aroylmethylbenzo[b]thiophenes. J. of Heterocyclic Chem. 21:353-359

569. **Rasmussen K, Booth GM, Lee ML** 1991 Mutagenicities of hydroxy-substituted carbazoles and dibenzothiophenes using the CHO/HGPRT assay. Environ. Tox. Chem. 10:1133-7

570. **Loquet C, Engelhardt U, Schaefer-Ridder M** 1985 Differentiated genotoxic response of carcinogenic and non-carcinogenic benzacridines and metabolites in rat hepatoma cells. Carcinogenesis 6:455-7

571. **Wright DJ, Robinson HK, Holder GM, Ryan AJ, Bonin AM, Baker RS** 1985 Metabolism of the carcinogen 7-methylbenz[c]-acridine in the rat. Xenobiotica 15:825-34

572. **West WR, Smith PA, Booth GM, Lee MM** 1986 Determination and genotoxicity of nitrogen heterocycles in a sediment from the Black River. Environ. Tox. Chem. 5:511-519

573. **Jacob J, Schmoldt A, Augustin C, Raab G, Grimmer G** 1991 Rat liver microsomal ring- and S-oxidation of thiaarenes with central or peripheral thiophene rings. Toxicology 68:181-194

574. **Kropp KG, Fedorak PM** 1998 A review of the occurrence, toxicity, and biodegradation of condensed thiophenes found in petroleum. Can. J. Microbiol. 44:605-22

575. **Jung DKJ, Klaus T, Fent K** 2001 Cytochrome P450 induction by nitrated polycyclic aromatic hydrocarbons, azaarenes, and binary mixtures in fish hepatoma cell line PLHC-1. Environ. Toxicol. Chem. 20:149-59

576. **Kumar S** 1995 A new abbreviated synthesis of 5-methylchrysene and its 2-hydroxy and 8-hydroxy derivatives. Tetrahedron Lett. 37:6271-4

577. **Kumar S** 1998 A new efficient route to the phenolic derivatives of chrysene and 5-methylchrysene, precursors to dihydrodiol and diol epoxide metabolites of chrysene and 5-methylchrysene, through Suzuki cross coupling reaction. J. Chem. Soc. Perkin Trans. J. 19:3157-60

578. **Kumar S, Kim TY** 2000 An improved and regiospecific synthesis of trans-3,4-dihydrodiol metabolite of benzobnaphtho2,1-dthiophene. J. Org. Chem. 65:3883-4

579. **Fertuck KC, Matthews JB, Zacharewski TR** 2001 Hydroxylated benzo[a]pyrene metabolites are responsible for *in vitro* estrogen receptor-mediated gene expression induced by benzo[a]pyrene, but do not elicit uterotrophic effects *in vivo*. Toxicol. Sci. 59:231-240

580. **Ogawa S, Taylor JA, Lubahn DB, Korach KS, Pfaff DW** 1996 Reversal of sex roles in genetic female mice by disruption of estrogen receptor gene. Neuroendocrinology 64:467-70

581. **Windahl SH, Vidal O, Andersson G, Gustafsson JA, Ohlsson C** 1999 Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERbeta -/- mice. J. Clin. Invest. 104:895-901

582. **Bauer E, Guo Z, Ueng Y-F, Bell LC, Zeldin D, Guengerich FP** 1995 Oxidation of benzo[a]pyrene by recombinant human cytochrome P450 enzymes. Chem. Res. Toxicol. 8:136-42

583. **Jacob J, Schmoldt A, Kohbrok W, Raab G, Grimmer G** 1982 On the metabolic activation of benz[a]acridine and benz[c]acridine by rat liver and lung microsomes. Cancer Lett. 16:297-306

584. **Jacob J, Schmoldt A, Grimmer G** 1986 The predominant role of S-oxidation in rat liver metabolism of thiaarenes. Cancer Lett. 32:107-116

585. **Murphy SE, Amin S, Coletta K, Hoffman D** 1992 Rat liver metabolism of benzo[b]naphtho[2,1-d]thiophene. Chem. Res. Toxicol. 5:491-5

586. **Nordqvist M, Thakker DR, Vyas KP, et al.** 1981 Metabolism of chrysene and phenanthrene to bay-region diol epoxides by rat liver enzymes. Mol. Pharmacol. 19:168-78

587. **von Angerer E, Biberger C, Leichtl S** 1995 Studies on heterocycle-based pure estrogen antagonists. Ann. N. Y. Acad. Sci. 12:176-91

588. **Golob T, Biberger C, Walter G, von Angerer E** 2000 Antiestrogenic activities of 3,8-dihydroxy-6,11-dihydrobenzo[a]carbazoles with sulfur-containing side chains. Arch. Pharm. (Weinheim) 333:305-11

589. **Southworth GR, Keffer CC, Beauchamp JJ** 1981 The accumulation and disposition of benz[a]acridine in the fathead minnow, *Pimephales promelas.* Arch. Environ. Contam. Tox. 10:561-9

590. **Modica R, Fiume M, Guaitani A, Bartosek I** 1983 Comparative kinetics of benz(a)anthracene, chrysene and triphenylene in rats after oral administration. I. Study with single compounds. Toxicol. Lett. 18:103-9

591. **Williams CL, Stancel GM** 1996 Estrogens and Progestins. In: Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG (eds) The Pharmacological Basis of Therapeutics, 9 ed. McGraw-Hill, New York, pp 1411-1440

592. **DeMayo FJ, Zhao B, Takamoto N, Tsai SY** 2002 Mechanisms of action of estrogen and progesterone. Ann N Y Acad Sci 955:48-59; discussion 86-8, 396-406

593. **Couse JF, Korach KS** 2001 Contrasting phenotypes in reproductive tissues of female estrogen receptor null mice. Ann N Y Acad Sci 948:1-8

594. **Lindberg MK, Alatalo SL, Halleen JM, Mohan S, Gustafsson JA, Ohlsson C** 2001 Estrogen receptor specificity in the regulation of the skeleton in female mice. J Endocrinol 171:229-36

595. **Geary N, Asarian L, Korach KS, Pfaff DW, Ogawa S** 2001 Deficits in E2-dependent control of feeding, weight gain, and cholecystokinin satiation in ER-alpha null mice. Endocrinology 142:4751-7

596. **Erlandsson MC, Ohlsson C, Gustafsson JA, Carlsten H** 2001 Role of oestrogen receptors alpha and beta in immune organ development and in oestrogen-mediated effects on thymus. Immunology 103:17-25

597. **Shughrue PJ, Lubahn DB, Negro-Vilar A, Korach KS, Merchenthaler I** 1997 Responses in the brain of estrogen receptor alpha-disrupted mice. Proc Natl Acad Sci U S A 94:11008-12

598. **Wang L, Andersson S, Warner M, Gustafsson JA** 2001 Morphological abnormalities in the brains of estrogen receptor beta knockout mice. Proc Natl Acad Sci U S A 98:2792-6

599. **Lee KH, Hess RA, Bahr JM, Lubahn DB, Taylor J, Bunick D** 2000 Estrogen receptor alpha has a functional role in the mouse rete testis and efferent ductules. Biol Reprod 63:1873-80

600. **Reid G, Denger S, Kos M, Gannon F** 2002 Human estrogen receptor-alpha: regulation by synthesis, modification and degradation. Cell Mol Life Sci 59:821-31

601. **Moggs JG, Orphanides G** 2001 Estrogen receptors: orchestrators of pleiotropic cellular responses. EMBO Rep 2:775-81

602. **Diel P** 2002 Tissue-specific estrogenic response and molecular mechanisms. Toxicol Lett 127:217-24

603. **McDonnell DP, Norris JD** 2002 Connections and regulation of the human estrogen receptor. Science 296:1642-4

604. **Lonard DM, Smith CL** 2002 Molecular perspectives on selective estrogen receptor modulators (SERMs): progress in understanding their tissue-specific agonist and antagonist actions. Steroids 67:15-24

605. **Watson CS, Campbell CH, Gametchu B** 2002 The dynamic and elusive membrane estrogen receptor-alpha. Steroids 67:429-37

606. **Levin ER** 2002 Cellular functions of plasma membrane estrogen receptors. Steroids 67:471-5

607. **Matthews J, Celius T, Halgren R, Zacharewski T** 2000 Differential estrogen receptor binding of estrogenic substances: a species comparison. J Steroid Biochem Mol Biol 74:223-34.

608. **Matthews JB, Fertuck KC, Celius T, Huang YW, Fong CJ, Zacharewski TR** 2002 Ability of structurally diverse natural products and synthetic chemicals to induce gene expression mediated by estrogen receptors from various species. J Steroid Biochem Mol Biol 82:181-94

609. **Blair RM, Fang H, Branham WS, et al.** 2000 The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol Sci 54:138-53

610. **Wang H, Masironi B, Eriksson H, Sahlin L** 1999 A comparative study of estrogen receptors alpha and beta in the rat uterus. Biol Reprod 61:955-64

611. **Emmen JM, Korach KS** 2001 Developing animal models for analyzing SERM activity. Ann N Y Acad Sci 949:36-43

612. **Zacharewski T** 1997 In vitro bioassays for assessing estrogenic substances. Environ Sci Technol 31:613-623

613. **Reel JR, Lamb IJC, Neal BH** 1996 Survey and assessment of mammalian estrogen biological assays for hazard characterization. Fundam Appl Toxicol 34:288-305

614. **Lauson HD, Heller CG, Golden JB, Sevringhaus EL** 1939 The immature rat uterus in the assay of estrogenic substances, and a comparison of estradiol, estrone and estriol. Endocrinology 24:35-44

615. **Jones RC, Edgren RA** 1973 The effects of various steroids on the vaginal histology in the rat. Fertility and Sterility 24:284-91

616. **Newbold RR, Jefferson WN, Padilla-Banks E** 2001 The mouse uterotrophic assay: other end points. Environ Health Perspect 109:A569-70

617. **Zacharewski TR, Fertuck KC, Fielden MR, Matthews JB** 2002 Mechanistically-based assays for the identification and characterization of endocrine disruptors. In: Vanden Heuvel JP, Greenlee WF, Perdew GH, Mattes W, Sipes IG, McQueen CA, Gandolfi AJ (eds) Cellular and Molecular Toxicology. Elsevier Science, Amsterdam, pp 559-582

618. **Bolt HM** 1979 Metabolism of estrogens--natural and synthetic. Pharmacol Ther 4:155-81

619. **Rozen S, Skaletsky H** 2000 Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365-86

620. **Shapiro RA, Xu C, Dorsa DM** 2000 Differential transcriptional regulation of rat vasopressin gene expression by estrogen receptor alpha and beta. Endocrinology 141:4056-64

621. **Hyder SM, Nawaz Z, Chiappetta C, Stancel GM** 2000 Identification of functional estrogen response elements in the gene coding for the potent angiogenic factor vascular endothelial growth factor. Cancer Res 60:3183-90

622. **Ikeda K, Orimo A, Higashi Y, Muramatsu M, Inoue S** 2000 Efp as a primary estrogen-responsive gene in human breast cancer. FEBS Lett 472:9-13

623. **Efron B, Tibshirani R, Storey JD, Tusher V** 2001 Empirical Bayes analysis of a microarray experiment. J. Am. Stat. Assoc. 96:1151-1160

624. **Benjamini Y, Hochberg Y** 1995 Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of The Royal Statistical Society B57:289-300

625. **Affymetrix** 2002 GeneChip Expression Analysis: Data Analysis Fundamentals.

626. **Geum D, Sun W, Paik SK, Lee CC, Kim K** 1997 Estrogen-induced cyclin D1 and D3 gene expressions during mouse uterine cell proliferation in vivo: differential induction mechanism of cyclin D1 and D3. Mol Reprod Dev 46:450-8

627. **Green AR, Parrott EL, Butterworth M, Jones PS, Greaves P, White IN** 2001 Comparisons of the effects of tamoxifen, toremifene and raloxifene on enzyme induction and gene expression in the ovariectomised rat uterus. J Endocrinol 170:555-64

628. **Anderson JN, Peck EJ, Jr., Clark JH** 1975 Estrogen-induced uterine responses and growth: relationship to receptor estrogen binding by uterine nuclei. Endocrinology 96:160-7

629. **Cheng SV, MacDonald BS, Clark BF, Pollard JW** 1985 Cell growth and cell proliferation may be dissociated in the mouse uterine luminal epithelium treated with female sex steroids. Exp Cell Res 160:459-70

630. **Lan NC, Katzenellenbogen BS** 1976 Temporal relationships between hormone receptor binding and biological responses in the uterus: studies with short- and long-acting derivatives of estriol. Endocrinology 98:220-7

631. **Choi D, Yoon S, Lee E, et al.** 2002 Characterization of cyclin D2 expression in human endometrium. Journal of the Society for Gynecologic Investigation 9:41-46

632. **Zhang Z, Laping J, Glasser S, Day P, Mulholland J** 1998 Mediators of estradiol-stimulated mitosis in the rat uterine luminal epithelium. Endocrinology 139:961-6

633. **Yang LS, Marshall A, Koide SS** 1994 Differential effect of estrogen on the production of cyclin B1, cdc2 p34 and c-fos protein in rat uterus. Endocr Res 20:377-86

634. **Horne MC, Goolsby GL, Donaldson KL, Tran D, Neubauer M, Wahl AF** 1996 Cyclin G1 and cyclin G2 comprise a new family of cyclins with contrasting tissue-specific and cell cycle-regulated expression. J Biol Chem 271:6050-61

635. **Parker NJ, Begley CG, Fox RM** 1994 Human R1 subunit of ribonucleotide reductase (RRM1): 5' flanking region of the gene. Genomics 19:91-6

636. **Maruyama T, Kitaoka Y, Sachi Y, et al.** 1997 Thioredoxin expression in the human endometrium during the menstrual cycle. Mol. Hum. Reprod. 3:989-993

637. **Weisz A, Bresciani F** 1993 Estrogen regulation of proto-oncogenes coding for nuclear proteins. Crit Rev Oncog 4:361-88

638. **Thompson CB, Challoner PB, Neiman PE, Groudine M** 1986 Expression of the c-myb proto-oncogene during cellular proliferation. Nature 319:374-80

639. **Suva LJ, Harm SC, Gardner RM, Thiede MA** 1991 In vivo regulation of Zif268 messenger RNA expression by 17 beta-estradiol in the rat uterus. Mol Endocrinol 5:829-35

640. **Tong W, Pollard JW** 1999 Progesterone inhibits estrogen-induced cyclin D1 and cdk4 nuclear translocation, cyclin E- and cyclin A-cdk2 kinase activation, and cell proliferation in uterine epithelial cells in mice. Mol Cell Biol 19:2251-64

641. **Hopwood D, Levison DA** 1976 Atrophy and apoptosis in the cyclical human endometrium. J Pathol 119:159-66

642. **Billing RJ, Barbiroli B, Smellie RM** 1969 The effect of oestradiol-17beta on the rate of synthesis of ribonucleic acid in rat uterus. Biochem J 114:37P-38P

643. **Tchernitchin A, Tchernitchin X, Galand P** 1975 Correlation of estrogen-induced uterine eosinophilia with other parameters of estrogen stimulation, produced with estradiol-17beta and estriol. Experientia 31:993-4

644. **McBride AE, Silver PA** 2001 State of the arg: protein methylation at arginine comes of age. Cell 106:5-8

645. **Das SK, Tan J, Raja S, Halder J, Paria BC, Dey SK** 2000 Estrogen targets genes involved in protein processing, calcium homeostasis, and Wnt signaling in the mouse uterus independent of estrogen receptor-alpha and -beta. J Biol Chem 275:28834-42.

646. **Salamonsen LA, Woolley DE** 1999 Menstruation: induction by matrix metalloproteinases and inflammatory cells. J Reprod Immunol 44:1-27

647. **Stewart PJ, Zaloudek CJ, Inman MM, Webster RA** 1983 Effects of dexamethasone and indomethacin on estrogen-induced uterine growth. Life Sci 33:2349-56

648. **Critchley HO, Kelly RW, Brenner RM, Baird DT** 2001 The endocrinology of menstruation--a role for the immune system. Clin Endocrinol (Oxf) 55:701-10

649. **Paul KG, Kumlien A, Jakobsson S, Brody S** 1967 Eosinophil granulocytes. I. Identification of the uterus peroxidase. Scand J Clin Lab Invest 20:11-6

650. **Ross R, Klebanoff SJ** 1966 The eosinophilic leukocyte. Fine structure studies of changes in the uterus during the estrous cycle. J Exp Med 124:653-60

651. **Finn CA** 1986 Implantation, menstruation and inflammation. Biol Rev Camb Philos Soc 61:313-28

652. **Sullivan DA, Wira CR** 1981 Estradiol regulation of secretory component in the female reproductive tract. J Steroid Biochem 15:439-44

653. **Hasty LA, Lambris JD, Lessey BA, Pruksananonda K, Lyttle CR** 1994 Hormonal regulation of complement components and receptors throughout the menstrual cycle. Am J Obstet Gynecol 170:168-75

654. **Padilla-Banks E, Jefferson WN, Newbold RR** 2001 The immature mouse is a suitable model for detection of estrogenicity in the uterotropic bioassay. Environ Health Perspect 109:821-6

655. **Fan JD, Wagner BL, McDonnell DP** 1996 Identification of the sequences within the human complement 3 promoter required for estrogen responsiveness provides insight into the mechanism of tamoxifen mixed agonist activity. Mol Endocrinol 10:1605-16

656. **Ino Y, Gotoh M, Sakamoto M, Tsukagoshi K, Hirohashi S** 2002 Dysadherin, a cancer-associated cell membrane glycoprotein, down-regulates E-cadherin and promotes metastasis. Proc Natl Acad Sci U S A 99:365-370

657. **Daikoku T, Matsumoto H, Gupta RA, et al.** 2003 Expression of Hypoxia-inducible Factors in the Peri-implantation Mouse Uterus Is Regulated in a Cell-

specific and Ovarian Steroid Hormone-dependent Manner. Evidence for differential function of HIFs during early pregnancy. J. Biol. Chem. 278:7683-7691

658. **Aaronson SA, Natori Y, Tarver H** 1965 Effect of estrogen on uterine ATP levels. Proc Soc Exp Biol Med 120:9-10

659. **Steplewski Z** 1966 Effect of actinomycin D on alkaline phosphatase induction by estrogen in uterine epithelium of spayed mice. Bull Acad Pol Sci Biol 14:387-90

660. **Yamada T, Horiuchi M, Dzau VJ** 1996 Angiotensin II type 2 receptor mediates programmed cell death. Proc Natl Acad Sci U S A 93:156-60

661. **Li XF, Ahmed A** 1996 Dual role of angiotensin II in the human endometrium. Hum Reprod 11 Suppl 2:95-108

662. **Mancina R, Susini T, Renzetti A, et al.** 1996 Sex steroid modulation of AT2 receptors in human myometrium. J Clin Endocrinol Metab 81:1753-7

663. **Zhang Z, Funk C, Roy D, Glasser S, Mulholland J** 1994 Heparin-binding epidermal growth factor-like growth factor is differentially regulated by progesterone and estradiol in rat uterine epithelial and stromal cells. Endocrinology 134:1089-94

664. **Pena A, Reddy CD, Wu S, et al.** 1993 Regulation of human ornithine decarboxylase expression by the c-Myc.Max protein complex. J Biol Chem 268:27277-85

665. **Tobias KE, Shor J, Kahana C** 1995 c-Myc and Max transregulate the mouse ornithine decarboxylase promoter through interaction with two downstream CACGTG motifs. Oncogene 11:1721-7

666. **Levy C, Glikman P, Vegh I, Mester J, Baulieu E, Soto R** 1984 Estradiol, progesterone and tamoxifen regulation of ornithine decarboxylase (ODC) in rat uterus and chick oviducts. Prog Clin Biol Res 142:133-44

667. **Curtis SW, Shi H, Teng C, Korach KS** 1997 Promoter and species specific differential estrogen-mediated gene transcription in the uterus and cultured cells using structurally altered agonists. J Mol Endocrinol 18:203-11

668. **Iyer R, Jenkinson CP, Vockley JG, Kern RM, Grody WW, Cederbaum S** 1998 The human arginases and arginase deficiency. J Inherit Metab Dis 21 Suppl 1:86-100

669. **Tabor CW, Tabor H** 1984 Polyamines. Annu Rev Biochem 53:749-90

670. **Heller JS, Fong WF, Canellakis ES** 1976 Induction of a protein inhibitor to ornithine decarboxylase by the end products of its reaction. Proc Natl Acad Sci U S A 73:1858-62

671. **Hayashi S, Fujita K** 1983 Antizyme and antizyme inhibitor of ornithine decarboxylase (rat liver). Methods Enzymol 94:185-93

672. **Yallampalli C, Dong Y-L** 2000 Estradiol-17{beta} inhibits nitric oxide synthase (NOS)-II and stimulates NOS-III gene expression in the rat uterus. Biol Reprod 63:34-41

673. **Rao VS, Chaves MC, Ribeiro RA** 1995 Nitric oxide synthase inhibition and the uterotrophic response to oestrogen in immature rats. J Reprod Fertil 105:303-6

674. **Pietila M, Alhonen L, Halmekyto M, Kanter P, Janne J, Porter CW** 1997 Activation of Polyamine Catabolism Profoundly Alters Tissue Polyamine Pools and Affects Hair Growth and Female Fertility in Transgenic Mice Overexpressing Spermidine/Spermine N1-Acetyltransferase. J. Biol. Chem. 272:18746-18751

675. **Freeman JJ, Williams MA** 1971 Estrogenic induction of uterine arginase activity. Horm Metab Res 3:352

676. **Sonoki T, Nagasaki A, Gotoh T, et al.** 1997 Coinduction of Nitric-oxide Synthase and Arginase I in Cultured Rat Peritoneal Macrophages and Rat Tissues in Vivo by Lipopolysaccharide. J. Biol. Chem. 272:3689-3693

677. **Durante W, Liao L, Reyna SV, Peyton KJ, Schafer AI** 2001 Transforming Growth Factor-{beta}1 Stimulates L-Arginine Transport and Metabolism in Vascular Smooth Muscle Cells : Role in Polyamine and Collagen Synthesis. Circulation 103:1121-1127

678. **Kushner PJ, Agard DA, Greene GL, et al.** 2000 Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74:311-7.

679. **Duan R, Porter W, Safe S** 1998 Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. Endocrinology 139:1981-90

680. **Cook JW, Hewett C, Hieger I** 1932 Coal tar constituents and cancer. Nature 130:926

681. **Markey CM, Michaelson CL, Veson EC, Sonnenschein C, Soto AM** 2001 The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A. Environ Health Perspect 109:55-60.

682. **Ashby J** 2001 Increasing the sensitivity of the rodent uterotrophic assay to estrogens, with particular reference to bisphenol A. Environ Health Perspect 109:1091-4

683. **Teng CT, Liu Y, Yang N, Walmer D, Panella T** 1992 Differential molecular mechanism of the estrogen action that regulates lactoferrin gene in human and mouse. Mol Endocrinol 6:1969-81

684. **Klinge CM, Bowers JL, Kulakosky PC, Kamboj KK, Swanson HI** 1999 The aryl hydrocarbon receptor (AHR)/AHR nuclear translocator (ARNT) heterodimer interacts with naturally occurring estrogen response elements. Mol Cell Endocrinol 157:105-19