Regulation of the estrogen receptor activity in human endometrium

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Abbreviations

Estradiol:	17β-estradiol: E_2
ER:	estrogen receptor
FSH:	follicle-stimulating hormone
LH:	luteinizing hormone
PR:	progesterone receptor
AF-1:	activation function domain 1
AF-2:	activation function domain 1
ERE:	estrogen responsive element
AP-1:	activator protein-1
Sp1:	specificity protein 1
NF-κB:	nuclear receptor kappa-B
EGF:	epidermal growth factor
IGF:	insuline-like growth factor
cAMP:	cyclic adenosine monophosphate
N-CoR:	nuclear receptor corepressor
SMRT:	silencing mediator for retinoid and thyroid hormone receptors
SRC-1:	steroid receptor coactivator-1
CBP:	CREB (cAMP responsive element binding protein) binding protein
HAT:	histone acetyl transferase
Co-IP:	co-immunoprecipitation
ChIP:	chromatin immunoprecipitation
RNA pol II:	RNA polymerase II
CDS:	coding sequence

Foreword

For six months I have been enrolled in real scientific research concerning the estrogen receptor and human endometrium and it has been a great and instructive experience. I learned not only how to do research, my interest in research increased with time and I became more experienced in performing several techniques.

Therefore I want to thank Patrick Groothuis for giving me the opportunity to run this training period as a part of the Endometrium group. Foremost I want to thank Andrea Romano not only for critically reviewing this thesis, but also to guide me during the training period. He supported me when I was insecure and emotional and if I had any question I could go to him. Next I want to thank Bert Delvoux for his joke ("What are you sinking about?"), but also for all the chats.

In the beginning I was only surrounded by men, but that changed when three female students arrived, also to run their training period. First of all I want to thank Caroline for supporting me when I was down and for all the chats we had. For the western blot and DOKA fun I want to thank Ann. And last but not least my thanks go to Annabel. All together, we had al lot of fun.

Furthermore I would like to thank the Department of Pathology, the Department of Obstetrics and Gynaecology and the Department of Molecular cell biology allowing me to do this research.

Special thanks go to my father who made it possible to complete this four years of study. Unfortunately this can not be experienced by my mother, but I now she watches over me. Last, my thanks go to my friends, and especially to my boyfriend Jurgen for his love and support, for always being there.

Abstract

The human endometrium is a specialized tissue that undergoes sequential phases of proliferation and secretory changes in order to support the implantation and growth of an embryo. In particular, estradiol has a central role in the coordination of these events. All major actions of estradiol are mediated by the estrogen receptor (ER). The classical mechanism of ER action involves estradiol binding and subsequent interaction between the activated ER and estrogen responsive elements (ERE) located in the promoters of target genes. However, ER can also regulate gene expression through interaction with other DNA-binding transcription factors, e.g. AP-1, Sp1 and NF-κB. Whatever the mechanism of ER activation, transcriptional activity requires the interaction of the ER with coregulator proteins (coactivators and corepressors) that are recruited at the level the target gene promoters. The objective of the present study was to study the regulation of ER-α activity and the role of coregulators in human endometrium. Two model systems were used:

1) The first consisted of a human endometrial cancer cell line. Two genes, known to be differentially regulated by estradiol in the human endometrium, were selected for investigation: GW112 and GADD45B. The promoters of both genes showed numerous putative regulatory motifs, including ERE half sites, Sp1, AP-1 and NF-κB sites indicating that ER-α could bind directly or indirectly to the promoters of these genes. The human endometrial cancer cell line RL95.2 was used to study the regulation of both genes. First, the interaction between ER-α and distinct coregulators was investigated in solution using co-immunoprecipitation. This showed that after estradiol treatment both coactivators and corepressors could interact with ER-α in solution. Thus, it could be hypothesized that selective recruitment of coregulators by ER-α occurs at the level of target gene promoters. To examine this hypothesis in the RL95.2 cells, chromatin immunoprecipitation (ChIP) could be used. However, this method had never been performed on these cells, therefore the ChIP protocol needed to be optimized.

2) The second model consisted of human endometrium to study all genes that are targeted by the ER-α in vivo. To address this question, the ChIP protocol can be coupled to genome-wide DNA micro-arrays. Also in this case, the protocol had to be optimized first.

In conclusion, the regulation of a particular gene by estradiol is thought to be not only influenced by the ligand but also by the cellular and promoter context. Knowledge of the mechanisms of estradiol and ER-α signaling to regulate gene expression are necessary to get a better understanding in the normal and abnormal endometrial physiology.

1   Introduction

The human endometrium, which forms the mucosal lining of the uterine cavity, is a dynamic tissue that, to prepare for implantation, undergoes well-characterized cycles of proliferation, differentiation and tissue breakdown in response to the endocrine and paracrine environmental changes. If fertilization occurs, the embryo is implanted into the endometrium and resides there for the rest of its development. If pregnancy fails to be established, the endometrium is shed and regenerates to prepare for the next possible implantation. Estradiol, the most potent and abundant estrogen, is one of the major steroid hormones involved in the regulation of these processes. However, the genes, molecular mechanisms and pathways by which estradiol and its estrogen receptor (ER) regulate these events are only partly understood, mainly because of the complexity and the large number of factors acting in concert. The main goal of this study is to get a better understanding in the mechanisms of estradiol action and ER signaling, and subsequently the regulation of gene expression.

1.1   The human endometrium

The human endometrium is constituted of simple columnar epithelium and underlain by a vascularized stroma. Surface epithelial cells are interspersed with glands lined by epithelial cells and extending from the surface in a tubular shape into the stroma. Two layers build up the endometrium: the superficial functionalis and the deeper located basalis. The functionalis is a thick layer that in response to blood levels of the ovarian hormones, estradiol and progesterone, undergoes cyclic changes and is shed during menstruation. The deeper narrow layer of the endometrium, the basalis, regenerates a new functionalis each menstrual cycle. At all times across the menstrual cycle this layer remains viable because of its continuous blood supply (1, 2).

1.1.1   The menstrual cycle

To prepare an ovarian follicle for ovulation and to prepare the uterine lining for possible implantation of a developing embryo, the ovaries and the endometrium go, respectively, through the ovarian cycle and the uterine cycle, together called the menstrual cycle. The

ovarian and uterine (or endometrial) cycles run parallel through the menstrual cycle. The uterine cycle can be divided into the menstrual, proliferative and secretory phase. The ovarian cycle can be divided into the follicular and the luteal phase. Figure 1 shows how the ovarian and the uterine cycles are coordinated. All phases of the menstrual cycle are regulated by two gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both released by the anterior pituitary (under the control of the hypothalamus). FSH and LH influence the blood levels of estradiol and progesterone, regulating the changes in the endometrial conformation across the menstrual cycle (1, 3-5).
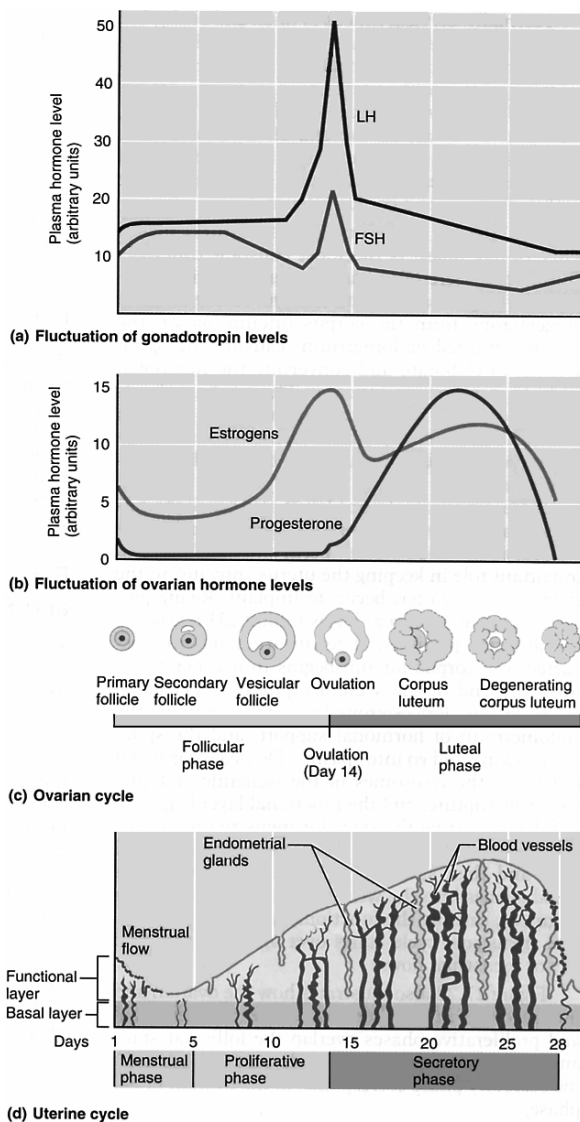
Figure 1. Correlation of anterior pituitary and ovarian hormones with structural changes in the ovary and uterus. The time bar at the bottom of the figure, going from cycle day 1-28 applies for all parts of this figure. (a) The fluctuating blood levels of the gonadotropins FSH and LH. These hormones regulate the events in the ovarian cycle. (b) The fluctuating blood levels of ovarian hormones estrogens and progesterone. These hormones cause endometrial changes of the uterine cycle. (c) Structural changes in the ovarian follicles during the ovarian cycle are correlated with endometrial changes in the uterine cycle. (d) The three phases of the uterine cycle: menstrual, proliferative and secretory phase. Briefly, during the menstrual phase the functional layer of the endometrium is shed. During and after this phase has passed, the functional zone of the endometrium is rebuild. Both phases occur before ovulation and together they correspond to the follicular phase of the ovarian cycle. The secretory phase, which begins immediately after ovulation, enriches the blood supply and provides the nutrients to prepare the endometrium to receive an embryo. This phase corresponds to the luteal phase of the ovarian cycle.

Proliferative (Follicular) phase

The proliferative phase spans from the end of menstruation until ovulation. During the early proliferative phase with little ovarian steroid activity present, the surface epithelium is thin, the glands are short and narrow, and the stroma is compact. Later in the follicular phase, the endometrium comes under estradiol dominance: the main changes that occur relate to the stimulatory action of estradiol on DNA synthesis and on mitotic activity. As a result, the endometrium proliferates, the mucosa thickens, the glands cells are mitotically active and enlarge, the stroma becomes well organized and the microvasculature is regenerated from the remaining arteriolar stump in the basalis. This leads to a relative hypertrophy of the uterine mucosa. Next to the stimulating effects of rising estradiol on the endometrium, it exerts also a positive feedback effect within the ovary. This feedback is essential because it allows the precise synchronism between the maturation of the dominant follicle (responsible for the estradiol signal) and the stimulation to ovulation (the preovulatory gonadotropin surge) (1, 3-5).

Secretory (Luteal) phase

The secretory phase commences after ovulation. Shortly after ovulation, estradiol levels decline. This is probably due to damage to the dominant estradiol-secreting follicle during ovulation. The ruptured follicle that remains after ovulation is transformed into a corpus luteum. This newly formed endocrine gland produces progesterone and estradiol almost immediately after it is formed. As the corpus luteum matures and progesterone secretion increases, the proliferative endometrium undergoes a secretory differentiation. During this process, several changes in the endometrium are induced: arrest of glandular proliferation, glandular and stromal cell differentiation, and elaboration of the arteries in the functionalis. The uterine glands of the secretory endometrium secrete nutritious glycogen into the uterine cavity. These nutrients sustain the embryo until it has implanted in the blood-rich endometrial lining. Furthermore, rising levels of progesterone in combination with estrogen exert a powerful feedback effect on the anterior pituitary that result in a decrease of the gonadotropins pulse frequency. Consequently, the development of new follicles is inhibited and the ovulation of additional oocytes is prevented (1, 3-5).

Menstrual phase

At the end of the secretory phase and in the absence of implantation, estrogen and progesterone secretion decreases dramatically due to the regression of the corpus luteum.

As a consequence of this hormonal withdrawal, the endometrium undergoes gradual necrotic ischemic changes. The release of highly potent proteolytic enzymes that help digestion of the functionalis and the vasoconstriction of vessels depriving the endometrium from oxygen and nutrients are responsible for the endometrial degeneration. Subsequently, an inflammatory process is initiated in which leukocytes invade the endometrium. Shortly, thereafter, an intense vasospasm causes expulsion of the functionalis, resulting in menstrual bleeding. At this point, the levels of the ovarian hormones are low, which ends the blockade of gonadotropin secretion. In turn, increasing levels of FSH and LH promote follicle growth and maturation, and estrogen secretion. The rise in estrogen blood levels stimulates the residual basal gland epithelium to proliferate in areas of complete denudation resulting in the reepithelialization of the endometrium. Endometrial stromal cells help in the replacement of shed endometrium. Later in the cycle, these cells play a supportive role in maintaining endometrial integrity until the next menstrual period (1, 3-6).

1.2   The estrogen receptor

As described above, the steroid hormones estradiol and progesterone play a central role in the regulation of all aspects of female reproductive activity. Together, they act at the level of the brain, ovary, and uterus to coordinate cyclic neuroendocrine gonadotropins production, ovulatory activity and uterine development in preparation for implantation of fertilized embryos.

The effects of both estradiol and progesterone are primarily mediated through their cognate receptors, the estrogen receptor and the progesterone receptor (PR). In the following sections of this report, the models that have been established concerning the mechanisms of estradiol and ER signaling are described.

The ER is an intracellular transcription factor that belongs to the nuclear receptor superfamily and it is expressed as two structurally related subtype, ER-α and ER-β, encoded by two distinct genes, ESR1 and ESR2 respectively. Both receptor proteins share a common structural architecture and have 6 regions, named A to F, each corresponding to a functional and structural domain (figure 2). The N-terminal A/B region is weakly conserved among the members of the nuclear receptor superfamily. It has a variable length,

and contains a transcriptional activation function domain (AF-1), a region of the receptor that contributes to ligand independent transcriptional activation of target gene and is involved in protein-protein interactions. There is poor conservation of the AF-1 domain of ER-α and ER-β. Furthermore, the AF-1 domain of the ER-β is less active (7, 8). The highly conserved DNA binding domain (C region) contains two zinc finger motifs, which mediate receptor dimerization, interaction with heat-shock proteins and binding of the receptor to specific DNA sequences. Downstream of the C region, a variable hinge region (D region) is followed by the E region or ligand binding domain, consisting of 12 α-helical and 2 very short β-turns. This domain mediates ligand binding, receptor dimerization, nuclear translocation and contains a coactivator recognition groove and a second transcriptional activation function domain (AF-2). Unlike AF-1, AF-2 is strictly ligand dependent and conserved among the members of the nuclear receptor superfamily. Finally, the F region forms the COOH-terminal of the receptor (9-13).
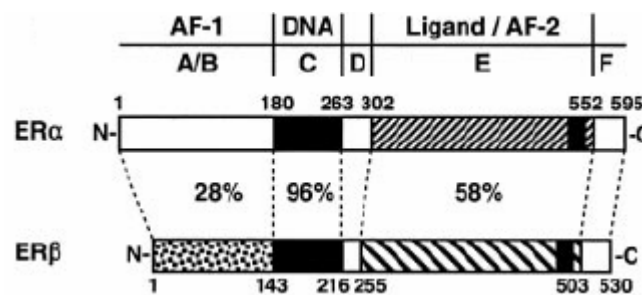
Figure 2. The structure of ER-α and ER-β. Both isoforms of the ER consist of 6 regions (A-F): the A/B region contains an activation function (AF-1), the C region forms the DNA binding domain, the D region is a variable hinge and the E region is the ligand binding site and contains the second activation function (AF-2), the F region forms the COOH-terminal. ER-α and ER-β are constituted of 595 and 530 amino acids, respectively. The degree of homology (%) with ER-α is given for each domain.

1.3   Mechanisms of estradiol and ER signaling

The complexity of estradiol and ER signaling mechanisms has substantially increased during the past decade. In the canonical genomic pathway of activation, estradiol enters the cell and binds to ER. Subsequently, a direct or indirect interaction between the activated ER and DNA specific sequences regulates the transcription of genes.

Not only do ligands regulate ER-dependent gene expression, ER can be transcriptionally activated by a variety of intracellular signaling pathways, mostly secondary to phosphorylation or other protein post-translational modifications. It is clear that a complex cross-talk between estradiol, ER activation and intracellular signaling pathways exists. Studying the mechanisms of these pathways is crucial to understand the variety of cell- and tissue-specific responses. Figure 3 gives an overview of the mechanisms of ER action.

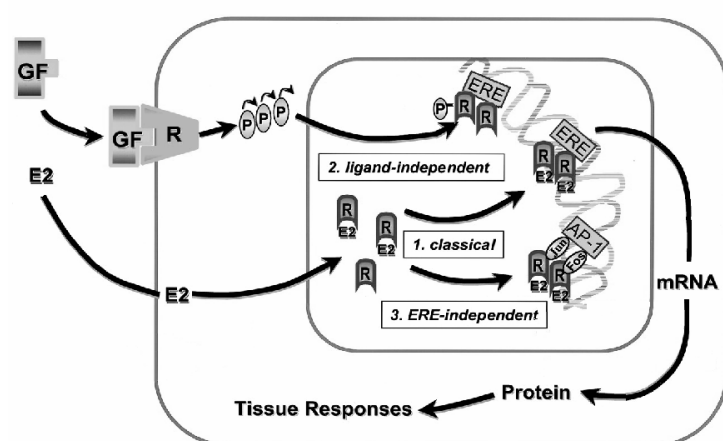### 1.3.1 The ligand-dependent ER signaling pathway

Unliganded ER proteins are associated with a large multiprotein complex of chaperones, including heat-shock protein 90, which maintains the receptor in an inactive state but keeps it prepared for hormone binding (14). Ligand binding induces a conformational change in the ER structure consisting in a shift orientation of α-helix 12. Upon binding of an agonist or an antagonist this shift vary (15, 16). This repositioning results in a novel conformation of the ligand binding domain, which is required for the interaction with co-activator proteins (co-activator recognition groove) (11). The conformational changes induced by an agonist allow the ER to dimerize (ER-α/ER-α, ER-β/ER-β, ER-α/ER-β) and to regulate gene expression by binding directly to specific DNA sequences, known as estrogen responsive elements (ERE) (8). These EREs are mostly located proximal to the transcriptional start sites in the promoters of specific genes (17). Comparison of promoter sequences of estrogen responsive genes led to the identification of the consensus sequence of the ERE, a palindrome of the 5′-AGGTCA-3′ motif separated by 3 base pairs (17, 18). These elements can be occupied by a liganded ER dimer, with one receptor interacting with each motif. Although a few promoters of estradiol target genes contain EREs that match the consensus sequence, e.g. the gene promoters of EBAG9, Efp and COX7A2L (17), most promoters contain imperfect palindromic elements or one or multiple ERE half-sites (19, 20). Both the sequence and the spatial organization of these ERE motifs are important for the affinity and the specificity of ER binding (21).

Next to the direct interaction with the DNA, the activated ER can also indirectly influence the regulation of gene expression through interaction with other transcription factors like activator protein-1 (AP-1; Jun/Fos complex), Specificity protein 1 (Sp1) or nuclear factor kappa-B (NF-κB), that, in turn, interact with the gene promoter (8, 17, 19, 20). In fact, several estrogen responsive genes contain a combination of ERE half-sites and sites where

ERs can indirectly interact with (22, 23). In summary, estradiol actions can be mediated by the interaction of ER with DNA or with DNA-bound transcription factors.

1.3.2   The ligand-independent ER signaling pathway

The expression of steroid target genes is determined not only by ligand-activation of the receptor, but also by activation through other paracrine and/or autocrine signals, including epidermal growth factor (EGF), insulin-like growth factor (IGF) (24, 25) and cAMP (26). Multiple kinases, such as mitogen-activated protein kinase and other protein kinases (protein kinase A and C), can be activated by these extracellular signals through binding to surface receptors (8). In turn, these kinases can induce phosphorylation of different sites in the ER, e.g. serine 118, modulating the transcriptional activity of the ER (24, 25).

Figure 3. The multifaceted mechanisms of estradiol and ER signaling. The biological effects of estradiol (E_2) are mediated through at least three ER pathways. 1) *Classical ligand-dependent:* E_2-ER complexes can bind to EREs in target promoters to regulate gene transcription and subsequent tissue responses. *2) ligand-independent:* extracellular signals such as growth factors (GF) can activate intracellular kinase pathways, leading to phosphorylation (P) and activation of ER at estradiol responsive gene promoters in a ligand-independent manner. *3) ERE-independent:* E2-ER complexes can also bind alternative response elements such as AP-1 sites through association with other DNA-bound transcription factors (Fos/Jun) to regulate gene expression.

1.3.3   Regulation of estradiol responsive gene expression by coregulators

Nuclear receptor coregulators are cellular factors recruited by AF-1 and AF-2 domains of the steroid hormone receptors that are essential in gene transcription. Several mechanisms have been suggested to explain how coregulators exert this control. A first mechanism postulates the recruitment of coregulators to the ER in a ligand-dependent manner. Binding of estradiol (agonist) to the ER induces conformational changes in receptor structure that promote the interaction between coactivator proteins and the AF-1 and AF-2 domains.

Such coactivators in turn promote chromatin remodelling and bridge the nuclear receptor-coactivator complex with general transcription machinery resulting in the formation of productive transcription initiation complexes. On the contrary, binding of an antagonist induces receptor conformational changes that make the AF domains nonpermissive to coactivator binding and instead promote interaction with corepressor proteins that inhibit transcriptional activity of the receptor (15, 16, 27).

A second mechanism that has been postulated by which coregulators can influence transcription consists in the chromatin remodelling, thereby changing the access of general transcription factors to the transcription initiation site. In absence of a ligand or when antagonist is bound to the receptor, a corepressors complex is recruited which induces deacetylation of histone tails leading to transcriptional repression (28). Examples of such corepressors are the nuclear receptor corepressor (N-CoR) and the related protein silencing mediator for retinoid and thyroid hormone receptors (SMRT) (8, 28-30). Addition of an agonist disrupts this repression complex in favour of association with a coactivator complex. Some co-activators, including the steroid receptor coactivator-1 (SRC-1), the CREB (for cAMP responsive element binding protein) binding protein (CBP) and its related protein p300, possess intrinsic histone acetyl transferase (HAT) activity and are recruited in a ligand-dependent manner (27, 28, 30). Histone acetylation reduces the DNA binding affinity of the protein and results in enhanced transcriptional activity. A recent study illustrated that ER-α-associated HATs are recruited to promote transcription in estradiol-responsive genes in a sequential fashion (31, 32).

1.4   The objectives and hypotheses

Estradiol can exert different effects in a variety of tissues e.g. in the endometrium, bone, breast tissue, cardiovascular system and central nervous system. These tissue-specific responses to estradiol result from regulation of different sets of genes. The ability of ER-α to interact with a variety of coactivator and corepressor proteins, together with the differential spatiotemporal expression of these coregulators, might play a key role in the regulation of different genes. Genes are thought to be transcriptionally stimulated or repressed by ER-α in response to estradiol through the selective recruitment of distinct sets of coregulators, coactivators and corepressors respectively. The objective of the present study is to understand the regulation of estradiol response and coregulator recruitment in the human endometrium. Steroid hormone based therapies are commonly used to treat

several diseases, thus, a better knowledge of the basic processes of the action of estradiol in the human endometrium are essential.

1.5 The research approach

To increase our understanding in the mechanisms of estradiol action in the human endometrium, two approaches were followed. The first approach was based on an in vitro model system (a human endometrial cancer cell line) used to study the regulation of the ER-α activity, including the binding of ER-α and the recruitment of coregulators, on different selected gene promoters known to be regulated by estradiol in vivo (approach 1). In the second approach the in vivo ER-α binding in human endometrium was studied (approach 2).

1.5.1 The regulation of the ER-α activity at the level of individual gene promoters using a model system (Approach 1)

The first step in investigating the regulation of the ER-α activity, and therefore the estradiol and ER-α signaling, in a model system is to identify and select some genes that are regulated by estradiol. This selection was based on preliminary micro-array data (Groothuis P, et al.). Gene expression analysis (micro-array) was performed in the human endometrium tissue collected either from the early proliferative (menstrual) phase and late proliferative phase or after 24 hours of culture in the presence of estradiol or vehicle. Several genes were regulated by estradiol and a few of them were involved in proliferation and growth arrest (table 1).

Table 1. A list of genes regulated by estradiol. Early proliferative (menstrual) and late proliferative endometrium were analyzed uncultured (in vivo) or cultured (in vitro) in the presence of estradiol for 24 hours. Both cultured and uncultured endometrium were analyzed with micro-array to find estrogen responsive genes.

Gene name	Protein name	Regulation by estradiol	
		In vivo	In vitro
Genes involved in the cell cycle			
CCNA1	Cyclin-A1	+	+
CCNB1	Cyclin-B1	+	
CCNB2	Cyclin-B2	+	
CDC2	Cell division control protein 2	+	
CDC20	Cell division cycle protein 20	+	
CDC6	Cell division control protein 6	+	
CDK10	Cell division protein kinase 10		+
CDKN1A	Cyclin-dependent kinase inhibitor 1	-	
CDKN2B	Cyclin-dependent kinase 4 inhibitor B		+
CDKN2C	Cyclin-dependent kinase 6 inhibitor	+	
CDKN3	Cyclin-dependent kinase inhibitor 3	+	
Gene involved in cell cycle arrest			
GADD45B	Growth arrest and DNA-damage-inducible protein beta	-	
Anti-apoptotic gene			
OLFM4	GW112, Olfactomedin 4	+	+

The estrogen responsive genes are divided into 3 categories: genes involved in cell cycle, cell cycle arrest and apoptosis. Genes positively regulated genes by estradiol are marked with a '+' sign, while negatively regulated genes are marked with a '-' sign.

The two most interesting genes that were candidate for further investigation were chosen based on their function and their response to estradiol. GW112, also called olfactomedin 4, is one of those genes that highly respond to estradiol in vivo as well as in vitro (early proliferative endometrium cultured in the presence of estradiol). Zhang et al found GW112 to be overexpressed in a number of human tumour types, especially those of the digestive system. These researchers described GW112 as a protein having anti-apoptotic effects that could promote tumour growth in a microenvironment that often is hypoxic, acidic or nutrient-deprived (33). Another gene of interest is the gene encoding for the growth arrest and DNA-damage-inducible protein beta (GADD45B; MyD118), which is involved in the regulation of cell cycle progression and apoptosis. Down regulation of this gene was seen when exposed to estradiol in vivo. Because GW112 and GADD45B were differentially expressed in response to the same agonist (estradiol) and because of their association with proliferation (the major event in the endometrium during the menstrual cycle), these two genes were the main focus of investigation.

Using bio-informatic tools (databases and software), the promoter sequences of GW112 and GADD45B were examined to predict regulatory sequences like the EREs, AP-1 and

Sp1 binding sites. Subsequently, an endometrial model system was searched to study the regulation of GW112 and GADD45B by estradiol. Four human endometrial cancer cell lines (ECC-1, Ishikawa, RL95.2 and AN$_3$CA) were analyzed. Subsequently the selected cell line could be used to explore the interaction of ER-α with coregulators (by co-immunoprecipitation, co-IP) and their recruitment at the level of selected gene promoters. One method for empirically examining the specific association of proteins with DNA in the context of living cells is called chromatin immunoprecipitation (ChIP). Briefly, in the ChIP procedure, cells are treated with formaldehyde to crosslink proteins that are in close association with DNA. Specific protein-DNA complexes can then be isolated by immunoprecipitation using an antibody that specifically binds the protein of interest. Following reversal of the crosslinking and purification of the protein-associated DNA, the specific DNA sequences can be examined by PCR with gene-specific primers. Most part of this project was dedicated to the optimization of the ChIP protocol before it could be applied on the cell line and human endometrium (see below) to study the ER-α binding and recruitment of coregulators.

1.5.2   ER-α binding in the human endometrium (Approach 2)

During the menstrual cycle, the human endometrium is exposed to different peripheral concentrations of follicular estradiol which in turn determine the changes in the growth and development of this tissue. These changes are mainly the result from modulations in gene expression. Since ER-α is primarily involved in these mechanisms, it is important to understand which gene promoters are targeted by ER-α at a genome-wide level. To examine the entire spectrum of in vivo DNA binding sites for ER-α, the ChIP procedure could be coupled to a whole-genome DNA micro array (ChIP-on-chip). Also in this case the ChIP protocol had to be optimized.

2   Materials and methods

In the following sections the materials used (human endometrial cancer cell lines and human endometrium) and the methods (bio-informatics, real-time PCR, western blot, co-immunoprecipitation and chromatin immunoprecipitation) performed are described.

2.1   Cell lines and cell culture

Four human endometrial cancer cell lines (ECC-1, Ishikawa, RL95.2 and AN_3CA were used in the present study. All cell lines were purchased from American Type Culture Collection (ATCC; Rockville, Md. USA). Cells were maintained in RPMI (Invitrogen Life Technologies, Inc., Carlsbad, CA) supplemented with 10 % foetal calf serum (Hyclone, Gent, Belgium), 2 mM L-glutamine, 2 mM Na-pyruvate, 100 U / L penicillin, 100 mg / L streptomycin).

2.2   Human endometrium

Human endometrium tissue was obtained from female volunteers either by aspiration biopsy using a Pipelle (Unimar Inc., Prodimed, Neuilly-Enthelle, France) or from hysterectomy specimens. All surgeries were performed for benign indications and an experienced pathologist observed no abnormalities in the collected tissues. Women were 20–45 years of age, did not receive any hormonal treatment and had normal, regular menstrual cycles of 25–35 days. In no case did women contribute more than one tissue sample. Dating of the tissue occurred according to clinical information of the start of the last menstrual period. Endometrium tissues were collected on different cycle days. After tissue collection, the tissue was treated as described later (see section *"Optimization of the chromatin immunoprecipitation to study binding of proteins to gene promoters"*). The study was approved by the Medical Ethical Committee of the University Hospital (Maastricht) and all participating women gave their written consent.

2.3   Steroid hormones

Estradiol (17β-estradiol) was purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands) and were dissolved in 100 % ethanol at different stock concentrations

(10^{-5} and 10^{-6} M). The anti-estrogen antagonist ICI 164384 was a gift from Organon, BV (Oss, The Netherlands).

2.4 Regulatory motif search in the promoter sequence of GW112 and GADD45B using bio-informatic tools

The NCBI database site was used to retrieve the nucleotide promoter sequences of the GW112 and GADD45B genes. These sequences were then analysed using different software to predict the binding of ER-α to the ERE palindrome, but also to find other regulatory motifs in the promoter sequences for binding of AP-1, Sp1 or NF-κB. Examples of these software programmes are TFSEARCH, NSITE and DNASIS MAX. Also motif patterns from literature were used to predict such binding sites.

2.5 Gene expression analysis using real-time PCR

ECC-1, Ishikawa, RL 95.2 and AN$_3$CA cells were grown to 40 - 60 % confluence (6 well culture plate) in phenol red-free RPMI (Invitrogen, Life Technologies, Inc., Carlsbad, CA) supplemented with 10 % steroid hormone-stripped foetal calf serum (c.c.pro GmbH, Neustadt, Germany) for at least 3 days. To check if the GW112, GADD45B and pS2 were estrogen responsive in these cell lines, cells were treated with ethanol (vehicle) or with 17-β-estradiol (10^{-8} M) for the indicating period of time. RNA was isolated using the Trizol reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's instructions. The RNA was subsequently treated with 3 U deoxyribonuclease I (MBI Fermentas GmbH, St. Leon-Rot, Germany) to remove residual genomic DNA. Complementary DNA (cDNA) was synthesised using 200 U M-MLV reverse transcriptase (Invitrogen, Life Technologies, Inc., Carlsbad, CA), 1 µg poly-T oligo (MBI Fermentas GmbH, St. Leon-Rot, Germany) and 1 mM dNTPs (MBI Fermentas GmbH, St. Leon-Rot, Germany). Five micrograms of total RNA were used for cDNA synthesis. To analyse the regulation of the selected genes by 17-β-estradiol, quantitative real-time PCR was carried out. The sequences of the used primer pairs are shown in table 2. Each real-time PCR reaction consisted of 10 ng cDNA, 12.5 µl SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA), and 40 nM forward and reverse primers. Reactions were carried out on the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) for 40 cycles (95 °C and 60 °C) after

an initial 10-min incubation at 95 °C. Experiments were performed for each sample in duplicate. Quantitative values were obtained from the threshold cycle number (Ct) at which the increase in the signal associated with exponential growth of PCR products using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The fold change in expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method (34). Briefly, the differences in the number of cycles, ΔCt, were determined as the difference between the target gene and the average of cyclophilin and β-actin within each experiment. Next, the ΔΔCt was calculated between the treated (estradiol) and control (vehicle) samples within each experiment. Subsequently the fold change could be calculated as FC= $2^{-\Delta\Delta Ct}$. The endogenous controls (cyclophilin A and β-actin) were used to normalize for the differences in the amount of cDNA added to each reaction. Finally, a t test was carried out to find significant differences (p-value < 0.05) between the mean fold change of all the groups (Simple Interactive Statistical Analysis; URL: http://home.clara.net/sisa/).

Table 2. Sequences of the primers used in the gene expression analysis.

Gene	Primer	Sequence
Cyclophilin A	Forward	TTCCTGCTTTCACAGAATTATTCC
	Reverse	GCCACCAGTGCCATTATGG
B-actin	Forward	CCTGGCACCCAGCACAAT
	Reverse	GCCGATCCACACGGAGTACT
GW112	Forward	TCTCCTAGCCCTTCTGTTCTTC
	Reverse	GGAGCCGGTGAAATTGGAAAAC
GADD45B	Forward	ATCGCCTCACAGTGGGGGT
	Reverse	CGTTGTCACAGCAGAAGGACTGG
pS2	Forward	CATCGACGTCCCTCCAGAAGAG
	Reverse	CTCTGGGACTAAATCACCGTGCTG

2.6   Protein expression of ER-α and coregulators in the four endometrial cancer cell lines

The different cell lines were characterized using whole cell protein extracts for the detection of the ER-α, the coactivators SRC-1, CBP and p300, and the corepressors N-CoR and SMRT. Cells were washed with ice-cold PBS and lysed in RIPA buffer (10 nM sodium phosphate pH 7.0, 150 mM NaCl, 2 mM EDTA, 1 % Na-deoxycholate, 1 % NP-40, 0.1 % SDS) supplemented with Complete[TM] protease inhibitor (Roche, Mannheim,

Germany). After determination of the protein concentration using the Bradford dye reagent (BCA assay; Uptima, Interchim, France), the same concentration of protein extract was used to load on an acrylamide gel (5 % polyacrylamide for the coregulators – 12 % for the ER) for SDS-PAGE. Subsequently the proteins were blotted on a nitrocellulose membrane (Protran, Schleicher and Schuell, Germany) using the mini protean-III system (Bio-Rad Laboratories, Hercules, CA, USA) as described by the manufacturer. To visualize the selected proteins different antibodies were used: monoclonal anti-ER-α antibody (F10), goat anti-SRC-1 antibody (C-20), rabbit anti-CBP antibody (A-22), rabbit anti-p300 antibody (N-15), goat anti-N-CoR antibody (C-20), and rabbit anti-SMRT antibody (H-300). All these antibodies were purchased from Santa Cruz Biotechnology (California, USA). For visualization of bound antibodies, HRP-conjugated rabbit-anti-mouse-antibodies (DAKO, Glostrup, Denmark), rabbit-anti-goat antibodies (DAKO, Glostrup, Denmark) or goat-anti-rabbit-antibodies (Pierce, Aalst, Belgium) and the super signal-R West-Femto kit (Pierce, Aalst, Belgium) were used.

2.7 Interaction analysis of ER-α and coregulators using co-immunoprecipitation

To investigate the interaction of ER-α to the different coregulators in solution, co-immunoprecipitation (co-IP) was used. This technique allows to precipitate proteins of interest with their interaction partners. Thus, IP with a specific coregulator can co-immunoprecipitate the ER-α, subsequently ER-α can be visualised using western blot.

RL 95.2 cells were grown to 80 % confluence (165 cm^2 culture flasks) in phenol red-free RPMI (Invitrogen, Life Technologies, Inc., Carlsbad, CA) supplemented with 10 % hormone-stripped foetal calf serum (c.c.pro GmbH, Neustadt, Germany). After 3 days of steroid hormone deprivation, cells were treated with ethanol (vehicle), estradiol (10^{-8} M) or a combination of estradiol (10^{-8} M) and ICI 164384 for 30 minutes at 37 °C in vitro. Cells were then scraped in PBS and mechanically lysed using a syringe to release all proteins. Subsequently, cell debris was pelleted and the protein concentration was measured (BCA assay; Uptima, Interchim, France). Equal amounts of protein (500 µg) were used in each co-IP. Prior to the addition of the indicated antibody for IP, stimulation was continued in silico. After overnight rotation, the antibody-protein complexes were precipitated by adding protein A beads. These beads were then washed in RIPA buffer for a few times. To elute the precipitated proteins from beads and antibodies, 120 µl RIPA buffer and 4 µl loading buffer (4 x concentrated New page sample buffer, 1 M DTT) per 10 µl protein

sample were added. Subsequently proteins were boiled for 10 minutes at 75 °C and 10 minutes centrifugation at maximum speed was done to pellet all beads. The supernatant containing the proteins of interest was used for analysis using the western blot technique as described earlier.

2.8 Optimization of the chromatin immunoprecipitation to study binding of proteins to gene promoters

Chromatin immunoprecipitation (ChIP) is a technique that allows the precipitation of proteins associated to DNA. Before studying the binding of ER-α to its target genes in vitro (in an endometrial cancer cell line) and in vivo (in human endometrium), the ChIP protocol had to be optimized.

2.8.1 ChIP applied on an endometrial cancer cell line

To study the regulation of individual genes known to be regulated by estradiol, binding of ER-α on their gene promoters was assessed using ChIP. For this procedure, the selected cell line was grown to 80 % confluence (165 cm^2 culture flasks) in phenol red-free RPMI (Invitrogen, Life Technologies, Inc., Carlsbad, CA) supplemented with 10 % hormone-stripped foetal calf serum (c.c.pro GmbH, Neustadt, Germany) for at least 3 days. Subsequently, cells were treated with ethanol (vehicle) or with 17-β-estradiol (10^{-8} M) for 50 minutes. Cells were then fixed (RPMI with 1 % formaldehyde, 10 minutes, room temperature) and washed with ice-cold PBS. Fixation was stopped by incubating 15 minutes with glycin (0.125 M). Cells were scraped in 2 ml of accutase (PAA, Laboratories GmbH, Austria) after 5 minutes of incubation at 4°C and 1 minute at 37 °C. Cells then were pelleted by centrifugation for 5 minutes at 4000 rcf and resuspended in 1 ml of cold PBS supplemented with Complete^{TM} protease inhibitor (Roche, Mannheim, Germany), again followed by centrifugation. To lyse the cells a cell lysis buffer (Active motif, Rixensart, Belgium; supplemented with Complete^{TM} protease inhibitor) was added. Nuclei were pelleted and subsequently lysed in 1ml shearing buffer (Active motif, Rixensart, Belgium; Complete^{TM} protease inhibitor) and sonicated for 5 minutes using a water bath sonicator. Debris was pelleted and aliquots of the nuclear lysates were kept at -80 °C for further analyses.

2.8.2 ChIP applied on human endometrium

To study the binding of ER-α on its target genes in vivo, the ChIP protocol had to be adjusted. After removal of the endometrium from the complete uteri, it was directly fixed with 1 % formaldehyde (10 minutes, room temperature). Subsequently the tissue was washed with ice-cold PBS and the crosslinking was stopped using glycin (0,125 M). To homogenize the tissue (in ice-cold PBS supplemented with Complete^{TM} protease inhibitor), a glass douncer was used in. The following step in the protocol consisted in the lysis of the tissue using a lysis buffer (5 mM PIPES ph 8.0, 85 mM KCL, 0.5 % NP-40 supplemented with Complete^{TM} protease inhibitor). Again the tissue was homogenized using a glass douncer, this was done to break the cell membranes more easily. Nuclei were subsequently lysed in a nuclear lysis buffer (50 mM Tris pH 8.1, 10 mM EDTA, 1 % SDS supplemented with Complete^{TM} protease inhibitor) and sonicated for 5 minutes using a water bath sonicator. Subsequently, cell debris was pelleted and aliquots of the nuclear lysates were kept at -80 °C for further analyses.

From this point on, the same protocol optimized for the ChIP on cell line was used. One aliquot of 20-50 µl was taken for checking the efficacy of the shearing and the DNA yield. Based on these parameters, the amount of DNA was adjusted by adding more or less DNA in each ChIP reaction. Next the samples were diluted in immunoprecipitation (IP) buffer (1 % Triton, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1) and pre-cleared (2 hours, 4 °C) with 100 µl of protein-G beads (Active motif, Rixensart, Belgium). Immunoprecipitation was preformed overnight in rotation at 4 °C with anti-RNA polymerase II (RNA pol II) antibodies 4H8 (1.5 µg unless indicated otherwise; Active motif, Rixensart, Belgium). A negative control was used in which no antibody was added. 100 µl Protein-G beads were added and the incubation was continued for 1.5 hours in rotation at 4 °C. Next, the precipitates were washed six times (100 mM Tris pH 8.0 containing 500 mM LiCl, 1 % NP-40, 1 % deoxy-cholic-acid) and chromatin-protein complexes were eluted from the beads with 100 µl 25 mM NaHCO$_3$ supplemented with 1% SDS. Formaldehyde crosslinking was reversed by addition of 4 µl 5 M NaCl and 10 U of RNase A (overnight incubation at 65 °C). Subsequently, samples were treated with proteinase-K (2 µl of proteinase-K (20 mg/ml), 2 µl of EDTA (500 mM, pH 8.0) and 2 µl of Tris-HCl (1 M pH 6.5)) for 2 hours at 42 °C. DNA was purified using the Qiaquick

reaction cleanup kit (Qiagen GmbH, Hilden, Germany). The DNA fragments pulled down in the IP reaction were analysed using PCR.

Binding of RNA pol II to the promoter region of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, known to be constitutively active, was used as positive control of the ChIP procedure, and it was assessed using primers that flank the RNA pol II site of the GAPDH promoter (ChIP positive primers, table 3). An aspecific negative control was also performed using the ChIP negative primer set (table 3). These primers flank a cytogenetic location 12 p13.3 where no transcription factors bind to. This locus should not be enriched by ChIP. Thus, PCR reactions with the negative control primers should give rise to equivalent amounts of product with no respect whether it is performed on DNA isolated by immunoprecipitation with anti-RNA pol II (anti-RNA pol II sample) or with no antibody (negative control). If this is not the case ChIP positive PCR signals should be normalized using the ChIP negative PCR signals. Products of ChIP positive and ChIP negative PCR reactions were separated on a 4 % gel. Band intensities were used to judge whether there was any enrichment in the immunoprecipitated DNA and for normalization. Band intensities were evaluated by eye or by using the ImageJ software. When using the ImageJ software, the fold change of enrichment for the GAPDH promoter locus could be calculated for the anti-RNA pol II samples. Using the following formulas this analysis could be performed: normalized intensity value= ((ChIP positive signal/ ChIP negative signal)/ input signal *100); fold change= normalized value of anti-RNA pol II sample / normalized negative control. This formula includes the normalization of all ChIP positive PCR signals with the ChIP negative signals and the normalization with the input (to compare between the samples). Subsequently, relative to the normalized negative control (no antibody), the normalized value of the anti-RNA pol II samples was used to calculate the fold change of enrichment.

Table 3. Sequences of the primers used for ChIP PCR.

Gene	Primer	Sequence
ChIP-positive	Forward	TACTAGCGGTTTTACGGGCG
	Reverse	TCGAACAGGAGGAGCAGAGAGCGA
ChIP-negative	Forward	ATGGTTGCCACTGGGGATCT
	Reverse	TGCCAAAGCCTAGGGGAAGA

3   Results

To study estradiol response in human endometrium we used two approaches. In the first approach an in vitro model system was used to study the regulation of ER-α activity on different selected gene promoters known to be regulated by estradiol in vivo (approach 1). In the second approach we studied ER-α binding in human endometrium (approach 2). Therefore, the results of this research project were subdivided into two parts.

3.1   The regulation of the ER-α activity at the level of individual gene promoters using an endometrial model system (Approach 1)

The ER-α-mediated regulation of specific genes, in particular of GW112 and GADD45B, was investigated using an endometrial cell line, since these genes were differentially regulated by estradiol in human endometrium in vivo (micro-array). First, the promoter sequences of these two genes were checked for regulatory motifs, e.g. ERE, AP-1 and Sp1 sites (Objective a). The suitable endometrial cell line was selected among four endometrial cancer cell lines based on the ER-α expression and the expression of the GW112 and GADD45B mRNA (Objective b). Subsequently the best model system was used to study the interaction between ER-α and coregulators in solution after treatment with an agonist and an antagonist using co-immunoprecipitation (Objective c). Finally, to study the ER-α binding and coregulator recruitment at the level of an individual promoter the chromatin immunoprecipitation (ChIP) method could be used. Given that the ChIP procedure was never performed in an endometrial cell line or in endometrium (see below), a preliminary optimization of the method was required (Objective d).

3.1.1   The prediction of regulatory motifs in the promoter of GW112 and GADD45B (Objective a)

One of the determinants that affect the efficiency of ER-α binding, and therefore the transcriptional activity is the promoter sequence of a gene. Via regulatory motif search, using databases (TFSEARCH, NSITE) and software (DNASIS MAX), the promoter could be analyzed for GW112 and GADD45B, examining 2000 base pairs upstream of the transcriptional start of the coding sequence (CDS). In figure 4 and 5 the promoter

sequences with the putative regulatory sites of these two genes are shown. The proximal promoter (approximately 1000 base pairs upstream of the CDS) of GW112 showed 3 half ERE sites, 1 Sp1 site and 1 AP-1 site. More distant no motifs were found. For the GADD45B promoter, more motifs were found compared to the GW112 promoter. In the proximal part of its promoter 2 Sp1 binding sites and 2 NF-κB binding sites were seen. More distant 4 Sp1- and 1 AP-1 binding site, and 2 half EREs were found. These results indicate that GW112 and GADD45B could be directly or indirectly regulated by ER-α.
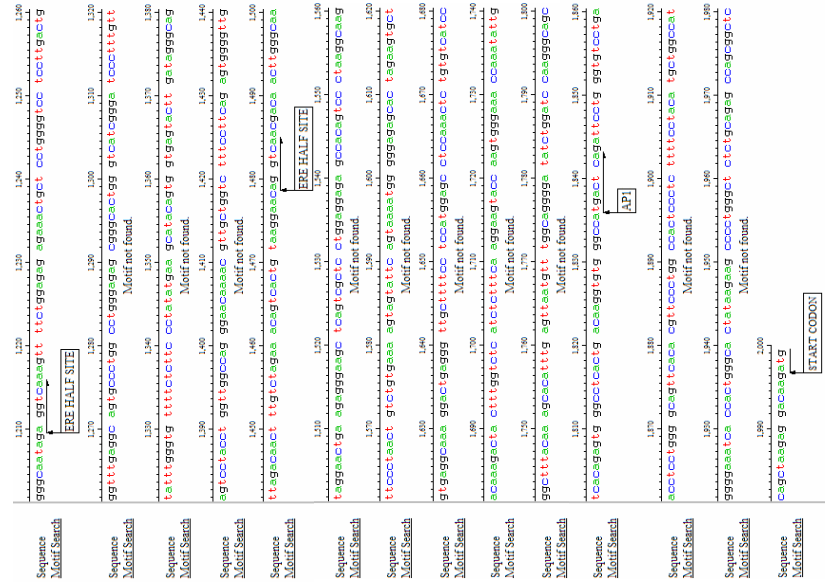
Figure 4. The promoter sequence of GW112 and its putative regulatory sequences. 2000 base pairs upstream of the 'atg' start of the coding sequence of the GW112 gene were analysed for putative regulatory sequences like the ERE, AP-1 and Sp1 sites using bio-informatics.
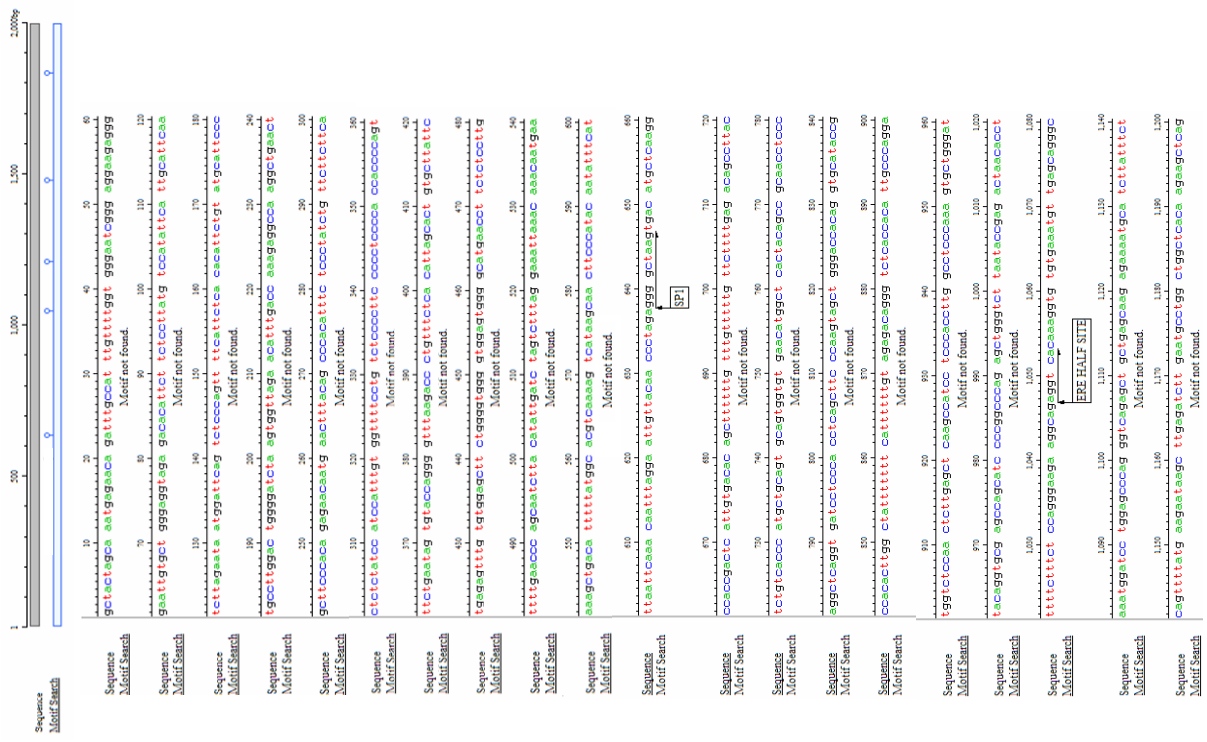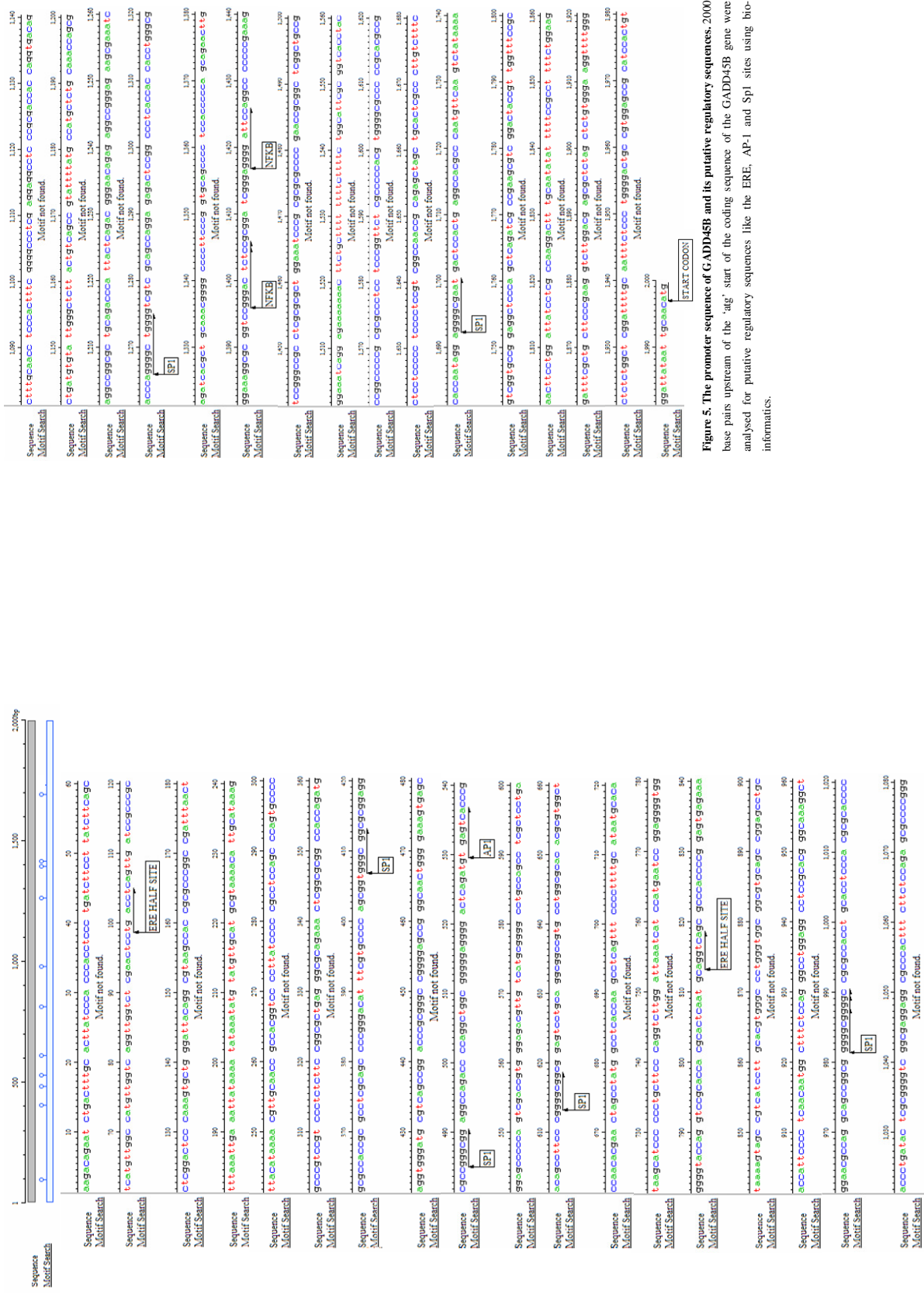
Figure 5. The promoter sequence of GADD45B and its putative regulatory sequences. 2000 base pairs upstream of the 'atg' start of the coding sequence of the GADD45B gene were analysed for putative regulatory sequences like the ERE, AP-1 and Sp1 sites using bio-informatics.

3.1.2   The search for the best endometrial model system for studying the regulation of GW112 and GADD45B gene expression in response to estradiol (Objective b)

To study the ER-α-mediated regulation of the genes GW112 and GADD45B in the endometrium, an endometrial model system was needed. This model system had to meet to the following criteria: a) the model system used had to be originating from human endometrium; b) the ER-α had to be expressed and c) the expression pattern of the two investigated genes had to be consistent with the micro-array data (described in the introduction) in which GW112 and GADD45B expression is respectively stimulated and repressed. Four endometrial cancer cell lines (ECC-1, Ishikawa, RL95.2 and AN$_3$CA) were selected as candidates for the model system because of their endometrial origin. The ECC-1, Ishikawa and RL95.2 cell lines originate all from a differentiated epithelial adenocarcinoma of the endometrium, while the AN$_3$CA cell line originates from lymph node metastasis of an undifferentiated human endometrial adenocarcinoma. These cell lines were analyzed for their ER-α expression using western blotting. The results are shown in figure 6. ER-α, a 65 kDa protein, was expressed in all cell lines.
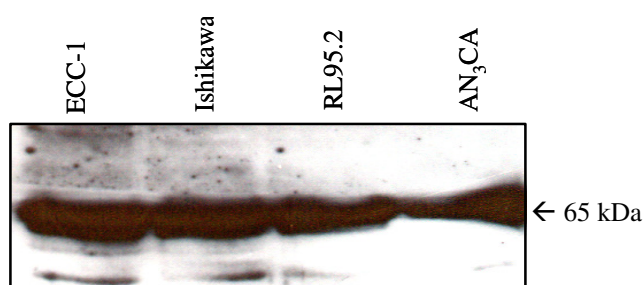
Figure 6. The expression of ER-α in the candidate model systems. Whole cell protein extracts of all candidate cell lines (ECC-1, Ishikawa, RL95.2 and AN$_3$CA) were analyzed for the expression of ER-α using western blotting.

To check whether the 4 cell lines expressed GW112 and GADD45B, all cell lines were cultured in steroid hormone-free medium, and afterwards exposed to estradiol (10^{-8} M) for 4 an 16 hours. To evaluate whether GW112 and GADD45B were regulated by estradiol the fold changes relative to untreated cells (vehicle alone; control group) were calculated for each gene and for each estradiol treatment (figure 7).
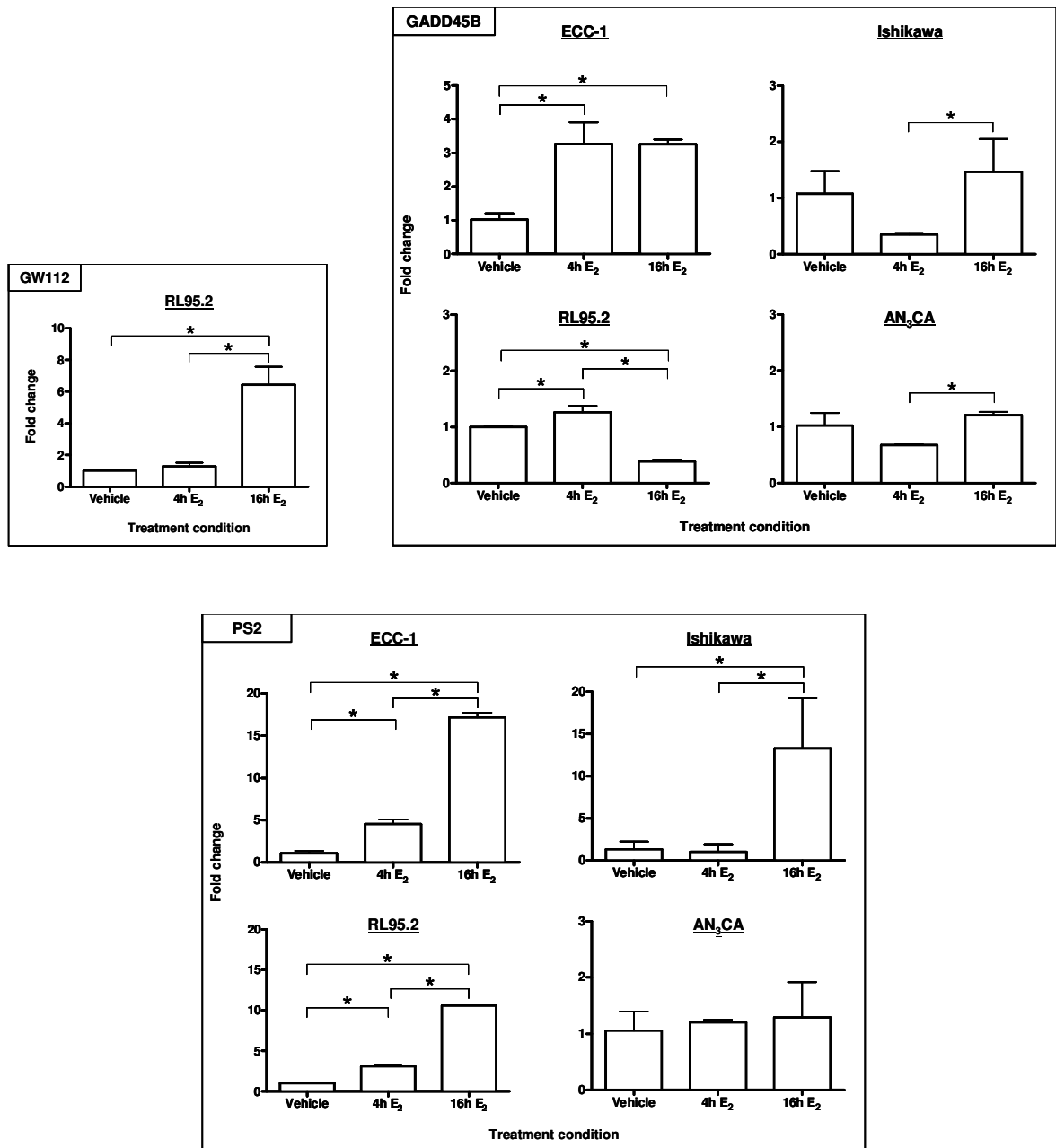
Figure 7. The effect of estradiol on the mRNA expression of GW112, GADD45B and pS2 in four human endometrial cancer cell lines. All studied cell lines (ECC-1, Ishikawa, RL95.2 and AN$_3$CA) were treated with vehicle (ethanol) or estradiol (E$_2$) for the indicated period of time (4 and 16 hours). Real-time PCR was performed on the cDNA of these samples. The data represent the fold change in target gene expression in cells treated with estradiol normalized to the average of two internal control genes (cyclophylin A and β-actin) and relative to cells treated with the vehicle. All experiments were carried out in duplo and the error bars represent the standard error of the mean. Significant differences (P < 0.05) between the groups (vehicle vs. 4 h estradiol, vehicle vs. 16 h estradiol and 4 h estradiol vs. 16 h estradiol) are indicated by an asterisk (*).

Interestingly among the four cell lines studied, only RL95.2 cells expressed GW112. The mRNA levels of this gene were upregulated compared the control group (P 0.004). In the

other cell lines the GW112 mRNA was not detectable. With regard to the expression of GADD45B, different responses were seen after treatment with estradiol in the cell lines. In Ishikawa cells and AN$_3$CA cells, the overall expression of this gene was not influenced by estradiol (P > 0.05); vehicle vs. 16 h estradiol). On the contrary, in ECC-1 cells the levels of GADD45B were increased by estradiol (P= 0.01) when compared with the control group. Whereas, in RL95.2 cells, this gene was negatively regulated after 16 hours of estradiol treatment (P= 0.003). The expression of pS2 was also analyzed. This gene is known to be positively regulated by estradiol, thus its expression was used to see if the cell lines responded correctly to estradiol. pS2 was not regulated by estradiol in the AN$_3$CA cells, while in all the other cell lines pS2 expression was upregulated (P < 0.05).

In conclusion, the RL95.2 cell line fulfilled all requirements for being an endometrial model system: a) endometrial origin; b) expression of ER-α; and c) GW112 and GADD45B gene regulation by estradiol. Therefore, RL95.2 was chosen for further studies.

In the previous experiments cells were cultured in medium supplemented with 10 % steroid hormone free-foetal calf serum (FCS). The presence of FCS affected the responses of GW112 and GADD45B to estradiol in a way that the trend of estradiol response remained, but the fold changes were larger when compared with cells cultured in absence of FCS and treated with estradiol (data not shown).

3.1.3 The expression of the coregulators in the selected endometrial model system

It was hypothesized that the recruitment of different coregulators by the ER-α is responsible for the differential expression of GADD45B and GW112 in response to the same ligand in the same model system. To know whether the chosen model system RL95.2 expressed a set of these coregulators including three coactivators (p300, CBP and SRC-1) and two corepressors (N-CoR and SMRT), a protein analysis was done using western blotting. The other cell lines (ECC-1, Ishikawa and AN$_3$CA) were also analyzed for most of these proteins. The expression of the three coactivators p300, CBP and SRC-1 are shown respectively in figure 8 a, b and c for RL95.2 and for some of the other cell lines. The corepressor N-CoR was analyzed for all cell lines and is shown in figure 8 d. The model system RL95.2 expressed not only the three coactivators, but also the corepressor

N-CoR. The expression of SMRT was not detected, probably because the antibody was not appropriate for western blotting (product information).
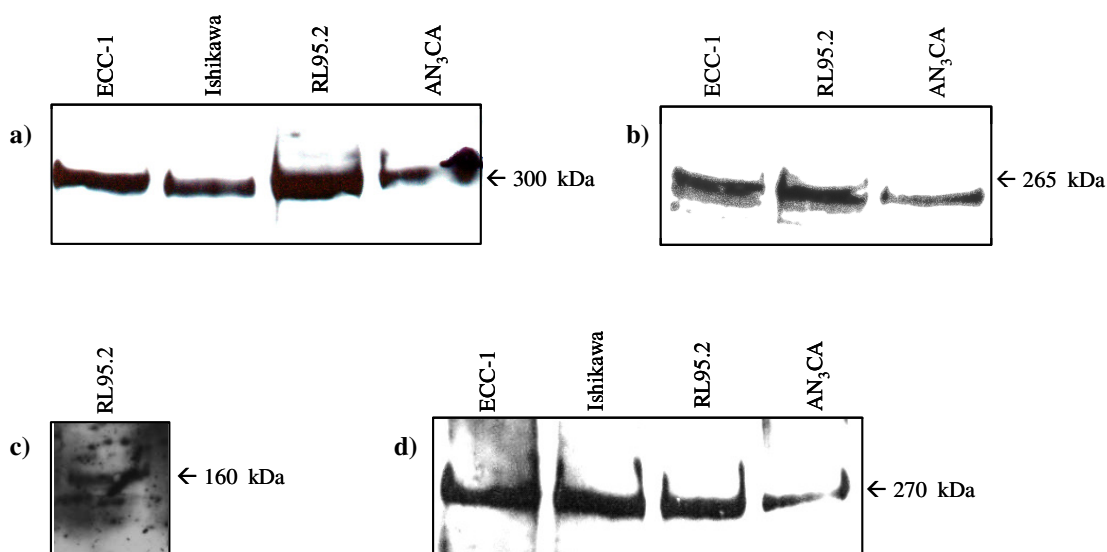

Figure 8. Protein expression of a set of coregulators. Whole cell protein extracts from most of the candidate model systems were separated using SDS page, and subsequently transferred to a nitrocellulose membrane. Next the protein of interest was visualized using a specific primary antibody, a HRP-conjugated secondary and the super signal-R West-Femto kit (Pierce, Aalst, Belgium). a) The expression of p300 (300 kDa) in all studied cell lines; b) The expression of CBP (256 kDa) in ECC-1, RL95.2 and AN$_3$CA; c) The expression of SRC-1 (160 kDa) in the RL95.2 cell line; d) The expression of N-CoR (270 kDa) in all studied cell lines.

3.1.4 *The effect of an agonist and an antagonist on the interaction of coregulators with ER-α in solution (Objective c)*

After knowing which coregulators are expressed, the interaction of those coregulators with ER-α were analyzed. Co-immunoprecipitation is a technique that allows the precipitation of a target antigen with its interaction partners. RL95.2 cells were cultured in steroid hormone-free medium for at least three days. Subsequently, the cells were exposed to an agonist (estradiol), a combination of an agonist (estradiol) and an anti-estrogen (ICI 164384) or the vehicle (ethanol) for 30 minutes in vitro. Subsequently the cells were collected, lysed and the stimulation with these ligands was continued in silico (in the cup). By western blot analysis the interaction of ER-α with each of these coregulators can be assessed. In figure 9 the results of the Co-IP are shown for each treatment condition. By comparison of the IP results under different stimulation conditions, it can be concluded that

in solution ER-α can interact with co-activators and corepressors independent of the treatment. It is also clear that only a small amount of ER-α was bound to the coregulators, while these proteins are all abundantly present in this cell line (figure 8). These results suggest that the recruitment of coregulators by an agonist or antagonist liganded ER-α might be controlled by the promoter context.
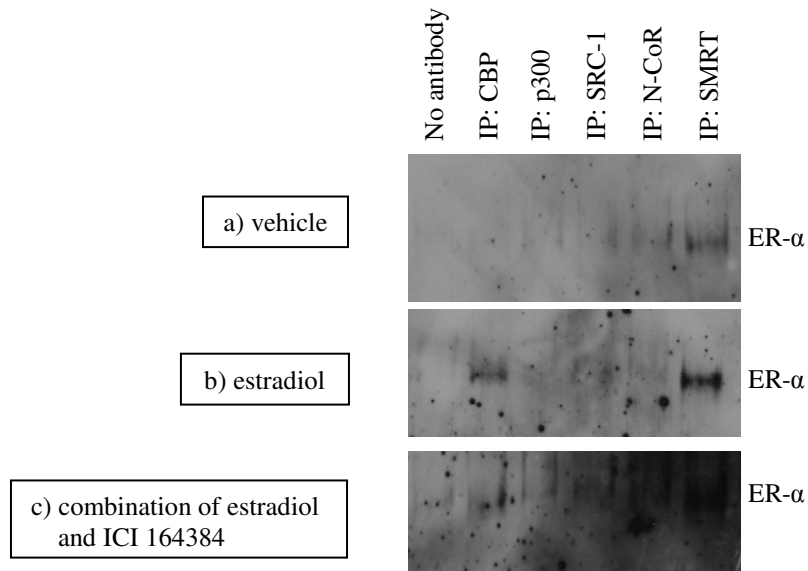
Figure 9. Analysis of the interaction between the coregulators and ER-α in different treatment conditions. RL95.2 cells cultured in steroid hormone free medium, were exposed for 30 minutes to a) vehicle (ethanol), b) estradiol (10^{-9} M), or c) anti-estrogen ICI 164384 (10^{-7} M) in combination with estradiol (10^{-9} M). Next cells were collected and further stimulated in silico. The co-IP allowed the precipitation of each coregulator, together with its interaction partners. Using western blot analysis the interaction partner of interest can be visualized, in this case ER-α. A negative control (no antibody) could be used to normalize for aspecific precipitation of proteins.

3.1.5 Optimization of the ChIP protocol for studying the binding of ER-α and the recruitment of coregulators to individual gene promoters in the model system (Objective d)

To study the ER-α binding and the selective recruitment of coregulators at specific gene promoter shown to be controlled by estradiol (GW112 and GADD45B) ChIP analysis can be performed. This procedure combines three steps: 1) in vivo formaldehyde crosslinking of whole cells that freezes protein-protein-DNA interactions; 2) chromatin shearing to create small fragments of protein-bound DNA; and 3) immunoprecipitation of the protein-

DNA complexes from sonicated extracts using specific antibodies against the protein of interest. PCR is then used to amplify the region to which these proteins are known to bind. Therefore the ChIP protocol had to be optimized at each step of the procedure, including the amount of starting material, the cell fixation, chromatin isolation, sonication shearing and the immunoprecipitation (IP) step.

Cell fixation

RL95.2 cells were used to perform all tests. These cells were grown to 80 % confluence on 165 cm² cell culture flask and fixed with formaldehyde to crosslink protein-protein and protein-DNA interactions after removing the culture medium. No washing step was included in the protocol before fixation. The interaction between transcription factors and DNA occurs within seconds, thus changes in the cell environment may disturb the interaction between proteins and DNA. However after removing the culture medium, a small layer of serum protein remains on the cells, and might interfere with the cell fixation by absorbing the formaldehyde. Therefore a few steps were changed during the course of the optimization: 1) a large volume of fix solution was used to dilute the serum proteins and to have an excess of formaldehyde; 2) incubation of the fix-solution was done on a rocking platform for 10 minutes at room temperature. It is important to not over-crosslink the proteins because giant crosslinked aggregates can be formed that can not be efficiently sonicated. Cells were washed thoroughly with PBS and the crosslinking was stopped (0.125 M glycin).

Chromatin isolation and sonication shearing

To isolate chromatin with the crosslinked proteins cells were harvested and lysed. To harvest the cells a few solutions where tried: PBS, a solution containing a mild trypsin (accutase) and scrape solution (Tris pH 9, DTT). The protocol based on the use of accutase gave the best result. From this point on, all solutions used were supplemented with protease inhibitor to prevent degradation of the proteins. Optimal lysis was achieved using the solutions provided with the ChIP-it kit (Active motif, Rixensart, Belgium). The sonication step to break the chromatin into small fragments was optimized to 5 minutes incubation in a water bath sonicator. One aliquot of chromatin was used to check the DNA shearing efficiency and the DNA concentration. Optimal shearing results in a smear of chromatin in the range of 500 – 1000 bp (figure 10).
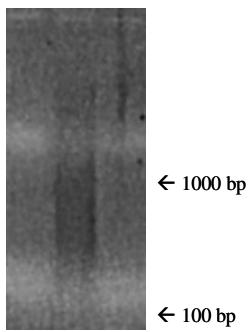
Figure 10. An example of optimally sheared chromatin. After chromatin isolation, the protein-crosslinked chromatin was sonicated for 5 minutes in a water bath sonicator. Subsequently the crosslinking was reversed, purified and loaded on a 2 % agarose gel. The smear of chromatin with fragments ranging from 500 – 1000 bp represents optimal shearing.

← 1000 bp

← 100 bp

The immunoprecipitation reaction

The protein-crosslinked chromatin was precleared with protein G beads to reduce aspecific background. From this precleared chromatin, one aliquot (10 µl into 90 µl H_2O) was kept as the 'input DNA', the remaining was used for the immunoprecipitation reaction. Based on the quantity of the sheared DNA observed, the amount of material used in the each IP reaction was adjusted. In the optimizing experiments, the IP was only performed using no antibody (negative control) and RNA polymerase II antibody (positive control; anti-RNA pol II). The input DNA served as a control for the PCR reaction. Two different primer sets were used to analyze the precipitated DNA: ChIP positive primers which flank the promoter of GAPDH, a constitutive active gene, and ChIP negative primers which flank a region in the genome where no transcription factors bind, only aspecific product is detected. The ChIP positive PCR performed on templates that were enriched for the GAPDH promoter locus (i.e. DNA precipitated with anti-RNA pol II) should yield more product than PCR performed on templates not enriched for the GAPDH promoter locus (i.e. DNA precipitated with no antibody). On the contrary, ChIP negative PCR should give rise to equivalent amounts of PCR product with no respect whether DNA obtained by IP with anti-RNA pol II or no antibody was used. If this is not the case, the ChIP positive signals should be normalized using the ChIP negative PCR to correct for the amount of DNA put in each reaction.

In figure 11, a ChIP result is shown. Part A shows the ChIP positive and negative PCR signals of sample series 1, 2 and 3. Each series contains one negative control (no antibody) and one positive control (anti-RNA pol II sample). Part B shows the fold changes of the ChIP positive signals of the anti-RNA pol II samples of each of the series. Together, these figures show if there is enrichment for the GAPDH promoter locus when immunoprecipitation is done with the anti-RNA pol II compared with the negative control

(no antibody). Samples series 1, 2 and 3 represent a titration of the amount of chromatin used in each IP reaction, 20 µl, 50 µl and 100 µl, respectively. Sample series 1 show a clear enrichment for the GAPDH promoter locus in the anti-RNA pol II sample. This sample uses an optimal chromatin-antibody ratio. In sample series 2 a titration of the RNA pol II antibody is performed. From this experiment can be concluded that when using 50 µl of chromatin the optimal concentration of anti-RNA pol II is 1.5 µg, or reproducibility is low. No enrichment is observed for the GAPDH promoter locus when immunoprecipitation was done with anti-RNA pol II in sample series 3.
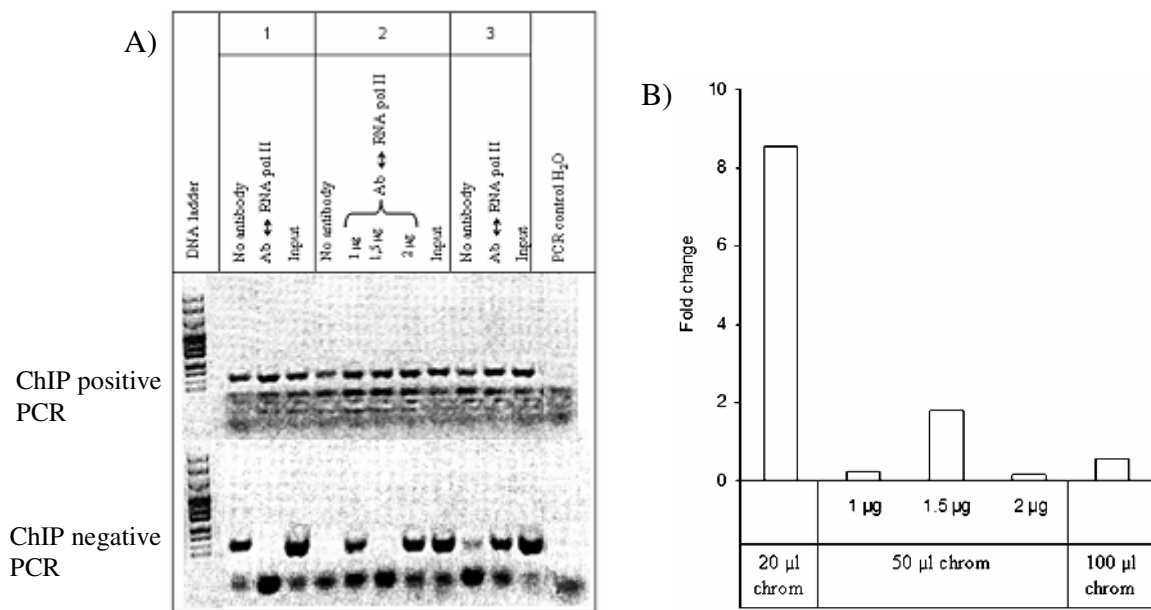
Figure 11. ChIP experiments using ChIP positive primers and ChIP negative primers on different samples. A) ChIP was performed on RL95.2 cells with a confluence of 80 % using the optimized protocol. Different amounts of isolated chromatin were put in each reaction ranging form 20 µl (1), 50 µl (2), and 100 µl (3). A titration of the concentration of the RNA pol II antibody used for precipitation was done in 3 reactions containing each 50 µl of isolated chromatin. PCR analysis of the DNA collected through ChIP is subsequently carried out. The amplified products were then loaded on a 4 % agarose gel. B) The fold changes of the ChIP positive signals of the anti-RNA pol II samples. The intensity of the ChIP positive and negative PCR signals (part A of the figure) were measured using the ImageJ software. Subsequently, the fold changes of the ChIP positive signals of the anti-RNA pol II samples were calculated using the following formula: normalized intensity value= ((ChIP positive signal/ ChIP negative signal)/ input signal *100); fold change= normalized value of the anti-RNA pol II sample/ normalized negative control (no antibody) (see section *"Materials and methods"*).

To end, the ChIP protocol, when optimized completely can be used to study the binding of ER-α to individual target genes, including GW112 and GADD45B. This technique also

allows the examination of the selective recruitment of coregulators to these gene promoters.

## 3.2   ER-α binding in the human endometrium (Approach 2)

The human endometrium undergoes dramatic changes during the menstrual cycle due to changes in estradiol and progesterone levels. One way to identify all target genes regulated by estradiol is to study the ER-α binding on a genome-wide scale in human endometrium throughout the menstrual cycle. This can be studied with the ChIP-on-chip method which combines the ChIP approach and whole-genome DNA micro-arrays. A comprehensive understanding of the ER-α-DNA interactions will increase our understanding of the mechanisms and the cellular events that occur during the menstrual cycle. However, ChIP on human endometrium was never performed before, thus the ChIP protocol had to be optimized.

3.2.1   Optimization of the ChIP protocol for studying the entire spectrum of in vivo DNA binding sites for ER-α

The ChIP protocol applied on the human endometrium had to be adjusted and optimized similarly to what has been described above for the cell line RL95.2: the fixation, the homogenization and lysis of the tissue, the sonication and shearing and the IP reaction.

The endometrium was removed from complete uteri and directly fixed with 1 % formaldehyde. After 10 minutes incubation at room temperature, the tissue was washed in ice-cold PBS and the crosslinking was stopped using glycin (0.125 M). Subsequently, homogenization of the endometrium was performed using a glass douncer in ice-cold PBS supplemented with protease inhibitor. Because human endometrium contains a large part of extracellular matrix a test was done in which the tissue was first incubated with a collagenase followed by homogenization. However the yield of isolated chromatin did not increase, therefore this step was excluded from the protocol. The following step in the protocol consisted in the lysis of the tissue resulting in the release of the cell nuclei. Nuclei were subsequently sonicated to fragment the chromatin with associated proteins (DNA fragments in the range of 500 – 1000 bp). From this point on the same protocol optimized for the ChIP on cell line RL.95.2 was used. Also in this case the shearing efficiency and the DNA yield was assessed and considered to adjust the amount of sheared chromatin

included in each IP reaction. In these preliminary experiments, the precipitation was performed using IgG (derived from mouse serum) and anti-RNA pol II. Subsequently, the precipitated DNA was purified. DNA isolated through the IgG ChIP was used a negative control (aspecific background), whereas DNA immunoprecipitated by anti-RNA pol II contained DNA fragments to which RNA pol II were bound in vivo. These templates were then amplified with PCR using different set of primers specific to different promoters (as described above). In figure 12, primers specific for the GAPDH promoter were used on ChIP reactions obtained using the RNA pol II antibodies and two different endometrial samples. For both endometrium samples enrichment was seen for the GAPDH promoter locus (i.e. DNA precipitated with anti-RNA pol II). The ChIP negative PCR (using primers which flank a region in the genome where no transcription factors bind) did not give any detectable signal, thus normalization was not needed.
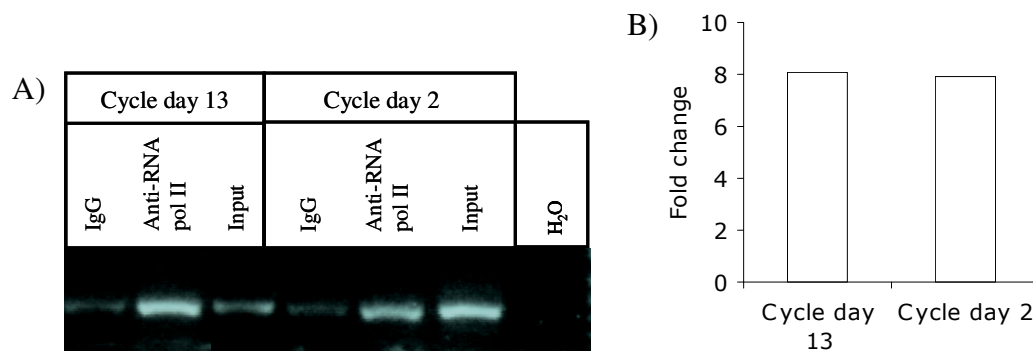
Figure 12. ChIP on endometrium samples (cycle day 13 and 2). A) ChIP was performed on endometrium using the optimized protocol, using a negative control antibody (IgG) and anti-RNA pol II. PCR analysis of the DNA pulled down through IP was subsequently carried out. The amplified products were loaded on a 4 % agarose gel. B) The fold changes of the ChIP positive signals of the anti-RNA pol II samples. The intensity of the ChIP positive PCR signals (part A of the figure) were measured using the ImageJ software. Subsequently, the fold changes of the ChIP positive PCR signals were calculated using the following formula. FC= (ChIP positive signal/ input signal *100)/ negative control (IgG) signal. Normalization of the ChIP positive signals was not needed, the ChIP negative signals were equal in all samples.

Although clear enrichments were obtained for several samples that were tested, reproducibility was still not optimal due to the complexity of the ChIP protocol. Therefore extra optimization is needed. When this technique will be completely optimized for the material (the endometrium), it can be exploited to perform the genome-wide identification of ER-α target genes in human endometrium throughout the menstrual cycle (ChIP-on-chip).

4   Discussion

During the menstrual cycle, the human endometrium undergoes cyclic changes (proliferation-differentiation-degeneration) that are controlled by the ovarian steroid hormones estradiol and progesterone. In particular, during proliferation, estradiol is dominant compared to progesterone that is required for later differentiation and decidualization (1, 3-6). The present study aims at investigating how estradiol controls proliferation in the human endometrium.

Estradiol actions are primarily mediated by its cognate receptor, the estrogen receptor (ER). The mechanisms of estradiol action in the human endometrium are only partly understood due to the complexity and the involvement of numerous factors. However, in response to estradiol, ER-α is not only capable of stimulating gene activity, but it can also exert its function as a competent transcriptional repressor. It is therefore hypothesized that other factors are involved in the regulation of gene expression. These factors, also called coregulators, can be subdivided into two major classes: the coactivators and corepressors. Coactivators and corepressors, together with the ER, are recruited at the promoters of target genes and affect the transcriptional activity in a positive or negative way, respectively. Differential coregulator recruitment by ER-α can thus explain why different target genes respond in different (opposite) ways to the same stimulus (i.e. estradiol). The objective of the present study was to understand how the response to estradiol is influenced by coregulator recruitment at the level of different target gene promoters in human endometrium.

The first part of the study focused on two particular genes that were differentially regulated in vivo in human endometrium in response to estradiol. One of those genes is GW112, which was positively regulated by estradiol. The other gene of interest was GADD45B. In response to estradiol, this gene was down regulated in the human endometrium. Both gene promoters contained several putative regulatory motifs that directly or indirectly could be occupied by ER-α. To investigate the regulation of these genes, the endometrial cancer cell line RL95.2 was used as an in vitro model system. Studying the interactions between ER-α and the different coregulators showed that in solution both coactivators and corepressors can interact with ER-α in a hormone-independent manner. Therefore it was hypothesized that the recruitment of distinct coregulators by ER-α occurs at the level of a gene promoter.

To answer specifically this question, chromatin immunoprecipitation (ChIP) can be used. ChIP allows to study coregulator recruitment at the level of chromatin (gene promoter) in the cell. However, the ChIP protocol had never been performed on the RL95.2 human endometrial cell line. Thus the protocol had to be optimized at all levels, including fixation, chromatin isolation, sonication and shearing, and the immunoprecipitation step. The second part of the present project aimed at investigating genes that are targeted by the ER-α in the human endometrium throughout the menstrual cycle at the genome wide level. Chromatin immunoprecipitation coupled to genome-wide DNA micro-arrays allows this kind of research. Also in this case, however, the ChIP protocol had to be optimized before it could be applied on endometrium.

To understand how GW112 and GADD45B are regulated by estradiol, the promoter sequences of both genes were analyzed for putative regulatory sites where ER-α could directly or indirectly (through interaction with other transcription factors) bind to. Recently it was shown that a large number of genes regulated by estradiol contain imperfect EREs or ERE half sites in combination with other transcription factor binding sites (19, 20).

The promoter of GW112 contains a Sp1 site adjacent to a ERE half site (figure 4). For other gene promoters binding of ER-α to half ERE was shown to enhance the subsequent binding of Sp1 and thus to enhance gene transcription (22, 35, 36). In other cases, binding of ER-α was suggested to synergistically cooperate with AP-1 binding to attract coactivators in response to estradiol.

The promoter of GADD45B contains two putative NF-κB regulatory elements (figure 5). Interestingly, it is reported that ER-α can inhibit the activity of NF-κB. However, the mechanism by which ER-α blocks the binding of this transcription factor to the gene promoter is controversial (37). Some researchers have suggested that ER-α forms a complex with NF-κB that prevents DNA binding (38). Others have suggested that estradiol blocks the degradation of the inhibitory subunit of NF-κB (39). In addition, the promoter of GADD45B contains a large number of Sp1 sites, two ERE half sites and one AP-1 site (figure 5). ER-α can therefore interact directly with these motifs to regulate gene expression.

Thus, the promoters of both genes contain multiple transcription factor binding sites that can synergize to regulate gene expression. However, all regulatory motifs found in the promoter sequences are based on predictive methods. Carroll and co-workers have shown that most of the predicted ERE do not really bind ER-α in vivo (18). Thus, the importance

of each of these putative regulatory motifs in the regulation gene expression has to be analyzed in the cell.

Among four endometrial cancer cell lines (ECC-1, Ishikawa, RL95.2 and AN$_3$CA), the RL95.2 cell line was selected as the in vitro model system for investigating estradiol action and ER-α signaling. Two reasons led to the selection of this cell line: RL95.2 cells expressed ER-α and showed the same expression profiles for GW112 and GADD45B as observed in the endometrium in vivo. The other 3 endometrial cell lines (ECC-1, Ishikawa and AN$_3$CA) were excluded from further analyses because, when studying the expression of GW112 and GADD45B and a gene known to be regulated by estradiol (pS2), different gene responses were observed. For example, in ECC-1 cells GADD45B was upregulated, on the contrary the same gene was downregulated in RL95.2 cells in response to the same ligand (estradiol). GW112 was only expressed in RL95.2 cells. And pS2 (used as a control for estradiol responsiveness in each cell line) was up regulated in ECC-1, Ishikawa and RL95.2 cells, however in AN$_3$CA cells this gene was not regulated by estradiol.

As mentioned, upon induction with estradiol of RL95.2 cells, GW112 resulted upregulated and GADD45B downregulated. Subsequent experiments, however, showed that the presence of serum in the culture medium affected the response of both genes to estradiol. It is known that a complex cross-talk between ER-α signaling, and other signaling pathways such as growth factors cascades exist and can affect gene expression. Thus it appears that both GW112 and GADD45B are regulated by estradiol in combination with other signaling pathways. Future investigations will be performed in which specific growth factor cascades will be inhibited.

It was hypothesized that the recruitment of different coregulators by the ER-α might be responsible for the differential expression of GADD45B and GW112 in response to the same ligand (estradiol) in RL95.2 cells. This cell line abundantly expressed all studied coregulators, including three coactivators (CBP, p300 and SRC-1) and one corepressor (N-CoR). Our interest was to examine how coregulators modified ER-α activation of GW112 and GADD45B, in response to estradiol.

First, the interaction in solution between distinct coregulators and ER-α was studied in response to different treatment conditions (estradiol/anti-estrogen) using co-immunoprecipitation.

RL95.2 cells were treated with different ER-α ligands. Pure agonist (estradiol), agonist plus antagonist (estradiol plus ICI 164384) or no ligand (vehicle alone) and co-immunoprecipitation was performed with different coactivators and corepressors. Only a small amount of ER-α was bound to the coregulators, although these proteins were all abundantly expressed in RL95.2 cells. Furthermore, all studied coregulators interacted with ER-α in vitro in a hormone independent manner. In fact, no enrichment of ER-α coactivator/corepressor complex formation was seen in the presence of agonist/antagonist, respectively. This could reflect differences between in vitro and in vivo binding conditions. Other studies (using GST pull-down assays) have also shown that N-CoR (40) and SMRT (41) can interact in a ligand-independent manner in vitro. These results were interpreted to indicate that unliganded ER-α was bound by N-CoR and SMRT and that anti-estrogen binding allows ER-α to retain its interaction with these corepressors, whereas estradiol stimulates corepressor dissociation.

There is evidence that upon binding of agonist like estradiol a conformational change in the structure of the ER-α is induced, resulting in the formation of a coactivator recognition groove (11, 15). The ability of the coactivators SRC-1, CBP and p300 to bind ER-α is dependent upon the integrity of a conserved motif consisting of a leucine-rich repeat (LXXLL, NR box) and on key hydrophobic residues in helix 12 within the ligand binding domain of ER-α (42). When an antagonist is bound to ER-α, the binding of coactivators is prevented and the recruitment of corepressors is facilitated. Furthermore, the DNA sequence where ER-α binds to also affects its structural conformation and thus the interaction with coregulators (43). Therefore to study the recruitment of coregulators by this receptor at the level of a gene promoter was one of the main goals of the present study.

The ChIP procedure can be used to study the ER-α binding and the selective recruitment of coregulators at specific gene promoter. Briefly, proteins bound to DNA in living cells are crosslinked using formaldehyde. After crosslinking, the extract is sonicated to shear the DNA into small fragments (ranging form 500-1000 bp). DNA fragments crosslinked to the protein of interest can be enriched using a protein specific antibody. After precipitation of the DNA-protein-antibody complexes, the formaldehyde crosslinking is reversed and the DNA is purified. Subsequently PCR can be performed on the precipitated DNA using primers that flank the region of the gene promoter where the protein of interest binds to. Until now ChIP has mostly been performed on breast cancer cells (MCF-7) to study the binding of ER-α and the recruitment of coregulators at different gene promoters. Different

protocols were therefore used to optimize the conditions to apply ChIP on the RL95.2 endometrial cancer cell line. The optimization included adjustments of the amount of starting material, cell fixation, chromatin isolation, sonication and shearing, and the immunoprecipitation (IP) step. A large part of the project was dedicated to ChIP optimization in cell lines and in tissue (see below). A high number of cells were necessary as starting material. Subsequently these cells were fixed in large volumes of fix solution containing formaldehyde. Over-crosslinking was prevented by including a washing step and addition of a glycin stop-fix solution. To isolate the protein-crosslinked chromatin, cells were lysed and finally the DNA was sheared (by sonication). The DNA yield after shearing determined the amount of DNA used in each IP reaction. Using the sheared chromatin, the IP reaction was performed to precipitate the DNA bound to the protein of interest. In the optimization tests, RNA pol II was used and the GAPDH promoter was analysed with PCR using specific primers.

Although a clear enrichment of DNA fragments specific to GAPDH promoter was obtained after IP with ant-RNA pol II, the protocol still needs some improvement due to low reproducibility of the results. Using different beads (dynabeads protein G) to pull down protein-DNA-antibody complexes may provide better performance decreasing aspecific background signals. When optimized and standardized completely, this technique can be used to study the binding of ER-α to its target genes such as GW112 and GADD45B. Furthermore, to identify the binding of known coregulators, preference and alternate binding, a second immunoprecipitation (re-ChIP) can be performed using antibodies against the coregulator of interest.

Some researchers have already investigated the binding of ER-α and the recruitment of several coregulators at the level of a few gene promoters (pS2, cathepsin D and c-myc) in breast cancer cells (MCF-7). Shang and co-workers found that ER-α and a number of coactivators rapidly associate with estradiol responsive promoters following estradiol treatment. These associations occurred in a cyclic fashion: cycles of ER-α complex assembly were followed by transcription. The same authors also showed that the p160 class of coactivators, including SRC-1, CBP and p300, were sufficient to determine gene transcription (32). These results were confirmed in other studies (31).

The second part of the present study was focussed on the identification of all target genes regulated by estradiol in the human endometrium throughout the menstrual cycle. When coupled with whole-genome DNA micro-array hybridization, ChIP can be used to examine

the entire spectrum of in vivo DNA binding sites for ER-α. Optimization of the ChIP protocol was needed, due to the fact that ChIP had never been performed on human endometrium. The conditions that were optimized to perform ChIP on RL95.2 cells were slightly changed. After the endometrium was removed from the uterus, it was crosslinked similarly to what was described for the cell line. Subsequently homogenization of the tissue (using a glass douncer) was necessary to transform the tissue into a single cell suspension. Thereafter, the cells were lysed and DNA was sheared. The IP was performed using the same protocol for the cell line and the tissue. As in the case for ChIP analysis on endometrial cancer cell lines, the reproducibility of results required further optimization. When all optimizations will be finished, this technique combined with the chip hybridization (ChIP-on-chip) has the potential to identify all gene promoters targeted by ER-α in the endometrium. The results will be combined with previous studies that have identified several genes regulated by estradiol in the endometrium (44, 45).

In conclusion, the variability in the types of estrogen-binding sequences and other transcription factor binding sites seen between the promoters of GW112 and GADD45B suggests that estrogen regulation of these genes may be related to particular promoter context. Furthermore, the degree to which liganded ER-α activates each particular promoter is probably cell-dependent. Thus, the overall cell context, including the cellular levels of ligands and receptors, the phosphorylation state of the various proteins, other transcription factor-binding sites in the promoter/enhancer, the chromatin structure of the regulatory region, and the spectrum of cellular coregulators, is critical for coupling the binding of ER-α to the activation or repression of gene expression. All these aspects determine the biological actions of estradiol in the human endometrium.

A detailed knowledge of the mechanisms regulating endometrial events involved in proliferation, differentiation, decidualization and embryo implantation, and menstruation are essential for understanding the mechanisms responsible for several common disturbances of the endometrium, such as abnormal menstrual bleeding, early pregnancy failure, infertility and cancers.

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