Mechanisms of Estrogen Sensitivity in the Human Endometrium

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A dissertation submitted to the faculty of The University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology and Laboratory Medicine, School of Medicine.

Chapel Hill
2007

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ABSTACT

Terrence Dairon Lewis: MECHANISMS OF ESTROGEN SENSITIVITY IN THE HUMAN ENDOMETRIUM

(Under the direction of David G. Kaufman, MD, PhD)

Endometrial cancer is the most common gynecologic malignancy, and a major cause of morbidity and mortality in women in the Western World. The American Cancer Society estimates that 41,200 cases will be diagnosed and 7,350 deaths will result from endometrial cancer in the United States in 2007. Unopposed estrogen exposure is the primary etiologic risk factor for developing this disease. However, the effects of estrogen and its receptors are not well defined within the human endometrium. The principal goal of this dissertation was to understand the individual contributions of the estrogen receptors (ERs) α and β within epithelial cells of the endometrium. To this end, three specific aims were developed to help further our understanding of the functions of these receptors in this tissue: (1) evaluate the expression of the receptors in normal, hyperplastic, and malignant tissue, (2) evaluate the effects of the receptor subtypes on the estrogen-inducible placental alkaline phosaphatase (ALPP) gene, and (3) to evaluate the effects of the receptors on proliferation of endometrial adenocarcinoma cells. Findings from the first aim indicate that an alteration in the normal ERα/ERβ ratio takes place in the stromal and epithelial compartments of the endometrium at the pre-malignant (hyperplastic) state. We believe that this change may be a key step that permits the endometrium to produce an exaggerated response to estrogens. Further studies evaluating the effects of the receptors on the ALPP gene used highly specific agonists of the

estrogen receptors, PPT and DPN, along with cell lines expressing one receptor or the other. These studies reveal that ERα, and not ERβ, is responsible for the upregulation of ALPP. Further studies utilizing inhibitors of both MAP-Kinase and (PI)3-Kinase, revealed that the upregulation of ALPP is at least in part due to these signaling pathways within this model system. In our final aim we learned that ERα, and not ERβ, is involved in estrogen-induced proliferation of endometrial epithelial cells. Furthermore, ERβ acts as an inhibitor of proliferation within this tissue by possibly inhibiting the cell cycle by regulating key components of the cell cycle machinery.

This dissertation is dedicated to all those, past and present, who fought and continue to fight for equality and civil rights.

Acknowledgements

First, I would like to recognize my parents, Francine McGriff and Ronald Lewis, for their unwavering love and support throughout my childhood and academic career. As the ancient African Proverb goes, “It takes a village to raise a child”, I would be negligent if I failed to recognize my grandparents, Rhett (deceased) and Alvada McGriff, and Herbert (deceased) and Juanita Lewis, in addition to a host of Aunts, Uncles, and Cousins who have all provided tremendous encouragement throughout my academic endeavors. Additionally, I would like to thank my extended family at New Destiny Fellowship, in Wilmington, Delaware. Without the collective support of my biological and extended family, this dissertation would not have been possible.

I would also like to recognize two persons whose influence led to my enrollment in the Department of Pathology and Laboratory Medicine at the University of North Carolina at Chapel Hill. The first of which is, Dr. Fatma Helmy, Professor and Director of the Delaware State University MARC Program. Without Dr. Helmy’s guidance and nurturing throughout my undergraduate career I would not be here at UNC. Her tough love approach to mentoring did not stop once I earned my degree at DSU, rather it has continued and is a reflection as to why many of her students receive graduate and professional degrees. Additionally, I would like to thank Dr. Henry Frierson, Director of Educational Support at the University of North Carolina at Chapel Hill, for supporting me through the SPGRE and RES programs. Without Dr. Frierson’s help throughout the application process, I most likely would not have entered this department. (I hope to never let either of you down!)

To my dissertation committee, I would like to thank each of you for spending countless hours discussing scientific facts, data, and future plans with me. Each of you are superior scientists and/or clinicians that serve as a source of inspiration for me. To the chair of my committee, Dr. David Kaufman, I would like to thank you for supporting me over the course of my graduate career. To Dr. Whang, I would like to thank you for your scientific expertise and for letting me know that "mother nature does not reveal her truths easily". To Dr. William "Bill" Coleman, I do not think words exist that express my gratitude for your guidance since you joined my dissertation committee. To Dr. Christopher Gregory, I would like to thank you for your help that started prior to your departure from the university. Last but not least, to Dr. Ruth Lininger, I would like to thank you for your open door policy and all of your help reviewing countless slides for Chapter II of this dissertation.

Finally, I would like to the entire Department of Pathology and Laboratory Medicine for supporting me throughout my training at UNC. I would like to personally thank Dr. J. Charles Jennette, Chair of Pathology, who has fostered a collaborative environment amongst the faculty and graduate students. Additionally, I would like to thank the support staff within the department including, Mrs. Dorothy Poteat, Mrs. Kia Barbee, Mrs. Penny Hawkins, and Ms. Denise Herndon for all of their help over the course of the past six years.

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LIST OF ABBREVIATIONS AND SYMBOLS

ALPP	Placental Alkaline Phosphatase
ChIP	Chromatin Immunoprecipitation
DPN	Diarylpropionitrile (ERβ agonist)
E_2	17β-Estradiol (Estrogen)
ERα	Estrogen Receptor Alpha (ESR1)
ERβ	Estrogen Receptor Beta (ESR2)
ERE	Estrogen Response Element
ERKO	Estrogen Receptor Knockout
IHC	Immunohistochemistry
IK-3H12	Ishikawa (Clone 3H12)
IK-3H12α	Ishikawa (Clone 3H12) Expressing ERα protein
IK-3H12αβ	Ishikawa (Clone 3H12) Expressing ERα and ERβ proteins
IK-3H12β	Ishikawa (Clone 3H12) Expressing ERβ protein
MAPK	Mitogen Activated Protein Kinase
MPP	Methyl-piperidino-pyazole (ERα antagonist)
mRNA	Messenger Ribonucleic Acid
(PI)3K	Phosphotidylinositol-3 Kinase
PPT	Propylpryazole-triol (ERα agonist)
qPCR	Quantitative Real Time Polymerase Chain Reaction
TMA	Tissue Microarray

Chapter I: Introduction

Uterine biology

Ontogeny of the uterus

During early embryogenesis, both males and females have undifferentiated gonads and have mesodermally derived Wolffian and Mullerian ducts. Wolffian ducts can develop into the male internal genitalia including the epididymus, seminal vesicles, and vas deferens. On the other hand, Mullerian ducts form the luminal and glandular epithelium of the uterus, in addition to the epithelial lining of the oviduct, cervix and upper vagina. Each Mullerian duct is surrounded by urogenital ridge mesenchyme that gives rise to the fibromuscular wall of the uterus, endometrial stroma and myometrium (Wynn and Jollie 1989). Additionally, this layer gives rise to the connective tissue and muscle layers of the oviduct, cervix, and upper vagina. Genetic information carried on the Y chromosome of XY embryos leads to the development of testes, which cause male internal and external genitalia to develop by producing testosterone and Mullerian Inhibiting Factor (MIF) (Gilbert 1997). In the absence of a Y chromosome, Mullerian duct development occurs by default and female internal genitalia result. The female external genitalia including the labia and clitoris are also the default. However, the external genitalia are not derived from the Mullerian ducts.

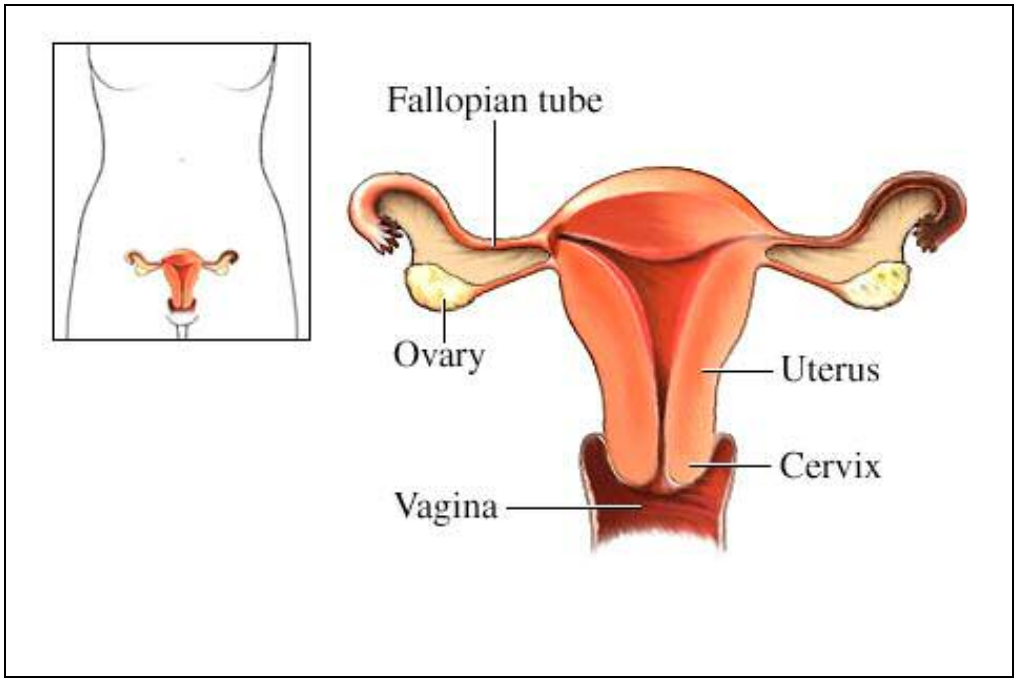

Figure 1.1. The Female Reproductive Organs

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Basic uterine anatomy

The female reproductive tract consists of the vagina, cervix (cervix uteri), uterus (corpus urteri), fallopian tubes, and ovaries. At the upper portion of the uterus the fallopian tubes and oviducts open, bilaterally, and extend outward to surround the ovaries. The cavity of the uterus is connected with the vagina directly via the cervical canal, which is located directly below the uterus and within by the cervix. The uterus of an adult woman who has not previously had children measures about 7.5 cm in length, 5 cm in breadth, at its upper part, and nearly 2.5 cm in thickness, weighing roughly 30 to 40 grams (Beckmann 2002). The uterus is a pear-shaped, thick-walled, muscular, hollow organ situated within the pelvic cavity between the bladder and rectum. The slight constriction between the apex and base which give the uterus its pear shape is known as the isthmus. The portion above the isthmus is termed the body and that below is the cervix. The biological role of this organ is to receive the blastocyst into its endometrial lining, nourish the developing blastocyst, and ultimately discharge the fetus at birth (Wynn and Jollie 1989). During pregnancy the uterus undergoes changes in size and structure to accommodate the needs of the developing embryo. Following birth the uterus returns to approximately its original size, but, typically remains somewhat enlarged.

The uterus can be divided into several structures including the body (corpus uteri), the cervix and cervical canal (cervical uteri), the endometrium, the myometrium, and the peritoneum. Most of the uterus is comprised of the corpus uteri, which makes up the top portion of the pear-shaped organ. The convex portion of the uterus in which the fallopian tubes are attached is known as the fundus. Below the isthmus, the uterus becomes conical in shape and terminates at the cervix. The cervical canal enters the vaginal canal providing

direct linkage between the uterus and the vagina. The uterus is comprised of three layers: the endometrium is the innermost lining of the organ. The myometrium, the middle layer, is a thick layer consisting of smooth muscle and the blood vessels that serve the organ. Finally, like abdominal organs, the uterus is covered by the peritoneum, a serous membrane composed of a layer of mesothelium and a thin layer of connective tissue.

The endometrium

As shown in Figure 1.1, the endometrium is the inner lining of the uterus, covering the uterine cavity. The endometrium contains two distinct layers, the basalis and the functionalis (Figure 1.2). The basalis directly contacts the myometrium and undergoes comparatively minor changes throughout the menstrual cycle. The functionalis, which is divided into the stratum compactum and stratum spongiosum, surrounds the lumen of the uterine cavity and undergoes extensive remodeling during the menstrual cycle. The functionalis of the endometrium consists of two distinct cell types: epithelial cells, which form the luminal surface and endometrial glands, and the surrounding stromal cells (Ludwig and Spornitz 1991).

The luminal epithelium is the internal lining of the endometrial cavity and is the internal site of contact between the implanting blastocyst and the maternal organ. The luminal layer contains cells with apical ciliation in addition to cells that lack ciliation. Ciliated cells increase from 1:30 to 1:15 during the early to late proliferative phase of the menstrual cycle before decreasing after day 20 to a final ratio of 1:50 (Glasser 2002; Wynn and Jollie 1989).

The glandular epithelium changes profoundly throughout the menstrual cycle. During the proliferative phase, estrogen increases glandular cell proliferation leading to an increase in the number of glandular structures. Additionally, the glandular cells have a poorly developed secretory apparatus including the Golgi apparatus, the smooth endoplasmic reticulum, and secretory vesicles. During the late proliferative phase the glands contain pseudostratified columnar cells. There are few mitotic cells but now cells have developed an elaborate secretory apparatus. As ovulation approaches there is an accumulation of glycogen in the cytoplasm of the glandular cells. During the early secretory phase the glandular epithelial cells transform into highly polarized cells actively involved in the production and secretion of complex secretory products at the apical surface (Glasser 2002). Additionally, there is a continuation of cytoplasmic glycogen deposition within the cell.

The endometrial stromal cells, also known as reticular cells, reside within the connective tissue along with granulated stromal cells and lymphocytes. The remaining connective tissue consists of an extracellular matrix with fibers and a gel-like polysaccharide ground substance. During the early proliferative phase the stromal cells resemble undifferentiated fibroblasts with mesenchymal characteristics. Through progression of the proliferative phase, stromal cells begin to take on the characteristics attributed to fibroblasts and begin to produce most of the matrix components (Wynn and Jollie 1989). Throughout the menstrual cycle, stromal cells remodel the matrix in response to various factors, including steroid hormones.

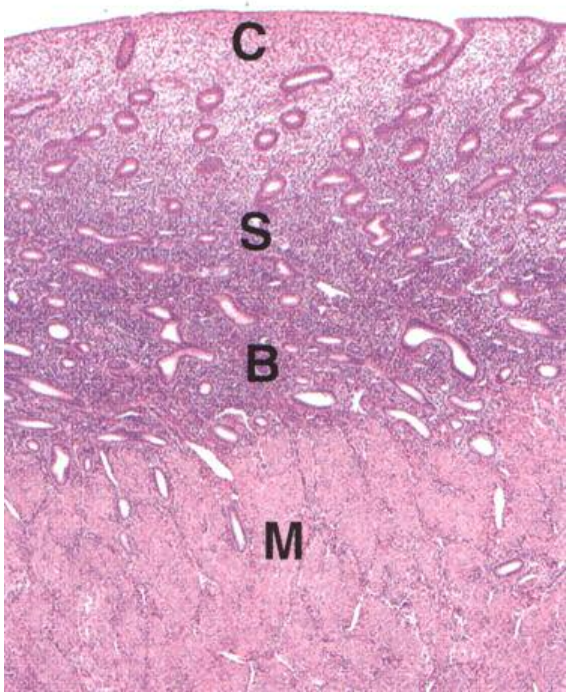

Figure 1.2. Normal Endometrial Histology. The endometrium contains two distinct regions termed the basalis (B) and the functionalis. The basalis resides above the myometrium (M), or smooth muscle layer of the uterus. The functionalis, or functional layer, is made up of the stratum compactum (C) and stratum spongiosum (S), which contains luminal and glandular epithelium, in addition to stromal cells.

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Endometrial vasculature

The endometrial blood supply is provided by a sophisticated network of vessels that undergo cycles of growth and regression during each menstrual cycle (Rogers 1996a). As shown in Figure 1.3, the uterine and ovarian arteries emanate from the myometrium and form the arcuate arteries, which give rise to the radial arteries. After passing through the junction between the myometrium and endometrium, the radial arteries branch into the basal and spiral arteries. As the name suggests, the basal arteries supply the basal layer while the spiral arteries supply the functional layer. The distinctive coiled appearance of the spiral arteries becomes more pronounced during the second half of the menstrual cycle known as the secretory phase (Rogers 1996b).

The basal layer and its vasculature remain relatively constant throughout the menstrual cycle. In contrast, the functional layer and its associated spiral artery vasculature continually change under the influence of circulating steroid hormones. Despite constant change within the functional layer, new vessel growth or angiogenesis is a tightly regulated process in the endometrium demonstrated by the fact that vascular density remains constant throughout the menstrual cycle (Rogers, et al. 1993). Angiogenesis is recognized to be an essential mechanism for tumor growth, invasion, and metastatic spread. Evidence gathered from endometrial cancer biopsies show an increase in the mean vessel density (MVD) compared to normal endometrial biopsies (de Gois Speck, et al. 2005; Puisoru, et al. 2006; Ribatti, et al. 2005; Stefansson, et al. 2006). This increase in vessel formation suggests that the vasculature in the endometrium may be critical to tumor development and progression in this complex tissue.

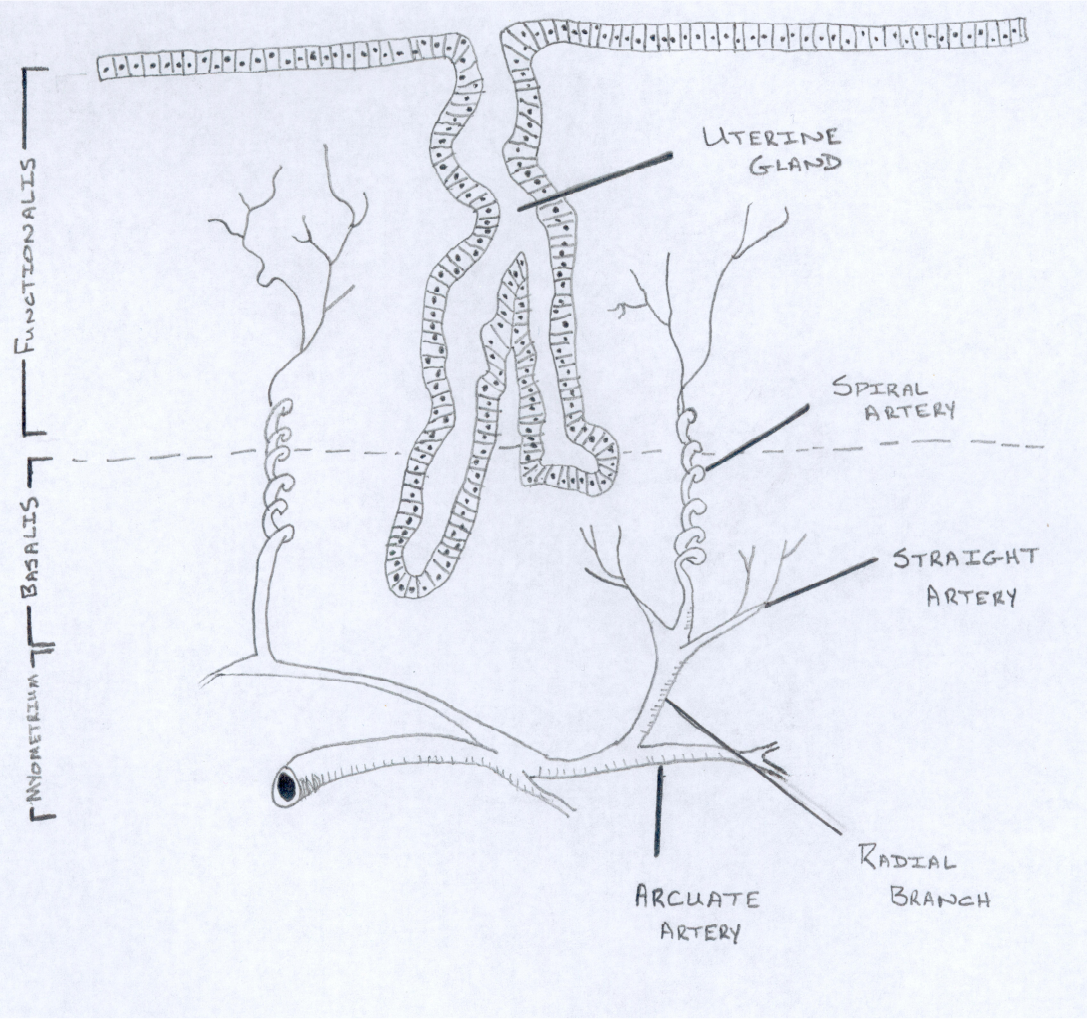

Figure 1.3. Schematic Drawing of the Endometrial Vasculature.

Ovarian control and the menstrual cycle

The menstrual cycle serves to prepare the endometrium for implantation of a fertilized ovum. In the absence of implantation, the cycle culminates with the shedding of the endometrial lining from the uterus. The steroid hormone estrogen plays an important role in the development of the female reproductive tract, pregnancy, and the menstrual cycle by providing a mitogenic stimulus. Progesterone, another steroid hormone, is also involved in the hormonal regulation of the menstrual cycle and pregnancy, but with effects that typically counteract the mitogenic effects of estrogen, leading to differentiation. The menstrual cycle stage can be distinguished by changes in both the ovary and endometrium.

The menstrual cycle is regulated primarily through the hypothalamus and anterior pituitary, which regulate the secretion of estrogen and progesterone from the follicular cells or the corpus luteum of the ovary. The hypothalamus releases Gonadotropin Releasing Hormone (GnRH) which stimulates the anterior pituitary to secrete Follicular Stimulating Hormone (FSH) or Lutenizing Hormone (LH). In turn, FSH and LH stimulate the secretion of estrogen and progesterone either by follicular cells or the corpus luteum.

Under the influence of FSH, the ovarian follicular cells develop and secrete estrogen during the follicular phase of the menstrual cycle. The gradual increase in estrogen in the follicular phase stimulates increased LH secretion from the anterior pituitary leading to ovulation. Following ovulation, LH induces the follicle to become the corpus luteum, (luteal phase) which will in turn secrete estrogen and progesterone during the second phase of the cycle. If pregnancy does not occur, the high levels of estrogen and progesterone provide constitutive feedback to inhibit secretion of GnRH, FSH, and LH. When LH levels decrease,

the corpus luetum regresses and fails to secrete estrogen or progesterone, leading to menstruation.

Endometrial changes during the menstrual cycle

For the endometrium, the menstrual cycle can be divided into the proliferative, secretory, and menstrual phases, which reflect this tissue's response to steroid hormones (Glasser 2002). During a normal cycle, ovarian follicles produce estrogen, and both estrogen and progesterone are produced by the corpus luteum, a small body formed by the follicular cells remaining in the ovary following ovulation. Estrogen and progesterone are not secreted at constant levels during the menstrual cycle. Instead, variations of the levels of the two hormones, estrogen and progesterone, are observed. These changes are directly responsible for alterations in the morphology and function of the endometrium during the menstrual cycle. Moreover, the expression of endometrial estrogen and progesterone receptor proteins are programmed in response to hormones throughout the cycle (Lessey, et al. 1988).

The proliferative phase, which follows menstration, is regulated by estrogen produced by ovarian follicles. During menstration, the upper two thirds of the endometrium are shed leaving the surface of the endometrium covered with epithelial cells proliferating from the basal glands (Glasser 2002). As the proliferative phase ensues, the endometrium becomes richly supplied with blood vessels that nourish the expanding tissue. Following ovulation, at approximately the mid-point of the menstrual cycle, the secretory phase begins as estrogen and progesterone are produced by the corpus luteum. Under the influence of the combination of steroid hormones there is an increase in the secretory differentiation of the endometrium, including the accumulation of glycogen and lipids within the glandular structures. If

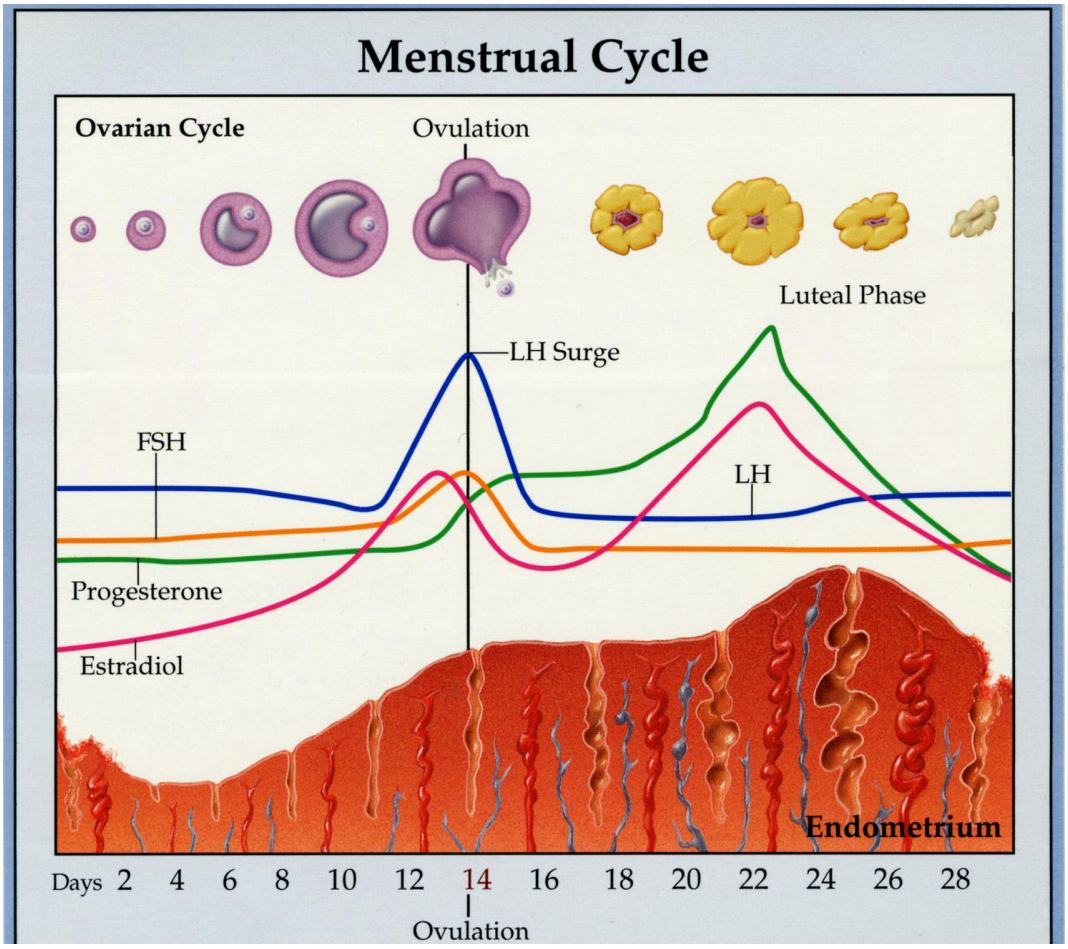

Figure 1.4. Schematic Representation of the Human Menstrual Cycle. The schematic shows both ovarian (top) and endometrial (bottom) changes throughout the normal human menstrual cycle. Hormones that are responsible for these changes are Follicular Stimulating Hormone (FSH), Lutenizing Hormone (LH), 17β-Estradiol (estrogen), and Progesterone.

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implantation occurs, human chorionic gonadotropin (hCG) is secreted by the developing placenta which maintains estrogen and progesterone secretion by the corpus luteum (Beckmann 2002). In the absence of implantation, the secretion of estrogen and progesterone dramatically decreases due to luteolysis, or cyclical regression of the corpus luteum. The spiral arteries that have grown into the endometrium contract and the upper endometrial lining, starved of its blood supply, dies and is shed from the uterus producing the bleeding associated with the menstrual phase. The cycle is then set to begin the proliferative phase and the rest of the cycle once again, continuing at approximately twenty-eight day intervals from adolescence to menopause.

Growth disorders of the endometrium: hyperplasia to neoplasia

Hyperplasia

Endometrial hyperplasia is defined as an overgrowth of both endometrial glands and stroma, and is characterized by a proliferative glandular pattern with or without different degrees of morphologic abnormality, or atypia (Mutter 2000). Proliferative patterns lacking cytologic atypia are classified as hyperplasia, while those displaying atypia are deemed atypical hyperplasia. Classification of both forms of hyperplasia can be elaborated by assessing the degree of architectural abnormalities. Hyperplasia and atypical hyperplasia lacking glandular complexity and crowding are designated simple hyperplasia and atypical hyperplasia, respectively. Hyperplasia and atypical hyperplasia with increased glandular complexity and crowding of the glands are called complex hyperplasia and complex atypical hyperplasia, respectively.

Increased estrogen levels, as found in polycystic ovarian syndrome (PCOS), estrogen-secreting ovarian tumors and obesity seem to increase the occurrence of hyperplasia (Montgomery, et al. 2004). The latter is particularly disturbing as the incidence of obesity in the United States is increasing. Atypical hyperplasia can be treated with either progestin's, to counteract the effects of estrogen, or hysterectomy (Jadoul and Donnez 2003). Without treatment, twenty-five percent of patients with atypical hyperplasia will develop endometrial cancer (Kurman, et al. 1985).

Neoplasia

Endometrial cancer is a major cause of morbidity and mortality in women and is the most common gynecologic malignancy of the female genital tract in the United States. The American Cancer Society estimates that 41,200 new cases will be diagnosed and 7,350 deaths will result from endometrial cancer in the United States in 2007. The incidence of endometrial cancer, like most cancers, is dependent on age. The median age of patients diagnosed with this disease is 63 years. Seventy-five percent of women diagnosed with endometrial cancer are post-menopausal.

Endometrial cancers are grouped into two broad categories. The most common endometrial cancers are known as type-1 or glandular endometrioid. Many are estrogen sensitive, low stage, and have an excellent prognosis. On the other hand, type-2 or serous-papillary carcinomas, which make up ten percent of endometrial cancers, commonly are estrogen insensitive, have a high stage when diagnosed, and carry a less favorable prognosis. Approximately eighty percent of all type-1 endometrial cancers are of the endometrioid type. Endometrioid lesions are classified as low or high grade by low-magnification assessment of

the amount of solid growth, the pattern of invasion, and the presence of tumor necrosis (Rubin and Farber 1999). Tumors are considered high grade if at least two of the following three criteria are met: more than fifty percent solid growth, diffusely infiltrative growth, and tumor cell necrosis.

As shown in Table 1.1 endometrial cancers are classified into several categories based on histopathology using guidelines presented by the World Health Organization (WHO) and the International Federation of Gynecology and Obstetrics (FIGO). The endometrioid cancers are those mentioned as type 1 cancer above and the type 2 cancers are the non-endometrioid cancers, particularly the papillary serous adenocarcinomas.

Unopposed stimulation of the endometrium by endogenous or exogenous estrogens is the classic etiological factor associated with the development of endometrial carcinoma (Rose 1996)**.** Stimulation of the endometrium with unopposed estrogens leads to hyperplasia, which increases the chance of development of atypical hyperplasia, and eventually type-1 endometrial cancer (Montgomery et al. 2004). Other risk factors for the development of this disease include estrogen-secreting ovarian tumors, Polycystic Ovarian Syndrome (PCOS), obesity, and diabetes mellitus. Each aforementioned factor is strongly associated with an increase in unopposed estrogens (Akhmedkhanov, et al. 2001; Glasser 2002; Hale, et al. 2002). Despite this knowledge, the molecular pathogenesis of endometrial cancer not clearly understood.

In addition to environmental factors in the development of endometrial cancer, there is now considerable evidence for certain genetic predispositions for the disease. Patients with Cowden's syndrome, an autosomal dominant trait with incomplete penetrance and variable expressivity, are characterized by germline mutations in the PTEN (phosphatase and tensin

Histopathologic classification

Endometrioid carcinoma
- Adenocarinoma
- Adenocanthoma
- Adenosquamous carcinoma

Non-endometrioid carcinoma
- Mucinous adenocarcinoma
- Papillary serous adenocarcinoma
- Clear-cell carcinoma
- Adenosquamous carcinoma
- Undifferentiated carcinoma
- Mixed carcinoma

Histological grade (G)

GX Grade cannot be assessed
G1 Well differentiated
G2 Moderately differentiated
G3 Poorly or undifferentiated

Surgical staging (Stage)

1A Tumor is limited to the endometrium
1B Invasion to less than half of the myometrium
1C Invasion equal to or more than half of the endometrium
2A Endocervical glandular involvement only
2B Cervical stromal invasion
3A Invasion of serosa of the corpus uteri and /or adnexa and/or positive cytology
3B Vaginal metastasis
3C Metastases to pelvic and/or para-aortic lymph nodes
4A Tumor invasion of bladder and/or bowel mucosa
4B Distant metastasis, including intra-abdominal metastases and/or inguinal lymph nodes

Table 1.1. FIGO Guidelines

homolog deleted on chromosome ten) tumor suppressor gene (Scheper, et al. 2006) (Kato, et al. 2001; Ramaswamy, et al. 1999). These patients are at a considerably higher risk of developing various cancers, including type-1 endometrial cancers, than the general population. At the molecular level, the most common abnormality associated with type-1 endometrial cancer is the mutation of the PTEN tumor suppressor gene, which occurs in approximately 80% of type 1 endometrial cancers (Eng 2003). The tumor suppressive activity of PTEN is a result of its ability to negatively regulate the phosphatidylinositiol (PI)3-kinase pathway. When receptor tyrosine kinases bind to their cognate ligands, their intracellular tyrosine kinase domain becomes activated, leading to autophosphorylation of certain tyrosines and recruitment of proteins with affinity for phosphotyrosine residues. (PI)3-kinase, through its p85 regulatory subunit, is translocated from the cytosol to the activated receptor tyrosine kinases and the activated (PI)3-kinase phosphorylates its main substrate phosphatidylinositol-4,5 bisphosphate (PIP_2) to phosphatidylinositol-3,4,5 triphosphate (PIP_3) at the D3 position of the inositol ring. Accumulation of PIP_3 leads to activation of downstream kinases such as the serine/threonine kinase Akt, which mediates cell survival and proliferation signals. PTEN was first thought to be a protein phosphatase, but was later shown to be a lipid phosphatase whose preferred *in vivo* substrate is PIP_3 (Maehama and Dixon 1998). The dephosphorylation of PIP_3 to PIP_2 by PTEN is the basis for its ability to negatively regulate the (PI)3-kinase pathway. Loss of PTEN expression is very common in a broad spectrum of human tumors and PTEN is among the most frequently mutated tumor suppressor gene in human tumors, perhaps only second to p53 (Ali, et al. 1999). The loss of PTEN results in constitutive activation of the (PI)3-kinase pathway and thus Akt, which induces cellular proliferation and decreased apoptosis (Madrid, et al. 2000;

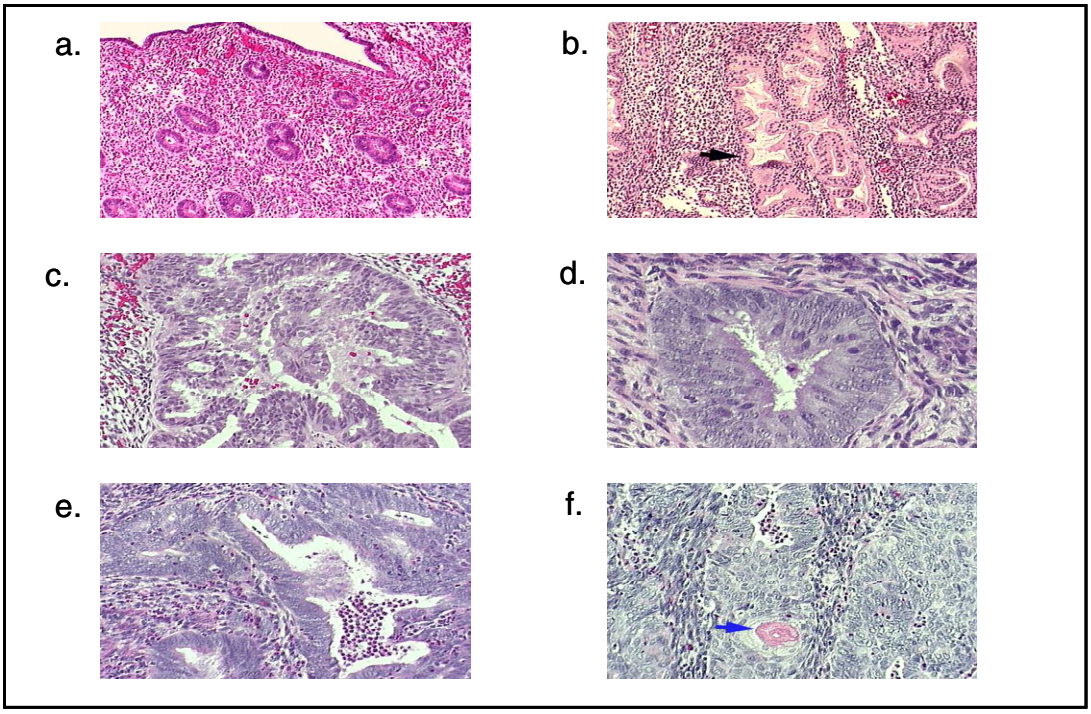

Figure 1.5. Abnormal Endometrial Histology. a) Normal proliferative phase showing normal tubular glands. b) Normal secretory phase with black arrow indicating a coiled gland, which is typical of this stage. c) Complex hyperplasia showing a single gland with complex architecture. d) Atypical hyperplasia showing nuclei with vesciculation, chromatin clearing. e) Adenocarcinoma reveals complex architecture and inflammatory cells in the lumen. f) Adenosquamous carcinoma reveals several malignant glands with inflammatory cells in the lumen. Blue arrow indicates a keratin pearl.

Images used with permission from Pathweb of the University of Conneticut

Myers, et al. 1998). PTEN is often lost in premalignant stages of endometrial carcinogenesis, suggesting that loss of PTEN is an important initiator of endometrial carcinogenesis (Mutter 2002; Mutter, et al. 2000; Ruhul Quddus, et al. 2002; Stoica, et al. 2003).

Mutations in the breast cancer susceptibility gene (BRCA1) have been associated with an increased risk for the development of endometrial cancer. In Thompson *et al.*'s cohort study (2002), it was found that women carrying a mutation in BRCA1 had a two-fold increased risk of developing endometrial cancer over the general population (Thompson and Easton 2002). Besides PTEN and BRCA1, other genetic factors for development of type-1 cancers include, but are not limited to, the KRAS2 oncogene, and germline mutations in DNA mismatch repair genes linked to hereditary non-polyposis colorectal cancers (HNPCC) (Enomoto, et al. 1993; Enomoto, et al. 1991; Lax, et al. 2000). Type-2 lesions are associated with mutations in TP53 and ERBB-2 (HER-2/neu) expression, and most are non-diploid (Okamoto, et al. 1991; Santin 2003).

Early studies on the uterotrophic effects of estrogen and the discovery of the estrogen receptor

The identification of estrogenic activity mediated by the ovary dates back to 1923 in the studies of Allen and Doisy, who induced estrus in female rats by injecting them with purified ovarian follicular extracts (Allen and Doisy 1983). Introduction of the purified exogenous extracts into spayed rats induced growth in mammary glands, and thickening and cornification of the vaginal walls. Further studies revealed that stimulation of the uterus with estrogens increased the synthesis of DNA, RNA, and proteins (Gorski, et al. 1965; Kaye, et al. 1971). Additionally, estrogen was shown to increase mitosis in the rat uterus (Kirkland, et

al. 1979). Subsequently, the mitogenic effects of estrogen were shown to be mediated through the induction of proto-oncogenes such as c-fos, c-jun, jun-B and jun-D, which act as transcription factors and stimulate gene expression through AP-1 DNA elements (Chiappetta, et al. 1992; Cicatiello, et al. 1992; Loose-Mitchell, et al. 1988; Nephew, et al. 1993a; Nephew, et al. 1993b; Webb, et al. 1990, 1993; Weisz and Bresciani 1988; Weisz, et al. 1990).

It was Jenson and Jacobson's preparation of tritiated (^{3}H) estradiol that allowed the discovery of specific target tissues. They also solidified the receptor concept, which was crucial in the identification of the first hormone receptor, known as the estrogen receptor (ER) (Jensen 1962; Toft and Gorski 1966). This group injected (^{3}H)-estradiol into immature rats and localized the labeled hormone in estrogen responsive tissues including the uterus and vagina. The radio-labeled hormone was also found in tissue which is now considered to be unresponsive to estrogen including the liver and kidneys. Moreover, estrogen metabolites were found in the blood. The non-metabolized form of estrogen was shown to be retained in target tissues suggesting that it associated with a specific estrogen-binding factor, later termed the ER, through which estrogen exerts its effects. In contrast, most unresponsive tissues lack high expression of the ER. Toft and Gorski later confirmed that the estrogen receptor was in fact a protein (Toft and Gorski 1966). The discovery that a cellular protein mediates the action of estrogen initiated a series of new studies to characterize the receptor protein. Collectively, studies on estrogen and its signaling pathways provide valuable insights concerning hormone action in the vertebrate endocrine system.

Estrogen receptor structure

The human Estrogen Receptor (hER) was originally cloned and sequenced in 1986 from the MCF-7 breast cancer cell line (Green, et al. 1986). The sequence was found to contain 595 amino acids with a predicted molecular weight of 66 kDa and its locus was later mapped to chromosome 6 band q25.1 (Menasce, et al. 1993).

The hER is a member of the steroid/thyroid hormone family of ligand-dependent transcription factors, which includes the thyroid receptor (TR), the androgen receptor (AR), the progesterone receptor (PR), the glucocorticoid receptor (GR), and the vitamin D receptor (VDR) (Evans 1988). The gene coding members of this superfamily have six distinct functional domains, designated A through F (Figure 1.6) (Beato, et al. 1995).

The N-terminal (A/B) domain of the nuclear receptor family is highly variable in sequence and length and usually contains a transactivation function, AF-1, which activates target genes by directly interacting with either components of the core transcriptional machinery, or coregulators that mediate signaling to downstream effectors. The AF-1 region of the ER has been implicated in ligand-independent activation.

The highly conserved DNA-binding domain, DBD, or (C) region contains two type II zinc fingers; the first of which is thought to confer binding specificity, while the second zinc finger is considered to be responsible for stabilizing binding to DNA through its interaction with the phosphate backbone on DNA (Umesono and Evans 1989; Zilliacus, et al. 1995). The C region also plays a role in hER dimerization when the receptor is bound to DNA (Schwabe, et al. 1993). The nuclear localization sequence for the hER is located downstream of the C region in the variable hinge region (D). The hinge region allows the hER protein to

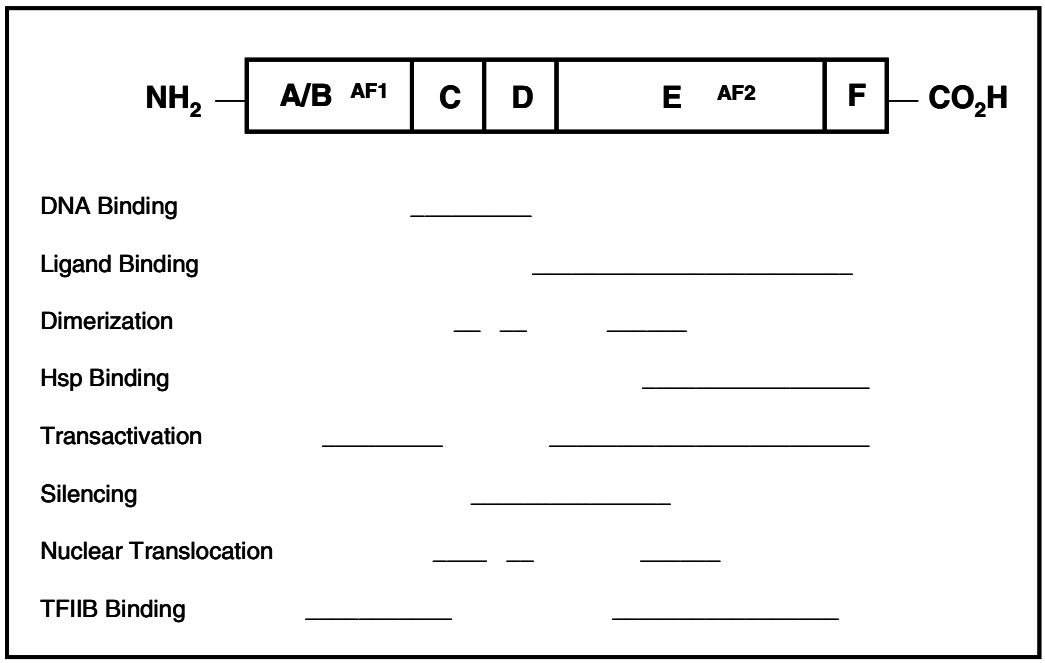

Figure 1.6. Schematic Representation of the Functional Domains of ERα. Members of this receptor family contain six functional domains, termed A-F. The location of the DNA binding (DBD) and ligand binding (LBD) domains are indicated. The positions of the activation function domains (AF-1 and AF-2) and the sites involved with dimerization are also shown. The nuclear localization and the hinge region are found in the D domain. Heat shock proteins and coactivators interact with the receptor through the E domain.

bend and/or alter its conformation on DNA in the presence of ligand(Kumar, et al. 1987; Picard, et al. 1990).

As compared to other domains, the ligand-binding domain, LBD, or (E) region is the largest (~250aa) and least conserved domain of the nuclear receptor family. This domain contains regions important for ligand binding, homodimerization, nuclear localization, activating function-2 (AF-2) transactivation, interaction with heat shock proteins such as HSP90 (in the absence of ligand) and interactions with coregulators (in the presence of ligand) (Glass, et al. 1997; Pratt 1998; Pratt and Toft 1997). The AF-2 region of the ER is directly activated in response to the binding of a ligand, and is therefore considered to be activated in a ligand dependent manner. Finally, at the C-terminal end of the receptor is the variable (F) region, for which no specific function has been identified, other than contributing to the transactivating capacity of the receptor (Enmark and Gustafsson 1999). The deletion of this region does not affect the function of the hER, as determined by reporter assays (Kumar et al. 1987).

Transcriptional activity of the estrogen receptors is mediated through two distinct domains termed activation function-1 (AF-1), located in the A/B domain, and activation function-2 (AF-2), located in the E domain. The AF-1 domain can influence transcription independently of ligand (Pham, et al. 1992; Tora, et al. 1989). In contrast, the activity of the AF-2 domain is ligand dependent (Pham et al. 1992; Tora et al. 1989). The function of AF-1 and AF-2 depends on several factors including the type of cell and promoter (Tzukerman, et al. 1994). The AF-1 region is also the site for ligand-dependent phosphorylation and may play a role in regulating ER transactivation by affecting the interaction of proteins involved with regulating ER transcription (Ali, et al. 1993; Le Goff, et al. 1994). Recently, a third

region with transcriptional activation activity (AF-2a), which functions without activation by ligand or external stimuli, was found in the N-terminal region of the E domain (Norris, et al. 1997).

Crystal structure of the ligand-binding domain

Important insight into the response of the ER to various compounds has come from studies of the ligand-binding domain (LBD) utilizing X-ray crystallography. These studies revealed that the ER LBD is similar to those described for other members of the nuclear receptor superfamily (Renaud, et al. 1995; Wagner, et al. 1995; Wurtz, et al. 1996). Studies on the ER revealed that its LBD consists of 12 α-helices (H1-H12), and a β-sheet forming a hydrophobic ligand-binding pocket. The α-helices of the ER LBD are arranged into a three-layer structure. The central portion of the structure is composed of helices 5, 6, 9, and 10, and is sandwiched between helices 1-4 and helices 7, 8, and 11 (Brzozowski, et al. 1997). The overall structure is conical with the ligand-binding interface located within a narrow portion of the pocket. Helix 12 is near the binding interface but points away from the conical structure. Upon estrogen binding, the LBD undergoes major conformational changes, resulting in the creation of a dimerization surface. This dimer interface consists of amino acid residues from H7, H8, H9, and H10/11(Tanenbaum, et al. 1998). During this conformational reorganization, helix 12 closes the binding pocket and creates a site where coactivators can interact with AF-2. Thus, ligand-induced repositioning of H12 appears to influence the recruitment of coactivators.

Many anti-estrogens, including Tamoxifen and Raloxifen, are able to bind within the same pocket of the LBD as 17β-estradiol. However, the resulting conformational change of

the LBD is different from that induced by 17β-estradiol, with the major difference being the positioning of H12. In the presence of antagonists, Tamoxifen and Raloxifene, helix 12 is displaced from the binding pocket, disrupting the site where coactivators interact with AF-2 (Brzozowski et al. 1997; Shiau, et al. 1998). This raises the possibility that this conformation of the LBD and particularly the positioning of H12 is determined by the chemical bound to the LBD.

Estrogen receptor subtypes

A second ER subtype, ERβ, was described in 1996 and the gene coding for it was mapped to chromosome 14 band q22-24 (Enmark, et al. 1997; Kuiper, et al. 1996). ERβ contains 530 amino acids with a predicted molecular weight of 59.2 kDa. The estrogen receptor beta (ERβ) shares significant homology with its counterpart hER, now known as ERα. Despite the homology between the receptors, two different chromosomes encode the receptors, which rules out the possibility that they are splice variants.

As shown in Figure 1.7, the gene for ERβ also has six domains comparable to those of the steroid/thyroid hormone family of ligand-dependent transcription factors, including ERα. There is remarkable conservation of the DNA-binding domain (DBD) between the two estrogen receptors, which suggests that they should be able to bind similar sequences within the promoters of estrogen-induced genes. The least conserved domain when comparing the two ER subtypes is the ligand-binding domain (LBD), which has 55% homology. This suggests that the receptors may bind ligands with different affinities. ERβ also lacks an efficient AF-1 region, which influences the interaction with coactivators that affect ligand responsiveness of the receptor (Hall and McDonnell 1999).

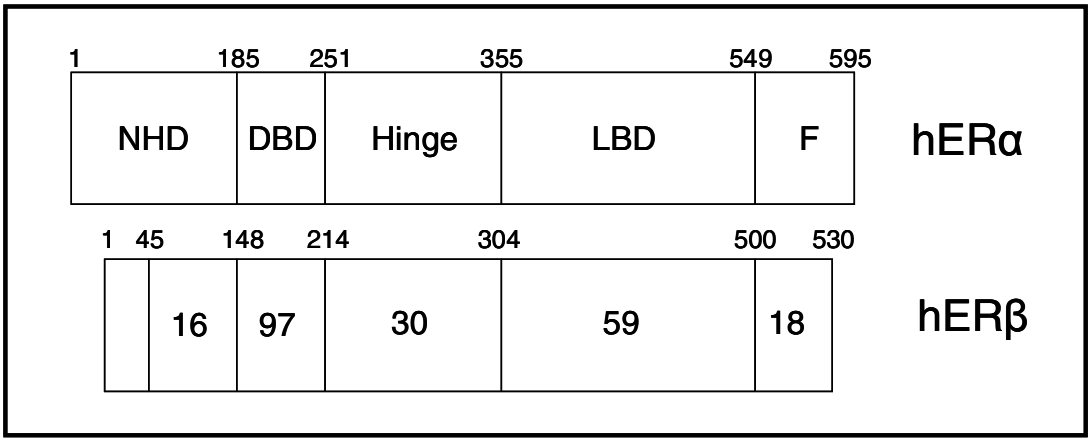

Figure 1.7. Schematic representation of the functional domains of ERα and ERβ. As members of the steroid/thyroid hormone family of receptors, the ERs are divided into six functional domains termed A-F. ERα is composed of 595 amino acids, while its counterpart ERβ is composed of 530 amino acids. Each functional domain is indicated in the boxes within ERα (See figure 1.6 for the names of the domains) and the percent homology between the receptors is indicated in ERβ. The DNA binding domain maintains significant homology between the receptors, while the ligand binding domain and N-terminal domain share less homology.

The physiologic relevance of ERβ is less well understood than its counterpart ERα. While ERα is abundantly expressed in estrogen responsive female tissues such as the ovaries, uterus, vagina, and mammary glands, ERβ appears to be highly expressed only in the ovaries.

Insight into the roles of the ERs from mouse models

Tremendous insight into the distinct roles of the estrogen receptor subtypes have come from the use of mouse models. In the immature uterus of wild-type mice, ERα and ERβ are equally expressed in both the epithelial (luminal and glandular) and stromal compartments. However, when these mice are treated with estrogen there is a decrease in ERβ expression within the stromal compartment, suggesting that estrogen negatively regulates this receptor in the mouse (Weihua, et al. 2000). As mice mature, ERα mRNA and protein become highly expressed in the uterine epithelial, stromal, and smooth muscle compartments. On the other hand, ERβ mRNA and protein are expressed at much lower levels than its counterpart within the same uterine compartments, suggesting that ERα is the dominant receptor in the mature tissue (Couse, et al. 1997; Shughrue, et al. 1998). When stimulated with estrogen, the wild-type rodent uterus reveals an induction in DNA synthesis and an increase in gene expression of estrogen responsive genes such as the progesterone receptor (PR), lactoferrin, and glucose-6-phosphatase dehydrogenase (Couse, et al. 1995; Kastner, et al. 1990; Liu and Teng 1992).

Data obtained from the use of mouse gene knockout technology has provided considerable insight into the distinct roles of the estrogen receptors in the rodent uterus. Dr. Oliver Smithies' and other laboratory groups have generated mice that carry null mutations in either or both ER subtypes in an effort to identify the roles of each receptor in normal

rodent physiology. A brief description of the phenotypic expression and general conclusions into the roles of both receptors elucidated from these studies is presented here.

The first ER knockout mice to be developed were those with null mutations in the ERα gene (ERαKO). These mice develop uteri, which contain the epithelial, stromal, and myometrial compartments (Lubahn, et al. 1993). However, the compartments within the organ are diminished in size and insensitive to estrogens, as they fail to induce DNA synthesis and estrogen responsive genes in response to pharmacologic doses of estrogen or a synthetic agonist, diethylstilbestrol (DES) as seen in wild-type littermates (Lubahn et al. 1993). The ERαKO mice are infertile, suggesting an important role for proper ERα signaling within the female reproductive tract. This finding suggests that ERα is an important mediator of both normal cellular proliferation and estrogen-mediated gene regulation within this organ.

ERβ knockout (ERβKO) mice develop normal uteri containing epithelial, stromal, and myometrial compartments (Krege, et al. 1998). However, unlike the ERαKO mice ERβKO mice maintain hormonally responsive uteri. These mice exhibit an increase in Ki-67 protein, a cell proliferation marker, and an exaggerated response to estradiol, suggesting that ERβ may play an important role in modulating the effects of ERα and may also provide an antiproliferative function in the immature uterus (Walker and Korach 2004). The ERβKO mice are considered subfertile as they have a reduction in the frequency and size of litters, as compared to wild-type littermates. Subfertility in these mice is not thought to be linked to the lack of ERβ activity in uterine function; rather, it is thought to be mediated by the loss of the receptor protein expression and activity in the ovary.

More recently, a group of mice was developed with an ERα and ERβ double knockout (ERαβKO) genotype. Mice containing the double knockout are viable and possess

an exaggerated ERαKO phenotype (Dupont, et al. 2000). It was noted that the mice have developed uteri with a reduction in the diameter and myometrial thickness of the organ. Despite carrying the normal uterine structures, ERαβKO mice are infertile indicating the importance of both ER subtypes in normal rodent uterine biology and reproduction.

Taken together, studies using the ERKO mice demonstrate specific phenotypes in the absence of either or both receptor subtypes within the uteri of mice. In ERαKO mice, which only express ERβ, investigators noted hypoplastic uteri that were insensitive to estradiol, suggesting that ERα is an important mediator of both cellular proliferation and estrogen mediated signaling within this tissue. On the other hand, increased cell proliferation and exaggerated response to estradiol in ERβKO mice, which express only ERα, suggests that ERβ may play an important role in modulating the effects of ERα and may also provide an antiproliferative function within the uterus (Walker and Korach 2004). Additionally, ERα can serve some essential roles of ERβ in fertility, but ERβ cannot substitute for ERα as ERαKO mice are infertile.

Stromal ERα is essential for 17β-estradiol induced epithelial proliferation

Over the past decade our understanding of the proliferative response of the epithelium of the uterus and prostate to mitogenic signaling from steroid hormones has increased tremendously (Cooke, et al. 1997; Cooke, et al. 1987). Work completed by Cooke *et. al.* (1997) provides strong evidence that the "epithelial ERα alone is neither necessary nor sufficient for uterine epithelial mitogenic response to 17β-estradiol" (Cooke et al. 1997). To reach this conclusion, stromal and epithelial cells from ERαKO mice (ko) and neonatal ER-positive wild-type (wt) BABL/c mice were used to construct tissue recombinants. The

recombinants were constructed to contain ERα in the epithelium and/or stroma, or to completely lack ERα expression: wt-stroma + wt-epithelium, wt-stroma + ko-epithelium, ko-stroma + ko-epithelium, and ko-stroma + wt-epithelium. Tissue recombinants were grown as grafts in female nude mice and treated with 17β-estradiol or vehicle.

The results revealed a similar increase in proliferation of tissue recombinants containing wt-stroma + wt-epithelium, and wt-stroma + ko-epithelium. This finding indicates that stromal cells release paracrine factors in response to estrogen, which lead to increased epithelial growth, even when the epithelium lacks ER expression. Furthermore, tissue recombinants containing ko-stroma + ko-epithelium, and ko-stroma + wt-epithelium, failed to induce proliferation in response to 17β-estradiol, despite the fact that the wt-epithelial cells have ERα expression.

The tightly regulated paracrine mediated response to steroid hormones in the normal uterus appears to be absent in disease settings, including hyperplasia and cancer. Rather than responding to paracrine-mediated factors emanating from stromal cells, epithelial cells begin to respond directly to 17β-estradiol. In fact, the epithelium begins to release paracrine factors that diminish the stromal compartment, indicating that the normal tissue microenvironment has been drastically altered. The exact mechanism of this change is not well defined within this tissue. However, I will provide a plausible mechanism whereby the stromal cells lose control and the epithelial cell gain the ability to respond to estrogens in Chapter II of this dissertation.

Decreased ERβ expression in cancer

Recent studies have shown alterations in the ERα/ERβ ratio in both estrogen responsive and nonresponsive cancers. Typically, alterations were demonstrated by showing a decrease in expression of ERβ mRNA, protein, or both in tumor versus normal tissues in several cancers including breast, ovary, colon, and prostate (Bardin, et al. 2004; Brandenberger, et al. 1998; Campbell-Thompson, et al. 2001; Fixemer, et al. 2003; Foley, et al. 2000; Horvath, et al. 2001; Latil, et al. 2001; Park, et al. 2003; Pujol, et al. 1998; Roger, et al. 2001; Rutherford, et al. 2000; Zhao, et al. 2003). A second mechanism for an alteration of the ratio between the receptors is an increase in ERα mRNA, protein, or both. Regardless of the mechanism, the ERα/ERβ ratio seems to increase in both estrogen sensitive and non-sensitive tissues during carcinogenesis, suggesting distinct, and possibly divergent, roles for ERα and ERβ (Leygue, et al. 1998).

Several groups have studied this phenomenon in estrogen responsive tissues, namely the breast and ovary, and have found an overall disruption in the ratio of ERα to ERβ in disease states associated with these tissues. A group using immunohistochemistry (IHC) to study this in the human breast found a higher percentage of ERβ positive epithelial cells in normal mammary glands when compared to those found in biopsies from patients with nonproliferative Benign Breast Disease (BBD), proliferative BBD, or carcinoma *in situ* (Roger et al. 2001). This study also noted an increase in ERα protein expression in diseased specimens, and an inverse correlation of ERβ with Ki-67, a marker of proliferation; together this may indicate that a loss in expression of ERβ leads to increased cellular proliferation (Roger et al. 2001).

Complementary studies addressing the status of the estrogen receptors in ovarian tissues have found alterations in the expression of ERβ mRNA and protein to be associated with ovarian disease. Normal ovaries express higher levels of ERβ protein than ERα, indicating that ERβ is the dominant receptor in this tissue (Kuiper et al. 1996). Data from many groups suggest that a proper balance between the two receptor subtypes is essential for the maintenance of normal ovarian function. Comparing biopsies of normal and metastatic ovarian cancer, one group demonstrated that both ERβ protein expression, as measured by Western immunoblot analysis, and mRNA were absent in samples of metastatic ovarian cancer (Rutherford et al. 2000). This finding suggests that a reduction or loss of the ERβ receptor is common in the development of metastatic ovarian disease.

In the human colon, which is generally thought to be an estrogen insensitive organ, epidemiological studies suggest that estrogens may decrease the risk of developing cancer (Calle, et al. 1995; Grodstein, et al. 1999). ERβ is expressed at higher levels in the colon than its counterpart ERα, again suggesting that ERβ is the dominant subtype in this tissue. Therefore, the protective effects of estrogen in this tissue may be mediated through the β receptor subtype. Using IHC and Quantitative Real Time-PCR (qPCR) several studies found that ERβ mRNA and protein expression decrease significantly in colon adenocarcinoma compared to their expression in the neighboring normal colonic epithelium (Campbell-Thompson et al. 2001). Furthermore, evidence suggesting a progressive decline in ERβ expression, which paralleled the loss of malignant colon cell differentiation, has been suggested by one group (Konstantinopoulos, et al. 2003).

In the development of prostate cancer, which has classically been thought to be an androgen mediated process, evidence now suggests that estrogens are important mediators in

the initiation and/or progression of the disease (Jarred, et al. 2000). A recent study found ERα and ERβ mRNA in all primary cultures of normal human prostate epithelial cells (Pasquali, et al. 2001). However, when primary malignant epithelial cells were assayed for expression of the ERs, the group found that only 17% of samples of cancer maintained expression of ERβ. Further studies used Western immunoblot analysis to determine the protein expression of the receptors within the same primary cultures. Ultimately, the findings suggested that expression of ERβ, and not ERα, is lost in primary cultures of malignant prostate epithelial cells.

Taken together, studies completed in the ovary, breast, colon, and prostate all suggest a decrease in ERβ mRNA and protein expression during carcinogenesis. An exact mechanism for a reduction or loss of ERβ has not been proposed or evaluated in great detail. However, these studies strongly suggest a protective role for ERβ against the mitogenic activities of estrogens in estrogen responsive and non-responsive tissues.

Literature on this phenomenon in the human endometrium is scarce and all fail to include hyperplastic tissue samples, which are precursors for type-1 endometrial cancers (Fujimoto, et al. 2002; Mylonas, et al. 2005; Utsunomiya, et al. 2000). Moreover, these studies tend to favor evaluating mRNA levels by *in situ* hybridization or the reverse transcriptase-polymerase chain reaction (RT-PCR), as opposed to evaluating the levels of receptor protein by IHC. Fujimoto *et. al.* (2002) provided results suggesting that there was no change in the ratio of the receptors (ERα to ERβ) between normal and malignant samples (Fujimoto et al. 2002). However, the same group found that the ratio in metatstatic lesions was significantly higher than in primary cancers. A separate study by Mylonas *et. al.* (2005) suggests that the ratio decreases significantly from normal to malignant, which indicates that

this change involves an increased proportion of ERβ (Mylonas et al. 2005). The final study evaluating the expression of the ER receptors was completed by Utsunomiya, *et. al.* (2000). This study used IHC, RT-PCR, and *in situ* hybridization to evaluate the levels of the receptor subtypes in normal and malignant samples. The results from this study revealed an increase in the mRNA levels of both receptors in the cytoplasm of carcinoma cells compared to normal cells (Utsunomiya et al. 2000). Thus, the primary concerns with previous studies are two-fold: first, they omit a critical step in endometrial cancer progression, and second, the data from each study seems to be inconsistent. The issue of ERβ expression in the progression of type-1 endometrial cancers will be addressed further in Aim 1 of this dissertation.

Reduction of proliferation by the estrogen receptor β

Although the specific actions of ERβ in cancer are not known, considerable evidence obtained from ERKO mice and the reduction/ablation of its protein in human cancer progression suggests that this receptor may have inhibitory effects on gene regulation and cellular proliferation. Recent work completed in MCF-7 and T47D, two ERα positive breast cell lines, suggest that introduction of an ERβ gene construct into cells inhibits breast cell proliferation and gene transcription (Paruthiyil, et al. 2004; Strom, et al. 2004). One group produced stable transformants of MCF-7 cells expressing ERβ or ERβcx, an isoform that is truncated at the C-terminal region. In this study, expression of either ERβ construct resulted in a reduced S phase population as compared to the parental cells (Omoto, et al. 2003). In an effort to elucidate the mechanism for these actions, several groups provide evidence that ERβ may reduce proliferation by inhibiting the cyclin D1 gene, a key mediator of the G_1-S

transition of the cell cycle (Strom et al. 2004). In fact, luciferase and transient expression studies conducted in HeLa cells, a cervical cancer cell line, suggested that treatment with 17β-estradiol increased cyclin D1 through ERα and decreased its expression through ERβ (Liu, et al. 2002). Furthermore, when both receptors were transiently expressed, ERβ maintained its ability to negatively regulate the cyclin D1 gene. Another laboratory evaluating proteins in breast cells by Western immunoblot analysis implicated negative regulation of several other proteins involved in regulation of the cell cycle, e.g. cyclin E and Cdc25A, by the ERβ (Strom et al. 2004). Additionally, it was reported that stable transformants expressing ERβ showed a reduction of cathepsin D and IGFBP4, two estrogen responsive genes, indicating that ERβ modulates ERα activity in breast cancer cell lines.

ER actions

Steroid receptor coactivators

Coactivators are proteins that bind to members of the steroid/thyroid hormone family of ligand-dependent transcription factors and modulate their functional activities. Over the past several years, the identification of coactivators has revolutionized our understanding of nuclear receptor action. Receptors, including the ER, glucocorticoid receptor (GR), and the progesterone receptor (PR), interact with specific coregulators (Robyr, et al. 2000). In fact, it was found that ER, PR, and GR could inhibit each other's transactivation when coexpressed in mammalian cells, suggesting that these receptors compete for functionally limiting amounts of transcription factors (Meyer, et al. 1989). In an attempt to identify these factors, the hER ligand-binding domain (LBD) was fused to glutathione-S-transferase (GST) and bound to glutathione affinity matrix (Cavailles, et al. 1994; Halachmi, et al. 1994; Hanstein,

et al. 1996). These studies identified a new class of hER binding proteins from mammalian cells with molecular weights of 140 KDa (RIP 140), 160 kDa (ERAP 160), and 300 kDa (p300). These proteins bound to the ER-LBD in the presence of ligand and in an AF-2 (ligand-dependent) manner (Glass et al. 1997). Further studies using the glucocorticoid receptor (GR) bound to a GR response element identified proteins from nuclear extracts that associated with the receptor (Eggert, et al. 1995). These proteins were termed GR-interacting proteins (GRIPS). Subsequently, Thyroid hormone Receptor (TR)-associated proteins (TRAPS) were identified by immunoprecipitation of an epitope-tagged TR (Fondell, et al. 1996). Some coactivators were also shown to be recruited in a ligand-independent manner by the AF-1 domain of the ER (Fondell et al. 1996; Tremblay, et al. 1999). The interaction between the nuclear receptors and coregulators has been linked to the LXXLL interacting motifs (where L is Leucine and X is any amino acid) of the coregulators with the AF-2 region of the receptor. Coactivators are believed to enhance transactivation of the nuclear receptors by bridging the receptor with the basal transcription machinery of the cell.

The p160 family of coactivators has been strongly implicated in the transcriptional activity of the ER. At least three subclasses of coactivators have been identified within the p160 family and are grouped based on their sequence homology: *SRC1* (steroid receptor coactivator-1)/NcoA-1 (Hong, et al. 1997; Onate, et al. 1995; Torchia, et al. 1997; Voegel, et al. 1998), *TIF2* (transcriptional intermediary factor 2)/GRIP1/NcoA-2 (Hong et al. 1997; Kim, et al. 1998; Voegel et al. 1998) and *AIB1* (advanced in breast cancer 1)/ACTR/pCIP/xSRC-3 (Anzick, et al. 1997; Chen and Evans 1995; Jenster 1998; Kim et al. 1998; Torchia et al. 1997). Coregulators function at the molecular level through enzymatic activities. Evidence suggests that *SRC1* and *AIB1* of the p160 family possess histone acetyl

transferase (HAT) activity *in vitro* (Jenster 1998; McKenna, et al. 1999). In addition, the p160 family members bind and recruit other coactivators with HAT activity, i.e. p300. HAT activity acetylates histones which assist in euchromatization, or "relaxation," of the chromatin structure. This change permits the basal transcription machinery to have access to the site, eventually leading to transcription or increased transcription.

Estrogen-response elements

After being activated by its ligand, the activated ER binds to estrogen-response elements found within the promoter of target genes. This binding induces transcription or repression of the gene. The classic ERE sequence was first discovered in the *Xenopus laevis* vitellogenin A2 promoter and was found to be composed of two palindromic half-sites separated by three nucleotides, 5'AGGTCAnnnTGACCT3', where n can be any nucleotide (Klein-Hitpass, et al. 1986). To date, the only human estrogen responsive genes that contain "perfect" response elements are EBAG9, COX7A2L, and EFP[a]/ZNF-147 (Bourdeau, et al. 2004; Ikeda, et al. 2000; Inoue, et al. 1993; Klinge 2001; Watanabe, et al. 1998).

More recently, a new subclass of response elements have been identified which appear to have diverged from known *Alu* sequences. The *Alu* family of short interspersed DNA elements are the most abundant mobile elements distributed throughout the human genome. McDonnell *et. al.* (2000) found that *Alu* elements have the potential to acquire the ability to function as estrogen receptor-dependent enhancers (Norris, et al. 1995). Generally speaking, the *Alu* responsive elements that confer estrogen responsiveness to a specific region generally contain one perfect ERE half-site.

Current model of the ER pathway

When not bound by its ligand, the estrogen receptor is located in the nucleus and is part of a multiprotein complex with several chaperone proteins, including heat shock proteins HSP90 and HSP70, p23, and immunophilins which serve to stabilize and/or mask the DNA binding domain of the receptor (Graumann and Jungbauer 2000; Pratt 1998; Pratt and Toft 1997; Smith and Toft 1993). When estrogen enters a responsive cell, it binds to the ligand-binding domain of the receptor, which undergoes a conformational change that results in the dissociation of the chaperone proteins (Pratt and Toft 1997). The release of the chaperones exposes the dimerization and AF-2 transactivation domains present in the E domain (Beekman, et al. 1993). As a result of a yet unknown mechanism, the estrogen/ER complexes form functionally active dimers that become-phosphorylated at specific amino acid residues within the AF-1 domain (Smith 1998). The activated ER dimers then bind specific sequences, estrogen response elements (EREs), within the promoters of target genes and initiate transcription or repression of those genes. Homodimerization between two ERα's or two ERβ's is well-recognized and accepted. However, there is no data supporting endogenous heterodimer formation. Heterodimerization of the two receptors has been observed in artificial systems in which each receptor was overexpressed and has only been hypothesized to occur *in vivo* (Chen and Evans 1995; Cowley, et al. 1997; Hall and McDonnell 1999; Pettersson, et al. 2000; Pettersson, et al. 1997).

Estrogens also regulate the transcription of genes that lack functional estrogen response elements (EREs) by modulating the activity of other transcription factors. The effects of estrogens in this instance are due to tethering of the active ER's to transcription factors, such as AP-1 or SP-1 within the promoter region of target genes (Webb, et al. 1995).

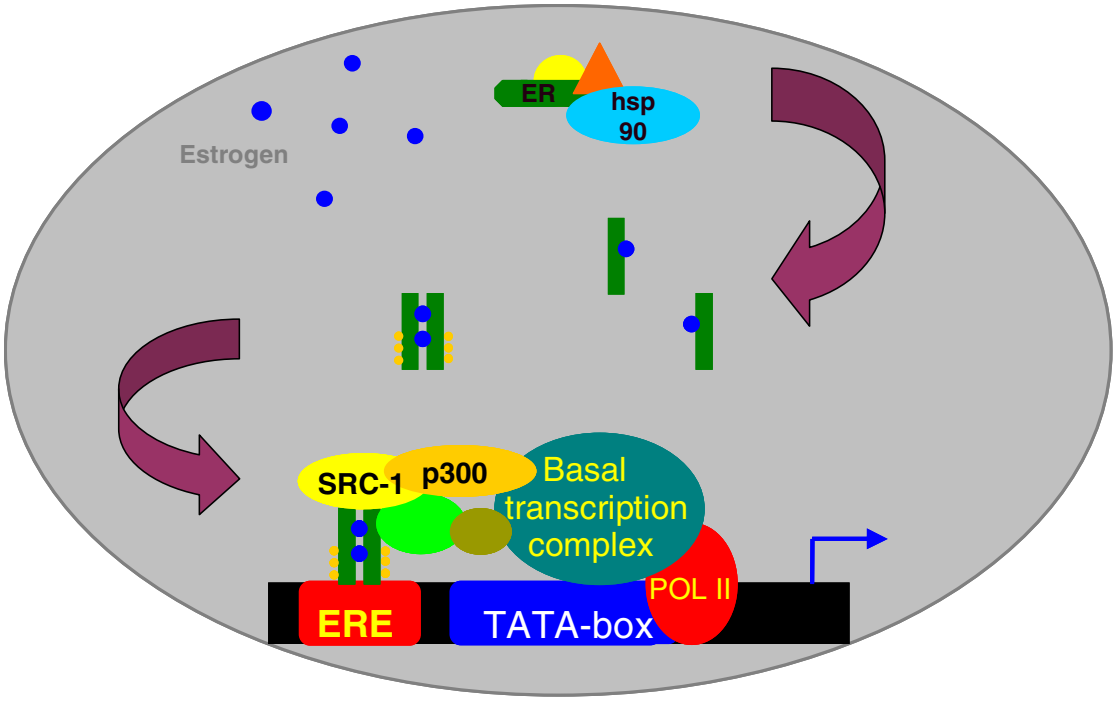

Figure 1.8. Estrogen Receptor Pathway. (1) The inactive ER resides in the nucleus as part of a protein complex that includes heat shock protein 90 (hsp 90). (2) Upon binding estrogen, the receptor dissociates from the protein complex. (3) The receptor dimerizes and becomes phosphorylated. (4) The dimer binds to specific DNA sequences termed estrogen response elements (ERE) and recruits coactivators to the promoter region of the gene. The ER regulates gene transcription by interacting with basal transcription factors and coactivators such as SRC-1 and p300.

Estrogen receptors have also been shown to interact with the nuclear factor kB (NFkB) (McKay and Cidlowski 1998; Shyamala and Guiot 1992).

Alternative pathways of the estrogen receptor

Ligand-independent activation of estrogen receptors

The ERs are phosphoproteins whose function can be altered by changes in phosphorylation in the absence of its endogenous ligand (Campbell, et al. 2001; Kato, et al. 1995; Kato, et al. 2000). Growth factors, i.e. epidermal growth factor (EGF) and insulin-like growth factor (IGF), have the ability to activate protein kinases, such as MAPK or (PI)3-kinase, which can induce estrogen-independent activities of the ERα by phosphorylating specific serine residues in the AF-1 region of the receptor.

Evidence supporting ligand-independent activation of the ER stems from an experiment showing that EGF induction of DNA and lipid synthesis in the uterus could be prevented by the anti-estrogen ICI 164384 (Kato et al. 2000). The elucidation of the mechanisms behind the crosstalk between these two independent receptors came from the observation that molecular events, i.e. activation of MAP-Kinase by EGFR complexes, could lead to phosphorylation and activation of the ERα at Ser^{118}, which is located in the AF1 domain of the protein (Bunone, et al. 1996; Kato et al. 1995; Kato et al. 2000). Phosphorylation at Ser^{118} results in activation of genes that are regulated by ER and not EGFR. Further data implicates activation of (PI)3- kinase by the EGFR complexes, which in turn phosphorylates ERα on Ser^{167} in the AF1 domain leading to activation of the ER(Campbell et al. 2001; Martin, et al. 2000).

Crosstalk between the ER pathway and growth factor pathways

The ligand-dependent estrogen-signaling pathway takes minutes or hours to increase protein synthesis by transcriptional activation. Recent data in the literature suggests that estradiol has other effects that cannot be explained by the ligand-dependent mechanism due to their rapid onsets. Many of these effects, deemed non-classic, have been linked to cell-surface forms of ER, which are thought to resemble nuclear ER (Kato et al. 1995; Song, et al. 2002; Watson, et al. 1999). There is a body of evidence that links estrogen cell surface receptors and activation of the mitogen activated protein kinase (MAPK) signaling cascade (Song et al. 2002; Song, et al. 2005). There have also been reports that link estrogen cell surface receptors with the rapid activation of the phosphatidylinositol (PI)3-kinase pathway (Stoica et al. 2003). The non-classic action of the ER provide a direct connection between estrogen and stimulation of anti-apoptotic/pro-cell proliferation pathways that have been strongly implicated in carcinogenesis.

Differential activation of the estrogen receptors

The development of several non-steroidal compounds, which have been found to be highly selective for one ER subtype over the other, have the potential to provide important insight regarding the differences between the functions of the two estrogen receptors *in vivo* and *in vitro* (Meyers, et al. 2001; Stauffer, et al. 2000). Non-steroidal compounds were synthesized and characterized as selective agonists or antagonists for ERα or ERβ based on their binding affinity and ability to transactivate gene constructs containing consensus EREs.

The first compound, diarylpropionitrile (DPN), is an agonist that has a selective effect on the ERβ (Meyers et al. 2001). It has been shown that DPN has a 100-fold preference for

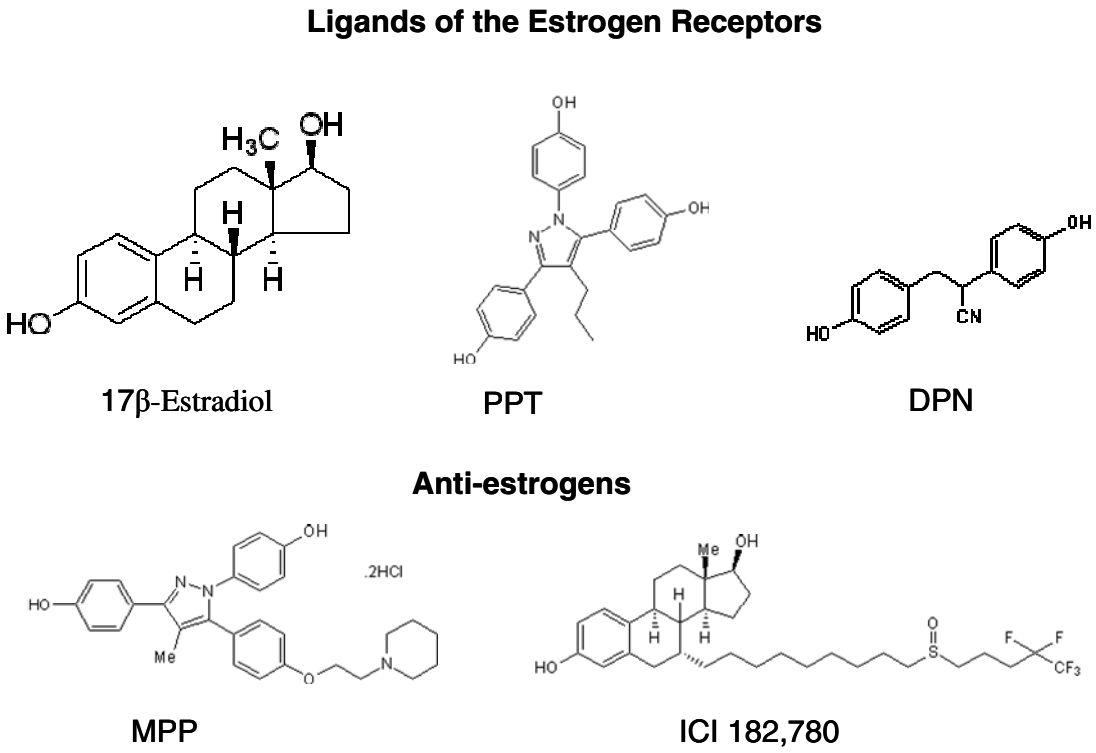

Figure 1.9. Molecular Structures of ER Agonists and Antagonists. Ligands of the ER used within this dissertation are 1) 17β-estradiol (estrogen), an agonist of both ER subtypes, 2) Propylpryazole-triol (PPT), an agonist of the ERα, and 3) Diarylpropionitrile (DPN), an agonist of ERβ. Anti-estrogens used within the studies herein are 1) Methyl-piperidino-pyazole (MPP), a specific inhibitor of ERα, and 2) ICI 182780, a potent inhibitor of both ERs.

ERβ over ERα (Harrington, et al. 2003). The second compound, propylpryazole-triol (PPT), is an agonist that was found to have a 400-fold preference for ERα over ERβ (Kraichely, et al. 2000). The ERα selectivity of PPT depends on its interaction with several regions of the ligand-binding domain (Harrington et al. 2003). Lastly, methyl-piperidino-pyazole (MPP) is a highly selective ERα antagonist. The basic side chain responsible for the activities of this compound is the same side chain found in Raloxifene, an ER antagonist. MPP has a 200-fold higher affinity for ERα over ERβ and is thus considered a highly selective, potent ERα antagonist.

Hypothesis and specific aims of this dissertation

Unopposed estrogens are considered to be the primary etiologic risk factor for developing type-1 endometrial cancer. The mechanisms of action of estrogen receptors in the human uterus are complex and not completely understood. However, evidence generated using mouse gene knockout models has provided tremendous insight into the divergent roles of the receptors in the uteri of rodents. Nevertheless, the function of the receptors in the mouse uterus may not completely correspond with their roles in the human. Therefore, it is imperative that the roles of each receptor be elucidated in human endometrial cells in an effort to understand the contributions of each receptor to estrogen-induced disease, particularly cancer.

Recent evidence suggests that the "normal" ERα/ERβ ratio is altered during human breast, ovarian, colon, and prostate cancer development. Because of similarities of endometrial cancer to these other neoplasms it is reasonable to suspect that a similar mechanism may apply. If this change also occurs in the endometrium it may lead us to a

mechanism whereby cells bypass the normal homeostatic constraints to induce proliferation, prevent apoptosis or both. Further observations accumulated using *in vitro* assays support the idea that a reduction of ERβ can lead to increased mitogenic signaling in response to 17β-estradiol. In these studies, introduction of ERβ attenuates 17β-estradiol's mitogenic signaling through ERα by direct or indirect mechanisms. Attenuation via the direct mechanism involves heterodimerization of the ERs. This dimer formation leads to a reduction in estrogen-dependent transactivation of estrogen-responsive luciferase constructs when compared to ERα homodimers (Cowley et al. 1997; Matthews and Gustafsson 2003). The indirect method involves recruitment of ERβ homodimers to the promoters of estrogen-responsive genes, which prevents mitogenic ERα homodimers, or ERα/ERβ dimers from inducing estrogen-dependent transcription. On the basis of these findings, I hypothesize that ERβ is a modulator of the activity of ERα in the human endometrium. If this is true, we should note a decrease in activity of estrogen-inducible genes, like placental alkaline phosphatase (ALPP), and proliferation when ERβ is introduced into endometrial epithelial cells.

Hypothesis 1. The ERα/ERβ ratio is altered as the human endometrium transitions from a normal to a neoplastic condition.

To test this hypothesis, I have:

i. Created Tissue Microarrays (TMA's) containing normal, hyperplastic, or neoplastic human endometrial biopsy samples.

ii. Stained separate TMA's with antibodies against the estrogen receptors α and β.

iii. Observed and quantified the expression of both estrogen receptor subtypes.

Hypothesis 2. ERα activation increases ALPP gene expression in human endometrial epithelial cells, while ERβ activation decreases ALPP expression.

To test this hypothesis, I have:

i. Used highly specific agonists of the ER's in the Ishikawa model system to elucidate the roles of each receptor in ALPP expression.

ii. Used the isogenic Ishikawa cell lines expressing ERα, ERβ, or both ER's to understand the role of both receptors on ALPP gene expression.

iii. Located EREs within the ALPP promoter and used Chromatin Immunoprecipitation (ChIP) Assays to determine if the receptor subtype(s) bind this region.

Hypothesis 3. ERα activity increases proliferation of human endometrial epithelial cells while ERβ activity acts as an inhibitor of proliferation.

To test this hypothesis, I have:

i. Used highly specific agonists of the ER's in the Ishikawa model system to elucidate the roles of each receptor in cell proliferation.

ii. Used an isogenic Ishikawa cell line, IK-3H12, which lacks expression of either ER subtype, I will create stable cell lines expressing one or both ER's.

iii. Used these stably transformed cell lines to distinguish the roles of each receptor in the proliferative responses of the endometrium.

iv. Evaluated the cell cycle progression of the IK-3H12 expressing either ERα or β in response to 17β-estradiol.

v. Evaluated estrogen-induced apoptosis by expressing ERα, ERβ or both in the IK-3H12 cells.

Chapter II. Determine the Relative Levels and Expression Patterns of ERα and ERβ in Normal, Hyperplastic, and Malignant Endometria

Abstract

Although estrogen is the leading etiologic factor in endometrial cancer, the mechanistic role of estrogen and its receptors (ERs) in endometrial carcinogenesis are not thoroughly understood. In the normal human endometrium, ERα and ERβ are expressed in the epithelial, stromal, and myometrial compartments. However, ERβ expression tends to be lower than that of ERα in the epithelial compartment. Since the loss of functional ERβ expression in the uterus is correlated with an exaggerated response to estrogen in estrogen-receptor beta knockout (ERβKO) mice, the work presented here investigated patterns of ERα and ERβ expression in normal, hyperplastic, and malignant human endometrial tissue samples. High density tissue microarrays were constructed using 29 cycling endometria (15 proliferative and 14 secretory), 29 hyperplastic endometria (15 complex and 14 complex with atypia), and 29 type-1 endometrioid cancer biopsies. ER expression was assayed by immunohistochemistry (IHC) using high-density tissue microarrays (TMAs) and Dako antibodies against the ERs. Epithelial and stromal cell nuclear and cytoplasmic ERα and ERβ immunostaining were assessed semiquantitatively on a scale ranging from 0 (no immunostaining) to 3 (strong immunostaining) in 100 nuclei or cells, yielding a score ranging from 0 to 300 for each core. The results contained herein reveal alterations of the

normal ERα/ERβ ratio in both the stromal and epithelial compartments of hyperplastic and neoplastic tissues. Specifically, alterations found in hyperplastic samples lead to an increase in ERα expression over that of ERβ. An alteration within the stroma of hyperplastic endometrial samples was observed as ERα expression remained constant (p=.6660), compared to normal, while ERβ expression decreased (p=.0001). Unlike the stromal compartment, the epithelium revealed an increase in ERα expression (p=.0001), compared to control, while ERβ expression remained constant (p=.5029), again indicating a mechanism whereby ERα expression is dramatically increased over ERβ. Furthermore, epithelial cells from malignant samples revealed a significant increase (p=.0005 and p=.0001) in the immunostaining of ERα and ERβ, respectively. Collectively, the findings described herein indicate alterations in the normal ERα/ERβ ratio in the hormonally responsive compartments of the endometrium. The observed alterations, specifically those in the hyperplastic samples, may provide a key mechanism which promotes estrogen-mediated carcinogenesis.

Introduction

Endometrial cancer is the most common gynecologic malignancy in the United States. According to the American Cancer Society approximately 41,200 women will be diagnosed and 7,350 women will die as a result of this disease in 2007. Approximately ninety percent of endometrial tumors arise from the luminal and glandular epithelium, while the remaining ten percent arise from stromal cells. Unopposed estrogen exposure is the primary etiologic risk factor for the development of endometrial hyperplasia, a precursor for Type-1 endometrial cancer (Montgomery et al. 2004; Rose 1996). The physiologic effects of estrogens are mediated through two distinct nuclear receptors called the estrogen receptor

(ER) α and β. The response to estrogens, in the normal endometrium, is a tightly regulated process involving the tissue microenvironment, specifically stromal and epithelial cells. However, in estrogen-dependent hyperplasia and cancers, there is a disruption of the normal regulatory interactions of the microenvironment thereby permitting epithelial cells to respond directly to steroid hormones (Bissell, et al. 1999). To understand the mechanisms of estrogen-dependent endometrial disease, it is imperative to uncover the individual contributions of the ERs within the microenvironment.

Over the past decade, our understanding of the proliferative response of the uterine epithelium to mitogenic signaling from steroid hormones has dramatically increased (Cooke et al. 1997). *In vitro* assays studying interactions between stromal and epithelial cells from the endometrium, breast, prostate, and testis revealed that paracrine factors released from normal stromal cells regulate the growth of normal epithelial cells when grown in coculture or when media taken from stromal cells grown in monoculture is introduced into cultures of epithelial cells growing in monoculture (Arnold, et al. 2001; Fong, et al. 1992; McGrath 1983; Skinner and Fritz 1985). Moreover, stromal cells release specific paracrine factors, in response to steroid hormones, which directly induce or repress epithelial cell proliferation (Cooke et al. 1997).

Cooke, *et. al.* (1997) provide strong evidence that the "epithelial ERα alone is neither necessary nor sufficient for uterine epithelial mitogenic response to 17β-estradiol" (Cooke et al. 1997). To reach this conclusion, stromal and epithelial cells from ERαKO mice (ko) and neonatal ER-positive wild-type (wt) BABL/c mice were used to construct tissue recombinants. The recombinants contained ERα in the epithelium and/or stroma, or to completely lack ERα expression: wt-stroma + wt-epithelium, wt-stroma + ko-epithelium, ko-

stroma + ko-epithelium, and ko-stroma + wt-epithelium. Subsequently, tissue recombinants were grown as grafts in female nude mice and treated with vehicle or 17β-estradiol.

The results revealed a similar increase in proliferation of tissue recombinants containing wt-stroma + wt-epithelium, and wt-stroma + ko-epithelium. This finding indicates that stromal cells release paracrine factors in response to estrogen, which lead to increased epithelial growth, even when the epithelium lacks ERα expression. Furthermore, tissue recombinants containing ko-stroma + ko-epithelium, and ko-stroma + wt-epithelium, failed to induce proliferation of the epithelium in response to 17β-estradiol, despite ERα expression in the wt-epithelial cells.

Studies using tissue recombinants have proved useful in our understanding of the normal response to steroid hormones. However, these studies do not address the individual contributions of ERα or ERβ in the stroma or epithelium of endometrium. Insight into the independent roles of the ERs came from studies using Estrogen Receptor Knockout (ERKO) mice (Dupont et al. 2000; Krege et al. 1998; Lubahn et al. 1993). Initial observations from these studies indicate specific phenotypes in the absence of either or both receptor subtypes within the uteri of mice. In ERαKO mice, which only express ERβ, investigators noted infertile mice with hypoplastic uteri that were insensitive to pharmacologic doses of 17β-estradiol, suggesting that ERα is an important mediator of both cellular proliferation and estrogen mediated signaling within this tissue. On the other hand, increased cellular proliferation and an exaggerated response to 17β-estradiol in ERβKO mice, which express only ERα, suggests that ERβ may play an important role in modulating the effects of ERα and may also provide an anti-proliferative function within the rodent uterus (Walker and Korach 2004). The information, in combination with the studies conducted by Cooke, *et al*

(1997) suggests that ERα in the stroma responds to estrogen and in turn releases paracine factors that induce proliferation of the epithelial compartment.

Following the phenotypic description of ERKO mice, many postulated that ERβ may be a modulator of the mitogenic effects of ERα within human tissues. If this was true, it was reasonable to hypothesize that the normal ratio between the two receptors would be altered during estrogen-dependent disease progression. With that hypothesis, several groups evaluated the expression levels of the ERs, by immunohistochemistry (IHC) and/or real time PCR (qPCR), to compare the ratio of the ERs in normal, hyperplastic, and neoplastic tissues. The described studies have been carried out in breast, ovary, prostate, and colon samples and have revealed an alteration in the "normal" ERα/ERβ ratio when normal tissues were compared against hyperplastic and malignant samples (Bardin et al. 2004; Brandenberger et al. 1998; Campbell-Thompson et al. 2001; Fixemer et al. 2003; Foley et al. 2000; Horvath et al. 2001; Latil et al. 2001; Park et al. 2003; Pujol et al. 1998; Roger et al. 2001; Rutherford et al. 2000; Zhao et al. 2003). These findings suggest that the maintenance of a "normal" ERα/ERβ ratio is critical to the normal homeostatic constraints in response to estrogens.

In summary, information derived from endometrial tissue recombinant studies, ERKO models and the ER ratio studies suggests several important points. First, there is an alteration of the normal homeostatic constraints in the endometrial microenvironment as the tissue transitions toward malignancy. Second, in the uteri of mice, ERβ may be a key modulator of the mitogenic effects of ERα. And third, an alteration of the ratio between the ERs may provide a mechanism whereby the epithelium obtains the ability to respond directly to steroid hormones. What is not known is whether there is an alteration of the ERα/ERβ ratio as the human endometrium progresses toward malignancy. Because of similarities of

endometrial cancer to the aforementioned cancers, it is reasonable to suspect that a similar mechanism may apply. Therefore, the goal of this study is to determine whether an alteration of the normal ERα/ERβ ratio takes place in endometrial microenvironment as it transitions from normal toward malignancy. The aim will be accomplished using immunohistochemistry and semiquantitative analysis to detect expression of the estrogen receptors.

A better understanding of how the epithelial compartment of the endometrium becomes hypersensitive to estrogens will yield important novel insights into endometrial carcinogenesis and may lead to the development of novel therapies for endometrial cancer or strategies to prevent malignant transformation of endometrial epithelial cells.

Materials and Methods

Patient and tissue samples. All endometrial specimens were acquired in compliance with the guidelines of the University of North Carolina at Chapel Hill Institutional Review Board (Application#: 05-PATH-866) and the Federal Health Insurance Portability and Accountability Act (HIPAA) protected health information regulations. Retrospective paraffin-embedded tissue samples were collected from the University of North Carolina Department of Pathology and Laboratory Medicine/Lineberger Comprehensive Cancer Center Tissue Procurement Core Facility following a key word search completed by Mr. Benjamin Aycock in the Office of Information Systems (OIS) within the School of Medicine. Specimens collected for the purpose of this study were stripped of patient identifiers and samples were assigned an anonymous research study number for record keeping. All research data were entered into a computer protected by password and present within a locked office. Additionally, all tissue samples were stored under appropriate conditions in locked

laboratories. A total of 87 endometrial specimens were used to create three independent tissue microarrays containing cycling, hyperplastic or malignant cores.

*Tissue microarray construction***.** Tissue microarray (TMA) technology was utilized for the proposed studies within this aim. This technology allows production of a single paraffin block containing multiple patient samples (Figure 2.1). A recent publication reported that the use of TMA technology for validating protein expression in the human endometrium maintained concordance between the TMA and whole slides (Fons, et al. 2006).

In this study, three independent high-density tissue microarrays (TMAs) were constructed using formalin-fixed, paraffin-embedded human endometrial specimens. Each TMA contained either normal cycling endometrium (proliferative and secretory), hyperplastic endometrium (complex and complex with atypia), or endometrial adenocarcinoma (endometrioid) samples.

To prepare tissue microarrays, paraffin blocks from patients identified only by study numbers assigned by the principal investigator were obtained for analysis. Six-micrometer sections were cut from "donor" paraffin blocks and stained with hematoxylin and eosin (H&E) using routine methods. Dr. Ruth Lininger, a surgical pathologist specialized in gynecologic pathology, evaluated each coded patient section and identified three representative areas of interest in each of the normal, hyperplastic, and adenocarcinoma biopsies. Representative areas of interest were encircled on each slide and 0.6mm tissue cores were excised from the corresponding sites in the paraffin donor block. The excised cores were subsequently implanted into a "recipient" paraffin block, with 1mm between each core, using a manually operated tissue microarray device (Beecher Instruments). Each TMA

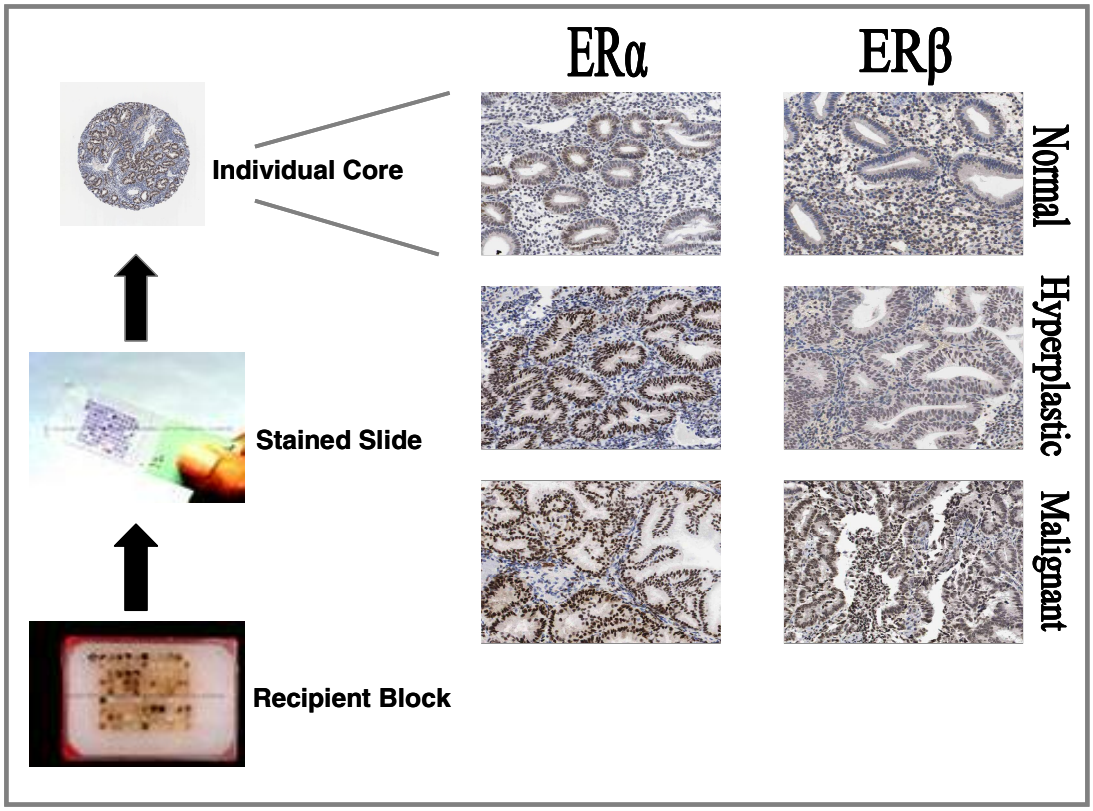

Figure 2.1. Schematic Representation of TMA Process. Recipient blocks containing multiple patient cores were sectioned, placed onto glass slides, and stained with individual antibodies against the ERs. After immunostaining, patient cores were excised digitally and stored as individual high resolution files. Examples of the digital files are located to the right of the figure.

was constructed with triplicate cores from each patient sample to produce an array containing approximately eighty-seven cores (Figure 2.1). The recipient block containing the arrayed cores was then cut into 4μm sections, transferred to glass slides and subsequently stained for ERα and ERβ.

*Immunochemistry***.** Preliminary studies to optimize primary antibody concentrations were carried out on normal and neoplastic breast sections. The unstained breast sections were deparaffinized with xylene and rehydrated through a series of graded alcohols. Antigen retrieval was carried out in Citra Plus Buffer (BioGenex) for 2 minutes at 120^{o}C. Endogenous peroxidases were blocked using 3% peoxidase in methanol for 10 minutes. Non-specific signal was blocked with normal horse serum (Vectastain Elite Kit – M) for 15 minutes at room temperature. Samples were incubated for 30 minutes at 37^{o}C with antibodies against the ERα (Dako, 1D5) at 1:35, or 1:20; or antibodies against ERβ at 1:20 or 1:10 (Dako, PPG5/10). Sections stained for ERα were incubated with Vectastatin LK (Vectastain Elite Kit - M) for 30 minutes, while sections reacted with anti-ERβ antibodies were incubated with Dako LSAB + System HRP. Following exposure to biotinylated secondary antibody, sections were visualized using streptavidin HRP (Dako LSAB+ System HRP). Reaction with DAB chromogen was carried out for 2 minutes and slides were counterstained with hematoxylin. Sections stained with antibodies against ERα at 1:20 and ERβ at 1:10 provided the optimal staining patterns, and will be used for further studies with TMA's.

Unstained TMA sections were processed as described above. Sections were incubated for 30 minutes at 37^{o}C with antibodies against the ERα (Dako, 1D5) at 1:20 or ERβ at 1:10 (Dako, PPG5/10). Normal and neoplastic breast sections were processed alongside the TMAs

and were used as positive controls for each antibody. Additionally, normal and neoplastic breast sections not receiving primary antibodies were used as controls for non-specific signal. An additional section of each tissue array was stained with H&E, using standard procedures (Figure 2.1).

*Image acquisition and analysis***.** Digital images of TMA's were captured using an Aperio Scanscope model T2 (Vista, CA) which allows one to scan an entire slide at high magnification and store the information digitally. TMA digital images were subsequently saved as .tiff image files and Aperio software (Vista, CA) was used to open and analyze each digital image. This software allows the user to evaluate the digital images at various magnifications. Subsequently, individual cores from each TMA were digitally separated using Aperio's TMA Lab software (Figure 2.1). After separation, each core was given a random identification number, which was known only by the principle investigator.

Randomized digital TMA cores were provided to an experienced observer (O. Harris Ford) as .tiff images. The files were analyzed by the observer so they were blinded to the type of antibody and to the identity of the tissue sections. Moreover, O. Harris Ford was blinded to the details and expected outcomes of the study. Additionally, Dr. Ruth A. Lininger reviewed random images to ensure that scoring was uniform. Epithelial and stromal cell nuclear and cytoplasmic ERα and ERβ immunostaining was semiquantitatively assessed on a scale ranging from 0 (no immunostaining) to 3 (strong immunostaining) in each of 100 nuclei or cells, yielding a score ranging from 0 to 300 for each feature for each specimen (Figure 2.2) (Majumder, et al. 2006).

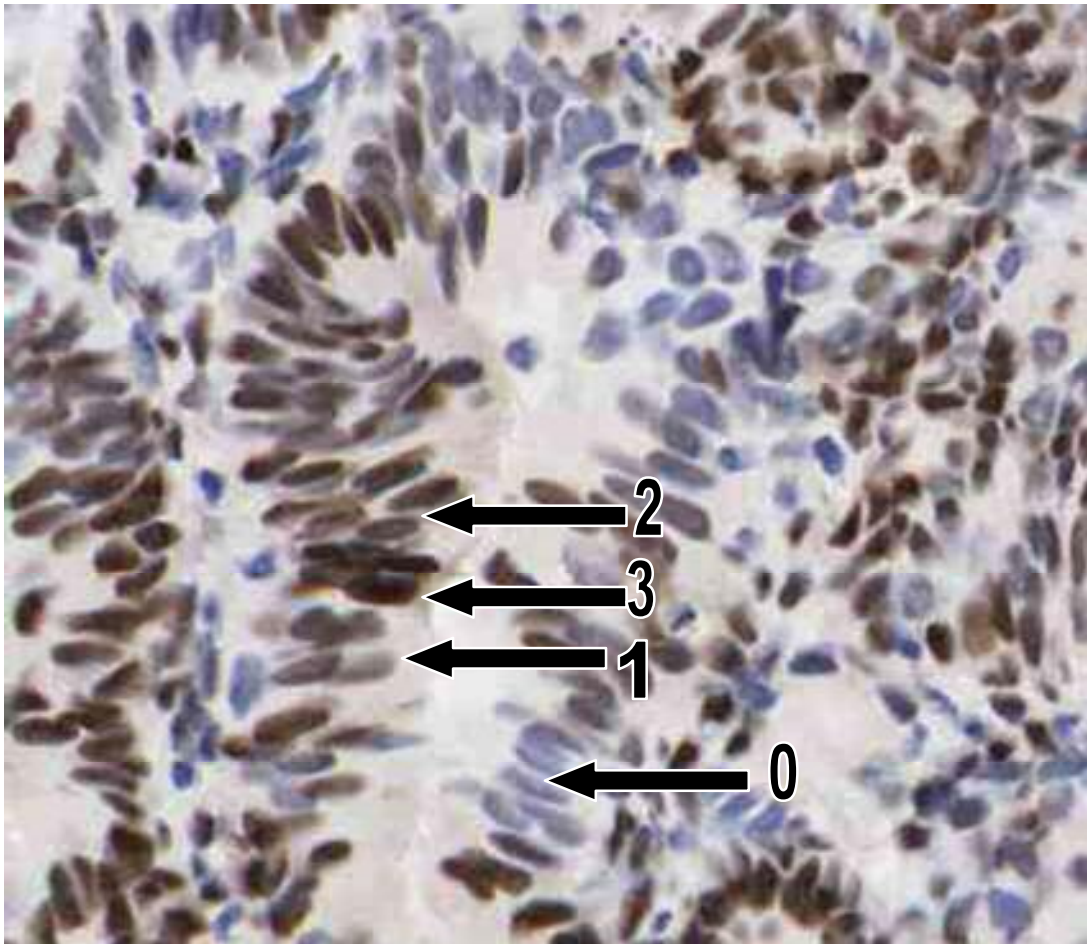

Figure 2.2. Examples of Nuclear Immunohistochemical Staining Intensities. Excised from core 36, indicating nuclei with immunoscores of 0, 1, 2 and 3. Nuclear intensity scoring was as follows: 0=hematoxylin only; 1=Light staining; 2=Moderate staining; 3=Intense staining.

*Statistical analysis***.** Data were obtained as the mean visual scores of ERα and ERβ immunostaining. Student's t test was used to compare ERα or ERβ nuclear and cytoplasmic immunostaining between normal and hyperplastic, normal and neoplastic, or hyperplastic and neoplastic samples. Following the initial Student's t test, ANOVA analysis (Tukey HSD Multiple Comparison test) was carried out to compare the staining between all three groups. Differences were considered to be significant at $P < 0.05$.

Results

*Internal Review Board (IRB) approval***.** An application was submitted to the University of North Carolina at Chapel Hill Institutional Review Board (IRB) for permission to obtain retrospective endometrial tissue biopsies to pursue the study of the expression levels of the estrogen receptors in the human endometrium. Following review of this application, the UNC-IRB granted approval (#: 05-PATH-866) to pursue the proposed studies for one calendar year.

Immumohistochemical Staining of the TMA's

*Cyclic endometrial samples***.** Immunohistochemical staining of cyclic endometrial samples with antibodies against the ER subtypes revealed staining in both the epithelial and stromal compartments. Maximum staining of ERα was seen in cores from the proliferative phase of the menstrual cycle and a reduction in immunostaining became apparent during the secretory phase of the menstrual cycle. Immunostaining for ERβ followed the same overall trend as its counterpart in the cycling endometria. However, ERβ expression remained considerably lower than that of ERα in the epithelial compartment. The observations in the

cycling endometria described are consistent with the findings of others (Lessey et al. 1988; Mylonas, et al. 2004).

*Stromal immunohistochemical staining***.** Nuclear ERα immunostaining in the stromal compartment revealed no significant change in immunostaining intensity between normal and hyperplastic cores (p=.6660). However, a significant decrease in staining intensity was observed (p=.0009) in the nuclei of stromal cells from malignant cores. Median intensity scores of 114 in normal (n=27), 129 in hyperplastic (n=20), and 46 in malignant (n=21) cores were obtained following data analysis (Figure 2.3a). On the other hand, nuclear ERβ immunostaining in the stromal compartment decreased in the hyperplastic cores (p=.0023) compared to normal, while malignant samples revealed a significant increase in immunostaining intensity as compared to normal (p=.0093). Median intensity scores of 162 in normal (n=22), 48 in hyperplastic (n=17), and 267 in malignant (n=22) cores were observed (Figure 2.3b). The range of scores for nuclear ERα and ERβ in the stromal cells of normal, hyperplastic, and neoplastic are shown in Figure 2.4 a-f.

Cytoplasmic ERα immunostaining in the stromal compartment revealed that approximately 5% of normal cells were positive, while there was no immunostaining detected in hyperplastic or malignant samples. On the other hand, 0% of normal stromal cells stained for cytoplasmic ERβ. However, an increase in ERβ cytoplasmic immunostaining, of 5.6% and 10%, in the hyperplastic and malignant samples was observed, respectively (Figures 2.3 c and d).

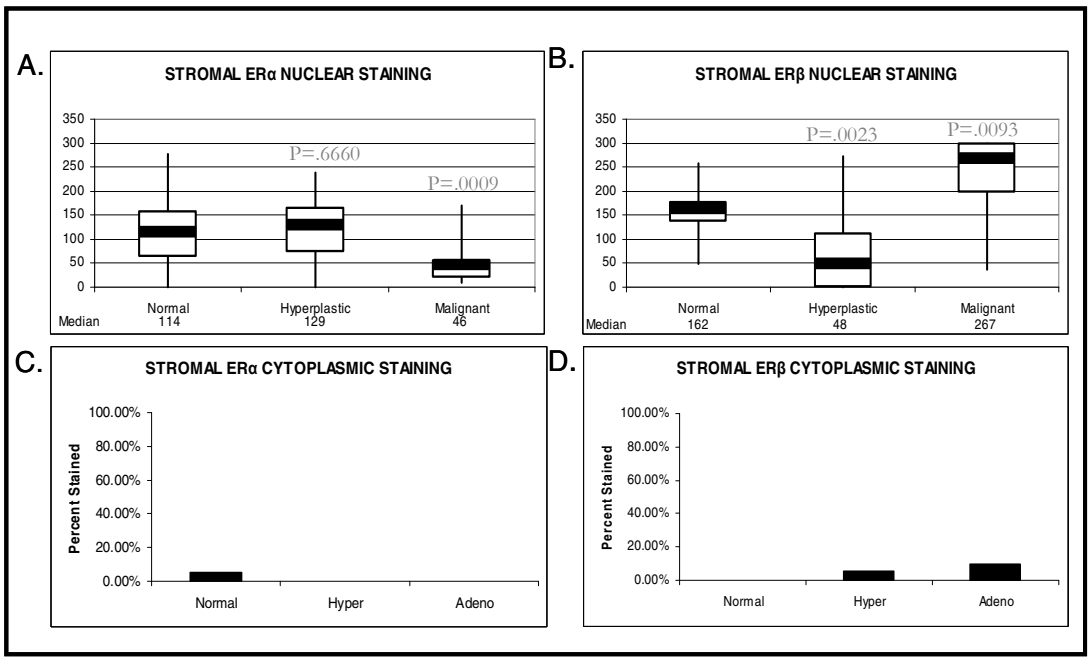

Figure 2.3. Results of ERα and ERβ Immunostaining in Endometrial Stromal Cells. Box and Whisker plots reveal the top and bottom quartiles, in addition to the minimum, maximum, and median scores for ER staining in the nuclei of stromal cells (A and B). Cytoplasmic staining of the stromal cells was recorded and graphed as percent positive cells (C and D).

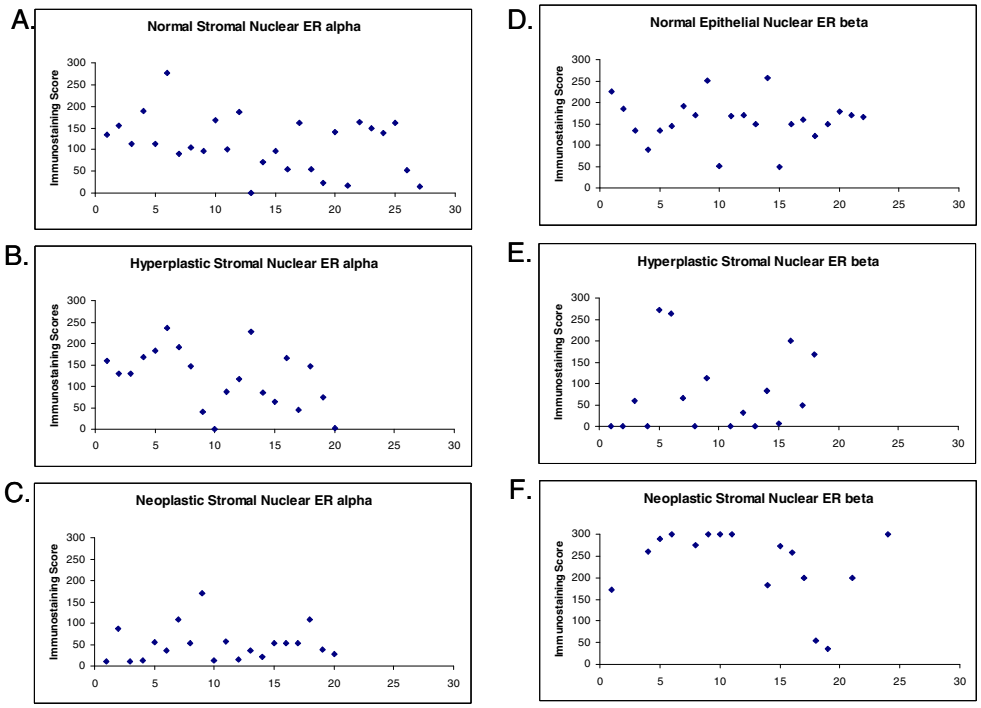

Figure 2.4. Distribution of Stromal Nuclear Immunostaining Scores. (A-C, Stained with antibodies for ERα) A) Normal B) Hyperplastic C) Neoplastic (D-F, Stained with antibodies for ERβ) D) Normal E) Hyperplastic F) Neoplastic. Immunointensity is located on the Y-axis and patient numbers are located on the X-axis.

*Epithelial immunohistochemical staining***.** Nuclear ERα immunostaining in the nuclei of epithelial cells revealed a significant increase in intensity in hyperplastic and malignant tissues (p=.0001 and p=.0005, respectively) as compared against normal tissues. Median intensity scores of 155, 281, and 243 where observed in normal (n=27), hyperplastic (n=20), and malignant (n=21) cores, respectively (Figure 2.5a). Unlike ERα nuclear staining in the epithelial compartment, ERβ remained constant in the hyperplastic samples before increasing dramatically in malignant cores (p=.0001). Median intensity scores for ERβ nuclear epithelial staining were 73, 119, and 300 in normal (n=22), hyperplastic (n=17), and malignant (n=22) cores, respectively (Figure 2.5b). The range of scores of nuclear ERα and ERβ nuclear staining scores are shown in Figure 2.6 a-f.

Epithelial cytoplasmic immunostaining for ERα resulted in 13% of normal cells and 61% of hyperplastic samples staining as positive. Interestingly, there was a reduction to 22% positive cells in the malignant samples. On the other hand, 55% of normal epithelial cells presented cytoplasmic ERβ staining, while hyperplastic samples revealed a slight reduction to 43%. Moreover, a significant increase in cytoplasmic ERβ staining in the malignant epithelial cells was observed, where 91% of epithelial cells were positive (Figures 2.5 c and d).

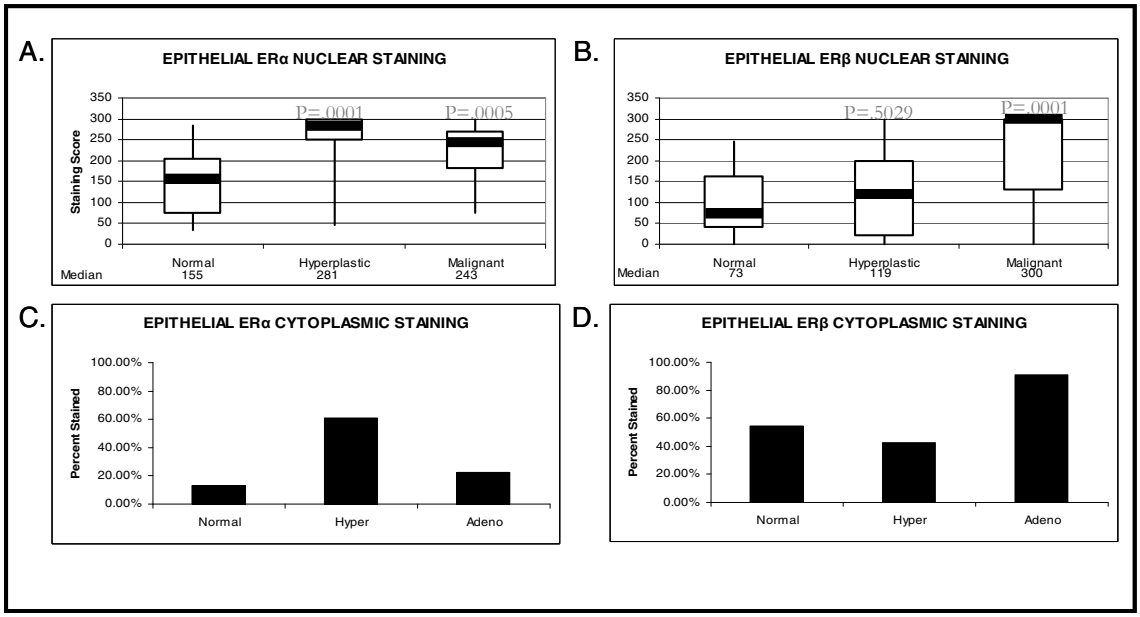

Figure 2.5. Results of ERα and ERβ Immunostaining in the Endometrial Epithelial Compartment. Box and whisker plots reveal the top and bottom quartiles, in addition to the minimum, maximum, and median scores for ER staining in the nuclei of epithelial cells (A and B). Cytoplasmic staining of the epithelial cells was recorded and graphed as percent positive cells (C and D).

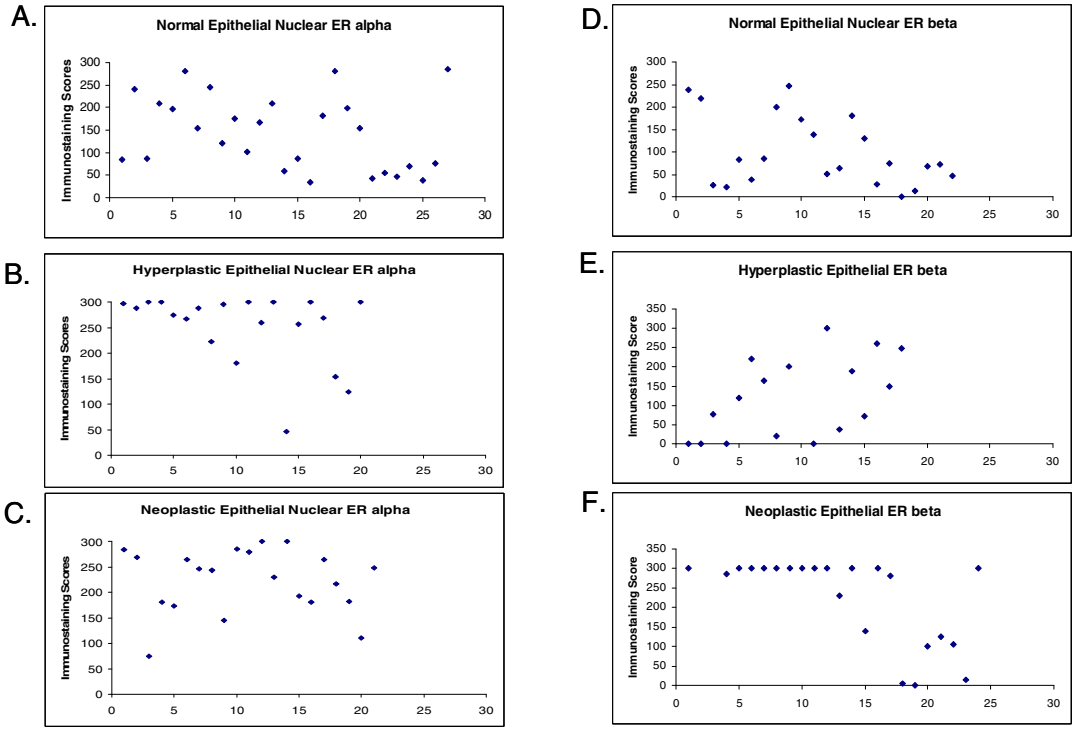

Figure 2.6. Distribution of Epithelial Nuclear Immunostaining Scores. (A-C, Stained against ERα) A) Normal B) Hyperplastic C) Neoplastic (D-F, Stained against ERβ) D) Normal E) Hyperplastic F) Neoplastic. Immunointensity is located on the Y-axis and the number of patients is located on the X-axis.

Discussion

Estrogens have been implicated in the initiation and/or progression of type-1 endometrial cancers for at least two decades (Rose 1996). The physiologic effects of estrogens are mediated by two distinct transcription factors known as the estrogen receptors (ERs) α and β (Enmark et al. 1997; Green et al. 1986; Kuiper et al. 1996). To date, the functions of the ERs in the normal and abnormal endometrium remain largely unresolved. However, evidence gathered using estrogen receptor knockout mice (ERKO) reveal that the ER subtypes may maintain divergent roles (Dupont et al. 2000; Krege et al. 1998; Kuiper et al. 1996). ERαKO mice display hypoplastic uteri that fail to respond to pharmacologic doses of estrogens, suggesting that ERα may be the key mediator of proliferation within this tissue. On the other hand, ERβKO mice have hyperplastic uteri that produce an increased response to estrogens, suggesting that ERβ may modulate ERα's stimulatory properties. Taken together, data from both *in vivo* and *in vitro* studies suggests that ERα and β have divergent roles with regard to gene regulation and proliferation.

In the current study, we assessed ERα and ERβ immunostaining in the nuclear and cytoplasmic compartments of normal, hyperplastic, and malignant endometrial stromal and epithelial cells. Unlike the epithelial compartment, the stromal compartment from normal endometrial cores revealed stronger immunostaining for ERβ than ERα. However, stromal cells from hyperplastic samples revealed a marked decrease in ERβ intensity and while ERα immunostaining remained constant, indicating that an alteration of the "normal" ERα/ ERβ ratio occurs within this compartment (Table 2.4). We believe this alteration of the ERα/ERβ ratio to be a key step in the initiation and/or progression of estrogen-dependent endometrial cancer. Work published by Arnold *et al.* has shown that paracrine factors from normal

	ERα	ERβ
Normal	112	158
Hyperplastic	122	77
Neoplastic	51	231

Table 2.2. Average Nuclear ER Immunoscores for Stromal Cells. Normal (n=27), Hyperplastic (n=20), and Neoplastic (n=21).

	ERα	ERβ
Normal	143	100
Hyperplastic	251	121
Neoplastic	222	222

Table 2.3. Average Nuclear ER Immunoscores for Epithelial Cells. Normal (n=27), Hyperplastic (n=20), and Neoplastic (n=21).

stromal cells regulate the proliferation of both normal and diseased endometrial epithelial cells *in vitro* in response to treatment with 17β-estradiol (Arnold et al. 2001; Arnold, et al. 2002). Therefore, alterations in the microenvironment of hyperplastic endometrial tissue must occur to allow the epithelial compartment to continue its abnormal growth in response to estrogens. Additionally, alterations in the expression profiles of the stromal receptors in malignant samples are probably not as significant because the stromal compartment becomes reduced in size at this stage of estrogen-dependent endometrial disease.

The epithelial compartment of normal endometrial tissues revealed stronger immunostaining for ERα than ERβ. However, within the hyperplastic tissue there was a dramatic increase in the immunoreactivity of ERα while ERβ immunostaining remained relatively constant, indicating that an alteration of the normal ratio had taken place (Table 2.3). Again, this alteration takes place at a critical junction for estrogen-dependent endometrial disease. The current literature suggests that ERα is a key stimulator of growth while ERβ is an inhibitor of growth in the human breast and ovary, and from these findings we believe this to be true also in the human endometrium. Finally, we believe that the final increase of ERβ in malignant tissues may be a protective response by the cell to curb uncontrolled growth in response to 17β-estradiol. However, cells at this stage of the disease may have acquired key cellular alterations to overcome ERβ's protective function and exploit any properties that may favor progression of the disease.

In these studies we observed that an alteration in the normal ERα/ERβ ratio takes place within the human endometrium during estrogen-dependent endometrial disease progression. The alteration always favored mitogenic signaling from ERα, in both the stromal and epithelial compartments of the endometrium, within hyperplastic samples

	Epithelial	Stromal
Normal	1.43 / 1	.71 / 1
Hyperplastic	2 / 1	1.6 / 1
Neoplastic	1 / 1	.2 / 1

Table 2.4. ERα to ERβ Nuclear Ratio in Stromal and Epithelial Cells of Normal, Hyperplatic, and Neoplastic Endometrial Biopsies.

	Epithelial	Stromal
Normal	0.7 / 1	1.4 / 1
Hyperplastic	0.48 / 1	0.63 / 1
Neoplastic	1 / 1	4.53 / 1

Table 2.5. ERβ to ERα Nuclear Ratio in Stromal and Epithelial Cells of Normal, Hyperplatic, and Neoplastic Endometrial Biopsies.

compared to normal samples. This finding suggests that the ER ratio must stay within certain boundaries to maintain a normal response to estrogens within the uterine microenvironment. This data also correlates with recent findings of increased p160 coactivator expression in the uteri of women with PCOS, who are likely to progress to endometrial hyperplasia and cancer, suggesting that ERs will have the necessary tools to induce transcription in response to estrogens (Gregory, et al. 2002). Additionally, Korach *et al.* noted that ERβKO mice have an exaggerated proliferative response to estrogens within the compartments of the uterus. The data presented within this chapter is consistent with these observations, in that an alteration of the ratio between the ERs leading to an abnormal increase of ERα expression over that of ERβ leads to increased cellular proliferation, as seen in hyperplasia, which is a precursor for type-1 endometrial cancer. Moreover, this finding may provide a mechanism whereby the epithelium overcomes the need for paracrine mediated factors, emanating from the stroma, to stimulate growth. Furthermore, the data contained herein is consistent with the hypothesis that ERβ is a modulator of ERα within the human endometrium. Finally, in order to elicit the mitogenic effects of ERα within this tissue there is an alteration of the homeostatic balance between the receptors in hyperplastic endometrial biopsies, a critical junction in estrogen-mediated endometrial disease progression.

Taken together, the data presented within this chapter suggests that the alteration in the ERα/ERβ ratio seen in the hyperplastic endometrial tissue setting, which is a key step toward estrogen-induced carcinogenesis within this tissue. This data also identifies the need to further investigate the potential role of both receptor subtypes in the regulation of endometrial epithelial cell proliferation and gene expression and will be addressed in chapters III and IV of this dissertation.

Chapter III: Characterization of the Roles of the Estrogen Receptors Alpha and Beta on Estrogen-Induced Gene Regulation

Abstract

Endometrial cancer is a major cause of morbidity and mortality in the western world. The primary risk factor for development of this disease is unopposed endogenous and exogenous estrogens. The mechanisms by which estrogen elicits its effects in the human endometrium are not well known. Herein, we provide evidence consistent with the hypothesis that Estrogen Receptor (ER) α is the primary receptor subtype involved in the upregulation of estrogen-inducible genes using the placental alkaline phosphatase (ALPP) gene as an example. Cytochemical and Quantitative-PCR (qPCR) for ALPP revealed that the ERα-selective agonist, Propylpyrazole triol (PPT), can induce ALPP message and activity, indicating that ERα is necessary to upregulate this gene. However, the ERβ-selective agonist, Diarylpropionitrile (DPN), failed to upregulate ALPP message or product. We have recently acquired an isogenic Ishikawa cell line, Ishikawa-3H12, which lack functional ER expression. Isogenic derivatives of Ishikawa-3H12 that stably express a single ER subtype have been constructed in order to complement the studies conducted with the ER agonists. These studies revealed that ERα, and not ERβ, is responsible for the upregulation of ALPP. Further studies utilizing U0126 and LY294002, inhibitors of MAP-Kinase and (PI)3-kinase, respectively, revealed that the upregulation of ALPP is at least in part due to these signaling

pathways in this endometrial adenocarcinoma model system. Taken together, the data suggests the existence of a membrane/cytoplasmic version of ERα, and that its activation results in the upregulation of ALPP via MAPK and (PI)3-kinase. Furthermore, ERβ activity within the same compartment does not negatively regulate ALPP.

Introduction

Endometrial cancer is a major cause of morbidity and mortality in women and is the most common gynecologic malignancy (Rose 1996). The American Cancer Society estimates that 41,200 new cases will be diagnosed and 7,350 deaths will result from endometrial cancer in the United States in 2007. The incidence of endometrial cancer, like most cancers, is dependent on age. The median age of patients diagnosed with this disease is 63 years. Seventy-five percent of women diagnosed with endometrial cancers are post-menopausal.

The predominant classic etiological factor associated with the development of endometrial carcinoma is exposure of the endometrium to endogenous and/or exogenous estrogens that are not modulated by progesterone (Rose 1996). Stimulation of the endometrium with excess and unopposed estrogen can lead to hyperplasia, which increases the risk of developing atypical hyperplasia and type 1 endometrial cancer (Montgomery et al. 2004). The physiologic effects of estrogens are mediated by two distinct transcription factors known as the estrogen receptor (ER) α and β. Currently, there are two pathways, classical and non-classical, by which the receptor subtypes are known to elicit their actions. In the well accepted classical pathway, the receptors are activated by ligand in the nucleus and in turn influence gene activation by binding to DNA in the promoter of responsive genes and recruiting coregulators which interact with the basal transcriptional machinery to induce

transcription of responsive genes (Graumann and Jungbauer 2000; Pratt 1998; Pratt and Toft 1997; Robyr et al. 2000; Smith 1998; Smith and Toft 1993). The less well accepted, non-classical pathway of ER action involves membrane/cytoplasmic forms of the ERs which are activated by ligand and in turn activate various cytoplasmic signaling cascades, such as (PI)3-kinase and MAP-Kinase to elicit transcription of estrogen responsive genes (Kim et al. 1998; Stoica et al. 2003). Although the mechanisms of ER action are known, their individual contributions in the regulation of target genes within the human endometrium are not well defined. Further evidence about the role of the two receptors has followed the development of estrogen receptor knockout (ERKO) mice which allows the function of the individual receptor subtypes to be evaluated.

ERα knockout (ERαKO) mice carry a null mutation within the ERα gene, and were the first model to show the phenotypic results of having only one receptor subtype. These mice exhibit developed uteri containing the usual epithelial, stromal, and myometrial cell types and endometrial and myometrial structures (Lubahn et al. 1993). However, the tissue components were reported to be diminished in size and their cells were insensitive to natural and synthetic estrogens. They fail to stimulate DNA synthesis and induce estrogen responsive genes in response to pharmacologic doses of estrogen or the synthetic agonist, diethylstilbestrol (DES), as is seen in wild-type littermates (Lubahn et al. 1993). This finding suggests that ERα is an important mediator of both normal cellular proliferation and estrogen-mediated gene regulation within the rodent uterus.

Unlike their counterparts, ERβ knockout (ERβKO) mice develop uteri with normal epithelial, stromal, and myometrial cells and normal sized endometrium and myometrium (Krege et al. 1998). The uteri of ERβKO mice maintain their responsiveness to estrogens. In

fact, these mice exhibit an increase in Ki-67 protein, a cell proliferation marker, and an exaggerated proliferative response to 17β-estradiol. These effects suggest that ERβ may be able to serve some of the effects of ERα in ERβKO mice yet normally serves an important role in modulating the effects of ERα. ERβ may also provide an antiproliferative function in the immature uterus (Walker and Korach 2004).

Distinguishing the functions of estrogen receptors α and β is crucial to understanding the mechanisms of estrogen-related disease. Such knowledge may help to elucidate how cell proliferation is normally regulated by ERα and ERβ the endometrium and how hypersensitivity to estrogens may contribute to endometrial carcinogenesis. To determine the roles of each ER in the transcriptional response to estrogens we chose to use the placental alkaline phosphatase (ALPP) gene, which is an estrogen-inducible gene whose product is easily detected using a cytochemical assay (Littlefield, et al. 1990). We studied ALPP induction in cells expressing ERα or ERβ or neither. Additionally, we utilized two commercially available highly specific agonists of the ERα and ERβ, PPT and DPN, respectively.

Materials and Methods

*Cell culture***.** Ishikawa endometrial adenocarcinoma cells, an established endometrial epithelial cancer cell line, were maintained in stromal medium (SM) consisting of a 1:1 mixture of Ham's F12 (GIBCO™, Invitrogen Corp.) and M199 basic medium (Sigma) supplemented with 5% bovine calf serum (BCS; Hyclone), 0.1%Mitoplus (BD Biosciences), 2μg/ml insulin (Sigma) and antibiotic/antimycotic (hereafter referred to as ABAM; source (GIBCO™). Ishikawa-3H12, 3H12α, 3H12β, and 3H12α/β cells were routinely maintained in

DMEM-F12 (GIBCO™) culture medium supplemented with 5% fetal calf serum, 200mM L-glutamine, and ABAM. Cells were transferred to steroid-free medium JAC-0.5 containing 1:1 F12/M199, ABAM, 0.5% charcoal-stripped fetal bovine serum (Hyclone), 0.25% ITS+™ (insulin-transferrin-selenium plus lipoic acid, Becton Dickinson), 0.1mM phosphorylethanolamine (Sigma) and 2mM glutamine (GIBCO™) prior to hormonal stimulation. The stromal medium and JAC 0.5 treatment media were phenol red-free. All cultures described in this report were maintained at 37°C in a humidified atmosphere of 5% CO_2.

*Alkaline phosphatase assay***.** On the day of the experiment, cells were seeded at a density of $7x10^3$ cells per well in a 96-well flat bottom microtiter plate (Costar) using stromal medium (SM) for the Ishikawa cells, and DMEM for the Ishikawa-3H12 cells. Test compounds were dissolved in ethanol and were diluted in JAC-0.5 medium (final concentration of ethanol, 0.1%). After addition of compounds (200µl/well) cells were incubated for 72 hours at 37°C in humidified air with 5% CO_2.

At the conclusion of the experiment, the medium was removed from the microtiter plates by inverting and shaking. 96-well plates, containing cells, were subsequently washed twice by immersing each plate into one liter of 1x PBS (0.15M NaCl, 10mM sodium phosphate, pH 7.4) (GIBCO™). Following the last wash, microtiter plates were blotted over clean paper towels. Methanol (Mallinckrodt Chemicals) was then added to each well of the plate and plates were placed at -70°C for 20 minutes and subsequently allowed to thaw at room temperature for five minutes. Methanol was then removed by shaking, and the 96-well plates, containing cells, were blotted over clean paper towels to remove residual methanol.

To each well, 100µl of ALPP substrate [p-nitrophenyl phosphate, pNPP (Sigma) 0.24mM $MgCl_2$, and 1mM diethanolamine (pH 9.8)] was then added to the cells. Plates were incubated at room temperature protected from light for one and a half to three hours. Following incubation, the intensity of developed color product was determined by reading the 96-well plates in a plate reader at 405nm.

*Chromatin immunoprecipitation (ChIP) assays***.** Ishikawa cells were cultured in steroid-free medium containing charcoal stripped fetal bovine serum (csFBS) and treated, with either 10^{-8}M 17β-estradiol or vehicle (ethanol), for indicated timepoints. ChIP assays using antibodies specific for acetylated histone H3 (Upstate), ERα (Santa Cruz), or ERβ (Santa Cruz) were performed. PCR was carried out using primers amplifying the putative estrogen response elements (EREs), 1 and 2, located 1.9 kb and 2.2 kb upstream of the start site of the ALPP gene, respectively. Cells were treated with 1% formaldehyde at room temperature for 15 minutes. Pellets were collected and lysed in 400µl lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris (pH 8.0)] plus protease inhibitors for 10 minutes on ice. Cells were then sonicated with 7 pulses of 3 seconds each using a Branson Sonifier 200 (Branson Sonifier). Sonification produced chromatin fragments of approximately 600 basepair (bp). Insoluble debris was removed by centrifugation and the soluble chromatin was diluted 2-fold with dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris (pH 8.1), and 167mM NaCl] plus protease inhibitors. 200µl of the diluted solution were precleared with protein A agarose beads (Upstate Biotechnology) for 30 minutes at 4°C, to prevent non-specific binding. In separate experiments, 800µl of the supernatant was incubated for 2 hours at room temperature with antibodies against acetylated histone H3 (Upstate Biotechnology),

ERα (Santa Cruz), or ERβ (Santa Cruz). 600µl of protein A agarose was added and the mixture was incubated overnight at 4°C. The next day, beads were washed sequentially with a low salt buffer [20mM Tris-HCl pH 8.0, 150mM NaCl, 0.1% Sodium Dodecyl Sulfate (SDS), 1% Triton X-100, 2mM Ethylenediaminetetraacetic acid (EDTA)], a high salt buffer [20mM Tris-HCl pH 8.0, 500mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA], a LiCl buffer [20mM Tris-HCl ph 8.1, 250mM LiCl, 500mM NaCl, 1% NP-40, 1% Deoxycholate, 2mM EDTA], and a TE buffer [10mM Tris-HCl pH 7.5, 1mM EDTA]. Immunocomplexes were eluted from protein A agarose beads with 250µl of elution buffer [1% SDS, 0.1M $NaHCO_3$] while rocking at room temperature for 15 minutes. Immunocomplexes were heated at 65°C for 4 hours to dissociate the DNA/protein cross-links. Organic extractions were carried out with phenol:chloroform:isoamyl alcohol (GIBCO™). DNA was precipitated using sodium acetate and resuspended in 50 µL water. 200µl of diluted soluble chromatin processed in the same way but without immunoprecipitation was termed "input DNA". PCR reaction conditions used to detect precipitated DNA fragments were as follows: 95^{o}C-12 minutes (activation of the polymerase), and cycled 35 times at, 94^{o}C-1 minute, 60^{o}C-1 minute, 72^{o}C-1 minute. The primer sequences were as follows: ERE-1 forward, 5'-TCTCG ACACC AGAAC ACAGC-3'; ERE-1 reverse, 5'-TACAG ATGCA TTTGG GTGA-3'; ERE-2 forward, 5'-ACCTG AGCTG CCTTT CTGAG-3'; ERE-2 reverse, 5'-CGGGT TTAAA TCAGG GAGAA-3'. PCR products were separated by electrophoresis on agarose gels, and visualized.

*RNA and quantitative real time PCR***.** Total cellular RNA was prepared from cells using the SV Total RNA Isolation kit (Promega Corp.). Total cellular RNA was used to produce first strand cDNA during the initial qPCR cycle. qPCR reactions were carried out

using Taqman universal PCR mastermix in the ABI PRISM 7700 system (Applied Biosystems) using the following reaction conditions: 48^{o}C-30 minutes (cDNA synthesis); 95^{o}C-10 minutes (Activation of the polymerase); and 35 cycles at 95^{o}C-15 seconds, and 60^{o}C-1 minute. The real time PCR probes used for this system were constructed with a reporter dye, 6-FAM^{TM} or 6-TET^{TM}, on the 5' end and a black hole quencher, TAMRA^{TM}, on the 3' end. The following primers and probe sequences were constructed in the Oligonucleotide Core Facility in the Department of Pathology and Laboratory Medicine for use in this assay: Placental alkaline phosphatase (ALPP) mRNA forward, 5'-GCT TCT TCC TCT TCG TGG A-3'; ALPP reverse 5'-TCT CAG TCAGTG CCC GGT A-3'; and ALPP Probe 5'-6- FAM^{TM} -CA TGA TGA CCA TGG TCG ATG CGA- TAMRA^{TM} -1-3'; β-Actin forward 5'-GGT CAT CAC CAT TGG CAA TG-3', β-Actin reverse 5'-TAG TTT CGT GGA TGC CAC AG-3', β-Actin Probe 5'-6- TET^{TM} -CA GCC TTC CTT CCT GGG CAT GGA- TAMRA^{TM} -1-3'. β-Actin mRNA levels were used to correct for RNA loading.

*Immunodetection of hormone receptors***.** Cultures were washed in cold saline buffer and scraped in 52µl/cm^2 of modified RIPA buffer [50mM Tris-HCl, pH 7.4; 150mM NaCl; 1 mM EDTA; 1% NP-40 detergent; 0.25% sodium deoxycholate; 1mM phenylmethanesulphonylfluoride (PMSF); 1µg/ml each of aprotinin, leupeptin, pepstatin; 1mM sodium orthovanadate and 1mM sodium fluoride]. The resuspended cells were transferred to a 1.5ml microcentrifuge tube and incubated on ice for 30 minutes, then centrifuged at 13,000xg for 30 minutes. The supernatants were assayed for total protein concentration using a bicinchonic acid assay (Pierce Chemical Co.). Proteins in the cell-free extracts were separated by SDS-PAGE in 10% polyacrylamide and electrotransferred onto a

nitrocellulose membrane. TBS/0.1% Tween-20 buffer [20mM Tris-HCl, pH 7.6; 73 mM NaCl; and 0.1% Tween-20] was used for all steps of the immunodetection and each step was preceded by three 5-minute washes at room temperature. Blocking of non-specific signal was achieved in 5% nonfat dry milk for 1 hour at room temperature. The blot was incubated overnight at 4ºC with primary antibody diluted 1:1000 in 5% BSA-TBS/0.1% Tween-20, then exposed to secondary antibody linked to horseradish peroxidase (Amersham) [1:2000 in 5% non fat dry milk in TBS/0.1% Tween-20] for 1 hour at room temperature. For protein detection, the blot was incubated in a luminol substrate (Pierce) for 5 minutes, covered in plastic wrap and exposed to X-ray film. Primary antibodies used were: anti-estrogen receptor α mouse monoclonal [1:1000 dilution; Cell Signaling Technologies, Inc.], anti-estrogen receptor β rabbit polyclonal [1:1000 dilution; Upstate].

*Retrovirus production and cell line construction***.** ESR1, the human gene encoding the estrogen receptor α protein was obtained from the laboratory of Dr. Donald P. McDonnell at Duke University. This gene cDNA was encoded within the pV16 expression vector (Clontech). The ESR1 gene was excised from pV16 by digesting with the EcoR1 restriction enzyme (New England Biolabs). The resulting digest was electrophoresed on agarose to separate the digested ESR1 gene fragment from the empty pV16 vector. The desired fragment was purified with Gene Elute minus ethidium bromide (Sigma) and subsequently ligated into the multiple cloning site of EcoR1 digested pQXCIN, a retroviral backbone with a neomycin resistance marker (BD Biosciences). Competent bacteria were transformed with the ligated pQXCIN-ERα constructs and grown on Luria Miller Agar plus ampicilian culture plates. Clones were picked and grown in Luria Broth (LB) media plus ampicilian. DNAs

were then isolated from the bacteria and digested with the Eco R1 restriction enzyme to ensure that the clone contained the cDNA insert. Following the identification of positive clones digests with the BGL II restriction enzyme (New England Biolabs) were conducted to ensure proper orientation of the gene cDNA sequence within the pQXCIN backbone. Following identification of properly oriented constructs, large scale preparations were conducted followed by DNA sequencing to ensure fidelity of the cDNA sequence.

The full length ESR2, the human gene encoding the estrogen receptor β protein was obtained from the laboratory of Dr. Sohaib Khan in the Department of Cell Biology at the University of Cincinnati. The ESR2 DNA was encoded on the pcDNA^{TM}3.1 expression vector. Sequencing was carried out to ensure that this DNA was the full-length ERβ cDNA and to ensure fidelity of the construct. The ESR2 sequence was amplified from the vector using primers, Forward- 5'-CCC GGA TCC ATG GAT ATA AAA AAC TCA C-3' and Reverse- 5'-CCC GGA TCC TCA CTG AGA CTG AGA CTG TGG GTT C-3', which placed Bam H1 restriction sites and Kozak fragments at the 5' and 3' ends of ESR2. PCR reactions were run with Proof-Start^{TM} Taq Polymerase (Qiagen) at 95^{o}C-15 minutes (activation of the polymerase), and cycled at 94^{o}C-30 seconds, 58^{o}C-30 seconds, and 72^{o}C-1.5 minutes for 32 cycles. PCR products were electrophoresed on agarose and excised. The ESR2 gene cDNA was purified with Gene Elute minus Ethidium Bromide (Sigma) and subsequently digested with the Bam H1 restriction enzyme (New England Biolabs). The desired fragment was ligated into the multiple cloning site of Bam H1 digested pQXCIP, a retroviral backbone with a puromycin resistance marker (BD Biosciences). Competent bacteria were transformed with the ERβ-pQXCIP construct and grown on Luria Miller Agar (GIBCO^{TM}) culture plates. Clones were picked and grown in Luria Broth (LB) media plus

ampicilian. DNAs were prepared and digests with Bam H1 were carried out to identify clones containing the cDNA insert. Following identification of positive clones, digests with BGL II and Eco RV restriction enzymes (New England Biolabs) were conducted to ensure proper orientation of the gene sequence within pQXCIP. Following identification of properly oriented constructs, DNA sequencing was carried out to ensure fidelity of the sequence.

As described above, the ESR1 and ESR2 DNA sequences were subcloned into the retroviral expression vectors pQCXIN and pQCXIP (BD Biosciences), respectively. Vesicular Stomatitis Virus-G pseudotyped, replication-incompetent retroviruses were produced by transient three-plasmid transfection into HEK-293T host cells (Soneoka, et al. 1995). Retroviruses were pelleted and resuspended in 1ml 1X Hanks Buffered Saline Solution (HBSS) plus Ca^{+}, Mg^{+}. Recipient cells, Ishikawa-3H12, were plated at a density of 50,000 cells and infected with the retroviruses preparations (1X-450uL or .1X-45ul) in the presence of 8µg/ml hexadimethrine bromide (Sigma) for 6 hours. This treatment was repeated the next day. Starting on day 3, transduced cells and a mock-infected control were treated with Neomycin (400µg/ml), pQXCIN-ERα, or Puromycin (400ng/ml), pQXCIP-ERβ, for 10 days. Following this interval, the control uninfected cells died and survivor cells in the infected culture were amplified and passed. Cells expressing ERα were named 3H12α and were used as a population, while those expressing ERβ were named 3H12β and were cloned revealing several clones expressing various levels of ERβ. All studies conducted within this dissertation were completed using 3H12β clone 13. A third cell line expressing both ERα and ERβ, 3H12αβ, was constructed using the 3H12β clone 13 cells and re-infecting them with the pQXCIN-ERα retroviral preparations. The cells were then subjected to selection with neomycin for 10 days. Survivor cells were amplified and used as a population.

Results

Cytochemical assay of placental alkaline phosphatase activity in estrogen-stimulated Ishikawa cells and Ishikawa-3H12 cells. To elucidate the roles of the two ERs on gene regulation we chose to use the placental alkaline phosphatase (ALPP) gene because the activity of the enzyme it encodes can be detected easily with a colorimetric cytochemical assay (Littlefield et al. 1990). Both conventional Ishikawa cells (ER expressing) and its derivative cell line Ishikawa-3H12 (null ER background) were treated with 17β-estradiol in concentrations ranging from 10^{-14} M to 10^{-5} M and ALPP activity was determined in the treated cells. The conventional Ishikawa cells responded to the 17β-estradiol treatment in a dose-dependent manner with the highest level of activity observed at 10^{-8} M (10nM). In contrast, the Ishikawa-3H12 cells, which do not express functional ERα or ERβ, did not respond to treatment with estrogen indicating that induction of ALPP expression depends on liganded ERα, or ERβ, or both (Figure 3.1).

Quantitative-PCR analysis of ALPP message in estrogen-stimulated Ishikawa cells. In an effort to demonstrate that the increase in alkaline phosphatase activity that was observed in estrogen-treated Ishikawa cells was the result of increased transcriptional activation, a quantitative real-time PCR (qPCR) was developed to detect placental alkaline phosphatase mRNA. Cells were grown in 10cm tissue culture plates and treated for 72 hours with 10^{-8} M 17β-estradiol +/- the powerful antiestrogen ICI 182780 (10^{-7} M), which blocks both the ERα and ERβ, or the highly specific ERα antagonist MPP (10^{-6} M). RNA was isolated using a Qiagen RNeasy kit and qPCR reactions were completed at the Oligonucleotide Synthesis Core Facility in the Department of Pathology and Laboratory

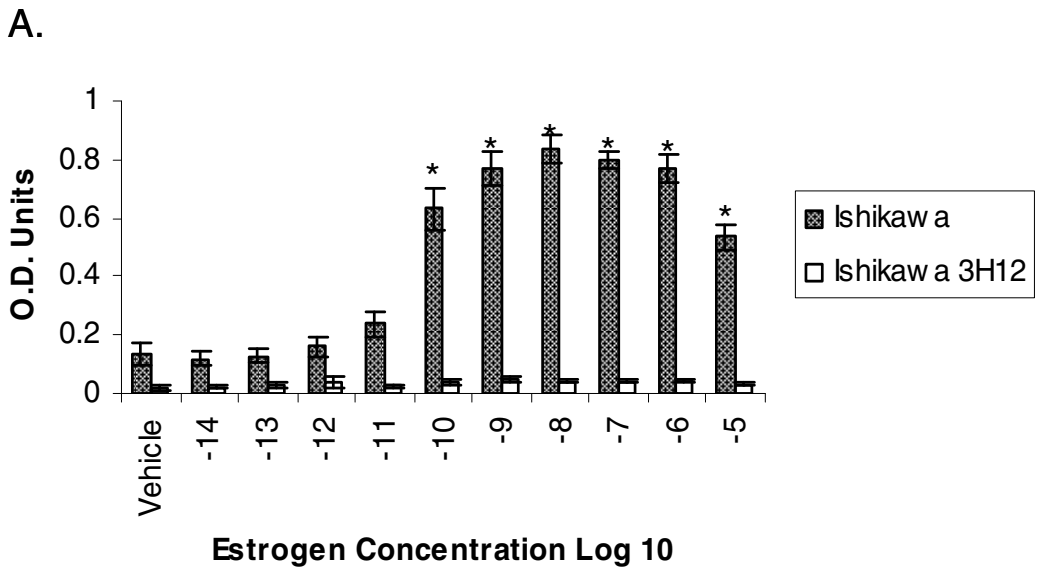

Figure 3.1. Induction of Alkaline Phosphatase Activity in Ishikawa, and not Ishikawa-3H12 Cells, by 17β-Estradiol. Ishikawa and IK 3H12 were seeded at 7000 cells/well and treated with increasing concentrations of 17β-estradiol (10^{-14}M to 10^{-5}M) for 72 hours. ALPP activity in the IK 3H12 cells is not upregulated due to the lack of either estrogen receptor.

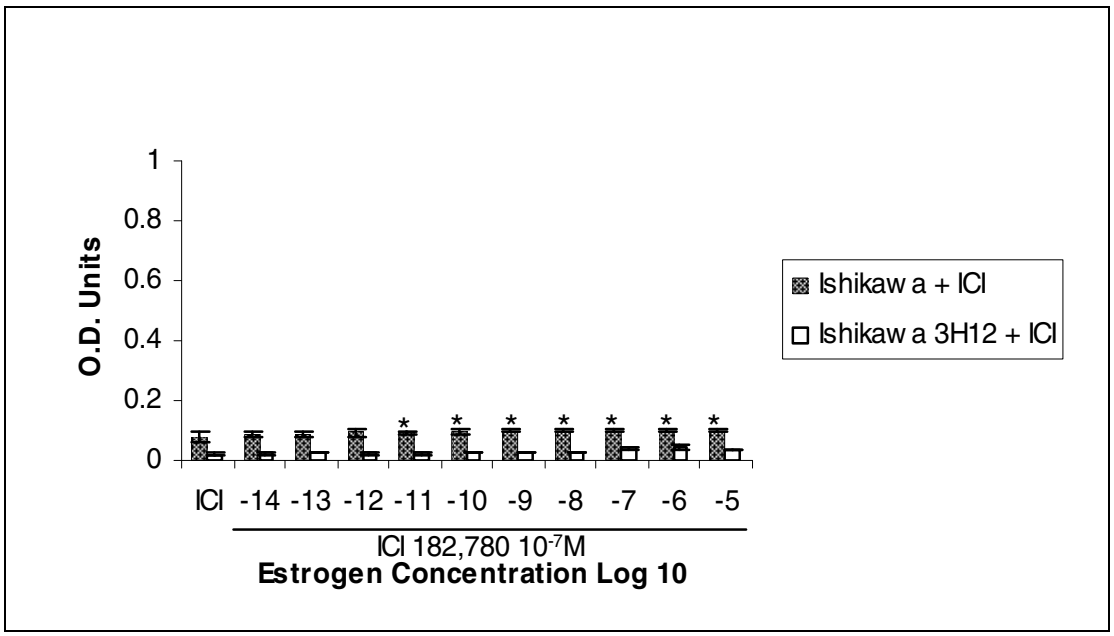

Figure 3.2. The Potent Anti-Estrogen, ICI 182780, Inhibits Upregulation of Alkaline Phosphatase Activity by Estrogen. Ishikawa and IK 3H12 were seeded at 7000 cells/well and treated with increasing concentrations of 17β-estradiol + ICI 182780 for 72 hours. ALPP activity in the IK 3H12 cells is not upregulated due to the lack of either estrogen receptor. See Figure 3.1 to compare the reduction in activity. * indicates $p<0.05$.

Medicine on an ABI Prism 7700. Treatment with 10^{-8}M 17β-estradiol induced a 14-fold induction in ALPP message, but this induction could be inhibited with the total ER inhibitor ICI 182780 or the ERα specific antagonist MPP (Figure 3.3). This data indicates that estrogen causes transcriptional upregulation of ALPP by an ER mediated process. Furthermore, studies using MPP, the ERα specific antagonist showing that the ALPP induction by 17β-estradiol is completely abrogated, indicates that ERα is the obligatory receptor for upregulation of the ALPP gene.

Upregulation of ALPP by the ERα selective agonists, PPT. PPT and DPN, specific agonists of the ERα and β, respectively, provided useful tools for the elucidation of the estrogen receptors roles. Studies in which conventional Ishikawa cells were treated with the ERα selective agonist, PPT, showed that the message and activity of ALPP was increased significantly above the levels seen in cells treated with vehicle (Figure 3.4). In contrast, conventional Ishikawa cells treated with the ERβ selective agonist, DPN, did not show an inhibition of basal levels of ALPP activity, and did not have significant levels of ALPP activity or message when compared with cells treated only with the vehicle (Figure 3.3). However, we did note that concentrations above 10^{-8}M DPN did increase ALPP activity. This increase is directly attributed to the ability of DPN to stimulate ERα at higher doses.

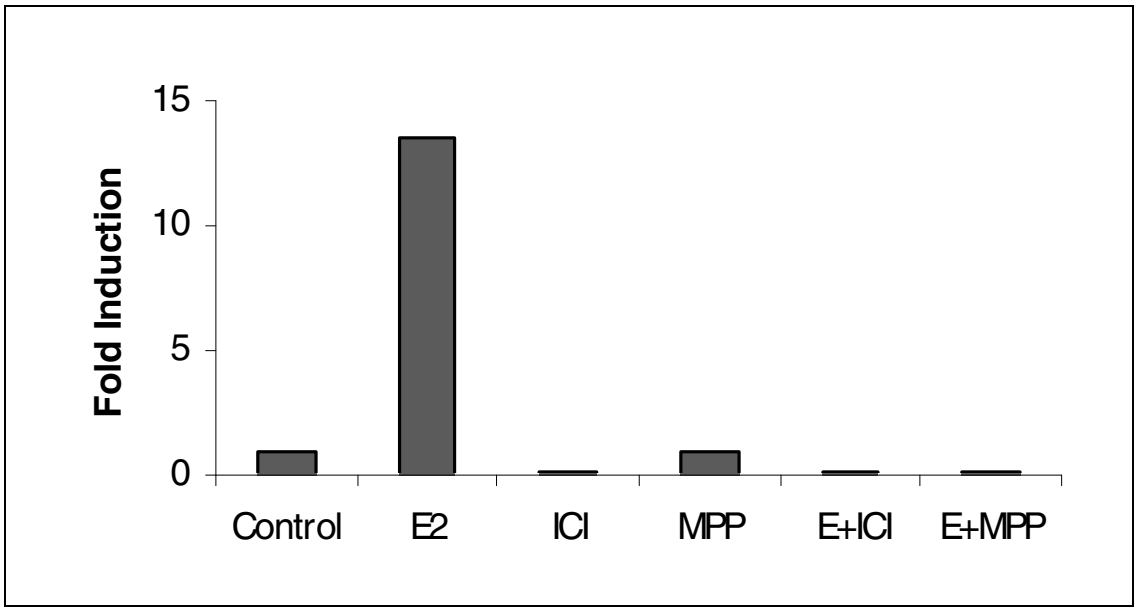

Figure 3.3. qPCR Reveals Inhibition of ALPP Message in Ishikawa Cells by ICI 182780 and MPP. Cells were treated with 10^{-8}M 17β-estradiol , +/- 10^{-7}M ICI 182780 (the total ER inhibitor) or 10^{-6}M MPP (ERα Inhibitor) for 72 hours. RNA was extracted and qPCR was performed for ALPP and β-Actin.

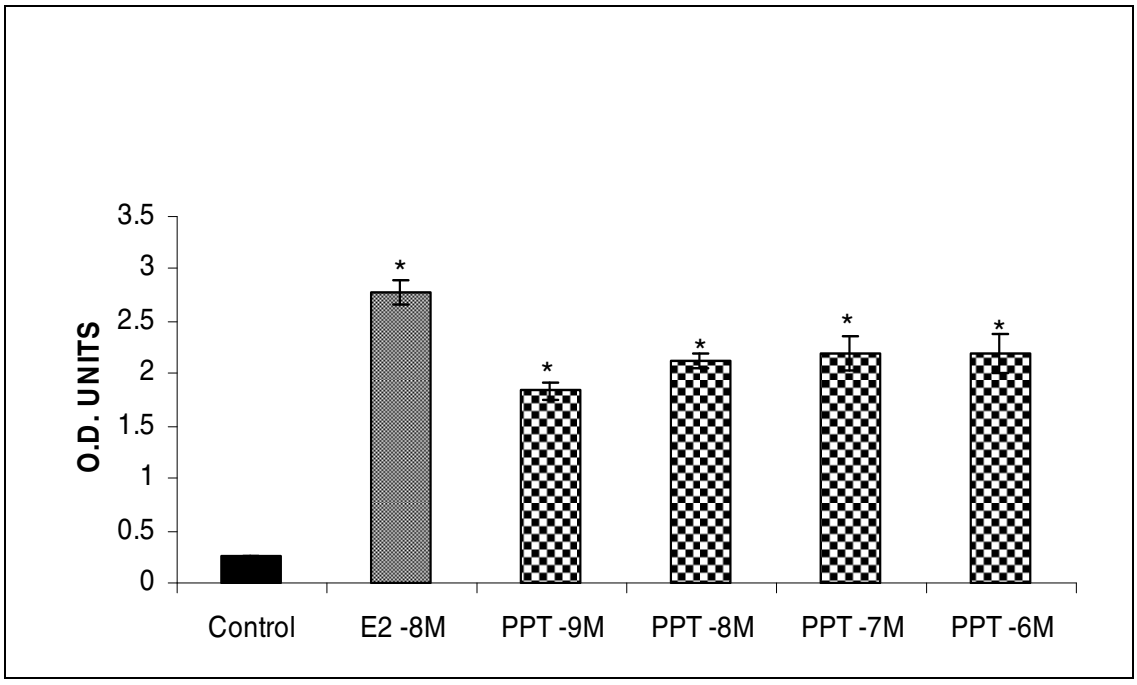

Figure 3.4. Signaling Through ERα is Involved in the Induction of ALPP Activity. Ishikawa cells were seeded at 7000 cells/well and treated with 10^{-8}M 17β-estradiol or increasing concentrations of the ERα agonist, PPT (10^{-9}M to 10^{-6}M), for 72 hours. ALPP activity was determined by colorimetric assay. The results show that ALPP activity is upregulated at least in part, by ERα. * indicates $p<0.05$.

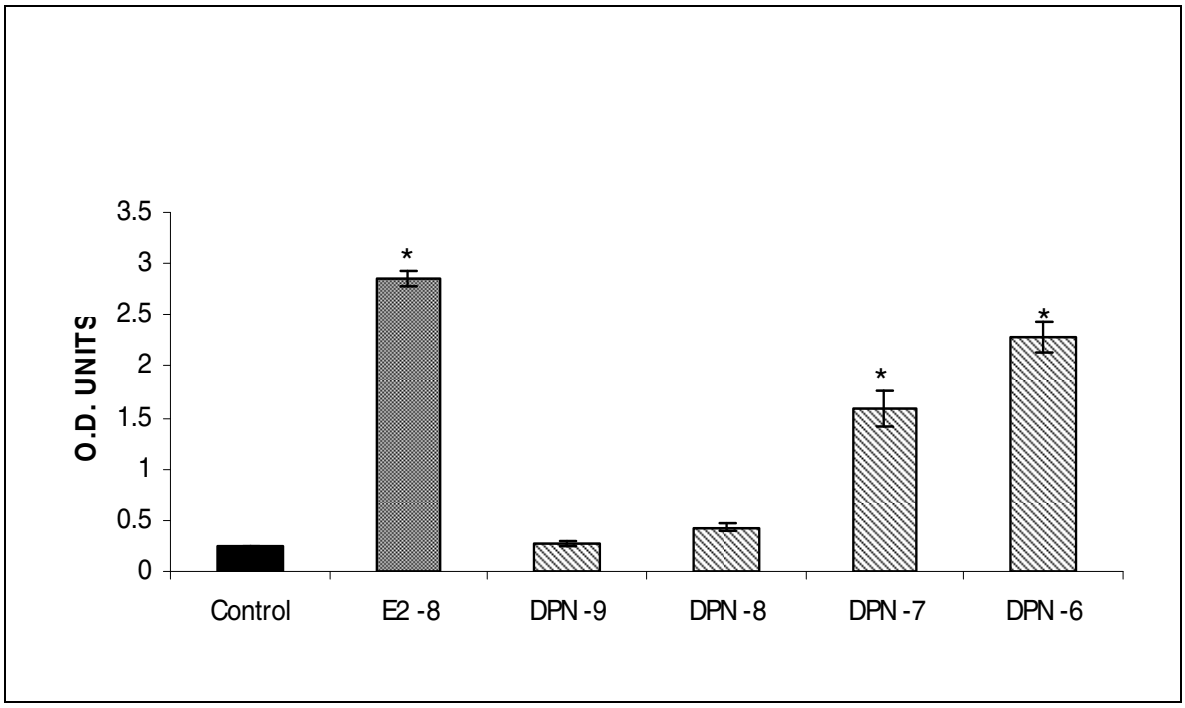

Figure 3.5. ERβ is Not Involved in Increased ALPP Activity. Ishikawa cells were seeded at 7000 cells/well and treated with 10^{-8}M 17β-estradiol or increasing concentrations of the ERβ agonist, DPN (10^{-9}M to 10^{-6}M), for 72 hours. ALPP activity increases as the concentration of DPN increases. However, we have shown that this effect is due to DPN binding the ERα by using the ERα inhibitor, MPP. Therefore, ALPP does not appear to be upregulated by ERβ. * indicates $p<0.05$.

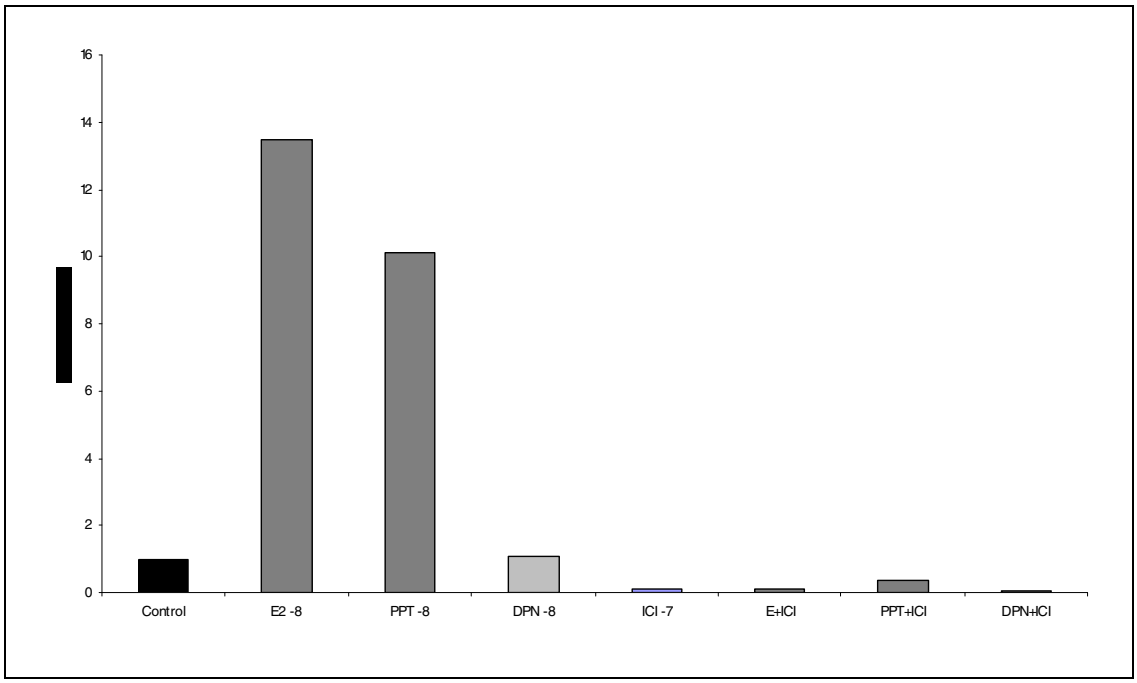

Figure 3.6. ALPP Message is Increased in Ishikawa cells by 17β-Estradiol and PPT. Ishikawa cells were treated with 10^{-8}M 17β-estradiol, 10^{-8}M PPT, or 10^{-8}M DPN +/- 10^{-7}M ICI 182780 for 72 hours. RNA was extracted and qPCR was performed for ALPP and β-Actin. ALPP message is increased in response 17β-Estradiol, and PPT, but not DPN. The increase can also be inhibited by ICI 182780, which indicates that upregulation is in fact mediated through the estrogen receptor(s).

*Construction of cell lines expression ERα, or ERβ or both by stable transfection of the ER subtypes into Ishikawa cells lacking functional ER activity***.** In an effort to understand the individual contributions of the ERs in the regulation of the estrogen-inducible ALPP we needed a model system in which cells express one or both receptor subtypes. The Ishikawa-3H12 cell line, which lacks functional expression of either ERα or ERβ, was stably transduced using retroviral vectors containing ERα, or ERβ (3H12α, 3H12β). ERα, expressing cells were selected with neomycin for 10 days and subsequently used as a population. In contrast, ERβ cells were selected with puromycin for 10 days and cloned, which revealed several clones with varying receptor protein expression levels. The studies contained herein utilized 3H12β clone-13, because its expression of ERβ was similar to that found in conventional Ishikawa cells. The Ishikawa-3H12β clone-13 was transduced with retroviral vectors containing ERα used to produce a third cell line expressing both receptor subtypes (3H12αβ). For the purpose of these studies, the 3H12αβ cell line was used as a population. The results of a Western immunoblot analysis using antibodies against the ERs reveal the expression phenotypes of the newly created cell lines as compared against Ishikawa cells (Figure 3.4).

*Regulation of ALPP by 17β-estradiol in Ishikawa cells and clones 3H12, 3H12α, and 3H12β***.** To elucidate the roles of the two estrogen receptors on ALPP gene regulation we utilized the cell clones constructed to express a single ER. Ishikawa, Ishikawa-3H12, 3H12α, and 3H12β cells were treated with 17β-estradiol ranging from 10^{-14} M to 10^{-5} M and then assayed colorimetrically to detect ALPP activity (Figure 3.8 a-d). As shown earlier (Figure 3.1), ALPP activity is upregulated in Ishikawa cells, but not in 3H12 cells. Treatment of the

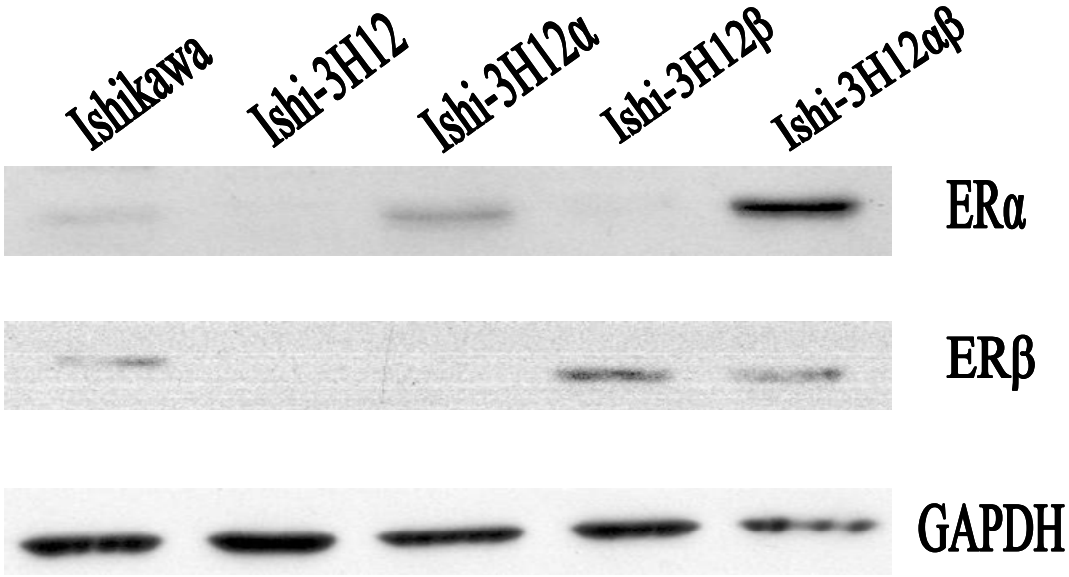

Figure 3.7. Protein Expression of ER Constructed Cell Lines. 30μg of total cell protein from Ishikawa, 3H12, 3H12α, 3H12β, and 3H12αβ populations were loaded and run on a SDS-page gel. Antibodies against ERα, ERβ, and GAPDH were used to determine protein expression within each cell line. (MCF-7 cell lysates were used as a positive control and are not shown.)

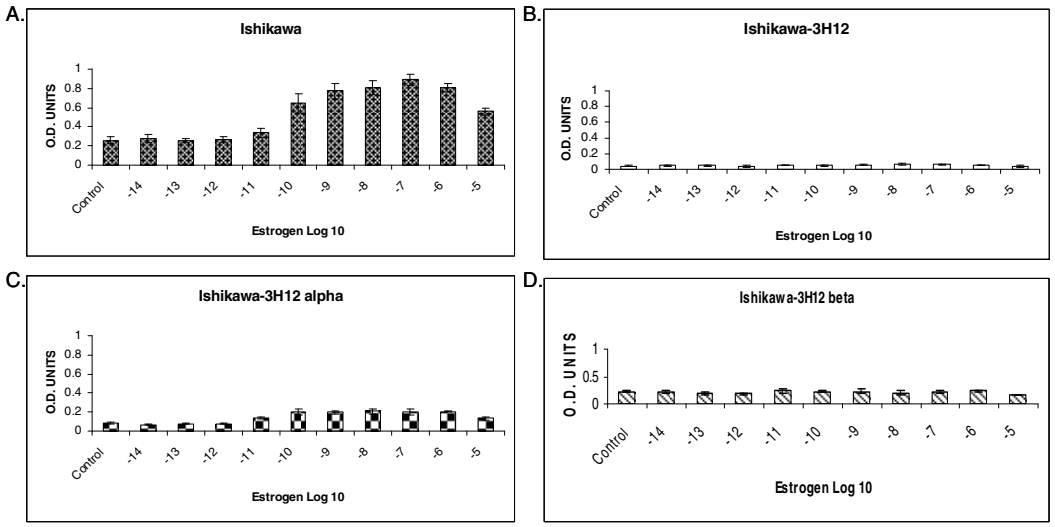

Figure 3.8. Induction of Alkaline Phosphatase Activity in Stable ER Expressing Cell Lines. Ishikawa, 3H12, 3H12α, and 3H12β cells were seeded at 7000 cells/well and treated with increasing concentrations of 17β-estradiol (10^{-14}M to 10^{-5}M) for 72 hours. A dose response in ALPP activity in is restored in the 3H12α, but not the 3H12β cells.

3H12α, 3H12β revealed gene induction by estrogen in the 3H12α but not the 3H12β. From this we conclude that ERα is the primary estrogen receptor responsible for the upregulation of ALPP. In cells expressing ERβ (3H12β) we detected a high background of ALPP activity, but ALPP activity did not respond to treatment with 17β-estradiol (Figure 3.8 d).

*Search for EREs in the promoter region of the placental alkaline phosphatase gene***.** As shown above, placental alkaline phosphatase is an easily measured estrogen-inducible gene. Most estrogen responsive genes contain one or more estrogen response elements (ERE) in their promoter regions and it is at these sites that estrogen receptors bind to DNA to exert their transcriptional activation role. The classic ERE was derived from the *Xenopus laevis* vitellogenin A2 promoter and was found to be composed of two palindromic half-sites separated by three nucleotides, 5'AGGTCAnnnTGACCT3', where n can be any nucleotide (Klein-Hitpass et al. 1986). More recently, a new subclass of EREs have been identified and are thought to be derived from Alu sequences (Norris et al. 1995).

Although placental alkaline phosphatase is a gene of choice for studying the estrogenic activity of various compounds in the endometrium, the regulatory region of this gene has not been described. To pursue this research objective, it was essential to first better characterize the promoter region of ALPP, specifically to identify ERE's present within it. To accomplish this goal, the Dragon ERE Finder program (http://sdmc.lit.org.sg/ERE-V2/index) was used to locate putative EREs within the 5' flanking region of the ALPP gene. The entire 5" flanking region of the ALPP gene was entered and the program identified two putative EREs, which were named ERE-1 and ERE-2. These elements are located 1973, and 2213 base pair, respectively, upstream of the TATA start site for ALPP. The sequence for

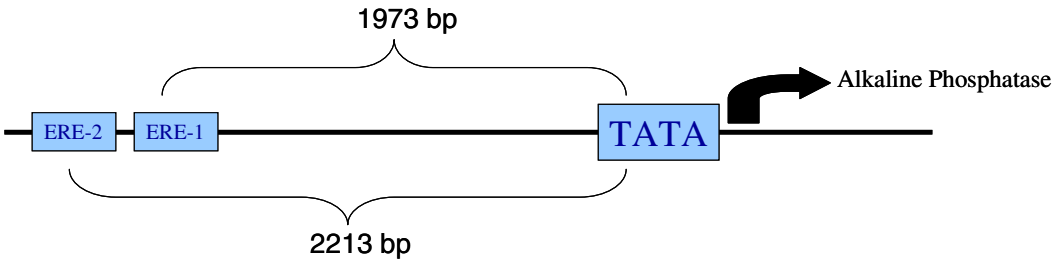

Figure 3.9. Schematic Representation of the Putative EREs Found in the 5' Flanking Region of ALPP. The Dragon ERE Finder software located two putative EREs, located on the complimentary DNA strand 1973 basepair (ERE-1; TG-GAACA-AGA-CACCC-TG) or 2213 basepair (ERE-2; GT-TGCCA-CCT-TGACC-CT) upstream from the TATA start site in the 5' flanking region of the ALPP gene.

ERE-1 is TG-GAACA-AGA-CACCC-TG and that for ERE-2 is GT-TGCCA-CCT-TGACC-CT. Both putative response elements were identified on the reverse compliment strand and are not considered classic EREs (Figure 3.9).

*Effect of estrogen on histone acetylation of EREs in the ALPP promoter region***.** Specific acetylation of histones associated with the promoter region of genes has been correlated with increased transcriptional activity. To understand if the putative response elements (ERE-1 and ERE-2) discovered above are in transcriptionally active regions of the genome in estrogen-treated cells we utilized a chromatin immunoprecipitation assay (ChIP) with anti-acetylated histone H3 antibodies. This technique exploits protein-DNA interactions to isolate and characterize specific sequence binding and was used here to evaluate the acetylation status of histone H3 in the newly identified estrogen response elements in the promoter regions of the ALPP gene. We found that both ERE-1 and ERE-2 primers amplified DNA when immuno-duplexes containing acetylated histone H3 were precipitated from Ishikawa cells, indicating that both response elements are associated with nucleosomes containing acetylated histones (Figure 3.10). This suggests the presence of a more open chromatin conformation, and therefore an active gene. We also evaluated acetylation of histone H3 in the conventional Ishikawa cells in response to 17β-estradiol. In this set of experiments we observed a rapid acetylation of histone H3 within 45 minutes indicating that both sites are located in transcriptionally active regions of the gene (Figure 3.10).

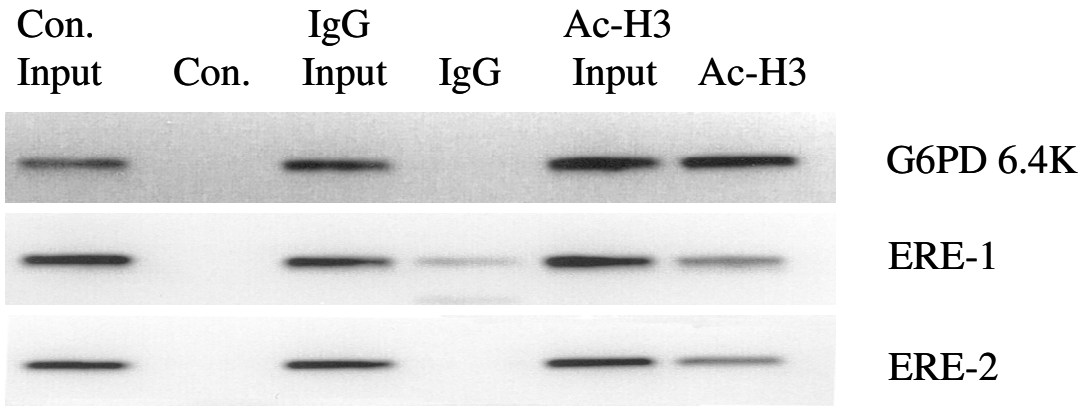

Figure 3.10. ChIP Assay Showing That ERE-1 and 2 are Located Within Transcriptionally Active Regions of the Genome. Chromatin from Ishikawa cells growing in 4% csFBS was crosslinked, sheared, immunoprecipitated with non-specific IgG or Anti-Ac-Histone H3 antibody. A control with no antibody was also added. PCR was carried out on the purified DNA and the resulting reactions were resolved on a 2% agarose gel stained with ethidium bromide (shown in reverse contrast). G6PD 6.4K is an area of the genome known to be associated with acetylated-histone H3, and is therefore used as a positive control.

Non-classical ER Signaling

*Activation of MAP-Kinase and (PI)3-Kinase in the Ishikawa cell line by 17β-estradiol***.** In an effort to determine whether 17β-estradiol upregulates ALPP expression via cytoplasmic signaling cascades we evaluated the ability of estrogen to induce phosphorylation (activation) of regulatory proteins in the MAP-Kinase and (PI)3-kinase pathways. Ishikawa cells were treated with 10^{-8}M 17β-estradiol for specific time intervals every 15 minutes for the first hour and then at 3, 6, 12, and 24 hours. The cells were harvested and cell lysates were subjected to Western immunoblot analysis using antibodies against both the native (unphosphorylated) and activated (phosphorylated) MAPK and (PI)3-kinase pathway proteins. The results revealed that treatment of conventional Ishikawa cells with 17β-estradiol lead to a rapid and biphasic activation of the MAPK pathway (Figure 3.11). Furthermore, the (PI)3-kinase pathway is constitutively activated in the Ishikawa cells due to the loss of the key regulator of the pathway, PTEN. However, treatment with 17β-estradiol leads to a further increase in phosphorylation of Akt as determined by Western immunoblot analysis (Figure 3.12).

*Effects of MAP-Kinase and (PI)3-Kinase inhibitors on the upregulation of ALPP by 17β-estradiol***.** Commercially available inhibitors of the MAPK (U0126) and (PI)3-K (LY294002) pathways provide useful tools for elucidating the roles of these cytoplasmic signaling pathways in assays of their biological function. Herein, studies evaluated the ability of 17β-estradiol to upregulate ALPP expression in the presence of U0126 (10μM) or LY294002 (10μM) (Figure 3.13). Treatment of Ishikawa cells with 10μM U0126 (Figure 3.13) or LY294002 (Figure 3.14) significantly reduced the upregulation of ALPP mRNA by

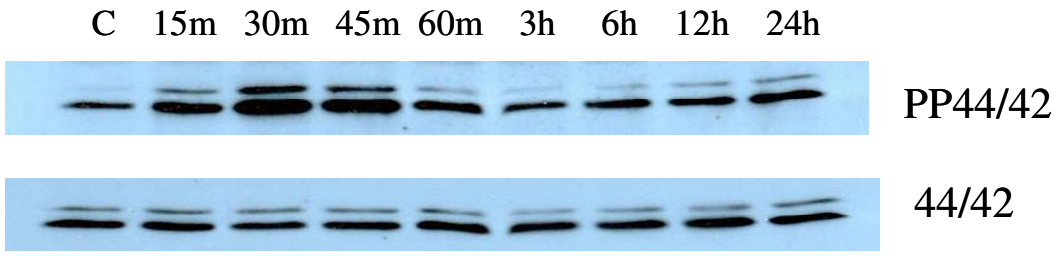

Figure 3.11. MAP-Kinase Induction by 17β-estradiol. Ishikawa cells were treated with 10^{-8}M 17β-estradiol for the indicated times. 30μg of total cell protein from the cells was loaded and run on a SDS-page gel. Antibodies against phosphorylated MAPK (PP44/42) or total MAPK (44/42), a loading control, were used to determine protein expression. This cell line showed a rapid response to treatment, which led to a biphasic activation of the MAPK pathway.

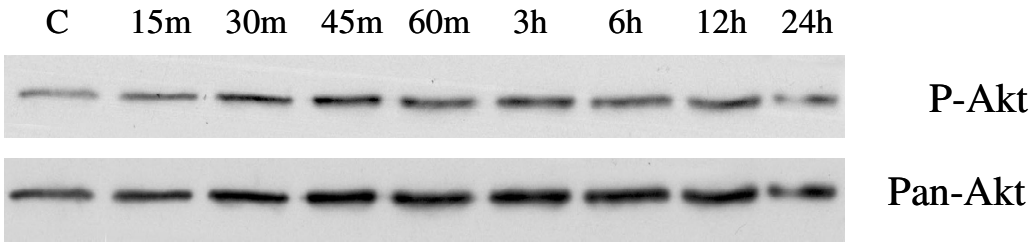

Figure 3.12. (PI)3-Kinase Induction by 17β-estradiol. Ishikawa cells were treated with 10^{-8}M 17β-estradiol for the indicated times. 30μg of total cell protein from the cells was loaded and run on a SDS-page gel. Antibodies against phosphorylated Akt (P-Akt) or total Akt (Pan-Akt), a loading control, were used to determine protein expression. This cell line showed a rapid response to treatment, which led to an increase in the phosphorylation of Akt.

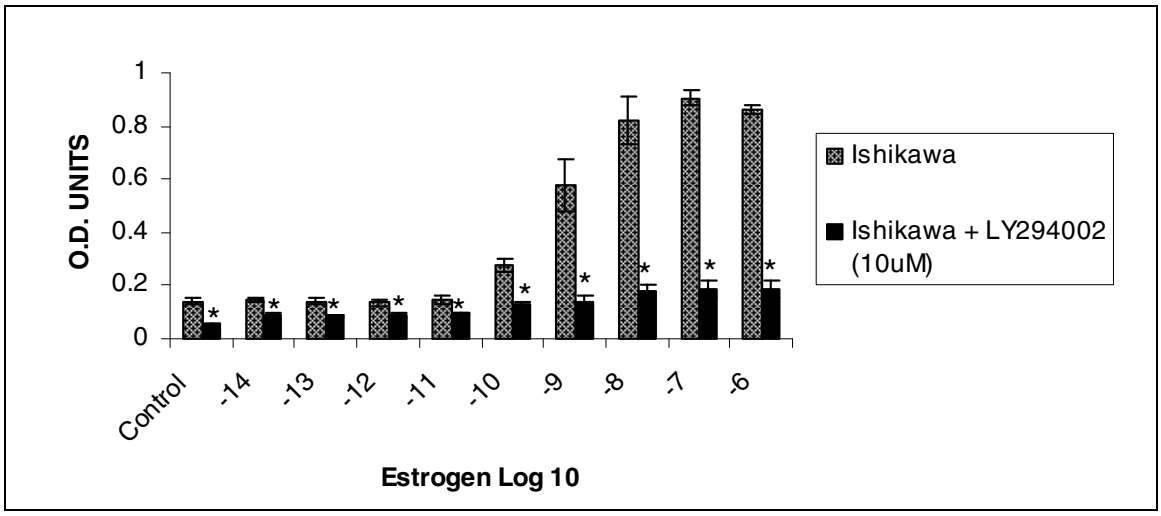

Figure 3.13. Inhibition of 17β-Estradiol Induced ALPP Activity by a PI3-K Inhibitor. Ishikawa cells were seeded at 7000 cells/well and treated with increasing concentrations of 17β-estradiol (10^{-14}M to 10^{-5}M) +/- 10μM of the PI3-Kinase inhibitor, LY294002 for 72 hours. Inhibition of the PI3-K pathway prevents upregulation of ALPP in response to 17β-estradiol. * indicates significant change ($p<0.05$) as compared to estrogen only treated cells.

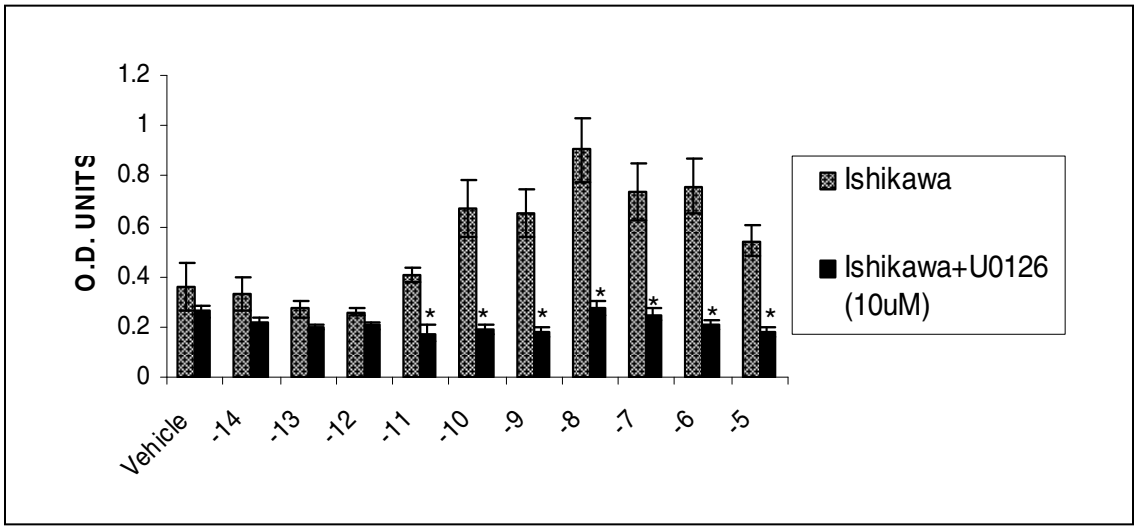

Figure 3.14. Inhibition of 17β-Estradiol Induced ALPP Activity by a MAP-K Inhibitor. Ishikawa cells were seeded at 7000 cells/well and treated with increasing concentrations of 17β-estradiol (10^{-14}M to 10^{-5}M) +/- 10μM of the MAP-Kinase inhibitor, U0126 for 72 hours. Inhibition of the MAP-K pathway ablates 17β-estradiol's ability to upregulate ALPP activity. * indicates significant change ($p<0.05$) as compared to estrogen only treated cells.

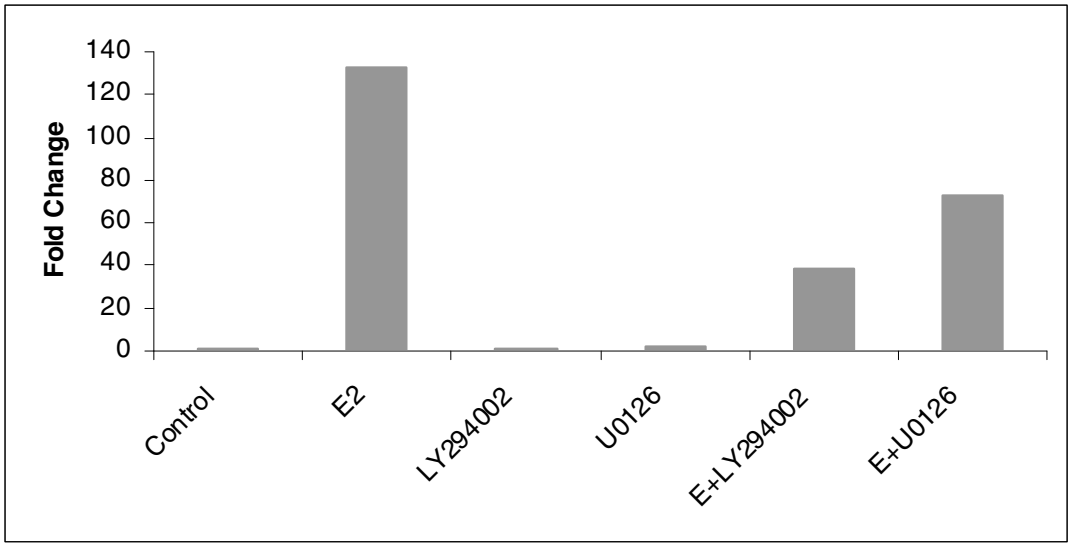

Figure 3.15. qPCR Reveals Inhibition of ALPP Message by PI3-K and MAP-K Inhibitors. Ishikawa cells were treated with 10^{-8}M 17β-estradiol +/- 10μM LY294002 or 10μM U0126 for 72 hours. RNA was extracted and qPCR was performed for ALPP and β-Actin

17β-estradiol. Since 10μM U0126 and LY294002 are considered somewhat cytotoxic, as they slightly reduce the number of Ishikawa cells as compared to nontreated cells, the cytochemical assay may not represent the best measure of the effects of both compounds on ALPP activity and/or expression. However, qPCR revealed that upregulation of ALPP message was significantly inhibited by both U0126 and LY294002 (Figure 3.15).

Discussion

The roles of the estrogen receptors α and β in signaling within the human endometrium are not completely understood. However, evidence accumulated in estrogen receptor knockout (ERKO) mice suggests that the receptor subtypes have different roles in the uteri of mice. ERα is a stimulator of proliferation and estrogen-responsive gene expression, while ERβ's actions are largely inhibitory. The activity of ERα has been studied extensively in the human breast, and these results have been shown to parallel the findings in the mouse models. However, the role of ERβ in the breast is less well understood. As the basis for this study, we hypothesized that ERα is stimulatory and ERβ is inhibitory with regard to gene regulation in the human endometrium.

Studies described herein were undertaken to understand the individual roles of the receptors in a human endometrial epithelial cell model system. We decided to use the easily measured estrogen-inducible alkaline phosphatase gene as an example of an ER regulated gene product (Littlefield et al. 1990). Utilizing specific agonists of the ER α and ERβ, we were able to show that signaling through ERα but not ERβ, lead to an upregulation in the activity of the ALPP gene product. Furthermore, studies using cell lines constructed to express either ERα or ERβ showed results comparable to those with agonists of the two

receptors. The results support our hypothesis that ERα is stimulatory with regard to induction of this gene product. Our results show that regulation through ERβ is not stimulatory, although they cannot support, nor disprove, the second portion of the hypothesis, which was ERβ is inhibitory with regard to regulation of this gene.

To understand if ERβ is inhibitory with regard to ALPP expression we needed to understand how the gene is regulated. A search for estrogen response elements (EREs) in the ALPP 5' flanking region identified two possible non-traditional elements (ERE-1 and ERE-2), which are located 1923 and 2213 base pair upstream of the start site for ALPP transcription, respectively. ChIP assays showed acyelation of histone H3 around the two putative EREs. However, ChIP assays using antibodies against the ERs failed to immunoprecipitate DNA encoding ERE-1 or 2. Despite these finding, the classic ER pathway cannot be ruled out, as ERE-1 and ERE-2 may not properly show the regulatory sequences within the promoter. The inability to confirm this pathway in the upregulation of ALPP may also result from a signal level which is below our threshold of detection.

Recent literature suggests that 17β-estradiol has other effects that cannot be explained by the ligand-dependent mechanism due to their rapid onsets (Kato et al. 1995; Song et al. 2002; Voegel et al. 1998; Watson et al. 1999). Many of these effects, deemed non-classic, have been linked to cell-surface forms of ER, which are thought to resemble nuclear ER (Kato et al. 1995; Song et al. 2002; Voegel et al. 1998; Watson et al. 1999). There is a body of evidence that links estrogen cell surface receptors and activation of the mitogen activated protein kinase (MAPK) and phosphatidylinositol (PI)3-kinase signaling cascades (Song et al. 2002; Song et al. 2005; Stoica et al. 2003). These non-classic actions of the ER could explain a direct connection between exposure to estrogen and stimulation of anti-apoptotic/pro

cellular proliferation pathways that have been strongly implicated in carcinogenesis. Since studies looking for liganded ER binding to ERE's in the ALPP gene promoter were inconclusive, we decided to use alternative methods to evaluate whether or not non-classic actions of the ER's were involved in the upregulation of the estrogen-inducible ALPP gene. We found that treatment of the Ishikawa cells with 17β-estradiol led to a rapid activation of MAPK. Furthermore, although the (PI)3-kinase pathway in the Ishikawa cell model system is constitutively activated due to the loss of the PTEN gene, treatment with 17β-estradiol led to a further increase in activity of the (PI)3-kinase pathway. Inhibitors of MAPK and (PI)3-kinase, U0126 and LY294002, respectively, both prevented estrogen-induced upregulation of ALPP message.

We have presented evidence that is consistent with the hypothesis that ERα induces expression of an estrogen-regulated gene in the human endometrium. Furthermore, upregulation of the estrogen-inducible gene, ALPP, occurs at least in part through the activation of the MAPK and (PI)3-kinase pathways in response to treatment with 17β-estradiol. This latter finding was surprising and strongly supports the existence of membrane and/or cytoplasmic forms of the estrogen receptors. Although, we were unable to demonstrate that ERβ is exerts an inhibitory function on the ALPP gene product, we can conclude that cytoplasmic forms of ERβ are not inhibitory with regard to ALPP induction.

Chapter IV: Characterization of the Roles of the Estrogen Receptors α and β on Proliferation of Endometrial Adenocarcinoma Cells

Abstract

Endometrial cancer is the most common gynecologic malignancy and is a major cause of morbidity and mortality in women in the Western World. The American Cancer Society estimates that 41,200 new cases will be diagnosed and 7,350 deaths will result from endometrial cancer in the United States alone in 2007. Unopposed estrogen exposure is the primary etiologic risk factor for developing this disease. However, the carcinogenic effects of estrogen and its receptors are not well defined within the human endometrium. The principal goal of this study is to understand the individual roles of the estrogen receptors (ERs) α and β in the proliferation of endometrial epithelial cells. To achieve this goal, we utilized two specific agonists of ERα and ERβ, Propylpryazole-triol (PPT) and Diarylpropionitrile (DPN), respectively. Treatment with PPT, DPN, and 17β-estradiol significantly increased proliferation of Ishikawa but not of Ishikawa-3H12 cells, an isogenic cell line that lacks functional ERα and ERβ expression. These results indicate that estrogen receptors are involved in estrogen-induced proliferation of Ishikawa endometrial epithelial cells. Furthermore, we developed cell lines which stably express the ER subtypes individually (3H12α and 3H12β) in the derivative of the Ishikawa-3H12 cell line. Compared to the parental cell line, in the absence of added 17β-estradiol, 3H12α cells showed a 103%

increase in proliferation while 3H12β cells revealed a 45% decrease in growth. Moreover, BrdU incorporation studies showed that 3H12β cells had a significantly reduced S-phase population as compared against parental 3H12 cells. Treatment of both the 3H12α and 3H12β cells with 17β-estradiol revealed significant increases in proliferation in response to estrogen treatment. Despite this observation, 3H12β cells never reached levels of proliferation seen in the parental cell line, suggesting that ERβ-mediated proliferation in response to 17β-estradiol is modest when compared to proliferation mediated by ERα. Taken together, these data strongly correlate with the findings in the breast and ovary, implicating a lack of effect or a protective role for ERβ in estrogen induced proliferation.

Introduction

Endometrial cancer is a major cause of morbidity and mortality in the United States. Exposure to unopposed estrogens is the primary etiologic risk factor associated with increased proliferation of epithelial cells leading to the development of endometrial hyperplasia and atypical hyperplasia. Atypical hyperplasia is often treated with progestin's to counteract the effects of estrogen or with hysterectomy (Jadoul and Donnez 2003). Without treatment, twenty-five percent of women with atypical hyperplasia will develop type-1 endometrial cancer (Kurman et al. 1985). Although the primary risk factor for the disease has been known for at least two decades, the precise mechanism of estrogen-induced carcinogenesis in this tissue has not been clearly elucidated.

The physiologic effects of estrogens are mediated by two distinct transcription factors known as the estrogen receptor (ER) α and β (Enmark et al. 1997; Green et al. 1986; Kuiper et al. 1996; Menasce et al. 1993). The estrogen receptor α and β genes share significant

homology. Despite the homology between these receptors, two different chromosomes encode them, which rules out the possibility that they are splice variants (Enmark et al. 1997; Kuiper et al. 1996; Menasce et al. 1993). There is remarkable conservation of the DNA-binding domain (DBD) between the two estrogen receptors, which suggests that they should be able to bind similar sequences within the promoters of estrogen-induced genes. The least conserved domain, when comparing the two ER subtypes, is the ligand-binding domain (LBD), which has 55% homology, suggesting that the receptors bind ligands with different affinities. ERβ also lacks an efficient activating function-1 (AF-1) region, which influences its interaction with coactivators that affect ligand responsiveness of the receptor (Hall and McDonnell 1999).

Although the exact roles of the receptor subtypes are not known in the human endometrium, evidence accumulated using estrogen receptor knockout (ERKO) mice has provided plausible roles for each receptor in the human uterus. ERαKO mice, which only express ERβ, developed hypoplastic uteri that were insensitive to 17β-estradiol, suggesting that ERα is an important mediator of both cellular proliferation and estrogen mediated signaling within this rodent tissue. On the other hand, the increased cellular proliferation and exaggerated response to 17β-estradiol seen in ERβKO mice, which express only ERα, suggest that ERβ may play an important role in modulating the effects of ERα and may also provide an antiproliferative function within the uterus (Walker and Korach 2004).

Since estrogen is extremely important in the development of endometrial cancer, it is imperative to understand the contribution of the subtypes of estrogen receptor in the initiation and progression of the disease. Most endometrial cell lines express both ER subtypes, making it difficult to elucidate the individual contributions of each receptor within these

cells. In this study the specific contributions of the individual ER subtypes were elucidated in Ishikawa cells, a moderately differentiated endometrial adenocarcinoma cell line, utilizing commercially available highly specific agonists of the two ER's. Additionally, we used the Ishikawa-3H12 cell line, an isogenic cell line that lacks functional expression of the ERs, to construct separate endometrial adenocarcinoma cell lines that express ERα, ERβ, or both ER subtypes.

Materials and Methods

*Cell culture***.** Ishikawa endometrial adenocarcinoma cells, an established endometrial epithelial cancer cell line, were maintained in stromal medium (SM) consisting of a 1:1 mixture of Ham F12 (GIBCO™) and M199 basic medium (Sigma) supplemented with 5% bovine calf serum (BCS; Hyclone), 0.1% Mitoplus (BD Biosciences), 2μg/ml insulin (Sigma) and antibiotic/antimycotic (hereafter referred to as ABAM; source (GIBCO™). Ishikawa-3H12, 3H12α, 3H12β, and 3H12α/β cells were routinely maintained in DMEM-F12 (GIBCO™) culture medium supplemented with 5% fetal calf serum, 200mM L-glutamine, and ABAM. Cells were transferred to steroid-free medium JAC-0.5 containing 1:1 F12/M199, ABAM, 0.5% charcoal-stripped fetal bovine serum (Hyclone), 0.25% ITS+™ (insulin-transferrin-selenium plus lipoic acid, Becton Dickinson), 0.1mM phosphorylethanolamine (Sigma) and 2mM glutamine (GIBCO™) prior to hormonal stimulation. The stromal medium and JAC-0.5 treatment media were phenol red-free. All cultures described in this report were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2.

*Immunodetection of Hormone Receptors***.** Cultures were washed in cold saline buffer and scraped in 52µl/cm^2 of modified RIPA buffer [50mM Tris-HCl, pH 7.4; 150mM NaCl; 1mM EDTA; 1% NP-40 detergent; 0.25% sodium deoxycholate; 1mM phenylmethanesulphonylfluoride (PMSF); 1µg/ml each of aprotinin, leupeptin, pepstatin; 1mM sodium orthovanadate and 1mM sodium fluoride]. The resuspended cells were transferred to a 1.5ml microcentrifuge tube and incubated on ice for 30minutes, then centrifuged at 13,000xg for 30 minutes. The supernatants were assayed for total protein concentration using a bicinchonicic acid assay (Pierce Chemical Co.). Proteins in the cell-free extracts were separated by SDS-PAGE in 10% polyacrylamide and electrotransferred onto a nitrocellulose membrane. TBS/0.1% Tween-20 buffer [20mM Tris-HCl, pH 7.6; 73mM NaCl; and 0.1% Tween-20] was used for all steps of the immunodetection and each step was preceded by three 5-minute washes at room temperature. Blocking was conducted in 5% nonfat dry milk for 1 hour at room temperature. The blot was incubated overnight at 4ºC with primary antibody diluted in 5% BSA- TBS/0.1% Tween-20, then exposed to secondary antibody linked to horseradish peroxidase (Amersham) [1:2000 in 5% non fat dry milk in TBS/0.1% Tween-20] for 1 hour at room temperature. For protein detection, the blot was incubated in a luminol substrate (Pierce) for 5 minutes, covered in plastic wrap and exposed to X-ray film. Primary antibodies used were: anti-estrogen receptor α mouse monoclonal [1:1000 dilution; Cell Signaling Technologies, Inc.], anti-estrogen receptor β rabbit polyclonal [1:1000 dilution; Upstate].

*Retrovirus production and cell line construction***.** ESR1, the human gene encoding the estrogen receptor α protein was obtained from the laboratory of Dr. Donald P. McDonnell at Duke University. This gene cDNA was encoded within the pV16 expression vector (Clontech). The ESR1 gene was excised from pV16 by digesting with the EcoR1 restriction enzyme (New England Biolabs). The resulting digest was electrophoresed on agarose to separate the digested ESR1 gene fragment from the empty pV16 vector. The desired fragment was purified with Gene Elute minus ethidium bromide (Sigma) and subsequently ligated into the multiple cloning site of EcoR1 digested pQXCIN, a retroviral backbone with a neomycin resistance marker (BD Biosciences). Competent bacteria were transformed with the ligated pQXCIN-ERα constructs and grown on Luria Miller Agar plus ampicilian culture plates. Clones were picked and grown in Luria Broth (LB) media plus ampicilian. DNAs were then isolated from the bacteria and digested with the Eco R1 restriction enzyme to ensure that the clone contained the cDNA insert. Following the identification of positive clones digests with the BGL II restriction enzyme (New England Biolabs) were conducted to ensure proper orientation of the gene cDNA sequence within the pQXCIN backbone. Following identification of properly oriented constructs, large scale preparations were conducted followed by DNA sequencing to ensure fidelity of the cDNA sequence.

The full length ESR2, the human gene encoding the estrogen receptor β protein was obtained from the laboratory of Dr. Sohaib Khan in the Department of Cell Biology at the University of Cincinnati. The ESR2 DNA was encoded on the pcDNA^{TM}3.1 expression vector. Sequencing was carried out to ensure that this DNA was the full-length ERβ cDNA and to ensure fidelity of the construct. The ESR2 sequence was amplified from the vector using primers, Forward- 5'-CCC GGA TCC ATG GAT ATA AAA AAC TCA C-3' and

Reverse- 5'-CCC GGA TCC TCA CTG AGA CTG AGA CTG TGG GTT C-3', which placed Bam H1 restriction sites and Kozak fragments at the 5' and 3' ends of ESR2. PCR reactions were run with Proof-Start^{TM} Taq Polymerase (Qiagen) at 95^{o}C-15 minutes (activation of the polymerase), and cycled at 94^{o}C-30 seconds, 58^{o}C-30 seconds, and 72^{o}C-1.5 minutes for 32 cycles. PCR products were electrophoresed on agarose and excised. The ESR2 gene cDNA was purified with Gene Elute minus ethidium bromide (Sigma) and subsequently digested with the Bam H1 restriction enzyme (New England Biolabs). The desired fragment was ligated into the multiple cloning site of Bam H1 digested pQXCIP, a retroviral backbone with a puromycin resistance marker (BD Biosciences). Competent bacteria were transformed with the ERβ-pQXCIP construct and grown on Luria Miller Agar (Gibco) culture plates. Clones were picked and grown in Luria Broth (LB) media plus ampicilian. DNAs were prepared and digests with Bam H1 were carried out to identify clones containing the cDNA insert. Following identification of positive clones, digests with BGL II and Eco RV restriction enzymes (New England Biolabs) were conducted to ensure proper orientation of the gene sequence within pQXCIP. Following identification of properly oriented constructs, DNA sequencing was carried out to ensure fidelity of the sequence.

As described above, the ESR1 and ESR2 DNA sequences were subcloned into the retroviral expression vectors pQCXIN and pQCXIP (BD Biosciences), respectively. Vesicular Stomatitis Virus-G pseudotyped, replication-incompetent retroviruses were produced by transient three-plasmid transfection into HEK-293T host cells (Soneoka et al. 1995). Retroviruses were pelleted and resuspended in 1ml 1X Hanks Buffered Saline Solution (HBSS) plus Ca^{+}, Mg^{+}. Recipient cells, Ishikawa-3H12, were plated at a density of fifty-thousand cells in a 100mm plate were infected with the retroviruses preparations (1X-

450uL or .1X-45ul) in the presence of 8 µg/ml hexadimethrine bromide (Sigma) for 6 hours. This treatment was repeated the next day. Starting on day 3, transduced cells and a mock-infected control were treated with Neomycin (400µg/ml), pQXCIN-ERα, or Puromycin (400ng/ml), pQXCIP-ERβ, for 10 days. During this interval, the control uninfected cells died. Survivor cells in the infected culture were amplified and used for experiments described herein. Cells expressing ERα were named 3H12α and were used as a population, while those expressing ERβ were named 3H12β and were cloned revealing several clones expressing various levels of ERβ. All studies conducted within this dissertation were completed using 3H12β clone 13. A third cell line expressing both ERα and ERβ, 3H12αβ, was constructed using the 3H12β clone 13 cells and re-infecting them with the pQXCIN-ERα retroviral preparations. The cells were then subjected to selection with neomycin for 10 days. Survivor cells were amplified and used as a population.

*Cellular proliferation***.** To assess proliferation, cells were seeded at a density of $5x10^5$ cells in 60mm tissue culture dishes (Corning). Test compounds were dissolved in ethanol and were diluted in JAC-0.5 medium (final concentration of ethanol, 0.1%), which was applied on days 0 and 2. Three representative dishes were counted as base line for the beginning of the assay. Cells were detached from the dishes using a 1:1 mix of trypsin:EDTA and Versene (GIBCO™) at 37°C for five minutes. Stromal medium was added to stop the reaction. Trypsonized cells were thoroughly mixed to obtain single cell suspensions, and enumerated with a Coulter Counter (Beckman Coulter, Inc., Fullerton, CA).

*BrdU incorporation assays***.** To assess cell cycle profiles, cells were seeded at a density of $5x10^5$ cells in 60mm tissue culture dishes (Corning, Corning, NY) in triplicate. 17β-estadiol was dissolved in ethanol and was diluted in JAC 0.5 medium (final concentration of ethanol, 0.1%), which was applied on days 0 and 2. BrdU stock solution was added to a final concentration of 10μM to each dish on day 4 and incubated with BrdU for 1 hour at 37°C. Cells were detached from the dishes using a 1:1 mix of trypsin:EDTA and Versene (GIBCO™) at 37°C for five minutes. JAC-0.5 medium was added to stop the reaction and trypsonized cells were pelleted at 1200 revolutions per minute (RPM) for 5 minutes at 4°C. Supernatant was removed and the cell pellet was washed with 1xPBS and cells were pelleted at 1200 RPM for 5 minutes at 4°C. Supernatant was removed and 1.5ml of cold 1xPBS was added while vortexing the cells followed by 3ml of cold 100% ethanol. Cells were stored in the dark at 4°C overnight.

Nuclei were placed into suspension by vortexing and were subsequently pelleted at 1000 RPM for 5 minutes at 4°C. Supernatant was removed and 3ml of 0.8% pepsin in 0.1 N hydrochloric acid (HCl) was added while vortexing. Nuclei were incubated at 37°C for 20 minutes. Nuclei were pelleted at 1000 RPM for 5 minutes at 4°C and supernatant was removed. 1.5ml of 2 N HCl was added to nuclei while being vortexed followed by a 20 minute incubation at 37°C. Nuclei were vortexed and 3ml of 0.1 M sodium borate was added. Nuclei were pelleted at 1000 RPM for 5 minutes at 4°C. Supernatant was removed and the nuclei were vortexed then 100μl of BrdU diluted 1:5 in IFA [10mM HEPES, 150mM NaCl, 4% fetal bovine serum, and 0.1% NaN_3] plus 0.5% Tween-20 was added. Nuclei were pelleted and RNase A (Roche Biomedical) was added to a final concentration of 5μg/ml. Propidium iodide was added to bring the concentration to 50μg/ml. Nuclei were then

incubated overnight at 4°C. Supernatant was removed from the nuclei following the overnight incubation at 4°C. Nuclei were vortexed and IFA plus 0.5% Tween-20 was added. Nuclei were transferred to appropriate tubes for the FACS machine and were analyzed the same day.

Results

Proliferative response to 17β-estradiol between the Ishikawa and Ishikawa-3H12 cell lines. Endometrial cancer has been linked to increased proliferation of the endometrial epithelial cells in response to unopposed estrogens (Kurman et al. 1985). To study this phenomenon in an *in vitro* model system we characterized the effects of physiologic concentrations of 17β-estradiol on proliferation of both Ishikawa and Ishikawa-3H12 cells. This treatment revealed that Ishikawa, and not IK 3H12, cells respond to 17β-estradiol treatment in a proliferative manner (Figure 4.1). Furthermore, the increase in proliferation, in response to estrogen, of Ishikawa cells can be inhibited using the total ERα/β inhibitor, ICI 182780 (Figure 4.2). This finding suggests that the estrogen receptors are indeed responsible for the increased proliferation of endometrial epithelial cells to physiologic concentrations of 17β-estradiol.

*Response of the Ishikawa cells to receptor-type specific ER agonists***.** The recent commercial release of specific agonists of the ERα and ERβ, PPT and DPN, respectively, has provided valuable new agents for the elucidation of the functions of each ER subtype. In this study, we utilized these agonists in the Ishikawa cell line to understand the contributions of each receptor subtype on proliferation. Treatment with PPT or DPN

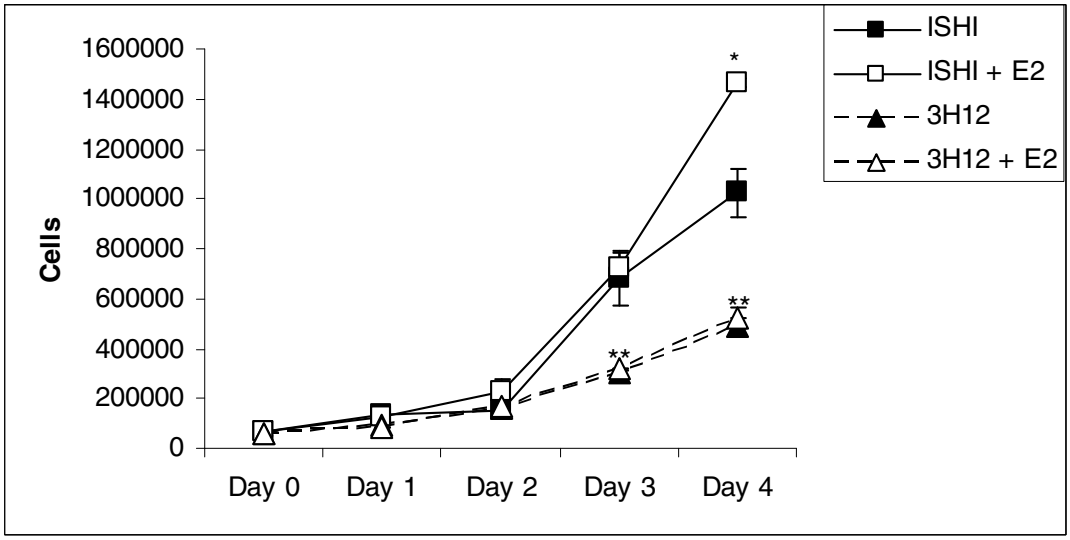

Figure 4.1. 17β-estradiol Treatment Increases Proliferation of Ishikawa and not Ishikawa-3H12 cells. Cells were treated with 10^{-8}M 17β-estradiol or vehicle (ethanol) and assayed on indicated days. Cells were harvested and enumerated by Coulter Counter®. * indicates significant change ($p<0.05$) as compared to vehicle treated cells. ** indicates significant change ($p<0.05$) as compared to Ishikawa cells.

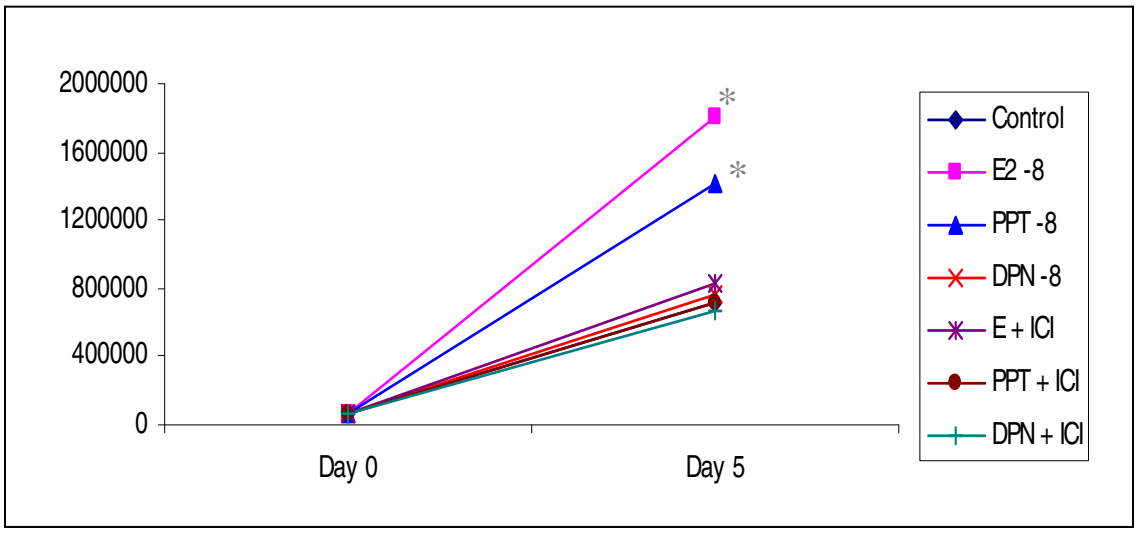

Figure 4.2. Ishikawa Proliferation is increased by ERα, but not by ERβ. Cells were treated with 10^{-8}M 17β-estradiol, 10^{-8}M PPT (ERα agonist), or 10^{-8}M DPN (ERβ agonist) +/- 10^{-7}M ICI 182780 (ER Inhibitor) and assayed on indicated days. Cells were harvested and enumerated by Coulter Counter®. * indicates significant change ($p<0.05$) as compared to vehicle treated cells.

revealed that activation of ERα, and not ERβ, could induce proliferation of the Ishikawa cells (Figure 4.2). Again, the effects of the ERα agonist, PPT, could be inhibited using the powerful antiestrogen ICI 182780, which blocks estrogen mediated effects on both ERα and ERβ. This shows that the response to the agonist is in fact mediated through the ERs (Figure 4.2). Taken together, this data suggests that ERα is the primary receptor involved in growth stimulation in response to estrogens. However, we cannot fully appreciate the individual contributions of each receptor subtype in this cell line, as the receptors are both expressed. To distinguish the effects of the two receptors, it would be necessary to study cells expressing only one or the other receptor.

*Construction of Ishikawa-3H12 expressing ERα and/or ERβ***.** Conclusions drawn from the use of the agonists remain subject to some question because these compounds are not completely specific. The development of cells that expresses either receptor alone is a logical means to verify and extend these results. We chose to use an isogenic cell line, Ishikawa-3H12, to pursue further studies. The Ishikawa-3H12 cells were obtained from Dr. Leen Blok and have been reported to lack functional expression of either ER subtype (Hanekamp, et al. 2003). We found that the Ishikawa 3H12 cell line lacks both mRNA and protein expression of both ER subtypes, which explains why they are incapable of responding to 17β-estradiol. To consider the individual roles of the ERs in carcinogenesis of the endometrium we must first investigate the individual roles of each receptor in proliferation. Therefore, the Ishikawa 3H12 cells were stably transfected using reterovirus specific for each ER subtype, either alone or together. As a result of these studies we obtained three new cell strains, named 3H12α, 3H12β, and 3H12αβ. To access the biology of

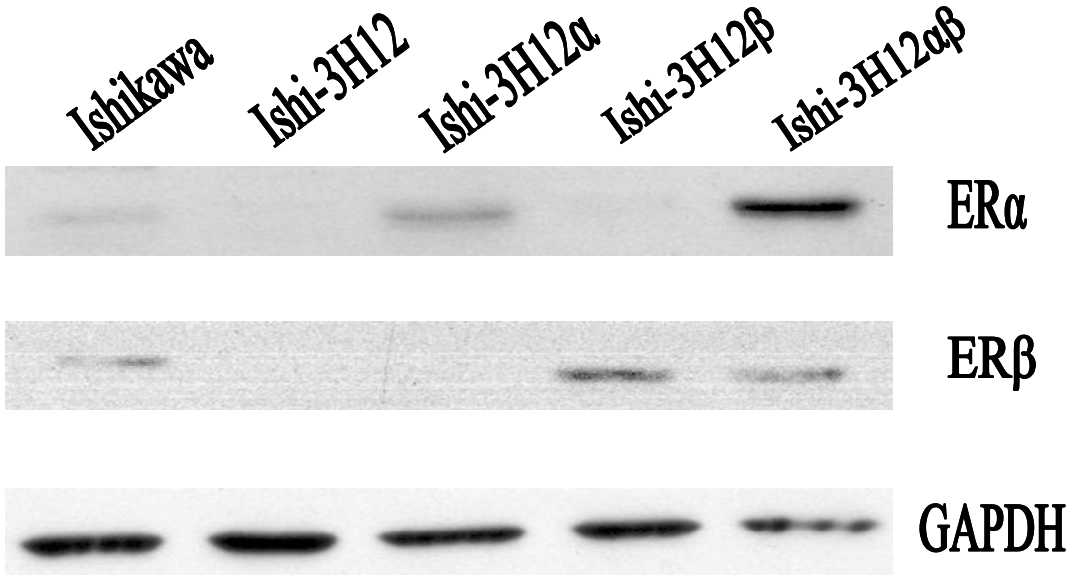

Figure 4.3. Protein Expression of ER Constructed Cell Lines. 30μg of total cell protein from Ishikawa, 3H12, 3H12α, 3H12β, and 3H12αβ populations were loaded and run on a SDS-page gel. Antibodies against ERα, ERβ, and GAPDH were used to determine protein expression within each cell line. (MCF-7 cell lysates were used as a positive control and are not shown.)

these cell strains, the presence of the receptor protein was confirmed by Western Immunoblot analysis (Figure 4.3).

*Response to 17β-estradiol by Ishikawa-3H12 cells expressing ER's***.** Construction of the ER expressing Ishikawa-3H12 cell lines (3H12α and 3H12β) has facilitated understanding of the functions of these receptors in endometrial adenocarcinoma cells. Cell proliferation assays revealed in 3H12α, cells expressing ERα, a 103% increase in growth when compared against the parental 3H12 cells after 4 days of culture. The expression of ERα in the 3H12 cells makes their constitutive proliferation rate comparable to that of the normal Ishikawa cells (Figure 4.4). The 3H12β cells that express ERβ had a 45% decrease in proliferation. This is significant as compared with the parental cell line, suggesting that ERβ activity reduces the proliferation of human endometrial adenocarcinoma cells (Figure 4.5).

Treatment of both ER constructed cell lines with 10^{-8}M 17β-estradiol revealed significant increases in growth over vehicle-treated cells. In fact, treatment of the 3H12α cells lead to a 40% increase in proliferation, which essentially restored the ability of the 3H12 cell lines to proliferate like Ishikawa cells (Figure 4.4). This finding indicates that ERα may be the primary receptor subtype responsible for the proliferative response to estrogens in Ishikawa endometrial epithelial cells. On the other hand, treatment of the 3H12β cell line with 17β-estradiol produced a 39% increase in proliferation (Figure 4.5). Although treatment of the 3H12β cells with physiologic concentrations of 17β-estradiol produce a significant increase in proliferation, these cells fail to reach the levels of proliferation seen in the parental strain, 3H12. Taken together, these observations suggest a stimulatory role for ERα and an inhibitory role for ERβ.

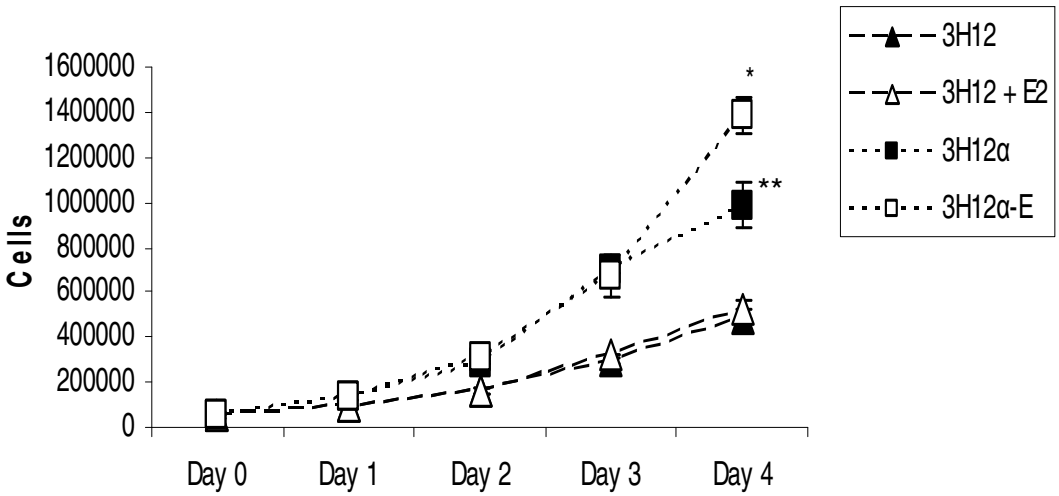

Figure 4.4. Comparison of 3H12 and 3H12α Cell Proliferation Rates. Cells were treated with 10^{-8}M 17β-estradiol or vehicle (ethanol) and assayed on indicated days. Cells were harvested and enumerated by Coulter Counter®. The result indicate that the reconstitution of ERα in the 3H12 cells leads to increased cellular proliferation and the ability to respond to estrogens. * indicates significant change ($p<0.05$) as compared to vehicle treated cells. ** indicates significant change ($p<0.05$) as compared to Ishikawa-3H12 cells.

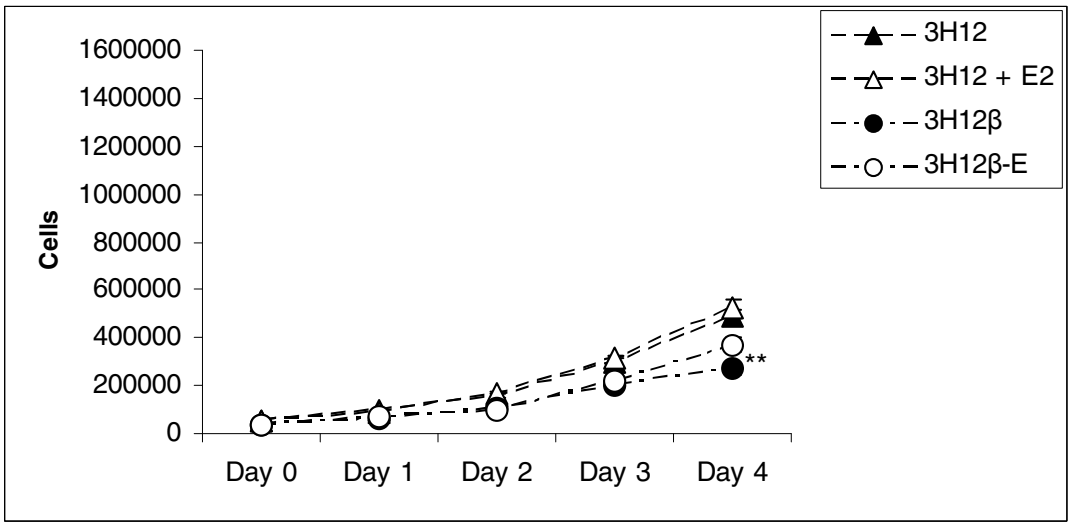

Figure 4.5. Comparison of 3H12 and 3H12β Cell Proliferation Rates. Cells were treated with 10^{-8}M 17β-estradiol or vehicle (ethanol) and assayed on indicated days. Cells were harvested and enumerated by Coulter Counter®. The results indicate that the reconstitution of ERβ in the 3H12 cells leads to a significant decrease in cellular proliferation. However, the 3H12β cells display a modest increase in proliferation in response to estrogens. ** indicates significant change ($p<0.05$) as compared to Ishikawa-3H12 cells.

*Cell cycle profiles of Ishikawa-3H12 ER expressing cells***.** The results of the proliferation assays utilizing the Ishikawa-3H12 cells expressing ERα or ERβ provided novel information regarding the roles of these receptors in human Ishikawa endometrial epithelial cells. The fact that introduction of ERα, in the absence of 17β-estradiol, restores the ability of the Ishikawa-3H12 cells to proliferation at rates comparable to that of conventional Ishikawa cells is remarkable. Furthermore, the introduction of ERβ significantly, in the absence of 17β-estradiol, reduced the ability of the Ishikawa-3H12 cells to proliferation. BrdU incorporation followed by flow cytometry assays were undertaken to evaluate the cell cycle profiles of these cells lines, in a search for possible mechanisms of the increase (3H12α cells) or decrease (3H12β cells) in cellular proliferation. The flow cytometry data showed no significant differences between the profiles of Ishikawa-3H12 and 3H12α cells (Figure 4.6 b and c). Unfortunately, this finding does not help us understand the mechanisms by which ERα expression in the Ishikawa-3H12 cells leads to increased proliferation. More puzzling is the fact that conventional Ishikawa cells have a greater population of cells in G1 and fewer cells in the synthesis (S)-phase and G2 phases of the cell cycle than Ishikawa-3H12 cells (Figure 4.6 a and b). However, a reasonable explanation for the conventional Ishikawa and the 3H12α cells may be that they simply progress through the cell cycle at a faster rate than the Ishikawa-3H12 cells.

Despite the data collected from conventional Ishikawa and 3H12α cells, flow cytometry data showed that Ishikawa-3H12 cells expressing ERβ (3H12β) had an S-phase population of 22% (Figure 4.6 b), while the parental strain (3H12) had an S-phase population of 34% (Figure 4.6 d). This observation is consistent with our findings that ERβ reduces proliferation of the Ishikawa cells. This finding is also consistent with the observation that

ERβ negatively regulates cyclin D1, a key component needed for the G1/S transition of the cell cycle, leading to a reduction in the S-phase population of cells thereby leading to a reduction in proliferation in T47D and MCF7 breast adenocarcinoma cells (Hartman, et al. 2006; Paruthiyil et al. 2004; Strom et al. 2004).

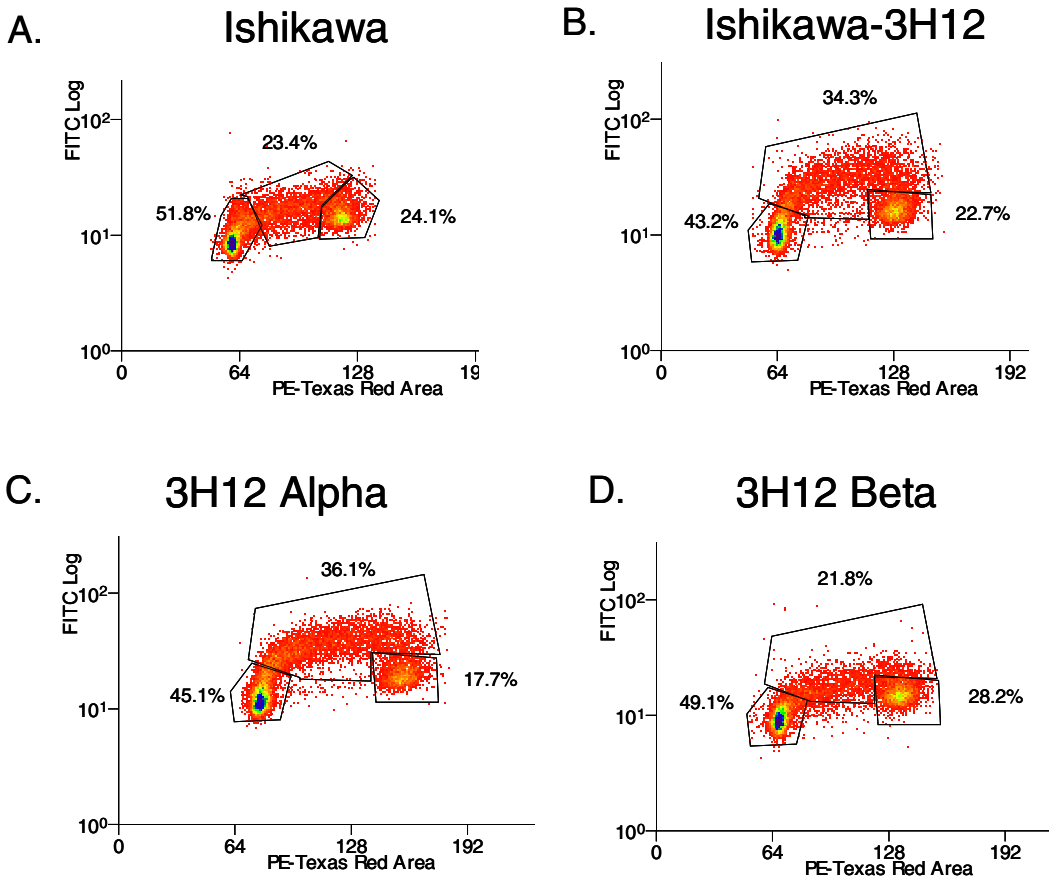

Figure 4.6. Comparison of Cell Cycle Profiles of Conventional Ishikawa, Ishikawa-3H12, ERα and ERβ expressing cells. Ishikawa (a), Ishikawa-3H12 (b), 3H12α (c), and 3H12β (d) cells following BrdU incorporation. 3H12β cells have fewer cells in the Synthesis (S)-phase of the cell cycle (22%) as compared to the partenal Ishikawa-3H12 cell line (34%). This indicates that introduction of ERβ may inhibit the G1 phase of the cycle.

Discussion

Up to the present, most studies on the function of the estrogen receptors have utilized transient transfections of the ERs or techniques to reduce the expression of the ERs, including the introduction of small interfering RNA (siRNA) or oligodeoxyribonucleotides (ODN) into endometrial target cells (Ali, et al. 2000; Taylor, et al. 2002; Zhang, et al. 2006). Although these techniques have proved to be useful by providing a foundation for further studies, data collected from them cannot provide a definitive answer with regard to specific receptor function. In the current study, we utilized a system in which the estrogen receptors were stably transfected into a line of cells manifesting an ER null-background. The level of expression of the ERs that were detected in the cell lines we constructed are not increased excessively, rather they are comparable to those in the Ishikawa cell line (Figure 4.3). The construction of the Ishikawa-3H12 derived cell lines expressing ERs (3H12α and 3H12β) has provided an alternative approach to studying the individual ER's that should aid our understanding of the functions of these receptors in endometrial adenocarcinoma cells.

Ishikawa endometrial adenocarcinoma cells, an established endometrial epithelial cancer cell line, have been used extensively to study the effects of estrogens and selective estrogen receptor modulators (SERMS) in the human endometrium. Recently, it was recognized that the Ishikawa cell line consisted of a heterogenous population containing cells with varying ER expression profiles (Nishida, et al. 1996). Subsequently, this cell line was cloned revealing several new cell lines with specific ER phenotypes (Nishida 2002; Nishida et al. 1996). In the current study, we utilized the original Ishikawa cells and the Ishikawa-3H12 clone, which lacks expression of either ER subtype (Nishida et al. 1996). Initial studies revealed a significant increase in the proliferation of the conventional Ishikawa cells and not

in the Ishikawa-3H12 clone, when they were treated with physiologic concentrations of 17β-estradiol. This finding, along with the results of the Western immunoblot analysis showing the absence of ERα or ERβ, validated that the 3H12 clone lacked functional ER expression, thereby rendering these cells incapable of responding to estrogens or SERMS via a receptor mediated mechanism.

In an effort to understand the contributions of the two different estrogen receptors, we also used two commercially available specific agonists of the ERα and ERβ, Propylpryazole-triol (PPT) and Diarylpropionitrile (DPN), respectively. Treatment of the 3H12 clone with the agonists revealed no change in proliferation as compared to vehicle treated samples, further solidifying the observation of a lack of ER expression (Figure 4.1). However, treatment of the conventional Ishikawa cells with either agonist revealed increases in proliferation, which could be prevented by treatment with the antiestrogen ICI 182780. These findings indicate that the ERα agonist can induce estrogen receptor-mediated proliferation of endometrial adenocarcinoma cells. Furthermore, we know from the studies using ICI 182780 that the effects of both agonists are in fact mediated through the ERs.

To confirm the findings obtained by using the highly specific agonists in the Ishikawa cell system, we created cell lines that express the ER subtypes individually. To complete this task we used the Ishikawa-3H12 clone, an isogenic cell line which lacks functional expression of either ER subtype (Hanekamp et al. 2003; Nishida et al. 1996). When cells expressing the receptors were tested, we noticed a restoration of proliferation in the 3H12α cells. In fact, these cells proliferated at approximately the same rate as the normal Ishikawa cell line, indicating that ERα is probably the primary receptor involved in stimulation of Ishikawa cell proliferation. On the other hand, the 3H12β cells showed a reduced capacity to

proliferate when compared against the parental 3H12 cell line, indicating that ERβ may have an opposing function in the context of this model system. However, when treated with 17β-estradiol, the 3H12β cells showed a modest but significant increase in proliferation, indicating that this receptor can also positively influence proliferation in response to ligand.

In summary, the data from this study suggests that ERα is the major receptor subtype involved in the constitutive and estrogen-induced proliferation of Ishikawa cells. Treatment of the Ishikawa cells with PPT, the highly specific ERα agonist, revealed a 121% increase in proliferation after 4 days of treatment (Figure 4.2). Additionally, its expression alone reconstitutes the exaggerated estrogen response seen in the conventional Ishikawa cell line (Figure 4.4). On the other hand, ERβ appears to reduce proliferation by impeding the G1/S transition of the cell cycle, possible due to decreased levels of cyclin D1 (Figure 4.6). However, without further studies we cannot rule out the possible involvement of other cell cycle proteins as Strom *et al.* (2004) suggested that many other components of the cell cycle associated with proliferation, e.g. cyclin E and Cdc25A, were decreased with ERβ expression in T47D breast cancer cells (Strom et al. 2004). Activation of this cell line with 17β-estradiol suggests that ERβ can induce proliferation in this model system. However, the estrogen-induced increase in proliferation observed in the 3H12β cells never reach the proliferation levels of the parental 3H12 cell line, indicating that the increase is at best modest and well controlled. Our observations are consistent with findings in the breast, ovary, prostate, and colon regarding ERβ's inhibitory effects in the epithelium of these tissues with respect to ligand-induced proliferation (Bardin et al. 2004; Brandenberger et al. 1998; Campbell-Thompson et al. 2001; Fixemer et al. 2003; Foley et al. 2000; Horvath et al. 2001; Latil et al.

2001; Park et al. 2003; Pujol et al. 1998; Roger et al. 2001; Rutherford et al. 2000; Zhao et al. 2003).

Chapter V: Experimental Conclusions

Alteration of the ERα/ERβ Ratio in the Human Endometrium

Recent in vitro studies focusing on the roles of the estrogen receptors α and β in the epithelium of breast suggests divergent roles for the receptor subtypes. An overwhelming majority of in vitro data suggests that ERα is stimulatory and ERβ is inhibitory to cell proliferation and differentiation in breast epithelium. Further studies in several human tissues have shown alterations in the ratio of ERα to ERβ in diseased specimens. Herein, we provide evidence that is consistent with findings of an altered ER ratio in human endometrial cells as has been seen in other human tissues, including breast, ovary, prostate, and colon. Utilizing TMA technology and immunohistochemistry (IHC), we were able to observe an alteration in the normal nuclear ERα/ERβ ratio in the stromal and epithelial compartments of the human endometrium. This alteration was observed in hyperplastic human endometrium, which is a critical phase in estrogen-dependent endometrial neoplasia, as twenty-five percent of the most severe cases progress into endometrial adenocarcinomas. Morover, the stromal and epithelial compartments of malignant samples revealed alterations in the "normal" ERα/ERβ ratio. Alterations of the ERα/ERβ ratio in the studies described here involve a relative increase in ERα, suggesting that this receptor subtype is primarily responsible for the increased proliferation seen in the hyperplastic and malignant tissues. At the same time the relative decrease in ERβ may remove an impediment to proliferation or a limiting control of

this process. Furthermore, this observation strongly suggests that maintaining a normal receptor ratio is essential to a normal response to estrogens within this tissue microenvironment.

Immunohistochemical studies that evaluated cytoplasmic staining of the receptors revealed patterns consistent with the hypothesis that a relatively high level of ERβ is found in normal endometrium and may serve to impede cell proliferation in the human endometrium. First, immunostaining of ERα in the cytoplasm of stromal cells showed that approximately 5% of normal cells were positive, while there was no cytoplasmic immunostaining detected in hyperplastic or malignant samples. On the other hand, 0% of normal stromal cells stained positively for cytoplasmic ERβ, while 6% of the cells in the hyperplastic and 10% of the cells in the malignant samples were positive (Figure 2.4).

Positive results for ERα immunostaining of the cytoplasm of epithelial cells were found in 13% of normal cells; 61% of hyperplastic samples; and 22% malignant cells. On the other hand, 55% of normal epithelial cells demonstrated cytoplasmic ERβ staining, while hyperplastic samples revealed a slight reduction to 43%. Furthermore, a significant increase in cytoplasmic ERβ staining in the malignant epithelial cells was observed, where 91% of epithelial cells were positive (Figure 2.5). The significance of changes in receptor levels in stromal cells from malignant samples must be considered with regard to the notable reduction in stromal cells in the immediate environment of the epithelial tumor cells.

The results obtained in pursuit of this aim are consistent with the hypothesis that normal conditions are defined by a balance between ERα and ERβ. ERβ is deduced to be an inhibitor of cell proliferation in the human endometrium. This deduction is based on the observation that the expression levels of ERβ decrease from their homeostatic level during

the progression of endometrial cancer and the increasing cell proliferation associated with this change. We have found significant alterations in the normal homeostatic expression of the receptor subtypes in hyperplastic endometrial tissue samples. We interpret these alterations as leading to an abnormal environment in which the relative level of ERα is increased over that of its counterpart ERβ. These observations appear to be consistent with the overall hypothesis regarding the independent roles of the receptors, in that ERα is a stimulator of growth while ERβ serves as an inhibitor of proliferation.

The Roles of the Estrogen Receptors α and β in Gene Regulation

In Chapter III, we present results that support the hypothesis that 17β-estradiol acts through ERα to exert an inductive effect on gene regulation in the human Ishikawa cells. This interpretation was based on observations using PPT, a specific agonist of ERα. It was also deduced from studies that used Ishikawa cells that express only the ERα (3H12α cell line). These studies showed that estrogenic induction on ERα increases both the quantity of mRNA transcribed from the placental alkaline phosphatase (ALPP) gene as well as ALPP activity in the cells. We were neither able to confirm nor deny the classic estrogen signaling pathway in the involvement of the regulation of ALPP. Our data, however, does provide evidence that upregulation of ALPP, our model estrogen-inducible gene, is mediated at least in part, through the activation of the MAPK and (PI)3-kinase pathways in response to treatment with 17β-estradiol. This finding provides support for the existence of membrane and/or cytoplasmic actions of the estrogen receptors. We were unable to conclude that ERβ is inhibitory with regard to the regulation of the ALPP gene induction using the specific agonist DPN or when using the 3H12β cell line, which only expresses the ERβ gene. However, we

can conclude that if ERβ functions in the cytoplasm it does not appear to be inhibitory with regard to the induction of ALPP gene expression.

The Roles of the Estrogen Receptors α and β in Cellular Proliferation

In Chapter IV, we present data consistent with the hypothesis that ERα is the key ER responsible for proliferation, while ERβ is inhibitory in Ishikawa epithelial cells. In an effort to understand the individual contributions of the receptors, we utilized two commercially available highly specific agonists of the ERα and ERβ, Propylpryazole-triol (PPT) and Diarylpropionitrile (DPN), respectively. Treatment of Ishikawa cells with PPT, but not DPN, revealed an increase in proliferation, which could be prevented with the ER inhibitor ICI 182780. These findings show that ERα is the primary receptor involved in the induction of estrogen receptor mediated proliferation of these endometrial adenocarcinoma cells.

To confirm the findings in studies that use the specific agonists for ERα and ERβ in Ishikawa cells, cell lines that express either ERα or ERβ individually (3H12α and 3H12β) were created and validated. After constructing cells expressing these receptors, restoration of proliferation in the cells expressing the ERα gene (3H12α) was observed. In fact, these cells proliferate at approximately the same rate as the normal Ishikawa cells, and much more rapidly than the 3H12 cells from which they were derived. Again this indicates that ERα is the primary receptor involved in Ishikawa cell proliferation. On the other hand, the 3H12β cells showed a reduced capacity to proliferate when compared to the parental 3H12 cell line, indicating that ERβ may have an opposing action on the proliferation of Ishikawa cells. When treated with 17β-estradiol, the 3H12β cells showed a modest but significant increase in

proliferation, indicating that this receptor can produce some positive influence on cell proliferation in response to the ligand.

Taken together, our results show that ERα is the major receptor subtype involved in the constitutive and estrogen-induced proliferation of Ishikawa cells. Treatment of the Ishikawa cells with PPT, the specific ERα agonist, produced a 121% increase in proliferation after 4 days of treatment. Additionally, the expression of ERα alone reconstitutes the exaggerated estrogen response seen in the Ishikawa cell line. On the other hand, ERβ appears to reduce the proliferation. Additionally, when cells expressing only ERβ (3H12β) were treated with BrdU and compared against the parental cells, we observed a significant reduction in the S phase population. Since ERβ is known to reduce the mRNA levels of cyclin D1, a key mediator of the G_1/S transition, it may act to impede cell cycle progression, possibly in the G_1 phase.

How We Addressed the Proposed Hypothesis

Our original hypothesis was that the Estrogen Receptor β acts as a modulator of the Estrogen Receptor α in the human endometrium. To address this hypothesis we pursued three Specific Aims as part of the original research proposal. These Aims and supporting evidence are as follows:

<u>Specific Aim I.</u> *Determine the Relative Levels and Expression Patterns of ERα and β in Normal, Hyperplastic, and Malignant Endometria.*

Herein we provide evidence of an alteration in the ratio of ERα to that of ERβ in human endometrial tissue as it transitions toward malignancy. There are two broad

explanations for the IHC findings contained within this dissertation. The first explanation is that alterations in the ER expression levels leading to alterations in the stromal and epithelial compartments are haphazard events that are meaningless with regard to carcinogenesis of the human endometrium. However, based on our current understanding of the biology of the ERs, estrogen being the classic etiologic risk factor type-1 endometrial lesions, and our statically significant results, we do not believe that these changes are simply haphazard events. The second explanation is that changes in the ER expression levels are in fact meaningful and reveal a possible mechanism that permits the endometrium to become hypersensitive to estrogens. When considering the second explanation, in concert with our current understanding of the functions of the ER subtypes in other tissue types, the general roles for the receptors in progression of endometrial cancer become apparent.

Using Dako® antibodies against the estrogen receptor subtypes we were able to observe alterations in the frequency and intensity of ER staining in both hyperplastic and malignant endometrial tissue samples. First, we were able to show an alteration from the normal ratio in stromal cells. In stromal cells from hyperplastic tissues, this change was observed as a decrease in ERβ intensity, while ERα remained the same as those in normal tissue samples (Chapter II). We observed a significant decrease in ERα immunostaining and a significant increase in ERβ immunostaining in stromal cells from malignant tissues. We believe that this may be an adaptive response by the stromal cells to constrain the proliferation of the epithelium in response to estrogens. However, the size of the stromal compartment in malignant endometrial tissue is diminished in size and therefore cannot produce the necessary paracrine factors to reduce the excessive proliferation of the epithelial compartment.

Additionally, we observed an alteration of the ratio in the epithelial compartment. Unlike the stroma, alterations in the epithelium was manifested by an increase in the intensity of ERα staining in the nuclei of epithelial cells from hyperplastic tissues and these elevated levels persisted in the epithelium of malignant samples. ERβ expression was the same in the normal and hyperplastic samples but increased significantly in malignant samples. One might speculate that the increase in expression of ERβ may be a result of the epithelial cells responding to modulate the high rate of proliferation in response to estrogens. However, we think that alterations/mutations in various signaling pathways, including (PI)3-kinase and MAPK, as a result of loss of the PTEN tumor suppressor gene, can help overcome any beneficial inhibitory effects that ERβ may have. Furthermore, we have shown in the studies in Aim 3 (Chapter IV) that ERβ activity reduces cellular proliferation. However, cells expressing this receptor can undergo a modest but significant increase in proliferation in response to estrogen. Therefore, it is possible that the endometrial adenocarcinoma cells may harness this response to further increase proliferation of epithelial cells in response to estrogens.

The work presented in this Specific Aim provides novel insight into mechanisms that may permit the human endometrium to become hypersensitive to estrogens. First, evidence utilizing immunohistochemistry (IHC) suggests an alteration in the ratio of ERα to ERβ in hyperplastic endometrial samples, which is consistent with findings in the breast, prostate, colon, and ovaries (Bardin et al. 2004; Brandenberger et al. 1998; Campbell-Thompson et al. 2001; Fixemer et al. 2003; Foley et al. 2000; Horvath et al. 2001; Latil et al. 2001; Park et al. 2003; Pujol et al. 1998; Roger et al. 2001; Rutherford et al. 2000; Zhao et al. 2003). Clinical findings and the current literature suggests that a small portion of endometrial hyperplasias

transition to malignancy. What is not known is whether the alteration in the ER profile is a determining factor in the transition of hyperplasia to cancer.

Specific Aim II. Characterize the Roles of the Estrogen Receptors α and β on Estrogen-Induced Gene Regulation in Human Endometrial Epithelial Cells.

Using agonists of the estrogen receptors and cell lines that express either receptor subtype alone, we have been able to elucidate the roles of the estrogen receptors on the regulation of the placental alkaline phosphatase gene (ALPP). We have shown that the ERα is the primary receptor involved in the upregulation of ALPP by utilizing highly specific agonists of the ER's. In the case of the ERα agonist, PPT, we noticed an increase in ALPP activity at concentrations as low as 10^{-10}M. Further studies using 3H12α cells revealed that these cells increase the expression of ALPP in response to physiologic concentrations of 17β-estradiol. Studies evaluating the regulatory role of ERβ showed that signaling through this receptor had no impact on ALPP. These studies included use of the ERβ agonist, DPN, and 3H12β cells, which express the β subtype only.

Additional studies were aimed at understanding how the ALPP gene was regulated at the molecular level. Two putative estrogen response elements (EREs) were identified within the 5' flanking region of ALPP. Chromatin Immunoprecipitation (ChIP) assays were unable to detect either ERE in DNA fragments precipitated with antibodies against the receptors. Although we were unable to precipitate the receptors bound to either ERE we are not able to exclude the possibility that these interactions are present, as levels of binding may be below the sensitivity of this assay. Further studies explored the non-classic activation of the ER's and the stimulation of the MAPK and (PI)3-kinase pathways in response to treatment with

17β-estradiol. We were able to inhibit the upregulation of ALPP by 17β-estradiol using inhibitors of either MAPK or (PI)3-kinase, U0126 or LY294002, respectively. These results suggest that both pathways are activated by ERα present in the membrane or cytoplasm of these cells. Furthermore, the data presented in Chapter III provides supporting evidence that endometrial epithelial cells contain membrane and/or cytoplasmic ERs which can activate cell survival cytoplasmic pathways such as (PI)3-kinase and MAPK to elicit effects on target genes.

An additional explanation for our observations may be that activation of the (PI)3-and MAP Kinase pathways by 17β-estradiol activates the ligand-independent (AF-1) region of nuclear ER through crosstalk thereby positively influencing transcription of the ALPP gene. However, the analysis of the 5' flanking region of the gene with the Dragon ERE Finder revealed two putative EREs located approximately 2000 basepair away from the TATA start site on the reverse complement strand. Moreover, this software predicted that both putative EREs would have an extremely low probability of being actual response elements. Therefore, regulation of the ALPP gene through nuclear ER, via the classic or non-classic pathway, may not be an accurate reflection of the normal biology of this gene. Furthermore, we were unable to demonstrate, by ChIP, that either of the ERs bound to the putative response elements. This suggests that crosstalk from the cytoplasmic signaling pathways to nuclear ER, which in turn regulates genes by binding to EREs, is unlikely to be a plausible explanation for these observations at this time.

An additional explanation comes from the idea that activation of the cytoplasmic pathway, MAPK, by growth factors has been implicated in stimulating the activity of p160 coactivator family members (Lopez, et al. 2001). As discussed in the Introduction to this

dissertation (Chapter I), the p160 proteins are critically important in ER-mediated transcription. Therefore, it may be reasonable to assume that inhibition of the (PI)3-Kinase and MAPK cytoplasmic pathways serves to reduce the activity of p160 coactivators, which would lead to a decrease in ER mediated transcription. However, this explanation may not fully explain the observations contained within Chapter III, as the current literature does not suggest that the ERs need active coactivators to induce transcription.

Introduction of ERα into 3H12 cells revealed an increase in ALPP activity over that of parental Ishikawa-3H12 cells. However, when compared to conventional Ishikawa cells, upregulation of ALPP by the 3H12α cells appears to be "weak", which may suggest that ERα is insufficient in the upregulation of this gene or that other required factors are missing. First, the evidence presented within this dissertation suggests that signaling through ERα is in fact sufficient to upregulate ALPP expression. Evidence presented within Chapter III utilized conventional Ishikawa cells and highly-specific ER agonists in combination with the ER inhibitor ICI 182780. These studies revealed that ERα, not ERβ, was responsible for upregulation of ALPP message and enzymatic activity. Furthermore, data gathered utilizing 3H12 cells expressing ERα or ERβ confirmed that ERα is the receptor responsible for inducing ALPP message and enzymatic activity.

The second explanation which may explain the "weak" induction of ALPP by estrogen is the notion that the parental Ishikawa-3H12 cell line lacks other required factors. This may provide a better explanation as to why the Ishikawa-3H12 cells "weakly" induce ALPP activity and message when compared against conventional Ishikawa cells. The current literature suggests the Ishikawa-3H12 cell-line not only lacks functional expression of the ERs, but also lacks expression of both progesterone receptor (PR) subtypes (Hanekamp et al.

2003; Nishida 2002; Nishida et al. 1996). Therefore, it is reasonable to propose that key components involved in the normal upregulation of the ALPP gene may also be absent from the Ishikawa-3H12 cells.

The third and final explanation with regard to the "weak" induction of ALPP in Ishikawa-3H12α cells as compared against conventional Ishikawa cells could come from differences in the subcellular localization of the ERs. Data presented within Chapter IV of this dissertation suggests that membrane and/or cytoplasmic versions of ERα have an important role in the induction of ALPP message and activity. Therefore, the 3H12α cells may not have the same number of membrane/cytoplasmic receptors as the conventional Ishikawa cells. This final point was not evaluated within this dissertation and needs to be addressed by immunofluorescence or Western immunoblot of proteins from cellular fractions.

Specific Aim III. Characterize the Roles of the Estrogen Receptors α and β on Cellular Proliferation in Human Ishikawa Endometrial Epithelial Cells.

Much of the work in the literature evaluating the roles of the estrogen receptors in human endometrial epithelial cells has implicated redundant roles for these receptors. Many of these studies utilized small interfering RNA (siRNA) to reduce the expression of one receptor, while attempting to study the biology of the second (Ali et al. 2000; Taylor et al. 2002; Zhang et al. 2006). The downfall with this approach is that cells maintain partial expression of the target receptor, which may contribute to the observed results. Additionally, separate studies have transiently overexpressed the ERs to study their biology. Unfortunately, overexpression studies suffer from problems analogous to those of experiments with the use of siRNA. The work presented in this dissertation using cells that express only one receptor

at a time allows for the elucidation of individual roles and provides evidence supporting the hypothesis that the ERs have distinct and divergent influences on the proliferation of human endometrial adenocarcinoma cells.

With the use of specific agonists of the estrogen receptors and cell lines that express either receptor subtype alone, we have been able to elucidate the roles of the estrogen receptors on proliferation of Ishikawa endometrial epithelial cells. First, we have shown that the ERα is the primary receptor involved in increased proliferation induced by 17β-estradiol. In the case of the ERα agonist PPT, we observed an increase in proliferation at concentrations equivalent to those physiologic for 17β-estradiol, 10^{-8}M. Further studies using 3H12α cells revealed that introduction of ERα leading to its expression in these cells could restore the ability of the cells to produce a significant response to 17β-estradiol. Further studies evaluating the role of ERβ in proliferation showed that treatment of conventional Ishikawa cells with 10^{-8}M DPN produced no significant change in proliferation. However, when cells expressing ERβ only (3H12β) were used, we observed that this receptor had an inhibitory effect on proliferation and cell cycle progression, even in the absence of 17β-estradiol treatment. The creation of new cell lines which express ERα, ERβ, or both have shown that the ERs possess different roles in the context of proliferation of the Ishikawa adenocarcinoma cells. This finding conflicts with previous reports in human endometrial cells, but parallels findings in human breast MCF-7 and T47D adenocarcinoma cells. (Hartman et al. 2006; Paruthiyil et al. 2004; Strom et al. 2004).

We recognize that the model system created for this dissertation may not completely reflect the normal biology of human endometrial epithelial cells. First, the use of a well-differentiated human endometrial adenocarcinoma cell line may not accurately reflect the ER

biology in normal human endometrial epithelial cells. However, we were limited to the use of abnormal cell lines because normal endometrial epithelial cell lines were not available at the beginning of this dissertation. Within the past few months a normal epithelial cell line, hTERT-Endometrial Epithelial Cells, has been made available to our laboratory. Although, the normal endometrial epithelial cell line expressing both ERs has been identified, it could not be used for studies expressing only one estrogen receptor at a time. Furthermore, isolating a normal cell line that lacks expression of the ERs would be virtually impossible, as the lack of one or both ERs would lead one to believe that the cells were abnormal.

Additionally, studies contained in this dissertation did not utilize co-culture systems, which would approximate better the normal biology of the uterus. However, the goal of this dissertation was to study the effects of 17β-estradiol, as opposed to paracrine mediated factors emanating from the stroma, on epithelial cells. Therefore, the use of the co-culture model system in this dissertation would not permit the elucidation of the roles of the estrogen receptors within our target cells. Because of our efforts and techniques this dissertation has yielded novel observations which will further our understanding of ER biology within epithelial cells of the human endometrium.

Stable reconstitution of the ERα or ERβ into 3H12 cells has provided an excellent model system for the elucidation of the roles of the receptor subtypes in endometrial adenocarcinoma cells. Figures 4.4 and 4.5 reveal the effects of both ERα and ERβ, respectively, on proliferation of endometrial adenocarcinoma cells. In both figures, we observe significant changes in proliferation in the absence of 17β-estradiol. That is, reconstitution of ERα significantly increased proliferation, while introduction of ERβ significantly decreased proliferation of the parental cell line. Throughout this dissertation I

have presented a case that the activation of the ERs is an extremely dynamic and complex process. With that in mind, there are several plausible explanations that may shed light on the underlying mechanisms by which the effects of the ERs are elicited in the absence of added estradiol in this model system. First, despite our efforts to remove all steroid hormones from the medium, via charcoal-stripping the fetal bovine serum, trace amounts of steroids may remain and activate the ERs through the classic pathway. Second, the ERs are phosphoproteins which can be stimulated/activated by various cytoplasmic signaling pathways, including (PI)3-Kinase and MAP-Kinase (Campbell et al. 2001; Kato et al. 1995; Kato et al. 2000). Many cytoplasmic signaling pathways have been shown to be stimulated by various growth factors, which may be contained within the tissue culture medium used for these studies. Therefore, it is plausible to postulate that activation of the receptors by the non-classic pathway may explain the observations with regard to significant changes in proliferation of the 3H12 cells in the absence of 17β-estradiol.

The Experimental Rigor with which the Hypothesis was Addressed

Our hypothesis was that ERβ acts as a modulator of ERα signaling in the human endometrium. We have tested this hypothesis in three series of experiments. First, we used immunohistochemistry to observe an alteration of the normal ERα/ERβ ratio in premalignant endometrial tissues. Prior to embarking on these studies we consulted with a biostatistian to determine the number of patient samples needed to achieve 95% power within our studies. To avoid observer bias in interpreting images, scorers were provided with digital images of tissue cores to be scored semi-quantitatively with only a coded study number to distinguish them. We elucidated the roles of the receptor subtypes on the estrogen-inducible alkaline

phosphatase gene expression and cellular proliferation of Ishikawa human endometrial epithelial cells, using commercially available highly specific agonists of the estrogen receptors. The activity of the two receptors was also evaluated using cell lines that express only one receptor subtype. Each experiment included within this dissertation was repeated at least three times and the results herein are representative of each experimental data set.

Future Directions

The studies performed in preparation of this dissertation have been geared primarily to elucidate the roles of the estrogen receptors α and β in the epithelial cells of the human endometrium. There are numerous experiments that could be done to more completely characterize the physiologic roles and molecular mechanisms of these receptors in this tissue. The additional studies may be classified into several broad categories: 1) further characterize the conditions that modify the ERα/ERβ ratio in the human endometrium 2) characterize the molecular effects of ERα and ERβ on more classic estrogen-responsive genes, including those known to contain EREs 3) further characterize the effects of ERα and ERβ on cell cycle progression in endometrial epithelial cells and 4) characterize the effects of the receptor subtypes on paracrine mediators from the stromal compartment.

A list of possible approaches for the study of these questions follows.

1) Since the risk of developing endometrial cancer is associated with unopposed estrogens and increased age, additional studies could evaluate the levels of the ERs in pre-, peri-, and post-menopausal women. If our data suggesting an alteration in the ratio of ERα to ERβ is correct we might expect to see a change as women age. Information taken from this

series of studies may further support our observations, and may be useful in developing strategies to prevent malignant transformation of the endometrium.

2) Additional studies could determine if the alteration in the expression of ERα and ERβ occurs prior to the development of morphologic hyperplasia in the human endometrium. A larger study could evaluate more independent stages of normal and diseased endometria rather than grouping into normal, hyperplastic, and malignant samples as was done in this study. We could include endometrial samples from the proliferative and secretory phases of the menstrual cycle, endometria from women predisposed to hyperplasia (i.e. Polycystic Ovarian Syndrome, Cowden's syndrome, simple hyperplasia, complex hyperplasia, complex hyperplasia with atypia, and endometrioid (Type-1) adenocarcinomas. Having more categories to evaluate for changes in the ERα/ERβ ratio could validate our current finding's and also determine how early in the course of adenocarcinoma development this ratio changes.

3) We could examine the roles of the receptors on genes known to contain classic EREs within their promoter to understand if the receptors continue to adhere to the overall hypothesis regarding their independent roles. There are several genes in which extensive characterization has been completed on the estrogen response elements contained within their promoters. These genes include, but are not limited to, Capthesin D and Cyclin D1. The latter is of great importance as it may play a critical role in the effects of the receptors on progression through the cell cycle.

4) We observed that the two estrogen receptors have different roles in the regulation of cell proliferation by our endometrial adenocarcinoma cells. This raises questions about how the two receptors function in the stimulation and/or inhibition of cell cycle progression.

To be better informed about this, we could evaluate the effects of each receptor on the progression of the cell cycle in specific endometrial cells. We could use BrdU incorporation and flow cytometry to determine the cell cycle profiles of the null and receptor expressing cells. We could then complete a systematic evaluation of cell cycle proteins that are involved in the inhibition or progression through the cycle including their expression and activation. This information may give us insight about why 17β-estradiol acting through ERα increases cellular proliferation above that in the receptor null parental cells, while 17β-estradiol acting through ERβ reduces proliferation of our cells.

5) It has recently been recognized that normal human endometrial epithelial cells do not proliferate directly in response to estrogens in vivo. Rather, the stromal cells respond to estrogens and releases paracrine mediators, which induce or repress cellular proliferation by endometrial epithelial cells. Therefore, we could evaluate the effect of stable overexpression of one receptor or the other in normal stromal cells to understand the independent roles of the receptors in the release of paracrine factors by the stromal cells. The use co-culture models or treating epithelial cells with media conditioned by stromal cells will allow us to test whether the actions of estrogen through the two receptors in the endometrial cells demonstrates that divergent functions of ERα and ERβ mediated estrogen responses provides a consistent basis for the observed features of endometrium in vivo.

Chapter VI. References

Akhmedkhanov A, Zeleniuch-Jacquotte A & Toniolo P 2001 Role of exogenous and endogenous hormones in endometrial cancer: review of the evidence and research perspectives. *Ann N Y Acad Sci* **943** 296-315.

Ali IU, Schriml LM & Dean M 1999 Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity. *J Natl Cancer Inst* **91** 1922-1932.

Ali S, Metzger D, Bornert JM & Chambon P 1993 Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. *Embo J* **12** 1153-1160.

Ali SH, O'Donnell AL, Balu D, Pohl MB, Seyler MJ, Mohamed S, Mousa S & Dandona P 2000 Estrogen receptor-alpha in the inhibition of cancer growth and angiogenesis. *Cancer Res* **60** 7094-7098.

Allen E & Doisy EA 1983 Landmark article Sept 8, 1923. An ovarian hormone. Preliminary report on its localization, extraction and partial purification, and action in test animals. By Edgar Allen and Edward A. Doisy. *Jama* **250** 2681-2683.

Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM & Meltzer PS 1997 AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* **277** 965-968.

Arnold JT, Kaufman DG, Seppala M & Lessey BA 2001 Endometrial stromal cells regulate epithelial cell growth in vitro: a new co-culture model. *Hum Reprod* **16** 836-845.

Arnold JT, Lessey BA, Seppala M & Kaufman DG 2002 Effect of normal endometrial stroma on growth and differentiation in Ishikawa endometrial adenocarcinoma cells. *Cancer Res* **62** 79-88.

Bardin A, Hoffmann P, Boulle N, Katsaros D, Vignon F, Pujol P & Lazennec G 2004 Involvement of estrogen receptor beta in ovarian carcinogenesis. *Cancer Res* **64** 5861-5869.

Beato M, Herrlich P & Schutz G 1995 Steroid hormone receptors: many actors in search of a plot. *Cell* **83** 851-857.

Beckmann CRB 2002 *Obstetrics and gynecology*. Philadelphia: Lippincott Williams & Wilkins.

Beekman JM, Allan GF, Tsai SY, Tsai MJ & O'Malley BW 1993 Transcriptional activation by the estrogen receptor requires a conformational change in the ligand binding domain. *Mol Endocrinol* **7** 1266-1274.

Bissell MJ, Weaver VM, Lelievre SA, Wang F, Petersen OW & Schmeichel KL 1999 Tissue structure, nuclear organization, and gene expression in normal and malignant breast. *Cancer Res* **59** 1757-1763s; discussion 1763s-1764s.

Bourdeau V, Deschenes J, Metivier R, Nagai Y, Nguyen D, Bretschneider N, Gannon F, White JH & Mader S 2004 Genome-wide identification of high-affinity estrogen response elements in human and mouse. *Mol Endocrinol* **18** 1411-1427.

Brandenberger AW, Tee MK & Jaffe RB 1998 Estrogen receptor alpha (ER-alpha) and beta (ER-beta) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: down-regulation of ER-beta in neoplastic tissues. *J Clin Endocrinol Metab* **83** 1025-1028.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA & Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389** 753-758.

Bunone G, Briand PA, Miksicek RJ & Picard D 1996 Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *Embo J* **15** 2174-2183.

Calle EE, Miracle-McMahill HL, Thun MJ & Heath CW, Jr. 1995 Estrogen replacement therapy and risk of fatal colon cancer in a prospective cohort of postmenopausal women. *J Natl Cancer Inst* **87** 517-523.

Campbell-Thompson M, Lynch IJ & Bhardwaj B 2001 Expression of estrogen receptor (ER) subtypes and ERbeta isoforms in colon cancer. *Cancer Res* **61** 632-640.

Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S & Nakshatri H 2001 Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *J Biol Chem* **276** 9817-9824.

Cavailles V, Dauvois S, Danielian PS & Parker MG 1994 Interaction of proteins with transcriptionally active estrogen receptors. *Proc Natl Acad Sci U S A* **91** 10009-10013.

Chen JD & Evans RM 1995 A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* **377** 454-457.

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** 113-123.

Cicatiello L, Ambrosino C, Coletta B, Scalona M, Sica V, Bresciani F & Weisz A 1992 Transcriptional activation of jun and actin genes by estrogen during mitogenic stimulation of rat uterine cells. *J Steroid Biochem Mol Biol* **41** 523-528.

Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, Taylor J, Lubahn DB & Cunha GR 1997 Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. *Proc Natl Acad Sci U S A* **94** 6535-6540.

Cooke PS, Young PF & Cunha GR 1987 Androgen dependence of growth and epithelial morphogenesis in neonatal mouse bulbourethral glands. *Endocrinology* **121** 2153-2160.

Couse JF, Curtis SW, Washburn TF, Lindzey J, Golding TS, Lubahn DB, Smithies O & Korach KS 1995 Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. *Mol Endocrinol* **9** 1441-1454.

Couse JF, Lindzey J, Grandien K, Gustafsson JA & Korach KS 1997 Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Endocrinology* **138** 4613-4621.

Cowley SM, Hoare S, Mosselman S & Parker MG 1997 Estrogen receptors alpha and beta form heterodimers on DNA. *J Biol Chem* **272** 19858-19862.

de Gois Speck NM, Focchi J, Alves AC, Ribalta JC & Osorio CA 2005 Relationship between angiogenesis and grade of histologic differentiation in endometrial adenocarcinoma. *Eur J Gynaecol Oncol* **26** 599-601.

Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P & Mark M 2000 Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* **127** 4277-4291.

Eggert M, Mows CC, Tripier D, Arnold R, Michel J, Nickel J, Schmidt S, Beato M & Renkawitz R 1995 A fraction enriched in a novel glucocorticoid receptor-interacting protein stimulates receptor-dependent transcription in vitro. *J Biol Chem* **270** 30755-30759.

Eng C 2003 PTEN: one gene, many syndromes. *Hum Mutat* **22** 183-198.

Enmark E & Gustafsson JA 1999 Oestrogen receptors - an overview. *J Intern Med* **246** 133-138.

Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M & Gustafsson JA 1997 Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* **82** 4258-4265.

Enomoto T, Fujita M, Inoue M, Rice JM, Nakajima R, Tanizawa O & Nomura T 1993 Alterations of the p53 tumor suppressor gene and its association with activation of the c-K-ras-2 protooncogene in premalignant and malignant lesions of the human uterine endometrium. *Cancer Res* **53** 1883-1888.

Enomoto T, Inoue M, Perantoni AO, Buzard GS, Miki H, Tanizawa O & Rice JM 1991 K-ras activation in premalignant and malignant epithelial lesions of the human uterus. *Cancer Res* **51** 5308-5314.

Evans RM 1988 The steroid and thyroid hormone receptor superfamily. *Science* **240** 889-895.

Fixemer T, Remberger K & Bonkhoff H 2003 Differential expression of the estrogen receptor beta (ERbeta) in human prostate tissue, premalignant changes, and in primary, metastatic, and recurrent prostatic adenocarcinoma. *Prostate* **54** 79-87.

Foley EF, Jazaeri AA, Shupnik MA, Jazaeri O & Rice LW 2000 Selective loss of estrogen receptor beta in malignant human colon. *Cancer Res* **60** 245-248.

Fondell JD, Ge H & Roeder RG 1996 Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proc Natl Acad Sci U S A* **93** 8329-8333.

Fong CJ, Sherwood ER, Braun EJ, Berg LA, Lee C & Kozlowski JM 1992 Regulation of prostatic carcinoma cell proliferation and secretory activity by extracellular matrix and stromal secretions. *Prostate* **21** 121-131.

Fons G, Hasibuan SM, Velden JV & Kate FJ 2006 Validation of tissue micro array technology in endometrioid cancer of the endometrium. *J Clin Pathol*.

Fujimoto J, Sakaguchi H, Aoki I, Toyoki H & Tamaya T 2002 Clinical implications of the expression of estrogen receptor-alpha and -beta in primary and metastatic lesions of uterine endometrial cancers. *Oncology* **62** 269-277.

Gilbert SF 1997 *Developmental biology*. Sunderland, Mass.: Sinauer Associates.

Glass CK, Rose DW & Rosenfeld MG 1997 Nuclear receptor coactivators. *Curr Opin Cell Biol* **9** 222-232.

Glasser S 2002 *The endometrium*. London ; New York: Taylor & Francis.

Gorski J, Noteboom WD & Nicolette JA 1965 Estrogen control of the synthesis of RNA and protein in the uterus. *J Cell Physiol* **66** Suppl 1:91-109.

Graumann K & Jungbauer A 2000 Quantitative assessment of complex formation of nuclear-receptor accessory proteins. *Biochem J* **345 Pt 3** 627-636.

Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P & Chambon P 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* **320** 134-139.

Gregory CW, Wilson EM, Apparao KB, Lininger RA, Meyer WR, Kowalik A, Fritz MA & Lessey BA 2002 Steroid receptor coactivator expression throughout the menstrual cycle in normal and abnormal endometrium. *J Clin Endocrinol Metab* **87** 2960-2966.

Grodstein F, Newcomb PA & Stampfer MJ 1999 Postmenopausal hormone therapy and the risk of colorectal cancer: a review and meta-analysis. *Am J Med* **106** 574-582.

Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C & Brown M 1994 Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* **264** 1455-1458.

Hale GE, Hughes CL & Cline JM 2002 Endometrial cancer: hormonal factors, the perimenopausal "window of risk," and isoflavones. *J Clin Endocrinol Metab* **87** 3-15.

Hall JM & McDonnell DP 1999 The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* **140** 5566-5578.

Hanekamp EE, Gielen SC, van Oosterhoud SA, Burger CW, Grootegoed JA, Huikeshoven FJ & Blok LJ 2003 Progesterone receptors in endometrial cancer invasion and metastasis: development of a mouse model. *Steroids* **68** 795-800.

Hanstein B, Eckner R, DiRenzo J, Halachmi S, Liu H, Searcy B, Kurokawa R & Brown M 1996 p300 is a component of an estrogen receptor coactivator complex. *Proc Natl Acad Sci U S A* **93** 11540-11545.

Harrington WR, Sheng S, Barnett DH, Petz LN, Katzenellenbogen JA & Katzenellenbogen BS 2003 Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. *Mol Cell Endocrinol* **206** 13-22.

Hartman J, Lindberg K, Morani A, Inzunza J, Strom A & Gustafsson JA 2006 Estrogen receptor beta inhibits angiogenesis and growth of T47D breast cancer xenografts. *Cancer Res* **66** 11207-11213.

Hong H, Kohli K, Garabedian MJ & Stallcup MR 1997 GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. *Mol Cell Biol* **17** 2735-2744.

Horvath LG, Henshall SM, Lee CS, Head DR, Quinn DI, Makela S, Delprado W, Golovsky D, Brenner PC, O'Neill G, et al. 2001 Frequent loss of estrogen receptor-beta expression in prostate cancer. *Cancer Res* **61** 5331-5335.

Ikeda K, Sato M, Tsutsumi O, Tsuchiya F, Tsuneizumi M, Emi M, Imoto I, Inazawa J, Muramatsu M & Inoue S 2000 Promoter analysis and chromosomal mapping of human EBAG9 gene. *Biochem Biophys Res Commun* **273** 654-660.

Inoue S, Orimo A, Hosoi T, Kondo S, Toyoshima H, Kondo T, Ikegami A, Ouchi Y, Orimo H & Muramatsu M 1993 Genomic binding-site cloning reveals an estrogen-responsive gene that encodes a RING finger protein. *Proc Natl Acad Sci U S A* **90** 11117-11121.

Jadoul P & Donnez J 2003 Conservative treatment may be beneficial for young women with atypical endometrial hyperplasia or endometrial adenocarcinoma. *Fertil Steril* **80** 1315-1324.

Jarred RA, Cancilla B, Prins GS, Thayer KA, Cunha GR & Risbridger GP 2000 Evidence that estrogens directly alter androgen-regulated prostate development. *Endocrinology* **141** 3471-3477.

Jensen EV 1962 On the mechanism of estrogen action. *Perspect Biol Med* **6** 47-59.

Jenster G 1998 Coactivators and corepressors as mediators of nuclear receptor function: an update. *Mol Cell Endocrinol* **143** 1-7.

Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H & Chambon P 1990 Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *Embo J* **9** 1603-1614.

Kato N, Kimura K, Sugawara H, Aoyagi S, Ikeda T & Horii A 2001 Germline mutation of the PTEN gene in a Japanese patient with Cowden's disease. *Int J Oncol* **18** 1017-1022.

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, et al. 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270** 1491-1494.

Kato S, Masuhiro Y, Watanabe M, Kobayashi Y, Takeyama KI, Endoh H & Yanagisawa J 2000 Molecular mechanism of a cross-talk between oestrogen and growth factor signalling pathways. *Genes Cells* **5** 593-601.

Kaye AM, Sheratzky D & Lindner HR 1971 Kinetics of DNA synthesis in immature rat uterus: age dependence and estradiol stimulation. *Biochim Biophys Acta* **261** 475-486.

Kim HJ, Lee SK, Na SY, Choi HS & Lee JW 1998 Molecular cloning of xSRC-3, a novel transcription coactivator from Xenopus, that is related to AIB1, p/CIP, and TIF2. *Mol Endocrinol* **12** 1038-1047.

Kirkland JL, LaPointe L, Justin E & Stancel GM 1979 Effects of estrogen on mitosis in individual cell types of the immature rat uterus. *Biol Reprod* **21** 269-272.

Klein-Hitpass L, Schorpp M, Wagner U & Ryffel GU 1986 An estrogen-responsive element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells. *Cell* **46** 1053-1061.

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

Konstantinopoulos PA, Kominea A, Vandoros G, Sykiotis GP, Andricopoulos P, Varakis I, Sotiropoulou-Bonikou G & Papavassiliou AG 2003 Oestrogen receptor beta (ERbeta) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumour's dedifferentiation. *Eur J Cancer* **39** 1251-1258.

Kraichely DM, Sun J, Katzenellenbogen JA & Katzenellenbogen BS 2000 Conformational changes and coactivator recruitment by novel ligands for estrogen receptor-alpha and estrogen receptor-beta: correlations with biological character and distinct differences among SRC coactivator family members. *Endocrinology* **141** 3534-3545.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA & Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci U S A* **95** 15677-15682.

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** 5925-5930.

Kumar V, Green S, Stack G, Berry M, Jin JR & Chambon P 1987 Functional domains of the human estrogen receptor. *Cell* **51** 941-951.

Kurman RJ, Kaminski PF & Norris HJ 1985 The behavior of endometrial hyperplasia. A long-term study of "untreated" hyperplasia in 170 patients. *Cancer* **56** 403-412.

Latil A, Bieche I, Vidaud D, Lidereau R, Berthon P, Cussenot O & Vidaud M 2001 Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays. *Cancer Res* **61** 1919-1926.

Lax SF, Kendall B, Tashiro H, Slebos RJ & Hedrick L 2000 The frequency of p53, K-ras mutations, and microsatellite instability differs in uterine endometrioid and serous carcinoma: evidence of distinct molecular genetic pathways. *Cancer* **88** 814-824.

Le Goff P, Montano MM, Schodin DJ & Katzenellenbogen BS 1994 Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. *J Biol Chem* **269** 4458-4466.

Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL & McCarty KS, Jr. 1988 Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J Clin Endocrinol Metab* **67** 334-340.

Leygue E, Dotzlaw H, Watson PH & Murphy LC 1998 Altered estrogen receptor alpha and beta messenger RNA expression during human breast tumorigenesis. *Cancer Res* **58** 3197-3201.

Littlefield BA, Gurpide E, Markiewicz L, McKinley B & Hochberg RB 1990 A simple and sensitive microtiter plate estrogen bioassay based on stimulation of alkaline phosphatase in Ishikawa cells: estrogenic action of delta 5 adrenal steroids. *Endocrinology* **127** 2757-2762.

Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, Price RH, Jr., Pestell RG & Kushner PJ 2002 Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. *J Biol Chem* **277** 24353-24360.

Liu Y & Teng CT 1992 Estrogen response module of the mouse lactoferrin gene contains overlapping chicken ovalbumin upstream promoter transcription factor and estrogen receptor-binding elements. *Mol Endocrinol* **6** 355-364.

Loose-Mitchell DS, Chiappetta C & Stancel GM 1988 Estrogen regulation of c-fos messenger ribonucleic acid. *Mol Endocrinol* **2** 946-951.

Lopez GN, Turck CW, Schaufele F, Stallcup MR & Kushner PJ 2001 Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. *J Biol Chem* **276** 22177-22182.

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** 11162-11166.

Ludwig H & Spornitz UM 1991 Microarchitecture of the human endometrium by scanning electron microscopy: menstrual desquamation and remodeling. *Ann N Y Acad Sci* **622** 28-46.

Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS, Jr. & Mayo MW 2000 Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. *Mol Cell Biol* **20** 1626-1638.

Maehama T & Dixon JE 1998 The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273** 13375-13378.

Majumder S, Liu Y, Ford OH, 3rd, Mohler JL & Whang YE 2006 Involvement of arginine methyltransferase CARM1 in androgen receptor function and prostate cancer cell viability. *Prostate* **66** 1292-1301.

Martin MB, Franke TF, Stoica GE, Chambon P, Katzenellenbogen BS, Stoica BA, McLemore MS, Olivo SE & Stoica A 2000 A role for Akt in mediating the estrogenic functions of epidermal growth factor and insulin-like growth factor I. *Endocrinology* **141** 4503-4511.

Matthews J & Gustafsson JA 2003 Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol Interv* **3** 281-292.

McGrath CM 1983 Augmentation of the response of normal mammary epithelial cells to estradiol by mammary stroma. *Cancer Res* **43** 1355-1360.

McKay LI & Cidlowski JA 1998 Cross-talk between nuclear factor-kappa B and the steroid hormone receptors: mechanisms of mutual antagonism. *Mol Endocrinol* **12** 45-56.

McKenna NJ, Xu J, Nawaz Z, Tsai SY, Tsai MJ & O'Malley BW 1999 Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. *J Steroid Biochem Mol Biol* **69** 3-12.

Menasce LP, White GR, Harrison CJ & Boyle JM 1993 Localization of the estrogen receptor locus (ESR) to chromosome 6q25.1 by FISH and a simple post-FISH banding technique. *Genomics* **17** 263-265.

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** 433-442.

Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS & Katzenellenbogen JA 2001 Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J Med Chem* **44** 4230-4251.

Montgomery BE, Daum GS & Dunton CJ 2004 Endometrial hyperplasia: a review. *Obstet Gynecol Surv* **59** 368-378.

Mutter GL 2000 Histopathology of genetically defined endometrial precancers. *Int J Gynecol Pathol* **19** 301-309.

Mutter GL 2002 Diagnosis of premalignant endometrial disease. *J Clin Pathol* **55** 326-331.

Mutter GL, Lin MC, Fitzgerald JT, Kum JB, Baak JP, Lees JA, Weng LP & Eng C 2000 Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. *J Natl Cancer Inst* **92** 924-930.

Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP & Tonks NK 1998 The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc Natl Acad Sci U S A* **95** 13513-13518.

Mylonas I, Jeschke U, Shabani N, Kuhn C, Balle A, Kriegel S, Kupka MS & Friese K 2004 Immunohistochemical analysis of estrogen receptor alpha, estrogen receptor beta and progesterone receptor in normal human endometrium. *Acta Histochem* **106** 245-252.

Mylonas I, Jeschke U, Shabani N, Kuhn C, Kriegel S, Kupka MS & Friese K 2005 Normal and malignant human endometrium express immunohistochemically estrogen receptor alpha (ER-alpha), estrogen receptor beta (ER-beta) and progesterone receptor (PR). *Anticancer Res* **25** 1679-1686.

Nephew KP, Polek TC, Akcali KC & Khan SA 1993a The antiestrogen tamoxifen induces c-fos and jun-B, but not c-jun or jun-D, protooncogenes in the rat uterus. *Endocrinology* **133** 419-422.

Nephew KP, Webb DK, Akcali KC, Moulton BC & Khan SA 1993b Hormonal regulation and expression of the jun-D protooncogene in specific cell types of the rat uterus. *J Steroid Biochem Mol Biol* **46** 281-287.

Nishida M 2002 The Ishikawa cells from birth to the present. *Hum Cell* **15** 104-117.

Nishida M, Kasahara K, Oki A, Satoh T, Arai Y & Kubo T 1996 Establishment of eighteen clones of Ishikawa cells. *Hum Cell* **9** 109-116.

Norris J, Fan D, Aleman C, Marks JR, Futreal PA, Wiseman RW, Iglehart JD, Deininger PL & McDonnell DP 1995 Identification of a new subclass of Alu DNA repeats which can function as estrogen receptor-dependent transcriptional enhancers. *J Biol Chem* **270** 22777-22782.

Norris JD, Fan D, Kerner SA & McDonnell DP 1997 Identification of a third autonomous activation domain within the human estrogen receptor. *Mol Endocrinol* **11** 747-754.

Okamoto A, Sameshima Y, Yamada Y, Teshima S, Terashima Y, Terada M & Yokota J 1991 Allelic loss on chromosome 17p and p53 mutations in human endometrial carcinoma of the uterus. *Cancer Res* **51** 5632-5635.

Omoto Y, Eguchi H, Yamamoto-Yamaguchi Y & Hayashi S 2003 Estrogen receptor (ER) beta1 and ERbetacx/beta2 inhibit ERalpha function differently in breast cancer cell line MCF7. *Oncogene* **22** 5011-5020.

Onate SA, Tsai SY, Tsai MJ & O'Malley BW 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270** 1354-1357.

Park BW, Kim KS, Heo MK, Ko SS, Hong SW, Yang WI, Kim JH, Kim GE & Lee KS 2003 Expression of estrogen receptor-beta in normal mammary and tumor tissues: is it protective in breast carcinogenesis? *Breast Cancer Res Treat* **80** 79-85.

Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL & Leitman DC 2004 Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Res* **64** 423-428.

Pasquali D, Rossi V, Esposito D, Abbondanza C, Puca GA, Bellastella A & Sinisi AA 2001 Loss of estrogen receptor beta expression in malignant human prostate cells in primary cultures and in prostate cancer tissues. *J Clin Endocrinol Metab* **86** 2051-2055.

Pettersson K, Delaunay F & Gustafsson JA 2000 Estrogen receptor beta acts as a dominant regulator of estrogen signaling. *Oncogene* **19** 4970-4978.

Pettersson K, Grandien K, Kuiper GG & Gustafsson JA 1997 Mouse estrogen receptor beta forms estrogen response element-binding heterodimers with estrogen receptor alpha. *Mol Endocrinol* **11** 1486-1496.

Pham TA, Hwung YP, Santiso-Mere D, McDonnell DP & O'Malley BW 1992 Ligand-dependent and -independent function of the transactivation regions of the human estrogen receptor in yeast. *Mol Endocrinol* **6** 1043-1050.

Picard D, Kumar V, Chambon P & Yamamoto KR 1990 Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors. *Cell Regul* **1** 291-299.

Pratt WB 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.

Pratt WB & Toft DO 1997 Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev* **18** 306-360.

Puisoru M, Fatu C & Fatu IC 2006 Histochemical evaluation of angiogenesis in endometrial adenocarcinoma. *Ann Anat* **188** 255-259.

Pujol P, Rey JM, Nirde P, Roger P, Gastaldi M, Laffargue F, Rochefort H & Maudelonde T 1998 Differential expression of estrogen receptor-alpha and -beta messenger RNAs as a potential marker of ovarian carcinogenesis. *Cancer Res* **58** 5367-5373.

Ramaswamy S, Nakamura N, Vazquez F, Batt DB, Perera S, Roberts TM & Sellers WR 1999 Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc Natl Acad Sci U S A* **96** 2110-2115.

Renaud JP, Rochel N, Ruff M, Vivat V, Chambon P, Gronemeyer H & Moras D 1995 Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid. *Nature* **378** 681-689.

Ribatti D, Finato N, Crivellato E, Marzullo A, Mangieri D, Nico B, Vacca A & Beltrami CA 2005 Neovascularization and mast cells with tryptase activity increase simultaneously with pathologic progression in human endometrial cancer. *Am J Obstet Gynecol* **193** 1961-1965.

Robyr D, Wolffe AP & Wahli W 2000 Nuclear hormone receptor coregulators in action: diversity for shared tasks. *Mol Endocrinol* **14** 329-347.

Roger P, Sahla ME, Makela S, Gustafsson JA, Baldet P & Rochefort H 2001 Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. *Cancer Res* **61** 2537-2541.

Rogers PA 1996a Endometrial vasculature in Norplant users. *Hum Reprod* **11 Suppl 2** 45-50.

Rogers PA 1996b Structure and function of endometrial blood vessels. *Hum Reprod Update* **2** 57-62.

Rogers PA, Au CL & Affandi B 1993 Endometrial microvascular density during the normal menstrual cycle and following exposure to long-term levonorgestrel. *Hum Reprod* **8** 1396-1404.

Rose PG 1996 Endometrial carcinoma. *N Engl J Med* **335** 640-649.

Rubin E & Farber JL 1999 *Pathology*. Philadelphia: Lippincott-Raven.

Ruhul Quddus M, Latkovich P, Castellani WJ, James Sung C, Steinhoff MM, Briggs RC & Miranda RN 2002 Expression of cyclin D1 in normal, metaplastic, hyperplastic endometrium and endometrioid carcinoma suggests a role in endometrial carcinogenesis. *Arch Pathol Lab Med* **126** 459-463.

Rutherford T, Brown WD, Sapi E, Aschkenazi S, Munoz A & Mor G 2000 Absence of estrogen receptor-beta expression in metastatic ovarian cancer. *Obstet Gynecol* **96** 417-421.

Santin AD 2003 HER2/neu overexpression: has the Achilles' heel of uterine serous papillary carcinoma been exposed? *Gynecol Oncol* **88** 263-265.

Scheper MA, Nikitakis NG, Sarlani E, Sauk JJ & Meiller TF 2006 Cowden syndrome: report of a case with immunohistochemical analysis and review of the literature. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **101** 625-631.

Schwabe JW, Chapman L, Finch JT & Rhodes D 1993 The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. *Cell* **75** 567-578.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA & Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95** 927-937.

Shughrue PJ, Lane MV, Scrimo PJ & Merchenthaler I 1998 Comparative distribution of estrogen receptor-alpha (ER-alpha) and beta (ER-beta) mRNA in the rat pituitary, gonad, and reproductive tract. *Steroids* **63** 498-504.

Shyamala G & Guiot MC 1992 Activation of kappa B-specific proteins by estradiol. *Proc Natl Acad Sci U S A* **89** 10628-10632.

Skinner MK & Fritz IB 1985 Testicular peritubular cells secrete a protein under androgen control that modulates Sertoli cell functions. *Proc Natl Acad Sci U S A* **82** 114-118.

Smith CL 1998 Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biol Reprod* **58** 627-632.

Smith DF & Toft DO 1993 Steroid receptors and their associated proteins. *Mol Endocrinol* **7** 4-11.

Soneoka Y, Cannon PM, Ramsdale EE, Griffiths JC, Romano G, Kingsman SM & Kingsman AJ 1995 A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucleic Acids Res* **23** 628-633.

Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R & Santen RJ 2002 Linkage of rapid estrogen action to MAPK activation by ERalpha-Shc association and Shc pathway activation. *Mol Endocrinol* **16** 116-127.

Song RX, Zhang Z & Santen RJ 2005 Estrogen rapid action via protein complex formation involving ERalpha and Src. *Trends Endocrinol Metab* **16** 347-353.

Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, Katzenellenbogen BS & Katzenellenbogen JA 2000 Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *J Med Chem* **43** 4934-4947.

Stefansson IM, Salvesen HB & Akslen LA 2006 Vascular proliferation is important for clinical progress of endometrial cancer. *Cancer Res* **66** 3303-3309.

Stoica GE, Franke TF, Moroni M, Mueller S, Morgan E, Iann MC, Winder AD, Reiter R, Wellstein A, Martin MB, et al. 2003 Effect of estradiol on estrogen receptor-alpha gene

expression and activity can be modulated by the ErbB2/PI 3-K/Akt pathway. *Oncogene* **22** 7998-8011.

Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J & Gustafsson JA 2004 Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci U S A* **101** 1566-1571.

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.

Taylor AH, al-Azzawi F, Pringle JH & Bell SC 2002 Inhibition of endometrial carcinoma cell growth using antisense estrogen receptor oligodeoxyribonucleotides. *Anticancer Res* **22** 3993-4003.

Thompson D & Easton DF 2002 Cancer Incidence in BRCA1 mutation carriers. *J Natl Cancer Inst* **94** 1358-1365.

Toft D & Gorski J 1966 A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proc Natl Acad Sci U S A* **55** 1574-1581.

Tora L, White J, Brou C, Tasset D, Webster N, Scheer E & Chambon P 1989 The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **59** 477-487.

Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK & Rosenfeld MG 1997 The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387** 677-684.

Tremblay A, Tremblay GB, Labrie F & Giguere V 1999 Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. *Mol Cell* **3** 513-519.

Tzukerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RB, Pike JW & McDonnell DP 1994 Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol Endocrinol* **8** 21-30.

Umesono K & Evans RM 1989 Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* **57** 1139-1146.

Utsunomiya H, Suzuki T, Harada N, Ito K, Matsuzaki S, Konno R, Sato S, Yajima A & Sasano H 2000 Analysis of estrogen receptor alpha and beta in endometrial carcinomas: correlation with ER beta and clinicopathologic findings in 45 cases. *Int J Gynecol Pathol* **19** 335-341.

Voegel JJ, Heine MJ, Tini M, Vivat V, Chambon P & Gronemeyer H 1998 The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways. *Embo J* **17** 507-519.

Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD & Fletterick RJ 1995 A structural role for hormone in the thyroid hormone receptor. *Nature* **378** 690-697.

Walker VR & Korach KS 2004 Estrogen receptor knockout mice as a model for endocrine research. *Ilar J* **45** 455-461.

Watanabe T, Inoue S, Hiroi H, Orimo A, Kawashima H & Muramatsu M 1998 Isolation of estrogen-responsive genes with a CpG island library. *Mol Cell Biol* **18** 442-449.

Watson CS, Norfleet AM, Pappas TC & Gametchu B 1999 Rapid actions of estrogens in GH3/B6 pituitary tumor cells via a plasma membrane version of estrogen receptor-alpha. *Steroids* **64** 5-13.

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** 721-726.

Webb DK, Moulton BC & Khan SA 1993 Estrogen induces expression of c-jun and jun-B protooncogenes in specific rat uterine cells. *Endocrinology* **133** 20-28.

Webb P, Lopez GN, Uht RM & Kushner PJ 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Mol Endocrinol* **9** 443-456.

Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M & Gustafsson JA 2000 Estrogen receptor (ER) beta, a modulator of ERalpha in the uterus. *Proc Natl Acad Sci U S A* **97** 5936-5941.

Weisz A & Bresciani F 1988 Estrogen induces expression of c-fos and c-myc protooncogenes in rat uterus. *Mol Endocrinol* **2** 816-824.

Weisz A, Cicatiello L, Persico E, Scalona M & Bresciani F 1990 Estrogen stimulates transcription of c-jun protooncogene. *Mol Endocrinol* **4** 1041-1050.

Wurtz JM, Bourguet W, Renaud JP, Vivat V, Chambon P, Moras D & Gronemeyer H 1996 A canonical structure for the ligand-binding domain of nuclear receptors. *Nat Struct Biol* **3** 87-94.

Wynn RM & Jollie WP 1989 *Biology of the uterus*. New York: Plenum Medical Book Co.

Zhang Y, Liao Q, Chen C, Yu L & Zhao J 2006 Function of estrogen receptor isoforms alpha and beta in endometrial carcinoma cells. *Int J Gynecol Cancer* **16** 1656-1660.

Zhao C, Lam EW, Sunters A, Enmark E, De Bella MT, Coombes RC, Gustafsson JA & Dahlman-Wright K 2003 Expression of estrogen receptor beta isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. *Oncogene* **22** 7600-7606.

Zilliacus J, Wright AP, Carlstedt-Duke J, Nilsson L & Gustafsson JA 1995 Modulation of DNA-binding specificity within the nuclear receptor family by substitutions at a single amino acid position. *Proteins* **21** 57-67.