INHIBITORY ACTIONS OF AH RECEPTOR AGONISTS AND INDOLE-CONTAINING COMPOUNDS IN BREAST CANCER CELL LINES AND MOUSE MODELS

A Dissertation

by

KELCEY MANAE BECKER WALKER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Toxicology

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Approved as to style and content by:

_____	_____
Stephen H. Safe (Chair of Committee)	Timothy Phillips (Member)
_____	_____
Robert C. Burghardt (Member)	Larry Johnson (Member)
_____	_____
Glen Laine (Head of Department)	Timothy Phillips (Chair of Toxicology Faculty)

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ABSTRACT

Inhibitory Actions of Ah Receptor Agonists and Indole-Containing Compounds in Breast Cancer Cell Lines and Mouse Models. (May 2004)

Kelcey Manae Becker Walker, B.S., College of William and Mary;

M.S., Virginia Tech University

Chair of Advisory Committee: Dr. Stephen H. Safe

The aryl hydrocarbon receptor (AhR) binds synthetic and chemoprotective phytochemicals, and research in this laboratory has developed selective AhR modulators (SAhRMs) for treatment of breast cancer. Activation of the AhR through agonists such as TCDD inhibits hormone activation of several E2-responsive genes in breast cancer cell lines. In this study, inhibition of E2-induced proliferation and gene expression by TCDD has been investigated in the uterus of wildtype, ERKO and AhRKO mice. Cyclin D1, DNA polymerase α, and VEGF mRNA levels are induced by E2 through ERα in the uterus as determined by in situ hybridization studies. TCDD down-regulated E2-induced cyclin D1 and DNA polymerase α expression, but not E2-induced VEGF expression, in wild-type mice, but not AhRKO mice, confirming the role of the AhR. Furthermore, protein synthesis was not necessary for induction of cyclin D1 or DNA polymerase α gene expression by E2 or inhibition of these

responses by TCDD. Therefore, AhR-ERα crosstalk directly regulates the expression of genes involved in cell proliferation in vivo.

AhR agonists induce down-regulation of ErbB family receptors in multiple tissues/organs suggesting possible inhibitory interactions with chemotherapeutic potential. Recently, it has been reported that the SAhRM 1,1',2,2'-tetramethyldiindolylmethane inhibited DMBA-induced mammary tumor growth in rats and also inhibited MAPK and PI3-K pathways in human breast cancer cells. BT-474 and MDA-MB-453 cell lines are ErbB2-overexpressing breast cancer cells that express functional AhR and exhibit constitutive activation of MAPK and PI3-K pathways. Therefore, 1,1',2,2'-tetramethyldiindolylmethane-induced inhibition of ErbB2 signaling was investigated in these cells lines and in the MMTV-c-neu mouse mammary tumor model, which overexpresses ErbB2 in the mammary gland. The growth of ErbB2 overexpressing cell lines and mammary tumors was inhibited by 1,1',2,2'-tetramethyldiindolylmethane; however, modulation of MAPK or PI3-K pathways and cell cycle proteins nor induction of apoptosis by 1,1',2,2'-tetramethyldiindolylmethane was observed in the ErbB2-overexpressing cell lines. Current studies are investigating mitochondrial effects of 1,1',2,2'-tetramethyldiindolylmethane in the ErbB2-overexpressing cell lines, as well as continuing studies on gene expression profiles in the mammary glands of MMTV-c-neu mice to better understand and identify critical genes that are responsible for ErbB2-mediated transformation and growth of cancer cells/tumors.

This work is dedicated to my husband, Shawn.

I couldn’t have asked for any more love and support…or children…through it all.

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CHAPTER I

INTRODUCTION

1.1 Breast cancer

1.1.1 General introduction with statistics

An estimated 1 in 8 women in the US will develop breast cancer, and it is the second leading cause of cancer-related deaths in women of the Western world (American, 2003). The American Cancer Society estimates that 267,000 new cases of breast cancer will be diagnosed and 39,800 women will die from breast cancer this year in the US. It is the most freqently diagnosed non-skin cancer for US women with 5-year survival rates of 97% for local breast cancers, 78% for regional metastasis and 23% for distant metastasis. Approximately 10% of breast cancer cases can be attributed to inheritance of mutations in genes such as BRCA1 and BRCA2; however, the majority of cases (~ 90%) occur in women with no familial history and the molecular basis and etiology of "sporadic" breast cancer is poorly understood (Claus et al., 1996).

1.1.2 Risk factors for breast cancer

A small percentage of all breast cancer cases (~ 10%) have a familial

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This dissertation follows the style and format of Gene.

pattern of incidence resulting from inheritance of germline mutations in high-penetrance genes such as *BRCA1/2* or other low-penetrance genes that have yet to be identified. Inheritance of mutated *BRCA1* or *BRCA2* carries a lifetime risk of 30-70% for cancer incidence with the variation in risk thought to be dependent upon genetic background and environmental factors. The cause of the remaining breast cancer cases, ~ 90%, is poorly understood, although multiple risk factors have been identified. The American Cancer Society includes increasing age, long menstrual cycle due to early menarche and late menopause, obesity after menopause, recent use of oral contraceptives or hormone replacement therapy, nulliparity or first pregnancy after the age of 30, and the consumption of one or more alcoholic beverages daily as risk factors for breast cancer (American, 2003).

1.1.2.1 Hormonal/environmental risk factors

Lifetime exposure to the female hormone estrogen is a major known risk factor for the development of breast cancer, and there is much evidence from studies of both familial and sporadic breast cancers that support the etiologic role of estrogen. Although few studies have looked at the effect of estrogen on the risk of breast cancer development in *BRCA1* mutation carriers, there is indirect evidence that increased exposure to estrogen increases breast cancer risk. For example, a bilateral prophylactic ovariectomy, which drastically decreases circulating estrogen levels in the body, will significantly decrease the risk of breast cancer in women that carry the *BRCA1* mutation (Rebbeck et al.,

1999). Other indirect evidence includes increased susceptibility to breast cancer from pregnancy (estrogen levels are 50-100 fold higher during pregnancy) (Johannsson et al., 1998; Jernstrom et al., 1999), a 4-fold increase in risk with a high body mass index (BMI) at age 12 (adipose tissue is a major source of estrogen without ovarian production) (Hilakivi-Clarke et al., 2002), and a possible increase in risk of breast cancer when oral contraceptives are taken prior to the first pregnancy (Ursin et al., 1997) in either women with a strong family history of breast cancer or with mutations in BRCA1/2.

There is also evidence that high estrogen exposure increases the risk of developing sporadic breast cancer, especially in post-menopausal women. Studies have shown that estrogen levels are higher in post-menopausal women that develop breast cancer than those that do not (Hankinson et al., 1998) and obesity leads to increased estrogen levels in post-menopausal women and this increases the risk of breast cancer (Yong et al., 1996). Multiple studies have shown that hormone replacement therapy, whether estrogen or estrogen + progestin treatment, during menopause increases the risk of breast cancer (Hoover et al., 1976; Colditz and Rosner, 2000; Schairer et al., 2000; Rossouw et al., 2002) and increased exposure to estrogen through the menstrual cycle, either by early age at menarche or by late age of menopause, increases the risk of developing breast cancer (Trichopoulos et al., 1972; Kampert et al., 1988; Hulka and Stark, 1995). Furthermore, a decrease in estrogen levels through a

bilateral ovariectomy decreases the postmenopausal risk of breast cancer and is a treatment for existing pre-menopausal disease (Kreiger et al., 1999).

Research has also investigated the effects of in utero exposure to estrogen on the risk of developing breast cancer and the results suggest that increased exposure to estrogen in utero also leads to increased risk of breast cancer (Trichopoulos, 1990). High birth weight has been linked to high maternal estrogen levels (Gerhard et al., 1987) and multiple studies suggest that high birth weight increases the risk of breast cancer development (Michels et al., 1996; Sanderson et al., 1996), although some studies suggest that this increase is observed only in pre-menopausal women (Potischman and Troisi, 1999). Maternal estrogen levels are also higher in pregnancies with twins compared to singletons (Duff and Brown, 1974), and twins have an increased risk of developing breast cancer (Braun et al., 1995; Weiss et al., 1997). In contrast pre-eclampsia and eclampsia during pregnancy are associated with lower maternal estrogen levels and offspring have a significantly lower risk of breast cancer (Ekbom et al., 1992). Animal studies have also shown that in utero exposure to E2 (Hilakivi-Clarke et al., 1997b), diethylstilbesterol (DES) (Walker, 1984), genistein (Hilakivi-Clarke et al., 1999), or diets high in n-6 polyunsaturated fatty acids (Walker, 1990; Hilakivi-Clarke et al., 1997b) increases the risk of developing mammary tumors in their offspring. One contradictory study showed that although Asian women have higher estrogen levels during pregnancy than Caucasian women, their offspring have a lower risk

of developing breast cancer (Lipworth et al., 1999). Differences in diet and the fact that non-pregnant Asian women have 40% lower levels of circulating estrogens than Caucasian women (Goldin et al., 1986) may have a more dominant effect on risk than the high estrogen levels during pregnancy.

Studies investigating a link between estrogen exposure and breast cancer risk during the reproductive years, either through menstrual cycling, pregnancy, or contraceptive use, have found that increased estrogen exposure is not always a risk for breast cancer development. Estrogens peak twice during the menstrual cycle, therefore a shorter cycle would lead to a higher number of cycles and more cumulative estrogen exposure. However, short menstrual cycle length dose not increase the risk of breast cancer development or recurrence (Garland et al., 1998; Titus-Ernstoff et al., 1998). Also, multiple studies have determined estrogen levels at different stages of the menstrual cycle and have found an altered pattern of estrogens during the luteal and follicular stages in high-risk women. In the luteal phase when estrogens peak twice, high risk women had lower estrogen levels; in the follicular phase when estrogen are normally low, high risk women had increased estrogen levels (Key et al., 1996).

Estrogen levels are 50-100 fold higher during pregnancy (Murr et al., 1974); however, some studies have shown that pregnancy is protective against breast cancer. Multiple pregnancies have been shown to decrease risk, and there is also a decreased risk for women who have a first full-term pregnancy before the age 20 (Murr et al., 1974; Yuan et al., 1988). Animal studies have

confirmed the protective effects of pregnancy. E2 alone or E2 + progesterone treatment to mimic pregnancy protects rats from chemically induced tumors (Guzman et al., 1999). In contrast, the first pregnancy after the age of 30 increases the risk of breast cancer development (MacMahon et al., 1970) and pregnancy after 25 year results in a short term increase in risk with up to a 20 fold increase during the first year after the pregnancy (Kelsey et al., 1993; Hsieh et al., 1994). It has been suggested that because estrogen during pregnancy stimulates ductal branching and extensive formation of more differentiated alveolar lobules in the mammary epithelium, pregnancy at a younger age leads to more differentiated cell types and this reduces the number of undifferentiated cells that could lead to cancer. However, as women age there is an increasing probability of initiated cells present in the mammary gland and estrogen exposure during pregnancy could induce these initiated cells to proliferate. Other studies have shown that high-birth weight babies, severe nausea/vomiting, and DES treatment during pregnancy, all of which are associated with increased estrogen exposure, lead to increased risk of breast cancer (Depue et al., 1987; Colton et al., 1993; Olsen and Storm, 1998). In contrast, pregnancy-induced hypertension, which is associated with lower estrogen levels, decreases the risk of breast cancer development (Hsieh et al., 1994). Studies have also shown the protective effects of breastfeeding against breast cancer. According to one study the relative risk of developing breast

cancer is decreased by 4.3% for every 12 months a woman breastfed (Collaborative, 2002).

Oral contraceptive use has also been studied as a possible risk factor for breast cancer. The use of oral contraceptives results in constant exposure to synthetic estrogens/progestins; however, circulating estrogens such as E2 and estrone are lower in these women compared to individuals who do not use oral contraceptives (O'Brien et al., 1997). Oral contraceptives induce proliferation in the human breast and long-term use of oral contraceptives leads to a 42-45% increase in risk of breast cancer up to the age of 45, but risk is not increased in women 45 years or older (Isaksson et al., 2001; Hilakivi-Clarke et al., 2002).

Environmental factors such as geographic location, diet, bodyweight, exercise, alcohol consumption, and exposure to endocrine disruptors have also been investigated as possible risk factors for breast cancer. Breast cancer risk is higher for women in Western countries such as the U.S. and the U.K. than for far Eastern countries such as Japan, China, and India (McPherson et al., 2000). Studies of migrants from Japan to Hawaii indicate that the rates of breast cancer for those migrants increased to the rates similar to those observed for breast cancer in the U.S. (Brinton and DeVesa, 1996). The change in breast cancer risk has been linked in part to an increase in dietary fat intake of the migrants (Kolonel, 1994; Lee et al., 1994). Most dietary studies have focused on the effects of a high-fat diet on breast cancer risk. Multiple studies have shown that a lower intake of fat in the diet decreases serum estrogen levels (Goldin et al.,

1982; Rose et al., 1987; Bennett and Ingram, 1990; Rose et al., 1993); however, there are conflicting results on whether a high-fat diet increases the risk of breast cancer. Most case-control studies and animal studies conclude that a high-fat diet does promote breast cancer (Freedman et al., 1990; Howe et al., 1990; Van't Veer et al., 1990; Richardson et al., 1991; Welsch, 1992); however, most cohort studies have not found a link between high-fat intake and increase risk of breast cancer (Willett et al., 1992; Hunter et al., 1996; Holmes et al., 1999). One pooled analysis of cohort studies did find a weak positive association between saturated fat intake and relative risk of breast cancer; however, no other type of fat was associated with breast cancer risk (Smith-Warner et al., 2001). Studies on bodyweight have found an inverse correlation between pre-menopausal bodyweight and breast cancer risk (Potischman et al., 1996; Cleary and Maihle, 1997; Huang et al., 1997; Trentham-Dietz et al., 1997). One study showed that a low pre-menopausal body mass index (BMI) results in a several fold increase in breast cancer risk. Furthermore, studies focused on bodyweight at puberty have found that lower BMI at puberty increases the risk of breast cancer (Hilakivi-Clarke et al., 2001), whereas higher weight or high fat consumption leads to a lower risk (Le Marchand et al., 1988; Parent et al., 1996; Huang et al., 1997; Magnusson et al., 1998).

Studies looking at the effects of exercise on breast cancer risk are not conclusive as to whether exercise can decrease breast cancer risk. One study does show that exercise can be protective against breast cancer in lean women,

but not in heavier women (Thune et al., 1997), and another study found a reduced risk of postmenopausal breast cancer associated with exercise early in life for women with low BMI at age 18 and those who have gained little or no weight in the adult years (Shoff et al., 2000). However, factors such as diet that often correspond with exercise levels could be confounding. Studies on alcohol consumption have shown that alcohol increases serum estrogen levels (Dorgan et al., 1994; Ginsburg et al., 1996; Muti et al., 1998; Purohit, 1998) and increases the risk of breast cancer development (Longnecker, 1994; Longnecker et al., 1995). Discovery of endocrine disrupting chemicals in our diet and environment and the ability of some to mimic estrogen have led to studies on whether exposure to endocrine disrupting chemicals affects the risk of developing breast cancer. Studies on organochlorine compounds do not show an increase in breast cancer risk with exposure and some studies even show lower pesticide levels in cases than in controls (Krieger et al., 1994; Lopez-Carrillo et al., 1996; Hunter et al., 1997; van't Veer et al., 1997). Furthermore, a meta-analysis on epidemiological studies of high soy intake showed that this high soy intake reduced pre-menopausal breast cancer risk (Hilakivi-Clarke et al., 2002).

1.1.2.2 Genetic risk factors

Approximately 10% of breast cancer cases can be attributed to familial incidence and research has focused on identifying the inherited genes responsible. The breast cancer susceptibility genes *BRCA1* and *BRCA2* were

identified in 1995 and highly penetrant mutations in these genes can account for 20-60% of familial breast cancer cases (Nathanson et al., 2001). First-degree relatives of breast cancer patients have a 2-fold greater risk for the development of breast cancer over the general population (Pharoah et al., 1997), and this cannot be accounted for by *BRCA1/2* alone (Peto et al., 1996). Although environmental factors may contribute to an increase in risk, other low-penetrant genetic variants may be important (Dunning et al., 1999). It has been predicted that a small number of other genetic variants (~ 4) may account for the remainder of familial risk (Easton, 1999).

Mutations in *BRCA1* or *BRCA2* lead not only to an increase in the risk of breast cancer, but also increased ovarian, prostate and pancreatic cancers (Rahman and Stratton, 1998). *BRCA1/2* mutations are described as highly penetrant with a lifetime risk for cancer incidence of 30-70% (Ford et al., 1998), some believe that variations in genetic backgrounds lead to the range in risk (Nathanson et al., 2001). Most mutations are small insertions or deletions, which are thought to lead to truncated forms of the proteins; however, many missense and nonsense mutations have also been described. Correlations between a particular mutation and a disease phenotype have not been convincing. Evidence suggests that the cancer risk associated with *BRCA1* and *BRCA2* mutations is influenced by additional factors, such as genetic background and environment and, therefore, particular mutations can not be

linked to specific disease phenotypes (Thorlacius et al., 1996; Gayther et al., 1997; Struewing et al., 1997).

BRCA1 and BRCA2 are very different in size and primary sequence; however, most evidence suggests they have common biological functions. They have similar patterns of expression and are expressed in many tissues in a cell-cycle dependent manner (Rajan et al., 1996; Bertwistle et al., 1997; Connor et al., 1997; Sharan and Bradley, 1997; Blackshear et al., 1998). Both proteins are localized to the nucleus in somatic cells where they coexist in subnuclear foci that redistribute after DNA damage (Chen et al., 1998b). Studies have confirmed a role for both proteins in response to DNA damage and there is evidence suggesting a role for BRCA1/2 in DNA double-strand break repair. Many studies suggest that BRCA1/2 proteins are important in activation of DNA damage checkpoints and that BRCA1 in particular contributes to DNA damage responses through interactions with enzymes involved in alterations of chromatin and DNA structure as well as transcriptional regulation of other genes involved in responses to DNA damage. Although much evidence points to a role for BRCA1/2 in DNA repair, it is still not clear what is the primary cause of mutations in the *BRCA1/2* genes leading to a disease state.

Many studies have investigated multiple other genes for possible low-penetrance breast cancer susceptibility alleles. The studies have focused mainly on polymorphisms in genes involved in steroid metabolism, such as *COMT*, *CYP17*, *CYP19*, *ER* and *PR* and carcinogen metabolism, such as

CYP1A1, *CYP2E1*, *GSTM1*, *GSTT1*, *GSTP1*, *NAT1* and *NAT2*, or common polymorphism of high-penetrance genes, such as *BRCA1* and *TP53* (Dunning et al., 1999). Results of these studies on low penetrance genes are inconclusive and often result in conflicting conclusions. Of the genes studied, *CYP19*, *GSTM1*, *GSTP1*, and *TP53* appear to be the strongest possibilities for low-penetrance breast cancer susceptibility genes (Dunning et al., 1999), although it is likely that the major low-penetrance familial risk genes are unknown.

1.1.3 Breast cancer therapy

Advances in early detection and treatment of both initial stage and advanced breast cancer have led to significant improvements in patient response and survival in recent years (American, 2003). Primary breast cancer is restricted to the breast and regional lymph nodes and can be removed surgically. Once removed adjuvant systemic therapy is generally appropriate due to the possibility of micrometastatic tumor deposits in other tissue. Adjuvant therapy can include chemotherapy, radiation therapy or endocrine therapy and combinations of at least two of these treatments are commonly prescribed (American, 2003). The basic criterion that determines the appropriate adjuvant therapy is the hormone receptor status of the tumor [estrogen receptor (ER) and progesterone receptor (PR)]. Receptor negative disease is rarely responsive to endocrine therapy and thus chemotherapy and radiation therapy are appropriate. For low risk receptor positive disease, observation or endocrine therapy may be appropriate; however, for the majority of receptor positive breast

tumors a combination of endocrine therapy and chemotherapy is used (Hortobagyi, 2002). The progression of metastatic breast cancer can vary considerably and the therapy of choice often depends on factors such as age, extent of metastasis, presence of co-morbid conditions and hormone receptor status of the tumors that indicate whether a patient is high or low risk (Hortobagyi, 2002). Low risk, hormone receptor positive disease is generally treated with endocrine therapy, whereas, for high-risk patients, chemotherapy in combination with endocrine therapy is the treatment of choice (Hortobagyi, 2002). Multiple endocrine therapies have been developed and many more are being tested. Due to development of new drugs and acquisition of resistance to some endocrine therapies, the most effective sequence for chemotherapeutic drug administration is still being determined and will depend on tumor type. Standard chemotherapy options include combinations of drugs such as cyclophosphamide, methotrexate and 5-fluorouracil (CMF); CMF with vincristine and prednisone; 5-fluorouracil, doxorubicin and cyclophosphamide or the taxanes paclitaxel and docetaxel. Many other chemical agents are effective for treatment of early and advanced breast cancer and there is extensive ongoing research on therapies that target specific molecular pathways of mammary tumor growth.

1.1.3.1 Endocrine therapy

In general endocrine therapy is targeted at interrupting estrogen-induced proliferation of tumor cells. The two main objectives of this type of therapy have

been to decrease estrogen levels, whether through ovarian ablation in premenopausal women or aromatase inhibitors in post-menopausal women, or to inhibit ER signaling with chemicals that bind ER directly such as selective estrogen receptor modulators (SERMs) (Figure 1.1). In addition, other chemicals indirectly inhibit estrogen receptor signaling through crosstalk with other receptors including some nuclear receptors and the aryl hydrocarbon receptor.

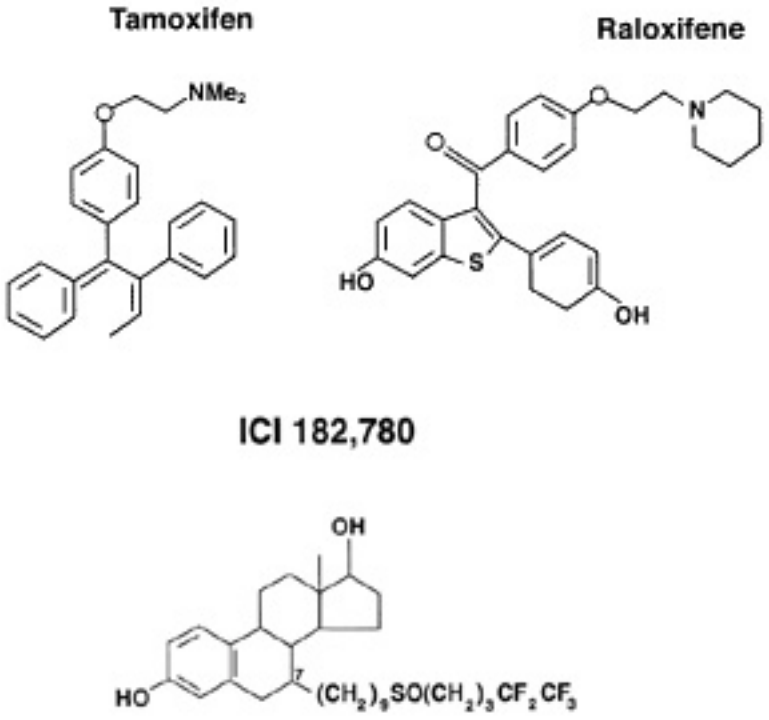


Figure 1.1: Structure of SERMs. (Adapted from Howell et al., 2000).

1.1.3.1.1 Tamoxifen

Tamoxifen was first developed in the 1960's as a potential anti-fertility drug; however, research in the 1970's discovered its potential as a novel antiestrogen for treatment of breast cancer (Harper and Walpole, 1967; Jordan, 1998; Jordan, 2003). Tamoxifen was approved by the USDA in 1978, and for 25 years has been the gold standard for first-line treatment of hormone-sensitive metastatic breast cancer and for adjuvant therapy for early breast cancer with ER+ tumors after removal. Tamoxifen is a non-steroidal triphenylethylene analog and its active metabolite, 4-hydroxytamoxifen, competes with estrogen for binding of the estrogen receptor with high affinity (Dorssers et al., 2001) (Figure 1.1). Because tamoxifen exhibits tissue-specific ER antagonist and agonist activity it is considered a SERM.

Standard 5 year post-operative treatment of breast cancer with tamoxifen decreases the annual odds of death by 25% and decreases the incidence of new contralateral breast cancer by 50% in ER- and/or PR-positive breast cancer (Early, 1998); however, treatment also results in a greater incidence of endometrial cancer (Fisher et al., 1996) and thromboembolis disease (Jaiyesimi et al., 1995; Fisher et al., 1996). Responsiveness to tamoxifen treatment is partially dependent upon the ER and PR status of the tumors. The highest response rate (70%) is seen in ER- and PR-positive tumors, ER-negative and PR-positive tumors have a response rate of 45% and ER-positive and PR-

negative tumors have a response rate of 34% (Clarke et al., 2001). Most studies report a response rate less than 10% for ER- and PR-negative tumors.

In advanced breast cancer, over half of the ER-positive tumors show some response to tamoxifen treatment (Jaiyesimi et al., 1995). A minor portion of patients treated with tamoxifen respond with complete remission of the disease and a majority of patients exhibit a partial remission or a stable disease for more than 6 months. The remaining patients exhibit either a short-term stable disease of less than 6 months or immediate disease progression and are considered intrinsically resistant to tamoxifen treatment (Howell et al., 1988; Robertson et al., 1989; Foekens et al., 1994). Even those patients initially responsive to therapy ultimately develop resistance to tamoxifen treatment and must be switched to second and third line endocrine therapy; however, those intrinsically resistant to tamoxifen are unlikely to respond to further second or third line endocrine therapy (Howell and Dowsett, 1997; Santen and Harvey, 1999).

The chemopreventative effects of tamoxifen are also being investigated. In one study, preventative treatment of high-risk women with tamoxifen decreased the risk of developing breast cancer in both pre-menopausal and post-menopausal women (Fisher et al., 1998). In contrast, two smaller studies did not show a similar decrease in breast cancer risk with tamoxifen treatment (Powles et al., 1998; Veronesi et al., 1998). However, differing criteria for admission, study design and size could account for the conflicting results and

needs to be further examined in order to determine whether tamoxifen is an effective chemopreventative agent for breast cancer.

1.1.3.1.2 Raloxifene

Multiple new SERMs have been developed in hopes of replacing tamoxifen with a drug that is equivalent or better than tamoxifen as an estrogen receptor antagonist in the breast, but that does not have the ER agonist activity of tamoxifen in endometrial tissue. Many of the newer SERMs exhibited activity and toxicity similar to or less than tamoxifen and have not been used to replace tamoxifen for the treatment of advanced disease (Howell et al., 2003); however, one SERM, raloxifene, has shown promise as a possible chemopreventative agent for breast cancer. Raloxifene is a benzothiophene derivative and is already approved for the treatment of post-menopausal symptoms of bone loss in women (Clarke et al., 2001) (Figure 1.1). Raloxifene is antiestogenic in both breast and endometrial tissue and estrogenic effects in bone, lipid metabolism and blood clotting. In a study of post-menopausal women with osteoporosis, raloxifene decreased the risk of invasive breast cancer by 76% during a three-year treatment. Also, raloxifene treatment did not increase the rate of endometrial cancer, but did increase the incidence of thromboembolic disease (Cummings et al., 1999; Cauley et al., 2001).

1.1.3.1.3 Fulvestrant (ICI 182,780)

Fulvestrant, or ICI 182,780, is an estrogen analog with a bulky side chain in the 7α position that prevents recruitment of co-activators to the ER complex

(Figure 1.1). Binding of fulvestrant to the receptor results in ubiquitination and degradation of the receptor leading to decreased estrogen-responsiveness. It has been termed a “pure” antiestrogen, however, it has been shown to activate transcriptional regulation by ERβ (Paech et al., 1997) and to exhibit estrogenic activity in normal and neoplastic mammary cells (Hilakivi-Clarke et al., 1997a; Kurebayashi et al., 1998). Fulvestrant is approved for treatment of post-menopausal women with hormone receptor-positive metastatic cancer following progression of the disease with first line endocrine therapy such as tamoxifen. Studies have shown that is it effective in 1 in 5 patients with resistance to tamoxifen (Howell et al., 2002; Osborne et al., 2002) and is as effective as anastrozole, an aromatase inhibitor, in second line treatment for advanced disease (Howell et al., 1996; Howell et al., 2003). Currently, the need for intramuscular injections is a limiting factor in the administration of fulvestrant and new orally bioactive estrogen analogs, such as ZK191703, are being developed.

1.1.3.1.4 Aromatase inhibitors

Aromatase inhibitors are being developed for the treatment of breast cancer in women without functional ovaries, whether through menopause or surgical removal. Aromatase is the enzyme that converts the androgens androstenedione and testosterone to the estrogens estrone and estradiol in the final step of estrogen synthesis. Estrogen synthesis occurs in peripheral tissues such as fat, muscle, skin, normal breast stromal cells, and breast tumor tissue in post-menopausal women, and aromatization is almost completely inhibited by

administration of aromatase inhibitors (Geisler et al., 2002) resulting in drastically reduced estrogen levels. However, aromatase inhibitors do not decrease estrogen production in the ovaries of pre-menopausal women. Third-generation aromatase inhibitors anastrolzole, letrozole and exemestane are very specific inhibitors and do not have the adverse effects of the first and second generation aromatase inhibitors (Figure 1.2). Anastrozole and letrozole are used in first and second line endocrine therapy for locally advanced and metastatic disease, and anastrozole is also used in adjuvant endocrine therapy with operable early breast cancer. Exemestane is used in second line therapy after progression of the disease with first line endocrine therapy.

There is evidence that third generation aromatase inhibitors are as effective or more effective than the traditional second line endocrine therapies, such as megestrol acetate and aminoglutethimide, and exhibit less toxicity (Buzdar et al., 1996; Jonat et al., 1996; Buzdar et al., 1997; Buzdar et al., 1998; Dombernowsky et al., 1998; Gershanovich et al., 1998; Goss et al., 1999; Kaufmann et al., 2000). Further studies showed that anastrozole and letrozole are more effective than tamoxifen in postmenopausal women for the treatment of advanced breast cancer (Bonneterre et al., 2000; Bonneterre et al., 2001; Mouridsen et al., 2001) and that anatrozole treatment as an adjuvant therapy in patients with operable breast cancer decreases the incidence of relapse and the development of new breast cancer compared to tamoxifen treatment (Baum et al., 2002). However, the third generation aromatase inhibitors are relatively new

and the longe term effects on hormone-responsive tissue such as the bone, cardiovascular system, pelvic floor, lipids, and brain are not known. There is some evidence that the steroidal exemestane may have lower antiestrogenic effects on normal tissue than the non-steroidal anastrozole and letrozole (Goss and Strasser, 2001).

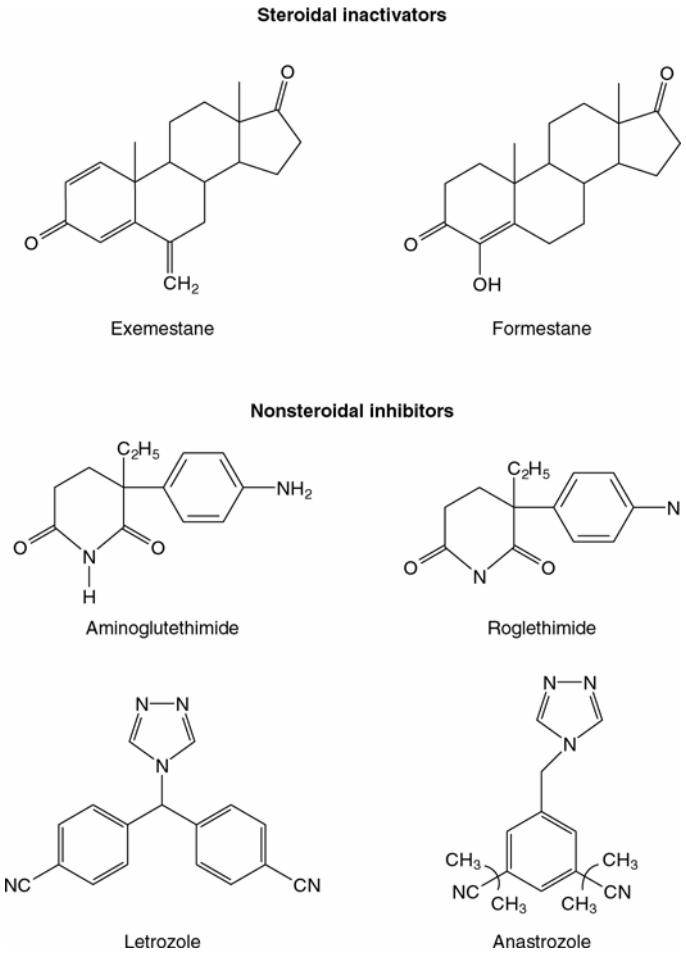


Figure 1.2: Structure of aromatase inhibitors. (Goss and Strasser, 2002).

1.1.3.1.6 Arylhydrocarbon receptor (AhR) agonists

The arylhydrocarbon receptor (AhR) is part of the basic helix-loop-helix family of transcription factors and is the only member that is ligand-activated. The AhR was first shown to bind 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an environmental toxicant that is a by-product of industrial waste and combustion of organic material, and other halogenated aromatic environmental contaminants. Ligand-dependent activation of the AhR is associated with a range of biochemical and toxic responses including induction of drug metabolizing enzymes, disruption of endocrine signaling, hepatotoxic responses such as porphyria, immunotoxiicty, developmental and reproductive toxicity, wasting syndrome, chloracne, tumor promotion and carcinogenesis (Safe, 2001). However, one interesting observation from a long-term feeding study of TCDD showed that although liver tumors were higher in female Spraque-Dawley rats, TCDD also decreased multiple age-related spontaneous tumors including estrogen-dependent uterine and mammary tumors (Kociba et al., 1978). This was one of the first indications that TCDD was able to block estrogen-induced responses and subsequent in vitro and in vivo research since has demonstrated inhibitory crosstalk between the AhR and ER signaling pathways. TCDD inhibits various E2-induced responses in vitro in breast cancer cell lines including cell proliferation, DNA synthesis, gene transcription, and cell cycle progression as well as E2-induced responses in the rodent uterus including wet weight increase, cell proliferation, gene transcription, peroxidase activity, and PR and

ER binding. The ability of TCDD to inhibit mammary tumor formation and growth has been confirmed in various age-dependent, as well as in carcinogen-induced mammary tumor models and in athymic nude mice with breast cancer cell xenografts. Furthermore, epidemiology studies of women exposed to TCDD in Sevesco, Italy show a lower incidence of breast and endometrial cancer (Bertazzi et al., 1993).

Studies on a series of alternate substituted alkyl polychlorinated dibenzofurans (PCDFs) typified by 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) demonstrated that these compounds bind the AhR with moderate affinity and exhibit both AhR agonist and antagonist activities (Figure 1.3). In the rodent model 6-MCDF inhibited many of the toxic responses associated with the AhR including TCDD-induced CYP1A1, porphyria, immunosupression, and cleft palate. However, 6-MCDF alone exhibited antiestrogenic activity and inhibited carcinogen-induced mammary tumor formation in Sprague-Dawley rats. Recently, an increasing number of studies have shown that various synthetic and structurally diverse naturally occurring compounds also exhibit tissue-specific AhR agonist and antagonist activites. These include phytochemicals that exhibit multiple chemoprotective and anticarcinogenic activities such as flavonoids, carotenoids, indole-3-carbinol and resveratrol (Bjeldanes et al., 1991; Jellinck et al., 1993; Chen et al., 1996; Gasiewicz et al., 1996; Gradelet et al., 1997; Casper et al., 1999; Seidel et al., 2000).

Inhibitory AhR-ER crosstalk results in downregulation of several estrogen-induced genes including pS2, c-fos, cathepsin, PR, and heat shock protein 27. Studies have also shown that AhR agonists induce down-regulation of ErbB1 protein and/or phosphorylation in multiple tissues/organs (Astroff et al., 1990; Guyda et al., 1990; Sewall et al., 1995; Zhang et al., 1995). The antiestrogenic properties as well as the possible downregulatory effect on the ErbB family of receptors by AhR agonist warrant the further development of selective AhR modulators (SAhRMs) for breast cancer chemotherapy where the compounds exhibit minimal toxicity but retain the antiestrogenic effects. Studies in this laboratory have focused on two series of SAhRMs: alternate-substituted alkyl PCDFs and ring-substituted diindolylmethanes (DIMs) that are able to inhibit mammary tumor growth in rodent models while exhibiting relatively low toxicity (McDougal et al., 1997; Chen et al., 1998a; McDougal et al., 2000; McDougal et al., 2001) (Figure 1.3).

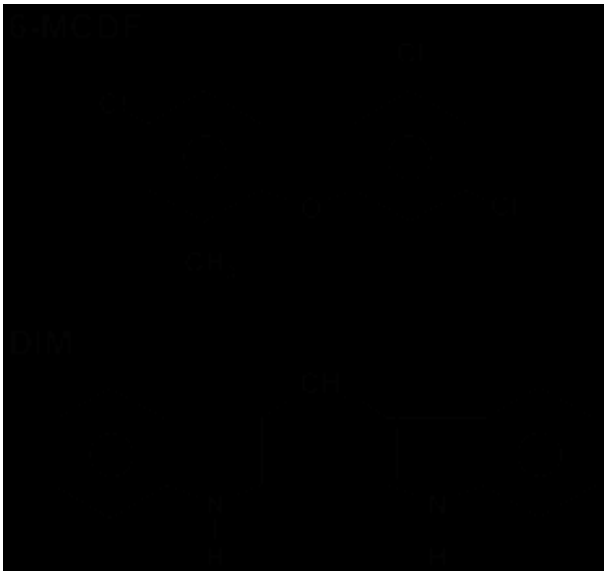


Figure 1.3: Structure of SAhRMs. (Safe, 2001).

1.1.3.2 Molecular targeting

1.1.3.2.1 Retinoids

Retinoids are natural and synthetic vitamin A analogs that possess antiproliferative, differentiative, immunomodulatory, and apoptosis-inducing properties in many cell types (Yang et al., 2002). Vitamin A must be acquired from the diet either as preformed vitamin A (retinol and retinyl ester) or as provitamin A carotenoids that are metabolized to vitamin A in the body, and subsequently converted into retinoic acid by oxidative enzymes (Paik et al., 2003). The two known natural retinoids are all-trans-retinoic acid and 9-cis retinoic acid. Retinoid signaling is largely through two types of nuclear retinoid receptors: retinoic acid receptor (RAR) and retinoid X receptor (RXR) that are part of the nulcear receptor superfamily and act as ligand activated transcription

factors that bind retinoic acid response elements (RAREs) (Pfahl, 1994; Mangelsdorf and Evans, 1995; Chambon, 1996). Cellular retinoid acid binding proteins (CRBP) I and II have also been shown to highly regulate the activity of retinoids (Li, 1999).

Multiple studies have shown aberrant retinoid signaling in breast cancer. (Jing et al., 1996; Widschwendter et al., 1997; Xu et al., 1997; Spinella and Dmitrovsky, 2000). Decreased expression of CRBP has been found both in breast cancer cell lines (Jing et al., 1996) and in tumors (Kuppumbatti et al., 2000), especially in early ductal carcinoma. Aberrant expression of RARs and RXRs has been observed in cultured breast cancer cells and tumors (Widschwendter et al., 1997; Xu et al., 1997; Lawrence et al., 1998; Ariga et al., 2000; Suzuki et al., 2001). RARβ is consistently downregulated or lost in breast cancer (Widschwendter et al., 1997; Xu et al., 1997) and loss of heterozygosity, as well as, hypermethylation of the promoter are thought to contribute to the silencing (Yang et al., 2001a; Yang et al., 2001b). Because many of the chemopreventative effects of retinoids are due to RARβ induction, RARβ expression may be used to identify tumors responsive to retinoid treatment and RARβ promoter demethylation could be used in therapy for some patients (Yang et al., 2002).

Epidemiology studies have given inconsistent results with respect to the benefits of vitamin A in breast cancer prevention. A large prospective study showed an inverse relationship between dietary vitamin A intake and breast

cancer incidence (Hunter et al., 1993); however, two smaller prospective studies showed no association between retinol or β-carotene intake and breast cancer (Kushi et al., 1996; Verhoeven et al., 1997). Inconsistancies between the studies and difficulties in determining vitamin A intake may account for the different results (Paik et al., 2003). Animal studies of mammary tumor formation and growth inhibition by retinoids have identified retinoids that may be effective against breast cancer and have defined windows for effective retinoid treatment (Paik et al., 2003). 4-Hydroxyphenylretinamide (4-HPR, retinamide), which accumulates in mammary tissue, effectively reduces mammary tumor incidence and tumor burden in animal models (Moon et al., 1979), and in carcinogen-induced mammary tumors the window for effective treatment with retinoids is early in tumor development as the anti-tumor activity of the retinoids decreased markedly when treatment began later in tumor progression (McCormick and Moon, 1982) (Figure 1.4). Combination treatments with retinoids (4-HPR or 9-cis retinoic acid) and endocrine therapies such as tamoxifen and raloxifene exhibit some synergistic effects in prevention of tumor formation in carcinogen-induced animal models (Ratko et al., 1989; Anzano et al., 1994; Anzano et al., 1996).

Clinical trials of retinoids have focused mainly on clinical safety; however, positive results for retinoid treatment are indicated. One trial of all-*trans*-retinoic acid in combination with tamoxifen indicated an “objective response” in patients that previously did not respond to tamoxifen alone. Another study of all-*trans*-

retinoic acid in postmenopausal, advanced, nonmetastatic breast cancer showed a reduction in tumor grade and elevated RARβ mRNA in tumors (Toma et al., 2000). A five year trial with 4-HPR treatment of women with stage 1 breast cancer or ductal carcinoma in situ showed no difference in overall recurrence of second breast cancer with or without treatement; however, re-evaluation which accounted for menopausal status indicated that 4-HPR treatment may be beneficial for premenopausal women (Veronesi et al., 1999). Further study of retinoid treatment for breast cancer is necessary and ongoing.

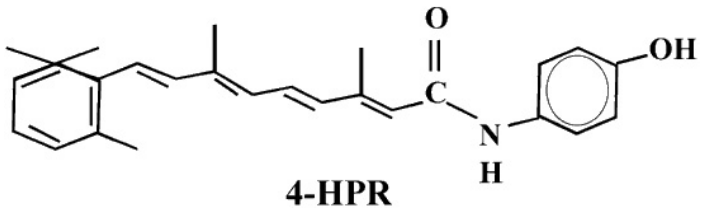


Figure 1.4: Structure of retinamide.

1.1.3.2.2 Angiogenesis

Angiogenesis is the process of new blood vessel development from existing vasculature. It is a multistep process in which endothelial cells proliferate and become motile, move towards protein angiogenic factors in hypoxic tissue, degrade the basement membrane and form primitive vessels (Harris, 1997). All tissues need oxygen and nutrients provided by the

vasculature; as tissue proliferates it requires parallel angiogenesis to provide oxygen and nutrients to maintain the growth. Angiogenesis occurs in normal tissue, especially during development, wound healing and menstrual cycling (Smith, 2001), as well as, in tumor tissue. New vessel growth is a tightly regulated process, especially in adults that is stimulated by numerous vascular growth factors, including vascular endothelial growth factor (VEGF), and is inhibited by factors such as angiostatin and endostatin (O'Reilly et al., 1994; O'Reilly et al., 1997; Ferrara, 2001). During tumorigenesis a tumor outgrows the capacity of the surrounding vasculature to provide oxygen and nutrients, therefore the center becomes hypoxic and produces hypoxia-dependent angiogenic factors that stimulate new vessel growth. Studies have shown that the growth of a tumor beyond 1-2 mm^3 is dependent upon new vascular development for growth, and if none is provided then the tumor will go into a dormant state (Folkman, 1995). It has been hypothesized that since angiogenesis is relatively low in normal tissue then anti-angiogenic treatment would be specific to tumors and significant research has focused on development of anti-angiogenic agents for the treatment of cancer.

The importance of angiogenesis in breast cancer has been demonstrated in multiple in vivo and clinical studies. One in vivo study showed that normal, healthy breast tissue showed no angiogenic potential, where as angiogenesis was induced in all of the breast carcinoma samples (Lichtenbeld et al., 1998). Furthermore, the use of anti-angiogenic agents such as microtubule disruptors,

VEGF receptor tyrosine kinase inhibitors and adenoviral expression of angiostatin in animal mammary tumor models have demonstrated the need for new vasculature for tumor growth (Klauber et al., 1997; Wedge et al., 2000; Gyorffy et al., 2001). Clinical breast cancer studies looking at angiogenesis as a prognostic indicator have shown an association between increased angiogenesis, measured by microvessel density, and decreased survival, decreased relapse-free survival, and an increase in metastases (Weidner et al., 1991; Weidner et al., 1992; Heimann et al., 1996).

There are three potential molecular targets for the development of anti-angiogenic agents: proteins and growth factors that stimulate angiogenesis, natural inhibitors, and survival factors necessary for maintainence of new vasculature. VEGF is a strong potential target of anti-angiogenic therapy because it induces endothelial cell proliferation and motility as well as increasing vascular permeability. One clinical trial treated metastatic breast cancer with a humanized recombinant mAb to VEGF (Miller, 2002). After 22 weeks of treatment 17% of the patients were responsive or stable and 3 patients continued treatment without progression of disease for over 12 months (Miller, 2002). Based on this study, anti-VEGF therapy shows potential as a possible treatment for breast cancer.

One difficulty in developing anti-angiogenic agents is to identify a molecular target that is necessary for disease progression, reliably measure the effects of therapy on the molecular target in clinical samples, and correlate that

with benefits to the patient. This is especially difficult with anti-angiogenic agents, because a simple measure of angiogenesis is not available. Furthermore, clinical trials on the benefits of anti-angiogenic agents must be carfully designed. Preclinical studies have shown that anti-angiogenic agents have the greatest potential as adjuvant therapy where prevention and delay in disease progression are the prime objectives (Miller, 2002). In contrast, anti-angiogenic therapy for metastatic disease may not result in a classical objective response and tumor involution seen with cytotoxic drugs since disease stabilization is the most likely outcome for these agents (Miller, 2002).

1.1.3.2.3 ErbB2

The human epidermal growth factor receptor (HER) family, also known as the ErbB family of receptors, is involved in normal growth and differentiation of the breast (Carraway et al., 1997) and has also been implicated in carcinogenesis (Slamon et al., 1989). The family includes genes that encode four tyrosine kinase, cell membrane receptors, ErbB1 (HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4(HER4) that are expressed at various levels in a variety of tissues. The receptors bind a range of growth factor ligands and activation via the tyrosine kinase region of the receptors mediates cell proliferation, differentiation, and survival (Salomon et al., 1995; Schlessinger, 2000; Simon, 2000). Dysregulation of ErbB signaling pathways can result from receptor gene amplifications or mutations that lead to an increase in receptor transcription, translation, or stabiltity altering the receptor protein expression

levels in the cells. ErbB2 overexpression in observed in 20-30% of breast tumors (van de Vijver et al., 1987; McCann et al., 1991) and is associated with aggressive tumor behavior and poor prognosis (Hynes and Stern, 1994). Patients with ErbB2 overexpressing tumors have a significantly shorter overall survival rate and time to relapse than patients with tumors that lack ErbB2 overexpression (Slamon et al., 1987; Slamon et al., 1989; Berchuck et al., 1990). One study showed that 92% of overexpression of ErbB2 in breast cancer was due to gene amplification (Pauletti et al., 1996), and overexpression is found in all stages of tumor development, but not in benign tissue (Allred et al., 1992). Much research has focused on the use of ErbB2 as a prognostic and predictive factor as wells as a target for breast cancer therapy.

Multiple approaches have been attempted to target the ErbB receptors including anti-ErbB specific monoclonal antibodies (mAbs), small molecular tyrosine kinase inhibitors, cytotoxic or immunoreactive agents coupled to anti-ErbB mAbs, and antisense technology targeted to the receptors. The mAb trastuzumab (Herceptin) has been the focus of most research targeting ErbB2 and is approved for use as a single agent for the treatment of patients with metastatic breast cancers that express ErbB2. Trastuzumab binds to the extracellular domain of ErbB2 with high affinity and causes internalization and degradation of the receptor and antibody-dependent cytotoxic responses depending on the level of ErbB2 expression (Baselga et al., 2001). One study of trastuzumab treatment in patients with high level ErbB2 tumor expression

showed 23% of patients had tumor shrinkage of 50% or more and an additional 8% developed stable disease for a period greater than 6 months (Seidman et al., 2001). Small molecular weight inhibitors of the tyrosine kinase activity of ErbB2 are also being investigated in animal models (Zhang et al., 1999); however, a second generation of more specific ErbB2 inhibitors are currently being developed for future use in clinical trials.

1.1.3.4 Peroxisome proliferator-activated receptor γ (PPARγ)

PPARγ is a member of the nuclear hormone receptor superfamily and is a ligand-activated receptor that heterodimerizes with RXR. The resulting heterodimer acts as a transcription factor that binds to peroxisome proliferator response elements (PPREs) in target gene promoters (Lehmann et al., 1995; Lemberger et al., 1996; Saltiel and Olefsky, 1996; Schoonjans et al., 1996b). In addition, RXR ligands in combination with PPARγ agonists increase transcriptional activity (Mukherjee et al., 1997). Two isoforms of PPARγ (PPARγ1 and PPARγ2) are produced from alternate promoter usage. PPARγ1 is expressed in multiple tissues including the breast and PPARγ2 is specific for adipocytes (Mueller et al., 1998). Natural ligands for PPARγ include the eicosanoid 15-deoxy-$\Delta^{12,14}$-prostaglandinJ_2 (PGJ_2) and certain unsaturated fatty acids. Multiple synthetic ligands called thiazolidinediones (TZDs) including rosiglitazone, pioglitazone, and troglitazone are currently used for the treatment of type II diabetes melitis (Figure 1.5). TZDs decrease insulin resistance by

increasing glucose uptake in peripheral tissue and decreasing hepatic glucose output.

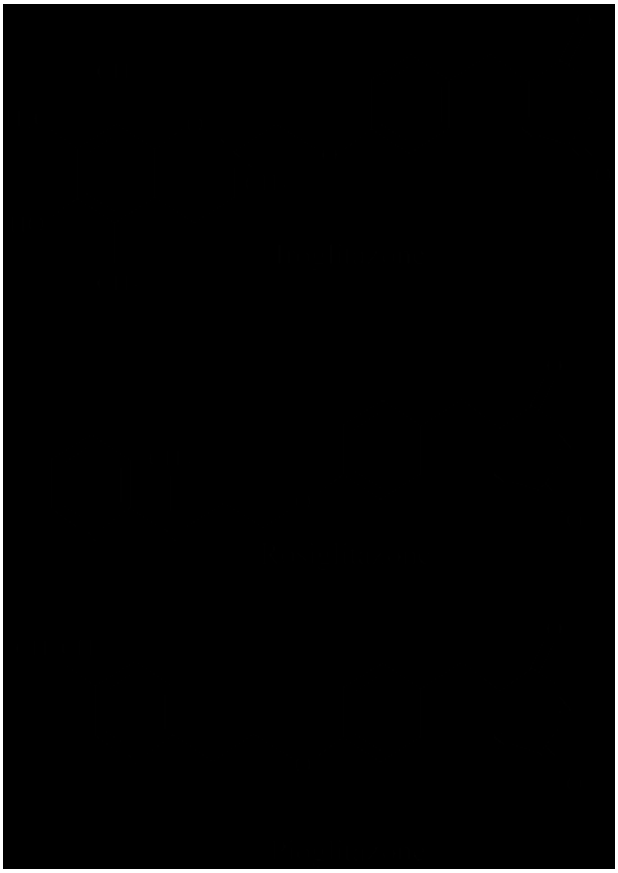


Figure 1.5: Structure of TZDs. (Martens et al., 2002).

Activation of PPARγ leads to transcriptional activation of numerous genes, especially those involved in metabolism and transport, differentiation of preadipocytes to adipocytes, as well as transcriptional repression of certain genes (Tontonoz et al., 1994a; Tontonoz et al., 1994b; Schoonjans et al., 1996a;

Jiang et al., 1998; Ricote et al., 1998; Li et al., 2000; Marx et al., 2000; Chawla et al., 2001). Antiproliferative and prodifferentiation activities of PPARγ ligands have been demonstrated in multiple cancers including breast cancer, however the target genes involved are unknown (Koeffler, 2003). Studies in different cancer cell lines have demonstrated that PPARγ agonists increased expression of cyclin-dependent kinase inhibitors $p21^{waf1}$ and $p27^{kip1}$, decreased cyclin D1 expression, decreased expression or activation of inflammatory cytokines and transcription factors such as TNF, IL-4, IL-1, and NFκB, and induced apoptosis (Karin and Delhase, 2000; Joyce et al., 2001). Furthermore, studies have shown that PPARγ-independent effects of PGJ_2 and TZDs may also contribute to their anticarcinogenic effects. In $PPAR\gamma^{-/-}$ ES cells TZDs deplete calcium stores causing activation of protein kinase R that phosphorylates and inactivates the α-subunit of eukaryotic initiation factor 2 (Palakurthi et al., 2001). A decrease in activity of translation initiation factors such as eukaryotic initiation factor 2 could contribute to the antiproliferative effects of these compounds. Furthermore, PGJ_2 inhibits the NFκB pathway in PPARγ-negative cells (Rossi et al., 2000; Straus et al., 2000). Therefore, PPARγ-dependent and independent pathways contribute to the anticarcinogenic effects of PPARγ ligands and contributions of these pathways may vary with cell context.

Breast cancer cells often express high levels of PPARγ and multiple in vitro and in vivo studies have demonstrated the potential for applications of

PPARγ ligands in treatment of breast cancer. TZDs inhibit proliferation and induce differentiation-like changes and can act synergistically with retinoids to inhibit proliferation and induce apoptosis in breast cancer cell lines in vitro and in xenografts on nude mice (Muller et al., 1988; Elstner et al., 1998). In vivo studies with carcinogen-induced mammary tumor models have shown that GW7845, a PPARγ ligand, inhibits tumor development in rats (Suh et al., 1999) and troglitazone inhibited tumor formation in mice. Combined treatment with retinoids plus troglitazone enhanced the inhibition of tumor formation (Mehta et al., 2000). Furthermore, carcinogen treatment of mice with a heterozygous germ-line deletion of PPARγ leads to a higher incidence of mammary tumor formation compared to wild-type mice, suggesting a protective role for PPARγ in the mammary gland (Girnun et al., 2002). These and other studies demonstrate the potential application of PPARγ ligands in breast cancer therapy, especially in conjunction with retinoid treatment.

1.1.3.5 Vitamin D_3 analogs

Vitamin D_3 is synthesized in the skin by the action of ultraviolet light and is then transformed by two metabolic steps in the liver and kidney to the biologically active 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Figure 1.6). Adequate synthesis of vitamin D_3 and dietary intake of calcium is necessary for skeletal health, and the active form of vitamin D_3 stimulates intestinal absorption of calcium and phosphate. The historical geographic distribution of rickets, a disease caused by vitamin D_3 deficiency, parallels that for some cancer rates

and enhanced sun exposure is also associated with lower mortality rates from prostate, breast and colon cancer (Garland and Garland, 1980; Garland et al., 1990; Gorham et al., 1990; Emerson and Weiss, 1992; Hanchette and Schwartz, 1992; Garland et al., 1999) implying a link between vitamin D_3 and cancer development. $1,25(OH)_2D_3$ acts through the vitamin D receptor (VDR), a member of the nuclear receptor superfamily. Ligand bound VDR heterodimerizes with other nuclear receptors, preferentially RXR, and regulates gene transcription by binding to vitamin D receptor response elements (VDREs) in gene promoters (Carlberg, 1995). Although the majority of VDR action is thought to be mediated through genomic pathways, non-genomic, rapid responses through a putative membrane receptor have been described (Nemere et al., 1998).

The VDR is expressed in normal breast tissue and is highly regulated during pregnancy and lactation (Mezzetti et al., 1987; Berger et al., 1988; Colston et al., 1988). Multiple studies have implicated a role for $1,25(OH)_2D_3$ in differentiation and milk production in the mammary gland (Mezzetti et al., 1988; Colston and Hansen, 2002). A high proportion of breast cancer tumors also express VDR (Freake et al., 1984; Eisman et al., 1986; Berger et al., 1987) and studies have reported a positive relation between VDR expression and disease-free survival (Colston et al., 1989; Berger et al., 1991). Epidemiology studies have also linked vitamin D3 and breast cancer risk. Studies have shown that vitamin D3 deficiency is associated with an increase in breast cancer risk

(Janowsky et al., 1999) and disease activity (Mawer et al., 1997) and that vitamin D3 intake and breast cancer risk have an inverse relationship (John et al., 1999; Shin et al., 2002). Furthermore, multiple studies have reported a link between polymorphisms of VDR and breast cancer risk (Curran et al., 1999; Lundin et al., 1999; Ingles et al., 2000; Bretherton-Watt et al., 2001).

The ability of $1,25(OH)_2D_3$ to inhibit the growth of multiple cancer cell lines including breast cancer cell lines in vitro led to development of a wide variety of synthetic analogs with the majority of modifications on the C,D-ring and the C-17 side chain (Hansen et al., 2001a; Colston and Hansen, 2002). The goal is to separate the growth regulation of the compound from the calcium mobilization and develop an analog with enhanced growth regulatory activity compared to the native compound but a weaker effect on calcium metabolism. Multiple analogs inhibit growth of breast cancer cells and various mechanisms of action have been reported including: regulation of cell cycle progression with arrest in G0/G1 (James et al., 1996; Simboli-Campbell et al., 1997; Wu et al., 1997; Hansen et al., 2001b), induction of morphological and biochemical features of apoptosis (Welsh, 1994; James et al., 1995; Simboli-Campbell et al., 1996; Narvaez and Welsh, 1997; Hansen et al., 2001b), modification of growth factor signaling [EGF (Koga et al., 1988; Desprez et al., 1991), amphiregulin (Akutsu et al., 2001), TGFβ (Koli and Keski-Oja, 1995; Mercier et al., 1996), and IGF-1 (Vink-van Wijngaarden et al., 1996; Xie et al., 1997; Xie et al., 1999a; Pirianov and Colston, 2001)], and a decrease in invasiveness and angiogenesis

(Oikawa et al., 1990; Majewski et al., 1993; Hansen et al., 1994; Majewski et al., 1996; Iseki et al., 1999).

EB1089 is a second-generation analog that has shown potential for breast cancer therapy (Figure 1.6). EB1089 has a conjugated double bond system in the side chain region and is 50 time more active than $1,25(OH)_2D_3$ in vitro but has reduced activity on calcium metabolism in vivo (Colston et al., 1992; Mathiasen et al., 1993). Mulitple studies in carcinogen-induced and breast cancer cell xenograft models in rodents have demonstrated a decrease in tumor progression after treatment with EB1089 (Colston et al., 1992; VanWeelden et al., 1998). Some have reported tumor regression and indicators of apoptosis (James et al., 1998; VanWeelden et al., 1998), as well as enhanced activity when combined with paclitaxel (Koshizuka et al., 1999a) or retinoic acid (Koshizuka et al., 1999b). Animal models for bone metastasis have also shown increased survival and decreased metastasis when treated with EB1089 (El Abdaimi et al., 2000). One phase I safety trial for EB1089 which included patients with advance breast cancer and colorectal cancer determined the maximum tolerated dose and found that 10 of the 36 patients developed hypercalceamia that later was resolved (Gulliford et al., 1998). Also, while no clear anti-tumor activity was observed, 4 of the 25 breast cancer patients showed disease stabilization for more than 3 months. Additional trials of EB1089 are underway and, in common with other vitamin D analogs, the biggest

obstacle for clinical applications is the level of hypercalcemic effects at the effective anticarcinogenic dose.

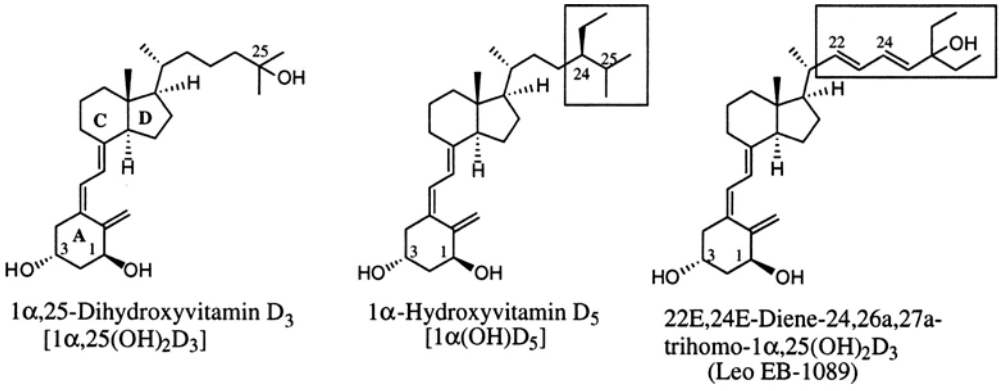


Figure 1.6: Structure of vitamin D_3 analogs. (Adapted from Guyton et al., 2003).

1.1.4 Mammary carcinogenesis

The process of carcinogenesis involves the three basic steps of initiation, promotion and progression. Initiation occurs when DNA is damaged and not properly repaired. DNA damage occurs regularly through exposure to chemicals, radiation and viruses and the majority of damage is eliminated through repair mechanisms or cell death. However, a minor portion of DNA damage that is not repaired can lead to permanent genetic damage that alters gene expression and results in the formation of neoplastic cells. Promotion occurs when a neoplastic cell is induced to proliferate and forms a premalignant lesion. The mechanism by which promotion leads to a growth advantage for

neoplastic cells is not clearly understood; however, tumor promoters usually alter signal transduction pathways that could result in dysregulation of growth signals. In the final step of progression mechanisms such as altered mitotic apparatus, telomere function (Blackburn, 1994), DNA hypomethylation, recombination, and gene transposition (Cheng and Loeb, 1993) lead to changes in chromosome structure (insertions, deletions, strand breaks) and genetic instability. These steps lead to formation of cancer cells, which exhibit uninhibited growth and the potential for metastasis. Cancer cells acquire several alterations that lead to malignant growth and these include: self-sufficiency in growth signals, insensitivity to growth inhibition, evasion of apoptosis, limitless replicative capability, angiogenic ability, and invasiveness (Hanahan and Weinberg, 2000).

Mammary carcinogenesis is also a progression through defined pathological stages that begins with atypical epithelial hyperplasia in which the epithelial cells proliferate and form multiple cell layers from the single cell layered epithelium (Figure 1.7). This progresses to in situ carcinoma in which the epithelial cells continue to proliferate and fill the mammary duct or lobule; however, with in situ carcinoma the basement membrane of the structure remains intact. Invasive carcinoma occurs when the basement membrane breaks down and epithelial cells invade the surrounding fatty and connective tissue where they can then spread to other parts of the body such as the lung, bone, or lymph nodes. Ductal epithelial cells are the most common targets for

development of breast cancer and ductal carcinoma in situ is estimated to account for 85% of the new in situ carcinoma cases in 2003 (American, 2003). The other major target for breast cancer development is the lobular epithelial cell and lobular carcinoma in situ accounts for most of the other 15% of new in situ carcinoma cases (American, 2003).

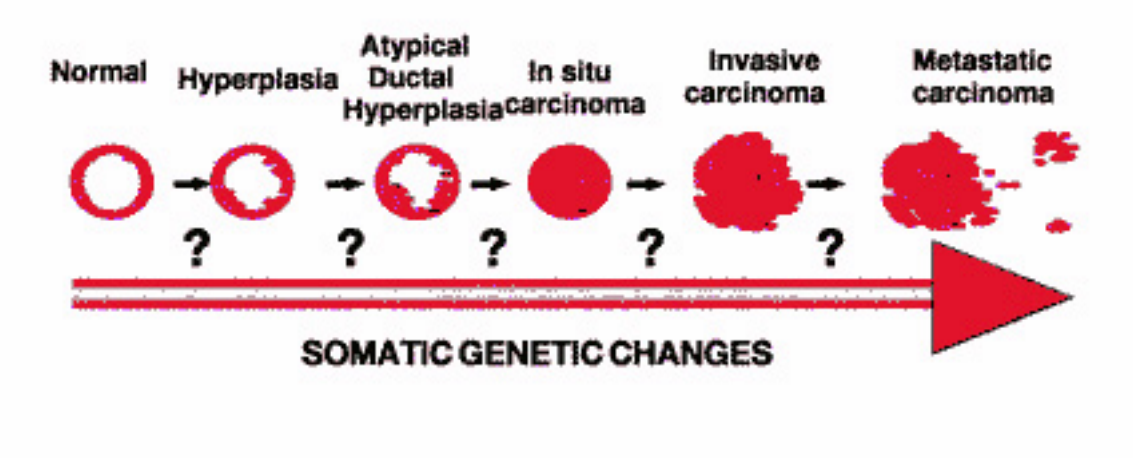


Figure 1.7: Progression of mammary carcinogenesis. (Polyak, 2001).

Hormones are involved in normal mammary development throughout gestation, puberty, pregnancy and lactation, and their involvement in development of breast cancer has also been established. Studies in carcinogen-induced and spontaneous mammary cancer models in rodents have shown that both estrogen and progesterone are able to initiate and promote mammary tumor formation and growth (Jabara et al., 1973; Welsch, 1985;

Robinson and Jordan, 1987). There are multiple possible mechanisms for hormonal initiation and promotion of mammary cancer. One mechanism for the initiation of mammary cancer by estrogen and other hormones is through metabolic activation of the hormones into reactive intermediates that are capable of adducting to DNA. One study demonstrated that in ER-negative cells treatment with E2 and DES resulted in chromosomal damage consistent with that observed in breast tumors (Russo, 2001). Therefore, E2 and DES are capable of DNA damage that may initiate tumor development in actual breast cancer cases and the damage is independent of ER signaling. Another mechanism for promotion of mammary tumor growth by estrogen is through the induction of epithelial cell proliferation. Multiple studies have demonstrated that estrogen induces mammary epithelial cell proliferation through the ER (Osborne et al., 1985; Katzenellenbogen et al., 1987). Premalignant atypical epithelial hyperplasia is characterized by intense epithelial cell proliferation and expression of ER in the presence of estrogen could give these cells a growth advantage (Hansen and Bissell, 2000). However, not all early lesions express ER so other unknown mechanisms of promotion must also contribute to breast cancer development.

1.2 Estrogen and estrogen receptors

1.2.1 Physiological role of E2

E2 is an important steroid hormone required for normal development and function of multiple organ systems including the reproductive, cardiovascular, and immune systems, as well as in aberrant growth such as mammary and uterine cancers. In the reproductive system, E2 functions in the synchronized phases of proliferation and differentiation in the uterus and in folliculogenesis and steroid production in the ovary. In the mammary gland, E2 is involved in ductal growth and stimulates formation of terminal end buds and proliferation of ductal epithelium.

1.2.1.1 Uterus

The uterus undergoes cyclic changes during the reproductive cycle in preparation for implantation of the embryo. Upon implantation the uterus undergoes massive differentiation of the stroma surrounding the implanted embryo. The uterus develops from the Mullerian ducts, which also give rise to the infundibula, oviducts, cervix, and anterior vagina (Gray et al., 2001). The uterine wall is composed of two compartments, the outer myometrium and the endometrium. The myometrium is the smooth muscle component of the uterine wall and consists of an inner circular smooth muscle layer and an outer longitudinal smooth muscle layer. The endometrium forms the mucosal lining of the uterus and is comprised of a stromal component and epithelial cells. The endomentium contains two types of epithelial cells the luminal and glandular

epithelial cells that form a single cell layer lining the lumen and glands of the uterus (Gray et al., 2001).

Preparation of the uterus for implantation is a complex process that involves synchronization of the embryo and is dependent on the multifunctional effects of both estrogen and progesterone on the uterine endometrium (Couse and Korach, 1999). Estrogen production that peaks at ovulation primes the uterus by inducing differentiation and proliferation of the luminal and glandular epithelial cells and increasing PR expression in the stroma and myometrium. The release of progesterone from the corpus luteum along with a physical stimulus of the uterine lining results in subsequent induction of decidualization of the stroma. Decidualization is a process of massive proliferation and differentiation of the stroma and localized increases in vascular permeability and edema leading to a swelling of the uterine stroma. Finally, attachment of the embryo to the uterine wall in the mouse is thought to be dependent upon a secondary increase in levels of E2.

1.2.1.2 Ovaries

The ovary can be divided into three main functional units: follicles, corpus lutea, and interstitial/ stromal tissue. A maturing follicle is comprised of an outer thecal layer surrounding multiple granulosa cell layers that together they act to encase the maturing oocyte. The follicles differentiate into corpus lutea after ovulation and the corpus lutea are vascularized structures that contain terminally differentiated thecal and granulosa cells. The interstitial or stromal tissue is the

matrix in which the follicles and corpus luteua are suspended and is the source of undifferentiated cells that eventually will form thecal and granulosa cells. Dedifferentaited thecal and granulosa cells from atretic follicles and regressed corpus luteua are also part of the stromal tissue. (Baird, 1984; Couse and Korach, 1999).

The two main functions of the ovaries are steroid production and folliculogenesis, and together they can be divided into two separate phases: the follicular phase of follicle maturation and E2 synthesis and the luteal phase that begins with ovulation in which the corpus luteum synthesizes large amounts of progesterone as well as E2. At the beginning of the follicular phase primordial follicles are recruited to form the primary follicles that grow and prepare for ovulation. Primary follicles develop through secondary, tertiary, and atretic or graffian stages that are each characterized by defined structure and function. The selection of follicles for ovulation in which some secondary-tertiary follicles will undergo atresia and others will continue development to the graffian or ovulatory stage is not well understood. A surge in the gonadotrophins LH and FSH leads to rupturing of the follicle and ovulation, the remaining thecal and granulosa cells of the follicle terminally differentiate to form a corpus luteum and the luteal phase begins (Richards, 1980; Couse and Korach, 1999).

Steroid synthesis occurs in the granulosa and thecal cells of the follicles, as well as the interstitial tissue, and is highly regulated by the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) that regulate the

expression and activity of the steroidogenic enzymes within the cells. For E2 production, LH stimulates the synthesis of androgens (androstenedione and testosterone) from cholesterol in the thecal cells through transcriptional and translational regulation of cholesterol side chain cleavage enzyme and 17α-hydroxylase/$_{C17\text{-}20}$ lyase (Couse and Korach, 1999). Androgens pass through the basement membrane to the granulosa cells where FSH stimulation and signaling from the oocyte regulate the aromatase-dependent conversion of androstenedione and testosterone to estrone and estradiol, respectively (Vanderhyden et al., 1993; Vanderhyden and Tonary, 1995; Vanderhyden and Macdonald, 1998). Estradiol is released into the follicular fluid and passes through the basement membrane to enter circulation. When ovulation occurs, luteinization of the follicle and differentiation of the thecal and granulosa cells leads to formation of the corpus luteum and altered expression and activity of the steroidogenic enzymes which now produce high levels of progesterone.

Numerous studies have described the effects of high levels of locally synthesized estrogen in the ovary that are thought to be essential for normal ovarian function. Multiple estrogenic effects have been described in granulosa cells including DNA synthesis and proliferation, increased ER expression, synthesis of IGF-1, increased gap junction size and number, attenuation of apoptosis and follicular atresia, and augmented response to FSH (Richards, 1975; Richards et al., 1976; Rao et al., 1978; Burghardt and Anderson, 1981; Farookhi and Desjardins, 1986; Hernandez et al., 1989; Wang and Greenwald,

1993; Hsueh et al., 1994; Bley et al., 1997). Overall, the effects of estrogen seem to increase the response of the follicles to gonadotrophins, resulting in increased estrogen synthesis (Zhuang et al., 1982; Wang and Greenwald, 1993). Thus estrogen appears to work in both autocrine and paracrine fashion in conjunction with gonadotrophins to regulate ovarian responses.

1.2.1.3 Mammary gland

The mammary gland is a dynamic structure that is continually changing throughout the reproductive life of females to form an extensive ductal gland capable of milk production. Development of the mammary gland is divided into 5 stages: embryonic/fetal, neonatal/prepubertal, pubertal, sexually mature adult, and pregnancy/lactation (Hovey et al., 2002). During embryonic and fetal development mammary epithelium invades from the nipple into a pad of fatty tissue called the mammary fat pad and forms a rudimentary, branched ductal network in the proximal corner of the fat pad. During the neonatal and prepubertal time period the mammary epithelium remains a rudimentary ductal network and grows isometrically with the rest of the body. At puberty ovarian hormones are released and terminal end buds (TEB) form from the distal ends of the mammary ducts that swell into the bulbous TEB structures with multiple layer of cuboidal epithelial cells. The TEBs proliferate and invade into the mammary fat. Growth and branching of the ductal network through bifurcation at the TEB or lateral bud formation from side branching continues until the extent of the fat pad is reached. Once the ends of the fat pad are reached the TEBs

regress. In sexually mature adult females the mammary gland is characterized by limited growth and lobule development or alveolar budding followed by regression with each menstrual or estrous cycle. Final maturation and differentiation of the mammary gland occurs during pregnancy and lactation. The mammary epithelium expands and differentiates into secretory, milk-producing, lobular alveoli and the fat cells dedifferentiate into preadipocytes. With cessation of suckling, the mammary gland regresses by apoptosis in a process called involution to return to the pre-pregnancy, adult stage of the mammary gland.

The ductal network of the mammary gland is composed of a single layer of luminal epithelial cells that lay on a layer of myoepithelial cells. A basement membrane separates the myoepithelial and luminal epithelial cells from the stromal tissue, which consists of stromal fibroblast cells, adipocytes, immune cells, and vasculature. The terminal end buds that form during puberty are bulbous structures that have muiltple layers of epitheial cells called body cells covered by a single layer of epithelial cells called cap cells that sit on a minimal basement membrane (Wiseman and Werb, 2002).

Mammary gland development from the pubertal stage to pregnancy and lactation is highly regulated by both reproductive and metabolic hormones (Neville et al., 2002). Estrogen is thought to play a major role in ductal morphogenesis during puberty, especially in TEB formation and cellular proliferation of the ductal epithelium resulting in ductal elongation (Neville et al.,

2002). Estrogen is also important for lobular and alveolar development during the adult reproductive cycle. Studies in ovariectomized mice have shown that exogenous treatment with E2 and progesterone induce proliferation in ductal and TEB epithelial cells leading to alveolar and ductal side branching as observed during the estrous cycle (Bresciani, 1968; Haslam, 1988).

1.2.2 ER subtypes

Estrogens modulate cellular changes through steroid hormone receptors called ERs. Thus far two ERs have been identified, ERα and ERβ (Figure 1.8). Separate genes located on different chromosomes encode the two receptors (Kuiper et al., 1996; Tremblay et al., 1997). Both receptors contain the A-F functional domains described for members of the nuclear receptor superfamily; however, the homology between the two ERs varies among these domains. The DNA binding region is highly conserved between ERα and ERβ, whereas the N'-terminal A/B domain that contains activation function-1 (AF-1) is not (Enmark et al., 1997). Amino acid residues critical for function of AF-2 are conserved between the receptors in the mouse and mutations lead to ligand-independent activation of both receptors (Tremblay et al., 1997; Giguere et al., 1998; Tremblay et al., 1998). Also, the site in the AF-1 region necessary for the ligand-independent activation of ERα by mitogen-activated protein kinase (MAPK) is present and functional in ERβ (Tremblay et al., 1997; Tremblay et al., 1998).

Figure 1.8: Schematics of human ERα and ERβ. (Katzenellenbogen et al., 2000).

Both receptors bind E2 and interact with ERE sequences; however distinct ligand binding profiles have been described (Katzenellenbogen and Katzenellenbogen, 2000; Katzenellenbogen et al., 2001; Meyers et al., 2001; Mortensen et al., 2001; Shiau et al., 2002) and the transactivation potential differs between the two receptors (Kuiper and Gustafsson, 1997; Paech et al., 1997; Barkhem et al., 1998; Kuiper et al., 1998; Montano et al., 1998; Hall and McDonnell, 1999). In most contexts, ERβ exhibits lower activity than ERα. Two studies have shown that 4-hydroxytamoxifen does not induce agonist activity with ERβ as it does with ERα (Tremblay et al., 1997; Watanabe et al., 1997) and studies on transcriptional activation through an AP-1 site showed that antiestrogens exhibited inhibitory activity with ERα and exhibited agonist activity with ERβ (Paech et al., 1997). Studies investigating ER activation of GC-rich

sites through Sp1 have also shown differential activity between ERα and ERβ (Saville et al., 2000).

Multiple studies have shown potential cooperative activity between ERα and ERβ through formation of heterodimers in vitro (Cowley et al., 1997; Pettersson et al., 1997; Tremblay et al., 1997; Ogawa et al., 1998); however, the significance of the findings depends upon identifying coexpression of the receptors in tissue. ERα and ERβ are expressed in the uterus, ovaries, lungs, male reproductive tract, thyroid, adrenals, bone, heart, and various regions of the brain; the prostate and the ovaries are the only tissues in which ERβ expression is higher than ERα (Shughrue et al., 1996; Arts et al., 1997; Byers et al., 1997; Couse et al., 1997; Kuiper et al., 1997; Brandenberger et al., 1998; Pau et al., 1998). ERα is also expressed in the pituitary, mammary gland, kidney, liver and skeletal muscle (Couse et al., 1997). Differences in expression patterns between species have also been found. ERβ is detected in the pituitary of the rat, human, and rhesus monkey, but not in the mouse (Couse et al., 1997; Mitchner et al., 1998; Pau et al., 1998; Petersen et al., 1998; Shupnik et al., 1998; Wilson et al., 1998); in the human mammary gland, ERβ has been detected in both normal and neoplastic human tissue and cell lines, whereas ERα is predominantly expressed in the mouse mammary gland (Couse et al., 1997; Dotzlaw et al., 1997; Enmark et al., 1997; Moore et al., 1998; Vladusic et al., 1998; Dotzlaw et al., 1999). Furthermore, in tissues such as the ovaries and

prostate where significant levels of ERβ expression have been found, the two receptors have distinct patterns of expression within particular cell types. In the ovaries ERβ is expressed in the granulosa cells whereas ERα is expressed in the thecal and interstitial regions (Hiroi et al., 1999; Rosenfeld et al., 1999). Similarly, in the prostate ERβ expression is detected in the epithelium and ERα is detected in the stroma (Kuiper et al., 1996). In contrast, one study has found co-localization of ERα and ERβ expression in the rat forebrain (Shughrue et al., 1998).

1.1.2.1 Generation of ER knockout mice

The generation of mice deficient in ERα (ERKO), ERβ (βERKO) and both receptors (αβERKO) have aided in deciphering the role of each receptor in the reproductive system and mammary gland of the mouse. ERKO and βERKO mice were generated through homolgous recombination. Targeted insertion of a 1.8 kb NEO sequence into exon 2 approximately 270 bp downstream of the translational start site resulted in disruption of the ERα gene (Lubahn et al., 1993). Similarly, for ERβ a 1.8 kb NEO sequence was inserted into exon 3 of the gene resulting in disruption of the sequence coding for the first zinc finger, which is necessary for the proper function of the receptor (Krege et al., 1998a). αβERKO mice were generated by mating of heterozygous mice from the ERKO and βERKO lines (Couse et al., 1999b).

Splice variants of the disrupted genes that may code for receptors with decreased functional activity have been detected in small amounts in both the ERKO and βERKO models and could complicate interpretation of data from the all the ERKO models (Couse et al., 1995; Krege et al., 1998b). One study comparing the ERKO model described here and a model generated by deletion of exon 2 (ERαΔ2KO) demonstrated that the ability of E2 to increase uterinte weights and induce production of nitric oxide was totally abrogated in the ERαΔ2KO mice, but was partially (uterine weights) or totally (nitric oxide) preserved in the ERKO mice (Pendaries et al., 2002). Furthermore, two splice variants with partially deleted A/B domains were detected in the uterus and one was detected in the aorta of the ERKO mice and could account for the partial response to E2.

1.1.2.2 Phenotypes of ER knockout mice

1.1.2.2.1 Uterus

Estrogen signaling is involved in preparation of the uterus for embryo implantation and for the actual implantation process. ERα is expressed in all compartments of the uterus and low levels of ERβ expression have also been described (Couse et al., 1997; Kuiper et al., 1997). Uteri from all three ERKO models possess normal structure with a myometrial compartment and an endometrial compartment consisting of both stroma and epithelium (Hewitt and Korach, 2003); however the ERKO and αβERKO uteri are immature and hypoplastic with fewer glands than the wild-type mice (Lubahn et al., 1993;

Couse et al., 1999b). Therefore, ERα and ERβ are not necessary for normal development of the uterus, but ERα is needed for maturation of the uterus. Uteri from ERKO and αβERKO mice are also not responsive to estrogen. Studies in ERKO mice have demonstrated that treatment with E2 does not induce typical uterine responses including increased weight, water imbibition or hyperemia, cell proliferation and DNA synthesis, or induction of estrogen responsive genes such as PR and lactoferrin (Lubahn et al., 1993; Couse et al., 1995). In contrast, βERKO mice undergo cyclic changes with ovarian hormones; however, the immature uterus has increased proliferative markers and exaggerated responses to estrogen (Krege et al., 1998a). These data indicate that ERα is necessary for estrogen-induced responses in the uterus, whereas ERβ may play an inhibitory role.

Multiple studies have described cross-talk between ER signaling and growth factor signaling in the uterus. Estrogen treatment increases epidermal growth factor (EGF) and its receptor (EGFR) (DiAugustine et al., 1988; Huet-Hudson et al., 1990), insulin-like growth factor-1 (IGF-1) (Couse and Korach, 1999), and transforming growth factor-α (TGF-α) (Nelson et al., 1992) and activates the IGF-1 signaling pathway (Richards et al., 1996) in the uterus. Furthermore, cotreatment with antibodies to EGF attenuates the uterine response to estrogen (Nelson et al., 1991) and treatment of IGF-1 knockout mice with E2 does not result in uterine epithelial cell mitosis (Adesanya et al., 1999), indicating that both EGF and IGF-1 are involved in the uterine response

to estrogen. Conversely, treatment with either EGF or IGF-1 results in estrogenic-like responses that include epithelial cell proliferation, induction of target genes, and increase in uterine weights (Nelson et al., 1991; Ignar-Trowbridge et al., 1992), and the estrogen antagonist ICI 164,384 decreases the uterine response to EGF (Ignar-Trowbridge et al., 1992). Therefore, ERKO mice were treated with EGF and IGF-1 to determine whether ERα is necessary for the growth factor-induced responses in the uterus. Although the ERKO mice express the growth factor receptors and c-fos induction indicated that the EGF signaling pathway was intact, neither EGF nor IGF-1 induced a mitogenic response in the uterine epithelium, indicating that ERα is necessary for the response (Curtis et al., 1996; Klotz et al., 2002).

Progesterone is essential for the decidual response in the mouse uterus and PR expression is highly regulated by estrogen; therefore the ability of the uterus to respond to progesterone was investigated in the ERKO mice. Although PR protein levels were approximately 60% of that seen in wild-type mice, progesterone treatment resulted in induction of progesterone-responsive genes in the uterus (Curtis et al., 1999). Furthermore, uterine decidualization can be experimentally induced with an estrogen-progesterone treatment regimen and injection of oil to induce 'trauma' to the epithelium and mimic implantation. Decidualization was observed in ERKO mice and progesterone alone also induced this response (Curtis et al., 1999). Therefore, the progesterone signaling pathway for gene expression and decidualization in the

uterus is functional without ERα. The ability of progesterone to induce decidualization in the ERKO uterus without estrogen 'priming' could be due to the inherently impaired uterine epithelium of the ERKO mice that is more sensitive to induced 'trauma' with oil injection than wild-type mice. Trauma to the uterus has been shown to induce decidualization without estrogen priming (Finn, 1965; Finn, 1966).

Estrogen signaling is also involved in implantation of the embryo in the uterus. Embryo implantions in ERKO mice utilized experimental transfer of donor embryos and hormone-priming because ERKO mice are anovulatory. Implantations were observed in control but not ERKO mice, indicating that ERα is necessary for implantation (Hewitt and Korach, 2003).

Many of the estrogenic effects induced in the adult uterine epithelium are highly dependent upon stormal-epithelial interactions. Estrogenic responses are observed in uterine epithelial cells that lack ERα (Couse and Korach, 1999). The generation of ERKO mice led to the development of tissue recombination experiments in which wild-type and ERKO tissue were combined to determine the cell types required to express ERα for estrogen action. Tissue recombination studies in the adult uterus have shown that ERα expression is required in the stromal tissue and not in the epithelium for E2-induced epithelial cell proliferation (Cooke et al., 1997) and that ERα expression in required in both the stromal and epithelial tissue for E2-induced secretion of lactoferrin and complement component C3 (Buchanan et al., 1999).

1.1.2.2.2 Ovaries

Estrogen and gonadotrophins coordinately induce steroid hormone production and folliculogenesis in the ovaries. Multiple studies have demonstrated ERα and ERβ expression in the ovaries with ERβ localizing mainly to the granulosa cells of growing follicles and ERα localizing to the interstitial and thecal regions (Hiroi et al., 1999; Rosenfeld et al., 1999; Sar and Welsch, 1999). The three ERKO models have been used to investigate the roles of ERα and ERβ in the ovaries.

The ovaries of neonatal and prepubertal ERKO female mice appear grossly normal and mature ovaries have a normal compliment of primordial follicles indicating that fetal and neonatal ovarian development and germ cell generation and migration are not dependent on ERα (Schomberg et al., 1999). In contrast mature ovaries are anovulatory with no corpus lutea present and exhibit enlarged, hemorrhagic and cystic follicles in ERKO mice (Schomberg et al., 1999). The cystic structures in the ERKO ovaries are also seen in mice that overexpress LHβ (Risma et al., 1995), and the development of the phenotype in ERKO mice was blocked by inhibtion of LH production (Couse et al., 1999b). Therefore, the increased LH levels seen in the ERKO mice are probably responsible for the cystic phenotype of the ovaries as opposed to a direct effect due to loss of ERα in the ovaries. Superovulation studies in ERKO mice have also shown that although ovulation cannot be induced in mature ovaries, immature ovaries can be induced to ovulate, although to a lesser extent than

observed in ovaries from wild-type mice (Couse et al., 1999a). Furthermore, follicles in the tertiary and pre-antral stages are present in the mature ovaries of ERKO mice indicating that recruitment of primordial follicles and the early stages of folliculogenesis do not require ERα. Attenuation of apoptosis and induction of LH receptors in granulosa cells of antral follicles, which are thought to be estrogen mediated, were also observed in ovaries of ERKO mice indicating that this response is also not mediated by ERα (Schomberg et al., 1999). Studies also examined the steroidogenic function of the ovaries and demonstrated that serum progesterone levels in ERKO mice were normal, whereas serum estrogen levels were increased (Couse and Korach, 1999).

Ovaries from βERKO mice are normal in size and morphology with a relatively normal intersitial/stromal compartment that contains follicles from various stages of follicular development. This indicates that like ERα, ERβ is not required for germ cell development or migration or for normal ovarian development. There was an increase in early atretic follicles and few corpus lutea indicating a reduction in complete folliculogenesis. Superovulation studies in βERKO mice showed that exogenous gonadotrophins induced ovulation; however, the ovulatory capacity was dramatically decreased compared to wild-type mice. The ovaries from superovulated βERKO mice contained numerous preovulatory, unruptured follicles suggesting that ovaries respond to the proliferative stimulation, but do not luteinize and rupture. The steroidogenic function of ovaries from βERKO mice appear to be normal, serum progesterone

and estrogen levels were similar to wild-type levels (Couse and Korach, 1999). The reduced ability of both the ERKO and βERKO mice to ovulate indicates that both receptors are required for efficient ovulation, although reduced ovulation was possible in the absence of each recpetor.

αβERKO mice exhibited an ovarian phenotype distinct from both the ERKO and βERKO mice. Normal follicles are present in the ovaries; however, follicles that lack oocytes and contain Sertoli-like cells and structures that are intermediate with characteristics of both types of follicles are observed (Couse et al., 1999b). It appears that the oocyte degenerates with age and the normal follicle transdifferentiates into a seminiferous tubule-like follicle. Simiar structures have been reported in aromatase knockout mice that are not able to produce estrogen and in mouse models that lack germ cells (Behringer et al., 1990; Britt et al., 2001). Therefore, ERα and ERβ may coordinately act to maintain oocyte integrity and prevent follicular degeneration.

1.1.2.2.3 Mammary gland

Estrogen is required for proper ductal morphogenesis of the mammary gland during puberty and may also play a role in alveolar development during the cycle and pregnancy (Neville et al., 2002). At birth the mammary gland of female ERKO mice exhibits the same rudimentary ductal tree structure with the epithelial and stromal portions as well as the connective tissue that is observed in wild-type mice, demonstrating that ERα is not required for normal gestational mammary gland development. During puberty the rudimentary ductal tree of the

mammary gland responds to hormones and elongates and branches to fill the mammary fat pad; however, the mammary gland of ERKO mice does not develop beyond the rudimentary ductal tree (Couse and Korach, 1999). Therefore, ERα is necessary for mammary gland elongation and growth during puberty. Maturation of the mammary gland during pregnancy involves further ductal growth and branching and development of lobular alveolar structures. However, the lack of ovulation in ERKO mice results in decreased progesterone levels that are insufficient for development of the lobular alveolar structures. Treatment of ERKO mice with progesterone showed that the ERKO mammary gland responds to progesterone and develops lobular alveolar structures (Hewitt and Korach, 2000) indicating that the ERKO mammary gland possesses tissue components necessary for further development but lack stimuli downstream of ERα. βERKO mice exhibit normal mammary gland development and βERKO mothers are able to nurse their pups (Couse and Korach, 1999). These data suggest that ERα is necessary for mammary gland elongation and development during puberty, and that ERβ is not required for mammary gland development or function.

Stromal-epithelial interactions are important for ductal elongation in the mammary gland. TEB formation and outgrowth in mice are dependent on interactions between epithelial cells and adipocytes or stromal fibroblasts (Daniel et al., 1984). Futhermore, the outermost proliferating cap cells of the TEB do not express ERα indicating that estrogen acts indirectly on the proliferating epithelial

cells either through the stromal or non-proliferating epithelial cells (Daniel et al., 1987). Tissue recombination studies using mammary gland epithelium and stroma from wild-type and ERKO mice have also shown that ERα expression in the stroma but not the epithelium is necessary for ductal growth (Cunha et al., 1997).

The role of estrogen in breast cancer is noted above (Section 1.1.2.1) and expression of ER and estrogen-dependent growth of human breast tumors is well established. The role of ERα in mammary tumorigenesis was investigated by crossing the ERKO mice with MMTV-Wnt-1 mice. MMTV-Wnt-1 is a transgenic line that expresses the Wnt-1 protooncogene in the mammary gland and nearly 100% incidence of mammary hyperplasia and lobuloalveolar adenocarcinoma is observed in one year-old females (Tsukamoto et al., 1988). The lobuloalveolar hyperplasia seen in the wild-type MMTV-Wnt-1 mice was also observed in ERKO-Wnt-1 mice; however, the extensive hyperplasia of the ductal epithelium and ductal elongation seen in the wild-type-Wnt-1 mice was decreased in ERKO-Wnt-1 mice. Expression of the Wnt-1 gene induced tumor formation on the ERKO background, but a 50% tumor incidence was seen in twice the time required for the response in wild-type-Wnt-1 mice. Therefore, the induction of hyperplasia and tumorigenesis in the mammary gland by Wnt-1 does not require ERα, however, ERα plays a role in promotion of the phenotypes and terminal end bud formation and ductal morphogenesis are ERα-dependent.

1.1.2.2.4 Hypothalamic-pituitary axis

The hypothalamus is located at the base of the brain above the pituitary and translates neuronal signals from the brain into humoral factors such as gonadotrophin releasing hormone (GnRH) that stimulate the function of the anterior pituitary via the hypothalamo-hypophyseal portal system (Couse and Korach, 1999). The pituitary produces and secretes the gonadotrophins follicle stimulating hormone (FSH) and luteininzing hormone (LH), the lactotroph prolactin (PRL), as well as other peptide hormones. The gonadotrophins stimulate gametogenesis and synthesis of steroid and peptide hormones in the gonads that then feed back to the hypothalamus and the pituitary to regulate FSH and LH production and secretion. FSH and LH are negatively regulated by estrogen (Gharib et al., 1990; Shupnik, 1996; Couse and Korach, 1999) and expression of ERα and ERβ in the pituitary and regions of the brain has been shown, although the levels vary between species (Couse et al., 1997; Mitchner et al., 1998; Pau et al., 1998; Petersen et al., 1998; Shupnik et al., 1998; Wilson et al., 1998). Female ERKO and αβERKO mice exhibit increases in FSHβ and LHβ mRNA levels in the pituitary and a similar increase in serum LH levels (Scully et al., 1997; Couse and Korach, 1999). In contrast the LH and FSH levels in βERKO females are normal indicating that ERα mediates the negative feedback of estrogen that regulates LH levels.

PRL acts on the female reproductive system to affect differentiation and function of the lactating mammary gland and to promote blastocyst implantation

through luteinization of the corpus luteum; furthermore, E2 regulates PRL synthesis and secretion (Maurer et al., 1990; Stefaneanu, 1997). Pituitary and serum hormone PRL mRNA levels are decreased in ERKO mice compared to wild-type animals indicating that ERα is required for PRL synthesis and secretion (Scully et al., 1997).

1.1.2.2.5 Bone

Bone is a dynamic tissue that is under constant absorption as a mineral source for the body and remodeling to maintain skeletal structure and strength. A link between estrogen and the dynamics of bone has been known since decreased estrogen levels are associated with osteoporosis, a disease characterized by loss of bone mass and strength due to a disruption of the equilibrium between bone absorption and remodeling (Couse and Korach, 1999). The beneficial effects of estrogen replacement therapy on the bone in postmenopausal women are well established (Barrett-Connor and Grady, 1998; Komm and Bodine, 1998); however, the mechanisms of estrogen action on the bone are unclear. Expression of ERα and ERβ in bone cell cultures (Arts et al., 1997; Onoe et al., 1997; Couse and Korach, 1999) and ERKO and βERKO mice have distinguished between the roles of both receptors in estrogen action on bone. In both males and females, differential effects on bone growth are seen in the three ERKO models. A decrease in long bone growth is seen in ERKO females; an increase in growth is seen in βERKO females and αβERKO females exhibit intermediate growth of long bones (Couse and Korach, 1999; Lindberg et

al., 2001) indicating a stimulatory role for ERα and inhibitory role for ERβ in long bone growth in females. Another study demonstrated an increase in cortical bone mineral associated with radial bone growth in βERKO females indicating a repressive role for ERβ in regulation of bone growth in females (Windahl et al., 1999). βERKO females are partially protected from age-related trabecular bone loss indicating a repressive action of ERβ in regulation of trabecular bone growth, and ERβ is not required for the protective effects of E2 on trabecular bone mineral density (Windahl et al., 1999; Windahl et al., 2001). Male βERKO mice do not exhibit any bone abnormalities, whereas, ERKO and αβERKO males exhibit decreased longitudinal and radial skeletal growth associated with decreased IGF-1 levels during growth and maturation (Windahl et al., 1999; Vidal et al., 2000) indicating that ERα has a stimulatory effect and ERβ does not have a role on bone growth in males.

1.1.2.2.6 Cardiovascular system

The protective effects of estrogen against cardiovascular disease has been demonstrated in various animal models and in multiple epidemiology studies documenting the reduced incidence of cardiovascular disease in postmenopausal women receiving estrogen replacement therapy (Nathan and Chaudhuri, 1997; Barrett-Connor and Grady, 1998). However, recent evidence from multiple trials and observational studies using estrogen/progestin combination therapy has shown an increase in cardiovascular disease including coronary heart disease, stroke and pulmonary embolism (Grodstein et al., 2000;

Simon et al., 2001; Viscoli et al., 2001; Grady et al., 2002; Rossouw et al., 2002). The increased risk of cardiovascular disease with combination estrogen/progestin therapy in postmenopausal women may be due to the prothrombotic and proinflammatory effects of progestins that outweigh any protective effects due to estrogen (Fletcher and Colditz, 2002). However, the mechanisms of the protective effects of estrogen vs. the detrimental effects of estrogen/progestin on the cardiovascular system are unclear.

Estrogen is thought to lower cholesterol through increased clearance of low-density lipoproteins (LDLs) from circulation via a mechanism that is dependent upon apoE protein expression (Nathan and Chaudhuri, 1997). Studies in ERKO mice have shown that ERα is necessary for E2-induced increases in apoE protein expression in serum (Srivastava et al., 1997). Multiple studies have investigated the role of the ERs and estrogen for inhibition of responses to vascular injury. E2 inhibits multiple types of responses to vascular injury in βERKO mice, but not in the ERαΔ2KO mice indicating the ERα and not ERβ is necessary for estrogen-dependent protection from response to vascular injury (Karas et al., 1999; Brouchet et al., 2001; Pare et al., 2002). Another study in ERKO mice demonstrated that ERα also mediates induction of nitric oxide production and inhibition of acetylcholine-elicited relaxation in the aorta by E2 (Darblade et al., 2002). βERKO mice have exhibited abnormalities in ion channels of vascular smooth muscle cells, the development of sustained systolic and diastolic hypertension due to age, as well as increased vasorelaxation and

augmented vasoconstricition with E2 treatment in blood vessels (Nilsson et al., 2000; Zhu et al., 2002).

1.2.3 Function of ER domains

ERα and ERβ contain the 6 functional domains characteristic of the nuclear receptor superfamily of transcription factors (Figure 1.9). The N'-terminal A/B domain is the least conserved between members of the superfamily; for example there is only 17% identity between human ERα and ERβ (White et al., 1987; Evans, 1988; Danielian et al., 1992; Enmark et al., 1997). The A/B domain is also known as AF-1 and is a ligand-independent transactivation domain. AF-1 interacts with the basal trancription machinery (Sadovsky et al., 1995) and several different coactivators (Endoh et al., 1999; Kobayashi et al., 2000; Wang et al., 2001a; Wu et al., 2001) and is a region of kinase-dependent phosphorylation required for ligand-independent activation of ER (Shibata et al., 1997). The C domain is the DNA-binding domain (DBD) and contains two zinc fingers that form a helix-loop-helix structure necessary for protein-DNA interactions between the receptor and DNA response elements. The first zinc finger fits into the major groove of DNA and is responsible for specific amino acid contacts with DNA and the second zinc finger is important for receptor homo- or heterodimerization and phosphate backbone contacts (Tsai and O'Malley, 1994). The DBD is the most highly conserved domain with 97% homology between the zinc finger regions of human ERα and ERβ (Enmark et al., 1997; Tremblay et al., 1997; Couse and Korach, 1999). The D domain is

approximately 30% conserved between the human ERs and contains the receptor nuclear localization signal (Enmark et al., 1997).

Figure 1.9: Functional domains of ER. (Shupnik, 2002).

The E domain contains the ligand binding domain (LBD) and AF-2 and has many functions including receptor dimerization, ligand binding (LBD), transcriptional activation (AF-2), and coactivator/corepressor binding. The E domain contains twelve α-helices arranged in an antiparallel sandwich with the insertion of a small β-sheet. Receptor dimerization is necessary for activation and mutational studies have shown that dimerization is mediated through helices 7-10 (Fawell et al., 1990; Lees et al., 1990). Ligand binding involves the β-sheet and helices 3,5,11,and 12 and the pocket formed for ligand binding contains a highly conserved region for stability and a highly variable region for ligand

specificity (Renaud and Moras, 2000). The AF-2 region, specifically helix 12, is critical for ligand-dependent transactivational activity (Danielian et al., 1992; Saatcioglu et al., 1993; Barettino et al., 1994; Durand et al., 1994). Ligand binding leads to repositioning of helix 12 and allows coactivator association (Renaud and Moras, 2000).

The C'-terminal F domain is not well conserved in the superfamily nor among ERs of different species; there is only 18% homology between human ERα and ERβ (Enmark et al., 1997). The function of the F domain is not well defined. Some studies indicate a role in transactivation of certain ligands through coregulatory function or dimerization (Montano et al., 1995; Peters and Khan, 1999). The proximity of the F domain to helix 12 indicates possible involvement in transactivation when helix 12 is repositioned upon ligand binding.

1.2.4 Mechanisms of ER action

Recent studies have provided evidence for multiple mechanisms of ER action (Figure 1.10). In the classic genomic pathway, E2 diffuses into the cell and binds to ER associated with heat shock protein 90 (hsp90). Ligand-bound ER undergoes conformational changes that lead to release of hsp90 and receptor homodimerization. The resulting ER dimers then bind to conserved palindromic estrogen response elements (EREs) in promoters of E2-responsive genes and along with a complex of other proteins and these interactions lead to induced gene expression (Kumar and Chambon, 1988). However, it has been shown that the classical pathway of ER activation does not explain E2-

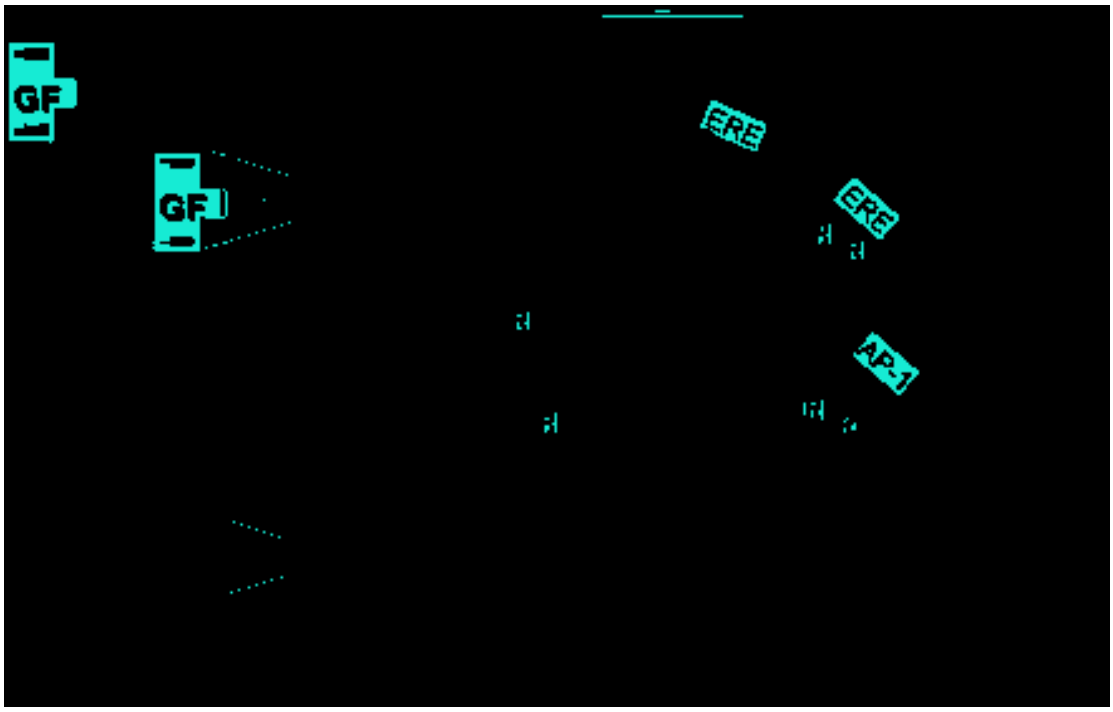


Figure 1.10: Multiple mechanisms of ER signaling. (Hall et al., 2001).

dependent gene expression (Paech et al., 1997; Saville et al., 2000), particularly for genes that do not contain consensus EREs. ER binds various transcription factors to modulate gene expression by protein-protein interactions (Day et al., 1990; Porter et al., 1997). E2 also induces rapid responses such as Ca^{2+} fluxes and kinase activation that are independent of the genomic pathway and involve membrane receptors (Revelli et al., 1998; Kelly and Levin, 2001). Moreover, growth factors induce kinase cascades that lead to phosphorylation and activation of ER in the absence of ligand (Katzenellenbogen and Norman, 1990; Kato et al., 1995; El-Tanani and Green, 1997). Multiple mechanisms of ER

action explain the wide range of cellular and genomic responses to ER activation.

1.2.4.1 Genomic

1.2.4.1.1 ER/Sp1

Signaling by multiple nuclear receptors (androgen receptor (Lu et al., 2000), PR (Gao et al., 2001), RARα receptor (Merchiers et al., 1999; Suzuki et al., 1999; Husmann et al., 2000), RXR receptor (Krey et al., 1995; Horie et al., 2001)) including the ER (Krishnan et al., 1994; Porter et al., 1996; Sun et al., 1998; Dong et al., 1999; Duan et al., 1999; Wang et al., 1999; Xie et al., 1999b; Vyhlidal et al., 2000; Xie et al., 2000; Castro-Rivera et al., 2001; Samudio et al., 2001) are mediated by nuclear receptor/Sp1 interaction with guanine-cytosine (GC)-rich promoter sequences. Specificity protein 1 (Sp1) is a housekeeping zinc-finger protein that contains three DNA binding regions that recognize GC-rich sites in the DNA with the core GGCGGG element. These GC-rich sites have been found in multiple promoters in the region between the transcription start site and 200 bp upstream and genome wide analysis indicates that the GC-rich sites are primarily found in regions associated with transcription initiation (Hapgood et al., 2001).

Early studies of estrogen responsive genes identified promoter regions that did not contain concensus or nonconcensus EREs but were still responsive to E2 [c-myc (Dubik and Shiu, 1992), cathepsin D (Krishnan et al., 1994), hsp 27 (Porter et al., 1996), and TGFα (Vyhlidal et al., 2000), *c*-fos (Duan et al., 1998),

adenosine deaminase (Xie et al., 1999b) and retinoic acid receptor α1 (Sun et al., 1998)]. In many of these genes, a DNA motif containing an ERE half-site and a GC-rich sequence that interacts with Sp1 was identified. It was hypothesized that E2-responsiveness was associated with cooperative interactions of ER and Sp1 [c-myc (Dubik and Shiu, 1992)]. Subsequent analysis of the cathepsin D and TGFα promoters demonstrated that both the ERE-half site and GC-rich site were necessary for estrogen responsiveness and ligand bound ER interacts with Sp1 proteins and the complex binds to the ERE-half and GC-rich sites, respectively [cathepsin D (Krishnan et al., 1994), TGFα (Vyhlidal et al., 2000)]. However, analysis of the hsp27 promoter showed that the ERE half-site was not necessary for activation by estrogen (Porter et al., 1996) and several genes that are E2-responsive require only GC-rich sites [*c*-fos (Duan et al., 1998), adenosine deaminase (Xie et al., 1999b), and retinoic acid receptor α1 (Sun et al., 1998), bcl-2 (Dong et al., 1999), DNA polymerase α (Samudio et al., 2001), thymidylate synthase (Xie et al., 2000), cyclin D1 (Castro-Rivera et al., 2001), and E2F1 (Wang et al., 1999)]. Studies of these promoters showed that ligand bound ER interacted with Sp1and regulated transcription through Sp1-binding to GC-rich promoter elements. Therefore, two mechanisms of ER/Sp1 transcriptional activation have been demonstrated: one dependent upon ER-DNA binding at ERE half-sites and one independent of ER binding to DNA. Since these early studies several laboratories have identified both ER-DNA binding dependent and independent activation of estrogen

responsive proteins by ER/Sp1 complexes [low density lipoprotein receptor (Li et al., 2001), human PR A (Petz and Nardulli, 2000), rabbit uteroglobulin gene (Scholz et al., 1998), vitellogenin A1 IO (Batistuzzo de Medeiros et al., 1997)].

Multiple studies have investigated protein-protein interactions between ER and Sp1. Initial studies used glutathione-S-transferase (GST)-fusion protein pull-down assays to show that ER interacts primarily with the C-terminal region of Sp1, and Sp1 interacts with multiple regions of ER including the AF1 and AF2 domains (Porter et al., 1997). Further studies demonstrated that the AF1 domain of ERα was necessary for transactivation of a GC-rich promoter element by ER and that the AF1 region of ERα functionally interacts with the zinc-finger region of Sp1 to form an active ERα/Sp1 complex (Saville et al., 2000).

1.2.4.1.2 ER/AP-1

AP-1 is a transcription factor and members of the AP-1 transcription factor family are referred to as immediate early genes because of their role in rapid transcriptional responses to extracellular signaling and their role in the transition from G0 to G1 phase of the cell cycle (Cohen and Curran, 1988; Lamph et al., 1988; Morgan and Curran, 1988). AP-1 is formed by homodimerization of Jun proteins, heterodimerization of Fos and Jun proteins, or heterodimerization of Fos-related antigen and Jun proteins. Several estrogen-responsive genes contain AP-1 promoter elements and E2 is able to increase or decrease transcriptional activation of these genes depending upon the promoter and cell context (Tzukerman et al., 1990; Doucas et al., 1991; Shemshedini et

al., 1991; Tzukerman et al., 1991; Webb et al., 1995; Kushner et al., 2000) (Figure 1.10).

Initial studies on activation of AP-1 promoter elements by E2 used the collagenase promoter in which the estrogen responsive region was identified as an AP-1 promoter element (Tzukerman et al., 1991; Webb et al., 1995). It was shown that ER activated the promoter without directly binding to DNA (Tzukerman et al., 1991) and that the activation at the AP-1 element involved ER interactions with Jun protein but not Fos protein or DNA (Webb et al., 1995). In vitro protein-protein interaction studies using co-immunoprecipitation and GST-fusion protein pull down assays have shown that the hinge region (amino acids 259-302) of ER interacts with the C'-terminal region of Jun; however, ER interaction with Fos has not been demonstrated. Furthermore, loss of ER-Jun interactions by disruption of amino acids 259-302 leads to loss of transcriptional activity and members of the p160 transcriptional coactivator family can enhance the activity of the complex (Teyssier et al., 2001). Also, recent identification of a coactivator of AP-1, CAPER, that specifically interacts with E2-bound ER, Jun and activating signal cointegrator-2 suggests biological significance for ER-Jun interactions (Jung et al., 2002). Evidence from these promoter and protein-protein interaction studies suggest that ER interacts with the Jun protein of the AP-1 transcription factor and activates AP-1 promoter elements in an ER-DNA binding independent manner. However, the mechanism of ER activation of AP-1 promoter elements is also ligand dependent and E2, tamoxifen, and ICI 164,384

all activate AP-1 promoter elements in HeLa cells. The regions of the ER that are necessary for ER/AP-1 activity are ligand-dependent. For example, tamoxifen required the DBD of ER for activation of the AP-1 element, whereas E2-dependent activation is also observed for ER containing deletion of the DBD (Webb et al., 1995).

1.2.4.2 Non-gemomic

The rapid induction of several E2-mediated responses may be due to a membrane associated ER that is responsible for multiple non-genomic mechanisms of action (Figure 1.10). Studies in various cell lines have described Ca2+ fluxes (Tesarik and Mendoza, 1995; Picotto et al., 1999; Stefano et al., 1999; Kelly and Levin, 2001), generation of cyclic nucleotides (Ropero et al., 1999; Teoh and Man, 2000), and activation of kinase cascades (Le Mellay et al., 1997; Doolan et al., 2000) that occur within seconds or minutes after treatment with E2. Recent studies have shown that ERα and ERβ rapidly activate the RAS-RAF-MAPK pathway through interactions with src (Migliaccio et al., 2000) and E2 induces phosphatidylinositol-3 kinase (PI3-K) activity which is inhibited by ICI182,780 in vascular endothelial cells (Simoncini et al., 2000). Rapid E2-dependent activation of nitric oxide synthase (NOS) has also been described in multiple cell types (Chen et al., 1999; Russell et al., 2000) and induction of NOS in vascular cells involves E2-dependent activation of MAPK and Akt pathways (Chen et al., 1999; Mendelsohn, 2000; Russell et al., 2000; Simoncini et al., 2000). Furthermore, a study using the c-fos promoter demonstrated that E2-

dependent activaiton of the serum response element (SRE) occurs through upregulation of the MAPK and PI3-K pathways resulting in phosphorylation and DNA binding of Elk-1 and Srf, respectively (Duan et al., 2001; Duan et al., 2002).

Although there is clear evidence for the existence of a membrane associated ER, convincing localization and isolation of an endogenous membrane-based ER has not been reported. It is not clear whether the membrane associated ER is ERα, ERβ or a new receptor. Associations of this receptor with the membrane have not been determined and it is noteworthy that neither ERα nor ERβ contain the hydrophobic, membrane-spanning regions or sites for post-translational lipid modification. One study in COS-1 cells demonstrated that transfected ERα and ERβ were able to incorporate into the cell membrane and mediate ligand-dependent activation of kinase cascades (Razandi et al., 1999) and multiple studies using antibodies to ER or E2-conjugated with BSA have shown punctate staining of the cell membrane (Norfleet et al., 2000; Russell et al., 2000; Duan et al., 2001). Furthermore, studies in MCF-7 cells have isolated receptors from plasma membrane extracts with antibodies to ERα and have identified 130, 110, 92 and 67 kDa proteins (Powell et al., 2001).

1.2.4.3 Ligand-independent ER activation

Ligand independent activation of ER by growth factors has also been reported (Figure 1.10). Growth factors activate kinase pathways such as MAPK, PI3-K, c-Jun N-terminal kinase (JNK), protein kinase A, B, or C (PKA, PKB,

PKC), and insulin receptor substrate 1 (IRS-1) and induce a multitude of cellular responses including cell cycle progression, cell proliferation, cell survival, and apoptosis. The ability of serum, insulin and insulin-like growth factor to activate ERα and an ERE-promoter construct without hormone treatment was the first indication of growth factor activation of ER (Katzenellenbogen and Norman, 1990) and this observation has been supported by subsequent studies. For example it has been shown that pS2 and PR mRNA levels are induced after treatment of MCF-7 cells with E2 or IGF-1 and both responses are inhibited by ICI 182,780 and wortmanin, a PI3-K inhibitor, indicating that induction is dependent on the PI3-K pathway and involves ER. Another study using an adensone deaminase (ADA) promoter reporter construct, which is activated via ER/Sp1 complexes, showed that gene activity was induced by EGF, IGF-1, and TGFα in MCF-7 cells transfected with ERα (Xie et al., 2001). Specific inhibitors of the MAPK pathway and overexpression of dominant-negative Ras, as well as mutation in serines 118 and 167 of ER inhibited IGF-1-dependent induction of the reporter construct indicating that IGF-1 activated ERα through the MAPK pathway (Xie et al., 2001) and serines 118 and 167 in ERα were required. Other studies have demonstrated that activation of ER by EGF and IGF-1 were AF-1 dependent and required phosphorylation of serine 118 (Kato et al., 1995; Bunone et al., 1996; Karas et al., 1999) and Akt, downstream of PI3-K, is involved in AF-1-dependent phosphorylation of ER. In contrast, studies with

cAMP analogs have reported AF-2-dependent activation of ER in various cell types (El-Tanani and Green, 1997).

Several studies have investigated activation of ERα by growth factors and this has also been observed for ERβ. For example, EGF induced reporter gene activity in cells transfected with an ERβ expression plasmid and an ERE reporter construct and the response was inhibited by antiestrogens and MAPK inhibitors (Tremblay and Giguere, 2001). This indicates that ligand-independent activation of ERβ by growth factors also involves kinase pathways, similar to that observed for ERα. Furthermore, in vitro assays have shown that ERβ is phosphorylated in the AF-1 domain, as has been reported for ERα.

In vivo animal studies also provide evidence for growth factor activation of ER. In studies with adult ovariectomized mice, EGF induces estrogen-like responses in the uterus including increased DNA synthesis, phosphatidylinositol turnover, PR, and lactoferrin (Nelson et al., 1991; Ignar-Trowbridge et al., 1992; Curtis et al., 1996) and cotreatment with ICI 182,780 reduces the uterine response to EGF (Ignar-Trowbridge et al., 1992) indicating the need for ER in EGF–induced uterine responses. Furthermore treatment of ERKO mice with EGF did not result in increased DNA synthesis or PR in the uterus; however, other EGF-mediated responses such as c-fos gene expression were observed in ERKO mice. These studies indicate that although the EGF signaling pathway is intact induction of estrogen-like responses by EGF require ERα and are not observed in ERKO mice (Curtis et al., 1996).

1.3 Aryl hydrocarbon receptor (AhR)

1.3.1 Structure and mechanism of action

The AhR is a ligand-activated transcription factor that binds a structurally diverse range of chemicals. It was initially identified as a receptor that bound hydrophobic environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs); however, recent studies have identified many naturally-occurring plant products including flavonoids, carotenoids, and phenolics that are AhR ligands. The AhR is a member of the basic-helix-loop-helix (bHLH)-Per,Arnt,Sim (PAS) family of transcription factors. The bHLH motif is located in the N-terminal region of the protein and is involved in DNA binding, heterodimerization, and interaction with the heat shock protein 90 (hsp90) (Figure 1.11). The nuclear localization signal (NLS) and nuclear export signal (NES) are also located within the bHLH region of the receptor. Adjacent to the C-terminal end of the bHLH motif is the PAS domain. It includes 2 imperfect repeats of 50 amino acids that are termed PAS A and PAS B. PAS A and PAS B form an interactive surface for heterodimerization and PAS B is also involved in ligand binding and hsp90 and Sp1 protein interactions. The C-terminal half of the receptor contains the Q-rich region which mediates the transactivation activity of the receptor and involves recruitment of CBP/p300 and RIP 140 coactivators.

Figure 1.11: Functional domains of AhR. (Mimura and Fujii-Kuriyama, 2003).

The mechanism of AhR action is similar to that described for other ligand-activated receptors and was derived from early studies on AhR-mediated induction of CYP1A1 gene expression (Swanson and Bradfield, 1993; Whitlock, 1993; Whitlock et al., 1996; Wilson and Safe, 1998) (Figure 1.12). The unbound hepatic AhR is located in the cytosol as a multi-protein complex containing two hsp90 molecules, the X-associated protein 2 (XAP2) (Meyer et al., 1998), and co-chaperone protein p23 (Kazlauskas et al., 1999). Following ligand binding the receptor undergoes a conformational change that exposes the NLS and results in translocation into the nucleus (Hord and Perdew, 1994; Pollenz et al., 1994). In the nucleus the ligand:AhR dissociates from the protein complex and heterodimerizes with a closely related nuclear bHLH-PAS protein called AhR nuclear translocator (Arnt) protein (Probst et al., 1993; Hankinson, 1995). The

heterodimer has a high affinity for DNA and binds dioxin response elements (DREs) in promoters of responsive genes. The consensus DRE contains an N T/G TGCGTG A/C C/G A/T A/G G/C N sequence in which the pentanucleotide core (GCGTG) is necessary for AhR/Arnt binding and the flanking sequence in important for transcriptional activation (Safe, 2001). Binding of the heterodimer to DNA leads to chromatin and nucleosome disruption, increased promoter accessibility, and ultimately gene transcription (Denison et al., 1988; Denison et al., 1989; Whitlock, 1999; Denison et al., 2002). Studies have demonstrated that other transcription factors such as Sp1 and coactivators such as CBP/p300, RIP140, and Src-1 are involved in the AhR/Arnt transcriptional activity (Ko et al., 1996; Kobayashi et al., 1996; Kobayashi et al., 1997; Kumar and Perdew, 1999; Kumar et al., 1999). The NES is responsible for cytoplasmic shuttling of receptors that do not bind Arnt or DNA and results in ubiquitination and proteasome degradation (Roberts and Whitelaw, 1999). The AhR-related factor, termed the AhR repressor (AhRR), has also been identified (Mimura et al., 1999). The AhRR is localized in the nucleus and forms heterodimers with Arnt that bind DREs but act as transcriptional repressors. The promoter region of AhRR also contains functional DREs and is inducible through the AhR, indicating that AhR and AhRR may form a regulatory feedback loop (Mimura et al., 1999).

Figure 1.12: AhR signaling. (Mimura and Fujii-Kuriyama, 2003).

1.3.2 Biological responses to AhR agonists

The most potent and best-characterized ligand for the AhR is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an HAH that is a byproduct of industrial processes and combustion of organic material. TCDD binds with high affinity to the AhR and induces a wide variety of biological and toxic responses. Most research has used TCCD as the prototypical AhR ligand to study the biological responses mediated by the AhR. TCDD indcues phase I and phase II xenobiotic metabolizing enzymes (XMEs) including cytochrome P450 (cyp) 1A1, cyp 1A2, cyp 1B1, NADP(H):oxidoreductase, class 3 aldehyde dehydrogenase, NAD(P)H: quinone reductase 1 and 2, glutathione-S-transferase Ya subunit, and UDP-

glucuronosyl transferase. Many of these induction responses involve AhR interactions with XREs in the promoters of the inducible genes (Paulson et al., 1990; Favreau and Pickett, 1991; Asman et al., 1993; Jaiswal, 1994). PAHs and HAHs that induce XMEs are often the substrates for some of the induced enzymes and the AhR mediated responses may have evolved to decrease the cellular levels of these compounds. Many of the induced metabolic pathways also generate electophilic intermediates from PAH substrates and these metabolites can be genotoxic. The induction of multiple genes involved in other cellular processes including cell proliferation (TGF-β, IL-1β, PAI-2), cell cycle regulation (p27, jun-B), apoptosis (Bax), DNA synthesis (DNA polymerase k), and AhR signaling (AhRR) by AhR agonists has also been described (Hankinson, 1995; Sogawa and Fujii-Kuriyama, 1997; Hahn, 1998; Kolluri et al., 1999; Mimura et al., 1999; Matikainen et al., 2001; Ogi et al., 2001). A microarray study in HepG2 cells showed that 108 genes were upregulated by TCDD (in the presence of cycloheximide) indicating that TCDD induces many other genes that have previously not been characterized (Frueh et al., 2001).

TCDD induces multiple cellular responses that are dependent on cell context. For example, TCDD induces cell proliferation and terminal differentiation in keratinocytes (Milstone and LaVigne, 1984; Choi et al., 1991; Gaido et al., 1992; Gaido and Maness, 1994) and both increases and decreases in proliferation rates in hepatocytes (Wiebel et al., 1991; Wolfle et al., 1993; Moolgavkar et al., 1996). TCDD induces apoptosis in thymocytes (McConkey et

al., 1988; McConkey and Orrenius, 1989; Kamath et al., 1997) and apoptosis as well as inhibition of UV-induced apoptosis in hepatocytes (Moolgavkar et al., 1996; Worner and Schrenk, 1996). Furthermore, the AhR can form protein complexes with retinoblastoma protein and these complexes lead to inhibition of cell cycle progression (Ge and Elferink, 1998; Puga et al., 2000; Elferink et al., 2001).

TCDD induces several well-characterized acute and chronic toxic responses including craniofacial abnormalities such as cleft palate in mice (Courtney and Moore, 1971) and a range of other embryotoxicities in other species including resorption, fetal mortality, and decreased fetal weights (Couture et al., 1990). Induction of cleft palate in mice by TCDD alters the proliferation and differentiation of the medial edge epithelium (Pratt et al., 1984; Abbott and Birnbaum, 1989), possibly through regulation of TGF-β3 (Pratt et al., 1985). TCDD induces thymic involution and immunosuppression at levels below those that cause systemic toxicity. Thymic atrophy can be induced with single low dose of TCDD and involution of the thymus is dependent upon AhR expression (Poland and Glover, 1980). Supression of cell-mediated immunity is also observed after treatment with low doses of TCDD and this response is mediated by non-lymphoid tissue (Nagarkatti et al., 1984). TCDD alters maturation and decreased mitogenic activity of thymic epithelium and it has been suggested that this may lead to the suppression of cell-mediated immunity (Greenlee et al., 1985). TCDD also affects humoral immunity through

suppression of B lymphocyte responses. TCDD treatment is required early in B cell activation; however, the inhibitory effects are seen later in the differentiation of B cells into plasma cells (Luster et al., 1988). TCDD induces tumors in various rodent models and the pattern of tumors is dependent upon the animal species and strain. Using the two-stage model of liver carcinogenesis, treatment with TCDD results in tumor promotion (Pitot et al., 1980) and chronic dietary studies in rats have shown that TCDD induces squamous cell carcinoma of the lungs, hard palate/nasal turbinates and tongue as well as hepatocellular carcinoma (Kociba et al., 1978; Goodman and Sauer, 1992). TCDD is not genotoxic (Poland and Glover, 1979; Geiger and Neal, 1981) and therefore the mechanism of TCDD induced carcinogenesis is unclear. TCDD could increase metabolism of other chemicals into carcinogens, increase lipid peroxidation, or alter cell proliferation. TCDD induces other toxic responses including altered lipid metabolism, epithelial hyperplasia and metaplasia, altered reproductive and endocrine function, porphyria, and wasting syndrome (Poland and Knutson, 1982; Bjerke et al., 1994; Kerkvliet, 1995; Oughton et al., 1995).

Although the molecular mechanisms for the induction of XMEs and multiple other genes involved in cell proliferation, differentiation, and apoptosis are known, the mechanisms by which the AhR mediates many toxic responses are unknown. PAHs and HAHs induce parallel dose-response curves for induction of XMEs whereas, only HAHs such as TCDD induce the toxic responses indicating that molecular mechanisms for the induction of XMEs and

toxicity may be different. One mechanism of TCDD-induced toxicity may be due to persistent transcriptional activation of genes regulated by the AhR; however, critical genes required for induced toxicity have not yet been identified. Furthermore, degenerate DREs or other low-affinity AhR DNA binding sites may be present in other genes and the persistent and potent HAHs such as TCDD may preferentially modulate expression of these genes. In addition, the molecular mechanisms may not be direct transcriptional regulation by AhR. Arnt also forms dimers with HIF-1α, a transcription factor involved in hypoxic responses and persistent activation of the AhR could sequester Arnt or other coactivators and decrease other signaling pathways such as hypoxia.

AhR agonists also cause tissue-specific inhibition of estrogen-induced responses (Kociba et al., 1978; Safe et al., 1998; Safe and McDougal, 2002) and SAhRMs have been developed for the treatment of breast cancer. Kociba and coworkers (Kociba et al., 1978) initially reported that age-dependent spontaneous mammary and uterine tumor formation in Sprague Dawley rats was inhibited in rats receiving TCDD in the diet. These correlated with epidemiology studies in Sevesco, Italy in which women accidentally exposed to TCDD exhibited lower rates of breast and endometrial cancer (Bertazzi et al., 1993; Bertazzi et al., 2001). Subsequent research in several laboratories has demonstrated that TCDD inhibits estrogen-induced responses in the rodent uterus, carcinogen-induced rodent mammary tumors and human breast/endometrial cancer cell lines. In the uterus TCDD and other AhR

agonists inhibit E2-induced increase in wet weight, cell proliferation, PR and EGF receptor binding, peroxidase activity, and EGF receptor and c-fos mRNA expression. In vitro work in breast cancer (MCF-7, T47D, MDA-MB-468) and endometrial cancer cell lines (ECC1, HEC1A) have shown that the AhR is expressed and agonists induce cyp1A1 expression. Studies in MCF-7 and T47D cells showed that TCDD inhibits E2-induced cell proliferation, DNA synthesis, PR mRNA and protein expression, pS2 mRNA and protein expression, cathepsin D mRNA and protein expression, prolactin receptor mRNA expression, vitelogenin A2 promoter activity and creatine kinase B promoter activity. TCDD also inhibited certain E2-induced cell cycle responses such as G0/G1 to S transition, retinoblastoma phosphorylation, cyclin-dependent kinase 2 and 4 phosphorylation, and cyclin D1 protein expression (Wang et al., 1998).

Multiple mechanisms of cross-talk between the AhR and ER signaling pathways have been proposed. Induction of cyp1A1 and cyp1A2 by TCDD results in rapid metabolism and cellular depletion of E2 in cell culture; however, the same effect was not observed in in vivo rodent studies (Gierthy et al., 1988; Badawi et al., 2000). TCDD also activates proteasome-dependent degradation of ERα (Wormke et al., 2000; Wormke et al., 2003) and cotreatment with E2 and TCDD results in low levels of ERα which may be limiting for transactivation (Figure 1.13). Several estrogen responsive genes contain inhibitory DRE (iDRE)

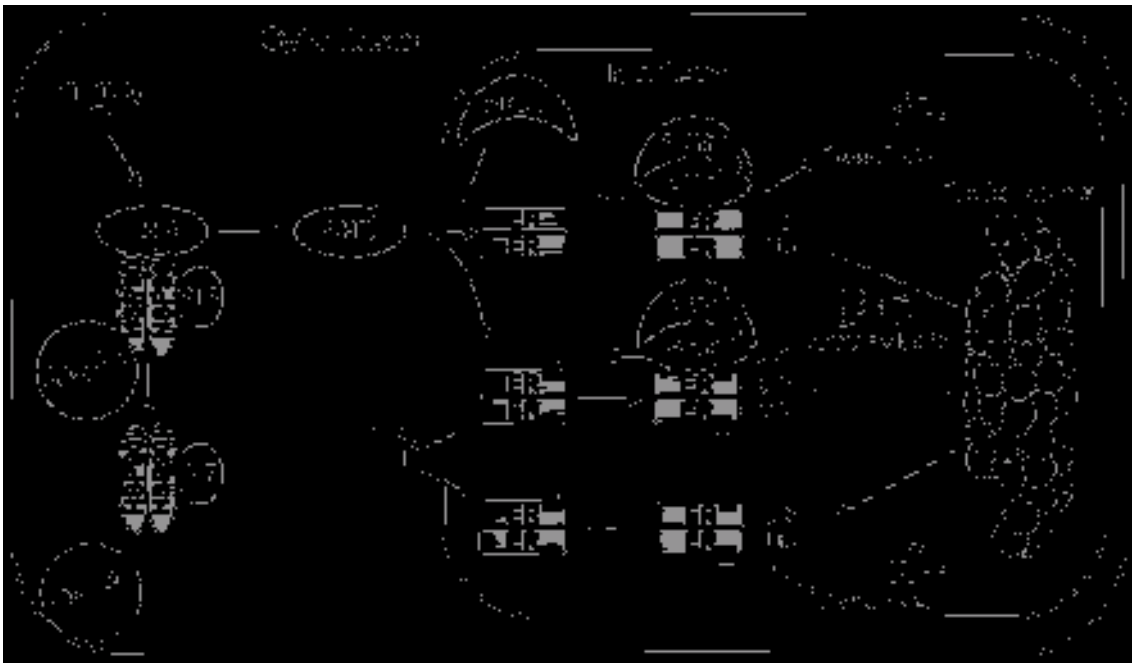


Figure 1.13: AhR-ER crosstalk through proteasome-mediated degradation. (Wormke et al., 2003).

sequences that interact with the AhR complex and this results in decreased E2-induced transactivation. Mutation of the core pentanulceotide DRE sequence leads to loss of inhibitory AhR-ER cross-talk (Gillesby et al., 1997; Duan et al., 1999; Porter et al., 2001; Wang et al., 2001b). However, several E2-induced genes inhibited by TCDD (eg. RARα1) do not have iDRE sequences in the promoters (Safe et al., 1998). It has also been suggested that competition for coactivators and other transcription factors or direct interactions between the AhR and ER may contribute to the inhibition of E2-induced responses by AhR agonists (Klinge et al., 2000; Carlson and Perdew, 2002; Ohtake et al., 2003).

1.3.3 Phenotype of AhR knockout (AhRKO) mice

AhRKO mice have been generated in several laboratories and the resulting animals develop some similar phenotypes and also a number of distinctive phenotypes (Fernandez-Salguero et al., 1995; Schmidt et al., 1996). The Gonzalez laboratory generated AhRKO mice by replacing exon 1 with a neomycin resistance gene resulting in the deletion of the translational start site and a protion of the basic amino acids involved in DNA binding (Fernandez-Salguero et al., 1995). The Bradfield laboratory generated AhRKO mice by replacing exon 2 with a neomycin resistance gene and the result was the deletion of the bHLH domain involved in heterodimerization and DNA binding (Schmidt et al., 1996). Both laboratories utilized ES cells from the 129 mouse; however, different substrains were utilized. C57BL/6 micewere also used in these studies; however, different breeding sources were utilized. In order to compare the two models the Gonzalez mice will be designated d1/d1 and the Bradfield mice will be designated d2/d2 based on the exon that was deleted in generation of the transgenic mice.

Certain phenotypes such as decreased liver size at 3-4 weeks of age and subtle hepatic portal fibrosis were seen in both transgenic models. Decreased constitutive expression of certain xenobiotic metabolizing enzymes (XMEs) and decreased body size during the first four weeks of age were observed in both the d2/d2 and d2/d2 AhRKO mice. Decreased fertility with difficulty in maintaining pregnancy, lactation, and rearing of pups to weaning was also

observed in these transgenic mice (Abbott et al., 1999). Induction of biological responses by TCDD and related AhR agonists was not seen in either AhRKO models. Cyp1A1 is not induced by TCDD in either the d1/d1 or d2/d2 AhRKO mice (Fernandez-Salguero et al., 1996; Schmidt et al., 1996) and a third AhRKO model was resistant to TCDD-induced teratogenesis and benzo(a)pyrene-induced carcinogenesis (Mimura et al., 1997; Shimizu et al., 2000). These studies indicate that the AhR is necessary for induction and basal expression and is also required for the teratogenic and chemical carcinogenic effects induced by TCDD and benzo(a)pyrene, respectively. It also indicates a role for the AhR in liver development and possibly some aspects of fertility.

Significant differences in phenotypic traits were also observed between the d1/d1 and d2/d2 AhRKO mice. Normal Mendelian distribution of the pups suggests that embryonic mortalities should not be observed in these mice; however, d1/d1 mice had a high mortality rate within the first 2 weeks of age whereas the d2/d2 had survival rates similar to their wild-type littermates (Fernandez-Salguero et al., 1995; Schmidt et al., 1996). Livers of the d1/d1 mice exhibited eosinophilia of the periportal hepatocytes, centrilobular hypercellularity, glycogen depletion, and inflammation of the bile duct, whereas livers of the d2/d2 mice exhibited prolonged extramedullary hematopoiesis and extensive microvesicular fatty metamorphosis within hepatocytes that completely resolved by 4-5 weeks of age (Fernandez-Salguero et al., 1995; Schmidt et al., 1996). The immune system of the d1/d1 mice also developed altered

pheonotypes (Fernandez-Salguero et al., 1995). The periarterial lymphatic sheaths of the spleen were small compared to wild-type mice and splenocyte numbers were decreased at 2-3 weeks and appeared to recover by week 8 and decreased again in mice 25-32 weeks of age. There were also fewer lymphoid cells in the peripheral lymph nodes in the d1/d1 mice. In contrast the spleens of the d2/d2 mice appeared normal except at 6 weeks of age when some mice exhibited high splenocyte numbers (Schmidt et al., 1996). Multiple factors could account for differences between d1/d1 and d2/d2 mice including the timing of these observations, the different targeting strategies leading to differential effects on the expression of neighboring genes, different substrain of ES cells, breeding of chimeras leading to different genetic backgrounds, and different environmental exposure of the colonies to xenobiotics and infectious agents (Lahvis and Bradfield, 1998). The discrepancies between the two AhRKO mouse models make if difficult to determine the role of the AhR in the different phenotypes.

d2/d2 AhRKO mice have been used to investigate the role of the AhR in mediating inhibition of estrogen-induced responses by TCDD in the uterus. TCDD inhibits estrogen-induced lactoferrin mRNA expression and proliferation in the luminal epithelium of the uterus in wild-type mice; however, TCDD does not inhibit either the increase in lactoferrin mRNA expression or increased labeling index in the epithelial cells induced by estrogen in the uterus of d2/d2 AhRKO mice (Buchanan et al., 2000). Furthermore, tissue recombination studies using

uterine cells from the d2/d2 AhRKO mice, as described with the ERKO mice, have shown that AhR expression in the stroma is necessary for TCDD-dependent inhibition of E2-induced proliferation of epithelial cells (Buchanan et al., 2000).

1.3.4 Diversity of agonists

An increasing number of studies have shown that the AhR binds structurally diverse compounds including phytochemicals that exhibit multiple chemoprotective and anticarcinogenic activities (Bjeldanes et al., 1991; Jellinck et al., 1993; Chen et al., 1996; Gasiewicz et al., 1996; Gradelet et al., 1997; Casper et al., 1999; Seidel et al., 2000). In general, the compounds can be divided into synthetic compounds or naturally occurring compounds that are produced in biological systems through natural processes. The naturally occurring compounds are primarily dietary phytochemicals or endogenous biochemicals.

1.3.4.1 Synthetic ligands

The planar, hydrophobic HAHs such as polyhalogenated dibenzo-p-dioxins, dibenzofurans, and biphenyls and PAHs such as 3-methylcholanthrene, benzo(a)pyrene, benzanthracene, and benzoflavones are the most extensively studied classes of AhR agonists (Figure 1.14). Moreover, many of these compounds are high affinity ligands for the AhR (Poland and Knutson, 1982; Gillner et al., 1993; Kafafi et al., 1993). The more stable HAHs are the most potent AhR agonists with binding affinities in the pM to nM range, whereas the

more labile PAHs have binding affinities in the nM to μM range. Structure-activity studies with these compounds initially demonstrated that AhR ligands tend to be co-planar with dimensions that fit within 14 A X 12 A X 5 A and high affinity binding was dependent upon particular electronic, steric and thermodynamic characteristics (Kafafi et al., 1993; Bonati et al., 1995; Waller and McKinney, 1995; Tuppurainen and Ruuskanen, 2000; Mhin et al., 2002). However, a large number of synthetic compounds such as SKF71739, 2(methylmercapto)aniline, omeprazole, and YH439 with structures and physiochemical properties different from the HAHs and PAHs also to bind the AhR and activate AhR-dependent gene expression (Denison and Heath-Pagliuso, 1998; Nagy et al., 2002). Therefore, the range of potential synthetic and natural AhR ligands has been continually expanding and includes diverse structural classes of compounds (Denison and Nagy, 2003).

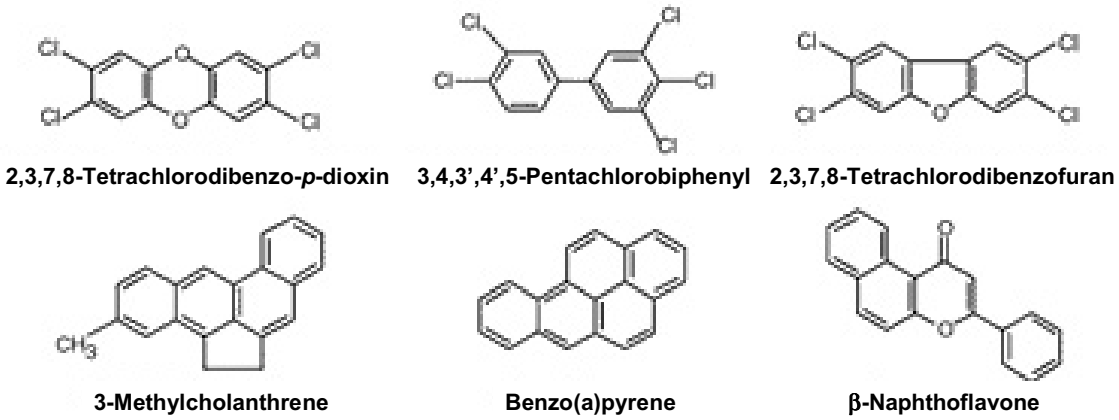


Figure 1.14: Structure of synthetic AhR ligands. (Adapted from Denison and Nagy, 2003).

1.3.4.2 Natural ligands

1.3.4.2.1 Dietary

A variety of naturally occurring dietary compounds activate or inhibit the AhR signaling pathways have also been reported. Dietary phytochemicals such as indole-3-carbinol (I3C) (Bjeldanes et al., 1991; Gillner et al., 1993), 7,8-dihydrorutacarpine (Gillner et al., 1989), dibenzoylmethanes (MacDonald et al.,

2001), curcumin (Ciolino et al., 1998), carotinoids (Gradelet et al., 1996a; Gradelet et al., 1996b) and flavonoids (Canivenc-Lavier et al., 1996; Ashida et al., 2000; Allen et al., 2001) competitively bind the AhR and/or activate AhR-dependent gene expression (Figure 1.15). Furthermore, dietary indoles such as I3C and tryptophan (Trp) can be converted in the mammalian digestive tract to more potent AhR ligands (Bjeldanes et al., 1991; Perdew and Babbs, 1991). Acid-catalyzed condensation of I3C results in the formation of indolo-[3,2-b]-carbazole (ICZ), which among the dietary compounds has perhaps the highest affinity for the AhR and is a potent inducer of AhR-dependent gene expression. 3,3'Diindolylmethane a dimeric condensation product of I3C is also an AhR agonist (Bjeldanes et al., 1991; Gillner et al., 1993; Jellinck et al., 1993).

Flavonoids such as flavones, flavanols, flavanones, and isoflavones are the largest group of dietary phytochemical AhR ligands. The majority of the flavonoids exhibit AhR antagonist activity; however, numerous agonists have also been identified (Canivenc-Lavier et al., 1996; Ashida et al., 2000; Allen et al., 2001). These compounds are found in fruits, vegetables, and teas and blood levels of flavonoids have been reported at concentrations sufficient to induce or inhibit AhR-dependent activity (Nakagawa et al., 1997; Paganga and Rice-Evans, 1997; de Vries et al., 1998).

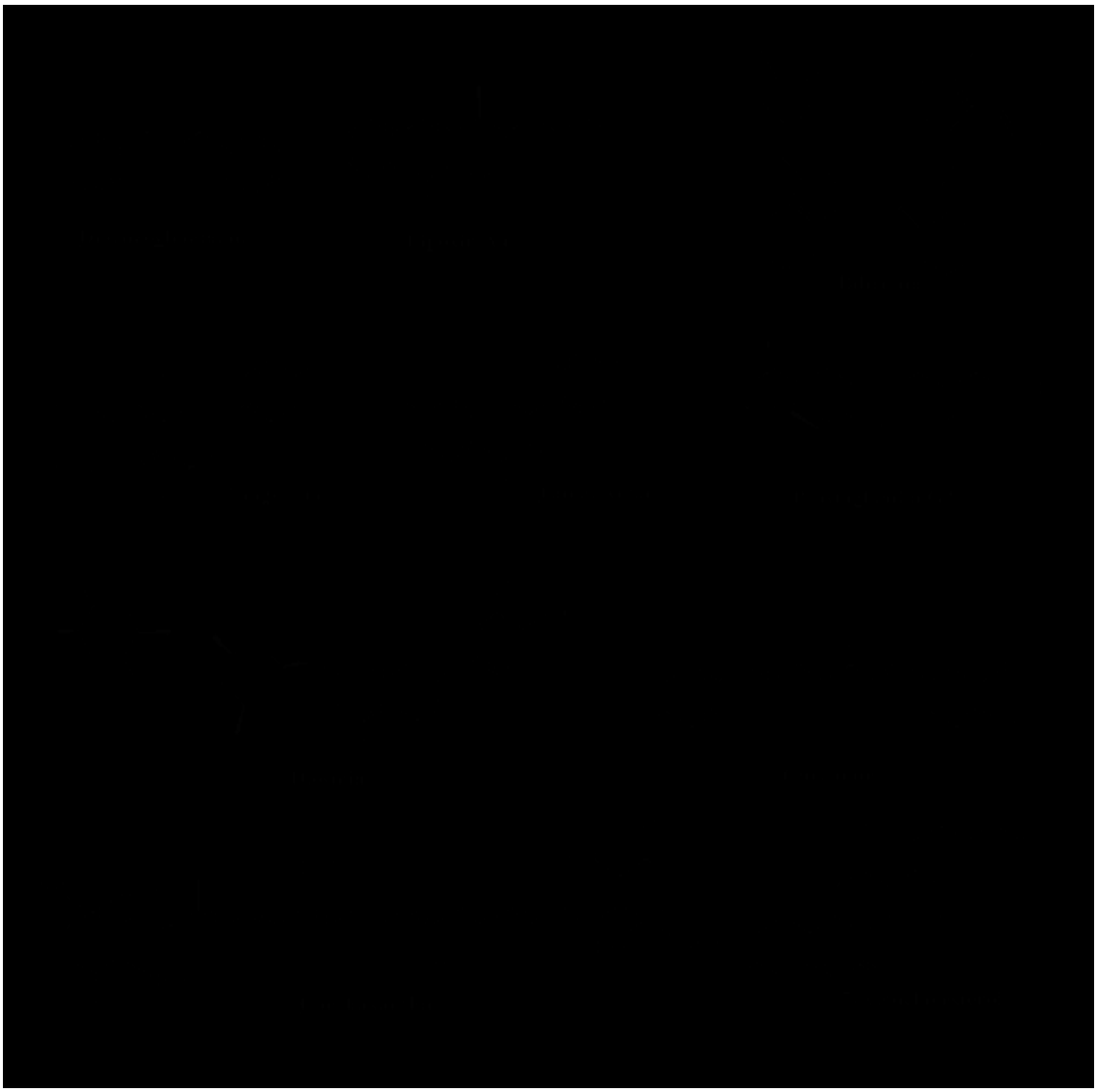


Figure 1.15: Structure of naturally occurring and endogenous ligands of the AhR.

(Denison and Nagy, 2003).

1.3.4.2.1 Endogenous

Although numerous synthetic and natural dietary ligands for the AhR have been identified, an endogenous, physiological ligand for the AhR has not been reported. The promiscuous ligand binding activity of the AhR suggests that there may be multiple endogenous ligands. Observations of AhR activation and receptor-dependent responses in the absence of endogenous ligand suggest that an endogenous ligand does exist. Nuclear AhR complexes have been identified in cells and tissue sections that have not been exposed to exogenous ligands (Abbott et al., 1994; Singh et al., 1996; Chang and Puga, 1998) and hydrodynamic shear stress conditions as well as methylcellulose suspensions of multiple cell types induce cyp1A1 expression (Sadek and Allen-Hoffmann, 1994; Monk et al., 2001; Denison et al., 2002). Also, disruption of the AhR through anti-sense or iRNA techniques results in decreased mouse blastocyst development and altered cell cycle regulation in cell culture (Paine, 1976; Peters and Wiley, 1995; Goerz et al., 1996; Abdelrahim et al., 2003). Furthermore, development of abnormalities in the liver and possibly the immune system of the AhRKO mice suggests that there is an endogenous ligand for the AhR in the mouse (Fernandez-Salguero et al., 1995; Schmidt et al., 1996).

Several structurally diverse endogenous biochemicals that bind the AhR and/or activate AhR-dependent gene expression have been identified. Endogenous compounds which contain indole structures activate the AhR and the majority are formed from Trp through biological or physiochemical

processes. Several Trp photooxidation products competitively bind the AhR and activate AhR-dependent gene expression (Rannug et al., 1987; Wei et al., 1999). One of the photoxidation products was identified as FICZ, a compound very similar in structure to ICZ which is formed from I3C (Bjeldanes et al., 1991; Denison and Nagy, 2003). It has been suggested that FICZ and other photooxidation products of Trp may be novel chemical messenger of light similar to other Trp-derived molecules such as indole acetic acid (involved in plant growth regulation) and seratonin (involved in circadian rhythms of mammals) (Wei et al., 1999). The AhR may be similar to other members of the PAS superfamily that are activated by light.

Trp metabolites such as tryptamine, indole acetic acid, and kyneurinines also activate the AhR signaling pathway. Although they have weak activity and normal blood concentrations are low, in abnormal conditions such as inhibition of monoamine oxidase activity levels of these compounds are sufficient to activate AhR signaling (Miller, 1997; Heath-Pagliuso et al., 1998). Indigo and indirubin are metabolites of Trp that can also be formed from cyp2A6/2C19/2E1-dependent metabolism of indoles (Gillam et al., 1999; Gillam et al., 2000). They were isolated from human urine and exhibited AhR activity in a yeast cell bioassay (Adachi et al., 2001). Although levels of these compounds in human serum have not been established, indigo and indirubin levels in fetal bovine serum were sufficient to activate the AhR in the yeast cell bioassay (Adachi et al., 2001).

Tetrapyroles such as bilirubin and biliverdin are another group of endogenous compounds that bind and activate the AhR (Figure 1.15). Persistent cyp1A1 expression in congenitally jaundiced Gunn rats suggested the presence of an endogenous AhR ligand (Kapitulnik and Gonzalez, 1993). Bilirubin, the primary breakdown product of heme, was present at high levels in the blood of Gunn rats and is sufficient to induce cyp1A1 expression and DRE-dependent activity in cultures at physiologically relevant concentrations (Kapitulnik and Gonzalez, 1993; Sinal and Bend, 1997; Phelan et al., 1998). Biliverdin, a precursor for bilirubin, also activates DRE-dependent gene transcription (Denison et al., 2002). Higher induction potency was observed for bilirubin and biliverdin in cells compared to in vitro systems suggesting these compounds may be converted to more potent metabolites in vivo (Denison et al., 2002).

Some arachidonic acid (AA) metabolites activate the AhR. Lipoxin A4, a lipoxygenase product of AA, binds the AhR and induces cyp1A1 and DRE-dependent gene expression at concentrations near physiological levels (Serhan and Sheppard, 1990; Schaldach et al., 1999) (Figure 1.15). Several prostaglandins, including prostaglandin G2, also bind the AhR and activate AhR-dependent gene expression, although only at concentrations >1 μM, which is much higher than physiological concentrations (Smith, 1989; Seidel et al., 2001) (Figure 1.15). However, it has been reported that prostaglandin concentrations can reach 5-10 μM near hepatocytes due to their secretion into the narrow

space of the Disse (Neuschafer-Rube et al., 1993). Studies have also demonstrated that several prostaglandins induced reporter expression 2-5 fold higher than the maximum inducing dose of TCDD, suggesting that prostaglandins may activate other pathways that augment AhR signaling.

Carotenoids including canthaxanthin, astaxanthin, and β-apo-8'-carotinal also induce cyp1A1 and other AhR-dependent gene expression (Astorg et al., 1994; Gradelet et al., 1996a; Gradelet et al., 1996b; Gradelet et al., 1997) (Figure 1.15). Although none of the endogenous retinoids exhibited AhR-dependent activity, synthetic retinoids activate the AhR and AhR-dependent gene expression, suggesting that endogenous retinoids or retinoid-like compounds may also affect AhR signaling (Soprano et al., 2001; Gambone et al., 2002). The endogenous oxysterol, 7-ketocholesterol competitively binds the AhR and act as an AhR antagonist (Savouret et al., 2001). Furthermore, concentrations of 7-ketocholesterol found in the blood are sufficient to inhibit TCDD-induced gene expression in culture (Savouret et al., 2001).

1.4 ErbB2 and ErbB receptor family

1.4.1 Structure and function

The epidermal growth factor receptor (EGFR) family, also known as the ErbB receptor family, plays a critical role in normal development and physiology as well as the growth of multiple human cancers including breast cancer. The ErbB family is comprised of 4 genes that encode 4 homologous tyrosine kinase

receptors, namely ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4 (Menard et al., 2000; Mendelsohn and Baselga, 2000; Olayioye et al., 2000; Sweeney et al., 2001). These receptors are phosphoglycoprotein transmembrane receptors that contain an extracellular ligand-binding domain, a single membrane-spanning region and an intracellular protein tyrosine kinase domain (Figure 1.16). The four receptors are differentially expressed in normal and cancerous tissues. ErbB2 plays a key role in coordinating with the other ErbBs in a complex signaling network that regulates cell growth, differentiation and survival (Yarden, 2001). ErbB2 is expressed in a wide variety of tissue except those of hematopoietic origin and is overexpressed in multiple types of cancer including breast cancer. ErbB2 is necessary for normal development and is expressed in the nervous system, bone, muscle, skin, heart, lungs, and intestinal epithelium of the human fetus (Coussens et al., 1985; Quirke et al., 1989). In the absence of ErbB2 in the mouse during development the trabeculae of the heart will not develop and the mice die during gestation (Meyer and Birchmeier, 1995). ErbB2 is also involved in normal mammary gland development and growth. ErbB ligands stimulate lobulo-alveolar development in the mouse mammary gland in expalnt cultures and in vivo (Yang et al., 1995; Jones et al., 1996; DiAugustine et al., 1997; Normanno and Ciardiello, 1997).

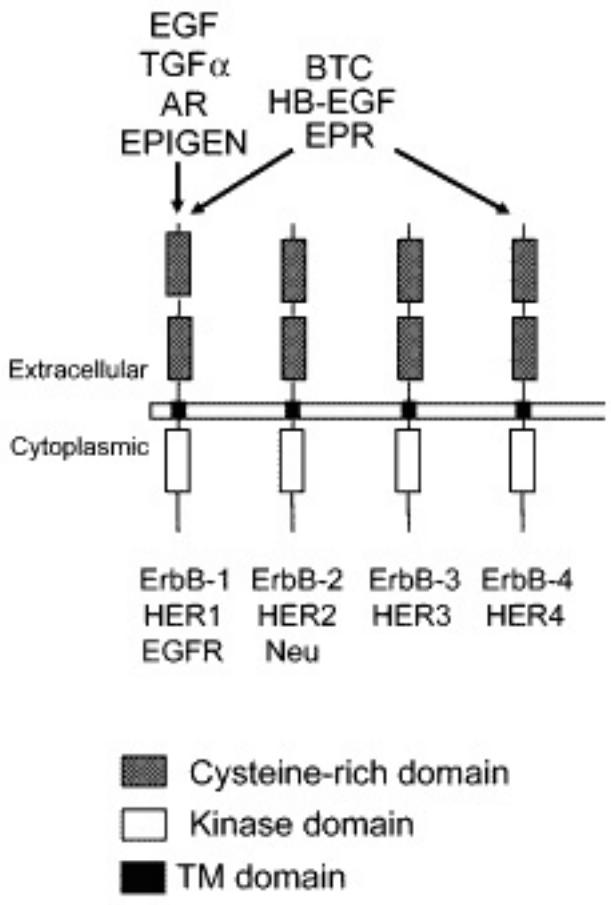


Figure 1.16: Stucture of the ErbB receptors. (Adapted from Harris et al., 2003).

1.4.2 ErbB ligands

Ligands for the ErbB family of receptors are members of the EGF-like peptide growth factor family that are produced as transmembrane precursors. The ectodomains are processed by proteolysis resulting in formation of soluble

growth factors. ErbB ligands contain an EGF-like motif of 50-55 amino acids with 6 highly conserved cysteine resudies that confer binding specificity. They can be divided into 3 classes of lignds based on receptor specificity. EGF, amphiregulin (AR), and TGFα bind ErbB1; beta cellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR) bind ErbB1and ErbB4; and neuregulins can be divided into two sub classes: neuregulins 1 and 2 bind ErbB3 and ErbB4 whereas neuregulins 3 and 4 bind ErbB4. Direct ligands for ErbB2 have not been described and studies suggest that differences in regions of the extracellular domain that contact the ligands between ErbB2 and the other ErbBs may account for their differences in ligand binding specificity (Cho and Leahy, 2002; Ogiso et al., 2002). Although ligands for ErbB2 have not been described ErbB2 is a coreceptor for many of the known ErbB ligands and is transactivated by many of the EGF-like ligands (Pinkas-Kramarski et al., 1996; Pinkas-Kramarski et al., 1997). ErbB ligands are bivalent molecules with two binding sites for the receptor. Neuregulin –1 contains a high affinity, narrow specificity binding site in the N-terminal region and a low-affinity, broad specificity binding site in the C-terminal region and ErbB2 preferentially binds the low affinity binding site (Tzahar et al., 1996; Yarden, 2001). This suggests that the ligands have high affinity binding sites that bind ErbB1, ErbB3, or ErbB4 and a low affinity site that recruits homo- or heterodimerization partners. Homo- and heterodimeric interactions between the ligand-bound ErbBs are not random; ErbB2 is the preferential dimerization partner for the ligand-bound ErbBs

(Tzahar et al., 1996; Burden and Yarden, 1997; Graus-Porta et al., 1997; Pinkas-Kramarski et al., 1998). Furthermore, dimerization with ErbB2 leads to higher ligand binding affinity as well as stonger and more sustained activation of signaling pathways compared to dimers that do not contain ErbB2 (Sliwkowski et al., 1994; Beerli et al., 1995; Graus-Porta et al., 1995; Karunagaran et al., 1996).

1.4.3 Activation of ErbB signaling pathways

Activation of ErbB pathways have been described as a signal transduction network with 3 layers: the input layer of ligands or growth factors; the cellular information processing layer of receptors, SH2-proteins, enzyme cascades, and trancription factors; and the output layer of cell growth differentiation or migration (Yarden, 2001). Ligand binding to ErbB family of receptors leads to homo- or heterodimerization and activation of the intrinsic kinase domain. Kinase activation results in phosphorylation of specific tyrosine residues on the cytoplasmic tail of the receptor that acts as docking sites for various signaling molecules. The signaling molelcules then activate signal transduction pathways such as the MAPK pathway and the PI-3K pathway (Beerli et al., 1995; Tzahar et al., 1996) (Figure 1.17). Studies using cell lines stably transfected with combinations of the ErbB receptors demonstrate that ErbB2-ErbB3 heterodimers give the highest mitogenic activity (Pinkas-Kramarski et al., 1996). ErbB3 lacks a functional kinase activation domain and, therefore, ErbB3 homodimers are catalytically inactive (Guy et al., 1994; Pinkas-Kramarski et al., 1996). However, the cytoplasmic tail of ErbB3 contains many docking

sites for signaling molecules. The lack of kinase activity may prevent overactivation of ErbB3 and the requirement of heterodimerization with ErbB2 for activity leads to increased control over signal transduction (Pinkas-Kramarski et al., 1996; Waterman et al., 1999).

Figure 1.17: ErbB2 signaling pathways. (Citri et al., 2003).

The prolonged signaling of ErbB2-containing heterodimers is partly due to the balance between the degradation and recycling pathways for the receptors. Ligand-bound receptors are endocytosed and the vesicles form early

endosomes. In the endosome the receptor and ligand are separated and sorting occurs in which the receptor is either recycled to the membrane or transported with the ligand to a lysosome for degradation (Yarden, 2001). An adapter protein c-Cbl controls the recycling/degradation pathway (Thien and Langdon, 1998). Along with the ligand, c-Cbl induces degradation of receptors in the early endosome by associating with the receptor and leading to ubiquitination and subsequent degradation in the lysosome (Levkowitz et al., 1998). ErbB1 homodimers are generally degraded by the lysosome, however, ErbB1 heterodimerized with ErbB2 is recycled to the cell membrane (Lenferink et al., 1998). c-Cbl binds strongly to ErbB1 and only weakly interacts with ErbB2, resulting in the differing balance in the degradation and recycling pathways (Levkowitz et al., 1996).

1.4.3.1 MAPK pathway

The MAPK signaling pathway is one of the main kinase cascades activated by ErbB receptors and is involved in signaling for both cell proliferation and apoptosis. The phosphyorylated tyrosines on the cytoplasmic tails of the receptors act as docking sites and form high affinity complexes with signaling molecules that link the receptor signaling to kinase cascades (Santen et al., 2002) (Figure 1.18). For example, the adaptor protein Shc can bind to the phosphorylated tyrosine residues, become phosphorylated, then further complex with the adaptor protein Grb-2. The guanine nucleotide exchange factor SOS then enters the complex and catalyzes the conversion of Ras-GDP to Ras-GTP.

Ras-GTP then initiates the signaling of the MAPK pathway. Ras-GTP interacts with and phosphorylates the first kinase in the MAPK pathway, Raf-1 or MAP kinase kinase kinase. Activated Raf-1 phosphorylates MEK 1/ 2 or MAP kinase kinase on two serine residues in the active loop resulting in activation of the kinase. MEK1/ 2 then activates MAP kinase or ERK1/2 through phosphorylation on tyrosine and threonine residues in the activation loop (Santen et al., 2002). Active ERK1 and 2 can then stimulate multiple downstream events involved in gene regulation including phosphorylation of other protein kinases, such as RSK proteins and MAP kinase interacting kinases 1 and 2, as well as transcription factors that lead to induction of cell proliferation. Active ERK 1 and 2 translocate to the nucleus and activate Elk-1, an ETS family domain protein that binds serum response elements and mediates transcription. ERK 1 and 2 also catalyze phosphorylation of c-Jun, c-Fos, and activating transcription factor-2 (Santen et al., 2002).

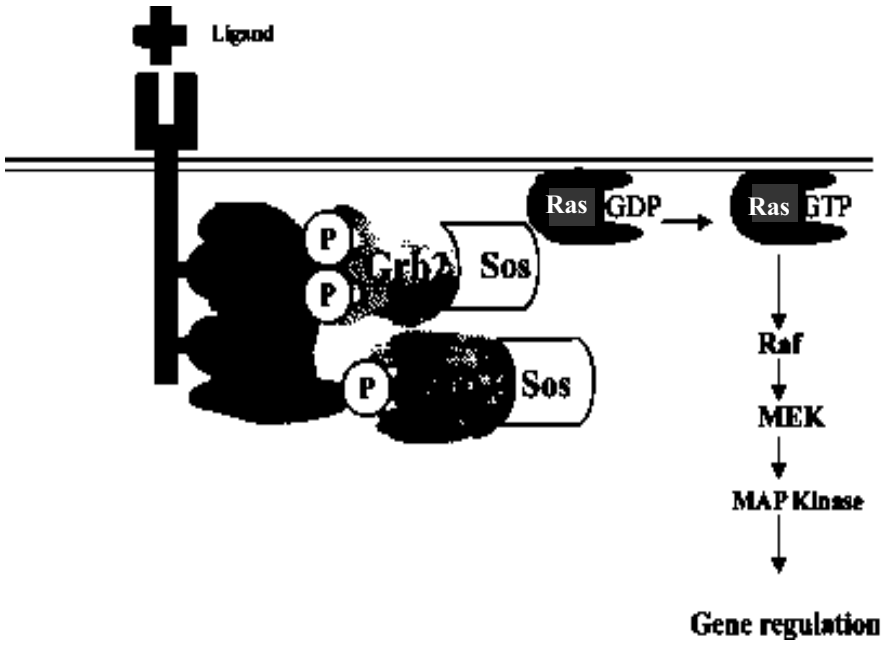


Figure 1.18: MAPK signaling pathway. (Santen et al., 2002).

1.4.3.2 PI3-K / Akt pathway

Activation of Akt, or protein kinase B (PKB), through the PI3-K pathway is the other major kinase signaling pathway activated by ErbB receptors. Akt is involved in signaling for cell survival and cell proliferation. The 85 kDa subunit of PI3-K binds phosphorylated tyrosine residues on cytoplasmic tails of activated ErbB receptors by a src homology 2 (SH2) domain and the 110 kDa catalytic subunit rapidly phosphorylates PIP2 to give PIP3 (Figure 1.19). Both phosphatidylinositol-dependent kinase (PDK) and Akt bind PIP3 through pleckstrin homology (PH) domains that recognize inositol lipids and PDK

phosphorylates Akt on threonine and serine residues. Activated Akt then translocates into the nucleus and phosphorylates target proteins involved in cell survival and cell proliferation. Glycogen synthase kinase (GSK) 3β phosphorylates cyclin D1 targeting it for degradation and inhibiting the cell cycle. Akt phosphorylates GSK 3β targeting it for degradation and allowing cyclin D1 to induce cell cycle progression. Akt also phosphorylates the protein BAD, preventing its binding to BCL-X and this resulting in inhibition of apoptosis and promotion of cell survival.

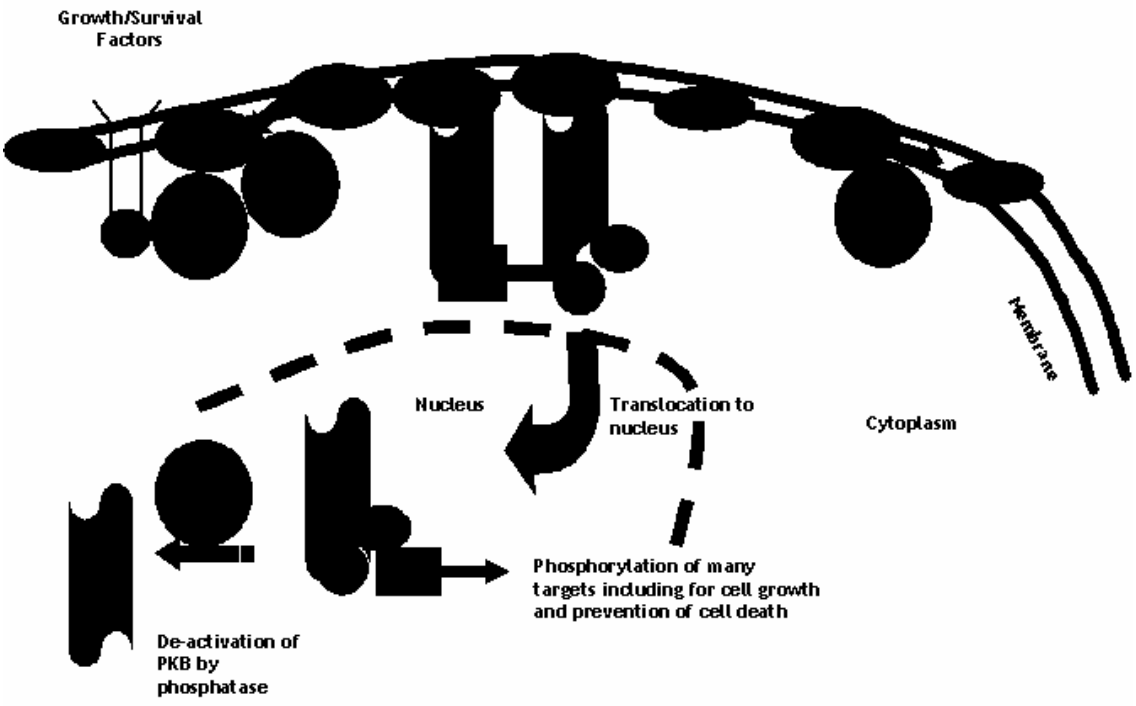


Figure 1.19: PI3-K/Akt signaling pathway. (Physiomics, 2003).

1.4.4 ErbB2 mouse mammary tumor models

ErbB2 overexpression has been observed in multiple tumor types including breast cancer, and is associated with high constitutive kinase activities. As discussed previously, ErbB2 overexpression has been reported in 20-30% of primary breast tumors (van de Vijver et al., 1987; McCann et al., 1991) and is associated with aggressive tumor behavior and poor prognosis (Hynes and Stern, 1994); however, the role of ErbB2 in tumor formation in unclear. Transgenic mice provide a useful model for assessing the role of an oncogene in tissue-specific tumor induction and growth in vivo. To study the role of a particular oncogene in mammary tumor formation, this gene can be fused to the mouse mammary tumor virus (MMTV) promoter, which drives expression in mammary tissue. The contruct can be introduced into the genome of mice and mammary tumor formation can be assessed. Transgenic mice carrying MMTV-c-myc and MMTV-v-Ha-ras fusion contructs develop solitary mammary adenocarcinomas in a stochastic manner that appears clonal in origin (Stewart et al., 1984; Leder et al., 1986; Sinn et al., 1987). This suggests that expression of c-myc or v-Ha-ras alone is not sufficient for transformation of mammary epithelial cells, and additional events are necessary for malignant transformation.

The rat homologue of ErbB2 was originally isolated from chemically-induced rat neuroblastoma and identified as c-neu (Shih et al., 1981). C-neu was isolated as an activated form that contained a single amino acid (valine to

glutamic acid) substitution in the transmembrane region of the receptor resulting in increased tyrosine kinase activity by inducing ligand-independent receptor dimerization (Bargmann et al., 1986). C-neu can also be activated by deletions in the extracellular region (Bargmann and Weinberg, 1988) or by overexpression (Di Fiore et al., 1987; Hudziak et al., 1987), as observed in human breast tumors. Therefore, multiple groups have used activated and wild-type c-neu fused to the MMTV promoter to develop transgenic mice and determine the role of neu in mammary tumor formation and growth (Muller et al., 1988; Bouchard et al., 1989; Guy et al., 1992).

The first studies developed transgenic mice expressing activated c-neu in the mammary epithelium using a recombinant plasmid with the MMTV LTR and cDNA encoding the activated c-neu protein (Muller et al., 1988). Four transgenic founder lines were produced and the transgene was passed onto the progeny and two of the lines expressed the transgene in organs assayed (Muller et al., 1988). Expression of c-neu in the mammary gland, parotid gland, Harderian-lacrimal gland and epididymis of the transgenic mice was consistent with previous studies using the MMTV promoter. The line designated TG.NF showed uniform expression of the transgene throughout the mammary gland and lactational defects were developed and followed by synchronous mammary tumor formation involving all mammary glands in all mice at 13-14 weeks of age (Muller et al., 1988). Examination of the surrounding mammary tissue showed a lack of normal mammary epithelium in the mammary glands of these transgenic

mice. The second line designated TG.NK expressed the transgene in a non-uniform, stochastic pattern of expression in mammary epithelium in the same gland and stochastic tumor formation adjacent to normal mammary epithelium starting around 25 weeks of age in female mice (Muller et al., 1988). However, while normal epithelium did not express the transgene, expression of the trangene within affected cells correlated morphologically with dysplasia and tumor formation. The kinetics of tumor formation and the lack of normal epithelium in the TG.NF, which has uniform expression of the transgene, suggest that the expression of activated c-neu is sufficient for transformation of the mammary epithelium (Muller et al., 1988). Mice from the TG.NK line are available from Charles River Laboratories and were used for our studies.

A second group also developed transgenic mice expressing the activated c-neu on the MMTV promoter; however, the kinetics of tumor formation differed from mice reported in the first study (Bouchard et al., 1989). Mammary tumors developed after 5 to 10 months of age in the majority of females in all transgenic lines established. The mammary tumors developed independently and ashynchronously, differing in size (Bouchard et al., 1989). Furthermore, transgene mRNA levels were highly expressed in morphologically normal mammary glands before the appearance of visible tumors (Bouchard et al., 1989). These results suggest that an additional genetic event is necessary for transformation of mammary epithelium that expresses activated c-neu. The contrasting results from the two studies could be due to the difference in the

structure of the transgene used to develop the mice. The transgene from the first group contained the rat 30 S sequence for the Harvey murine sarcoma viral gemone between the MMTV LTR and the cDNA for c-neu; in contrast the MMTV LTR and the c-neu cDNA were adjacent to each other in the transgene used by the second group (Bouchard et al., 1989). Furthermore, undetected modifications to the transgene could have been generated during manipulation and changed the biological properties of one of the transgenes (Bouchard et al., 1989).

Studies of transgenic mice with the wild-type c-neu gene on the MMTV promoter have shown that overexpression of unactivated c-neu in the female mammary epithelium resulted in focal mammary tumor formation that metastasized with high frequency (Guy et al., 1992). The pattern of tumor formation and high level transgene expression in normal mammary epithelium in these studies suggest that additional events are necessary for transformation of mammary epithelial cells that overexpress unactivated c-neu (Guy et al., 1992). However, results showing that mammary tumors express higher tyrosine kinase activity than the surrounding normal epithelium together with results from the first MMTV-c-neu transgenice mice (that express activated c-neu) indicate that activation of c-neu leading to higher tyrosine kinase activity could lead to transformation (Muller et al., 1988; Guy et al., 1992).

Transgenic mice overexpressing oncogenes in knockout mice have been used to determine the role of cyclin D1 in transformation of the mammary

epithelium. Cyclin D1 is involved in the transition from G0/G1 to S phase of the cell cycle and both the *ras* and *neu* oncogenes regulate cyclin D1 expression through the MAPK pathway (Filmus et al., 1994; Albanese et al., 1995; Liu et al., 1995; Lavoie et al., 1996; Lee et al., 2000). One study crossed the MMTV-v-Ha-ras, MMTV-c-neu, MMTV-c-myc, and MMTV-Wnt-1 transgenic mice with cyclin D1 knockout mice to determine whether cyclin D1 expression was necessary for tumor formation in the mice (Yu et al., 2001). MMTV-v-Ha-ras and MMTV-c-neu mice that did not express cyclin D1 did not develop mammary tumors, whereas the MMTV-c-myc and MMTV-Wnt-1 mice that did not expresss cyclin D1 did develop mammary tumors (Yu et al., 2001). Similarly, tumors and mammary epithelial cells from the MMTV-v-Ha-ras and MMTV-c-neu did not express cyclin D2, whereas tumors and mammary epithelial cells from MMTV-c-myc and MMTV-Wnt-1 mice expressed cyclin D2 (Yu et al., 2001). These studies indicate that while the oncogenes c-myc and Wnt-1 bypass the need for cyclin D1 expression for cell cycle progression, most likely through the expression of cyclin D2, c-neu and v-Ha-ras oncogenes are dependent upon cyclin D1 expression for cell cycle progression. This is probably due to their inability to induce the expression of cyclin D2 or other cyclins that compensate for cyclin D1. Further studies in mice show that replacement of cyclin D1 with cyclin E on the cyclin D1 promoter and overexpression of ras and c-neu on the MMTV promoter induced mammary tumor formation (Yu et al., 2001). These results indicate that cyclin E can compensate for cyclin D1 in ras- and c-neu- induced

cell cycle progression when the cyclin D1 promoter was used for expression of cyclin E.

1.5 Objectives

1.5.1 Objective 1

E2-induced cell proliferation is well characterized in vitro in breast cancer cells and in vivo in the rodent uterus, and this is accompanied by induction of several genes associated with cell proliferation such as c-myc, c-fos, ornithine decarboxylase, and cyclin D1. Recently, it has been shown that E2 induces several genes associated with purine/pyrimidine and DNA synthesis, as well as the growth factor VEGF in breast cancer cells. Therefore, the immature mouse uterus will be used as an in vivo model to investigate E2-induced expression of genes involved in cell proliferation. The organ structure and multiple cell types of the uterus complicate gene expression studies, compared to in vitro studies which involve a single cell type. In order to localize changes in expression to specific regions or cell types, gene expression in the uterus will be analyzed by in situ hybridization. There is evidence in the uterus that E2 binds ER in stromal or epithelial cells and induces expression of paracrine factors that then induce proliferation in neighboring cells or other cell types. In order to differentiate direct induction of gene expression by E2 from indirect induction of gene expression by a paracrine factor, protein synthesis will be inhibited by

cycloheximide prior to E2 treatment. Furthermore, to determine whether ERα is necessary for E2-induced gene expression, ERKO mice will also be used.

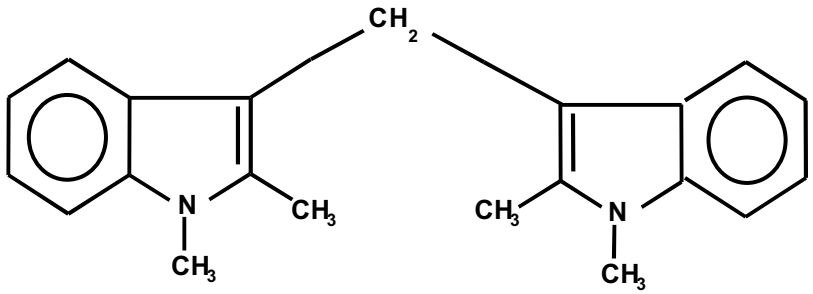
Crosstalk between AhR and ER has been characterized in vitro in breast cancer cell lines. Activation of the AhR through agonists such as TCDD down-regulate induction of several E2-responsive genes including those required for cell proliferation. Therefore, E2-induced proliferation in the immature mouse uterus will be used as a model to determine whether AhR agonists inhibit induction of gene expression by E2 in vivo. Furthermore, AhRKO mice will be used to determine whether AhR is necessary for these responses and cycloheximide-dependent inhibition of protein synthesis will determine whether new protein synthesis is necessary for AhR-mediated inhibitory responses.

1.5.2 Objective 2

Overexpression and amplification of ErbB2 have been implicated in development of aggressive forms of human breast cancer. In vitro studies in BT-474 ErbB2 overexpressing human breast cancer cells indicate that modulation of cyclin D1 and p27 through both the MAPK and PI3K/Akt pathways allows G1 to S phase transition; in vivo studies crossing MMTV-c-neu and cyclin D1-/- mice indicate that cyclin D1 is necessary for ErbB2 induced mammary tumor formation (Lenferink et al., 2001; Yu et al., 2001). However the mechanism of ErbB2 transformation and growth of cells is not completely understood. The MMTV-c-neu mouse mammary tumor model expresses a mutated form of the rat c-neu (ErbB2) protein under the mouse mammary tumor

virus resulting in a well established progression of mammary carcinogenesis with palpable mammary tumors forming around 25 weeks of age (Muller et al., 1988). Therefore, gene expression profiles during the progressive stages of mammary carcinogenesis in MMTV-c-neu mice will be analyzed to better understand how ErbB2 overexpression transforms cells and results in tumor formation.

Previous studies have shown that AhR agonists induce down-regulation of ErbB1 protein and/or phosphorylation in multiple tissues/organs (Astroff et al., 1990; Guyda et al., 1990; Sewall et al., 1995; Zhang et al., 1995), suggesting possible inhibitory interactions with chemotherapeutic potential. Several ErbB2-overexpressing breast cancer cells such as the BT-474 and MDA-MB-453 cell lines express a functional AhR and exhibit constitutive activation of both the MAPK and PI3K pathways. Recently, it has been reported that the SAhRM 1,1',2,2'-tetramethyldiindolylmethane (1,1',2,2'-tetraMethDIM, Figure 1.20) inhibited DMBA-induced mammary tumor growth in rats and also inhibited MAPK and PI3K pathways in MCF-7 human breast cancer cells. The proposed studies will investigate inhibitory AhR-ErbB2 interactions of 1,1',2,2'-tetraMethDIM in these cells lines and investigate the mechanism of growth inhibition. A parallel approach using MMTV-c-neu transgenic mice that overexpress ErbB2 in the mammary gland will also be carried out to determine the inhibitory effects of 1,1',2,2'-tetraMethDIM on ErbB2-induced tumor formation.



1,1',2,2'-tetraMethDIM

Figure 1.20: Structure of 1,1',2,2'-tetraMethDIM.

CHAPTER II

MATERIALS AND METHODS

2.1 Animals

Female B6C3F1 mice and MMTV-c-neu mice were obtained from Charles River Laboratories (Wilmington, MA). Twenty-five day old female AhRKO and ERKO mice were obtained from in house colonies. The mice were kept in temperature controlled conditions room with a 14 h light and 10 h dark photocycle. Rodent chow and water was supplied ad libitum.

2.2 Treatment and tissue processing

For uterine studies, twenty-five day-old mice were injected intraperitoneally with either 200 ng of E2 (Sigma, St Louis, MO) in 100 μl of corn oil, 1 μg of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in 100 μl oil, E2 + TCDD, or corn oil alone. Some mice also received 75 mg/kg of cycloheximide (Sigma) prior to treatment with E2 and TCDD. One, three, six, or twelve hours after treatment the mice were euthanized by CO_2 asphyxiation. The uteri were removed, fixed in 4% paraformaldehyde (EMS, Fort Washington, PA) overnight, washed with 70% ethanol and paraffin embedded. For MMTV-c-neu studies, female MMTV-c-neu and FVB mice at 6, 12, and 18 weeks of age were euthanized by CO_2 asphyxiation and mammary glands were removed or twenty-

two week old MMTV-c-neu mice were treated with 10 mg/kg 1,1',2,2'-tetraMethDIM or corn oil by gavage and palpable mammary tumor were measured every other day. After 28 days the mice were euthanized by CO_2 asphyxiation and mammary gland and lungs were removed, fixed in 4% paraformaldehyde or 10% formaldehyde (Sigma) and paraffin embedded.

2.3 Probe synthesis for in situ hybridization

Radiolabeled cRNA probes were generated from linearized cDNA plasmids using in vitro transcription by T7, T3 or SP6 RNA polymerases (Promega, Madison, WI) with [^{35}S]-UTP (NEN, Boston, MA). The cyclin D1 cDNA in pBluescript was a gift from Dr. Charles Sherr (St. Jude Children's Research Hospital, Memphis, TN) and the pcDEB-M180K DNA polymerase α cDNA construct was a gift from Dr. Fumio Hanaoka (The Institute of Physical and Chemical Research, RIKEN, Saitama, Japan). The DNA polymerase α cDNA fragment was cloned into pcDNA3.0 and then used to make cRNA probes. The VEGF cDNA fragment was amplified by RT-PCR, cloned into pcDNA3.0, and sequenced in this lab.

2.4 In situ hybridization

Sections (0.5 μM) of paraffin-embedded tissue were placed on positively charged slides. The sections were deparaffinized with three 5 min washes with the xylene substitute Hemo-De (Fisher Scientific, Pittsburgh, PA), re-hydrated

with a graded series of 5 min ethanol baths (100% x 3, 95% x 2, 70% X1) and post-fixed with 4% paraformaldehyde in PBS for 20 min. The sections were then digested with proteinase K (Roche, Indianapolis, IN)(20 μg/ml) in a digestion buffer (50 mM Tris, 5 mM EDTA, pH 8) for 7.5 min, re-fixed for 5 min with 4% paraformaldehyde in PBS, washed with DEPC-treated water for 30 sec, rinsed in PBS, and dehydrated with a graded series of ethanol baths and air dried at room temperature. Sense and antisense radiolabeled cRNA probes were denatured in hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl [pH 8], 5mM EDTA [pH 8], 10 mM sodium phosphate [pH 8], single-strength Denhardt's solution, 10% dextran sulfate, 0.5 mg/ml yeast RNA, 100 mM dithiothreitol) at 70^{o}C for 10 min and added to sections. Slides were hybridized with the cRNA probes (5 x 10^{6} cpm/slide) for 18 h at 55^{o}C in a humidified chamber containing 50% formamide/5X SSC. Slides were washed for 30 min in 5X SSC/10 mM β-mercaptoethanol (βME) at 55^{o}C, 20 min in 50% formamide/2X SSC/50 mM βME at 65^{o}C, 10 min in 1X TEN (0.05 M NaCl, 10 mM Tris [pH 8], 5 M EDTA) at room temperature, and three 10 min changes of 1X TEN at 37^{o}C. Slides were digested with DNase-free RNase (Promega) (10 μg/ml) in 1X TEN at 37^{o}C for 10 min to remove nonspecifically bound probe, then washed for 15 min in 1X TEN at 37^{o}C, 20 min in 50% formamide/2X SSC/50 mM βME at 65^{o}C, 15 min in 2X SSC at room temperature, 12 min in 0.1X SSC at room temperature, 5 min in 70% ethanol/0.3 M ammonium acetate at room temperature, 1 min in 95% ethanol/0.03 M ammonium acetate at room temperature, and two 1 min changes

in 100% ethanol. Slides were coated with Kodak NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY), developed 3-4 weeks later with Kodak D-19 developer (Eastman Kodak), counterstained with Harris Modified Hematoxylin (Fisher Scientific), dehydrated with a graded series of ethanol to Hemo-De, and fixed with cover slips by Permount (Fisher Scientific). The slides were evaluated by both brightfield and darkfield microscopy with a Zeiss Axioplan 2 Photomicroscope (Carl Zeiss Inc, Thornwood, NY) and digital images were captured. Representative photomicrogrphs are shown for each probe and treatment group.

2.5 Histopathology

Animals were euthanized by CO_2 asphyxiation and the #1 and #3 mammary glands were removed, fixed in 4% paraformaldehyde, and processed by the Hisotpathology Lab. Stained tissue sections were analyzed by a board certified Pathologist.

2.6 Microarray analysis

Animals were euthanized by CO_2 asphyxiation and the #4, #5, and part of #3 mammary glands were removed. RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer protocol, resuspended in RNA Storage Solution (Ambion, Austin, TX), and concentration and purity determined by spectrophotometry. Microarray analysis was determined using

Affymetrix MG-U74Av2 chip according to manufacturers protocol. Gene expression data was analyzed using GeneSpring version 6.0 (Silicon Genetics, Redwood City, CA). Genes up-regulated or down-regulated greater than two-fold were grouped based on known gene ontology using DAVID a web-based, client/server application (http://david.niaid.nih.gov/david/upload.asp) that allows users to access a relational database of functional annotations (Dennis et al., 2003). Functional annotations are derived primarily from LocusLink and the annotation data used by DAVID is updated weekly. It should be noted that DAVID may classify genes under more than one category since many genes are known to have more than one function.

2.7 Cell culture

All cell lines were obtained from ATCC (Manassas, VA). BT-474 human breast cancer cells were cultured in DME/F-12 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), sodium pyruvate (0.11 g/L), insulin (10 mg/L), and antibiotic/antimyotic solution. MDA-MB-453 cells were maintained in RPMI medium (Sigma) supplemented with 10% fetal bovine serum, sodium bicarbonate (1.5 g/L), Hepes (2.38 g/L), sodium pyruvate (0.11 g/L), glucose (4.5 g/L), and antibiotic/antimyotic solution. All cells were maintained at 37C in a 5% CO_2 atmosphere.

2.8 Cell proliferation

Cells were seeded for experiments in DME/F-12 medium without phenol red (Sigma) containing 2.5% charcoal-stripped FBS, sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), sodium pyruvate (0.11 g/L), insulin (10 mg/L), and antibiotic/antimyotic solution in triplicate in 6 or 12 well plates. Twenty-four hours after seeding cells were dosed with 1,1',2,2'-tetraMethDIM at 2.5, 5.0 and 10.0 μM either in DME/F-12 media without phenol red supplemented with 2.5% or 5.0% charcoal-stripped FBS or in the medium in which the cells were maintained. Dosing medium was changed every 48 h throughout the assay and throughout the time course each well of cells was trysinized, placed in Isoton II (Fisher Scientific), and counted by the Coulter Z1 cell counter in duplicate. Results are expressed as means ± SE for three replicate experiments for each treatment group.

2.9 Cell cycle analysis

Cells were seeded for experiments in DME/F-12 medium without phenol red containing 2.5% charcoal-stripped FBS, sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), sodium pyruvate (0.11 g/L), insulin (10 mg/L), and antibiotic/antimyotic solution in triplicate in 6 well plates. Twenty-four hours after seeding cells were treated with 2.5, 5.0 and 10.0 μM 1,1',2,2'-tetraMethDIM in 2.5% DME/F-12 and dosing medium was changed every 48 h. Forty-eight hours after treatment the cells were collected and place in propidium iodide staining

solution [sodium citrate 4 mM, triton X-100 0.1%, propidium iodide 50 mg/ml (Sigma), RNase One 30U/ml (Promega)] for 10 min at 37^{o}C in the dark. NaCl was then added for a final concentration of 0.15 M and samples stored at 4^{o}C in the dark until analyzed by flow cytometry. Results are expressed as means ± SE for three replicate experiments for each treatment group.

2.10 Western blot analysis

Cells were seeded in DME/F-12 medium without phenol red containing 2.5% charcoal-stripped FBS. After treatment for 12 and 24 hours, cells were harvested in 60 to 200 μl of 1X Laemmli buffer (50 mM Tris-Cl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM DTT) depending upon experiments and equal volumes of whole cell lysates were boiled for 5 min and used for Western blots. After 40 hour and 4 day treatments, cells were lysed in ice cold lysis buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA) supplemented with protease inhibitor cocktail (Sigma). Cells lysates were scraped into 1.5 ml eppendorf tubes and incubated on ice for 1 hour with intermittent vortexing. Cell lysates were then centrifuged for 15 min at 40,000 x g and the supernatants used for Western blot analysis. Protein concentrations were measured with Bradford reagent according to standard protocol and equal amounts of protein from each treatment group were boiled in 1X Laemmli buffer for 2 min prior to loading. Proteins from both lysis protocols were separated by 10% SDS-PAGE, and

electrophoresed to PVDF membrane (BioRad, Hercules, CA). Membranes were blocked in Blotto [5% milk +TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) + 0.05% Tween 20] and probed with primary antibodies ErbB2 (neu), p-ERK1/2, ERK1/2, p-Akt, Akt, cyclin D1, p27, ERα (G-20), AhR (H-211), and cyp1A1 at 1:1000 in Blotto or p21 (rabbit polyclonal) at 1:400 in Blotto (antibodies were purchased from Santa Cruz Biotechnologies, Santa Cruz, CA). Membranes were washed with TBS + 0.05% Tween 20 and following incubation with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnologies) at 1:5000 in Blotto, immunoglobins were visualized using the ECL detection system (Perkin Elmer, Boston, MA). For Western blots with PARP antibody (Santa Cruz Biotechnologies) membranes were blocked in 1% bovine serum albumin (BSA) + 1X PBS + 0.04% Tween 20, probed with the PARP antibody at 1:200 dilution in 1% BSA + 1X PBS + 0.04% Tween 20 overnight, washed with 1X PBS + 0.04% Tween 20, probed with peroxidase-conjugated mouse IgG_{2a} (Santa Cruz Biotechnologies) at 1:5000 in 1% BSA + 1X PBS + 0.04% Tween 20 for 4 h and immunoglobins were visualized using the ECL detection system (Perkin Elmer).

2.11 Immunoprecipitation

Cells were seeded in DME/F-12 medium without phenol red containing 2.5% charcoal-stripped FBS. After 24 h cells were treated with 10 μM 1,1',2,2'-tetraMethDIM, 10 nM E2, 10 μM U0126, or 10 μM LY294002 and treatment media was changed after 48 h. Fours days after the beginning of treatment, the

media was removed and cells were lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA) supplemented with protease inhibitor cocktail (Sigma). Cells lysates were scraped into 1.5 ml eppendorf tubes and incubated on ice for 1 hour with intermittent vortexing. Cell lysates were then centrifuged for 15 min at 40,000 x g and the supernatants used for immunoprecipitations. Protein concentrations in the supernatants were measured using Bradford reagent according to standard protocols. For immunoprecipitations, 350 mg of protein was precleared with 30 μl of Protein A/G-agarose Plus beads (Santa Cruz Biotechnologies) in 1 ml 1X phosphate buffered saline (PBS) rocking for 1 hour and 15 min at 4°C. Beads were centrifuged at 700 x g for 3 min and 1200 μl of supernatant was transferred to a new tube. Two micrograms of ErbB2 (Santa Cruz Biotechnologies) or normal rabbit IgG (Santa Cruz Biotechnologies) antibody plus 30 μl of Protein A/G-agarose Plus beads were added to the samples and incubated overnight at 4°C with gentle shaking. For washing, the beads were centrifuged at 700 x g for 3 min, supernatant removed, 1 ml RIPA buffer [50 mM Tris-HCl (pH 7.5), 15mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] added, and rocked for 30 sec. The wash was then repeated with 1 ml of 1X PBS, the beads were pelleted again at 700 x g for 3 min, supernatant removed, and 50 μl of 1X Laemmli buffer (50 mM Tris-Cl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM DTT) was added to each sample. Immunoprecipitated samples were boiled for 5 min prior to loading and 50 μg of the total protein for

each sample was loaded in 1X Laemmli buffer after boiling for 2 min. The proteins were separated on a 10% SDS-PAGE gel and electrophoresed to PVDF membrane (BioRad). Membranes were blocked in 1% milk + 1% BSA +TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) + 0.05% Tween-20, probed with anti-p-Tyr antibodies (Santa Cruz Biotechnologies) at 1:1000 for 4 h, washed with TBS + 0.05% Tween 20, probed with peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnologies) antibodies at 1:5000 for 3 h, washed with TBS + 0.05% Tween 20, and immunoglobins were visualized using the ECL detection system (Perkin Elmer). Membrane was stripped for 45 min at 65^{o}C in stripping solution (62.5 mM Tris-HCl, 2% SDS, 10 mM β-mercaptoethanol, pH 6.8), rinsed with dH_2O, incubated in dH_2O overnight at 4^{o}C, and reprobed with ErbB2, p-ERK1/2, ERK1/2, p-Akt, and Akt antibodies as described above for Western blots.

2.12 Caspase-3 apoptosis assay

Cells were seeded for experiments in DME/F-12 medium without phenol red containing 2.5% charcoal-stripped FBS, sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), sodium pyruvate (0.11 g/L), insulin (10 mg/L), and antibiotic/antimyotic solution in triplicate in 96 well plates. Twenty-four hours after seeding, cells were treated with 2.5, 5.0 and 10.0 μM 1,1',2,2'-tetraMethDIM; 0.1 μM okadaic acid; or 3 μM camptothecin in 2.5% DME/F-12. After 24, 48, and 72 h the cells were washed with 1X PBS and lysed with cell

lysis buffer (10 mM Tris-HCL, 10 mM $NaH_2PO_4/NaHPO_4$ (pH7.5), 130 mM NaCl, 1% triton X-100, 10 mM $NaPP_i$) for 30 min on ice. Ac-DEVD-AMC fluoroganic substrate (BD Biosciences, San Jose, CA) at a final concentration of 20 μM and protease assay buffer (20 mM HEPES (pH 7.5), 10% glycerol, 2 mM DTT) were added to the cell lysates and incubated for 1 hour at 37°C in the dark. The release of AMC by cleavage with active caspase-3 in the cell lysates was measured with an excitation wavelength of 390 nm and an emission wavelength of 460 nm. Results are expressed as means ± SE for three replicate experiments for each treatment group.

2.13 Lactose dehydrogenase (LDH) cytotoxicity assay

Cells were seeded for experiments in DME/F-12 medium without phenol red containing 2.5% charcoal-stripped FBS, sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), sodium pyruvate (0.11 g/L), insulin (10 mg/L), and antibiotic/antimyotic solution in triplicate. Twenty-four hours after seeding cells were treated with 2.5, 5.0 and 10.0 μM 1,1',2,2'-tetraMethDIM or 0.005, 0.05, or 5 mM phenol in 2.5% DME/F-12. Two or 4 days after treatment 20 μl of supernatant was removed from each well to be assayed. Three control wells were lysed with 0.3% Triton X-100 3 to 6 h prior to assay to measure maximum release of LDH into the supernatant. Total lysis of cells was confirmed under the microscope. Two hundred microliters of phosphate buffer ($NaH_2PO_4/NaHPO_4$, pH7.5) with 1.22 mM pyruvate (Sigma) and 8 μl of NADH (Sigma) at 12.4 mg/ml

were added to the cell supernatant and incubated at 37^{o}C for 30 min. The absorbance at 390 nm was measured to determine the decrease in NADH concentration resulting from LDH converting pyruvate to lactate. Controls of pyruvate alone, pyruvate + NADH alone, and pyruvate + NADH + DME-F-12 without cells were used for baseline measurements of minimum and maximum absorbance and wells lysed with Triton X-100 were used to measure the maximum decrease in absorbance due to total cell lysis. Results are expressed as means ± SE for three replicate experiments for each treatment group.

2.14 Mammalian one-hyrbrid transfections

Cells were seeded for experiments in DME/F-12 medium without phenol red containing 2.5% charcoal-stripped FBS, sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), sodium pyruvate (0.11 g/L), insulin (10 mg/L), and antibiotic/antimyotic solution in 12 well plates. Twenty-four hours after seeding cells were transfected by the calcium phosphate method with 100 ng of the empty pM, Elk1-Gal4, SRF-Gal4, SMAD2-Gal4, or SMAD4-Gal4 fusion protein expression plasmids and 1 μg of the Gal4-luciferase reporter vector. Five hours after transfection the cells were washed with 1X PBS, shocked with 25% glycerol in 1X PBS for 15 sec, rinsed once with 1X PBS and treated for 36 h. Cells were harvested by scraping the plates in 100 μl of 1X lysis buffer. Twenty microliters of the cell lysate was used for performing luciferase assays (Promega) on a Lumicount Luminometer (Packard Instrument Co., Downers

Grove, IL). Total protein in each sample was measured with Bradford reagent and normalized luciferase values were calculated by dividing the luciferase value by total protein for a given sample. Results are expressed as means ± SE for three replicate experiments for each treatment group.

2.15 Statistical analysis

Statistical significance was determined by analysis of variance and student's t-test. Treatment groups were considered statistically different if $p < 0.05$. Results are expressed as means ± standard error (SE) for at least three replicate experiments for each treatment group.

CHAPTER III

RESULTS

3.1 ERα-AhR crosstalk in uterine gene expression

3.1.1 E2-induced expression of genes involved in cell proliferation in the mouse uterus

E2-induced cell proliferation is well characterized in vitro in breast cancer cells and in vivo in the rodent uterus and is associated with the induction of several genes including c-myc, c-fos, ornithine decarboxylase, and cyclin D1 (Dubik and Shiu, 1992; Duan et al., 1998; Castro-Rivera et al., 2001). Recently, it has been shown that E2 induces several genes associated with purine/pyrimidine and DNA synthesis, as well as the growth factor VEGF, in breast cancer cell lines (Samudio et al., 2001; Stoner et al., 2004). In order to investigate in vivo induction of gene expression by E2 and to localize the expression within the uterus the immature mouse uterus was used as an in vivo model for studying hormonal regulation of cell proliferation. The organ structure and multiple cell types of the uterus can complicate gene expression studies using whole organ extracts, compared to in vitro studies that involve single cell types and lack organ structure. Therefore in situ hybridization was used to

localize changes in mRNA levels to specific regions or cell types in the rodent uterus.

Initial studies investigated the time course induction of gene expression by E2 by determining increased mRNA levels in specific regions of the uterus. Inbred B6C3F1 mice were treated with E2 by intraperitoneal injection and the uterus was removed 1, 3, 6, and 12 hours later for analysis. Induction of cyclin D1 by E2 is well characterized in breast cancer cell lines and previous studies have used whole organ extracts to investigate induction of cyclin D1 by E2 in vivo in the uterus (Geum et al., 1997; Wang et al., 1998; Castro-Rivera et al., 2001; Buchanan et al., 2002). This study localized the highest induction of cyclin D1 gene expression to the luminal epithelium. Three hours after treatment with E2 cyclin D1 probes showed a generalized staining of the cyclin D1 mRNA throughout the stroma of the uterus that was consistently higher than

observed in tissue from control (corn oil-treated) sections. (Figure 3.1, E-F compared to G-H). Six and 12 hours after treatment with E2 there was intense staining of cyclin D1 mRNA in the luminal epithelium of the uterus with low levels of generalized staining in the stroma (Figure 3.1, I-J v. K-l and M-N v. O-P). These data are consistent with a study showing increased cyclin D1 mRNA levels by Northern blot analysis 6 and 12 hours after treatment with E2 (Geum et al., 1997). Furthermore, previous studies have shown that although cell proliferation is primarily observed in the luminal epithelium in the adult uterus, proliferation is seen in both the stroma and the epithelium of the immature uterus (Quarmby and Korach, 1984). Therefore, the induction of cyclin D1 in the stroma and the luminal epithelium seen in this study is consistent with studies that show proliferation of cells in both the stroma and epithelium in the immature uterus.

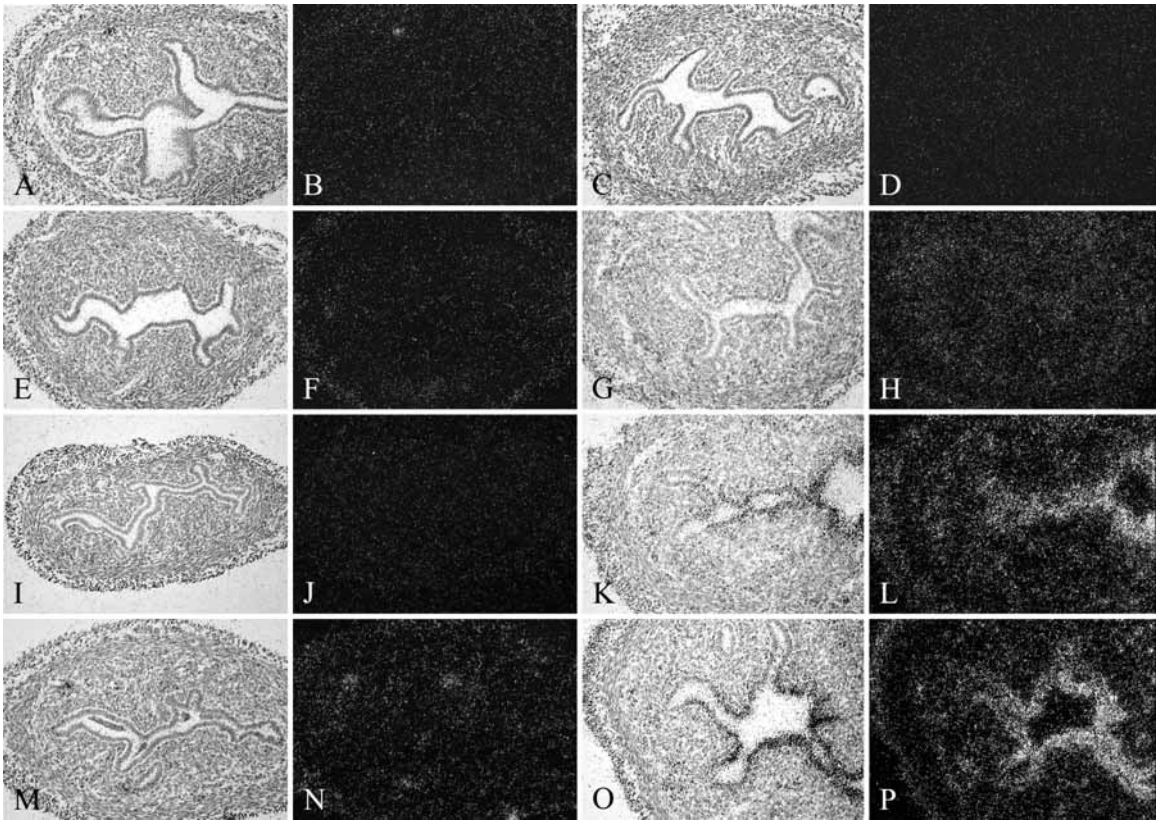


Figure 3.1: Time-dependent expression of cyclin D1 mRNA after treatment with E2 in B6C3F1 mice. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle (A-B, E-F, I-J, M-N) or 200 ng of E2 in corn oil (C-D, G-H, K-L, O-P) (n=4 per treatment). Cyclin D1 mRNA levels were determined by *in situ* hybridization of uterine sections taken 1 hour (A-D), 3 hours (E-H), 6 hours (I-L), and 12 hours (M-P) after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for cyclin D1 mRNA. Stained sections were analyzed by brigthfield (A, C, E, G, I, K, M, O) and darkfield (B, D, F, H, J, L, N, P) microscopy. A representative section of each treatment and time point is shown.

The growth factor, VEGF, is involved in embryonic vascular development and postnatal angiogenesis and studies in the rat uterus have shown that treatment with E2 results in rapid upregulation of VEGF mRNA in the stromal compartment within 1 hour after treatment. Furthermore, it has been shown in humans and non-human primates that VEGF expression in the uterus is hormonally regulated throughout the menstrual cycle and induction of VEGF by E2 in breast cancer cell lines is context dependent (Stoner et al., 2000; Nayak and Brenner, 2002; Sugino et al., 2002; Stoner et al., 2004). Analysis of the human VEGF promoter shows that E2 downregulates VEGF mRNA levels and promoter activity in Hec1A endometrial cancer cells whereas hormone-induced transactivation was observed in ZR-75 breast cancer cells with treatment (Stoner et al., 2000; Stoner et al., 2004). In the present studies an early induction of VEGF mRNA in stromal cells of the uterus was observed as previously reported in the rat uterus (Hyder et al., 2000). As shown in Figure 3.2, within 1 hour after treatment with E2 there was an increase in staining of VEGF mRNA in the stromal compartment (Figure 3.2, A-B vs. C-D). The increased staining was also seen 3 hours after treatment with E2 (Figure 3.2, E-F vs. G-H). VEGF mRNA staining decreased after 6 hours and returned to control levels 12 hours after treatment with hormone (Figure 3.2, I-J vs. K-L and M-N vs. O-P).

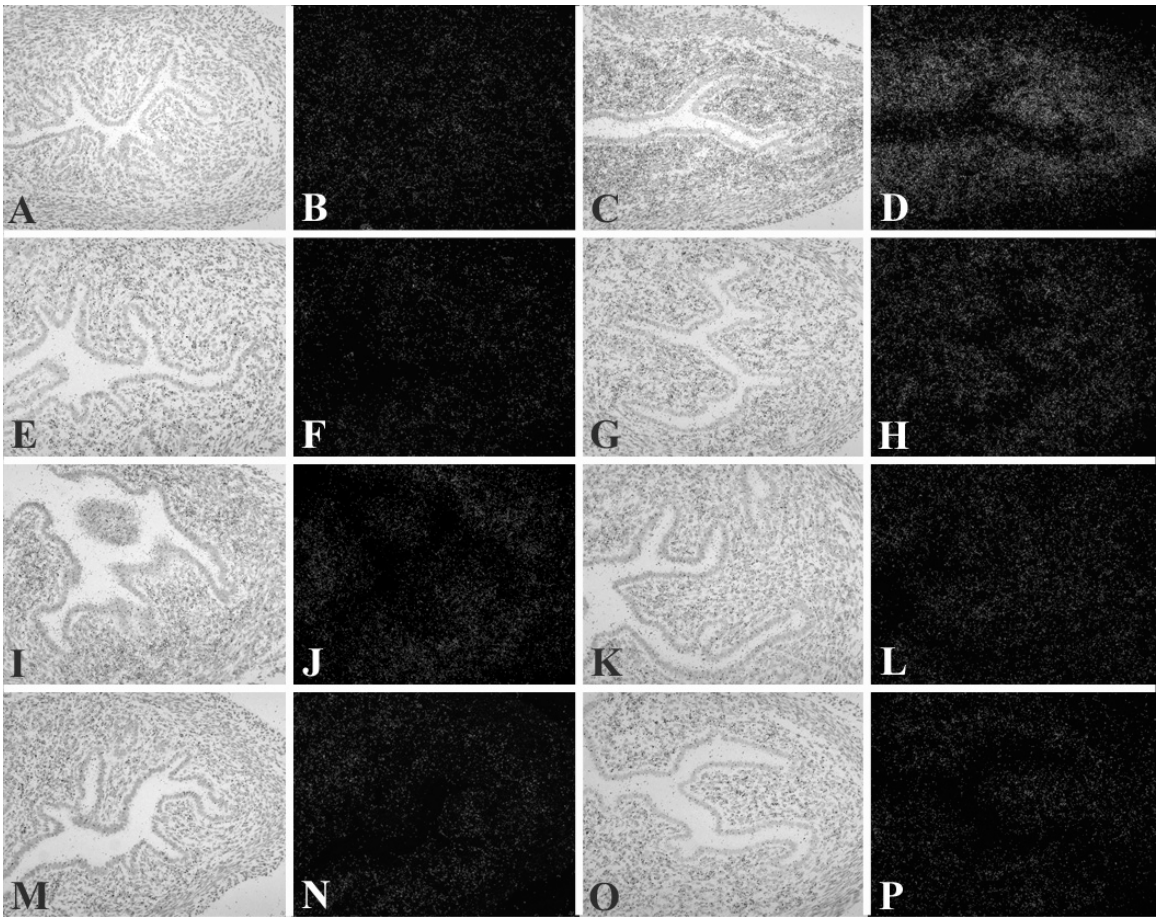


Figure 3.2: Time-dependent expression of VEGF mRNA after treatment with E2 in B6C3F1 mice. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle (A-B, E-F, I-J, M-N) or 200 ng of E2 in corn oil (C-D, G-H, K-L, O-P) (n=4 per treatment). VEGF mRNA levels were determined by *in situ* hybridization of uterine sections taken 1 hour (A-D), 3 hours (E-H), 6 hours (I-L), and 12 hours (M-P) after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for VEGF mRNA. Stained sections were analyzed by brightfield (A, C, E, G, I, K, M, O) and darkfield (B, D, F, H, J, L, N, P) microscopy. A representative section of each treatment and time point is shown.

DNA polymerase α, which is necessary for DNA synthesis in S phase of the cell cycle, is also induced by E2 in vitro in breast cancer cell lines (Samudio et al., 2001); however, the involvement of DNA polymerase α in E2-induced proliferation in the mouse uterus has not previously been characterized. An initial time course study showed that E2 induced expression of the 180 kd catalytic subunit of DNA polymerase α in the mouse uterus and the temporal pattern of expression was similar to that of cyclin D1. One and 3 hours after treatment with E2 there was a consistent staining of the DNA polymerase α 180 kd catalytic subunit mRNA in the stromal cells compared to control sections (Figure 3.3, A-B vs. C-D and E-F vs. G-H). Six and 12 hours after hormonal treatment there was an increase in staining of the DNA polymerase α 180 kd catalytic subunit mRNA in the uterine luminal epithelial cells compared to controls and the general staining of the stroma was also observed (Figure 3.3, I-J vs. K-L and M-N vs O-P). The time course of E2-induced expression of the catalytic subunit of DNA polymerase α was consistent with the timing of E2-induced cell cycle progression shown in previous studies in which [^{3}H]thymidine incorporation (and DNA synthesis) was initiated approximately 6 hours after treatment with E2 in the immature rat or mouse uterus and this response peaked around 16 hours after hormone treatment.

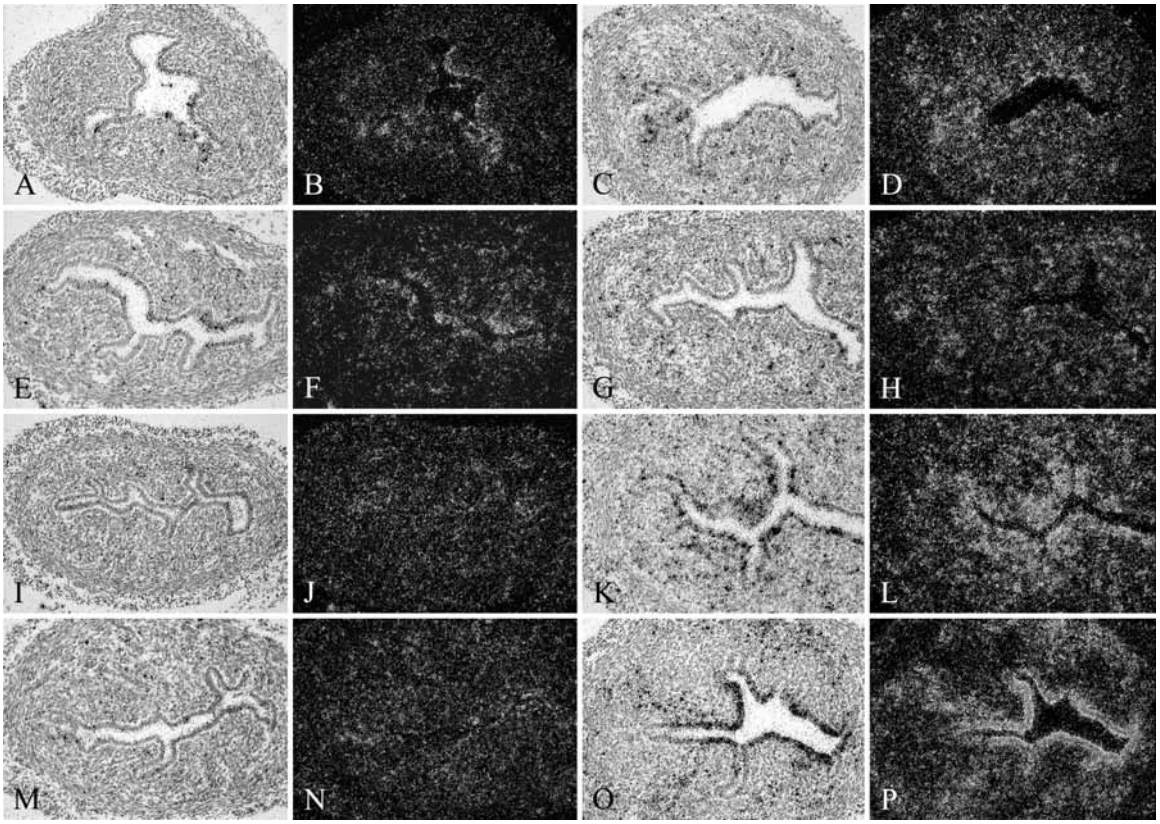


Figure 3.3: Time-dependent expression of DNA polymerase α catalytic subunit mRNA after treatment with E2 in B6C3F1 mice. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle (A-B, E-F, I-J, M-N) or 200 ng of E2 in corn oil (C-D, G-H, K-L, O-P) (n=4 per treatment). DNA polymerase α catalytic subunit mRNA levels were determined by *in situ* hybridization of uterine sections taken 1 hour (A-D), 3 hours (E-H), 6 hours (I-L), and 12 hours (M-P) after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for DNA polymerase α catalytic subunit mRNA. Stained sections were analyzed by brigthfield (A, C, E, G, I, K, M, O) and darkfield (B, D, F, H, J, L, N, P) microscopy. A representative section of each treatment and time point is shown.

3.1.2 Requirement of ERα for E2-induced gene expression in the mouse uterus

E2-induced responses are mediated through steroid hormone receptors and to date, two estrogen receptors have been identified, estrogen receptor α (ERα) and estrogen receptor β (ERβ). Both ERα and ERβ are expressed in the uterus; however, the localization and levels of expression within uterine cell types are variable. The generation of mice deficient in ERα (ERKO), ERβ (βERKO) and both receptors (αβERKO) have aided in deciphering the role of each receptor in the uterus. αERKO mice have a hypoplastic uterus that is not responsive to E2 (Lubahn et al., 1993). In contrast, βERKO mice have a normal uterus that undergoes cyclic changes with ovarian hormones; however, the immature uterus expresses increased proliferative markers and exaggerated responses to E2 (Krege et al., 1998a). These data indicate that ERα is necessary for E2-induced responses in the uterus, whereas ERβ may play an inhibitory role. Therefore, we used the ERKO mice to determine whether or not ERα is necessary for E2-induced gene expression of cyclin D1, VEGF and DNA polymerase α in the uterus characterized in Figures 3.1-3.3.

Twenty-five day old ERKO$^{+/+}$ (ERα wild-type) and ERKO$^{-/-}$ (ERα knockout) were treated with E2 or corn oil and the uterus was removed at 1, 6 and 12 hours later for analysis. Overall, a loss of E2-induced gene expression was seen in the ERKO$^{-/-}$ mice (Figures 3.4-3.8): The staining of cyclin D1 mRNA in the uterus differed between the ERKO$^{+/+}$ mice and ERKO$^{-/-}$ mice. As seen in

the control sections from the 6 hour timepoint, cyclin D1 probes exhibit a higher level of staining in the epithelium, stroma, and myometrium of the uterus in ERKO$^{+/+}$ mice compared to ERKO$^{-/-}$ mice (Figure 3.4, A-B v. E-F); although no difference is seen between the controls from the 12 hour timepoint (Figure 3.5, A-B v. E-F). Six hours and 12 hours after treatment with E2, an increase in staining of the luminal epithelium of the uterus is observed compared to control treatment in the ERKO$^{+/+}$ mice, and this was comparable to that seen in the B6C3F1 mice after treatment with E2 (Figures 3.4 and 3.5, A-B v. C-D). However, the staining of the uterus from the E2 treated ERKO$^{-/-}$ mice does not differ from that of the ERKO$^{-/-}$ control mice after 6 or 12 hours of treatment (Figures 3.4 and 3.5, E-F v. G-H) showing that ERα is necessary for E2-induced cyclin D1 gene expression in the uterus. A similar pattern of staining is seen for the 180 kd catalytic subunit of DNA polymerase α mRNA. An increase in staining for the 180 kd catalytic subunit of DNA polymerase α mRNA is observed in all three uterine layers of control ERKO$^{+/+}$ mice compared to control ERKO$^{-/-}$ mice from the 6 hour timepoint (Figure 3.6, A-B v. E-F);

although no difference is seen between the controls from the 12 hour timepoint (Figure 3.7, A-B v. E-F). Furthermore, six and 12 hours after treatment with E2 the ERKO$^{+/+}$ mice exhibited an increased staining of the DNA polymerase α catalytic subunit mRNA in the luminal epithelium compared to controls (Figures 3.6 and 3.7, A-B v. C-D). However, increased staining was not observed in the ERKO$^{-/-}$ mice after treatment with E2 (Figure 3.6 and 3.7, E-F v. G-H). Uterine sections from ERKO$^{+/+}$ and ERKO$^{-/-}$ mice stained for VEGF mRNA also demonstrated that ERα is necessary for E2-induced VEGF expression. One hour after treatment with E2 the ERKO$^{+/+}$ mice exhibited an increased staining for VEGF mRNA in the stromal cells (Figure 3.8, A-B v. C-D), however no difference in staining was observed in the ERKO$^{-/-}$ mice treated with E2 (Figure 3.8, E-F v. G-H).

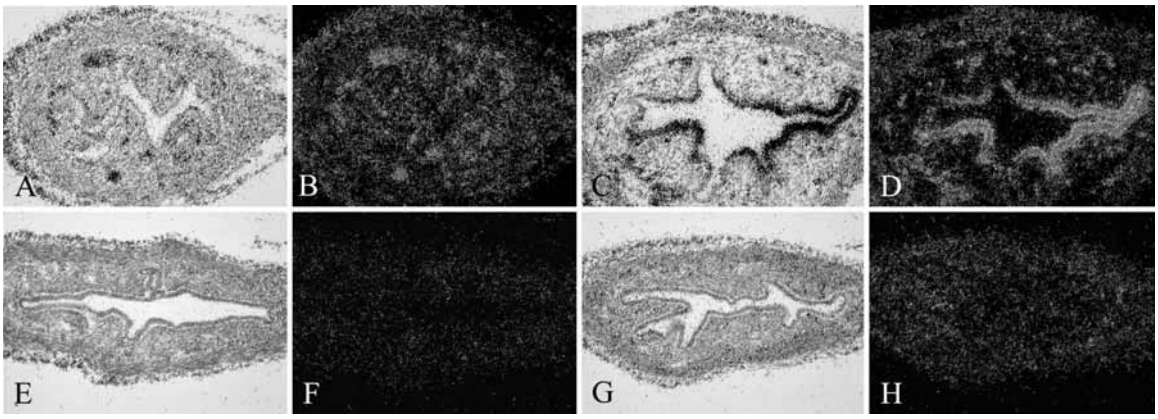


Figure 3.4: Expression of cyclin D1 mRNA six hours after treatment with E2 in ERKO$^{+/+}$ and ERKO$^{-/-}$ mice. Twenty-five day old female ERKO$^{+/+}$ (A-D) or ERKO$^{-/-}$ (E-H) mice were treated with the corn oil vehicle (A-B, E-F) or 200 ng of E2 in corn oil (C-D, G-H) (n is 3-5 per treatment). Cyclin D1 mRNA levels were determined by *in situ* hybridization of uterine sections taken 6 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for cyclin D1 mRNA. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment and genotype is shown.

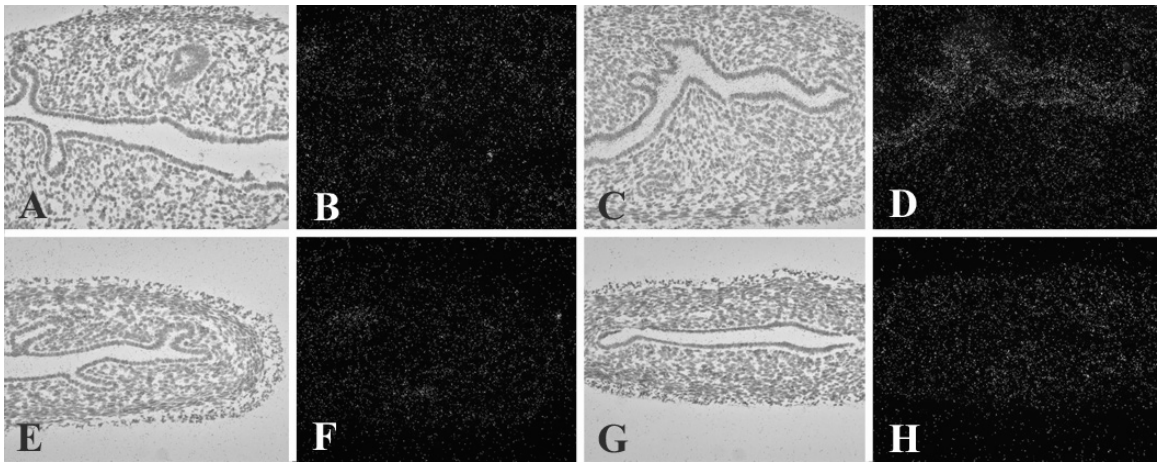


Figure 3.5: Expression of cyclin D1 mRNA twelve hours after treatment with E2 in ERKO$^{+/+}$ and ERKO$^{-/-}$ mice. Twenty-five day old female ERKO$^{+/+}$ (A-D) or ERKO$^{-/-}$ (E-H) mice were treated with the corn oil vehicle (A-B, E-F) or 200 ng of E2 in corn oil (C-D, G-H) (n is 3 per treatment). Cyclin D1 mRNA levels were determined by *in situ* hybridization of uterine sections taken 12 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for cyclin D1 mRNA. Stained sections were analyzed by brigthfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment and genotype is shown.

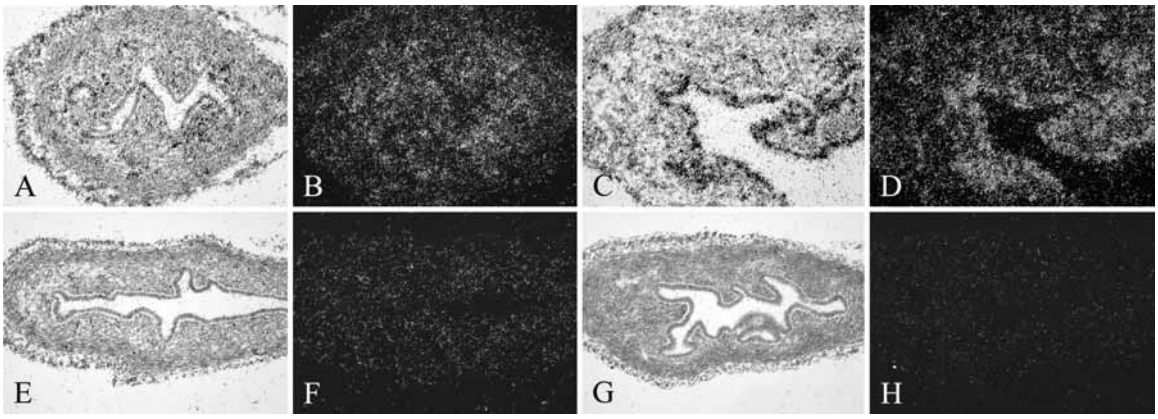


Figure 3.6: Expression of DNA polymerase α catalytic subunit six hours after treatment with E2 in ERKO$^{+/+}$ and ERKO$^{-/-}$ mice. Twenty-five day old female ERKO$^{+/+}$ (A-D) or ERKO$^{-/-}$ (E-H) mice were treated with the corn oil vehicle (A-B, E-F) or 200 ng of E2 in corn oil (C-D, G-H) (n is 3-5 per treatment). DNA polymerase α catalytic subunit mRNA levels were determined by *in situ* hybridization of uterine sections taken 6 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for DNA polymerase α catalytic subunit mRNA. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment and genotype is shown.

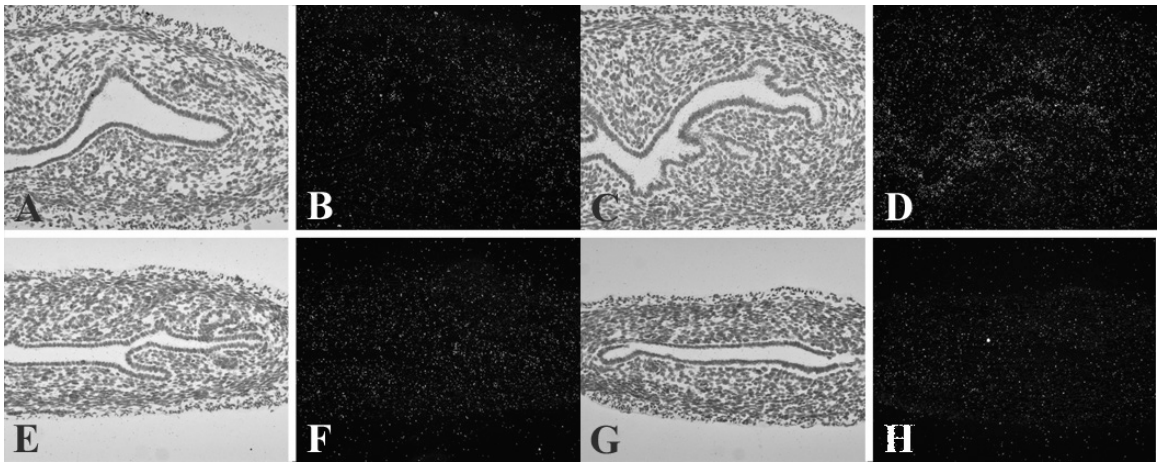


Figure 3.7: Expression of DNA polymerase α catalytic subunit twelve hours after treatment with E2 in ERKO$^{+/+}$ and ERKO$^{-/-}$ mice. Twenty-five day old female ERKO$^{+/+}$ (A-D) or ERKO$^{-/-}$ (E-H) mice were treated with the corn oil vehicle (A-B, E-F) or 200 ng of E2 in corn oil (C-D, G-H) (n is 3 per treatment). DNA polymerase α catalytic subunit mRNA levels were determined by *in situ* hybridization of uterine sections taken 12 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for DNA polymerase α catalytic subunit mRNA. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment and genotype is shown.

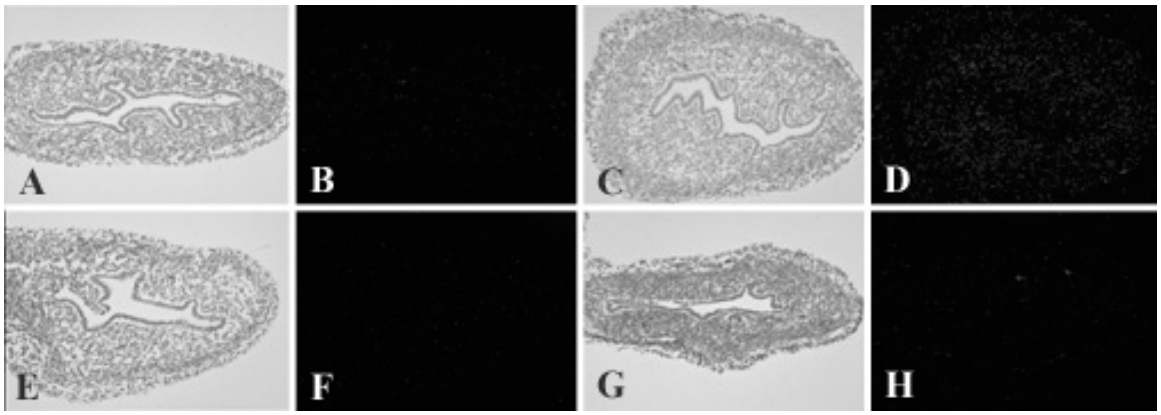


Figure 3.8: Expression of VEGF one hour after treatment with E2 in ERKO$^{+/+}$ and ERKO$^{-/-}$ mice. Twenty-five day old female ERKO$^{+/+}$ (A-D) or ERKO$^{-/-}$ (E-H) mice were treated with the corn oil vehicle (A-B, E-F) or 200 ng of E2 in corn oil (C-D, G-H) (n is 3-4 per treatment). VEGF mRNA levels were determined by *in situ* hybridization of uterine sections taken 1 hour after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for VEGF mRNA. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment and genotype is shown.

3.1.3 Inhibition of E2-induced gene expression in the mouse uterus by AhR agonist TCDD

Crosstalk between AhR and ER has been characterized in vitro in breast cancer cell lines and in vivo in various rodent models. Research in several laboratories has demonstrated that TCDD inhibits estrogen-induced responses in the rodent uterus, carcinogen-induced rodent mammary tumors and human breast/endometrial cancer cell lines. Activation of the AhR through agonists such as TCDD has been shown to downregulate induction of several E2-responsive genes including those required for cell proliferation. Therefore, we further investigated inhibitory AhR-ERα crosstalk in the mouse uterus to determine whether TCDD would inihibit the E2-induced gene expression of cyclin D1, VEGF or DNA polymerase α as characterized above.

Twenty-five day old B6C3F1 mice were treated with corn oil, E2, TCDD (1 μg) or E2+TCDD and 1, 3, 6, and 12 hours later the uterus was removed for

analysis. Previous studies have shown that treatment with TCDD inhibits E2-induced increase in uterine wet weights. Therefore, uterine wet weights were used as a positive control for the activity of TCDD as an inhibitor of E2-induced responses. As shown in Figure 3.9, uterine wet weights were similar between treatment groups after compound administration for 1 hour. Three, 6 and 12 hours after treatment with E2 uterine weights were increased compared to control mice and wet weights of uteri from animals treated with E2+TCDD were decreased compared to mice treated with E2 alone at 6 and 12 hours. Therefore, our TCDD dose and treatment regimen showed that within the 12 hour treatment period TCDD inhibited the E2-induced increase in uterine wet weights. These data are comparable to previous studies where longer treatment periods of E2 and TCDD were used to study inhibitory AhR-ERα interactions in the uterus (Ramamoorthy et al., 1999).

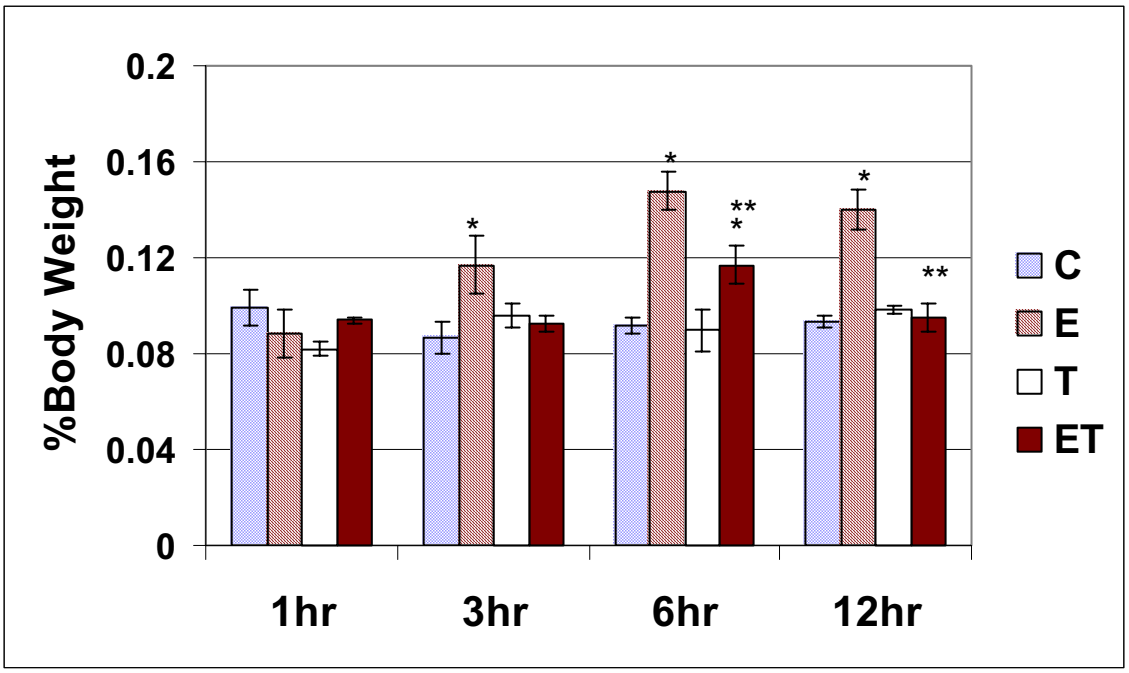


Figure 3.9: Uterine weights after treatment with E2 and TCDD in B6C3F1 mice.

Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle, 200 ng of E2, 1 μg of TCDD, or E2 + TCDD (n=4 per treatment). Mice were weighed and uteri were removed and weighed 1, 3, 6, and 12 hours after treatment. Uterine weights are presented as mean ± SE and are a percentage of total body weight. Significant ($p<0.05$) induction by E2 (*) and inhibition in the cotreatment (ET) (**) groups is indicated.

The ability of TCDD to inihibit E2-induced cyclin D1, VEGF and DNA polymerase α expression was also investigated. Six hours after treatment, cyclin D1 probes stained intense areas of the stroma, localizing to the glandular epithelium in the sections from control mice and TCDD treated mice (Figure 3.10, A-B and C-D) and staining of cyclin D1 mRNA of the luminal epithelium is increased in uterine sections from E2 treated and E2 + TCDD treated mice compared to the sections from controls (Figure 3.10, A-B v. E-F or G-H). However, the intensity of staining in the luminal epithelial cells is slightly higher in the E2 sections compared to sections from animals treated with E2 + TCDD (Figure 3.10, E-F v. G-H). Twelve hours after treatment, uteri from control and TCDD treated mice still showed intense areas of cyclin D1 mRNA staining that localized to glandular epithelium (Figure 3.11, A-B and C-D); the luminal epithelial staining of cyclin D1 mRNA was increased in E2 and E2+TCDD treated mice compared to control and TCDD treated mice (Figure 3.11, A-B and C-D v. E-F or G-H). However, the differences in staining intensities of cyclin D1 mRNA in the luminal epithelium between the E2 and E2+TCDD treated mice is greater after cotreatment for 12 hours compared to the 6 hour treatment group (Figure 3.11, E-F v. G-H compared to Figure 3.10, E-F v. G-H). These data demonstrate that TCDD decreased induction of cyclin D1 gene expression by E2 in the luminal epithelium of the mouse uterus and the inhibitory response was highest 12 hours after cotreatment with E2 + TCDD.

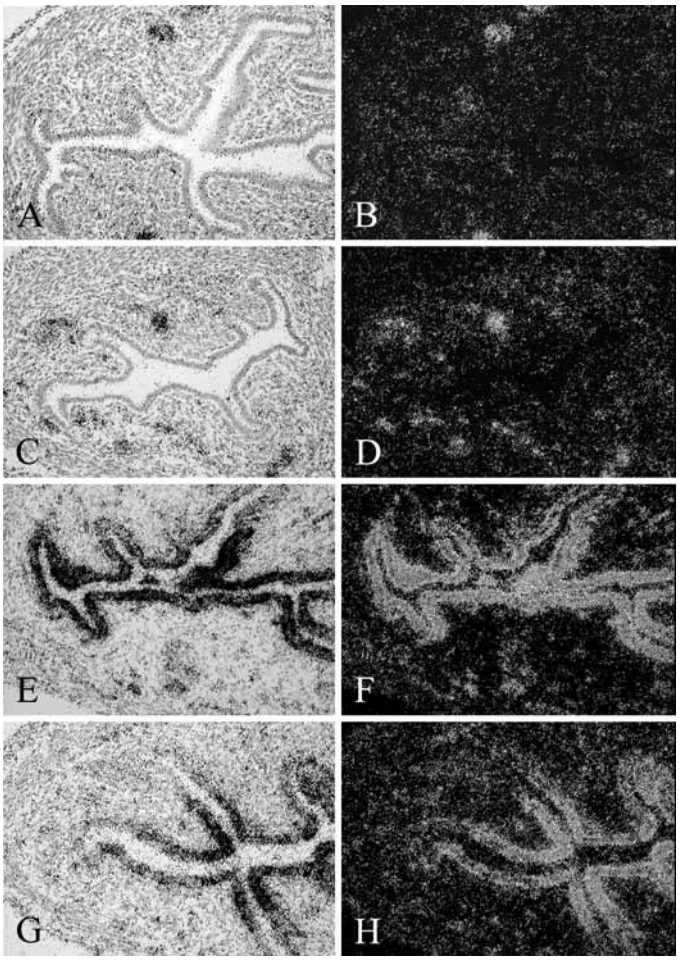


Figure 3.10: Expression of cyclin D1 mRNA six hours after treatment with E2 and TCDD in B6C3F1 mice. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle, (A-B) 1 μg of TCDD (C-D), 200 ng of E2 (E-F), or E2 + TCDD (G-H) (n=4 per treatment). Cyclin D1 mRNA levels were determined by *in situ* hybridization of uterine sections taken 6 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for cyclin D1 mRNA. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment is shown.

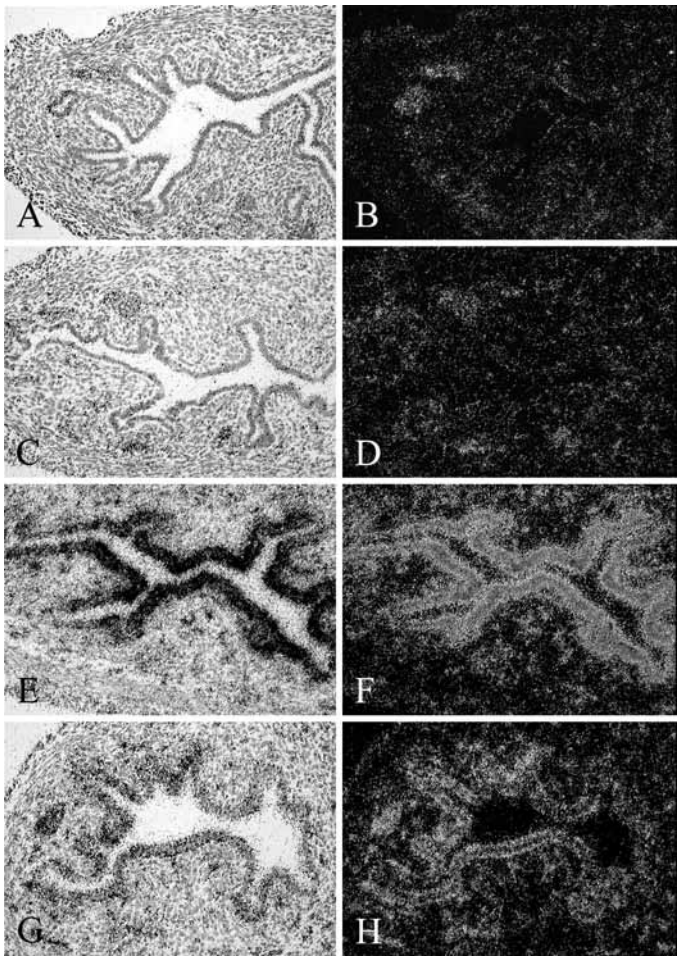


Figure 3.11: Expression of cyclin D1 mRNA twelve hours after treatment with E2 and TCDD in B6C3F1 mice. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle, (A-B) 1 μg of TCDD (C-D), 200 ng of E2 (E-F), or E2 + TCDD (G-H) (n=4 per treatment). Cyclin D1 mRNA levels were determined by *in situ* hybridization of uterine sections taken 12 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for cyclin D1 mRNA. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment is shown.

Uterine sections from control and TCDD treated mice showed similar low levels of staining of the 180 kd catalytic subunit of DNA polymerase α mRNA throughout the stromal cells 6 and 12 hours after treatment (Figures 3.12 and 3.13, A-B and C-D). Uterine sections from mice treated with E2 alone and E2+TCDD showed an increase in staining of DNA polymerase α catalytic subunit mRNA in the luminal epithelial cells compared to controls (Figures 3.12 and 3.13, A-B v. E-F or G-H). However, the sections from E2+TCCD treated mice had a much lower staining intensity the luminal epithelium when compared to the staining intensity in sections from the E2-treated mice (Figures 3.12 and 3.13, E-F v. G-H). An increase in staining of DNA polymerase α catalytic subunit mRNA in the stroma is also seen in the sections from E2-treated mice when compared to control and E2+TCDD mice (Figures 3.12 and 3.13, E-F v. A-B or G-H). Therefore, as with cyclin D1, TCDD inhibited E2-induced expression of the 180 kd catalytic subunit of DNA polymerase α gene in the mouse uterus. However, six hour after cotreament TCDD inhibits E2-induced expression of the DNA polymerase α gene to a greater extent than observed for inhibition of E2-induced cyclin D1 expression at the same time point.

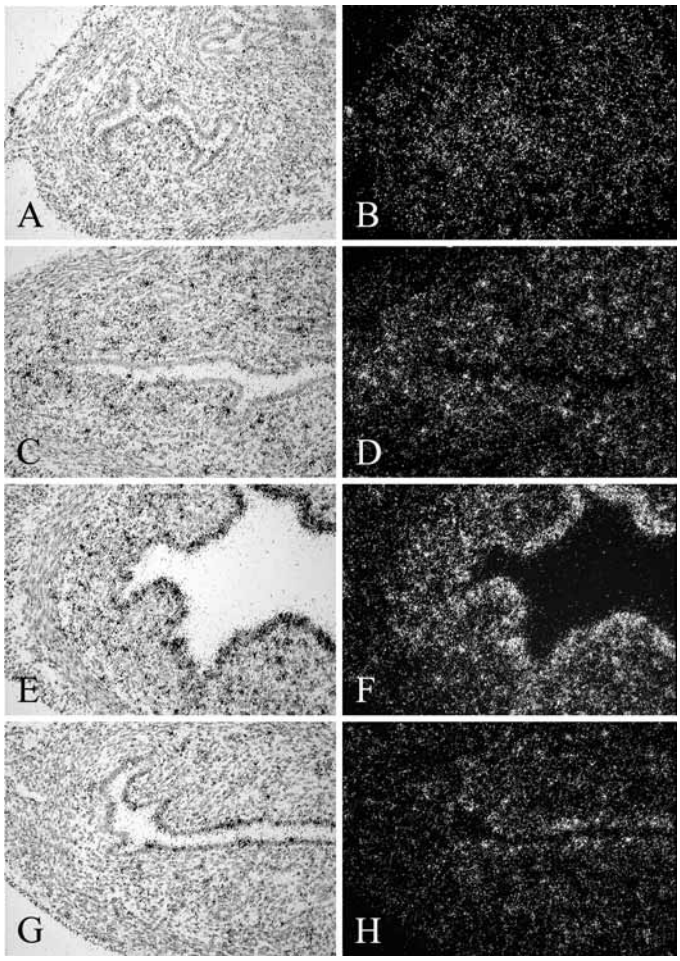


Figure 3.12: Expression of DNA polymerase α catalytic subunit mRNA six hours after treatment with E2 and TCDD in B6C3F1 mice. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle (A-B), 1 μg of TCDD (C-D), 200 ng of E2 (E-F), or E2 + TCDD (G-H) (n=4 per treatment). DNA polymerase α catalytic subunit mRNA levels were determined by *in situ* hybridization of uterine sections taken 6 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment is shown.

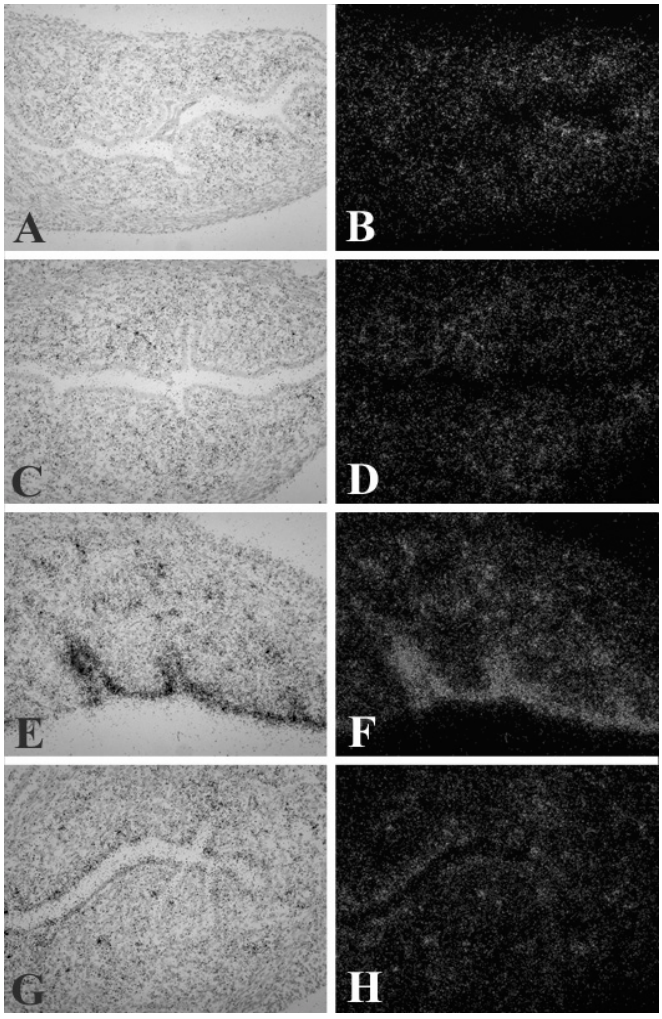


Figure 3.13: Expression of DNA polymerase α catalytic subunit mRNA twelve hours after treatment with E2 and TCDD in B6C3F1 mice. Twenty-five day old female B6C3F1 mice were treated with corn oil (A-B), 1 μg of TCDD (C-D), 200 ng of E2 (E-F), or E2 + TCDD (G-H) (n=4 per treatment). mRNA levels were determined by *in situ* hybridization utilizing [^{35}S]-labeled cRNA sense or antisense probes for DNA polymerase α catalytic subunit. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment is shown.

The timing and localization of E2-induced VEGF gene expression is different from that observed for cyclin D1 and DNA polymerase α and the ability of TCDD to inhibit the E2-induced response in our model is also different. One and 3 hours after treatment both the control and TCDD treated mice show low levels of staining of VEGF mRNA throughout the stroma (Figures 3.14 and 3.15, A-B and C-D). Treatment with E2 induced an increase in staining of VEGF mRNA throughout the stroma when compared to the control and TCDD treated mice and, as observed for the B6C3F1, the increase was higher 1 hour after treatment than after 3 hours (Figures 3.14 and 3.15, A-B and C-D v. E-F). E2+TCDD treatment showed a similar increase in staining of VEGF mRNA in the stroma compared to the control and TCDD treated mice (Figures 3.14 and 3.15, A-B and C-D v. G-H) and no difference in staining intensity was seen between the E2 and E2+TCDD treatment groups after 1 and 3 hours of treatment (Figures 3.14 and 3.15, E-F v G-H). Thus TCDD did not inhibit E2-induced VEGF gene expression one hour after cotreatment with both compounds.

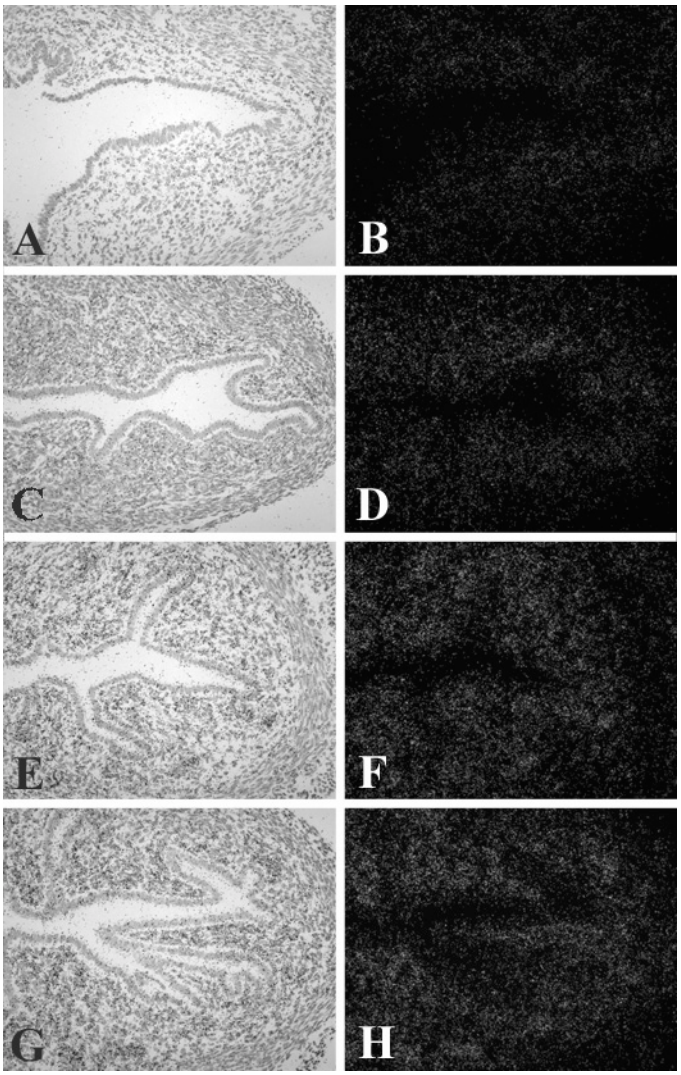


Figure 3.14: Expression of VEGF mRNA one hour after treatment with E2 and TCDD in B6C3F1 mice. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle (A-B), 1 μg of TCDD (C-D), 200 ng of E2 (E-F), or E2 + TCDD (G-H) (n=4 per treatment). VEGF mRNA levels were determined by *in situ* hybridization utilizing [^{35}S]-labeled cRNA sense or antisense probes. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment is shown.

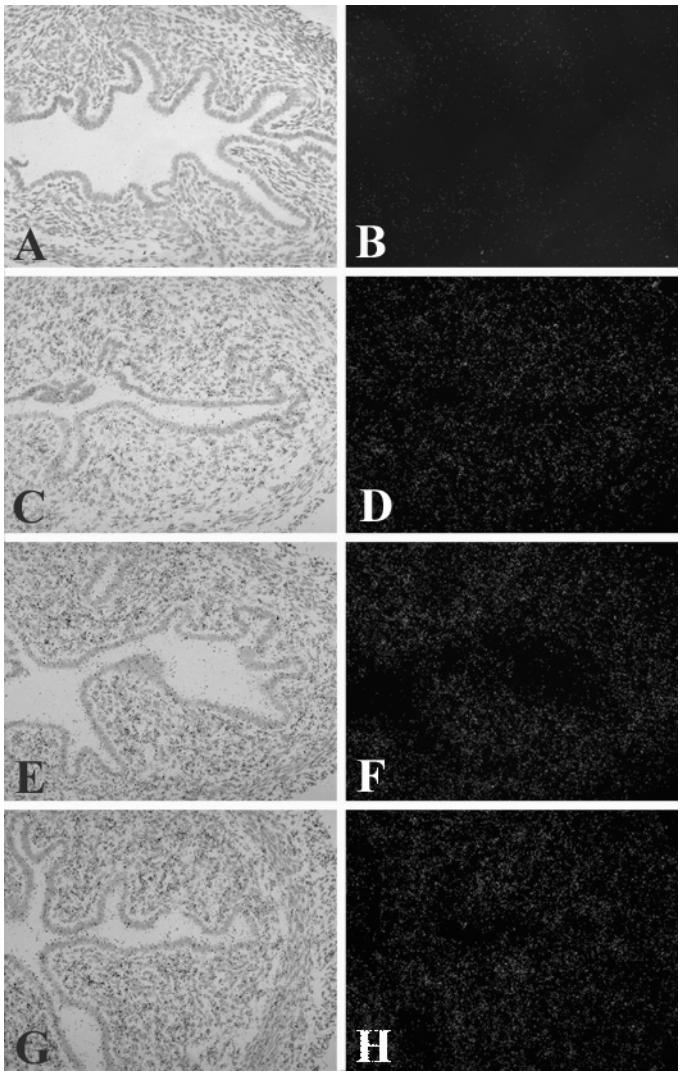


Figure 3.15: Expression of VEGF mRNA three hours after treatment with E2 and TCDD in B6C3F1 mice. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle (A-B), 1 μg of TCDD (C-D), 200 ng of E2 (E-F), or E2 + TCDD (G-H) (n=4 per treatment). VEGF mRNA levels were determined by *in situ* hybridization utilizing [^{35}S]-labeled cRNA sense or antisense probes. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment is shown.

3.1.4 Requirement of AhR for inhibition of E2-induced gene expression by AhR agonist

The AhR was initially identified as a receptor that bound the environmental toxicant 2,3,7,8-tetrachlorodobenzo-p-dioxin (TCDD) with high affinity and studies with AhR knockout mice have confirmed a role for this protein in mediating TCDD-induced toxicity (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Mimura et al., 1997). AhRKO mice have also been used to determine the role of AhR in the ability of TCDD to inhibit E2-induced responses (Buchanan et al., 2000). In this study, we have used AhRKO mice to investigate the role of the AhR in the mouse uterus in mediating the antiestrogenic effects of TCDD associated with inhibition of E2-induced cyclin D1 and DNA polymerase α gene expression. Twenty-five day old AhRKO females were treated with corn oil, E2, TCDD, or E2+TCDD and uteri were removed for analysis 6 and 12 hours after treatment.

In AhRKO mice, TCDD did not inhibit E2-induced cyclin D1 and DNA polymerase α gene expression. As indicated in the control sections from the 6 hour timepoint, probes for cyclin D1 mRNA stain intense areas of the stroma in uterine sections from AhRKO mice, generally localizing to the glandular epithelium (Figure 3.16, A-B). Six hours after treatment, cyclin D1 probes intensely stain the luminal epithelium of uterine sections from E2 and E2 + TCDD treated mice compared to sections from control mice (Figure 3.16, A-B v. C-D, E-F) and there was no difference in staining intensities of the luminal

epithelium sections from E2 and E2+TCDD treatment groups (Figure 3.16, C-D v. E-F). After 12 hours of treatment a similar staining pattern for cyclin D1 mRNA in seen. E2 and E2+TCDD increased staining of the luminal epithelial cells compared to controls (Figure 3.17, A-B v. C-D, E-F) and there is no difference in staining intensity between the two treatments (Figure 3.17, C-D v E-F). Sections from the uteri of control and TCDD treated mice at 6 hours of treatment stained for the 180 kd catalytic subunit of DNA polymerase α mRNA show a low level of staining throughout the stroma (Figure 3.18, A-B and C-D). Sections from E2 and E2+TCDD treated mice six and 12 hours after treatment stained for the DNA polymerase α catalytic subunit mRNA exhibited a comparable increase in staining of the stroma as well as an increase in staining along the luminal epithelial cells compared to the controls (Figures 3.18 and 3.19, A-B v E-F or G-H). However, as observed for staining of cyclin D1 mRNA, differences in staining intensities of the DNA polymerase α catalytic subunit mRNA in the stroma or luminal epithelium were not observed in sections from E2 and E2+ TCDD treated mice (Figures 3.18 and 3.19, E-F v. G-H). These results indicate that the AhR is necessary for inhibition of E2-induced cyclin D1 and 180 kd catalytic subunit of DNA polymerase α gene expression by TCDD in the mouse uterus.

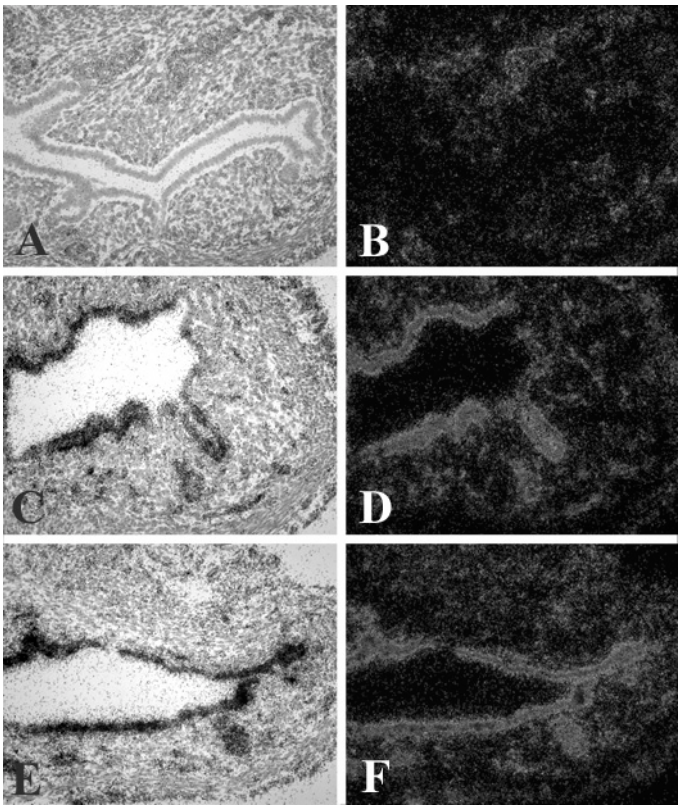


Figure 3.16: Expression of cyclin D1 mRNA six hours after treatment with E2 and TCDD in AhRKO mice. Twenty-five day old female AhRKO mice were treated with the corn oil vehicle (A-B), 200 ng of E2 (C-D), or E2 + TCDD (E-F) (n is 5-6 per treatment). Cyclin D1 mRNA levels were determined by *in situ* hybridization of uterine sections taken 6 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for cyclin D1 mRNA. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment is shown.

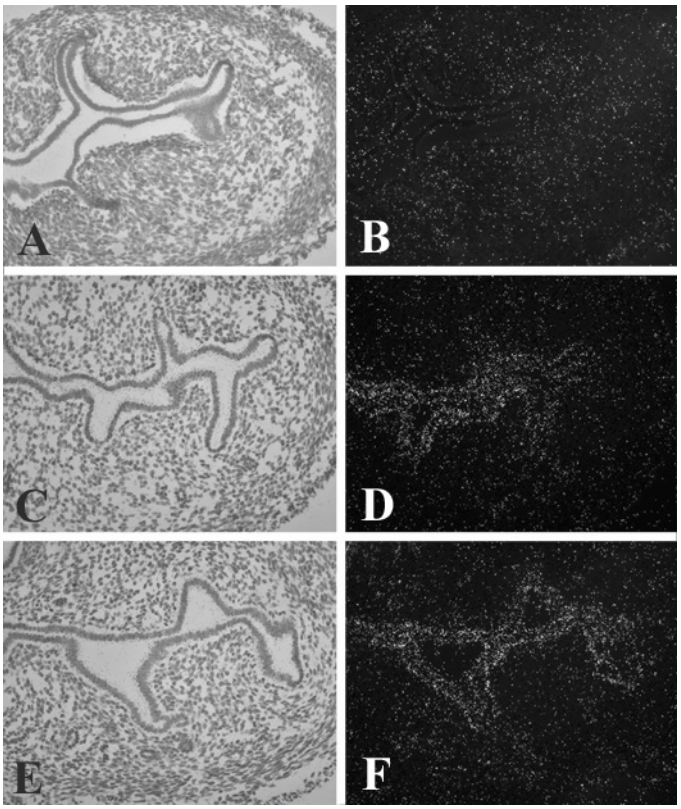


Figure 3.17: Expression of cyclin D1 mRNA twelve hours after treatment with E2 and TCDD in AhRKO mice. Twenty-five day old female AhRKO mice were treated with the corn oil vehicle (A-B), 200 ng of E2 (C-D), or E2 + TCDD (E-F) (n is 2-3 per treatment). Cyclin D1 mRNA levels were determined by *in situ* hybridization of uterine sections taken 12 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for cyclin D1 mRNA. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment is shown.

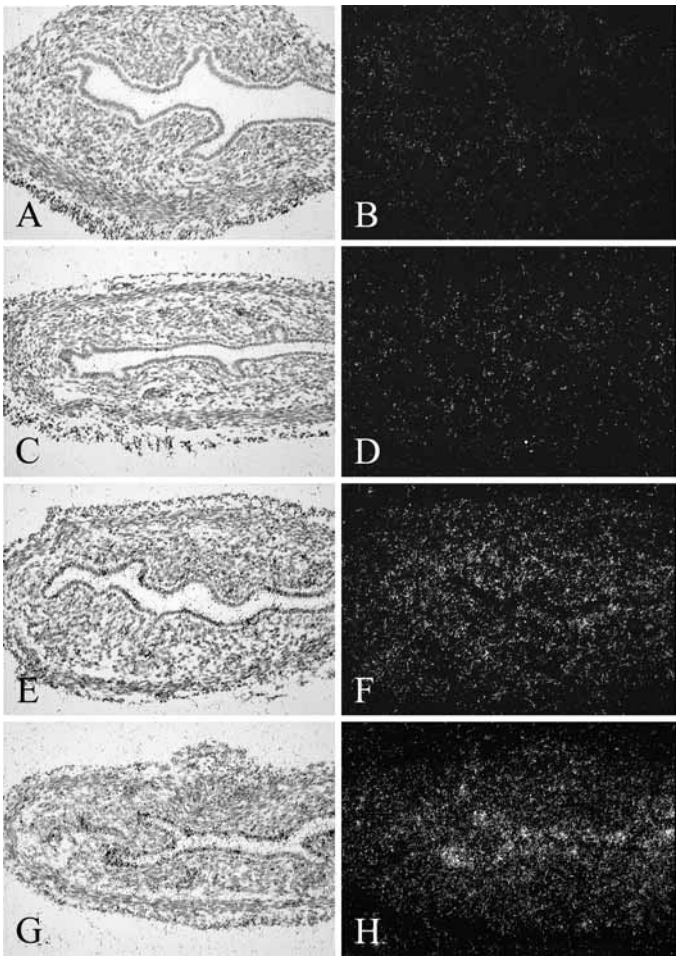


Figure 3.18: Expression of DNA polymerase α catalytic subunit mRNA six hours after treatment with E2 and TCDD in AhRKO mice. Twenty-five day old female AhRKO mice were treated with the corn oil vehicle (A-B), 1 μg of TCDD (C-D), 200 ng of E2 (E-F), or E2 + TCDD (G-H) (n is 5-6 per treatment). DNA polymerase α catalytic subunit mRNA levels were determined by *in situ* hybridization of uterine sections taken 6 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment is shown.

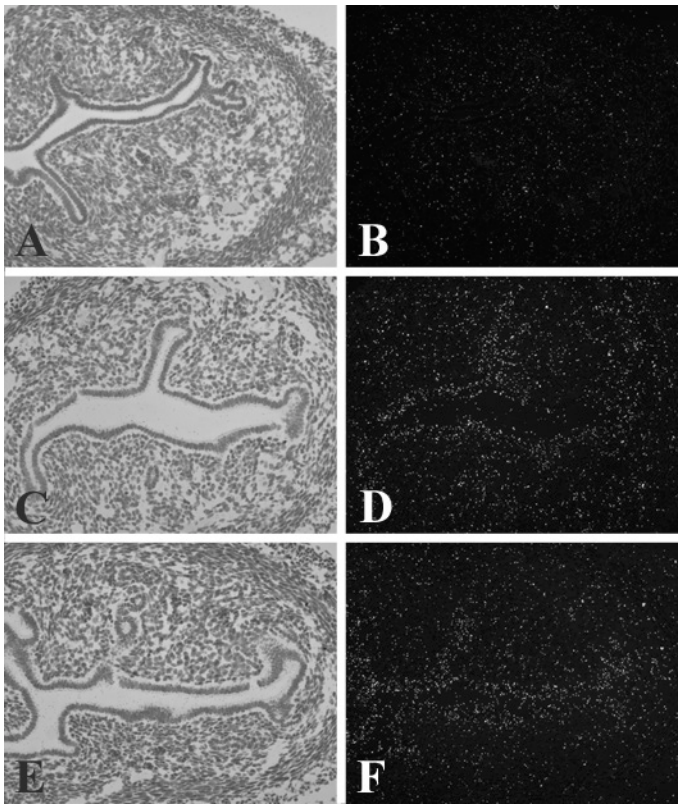


Figure 3.19: Expression of DNA polymerase α catalytic subunit mRNA twelve hours after treatment with E2 and TCDD in AhRKO mice. Twenty-five day old female AhRKO mice were treated with the corn oil vehicle (A-B), 200 ng of E2 (C-D), or E2 + TCDD (G-H) (n is 2-3 per treatment). DNA polymerase α catalytic subunit mRNA levels were determined by *in situ* hybridization of uterine sections taken 12 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes for DNA polymerase α catalytic subunit. Stained sections were analyzed by brightfield (A, C, E, G) and darkfield (B, D, F, H) microscopy. A representative section of each treatment is shown.

3.1.5 Stromal-epithelial interactions necessary for E2-induced gene expression and inhibition of E2-induced gene expression by AhR agonist

There is evidence that E2 can bind to ER in stromal or epithelial cells of the uterus and induce expression of paracrine factors that then enhance proliferation in neighboring cells or other cell types. Furthermore, Buchanan and coworkers have shown that in uterine tissue from adult mice AhR expression is necessary in the stromal cells and not the epithelial cells in order for AhR agonists to inhibit E2-induced responses of the epithelium (Buchanan et al., 2000). Therefore, in order to differentiate between direct induction of gene expression by E2 from indirect induction of gene expression by a paracrine factor we have used the protein synthesis inhibitor cycloheximide. In addition this inhibitor was also used to distinguish between direct inhibition of E2-induced responses by AhR from indirect inhibition by paracrine factors.

Treatment with TCDD induces high levels of protein synthesis of multiple drug metabolizing enzymes in the liver, including cytochrome p450 1A1 (cyp1A1). Therefore, the induction of cyp1A1 protein in the liver was used as a control for protein synthesis inhibition by cycloheximide and was determined by Western blot analysis of protein isolated from liver microsomes. As seen in

Figure 3.20, mice that were not pretreated with cycloheximide exhibited high levels of cyp1A1 protein expression in the liver 12 hours after treatment with TCDD or E2+TCDD. In contrast mice pretreated with cycloheximide for 1 hour exhibited little or no induction of cyp1A1 protein expression 12 hours after treatment with TCDD or E2+TCDD, except for mouse #2 in the E2+TCDD treatment group (Figure 3.20). Uterine wet weights were also measured as an indication of general uterine responses to treatments. Without cycloheximide pretreatment, uterine weights from mice treated with E2 for 12 hours were increased compared to control mice and mice treated with TCDD or E2+TCDD (Figure 3.21). In mice pretreated with cycloheximide uterine weights were increased in mice treated with E2 and E2+TCDD but not changed in mice treated with TCDD alone suggesting that the protein synthesis inhibitor blocked the antiestrogenic effects of TCDD (Figure 3.21).

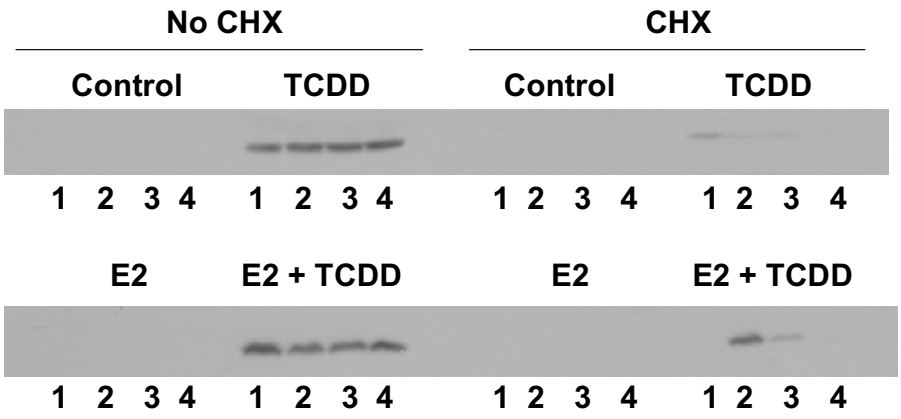


Figure 3.20: Cyp1A1 protein levels in liver microsomes 12 hours after treatment with E2 and TCDD with and without cycloheximide. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle, 1 μg of TCDD, 200 ng of E2 or E2 + TCDD with or without 1 hour pretreatment of 0.75 mg of cycloheximide (n is 4 per treatment). Cyp1A1 protein levels were determined by Western blot analysis of protein isolated from liver microsomes.

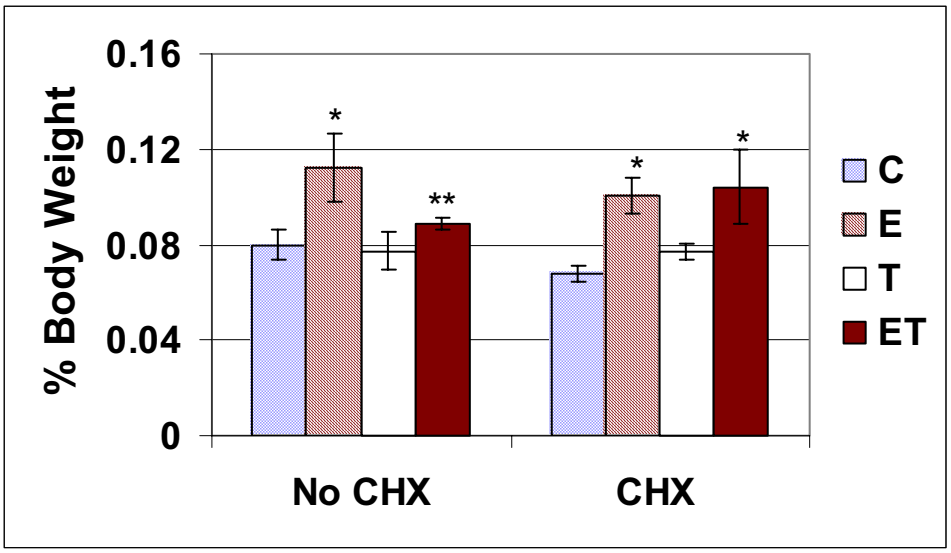


Figure 3.21: Uterine weights after treatment with E2 and TCDD with and without cycloheximide. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle, 1 μg of TCDD, 200 ng of E2 or E2 + TCDD with or without 1 hour pretreatment of 0.75 mg of cycloheximide (n is 4 per treatment). Mice were weighed and uteri were removed and weighed 12 hours after treatment. Uterine weights are presented as mean ± SE and are a percentage of total body weight. Induction by E2 (*$p<0.05$) and inhibition in the cotreatment (ET) (**$p<0.07$) groups is indicated.

As shown in Figures 3.22 and 3.23, pretreatment with cycloheximide did not affect E2 induction of cyclin D1 or 180 kD DNA polymerase α catalytic subunit mRNA in luminal epithelial cells of the uterus. With and without cycloheximide pretreatment, after 12 hours of treatment with E2 there is a similar increase in staining of cyclin D1 mRNA in the luminal epithelial cells of the

uterus compared to sections of the uterus from control mice (Figure 3.22, A-D v. I-L). Furthermore, with and without pretreatment of cycloheximide, treatment with E2 for 12 hours also resulted in similar increased staining of the luminal epithelial cells for 180 kD catalytic subunit of DNA polymerase α mRNA compared to uterine sections from the controls (Figure 3.23, A-D v. I-L).

Pretreatment with cycloheximide also did not affect the inhibition of E2-induced cyclin D1 and 180 kD DNA polymerase a catalytic subunit mRNA by TCDD. With and without pretreatment of cycloheximide, treatment with E2+TCDD resulted in a slight increased staining for cyclin D1 mRNA in the stroma and luminal epithelial cells compared to control mice and mice treated with TCDD alone (Figure 3.22, A-D and E-H v. M-P), however the staining was less intense than the staining in uterine sections from mice treated with E2 alone (Figure 3.22, I-L v. M-P). Similarly, with and without pretreatment of cycloheximide, staining for the 180 kD DNA polymerase a catalytic subunit was increased along the luminal epithelial cells of uterine sections from mice treated with E2+TCDD when compared to sections from control mice and mice treated with TCDD (Figure 3.23, A-D and E-H v. M-P); however, the intensity of staining was lower than the staining of the luminal epithithelial cells in sections from mice treated with E2 alone (Figure 3.23, I-L v. M-P).

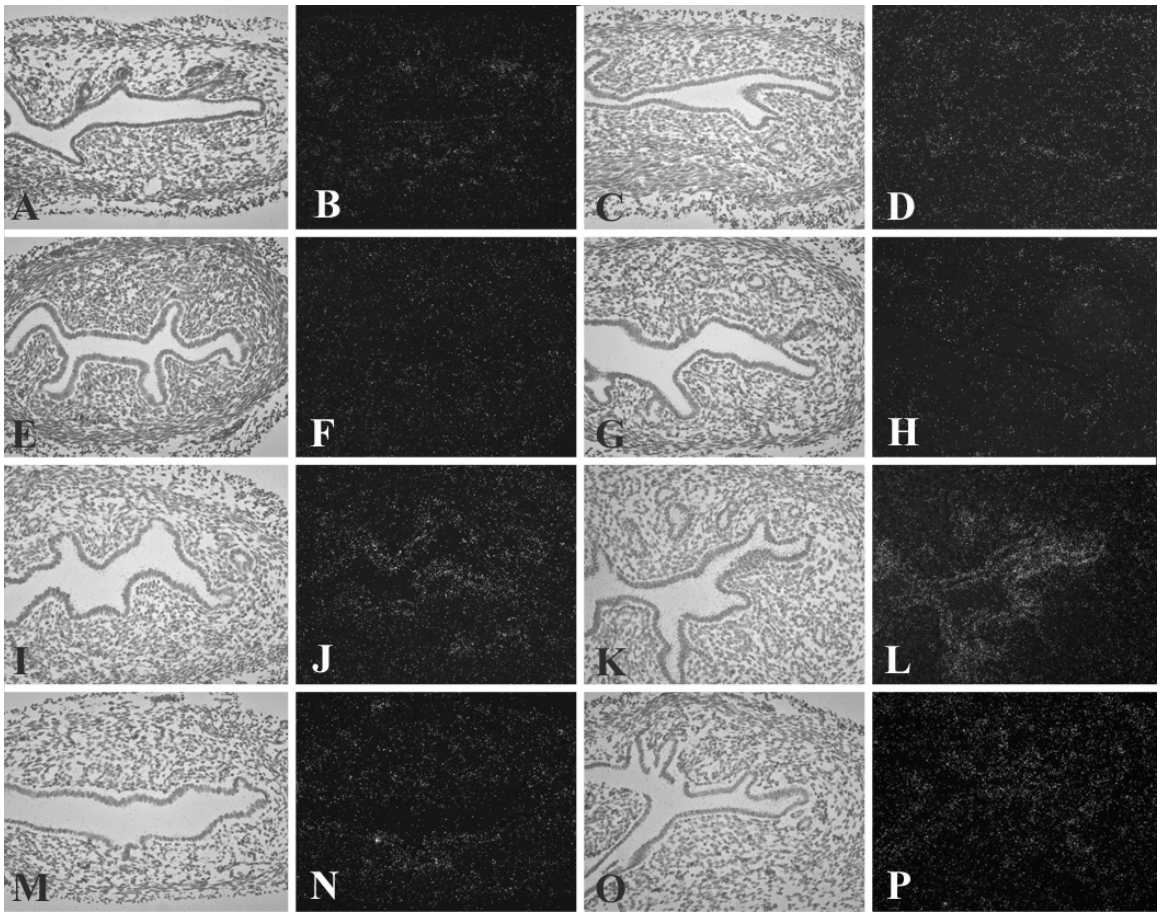


Figure 3.22: Expression of cyclin D1 mRNA twelve hours after treatment with E2 and TCDD with and without cycloheximide treatment. Twenty-five day old female B6C3F1 mice were treated with the corn oil vehicle, (A-D), 1 μg of TCDD (E-H), 200 ng of E2 (I-L) or E2 + TCDD (M-P) (n is 4 per treatment) with (A-B, E-F, I-J, M-N) or without (C-D, G-H, K-L, O-P) 1 hour pretreatment of 0.75 mg of cycloheximide. Cyclin D1 mRNA levels were determined by *in situ* hybridization of uterine sections taken 12 hours after treatment utilizing [^{35}S]-labeled cRNA sense or antisense probes. Stained sections were analyzed by brightfield and darkfield microscopy. A representative section of each treatment is shown.

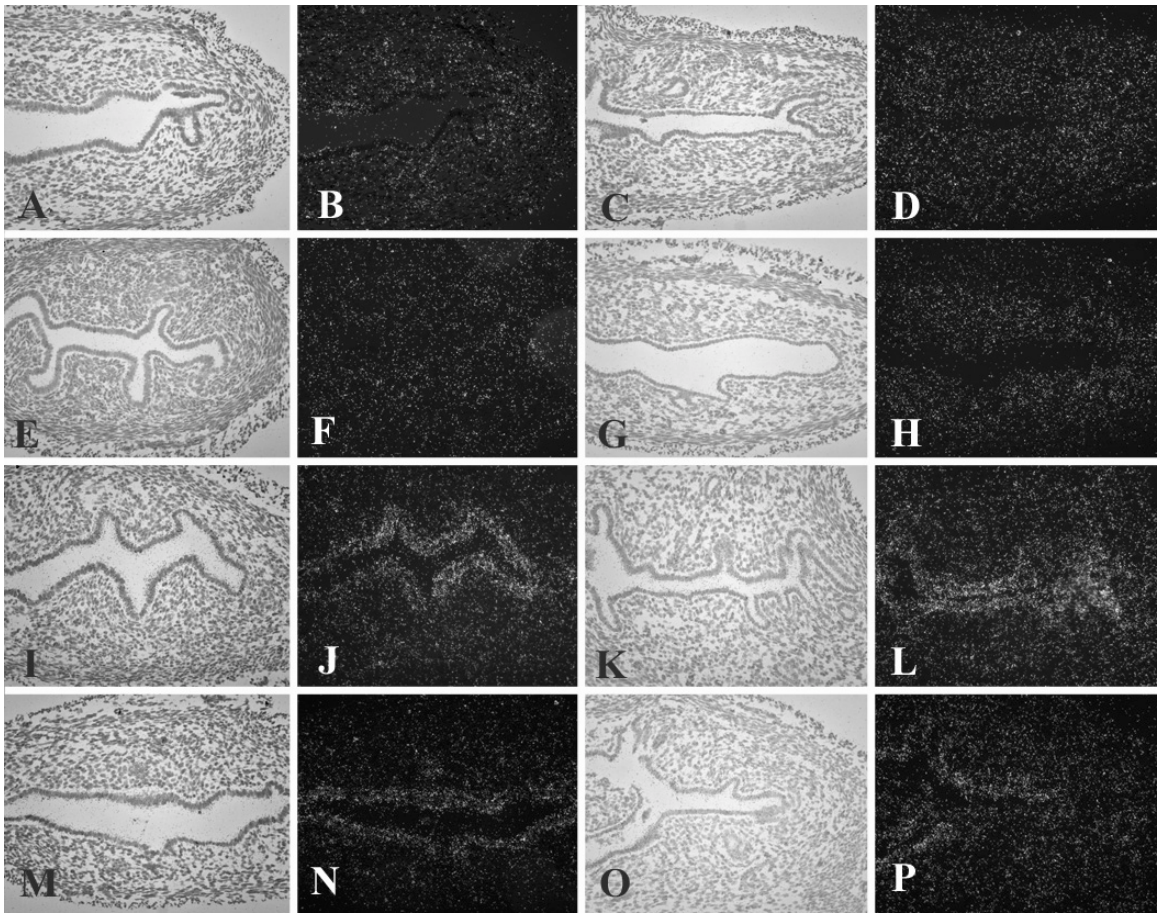


Figure 3.23: Expression of DNA polymerase α catalytic subunit mRNA twelve hours after treatment with E2 and TCDD with and without cycloheximide treatment. Twenty-five day old female B6C3F1 mice were treated with corn oil vehicle, (A-D), 1 μg of TCDD (E-H), 200 ng of E2 (I-L) or E2 + TCDD (M-P) (n is 4 per treatment) with (A-B, E-F, I-J, M-N) or without (C-D, G-H, K-L, O-P) 1 hour pretreatment of 0.75 mg of cycloheximide. DNA polymerase α catalytic subunit mRNA levels were determined by *in situ* hybridization utilizing [^{35}S]-labeled cRNA sense or antisense probes. Stained sections were analyzed by brightfield and darkfield microscopy. A representative section of each treatment is shown.

3.2 ErbB2-induced tumor development and inhibition of ErbB2-mediated responses by 1,1',2,2'-tetramethyldiindolylmethane (1,1',2,2'-tetraMethDIM)

3.2.1 Characterization of gene expression that leads to ErbB2-induced tumor development

Overexpression and amplification of ErbB2 have been implicated in development of aggressive forms of human breast cancer and many other tumor types. In vitro studies in BT-474 ErbB2 overexpressing human breast cancer cells indicate that modulation of cyclin D1 and p27 through both the MAPK and PI3K/Akt pathways allows G1 to S phase transition; and in vivo studies crossing MMTV-c-neu and cyclin D1-/- mice indicate that cyclin D1 is necessary for ErbB2 induced mammary tumor formation (Lenferink et al., 2001; Yu et al., 2001). However the mechanism of ErbB2 transformation of cells is not understood. The MMTV-c-neu mouse mammary tumor model expresses a mutated form of the rat c-neu (ErbB2) protein under the mouse mammary tumor virus promoter and in this transgenic model there is a well established progression of mammary carcinogenesis with palpable mammary tumors forming around 25 weeks of age (Muller et al., 1988). Therefore, analysis of gene expression profiles during the progressive stages of mammary carcinogenesis in MMTV-c-neu mice will provide insights on ErbB2-induced genes that contribute to enhanced tumor formation and growth.

Mammary glands have been removed for RNA isolation and histopathological analysis from female MMTV-c-neu and FVB control mice at 6,

12, and 18 weeks of age; however, only tissue from the 6 week old mice has been analyzed in preliminary studies. The #1 and #2 mammary glands were isolated for histopathological analysis because the location of the glands makes it difficult to isolate the mammary tissue without muscle and other surrounding tissue included, and inclusion of surrounding tissue for RNA isolation and microarray analysis could alter gene expression profiles. Histopathological analysis of mammary glands from the MMTV-c-neu mice and FVB mice showed no differences between the control and the transgenic mice at 6 weeks of age. The glands were normal with no indication of transformation in the mammary epithelium. These results agree with the original description of the TG.NK line by Muller and coworkers in which stochastic expression of ErbB2 and mammary epithelial transformation was not observed until mice were older (Muller et al., 1988).

Microarray analysis comparing gene expression in the mammary of FVB and MMTV-c-neu mice at 6 weeks of age was performed using the Affymetrix MG-U74Av2 chip. A summary of the gene expression profiles is shown in Table 3.1. Thirty-two genes were up-regulated greater than 2 fold in the MMTV-c-neu mice compared to the FVB background mice and 23 genes were down-regulated greater than 2 fold. The genes that were up-regulated or down-regulated greater than 2-fold were classified under molecular function/pathways based on gene ontology using tools from the web-based bioinformatics resource David (http://david.niaid.nih.gov/david/upload.asp). The results (Tables 3.2 and 3.3)

indicate that catalytic activity and binding were the two classifications that contained most genes up-regulated and down-regulated. Several up-regulated genes in the transgenic mice are also involved in development, signal transduction and transporter activity (Table 3.2) and multiple down regulated genes exhibited transporter activity and enzyme regulation (Table 3.3). Further analysis of gene expression changes in the MMTV-c-neu mice compared to the FVB background at six weeks of age is in progress.

Based on the minimal variation in gene expression found within the FVB and MMTV-c-neu groups at 6 weeks of age samples from 2 MMTV-c-neu and 2 FVB mice 12 and 18 weeks will be analyzed in future studies. Table 3.4 indicates the detection of gene expression within the FVB and MTVV-c-neu groups and the scatter graphs in Figure 3.24 compare the three animals within the MMTV-c-neu group showing good correlation in gene expression between animals. Similar correlation was found between the FVB animals. Therefore, 2 samples per group at each the 12 week and 18 week time points should allow delineation of differences in gene expression between the FVB and MMTV-c-neu mice.

Table 3.1: Summary of gene expression profiles in mouse mammary tissue at 6 wks based on analysis with GeneSpring software. RNA was isolated from mammary tissue of FVB and MMTV-c-neu at 6 weeks of age and gene expression analyzed using the Affymetrix MG-U74Av2 microarray chip.

Total samples analyzed:	2 samples of FVB control 3 samples of MMTV-c-neu
Total genes on Affytmetrix MG-U74Av2 chip:	12,520
Total genes changed after removing genes with same levels under both conditions	1,187
Total genes up-regulated among genes changed:	741
Total genes down-regulated among genes changed:	446
Total genes up-regulated greater than 2 fold:	32
Total genes down-regulated greater than 2 fold:	23

Table 3.2: Classification of MMTV-c-neu genes up-regulated greater than 2 fold. RNA was isolated from mammary tissue of FVB and MMTV-c-neu at 6 weeks of age and gene expression analyzed using the Affymetrix MG-U74Av2 microarray chip and GeneSpring software comparing MMTV-c-neu and FVB mice.

CLASSIFICATION	GENENAME	GenBank	Fold Change
Catalytic Act.	glucan (1,4-alpha-), branching enzyme 1	AI854404	2.03
	ATP citrate lyase	AW121639	2.2
	ATP-binding cassette, sub-family D (ALD), member 2	Z48670	2.09
	apolipoprotein B editing complex 2	AW124988	2.25
	aldehyde dehydrogenase family 1, subfamily A7	U96401	2.1
	neural precursor cell expressed, developmentally down-regulted gene 4	AV365271	4.07
	ELOVL family member 6, elongation of long chain fatty acids	AW122523	2.72
	ELOVL family member 6, elongation of long chain fatty acids	AI839004	2.5
	methylmalonyl-Coenzyme A mutase	X51941	2.13
	malic enzyme, supernatant	J02652	2.37
Binding	ATP citrate lyase	AW121639	2.2
	ATP-binding cassette, sub-family D (ALD), member 2	Z48670	2.09
	nuclear receptor subfamily 1, group D, member 1	AI834950	2.07
	apolipoprotein B editing complex 2	AW124988	2.25
	growth differentiation factor 8	U84005	2.34
	neural precursor cell expressed, developmentally down-regulted gene 4	AV365271	4.07
	retinol binding protein 4, plasma	U63146	2.34
Development	frizzled homolog 4 (Drosophila)	U43317	3.23
	RIKEN cDNA A430096B05 gene	AV373294	2.28
	patched homolog	AI848841	2.94
	angiomotin like 2	AI854404	2.03
Signal Transducer	nuclear receptor subfamily 1, group D, member 1	AI834950	2.07
	frizzled homolog 4 (Drosophila)	U43317	3.23
	growth differentiation factor 8	U84005	2.34
	patched homolog	AI848841	2.94
Transporter Act.	ATP-binding cassette, sub-family D (ALD), member 2	Z48670	2.09
	retinol binding protein 4, plasma	U63146	2.34
	hemoglobin alpha, adult chain 1	AV003378	2.45
Behavior	nuclear receptor subfamily 1, group D, member 1	AI834950	2.07
Cell Adhesion Mol.	RIKEN cDNA A430096B05 gene	AV373294	2.28
Transcript. Regulator	nuclear receptor subfamily 1, group D, member 1	AI834950	2.07
Unclassified	interferon-induced protein with tetratricopeptide repeats 1	U43084	3.27
	pentaxin related gene	X83601	3.06
	RIKEN cDNA 2310034L04 gene	AI839116	2.07
	RIKEN cDNA 2700089E24 gene	AI131982	2.14
	RIKEN cDNA 6330514M23 gene	AW048944	2.09
	RIKEN cDNA B430320C24 gene	AI606967	2.79
	cytotoxic T lymphocyte-associated protein 2 beta	X15592	2.17
	beta-1-globulin	V00722	2.6
	small inducible cytokine A2	M19681	2.36
	small inducible cytokine A7	X70058	2.3
	hemoglobin beta, adult major chain	J00413	2.18

Table 3.3: Classification of MMTV-c-neu genes down-regulated greater than 2 fold. RNA was isolated from mammary tissue of FVB and MMTV-c-neu at 6 weeks of age and gene expression analyzed using the Affymetrix MG-U74Av2 microarray chip and GeneSpring software comparing MMTV-c-neu and FVB mice.

CLASSIFICATION	GENENAME	GenBank	Fold Change
Binding	glycerol kinase	U48403	2.1
	casein gamma	D10215	7.87
	nuclear factor I/A	D90173	2.04
	myeloblastosis oncogene	M12848	2.07
	capping protein (actin filament) muscle Z-line, beta	U10407	2.56
	fatty acid binding protein 3, muscle and heart	X14961	3.57
	chemokine (C-C motif) ligand 19	AW120505	2.15
	apolipoprotein D	X82648	2.02
Catalytic Act.	glycerol kinase	U48403	2.1
	reelin	U24703	2.1
	solute carrier family 27 (fatty acid transporter), member 2	AF072757	3.46
	solute carrier family 27 (fatty acid transporter), member 1	U15976	2.04
	phosphoglycerate mutase 2	AF029843	2.12
	cyclin B2	X66032	2.42
	creatine kinase, mitochondrial 2	AI181132	2.51
Transporter Act.	fatty acid binding protein 3, muscle and heart	X14961	3.57
	solute carrier family 27 (fatty acid transporter), member 1	U15976	2.04
	apolipoprotein D	X82648	2.02
Enzyme Regulator Act.	cyclin B2	X66032	2.42
	extracellular proteinase inhibitor	X93037	2.28
Behavior	reelin	U24703	2.1
Cell Adhesion Mol. Act.	reelin	U24703	2.1
Cellular Process	antigen identified by monoclonal antibody Ki 67	X82786	2.38
Chaperone Act.	DnaJ (Hsp40) homolog, subfamily B, member 1	AB028272	2.27
Development	reelin	U24703	2.1
Signal Transducer Act.	chemokine (C-C motif) ligand 19	AW120505	2.15
Structural Molecule Act.	reelin	U24703	2.1
Transcription Regulator	nuclear factor I/A	D90173	2.04
Unclassified	H19 fetal liver mRNA	X58196	4.18
	casein alpha	M36780	3.28
	casein beta	X04490	8.83
	unncoupling protien, mitochondrial	M21247	2.5
	small proline-rich protein 2a	AJ005559	2.35

Table 3.4. Detection of gene expression among animals within the FVB and the MMTV-c-neu groups. RNA was isolated from mammary tissue of FVB and MMTV-c-neu at 6 weeks of age and gene expression analyzed using the Affymetrix MG-U74Av2 microarray chip.

Animals	% Present	% Absent	% Marginal
FVB1	47.2	50.2	2.6
FVB2	44.1	53.2	2.7
MMTV-c-neu1	48.3	49.1	2.5
MMTV-c-neu2	41.7	55.9	2.4
MMTV-c-neu3	47.4	50.1	2.5

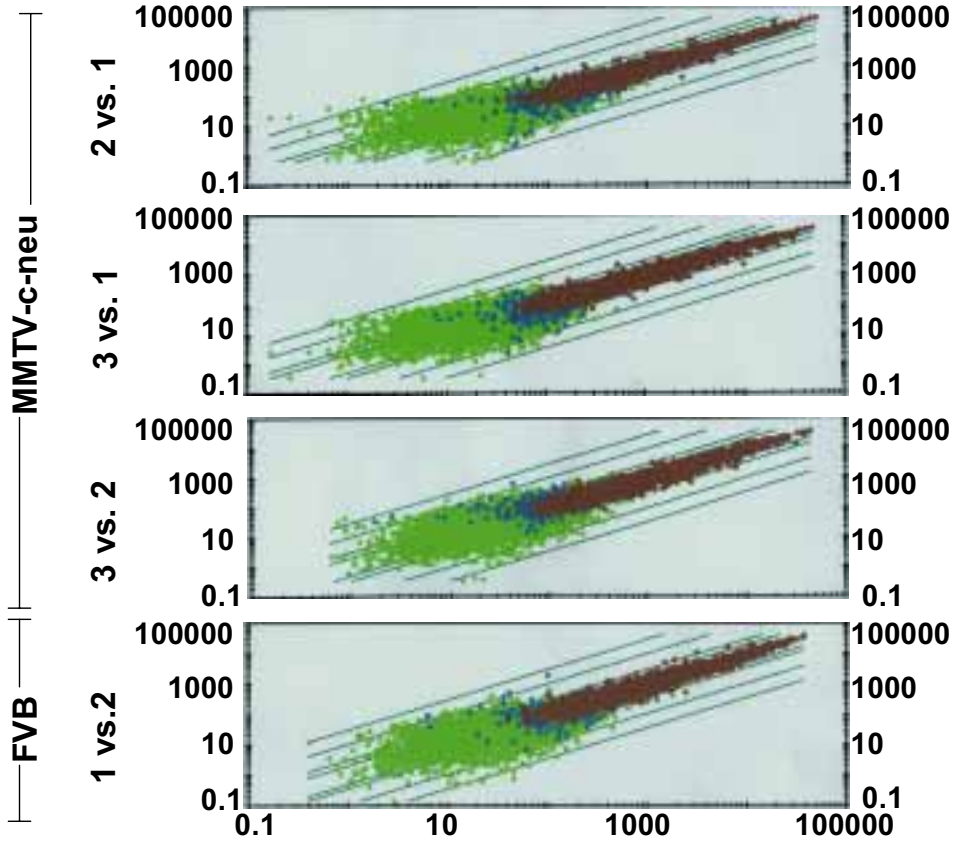


Figure 3.24: Scatter graphs comparing gene expression within the MMTV-c-neu and FVB groups. RNA was isolated from mammary tissue of FVB and MMTV-c-neu at 6 weeks of age and gene expression analyzed using the Affymetrix MG-U74Av2 microarray chip. Scatter graphs were plotted to compare gene expression patterns between animals within the groups.

3.2.2 Inhibition of ErbB2-induced growth in vitro by 1,1',2,2'-tetraMethDIM, an AhR agonist

Recently, it has been reported that 1,1',2,2'-tetraMethDIM, an AhR agonist, inhibited DMBA-induced mammary tumor growth in rats (McDougal et al., 2001) and in in vitro studies, this compound inhibited constitutively active MAPK and p110 (catalytic subunit of PI3-K). Breast cancer cell lines that overexpress ErbB2 exhibit constitutive activation of both the MAPK and PI3K pathways. Therefore, the ability of 1,1',2,2'-tetraMethDIM to inhibit the growth of ErbB2 overexpressing cells in vitro was investigated. Initial studies examined the ability of 1,1',2,2'-tetraMethDIM to inhibit growth of BT-474 and MDA-MB-453 human breast cancer cell lines, which overexpress ErbB2, and also determine their effects on E2- and heregulin (an ErbB2 agonist)- induced growth in MCF-7 human breast cancer cells. All three cell lines express a functional AhR.

BT-474 and MDA-MB-453 cells were grown over a 5-7 day period in media containing 2.5 or 5% charcoal-stripped fetal bovine serum or 10% untreated fetal bovine serum and treated with various concentrations of 1,1',2,2'-tetraMethDIM. The ability of 1,1',2,2'-tetraMethDIM to inhibit the growth of both BT-474 and MDA-MB-453 cells was dependent upon the serum in the treatment media. BT-474 and MDA-MB-453 cells cultured in 10% serum grew at a much higher rate than cells grown in the other media; moreover, 1,1',2,2'-tetraMethDIM did not inhibit the growth at the concentrations (2.5 to 10 μM)

used in this study (Figure 3.25). After treatment of BT-474 cells grown in media containing 5% charcoal-stripped serum with 1,1',2,2'-tetraMethDIM growth was inhibited 5 and 7 days after treatment with 10 μM 1,1',2,2'-tetraMethDIM. In contrast 2.5 or 5 μM 1,1',2,2'-tetraMethDIM did not affect growth over the time course of this study (Figure 3.26). MDA-MB-453 cells grown in media containing 5% charcoal-stripped serum were treated with 5 and 10 μM 1,1',2,2'-tetraMethDIM and cell growth was inhibited after 7 days; 5 and 10 mM 1,1',2,2'-tetraMethDIM did not significantly inhibit growth at earlier time points and 2.5 μM 1,1',2,2'-tetraMethDIM had no effect on cell growth over the 7 day period (Figure 3.26). The highest inhibition of growth by 1,1',2,2'-tetraMethDIM was observed in cells grown in media containing 2.5% charcoal-stripped serum. Five and 10 μM 1,1',2,2'-tetraMethDIM inhibited growth of BT-474 cells after 5 and 7 days in media containing 2.5% charcoal-stripped serum (Figure 3.27). Growth of MDA-MB-453 cells was also inhibited by different concentrations of 1,1',2,2'-tetraMethDIM when cells are grown in 2.5% charcoal-stripped serum (Figure 3.27).

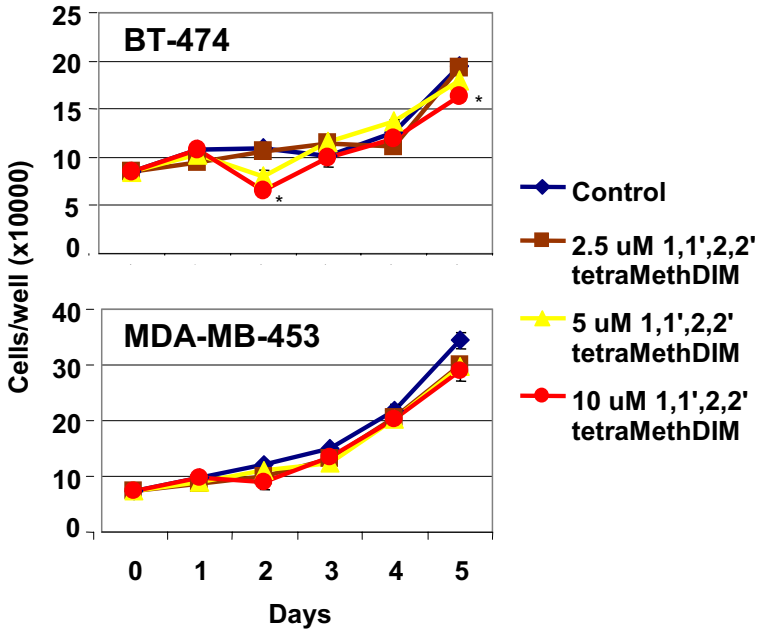


Figure 3.25: BT-474 and MDA-MB-453 cell proliferation in media containing 10% FBS. Cells were seeded in media containing 10% FBS and treated with DMSO, 2.5, 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM over 5 days. Cells were counted everyday. The mean ± SE is plotted (n=3) and significant ($p<0.05$) changes are indicated (*).

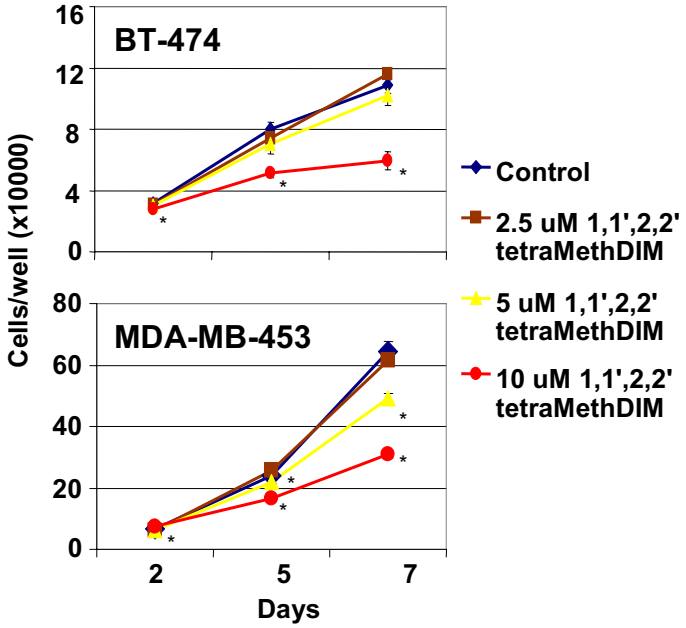


Figure 3.26: BT-474 and MDA-MB-453 cell proliferation in media containing 5% charcoal-stripped FBS. Cells were seeded in media containing 5% charcoal-stripped FBS and treated with DMSO, 2.5, 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM over 7 days. Cells were counted on day 2, 5, and 7. The mean ± SE is plotted (n=3) and significant ($p<0.05$) changes are indicated (*).

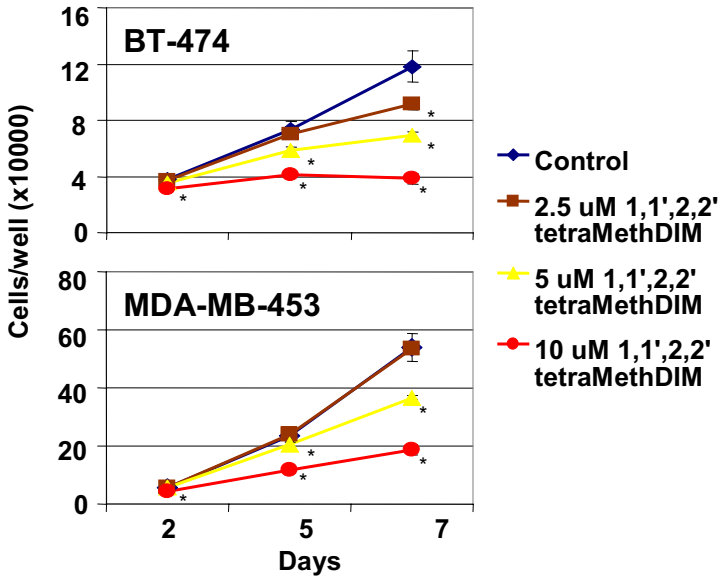


Figure 3.27: BT-474 and MDA-MB-453 cell proliferation in media containing 2.5% charcoal-stripped FBS. Cells were seeded in media containing 2.5% charcoal-stripped FBS and treated with DMSO, 2.5, 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM over 7 days. Cells were counted on day 2, 5, and 7. The mean ± SE is plotted (n=3) and significant ($p<0.05$) changes are indicated (*).

The ability a 1,1',2,2'-tetraMethDIM to inhibit baseline, as well as, E2- and heregulin-induced growth was investigated in MCF-7 cells. MCF-7 cells were grown in serum-free media alone or in serum-free media plus 1 nM E2 or 2.5 ng/ml heregulin and the growth inhibitory effects of 0, 2.5, 5, or 10 μM 1,1',2,2'-tetraMethDIM over a six day period were determined. MCF-7 cells treated with 0 or 2.5 μM 1,1',2,2'-tetraMethDIM had similar growth curves, whereas 5 and 10 μM 1,1',2,2'-tetraMethDIM inhibited growth of MCF-7 cells (Figure 3.28). One nanomolar E2 induced growth of MCF-7 cells compared to solvent treated MCF-7 cells and 1,1',2,2'-tetraMethDIM inhibited E2-induced growth of MCF-7 cells in a concentration-dependent manner (Figure 3.28). An induction of growth was not seen in the heregulin treated MCF-7 cells compared to the solvent-treated control cells until 6 days after treatment. Similarly, inhibition of growth by all three doses of 1,1',2,2'-tetraMethDIM was not observed until 6 days of treatment (Figure 3.28). Although heregulin did not induce growth of MCF-7 cells after 2 and 4 days of treatment, cells treated with 5 and 10 μM 1,1',2,2'-tetraMethDIM + heregulin showed an increased growth, whereas cells treated with 5 and 10 μM 1,1',2,2'-tetraMethDIM alone did not (Figure 3.28).

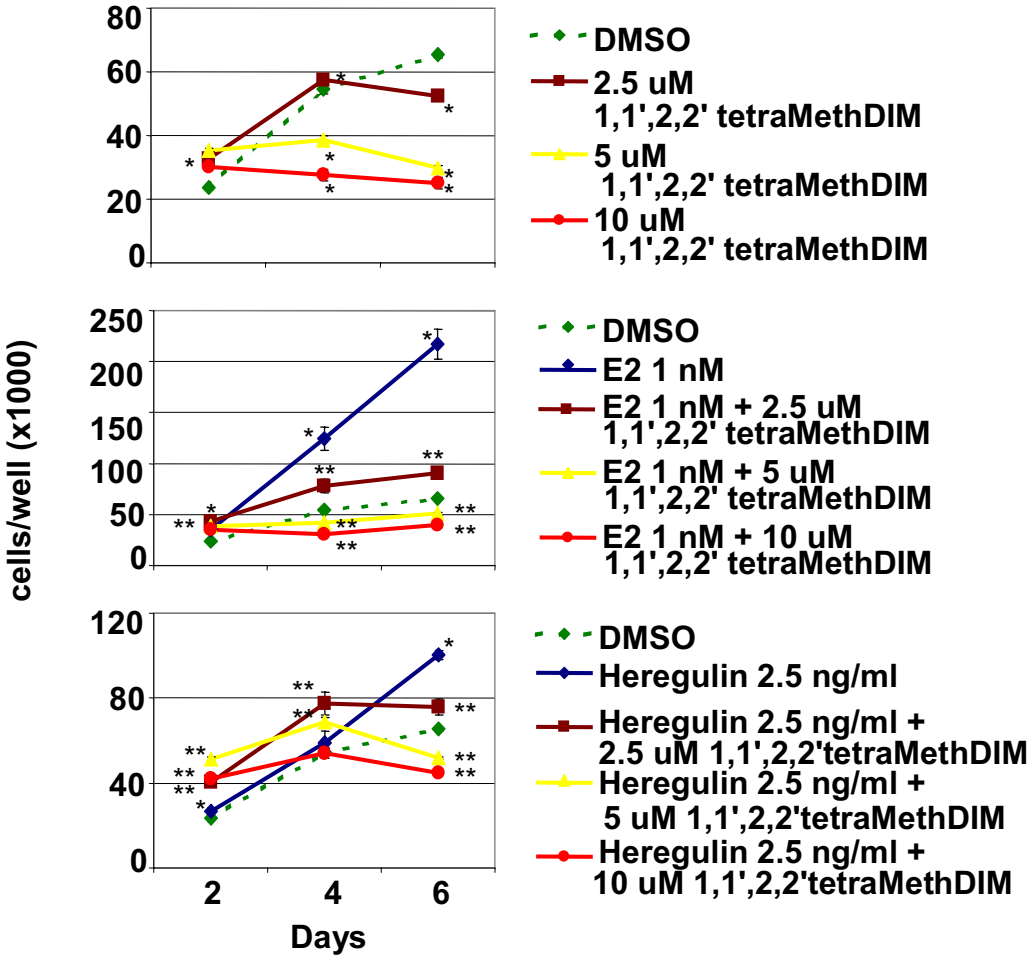


Figure 3.28: MCF-7 cell proliferation in serum-free media. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed in serum-free media with DMSO, E2 or heregulin plus 2.5, 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM over 6 days. Cells were counted on day 2, 4, and 6. The mean ± SE is plotted (n=3) and significant ($p<0.05$) changes are indicated (*).

3.2.3 Mechanism of growth inhibition by 1,1',2,2'-tetraMethDIM in BT-474 and MDA-MB-453 cells – cytotoxicity, induction of apoptosis, or cell cycle arrest

Cancer cell growth is inhibited either by cell death or inhibition of cell division or both, and cell death occurs through cytotoxicity, induction of apoptosis, or a combination of the two processes. We investigated whether treatment of BT-474 or MDA-MB-453 cells with 1,1',2,2'-tetraMethDIM resulted in cytotoxicity, cell cycle arrest , or apoptosis (measured by induction of caspase 3 activity or cleavage of PARP protein). BT-474 and MDA-MB-453 cells were cultured in media containing 2.5% charcoal-stripped serum and treated with 1,1',2,2'-tetraMethDIM (Figure 3.27). Cytotoxicity was measured using the lactose dehydrogenase (LDH) cytoxicity assay. The amount of LDH in the media provides a relative measure of cells that have lysed due to cytotoxicity. The LDH released from these cells is determined by measuring the conversion of $NADH^+$ + pyruvate to NAD^+ + lactate and the subsequent decrease in the absorbance of $NADH^+$. Therefore, a decrease in absorbance from the pyruvate + $NADH^+$ control indicates the presence of LDH in the sample and thus cytoxicity. Cytotoxicity was measured 2 and 4 days after treatment of BT-474 cells with 2.5, 5, and 10 μM 1,1',2,2'-tetraMethDIM. Treatment with 0.005, 0.05 and 5 mM phenol was used as a positive control for cytotoxicity in the assay and Triton-X was used as a measure of the maximum decrease in absorbance with complete cell lysis. Treatment with 5 mM phenol for 2 days resulted in cytotoxicity as indicated by a decrease in absorbance compared to the pyruvate

+ $NADH^+$ control and after 4 days all three doses of phenol induced cytoxicity (Figure 3.29). There was no indication that 1,1',2,2'-tetraMethDIM was cytotoxic at any dose (Figure 3.29). Similarly, after 2 days of treatment of MDA-MB-453 cell with 1,1',2,2'-tetraMethDIM there was no indication of cytoxicity at any dose (Figure 3.30).

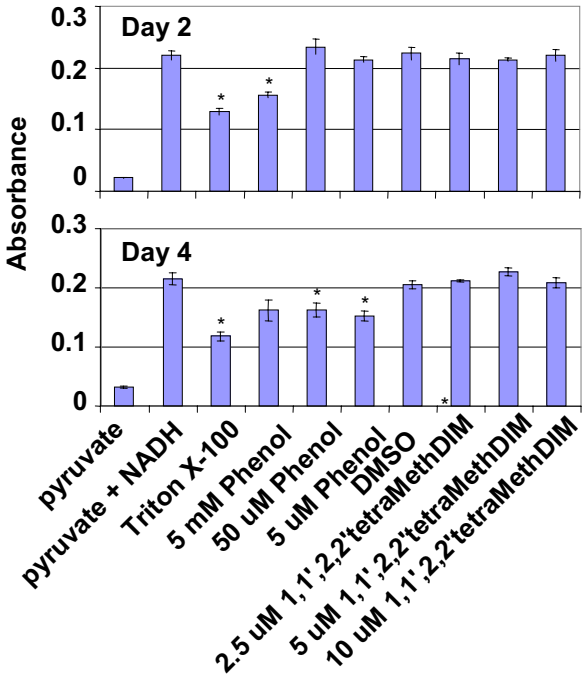


Figure 3.29: LDH cytotoxicity assay in BT-474 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 2.5, 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM; 0.005, 0.05 or 5 mM phenol over 4 days. Aliquots of media were removed from the cells on day 2 and 4 and analyzed using the LDH cytotoxicity assay. The mean ± SE is plotted (n=3) and significant ($p<0.05$) changes are indicated (*).

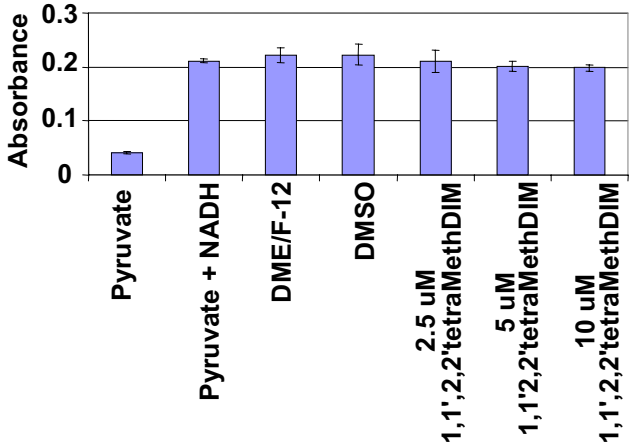
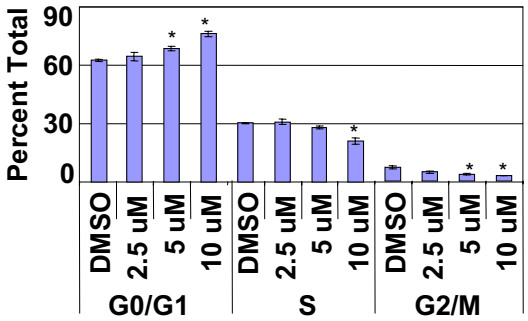


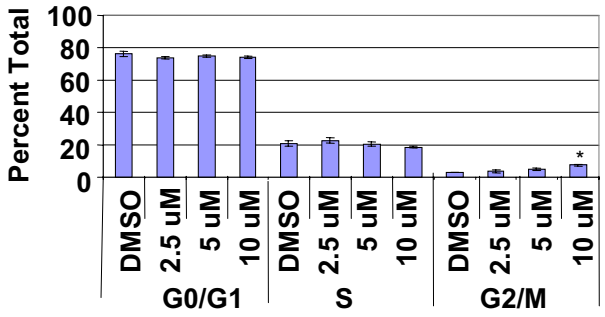
Figure 3.30: LDH cytotoxicity assay in MDA-MB-453 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO, 2.5, 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM over 2 days. Aliquots of media were removed from the cells on day 2 and analyzed using the LDH cytotoxicity assay. The mean ± SE is plotted (n=3) and significant ($p<0.05$) changes are indicated (*).

In order to determine whether inhibition of cell growth by 1,1',2,2'-tetraMethDIM was due to cell cycle arrest, cell cycle progression was measured 4 days after treatment of BT-474 and MDA-MB-453 cells with 1,1',2,2'-tetraMethDIM. Cells were stained with propidium iodide to measure DNA content and analyzed by flow cytometry. The percentage of cells in G0/G1 phase of the cell cycle after 4 days of treatment was increased in BT-474 cells treated with 10 μM 1,1',2,2'-tetraMethDIM and the percentage of cells in S and G2/M phases of the cell cycle was decreased (Figure 3.31). Treatment of BT-474 cells with 5 μM 1,1',2,2'-tetraMethDIM showed a trend similar to that observed with 10 μM 1,1',2,2'-tetraMethDIM (Figure 3.31). Therefore, inhibition of BT-474 cell growth by 1,1',2,2'-tetraMethDIM is at least partially due to an increase in the percentage of cells in G0/G1 phase of the cell cycle. This effect may be related to an increase in time that the overall cell population spends in G0/G1, thereby extending the time for cell division. It is also possible that a certain cell population exits from the cell cycle and arrests in G0/G1 so that fewer cells are undergoing cell division. 1,1',2,2'-tetraMethDIM treatment did not result in a change in the percentage of MDA-MB-453 cells in G0/G1 at any dose (Figure 3.32). In contrast a doubling of the percentage of MDA-MB-453 cells in G2/M phase of the cell cycle occurred after treatment with 10 μM 1,1',2,2'-tetraMethDIM (Figure 3.32). Therefore, the effect of 1,1',2,2'-tetraMethDIM on percent distribution of MDA-MB-453 cells in phases of the cell cycle is different from that observed for BT-474 cells.



		Average	Std. Error
G0/G1	DMSO	62.61	0.560
	2.5 uM 1,1',2,2'tetraMethDIM	64.51	2.016
	5 uM 1,1',2,2'tetraMethDIM	68.50	1.066
	10 uM 1,1',2,2'tetraMethDIM	76.18	1.444
S	DMSO	30.28	0.282
	2.5 uM 1,1',2,2'tetraMethDIM	30.96	1.415
	5 uM 1,1',2,2'tetraMethDIM	28.15	0.788
	10 uM 1,1',2,2'tetraMethDIM	21.12	1.490
G2/M	DMSO	7.10	0.825
	2.5 uM 1,1',2,2'tetraMethDIM	4.54	0.694
	5 uM 1,1',2,2'tetraMethDIM	3.35	0.373
	10 uM 1,1',2,2'tetraMethDIM	2.70	0.142

Figure 3.31: Cell cycle analysis in BT-474 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 2.5, 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM over 4 days. Cells were stained with propidium iodide and DNA content was analyzed by flow cytometry. The mean ± SE is plotted (n=3) and significant ($p<0.05$) changes are indicated (*).



		Average	Std. Error
G0/G1	DMSO	76.26	1.561
	2.5 uM 1,1',2,2'tetraMethDIM	73.80	0.747
	5 uM 1,1',2,2'tetraMethDIM	74.84	0.706
	10 uM 1,1',2,2'tetraMethDIM	74.14	0.858
S	DMSO	20.75	1.581
	2.5 uM 1,1',2,2'tetraMethDIM	22.64	1.580
	5 uM 1,1',2,2'tetraMethDIM	20.36	1.192
	10 uM 1,1',2,2'tetraMethDIM	18.53	0.522
G2/M	DMSO	2.99	0.101
	2.5 uM 1,1',2,2'tetraMethDIM	3.56	1.036
	5 uM 1,1',2,2'tetraMethDIM	4.81	0.709
	10 uM 1,1',2,2'tetraMethDIM	7.33	0.402

Figure 3.32: Cell cycle analysis in MDA-MB-453 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 2.5, 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM over 4 days. Cells were stained with propidium iodide and DNA content was analyzed by flow cytometry. The mean ± SE is plotted (n=3) and significant ($p<0.05$) changes are indicated (*).

Caspase 3 activity and cleavage of the PARP protein were used as indicators of 1,1',2,2'-tetraMethDIM-mediated apoptosis as a mechanism for inhibition of BT-474 or MDA-MB-453 cell growth. As an early indicator of apoptosis, caspase 3 activity was measured 24, 48, and 72 hours after treatment. Caspase 3 activity was determined using the Ac-DEVD-AMC

fluorogenic substrate that fluoresces when cleaved by active caspase 3 protein, and camptothecin was used as a positive control for induction of apoptosis. After treatment for 24 hours neither camptothecin nor any dose of 1,1',2,2'-tetraMethDIM resulted in increased fluorescence indicating that caspase 3 activity was not increased in BT-474 or MDA-MB-453 cells (Figures 3.33 and 3.34). In contrast, treatment with camptothecin for 48 and 72 hours increased caspase 3 activity, whereas 1,1',2,2'-tetraMethDIM did not affect caspase activity in BT-474 or MDA-MB-453 cells (Figures 3.33 and 3.34). Cleavage of the PARP protein is also an indicator of apoptosis and a downstream substrate of the caspase cascade. Cleavage of PARP was determined by Western blot analysis of whole cell lysates isolated after treatment of BT-474 or MDA-MB-453 cells with 1,1',2,2'-tetraMethDIM for 40 hours. Treatment with 10 μM 1,1',2,2'-tetraMethDIM did not result in cleavage of the PARP protein in either BT-474 or MDA-MB-453 cells, whereas okadaic acid, a known inducer of apoptosis, induced cleavage of PARP (Figure 3.35). Therefore, these data suggest that 1,1',2,2'-tetraMethDIM does not induce apoptotic pathways associated with PARP cleavage in BT-474 or MDA-MB-453 cells. The data suggests that the growth inhibitory effects of 1,1',2,2'-tetraMethDIM must be associated with other pathways.

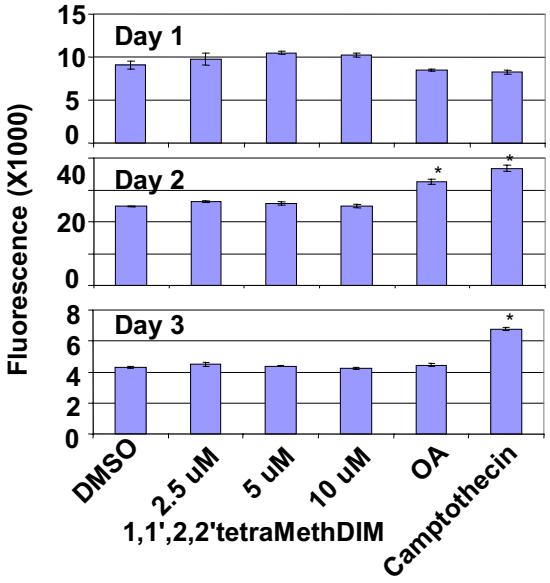


Figure 3.33: Induction of caspase 3-dependent activity in BT-474 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 2.5, 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM; 0.1 μM okadaic acid; or 3 μM camptothecin. On days 1, 2, and 3 after treatment cells were lysed and assayed for caspase 3 activity. The mean ± SE is plotted (n=3) and significant ($p<0.05$) changes are indicated (*).

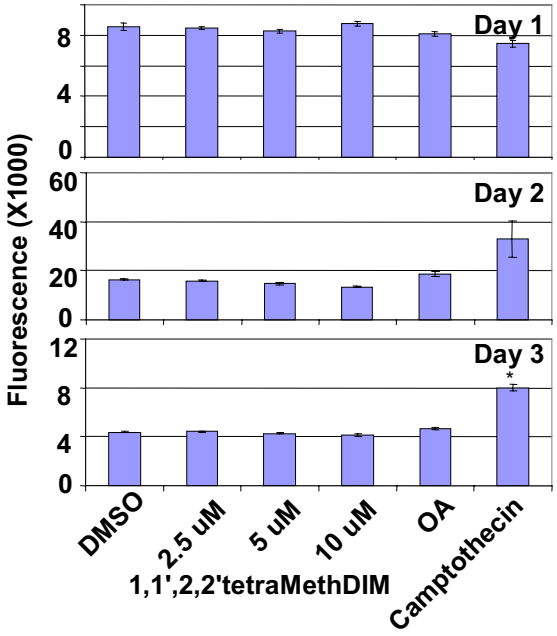


Figure 3.34: Induction of caspase 3-dependent activity in MDA-MB-453 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 2.5, 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM; 0.1 μM okadaic acid; or 3 μM camptothecin. On days 1, 2, and 3 after treatment cells were lysed and assayed for caspase 3 activity. The mean ± SE is plotted (n=3) and significant ($p<0.05$) changes are indicated (*).

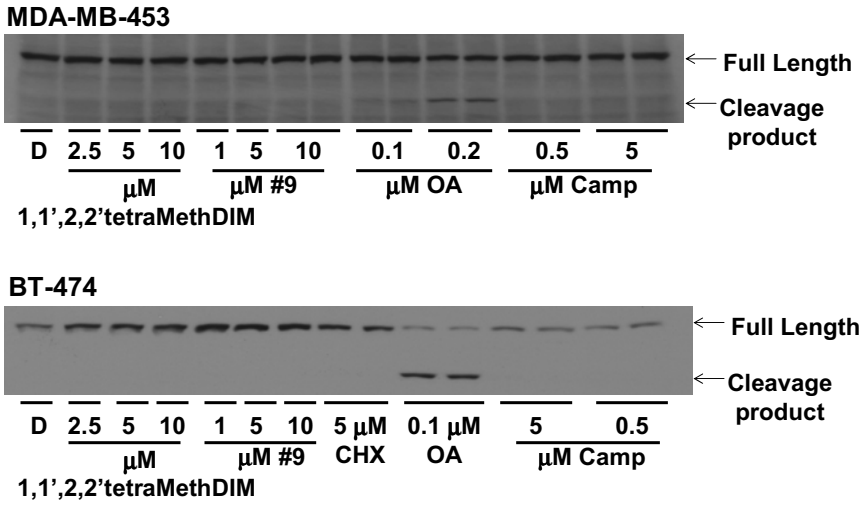


Figure 3.35: Induction of PARP cleavage in MDA-MB-453 and BT-474 cells.

Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 2.5, 5, or 10.0 μM 1,1',2,2',tetraMethDIM; 1, 5, or 10 μM compound #9; 5 μM CHX; 0.1 or 0.2 μM okadaic acid (OA); or 0.5 or 5 μM camptothecin (Camp). Forty hours after treatment the cells and pelleted supernatant were treated with high salt lysis buffer and protein used for Western blot analysis of PARP.

3.3.5 No evidence for inhibition of MAPK or PI3K pathways by 1,1',2,2'-tetraMethDIM in BT-474 or MDA-453 cell lines

Previous studies in BT-474 cells indicate that modulation of cyclin D1 and p27 through both the MAPK and PI3K/Akt pathways allows G1 to S phase transition (Lenferink et al., 2001). In our studies 1,1',2,2'-tetraMethDIM increases the percentage of BT-474 cells in G0/G1; therefore, we investigated whether or not 1,1',2,2'-tetraMethDIM inhibits the MAPK or PI3K/Akt pathways or affects downstream activation of cyclin D1 and p27. Western blots were used determine phosphorylation of ErbB2 as an initial step in the activation of both the MAPK and PI3K/Akt pathways, and the phosphorylation of ERK1/2 and Akt1 as downstream components of each pathway, respectively. After treatment of BT-474 cells with 1,1',2,2'-tetraMethDIM, U0126 (an inhibitor of the MAPK pathway), or LY294002 (an inhibitor of the PI3K/Akt pathway) for 4 days, protein was isolated and immunoprecipitated with antibodies to ErbB2 or IgG as a control.

The precipitated protein was probed with an antibody to phosphorylated tyrosine (p-tyr) to determine the level of ErbB2 phosphorylation on tyrosine residues. After 4 days of treatment with 1,1',2,2'-tetraMethDIM, U0126, or LY294002 BT-474 cells did not exhibit any change in phosphorylation of ErbB2 on tyrosine residues and none of the treatments affected overall ErbB2 protein levels (Figure 3.36). Immunoprecipitation with the control IgG antibody did not pull down non-specific proteins with p-try residues. Aliquots from the same protein samples were also used in Western blots analysis for phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, phosphorylated Akt1 (p-Akt1), and Akt. 1,1',2,2'-tetraMethDIM did not affect the phosphorylation of ERK1/2 or Akt1 or the overall level of the ERK1/2 or Akt proteins (Figure 3.36). LY294002, a specific inhibitor of the PI3K/Akt pathway, decreased phosphorylation of Akt, and U0126, a specific inhibitor of MAPK pathway, decreased the phosphorylation ERK1/2.

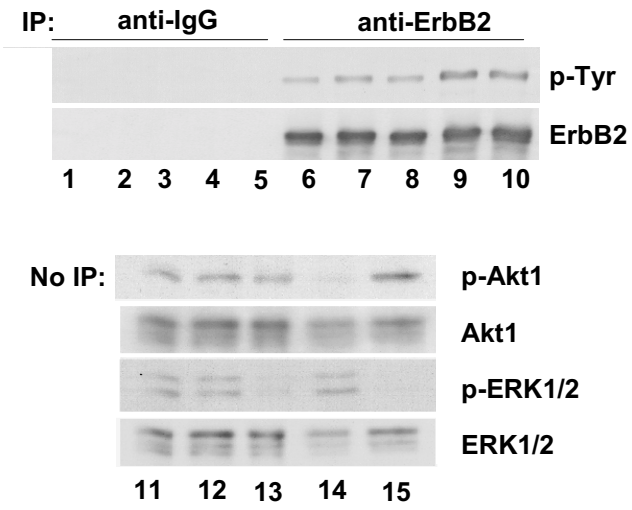


Figure 3.36: ErbB2, ERK, and Akt phosphorylation in BT-474 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO (Lanes 1, 6, 11); 10.0 μM 1,1',2,2',tetraMethDIM (Lanes 2, 7, 12); 1 nM E2 (Lanes 3, 8, 13), 10 μM U0126 (Lanes 4, 9, 14) or 10 μM LY294002 (Lanes 5, 10, 15). After 4 days of treatment, the cells were lysed in high salt lysis buffer and proteins used for immunoprecipitation and Western blot analysis. Representative Western blots are shown.

In a separate experiment BT-474 and MDA-453 cells were treated with 2.5, 5, and 10 μM 1,1',2,2'-tetraMethDIM for 4 days and ERK and Akt phosphorylation and cyclin D1 and p27 protein levels were determined by Western blot analysis of whole cell lysates. As indicated in Figure 3.36, 1,1',2,2'-tetraMethDIM does not affect ERK1/2 or Akt1 phosporylation, whereas the specific inhibitors, U0126 and LY294002 decreased constitutive levels of ERK1/2 and Akt1 phosphorylation, respectively, in BT-474 cells (Figure 3.37). In MDA-MB-453 cells 1,1',2,2'-tetraMethDIM did not affect the phosphorylation of ERK1/2 or Akt1; suprisingly, U0126 and LY294002 also did not inhibit the phosphorylation of ERK1/2 and Akt (Figure 3.38). ERK1/2 phosphorylation was actually increased in MDA-MB-453 cells by treatment with 10 μM 1,1',2,2'-tetraMethDIM, U0126 and LY294002 for 4 days. Cyclin D1 protein levels were not affected after treatment of BT-474 cell with 1,1',2,2'-tetraMethDIM for 4 days; however, treatment with LY294002 decreased cyclin D1 protein (Figure 3.37).

In MDA-MB-453 cells treated with 10 μM 1,1',2,2'-tetraMethDIM cyclin D1 protein levels were slightly increased and LY294002 treatment decreased cyclin D1 protein as observed in BT-474 cells (Figure 3.38). p27 protein levels were slightly increased in both BT-474 and MDA-MB-453 cells by treatment with 1,1',2,2'-tetraMethDIM in a dose dependent manner, as well as by L294002 treatment in BT-474 cells and DIM treatment in MDA-MB-453 cells after 4 days (Figures 3.37 and 3.38).

In order to determine whether an early change in cyclin D1 or the cell cycle inhibitors p21 or p27 correlated with the decrease in cell growth, cyclin D1, p21 and p27 protein levels were determined by Western blot analysis using whole cell lysates from BT-474 and MDA-MB-453 cells treated with 5 and 10 μM 1,1',2,2'-tetraMethDIM. However, after 12 and 24 hours of treatment with 1,1',2,2'-tetraMethDIM cyclin D1, p21 and p27 protein levels changed minimally (Figures 3.39 and 3.40).

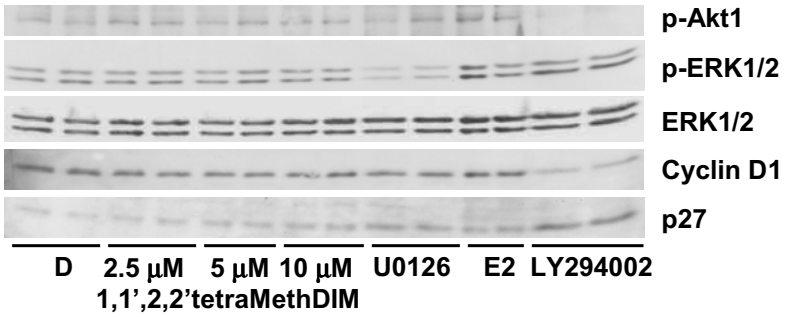


Figure 3.37: MAPK, PI3-K and cell cycle protein levels after 4 days of treatment in BT-474 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 2.5, 5, or 10.0 μM 1,1',2,2',tetraMethDIM; 10 μM U0126; 1 nM E2; or 10 μM LY294002. After 4 days of treatment, the cells were lysed in high salt lysis buffer and proteins used for Western blot analysis. Representative Western blots are shown.

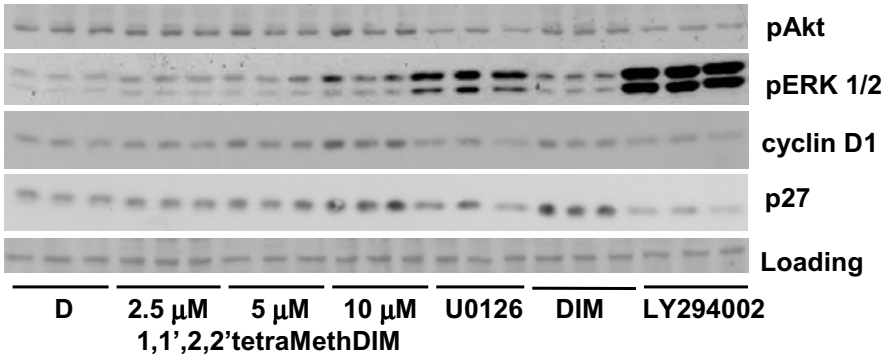


Figure 3.38: MAPK, PI3-K and cell cycle protein levels after 4 days of treatment in MDA-MB-453 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 2.5, 5, or 10.0 μM 1,1',2,2',tetraMethDIM; 10 μM U0126; 1 nM E2; or 10 μM LY294002. After 4 days of treatment, the cells were lysed in high salt lysis buffer and proteins used for Western blot analysis. Representative Western blots are shown.

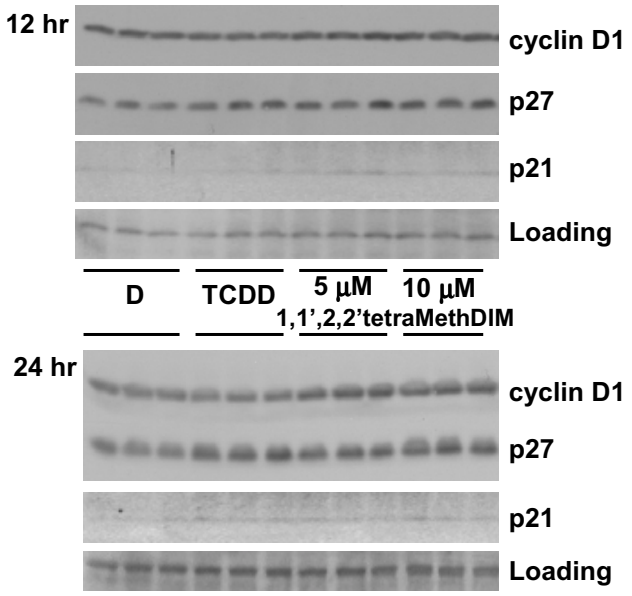


Figure 3.39: Cell cycle protein levels after 12 and 24 hours of treatment in BT-474 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 5 or 10.0 μM 1,1',2,2',tetraMethDIM; or 10 nM TCDD. After 12 and 24 hours of treatment, the cells were lysed in 1X laemmli buffer and whole cell lysates used for Western blot analysis. Representative Western blots are shown.

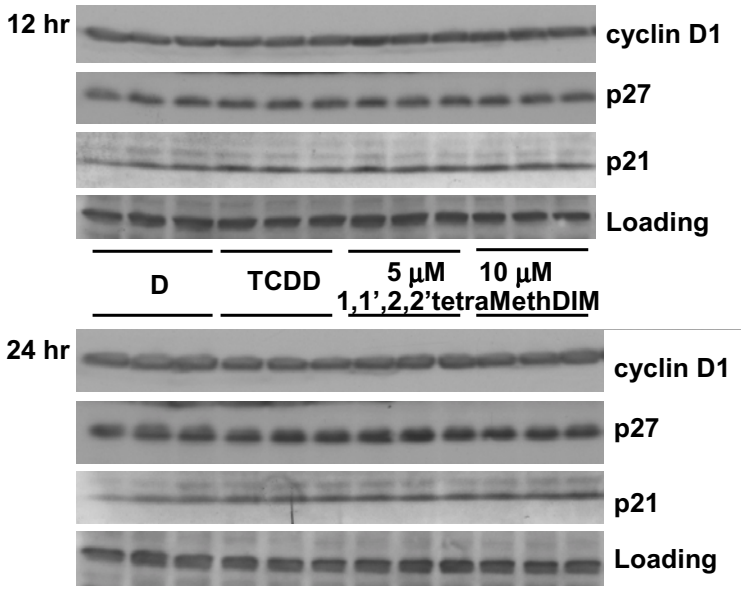


Figure 3.40: Cell cycle protein levels after 12 and 24 hours of treatment in MDA-MB-453 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 5 or 10.0 μM 1,1',2,2',tetraMethDIM; or 10 nM TCDD. After 12 and 24 hours of treatment, the cells were lysed in 1X laemmli buffer and whole cell lysates used for Western blot analysis. Representative Western blots are shown.

1,1',2,2'-tetraMethDIM did not inhibit the phosphorylation of ErbB2 in BT-474 cells and inhibition of downstream components, ERK1/2 and Akt, also was not observed in BT-474 or MDA-MB-453 cells by Western blots. It is possible that 1,1',2,2'-tetraMethDIM is acting downstream of activated ERK1/2 and Akt. Therefore, we examined the effects of 1,1',2,2'-tetraMethDIM on activation of Elk-1 and Srf using chimeric GAL4-fusion proteins and a construct containing 5 tandem GAL4 response elements (pGAL4). Elk-1 and Srf are downstream targets of MAPK and Akt respectively and activation of these transcription factors is required for induction of several growth-regulatory genes (references). MDA-MB-453 cells were used for the transfection studies due to poor transfection efficiencies observed in BT-474 with several transfection reagents including calcium phosphate, Lipofectamine (Invitrogen), and Superfect (Qiagen). MDA-MB-453 cells were transfected with pGAL4 and either an empty pM (GAL4) expression vector or one expressing a Srf-GAL4 fusion protein or an Elk1-GAL4 fusion protein. Active Srf- or Elk1-GAL4 fusion proteins will bind to the GAL4 promoter and induce expression of luciferase protein, therefore a luciferase assay can be used as an indirect measure of Srf and Elk1 activation. The Elk1-GAL4 fusion vector did not increase luciferase activity above the empty pM vector control (Figure 3.41). The assay depends on activation of the Srf-GAL4 and Elk1-GAL4 fusion proteins by constitutively active PI3K/Akt and

MAPK pathways in the MDA-MB-453 cells. Because the Elk1-GAL4 fusion protein was not activated by the MAPK pathway in the MDA-MB-453 cells, we were unable to further determine inhibition of Elk1-dependent activity by 1,1',2,2'-tetraMethDIM using this assay. The Srf-GAL4 fusion vector showed increased luciferase activity above the empty pM vector and treatment with LY294002 inhibited luciferase activity; however treatment with 1,1',2,2'-tetraMethDIM increased luciferase activity above the DMSO control (Figure 3.41). Therefore, 1,1',2,2'-tetraMethDIM does not inhibit activation of Srf in MDA-MB-453 cells in transfection studies, but surprisingly induces activity. These results indicate that activation of Srf and Elk-1 was not inhibited by 1,1',2,2'-tetraMethDIM suggesting that the growth inhibitory effects of this compound were not related to direct inhibition of ErbB2, Akt, or MAPK kinase signaling pathways.

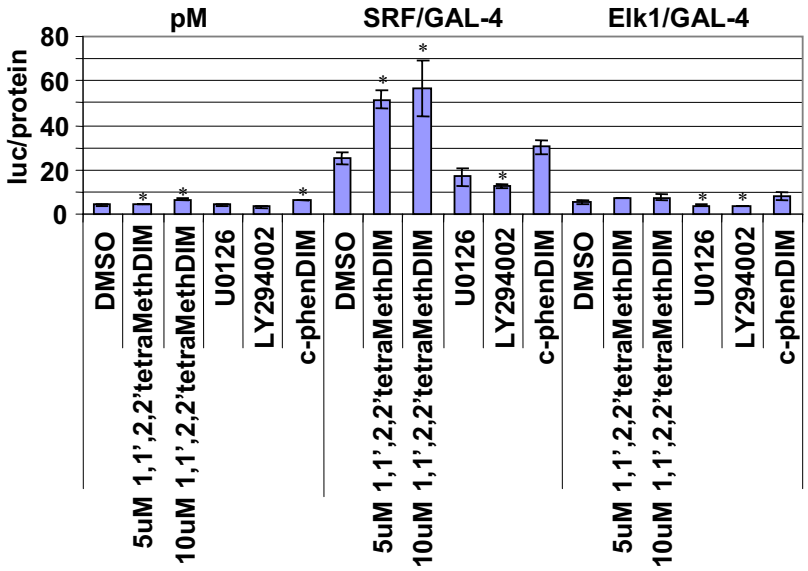


Figure 3.41: Srf and Elk1 activity in transient transfection assays with MDA-MB-453 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and transfected with the empty pM vector, the Srf-Gal4 expression plasmid or the Elk1-Gal4 expression plasmid and the Gal4 promoter-luciferase reporter plasmid using the standard calcium phosphate method. The transfected cells were dosed with DMSO; 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM; 10 μM U0126; 10 μM LY294002; or 10 μM 1,1-bis(3'-indolyl)-1-(phenyl)methane (c-phenDIM). Thirty-six hours after treatement the cells were lysed and assayed for luciferase activity and total protein concentration. Luciferase activity was normalized to total protein concentration for each sample and mean ± SE is plotted (n=3). Significant ($p<0.05$) changes are indicated (*).

TGFβ1 induces growth arrest and apoptosis in many cell types and these effects are mediated through kinase receptors and downstream intracellular effectors termed SMADs. TGFβs activate serine/threonine kinase receptors that propagate the signal through phosphorylation of SMAD2 and 3. Active SMAD2 and 3 then bind to SMAD4 and the resulting complexes localize to the nucleus and interact with specific promoter sequences to control gene expression. Therefore, the potential role of the TGFβ/SMAD pathway in mediating inhibition of cell proliferation by 1,1',2,2'-tetraMethDIM was further investigated using a mammalian one-hybrid system to determine the activation of SMAD2- and SMAD4-GAL4 fusion proteins. MDA-MB-453 cells were transfected with pGAL4 and either an empty pM expression vector or vectors expressing SMAD2-GAL4 or SMAD4-GAL4 fusion proteins. Transfection with the SMAD2 and SMAD4 fusion proteins increased the luciferase activity above that of the pM empty

vector, with higher activities observed for SMAD4 compared to SMAD2 (Figure 3.42). TGFβ1 was used as a positive control to show that activation of the SMAD pathway in the MDA-MB-453 cells leads to activation of the transfected fusion proteins and thus increased luciferase activity. Treatment of MDA-MB-453 cells with 10 ng/ml TGFβ1 induced transactivation in cells transfected with SMAD2-GAL4 whereas 5 or 10 μM 1,1',2,2'-tetraMethDIM, DIM, and MCDF had minimal effects (Figure 3.42). Ten ng/ml TGFβ1 also induced luciferase activity in cells transfected with SMAD4-GAL4 and 5 and 10 μM 1,1',2,2'-tetraMethDIM (but not DIM or MCDF) also induced transactivation. These results indicate that one mechanism of growth inhibition of MDA-MB-453 cells by 1,1',2,2'-tetraMethDIM may be through activation of the TGFβ1/SMAD pathway downstream of SMAD2 and upstream of SMAD4.

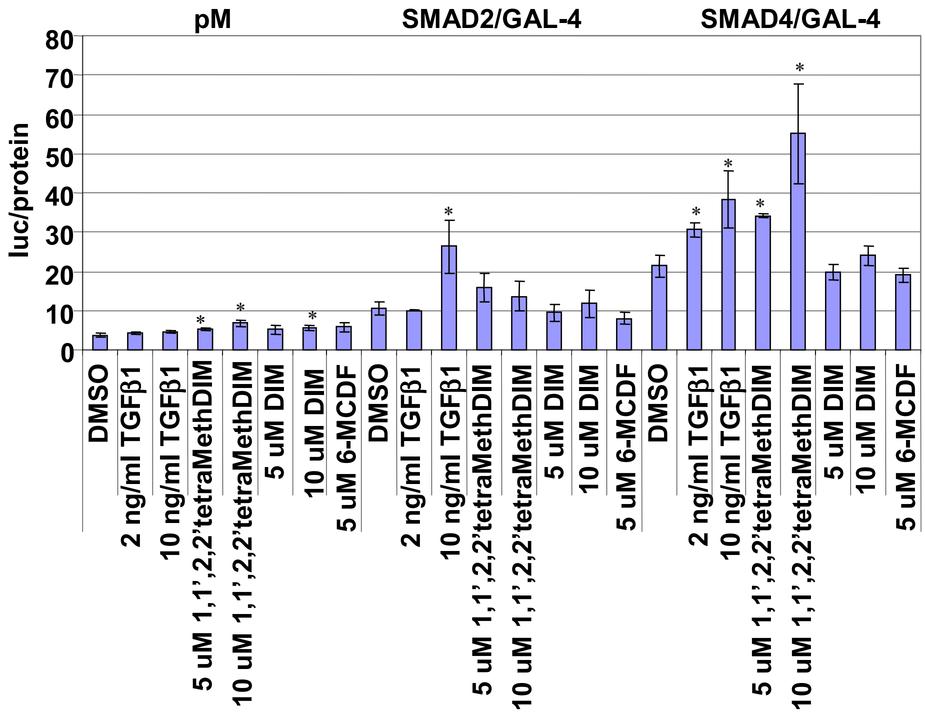


Figure 3.42: SMAD2 and SMAD4 activity in transient transfection assays with MDA-MB-453 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and transfected with the empty pM vector, the SMAD2-Gal4 expression plasmid or the SMAD4-Gal4 expression plasmid and the Gal4 promoter-luciferase reporter plasmid using the standard calcium phosphate method. The transfected cells were dosed with DMSO; 2ng/ml or 10 ng/ml TGFβ1 (PeproTech, Inc.); 5.0 or 10.0 μM 1,1',2,2',tetraMethDIM; 5 or 10 μM DIM; or 5 μM 6-MCDF. Thirty-six hours after treatment the cells were lysed and assayed for luciferase activity and total protein concentration. Luciferase activity was normalized to total protein concentration for each sample and mean ± SE is plotted (n=3). Significant ($p<0.05$) changes are indicated (*).

3.3.6 Downregulation of the AhR and induction of cyp1A1 by 1,1',2,2'-tetraMethDIM compared to other AhR agonists in MCF-7, BT-474 and MDA-MB-453 cells

AhR agonists exhibit different receptor binding affinities as well as different potencies as inducers of AhR-dependent responses such as downregulation of the receptor itself and induction of cyp1A1 protein. These studies compared the effects of TCDD, 6-MCDF, DIM and 1,1',2,2'-tetraMethDIM on downregulation of the AhR and induction of cyp1A1 protein in MCF-7, BT-474 and MDA-MB-453 cells. Cells were treated for 24 hours and whole cell lysates were analyzed by Western blot analysis to compare AhR, cyp1A1 and ERα protein levels in the various treatment groups. Treatment of MCF-7 cells with TCDD and 6-MCDF resulted in a high induction of cyp1A1 protein accompanied by downregulation of AhR protein (Figure 3.43). 1,1',2,2'-tetraMethDIM exhibited minimal induction of cyp1A1 in MCF-7 cells; however, this was not accompanied by downregulation of AhR protein. DIM and E2 had no effect on cyp1A1 or AhR proteins; however, treatment with TCDD, 6-MCDF, DIM, and E2 all enhanced downregulation of ERα in MCF-7 cells compared to untreated controls. In contrast, treatment with 1,1',2,2'-tetraMethDIM did not affect ERα levels (Figure 3.43). In BT-474 cells all the AhR agonists induced

cyp1A1 protein expression with similar induction potencies for 10 nM TCDD, 10 μM 1,1',2,2'-tetraMethDIM, and 5 μM 6-MCDF, whereas lower levels were observed in cells treated with DIM. In contrast, only TCDD and 6-MCDF downregulated AhR protein (Figure 3.44). DIM treatment did not induce downregulation of AhR. BT-474 cells did not express levels of ERα protein as determined in Western blot analysis. Previous reports of ERα expression in BT-474 cells are conflicting, some studies report expression of low levels of ERα and others refer to BT-474 cells as ERα negative cells lines (Elstner et al., 1995; Grunt et al., 1995; Magklara et al., 2000). MDA-MB-453 cells exhibited the lowest level of response to the AhR agonists. Only TCDD induced cyp1A1 protein and downregulated AhR protein (Figure 3.45). MDA-MB-453 cells have previously been characterized as an ERα negative cell line, and ERα was not detected in this study.

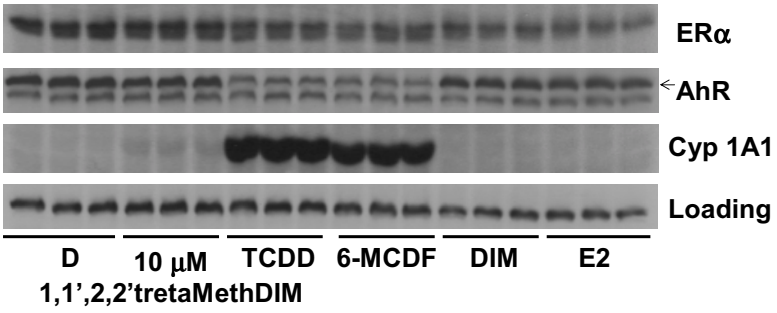


Figure 3.43: AhR downregulation and Cyp1A1 induction in MCF-7 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 10.0 μM 1,1',2,2',tetraMethDIM; 10 nM TCDD, 5 μM 6-MCDF, 10 μM DIM, or 1 nM E2. After 24 hours of treatment, the cells were lysed in 1X laemmli buffer and whole cell lysates used for Western blot analysis. Representative Western blots are shown.

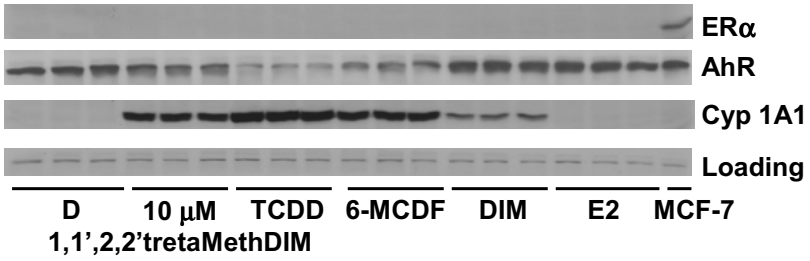


Figure 3.44: AhR downregulation and Cyp1A1 induction in BT-474 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 10.0 μM 1,1',2,2',tetraMethDIM; 10 nM TCDD, 5 μM 6-MCDF, 10 μM DIM, or 1 nM E2. After 24 hours of treatment, the cells were lysed in 1X laemmli buffer and whole cell lysates used for Western blot analysis. A sample of DMSO treated MCF-7 whole cell lysates is also loaded as a positive control for ERα protein. Representative Western blots are shown.

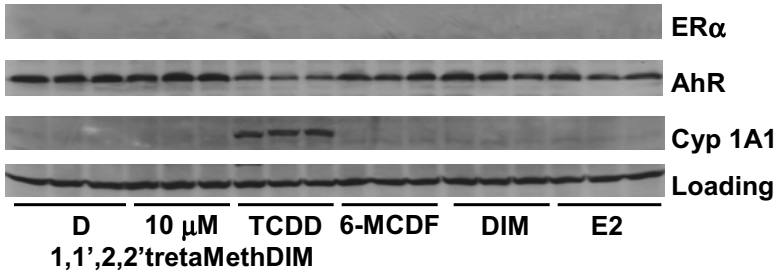


Figure 3.45: AhR downregulation and Cyp1A1 induction in MDA-MB-453 cells. Cells were seeded in media containing 2.5% charcoal-stripped FBS and dosed with DMSO; 10.0 μM 1,1',2,2',tetraMethDIM; 10 nM TCDD, 5 μM 6-MCDF, 10 μM DIM, or 1 nM E2. After 24 hours of treatment the cells were lysed in 1X laemmli buffer and whole cell lysates used for Western blot analysis. Representative Western blots are shown.

3.3.7 Inhibition of tumor development and growth in MMTV-c-neu mice by 1,1',2,2'-tetraMethDIM

The MMTV-c-neu mouse mammary tumor model expresses a mutated form of the rat c-neu (ErbB2) protein under the mouse mammary tumor virus promoter. This murine model exhibits well characterized morphological changes in the mammary gland with palpable mammary tumors forming around 25 weeks of age (Muller et al., 1988). Recently it has been reported that 1,1',2,2'-tetraMethDIM inhibited DMBA-induced mammary tumor growth in rats (McDougal et al., 2001) and in vitro data in this study show that 1,1',2,2'-tetraMethDIM inhibits growth of breast cancer cell lines that overexpress ErbB2 (Figures 3.26 and 3.27). Therefore, an initial study examined the potential antitumor activity of 1,1',2,2'-tetraMethDIM as an inhibitor of development and growth of tumors in MMTV-c-neu mice. MMTV-c-neu mice were dosed with 1,1',2,2'-tetraMethDIM (10 mg/kg) or a corn oil control every other day for 4 weeks starting at 22 weeks of age. Mice were palpated for tumors at each

dosing and after initial detection tumors volumes were determined every second day. After 4 weeks, tumors were removed for final measurement and fixed for analysis and further studies. The results (Figure 3.46) show that a mouse from the control group developed a tumor within 2 days after initiating treatment and after 4 weeks all the control (untreated) mice developed at least one tumor. Tumors did not appear in the 1,1',2,2'-tetraMethDIM treatment group until 14 days after initial treatment and one of the mice treated with 1,1',2,2'-tetraMethDIM did not develop mammary tumors by the end of the study. Two of the control mice developed fast growing tumors 12 and 24 days into the study compared to the slow growing tumors that appeared in the 1,1',2,2'-tetraMethDIM treated mice, only one of which grew to more than 200 cm^3 after 12 days of growth (Figure 3.46). This initial study indicates that 1,1',2,2'-tetraMethDIM may decrease the incidence and growth of mammary tumors in MMTV-c-neu mice and warrants further research.

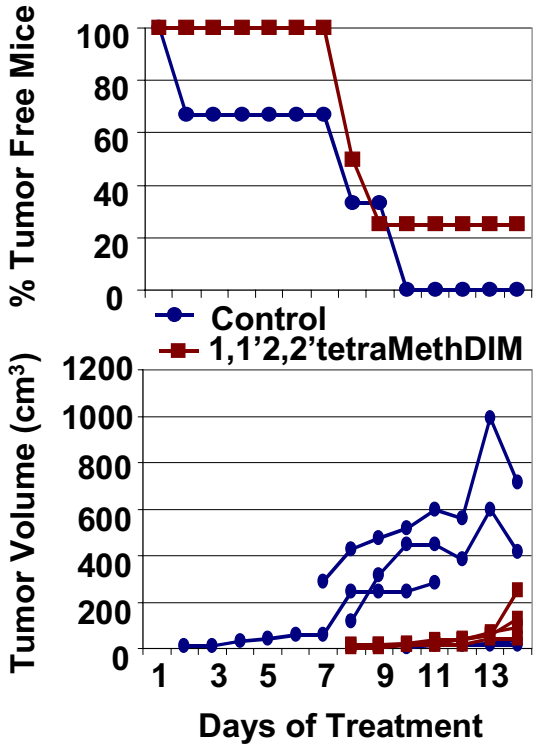


Figure 3.46: Tumor incidence and growth in MMTV-c-neu mice. Twenty-two week old MMTV-c-neu mice were treated with 10 mg/kg 1,1',2,2'-tetraMethDIM or corn oil by gavage and palpable mammary tumor were measured and recorded every other day for 28 days. The percent tumor free mice and tumor volumes are plotted. (n=3 control, n=4 1,1'2,2'tetraMethDIM).

CHAPTER IV

DISCUSSION AND CONCLUSIONS

4.1 AhR-ERα crosstalk in uterine gene expression

Hormone or mitogenic polypeptide stimulation of the rodent female reproductive tract has been extensively investigated in both immature and ovariectomized rodents and in ERα and ERβ knockout mouse models. A comparison of E2-responsiveness in uteri from ERKO and βERKO mice suggests that ERα plays a dominant role in normal uterine development (Lubahn et al., 1993; Krege et al., 1998a; Couse and Korach, 1999; Hewitt and Korach, 2003). Moreover, there is evidence from studies in βERKO mice that ERβ may inhibit ERα-induced responses; for example, in βERKO mice (compared to wildtype mice) enhanced E2-responsiveness and higher levels of progesterone receptor and Ki-67, a proliferation associated protein, were observed (Weihua et al., 2000). In contrast, uteri from ERKO mice are poorly developed and hypoplastic, and after treatment with E2 the prototypical increase in uterine wet weight and vaginal cornification are not observed (Lubahn et al., 1993). Growth factors such as EGF also activate ERα-dependent pathways/genes in the mouse uterus and EGF induces increases in uterine DNA synthesis and progesterone receptor mRNA levels in wildtype but not ERKO mice (Curtis et al., 1996). In contrast, some estrogenic responses such as

induction of c-fos are induced by EGF in both wildtype and ERKO mice demonstrating an ERα-independent pathway for c-fos gene expression by EGF in the mouse uterus. Recent studies show that E2, estrogenic metabolites, or synthetic estrogens also induce expression of multiple genes that are independent of ERα and ERβ and their induction is not inhibited by 'pure' antoestrogens such as ICI 182,780 (Das et al., 1997; Banerjee et al., 2003; Lee et al., 2003).

Cyclin D1 plays a critical role in cell proliferation and progression of cells from G_0/G_1 through S phase of the cell cycle and DNA polymerase α is necessary for DNA synthesis in S phase of the cell cycle. Both genes are induced by E2 and cotreatment with TCDD inhibits the E2-induced response in vitro in breast cancer cell lines through ERα. E2-induced cyclin D1 expression in the mouse uterus has also been demonstrated (Geum et al., 1997; Wang et al., 1998; Castro-Rivera et al., 2001; Samudio et al., 2001; Buchanan et al., 2002). Therefore, we hypothesized that cyclin D1 and DNA polymerase α would be appropriate model genes for investigating inhibitory AhR-ERα crosstalk in wildtype and transgenic mice. The results in Figure 3.1 and 3.3 demonstrate that treatment with E2 induced uterine cyclin D1 and the 180 kDa catalytic subunit of DNA polymerase α gene expression in B6C3F1 mice, and maximal expression was observed 6-12 h after treatment with E2 in the luminal epithelial cells. These results for cyclin D1 gene expression agree with previous studies that demonstrated maximal increase in total uterine cyclin D1 expression 6-12 h

after treatment with E2 and the time course of both cyclin D1 and 180 kDa catalytic subunit of DNA polymerase α gene expression correlates with previous studies showing maximal proliferation 12 to 24 hours after single treatment with E2 (Quarmby and Korach, 1984; Couse et al., 1997; Geum et al., 1997). Furthermore, the lack E2-induced cyclin D1 and DNA polymerase a catalytic subunit mRNA expression in uteri from ERKO-/- mice (Figures 3.4-3.7) demonstrate the requirement for ERα in the E2-induced response.

A slight induction of cyclin D1 and DNA polymerase α catalytic subunit gene expression was seen in the stromal compartment 3 h after treatment and persisted through 12 h in the B6C3F1 mice (Figures 3.1 and 3.3). In contrast, there was considerable diffuse background staining and staining of glands for cylin D1 mRNA in the uterine stroma of untreated ERKO+/+ mice and AhRKO mice at 6 hours after treatment (Figures 3.4 and 3.16) and for DNA polymerase α catalytic subunit mRNA in the uterine stroma of untreated ERKO+/+ mice (Figure 3.6) and this did not change after treatment with E2. Similarly, after 12 h treatment in the ERKO +/+ and AhRKO mice, although the overall staining of the uterine tissue for cyclin D1 and DNA polymerase α catalytic subunit was weaker, an induction of cyclin D1 gene expression was not seen in the stromal compartment of the uterus with hormone treatment in either mouse model (Figure 3.5 and 3.17). Induction of DNA polymerase α catalytic subunit gene expression was not observed in the stromal compartment of ERKO+/+ mice (Figures 3.7). In contrast, DNA polymerase α catalytic subunit mRNA was

induced in uterine stromal cells of AhRKO mice after 6 h treatment and some induction was also detected after 12 h (Figures 3.18 and 3.19). Since ERα is expressed in both uterine stromal and epithelial cells (Couse and Korach, 1999) and E2 induces proliferation in both stromal and epithelial cells of the immature mouse uterus (Quarmby and Korach, 1984), it is not surprising that hormone-induced cyclin D1 and DNA polymerase a catalytic subunit gene expression is observed primarily in luminal epithelium but also noted in stromal uterine cells of the immature mouse uterus in B6C3F1 mice. Higher glandular development and glandular epithelial staining for cyclin D1 mRNA were observed in the uteri from ERKO+/+ and AhRKO mice (Figures 3.4, 3.5, 3.16, and 3.17) and even the B6C3F1 mice used for E2 and TCDD cotreatment studies (Figures 3.10 and 3.11) compared to the uteri from B6C3F1 mice used in the initial timecourse studies (Figure 3.1). Proliferation is seen only in the luminal epithelium of mature adult uteri, and since glandular development is a sign of maturation, perhaps the uteri of these mice are slightly more developed and thus E2-induced proliferation and the need for cyclin D1 and DNA polymerase α catalytic subunit expression in the stroma is reduced.

Previous studies using tissue recombination of stromal and epithelial cells from wildtype and ERKO mice show that in ovariectomized nude mice bearing stromal and epithelial tissue recombinants as subrenal capsule grafts, hormone-induced DNA synthesis /proliferation was primarily dependent on stroma from ER-postive animals (Cooke et al., 1997; Buchanan et al., 1999). It was

concluded that “epithelial ER is neither necessary nor sufficient for E2-induced uterine epithelial proliferation” (Cooke et al., 1997), and the proposed model for epithelial cell proliferation was associated with a paracine pathway and dependent on induction of ERα-dependent stromal factors. Nevertheless, other E2-induced genes such as lactoferrin and complement C3 can be induced directly in uterine epithelial cells (Buchanan et al., 1999).

Previous studies by Geum and coworkers (Geum et al., 1997) show that E2 induced cyclin D1 mRNA levels in uterine tissue of immature female ICR mice, and their time course study for cyclin D1 mRNA was comparable to the results illustrated in Figure 3.1 with maximal induction observed after 6-12 hours. Moreover, since induction of cyclin D1 mRNA is primarily localized to the luminal epithelial cells with only slight induction in the stromal cells (Figure 3.1), induced cyclin D1 mRNA in whole uterine extracts must be primarily due to increased cyclin D1 mRNA in epithelial cells (Geum et al., 1997). It was also reported that the protein synthesis inhibitor cycloheximide did not affect hormonal activation of uterine cyclin D1 (Geum et al., 1997) suggesting that induction of cyclin D1 by E2 in luminal epithelial cells observed in this study may be due to a direct effect on the epithelial cells and not related to ERα-dependent stromal paracrine factors. Similarly, our studies demonstrate that treatment with cycloheximide does not affect E2-induced cyclin D1 or DNA polymerase α catalytic subunit gene expression in the luminal epithelial cells (Figures 3.22 and 3.23) or E2-induced increase in uterine wet weights (Figure 3.21). This suggests that the

stromal cell-dependent proliferation of uterine epithelial cells observed in tissue recombination studies may not involve cyclin D1 or DNA polymerase α but other genes/proteins critical for cell proliferation. However, the tissue recombination studies use stromal and epithelial cells from adult mice and our study as well as the study by Guem and coworkers used immature mice (Cooke et al., 1997; Geum et al., 1997; Buchanan et al., 1999). Therefore, the difference in the requirement for paracrine factors from the stromal for epithelial responses could also be due to the different developmental stages of the uterine tissue studied. Differences in the ability of the adult and immature uterus to respond to treatment with E2 have been demonstrated previously. Stromal and epithelial uterine tissue proliferate in response to E2 in the immature mouse, whereas stromal uterine tissue in adult mouse loses the ability to respond and only the epithelium proliferates in response to hormone. Therefore, ERα signaling in the mature adult uterus differs from that of the immature uterus and could account for the difference in direct vs. indirect action of E2 on the luminal epithelial cells.

Insufficient inhibition of protein synthesis by cycloheximide treatment may also have allowed a high enough level of paracrine factors to be produced to elicit an E2-induced response in the luminal epithelial cells of the uterus. Guem and coworkers (Geum et al., 1997) did not include a positive control in their study demonstrating that the dose of cycloheximide was sufficient to inhibit protein synthesis (Geum et al., 1997). We used the inhibition of TCDD-induced cyp1A1 protein expression in the liver as a control for cycloheximide treatment.

Protein expression of cyp1A1 was inhbited in the TCDD and TCDD+E2 mice treated with cyclohexmide compared to those not treated with cylcloheximide; however, 4 out of the 8 TCDD and TCDD+E2 animals treated with cycloheximide do show a low level of cyp1A1 expression compared to the absence of any cyp1A1 protein without TCDD treatment (Figure 3.20). Therefore, a low level of protein synthesis occurred in the livers of some animals treated with cycloheximide and may also have occurred in the uterus. It could be argued that even partial inhibiton of protein synthesis would lead to lower levels of paracrine factors produced and a decrease in the luminal epithelial response to E2; however, a threshold level may be all that is necessary for a full luminal epithelial response. Many in vivo studies with cycloheximide treatment limit the time course to 6 hours to ensure that maximal inhibiton of protein synthesis throughout the study because the effectiveness of cycloheximide may decrease at later timepoints.

Previous studies have demonstrated that activation of the AhR complex by TCDD and related compounds results in inhibitory AhR-ERα crosstalk in the rodent uterus and in breast cancer cells (Wang et al., 1998; Safe and McDougal, 2002). The results in Figures 3.10 and 3.11 show that in B6C3F1 mice cotreated with E2+TCDD, there was decreased luminal epithelial cyclin D1 mRNA expression after 6 or 12 h compared to animals treated with E2 alone, and the inhibitiory response was more pronounced after 12 h. Similarly, Figures 3.12 and 3.13 show that cotreatment with E2+TCDD in B6C3F1 mice resulted in

a decreased luminal epithelial DNA polymerase α catalytic subunit mRNA expression at 6 and 12 h compared to animals treated with E2 alone; moreover, the inhibitory response was as effective at 6 h as it was at 12 h. In contrast, TCDD did not inhibit activation of cyclin D1 or DNA polymerase a catalytic subunit gene expression in AhRKO mice (Figures 3.16-3.19) confirming a role for the AhR in mediating this inhibitory response. Using the tissue recombination approach with uterine stroma and epithelium from wildtype and AhRKO mice, Buchanan and coworkers showed that inhibition of E2-induced uterine epithelial cell proliferation and lactoferrin mRNA levels by TCDD was dependent on the stromal AhR (Buchanan et al., 2000). We have demonstrated that in the immature female mouse model, E2 acts directly on the luminal epithelial cells and does not require paracrine factors from the stroma; therefore, if the AhR is required in the stroma then stromal-epithelial cell interactions are necessary for the inhibitory response.

Several possible mechanisms of inhibitory AhR-ERα crosstalk have been described including direct interaction of the AhR complex with 5'-promoter inhibitory dioxin response elements, competition with ERα for common coregulatory proteins, activation of proteasome-dependent degradation of ERα, or induction /activation of nuclear factors that inhibit hormone activation of gene expression (Gierthy et al., 1988; Badawi et al., 2000; Klinge et al., 2000; Carlson and Perdew, 2002; Ohtake et al., 2003; Wormke et al., 2003). Most of these inhibitory pathways are intracellular, whereas a stromal influence on the

inhibitory AhR-ERα crosstalk must induce other paracrine-dependent inhibitory pathways. In contrast, Figures 3.22 and 3.23 show that inhibition of protein synthesis by cycloheximide treatment does not affect the inhibition of E2-induced cyclin D1 or DNA polymerase α catalytic subunit gene expression by TCDD. Therefore, the inhibitory AhR-ERα crosstalk for gene expression in these studies most likely involves an intracellular mechanism rather than a paracrine dependent pathway.

The conflicting results between our AhR-ERα cross talk studies and the tissue recombination studies are analogous to the differences between the results of our studies on E2-induced luminal epithelial responses and the tissue rescombination studies. Primarily, the uteri used were at different levels of development and the requirements for AhR-ERα crosstalk may change as ERα signaling changes in the maturing uterus. It could be that the AhR is required in the stroma of adult tissue because E2-induced responses depend on stromal ERα and induced stromal factors. Tissue recombination studies in the adult uterus have demonstrated that ERα and the AhR are necessary in the stroma for E2-induction of luminal epithelial responses and for inhibition of the responses by TCDD. Therefore, ERα and AhR receptors are required in the same cell types and the mechanism of AhR-ERα crosstalk could be one of the intracellular inhibitory mechanisms that have been described. However, if E2 is acting directly on the luminal epithelial cells in the immature uterus to induce a response, then tissue recombination studies with immature uteri may show that

the AhR is required in the luminal epithelial cells to inhibit the E2-induced response through one of the intracellular inhibitory mechanisms described. Furthermore, as described above for E2-induced responses in the luminal epithlelium, incomplete inhibtion of protein synthesis could also account for the conflicting results. A low level of paracrine factors produced in the stroma may be sufficient to inhibit E2-induced responses in the luminal epithelial cells.

The growth factor VEGF is involved in postnatal angiogenesis and studies in humans and non-human primates have shown regulation of VEGF throughout the menstrual cycle to prepare the stromal compartment of the uterus for implantation (Nayak and Brenner, 2002; Sugino et al., 2002). Previous studies in the rat uterus have shown that treatment with E2 results in rapid upregulation of VEGF mRNA in the stromal compartment within 1 hour after treatment. The localization and timing of E2-induced VEGF expression in the uterus described in previous reports differed from that of cyclin D1 and therefore, it was used as a third model gene to investigate AhR-ERα crosstalk in wildtype and transgenic mice. Initial studies in B6C3F1 mice demonstrated E2-induced expression patterns similar to that seen previously in the rat (Figure 3.2). VEGF mRNA was induced throughout the stroma of the uterus with maximal expression 1 and 3 h after treatment. Similarly, VEGF gene expression was induced by E2 in the stroma of ERKO+/+ uteri; however, no induction of VEGF gene expression was seen with hormone treatment in ERKO-/- mice (Figure 3.8) demonstrating the requirement for ERα in the E2-induced gene expression in the stromal tissue. In

contrast to the ability of TCDD to inhibit E2-induced cyclin D1 and DNA polymerase α gene expression, TCDD did not inhibit E2-induced VEGF gene expression in the uterine stroma after 1 or 3 h (Figures 3.14 and 3.15). The induction of VEGF mRNA by E2 differed from E2-induced cyclin D1 and DNA polymerase α gene expression in both timing, with maximal induction at 1 to 3 h vs. 6 to 12 h, and localization, with strong stromal induction vs. slight stromal induction and strong luminal epithelial induction. TCDD inhibited E2-induced DNA polymerase α catalytic subunit gene expression in the stromal cells of B6C3F1 mice (Figures 3.12 and 3.13); therefore the lack of TCDD inhibition of E2-induced VEGF gene expression is likely not due to the stromal location of the mRNA induction. However, the rapid induction of VEGF mRNA by E2 could account for the inability of TCDD to inhibit the response in our experimental model. Mice were cotreated with TCDD and E2 at the same time. It is possible that AhR-mediated inhibition of hormone-induced VEGF expression requires more prolonged treatment with TCDD and induction of inhibitory factors. Future studies will investigate this possibility.

4.2 ErbB2-induced tumor development in MMTV-c-neu mice

ErbB2 and the ErbB family of receptors are involved in normal growth and differentiation of the breast (Carraway et al., 1997) and have been implicated in carcinogenesis as well (Slamon et al., 1989). Activation via the tyrosine kinase region of the receptors mediates cell proliferation, differentiation, and survival

(Salomon et al., 1995; Schlessinger, 2000; Simon, 2000). Dysregulation of ErbB2 signaling pathways can result from receptor gene amplifications or mutations that lead to an increase in receptor transcription, translation, or stability altering the receptor protein expression levels in the cells. ErbB2 overexpression in observed in 20-30% of breast tumors (van de Vijver et al., 1987; McCann et al., 1991) and is associated with aggressive tumor behavior and poor prognosis (Hynes and Stern, 1994). One study showed that 92% of overexpression of ErbB2 in breast cancer was due to gene amplification (Pauletti et al., 1996) and overexpression is found in all stages of tumor development, but not in benign tissue (Allred et al., 1992). However, the role of ErbB2 in tumor formation is unclear.

Transgenic mice provide a useful model to assess the role of oncogenes in tissue-specific tumor induction and growth in vivo. To study the contribution of an oncogene to mammary tumor formation, a gene can be fused to the MMTV promotor, which drives expression in mammary tissue, and the construct introduced into the genome of mice. Tumor formation and growth can then be assessed to determine the effects of overexpression of the oncogene. Studies of transgenic mice carrying MMTV-c-myc and MMTV-v-Ha-ras fusion contructs have shown development of solitary mammary adenocarcinomas in a stochastic manner that appear clonal in origin (Stewart et al., 1984; Leder et al., 1986; Sinn et al., 1987). This indicates that the expression of c-myc or v-Ha-ras alone is not sufficient for transformation of mammary epithelial cells, additional events are

necessary for malignant transformation. Multiple transgenic lines carrying MMTV-c-neu (ErbB2), either an activated or a wildtype c-neu, have been developed to assess the role of ErbB2 expression and activation in mammary tumor formation. Some transgenic MMTV-c-neu lines indicate that further events in addition to expression of an activated ErbB2 are necessary for mammary epithelial cell transformation; however, multiple other lines indicate that overexpression of an activated ErbB2 receptor is sufficient for mammary epithelial cell transformation (Muller et al., 1988; Bouchard et al., 1989; Guy et al., 1992).

For these studies we used an MMTV-c-neu line expressing an activated ErbB2 receptor available from Charles River Laboratories that was developed by Muller and coworker (Muller et al., 1988) and designated TG.NK in the original publication. Studies of this line and others developed by Muller and coworkers indicated that expression of the activated ErbB2 receptor is sufficient for transformation and tumor development in mammary epithelium (Muller et al., 1988). Expression of activated ErbB2 is found in transformed epithelium but not in normal mammary epithelium in these mice and expression of the activated ErbB2 receptor occurs in a stochastic manner as the mice age so that transformed epithelium and tumors are found surrounded by normal mammary epithelium (Muller et al., 1988). These transgenic mice provide a consistent model in which activated ErbB2 leads to transformation of the mammary

epithelium and eventual tumor formation around 25 weeks of age in 100% of the animals.

In order to better understand how ErbB2 leads to transformation of the mammary epithelium we wanted to determine changes in gene expression profiles that occur during transformation in these MMTV-c-neu mice. Therefore, mammary glands were collected at 6, 12 and 18 weeks of age from MMTV-c-neu mice and from FVB mice, which is the background strain of the MMTV-c-neu transgenic line. Theoretically, in the mammary gland of young mice the activated ErbB2 is not expressed and the mammary epithelium is normal, as the mice age activated ErbB2 begind to be expressed in certain mammary epithelial cells leading to changes in gene expression that result in transformation of the cells, and the older the mice, the more cells expressing activated ErbB2 and the higher the levels of changes in gene expression. For these studies we are assuming the expression of the activated ErbB2 is random throughout the mammary epithelium of all the mammary glands. Mammary glands #1 and #2 were used for histological analysis because their location made isolation of mammary tissue without other contaminating tissue difficult and mammary gland #3, #4, and #5 were used for RNA isolation and microarray analysis of gene expression. Histological analysis of serial sections of the mammary gland can assess the level of transformation of the mammary epithelium at each time point and the changes in the histopathology can be correlated with changes in gene expression profiles in the mammary glands at each time point. Because ErbB2

is not available on the commercial Affymetrix or CodeLink microarray chips it is necessary to also analyze ErbB2 expression levels by RT-PCR and immunohistochemistry to compare differences in ErbB2 expression between mice and mammary glands with differences in gene expression profiles.

Initial histopathological analysis of the mammary glands of 6 week old MMTV-c-neu and FVB found no differences between the transgenic and the background mice. There was no indication of transformation in the mammary epithelium of the MMTV-c-neu mice. Furthermore, there was little change in the gene expression profies of the mammary glands between the MMTV-c-neu and FVB mice based on microarray analysis using the Affytmetrix MG-U74Av2 chip and expression analysis using GeneSpring expression analysis software. As shown in Table 3.1, of the 12,520 genes only 32 were upregulated greater than two fold and only 23 were downregulated greater than two fold when comparing three MMTV-c-neu and two FVB mice and few of these genes were changed greater than 3 fold (Tables 3.2 and 3.3). Initial analysis has demonstrated little variation in gene expression within the MMTV-c-neu and FVB mice indicating that two animals per group may be enough for analysis at the 12 and 18 week time points (Table 3.4, Figure 3.24). However, the 12 and 18 week old animals will also be going through the estrous cycle and changes in hormone levels may lead to changes in mammary gland gene expression profiles within the MMTV-c-neu and FVB groups resulting in more variation and the need for more animals within each group for analysis. Further analysis of gene expression changes at

6 weeks of age and analysis of gene expression in the mammary glands of mice at 12 and 18 weeks of age will increase our understanding of ErbB2-induced mammary epithelial cell transformation.

4.3 Inhibition of ErbB2-mediated responses by 1,1',2,2'-tetraMethDIM, an AhR agonist

The antiestrogenic properties as well as the possible downregulatory effect on the ErbB family of receptors by AhR agonist warrant the further development SAhRMs for breast cancer chemotherapy where the compounds exhibit minimal toxicity but retain the antiestrogenic/antitumorigenic effects. One series of SAhRMs our lab has focused on is ring-substituted diindolylmethanes (DIMs) that are able to inhibit mammary tumor growth in rodent models while exhibiting relatively low toxicity (McDougal et al., 1997; Chen et al., 1998a; McDougal et al., 2000; McDougal et al., 2001). Recently it has been reported that the SAhRM 1,1',2,2'-tetraMethDIM inhibited DMBA-induced mammary tumor growth in rats and also inhibited constitutively active MAPK and p110 (catalytic subunit of PI3-K) in vitro in MCF-7 human breast cancer cells. Several ErbB2-overexpressing breast cancer cells such as the BT-474 and MDA-MB-453 cell lines express a functional AhR and exhibit constitutive activation of both the MAPK and PI3K pathways. Therefore we investigated inhibitory AhR-ErbB2 interactions of 1,1',2,2'-tetraMethDIM in BT-474, MDA-MB-453 and MCF-7 cells lines.

Initially we determined whether 1,1',2,2'-tetraMethDIM would inhibit the growth of BT-474 or MDA-MB-453 cells in presence of low levels (2.5% and 5%) of charcoal-stripped fetal bovine serum or high levels (10%) of untreated fetal bovine serum, and whether 1,1',2,2'-tetraMethDIM would inhibit the basal, E2-induced or heregulin-induced growth of MCF-7 cells. Results in Figures 3.25-3.27 show that 1,1',2,2'-tetraMethDIM inhibits the growth of both BT-474 and MDA-MB-453 cells in low levels of serum, but not in the presence of 10% FBS. Most likely the high level of growth stimulatory factors present in that amount of untreated FBS masked the inhibitory actions of 1,1',2,2'-tetraMethDIM in the cells. Furthermore, as seen in Figure 3.28, 1,1',2,2'-tetraMethDIM was able to inhibit basal growth of MCF-7 cells, as well as E2 and heregulin induced growth.

Cell growth can be inhibited by cytotoxicity leading to cell lysis, alterations in the cell cycle leading to increases in the length of stages of the cell cycle or cell cycle arrest, or induction of apoptosis leading to cell death. Results in Figures 3.29 and3.30 show that 1,1',2,2'-tetraMethDIM did not induce cytotoxicity at any of the doses used in either cell line. Analysis of apoptosis induction demonstrated that 1,1',2,2'-tetraMethDIM did not activate apoptosis pathways that involved caspase-3 activation or PARP cleavage in either the BT-474 or the MDA-MB-453 cells (Figures 3.33-3.35). However, in order to fully exclude apoptosis as one of the mechanisms of 1,1',2,2'-tetraMethDIM growth inhibition, further analysis with other apoptosis assays is necessary.

Alterations in the timing of the cell cycle are apparent by changes in the percentage of cells in each stage of the cycle at a particular time. We analyzed cell cycle progression after 4 days of treatment because this time point was just prior to time points in which increasing differences in cell numbers were seen in the cell proliferation assays (Figure 3.27). As seen in Figure 3.31, treatment with 1,1',2,2'-tetraMethDIM resulted in an increase in the percentage of BT-474 cells in G_0/G_1 stage of the cell cycle. These results indicate that 1,1',2,2'-tetraMethDIM alters cell cycle progression in BT-474 cells resulting in a longer time spent in G_0/G_1 for the overall cell population or an arrest of a small population of the cells in G_0/G_1. In contrast, 1,1',2,2'-tetraMethDIM did not affect the cell cycle progression in MDA-MB-453 cells (Figure 3.32). The cell cycle proteins cyclin D1, p21 and p27 were also analyzed to determine any effects 1,1',2,2'-tetraMethDIM had on the cell cycle. Although cyclin D1 and p21 levels were not affected by treatment with 1,1',2,2'-tetraMethDIM, p27 levels were increased in a dose dependent manner after 4 days of treatment in both BT-474 and MDA-MB-453cells (Figure 3.37 and 3.38). Therefore, it seems that one mechanism of growth inhibition by 1,1',2,2'-tetraMethDIM could be through increased expression of the inhibitory protein p27. In BT-474 cells the increased p27 levels could be the cause of the altered cell cycle progression detected in BT-474 cells with an increase in cells in G_0/G_1. However, previous studies in BT-474 cells have shown that modulation of both cyclin D1 and p27 was

necessary for inhibition of cell growth by the ErbB kinase inhibitor AG1478 (Lenferink et al., 2001).

Both BT-474 and MDA-MB-453 cells have constituitively active MAPK and PI3-K pathways that regulate cell proliferation and apoptosis and inhibition of cell proliferation in these cells is likely due to inhibition of one of the kinase pathways. We analyzed phosphorylation of ErbB2, ERK1/2, and Akt to determine if 1,1',2,2'-tetraMethDIM inhibited either of these kinase pathways leading to decreased phosphorylation of the receptor or signaling molecules. However, 1,1',2,2'-tetraMethDIM did not decrease phosphorylation of ErbB2 receptor or ERK or Akt signaling molecules (Figures 3.36-3.38). Furthermore, MDA-MB-453 cells were used in transfections for the mammalian one-hybrid assay with Srf and Elk fusion proteins to determine if 1,1',2,2'-tetraMethDIM acted downstream of ERK and Akt to inhibit either kinase pathway. Srf and Elk are transcription factors downstream of the PI3-K and MAPK pathways; however, Elk was not activated in the MDA-MB-453 cells and Srf activation was not affected by 1,1',2,2'-tetraMethDIM treatment (Figure 3.41). Therefore, we found no evidence that 1,1',2,2'-tetraMethDIM was inhibiting cell growth in BT-474 or MDA-MB-453 cells through inhibition of either the MAPK or PI3-K pathways.

TGFβ1 is an inhibitor of the cell proliferation as well as an inducer of apoptosis in many cell types. The intracellular SMAD proteins mediate the downstream signal of TGFβ1 with SMAD2 and 3 activated through

phosphorylation and binding to SMAD4 to regulate transcription. In our studies using the SMAD2 and SMAD4 fusion protein with the mammalian one-hybrid assay in MDA-MB-453 cells, 1,1',2,2'-tetraMethDIM increased the activity of SMAD4, but did not affect SMAD2 activity (Figure 3.42). Therefore, 1,1',2,2'-tetraMethDIM may inhibit cell growth through induction of TGFβ1/SMAD signaling downstream of SMAD2 and upstream of SMAD4. However, a better system to test the activity of the SMAD proteins may be necessary because the basal activity and the induction by TGFβ1 of the SMAD2 fusion protein were relatively low.

Ongoing studies with several ring-substituted DIMs and DIM indicate that these compounds inhibit growth of ER-negative breast cancer cells and other ER-independent cell lines. Minimal effects on cell cycle genes and kinase pathways have been observed as reported in this study for 1,1',2,2'-tetraMethDIM in BT-474 and MDA-MB-453 cells. Recent data suggests that the DIM group of compounds may target the mitochondria and induce apoptotic or necrotic death pathways and these are currently being investigated.

1,1',2,2'-tetraMethDIM and other methyl-substituted DIM analogues are classified as SAhRMs. The compounds bind the AhR transforming the rat hepatic AhR into a DNA binding form and inhibit E2-induced cell proliferation in T47D cells as well as certain E2-induced uterine responses in mice; however, they do not induce cyp1A1 promoter activity in vitro in T47D cells (McDougal et al., 2001). We wanted to determine the AhR agonist activity of 1,1',2,2'-

tetraMethDIM and other AhR ligands such as TCDD, 6-MCDF, and DIM in BT-474 and MDA-MB-453 cells because differential activation of the AhR could account for different mechanisms of growth inhibition between the cell lines. Downregulation of AhR protein and induction of cyp1A1 protein were used as measures of AhR agonist activity. As seen in Figure 3.44, TCDD, 1,1',2,2'-tetraMethDIM, 6-MCDF, and DIM induced cyp1A1 protein with TCDD as the most potent inducer and DIM as the weakest inducer in BT-474 cells. TCDD, 6-MCDF, and 1,1',2,2'-tetraMethDIM also downregulated AhR protein levels in BT-474 cells; however, the downregulation by 1,1',2,2'-tetraMethDIM was very slight. In contrast, only TCDD induced cyp1A1 protein and downregulated AhR protein in MDA-MB-453 cells (Figure 3.45). The AhR agonist activity of the compounds in MCF-7 cells fell between activity in the BT-474 and MDA-MB-453 cells. TCDD and 6-MCDF induced high increases in cyp1A1 protein and easily detectable decreases in AhR protein, whereas 1,1',2,2'-tetraMethDIM induced very low levels of cyp1A1 protein and did not downregulate the AhR in MCF-7 cells (Figure 3.43). Furthermore, ERα downregulation was induced by treatment with TCDD, 6-MCDF and DIM, but not 1,1',2,2'-tetraMethDIM in MCF-7 cells. The differences in AhR agonist activity in the cell lines could be caused by varying levels of AhR or cofactor expression in the cells and could result in activation of differential signaling and thus different mechanisms of cell growth inhibition.

In addition to the in vitro studies, a preliminary study using MMTV-c-neu transgenic mice that overexpress ErbB2 in the mammary gland was also carried out to determine the inhibitory effects of 1,1',2,2'-tertaMethDIM on ErbB2-induced tumor formation. Although the number of mice used for the study was low, the results in Figure 3.46 indicate that 1,1',2,2'-tetraMethDIM may inhibit ErbB2-induced tumor growth in mice. Future studies using more mice should focus on earlier treatment with 1,1',2,2'-tetraMethDIM or related compounds to determine if the compounds are able to decrease tumor incidence or on treatment once palpable tumors arise to determine whether or not the compounds are able to inhibit the growth of detectable tumors.

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VITA

Kelcey Manae Becker Walker
15712 Racine Cove
Austin, TX 78717
512-255-3475

Education

College of William and Mary, Williamsburg, VA, B.S., May 1995, Biology

Virginia Tech University, Blacksburg, VA, M.S., Aug. 1999, Veterinary Medical Sciences (Immunology)

Texas A&M University, College Station, TX, Ph.D., May 2004, Toxicology

Professional Experience

1999-2004: Graduate Research Assistant, Faculty of Toxicology, Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University

1997-1999: Graduate Research/Teaching Assistant, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech University, Blacksburg, Virginia

1995-1997: Study Technician III, Post-Life Laboratory, Covance Laboratories Inc., Vienna, VA

1994-1995: Undergraduate Research Assistant, Population Biology and Endocrinology Laboratory, Department of Biology, College of William and Mary, Williamsburg, VA

Scholarships and Awards

2001 George T Edds Toxicology Award for outstanding toxicology student, College of Veterinary Medicine, Texas A&M University

2000-2004: NIEHS Toxicology Training Grant Fellowship, Faculty of Toxicology, Texas A&M University

1999: Regents Fellowship, Office of Graduate Studies, Texas A&M University